The Combined Effects of Young’s Modulus and Low Oxygen Tension on Human Induced Pluripotent Stem Cells

Thesis Submitted to University College London for the Degree of
Doctor of Philosophy in Biochemical Engineering

by

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Declaration

I, Nuttinee Yongsanguanchai confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

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Abstract

Human Induced Pluripotent Stem Cells (hiPSCs) have the ability to differentiate into any adult cell type. One of the major challenges in pluripotent stem cell culturing is to improve the yield and efficiency of differentiated cells. Conventional cell culture approaches employ atmospheric oxygen tension (20% O₂) and hard tissue culture polystyrene plastic surfaces (Young’s modulus value of 3 x 10⁶ kilopascals). Recent studies have shown that the differentiation of hiPSCs can be influenced and improved by mimicking Young’s moduli or oxygen tension environment experienced by cells in vivo. Based on existing literature, our aim was to expand on the research by mimicking in vivo conditions by combining both factors of Young’s modulus and low oxygen tension during hiPSCs differentiation. The combination of Young’s modulus and low oxygen tension for hiPSCs culture have not been investigated before. In this thesis, the hiPSCs monolayer cultures and aggregates, known as embryoid bodies (EBs), were differentiated for 8 days on polydimethylsiloxane (PDMS) gels with a range of Young’s modulus: 0.2, 2, 16, 64 kilopascals to mimic in vivo conditions. The hiPSCs cells grown on the Young’s modulus: 0.2, 2, 16, 64 kilopascals were also grown both at 2% and 20% oxygen. We discovered that the combination of mechanical environment and low oxygen tension had a significant effect on hiPSCs differentiation. The combination of soft substrates (0.2 kilopascals) and 2% oxygen resulted in an upregulation of endoderm associated genes sry-related HMG box 17 (SOX17), forkhead box A2 (FOXA2) and alpha-fetoprotein (AFP). Whilst both monolayer and EB cultures showed similar responses, the upregulation of these genes was significantly higher in EB cultures, indicating that higher cell-cell contact presented in EB cultures plays
a critical role in hiPSCs differentiation. This data indicates that the novel combination of both oxygen and Young’s modulus has the potential to produce endoderm derivatives, such as pancreatic, endothelial and liver cells, which have important roles in drug testing, drug discovery and clinical applications.
Impact Statement

The use of human induced pluripotent stem cells (hiPSCs) in research has great potential and impact to the pharmaceutical and medical industry as they are a tool used for disease modelling, drug discovery and drug screening. Additionally, for clinical applications, they can provide treatment caused by injury, diseases and disorders that currently have no treatment, such as neurons for spinal cord injury, cardiomyocytes for myocardial infarction or even potentially replace injuries or failing organs.

The field is currently facing multiple challenges on differentiation. Therefore, an alternative effective platform for cell differentiation than current conventional methods would have to be considered. Conventional cell culturing in vitro conditions are significantly different than the conditions found in vivo. Conventionally, cells are grown in atmospheric oxygen of 20% O₂ and tissue culture plastic which have Young’s modulus value of 3 x 10⁶ kilopascals, this makes it harder than anything in the human body. In this thesis, we aim to mimic in vivo conditions by combining Young’s modulus and low oxygen tension during the differentiation of hiPSCs. Although mimicking in vivo conditions using a single factor have been widely studied in literature, that we know of, there have been no studies on the effect of Young’s modulus and oxygen tension combined. Therefore, the novelty of this thesis is investigating the combination of Young’s modulus and low oxygen tension on differentiation of hiPSCs culture. Our aim is to provide a better alternative cell differentiation platform that could be more efficient and less costly than conventional methods, potentially improving hiPSCs differentiation into
functional cell types for research and clinically relevant populations such as cardiomyocytes, neurons, beta cells and hepatocytes.

The results from this research have shown that the combined manipulation of two factors, Young’s modulus and oxygen tension had a highly significant impact on the cell differentiation outcome. Here we focused on the differentiation of cells through culturing on soft Young’s modulus and low oxygen tension using a spontaneous differentiation protocol as well as limited growth factors using an adapted directed differentiation protocol. These findings could be studied and further applied for any cell type differentiation and consequently improve cell differentiation efficiency.
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List of Abbreviations

*AFP* - Alpha Fetoprotein

*ANOVA* - Analysis of variance

*bFGF* - Basic fibroblast growth factor

*BMP* - Bone Morphogenetic Protein

*cDNA* - Complementary deoxyribose nucleic acid

*CXCR4* - Chemokine (C-X-C motif) receptor 4

*DAPI* - 4',6-diamidino-2-phenylindole

*DMEM* - Dulbecco’s Modified Eagle Medium

*DMSO* - Dimethyl sulfoxide

*DNA* - Deoxyribonucleic acid

*EB* - Embryoid bodies

*ECM* - Extracellular Matrix

*EDTA* - Ethylenediaminetetraacetic acid

*ERK* - Extracellular Signal-Regulated Kinases

*FBS* - Fetal Bovine Serum

*FGF* - Fibroblast Growth Factor

*FOXA2* - Forkhead box A2

*GXG* - Gelatin Crosslinked with Glutaraldehyde

*hESC* - Human embryonic stem cell

*HIF* - Hypoxia inducible factor

*hiPSC* - Human induced pluripotent stem cells

*hMSC* - Human mesenchymal stem cell

*HPC* - Hematopoietic progenitor cells
hPSC - Human pluripotent stem cells

ICM - inner cell mass

KOSR - Knockout serum replacement

LIF - Leukemia Inhibitory Factor

MEF - Mouse embryonic fibroblast

MSX1 - MSH homebox 1

NaBH4 - Sodium Borohydride

NEAA - non-essential amino acids

O2 - Oxygen

OCT-4 - Octamer-binding transcription factor 4

PA - Polyacrylamide

Pax 6 - Paired box protein 6 gene

PBS - Phosphate buffered saline

PDMS - Polydimethylsiloxane

PDX1 - Pancreatic and Duodenal Homeobox 1

PFA - paraformaldehyde

RNA - Ribonucleic acid

ROCK - rho kinase inhibitor

RPM - revolution per minute

RT-PCR - Real time polymerase chain reaction

A-SMA - Smooth muscle actin

SOX17 - Sex determining region Y-box 17

SSEA1 - Stage-specific embryonic antigen 1

SSEA3 - Stage-specific embryonic antigen 3
**SSEA4**- Stage-specific embryonic antigen 4

**TCP** - Tissue Culture Polystyrene

**TRA 1-60**- Tumour resistance antigen 1-60

**TRA1-81**- Tumour resistance antigen 1-81

**VEGFA** – Vascular endothelial growth factor A

**VEGFB** - Vascular endothelial growth factor B

**VEGFR-2** - Vascular endothelial growth factor receptor 2

**α - SMA** – Alpha smooth muscle actin
Chapter 1: Introduction

1.1 Regenerative Medicine and Stem Cells

Regenerative medicine is an interdisciplinary field of medicine and research which aims to restore function in cells, tissues and organs by growing cells and replacing them (Haseltine, 2001; Mason and Dunnill, 2008). Stem cell research is part of the regenerative medicine field. In addition to the regenerative medicine field, stem cell research also plays a critical role in the pharmaceutical industry for drug modelling, drug toxicity screens, disease modelling and drug discovery.

The term “stem cell” was believed to be first introduced by zoologist Ernst Häckel in 1868 (Haeckel, 1868) as a term to describe “all multicellular organisms evolved from a unicellular organism”. A human’s stem cell’s potency can be categorised into five groups: totipotent, pluripotent, multipotent, oligopotent and unipotent (figure 1.1) (Morrison, Uchida and Weissman, 1995; Potten, 1996; Gökhan, Song and Mehler, 1998; Slack, 2000; Ko et al., 2009; Zhang, Hu and Athanasiou, 2009; Zipori, 2009). In general, stem cells are identified by their unique ability to self-renew in an undifferentiated state and to differentiate into specialised cells. Stem cells are categorised into the three types of potency: totipotent, pluripotent and multipotent stem cells. Totipotent stem cells can differentiate into any cells, including the cells that form extra-embryonic tissues such as placental cells, and therefore have the potential to form whole organisms (Gardner, 1985). They emerge immediately after fertilisation before differentiating into pluripotent stem cells (PSCs). pluripotent stem cells form the three germ layers (endoderm, ectoderm and mesoderm) which can differentiate into any specialised cell in the body (Nicholas and Hall, 1942; Thomson et al., 1998). Multipotent stem cells are
limited in their differentiation capability and can only differentiate into specific
lineages (Quesenberry and Levitt, 1979). Multipotent stem cells are found in
specialised compartments known as niches, which are found throughout the body
in most organs, example of niches in the body includes skin, bone marrow, central
nervous system, digestive systems and muscles (Ferraro et al., 2010; Genbacev,
1997)

In this thesis, we used induced pluripotent stem cells, which are a type of
pluripotent stem cells. There are two types of pluripotent stem cells: embryonic
stem cells (Evans and Kaufman, 1981; Martin, 1981; Thomson et al., 1998) and
induced pluripotent stem cells (Takahashi and Yamanaka, 2006; Okita, Ichisaka
and Yamanaka, 2007). The following section will introduce human pluripotent stem
cells and the importance of oxygen tension and Young's modulus to cell culturing.
Figure 1.1 Diagram of stem cell pluripotency

Diagram explaining the five category of stem cell’s potency: totipotent, pluripotent, multipotent, oligopotent and unipotent
1.1.1 Human Embryonic Stem Cells (hESCs)

As mentioned in the previous section, embryonic stem cells (ESCs) are a type of pluripotent stem cells (PSCs). These cell types are derived from the inner cell mass of blastocysts. In development, blastocysts form during embryo development, three days after fertilisation in mice (Evans and Kaufman, 1981; Martin, 1981) and at day five in humans (Thomson et al., 1998). The first mouse embryonic stem cell (mESCs) lines were isolated in 1981 (Evans and Kaufman 1981; Martin 1981), and the first human embryonic stem cell (hESCs) lines was isolated in 1988 (Thomson et al., 1998). In the past, cell culturing of mESCs and hESCs were carried out on inactivated mouse embryonic fibroblasts (MEFs) with the addition of anti-human serum or guinea pig complement (Martin, 1981; Bosma, Custer and Bosma, 1983; Thomson et al., 1998; Reubinoff et al., 2000; Cowan et al., 2004; Ellerström et al., 2006). The MEFs were required in the co-culture as they provided the ESCs with a matrix to grow on and in addition, secreted growth factors such as bFGF into the medium which helped with the pluripotency(Martin and Evans, 1975; Evans and Kaufman, 1981; Martin, 1981; Thomson et al., 1998). However, xeno contamination makes it unsuitable for clinical applications, this is due to the presence of animal components and potential pathogens in the culture. Therefore, pluripotent stem cells are now more commonly grown on feeder-free cultures where xeno-free extracellular matrices such as vitronectin is used instead of MEFs (McElroy and Reijo Pera, 2008).

There are two types of pluripotent states found initially in mESCs, and were distinguished as the Naïve state which represent mESCs derived from the inner cell
mass of pre-implantation blastocysts and Primed states which are mESCs from post-implantation epiblast cells, both these cell types are pluripotent and can therefore differentiate into any stomatic cell type, but they have different properties (Chenoweth et al., 2007). The difference between these two pluripotent states are that mESCs Naïve cells are able to produce chimeras and be passaged as single cells, whereas Primed state cells cannot. Other differences between the Naïve and Primed cells includes morphology differences, gene expression, cloning efficiency and different energy generation pathway. Naïve cells have a more round morphology, higher cloning efficiency than Primed cells, additionally, Naïve cells produces energy via both glycolysis and oxidative phosphorylation, whereas Primed cells can only produce energy by glycolysis (Chenoweth et al., 2007; Nichols and Smith, 2009; Davidson et al., 2015; Takahashi et al., 2018). Human embryonic stem cells and human induced pluripotent stem cells are considered to be equivalent to the Primed pluripotent state. In human pluripotent stem cells, there are multiple studies that have induced the Primed cells into the Naïve state (Hanna et al., 2010; Gafni et al., 2013; Ware et al., 2014; Valamehr et al., 2014), however, presently there are still no established protocol where these Naïve hESCs.

1.1.2 Human Induced Pluripotent Stem Cells (hiPSCs)

Another type of pluripotent stem cells are induced pluripotent stem cells (iPSCs). Induced pluripotent stem cells are derived by reprogramming somatic cells such as fibroblasts using four transcription factors (Takahashi and Yamanaka, 2006). Other somatic cells that have been reprogrammed include blood T-lymphocytes (Brown et al., 2010), peripheral blood mononuclear cells (PBMCs) (Kim et al., 2016), multipotent stem cells (Sun et al., 2009) and neural stem cells
(Kim et al., 2008). The first iPSCs were generated using retroviral vectors to overexpress four transcription factors (OCT4, Sox2, Klf4 and c-MYC) by Yamanaka and his team in mouse and in human cells the year after (Takahashi and Yamanaka, 2006; Takahashi et al., 2007). This hiPSCs is a major discovery as they can be used for autologous cell therapy as well as complimenting, and potentially replacing the use of hESCs. Yamanaka and his team demonstrated that these iPSCs derived from mouse and human fibroblasts were indeed pluripotent in nature. Yamanaka et al., demonstrated the iPSCs are pluripotent by proving that the cells showed pluripotency marker genes, direct differentiation, teratoma formation and generation of mice chimeras with mouse iPSCs (Takahashi and Yamanaka, 2006; Takahashi et al., 2007).

Subsequent studies showed that there is a wider range of transcription factors and methods that can be used for the reprogramming. These include Klf4 and c-MYC with NANOG and Lin28 (Yu et al., 2007) that can be combined to achieve similar outcomes. Moreover, reprogramming methods can be carried with just two factors, OCT4 and SOX2 (Huangfu et al., 2008) and even just one gene, OCT4 (Zhu et al., 2010). The latter is of greater benefit as it reduces the occurrence of mutagenesis caused by viral integration. Multiple non-integrating methods have been explored to reduce this viral integration, including the use of Sendai viral vectors (Fusaki et al., 2009; Ban et al., 2011), recombinant proteins (Zhou et al., 2009), episomal plasmid DNA (Hu and Slukvin, 2013; Okita et al., 2013), polycistronic virus (Carey et al., 2009), adenovirus (Okita et al., 2008; Zhou et al., 2009), oirP/EBNA1-based episomal vectors (Chang et al., 2009), piggyback (Kaji et al., 2009), minicircles vectors (Jia et al., 2010; Narsinh et al., 2011), mRNA
Recent developments in hiPSCs reprogramming focuses on using and improving non-integrative reprogramming methods for various somatic cell types. These includes the use of Sendai viral vectors to reprogram of hiPSCs from human urine samples (Uhm et al., 2017), Sendai viral vectors to reprogram hiPSCs from 3000 cells of cryopreserved human blood (Zhou et al., 2015), polycistronic viral vectors to reprogram hiPSCs from human adipose derived stem cell (Qu et al., 2012). Additionally, hiPSCs reprogramming efficiency have been explored. Worringer et al., (Worringer et al., 2015) found that the inhibition of Let-7 (mRNA) resulted in a 2-fold increase in successful reprogrammed hiPSCs compared to using Yamanaka’s factors alone. It was also shown by Liu et al (Liu et al., 2013) that sequential introduction of the Yamanaka’s factors every 36 hours over 4 days resulted in a 5-fold reprogrammed hiPSCs yield compared to introducing the Yamanaka’s factors all at once. These hiPSCs reprogramming methods and origin somatic cell type moves toward a more efficient, safe and easier production of hiPSCs for the medical and clinical industry. For both medical and clinical use of human pluripotent stem cells. One of the most important factors in hPSCs research is to test the hPSCs pluripotency.

The pluripotent state or “stemness” of PSCs can be characterised by positive expression of pluripotency markers OCT4 (Nichols et al., 1998), Sox2 (Avilion et
Along with their pluripotency, these PSCs must have the ability to differentiate into the three germ layers. They can either differentiate in a monolayer culture or form spherical aggregates called embryoid bodies (EBs) (Itskovitz-Eldor et al., 2000). Additional methods in proving pluripotency is to form teratomas in vivo (Draper et al., 2004; Valbuena et al., 2006) and in the cause of mouse cells, to transplant cells into blastocysts to investigate germ line contribution in chimeras (ISSCR International Human Embryonic, 2007).

Since the discovery of hiPSCs, a lot of research has been carried out in trying to use cells differentiated from hiPSCs for drug testing, drug screening, cytotoxicity testing, drug metabolism modelling (Liang et al., 2010; Zhang et al., 2012; Lee et al., 2013; Yu et al., 2018), in vitro modelling (Marchetto et al., 2010; Rashid et al., 2010; Zhang et al., 2011), disease modelling (Park et al., 2008; Ebert et al., 2009; Urbach et al., 2010) and potential cell therapy as these iPSCs have great potential. Examples of functional cells generated from hiPSCs include human cardiomyocytes (Zhang et al., 2009), human vascularised liver organoids (Takebe et al., 2013) and human neurons (Matsumoto et al., 2016). These cells are shown to be functional, however they are found to be immature cells and are different from adult functional cells, and therefore requires more studies on the maturation of these cells. Additionally, there are also studies to discover the treatment for illnesses such as retinal and macular degeneration (Okamoto and Takahashi, 2011), Type 1 and 2 diabetes (Maehr et al., 2009; Mayhew and Wells, 2010; Teo, Wagers and Kulkarni, 2013) and spinal cord injuries (Tsuji et al., 2010; Nori et al.,
There are also research using hiPSCs to model genetic disorders and diseases including Down syndrome (Park et al., 2008; Chen et al., 2014; Huo et al., 2018), Parkinson’s disease (Jiang et al., 2012), Alzheimer’s disease (Yagi et al., 2012; Kondo et al., 2013), Huntington’s Disease (Zhang et al., 2010; Juopperi et al., 2012) and schizophrenia (Brennand et al., 2011; 2015). A summary for cell and disease modelling studies can be seen in Table 1.1. For clinical trials to be approved, strict rules and regulations must be met to ensure patient safety is absolutely resolute, hiPSCs are currently moving in that direction with the first few hiPSCs and hESCs clinical trials currently taking place, as hiPSCs and hESCs are generally used in tandem for studies. The first being a trial on autologous hiPSC derived retinal pigment epithelial cells forming sheets of implantable cells to treat patients with macular degeneration (Cyranoski, 2013; Kamao et al., 2014). And according to the World Health Organisation (WHO) International Clinical Trials Registry Platform, there are also ongoing clinical trials on macular degeneration, vasodenerative repair as well as multiple safety and efficiency trails (World Health Organization, 2019).

Other applications of hiPSCs can be found in the pharmaceutical industry to produce disease models (Park et al., 2008; Briggs et al., 2013) and accelerate drug discovery for preclinical drug testing (Tanaka et al., 2009) to investigate how a drug affects specialised cells and whether it causes adverse effects. These studies such as drug metabolism modelling are most useful in helping in vitro studies of drugs before pre-clinical measures such as in animal and human trials (Liang et al., 2010; Zhang et al., 2012; Lee et al., 2013; Yu et al., 2018). In addition to the derivation of healthy iPSCs from diseased patients (Dimos et al., 2008), iPSCs can also be
derived from the patients diseased cells and be studied in vitro to establish what's known as “disease in a dish” (Badger et al., 2014; Xie and Zhang, 2014; Zanella, Lyon and Sheikh, 2014; Kamdar et al., 2015; Smith et al., 2016) or even form organoids which are aggregates of self-organised cell types similar to an organ in vivo including cerebral organoids (Lancaster et al., 2013), livers organoids (Takebe et al., 2013), lungs organoids (Dye et al., 2015), intestinal organoids (McCracken et al., 2014) and kidney organoids (Takasato et al., 2015). These techniques are tremendously useful as they allow the study of the underlying mechanisms involved in various illnesses and diseases to discover more effective treatments and identify potential cures.
<table>
<thead>
<tr>
<th>Cell types (hiPSCs derived)</th>
<th>Reference</th>
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<tbody>
<tr>
<td>Cardiomyocytes</td>
<td>(Zhang et al., 2009)</td>
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<tr>
<td>Hepatocytes</td>
<td>(Takebe et al., 2013)</td>
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<tr>
<td>Neurons</td>
<td>(Meijer et al., 2019)</td>
</tr>
<tr>
<td>Endothelial cells</td>
<td>(Olmer et al., 2018)</td>
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<tr>
<td>Skeletal Muscle cells</td>
<td>(Miyagoe-Suzuki and Takeda, 2017)</td>
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<tr>
<td>Organoids</td>
<td>Reference</td>
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<tr>
<td>Cerebral Organoids</td>
<td>(Lancaster et al., 2013)</td>
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<tr>
<td>Livers Organoids</td>
<td>(Takebe et al., 2013)</td>
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<tr>
<td>Lungs Organoids</td>
<td>(Dye et al., 2015)</td>
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<tr>
<td>Intestinal Organoids</td>
<td>(McCracken et al., 2014)</td>
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<tr>
<td>Kidney Organoids</td>
<td>(Takasato et al., 2015).</td>
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<tr>
<td>Genetic Disorders modelling</td>
<td>Reference</td>
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<tr>
<td>Down Syndrome</td>
<td>hiPSCS derived neurons and astrocytes from patients with Down Syndrome (Park et al., 2008; Chen et al., 2014; Huo et al., 2018)</td>
</tr>
<tr>
<td>Amyotrophic Lateral Sclerosis (ALS)</td>
<td>hiPSCS derived motor neurons cells from patients with ALS (Dimos et al., 2008; Sances et al., 2016)</td>
</tr>
<tr>
<td>Schizophrenia</td>
<td>hiPSCS derived neural progenitor cells from patients with schizophrenia (Brennand et al., 2011; 2015)</td>
</tr>
<tr>
<td>Parkinson's Disease</td>
<td>hiPSCS derived neurons from patients with Parkinson's Disease (Jiang et al., 2012)</td>
</tr>
<tr>
<td>Alzheimer's Disease</td>
<td>hiPSCS derived neurons and astrocytes from patients with Alzheimer's Disease (Yagi et al., 2012; Kondo et al., 2013)</td>
</tr>
<tr>
<td>Huntington's Disease</td>
<td>hiPSCS derived Neural stem cells and astrocytes cells from patients with Huntington's Disease (Zhang et al., 2010; Juopperi et al., 2012)</td>
</tr>
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</table>
1.1.3 Differences and Similarities Between Human Embryonic Stem Cells (hESCs) and Human Induced Pluripotent Stem Cells (hiPSCs)

Although induced pluripotent stem cells (iPSCs) are reprogrammed cells, they are similar to embryonic stem cells (ESCs). However, minor differences have been identified, such that iPSCs retain an “epigenetic memory” upon being reprogrammed (Marchetto et al., 2009; Ghosh et al., 2010; Hu et al., 2010). Epigenetic memory is believed to be residual DNA methylation as a by-product of incomplete reprogramming, where some genes from the original somatic cells were left. However, the residual DNA does not affect hiPSCs reprogramming as reprogramming can still be successful (Kim et al., 2011; Lister et al., 2011; Ohi et al., 2011). For example, iPSCs derived from hematopoietic progenitor cells formed more hematopoietic colony-forming cells than iPSCs derived from fibroblasts (Kim et al., 2010). However, this is deemed minor as it only affects spontaneous differentiation yields and these differences do not affect the iPSCs ability to differentiate into the three germ layers. This epigenetic memory, however, can be advantageous in some applications where the desired differentiated cells are the same as the cells of origin. Other studies also found differences in teratoma formation (Miura et al., 2009), microRNA gene expression (Chin et al., 2009) and DNA methylation (Doi et al., 2009; Altun, Loring and Laurent, 2010; Narsinh, Plews and Wu, 2011; Nazor et al., 2012). Despite the differences stated above, other studies have proven they are similar and there were no differences between the differentiation capabilities between ESCs and iPSCs (Kiss et al., 2011) and therefore hiPSCs should be classed in the same cell category as hESCs (Kim et
al., 2008; Guenther et al., 2010; Newman and Cooper, 2010; Bock et al., 2011; Mallon et al., 2014).

