Comparability & reimbursement for the translation of scalable, automated stem cell cultures

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Additional Information:

- A Doctoral Thesis. Submitted in partial fulfilment of the requirements for the award of Doctor of Philosophy of Loughborough University.

Metadata Record: https://dspace.lboro.ac.uk/2134/20178

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Comparability & Reimbursement for the Translation of Scalable, Automated Stem Cell Cultures

by

Peter Archibald

A thesis submitted to
Loughborough University
for the degree of

Doctor of Philosophy

Centre for Biological Engineering
Wolfson School of Mechanical & Manufacturing Engineering
Loughborough University
October 2015

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I wish to acknowledge Professor David Williams and Dr Amit Chandra for their support throughout the duration of this work, their supervision throughout this research, and their guidance on the direction of this work. I would also like to acknowledge the TAP Biosystems (Royston, UK) for providing funding that contributed to the laboratory experiments performed in this research, and I would like to thank Dr Dave Thomas (TAP Biosystems) for providing supervision and guidance throughout the comparability experimental work. Furthermore, I would like to acknowledge LGC Molecular & Cell Biology Team (Teddington, UK) and the Institute for Stem Cell Therapy and Exploration of Monogenic Diseases (I-Stem, Evry, France) for their collaboration and contributions to the hMSC and hiPSC comparability experiments, respectively. Additionally, I would like to thank LGC Standards (Teddington, UK) for providing the Short Tandem Repeat (STR) profiling for each cell type, through their Cell Line Authentication (CLA) service.

The author would also like to recognise Dr Jessica Beckwith (Formerly of University of Minnesota, Minneapolis, USA), Professor Henk-Jan Schuurman (SchuBiomed Consultancy, Utrecht, Netherlands), and Professor John Nyman (University of Minnesota) for providing the raw cost data used within the cost-effectiveness analyses and sensitivity analyses. I would also like to thank Dr Kourosh Saeb-Parsy (University Lecturer & Honorary Consultant in Transplant Surgery, Addenbrooke’s Hospital, Cambridge, UK) for providing a clinical perspective to the cost-effectiveness analyses, and Dr Elangovan Gajraj (Technical Adviser, National Institute for Health and Care Excellence Scientific Advice Team, UK) for advising, and critiquing both the methodologies and findings of the cost-effectiveness analyses.

I would like to thank Dr Robert Thomas (Loughborough University, Loughborough, UK) for providing valuable guidance with regards experimental methods and data interpretation during both the first and second year viva voce. Finally, I would like to thank Thomas Heathman, for performing the appropriate manual cell culture processes on the few occasions that I was not available and for his assistance during the hMSC flow cytometry analyses; Alex Chan, for his assistance in developing the Kynurenine quantification assay; as well as Dr Forhad Ahmed and Dr Rachel Bailey (All Loughborough University), for their assistance during the Ruler hESC and hiPSC flow cytometry analyses.
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Chapter 1: Introduction

1.1 Themes
The research in this thesis focuses primarily on two critical challenges that inhibit the late stage translation of cell-based therapies and Regenerative Medicines (RM). These include product comparability after a change in manufacturing process or site; and the reimbursement of RM, in particular those which target multiple simultaneous indications, or ‘Multimorbidity’. The automation and standardisation of stem cell cultures also represent key themes of this thesis, which may facilitate the development of scalable, reproducible manufacturing processes for cell-based therapies. Furthermore, given the current uncertainty regarding the characterisation and potency of Human Mesenchymal Stromal (or Stem) Cells (hMSCs) that has inhibited the successful clinical translation of hMSC-based products, understanding the characterisation and putative modes of action of these cells was also a priority throughout this research. Also, due to the increasing number of Human Embryonic Stem Cell (hESC) derived therapies progressing towards market, and the industry-wide shift towards Human Induced Pluripotent Stem Cells (hiPSC) as an alternative to hESCs, the measurement of the growth and characterisation of these cells types represents an important method of demonstrating product comparability after alternative manufacturing process steps in the present thesis. Finally, due to the potential of hiPSCs as a source of large numbers of hMSCs, the culture conditions required to direct the differentiation of hiPSCs to hMSCs are explored.

1.2 Introduction Chapter Structure
Firstly, the context of this research and the key themes in this thesis are described in order to give a background to this research. Subsequently, the specific aims of this research are highlighted in the order in which the thesis chapters are presented. Finally, the thesis structure is described, the contribution of this research to the field is identified, and the appropriate acknowledgements are made.

1.3 Research Context
Regenerative Medicines have the potential to “replace or regenerate human cells, tissues or organs, to restore or establish normal function” (Mason & Dunnill, 2008). The term Regenerative Medicine is broad, and generally refers to cell-based therapies, of autologous, allogeneic or xenogeneic nature, that activate or replace existing, physiological cell populations. The potential market for RM in healthcare could be significant, and it has
previously been reported that 1 in 3 people in the United States could potentially benefit from access to such therapies (Harris, 2009). Furthermore, with a global industry revenue that has already reached over US$1 billion (Mason et al, 2012) and the number of active clinical trials at approximately 1,342 as of January 2014 (Heathman et al, 2015), great strides have been made towards the translation of cell-based therapies into healthcare. RM also represents a rapidly moving field, and this is demonstrated by the 7% increase in the number of Stem Cell research publications from 2008 to 2012 (van Servellen & Oba, 2014), and the 130% increase in the number of newly registered cell therapy trials from 2011 to 2014 (Bersenev, 2015). The evolving nature of Regenerative Medicine is epitomised by the dramatic increase and growing prominence of iPSC research after the discovery of this cell type in 2006, with a 77% increase in iPSC research publications from 2008 to 2012 (van Servellen & Oba, 2014).

However, at present a number of challenges regarding the development, manufacture, regulation, reimbursement, and delivery of RMs have inhibited their commercialisation and adoption. This research focuses primarily on a number of key aspects of the manufacturing, regulatory and reimbursement hurdles that have hindered the translation of cell-based therapies.

1.3.1 ‘Comparability’

A major factor that has contributed to both the manufacturing and regulatory challenges associated with RMs, involves the demonstration of product comparability after manufacturing changes. The term ‘comparability’ encompasses equivalence between products or processes. The existing regulatory structure in both Europe and the U.S, as part of the Chemistry, Manufacturing and Control component of regulatory applications, imposes a requirement to establish and maintain comparability between manufacturing sites or when changes are made to the manufacturing process (Hourd et al, 2014). The U.S Food and Drug Administration (FDA) outline that a comparability protocol represents a plan for assessing the effect of specific chemistry, manufacturing and control changes upon the identity, strength, quality, purity and potency of a specific drug product, as these factors relate to the safety and effectiveness of the product (Food and Drug Administration, 2003). Furthermore, the International Conference of Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) explain that a comparability exercise should provide analytical evidence that a product has highly similar quality attributes both before and after manufacturing process changes, with no adverse impact on safety or efficacy (ICH, 2004).
Within biopharmaceuticals, this term is often used to describe likenesses between cell culture or manufacturing platforms, processes, process steps, or manufacturing sites. For example, during process development, product equivalence must be demonstrated after ‘scale-up’ or ‘scale-out’ of the process. Furthermore, comparability must also be confirmed when multiple manufacturing sites are established, or when changes to the manufacturing process occur post-launch, including alterations to the manufacturing facility, changes to cell banks, deviations from the target product profile, or changes to raw materials or reagents. Over the course of the life cycle of a cell-based therapy, changes in the starting material, reagents or manufacturing processes are inevitable (Salmikangas et al, 2015).

Without comparability, a company will be limited to a single manufacturing site. Therefore, the issue of comparability across multiple manufacturing sites is critical and should be considered by manufacturers early in the RM product development process, as recommended by the Regenerative Medicine Expert Group (2015).

Comparability can be demonstrated through a variety of measures, including product characterisation, stability, purity, safety and potency, although the regulatory data requirements will depend upon the nature and extent of the changes to the manufacturing process, as well as the stage of product development (Hourd et al, 2014). If the outcome of the comparability exercise indicates that product characteristics, safety, efficacy, or pharmacological effect have been altered by a change in the manufacturing process, a ‘bridging study’ must be performed, which may involve further in vivo animal or clinical studies (Schlegel & Bobinnec, 2013). Without measures of in vivo biological activity or surrogate measures of potency, non-clinical or clinical testing (or both) will be required to determine product comparability (Bravery et al, 2013).

In the present research, the comparability between manual and automated cell culture process steps is examined by comparing cell growth, viability, stability, surface marker expression and functionality of multiple cell types when either manual or automated process steps are utilised (Chapters 7-9). The cell types utilised include Human Mesenchymal Stem (or Stromal) Cells (hMSCs), a ‘Ruler’ Human Embryonic Stem Cell (hESC) line (EC 2102Ep), and a Human Induced Pluripotent Stem Cell (hiPSC) line (VAX001024c07).

Additionally, the comparability between culture processes at multiple sites is explored. Specifically, a planned protocol transfer was undertaken between the Institute for Stem cell Therapy and Exploration of Monogenic diseases (I-Stem) (Evry, France) and Loughborough
University (Loughborough, UK) in order to demonstrate that the automated culture of hiPSCs can be performed at multiple sites, using TAP Biosystems’ (Royston, UK) CompacT SelecT automated cell culture platform (Chapter 9).

To further examine hiPSC culture process step comparability, the seeding and selection efficiency of hiPSCs (VAX001024c07), cultured using alternative process steps, in MSC medium was compared. This allowed for a brief examination of the comparability in hiPSC to MSC differentiation capacity between hiPSCs cultured utilising either manual or automated process steps, as well as the examination of the effect of variation in starting material upon hiPSC to MSC differentiation. Furthermore, the comparability between hiPSCs cultured in MSC medium and adult hMSCs in terms of cell morphology, diameter and trilineage differentiation is examined.

Furthermore, the comparability between Flow Cytometry analyses, performed at multiple sites through a planned protocol transfer, for hMSCs cultured utilising manual or automated cell culture process steps was examined (Chapter 7). Analysis of hMSC phenotype through Flow Cytometry was performed at both Loughborough University and LGC (Molecular & Cell Biology Team, Teddington, UK) in order to determine whether variability exits between analyses performed in multiple, independent laboratories and by multiple operators.

1.3.2 ‘Automation’

Within this work, the term ‘automation’, when used in reference to the utilisation of an automated process, refers to the almost complete replacement of manual process steps with automated process steps, performed by automatic equipment, with a minimal number of manual process steps remaining. In this instance, automation does not refer to the automation of a single process step or the mechanisation of any steps, in which process steps are performed by users with machinery. Rather, in the present work, the majority of culture process steps are performed by automatic equipment.

Within bioprocessing, process automation has been identified as a method of enabling increased sterility, reduced human error, increased productivity, improved product comparability, and therefore greater regulatory compliance (Brindley, Wall & Bure, 2013). Furthermore, despite the significant upfront capital expenditure required, the cost savings of an automated process, over a manual process, outweigh the initial risk (Brindley, Wall & Bure, 2013).
In Chapter 2, the progress that has been made towards the automation of pluripotent stem cell cultures is analysed in the form of a literature review. Furthermore, as presented in Chapters 7-9, the automated culture of hMSCs, ‘Ruler’ hESCs, and hiPSCs was performed and the comparability between manual and automated process steps was compared.

1.3.3 ‘Human Mesenchymal Stromal Cells (hMSCs)’

A significant proportion of this research focused upon hMSCs and their mode of action (MoA), in addition to examining their comparability after utilisation of alternative process steps. The translation of therapies based upon this cell type has advanced significantly in recent years, and this is reflected in the 382 clinical trials involving hMSC-based therapies, targeting over 18 different indications, active in 2014 (Heathman et al, 2015).

Initially, in the present research, a literature review was performed in which hMSC characterisation, mode of action, source specificity and effects of expansion were explored, and this is presented in Chapter 2. Furthermore, during the hMSC process step comparability study, the paracrine functionality of these cells after culture using alternative process steps was examined, and is presented in Chapter 7. Given the significant uncertainty surrounding hMSC mode of action, the examination of comparability in hMSC functionality, after the alteration of a single process step in the manufacturing process, represents an important area of research.

The implications of changes to the UK reimbursement environment upon the likelihood of reimbursement for cell-based therapies which may target multiple indications simultaneously, such as hMSC-based therapies, is also explored in both a literature review chapter (Chapter 3) and a modelling chapter (Chapter 4), and this is discussed further in sections 1.3.5 and 1.3.6.

Finally, the literature regarding the differentiation of hiPSCs to hMSCs (hiPSC-MSCs) is reviewed (Chapter 2); the culture conditions required to permit hiPSC survival and selection in MSC medium is explored using an unplanned protocol transfer between I-Stem and Loughborough University (Chapter 10); and the seeding and selection efficiency of hiPSCs, cultured using alternative process steps, in MSC medium is compared in order to examine the effect of differences in starting material upon hiPSC to MSC differentiation (Chapter 10). The comparability between hiPSC-MSCs and adult hMSCs, in terms of cell morphology, size and trilineage differentiation, is also examined (Chapter 10). The derivation of hMSCs from hiPSCs may represent an interesting prospect for the generation of large numbers of autologous, or Human Leukocyte Antigen (HLA) matched cells.
1.3.4 ‘Standardisation’

In addition to investigating product and process comparability, this research also explores the ‘standardisation’ of cell cultures. Standardisation can refer to the development of written standards, including codes of practice, procedures, and agreed definitions or methods between stakeholders. However, it has been proposed that the development of well-characterised ‘reference’, or ‘ruler’, cell lines, with sufficient precision, is fundamental to establishing methods of demonstrating comparability (Hourd et al, 2014). This could then be applied to the standardisation of cell lines, culture methods and assays. The utilisation of reference materials allows for the confirmation of assay performance, the identification of the relative potency of a batch of cell therapy product, the understanding of assay variation, and the mitigation of assay and process drift (Bravery et al, 2013). Reference materials also facilitate the identification of whether the cause of drift in a product measurement is attributed to a drift in the assay or in the process (Bravery et al, 2013). It has also been noted that standardised approaches to culture and characterisation will be important to determine differences between cell lines (Barry et al, 2015). Furthermore, the utilisation of reference standards is valuable for the demonstration of comparability after a change in the manufacturing process (Schlegel & Bobinnec, 2013). Currently, international reference standards or materials do not exist for cell therapy products. However, in recent years, research focusing upon the identification of reference cell lines for multiple stem cell types, including hESCs (Josephson et al, 2007) and hMSCs (Deans, 2015), has been undertaken.

A typical approach to the development of reference material in manufacturing would be to allocate a representative batch of product, which is used as the master reference material, and, consequently, working reference material can be qualified and used for batch release, comparability and other needs (Bravery et al, 2013). However, for cell therapy products, the manufacture of a large quantity of product, in order to allow for the generation of reference materials, may not be possible.

Linking the use of these reference materials or cell lines to automated processing systems could provide a method of comparison between manufacturing processes at multiple sites, or between processing equipment (Hourd et al, 2014). The establishment of reference standards, as well as the removal of manual processing and the comprehensive characterisation of cell lines, has been identified as one of a number of essential steps towards the development of a commercial cell therapy process (Wall & Brindley, 2013).
The present research reviews the available literature regarding the standardisation of pluripotent stem cell cultures (Chapter 2), and investigate the comparability between a ‘Ruler’ hESC line, known as Embryonal Carcinoma 2102Ep (EC 2102Ep), cultured using alternative process steps (Chapter 8).

1.3.5 ‘Reimbursement’

As mentioned previously, in addition to addressing the manufacturing and comparability challenges, this research also focuses upon the reimbursement challenges that are faced when commercialising cell-based therapies, specifically within the UK.

The term ‘reimbursement’ refers to the mechanism through which the National Health Service (NHS), and others, pay manufacturers for their new healthcare technologies. It is apparent that the UK reimbursement landscape is evolving and that the complex reimbursement pathways in place in the UK can act as a barrier to the adoption of new products. Within the last decade, an alternative system of price-setting for new medicines has been proposed, known as ‘Value-Based Pricing’ (VBP), and was set to be introduced in the UK by January 2014 (Office for Fair Trading, 2007; Department of Health, 2010). This VBP approach would set the price for new treatments based upon the price at which the customer values the product relative to other products. However, as a result of ambiguity within the methodology and concerns voiced by key stakeholders, the VBP scheme was adapted into an alternative system, known as ‘Value-Based Assessment’ (VBA), which aligned with the current system and was to be introduced in autumn of 2014 (Department of Health & ABPI, 2013). Furthermore, in September 2014, after review by the National Institute for Health & Care Excellence (NICE) and public consultation, it was recommended that no change to the technology appraisal methodology be made in the short term, and therefore that the current reimbursement system be maintained for the foreseeable future (National Institute for Health and Care Excellence, 2014).

Therefore, the present research reviews these changes to the UK reimbursement approach and proposes how these changes may have impacted the reimbursement of RMs (Chapter 3). Moreover, economic models are utilised to explore the consequences of changes to the UK appraisal methodology upon the likelihood of reimbursement for cell-based therapies. Specifically, an illustrative study was undertaken in which a number of cost-effectiveness analyses of an exemplar cell-based therapy, specifically Allogeneic Islet Transplantation, were performed using either current HTA approaches, the proposed Value-Based Pricing
(VBP) approach, and the more recently developed Value-Based Assessment (VBA) approach (Chapter 5). Furthermore, sensitivity analyses were performed in order to explore the effect of prospective price and efficacy targets upon the Incremental Cost-Effectiveness Ratio (ICER) and reimbursement potential of a hiPSC derived Beta Cell Therapy.

1.3.6 ‘Multimorbidity’

The final recurring theme within this thesis involves the potential of regenerative medicines, such as an MSC-based therapy, to treat patients with multiple simultaneous indications, or ‘Multimorbidity’, and the methods of reimbursement and early economic analysis that could be applied to such therapies. More specifically, the need for improvements in the treatment of multimorbid patients is discussed, the way in which proposed changes to the UK reimbursement system may have influenced the likelihood of therapies which target multiple indications simultaneously achieving reimbursement is proposed, and the way in which early economic analyses could be modified to account for therapies which target multimorbid patients is explored.

It has been proposed that if Regenerative Medicine products, such as hMSC-based therapies, could repair or replace diseased or damaged cells, potentially curing disease or better managing the cause, this would diminish or delay the onset of associated conditions (Prescott, 2011; Prescott, 2012). In Chapter 3, the potentially favourable impact of the changes to the UK reimbursement system, associated with the introduction of VBP, upon the reimbursement of therapies which target multiple simultaneous indications is explored. Furthermore, Chapter 4 focuses upon the adaptation of early economic analysis methods, in particular the ‘Headroom Method’, in order to account for technologies which treat multiple indications simultaneously. The Headroom Method uses optimistic assumptions to determine whether a product in development would be cost-effective if it provides the maximum improvement over incumbent technologies. The adaptations to the Headroom Method, proposed in Chapter 4, involved the development of a number of Headroom variants, using Insulin Dependent Diabetes Mellitus (IDDM) as a case study, and the presentation of those which are most promising.

1.4 Research Aims

The research aims below are outlined in the order in which the thesis chapters are presented.
Chapter 1: Introduction

- To review the development and adaptation of the Value-Based Pricing (VBP) scheme that was proposed for the appraisal and reimbursement of new health technologies. Also, to hypothesise how the VBP scheme, and how modifications to this scheme, may have impacted the reimbursement of RMs. This represents an important consideration of the translation-led research performed in the present thesis, as changes to reimbursement are likely to influence the price, and therefore required cost, of cell therapy products.

- To review the way in which multimorbid patients are treated through current pathways within the healthcare system, and to examine whether the implementation of VBP may favour the reimbursement of treatments which target multiple indications simultaneously, for example an MSC-based therapy. Also, to undertake a modelling activity in order to adapt the ‘Headroom Method’, allowing for the early economic analysis of healthcare technologies that target multiple simultaneous indications

- To perform economic modelling to compare cost-effectiveness analyses of an exemplar cell-based therapy using the ‘current’ appraisal methodology, and the proposed VBP and VBA (Value-Based Assessment) approaches. Also, to perform efficacy and cost sensitivity analyses in order to provide reimbursement perspectives for an emerging cell therapy, specifically a hiPSC derived Beta Cell Therapy

- To determine the effect of alternative cell culture process steps, and residual dissociation agent, upon hMSC growth, viability, morphology, stability, surface marker expression and functionality as an instance of comparability

- To determine the effect of alternative cell culture process steps, and residual dissociation agent, upon the growth, viability, morphology, stability and pluripotency marker expression of a Ruler hESC line (EC 2102Ep) and a hiPSC line (VAX001024c07) as further instances of comparability

- To examine the effect of numerous components of hiPSC to MSC differentiation protocols; including MSC culture medium composition, and Rho-associated protein kinase (ROCK) inhibitor pre-treatment; upon the early stages of hiPSC (VAX001024c07 line) to MSC differentiation through the measurement of hiPSC seeding and selection efficiency. Also, to further examine process step comparability, the seeding and selection efficiency of hiPSCs, cultured using alternative process steps, in MSC medium is compared in order to determine the effect of variation in starting material upon hiPSC to MSC differentiation
To examine the comparability of hiPSC derived MSCs (hiPSC-MSCs) and adult hMSCs through the comparison of cell morphology, diameter and trilineage differentiation

1.5 Thesis Structure

1. Introduction

In the introductory chapter, the context and background of this research have been described and the recurrent themes of ‘automation’ and ‘comparability’ have been defined. Furthermore, the aims of the research are outlined.

2. Literature Review- The Characteristics, Automation, and Standardisation of Human Mesenchymal Stromal Cell (hMSCs), Human Embryonic Stem Cell (hESC), and Human Induced Pluripotent Stem Cell (hiPSC) Culture

This chapter reviews the available literature on the characterisation, mode of action, source specificity and effects of expansion of Human Mesenchymal Stromal Cells (hMSCs). Additionally, this chapter describes the available reprogramming methods for the generation of hiPSCs, the characteristics of these cells, and the progress that has been made to automate the culture of this cell type. Furthermore, this chapter reviews the current literature examining hMSCs derived from the differentiation of Human Induced Pluripotent Stem Cells (hiPSCs), or hiPSC-MSCs. Specifically, the protocols for differentiation, the characterisation, and the potential epigenetic memory of hiPSC-MSCs are explored. Finally, this review highlights the defining characteristics of Human Embryonic Stem Cells (hESCs), examine the progress that has been made to automate the culture of this cell type, and discuss the need for standardisation of pluripotent stem cell cultures.

3. A Systematic Literature Review of the Transformation of the UK Reimbursement Environment, the Remodelling of VBP over a Two Year Period & the Potential Implications for Regenerative Medicines which Target Multiple Simultaneous Indications

The third chapter consists of a systematic review of the proposed Value-Based Pricing (VBP) scheme for the reimbursement of new health technologies. This review summarises the ‘current’ UK reimbursement environment as well as the components and drawbacks of the VBP scheme. Additionally, this review describes the current treatment and care pathways for multimorbid patients and proposes how the introduction of VBP may have favoured treatments which target multiple simultaneous indications. Finally, this chapter describes the
adaptation of the VBP scheme over time and the way in which these changes may impact the reimbursement of unconventional technologies, such as RMs.

4. The Adaptation & Modelling of the ‘Headroom Method’ for Products Which Target Multiple Simultaneous Indications: Using Insulin Dependent Diabetes Mellitus as a Case Example

The fourth chapter in the present thesis represents a brief, but novel and exploratory, modelling study which acts as a precursor to the comprehensive economic analyses performed in Chapter 5. A background to the ‘Headroom Method’ for early economic analysis is provided, the issues with care for multimorbid patients is further discussed, and a number of methods of adaptation for the Headroom Method, in order to account for technologies which treat multiple indications simultaneously, are presented using Type I Diabetes as a case study. Finally, the Headroom Method variant that most adequately combines the multiple Headrooms associated with the treatment of multiple simultaneous indications is identified.

5. The Incorporation of ‘Value-Based’ Appraisal Methodologies into Cost-Effectiveness Analyses: An Illustrative Study of Allogeneic Islet Transplantation and the Implications for hiPSC derived Beta Cell Therapies

In this chapter, an economic modelling activity is undertaken in which the results of multiple cost-effectiveness analyses of Allogeneic Islet Transplantation for the treatment of Insulin Dependent (Type I) Diabetes Mellitus (IDDM); using either current HTA approaches, the proposed Value-Based Pricing (VBP) approach, and the more recently developed Value-Based Assessment (VBA) approach; are compared. Furthermore, sensitivity analyses were performed in order to identify the possible effect of improved efficacy or cost in hiPSC derived Beta Cell therapy, versus Allogeneic Islet Transplantation, upon the time required to reach the ‘breakeven’ point and to become cost saving. These cost-effectiveness and sensitivity analyses were performed with guidance from Dr Elangovan Gajraj (Technical Adviser, NICE Scientific Advice Team, UK) and Dr Kourosh Saeb-Parsy (University Lecturer & Honorary Consultant in Transplant Surgery, Addenbrooke’s Hospital, Cambridge, UK).
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6. Materials & Methods

In the ‘Materials & Methods’ Chapter, the methods, equipment, and reagents used in the hMSC, Ruler hESC line (EC 2102Ep), and hiPSC (VAX001024c07) process comparability experiments are described. Furthermore, the materials and methods utilised to differentiate hiPSCs into MSCs, and to compare hiPSC-MSCs and adult hMSCs, are outlined.

7. Comparison of a Manual, Centrifugation & an Automated, Non-Centrifugation Cell Culture Process Step for Human MSCs

The first experimental results chapter compares the effects of manual (Centrifugation) and automated (Non-Centrifugation) cell culture process steps upon hMSC growth, viability, morphology, surface marker expression, Short Tandem Repeat (STR) profile, colony-forming potential, trilineage differentiation, and paracrine functionality. Furthermore, the comparability between hMSC flow cytometry and surface marker expression analyses performed at independent laboratories is explored. This study was performed in collaboration with TAP Biosystems (Royston, UK) and LGC (Teddington, UK).

8. Comparison of a Manual, Centrifugation & an Automated, Non-Centrifugation Cell Culture Process Step for a ‘Ruler’ Human ESC line

The next experimental results chapter compares the effects of manual (Centrifugation) and automated (Non-Centrifugation) cell culture process steps upon the growth, viability, morphology, STR profile, and pluripotent marker expression of a Ruler hESC line (EC 2102Ep). This study was performed in collaboration with TAP Biosystems.

9. Comparison of a Manual, Centrifugation & an Automated, Non-Centrifugation Cell Culture Process Step for a Human iPSC line

Similarly to previous chapters, Chapter 9 compares the effects of manual (Centrifugation) and automated (Non-Centrifugation) cell culture process steps upon the growth, viability, morphology, STR profile, and pluripotent marker expression of a hiPSC line (VAX001024c07). This experimental work was performed in collaboration with TAP Biosystems and the Institute for Stem cell Therapy and Exploration of Monogenic diseases (I-Stem, Evry, France).

10. The Exploration of Culture Conditions and Comparability in Starting Material for the Early Stages of hiPSC to MSC Differentiation
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The final results chapter, in which an unplanned protocol transfer was undertaken between I-Stem and Loughborough University, examines the effect of MSC culture medium composition, and the effect of ROCK inhibitor upon the seeding and selection efficiency of hiPSCs. Furthermore, to further examine process step comparability, the seeding and selection efficiency of hiPSCs, cultured using either manual or automated process steps, in MSC medium was compared. This allowed for the effect of differences in starting material upon the early stages of hiPSC to MSC differentiation to be determined. Additionally, the morphology, cell diameter and trilineage differentiation of hiPSC-MSCs was compared to that of adult hMSCs. This chapter represents a brief, exploratory study into the effect of numerous components of hiPSC to MSC differentiation protocols upon the early stages of differentiation of hiPSCs to MSCs, which was performed in collaboration with I-Stem.

11. Conclusions

The final chapter summarises the main findings, as well as the conclusions that can be drawn, from the literature review, modelling and experimental research presented in this thesis. The conclusions from the literature review and modelling research, regarding the reimbursement of RMs and the impact of the changes to the UK health technology appraisal methodology upon the reimbursement of cell-based therapies, are summarised. Furthermore, a multi-faceted comparison of the manual (Centrifugation) and automated (Non-Centrifugation) process steps, through the measurement of a number of product and process parameters, is presented and the uncertainty surrounding the importance of the centrifugation cell culture process step is addressed.

1.6 Contribution of research

• The transformation of Value-Based Pricing (VBP) over recent years was reviewed, and it was illustrated that the proposed Value-Based approaches would have been likely to be favourable for the reimbursement of RMs. The way in which these proposed changes may have impacted the UK reimbursement environment and methodology has not previously been rigorously examined, particularly for RMs.

• The impact of the introduction of the proposed value-based methodologies upon the reimbursement of therapies which target multiple indications was explored, for which it was determined that value-based approaches may have again been favourable.
• A number of novel Headroom Method ‘variants’ were developed in order to allow for the early economic analysis of healthcare technologies which target multiple simultaneous indications, which in turn could allow for a more accurate representation of the value and reimbursement potential of such products to be modelled and determined. An exploratory study of this nature has not previously been undertaken.

• Numerous cost-effectiveness analyses of Allogeneic Islet Transplantation were performed, using each of the current and value-based approaches, in order to model the effect of the proposed VBP & VBA schemes upon the likelihood of reimbursement of an exemplar cell-based therapy. This demonstrated that the proposed value-based methodologies would have been favourable for the reimbursement of an exemplar cell-based therapy by reducing its Incremental Cost-Effectiveness Ratio (ICER). The literature in which the VBP approach has been utilised to appraise healthcare technologies is limited (Girling et al, 2010; Koerber, Rolauffs & Rogowski, 2013), and has not previously been accomplished using the VBA approach.

• Efficacy and cost sensitivity analyses, based upon the Allogeneic Islet Transplantation data, were performed in order to identify the targets required for a hiPSC-derived Beta Cell therapy to be considered cost-effective. These models highlighted that the requirement for immunosuppression, the duration of graft function, and the rate of graft failure will determine the likelihood of reimbursement for a hiPSC-derived Beta Cell therapy. Sensitivity analyses of this kind have not previously been performed for any cell-based therapy.

• The comparability between hMSCs, ‘Ruler’ hESCs, and hiPSCs cultured using alternative process steps was examined by measuring a number of key parameters that are representative of growth, stability, characterisation and functionality. These parameters included cell morphology, diameter, aggregation, yield, viability, Short Tandem Repeat STR profile, phenotype, colony forming potential, differentiation, and cytokine secretion. It was observed that, although a number of exceptions were observed in the case of hiPSCs, the utilisation of an automated, Non-Centrifugation process step generated cell populations with comparable stability, characterisation and viability. However, in general, the culture processes based upon the Non-Centrifugation process step generated a greater cell yield, although this may have been associated with measurement variability. A multi-
parameter analysis measuring the comparability between alternative cell culture process steps using multiple cell types has not previously been accomplished.

- The comparison of numerous MSC medium compositions revealed that the DMEM & 20% HPL composition was the most effective for seeding and selection of hiPSCs in MSC medium. The utilisation of animal serum-free medium compositions for the differentiation of hiPSCs to MSCs has only previously been explored in a single study (Luzzani et al, 2015). The examination of alternative ROCK inhibitor pre-treatment regimens identified that pre-treatment allowed for the survival of hiPSCs in MSC culture medium, and that extended treatment increased hiPSC survival. Research regarding the influence of ROCK inhibitor pre-treatment regimens upon hiPSC survival in MSC medium is currently lacking. The comparison of the culture of hiPSC starting materials in MSC medium identified that significant variability in seeding and selection efficiencies, as well as cell diameter, were observed between hiPSC populations initially expanded using either manual or automated process steps. The influence on hiPSC starting material upon hiPSC seeding and selection efficiency in MSC medium has not previously been explored.

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1.8 List of Equations

- The Headroom Method (Section 4.1.1)
  - Maximum change in Effectiveness (MaxΔQALY)
    \[ \text{MaxΔQALY} = (1 - \text{Health utility of Gold Standard}) \times \text{Duration of Benefit (Years)} \]
  - Headroom (MaxΔCost)
    \[ \text{Headroom (MaxΔCost)} = \text{WTP Threshold} \times \text{MaxΔQALY} \]
  - Return on Investment/Revenue
    \[ \text{Revenue (ROI)} = (\text{MaxΔCost} - \text{COGs}) \times \text{Volume} \]

- Determination of the Incremental Cost-Effectiveness Ratio (ICER) (Section 5.2.1)
  - Quality-Adjusted Life Years (QALYs) Gained
    \[ \text{QALYs gained} = \text{Health Utility (0 to 1 scale)} \times \text{duration (years) of that state} \]
  - Change in QALYs (ΔQALY)
    \[ \Delta\text{QALY} = \text{New Treatment QALYs} - \text{‘Gold Standard’ QALYs} \]
  - Change in Cost (ΔCost)
    \[ \Delta\text{Cost} = \text{Cost of new treatment - Cost of ‘Gold Standard’ Treatment} \]
  - Incremental Cost-Effectiveness Ratio (ICER)
    \[ \text{ICER} = \frac{\Delta\text{Cost}}{\Delta\text{QALY}} \]

- Assessment and weighting of the Burden of Illness (BoI) under Value-Based Pricing (Section 5.3.2)
  - QALYs lost through life expectancy
    \[ \text{QALYs lost through life expectancy} = (\text{Average Life expectancy} - \text{Life expectancy of a patient}) \times \text{Average QoL of individual without indication} \]
  - QALYs lost through reduced Quality of life (QoL)
    \[ \text{QALYs lost through reduced Quality of life (QoL)} = \text{Remaining years of life (Patient Life expectancy-patient age)} \times \Delta\text{QoL (Healthy QoL-Patient QoL)} \]
  - Total BoI
    \[ \text{Total BoI} = \text{QALYs lost through life expectancy} + \text{Burden of Illness (BoI) during life} \]
  - BoI Weight per QALY gained
    \[ \text{BoI Weight per QALY gained} = [\text{BoI x Fixed rate of QALY value for BoI (5%)}] + 1 \]
  - BoI-adjusted additional QALY gain

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BoI-adjusted additional QALY gain = Incremental QALY gains from new treatment x BoI Weight per QALY gained

- Bol-adjusted ICER
  
  Bol-adjusted ICER = ∆Cost between new & incumbent technology/BoI-adjusted additional QALY gain

- Assessment and weighting of the Wider Societal Benefit (WSB) under VBP (Section 5.3.4)
  
  - WSB-adjusted QALY gain
    
    WSB-adjusted QALY gain = Net Production per QALY gained (From the DoH Template Spreadsheet)/Proposed WSB exchange rate

  - Bol- & WSB-adjusted QALY gain
    
    Bol- & WSB-adjusted QALY gain = Bol-adjusted QALY gain + WSB-adjusted QALY gain

  - WSB per QALY
    
    WSB per QALY = WSB-adjusted QALY gain / Incremental QALY gains from new treatment

- Determination of the BoI and Weighting of the ICER under Value-Based Assessment (VBA) (Section 5.4.2)
  
  - Number of years of life remaining for healthy individual
    
    Number of years of life remaining for healthy individual = Normal Life Expectancy - Patient Age

  - QALYs remaining for a healthy individual
    
    QALYs remaining for a healthy individual = Years of life remaining x Normal QoL/Health utility

  - Number of years remaining for patient without treatment
    
    Number of years remaining for patient without treatment = Patient Life Expectancy – Patient Age

  - QALYs remaining for patient without treatment
    
    QALYs remaining for patient without treatment = Number of years remaining for patient without treatment x Patient QoL or Health utility

  - Expected loss of QALYs without of treatment
    
    Expected loss of QALYs without of treatment = QALYs remaining for a healthy individual - QALYs remaining for patient without treatment

  - Proportion of healthy individual QALYs remaining
    
    Proportion of healthy individual QALYs remaining = Expected loss of QALYs without of treatment / QALYs remaining for a healthy individual

  - Proportional Shortfall
    
    Proportional Shortfall = 100 (%) - Proportion of healthy individual QALYs remaining

- Determination of the Wider Societal Impact (WSI) and Weighting of the ICER under VBA (Section 5.4.3)
  
  - Absolute Societal Shortfall
    
    Absolute Societal Shortfall = Expected QALYs without disease - Expected QALYs with disease

- Colony Forming Efficiency of hMSCs (Section 7.2.7)
  
  Colony Forming Efficiency = (Number of colonies/250 cells seeded) x 100
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- Determination of hMSC, Ruler hESC & hiPSC Population Doublings (Sections 7.3.8, 8.3.5, 9.3.5)
  - Population Doubling Time (PDT)
    \[
    PDT = \frac{\text{Time of Final Cell Count (Days)} - \text{Time of Seeding (Days)}}{3.32 \times (\log(\text{Final Cell Yield}) - \log(\text{Number of Cells Seeded}))}
    \]
  - Cumulative Population Doublings (CPDs)
    \[
    CPDs = \frac{\text{Time of Final Cell Count (Days)} - \text{Time of Seeding (Days)}}{PDT \text{ (Days)}}
    \]

1.9 List of Frequently Used Abbreviations

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1.10 List of Journal Publications


1.10.2 Co-Authored Papers


1.10.3 Publications in Progress

The aim of the present chapter is to review the literature regarding the characterisation, functionality, and manufacture of hMSCs, hESCs, hiPSCs, and hiPSC-MSCs, and to highlight the areas in need of future research.

2.1 Introduction

Due to the significant focus upon Human Mesenchymal Stromal Cells (hMSCs) in the present thesis, the current literature pertaining to MSC characterisation, variation between tissue sources, effect of culture expansion, and mode of action in a physiological environment are first summarised and discussed. Additionally, the progress towards the scalability and automation of MSC manufacture is reviewed. Areas of future promise within MSC research are highlighted, and a novel paradigm, involving the utilisation of MSCs in the treatment of a range of indications and associated complications, are then presented. The reimbursement considerations for this paradigm are also explored in section 3.3.3.2 and Chapter 4.

Given the research undertaken in the present thesis regarding the exploration of the differentiation process for, and the reimbursement of, hiPSC derived products, the characterisation of Induced Pluripotent Stem Cells (iPSCs), the available methods for the reprogramming of adult cells into iPSCs, the development of automated and scalable iPSC culture methods, and the challenges that may impede the scalable manufacture of these cells was next reviewed. Further, this review goes on to describe the availability of hiPSCs as a source of MSCs, the limitations of deriving MSCs from iPSCs, the differentiation protocols that have been developed, the characterisation and functionality of hiPSC-MSCs, and the epigenetic memory that may remain after deriving MSCs from iPSCs. Finally, the characterisation of Human Embryonic Stem Cells (hESCs), as well as the automation, scalability and standardisation of hESC culture, is discussed in this chapter.

2.2 Human Mesenchymal Stromal Cells (hMSCs)

2.2.1 Background

A number of labels have been attributed to MSCs in the past, including ‘Colony-Forming Unit-Fibroblasts’ (CFU-F) and ‘Mesenchymal Stem Cells’, as discussed in section 2.2.2, and
there has been great uncertainty surrounding the true source, identity, and in vivo function of these cells.

There are a number of companies currently involved in the development of such MSC-based regenerative medicine products, the most successful of which include Athersys Inc’s MultiStem®, Pluristem Therapeutics Inc’s PLX cells, and Osiris Therapeutics Inc’s Prochymal®, however both Athersys Inc’s and Osiris’ product have recently failed to meet the primary clinical endpoint. Athersys Inc’s MultiStem® product did not show a significant improvement in Phase II trials for both Ulcerative Colitis and Ischaemic Stroke (Athersys Inc, 2014; Athersys Inc, 2015), and similar results were found in Osiris’ Phase III clinical trial of their Prochymal® therapy for Graft versus Host Disease (Galipeau, 2013). The basis of each of these therapies is similar as the cell types and tissue sources are comparable. Each of these technologies utilises cells from a population known as ‘Mesenchymal Stromal Cells’ (MSCs), which can be derived from a variety of tissues throughout the body, obtained through isolation from an allogeneic source. Although both Athersys Inc and Osiris Therapeutics Inc isolate their cell population from the bone marrow of a qualified donor for their MultiStem® and Prochymal® respectively, Pluristem Therapeutics Inc’s PLX cells are derived from donor placental tissue, following childbirth, which would otherwise be discarded as medical waste. The way in which the tissue source affects the characteristics of these cells is discussed further in section 2.2.4.

Furthermore, in recent years MSC-based therapies have been applied to a variety of indications, including cardiovascular, pulmonary, neurological, metabolic, skeletal, inflammatory and immune disorders. However, although these products aim to target indications individually, given the recent paradigm shift in the understanding of the mode of action of these cells, as described later in section 2.2.3, it may be feasible to suggest that these products could target a number of these indications simultaneously.

This capacity for simultaneous therapy may represent an opportunity to treat those patients afflicted with a number of indications. The co-existence of two or more chronic conditions, where one is not necessarily more central than the others, is known as ‘Multimorbidity’ (Boyd & Fortin, 2010). Currently, one in four adults suffers from two or more chronic conditions, and very few standardised procedures are in place for the treatment of multimorbid patients (Boyd & Fortin, 2010). Instead, the UK currently adopts a single-
disease based approach to care delivery for these patients, which can lead to increased healthcare costs and utilisation (Glynn et al, 2011; Nagl et al 2012), as well as potentially duplicative and unsafe treatment (Barnett et al, 2012). Therefore, if MSC-based products do in fact have the capacity to target multiple indications simultaneously, this could represent a step change in the treatment available to multimorbid patients.

Regardless of the clinical indication targeted, improvements in the measurement of quality and potency must be made in order to determine appropriate critical quality attributes (CQAs) and potency assays, and to therefore ensure the safety and efficacy of an MSC-based product. This is apparent from the number of clinical trials involving MSC-based products which have failed to meet their primary endpoints (Osiris Therapeutics Inc, 2009; Athersys Inc., 2014; Athersys Inc, 2015).

However, progression in the understanding of MSC basic characterisation and mode of action must first be achieved. Therefore, the focus of this review revolves around, and aims to tackle, the ambiguity surrounding this cell type and provide clarification regarding the characterisation, tissue-specific sources, and mode of action of MSCs. This chapter also examines the effects of extended culture and expansion of these cells.

2.2.2 Characterisation

The cell population known as ‘Mesenchymal Stromal Cells’ (MSCs), identified by Friedenstein and colleagues in the 1970s, was first described as an adherent, non-haematopoietic cell type, present in the bone marrow, with the capacity to form fibroblastic colonies in vitro. This population was given the name ‘Colony-Forming Unit-Fibroblasts’ and were found to have adipogenic, chondrogenic and osteogenic differentiation potential. Lanotte et al (1981) proceeded to discover the bone marrow stromal location of these cells, which led to the term ‘Stromal Cells’, and became the prominent label for this population. A number of years later, the existence of a cell type, known as ‘Stromal Stem Cells’, which gave rise to stromal cells and resided at the top of the stromal system hierarchy, was proposed by Owen (1988). In the early 1990’s, this cell population was reported, by Caplan (1991), to be capable of differentiation into all cells of the mesodermal lineage, and thus gained the name ‘Mesenchymal Stem Cells’, which consequently stimulated a wealth of research into their role in tissue regeneration. Despite the on-going debate surrounding the true stemness of the cells, as discussed later in this section, this label has been maintained and is often still
used. However, as a result of the International Society for Cellular Therapy’s (ISCT) definition (Dominici et al, 2006) and a general shift in consensus regarding this issue, these cells are now more often known as ‘Multipotent Mesenchymal Stromal Cells’, whilst maintaining their ‘MSC’ acronym (Phinney & Sensebe, 2013). However, despite the shift away from the ‘stem cell’ terminology, great confusion regarding the true identity and source of these cells remains, and hence a number of alternative nomenclatures exist to describe subpopulations of this cell type or populations with marginally differing characteristics, including ‘Adipose-tissue Derived Stem Cells’ (ADSCs), ‘Muscle-Derived Stem Cells’ (MDSCs), and ‘Multipotent Adult Progenitor Cells’ (MAPCs) (Chen et al, 2009; Corselli et al, 2010). This latter term is used to describe the cell type isolated from donor bone marrow in Athersys Inc’s MultiStem® cell therapy. MAPCs have been proposed to be MSC progenitors, displaying a level of pluripotency whilst expressing a similar cell surface phenotype to that of MSCs (Jiang et al, 2002; Jacobs et al, 2013). Recent studies have also analysed the secretome of these cells as well as their immunomodulatory capacity in humans (Burrows et al, 2013; Soeder et al, 2015). Pluristem Therapeutics Inc. derive a similar cell type from donor placental tissue for their PLX cells and classify these cells as ‘Mesenchymal-like Adherent Stromal Cells’ or ‘ASCs’. Despite the disparity in nomenclature, all of these cell types are similar and can essentially fall into the category of MSCs, although differences between tissue sources may exist and this is discussed in section 2.2.4. This diversity in the terminology used to define this cell population highlights the difficulty associated with its identity and characterisation, and this issue will now be addressed.

Currently, the basic characteristics of MSCs, as defined in the ISCT definition paper (Dominici et al, 2006), are based upon the surface marker expression and the activity of these cells in culture. This paper outlines a number of criteria for the identification of these cells, including their adherence to culture plastic, their positive expression of CD105, CD73, and CD90, their lack of CD45, CD34, CD14 or CD11b, CD79α or CD19, and HLA-DR expression, and finally their osteogenic, chondrogenic and adipogenic differentiation potential in vitro. However, although the use of these parameters during isolation may result in an enriched MSC population, these methods may be flawed due to their lack of accuracy and lack of specificity to the MSC cell type. It is also clear that, as described further in this review, these parameters are not representative of the in vivo mode of action of these cells.
Prior to the publication of the ISCT MSC definition, the isolation of these cells utilised a number of antibodies which were believed to react specifically with markers expressed on MSCs alone. In the early 1990’s, Simmons & Torok-Storb (1991) identified Stro-1 as the first antibody reactive with non-haematopoietic progenitors from the bone marrow, discovering that CFU-Fs were exclusively present in the Stro-1⁺ cell population. Towards the end of this decade, a further two antibodies, namely SB-10 (Bruder et al, 1998) and SH-2 (Barry et al, 1999), were found to distinguish undifferentiated MSCs from other cell types in bone marrow aspirate by reacting with CD166 and CD105 respectively. A further two antibodies (SH-3 and SH-4), which recognise epitopes on the MSC antigen CD73, were identified and found to not recognise haematopoietic cells or osteocytes (Barry et al, 2001). Finally, the identification of negative markers led to the development of a CD34/CD45/CD11b immunodepletion method capable of generating purified MSC preparations (Ortiz et al, 2003). However, it has been acknowledged that these antibodies and isolation methods may not be specific to MSCs and a number of the targeted markers may be expressed on a variety of cells (Barry & Murphy, 2004).

This lack of specificity in isolation techniques has stimulated the examination of the MSC surface phenotype in greater detail, and investigations after the publication of the ISCT definition paper have determined that in fact these cells express a greater number of markers than previously thought. Antigens which represent adhesion molecules (CD44, CD50, CD54, CD56, CD102, CD106, CD146, CD166), integrins (CD11a, CD18, CD29, CD49a-f, CD51, CD61, CD104), selectins (CD62E, CD62L, CD62P), chemokine receptors (CD117, CD119, CD121a, CD123, CD124, CD126, CD127, CD140a, CD140b) and membrane bound receptors involved in apoptosis (CD95, CD178, CD120a, CD12b) have all been identified (Rojewski, Weber & Schrezenmeier, 2007). This study also revealed that the widely accepted negative markers CD133 and CD34 may in fact be prospective markers for MSCs prior to differentiation. These findings highlight the uncertainty surrounding the marker expression and surface phenotype of these cells. The authors (Rojewski, Weber & Schrezenmeier, 2007) also outline the need for a panel of positive markers for MSCs expanded from every tissue, and the testing of negative markers of cells which may have accidentally been co-cultured or co-isolated with MSC samples. It has also been suggested that, due to the intrinsic heterogeneity of MSCs, it may be more relevant to identify subsets of markers that denote
functional differences in populations, rather than focusing upon identifying the phenotype of ‘true’ MSCs (Phinney & Sensebe, 2013).

Recently, more advanced characterisation techniques have been utilised to analyse the membrane proteome of human bone marrow derived MSCs, and has helped to establish common characteristics shared between culture expanded MSCs and *in vivo* cell populations which have previously been lacking. Rasini and colleagues (2013) discovered overlapping *in vivo* expression of CD10, CD73, CD140b, CD146, GD2, and CD271 markers which characterise *ex vivo* expanded MSCs. This study (Rasini et al, 2013) found that GD2 was expressed exclusively on MSCs in healthy marrow, and that CD73⁺, CD271⁺, and CD10⁺ cells could be isolated without CD45 expression and therefore may represent bona fide MSCs *in vivo*, although this may be a simplistic view. Further, Mindaye et al (2013) utilised a combination of liquid chromatography MALDI and ESI tandem mass spectrometry to examine the marker expression of MSCs isolated from 4 donors. This investigation revealed a range of previously unknown CD markers expressed on the surface of MSCs, including CD5L, CD30, CD35, CD101, CD135, CD136, CD158D, CD163D, CD206, CD243, CD247, CD318, CD351, and CD355, and resulted in the creation of an extensive list of CD markers identified in culture-expanded human bone marrow derived MSCs. Despite these recent advances in characterisation, these findings are yet to be substantiated and variation in expression profiles between studies remains to be established.

The findings regarding the expression of the CD34 antigen reflects this heterogeneity of MSCs and variation in methodology between investigations particularly well and is discussed in a recent publication by Lin and colleagues (2012). They explain that CD34 is an established negative marker for MSCs and an established haematopoietic marker, however the existence of CD34⁻ Haematopoietic Stem Cells (HSCs) is well documented. It is also highlighted that the Stro-1 isolation technique, developed by Simmons & Torok-Storb (1991), was generated using CD34⁺ bone marrow-derived cells as immunogens, and this same study found that over 95% of CFU-Fs were CD34⁺. However, a number of years later Pittinger et al (1999) reported the isolation of CD34⁻ MSCs based upon culture expanded cells rather than those derived from the bone marrow. Lin et al (2012) explain that, as the culture of MSCs leads to the disappearance of the CD34 antigen and that most characterisation studies have compared culture expanded MSCs to HSCs in suspension, the flawed concept that MSCs lack CD34 expression whereas HSCs express this antigen has become accepted. The authors (Lin
et al, 2012) continue to propose that CD34⁺ progenitor cells may circulate in the body and after entering the bone marrow may become CD34⁻ stromal fibroblast-like cells or MSCs, explaining their CD34⁻ phenotype. However, the concept of a haematopoietic-mesenchymal stem cell remains controversial. Various other investigators have claimed to have isolated MSCs directly from the bone marrow and discovered their defining phenotypes, including CD271⁺ (Jones & McGonagle, 2008) and CD73⁺ CD45⁻ (Rasini et al, 2013), however the in vivo counterpart of these cells remains undetermined and is discussed later in section 2.2.4.

The antigens expressed on the MSC membrane also form their immune phenotype, and these cells have been found to express HLA I, whilst lacking HLA II and co-stimulatory molecules including CD40, CD80 and CD86 (Chamberlain et al, 2007). They have also been shown to express a variety of integrins, integrin receptors, including CCR1, CCR7, CCR9, CXCR4, CXCR5, and CXCR6, growth factor receptors, including bFGFR, PDGFR, EGFR, TGFβIR/IIR, and cell adhesion molecules, including VCAM-1, ICAM1/2, ALCAM-1, L-selectin, CD105, and CD44 (reviewed by Docheva et al, 2007).

In addition to the lack of clarity regarding the surface phenotype of MSCs, issues also surround the ISCT parameter of the plastic adherence of MSCs. Although the majority of cells isolated from tissue samples are not adherent to culture plastic, this property is not exclusive to MSCs, and it has been indicated that a number of other cell types, including pre-B-cell progenitors, and granulocytic and monocytic precursors, may also possess this capacity (Docheva et al, 2007; Prockop, 2009; Alt et al, 2011). This lack of plastic adherence exclusivity further disrupts attempts to develop an adequate isolation methodology for this cell type.

The final ISCT parameter refers to the trilineage differentiation potential of MSCs, and despite their well-documented in vitro adipogenic, chondrogenic and osteogenic differentiation capacity, this capability is yet to be definitively proven in vivo (Haynesworth et al, 1992; Pittinger et al, 1999; Mackay et al, 1998). Further studies have examined the pluripotency of MSCs and have successfully induced the differentiation of these cells into those of non-mesenchymal lineages, including cells of the neuroectoderm, visceral mesoderm and endoderm (Woodbury et al, 2000; Jiang et al, 2002; Kopen et al, 1999). However, it has been highlighted that the daughter cells generated after transdifferentiation share only some of the morphological, phenotypic and functional characteristics of cells of the specific tissue.
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and therefore could only be considered to be similar to those of the relevant origin as oppose to belonging to it (Fernandez-Vallone et al, 2013). Further, the innate ability of MSCs to transdifferentiate into cells outside of the mesenchymal lineage has not been proven, and may in fact result from chemically induced reprogramming of the cells in culture (Bianco et al, 2013). This multipotentiality may also depend upon a number of intrinsic factors, including genetics, and extrinsic factors, including the local tissue microenvironment, various growth factors, and certain nutrients (Fernandez-Vallone et al, 2013).

As mentioned previously in this section, there has been great debate regarding the true stemness of MSCs and whether they can truly self-renew. Parker and colleagues (2004) discuss the issue in detail and outline that multipotential cells should not be known as stem cells, as they do not have an unlimited self-renewal capacity, and should therefore be known as progenitors. The authors continue to explain that MSCs have a limited replicative capacity and age, and lack the telomerase enzyme that maintains telomere length after proliferation. This proliferative limitation is known as ‘Replicative Senescence’ or the ‘Hayflick Limit’, determines the number of divisions a cell can undertake, and is discussed in greater detail in section 2.2.5. As stem cells have an almost infinite proliferative capacity, this would suggest that MSCs are in fact progenitor cells of the mesenchymal lineage.

However, evidence supporting the self-renewal capacity of MSCs does exist (Sacchetti et al, 2007; Dennis et al, 1999; Bruder et al, 1997; Colter et al, 2000), as well as evidence for the multipotentiality of these cells, as discussed previously. Nonetheless, due to the heterogeneity of MSC populations, the limited evidence (Smith et al, 2004; Muraglia, Cancedda & Quarto, 2000; Sarugaser et al, 2009) demonstrating the self-renewal of definitive single cell-derived clonal populations from a mesenchymal source, and, as previously highlighted, the potentially flawed findings regarding the transdifferentiation of these cells, it is difficult to justify the ‘stem cell’ nomenclature attributed to MSCs.

It has also been proposed that not all cells within an MSC population will have self-renewal and transdifferentiation capacity, and that populations of cultured MSCs may not represent a stem cell culture, but rather a culture of cells in which a subset may represent stem cells (Docheva et al, 2007; Tolar et al, 2010). It has been suggested that this small subset of stem cell-like MSCs may exist as MAPCs, which represent around 1% of this population and yield continuously growing cultures (Baddoo et al, 2003). This cell population is reported to
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present a CD44⁺ Leukaemia Inhibitory Factor receptor (LIFR)⁺ Oct-4⁺ SSEA-1⁻ c-kit⁻ phenotype, to be unobtainable directly from bone marrow, and to often harbour abnormal karyotypes which may contribute to their plasticity (Baddoo et al, 2003; Phinney & Prockop, 2007). However, these MAPCs may not be the only population which are similar to that of MSCs and that may cause confusion during the isolation and characterisation of these cells.

An investigation by Alt and colleagues (2011) discovered that human skin-derived fibroblasts (hSDFs) had a similar spindle-like morphology, expressed similar MSC and fibroblastic antigens, displayed a similar gene expression profile, and exhibited similar adherence to culture plastics to that of human adipose-derived MSCs. The authors (Alt et al, 2011) also identified that these hSDFs displayed trilineage differentiation and colony forming capacity, albeit to a lesser extent than that of the adipose-derived MSCs. The findings of this publication suggest that the expression of a number of MSC markers, including CD44, CD90, and CD105, as well as the capability to adhere to tissue culture plastic may not represent unique characteristics of MSCs.

Although the supporting evidence for the self-renewal capacity and multipotentiality of MSCs may be limited, a number of in vitro (Riekstina et al, 2009; Gang et al, 2007; Alt et al, 2011; Beltrami et al, 2007; Greco, Liu & Rameshwar, 2007) and in vivo (Rasini et al, 2013) studies have identified the expression of embryonic markers which contribute to the stemness of embryonic stem cells (ESCs), including Oct-4, Nanog and SSEA-4, by MSCs. Therefore, despite the failure to adequately demonstrate the self-renewal and multipotentiality of MSCs, and the existence of conflicting evidence regarding embryonic marker expression (Mueller et al, 2009; Pierantozzi et al, 2010; Jeschke et al, 2011), these findings may suggest that MSCs possess some stem cell-like properties, and Riekstina and colleagues (2009) proposed that MSCs may represent pluripotent cells which have been deposited in tissue throughout the body during development, although little evidence is given to support this hypothesis.

From the literature described, it is clear that adequate understanding may be lacking in the three widely utilised in vitro MSC characterisation parameters, which were proposed in the ISCT definition paper, as well as many of the in vivo characteristics of these cells, and therefore more definitive research may be required to define these cells both in culture and in their natural physiological location. However, it may be that this definition is too simplistic and that a greater number of criteria are required to successfully identify the MSC population. Later in this review (Section 2.6.1), a new paradigm for the characterisation of these cells,
which focuses on the determination and quantification of functionality rather than upon phenotypic identification alone, is presented. If the functionality of MSCs is to be considered in their characterisation, it is important to understand the mechanisms through which these cells act in vivo and thus the available literature relating to this will now be reviewed.

2.2.3 Mode of Action

It was first discovered by Dexter et al (1977), demonstrated further by Majumdar et al (1998), and is now well established, that MSCs play a supporting role in haematopoiesis in the bone marrow by creating an appropriate microenvironment through the release of cytokines, chemokines, growth factors, metalloproteinases, MMP inhibitors and extracellular matrix (ECM) components (reviewed by Fernandez-Vallone et al, 2013). However, over the past 20 years, the dominant theory regarding MSC function in vivo has been that of direct cell replacement and tissue specific differentiation, as first proposed by Caplan (1991).

This concept has been supported by a number of studies in which MSC transplantation led to cell engraftment at the site of injury. However, although in vitro experiments have demonstrated multipotency, in vivo evidence of differentiation is limited to animal studies in which the therapeutic effects of MSCs upon cardiac tissue is examined. These studies identified MSC transdifferentiation into cardiomyocytes and endothelial cells in mice (Jiang et al, 2006) and bovine (Nakamura et al, 2007) models, albeit sporadic and milieu-dependent differentiation (Al-Khaldi et al, 2003). It has also been recognised that only a very small number, around 0.07%, of these transplanted cells actually differentiate into cardiomyocytes (Martin-Rendon et al, 2008). There may also be a possibility that partial cellular reprogramming of MSCs, leading to the acquisition of some of the relevant characteristics of the desired lineage, may contribute to the tissue reparative therapeutic effect of these cells (Rose et al, 2008). However, as highlighted by Caplan & Dennis (2006), the therapeutic effects of MSCs in cardiac tissue are likely to be caused by the interaction of a number of alternative factors, which could include neovascularisation, inhibition of scarring, decreased cardiomyocyte apoptosis, and increased nerve sprouting via the differentiation of MSCs into cells which overexpress neurotrophic or other growth factors (Pak et al, 2003), as well as direct differentiation into cardiomyocytes. Conflicting evidence to this differentiation concept in cardiac tissue does exist, and suggests that these beneficial effects of MSCs may occur too rapidly (<72 hours) to be associated with cellular regeneration (Gnecchi et al, 2008).
However if this direct cell replacement does in fact occur in cardiac tissue repair, it is likely that this would be considered to be the minor mechanism of action of these cells.

As mentioned previously in section 2.2.2, the trilineage potential, and to a certain extent the multilineage potential, of the MSC population has been shown in culture however is yet to be demonstrated adequately in vivo, and in the search for the true utility of these cells in situ, recent focus has shifted away from this concept. This shift was initiated after a number of reports established that MSC therapy had a positive influence upon tissue repair despite low or transient levels of cell engraftment in the tissue (reviewed by Phinney & Prockop, 2007). It had previously been demonstrated, in the mid-1990’s, that cultured MSCs have the capacity to secrete a number of cytokines into the local microenvironment (Haynesworth et al, 1996). However, this paracrine function was initially attributed to the direction and control of MSC differentiation by the authors.

However, by the mid-2000’s it was determined that, in combination with MSC differentiation, these cytokines, along with a number of growth factors, may also aid tissue repair and suppress immune reaction (Caplan & Dennis, 2006). The authors further defined the role of these bioactive molecules by describing two methods in which they can act. Caplan & Dennis (2006) proposed that these factors can exert effects directly in an autocrine manner, causing intracellular signalling, or indirectly in a paracrine manner, causing nearby cells to secrete functionally active agents. However, it was not until a review by Phinney & Prockop (2007) that it was recognised that the major therapeutic mode of action of MSCs is in fact the secretion of soluble factors as oppose to their transdifferentiation potential. In recent years, this paracrine in vivo mode of action of MSCs has been increasingly accepted and has become the dominant concept, with intense research in the area and the majority of MSC-based therapies in recent clinical trials have utilised these cells for their secretion of bioactive factors rather than their differentiation potential (Caplan & Correa, 2011). These paracrine properties of MSCs have more recently been categorised into trophic, immunomodulatory, anti-scarring, and chemoattractant effects, with the trophic effects being further divided into anti-apoptotic, supportive or mitotic, and angiogenic (da Silva Meirelles et al, 2009). These mechanisms are described further in section 2.2.3.3.

Therefore, as discussed previously, since the discovery of the Mesenchymal Stromal Cell type, there have been a number of prevailing theories aimed at defining the physiological
mode of action of these cells. This is aptly described by Prockop & Oh (2011), who outline three paradigms for the functional utility of these cells that have been proposed over the years:

- Initial theory- MSC populations make up a feeder layer which provides a niche for the culture of haematopoietic cells
- Second theory- MSC populations are composed of reparative cells which are able to engraft in injured tissue and differentiate in order to replace damaged cells.
- Most recent theory- MSCs transiently appear in injured tissue during which their crosstalk with injured cells, immune and inflammatory-mediating gene upregulation, stimulation of endogenous stem/progenitor cell proliferation, and transfer of vesicular components enable them to limit tissue destruction and enhance repair. This third paradigm is examined further in sections 2.2.3.1 - 2.2.3.3.

2.2.3.1 MSC Homing

One of the properties of mesenchymal stromal cells, which may be fundamental to their therapeutic effect in vivo, is their ability to home to sites of tissue injury. The mechanism behind this migration involves the secretion of a number of cytokines, chemokines and growth factors from the site of tissue damage, which act in a dose-dependent fashion, as shown in vitro, to attract MSCs towards the relevant area (da Silva Meirelles et al, 2009).

It has been well established that Stromal-cell-derived factor 1 (SDF-1) (Askari et al, 2003; Yamaguchi et al, 2003), Granulocyte colony-stimulating factor (G-CSF) (Kawada et al, 2004) and Tumour Necrosis Factor-alpha (TNF-α) (Ponte et al, 2007) play an important role in MSC migration towards injured tissue. More recently, a variety of further cytokines and growth factors have been associated with the migration of MSCs, including TNF-Related Apoptosis-Inducing Ligand (TRAIL), Receptor Activator of Nuclear factor Kappa-B Ligand (RANKL), Platelet-Derived Growth Factor (PDGF), Interleukin-17 (IL-17), Basic Fibroblast Growth Factor (bFGF), Interferon-gamma (IFN-γ), Insulin-like Growth Factor (IGF), Transforming Growth Factor-beta (TGF-β), Epidermal Growth Factor (EGF) and Erythropoietin (EPO) (reviewed by Fernandez-Vallone et al, 2013). The MSCs detect these stimuli through expression of the corresponding receptors, which include CXC Chemokine Receptor type 4 (CXCR4) (Zazag et al, 2005; Müller et al, 2001), CC Chemokine Receptor type 2 (CCR2) (Belama-Belada et al, 2008), TRAIL receptors (DR5 and DcR2), RANK, PDGF receptor (Type α and β), IL-17 receptor and EPO receptor (reviewed by Fernandez-
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Vallone et al, 2013). Ceradini et al (2004) describe the CXCR4 homing axis in greater detail, identifying Hypoxia-Inducible Factor-1 (HIF-1) as a mediator for the recruitment of this receptor, which may contribute to impaired homing ability and tissue repair with age due to a decreased in HIF-1 expression (Rivard et al, 2000).

This homing mechanism has been examined extensively in the cardiac tissue, and it has been identified that, upon injury, cardiac cells secrete proteases and express Monocyte chemoattractant protein-1 (MCP-1). These proteases interact with the surrounding collagen matrix and digest it into fragments which are chemotactic for MSCs (Schmidt et al, 2006; Ji et al, 2004). The expression of MCP-1 promotes MSC transmigration and homing as expressed CCR2 on the MSC membrane is the receptor for MCP-1 (Ji et al, 2004; Damas et al, 2000).

The transmigration and chemotaxis of MSCs into the damaged tissue site relies upon a number of integrins (β-1 and α-4), adhesion molecules (Vascular Cell Adhesion Molecule-1 (VCAM-1 and ELAM-1) and metalloproteinases (MMP-2), and upon both carbohydrate-lectin interactions and Intα4β1-dependent binding (Röster et al, 2006). The interaction of these components facilitates the adhesion and tethering of circulating MSCs to the vascular endothelium, the movement of these cells along this membrane, and finally the invasion of the endothelium by the MSCs. After the MSCs undergo leucocyte-like diapedesis through the capillary wall, their migration towards the site of tissue injury or inflammation can occur.

It has also been highlighted in a number of studies (Terashima et al, 2005; Wong & Fish, 2003; Belema-Bedada et al, 2008) that the homing of MSCs can be enhanced by a positive feedback loop which amplifies the signal that attracts MSCs towards the specific site. This feedback loop revolves around the SDF-1 axis, and, after initial homing initiation through SDF-1 secretion, Vascular Endothelial Growth Factor (VEGF) recruits more MSCs by inducing further SDF-1 secretion (Grunewald et al, 2006).

However, despite the substantial evidence demonstrating the capability of this cell type to migrate towards areas of injury, it has been proposed that the efficiency of this homing is very low (1-2%) (Caplan & Bruder, 2001; Ma, 2010), that these cells in fact only make a transient appearance at the site of injury, and a large proportion of infused MSCs may become trapped in the lung rather than migrate to the relevant site (Prockop & Oh, 2011). Yet, in spite of the brief appearance of a small number of MSCs, infusion of these cells can still
have a beneficial effect upon tissue damage, which is believed to be mediated by the upregulation of TNF-α stimulated gene/protein 6 (TSG-6) which reduces inflammation (Lee et al, 2009; Prockop & Oh, 2011). This may indicate that the primary mode of action of hMSCs in vivo is the paracrine secretion of trophic factors which promote regeneration through a systemic effect.

The ability of MSCs to migrate and target a specific site of injury could be advantageous in the treatment of simultaneous indications, allowing infused MSCs to respond to injury stimuli, travel to relevant tissues and exert their therapeutic effects. However, currently, little evidence exists to suggest that these cells have the capacity to migrate towards a number of injury sites and produce multiple beneficial outcomes, and therefore further investigation is required. However, it has been proposed that all tissues possess vascular ‘zip codes’ (Arap et al, 2002), which would allow MSCs to be aimed specifically at affected areas.

2.2.3.2 Immune Modulation

As previously described in section 2.2.3, it has been revealed that the mechanism behind the therapeutic effects of MSC-based treatments may not be via direct cell replacement and differentiation, and may in fact be through paracrine effects upon other cells. MSCs have the capacity to exert these effects upon cells of various tissues throughout the body, however it has been recently determined that one of the most significant components which contributes to the beneficial effects of these cells is their ability to modulate the immune system.

This immunomodulatory phenomenon of MSCs was first observed in the late 1990’s in a number of in vitro studies (Klyushnenkova, Mosca & McIntosh, 1998; McIntosh et al, 1999), however it was later described in detail by Le Blanc and colleagues (2003). In this study it was determined that MSCs suppress T cell proliferation, in a dose dependent fashion, in mixed lymphocyte cultures. The authors also observed that, when MSCs were present in low concentrations, they appeared to have the opposite effect and increased lymphocyte activation. This discrepancy in immune modulation between MSC populations has been investigated further by Waterman and colleagues (2010), and supported more recently by Gazdic and colleagues (2015), and it was discovered that MSCs can be polarised into two phenotypes due to their Toll-like receptor (TLR) signalling. The authors explain that the first population of MSCs (MSCs1) are mediated by TLR4 and release cytokines, including IL-6 and IL-8, which have a pro-inflammatory effect. However, in contrast, MSCs2 are mediated by TLR3 and
release molecules, including IL-10, Indoleamine 2,3 dioxygenase (IDO) and TSG-6, which have an immunosuppressive effect.

Since the discovery of MSCs’ effects upon the immune system, this mechanism has been examined further and a number of cytokines, chemokines, growth factors and enzymes, expressed by MSCs, have been found to be responsible for exerting this effect. IDO is one such key enzyme which, when upregulated by MSCs, leads to the accumulation of toxic metabolites resulting in the suppression of T-cell, B-cell and dendritic cell responses (Mellor & Munn, 2004; Romieu-Moure, Coutu & Galipeau, 2012). Another molecule found to be central to the immunosuppressive effects of MSCs is Nitric Oxide (NO) which, when expressed by MSCs in response to Interferon-Gamma (IFN-γ), induces T-cell suppression (Sato et al, 2007; Ren et al, 2008). A number of other expressed molecules, including Prostaglandin E2 (PGE-2), TGF-β1, LIF, Hepatocyte Growth Factor (HGF), Human Leucocyte Antigen-G5 (HLA-G5), Nitric Oxide Synthase (NOS), Heme-oxygenase (HO), IL-6, VEGF and TSG-6, have also been linked to the modulatory effects of MSCs over the immune system.

The expression of these factors secreted by MSCs is believed to be stimulated by the combination of a number of cytokines and direct cell-to-cell contact, although it has been proposed that the former is of greater significance (Le Blanc et al, 2003; da Silva Meirelles, Caplan & Nardi, 2008). The significant interaction between the two has been aptly demonstrated in a publication by Sheng et al (2008), in which the authors describe the mechanism in which the presence of IFN-γ promotes MSC immune suppression via the upregulation of the inhibitory surface molecule B7-H1 which is known to be involved in binding between immune cells.

The bioactive molecules, mentioned previously, affect many cells of the immune system via a vast array of mechanisms. It is well established, and has been reviewed in many publications (Le Blanc & Ringden, 2007; Tolar et al, 2010; Chen et al, 2011), that MSCs influence both innate and adaptive immunity through suppression of T-cell proliferation, particularly CD4+ and CD8+ T-cells (Di Nicola et al, 2002), dendritic cell maturation, B-cell maturation and proliferation, and Natural Killer cell (NK cell) proliferation and cytotoxicity. It has also been identified that MSCs stimulate the generation of regulatory CD4+CD25+ T-cells (Maccario et al, 2005), reduce the expression of chemokine receptors upon, and therefore migration of,
B-cells (Corcione et al, 2006), detrimentally affect neutrophil survival (Raffaghello et al, 2008), and inhibit differentiation and maturation of Antigen Presenting Cells (APCs) (Jiang et al, 2005). Finally, it has also been reported that MSCs can induce macrophage reprogramming and modify these cells from a pro-inflammatory M1 phenotype to an anti-inflammatory M2 phenotype (Németh et al, 2009).

In order to exert these effects upon cells of the immune system, MSCs secrete a variety of chemoattractant factors which target monocytes, eosinophils, neutrophils, basophils, memory and naïve T-cells, B-cells, NK cells, dendritic cells, and haematopoietic and endothelial progenitors. These molecules include MCP-1, MIP-1α, MIP-1β, RANTES, MCP-3, MIP-3α, eotaxin-3, fractalkine, ENA-78, i-TAC, GROα, SDF-1, IL-8, GROβ, and IP-10 (reviewed by da Silva Meirelles, Caplan & Nardi, 2008).

Additionally, two negative feedback loops have been found to modulate the influence of MSCs upon the immune system and pathway of inflammation (Prockop, 2013). Firstly, pro-inflammatory mediators secreted by ‘sensor’ cells of the immune system stimulate MSCs to increase their secretion of PGE-2 which drives the transition of resident macrophages from their M1 to M2 phenotype and secrete anti-inflammatory mediators, which inhibit the actions of ‘effector’ cells. The second negative feedback loop, also activated by mediators secreted by sensor cells, involves the increased expression of a number of genes, including TSG-6. This increased TSG-6 expression then acts to reduce the secretion of pro-inflammatory mediators, including TNF-α, by resident macrophages.

More recently, it has been suggested that native MSCs, located in blood vessels, generate a local curtain of bioactive molecules in order to inhibit the entrance of cells from the host’s immune system to the site of injury (Caplan & Sorrell, 2015). From the front of the cells, away from the area of damage, activated MSCs produce this curtain in order to inhibit an autoimmune reaction. Furthermore, from the back of these activated MSCs, molecules which stimulate regeneration of the injury site are released.

However, it would not be possible for MSCs to exercise their mode of action, upon both areas of tissue damage and the immune system, if they were recognised and a host immune response was induced. It has become accepted, and is acknowledged in a number of papers (LeBlanc et al, 2003; reviewed by Pourrajab et al, 2011), that transplanted MSCs escape recognition by alloreactive T-cells and are unable to stimulate allogeneic T-cell proliferation.
It is believed that the lack of ABO blood group antigen expression (Schäfer et al, 2011), and the lack of costimulatory antigen expression, including CD86 (Krampera et al, 2003), are responsible for the immune privileged nature of these cells. These costimulatory antigens are recognised as being essential for the signalling of T-cell activation.

The expression of Human Leucocyte Antigens (HLA) is also important in the stimulation of host immune responses, however there is on-going debate regarding the expression of these by MSCs, with evidence of both intermediate (Le Blanc et al, 2003) and low (Potian et al, 2003) HLA class 1 expression reported. In contrast, it is generally accepted that MSCs express low levels of HLA class 2 antigens and Fas Ligand (reviewed by Fernandez-Vallone, 2013; Le Blanc et al, 2003).

Therefore, due to the immunomodulatory and immune privileged nature of transplanted MSCs, these cells represent a potential allogeneic therapy for a number of disorders, including those of the immune system (Griffin, Ritter & Mahon, 2010). This would attenuate the issues with immune rejection and obtaining adequate cell numbers from a single donor associated with autologous therapies. This potential has been acknowledged by those companies responsible for the development of MSC-based therapies, including Osiris Therapeutics Inc and Athersys Inc, who have targeted indications such as Graft Versus Host Disease and Crohn’s disease, which are related to immunity and inflammation using allogeneic MSCs.

However, the variation in immunomodulatory potential of MSCs between donors may affect the potency of these allogeneic therapies. Galipeau (2013) explains that the magnitude of MSC IDO upregulation, in response to IFN-γ, correlates with immunosuppressive function of these cells and may differ between donors. Therefore, the potency of an MSC-based therapy may dependent upon the whether the donor is a high or low IDO inducer. However, despite this potential potency disparity, which requires further examination, the use of these cells still represents a prospective therapy for those patients with a number of indications simultaneously, including those afflicted with inflammatory or immune disorders. It has been identified that the conditioned medium of MSCs can contribute to healing and repair gut injury models through the pleiotropic action of secreted trophic factors, which contribute to the wound healing, anti-inflammatory, proliferative, and tissue remodelling processes (Watanabe et al, 2014). Although Caplan & Correa (2011) also provide favourable evidence
for a combined mode of action by suggesting that MSCs first inhibit immune cells, which survey tissue damage, and then proceed to secrete bioactive molecules, in order to establish a regenerative microenvironment, the potential for simultaneous immunomodulatory and trophic effects using MSCs represents an area which calls for further investigation.

Based upon the available literature and the consensus of the field, the ISCT have proposed a standardised approach for the assessment of the immunoregulatory properties of human MSCs comprising of seven guidelines; including the utilisation of IFN-γ ± TNF-α as in vitro priming agents, the examination of IDO response as part of an in vitro priming assay, and the clinical analysis of the immune response against MSC transplantation (Krampera et al, 2013).

2.2.3.3 Trophic Effects

As discussed previously in section 2.2.3, it has become accepted that the primary therapeutic mode of action of MSCs in vivo involves the secretion of soluble factors which act to aid tissue repair and suppress circulating immune cells (Phinney & Prockop, 2007; Caplan & Dennis, 2006). The paracrine secretions of MSCs can affect various host cell types through a number of mechanisms which will now be discussed in detail.

The first method by which MSCs induce tissue repair at sites of injury is through the stimulation of new blood vessel formation and the organisation of vascular networks (Au et al, 2008). This angiogenic effect has been found to be modulated by the secretion of pro-angiogenic factors by MSCs, including bFGF, VEGF, Placental Growth Factor (PIGF), MCP-1, IL-6, and ECM molecules (Kinnaird et al, 2004; Hung et al, 2007; Sorrell, Baber & Caplan, 2009). Phinney & Prockop (2007) also indicated that these factors interact with a number of proteins, also expressed by MSCs and which modulate endothelial cell migration, to induce capillary proliferation, expansion of the sinusoidal space, and vessel remodelling. Finally, it has also been discovered that MSCs may play a role in new blood vessel stabilisation by entering the perivascular location and becoming ‘pericytes’ (Sorrell, Baber & Caplan, 2009; Traktuev et al, 2008), which is discussed in more detail later in section 2.2.4.

It has also become well established that, not only do MSCs secrete pro-angiogenic factors, but they also produce large amounts of anti-apoptotic factors which act to limit the field of damage at the site of injury, especially if it results in ischaemia (Nagaya et al, 2004; Hung et al, 2007). A number of molecules, including VEGF, HGF, IGF-1, Stanniocalcin-1 (STC-1), TGF-β, bFGF, Granulocyte Macrophage Colony-Stimulating Factor (GM-CSF) (Tögel et al,
2007; Block et al, 2009; Rehman et al, 2004), have been associated with this inhibition of apoptosis and protection of vulnerable host cells.

The activation of endogenous tissue specific stem or progenitor cells has also been discovered as a mechanism through which bioactive molecules secreted by MSCs induce repair. It has been determined that MSCs express mitogens, including Stem Cell Factor (SCF), LIF, IL-6, Macrophage Colony Stimulating Factor (M-CSF), SDF-1, and Angiopoietin-1 (Sugiyama et al, 2006; Ohab et al, 2006), which stimulate the proliferation and differentiation of these progenitor cells leading to the repair of damaged tissue.

The final, widely acknowledged paracrine mechanism by which MSCs induce a therapeutic effect is through the inhibition of scar tissue formation. It has been determined that this cell population secrete anti-fibrotic factors including bFGF, HGF, and Adrenomedullin (Suga et al, 2008; Li et al, 2009). It has also been observed that the degradation of collagen is mediated by MMPs, that these MMPs are secreted by MSCs, and that these cells therefore act to reduce fibrosis and scar formation (Jugdutt, 2003).

The means through which MSCs are attracted and home to sites of injury have previously been discussed in section 2.2.3.1, however one particular in vivo environment which activates the paracrine mechanisms of these cells is that of ischaemic injury. MSCs home to hypoxic tissues, secrete cytokines, promote angiogenesis and tissue repair, enhance the proliferation of endogenous progenitor cells, and protect new cells in the ischaemic environment (reviewed by Rastegar et al, 2011). It has been identified in a study by Liu and colleagues (2008) that hypoxia causes MSCs to activate the proliferation and migration of microvascular endothelial cells, and to overexpress HIF-1 which increases VEGF production. This acts to repair or rebuild hypoxic vessels. The examination of hypoxic cardiac tissue has also revealed that oxidative stress stimulates MSCs to secrete cytokines and growth factors, including SDGF-1, Secreted Frizzled-Related Protein (SFRP-2), IL-10, TSG-6, and VEGF, which increase the survival and repair of cardiomyocytes (Li et al, 2009).

Another proposed mechanism for restoring the function of ischaemic cells involves the transfer of vesicular components, which include mitochondria and microRNAs, from MSCs to cells in the hypoxic environment (Spees et al, 2006). Extracellular vesicles have also been found to carry over a vast number of proteins, nucleic acids and lipids (Kim et al, 2011; Lai et al, 2015) It has been identified that multiple classes of extracellular vesicle are secreted by
MSCs, including microvesicles, microparticles and exosomes (Lai et al, 2015). The capacity of these MSC secreted vesicles to communicate with, and transfer their components to, multiple cell types within the microenvironment at the site of injury has led to their utilisation as cell-free therapies, and is under examination for the treatment of a number of indications, including Myocardial Infarction and Cardiovascular Disease (Baglio, Pegtel & Baldini, 2012; Lai et al, 2010; Lai et al, 2011).

The potential utility of MSCs in the treatment of cancer has also been explored, and substantial evidence exists to support their potential anti-neoplastic effect. It has been discovered that high concentrations of paracrine growth factors, including IL-8, TGF-β1, and VEGF, recruit MSCs towards sites of tumour growth (Birnbaum et al, 2007). It has also been found that MSCs slow tumour proliferation by secreting DKK-1 (Qiao et al, 2008), an alleged tumour suppressor, and by increasing apoptosis and cell cycle arrest of cancer cells (Lu et al, 2008). It also appears that MSCs may promote the immune response against cancerous cells through endocytosis of tumour associated antigens which enhances the CD8 mediated anti-tumour response (Ma et al, 2008). However, conflicting evidence has been discovered which suggests that MSCs may promote survival and metastasis of cancer cells, stimulate angiogenesis at tumour sites, and enhance tumour growth (Karnoub et al, 2007). Houghton et al (2004) have also determined that MSCs may act as precursors to cancer cells in a gastric cancer model. Therefore, the cancer promoting potential of this cell population must be carefully examined, and further clinical investigation is required.

Despite the evident potential for these cells in therapy for a variety of indications, their recently identified paracrine mode of action represents an evolving concept with a lack of substantial clinical evidence, and therefore requires further clarification. Also, the clinical application of MSC-based therapies still faces a number of challenges including, as highlighted by Ma (2010), low cell survival after transplantation, limited homing capacity, low grafting efficiency, lack of defining markers, cell population heterogeneity, low occurrence in bone marrow, and the detrimental effects of expansion and cryopreservation. Variation may also be present between donors, with Caplan & Bruder (2001) recognising that the level of bioactive molecule secretion may vary between hosts possibly as a result of genotype. It has also been suggested that further complexity may exist with regards to the paracrine function of MSCs, in that many of their secreted regulatory molecules are expressed specifically by subpopulations of these cells which explains their broad therapeutic
efficacy (Phinney & Prockop, 2007). Therefore, greater precision is required regarding the processing, characterisation and *in vivo* mechanisms of this cell type.

However, regardless of the difficulties associated with the development and clinical utility of MSC-based products, these regenerative therapies offer substantial potential and may represent a step change in the treatment of not only a number of diseases, but also their associated comorbidities. Due to the bioactive molecule secreting function, pharmaceutical-like mode of action and targeting capability of MSCs *in vivo*, the number of cells required to induce tissue repair at multiple sites of injury may be lower than if the primary mode of action was that of tissue specific differentiation. Therefore, the secretion of trophic factors, which act to aid tissue repair, reduce inflammation, and suppress circulating immune cells, could be more applicable to multiple simultaneous therapy. Also, the hypoimmunogenic nature of these cells may circumvent issues regarding the collection of adequate cell numbers from a single donor, associated with autologous therapy, and the possibility of immune rejection. Therefore, their clinical utility would not be restricted to a single host and their use as an allogeneic therapy may offer great promise.

The significance this newly discovered mode of action of MSCs could have upon healthcare has not gone unnoticed, and it has been proposed that, as the mechanisms through which these cells are therapeutic no longer centres around the potentially dangerous and uncontrollable differentiation of these cells, the safety of MSC-based products may be greater and therefore warrants more rapid adoption into clinical practice (Caplan & Correa, 2011). However, it is likely that greater clinical efficacy and safety data will be required before this potential is realised. Particularly in the case of the treatment of simultaneous indications, where the capacity to target multiple diseases simultaneously, simultaneously modulate immunity and induce tissue repair, or even to generate multiple paracrine effects is yet to be thoroughly investigated. The cytokine secreting function and pharmaceutical-like mode of action of hMSCs when used therapeutically *in vivo* could, in theory, be more feasible for the treatment of multimorbidity than the direct cell replacement mechanism, as the number of cells required to differentiate into each relevant tissue specific cell type in order to induce repair would be high. Although Caplan & Correa (2011) have indicated that transplanted MSCs may first act to suppress immune reactions and then proceed to exert trophic effects upon host cells, and a single study has revealed that transplanted MSCs may have the capacity to simultaneously target diabetes and cardiovascular complications in rats (Abdel
Aziz et al, 2008), further evidence is required if MSC-based therapies are to realise their potential in the treatment of multimorbidity.

As discussed previously in section 2.2.2, although the surface markers and morphology of MSCs have been extensively examined, the determination of the identity and in vivo function of this cell population has been difficult. Variations between donors, sources and culture conditions all contribute to the already heterogeneous nature of these cells and the difficulties associated with characterisation. Recently, Galipeau (2013) has argued that surface markers have little utility in determining cellular functions and subsequent clinical efficacy. Therefore, in order to ensure these products have the desired potency in vivo, the cells must be characterised in vitro in a manner that reflects their mode of action and consequent clinical benefit. Consequently, the development of functional assays which identify and quantify the paracrine function of populations of MSCs is required. Carmen et al (2012) propose that the mimicking of in vivo paracrine responses of these cells could be used to measure potency in vitro. The authors (Carmen et al, 2012) continue to describe the need for indication specific potency assays for cell therapies which target multiple clinical indications. A number of groups have developed assays to predict the potency of MSCs for a variety of the putative modes of action of these cells, including immunosuppression, bone formation, angiogenesis and trilineage differentiation (Bloom et al, 2015; Janicki et al, 2011; Lehman et al, 2012; Russell et al, 2011). However, the development of a consistent potency assay to predict MSC function in humans has yet to be achieved.

It is clear that the need for improved characterisation and potency analyses to identify and quantify the paracrine function of MSC-based therapies which target single or multiple indications is beginning to be recognised, however it is evident that further research is necessary.

2.2.4 Source Specificity

Historically, it was believed that MSCs were present in virtually all post-natal tissues throughout the body, and cells resembling MSCs have been isolated from bone marrow (Friedenstein, Piatetzky-Shapiro & Pertakova, 1966), periosteum (O’Driscoll et al, 2001), trabecular bone (Nöth et al, 2002), adipose tissue (De Ugarte et al, 2003), synovium (De Bari et al, 2003), skeletal muscle (Jankowski, Deasy & Huard, 2002), deciduous teeth (Miura et al, 2003), skin (Shih et al, 2005) and lungs (Noort et al, 2002). MSC-like cells have also been
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derived from Umbilical Cord Blood (UCB) (Erices et al, 2000), Umbilical Cord Wharton’s Jelly (Wang et al, 2004), Amniotic fluid (Nadri & Soleimani, 2007), Placental tissue (Scherjon et al, 2004) as well as a variety of foetal tissues (Campagnoli et al, 2001). However, it was recently highlighted that the presence of homogenous MSCs in virtually all post-natal organs has never been conclusively proven (Bianco et al, 2013).

It was also believed that little difference existed between MSCs from different tissue sources. A number of studies in the early 2000’s examined the presence of variation in morphology, phenotype and differentiation potential between cultured MSC populations isolated from various tissues. De Ugarte and colleagues (2003) reported little difference between cells from bone marrow and those from adipose tissue in terms of yield, growth kinetics, cell senescence, multi-lineage differentiation capacity and gene transduction. A study by Kern et al (2006) also examined the difference between MSCs from adipose and bone marrow, as well as UCB, and found no obvious difference in morphology or immune phenotype. However, the authors (Kern et al, 2006) did find subtle differences in isolation success, culture viability, proliferation capacity, differentiation potential and marker expression. They determined that the isolation of bone marrow-derived and UCB-derived MSCs was more successful than that of adipose-derived MSCs, that UCB-derived MSCs could be cultured longer than their bone marrow and adipose-derived counterparts, and that UCB-derived MSCs had a higher proliferation capacity. Also, Kern and colleagues discovered that the adipogenic potential of bone marrow and adipose-derived MSCs was greater than that of the UCB-derived cells, although little difference was observed between the osteogenic and chondrogenic capacity of cells from each tissue source. Lastly, they determined that CD90, CD105 and CD106 expression differed between sources, and highlighted that these antigens are associated with haematopoiesis and cell migration. More recently, an extensive list of expressed markers that vary between tissue sources has been produced, leading to the proposition that significant disparity in expression profiles of MSCs from different sources does exist and that panels of positive markers from each tissue source should be established in order to aid characterisation (Rojewski, Weber & Schresenmeier, 2007).

Musina, Bekchananova & Sukikh (2005) examined a wider variety of tissue sources, including skin, placental and thymus, and found that skin and placenta provided cells similar to that of other established MSC sources, despite small differences in CD44, CD13 and CD10 expression, whereas cells derived from the thymus differed morphologically, in marker
expression, and exhibited spontaneous neural differentiation. Finally, a Japanese research group (Sakaguchi et al, 2005) also studied synovial and periosteal tissue as possible sources of MSCs, concluding that cells from these sources exhibited greater osteogenesis and chondrogenesis than adipose-derived cells, similar to that of bone marrow-derived cells, and displayed similar adipogenesis to both bone marrow and adipose-derived MSCs.

However, contrary to this popular belief that tissue source minimally affects MSC characteristics, more recent thinking suggests that significant variation in characteristics, long term growth kinetics, expression profile, and function may exist between MSCs from different sources (Phinney & Prockop, 2007; Wagner et al, 2008; Rastegar et al, 2011; Keating, 2012). Nesselmann et al (2008) have also proposed that MSCs from different tissues may be primed towards specific lineages, and thus different organs have different subpopulations of these cells each with varying characteristics, behaviour, differentiation potential and function. Caplan (2008) has also highlighted that, in culture, MSCs from different tissues exhibit distinct sensitivities to inductive bioactive molecules reflecting their tissue origin. This has been demonstrated by Estes and colleagues (2006) who found that, in order to induce chondrogenesis, bone marrow-derived MSCs must be supplemented with TGF-β, whereas adipose derived-MSCs must be supplemented with additional BMP-6.

These issues regarding the tissue source of MSCs may also contribute and exacerbate the difficulties surrounding the characterisation and in vivo identification of this cell type. Bianco and colleagues (2008) have proposed that cell characteristics may be dictated by the local tissue microenvironment causing the lack of a defining antigenic marker for MSCs which represents one of the major difficulties associated with MSC characterisation. Also, the identification of the in vivo representatives of MSCs may be affected by the examination of these cells in culture, as markers, which are often utilised in the search for MSCs in situ, may be determined by culture conditions (da Silva Meirelles et al, 2009). It has also been highlighted that the ‘distinct’ subpopulations of these cells, including MAPCs, MDSCs, ADSCs, have only been derived from their tissue of origin and examined retrospectively in culture, which provides little indication as to their native identity, frequency, and anatomic location (Chen et al, 2009; Corselli et al, 2010).

However, recently a hypothesis has been proposed which addresses the issue of MSC in vivo identity and may explain the ubiquitous presence of these cells. This hypothesis, first
presented and examined by Crisan et al (2008), suggests that MSCs exist as perivascular cells in vivo, known as pericytes, and surround blood vessels. They have also been referred to as ‘Rouget’, ‘Mural’ or ‘Periendothelial’ cells, and have been labelled differently depending upon the tissue from which they are isolated (e.g. ‘Perisinusoidal’ cells in sinusoids, ‘Adventitial Reticular’ cells (ARCs) in bone marrow, ‘Ito’ or ‘Stellate’ cells in the liver, or ‘Mesangial’ cells in kidney glomeruli) (Chen et al, 2009; da Silva Meirelles et al, 2009). The location of these cells upon blood vessels, and the presence of vascular structures within almost every tissue within the body may explain the omnipresence of multilineage progenitors in multiple organs (Crisan et al, 2008).

It has been described that these cells have multiple physiological functions including the regulation of blood flow, the stabilisation of vessels, and the maintenance of tissue homeostasis (Hirschi & D’Amore, 1996; da Silva Meirelles, Chagastelles & Nardi, 2006). It has also been proposed that this cell population may have local functions in the tissue microenvironment (Caplan & Correa, 2011) and may include progenitor cells capable of differentiation into Mesenchymal lineages (Farrington-Rock et al, 2004). However, with the recent association of pericytes with MSCs, it has also been indicated that these cells may induce paracrine effects through the secretion of bioactive molecules in a similar manner to that of the previously discussed mode of action for MSCs. A number of publications have reviewed the pericyte activation mechanism (Caplan & Correa, 2011; Chen et al, 2009; Corselli et al, 2010; da Silva Meirelles, Caplan & Nardi, 2008; da Silva Meirelles et al, 2009) explaining that, after tissue damage, pericytes are first liberated from their perivascular niche and proliferate. Contrary to the proposed MSC paracrine mode of action, these cells may initially support immune cells in their response to injury. However, as the local microenvironment changes, the expression profile of the pericytes also changes and they give rise to MSCs, a transition believed to be coordinated by cues from the perivascular niche. These MSCs can then utilise their capacity to migrate and secrete bioactive molecules in order to exert trophic effects upon host cells and modulate the immune reaction to injury, as previously discussed in section 2.2.3.2.

The breakthrough publication by Crisan and colleagues (2008) uniquely identified these pericytes and their expression of a CD146+NG2+PDGF-β+ALP+CD34- vWF-CD144- phenotype. It was also discovered that the perivascular cells surrounding venules and arterioles expressed α-smooth muscle actin (α-SMA) whereas those surrounding capillaries
often did not. As $\alpha$-SMA has been associated with the contractility of vessels, this may explain their function in controlling blood flow. Further, this study compared the antigenic markers expressed on the surface of pericytes to those of MSCs, and it was determined that pericytes from various sources expressed all markers typical of MSCs, including CD10, CD13, CD44, CD73, CD90, CD105, CD108, CD109, CD140b, CD164, CD166, CD138, CD340, CD349, SSEA-4, and HLA-CL1, and lacked similar markers to MSCs, including CD56, CD106, CD133, CD324, CD326, CD344, and HLA-DR. This study also discovered that pericytes exhibited similar in vitro trilineage potential to that of MSCs.

These findings have been supported by a number of studies (Corselli et al, 2010; Chen et al, 2009) and have been further reinforced by the similarity in functional properties between these two cell types (Covas et al, 2008), the recognition of the MSC identifier antibody STRO-1 by pericytes (Chen et al, 2009), and the correlation between MSC frequency and vascular density (da Silva Meirelles et al, 2009). These similarities between the two cell types have led to the hypothesis that all MSCs are pericytes (Caplan, 2008). However, Caplan (2008) acknowledges that not all pericytes may not necessarily be MSCs, and the discovery of haematopoietic and neural stem cells within the perivascular niche confirms this (Hirschi & D’Amore, 1996). Closer examination of the data reveals that differences between pericytes and MSCs may exist including marked differences in expression levels of lineage restricted mRNAs (Covas et al, 2008), a lack of contractility in MSCs (da Silva Meirelles, Chagastelles & Nardi, 2006), and a lack of evidence of self-renewal in pericytes (da Silva Meirelles et al, 2009).