Even though iPSCs and ESCs are classed in the same category, iPSCs are preferred as they can be used for autologous treatments as well as not having any ethical concerns, unlike ESCs. Furthermore, ESCs are susceptible to immune rejection (Thomson, 1998; Kaufman, Odorico and Thomson, 2000; Swijnenburg et al., 2005, 2008), therefore an alternative option would be the use of major histocompatibility complex (MHC) or human leukocyte antigen (HLA) matched ESCs (Kim et al., 2007) if not using iPSCs (Sugita et al., 2016). Major histocompatibility complex (MHC) and human leukocyte antigen (HLA) are surface proteins which the body uses to identify and to elicit an immune response, therefore MHC/HLA matched stem cells would not require immunosuppression post-transplantation, there are hiPSCs banks being set up in order to provide matched MHC/HLA (Garreta et al., 2018). In Japan, Nakatsuji et al., (Nakatsuji et al., 2008) modelled that 50 homozygous hiPSCs lines would be required in the Japanese hiPSCs bank to provide HLA matched hiPSCs covering 90.7% of the Japanese population. Additionally, to cover 93% of the UK population, 150 homozygous hiPSCs cell lines would be required (Taylor et al., 2005; De Sousa et al., 2017). Another alternative would be “universal” hiPSCs line consisted of inactivated MHC and therefore would stop the body’s immune system from recognising it as foreign (Shani and Hanna, 2019), Alternatively, other hiPSCs methods such as hiPSCs generated via somatic cell nuclear transfer (SCNT) (Campbell et al., 1996), the process where the nucleus is transplanted into a nucleus removed egg cell, an example of SCNT that is commonly known is the three parent embryo (Zhang et
Due to the reasons above, this research will be focusing on the use of hiPSCs.

1.1.4 Pluripotency of Pluripotent Stem Cells

Specific signalling pathways have been identified to directly influence the pluripotency and self-renewal hPSCs, these include TGF-β, which signals through Smad2/3 and FGFR activating the Akt and MAPK pathways, these pathways maintain pluripotency in hPSCs. Another pathway that promotes pluripotency is the Wnt pathway via β-catenin activation (Ogawa et al., 2006; Kurek et al., 2015). Signalling through these pathways leads to the expression of OCT4 (Nichols et al., 1998), Sox2 and NANOG (Chambers et al., 2003). OCT4, SOX2 and NANOG are key transcription factors for pluripotency and self-renewal.

Although much research has been done on mouse embryonic stem cells (mESCs), it is known that human and mouse embryonic stem cells exhibit some key differences. Firstly, as mentioned earlier, there are Naïve and Primed states of pluripotency in mESCs whereas hESCs and hiPSCs are classes as equivalent to Primed mESCs. Additionally, hESCs do not exhibit their pluripotency pathway through Leukaemia Inhibitory Factor (LIF) and Stat3 pathways and IGF insulin do not promote its self-renewal (Sato et al., 2004). Even though mESCs express the same pluripotency transcription factors, these examples show that mouse and human stem cells are different and therefore different experimental results can be expected. For example, SSEA1 is a marker used to assess pluripotency in mESCs and the SSEA3/4 marker is used to indicate early differentiation in mESCs whereas, in hESCs, expression of SSEA1 signifies differentiation and expression of SSEA3/4 signifies pluripotency (Ginis et al., 2004). Moreover, they also differ in their ability
to differentiate into trophectoderm and early embryo development (Thomson et al., 1995; Thomson, 1998).

1.1.5 Differentiation of Pluripotent Stem Cells

Multipotent stem cells can be found in niches all over the body. Multipotent stem cells are cells that can self-renew and differentiate into a limited cell type and are required as somatic cells are continuously replaced; the turnover rate of these cells varies depending on the cell type and function. For example, intestine epithelial cells have a short life span of 2-4 days (Grossmann et al., 2000) and haemoglobins up to 4 months (Mock et al., 1999; Lichtman et al., 2000).

Differentiation of stem cells occurs through the altering of gene expression patterns from one cell type to another. Transcription factors play an important role in determining which genes are expressed, and therefore the fate of the cells (Itskovitz-Eldor et al., 2000; Schuldiner et al., 2000). As mentioned earlier, there are three main germ layers that cells form before further differentiating into specialised cells (stomach functioning cells). The ectoderm layer differentiates into nervous system and epidermis derivatives; the mesoderm differentiates into muscle, connective tissue, circulatory and skeletal system; and the endoderm layer differentiates into the digestive and respiratory system, liver and pancreas as well as the urethra, bladder and the reproductive system (Svajger and Levak Svajger, 1976; Pansky, 1982).

In human and mouse, Cell differentiation is initiated via the BMP4 pathway inducing differentiation by using Smad1/5/8 and inhibiting pluripotency transcription factors Oct-4, Sox2 and NANOG (Vallier et al., 2009). The cells then become specialised cells via lineage-specific pathways. However, there are differences
between hESCs and mESCs in terms of their response to inducing directed differentiation. For example, hESCs and mESCs directed to differentiate into cardiomyocytes (using the same protocol) found that both cell lines produced cardiomyocytes. However, the human cell line produced cardiomyocytes with a slower beating rate than the mouse line and the human cardiomyocytes were able to beat continuously for over 3 months whereas the mouse cardiomyocytes lasted less than a month (Mu et al., 2014). Another study was able to produce cardiomyocytes from mESCs and hPSCs under the same hypoxic conditions found that hPSCs have a lower differentiation rate and maintain pluripotency for longer compared to the mESCs (Fynes et al., 2014). Fynes et al., believed that the pluripotency maintenance and self-renew in hPSCs are neutralised by HIF1α in mESCs culture by the blocking of self-renewal LIFR/STAT3 pathway and therefore causes differentiation.

Common procedures for cell differentiation are the production of embryoid bodies (EBs) and monolayer cultures. EBs are small round three dimensional aggregates that contain cells of all three germ layers, used in research to mimic embryogenesis (Risau et al., 1988; Itskovitz-Eldor et al., 2000). Monolayer differentiation involves cells are grown in a two-dimensional layer rather than three dimensional in EBs. Both methods will be used in this thesis, as these methods will expose cells to the substrate and environment differently. There are two types of differentiation: spontaneous differentiation occurs when cells are allowed to differentiate without the addition of components such as growth factors and small molecules that would directly promote lineage-specific differentiation and directed differentiation is when growth factors and small molecules are used. The thesis
aims to carry out spontaneous differentiation of hiPSCs cells by mimicking in vivo environment such as in vivo Young’s modulus values as well as mimicking embryogenesis conditions with low oxygen conditions.

1.1.6 Early Embryo Development

In the previous section, we covered differentiation of pluripotent stem cells, here we will explore embryogenesis in human, it’s condition and how the three germ layers are developed in vivo. Embryogenesis is the term used to describe the development and formation of embryos (Crick, 1970; Martin, 1981). It begins once the egg has been fertilised. The fertilised egg becomes a zygote, the zygote then undergoes multiple divisions called cleavage; this is the first stage of blastulation, the term for the formation of a blastocyst. Cleavage is the process when multiple cell divisions occur, however there is no significant growth and at the end of cleavage the zygote is still the same size (Abbott, 1936; Ducibella and Anderson, 1975; Graham and Deussen, 1978; Johnson and Ziomek, 1981; Keith L. Moore, Mark G. Torchia, 2004). This process is carried out while it travels down the fallopian tube towards the uterus taking around 7 days. Once the blastocyst is formed, it consists of the outer layer of trophoblasts, a blastocyst cavity and an inner cell mass. The inner cell mass (ICM) is an important cell population as it gives rise to all the embryonic germ layers (Copp, 1979; Graham and Lehtonen, 1979; Johnson and Ziomek, 1981)(Figure 1.2). At day 7-8, the blastocyst implants itself to the uterus wall. During implantation, the two layers of bilaminar embryonic discs: (epiblast and hypoblast) are developed from the ICM (Figure 1.3). The epiblast forms the embryonic ectoderm and primitive streak whereas the hypoblast develops the endoderm.
Gastrulation is the process when germ layers are formed. This process occurs once the blastocyst has been formed (Sugihara et al., 1998) and usually occurs three weeks after fertilisation. The three germ layers formed are endoderm, mesoderm and ectoderm. The ectoderm layer differentiates into nervous system and epidermis derivatives; the mesoderm differentiates into muscle, connective tissue, circulatory and skeletal system; and the endoderm layer differentiates into the digestive and respiratory system and inner organs including the reproductive system (Skreb, Svajger and Levak Svajger, 1976; Pamsky, 1982; Matsui, Zsebo and Hogan, 1992).
Figure 1.2 The early embryo development, showing a drawing of the human reproductive tract and the first 14 days of embryo development.

Starting from fertilisation to implantation. Once fertilisation occurs, the zygote undergoes multiple rapid divisions called the cleavage process until the blastocyst is form and implantation occurs. (Mundy and Vilchez, 2017).
Figure 1.3 The early embryo development after implantation.

Diagram shows gastrulation, this is when the formation of the three germ layers occurs and gets organised. The three germ layers are endoderm, ectoderm and mesoderm. Endoderm give rise to the internal germ layers and forms internal organs and the lining of the digestive system. Ectoderm give rises to the brain, nervous system, external tissues and skin. The Mesoderm produces the circulatory system, muscle and skeletal system. (Jones, 2019).
1.2 The Role of Oxygen in Early Embryo Development

The following section summarises the literature review of low oxygen tension during early embryo development and studies that have been carried out. The reason why low oxygen tension is such an important factor to investigate is that in adult *in vivo* microenvironment as well as embryonic development, cells are exposed to a lower oxygen tension than the atmospheric tension (20% oxygen).

Conventional *in vitro* cell culturing occurs at an atmospheric oxygen tension of 20% v/v. However, the oxygen tension found in the somatic cells varies throughout the human body, ranging from 2%-9% (Brahimi-Horn and Pouysségur, 2007), 1-8% in various stem cell niches present around the body and 12% in arterial blood (Csete, 2005). It can be as low as 0%-4% in the bone marrow and 0.5%-7% in the brain (Ivanovic, 2009). Therefore, atmospheric conditions are not appropriate for stem cell cultures. Additionally, as mention in Section 1.1.1 ESCs are derived from the inner cell mass found in the blastocysts. The blastocyst is fully developed on the 5th day after fertilisation where it travels down the fallopian tube to be implanted in the uterus (Figure 1.2) (Thomson 1998). At this stage, the embryo has not yet implanted itself into the uterus and the oxygen supply is severely limited. The oxygen supply available within the fallopian tube was found to be between 1.5-8.7% (Fischer and Bavister, 1993; Genbacev *et al*., 1997). In addition, the oxygen concentration in the human uterine surface increases from 2.4% in week 7-10 to 8% in week 11 of pregnancy; this is due to the established circulatory system which allows for the maternal blood to be received (Rodesch *et al*., 1992). Prior to the circulatory system being established, the embryo is exposed to only the nutrients available in the fluid surrounding the uterus (Hustin and Schaaps, 1987; Rodesch
et al., 1992; Fischer and Bavister, 1993). Due to this, it is believed that hypoxic conditions are important to pluripotent stem cell cultures, as these conditions would mimic the oxygen tensions present within the inner cell mass and embryogenesis in vivo. To reiterate this point, it was found that higher cell count inner cell mass was discovered in bovine blastocysts developed at 2% than ones developed at 7% or 20% in vitro via immunostaining with Hoechst nuclei stain and counting using a fluorescence microscope (trophectoderm was also stain with Hoechst and propidium iodide, a trophectoderm stain to distinguish between inner cell mass and trophoderm cells) (Harvey et al., 2004). In addition, a study conducted by Yoshida et al., (Yoshida et al., 2009a) also found that deriving hiPSCs under hypoxic conditions resulted in greater efficiency of iPSCs colony formation. And in the same study, found MEF colonies (used as feeders) cultured under 5% $O_2$ showed a 7.4-fold increase on day 20 compared to just a 4.2 fold increase on day 24 for colonies grown at 20% $O_2$.

1.2.1 The Role of Hypoxia Inducing Factors (HIFs) in the Cellular Environment

Hypoxia inducing factors (HIFs) plays a key role within cells cultured in low oxygen tension. Hypoxia inducing factors are heterodimeric (proteins composed of two polypeptide chains) transcription factors and become activated in a low oxygen environment (Semenza et al., 1991; Semenza, 1999; Ke and Costa, 2006). There are two sub-units in HIF: the HIF-α and HIF-β (Gu et al., 1998; O'Rourke et al., 1999; Semenza, 1999; Srinivas et al., 1999). The HIFs family consists of HIF1, HIF2, HIF3 α and β subunits. In 20% oxygen (normoxia) HIF α subunits (HIF-1α,
HIF-2α and HIF-3α) are hydroxylated (oxidative degradation by a hydroxyl group) by the Prolyl hydroxylase domain (PHD) enzymes, but this does not happen in hypoxia (Figure 1.4). Under hypoxic conditions, PHDs are inhibited and therefore the HIF-1α/2α are not hydroxylated (Maxwell et al., 1999; Ohh et al., 2000; Mahon, Hirota and Semenza, 2001; Koivunen et al., 2004). The HIF α subunits then dimerize with HIF-1β and bind to DNA to activate target gene transcriptions to respond to O₂ deprivation (Dengler, Galbraith and Espinosa, 2014).

The HIF regulating mechanism in stem cell behaviour remains unclear. However, as low oxygen tension is also an environment for cancer cells, there are multiple studies on HIF 1α regulating mechanism in cancer cells, where it is shown that the mTOR pathway is a positive regulator of HIF-1α (Liu et al., 2008). Moreover, there are also other hypoxia responsive genes including glucose transporter 1 (GLUT1), Erythropoietin (EPO), Vascular endothelial growth factor (VEGF) and lactate dehydrogenase A (LDHA) which are induced during the up-regulation of HIF (Van Tuyl et al., 2005; Fryer and Simon, 2006; Provot et al., 2007; Dunwoodie, 2009) and then affect cell’s behaviour including tumorigenesis (Ratcliffe, 2013), metabolism (Takubo et al., 2010, 2013), angiogenesis (Rey and Semenza, 2010), erythropoiesis (Rey and Semenza, 2010) and differentiation (Millman, Tan and Colton, 2009; Mohyeldin, Garzón-Muvdi and Quiñones-Hinojosa, 2010; Ng et al., 2010).
1.2.2 The Effect of Oxygen on Pluripotency

There are several studies on the effect of low oxygen environment in cell culture. Experiments under hypoxic (<20% oxygen) conditions for the maintenance of hESCs have found that cells grown under 3% and 5% O$_2$ conditions cause little morphological differences compared to cells grown under normoxia (20% O$_2$) at 7 days. However, at day 9 cells grown under 20% O$_2$ exhibit signs of differentiation morphology and revealed decreased expression of OCT4 and SSEA4 pluripotency markers whereas hESCs cells cultured in 3% and 5% conditions did not and still had expressed pluripotency markers OCT4 and SSEA4 (Ezashi, Das and Roberts, 2005). The morphology of the hESCs under hypoxic 5% O$_2$ conditions appeared
more compacted, enabling them to be passaged as per usual once a certain confluency was reached; whereas in 20% O$_2$ the hESCs colonies showed signs of differentiation at day 4 and had to be passaged prematurely in order to limit spontaneous differentiation (Forristal et al., 2010). This was confirmed when Fynes et al., (Fynes et al., 2014) showed that ‘conditioning hiPSCs and mESCs cells’ at 2% O$_2$ tension for three passages improved maintenance of hiPSC pluripotency and decreased signs of spontaneous differentiation. Moreover, studies have also demonstrated human progenitor cells cultured in low oxygen tension (1% O$_2$ and 3% O$_2$ respectively) were better in pluripotency maintenance than in 20% O$_2$ (Ivanovic et al., 2000; Studer et al., 2000), it was also found that there was an increase in pluripotency markers expression (OCT4, SOX2 and NANO2), proliferation rate, cell recovery and reduction in chromosomal aberrations in hESCs, hiPSCs, mESCs cells cultured in hypoxia (Ezashi, Das and Roberts, 2005; Covello et al., 2006; Forsyth et al., 2006; Gibbons, Hewitt and Gardner, 2006; Millman, Tan and Colton, 2009; Yoshida et al., 2009; Forristal et al., 2010; Correia et al., 2014; Sato et al., 2014; Sugimoto et al., 2018).

Another study investigating the long term biochemical and morphological effects of culturing hESCs in 3%, 12% and 20% O$_2$ conditions (mimicking the reproductive tract 3% and arterial blood(12%)for 20 days found that cells grown at 12% O$_2$ still had traditional hESCs morphology of round, tightly packed cells with large nuclei and expressed high levels of pluripotency markers OCT4 and NANO2 compared to 3% and 20% O$_2$ (Lim et al., 2011). Moreover, there was a reduction in chromosomal abnormalities (aneuploidy) in cultures grown at 12% (8.3% aneuploidy compared to 25% at 20% O$_2$ normoxia). Chromosomal abnormalities
are considered bad as, it can lead to unreliable cell behaviour and undesired cancerous cells (Na et al., 2014). Expression of hypoxia-inducible factors (HIF)-2α was highest in cells grown at 12% O₂. HIF-2α is believed to bind to the OCT4 promoter region directly and therefore overexpression of HIF-2α upregulates OCT4 expression (Covello et al., 2006). Table 1.2 shows a summary of the effects of low oxygen tension on hiPSCs and hESCs stem cell pluripotency.
<table>
<thead>
<tr>
<th>Cell type</th>
<th>Conditions</th>
<th>Result (compared to 20% O₂)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>hESCs</td>
<td>1, 3 and 5% O₂</td>
<td>decrease in differentiated cells around colonies, increase expression of SSEA4 and OCT4; increased EBs formation at 1%</td>
<td>(Ezashi, Das and Roberts, 2005)</td>
</tr>
<tr>
<td>hESCs</td>
<td>5% O₂</td>
<td>Decreased in spontaneous differentiation during maintenance</td>
<td>(Forristal et al., 2010)</td>
</tr>
<tr>
<td>hESCs and hiPSCs</td>
<td>2% O₂</td>
<td>increased expression of pluripotency marker TRA1-60 and SSEA4</td>
<td>(Mattieu et al., 2013)</td>
</tr>
<tr>
<td>hESCs</td>
<td>2% O₂</td>
<td>increased clonal recovery; decreased chromosomal abnormalities</td>
<td>(Forsyth et al., 2006)</td>
</tr>
<tr>
<td>hESCs</td>
<td>3 and 12% O₂</td>
<td>Increased expression of pluripotency markers OCT4,</td>
<td>(Lim et al., 2011)</td>
</tr>
<tr>
<td>hiPSCs</td>
<td>5% O₂</td>
<td>Increased in cell colonies, increased expression of pluripotency markers OCT4, SOX2 and NANO.G.</td>
<td>(Sugimoto et al., 2018)</td>
</tr>
<tr>
<td>hiPSCs</td>
<td>1 and 5% O₂</td>
<td>enhances reprogramming efficiency of iPSCs and increased expression of pluripotency marker alkaline phosphatase</td>
<td>(Yoshida et al., 2009)</td>
</tr>
<tr>
<td>hESCs</td>
<td>5% O₂</td>
<td>Increased in proliferation rate</td>
<td>(Ludwig et al., 2006)</td>
</tr>
<tr>
<td>hiPSCs</td>
<td>2% O₂</td>
<td>improved maintenance of hiPSC pluripotency and decreased signs of spontaneous differentiation</td>
<td>(Fynes et al., 2014)</td>
</tr>
<tr>
<td>hESCs</td>
<td>1, 5, 10 and 15% O₂</td>
<td>Increased expression of pluripotency markers NANOG and NOTCH1</td>
<td>(Prasad et al., 2009)</td>
</tr>
</tbody>
</table>
1.2.3 The Effect of Oxygen on Differentiation

Apart from hypoxic conditions which maintain cells in an undifferentiated state, it was also found that it is beneficial for the differentiation of cells. For example, Ezashi et al., (Ezashi, Das and Roberts, 2005) observed that hESCs grown in hypoxic conditions produced more EBs with 16% of those EBs successfully attached to the 6-well plate and developed outgrowth compared to <2% of EBs cultured under normoxia (atmospheric 20% O$_2$) within the same study. This also correlates with the findings from Lim et al., (Lim et al., 2011), which showed that EBs produced from hESCs has grown in hypoxia lead to higher expression of endodermal markers compared to cultures grown in 20% O$_2$. These markers were also present on the EBs outer cell layer rather than within the EBs, suggesting the cells may have differentiated into primitive endoderm, a germ layer that forms extraembryonic endoderm such as the placenta.

This environment also enhanced via directed differentiation the generation of neuronal precursors cells (mESCs cells expressing neuronal markers βIII tubulin and MAP2) with a 55-fold increase in gene expression for βIII tubulin and 114 fold increase for MAP2 in mESCs grown at 2% O$_2$ (Mondragon-Teran, Lye and Veraitch, 2009). This was also the case in another study (Bae et al., 2012), which investigated hESCs and hiPSCs growth in normoxia and hypoxia at 20% and 2% oxygen tensions which showed that hypoxic conditions enhance the generation of neural retinal progenitor cells. These cells grown in 2% oxygen undergo less spontaneous differentiation and EB’s cultured at 2% expressed a photoreceptor marker for muller glial and bipolar cells cone-rod homeobox (CRX), a photoreceptor marker.
marker for muller glial and bipolar cells, whereas the EBs grown in 20% O\textsubscript{2} did not express CRX. This signifies that hypoxic conditions do promote directed differentiation of neuronal precursors cells. Moreover, there was increased differentiation into the ectodermal lineages. Studies also found that hypoxic conditions reduce apoptosis in neural stem cell cultures (Schänzer \textit{et al.}, 2006) and reduce chromosomal abnormalities (Forsyth \textit{et al.}, 2006). Other cell types differentiated in a hypoxia environment include retinal progenitor cells (Bae \textit{et al.}, 2012; Garita-Hernández \textit{et al.}, 2013), endothelial cells (Ramírez-Bergeron \textit{et al.}, 2004, 2006) and hepatocytes (Prasajak and Leeanansaksiri, 2013; Cai, 2014; van Wenum \textit{et al.}, 2018).