Finally, variation may also exist between pericytes and it has been recognised that not all blood vessels are identical, and therefore not all pericytes are identical and there may be a range of phenotypes throughout the body (Diaz-Flores et al, 2009; da Silva Meirelles et al, 2008). Also, pericytes from different sources have been found to show some differences in differentiation potential (da Silva Meirelles, Chagastelles & Nardi, 2006; da Silva Meirelles et al, 2008) and gene expression profiles (Covas et al, 2008). This variation between pericyte sources again raises questions regarding source specificity and whether an optimal source of MSCs or pericytes exists. This could substantially affect isolation, characterisation, and functionality of MSC-based products and suggests that more conclusive research into the in vivo identity of these cells is still required.
2.2.5 Effects of Expansion

It is well understood that the culture and expansion of donor cells, including MSCs, is often required in order to obtain the relevant cell numbers required for an autologous or allogeneic cellular therapy. However, it has been indicated that a number of culture parameters, including nutritional level, cell confluence, oxygen level, number of passages, and plastic surface quality, are likely to influence MSC properties, behaviour and therapeutic potential (Barrilleaux et al, 2006; Prockop, 2009). Therefore, the extended culture of cells in vitro may affect certain characteristics and functions of these cells, which may be detrimental to the quality and safety of the cell product being developed.

In the past, due to the uniform appearance of fibroblast-like cells in confluent cultures and the retained homogenous appearance of these cells after multiple passages as high density cultures, it was believed that all cultures of MSCs were homogenous. However, it was eventually established that the nature of these cells may in fact change over time and can be classified into a number of categories (Mets & Verdonk, 1981; Sekiya et al, 2002). Mets & Verdonk (1981) suitably describe this transition and explain that early (Type 1) MSCs adhere and proliferate rapidly in culture, reflecting their release from their in vivo microenvironments which limit them. This rapid growth phase is believed to be driven by the release of the Wnt signalling pathway inhibitor DKK-1. During this stage, MSCs express surface proteins, including α6-integrin and podocalyxin-like protein (PODXL), which are associated with anti-adhesion and cell motility. However, as colonies of these cells expand and enter later (Type 2) stages, DKK-1, PODXL and related protein expression decreases, and the colonies enter a near stationary phase as they move towards senescence. It has been proposed that these dramatic changes to MSCs during expansion can be explained by the ability of these cells to generate their own in vitro microenvironment or niches when plated at a low density to generate single-cell derived colonies, with subpopulations of cells acting as nurse cells for other subpopulations (Prockop, 2009).

It has also been observed that, as colonies form in culture, MSCs from inner regions are often distinct from outer regions and that different microenvironments or niches may exist between colonies of cells (Sekiya et al, 2002; Ylöstalo, Bazhanov & Prockop, 2008). Inner regions have been found to proliferate more slowly, express proteins associated with differentiation, although this differentiation is reversible at this stage, and exhibit differences in transcriptomes compared to outer regions.
In order to culture cells \textit{ex vivo}, the medium, in which these cells grow and proliferate, requires supplementation with serum which is often xenogeneic in nature. Fetal Bovine serum (FBS) or Fetal Calf serum (FCS) are frequently used in cell culture, however the effect this supplementation may have upon the donor cells can at times be neglected. A number of studies have compared the effect of the use of xenogeneic serum to the use of human derived sources, including Human Platelet Lysate (HPL) and Autologous serum, upon the features of MSCs. Azouna et al (2012) discovered that HPL could replace the use of FBS in culture whilst maintaining the MSC phenotype, increasing the growth rate of cell populations, producing larger, more densely packed colonies of cells, and reducing the risk of an adverse response to the transplantation of these cells. Various publications have also commented on the risk associated with the use of xenogeneic serum highlighting that the use of FBS may be undesirable due to the risk of viral and prion disease transmission and the risk of xenogeneic immune response to the culture cells due to their increased immunogenicity (Horwitz et al, 2002; Shahdadfar et al, 2005; Sundin et al, 2007). The authors (Shahdadfar et al, 2005) found, as supported by Kocaoemer and colleagues (2007), that MSCs cultured in autologous serum proliferated faster, exhibited more stable gene expression, and retained their multipotency for longer than those cultured in FBS. It was also established that MSCs in autologous serum displayed faster population doubling times and no significant morphological differences compared to those expanded in FBS. However, the use of FBS induced greater cell numbers, the overexpression of genes associated with prolongation of the cell cycle, including growth arrest-specific 1 and antiproliferative protein 1, and may result in greater immunosuppressive potential of cultured MSCs due to the upregulation of prostaglandin synthase genes. Although conflicting evidence, suggesting that expansion may reduce MSC immunosuppressive properties as well as increasing the risk of immune reaction thereby limiting \textit{in vivo} survival and function, also exists (Moll et al, 2012).

However, changes to the features of cultured cells do not arise due to the effects of the culture parameters alone. It is understood that MSCs, like most other cell types, are subject to the Hayflick limit, at around 50 population doublings, after which they cease to divide and enter replicative senescence (Izadpanah et al, 2006; Jiang et al, 2002). The rate at which this replicative senescence occurs may vary between donors. It has been proposed that donor age may affect the rate of senescence and it has been discovered that a negative correlation may exist between donor age and proliferative capacity (Stenderup et al, 2003; Cristofalo et al,
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1998; Mareschi et al, 2006). Furthermore, increased donor age has been associated with increased expression of senescence markers, specifically p53 and p21, in bone marrow-derived MSCs (Stolzing et al, 2008).

The direct cause of this senescence remains unknown, however it has been proposed that it may result from a purposeful, gene-driven programme, from random, accidental events, or from a combination of the two (Hayflick, 2007). Telomere length and telomerase activity have also been linked to senescence and cellular ageing. It is well established that, as a result of expansion, the length of the telomeres on the chromosomes in MSCs shortens and the activity of the telomerase enzyme, responsible for maintenance of telomere length, decreases (O’Hare et al, 2001; Di Donna et al, 2003; Bernardo et al, 2007; Baxter et al, 2004). Although there is a link between telomere length and senescence, there is a lack of evidence to suggest that it is the cause. Associations between senescence and DNA damage, accumulation of cyclin-dependent kinase inhibitor p16INK4a, and oxidative stress have also been observed (Ho, Wagner & Mahlknecht, 2005; Janzen et al, 2006; Kiyono et al, 1998).

The effect of senescence upon MSCs has been well documented and the impairment of multilineage differentiation potential, proliferative capacity and functional capacity has been reported (Bonab et al, 2006; Wagner et al, 2008). Both Bonab et al (2006) and Wagner and colleagues (2008) also established that the prolonged culture of MSCs can slowly affect their morphology, causing the cells to display a larger, flatter and more irregular shape with restricted nuclei, granular cytoplasm containing many inclusions, and cellular debris.

Wagner et al (2008) further examined the changes associated with senescence by comparing the gene and microRNA expression of senescent passages to that of earlier passages. It was discovered that several genes, including GPNMB, RAMP, PERP, LY96, STAT1, and PRNP, and microRNAs, including hsa-mir-371, hsa-mir-369-5P, hsa-mir-29c, hsa-mir-499, and hsa-mir-217, were upregulated with a small number of genes downregulated, including HAS1, ID1, and TNFSF11, as a result of expansion. The authors (Wagner et al, 2008) went on to describe that the upregulated genes in senescent cells were integral to the membrane, receptor activity, cell adhesion, vacuole and lysosome, and that this upregulation began in very early passages. These findings support the observed phenotypic changes associated with cells in later passages, and suggest that senescence may begin early in the culture of MSCs.
Despite the substantial changes to MSC features as a result of extensive culture, as previously described, there is evidence to suggest that this may not have a significant effect upon the immunophenotype of these cells, and that they may maintain the relevant surface markers which define them (Bonab et al, 2006; Carmen et al, 2012). However, contradictory data showing that the level of surface antigens detected in early passages was much higher than that of later passages has been discovered, which led to the authors proposing that the composition and level of surface markers may vary over long-term expansion (Wagner et al, 2008). For example, the STRO-1 antigen has been found to be progressively lost in human MSCs with time in culture (Ishii et al, 2005), and STRO-1^- MSC subsets have been identified in bone marrow (Bensidhoum et al, 2004). The cause of this alteration to marker expression during MSC culture is yet undetermined, however it has been proposed that the presence of other cell types and their secretion of various factors during initial passages may be responsible for the expression of certain markers by MSCs in vitro (Gronthos et al, 2001).

Features of cell culture including prevention of cell adhesion, oxygen tension, temperature, and medium composition have also been linked to immunophenotypic changes (Vergidis, 2006). As current methods of MSC characterisation revolve around the identification of surface markers, it is clear that the prolonged expansion of these cells may lead to complications and could be detrimental to product purity and quality. Wagner et al (2008) develop this further, describing that, as extended culture also affects MSC morphology and differentiation potential, expansion affects all parameters currently used in the characterisation of these cells. Further, investigations into changes in cytokine secretion during expansion also suggest that, if functional assays which identify and quantify the relevant bioactive molecules secreted by these cells in order to exert a therapeutic effect are to be developed, the culture of MSCs must be minimised or monitored. Carmen and colleagues (2012) discovered a difference in cytokine secretion, including IL-6, IL-8, VEGF, and IDO, with each MSC population doubling, which could lead to difficulties regarding the characterisation of these cells for their mode of action and the maintenance of product quality.

The in vivo homing capacity of transplanted MSCs may also be inhibited as a result of extended culture and has been adequately demonstrated using murine cells (Rombouts & Ploemacher, 2003). A variety of expressed molecules and surface markers have been associated with the migratory capacity of MSCs, two of which, CD49d and CD44, are involved in the binding of these cells to the endothelium and have been found to be sensitive
to the proteolytic action of trypsin which is used during the harvest of MSCs from culture plates (Nolte et al, 2002). This migratory capacity is also reliant upon the chemotaxis of MSCs, however it has been determined that the serial passaging of these cells reduces their expression of adhesion molecules, expression of chemokine receptors, and chemotactic response to chemokines (Toma et al, 2009; Wall, Bernacki & Loboa, 2007; Kretlow et al, 2008).

Another potential deleterious outcome of extended MSC culture is the alteration of various genes and the resulting increased risk of tumour formation with transplantation. It has been suggested that MSCs and other stem cells can undergo spontaneous transformation in a malignant manner during extended culture and as a result of the manufacturing process (Rubio et al, 2005; Wuchter et al, 2015). This has been observed after in vivo transplantation into mice, and the formation of fibrosarcomas was demonstrated (Miura et al, 2005). Rubio and colleagues (2005) propose that this transformation may be associated with chromosomal abnormalities, increased telomerase activity, and increased c-myc expression. However, extended culture of human MSCs has failed to reveal any chromosomal alterations, despite the alteration of cell cycle, protein ubiquitination and apoptosis gene expression (Izadpanah et al, 2008). Unless extensively expanded in stressful conditions, these cells have not been found to develop significant genomic instability or become tumorigenic (Dahl et al, 2008). However, despite the lack of evidence demonstrating the tumorigenic potential of these cells in vivo, the substantial amount of gene and expression alteration, as a result of culture, that has been demonstrated may still increase the risk associated with the transplantation of these MSCs.

Therefore, as a result of the observed effects of cell culture described previously, it can be deduced that the prolonged culture of MSCs could affect their functional capacity, beneficial effects in vivo, and safety. It is therefore clear that the alterations to cellular characteristics associated with the culture parameters or the senescence of cultured cells must be considered when MSCs are to be expanded for either experimental or clinical application. These effects of MSC expansion have led to the suggestion that MSCs in culture should not be considered equal to those under physiological conditions in vivo (Meirelles, Caplan & Nardi, 2008), and that their in vitro phenotype may be a phenomenon resulting from their culture (Keating, 2012).
However these changes may be advantageous in certain situations and could be utilised. A number of methods of MSC preconditioning, which may increase the secretion of certain bioactive molecules or prime cells towards a particular mode of action, have been identified. Hypoxia preconditioning has been developed as a method of *in vivo* injury simulation and has been linked to improved survival of cells, increased anti-oxidant enzyme production, reduced pro-oxidant production, greater expansion capacity, greater differentiation capacity, improved migratory capacity, and increased VEGF secretion (Peterson et al, 2011; Fehrer et al, 2007; Wang et al, 2008; Rosova et al, 2008; Li et al, 2007). The pre-treatment of MSCs with growth factors also represents an opportunity to facilitate differentiation or enhance the cells anti-apoptotic effects (Nesselmann et al, 2008). Meirelles and colleagues (2009) also highlighted that the supplementation of IFN-γ during MSC culture may also enhance the immune modulatory properties of these cells. Genetic engineering could also be utilised to manipulate MSCs to secrete a particular gene product, enhance survival, alter cell homing or to direct differentiation (Nesselmann et al, 2008). However, substantial genetic manipulation of this kind may incur substantial regulatory burden and would be unlikely to gain approval as a preconditioning method for most MSC-based therapies. Finally, pharmacological methods and physical methods, including cell-to-cell interaction, shear stress, and MSC spheroid formation, have also been tested, and, although pharmacological preconditioning has shown little efficacy, physical manipulations have had some success (Ranganath et al, 2012).

### 2.2.6 Automated & Scalable Manufacture of hMSCs

Despite the significant challenges that exist in the characterisation of these cells, hMSCs represent a clinically relevant cell type and a number of hMSC-based therapies are advancing towards the clinic. However, the scalable manufacture of such therapies remains a challenge, and manual cell culture methods may not be capable of achieving the required cell numbers without significant workload, cost and variability (Thomson, 2007).

The development of automated adherent cell culture platforms, for example TAP Biosystems’ CompacT SelecT, has made automation a viable alternative to manual cell culture processes. Thomas and colleagues (2007) demonstrated that this automated platform can be used to generate hMSCs of similar characteristics to those cultured manually. Alternative automated processes have been established, for example the Quantum Cell Expansion System (Terumo BCT, CO, USA) which is comprised of an automated, hollow-fibre bioreactor system. It has
been demonstrated that $1 \times 10^7$ Bone-marrow derived MSCs, displaying typical MSC characteristics, can be obtained when these cells were cultured in Human Platelet Lysate (HPL) within the Quantum system (Rojewski et al, 2013).

Although multiple manufacturing platforms have been developed for the expansion of hMSCs, Stirred Tank Bioreactor based systems, in which cells are cultured on microcarriers, currently offer the greatest cell yield (Rowley et al, 2012; Simaria et al, 2014). Furthermore, serum-free microcarrier processes have been developed, which have demonstrated greater yields of hMSCs compared to serum-based processes, and typical characteristics (Heathman et al, 2015).

### 2.3 Induced Pluripotent Stem Cells (iPSCs)

#### 2.3.1 Background

The derivation of pluripotent stem cells from adult somatic cells through epigenetic reprogramming was first discovered by Yamanaka and colleagues (2006) and has the potential to revolutionise drug discovery, disease modelling and cellular therapy.

The generation of these Induced Pluripotent Stem Cells (iPSCs) was achieved by the overexpression of the appropriate transcription factors, including Oct3/4, Sox2, Klf4, and c-Myc, each of which were delivered via retroviral vectors. However, since the discovery of iPSC generation, many alternative methods of reprogramming have been developed in an attempt to improve upon the low reprogramming efficiency and consistent integration of reprogramming vectors into the infected cell’s genome, as seen with integrating reprogramming methods. The various methods which have been adopted to produce iPSCs for human cells are discussed later in section 2.3.2.

iPSCs have a number of key advantages over more lineage restricted somatic cell types. Firstly, as these cells have been reprogrammed into a pluripotent state, they possess the capacity to differentiate into cells from all three germ layers and therefore represent a source of a wide range of cell types. Also, these cells are almost indefinitely expandable *ex vivo* and therefore provide an inexhaustible source of pluripotent or differentiated cells (Bajpai et al, 2012; Lian et al, 2010). Yet, these advantages are shared with Embryonic Stem Cells (ESCs), the pluripotent cells derived from the developing embryo, which were discovered over 50 years ago. However, iPSCs differ from ESCs in that, as the reprogramming process is not limited to a single somatic cell type, any adult tissue source can be utilised to generate a
population of iPSCs. Also, these cells may not share the same ethical issues as ESCs, as they are not derived from embryonic sources which are associated with a number of ethical concerns. Furthermore, as iPSCs are derived from adult tissue, these cells represent a potential source of autologous cells which may not elicit an immune response. This lack of immunogenicity was demonstrated in a study by Schnabel and colleagues (2014), in which it was determined that iPSCs induced a lower *in vitro* T-Cell response than that of MSCs, for which the T-Cell response was also negligible. However, it has been previously identified that iPSCs derived from Mouse Embryonic Fibroblasts (MEFs) were immune-rejected after re-introduction into donor mice, likely due to abnormal gene expression (Zhao et al, 2011). Novel methods of preventing iPSC graft rejection have been proposed, including the induction of immunological tolerance by generating ‘inducible Treg cells’ by co-culturing these cells with iPSCs and conditioning them to regulate graft acceptance (Boyd et al, 2012). Another safety concern shared by both of these pluripotent cell types is the characteristic teratoma formation observed after *in vivo* transplantation (Yu et al, 2007; Takahashi et al 2007). This represents a major barrier to the development of safe and effective pluripotent cell therapies, and perhaps suggests that ESCs and iPSCs would be more applicable as sources of more lineage restricted cells.

### 2.3.2 Methods of iPSC Reprogramming

The earliest iPSC reprogramming studies (Takahashi & Yamanaka, 2006) utilised retroviral vectors as a delivery system for the four transcription factors in order to induce the expression of the pluripotent markers. The reprogramming efficiency of this method in human cells was 0.01-0.02%, although, by increasing the number of transcription factors transfected, this increased to 0.25% (Park et al, 2008). However, this method only infects dividing cells, and therefore there was a shift towards lentiviral delivery systems in order to target both dividing and non-dividing cells and to increase reprogramming efficiency. Also, within the initial lentiviral vectors used to reprogram iPSCs, the introduction of loxP sites provided a substrate which allowed most of the transgene sequences to be removed by Cre-mediated recombination. However, initially there were concerns about the differing levels of reprogramming factor expression which may have contributed to the very low reprogramming efficiency (0.0004%) observed when using this method (Papapetrou et al, 2009; Chang et al, 2009). Yet despite the achievement of improved reprogramming efficiencies (0.1-1.5%) in humanised vectors and the discovery of integrated transgene
excision, the clinical use of cells derived using the lentiviral reprogramming method is still unlikely due to the small portions of transgene which remain after excision.

A more recent integrating reprogramming method involving a vector known as the piggyBac transposon, integrates into the chromosomes in the presence of piggyBac transposase allowing reprogramming factors to be co-transfected. This method also allows the vector to be cleanly excised and generate iPSCs at an efficiency of 0.02-0.05% (Kaji et al, 2009). However, the viability of this method is hindered due to the additional step required for excision and the lack of published data on piggyBac insertion excision from human cells.

Advances in iPSC reprogramming have led to the discovery of non-integrating, zero-footprint methods which do not leave transgene sequences within the recipient cell’s genome and may therefore be more applicable to the generation of clinically relevant iPSCs. Non-integrating viruses, including adenovirus and sendai virus, have been utilised as vectors for the pluripotency transcription factors with efficiencies of 0.0002% and 1% efficiency in human cells respectively (Stadtfeld et al, 2008; Fusaki et al, 2009). It is clear that the sendai RNA virus has a much greater reprogramming efficiency than that of adenovirus methods, and this method is also advantageous in the generation of translational-grade iPSCs in that large amounts of protein can be produced without the virus entering the nucleus of the infected cell and can therefore be diluted out of the cell very quickly. Consequently, due to its relatively high reprogramming efficiency and lack of transgene footprint, the sendai virus method is one of the most attractive options for the production of iPSCs for clinical use.

Plasmids, which are small, circular segments of DNA, have also been demonstrated to act as zero-footprint vectors for the pluripotency transcription factors when transfected into human cells. Episomal plasmids have been found to reprogram human cells with an efficiency of 0.0003-0.035% depending upon the initial cell source (Yu et al, 2009; Hu et al, 2011). Hu and colleagues (2011) also discovered that the addition of thiazovivin enhanced reprogramming by more than ten times. In addition, recent construction of more advanced episomal vectors has not only increased reprogramming efficiency to 0.02% in certain cell types (Chou et al, 2011), but also led to the development of feeder-free culture systems which reduce the need for xenogenic components, a factor which has inhibited the translation of cell therapies in the past. Plasmids have also been adapted, by removing the plasmid backbone, to form minicircle vectors which have been used to successfully reprogram human cells (Jia et
al, 2010; Narsinh et al, 2011). However, with a reprogramming efficiency of 0.005% and a significant lack of data regarding the reprogramming of alternative cell types, further validation is required before this method becomes established.

Protocols in which the reprogramming efficiency of integrating, lentiviral vector methods have been enhanced through the co-transfection with miRNA have been generated, and a reported 10 to 15-fold increase in efficiency has been reported (Subramanyam et al, 2011). Although this highly efficient integrating method is not ideal for the production of clinical grade iPSCs, the use of three mature miRNAs, namely mir-200c, mir302s, and mir-369s, to reprogram human cells without the use of a viral delivery system may be a viable alternative and has demonstrated an efficiency of 0.002% (Miyoshi et al, 2011). However, currently only a single study has successfully reprogrammed iPSCs through this method and further research is required in order to obtain data regarding its validity.

A number of alternative zero-footprint, vector-free reprogramming methods have been devised, with mixed results reported, although early difficulties regarding the delivery across the plasma membrane and the elicitation of an immune response have been encountered. The direct expression of reprogramming factors as proteins has been found to produce transgene-free iPSCs with a reprogramming efficiency of 0.001% in human cells (Kim et al, 2009). The expression of reprogramming factors as mRNAs can also achieve zero-footprint reprogramming, however the efficiency of this is far greater (1.4%) and can be further increased by adding an additional reprogramming factor (Lin28) to the original four, adding valproic acid to the cell culture medium, and culturing cells in a hypoxic environment (Warren et al, 2010). However, the validity of these reprogramming protocols is questionable as they are commonly labour intensive, costly, and require specialist skills to complete successfully.

Therefore, due to the low efficiencies and lack of evidence supporting alternative zero-footprint methods, including adenovirus, protein, mRNA and miRNA, it is likely that episomal plasmid and sendai virus protocols represent the greatest prospects for generating clinically relevant iPSCs.

2.3.3 Automated iPSC culture

If cell therapies derived from Human iPSCs (hiPSCs) are to gain adoption and become readily available in healthcare, consistent and scalable manufacturing processes will be
essential. Although in recent years significant advances have been made towards the development of allogeneic stem cell therapies, derived from hMSCs and hESCs, autologous hiPSC-derived therapies hold significant promise. In the case of allogeneic cell products, it is likely that transitioning to a suspension, microcarrier or perfusion based culture within a bioreactor system would be the most effective manufacturing platform in order to yield the greatest number of cells (Heathman et al, 2015; Rowley et al 2012). However, for an autologous cell therapy, in which less expansion and fewer cells per batch are required (Ährlund-Richter et al, 2009; Mason & Dunnill, 2009), an automated platform may allow for the required consistency and low cost of goods for the development of an efficient manufacturing process. Nonetheless, monolayer culture-based automated platforms represent a feasible option for manufacturers developing either allogeneic or autologous cell therapy products due to the level of comparability with manual manufacturing processes, as demonstrated by Thomas and colleagues (2007). Additionally, the utilisation of such automated platforms may reduce the product comparability risks that are associated with manufacturing platform transfers. Experimental research to quantify the comparability between manual and automated process steps for the culture of numerous cell types is presented in Chapters 7-9 of the present thesis.

However, similarly to Human ESCs (hESCs), the development of an automated hiPSC culture platform is hindered by the inherent sensitivity of these cells, due to their tendency to change state during the culture process, which also makes obtaining consistent results with these cells a challenge (Koike et al, 2012). Despite the significant hurdles, a small number of research groups have published studies demonstrating the successful, automated culture of iPSCs. Marx and colleagues (2013) reported the development of a fully automated platform which allowed for the reprogramming of fibroblasts into iPSCs, the culture of iPSCs in colonies, the quality control of cultures, and the harvesting of iPSCs using an incorporated AVISO (Jena, Germany) CellCelector device. The CellCelector device has been found to enable efficient identification and isolation of ESC and iPSC colonies (Haupt et al, 2012).

Taking a different approach, Koike et al (2012) developed an automated platform for the culture and passage of murine iPSCs using an incubated stack system, for culture plate incubation, a vibrating cell detachment system, to allow for the single cell passage of iPSC colonies, and a series of peristaltic pumps to allow for liquid handling of dissociation agents,
PBS and culture medium. The authors (Koike et al, 2012) also reported that, after 4 weeks of automated culture of iPSC colonies, pluripotent marker expression was maintained.

Another automated platform on which iPSC colonies have been cultured and passaged is TAP Biosystem’s (Royston, UK) CompacT SelecT. This platform utilises an incubated rotating carousel, for T175 flask incubation, a processing chamber, in which a robotic arm can perform flask handling and pipetting processes, a series of peristaltic pumps, for liquid handling, and an incorporated Cedex Automated cell counter (Roche, Basel, Switzerland). Soares and colleagues (2014) utilised this platform to culture and perform aggregate-based passages of hiPSCs in feeder free conditions and demonstrated that hiPSCs could be passaged using an automated system without losing their pluripotency. However, the authors (Soares et al, 2014) recommended some adaptations to the incubator and pipetting systems to improve the reproducibility of the process. The CompacT SelecT automated platform has also been utilised to culture hiPSC derived Neuroepithelial-like Stem Cells for High-Throughput Screening applications, and it was identified that the automated culture process yielded a greater cell number compared to the manual process (Mclaren et al, 2012).

2.3.4 Scalable manufacture of iPSCs

The utilisation of bioreactor-based systems, including perfusion, microfluidic, rotary and stirred-tank bioreactors, has been adopted for stem cell bioprocessing based upon the success in the production of antibodies and recombinant proteins (Serra et al, 2012). These platforms, in particular the 3D suspension systems, offer improved scalability, improved control, and more accurate representation of the physiological environment compared to adherent culture systems. However, little published research is currently available regarding the scalable manufacture of hiPSCs.

Initial studies investigating the suspension culture of iPSC discovered that typical pluripotent features were maintained over 20 passages, including a stable karyotype and pluripotent marker expression, when cells were cultured at small scale in petri dishes (Amit et al, 2010). In this study (Amit et al, 2010), when iPSCs were cultured for two weeks, cell spheroids reached 500μm in diameter and apoptosis increased to 14%. The authors identified that most of these apoptotic cells were located in the centre of the spheroids, and that cell death was likely due to poor diffusion.
More recently, larger scale iPSC suspension cultures have been generated with successful maintenance of morphology, pluripotency, and karyotype. Shafa and colleagues (2011) cultured murine iPSCs in 125ml suspension bioreactors over 8 passages, achieving a 55 fold expansion as well as >80% viability throughout the culture process. Olmer et al (2012) utilised the DASGIP® four parallel 250ml bioreactor systems (Eppendorf, Hamburg, Germany) with an adapted propeller to culture hiPSCs derived from cord-blood endothelial cells. In this study, a 5.5 fold expansion was achieved over a week of culture with no change in pluripotency.

Suspension culture has not only been applied to the expansion of iPSCs, but also the reprogramming of adult cells into iPSCs. Fluri and colleagues (2012) successfully reprogrammed fibroblasts in suspension in serum- and feeder-free conditions. It was also determined that these reprogrammed cells could differentiate into the three germ layers, and that a high correlation between the gene expression of fibroblasts reprogrammed in adherent and suspension culture existed.

Although previous studies have primarily used aggregate-based systems for the suspension culture of iPSCs, the culture strategy and nature of cell attachment is likely to differ depending upon cell characteristics and the scale of the manufacturing process.

Serra and colleagues (2012) comprehensively describe the benefits and limitations of aggregate-, microcarrier- and encapsulation-based systems for the suspension culture of pluripotent stem cells. Although aggregate culture can be scalable, reproducible, maintain cell to cell contact, and result in high differentiation efficiency and cell yield, difficulties in controlling culture outcome, controlling aggregate size, harvesting as single cells, and preventing cell damage due to physical forces exist. Similarly to aggregate-based systems, microcarrier-based culture can be scalable, highly reproducible, and generate in high cell yields. However, microcarrier culture also may not limit gas and mass diffusion and allows for a high surface to volume ratio. The limitations of this method include cell aggregation, the requirement for cell and bead separation, cell damage due to physical forces, the material costs of microcarriers and the xenogenic coatings required. Finally, cell encapsulation culture may also allow for scalability, reproducibility, a high surface to volume ratio, a high cell yield, protection from physical forces and the complete maintenance of cell to cell and cell to matrix interactions. However, difficulties monitoring and visualising encapsulated cells, the
limited mass and gas diffusion, the poor harvesting of cells after encapsulation, and the high material costs associated with encapsulation represent major barriers for the development of a scalable manufacturing process.

2.4 iPSC derived MSCs

2.4.1 Background

Recently, there has been increasing focus upon iPSCs as a source of large numbers of autologous MSCs, although this may also represent a significant step in the development of allogeneic MSC therapies due to the low immunogenicity of MSCs.

iPSC derived MSCs may also mitigate many of the challenges associated with the development of adult MSC-based therapies; including their limited availability in vivo, decline in quality and quantity over time, and the requirement for invasive extraction which may result in donor site morbidity (Chen et al, 2012; Giuliani et al, 2011; Hynes et al, 2013). However, there are numerous safety and economic limitations involved in the utilisation of iPSCs as an MSC source. With regards to the safety of iPSC-MSC therapies, the contamination of iPSC-MSC populations with undifferentiated cells could significantly increase the risk of teratoma formation, the iPSC reprogramming process prior to MSC differentiation may lead to gene disruption, and the use of feeder cells during the expansion process may contain pathogens and thus may contaminate the cell population (Jung, Bauer & Nolta, 2012; Villa-Diaz et al, 2012). The immunogenicity of MSCs derived from iPSCs after transplantation in humans has yet to be determined and requires further investigation, however a number of studies have reported a lack of immune response to pluripotent stem cell derived cells after transplantation in mice (Guha et al, 2013; Araki et al, 2013; Li et al, 2004; Drukker et al, 2006). More recently, it has been reported that, compared to adult BM-MSCs, iPSC-MSCs have an enhanced immune privilege after transplantation; as demonstrated by increased cell survival post-transplantation, reduced inflammatory cell accumulation, and hiPSC-MSC insensitivity to IFN-γ-induced HLA-II expression (Sun et al, 2015). Finally, there may also be economic consequences to the differentiation of iPSCs intoMSCs including complex cell sorting, extensive culture time and related labour cost, low efficiency of differentiation and yield of MSCs (Liu et al, 2012).
2.4.2 iPSC to MSC Differentiation Protocols

In order to differentiate iPSCs into MSCs, a variety of different protocols have been utilised each of which have subtle differences. However, generally these protocols follow one of two different fundamental rationales, namely single-cell or colony culture and differentiation.

The single-cell culture method often shares a number of processes to that of adherent cell passaging including enzymatic dissociation, centrifugation and single-cell seeding, however a number of distinct differences are required to differentiate iPSCs down the required lineage. The studies which have derived MSCs through the single-cell method (Liu et al, 2012; Zou et al, 2013; Chen et al, 2012; Lian et al, 2010) have often used compounds to prevent spontaneous differentiation prior to directed MSC differentiation, including Rho-associated protein kinase (ROCK) inhibitors, which improve the survival of single-cell, dissociated pluripotent cells (Watanabe et al, 2007), or TGFβ/Activin receptor-like kinase (ALK) inhibitors, which inhibit SMAD2/3 phosphorylation, maintains pluripotency marker expression and therefore prevents unwanted differentiation (Chen et al, 2012).

Alternatively, a number of studies have utilised colony or Embryoid Body (EB) culture and differentiation methods (Himeno et al, 2013; Chen et al, 2012; Villa-Diaz et al, 2012; Giuliani et al, 2011) in order to derive MSCs from iPSCs. This method commonly involves the maintenance of iPSCs in colonies, the manual picking or detachment of colonies, and the re-plating of iPSC colonies. This method also differs from the single-cell method in that differentiation into fibroblastic MSC-like cells occurs at the edges of the colonies, from which these cells grow outwards, rather than at a single-cell level across the culture.

However, both the single-cell and colony protocols share a similar method through which they induce the differentiation of iPSCs into MSCs. This involves the gradual replacement of iPSC culture medium with MSC culture medium; which frequently contains Basal Alpha-MEM, 10% FBS, L-Glutamine, and Non-Essential Amino Acids. As the single-cell or colony cultures grow in this medium, differentiation occurs and they begin to exhibit a fibroblastic morphology and sub-confluency is often reached within a week (Liu et al, 2012).

Comparative studies of the two iPSC to MSC differentiation protocols have been performed, with Chen and colleagues (2012) finding that EB- and single-cell-derived iPSC-MSCs were morphologically similar, expressed similar surface markers and had similar differentiation capacity. However, the authors (Chen et al, 2012) discovered that the single-cell method,
which utilises a TGFβ/ALK inhibitor, allows for shorter culture period than EB based protocols and also avoids the initial heterogeneity of the cell populations generated using the EB method. However, although Liu et al (2012) found that single-cell and EB-derived MSCs both expressed typical MSC markers, they observed that iPSCs cultured in colonies on fibrillar collagen coating did not induce differentiation into a spindle-like morphology and uncontrolled differentiation occurred. Nevertheless, there is a lack of data comparing the properties of MSCs derived from single-cell or colony iPSC differentiation protocols, and therefore further research is required in order to determine which method is optimal.

Recent studies have also explored controlled differentiation using polymer coated culture surfaces. Liu and colleagues (2012) successfully derived MSCs, which expressed typical MSC surface markers and displayed trilineage differentiation potential, from iPSCs through culture on a fibrillar collagen coating. A limitation of this study however was that dexamethasone was utilised during the culture and differentiation process which may have contributed to directing the iPSCs down the Mesenchymal lineage. Villa-Diaz et al (2012) also explored the use of synthetic substrates to direct iPSC differentiation which would also reduce the use of human feeder cells and extracellular matrix coatings which are likely to be undefined, expensive, immunologically incompatible, contain pathogens, and have batch to batch variations. The authors (Villa-Diaz et al, 2012) found that when iPSCs grown in colonies were differentiated into MSCs on a Poly[2-(methacryloyloxy) ethyl dimethyl-(3-sulfopropyl)ammonium hydroxide (PMEDSAH) coated surface an initial population of heterogenous MSCs developed, although by passage 2 >90% of the total cell population had acquired a fibroblast-like morphology. These iPSC-MSCs expressed typical MSC markers, displayed minimal pluripotency marker expression, maintained a normal karyotype over multiple passages, displayed colony forming capacity, demonstrated trilineage differentiation, and formed new bone in an in vivo mouse model. These early experiments demonstrate that polymer coatings may represent an efficient method of directing pluripotent cell differentiation towards the Mesenchymal lineage in the future.

However, currently none of these methods of iPSC differentiation are established and improvements in the efficiency and consistency of differentiation is required if iPSC derived products are to comply with Good Manufacturing Practice (GMP) standards.
The mechanism behind the differentiation of iPSCs to MSCs, through serial passaging and culture, is currently unclear, however it has been proposed that the addition of serum, which contains a variety of cytokines, growth factors and metabolites, to the culture medium may influence this process (Zou et al, 2013). Therefore, further investigation is required in order to identify the factors responsible for stimulating iPSC to MSC differentiation.

The influence of the method of iPSC reprogramming upon their differentiation into MSCs also requires further examination, as this process can leave an epigenetic memory which may interfere with efforts to direct differentiation (Kim et al, 2010).

2.4.3 iPSC-MSC Characterisation

MSCs derived from iPSCs have been well characterised and the results of many studies have indicated that iPSC-MSCs have a similar surface marker expression profile to that of adult MSCs with positive CD73, CD90, CD105, CD146, CD44 and HLA-ABC expression and negative CD45, HLA-DR, and CD34 expression (Bajpai et al, 2012; Chen et al, 2012; Giuliani et al, 2011; Himeno et al, 2013; Liu et al, 2012; Villa-Diaz et al, 2012; Zou et al, 2013). Although, Liu and colleagues (2012) did find that CD90 expression increased with each passage, and a surface marker expression shift after 120 population doublings of iPSC-MSCs was reported by Lian et al (2010). However, disparity exists with regards the expression of pluripotency markers. A number of experiments have found that after iPSCs differentiate into MSCs they no longer express the pluripotency markers Oct3/4, TRA-181 or Nanog (Chen et al, 2012; Giuliani et al, 2011; Lian et al, 2010; Villa-Diaz et al, 2012). However, maintained pluripotency marker expression after differentiation has been reported in a few isolated studies (Zou et al, 2013; Villa-Diaz et al, 2012).

The differentiation capacity of iPSC derived MSCs has also been thoroughly investigated and compared to that of adult MSCs with a similar trilineage differentiation potential demonstrated in a number of studies (Bajpai et al, 2012; Giuliani et al, 2011; Himeno et al, 2013; Hynes et al, 2013; Zou et al, 2013). However, in some cases an unequal propensity for differentiation towards certain lineages has been found. It has been proposed that, although their differentiation towards adipose and bone is efficient, the differentiation of iPSC-MSCs towards cartilage is most efficient (Lian et al, 2010). However, later experiments, undertaken by Chen and colleagues (2012) and Villa-Diaz and colleagues (2012), discovered a greater propensity for osteogenic and chondrogenic differentiation in iPSC derived MSCs. Contrary
Chapter 2: Literature review- The Characterisation, Automation, and Standardisation of Human Mesenchymal Stromal Cell (hMSCs), Human Embryonic Stem Cell (hESC), and Human Induced Pluripotent Stem Cell (hiPSC), and hiPSC derived MSC (hiPSC-MSC) Culture

to this, Liu et al (2012) discovered that iPSC-MSCs did not form fully mature osteoblasts after differentiation despite staining positive for calcium deposition and positive in Alcian Blue and Oil Red O, indicating chondrogenesis and adipogenesis respectively. These discrepancies in differentiation potential have been linked to donor variation, the detrimental effects of repeated passaging, and the epigenetic memory of reprogrammed cells (Liu et al, 2012; Villa-Diaz et al, 2012), the latter of which is discussed in section 2.4.5.

The genetic stability of differentiated iPSCs is required if translation into cell therapy is to be achieved, and this has been demonstrated in cultured iPSC-MSCs, over multiple passages, by a number of investigators (Chen et al, 2012; Lian et al, 2010; Villa-Diaz et al, 2012; Zou et al, 2013). However, it has been reported that, after extended passage (>45 passages) of iPSC derived MSCs, random chromosomal aberrations begin to occur (Lian et al, 2010). Although iPSC derived MSCs have been demonstrated to be karyotypically normal, it has been observed that they exhibit up to ten times greater telomerase activity than their adult counterparts (Lian et al, 2010; Zou et al, 2013). This finding may explain the higher proliferation capacity of iPSC-MSCs compared to adult MSCs reported by numerous investigators (Bajpai et al, 2012; Chen et al, 2012; Lian et al, 2010; Zou et al, 2013). Greater cell density and lower rate of senescence, which correlated with a lower expression of senescence marker p21, of iPSC-MSCs in culture has also been identified (Bajpai et al, 2012).

2.4.4 iPSC-MSC Functionality

In recent times, a relatively small number investigations into the functionality of iPSC derived MSCs in animal and in vitro models have been undertaken in order to determine their mode of action and potential clinical utility. The mechanisms identified in the limited published literature can be categorised into three distinct areas, namely paracrine effects, differentiation, and immunosuppression, and align closely with those associated with adult MSCs.

Despite the frequently reported in vitro trilineage differentiation potential of iPSC-MSCs, the demonstration of the differentiation of iPSC-MSCs into cell types with in vivo functionality is currently limited to a few cell types. Osteogenesis of iPSC-MSCs onto transplanted scaffolds (Zou et al, 2013) and in an in vivo animal model (Villa-Diaz et al, 2012) has been discovered, and the new bone tissue has been found to support haematopoiesis, stimulate ECM formation, encourage mineralisation and successfully engraft into host tissue. Also, Bajpai and
colleagues (2012) successfully generated functional, contractile vascular smooth muscle cells, which may have potential value in blood vessel formation, from iPSC-MSCs. Finally, the regeneration of periodontal defect areas using iPSC-MSC treatment, in which the cells engrafted and differentiated within the damaged area, has also been reported (Hynes et al, 2013).

The second putative mode of action of iPSC-MSCs investigated within the body of research immunosuppression and regulation of the immune response. Giuliani et al (2011) identified that iPSC-MSCs effectively impaired NK cell activity and proliferation and reduced the expression of the inflammatory cytokine IFN-$\gamma$ in a similar manner to that of bone marrow derived MSCs and embryonic stem cell derived MSCs. It was also observed that iPSC-MSCs reduced NK cell immunological synapase formation, which leads to cytolysis and granule secretion, to a greater extent than ES-MSCs. Finally, the authors (Giuliani et al, 2011) examined the expression of HLA-G, PGE-2 and IDO, which have previously been linked to NK cell inhibition, by MSCs from each source in NK cell co-culture and found that those MSCs derived from the pluripotent cells exhibited an increased expression of HLA-G and PGE-2.

The final mechanism which has been briefly explored in the literature relating to iPSC-MSC functionality involves the paracrine secretion of bioactive factors, which has become acknowledged as the predominant mode of action of adult MSCs in vivo, as discussed in section 2.2.3. However, recent research into the trophic effects of iPSC-MSCs has focused primarily on the angiogenic potential of these cells. In 2010, a Chinese research group (Lian et al, 2010) compared iPSC-MSC mediated neovascularisation to that of BM-MSCs and revealed that iPSC-MSCs displayed a greater capacity for endothelial and smooth muscle differentiation; expressed greater levels of SDF-1, SCF, and bFGF; reduced inflammation to a greater extent; demonstrated increased tubular formation, myogenesis, and neovascularisation; and therefore induced superior blood flow restoration and reduction in fibrosis in a murine hind limb ischaemia model than did BM-MSCs. More recently, Himeno and colleagues (2013) reported a similar pattern secreted factors between iPSC-MSCs and somatic MSCs, with only PDGF-A expressed significantly higher in iPSC-MSCs. The use of mouse models of diabetic polyneuropathy determined that MSCs from both sources successfully restored blood flow to the plantar skin.
2.4.5 Epigenetic Memory of iPSC-MSCs

The findings discussed in section 2.4.4, regarding iPSC-MSC functionality, may suggest that these cells offer great potential as a source of MSCs suitable for clinical use. However, one of the major barriers to the translation of iPSC derived therapies is the possible detrimental effects of their epigenetic memory upon their efficacy and safety *in vivo*. It has been proposed that epigenetic markers retained after reprogramming may negatively affect the differentiation potential and functionality of these cells (Jung, Bauer & Nolta, 2012). Villa-Diaz and colleagues (2012) suggested that this epigenetic memory may explain the unequal differentiation capacity and the diminished adipogenic potential they witnessed in iPSC derived MSCs.

This epigenetic carryover has been investigated in further detail by Kim et al (2010), who draw four major conclusions from their research which may help advance our understanding of this phenomenon. Firstly, their results indicate that the tissue source of the starting material may significantly affect the efficiency and precision of reprogramming, with murine blood cells yielding more typical iPSCs than dermal fibroblasts. Also, they identified that substantial differences in DNA methylation exist between iPSCs and ES cells. By examining the differentiation potential of non-haematopoietic cells the authors demonstrated that epigenetic memory and residual methylation restricts the lineages down which iPSCs can differentiate. Thirdly, by differentiating iPSCs down a certain lineage, returning these differentiated cells to a pluripotent state and then re-differentiating these iPSCs back down the same lineage, they determined that the differentiation potential and methylation profile of iPSCs can be reset. Finally, Kim and colleagues propose that the epigenetic differences between iPSCs and ES cells may only manifest after the pluripotent cells differentiate once the specific loci that retain residual epigenetic markers are expressed, which may influence cell fate.

However, despite the obstacles associated with iPSC epigenetic memory, it is clear that these cells represent an attractive source of MSCs, which have been found to exhibit similar characteristics to adult MSCs and may exert clinical benefit equal to, or greater than, adult MSCs through multiple mechanisms *in vivo*. It is also feasible that, with further research, an optimal tissue source of cells for reprogramming into iPSCs with the capacity to effectively differentiate into MSCs could be identified. This would allow the considerable potential of
iPSCs as a source of MSCs to be utilised and may accelerate the translation and commercialisation of MSC therapies.

2.5 Human Embryonic Stem Cells (hESCs)

2.5.1 Background

Human Embryonic Stem Cells (hES cells), which were first derived by Thomson and colleagues in 1998 (Thomson et al, 1998), represent a potential unlimited source of cells for cell-based therapies and also represent a valuable model for early human development. These self-renewing and pluripotent cells are characterised by the following (Hoffman & Carpenter, 2005):

- Isolated from the inner cell mass of the blastocyst
- Proliferate extensively in vitro
- Maintain normal euploid karyotype over extended culture
- Differentiate into derivatives of all three germ layers
- Show telomerase activity
- Highly express Oct4, a transcription factor which plays a critical role in pluripotency

A number of other antigens that are commonly expressed antigens have been identified by The International Stem Cell Initiative in their 2007 paper (Adewumi et al, 2007), which compared the characterisation of a range of hES cell lines, including SSEA-3, SSEA-4, TRA-1-60, TRA-1-81, GCTM2, GCT343, CD9, CD90, Tissue-nonspecific Alkaline Phosphatase (ALP), HLA I, Nanog, TDGF1, DNMT3B, GABRB3, and GDF3.

However, although surface marker expression and characterisation have been linked to pluripotency, this antigen profile is not necessarily indicative of differentiation capacity in hES cells. The most commonly utilised methods to determine pluripotency is through the formation of Embryoid Body formation in vitro and through teratoma formation in vivo after injection into immunocompromised mice.

2.5.2 Automation of hESC culture

The capability of these cells to differentiate into cells from all three germ layers clearly represents an exciting opportunity for the cell therapy field, however in order to effectively exploit the great potential of this cell type, these cells must be reproducibly derived, manipulated and differentiated into the desired cell type at the relevant scale (Hussain et al,
The automated culture of these cells may hold the key to navigating such issues through the improvement of quality control, scalability, reproducibility, and the economics of the culture process (Archer & Williams, 2005). Automated platforms may allow for the control of cell density, fluid flow, centrifugal force, pH and temperature utilised during the dissociation and passage processes, to which hES cells are particularly sensitive with variations in these parameters being known to lead to shifts in hES cell phenotype and cause spontaneous differentiation (Veraitch et al, 2008). Such parameters often remain uncontrolled in manual culture protocols and inter-individual variation, leading to possible genomic instability, is commonplace in many laboratories (Terstegge et al, 2007). Therefore, adequate standardisation and reproducibility required for the scalable processing of hES cells may not be achievable through manual processing. However, due to the sensitivity of these cells, the potential contamination risk in antibiotic-free cultures necessary to produce a translatable product, the need for visual examination of the growth and morphology of these cells, and the often complex co-culture and differentiation protocols associated with this cell type, the automation of the hES cell culture process faces many hurdles (Terstegge et al, 2007).

Despite the challenges associated with the development of an appropriate automated hES cell culture system, a number of investigators have reported significant advances towards the generation of such a platform with a variety of methods being utilised. Automated microwell plate-based (Terstegge et al, 2007; Hussain et al, 2013), tissue culture flask-based (Thomas et al, 2008), and hollow-fibre bioreactor-based (Roberts et al, 2012) platforms have been utilised to successfully culture hES cells, as well as tissue chopper-based, partially automated systems (Joannides et al, 2006), in which an automated tissue chopper was employed to split hES cell colonies. In each of these studies, comparable growth rates, pluripotency marker expression and differentiation capacity were observed in hESCs cultured using an automated process compared to that of cells cultured manually.

2.5.3 Scalable manufacture of hESCs

As with hiPSCs, 3D suspension systems may offer a viable alternative to 2D adherent systems for the culture of hESCs, and may allow for improvements in scalability and control.

Mouse ESCs (mESCs) have been readily cultured in in 125ml stirred vessels, with shear stress being found to maintain pluripotency (Gareau et al, 2010). Furthermore, the expansion and differentiation of mESCs into cardiomyocytes has been demonstrated up to a 2L scale
Chapter 2: Literature review- The Characterisation, Automation, and Standardisation of Human Mesenchymal Stromal Cell (hMSCs), Human Embryonic Stem Cell (hESC), and Human Induced Pluripotent Stem Cell (hiPSC), and hiPSC derived MSC (hiPSC-MSC) Culture

(Zandstra et al, 2003). The addition of Leukaemia Inhibitory factor (LIF), which activates the JAK/Stat pathway, largely prevents mESC differentiation in both conventional plastic adherent 2D cultures as well as in aggregates in 3D suspension cultures (Singh, Mok & Zweigerdt, 2011). However, unlike mESCs, hESCs tend to readily differentiate when cultured as aggregates in stirred suspension bioreactors (Kehoe et al, 2010). Furthermore, LIF does not maintain the self-renewal of hESCs (Singh, Mok & Zweigerdt, 2011). Although single cell passaging has been successful in mESC cultures, this method is detrimental to the viability and self-renewal of hESCs without the addition of Rho-Kinase (ROCK) inhibitor (Kehoe et al, 2010).

Early hESC suspension culture studies utilised slow-turning lateral vessels and high-aspect rotating vessels which allowed for significant cell expansion, but was found to cause large amounts of aggregation of Embryoid Bodies (EBs) (Gerecht-Nir et al, 2004). Initial hESC spinner flask culture studies yielded EBs containing cells of all germ layers, which demonstrates the importance of optimising and maintaining bioreactor operating conditions (Yirme et al, 2008). It has been noted that hESCs in suspension can form aggregates of karyotypically stable cells within 24 hours, although un-aggregated cells, with a low viability (<10%) will also be present (Singh, Mok & Zweigerdt, 2011).

More recently, Hunt and colleagues (2013) cultured hESCs in nine duplicate 125ml stirred suspension bioreactors in order to explore the effect of seeding density and agitation rate upon a variety of cell characteristics. After determining their optimal conditions for cell yield and viability, specifically a medium agitation rate (100rpm) and a low seeding density (2x10⁴ cells/ml), it was observed that these conditions yielded aggregates of hESCs that maintained their differentiation capacity, stable karyotype, and pluripotency marker expression.

The suspension culture of encapsulated hESCs and hESCs attached to microcarriers has also been examined as alternatives to the culture of these cells as EBs or aggregates. The encapsulation of hESCs has been found to allow for 260 days of culture whilst maintaining pluripotency marker expression (Siti-Ismail et al, 2008). Microcarrier culture of hESCs has been demonstrated to allow for cell growth at a comparable rate to adherent culture, maintain the expression of pluripotency markers, maintain differentiation potential into the three germ layers, and improve recovery post-thaw (Nie et al, 2009). Furthermore, stirred microcarrier systems, in which animal and human component-free medium was utilised, has been shown
to yield $1.6 \times 10^8$ cells within 6 days of culture whilst maintaining hESC pluripotency (Marinho et al, 2013).

However, cell harvesting from microcarriers is typically difficult, particularly for hESCs which aggregate significantly, and represents a considerable challenge for the scalable manufacture of pluripotent stem cells. Furthermore, for the generation of clinically relevant cells, xeno-free reagents and microcarrier coatings will be required which may add additional cost and require further investigation into the effect of transitioning to a xeno-free environment.

2.5.4 Standardisation of hESC culture

It has been proposed that the development of well-characterised reference cell lines, with sufficient precision, is fundamental to establishing methods of demonstrating comparability, and that linking their use to automated processing systems could provide a straightforward method for the comparison of manufacturing processes at multiple sites or between processing equipment (Hourd et al, 2014). Therefore, the identification of such a reference cell line would allow for the comparison of hES cells, which has previously been complicated by variations in culture conditions, feeder cells and spontaneous differentiation, as well as the development of standardised assays (Josephson et al, 2007). Thus, the utilisation of a reference hESC line as a ‘metric’ may also enable comparability between cell lines, culture methods and assays.

The Embryonal Carcinoma cell line known as EC 2102Ep has been proposed as a reference hESC line, and was originally derived from a surgical specimen of a primary testicular germ-cell tumour containing EC and yolk sac elements (Andrews et al, 1982; Wang et al, 1980). The antigen expression profile of these cells is very similar to that of conventional hES cells, with the strong expression of Oct3/4, SSEA-4, TRA-1-81, TRA-1-60 and TRA-1-85 previously reported (Josephson et al, 2007). These authors (Josephson et al, 2007) also identified the positive expression of ALP and a lack of SSEA-1 expression, a marker commonly associated with differentiating hES cells. Josephson and colleagues (2007) also discovered that this expression profile did not change over 10 passages. Therefore, it is clear that these cells do not readily differentiate in culture which makes them a useful tool in the development of an automated cell culture platform for hES cells. The relative ease of the culture of these EC 2102Ep cells, unlike the majority of hES cell lines, also makes them
Chapter 2: Literature review- The Characterisation, Automation, and Standardisation of Human Mesenchymal Stromal Cell (hMSCs), Human Embryonic Stem Cell (hESC), and Human Induced Pluripotent Stem Cell (hiPSC), and hiPSC derived MSC (hiPSC-MSC) Culture

attractive for the optimisation of an automated system. These cells have been successfully cultured as a monolayer in Dulbecco’s Modified Eagle Medium, with 10% Foetal Bovine Serum, 4mM Glutamine and without feeder cells (Andrews et al, 1982; Josephson et al, 2007).

The characteristic hES marker expression that the EC 2102Ep cells display, combined with their lack of spontaneous differentiation and relative ease of culture, led to the proposal of this cell line as a reference standard, or ‘Ruler’, for hES cell research. A Ruler cell line can be used as a reference standard, for a variety of assays, for other cell lines to be measured against. When compared to alternative candidate hES Ruler cell lines, it was determined that, although the EC 2102Ep cell line was less similar than alternative cell lines with regards global gene and microRNA expression, this cell line expressed more of the microRNAs associated with classical hES cells, as well as fewer markers of differentiated fates (Josephson et al, 2007). Therefore, the EC 2102Ep cell line has been identified as the most promising candidate in the identification of a representative hES Ruler cell line.

2.6 Summary and Future Research

2.6.1 hMSCs

This literature review has discussed the lack of suitable MSC characterisation parameters and methodologies currently available, described the paradigm shift towards explaining the mode of action of these cells by a paracrine effect, explained the difficulty associated with their isolation from various sources, outlined a new hypothesis regarding the perivascular location of these cells in vivo, examined the deleterious effects associated with their prolonged in vitro expansion, described the variety of platforms available for hMSC manufacture, indicated the potential clinical utility of MSCs in the treatment of multiple simultaneous indications, and highlighted the need for functional assays which identify and quantify the biomarker secretion of MSCs in order to determine their utility.

A recent study has proposed the identification of a MSC reference cell line in order to redefine the MSC minimal criteria to include a greater number of parameters, including growth characteristics, marker expression, gene expression, secretome assays, metabolite expression, miRNA and IncRNA expression, differentiation assays, engraftment assays, stability after cryopreservation (Tanavde et al, 2015). Therefore, further research is required
to identify a suitable MSC reference material and to obtain sufficient data sets for each parameter.

After examining the literature, the areas which appear to offer the greatest potential for the development of functional assays for MSCs involve the analysis of the transcriptome and the secretome of these cells. These methods examine the gene transcripts and RNA molecules produced, and the proteins secreted from the cell respectively. In particular, the study of the MSC secretome could induce significant advances in this field, although a number of hurdles exist. Although studies have begun to interrogate the secretome of MSCs (Chiellini et al, 2008; Lee et al, 2009; Choi et al, 2010; Estrada et al, 2009; Kim et al, 2013), further research is required in order to link the MSC secretome profile to the in vivo functionality of these cells.

The examination of the MSC secretome has also led to the utilisation of cell-free cytokine therapies, including the transfusion of VEGF, G-CSF, EPO, IGF-1, and SDF-1. Furthermore, the movement away from cell therapies and towards cell-derived therapies, based upon secreted growth factors or cytokines, has been proposed and has been labelled as ‘Regenerative Pharmacology’ (Choudhury & Mathur, 2013). As discussed previously in section 2.2.3.3, the utilisation of MSC secreted extracellular vesicles in cell-free therapy also holds promise. However, it has been illustrated that, in order to achieve a similar beneficial effect to that of MSC-based therapies, high doses of these factors and simultaneous infusion of multiple cytokines may be required. Considering this, the sustained pharmacokinetics, synergy from multiple factors, and capacity for systemic infusion make MSCs more amenable to repeated dosing than these proposed cell-free cytokine therapies (Ranganath et al, 2012).

The identification of relevant biomarkers and the development of functional assays capable of quantifying the paracrine activity of MSC populations could represent significant advances in MSC characterisation and potency measurement. However, it is clear that a number of challenges are yet to be overcome, especially given the range of indications for which MSCs are utilised and the likely need for these cells to possess multiple critical functions for each application (Ren et al, 2011). It is also apparent that improvements in in vitro and animal models, as well as further in vivo and clinical studies, are required in order to further develop our understanding of MSC mode of action and to identify the most suitable clinical
indications towards which further research can be targeted. A review by Ranganath et al (2012) made a number of intriguing observations with regards to the variation between the \textit{in vivo} and \textit{in vitro} characteristics of MSCs. They noted that the \textit{in vitro} MSC secretome is likely to differ substantially to the \textit{in vivo} secretome due to the different microenvironments, that control over the cytokine production of these cells after transplantation is likely to be limited, and that the \textit{in vitro} secretome observed must consider the serum used in culture as it may contain overlapping components which can interfere with the detection of secreted biomarkers.

As discussed in section 1.3.1, product comparability after changes to the manufacturing process represents a major manufacturing and regulatory challenge for all cell therapy products. Therefore, given the clinical relevance of the hMSC cell type, further research is required in order to examine the effect of changes in the hMSC manufacturing process upon the product. In the present thesis (Chapter 7), the effect of a change from a manual to an automated cell culture process step upon the growth, stability, characterisation and functionality of hMSCs will be examined, and the comparability between these process steps will be measured.

The evolving nature of the UK reimbursement environment may influence the likelihood of RMs achieving reimbursement. As described in sections 2.2.1 and 2.2.3.3, hMSC-based therapies may hold promise for the treatment of multiple simultaneous indications, a paradigm that does not fit within the current, single-indication based reimbursement structure. Therefore, to address these reimbursement challenges, the present thesis examines the effect of the proposed changes to the current UK reimbursement upon the reimbursement of RMs (Chapter 3), and explores the adaptation of a method of early economic assessment to account for treatments which target multiple simultaneous indications (Chapter 4).

2.6.2 hiPSCs

In this chapter, the advantages of hiPSCs over hESCs were summarised, the uncertainty regarding the immunogenicity of transplanted hiPSCs was described, the available methods of iPSC reprograming were discussed with the most promising methods highlighted, and the progress towards the development of automated iPSC culture processes, as well as alternative scalable processes, was outlined.
Despite some improvements in reprogramming efficiency and the creation of alternative reprogramming methods, more efficient reprogramming could facilitate the development of improved iPSC manufacturing processes. Additionally, from this literature review it is apparent that further research is required to examine residual epigenetic carryover after reprogramming into iPSCs. The auto-immune response to iPSCs, of either autologous or allogeneic nature, also requires further investigation in order to achieve the clinical translation of iPSC derived therapies. Finally, despite the successful utilisation of automated platforms to culture hiPSCs, the manufacturing challenge of comparability after a manufacturing process change has yet to be thoroughly investigated for this cell type. Therefore, Chapter 9 of the present thesis will measure the comparability between manual and automated process steps for the culture of hiPSCs, and determine the effect of this process step change upon hiPSC growth, stability, and characterisation.

2.6.3 hiPSC-MSCs

The present review has outlined the advantages and limitations of iPSCs as a source of MSCs, described the various protocols that have been generated for the differentiation of iPSCs to MSCs, discussed how the characterisation and functionality of hiPSC-MSCs compares to that of adult MSCs, and explored how the epigenetic memory of these cells may influence their trilineage differentiation.

Similarly to iPSCs, the epigenetic carryover and immunogenicity of cells derived from iPSCs remain areas of significant uncertainty. Conflicting data has been published regarding the immunogenicity of iPSC-MSCs, and greater clarity is required to enable the clinical translation of products based upon iPSC derived cells. The epigenetic carryover after the differentiation of iPSCs may influence the immunogenicity of these cells, as well as the in vivo functionality, and therefore further research is required. With regards the iPSC to MSC differentiation process, it remains unclear as to the precise stimulus within the process that causes differentiation to occur, and the identification of this stimulus may facilitate process optimisation. In order to comply with current regulation, it is likely that a hiPSC-MSC manufacturing process will be required to transition away from serum based culture, and therefore the effectiveness of animal serum-free culture medium formulations in iPSC to MSC differentiation must be investigated. Although the differentiation of hiPSCs to MSCs has recently been achieved (Luzzani et al, 2015), further research is required in order to validate a serum-free differentiation process, and therefore this is examined in the present
thesis (Chapter 10). Finally, although a significant body of research has been published regarding the differentiation of iPSCs to MSCs, a scalable differentiation process has yet to be developed.

2.6.4 hESCs
Finally, this chapter identified the primary characteristics that are representative of hESCs, summarised the progress that has been made in the utilisation of automated or alternative scalable platforms for the culture of these cells and described their comparability to manually cultured cells, discussed the value that the identification of a reference hESC line may add, and described the characteristics of the proposed reference hESC line known as EC 2102Ep.

As with hMSCs and hiPSCs, although the automated culture of hESCs has been demonstrated, the manufacturing challenge of comparability after a manufacturing process change requires further investigation. Thus, in the present thesis (Chapter 8), the effect of a manual to automated process step change upon the growth, stability, and characterisation of a Ruler hESC line will be determined, and the comparability between process steps will be measured. Furthermore, the selection of a reference hESC line may offer significant benefits in process and assay development. Therefore, in the present research (Chapter 8), the suitability of the EC 2102Ep cell line as a reference cell line is explored.
Chapter 3: A Systematic Literature Review of the Transformation of the UK Reimbursement Environment, the Evolution of VBP over a Two Year Period & the Potential Implications for Regenerative Medicines which Target Multiple Simultaneous Indications

The aim of this chapter is to describe the current UK reimbursement environment, review the way in which the Value-Based Pricing (VBP) scheme has been modified since its proposal, and propose the way in which the introduction of such schemes may have impacted the reimbursement of regenerative medicines, in particular those which target multiple simultaneous indications. A systematic review of this nature has not previously been performed.

3.1 Introduction

It is well established that Regenerative Medicines (RMs) offer the capacity to restore or establish normal function through the replacement or regeneration of human cells, tissues or organs (Mason & Dunnill, 2008). However, it has also been proposed that if such therapies, by replacing or repairing damaged or diseased cells, could cure, or better manage the underlying cause, of diseases, then this could attenuate or delay the onset of associated conditions, or ‘Multimorbidities’ (Prescott, 2011). Therefore, RM, if successful, may hold the potential to revolutionise healthcare and, if this opportunity can be capitalised, could generate great benefits for both stakeholders and patients alike.

However, as cellular therapies progress towards market and the clinic, there are substantial barriers that must be overcome in order to facilitate adoption into clinical practice and to achieve commercialisation in the UK. These include access to capital; lack of clinical data and long term evidence; clinician acceptance; NHS culture; and healthcare reimbursement, the latter of which is the major focus of this chapter.

The complex reimbursement pathways in place in the UK healthcare system can act as a significant barrier to the adoption and diffusion of products into clinical practice. The issues with the current system have been recognised by the authorities responsible for pricing within healthcare, including the Department of Health, the National Institute for Health and Clinical Excellence (NICE), as well as the Office for Fair Trading, and a new system of medicines pricing, known as ‘Value-Based Pricing’, was proposed and was set to be introduced in the UK by January 2014 (Office For Fair Trading, 2007; Department of Health, 2010). However,
as a result of the ambiguity surrounding its definition, the difficulties associated with its implementation, and the immediate voicing of concerns from key stakeholders, in recent years the new VBP scheme has been progressively eroded to the point where it effectively does not disturb the status quo.

Therefore, the following systematic review summarises the infrastructure, procedures and barriers within the current UK healthcare reimbursement environment and describes how this was proposed to change once the new ‘Value-Based Pricing’ (VBP) system was introduced. It goes on to describe the possible limitations of this new method of pricing and how it may have affected the adoption, reimbursement and pricing of regenerative medicine products. The impact of these proposed changes to the UK reimbursement methodology upon the cost-effectiveness analysis, and therefore the likelihood of achieving reimbursement, is also quantitatively modelled in Chapter 5.

The present chapter also examines the issue of Multimorbidity, including the current procedures and treatments in place within the UK healthcare system, and the potential impact the introduction of a VBP scheme could have exerted upon the likelihood of successful reimbursement of novel therapies which may target multiple simultaneous indications, for example hMSC-based therapies. The adaptation of a method of early cost-effectiveness analysis to account for therapies with the potential to target multiple simultaneous indications, such as hMSC-based therapies, is explored and modelled in Chapter 4.

Finally, this review describes the way in which the proposed VBP concept has been progressively dismantled, and discusses the potential implications of these changes for the reimbursement of regenerative medicines.

Therefore, a systematic literature review of available data was performed in order to briefly describe the current UK reimbursement environment, outline the characteristics and mechanisms behind VBP, evaluate how value-based pricing has been modified since its proposal, and propose how its introduction may impact regenerative medicine, particularly those products which target multiple simultaneous indications. However, due to a lack of available literature, information regarding recent alterations to the VBP scheme, and the possible implications of a change in pricing scheme upon RM, is mostly anecdotal and represents a review of the limited literature available as well as personal commentaries. Furthermore, this evolution of UK reimbursement was ongoing during the period in which
the work in this thesis was performed, demonstrating the rate of change in the field. A comprehensive literature review of this nature has not previously been published.

### 3.2 Methods

#### 3.2.1 Review Question and Study Protocol

This protocol-driven, systematic review is reported closely following the Preferred Reporting Items for Systematic Reviews and Meta-Analyses: The PRISMA Statement (Moher, 2009). The key research questions addressed in this literature review include “What are the current barriers in the UK healthcare reimbursement system?”, “How was the initial mission for VBP to be implemented?”, “How may the introduction of VBP affect the reimbursement of regenerative medicine products which target multiple simultaneous indications?”, “How has the VBP scheme changes since its proposal?”, and “How may the changes to VBP affect the reimbursement of regenerative medicine products which target multiple simultaneous indications?”.

#### 3.2.2 Eligibility Criteria

Literature referring primarily to UK reimbursement was the focus of this review, whereas articles referring to reimbursement outside of the UK were excluded. Any literature discussing both regenerative medicine and VBP was prioritised.

#### 3.2.3 Search Strategy

The databases through which the literature search was performed included ‘MEDLINE’, ‘Web of Knowledge’, ‘Science Direct’, and ‘British Medical Journal’. The search terms used to identify relevant literature included “Value-Based Pricing”, “Regenerative Medicine”, “Reimbursement”, “Multimorbidity”, “Value-Based Assessment”, “NICE”, “Willingness to Pay Threshold”, “Health Technology Appraisal”, “Pharmaceutical Price Regulation Scheme”, “Orphan Diseases”, “UK pricing agreement”, “Wider societal benefit”, and “NHS Resistance to Change”. Additional articles were discovered by searching the references of relevant, eligible papers.

#### 3.2.4 Data Abstraction & Synthesis of Results

The relevant, selected literature was independently examined and reviewed by the single author of the current review. Due to the qualitative nature of the isolated data, key statements and messages conveyed in the articles were noted and stored. This information was then
grouped depending upon the subject matter, and was often collated in terms of arguments for and against a certain point.

3.2.5 Study Selection

53 articles were selected as eligible for inclusion in this review, with over 100 articles, published between 1998 and 2015, screened for potential inclusion.

![Figure 1: Category & Publication date of articles selected for inclusion in this review.](image)

3.2.6 Limitations of the Literature Review

Due to the use of a single reviewer, an increased risk of bias may exist within this review and therefore there is an increased risk that inappropriate inclusion or exclusion may have occurred.

However, the main limitation of this review relates to the limited available literature regarding the recent changes to VBP and how any changes to the pricing system may affect the reimbursement of regenerative medicines which target multiple simultaneous indications. This scarcity in data sources is due to the developments in this area being recent, and often rapid, and due to the novelty of the subject area. Although these proposed changes to the UK reimbursement environment were often newsworthy, sufficient time did not pass for relevant research papers to be published and included in the present research.
Chapter 3: A Systematic Literature Review of the Transformation of the UK Reimbursement Environment, the Evolution of VBP over a Two Year Period & the Potential Implications for Regenerative Medicines which Target Multiple Simultaneous Indications

3.3 Results

3.3.1 Current UK Reimbursement

3.3.1.1 Aspects and Criticisms of the UK Healthcare system

The main authority that oversees reimbursement in the UK is the National Institute for Health and Care Excellence, or NICE. One of the primary functions of NICE is the appraisal and evaluation of new medical products, although the development of quality standards and the development of clinical practice guidelines also come under their remit. NICE also has established processes to identify new products that may have impact upon NHS, enable evidence of clinical and cost-effectiveness to be collated to inform judgement on the value of treatment, and issue guidance on whether a treatment can be recommended for routine use by the NHS (Parton, 2011, pp373).

It has been well reported (Department of Business Innovation & Skills, 2011; Ginty et al, 2010; Ginty et al, 2011; Plagnol et al, 2009; Rowley & Martin, 2009) that NICE, the systems in place within it, and the healthcare system around it, are considered to have difficulty accepting high up-front costs for new technologies, which may be a particularly relevant barrier to the adoption of regenerative medicines into the UK healthcare system, due to the high initial costs of such products. Rose & Williams (2012) explain that current NHS targets appear to be more focused upon improving patient throughput and cost-effectiveness than quality of care, and that price is a much more significant driver of decisions than in other healthcare systems. Ginty et al (2010) remark that the UK healthcare system, including NICE, may prioritise cost-effectiveness over clinical utility. This paper (Ginty et al, 2010) also highlights that the current perspective among members of the regenerative medicine and cell therapy industry is that the entire healthcare system in the UK is risk averse and conservative. This characteristic may affect the likelihood of innovative products, such as cell therapies, being used in the clinic and reaching patients.

The analyses of new technologies and treatments which NICE perform, known as Health Technology Appraisals (HTAs), provides the basis for most reimbursement decisions in UK healthcare. These HTAs could be viewed as a method of ‘rationing’ healthcare and informing resource allocation decisions (Drummond et al, 2008), and this could be considered to be one of the most important functions of HTAs (Pugatch & Ficai, 2007). This ‘rationing’ of healthcare has been observed in the past where, in order to fund NICE guidance, local authorities have had to cut existing services or not implement new ones, which could affect
the health of their patients (Towse & Raftery, 2009). Towse (2010) indicates the likely effect of inaccurate cost-effectiveness assessments, explaining that if NICE exaggerates the cost-effectiveness of a therapy to allow access for a particular sub group, then costs must be cut elsewhere and therefore an unknown sub group will not have access to an unknown treatment. This national control of healthcare allocation raises ethical concerns, and stems from the desire to control healthcare costs.

Further concerns have recently been raised regarding local healthcare purchasing decisions. In the past, once a product had been recommended for general use by NICE, after the completion of an appraisal, Primary Care Trusts (PCTs) were obliged to make funding available in order to purchase the technologies. However, in recent years, with the increasing responsibility of Care Commissioning Groups (CCGs), this requirement has been attenuated to the extent that CCGs, formerly known as General Practice (GP) consortia and which replaced PCTs, can now overrule NICE decisions and often may adopt if there is considerable patient demand, despite a negative NICE appraisal. Webb (2011) commented on this issue indicating that GP consortia may also choose not to purchase some of the more costly, but cost-effective, new products which could reignite concerns regarding ‘postcode prescribing’ which have been prevalent in the past. This ability to overrule NICE may have led to further, local rationing of healthcare and variations in patient care around the country, however NHS reorganisation has seen the legal obligation to fund NICE recommended treatment return.

The main component of these HTAs, performed by NICE, is cost-effectiveness analysis. In order to calculate how cost-effective a new therapy may be, NICE must determine the ‘incremental cost per QALY’ of the new product. Quality-Adjusted Life Years, or QALY, is a measure of “the quantity and quality of life generated by healthcare” (McAteer & Lilford, 2009). The quantity of resulting life is expressed in years and the quality of life generated is expressed in health utilities, with 0 representing death and 1 indicating perfect health. The use of the QALY unit has been criticised (Camps-Walsh, Aivas & Barratt, 2009) as it is considered to be a one size fits all approach which does not take into account patient variation, ability to live independently for longer, or return to work after treatment. Therefore, as cell therapy products may involve or result in such characteristics, the applicability of this unit is questionable. This is reiterated by Ginty and colleagues (2010) who explain that it may be difficult to quantify the reimbursable value of cell based therapies using QALY as
improvements in patient function and utility, and the reduced need for expensive care or equipment as a result of these therapies, is not accounted for in the QALY model.

Once the QALY of the new product has been specified, it is then compared to that of the current gold standard treatment and the difference is calculated (ΔQALY). The difference in cost between the current gold standard and the new therapy is then calculated, and this is divided by the change in effectiveness, or ΔQALY. From these calculations, an ‘Incremental Cost-Effectiveness Ratio’ (ICER), or ‘Incremental Cost per QALY’, is established. Finally, in order to decide whether this new product is cost-effective and whether a recommendation for reimbursement will be considered, the ICER is compared to the NICE, or Willingness To Pay (WTP), threshold. In the UK this threshold is set by NICE, and is between £20,000 and £30,000 per QALY. The cost per QALY of the new therapy must be below this threshold to be considered cost-effective without the requirement for additional supporting evidence. In recent years, there has been much controversy surrounding this NICE threshold. It has been indicated (Appleby, Devlin & Parkin, 2007; Collier, 2008) that the value of QALY is not consistent throughout the NHS and that the value may vary throughout the country. There is also concern with the lack of alteration to the threshold since its inception in 1999 (Appleby, Devlin & Parkin, 2007) and the fact that the threshold appears to be arbitrary with no basis in scientific theory or evidence (Appleby, Devlin & Parkin, 2007; Parton, 2011, pp374; Collier, 2008; Towse & Raftery, 2009). There is debate as to whether, and how, this threshold should change, and there is significant support for both an increase (Towse & Raftery, 2009) and decrease (Appleby, Devlin & Parkin, 2007; Towse & Raftery, 2009; Webb, 2011) to the threshold. Any change to the NICE cost-effectiveness threshold could have a substantial impact upon future reimbursement decisions and adoption of new therapies into the NHS, especially for those with high initial costs such as cell therapies. It is likely that any increase in the threshold would be beneficial to regenerative medicine products, however, if the threshold were to be decreased, translation of cell therapies from the laboratory to the clinic could be hindered further.

Despite the current issues with NICE, many of which have been explained above, it is still considered to be an international model for cost-effectiveness analysis, and prices set by NICE are often used as reference prices by countries around the world. Despite a positive NICE appraisal decision not guaranteeing reimbursement, utilisation or revenue (Rose &
Williams, 2012; Medical Research Council, 2012), it is considered to be a good indicator of quality and value in a product, particularly for clinicians (Rose & Williams, 2012).

As described earlier, there are significant barriers to regenerative medicine products within NICE, however the reimbursement challenges do not solely reside within this particular authority. The organisations under the control of which NICE acts, for example the Department of Health, must seriously consider implementing cultural and structural changes to the NHS and introducing new pathways for translational technologies, such as regenerative medicines, if these products are to be introduced into the clinic and the benefits to patients are to be realised (Parton, 2011, pp.370). However, such changes may be beginning to occur, and it appears that NICE may be leading the way. In the recent VALUE report, Parton (2012, pp.23) outlines that the initial thinking has been laid out regarding how the adoption of cell therapies can be optimised. Parton (2012) continues to explain that NICE were considering initially directing regenerative medicine products through the ‘Interventional Procedures’ pathway and consequently reviewing safety and efficacy before taking further action. The recently established NICE highly specialised technologies programme and NHS specialised services may also represent routes to adoption for regenerative medicines which very small patient populations. These changes signify the willingness of the UK healthcare system to adopt innovative regenerative medicine technologies, although, if such products are to treat patients on a large scale and revolutionise healthcare, entirely new pathways may be required to streamline translation. However, Parton (2012) also highlights that if this impact on regenerative medicine in healthcare is to be achieved, good clinical follow up data is key.

A report from the Medical Research Council (2012) also commented on the existing pathways into clinical practice which are in place for regenerative medicines, outlining that such therapies are not appropriately assessed using short term outcome measures, and that benefits that fall outside of healthcare are often not captured during assessment. The report (Medical Research Council, 2012) recommends that new appraisal procedures be introduced which include the full cost and savings across the entire treatment path and patient journey for novel therapies. The remedi project report (Rowley & Martin, 2009) also suggested that novel pricing schemes, such as provisional reimbursement or outcome-based reimbursement, may offer potential routes into healthcare by allowing for ‘risk sharing’ between industry and healthcare providers, which may provide incentives for innovative technologies to be adopted. More recently, the importance of the development of risk sharing schemes and innovative
business models between cell therapy manufacturers and the NHS has been affirmed (Regenerative Medicine Expert Group, 2015). A novel pricing scheme was proposed for implementation in the UK, the success of which is described in section 3.3.4.

Budgeting within the NHS has also been identified as a significant barrier for translation of cell therapies into clinical practice. In the VALUE report, Prescott (2012, pp.15) described that NHS budgets are managed annually, and thus, if a therapy is relatively expensive yet the benefits are accrued over a timescale that goes beyond the budget cycle, then adoption of these products may be limited. This near sighted approach to reimbursement is also present within hospitals, in that they are less concerned with the long term wellbeing of patients and more with the period in which they are liable. This is described as hospital ‘short-termism’ (Plagnol et al, 2009; Rowley & Martin, 2009).

Another issue regarding budgeting which can often affect reimbursement of therapies relates to the segmentation of departments within healthcare, known as ‘silo budgeting’. This refers to the paradigm that often, within the NHS, cost is accumulated in one department, yet the benefits are accrued in another (McAteer & Lilford, 2009). Rose & Williams (2012) apply this to cell therapy products explaining that “reimbursement of such products is difficult to justify as they are marketed to a payer who is not the beneficiary”. They (Rose & Williams, 2012) go on to explain that this often deters decision makers from investing in products that would be of benefit to the entire system. The issue of silo budgeting may also detrimentally affect the manufacturers of innovative RM products which have multiple-applications looking to enter their product into UK healthcare market. Products which can be utilised for more than one single indication may have to complete assessments and comply with the relevant regulations for more than one department of the healthcare system, and therefore may require multiple cost-effectiveness analyses and documents for the different departments (McAteer & Lilford, 2009). This would likely increase the regulatory burden upon them and increase expenditure. The effect of a transition towards value-based appraisal methodologies upon the reimbursement of therapies which simultaneously target multiple indications is discussed in section 3.3.3.2, and the adaptation of a method of early cost-effectiveness analysis to account for such therapies is presented in Chapter 4.

Lack of competition within healthcare has been highlighted as an undesirable characteristic of UK healthcare. The UK is considered to be a single-payer market, with the majority of
healthcare provision being undertaken by the NHS, and a minimal amount being performed privately, for example by The British United Provident Association Limited (Bupa) (London, UK). Therefore, hospitals and providers are not required to compete with one another in order to attract patients and earn revenue, unlike in other countries such as the US which have multiple private payers. Lack of competition is often thought to stagnate the industry and hinder innovation, as providers do not need to try to improve the quality of care they provide by funding treatments that would be considered expensive and risky, such as cell therapies. Plagnol et al (2009) explain that countries which are primarily privately structured are thought to offer a greater potential for reimbursement of regenerative medicine products, as providers compete among each other and want to take advantage of the latest technologies in order to attract the most patients, and therefore revenue.

The desire of clinicians to access the latest technologies and the subsequent clinical acceptance of new innovative products is thought to be another barrier within UK healthcare (Plagnol et al, 2009). Therefore a positive opinion may be important when clinicians are consulted regarding reimbursement decisions and the early involvement of clinicians could be vital (Ginty et al, 2011). However, healthcare professionals can often be a source of healthcare innovation and may have a demand for more efficient tools (Williams et al, 2008). Also, it has been suggested that surgeons may adopt new technologies, with encouragement from patients, despite little evidence of either efficacy or superiority over existing treatments (Wilson, 2006). This demonstrates that patient demand, often voiced through patient advocacy groups, can act as an important stimulus for adoption and could be a possible route into practice for some therapies.

3.3.1.2 Industrial Perspective

One of the most significant hurdles in translating new products into clinical treatments is the lack of understanding and cooperation between regenerative medicine companies looking to introduce their product into the UK healthcare system and the decision makers within the system. It is also clear that those within the private sector lack the relevant knowledge and expertise to facilitate adoption of their products, and are often uncertain regarding regulation and reimbursement (Plagnol et al, 2009). Plagnol et al (2009) also highlighted that those within industry perceived HTAs as unpredictable and that there was a lack of transparency regarding reimbursement decisions. It is believed lack of reimbursement decision clarity has hindered the success of cell therapy translation. One of the first cell therapy products, known
as Carticel® (Genzyme, MA, USA) was approved by the FDA as long ago as 1997, yet since this date very few regenerative medicine products have received positive HTA decisions and have reached the healthcare market (Warren & Matthews, 2012, pp.29). The VALUE report (Warren & Matthews, 2012, pp.29) also indicated that there is yet to be a significant volume of literature on HTA methods for cell therapies produced. This could make details regarding such decisions very difficult to come by for cell therapy companies, especially SMEs who are likely to be new to such pathways. It has also been recognised that product assessments are costly and take time, which can be detrimental to a company’s business plans. Thus many companies have called for clearer and more rapid pathways to be created for innovative cell based therapies (Plagnol et al, 2009; Collier, 2008). Representatives from the UK private healthcare sector, specifically Bupa, have acknowledged this need and created a specific algorithm which has been adapted from that used for the Interventional Procedures pathway, down which NICE plans to direct regenerative medicines (Warren & Matthews, 2012, pp.31). This novel process can more rapidly evaluate cell based therapies in order to inform reimbursement decisions, reducing decision times from weeks to hours. If such a pathway were to be implemented into the public sector reimbursement decision process, it would streamline adoption of cell based products and could substantially improve the market potential for UK regenerative medicine companies and increase the availability of such products within the NHS.

It is unsurprising that regenerative medicine companies looking to access the UK public healthcare market have numerous criticisms regarding the reimbursement processes in place, however the decision makers within the system also have criticisms regarding the applications provided by these companies. In a presentation given by a representative of RTI Health Solutions (Spinner, 2011), the main HTA agency criticisms of cell therapy reimbursement applications were outlined. HTA agencies indicate that the major issues with applications include poor trial design, insufficient trial duration, concerns regarding safety, and a lack of evidence of an incremental increase in efficacy or cost-effectiveness over the current gold standard treatments. However, companies have found collecting such detailed evidence to be difficult (McAteer & Lilford, 2009) and believe that the amount of data required is becoming unrealistic, especially for SMEs with limited funding (Plagnol et al, 2009). This requirement of data for reimbursement decisions has been highlighted by many studies in the literature, and the current opinion suggests that there is also a lack of data on...
indirect costs (Mason & Dunnill, 2008), which is considered detrimental for novel cell based therapies with high initial costs yet little on-going expenditure, a lack of long term benefit data (Ginty et al, 2010), and a requirement for a significant evidence base if cell therapies are to be integrated into clinical practice (Rowley & Martin, 2009).

As mentioned previously, the competition with current gold standard treatments, and the evidence of superiority over these treatments required, can hinder reimbursement prospects for many regenerative medicines. It has therefore been suggested that, in order to improve ease of access to market in the UK and reimbursement prospects, companies should target unmet clinical need (Ginty et al, 2011). It has been observed (Rowley & Martin, 2009) that cell therapies are rarely used in clinical practice, however when they are, it is often only once other treatments have failed as, compared to the gold standard treatments, cell therapies are often relatively expensive. Thus, to demonstrate step change in therapy outcomes as a result of regenerative medicine use, niche conditions where there is an unmet need should be addressed. Plagnol et al (2009) even went as far as saying that today’s barriers to adoption for cell therapies would not be present if such products targeted unmet needs. Prescott (2012, pp.15) also identified ‘orphan diseases’, those which are rare with few treatments, as a potential route into healthcare for regenerative medicines, as this would have little impact upon healthcare budgets. Due to the low patient numbers and likely high cost of treatment, these therapies are unlikely to be highly profitable; however this may represent a more straightforward route to adoption prior to branching into, and undertaking clinical trials for, indications with greater patient numbers.

The uncertainty surrounding reimbursement of new products is often neglected throughout the product development cycle and usually only considered after product completion when it is due to be launched. This can lead to funds being misguided channelled into products that may never reach the market. Early cost-effectiveness analysis and use of a model, such as the ‘Headroom Method’ developed by McAteer & Lilford (2009), can prevent this and provide data to allow investors to make informed decisions regarding funding. The utilisation and adaptation of this method is discussed in Chapter 4.

3.3.2 Introduction of a ‘Value-Based Pricing’ Approach

As discussed in section 3.1, the government were preparing to introduce a new pricing scheme for medicines in the UK as of January 2014. This aim of this new scheme, known as
‘Value-Based Pricing’ (VBP), was to improve patient outcomes, allow better access to medicines, stimulate innovation, improve value for money of treatments, facilitate more efficient use of resources, and account for a broader range of factors, other than cost-effectiveness, when pricing medicines (Easley & Tang, 2011). VBP also aimed to avoid setting a high cost for medicines that will only have a moderate benefit (Webb, 2011).

The idea behind VBP was to price treatments based upon the price at which the customer values a product relative to other products. Camps-Walsh, Aivas & Barratt (2009) explain that pricing new products based upon the perceived value of customers is an ideal method, however, due to the complexity of the healthcare market, this may be difficult to implement within the NHS. Therefore, they (Camps-Walsh, Aivas & Barratt, 2009) suggest an alternative definition which explains that VBP sets a “price that reflects value to patients, carers, society and the economy which delivers health benefits that exceed the health predicted to be displaced elsewhere in the NHS and in welfare, due to their additional cost”. Simply put, VBP was to account for the benefits of products exhibited throughout the patient cycle, and set a price for products that ensures that the incremental health benefits of the new treatment are greater than the health that will be lost as a result of channelling more funds into the new product and away from other departments within the healthcare system. These aims are well summarised by Claxton et al (2008), who explain that, “in the short term, VBP ensures that products are only approved at a price that ensures the benefits exceed health displaced elsewhere” however, “in the long term, will provide incentives for manufacturers to develop innovative technologies that are more likely to be of value to the NHS and be cost-effective”. This clear prioritisation of innovative therapies which provide significant benefit to patients could have favoured products with regenerative potential, such as cell therapies, and may have improved reimbursement prospects for such products, as discussed further in section 3.3.3.2.

However, this concept of ‘value’ to the customer is not straightforward, and will vary depending on who is asked. A large disparity could exist between what the NHS ‘values’ and what patients and other stakeholders ‘value’. However, in a survey of non-government stakeholders, it was believed that ‘value’ should reflect the value to patients and the wider costs to society and the economy (Camps-Walsh, Aivas & Barratt, 2009).
This chapter goes on to describe how the change to VBP could have affected future reimbursement and pricing of new medicines in the UK (Sections 3.3.2.1- 3.3.2.3), however, due to the lack of literature on the subject, the specific effects of the introduction of this new pricing regime upon reimbursement of regenerative medicines is implied and is based upon the literature available for the possible effects of VBP upon generalised medicines and pharmaceuticals (Section 3.3.3.2). This scheme would likely have been attractive for RMs as they can be expected to represent a cure.

Also, the adaptation and dilution of the VBP initiative that occurred, primarily due to an inherent resistance to change within the NHS and pressure from the pharmaceutical industry, is discussed later in section 3.3.4. Given the adaptation, and eventual rejection, of the value-based methodologies, the specific mechanisms that were originally proposed are outlined, however the suggested effects of the scheme, which are hypothetical, can only be postulated.

3.3.2.1 Implementation of Value-Based Pricing

Firstly, the possible mechanisms and manner in which VBP was to be implemented are highlighted, as the system and process changes that were to occur would have significantly affected the reimbursement of therapies. These changes are important to consider if the introduction of a regime similar to that of the original VBP mission is to be re-examined in the future, and, as discussed in section 5.1, it has been indicated that the approval process will be re-examined in the future.

As the introduction of VBP was to impact upon the way in which medicines are priced and reimbursed, the majority of system changes would have revolved around the organisation which plays a central role in reimbursement in the UK, known as NICE. As discussed earlier in section 3.3.1, the cost-effectiveness threshold used by NICE has been heavily criticised. However, the intent of the implementation of VBP was to shift this threshold from a predominantly NICE determined method to a government defined threshold and price setting method based upon unmet clinical need and disease burden. Although this new price setting method may have introduced more definitive system wide prices, reducing the uncertainty that was associated with cost per QALY threshold, it has been suggested that introducing government price setting could compromise VBP (Towse, 2010). However, NICE was to remain central to reimbursement decisions (Webb, 2011) and was to be given powers to account for savings beyond the borders of the healthcare system when assessing cost-
effectiveness (Rose & Williams, 2012). Also, in the past NICE have given positive or negative decisions regarding reimbursement of new technologies. However, in the proposed system change, NICE was no longer be obliged to say ‘no’ to expensive treatments, but could tell companies at what price they will say ‘yes’ (Webb, 2011), thereby setting a target for manufacturers.

A number of methods of VBP implementation were proposed, including an ‘ex post’ system, where ‘free pricing’ occurs at launch and is followed by reviews of cost-effectiveness in order to set the maximum price, or an ‘ex ante’ system, whereby products with sufficient cost-effectiveness evidence are ‘fast tracked’ for assessment and maximum price reflecting benefits is set when the decision to reimburse is chosen (Camps-Walsh, Aivas & Barratt, 2009). Each system has its own benefits and drawbacks, as described by Kavanos et al (2010). ‘Ex post’ is considered to offer a continuation of rapid access to medicines and was considered to be the preferred option for industry, due to the initial ‘free pricing’ which would offset future costs. However this system would put the NHS in a weaker bargaining position, as the withdrawal of products is not considered such a credible threat. An ‘ex ante’ procedure would not reimburse cost ineffective treatments, would provide the NHS with a better bargaining position, could increase the uptake of cost-effective medicines, and remove the timely NICE referral process. On the other hand, this ‘ex ante’ mechanism could have led to lengthy negotiations in the absence of sufficient evidence, reduce the adoption of products which could have future application and/or lead to further innovation, and increase manufacturer risk. It is clear that neither system is perfect, and therefore it is likely that a combination of both methods would be more suitable. A number of sources have implied an ‘ex ante’ price premium at launch followed by regular ‘ex post’ assessments would be the most beneficial mechanism of price setting (Claxton, 2007; Kavanos et al, 2010).

The pricing structure and negotiation methods that could be implemented for a VBP system must also be acknowledged, and these are comprehensively described and analysed by Claxton (2007). The author highlights the need for analysis of cost-effectiveness by subgroup, explaining that, within a single indication and a single population group, if the price of a technology is set so that the ICER is just equal to the NICE threshold, then there will be no net benefit of the technology to the NHS, illustrated in Figure 2a (below). However, within a single indication, cost-effectiveness may vary between sub-groups. Therefore, the price set for a technology and the possible ‘coverage’, or number of patients it can target, can be
varied. A higher price would result in a smaller patient coverage, whereas a lower price will increase the possible market for the product. This sub-group pricing method is illustrated in Figure 2b (below).

Figure 2a & b: Sub-group pricing—Claxton, K., 2007. OFT, VBP:QED?. Health Economics. 16(6), pp.545-558

Claxton (2007) also suggests the most favourable pricing method for both the NHS and the manufacturer in the short and medium term. Pricing ‘at the margin’ involves setting a price for the lowest cost-effective sub-group and therefore the greatest coverage. For the higher prices, the health benefits from the technology may just outweigh the health displaced elsewhere, however, at the lower price range, the manufacturer would be given unrestricted coverage approval, the net benefit to the NHS would be larger and the manufacturer’s revenue may increase.

Finally, Claxton (2007) outlines two possible negotiation strategies which could be employed after a pricing system change and which could affect the prices obtained and therefore the adoption of new treatments into healthcare. Firstly, the NHS could offer companies free choice from a ‘menu’ of prices and the associated coverage with their choice. However, this may be limited in that manufacturers will tend to choose higher prices with less coverage, whereas the NHS would prefer lower prices with greater coverage. Secondly, the NHS could offer a ‘take it or leave it’ deal, at full coverage and the lowest price, or nothing at all. However, the political consequences of this method could be substantial, and thus it is suggested that the pricing authority must be politically independent.

Although the new VBP scheme was aimed at increasing patient access, in 2012 the Secretary of State for Health at the time, Andrew Lansley, suggested that no more than 20 new products were to be assessed annually. This could have represented a rate limiting step in the achievement of reimbursement in the UK, and signifies the early recognition of the difficulty
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associated with the introduction of VBP. Also, products already on the market would not have undergone re-evaluation under VBP as NICE stated that they would not have the capacity to reassess products already on the market (National Institute for Health and Clinical Excellence, 2011).

3.3.2.2 ‘Weighting’ the Cost-Effectiveness Threshold

As mentioned previously in section 3.3.2, the proposed VBP scheme attempted to integrate other factors, not only cost-effectiveness, into HTAs and to consider these factors when making reimbursement decisions by giving them ‘weighting’. This method of analysis would have again used a basic cost-effectiveness threshold, set by the government rather than NICE, however, this threshold would have been flexible and could have been altered depending on how the product addressed a number of factors. Accounting for these alternative factors would consequently reduce the emphasis upon cost-effectiveness within NICE appraisals of new health technologies, which has frequently been criticised. The scale at which the new therapy provides benefit in these areas would determine how the threshold was altered and the threshold would be ‘weighted’ accordingly. Claxton et al (2008) expressed concern regarding this threshold, suggesting that it must be accurate, and, if the pricing scheme is poorly specified, the future evidence base for new therapies could have been damaged.