Another cell type that benefits from hypoxic conditions are chondrocytes. EBs produced from hESCs were subjected to chondrogenic differentiation in 2% O\textsubscript{2} had better biomedical functionality as measured by the increased production of glycosaminoglycan, collagen I and collagen II, which are components necessary for cartilage formation (Koay \textit{et al.}, 2008). Mesodermal lineage investigated in oxygen-deprived conditions saw a higher yield in cardiomyocytes. Differentiation efficiency in 5% O\textsubscript{2} between day 0-2 was higher in all seven hiPSCs lines tested and with the addition of polyvinyl alcohol (PVA) to increase cardiomyocytes contraction saw 94.7% of cells contracting (Burridge \textit{et al.}, 2011).

Evidently, as shown in this section, oxygen is an important factor in stem cell biology. Extensive research has established that the use of different oxygen tensions mimicking \textit{in vivo} conditions can be used to maintain, expand and promote efficient differentiation of cells. Table 1.3 shows a summary of the effects of low oxygen tension on stem cell differentiation. In this thesis, we will investigate the
combined effects of Young's modulus and low oxygen tension on hiPSCs differentiation, the novelty of the thesis is that the combination of such factors has not been investigated before. Apart from oxygen tension, another factor that effects adherent cell culture is the extracellular matrix the cells are cultured on. The substrate elasticity is measured by Young’s modulus in Pascals (Pa). Young’s modulus is the ratio between the force (stress) applied to a material and the resistance (strain) produced (Young, 1807). It is believed that adherent cells sense the substrate properties through mechanotransduction.
<table>
<thead>
<tr>
<th>Origin cell type</th>
<th>Conditions</th>
<th>Result (compared to 20% O$_2$)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>hESCs</td>
<td>1 and 5% O$_2$</td>
<td>Increased expression of endothelial markers VEGFA, VEGFB and VEGFR-2</td>
<td>(Prado-Lopez et al., 2010)</td>
</tr>
<tr>
<td>hESCs</td>
<td>3 and 12% O$_2$</td>
<td>Increased expression of endodermal markers FOXA2, SOX17, AFP, and GATA4</td>
<td>(Lim et al., 2011)</td>
</tr>
<tr>
<td>mESCs</td>
<td>2% O$_2$</td>
<td>Increase in gene ectoderm expression of βIII TUBULIN and MAP2</td>
<td>(Mondragon-Teran, Lye and Veraitch, 2009)</td>
</tr>
<tr>
<td>hESCs and hiPSCs</td>
<td>2% O$_2$</td>
<td>Increased expression of ectoderm CRX</td>
<td>(Bae et al., 2012)</td>
</tr>
<tr>
<td>hESCs and hiPSCs</td>
<td>5% O$_2$</td>
<td>Increased expression of vascular cells marker VEcad and CD31</td>
<td>(Kusuma et al., 2014)</td>
</tr>
<tr>
<td>hESCs</td>
<td>2% O$_2$</td>
<td>Increased production of collagen I, collagen II and glycosaminoglycans in chondrocytes</td>
<td>(Koay et al., 2008)</td>
</tr>
<tr>
<td>hESCs</td>
<td>5% O$_2$</td>
<td>Increased in amount of EBs spontaneously contracting (cardiomyocytes)</td>
<td>(Yang et al., 2008)</td>
</tr>
<tr>
<td>hESCs</td>
<td>0.1% O$_2$</td>
<td>&quot;hypoxic preconditioning&quot; for 12 hours resulting in increased of neuronal cells</td>
<td>(Francis and Wei, 2010)</td>
</tr>
<tr>
<td>hMSCs</td>
<td>5% O$_2$</td>
<td>Increased in hepatocyte-like cells yield</td>
<td>(Prasajak and Leeanansaksiri, 2013)</td>
</tr>
<tr>
<td>hiPSCs</td>
<td>10% O$_2$</td>
<td>Increased in hepatocytic differentiation yield</td>
<td>(Ayabe et al., 2018)</td>
</tr>
<tr>
<td>hESCs</td>
<td>4% O$_2$</td>
<td>Increased in cardiomyocytes differentiation yield</td>
<td>(Niebruegge et al., 2009)</td>
</tr>
</tbody>
</table>
1.3 Mechnotransduction

Apart from oxygen, another environmental factor that has been widely studied is the effect of substrate stiffness on cell behaviour. Mechanosensing and mechanotransduction are important factors to consider in cell culture. These are the processes by which the cells sense, interact and respond to their surrounding mechanical environment. Studies using substrates to influence cell outcome dates back to 1977, where mouse epithelial cells were grown on different substrates and resulted in cells with different morphology (Emerman and Pitelka, 1977). These environmental cues could include the density and topography of the matrix that the cells are growing on, the physical forces such as stretching and shear stress, cell-to-cell interactions, gaseous conditions and metabolites in the media (Hemsley et al., 2011). Some cells, including hPSCs, are adherent cells and need to adhere to a surface (Folkman and Moscona, 1978). The properties of the surface affect this cell adhesion and cell behaviour.

One variable that has been studied is the elasticity of the matrix. Elasticity of a material is the measurement of the deformation when stress is applied to the material. The types of elastic moduli (Pascals, Pa) are Young’s modulus ($E$), bulk modulus ($K$) and shear modulus ($G$). Young’s modulus is the ratio between the force (tensile stress) applied to a material and the resistance (tensile strain) produced (Young, 1807). Bulk modulus is the measurement of elasticity in three dimensions when deformed in all directions uniformly. It is the ratio of uniform volumetric stress over uniform volumetric strain (Anderson, 1969). Shear modulus
describes the force deforming a material where the on a particular side and not the other, an example of shear modulus is friction. Shear modulus is the ratio of shear stress over shear strain (IUPAC, 1997). A summary diagram of the different elastic moduli can be seen in Figure 1.5. Another type of stress and strain ratio is the complex modulus. It is a measurement of strain to strain that is measured under vibration conditions in viscoelastic materials. In viscoelastic materials, there are storage modulus and loss modulus of the viscoelastic material that can be defined. Where, elastic moduli in materials are classed as storage energy and loss moduli is the energy dissipated as heat in vicious properties (when vibrated) (tensile storage modulus, E′ and tensile loss modulus E″. Similarly, shear storage modulus, G′, shear loss modulus G″) (Sabbagh et al., 2000). However, in this thesis we will be using the elasticity moduli of Young’s modulus as the substrates are solid materials.
Figure 1.5 Summary diagram of the different elastic moduli

Diagram describing different types of elastic moduli, where i) Young’s modulus, ii) shear modulus and iii) bulk modulus. (Turowec., 2013)
Different biological samples of the same cells and tissue types can have varying Young’s modulus due to disease (Li et al., 2008), the way they developed (Benson-Martin et al., 2006; Murayama et al., 2006), and age (Pailler-Mattei, Bec and Zahouani, 2008). For example, there were differences in cell elasticity between hESCs and hiPSCs derived from different somatic cells (Kiss et al., 2011). Table 1.4 shows a list of Young’s moduli of various cell/tissue type. This identifies the issue that conventional cell culturing takes place on tissue culture polystyrene (TCP) with Young’s modulus values over $10^7$ fold higher than in vivo environments (Figure 1.6). The influence of substrates on cells can initially be detected by simply observing the cell morphology when grown on substrate of different Young’s modulus (Figure 1.6ii). Cells grown on soft substrates tend to be clumped together and do not readily spread whereas cells seeded on stiff substrates are prone to spreading and exhibit more focal adhesions (Pelham and Wang, 1997)(Figure 1.6ii). This was also seen on ESCs (Chowdhury et al., 2010). Lots of studies have been carried out on the effect of Young’s modulus on cell culture and it was discovered that Young’s modulus does affect not only the cell’s morphology (Pelham and Wang, 1997; Kurima et al., 1998), but also their organisation (Engler et al., 2008), migration speed (Lo et al., 2000; Peyton and Putnam, 2005), protein expression (Collinsworth et al., 2002) and gene expression (Chowdhury et al., 2010).
Table 1.4 Young’s modulus values of various human tissues as found in the literature

<table>
<thead>
<tr>
<th>Source</th>
<th>Young’s Modulus (kPa)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human zona pellucida (of embryos)</td>
<td>2.4 ± 1.2</td>
<td>(Khalilian et al., 2010)</td>
</tr>
<tr>
<td>Human zona pellucida (of oocytes)</td>
<td>1.1 ± 0.5</td>
<td>(Khalilian et al., 2009)</td>
</tr>
<tr>
<td>Human amniotic membrane</td>
<td>2300 ± 700 (2.3 ± 0.7MPa)</td>
<td>(Benson-Martin et al., 2006)</td>
</tr>
<tr>
<td>hESCs</td>
<td>1.2 ± 0.4</td>
<td>(Hammerick et al., 2011; Kiss et al., 2011)</td>
</tr>
<tr>
<td>hASCs-hiPSCs</td>
<td>0.9 ± 0.4</td>
<td>(Hammerick et al., 2011)</td>
</tr>
<tr>
<td>Fibroblast-hiPSCs</td>
<td>1.3 ± 0.5</td>
<td>(Hammerick et al., 2011)</td>
</tr>
<tr>
<td>hASCs</td>
<td>5.2 ± 1.0</td>
<td>(Hammerick et al., 2011)</td>
</tr>
<tr>
<td>Fibroblasts</td>
<td>2.9 ± 1.0</td>
<td>(Hammerick et al., 2011)</td>
</tr>
<tr>
<td>Smooth muscle</td>
<td>8.8 ± 1.3</td>
<td>(Engler et al., 2004)</td>
</tr>
<tr>
<td>Skeletal muscle</td>
<td>13.0 ± 5.0</td>
<td>(Engler et al., 2006; Gilbert et al., 2010a)</td>
</tr>
<tr>
<td>Cardiac muscle</td>
<td>15.0 ± 5.0</td>
<td>(Sinkus, 2014)</td>
</tr>
<tr>
<td>Cartilage</td>
<td>900</td>
<td>(Trickey, Lee and Guilak, 2000)</td>
</tr>
<tr>
<td>Osteoids (crosslinked collagen)</td>
<td>32 ± 8.0</td>
<td>(Engler et al., 2007)</td>
</tr>
<tr>
<td>Bone</td>
<td>1800000 ± 0.2 (1.8 ± 0.2GPa)</td>
<td>(Smith and Walmsley, 1959)</td>
</tr>
<tr>
<td>Epithelial</td>
<td>7.0 ± 6.5</td>
<td>(Berdyyeva, Woodworth and Sokolov, 2005)</td>
</tr>
<tr>
<td>Lung</td>
<td>0.7</td>
<td>(De Wilde et al., 1981)</td>
</tr>
<tr>
<td>Pancreas</td>
<td>1.6 ± 0.25</td>
<td>(Rubiano et al., 2018)</td>
</tr>
<tr>
<td>Liver</td>
<td>6.4 ± 0.8</td>
<td>(Yeh et al., 2002)</td>
</tr>
<tr>
<td>Brain</td>
<td>0.2 ± 0.2</td>
<td>(Flanagan et al., 2002; Clatz et al., 2005; Schiavone et al., 2009)</td>
</tr>
<tr>
<td>Tissue culture polystyrene (TCP)</td>
<td>30000000 (3GPa)</td>
<td>(Bonaccurso, Cappella and Graf, 2006)</td>
</tr>
</tbody>
</table>
Figure 1.6 Young’s modulus scale of various human tissues in kilopascals (kPa).

Where i) Young’s modulus scale of human tissues in kPa and ii) the morphology of cell on different substrate stiffness
Although the definite pathways relating to the effect substrates have on cells are not fully understood, we do know that cells adhere to a matrix through clusters of various anchorage proteins (talin, vinculin, paxillin and α-actinin) called integrins. These integrins are found at the end of the actin cytoskeleton and connect to the β-unit of the extracellular head region (there are two extracellular heads consisting of an α and β-unit). The head region of the α and β-unit binds to the extracellular protein of the matrix coating; these sites are called focal adhesions (Jacot, McCulloch and Omens, 2008; Baker, Bonnecaze and Zaman, 2009; Jacot, Martin and Hunt, 2010; Ivaska, 2012). To date, 24 integrins have been identified (Baker and Zaman, 2010).

The cytoskeleton can ‘sense’ the surface elasticity by applying pulling forces through the focal adhesions. This is carried out by non-muscle myosin II (NMMII), an actin binding protein which can contract and retract the actin cytoskeleton (Tamada, Sheetz and Sawada, 2004).

The cytoskeleton consists of a large network of stress fibres of polymerised F-actin that is in the cell. It is made up of microtubules, actin filaments and intermediate filaments and it dictates the morphology of the cells (Girard and Nerem, 1995; T. Yeung et al., 2005; Wang, 2007; Wang, Tytell and Ingber, 2009; Stricker, Falzone and Gardel, 2010). Microtubules are polymers of tubulin and are important in maintaining the stability of the cell’s shape and structure (Kirschner and Mitchison, 1986; Desai and Mitchison, 1997). However, Intermediate filaments do not contribute to the cell’s response on mechanotransduction or motility; its role is to provide stability to the plasma membrane of the cell and resist any cell deformations caused cell-cell contact or extracellular matrix (Lodish et al., 2000).
It has been established that matrix stiffness and cytoskeletal tension are related as integrins are regulated by growth factors, Rho GTPase proteins and ERK proteins. Rho GTPase regulates myosin activity. On stiff substrates, the formation of focal adhesion sites is promoted, leading to elevations in ERK and Rho GTPase proteins and resulting in more tension in the cytoskeleton (Paszek et al., 2005). This, in turn, can alter both the physical and chemical activity within the cells. Another effect on the cell, is that the cytoskeleton is linked to the nucleus (via a linker to nucleoskeleton and cytoskeleton complex) and therefore can affect the nucleus cytoskeleton and gene expression (Wang, Tytell and Ingber, 2009).

Conventionally cells are grown on tissue culture polystyrene (TCP) (3GPa) over $10^6$ times stiffer than the embryo (Khalilian et al., 2010). Multiple tuneable polymers can be used for cell culture to determine the effects of Young’s modulus on cell behaviour. However, materials used for cell culture would have to possess certain qualities such as compatibility to cell culture, hydrophilic and inert for the cells to adhere, survive and grow. As mention earlier and in Section 1.1.1, substrates are coated with protein in which the cell’s integrin attach to. There are currently a variety of commercially available protein that are used in cell culture such as collagen, elastin, fibronectin, laminin, vitronectin, poly-lysine and Matrigel. Despite the coating, the cells can sense the substrate it has been shown by Yeh et al., (Yeh et al., 2017) that soft substrate effects the mouse mammary gland epithelial cells regardless of the substrate being coated with collagen, poly-L-lysine, fibronectin or Matrigel. And Trappmann et al., (Trappmann et al., 2012) reported that human keratinocytes grown on fibronectin and collagen coated polyacrylamide gels had the same results. Additionally, all cell culture studies use
protein coatings on their substrates and their results from indicates that the cells can sense the substrate stiffness.

1.3.1 The Effect of Young’s Modulus on Pluripotency

The effects of cell-matrix interactions have been investigated; studies suggest that maintaining undifferentiated stem cells is challenging in general. Chowdhury et al., (Chowdhury et al., 2010) found that mESCs cultured on soft substrates that mimic mouse embryos promotes self-renewal and pluripotency, even in the absence of Leukemia inhibitory factor (LIF), an essential factor in regulating mESC pluripotency and self-renewal. Moreover, muscle stem cells (MuSCs) grown on hydrogels with the Young’s modulus of 12kPa that mimic muscle tissue could self-renew and 25% of mice transplanted with the MuSCs that were grown on 12kPa were engrafted compared to 0% on MuSCs cultured on plastic (Gilbert et al., 2010b). However, this has not been reflected in human stem cells as all research so far using human cells reported no significant differences in pluripotency between hPSCs grown on various Young’s modulus (Evans et al., 2009; Keung et al., 2012; Przybyla, Lakins and Weaver, 2016). The effect of Young’s modulus on pluripotency will not be investigated in this thesis apart from characterising cell culture on the substrates as this thesis will be looking at the effects of combining Young’s modulus and low oxygen tension to hiPSCs differentiation.

1.3.2 The Effect of Young’s Modulus on Differentiation

Multiple studies on soft substrates have been shown to affect stem cell differentiation. Human mesenchymal stem cells (hMSCs) (Engler et al., 2006), hPSCs (Keung et al., 2012), mESCs (Ali et al., 2015), mouse neural stem cells (mNSCs) (Saha et al., 2008; Jiang et al., 2015) all grown on soft substrates
mimicking brain tissue have been shown to promote neuronal differentiation. Moreover, hMSCs were allowed to spontaneously differentiate on three different elasticities mimicking brain (0.1-1kPa), muscle (8-17kPa) and bone (25-40kPa) tissue showed that there was a 5-fold increase in neurogenic expression on 0.1-1kPa substrates, 6-fold increase in myogenic expression on 8-17kPa substrates and 4-fold increase in osteogenic expression (Engler et al., 2006).

Keung et al., (Keung et al., 2012) investigated the effect of neuronal directed differentiation on hESCs and hiPSCs grown on 0.1, 0.7 and 75kPa polyacrylamide gels. They found that hPSCs grown on 0.1kPa resulted in the emergence of early ectodermal markers after 9 days and the highest expression of PAX6 and SOX1 genes, which are of neuronal lineage. This research implied that cells only need to be grown on soft substrates for 5-days to have an effect on neuronal expression.

Another study (Ali et al., 2015a) on mouse embryonic stem cells (mESCs) on gelatin cross linked with glutaraldehyde (GXG) ranging from 2-35 kPa and in this case, there was an increase in neural differentiation yield on soft substrates that mimic mouse blastocysts and brain tissue. There was also high gene expression of βIII TUBULIN in cells grown on 0.5kPa gels and higher yield of neuron differentiation from cells grown on 0.1-0.5kPa gels and glial grown on 1-10kPa gels.

In a study that looked at how the elasticity of the substrate affected the growth and differentiation of mouse neural stem cells, single cells were seeded on 3 different types of matrices: TCP (3GPa), Tiansu silicone (4.3MPa) and SMI silicone (2.3MPa). 7 days after seeding, it was found that SMI and Tiansu had higher cell differentiation rates than TCP and that there was a higher yield of astrocytes in samples grown on the softer SMI silicone (Jiang et al., 2015).
Additionally, 20-40% of cardiomyocytes were beating on soft substrates that mimic heart elasticity at ≤11kPa compared to 2-8% of the cardiomyocytes on 34kPa matrix (Engler et al., 2008). When mESCs were grown on a stiff material (2.7MPa) had a higher yield in mesendoderm gene expression as well as osteogenic differentiation than softer substrates (Evans et al., 2009). Mouse embryonic stem cells were also plated to spontaneously differentiate for 5 days on soft substrates (0.04-0.2kPa) and revealed high gene expression in AFP and SOX17, endoderm markers with a particularly high AFP expression at 0.013 and 0.171kPa with no significant difference in pluripotency or other germ layer markers (Jaramillo et al., 2015)

Another study (Al-Rekabi and Pelling, 2013) looked at how myoblasts reacted when a 10nN force was applied while cultured on GXG matrices on varying elasticities of 16-51kPa, which constitute the elasticity range of resting mouse muscle, and 64-89 kPa which is the elasticity range of active mouse muscle. The Young’s modulus values were confirmed by an atomic force microscop. They found that the myoblasts only generate a significant increase in traction from the force applied when the substrate is of the same elasticity as active mouse muscle tissue (38-89 kPa) and no significant responses were found on substrate elasticities of less than 64 kPa. They further investigated the effect of the cell morphology by inhibiting rho-kinase (ROCK) and myosin-II (MyoII), which are key factors that influence the morphology of the actin cytoskeleton and focal adhesion of the cell, and found that the cells lack structure, stress fibres and decrease in traction force. This study proves that the extracellular environment does affect the cell significantly.
A study by Trappmann et al. (Trappmann et al., 2012), investigated the behaviour of human epidermal stem cells cultured on polydimethylsiloxane (PDMS) with a range of stiffness between 0.1 Pa-2.3 MPa and polyacrylamide (PA) between 0.5 kPa to 740 kPa. It was found that the keratinocytes differentiated less on the softer matrices and they could adhere to the surface, however they did not spread or form a cortical actin cytoskeleton required for differentiation. As the level of stiffness was increased the cells were observed to be spreading more until reaching a plateau cell area of 2000 µm² at 115 kPa, measured by staining the cells with actin and measuring the adhesive cell area. They also found that the difference in the stiffness of the matrix results in an alteration in the extracellular-signal-related kinase (ERK)/mitogen-activated protein kinase (MARK) signalling pathway due to the lack of spreading.

It has been established that soft substrates influence hPSCs mesoderm differentiation via the wnt/β-catenin pathway. This is because β-catenin is repressed as well as being degraded in cells seeded on stiff substrates and therefore reduces mesodermal differentiation. However, mesodermal expression was found to be the highest in soft substrates of 0.4 kPa. It is believed that the soft substrate mimics Young’s modulus of the gastrulation stage of embryo development and that during gastrulation the mesodermal layer was identified (Przybyla, Lakins and Weaver, 2016).