The factors, that were considered and to which weighting would have been given include:

- Medicines that treat diseases where there is a greater ‘burden of illness’. The more the therapy targets an unmet clinical need, the more the threshold will be increased
- Medicines that demonstrate greater therapeutic innovation and improvements on the current gold standard treatments
- Medicines that demonstrate wider societal benefits outside of healthcare

The first factor, relating to burden of illness and unmet clinical need, for which Figure 3 (below) illustrates a possible assessment framework, could have been particularly relevant to cell based therapies. As highlighted earlier in section 3.3.1.2, orphan diseases and areas of unmet need have been identified as possible routes into healthcare for regenerative medicines, and therefore, if companies begin to target these areas and conditions, then the price and reimbursement they could have received may have been greater than in past reimbursement systems. It is also plausible that the regenerative nature of these therapies could provide more realistic prospects of treating rare conditions, and those for which there is no current cure,
including chronic diseases, than other forms of therapy. Ginty and colleagues (2010) explained that regenerative medicines have the capacity to improve quality of life which may improve patient function and utility, and therefore reduce burden of illness and need for expensive care and equipment. They (Ginty et al, 2010) acknowledge that this was not accounted for in past QALY based models. Therefore, if regenerative medicines have the potential to reduce the burden of illness, it would be logical to suggest that they could have gained extra weighting in the proposed VBP scheme. It is also important to consider the methods of unmet need assessment. If epidemiology is used to evaluate unmet need, then the treatment of rarer conditions may have been prejudiced against, which may have hindered the achievement of reimbursement for cell based therapies if they were to target orphan diseases (Easley & Tang, 2011).

The second factor, for which weighting would have been given, involved the prioritisation of innovative treatments which improve upon the current gold standard therapies. Giving substantial weighting to therapies that demonstrate an improvement on the current, most commonly used treatments could have, in theory, increased prices obtained for cell based therapies and regenerative medicines, as the adoption of these innovative products into the NHS is often hindered, not by the lack of improvement in quality of results, but by their high initial cost and inability to demonstrate cost-effectiveness. However, in practice, implementing such a weighting system and obtaining an increased price from it, could have

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in fact been challenging due to the difficulty in providing evidence of superiority to meet the unrealistic expectations of the reimbursement authorities, as explained earlier in section 3.3.1.2 (Spinner, 2011; McAteer & Lilford, 2009; Plagnol et al, 2009).

The final factor, for which weighting was considered, related to the wider societal benefits that may occur as a result of a treatment. This aimed to allow for an increase in the price set for products which give benefit or reduce costs which are borne outside of healthcare. It may be obvious to suggest that, due to the usually shorter treatment period when using regenerative medicines compared to other methods of care, their use could reduce the length of hospital stay, length of nursing time and amount of dressing used (Plagnol et al, 2009), and thus reduce healthcare expenditure. However, outside of healthcare, despite chronic diseases being a major economic burden upon healthcare systems, reduced productivity of employees is in fact the most substantial economic burden of chronic disease (Prescott, 2012, pp.14). Therefore, if regenerative medicines have the capability to restore health, the time to return to work and the dependence upon social care of patients may be reduced (Prescott, 2012, pp.14). Also, the increased absenteeism, reduced worker capacity and worker productivity, as a result of chronic disease, could be attenuated (Prescott, 2011).

However, as beneficial to innovative therapies and favourable to regenerative medicines this weighting seemed, in practice the implementation of such a scheme could have presented challenges due to the current lack of direct and indirect cost data for new treatments, such as cellular based therapies, with high initial costs but low on-going expenditure (Mason & Dunnill, 2008). Also, prioritising treatments which enable wider societal benefits raises questions as to what exactly would be defined as a societal benefit under VBP. Factors including an overall decrease in the loss of the country’s economic output due to improved health and reduced disease (Prescott, 2011); patient age and disease type (Camps-Walsh, Aivas & Barratt, 2009); restoration of function to patients whom contribute the greatest burden upon healthcare, specifically elderly and obese, thus reducing pensions and increasing workforce size (Mason & Dunnill, 2008); and ‘multimorbidity’ (Prescott, 2011) have all been suggested in the literature. In the paper by Prescott (2011), it is described that the inclusion of such factors would have been implausible and that, although many of these suggested factors have cause to be accounted for when weighting the threshold for new products, some may be difficult to calculate, require extensive evidence or long clinical trials in order to establish the time period of benefit, which would increase assessment time, complexity and cost. However,
although complexity of calculation may be a limiting factor to the use of numerous beneficial wider societal factors in new product assessment, it is not specific to this category. Easley & Tang (2011) have indicated that this complexity of weighting calculation may be true of all the proposed factors to be used for weighting the cost-effectiveness threshold, and is a possible drawback of the VBP process. Further potential drawbacks are discussed in the next section, and these are also summarised, along with concerns regarding the current reimbursement environment, in Appendix 13.1.

The latter of these suggested factors from the literature may have particular relevance to regenerative medicines, in that initial use of a cell based therapy may delay or completely diminish the need for treatment of the multimorbid patient in the future (Prescott, 2011). This condition, and how it could have been affected by VBP, is discussed in more detail in section 3.3.3.1.

3.3.2.3 Potential drawbacks of Value-Based Pricing

As described above, a number of concerns regarding the implementation and calculation of the threshold weighting system have been voiced, and NICE have indicated that VBP may have led to double counting of benefits already accounted for in their cost-effectiveness analyses (National Institute for Health and Clinical Excellence, 2011). Also, despite the apparent potential benefits of the introduction of a VBP scheme to new healthcare products such as regenerative medicines, the literature suggests that there were some who were also concerned with the possible effects that VBP could have had upon reimbursement, adoption, the NHS as a whole and the entire UK economy.

Two main papers, both written after the proposal of a VBP system by OFT (2007) yet prior to the DoH’s statement regarding its implementation (2010), examined the potential limitations of the proposed VBP regime. The first, written by Claxton et al (2008), discussed the detrimental effects that introducing the new pricing scheme may have predominantly on a nationwide healthcare market level. This paper criticises the new pricing system by describing the effect it could have had upon products which do not fulfil an unmet clinical need. The authors explain that VBP may have disadvantaged treatments which are ‘second to market’ and that they may not have received a similar weighting to that of the incumbent and therefore would not have commanded a similar price. They also argue that innovative technologies, which may lead to the development of more valuable technologies in the future,
may not have been recognised in the proposed VBP scheme and would therefore be unlikely to obtain adoption. Claxton et al (2008) describe that the possibility exists that VBP may have led to reduced NHS spend, due to the reallocation of revenue from less to more valuable treatments which are likely to be more uncommon. However, the authors also emphasise that, if a substantial number of new treatments of greater value are created, then national spend may have in fact increased.

The second paper (Camps-Walsh, Aivas & Barratt, 2009) identified some of the possible disadvantages of the VBP method on a smaller scale, highlighting the more specific and product level effects that the introduction of VBP may have incurred. As mentioned previously in section 3.3.1.2, stakeholders in industry believe that they have already reached the limit of their evidence generating capabilities, whilst still being commercially viable (Easley & Tang, 2011). However, Camps-Walsh, Aivas & Barratt (2009) propose that VBP would increase the requirement for manufacturers to collect evidence regarding effectiveness, long term effects, possible side effects, possible economic and social effects, and potential new indications of their products. This would have increased the time and cost of collecting evidence for appraisal.

This paper (Camps-Walsh, Aivas & Barratt, 2009) also outlines two possible underlying aims and rationale behind this proposed pricing method. The first was a genuine attempt to reward innovative treatments resulting in substantial benefit and improved health outcomes for patients. The second being an effort to control and cut costs by reducing the prices paid by the healthcare service to companies for treatments. It has been recognised that the introduction of a pricing system based upon ‘value’ could have reduced the prices, obtained by large pharmaceutical companies, for premium price, branded drugs which are not substantially better than competitors and do not offer significantly greater health outcomes (Claxton, 2007). This substantial risk for pharmaceutical companies may have contributed to the adaptation of the proposed VBP scheme which has taken place in recent years, and is discussed in greater detail in section 3.3.4. It was also calculated that a cut in expenditure on premium drugs could lead to NHS savings of £500 million (Claxton, 2007; Kavanos et al, 2010). Camps-Walsh, Aivas & Barratt (2009) also hypothesise that if the aim of VBP was in fact to cut costs, then it may have led to increased prices at launch to cover NICE appraisal costs, further adoption delays, pricing instability, greater expense and bureaucracy for companies, and impact upon worldwide pricing due to the regular use of the UK as an
international benchmark price. This reduction in prices in other countries could have affected company returns across the world. However, the counter to this argument is also provided, and it is proposed that this reduction in worldwide pricing may not have occurred and VBP may have in fact caused companies to think more carefully about the evidence they collect and the products in which they invest, which may have stimulated increased innovation.

The final major criticism that this paper presents suggests that, if VBP was to be introduced as a cost-cutting exercise, this may have added uncertainty to company return on investment due to the complex weighting system. They (Camps-Walsh, Aivas & Barratt, 2009) also found that there was a consensus among stakeholders that the obtainable price for new technologies would likely be reduced with the introduction of VBP. This stakeholder resistance, as discussed later in this review, may also have contributed to the changes that VBP has undergone in recent years. The uncertainty in return on investment could have deterred medical companies from attempting to access the UK market in the future, led to delayed UK product launch, resulted in the UK becoming a ‘second market’ (Towse, 2010), and caused the relocation of companies away from the UK (Claxton et al, 2008). The Medical Research Council (2012) has acknowledged that, given the state of the reimbursement environment in the UK, the development of regenerative medicines abroad may become common. This may have been further promoted by recent changes to the regulatory system in Japan. It has therefore been highlighted that this shift away from the UK could affect both patients and the economy (Towse, 2010). Camps-Walsh, Aivas & Barratt (2009) proposed that delaying product launch in the UK could potentially lead to a decrease in the number of clinical trials, amount of research and development, and clinical trial funding; a delay or lack of availability of new treatments, and therefore delayed access and reduced health outcomes for UK patients; reduced UK medical company employment; and reduced UK medical company investment.

It may be logical to assume that, under VBP, a reduction in prices may have occurred for many products and that this could significantly impact industries, such as pharmaceuticals, where there are large numbers of premium price treatments which do not fulfil unmet need or display significant improvements over other products. Companies may have also had difficulty presenting evidence of the required quality to obtain higher prices. However, whether the reduced pricing associated with VBP would have occurred for novel therapies, such as regenerative medicines, is not so obvious, although given the potential for these
technologies to meet the criteria for increasing the threshold weighting, there can be optimism that prices set for these products in the UK may not have been significantly diminished if VBP was to be introduced. However, the impact of recent modifications to VBP upon the reimbursement of regenerative medicines, and therapies which target multiple simultaneous indications, is yet to be determined and is discussed further in section 3.3.4.6.

3.3.3 Multimorbidity

At the 2012 NICE Annual Conference, the NICE chairman at the time, Sir Michael Rawlins, identified multimorbidity as an area in need of incorporation into NICE guidelines, and that NICE must move beyond giving advice to GPs based upon single conditions.

In their definitions, multimorbidity and comorbidity are very similar in that they both refer to the presence of multiple diseases or conditions in a single patient. It seems obvious to suggest that the difference between the two is simply a difference in the number of simultaneous conditions, however the difference appears to be more subtle than this. Nagl et al (2012) accurately describe this by giving more detailed definitions of each. They intimate that comorbidity “examines the presence of any additional disease pattern regarding a patient who is affected by the index disease under study” and define multimorbidity as “the parallel existence of two or more diseases within a patient”. The discrete disparity between the two definitions indicates that multimorbidity refers to the multiple conditions as having equal importance, whereas comorbidity describes them as being secondary to the major illness, and this reflects the perspective of how multimorbidity should be treated. Multimorbidity can be clearly demonstrated using an example such as Non-Insulin Dependent Diabetes Mellitus (NIDDM). This condition is associated with numerous circulatory disorders; such as Coronary Heart Disease, stroke, peripheral arterial disease, cardiomyopathy, and congestive heart failure; as well as disorders of the eyes, kidneys and nerves (Buse et al, 2007). These associated complications can occur as a consequence of NIDDM, or often as a result of the lifestyle choices responsible for the onset of NIDDM.

The prevalence of multimorbidity is thought to be very high in modern society, however the exact figures often vary depending upon the population under examination. Increasing age and lower affluence, which can vary between populations, are significant risk factors for the onset of multimorbidity (Barnett et al, 2012). Guthrie et al (2012) found that, in more deprived areas, multimorbidity can develop 10-15 years earlier than in more affluent areas.
Chapter 3: A Systematic Literature Review of the Transformation of the UK Reimbursement Environment, the Evolution of VBP over a Two Year Period & the Potential Implications for Regenerative Medicines which Target Multiple Simultaneous Indications

Nagl et al (2012) commented on the effect of age, explaining that “an increased life expectancy is accompanied by chronic illness which entails coexisting morbidities”. Due to this increased susceptibility in the elderly and the ageing of society, it is thought that the prevalence of multimorbidity is rising (Haggerty, 2012), making it a pertinent issue in modern healthcare. Therefore, any changes in healthcare, such as the introduction of VBP, should look to take this issue into account. Whether the proposed VBP scheme, or the recently modified version of this system, would have acknowledged this problem is examined in section 3.3.4.6.

However, the estimated prevalence of multimorbidity within populations is often inaccurate and can vary substantially depending on the way in which multimorbidity is defined and the method of data collection used (Glynn et al, 2011). It has also been reported that overestimation of prevalence can occur due to use of clinician records rather than a representative sample (Barnett et al, 2012), and underestimation can occur due to lack of reporting (Glynn et al, 2011).

This review now goes on to describe how those patients afflicted with multimorbidity interact with the healthcare system and the way in which treatment of these patients occurs. The way in which the proposed VBP system may have had an impact upon the reimbursement and adoption of treatments which may significantly benefit the multimorbid, specifically regenerative medicines, is also discussed. However, due to the limited volume of literature regarding the interaction of VBP, multimorbidity and regenerative medicines, a large proportion of this discussion represents speculation and conjecture.

3.3.3.1 Current Treatment of Multimorbidity

Currently, there are few standardised procedures in place to treat and manage multimorbid patients within UK healthcare, and it is clear that clinicians have a very limited knowledge of how to treat these patients (Boyd & Fortin, 2010). UK healthcare currently adopts a single disease approach, where care of the multimorbid focuses upon one disease at a time, and care provision is fragmented (Barnett et al, 2012). It has been indicated that this narrow approach to healthcare increases healthcare costs and utilisation (Glynn et al, 2011; Nagl et al, 2012). This highlights the substantial need for an integrated clinical plan, continuity of care (Nagl et al, 2012; Haggerty 2012), and better communication between departments of the healthcare system, between which these patients are often transferred, and it has been suggested that
patients with multimorbidity could benefit from having a dedicated physician responsible for
the coordination of their care (Barnett et al., 2012; Haggerty, 2012). The use of many services
by each multimorbid patient, in order to manage each individual disease with which they are
afflicted, can lead to treatment becoming duplicative and unsafe for patients (Barnett et al,
2012) as the treatment of one condition may cause undesirable consequences with regards
another (Campbell-Scherer, 2010).

The increased cost of multimorbidity has been examined in a number of studies, and cost has
been shown to increase exponentially with the number of chronic conditions (Campbell-
Scherer, 2010; Glynn et al., 2011; Nagl et al., 2012). Examples of cost data which
demonstrates this include the seven times greater cost of multimorbid patients than those with
a single chronic condition (Prescott, 2012, pp.13); a variation of 11.5% in cost explained by
multimorbidity, within advanced elderly population, which appeared to be a stronger
association than age (Nagl et al., 2012); and an increase of one chronic condition within a
multimorbid patient being associated with a €563 increase in cost per year (Nagl et al., 2012).
However, the cost associated with multimorbidity may vary depending upon the population
under examination, the accuracy of the data available, and whether indirect costs were taken
into account (Glynn et al., 2011).

Another difficulty associated with the treatment of patients afflicted with multimorbidity is
the need to detect and diagnose these patients as early as possible in order to effectively treat
them. Aarts et al (2010) describe this further stating that “early identification and monitoring
of people with multimorbidity could provide opportunities for controlling the negative health-
related consequences of multimorbidity”.

Current clinical trial protocols also represent a significant barrier to the improvement in care
for those patients with numerous, simultaneous medical conditions. The extent to which
samples selected for clinical trials are representative could determine the care which
multimorbid patients receive, and may represent an area of opportunity for novel therapies,
such as regenerative medicines. It has been well reported (Barnett et al., 2012; Campbell-
Scherer, 2010) that trials for new therapies and technologies often exclude the elderly and
multimorbid due to the difficulty and risk associated with their treatment. Those afflicted
with multimorbidity are then often prescribed these treatments, if they are affected with the
relevant medical condition, despite the presence of associated, and potentially conflicting,
illnesses and the lack of efficacy evidence in multimorbid patients. This raises concerns regarding the applicability of therapies, for which the trials excluded those with multimorbidity, for the treatment of the multimorbid. As mentioned previously, due to the growing prevalence of multimorbidity and its burden upon healthcare, the inclusion of affected patients in clinical trials should be considered in order to identify and establish improved therapies for the treatment of multimorbidity. Any change in sample selection for clinical trials involving the inclusion of the multimorbid could represent a substantial opportunity for regenerative therapies, such as cell therapies, due to their potential to restore normal health and function.

Other solutions and processes aimed at counteracting the issues present within the current single disease based approach, have been suggested in the literature. Campbell-Scherer (2010) suggests the use of a ‘payoff time calculation’, which determines the “minimum elapsed time until the cumulative incremental benefits of an intervention exceed their cumulative incremental harms, weighed against the patient’s comorbidity-adjusted life expectancy”. It has also been highlighted that tailoring clinical guidelines at the point of care (Braithwaite et al, 2007), and the collation of relevant guidelines for different conditions in order to identify synergies, cautions and contradictions (Guthrie et al, 2012) could improve the fragmented healthcare system currently in place.

### 3.3.3.2 Effect of VBP upon treatments which target Multimorbidity

It is plausible to suggest that innovative technologies with regenerative capabilities, such as cell therapies, may diminish or postpone the development of multimorbidities or attenuate the severity of the conditions and their symptoms. This opportunity has been recognised by one particular author, in multiple sources, and the consequences regarding pricing of such therapies were questioned. Prescott (2011; 2012, pp.14) proposes; as part of the national, TSB funded VALUE project which aimed to tackle issues such as the adoption and reimbursement of RMs; that if regenerative medicines could repair or replace diseased or damaged cells, and potentially ‘cure’ disease or better manage the cause, this would diminish or delay the onset of associated conditions. The author goes on to explain that this restoration of normal function could lead to cost benefits over time, due to the lack of, or delayed, requirement to treat and care for those with associated medical conditions which could reduce indirect costs. This brings about questions regarding how treatments such as regenerative medicines should be valued. Should these indirect savings and the reduced health and social
care burden be accounted for when assessing such products? Prescott (2011) argues that, if these products are to be appropriately assessed, in principal, these benefits should be taken into consideration. However, the author also acknowledges that sufficient evidence of this would be required and the length of time for which these benefits are sustained must also be recognised. To collect such a significant amount of evidence would require extended clinical trials, and this would increase application time and complexity for companies, therefore increasing cost to the extent to which it may become prohibitively expensive (Prescott, 2011).

However, if the original plans for a VBP system were to be fully implemented and sufficient supportive evidence could be displayed, the wider societal benefits of the use of novel cell therapies for the treatment of the multimorbid could have induced a substantial weighting upon the cost-effectiveness threshold used in appraisals to the benefit of the new products. It has been noted that treatment with regenerative medicine products will often reduce the length of hospital stay, length of nursing time and amount of dressing used (Plagnol et al, 2009), therefore reducing healthcare expenditure in the long term. The potential regenerative nature of these products also has the potential to attenuate the economic burden of chronic diseases, improving productivity and time to return to work and reducing dependence upon social care (Prescott, 2012, pp.14). Clearly the use of these products in healthcare could induce a number of wider societal benefits, however, in the case of therapies which target multiple indications simultaneously, this advantage could be even greater, as those with multimorbidity have greater self-care and social care needs (Boyd & Fortin, 2010). This could have increased the threshold making the reimbursement, and likelihood of reaching the UK healthcare market, of these products more feasible.

Also, the regenerative potential, and capability to diminish or delay the onset of associated medical conditions, of these therapies, could reduce patient ‘burden of illness’. It could also be suggested that, due to the high prevalence of multimorbidity and the inadequacy, and potentially detrimental effect, of using multiple treatments concurrently, the treatment of multimorbidity could be considered an unmet need. As these factors were also proposed to be accounted for in VBP, the ability to restore normal function and reduce the severity of symptoms may have also influenced the cost-effectiveness threshold through a positive weighting. Finally, it is also possible that, due to the poor current standard of care for patients afflicted with multimorbidity and the potentially regenerative nature of cell therapies, these
products may represent an improvement upon the gold standard treatment and thus may have also received a more positive weighting as a result.

Therefore, it is conceivable that, the introduction of the proposed VBP method could have led to the increased consideration and positive weighting for regenerative medicines which target and treat multimorbid patients. It would also suggest that under a VBP scheme, along with orphan diseases, multimorbidity may have represented a niche for which cell therapies companies may have wished to aim, and may have acted as a potential route into UK healthcare. However, as discussed later in this chapter, a number of alterations to the original VBP mission have occurred since its proposal which may significantly impact the reimbursement of these products, and therefore these changes could determine the future adoption of regenerative medicine products. This highlights the dynamism of the field and the rapid changes of incentive for manufacturers.

3.3.4 The transformation of VBP over time & the maintenance of PPRS

3.3.4.1 Initial Reactions to VBP

The proposition of a shift in pricing scheme away from previous models of pricing negotiation between the manufacturer and the state towards a value-based approach was quickly met with reservations from a significant number of stakeholders. The list of initial concerns was substantial, as discussed previously in section 3.3.2.3, these included apprehension regarding increased government control of price setting (Towse, 2010), difficulty determining a unanimous definition of ‘value’, a lack of data concerning the indirect cost of medicines (Mason & Dunnill, 2008), increased data requirements to satisfy reimbursement authorities and inform appraisals (Spinner, 2011), and uncertainty of a return on investment in a new medicine (Camps-Walsh, Aivas & Barratt, 2009). The main drawbacks of VBP highlighted, however, involved the UK healthcare market and were expressed by those stakeholders within the pharmaceutical industry. Many within the industry feared that the introduction of VBP would decrease the price that the NHS pays for traditionally premium price, branded drugs from which pharmaceutical companies make significant profit (Claxton, 2007). Also, it was unclear as to how the change to VBP would affect new medicines from other areas of the healthcare industry. These fears of stakeholders from the pharmaceutical industry were compounded with the suggestion of international repercussions of a decrease in medicines pricing in the UK, due to the international pricing benchmark status of NICE (Camps-Walsh, Aivas & Barratt, 2009). Concerns that the UK
may become a second market for the launch of new medicines were also expressed, as a consequence of the low pricing in the VBP scheme, and this may in turn have an impact upon R&D funding (Towse, 2010).

3.3.4.2 The Dilution of VBP

In the following months, as a result of the extensive criticism of the VBP initiative, the absence of purposeful leadership, and the relatively short timeline designated for its implementation, the government began to retreat on its proposals and compromises began to occur (Department Of Health, 2011). In the initial strategy, the new VBP scheme would apply to all medicines, however, by 2011, this had been swiftly reduced to apply only to new products after the scale of the initial plans were realised and their lack of feasibility was apparent. It was decided that drugs that were already established in the NHS would remain under the Pharmaceutical Price Regulation Scheme (PPRS) agreement in which companies can maintain free pricing of products which allows them to incorporate significant profit margins. Although the plans to re-appraise and re-negotiate all medicines used within the NHS had been ambitious, this was the first case in which VBP had succumbed to stakeholder pressure. A number of key withdrawals by the government followed this decision, most significantly the discarding of the proposal for an expert panel to perform value assessments and the consequent delegation of this role to NICE in early 2013 (Raftery, 2013). Although NICE are clearly best positioned to perform such assessments, this move saw the change to VBP becoming described as an alteration to the current NICE cost/QALY framework rather than an overhaul. This retraction of the original VBP mission was further continued and, due to constant lack of consensus, it was announced in October 2013 that the introduction of the VBP scheme was to be delayed until late 2014.

However, in November 2013, a drastic turnaround to government’s proposed VBP scheme occurred, and alterations were made to fit the assessment of ‘value’ within the current pricing framework and consequently render the scheme unrecognisable in comparison to the original mission. Although VBP was initially designed to replace PPRS, it was announced in a Department of Health (DoH) and Association of the British Pharmaceutical industry (ABPI) (2013) White paper that VBP would be integrated into the PPRS as of the autumn of 2014, and was to be known as Value-Based Assessment (VBA). This VBA methodology was to be combined with statutory schemes, including patient access schemes, and the previous PPRS to form the UK pricing agreement which would be maintained from late 2014 until 2018. As
mentioned previously, the value assessments were to be performed by NICE, however NICE would not have been responsible for price setting and negotiation, which would have remained under the remit of the DoH. It was also specified that the NICE threshold would remain at the current level for the duration of the UK pricing agreement. In late January 2014, NICE laid out its strategy for the assessment of value in new medicines, and these were opened to public consultation in February 2014. As a result of this meeting, NICE decided to incorporate burden of illness calculations into their QALY system, however they chose to reject the government’s proposal of the integration of wider societal benefits as it could not be comfortably integrated into NICE technology appraisals (National Institute for Health and Care Excellence, 2014). Alternatively, they proposed the introduction of ‘wider societal impact’ assessment, a calculation which would involve the measurement of the proportional and absolute QALY shortfall experienced as a result of having a condition with current treatment. This VBA strategy appeared to be a continuation of the NICE cost per QALY system, demonstrating NICE’s reluctance to adapt and further transforming and diluting VBP into an entity which more closely resembled the current model.

The PPRS was to persist in a similar fashion to that of previous pricing schemes, within which many stakeholders have benefited and are now entrenched, and was to serve as a component of the latest UK pricing agreement. PPRS allows for free pricing of medicines, where companies can set a price for their product which includes profit margins, as well as negotiation between the manufacturer and payer. One of the key features of PPRS is the cap which is set upon company growth in income from sales annually (Parliamentary Office of Science & Technology, 2010). This requires companies to pay the UK government once they exceed a specified level of growth, relative to the amount by which they exceed this pre-agreed level. This is seen as mutually beneficial as it provides predictability for the government and does not require a change of company list prices. This favouritism of the PPRS mechanism is likely to have played a significant role in the dilution of the original vision for VBP.

3.3.4.3 Favourable Compromise

Whilst attending the Westminster Health Forum’s Keynote Seminar regarding ‘The developing role of NICE and the future of value-based pricing’ in January 2014, which took place after the decision to move towards a VBA approach had been made, the opinion of key stakeholders, regarding the changes to VBP and the future of VBA, was gathered (Personal
Communications, 2014). Based upon the opinions expressed by key stakeholders from the NHS, NICE, a variety of charities, academia, and the pharmaceutical industry; it is clear that the proposed VBP scheme was not considered to be favourable, even seven years since its proposal. A number of attendees, including Julia Manning (Chief Executive, 2020 Health) and Eric Low (Chief Executive, Myeloma UK), commented that they were concerned about the VBP scheme from the outset and that they were also pleased to see the continuation of PPRS. In a keynote presentation by Paul Catchpole (Value & Access Director, Association of the British Pharmaceutical Industry), it was identified that the preservation of free pricing in PPRS was critical to maintain the UK as a first launch market for new medicines, which may be important for the UK economy, and that accounting for a broader definition of value within PPRS was a good compromise. The delay in the introduction of value assessments was viewed positively by stakeholders, including Julia Manning and Drew Lindon (formerly Head of Policy and Campaigns, Prostate Cancer UK), as it would allow for more time to form a consensus regarding the definition of wider societal benefits and as the VBP scheme, as originally proposed, was perceived as a drastic change requiring more consideration. Generally, the concerns regarding value-based assessments remained similar to those that were expressed upon the proposal of VBP, although these were moderated by the adaptation to VBA and the integration with PPRS. The apprehensions regarding the assessment of wider societal impact remained present and, in particular, Julia Manning expressed fears that this method of assessment for medicines may disadvantage elderly patients, nearing the end of life, that contribute less economically, and may have prevented them from accessing more expensive treatments. However, the PPRS and UK pricing agreement was recognised as favouring innovation, as new medicines would not be accounted for in government ‘claw backs’ once companies exceed the pre-agreed level of growth, as noted by Paul Catchpole. Nevertheless, it is difficult to see the proposed integration of VBA within PPRS as anything other than a compromise and an attempt to resist significant change by stakeholders.
3.3.4.4 Rejection of VBA

Despite the progress made to outline the VBA methodologies for the weighting of the ICER of a new technology and the NICE threshold, based upon the burden of illness and wider societal impact (NICE DSU, 2013; NICE DSU, 2013), debate continued and significant stakeholder resistance remained. As a result, after the results of the public consultation were determined and a NICE review was completed, in September 2014, NICE recommended that no changes to the technology appraisal methodology be made in the short term, and therefore the current reimbursement system will be maintained for the foreseeable future (NICE, 2014). A summary table and timeline describing the changes to the VBP scheme over time is presented in Appendix 13.2.

It is unlikely that any value-based appraisal methodologies will be implemented in the future given the significant debate, stakeholder resistance and implementation challenges. However, the topic of reimbursement methodology is likely to be revisited in the future, as described in section 5.1. Although the cost per QALY system can be expected to be maintained, the streamlining of the approval process, the development of innovative business models, and the prospect of risk sharing may be addressed.

3.3.4.5 NHS Resistance to Change

It would be overly critical to lay the blame for the degradation of VBP and VBA entirely at the feet of the pharmaceutical industry, the products of which make up around £12 billion or 10% of total NHS spending. However, with the proposal of VBP, the risk to the price of branded drugs and to the profit margins of their manufacturers was undeniable, and the
opposition of ‘Big Pharma’ was unmistakable. One of the most considerable factors that has led to the abandonment of VBP and VBA is the inherent resistance to change within the NHS which causes delays and further consultation in the majority of proposed NHS modifications, which has been reported as far back as 1998 (Plamping, 1998). A more recent demonstration of this resistance to change was observed during the transition from Primary Care Trusts (PCTs) to Care Commissioning Groups (CCGs) in 2010. Considering the interests of the pharmaceutical industry and the resilience of the NHS in preserving the status quo, PPRS is harmonised with the views of the key stakeholders, allowing free pricing of new products for pharmaceutical companies, the receipt of payments in cash by the government from companies that exceed the cap on allowed growth, and the lack of significant changes in transactions between the NHS and the pharmaceutical industry. Therefore, it is perhaps no surprise that PPRS has been maintained whilst VBP has been progressively dismantled and adapted to fit with current pricing systems.

3.3.4.6 Reflections of VBA upon Regenerative Medicine and new unconventional technologies

The implications of the failure to introduce the VBP scheme could have been substantial, and here the impact of the VBA scheme upon the reimbursement of new products outside the conventional pharmaceutical sector, for instance regenerative medicines, is considered. Due to the relatively early stage development of the RM industry, compared to that of the pharmaceutical industry, stakeholders are currently attempting to establish reimbursement and adoption pathways for RMs in the UK, and therefore the recent proposal and deterioration of VBP may have only added to the uncertainty they face. Earlier in this chapter (Section 3.3.1.2), where the possible consequences of the proposed VBP system for the reimbursement of RMs were addressed, it was suggested that this change in pricing scheme may be advantageous for RMs due to the introduction of the NICE threshold ‘weighting’ system. However, whether VBA would have increased the likelihood of reimbursement for RMs has yet to be determined. The inclusion of wider societal impact in NICE value assessments may have also been favourable for RMs, however in the proposed UK pricing agreement these benefits may not have had as much influence upon pricing as they may have done in VBP. Burden of illness and unmet clinical need would have again been accounted for in VBA, and therefore these factors may have again been favourable for RM products during appraisals. However ‘therapeutic innovation’ would not have been favoured under VBA as it would have been in VBP, which may have hindered the reimbursement potential of RMs.
Previously, in section 3.3.3.2, it was highlighted that the introduction of VBP may have been advantageous to RMs which target numerous indications simultaneously, as addressing the ‘multimorbidity’ issue may have fulfilled each of the three factors within the VBP weighting system and therefore would have reflected positively on RM pricing. Furthermore, during a keynote talk given by Gavin Lewis (Head of Pricing and Market Access, Region Europe, Roche) at the Westminster Health Forum’s Keynote Seminar (Personal Communication, 2014), it was recommended that value-based assessments reflect the value of the product across all indications rather than in the lowest value indication alone. Given the inclusion of a number of the main VBP weighting criteria in the VBA appraisal methodology, although not to the same degree, it is likely that VBA would have also been favourable for the reimbursement of RMs that target multiple indications simultaneously.

However, with the rejection of the VBA scheme, it is unlikely that RM technologies will receive any additional weighting in the appraisal process or reimbursement decisions. This would suggest that innovative business models, risk sharing strategies between manufacturers and the NHS, and novel adoption and reimbursement pathways, such as the NICE ‘Highly Specialised Technologies’ programme which was introduced on a trial basis in early 2014, will become increasingly important for the reimbursement of high-value, unconventional technologies. Furthermore, the rejection of the proposed value-based appraisal methodologies increases the impetus on manufacturers to deliver cost-effective products if they are to achieve reimbursement through the typical HTA pathways.

Also, whilst attending the Keynote Seminar, Eric Low (Chief Executive, Myeloma UK) noted that innovation in care is currently lacking in the NHS and it may in fact be de-incentivised (Personal Communication, 2014). This may inhibit the adoption of new healthcare technologies such as RMs. This discouragement of innovation may be associated with the resistance to change which is characteristic of the NHS, which in itself may hinder the translation of RM therapies by impeding changes to care pathways, care commissioning, and reimbursement. Thus, accelerated routes to market, for example patient access schemes, may be influential in providing evidence to support the adoption of RMs in the NHS and to counteract the innate resistance to change.

As of January 2014, an advisory group of key stakeholders from NHS organisations, the RM industry, and academia, known as the Regenerative Medicine Expert Group (RMEG), was
established with the aim of developing an NHS RM delivery readiness strategy and action plan to the government in order to accelerate the translation of RM therapies in the UK. This action plan encompassed a number of interacting elements within the translational pathway for RMs into the NHS including manufacture, regulation and reimbursement. As a result of the RMEG discussions, it was recommended that ‘mock’ technology appraisals of regenerative medicine products be performed, and that NICE consider the findings of these appraisals and whether changes to the appraisal methods may be required (Regenerative Medicine Expert Group, 2015). Based upon this recommendation, a cost-effectiveness analysis was performed in the present thesis (Chapter 5) for an exemplar cell-based therapy, specifically Allogeneic Islet Transplantation, using current and value-based appraisal methodologies. Furthermore, sensitivity analyses were undertaken to explore the data requirements for the appraisal, as well as the potential cost-effectiveness, of a hiPSC derived Beta cell therapy, as presented in section 5.5.

3.4 Conclusions

Currently, the UK healthcare system has numerous limitations with regards to the adoption and reimbursement of novel cell therapies, including difficulty accepting high initial costs, varying local adoption, applicability of the current cost per QALY model, suitability of the NICE threshold, silo budgeting, hospital short-termism, lack of competition, varying clinical adoption, lack of reimbursement decision clarity, and unrealistic evidence requirements.

However, steps are being made to adapt the healthcare system in order to accommodate such innovative technologies, and NICE are beginning to develop pathways for regenerative medicines. However, these proposed pathways are, at present, only adaptations of current procedures and new pathways may be required if cell therapies are to be adopted efficiently.

The proposed change in pricing method and infrastructure to a ‘Value-Based Pricing’ system may have represented a signal that the healthcare system was looking to welcome innovation in order to improve patient health outcomes, which may have been an encouraging development for the regenerative medicine industry. The ‘weighting’ system may have increased the cost-effectiveness threshold for cell therapies, due to their ability to reduce ‘burden of illness’, display improvements on the gold standard of care, and produce wider societal benefits, despite the requirement for substantial evidence of this over a significant length of time. Yet a variety of concerns regarding the implementation of such a pricing
system were voiced upon its proposal, and it was believed that it would have had a detrimental impact upon the UK economy, international pricing and the private sector. Large pharmaceutical companies were also apprehensive that VBP would have led to a reduction in price for premium drugs which are not cost-effective and do not deliver significant improvements in patient health outcomes. This may well have been the case, however it could have also been argued that VBP represented a positive outcome, may have stimulated innovation within companies, and may have benefited technologies such as regenerative medicines. Nonetheless, as a result of this significant stakeholder criticism upon its proposal, as well as a lack of leadership and short timeline designated for its introduction, the plans for the introduction of the VBP scheme were increasingly modified to the point where fitted with the incumbent reimbursement structures, and eventually were rejected.

Orphan diseases and areas of unmet need have been identified as areas that the regenerative medicine industry may wish to target in order to streamline their route to market and display a step change in health outcomes. However, multimorbidity may have also been an area of opportunity for the industry, in particular hMSC-based therapies. The increasing burden of multimorbidity upon healthcare and the capability of cellular therapies to fulfil the three factors for weighting the cost-effectiveness threshold make this a niche in which regenerative medicines may have prospered under VBP. Under the modified VBA & PPRS approach, considering the proposed removal of the weighting framework for ‘therapeutic innovation’ and the adaptation of the ‘wider societal benefits’ framework, it could be suggested that the appraisal methodology was less favourable for the reimbursement of RMs than VBP, yet it is likely that this adapted scheme would have been more favourable than current reimbursement systems. However, with the rejection of both of these value-based appraisal schemes, the introduction of alternative pathways or risk sharing models may become increasingly important for the future reimbursement of RMs.

This chapter has reviewed the way in which the proposed VBP scheme was to be introduced, described the possible impact of this regime, outlined the ways in which VBP has been transformed in recent times, illustrated how the newly created VBA method was likely to be implemented, proposed how VBA may have impacted the reimbursement of regenerative medicines and those therapies which target multimorbid patients, and identified the importance of future schemes or pathways in the reimbursement of RMs. The recent Regenerative Medicine Expert Group report (2015) recommended that mock technology
appraisals be undertaken for RM products, in order to examine the relevance of current appraisal methods for such products. Therefore, in the present thesis (Chapter 5) cost-effectiveness analyses for an exemplar cell-based therapy, specifically Allogeneic Islet Transplantation, was undertaken using current and value-based appraisal methodologies. Sensitivity analyses were also performed to examine the cost-effectiveness of a hiPSC derived Beta cell therapy product, as presented in section 5.5.
Chapter 4: The Adaptation & Modelling of the ‘Headroom Method’ for Products Which Target Multiple Simultaneous Indications: Using Insulin Dependent Diabetes Mellitus as a Case Example

The aim of the present chapter is to explore the adaptation of the ‘Headroom Method’ of early economic analysis in order to account for therapies which target multiple simultaneous indications, using an MSC-based therapy targeting Insulin Dependent Diabetes (IDDM) as a case study. No previous research on the adaptation of the Headroom Method to account for treatments which target multimorbidity has been published.

4.1 Introduction

Therapies capable of simultaneously targeting multiple indications may offer a possible step change in the treatment of multimorbidity, the prevalence of which is increasing (Haggerty, 2012). Diabetes Mellitus is an indication which is commonly associated with a number of complications and comorbidities. Diabetic patients have been found to have twice the risk of developing a range of Cardiovascular Diseases (CVD) compared to healthy individuals (Emerging Risk Factors Collaboration, 2010). Furthermore, diabetes has also been associated with kidney disease, retinopathy, neuropathy, depression and dementia.

One example of a therapy capable of targeting multiple indications may lie in the transplantation of human Mesenchymal Stromal Cells (hMSCs) which are capable of homing to sites of injury, modulating immune responses, and secreting a variety of bioactive factors to aid in tissue repair. Although there is evidence to suggest that MSC therapy may improve health in diabetic patients through their differentiation into insulin producing cells, with MSC treatment having been found to lower serum glucose and increase serum insulin in a rat model (Abdel Aziz et al, 2008), their immunomodulatory and pro-angiogenic functions may be the most promising modes of action for combination therapy (Dominguez-Bendala et al, 2011). MSCs may be capable of attenuating autoimmune reactions to host islets through their immunomodulatory mode of action (Davis et al, 2012). Furthermore, MSC therapy has been demonstrated to increase the density of cardiac nerve fibres, therefore attenuating diabetic cardiac autonomic neuropathy in diabetic rats (Wang et al, 2013). Treatment with MSCs has also been shown to improve the symptoms of a number of other common diabetic complications, including diabetic cardiomyopathy, diabetic polyneuropathy, diabetic nephropathy, cardiac function and diabetic wounds (Volarevic et al, 2011). However, further research examining the treatment of diabetes and associated complications is required, with
only a single study having reported improvements in diabetic complications as well as in the underlying indication (Abdel Aziz et al, 2008).

Difficulties are likely to arise when measuring the true value of such technologies, as many of their benefits may occur outside of acute healthcare and may accrue over time in a social care setting. The proposed introduction of the ‘Value-Based Pricing’ scheme in the UK offered the potential for these therapies to obtain a price which aligned with their value. However, if these products are to be reimbursed, they must be cost-effective, and it has therefore been proposed that early cost-effectiveness analysis performed by suppliers can inform investment decisions and help to guide funding towards products which are likely to gain adoption. One such calculation, known as the ‘Headroom Method’ (Cosh et al, 2007; McAteer et al, 2007), has been developed, however the adaptation of this method for products which simultaneously target multiple indications has yet to be undertaken, and therefore this chapter focuses upon this challenge.

In this chapter, the example of an MSC-based therapy targeting Insulin Dependent Diabetes Mellitus (IDDM), and its associated comorbidities, is utilised. For this example a number of possible variants of the original Headroom Method are proposed which account for the treatment of simultaneous indications. These include ‘Mean Utility & Duration of Benefit’, ‘Average of indication specific Headrooms’, ‘Maximum Headroom’, ‘Minimum Headroom’, ‘Greatest Product Opportunity’ and ‘Shortest Duration’ variants. The work in this chapter indicates the potential utility of such Headroom Method variants, however the availability and accuracy of comorbidity data available in the literature limited the ability to test these proposed variants.

The present chapter represents a novel, exploratory study into the feasibility of the adaptation of an early cost-effectiveness analysis method in order to account for treatments which target multiple simultaneous indications. No previous research on the adaptation of the Headroom Method to account for treatments which target multimorbidity has been published.

4.1.1 The Headroom Method

The Headroom Method, first developed by the University of Birmingham group and described in two of their 2007 publications (Cosh et al, 2007; McAteer et al, 2007), is a technology appraisal method which helps stakeholders “avoid misguidedly investing in those technologies that will never be cost-effective”. Cost-effectiveness has become an increasingly
important factor considered by healthcare providers and reimbursement authorities, and
evidence of cost-effectiveness can influence stakeholders substantially, encouraging a
positive adoption decision. A number of authors have acknowledged that often the National
Institute for Health and Clinical Excellence (NICE), the authority responsible for Health
Technology Appraisals (HTAs), may prioritise cost-effectiveness over clinical utility in their
appraisals (Rose & Williams, 2012; Ginty et al 2010). Therefore, providing adequate cost-
effectiveness data for new health technologies is vital for gaining successful adoption and
reimbursement in the UK.

NICE cost-effectiveness decisions are based upon an approach known as the ‘Incremental
Cost-effectiveness Ratio’ (ICER), which compares the difference in the effectiveness and
cost between a new health technology and that of the current ‘gold standard’ treatment. This
calculation therefore determines the extra cost per extra unit of benefit of the new technology.
Finally, in order to determine whether the new product can be considered cost-effective, the
increase in price per extra unit of benefit (ICER) is compared to a Cost per Quality Adjusted
Life Years (Cost per QALY) threshold. In the UK, this threshold is currently set by NICE and
is considered to be between £20,000 to £30,000 per QALY. If the ICER of the new
technology is below £20,000 per QALY, then it would be considered to be cost-effective and
would be recommended for reimbursement. If the ICER is between £20,000 and £30,000 per
QALY, then other factors may need to be present to positively influence the reimbursement
decision. Finally, if the ICER is above £30,000 per QALY, then it is unlikely that this
treatment would be reimbursed and strong additional evidence would be required in order to
gain acceptance.

As demonstrated above, cost-effectiveness analysis is typically required by the demand side
in order to inform decision making after the product has been developed. However, McAteer
& Lilford (2009) argue that such health economics analysis should be performed by the
supply side during early stage development. However, at this stage, little effectiveness
evidence will be available, and therefore this approach utilises optimistic assumptions
regarding incremental effectiveness. Rather than calculating how cost-effective the new
treatment will be, this method estimates the cost at which the new treatment would be
considered cost-effective if its greatest potential efficacy was achieved. This value can then
be used as the “maximum cost that the technology can be brought to market and still be
considered cost-effective” (McAteer & Lilford, 2009). This data can then be used to advise investment decisions and to guide funds towards those products with the greatest potential.

In order to calculate the Headroom, or $\text{Max}\Delta\text{Cost}$, first the maximum change in effectiveness, or $\text{Max}\Delta\text{QALY}$, must be determined. Effectiveness is determined by measuring a patient’s health state, or health utility, after treatment, which is ranked on a scale of 0 (death) to 1 (perfect health). In order to calculate the maximum change in effectiveness, the health utility associated with the gold standard treatment is subtracted from 1, as the effectiveness of the new technology is assumed to be perfect. This is then multiplied by the duration of benefit which, if there is no effect upon life expectancy between the two treatments, is considered to be the length of time for which the health utilities differ, and therefore the duration of benefit of the gold standard is used.

$$\text{Max}\Delta\text{QALY} = (1 - \text{Health utility of Gold Standard}) \times \text{Duration of Benefit (Years)}$$

Once the effectiveness gap between the two products has been calculated, the Headroom can be calculated by multiplying the $\text{Max}\Delta\text{QALY}$ by the Cost per QALY threshold of the payer which, in the case of NICE in the UK, is £20,000 to £30,000.

$$\text{Headroom} (\text{Max}\Delta\text{Cost}) = NICE \text{ Threshold} \times \text{Max}\Delta\text{QALY}$$

Once the Headroom has been determined, stakeholders can continue to calculate the potential Return on Investment (ROI) of the new technology, make an informed decision regarding investment or can perform a formal value of investment analysis. In order to calculate ROI, the expected cost of goods per treatment is subtracted from the Headroom, which is then multiplied by the expected sales. However, this ROI may be affected by the rarity of the target indication, or it’s prevalence in countries which cannot support the high cost of the new treatments.

$$\text{Revenue} (\text{ROI}) = (\text{Max}\Delta\text{Cost} - \text{COGs}) \times \text{Volume}$$

As described above, the Headroom Method determines the maximum potential cost of bringing a single new product with a single application to market whilst still being considered cost-effective. However, if a product has multiple target indications and applications, then it will therefore have a number of Headrooms, with each indication targeted being compared to the health utilities and durations of benefit of the respective gold standard treatment in that
particular area. This is not accounted for in the Headroom Method calculations, and therefore may cause complications and require adaptation to the method if it is to be applicable to such products.

Although the current model may require alteration in order to incorporate the analysis of products which target multiple indications, the authors (McAteer & Lilford, 2009) have considered this difficulty explaining that “each application may have a different ICER and headroom” and that “the first application might not necessarily be the ‘big one’”, therefore suggesting that each Headroom should be considered on an individual indication basis. This suggestion would fulfil the necessary requirements if the technology were to be developed and marketed individually and separately for each indication, and each indication targeted would therefore have its own specific headroom describing the cost-effectiveness of the product used in that area. However, this consideration also reaches difficulties when the mechanism of action of the new technology under development incorporates the targeting of a number of indications simultaneously. Here, the cost-effectiveness analysis should integrate the analysis of a number of individual indications into a combined Headroom for the single product. Therefore, a more holistic view must be taken, with the treatment of the patient as a whole, rather than each individual condition, being considered.

As discussed in sections 2.2.1, 2.2.3.3 & 4.1, hMSC-based therapies, of which a number have begun to reach the market, may have the potential to target a number of indications simultaneously. The effect of proposed changes to the UK health technology appraisal methodology upon the reimbursement of such therapies is also discussed in sections 3.3.3.2 and 3.3.4.6. If such products are to be accurately analysed in a way which takes into account their full benefits, and are to eventually achieve adoption into the healthcare market, the development of a health economics evaluation method which can combine the analysis of a number of indications would represent a valuable tool. The aim of this chapter is to begin to examine how this method of economic analysis can be modified to account for treatments which target multiple indications simultaneously.

4.2 Methods: Adapting the Headroom Method

In order to accommodate the putative simultaneous functionality of a hMSC-based product within the Headroom Method, slight alterations to, or further calculations in addition to, the existing method are required. Here a number of variants of the traditional Headroom Method
were examined, for a typical example of multimorbidity, in order to demonstrate the way in which this method can be adapted to analyse treatments which target a number of conditions. The health utility and duration of benefit data required for this example was obtained from a study in which the effects of Insulin Dependent Diabetes Mellitus (IDDM), or Type I Diabetes, treatment was modelled over a 20 year time horizon (Beckwith et al, 2012).

Due to the optimistic nature of the Headroom Method calculations, the upper limit of the £20,000 to £30,000 per QALY NICE threshold was utilised in order to determine the maximum cost at which the new treatment would be considered cost-effective.

4.2.1 Multimorbidity Example

In this example, the cost-effectiveness of a new therapy which targets IDDM is performed, and for which a number of comorbidities are associated. Many of the simultaneous indications associated with IDDM cannot be treated directly and are only preventable through adequate management of the patient’s blood glucose levels. In this case, the associated conditions, for which the maximum potential cost of a new treatment which targets these indications are determined, include Diabetic Neuropathy and Hypoglycaemia unawareness. Neither of these conditions has an adequate gold standard treatment and they usually rely on accurate blood glucose management by the patient themselves. Therefore, normal insulin treatment is used as the gold standard for both IDDM itself and for hypoglycaemia unawareness. However, as neuropathy can often occur in the distal portions of the limbs and, if severe enough, can often lead to amputation, amputation is utilised as the comparator for the treatment of neuropathy. For this example, estimates for the health utilities and the duration of benefit were taken from Beckwith et al (2012) and are shown in the table below (Figure 5). Beckwith and colleagues (2012) modelled the health utility of patients with a number of comorbidities over a 20 year time horizon, and therefore this value (20 years) is used as the duration of benefit for each of the indications considered in the present research. However, ideally a more accurate measure of duration of efficacy of the gold standard treatment would be available, as it cannot be assumed that the new therapy would be effective over this 20 year period.
Chapter 4: The Adaptation & Modelling of the ‘Headroom Method’ for Products Which Target Multiple Simultaneous Indications: Using Insulin Dependent Diabetes Mellitus as a Case Example

<table>
<thead>
<tr>
<th>Indication</th>
<th>Insulin Dependent Diabetes Mellitus</th>
<th>Hypoglycaemia Unawareness</th>
<th>Neuropathy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment</td>
<td>Insulin treatment</td>
<td>Insulin treatment</td>
<td>Amputation</td>
</tr>
<tr>
<td>Health Utility</td>
<td>0.81</td>
<td>0.75</td>
<td>0.64</td>
</tr>
<tr>
<td>Duration of Benefit</td>
<td>20 years</td>
<td>20 years</td>
<td>20 years (Lifetime)</td>
</tr>
</tbody>
</table>

Figure 5: IDDM example gold standard treatment, health utility and duration of benefit for each of the simultaneous conditions

4.3 Results: Headroom Variants

- **Headroom Variant 1 - Mean utility & duration of benefit**
  1. Mean Utility=0.81+0.75+0.64=2.2/3=0.73
  2. ΔUtility=1-0.73=0.27
  3. Mean Duration of benefit=20 years
  4. MaxΔQALY=0.27x20 years=5.4
  5. Headroom=5.4x£30,000=£162,000

Although, in the current example, there was no difference between the duration of benefit for each indication, using this method would allow the resulting Headroom for the new treatment to account for high or low potential improvements in health utility values and for high or low duration of benefit values, which may either significantly improve or be detrimental to the patient’s health status and may have a significant impact upon the economic analysis of the new product. This may create a more realistic estimation of maximum potential cost of the new combined treatment, by increasing or decreasing the mean Headroom. However, this attribute may be unfavourable in that high or low health utility or duration of benefit data associated with one of the complications may adjust the mean to a greater extent than the actual effect this outlier has on the health status of the patient as a whole, therefore increasing or decreasing the effectiveness gap between the gold standards and the new therapy. This may create an estimation which is less representative of the true benefit of the combined treatment, and highlights the need for accurate data.

- **Headroom Variant 2 - Average Headroom using individual Headroom for each indication**
  1. IDDM Headroom=1-0.81=0.19x20 years=3.8x£30,000=£114,000
Chapter 4: The Adaptation & Modelling of the ‘Headroom Method’ for Products Which Target Multiple Simultaneous Indications: Using Insulin Dependent Diabetes Mellitus as a Case Example

2. Hypoglycaemia Unawareness Headroom=1-0.75=0.25x20
   years=5x£30,000=£150,000
3. Neuropathy Headroom=1-0.64=0.36x20 years=7.2x£30,000=£216,000
4. Sum of Headrooms=£480,000
5. Mean Headroom=£223,290/3=£160,000

As each indication is usually treated separately and has its own health utility, perhaps the change in effectiveness and Headroom could be determined for each indication individually, for which the mean could be taken in order to produce an average Headroom for the new multimorbidity treatment? Although this method may give greater representation to each indication, it may also exaggerate any skewness in the data which could therefore affect the average.

- **Headroom Variant 3- Maximum Headroom**
  1. IDDM Headroom=1-0.81=0.19x20 years=3.8x£30,000=£114,000
  2. Hypoglycaemia Unawareness Headroom=1-0.75=0.25x20
     years=5x£30,000=£150,000
  3. Neuropathy Headroom=1-0.64=0.36x20 years=7.2x£30,000=£216,000

The Headroom method is defined by McAteer & Lilford (2009) as an approach which estimates the “maximum cost that a technology can be brought to market and still be considered cost-effective”. Therefore, for a product which targets multiple indications, it would seem logical to use the largest Headroom as a guide for the extra cost above that of the current gold standard. McAteer & Lilford (2009) also recognise this explaining that, as discussed earlier in section 4.1.1, the Headroom should be considered on an individual indication basis, however the issue with this technique being inappropriate for simultaneous treatment has also been described previously in section 4.1.1. Furthermore, although this largest Headroom data may give confidence to developers, presenting them with a greater potential market, an overestimation of maximum cost could also increase the risk of product failure as profits may not match expectations.

- **Headroom Variant 4- Minimum Headroom**
  4. IDDM Headroom=1-0.81=0.19x20 years=3.8x£30,000=£114,000
  5. Hypoglycaemia Unawareness Headroom=1-0.75=0.25x20
     years=5x£30,000=£150,000
Chapter 4: The Adaptation & Modelling of the ‘Headroom Method’ for Products Which Target Multiple Simultaneous Indications: Using Insulin Dependent Diabetes Mellitus as a Case Example

6. Neuropathy Headroom=1-0.64=0.36x20 years=7.2x£30,000=£216,000

Using the lowest headroom data may not be the most relevant method given the optimistic nature of the Headroom Method, may not produce the most accurate estimation and may result in many projects being incorrectly discontinued, and therefore should not be used as the sole determinant of whether a product should gain investment. However, utilising this data would induce less risk upon developers, could be used as a ‘worst case scenario’ and could therefore be very useful to investors during product development.

• **Headroom Variant 5- Greatest product opportunity**
  1. Lowest Health utility=0.64 (Neuropathy)
  2. Longest Duration of benefit= 20 years
  3. Headroom=1-0.64=0.36x20 years=7.2x£30,000=£216,000

Although the Maximum Headroom adaptation may offer the value for the greatest opportunity if the product were to specifically target a single indication, this greatest opportunity method utilises the most favourable values, from the indications being targeted, in order to calculate the best possible outcome of the new treatment. This method uses the health utility of the indication where the greatest potential benefit in health status is available, and the duration of benefit where the known effectiveness of the gold standard comparison is longest. In the example shown, this greatest potential method happens to correlate with the maximum headroom (Variant 3), however this often may not be the case in reality.

• **Headroom Variant 6- Shortest Duration method**
  1. Mean Utility=0.81+0.75+0.64=2.2/3=0.73
  2. Shortest Duration=20 years
  3. Headroom=1-0.73=0.27x20 years=5.4x£30,000=£162,000

This variant is another method of analysing new products in a conservative manner and can give a baseline headroom with which products with great potential can be rapidly identified. Here the average of the health utilities is used in order to calculate the mean difference effectiveness gap, and therefore this method would be representative of the potential of a new product which is capable of treating each of the indications. However, the shortest duration of benefit, rather than the mean, is used in order to represent a treatment which is effective for each indication, but only for the shortest duration before one or more of the indications
reoccurs. Although in the current example the shortest duration of benefit does not differ from the longest, in reality a significant difference between the shortest and longest could exist.

<table>
<thead>
<tr>
<th>Headroom Variant</th>
<th>Method</th>
<th>Headroom outcome for IDDM example</th>
</tr>
</thead>
</table>
| 1. Mean Utility & Duration of Benefit | 1. Mean Health Utility  
2. ΔUtility=1-Mean Utility  
3. Mean Duration of Benefit  
4. MaxΔQALY=ΔUtility x Mean Duration of Benefit  
5. Headroom=MaxΔQALYxNICE Threshold | £162,000 |
| 2. Average of indication specific Headrooms | 1. Indication 1 Headroom  
2. Indication 2 Headroom  
3. Indication 3 Headroom  
4. Sum of Headrooms  
5. Mean of Headrooms | £160,000 |
| 3. Maximum Headroom | 1. Indication 1 Headroom  
2. Indication 2 Headroom  
3. Indication 3 Headroom  
4. Use largest Headroom | £216,000 |
| 4. Minimum Headroom | 1. Indication 1 Headroom  
2. Indication 2 Headroom  
3. Indication 3 Headroom  
4. Use smallest Headroom | £114,000 |
| 5. Greatest Product Opportunity | 1. Lowest Health Utility available  
2. Longest Duration of Benefit available  
3. Calculate Headroom using these values | £216,000 |
| 6. Shortest Duration Method | 1. Mean Health Utility  
2. Shortest Duration of Benefit available  
3. Calculate Headroom using these values | £162,000 |

**Figure 6:** Summary table of proposed Headroom method variants and the outcomes for the IDDM example

### 4.4 Discussion

#### 4.4.1 Rejected Variants

During the initial analysis and testing of potential adaptations to the Headroom Method, a number of possible methods were discarded due to unfeasibility. For example, a total Headroom Method was considered in which the sum of the effectiveness gaps, durations of benefit and, therefore, Headrooms for each indication were used. However, the cumulative nature of this method may result in health utilities and potential improvements upon the gold standard of over 1 and therefore, as a health utility of 1 represents perfect health, this adaptation is not feasible.
It was then proposed that, rather than using the sum of the effectiveness gaps and the durations of benefits, only the Headroom for each indication should be taken and totalled to give a cumulative Headroom. However, although consolidating each individual Headroom into one may appear to give the maximum potential cost at which the new treatment, which targets a number of indications simultaneously, would be considered cost-effective, the use and accumulation of the effectiveness gaps for each indication may not be representative. In a multimorbid patient, the improvements in health seen with treatment of each condition would interact, and the successful treatment of a single condition may not necessarily restore the patient to full health, and a health utility of 1, in the presence of other indications. However, the beneficial effects of treatment upon one indication may positively influence the disease state associated with another, as often co-occurring conditions in multimorbidity are dependent upon one another. In many cases the occurrence of a key underlying ‘instigator’ condition, for example IDDM, can lead to a cascade of numerous comorbidities, and therefore any treatment which reduces the burden of the instigator condition may also reduce the burden of the associated comorbidities.

Other methods involving differences and average differences between the health utilities induced by the gold standard treatments were also suggested, however these too were unfeasible due to the use of health utilities greater than 1, and their potential to reward new products for which large disparities in health benefits between gold standard treatments exist between indications.

4.4.2 Further Concerns
The use of therapies which have the capacity to treat a number of indications simultaneously would likely reduce the number of different treatments required to manage a patient’s health, which in turn would be likely to reduce healthcare costs, duplication of treatment, and the likelihood of detrimental interaction between treatments. The removal of the possibility of such interactions could reduce the risk of harmful side effects, and increase the safety and efficacy of the care these patients receive. If this detrimental effect of treatment interaction upon patient health is to be accommodated in the adaptation of the Headroom Method, it would require alteration of the health utilities associated with the gold standard treatments, and cannot be factored into the modified calculations as the interactions between treatments will differ in each multimorbidity scenario. However, the possibility of a synergistic effect of concurrent therapies may also exist. This also cannot be included in the Headroom method.
adaptations due to its complexity, and must be factored into the health utilities associated with these treatments. Therefore there is a requirement for better, more accurate data regarding the effect of duplicative treatment and concurrent therapies upon the health utilities of available treatments.

Also, as simultaneous therapies, such as MSC-based products, are likely to require few doses, issues surrounding dosage regimen, or treatment changes due to lack of efficacy or unwanted side effects, are likely to be reduced. If such changes are to be taken into account in the modified calculations for the Headroom Method, the durations of benefit of the gold standard treatments would require adjustment, which, again, would require more accurate data which could be collected after treatment for example. These changes must also be performed on an individual case basis as the treatment regimen may change depending on the indications involved.

Towards the end of the Headroom Method calculations, prior to the determination of the ROI but after an initial Headroom has been established, the opportunity exists to account for any cost savings that the new therapy may incur. Therefore, as cell-based therapies which have the capacity to target multiple indications simultaneously are likely to reduce hospital stay, reduce social care, reduce recurrence, and thus reduce long-term healthcare costs, these savings could be added to the initial headroom. For example, as described in McAteer’s Thesis (McAteer, 2010), prevention of recurrence can be accounted for by including the cost of re-treatment in the final headroom.

Finally, when adapting the Headroom Method to allow for evaluation of a therapy which simultaneously targets a number of indications, difficulties exist when health utilities and durations of benefit are used which overlap between indications. The concerns with taking the health utility associated with one indication and the duration of benefit associated with another were present in both discarded and proposed methods (Variant 5). Whether these concerns are warranted is yet to be determined and requires further evaluation through testing with realistic and more accurate data.

In order to test these Headroom variants thoroughly, highly accurate data relating to the health utilities and durations of benefit of the gold standard treatments for a number of commonly co-occurring indications should be utilised in order to calculate the Headroom for a new treatment which targets these indications simultaneously. The economic data regarding
developmental costs of the new product can then be compared to this Headroom calculation in order to examine the viability of this new product and to examine the compatibility of appraisal methods, such as the Headroom method, with treatments which target numerous indications simultaneously. Health utility data concerning multimorbid patients affected with various ‘subsets’ of indications and who currently utilise a number of gold standard therapies to manage their health would be extremely valuable, although the availability of this would diminish the need for adaptation to the Headroom Method. It is also clear that the collection of more accurate data regarding the duration of benefit of gold standard treatments is necessary. However, currently, accurate data relating to comorbidities is difficult to obtain due to the complexity and interaction of the multiple indications, and due to the variation between patients in indications with which they are simultaneously afflicted.

4.4.3 Allogeneic Islet Transplantation (Edmonton Protocol) Example

As demonstrated by the multimorbidity example used in the present study, Diabetes Mellitus is a particularly common indication with which multiple comorbidities are associated, and is of growing concern in modern healthcare. Often, the treatment of IDDM is not adequately effective, and, in the case of patients afflicted with a number of diabetes complications, an array of therapies and pharmaceuticals are often prescribed and these can regularly change. However, the treatment of IDDM and associated complications often focuses upon the control of blood glucose and insulin therapy alone. This is due to the lack of functioning beta cells, as oppose to the glucose insensitivity of beta cells in Non-Insulin Dependent Diabetes Mellitus (NIDDM), and therefore the severity of the underlying indication and its effect upon comorbidities. This lack of function makes IDDM an applicable target indication for regenerative therapies, and currently there are three such approaches.

The use of artificial pancreas therapy, isolated islet transplantation, and solid organ (pancreas) transplantation has each shown promise. Within the category of isolated islet transplantation, one treatment that has received particular interest is the transplantation of cadaveric islets, for which the Edmonton protocol was created by Shapiro et al (2000). More recently, alternative sources of Beta cells, the insulin producing cells of islets, have been explored. For example, functional Beta cells have been derived from pluripotent stem cells in a number of studies (Zhang et al, 2009; Rezania et al, 2012; Rezania et al, 2014). Treatments such as these offer great potential in the restoration of health for patients with IDDM, however further research into the cost-effectiveness of these therapies is required. Beckwith et al (2012) previously
determined that Allogeneic Islet Transplantation was more effective than standard insulin treatment and induced cost savings after 9-10 years. In the present thesis (Chapter 5), both current and value-based appraisal methodologies are utilised to examine the cost-effectiveness of Allogeneic Islet Transplantation. Furthermore, the potential cost-effectiveness of a Human Induced Pluripotent Stem Cell (hiPSC) derived Beta Cell therapy is explored in section 5.5.

However, current regenerative approaches to IDDM therapy target the underlying condition alone and do not aim to treat any related complications. Whereas, as previously discussed in section 4.1, hMSC-based therapies may have clinical utility with regards simultaneously targeting multiple indications. Therefore, IDDM may represent a target indication for such a combined therapy, and evidence exists to suggest that MSCs may be valuable in treatment of diabetes and its associated complications (Abdel Aziz et al, 2008; Volarevic et al, 2011).

4.5 Conclusions

After the exploration of the adaptation of the Headroom Method, and the proposal of a number of Headroom Method variants, it would appear that using the mean health utility and mean duration of benefit to calculate a mean Headroom (Variant 1) may be the most relevant to calculate an average value for the maximum price at which the product can be considered cost-effective. However, it may be relevant for developers to consider the maximum Headroom (Variant 3) in order to examine a product’s single indication potential and to prevent projects being abandoned too readily. It may also be relevant for developers to examine ‘worst case scenarios’ and to calculate the minimum Headroom (Variant 4) in order to critically analyse their product. However, due to the concerns regarding the use of overlapping health utilities and durations of benefit between indications within a Headroom variant, these methods cannot be recommended until this interaction has been studied further. Further research, utilising more accurate health utility data for multimorbid patients, is required to accurately test the proposed Headroom Method variants.

It is apparent that further validation of these variants, using more accurate health utility data for associated comorbidities, is required, and this was not possible in the present research. However, to further explore the reimbursement of RMs and cell-based therapies, the cost-effectiveness of Allogeneic Islet Transplantation was examined using current and value-based appraisal methodologies, and is presented in Chapter 5. Furthermore, the potential cost-
effectiveness of a hiPSC-derived Beta Cell therapy was explored in section 5.5. These analyses follow the Regenerative Medicine Expert Group recommendation for the performance of a number of ‘mock’ appraisals for cell-based therapies (Regenerative Medicine Expert Group, 2015), and were validated by a NICE representative (Dr Elangovan Gajraj, Technical Advisor, NICE Scientific Advice Team, UK).
Chapter 5: The Incorporation of ‘Value-Based’ Appraisal Methodologies into Cost-Effectiveness Analyses: An Illustrative Study of Allogeneic Islet Transplantation and the Implications for Human Induced Pluripotent Stem Cell (hiPSC) derived Beta Cell Therapies

The aim of this chapter is to determine the cost-effectiveness of Allogeneic Islet Transplantation over a 20 year time horizon, using current Health Technology Appraisal (HTA) methods as well as VBP and VBA approaches. The chapter also explores the cost-effectiveness of a hiPSC derived Beta Cell therapy for the treatment of Insulin Dependent Diabetes Mellitus (IDDM) using sensitivity analyses. The analyses presented in this chapter were performed with the guidance of Dr Elangovan Gajraj (Technical Adviser, National Institute of Health and Care Excellence (NICE) Scientific Advice Team) and Dr Kourosh Saeb-Parsy (University Lecturer & Honorary Consultant in Transplant Surgery, Addenbrooke’s Hospital, Cambridge, UK). The literature in which the VBP approach has been utilised to appraise healthcare technologies is limited, and neither a mock HTA using a VBA approach, nor a sensitivity analysis exploring the cost-effectiveness of a hiPSC derived Beta cell therapy, has previously been published.

5.1 Introduction

5.1.1 Proposed Changes to UK Reimbursement

At present, the analysis of new healthcare technologies in the UK, known as Health Technology Appraisals (HTAs), is performed by the National Institute for Health and Care Excellence (NICE) and provides the basis for most reimbursement decisions in the UK healthcare system, as discussed in section 3.3.1. The main component of these HTAs is the cost-effectiveness analysis of new technologies, although a number of other factors are also considered during the appraisal process. In order to calculate how cost-effective a new technology may be, the ‘Incremental Cost-Effectiveness Ratio’ (ICER), or ‘Incremental Cost per Quality-Adjusted Life Year (QALY)’ must be determined. This ICER value is then compared to the NICE threshold, currently set between £20,000 and £30,000 per QALY, to inform the decision making process and to therefore determine whether a recommendation for the reimbursement of the new technology will be considered. A favourable recommendation from NICE becomes increasingly unlikely, and the requirement for stronger supporting evidence outside of the cost-effectiveness analysis becomes increasingly
necessary, the further above the £20,000 per QALY threshold the ICER for a new technology falls.

As discussed further in section 3.3.2, the Secretary of State for Health in 2011, Andrew Lansley, put forward a proposal for a transition to a Value-Based Pricing (VBP) approach after the expiry of the Pharmaceutical Price Regulation Scheme (PPRS) at the end of 2013. The initial plans for this transformation of the pricing system involved the introduction of a scheme in which medicines would be priced based upon their value to the customer, placing less emphasis upon the cost-effectiveness analysis as well as reducing the influence of pricing negotiations between the manufacturer and the state. The primary aim of the transition to a VBP scheme was to include a wider assessment of the way in which medicines deliver benefit to patients and society through the implementation of a ‘weighting’ system which would supplement NICE’s cost-effectiveness analysis during their appraisal process.

The proposition of an overhaul of the current reimbursement system was met with significant stakeholder resistance, as described in section 3.3.4. In particular, those within the pharmaceutical industry, which represents one of the largest and most influential NHS stakeholder groups, did not respond positively to the proposed change. Within eight months of the date on which the plans for the introduction of a VBP approach were outlined, the government began to retreat on its original proposals due to the extensive criticism of the change, the absence of purposeful leadership and the relatively short timeline designated for its implementation. As of November 2013, the continued failure of the VBP scheme to gain momentum led to its adaptation into a Value-Based Assessment (VBA), which would be integrated into the PPRS from autumn 2014. The VBA scheme would be used to inform reimbursement decisions, rather than determining the ICER, with PPRS remaining at the core of price setting methods.

Therefore, in both the current appraisal scheme and VBA approach, PPRS would allow for price negotiations after an appraisal and ICER calculations had been performed to determine the most acceptable ICER and price. With regards to regenerative medicines and cell-based therapies, although it is unlikely that these technologies would be reimbursed through the PPRS, it may be that, if significant adoption were achieved, an alternative scheme to PPRS would be put in place to determine the pricing of these therapies. For example, the NICE highly specialised technologies programme, which is currently in development, will appraise...
new or existing technologies that target ‘ultra-orphan’ indications and that would not typically be considered cost-effective (PMLive.com, 2014). Additionally, NHS Specialised Services commission therapies which target a very small patient population, provided that the cost is sufficiently low and the required number of clinicians and hospitals are available, and this pathway may therefore represent an alternative route to adoption for regenerative medicines (Regenerative Medicine Expert Group, 2015; Kefalas, 2014). Furthermore, commissioning through NHS Specialised Services does not require a NICE technology appraisal, although a positive NICE recommendation can significantly speed up Specialised Services decision making (Kefalas, 2014). Alternatively, the development of innovative business models involving risk sharing schemes between manufacturers and the NHS has been proposed (Regenerative Medicine Expert Group, 2015). However, the approach to reimbursement may differ between allogeneic and autologous therapies, each of which utilise varying manufacturing and delivery processes. This disparity between allogeneic and autologous approaches has been identified as one of the key barriers to adoption of regenerative medicines by the NHS (Regenerative Medicine Expert Group, 2015).

As of September 2014 and as a result of NICE review and public consultation, NICE recommended that no changes to the technology appraisal methodology should be made in the short term, and therefore the current reimbursement system will be maintained for the foreseeable future (NICE, 2014). Nonetheless, although no changes are to occur at present, it is likely that the topic of reimbursement methodology will be revisited in the future. Although it is unlikely that any alterations to the cost per QALY system will be made in the near future, the prospect of streamlining the approval process may be addressed.

However, it may still be beneficial to consider the impact that the proposed Value-Based approaches may have had upon reimbursement decisions, as the matter of the incorporation of burden of illness and wider societal impact into HTAs remains unresolved and in the interest of the public. Additionally, it is apparent that innovation remains both a challenge and a priority, with the UK Government Chief Scientific Adviser, Sir Mark Walport, recently creating an annual innovation report on behalf of the Government Office for Science (Government Office for Science, 2014), and therefore the exploration of methods to promote the adoption of innovative technologies may help to accelerate innovation in healthcare. As the proposed Value-Based reimbursement approaches aimed to facilitate innovation, developing a better understanding of the mechanisms and issues associated with these
approaches may inform future models. Thus, in the present study, a case study in which a mock HTA of Allogeneic Islet Transplantation for Insulin Dependent Diabetes Mellitus (IDDM), or Type I Diabetes, is performed using the Current Technology Appraisal Approach, as well as the proposed Value-Based Pricing and Value-Based Assessment approaches.
Chapter 5: The Incorporation of ‘Value-Based’ Appraisal Methodologies into Cost-Effectiveness Analyses: An Illustrative Study of Allogeneic Islet Transplantation and the Implications for Human Induced Pluripotent Stem Cell (hiPSC) derived Beta Cell Therapies

Figure 7: Current HTA, Proposed Value-Based Pricing, and Value-Based Assessment Approaches


Value Based Pricing Approach: NATIONAL INSTITUTE FOR HEALTH AND CLINICAL EXCELLENCE DECISION SUPPORT UNIT., 2013. Department of Health Proposals for Including Burden of Illness into Value Based Pricing: A Description and Critique.
5.1.2 Diabetes & Islet Transplantation

Diabetes has been reported to affect approximately one in 17 people in the UK, with 3.2 million individuals diagnosed, and approximately 630,000 remaining undiagnosed, in 2013 (Diabetes UK, 2014). Furthermore, this report approximated that 10% of diabetic patients in the UK have Type I diabetes (IDDM) and 90% have Type II diabetes, or Non-Insulin Dependent Diabetes Mellitus (NIDDM).

Importantly, diabetes has been linked to a number of chronic complications and comorbidities. Diabetic patients have been found to have twice the risk of developing a range of Cardiovascular Diseases (CVD) compared to healthy individuals (Emerging Risk Factors Collaboration, 2010). It has also been reported that CVD is responsible for 44% of fatalities in IDDM and 52% in NIDDM (Morrish et al, 2001). Furthermore, treatment of diabetes with exogenous insulin is not associated with a reduction in the risk of long term cardiovascular complications (Fiorina et al, 2008; Nathan et al, 2005).

Additionally, it has been estimated that one in four diabetes patients will develop kidney disease during their lifetime (The Health and Social Care Information Centre, 2013). Further, one quarter of end-stage renal disease is caused by diabetes (Gilg et al, 2012). Kidney disease has also been associated with 21% of deaths in Type I and 11% of deaths in Type II diabetes (Morrish et al, 2001). Therefore, a curative treatment for diabetic patients at risk of renal failure may reduce their mortality risk. Further complications that are commonly related to diabetes include Retinopathy, Neuropathy, Depression and Dementia.

IDDM, caused by insufficient physiological insulin production, is usually treated with the utilisation of regular exogenous insulin injection. However, in 2000, Shapiro and colleagues demonstrated that the transplantation of islets from the pancreata of brain-dead donors was capable of restoring insulin independence in IDDM patients. Although this represents a promising cell-based therapy, long term immunosuppressive therapy is required in conjunction with allogeneic islet transplantation and graft failure within a year of transplantation may occur, with a 28% complete graft loss after 1 year previously demonstrated (Shapiro et al, 2006). However, although insulin independence may not be sustainable in the long term, the transplanted islets may still provide protection from severe hypoglycaemia and hypoglycaemia unawareness, as well as maintain a normal level of glycated haemoglobin. Furthermore, recent developments in allogeneic islet transplantation
protocols have yielded significant improvements in insulin independence rates, with increases in three-year insulin independence rates from 27% in 1999-2002 to 44% in 2007-2010 (Barton et al, 2012). Although insulin independence after islet transplantation may not be maintained in the long term, 50% graft survival over five years has been reported in a previous study when combined with T-cell depleting antibody treatment and TNF-α inhibition, and this was found to be comparable to that of whole pancreas transplantation (Bellin et al, 2012).

Furthermore, it has been reported that over 25% of diabetic patients reliant upon intensive insulin therapy suffer from more than one episode of severe hypoglycaemia per year, and hypoglycaemia unawareness accounts for 4% of deaths in IDDM patients (Cryer, 2005). One of the main clinical outcomes of islet transplantation, that has a positive impact upon patient quality of life (QoL), is the almost complete elimination of hypoglycaemic episodes (Johnson et al, 2004). Further, in a study of 112 patients, a reduction in severe hypoglycaemic episodes from 82% per year to 5% per year following islet transplantation has been reported (NICE, 2008).

Although Allogeneic Islet Transplantation has primarily been utilised for the treatment of IDDM patients, it is also feasible that this therapy could be applied to the treatment of NIDDM patients, and therefore this may increase the potential market size. However, NIDDM has been strongly associated with lifestyle choices and therefore Allogeneic Islet Transplantation may not represent a curative treatment (Fujimoto, 1996).

However, the availability of pancreatic tissue for transplantation is limited, with only 456 pancreas donors, from either brain dead individuals or those who have suffered circulatory death, in the UK in 2013/14 (NHS Blood & Transplant, 2013). During that time, 270 patients were placed on the transplant list and 246 pancreas or islet transplantations were performed. These pancreatic transplantations are often combined with Kidney transplantation (NHS Blood & Transplant, 2013), and therefore, the availability of a curative diabetes treatment would significantly reduce the incidence of renal failure and the mortality risk in diabetic patients.

Alternative treatment options to Allogeneic Islet Transplantation and insulin therapy are available for diabetic patients and include whole pancreas transplantation and insulin pumps, which allows for real-time glucose sensing.
The present study examines whether the efficacy of Allogeneic Islet Transplantation over a 20 year period is sufficient to warrant its increased initial cost, in comparison to insulin therapy, by utilising the current HTA methods, and the proposed VBP and VBA approaches. This allowed any possible differences in the appraisal outcomes and reimbursement decisions between each approach to be indicated. However, as the proposed methodologies were not completed or implemented, a number of assumptions were made throughout the VBP and VBA approaches.

Finally, the feasibility of a Human Induced Pluripotent Stem Cell (hiPSC) derived Beta Cell therapy for the treatment of IDDM is explored by examining the cost-effectiveness of this technology, in comparison to Allogeneic islet Transplantation. Sensitivity analyses were performed, in which the cost and efficacy data of Allogeneic Islet Transplantation was manipulated, to determine the impact of improvements in efficacy and increased cost upon the reimbursement potential of such a therapy.

5.2 Cost-Effectiveness Analysis: Current HTA Approach

The reimbursement decision making process during current HTAs is performed by an Appraisal Committee which acts as an independent advisory body for NICE. These committees consist of NHS employees, academics from relevant research disciplines, members of the pharmaceutical industry, members of the medical device industry, as well as patients and patient representatives (NICE, 2013). The Appraisal Committee also takes into account the views of clinical specialists, commissioning experts and patient experts during the committee meetings.

As illustrated in Figure 7 (above), there are five primary components of current health technology appraisals used to make judgements about the acceptability of the technology as an effective use of NHS resources. As the ICER increases above the £20,000 per QALY NICE threshold, the committee will need identify an increasingly stronger case in support of the new technology, using the five components, in order for a positive reimbursement decision to be made. These components include:

- The degree of certainty of the ICER for the new technology
- Whether the HRQoL benefits of the new technology have been inadequately captured
• The innovative nature of the new technology which has not been captured in QALY measures. The supporting evidence for which should be substantial, demonstrable and distinctive

• The non-health related objectives of the NHS, specifically:
  o Whether the decision would have a bearing on broader social considerations to the extent that these are covered by NICE’s principles on social value judgements
  o Whether a substantial proportion of the cost savings are incurred outside of the NHS, and personal and social services, or are associated with significant benefits outside of a patient’s health
    • However, this is only considered when requested specifically by the DoH

• The life extension properties of the new technology at the end of life

With regards to the non-health related objectives of the NHS, techniques exist to quantitatively measure the trade-off between health benefits and non-health benefits. However, at present, the introduction of these techniques is considered unsuitable. Therefore, the committee takes non-health objectives of the NHS into account by considering the extent to which society may be prepared to forego health gain in order to achieve other benefits that are not health related.

One of the most significant roles of the appraisal committee is to decide upon the most realistic ICER for the new technology after an independent academic group has performed the cost-effectiveness modelling and ICER analysis. It has recently been reported that cost-effectiveness, as determined through ICER calculations, correctly predicts 82% of NICE appraisal decisions (Dakin et al, 2015). Outside of this decision-making process, no formal calculations are used to determine the influence of each of the components of the current HTA approach upon the reimbursement decision. For the new technology, each component is deliberated upon, and a consensus is reached, by the appraisal committee as to whether any of the five components should have a bearing upon the reimbursement decision. However, it is within the authority of the Appraisal Committee to apply a maximum of a 1.5 times weighting upon the lower boundary of the NICE threshold (£20,000 per QALY) when considering the majority of the components outside of the ICER of the new technology (NICE, 2014). Additionally, as described later in section 5.2.5, the maximum weighting
applicable for treatments that meet the ‘End of Life’ criteria is 2.5 times the lower boundary of the NICE threshold.

Within the present study, in order to perform a cost-effectiveness analysis for Allogeneic Islet Transplantation, based upon the NICE HTA parameters, a number of assumptions were made due to data availability and in order to avoid unnecessary complexity. These included:

- A maximum time horizon of 20 years was utilised
  - This was based upon the time horizon of the annual cost data for Allogeneic Islet Transplantation and Insulin Therapy provided by Beckwith and colleagues (2012)
- A conservative assumption was made to not incorporate the clinical and cost benefits from avoiding long-term complications after Allogeneic Islet Transplantation into this analysis
- Allogeneic Islet Transplantation complete graft failure rates per year were based upon those utilised within the Beckwith et al study (2012). Complete graft failure rates were reported to be 0% over one year, 17% over five years, 24% over 10 years, and 30% over 20 years. The proportion of patients with full graft function was reported to be 93% over one year, 47% over five years, 27% over ten years, and 7.5% over 20 years. The graft survival rates utilised in the present study are comparable to those described in recent longitudinal studies (Barton et al, 2012; Bellin et al, 2012).
  - Patients with full graft function are defined as being completely insulin independent, whereas patients with complete graft failure are defined as no longer presenting detectable levels of C-peptide (Beckwith et al, 2012)
- The NICE ‘reference case’ methods were not utilised to derive the health utility and cost values, or the time horizon, utilised in the present analysis
- When determining the impact of a new treatment upon the Net Production (£ per QALY gained) of a single IDDM patient within the VBP assessment, using the Department of Health Wider Societal Benefit (WSB) calculation template spreadsheet, it was assumed that Allogeneic Islet Transplantation contributed minimally to life extension (1% QALY gains through life extension)
5.2.1 Assessment of the manufacturer’s Value Dossier, including cost-effectiveness analysis through the determination of the Incremental Cost-Effectiveness Ratio (ICER)

In order to perform the ICER calculations for Allogeneic Islet Transplantation, the QALYs gained from both the new (Allogeneic Islet Transplantation) and ‘Gold Standard’ (Insulin Therapy) must first be identified. This calculation is based upon the patients’ health state after treatment, on a scale of 0 to 1, and the duration for which they are in that health state. Generally, the duration values are equal for both the new and ‘Gold Standard’ treatments, unless the new treatment extends patient life expectancy.

\[
\text{QALYs gained} = \text{Health Utility (0 to 1 scale) x duration (years) of that state}
\]

The QALYs utilised in the present study are based upon the results of the Monte Carlo simulations performed by Beckwith et al (2012).

**Allogeneic Islet Transplantation QALYs gained** = 10.7 QALYs over 20 years (Beckwith et al, 2012)

**Insulin Therapy QALYs gained** = 9.2 QALYs over 20 years (Beckwith et al, 2012)

Next, the change in QALYs must be determined by subtracting the QALYs gained from the ‘Gold Standard’ treatment from the QALYs gained for the new treatment, as shown in the formula below.

\[
\Delta \text{QALY} = \text{New Treatment QALYs} - \text{‘Gold Standard’ QALYs}
\]

\[
\Delta \text{QALY} = 10.7 - 9.2 = 1.5 \text{ QALYs}
\]

The change in cost, due to the introduction of the new treatment, is calculated by subtracting the cost of the ‘Gold Standard’ treatment from the cost of the new treatment, as shown in the formula below. In the current reimbursement system, the price proposed by the manufacturer is used as the cost values for the new treatment during ICER calculations. For the change in cost calculations within the present appraisal, allogeneic islet transplantation is viewed as an interventional procedure, for which the cost of the treatment is utilised, rather than as a new product from a manufacturer, in which the price with an incorporated profit margin would be incorporated. However, although cost-effectiveness analyses are not routinely incorporated into NICE interventional procedures guidance (NICE, 2009) and given the high costs
associated with Allogeneic Islet Transplantation, it is likely that an economic evaluation would be incorporated into an appraisal of this procedure.

\[ \Delta \text{Cost} = \text{Cost of new treatment} - \text{Cost of ‘Gold Standard’ Treatment} \]

\[ \Delta \text{Cost} = £321,704.20 - £410,870.63 = -£89,166.44 \] over 20 years (Beckwith et al, 2012)

The results of this appraisal indicate that adopting Allogeneic Islet Transplantation to treat IDDM would generate a 1.5 QALY gain compared to using Insulin Treatment, whilst saving the payer approximately £89,000, over a 20 year period. However, the initial, one-off costs of approximately £60,000 for Islet Transplantation, attributed to organ donation, islet manufacture, screening, and the performance of the medical procedure, may be prohibitively expensive. Although, provided sufficient certainty in the long-term cost-saving nature of the new technology exists, high initial costs may not be considered unreasonable. With regards patient QoL, it must also be noted that, in addition to the 1.5 QALY gain observed over a 20 year period, islet transplantation has been previously reported to reduce pain scores by up to 70% and to result in over 90% patient satisfaction (Garcea et al, 2013).

The 20 year QALY and cost data for both Insulin therapy and Allogeneic Islet Transplantation utilised in the present analysis, was generated, using Markov and Monte Carlo modelling, and published by Beckwith and colleagues (2012). The cost data was originally published in U.S dollars ($), and these values were converted into British Pound Sterling (£) using a conversion rate of £0.62 per dollar (October 2014). Although the 20 year costs of Allogeneic Islet Transplantation and Insulin Therapy, generated by Beckwith and colleagues (2012), are likely to be specific to the healthcare system from which the initial cost estimates were taken, this data was applied to the UK healthcare system and utilised in the present study as it represents the most comprehensive long-term analysis of the costs of Allogeneic Islet Transplantation.

The formula for the calculation of the ICER for Allogeneic Islet Transplantation, as shown below, incorporates the results of the two previous calculations, dividing the change in QALYs gained by the change in cost.

\[ \text{ICER} = \Delta \text{Cost} / \Delta \text{QALY} \]
After calculating the ICER for Allogeneic Islet Transplantation using a 20 year time horizon, it is apparent that this intervention is associated with both cost savings and QALY gains, and therefore ‘dominates’ the comparator. Although negative ICERs lack meaning, the 20 year ICER calculation, which induces a negative ICER, is shown below.

\[ 20 \text{ year ICER} = -£89,166.44/1.5 \text{ QALYs} = -£59,087.70/\text{per QALY} \]

From the results of the ICER calculations, it is clear that the introduction of Allogeneic Islet Transplantation for the treatment of IDDM would be considered to be cost saving and would dominate the comparator (Insulin Therapy) over an approximate time horizon of 9 years. However, as shown in the sensitivity analyses (Figures 8 & 11), in the short term (<9 years) this treatment would be unlikely to be cost saving. Additionally, if the costs of this procedure increase above those utilised in the present analysis, a longer period would be required in order to realise the cost savings and before the intervention dominated the comparator. However, if the treatment of diabetic patients with Allogeneic Islet Transplantation
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...successfully induced significant clinical benefit for approximately a decade, the likelihood of long-term cardiovascular complications may be significantly reduced (Fiorina et al, 2003; Fiorina et al, 2005).

By utilising the data regarding the ΔQALY between Insulin Therapy and Allogeneic Islet Transplantation, and the NICE threshold of £20,000 per QALY, the maximum cumulative cost of Allogeneic Islet Transplantation required to achieve cost-effectiveness can be determined and is illustrated in Figure 9 (below). It is apparent from the cumulative cost data that, given the efficacy of the treatment, Allogeneic Islet transplantation is too expensive to be considered cost-effective in the short term. However, after 9 years, the cumulative cost falls below the maximum cumulative cost and could therefore be considered cost-effective, as is also demonstrated in the ICER sensitivity analysis (Figure 8). In the long-term, with a time horizon of 20 years, a cumulative cost of £441,051.63 could be incurred with the treatment remaining cost-effective. Both the maximum cumulative cost and the actual cumulative cost assume the same efficacy of transplantation.

![The Maximum Cumulative Cost of Allogeneic Islet Transplantation Required to Achieve Cost-Effectiveness](image)

**Figure 9:** Comparison of the maximum cumulative cost to fall below the NICE threshold (£20,000 per QALY) and the actual cumulative cost of Allogeneic Islet Transplantation. The efficacy of treatment was assumed to be the same for both the maximum and actual cumulative costs.

Within the published study by Beckwith and colleagues (2012), rates of full graft function of 93% over one year, 47% over five years, 27% over ten years, and 7.5% over 20 years were assumed. This data, used within the Markov models (Beckwith et al, 2012), regarding the...
probability of graft function was obtained from the Diabetes Control and Complications Trial conducted between 1990 and 1993 (The Diabetes Control and Complications Trial Research Group, 1997), as this was considered to represent the most accurate estimates at the time. At the time of the publication of Beckwith et al study (2012), these graft survival rates were considered to be optimistic. However, given the recent improvements in Allogeneic Islet Transplantation protocols and graft survival, with up to 50% graft survival reported after 5-years (Bellin et al, 2012), these values can be considered more representative. If graft survival rates were to improve further, and the cost effectiveness analyses performed in the present study were based upon these improved graft survival rates, the time point at which the intervention would become cost saving and would dominate the comparator would extend.

Due to the uncertainty of Allogeneic Islet Transplantation graft survival over time, it may be appropriate to consider the consequences of a short time horizon. Therefore, in addition to the 20 year time horizon utilised in the present analysis, the cost-effectiveness of Allogeneic Islet Transplantation was also examined over a conservative 5 year time horizon, based upon the duration of follow-up in a previous international trial (Shapiro et al, 2006). A conservative time horizon, as specified by the appraisal committee, can also be based upon the duration of efficacy of similar products, although this evidence may not exist for regenerative medicines.

If the time horizon is limited to 5 years, due to the uncertainty of graft survival over time, it is unlikely that Allogeneic Islet Transplantation would be considered cost-effective with an
ICER of £128,000 per QALY, as demonstrated in Figure 11. Furthermore, the maximum five-year cumulative cost for Allogeneic Islet Transplantation to be considered cost-effective would be £73,141.37, whereas the actual cost is £119,224.06, as demonstrated in Figure 9. Therefore, greater evidence of long-term graft survival is required before the 9 year time horizon and the corresponding ICER would be considered to have sufficient certainty and thus for Allogeneic Islet Transplantation to be considered cost-effective.

![The Incremental Cost-Effectiveness Ratio (ICER) of Allogeneic Islet Transplantation over a 5 year period](image)

**Figure 11:** Sensitivity analysis illustrating the Incremental Cost-Effectiveness Ratio (ICER) of Allogeneic Islet Transplantation with the time horizon limited to 5 years, compared to the NICE Threshold (£20,000 per QALY).

After the determination of the ICER of the new treatment, a decision on the adequacy of the evidence provided and the certainty of the ICER is required. Although the data provided in the Beckwith et al (2012) study was modelled over a 20 year period and gives an indication of long-term costs, the significant rate of graft failure after transplantation creates uncertainty in the accuracy of the ICER and may therefore impact the likelihood of a positive reimbursement decision from the appraisal committee. Therefore, it is apparent that the duration of graft survival is a major determinant of cost-effectiveness, and that resolving uncertainty in this parameter, through longer term data collection in a larger group of patients, would increase the plausibility of estimates of cost-effectiveness.
5.2.2 Decision regarding whether Health Related Quality of Life (HRQoL) was inadequately captured

It is likely that the HRQoL was adequately represented in the health utility and QALY data utilised in the ICER calculations. However, although HRQoL is often accounted for in the determination of health utilities and QALYs for a new treatment, this may not fully capture social or psychological effects, for example the fear of hypoglycaemia in diabetic patients (Currie et al, 2006).

Various studies, including the GRAGIL (Benhamou et al, 2009) and MIAMI (Tharavani et al, 2008) trials, have examined the HRQoL associated with Islet Transplantation, accounting for factors outside of physical health. The MIAMI study reported improved physical functioning up to 6 years post-transplantation and improved social functioning up to 5 years post-transplantation, and these findings have been supported by a number of other studies (Benhamou et al, 2009; Barshes et al, 2005). The MIAMI study also identified no sustained change in mental health problems, energy levels or fatigue after Islet Transplantation. Toso and colleagues (2007) demonstrated that fewer emotional problems were reported post-transplantation, and propose that this is likely to be due to the improved management of hypoglycaemic events. Glucose control stability has previously been described as the main beneficial factor of Islet Transplantation which influences patient QoL (Poggioli et al, 2006).

Although these studies did not utilise the EQ-5D health outcome measurement instrument, which is considered to be NICE’s preferred method of measurement, they did utilise a number of established methods of health outcome data collection, including the Health Status Questionnaire 2.0 (HSQ 2.0), the 36-Item Short Form Health Survey (SF-36), and the Hypoglycaemia Fear Index.

However, although successful islet transplantation may abolish the requirement for regular, intensive insulin therapy, patients will still require regular immunosuppressive treatment or medication in order to prevent graft rejection. The use of these medications may have significant side effects, and the long-term effects of which are under examination. Such immunosuppressive treatment is commonly performed after transplantation and any negative effects of this therapy are likely to have been considered within health utility and QALY estimates.

Therefore, the improved management of hypoglycaemic events and the resulting improved HRQoL after Islet Transplantation, which is unlikely to be included in health utility and
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QALY calculations, should be considered by the Appraisal Committee during decisions regarding inadequately captured HRQoL. It is therefore likely that increased allowance would be applied to the NICE threshold for Islet Transplantation, however, if the 20 year ICER for this treatment were to be considered accurate by the appraisal committee, this treatment may be reimbursed regardless of HRQoL that has been inadequately captured as the ICER falls significantly below the £20,000 per QALY NICE threshold. After considering the results of the sensitivity analysis (Figures 8 & 11), although the Appraisal Committee may be likely to consider the HRQoL inadequately captured evidence in their decision making process and given the uncertainty regarding the duration of graft function, this consideration is unlikely to be sufficient to allow for a positive reimbursement decision if a short time horizon (<9 years) is considered.

5.2.3 Decision regarding whether the technology is significantly innovative in nature

In a study for NICE, by Sir Ian Kennedy (Kennedy, 2009), it was outlined that a new technology would be considered innovative if it met three initial criteria during appraisals:

- The technology is new
- The new technology constitutes an improvement upon existing products
- The technology offers a step-change in terms of outcomes for patients

Given its potentially curative nature, the potential development of insulin independence after treatment, and the recent advancements in the derivation, purification and delivery of islets (Pavlakis & Khwaja, 2007; McCall & Shapiro, 2012; Johnson & Jones, 2012), Allogeneic Islet Transplantation for the treatment of IDDM could be considered to be significantly innovative in comparison to the ‘Gold Standard’ Insulin Treatment. As demonstrated in Figure 8, eliminating the requirement for regular insulin therapy and glucose monitoring may induce a significant cost saving for the payer of approximately £60,000 over a 20 year period.

Additionally, the isolation of Islets of Langerhans from cadaveric donor pancreas and their infusion into a patient’s hepatic portal vein, in order to restore beta cell function and the maintenance of blood glucose, can be considered significantly novel in comparison to the ‘Gold Standard’ therapy for IDDM of insulin therapy. After pioneering research in rodents by Paul Lacy throughout the 1960s, Dr James Shapiro and colleagues developed and published the first standardised protocol, known as the Edmonton Protocol, for islet transplantation in July 2000 (Shapiro et al, 2000) and the results of the first international trial of this protocol
were published in September 2006 (Shapiro et al, 2006). This approach has a curative potential for patients, albeit with subsequent immunosuppressive medication, and may contribute to the future development of a cure for IDDM. Recent research has been seeking methods of allowing immune tolerance of transplanted islets, including their encapsulation in biocompatible materials (Teramura et al, 2007; Beck et al, 2007), and future developments in this area hold significant promise.

Thus, as Allogeneic Islet Transplantation could be considered to be ‘new’, to constitute an improvement upon existing products, with full graft function, and would represent a step-change in treatment outcomes compared to insulin therapy, the innovative nature of islet transplantation should be considered by the Appraisal Committee during their deliberation. Therefore, greater allowance in the NICE threshold may be permitted for Allogeneic Islet Transplantation, if the 20 year ICER for this treatment were to be considered accurate by the appraisal committee, this treatment may be reimbursed regardless of its innovative nature as the ICER falls significantly below the £20,000 per QALY NICE threshold. However, after consideration of the results of the sensitivity analyses (Figures 8 & 11), although the innovative nature of this treatment may support its reimbursement, it may not be sufficient to facilitate a positive reimbursement decision if the benefits of Allogeneic Islet Transplantation are accrued for <9 years. Furthermore, it must be acknowledged that the duration of graft function and rate of graft failure place significant uncertainty upon the ICER.

5.2.4 Decisions on other factors unrelated to health

During HTAs and reimbursement decisions, non-health related objectives of the NHS are considered. A number of parameters, that are typically given additional weighting when making judgements, have been defined, and include the severity of the underlying illness, disadvantaged populations, children, and stakeholder persuasion (Rawlins et al, 2010). It is likely that IDDM patients would strongly support the adoption of islet transplantation, particularly due to the potential for insulin independence. The World Health Organisation estimates that between 11 and 22 million people worldwide suffer from IDDM (WHO, 2013). Therefore a significant patient population exists and this may have an influence upon Appraisal Committee decisions. Thus, the committee may consider applying increased allowance to the NICE threshold. Although this increased allowance may not be necessary to induce a positive reimbursement decision when the benefits of islet transplantation are
accrued over a 20 year period, it is unclear whether any increased allowance would be sufficient when the benefits are accrued for fewer than 9 years.

5.2.5 Determination of life extension during end of life treatment

Currently, NICE appraisal committees are asked to consider applying a weight to QALYs gained for people at the end of their life (<2 years) and who are likely to gain at least 3 months of overall survival from treatment (NICE Centre for Health Technology Evaluation, 2014). A weighting upon ICERs of up to 2.5x can currently be applied by appraisal committees for ‘end of life’ treatments. This weighting allows ICERs of up to 2.5x the £20,000 per QALY NICE threshold to be considered as acceptable by the appraisal committees, depending upon the number of QALYs gained at the end of life through treatment. However, Allogeneic Islet Transplantation is not a treatment that is regularly utilised, and does not generally extend life, at the end of a patient’s life. Therefore, due to the lack of life extension of this treatment at the end of life, no additional consideration for this would be made by the Appraisal Committee.

5.3 Cost-Effectiveness Analysis: Proposed Value-Based Pricing (VBP) Approach

As described previously in sections 3.3.2.2 & 5.1, the transition to a VBP scheme involved the increased prioritisation of three key parameters within the HTA process, including burden of illness, the innovative nature of a new technology, and the wider societal benefit associated with the new technology. Two of these components, specifically burden of illness and wider societal benefit, were not previously considered in HTAs in a significant or quantitative manner.

It must be noted that this VBP approach would have maintained the utilisation of an ICER to determine the cost-effectiveness of a new technology. However, this ICER would have been weighted based upon burden of illness and the wider societal benefit of the new technology. The VBP model would have utilised the initial price set by the manufacturer to determine the weighted ICER which would in turn inform pricing negotiations between the manufacturer and the government. If the weighted ICER for new technology was not favourable, the manufacturer would have the opportunity to reconsider their pricing, and therefore if the opportunity to reduce the price of the technology under appraisal was taken, the cost-effectiveness analysis would be repeated using the new, lower price. (National Institute for
Health and Clinical Excellence, 2011). One of the most significant changes that would have occurred under VBP was the exclusion of PPRS in pricing negotiations.

5.3.1 Assessment of the manufacturer’s Value Dossier, including cost-effectiveness analysis through the determination of the Incremental Cost-Effectiveness Ratio (ICER)

After calculating the ICER for Allogeneic Islet Transplantation using a 20 year time horizon, it is apparent that this intervention is associated with both cost savings and QALY gains, and therefore ‘dominates’ the comparator. Although negative ICERs lack meaning, the 20 year ICER calculation, which induces a negative ICER, is shown below.

\[ \text{20 year ICER} = \frac{-£89,166.44}{1.5 \text{ QALYs}} = -£59,087.70 \text{ per QALY} \]

However, as demonstrated in the earlier sensitivity analysis (Section 5.2.1), in the short term (<9 years), this treatment is unlikely to be considered to be cost saving. Additionally, as evidence exists to suggest that graft survival may not be as high as that incorporated into the analysis performed by Beckwith and colleagues (2012), a 9 year duration is unlikely to be cost saving (Shapiro et al, 2006), and therefore significant uncertainty surrounds this analysis.

5.3.2 Assessment and weighting of the Burden of Illness (BoI)

Burden of Illness (BoI) can be described as a the projected number of QALYs for a patient treated through current methods, subtracted from the expected number of QALYs for an individual of the same gender and age without the condition (NICE Decision Support Unit, 2013).

The Department of Health proposed that Burden of Illness could be calculated by subtracting the life expectancy of a patient from the gender and age adjusted life expectancy of a healthy individual, and multiply this by the Quality of Life (QoL) (%) that they would have been expected to have without the condition (NICE Decision Support Unit, 2013). For example, a 20 year old male IDDM patient with a current QoL of 81%, using the health utility data from Beckwith et al (2012), would be expected to live until age 59 with current treatment (Department of Health, 2001). Without the condition, they would have been expected to live until 79 years of age (Office for National Statistics, 2013) with a QoL of 91% of normal (Beckwith et al, 2012).
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| QALYs lost through life expectancy | 79-59 years × 91% QoL = 18.2 QALYs |
| QALYs lost through reduced Quality of life (QoL) | (59 years - 20 years) × (91% - 81%) = 3.9 QALYs |

Total BoI = QALYs lost through life expectancy + QALYs lost through reduced QoL

Total BoI = 18.2 QALYs + 3.9 QALYs = 22.1 QALYs

Additionally, if, for example, a treatment maintained a patient’s life expectancy but increased their QoL by 10% for 5 years, then a 0.5 QALY gain (5 years × 10% QoL) would be added to the BoI calculations. A relatively simple approach to valuing a BoI was proposed by the DoH, with each QALY loss valued at a fixed rate (5%).

It is apparent that, as this calculation determines the burden of illness for a typical IDDM patient and does not distinguish between treatments, no difference in the BoI would be
observed between patients that received Allogeneic Islet Transplantation and those that received Insulin Therapy.

**BoI Weight per QALY gained** = \[\text{BoI} \times \text{Fixed rate of QALY value for BoI (5%)}] + 1

<table>
<thead>
<tr>
<th>BoI Weight per QALY gained = (22.1 QALYs x 0.05) + 1 = 2.105</th>
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**BoI-adjusted additional QALY gain** = Incremental QALY gains from new treatment \( \times \) BoI Weight per QALY gained

**BoI-adjusted additional QALY gain** = 1.5 QALYs \( \times \) 2.105 = 3.158 QALYs

However, with Islet Transplantation producing 0.03, 0.43 or 0.93 QALYs over 1, 5 or 10 years respectively, the BoI adjusted QALY gain would equate to 0.063, 0.905, or 1.96 QALYs respectively.

It has been indicated that society values the two components of BoI, Quality- (QoL) and Length-of Life (LoL), differently, with LoL being valued higher than QoL (Brazier et al, 2013). Therefore, QALY gains from QoL and LoL could be separated and the relevant LoL and QoL BoI weights could be applied respectively. However, it was not specified whether a one- or two-component weighting approach would be utilised under VBP, and therefore, in the present study, BoI weighting will be determined using the method outlined in the NICE proposals for Wider Societal Benefits (NICE Decision Support Unit, 2013).

The DoH proposals also outline that under VBP, a single NICE threshold universally applied to all new technologies would no longer exist and that this threshold would be adjusted depending upon the amount of BoI-adjusted QALY gains a new technology would theoretically displace, due to resource allocation away from incumbent technologies, and produce. Technologies that produce more BoI-adjusted QALY gains than they displace should be compared against a higher NICE threshold, as the health gains they produce are not entirely recognised under the current QALY calculations. Conversely, a lower NICE threshold should be utilised for technologies that displace more BoI-adjusted QALY gains than they produce.
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The NICE threshold of £20,000 per QALY represents the value that, when spent, displaces 1 QALY elsewhere in the NHS. If, for example, a treatment incurs a health gain of 2 QALYs and an ICER of £10,000 per QALY, the treatment is generating 2 QALYs where only 1 QALY is lost elsewhere in the NHS, and therefore there is a net gain of 1 QALY when £20,000 is spent. However, under VBP, as the value of a displaced QALY may vary depending upon the indication and the burden of illness, then the cost-effectiveness threshold must be manipulated.

**BoI-adjusted ICER** = \( \frac{\Delta \text{Cost between new & incumbent technology}}{\text{BoI-adjusted additional QALY gain}} \)

After adjustment for BoI, the intervention will continue to induce both QALY gains and cost savings, over a 20 year time horizon, and will therefore dominate the comparator. This is demonstrated in the negative ICER calculation below:

**BoI-adjusted 20-year ICER** = -£89,166.44 (\( \Delta \text{Cost between Allogeneic Islet Transplantation & Insulin Therapy} \))/3.158 QALYs = -£28,235.10 per QALY

If the BoI-adjusted ICER is adjusted for the short term benefits of Allogeneic Islet Transplantation over 1, 5 or 10 years, using cost differences of £65,917.23, £54,641.91, £2,402.85 and BoI-adjusted QALYs of 0.063, 0.905, and 1.96, the BoI-weighted ICER would equate to £1,046,305.24, £60,377.80 or £1,225.94 per QALY. The relationship between the BoI-adjusted ICER for Allogeneic Islet Transplantation and the time horizon considered is illustrated in Figure 15, and the raw data is presented in Appendix 13.4.

### 5.3.3 Assessment of therapeutic innovation

In agreement with the Department of Health’s proposal for Value-Based Pricing, NICE acknowledged that it is important to encourage the development of new medicines in areas that will be of greatest value to the NHS (National Institute for Health and Clinical Excellence, 2011). However, NICE identified that current HTAs already account for the innovative nature of a new technology to a certain extent, through their technology appraisals and reflected in improved health outcomes, and that the introduction of a weighting for innovative technologies may lead to ‘double counting’ of these benefits. Nonetheless, in some instances weighting may be applicable where the product may have wider benefits in the future. Although no formal measure of therapeutic innovation was defined, and given the
innovative and potentially curative nature of allogeneic islet transplantation, it is likely that this would have been considered in VBP appraisals and reimbursement decisions, and may have provided additional support in favour of a positive reimbursement decision for Allogeneic Islet Transplantation. However, as discussed previously in section 5.2.3, if the duration of islet graft function is under 9 years, then the consideration of the innovative nature of this therapy may not be sufficient to support a positive reimbursement decision.

5.3.4 Assessment and weighting of the Wider Societal Benefit (WSB)

The Department of Health defined Wider Societal Benefits (WSB) as ‘the difference between the amount of resources a patient contributes to society whilst afflicted with the condition (production) and the amount they use (consumption)’ (National Institute for Health and Clinical Excellence Decision Support Unit, 2013). They define ‘production’ as the addition of paid labour, or employment, and unpaid labour, for example childcare. ‘Consumption’ is defined as the combination of informal care; formal care; personal paid consumption; including consumables; personal unpaid consumption, for example cleaning; informal childcare and government services unrelated to health. More specifically, WSBs can be defined as the net economic contribution of a patient to society. If a treatment generates more WSBs than it displaces, then the cost-effectiveness should increase and this should be reflected by an increase in the NICE threshold for that particular treatment. Conversely, a therapy that displaces more WSBs than it produces should be compared against a lower NICE threshold.
The table below (Figure 14) outlines the formulae required to calculate each component of patient production and consumption, which can then be used to determine the patient’s net production. A substantial amount of the data required for these formulae, obtained from public sources such as the Annual Survey of Hours and Earnings (Office for National Statistics, 2012), the Time Use Survey (Office for National Statistics, 2000), the Adult Social Care Survey (NHS Information Centre, 2012), the Living Costs and Food Survey (Office for National Statistics, 2011), and the Public Expenditure Statistical Analysis (HM Treasury, 2012), has been collated and incorporated into a single template spreadsheet by the Department of Health. The template spreadsheet can be accessed by contacting the Department of Health (Gavin Roberts, Departments of Health, gavin.roberts@dh.gsi.gov.uk). By entering the relevant values for the Age, Gender, International Classification of Disease tenth revision (ICD-10) code, QoL, QoL increment, and Percentage of QALY gains from life extension, for the relevant patient population, into this spreadsheet, the Net Production (£ per calendar month) of treated and untreated patients can be calculated and the Net production per QALY gained for a new treatment can be determined. An example of the way in which this spreadsheet was utilised in the present study is illustrated in both Appendices 13.5.1 and 13.5.2.
An exchange rate has been proposed in the NICE proposal for Wider Societal Benefits in the VBP scheme, allowing the conversion of WSBs into a ‘QALY-worth equivalent’ which can then be combined into a single QALY value (NICE Decision Support Unit, 2013). This exchange rate equates to £60,000 per additional QALY gained, and can be utilised to determine a WSB-adjusted QALY.

**WSB-adjusted QALY gain** = Net Production per QALY gained (From the DoH Template Spreadsheet)/Proposed WSB exchange rate

**WSB-adjusted QALY gain** = £22,064 per QALY (Net Production per QALY gained for Allogeneic Islet Transplantation [See Appendix 13.5.2])/ £60,000 per additional QALY = 0.368 QALYs

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**Figure 14**: Table of the components and formulas for WSB Modelling. Components account for: A= Age groups, G= Gender, Q= QoL, I= ICD-10 Classification. See Appendix 13.3 for definitions of WSB model components.

<table>
<thead>
<tr>
<th>Component of Production or Consumption</th>
<th>Formula</th>
</tr>
</thead>
<tbody>
<tr>
<td>Paid Production</td>
<td>Paid Labour$<em>{AGO}$ = Productivity$</em>{AQ}$ x Wage per Month$_{AQ}$ x Oncosts</td>
</tr>
<tr>
<td>Unpaid Production: Childcare</td>
<td>Hours at Full Health$<em>{AQ}$ x Net Hourly Wage x (1- Sick Rate$</em>{AQ}$)</td>
</tr>
<tr>
<td>Unpaid Production: Informal Care</td>
<td>Hours at Full Health$<em>{AQ}$ x Net Hourly Wage x (1- Sick Rate$</em>{AQ}$)</td>
</tr>
<tr>
<td>Unpaid Production: All other Unpaid Production</td>
<td>Hours at Full Health$<em>{AQ}$ x Net Hourly Wage x (1- Sick Rate$</em>{AQ}$)</td>
</tr>
<tr>
<td>Formal &amp; Informal Care</td>
<td>(Probability of using Residential Care$<em>{AQ}$ x Cost of Residential Care) + (Probability of using Non-Residential Care$</em>{AQ}$ x Cost of Non-Residential Care) + (Days of Informal Care$<em>{AGIQ}$ x Hours of Informal Care per Day$</em>{AQ}$ x Net Wage)</td>
</tr>
<tr>
<td>Personal Unpaid Consumption</td>
<td>Estimated with respect to patient age. Average value per month estimated at £1,210 per month</td>
</tr>
<tr>
<td>Personal Paid Consumption</td>
<td>Estimated in relation to Age and QoL</td>
</tr>
<tr>
<td>Informal Childcare</td>
<td>Estimated based upon QoL and programmed into ‘Template Spreadsheet’</td>
</tr>
<tr>
<td>Government Services</td>
<td>(Government Expenditure- Government Expenditure on transfers payments- Government Expenditure on Public Goods)$_{A}$</td>
</tr>
</tbody>
</table>
Therefore, by utilising the WSB-adjusted QALY gain for Allogeneic Islet Transplantation, the BoI- & WSB-adjusted QALY gain and BoI- & WSB-adjusted ICER can be determined.

**BoI- & WSB-adjusted QALY gain =** BoI-adjusted QALY gain + WSB-adjusted QALY gain

**BoI- & WSB-adjusted QALY gain =** 3.158 + 0.368 = **3.526 QALYs**

When the Islet Transplantation procedure is only considered to be effective for 1, 5 or 10 years, this WSB and BoI-adjusted QALY equates can be calculated as 0.431, 1.273, or 2.328 respectively.

After adjustment for both BoI and WSB, the intervention will continue to induce both QALY gains and cost savings, over a 20 year time horizon, and will therefore dominate the comparator. This is demonstrated in the negative ICER calculation below.

**BoI- & WSB-adjusted 20-year ICER =** -£89,166.44 (ΔCost between Allogeneic Islet Transplantation & Insulin Therapy)/3.526 (BoI- & WSB-adjusted QALY gain) = **-£25,288.27 per QALY**

However, using the cost differences of £65,917.23, £54,641.91, £2,402.85 and BoI- & WSB-adjusted QALY gains of 0.431, 1.273, 2.328 over 1, 5 or 10 years, the WSB and BoI-adjusted ICER equates to £152,940.21, £42,923.73, or £1,032.15 respectively. The relationship between the BoI- & WSB-adjusted ICER and the time horizon considered is plotted in Figure 15, and the raw data is presented in Appendix 13.4.

In the NICE WSB proposal document (NICE Decision Support Unit, 2013), a formula for the incorporation of BoI and WSBs weightings for a new technology into an adjusted NICE threshold was outlined (below).

**Adjusted NICE Threshold =** Current NICE Threshold x [(1+BoI Weight+WSBs per QALY)/(1+dBoI weight+dWSBs per QALY)]

In the formula above, BoI weight is expressed as a percentage, and the WSBs per QALY are calculated by dividing the WSB adjusted QALY by the total number of QALYs gained for the new technology. The ‘d’ represents the equivalent values for the displaced technology.

The WSB per QALY value, from the formula above, can be calculated as demonstrated below:
WSB per QALY = WSB-adjusted QALY gain / Incremental QALY gains from new treatment


WSB per QALY = \( \frac{0.368 \text{ QALYs} \text{ (WSB-adjusted QALY gain for allogeneic islet transplantation)}}{1.5 \text{ QALYs} \text{ (Incremental QALY gains for allogeneic islet transplantation over 20 years)}} = 0.245 \)

The WSBs per QALY for the displaced treatment, which in the present case could hypothetically be insulin therapy, would be calculated using the formulae above and by comparing insulin therapy to a previously displaced therapy, which for IDDM is likely to be dietary control. However, the displaced therapy cannot be truly known, and this has been acknowledged as an uncertainty by the NICE Decision Support Unit (NICE Decision Support Unit, 2013). Also, as it remains unclear as to how the proposed BoI weight (%), specific to Allogeneic Islet Transplantation and Insulin Therapy, would have been determined, the Adjusted NICE threshold cannot be calculated in the present study. The 5% weighting proposed for the BoI calculations cannot be utilised here, as this value was to act as a fixed exchange rate for QALYs lost.

Finally, it must also be acknowledged that, from the example of Allogeneic Islet Transplantation in which a 20 year time horizon was utilised, as the QALYs gained from the new treatment is adjusted for BoI and WSB, the BoI- and WSB-adjusted ICER increases, becoming less negative, and moving closer towards the NICE threshold. It would be expected that, after the adjustment of the incremental QALY gain for BoI and WSB, the adjusted ICER would decrease moving further below the NICE threshold of £20,000 per QALY. This incorrect function of the formulae demonstrates the inconsequential nature of negative ICERs and the reason why negative ICERs are not routinely utilised. However, it is clear that this unexpected increase in the ICER after BoI and WSB adjustment occurs as these calculations do not account for cost saving treatments with a negative ICER, and that, when ICERs fall below zero, the Adjusted-ICER formulae would require adaptation. The correct function of these formulae is demonstrated when a shorter length of allogeneic islet transplantation graft survival is utilised, and where positive ICER values are observed. In these instances, the ICERs can be correctly adjusted, becoming smaller and falling closer to the £20,000 per QALY NICE threshold.
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5.4 Cost-Effectiveness Analysis: Preliminary Value-Based Assessment Approach

In the adapted VBA scheme, burden of illness (BoI) was again to be incorporated into HTAs, and it was proposed that a proportional shortfall approach be utilised. This concept of a proportional QALY shortfall was considered to be consistent with NICE’s current End of Life component (NICE Centre for Health Technology Evaluation, 2014). In contrast, as previously discussed in section 3.3.4.2, the wider societal benefit component proposed in the VBP approach was to be replaced with a wider societal impact (WSI) component. This would be calculated using an absolute QALY shortfall approach in order to determine whether greater allowance should be given to the ICER for the new technology (NICE Centre for Health Technology Evaluation, 2014).

Under the VBA scheme, although BoI and WSI would have been incorporated into HTAs, the majority of the components of the current appraisal system, including ICER certainty; HRQoL inadequately captured; innovation; non-health related objectives of the NHS, would have also been maintained. Additionally, although the End of Life criteria was not included in the initial VBA proposal, the DoH recommended that it be included in the new scheme (NICE, 2014). With regards the weighting of each HTA component, it was indicated in the original VBA consultation document that a maximum cumulative modifier of 2.5 may be
applied to new technologies that fulfil all the relevant criteria (NICE Centre for Health Technology Evaluation, 2014). However, as a result of public discussion and consultation with the DoH, it was recommended that the 1.5 times modifier, currently utilised for each HTA component, be maintained, with only the End of Life component holding a modifier of 2.5 (NICE, 2014).

It has recently been acknowledged, that under the VBA approach, it is likely that the outcomes for most new products would have been similar to what would have been expected under previous reimbursement models (Garau, 2014). This is likely to have been the case due to the maintenance of PPRS, and the reduction of the impact of BoI and WSI components upon the weighting of the ICER.

5.4.1 Determination of the Incremental Cost-Effectiveness Ratio (ICER)

In concordance with the current HTA approach, the VBA scheme would have utilised the manufacturer’s price to inform the cost values during the ICER calculations for the new technology. Additionally, PPRS would have again been at the core of price setting, and would have allowed for price negotiations after the HTA had determined the most acceptable ICER and price.

After calculating the ICER for Allogeneic Islet Transplantation using a 20 year time horizon, it is apparent that this intervention is associated with both cost savings and QALY gains, and therefore ‘dominates’ the comparator. Although negative ICERs lack meaning, the 20 year ICER calculation, which induces a negative ICER, is shown below.

\[20 \text{ year ICER} = -£89,166.44/1.5 \text{ QALYs} = -£59,087.70 \text{ per QALY}\]

From the results of the ICER calculations, it is clear that the introduction of Islet Transplantation for the treatment of IDDM would be considered to be cost saving over a 20 year period when compared to the current gold standard. However, as demonstrated in the sensitivity analyses (Figures 8 & 11), this treatment is unlikely to be considered to be cost saving in the short term (<9 years).
5.4.2 Determination of the Burden of Illness (BoI) and Weighting of the ICER

Burden of Illness is defined as “the total amount of future health lost for people with a condition, measured in QALYs” (NICE Centre for Health Technology Evaluation, 2014). The point from which the health loss is determined would be the moment when intervention with the technology under appraisal would be undertaken. Although an absolute QALY shortfall approach was considered, in which the reduced number of QALYs of a patient were subtracted from the total QALYs that people of the same age and gender distribution would be expected to have, a proportional QALY shortfall approach was put forward by NICE, where the total QALYs for a patient is measured relative to those expected for a person of the same age and gender distribution, in order to calculate the burden of illness. Although a proportional QALY shortfall is not considered to be particularly sensitive to the age at diagnosis, it is consistent with the current ‘end of life’ approach and would have therefore comfortably integrated into the NICE technology appraisal methodologies.

Similarly to the ‘end of life’ treatment modifier in the current appraisal scheme, a maximum combined modifier of 2.5 was initially proposed for treatments that fulfil the criteria for each of the components of the VBA scheme (NICE Centre for Health Technology Evaluation, 2014). Although this would not have extended the maximum acceptable ICER above the £50,000 per QALY threshold currently in place for technologies that fulfil the End of Life criteria, it would allow new treatments, for which the ICER falls above the £20,000 per QALY threshold, to be considered acceptable by the appraisal committee. However, as described previously in section 5.4, more recently the DoH advised NICE to maintain the 1.5 times modifier for the majority of VBA components (NICE, 2014).

Regardless, no definitive guidance regarding the application of BoI weighting upon the NICE threshold was issued by the Department of Health, and this may have been considered during the Appraisal Committee’s deliberations. Therefore, in the present study, the proportional QALY shortfall is calculated for the treatment of IDDM patients with Allogeneic Islet Transplantation and the weighting is speculated. The calculations below, as in the Value-Based Pricing calculations, utilise a 20 year old patient, with a life expectancy of 59 years, as an example, as well as healthy individual life expectancy and health utility values of 79 years and 0.91 respectively.
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Number of years of life remaining for healthy individual = Normal Life Expectancy - Patient Age

Number of years of life remaining for healthy individual = 79-20 years = 59 years

QALYs remaining for a healthy individual = Years of life remaining x Normal QoL/Health utility

QALYs remaining for a healthy individual = 59 years x 0.91 (Normal Health Utility) = 53.69 QALYs

Number of years remaining for patient without treatment = Patient Life Expectancy – Patient Age

Number of years remaining for patient without treatment = 59 years - 20 years = 39 years

QALYs remaining for patient without treatment = Number of years remaining for patient without treatment x Patient QoL or Health utility

QALYs remaining for patient without treatment = 39 years x 0.81 (Health Utility of IDDM patient with insulin therapy) = 31.59 QALYs

Expected loss of QALYs without of treatment = QALYs remaining for a healthy individual - QALYs remaining for patient without treatment

Expected loss of QALYs without of treatment = 53.69 - 31.59 QALYs = 22.1 QALYs

Proportion of healthy individual QALYs remaining = Expected loss of QALYs without of treatment / QALYs remaining for a healthy individual
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Proportion of healthy individual QALYs remaining = \frac{22.1 \text{ QALYs}}{53.69 \text{ QALYs}} = 0.412 \times 100 = 41.2\%

Proportional Shortfall = 100\% - Proportion of healthy individual QALYs remaining

Proportional Shortfall = 100\%-41.2\% = 58.8\%

As discussed previously, no method of weighting the ICER or NICE threshold based upon this proportional shortfall was proposed and this may have been determined by Appraisal Committee deliberation. However, it can be assumed that, as this shortfall is significantly greater than the average (9\% shortfall) (Garau, 2014), the ICER the treatment of IDDM patient using Allogeneic Islet Transplantation would be compared to a higher NICE threshold (>\£20,000 per QALY) or that greater allowance would be applied to this threshold. However, this methodology could be considered ambiguous and may not adequately inform an appraisal committee during the decision-making process.

5.4.3 Determination of the Wider Societal Impact and Weighting of the ICER

Within current HTAs, the impact of a disease outside of the health of a patient can be considered, however, in practice, the capacity of the committee to do this is often limited. By incorporating a formal measure of the contribution or burden of a patient, with or without treatment, upon society, the potential range of benefits of a new treatment may be more comprehensively represented. This may be favourable for technologies which have the potential to restore a patient’s health and consequently their ability to contribute to society.

As mentioned previously in section 3.3.4.2, it was outlined in the VBA consultation paper that the framework for wider societal benefit (WSB) could not be comfortably integrated into NICE appraisals without significant alteration as well as mitigation of effects that would have been considered unacceptable to society, in particular age-related productivity bias (NICE Centre for Health Technology Evaluation, 2014). Therefore, it was proposed that WSB be replaced by a wider societal impact (WSI) framework in the VBA scheme. Rather than determine the difference between net production and consumption per patient, as with WSB, the WSI framework would utilise an absolute, societal shortfall approach in which the health lost by patients as a result of their condition is utilised to determine the wider societal impact. This shortfall would represent the loss in a patient’s ability to engage with society as a result
their condition, compared to the capacity to engage with society of an unaffected individual, with QALYs used as an indirect measure of societal shortfall. Although patient age will inevitably affect the ability of a person to contribute to society, age was not to be used as the basis of HTA decisions.

As discussed previously in section 5.4, it was suggested that a maximum cumulative modifier of 2.5 could be applied to treatments which fulfil each of the VBA component criteria, including BoI and WSI, although the DoH recommended maintaining the current 1.5 times weighting for all components besides End of Life. However, as with the BoI weighting, no guidance was released describing how WSI would have adjusted the NICE threshold. Therefore, in the present study, the absolute, societal QALY shortfall is determined and the weighting is conjectured.

**Absolute Societal Shortfall** = Expected QALYs without disease - Expected QALYs with disease

**Absolute Societal Shortfall** = 53.69 - 31.59 QALYs = **22.1 QALYs**

Although no method of weighting the ICER or NICE threshold based upon this absolute shortfall was proposed, it can be assumed that, as this shortfall is significantly greater than average (2.1 QALY shortfall) (Garau, 2014), the ICER for the treatment of IDDM patient using Allogeneic Islet Transplantation would be compared to a higher NICE threshold (£20,000 per QALY) or that greater allowance would be applied to this threshold. However, as with the BoI proportional shortfall value, this outcome could be considered ambiguous and may not adequately inform appraisal committees.

5.4.4 Decision regarding whether Health related Quality of Life (HRQoL) was inadequately captured

No changes to the method of determining whether HRQoL has been inadequately captured within a HTA were proposed (Garau, 2014). Therefore, the improved HRQoL, and in particular the improved management of hypoglycaemic events, after Islet Transplantation, which is unlikely to be included in health utility and QALY calculations, should be considered in reimbursement decisions. Therefore, it is likely that increased allowance would be applied to the NICE threshold for Islet Transplantation, however, if the 20 year ICER for this treatment were to be considered accurate by the appraisal committee, this treatment may be reimbursed regardless of HRQoL that has been inadequately captured as the ICER falls.
significantly below the £20,000 per QALY NICE threshold. Given the results of the sensitivity analyses (Figures 8 & 11), although the Appraisal Committee may be likely to consider the HRQoL inadequately captured evidence in their decision making process, this consideration is unlikely to be sufficient to allow for a positive reimbursement decision for if the benefits are accrued for less than 9 years.

5.4.5 Decision regarding whether the technology is significantly innovative in nature

The decision making process for determining the significance of the innovative nature of a new technology was to remain similar to previous HTA approaches, however, under the VBA approach, ‘a step change in the management of a condition’ would have been given greater consideration (Garau, 2014). Therefore, the innovative nature of islet transplantation should be considered when making a reimbursement decision under VBA. Additionally, it may be the case that extra consideration should be given to allogeneic islet transplantation given that this treatment may represent a step change in the management of IDDM in comparison to standard insulin therapy. If the 20-year ICER, in which the allogeneic islet transplantation remains successful, were to be utilised in reimbursement decision making, this additional supporting evidence may not be necessary to facilitate a positive reimbursement decision. However, from the sensitivity analysis data, it is unlikely that this additional consideration would provide further support for a positive reimbursement decision if the time horizon is less than 9 years.

5.4.6 Decisions on other factors unrelated to health

Once again, the methods of determining to what extent a new technology fulfils the non-health related objectives of the NHS will not be significantly altered compared to previous appraisal methods. Therefore, the significant IDDM patient population that exists would be likely to have an influence upon reimbursement decisions under VBA.
<table>
<thead>
<tr>
<th>HTA Methodology</th>
<th>Component of HTA</th>
<th>5 years: £127,679.50</th>
<th>20 years: £59,087.70</th>
<th>5 years: £60,377.80</th>
<th>20 years: £28,235.10</th>
<th>5 years: £42,923.73</th>
<th>20 years: £25,288.27</th>
</tr>
</thead>
<tbody>
<tr>
<td>Current</td>
<td>ICER &amp; Certainty (Quantitative &amp; Qualitative)</td>
<td>HRQoL Inadequately Captured (Qualitative)</td>
<td>Innovative Nature of technology (Qualitative)</td>
<td>Non-Health related objectives of the NHS (Qualitative)</td>
<td>Life Extension at end of life (Quantitative &amp; Qualitative)</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5 years: Dominating</td>
<td>Some inadequately captured improvements in HRQoL (e.g. management of hypoglycaemic events)</td>
<td>Potentially regenerative therapy. Innovative in comparison to gold standard (Insulin Therapy)</td>
<td>Significant patient population</td>
<td>N/A to this technology</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>VBP</td>
<td>ICER &amp; Certainty (Quantitative &amp; Qualitative)</td>
<td>Burden of illness (BoI) (Quantitative)</td>
<td>Therapeutic Innovation (Qualitative)</td>
<td>Wider Societal Benefit (WSB) (Quantitative)</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5 years: Dominating</td>
<td>Bol-adjusted ICER: 5 years: £60,377.80</td>
<td>Potentially regenerative therapy. Innovative in comparison to gold standard (Insulin Therapy)</td>
<td>Bol- &amp; WSB-adjusted ICER: 5 years: £42,923.73</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td></td>
<td>20 years: Dominating</td>
<td>Bol-adjusted ICER: 20 years: £28,235.10</td>
<td>-</td>
<td>20 years: Dominating (-£25,288.27)</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>VBA</td>
<td>ICER &amp; Certainty (Quantitative &amp; Qualitative)</td>
<td>Burden of illness (BoI) (Quantitative)</td>
<td>No weighting method proposed</td>
<td>Wider Societal Impact (WSI) (Quantitative)</td>
<td>No weighting method proposed</td>
<td>HRQoL Inadequately Captured (Qualitative)</td>
<td>Innovative Nature of Technology (Qualitative)</td>
</tr>
<tr>
<td></td>
<td>5 years: Dominating</td>
<td>Proportional QALY shortfall: 58.8% Greater than average shortfall</td>
<td>Absolute QALY shortfall: 22.1 QALYs Greater than average shortfall</td>
<td>Some inadequately captured improvements in HRQoL (e.g. management of hypoglycaemic events)</td>
<td>-</td>
<td>Potentially regenerative therapy. Innovative in comparison to gold standard (Insulin Therapy)</td>
<td>Significant patient population</td>
</tr>
</tbody>
</table>

Figure 16: Summary Table of HTA Components & Outcomes for Allogeneic Islet Transplantation for each of the Current, VBP & VBA Appraisal Methodologies.
5.5 Recent Developments in Pluripotent Stem Cell derived Beta cells

Since the development of the Edmonton Protocol (Shapiro et al, 2000), in which islets from brain-dead donors are transplanted into diabetic patients, there has been an increasing emphasis on identifying alternative sources of islets, due to the issue of donor and tissue availability associated with Autologous and Allogeneic Islet transplantation (Markmann et al, 2003). Within the last decade, the possibility of deriving functional beta cells, the insulin secreting cells of pancreatic islets, from pluripotent stem cells has been explored.

A significant body of research has been published regarding the generation of functional Human Embryonic Stem Cell (hESC) derived beta cells (Zhang et al, 2009; Rezania et al, 2012; Rezania et al, 2014). In recent years, Douglas Melton and his colleagues at Harvard University have successfully derived mature, human pancreatic beta cells \textit{in vitro} and these cells are currently under examination in primate models of diabetes (Harvard Stem Cell Institute, 2014). An alternative strategy for the manufacture insulin producing cells from hESCs has been developed by a company known as ViaCyte (CA, USA), involving the differentiation of these cells into pancreatic endodermal cells, which, after encapsulation and transplantation, mature into insulin producing beta cells (Kelly et al, 2011; Schulz et al, 2012). Animal trials of this combination product have been completed and a Phase I/II trial in humans is underway (ViaCyte, 2014).

With the recent discovery of Human Induced Pluripotent Stem Cells (hiPSCs) by Yamanaka and colleagues (Takahashi & Yamanaka, 2006), significant advances have been made in differentiating these cells into functional beta cells. The transplantation of these differentiated cells into animal models of diabetes has also been explored and it has been determined that blood glucose is normalised after the production of insulin by beta cells in response to glucose (Jeon et al, 2012; Alipio et al, 2010; Pagliuca et al, 2014). One potentially significant advantage of the derivation of beta cells from hiPSCs rather than hESCs is the autologous nature of these hiPSC derived cells. Human Leukocyte Antigen (HLA) matching, or the derivation of source material from the patient, may mitigate alloimmune reactions to transplanted cells. Differentiated cells derived from pluripotent stem cells, including iPSCs, have been found not to induce an immune reaction in mice (Guha et al, 2013; Araki et al, 2013; Li et al, 2004; Drukker et al, 2006), which may suggest that iPSCs represent a valuable source of a variety of cell types for cell-based therapies. However, it has previously been identified that iPSCs derived from Mouse Embryonic Fibroblasts (MEFs) were immune-
rejected after re-introduction into donor mice, likely due to abnormal gene expression (Zhao et al, 2011). Differences in the immunogenicity of hiPSC derived cell types may exist, and it has been reported that hiPSC derived retinal pigment epithelial cells are immune tolerated, whereas hiPSC derived smooth muscle cells are highly immunogenic, in a humanised mouse model (Zhao et al, 2015).

However, the autoimmune destruction of native pancreatic beta cells that occurs in IDDM may represent an additional challenge, with graft failure after Allogeneic Islet Transplantation often associated with the immune clearance of transplanted cells (National Institute for Health and Clinical Excellence, 2008). It is conceivable that autoimmune reactions in IDDM patients against transplanted autologous pluripotent derived beta cells may lead to recurrence of the condition (Holditch, Terzic & Ikeda, 2014). Such autoimmune rejection would be likely to limit the efficacy and safety of a hiPSC derived Beta Cell therapy in the absence of immunosuppression, and therefore such a therapy would be unlikely to achieve adoption into healthcare. Additionally, long-term teratoma formation after the transplantation of hiPSC derived cells may represent an additional safety risk, and this has recently been identified in a mouse spinal cord model after hiPSC derived neural cell transplantation (Nori et al, 2015). However, this was not observed after hiPSC derived beta cell transplantation in mice (Jeon et al, 2012).

Nevertheless, it is yet unknown whether the transplantation of beta cells derived from autologous hiPSCs would induce an autoimmune reaction, and further investigation is required. A recent investigation into the immunogenicity of iPSC-derived organs determined that transplanted iPSC derived islets were not rejected by the recipient immune system in mice (Wang et al, 2015), however further research is required. An approach to avoiding the autoimmune clearance of pluripotent stem cell derived beta, or insulin producing, cells, that is currently under investigation in early clinical trials and animal studies (Harvard Stem Cell Institute, 2014; ViaCyte, 2014), is the encapsulation of these cells in order to provide protection from the immune system whilst allowing the secretion of the appropriate molecules. Besides immunosuppressive therapy, cell encapsulation currently represents the most promising method of avoiding autoimmune rejection of transplanted pluripotent stem cell derived beta cells that may occur in IDDM patients. However, although the safety and efficacy of encapsulated hESC derived insulin producing cells has recently been demonstrated in mice (Agulnick et al, 2015), there is currently a lack of clinical data
regarding the efficacy of encapsulated pluripotent stem cell derived beta cells for the treatment of IDDM.

Provided that adequate clinical evidence supporting the avoidance of immune rejection after hiPSC derived Beta Cell therapy can be generated, and given that the cost of immunosuppression is one of the most significant contributors to the estimated $19,000 yearly costs associated with Allogeneic Islet Transplantation (Beckwith et al, 2012), the derivation of functional beta cells from hiPSCs may significantly reduce the annual costs after transplantation. Although the manufacturer of a hiPSC derived Beta Cell technology may not offer immunosuppressive therapy along with their product, it is likely that, if immunosuppressive therapy is required, the cost would be accounted for in a HTA of their technology as part of the care pathway. However, if it can be demonstrated that treatment with hiPSC derived Beta Cells does not require immunosuppression, the ICER for this product may be reduced and patient QoL may improve, which may lead to a lower Incremental Cost-Effectiveness Ratio (ICER) and therefore a more cost-effective therapy.

If adequate long-term data of the superiority of the duration of graft function for hiPSC derived beta cells over Allogeneic Islets can be demonstrated, this may provide greater certainty to the Incremental Cost-Effectiveness Ratio (ICER) for this technology. However, as observed with Allogeneic Islet Transplantation, the duration of graft function is likely to be a key uncertainty in hiPSC Beta Cell therapy efficacy and cost-effectiveness. If a lower annual cost for a hiPSC derived beta cell product compared to Allogeneic Islet Transplantation is observed, due to lack of the requirement for immunosuppressive treatment, this would be likely to result in a lower ICER, compared against insulin therapy, for the hiPSC derived product. This assumes that the efficacy is equal to, or greater than, Allogeneic Islet Transplantation and that the cost of the hiPSC Beta Cell Therapy is not prohibitively expensive. This ICER reduction may result in a lower ‘breakeven point’, compared to the 9 year time horizon required for Allogeneic Islet Transplantation, although this will be dependent upon the initial cost of the therapy. Figure 17 demonstrates the effect that varying manufacturing costs and the avoidance of the cost of immunosuppression may have upon an estimated, prospective ICER for a hiPSC derived Beta Cell therapy. Due to the lack of available clinical data regarding a hiPSC derived beta cell product, greater improvements in patient QoL compared to Allogeneic Islet Transplantation cannot be accurately incorporated into these analyses. However, a sensitivity analysis (Figure 18) has been generated to
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estimate the effect of greater QALY gains after hiPSC derived beta cell therapy compared to Allogeneic Islet Transplantation, using Insulin therapy as the ‘gold standard’ comparator in both cases.

An additional advantage of a hiPSC derived beta cell therapy is that this approach would not be limited by the availability of organ donors, unlike an Allogeneic Islet Transplantation approach which often requires multiple donors (Shapiro et al, 2000). In 2013/14, 456 pancreas donors, from either brain dead individuals or those who have suffered circulatory death, were available in the UK, of which only 270 patients were placed on the transplant list (NHS Blood & Transplant, 2013). Therefore, if the number of Allogeneic Islet Transplantations performed were to increase, the number of available pancreas or islet donors would be rapidly depleted. In contrast, the utilisation of hiPSC derived beta cell would not reduce the number of pancreas donors, and would not diminish the number of organs available for whole pancreas transplants.

However, the initial costs associated with generating beta cells from hiPSCs would likely be far greater than the derivation of source material for Allogeneic Islet Transplantation, which is already considered to have high upfront costs. The development of hiPSC derived beta cells would be likely to face a number of costly challenges, including the creation of a hiPSC bank, the optimisation of the hiPSC differentiation process, and the development of a scalable manufacturing process. Furthermore, for an encapsulated hiPSC derived Beta Cell therapy product, the development, testing, optimisation, and manufacture of an appropriate cell encapsulation device will add additional cost, although this is unlikely to be as significant as the cell banking, differentiation and manufacturing costs.

It has been noted that the cost of generating, testing and expanding research-grade hiPSC lines is approximately $10,000 to $20,000, however, when creating a hiPSC line for clinical use, this cost would be likely to rise to approximately $800,000 (Bersenev, 2014). Therefore, in order to create one or more Master Cell banks, and to perform the necessary comparability work to demonstrate the equivalence of each hiPSC line, the cost is likely to have escalated above $1,000,000 (Bersenev, 2014).

Developing a scalable differentiation process is likely to represent the most challenging process step in the manufacture of functional beta cells from hiPSCs. The efficiency of the differentiation process raises a number of concerns including, not only the presence of
undifferentiated hiPSCs in the final product (Carpenter et al, 2009), but also the number of hiPSCs required to generate a sufficient dose of beta cells for transplantation. In the published literature, the expression of Insulin in response to glucose is the most commonly used potency marker for functional differentiated beta cells. The majority of these studies have reported the percentage of differentiated cells expressing Insulin to be between 0.7 and 17% (Thatava et al, 2011; D’Amour et al, 2008; Kunisada et al, 2012; Pellegrini et al, 2015), although Alipio and colleagues (2010) discovered that this percentage increased to 50% after 20 days of culture. Therefore, due to the relatively low yield of insulin producing cells derived from hiPSC cultures, a large number of these cells would be required to create a sufficient dose of pancreatic beta cells. Thus, significant expense will be required to generate large numbers of hiPSCs in order to derive sufficient beta cells using a low differentiation efficiency, or in order to optimise the differentiation process and improve the beta cell yield.

Additionally, the scalability of both the differentiation and manufacturing processes would be likely to incur substantial cost to the manufacturer, with consumables utilisation increasing significantly. For example, it has previously been estimated that the cost of cell culture medium will comprise approximately 24% of the cost of goods in a manufacturing process that provides 1,000 doses of cells per year, whereas this percentage of cost of goods will increase to approximately 58% when the number of doses is increased to 100,000 per year (Davie & Brindley, 2012). If the generation of beta cells from hiPSCs were based upon the creation of a number of hiPSC ‘haplobanks’ and the generation of HLA matched hiPSC derived beta cells, it is likely that the initial cost of generating a scalable manufacturing process would be large. However, this approach may allow for a large patient throughput. On the other hand, if an autologous approach were to be utilised, in which hiPSCs and differentiated beta cells were created from donated patient tissue, a similar scale of manufacturing process to that of a ‘haplobank’ approach may not be required, however an autologous approach may not be feasible to treat a large number of patients at a reasonable cost (Mason & Dunnill, 2009).

Although the cost of developing the cell banking, differentiation, and manufacturing processes would not be directly incorporated into the price of the hiPSC derived beta cell product, the manufacturers of this technology are likely to incorporate a profit margin on top of the initial cost of manufacture. Therefore, although the total process development and manufacturing costs would not be directly incorporated into ICER calculations, the value
utilised in ICER calculations would likely represent the cost of manufacture for one unit, amortisation of R&D costs, and the incorporated profit margin. This would differ from the value for Allogeneic Islet Transplantation which, as it is generally administered as an interventional procedure, would not incorporate a profit margin. Thus, in the short term, the ICER for a hiPSC derived beta cell product would be likely to be significantly greater than that of an Allogeneic Islet Transplantation procedure, assuming that the efficacy of the two technologies were similar. However, as discussed previously, if the cost of immunosuppression can be avoided, the ICER for the hiPSC derived therapy may be lower in the longer term. The effect of the incorporation of a 30%, 50% or 70% profit margin upon the ICER of a hiPSC derived Beta Cell therapy is demonstrated in Figure 17 (below). Figure 17 also demonstrates the effect of increasing the manufacturing costs ($200,000) and avoiding the cost of immunosuppression may have upon an estimated, prospective ICER for a hiPSC derived Beta Cell therapy.

5.5.1 Pluripotent Stem Cell derived Beta Cell Cost-Effectiveness Sensitivity Analyses

The generation of a sensitivity analysis to examine a prospective ICER for a hiPSC derived Beta Cell therapy, based upon the Allogeneic Islet Transplantation cost and efficacy data, allows the effect of an increased initial cost and a reduced annual cost, due to the lack of required immunosuppression, upon the required duration of graft function, in order to achieve cost-effectiveness, to be illustrated and explored. The comparator, or ‘Gold Standard’, for the hiPSC derived Beta Cell therapy ICER sensitivity analysis was Insulin Therapy, as with the analysis of the Allogeneic Islet Transplantation ICER.

In order to perform this sensitivity analysis for an estimated hiPSC derived Beta Cell therapy ICER, a number of optimistic assumptions were made due to the lack of available long-term clinical data and cost data for the treatment of Diabetes patients with a hiPSC derived Beta Cell product. It was assumed that the efficacy of a hiPSC derived Beta Cell therapy for the treatment of IDDM was equal to that of Allogeneic Islet Transplantation, and therefore the QALY gains and incremental effectiveness utilised were identical. However, it should be recognised that the health state of IDDM patients after hiPSC derived Beta Cell therapy may be greater than that of Allogeneic Islet Transplantation, due to the possible avoidance of the requirement for immunosuppressive therapy and greater duration of graft function. Therefore, the incremental effectiveness, when compared to standard insulin therapy, may be greater.
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than that of Allogeneic Islet Transplantation. However there is no published clinical data available for a hiPSC derived Beta Cell therapy to support this conclusion.

For the first hiPSC derived Beta Cell therapy sensitivity analysis, it was assumed that the efficacy of such a product for the treatment of IDDM was equal to that of Allogeneic Islet Transplantation, and therefore the QALY gains and incremental effectiveness utilised were identical. The initial cost of goods for a hiPSC derived beta cells product was also assumed to be equal to that of Allogeneic Islet Transplantation ($99,629/£61,770), as there is little available data on the likely cost or price of such a product, and this value would not be considered highly unrealistic. The cumulative cost data was then manipulated in order to examine the effect of an increased cumulative cost, in comparison to Allogeneic Islet transplantation, or the avoidance of immunosuppressive treatment costs, upon the ICER over a 20 year time horizon (Figure 17).

In order to account for the lack of immunosuppressive therapy, the annual cost of immunosuppressive therapy, assumed to be $19,000 (Beckwith et al, 2012), was subtracted from the annual cumulative cost of therapy. However, this cost also includes the cost of consultations and associated procedures, and therefore may not be truly accurate.

To generate ICERs in which 30%, 50%, and 70% profit margins were incorporated, the cost of goods remained the same ($99,629/£61,770), however the price of the product was increased (30%, 50% or 70%) and this was discounted based upon inflation rates. The final ICER within the first sensitivity analysis (Figure 17) assumes an initial cost of goods of $200,000 (£124,000), rather than $99,629 (£61,770), and includes the annual immunosuppressive therapy costs. Recently, an autologous cellular immunotherapy known as Sipuleucel-T, or Provenge® (Dendreon, Seattle, USA), was not considered by NICE to be cost-effective with a £47,000 cost per patient (NICE, 2015). Therefore, an initial cost of $200,000 would be considered high.

For the 30%, 50%, and 70% profit margin ICERs, as well as the $200,000 initial cost ICER, a 1.3% annual discount rate, based upon the current UK inflation rates (October 2014) (Office for National Statistics, 2014), was applied to the initial cost when included in the cost calculations for each year of the analysis.
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The second hiPSC derived Beta Cell therapy sensitivity analysis (Figure 18) assumed that the cost of this new therapy would be the same as Allogeneic Islet Transplantation, and therefore the ∆Cost compared to Insulin therapy would be the same. Furthermore, it was assumed that immunosuppression would not be required and this annual cost ($19,000) was subtracted from the cumulative cost. However, in order to estimate the effect of an improved efficacy upon the ICER, the ∆QALYs was increased proportionally in order to produce a 2 or 2.5 QALY gain over the 20 year time horizon. The resultant ICERs were then compared to the ICER in which a 1.5 QALY gain is observed, as with Allogeneic Islet Transplantation.

![Estimated Prospective Incremental Cost-Effectiveness Ratio (ICER) targets for a hiPSC derived Beta Cell therapy over a 20 year period](image)

**Figure 17:** Sensitivity analysis illustrating an estimate of a prospective Incremental Cost-Effectiveness Ratio (ICER) for a hiPSC derived Beta Cell therapy compared to the NICE Threshold (£20,000 per QALY), over a 20 year time horizon. The impact of the incorporation of profit margins (30%, 50%, and 70%), the avoidance of the requirement for immunosuppressive therapy, and an increase in manufacturing costs ($200,000) is demonstrated.

As expected, the results of the sensitivity analysis above would suggest that, when similar cost and efficacy data to Allogeneic Islet Transplantation are assumed, a time horizon of 9 years would be required for a hiPSC derived Beta Cell product to become cost saving. Additionally, a sufficiently low graft failure rate would be required in order to provide adequate certainty in the ICER and clinical efficacy after extrapolation of the clinical trial data to a 9 year time horizon. In the present study, the assumed graft failure rates for Allogeneic Islet Transplantation were 0% over one year, 17% over five years, 24% over 10...
years, and 30% over 20 years, based upon Beckwith and colleagues (2012) simulations. It is likely that significantly lower graft failure rates than assumed for Allogeneic Islet Transplantation would be required in order to create sufficient certainty in a ≤9 year time horizon.

Depending upon the value proposition of the new technology, the clinical trial data may also be required to demonstrate a lack of need for immunosuppressive therapy. If a hiPSC derived Beta Cell product could be manufactured and delivered at a similar cost to an Allogeneic Islet Transplantation procedure ($99,629/£61,770) whilst avoiding the requirement for immunosuppressive therapy, then, in order to achieve an ICER that falls below the NICE threshold, a time horizon of 8 years would be sufficient to incur cost savings. Therefore the long-term clinical data, and estimated duration of full graft function, required to achieve a positive reimbursement decision is likely to be reduced.

However, if profit margins of 30%, 50%, or 70% of the cost of goods are incorporated, with a 1.3% per annum discount rate, then the required time horizon increases to 9, 10 or 11 years respectively. Finally, if an initial cost of goods of $200,000 was required to manufacture the product, and if immunosuppressive therapy remained a requirement, a time horizon of approximately 14 years must be demonstrated in order reach the ‘breakeven point’ for cost savings. However, if the cumulative cost of therapy is discounted at a rate of 1.3% per annum, the realistic time horizon to achieve cost savings would remain at approximately 11 years.

It must be acknowledged that the results of the above sensitivity analysis (Figure 17) are based upon the assumption that a hiPSC derived Beta Cell product would achieve a similar efficacy, and therefore QALY gain, to that of Allogeneic Islet Transplantation. Due to the lack of available clinical data regarding a hiPSC derived Beta Cell product, greater improvements in patient QoL compared to Allogeneic Islet Transplantation cannot be accurately incorporated into these analyses. However, a hiPSC derived Beta Cell therapy may yield an improved health state compared to Allogeneic Islet Transplantation, provided that the requirement for immunosuppressive therapy can be avoided and, consequently, a greater duration of graft function can be achieved. Therefore, the incremental effectiveness, when compared to standard insulin therapy, may be greater than that of Allogeneic Islet Transplantation. A sensitivity analyses (Figure 18) was performed in order to estimate the
effect of greater QALY gains after hiPSC derived Beta Cell therapy compared to Allogeneic Islet Transplantation, using Insulin therapy as the ‘gold standard’ comparator.

As illustrated in Figure 18, if the efficacy of a hiPSC derived Beta Cell product, which avoided the requirement for immunosuppressive therapy and achieved a similar cost of goods to that of Allogeneic Islet Transplantation ($99,629/£61,770), was greater than that of Allogeneic Islet Transplantation, for example inducing a QALY gain of 2.5 QALYs over a 20 year time horizon, then the duration required to dominate the comparator would fall to 8 years. The costs of treatment are assumed to be the same as that of Allogeneic Islet Transplantation, however the annual cost of immunosuppression is removed. The comparator, or ‘Gold Standard’, for the hiPSC derived Beta Cell therapy ICER sensitivity analysis was Insulin Therapy, as with the analysis of the Allogeneic Islet Transplantation ICER.

It is apparent from the sensitivity analyses in the present study that, due to the early development of pluripotent derived Beta cell products, both the cost and efficacy of such a technology are currently unknown and that these may improve as scientific and manufacturing expertise advance. As observed with Allogeneic Islet Transplantation, the
duration of graft function is likely to be a key uncertainty in hiPSC Beta Cell therapy efficacy and cost-effectiveness. Therefore, the cost and efficacy data utilised in Figures 17 and 18 represent major uncertainties.

It should also be recognised that, although a hiPSC derived Beta Cell Product may fulfil many of the current, VBP and VBA appraisal components to the same extent as Allogeneic Islet Transplantation, it may be that such a technology would be considered more innovative due to the hiPSC differentiation process, the possible avoidance of the requirement for immunosuppressive therapy and the potentially allogeneic nature of the Beta Cells based upon HLA matching. Additionally, it may be that a hiPSC derived therapy would satisfy a number of Non-Health related objectives of the NHS, for example incentives around Regenerative Medicine as it has previously been identified as one of the eight great technologies in which the UK could become a leader (HM Government, 2012), although NICE may not consider this within its remit. Therefore, a hiPSC derived Beta Cell Therapy has the potential to induce greater allowance upon the NICE threshold when assessed through current, VBP or VBA appraisal schemes.

Therefore, the results of the hiPSC derived Beta Cell ICER sensitivity analyses reveal that the initial cost of goods, the requirement for immunosuppressive therapy, and the demonstration of long term efficacy and graft survival will have a significant impact upon the likelihood of reimbursement for a hiPSC derived Beta Cell therapy.

5.6 Discussion & Conclusions

From the results of the ICER calculations and the ICER sensitivity analysis for the utilisation of allogeneic islet transplantation for the treatment of IDDM patients over a 20 year period (Figure 8), it is apparent that this treatment would only be cost-effective if the time horizon was 9 years or greater. If the time horizon falls under 9 years, then it becomes unlikely that a positive reimbursement decision would be achieved. Furthermore, given the clinical evidence to date, regarding the duration of graft function after Allogeneic Islet Transplantation, it is unlikely that a time horizon of 9 years would be achieved. By reducing the time horizon, in order to increase ICER certainty, the likelihood of achieving cost-effectiveness decreases, as illustrated in Figure 11. However, if the time horizon is considered to be over 9 years, it is likely that a cost saving, in comparison to insulin therapy, would be induced. Additionally, if
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A 20-year time horizon is considered, then it may incur a cost-saving to the payer of approximately £59,000.

As a result of these mock appraisals, utilising the current approach, VBP scheme, and VBA system, it can be concluded that:

- Allogeneic Islet transplantation may be considered cost-effective over a 9 year time horizon and would incur significant cost savings (£59,000) over the gold standard over a 20 year time horizon (Figure 8)
  - However, given the available clinical data regarding graft failure rates after Allogeneic Islet Transplantation, significant uncertainty exists regarding an ICER with a 9 year time horizon. Improved graft failure rates would be required to create sufficient certainty in a 9 year time horizon
  - The duration of graft survival after Allogeneic Islet Transplantation represents the greatest uncertainty influencing cost-effectiveness in the present study
  - When a shorter time horizon is considered (5 years), for Allogeneic Islet Transplantation to be considered cost-effective the cumulative cost must be £73,141.37 or less, whereas the actual cumulative cost of the treatment is £119,224.06.
- The ICER for a new product is likely to be the main determining factor in HTA decisions regardless of the appraisal methodology utilised
  - Particularly in the current reimbursement system where the additional components of HTA decisions are not quantified and are informally determined by the appraisal committee
  - A summary table illustrating the outcome of each HTA component, within each of the three appraisal methodologies, for Allogeneic Islet Transplantation is shown in Figure 16
- The weightings applied to the ICER under the VBP scheme would have been beneficial to the likelihood of a positive reimbursement decision, as they would have been likely to reduce the ICER and may have increased the NICE threshold
  - This reduction of the ICER is demonstrated for allogeneic islet transplantation over a 20 year period in the Adjusted-ICER sensitivity analysis (Figure 15)
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- The VBA approach may have been favourable for high cost therapies, although not to the same extent as the VBP approach, due to the increased allowance applied to the NICE threshold based upon the BoI and WSI QALY shortfalls
  - As no proposal for a quantitative measure of BoI and WSI were included in the VBA scheme, it is unlikely that this approach would have been as favourable as VBP for high cost therapies, and would have been less likely to result in a positive reimbursement decision
- Although the introduction of a VBP or VBA scheme may have increased the number of positive reimbursement decisions for high cost therapies, it may have also significantly increased NHS spending and distributed resource away from other areas of the healthcare system

Looking to the future, it is unlikely that these value-based approaches will be revisited without significant evidence and greater certainty around the calculations of burden of illness and wider societal benefit or impact. Therefore, it is likely that, for the foreseeable future, the current reimbursement scheme, based upon QALY and ICER methodologies, will be maintained. However, it has been suggested that alternative modifications to the current reimbursement scheme will be investigated in the future, and that these will particularly focus upon addressing the issue of uncertainty of evidence in appraisals and streamlining the approval process (Hawkes, 2014). Alternative commissioning pathways, including ‘Hospital Exemptions’ and ‘Patient Access Schemes’, may be explored as methods of gaining suitable evidence to inform HTAs and increase certainty in decision making.

With regards future developments for the treatment of IDDM patients, it is clear that a number of scientific and manufacturing challenges remain before the successful translation and adoption of hiPSC derived beta cell therapies can be achieved. However, if these pluripotent derived beta cells can be transplanted without the requirement for immunosuppressive therapy, this may lead to an increased efficacy and increased duration of graft function. Therefore, if the incursion of cost due to immunosuppressive treatment can be avoided and the duration of graft function can be increased, in comparison to Allogeneic Islet Transplantation, the time to reach the ‘breakeven point’ may reduce and an increased certainty in the time horizon may be achieved. This may lead to such a technology being considered cost-effective in the long-term, and would therefore encourage a positive reimbursement decision. However, it is imperative that sufficient clinical trial data,
demonstrating adequate certainty in the graft failure rate, the lack of requirement for immunosuppressive therapy, and the time horizon considered, is collected. Although, in principal, public healthcare systems, such as the NHS, have a lifetime perspective on patients (Bravery, 2012), the time horizon considered must reflect the realistic duration of benefit of a treatment, and therefore may represent a more conservative estimate.

From the hiPSC derived Beta Cell therapy sensitivity analyses, it was determined that, by avoiding the annual cost of immunosuppressive therapy, such a product would become cost saving after 8 years, assuming a similar initial cost to Allogeneic Islet Transplantation. If the initial cost of a hiPSC derived Beta Cell therapy reached $200,000, and the need for immunosuppressive therapy remained, the ‘breakeven point’ to become cost saving would increase to 11 years. If the efficacy of a hiPSC derived Beta Cell product increased to 2.5 QALYs over a 20 year time horizon, without the requirement for immunosuppressive therapy and at a similar cost to Allogeneic Islet Transplantation, the duration required to dominate the comparator (Insulin Therapy) would fall to 8 years.

5.7 Summary

In this chapter, numerous analyses were performed, using current HTA or value-based appraisal methodologies, in order to quantitatively model the cost-effectiveness of Allogeneic Islet Transplantation over a 20-year time horizon, the difference in cost-effectiveness outcome between appraisal methodologies, and the cost-effectiveness of a hiPSC derived Beta Cell therapy.

From these analyses, it was determined that Allogeneic Islet transplantation may be considered cost-effective over a 9 year time horizon, incurring significant cost savings over a 20 year time horizon. Also, the weightings applied to the ICER under both VBP and VBA were likely to be favourable for the reimbursement of high cost therapies, with VBP being most beneficial. Finally, this chapter highlights that the collection of sufficient clinical trial data; demonstrating adequate certainty in the graft failure rate, the time horizon considered, and the lack of requirement for immunosuppressive therapy; is imperative for the achievement of a positive reimbursement decision for a hiPSC derived Beta Cell therapy.

5.8 Limitations of the present study

- The present analysis was based primarily upon the Markov and Monte Carlo modelling performed and published by Beckwith and colleagues (2012)
The cost data generated by Beckwith and colleagues (2012), was based upon the U.S healthcare system and may not be directly applicable to the UK healthcare system.

The time horizon of the analysis for both Allogeneic Islet Transplantation and hiPSC Beta Cell therapy was limited to 20 years, due to the availability of accurate data, and this may not represent the lifetime of the treatment or the full duration of graft function.

The clinical and cost benefits of avoiding long term complications were not included in the present analysis.

As is apparent from the results of the present study, Allogeneic Islet Transplantation would be considered to be cost saving in the long term and therefore may not be appraised using the typical HTA parameters that were utilised in the present analysis.

The present analysis does not incorporate an accurate or comprehensive economic analysis, as would be included in a NICE HTA.

Neither the Value-Based Pricing nor the Value-Based Assessment reimbursement models were implemented by the UK government and therefore cannot be fully evaluated.

Currently, a lack of data regarding the cost and efficacy, including the avoidance of autoimmune rejection, of a hiPSC derived Beta Cell therapy exists, and therefore the values utilised in the present study are speculative.

**5.9 Future Research**

Future studies examining the cost-effectiveness of Allogeneic Islet Transplantation should aim to incorporate the cost savings associated with the avoidance of long term complications as a result of this treatment, in order to give a more accurate representation of its value.

Furthermore, in order to perform more accurate cost-effectiveness analyses of a hiPSC derived Beta Cell therapy, future research should aim to generate adequate long term cost and efficacy data, with a particular focus upon the rate of graft failure in patients and the need for immunosuppressive therapy.
Chapter 6: Materials & Methods

In the present chapter, the materials and methods utilised in the experimental research of this thesis, as presented in Chapters 7-10, are described. This encompasses the experiments in which the comparability between manual and automated process steps for the culture of hMSCs, Ruler hESCs, and hiPSCs was examined, as well as the experiments in which the differentiation of hiPSCs to MSCs was explored. The materials and methods in this chapter describe the cell lines, culture medium, automated and manual culture protocols, cryopreservation protocols, pre-treatment regimen, cell culture for various assays, analytical techniques, and statistical analyses utilised. More specific descriptions of the materials and methods are presented in each chapter.

6.1 Cell Lines

The hMSC line used in the present study was initially derived from Lonza (Lonza, Basel, Switzerland) as Human Bone-Marrow Aspirate obtained from healthy donors, and from which Human Bone Marrow Mononuclear Cells (BM-MNCs) were derived. These cells were then cultured in a monolayer on tissue culture plastic to remove any non-adherent cells. After 2 weeks of culture, the cells were cryopreserved and stored as P0 hMSCs.

Two cryovials of the ‘Ruler’ hES Cell line (EC 2102Ep) (P48) were initially purchased from GlobalStem® (Gaithersburg MD, USA) and were placed in cryostorage prior to expansion and the creation of an Optimisation and Experimental cell bank.

After the generation of Material Transfer Agreement (MTA), between Loughborough University and the Institute for Stem cell Therapy and Exploration of Monogenic diseases (I-Stem) (Evry, France), the hiPSC line (VAX001024c07) (P22+9) was delivered to Loughborough University, Centre for Biological Engineering and placed in cryostorage before the generation of a cell bank. The passage number P22+9 refers to the culture of the hiPSC line for 22 passages on a feeder cell layer followed by the transfer of these cells onto a Matrigel attachment substrate and the culture of these hiPSC line on the attachment substrate for 9 passages.

hiPSC-MSCs were derived through the culture of the VAX001024c07 hiPSC line in MSC culture medium, on tissue culture plastic, as discussed below in sections 6.2 & 6.4.
6.2 Cell Culture Medium

For the culture of hMSCs and Ruler hES Cells, Life Technologies (Thermo Fisher Scientific, Waltham, USA) Dulbecco’s Modified Eagle’s Medium (DMEM), High Glucose, with GlutaMAX™ was supplemented with 10% Qualified Fetal Bovine Serum (FBS) (Gibco®, Life Technologies).

The culture of hiPSCs was performed using Stem Cell Technologies (Vancouver, Canada) mTESR™1 basal medium supplemented with mTESR™1 5X supplement. Additionally, Stem Cell Technologies Y-27632 ROCK inhibitor (10µM) was added to the culture medium 1 hour prior to passaging, during passaging or seeding, and 24 hours after passaging or seeding.

hiPSC-MSCs were derived through the culture of the VAX001024c07 hiPSC line, obtained from cryostorage or post-passage, in MSC medium on tissue culture plastic. Furthermore, once hiPSC-MSCs were derived, these cells were cultured in MSC medium. MSC medium compositions included DMEM High Glucose GlutaMAX™ (Life Technologies) & 10% Fetal Bovine Serum (FBS) (Gibco®, Life Technologies), DMEM High Glucose GlutaMAX™ & 20% (FBS), Knockout™ DMEM (Life Technologies) & 20% FBS, DMEM High Glucose GlutaMAX™ & 10% Stemulate™ HPL (Cook Regentec, Indiana, USA), DMEM High Glucose (Lonza, Basel, Switzerland), 2mM UltraGlutamine (Lonza) & 20% Stemulate™ HPL, Knockout™ DMEM & 10% Stemulate™ HPL, and Knockout™ DMEM & 20% Stemulate™ HPL.

6.3 Cell Resuscitation

Cryovials of hMSCs (P3), Ruler hES Cells (P51) and hiPSCs (P22+9) used in the present study were removed from cryostorage, rapidly thawed in a 37°C water bath, and transferred into a Biosafety cabinet. The contents of the vials were transferred into centrifuge tubes, resuspended in pre-warmed (37°C) culture medium, and centrifuged (Sigma Refrigerated Centrifuge 3-16PK), for which the Relative Centrifugal Force (RCF) used was dependent upon the cell type (See Appendix 13.8). The supernatant was then aspirated in order to isolate a cell pellet which was then resuspended in 5ml of pre-warmed (37°C) culture medium, or 10ml of complete mTESR1 medium in the case of hiPSCs. The cells were then seeded on tissue culture plastic, at a relevant density and in a relevant volume of complete culture medium depending upon the cell type and the surface area of the tissue culture plastic.
6.4 Manual Monolayer Cell Culture

During the manual cell culture process, once the cells had been seeded onto tissue culture plastic, the cells were placed within a humidified CO₂ controlled (5%) incubator. For hMSCs, Ruler hESCs, and hiPSC-MSCs complete medium exchanges were performed every 48 or 72 hours, with complete culture medium pre-warmed to 37°C. However, with regards the hiPSC line, complete medium exchanges were performed daily with mTESR1 basal medium supplemented with the mTESR1 5x supplement warmed to room temperature. Additionally, as previously described in section 6.2, Y-27632 ROCK inhibitor was added to the culture medium 1 hour prior to passaging, during passaging or seeding, and 24 hours after passaging or seeding. Once the cells had reached approximately 80-90% confluency, which was confirmed using microscopy, they were passaged into new tissue culture flasks.

6.4.1 Cell Dissociation

In order to passage the hMSC and Ruler hESC lines manually, the spent culture medium was first aspirated and the cells were washed with Phosphate Buffered Saline (PBS) (Lonza), whereas in the case of hiPSCs the cells were washed with Accutase. Wash steps for hiPSC-MSCs were performed using PBS or Accutase, depending on the duration of culture. A dissociation agent, either Trypsin 0.05% EDTA (Life Technologies), Trypsin 0.25% EDTA (Life Technologies), or Accutase, for hMSCs, Ruler hESCs, and hiPSCs respectively, was then applied and the flask was incubated in a humidified, CO₂ controlled (5%) incubator at 37°C in order to detach the cells from the tissue culture plastic. Either Accutase or Trypsin 0.05% EDTA was utilised for the dissociation of hiPSC-MSCs. After the cells were manually agitated to ensure dissociation from the tissue culture plastic, the dissociation agent was neutralised through the addition of complete culture medium and the cell suspension was transferred into a centrifuge tube.

6.4.2 Centrifugation & Cell Counting

The cell suspension was then centrifuged, at an appropriate RCF for the cell type, for 5 minutes at room temperature, and the supernatant generated was aspirated. The isolated cell pellet was then resuspended in an appropriate volume of culture medium, and a sample of the cell suspension was taken in order to determine the viable cell number using either the Cedex Automated Cell Counter (Roche, Switzerland) or the Nucleocounter NC-3000 (ChemoMetec, Allerod, Denmark). However, with regards the hiPSC line, after neutralisation of the dissociation agent with compete mTESR1 culture medium, the cell suspension is thoroughly
mixed and an automated cell count is performed using the Cedex Automated Cell Counter. An appropriate cell number is then isolated from the cell suspension, by transfer to an empty tissue culture flask, and further diluted in complete mTESR1 culture medium to the appropriate cell density (3.5x10^5 cells/ml).

6.4.3 Cell Seeding
In the case of the hMSCs, the cells were seeded into pre-warmed (37°C) culture medium, within fresh daughter tissue culture flasks, at a density of approximately 7.5x10^5 cells per daughter flask. Similarly, the hiPSCs were seeded into daughter flasks at a density of 3.5x10^6 cells per T175 flask (2x10^4 cells/cm²) into complete mTESR1 medium with Y-27632 ROCK inhibitor (10µM). A seeding density of 4.5x10^4 or 6x10^3 cells/cm² was utilised for the seeding of hiPSC-MSCs depending upon the time in culture, with a cell density of 4.5x10^4 cells/cm² utilised in early passages. Whereas, in the case of the Ruler hES Cells, the cells were passaged based upon a 1:3 tissue culture flask split ratio.

6.5 CompacT SelecT Preparation & Calibration
Prior to the performance of any automated protocol on the TAP Biosystems’ (Royston, UK) CompacT SelecT platform, the machine was prepared for use by ensuring the relevant number of pipette tips were loaded, sufficient new BD Falcon™ T175 flasks (BD Biosciences, San Jose, USA) were available, and that the adequate volume of reagents were loaded. The preparation of cell culture reagents involved the dispensing of the required volume of reagents into an adequate number of sterile reagent bottles (Thermo Fisher Scientific, Waltham, USA), within a Biosafety Cabinet, and the attachment of sterile plastic tubing (Watson-Marlow Pumps, Falmouth, UK) with adequate bore to allow for the required volume of reagents to be pumped using the peristaltic pump system.

In order to ensure that the required volumes of reagent are dispensed throughout each protocol, a calibration step is performed during the preparation process prior to the performance of each CompacT SelecT protocol. This step involved priming of the plastic tubing with the relevant reagent, followed by the dispensing of a small volume of the reagent (e.g 10ml) into a T175 tissue culture flask. This flask was then exported and the contents were weighed, using digital scales, to determine the volume of liquid that was in fact dispensed, assuming that 1ml of reagent weighs approximately 1 gram. This value can then be entered into the CompacT SelecT software in order to calibrate the peristaltic pump system.
6.6 Automated Monolayer Cell Culture

Automated cell culture was performed using TAP Biosystems’ CompacT SelecT automated cell culture platform. For the automated cell culture of hMSCs and Ruler hESCs, once the cells had been seeded, using either a manual or automated process, onto a barcoded BD Falcon™ T175 flask (BD Biosciences, San Jose, USA), the cells were placed into the carousel within the in-built humidified CO₂ controlled (5%) incubator of the CompacT SelecT automated cell culture platform. Similarly to the manual cell culture process, complete medium exchanges were performed every 24, 48 or 72 hours, depending upon the cell type, with complete culture medium pre-warmed to 37°C. Once the cells had reached approximately 80-90% confluency, which was confirmed using microscopy, they were passaged into new tissue culture flasks using automated protocols. During these cell type-specific automated protocols, the spent culture medium was first poured off and the cells were washed with the Phosphate Buffered Saline (PBS) pumped liquid. Next, sufficient dissociation agent, specifically Trypsin 0.05% EDTA, Trypsin 0.25% EDTA depending upon the cell type, to fully coat the tissue culture flask was applied and then immediately poured off until only a residual coating remained. The cells with residual dissociation agent coating were then incubated in a humidified, CO₂ controlled (5%) incubator in order to detach the cells from the tissue culture plastic. The residual dissociation agent was then neutralised using complete growth medium and the flask was agitated by the CompacT SelecT robot arm to ensure dissociation of the cells from the tissue culture plastic. The viable cells were counted using the Cedex Automated Cell Counter (Roche, Switzerland) and these counts
were used to seed new daughter tissue culture flasks containing pre-warmed (37°C) culture medium at a density of $7 \times 10^5$ cells per daughter flask in the case of hMSCs, and $2 \times 10^4$ cells per cm$^2$ of tissue culture plastic in the case of hiPSCs. However, in the case of Ruler hES Cells, the cells were passaged based upon a 1:3 tissue culture flask split ratio.

The automated process utilised for the culture of hiPSCs differed slightly from that of the hMSCs and Ruler hESCs, with the protocols generated by the researchers at I-Stem. However, these protocols required some adaptation as they involved the handling of T75 tissue culture flasks, as well as T175 flasks, and therefore, due to the lack of T75 flask handling capability on the CompacT SelecT located in the laboratories of Loughborough University’s Centre for Biological Engineering, these protocols required adaptation to utilise T175 flasks only. Initially, 2 vials of hiPSCs (4x10$^6$ cells/vial) were thawed, pooled, and diluted in complete mTESR1 medium with Y-27632 ROCK inhibitor in a 50ml centrifuge tube, which was placed into the static holder of the CompacT SelecT. The pooled hiPSCs were then seeded onto a Matrigel coated T175 flask using an automated process, in which the cell suspension was diluted in complete mTESR1 medium to $3.5 \times 10^5$ cells/ml and 10ml of this suspension was transferred into the Matrigel coated flask ($3.5 \times 10^6$ cells/T175 flask). For this cell type, as with the manual culture process, medium exchanges with complete mTESR1 medium, and Y-27632 ROCK inhibitor when required, were performed every 24 hours. Once the hiPSCs had reached approximately 80% confluency, which was confirmed using microscopy, they were passaged into new tissue culture flasks using automated protocols. These automated protocols included initial removal of the spent culture medium and the washing of the cells with Accutase. After washing, further Accutase was added and the cells were incubated at 37°C for 10 minutes in order to dissociate them. The cells were then agitated and the dissociation agent was then neutralised with complete mTESR1 medium and Y-27632 ROCK inhibitor. The viable cells were counted using the Cedex Automated Cell Counter, and these counts were used to transfer $8 \times 10^6$ cells into an empty flask. This cell suspension was then diluted to $3.5 \times 10^5$ cells/ml and 10mls of this suspension was transferred into daughter Matrigel Coated T175 flasks ($3.5 \times 10^6$ cells/flask).

The processes described above were utilised during the Non-Centrifugation experimental runs, however this process was altered for the Centrifugation experimental runs to incorporate the centrifugation process step. Process diagrams demonstrating the differences
between the Centrifugation and Non-Centrifugation processes are presented in sections 7.2.3, 8.2.4, and 9.2.4, for the hMSC, Ruler hESC and hiPSC experiments respectively.

The Non-Centrifugation processes for hMSCs, Ruler hESCs and hiPSCs were adapted in order to incorporate a pre-centrifugation cell count, a centrifugation process step, and a post-centrifugation cell count. To passage cells using the centrifugation process, a Pre-Centrifugation protocol was utilised where the cells were washed, dissociated and the dissociation agent was neutralised, similarly to the Non-Centrifugation process. Following this Pre-Centrifugation process, the flask, containing the cells undergoing passage, was outfeeded, the cells were transferred into a 50ml centrifuge tube and centrifuged at the relevant RCF depending upon the cell type (See Appendix 13.8). Once centrifuged into a cell pellet, the cells were resuspended in fresh culture medium and reintroduced into the outfeeded flask. This flask was then reimported back into the CompacT SelecT and a Post-Centrifugation protocol was performed in order to obtain a Post-Centrifugation cell count and to passage the mother flask into new daughter flasks.

For each of the hMSC, Ruler hESC & hiPSC lines, four Centrifugation and Non-Centrifugation batches of cells were generated.

6.7 Cell Cryopreservation

In order to cryopreserve the cells produced during cell banking and both the Centrifugation and Non-Centrifugation experiments, the cells were dissociated using reagents and automated protocols relevant to the cell type. The dissociated cells from each flask were then pooled into a single flask. An automated cell count was then taken, using the Cedex automated cell counter, and the total viable cell number was then calculated. The cell suspension was then aliquoted into 50ml centrifuge tubes and centrifuged at a RCF relevant to the cell type. The supernatant was then aspirated and the cell pellet was resuspended in the relevant type and volume of cryopreservation medium depending upon the cell type and the concentration of cells required.

For the cryopreservation of hiPSC-MSCs, cells were manually dissociated, centrifuged, and a cell count was performed using the Nucleocounter NC-3000 cell counter. The supernatant was aspirated and the cell pellet was then resuspended in the appropriate cryopreservation medium at an appropriate concentration.
Both hMSCs and Ruler hESCs (EC 2102Ep) were cryopreserved in 90% Fetal Bovine Serum with 10% Dimethyl Sulfoxide (Sigma Aldrich, St Louis, USA) whereas hiPSCs (VAX001024c07) and hiPSC-MSCs were cryopreserved in CryoStor® CS-10 medium (Stem Cell Technologies). The cell suspension was then transferred into the required number of cryovials. In general, 1ml of cells suspended in cryopreservation medium was stored in each cryovial, although the number of cells per cryovial varied depending upon the cell type. The cryovials were then placed into ‘Mr Frosty’ containers and stored at -80˚C for 24 hours before being transferred into liquid nitrogen cryostorage.

6.8 Pre-Treatment of hMSCs for paracrine assays

Baseline, Post-Centrifugation, and Non-Centrifugation hMSC samples were pre-treated using three methods.

Firstly, hMSCs were treated with two pro-inflammatory cytokines, specifically Tumour Necrosis Factor Alpha (TNF-α) and Interferon Gamma (IFN-γ) (Sigma Aldrich), in order to induce an anti-inflammatory response. In order to treat the hMSC samples, cryopreserved Baseline, Post-Centrifugation and Non-Centrifugation hMSCs were thawed and manually seeded in pre-warmed (37˚C) complete culture medium, using the methods described above, at a density of 7x10^5 cells per tissue culture flask. After 72 hours, the spent culture medium in each tissue culture flask was replaced with fresh culture medium containing 10ng/ml IFN-γ and 15ng/ml TNF-α. The hMSCs were then cultured in this culture medium supplemented with pro-inflammatory cytokines for a further 72 hours in order to pre-treat the cells. Once the cells have been treated for 72 hours, the culture medium conditioned by the hMSCs was collected and stored in a -80˚C freezer.

The second method of pre-treatment, involved exposing the Baseline, Post-Centrifugation, and Non-Centrifugation hMSCs to hypoxia (1% O₂) in order to induce a pro-angiogenic response. To hypoxically pre-treat the hMSCs, cryopreserved Baseline, Post-Centrifugation and Non-Centrifugation hMSCs were thawed and manually seeded in pre-warmed (37˚C), complete culture medium, using the methods described above, at a density of 1x10^6 cells per T175 tissue culture flask. After 72 hours, the spent culture medium in each tissue culture flask was replaced with fresh, pre-warmed (37˚C), pre-conditioned (1% O₂ overnight), complete culture medium and each of the tissue culture flasks were placed in a humidified (37˚C) hypoxic (1% O₂) incubator. The hMSCs were then cultured in this hypoxic environment for a further 48 hours in order to pre-treat the cells. Once the cells had been
treated for 48 hours, the culture medium conditioned by the hMSCs was collected and stored in a -80°C freezer.

Control samples, or No Treatment (NT) groups, of Baseline, Post-Centrifugation and Non-Centrifugation hMSCs were manually seeded in pre-warmed (37°C) complete culture medium, using the methods described above, at a density of 7x10^5 cells per tissue culture flask. After 72 hours, the spent culture medium in each tissue culture flask was replaced with fresh culture medium and the tissue culture flasks were placed back into a humidified (37°C) CO_2 controlled (5% CO_2) incubator. After a further 72 hours, the culture medium conditioned by the hMSCs was collected and stored in a -80°C freezer.

6.9 Cell Culture Protocol for hMSC Colony Forming Unit Assay

In order to generate sufficient cells for the performance of the Colony Forming Unit Assay, Baseline (P2), Centrifugation (P5) and Non-Centrifugation hMSCs (P5) hMSCs were seeded in separate T175 flasks (4000 cells/cm^2), a medium exchange was performed after 3 days, and, after 6 days, the cells were dissociated, centrifuged, resuspended in DMEM with 10% FBS, and a cell count was performed. Next, 250 cells from each condition were seeded in separate Nunc™ T25 flasks (Thermo Fisher Scientific, MA, USA) in 8mL of DMEM FBS. Additionally, a replicate of each condition was seeded. The cells were then cultured for 14 days with a medium exchange performed every 5 days.

6.10 Protocol for Differentiation of hiPSCs to hMSCs (hiPSC-MSCs)

Protocols for the differentiation of hiPSCs to MSCs were initially based upon previous work performed at I-Stem. These protocols were primarily based upon seeding hiPSCs, on Gelatin or Matrigel coated tissue culture plastic, at a high cell density (4.5x10^4 cells/cm^2) in MSC medium, consisting of Knockout (KO-) DMEM, 1% GlutaMAX™, 20% Fetal Bovine Serum (FBS) & 10mM Y-27632 ROCK inhibitor, and culturing these cells for two weeks with complete medium exchanges, without Y-27632 ROCK inhibitor, performed on alternating days. After this two week culture period, the cells are passaged and seeded in MSC medium, on tissue culture plastic, at a lower cell density (6x10^3 cells/cm^2) and cultured for a further four days before the cells are passaged once again. In the present study, the enzyme utilised for cell dissociation varied based upon the time in culture, with Accutase used in early passages and Trypsin 0.05% EDTA used in later passages. Frequent passages are continued for a further three passages cumulating in a culture process of 30 days. The utilisation of alternative medium compositions was also examined in the present research, including
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DMEM & 10% FBS, DMEM & 20% FBS, KO-DMEM & 20% FBS, DMEM & 10% HPL, DMEM & 20% HPL, KO-DMEM & 10% HPL, and KO-DMEM & 20% HPL.

6.11 Cell Culture Protocol for Trilineage Differentiation of hMSCs & hiPSC-MSCs

In order to generate sufficient cells for the performance of the trilineage differentiation protocols, Baseline (P2), Centrifugation (P5) and Non-Centrifugation (P5) hMSCs were seeded in separate T175 flasks (4000 cells/cm$^2$) and cultured for 6 days with a medium exchange performed after 3 days. After 6 days, the cells were dissociated, centrifuged, resuspended in DMEM with 10% FBS, and a cell count was performed.

To derive hiPSC-MSCs for the trilineage differentiation assay, hiPSCs were seeded at 4.5x10$^4$ cells/cm$^2$ in MSC medium (KO-DMEM & 20% FBS) and cultured over a single passage for a total of 16 days. After this time, the cells were dissociated, centrifuged, resuspended in culture medium, and a cell count was performed.

In order to induce Adipogenic and Osteogenic differentiation, hMSCs from each condition, as well as hiPSC-MSCs, were seeded in 12-well plates at 10,000 cells/cm$^2$ in 1mL of the relevant culture medium. A replicate of each of the wells seeded with Baseline hMSCs, Centrifugation hMSCs, Non-Centrifugation hMSCs, or hiPSC-MSCs were also seeded. After 3 days, the culture medium was replaced with either StemPro® Adipogenic or Osteogenic differentiation medium (Life Technologies).

In order to induce Chondrogenic differentiation, hMSCs from each condition, as well as hiPSC-MSCs, were suspended in StemPro® Chondrogenic differentiation medium at 16x10$^6$ cells/mL and 5-6 x 2μL droplets of suspension were added to the relevant number of wells in a 12-well plate. A replicate of each of the wells seeded with Baseline hMSCs, Centrifugation hMSCs, Non-Centrifugation hMSCs, or hiPSC-MSCs were also seeded. After 30 minutes, 1mL of Chondrogenic differentiation medium was added to each well.

For the hMSCs from each of the conditions, or hiPSC-MSCs, undergoing either Adipogenic, Osteogenic or Chondrogenic differentiation, the cells were cultured for 14 days, with a differentiation medium exchange performed every 3-4 days.
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6.12 Analytical Techniques

6.12.1 Microscopy

In order to examine the morphology and determine the confluency of cell populations in culture, tissue culture flasks were examined under the Nikon (Chiyoda, Japan) Eclipse Ti Inverted Light microscope. Various magnifications were utilised depending upon the detail of the image required and the size of the cells examined. The attached digital camera was used to save images of the tissue culture flask being observed.

6.12.2 STR Analysis

STR analyses of each of the cell lines were performed by LGC Standards (Teddington, UK) as part of their Cell Line Authentication (CLA) analysis. This involves the use of the Powerplex® 16 HS service, in which 16 loci are analysed, in order to generate a Short Tandem Repeat (STR) profile for each cell sample, which is presented as both an Electropherogram (EPG) and a results table. The number of STRs at each allele of each locus, as presented in the results tables, is compared in order to identify peak area differences, below-threshold peaks and stutter peaks, which allows for the determination of significant differences in STR expression between cell samples.

6.12.3 Flow Cytometry

Flow Cytometry analysis was performed on Baseline, Centrifugation and Non-Centrifugation hMSC, Ruler hESCs, and hiPSCs.

6.12.3.1 Cell Culture/Preparation for Flow Cytometry

Cells were thawed, the cryoprotectant was removed, and the cells from each of the conditions were seeded in separate tissue culture flasks at an appropriate cell density. The cells were then cultured until they reached approximately 80% confluency with complete medium exchanges performed at the appropriate times. Once confluent, the cells were dissociated with the appropriate dissociation agents, a cell count was performed, and the cells were resuspended in the relevant culture medium at the appropriate cell density. Next, the appropriate number of cells from each sample was added to the relevant number of wells in a 96 well plate or flow cytometry tubes, which were then centrifuged to obtain cell pellets. Cells were then repeatedly washed with wash buffer or stain buffer and centrifuged, and the supernatant was aspirated after each wash. The appropriate antibodies, antibody cocktails, and controls, as discussed in section 6.12.3.2 below, were then added to the samples from each condition following the manufacturer’s instructions. The appropriate control beads were
prepared following the manufacturer’s instructions. Blank or unstained cell samples and repeats of each sample were also prepared. Finally, further wash steps were performed before the cells were resuspended in stain buffer prior to flow cytometry analysis.

6.12.3.2 Flow Cytometry Analysis

For each cell type, certain surface markers specific to each cell type were selected that, after the percentage expression was determined, would allow for the purity of each cell population to be calculated. To analyse the surface marker expression of Baseline, Centrifugation, and Non-Centrifugation hMSCs, Ruler hESCs & hiPSCs, antibodies, or antibody cocktails, for the surface markers relevant to each cell type were used. In the case of the hMSC surface marker expression analysis, multicolour flow cytometry was performed, utilising pre-conjugated positive and negative antibody cocktails for the primary CD markers that constitute the ISCT minimum criteria for hMSCs (Dominici et al, 2006), which allowed for multiparameter analysis of surface marker expression at the single cell level. For both hiPSC and Ruler hESC samples, multicolour flow cytometry was also performed using three fluorochrome-conjugated antibodies for each cell type to distinguish pluripotent cells from differentiated cells. Flow cytometry analysis was performed on the BD FACSCanto II (BD Biosciences), using the FACSDiva software version 6.1.3. For analysis, the flow cytometry data was exported in FCS 3 format and analysed using FlowJo software v10.

6.12.4 Automated Cell Count

Cell counts, viability and aggregation were determined via Trypan Blue (Sigma Aldrich, St Louis, USA) exclusion, using the Cedex Automated Cell Counter contained within the CompacT SelectT automated cell culture platform. 1mL cell suspension samples were taken from the relevant cultures and transferred into the Cedex Automated Cell Counter inlet within the CompacT SelectT negative pressure airflow cabinet where they then flow into the automated cell counter. The automated cell counter would then generate 30 viable images in which the total cell number, viable cell number and percentage of viable cells would be calculated for each image and averaged across all 30 images in order to generate a Total Cell Number (x10^5 cells/ml), Viable Cell Number (x10^5 cells/ml) and Viability (%) for the sample.

6.12.5 Staining Protocol for hMSC Colony Forming Unit Assay

After 14 days of culture, the culture medium of each T25 flask was aspirated and the cells were washed with PBS. Cells were then fixed by treatment with 4% Paraformaldehyde (PFA) (Sigma Aldrich) for 30 mins. After fixation, each flask was washed twice with distilled water
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and 1% crystal violet solution (Sigma Aldrich) was added for 30 mins. Finally, each flask was again washed twice with distilled water. Colonies were then visualised and the number of colonies were counted. Small colonies were considered to consist of 25-50 cells, medium colonies were considered to consist of 50-100 cells, and large colonies were considered to consist of >100 cells. Colony Forming Efficiency was determined using the following calculation:

\[
\text{Colony Forming Efficiency} = \left( \frac{\text{Number of colonies}}{\text{250 cells seeded}} \right) \times 100
\]

6.12.6 Staining Protocol for examination of Trilineage Differentiation of hMSCs & hiPSC-MSCs

For the staining of cultures after 14 days of adipogenic differentiation, the differentiation culture medium was aspirated and the cells were washed with PBS. Next, the cells were fixed with 4% PFA for 20-30 mins at room temperature. After fixation, cells were washed three times with deionised water and 60% Isopropanol (IPA) (Sigma Aldrich) was added for 5 mins. IPA was then removed and sterile-filtered Oil Red O working solution (3 parts Oil Red O stock solution & 2 parts deionised water) was applied for 5 mins at room temperature. Oil Red O stock solution was generated by adding Oil Red O powder (Sigma Aldrich) to IPA. Finally, Oil Red O working solution was removed, cultures were washed with deionised water until clear, and stained cells were visualised using light microscopy.

For the staining of cells after chondrogenic differentiation, differentiation culture medium was aspirated after 14 days and cells were washed with PBS. Cells were then fixed with 4% PFA for 20 mins. After fixation, cells were washed with PBS and Alcian Blue solution was added for 30 mins. Alcian Blue solution was generated by adding Alcian Blue powder (Sigma Aldrich) to 0.1N Hydrochloric Acid (HCL) (Sigma Aldrich). The cells were then rinsed three times with 0.1N HCL and distilled water was added to dilute the acidity. Cells were then visualised using light microscopy.

For the staining of hMSCs or hiPSC-MSCs after 14 days of osteogenic differentiation, the differentiation culture medium was aspirated and the flasks were rinsed with PBS. Next, 4% PFA was added for 10 mins in order to fix the cells. After fixation, cells were rinsed with PBS and 2.5% Silver Nitrate solution (Sigma Aldrich) was applied for 30 mins at room temperature under UV light. After UV treatment, the cells were washed three times with deionised water and treated with 4% Napthol AS-MX Phosphate Alkaline solution (Sigma Aldrich) in Fast Violet solution for 45 minutes at room temperature in the dark. Fast Violet
solution was generated from Fast Violet Salt (Sigma Aldrich). After 45 minutes of room temperature incubation, the cells were washed three times with deionised water and visualised using light microscopy.

The methods utilised in the present study for the determination of hMSC and hiPSC-MSC trilineage differentiation follow those recommended in the ISCT MSC minimal criteria (Dominici et al, 2006).