These examples show that substrate elasticity does influence cells in many ways, and it is, therefore, important to better understand the effects of substrate elasticity on the cells in order to improve cell culturing techniques. Table 1.5 shows a summary of the effects of Young’s modulus on stem cell differentiation. In this
thesis, we will investigate the combined effects of Young’s modulus and low oxygen
tension on hiPSCs differentiation, the novelty of the thesis is that the combination
of such factors has not been investigated before.
Table 1.5 Effects of Young's modulus conditions on stem cell differentiation

<table>
<thead>
<tr>
<th>Origin Cell Type</th>
<th>Conditions</th>
<th>Result (compared to TCP)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>hMSCs</td>
<td>0.1-1 kPa</td>
<td>increase in neurogenic expression</td>
<td>(Engler et al., 2006)</td>
</tr>
<tr>
<td>hMSCs</td>
<td>8-17 kPa</td>
<td>increase in myogenic expression</td>
<td>(Engler et al., 2006)</td>
</tr>
<tr>
<td>hMSCs</td>
<td>25-40 kPa</td>
<td>increase in osteogenic expression</td>
<td>(Engler et al., 2006)</td>
</tr>
<tr>
<td>hESCs and hiPSCs</td>
<td>0.1, 0.7 and 75 kPa</td>
<td>increase expression neuronal markers of PAX6 and SOX1 genes</td>
<td>(Keung et al., 2012)</td>
</tr>
<tr>
<td>mESCs</td>
<td>2, 15 and 35 kPa and</td>
<td>increase in neuronal differentiation</td>
<td>(Ali et al., 2015)</td>
</tr>
<tr>
<td>mNSC</td>
<td>0.1 -0.5 kPa</td>
<td>increase in neuronal differentiation</td>
<td>(Saha et al., 2008)</td>
</tr>
<tr>
<td>mNSC</td>
<td>2.3 MPa and 4.3 MPa</td>
<td>increase in yield of astrocytes</td>
<td>(Jiang et al., 2015)</td>
</tr>
<tr>
<td>hMSCs</td>
<td>11 kPa</td>
<td>increase in beating cardiomyocytes</td>
<td>(Engler et al., 2008)</td>
</tr>
<tr>
<td>mESCs</td>
<td>2.7 MPa</td>
<td>increase in meso-endoderm gene expression as well as osteogenic differentiation</td>
<td>(Evans et al., 2009).</td>
</tr>
<tr>
<td>mESCs</td>
<td>0.04-0.2 kPa)</td>
<td>increase endoderm markers expression in AFP and SOX17</td>
<td>(Jaramillo et al., 2015)</td>
</tr>
<tr>
<td>hESCs</td>
<td>0.4 kPa</td>
<td>increase in mesodermal expression</td>
<td>(Przybyla, Lakins and Weaver, 2016).</td>
</tr>
<tr>
<td>hMSCs</td>
<td>42 kPa</td>
<td>increase in osteogenic differentiation</td>
<td>(Shih et al., 2011)</td>
</tr>
<tr>
<td>hMSCs</td>
<td>40 kPa</td>
<td>increase in osteogenic differentiation</td>
<td>(Xue et al., 2013)</td>
</tr>
<tr>
<td>hiPSCs</td>
<td>9 kPa</td>
<td>increased in cardiac differentiation</td>
<td>(Wang et al., 2019)</td>
</tr>
<tr>
<td>hESCs</td>
<td>2.1 kPa</td>
<td>Increased in beta-like cell</td>
<td>(Narayanan et al., 2013)</td>
</tr>
</tbody>
</table>
1.4 Thesis Aims and Objectives

In summary, human embryonic stem cells (hESCs) and human induced pluripotent stem cells (hiPSCs) are a type of pluripotent stem cells, cells that are able to self-renew and differentiate into any stomatic cell within the human body. Conventionally, hESCs and hiPSCs are cultured in atmospheric 20% oxygen, however, the oxygen tension in vivo is lower than 20%. In vivo, the oxygen tension is between 0-12%, moreover, the oxygen tension in the fallopian tube 1.5% - 8.7%, this is where the inner cell mass of the blastocyst is found in vivo. The inner cell mass is used for hESCs derivation. Due to these in vivo oxygen tension, it is believed that low oxygen tension plays an important role in human pluripotent stem cells pluripotency and differentiation. Existing studies on hiPSCs and hESCs grown in low oxygen tension (< 20% oxygen) demonstrated increased hiPSCs reprogrammed efficiency and maintenance of pluripotency (Table 1.2) and higher yield of desired differentiated cells (Table 1.3). Additionally, as human pluripotent stem cells are anchorage dependent cells they have to be grown on a substrate. Conventionally, cells are grown on tissue culture plastic which have a Young’s modulus value of 3x10^6 Pa, this is much harder than anything in vivo conditions (Table 1.4). Existing studies on hiPSCs and hESCs grown on various Young’s modulus shows that substrate stiffness has an effect on cell morphology and differentiation outcome (table 1.5). To date, there have been studies on how Young’s modulus and low oxygen tension independently affect cell pluripotency, phenotype and differentiation (Table 1.2, 1.3 and 1.5). However, there have not yet been any studies on the combined effects of Young’s modulus and low oxygen tension on hiPSCs and therefore we aim to mimic in vivo conditions (relevant to
both of these factors). The physiological relevant Young’s modulus values this thesis will be investigating with are in the following ranges (as mentioned in Section 1.3 and Table 1.5): 0.1-0.5kPa (brain) (Flanagan et al., 2002; Clatz et al., 2005; Schiavone et al., 2009), 1-3kPa (zona pellucida of embryos) (Khalilian et al., 2010), 10-20kPa (muscle) (Engler et al., 2004, 2006; Gilbert et al., 2010b) and >30kPa (osteoid) (Takai et al., 2005; Engler et al., 2007). Additionally, based on literature reviewed in Section 1.2, this thesis will be using 2% oxygen as the low oxygen environment.

The objectives of this thesis are to study the novel combined effects of Young’s modulus and low oxygen tension on hiPSCs as it is hypothesised that the combined effects of these factors would have significant impact on cell phenotype, interactions and differentiation of the three germ layers. In turn, to find the optimal environmental conditions to propagate and differentiate hiPSCs into various lineages. The results obtained from this study in collaboration with other studies could then be used to develop a better understanding of cell culturing which can facilitate the design of improved culturing bioprocessing platform. We hope to provide a novel and cheaper alternative to stem cell differentiation as growth factors and small molecules that are required for direct differentiation are costly.

Objectives for each experimental chapter are as follows:

**Chapter 3: Characterisation of human induced pluripotent stem cells.**

In this chapter, we aim to characterise the human induced pluripotent stem cell lines (cord blood and MSU001 lines) to prove their pluripotency. This will be carried out by immunocytochemistry, flow cytometry and differentiation into the three germ
layers via spontaneous differentiation of hiPSCs monolayer and embryoid bodies (EBs). Cells were analysed using flow cytometry and immunocytochemistry for pluripotent and differentiation markers.

Chapter 4: Evaluation of Substrate and Characterisation.

The aim of this chapter is to produce and investigate different types of substrates, with the desired Young’s modulus values. Substrates produced will have their Young’s modulus value measured using Atomic Force Microscopy (AFM). Cells were also grown on the substrates and characterised to ensure they are able to grow on these matrices.

Chapter 5: The Effects of Young’s Modulus and Low Oxygen Tension on Human Induced Pluripotent Stem Cell Spontaneous Differentiation in a 2D Monolayer Culture.

Once substrates have been characterised, in this chapter our aim is to investigate the differentiated outcome of hiPSCs monolayer culture influenced by Young’s modulus values of 0.2, 2, 16, 64 kPa and TCP and low oxygen tension of 2% O₂. Cells were analysed using RT-qPCR, flow cytometry and immunocytochemistry for markers of the three germ layers. We hypothesised that the combination of Young’s modulus and low oxygen tension will have an impact on germ layer formation, and we will analyse the data sets to determine the most favourable condition for each germ layer.

Chapter 6: The Effects of Young’s Modulus and Low Oxygen Tension on Human Induced Pluripotent Stem Cell Spontaneous and Directed Differentiation in Three Dimensional Embryoid Bodies (EBs) Culture.
Lastly, in this chapter we aim to investigate the combined effects of Young's modulus and low oxygen using 3D Embryoid bodies (EBs) culture and study the differentiated outcome of hiPSCs grown on Young's modulus values of 0.2, 2, 16, 64 kPa and TCP in 2% O₂. Cells will be analysed using RT-qPCR, flow cytometry and immunocytochemistry. We hypothesised that results from EBs would have an impact on germ layer formation due to cell-cell contact. We aim to distinguish the optimum combination for each germ layer. Following on from spontaneous differentiation of EBs, directed differentiation will be implemented with the addition of ACTIVIN A to cells grown on 0.2kPa in 2% Oxygen.
2 Chapter 2: Materials and Methods

2.1 Stem Cell Culture

2.1.1 Maintenance of Feeder-free Human Induced Pluripotent Stem Cell (hiPSCs) Lines

Human induced pluripotent stem cells (hiPSCs) cord blood line which was derived episomally from CD34+ cord blood with seven expressed factors (OCT4, Sox2, Klf4, NANOG, Myc, Lin28 and SV40 T) was purchased from Life Technologies (Carlsbad, CA, USA, catalogue number A18945). The line was initially grown on feeders and was adapted to a freeder free culture by Life Technologies, the cell line when purchased was feeder free. Experiments were carried out when cells were cultured from passage 30 to 64. The hiPSCs (MSU001) were derived from fibroblasts by Professor Jose B. Cibelli’s laboratory (Michigan State University) were acquired from the Spanish stem cell bank. The MSU001 cell line was generated through the overexpression of Oct-4, SOX2, NANO and Lin28 using lentivirus vector and then adapted to feeder-free conditions. Feeder-free adaptation consisted of removing mouse embryonic fibroblast (MEF) and replacing them with vitronectin as the extracellular matrix the hiPSCs were cultured on. The adapted cells were cultured in Essential 8™ (E8) media (Gibco, Thermo Fisher Scientific, Waltham, MA, USA, catalogue number A1517001) on vitronectin coated T-25 flasks (Thermo Fisher Scientific, Waltham, MA, USA, catalogue number 15217915) (Gibco, Thermo Fisher Scientific, Waltham, MA, USA, catalogue number A31804). Vitronectin was diluted in 0.1% v/v phosphate buffer saline without calcium and
magnesium (PBS) (LONZA, Basal, Switzerland, catalogue number BE17-516F) and incubated for one hour at room temperature prior to cell culture.

2.1.2 Feeder-free Sub-culture of Human Induced Pluripotent Stem Cell (hiPSCs) Lines

Upon 70-80% confluency at day 4, cells were passaged at a ratio of 1:3 (hiPSCs seeding density 0.7 x 10^6). As previously described (Section 2.1.1), T-25 flasks were coated with 2 mL 0.5mg/mL vitronectin (Gibco, Thermo Fisher Scientific, Waltham, MA, USA, catalogue number A31804) in 0.1% v/v phosphate buffer saline (PBS) (LONZA, Basal, Switzerland, catalogue number BE17-516F) for an hour prior to passaging. E8 media was removed from the flask, the flask was washed with PBS, then 2 mL of 0.5 mM Ethylenediaminetetraacetic acid (EDTA) (Life Technologies, Carlsbad, CA, USA, catalogue number 15575-038) was used to dislodge clumps of cells by leaving the cells in EDTA for 5 minutes at room temperature. EDTA was then removed, and 6 mL of E8 media was used to gently wash and dislodge the attached cells from the flask. Equal volumes of cell suspension were aliquoted into new T-25 flasks. For hiPSCs maintenance the hiPSCs Cells were not passaged with ROCK inhibitor. E8 media was topped up to reach a total volume of 7 mL and immediately placed in the incubator (Thermo Fisher Scientific, Waltham, MA, USA) at 37 °C and 5% v/v CO2. No media was changed within the first 48 hours to allow cell adhesion. After that daily media change occurred until cells were 80% confluent at day 4.
2.1.3 Freezing Feeder-free Human Induced Pluripotent Stem Cells (hiPSCs)

E8 media was removed from flasks and washed once with PBS. Two mL of EDTA was added and incubated at room temperature for three to five minutes to dissociate the cells. After the removal of EDTA, 6 mL of E8 media was added to dislodge the cells. They were then pelleted by centrifugation at 1500 rpm for five minutes at 4 °C, at this stage, cells are centrifuged as we want to remove the E8 media from the cell suspension. The supernatant was removed, and the cell pellet was resuspended by flicking the centrifuge tube gently. mFreSR™ (Stemcell Technologies, Vancouver, Canada, catalogue number 05854) was added to pellet at 2 mL per flasks used. mFreSR cell solution was pipetted into Nalgene™ cryogenic tubes (Thermo Fisher Scientific, Waltham, MA, USA, catalogue number 11740573) at 1 mL per tube on ice and placed immediately into a Mr Frosty™ Freezing container (Thermo Fisher Scientific, Waltham, MA, USA, catalogue number 11315674) and stored at -80 °C for three days before being transferred to liquid nitrogen for long term storage.

2.1.4 Thawing Feeder-free Human Induced Pluripotent Stem Cells (hiPSCs)

T25 flasks were coated with 2 mL 0.5 mg/mL vitronectin in 0.1% v/v PBS one-hour prior to thawing. E8 media was left at room temperature for an hour to warm up. Frozen cells in cryogenic vials retrieved from liquid nitrogen storage were thawed in a water bath at 37 °C for 2 minutes at room temperature, once thawed the cell suspension was quickly transferred into a falcon tube and topped up with 6 mL E8 media. The suspension was centrifuged at 1500 rpm for three minutes at room temperature. The supernatant was removed, and the cells were gently dislodged using 6 mL of fresh E8 media before being transferred into a vitronectin
coated T25 flask and immediately placed in the incubator at 37°C and 5% v/v CO₂. Cells were left to settle and adhere for 48 hours. Daily media change was carried out until cells were 80% confluent.

2.2 Cell count

2.2.1 Cell Dissociation for Cell Counting

In order to assess viability and cell numbers, cells were detached from the flasks. E8 Media was removed from the flask and washed once with PBS. Once washed, 2mL of TrypLE™ (Gibco, Thermo Fisher Scientific, Waltham, MA, USA, catalogue number 34421) was added into the flask and incubated at 37°C for three minutes. TrypLE™ was used for single cell dissociation. To inactivate the TrypLE™, 6mL of E8 media was added before the suspension was transferred into a falcon tube and centrifuged at 1500 rpm for five minutes. After centrifugation, the supernatant was aspirated, and the cell pellet was gently dissociated by flicking the bottom of the falcon tube. Fresh 2 mL E8 media was added into the tube with 10μL of 0.4% tryphan blue (Sigma-Aldrich, Gillingham, UK, catalogue number 72571) and cells were counted manually using a haemocytometer (Thermo Fisher Scientific, Waltham, MA, USA) or automatically by the Vicell machine (Beckman Coulter, Brea, CA, USA).

2.2.2 Haemocytometer and Vi-cell

Cells were counted to calculate the volume required for desired seeding density. 10 µL of single cells in suspension was stained with 10 µL of 0.4% tryphan blue (Sigma-Aldrich, Gillingham, UK, catalogue number 72571) and were counted manually using a haemocytometer. The haemocytometer was placed under a phase contrast microscope and viewed under a 10x objective. The single cells
present within the haemocytometer grid were counted manually by counting the live cells which were bright, and dead cells were stained blue. The number of viable cells was divided by the number of grids counted, multiplied by $2 \times 10^4$ to calculate the viable cells/mL. (Absher., 1973).

Alternatively, the Vi-Cell XR Cell Viability Analyser (Beckman Coulter, Brea, CA, USA) is used. 1mL of single cell suspension was collected into a Vi-cell collection tube (Beckman Coulter, Brea, CA, USA) and placed in a Vi-Cell XR Cell Viability for automated cell counting. The Vi-cell measures the cell concentration and viability by the automatic addition of trypan blue and taking 50 images of the sample.

### 2.2.4 Cell Seeding

The volume of cell suspension required for desired cell density was calculated. A seeding density for a 6-well plate at $3 \times 10^5$ cells per well was used. The volume was calculated using the formula (Absher, 1973).

$$V_1 = \frac{(V_2 \times C_2)}{C_1}$$

Where:

$V_1 =$ Volume required to achieve the desired cell density

$V_2 =$ total seeding volume

$C_1 =$ Cell count ($x10^4$ cells/mL)

$C_2 =$ cell concentration required to achieve seeding density desired

Following calculation, $V_1$ was dispensed and diluted to a volume of $V_2$ and seeded onto the 6-well plate. Once seeded the 6-well plate was swirled in a figure
of eight motion to disperse the cells within the wells and immediately placed in the incubator at 37°C and 5% v/v CO₂.

### 2.3 Differentiation of Feeder-free Human Induced Pluripotent Stem Cells (hiPSCs) in Monolayer

#### 2.3.1 Human Induced Pluripotent Stem Cells (hiPSCs) Media for Spontaneous Differentiation

Spontaneous differentiation was assessed using two methods: two-dimension monolayer and three-dimension embryoid bodies formation. The media was filtered using a filtered media bottle (Thermo Fisher Scientific, Waltham, MA, USA) and stored at 4°C. For experiments requiring the use of single cells in suspension an addition of 10µM/L Rho-associated protein kinase (ROCK) inhibitor (Sigma-Aldrich, Gillingham, UK, catalogue number SCM075) was added to the hiPSCs media for just the first day. ROCK inhibitor is used to help hiPSCs cells to attach as single cells on the substrate as hiPSCs can go into apoptosis when they are not in clumps (Claassen et al., 2009). Spontaneous differentiation media consisted of DMEM (DMEM/F12 plus glutamax) (Gibco, Thermo Fisher Scientific, Waltham, MA, USA, catalogue number 11320033) and supplemented with 20% Knock out serum replacement (KOSR) (Sigma-Aldrich, Gillingham, UK), 1% non-essential amino acids (NEAA) (Sigma-Aldrich, Gillingham, UK) and 0.1% β-Mercapthanol (Sigma-Aldrich, Gillingham, UK).
2.3.2 Culturing of Human Induced Pluripotent Stem Cells (hiPSCs) in Monolayer for Differentiation

Spontaneous differentiation media with an addition of 10µM/L of ROCK inhibitor was left at room temperature for 30-60 minutes prior to cell seeding to warm up. 6-well plates (Thermo Fisher Scientific, Waltham, MA, USA, catalogue number 10396482) were coated with Matrigel™ matrix (Corning Inc., Corning, NY, USA, catalogue number 7341440) 1:30 (protein concentration between 8 to 11 mg/mL, as Matrigel™ has batch variation) at room temperature for two hours before cell seeding.

To harvest cells from confluent T25 flasks, E8 media was removed, and the flask was washed with PBS. Then 2 mL of TrypLE™ (Gibco, Thermo Fisher Scientific, Waltham, MA, USA) was added into the confluent flask and incubated at 37°C for three minutes to dissociate the cells. Then 6 mL of spontaneous differentiation media with ROCK inhibitor was added to deactivate the TrypLE™ and also used to gently wash in order to detach the cells from the flask surface. The suspension was then placed in a falcon tube through a 40 µm strainer (Thermo Fisher Scientific, Waltham, MA, USA, catalogue number 11587522) and centrifuged at 1500 rpm for five minutes. The supernatant was removed, and the cell pellet was gently dissociated by flicking. Subsequently, 6 mL of fresh, spontaneous differentiation media with ROCK inhibitor was dispensed into the tube, and the desired volume of cell suspension was taken based on prior calculations made for required seeding density (3 x 10^5 cells per 6-well plate). Spontaneous differentiation media was topped up to achieve 2mL per well. Once seeded, the 6-well plate was swirled in a figure eight motion to disperse the cells and immediately
placed in the incubator 37°C and 5% v/v CO₂. Media change occurred every 48 hours.

2.3.3 Monolayer Culture in Low Oxygen

The same procedure was carried out for cell seeding and media used, cells were however placed in a 2% O₂ incubator (Thermo Fisher Scientific, Waltham, MA, USA) at 37°C and 5% v/v CO₂.

2.4 Differentiation of Human Induced Pluripotent Stem Cells (hiPSCs) by Embryoid Bodies (EBs) Aggregates

2.4.1 Embryoid Bodies (EBs) Formation

Spontaneous differentiation media (Section 2.3.1) with 10 µM/L ROCK inhibitor was warmed up at room temperature 60 minutes prior to using. Prior to cell seeding, 1 mL Anti-Adherence Rinsing Solution were dispensed into each AggreWell™ 800 (Stemcell Technologies, Vancouver, Canada catalogue number 07010) and centrifuged at 4000 rpm for five minutes to remove all air bubbles. This was then repeated with 0.5 mL spontaneous differentiation media were dispensed into each AggreWell™ 800 plates and centrifuged at 4000 rpm for five minutes to remove all air bubbles.

To harvest cells from T25 flasks, E8 media was removed from the flask and washed with PBS followed by the addition of 2 mL of TrypLE™. The flask was incubated at 37°C for three minutes before 6 mL of spontaneous differentiation media with ROCK inhibitor was added to deactivate the TrypLE™ and used to gently wash to detach the cells from the flask surface. The single cell suspension containing the hiPSCs cells was then passed through a 40 µm strainer (to de-clump
the cells) placed in a 50 mL falcon tube and centrifuged at 1500 rpm for five minutes at room temperature. The supernatant was removed, and the cell pellet was gently dissociated by flicking. Then 6 mL of fresh, spontaneous differentiation media with ROCK inhibitor was dispensed into the tube, and the desired volume of cell suspension was removed and $3 \times 10^6$ cells were seeded into each AggreWell™ well based on prior cell seeding density calculations in order to obtain 400 µm EBs with 10,000 cells. Media was added to reach 2 mL per AggreWell™. The plates were centrifuged at 400 rpm for three minutes for the cells to accumulate at the bottom of the AggreWell™. They were then placed in the incubator at 37°C and 5% v/v CO₂ for 24 hours.

2.4.2 Harvesting and Differentiation of Embryoid Bodies (EBs)

The day after seeding the EBs were harvested. Matrigel matrix was diluted at a ratio of 1:30 (protein concentration between 8 to 11 mg/mL) in spontaneous differentiation media, the 6-well plate and was left to coat for two hours before EBs seeding. To dislodge the EBs in the AggreWell™, the media solution in the AggreWell™ was pipetted up and down to dissociate the EBs from the AggreWell™. The media containing EBs were filtered using a 40 µm strainer to separate the single cells from the EBs. The separated EBs were then transferred to a 6-well plate. A dissecting microscope was used to collect 30 EBs per well to be seeded onto the Matrigel-coated 6-well plate. The seeding density of 30 EBs was required as this is the same cell density as the monolayer seeding density of $3 \times 10^5$ cells per well of a 6-well plate ($10,000 \text{ cells per EBs} \times 30 \text{ EBs} = 3 \times 10^5 \text{ cells per well}$). Once the EBs were collected and seeded, spontaneous differentiation media was added to each well to reach 2 mL. The 6-well plates were swirled gently
in a figure of eight motion to disperse the EBs within the wells and placed in the incubator at 37°C and 5% v/v CO₂. The EBs were cultured with spontaneous differentiation media, and media change occurred every 48 hours. Once the experiment was completed, cells were fixed and prepared for further analysis.

2.4.3 Embryoid Bodies (EBs) Culture in Low Oxygen

The same procedure was carried out for cell seeding and media used; cells were however placed in a 2% O₂ incubator.

2.4.4 Directed Differentiation Media

For endoderm directed differentiation experiments, the same cell culture protocol was carried out on Cytosoft and tissue culture plastic 6 well plates, with an addition of 100 ng/ml of ACTIVIN A (PeproTech, United Kingdom, catalogue number AF12014E) to the spontaneous differentiation media (Section 2.3.1) to induce definitive endoderm differentiation.

2.5 Immunocytochemistry

In order to assess the stemness and differentiation markers, immunocytochemistry was used as a qualitative method for protein expression. This protocol was used on both the monolayer and EBs cell cultures.