6.12.7 Determination of the paracrine functionality of hMSCs

Once the Baseline, Centrifugation or Non-Centrifugation cells had been pre-treated using the relevant method, as described above, and the conditioned medium had been collected, it was then analysed to photometrically determine the concentration of various bioactive factors. Prostaglandin-E2 (PGE-2) concentration and Indoleamine 2,3 Dioxygenase (IDO) activity were measured in order to determine the anti-inflammatory response of Baseline, Centrifugation and Non-Centrifugation hMSCs. Whereas, Vascular Endothelial Growth Factor (VEGF) concentration was measured to determine the pro-angiogenic response of hMSCs from each condition.

The concentrations of both PGE-2 and VEGF were determined using Invitrogen (Life Technologies, Thermo Fisher Scientific, Waltham, USA) Enzyme Linked Immunosorbant Assay (ELISA) kits specific to each of these factors. The manufacturer’s guidelines were followed in order to photometrically determine the concentration of these factors in each of the conditioned medium samples. Further details regarding the methods used during the performance of each of the ELISAs are discussed in section 7.2.9.

However, in order to determine the concentration of IDO in conditioned medium samples, a photometric assay to determine the concentration of kynurenine was utilised. Kynurenine is a metabolite generated from the catabolism of tryptophan by the IDO enzyme, and can therefore act as a surrogate marker of IDO activity. The reagents used in this assay, including Tricholoroacetic Acid (TCA), p-Dimethylbenzaldehyde, and Glacial Acetic Acid, were purchased from Sigma Aldrich (St. Louis MO, USA). Further details regarding the methods used during the performance of this photometric assay are discussed in section 7.2.9.

6.13 Statistical Analyses

For the statistical analysis of all the data reported, IBM (Armonk NY, USA) SPSS statistical software was utilised. For the statistical analysis of the Viable Cell Density, Viable Cell Yield,
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and Viability data of Pre-Centrifugation, Post-Centrifugation and Non-Centrifugation hMSCs, Ruler hES Cells, and hiPSCs within each passage, One-Way Analysis of Variance (ANOVA) multiparameter analyses were used. One-Way ANOVAs were used to assess the significance of differences in the Standard Deviations (SD) of the Viable Cell Densities, Viable Cell Yields, and Viabilities of hMSCs, Ruler hES Cells, and hiPSCs in each of the four batches, cultured using Pre-Centrifugation, Post-Centrifugation, and Non-Centrifugation process steps, in the second passage. One-Way ANOVAs were also used to determine the significance of any differences in the Kynurenine, PGE-2, and VEGF concentrations between hMSC conditioned medium samples in their respective assays. One-Way ANOVAs were again utilised in order to determine the significance of differences in cell density, seeding/selection efficiency, viability and diameter between medium compositions and hiPSC starting materials.

Additionally, Two-Way ANOVAs were used to assess the significance of any differences in the Viable Cell Density, Viable Cell Yield, Population Doubling Time, Viability, Cell Diameter, and Aggregate Rate of Pre-Centrifugation, Post-Centrifugation and Non-Centrifugation hMSCs, Ruler hESCs, and hiPSCs observed across all passages.

For all ANOVAs performed, the cutoff value for statistical significance (p) was set at 0.05, and Tukey’s Honest Significant Difference (HSD) Post-Hoc Tests were used to perform multiple comparisons of the growth and functionality data for each cell line.

However, although the majority of statistical analysis was performed using IBM SPSS statistical software, Minitab® (State College PA, USA) 17 statistical software was utilised to perform a Gage R&R ANOVA in order to compare manual and automated cell counts of the Cedex Automated Cell Counter Images for each of the dissociation conditions during the Microwell plate experiment within the Ruler hES Cell protocol transfer. Within this analysis, 13 ‘Parts’, 2 ‘Operators’ and 3 ‘Repeats’ were selected and compared. The 13 ‘Part’ parameters represented the 13 dissociation conditions tested in the Microwell plate experiment. The 2 ‘Operator’ parameters represented the Manual and Automated cell counts of the images generated by the Cedex Automated Cell Counter. Finally, the 3 ‘Repeat’ parameters represented the number of selected images within a single cell count, from the 30 images generated by the Cedex Automated Cell Counter, which were counted manually and automatically.
Chapter 7: Comparison of a Manual, Centrifugation & an Automated, Non-Centrifugation Cell Culture Process Step for Human MSCs

In collaboration with TAP Biosystems (Royston, UK) & LGC Molecular and Cell Biology Team (Teddington, UK)

The aim of this chapter is to determine the comparability between a manual, Centrifugation and an automated, Non-Centrifugation hMSC culture process step using a number of process and product parameters, through which cell growth, stability, characterisation, and functionality are measured. Furthermore, the comparability between hMSC flow cytometry analyses performed at multiple independent laboratories is examined.

7.1 Introduction

Mesenchymal Stromal cells (MSCs) were first described by Friedenstein and colleagues in the 1970s as an adherent, non-haematopoietic cell type, present in the bone marrow, with the capacity to form fibroblastic colonies in vitro, and were assigned the name ‘Colony-Forming Unit-Fibroblasts’ (Friedenstein et al, 1970). After two decades of research, the stromal location and trilineage differentiation potential of this cell population were determined, and in the early 1990’s Caplan (1991) reported that these cells were capable of differentiating into all cells of the mesodermal lineage, and thus labelled them ‘Mesenchymal Stem Cells’.

However, due to the ongoing debate regarding their ‘stemness’, in vivo identity, and tissue source, a variety of nomenclatures have been proposed as alternatives and there is currently a lack of consensus, although the acronym ‘MSC’ remains prevalent. There is also enduring uncertainty surrounding the characterisation of this cell population, with a lack of unique surface marker or surface marker profile identified. Despite this uncertainty, the ISCT published a definition paper (Dominici et al, 2006), outlining three basic criteria for the identification of MSCs, which included their adherence to culture plastic, their positive expression of CD105, CD73, and CD90, their lack of CD45, CD34, CD14 or CD11b, CD79α or CD19, and HLA-DR expression, and their osteogenic, chondrogenic and adipogenic differentiation potential in vitro. However, although this definition may lack accuracy and specificity to the MSC cell type, it represents a step closer to defining the MSC phenotype. More recently attempts have been made to characterise MSCs based upon their proteomic profile (Mindaye et al, 2012), and it is likely that an accurate protein expression profile could significantly improve upon the current definition of an MSC.
However, this current definition of human MSCs does not account for the *in vivo* mode of action of this cell population, and it is likely that a population of cells exhibiting each of the basic characteristics of an MSC would not act homogenously *in vivo*. As previously described in section 2.2.3, by the mid-2000’s it was determined that, in combination with the traditional MSC differentiation mode of action, various cytokines and growth factors secreted by MSCs may aid tissue repair and suppress immune reaction (Caplan & Dennis, 2006). Phinney & Prockop (2007) further recognised that this secretion of soluble factors may in fact represent the major therapeutic mode of action of MSCs *in vivo*. Although currently any definition of the various MSC therapeutic effects is lacking, recent years have seen the initial developments in the characterisation of MSC mode of action with the ISCT proposing a series of assays capable of determining human MSC immune regulatory properties (Krampera et al, 2013). Furthermore, a recent study has proposed the development of a MSC reference standard to allow for the redefinition of the MSC minimal criteria, which would consist of 9 primary parameters defining MSC growth, characterisation, functionality, and recovery after cryopreservation (Tanavde et al, 2015). However, substantial progress is required before *in vivo* MSC function is fully understood and before comprehensive potency assays for each of the MSC putative mechanisms of action can be developed.

In the present study, the expression of various cytokines and growth factors which have been associated with a number of the more established MSC modes of action, including immune modulation, angiogenesis, and anti-apoptosis were analysed. In particular, the hMSC-mediated secretion of Prostaglandin E-2 (PGE-2) and Vascular Endothelial Growth Factor (VEGF) into the cell culture medium, as well as the hMSC-mediated Indoleamine 2,3 dioxygenase (IDO) activity, by Baseline, Centrifugation, and Non-Centrifugation hMSCs after pre-treatment was examined.

PGE-2 has been highlighted as a central mediator of the inhibitory effects of MSCs upon immune cells (Meirelles et al, 2009). It has been discovered (Prockop & Oh, 2001; Prockop, 2013) that, in response to pro-inflammatory mediators, MSCs secrete PGE-2 which requires intimate association or cell to cell contact to bind to the EP2 and EP4 receptors of resident macrophages. The binding of PGE-2 to host macrophages drives the transition of these cells from their classical M1, pro-inflammatory phenotype towards an M2, anti-inflammatory phenotype in which these macrophages secrete anti-inflammatory mediators, including IL-10 and IL-1 receptor agonist (Prockop, 2013).
Culture expanded hMSC IDO secretion, stimulated by pro-inflammatory Interferon Gamma (IFN-γ) (Krampera, 2011), has been strongly linked to the suppression of T, B and Dendritic cells (Galipeau, 2013). Natural Killer (NK) cell function has also been found to be inhibited by both IDO and PGE-2 secretion by MSCs (Yagi et al, 2010). The suppression of T cell response, through the upregulation of IDO, has been associated with the depletion of tryptophan and the accumulation of toxic metabolites (Tolar et al, 2010). More recently, IDO has been found to be a central effector of MSC T cell suppressive function and a strong correlation between magnitude of IDO expression and suppression of T cell proliferation has been discovered (Menard et al, 2013).

The Hypoxic culture and preconditioning of hMSCs, in which ≤1% O₂ is commonly used, induces the overexpression of VEGF, an established pro-angiogenic factor (Liu et al, 2008; Rehman et al, 2004; Hu et al, 2008; Li et al, 2007; Tögel et al, 2007; Chacko et al, 2010). A number of these studies have identified VEGF as the most critical factor responsible for the angiogenic properties of MSCs in vivo (Rehman et al, 2004; Hu et al, 2008; Li et al, 2007; Tögel et al, 2007; Chacko et al, 2010; Al-Khaldi et al, 2003). Furthermore, the expression of this protein by MSCs has also been associated with an anti-apoptotic effect upon host cells, improving cell survival in a number of studies (Rehman et al, 2004; Tögel et al, 2007).

Therefore, it is clear that the expression of PGE-2, IDO and VEGF are crucially important for the anti-inflammatory, immune modulatory, angiogenic and anti-apoptotic MSC modes of action. Thus, the measurement of these paracrine factors in the present study, after hMSC priming, may allow for the determination and comparison of the functional activity of hMSCs cultured using either manual or automated process steps.

The measurement of the expression of the cytokines associated with MSC immune modulation, specifically PGE-2 and IDO, was stimulated through the pre-treatment of hMSCs, from each of the Baseline, Centrifugation, and Non-Centrifugation conditions, with the inflammatory molecules IFN-γ and Tumour Necrosis Factor alpha (TNF-α) at the concentrations recommended in the recent ISCT paper which aims to highlight the optimal methods of accurately measuring the immune modulatory capacity of MSCs (Krampera et al, 2013). The expression of the pro-angiogenic and anti-apoptotic factor VEGF was stimulated through the hypoxic pre-treatment of hMSCs from all three conditions, following the protocols outlined in a number of previous studies in which MSCs have been pre-treated in
hypoxia (Wei et al, 2012; Hu et al, 2008; Chacko et al, 2010). The methods of pre-treatment and measurement of bioactive factor expression are described in greater detail in section 7.2.9.

Despite the significant challenges that exist in the characterisation of MSC therapies, this cell population holds great promise in the treatment for a variety of indications, including cardiovascular, pulmonary, neurological, metabolic, skeletal, inflammatory and immune disorders. However, considerable hurdles are also present in the manufacture of hMSC-based therapies, including the requirement for a large number of cells for cell therapy, and the significant workload, cost and variation that is associated with manual cell culture (Thomson, 2007). The development of automated adherent cell culture platforms, for example TAP Biosystems’ CompacT SelecT, have made automation a viable alternative to manual cell culture processes, however the examination of the way in which differences between manual and automated cell culture processes may affect relevant cell culture parameters is limited to a few studies and further investigation is required (Hussain et al, 2013; Terstegge et al, 2007; Thomas et al, 2007; Thomas et al, 2009).

Many of the process steps within the CompacT SelecT automated hMSC passage protocols utilised in the present study are very similar to that of a manual passage, however these two culture methods differ in one key process step. During manual cell culture, once the dissociation agent (e.g. Trypsin EDTA) has been applied and the cells have been incubated, the enzyme is neutralised, the suspension is centrifuged and the supernatant is aspirated in order to isolate a cell pellet. However, during automated cell culture, once the dissociation agent has been applied, it is immediately poured off so that only a residual amount remains. The cells, and remaining enzyme, are then incubated and culture medium is then applied in order to neutralise the dissociation agent.

Recently, a method for determining residual levels of Trypsin in cell-based products has been developed with high sensitivity and the capacity for a broad range of detection (Braatz et al, 2015). However, the specific effect of dissociation agent carryover and lack of centrifugation in the automated cell culture process has yet to be investigated. Therefore, the research in the present chapter aims to compare the effects of Centrifugation and Non-Centrifugation process steps upon hMSC number, viability, surface marker expression, Short Tandem Repeat (STR) profile, trilineage differentiation and paracrine function. In order to facilitate direct comparison, and to measure and establish process comparability, only the centrifugation and dissociation process steps differed between the manual and automated culture methods in
order to minimise any manual variation. This novel investigation into the effects of these two key process steps will help to improve the understanding of the influence of automated cell culture upon various cell culture parameters and may enable further optimisation of this process in the future. The null hypothesis for these experiments was that no significant difference in cell yield, viability, surface marker expression, STR profile, trilineage differentiation or paracrine function, would be observed for hMSCs cultured utilising either manual or automated process steps.

Furthermore, the present study explores the comparability between multiple flow cytometry and surface marker expression analyses of hMSCs. It is apparent that significant inter-individual variation and differences in methods of analysis exist between users and laboratories, and that these are likely to contribute to the variability in the results of flow cytometry analyses observed between laboratories (Zenger et al, 1998). Therefore, by comparing the results of hMSC flow cytometry analyses performed at both Loughborough University and LGC (Teddington, UK), the effect of this inter-laboratory variability was examined.

Finally, a hMSC culture protocol transfer between Loughborough University and LGC was performed. However, as no CompacT SelecT was available at LGC, the automated Centrifugation and Non-Centrifugation protocols were replicated manually. This allowed for the comparison of hMSC phenotype after culture utilising either automated or manual variations of the Centrifugation and Non-Centrifugation process steps. Therefore, in order to demonstrate the effect of manual and automated processing upon hMSC phenotype, the comparability between manual and automated versions of the Centrifugation and Non-Centrifugation process steps was investigated.

7.2 Methods

In this section, the specific materials and methods utilised for the hMSC experiments, which are presented in this chapter, are outlined in greater detail than those described in the Materials & Methods Chapter (Chapter 6).

7.2.1 hMSC Culture- Initial expansion

- Lonza (Basel, Switzerland) hMSC vial (P0) (1x10^6 cells) removed from cryostorage and thawed.
The cryopreservation medium was removed and the hMSCs were manually seeded into BD Falcon™ Barcoded T175 flasks (BD Biosciences, San Jose, USA), containing DMEM HG GlutaMAX™ (Life Technologies, Thermo Fisher Scientific, Waltham, USA) supplemented with 10% FBS (Gibco®, Life Technologies).

- DMEM-based culture medium, supplemented with 10% FBS, was selected for this study as it is representative of the hMSC culture conditions that are commonly utilised in research laboratories at present, and therefore allows for comparability between studies.

A medium exchange was performed on all hMSC flasks every 3 days after seeding or passage.

- Once confluent (~6 days), the mother flask was manually passaged into 4 new daughter flasks (1 flask→4 new flasks).
  - Similarly to the choice of culture medium, a 1:4 split ratio was utilised throughout this study as it is representative of the hMSC adherent culture protocols commonly utilised in research laboratories at present.

Once confluent, the 4 daughter flasks were imported into the CompacT SelecT automated cell culture platform and each confluent daughter flask was passaged into 4 new granddaughter flasks using Non-Centrifugation protocol (4 flasks→15 new flasks).

- One flask did not receive enough medium during passage and was disposed.

Once the granddaughter flasks were confluent all flasks were exported from the CompacT SelecT and the cells were manually prepared for cryopreservation.

- Notified on the day of cryostorage procedure that pressurised gas system had malfunctioned and therefore CompacT SelecT could not be used.

- hMSCs were washed and dissociated from the flask using PBS (Lonza) and Trypsin EDTA (0.05%) (Life Technologies) respectively, incubated for 5 minutes and neutralised using 6ml of DMEM GlutaMAX™ High Glucose with 10% FBS.

- The suspension was then centrifuged at 276 RCF for 5 minutes in order to isolate a cell pellet, and this was resuspended in 3ml of freeze medium (90% FBS with 10% Dimethyl Sulfoxide (Sigma Aldrich, St Louis, USA).

- 1.5ml of this suspension was transferred into each cryovial in order to generate a bank of 30 cryovials with ~2x10^6 hMSCs/vial. Cryovials were transferred to ‘Mr Frosty’ container and stored at -80°C for 24 hours before being transferred to cryostorage.
• The cells generated during the initial expansion represent ‘Baseline’ hMSCs (P2), to which the hMSCs cultured utilising Centrifugation or Non-Centrifugation process steps were compared.

7.2.2 hMSC Culture- Centrifugation Experiment- 4 experiment runs

• 1 banked hMSC vial (P2) removed from cryostorage and thawed.

• After the cryopreservation medium was removed, these hMSCs were transferred into a Barcoded T175 flask, containing DMEM HG GlutaMAX™ with 10% FBS, which was then imported into the CompacT SelecT, and a seeding protocol was initiated.
  o hMSCs were seeded at a cell density of $0.7 \times 10^5$ cells/ml.

• A medium exchange was performed every 3 days after seeding or passage.

• Once confluent (~6 days), a hMSC Pre-Centrifugation protocol was used to detach cells from the seeded flask, to obtain cell count data (Viable Cell Density, Viability, Cell Diameter & Aggregate Size), and to outfeed the flask undergoing passage.
  o Viable Cell Yield was calculated by multiplying the Viable Cell Yield (cells/ml) by the volume of cell suspension.

• A manual centrifugation procedure (400 RCF x 5mins) was then performed on the cells from the outfed flask in order to isolate a cell pellet and to resuspend in fresh culture medium within the same flask.

• This flask was then imported back into the CompacT SelecT and a Post-Centrifugation protocol was performed to passage the hMSCs into 4 new daughter flasks (1 flask $\rightarrow$ 4 new flasks), each seeded with $7 \times 10^5$ cells, and obtain cell count data.

• Once each of the 4 daughter flasks reached confluence the same method as above was performed in order to passage each flask into 4 new granddaughter flasks (4 flasks $\rightarrow$ 16 new flasks) and to obtain cell count data.

• Once 16 granddaughter flasks reached confluence, all 16 flasks were pooled into one single flask, using a Pool protocol, and the cells were isolated using centrifugation, resuspended in cryopreservation medium (90% FBS & 10% DMSO) and cryopreserved in a sufficient number of cryovials.

• The required amount of cells were then thawed and used for post-manual characterisation and functional assays.

• Also, $2 \times 10^6$ of the cryopreserved cells were suspended in transport buffer and shipped to LGC Standards for post-manual CLA and comparison to the baseline CLA analysis.
• Only 1 batch/run was utilised for characterisation, CLA and functional assays.
  o No cross-batch/run pooling.

• The above methods were repeated (excluding characterisation, CLA and functional assays) another 3 times in order to complete 4 Centrifugation experiment runs.

7.2.3 hMSC Culture- Non-Centrifugation Experiment- 4 experiment runs

• 1 banked hMSC vial removed from cryostorage and thawed.

• After the cryopreservation medium was removed, the hMSCs were transferred into a Barcoded T175 flask, containing DMEM HG GlutaMAX™ with 10% FBS, which was then imported into the CompacT SelecT, and a seeding protocol was initiated.
  o hMSCs were seeded at a cell density of \(0.7 \times 10^5\) cells/ml.

• A medium exchange was performed every 3 days after seeding or passage.

• Once confluent (~6 days), a hMSC Non-Centrifugation protocol, involving a Trypsin pour-off step, was used to passage the seeded flask into 4 new daughter flasks (1 flask \(\rightarrow\) 4 new flasks), each seeded with \(7 \times 10^5\) cells, and obtain cell count data.

• Once each of the 4 daughter flasks reached confluency, the same protocol as above was used to passage each flask into 4 new granddaughter flasks (4 flasks \(\rightarrow\) 16 new flasks) and obtain cell count data.

• Once 16 granddaughter flasks reached confluency, all 16 flasks were pooled into one single flask and the cells were isolated using centrifugation, resuspended in cryopreservation medium (90% FBS & 10% DMSO) and cryopreserved in a sufficient number of cryovials.

• The required amount of cells were then thawed and used for post-automated characterisation and functional assays.

• Also, \(2 \times 10^6\) of the cryopreserved cells were suspended in transport buffer and shipped to LGC Standards for post-automated CLA and comparison to the baseline CLA analysis.

• Only 1 batch/run was utilised for characterisation, CLA and functional assays.
  o No cross-batch/run pooling.

• The above methods were repeated (excluding characterisation, CLA and functional assays) another 3 times in order to complete 4 Non-Centrifugation experiment runs.
7.2.4 Surface Marker Expression

7.2.4.1 Loughborough University cell culture and preparation for flow cytometry analysis of hMSCs cultured using CompacT SelecT automated Centrifugation & Non-Centrifu- 
gation process steps

- 1 vial of Baseline (P2), Centrifugation (P5), and Non-Centrifu- 
gation (P5) hMSCs were thawed, the cryoprotectant was removed, and the cells from each of the 
conditions were seeded in separate T175 flasks at a density of 4x10^3 cells/cm^2 (7x10^5 
cells/T175 flask) in DMEM with 10% FBS.
- The cells were cultured until they reached approximately 80% confluency (~6 days) 
with a complete medium exchange performed after 72 hours.
- Once confluent, the cells were dissociated, using Trypsin EDTA, a cell count was 
performed, and the cells were resuspended in DMEM with 10% FBS at a 
concentration of 5x10^5 cells/ml.
- Next, 200μl of cell suspension (1x10^5 cells) was plated per well in the relevant 
number of wells in a 96 well plate, and this was centrifuged to obtain cell pellets.
- The cells were then washed twice with stain buffer (BD Pharmingen™, BD 
Biosciences), centrifuging and aspirating the supernatant after each wash.
- Isotype positive control cocktail, isotype negative control cocktail, hMSC positive 
pre-conjugated antibody cocktail, hMSC negative pre-conjugated antibody cocktail, 
Isotype Drop-in Control, and Anti-Human CD44 Drop-in components (BD Stemflow™, BD 
Biosciences) were then added to the samples from each condition 
following the manufacturer’s instructions. Blank cell samples were also plated, and 
samples from each condition, plated with each component, were repeated three times.
• Finally, the wash step was repeated a further two times for each sample, before the cells were resuspended in 250μl of stain buffer prior to flow cytometry.

7.2.4.2 Loughborough University flow cytometry analysis of hMSCs cultured using CompacT SelecT automated Centrifugation & Non-Centrifugation process steps

To determine the immunophenotype of Baseline, Centrifugation, and Non-Centrifugation hMSCs, multicolour flow cytometry was performed utilising positive and negative antibody cocktails for the primary CD markers that constitute the ISCT minimum criteria for hMSCs (Dominici et al, 2006). This included the positive expression of CD105, CD90, CD73, and CD44; and the lack of CD34, CD45, CD11b, CD19, and HLA-DR expression. The utilisation of pre-conjugated antibody cocktails allowed for multiparameter analysis of surface marker expression at the single cell level. This method has been previously validated by colleagues at Loughborough University (Chan et al, 2014).

The hMSC samples were analysed on the BD FACSCanto II (BD Biosciences) which was operated using the FACSDiva software version 6.1.3. For analysis, the flow cytometry data was exported in FCS 3 format and analysed using FlowJo software v10.

7.2.5 Comparability in Surface Marker Expression

7.2.5.1 LGC hMSC culture using manual Centrifugation & Non-Centrifugation process steps for flow cytometry analysis

Passage 2 hMSCs were distributed from Loughborough University to LGC. From passage 3 to 5, hMSCs were cultured and expanded using manual protocols mimicking that of the manual (Centrifugation) and automated (Non-Centrifugation) process steps performed at Loughborough University. In order to perform the manual Centrifugation and Non-Centrifugation culture processes, the automated protocols, as described previously in section 7.1, were replicated as closely as possible, with similar reagents and process steps, but without the use of the CompacT SelecT automated cell culture platform. In contrast to the automated processes, cell counting was performed using the Vi-Cell XR (Beckman Coulter, Brea, USA) during both the manual Centrifugation and Non-Centrifugation processes, rather than using the Cedex automated cell counter. Also, during the cryopreservation process for each passage (P5-8), approximately 50% of the pooled cells were banked at 1x10^6 cells per ml in DMEM with 10% FBS and 10% DMSO. Further passages were initiated by seeding multiple T175 flasks with the remaining pooled cells at a density of 7x10^5 cells per T175 flask.
7.2.5.2 LGC cell preparation for flow cytometry analysis of hMSCs cultured using manual Centrifugation & Non-Centrifugation process steps

- Pre-banked hMSCs (P5) were analysed directly after cryopreservation in liquid nitrogen.
- After rapid thawing at 37°C, the cells were washed once, with wash solution (BD Bioscience), centrifuged at 1200 RPM for 5 minutes, and fixed in 1x cell fixation buffer (BD Biosciences) following the manufacturer’s instructions.
- Next, fixed cells were incubated in Fc blocking reagent (BioLegend, San Diego, USA) for 15 minutes at room temperature, and 2x10^5 cells were incubated at 4°C for 30 minutes, whilst protected from light, with the selected group of antibody/isotype control cocktails in staining buffer (BioLegend).
- After two wash steps with wash solution, the cells were centrifuged at 6500 RPM for 2 minutes, resuspended in fresh cold PBS and transferred into 5 ml polystyrene round-bottom flow cytometry tubes (BD Biosciences).
- The samples were kept on ice covered with foil during measurement sessions.

7.2.5.3 LGC flow cytometry analysis of hMSCs cultured using manual Centrifugation & Non-Centrifugation process steps

To identify the correct phenotypic pattern of BM isolated hMSCs, immunophenotyping was performed by flow cytometry utilising the most commonly used monoclonal antibodies based upon the literature (See Figure 21 & Appendix 13.7.1). The expression level of different surface markers was analysed by direct immunolabeling approach. Staining was performed on passage 5 cells from each condition (CEN and NC) using the selected group of antibody cocktails with the corresponding isotype controls (Figure 21). This enabled target cells to be labelled and analysed on the flow cytometer for the designated group of antibodies simultaneously.

<table>
<thead>
<tr>
<th>Markers</th>
<th>Group 1</th>
<th>Group 2</th>
<th>Group 3</th>
<th>Group 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>GENERIC</td>
<td>CD9</td>
<td>CD44</td>
<td>CD98</td>
<td>CD29</td>
</tr>
<tr>
<td>STRESS</td>
<td>CD63</td>
<td></td>
<td>CD95</td>
<td></td>
</tr>
<tr>
<td>MSC&quot;+&quot;</td>
<td>CD105</td>
<td>CD90</td>
<td>CD73</td>
<td>CD146</td>
</tr>
<tr>
<td>MSC&quot;-&quot;</td>
<td>CD34</td>
<td>CD45 HLA-DR</td>
<td>CD11b</td>
<td></td>
</tr>
</tbody>
</table>

**Figure 21**: Table of selected surface markers for LGC hMSC cell immunophenotyping. Antibodies were categorized in groups as “generic”, “stress”, “MSC positive (+)” and MSC negative (-) markers. Pre-conjugated antibodies were selected to generate four groups of antibody cocktails.
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LGC also analysed hMSC samples on the BD FACSCanto II (4-2-2 configuration optical filters) (BD Biosciences) fitted with an octagon-488 nm blue laser, trigon-633 nm red laser and trigon-405 nm violet laser, operated through the FACSDiva software version 6.1.3. The data was exported in FCS 3 format and analysed using FlowJo software v10. Quality control was performed daily using C&ST beads (BD Biosciences). Machine sensitivity was tested using SPHERO™ Ultra Rainbow fluorescent particles (6-peak beads) (Spherotech, Lake Forest, USA). Compensation was set in FACSDiva software using compensation particles, labelled with corresponding antibodies (BD Biosciences).

An acquisition threshold was set on FSC to eliminate debris. SSC-H and SSC-W were used to eliminate doublets. A total of 10,000 events were acquired per run. Expression level of selected markers was evaluated by determining the % of positive populations.

7.2.6 Cell Line Authentication (CLA) & Short Tandem Repeat (STR) Profiling

- STR profiling was performed by LGC standards (Teddington, UK) through their CLA service.
- Samples were prepared for transport and CLA analysis following the protocol provided by LGC standards.
- 1 vial of baseline hMSCs, 1 vial of Post-Centrifugation hMSCs, and 1 vial of Non-Centrifugation hMSCs were removed from cryostorage, thawed and centrifuged.
- The supernatant was then aspirated and a PBS wash was applied, in order to remove any remaining freeze medium.
- The samples were again centrifuged and the supernatant aspirated in order to isolate the cell pellets.
- Finally, the samples were resuspended in 400μl of transport buffer, provided by LGC standards, which lyses the cells and preserves the gDNA.
- The samples were then packaged and sent to LGC Standards, where the CLA analysis was undertaken and the STR profiles for each sample was compared.

7.2.7 Colony Forming Unit (CFU) Assay

- To generate sufficient cells for CFU assay, 1 vial of Baseline (P2), Centrifugation (P5) and Non-Centrifugation hMSCs (P5) hMSCs were thawed, the cryoprotectant removed, and the cells seeded in separate T175 flasks (4000 cells/cm²). Two repeats were utilised for each condition.
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- A medium exchange was performed after 3 days, and, after 6 days, the cells were dissociated, centrifuged, resuspended in DMEM with 10% FBS, and a cell count was performed.
- 250 cells from each condition were seeded in separate Nunc™ T25 flasks (Thermo Fisher Scientific, MA, USA) in 8mL of DMEM FBS. A replicate of each condition was also seeded.
- The cells were cultured for 14 days with a complete medium exchange performed every 5 days.
- To stain, firstly, the culture medium of each T25 flask was aspirated and the cells were washed with PBS.
- Cells were then fixed by treatment with 4% Paraformaldehyde (PFA) (Sigma Aldrich) for 30 mins.
- Each flask was then washed twice with distilled water and 1% crystal violet solution (Sigma Aldrich) was added for 30 mins.
- Each flask was again washed twice with distilled water, and colonies were then visualised and the number of colonies were counted.
- Small colonies were considered to consist of 25-50 cells, medium colonies were considered to consist of 50-100 cells, and large colonies were considered to consist of >100 cells.
- Colony Forming Efficiency was determined using the following calculation:
  \[ \text{Colony Forming Efficiency} = \frac{\text{Number of colonies}}{250 \text{ cells seeded}} \times 100 \]

7.2.8 Trilineage Differentiation Capacity

- 1 vial of Baseline (P2), Centrifugation (P5) and Non-Centrifugation hMSCs (P5) hMSCs were thawed, the cryoprotectant removed, and the cells seeded in separate T175 flasks (4,000 cells/cm²).
- The cells were cultured for 6 days with a medium exchange performed after 3 days.
- After 6 days, the cells were dissociated, centrifuged, resuspended in DMEM with 10% FBS, and a cell count was performed.
- In order to induce Adipogenic and Osteogenic differentiation, hMSCs from each condition were seeded in 12-well plates at 10,000 cells/cm² in 1mL of DMEM with 10% FBS. A replicate of each of the wells seeded with Baseline, Centrifugation or Non-Centrifugation hMSCs were also seeded. After 3 days, the DMEM with 10% FBS was
replaced with either StemPro® Adipogenic or Osetogenic differentiation medium (Life Technologies).

- In order to induce Chondrogenic differentiation, hMSCs from each condition were suspended in StemPro® Chondrogenic differentiation medium at 16x10^6 cells/mL and 5-6 x 2μL droplets of suspension were added to the relevant number of wells in a 12-well plate. A replicate of each of the wells seeded with Baseline, Centrifugation or Non-Centrifugation hMSCs were also seeded. After 30 minutes, 1mL of Chondrogenic differentiation medium was added to each well.

- For the hMSCs from each of the conditions undergoing either Adipogenic, Osteogenic or Chondrogenic differentiation, the cells were cultured for 14 days, with a differentiation medium exchange performed every 3-4 days.

7.2.8.1 Adipogenic Differentiation Staining

- After 14 days of adipogenic differentiation, the differentiation culture medium was aspirated and the cells were washed with PBS.

- The cells were then fixed with 4% PFA for 20-30 mins at room temperature. After fixation, cells were washed three times with deionised water and 60% Isopropanol (IPA) (Sigma Aldrich) was added for 5 mins.

- IPA was then removed and sterile-filtered Oil Red O working solution (3 parts Oil Red O stock solution & 2 parts deionised water) was applied for 5 mins at room temperature. Oil Red O stock solution was generated by adding Oil Red O powder (Sigma Aldrich) to IPA.

- Oil Red O working solution was then removed, cultures were washed with deionised water until clear, and stained cells were visualised using light microscopy.

7.2.8.2 Chondrogenic Differentiation Staining

- Differentiation culture medium was aspirated after 14 days and cells were washed with PBS.

- The cells were then fixed with 4% PFA for 20 mins. After fixation, cells were washed with PBS and Alcian Blue solution was added for 30 mins. Alcian Blue solution was generated by adding Alcian Blue powder (Sigma Aldrich) to 0.1N Hydrochloric Acid (HCL) (Sigma Aldrich).

- The cells were then rinsed three times with 0.1N HCL, distilled water was added to dilute the acidity, and the cells were then visualised using light microscopy.
7.2.8.3 Osteogenic Differentiation Staining

- After 14 days of osteogenic differentiation, the differentiation culture medium was aspirated and the flasks were rinsed with PBS.
- 4% PFA was then added for 10 mins in order to fix the cells. After fixation, cells were rinsed with PBS and 2.5% Silver Nitrate solution (Sigma Aldrich) was applied for 30 mins at room temperature under UV light.
- The cells were then washed three times with deionised water and treated with 4% Naphthol AS-MX Phosphate Alkaline solution (Sigma Aldrich) in Fast Violet solution for 45 minutes at room temperature in the dark. Fast Violet solution was generated from Fast Violet Salt (Sigma Aldrich).
- The cells were then washed three times with deionised water and visualised using light microscopy.

7.2.9 Paracrine Functionality Assays

7.2.9.1 Collection of Conditioned Medium

- Baseline, Centrifugation, and Non-Centrifugation hMSCs were each seeded in T175 flasks, each containing DMEM HG GlutaMAX™ with 10% FBS, at a density of 1x10⁶ cells/flask.
  - 3 vials from each condition were seeded.
- These 9 flasks were divided into 3 groups of 3 T175 flasks, with each group containing one flask from each of the Baseline, Centrifugation, and Non-Centrifugation conditions.
  - The 3 groups consisted of ‘Inflammatory Pre-Treatment’ (IN-PT), ‘Hypoxic Pre-Treatment’ (HY-PT), and No Treatment (NT) groups.
- After 3 days of culture, a medium exchange was performed for each of the 9 flasks. The medium was aspirated from all hMSC flasks, and the flasks in each of the groups were specifically pre-treated.
  - The NT hMSCs were again treated with DMEM HG GlutaMAX™ with 10% FBS and incubated at 37°C at 5% CO₂ for a further 72 hours.
  - The IN-PT hMSCs were treated with DMEM HG GlutaMAX™ with 10% FBS, as well as 10ng/ml IFN-γ (Sigma Aldrich) and 15ng/ml TNF-α (Sigma Aldrich) for a further 72 hours.
    - These conditions were selected based upon the ISCT guidelines for immunological characterisation of MSCs (Krampera et al, 2013).
The HY-PT hMSCs were treated with DMEM HG GlutaMAX™ with 10% FBS, which had been pre-conditioned at 1% O₂ overnight, and were incubated at 37°C at 1% O₂ for a further 48 hours.

- These conditions were selected based upon established hypoxic hMSC preconditioning methods (Wei et al, 2012; Hu et al, 2008; Chacko et al, 2010; Potier et al, 2007). Chacko and colleagues (2010) demonstrated, through hypoxic pre-treatment for 72 hours, that VEGF expression was greatest after 48 hours of pre-treatment.

After the hMSC populations were exposed to either the NT, IN-PT, or HY-PT conditions, the conditioned medium from each flask was collected and stored at -80°C.

### 7.2.9.2 PGE-2 Quantification

- The Prostaglandin E2 Enzyme-Linked Immunosorbant Assay (ELISA) was performed following the manufacturer’s directions (Life Technologies™, Invitrogen™ Novex® Prostaglandin E2 Human ELISA Kit).
- All buffers, Tracers and Antibodies were prepared as described in the manufacturer’s directions. Prostaglandin E2 Standard was prepared at the recommended concentrations, however culture medium rather than Tris Buffer was used for the dilution of the standard curve. This is recommended in the manufacturer’s directions if assaying culture medium samples.
- Blank (2 wells), Non-Specific Binding (2 wells), Maximum Binding (3 wells), Total Activity (1 well), Standard (16 wells- 8 standards in duplicate), and Sample wells (18 wells- 6 wells in triplicate) were prepared as indicated in the manufacturer’s guidelines.
- After incubation for 90 minutes in the dark, on an orbital shaker, the absorbance was measured using a plate reader at a wavelength of 410 nm.

### 7.2.9.3 Kynurenine Quantification & IDO activity measurement

- The enzyme Indoleamine 2,3-dioxygenase (IDO) converts tryptophan to kynurenine, and the following assay was used to photometrically determine the concentration of kynurenine in Inflammatory Pre-Treated (IN-PT) or No Treatment (NT) conditioned medium samples from each of the Baseline, Centrifugation, and Non-Centrifugation hMSC groups.
This assay has been successfully utilised in a number of previous studies in order to quantify IDO activity for a number of cell types, including hMSCs (Meisel et al, 2004; Feng & Taylor, 1989; Takikawa et al, 1988; Matin et al; 2006).

- 50µl of 30% Trichloroacetic Acid (TCA) (Sigma Aldrich) was added to 150µl of each of the conditioned medium samples as well as a fresh DMEM with 10% FBS sample.
  - The TCA and medium samples were incubated for 15 minutes at 50°C and centrifuged at 73 RCF for 5 minutes in order to hydrolyse N-formylkynurenine to kynurenine.

- 75µl of the supernatant from each of the samples was then transferred to a 96-well plate.
- A serial dilution of 100µm kynurenine solution (Sigma Aldrich) was used as a standard (100µm→0µm) to allow for the generation of a standard curve.
- 75µl of Ehrlich’s reagent (1% p-dimethylbenzaldehyde in glacial acetic acid) (Sigma Aldrich) was added to each well and the plate was incubated for 10 minutes at room temperature.
- Finally, the fluorescence of each well was determined by measuring the absorbance at 492nm.
- Each sample was measured in duplicate and a repeat of the assay was performed in order to increase the reliability of the data.
  - Sample data was normalised against the average absorbance of two medium (DMEM HG GlutaMAX™ with 10% FBS) blanks.
  - Medium normalised values were then standardised utilising the Kynurenine Standard curve and the average values were then generated.

7.2.9.4 VEGF Quantification

- The VEGF Enzyme-Linked Immunosorbant Assay (ELISA) was performed following the manufacturer’s directions (Life Technologies™, Invitrogen™ Novex® Human VEGF ELISA Kit).
- All Buffers, Tracers and Antibodies were prepared as described in the manufacturer’s directions. VEGF Standard was diluted in Standard Diluent Buffer and the manufacturer’s directions were followed in order to generate a standard curve.
- Chromogen Blank (2 wells), Zero (2 wells), Standard (16 wells- 8 standards in duplicate), and Sample wells (18 wells- 6 wells in triplicate) were prepared as indicated in the manufacturer’s guidelines.
• After a final incubation of 30 minutes at room temperature, in the dark, the absorbance was measured using a plate reader at a wavelength of 450 nm.

• VEGF concentration in each sample was determined by plotting the absorbance of samples against the VEGF standard curve. In order to correct for the 1:2 dilution of samples performed during the assay, VEGF concentrations of each sample were multiplied by 2.

7.2.10 Statistical Analyses
Experimental data regarding the Viable Cell Density, Viable Cell Yield, and Viability of Pre-Centrifugation, Post-Centrifugation and Non-Centrifugation hMSCs within each passage was assessed using One-Way Analysis of Variance (ANOVA) multiparameter analysis, utilising the IBM (Armonk, USA) SPSS statistical software, to determine significant differences. One-Way ANOVAs were also used to assess the significance of differences in the Standard Deviations (SD) of the Viable Cell Densities, Viable Cell Yields, and Viabilities of hMSC Pre-Centrifugation, Post-Centrifugation, and Non-Centrifugation samples, in each of the four batches, from the second passage. Two-Way ANOVAs were used to assess significant differences in the Viable Cell Density, Viable Cell Yield, Population Doubling Time, Viability, and Cell Diameter of Pre-Centrifugation, Post-Centrifugation and Non-Centrifugation hMSCs across all passages. Finally, One-Way ANOVAs were used to determine the significance of any differences in the Kynurenine, PGE-2, and VEGF concentrations of Untreated and Pre-Treated Baseline, Centrifugation, and Non-Centrifugation hMSCs in their respective assays. The cutoff value for statistical significance (p) was set at 0.05. Tukey’s Honest Significant Difference (HSD) Post-Hoc Tests were used to perform multiple comparisons of the hMSC growth and paracrine functionality data.
7.3 Results

7.3.1 Morphology

Figures 22 & 23 (above) demonstrate that no difference in morphology was observed between hMSCs cultured utilising manual or automated process steps, and that both hMSC populations exhibited a spindle shaped, fibroblast-like morphology.
7.3.2 Cell Diameter

As illustrated in Figure 24, no significant difference in cell diameter was observed between passages or between process steps, and hMSC diameter remained consistently around 20μm.

7.3.3 Surface Marker Expression

The ISCT minimal criteria for the phenotypic expression of hMSCs indicates that ≥95% of the cell population should express CD105, CD73 and CD90, and that ≤2% of the cell population should express CD45, CD34, CD11b, CD19, or HLA-DR (Dominici et al, 2006). As demonstrated in Figures 25, 26 & 27, the multicolour analysis performed in the present study allowed for the demonstration of the co-expression of multiple hMSC positive markers, as well as the lack of co-expression of multiple negative markers and haematopoietic antigens, on single cells. This method conforms to that recommended in the ISCT minimal criteria (Dominici et al, 2006). Furthermore, no significant difference in positive or negative expression was observed between hMSCs cultured utilising alternative process steps, and between early (P2) and late (P5) passage hMSCs, with all populations expressing ≥95% positive marker expression and ≤2% negative marker expression, as illustrated in the scatter plots presented below (Figures 25, 26 & 27).
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Figure 25: Scatter plots demonstrating Loughborough University multicolour flow cytometric analysis of positive and negative surface marker co-expression of Baseline hMSCs (P2).

Figure 26: Scatter plots demonstrating Loughborough University multicolour flow cytometric analysis of positive and negative surface marker co-expression of Centrifugation hMSCs (P5).
7.3.4 Comparability in Surface Marker Expression

The immunophenotype of hMSCs cultured in the present study met many of hMSC surface marker expression criteria when analysed by flow cytometry at LGC (See Figure 28 & Appendix 13.7.2), with significant expression of the primary hMSC positive markers (CD105, CD73, and CD90) observed. However, both CD105 and CD146 expression was found to be lower than expected, with CD105 expression falling below the threshold set within the ISCT minimal criteria. The typical hMSC negative markers CD45, CD11b and HLA-DR conformed to the ISCT minimal criteria, with expression observed at <2%, whilst CD34 was detected in a higher proportion of cells. Examined generic and stress-linked surface markers were expressed at high levels in hMSCs cultured under both conditions (>46-60%).

The LGC flow cytometric analysis of hMSCs, cultured using either the manually replicated Centrifugation or Non-Centrifugation process steps over two passages, revealed a similar level of surface marker expression in both conditions. However, by comparing the results of the Loughborough University and LGC Flow Cytometry analyses, it is apparent that some
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differences in CD105 and CD34 expression were demonstrated, with lower percentage expression of CD105 and higher percentage expression of CD34 observed in LGC’s analysis. These findings are discussed further in section 7.4.

**Figure 28:** LGC flow cytometric analysis of surface marker profiles of Post-Centrifugation (Post-Cent) and Non-Centrifugation (Non-Cent) bone marrow-derived hMSCs (P5). The mean percentage expression level of surface markers of BM-MSC cells (P5), and error bars, reflecting three technical replicates per sample, are plotted (SD).
7.3.5 Cell Line Authentication (CLA) & Short Tandem Repeat (STR) Profiling

**Figure 29 (above)**: hMSC Baseline Electropherogram Short Tandem Repeat (STR) Profile

**Figure 30 (above)**: hMSC Post-Centrifugation Electropherogram Short Tandem Repeat (STR) Profile
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Figure 31 (above): hMSC Non-Centrifugation Electropherogram Short Tandem Repeat (STR) Profile

<table>
<thead>
<tr>
<th>Loci</th>
<th>hMSC Profile</th>
</tr>
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<tr>
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</tr>
<tr>
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</tr>
</tbody>
</table>

Figure 32 (above): Baseline, Centrifugation & Non-Centrifugation hMSC alleles at each locus within Short Tandem Repeat (STR) Profile
Figures 29, 30 & 31 demonstrate that no significant difference was observed between the Short Tandem Repeat (STR) profiles of the Baseline, Post-Centrifugation, and Non-Centrifugation hMSCs, as determined through the comparison of the number of STRs at each allele of each locus between hMSC samples. Figure 32 outlines the hMSC allele expression at each locus profiled, and these were found to be identical between Baseline, Centrifugation and Non-Centrifugation samples. These results were determined by comparing the STR Electropherograms (EPG) of hMSCs cultured using manual and automated process steps to that of hMSCs generated in the initial cell bank. This data suggests that neither the automated nor the manual process steps elicited an alteration in the pattern of short tandem repeats expressed in the hMSC DNA.

However, as illustrated in the figures above, differences in the level of STR expression between Baseline, Non-Centrifugation, and Post-Centrifugation hMSCs may exist. The EPG charts above demonstrate that both the Post-Centrifugation and Non-Centrifugation samples generated greater peaks in fluorescence at all loci compared to those of the Baseline sample. The above figures also reveal that the Non-Centrifugation sample produced a greater fluorescence at all loci compared to the Post-Centrifugation sample.
7.3.6 Colony Forming Unit Assay

From the Colony Forming Unit Assays performed for Baseline (Figure 33), Centrifugation (Figure 34) & Non-Centrifugation (Figure 35) hMSCs, it is apparent that hMSCs from all conditions generated colonies when seeded at a low density (10 cells/cm²). Furthermore, it was demonstrated that hMSCs from each of the conditions exhibited a similar colony forming potential (Figure 36). However, these results may also highlight trends for a reduced Colony Forming Efficiency after culture using the Centrifugation process step, and for a decrease in Colony Forming Efficiency after multiple passages.
7.3.7 Trilineage Differentiation Capacity

**Figure 37 (Left):** Oil Red O Staining of Baseline (Ba) hMSC Adipogenic Differentiation. **Figure 38 (Centre):** Alcian Blue Staining of Baseline (Ba) hMSC Chondrogenic Differentiation. **Figure 39 (Right):** Von Kossa Staining of Baseline (Ba) hMSC Osteogenic Differentiation. White arrows indicate areas of differentiation.

**Figure 40 (Left):** Oil Red O Staining of Centrifugation (Ce) hMSC Adipogenic Differentiation. **Figure 41 (Centre):** Alcian Blue Staining of Centrifugation (Ce) hMSC Chondrogenic Differentiation. **Figure 42 (Right):** Von Kossa Staining of Centrifugation (Ce) hMSC Osteogenic Differentiation. White arrows indicate areas of differentiation.

**Figure 43 (Left):** Oil Red O Staining of Non-Centrifugation (NC) hMSC Adipogenic Differentiation. **Figure 44 (Centre):** Alcian Blue Staining of Non-Centrifugation (NC) hMSC Chondrogenic Differentiation. **Figure 45 (Right):** Von Kossa Staining of Non-Centrifugation (NC) hMSC Osteogenic Differentiation. White arrows indicate areas of differentiation.
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After the staining of Baseline, Centrifugation & Non-Centrifugation hMSC populations, cultured in adipogenic, chondrogenic or osteogenic conditions, it is apparent that hMSCs from all conditions exhibited trilineage differentiation capacity (Figures 37-45). The Oil Red O staining (Figures 37, 40 & 43) demonstrates that hMSCs from all conditions differentiated into adipocytes and generated lipid droplets (red staining). The Alcian Blue staining (Figures 38, 41 & 44) indicates that hMSCs from all conditions differentiated into chondrocytes and generated cell micromasses (dark blue spheres) and glycosaminoglycans (blue staining). Finally, the von Kossa staining (Figures 39, 42 & 45) illustrates that hMSCs from all conditions differentiated into osteoblasts forming large calcium deposits (black stained areas).

Therefore, these results demonstrate that the Baseline, Centrifugation and Non-Centrifugation hMSCs meet the ISCT minimal criteria for the in vitro differentiation of MSCs (Dominici et al, 2006), which was determined using the recommended staining methods.

7.3.8 Growth Data

![Graph showing Mean Viable Cell Densities for hMSC Batches 1-4 Centrifugation & Non-Centrifugation](image)

**Figure 46:** The Means of the Viable Cell Densities of hMSCs, from all four Batches (n=4), counted Pre-Seeding, Pre-Centrifugation, Post-Centrifugation, and Non-Centrifugation over two passages. Outliers are plotted as well as Standard Deviations which are shown as error bars. Asterisks (*) denote significance (p=0.05).
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After culturing hMSCs over two passages, whilst utilising manual and automated process steps, it was determined that there was no significant difference in Viable Cell Density between hMSCs after Centrifugation or Non-Centrifugation cell culture methods across all four batches (p=0.999). This is presented in Figure 46, in which the Mean Viable Cell Densities for hMSCs after seeding, Pre-Centrifugation, Post-Centrifugation, and Non-Centrifugation, across all four batches, and over two passages are plotted. The Box plot shown in Figure 47 (above) refers specifically to the second passage and illustrates the variation in the Viable Cell Density of Pre-Centrifugation, Post-Centrifugation, and Non-Centrifugation hMSCs, across all four batches.
Figure 48: A Box Plot showing the Mean, Interquartile range, and Range of Viable Cell Densities for Pre-Centrifugation, Post-Centrifugation, and Non-Centrifugation hMSCs in the 2nd Passage (P5) within each of the four Batches. Outlier=
Although no measurable distinction in Mean Viable Cell Density was demonstrated, this chart may highlight a non-significant trend for a slightly greater Viable Cell Density obtained through utilisation of the automated process, however, further experiments would be required in order to confirm this trend. This data may also indicate that the centrifugation process step increased the obtainable Viable Cell Density of hMSC cultures, and it was observed that the Post-Centrifugation Mean Viable Cell Density (p=0.009), as well as the Non-Centrifugation Mean Viable Cell Density (p=0.008), was significantly greater than that of the Pre-Centrifugation samples in the second passage. This pattern of increased Viable Cell Densities in Post-Centrifugation (p=0.013) and Non-Centrifugation (p=0.07) hMSCs compared to Pre-Centrifugation hMSCs, were consistent over all passages. However, due to the differences in the volume of culture medium in which the cells are suspended, this trend may not represent a substantial difference in total cell number or yield. These potential patterns are discussed further in this section as well as in section 7.4.

However, Figures 46, 47 & 48 illustrate that, despite producing Viable Cell Densities which were similar to those obtained through the manual process, the automated process step may have exhibited a trend for a greater variation in Viable Cell Densities, in each batch, compared to the manual process step both prior to or after centrifugation. However, after statistical analysis of the SDs of the Viable Cell Density data in the second passage, it was determined that this pattern was not significant in the second passage (p=0.069).
In the chart above (Figure 49), the average number and size of viable hMSC aggregates from the Pre-, Post- & Non-Centrifugation samples from Batches 1-4, across two passages, are plotted. This data demonstrates that after centrifugation, an increase in the number of cells present in smaller aggregate sizes occurred. This suggests that the centrifugation process may be reducing larger aggregates of cells into smaller aggregates or into single cells. However, the lack of a greater number of cells in larger aggregates in the Pre-Centrifugation samples may not support this conclusion. It is also apparent that no difference in live cell aggregate size was observed when either the Centrifugation or Non-Centrifugation process steps were utilised.
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The chart above (Figure 50) illustrates the average Viable hMSC Yield of Centrifugation and Non-Centrifugation flasks from all four batches over two passages. Figure 51 (below) also highlights the Average Cumulative Viable hMSC Yield per Batch, of all four batches, over P3 (Upon seeding the Mother Flask), P4 (One confluent flask) and P5 (Four confluent flasks). These results demonstrate that no significant difference between Viable hMSC Yield obtained utilising Pre-Centrifugation, Post-Centrifugation, and Non-Centrifugation process steps was observed in each passage or across all passages. This therefore suggests that the increased Non-Centrifugation Viable Cell Density compared to the Pre-Centrifugation Viable Cell Density observed in the present study, as shown in Figure 46, was due to a difference in the volume of culture medium in which the cells were suspended. However, these results may indicate that the Viable hMSC Yield generated whilst utilising the Non-Centrifugation process was greater than the Viable hMSC Yield generated through the manual process, represented in the Post-Centrifugation measurements, although this trend was not significant (p=0.999).

**Figure 50**: The Average Centrifugation & Non-Centrifugation Viable hMSC Yield per flask for all four batches (n=4) for both the first (4 Replicates) and second (16 Replicates) experimental passages. Outlier flasks are plotted as well as Standard Deviations which are shown as error bars.
Figure 50 also demonstrates that the process of centrifugation may in fact decrease Viable hMSC Yield, although this pattern lacks significance (p=0.524), and that the increase in Viable Cell Density after centrifugation observed previously was due to the lower volume of culture medium in which the cells were resuspended Post-Centrifugation.

Additionally, the Viable hMSC Yield results from the present study indicate that the Viable Cell Yields in Passage 4 were significantly greater than those in Passage 5 (p=0.036). This finding implies that the growth rate of the hMSCs decreased over two passages regardless of the process steps utilised.

Finally, although the trends in the Viable hMSC Yield data also suggest that the variation in the Non-Centrifugation cell counts may have been greater than that of Pre-Centrifugation and Post-Centrifugation cell counts, no significant difference between the SDs of the Viable hMSC Yields, in the second passage, was observed. Therefore, these results illustrate that no significant difference in the variation in Viable hMSC Yield between process steps, in the second passage, was found.
The Figure above (Figure 52) illustrates the mean Population Doubling Times (PDTs) for Pre-Centrifugation and Non-Centrifugation hMSCs at Day 6 in Passage 4 & 5. Pre-Centrifugation hMSC data was utilised, rather than Post-Centrifugation hMSC data, in order to exclude the effect of the centrifugation process upon the cell count data. From the mean PDTs, it was determined that the PDT of Centrifugation hMSC populations may have been shorter than that of Non-Centrifugation hMSC populations, and therefore that the growth rate may have been greater. Furthermore, this data may suggest that the PDT was more consistent in Centrifugation hMSC populations compared to Non-Centrifugation hMSC populations. However, these trends were not found to be statistically significant, and therefore it can be concluded that no significant difference was observed between process steps. Finally, Figure 52 may also indicate that the growth rate of both Centrifugation and Non-Centrifugation hMSCs decreased over multiple passages.

Mean cumulative population doublings (CPDs) of 3.65 and 3.26 were achieved after culture over two passages utilising the Centrifugation and Non-Centrifugation process steps respectively. Concordantly with the PDT calculations, the Mean CPDs were calculated based upon Pre-Centrifugation and Non-Centrifugation cell counts.
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The PDTs and CPDs for Pre-Centrifugation and Non-Centrifugation hMSC samples were determined using the following formulae:

\[
PDT = \frac{[\text{Time of Final Cell Count (Days)} - \text{Time of Seeding (Days)}]}{[3.32 \times (\log(\text{Final Cell Yield}) - \log(\text{Number of Cells Seeded}))]} \\
CPDs = \frac{[\text{Time of Final Cell Count (Days)} - \text{Time of Seeding (Days)}]}{PDT \text{ (Days)}}
\]

Figure 53, as shown above, summarises the Mean Viabilities for hMSCs after seeding, Pre-Centrifugation, Post-Centrifugation, and Non-Centrifugation, across all four batches, and over two passages. This chart indicates that no significant difference in the Viability of hMSC cultures, and the SDs for these Viabilities, was observed between centrifugation and non-centrifugation cell culture methods.
7.3.9 Paracrine Functionality Assays

After performing a photometric assay of kynurenine concentration, a marker of IDO activity, it was determined that untreated hMSCs did not express IDO, as expected, and no significant difference between the IDO activity of untreated Baseline, Centrifugation or Non-Centrifugation hMSCs was observed. Figure 54 (below) also illustrates that inflammatory pre-treatment significantly increased IDO activity of all hMSCs, which therefore demonstrates that hMSCs cultured utilising either manual or automated process steps expressed IDO in response to IFN-γ stimulation, as is typical of hMSCs (Krampera, 2011). The results of this assay may also indicate a non-significant trend for the kynurenine concentration in pre-treated Centrifugation (p=0.763) and Non-Centrifugation (p=0.273) hMSC conditioned medium to be reduced compared to that of pre-treated Baseline hMSC conditioned medium, which would suggest that the IDO activity of the hMSCs decreased over two passages. The results of this assay may also indicate that the kynurenine concentration in the inflammatory pre-treated Centrifugation hMSC conditioned medium may be greater than that of inflammatory pre-treated Non-Centrifugation hMSC conditioned medium (p=0.943), which would suggest that the IDO activity of pre-treated hMSCs cultured utilising the Centrifugation process step was greater than that of pre-treated hMSCs cultured utilising the Non-Centrifugation process step. However, given the range of data, lack of significance of these trends, and the inconsistency in the measurement accuracy for this particular assay, there is not sufficient evidence to confirm these patterns.

The results of the Prostaglandin E2 ELISA, as shown in Figure 55, illustrates that the conditioned medium of all untreated hMSCs contained little PGE-2, which therefore demonstrates that, similarly to IDO, hMSCs do not constitutively express significant amounts of PGE-2. The data presented in Figure 55 also suggests that the untreated Baseline hMSCs may secrete more PGE-2 than untreated Centrifugation (p=0.625) and Non-Centrifugation (p=0.643) hMSCs. This may indicate that the culture of hMSCs, using either manual or automated process steps, over two passages, may decrease the constitutive secretion of PGE-2. However, this difference between the PGE-2 expression of each of the untreated hMSC conditioned medium samples was not significant.
Figure 54: Graph illustrating the average, DMEM FBS corrected, concentration of Kynurenine in Baseline, Centrifugation and Non-Centrifugation hMSC conditioned medium samples after No Treatment (NT) or Inflammatory Pre-Treatment (IN-PT). Samples were measured in duplicate, the assay repeated, and the average and SD’s were plotted. Asterisks (*) denote significance over untreated samples (p=0.05)
Figure 55: Graph illustrating the average concentration of Prostaglandin E2 (PGE-2) in Baseline, Centrifugation and Non-Centrifugation hMSC conditioned medium samples after No Treatment (NT) or Inflammatory Pre-Treatment (IN-PT). The samples were measured in triplicate, and the averages and SD’s were plotted. Asterisks (*) denote significance over untreated samples (p=0.05) and Number signs (#) denote significance over pre-treated samples (p=0.05).
The results of the PGE-2 ELISA also illustrate that each of the pre-treated hMSC samples exhibited a significantly increased concentration of PGE-2 compared to the untreated hMSC samples, demonstrating that, similarly to IDO, an inflammatory microenvironment is required to stimulate significant PGE-2 expression. The increased secretion of PGE-2 by all pre-treated hMSC groups also indicates that, regardless of the cell culture process steps utilised, all of the cells demonstrated an immunosuppressive response to an inflammatory microenvironment.

From the PGE-2 ELISA data in the present study, it can also be deduced that the inflammatory pre-treatment of the Non-Centrifugation hMSCs induced a significantly greater secretion of the immunomodulatory factor PGE-2 compared to that of Baseline (p=0.00) and Centrifugation (p=0.00) hMSCs. Also, no significant difference between the PGE-2 expression of pre-treated Baseline and Centrifugation hMSCs was observed (p=0.885). These findings contradict those of the IDO assay, and suggest that the culture of hMSCs, over two passages, utilising either Centrifugation or Non-Centrifugation process steps had no effect upon the PGE-2 secretion, and therefore the immuunosuppressive potential, of hMSCs. This data may also indicate that the utilisation of automated process steps may promote an increased production of PGE-2 compared to Baseline or Centrifugation hMSCs.
Average VEGF Concentration in Untreated and Hypoxia Pre-Treated Baseline, Centrifugation & Non-Centrifugation hMSC Conditioned Medium

**Figure 56:** Graph illustrating the average concentration of Vascular Endothelial Growth Factor (VEGF) in Baseline, Centrifugation and Non-Centrifugation hMSC conditioned medium samples after No Treatment (NT) or Hypoxic Pre-Treatment (HY-PT). The samples were measured in triplicate, and the averages and SD’s were plotted. Asterisks (*) denote significance over all samples (p=0.05) and Number signs (#) denote significance over pre-treated samples (p=0.05)
As illustrated in Figure 56 (above), the results of the VEGF ELISA demonstrated that the conditioned medium of all untreated hMSCs contained substantial concentrations of VEGF, and therefore that, in contrast to IDO and PGE-2, hMSCs constitutively expressed VEGF. Additionally, untreated Baseline hMSCs secreted significantly greater concentrations of VEGF compared to untreated Centrifugation (p=0.06), untreated Non-Centrifugation (p=0.02), Hypoxia pre-treated Baseline (p=0.00), Hypoxia pre-treated Centrifugation (p=0.039), and Hypoxia pre-treated Non-Centrifugation (p=0.00) hMSCs. This may suggest that both the manual and automated process steps for the culture of hMSCs, over two passages, significantly decreased the capacity of these cells to secrete VEGF under normal oxygen concentrations (20%), or ‘normoxia’.

These results also indicate that hypoxic pre-conditioning, at 1% O₂, did not significantly increase hMSC VEGF production. No significant difference in VEGF concentration of hMSC conditioned medium samples was observed between untreated Centrifugation and untreated Non-Centrifugation hMSCs compared to all Hypoxia pre-treated hMSCs. However, these results illustrate that Hypoxia pre-treated Centrifugation hMSCs may have produced a greater angiogenic response to hypoxic preconditioning compared to Hypoxia pre-treated Baseline (p=0.09) hMSCs, and produced a significantly greater response compared to Non-Centrifugation hMSCs (p=0.05), by secreting a greater concentrations of VEGF into the culture medium.

7.4 Discussion

One of the preliminary, and most basic, methods of characterising a cell population is through visual examination of a culture under a microscope. Although this method does not give an accurate representation of the identity of the cells and does not give any quantifiable measure of the characteristics of the cells, it can be used as a rudimentary method of verifying that the cells have not differentiated or undergone any substantial transformations.

It is clear from the microscopy images, shown in Figures 22 & 23 that both of the cell culture methods generated cells with a classical spindle-shaped, fibroblast-like hMSC morphology and that no significant difference in morphology, between the cells from the Centrifugation batches and the Non-Centrifugation batches, was observed. This would suggest that neither the centrifugation step in the manual process, nor the trypsin carryover in the automated process, had a substantial effect upon the morphology of the hMSCs.
Furthermore, the cell diameter data demonstrated that no significant difference was observed in the size of hMSCs between process steps and passages. This lack of change in cell diameter contradicts previously reported increases in hMSC diameter over multiple passages (Lo Surdo et al, 2012; Lo Surdo et al, 2013). This consistency in cell diameter demonstrated in the present research provides further evidence to suggest that neither manual nor automated process steps significantly changed the morphology of the hMSCs. Cell size may be an important consideration for hMSC transplantation, and small cells may have a greater migratory capacity through tissues (Jung et al, 2012).

The ISCT minimal criteria for the phenotypic expression of hMSCs indicates that ≥95% of the cell population should express CD105, CD73 and CD90, and that ≤2% of the cell population should express CD45, CD34, CD11b, CD19, or HLA-DR (Dominici et al, 2006). Although this surface marker profile does not uniquely identify hMSC populations, it is generally accepted that all hMSC populations should co-express the appropriate percentage of these antigens.

From the results of the multicolour flow cytometry analysis performed in the present study, it is clear that Baseline (P2), Centrifugation (P5), and Non-Centrifugation (P5) hMSC populations each co-expressed the required hMSC positive markers at the appropriate percentage (≥95%) and lacked co-expression of the required hMSC negative markers and haematopoietic markers at the required percentage (≤2%). Additionally, no significant difference in positive or negative expression was observed between hMSCs cultured utilising alternative process steps, and between early (P2) and late (P5) passage hMSCs, with all populations expressing ≥95% positive marker expression and ≤2% negative marker expression. Lo Surdo and colleagues (2013) have previously reported similar findings, identifying no change in the phenotype of hMSCs from multiple donors over multiple passages.

Therefore, the Loughborough University flow cytometry data indicates that all hMSC populations fulfilled the ISCT basic criteria for immunophenotype and that culture utilising alternative process steps had no influence upon hMSC surface marker expression. Furthermore, although the Viable hMSC Yield data may indicate that the rate of proliferation may decrease in later passages, the flow cytometry analysis demonstrates that no difference in surface marker expression was observed in later passage hMSCs compared to earlier passage hMSCs.
In addition to the ISCT minimal criteria, it has been identified that hMSCs can express a number of surface markers, including CD9, CD29, CD44, CD63, CD99, CD106, CD146 and often lack expression of CD14, CD34, CD45 and CD133 (Nery et al, 2013). However, conflicting data exists regarding the positivity or negativity of a number of CD markers for hMSCs. One of the most debated markers is CD34, which has been identified as a hMSC marker in adult adipose tissue (Zimmerlin et al, 2010).

After LGC performed flow cytometry analysis of hMSCs cultured by replicating the Centrifugation and Non-Centrifugation process steps manually, it was determined that the majority of hMSC positive and negative markers were expressed at the levels defined in the ISCT minimal criteria. However, both CD105 and CD146 expression, both typically hMSC positive markers, was lower than expected, with CD105 expression falling below the 95% expression threshold. Additionally, CD34 was detected in a higher proportion of cells than expected. Examined generic and stress-linked surface markers were also expressed at high levels in hMSCs cultured using both the manually replicated Centrifugation and Non-Centrifugation conditions (>46-60%).

The low expression of CD146 (MCAM) by both Centrifugation and Non-Centrifugation hMSCs may reflect hMSC heterogeneity due individual donor variability. Alternatively, lower CD146 expression may reflect the later passage number of hMSCs used for flow cytometry analysis as this marker has been associated with cell that possess a shorter doubling time (Russell et al, 2013).

As previously described in this section, there is significant uncertainty regarding the CD34 expression of hMSCs. Although CD34 negativity is outlined in the ISCT minimal criteria, more research on these cells has indicated that the lack of CD34 expression may be an in vitro artefact and these cells may in fact be CD34+ in their in vivo niche (Lin et al, 2012). In the present study, a high percentage expression of the hMSC “negative” marker CD34 was observed in both culture conditions.

The LGC flow cytometric analysis of hMSCs, cultured using either the manually replicated Centrifugation or Non-Centrifugation process steps over two passages, revealed a similar level of surface marker expression in both conditions. However, by comparing the results of the Loughborough University and LGC Flow Cytometry analyses, it is apparent that some differences in CD105 and CD34 expression were demonstrated, with lower percentage
expression of CD105 and higher percentage expression of CD34 observed in LGC’s analysis. This may highlight the significance of the utilisation of alternative methods of analysis and inter-individual variation upon the results obtained from flow cytometry. Furthermore, this highlights the need for standardisation and calibration of flow cytometry methodologies and equipment. However, the differences in culture protocols between sites, with LGC manually replicating the automated process steps, may have also influenced this variation in surface marker expression.

Short Tandem Repeat (STR) profiling is a method used to amplify and compare specific loci on the DNA of multiple cell populations, and is often used in forensic analysis (Butler, 2006; Masters et al, 2001). An STR is a unit of multiple nucleotides which are repeated many times in sequence along the length of a DNA strand, and by counting the number of these repeating units at specific loci within the DNA, an individual profile of the sample can be generated. The STR profile analysis used in this study was provided by LGC Standards as part of their Cell Line Authentication (CLA) Service, and this is used to measure the difference in STRs, at each allele of 16 loci within the genome, between samples. These analyses allowed for the determination of the stability of hMSC populations after culture using alternative process steps. The output of each of these analyses is both an Electropherogram (EPG), illustrating the relative expression of STRs at each loci as peaks in fluorescence on the EPG, and a results table, outlining the number of STRs at each allele of each locus.

After the performance of an STR profile for each of the Baseline, Post-Centrifugation, and Non-Centrifugation hMSC samples, the EPGs of the Post-Centrifugation and Non-Centrifugation samples were compared to that of the Baseline sample, created in the initial cell bank, in order to determine whether either the manual or the automated process steps had any significant effect upon the STR profiles of the hMSCs, and therefore their genome or identity. Comparison of the STR profiles of the Post-Centrifugation and Non-Centrifugation hMSC samples against the Baseline samples, as shown in Figures 29, 30 & 31, indicated that there was no significant difference between any of the samples, and that each expressed the same number of STRs at each allele of the same loci. This suggests that neither the automated nor the manual process steps induced an alteration in the loci at which the hMSCs expressed peak numbers of STRs in their DNA.

However, the comparison of the EPGs of the Baseline, Non-Centrifugation, and Post-Centrifugation hMSC samples highlights the existence of possible differences in the level of
STR expression. The EPG charts suggest that both the Post-Centrifugation and Non-Centrifugation samples generated greater peaks in fluorescence at each of the loci where significant STR expression was present, with the Non-Centrifugation sample producing the greatest fluorescence, compared to those of the Baseline hMSC sample. It is unclear as to the cause of the varying levels of expression between samples, and whether this variation represents a significant alteration in the STR profile of the hMSCs. The experimental protocols for this method of measurement would suggest that a greater peak on the EPG represents a stronger fluorescence, and therefore that more copies of the STR exist at each locus. On the other hand, it is also apparent that variations in fluorescence may occur between experimental runs, due to differential amplifications of the alleles or primer-binding site mutations (Clayton et al, 1998), and therefore examining differences in peak sizes may be redundant. However, under LGC Standards guidelines, the variations in peak size between the three hMSC samples provided in this experiment are not considered to represent a significant change in the identity of a cell sample, and this is supported by the lack of difference observed in the number of STRs present at each allele of each locus between samples. Therefore, each of the samples is considered to have identical STR profiles. Although previous investigations have demonstrated the development of genomic mutations in culture Human Umbilical Cord MSCs (Wang et al, 2013), the results of the present study support previous findings regarding the genetic variances in culture expanded Bone Marrow derived hMSCs, with Cai and colleagues (2014) identifying that genetic mutations were very infrequent (0.1-1%), and that these cells exhibit a stable genomic composition, in early passages (P1-8).

When first discovered by Friedenstein and colleagues (1970), MSCs were assigned the name ‘Colony-Forming Unit-Fibroblasts’, due to their capacity to form fibroblastic colonies in vitro. Therefore, the examination of the colony forming potential of these cells represents one of the most fundamental methods of hMSC characterisation. Furthermore, colony formation has been highlighted as an important assay for the determination of quality of hMSC preparations (Pochampally, 2008).

By comparing the Colony Forming Efficiency of Baseline, Centrifugation & Non-Centrifugation hMSCs, it was determined that no difference in the colony forming potential of hMSCs was observed between conditions. Therefore, it can be concluded that neither the manual nor the automated process steps influenced the colony forming potential of hMSCs.
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However, trends for a lower colony forming efficiency in Centrifugation hMSCs, as well as a greater colony forming efficiency in Baseline hMSCs, may exist. These trends would suggest that the centrifugation process had a negative impact upon colony forming potential, and that, in concurrence with previous studies (Izadpanah et al, 2006; Lo Surdo et al, 2012; Lo Surdo et al, 2013), hMSC colony forming potential decreases over time in culture. Furthermore, it was also observed that Baseline hMSCs produced larger colonies than Centrifugation and Non-Centrifugation hMSCs. This would suggest that Baseline hMSCs displayed a higher growth rate than hMSCs cultured using either manual or automated process steps, which is to be expected for cells from an earlier passage (Bonab et al, 2006).

However, it must be acknowledged that substantial conclusions regarding observed trends cannot be drawn due to the manual nature of colony number data collection, the small number of repeats performed (n=2), and the significant inherent heterogeneity between hMSC populations. Nonetheless, the colony forming efficiency values observed in the present experiment are approximately similar to previous studies in which early passage hMSCs, also seeded at 10 cells/cm², were found to have an efficiency of 35% (±10%) (Sekiya et al, 2002).

In addition to their fibroblastic morphology, appropriate surface marker profile, and colony forming potential, MSCs typically have the potential to undergo trilineage differentiation in vitro into adipocytes, chondrocytes and osteocytes (Dominici et al, 2006).

After culture in adipogenic, chondrogenic or osteogenic conditions in the present study, it was determined that Baseline, Centrifugation and Non-Centrifugation hMSCs each exhibited trilineage differentiation capacity. Oil Red O staining was used to examine the differentiation of hMSCs into adipocytes and the formation of lipid droplets. Alcian Blue staining was used to identify differentiation into chondrocytes, the generation of micromasses, and the presence of glycosaminoglycans. Finally, the von Kossa staining method was utilised to identify the differentiation of hMSCs into osteoblasts and the deposition of calcium. It was determined that Baseline, Centrifugation and Non-Centrifugation hMSCs each demonstrated adipogenic, chondrogenic and osteogenic differentiation.

Difficulty exists when drawing conclusions regarding differences in the trilineage differentiation potential of hMSCs from each condition, due to the qualitative nature of the methods utilised. However, after determining the trilineage differentiation potential of
hMSCs from each condition, as well as their plastic adherence, fibroblastic morphology, and appropriate surface marker profile, it can be confirmed that Baseline, Centrifugation and Non-Centrifugation hMSCs each meet the ISCT minimal criteria for defining multipotent MSCs (Dominici et al, 2006). Each of these criteria was determined using the recommended methodology.

With regards the growth of hMSCs cultured utilising automated or manual process steps, it was observed that no significant difference between the Viable Cell Density of cell populations cultured using either process, over two passages and across four batches. However, although no measurable difference in Mean Viable Cell Density was demonstrated, the data may illustrate a trend towards a slightly greater Viable Cell Density when the hMSCs are passaged using the automated, Non-Centrifugation method. However, after determining the Viable hMSC Yield per flask, it is apparent that this increased Viable Cell Density achieved when utilising the Non-Centrifugation process is in fact a result of the lower volume of culture medium in which the Non-Centrifugation hMSCs were suspended (15ml) compared to the Pre-Centrifugation populations (21ml). However, although the yield data does not support the pattern of an increased viable cell density which resulted from the utilisation of the Non-Centrifugation process steps, it does illustrate that no significant difference in the total number of viable cells present Pre-Centrifugation or after the Non-Centrifugation process was observed. The Viable hMSC Yield also demonstrates that, although it may appear that the centrifugation process could have a detrimental effect upon the total number of viable cells obtained resulting in a lower total yield compared to the automated process, this trend lacked significance.

Therefore, from the data presented in the present study it can be concluded that no significant difference between the obtainable Viable Cell Density and Viable Cell Yield of hMSC populations cultured using manual or automated process steps, although a trend for a greater obtainable Viable Cell Density, and therefore Viable Cell Yield, in hMSCs cultured using the automated process may exist. Thus, it can also be concluded that the presence of residual Trypsin carried over during the automated dissociation process step had no detrimental effect upon the number of obtainable cells compared to when this residual dissociation agent was removed using a manual centrifugation process step, commonly utilised in traditional cell culture protocols.
The Viable hMSC Yield data from the present study also demonstrates that the average Viable Cell Yield per flask may have been lower after the second passage compared to Viable Cell Yield after the first passage, regardless of whether the manual or automated process steps were utilised. This indicates that the growth rate of these cells may have decreased over two passages, resulting in a lower Viable Cell Yield, which supports the findings of previous investigations (Thomas et al, 2007). This would suggest that the cells had begun to enter senescence over the duration of their culture over two passages, and thus their growth rate began to decrease. The phenotypic changes associated with senescence have been reported as being present early in the culture of hMSCs (Wagner et al, 2008), however the cause of this phenomenon remains unclear. It has previously been proposed that a purposeful, gene-driven programme triggered by random, accidental events may be responsible for the initiation of senescence (Hayflick, 2007), and the shortening of telomere length and reduction in Telomerase activity have been strongly implicated (O’Hare et al, 2001; Di Donna et al, 2003; Bernardo et al, 2007; Baxter et al, 2004). Although, in the present study, no significant change in the STR profile of the hMSCs over multiple passages was reported, indicating that no significant change in genomic identity occurred in these cells, it remains unclear as to whether significant changes in the gene expression of these cells occurred throughout their culture. However, it can be concluded that the hMSC growth rate decreased in a similar fashion regardless of whether manual or automated process steps were utilised, which is indicative of a similar level of senescence.

During the manual hMSC culture processes in this study, cell counts were performed prior to, and immediately after, the centrifugation step in order to determine the effect upon cell number and viability. By comparing the Viable Cell Densities of hMSC populations Pre-Centrifugation and Post-Centrifugation, the data would suggest that the cell counts were significantly greater in the Post-Centrifugation populations than the Pre-Centrifugation populations, which would suggest that the manual process step increased the number of viable cells in the hMSC populations. It is self-evident that the process of centrifugation cannot increase the number of viable cells, therefore an alternative explanation may be that the centrifugation process disperses larger aggregates of cells that were present prior to centrifugation, and therefore increases the number of single cells or cells grouped in small aggregates, as shown in Figure 49. However, although a reduction in particle size after the centrifugation of mammalian cells has previously been reported (Westoby et al, 2010), this hypothesis is not entirely supported by the hMSC aggregate data in the current experiment, as
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there is a lack of a greater number of larger cell aggregates in the Pre-Centrifugation hMSC populations. Additionally, the live cell aggregate data in the present study indicates that no difference in the size of hMSC aggregates was observed between Centrifugation and Non-Centrifugation process steps. However, the larger standard deviations in live cell aggregate size after the Non-Centrifugation process step may also demonstrate the greater variability in the automated process step, as are discussed later in this section.

Although the hMSC aggregate data does not adequately clarify the mechanism responsible for the increased Viable Cell Density observed after centrifugation, the Viable hMSC Yield data may in fact explain this phenomenon. After determining the Viable hMSC Yield of Pre-Centrifugation, Post-Centrifugation and Non-Centrifugation populations, it is apparent that the number of viable cells does not significantly change and that the patterns in this data may suggest that it does in fact decrease. The likely cause of the increase in Viable Cell Density observed was the difference in the volume of culture medium in which the cells were suspended at the time of the cell count. At the time of the Pre-Centrifugation cell count, the hMSCs are suspended in a 21ml suspension of Trypsin EDTA and culture medium. In contrast, at the time of the Post-Centrifugation and Non-Centrifugation cell counts, the hMSCs have been resuspended in 15ml of culture medium after trypsinisation. This therefore results in a significantly increased Viable Cell Density in the Post-Centrifugation and Non-Centrifugation cell counts, despite little difference, or a decrease, in the total number of viable cells between populations.

Although the differences in the volume of culture medium in which the cells are suspended may explain the increased Viable Cell Density after centrifugation, this does not explain the non-significant trend towards a reduced Viable Cell Yield observed after Centrifugation. It may be that the Relative Centrifugal Force (RCF) used in the present study was sufficiently high to induce cell death and to therefore reduce the Viable Cell Yield, although the lack of a significant change in viability does not support this. Alternatively, it may be that the RCF used was insufficient to create a cell pellet containing all the viable cells and therefore a fraction of the viable hMSCs were aspirated and discarded with the supernatant. Although the RCF and duration of centrifugation utilised in the present study is recommended in industrial cell culture protocols (See Appendix 13.8), it may be that further optimisation is required to determine an optimal RCF and duration of centrifugation for the recovery of a higher proportion of the Viable hMSC Yield. However, this represents a challenging endeavour, and
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it has been previously highlighted that RCFs which are sufficient to achieve high cell yields are often damaging, and that RCFs that yield high viability often produce low yields (Katkov & Mazur, 1999). Therefore, the exploration of alternatives to centrifugation may represent a valuable area of research. Although the centrifugation process step may reduce the obtainable Viable Cell Yield, when the standard deviations of the Pre- and Post-Centrifugation Viable Cell Yields are compared, it appears that it may reduce the variability in the cell count. This may be attributed to the increased number of small aggregates observed after the centrifugation process step which may therefore lead to more reliable cell counts and more consistent seeding of daughter flasks.

However, although no apparent difference in Viable Cell Density or Yield, in each batch over two passages, between hMSCs cultured utilising manual or automated process steps was observed, the results of this experiment suggest that utilisation of the Non-Centrifugation process step may produce greater variability in Viable Cell Densities and Yields, over two passages, compared to the Centrifugation process step when measuring either Pre- or Post-Centrifugation. Previous studies have demonstrated that a fully automated cell culture process is typically more consistent in comparison to a fully manual cell culture process (Liu et al, 2010; Terstegge et al, 2007). This is likely to also be the case with the automated cell culture process used in the present study, in which the CompacT SelecT platform was utilised, however the Viable Cell Density and Yield data may indicate that greater variability exists when utilising the Non-Centrifugation process step compared to the Centrifugation process step within the automated process. Regardless, statistical analysis revealed that this trend was not significant and therefore the possible trend for increased variability when utilising the Non-Centrifugation process step cannot be confirmed in the present study. However, the standard deviations in the live cell aggregate data, illustrated in Figure 48, may also demonstrate the variability observed when the Non-Centrifugation process step was utilised.

It is likely that any variation when utilising the automated process steps can be attributed to a single stage in the CompacT SelecT automated protocol, namely the seeding of daughter flasks. Within this step, a number of sub-processes may be linked to the lack of consistency in Viable Cell Density, and resultant Viable Cell Yield, observed in the automated process. The first relates to the variation in the time taken for the flask carousel to rotate and for the relevant new flask column to be located when selecting a new daughter flask to be seeded. This may lead to variations in the time between mixing steps and in the duration in which the
cells remain in the mother flask, leading to differences in the adherence of cells to the mother flask and therefore a variation in the number of cells transferred into the daughter flask. Another potentially compounding factor in the automated process may be the difference in the length of time the cells remained in the mother flask between the first daughter flask seeded and the fourth daughter flask seeded, when the daughter flasks are split at a 1:4 split ratio. This may also lead to either an increased adherence of cells and a reduced number of cells seeded in the final daughter flask, or an increased settling of cells out of suspension and an increased number of cells seeded into the final flask, therefore causing a variation in the obtainable Viable Cell Density and Viable Cell Yield between flasks. Although these subprocesses are also present within the manual process used in this experiment, the more thorough mixing and resuspension of cells after the centrifugation step, which was performed manually, may counteract the detrimental effects of the variability in time taken to seed daughter flasks by producing a more evenly mixed suspension of cells.

The failure to generate a homogenous single cell suspension in flasks undergoing a passage using the Non-Centrifugation process step is likely to have created differences in the number of cells seeded into new daughter flasks. Also, the inadequate mixing of hMSC suspensions when utilising the Non-Centrifugation process step may have also led to sampling process errors. Without sufficient resuspension of hMSC populations, it is likely that heterogeneity in cell counts existed within suspensions, when multiple samples are taken from within a single suspension, and between suspensions, when a single sample is taken from multiple suspensions, due to differences in cell aggregation, settling and adherence.

Therefore, further optimisation studies may be required to develop a protocol with sufficient mixing steps to generate a single cell suspension and to allow for more consistent hMSC seeding.

After the determination of the Population Doubling Times (PDTs) of hMSCs after 6 days, cultured using either manual or automated process steps, it was identified that the PDT of Centrifugation hMSCs may have been lower than that of Non-Centrifugation hMSCs. Additionally, greater variability in PDT may have been observed in Non-Centrifugation hMSC populations. These trends would suggest that the Non-Centrifugation process step, and the residual dissociation agent carryover, may detrimentally effect upon hMSC PDT. However, these trends were not found to be statistically significant. Therefore, it can be concluded that no significant difference in PDT was observed between hMSCs cultured using
manual or automated process steps, and that the residual dissociation agent carryover had no effect upon hMSC proliferation.

It was also identified that the PDT of hMSCs, regardless of the process step utilised, may have increased over multiple passages. This observation is supported by previous work in which comparable PDTs, as well as similar increases in PDT over multiple passages, were reported (Jin et al, 2013).

The data from the present study also demonstrates that no significant difference between the viability of Pre-Centrifugation, Post-Centrifugation, or Non-Centrifugation hMSC samples over two passages, which would therefore suggest that the enzyme carryover in the Non-Centrifugation process step had no detrimental effect upon cell viability. Although these results indicate that the viability of Non-Centrifugation hMSC samples measured at Seeding and in the first automated passage (P4) may have been lower than Pre- and Post-Centrifugation hMSC samples, this trend was not significant and was not observed in the second passage (P5). From the results of this experiment, it was also determined that no significant difference between Pre-Centrifugation and Post-Centrifugation hMSC viability was observed and therefore that the process of centrifugation had no effect upon hMSC viability. The viability data also illustrates that no significant difference in the variation of hMSC viabilities within each group was observed between Pre-Centrifugation, Post-Centrifugation and Non-Centrifugation hMSC populations over two passages. This variation was approximately 4% (±2%) viability in each group.

However, although most of the average viabilities for the Pre-Seeding, Pre-Centrifugation, Post-Centrifugation; and Non-Centrifugation groups across all batches and passages were similar; most of the patterns present in the hMSC viability data were negligible and non-significant; and the variation across all four batches was similar for each group, small changes in cell viability may be noteworthy and could be detrimental to a cell therapy manufacturing process. Although it does not necessarily determine functionality (Galipeau, 2013), cell viability is often considered a primary measurement for determining the potency of cellular therapies after transplantation, and this must be accurate so that product dose can be determined (Williams et al, 2012). Therefore, depending upon hMSC therapy mode of action and disease target, which determine the tolerance and specifications in place for hMSC viability, the acceptability of a variation in cell viability of approximately 4% within the manual or automated process, as seen in the present study, may differ. However, it must be
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acknowledged that the Cedex automated cell counter utilised in the present study incorporates a maximum intra-sample variability in cell viability of ±3% (Roche, 2003). Furthermore, given that the U.S food and Drug Administration (FDA) have previously recommended a cell viability of greater than 80% for an approved cell therapy product (FDA & Genzyme, 2007), it is likely that the viabilities measured in the present would often be considered acceptable.

The expression of IDO by hMSCs has been associated with the immunosuppressive properties of these cells, and this enzyme has been found to be one of the key bioactive factors responsible for the suppression of T, B, Dendritic and NK cells (Galipeau, 2013; Yagi, 2010; Menard et al, 2013). Waterman and colleagues (2010) discovered that, after Toll-like receptor 3 (TLR3) priming, hMSCs exhibited an immunossuppressive phenotype during which these cells demonstrated an increased IDO expression which acted to suppress T lymphocyte activation. Meisel et al (2004) previously described the mechanism through which the hMSC secreted IDO inhibits T-cell activation, with their findings indicating that the expression of IDO, which converts tryptophan to kynurenine, by hMSCs significantly inhibited allogeneic T-cell responses, that the addition of tryptophan restored T-cell proliferation, and therefore that it is the IDO-mediated catabolism of tryptophan to kynurenine that is responsible for the inhibitory effects of hMSC secreted IDO upon T-cell activity. Also, more recently, Francois and colleagues (2012) determined that tryptophan inhibition abolished hMSC suppression of T cell proliferation, that the level of IDO expression was correlated to hMSC immunosuppressive potential, and that hMSC secreted IDO caused monocytes to differentiate into immunosuppressive macrophages.

However, it is generally accepted that hMSCs must be stimulated by pro-inflammatory cytokines, particularly IFN-γ, in order to upregulate IDO expression (Krampera, 2006; Meisel et al, 2004). The data from the present study, in which kynurenine concentration was measured photometrically, supports this hypothesis and it was determined that untreated hMSCs do not constitutively express IDO and that pre-treatment with inflammatory cytokines, specifically IFN-γ and TNF-α, was required to stimulate the expression of IDO by hMSCs. As untreated hMSCs did not express IDO, and therefore did not induce significant breakdown of tryptophan or the resultant increase in kynurenine concentration, no significant difference between untreated Baseline, Centrifugation or Non-Centrifugation hMSCs was observed.
As previously described in section 7.3.9, all pre-treated hMSC samples exhibited significantly increased kynurenine concentrations, and therefore increased IDO activity, compared to untreated hMSC samples. The IDO activity observed in all inflammatory pre-treated hMSC conditioned medium samples was sufficient to be of clinical relevance, with Tattevin and colleagues (2010) previously reporting that a kynurenine concentration of ≤20μM was correlated with an increased anti-inflammatory response in sepsis patients. Therefore, as the results of the present study indicate that each of the inflammatory pre-treated hMSC conditioned medium samples exhibited a kynurenine concentration of >20μM, it is clear that each of the inflammatory pre-treated hMSC populations produced a clinically relevant immunosuppressive effect.

However, the results of the present study may highlight a number of potential differences in the IDO activity of hMSCs from the initial cell bank or after culture utilising either automated or manual process steps. The data may illustrate the existence of a trend for increased kynurenine concentration in inflammatory pre-treated Baseline hMSCs compared to both the Centrifugation and Non-Centrifugation hMSCs. This may indicate that over two passages the IDO activity of the hMSCs, in response to inflammatory pre-treatment, decreased, which would suggest that these cells, regardless of the process steps used, decreased their expression of this particular immunosuppressive factor. However, due to the lack of significance of these findings and the inconsistency in the measurement accuracy for the IDO assay, this trend cannot be confirmed.

The results of the IDO assay may also demonstrate a small non-significant difference in IDO activity between hMSCs cultured utilising either manual or automated cell culture process steps. The trends in the data may suggest that the utilisation of the Centrifugation process results in an increased kynurenine concentration in the hMSC conditioned medium compared to the conditioned medium of hMSCs cultured utilising the Non-Centrifugation process. From this pattern it could be deduced that the fully automated culture process, which lacks a centrifugation step, may be detrimental to the IDO activity of hMSCs and therefore may influence their immunosuppressive effects. However, once again, due to the lack of measurement accuracy, small magnitude of difference, and lack of significance observed, these trends cannot be confirmed and the conclusions that can be drawn are limited.

Therefore, from the IDO assay performed in the present study, it can be concluded that untreated hMSCs do not constitutively express IDO and that inflammatory pre-treatment is
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required in order to induce IDO secretion. It can also be proposed that the culture process, regardless of the process step utilised, may have a detrimental impact upon hMSC IDO expression after inflammatory pre-treatment, and that the automated Non-Centrifugation process step may have a slightly greater detrimental impact upon IDO activity compared to the manual Centrifugation process step. Given the correlation between IDO production and hMSC immunosuppressive potential (Francois et al, 2012), any effect of the culture process upon hMSC IDO expression may significantly impact their clinical efficacy, which would represent a major drawback for the manufacture of hMSC-based therapies. However, due to the inconsistency of the measurement accuracy for the IDO assay, the small differences observed and the lack of significance, there is insufficient evidence to fully support the trends observed in this study.

In addition to the variation within the IDO assay, it must also be recognised that the innate variability between hMSC populations may have been likely to influence the results of this assay. For example, variability between donors, including age and gender, has been found to significantly influence hMSC gene expression and immune-suppressive function (Mindaye et al, 2013; Siegel et al, 2013). It has also been observed that the expansion of hMSCs in monolayer culture can lead to alterations in gene expression, therefore creating differences in gene expression profiles between populations (Izadpanah et al, 2008; Lee et al, 2013). Therefore, the heterogeneous nature of these cells may have contributed to the non-significant variability in IDO activity, between hMSCs cultured utilising each of the alternative process steps, observed in the present study.

Further investigation, utilising a more accurate measurement system with more samples over a greater number of passages, would be required in order to draw more accurate conclusions regarding the IDO expression of pre-treated Baseline, Centrifugation and Non-Centrifugation hMSCs. Nevertheless, the results of the IDO assay in the present study demonstrate the IDO expression capacity, and thus the immunosuppressive properties, of hMSCs cultured utilising alternative process steps as well as highlighting the possible detrimental effects of culture upon hMSC IDO activity.

The PGE-2 mediated immunosuppression of hMSCs was first observed in a mouse sepsis model in which bone marrow derived stromal cells were administered (Nemeth et al, 2008). It has since been determined that pro-inflammatory mediators activate sensors on the MSC membrane causing upregulation of the expression of COX2, and other arachidonic acid
pathway components, by the MSCs, which in turn increases their PGE-2 secretion (Prockop, 2013). This PGE-2 negative feedback loop, for which intimate association or cell-to-cell contact may be required, stimulates the transition of resident macrophages from their classical pro-inflammatory phenotype towards an anti-inflammatory phenotype, in which the cells secrete anti-inflammatory mediators such as IL-10 and IL-1 receptor agonist (Prockop, 2013). PGE-2 has also been found to suppress Dendritic Cell maturation, T-lymphocyte activation, T-lymphocyte proliferation, and T-lymphocyte cytokine secretion (Yanez et al, 2010; Najar et al, 2010). Additionally, blocking the synthesis or activity of PGE-2, using either indomethacin or PGE-2 blocking antibody, has also been found to prevent the immunosuppressive effects of MSCs and allow lymphocyte proliferation to be restored (Prasanna et al, 2010; Solchaga & Zale, 2012).

The concentration at which PGE-2 is secreted by hMSCs may be critical in the magnitude of immunosuppressive response. Solchaga & Zale (2012) discovered that PGE-2 accurately correlated with the immunosuppressive capacity of hMSCs, and thus that increased PGE-2 expression resulted in a greater immunosuppressive effect. In this investigation (Solchaga & Zale, 2012), alternative trophic factors, including TGF-β, IL-10, VEGF & HGF, were also screened, however none of these were found to adequately correlate with hMSC mediated immunosuppression. Therefore, any differences in the concentration of PGE-2 secreted by Baseline, Centrifugation, or Non-Centrifugation hMSCs in response to pro-inflammatory cytokines in the present study may be significant and may represent a substantial change in the immunosuppressive function of these cells.

In previous experiments, Najar et al (2010) reported that hMSCs derived from bone marrow, Wharton’s jelly, and adipose tissue constitutively produced PGE-2. The results of the present study suggest that although Baseline hMSCs may constitutively secrete PGE-2, the concentration at which the PGE-2 was produced was very low in comparison to inflammatory pre-treated cells. The data also indicates that the constitutive expression of PGE-2 in untreated cells may decrease with culture over two passages, as the concentration of PGE-2 in the conditioned medium of untreated Centrifugation and Non-Centrifugation hMSCs may have been lower than in that of the untreated Baseline hMSCs, although this was not a significant trend.