2.5.1 Fix, Permeabilisation and Blocking

Media was removed from wells and washed once with PBS. Cells were fixed with 2 mL of 4% para-formaldehyde (PFA) (Sigma-Aldrich, Gillingham, UK, catalogue number 158127) in PBS for 30 minutes at room temperature. To produce 4% PFA stock solution, 40g of PFA powder was added to 800 mL of 1x PBS and placed on a stir plate until fully dissolved in a fume hood. The stock solution was then
aliquoted and stored at -20°C.

PFA was removed, and cells were washed with PBS three times. Permeabilisation was required only when staining for intracellular markers; it was done by adding 2 mL of 0.1% Triton X-100 (Sigma-Aldrich, Gillingham, UK) in PBS to the wells for ten minutes at room temperature. Once triton was removed, the wells were washed with PBS three times. Cells were then treated with a blocking solution consisting of 5% foetal bovine serum (FBS) (Thermo Fisher Scientific, Waltham, MA, USA, catalogue number 26140079) in PBS and left for 30 minutes at room temperature.

2.5.2 Primary and Secondary Staining

Primary antibodies (table 2.1) were diluted in a ratio of 1:300 in 2% v/v foetal bovine serum (FBS) in PBS, they were added into the wells and left overnight at 4°C. The list of antibodies used is summarised in Tables 2.1 – 2.2. Primary antibodies were removed, and wells were washed with PBS three times. Secondary antibodies (Table 2.2) were diluted in a ratio of 1:300 with 2% FBS in PBS wash buffer and added into the wells and left at room temperature under foil for one hour. Secondary antibodies were removed, and wells were washed with PBS three times. Following this, a 1:3000 dilution of 4′, 6′-diamidino-2-phenylindole (DAPI) (Thermo Fisher Scientific, Waltham, MA, USA, catalogue number D1306) with 2% v/v FBS in PBS wash buffer were used to stain the cell’s nuclei and incubated at room temperature for ten minutes. The DAPI solution was aspirated, and cells were washed with PBS three times with PBS left in the wells after the third wash, this was to prevent the cells drying out. Blocking solution was used as isotype control as well as secondary only staining, they were carried out in every experiment as
the negative control to ensure there are no non-specific bindings. Immunocytochemistry and phase contrast imaging was carried out using an EVOS™ FL cell imaging system microscope (Thermo Fisher Scientific, Waltham, MA, USA)
Table 2.1 List of primary antibodies used for stemness and differentiation characterisation of Human Induced Pluripotent Stem Cells (hiPSCs) for immunocytochemistry

<table>
<thead>
<tr>
<th>Primary antibody</th>
<th>Manufacturer</th>
<th>Dilution</th>
<th>Marker</th>
<th>Catalogue number</th>
<th>Intracellular or Extracellular</th>
</tr>
</thead>
<tbody>
<tr>
<td>OCT3/4</td>
<td>Abcam (Cambridge, UK)</td>
<td>1:300</td>
<td>Pluripotency</td>
<td>AB238602</td>
<td>Intracellular</td>
</tr>
<tr>
<td>SSEA3</td>
<td>Abcam (Cambridge, UK)</td>
<td>1:300</td>
<td>Pluripotency</td>
<td>AB16286</td>
<td>Extracellular</td>
</tr>
<tr>
<td>NANOG</td>
<td>Abcam (Cambridge, UK)</td>
<td>1:300</td>
<td>Pluripotency</td>
<td>AB238602</td>
<td>Intracellular</td>
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<td>TRA1-81</td>
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<td>AB16288</td>
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<td>SSEA1</td>
<td>Abcam (Cambridge, UK)</td>
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<td>Early differentiation</td>
<td>AB238602</td>
<td>Extracellular</td>
</tr>
<tr>
<td>NESTIN</td>
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<td>1:300</td>
<td>Ectoderm</td>
<td>MA1-110</td>
<td>Intracellular</td>
</tr>
<tr>
<td>βIII TUBULIN</td>
<td>Abcam (Cambridge, UK)</td>
<td>1:300</td>
<td>Endoderm</td>
<td>AB78078</td>
<td>Intracellular</td>
</tr>
<tr>
<td>SOX17</td>
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<td>Endoderm</td>
<td>AB84990</td>
<td>Intracellular</td>
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<tr>
<td>FOXA2</td>
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<td>1:300</td>
<td>Endoderm</td>
<td>AB208376</td>
<td>Intracellular</td>
</tr>
<tr>
<td>AFP</td>
<td>Abcam (Cambridge, UK)</td>
<td>1:300</td>
<td>Endoderm</td>
<td>AB3980</td>
<td>Intracellular</td>
</tr>
<tr>
<td>BRACHYURY</td>
<td>Invitrogen (Paisley, UK)</td>
<td>1:300</td>
<td>Mesoderm</td>
<td>X1AO2</td>
<td>Intracellular</td>
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<tr>
<td>Phalloidin</td>
<td>Invitrogen (Paisley, UK)</td>
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<td>Cytoskeleton</td>
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<td>Intracellular</td>
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<td>Primary antibody</td>
<td>Manufacturer</td>
<td>Dilution</td>
<td>Catalogue number</td>
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<tr>
<td>Alexa Fluor® 488 Goat anti-Mouse IgG</td>
<td>Thermo Fisher Scientific (Waltham, MA, USA)</td>
<td>1:300</td>
<td>A11001</td>
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<td>Alexa Fluor® 488 Goat anti-Mouse IgM</td>
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<td>Thermo Fisher Scientific (Waltham, MA, USA)</td>
<td>1:300</td>
<td>A21044</td>
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### 2.7 Flow Cytometry

#### 2.7.1 Live Staining

To maintain cell viability, as cells were alive this protocol was carried out on ice to keep the cells alive. Media was removed from wells and washed with PBS once. Then 2 mL of TrypLE™ was added and incubated at 37°C for three minutes to dissociate the cells. After 3 minutes, 6 mL of medium was added to inactivate the TrypLE™ and to aspirate and break up cells to obtain a single cell suspension. A sample was taken for cell counting and equally divided into a number of samples required to be separate for staining of multiple markers. Cells were pelleted by centrifugation at 1500 rpm for five minutes at 4°C. The supernatant was removed, and the cell pellet was dispersed by flicking gently. For intracellular markers which required the permeabilisation of cells, 2 mL of 0.1% Triton X-100 (Sigma-Aldrich, Gillingham, UK, catalogue number X100-100ML) in PBS was added to the wells for
ten minutes at 4°C. To wash, cells were pelleted by centrifugation at 1500 rpm for five minutes at 4°C. The supernatant containing triton was removed, and the cell pellet was dispersed by being flicked gently and replaced with 2% FBS in PBS. PBS washes were carried out three times. The desired antibodies (Tables 2.3-2.5) were diluted in a ratio of 1:300 with 2% FBS in PBS wash buffer, 1 mL was added into the tubes and left 4°C for 1 hour at 4°C. Cells were washed by adding 2% FBS in PBS and centrifuged at 1500 rpm for 5 minutes at 4°C. The supernatant was removed, the cell pellet was dispersed by being flicked gently. Cells were washed by adding 2% FBS in PBS wash buffer and spun at 1500 rpm for five minutes at 4°C. Cells were kept in wash buffer suspension. Flow cytometry was carried out using a C6 Accuri flow cytometer (BD Biosciences, San Jose, CA USA). If required after live staining, cells can then be fixed with 4% PFA for 30 minutes at room temperature. To wash, cells were pelleted by centrifugation at 1500 rpm for five minutes at 4°C. The supernatant containing PFA was removed, and the cell pellet was dispersed by being flicked gently and replaced with 2% FBS in PBS. This can be stored at 4°C overnight for next day Flow Cytometry analysis.
Table 2.3 List of conjugated antibodies used for pluripotency and differentiation characterisation of Human Induced Pluripotent Stem Cells (hiPSCs) for Flow Cytometry

<table>
<thead>
<tr>
<th>Primary antibody</th>
<th>Manufacturer</th>
<th>Catalogue number</th>
<th>Dilution</th>
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<tr>
<td>Oct3/4</td>
<td>(BD Biosciences, San Jose, CA USA)</td>
<td>560794</td>
<td>20 µL per 5 x 10^5 cells</td>
</tr>
<tr>
<td>SSEA-4</td>
<td>(BD Biosciences, San Jose, CA USA)</td>
<td>560796</td>
<td>20 µL per 5 x 10^5 cells</td>
</tr>
<tr>
<td>SSEA1</td>
<td>(BD Biosciences, San Jose, CA USA)</td>
<td>560142</td>
<td>20 µL per 5 x 10^5 cells</td>
</tr>
<tr>
<td>SSEA3</td>
<td>BD Biosciences, San Jose, CA USA)</td>
<td>560461</td>
<td>20 µL per 5 x 10^5 cells</td>
</tr>
<tr>
<td>AFP</td>
<td>(BD Biosciences, San Jose, CA USA)</td>
<td>563002</td>
<td>20 µL per 5 x 10^5 cells</td>
</tr>
<tr>
<td>BRACHYURY</td>
<td>(R&amp;D Systems, USA)</td>
<td>IC2085P</td>
<td>20 µL per 5 x 10^5 cells</td>
</tr>
<tr>
<td>SOX17</td>
<td>(BD Biosciences, San Jose, CA USA)</td>
<td>561591</td>
<td>20 µL per 5 x 10^5 cells</td>
</tr>
<tr>
<td>βIII TUBULIN</td>
<td>(BD Biosciences, San Jose, CA USA)</td>
<td>560394</td>
<td>20 µL per 5 x 10^5 cells</td>
</tr>
<tr>
<td>PerCP-Cy5.5 Mouse IgG1, κ Isotype Control</td>
<td>(BD Biosciences, San Jose, CA USA)</td>
<td>552834</td>
<td>20 µL per 5 x 10^5 cells</td>
</tr>
<tr>
<td>PE Goat IgG Isotype Control</td>
<td>(R&amp;D Systems, USA)</td>
<td>IC108P</td>
<td>20 µL per 5 x 10^5 cells</td>
</tr>
<tr>
<td>PE Mouse IgG2a, K Isotype Control</td>
<td>(BD Biosciences, San Jose, CA USA)</td>
<td>558595</td>
<td>20 µL per 5 x 10^5 cells</td>
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<tr>
<td>PE Mouse IgG1, K Isotype Control</td>
<td>BD Biosciences, San Jose, CA USA)</td>
<td>554680</td>
<td>20 µL per 5 x 10^5 cells</td>
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<tr>
<td>PE Mouse IgM, K Isotype Control</td>
<td>(BD Biosciences, San Jose, CA USA)</td>
<td>555584</td>
<td>20 µL per 5 x 10^5 cells</td>
</tr>
<tr>
<td>Alexa Fluor® 647 Mouse IgG3, κ Isotype Control</td>
<td>(BD Biosciences, San Jose, CA USA)</td>
<td>560803</td>
<td>20 µL per 5 x 10^5 cells</td>
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</tbody>
</table>
2.8 Quantitative Polymerase Chain Reaction (RT-qPCR)

2.8.1 RNA Extraction

RNA extraction was carried out according to manufacturer’s instructions, using the RNeasy Micro kit (Qiagen, Netherlands, catalogue number 74004). Briefly:

1. 350 µl of RLT buffer was added to pelleted cells. The lysate was transferred into a Qiashredder column (Qiagen, Netherlands, catalogue number 79654) and centrifuged at 15000 rpm for two minutes. 350 µL of 70% Ethanol was added to the homogenised lysate in the collection tube and mixed thoroughly by pipetting.

2. The 700µL mixture was transferred to a RNeasy spin column (RNeasy Micro kit, Qiagen, Netherlands, catalogue number 74004) and centrifuged for 15 seconds at 10,000 rpm. The flow-through in the collection tube was discarded and replaced with a new collection tube.

3. Then 350 µL buffer RW1 (RNeasy Micro kit, Qiagen, Netherlands, catalogue number 74004) was added to the RNeasy spin column and centrifuged for 15 seconds at 10,000 rpm, the flow-through in the collection tube was discarded and replaced with a new collection tube.

4. A stock solution of 10µL DNAse 1 to 70µL buffer RDD was made prior. The 80µL of DNAse and buffer RDD mix was added to RNeasy spin column and left to incubate at room temperature for 15 minutes.
5. After incubation, 350µL of buffer RW1 was added to the RNeasy spin column and centrifuged for 15 seconds at 10,000 rpm. The flow-through in the collection tube was discarded and replaced.

6. 500µL of buffer RPE was added to RNeasy spin column and centrifuged for 15 seconds at 10,000 rpm; the flow-through was discarded. 500µL of buffer RPE was added to RNeasy spin column and centrifuged for two minutes at 10,000 rpm. The flow-through was discarded and the collection tube replaced. The RNeasy spin column was centrifuged for an additional 1 minute at full speed. The collection tube was discarded and replaced with new a 1.5mL collection tube (RNeasy Micro kit, Qiagen, Netherlands, catalogue number 74004).

7. Then 40µL of RNase-free water (RNeasy Micro kit, Qiagen, Netherlands, catalogue number 74004) was added to the RNeasy spin column membrane and centrifuged for 1 minute at 10,000 rpm. The RNeasy spin columns were discarded, and the RNA samples diluted in RNase-free water were collected in the collection tubes and placed on ice. RNA concentration measurement was carried out using the Nanodrop ND-1000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). RNA samples diluted in RNase-free water were stored in -80°C.

2.8.2 Nanodrop Spectrophotometer

A Nanodrop ND-1000 spectrophotometer was used to carry out measurements of RNA concentrations. To measure, 1 µL of RNase-free water was used to blank the machine prior to measuring samples. Then 1 µL of each RNA
template samples were dispensed onto the reader and recorded. The nanodrop spectrophotometer carried out measurements via absorbance at wavelengths of 260/280nm. The RNA templates were placed on ice for cDNA synthesis or stored at -80°C.

2.8.3 cDNA Synthesis: Genomic DNA Elimination and Reverse Transcriptase Polymerase Chain Reaction

Prior to cDNA synthesis, the RNA concentration readings carried out via the spectrophotometer and the volume required of RNA template for 1 µg of RNA were calculated and pipetted. This was carried out using the QuantiTect Reverse Transcription Kit (Qiagen, Netherlands, catalogue number 205310) according to manufacturer’s instructions.

1. The RNA template samples, reverse transcriptase (QuantiTect Reverse Transcription Kit Qiagen, Netherlands, catalogue number 205310), RT buffer (QuantiTect Reverse Transcription Kit Qiagen, Netherlands, catalogue number 205310), RNase-free water (QuantiTect Reverse Transcription Kit Qiagen, Netherlands, catalogue number 205310) and gDNA wipeout buffer (QuantiTect Reverse Transcription Kit Qiagen, Netherlands, catalogue number 205310) were thawed on ice.

2. Genomic DNA elimination reaction was carried out on ice. The stock solution consisted of gDNA wipeout buffer, RNase-free water and template RNA were made and aliquoted to reduce pipetting inaccuracy. (For six genes across five samples: 0.2kPa, 2kPa, 16kPa, 64kPa, TCP and no reverse transcriptase controls, a stock solution
was made for 35 reaction wells with an addition of 10% volume). For each reaction well 70µL Eppendorf tubes (Eppendorf, Hamburg, Germany, catalogue number 0030120086) were used. For the genomic DNA elimination reaction, each sample was made up to 14µL containing: 2µL gDNA wipeout buffer, x µL template RNA (where x is the volume required RNA template for 1mg RNA) and 12-x µL of RNase-free water (to reach a desired total volume of 14µL). The Eppendorf tubes were centrifuged at 10,000 rpm for five seconds and transferred to the T100 thermocycler (Bio-Rad Laboratories, Hercules, CA, USA) to be incubated at 42°C for two minutes.

3. For reverse transcription, a stock solution of master mix consisted of: 1µL reverse transcriptase, 4µL RT buffer and 1µL RT primer mix were made volume to reduce pipetting inaccuracy. (For six genes across five samples: 0.2kPa, 2kPa, 16kPa, 64kPa and TCP, a stock solution was prepared for 30 reaction wells with an addition of 10% volume).

4. An additional stock solution of a master mix without reverse transcriptase was also made up for each sample as no reverse transcriptase control (for five samples: 0.2kPa, 2kPa, 16kPa, 64kPa and TCP; a stock of 5 reaction wells were made with an additional 10% volume). A total volume of 20µL was made by dispensing 6µL of the reverse transcription master mix into each of the Eppendorf tubes containing the 14µL gDNA elimination reaction mixture made prior.

5. The tubes were centrifuged at 10,000 rpm for 5 seconds and transferred to the T100 thermocycler to be incubated at 42°C for 15
minutes, followed by incubation at 95°C for three minutes. The cDNA samples were placed on ice for the PCR plate setup or stored at -20°C.

2.8.4 PCR plate setup

Quantitect SYBRGreen (Qiagen, Netherlands, catalogue number 204145), cDNA, Quantitech pre-validated primer assays (Qiagen, Netherlands) and RNase-free water were thawed on ice and prepared according to manufacturer’s instructions.

1. Quantitect Mastermix stock solution of each gene was prepared, which consisted of 37.5µL Quantitech pre-validated primers (Table 2.6), 75µL RNase free water and 187.5µL SYBRGreen.

2. Then 15µL of the master mix was dispensed into each reaction well (for a full 96 well PCR plate, a total of 12 reaction wells were required per gene). Subsequently, 5µL of sample cDNA was aliquoted into the wells of the Hard-Shell Low Profile Thin-Wall 96 Well Skirted PCR plates (Bio-Rad Laboratories, Hercules, CA, USA, catalogue number HSP9601), and the plate was covered with plastic film cover (Bio-Rad Laboratories, Hercules, CA, USA, catalogue number MSC1001) and centrifuged at 4000 rpm for 2 minutes to remove all air bubbles.

3. The PCR plate was placed into a CFX Connect Real-time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA) for analysis using the “quantitect” protocol. Internal control genes candidates were investigated before in this laboratory and based on those previous studies, all screenings were carried out along with β-
Actin as the house-keeping gene. Each reaction plate also contains no reverse transcriptase control (NRT) and no template control (NTC) to make sure the samples are not contaminated or to detect the presence of primer dimer formation.

2.8.5 Quantification

Quantitative results were exported from the CFX Manager 3.0 (Bio-Rad Laboratories, Hercules, CA, USA) and fold change calculations were done using Microsoft Excel (Microsoft, Redmond, WA, USA) using the $2^{-\Delta\Delta Ct}$ calculation (Pfaffl, 2001).

Where

$$\Delta\Delta Ct = [(Ct \text{ gene of interest sample A} - Ct \text{ control sample A})$$
$$- (Ct \text{ gene of interest sample B} - Ct \text{ control sample B})]$$

(Ct refers to the threshold cycle, the number of PCR cycles required for the threshold to be overcome by the sample. Raw Ct values were all first ‘corrected’ by normalising them against the housekeeping gene (β-actin). They were subtracted by the corrected average of the control Ct result of that gene.)
2.8.6 Statistical Analysis

Statistical analysis was carried out using the PRISM GraphPad software (San Diego, CA, USA). Two-way ANOVA were used to analyse RT-qPCR results based on how many variables were involved in the experiment. Results where \( p<0.05 \) (*) values are accepted as significant, \( p<0.01 \) (**) as very significant,
p<0.001 (*** as highly significant and p<0.0001 (****) as extremely significant. All experiments were carried out in biological triplicates.

2.9 Mycoplasma and Karyotyping

Both cell lines MSU001 and Cord Blood hiPSCs were routinely sent for mycoplasma testing (Surrey Diagnostics, Cranleigh, UK). They were both also sent for karyotyping using G banding, and up to 20 cells were analysed (Figures 2.1 and 2.2). After all experiments were conducted, the cell samples of late passages were sent for karyotype abnormalities. Karyotype abnormalities testing was carried out by Cell Guidance System (Babraham Research Campus, Cambridge, UK).
Figure 2.1 hiPSCs (cord blood) karyotype.

(A) passage 40, (B) at passage 63 indicated normal karyotype. Normal G banded chromosomes for all human iPSCs samples showing 46, XX karyotype with no visual chromosomal aberrations in samples of 20 cells examined.
Figure 2.2 hiPSCs (MSU001) karyotype.

Passage 30 indicated normal karyotype. Normal G banded chromosomes for all human iPSCs samples showing 46, XX karyotype with no visual chromosomal aberrations in samples of 20 cells examined.
2.10 Substrates

In-house production of extracellular substrates was attempted. A summary can be seen in Table 2.5.

<table>
<thead>
<tr>
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<th>In-house production</th>
<th>Catalogue number</th>
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<tr>
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<td>In-house production</td>
<td></td>
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<tr>
<td>Polydimethylsiloxane (PDMS)</td>
<td>In-house production</td>
<td></td>
</tr>
<tr>
<td>Poly-ε-Lysine (PεK) polymer</td>
<td>In-house production</td>
<td></td>
</tr>
<tr>
<td>Excellness</td>
<td>Commercially available from Excellness LTD (Lausanne, Switzerland)</td>
<td>SSL16-EC EA</td>
</tr>
<tr>
<td>Softwell</td>
<td>Commercially available from by Matrigen (Brea, CA, USA)</td>
<td>SW6-EC-12EA</td>
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<tr>
<td>Cytosoft</td>
<td>Commercially available from Advanced Biomatrix (San Diego, CA, USA)</td>
<td>5141-5EA, 5165-5EA, 5145-5EA</td>
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</tbody>
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2.10.1 Gelatin Crossedlinked with Glutaraldehyde (GXG)

GXG protocol was adapted from Ali et al., (Ali et al., 2015a) and Al-Rekabi et al., (Al-Rekabi and Pelling, 2013).

2.10.1.1 Preparation of GXG stock solution

Gelatin powder (Sigma-Aldrich, Gillingham, UK, catalogue number G1890) was weighed out in 3, 4 or 6% w/v (in correspondence to grams of gelatin in 1L solution). Gelatin powder was dissolved in Milli-Qwater. The solutions were autoclaved and stored at 4°C.

2.10.1.2 Preparation of GXG matrix

The gelatin stock solution was placed in a 37°C water bath for 15 minutes. 15mL of the stock solution was transferred to a tube, and 5 μL/mL glutaraldehyde (Sigma-Aldrich, Gillingham, UK, catalogue number G7651) was added to the
solution. The mixture was vortexed, and 1 mL was added to each well of a 6-well plate. The GXG coated plates were left overnight in a humidity chamber at 4°C. The next day, the plates were washed three times with PBS. As glutaraldehyde is toxic, sodium borohydride (Sigma-Aldrich, Gillingham, UK, catalogue number 452882-25) was used to react with the excess glutaraldehyde. Sodium borohydride powder was dissolved in PBS over ice at a concentration of 1 mg/mL of PBS. Then 2.25 mL of the diluted sodium borohydride solution was added to each well of the 6-well plate (0.25 mL per cm²) using a syringe and a 0.40 µm filter. The well-plates were kept on ice for an hour while reduction occurs. The GXG matrix was washed vigorously with PBS for five washes or until all the bubbles have dissipated. The wells were kept in PBS and stored overnight at 4°C in the humidity chamber. The next morning, they were washed three times with PBS, cell culture media was added to the wells and incubated for four hours prior to the start of the experiment to equilibrate.