Previous studies have revealed that the stimulation of hMSCs with certain factors increased their PGE-2 secretion, similarly to what has been observed with IDO. Waterman and
colleagues (2010) determined that Toll-like Receptor 4 (TLR4) priming polarised hMSCs to a pro-inflammatory phenotype and TLR3 caused hMSCs to exhibit an anti-inflammatory phenotype, in which they secreted immunosuppressive mediators such as PGE-2 and IDO. TNF-α has also been identified as a pro-inflammatory cytokine which increases the expression of PGE-2 by hMSCs (Solchaga & Zale, 2012).

The data from the present study supports the findings from previous studies and indicate that the pre-treatment of hMSCs, with the pro-inflammatory cytokines TNF-α and IFN-γ, induced a significant increase in the secretion of PGE-2 regardless of the culture process steps utilised. The concentration of PGE-2 secreted into the culture medium by each of the pre-treated hMSC samples was substantial, and therefore all of the hMSC groups displayed an immunosuppressive response to an inflammatory microenvironment. Therefore, it can be concluded that inflammatory pre-treatment is required to stimulate significant hMSC PGE-2 secretion. Previous experiments (Bouffi et al, 2010; Zafranskaya et al, 2013) have reported a similar magnitude of PGE-2 secretion in mice and human MSCs after alternative pre-treatment and co-culture, however Zafranskaya and colleagues (2013) observed that a tenfold greater PGE-2 concentration than was observed in the present study (10,000pg/ml) was required to induce a significant suppression of T cell proliferation in MS patients.

The PGE-2 concentration data from the present study also illustrates that the inflammatory pre-treated Non-Centrifugation hMSCs produced significantly greater levels compared to pre-treated Baseline or Centrifugation hMSCs. It is also apparent that no significant difference between the PGE-2 concentration in the pre-treated Baseline hMSC conditioned medium and the pre-treated Centrifugation hMSC conditioned medium was observed.

Therefore, from the results regarding the hMSC PGE-2 expression it can be concluded that pre-treatment with pro-inflammatory cytokines was required to induce significant PGE-2 secretion in all hMSC groups, although culture over two passages may decrease constitutive PGE-2 expression. It can also be deduced that, contrary to the IDO assay results, culture over two passages using either the manual or the automated culture process steps did not decrease the hMSC immunosuppressive response to pro-inflammatory cytokines, and that the utilisation of the Non-Centrifugation process steps may in fact increase the secretion of PGE-2 in response to an inflammatory microenvironment. It can be hypothesised that this increased PGE-2 concentration in the conditioned medium of pre-treated Non-Centrifugation hMSCs was due to an increase in the immunosuppressive capacity of these cells, or perhaps
due to the more pro-inflammatory conditions in the culture when utilising the Non-Centrifugation process steps, resulting in a greater anti-inflammatory response. Further investigation would be required to identify the cause of the effects of the Non-Centrifugation process steps upon the PGE-2 secretion of inflammatory pre-treated hMSCs.

It has previously been demonstrated, both in vitro and in vivo, that hMSCs have the capacity to stimulate new blood vessel formation, and it has therefore become widely recognised that these cells have a pro-angiogenic function (Phinney & Prockop, 2007; Keating, 2012; Caplan & Dennis, 2006; da Silva Meirelles et al, 2009; Rastegar et al, 2011). As discussed earlier in section 7.1, Vascular Endothelial Growth Factor (VEGF) has been strongly linked, by a number of authors in a variety of studies, to the angiogenic potential of hMSCs (Caplan, 2008; Caplan & Correa, 2011; Fernandez-Vallone et al, 2013; Pourrajab, Forouzannia & Tabatabaee, 2011). Additionally, it has been proposed that this growth factor plays a critical role in hMSC pro-angiogenic function (Rehman et al, 2004; Hu et al, 2008; Li et al, 2007; Tögel et al, 2007; Chacko et al, 2010; Al-Khaldi et al, 2003).

A significant body of research regarding the preconditioning of hMSCs in low oxygen concentrations has also been generated. From this research, it has been identified that, although hMSCs have been found to constitutively express VEGF in normal oxygen concentrations, exposing these cells to a low partial pressure of oxygen, or hypoxia, stimulates the increased expression of VEGF (Liu et al, 2008; Rehman et al, 2004; Hu et al, 2008; Li et al, 2007; Tögel et al, 2007; Chacko et al, 2010; Linero & Chaparro, 2014). Therefore, it has been concluded that by pre-treating hMSCs in hypoxic conditions the expression of VEGF by these cells will be increased, which will in turn improve their pro-angiogenic function. A number of investigators have demonstrated the increased angiogenic potential of hypoxia preconditioned hMSCs and have found that these pre-treated cells increase endothelial cell growth in vitro (Rehman et al, 2004; Tögel et al, 2007) and improve perfusion in vivo in hind limb ischaemia models in rodents (Rehman et al, 2004; Hu et al, 2008).

In the present study, the results of the VEGF ELISA concur with the literature and demonstrate that untreated hMSCs constitutively express VEGF. However, it was also observed that both Centrifugation and Non-Centrifugation hMSCs secreted significantly lower concentrations of VEGF compared to Baseline hMSCs. This may suggest that, regardless of the process step utilised, the culture of these cells over two passages decreased
their VEGF expression under normoxic conditions. However, it has previously been demonstrated that both rat and human MSCs maintain, or even increase, their VEGF secretion over multiple passages (Al-Khaldi et al, 2003; Kagiwada et al, 2008). Additionally, this same pattern was not observed when the hMSCs were cultured under hypoxic conditions.

Contrary to the majority of the available literature, the results of the present study indicated that hypoxic preconditioning did not significantly increase the concentration of VEGF secreted into the culture medium by Baseline, Centrifugation or Non-Centrifugation hMSC populations in comparison to the untreated hMSCs. Although substantial evidence supporting increased VEGF expression by hypoxic preconditioned hMSCs exists, a failure to demonstrate this pattern has been previously reported in hMSCs cultured as a monolayer at 1% and 5% O₂ (Potapova et al, 2007; Paquet et al, 2015).

However, it was observed that hypoxia pre-treated Centrifugation hMSCs secreted the greatest concentration of VEGF into the culture medium in response to the low oxygen concentration and therefore produced the greatest angiogenic response to hypoxia. This finding may indicate that the centrifugation process step has the capacity to increase the expression of VEGF by hMSCs. However, although hypoxic preconditioning may facilitate this increase cooperatively with the Centrifugation process, as this finding was not observed in the untreated Centrifugation hMSC samples, this hypothesis may be flawed.

Also, as discussed previously in this section with regards the results of the IDO assay, it must be acknowledged that, despite the differences in VEGF and PGE-2 expression between hMSCs cultured using each of the process steps reported in the present study, the heterogeneous nature of hMSC populations, often observed due to donor heterogeneity and inconsistency in the manufacturing process (Bloom et al, 2015), may significantly influence these results. This heterogeneity of hMSC cultures may have also contributed to the lack of increased VEGF secretion after hypoxic preconditioning observed in all samples, which is commonly reported in the literature.

To summarise the results of the multiple hMSC paracrine functionality assays, it is clear that untreated hMSCs do not constitutively exhibit significant IDO and PGE-2 expression, and that pro-inflammatory cytokine pre-treatment is required to stimulate significant secretion of these immunomodulatory factors. It is also apparent that no significant difference in the concentration of IDO and PGE-2 in the conditioned medium of inflammatory pre-treated
hMSCs from the initial cell bank and of those cultured using each of the alternative process steps was observed, with one exception. In the case of the inflammatory pre-treated Non-Centrifugation hMSCs, significantly greater levels of PGE-2 were observed in the conditioned medium, which may suggest that this process step may encourage or facilitate the increased secretion of PGE-2 by hMSCs in response to inflammatory pre-treatment. With regards the expression of VEGF, it was demonstrated that untreated hMSCs constitutively secrete significant concentrations of VEGF. However, the results of the VEGF ELISA illustrate that the hypoxic pre-treatment utilised in the present study did not significantly increase hMSC VEGF expression, and that untreated Baseline hMSCs and hypoxia pre-treated Centrifugation hMSCs secreted significantly greater levels of VEGF, with untreated Baseline hMSCs expressing the greatest concentration. These hMSC VEGF results contradict the majority of the available literature in which the effect of hypoxic pre-treatment upon hMSC VEGF expression has been investigated, although a previous study has also failed to demonstrate any difference between untreated and hypoxia pre-treated hMSC VEGF expression (Potapova et al, 2007).

Finally, with regards the analytical techniques used to measure the paracrine marker expression of each of the hMSC populations, although expression was not measured on a per cell basis, each of the hMSC populations were derived from the same donor, each of the populations were in culture for the same duration, and comparable growth rates between the manual and automated processes were previously demonstrated. In the Viable Cell Yield data presented from the present study, a trend for a decrease in hMSC yield after multiple passages may have been observed, which suggests that differences in growth rate may have been observed between early and late passage hMSCs, However, this trend was not found to be significant and therefore it cannot be proposed that significant differences in cell numbers was observed between each of the hMSC populations analysed for their paracrine marker expression.

It must also be stressed that the inherent heterogeneity of hMSCs is likely to result in variability in protein expression between populations and therefore may have significantly influenced the results of the paracrine assays observed in the present study. Furthermore, measurement accuracy may significantly impact the results of the functional assays performed in the present study. Recently, up to 10% variation in the results of ELISA based assays for Multipotent Adult Progenitor Cell (MAPC) cytokine secretion between operators,
days, or both was reported (Porat et al, 2015). These ELISA based assays were considered to be consistent despite the 10% variability observed between different operators and days.

The comparability between Baseline hMSCs, and those cultured using Centrifugation and Non-Centrifugation process steps is summarised in Figure 57 (below), in which the results for each of the parameters measured are outlined.
<table>
<thead>
<tr>
<th>hMSC Condition</th>
<th>Parameter</th>
<th>Baseline (P2)</th>
<th>Pre-Centrifugation (P5)</th>
<th>Post-Centrifugation (P5)</th>
<th>Non-Centrifugation (P5)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Morphology: Spindle shaped, fibroblast-like</td>
<td>Cell Diameter: 19.51μm (±0.68μm)</td>
<td>Cell Diameter: 19.84μm (±0.66μm)</td>
<td>Cell Diameter: 20.13μm (±0.75μm)</td>
<td>Cell Diameter: 20.13μm (±0.75μm)</td>
</tr>
<tr>
<td></td>
<td>Viable Cell Yield: -</td>
<td>Viable Cell Yield: 2.2x10^6 (±0.54x10^6)</td>
<td>Viable Cell Yield: 2.06x10^6 (±0.25x10^6)</td>
<td>Viable Cell Yield: 2.2x10^6 (±0.66x10^6)</td>
<td>Viable Cell Yield: 2.2x10^6 (±0.66x10^6)</td>
</tr>
<tr>
<td></td>
<td>Population Doubling Time: 3.68 Days (±0.79 Days)</td>
<td>Population Doubling Time: 3.94 Days (±0.48 Days)</td>
<td>Population Doubling Time: 3.96 Days (±1.28 Days)</td>
<td>Population Doubling Time: 3.96 Days (±1.28 Days)</td>
<td>Population Doubling Time: 3.96 Days (±1.28 Days)</td>
</tr>
<tr>
<td></td>
<td>Viability: 97.6% (2.18%)</td>
<td>Viability: 97.95% (±1.78%)</td>
<td>Viability: 97.8% (±1.7%)</td>
<td>Viability: 97.8% (±1.7%)</td>
<td>Viability: 97.8% (±1.7%)</td>
</tr>
<tr>
<td></td>
<td>STR Profile: NSD</td>
<td>STR Profile: NSD</td>
<td>STR Profile: NSD</td>
<td>STR Profile: NSD</td>
<td>STR Profile: NSD</td>
</tr>
<tr>
<td></td>
<td>Colony Forming Unit Potential: 20.6% (±2.5%)</td>
<td>Colony Forming Unit Potential: 13.4% (±0.8%)</td>
<td>Colony Forming Unit Potential: 18.2% (±3.1%)</td>
<td>Colony Forming Unit Potential: 18.2% (±3.1%)</td>
<td>Colony Forming Unit Potential: 18.2% (±3.1%)</td>
</tr>
<tr>
<td></td>
<td>IDO Activity: 48.01μM (±24.61μM) (NSD)</td>
<td>IDO Activity: 32.17μM (±4.23μM) (NSD)</td>
<td>IDO Activity: 21.54μM (±2.36μM) (NSD)</td>
<td>IDO Activity: 21.54μM (±2.36μM) (NSD)</td>
<td>IDO Activity: 21.54μM (±2.36μM) (NSD)</td>
</tr>
<tr>
<td></td>
<td>PGE-2 Secretion: 1129.49 pg/ml (±117.56 pg/ml) (NSD)</td>
<td>PGE-2 Secretion: 1190.85 pg/ml (±50.99 pg/ml) (NSD)</td>
<td>PGE-2 Secretion: 1710.60 pg/ml (±93.94 pg/ml) (SD)</td>
<td>PGE-2 Secretion: 1710.60 pg/ml (±93.94 pg/ml) (SD)</td>
<td>PGE-2 Secretion: 1710.60 pg/ml (±93.94 pg/ml) (SD)</td>
</tr>
<tr>
<td></td>
<td>VEGF Secretion: 621.39 pg/ml (±54.5 pg/ml) (NSD)</td>
<td>VEGF Secretion: 894.40 pg/ml (±164.14 pg/ml) (SD)</td>
<td>VEGF Secretion: 589.11 pg/ml (±24.33 pg/ml) (NSD)</td>
<td>VEGF Secretion: 589.11 pg/ml (±24.33 pg/ml) (NSD)</td>
<td>VEGF Secretion: 589.11 pg/ml (±24.33 pg/ml) (NSD)</td>
</tr>
</tbody>
</table>

**Figure 57:** Summary table of parameters used to examine comparability between Baseline (P2), Pre-Centrifugation (P5), Post-Centrifugation (P5) and Non-Centrifugation (P5) hMSCs. Average values are presented and ± denotes Standard Deviations. IDO activity and PGE-2 secretion values represent data taken after inflammatory pre-treatment. VEGF secretion values represent data taken after hypoxic pre-treatment. NSD= No Significant Difference. SD= Significant Difference
Figure 58 (below) illustrates the proportional means for each parameter measured, which allows for the consistency of Centrifugation and Non-Centrifugation hMSC measurements to be examined for each parameter. Furthermore, the proportional standard deviations are shown, which facilitates further comparison of the consistency of Centrifugation and Non-Centrifugation measurements by comparing the variability within each parameter. The comparison of proportional standard deviations also allows for the variability between parameters to be compared. This allows process and product control to be identified.

However, it must be acknowledged that the standard deviations for Centrifugation and Non-Centrifugation hMSC IDO Activity, PGE-2 Secretion, and VEGF Secretion are not representative of biological replicates, between populations, but rather represent technical replicates within populations. Nonetheless, this data does not incorporate inter-passage or inter-batch variability.

From the data presented in Figure 58, it can be determined that the Cell Diameter, Viability, and PGE-2 Secretion parameters were well controlled. However, it was observed that greater variability was present in the Viable Cell Yield, Population Doubling Time, and IDO Activity data.
The normalised means and standard deviations data also indicate that the Non-Centrifugation hMSC IDO Activity and VEGF Secretion data was less variable than the Centrifugation hMSC data. Additionally, the Viable Cell Yield, Population Doubling Time, and Colony Forming Unit Potential data was found to be less variable in Centrifugation hMSC measurements compared to Non-Centrifugation hMSC measurements.

However, it should also be noted that, in the case of the Viable Cell Yield and Population Doubling Time data, the standard deviation of Centrifugation hMSCs fell within that of Non-Centrifugation hMSCs. Furthermore, the standard deviations of the Centrifugation and Non-Centrifugation hMSC Cell Diameter and Viability data were found to overlap, indicating that the variability between process steps was not significant.

However, the Colony Forming Unit Potential, IDO Activity, PGE-2 Secretion, and VEGF Secretion standard deviations for Centrifugation and Non-Centrifugation were not found to overlap, which would indicate that the functionality of hMSCs was not comparable between cells cultured utilising either manual or automated process steps.

7.5 Conclusions
In the present chapter, quantitative experiments have been performed in order to determine the comparability between a manual and an automated process step for the culture of hMSCs. These experiments allow for process experience to be gained, for the variation between alternative process steps to be compared, and for process comparability to be examined after a change to a single manufacturing process step, which therefore contributes to the second challenge addressed in this thesis regarding comparability in cell-based therapies.

The results of these experiments demonstrate that hMSCs, cultured using both manual and automated process steps, displayed comparable morphology, stability, yield, viability, and characterisation, with all hMSCs meeting the ISCT minimal criteria (Dominici et al, 2006). However, a trend for greater variability in hMSC yield when the automated, Non-Centrifugation process step was utilised may exist. Furthermore, by utilising surrogate potency assays for a number of putative hMSC modes of action, it was determined that the functionality of hMSCs cultured using alternative process steps was not comparable. This highlights the challenge of achieving comparability in hMSC potency after a change in the manufacturing process.
Future research may include in-process measures of cell growth and characterisation, as well as further examination of hMSC functionality, due to the inherent variability within the ELISA based assays utilised in the present research. As discussed in section 11.8.2, further experiments are also required to compare a fully manual to a fully automated manufacturing process, and to determine the specification limits of a fully automated manufacturing process.
Chapter 8: Comparison of a Manual, Centrifugation & an Automated, Non-Centrifugation Cell Culture Process Step for a ‘Ruler’ Human ESC line

In collaboration with TAP Biosystems (Royston, UK)

The aim of the present chapter is to examine the comparability between manual and automated process steps for the culture of a ‘Ruler’ hESC line, through the measurement of a number of process and product specifications which are representative of cell growth, stability, and characterisation.

8.1 Introduction

Human Embryonic Stem Cells (hESCs), which were first derived by Thomson and colleagues in 1998 (Thomson et al, 1998), are self-renewing and pluripotent cells which represent a potential unlimited source of cells for cell-based therapies, as well as a model for early human development.

However, manual culture protocols and inter-operator variability often lead to genomic instability in hESC cultures (Terstegge et al, 2007). Automated cell culture platforms allow for the control of cell density, fluid flow, centrifugal force, pH and temperature utilised during the dissociation and passage processes, to which hESCs are particularly sensitive, and variations in these parameters have been found to cause shifts in hESC phenotype and spontaneous differentiation (Veraitch et al, 2008). However, the development of automated hESC culture processes faces a number of challenges, including the sensitivity of these cells, the high risk of contamination, the need for visual examination of cultures, and the often complex culture and differentiation protocols required (Terstegge et al, 2007).

The enzymatic dissociation and centrifugation process steps often differ between manual and automated hESC culture processes. Previous studies have examined the effects of manual processing steps, in particular the centrifugation step, upon murine ESC culture (Veraitch et al, 2008; Wong, 2009). Thomas and colleagues (2008) have also compared the effects of manual and automated protocols upon hESC culture. However, the effect of alternative methods used in these two key stages upon the growth and quality of hESCs has yet to be examined. Therefore, the present study aims to determine the effect of centrifugation and non-centrifugation process steps upon the pluripotency marker expression, STR profile, growth and viability of a hES cell line.
The hESC line that is to be cultured in this experiment is known as the Embryonal Carcinoma (EC) 2102Ep cell line which was originally derived from a surgical specimen of a primary testicular germ-cell tumour containing EC and yolk sac elements (Andrews et al, 1982; Wang et al, 1980). As previously described in section 2.5.4, this cell line displays a similar antigen expression profile to that of traditional hESCs, and this phenotype can be maintained for over 10 passages (Josephson et al, 2007). Additionally, these cells have been successfully cultured as a monolayer in Dulbecco’s Modified Eagle Medium, with 10% Foetal Bovine Serum, 4mM Glutamine and without feeder cells (Andrews et al, 1982; Josephson et al, 2007).

Therefore, the lack of spontaneous differentiation and the relative ease of culture of these cells, in comparison to other hESC lines, may allow this cell line to be used as a tool for the development and optimisation of an automated process. As discussed in section 2.5.4, these characteristics have led to the proposal of the EC 2102Ep cell line as a ‘Reference Standard’, or ‘Ruler’ for hESC research. When compared to alternative candidate hESC Ruler lines, the EC 2102Ep cell line has been found to express more of the microRNAs associated with classical hESCs, as well as fewer markers of differentiated fates, and has therefore been proposed as the most promising candidate cell line (Josephson et al, 2007).

Hourd and colleagues (2014) highlighted that the identification of a well-characterised reference cell line is fundamental to establishing methods of demonstrating comparability, and that linking the use of this cell line to automated processing systems could provide a straightforward method for the comparison of manufacturing processes at multiple sites or between processing equipment. In a recent study, the generation of hMSC reference material has been explored, and a source of this standard cell line has been proposed (Tanavde et al, 2015). With regards hESCs, comparisons of numerous cell lines have been impeded by variations in culture conditions, feeder layers and rate of spontaneous differentiation. However, the selection of a reference hESC line may overcome these difficulties, enabling the development of standardised assays, as well as the comparability between cell lines, protocols and assays.

Therefore, if, in the present study, the EC 2102Ep cell line can be successfully cultured on an automated platform with no significant change in a number of key attributes, this would confirm that an automated process is a viable and equivalent option for the culture of hES cells. Also, the scalable culture of a reference cell line would be necessary in order to provide sufficient cells for its global dissemination, and therefore, an automated process would be of
great value, if it can be demonstrated that the characteristics of these cells are maintained. Furthermore, this chapter will measure and establish the comparability between Centrifugation and Non-Centrifugation process steps, through the examination of a number of parameters. The null hypothesis for this study was that no significant difference in cell yield, viability, pluripotency marker expression, and STR profile would be observed for the Ruler hESCs cultured utilising either manual or automated process steps. This hypothesis assumes that further process optimisation is not required after the performance of a process and protocol transfer to allow for the automated culture of EC 2102Ep cells using Centrifugation and Non-Centrifugation process steps, which is described further in this section and in section 8.2.2.

Although the manual culture of the EC 2102Ep cell line has been successfully performed in a number of studies (Andrews et al, 1982; Wang et al, 1980; Sperger et al; 2003; Josephson et al; 2007), these cells have not previously been cultured utilising an automated platform such as the CompacT Select, and therefore the translation of the manual cell culture process into an automated protocol is yet to be undertaken. The generation of an automated protocol requires the adaptation of a number of critical processes, in particular, the centrifugation step that is frequently utilised during manual passage must be removed due to the lack of an incorporated centrifuge within the CompacT Select. Thus, these process and protocol transfer steps represent a significant and critical stage in the completion of this experiment, involving the examination of a number of process steps. The decisions involved in this protocol transfer stage are described in greater detail later in section 8.2.2.

8.2 Methods

In this section, the specific materials and methods utilised for the Ruler hESC experiments, which are presented in this chapter, are outlined in greater detail than those described in the Materials & Methods Chapter (Chapter 6).

8.2.1 Ruler EC 2102Ep Culture- Initial expansion

The protocol provided by GlobalStem® for the culture and passage of the Human Embryonal Carcinoma 2102Ep cell line was originally designed to be compatible with the use of tissue culture plastic with a surface area of 75cm² (T75 flask) or smaller. This was apparent as each vial provided contained 5x10⁶ cells, which is the minimum number of cells required to fulfil the minimum plating density (6.7x10⁴ cells/cm²) for a T75 flask. Therefore, significant expansion (3 passages), and banking of these cells at higher densities per vial than originally
supplied, was required. These cells were expanded manually, in order to generate enough cells for both a protocol transfer onto the CompacT SelecT automated cell culture platform and the experimental work to be performed on this cell line. The chosen number of cells per vial when banking (2x10^7 cells/vial) was selected to allow for the presence of excess cells when seeding a T175 flask in the CompacT SelecT using a protocol which seeds each flask with the specified number of cells/cm^2 (6.7x10^4 cells/cm^2 or 1.1725x10^7 cells/T175 flask).

- 2 vials of GlobalStem EC 2102Ep cells (P48) were removed from cryostorage, thawed and each seeded manually onto a BD Falcon™ T75 flask (BD Biosciences) at a density of 6.7x10^4 cells/cm^2 in DMEM HG GlutaMax™ with 10% FBS.
- A medium exchange was performed every 2 days after seeding or passage.
- Once confluent, after 3 days, the seeded flasks were each passaged manually into a daughter BD Falcon™ T175 flask at a density of 6.7x10^4 cells/cm^2.
  - Cells were washed and dissociated using PBS and Trypsin EDTA (0.25%) (Life Technologies) respectively. Trypsinised cells were incubated for 7 minutes, or until it was visually confirmed that the cells had detached, at 37˚C and 5% CO2.
- Once confluent, the daughter flasks were each passaged into 4 granddaughter T175 flasks at the required cell density (2→8 new flasks).
  - A 1:4 split ratio was utilised due to the difficulty associated with obtaining an accurate cell count, as a result of the inability to disperse clumps of cells to create a single cell suspension.
- Once the 8 granddaughter flasks had reached confluency, the cells were again passaged at a 1:4 split ratio into 32 great-granddaughter flasks (8→32 new flasks).
- Once the 32 great-granddaughter flasks reached confluency, the cells were prepared for cryopreservation.
  - EC 2102Ep cells were dissociated as described earlier in this section, were neutralised using DMEM HG GlutaMAX with 10% FBS, and were pooled into a single T175 flask.
  - The cell suspension was then split into 8x50ml centrifuge tubes which were then centrifuged at 270 RCF for 5 minutes in order to isolate a cell pellet. Next, these cell pellets were resuspended in freeze medium (90% FBS with 10% Dimethyl Sulfoxide) and 1ml of the suspension was transferred into each cryovial. Cryovials were transferred to ‘Mr Frosty’ container and stored at -80˚C for 24 hours before being transferred to cryostorage.
33 cryovials of EC 2102Ep cells (P52) were isolated in total, of which 16 cryovials made up the Experimental Bank and 17 vials made up the Optimisation Bank.

8.2.2 Ruler EC 2102Ep Manual to Automated Process and Protocol Transfer

During the EC 2102Ep manual cell banking process, particularly during the passage of these cells, it was observed that the recommended enzymatic dissociation step was not adequate to dissociate all of the cells which had adhered to the culture flask. In the passage protocol provided by GlobalStem®, it is proposed that 1-2 minutes of incubation with 0.25% Trypsin EDTA is sufficient to detach all of the cells from the culture plastic. However, after visual examination it became clear that this process step was not adequate and that longer incubation, or utilisation of alternative dissociation agent, was required. Therefore, after the utilisation of longer incubation durations, it was discovered that, in combination with vigorous agitation, 6-7 minutes of incubation was more favourable for detaching the majority of the adhered cell population.

However, although increasing the incubation period improved the detachment of cells, this method did not disperse the clumps of cells that were present after detachment to any greater extent than a shorter incubation period. This issue with cell aggregation also persisted despite increasing the number of mixing steps performed. Cell aggregation after the dissociation and mixing steps is detrimental when performing a cell count, as the Cedex automated cell counter software cannot accurately count the number of cells in a suspension when they are aggregated, and the value generated is often significantly lower than expected. Additionally, although the Cedex Automated cell counter measures the number and size of EC 2102Ep aggregates, significant cell aggregation may cause inaccuracy in these aggregate number and size measures, and therefore the aggregate data produced represents an estimate. This causes significant difficulties when seeding new flasks during a passage, as well as when generating the growth data for each batch during experiments, and thus is a challenge that must be overcome during the process and protocol transfer in order to generate a viable automated protocol for this cell line. Therefore, a number of possible solutions were identified, and these were tested during the process and protocol transfer. The options proposed to allow for the automated passage and seeding of EC 2102Ep cultures included:

1. The utilisation of split ratios (1:3 or 1:4) for the passage and seeding protocols. This would involve the standardisation of culture duration.
2. The utilisation of the Cedex cell counter, accepting that the cell counts were unlikely to be accurate, and adjusting the seeding density of the new flasks to a lower value accordingly.

The options proposed to allow for the generation of accurate EC 2102Ep cell growth data included:

1. The utilisation of the Cedex cell counter to collect images of cell suspensions, and the replacement of the automated image analysis with manual cell counting, for which a consensus of 2-3 counts by different individuals would be obtained. However, this process step would increase the manual intervention required during automated culture.

2. The utilisation of the Cedex cell counter to collect images of the cell suspension, and the utilisation of an alternative imaging software to generate more accurate cell counts.

3. The isolation of a cell sample from the dissociated cell suspension prior to the performance of a cell count and, after the completion of the passage using a split ratio, the dilution and mixing of the sample in PBS or Trypsin EDTA to further disperse the cell aggregates. After sample dilution, the Cedex cell counter can be utilised to generate more accurate cell counts. However, it was proposed that the ‘Total Cell Density’ be utilised for cell counts after dilution, rather than the ‘Viable Cell Density’, as the viability of the cells may be affected during the dilution process, particularly if Trypsin EDTA is used.

4. The utilisation of more vigorous methods of cell dissociation, for example TrypLE™ (Life Technologies), or longer durations of incubation with Trypsin EDTA. However, as described above, this may be detrimental to the viability of the cells and therefore the ‘Total Cell Density’ may be a more relevant measure of cell growth.

After extensive discussion with colleagues and collaborators, it was decided that the utilisation of a split ratio, with a standardised culture time, would be most suitable passage method due to the difficulties associated with obtaining accurate cell counts. This would also reduce the manual intervention required to perform an automated protocol, which may reduce the variation and processing time associated with manual processing (Liu et al, 2010; Thomas et al, 2008). The elimination of manual intervention is also essential to generate a fully automated protocol, which was necessary in these experiments to compare manual and automated process steps for the culture of this cell line. However, although it was aimed to minimise manual intervention, the seeding of mother flasks remained a manual process due to the inability to obtain accurate cell counts. Inaccurate cell counts may lead to low or
inconsistent seeding densities which may substantially affect the characteristics and growth of the EC 2102Ep cell populations. Therefore, the decision was made to seed each mother flask with one full cryovial of these cells, which were stored at approximately 2x10^7 cells/vial. Although the protocol for the culture of the EC 2102Ep cell line recommends a seeding density of 1.1725x10^7 cells/T175 flask, it was agreed that an increased seeding density was favourable compared to an inconsistent seeding density.

It was also decided that, in order to obtain a cell growth data as a measure of the quality of the cultures, cell samples would be removed from culture during a passage, and plated in microwell plates. These samples would then be exposed to more vigorous mixing steps and extended periods of incubation with the dissociation agent, in order to break up any cell aggregates. However, although it was initially proposed that the Cedex Cell counter would be used to measure the ‘Total Cell Density’, due to the possible detrimental effects of this extended exposure to enzymatic dissociation upon cell viability, it was determined that the more vigorous dissociation steps had no significant effect upon cell viability, as demonstrated in Figure 62, and therefore ‘Viable Cell Density’ was utilised.

In order to assess the validity of the split ratio passage and vigorous cell aggregate dispersion methods, a number of experiments were undertaken during process and protocol transfer. Firstly, 5 flasks of EC 2102Ep cells were seeded in T175 flasks and imported into the CompacT SelecT. Medium exchanges and passages were performed, as well as visual examination of the cells to assess the confluency of the cell populations, in order to determine the relevant split ratio and duration of culture to achieve a suitable cell yield per flask. Secondly, an experiment using samples from these EC 2102Ep cell populations was also performed in multi-well plates in order to determine the most effective method of dispersing cell clumps and obtaining accurate cell counts. A test run of the adapted automated protocols for the culture, and the manual method of cell counting in multi-well plates, were then performed prior to the initiation of the Centrifugation and Non-Centrifugation experiment.

As a result of the experiments performed during the process and protocol transfer steps, it was determined that a 3 day passage protocol, with a medium exchange after 48 hours and visual examination throughout, was suitable for EC 2102Ep cells, seeded in T175 flasks, to reach sufficient confluency (80-90%). Also, after performing an automated passage on 3 confluent T175 flasks of these cells, in which each of the flasks was passaged at either a 1:3, 1:4, or 1:5 split ratio, it was deduced that a 1:3 split ratio was most suitable for seeding new
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T175 flasks at a sufficient density to obtain confluency within 3 days, and for maintaining a consistent seeding density between daughter flasks. A 1:3 split ratio was selected after the visual examination of daughter flasks seeded during passages at 1:4 and 1:5 split ratios revealed that only the first three daughter flasks could be seeded consistently and at sufficient density, and that EC 2102Ep cells may undergo morphological changes at higher split ratios with lower seeding densities, as shown in Figures 59a, 58b, and 58c, that has been previously associated with the differentiation of these cells (Andrews et al, 1982).

To determine a suitable method for the dispersion of cell aggregates, which was required to obtain an accurate cell count for the EC 2102Ep cell line, a Multi-well plate experiment was undertaken, whereby the cells were suspended in various dissociation agents for a variety of durations, and using a varying number of mixing steps prior to the performance of a cell count. The method for this experiment is outlined below:

- The medium of two confluent T175 flasks was aspirated, and a PBS wash step, followed by the addition of 10ml of 0.25% Trypsin EDTA, was applied to each flask.
- The first T175 flask was incubated for 15 minutes, vigorously agitated and visually examined before the cell suspension was removed and diluted in PBS, at a ratio of 1:4ml.
- The second T175 flask was incubated for 7 minutes, vigorously agitated and visually examined before the cell suspension was removed, centrifuged at 270 RCF for 5 minutes, and resuspended in TrypLE.
  - The cell suspension was then plated into 9 wells (3ml/well) of a 12 well plate and exposed to a variety of conditions, as described in Figure 60 below.
- The remainder of the cells suspended in TrypLE were again centrifuged at 270 RCF for 5 minutes. They were then however resuspended in 9ml 0.25% Trypsin EDTA, plated in

![Figures 59a (Left), b (Middle), and c (Right): Microscopy images (40x Magnification) of EC 2102Ep cells seeded at 1:3 (Figure 58a), 1:4 (Figure 58b), and 1:5 (Figure 58c) split ratios.](image-url)
the final 3 wells of the 12 well plate (3ml/well), and exposed to a range of conditions as described in Figure 60 below.

- 1ml samples were then taken from each well, or suspension, and used to perform a cell count on the Cedex automated Cell Counter.

![Figure 60](image)

*Figure 60:* 12 Well plate diagram demonstrating the conditions used in each well to disperse cell clumps during the Multi-well plate experiment.

In order to generate a cell count, the Cedex automated cell counter generates 30 viable images, the number of viable cells on each image are then counted and used to calculate the number of viable cells/ml. Therefore, in order to analyse the effect of the various conditions in the Multi-well plate experiment upon the aggregation of cells, three images per cell count, which exhibited the most cell aggregation, were selected and the number of viable cells in these images were manually counted, as shown Figure 61 below. This method was sufficient to allow for manual and automated counts to be obtained that were representative of the level of cell aggregation that was present within the various samples. These manual counts were then compared to the automated cell counts in order to determine which of the conditions was most effective in dispersing the cell aggregates. Due to the inability of the Cedex automated cell counter to accurately count the number of cells present in an aggregate, the conditions in which the automated cell counts most closely matched the manual counts demonstrated the greatest capability to disperse cell aggregates, as the counts from these conditions are likely to exhibit the fewest number of cell aggregates.
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Through the comparison of the manual and automated cell counts, it was determined that, after the dissociation of EC 2102Ep cells from the T175 flasks, the treatment of these cells with 0.25% Trypsin EDTA, with a 5 minute incubation period and 10 mixing steps (Flask 2, Well 10), in a multi-well plate provided the most effective dispersion of cell aggregates. This is demonstrated in Figures 63 to 65 (below), in which the manual and automated cell counts, for the 3 selected images, from each cell count are compared. It is also clear that extended

<table>
<thead>
<tr>
<th>Flask &amp; Well</th>
<th>Flask 1</th>
<th>Flask 2, Well 1</th>
<th>Flask 2, Well 2</th>
<th>Flask 2, Well 3</th>
<th>Flask 2, Well 4</th>
<th>Flask 2, Well 5</th>
<th>Flask 2, Well 6</th>
<th>Flask 2, Well 7</th>
<th>Flask 2, Well 8</th>
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<th>Flask 2, Well 10</th>
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<tr>
<td>Viability (%)</td>
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<td>95.4</td>
<td>87.3</td>
<td>95.1</td>
<td>92.6</td>
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<td>92.8</td>
<td>96.9</td>
<td>96.6</td>
<td>89.5</td>
</tr>
</tbody>
</table>

Figure 61: A diagram demonstrating an example of 3 Cedex automated cell counter images, within a single cell count, with the most significant cell aggregation.

Figure 62: A table showing the total percentage of viable cells in the cell counts for each condition in the multi-well plate experiment.
periods of incubation of EC 2102Ep cell populations in dissociation reagent had little effect upon the viability of these cells, as shown in Figure 62 (above). Figure 66 (below) also highlights the conditions, in the multi-well plate experiment, which exhibited the least variability between manual and automated cell counts. The analysis of this data indicates that the utilisation of 0.25% Trypsin EDTA, with an incubation period of 5 minutes and 10 mixing steps, produced the most consistent cell counts and is therefore most likely to reduce cell aggregation significantly compared to the other conditions tested. The Viable and Total Cell Densities calculated for each of the conditions in the multi-well plate experiment are presented in Appendix 13.14.

The derivation of a representative cell count for the entire cell population from the cell counts obtained from the cell aggregate dissociation process requires this count to be multiplied by 3, as the 1ml cell count sample was taken from a 3ml suspension of cells and 0.25% Trypsin EDTA. To obtain a Total Viable Cell Number, this value then must be multiplied by 15, as the cells taken for cell aggregate dissociation were obtained from a 15ml suspension of cells and DMEM HG GlutaMAX with 10% FBS.

**Figure 63:** A graph to show a comparison of manual and automated cell counts for the first selected images.
Figure 64: A graph to show a comparison of manual and automated cell counts for the second selected images.

Figure 65: A graph to show a comparison of manual and automated cell counts for the third selected images.
Figure 67 (below) illustrates the results of a Gage R&R Analysis of Variance (ANOVA) for the Manual and Automated Cell Counts of the Cedex Automated Cell Counter Images, from each of the dissociation conditions. The main conclusions that can be drawn from this statistical analysis are that the differences in the cell counts between the various dissociation conditions explains most of the variation in this analysis, that the variability between the cell counts of the same images measured using manual or automated methods explains the smallest proportion of the variation in this analysis, and that the variation in the measurement system contributes to the total variation in this analysis to a greater extent than the variability in the number of cells present in each image within a Cedex cell count for a particular dissociation condition. It can also be confirmed that the Flask 2, Well 10 condition, where Trypsin EDTA is applied for 5 minutes and 10 mixing steps are applied, exhibited the least variation between manual and automated cell counts, and therefore that this method reduces the cell aggregation to the greatest extent and allows for the most accurate cell count to be obtained.

Figure 66: A graph to show the difference between manual and automated cell counts, for each of the selected images, across the various conditions.
In order to assess the Centrifugation and Non-Centrifugation automated protocols generated (see Appendices 13.13.1, 13.13.4 & 13.13.5), which employ a 1:3 split ratio, these protocols were utilised in an initial experiment whereby 2 T175 flasks of EC 2102Ep cells were seeded and cultured over three days before passaging using either the Centrifugation or Non-Centrifugation process steps. The effectiveness of the initial trypsinisation step, the process of seeding new daughter flasks, and the adapted method of cell aggregate dispersion and cell counting was then determined. The Centrifugation and Non-Centrifugation processes are described further in sections 8.2.3 and 8.2.4.

During the Centrifugation and Non-Centrifugation protocols utilised to passage both T175 flasks of EC 2102Ep cells, the cells were incubated for 7 minutes in either 6ml of 0.25% Trypsin EDTA or the residual 0.25% Trypsin EDTA remaining after the dissociation agent was poured off, during the manual and automated processes respectively. It was determined
that both of these initial enzymatic dissociation methods used during either of the passage protocols, were sufficient to remove the majority of the EC2102Ep cells from the tissue culture plastic, with very few cells remaining in the mother flask after each passage.

Also, as a result of this initial experiment, it was determined, through the visual examination of daughter flasks 1 day after seeding, that each of the daughter flasks were seeded approximately equally (See Figures 68a & b below). This initial experiment also demonstrated that the method of cell aggregate dispersion and cell counting, which was selected from the multi-well plate experiment, was adequate to significantly disperse cell clumps and therefore obtain a more accurate cell count.

8.2.3 Ruler EC 2102Ep Culture: Centrifugation Experiment

- 1 banked EC 2102Ep vial removed from cryostorage.
- After thawing, these cells were resuspended in complete medium, centrifuged, the supernatant aspirated. The cells were then resuspended in DMEM HG GlutaMAX with 10% FBS and the cell suspension was transferred into a Barcoded T175 flask containing complete medium which was then imported into the CompacT SelecT.
- A medium exchange was performed every 48 hours after seeding or passage.
- 72 hours after seeding, once the cells had reached 80-90% confluence, a hES Ruler Pre-Centrifugation protocol was used to detach cells from the seeded flask, suspend the cells in complete medium, and outfeed the flask undergoing passage.
- A 1ml sample of this cell suspension was taken and the cell aggregate dissociation method was utilised to obtain a Pre-Centrifugation cell count.
3ml of complete medium was added to the sample, which was then centrifuged to isolate a cell pellet (270 RCF x 5 mins), resuspended in 3ml of 0.25% Trypsin EDTA, plated in a 12 well plate and incubated for 5 minutes.

10 mixing steps were performed upon the cell suspension after incubation and the Cedex automated cell counter was used to derive a Pre-Centrifugation cell count.

Cell Counts were utilised throughout in order to obtain Viable Cell Density, Viability, Cell Diameter, and Aggregate Size data. Viable Cell Yields were calculated by multiplying the Viable Cell Density by the volume of the cell suspension.

Next, a manual centrifugation procedure (270 RCF x 5mins) was performed in order to isolate a cell pellet and to resuspend the cells in complete medium. A 1ml sample of this suspension was then taken and the cell aggregate dissociation method was utilised in order to obtain a Post-Centrifugation cell count.

The same method as in the Pre-Centrifugation cell aggregate dissociation and cell count was utilised at this stage.

The remaining cell suspension was reintroduced into the outfeeded flask, imported back into the CompacT SelecT, and a Post-Centrifugation protocol was performed to passage the EC 2102Ep cells using a 1:3 split ratio (1→3 new flasks).

72 hours after each of the 3 daughter flasks were seeded and once they had reached confluency, the same method as above was utilised in order to passage each daughter flask into 3 granddaughter flasks (3 flasks→9 new flasks) and to obtain Pre- and Post-Centrifugation cell count data.

72 hours after each of the 9 granddaughter flasks were seeded and once they had reached confluency, all 9 flasks were pooled into a single flask, using a Pool protocol, and the cells were prepared for cryopreservation by suspending in cryopreservant medium (90% FBS & 10% DMSO), transferred into cryovials and stored in liquid nitrogen.

The required amount of cells were then thawed and used for post-manual characterisation.

Also, one vial of the cryopreserved EC 2102Ep cells were suspended in transport buffer and shipped to LGC Standards for post-manual CLA and comparison to the baseline CLA analysis.

Only 1 batch/run was utilised for characterisation and CLA analysis.

No cross-batch/run pooling.
• The above methods were repeated (excluding characterisation and CLA analysis) another 3 times in order to complete 4 Centrifugation experiment runs.

8.2.4 Ruler EC 2102Ep Culture: Non-Centrifugation Experiment

• 1 banked EC 2102Ep vial removed from cryostorage.
• After thawing, these cells were suspended in complete medium, centrifuged, the supernatant aspirated, then resuspended in DMEM HG GlutaMAX with 10% FBS. The EC 2102Ep cell suspension was then transferred into a T175 flask containing complete medium which was then imported into the CompacT SelecT.
• A medium exchange was performed every 48 hours after seeding or passage.
• 72 hours after seeding, once the cells had reached 80-90% confluency, a hES Ruler Non-Centrifugation protocol, involving a Trypsin pour-off step, was used to passage the mother flask, at a 1:3 split ratio, into 3 daughter flasks (1→3 new flasks).
  - After the Non-Centrifugation protocol was performed, a 1ml sample of the cell suspension remained in the mother flask, and this was used to obtain a Non-Centrifugation cell count using the cell aggregate dissociation method.
    - 3ml of complete medium was added to the sample, which was then centrifuged to isolate a cell pellet (270 RCF x 5 mins), resuspended in 3ml of 0.25% Trypsin EDTA, plated in a 12 well plate and incubated for 5 minutes.
    - 10 mixing steps were performed upon the cell suspension after incubation and the Cedex automated cell counter was used to derive a Non-Centrifugation cell count.
• 72 hours after each of the 3 daughter flasks were seeded and once they had reached confluency, the same Non-Centrifugation protocol and cell aggregate dissociation and cell counting method as above was utilised in order to passage each daughter flask into 3 granddaughter flasks (3 flasks→9 new flasks) and to obtain Non-Centrifugation cell count data.
• 72 hours after each of the 9 granddaughter flasks were seeded and once they had reached confluency, all 9 flasks were pooled into a single flask, using a Pool protocol, and the cells were frozen in freeze medium and stored in cryostorage.
• The required amount of cells were then thawed and used for post-automated characterisation.
- Also, one vial of the cryopreserved EC 2102Ep cells were suspended in transport buffer and shipped to LGC Standards for post-automated CLA and comparison to the baseline CLA analysis.
- Only 1 batch/run was utilised for characterisation and CLA analysis.
  - No cross-batch/run pooling.
- The above methods were repeated (excluding characterisation and CLA analysis) another 3 times in order to complete 4 Non-Centrifugation experiment runs.

8.2.5 Pluripotency Marker Expression
- One vial of each of the Baseline, Centrifugation and Non-Centrifugation EC 2102Ep cells was thawed, the cryoprotectant was removed, and the cells were resuspended in DMEM 10% FBS with added UltraGlutamine (2mM) (Lonza, Basel, SUI). A cell count was performed and EC 2102Ep cells were seeded in T75 flasks at 67,000 cells/cm² (5.025x10⁶ cells/flask). The cells were then cultured until ~80% confluent with medium exchanges performed every 48 hours.
- EC 2102Ep cells were then prepared for multicolour flow cytometry and analysed following the manufacturer’s instructions (BD Stemflow, BD Biosciences), as follows:
  - Once confluent, the cell monolayer was washed with PBS, the cells were dissociated using 0.25% Trypsin EDTA, the cells were washed with PBS, and a cell count was performed for each population.

Figure 69: Diagram illustrating the differences between the manual and automated EC 2102Ep passage process steps.
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- The cells were then fixed using Cytofix Fixation Buffer (4% PFA) (BD Biosciences), at a concentration of 1x10⁷ cells/ml, and incubated at room temperature for 20mins.

- The cells were then washed with Perm/Wash Buffer (BD Biosciences), resuspended in Perm/Wash buffer at a concentration of 1x10⁷ cells/ml, and incubated for 10mins at room temperature to permeabilise.

- 100μL of permeabilised cells from each condition were added to separate polystyrene flow cytometry tubes (BD Biosciences) and placed in the dark at room temperature for later analysis.

- To stain the permeabilised cells remaining for each condition, 100μL of permeabilised cells was added to two separate flow cytometry tubes. To one tube, labelled ‘Specific Stain’, 20μL of the PerCP-Cy5.5 Oct 3/4, PE SSEA-1 and Alexa Fluor 647 SSEA-4 antibodies were added. To the other, labelled ‘Isotype Control’, 20μL of the PerCP-Cy5.5, PE and Alexa Fluor 647 isotype controls were added. Replicates of each tube were generated.

- The tubes were then mixed and incubated in the dark at room temperature for 30mins. After incubation, each tube was washed twice in Perm/Wash buffer and resuspended in 350μL of stain buffer (BD Biosciences).

- To prepare the control beads, 100μL of Perm/Wash buffer and 1 drop of negative beads were added to four flow cytometry tubes (Negative, PerCP, PE, Alexa Fluor 647). One drop of the Anti-Mouse positive beads and 20μL of the appropriate antibody was then added to the relevant tubes. After incubation at room temperature in the dark for 30mins, the beads were washed twice with Perm/Wash buffer and resuspended in 350μL of stain buffer.

- The EC 2102Ep samples were analysed on the BD FACSCanto II (BD Biosciences) which was operated using the FACSDiva software version 6.1.3. The beads and unstained cells were analysed first in order to generate controls, and this was followed by the subsequent multiparameter analysis of the Baseline, Centrifugation and Non-Centrifugation EC 2102Ep cells at the single cell level. For analysis, the flow cytometry data was exported in FCS 3 format and analysed using FlowJo software v10.
8.2.6 CLA & STR Profiling

- CLA analysis was performed by LGC standards.
- Samples were prepared for transport and CLA analysis following the protocol provided by LGC standards.
- 1 vial of baseline EC 2102Ep cells, 1 vial of Post-Centrifugation EC 2102Ep cells, and 1 vial of Non-Centrifugation EC 2102Ep cells were removed from cryostorage, thawed and centrifuged.
- The supernatant was then aspirated and a PBS wash was applied, in order to remove any remaining freeze medium.
- The samples were again centrifuged and the supernatant aspirated in order to isolate the cell pellets.
- Finally, the samples were resuspended in 400μl of transport buffer, provided by LGC standards, which lyases the cells and preserves the gDNA.
- The samples were then packaged and sent to LGC Standards, where the CLA analysis was undertaken and the STR profiles of each sample were compared.

8.2.7 Statistical Analysis

Experimental data regarding the Viable Cell Density, Viable Cell Yield, and Viability of Pre-Centrifugation, Post-Centrifugation and Non-Centrifugation EC 2102Ep cells within each passage was assessed using One-Way Analysis of Variance (ANOVA) multiparameter analysis, utilising the IBM SPSS statistical software, to determine significant differences. One-Way ANOVAs were also used to assess the significance of differences in the Standard Deviations (SD) of the Viable Cell Densities, Viable Cell Yields, and Viabilities of EC 2102Ep cells, in each of the four batches, cultured using Pre-Centrifugation, Post-Centrifugation, and Non-Centrifugation process steps in the second passage. Two-Way ANOVAs were used to assess significant differences in the Viable Cell Density, Viable Cell Yield, Population Doubling Time, Viability, and Cell Diameter of Pre-Centrifugation, Post-Centrifugation and Non-Centrifugation EC 2102Ep cells across all passages. The cutoff value for statistical significance (p) was set at 0.05. Tukey’s Honest Significant Difference (HSD) Post-Hoc Tests were used to perform multiple comparisons of the EC 2102Ep cell growth data.
8.3 Results

8.3.1 Morphology

From the microscopy images above (Figures 70a & b), it is clear that no difference in the morphology of EC 2102Ep cells after passage utilising either the Centrifugation or Non-Centrifugation process step was observed.

8.3.2 Cell Diameter

**Figure 71:** EC 2102Ep Batches 1-4 Centrifugation & Non-Centrifugation Average Cell Diameter. Standard Deviations are shown as error bars.
No significant difference in EC 2102Ep cell diameter was observed between passages or between process steps, as illustrated in Figure 71 (above). However, it is apparent that significant variability in cell diameter was observed in the 1st passage when the Non-Centrifugation process step was utilised.

8.3.3 CLA & STR Profiling

Figure 72: EC 2102Ep Baseline Electropherogram Short Tandem Repeat (STR) Profile.

Figure 73: EC 2102Ep Centrifugation Electropherogram Short Tandem Repeat (STR) Profile.
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**Table 74**: Electropherogram Short Tandem Repeat (STR) Profile

<table>
<thead>
<tr>
<th>Loci</th>
<th>EC 2102Ep Profile</th>
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<tbody>
<tr>
<td>Amelogenin</td>
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<td>FGA</td>
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</table>

**Figure 74**: EC 2102Ep Non-Centrifugation Electropherogram Short Tandem Repeat (STR) Profile.

**Figure 75 (above)**: Baseline, Centrifugation & Non-Centrifugation EC 2102Ep alleles at each locus within Short Tandem Repeat (STR) Profile. * Denotes Peak Area Difference (PAD) between Baseline and Centrifugation EC 2102Ep cells.
Figures 72, 73 & 74 demonstrate that no significant difference between the Short Tandem Repeat (STR) profiles of the Baseline, Post-Centrifugation, and Non-Centrifugation EC 2102Ep cells was observed. Figure 75 outlines the number of STRs at each allele of each locus profiled for the EC 2102Ep cells, and these were found to be comparable between Baseline, Centrifugation and Non-Centrifugation EC 2102Ep cells. These results were determined by comparing the STR Electropherograms (EPG), as well as the number of STRs at each locus, of EC 2102Ep cells cultured using both manual and automated process steps to that of EC 2102Ep cells generated in the initial cell bank. These findings indicate that neither the automated nor the manual process steps induced an alteration in the pattern of short tandem repeats expressed in the EC 2102Ep cell DNA. However, Figure 75 highlights the existence of a Peak Area Difference (PAD) between Baseline and Centrifugation EC 2102Ep cells at the TH01 locus. This single PAD is not considered to represent a significant difference between the STR profiles of Baseline and Centrifugation EC 2102Ep cells.

Although the above figures may also illustrate differences in the level of STR expression between Baseline, Non-Centrifugation, and Post-Centrifugation EC 2102Ep cells, these differences are very small in magnitude and it is difficult to discern any significant trends between each of the EPGs.

8.3.4 Pluripotency Marker Expression

![Figure 76 (above): Scatter plots demonstrating multicolour flow cytometric analysis of pluripotency and differentiation marker co-expression of Baseline EC 2102Ep cells (P52).]
By analysing the immunophenotype of the Baseline, Centrifugation and Non-Centrifugation EC 2102Ep cells, it was determined the expression of SSEA-4 and Oct 3/4 pluripotency markers was high in all EC 2102Ep cell populations, with >99% SSEA-4 and >80% Oct 3/4 expression in each population. It was also identified that a high percentage of cells in each population co-expressed these pluripotency markers (>86%). Furthermore, the expression of SSEA-1, a marker which identifies differentiated cells, was found to be very low in all EC 2102Ep cell populations (<0.5%). These findings demonstrate that each of the Baseline, Centrifugation and Non-Centrifugation EC 2102Ep cell populations predominantly expressed a pluripotent phenotype.

However, by comparing the multicolour flow cytometry data for each of the EC 2102Ep cell populations, it was observed that Non-Centrifugation cell populations may have exhibited
lower Oct 3/4 expression compared to both the Baseline and Centrifugation populations, although this difference was small (<0.45%). Concordantly, the expression of the SSEA-1 differentiation marker may have been higher in Non-Centrifugation EC 2102Ep cell populations compared to Baseline and Centrifugation populations, although, again, this difference was small (<0.3%). Therefore, this reduced pluripotency marker expression and increased differentiation marker expression may suggest that the utilisation of the Centrifugation process step maintained the pluripotent phenotype of these cells more effectively than the Non-Centrifugation process step. However, it is apparent that these differences in pluripotency and differentiation marker expression observed between EC 2102Ep cell populations was negligible, and that no difference in pluripotency marker co-expression was observed between Ruler hESC conditions.

8.3.5 Growth Data

**Figure 79:** The Means of the Viable Cell Densities for Pre-Centrifugation, Post-Centrifugation & Non-Centrifugation EC 2102Ep cells over two passages. Standard Deviations are plotted as error bars and asterisks (*) denote significance (p=0.05).
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From the EC 2102Ep cells Viable Cell Density data, as illustrated in Figures 79 & 80, it is apparent that a significantly greater Viable Cell Density was observed in Non-Centrifugation cell counts compared to Pre-Centrifugation (p=0.00) and Post-Centrifugation (p=0.00) cell counts. This would suggest that the culture of EC 2102Ep cells using automated process steps resulted in a significantly greater number of cells than when manual process steps are utilised.

These results also suggest that the centrifugation process may increase the Viable Cell Density of EC 2102Ep cell samples, however this pattern was non-significant (p=0.395). Although this trend may suggest that an increased number of cells were present after centrifugation, this is unlikely and it is more likely that the centrifugation and manual resuspension processes may produce a more evenly mixed cell suspension compared to cell suspensions prior to centrifugation. However, it may be that the difference between the volumes of medium in which the cells are suspended Pre-Centrifugation (21ml) and Post-Centrifugation (15ml) is responsible for the patterns observed in the present study.

The data presented in Figures 79 & 80 may also demonstrate that the variability in the Viable Cell Densities measured after the Non-Centrifugation process steps may be greater than the...
variability in Pre-Centrifugation (p=0.473) or Post-Centrifugation (p=0.398) samples. However, this difference in the variation in Viable Cell Densities was found to be non-significant regardless of whether outliers were included or excluded.

This pattern of greater Viable Cell Densities, as well as increased variability, obtained when utilising the automated, Non-Centrifugation process steps compared to when the manual, Pre- and Post-Centrifugation process steps are utilised is also supported when the individual batch data is examined, as shown in Figure 81 (below). However, a significant proportion of this variation could be attributed to the first batch of EC 2102Ep cells cultured using Non-Centrifugation process steps, in which manual intervention was required for the passage of a single flask within the batch due to an equipment error which resulted in the application of an insufficient volume of Trypsin EDTA to the cells and therefore a lack of adequate cell dissociation.
Figure 81: A Box Plot showing the Mean, Interquartile range, and Range of Viable Cell Densities for Pre-Centrifugation, Post-Centrifugation, and Non-Centrifugation EC 2102Ep cells in the 2nd Passage (P54, 3 Replicates per batch) within each of the four Batches (n=4).
From the Estimated Live Cell Aggregate Data, illustrated in Figure 82 (above), it can be determined that the manual dissociation method employed to obtain more accurate cell counts allowed for the generation of cell suspensions containing primarily small cell aggregates of approximately 1-5 cells in size. Furthermore, it is apparent that the process of centrifugation increased the number of very small aggregates, of 1-3 cells in size. Finally, it can be deduced that the Non-Centrifugation EC 2102Ep samples contained greater numbers of small cell aggregates, 1-6 cells in size, compared to the Pre- or Post-Centrifugation samples. This may suggest that the Non-Centrifugation process step generated greater cell numbers compared to the Centrifugation process step, however the larger standard deviations observed in the Non-Centrifugation samples suggests that cell aggregation and settling may have influenced these results and support the trend for greater variability in Viable Cell Density and Yield, as discussed in section 8.4. The raw estimated live cell aggregate size data and standard deviations are presented in Appendix 13.15.
The EC 2102Ep Viable Cell Yield results, as shown in Figures 83 (above) & 84 (below), demonstrate that the utilisation of the Non-Centrifugation process steps generated a significantly greater number of viable cells compared to the number of viable cells present Pre- (p=0.00) or Post-Centrifugation (p=0.00). The Viable Cell Yield data also indicates that there was no significant change in the growth rate of the EC 2102Ep cells over two passages (p=0.999). From this data, it is again apparent that greater variation may exist when the automated, Non-Centrifugation process steps are utilised, and this is illustrated by the non-significant trend for greater SDs of the Non-Centrifugation data points groups compared to the Pre- and Post-Centrifugation data points.

The results of the present study may also suggest that the process of centrifugation may reduce the Viable Cell Yield of the EC 2102Ep samples. From this pattern it can be proposed that the centrifugation process may have a detrimental impact upon the cell populations resulting in a lower number of viable cells. However, this trend cannot be confirmed as it was not significant (p=0.492).
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**Figure 84**: The Average Centrifugation & Non-Centrifugation Viable EC 2102Ep Cumulative Yield per Batch, of all four batches, at Passage 52 (Seeding Mother Flask), 53 (1 confluent flask per Batch), and 54 (3 confluent flasks per Batch). Standard Deviations are shown as error bars.

**Figure 85**: The Mean Population Doubling Times of Centrifugation & Non-Centrifugation EC 2102Ep cells, across all four batches (n=4), at Passage 53 (4 Replicates) and 54 (12 Replicates). Standard Deviations are shown as error bars. * Denotes Significance.
Figure 85 (above) illustrates the mean Population Doubling Times (PDTs) for Pre-Centrifugation and Non-Centrifugation EC 2102Ep cells at Day 3 in Passage 53 & 54. Data regarding Pre-Centrifugation EC 2102Ep cells was utilised, rather than that of Post-Centrifugation EC 2102Ep cells, in order to exclude the effect of the centrifugation process upon the cell count data. From the PDT data, it was observed that the PDT of Non-Centrifugation EC 2102Ep cells was significantly shorter than that of Centrifugation cells (p=0.00), which would indicate that the growth rate of the Non-Centrifugation EC 2102Ep populations was greater. Furthermore, no change in PDT was demonstrated over multiple passages.

Mean cumulative population doublings (CPDs) of 5.17 and 7.69 were achieved after culture over two passages utilising the Centrifugation and Non-Centrifugation process steps respectively. Similarly to the PDT calculations, the Mean CPDs were calculated based upon Pre-Centrifugation and Non-Centrifugation cell counts.

The PDTs and CPDs for Pre-Centrifugation and Non-Centrifugation EC 2102Ep samples were determined using the following formulae:

\[ PDT = \frac{[\text{Time of Final Cell Count (Days)} - \text{Time of Seeding (Days)}]}{[3.32 \times (\log(\text{Final Cell Yield}) - \log(\text{Number of Cells Seeded}))]} \]

\[ CPDs = \frac{[\text{Time of Final Cell Count (Days)} - \text{Time of Seeding (Days)}]}{\text{PDT} \text{ (Days)}} \]
From the EC 2102Ep viability data (Figure 86, above), it can be concluded that no significant difference was observed between the viability of cells after Pre-Centrifugation, Post-Centrifugation or Non-Centrifugation process steps. Also, no significant difference between the variation in the viabilities of Pre-Centrifugation, Post-Centrifugation and Non-Centrifugation samples was observed.

8.4 Discussion

The visual examination of the morphology of cells in culture, using microscopy, allows for rudimentary characterisation and determination of the identity of the cell population. Although this method is not quantitative, it can act as a basic method for the identification of cells undergoing differentiation, transformation or apoptosis.

From the microscopy images taken throughout the present study, as illustrated in Figures 70a & b, it is apparent that no difference in the morphology of EC 2102Ep cells cultured utilising the Centrifugation or the Non-Centrifugation process steps was observed. All of the EC 2102Ep cells demonstrated a round morphology, with a relatively large nucleus and a tendency to grow in clusters, as has been previously reported (Andrews et al, 1982).
indicates that these cells proliferated in culture in a similar manner regardless of whether manual or automated cell culture process steps were employed.

Additionally, the cell diameter data (Figure 71) indicates that no significant difference was observed between the size of the EC 2102Ep cells in each passage and each of the process steps. However, a trend for greater variability in cell diameter after culture using the Non-Centrifugation process step, in the first passage, was demonstrated, which may indicate the automated process step influenced cell diameter. However, this trend was not observed across all passages. Therefore, it can be concluded that Ruler hESCs cultured using manual or automated process steps were of a similar size and morphology, and that neither of the process steps significantly impacted these parameters.

The utilisation of multicolour flow cytometry allowed for the co-expression of pluripotency and differentiation markers in Baseline, Centrifugation and Non-Centrifugation EC 2102Ep cell populations to be examined at the single cell level. The expression of the transcription factor Octamer-binding transcription factor 3/4 (Oct 3/4 or POU5F1) and the surface antigen Stage-Specific Embryonic Antigen-4 (SSEA-4) were measured in order to determine the pluripotency marker expression of EC 2102Ep cell populations. The expression of the SSEA-1 cell surface antigen was determined to identify the level of differentiation marker expression in EC 2102Ep populations. The International Stem Cell Initiative has previously characterised 59 hESC lines and found that both SSEA-4 and Oct 3/4 are highly expressed in all cell lines, and that low expression of SSEA-1 is observed in undifferentiated cells (Adewumi et al, 2007).

In the present study, strongly positive expression of both SSEA-4 and Oct 3/4 was observed in Baseline, Centrifugation and Non-Centrifugation populations, and that over 86% of cells in each population co-expressed these pluripotency markers. Furthermore, negligible expression of the differentiation marker SSEA-1 was identified in each EC 2102Ep cell population (<0.5%). Therefore, it can be concluded that the majority of EC 2102Ep cells in each of the Baseline, Centrifugation and Non-Centrifugation populations expressed a pluripotent phenotype.

Similarly to previous studies in which the pluripotency and differentiation marker expression of EC 2102Ep cells was examined (Josephson et al, 2007), over 99% positive expression of SSEA-4 and negligible SSEA-1 expression was observed in each of the EC 2102Ep
populations in the present study. However, EC 2102Ep populations in the present study exhibited marginally lower Oct 3/4 expression than previously reported for this cell line (98%) (Josephson et al, 2007). In comparison to conventional hESC lines, such as the HUES7 line, similar positive expression of the pluripotency markers SSEA-4 (>96%) and Oct 3/4 (92%) has been previously reported (Brosnan et al, 2013; Bajpai et al, 2008). However, greater expression of the differentiation marker SSEA-1 has been identified in the HUES7 line (Brosnan et al, 2013).

Nonetheless, lower expression of the pluripotency marker Oct 3/4 (<4.5%), as well as higher expression of SSEA-1 (<0.3%), was identified in Non-Centrifugation EC 2102Ep cell populations when compared to Baseline and Centrifugation populations. These findings may suggest that a greater number of cells expressing markers of differentiation were present in Non-Centrifugation populations, and therefore that the manual, centrifugation process step maintained the pluripotent phenotype of EC 2102Ep cell populations more effectively than the automated, Non-Centrifugation process step. This would indicate that the dissociation agent carryover, which occurs during the Non-Centrifugation process step, may influence EC 2102Ep pluripotency marker expression. Previous investigations have also reported significant differences in Oct 3/4 expression in hESCs after dissociation with a number of enzymes, with Trypsin inducing the greatest detrimental effect (Bajpai et al, 2008), which would indicate that dissociation agent exposure, and choice of enzyme, may influence hESC pluripotency marker expression. However, it is clear that these differences in pluripotency and differentiation marker expression between EC 2102Ep cell populations observed in the present study were negligible, and that no difference in pluripotency marker co-expression was observed between Ruler hESC conditions. Therefore, it can be concluded that the phenotype of EC 2102Ep cell populations from each condition was comparable.

As described in Chapters 6 and 7, Short Tandem Repeat (STR) profiling is a method used to amplify and compare specific loci on the DNA of multiple cell populations, and is often used in forensic analysis (Butler, 2006; Masters et al, 2001). In the present study, the STR profile analysis was provided by LGC Standards as part of their Cell Line Authentication (CLA) Service, and this is used to measure the difference in STRs at 16 loci within the genome between samples illustrated in an Electropherogram (EPG). These analyses allowed for the determination of the stability of EC 2102Ep populations cultured using alternative process steps.
Once the STR profiles had been generated for each of the Baseline, Post-Centrifugation, and Non-Centrifugation EC 2102Ep samples, the EPGs of the Post-Centrifugation and Non-Centrifugation samples were compared to that of the Baseline sample in order to determine whether either the manual or the automated process step had any significant effect upon the STR profiles, and therefore the genome or identity, of the EC 2102Ep cells. The comparison of the STR profiles of the Post-Centrifugation and Non-Centrifugation EC 2102Ep samples against the Baseline samples, as shown in Figures 72, 73 & 74, demonstrated that there was no significant difference between any of the samples, and that each expressed peaks in STRs at the same loci. However, as demonstrated in Figure 75, a Peak Area Difference (PAD) was observed in the Centrifugation EC 2102Ep sample compared to the Baseline sample at the TH01 locus. This indicates that the alleles at the TH01 locus amplified differently within each sample and therefore produced peaks with a different area. Nonetheless, the alleles of cell lines often do not amplify in the same manner and these PADs are more common in cell line samples and cancer cells (Reid et al, 2004). Therefore, this single PAD between the Baseline and Centrifugation EC 2102Ep samples does not represent a significant difference in STR profile. This indicates that neither the automated nor the manual process steps created alterations in the loci at which the EC 2102Ep cells expressed peak numbers of STRs in their DNA.

By comparing the EPGs of the Baseline, Post-Centrifugation, and Non-Centrifugation EC 2102Ep cell samples it is apparent that possible differences in the level of STR expression may exist. As discussed in previous chapters, these differences in peak size between samples would suggest that the greater peaks on the EPGs represent a stronger fluorescence, and therefore that more copies of the STR exist at each locus. However, variations in fluorescence between experimental runs, due to differential amplifications of the alleles or primer-binding site mutations (Clayton et al, 1998), may represent a confounding factor and therefore the differences in peak size observed may represent experimental error between runs. Under LGC Standards guidelines, the variations in peak size between the three EC 2102Ep cell samples provided in this experiment are not considered to represent a significant change in the identity of a cell sample and therefore each of the samples are considered to have identical STR profiles.

Previous studies have yielded contradictory results with regards to the genomic stability of cultured hES cells. Catalina and colleagues (2009) cultured multiple hES cell lines for
extended periods, utilising mechanical dissociation methods, and discovered that these cells displayed normal karyotypes throughout. Methods of enzymatic passage have also been developed which allow for the maintenance of normal karyotypes for up to 2 years (Suemori et al, 2006). More recently, the effects of mechanical and enzymatic dissociation methods upon the genomic stability of hESCs has been examined, and it was determined that the occurrence of genomic instability was similar in each method (Tosca et al, 2015).

In a 2004 publication by Buzzard and colleagues (Buzzard et al, 2004), the authors indicated that despite frequent cytogenetic assessment of numerous hES cell lines, a karyotypic change had only once been observed in the Embryonic Stem Cell International Laboratories (Melbourne, Australia) and this was detected in an early passage population (P5). These authors (Buzzard et al, 2004) continued to outline that they had never identified non-cumulative aneuploidies in hES cells that had been previously reported by other investigators. Mitalipova et al (2005) reported similar findings with the maintenance of normal karyotypes and gene expressions for around 50 passages, however the authors also identified that extended periods of culture and different methods of dissociation can induce abnormal karyotypes in hES cell lines. These findings have been supported by various studies in which the genomic stability of cultured hES lines was examined. Maitra and colleagues (2005) examined the genomic stability of 9 late passage hES cell lines and discovered that 8 of these exhibited one or more genomic alterations. A 2006 study (Imreh et al, 2006) also observed karyotypic changes in 100% of cells of 1 hES cell line after culture on tissue culture plastic without feeder cells. Finally, Draper et al (2003) found that karyotypic changes involving the gain of chromosome 17q in three cultured hES cell lines.

However, although the karyotype and STR profile of the hES Ruler Cell line EC 2102Ep has previously been investigated (Josephson et al, 2007), the genomic stability of this cell line over multiple passages has not previously been examined. Therefore, from the results of the present study, although this hES Ruler Cell line is not truly representative of most hES cells, it is apparent that this cell line does not undergo any significant genomic alterations as a result of multiple passages using either manual or automated process steps and therefore may represent a useful tool for the comparison of hES cell lines and the validation of assays.

From the cell counts and growth data for the EC 2102Ep cells in the present study, it was observed that the Viable Cell Density of cell populations measured after the Non-Centrifugation process was significantly greater than those measured Pre-Centrifugation or
Post-Centrifugation. This would suggest that the utilisation of the automated, Non-Centrifugation process steps is more favourable for the culture of the EC 2102Ep cells compared to the manual, Centrifugation process steps. The results of the present study may also indicate that the Viable Cell Density of EC 2102Ep Post-Centrifugation cell populations were greater than that of Pre-Centrifugation cell populations. From this trend it can be proposed that the Centrifugation process may increase the Viable Cell Density of EC 2102Ep populations, although this trend was not significant.

However, the trends in the Viable Cell Density data observed in this experiment are likely to be substantially influenced by the differences in the volume of culture medium in which the cells are suspended. At the time of the Pre-Centrifugation cell counts, the EC 2102Ep cells are suspended in 21ml of culture medium. Whereas, at the time of the Post-Centrifugation and Non-Centrifugation cell counts, the cell populations are suspended in 15ml and 30ml respectively. This will significantly impact the number of cells per millilitre of suspension.

Another possible pattern that can be identified from the Viable Cell Density data is the trend for a greater variation in the Viable Cell Densities of Non-Centrifugation cell populations compared to Pre-Centrifugation and Post-Centrifugation populations. Although this trend was found to be non-significant, a similar non-significant trend was demonstrated when the data for each individual batch was analysed. From the individual batch data, as shown in Figure 81, it is clear that, although the variability in most of the Non-Centrifugation batches may be greater than that of the Pre- and Post-Centrifugation batches, the counts from the first batch of the Non-Centrifugation cells may have contributed significantly to the total variation for the Non-Centrifugation samples. It is likely that this increased variation in Viable Cell Densities in the first Non-Centrifugation batch may have been influenced by the manual intervention undertaken, in order to passage one of the three flasks during the second passage, which was required due to an equipment malfunction on the CompacT Select during the second passage.

It is also likely that the increased Viable Cell Density variation in the first Non-Centrifugation batch may have been affected by the adherence of the EC 2102Ep cells to the base of the T175 mother flask. Towards the end of the Non-Centrifugation passage protocol, once all of the new daughter flasks have been seeded, the mother flask is exported with around 1ml of cell suspension remaining in the flask. This remaining cell suspension is then used to obtain the cell count for the mother flask. However, as this sample represents the
residual cell suspension from the passage, it is likely that a significant number of cells will have settled towards the base of the mother flask. As this settling of cells is unlikely to be uniform for each of the Non-Centrifugation mother flasks passaged, it may be that this contributed to the trend for an increased variation and increased Viable Cell Densities when the Non-Centrifugation process step was utilised.

The estimated live cell aggregate data for the EC 2102Ep samples indicates that the process of centrifugation may have increased the number of small aggregates, of 1-3 cells in size. Thus, although the centrifugation process step may reduce the obtainable viable cell yield, the reduction in aggregate size may have influenced the reduced standard deviations in Viable Cell Yield observed in the Post-Centrifugation samples. Therefore, the utilisation of a centrifugation process step may allow for more accurate cell counting and improved consistency of seeding daughter flasks. Additionally, it is clear that the Non-Centrifugation process step yielded a greater number of small cell aggregates, of 1-6 cells in size, compared to the Pre- and Post-Centrifugation samples. This may indicate that the Non-Centrifugation process step yielded a greater number of cells, however the larger standard deviations in the number of aggregates observed in the Non-Centrifugation samples suggests that cell aggregation and settling may have influenced these results and support the trend for greater variability in Viable Cell Density and Yield.

Once the Viable Cell Yield had been calculated for the EC 2102Ep cell populations, it was determined that the average Non-Centrifugation Viable Cell Yield was significantly greater than that of the Pre-Centrifugation and Post-Centrifugation populations. From this finding it can be concluded that a significantly greater number of cells were present in the Non-Centrifugation EC 2102Ep samples. However, as previously described in this section, a substantial number of cells may settle at the base of the mother flasks prior to performance of the Non-Centrifugation cell counts. Therefore, it may be that, when this residual cell suspension is used to obtain a cell count, the cell count could be significantly greater than what would be expected for that cell population and may not be representative. Therefore, although it can be concluded that, in the present study, the culture of EC 2102Ep cells using automated process steps generated samples with significantly greater Viable Cell Densities, it cannot be stated for certain that the utilisation of Non-Centrifugation process steps was more favourable and produced a greater number of cells than the manual, Centrifugation process steps.
Contrary to the Viable Cell Density data, the Viable Yield data, as illustrated in Figures 83 & 84, highlights a trend for a decrease in the number of cells after Centrifugation. This non-significant trend for a decrease in the Viable Cell Yield after Centrifugation, as was also observed in the hMSC experiment, supports the proposition that the volume of culture medium in which the cells were suspended substantially affected the Viable Cell Density results. Therefore, it is possible that an excessively high Relative Centrifugal Force (RCF), causing damage to the cells, or an insufficient RCF, incapable of creating an adequate cell pellet, was utilised causing the centrifugation process to have a detrimental effect upon the Viable Cell Yield. However, the lack of a significant detrimental effect of the centrifugation process upon cell viability suggests that the latter is more likely, although further investigation is required to determine whether this trend is noteworthy and to identify the cause of this pattern.

Finally, from the EC 2102Ep Viable Cell Yield data, it can be concluded that no significant change in the growth rate of these cells was observed over two passages. This would suggest that the cells had not begun to enter senescence, which given the immortalised nature of these cells was an expected result, and that both the cells cultured utilising both the manual and automated process steps maintained their growth rate over multiple passages.

From the Population Doubling Times (PDTs) of EC 2102Ep cells after 3 days (Figure 85), cultured utilising either manual or automated process steps, it was observed that the Non-Centrifugation EC 2102Ep cells exhibited a significantly shorter population doubling time than that of Centrifugation cells. However, as discussed previously in this section, this may have been influenced by the occurrence of cell settling prior to the performance of a cell count during the Non-Centrifugation process, which may have led to unrepresentative cell count data. Despite this possible measurement inaccuracy, it was determined that the PDT of EC 2102Ep cells did not change over multiple passages regardless of the process step utilised. This demonstrates that all EC 2102Ep cells maintained their proliferative rate over multiple passages, which is to be expected for immortalised cell lines. Furthermore, the PDTs of Ruler hESCs observed in the present study was found to be similar to previously reported 24-48 hour PDTs for hESCs (Cowan et al, 2004).

The viability data for the EC 2102Ep cells demonstrates that no significant difference in cell viability between the Pre-Centrifugation, Post-Centrifugation and Non-Centrifugation cell samples was observed, and this lack of difference in cell viability was observed in both
passages. Also, no difference in the variability in cell viabilities was measured between Pre-
Centrifugation, Post-Centrifugation and Non-Centrifugation samples. From these results, it
can be concluded that neither the manual nor the automated cell culture process steps had a
significant detrimental effect upon the viability of EC 2102Ep cells.

The comparability between Baseline Ruler hESCs, and those cultured using Centrifugation
and Non-Centrifugation process steps is summarised in Figure 87 (below), in which the
results for each of the parameters measured are highlighted.
### Table: Summary of Comparison Parameters

<table>
<thead>
<tr>
<th>Condition</th>
<th>Morphology</th>
<th>Cell Diameter</th>
<th>Viable Cell Yield</th>
<th>Population Doubling Time</th>
<th>Viability</th>
<th>STR Profile</th>
<th>Pluripotency Marker Expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline (P51)</td>
<td>Spherical morphology forming tightly packed clusters</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>NSD</td>
<td>86.6% SSEA-4 &amp; Oct 3/4 Co-Expression &lt;0.5% SSEA-1 Expression</td>
</tr>
<tr>
<td>Pre-Centrifugation (P54)</td>
<td>Comparable</td>
<td>17.98μm (±0.59μm)</td>
<td>6.28x10^7 (±1.03x10^7)</td>
<td>0.83 Days (±0.05 Days)</td>
<td>99.4% (±0.21%)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Post-Centrifugation (P54)</td>
<td>Comparable</td>
<td>17.95μm (±0.41μm)</td>
<td>5.334x10^7 (±0.66x10^7)</td>
<td>0.88 Days (±0.55 Days)</td>
<td>99.3% (±0.28%)</td>
<td>NSD</td>
<td>87.2% SSEA-4 &amp; Oct 3/4 Co-Expression &lt;0.5% SSEA-1 Expression</td>
</tr>
<tr>
<td>Non-Centrifugation (P54)</td>
<td>Comparable</td>
<td>18.21μm (±0.92μm)</td>
<td>16.7x10^7 (±3.83x10^7) (SD)</td>
<td>0.62 Days (±0.08 Days) (SD)</td>
<td>99.2% (±0.27%)</td>
<td>NSD</td>
<td>86.7% SSEA-4 &amp; Oct 3/4 Co-Expression &lt;0.5% SSEA-1 Expression</td>
</tr>
</tbody>
</table>

**Figure 87**: Summary table of parameters used to examine comparability between Baseline (P51), Post-Centrifugation (P54) and Non-Centrifugation (P54) Ruler hESCs. Average values are presented and ± denotes Standard Deviations. NSD= No Significant Difference. SD= Significant Difference.
Figure 88 (below) illustrates the proportional means for each parameter measured, which allows for the consistency of Centrifugation and Non-Centrifugation Ruler hESC measurements to be examined for each parameter. The proportional standard deviations are also presented, which facilitates further comparison of the consistency of Centrifugation and Non-Centrifugation measurements by comparing the variability within each parameter. The comparison of proportional standard deviations also allows for the variability between parameters to be compared, allowing process and product control to be identified.

Figure 88, demonstrates that the variability in both EC 2102Ep Cell Diameter and Viability was low, and therefore that both of these parameters were well controlled. However, this figure also highlights that Viable Cell Yield was variable in both Centrifugation and Non-Centrifugation Ruler hESCs. Figure 88 (above) also illustrates that differences were observed in both the Viable Cell Yield and Population Doubling Time mean and standard deviation when comparing Centrifugation and Non-Centrifugation EC 2102Ep cells. Centrifugation cells exhibited a lower mean and a smaller standard deviation in Viable Cell Yield, whereas Non-Centrifugation cells exhibited a lower mean but greater standard deviation in Population Doubling Time. This would indicate that, although Centrifugation EC 2102Ep cells may have
exhibited a lower Viable Cell Yield, greater consistency was obtained using the manual process step, as described earlier in both this section and section 8.3.5 (Figure 83). Furthermore, this would suggest that, although Non-Centrifugation EC 2102Ep cells achieved a shorter Population Doubling Time, their proliferative rate was more variable. Therefore, given the lack of overlap between the Viable Cell Yield and Population Doubling Time standard deviations for Centrifugation and Non-Centrifugation Ruler hESCs, there could be considered to be a lack of comparability in these parameters between process steps. These findings indicate that this cell line is not suitable as a standard to compare manual and automated culture processes, given the significant influence of the alternative process steps upon performance. Also, in order to utilise the EC 2102Ep cell line as a standard for the automated, Non-Centrifugation process; cell populations must be generated using this fully automated process. Thus, it is apparent that further research is required to generate a hESC standard that is consistent between both manual and automated culture processes. However, the discrepancy between the means of Centrifugation and Non-Centrifugation Viable Cell Yields and Population Doubling Times may be associated with sampling variability, which may be attributed to the cell settling observed during the Non-Centrifugation process, as discussed previously in this section. Therefore, further work may be required to optimise the Non-Centrifugation process step in order to reduce cell aggregation and settling.

The normalised means and standard deviations data also demonstrates that the standard deviation of Centrifugation EC 2102Ep Cell Diameter data falls within that of Non-Centrifugation Cell Diameter data. Furthermore, it was also observed that the variability in Centrifugation and Non-Centrifugation EC 2102Ep Viability, expressed as standard deviations, overlap. This would suggest that little variability between Centrifugation and Non-Centrifugation Ruler hESCs was observed in these parameters.

### 8.5 Conclusions

In this chapter, after a process and protocol transfer, quantitative experiments have been performed in order to determine the comparability between manual and automated process steps for the culture of a ‘Ruler’ hESC line. This allowed for further process experience to be gained and for additional evidence regarding process comparability after a change to a single process steps to be collected. The research in the present chapter therefore contributes to the second challenge addressed in this thesis regarding comparability in cell-based therapies. Furthermore, the present chapter examines the comparability between alternative process
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steps for the culture of a candidate reference standard, or ‘Ruler’, hESC line, which may represent a valuable tool in the development of hESC manufacturing processes.

From these experiments, it was determined that comparability was achieved in Ruler hESC morphology, stability, viability and characterisation between manual and automated process steps. A lack of comparability was observed in Ruler hESC yield and population doubling time between process steps, with a greater cell yield and shorter population doubling time observed after culture utilising the automated process step. Additionally, a trend for greater variability in Ruler hESC yield and population doubling time after culture using the Non-Centrifugation process step may exist. However, these findings may have been associated with cell aggregation and settling observed during the Non-Centrifugation process step.

Therefore, it can be concluded that, although the Ruler hESC line is amenable to culture on an automated platform, it can only be used as a reference standard for the stable parameters in both the manual and automated processes, specifically morphology, stability, viability and characterisation. It is clear that further work is required in order to optimise the Non-Centrifugation process step for the EC 2102Ep cell line, and to generate a consistent hESC standard for both manual and automated culture processes. Furthermore, further work is required in order to remove the requirement for an additional manual dissociation step after both the manual and automated process steps by reducing cell aggregation throughout the dissociation process. Further research should also examine the comparability between the Ruler EC 2102Ep hESC line and alternative hESC lines, as well as further exploring alternatives to the Centrifugation process step.
Chapter 9: Comparison of a Manual, Centrifugation & an Automated, Non-Centrifugation Cell Culture Process Step for a Human iPSC line

In collaboration with TAP Biosystems (Royston, UK) & Institute for Stem cell Therapy and Exploration of Monogenic diseases (CECS, I-Stem, Evry, France)

The aim of this chapter is to determine the comparability between manual and automated hiPSC culture process steps by measuring product and process specifications representative of cell growth, stability, and characterisation.

9.1 Introduction

The epigenetic reprogramming of adult somatic cells into pluripotent stem cells, known as Induced Pluripotent Stem Cells (iPSCs), was first achieved by Takahashi and Yamanaka (2006) through the overexpression of a number of transcription factors, including Oct3/4, Sox2, Klf4, and c-Myc, which were delivered via retroviral vectors. The progression that has been made in iPSC reprogramming is discussed in Chapter 2, and it is apparent that further research is required in order to improve reprogramming efficiency above 1.5%.

Not only do iPSCs share the key advantages of pluripotent stem cells over more lineage restricted somatic cell types, including the capacity to differentiate into cells from all three germ layers and almost unlimited expansion in vitro; but they also can be derived from any adult tissue source, do not share the major ethical concerns associated with Human Embryonic Stem Cells (hESCs), and may represent a source of autologous cells which do not elicit an immune response. Additionally, ‘haplobanking’ may allow for the generation of numerous Human iPSC (hiPSC) banks, each of which is matched to a Human Leukocyte Antigen (HLA) haplotype present within in a population. These hiPSC banks can then be utilised to generate HLA matched cell therapies for large numbers of patients with the relevant haplotype, which therefore exhibit a reduced immunogenicity. Although there is currently a lack of clarity regarding the immunogenicity of iPSC derived cells, as discussed further in sections 2.4.1 and 5.5, these cells are likely to be highly valuable in drug discovery, in vitro disease modelling, and cell therapy.

However, if cell therapies derived from hiPSCs are to gain adoption and become readily available in healthcare, consistent and scalable manufacturing processes will be essential. Lack of control and inconsistency in critical parameters during manual cell culture often leads to significant inter-individual variation in many laboratories (Terstegge et al, 2007). In
contrast, the automation of cell culture holds significant promise for the improvement of quality control, scalability, reproducibility and economics of the process by controlling cell density, fluid flow, centrifugal force, pH and temperature (Archer & Williams, 2005; Veraitch et al, 2008). Although suspension, microcarrier or perfusion based bioreactor systems are likely to be more effective manufacturing platforms for allogeneic cell products in order to achieve the greatest cell yield, automated platforms may allow for the consistent manufacture of autologous cell products at a low cost. Furthermore, the level of comparability with manual manufacturing processes and the reduction of product comparability risk, which is often associated with manufacturing platform transfers, make monolayer culture-based automated platforms a feasible option for manufacturers of allogeneic or autologous cell therapy products. However, as with hESCs, the development of an automated hiPSC culture platform is hindered by the inherent sensitivity of these cells. Therefore, further research is required before the development of a consistent hiPSC manufacturing process can be achieved.

In the present study, a novel investigation comparing the effects of alternative process steps upon the growth and characteristics of hiPSCs will allow for an improved understanding of the effects of the automated process upon these cells and may allow for further optimisation in the future. The CompacT SelecT automated cell culture platform was utilised and the automated protocols for the seeding of a hiPSC line (VAX001024c07) in T75 flasks, the passage and seeding of these cells into T175 flasks, the performance of medium exchanges for cell populations cultured in T175 flasks, and the harvest of confluent cell populations in multiple T175 flasks were provided by I-Stem (Evry, France). Therefore, by undertaking a protocol transfer and replicating the VAX001024c07 culture processes at an alternative location (Centre for Biological Engineering, Loughborough University, UK), comparability between sites can be demonstrated. For biological products where multiple manufacturing sites are necessary, the generation of comparable products across these sites is crucial in order to achieve regulatory approval (Chirino & Mire-Sluis, 2004), which may often be the case for autologous cell therapies.

However, the transferred protocols provided required adaptation due to the lack of T75 flask handling capability and a lack of an incorporated Incucyte™ (Essen BioScience, Michigan, USA), for automated measurement of culture confluency, on the platform located in the Centre for Biological Engineering (Loughborough University, UK) laboratories. New protocols were generated to allow for the initial seeding of T175 flasks and for the passage of
1xT175 flask into 2xT175 flasks. Daily microscopy was utilised to measure culture confluency, and a number of test experimental runs were performed to determine the required confluency prior to passage and the duration of culture required to reach confluency. The automated protocols were also adapted to allow for the incorporation of the centrifugation process step in the manual process arm of the Centrifugation and Non-Centrifugation experiment.

The recommended passage procedure for the VAX001024c07 hiPSC line involves the dissociation of the cells into a single cell suspension and their re-seeding onto fresh, Matrigel-coated tissue culture plastic as single cells. Significant debate exists regarding whether pluripotent stem cells should be cultured and passaged in colonies or as a single cell suspension. A number of papers have identified that the dissociation of pluripotent stem cells, particularly hESCs, into a single cell suspension can lead to apoptosis (Mitalipova et al, 2005; Ohgushi et al, 2010). In particular, E-cadherin mediated cell to cell cohesion has been proposed as playing an important role in the survival and self-renewal of pluripotent stem cells, and the breakdown of this interaction during the dissociation step has been linked to the detrimental effects of enzymatic passaging (Li et al, 2012). However, conflicting data has been published demonstrating the maintenance of hESC morphology over 10 passages despite trypsinisation into single cells (Di Stefano et al, 2010). The generation of single cell suspensions has also been highlighted as an important factor in the maintenance of pluripotency in iPSCs (Koike, 2012).

Although the feasibility of scalable, automated culture of iPSCs in aggregates utilising the CompacT SelecT automated cell culture platform has previously been demonstrated (Soares et al, 2014), this method is not achievable without significant process development and optimisation. Therefore, in the present study, the hiPSCs were seeded as single cells, cultured as a monolayer and dissociated into a single cell suspension. Additionally, the VAX001024c07 hiPSC line was previously routinely cultured as single cells and the automated protocols for the culture of these cells were provided by I-Stem. As part of the automated culture process for the VAX001024c07 hiPSC line, Rho-associated protein kinase (ROCK) inhibitor was added to the culture during the initial seeding, passage and cryopreservation processes, as well as on Day 1 after initial seeding or passage. ROCK inhibitor has been shown to allow for the survival and maintenance of pluripotency of pluripotent stem cells after dissociation (Watanabe et al, 2007). The addition of ROCK
inhibitor was particularly important for the successful culture of the VAX001024c07 hiPSC line on the CompacT SelecT platform as colony culture was not feasible.

Furthermore, ROCK inhibitor has been demonstrated to improve both hESC and hiPSC viable cell recovery, colony formation and cell attachment after cryopreservation (Li et al, 2008; Martin-Ibanez et al, 2008; Li et al, 2009; Claassen et al, 2009; Mollamohammadi et al, 2009). However, as discussed later in section 9.4, although hiPSC recovery after cryopreservation may have been improved with the addition of ROCK inhibitor, the recovery of hiPSCs was still negatively affected by the cryopreservation process.

Therefore, the aim of the present study was to measure and establish the comparability between process steps and manufacturing sites for the culture of the hiPSCs. This investigation examined the effects of manual and automated process steps upon the viable cell yield, viability, aggregate rate, STR profile and pluripotency marker expression of the VAX001024c07 hiPSC line. The null hypothesis for this study was that no significant difference in cell yield, viability, aggregate rate, STR profile and pluripotency marker expression would be observed between the hiPSCs cultured utilising either the Centrifugation or the Non-Centrifugation process steps.

9.2 Methods

In this section, the specific materials and methods utilised for the hiPSC experiments, which are presented in this chapter, are outlined in greater detail than those described in the Materials & Methods Chapter (Chapter 6).

9.2.1 hiPSC Culture- Initial Expansion

The initial expansion of the hiPSCs was performed manually, following the automated process steps and parameters as closely as possible, whilst the initial testing and adaptation of the I-Stem protocols was performed. Due to the large number of cells obtained from each passage, a single passage was performed and only four T175 flasks were pooled during the cell banking process to generate a sufficient number of cells. A sufficient number of cells per vial (4x10^6 cells) were banked in order to allow for the presence of excess cells when initially seeding a T175 flask in the CompacT SelecT using a protocol which seeds each flask with the specified number of cells/cm² (2x10^4 cells/cm² or 3.5x10^6 cells/T175 flask)

- 2 vials of I-Stem VAX001024c07 hiPSCs (P22+9) were removed from cryostorage, thawed and each seeded manually onto a Matrigel-coated T75 flask at a density of 2x10^4
cells/cm² (1.5x10⁶ cells/T75 flask) in complete mTESR™1 medium (StemCell Technologies, Vancouver, Canada). Matrigel™ (BD Biosciences, San Jose, USA) was diluted with Knockout™ DMEM (322.5μg/ml) (Life Technologies, Thermo Fisher Scientific, Waltham, USA).

- A complete mTESR1 medium exchange was performed each day for every T75 or T175 flask after initial seeding or passage.
  - A medium exchange with ROCK inhibitor (10mM) (Y-27632, StemCell Technologies, Vancouver, Canada) was performed the day after both initial seeding and passage, as well as 1 hour prior to passage.

- Once the cells reached ~80% confluency, after approximately 7 days, the mother flasks were each passaged into two Matrigel-coated T175 flasks (2 T75 flasks → 4 T175 flasks) at a density of 2x10⁴ cells/cm² (3.5x10⁶ cells/T175 flask).
  - The cells were washed and dissociated with Accutase™ (StemCell Technologies) and were incubated for 10 minutes, or until it was visually confirmed that the cells had detached, at 37°C and 5% CO₂.

- Once the four daughter flasks reached ~80% confluency, the cells were prepared for cryopreservation.
  - The hiPSCs were dissociated as described previously, neutralised with 12ml of mTESR1 medium, and were pooled into a single T175 flask for every two T175 flasks with 10mM of ROCK inhibitor.
  - A cell count was performed, each of the two pooled cell suspensions were aliquoted into two 50ml centrifuge tubes, and the cells were centrifuged at 276 RCF for 10 minutes at 4°C.
  - The supernatant was then aspirated and the cells were resuspended in cold (2-8°C) Cryostor® CS10 (StemCell Technologies) freeze medium at 4x10⁶ cells/ml.
  - 1ml of the cell suspension was then transferred into each cryovial (4x10⁶ cells/vial). These cryovials were then placed into a ‘Mr Frosty’ container and stored at -80°C for 24 hours before being transferred into cryostorage.
  - A total of 101 cryovials of I-Stem VAX001024c07 hiPSCs were generated to make up the cell bank (P22+11).
9.2.2 hiPSC Protocol Transfer- Determination of required confluency at passage

As previously described in section 9.1, the original automated protocols for the culture of the VAX001024c07 hiPSC line were provided by I-Stem. These protocols required adaptation to allow for the initial seeding of T175 flasks, due to the lack of T75 handling capability of the CompacT SelecT used in the present study, and involved the maintenance of the initial seeding density (2x10^4 cells/cm^2), although the appropriate seeding density was explored in the protocol transfer experiments reported in this section. New T175 flask passage protocols were also generated, as the protocols provided by I-Stem were designed to allow for the passage of 1xT75 to 2xT175 flasks. Furthermore, rather than utilise the 2xT175 to 4xT175 flask passage protocol in order to passage two daughter flasks, it was decided that the 1xT175 to 2xT175 passage protocol would be utilised for each of the two daughter flasks. This method would isolate 8x10^6 cells from each daughter flask and seed two granddaughter flasks at the appropriate cell density, rather than pooling all the cells from each of the two daughter flasks. In order to pool the cells from each of the two daughter flasks and dilute to the relevant concentration (1x10^6 cells/ml), a significant volume of culture medium would have been required and this would have unnecessarily increased resource use and cost. Although it could be argued that the proposed pooling process during the passage protocols would reduce the heterogeneity between cultures, the cells were pooled prior to cryopreservation and therefore variability between vials was minimised. In addition to the adaptation of the automated passage protocols in order to account for flask surface area and to remove pooling, the passage protocols were also altered to allow for the incorporation of the centrifugation process step during the Centrifugation experimental runs.

However, one of the most significant required changes to the culture processes developed by I-Stem, involved the transition from automated confluency measurement, using the Incucyte (Essen Biosciences, MI, USA), to manual confluency measurement, using microscopy. This adaptation was required due to the lack of an available inbuilt Incucyte in the CompacT SelecT utilised in the present study. Therefore, hiPSC confluency was assessed manually using microscopy to determine the point at which the cells required passage. In order to determine the approximate duration of culture required to achieve the recommended 80% confluency necessary before passaging, and to allow the operators to gain experience of the nature of this hiPSC at confluence, a protocol transfer experiment was performed. Although the utilisation of manual confluency measurement increased the risk of passaging cell populations that were under- or over-confluent, the process experience gained throughout the
protocol transfer mitigated this risk. This process experience also allowed approximate cell population confluency to be correlated with time in culture, as described further in this section.

In this protocol transfer experiment, two T175 flasks were seeded at 2x10^4 cells per cm^2 (3.5x10^6 cells/flask) using the automated seeding protocol. A medium exchange was performed every 24 hours for each flask, adding ROCK inhibitor (10mM) when appropriate. Additionally, microscopy images were taken every 24 hours in order to monitor cell growth and the nature of the cultures. These cells were allowed to proliferate until they reached and appropriate confluency, at which point the cells were passaged into two new daughter flasks per mother flask, and a cell count was measured.

However, from this experiment it was determined that recovery of hiPSCs after cryopreservation was poor, illustrated by the low cell adherence and low cell density early in the hiPSC cultures. The poor recovery of pluripotent stem cells after cryopreservation has been widely reported in the literature, and although the addition of ROCK inhibitor to pluripotent stem cell cultures has been found to improve the percentage of viable cells after cryopreservation, the achievement of over 80% viable cell recovery is rare (Li et al, 2009; Martin-Ibanez et al, 2008; Claassen et al, 2009). With regards the recovery of hiPSCs after cryopreservation, a number of studies have reported viable cell recovery percentages ranging from 20-60% depending upon the cryoprotective agent and freezing method utilised (Mollamohammadi et al, 2009; Katkov et al, 2001; Nishigaki et al, 2011). Given the freezing method utilised in the present study, it is likely that a DMSO-based freezing medium, such as the CryoStor CS-10 medium (Stem Cell Technologies) used in the protocols for the cryopreservation of the VAX001024c07 hiPSC line, would be one of the most effective cryoprotective agents (Katkov et al, 2011).

Once the cells had been seeded after cryopreservation, it was noted that the cell density appeared to be lower than expected, despite the use of a cell count to seed the appropriate number of cells (3.5x10^6 cells/T175 flask). It was observed that the number or cells per cm^2 of daughter flasks seeded after a passage was significantly greater than that of mother flasks seeded after cryopreservation. The differences in cell adherence after seeding are illustrated in Figures 89a-d. This led to slower cell growth and therefore lower cell yield after thawing compared to after a passage when comparing the same duration of culture, as demonstrated in Figure 93.
It was also noted that, once the cells had been seeded after cryopreservation and were approaching confluency, the cells began to aggregate and grow in a three-dimensional manner, growing upwards and on top of one another, rather than as a monolayer into the empty surfaces of the tissue culture flask. This phenomenon was identified to occur at around Days 6-8 of culture and can be identified in Figures 91a-d. When the hiPSCs were allowed to proliferate further after this point, for example until Day 10 after initial seeding, although a greater cell yield and greater confluency across the flask would be obtained, the cell populations exhibited a less uniform morphology, indicative of greater numbers of spontaneously differentiated cells; and a greater amount of cell aggregation occurred. It is also possible that these observed changes may have had a detrimental effect upon the characteristics of these cells, for example pluripotent marker expression. In contrast, earlier stage cultures, for example at Day 7 (Figure 91a), despite delivering lower cell yields and a lower overall coverage of the tissue culture flask, exhibited a more uniform cell morphology, indicative of fewer spontaneously differentiated cells, and a lower amount of cell aggregation, which may provide less variation and greater homogeneity of cells compared to later stage cultures.

Figures 89a, b, c, d: Microscopy images of hiPSC Non-Centrifugation Mother (10x magnification) (Top, Left), Centrifugation Mother (10x magnification) (Top, Right), Centrifugation Daughter (4x Magnification) (Bottom, Left), and Non-Centrifugation Daughter (10x magnification) (Bottom, Right) flasks at Day 1 after initial seeding.
Therefore, from this protocol transfer experiment, it was determined that, in order to obtain hiPSC populations with consistent growth and morphology prior to the commencement of the Centrifugation and Non-Centrifugation comparability experiment, and to avoid the detrimental effects of poor cell recovery after cryopreservation, a pre-comparability experiment passage, referred to as the ‘Pre-Experimental passage’, would be performed. Initially, the appropriate number of cryovials was thawed and hiPSCs were seeded into a T175 flask using the automated seeding protocol. These cells were allowed to proliferate until they reached sufficient confluency for the cells to have formed large colonies and the cells...
had begun to aggregate, at which point the cells were passaged into a new daughter flask. After this pre-experimental passage had been performed, the Centrifugation and Non-Centrifugation comparability experimental runs were initiated.

It can be concluded that the improved cell recovery observed in daughter flasks, and the more even distribution of cells across the tissue culture flask after a passage, led to a more even coverage of cells with less cell aggregation compared to mother flasks seeded after cryopreservation, in which significant cell death and lack of adherence is observed. Whereas, in the case of mother flasks seeded after cryopreservation, hiPSCs tended to form colonies, with significant aggregation and three dimensional growth, yielding large areas of the tissue culture flask with a low cell density. Thus, it is clear that the cells seeded after a passage reached 80% confluency in a monolayer more rapidly than those seeded after cryopreservation.

In addition to comparing the proliferation of hiPSCs after either cryopreservation or passage, the effect of increasing the seeding density after cryopreservation was examined. The original seeding density, outlined in the I-Stem culture protocols for the VAX001024c07 hiPSC line (2x10^4 cells/cm²), was compared to an increased seeding density of 2.7x10^4 cells/cm². From this comparison, it is apparent that, although the increased seeding density did not reduce the culture duration required to achieve 80% confluency (~10 days), a more homogenous hiPSC population with a more evenly distributed monolayer was generated, as illustrated in Figures 92a & b (below). Although the growth characteristics of the hiPSC populations may have improved with an increased seeding density after cryopreservation, this does not remove the requirement for a pre-experimental passage to ensure an accurate seeding density at the initiation of the alternative process step comparability experiment. However, these findings suggest that a greater seeding density should be utilised during the pre-experimental passage to generate a more homogenous hiPSC population. Therefore, an initial seeding density of 2.7x10^4 cells/cm² (4.725x10^6 cells/flask) was utilised post-cryopreservation throughout the experiment, rather than the recommended 2x10^4 cells/cm².
Furthermore, a complete medium exchange, including the addition of ROCK inhibitor, was performed shortly after seeding (~4 hours), once the viable cells had adhered, in order to remove any dead or non-adherent cells that may detrimentally affect the growth of the viable hiPSC population via apoptotic signalling.

**Figures 92a & b:** Microscopy images of a hiPSC Non-Centrifugation Mother flask (2x10^4 cells/cm²) at Day 10 (10x Magnification) (a) and a Mother flask (2.7x10^4 cells/cm²) at Day 10 (10x magnification) (b) after initial seeding. White arrows indicate areas of differentiated cells.
Figure 93: Viable Cell Yield of Centrifugation & Non-Centrifugation hiPSCs after varying durations of culture. A comparison of hiPSC Viable Cell Yield after thawing and after passage.
9.2.3 hiPSC Culture- Centrifugation Experiment

- As described in section 9.2.2, a pre-experimental passage and initial seeding protocol were utilised in order to mitigate the detrimental effects of cryopreservation.

- Two vials of hiPSCs (4x10^6 cells/cryovial) were thawed, pooled and suspended in 10ml of pre-warmed (37˚C) mTESR1 medium.
  - 2 vials pooled for initial seeding due to lack of recovery of sufficient cell numbers from a single cryovial (4x10^6 cells/vial) to seed a T175 flask at the appropriate seeding density (4.75x10^6 cells/T175 flask).

- The cell suspension was then centrifuged at 276 RCF for 5 minutes, and the supernatant was aspirated to remove any cryopreservation medium. The cell pellet was then resuspended in 10ml complete mTESR1 medium with 10µl ROCK inhibitor (10mM) and transferred into a 50ml centrifuge tube.

- This 50ml centrifuge tube, containing the pooled hiPSCs, was placed in the static holder of the CompacT SelecT and an automated seeding protocol was performed.
  - In this seeding protocol, the cells are mixed, a cell count is performed, the cells are diluted with mTESR1 medium to 2.375x10^5 cells/ml, and 20ml of this suspension (4.75x10^6 cells) is transferred into a new Matrigel coated T175 flask.

- A medium exchange was performed approximately 4 hours after seeding, once the viable cells had adhered to the flask, to remove dead or non-adherent cells.

- 24 hours after initial seeding, a medium exchange with 27ml of mTESR1 and 3ml of 10µM ROCK inhibitor solution (2.97ml mTESR1 & 30µl ROCK inhibitor) was performed. After Day 1, a medium exchange with 30ml of mTESR1 was performed every 24 hours.

- Once the cells reached approximately 80% confluency (~10 days), determined visually using microscopy, a Pre-Experimental passage was performed and an automated Pre-Centrifugation protocol was utilised in order to dissociate the cells with Accutase (10 minute incubation), agitate any non-dissociated cells, quench with mTESR1 and perform a cell count.
  - Cell counts were utilised to obtain Viable Cell Density, Viability, Cell Diameter, and Aggregation Rate data. Viable Cell Yields were calculated by multiplying the Viable Cell Density by the volume of the cell suspension.
  - The T175 flask containing the dissociated cells was then outfed, and the cell suspension transferred into a 50ml centrifuge tube for centrifugation (276 RCF x
5mins). After centrifugation, the supernatant was aspirated, the cell pellet was resuspended in 25ml of mTESR1, the cells were thoroughly mixed and reintroduced into the outfedded T175 flask, and the flask was imported back into the CompacT SelecT.

- Next, an automated Post-Centrifugation protocol was utilised to perform a cell count, add 3ml of 10μM ROCK inhibitor solution, isolate 8x10⁶ cells, dilute isolated cells to 3.5x10⁵ cell/ml, seed one Matrigel-coated daughter flask (1→1 T175 flasks) with 3.5x10⁵ cells and add additional mTESR1 and ROCK inhibitor solution to each daughter flask. The process diagram presented in Figure 94 demonstrates the differences between the manual, Centrifugation and the automated, Non-Centrifugation processes.

- 24 hours after seeding of the daughter flasks, a medium exchange with 27ml of mTESR1 & 3ml of 10μM ROCK inhibitor solution was performed for each daughter flask. After Day 1, a medium exchange with 30ml of mTESR1 was performed every 24 hours.

- Once the cell populations had reached approximately 80% confluency (~Day 7), determined visually using microscopy, identical Pre-Centrifugation, Centrifugation and Post-Centrifugation protocols were performed in order to obtain cells counts and to passage each daughter flask into 2 new granddaughter flasks (1→2 T175 flasks).

- Daily medium exchanges were again performed, with 10μM ROCK inhibitor solution added on Day 1.

- Once 80% confluent (~Day 7), identical Pre-Centrifugation, Centrifugation and Post-Centrifugation protocols were performed in order to obtain cells counts and to passage each daughter flask into 4 new great-granddaughter flasks (2→4 T175 flasks).*

- Daily medium exchanges were again performed, with 10μM ROCK inhibitor solution added on Day 1.

- Once 80% confluent (~Day 7), the cells within the four Centrifugation great-granddaughter flasks were harvested for cryopreservation using an automated final dissociation protocol.
  - After a 1 hour pre-treatment with 10μM ROCK inhibitor solution, the culture medium of each flask was poured off, an Accutase wash step was performed, and Accutase was applied in order to dissociate the cells using a 10 min incubation period at 37˚C. The dissociation step for all four flasks was performed in parallel.

* Centrifugation Granddaughter flask cell count data could not be collected for the fourth batch due to a malfunction of the Cedex Automated Cell Counter.
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- After incubation, mTESR1 was added in order to quench the dissociation agent and the flasks were agitated in order to ensure dissociation. Detached cells in suspension from each of the four flasks were then pooled into an empty flask, and the pooled flask was outfeeded from the CompacT Select.

- After collecting the outfed flask, a cell count was performed, 1/8th of the cell suspension was isolated and the isolated suspension was centrifuged at 4°C. Next, the supernatant was aspirated, and the cell pellet was resuspended in Cryostor® CS-10 (StemCell Technologies) freeze medium at 2.7x10^6 cells/ml. 1.5ml of cells suspended in freeze medium was added into each cryovial (4x10^6 cells/vial). The cryovials were then placed in a ‘Mr Frosty’ container and stored at -80°C for 24 hours. After 24 hours, cryovials were transferred into liquid nitrogen cryostorage.

- This entire culture process was repeated a further three times in order to complete a total of four batches, and no cross-batch pooling was performed.

- A single hiPSC batch was selected for characterisation and STR profiling.

9.2.4 hiPSC Culture- Non-Centrifugation Experiment

- As with the Centrifugation experiment, a pre-experimental passage and initial seeding protocol were utilised in order to mitigate the detrimental effects of cryopreservation.

- Two vials of hiPSCs (4x10^6 cells/cryovial) were thawed, pooled and suspended in 10ml of pre-warmed (37°C) mTESR1 medium.
  - 2 vials pooled for initial seeding due to lack of recovery of sufficient cell numbers from a single cryovial (4x10^6 cells/vial) to seed a T175 flask at appropriate seeding density (4.75x10^6 cells/T175 flask).

- The cell suspension was then centrifuged at 276 RCF for 5 minutes, and the supernatant was aspirated to remove any cryopreservation medium. The cell pellet was then resuspended in 10ml complete mTESR1 medium with 10µl ROCK inhibitor (10mM) and transferred into a 50ml centrifuge tube.

- This 50ml centrifuge tube, containing the pooled hiPSCs, was placed in the static holder of the CompacT Select and an automated seeding protocol was performed.
  - In this seeding protocol, the cells are mixed, a cell count is performed, the cells are diluted with mTESR1 medium to 2.375x10^5 cells/ml, and 20ml of this suspension (4.75x10^6 cells) is transferred into a new Matrigel coated T175 flask.

- A complete medium exchange, with 27ml of mTESR1 and 3ml of ROCK inhibitor solution (2.97ml mTESR1 & 30µl ROCK inhibitor (10mM), was performed.
approximately 4 hours after seeding, once the viable cells had adhered to the flask, to remove dead or non-adherent cells.

- 24 hours after initial seeding, a medium exchange with 27ml of mTESR1 and 3ml of ROCK inhibitor solution (2.97ml mTESR1 & 30µl ROCK inhibitor (10mM) was performed. After Day 1, a medium exchange with 30ml of mTESR1 was performed every 24 hours.

- Once the cells reached approximately 80% confluency (~10 days), determined visually using microscopy, a Pre-Experimental passage was performed using an automated Non-Centrifugation protocol order to dissociate the cells with Accutase (10 minute incubation), agitate any non-dissociated cells, quench with mTESR1, add 3ml of 10µM ROCK inhibitor solution, perform a cell count, isolate 8x10^6 cells, dilute isolated cells to 3.5x10^6 cell/ml, seed one Matrigel-coated daughter flask (1\rightarrow 1 T175 flasks) with 3.5x10^6 cells and add additional mTESR1 and ROCK inhibitor solution to each daughter flask. The differences between the Centrifugation and Non-Centrifugation processes are illustrated in Figure 94.

- 24 hours after seeding of the daughter flasks, a medium exchange with 27ml of mTESR1 & 3ml of 10µM ROCK inhibitor solution was performed for each daughter flask. After Day 1, a medium exchange with 30ml of mTESR1 was performed every 24 hours.

- Once the cell populations had reached approximately 80% confluency (~Day 7), determined visually using microscopy, an identical Non-Centrifugation protocol was performed in order to obtain cells counts and to passage each daughter flask into 2 new granddaughter flasks (1\rightarrow 2 T175 flasks).

- Daily medium exchanges were again performed, with 10µM ROCK inhibitor solution added on Day 1.

- Once 80% confluent (~Day 7), identical Pre-Centrifugation, Centrifugation and Post-Centrifugation protocols were performed in order to obtain cells counts and to passage each daughter flask into 4 new great-granddaughter flasks (2\rightarrow 4 T175 flasks).*

- Daily medium exchanges were again performed, with 10µM ROCK inhibitor solution added on Day 1.

- Once 80% confluent (~Day 7), the cells within the four Non-Centrifugation great-granddaughter flasks were harvested for cryopreservation using an automated final dissociation protocol.

* Non-Centrifugation Granddaughter flask cell count data could not be collected for the fourth batch due to a malfunction of the Cedex Automated Cell Counter
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- After a 1 hour pre-treatment with 10μM ROCK inhibitor solution, the culture medium of each flask was poured off, an Accutase wash step was performed, and Accutase was applied in order to dissociate the cells using a 10 min incubation period at 37°C. The dissociation step for all four flasks was performed in parallel.
- After incubation, mTESR1 was added in order to quench the dissociation agent and the flasks were agitated in order to ensure dissociation. Detached cells in suspension from each of the four flasks were then pooled into an empty flask, and the pooled flask was outfeeded from the CompacT SelecT.

- After collecting the outfeeded flask, a cell count was performed, 1/8th of the cell suspension was isolated and the isolated suspension was centrifuged at 4°C. Next, the supernatant was aspirated, and the cell pellet was resuspended in Cryostor® CS-10 (StemCell Technologies) freeze medium at 2.7x10^6 cells/ml. 1.5ml of cells suspended in freeze medium was added into each cryovial (4x10^6 cells/vial). The cryovials were then placed in a ‘Mr Frosty’ container and stored at -80°C for 24 hours. After 24 hours, cryovials were transferred into liquid nitrogen cryostorage.
- This entire culture process was repeated a further three times in order to complete a total of four batches, and no cross-batch pooling was performed.
- A single hiPSC batch was selected for characterisation and STR profiling.

**Figure 94:** Diagram illustrating the differences between the manual and automated hiPSC passage process steps.
9.2.5 Pluripotency Marker Expression

- One vial of each of the Baseline, Centrifugation and Non-Centrifugation hiPSCs was thawed, the cryoprotectant was removed, and the cells were resuspended in complete mTESR1. A cell count was performed and hiPSCs were seeded in T75 flasks at 27,000 cells/cm² (2.025x10⁶ cells/flask). The cells were then cultured until ~80% confluent with medium exchanges performed daily.

- hiPSCs were then prepared for multicolour flow cytometry and analysed following the manufacturer’s instructions (BD Stemflow, BD Biosciences), as follows:
  - Once confluent, the cell monolayer was washed with PBS, the cells were dissociated using Accutase, the cells were washed with PBS, and a cell count was performed for each population.
  - The cells were then fixed using Cytofix Fixation Buffer (4% PFA) (BD Biosciences), at a concentration of 1x10⁷ cells/ml, and incubated at room temperature for 20mins. The cells were then washed and resuspended in stain buffer (BD Biosciences) at a concentration of 1x10⁷ cells/ml.
  - 100µL of unstained cells from each condition were added to separate polystyrene flow cytometry tubes (BD Biosciences) and placed in the dark at room temperature for later analysis.
  - To stain the fixed cells remaining for each condition, 100µL of fixed cells was added to two separate flow cytometry tubes. To one tube, labelled ‘Specific Stain’, 20µL of the FITC SSEA-1, PE SSEA-3 and Alexa Fluor 647 TRA-1-81 antibodies were added. To the other, labelled ‘Isotype Control’, 20µL of the FITC, PE and Alexa Fluor 647 isotype controls was added. Replicates of each tube were generated.
  - The tubes were then mixed and incubated in the dark on ice for 30mins. After incubation, each tube was washed twice in stain buffer and resuspended in 350µL of stain buffer.
  - To prepare the control beads, 100µL of stain buffer and 1 drop of negative beads were added to four flow cytometry tubes (Negative, FITC, PE, Alexa Fluor 647). One drop of the appropriate positive beads and 20µL of the appropriate antibody was then added to the relevant tubes. After incubation at room temperature in the dark for 30mins, the beads were washed twice with stain buffer and resuspended in 350µL of stain buffer.
The hiPSC samples were analysed on the BD FACSCanto II (BD Biosciences) which was operated using the FACSDiva software version 6.1.3. The beads and unstained cells were analysed first in order to generate controls, and this was followed by the subsequent analysis of the Baseline, Centrifugation and Non-Centrifugation hiPSCs at the single cell level. For analysis, the flow cytometry data was exported in FCS 3 format and analysed using FlowJo software v10.

9.2.6 CLA & STR Profiling

- STR profiling was performed by LGC standards (Teddington, UK) through their CLA service.
- Samples were prepared for transport and CLA analysis following the protocol provided by LGC standards.
- 1 vial of baseline hiPSCs, 1 vial of Post-Centrifugation hiPSCs, and 1 vial of Non-Centrifugation hiPSCs were removed from cryostorage, thawed and centrifuged.
- The supernatant was then aspirated and a PBS wash was applied, in order to remove any remaining freeze media.
- The samples were again centrifuged and the supernatant aspirated in order to isolate the cell pellets.
- Finally, the samples were resuspended in 400μl of transport buffer, provided by LGC standards, which lyses the cells and preserves the gDNA.
- The samples were then packaged and sent to LGC Standards, where the CLA analysis was undertaken and the STR profiles for each sample were compared.

9.2.7 Statistical Analyses

Experimental data regarding the Viable Cell Density, Viable Cell Yield, Population Doubling Time, Viability, Cell Diameter, and Aggregate Rate of Pre-Centrifugation, Post-Centrifugation and Non-Centrifugation hiPSCs across all passages was analysed using Two-Way Analysis of Variance (ANOVA) multiparameter analyses, through the IBM SPSS statistical software, to determine significant differences. Furthermore, One-Way ANOVAs were utilised to assess the significance of differences in Viable Cell Density, Viable Cell Yield, and Viability of Pre-Centrifugation, Post-Centrifugation and Non-Centrifugation hiPSCs within the 2nd passage. One-Way ANOVAs were also used to assess the significance of differences between the Standard Deviations (SD) of the Viable Cell Densities, Viable Cell
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Yields, and Viabilities of Pre-Centrifugation, Post-Centrifugation, and Non-Centrifugation hiPSCs, in each of the four batches, from the second passage. The cutoff value for statistical significance (p) was set at 0.05. Tukey’s Honest Significant Difference (HSD) Post-Hoc Tests were used to perform multiple comparisons of the hMSC growth and paracrine functionality data.

9.3 Results

9.3.1 Morphology

By comparing hiPSC populations early in culture, after seeding (Days 0-1) during which Rho-associated protein kinase (ROCK) inhibitor was added at each medium exchange, and later in culture (≥Day 2), during which ROCK inhibitor was not added, it is clear that ROCK inhibitor has an impact upon cell morphology. ROCK inhibitor is added to pluripotent stem cell cultures in order to allow for survival and to maintain pluripotency after dissociation (Watanabe et al, 2007). When ROCK inhibitor was added to early cultures (Days 0-1), the cell populations consisted primarily of single cells with multiple long, thin lamellipodia (Figures 95 & 96). However, in later cultures when ROCK inhibitor was not added (≥Day 2), the cells clustered together and formed small colonies, demonstrating a rounded morphology (Figures 97 & 98). Furthermore, when ROCK inhibitor is removed, there appears to be an increase in cell death which may result in a small reduction in cell number and confluency.

Also, over the course of the culture period, spontaneous differentiation of the hiPSCs was observed, and these differentiated cells were identified visually by their larger size, larger nuclei, and location towards the edges of undifferentiated cell colonies. However, these differentiated cells represented a small proportion of the total cell population. Differences in the level of spontaneous differentiation throughout culture were not apparent between Centrifugation and Non-Centrifugation hiPSC cultures.

When hiPSC populations are compared after Centrifugation and Non-Centrifugation process steps, it is apparent that no difference in morphology is observed both early (Day 0-1), with ROCK inhibitor, and later in culture (≥Day 2), without ROCK inhibitor. However, hiPSCs cultured using the Non-Centrifugation process step appeared to form colonies more rapidly than cells cultured using Centrifugation process step, despite the addition of ROCK inhibitor early in culture (Days 0-1).
Figure 95 (Left): Batch 3 hiPSC Centrifugation (Ce) Daughter Flask Day 1. Figure 96 (Right): Batch 3 hiPSC Non-Centrifugation (NC) Daughter Flask Day 1.

Figure 97 (Left): Batch 3 hiPSC Centrifugation (Ce) Daughter Flask Day 4. Figure 98 (Right): Batch 3 hiPSC Non-Centrifugation (NC) Daughter Flask Day 4.

9.3.2 Cell Diameter

<table>
<thead>
<tr>
<th>hiPSC Batches 1-4 Centrifugation &amp; Non-Centrifugation Average Cell Diameter</th>
</tr>
</thead>
<tbody>
<tr>
<td>Average Cell Diameter (μm)</td>
</tr>
<tr>
<td>Pre-Exp. Pre-Cent. Pre-Exp. Post-Cent. Pre-Exp. Non-Cent. 1st Passage Pre-Cent. 1st Passage Post-Cent. 1st Passage Non-Cent. 2nd Passage Pre-Cent. 2nd Passage Post-Cent. 2nd Passage Non-Cent.</td>
</tr>
</tbody>
</table>

Figure 99: Batches 1-4 (n=4) Pre-Centrifugation, Post-Centrifugation & Non-Centrifugation hiPSC Average Cell Diameter over the Pre-Experimental (4 Replicates), 1<sup>st</sup> (4 Replicates), and 2<sup>nd</sup> Passages (6 Replicates). Standard Deviations are plotted as error bars. * Denotes significance over 1<sup>st</sup> and 2<sup>nd</sup> Passages (p=0.05).
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After comparing the cell diameter of Pre-Centrifugation, Post-Centrifugation and Non-Centrifugation hiPSCs from the Pre-Experimental, 1st and 2nd passages, it was determined that cells from the Pre-Experimental passage, cultured after cryopreservation, were significantly larger than cells from the 1st and 2nd passages regardless of the process step utilised (p=0.000) (Figure 99). No significant difference in cell diameter was found between process steps.

9.3.3 CLA & STR Profiling

Figure 100 (above): VAX001024c07 hiPSC Baseline Electropherogram Short Tandem Repeat (STR) Profile.

Figure 101 (above): VAX001024c07 hiPSC Post-Centrifugation Electropherogram Short Tandem Repeat (STR) Profile.
Figure 102 (above): VAX001024c07 hiPSC Non-Centrifugation Electropherogram Short Tandem Repeat (STR) Profile.

<table>
<thead>
<tr>
<th>Loci</th>
<th>VAX001024c07 Profile</th>
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<tbody>
<tr>
<td>Amelogenin</td>
<td>X,X</td>
</tr>
<tr>
<td>D3S1358</td>
<td>15,16</td>
</tr>
<tr>
<td>TH01</td>
<td>6,6</td>
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</tr>
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<td>13,19</td>
</tr>
<tr>
<td>Penta E</td>
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</tr>
<tr>
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</tr>
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<td>D13S317</td>
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<td>TPOX</td>
<td>10,10</td>
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<tr>
<td>FGA</td>
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</table>

Figure 103 (above): Baseline, Centrifugation & Non-Centrifugation VAX001024c07 alleles at each locus within Short Tandem Repeat (STR) Profile
Figures 100, 101 & 102 demonstrate that no significant difference was observed between the Short Tandem Repeat (STR) profiles of the Baseline, Post-Centrifugation, and Non-Centrifugation hiPSCs, as determined through the comparison of the number of STRs at each allele of each locus between hiPSC samples. Figure 103 outlines the hiPSC STR expression at each allele or each locus profiled, and these were found to be identical between Baseline, Centrifugation and Non-Centrifugation samples. This data suggests that neither the automated nor the manual process steps elicited an alteration in the pattern of short tandem repeats expressed in the hiPSC DNA. Differences in fluorescence peak size between samples may have been observed, however, as described in previous chapters, this does not represent a significant difference between STR profiles.

9.3.4 Pluripotency Marker Expression

![Figure 104](above): Scatter plots demonstrating multicolour flow cytometric analysis of pluripotency and differentiation marker co-expression of Baseline hiPSCs (P22+11).

![Figure 105](above): Scatter plots demonstrating multicolour flow cytometric analysis of pluripotency and differentiation marker co-expression of Centrifugation hiPSCs (P22+14).
Analysis of the immunophenotype of Baseline, Centrifugation and Non-Centrifugation hiPSCs revealed that the expression of the pluripotency markers SSEA-3 and TRA-1-81 was high in all hiPSC populations. Furthermore, the majority of cells in each hiPSC population co-expressed these pluripotency markers (>50%). It was also identified that the expression of SSEA-1, a marker of differentiated cells, was low in all hiPSC populations (<20%). These findings indicate that each of the Baseline, Centrifugation and Non-Centrifugation hiPSC populations contained predominantly pluripotent cells.

However, after comparing the multicolour flow cytometry data of hiPSC populations from each condition, it was observed that Centrifugation hiPSC populations exhibited a reduced pluripotency marker expression, and an increased differentiation marker expression, compared to both Baseline and Non-Centrifugation hiPSC populations. This reduced pluripotency marker expression and increased differentiation marker expression in Centrifugation hiPSC populations compared to other conditions would suggest that more differentiated cells were present in Centrifugation populations, and that the utilisation of the Non-Centrifugation process step maintained hiPSC pluripotency more effectively than the utilisation of the Centrifugation process step.
9.3.5 Growth Data

From the Viable Cell Density data presented above (Figure 107), it was determined that the Viable Cell Density of hiPSCs in the 1\textsuperscript{st} (P35/P22+13) (p=0.003) and 2\textsuperscript{nd} (P36/P22+14) (p=0.000) passages were significantly greater than in the Pre-Experimental passage (P34/P22+12). However, no significant difference was observed between process steps over all three passages. Furthermore, no significant difference in the variability in Viable Cell Densities between process steps in the 2\textsuperscript{nd} passage (P36/P22+14) was identified, as demonstrated by differences between Standard Deviations.
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**Figure 108:** Box plot showing the Mean, Interquartile range, and Range of Viable Cell Densities for Pre-Centrifugation, Post-Centrifugation & Non-Centrifugation hiPSCs in the 2ndPassage (P36/P22+14, 2 Replicates per batch) over three batches (n=3).

**Figure 109:** Bar chart showing the Mean and Standard Deviation of Viable Cell Densities for Pre-Centrifugation, Post-Centrifugation & Non-Centrifugation hiPSCs in the 2nd Passage (P36/P22+14, 2 Replicates per batch) within each of three batches (n=3).
In Figure 108, the Mean, Interquartile Range, and Range of Pre-Centrifugation, Post-Centrifugation and Non-Centrifugation hMSCs in the 2nd passage and across 3 batches are plotted. In Figure 109, the Mean and Standard Deviation of Pre-Centrifugation, Post-Centrifugation and Non-Centrifugation hMSCs in the 2nd passage within each of three batches are plotted. Only three batches were plotted due to lack of data from the fourth batch of the 2nd passage caused by a malfunction of the Cedex Automated Cell Counter.

From Figures 108 & 109, it is apparent that no significant difference in Viable Cell Density in the 2nd passage, over each of the 3 batches analysed, was observed. Additionally, there was no significant difference in the Viable Cell Density of hiPSCs between process steps, within the 2nd passage.

As illustrated in Figure 110 (above), the Viable Cell Yields of hiPSC populations in the 1st (P35/P22+13) (p=0.003) and 2nd (P36/P22+14) (p=0.000) passage were significantly greater than those in the Pre-Experimental (P34/P22+12) passage regardless of the process step utilised. Pre-Centrifugation hiPSC populations demonstrated significantly greater Viable Cell
Yields compared to Post-Centrifugation hiPSC populations both over all passages (p=0.007), as well as in the 2\textsuperscript{nd} passage alone (p=0.008). Furthermore, Non-Centrifugation hiPSC populations exhibited significantly greater Viable Cell Yields compared to Post-Centrifugation hiPSC populations over all passages (p=0.028), however this was not the case in the 2\textsuperscript{nd} passage alone (p=0.204). It is also apparent from the Viable Cell Yield data, that no significant difference in the variability in Viable Cell Yields between process steps in the 2\textsuperscript{nd} passage was observed, as demonstrated by the differences between Standard deviations.

Figure 111 (below) illustrates the Average Cumulative Viable hiPSC Yield per Batch, over each of the four batches and three passages.

**Figure 111**: The Average Centrifugation & Non-Centrifugation hiPSC Cumulative Viable Cell Yield per batch over the Pre-Experimental (P34/P22+12, 4 Replicates), 1\textsuperscript{st} (P35/P22+13, 4 Replicates), and 2\textsuperscript{nd} (P36/P22+14, 6 Replicates) passages, and across four batches (n=4). Standard Deviations are plotted as error bars.
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Figure 112 (above) illustrates the mean Population Doubling Times (PDTs) for Pre-Centrifugation and Non-Centrifugation hiPSCs at the time of Passage or harvest (Day 7 or 10) in the Pre-Experimental, 1st and 2nd Passages. Pre-Centrifugation hiPSC data was utilised, rather than Post-Centrifugation hiPSC data, in order to exclude the effect of the centrifugation process upon the cell count data. From the mean PDTs, no significant difference was observed between PDT of Centrifugation and Non-Centrifugation hiPSCs over multiple passages. However, it was determined that hiPSC populations in the Pre-Experimental passage, after cryopreservation, exhibited a significantly longer PDT than hiPSC populations in 1st (p=0.007) and 2nd (p=0.001) passages. This would indicate that the cryopreservation process is detrimental to hiPSC proliferation and that one passage is required for hiPSCs to recover their proliferative rate. However, after the Pre-Experimental passage, hiPSC population maintained their PDT over multiple passages.

Mean cumulative population doublings (CPDs) of 17.61 and 17.72 were achieved after culture over three passages utilising the Centrifugation and Non-Centrifugation process steps respectively. Similarly to the PDT calculations, Mean CPDs were calculated based upon Pre-Centrifugation and Non-Centrifugation cell counts.
The PDTs and CPDs for Pre-Centrifugation and Non-Centrifugation hiPSC samples were determined using the following formulae:

\[
PDT = \frac{\text{Time of Final Cell Count (Days) – Time of Seeding (Days)}}{3.32 \times (\log(\text{Final Cell Yield}) – \log(\text{Number of Cells Seeded}))}
\]

\[
CPDs = \frac{\text{Time of Final Cell Count (Days) – Time of Seeding (Days)}}{\text{PDT (Days)}}
\]

In Figure 113 (above), the average number and size of viable hiPSC aggregates from Pre-, Post- & Non-Centrifugation populations in Batches 1-4, over three passages, are plotted. The raw hiPSC estimated live cell aggregate size data is presented in Appendix 13.17. Figure 113 suggests that greater numbers of aggregates were present in Pre-Centrifugation samples compared to Post-Centrifugation samples, regardless of the aggregate size. The aggregate number and size data also indicates that the number of aggregates in Non-Centrifugation samples was similar to that of Pre-Centrifugation samples, but greater than that of Post-Centrifugation samples.
The chart above (Figure 114) illustrates the average aggregate rate of Pre-, Post- & Non-Centrifugation hiPSCs, from all four batches, over three passages. These results indicate that the aggregate rate of hiPSC populations in the Pre-Experimental passage were significantly higher than those from the 1st (p=0.001) and 2nd passages (p=0.017), regardless of the process step utilised. Significantly greater aggregation was also observed in Pre-Centrifugation hiPSC samples when compared to Post-Centrifugation samples over all passages (p=0.05). Additionally, Non-Centrifugation hiPSC populations were also found to have significantly greater aggregate rates compared to Post-Centrifugation populations across all passages (p=0.000).
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The Viability data, as presented in Figure 115 (above), demonstrates that no significant difference in hiPSC Viability was observed between passages. However, Post-Centrifugation hiPSC populations were found to have a significantly greater Viability compared to Non-Centrifugation populations across all passages (p=0.000) and batches. In the 2nd passage alone, both Pre-Centrifugation (p=0.005) and Post-Centrifugation (p=0.000) samples demonstrated significantly greater Viabilities compared to Non-Centrifugation samples. Furthermore, significantly greater hiPSC viability was observed in Post-Centrifugation samples compared to Pre-Centrifugation samples in the 2nd passage (p=0.045). Finally, no significant difference in the variability in hiPSC Viability between process steps was observed, as demonstrated by the differences between the Standard Deviations.

Figure 115: The Mean Viability of Pre-Centrifugation, Post-Centrifugation & Non-Centrifugation hiPSC samples over the Pre-Experimental (P34/P22+12, 4 Replicates), 1st (P35/P22+13, 4 Replicates), and 2nd (P36/P22+14, 6 Replicates) passages, and across four batches (n=4). Standard Deviations are plotted as error bars. * Denotes significance over Non-Centrifugation hiPSCs (p=0.05). # Denotes significance over Pre-Centrifugation hiPSCs in the 2nd passage (p=0.05).
9.4 Discussion

From the protocol transfer performed for the VAX001024c07 hiPSC line, prior to the comparison of manual and automated process steps, it can be concluded that the cryopreservation and thawing processes have a detrimental effect upon the growth of these cells. This is highlighted by the low adherence, and the resulting low cell density, after seeding from cryopreservation. The detrimental effect upon Viable Cell Density and Viable Cell Yield in the Pre-Experimental passage also illustrates this phenomenon, as discussed later in this section.

However, the protocol transfer experiment also revealed that the detrimental effects upon hiPSC growth associated with cryopreservation were partly mitigated by increasing the initial seeding density. This led to a more even distribution of cells across the flasks and more uniform cell morphologies, with fewer differentiated cells. Although, spontaneous differentiation of hiPSCs did occur over the course of the culture period, as confirmed by visual examination, the number of differentiated cells was low in comparison to the number of undifferentiated cells.

Both the protocol transfer and process step comparison experiments also demonstrated that, after the removal of ROCK inhibitor, hiPSCs formed small colonies and therefore that ROCK inhibitor maintained hiPSC populations as single cells. These findings support those of previous authors, and it has been reported that the addition of ROCK inhibitor significantly increased growth and resulted in colony formation of pluripotent stem cells when seeded as single cells (Watanabe et al, 2007). However, it must be recognised that, after the removal of ROCK inhibitor on Day 2, significant cell death occurred, which is likely to substantially reduce the number of viable cells. Although, the detrimental effect of the removal of ROCK inhibitor during culture upon viable cell number has yet to be thoroughly investigated, Krawetz and colleagues (2010) reported a brief decrease in Viable Cell Density after the removal of ROCK inhibitor when culturing hESCs in suspension bioreactors.

As discussed in sections 7.4 and 8.4, the visual examination of cell morphology represents one of the fundamental methods of characterisation, despite the limitations of this method. In the case of pluripotent stem cells, such as hiPSCs, the examination of the morphology of cell populations could be considered a valuable technique for the early identification of differentiated cells. Further research exploring the utilisation of morphology changes in
pluripotent stem cell populations may allow for the development of a method of in-process characterisation, as further discussed in section 11.8.3.

After visual examination using light microscopy, it was determined that single cells with multiple long, thin lamellipodia were generated early in the culture period with the addition of ROCK inhibitor (Days 0-1), and that cell populations observed similar morphologies during this period, regardless of whether the Centrifugation or Non-Centrifugation process step was utilised (Figures 95 & 96). However, after the removal of ROCK inhibitor (≥Day 2), hiPSCs formed small colonies with a rounded morphology. Although no difference was observed between the morphology of these cells when either Centrifugation or Non-Centrifugation process steps were utilised (Figures 97 & 98), it appeared that hiPSCs cultured using the Non-Centrifugation process step formed small colonies more rapidly than those cultured using the Centrifugation process steps. This may suggest that cells cultured using the automated process step may require greater cell to cell interaction. Additionally, it must be noted that no difference in the level of hiPSC spontaneous differentiation was observed during culture, which would indicate that no difference in proportion of pluripotent cells was observed between hiPSC populations cultured using either automated or manual process steps.

In addition to similarities in cell morphology, no significant difference in cell diameter was observed between Pre-, Post- & Non-Centrifugation hiPSCs populations, demonstrating that neither the automated nor the manual process steps significantly influenced cell size. However, it was determined that the cell diameter of hiPSCs from the Pre-Experimental passage (P34/P22+12) was significantly greater than cells from the 1st (P35/P22+13) and 2nd (P36/P22+14) passages, regardless of the batch or process step. This difference in cell diameter further highlights the impact of the cryopreservation process upon hiPSC populations. It may be that the low cell density, caused by the detrimental effect of cryopreservation, provides each cell with a greater surface area to occupy and less cell-to-cell contact, which leads to cells exhibiting larger diameters. Furthermore, reduced cell size has been associated with increased proliferation (Su & O’Farrell, 1998), and therefore the larger cell size observed after thawing may be attributed to the detrimental effect of cryopreservation upon hiPSC growth rate.

In the present study, Short Tandem Repeat (STR) profiles were obtained through LGC Standards as part of their Cell Line Authentication (CLA) Service, in which the difference in STRs at 16 loci within the genome was measured and compared between samples. This
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Analysis was utilised to assess the stability of hiPSC populations after culture using alternative process steps. As discussed in sections 7.4 and 8.4, STR profiling amplifies and compares specific loci on the DNA of multiple cell populations, and is often used in forensic analysis (Butler, 2006; Masters et al, 2001).

The comparison of the STR profiles of the Post-Centrifugation and Non-Centrifugation hiPSC samples against the Baseline samples, as illustrated in Figures 100, 101 & 102, identified no significant difference between samples, due to the expression of peaks in STRs at the same loci. Figure 103 outlines the STR expression of Baseline, Centrifugation and Non-Centrifugation hiPSCs at each allele of each locus measured, and indicates that no difference was observed between samples. Thus, it can be concluded that neither the automated nor the manual process steps created alterations in the number of STRs at each of the loci measured for the hiPSC samples.

The gene expression profile of hiPSCs has been discovered to be unique compared to hESCs, however, after prolonged culture, it may become more similar to that of hESCs (Chin et al, 2009). These differences in gene expression may be associated with hiPSC source material, derivation method and culture conditions (Martins-Taylor & Xu, 2012). Furthermore, although the majority of the epigenome of hiPSCs is similar to that of hESCs, differences may exist including DNA methylation that remains from the somatic cells from which the hiPSCs were derived (Chen, Zhao & Xu, 2012). Previous studies have also reported the occurrence of genetic changes in hiPSCs after prolonged culture, although these may not be as frequent as in hESCs (Martins-Taylor & Xu, 2012). Additionally, the International Stem Cell Initiative (2011) has reported that late passage pluripotent stem cells are twice as likely to exhibit genetic changes compared to early passage cells. Such alterations were not observed in the present study, which is likely to be due to the lack of prolonged culture of hiPSC populations.

As with the hMSC and EC 2102Ep cell population STR profiles previously examined in Chapters 7 and 8, possible differences in the level of STR expression may have been observed between hiPSC samples, suggesting that more copies of the STR existed at certain loci. However, as discussed in section 7.4, variations in fluorescence between experimental runs may contribute to these variations in peak size, which are not considered significant under LGC guidelines.
Using multicolour flow cytometry, the co-expression of pluripotency and differentiation markers in Baseline, Centrifugation and Non-Centrifugation hiPSC populations was determined at the single cell level. The expression of the cell surface antigens Stage-Specific Embryonic Antigen-3 (SSEA-3) and Tumour-Related Antigen-1-81 (TRA-1-81) were measured in order to determine the pluripotency of hiPSC populations. Measurement of the expression of the SSEA-1 cell surface antigen was utilised to determine the level of differentiation in hiPSC populations. Human iPSCs have been previously demonstrated to express these typical pluripotency markers and to lack the expression of the SSEA-1 differentiation marker (Takahashi et al, 2007).

In the present study, it was determined that the Baseline, Centrifugation and Non-Centrifugation hiPSC populations exhibited high expression of SSEA-3 and TRA-1-81 pluripotency markers and that the majority of each population co-expressed these antigens (>50%). Furthermore, these populations demonstrated low expression of the SSEA-1 differentiation marker. Therefore, it can be concluded that each of the hiPSC populations contained predominantly pluripotent cells. The percentage co-expression of pluripotency and differentiation markers identified for the majority of hiPSC populations in the present study was similar to that of alternative pluripotent stem cell lines reported in previous studies (Atkinson et al, 2012).

However, lower expression of the SSEA-3 and TRA-1-81 pluripotency markers, and higher expression of the SSEA-1 differentiation marker, in Centrifugation hiPSC populations was demonstrated when compared to Baseline and Non-Centrifugation populations. This would indicate that a greater number of differentiated cells were present in Centrifugation hiPSC populations. Therefore, it can be proposed that the automated, Non-Centrifugation process step maintained the pluripotency of hiPSC populations more effectively than the manual, Centrifugation process step. Previous studies have also identified a decrease in pluripotency marker expression, specifically Oct4, after centrifugation, and that, by increasing the centrifugal force up to 1,000 RCF, the pluripotency marker expression decreases further (Veraitch et al, 2008; Wong, 2008).

Although the multicolour flow cytometry analysis of hiPSC populations was performed upon cells that were seeded post-cryopreservation and cultured until confluent, and therefore may not have recovered from cryopreservation as discussed later in this section, this method was
consistent between hiPSC conditions, and therefore differences in antigen expression between conditions cannot be attributed to the analytical method.

When comparing the growth of hiPSC populations after culture using manual or automated process steps, it was observed that samples measured in the 1st and 2nd passages had significantly greater Viable Cell Densities than those measured in the Pre-Experimental passage, regardless of the process step utilised. However, it must be noted that differences in the volume of culture medium in which the cells were suspended may influence the Viable Cell Densities measured. Nonetheless, a similar pattern was also reported for the Viable Cell Yield data, and this further demonstrates the detrimental effect of cryopreservation upon hiPSC populations. Therefore, both the Viable Cell Density and Yield data indicate that the VAX001024c07 hiPSC line requires one passage to recover, and return to a normal proliferative rate, after cryopreservation. Yet, despite the decreases in Viable Cell Density and Yield after cryopreservation, no significant difference was observed between hiPSC Viability in the Pre-Experimental, 1st and 2nd passages.

Although no significant difference in Viable Cell Density was observed between Pre-, Post- & Non-Centrifugation hiPSC populations, once the Viable Cell Yields had been determined, a number of significant findings arose. In addition to the greater Viable Cell Yields observed in the 1st and 2nd passages, when compared to the Pre-Experimental passage, it was determined that Post-Centrifugation hiPSC Viable Cell Yields were significantly lower than Pre-Centrifugation hiPSC Viable Cell Yields over all passages. This suggests that the centrifugation process may have a detrimental effect upon cell yield, which may be due to an insufficient RCF to generate an adequate cell pellet or the aspiration of cells when removing the supernatant after centrifugation. However, the RCF used in the present experiment is similar to that which is recommended for use during the culture process for iPSCs and other stem cell types (See Appendix 13.8). Although the supernatant was carefully aspirated after the centrifugation process, this step is difficult to standardise and may have influenced Post-Centrifugation cell counts.

Furthermore, the Viable Cell Yield of Non-Centrifugation hiPSC populations were found to be significantly greater than Post-Centrifugation hiPSC populations across all passages. Although no significant difference in Viable Cell Yield between Pre-Centrifugation and Non-Centrifugation hiPSC populations was observed, given the reduction in Viable Cell Yield
after the centrifugation process, it is apparent that a greater Viable Cell Yield is achievable when the Non-Centrifugation process step is utilised.

It should also be recognised that neither Viable Cell Density nor Yield decreased over the three passages, which indicates that the growth rate was maintained. This would suggest that, unlike the decrease in Viable Cell Yield over multiple passages observed in hMSCs (Chapter 7), hiPSC growth rate does not decrease with multiple passages, and that they demonstrate the unlimited self-renewal that is characteristic of pluripotent stem cells (Thomson et al, 1998).

Furthermore, by comparing the standard deviations of Viable Cell Densities and Yields for Pre-, Post- & Non-Centrifugation hiPSC populations, it was determined that no significant difference in the variability in Viable Cell Density or Yield was observed between process steps. This suggests that there was no difference in the consistency of the manual and automated process steps. However, non-significant trends for a lower variability in Viable Cell Density in Non-Centrifugation hiPSC samples compared to Pre- and Post-Centrifugation samples, as well as a lower variability in Viable Cell Yield in Non-Centrifugation samples compared to Post-Centrifugation samples, may exist. These non-significant trends may suggest that the Non-Centrifugation process step allowed for hiPSC populations with more consistent cell numbers to be manufactured. This is supported by the conclusions of previous investigations in which it was identified that automated cell culture process steps are often more consistent than manual cell culture process steps (Liu et al, 2010; Thomas et al, 2009; Terstegge et al, 2007).

Given that the protocol for the culture of the VAX001024c07 hiPSC line is based upon the seeding of single cells, and that these cells have a tendency form small colonies over the duration of culture, the determination of cell aggregation may represent a valuable measurement for the comparison of alternative process steps in the culture of this cell type. By examining the number and size of live cell aggregates, it was apparent that greater numbers of aggregates of all sizes were present in Pre-Centrifugation hiPSC samples compared to Post-Centrifugation samples. Additionally, this data indicates that the number of aggregates of all sizes in Non-Centrifugation samples was similar to Pre-Centrifugation samples, but greater than Post-Centrifugation samples.

The results of the analysis of aggregate rates also supports these patterns, and it was determined that the aggregate rate was significantly higher in Pre-Centrifugation hiPSC
samples compared to Post-Centrifugation samples across all passages. This suggests that the process of centrifugation reduces the amount of cell aggregation, and therefore may indicate that the centrifugation process step is beneficial for the generation of a single cell suspension which is required for seeding of the VAX001024c07 hiPSC line. Furthermore, it was determined that the aggregate rate was significantly higher in Non-Centrifugation samples compared to Post-Centrifugation samples across all passages. This finding would indicate that, due to the lack of an incorporated centrifugation process step, the automated, Non-Centrifugation process step does not allow for as much aggregate dissipation as the manual, Centrifugation process step. It has previously been identified that the dispersion of hiPSC aggregates represents the most difficult step to automate when developing a hiPSC culture protocol using the CompacT SelecT automated cell culture platform (Soares et al, 2014). This may therefore negatively impact the capacity for single cell seeding when utilising the automated process step, however the incorporated mixing steps performed after the Non-Centrifugation cell count may partly mitigate this increased aggregation. Although this increased aggregation in Non-Centrifugation hiPSC populations was not found to influence hiPSC growth or characterisation, it may have led to the more rapid formation of colonies observed early in Non-Centrifugation cultures.

Although the increased hiPSC aggregation in Non-Centrifugation populations may not have detrimentally affected the culture of these cells, it may have influenced the consistency of cell counts which could lead to variability in cell counts between, and within, cell populations. This is due to the detrimental effect of aggregation upon the accuracy of cell counts performed by Cedex automated cell counter. This aggregation may therefore influence the Viable Cell Density and Yield data obtained for the Non-Centrifugation populations, as well the accuracy of seeding daughter flasks. However, the low variability in Viable Cell Density and Yield for Non-Centrifugation samples does not support this suggestion.

Furthermore, Aggregate Rate was found to be significantly greater in hiPSC populations in the Pre-Experimental passage compared to the 1\textsuperscript{st} and 2\textsuperscript{nd} passages. Similarly to the Viable Cell Density and Yield data, this once again demonstrates the impact of the cryopreservation process upon hiPSC culture. This increased aggregation in the first passage after cryopreservation and when utilising the Non-Centrifugation process step may be detrimental to the single cell culture of hiPSCs, and may therefore have significant implications for the development of an automated manufacturing process in which single cell culture is utilised and a centrifugation step is not incorporated. The greater Aggregate Rate observed in Pre-
Experimental passages may also highlight the need for cell to cell interaction after cryopreservation. To determine whether this is directly due to the negative impact of the cryopreservation process, or due to the low cell density associated with low cell adherence, after cryopreservation requires further investigation.

After determining the Population Doubling Time (PDT) of hiPSC populations prior to passage or harvest, cultured using either manual or automated process steps, no significant difference in PDT was observed between Centrifugation and Non-Centrifugation hiPSCs across all passages. This indicates that the residual dissociation carryover did not influence hiPSC growth rate. However, it was determined that the PDT of both Centrifugation and Non-Centrifugation hiPSCs was significantly longer in the Pre-Experimental passage compared to the 1st and 2nd passages. This once again highlights the detrimental impact of the cryopreservation process upon the proliferation of hiPSC populations, and the need for culture over one passage in order to recover hiPSC proliferative rate. Nonetheless, after the Pre-Experimental passage, it was observed that hiPSC populations maintained their growth rate over multiple passages, as expected for self-renewing pluripotent stem cells. The PDTs of hiPSCs in the present study were comparable to those previously reported for pluripotent stem cells, specifically hESCs (Cowan et al, 2004), but were found to be shorter than those reported in the seminal iPSC research (~46 hours) (Takahashi et al, 2007).

As mentioned previously in this section, no significant difference was observed between the Viability of hiPSC populations in the Pre-Experimental, 1st or 2nd passages. This indicates that, although cryopreservation may impact initial cell recovery and growth, no difference in the number of dead or non-viable cells was observed at each passage. However, it must be noted that the daily medium exchanges performed may reduce the number of dead cells present throughout culture.

It was also demonstrated that the Viability of Non-Centrifugation hiPSC samples was significantly lower than that of Post-Centrifugation samples across all passages, and was significantly lower than that of Pre-Centrifugation samples in the 2nd passage. This would suggest that the Non-Centrifugation process step negatively impacted the Viability of hiPSC populations, and this may be linked to the residual dissociation agent carryover. However, it must be pointed out that the difference between the mean Viabilities for Post-Centrifugation and Non-Centrifugation samples was ≤2.2%; and that the Viability of Non-Centrifugation populations remained above 94% throughout the experiment, which is significantly greater
than the minimal cell viability (80%) for cell therapy products outlined by the FDA (FDA & Genzyme, 2007). This difference between mean viabilities for Post-Centrifugation and Non-Centrifugation samples is also below the maximum intra-sample variability in cell viability for the Cedex automated cell counter (±3%) (Roche, 2003). Furthermore, this difference in Viability between hiPSC populations cultured using alternative process steps was similar to the non-significant difference observed between process steps within the first passage of the hMSC experiment (Chapter 7).

The Viability of Post-Centrifugation hiPSCs was also identified to be significantly greater than Pre-Centrifugation hiPSCs in the 2nd passage. This trend was also present in the Pre-Experimental and 1st passages, although not significant. This would suggest that the process of centrifugation improved the Viability of hiPSC populations. Improvements in Viability after the process of centrifugation may be attributed to the removal of debris and non-viable cells, however further research is required in order to identify the cause of this change. Increased viability of cell suspensions after centrifugation has been previously demonstrated, and it was also determined that longer centrifugation times resulted in greater cell viabilities (Kreamer et al, 1986).

When the standard deviations of hiPSC Viabilities were compared, no significant difference was identified. This suggests that no difference in the consistency and variability of hiPSC Viability was observed between process steps. However, trends for increased variability in hiPSC samples from the Pre-Experimental passage compared to the 1st and 2nd passages, and for greater variability in the viability of Non-Centrifugation hiPSC populations compared to Pre- and Post-Centrifugation populations across all passages, may be apparent. Nevertheless, these trends were not found to be significant, and it can be concluded that no difference in the variability of hiPSC Viability was observed between Pre-, Post-, and Non-Centrifugation samples over all passages.

The comparability between Baseline hiPSCs, and those cultured using Centrifugation and Non-Centrifugation process steps is summarised in Figure 116 (below), in which the results for each of the parameters measured are highlighted.
## Chapter 9: Comparison of a Manual, Centrifugation & an Automated, Non-Centrifugation Cell Culture Process Step for a Human iPSC line

<table>
<thead>
<tr>
<th>hiPSC Condition</th>
<th>Morphology</th>
<th>Parameter</th>
<th>STR Profile</th>
<th>Pluripotency Marker Expression</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td><strong>Morphology</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline (P22+11)</td>
<td>With ROCK inhibitor (Day 0-1): Single cells with multiple long, thin lamellipodia</td>
<td>-</td>
<td>-</td>
<td>NSD</td>
</tr>
<tr>
<td></td>
<td>Without ROCK inhibitor (Day 2-7): Form tight clusters of cells with rounded morphology</td>
<td>-</td>
<td>-</td>
<td>67.8% SSEA-3 &amp; TRA-1-81 co-expression &lt;10% SSEA-1 expression</td>
</tr>
<tr>
<td>Pre-Centrifugation (P22+14)</td>
<td>Comparable</td>
<td>14.88μm (±0.36μm)</td>
<td>42.97% (±5.31%) (SD)</td>
<td>113.13x10⁶ (±12.23x10⁶) (SD) 0.98 Days (±0.02 Days) 97.15% (±0.55%) (SD) NSD - -</td>
</tr>
<tr>
<td>Post-Centrifugation (P22+14)</td>
<td>Comparable</td>
<td>14.64μm (±0.19μm)</td>
<td>36.97% (±4.39%) (SD)</td>
<td>91.29x10⁶ (±12.26x10⁶) (SD) 1.03 Days (±0.03 Days) 97.88% (±0.39%) (SD) NSD - -</td>
</tr>
<tr>
<td>Non-Centrifugation (P22+14)</td>
<td>Comparable</td>
<td>15.08μm (±0.14μm)</td>
<td>45% (±3.67%) (SD)</td>
<td>102.32x10⁶ (±6.24x10⁶) (SD) 1.00 Days (±0.01 Days) 95.67% (±0.79%) NSD - -</td>
</tr>
</tbody>
</table>

**Figure 116**: Summary table of parameters used to examine comparability between Baseline (P22+11), Pre-Centrifugation (P22+14), Post-Centrifugation (P22+14) and Non-Centrifugation (P22+14) hiPSCs. Average values are presented and ± denotes Standard Deviations. NSD= No Significant Difference. SD= Significant Difference.
Figure 117 (below) examines the consistency of Centrifugation and Non-Centrifugation hiPSC measurements by illustrating the proportional means for each parameter measured. Furthermore, the proportional standard deviations are shown, which facilitates further comparison of the consistency of Centrifugation and Non-Centrifugation measurements by comparing the variability within each parameter. The comparison of proportional standard deviations also allows for the variability between parameters to be compared. This allows process and product control limits to be identified.

![Normalised Means and Standard Deviations for each hiPSC Parameter](image)

**Figure 117**: Normalised Means and Standard Deviations for each of the Centrifugation & Non-Centrifugation hiPSC parameters measured. Proportional Standard Deviations are shown as error bars. Asterisks (*) denote significance of Centrifugation over Non-Centrifugation, or vice versa (p=0.05).

Figure 117 indicates that Cell Diameter, Viability and Population Doubling Time of Centrifugation and Non-Centrifugation hiPSCs demonstrated low variability and were therefore well controlled, whereas substantial variability was observed in the Viable Cell Yield and Aggregate Rate parameters.

The data presented in Figure 117 also illustrates that the standard deviation of Non-Centrifugation hiPSC Cell Diameter falls within that of Centrifugation hiPSC Cell Diameter, which suggests that little variability was observed between the Cell Diameter of Centrifugation and Non-Centrifugation hiPSCs. Furthermore, Figure 117 highlights that
significant overlap was observed between the Viable Cell Yield, Population Doubling Time, and Aggregate Rate standard deviations of Centrifugation and Non-Centrifugation hiPSCs.

The normalised means and standard deviations also demonstrate that differences in the variability in Centrifugation and Non-Centrifugation hiPSC Viable Cell Yield and Aggregate rate parameters were observed, with smaller standard deviations, and therefore greater consistency, in these parameters associated with Non-Centrifugation hiPSCs.

Finally, some difference may have been observed between Centrifugation and Non-Centrifugation hiPSC Viabilities, with differences in the means and standard deviations, which do not overlap, observed. Significant differences in hiPSC Viabilities between Centrifugation and Non-Centrifugation hiPSCs were also identified in section 9.3 (Figure 115). This may indicate that hiPSCs cultured using Centrifugation or Non-Centrifugation process steps were not comparable in terms of cell viability. However, as discussed earlier in this section, the observed difference between the mean Post-Centrifugation and Non-Centrifugation hiPSC Viabilities was small (≤2.2%), and the Viability of Non-Centrifugation populations remained above 94% throughout the experiment, which is significantly greater than the FDA prescribed minimal cell viability for cell therapy products (80%) (FDA & Genzyme, 2007).

9.5 Conclusions

After the performance of a protocol transfer, the quantitative experiments presented in the present chapter were performed in order to determine the comparability between a manual and an automated process step for the culture of hiPSCs. This allowed for process comparability, after a change to a single process step, to be examined and for further process experience to be gained, therefore contributing to the second challenge addressed in this thesis regarding comparability in cell-based therapies.

The experiments demonstrated that comparable morphology, stability, and population doubling time were observed between hiPSCs cultured using manual and automated process steps. Some distinction was observed in hiPSC phenotype, yield, aggregate rate, and viability between cells cultured using manual and automated process steps; with Non-Centrifugation hiPSC populations exhibiting a greater cell yield, greater aggregate rate, and lower cell viability compared to Centrifugation hiPSCs. Furthermore, although comparable levels of hiPSC spontaneous differentiation were observed between Centrifugation and Non-Centrifugation hiPSC populations after visual examination, increased pluripotency marker
expression and decreased differentiation marker expression was identified in Non-Centrifugation hiPSC populations. This may indicate that the Non-Centrifugation process step was more favourable for the maintenance of pluripotency in hiPSC cultures. However, contrary to the trends observed in the hMSC and Ruler hESC experiments, a trend for a decreased variability in cell yield was observed after the utilisation of the Non-Centrifugation process step. The experiments in this chapter also highlight the detrimental effect of the cryopreservation and thawing processes upon hiPSC culture, with significantly greater cell diameters, lower yields, longer population doubling times, and greater aggregate rates observed in populations in the first passages post-thaw.

Future research, utilising in-process measures of hiPSC growth and characterisation, may provide greater evidence of process comparability. Further work is also required to optimise the hiPSC automated, Non-Centrifugation process step in order to reduce cell aggregation. Also, further examination of the Centrifugation process step is necessary to identify the cause of the reduction in hiPSC yield after centrifugation. Additionally, the examination of the differentiation of Centrifugation and Non-Centrifugation hiPSCs into the three ‘Germ Layers’ may allow for further comparison of the pluripotency of these cells after culture using alternative process steps. It is also apparent from the present chapter, that further work is required in order to improve hiPSC recovery after cryopreservation and thaw. Finally, as discussed in greater detail in section 11.8.3, further research investigating the utilisation of hiPSC morphology changes as an early indicator of spontaneous differentiation may facilitate the development of a rudimentary method of in-process characterisation for hiPSC populations.
Chapter 10: The Exploration of Culture Conditions and Comparability in Starting Material for the Early Stages of hiPSC to MSC Differentiation

In collaboration with the Institute for Stem cell Therapy and Exploration of Monogenic diseases (CECS, I-Stem, Evry, France)

The aim of the present chapter is to develop further understanding regarding a number of components of hiPSC to MSC differentiation protocols through the investigation of the effect of MSC medium composition, ROCK inhibitor pre-treatment regimen, and hiPSC starting material upon the early stages of hiPSC to MSC differentiation. This chapter also briefly examines the comparability between hiPSC-MSCs and adult hMSCs. The impact of each of these parameters upon the hiPSC to MSC differentiation process has not been rigorously explored in previous literature. An unplanned protocol transfer for the hiPSC to MSC differentiation process was also undertaken between independent laboratories at I-Stem and Loughborough University (Loughborough, UK).

10.1 Introduction

As described in previous chapters, Induced Pluripotent Stem Cells (iPSCs) can be derived through the reprogramming of adult somatic cells, which is achieved through the delivery of a number of transcription factors via retroviral vectors (Takahashi & Yamanaka, 2006). These cells share a number of key characteristics with Embryonic Stem Cells (ESCs), including pluripotency and almost unlimited expansion in vitro. However, iPSCs also have a number of unique advantages over alternative pluripotent stem cell types, including the capacity for derivation from any adult tissue, a lack of the major ethical concerns associated with ESCs, and the potential of these cells as a source of cells for autologous transplantation.

Mesenchymal Stromal Cells (MSCs), first described by Friedenstein and colleagues (1970), have been characterised using a number of basic criteria, including their adherence to tissue culture plastic; their positive expression of CD105, CD73 and CD90; their lack of CD45, CD34, CD14 or CD11b, CD79α or CD19, and HLA-DR expression; and their osteogenic, chondrogenic, and adipogenic differentiation potential in vitro (Dominici et al, 2006). However, these minimal criteria are not representative of the in vivo mode of action of these cells, and it has been recognised that the secretion of soluble factors may in fact represent the major therapeutic mechanism of action of MSCs (Phinney & Prockop, 2007).
Chapter 10: The Exploration of Culture Conditions and Comparability in Starting Material for the Early Stages of hiPSC to MSC Differentiation

The development of adult hMSC-based therapies faces a number of significant challenges, including the limited availability of these cells \textit{in vivo}, the decline in MSC quality and quantity over time in culture, the reduced MSC proliferative capacity correlated with increased donor age, and the requirement for invasive extraction which may result in donor site morbidity (Chen et al, 2012; Stenderup et al, 2003; Giuliani et al, 2011; Hynes et al, 2013). Therefore, the identification of alternative sources of MSCs, to which these limitations may not apply, could allow for an improvement in both the accessibility and quality of the source material for a hMSC-based product.

The derivation of MSCs through the differentiation of iPSCs (iPSC-MSCs) may diminish the challenges associated with the derivation of these cells from adult tissue, with the unlimited expansion capacity and rapid proliferation of iPSCs allowing for the generation of large numbers of autologous or allogeneic hMSCs. It has recently been proposed that an iPSC-MSC line may be suitable as a MSC reference cell line, given the strong correlation ($R^2>0.93$) between the gene expression profiles of iPSC derived MSCs and those derived from various tissue sources (Tanavde et al, 2015).

However, the contamination of MSC populations with undifferentiated iPSCs, the disruption of gene expression during the iPSC reprogramming process, the contamination from pathogens contained within feeder cells utilised during the iPSC expansion process, and the immunogenicity of iPSC-derived cells all represent potential risks to the safety of an iPSC derived MSC product (Jung, Bauer & Nolta, 2012; Villa-Diaz et al, 2012). Although various investigations have reported a lack of significant host immune response after the transplantation of cells derived from pluripotent stem cells (Guha et al, 2013; Araki et al, 2013; Li et al, 2004; Drukker et al, 2006), further research is required in order to determine the immunogenicity of iPSC-MSCs. However, recent investigations have identified that, compared to adult BM-MSCs, iPSC-MSCs induce a reduced immune response after transplantation (Sun et al, 2015).

The generation of a hiPSC-MSC product may also pose additional economic and manufacturing challenges, due to the complex cell sorting required, the extensive culture time required, the high cost including labour, the low efficiency of iPSC differentiation, and the resulting low yield of MSCs (Liu et al, 2012). As discussed in Sections 5.5 & 9.1, ‘haplobanking’ may require the generation numerous hiPSC banks, each corresponding to a...
HLA haplotype within a population. These cells can then be utilised to generate HLA matched hiPSC derived cell therapies for a large number of patients with the appropriate haplotype, whilst exhibiting a reduced immunogenicity. This approach may require greater up-front investment compared to an autologous approach, but may allow for a higher patient throughput.

Currently, the protocols generated to derive MSCs from iPSCs can be categorised based upon the method of passaging utilised, specifically single-cell culture or colony culture. However, controlled differentiation through the use of biomaterials has also been explored (Liu et al, 2012). The iPSC to MSC differentiation process, regardless of the passage method utilised, is primarily based upon the transition from pluripotent stem cell culture medium to MSC culture medium. The underlying mechanism behind this differentiation process is currently unclear, however it has been proposed that the addition of serum, containing a variety of cytokines, growth factors and metabolites, to the culture medium may influence this process (Zou et al, 2013). A variety of culture medium compositions have been employed in order to generate MSC populations from iPSCs, the basal media for which have included Basal Alpha-MEM, DMEM/F12, and DMEM. The literature regarding the methods of iPSC to MSC differentiation has been discussed in Chapter 2.

One of the primary aims of the present study is to examine the effect of MSC medium composition, including serum-free compositions, upon the early stages of hiPSC to MSC differentiation, through the measurement of selection and seeding efficiencies of hiPSCs (VAX001024c07). Selection efficiency is defined in the present research as the number of live cells present in MSC medium-based culture after the removal of ROCK inhibitor, and is expressed as a percentage of the seeding density. Seeding efficiency is described in the present research as the number of live cells present after the seeding of hiPSCs in MSC culture medium, and is expressed as a percentage of the seeding density.

Initial experiments in the present research were based upon the performance of an initially unanticipated, or unplanned, protocol transfer between I-Stem (Evry, France) and Loughborough University (Loughborough, UK), in order to replicate the hiPSC to MSC differentiation process developed at I-Stem. This protocol transfer was undertaken without extensive preparation and previous generation of a comparability protocol, unlike those undertaken during the process step comparability experiments (Chapters 7-9), and was
therefore unplanned. However, due to a failure to replicate the I-Stem differentiation protocols, with poor survival of hiPSC populations over time during the adaptation to MSC culture medium, as well as the limited time available, this study focused upon the early stages of hiPSC to MSC differentiation and adaptation to MSC culture medium.

Previous studies have primarily utilised animal-derived serum, specifically Fetal Bovine Serum (FBS), as part of their medium compositions, and the effectiveness of serum-free medium compositions has yet to be thoroughly investigated. Luzzani and colleagues (2015) successfully derived MSCs from pluripotent stem cells using Alpha-MEM supplemented with 10% platelet lysate, however further research is required to explore the possibility of a serum-free differentiation protocol. Therefore, the effectiveness of numerous serum-free MSC culture medium compositions for the differentiation of hiPSCs to MSCs is also examined in this chapter.

Furthermore, the effect of Rho-associated protein kinase (ROCK) inhibitor upon the selection efficiency of hiPSCs in MSC medium is investigated. ROCK inhibitor is utilised in pluripotent stem cell culture in order to improve the survival of single-cells after dissociation (Watanabe et al, 2007). Thus, in the present research, the effect of the removal of ROCK inhibitor from culture upon the survival and selection of iPSCs in MSC medium is explored.

Additionally, the trilineage differentiation potential of hiPSCs, cultured in MSC medium and termed ‘hiPSC-MSCs’, is examined, in order to characterise these cells and to determine their comparability to adult hMSCs. The utilisation of more accurate methods to characterise these cell populations was planned, including the flow cytometric analysis of cell surface markers, however given the lack of success in the derivation of MSC-like populations from hiPSCs, after adaptation to MSC culture medium in culture, these analyses were not undertaken.

Finally, as a continuation of Chapter 9, in which the comparability between hiPSCs cultured using alternative process steps was determined, the comparability between process steps is further examined by comparing the seeding and selection efficiencies of hiPSCs cultured using manual or automated process steps in order to determine the effect of differences in source material upon hiPSC to MSC differentiation.

The present chapter therefore represents a novel investigation into the effect numerous components of hiPSC to MSC differentiation protocols; including MSC medium composition,
Chapter 10: The Exploration of Culture Conditions and Comparability in Starting Material for the Early Stages of hiPSC to MSC Differentiation

ROCK inhibitor pre-treatment regimen, and hiPSC starting material; upon the early stages of hiPSC to MSC differentiation, as well as a brief exploration into the comparability between hiPSC-MSCs and adult hMSCs.

10.2 Methods

10.2.1 hiPSC to MSC Differentiation Protocol Development

Initially, the hiPSC to MSC differentiation protocols examined here were based upon previous work performed at I-Stem, during which their researchers developed a preliminary protocol for the differentiation of hiPSC lines into MSCs, through the performance of an unplanned protocol transfer between I-Stem and Loughborough University. The I-Stem protocol was primarily based upon seeding hiPSCs (VAX001024c07), on Gelatin or Matrigel coated tissue culture plastic, at a high cell density (4.5x10^4 cells/cm²) in MSC medium, consisting of Knockout (KO-) DMEM, 1% GlutaMAX™, 20% Fetal Bovine Serum (FBS) & 10mM ROCK inhibitor, and culturing these cells for two weeks with complete medium exchanges, without ROCK inhibitor, performed on alternating days. After this two week culture period, the cells are passaged and seeded in MSC medium, on tissue culture plastic, at a lower cell density (6x10^3 cells/cm²) and cultured for a further four days before the cells are passaged once again. These frequent passages are continued for a further three passages cumulating in a culture process of 30 days.

To examine whether more usual hMSC culture conditions were capable of stimulating the differentiation of hiPSCs to MSCs, the hMSC culture medium components previously employed in the hMSC comparability experiments of the present thesis (Chapter 7) were utilised as an alternative to the culture medium components recommended by I-Stem. The culture medium components recommended in the I-Stem iPSC to MSC differentiation protocol would not be considered to be typical of hMSC culture medium. Therefore, DMEM High Glucose (HG) GlutaMax™ (Life Technologies), supplemented with 10% FBS (Life Technologies), was utilised to allow for comparability in culture medium components between hiPSC-MSC and hMSC experiments. It has been previously demonstrated that high glucose concentration does not negatively influence hMSC growth or paracrine mode of action (Weil et al, 2009).

In addition to the culture medium compositions utilised in the I-Stem protocol and in previous hMSC experiments, further MSC medium compositions were tested in order to
identify the combination of medium components which promoted the greatest cell survival and differentiation towards MSCs. The utilisation of an increased concentration of FBS (DMEM HG GlutaMAX™ & 20% FBS) was examined in order to determine whether the addition of greater concentrations of a variety of proteins, cytokines, and growth factors within the animal serum would more effectively stimulate hiPSC differentiation towards MSCs.

In order to investigate the effectiveness of animal serum-free culture medium compositions in the differentiation of hiPSCs to MSCs, Human Platelet Lysate (HPL) was utilised as a replacement for FBS. Four serum-free medium conditions were compared, in which the basal medium and the concentration of HPL were varied. These included KO-DMEM (Life Technologies) & 10% Stemulate™ HPL (Cook Regentec, Indiana, USA); KO-DMEM & 20% Stemulate™ HPL; DMEM HG GlutaMAX™ & 10% Stemulate™ HPL; and DMEM HG (Lonza), 2mM UltraGlutamine (Lonza) & 20% Stemulate™ HPL. The differentiation medium conditions tested in the present study are summarised in Figure 118 (below).

<table>
<thead>
<tr>
<th>Differentiation Medium Name</th>
<th>Differentiation Medium Components</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMEM 10% FBS</td>
<td>DMEM High Glucose GlutaMAX™ (Life Technologies) &amp; 10% Fetal Bovine Serum (Gibco®, Life Technologies)</td>
</tr>
<tr>
<td>DMEM 20% FBS</td>
<td>DMEM High Glucose GlutaMAX™ (Life Technologies) &amp; 20% Fetal Bovine Serum (Gibco®, Life Technologies)</td>
</tr>
<tr>
<td>KO-DMEM 20% FBS</td>
<td>Knockout™ DMEM (Life Technologies) &amp; 20% Fetal Bovine Serum (Gibco®, Life Technologies)</td>
</tr>
<tr>
<td>DMEM 10% HPL</td>
<td>DMEM High Glucose GlutaMAX™ (Life Technologies) &amp; 10% Stemulate™ HPL (Cook Regentec, Indiana, USA)</td>
</tr>
<tr>
<td>DMEM 20% HPL</td>
<td>DMEM High Glucose (Lonza), 2mM UltraGlutamine (Lonza) &amp; 20% Stemulate™ HPL (Cook Regentec, Indiana, USA)</td>
</tr>
<tr>
<td>KO-DMEM 10% HPL</td>
<td>Knockout™ DMEM (Life Technologies) &amp; 10% Stemulate™ HPL (Cook Regentec, Indiana, USA)</td>
</tr>
<tr>
<td>KO-DMEM 20% HPL</td>
<td>Knockout™ DMEM (Life Technologies) &amp; 20% Stemulate™ HPL (Cook Regentec, Indiana, USA)</td>
</tr>
</tbody>
</table>

Figure 118: A table outlining the name, components, and component suppliers of each hiPSC to MSC differentiation medium composition tested.

The effect of the duration of ROCK inhibitor pre-treatment upon the survival and differentiation of hiPSCs in MSC medium was also examined in the present research. To
investigate ROCK inhibitor pre-treatment, the survival, characteristics and proliferation of hiPSCs after two pre-treatment regimens were compared. These pre-treatment regimens included hiPSC seeding with ROCK inhibitor and removal after two days; and hiPSC seeding with ROCK inhibitor, with the addition of ROCK inhibitor after two days, and removal after four days.

10.2.2 Trilineage Differentiation of hiPSC-MSCs

Trilineage differentiation was examined in hiPSCs selected through the culture of these cells in MSC medium for 16 days (KO-DMEM & 20% FBS), over a single passage. Although these cells were not characterised to an equivalent level to the hMSC populations from the process comparability experiments in this thesis (Chapter 7), and it was not fully determined that these cells were truly representative of hMSCs, these cells were designated as ‘hiPSC-MSCs’ to allow for distinction between hiPSCs and hiPSCs culture in MSC medium conditions.

After this culture period, the cells were dissociated, centrifuged, resuspended in MSC medium, and a cell count was performed. Trilineage differentiation was determined using similar methods to those utilised in the hMSC process step comparability experiments performed in the present research (Section 7.2.8). These methods are also described in further detail in the Materials & Methods Chapter (Sections 6.11 & 6.12.6).

Briefly, to induce Adipogenic and Osteogenic differentiation, the dissociated cells were seeded in 12-well plates at 10,000 cells/cm² in 1mL of MSC medium. A replicate of each of the wells was also seeded. After 3 days, the MSC medium was replaced with either StemPro® Adipogenic or Osteogenic differentiation medium (Life Technologies). In order to induce Chondrogenic differentiation, dissociated cells were suspended in StemPro® Chondrogenic differentiation medium at $16 \times 10^6$ cells/mL and 5-6 x 2μL droplets of suspension were added to a well of a 12-well plate. A replicate of this well was also seeded. After 30 minutes, 1mL of Chondrogenic differentiation medium was added to these wells. For each of the Adipogenic, Osteogenic or Chondrogenic differentiation conditions, the cells were cultured for 14 days, with a differentiation medium exchange performed every 3-4 days.

After 14 days of culture in differentiation conditions, the medium in each well was aspirated and the cells were washed with PBS. The cells were then fixed with 4% PFA and washed with either PBS or 60% Isopropanol (IPA) depending upon the condition. Next, the relevant
stain was applied to each well. For Adipogenic differentiation, Oil Red O solution (Sigma Aldrich) was applied, whereas the Chondrogenic differentiation protocol utilised Alcian Blue solution (Sigma Aldrich), and the Osteogenic differentiation protocol utilised 2.5% Silver Nitrate solution (Sigma Aldrich). After 5 mins of room temperature incubation, Oil Red O solution was removed from Adipogenic differentiation wells, the wells were washed with deionised water and the stained cells were visualised using light microscopy. After 30 mins of Alcian Blue staining at room temperature, the Chondrogenic differentiation wells were rinsed three times with 0.1N HCL, distilled water was added, and the cells were visualised using light microscopy. Finally, after 30 mins of staining with 2.5% Silver Nitrate solution at room temperature under UV light, the cells were washed three times with deionised water, treated with 4% Napthol AS-MX Phosphate Alkaline solution (Sigma Aldrich) in Fast Violet solution (Sigma Aldrich) for 45 mins at room temperature in the dark, washed three times with deionised water, and visualised using light microscopy.

10.2.3 Comparison of Differentiation Medium Compositions

To compare the hiPSC seeding and selection efficiency in each MSC medium composition, as well as the effect of ROCK inhibitor pre-treatment, a multi-well plate experiment was performed in which cells were harvested after culture in various medium compositions and pre-treatment regimens. The MSC medium compositions utilised were selected based upon the hiPSC to MSC differentiation protocol development experiments.

Three MSC medium compositions and two pre-treatment regimens were compared, which culminated in the comparison of a total of six culture conditions. The three selected MSC medium compositions included KO-DMEM 20% FBS, KO-DMEM 20% HPL, and DMEM 20% HPL. The two ROCK inhibitor pre-treatment regimens included seeding without ROCK inhibitor, and seeding with ROCK inhibitor followed by a complete medium exchange after two days without the addition of ROCK inhibitor. Each of the wells of two 6-well plates, previously coated with Matrigel (BD Biosciences), were seeded with hiPSCs (4.5×10^4 cells/cm²), which were suspended in one of the three selected medium compositions, either with or without ROCK inhibitor. An additional repeat of each of the six culture conditions was also performed, culminating in a total of 12 wells.

Seeding of hiPSCs (VAX001024c07) was performed by thawing three vials of hiPSCs, from the previously generated master cell bank (‘Baseline’), at 37°C, suspending cells from each
vial in one of the three MSC medium compositions, centrifuging the cells at 276 RCF for 5 mins, aspirating the supernatant to remove the cryoprotectant, resuspending the cells in the relevant MSC medium composition at the appropriate cell density, and seeding the appropriate number of cells in each well of the 6-well plates (4.32x10^5 cells/well).

The wells seeded without ROCK inhibitor were sacrificially harvested after two days in order to determine the hiPSC seeding efficiency in various MSC medium compositions without the addition of ROCK inhibitor. The wells seeded with ROCK inhibitor were cultured for two days in the presence of ROCK inhibitor, after which a complete medium exchange was performed without the addition of ROCK inhibitor. After a further two days of culture without the presence of ROCK inhibitor, the cells were sacrificially harvested in order to determine the hiPSC selection efficiency in the chosen MSC medium compositions after ROCK inhibitor pre-treatment.

Cells were dissociated by aspirating the spent culture medium, performing a wash step using Accutase (StemCell Technologies, VA, Canada), applying further Accutase, and incubating the cells for 10 mins at 37˚C. Dissociated cells were then suspended in the relevant MSC culture medium in order to quench the dissociation agent.

Cell morphology was examined throughout culture, harvested cells were counted, and the viability and average cell diameter of each harvested population was determined using the Nucleocounter NC-3000 (ChemoMetec, Allerod, Denmark). The hiPSC seeding and selection efficiencies in MSC medium, calculated based upon the cell counts of harvested cell populations, were utilised as surrogate markers of hiPSC to MSC differentiation in the present study. This allowed for the effectiveness of each MSC medium composition, and each ROCK inhibitor pre-treatment regimen, to be rapidly compared.

10.2.4 Comparison of hiPSC Starting Material

To compare the hiPSC seeding and selection efficiency of hiPSCs (VAX001024c07), cultured utilising either manual or automated process steps, a multi-well plate experiment was performed in which cells, previously cultured using alternative process steps, were harvested after various durations of culture in MSC culture medium.

The MSC medium composition that yielded the greatest seeding and selection efficiencies in the previous experiment (Section 10.2.3) was selected and utilised for the comparison of
hiPSC starting material. Specifically, the medium composition selected was DMEM & 20% HPL. This allowed for further examination of the effectiveness of animal serum-free MSC culture medium compositions in the differentiation of hiPSCs to MSCs, as well as the effect of variations in starting material upon hiPSC to MSC differentiation.

The seeding and selection efficiencies of three hiPSC starting materials were compared by seeding cells (4.5x10^4 cells/cm²) in MSC culture medium with ROCK inhibitor, followed by either performing a sacrificial harvest after two days, to determine the seeding efficiency; or after four days following a complete medium exchange without the addition of ROCK inhibitor after two days, in order to determine the selection efficiency. The three hiPSC starting materials included ‘Baseline’ hiPSCs, from the master cell bank, ‘Centrifugation’ hiPSCs, cultured using a manual centrifugation cell culture process step, and ‘Non-Centrifugation’ hiPSCs, cultured using an automated Non-Centrifugation cell culture process step. Baseline, Centrifugation and Non-Centrifugation hiPSCs utilised in the present experiment were obtained from the same cell banks and experimental batches as those analysed in the hiPSC process step comparability experiment (Chapter 9). Furthermore, Centrifugation and Non-Centrifugation hiPSCs were obtained from the same experimental batch, therefore limiting cross-batch variability.

Therefore, by comparing three hiPSC starting materials and two pre-treatment regimens, a total of six conditions were examined. Eighteen wells of three 6-well plates, previously coated with Matrigel, were seeded with Baseline, Centrifugation or Non-Centrifugation hiPSCs suspended in DMEM & 20% HPL medium with ROCK inhibitor. Two additional repeats of each of the six conditions were also performed, culminating in a total of 18 wells.

Seeding of hiPSCs was performed in a similar manner to the previous comparison of differentiation medium conditions experiment (Section 10.2.3). Cells in all wells were cultured in MSC medium for two days in the presence of ROCK inhibitor. To determine the seeding efficiency of each hiPSC starting material in serum-free MSC medium, a sacrificial harvest of wells containing each starting material, as well as the two additional repeats (9 wells in total), was performed after two days of culture. After a complete medium exchange without ROCK inhibitor, and a further two days of culture, a sacrificial harvest of the remaining 9 wells was performed in order to determine the selection efficiency of each hiPSC starting material in serum-free MSC medium. Cell dissociation was performed in a similar
manner to the previous comparison of differentiation medium compositions experiment (Section 10.2.3), however all cells were resuspended in the same culture medium after centrifugation (DMEM & 20% HPL). Cell morphology was examined throughout culture, harvested cells were counted, and the viability and average cell diameter of each harvested population was determined.

10.2.5 Statistical Analyses
For the statistical analyses of seeding efficiency, selection efficiency, viability and cell diameter of hiPSCs cultured in different medium compositions and derived from different starting materials, IBM SPSS statistical software was utilised. One-Way Analysis of Variance (ANOVA) multiparameter analyses were utilised in order to determine the significance of any differences in cell density, seeding/selection efficiency, viability and diameter between medium compositions and hiPSC starting materials.

For all ANOVAs performed, the cutoff value for statistical significance (p) was set at 0.05, and Tukey’s Honest Significant Difference (HSD) Post-Hoc Tests were used to perform multiple comparisons of the growth and functionality data for each cell line.

10.3 Results
10.3.1 hiPSC to MSC Differentiation Protocol Development
The I-Stem hiPSC to MSC differentiation protocol, based upon culture in KO-DMEM & 20% FBS, was repeatedly replicated in numerous cultures within the present study, however this protocol could not be successfully reproduced and the differentiation of hiPSCs to MSCs could not be achieved using this method (Figures 119 & 120). This was due to low cell survival over culture and the significant cell apoptosis observed after seeding, during culture or after a passage. This failure to replicate the protocols of an independent laboratory highlights the challenges associated with unplanned protocol transfers between laboratories and achieving multi-site comparability.
Therefore, the utilisation of alternative MSC medium compositions for the differentiation of hiPSCs to MSCs was examined, in order to permit an experimental programme investigating the effectiveness of numerous MSC medium compositions and ROCK inhibitor pre-treatment regimens for the differentiation of hiPSCs to MSCs, as well as the effect of hiPSC starting material upon differentiation. As discussed previously in section 10.2.1, a more typical MSC medium composition (DMEM & 10% FBS) was tested, however it was observed that significant cell death frequently occurred after a short duration of culture in this condition (Figure 121), and therefore this MSC medium composition was not effective for the survival and differentiation of hiPSCs.

The replacement of FBS with an equal concentration of animal serum-free culture medium supplement, specifically HPL, did not yield significantly greater cell survival when combined with DMEM basal medium (Figure 122). However, it was observed that the addition of
greater concentrations of HPL (20%), to either KO-DMEM or DMEM basal medium, resulted in greater cell survival and selection over culture compared to the addition of 20% FBS (Figures 122-128), and may therefore be more effective for the differentiation of hiPSCs to MSCs. Furthermore, by comparing hiPSCs cultured in numerous MSC medium compositions, it was observed that DMEM & 20% HPL permitted the greatest cell survival and proliferation (Figures 125-128).

Figure 122: hiPSCs seeded and cultured in DMEM & 10% HPL medium (P1) (Day 5). White arrows indicate areas of live cells.

Figure 123 (Left) hiPSCs seeded and cultured in KO-DMEM & 20% HPL medium (P1) (Day 4).
Figure 124 (Right): hiPSCs seeded and cultured in KO-DMEM & 20% HPL medium over one passage (P2) (Day 2).
The duration of ROCK inhibitor pre-treatment was also explored, and it was observed that seeding hiPSCs in MSC medium without the addition of ROCK inhibitor did not permit cell survival or proliferation (Figure 129). It was also observed that, by seeding hiPSCs with ROCK inhibitor and removing it from the culture medium after two days, cell survival was found to be greater over the first two days (Figure 130). However, although there was limited cell survival after two days, significant cell death occurred once ROCK inhibitor was removed on Day 2 (Figure 130). With the addition of ROCK inhibitor at seeding and after two days in culture, greater cell survival was observed through visual examination over the duration of culture (Figure 131). Once ROCK inhibitor was removed after four days, although significant cell death occurred once again, cell survival was greater than previous ROCK inhibitor pre-treatment regimens.
The addition of ROCK inhibitor when seeding cells after a passage was found to improve cell survival compared to the seeding of cells after a passage without ROCK inhibitor (Figures 132 & 133). Furthermore, it should also be noted that the survival of hiPSCs after seeding in MSC medium was significantly greater when the hiPSCs were harvested from culture (Figures 132 & 133) rather than thawed after cryopreservation (Figure 134). This further highlights the detrimental effect of cryopreservation upon hiPSCs, as discussed in Chapter 9.
The comparison of the morphology of hiPSCs cultured in pluripotent stem cell medium, as presented in Section 9.3.1, and those cultured in MSC medium revealed that discrepancy existed between these cell populations.

The seeding of hiPSCs (VAX001024c07), on tissue culture plastic coated with Matrigel™ (BD Biosciences), in typical pluripotent stem cell culture medium (mTESR™1, StemCell Technologies) supplemented with 10mM ROCK inhibitor (Y-27632, StemCell Technologies) generated cell populations that consisted primarily of single cells with multiple long, thin lamellipodia. After the removal of ROCK inhibitor from these cultures, hiPSCs clustered together and formed small colonies, exhibiting a rounded morphology.
However, after seeding hiPSCs in numerous MSC medium compositions, it is apparent that an alternative cell morphology was observed. Cells cultured in MSC medium displayed a more fibroblastic morphology (Figures 122-128 & 130-134), which is considered to be representative of hMSCs, compared to those cultured in pluripotent stem cell medium. Therefore, it is apparent that the culture of hiPSCs in MSC medium significantly altered the morphology of these cells. Furthermore, changes in cell morphology, distribution, and cell to cell interaction due to the removal of ROCK inhibitor from culture, as observed during the culture of hiPSCs in pluripotent stem cell medium, was not observed during the culture of these cells in MSC medium conditions. Therefore, although ROCK inhibitor may influence the survival of hiPSCs in MSC medium, it does not appear to affect cell morphology.

It must also be noted that the proliferation of hiPSCs was significantly slower in MSC culture medium compared to pluripotent stem cell culture medium, which is to be expected as human pluripotent stem cell differentiation is associated with decreases in proliferation (Peterson & Loring, 2014). Furthermore, it should be acknowledged that variability in the survival and proliferation of hiPSCs in MSC medium was observed between flasks, and therefore further work may be required in order to improve the consistency of the differentiation process.

10.3.2 Trilineage Differentiation of hiPSC-MSCs

After the culture of hiPSC-MSCs in adipogenic, osteogenic or chondrogenic culture conditions, it was determined that these cells demonstrated trilineage differentiation capacity. The Oil Red O staining (Figures 135a & b) illustrates that these cells differentiated into adipocytes and generated lipid droplets (Red Staining). Although the amount of lipid droplet formation was not substantial, adipogenic differentiation was demonstrated nonetheless. The differentiation of these hiPSC-MSCs into chondrocytes, the generation of cell micromasses (dark spheres), and the formation of glycosaminoglycans (blue staining) was apparent after the Alcian Blue staining protocol (Figures 136a & b). Finally, the von Kossa staining (Figures 137a & b) demonstrates that these cells differentiated into osteoblasts and formed large calcium deposits (black stained areas).

Therefore, these results demonstrate that hiPSCs selected through culture in MSC medium (KO-DMEM & 20% FBS) exhibited trilineage differentiation capacity in vitro. Trilineage differentiation is a characteristic that is considered to be representative of hMSCs (Dominici et al, 2006), and therefore these findings may indicate that the hiPSCs, cultured in MSC
medium, exhibited hMSC characteristics. However, the present study is limited in that the hiPSCs were cultured in MSC medium over a single passage prior to trilineage differentiation. Therefore, it remains unclear whether the trilineage differentiation potential observed in the present study can be attributed to the trilineage differentiation capacity of hiPSC derived MSCs, or to the residual pluripotency of hiPSCs. Consequently, further examination of the trilineage differentiation potential of hiPSC-MSCs after a longer duration in culture may be required.

These findings may also indicate that these hiPSC-MSCs did not undergo adipogenic differentiation as readily as chondrogenic and osteogenic differentiation. However, this observation cannot be confirmed in the present study, and further research examining the gene expression of these cells when undergoing trilineage differentiation will be required.