After four hours, the media was aspirated, and the matrices were coated with vitronectin solution for an hour. Finally, the vitronectin solution was removed, and cells were seeded onto the matrices.

2.10.2 Polydimethylsiloxane (PDMS)

The PDMS protocol was adapted from Evans et al., (Evans et al., 2009), Ahmed et al., (Ahmed et al., 2011) and Brown et al., (Brown et al., 2006). The substrate was produced by combining the curing agent elastomer (Sigma-Aldrich, Gillingham, UK, catalogue number 761028) with Sylgard 184 (Sigma-Aldrich, Gillingham, UK, catalogue number 761028) in various ratios to form matrices with different Young's moduli. The ratios used were 1:9, 1:20, 1:30, 1:40, 1:50 and 1:60.
Once the elastomer and Sylgard 184 had been thoroughly mixed, they were placed in a vacuum chamber and left for 30 minutes (or until all air bubbles had been removed). The PDMS mixture was carefully transferred into 6-well plates and left to cure in an oven at 60°C overnight or until solidified (depending on component ratios). Once the cross-links had taken place, the PDMS 6-well plate was removed from the oven and placed under a UV lamp for 2 hours to sterilise. After the wells were washed with PBS three times, 2 mL of fresh E8 media was dispensed into each well and left for four hours in the 37°C, 5% v/v CO₂ incubator for equilibration. After four hours E8 media was removed, and the PDMS 6-well plates were coated with vitronectin prior to cell seeding.

2.10.3 Cytosoft

Silicon-based polymer, Cytosoft was provided by Advanced Biomatrix (San Diego, CA, USA) in 6-well plates with Young's modulus values of 0.2kPa, 0.5kPa, 2kPa, 8kPa, 16kPa, 32kPa and 64kPa.

2.10.4 Poly-ε-Lysine (PεK) polymer

Protocol was shown by Spheritech Ltd (Runcorn, UK)(Gallagher et al., 2016).

2.10.4.1 Preparation of PεK stock solution.

20.45g of PεK powder (Zhengzhou Bainafo bioengineering Co., Ltd., Zhengzhou city, Henan, China, catalogue number 28211) was weighed out in a 100mL glass bottle. Then 70 mL of water was added into PεK and put on a roller to dissolve completely. Subsequently, 12.2 g of Acid 1 (Brasilic acid) (VWR International Ltd., Leicestershire, UK, catalogue number U6015G) and 1.22g of Acid 2 (Sabasic acid) (Alfa Aesar, Lancashire, UK, catalogue number A14158) was
weighed out and placed in another 100mL glass bottle followed by the addition of 10mL of water and 12.8mL of 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide HCl (Carbosynth, Berkshire, UK, catalogue number FD05800) before being placed onto a sonicator.

Once the PεK is dissolved, pH was measured using a pH probe, and drops of 5.0M Sodium Hydroxide (Sigma-Aldrich, Gillingham, UK, catalogue number 221465) were added until the pH level is 7.1. The pH 7.1 PεK solution was poured into a 100mL measuring cylinder and topped up with water to 100mL. This solution was filtered using a syringe (Fisher Scientific, Waltham, MA, USA, catalogue number S7510) and 0.45µm filter (Merck Millipore, Billerica, MA, USA, catalogue number HAWP04700) into a new sterile bottle. Once the di-acid solution was dissolved, it was transferred into a clean 100mL measuring cylinder and again topped up with water to reach 100mL. This solution was also filtered into a new sterile bottle using a syringe and 0.45µm filter. Meanwhile, 5% Tween 80 (Sigma-Aldrich, Gillingham, UK, catalogue number 9005656) solution was made with sterile water. Stock solutions were stored at room temperature for up to two weeks.

2.10.4.2 Preparation of PεK polymer

Prior to the preparation of PεK polymer, Microsoft Excel (Microsoft, Redmond, WA, USA) 2 was used to calculate based on existing calculation, the volume of PεK, acid 1, acid 2 and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide HCl required to achieve various Young’s moduli. The components where weighed where 690 mg of N-Hydroxysuccinimide (NHS) (Carbosynth, Berkshire, UK, catalogue number FC19777) and 6 g of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDCI) (Sigma-Aldrich, Gillingham, UK,
catalogue number 03449) were weighed out in separate tubes. PεK from stock solution and 11.3 mL di-acid stock solution were measured out into a new separate falcon tube. 40 µL 5% tween80 was added into the PεK and di-acid solution. The PεK, di-acid and tween80 solution were topped up with distilled water until it reached half of the desired total. 2 mL of water was added into the EDCI tube and NHS tube; the tubes were shaken to make sure they dissolved completely. The EDCI solution was filtered using a syringe and 0.45µm filter. The same syringe and 0.40 µm filter were used to filter the NHS solution into the falcon tube that contains the EDCI solution. The solution was topped up with distilled water until it reached half of the desired total volume. As the solution reacted within five minutes the next step was performed straight away.

The polymer was made by mixing the EDCI and NHS solution with the PεK, di-acid and tween80 solution and tube was inverted gently 3-5 times to mix the solution thoroughly. The mixed solution was swiftly poured onto the mould surface and check that there were no air bubbles. The polymer was left to set for at least 5 hours or overnight at room temperature.

2.10.4.3 Washing protocol

After the polymers were left for at least 5 hours to set, they were washed with water and left to soak. Polymers were soaked for five minutes during each washing step, for glass slides and 15 minutes for big sheets. The water was aspirated and replaced with 0.25M NaOH and left to soak. Water was added and left to soak before being aspirated. The above steps were repeated (total of two washes) and water was left in the mould after the second time.
2.10.4.4 Cell Seeding

Prior to cell seeding, the polymer was soaked in 70% ethanol to sterilise for 15 minutes. Ethanol was aspirated, and the polymer was washed three times with PBS. Cell culture media was added and left overnight in the incubator to equilibrate the polymer. After equilibrium, media was removed, and a vitronectin solution was used to coat the polymer for an hour before cells were seeded.

2.10.4.5 Salinising glass

The PεK was produced on glass, once it solidified it was then removed from glass and put into a 6-well plate. The salinising glass process was applied to the glass so that the polymer attached itself to the glass. Glass surfaces were soaked with 0.25 M/L of hydrochloric acid (HCl) (Sigma-Aldrich, Gillingham, UK, catalogue number 258148) and placed on a shaking plate for five minutes. Hydrochloric acid was removed, and water was added to soak the glass surfaces on a shaking plate for five minutes. Water was replaced with acetone and left on a shaking plate. The acetone (Sigma-Aldrich, Gillingham, UK, catalogue number 179124) were aspirated, and the glass surfaces were dried using hot air. Glass surfaces were soaked with 2% v/v Silane (Sigma-Aldrich, Gillingham, UK, catalogue number 7803625) in acetone and left on a shaking plate for five minutes. 2% v/v Silane solution was removed, and water was added to soak the glass surfaces and left on shaking plate for five minutes. Water was replaced with acetone and left on the shaking plate for five minutes. Acetone was removed, and glass surfaces were dried with hot air and kept at room temperature.
2.10.5 Excellness

Excellness hydrogels were obtained from Excellness LTD (Lausanne, Switzerland) in 24 well plates and 35 mm petri dishes with Young’s modulus values of 2kPa, 16kPa, 25kPa and 64kPa.

2.10.6 Softwell

Softwell hydrogels were provided by Matrigen (Brea, CA, USA) in 24 well plates and 16 well glass chamber slides with Young’s modulus of 0.2kPA, 4kPa, 25kPa and 50kPa.

2.11 Atomic force microscope (AFM)

AFM measurements were carried out in fluid with a JPK Nanowizard 1 (JPK Instruments, Berlin, Germany) mounted on an Olympus IX71 inverted microscope (Olympus, Tokyo, Japan). MLCT cantilevers (Bruker, Camarillo, CA, USA, catalogue number MLCT) with a spring constant of 0.01N/m and a tip radius of 0.55µm were used for contact mode. The force measurements were carried out at room temperature.

2.11.1 Beads attachment

As substrate may be soft, beads attachment is required to prevent cantilever piercing through the substrates (Figure 2.3). Cantilever calibration must be carried out prior to beads attachment, this is to measure the spring constants of the cantilever for accurate and reliable results. The desired cantilever was mounted on the fluid cell, and the AFM was set up following the manufacturer’s protocol. A glass slide with a drop of UV curing glue (Henkel, Düsseldorf, Germany) on one edge and desired beads (15µm radius) on the other edge was mounted on the AFM
microscope. The cantilever was lowered slowly until the tip of the cantilever contacted the glue and was elevated right after. Once the cantilever tip had been covered in glue, the glass slide was adjusted to locate a lone bead. The bead was carefully positioned directly below the cantilever tip; the cantilever was lowered 5µm at a time until the glued cantilever tip contacted the bead. Once contact was made, the cantilever was removed and placed under UV light for curing (JPK Instruments, 2012).

(i)

Figure 2.3 Diagram showing the application of Atomic Force Microscope (AFM).
(i) Bead attachment on cantilever tip and (ii) how Young’s modulus value of substrate is measured using the Atomic Force Microscope (AFM).
2.11.2 Calculations of AFM measurements

The force-displacement curves produced by AFM were used to calculate Young's modulus of the sample by using the Hertz mode (Hertz, 1882; Borodich, 1993):

\[ F = \frac{4}{3} \frac{E}{1 - \nu^2} \sqrt{R \delta^2} \]

Where \( F \) is the indentation force, \( E \) is Young's modulus, \( \nu \) is the Poisson ratio (0.5 for soft biological materials) (Vinckier and Semenza, 1998), \( R \) is the radius of the indenting sphere (15\( \mu \)m), and \( \delta \) is the indentation depth. Calculations were carried out using the JPK Data processing software (JPK Instruments, Berlin, Germany).
Chapter 3: Characterisation of Human Induced Pluripotent Stem Cells (hiPSCs)

3.1 Introduction

Human induced pluripotent stem cells (hiPSCs) are classified as pluripotent stem cells. iPSCs are somatic cells reprogrammed back into a stem cell-like state using four transcription factors known as Yamanaka's factors (Takahashi and Yamanaka, 2006). These cells are capable of proliferation, self-renew and differentiation into the three germ layers (Mesoderm, Endoderm and Ectoderm) (Kannagi et al., 1983; Andrews et al., 1984; Carpenter, Rosler and Rao, 2003; Hoffman and Carpenter, 2005). The hiPSCs were originally reprogrammed and cultured on inactivated mouse fibroblast (MEFs) (Evans and Kaufman, 1981; Martin, 1981; Thomson et al., 1998) provides an extracellular matrix support and secretion of growth factors for pluripotency maintenance is needed. However, there has been a significant shift away from animal-derived materials due to contamination concerns and most of the protocols have been adapted to become xeno-free. Cell lines have also been adapted to feeder-free environment (Nakagawa et al., 2014). Xeno-free cell culturing is also preferred in Good Manufacturing Practice (GMP) for human clinical trials (Sjögren-Jansson et al., 2005; Klimanskaya et al., 2006; Ludwig, Bergendahl et al., 2006; Akopian et al., 2010; Steiner et al., 2010).

To ensure that pluripotency is maintained during cell culture, it is important to prove the pluripotency before the experiments are conducted. Methods for such characterisation are confirmation of the stemness markers, ability to differentiate
and form embryoid bodies (EBs), teratoma formation, chimera production, DNA fingerprinting, genome analysis and mycoplasma testing (Till and McCulloch, 1980; Evans and Kaufman, 1981; Martin, 1981; Potten and Loeffler, 1990; Thomson et al., 1998; Amit et al., 2000; Crook, Hei and Stacey, 2010).

For this thesis, feeder-dependent hiPSCs were adapted to feeder-free cultures in-house by Dr Rana Khalife. Characterisation of the cell lines was carried out using immunocytochemistry, flow cytometry and their ability to differentiate into the three-germ layers via EBs formation and monolayer culture. The passages used for each cell line were between passages 39-60 (Cord blood) and 28-30 (MSU001).

3.2 Aims and Objectives

This chapter aims to characterise the feeder-free human induced pluripotent stem cell lines and ensure that pluripotency was maintained. The detailed objectives are:

- To Assess stemness of hiPSCs (Cord blood) and hiPSCs (MSU001) by:
  1. Immunostaining the pluripotency markers: OCT4, SSEA3, SSEA4, TRA160 and Tra181.

- Ensure the ability of both hiPSCs line’s differentiation ability to all the three germ layers by:
  1. Single cells in suspension spontaneous differentiation via immunocytochemistry
2. Embryoid bodies spontaneous differentiation via immunocytochemistry

3.3 Results

3.3.1 Morphological Analysis of Human Induced Pluripotent Stem Cells (hiPSCs) in their Undifferentiated State

In this thesis, all hiPSCs used are feeder-free cultures. The hiPSCs (cord blood) was purchased as feeder-free cultures and hiPSCs (MSU001) were adapted to a feeder-free culture in-house. Both cell lines were expanded and stored in liquid nitrogen as cell bank for the laboratory. The cells were cultured in vitronectin coated T25 flask using Essential 8™ Medium and were passaged every 4 days. In their undifferentiated state, hiPSCs have a specific morphology: hiPSCs colonies consisted of small round cells with a high nucleus to cytoplasm ratio, they grew in tightly packed colonies with well-defined edges (Healy and Ruban, 2015). Figure 3.1 shows the phase contrast images of hiPSCs Cord Blood (i) and MSU001 (ii), which showed typical stem cell morphology.

Figure 3.1 Phase contrast of Cord Blood hiPSCs colonies.
(i) hiPSCs (cord blood) (ii) hiPSCs (cord (MSU001) showing hiPSCs colonies consisted of small round cells with a high nucleus to cytoplasm ratio, they grew in tightly packed colonies with well-defined edges. All scale bars = 400µm. Images were taken at 100X ocular lens magnification using Evos® XL magnification using Evos® XL.
3.3.2 Characterisation of Human Induced Pluripotent Stem Cells (hiPSCs) by immunocytochemistry

Immunocytochemistry was performed to determine the presence and expression of pluripotency markers. Once the cell culture reached 80% confluency, the cells were fixed, and stained according to the protocol mentioned in Materials and Methods (Section 2.5). The markers chosen for characterisation were pluripotency markers of OCT4, TRA1-81, SSEA3 and early differentiation marker of SSEA1 (data not shown). DAPI was also used to identify the cell’s nucleus and distinguish the marker location in relation to the nucleus. These images (Figure 3.2-3.3) demonstrate a positive staining for intracellular staining OCT4 and extracellular staining: SSEA3, TRA160 and TRA1-81. Both cell lines exhibit positive expression for the pluripotency markers and negative results for SSEA1. This indicates that the cells cultured are pluripotent by showing stemness. Pluripotency marker TRA1-81 and TRA160 was used on hiPSCs (cord blood) and hiPSCs (MSU001) respectively and not on both due to availability of the antibodies at the time when assays were carried out.
Figure 3.2 Immunocytochemistry analysis of hiPSCs (cord blood) for cell characterisation.

(A) Pluripotency markers were tested: (i) phase contrast (ii) TRA1-81 (iii) DAPI. (B) Pluripotency marker SSEA3: (i) phase contrast (ii) SSEA3 (iii) DAPI. (C) Pluripotency marker OCT4: (i) phase contrast (ii) OCT4 (iii) DAPI. (D) Secondary only staining was carried out as control: (i) secondary only mouse IgG (ii) secondary only mouse IgM. All scale bars = 400µm. Images were taken at 100X ocular lens magnification using Evos® XL.
Figure 3.3 Immunocytochemistry analysis of hiPSCs (MSU001) for cell characterisation.

(A) Pluripotency marker OCT4: (i) phase contrast (ii) OCT4 (iii) DAPI. (B) Pluripotency marker TRA160: (i) phase contrast (ii) TRA160 (iii) DAPI. (C) Pluripotency marker SSEA3: (i) phase contrast (ii) SSEA3 (iii) DAPI

(D) Secondary only staining was carried out as control: (i) secondary only mouse IgG (ii) secondary only mouse IgM. All scale bars = 400µm. Images were taken at 100X ocular lens magnification using Evos® XL.
3.3.3 Characterisation of Human Induced Pluripotent Stem Cells (hiPSCs) by Flow Cytometry

Flow cytometry was used to assess pluripotency quantitatively. The markers OCT4, SSEA4 and SSEA1 antibodies were used to stain the hiPSCs. Flow cytometry graphs were gated using isotype as negative controls to identify any non-specific binding of the antibodies (Herzenberg et al., 2006). Figure 3.4 shows hiPSCs (Cord Blood) where 98.2% of cells were positive for SSEA4, 99.4% positive for OCT4 and 0.1% positive for SSEA1. hiPSCs (MSU001) (Figure 3.5) also showed similar results of 82.5% and 88.3% positive for SSEA4 and OCT4 and 0.1% positive for SSEA1. These results correlate with the immunocytochemistry data and confirm that the cells are pluripotent.
Figure 3.4: Flow cytometry analysis hiPSCs (cord blood).

Showing positive for pluripotency markers OCT 4 and SSEA3/4 and negative for early differentiation marker SSEA1. (A) (i) OCT4 Isotype (ii) OCT4, (B) (i) SSEA3/4 Isotype (ii) SSEA3/4 (C) (i) SSEA1 Isotype and (ii) SSEA1. Gating carried out based on isotype controls.
Figure 3.5 Flow cytometry analysis hiPSCs (MSU001).

Showing positive for pluripotency markers OCT4 and SSEA3/4 and negative for early differentiation marker SSEA1. (A) (i) OCT4 Isotype (ii) OCT4, (B) (i) SSEA3/4 Isotype (ii) SSEA3/4 (C) (i) SSEA1 Isotype and (ii) SSEA1.
3.3.4 Characterisation of Human Induced Pluripotent Stem Cells (hiPSCs) by Embryoid Bodies (EBs) Aggregates Spontaneous Differentiation

Another method to characterise the hiPSCs is to confirm the hiPSCs’ potential to form embryoid bodies (EBs) and subsequently differentiate into a mixture of all the three germ layers within the aggregates *in-vitro* (Itskovitz-Eldor *et al.*, 2000). Both hiPSCs lines were used to form EBs using AggreWell™ plates. After 24 hours, the EBs were harvested and seeded onto a TCP 6-well plate coated with Matrigel. Cells were grown for ten days to allow them to differentiate into the three germ layers, with phase contrast images were taken daily. After ten days, the cells were fixed, and immunocytochemistry analysis was carried out for the three germ layers: *BRACHYURY* (mesoderm), *NESTIN* (ectoderm) and *SOX17* (endoderm).

The formation of EBs is shown in Figure 3.6. Figure 3.7-3.8 showed the immunocytochemistry images of the three germ layers, *BRACHYURY* (mesoderm), *SOX17* (endoderm) and *NESTIN* (ectoderm). These images validate that the differentiated cells plated from the EBs were positive for all three germ layers markers. This proved that the hiPSCs were able to form EBs as well as differentiate into cells within the three germ layers.
Figure 3.6 hiPSCs differentiation EB formation using AggreWell™ plates.

(i) day 0 (ii) day 1. *Images shown of Cord blood EBs, the same procedure was carried out for all cell lines. (B) Phase contrast images of EBs produced from hiPSCs (cord blood) seeded on matrigel coated 6-well plate at and allowed to spontaneously differentiate using hPSCs media (i) day 0 (ii) day 4 (ii) day 8. (C) Phase contrast images of EBs produced from hiPSCs (MSU001) seeded on matrigel coated 6-well plate at and allowed to spontaneously differentiate using hPSCs media (i) day 0 (ii) day 4 (ii) day 8. All scale bars = 400µm. Images were taken at 100X ocular lens magnification using Evos® XL.
Figure 3.7 images of hiPSCs (cord blood) differentiation via EB formation using AggreWell™ plates

(A) Immunocytochemistry images of fixed EBs seeded on 6-well plates stained for endoderm germ layer marker: (i) phase contrast (ii) SOX17 (iii) DAPI. (B) Immunocytochemistry staining for ectoderm germ layer: (i) phase contrast (ii) NESTIN (iii) DAPI. (C) Immunocytochemistry staining for mesoderm germ layer: (i) phase contrast (ii) BRACHYURY (iii) DAPI. (D) Secondary only staining was carried out as control: (i) secondary only mouse IgG (ii) secondary only mouse IgM. All scale bars = 400µm. Images were taken at 100X ocular lens magnification using Evos® XL.
Figure 3.8 images of hiPSCs (MSU001) differentiation via EB formation using AggreWell™ plates.

(A) Immunocytochemistry staining for ectoderm germ layer: (i) phase contrast (ii) NESTIN (iii) DAPI. (B) Immunocytochemistry images of fixed EBs seeded on 6-well plates stained for endoderm germ layer marker: (i) phase contrast (ii) SOX17 (iii) DAPI. (C) Immunocytochemistry staining for mesoderm germ layer: (i) phase contrast (ii) BRACHYURY (iii) DAPI. (D) Secondary only staining was carried out as control: (i) secondary only mouse IgG (ii) secondary only mouse IgM. All scale bars = 400µm. Images were taken at 100X ocular lens magnification using Evos® XL.
3.3.5 Characterisation of Human Induced Pluripotent Stem Cells (hiPSCs) by Monolayer Spontaneous Differentiation

Monolayer differentiation using single cells in suspension is an alternative to EB formation as multiple differentiation protocols uses this method for various cell type including cardiomyocytes (Batalov and Feinberg, 2015), osteoblasts (Maeno et al., 2005), smooth muscle cells (Huang et al., 2006), retinal pigment epithelium cells (Idelson et al., 2009) and hepatocytes (Hay et al., 2007). This experiment was carried out to investigate the spontaneous differentiation capabilities of single cells in suspension and to determine if cells differentiate into the three germ layers. It was carried out as part of hiPSCs characterisation. Cord blood and MSU001 hiPSCs were dissociated into single cells and seeded at a density of 3x10^5 cells per well of Matrigel-coated 6-well plate.

Figure 3.9 shows the phase contrast of the single cells in suspension from 3-day differentiation when the culture was confluent prior to fixation for immunocytochemistry. Figure 3.9-3.10 shows the immunocytochemistry analysis from monolayer spontaneous differentiation. The markers used for the three germ layers were BRACHYURY for mesoderm, SOX17 for endoderm and NESTIN for ectoderm. The immunocytochemistry images show the cells were positive for all three germ layers. This proves that the hiPSCs were able to differentiate into cells within the three germ layers in monolater conditions.
Figure 3.9 Images of hiPSCs (Cord blood) monolayer on Matrigel.