**Figure 135a (Left) & b (Right):** Oil Red O Staining of hiPSC-MSC Adipogenic Differentiation (White Arrows).

**Figure 136a (Left) & b (Right):** Alcian Blue Staining of hiPSC-MSC Chondrogenic Differentiation. White arrows indicate micromass formation. Black arrows indicate glycosaminoglycan staining.
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10.3.3 Comparison of Differentiation Medium Compositions

**Figure 137a (Left) & b (Right):** von Kossa Staining of hiPSC-MSC Osteogenic Differentiation (White arrows).

**Figure 138:** hiPSC Live Cell Density measured after two days in culture (Seeding Efficiency), or after four days in culture (Selection Efficiency), with a complete medium exchange without ROCK inhibitor performed on Day 2, in three MSC medium compositions. The three MSC medium compositions included KO-DMEM & 20% FBS, KO-DMEM & 20% HPL, and DMEM & 20% HPL. Standard Deviations are presented as error bars. * denotes significance over KO-DMEM FBS (Day 2), and # denotes significance over KO-DMEM FBS (Day 2 & 4) and KO-DMEM HPL (Day 2 & 4) (p=0.05).
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Figure 139: hiPSC Seeding and Selection Efficiency after two days in culture (Seeding Efficiency), or after four days in culture (Selection Efficiency), with a complete medium exchange without ROCK inhibitor performed on Day 2, in three MSC medium compositions. The three MSC medium compositions included KO-DMEM & 20% FBS, KO-DMEM & 20% HPL, and DMEM & 20% HPL. Standard Deviations are presented as error bars. * denotes significance over KO-DMEM FBS (Day 2), and # denotes significance over KO-DMEM FBS (Day 2 & 4) and KO-DMEM HPL (Day 2 & 4) (p=0.05).
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After seeding hiPSCs in each MSC culture medium composition, without the addition of ROCK inhibitor, and culturing the cells for two days, it was determined that the hiPSCs do not adhere or survive in culture (0% Seeding Efficiency). This once again highlights the importance of the addition of ROCK inhibitor when seeding hiPSCs after cryopreservation, previously observed in Chapter 9, and indicates that MSC culture medium compositions are not capable of promoting hiPSC survival after cryopreservation without the addition of ROCK inhibitor.

The seeding of hiPSCs in KO-DMEM & 20% FBS medium, with the addition of ROCK inhibitor, resulted in a low cell density after two days (1.35% Seeding Efficiency). Furthermore, the cell density in this medium condition decreased after a further two days in culture, resulting in a selection efficiency of 0.33%. This indicates that the majority of

<table>
<thead>
<tr>
<th>Condition</th>
<th>Live Cell Density (Cells/cm²)</th>
<th>Viability (%)</th>
<th>Seeding/Selection Efficiency (%)</th>
<th>Cell Diameter (µm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Seeding Density (Day 0)</td>
<td>45,000</td>
<td>92.6 (±1.96)</td>
<td>-</td>
<td>14 (±0.75)</td>
</tr>
<tr>
<td>KO-DMEM FBS ROCK (Day 2)</td>
<td>609.7 (±83.5)</td>
<td>68.75 (±0.2)</td>
<td>1.35 (±0.19)</td>
<td>14.75 (±0.21)</td>
</tr>
<tr>
<td>KO-DMEM HPL ROCK (Day 2)</td>
<td>4,409.4 (±322.6)*</td>
<td>94.5 (±0.4)*</td>
<td>9.8 (±0.72)*</td>
<td>16.95 (±0.35)*</td>
</tr>
<tr>
<td>DMEM HPL ROCK (Day 2)</td>
<td>6,968.8 (±221)*</td>
<td>91.9 (±0.7)*</td>
<td>15.49 (±0.49)*</td>
<td>17.8 (±0.71)*</td>
</tr>
<tr>
<td>KO-DMEM FBS Selection (Day 4)</td>
<td>150.4 (±42.1)</td>
<td>57.25 (±13.4)</td>
<td>0.33 (±0.09)</td>
<td>N/A</td>
</tr>
<tr>
<td>KO-DMEM HPL Selection (Day 4)</td>
<td>3,058.3 (±16.2)</td>
<td>88.05 (±0.2)</td>
<td>6.80 (±0.04)</td>
<td>19.05 (±0.35)</td>
</tr>
<tr>
<td>DMEM HPL Selection (Day 4)</td>
<td>13,901 ±(1922.4)#</td>
<td>91.2 (±0.6)*</td>
<td>30.89 (±4.27)#</td>
<td>19.85 (±0.21)</td>
</tr>
</tbody>
</table>

Figure 140: Mean hiPSC Live Cell Density, Viability, Seeding/Selection Efficiency, and Cell Diameter after two days in culture (Seeding Efficiency), or after four days in culture (Selection Efficiency), with a complete medium exchange without ROCK inhibitor performed on Day 2, in three MSC medium compositions. The three MSC medium compositions included KO-DMEM & 20% FBS, KO-DMEM & 20% HPL, and DMEM & 20% HPL. Standard Deviations are shown in brackets. * denotes significance over KO-DMEM FBS (Day 2), # denotes significance over KO-DMEM FBS (Day 2 & 4) and KO-DMEM HPL (Day 2 & 4), and • denotes significance over KO-DMEM FBS (Day 2 or 4) (p=0.05).
hiPSCs did not survive in KO-DMEM & 20% FBS culture medium and that significant cell apoptosis continued after the removal of ROCK inhibitor.

Compared to the KO-DMEM & 20% FBS medium composition, the utilisation of the KO-DMEM & 20% HPL medium composition yielded a significantly greater cell density after seeding (9.8% Seeding Efficiency) (p=0.001). However, similarly to the KO-DMEM & 20% FBS condition, cell density decreased after a further two days in culture after the removal of ROCK inhibitor (6.8% Selection Efficiency). Nonetheless, it is apparent that cell density remained greater in the KO-DMEM & 20% HPL condition compared to the KO-DMEM & 20% FBS condition.

Finally, the cell density of hiPSCs seeded in the DMEM & 20% HPL culture medium composition was significantly greater than KO-DMEM & 20% FBS (p=0.00) and KO-DMEM & 20% HPL (p=0.003) compositions (15.49% Seeding Efficiency). Additionally, cell density after a further two days in culture, after the removal of ROCK inhibitor, increased, yielding a selection efficiency of 30.89%, which was significantly greater the KO-DMEM & 20% FBS (p=0.002) and KO-DMEM & 20% HPL (p=0.005) compositions. These findings not only indicate that the DMEM & 20% HPL condition promoted cell survival most effectively, but also that this condition allowed for cell proliferation without the addition of ROCK inhibitor.

By comparing the viability of hiPSC populations seeded and cultured in MSC medium, it was observed that the KO-DMEM & 20% FBS yielded a significantly lower cell viability compared to cells measured after seeding in KO-DMEM & 20% HPL (p=0.019) and after both seeding and selection in DMEM & 20% HPL (p=0.025 and p=0.044). Culture of hiPSCs in the KO-DMEM & 20% HPL and DMEM & 20% HPL compositions resulted in similar cell viabilities with no significant difference observed after both seeding or selection. However, by comparing the cell diameter of hiPSCs seeded and cultured in MSC medium, it can be identified that the KO-DMEM & 20% HPL (p=0.038) and DMEM & 20% HPL (p=0.015) compositions generated significantly larger cells than the KO-DMEM FBS composition, and that the DMEM & 20% HPL composition may generate larger cells compared to other medium compositions, although the significance of this trend could not be determined due to a limited number of measurements.
This therefore indicates that the DMEM & 20% HPL medium composition was the most effective of those examined for the survival and selection of hiPSCs in MSC culture medium.

With regards cell morphology after culture in numerous MSC medium compositions, it was observed that hiPSCs cultured in all MSC medium compositions differed significantly from that of cells cultured in pluripotent stem cell medium, and a transition towards a morphology more closely resembling that of hMSCs may have occurred (Figures 141-143). However, no difference in cell morphology was observed between the KO-DMEM & 20% FBS, KO-DMEM & 20% HPL, and DMEM & 20% HPL medium compositions.

Figure 141 (Top Left): hiPSCs seeded and cultured in DMEM & 20% HPL (P1) (Day 4). Figure 142 (Top Right): hiPSCs seeded and cultured in KO-DMEM & 20% HPL (P1) (Day 2). Figure 143 (Above): hiPSCs seeded and cultured in KO-DMEM & 20% FBS (P1) (Day 4).
10.3.4 Comparison of hiPSC Starting Material

**Figure 144:** Live Cell Density of different hiPSC starting materials measured after two days in culture (Seeding Efficiency), or after four days in culture (Selection Efficiency), with a complete medium exchange without ROCK inhibitor performed on Day 2, in MSC medium (DMEM & 20% HPL). The three hiPSC starting materials included cells from the master cell bank (‘Baseline’), after culture utilising the Centrifugation process step (‘Centrifugation’), and after culture utilising the Non-Centrifugation process step (‘Non-Centrifugation’). Standard Deviations are presented as error bars. * denotes significance over Baseline and Non-Centrifugation hiPSCs (Day 2 & 4), and # denotes significance over Baseline hiPSCs (Day 4) (p=0.05).
Figure 145: Seeding and Selection Efficiency of hiPSC starting materials after two days in culture (Seeding Efficiency), or after four days in culture (Selection Efficiency), with a complete medium exchange without ROCK inhibitor performed on Day 2, in MSC medium (DMEM & 20% HPL). The three hiPSC starting materials included cells from the master cell bank (‘Baseline’), after culture utilising the Centrifugation process step (‘Centrifugation’), and after culture utilising the Non-Centrifugation process step (‘Non-Centrifugation’). Standard Deviations are presented as error bars. * denotes significance over Baseline and Non-Centrifugation hiPSCs (Day 2 & 4), and # denotes significance over Baseline hiPSCs (Day 4) (p=0.05).
To examine the differences in seeding and selection efficiency between hiPSC starting materials, cells were seeded and cultured in the MSC medium selected as the most effective from the previous comparison of medium compositions experiment (DMEM & 20% HPL) (Section 10.3.3).

The comparison of seeding and selection efficiencies of hiPSC starting materials revealed that the cell density of Baseline hiPSCs was significantly lower than Centrifugation hiPSCs after both seeding (p=0.004) and selection (p=0.00), with a seeding efficiency of 23.55% and a selection efficiency of 77.13%. The cell density of Non-Centrifugation hiPSCs was found to be significantly greater than that of Baseline hiPSCs after selection (p=0.001), with a
selection efficiency of 182.1%. Finally, it was determined that the cell density of Centrifugation hiPSCs was significantly greater after seeding and selection than both Baseline (p=0.004 and p=0.00) and Non-Centrifugation (p=0.017 and p=0.00) hiPSCs, with a seeding efficiency of 57.5% and a selection efficiency of 341.7%.

Therefore, as also observed in the previous comparison of medium compositions experiment (Section 10.3.3), the DMEM & 20% HPL medium composition permitted the survival, proliferation, and selection of hiPSCs, both in the presence of ROCK inhibitor and after it had been removed from the culture. However, differences in the seeding and selection efficiencies between hiPSC starting materials were observed. As previously described in this section, hiPSCs previously cultured using a manual, Centrifugation process step exhibited the greatest seeding and selection efficiency in serum-free MSC medium, whereas Baseline hiPSCs exhibited the lowest.

By comparing the viability of hiPSCs seeded and cultured in serum-free MSC medium, it was observed that no significant difference in viability was observed between hiPSC starting materials after both seeding and selection. Comparison of cell diameters revealed no significant difference between hiPSC starting materials after seeding, however, after selection, cell diameter may vary between starting materials. It was determined that Baseline hiPSCs displayed significantly larger cell diameters than Centrifugation (p=0.001) and Non-Centrifugation (p=0.04) hiPSCs after selection, with Non-Centrifugation hiPSCs also exhibiting significantly greater cell diameters than Centrifugation hiPSCs after selection (p=0.035).

These results indicate that variation in hiPSC input material is likely to influence the differentiation of hiPSCs to MSCs.

By comparing the morphology of cell populations from different hiPSC starting materials through visual examination, it was determined that the morphology of cells cultured in MSC medium, from each starting material, differed significantly from hiPSCs cultured in pluripotent stem cell medium (Figures 147-149). However, no difference in morphology between Baseline, Centrifugation and Non-Centrifugation hiPSCs was observed during culture in MSC medium.
Chapter 10: The Exploration of Culture Conditions and Comparability in Starting Material for the Early Stages of hiPSC to MSC Differentiation

**Figure 147 (Top Left):** Baseline hiPSCs seeded and cultured in DMEM & 20% HPL (P1) (Day 2).

**Figure 148 (Top Right):** Centrifugation hiPSCs seeded and cultured in KO-DMEM & 20% HPL (P1) (Day 2). **Figure 149 (Above):** Non-Centrifugation hiPSCs seeded and cultured in KO-DMEM & 20% FBS (P1) (Day 2).
10.4 Discussion

As discussed in sections 10.1 and 10.2.1, the initial investigations into the differentiation of hiPSCs to MSCs in the present study were based upon protocols provided by I-Stem. However, this protocol could not be successfully reproduced, due to the low cell survival and significant apoptosis observed throughout culture, and therefore hiPSC to MSC differentiation could not be achieved using this method. Failure to replicate the protocols of an independent laboratory demonstrates the challenges associated with protocol transfers between laboratories and the achievement of multi-site comparability, which are also highlighted in sections 7.3.4 and 9.2.2. Current regulation in both Europe and the U.S requires the establishment and maintenance of comparability between manufacturing sites, as part of the Chemistry, Manufacturing and Control component of regulatory applications (Hourd et al, 2014). Therefore, the failure to replicate manufacturing protocols for a cell therapy product at multiple sites is likely to hinder regulatory applications. The protocol transfer for the hiPSC to MSC differentiation process, as presented in this chapter, was unplanned as it was initially unanticipated and extensive preparation was not performed, unlike those performed in Chapters 7 & 9. This therefore demonstrates the importance of undertaking significant planning, and developing significant understanding of the protocol, prior to performance of a protocol transfer.

The utilisation of a typical MSC culture medium composition, specifically DMEM & 10% FBS, was also found to be unsuitable for the differentiation of hiPSCs to MSCs, as this condition did not permit cell survival. By transitioning away from animal serum-based medium supplements to an animal serum-free medium supplement, specifically HPL, the survival of hiPSCs seeded in MSC medium, based upon either KO-DMEM or DMEM basal medium, improved. Furthermore, it was observed that, by increasing the FBS or HPL concentration in the MSC medium, the survival and proliferation of hiPSCs in MSC medium increased. Previous studies have reported similar findings after comparing high and low dose FBS concentrations in murine pluripotent stem cell cultures (Chaudhry et al, 2008), and reduced proliferation in response to low medium supplement concentrations is likely due to the presence of less protein and fewer signalling growth factors in the culture medium (Page et al, 2014). By increasing the serum concentration of culture medium, the concentration of various growth factors, adhesion factors, trace elements, minerals, vitamins, lipids, hormones,
amino acids, binding proteins, buffers, protease inhibitors, protection factors (e.g. albumin), and antitoxins is increased (Jung et al, 2012).

It was also determined that hiPSCs cultured in MSC medium displayed a morphology that differed significantly from that of hiPSCs cultured in typical pluripotent stem cell medium conditions. A more fibroblastic morphology, which is considered to be more representative of hMSCs, was observed in hiPSCs cultured in MSC medium. Whereas, during hiPSC culture in pluripotent stem cell medium, single cell populations demonstrating multiple long, thin lamellipodia were observed when cultured with ROCK inhibitor, and small colonies of hiPSCs with rounded morphologies were observed after the removal of ROCK inhibitor. These findings demonstrate that the culture of hiPSCs in MSC medium significantly altered the morphology of these cells. Therefore, as discussed previously in section 9.4, further research is required to examine the potential of the utilisation of changes in morphology as an early indicator of hiPSC differentiation may facilitate the development of a preliminary method of in-process characterisation.

It was also demonstrated that ROCK inhibitor pre-treatment, did not influence the cell morphology, distribution, and cell to cell interaction of hiPSCs cultured in MSC medium, unlike when these cells are cultured in pluripotent stem cell medium. Therefore, although ROCK inhibitor may influence the survival of hiPSCs in MSC medium, it does not appear to affect cell morphology. Furthermore, neither MSC medium composition nor hiPSC starting material significantly influenced the morphology of hiPSCs when cultured in MSC medium.

After the comparison of the survival and proliferation of hiPSCs in multiple MSC culture medium compositions, it was determined that the DMEM & 20% HPL composition was the most effective. The cell density after both seeding and selection was greatest in the DMEM & 20% HPL composition compared to other MSC medium compositions, and, unlike other conditions, DMEM & 20% HPL permitted cell proliferation after the removal of ROCK inhibitor. Culture of hiPSCs in DMEM & 20% HPL also yielded cell populations with high viabilities (>90%) and cells with larger cell diameters compared to other medium compositions. In section 7.3.2, the cell diameter of hMSCs was reported to be approximately 20µm, whereas, in section 9.3.2, the cell diameter of hiPSCs was reported to be approximately 15µm. Therefore, this would suggest that a larger cell diameter may be more representative of the morphology of hMSCs, and that the DMEM & 20% HPL medium
composition may generate cells that exhibit a morphology more closely representative of hMSCs. However, cell size has been demonstrated to vary with differences in partial pressure of CO₂, osmolality, and pH (deZengotita, Schmelzer & Miller, 2002), and therefore these findings cannot be considered conclusive.

The comparison of a number of ROCK inhibitor pre-treatment regimens allowed for the requirement for ROCK inhibitor pre-treatment, as well as the effectiveness of each of these regimens, to be determined. After seeding hiPSCs in various MSC medium compositions without the addition of ROCK inhibitor, it was observed that hiPSCs did not adhere to tissue culture plastic or survive. It can therefore be concluded that the addition of ROCK inhibitor is required when seeding hiPSCs in MSC medium after cryopreservation.

It was also observed that, not only does ROCK inhibitor improve hiPSC recovery post-thaw, but also that the addition of ROCK inhibitor to MSC culture medium improved the recovery of hiPSC-MSCs after a passage, and that, similarly to typical hiPSC culture, cell survival was greater after a passage than after cryopreservation. This is likely due to the detrimental effects of cryopreservation upon pluripotent stem cells; including activated apoptosis, disruption of cell to cell matrix adhesions, and elevated Reactive Oxygen Species (ROS) (Li & Ma, 2012); as well as the capacity of ROCK inhibitor to allow for the improved cell survival after dissociation (Watanabe et al, 2007).

By increasing the duration of ROCK inhibitor pre-treatment to four days, improvements in cell survival were observed in all MSC medium conditions. Although ROCK inhibitor pre-treatment regimens of longer durations were found to improve cell survival, it is unclear whether such regimens will inhibit hiPSC differentiation and maintain their pluripotency. The treatment of pluripotent stem cells with ROCK inhibitor has been previously demonstrated to maintain their pluripotency after dissociation (Watanabe et al, 2007).

It must also be noted that, despite the improved survival of hiPSCs in MSC medium with longer durations of ROCK inhibitor pre-treatment, significant cell death occurs after the removal of ROCK inhibitor regardless of the duration of pre-treatment. A similar decrease in cell density after the removal of ROCK inhibitor has previously been reported in the suspension culture of embryonic stem cells (Krawetz et al, 2010). However, longer pre-treatment durations may mitigate the cell apoptosis that occurs after the removal of ROCK inhibitor.
The culture of hiPSC-MSCs, previously cultured in MSC medium over a single passage, in adipogenic, chondrogenic and osteogenic differentiation medium allowed for the examination of the trilineage differentiation potential of these cells. It was determined that these cells successfully demonstrated trilineage differentiation, which is considered to be a typical hMSC property, although adipogenic differentiation may have been less significant. A reduced trilineage differentiation capacity of pluripotent stem cell derived MSCs compared to BM-MSCs has been previously demonstrated (Brown, Squire & Li, 2014). However, it is unclear whether the trilineage differentiation observed in the present research was associated with the trilineage differentiation potential of hiPSC-MSCs or the residual pluripotency of hiPSCs.

The effect of variations in hiPSC starting material upon the seeding and selection efficiencies of hiPSCs in MSC serum-free culture medium was determined by seeding and culturing Baseline, Centrifugation and Non-Centrifugation hiPSCs in DMEM & 20% HPL medium. As discussed previously in this section, this medium composition was found to be the most effective for the survival and proliferation of hiPSCs in MSC medium. After the comparison of different hiPSC starting materials, it was determined that Centrifugation hiPSCs exhibited the greatest cell density after both seeding and selection, whereas Baseline hiPSCs exhibited the lowest at both time points. However, it must be noted that the cell density of hiPSCs from each starting material was greater after selection, in which ROCK inhibitor is removed from the cultures, and a further two days of culture was performed. This would indicate that all hiPSC starting materials exhibited cell proliferation after the removal of ROCK inhibitor. As previously described in this section, ROCK inhibitor has been found to improve the survival of pluripotent stem cells after dissociation, as well as maintaining their pluripotency (Watanabe et al, 2007). Therefore, it is likely that the removal of ROCK inhibitor from hiPSC cultures, in culture medium conditions not optimised for pluripotent stem cell culture, would lead to significant hiPSC apoptosis, as demonstrated in section 10.3.1. Alternatively, the removal of ROCK inhibitor may select for cells that have adapted to the MSC culture medium conditions and those which may have differentiated. Therefore, it can be proposed that, as all hiPSC starting materials demonstrated cell proliferation after the removal of ROCK inhibitor, a proportion of each starting material cell population exhibited adaptation to the MSC culture medium conditions.
Further variations between hiPSC starting materials were observed, with lower cell diameters observed in Centrifugation and Non-Centrifugation hiPSCs after selection in MSC medium. This may indicate that differences in cell diameter correlate with cell proliferation, with smaller cells proliferating more rapidly and larger cells proliferating more slowly. A similar inverse correlation between cell size and cell proliferation has been previously observed in a number of investigations (Christodoulou et al, 2013; Majore et al, 2009; Su & O’Farrell, 1998). As highlighted previously in section 7.4, smaller cells may be advantageous for hMSC transplantation allowing for greater migration through tissues (Jung et al, 2012).

Although no significant difference in cell diameter was observed between hiPSC starting materials after seeding, or in cell viability between each hiPSC starting material after seeding and selection (>92%), the observed cell diameters of hiPSCs after both seeding and selection in MSC medium were greater than those measured during hiPSC seeding. This may indicate a shift towards a larger, hMSC morphology after culture in MSC serum-free medium. However, as previously discussed in this section, a number of culture parameters can influence cell size.

These findings regarding hiPSC starting materials indicate that significant variability in hiPSC seeding and selection efficiencies between starting materials existed; that the culture of hiPSCs utilising alternative process steps may improve the survival and selection of these cells when exposed to MSC culture medium; that cells with improved survival and adaptation in MSC culture medium may be generated when the Centrifugation cell culture process step is utilised; and that larger cell diameters, considered to be more representative of the morphology of hMSCs, were observed in Baseline and Non-Centrifugation hiPSCs after selection. The greater cell survival observed in Centrifugation hiPSC populations may indicate that the Centrifugation process step conditions cells for, or selects cells based upon, increased durability; or that the residual dissociation agent carryover that occurs during the Non-Centrifugation hiPSC culture process step may have a detrimental effect upon the early stages of hiPSC to MSC differentiation.

The difficulties in the generation of a successful hiPSC to MSC differentiation process observed in the present study highlights the considerable challenges associated with the development of transferable and scalable hiPSC differentiation processes and hiPSC derived cell therapy product manufacturing processes. Due to the often low differentiation efficiency,
a large number of hiPSCs must be generated in order to derive sufficient cell numbers for a cell therapy product. Furthermore, increasing the scale of differentiation and manufacturing processes for a hiPSC derived cell therapy is likely to incur substantial cost to the manufacturer, with significant increases in consumables utilisation for example.

The exploratory research performed in the present study may be representative of the early stages of process development for a hiPSC derived cell therapy product. However, the comparison of the effectiveness of such a variety of MSC culture medium compositions in the differentiation of hiPSCs to MSCs has not previously been performed. The examination of the effectiveness of animal serum-free medium compositions for the differentiation of hiPSCs to MSCs has only previously been explored in a single study (Luzzani et al, 2015), and therefore this research supports the feasibility of a serum-free differentiation process. Additionally, the investigation of the effect of variation in hiPSC starting material upon the hiPSC to MSC differentiation process has not previously been undertaken.

Further work is required in order to facilitate the optimisation of the hiPSC to MSC differentiation process, and the step-wise introduction of MSC medium to hiPSC culture should be explored as a method of improving cell adaptation. Similar step-wise approaches are utilised to adapt mammalian cell cultures to serum-free medium (Hernandez & Fischer, 2006). Future research regarding hiPSC-MSCs should also focus upon the determination of the stimulus that drives hiPSC to MSC differentiation, the scalability and automation of the differentiation process, and the comparability of these cells to adult hMSCs in terms of characterisation and functionality. Furthermore, in order to successfully replicate the I-Stem hiPSC to MSC differentiation protocol for the VAX001024c07 hiPSC line, a more carefully planned protocol transfer should be undertaken.

10.5 Conclusions

In this chapter, a number of qualitative and quantitative experiments have been performed in order to determine the effect of MSC culture medium composition, ROCK inhibitor pre-treatment regimen, and hiPSC starting material upon the early stages of hiPSC to MSC differentiation. This allowed for further understanding of these components of hiPSC to MSC differentiation protocols to be developed. Furthermore, the trilineage differentiation of hiPSC-MSCs was examined. These exploratory experiments; involving a complex, unplanned protocol transfer; process comparability; and comparability between starting
Chapter 10: The Exploration of Culture Conditions and Comparability in Starting Material for the Early Stages of hiPSC to MSC Differentiation

materials; contribute to the second challenge addressed in the present thesis regarding comparability in cell-based therapies.

These experiments determined that the DMEM & 20% HPL medium composition yielded the greatest cell survival and selection after the removal of ROCK inhibitor. Furthermore, it was observed that longer ROCK inhibitor pre-treatment regimens increased hiPSC survival in MSC medium. It was also demonstrated that differences were observed in hiPSC seeding and selection efficiency in MSC medium between hiPSC starting materials, indicating that the process steps utilised for hiPSC expansion are likely to influence the differentiation of these cells towards hMSCs. Finally, it was demonstrated that, similarly to adult hMSCs, hiPSC-MSCs displayed trilineage differentiation capacity.

The development of an effective hiPSC to MSC differentiation process was not achieved in the present research, and further work towards the optimisation and scalability of this process is required in order to facilitate the development of a hiPSC-MSC manufacturing process. Little analysis of cell characterisation, stability and functionality was performed in the present experiments, and further research would allow for greater evidence regarding the comparability between hiPSC-MSCs and adult hMSCs to be collected. Furthermore, although these experiments highlighted the significant difference between the morphology of hiPSCs cultured in pluripotent stem cell medium and those cultured in MSC medium, future research examining the feasibility of morphology changes as an early indicator of hiPSC to MSC differentiation may allow for the development of a straightforward method of in-process characterisation. Given the duration of culture required for the hiPSC to MSC differentiation process, the time required to perform the protocol transfer and to develop an understanding of the differentiation protocol, and the time available to undertake this research, it was not possible to develop an effective hiPSC to MSC differentiation process or to perform more rigorous characterisation of the hiPSC-MSCs generated.
Chapter 11: Summary & Conclusions

This chapter presents the summary, conclusions and limitations of this thesis, as well as highlighting opportunities for future research. It consists of a chapter by chapter summary, followed by a challenge-based summary relating to the two major challenges addressed in this thesis, which link key issues for the translation of stem cell-based therapies emerging from this fast-moving scientific field. These summaries are followed by conclusions regarding the two major challenges addressed; specifically the reimbursement of, and comparability in, cell-based therapies. The conclusions concerning the reimbursement of cell-based therapies are drawn across chapters, and those regarding the comparability between alternative cell culture process steps are presented both as cell-type specific conclusions, and as overarching conclusions regarding the Centrifugation and Non-Centrifugation process steps. The chapter closes with a discussion of the limitations of the present research, and the proposal of a number of areas of further research to build upon the findings of this thesis.

11.1 Summary

11.1.1- Chapter Summary

In this thesis, the characterisation, mode of action, source specificity and effects of in vitro expansion of Human Mesenchymal Stromal Cells (hMSCs) have been reviewed (Chapter 2). Additionally, the various reprogramming methods created for the generation of Human Induced Pluripotent Stem Cells (hiPSCs) were described and the progress towards the automated manufacture of hiPSCs summarised (Chapter 2). The protocols described in the published literature for the differentiation of iPSCs into MSCs were also summarised, and the characterisation and potential epigenetic carryover of iPSC-MSCs was discussed (Chapter 2). Chapter 2 also reviews the characteristics of Human Embryonic Stem Cells (hESCs), and the automated culture platforms and processes that have been applied to this cell type.

In Chapter 3, the reimbursement environment, current during this research, was described, the proposed change to Value-Based Pricing (VBP) discussed, the transformation of VBP identified, and the impact of the VBP upon the reimbursement of Regenerative Medicines (RMs) which target multiple simultaneous indications postulated. The adaptation of the ‘Headroom Method’, in order to account for such therapies capable of treating multiple simultaneous indications, was also examined in an exploratory study, as presented in Chapter 4. A cost-effectiveness analysis of Allogeneic Islet Transplantation, when employed for the treatment of Insulin Dependent Diabetes Mellitus (IDDM), was performed utilising the
current, VBP & Value-Based Assessment (VBA) methodologies (Chapter 5). Further, sensitivity analyses were undertaken to propose cost and efficacy targets for a hiPSC-derived Beta Cell therapy (Chapter 5). These cost-effectiveness and sensitivity analyses, as presented in Chapter 5, were performed with support from Dr Elangovan Gajraj (Technical Adviser, National Institute of Health and Care Excellence (NICE) Scientific Advice Team), in order to ensure that these analyses were well informed.

The experimental work in the present thesis contributed to the overarching theme of comparability in cell-based therapies through the comparison of the effects of manual and automated cell culture process steps upon a variety of product and process parameters representative the growth, viability, stability, characterisation and functionality of hMSCs, ‘Ruler’ hESCs, and hiPSCs, (Chapters 7-9). All of the process step comparability experiments were undertaken in collaboration with TAP Biosystems, with LGC and I-Stem also collaborating on the hMSC and hiPSC process step comparability experiments respectively. Finally, an exploratory study into the effect of numerous differentiation protocol components upon the early stages of hiPSC to MSC differentiation was undertaken in collaboration with I-Stem, and the comparability in hiPSC selection when cultured in MSC culture medium, between hiPSCs cultured using manual or automated process steps, was also examined. This allowed the effect of differences in starting material upon hiPSC to MSC differentiation to be explored (Chapter 10).

11.1.2- Challenge-Based Summary

This thesis has explored two major, interlinked challenges associated with the translation of stem cell-based therapies.

11.1.2.1- Reimbursement Challenge

The first challenge is the achievement of reimbursement for stem-cell based therapies, which can influence pricing, and therefore have consequences upon affordable manufacturing costs. Changes in the UK reimbursement landscape, with a shift towards VBP, were likely to influence the reimbursement of regenerative medicines. As described in Chapter 3, the transition towards such value-based appraisal methodologies faced significant resistance from stakeholders. However, after the performance of cost-effectiveness analyses using current and value-based appraisal methodologies, as presented in Chapter 5, it was demonstrated that the value-based methodologies were likely to have been favourable for the reimbursement of an exemplar cell-based therapy, specifically Allogeneic Islet Transplantation.
Chapter 11: Summary & Conclusions

The proposal of VBP also highlighted the importance of a holistic approach to patient care across care pathways, and that, in order to utilise such an approach, the treatment of patients with multiple simultaneous indications, or multimorbidity, must be addressed. Therefore, in the present research (Chapter 4), a number of variants were proposed as a preliminary study to explore the adaptation of the Headroom Method of early cost-effectiveness analysis to account for treatments capable of targeting multiple simultaneous indications. Furthermore, due to their putative, pleiotropic in vivo mode of action and, consequently, their potential to target multiple simultaneous indications, hMSCs represent a major focus of the present thesis. The comparability in growth, viability, stability, characterisation and putative modes of action, between hMSCs cultured using either manual or automated process steps, was measured in Chapter 7.

Due to the promising and fast-growing nature of the hiPSC field, the reimbursement potential and cost-effectiveness of a hiPSC derived product, specifically a hiPSC derived Beta Cell therapy, is also explored in the present thesis (Chapter 5). This was undertaken using sensitivity analyses based upon the current NICE appraisal methodology and Allogeneic Islet Transplantation cost-effectiveness data, and was informed by the input of a NICE representative (Dr Elangovan Gajraj, Technical Adviser, NICE Scientific Advice Team). As highlighted in Chapter 5, there is currently a requirement for greater long-term cost and efficacy data for hiPSC derived products. Therefore, the performance of accurate cost-effectiveness analyses is currently inhibited and the likelihood of achieving reimbursement for such products is diminished. Furthermore, the results of the hiPSC derived Beta Cell therapy product sensitivity analyses (Chapter 5) indicate that certainty in the duration of graft survival and the avoidance of the requirement for immunosuppressive therapy are key to achieving a favourable reimbursement decision; and that a lack of certainty may reduce the time horizon that can be considered in appraisals.

11.1.2.2 Comparability in Cell-Based Therapies

The second challenge addressed in this thesis concerns comparability in cell-based therapies between sites and between manufacturing process steps. In the present research a number of quantitative experiments were undertaken to determine the consequences of a change in a single manufacturing process step for multiple clinically relevant cell types, specifically hMSCs and hiPSCs; adding to our understanding of comparability after a process change, and of methods to measure and address comparability. To examine the effect of a change in a single manufacturing process step, a semi-automated culture process, which incorporated a
manual Centrifugation process step, and a fully automated culture process, which incorporated an automated Non-Centrifugation process step, were compared for each cell type. In order to determine the consequences of this process step change, multiple product (e.g. phenotype) and process (e.g. viable cell yield) parameters were measured. It is important to identify both product and process comparability as, although the regulators may be primarily focused upon product consistency, the achievement of product consistency often demands process consistency.

After the comparison of Centrifugation and Non-Centrifugation hMSC populations, as presented in Chapter 7, it was determined that all hMSC populations fulfilled the ISCT minimal criteria. Furthermore, all hMSC populations were found to be comparable in terms of morphology, stability, yield and viability. However, significant variability between populations was observed in measures of putative hMSC modes of action.

The comparability between process steps was also examined using a candidate hESC reference standard cell line (EC 2102Ep), in order to determine the feasibility of the utilisation of this cell line as a ‘Ruler’ to assist process development (Chapter 8). Although a greater cell yield was obtained using the automated, Non-Centrifugation process step, cell aggregation and settling during the Non-Centrifugation process step may have caused significant measurement variability in the cell counts obtained. Therefore, without further work, the EC 2102Ep cell line, when prepared using an automated culture process, can only be used as a reference for the critical quality attributes (CQAs) that were found to be stable; including morphology, cell diameter, viability, STR profile, and pluripotency marker expression.

As described in Chapter 9, comparability in hiPSC morphology, stability and population doubling time was observed between hiPSCs cultured utilising either Centrifugation or Non-Centrifugation process steps. However, differences in hiPSC yield, viability, phenotype and aggregate rate were identified between Centrifugation and Non-Centrifugation hiPSCs. Importantly, contrary to other cell types examined in the present research, a trend for decreased variability in cell yield was observed in hiPSCs cultured using the Non-Centrifugation process step.

The comparability between hMSC flow cytometry analyses performed at two independent laboratories was determined in Chapter 7, with variability in multiple surface markers
observed between laboratories; and an automated hiPSC culture protocol transfer was undertaken between laboratories in Chapter 9, in order to explore multi-site comparability.

The penultimate chapter of this thesis (Chapter 10) represents an exploratory study involving a protocol transfer, process comparability, and comparability between starting materials; in which the generation of hMSCs from hiPSCs was investigated. From this exploratory research performed in Chapter 10, it was determined that a serum-free culture medium composition yielded the greatest hiPSC survival and selection in MSC medium conditions, that longer durations of ROCK inhibitor pre-treatment promoted greater hiPSC survival in MSC culture medium, and that variability in hiPSC seeding and selection efficiency between hiPSC starting materials exists.

In addition to the individual chapter specific conclusions and the conclusions drawn relating to the two major challenges addressed, this research has identified many of the data requirements for the demonstration of cost-effectiveness, in order to achieve reimbursement, and comparability after a manufacturing process change, both of which represent critical challenges for the translation of stem cell-based therapies.

11.2 Reimbursement Conclusions

From the literature review regarding the transformation of the UK reimbursement environment (Chapter 3); and the cost-effectiveness analyses of Allogeneic Islet Transplantation for the treatment of IDDM performed using current, VBP, and VBA appraisal methodologies (Chapter 5); it can be concluded that the introduction of VBP would have been likely to be favourable for the reimbursement of Regenerative Medicines (RMs), as well as therapies which target multiple simultaneous indications, or Multimorbidity. The multiple pleiotropic modes of action that have been attributed to hMSCs suggest that a hMSC-based therapy may have potential in the treatment of multimorbid patients. Chapters 3 & 4 addressed the reimbursement challenges faced by such hMSC-based products, and Chapter 7 addressed the manufacturing challenge of comparability after a change in the manufacturing process for these cells.

Value-Based Appraisal methodologies may have been favourable for RMs due to a reduction in the Incremental Cost-Effectiveness Ratio (ICER), and an increase in the NICE Cost per QALY threshold, which would have occurred for these technologies under the VBP scheme due to their fulfilment of multiple criteria, including Wider Societal Benefits (WSB) and Burden of Illness (BoI). Further, it is also likely that the VBA scheme would have been
favourable for these therapies, although to a lesser extent than VBP. Cost-effectiveness analyses of a cell-based therapy using VBP and VBA methodologies, as performed in the present thesis, have not previously been undertaken.

The present thesis has also demonstrated that the Headroom Method of early cost-effectiveness analysis can be adapted to account for therapies which target Multimorbidity, such as hMSC-based therapies, as presented in Chapter 4. However, it is apparent that further research, utilising more accurate health utility data for multimorbid patients, is required in order to test the Headroom variants proposed. The most promising adaptation was found to be the ‘Mean utility & duration of benefit' variant. The extension of the Headroom Method to account for therapies which target multiple simultaneous indications has not previously been undertaken.

The derivation of cells through the differentiation of hiPSCs offers the potential for the generation of large numbers of autologous or allogeneic cells for cell-based therapy. The challenge of reimbursement for cell-based therapies, and in particular hiPSC derived therapies, is further addressed in Chapter 5, in which sensitivity analyses are performed in order to propose cost and efficacy targets for a hiPSC-derived Beta Cell therapy. These analyses demonstrated the importance that the avoidance of immunosuppression, the achievable duration of graft function, and achievable rate of graft failure may have upon the likelihood of reimbursement for such a cell-based therapy. The findings of these sensitivity analyses were based upon the assumption that a hiPSC-derived Beta Cell therapy would achieve a similar cost and efficacy to that of Allogeneic Islet Transplantation. However, increases or decreases in cost or efficacy would alter the cost-effectiveness of such a product. Sensitivity analyses of this kind have not previously been performed for a hiPSC-derived Beta Cell therapy, and there is currently a lack of long-term cost and efficacy data for hiPSC-derived products, which may impede cost-effectiveness analyses, and therefore reimbursement, for such products. The modelling results presented in Chapter 5 highlight the particular need for adequate long-term data regarding the duration of graft survival and the avoidance of the requirement for immunosuppressive therapy in order to create sufficient certainty in the cost-effectiveness analyses.

Both the systematic literature review (Chapter 3) and the cost-effectiveness analyses of Allogeneic Islet Transplantation (Chapter 5) highlight that the methodology utilised in appraisals may act as an incentive for innovators, directing product development, and may
influence investment decisions. Finally, the cost-effectiveness analyses performed in Chapter 5 also indicate that, without sufficient certainty in the duration of graft survival, the time horizon considered in appraisals would be limited. This is demonstrated in section 5.2.1, in which the ICER for Allogeneic Islet Transplantation with a time horizon limited to 5 years is presented.

11.3- Cell Type-Specific Process Step Comparability Conclusions

11.3.1 hMSC Comparability Conclusions

After comparing and quantifying the effects of alternative process steps upon hMSC growth, characterisation and functionality, it was determined that no significant difference in hMSC morphology, cell diameter, phenotype, STR profile, yield, population doubling time or viability was observed, over multiple passages, between cells cultured using either manual or automated process steps. Furthermore, all hMSC conditions displayed Colony Forming Unit (CFU) Potential and trilineage differentiation capacity, which are typical of hMSC populations. Therefore, hMSCs cultured utilising both manual and automated process steps met the ISCT minimal criteria (Dominici et al, 2006), and could therefore be considered to display comparable characteristics. Although the methods for the determination of CFU potential and trilineage differentiation utilised in the present study were not able to accurately quantify these parameters and could be considered subjective, these methods are representative of those recommended by the ISCT (Dominici et al, 2006) and allowed for confirmation that hMSC CFU potential and trilineage differentiation remained after culture using alternative process steps. Therefore, it can be concluded that the utilisation of the Non-Centrifugation process step, in which residual dissociation agent remains within the culture, did not significantly influence these measures of hMSC growth and characterisation. However, a non-significant trend for greater variability in hMSC viable cell yield was observed when the automated process step was utilised, which would suggest that the culture process incorporating the Non-Centrifugation process step produced hMSC populations with less consistency in viable cell yield. However, this increased variability in hMSC yield may not be observed when comparing a fully automated manufacturing process, including the Non-Centrifugation process step, to a fully manual manufacturing process.

By exploring hMSC paracrine functionality using ELISA- and fluorescence-based assays, it was determined that hMSCs from all conditions responded to inflammatory pre-treatment by increasing IDO activity and PGE-2 secretion. However, a trend for a greater IDO response in
Baseline hMSCs, as well as significantly greater PGE-2 secretion in inflammatory pre-treated Non-Centrifugation hMSCs, was observed. Additionally, hMSCs from all conditions expressed VEGF both constitutively and in response to hypoxic pre-treatment. However, significantly greater VEGF expression was found in untreated Baseline hMSCs and hypoxia pre-treated Centrifugation hMSCs. Therefore, it can be concluded that a lack of comparability was observed in hMSC IDO activity, PGE-2 secretion, VEGF secretion between manual and automated process steps. The comparability between Post-Centrifugation and Non-Centrifugation hMSCs is summarised in Figure 150.

Despite the trends observed in the ELISA-based assays of hMSC paracrine functionality, it must be noted that inherent variability is often observed in assays of this nature, and that inherent heterogeneity is common between hMSC populations. Therefore, despite utilising surrogate potency assays to assess the functionality of hMSCs cultured utilising alternative process steps, these challenges highlight the current difficulties in developing accurate and consistent potency assays for this cell type.

Finally, by comparing the flow cytometry data obtained from two separate laboratories, specifically Loughborough University and LGC, it is apparent that the reported phenotype of hMSC populations can differ between laboratories. Minor decreases in CD105 and increases in CD34 expression were reported in LGC’s analysis in comparison to the results obtained at Loughborough University. This further highlights the challenges of achieving process and product comparability between multiple sites. However, it should be recognised that differences in culture and flow cytometry methodologies were present between laboratories, in particular automated culture protocols were manually replicated at LGC.

11.3.2 Ruler hESC Comparability Conclusions

From the Centrifugation and Non-Centrifugation ‘Ruler’ hESC (EC 2102Ep) cell culture process step comparability experiment, it was demonstrated that no significant difference in EC 2102Ep cell morphology, cell diameter, STR profile and viability was observed between cells cultured using either manual or automated process steps, over multiple passages.

By comparing the pluripotency marker expression of EC 2102Ep cell populations cultured using alternative process steps, it was determined that all populations highly expressed typical pluripotency markers, and that the co-expression of these markers was high in all populations. Additionally, differentiation marker expression was found to be low in all
populations. Therefore, although each population exhibited heterogeneity in cell phenotype, each EC 2102Ep population predominantly contained cells with a pluripotent phenotype.

However, the Non-Centrifugation EC 2102Ep cell populations exhibited marginally lower Oct 3/4 pluripotency marker expression (<4.5%), as well marginally higher SSEA-1 differentiation marker expression (<0.3%), compared to Baseline and Centrifugation EC 2102Ep cell populations. However, it is apparent that these differences in pluripotency marker and differentiation marker expression observed between EC 2102Ep cell populations were negligible, and that no difference in pluripotency marker co-expression was observed between Ruler hESC conditions. Therefore, it can be concluded that the phenotype of EC 2102Ep cell populations from each condition was comparable.

It was also determined that the culture of EC 2102Ep cells using the automated process step generated a significantly greater viable cell yield and shorter population doubling time compared to those cultured using the Centrifugation process step. This would indicate that the automated process step was more favourable for the growth of the EC 2102Ep populations. However, difficulties in achieving a representative cell count, due to the settling and aggregation of cells during the fully-automated Non-Centrifugation process, may have contributed to these findings.

The EC 2102Ep viable cell yield data also indicated that greater variability in viable cell yield may have been observed in Non-Centrifugation populations compared to Centrifugation populations. This would suggest that the automated process step generated EC 2102Ep cell populations with less consistent viable cell yields compared to the manual process step. However, this trend was found to be non-significant, and a large proportion of the variability in the second passage could be attributed to a single batch in which a malfunction occurred. Figure 150 summarises the comparability between Post-Centrifugation and Non-Centrifugation Ruler hESCs.

After culturing the EC 2102Ep cell line on the CompacT SelecT automated cell culture platform, utilising both manual and automated process steps, it can be confirmed that this proposed Ruler hESC line is amenable to automated cell culture. However, for this particular automated platform, this candidate Ruler cell line should only be used as a reference for the hESC CQAs that were demonstrated as being stable and comparable between process steps; which included morphology, cell diameter, viability, STR profile, and pluripotency marker expression. Therefore, although this hESC Ruler cell line may represent a valuable tool for
the validation of automated cell culture platforms, further work is required to overcome the
difficulties associated with the aggregation and settling of these cells after dissociation within
the automated process step, in order to increase the accuracy of automated cell counts and to
permit wider use of this automated platform for the culture of this cell line.

11.3.3 hiPSC Comparability Conclusions

The comparison of manual and automated hiPSC culture process steps revealed no significant
difference, and therefore comparability, in hiPSC morphology, cell diameter, STR profile,
and population doubling time between cells cultured utilising either Centrifugation or Non-
Centrifugation process steps, over multiple passages.

With regards hiPSC morphology, although all populations contained single cells, with
multiple long projections, in the presence of ROCK inhibitor, and formed tight clusters of
rounded cells without the presence of ROCK inhibitor, it was observed that hiPSCs cultured
using the Non-Centrifugation process step appeared to form colonies more rapidly than cells
cultured using the Centrifugation process step, although this is unlikely to have influenced
viable cell yield. This observation may indicate that cell to cell interaction is important for the
growth of cell populations cultured using the automated process step and, therefore, those
which are exposed to residual dissociation agent.

The comparison of hiPSC pluripotency marker expression revealed that all hiPSC
populations contained predominantly pluripotent cells, although heterogeneity in cell
phenotype was observed in each population. All hiPSC populations highly expressed typical
pluripotency markers, and the co-expression of these markers was high in all populations.
Differentiation marker expression was also found to be low in all populations.

Centrifugation hiPSC populations exhibited lower expression of typical pluripotency markers,
with 20.5% lower pluripotency marker co-expression; and greater differentiation marker
expression, with up to 15.21% greater SSEA-1 expression, compared to Non-Centrifugation
hiPSC populations. This would suggest that the Centrifugation hiPSC populations contained
greater numbers of differentiated cells and that the automated, Non-Centrifugation process
step maintained the pluripotency and purity of the VAX001024c07 hiPSC line more
effectively than the manual, Centrifugation process step.

When comparing hiPSC growth after culture using either manual or automated process steps,
it was observed that Non-Centrifugation hiPSC populations exhibited significantly greater
viable cell yields than Post-Centrifugation populations. This would indicate that the utilisation of the automated cell culture process step yielded significantly greater viable cell numbers than culture using the manual process step, and therefore that the automated process step was more favourable in terms of hiPSC yield. It was also determined that Pre-Centrifugation hiPSC samples exhibited significantly greater cell yields compared to Post-Centrifugation samples, which suggests that the centrifugation process may be detrimental to the final viable cell yield. Furthermore, in addition to the significantly greater viable hiPSC yield observed in Non-Centrifugation populations, less variability may have also been observed in these populations in comparison to Post-Centrifugation populations. Therefore, the utilisation of the automated process step may generate hiPSC populations with more a consistent cell yield compared to the utilisation of the manual process step. However, this trend was not found to be significant.

With regards the effect of manual and automated process steps upon hiPSC viability, it was determined that Post-Centrifugation hiPSC populations exhibited significantly greater viabilities than Non-Centrifugation populations across all passages. It was also identified that the viability of Post-Centrifugation hiPSC populations was significantly greater than that of Pre-Centrifugation populations in the final passage. These findings indicate that the automated process step may be detrimental to hiPSC viability, however the viability of Non-Centrifugation hiPSC populations remained >94% throughout, which is significantly above the minimal cell viability (80%) for cell therapy products outlined by the FDA (FDA & Genzyme, 2007). Furthermore, the difference observed in hiPSC viability between alternative process steps was <2.2%, which is within the maximum intra-sample variability in viability for the Cedex automated cell counter (±3%) (Roche, 2003). These results may also suggest that the Centrifugation process step improved the viability of hiPSC populations, which may indicate that viable cells are positively selected during this process step.

The rate of hiPSC aggregation was also compared between process steps, and it was demonstrated that the aggregate rate of Non-Centrifugation hiPSCs was significantly greater than that of Post-Centrifugation hiPSCs. This would indicate that significantly greater aggregation occurred as a result of the automated culture process, which may influence the accuracy of cell counts and the seeding of hiPSC daughter flasks. Additionally, Pre-Centrifugation hiPSCs exhibited a significantly greater aggregate rate than Post-Centrifugation hiPSCs. Therefore, it can be concluded that the Centrifugation process step; which involves centrifugation, aspiration of the supernatant, and the resuspension of cells in
fresh culture medium; effectively dispersed cell aggregates forming a more homogenous single cell suspension. The comparability between Post-Centrifugation and Non-Centrifugation hiPSCs, determined using key product and process parameters, is summarised in Figure 150.

One of the most significant findings of the hiPSC process step comparability experiment related to the effect of cryopreservation upon hiPSC characteristics and growth. It was determined that hiPSCs in the Pre-Experimental passage, after cryopreservation, displayed significantly greater cell diameters, lower yields, longer population doubling times, and greater aggregate rates compared to cell populations from other passages. These findings highlight the detrimental impact of cryopreservation and thawing upon hiPSC manufacturing processes, the need for culture over a single passage in order to recover typical hiPSC characteristics and growth, and the need for optimisation of the hiPSC cryopreservation process. However, despite the numerous changes observed in the first passage after cryopreservation, hiPSC viability was not affected and did not significantly differ from later passages.

**11.4 Conclusions regarding the Exploration of Culture Conditions and Comparability in Starting Material for the Differentiation of hiPSCs to MSCs**

In Chapter 10, an exploratory study was undertaken to examine the effect of MSC medium composition, ROCK inhibitor pre-treatment regimen, and hiPSC starting material upon the early stages of hiPSC to MSC differentiation, in order to develop further understanding of hiPSC to MSC differentiation protocols.

By comparing a number of different MSC culture medium compositions, it was determined that transitioning from animal serum (FBS) based compositions to animal serum-free compositions (HPL) appeared to improve hiPSC survival in MSC medium. Furthermore, it was observed that increasing the concentration of either FBS or HPL also improved hiPSC survival in MSC medium, that the DMEM & 20% HPL MSC medium composition allowed for the greatest cell survival and proliferation of those examined, and that this medium composition yielded the greatest viabilities and cell diameters of those examined. A larger cell diameter may be representative of a shift towards the morphology of hMSCs, and away from that of hiPSCs, given the typically larger size of hMSCs compared to hiPSCs.

The visual examination of cell morphology also revealed that hiPSCs cultured in MSC medium displayed a more fibroblastic morphology, which is considered to be representative
of hMSCs, compared to cells cultured in pluripotent stem cell medium. Although the culture of hiPSCs in MSC medium may have altered the morphology of these cells, no difference in cell morphology was observed between each of the MSC medium compositions. Further research is required in order to quantify and model these morphology changes, which may facilitate the utilisation of changes in cell morphology as an early indicator of hiPSC differentiation and as a method of in-process characterisation.

The examination of ROCK inhibitor pre-treatment regimens revealed that the seeding of hiPSCs in MSC medium without the addition of ROCK inhibitor does not permit cell survival; that a longer duration of ROCK inhibitor pre-treatment improved hiPSC survival; that, regardless of the duration of ROCK inhibitor pre-treatment, the removal of ROCK inhibitor from culture was associated with significant cell death; and that no change in cell morphology, distribution, or cell to cell interaction was observed after the removal of ROCK inhibitor from culture.

Trilineage differentiation capacity of hiPSCs seeded in MSC medium, and cultured over a single passage, was also demonstrated, and this is considered to be a typical characteristic of hMSCs. However, it is unclear whether the trilineage differentiation potential demonstrated was associated with the trilineage differentiation capacity of hiPSC-MSCs or the residual pluripotency of hiPSCs.

Finally, the comparison of the seeding and selection efficiencies of hiPSC starting materials identified that Centrifugation hiPSCs exhibited significantly greater cell densities than Baseline and Non-Centrifugation hiPSCs after both seeding and selection. Furthermore, although no significant difference in cell viability after seeding and selection, and in cell diameter after seeding, was observed, Baseline and Non-Centrifugation hiPSCs exhibited significantly larger cell diameters than Centrifugation hiPSCs after selection in MSC culture medium. These results highlight the variability in hiPSC response to seeding and selection in MSC culture medium between hiPSC starting materials. However, despite this variability, no difference in morphology between Baseline, Centrifugation and Non-Centrifugation hiPSCs was observed through visual examination during culture in MSC medium, although the morphology of these cells differed significantly from that of hiPSCs cultured in pluripotent stem cell medium.
**Table summarising the comparability between alternative process steps for hMSCs, Ruler hESCs and hiPSCs determined using numerous parameters. Asterisk (*) denotes significance over other conditions (p=0.05). Number Sign (#) denotes significance of Non-Centrifugation hiPSCs over Baseline hiPSCs (p=0.05). NSD= No Significant Difference (p>0.05).**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>hMSCs</th>
<th>‘Ruler’ hESCs</th>
<th>hiPSCs</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cell Type</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Morphology</td>
<td>Spindle shaped, fibroblast-like</td>
<td>Spherical morphology forming tightly packed clusters</td>
<td>hiPSCs *With ROCK inhibitor (Day 0-1): Single cells with multiple long, thin lamellipodia. hiPSCs *Without ROCK inhibitor (Day 2-7): Form tight clusters of cells with rounded morphology</td>
</tr>
<tr>
<td>Cell Diameter</td>
<td>19.84μm (±0.66μm)</td>
<td>20.13μm (±0.75μm)</td>
<td>18.11μm (±0.41μm)</td>
</tr>
<tr>
<td>Viable Cell Yield</td>
<td>P5: 2.06x10⁶ (±0.25x10⁶)</td>
<td>P5: 2.2x10⁶ (±0.66x10⁶)</td>
<td>P54: 5.33x10⁷ (±0.66x10⁷)</td>
</tr>
<tr>
<td>Viability</td>
<td>P5: 97.6% (±0.17%)</td>
<td>P5: 97.8% (±0.3%)</td>
<td>P54: 99.3% (±0.28%)</td>
</tr>
<tr>
<td>STR Profile</td>
<td>NSD in STR expression pattern</td>
<td>NSD in STR expression pattern</td>
<td>NSD in STR expression pattern</td>
</tr>
<tr>
<td>Phenotype</td>
<td>Meet ISCT minimal criteria</td>
<td>Meet ISCT minimal criteria</td>
<td>87.2% SSEA-4 &amp; Oct 3/4 Co-Expression &lt;0.5% SSEA-1 Expression</td>
</tr>
<tr>
<td>Functionality</td>
<td>Trilineage Differentiation:</td>
<td></td>
<td>86.7% SSEA-4 &amp; Oct 3/4 Co-Expression &lt;0.5% SSEA-1 Expression</td>
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<tr>
<td></td>
<td>Post-Centrifugation &amp; Non-Centrifugation</td>
<td></td>
<td>50.3% SSEA-3 &amp; TRA-1-81 co-expression &lt;20% SSEA-1 expression</td>
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<tr>
<td></td>
<td>hMSCs displayed adipogenic, chondrogenic &amp; osteogenic differentiation capacity</td>
<td></td>
<td>70.8% SSEA-3 &amp; TRA-1-81 co-expression &lt;5% SSEA-1 expression</td>
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<tr>
<td></td>
<td>Colony Forming Unit Potential:</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Post-Centrifugation= 13.4% (±0.8%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Non-Centrifugation= 18.2% (±3.1%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Immunosuppression:</td>
<td>N/A</td>
<td>N/A</td>
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<td></td>
<td>NSD in IDO activity after TNF-α &amp; IFN-γ pre-treatment</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>Significantly greater PGE-2 expression in Non-Centrifugation hMSCs after TNF-α &amp; IFN-γ pre-treatment*</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Angiogenesis:</td>
<td>N/A</td>
<td>N/A</td>
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<tr>
<td></td>
<td>Significantly greater VEGF expression in Centrifugation hMSCs after Hypoxic preconditioning (1% O₂)*</td>
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</table>

**Figure 150:** Table summarising the comparability between alternative process steps for hMSCs, Ruler hESCs and hiPSCs determined using numerous parameters. Asterisk (*) denotes significance over other conditions (p=0.05). Number Sign (#) denotes significance of Non-Centrifugation hiPSCs over Baseline hiPSCs (p=0.05). NSD= No Significant Difference (p>0.05).
Chapter 11: Summary & Conclusions

11.5 Conclusions regarding Centrifugation & Non-Centrifugation Process Steps

The comparison of manual and automated process steps for the culture of numerous cell types allowed for process experience to be gathered, for process performance to be examined, and for the data requirements for the demonstration of comparability after a process change to be highlighted. Experiments of a similar nature to those performed in the present study are likely to be undertaken during the process development stages of cell therapy product manufacturing after a process or platform change has been made, which often occur prior to Phase III clinical trials. Such comparative studies are likely to represent engineering runs performed prior to Process Qualification (PQ) runs in order to determine process performance and to confirm that the newly developed process can be executed within the required limits. The PQ stage is then utilised to verify process performance. Therefore, the experiments performed in the present research, in which Centrifugation and Non-Centrifugation cell culture process steps were compared for multiple cell types, are representative of the experimental manufacturing runs that would be performed prior to PQ, after the decision to change a single, key manufacturing process step has been made.

By comparing the results of the hMSC, Ruler hESC and hiPSC process step comparability experiments, general conclusions can be made regarding the Centrifugation and Non-Centrifugation process steps. Each of these process steps are associated with risks that may be detrimental to cell populations, including cell damage due to centrifugal force or residual enzyme carryover, and the effect of the utilisation of these two process steps upon key product and process parameters has been determined. It was determined that cell morphology and STR profile were comparable between manual and automated process steps across all cell types. Furthermore, it was demonstrated that no difference in hMSC or Ruler hESC phenotype was observed between process steps, however a decreased pluripotency marker expression, and increased differentiation marker expression, was observed in Centrifugation hiPSCs. These findings therefore indicate that cell characterisation and stability was similar between cells cultured utilising alternative process steps.

It was also determined that Cell Diameter and Viability were well controlled parameters across all experiments and cell types. Although cell diameter could be considered to be a stable parameter, and that significant changes may be associated with a number of variables, for example osmolality, this control gives an indication that cell morphology does not significantly vary when alternative cell culture process steps are utilised. The low variability in cell population viability indicates that the number of apoptotic cells in each population was
similar both within, and between, process steps. However, in the case of hiPSCs, although little variability in the cell viability parameter was demonstrated within each process step, a significant difference in this parameter was observed between process steps which may indicate a lack of comparability.

A trend may exist between cell types for a greater viable cell yield in cell populations cultured using the automated process step compared to those cultured using the manual process step. This trend was found to be significant in Ruler hESC and hiPSC populations, but non-significant in hMSC populations. This would demonstrate that the utilisation of the fully automated culture process yielded a greater number of cells than the process in which the Centrifugation process step was incorporated. However, non-significant trends for greater variability in viable cell yield were observed in two of the three cell types when the Non-Centrifugation process step was employed. This highlights that the impact of cell settling and aggregation, during the Non-Centrifugation dissociation process step, upon the accuracy of the automated cell counts may have influenced the cell yield data obtained. The challenges associated with the determination of accurate cell counts during the Non-Centrifugation dissociation process step represent a recurring theme throughout the comparability experiments performed in the present research, and further work may be required in order to optimise the number of mixing steps performed prior to the cell count, during the fully-automated culture process.

The comparison of the seeding and selection efficiencies of hiPSC starting materials in MSC culture medium demonstrated variability between hiPSCs cultured using alternative process steps. It was observed that Centrifugation hiPSCs exhibited significantly greater seeding and selection efficiencies compared to Baseline and Non-Centrifugation hiPSCs, and that Baseline hiPSCs exhibited the lowest efficiencies. It was also identified that Centrifugation hiPSCs exhibited significantly smaller cell diameters after selection compared to Baseline and Non-Centrifugation hiPSCs. However, no significant difference in cell viability was observed between hiPSC starting materials after seeding or selection. These findings indicate that variability in hiPSC seeding and selection efficiencies between starting materials existed; that the expansion of hiPSCs using either manual or automated process steps may improve the survival and selection of these cells when exposed to MSC culture medium; that the Centrifugation cell culture process step may generate cells with improved survival and adaptation in MSC culture medium; and that Baseline and Non-Centrifugation hiPSCs
exhibited larger cell diameters after selection, which may be more typical of the morphology of hMSCs.

In conclusion, these findings indicate that generally, in comparison to a more typical Centrifugation cell culture process step, the Non-Centrifugation cell culture process step generated cells with comparable stability, characterisation, and viability; despite the residual dissociation agent carryover. A number of exceptions to this conclusion were observed in the case of hiPSCs. Furthermore, in general, the culture processes based upon the Non-Centrifugation process step generated a greater cell yield, although this may have been associated with increased cell settling during the Non-Centrifugation process step. However, further research is required to examine the variability in hMSC functionality and hiPSC to MSC differentiation between cells cultured using alternative manufacturing process steps, and to determine the implications of this variability upon product safety and efficacy. It is also apparent that further work is required in order to optimise the Non-Centrifugation process step in order to generate more consistent cell yields. Furthermore, to facilitate a process transfer towards a fully automated manufacturing process, future work should build upon the process comparability risks identified in the present research in order to identify the specification limits of a fully automated manufacturing process for each cell type, as discussed further in section 11.8.2.

Although each of the comparability experiments performed represents a brief comparability study, the present research presents valuable insight into the comparability between manual and automated cell culture process steps, which is required for the demonstration of equivalence when developing a scalable cell therapy manufacturing process in order to comply with regulatory requirements (Hourd et al, 2014). In the present research, a number of analytical methods were employed in order to assess the differences in identity, quality, purity and potency of multiple cell types when alternative process steps were utilised, and therefore the effect upon safety and effectiveness was determined through surrogate assays. These comparability studies therefore conform to the definition of a comparability protocol as defined by the FDA (Food and Drug Administration, 2003), although these guidelines has recently been withdrawn (Federal Register, 2015).

11.6 Multi-site Comparability Conclusions

By performing flow cytometric analysis of hMSCs, cultured using the Centrifugation or Non-Centrifugation process steps (Loughborough University) or by manually replicating these
processes (LGC), at two independent laboratories and comparing the phenotypes observed, differences in CD105 and CD34 expression measured between sites were encountered. Lower expression of the positive hMSC marker (CD105) and higher expression of the negative hMSC marker (CD34) was observed in the analysis performed by LGC compared to that performed at Loughborough University. Although these findings may highlight the influence of differences in culture protocols between sites, the effect of inter-individual variation and differences in analysis methods upon flow cytometry results are also likely to have contributed to this variability in surface marker expression. Furthermore, it is apparent that a requirement exists for the standardisation of flow cytometry methodologies.

The comparability between multiple laboratories was further explored in the present research through the performance of multiple hiPSC protocol transfers between sites, specifically between I-Stem and Loughborough University. Automated seeding, medium exchange, passage, and harvest protocols for the culture of hiPSCs (VAX001024c07) were transferred from I-Stem to Loughborough University. Despite the requirement for the some adjustment, adaptation and optimisation of a number of these protocols, each of these protocols was readily transferred between sites and was successfully replicated.

However, as presented in Chapter 10, after the protocol transfer of the hiPSC (VAX001024c07) to MSC differentiation process from I-Stem to Loughborough University, it was determined that this protocol could not be successfully replicated. This failure to reproduce the protocols of another laboratory highlights the challenges that are faced in the achievement and demonstration of multi-site comparability, with significant amounts of data and resource required. Furthermore, the difficulties faced in the present research, regarding the replication of externally generated protocols, underlines the need for significant planning prior to the performance and execution of a protocol transfer.

11.7 Limitations of Research

The utilisation of more accurate health utility, duration of benefit and economic data, relating to the treatment of multimorbid patients, is required in order to perform more precise early cost-effectiveness analyses for treatments which target multiple simultaneous indications. This would also allow for greater validation of the Headroom variants proposed for treatments capable of targeting multiple simultaneous indications.

Furthermore, with the recent progression of Pluripotent Stem Cell-derived Beta Cell or Pancreatic Progenitor therapies, for example Viacyte’s VC-01™ product, the utilisation of
more accurate cost of goods and differentiation efficiency data in the future is likely to allow for more detailed cost-effectiveness analysis of a hiPSC-derived Beta Cell therapy than was achievable in the present research.

To further examine the comparability of manual and automated cell culture process steps, greater numbers of repeats, batches and passages than undertaken in the present research could be performed for each cell type in order to achieve greater statistical power. Additionally, further analysis of the growth rate of each cell type over the course of culture, using either manual or automated process steps, could be utilised to further examine the comparability between process steps. Further in-process characterisation and measurements may also allow for more detailed analysis of the effect of alternative process steps over the course of the manufacturing process. The comparison of fully manual and fully automated manufacturing processes for each cell type may provide further context for the data generated in the present research. However, such detailed analyses may only be necessary during the process development stages of a cell-based therapy. Further work is also required in order to address the cell count measurement variability that is observed during the Non-Centrifugation process step due to cell aggregation and settling, particularly in the case of the Ruler hESC line, and to optimise this process step through the reduction of this cell aggregation.

Additional analysis of hMSCs, cultured using alternative process steps, through the utilisation of alternative laboratory techniques, for example gene expression assays, may have also provided further insight into the comparability in hMSC mode of action. The utilisation of alternative methods of protein quantification was planned, however complications with the molecular weight of the proteins measured prevented the completion of these analyses.

In order to more robustly examine how representative the proposed ‘Ruler’ hESC line is of conventional hESCs, comparisons against an alternative hESC line could be performed. The data obtained in the present research regarding the culture of the EC 2102Ep line on the CompacT SelecT automated platform could be utilised as a comparator against alternative hESC lines culture on this platform in the future. Further validation of analytical techniques and culture processes for hESCs using the EC 2102Ep line as a ‘Ruler’ would also contribute to the progression of standardisation of hESC culture and analysis.

Although the analysis of pluripotency marker expression provides substantial evidence of the pluripotency of hPSCs, more conclusive evidence can be obtained through the examination the differentiation of these cells into the three germ layers. Therefore, the differentiation of
Centrifugation and Non-Centrifugation hiPSCs into the three germ layers could provide additional evidence of the comparability between process steps with regards hiPSC pluripotency. It is also apparent from the present research that further work is required in order to optimise the cryopreservation process for hiPSCs and to therefore improve the recovery of these cells.

To further advance the preliminary work exploring the differentiation of iPSCs into MSCs undertaken in the present research, experiments involving the optimisation of the differentiation process, in order to improve differentiation efficiency; the scalable differentiation of iPSCs to MSCs; and the expansion of these differentiated cells may facilitate the development of a scalable hiPSC-MSC manufacturing process. Finally, further examination of hiPSC-MSC stability, phenotype, characterisation, and functionality would give valuable insight into safety and efficacy of these cells, as well as their comparability to adult hMSCs.

11.8 Further Research

11.8.1 Data Requirements for the Reimbursement of a hiPSC Derived Beta Cell therapy
The sensitivity analyses performed in Chapter 5, exploring the reimbursement potential of a hiPSC derived Beta cell therapy, represent a prospective cost-effectiveness analysis of such a product, based upon the cost and efficacy data available for Allogeneic Islet Transplantation. Neither long-term nor short-term cost and efficacy data is currently available for a hiPSC derived Beta cell therapy product, and therefore accurate cost-effectiveness analyses cannot be performed. In order to achieve a positive reimbursement decision, particularly in the UK, this data must be present as part of a Health Technology Appraisal (HTA) submission.

In the present research, as presented in Chapter 5, it has been identified that, in addition to providing sufficient cost and efficacy data, the clinical trial data collected for a hiPSC derived Beta Cell therapy must demonstrate adequate certainty in the graft failure rate, the time horizon considered, and the avoidance of the requirement for immunosuppressive therapy. This evidence becomes crucial if the time horizon considered during appraisals is reduced due to the uncertainty in long-term graft survival, as demonstrated in section 5.2.1 by the utilisation of a limited, practical time horizon for Allogeneic Islet Transplantation of 5 years. Therefore, further research is required to collect sufficient data regarding the long-term efficacy, long-term cost, graft failure rate, and avoidance of the requirement of immunosuppressive therapy for a hiPSC derived Beta Cell therapy.
Chapter 11: Summary & Conclusions

11.8.2 Fully Automated Manufacturing Process Comparability Experiments

The process step comparability experiments undertaken in the present research (Chapters 7-9) are representative of the experimental manufacturing or engineering runs that would be performed, after a change to a single manufacturing process step has been made, in order to gain process experience. These studies also determined the process variation encountered, and the variables of greatest sensitivity, when either a Centrifugation or Non-Centrifugation process step is utilised, by measuring numerous product and process parameters.

A risk-based approach to manufacturing is recommended for cell-based therapies in order to limit the extent of the non-clinical and clinical data to be included in applications for market authorisation (Salmikangas et al, 2015). The process step comparability experiments described in the present thesis identify the risks associated with the change of a single manufacturing process step. However, after a process transfer from an entirely manual to an entirely automated manufacturing process, due to increased scalability and reduced process variability associated with automated processes, further experiments examining the overall performance of a fully automated manufacturing process are required prior to the PQ stage of process validation. Therefore, building upon the risks identified in the single process step comparability experiments from the present research, future research should aim to identify the specification limits of a fully automated manufacturing process for each clinically relevant cell type by quantifying the overall variation that is achievable.

11.8.3 hiPSC Morphology Changes for In-Process Characterisation

The present research (Chapters 9 & 10) highlights the significant difference between the morphology of hiPSCs cultured in pluripotent stem cell medium and those cultured in MSC medium, and that the latter may adopt a more fibroblastic morphology that is representative of hMSCs (Section 10.3.1). Furthermore, the change in hiPSC morphology after the removal of ROCK inhibitor, the desired morphology of hiPSC populations, and the morphology of spontaneously differentiated cells in hiPSC cultures were identified in pluripotent stem cell culture conditions (Chapter 9).

The performance of further experiments to quantify and model hiPSC morphology changes may allow for the feasibility of using these changes as an early indicator of unwanted or directed differentiation within hiPSC populations to be explored. Recently, Phadnis and colleagues (2015) demonstrated that the dynamic behavioural patterns of hPSCs can be measured and quantified using time-lapse imaging, that the outcome of the next cellular
division of these cells can be predicted using algorithmic statistics, and that the migratory movement of these cells is predictive of their survival and pluripotency. However, further work is required in order to capitalise on such research, and to facilitate the development of a straightforward method of in-process characterisation for pluripotent stem cell populations.
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### Chapter 13: Appendices

#### 13.1 Summary Table of Concerns regarding Current Reimbursement and VBP

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<tr>
<th>Area of Concern</th>
<th>Current UK Reimbursement Concerns</th>
<th>Concerns carried into new VBP system</th>
<th>Specific VBP concerns</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthcare</td>
<td>• Risk averse &lt;br&gt;• Difficulty accepting high upfront costs &lt;br&gt;• ‘Postcode prescribing’ &lt;br&gt;• Focus on patient throughput &lt;br&gt;• Annual NHS budgeting &lt;br&gt;• Hospital ‘short-termism’ &lt;br&gt;• Silo budgeting</td>
<td>• Adoption delays &lt;br&gt;• Lack of competition within healthcare &lt;br&gt;• Clinical adoption</td>
<td>• Reduced NHS spend</td>
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<td>Appraisal Method</td>
<td>• Suitability of QALY model &lt;br&gt;• Does not account for benefits outside the healthcare system &lt;br&gt;• Lack of reimbursement decision clarity &lt;br&gt;• Lack of industry knowledge regarding HTAs</td>
<td>• Lack of indirect cost data &lt;br&gt;• Assessment time, complexity, and cost &lt;br&gt;• High evidence and trial data requirements</td>
<td>• Limited number of products assessed &lt;br&gt;• Lack of re-evaluation for adopted products &lt;br&gt;• Prejudice of epidemiology for burden of illness &lt;br&gt;• Factors chosen for wider societal benefits &lt;br&gt;• Difficulty calculating weightings &lt;br&gt;• ‘Double counting’ of benefits</td>
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<td>Threshold</td>
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<td>• Accuracy of cost effectiveness threshold (From NICE to government set threshold)</td>
<td>• Government price setting</td>
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<td>Regenerative Medicine</td>
<td>• Lack of specific adoption pathways for RM &lt;br&gt;• Difficulty accepting high up-front costs in healthcare &lt;br&gt;• Suitability of QALY model</td>
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<td>• Onus on manufacturers to collate evidence &lt;br&gt;• Disadvantage ‘second to market’ products &lt;br&gt;• Future innovative value of products not recognised &lt;br&gt;• Impact upon ‘Big Pharma’ (Reduced price for premium price, branded drugs) &lt;br&gt;• Higher prices at launch &lt;br&gt;• Pricing instability &lt;br&gt;• Uncertainty to ROI</td>
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<tr>
<td>Private sector</td>
<td>• Lack of industry knowledge regarding HTAs &lt;br&gt;• Lack of understanding between industry and payer</td>
<td>• Lack of access to capital</td>
<td></td>
</tr>
<tr>
<td>Economy</td>
<td></td>
<td>-</td>
<td>• Impact on worldwide pricing &lt;br&gt;• Delayed UK product launch &lt;br&gt;• Company relocation outside UK &lt;br&gt;• Reduced R&amp;D and trial funding</td>
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</table>
### 13.2 VBP, PPRS & VBA Summary Table and Timeline

<table>
<thead>
<tr>
<th>VBP</th>
<th>PPRS &amp; VBA</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Pricing</strong></td>
<td></td>
</tr>
<tr>
<td>• Government pricing based upon ‘value’</td>
<td>• Free pricing of new medicines with assessments of value</td>
</tr>
<tr>
<td><strong>Value Assessment</strong></td>
<td></td>
</tr>
<tr>
<td>• Assessments of:</td>
<td></td>
</tr>
<tr>
<td>o ‘Therapeutic innovation’</td>
<td></td>
</tr>
<tr>
<td>o Improvement upon current Gold Standard</td>
<td></td>
</tr>
<tr>
<td>o Burden of Illness</td>
<td></td>
</tr>
<tr>
<td>o Unmet Need</td>
<td></td>
</tr>
<tr>
<td>o Wider Societal Benefits¹</td>
<td></td>
</tr>
<tr>
<td>• Assessments of:</td>
<td></td>
</tr>
<tr>
<td>o Burden of Illness</td>
<td></td>
</tr>
<tr>
<td>o Unmet Need</td>
<td></td>
</tr>
<tr>
<td>o Wider Societal Impact²</td>
<td></td>
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<tr>
<td><strong>NICE Threshold Weighting</strong></td>
<td></td>
</tr>
<tr>
<td>• Weighting upon the NICE Threshold</td>
<td>• NICE Threshold remains at current level³</td>
</tr>
<tr>
<td><strong>Stakeholder Resistance</strong></td>
<td></td>
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<tr>
<td>• Significant resistance from stakeholders</td>
<td>• Diminished resistance from stakeholders</td>
</tr>
<tr>
<td></td>
<td>o Concerns remain regarding wider assessments of value</td>
</tr>
</tbody>
</table>

### Timeline

- **February 2007**: OFT propose VBP approach for pharmaceuticals⁴
- **March**: Only new medicines would be assessed under VBP. Drugs already on the market would remain under PPRS¹
- **March**: Delegation of value assessment role to NICE⁵
- **October**: Delay of introduction of VBP from January 2014 to late 2014⁷
- **February**: Public consultation on VBA opened²
- **March**: Withdrawal of the proposal for an expert panel tasked with performing value assessments¹
- **November**: Change to VBA and integration with PPRS³
- **September**: Rejection of VBA⁸
13.2.1 Summary Table and Timeline References

1- Department of Health 2011- A new value-based approach to the pricing of branded medicines: A consultation

2- National Institute For Health And Care Excellence., 2014. Value Based Assessment of Health Technologies
   http://www.nice.org.uk/media/B00/0E/January2014PublicBoardMeetingAgendaAndPapers.pdf


4- Office of Fair Trading 2007- The Pharmaceutical Price Regulation Scheme: An OFT Market Study

5- Department of Health 2010- Equity and excellence: Liberating the NHS

6- Secretary of State for Health 2013- The Government’s Response to the Health Select Committee’s Eighth Report of Session 2012-13 on the National Institute for Health and Clinical Excellence
   http://www.hsj.co.uk/news/finance/delay-in-pricing-plan-for-new-drugs/5063963.article#.UzqR8PldWSo

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13.3 Definitions of Components of Wider Societal Benefits (WSBs) Model

**Paid Production** - Value of labour provided by the patient. Productivity refers to the proportion of working time spent actually working and on-costs refer to employer overheads.

**Unpaid Production** - Consists of the production of both active and passive childcare, informal care, and all other unpaid production (e.g. domestic work)

**Formal Care** - Consumption of residential and non-residential care paid for by either the individual or by the state

**Informal Care** - Support provided by family or friends which is estimated separately for adults and children

**Personal Unpaid Consumption** - Unpaid consumption not related to health care, for example domestic work

**Personal Paid Consumption** - Consumption of utilities or activities for which the individual pays, for example the purchase of food, housing or leisure activities

**Informal Childcare Consumption** - Consumption of all paid childcare (e.g. childminders) and unpaid childcare (e.g. from parents)

**Government Services Consumption** - Consumption of government services that can be related to a given person, given the patient’s age, including education, other health care, law courts and prisons

13.4 BoI-Adjusted and BoI & WSB-Adjusted Incremental Cost-Effectiveness Ratio (ICER) for Allogeneic Islet Transplantation over a 20 year Time Horizon

<table>
<thead>
<tr>
<th>Time Horizon (Years)</th>
<th>ICER (£ per QALY)</th>
<th>BoI-adjusted ICER (£ per QALY)</th>
<th>BoI &amp; WSB-adjusted ICER (£ per QALY)</th>
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<td>1</td>
<td>£1,999,348.47</td>
<td>£1,046,305.24</td>
<td>£152,940.21</td>
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<tr>
<td>5</td>
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<td>£60,377.80</td>
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<tr>
<td>10</td>
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<td>£1,225.94</td>
<td>£1,032.15</td>
</tr>
<tr>
<td>20</td>
<td>-£59,087.70</td>
<td>-£28,235.10</td>
<td>-£25,288.27</td>
</tr>
</tbody>
</table>
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13.5 Screenshots Illustrating the Utilisation of the Department of Health Wider Societal Benefit (WSB) Calculation Template Spreadsheet

13.5.1 Determination of the Net Production (£ pcm) for a Single Patient

- For an average IDDM patient the inputs include:
  - Age = 41 years
  - Gender = Male
  - ICD Code = E10
  - QoL = 0.81 (Beckwith et al, 2012)
13.5.2 Determination of the Impact of a New Treatment upon the Net Production (£ per QALY gained) of a Single Patient

- For an average IDDM patient receiving an Allogeneic Islet Transplantation:
  - Age = 41
  - Gender = Male
  - ICD Code = E10
  - QoL = 0.810 (Beckwith et al, 2012)
  - QoL increment = 0.01
  - % QALY gains coming through life extension = 1%

    Allogeneic Islet Transplantation assumed to minimally contribute to life extension
13.6 hMSC Automated Culture Protocols

13.6.1 hMSC Seeding Protocol

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13.6.2 hMSC Non-Centrifugation Passage Protocol

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13.6.4 hMSC Post-Centrifugation Passage Protocol

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13.6.5 hMSC Medium Exchange Protocol

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finaldispensespeed = "5ml/s"/>
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dispensespeed = "1ml/s"
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13.6.6 hMSC Pooling Protocol

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volume = "6ml"/>
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13.7 LGC Flow Cytometry Assays

13.7.1 List of Selected Antibodies and Reagents

<table>
<thead>
<tr>
<th>CD markers</th>
<th>Supplier</th>
<th>Cat No</th>
<th>Isotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>APC/Cy7 anti-human CD29</td>
<td>BioLegend</td>
<td>303014</td>
<td>Mouse IgG1, k</td>
</tr>
<tr>
<td>Alexa fluor 647 anti-human CD45</td>
<td>BioLegend</td>
<td>304018</td>
<td>Mouse IgG1, k</td>
</tr>
<tr>
<td>Pacific Blue antihuman CD95</td>
<td>BioLegend</td>
<td>305619</td>
<td>Mouse IgG1, k</td>
</tr>
<tr>
<td>Alexa Fluor 488 anti-human HLA-DR</td>
<td>BioLegend</td>
<td>307620</td>
<td>Mouse IgG2a, k</td>
</tr>
<tr>
<td>PE anti-human CD9</td>
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<td>Mouse IgG1, k</td>
</tr>
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<td>FITC anti-human CD98</td>
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<td>Mouse IgG1, k</td>
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<td>PE-anti-human CD73</td>
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<td>Alexa Fluor 488 anti-human Ki67</td>
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<tr>
<td>PE anti-human CD11b</td>
<td>BioLegend</td>
<td>301306</td>
<td>Mouse IgG1, k</td>
</tr>
<tr>
<td>APC anti-human Vimentin</td>
<td>R&amp;D Systems</td>
<td>IC2105A</td>
<td>Rat IgG2A</td>
</tr>
<tr>
<td>Negative control (BSA)</td>
<td>BD</td>
<td>560497</td>
<td>Anti-mouse IgK</td>
</tr>
<tr>
<td>Compensation Plus (7.5uM)</td>
<td>BD</td>
<td>4660497</td>
<td>Anti-mouse IgK</td>
</tr>
<tr>
<td>PBS</td>
<td>SIGMA</td>
<td>D8537-500ML</td>
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<td>BioLegend</td>
<td>422302</td>
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</table>
13.7.2 Positive CD105 and Negative CD45 Expression of Centrifugation and Non-Centrifugation hMSCs (P5) after LGC Flow Cytometric Analysis

- Dark blue (Non-Centrifugation) and light blue (Centrifugation) histograms represent the fluorescent intensity of cells stained with the indicated antibodies and the black histogram represents the negative control cells, which were stained with a non-immunoreactive isotype control antibody.

13.8 Standard Centrifugation Conditions for Various Cell Types

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Standard Centrifugation conditions</th>
</tr>
</thead>
</table>
| hOST      | Transfer to 15ml or 50ml centrifuge tube  
|           | Relative Centrifugal Force- 220 RCF  
|           | Time- 5 min  
|           | Temperature- Not Specified  
|           | **Source**- Bernstein Protocol  
| hMSC (Bone Marrow derived) | Transfer to 15ml conical centrifuge tube  
|           | Relative Centrifugal Force - 400 RCF  
|           | Time- 5 min  
|           | Temperature- Not Specified  
|           | **Source**- R&D Systems  
|           | [http://www.rndsystems.com/literature_ccm004.aspx](http://www.rndsystems.com/literature_ccm004.aspx) |
| hES (Cultured on Irradiated Mouse Embryonic Fibroblasts) | Transfer to 15ml centrifuge tube  
|           | Relative Centrifugal Force - 200 RCF  
|           | Time- 4 min  
|           | Temperature- Not Specified  
|           | **Source**- R&D Systems  
|           | [http://www.rndsystems.com/literature_IMEF.aspx](http://www.rndsystems.com/literature_IMEF.aspx) |
| iPSC (Reprogrammed Fibroblasts) | Transfer to 15ml conical centrifuge tube  
|           | Relative Centrifugal Force - 200 RCF  
|           | Time- 4 min  
|           | Temperature- Not Specified  
|           | **Source**- Life Technologies  
|           | [http://tools.invitrogen.com/content/sfs/manuals/reprogramming_fibroblasts_w_cytotune.pdf](http://tools.invitrogen.com/content/sfs/manuals/reprogramming_fibroblasts_w_cytotune.pdf) |

As temperature is not specified, it was assumed that room temperature was used. The centrifugation of cells at room temperature (21°C) is supported by previous work in a thesis from UCL by Ju Wei Wong (2008), which demonstrates that centrifugation at 21°C results in lower cell losses than at higher or lower temperatures.
13.9 Initial IDO/Kynurenine Assay

13.9.1 Results of Initial IDO/Kynurenine Assay

Kynurenine Concentration in Untreated and Inflammatory Pre-Treated Baseline, Centrifugation & Non-Centrifugation Conditioned Medium
13.9.2 Initial IDO/Kynurenine Assay Standard Curve

\[ y = 0.0016x - 0.0197 \]
\[ R^2 = 0.9966 \]

Absorbance (nm) vs. Kynurenine Standard Concentration (μm)

13.10 Repeated IDO/Kynurenine Assay

13.10.1 Repeated IDO/Kynurenine Assay Standard Curve

\[ y = 0.0013x + 0.002 \]
\[ R^2 = 0.9982 \]

Absorbance (nm) vs. Kynurenine Standard Concentration (μm)
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13.10.2 Results of Repeated IDO/Kynurenine Assay

Kynurenine Concentration in Untreated and Inflammatory Pre-Treated Baseline, Centrifugation & Non-Centrifugation Conditioned Medium
13.11 PGE-2 ELISA Standard Curve

![PGE-2 Standard Curve](image)

\[ y = -8E-07x^2 + 0.0029x + 0.0525 \]

\[ R^2 = 0.9795 \]

13.12 VEGF ELISA Standard Curve

![VEGF Standard Curve](image)

\[ y = -8E-07x^2 + 0.0029x + 0.0525 \]

\[ R^2 = 0.9999 \]
13.13 Ruler hESC Automated Culture Protocols

13.13.1 Ruler hESC 1:3 Split Ratio Non-Centrifugation Passage Protocol

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13.13.2 Ruler hESC 1:4 Split Ratio Non-Centrifugation Passage Protocol

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        <incubate period = "7m"/>
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dispensespeed = "1ml/s"
pause = "2s"/
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13.13.3 Ruler hESC 1:5 Split Ratio Non-Centrifugation Passage Protocol

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<dispense liquid = "PETE-Trypsin EDTA" volume = "6ml"/>
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toname = "output"
volume = "10ml"
fromheight = "2mm"
toheight = "20mm"
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dispensespeed = "1ml/s"
pause = "2s"/>
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volume = "10ml"/>
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volume = "6ml"/>
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volume = "15ml"/>
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volume = "10ml"
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mixspeed = "5ml/s"
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13.13.5 Ruler hESC 1:3 Split Ratio Post-Centrifugation Passage Protocol
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repeat = "2"
fromheight = "2mm"
toheight = "50mm"
mixspeed = "5ml/s"
finaldispensespeed = "5ml/s"/>
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volume = "16ml"/>
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finaldispensespeed = "5ml/s"/>
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fromheight = "2mm"
toheight = "20mm"
aspiratespeed = "1ml/s"
dispensesspeed = "1ml/s"
pause = "2s"/>
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13.14 Multi-Well Plate Experiment: Table of Viable & Total Cell Densities across all Dissociation Conditions

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<tr>
<th>Flask &amp; Well</th>
<th>Flask 1</th>
<th>Flask 2, Well 1</th>
<th>Flask 2, Well 2</th>
<th>Flask 2, Well 3</th>
<th>Flask 2, Well 4</th>
<th>Flask 2, Well 5</th>
<th>Flask 2, Well 6</th>
<th>Flask 2, Well 7</th>
<th>Flask 2, Well 8</th>
<th>Flask 2, Well 9</th>
<th>Flask 2, Well 10</th>
<th>Flask 2, Well 11</th>
<th>Flask 2, Well 12</th>
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</thead>
<tbody>
<tr>
<td>Viable Cell Density (x10^5 cell/ml)</td>
<td>13.5</td>
<td>9.93</td>
<td>7.95</td>
<td>7.49</td>
<td>5.19</td>
<td>9.89</td>
<td>8.65</td>
<td>6.61</td>
<td>7.47</td>
<td>6.07</td>
<td>9.08</td>
<td>8.26</td>
<td>2.53</td>
</tr>
<tr>
<td>Total Cell Density (x10^5 cells/ml)</td>
<td>13.72</td>
<td>10.40</td>
<td>9.10</td>
<td>7.88</td>
<td>5.61</td>
<td>10.49</td>
<td>9.40</td>
<td>7.10</td>
<td>7.97</td>
<td>6.55</td>
<td>9.37</td>
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### Table of Ruler hESC Estimated Live Cell Aggregate Sizes

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<tr>
<th>Process Step</th>
<th>One</th>
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<th>Three</th>
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<th>Five</th>
<th>Six</th>
<th>Seven</th>
<th>Eight</th>
<th>Nine</th>
<th>Ten</th>
<th>Eleven</th>
<th>Twelve</th>
<th>Thirteen</th>
<th>Fourteen</th>
<th>Fifteen</th>
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<tbody>
<tr>
<td>1st Passage</td>
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<td>194.50</td>
<td>125</td>
<td>88.75</td>
<td>58.25</td>
<td>31.5</td>
<td>17.75</td>
<td>13.5</td>
<td>10</td>
<td>8</td>
<td>3 ±6</td>
<td>0 ±0</td>
<td>0 ±0</td>
<td>0 ±0</td>
</tr>
<tr>
<td></td>
<td>±133.10</td>
<td>±58.74</td>
<td>±41.07</td>
<td>±62.98</td>
<td>±29.83</td>
<td>±18.12</td>
<td>±21</td>
<td>±10.14</td>
<td>±17.23</td>
<td>±8.16</td>
<td>±10.10</td>
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<td></td>
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<td></td>
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<tr>
<td>Post-Cent.</td>
<td>538</td>
<td>226.25</td>
<td>151.25</td>
<td>91</td>
<td>53.5</td>
<td>37.5</td>
<td>38.5</td>
<td>4</td>
<td>13.25</td>
<td>10</td>
<td>2.75</td>
<td>9 ±16.97</td>
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<tr>
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<td>±86.15</td>
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<td>±24.7</td>
<td>±24.25</td>
<td>±28.3</td>
<td>±20.24</td>
<td>±21.67</td>
<td>±10.07</td>
<td>±14.29</td>
<td>±11.54</td>
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<tr>
<td>Non-Cent.</td>
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<td>547</td>
<td>355.5</td>
<td>209.75</td>
<td>140</td>
<td>74</td>
<td>47</td>
<td>40</td>
<td>22.5</td>
<td>7.5</td>
<td>5.5</td>
<td>6 ±12</td>
<td>2.75 ±5.5</td>
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<td>6 ±12</td>
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<tr>
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<td>±177.34</td>
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<td>±39.27</td>
<td>±28.47</td>
<td>±33.27</td>
<td>±15</td>
<td>±6.35</td>
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<td>2nd Passage</td>
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<td>1.5</td>
<td>3.33</td>
<td>1.83</td>
<td>0 ±0</td>
<td>1.08</td>
<td>0 ±0</td>
<td>0 ±0</td>
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<tr>
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<td>±81.47</td>
<td>±50.56</td>
<td>±47</td>
<td>±35.09</td>
<td>±33.46</td>
<td>±25.26</td>
<td>±12.43</td>
<td>±14.22</td>
<td>±3.5</td>
<td>±6.5</td>
<td>±4.28</td>
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<tr>
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<td>93.25</td>
<td>47.17</td>
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<td>6.67</td>
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<tr>
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<td>±69.87</td>
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<td>±14.73</td>
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<td>±9.39</td>
<td>±2.89</td>
<td>±6.35</td>
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</tr>
<tr>
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<td>70.08</td>
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<td>23.33</td>
<td>13.42</td>
<td>9 ±7.8</td>
<td>3.67</td>
<td>2.92</td>
<td>1.08</td>
<td>1.17 ±4.04</td>
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</tr>
<tr>
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<td>±20.88</td>
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<td>±5.28</td>
<td>±3.75</td>
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</tbody>
</table>
13.16 hiPSC Automated Culture Protocols

VAX001024c07 CompacT Select culture protocols cannot be disclosed due to confidentiality, however the flow charts below outline the process steps utilised in each protocol.

13.16.1 hiPSC Count and Seed 1xT175 (27,000 cells/cm²) Protocol
13.16.2 hiPSC Medium Exchange with ROCK inhibitor (NxT175) Protocol

13.16.3 hiPSC Medium Exchange without ROCK inhibitor (NxT175) Protocol
13.16.4 hiPSC Pre-Centrifugation Passage (1xT175) Protocol
13.16.5 hiPSC Pre-Experiment Post-Centrifugation Passage (1xT175→1xT175) Protocol
13.16.6 hiPSC Pre-Experimental Non-Centrifugation Passage (1T175→1xT175) Protocol
13.16.7 hiPSC Post-Centrifugation Passage (1xT175→2xT175) Protocol
13.16.8 hiPSC Non-Centrifugation Passage (1xT175 → 2xT175) Protocol
13.16.9 hiPSC Final Dissociation (4xT175) Protocol
### Chapter 13: Appendices

#### 13.17 Table of hiPSC Estimated Live Cell Aggregate Size

<table>
<thead>
<tr>
<th>Process Step</th>
<th>One</th>
<th>Two</th>
<th>Three</th>
<th>Four</th>
<th>Five</th>
<th>Six</th>
<th>Seven</th>
<th>Eight</th>
<th>Nine</th>
<th>Ten</th>
<th>Eleven</th>
<th>Twelve</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-Exp.</td>
<td>1943 ±847.46</td>
<td>1026.75 ±373.35</td>
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<td>10 ±0</td>
<td>5.5 ±7.78</td>
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<tr>
<td>Pre-Cent.</td>
<td>1812.5 ±818.43</td>
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<td>299.75 ±72.91</td>
<td>86.75 ±5.92</td>
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<td>6 ±10.39</td>
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</tr>
<tr>
<td>Pre-Exp.</td>
<td>2197 ±550.12</td>
<td>1174 ±315.66</td>
<td>446.75 ±94.95</td>
<td>158.25 ±50.33</td>
<td>56.5 ±15.15</td>
<td>17.75 ±14.29</td>
<td>7 ±5.72</td>
<td>2 ±4</td>
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<tr>
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<tr>
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</tr>
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<td>207.33 ±83.83</td>
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<td>10.25 ±11.64</td>
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<tr>
<td>Pre-Cent.</td>
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<td>1346.83 ±128.45</td>
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<td>160.67 ±50.60</td>
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<td>1.33 ±3.27</td>
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<td>0 ±0</td>
<td>0 ±0</td>
<td>0 ±0</td>
</tr>
<tr>
<td>Non-Cent.</td>
<td>2561 ±101.50</td>
<td>1346.83 ±128.45</td>
<td>435.33 ±98.10</td>
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<td>55.17 ±21.54</td>
<td>17.67 ±6.41</td>
<td>4.17 ±5.23</td>
<td>1.33 ±3.27</td>
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