(A) Immunocytochemistry images of fixed monolayer seeded on 6-well plates allowed to spontaneously differentiate using hiPSCs media stained for endoderm germ layer marker: (i) phase contrast (ii) SOX17 (iii) DAPI.  (B) Immunocytochemistry staining for ectoderm germ layer: (i) phase contrast (ii) NESTIN (iii) DAPI.  (C) Immunocytochemistry staining for mesoderm germ layer: (i) phase contrast (ii) BRACHYURY (iii) DAPI.  (D) Secondary only staining was carried out as control: (i) secondary only mouse IgG (ii) secondary only mouse IgM.  All scale bars = 400µm.  Images were taken at 100X ocular lens magnification using Evos® XL.
Figure 3.10 Images of hiPSCs (MSU001) monolayer on Matrigel.

(A) Immunocytochemistry images of fixed single cells in suspension seeded on 6-well plates allowed to spontaneously differentiate using hiPSCs media stained for endoderm germ layer marker: (i) phase contrast (ii) SOX17 (iii) DAPI. (B) Immunocytochemistry staining for ectoderm germ layer: (i) phase contrast (ii) NESTIN (iii) DAPI. (C) Immunocytochemistry staining for mesoderm germ layer: (i) phase contrast (ii) BRACHYURY (iii) DAPI. All scale bars = 400µm. (D) Secondary only staining was carried out as control: (i) secondary only mouse IgG (ii) secondary only mouse IgM. All scale bars = 400µm. Images were taken at 100X ocular lens magnification using Evos® XL.
3.3.6 Discussion

It is important to characterise a stem cell line prior to any experiments as they are prone to differentiation in culture (Geraghty et al., 2014; International Stem Cell Initiative, 2018). Pluripotent stem cells should exhibit the same pluripotent and differentiation markers despite their minor differences in their morphology and growth profiles (Hoffman and Carpenter, 2005; Adewumi et al., 2007; Skottman, Narkilahti and Hovatta, 2007).

We have shown that both hiPSCs (cord blood between passages 39-60) and hiPSCs (MSU001 between passages 28-30) expressed typical hiPSCs characteristics through their morphology (Figure 3.1), immunocytochemistry (Figure 3.2-3.3) and flow cytometry (Figure 3.4-3.5). Both cell lines had similar hiPSCs morphology, which consisted of compact and tightly packed spherical cells containing significantly larger nuclei in comparison to the cytoplasm (Takahashi et al., 2007; Yu et al., 2007). These undifferentiated pluripotent compacted cell populations exist in a cluster which forms distinct colonies (Pera, Reubinoff and Trounson, 2000; Reubinoff et al., 2001; Tavakoli et al., 2009; Steiner et al., 2010). Additionally, the cell lines were able to successfully proliferate and self-renew, this was shown by their expression of pluripotency markers OCT4 and SSEA3 and TRA161 and the absence of early differentiation marker SSEA1 (Okamoto et al., 1990; Schöler et al., 1990; Thomson et al., 1998; Reubinoff et al., 2000; Pan et al., 2002; Rosler et al., 2004). Another characteristic of pluripotent stem cells is their ability to differentiate into the three germ layers (Thomson et al., 1998; Takahashi et al., 2007). Cord blood and MSU001 hiPSCs were able to express BRACHYURY (Mesoderm), SOX17 (Endoderm) and NESTIN (Ectoderm) through embryoid
bodies formation as well as single cells in suspension (monolayer) culturing methods.

In summary, it was of utmost importance immunocytochemistry, and flow cytometry characterisation was carried out for these cell lines prior to any experiments. Both hiPSCs lines expressed pluripotency markers and did not express the differentiation markers when in their undifferentiated state. They were able to differentiate into the three germ layers in both monolayer and EB state. Additionally, routinely sent for mycoplasma testing and karyotyping where they were negative for mycoplasma contamination and showed no abnormalities in karyotyping (Section 2.9). This gave us enough evidence to conclude that they are indeed pluripotent stem cells and we are confident to use them in our research.
4 Chapter 4: Evaluation and Characterisation of Different Biomaterials for Controlling Young’s Modulus on Human Induced Pluripotent Stem Cells (hiPSCs)

4.1 Introduction

The initial aim and challenges in this chapter were to develop a set of substrates suitable for cell culture that would mimic the desired range of Young’s modulus values. Young’s modulus is a measurement of elasticity. In the body, Young’s modulus values in vivo vary greatly; however, conventional cell culture procedures are carried out using tissue culture plastic (TCP) which has Young’s modulus value of 3GPa (Bonaccurso, Cappella and Graf, 2006). As summarised in Chapter 1, studies have shown cells that Young’s modulus of substrate has an effect on cell culture’s morphology (T. Yeung et al., 2005; Vertelov et al., 2016) and differentiation outcome (Engler et al., 2006).

As our thesis aim was to mimic in vivo conditions through four physiological relevant Young’s modulus values we aimed to achieve were 0.1-0.5kPa (brain) (Flanagan et al., 2002; Clatz et al., 2005; Schiavone et al., 2009), 1-3kPa (zona pellucida of embryos) (Khalilian et al., 2010), 10-20kPa (muscle) (Engler et al., 2004, 2006; Gilbert et al., 2010b) and >30kPa (osteoid) (Takai et al., 2005; Engler et al., 2007). All substrates are coated with vitronectin for hiPSCs maintenance and Matrigel™ for differentiation, it has been shown by Yeh et al., (Yeh et al., 2017) that soft substrate effects the mouse mammary gland epithelial cells regardless of the substrate being coated with collagen, poly-L-lysine, fibronectin or Matrigel™,
Substrate coatings is commonly used in cell culture and have also been used in studies of Young’s modulus on cell culture in literature. Therefore, it is believed that the substrate stiffness can affect cell culture despite substrate coatings.

Initially, it was essential to characterise the different substrates used. Characterisation was established using Atomic Force Microscopy (AFM) and cell culturing was carried out on six different substrates.

We examined three substrates produced in-house and three commercially available products. The first substrate was gelatine crosslinked with glutaraldehyde (GXG), which was produced in-house (Materials and Methods Section 2.10.1). The three different concentration of each gelatin stock solution (3%, 4% and 6%) was adapted from (Ali et al., 2015). The gelatin percentage results in a different Young’s modulus (Table 4.1). Once the substrate was produced, its Young’s modulus value was measured using the Atomic Force Microscopy (AFM) as well as seeding hiPSCs cord blood on them to determine their compatibility.

The next substrate we investigated was Polydimethylsiloxane (PDMS), a silicone-based material commonly used in cell culture. PDMS has been widely used in cell culture due to its bio-compatibility (Keough et al., 1985; Bordenave et al., 1992; van Kooten, Whitesides and von Recum, 1998; Leclerc, Sakai and Fujii, 2003; Mata, Fleischman and Roy, 2005). The PDMS was tuneable based on the ratio of the base elastomer and the curing agent, the standard mixing ratio for PDMS is 1:10 between the curing agent to the base elastomer. Six different ratios were produced by adaptation of the ratios presented by Gray (Gray, Tien and Chen, 2003) (1:10, 1:20, 1:30, 1:40, 1:50 and 1:60) (Materials and Methods Section 2.10.2). It was found that at high elastomer ratios of above 1:30 the PDMS could
not crosslink and was unable to cure after long durations in the oven and remained viscous. This correlates with reports in the literature in PDMS used in microfluidic devices at a ratio of 1:10 (Dodge et al., 2006).

We also investigated PɛK polymer, a novel polymer which was produced in-house with training from Spheritech LTD. The PɛK polymer is adjustable by altering its density, the type and type of acid in the stock solution (Materials and Methods Section 2.10.3). The longer the acid chain, the softer the polymer due to the lower number of cross-linkages. Four different samples were produced with altering components to try achieved low Young’s moduli (Table 4.2).

The commercially available products used were: Excellness, Soft Well and Cytosoft. Excellness plates are silicone-based substrates obtained from Excellness LTD (Switzerland). Excellness LTD offers Excellness substrates with 2, 5, 10, 15, 30 and 100kPa. Excellness has been used in culturing human amniotic fluid-derived stem cells (Skardal et al., 2012), human mesenchymal stromal cells (human mesenchymal stem cells) (hMSCs) (Talele et al., 2015) and human dermal fibroblast (Klingberg et al., 2018).

Another substrate with various Young’s moduli products is Softwell hydrogels, which were purchased from Matrigen LTD. Softwell is a polyacrylamide hydrogel available with Young’s modulus values of 0.2, 0.5, 1, 2, 8, 12, 25 and 50kPa. Research using softwells includes hMSCs (Yang et al., 2016) and human lung adenocarcinoma epithelial cells (Chang et al., 2015).

Finally, the third commercially available substrate investigated was purchased from Advanced Biomatrix (USA), Cytosoft. It is a Polydimethylsiloxane (PDMS) polymer available with Young’s modulus values of 0.2, 0.5, 2, 8, 16, 32
and 64kPa. Studies carried out on these substrates includes human foreskin fibroblasts (Paul et al., 2017) and human lung fibroblasts (Asano et al., 2017). AFM measurements of these commercially available substrates were not required as they had been tested by the companies.

Once we characterised the substrate, we investigated if Young’s modulus affects pluripotency to correlate with findings in literature that Young’s modulus value has no effect on cell pluripotency (Evans et al., 2009; Keung et al., 2012; Przybyla et al., 2016). As mentioned in earlier chapters, extracellular matrix has an impact on cell morphology, activity and differentiation outcome. This is due to the fact that in vivo ECM is different to in vitro conditions. Conventionally, in vitro cell cultures are maintained on tissue culture plastic which has a Young’s modulus value of 3GPa (Callister et al., 2012). A change in Young’s modulus has been shown to improve in vitro differentiation outcomes in a variety of cell types including human MSCs (Engler et al., 2006) and hiPSCs (Sun et al., 2012) and hESCs (Arshi et al., 2013; Narayanan et al., 2013). However, studies investigating Young’s modulus and cell pluripotency have shown that Young’s modulus has no effect on cell culture’s pluripotency (Keung et al., 2012).

4.2 Aims and Objectives

This chapter aimed to determine which substrate would be best for culturing hiPSCs (cord blood). Only hiPSCs (cord blood) was used in these experiments to reduce overall costs, hiPSCs (MSU0001) will be used as a different cell line repeat of important experiment to show the results are not cell line specific.

The detailed objectives were as follows:

- To characterise Young’s modulus values on various ECM substrate
• To establish cell culture on substrates.
• To characterise hiPSCs pluripotency on substrates using analytical methods of:
  • Immunocytochemistry of pluripotent marker OCT4.
  • Flow cytometry using pluripotency markers OCT4, SSEA3 and differentiation marker SEA1.
  • RTq-PCR of pluripotency markers OCT4, FGF4 and differentiation markers of NESTIN (Ectoderm), SOX17SOX17 (Endoderm) and BRACHYURY (Mesoderm).

Experiments in this chapter was carried out with hiPSCs (cord blood).

4.3 Results
4.3.1 Atomic Force Microscopy (AFM)

Atomic Force Microscope (AFM) was used to measure Young’s modulus of GXG and PDMS substrates produced in-house. MLCT cantilevers were used to apply force onto the substrates. Young’s modulus value of each substrate was calculated by the area of the cantilever tip and force curve (Figure 2.3) using the Hertz model (Materials and Methods Section 2.11.2). GXG production and measurements were repeated multiple times without successfully achieving the low Young’s modulus (0.1-0.5kPa brain, 1-3kPa embryo) that we aimed for (Table 4.1) because, with a lower gelatin stock solution, the GXG did not solidify.

On PDMS substrates, AFM measurements were carried out at a ratio of 1:20 and 1:30. As sample formulated at a ratio of 1:40, 1:50 and 1:60 were too viscous and did not solidify and therefore were not able to measure the Young’s modulus
or carry out cell culture on. PDMS ratios of 1:20 and 1:30 was measured using AFM, calculated results were 422kPa for 1:20 and 136.6kPa for 1:30. PEEK polymer produced were porous and cells were able to pass through the pores, this was discovered when the cells were stained with immunocytochemistry markers. This made it a three-dimensional substrate and not a two-dimensional substrate which was not what this thesis is working on, therefore AFM measurements was not carried out as we were not focusing on three-dimensional substrate.

Table 4.1 GXG Young’s modulus measurements

<table>
<thead>
<tr>
<th>Concentration (% w/v)</th>
<th>Young’s modulus measured (kPa) (n=3 biological repeats where ± represents standard deviation from mean)</th>
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<tbody>
<tr>
<td>GXG: 3% dissolved in PBS</td>
<td>7.38 ± 3.2</td>
</tr>
<tr>
<td>GXG: 4% dissolved in Milli-Q water</td>
<td>31.10 ± 3.8</td>
</tr>
<tr>
<td>GXG: 6% dissolved in Milli-Q water</td>
<td>40.46 ± 1.4</td>
</tr>
</tbody>
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Table 4.2 PDMS Young’s modulus measurements

<table>
<thead>
<tr>
<th>Concentration (% w/v)</th>
<th>Young’s modulus measured (kPa) (n=3 biological repeats where ± represents standard deviation from mean)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PDMS 1:20</td>
<td>422 ± 2.6</td>
</tr>
<tr>
<td>PDMS 1:30</td>
<td>136.6 ± 1.8</td>
</tr>
</tbody>
</table>

4.3.2 Cell attachment on Different Substrates and Establishment of Cell Culture
Cell attachment was studied in order to determine whether cell culture was feasible on these substrates. Cell attachment was studied on all six different types of substrates (GXG, PDMS, PεK, Softslide, Excellness and Cytosoft) (Table 4.3). All substrates were coated with vitronectin before seeding and hiPSCs were seeded at a density of $2 \times 10^5$ cells per 6-well plate. Phase contrast images (Figure 4.2) shows floating cells and no attachment of cells on all GXG substrates. It also showed the majority of cells that did attach were single cells and had a differentiated morphology of narrow and elongated shapes which were similar to fibroblast-like cells.

Due to the higher ratios of PDMS not curing, after the cells were seeded, we found that the PDMS had dissolved and became mixed with the media solution. Phase contrast images at 24 hours show a lack of cell attachment (Figure 4.3). Further to this, the PDMS was not able to achieve Young’s modulus values. Hence, we decided to not continue with the use of PDMS.

As the PεK polymer was opaque, phase contrast imaging could not be carried out. The cells were fixed and stained with phalloidin and attachment was observed under the microscope (Figure 4.4). Although fluorescence imaging shows cell attachment, the colonies were few and small on day 4. It was decided that PεK polymer would not be used for further experiments due to it being opaque as well as it is a porous polymer making it a 3D substrate which was not our aim.

Due to the thickness of the Softslide hydrogels, the cells could not be visualised using the EVOS light microscope, and therefore, the cells were fixed and stained with phalloidin (Figure 4.5). Immunocytochemistry shows very small colonies of cells present on the Softwell hydrogel after four days of culturing. This
signifies that the cells have difficulty growing on this particular hydrogel and therefore Softwell was not chosen as a substrate for future experiments.

Figure 4.6 shows that iPSCs were able to attach to the Excellness substrate. Cells attached showed the desired morphology of undifferentiated cells. Cell attachment was observed at different Young’s modulus substrates.

On Cytosoft, cells attached displayed the desired morphology of undifferentiated cells. It was found after 24 hours that the cells were able to attach to all of the different Young’s modulus substrates (Figure 4.7)

Figure 4.1 Phase contrast images of hiPSCs seeded after 24 hours GXG.
(i) TCP control and GXG (ii) 3% gelatin GXG (iii) 4% gelatin GXG (iv) 6% gelatin GXG. Scale bar = 400µm. Images were taken at 100X ocular lens magnification using Evos® XL.
Figure 4.2 Phase contrast images of hiPSCs after 24 hours on PDMS.

(i) TCP control and PDMS (ii) 40:1 PDMS (iii) 50:1 PDMS. Scale bar = 400µm. Images were taken at 100X ocular lens magnification using Evos® XL.
Figure 4.3 Phase contrast image of hiPSCs after 48 hours on PɛK polymer.

(i) TCP control and phalloidin stained cells on (i) PɛK polymer (as cells cannot be seen using phase contrast due to the opaque polymer). Scale bar = 400µm. Images were taken at 100X ocular lens magnification using Evos® XL.
Figure 4.4 Phase contrast image of hiPSCs after 4 days on Softslide.

(i) TCP control and phalloidin stained cells on (ii) 50kPa, (iii) 25kPa, (iv) 4kPa and (v) 0.2kPa Softslide polymers (as cells cannot be seen using phase contrast due to the opaque polymer). Scale bar = 400µm. Images were taken at 100X ocular lens magnification using Evos® XL.
Figure 4.5 Phase contrast images of hiPSCs on Excellness.

(i) TCP control and Excellness at Day 4 (ii) 2kPa (iii) 30 kPa. Scale bar = 400µm. Images were taken at 100X ocular lens magnification using Evos® XL.
Figure 4.6 (A) Phase contrast images of hiPSCs on Cytosoft.

(i) TCP control and Cytosoft at day 4 (ii) 0.2kPa (iii) 2kPa (iv) 8 kPa (v) 16kPa (vi) 32kPa (vii) 64kPa. Scale bar = 400µm. Images were taken at 100X ocular lens magnification using Evos® XL.
### Table 4.3 Summary of cell attachment on substrates

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Production</th>
<th>Cell attachment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gelatin crosslinked with glutaraldehyde (GXG)</td>
<td>In-house production</td>
<td>No</td>
</tr>
<tr>
<td>Polydimethylsiloxane (PDMS)</td>
<td>In-house production</td>
<td>No</td>
</tr>
<tr>
<td>Poly-ε-Lysine (PεK) polymer</td>
<td>In-house production</td>
<td>Yes, however, hiPSCs colonies attached were very small compared to TCP cultured colonies</td>
</tr>
<tr>
<td>Excellness</td>
<td>Commercially available from Excellness LTD (Lausanne, Switzerland)</td>
<td>Yes, however, softest Young’s modulus available is 2kPa</td>
</tr>
<tr>
<td>Softwell</td>
<td>Commercially available from Matrigen (Brea, CA, USA)</td>
<td>Yes, however, hiPSCs colonies attached were very small compared to TCP cultured colonies</td>
</tr>
<tr>
<td>Cytosoft</td>
<td>Commercially available from Advanced Biomatrix (San Diego, CA, USA)</td>
<td>Yes</td>
</tr>
</tbody>
</table>

#### 4.3.3 Morphological Analysis of Human Induced Pluripotent Stem Cells (hiPSCs) on Cytosoft Substrates

From all the six substrates investigated, Excellnes and Cytosoft showed cell attachment and proliferation. However, it was decided to use Cytosoft in this thesis as Cytosoft was readily available and offers a large range of Young’s moduli. Additionally, Excellness softness Young’s modulus value was 2kPa and we require 0.2kPa in this thesis. The first observation was the morphology of the cells attached to the substrates. Phase contrast images revealed that the cells were able to attach
to all substrate conditions as previously stated, as well as expand for the duration of days required, which in this thesis was 8 days. The cell morphology was of typical stem cell-like morphology of a round cell with a high ratio of cytoplasm to nuclei compacted together in a colony. The cell morphology was consistent throughout all Young’s modulus conditions, with minor differences between colony sizes in different Young’s modulus values. Softer substrates (0.2 and 2kPa) showed smaller and rounder colonies from the other substrates. Figure 4.7 shows clearly that there was less cell spreading between softer conditions and tissue culture plastic. The phase contrast image also showed more overlaying of cells grown on TCP compared to the Cytosoft substrates (Figure 4.7). Phase contrast images (Figure 4.8) taken would be used to aid immunocytochemistry images, flow cytometry and qPCR analysis results to draw conclusions.
<table>
<thead>
<tr>
<th>Day 2</th>
<th>Day 5</th>
<th>Day 8</th>
</tr>
</thead>
<tbody>
<tr>
<td>TCP</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(i)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>64kPa</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(ii)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>16kPa</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(iii)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2kPa</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(iv)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.2kPa</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(v)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Figure 4.7 Phase contrast images of hiPSCs on Cytosoft at time point day 2, 5 and 8.

TCP and four Cytosoft materials (64 kPa, 16 kPa, 2 kPa and 0.2 kPa) at time point day 2, 5 and 8. Cells exhibited stem cell-like morphology, softer substrates had smaller colonies. All scale bars = 400 µm. Images were taken at 100X ocular lens magnification using Evos® XL.
4.3.4 Immunocytochemistry Characterisation of Human Induced Pluripotent Stem Cells (hiPSCs) on Cytosoft Substrates

Although phase contrast images display an over confluent and multilayered culture on TCP than the Cytosoft wells, the immunocytochemistry staining shows positive OCT4 for all conditions at all time points (Figure. 4.8) indicating all cultures are pluripotent. Immunostaining was carried out at day 5 to prove cell’s pluripotency, day 5 is the time point where hiPSCs conventionally cultured on tissue culture plastic are passaged.

All five Young’s modulus condition shows similar fluorescence images, and this alone cannot be used to draw conclusion as there were no significant differences as well as only one marker being used. Immunocytochemistry images are used as a supporting data in conjunction with flow cytometry data and RT-qPCR, and therefore OCT4 was used as the immunocytochemistry marker to reduce cost of consumables.
Figure 4.8 OCT4 Immunocytochemistry of hiPSCs on Cytosoft.

Immunocytochemistry images of TCP and four Cytosoft materials (64 kPa, 16 kPa, 2 kPa and 0.2 kPa) at day 5. For each Young’s modulus condition phase contrast and immunocytochemistry for OCT4. Images show positive staining. All scale bars = 400 µm. (F) Secondary only staining was carried out as control: (i) secondary only mouse IgG (ii) secondary only mouse IgM. Blocking solution was used as isotype control. Images were taken at 100X ocular lens magnification using Evos® XL.
4.3.5 Flow Cytometry Characterisation of Human Induced Pluripotent Stem Cells (hiPSCs) on Cytosoft Substrates

In addition to immunocytochemistry, cells were harvested at day 5 for flow cytometry analysis. Cells were harvested at day 5 as from previous experiments phase contrast images showed that the cultures were 80% confluent and had hiPSCs undifferentiated morphology, from day 5 onwards cells were growing on top of each other and displaying differentiated morphology. Additionally, when maintaining hiPSCs cultures cells are usually passaged at 80% confluency usually at day 4/5. Each condition was stained with pluripotency markers OCT4, SSEA3 and early differentiation marker SSEA1. Figure 4.9 shows OCT4 results for all Young’s modulus conditions at day 5. The results show 75.9%, 75.9%, 85.6%, 81.1% and 82.7% positive for OCT4 at day 5 from TCP, 64 kPa, 16 kPa, 2 kPa and 0.2 kPa respectively. This was also supported by the positive expression of SSEA3, another pluripotency marker from 74.5% (TCP), 66.5% (64 kPa), 54.9% (16kPa), 54.3% (2 kPa) and 79.5% (0.2 kPa) at day 5 (Figure 4.10). Results from early differentiation marker, SSEA1 shows <2% for all conditions (Figure 4.11). A slight increase of SSEA1 shows that as cells lose their pluripotency, they gain a differentiation marker. However, as SSEA1 is an early differentiation marker it would not be positive on cells that have differentiated into a later stage (Draper et al., 2002). The flow cytometry data from all conditions are similar and therefore this suggests that there were no differences between the conditions, and the cells grown on Cytosoft substrates are pluripotent.
Figure 4.9 Flow cytometry analysis showing hiPSCs (cord blood) at day 5 positive for pluripotency marker OCT4.

Where (i) OCT4 Isotype (ii) TCP, (iii) 64kPa, (iv) 16kPa, (v) 2kPa and (vi) 0.2kPa.
Figure 4.10 Flow cytometry analysis showing Cord Blood hiPSCs (cord blood) at day 5 positive for pluripotency marker SSEA3.

Where (i) SSEA3 Isotype (ii) TCP, (iii) 64kPa, (iv) 16kPa, (v) 2kPa and (vi) 0.2kPa.
Figure 4.11 (A) Flow cytometry analysis showing hiPSCs (cord blood) at day 5 negative for early differentiation marker SSEA1.

Where (i) SSEA1 Isotype (ii) TCP, (iii) 64kPa, (iv) 16kPa, (v) 2kPa and (vi) 0.2kPa.
4.3.6 RT-qPCR of Human Induced Pluripotent Stem Cells (hiPSCs) on Cytosoft Substrate

An additional study to determine if Young’s modulus had an effect on pluripotency gene regulation was carried out using RT-qPCR. The genes used for RT-qPCR analysis were based on availability of the genes at the time of running the assays. Genes used were pluripotency genes: OCT4, FGF4 (Yuan et al., 1995; Adewumi et al., 2007) and differentiated genes BRACHYURY (Mesoderm), SOX17 (Endoderm) and NESTIN (Ectoderm), these genes were chosen as they are commonly used in literature. β-actin was used as a housekeeping gene. Cells were harvested at day 5 and raw Ct values were normalised with β-actin and corrected using TCP condition as control. Statistical analysis 2-way ANOVA was applied to these results (Figure. 4.12-4.16) (where significant difference *p<0.05, very significant difference **P<0.01 and extremely significant difference ***P<0.001). Calculations showed that there were no significant differences between the conditions for both pluripotency markers OCT4 and FGF4, these results correlate with flow cytometry data and immunocytochemistry images as well as in literature that Young’s modulus does not have an effect on hiPSCs pluripotency (Keung et al., 2012). This data suggests that pluripotency expression is not solely governed by Young’s modulus value of the ECM.
Figure 4.12 RT-qPCR analysis showing mean value of gene expressions for pluripotency marker *OCT4* on Cytosoft.

*OCT4* on TCP and four Cytosoft materials (64 kPa, 16 kPa, 2 kPa and 0.2 kPa) for hiPSCs maintained in E8 media at day 5. Statistical analysis carried out with 2-way ANOVA and where appropriate with post hoc testing using Tukey method using GraphPad Prism 7.03, n = 3 (biological triplicates), error bars represents standard deviation from mean. *p<0.05, **p<0.01, ***p<0.001 and ****P<0.0001 showing significance difference between conditions. Expression was normalised with β-actin (housekeeping gene) and then corrected with undifferentiated hiPSCs using 2^\(-\Delta\Delta Ct\).
Figure 4.13 RT-qPCR analysis showing mean value of gene expressions for pluripotency marker *FGF4* on Cytosoft.

*FGF4* on TCP and four Cytosoft materials (64 kPa, 16 kPa, 2 kPa and 0.2 kPa) for hiPSCs maintained in E8 media at day 5. Statistical analysis carried out with 2-way ANOVA and where appropriate with post hoc testing using Tukey method using GraphPad Prism 7.03, n = 3 (biological triplicates), error bars represents standard deviation from mean. *p<0.05, **p<0.01, ***p<0.001 and ****P<0.0001 showing significance difference between conditions. Expression was normalised with β-actin (housekeeping gene) and then corrected with undifferentiated hiPSCs using 2^{-ΔΔCt}. 
Figure 4.14 RT-qPCR analysis showing mean value of gene expressions for early ectoderm marker *NESTIN* on Cytosoft.

*NESTIN* on TCP and four Cytosoft materials (64 kPa, 16 kPa, 2 kPa and 0.2 kPa) for hiPSCs maintained in E8 media at day 5. Statistical analysis carried out with 2-way ANOVA and where appropriate with post hoc testing using Tukey method using GraphPad Prism 7.03, n = 3 (biological triplicates), error bars represents standard deviation from mean. *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001 showing significance difference between conditions. Expression was normalised with β-actin (housekeeping gene) and then corrected with undifferentiated hiPSCs using $2^{-\Delta\Delta Ct}$. 
Figure 4.15 RT-qPCR analysis showing mean value of gene expressions for early entoderm marker SOX17 on Cytosoft.

SOX17 on TCP and four Cytosoft materials (64 kPa, 16 kPa, 2 kPa and 0.2 kPa) for hiPSCs maintained in E8 media at day 5. Statistical analysis carried out with 2-way ANOVA and where appropriate with post hoc testing using Tukey method using GraphPad Prism 7.03, n = 3 (biological triplicates), error bars represents standard deviation from mean. *p<0.05, **p<0.01, ***p<0.001 and ****P<0.0001 showing significance difference between conditions. Expression was normalised with β-actin (housekeeping gene) and then corrected with undifferentiated hiPSCs using 2^{-∆∆Ct}. 
Figure 4.16 RT-qPCR analysis showing mean value of gene expressions for early mesoderm marker *BRACHYURY* on Cytosoft.

*BRACHYURY* on TCP and four Cytosoft materials (64 kPa, 16 kPa, 2 kPa and 0.2 kPa) for hiPSCs maintained in E8 media at day 5. Statistical analysis carried out with 2-way ANOVA and where appropriate with post hoc testing using Tukey method using GraphPad Prism 7.03, n = 3 (biological triplicates), error bars represents standard deviation from mean. *p<0.05, **p<0.01, ***p<0.001 and ****P<0.0001 showing significance difference between conditions. Expression was normalised with β-actin (housekeeping gene) and then corrected with undifferentiated hiPSCs using $2^{-\Delta\Delta Ct}$. 
4.4 Discussion

It is known that extracellular matrix (ECM) has an effect on cell culture. Young’s modulus is a aspect of ECM that has been widely studied (Bissell and Aggeler, 1987; Ingber, 1993; Wang and Ingber, 1994; Choquet, Felsenfeld and Sheetz, 1997; Lo et al., 2000; Sarasa-Renedo and Chiquet, 2005). Studies have shown that external factors such as Young’s modulus value an influence on cell morphology (Tony Yeung et al., 2005; Vertelov et al., 2016) and the differentiation outcome (Engler et al., 2006). This is due to the difference in Young’s modulus in vivo and in vitro. In this thesis, we want to investigate cell culturing on Young’s modulus and low oxygen tension. The Young’s modulus chosen for our substrates were 0.2, 2, 16 and 64 kPa to mimic in vivo conditions of embryo (Khalilian et al., 2009), brain (Flanagan et al., 2002; Clatz et al., 2005; Schiavone et al., 2009), muscle (Engler et al., 2004; Engler et al., 2006; Gilbert et al., 2010) and bone (Takai et al., 2005; Engler et al., 2007) to test if cell culture and cell differentiation efficiency could be enhanced (Section 1.3).

Multiple substrates were produced and investigated, commercially available products used were Excellness, Softwell and Cytosoft and in-house produced substrates were GXG, PDMS and PƐK. The substrates produced in-house were not able to achieve the Young’s modulus values we aimed for after characterising using an AFM. Moreover, cell culture could not be established on in-house substrates as cell attachment was been observed. On GXG substrates, most cells that did attach were single cells and had a differentiated morphology of stretched fibroblast-like cells-- possibly as a result of stress (Takahashi et al., 2007).
In-house production of GXG and PDMS were both unsuccessful in achieving the low Young’s modulus value as well as establishing cell culture. The phase contrast images revealed a lack of cell attachment. It is believed that this could be due to the coating of the PDMS (Lee et al., 2004). For PƐK polymer, it was discovered by immunocytochemistry of hiPSCs culture that the substrate contains large pores and therefore a three-dimensional matrix was created instead of a two-dimensional matrix. Due to these reasons, it was decided that the in-house substrates attempts were unsuccessful and would not be used in further experiments.

Three commercially available products were then investigated: Excellness, Softwell and Cytosoft. It was decided that Cytosoft plates will be used in this thesis as cell culture was established and the hiPSCs morphology was maintained. Additionally, Cytosoft is a PDMS polymer, although we were unable to produce our own PDMS, PDMS is widely used in research (Lee et al., 2004; Mata, Fleischman and Roy, 2005; Palchesko et al., 2012; Raczkowska et al., 2016). Through characterisation, Excellness and Cytosoft showed successful hiPSCs culture (Figure 4.6-4.7). However, Cytosoft was chosen over Excellness due to the fact that Excellness softness Young’s modulus value was 2kPa and we require 0.2kPa in this thesis. Additionally, Cytosoft also offers a large range of Young’s moduli (0.2, 0.5, 2, 8, 16, 32 and 64kPa).

A characterisation of pluripotency of the hiPSCs cell cultured on the Cytosoft substrates was carried out. Pluripotency characterisation of hiPSCs on ECM was a crucial step to determine if the hiPSCs population was able to maintain and self-renew on Cytosoft before further experiments were carried out. This step was also
carried out to confirm that the results obtained correlates with literature. Literature states that Young’s modulus value has no effect on cell pluripotency (Evans et al., 2009; Keung et al., 2012; Przybyla et al., 2016).

Cytosoft substrate with Young’s modulus values of 0.2, 2, 16 and 64kPa were used to represent embryo, brain, muscle and bone as previously mentioned. The results show hiPSCs cultured on soft substrates show less spreading. This correlates with literature on how cell colonies tend to not spread on soft substances due to lack of actin stress fibres on soft surfaces and it is related to cell anchorage and cell spreading (Yeung et al., 2005).

Regarding pluripotency characterisation of hiPSCs on Cytosoft substrates, all conditions showed positive immunocytochemistry staining of OCT4, a known pluripotency marker. Further quantitative analyses were carried out using flow cytometry and RT-qPCR. With flow cytometry, there was a consistently high level of OCT4 and SSEA3 expression in all conditions and no expression of early differentiation marker SSEA1. This also correlates with RT-qPCR gene expression whereby after applying 2-way ANOVA analysis on the data, it shows no significant differences between all 4 Cytosoft conditions (0.2, 2, 16 and 64kPa) to TCP control. Collectively, the data from phase contrast images, immunocytochemistry, flow cytometry and RT-qPCR indicates that Cytosoft substrates are able to maintain hiPSCs culture in their pluripotent state and that substrate stiffness does not have an effect on hiPSCs pluripotency as conditions investigated were pluripotent.
Chapter 5: The effects of Young’s Modulus and Low Oxygen Tension on 2D Monolayer of Human Induced Pluripotent Stem Cells (hiPSCs) – Spontaneous Differentiation

5.1 Introduction

\textit{In vivo} embryonic development of mammals occurs in a low oxygen microenvironment (1.5-8% O\textsubscript{2}) (Fischer and Bavister, 1993; Genbacev \textit{et al.}, 1997). Once the egg is fertilized, it travels down the fallopian tube for 5-7 days during which the oxygen tension is 1.5-8% O\textsubscript{2}, to be implanted in the uterus (Rodesch \textit{et al.}, 1992). It has been shown the first trimester of pregnancy the oxygen tension of the human uterine surfaces increases from 2% to 7% O\textsubscript{2} (Rodesch \textit{et al.}, 1992). During the journey, the zygote undergoes rapid and multiple cellular divisions which then becomes a blastocyst. Human embryonic stem cells are derived from the inner cell mass of blastocyst at this stage. \textit{In vivo}, the blastocyst then implants itself to the uterus wall and establishes a blood circulation system with the mother (Viganò \textit{et al.}, 2003). The oxygen tension during this period is low and has a significant impact on embryonic development as mentioned in Chapter 1 (Jauniaux \textit{et al.}, 2000; Jauniaux, Gulbis and Burton, 2003; Staun-Ram and Shalev, 2005; Liu \textit{et al.}, 2008; Zhou \textit{et al.}, 2012).

The effects of low oxygen tension have been widely investigated on pluripotency maintenance and differentiation to mimic the uterus environment (Karagenc \textit{et al.}, 2004; Tejera \textit{et al.}, 2012; Hu and Yu, 2017; Soares, Iqbal and Kozai, 2017). Stem cells cultured at low oxygen (<5% O\textsubscript{2}) have positive effects on
stem cell self-renewal (Ezashi, Das and Roberts, 2005; Westfall et al., 2008; Abaci et al., 2010; Guo et al., 2013; Zhi et al., 2018). Upon differentiation, hESCs cultured at 3% O$_2$ had a higher differentiation outcome into oligodendrocytes and chondrogenic precursor cells (Khan, Adesida and Hardingham, 2007; Koay et al., 2008; Stacpoole et al., 2013). Moreover, low oxygen exposure during culturing has also been reported to result in an increase of endothelium formation (Prado-Lopez et al., 2010), cardiomyocytes (Niebruegge et al., 2009) and neuronal cells (Mondragon-Teran, Lye and Veraitch, 2009; Mondragon-Teran et al., 2013). These examples highlight the significance of low oxygen tension as a microenvironment.

On top of low oxygen tension, another environmental factor that has also been widely studied is the effects of extracellular matrix on cell culture. It has been shown that different substrate stiffness that mimics in vivo conditions can influence the cell to differentiate into that cell type (Ingber, 1993; Pelham and Wang, 1997; Lo et al., 2000). Studies have shown that external factors such as substrate stiffness influence cell morphology (Yeung et al., 2005; Vertelov et al., 2016) and differentiation outcome. For instance, MSCs grown on hard substrates that mimics bone had a higher yield in osteogenic cells. Substrate that mimics muscle has a higher yield of muscle cells and soft substrate that mimic brain had a higher yield of neuronal cells (Engler et al., 2006).

After the characterisation of our substrate (Chapter 4) where it was decided that Cytosoft would be used in the experiments, we wanted to test the effect of Young’s modulus and low oxygen tension on hiPSCs spontaneous differentiation. We hypothesized that the combined effects of Young’s modulus and low oxygen
tension would lead to more efficient differentiation down the germ layers or a bias towards a germ layer.

Cells were cultured as a monolayer, this would mean the cells were equally exposed to the substrate and oxygen tension as in three-dimensional aggregates such as embryoid bodies, not all cells would be in contact with the substrate nor would the cells be exposed to the same oxygen and nutritional environment.

5.2 Aims and Objectives

The aims and objectives of this chapter were to investigate the combined effects of low oxygen tension and Young’s modulus on two-dimensional (2D) cell culture. hiPSCs were allowed to spontaneously differentiate for eight days (based on previous experiments where cells showed differentiated morphological changes on day 8) and samples were taken for analysis at days four and eight. Only hiPSCs (cord blood) was used in these experiments to reduce overall costs, hiPSCs (MSU0001) will be used as a different cell line repeat of important experiment to show the results are not cell line specific.

The detailed objectives were:

- To study the impact of Young’s modulus (64kPa, 16kPa, 2kPa and 0.2kPa) and low oxygen tension (2% O₂) on cell attachment and viability of monolayer hiPSCs culture
- To investigate spontaneous differentiation on cells grown on various substrates at low oxygen tension (2% O₂) using analytical methods of:
  - RT-qPCR panel of differentiation markers
    - endoderm: AFP and SOX17
    - mesoderm: BRACHYURY and MSX1
- ectoderm: $\beta_{III}$ TUBULIN and PAX6

- Immunocytochemistry of differentiation markers of the three-germ layers: SOX17, $\beta_{III}$ TUBULIN and BRACHYURY.

- Flow cytometry using differentiation markers of the three-germ layers: SOX17, AFP and BRACHYURY.

All experiments in this chapter were carried out using hiPSCs (cord blood).

5.3 Results

5.3.1 Experimental plan

Studies have been carried out on the effects of low oxygen tension as well as the effects of Young's modulus on stem cell differentiation, however, as of now, there has been no study on the combined effects of low oxygen tension and Young's modulus values. Here, we investigated the combined effects of Young's modulus and 2% low oxygen tension on hiPSCs (cord blood). Here, 2% oxygen tension was chosen based on \textit{in vivo} conditions and prior studies in literature (Section 1.2) Cells were cultured for four and eight days in various Young's modulus and oxygen tension conditions and investigated how these conditions regulate the hiPSCs into the three germ layers.

Figure 5.1 summarises the spontaneous differentiation protocol used in this chapter. Undifferentiated hiPSCs (cord blood) used were previously characterised in Chapter 3, hiPSCs cultured all showed typical stem cell morphology and expressed pluripotency markers (Chapter 3). For the differentiation protocol, single cells in suspension were seeded on Matrigel-coated tissue culture plastic (TCP) and Cytosoft 6-well plates and allowed to spontaneously differentiate as a monolayer. Matrigel™ is used in this protocol for hiPSCs differentiation whereas
vitronectin was used for hiPSCs maintenance. We first investigated using a monolayer protocol, so cells are equally exposed to Young’s modulus of the substrate. A total of 10 different conditions (TCP, 64, 16, 2 and 0.2kPa in 20% O₂ and TCP, 64, 16, 2 and 0.2kPa in 2% O₂) were cultured at the same time using TCP in 20% O₂ as a control. The hiPSCs were seeded in the form of monolayer on Matrigel-coated 0.2kPa (brain), 2kPa (embryo), 16kPa (muscle), 64kPa (osteoid) Cytosoft and TCP 6-well plates at a density of 2x10⁵ cells per well. Cells were maintained with hPSCs spontaneous differentiation media at 2% O₂ and 20% O₂, the analysis took place at time points day four and eight. Day four and eight was chosen based on previous experiments where cells showed differentiated morphological changes on day 8, in addition to day 8, day 4 was chosen to investigate early differentiation. Cells were harvested to investigate their differentiated gene expression. The markers used in this chapter can be seen in table 5.1. Cultures underwent daily media changes.
Figure 5.1 2D monolayer spontaneous differentiation protocol experimental setup.

Cells were seeded on Matrigel coated TCP, 64, 16, 2 and 0.2kPa 6-well plates and incubated for 4 and 8 days in 20% O₂ and 2% O₂.

Table 5.1 List of markers used in this chapter

<table>
<thead>
<tr>
<th>Marker</th>
<th>RT-qPCR</th>
<th>Flow Cytometry</th>
<th>Immunocytochemistry</th>
</tr>
</thead>
<tbody>
<tr>
<td>Endoderm</td>
<td>AFP, SOX17 and FOXA2</td>
<td>AFP</td>
<td>AFP</td>
</tr>
<tr>
<td>Ectoderm</td>
<td>βIII TUBULIN and PAX6</td>
<td>Not available</td>
<td>βIII TUBULIN</td>
</tr>
<tr>
<td>Mesoderm</td>
<td>BRACHYURY and MSX1</td>
<td>BRACHYURY</td>
<td>α - SMA</td>
</tr>
</tbody>
</table>
5.3.2 Cell morphology

The cell’s morphology is important, as it is a clear characteristic difference between cells. The morphology of a cell is result of the cytoskeleton structure, which is a network of filaments and protein (Dos Remedios et al., 2003). Changes in the cell morphology caused by ECM conditions are known to affect the cell’s differentiation outcome (Discher, Janmey and Wang, 2005; Engler et al., 2006). Phase contrast images (Figure 5.2) show hiPSCs grown on TCP, 64, 16, 2 and 0.2kPa under 20% and 2% O\textsubscript{2}. Figure 5.3 shows that although there are no noticeable differences between TCP, 64kPa and 16kPa there are distinguishable differences between those three conditions to the softer substrates of 2kPa and 0.2kPa. Although the cells were seeded in a monolayer culture, it can be observed that cells on 2kPa and 0.2kPa were clumped together in small colonies whereas in the other conditions the cells were confluent. The morphology of cells in 2kPa and 0.2 kPa were similar in 20% O\textsubscript{2} than 2% O\textsubscript{2}.

Cells were more confluent at day 8 and there was no noticeable difference that can be seen between TCP, 64kPa and 16kPa. However, cells in those three conditions were less overgrown at 2% O\textsubscript{2} compared to 20% O\textsubscript{2}. This also correlates with cell viability data (Figure 5.17). For conditions on 2kPa and 0.2kPa substrates, a congregation of neuronal-like structures were consistently seen throughout all the wells. This was consistent between the two oxygen tension. This confirms that Young’s modulus affects cell morphology. Moreover, the morphology of cells in each condition exhibited different cell size, shape and spreading leading to the conclusion that they are possibly different cell types. Subpopulations of elongated cells, mesenchymal-like cells, endodermal-like cells and epithelial-like cells are
noticeable and more common in various conditions (Figure 5.4). Morphology of sub-populations were seen throughout all the conditions. However, examples of sub-populations are shown in Figure 5.4 where Figure 5.4i shows a morphology of star shaped cells in 16kPa in 20% O₂, Figure 5.4ii striated cells in 2kPa in 2% O₂, Figure 5.4iii of spindle shaped cells in 16kPa in 2% O₂, Figure 5.4iv shows cobble stone shaped endoderm-like cells grown in 0.2kPa in 2% O₂, MSC-like cells in Figure 5.4v in 64kPa 20% O₂, Figure 5.4vi of primitive streak-like cells in 0.2kPa 2% O₂ and MSC progenitors-like cells in 64kPa in 2% O₂ (Figure 5.4vii).

Figure 5.5 exhibits the difference in cell morphology at day 2 between conditions in 20% O₂ and 2% O₂. Images was taken at day 2 so that the cells morphology can be clearly seen, as density of the cells increases, and the culture becomes confluent. It can be clearly seen in Figure 5.5 the morphology of cells in 20% O₂ have accentuated membrane regions compared to 2% O₂. In this figure, the cell’s membrane spread less in 2% O₂ than 20% O₂. This spread is the cell’s actin filaments (Pollard and Borisy, 2003).
Figure 5.2 Phase contrast images of differentiated hiPSCs on Cytosoft at day 4.

For each Young’s modulus condition (A) (i) and (B) (i) 20% $O_2$ (A) (ii) and (B) (ii) 2% $O_2$. All scale bars = 400 μm. Images were taken at 100X ocular lens magnification using Evos® XL.
Figure 5.3 Phase contrast images of differentiated hiPSCs on Cytosoft materials at day 8. For each Young’s modulus condition (A) (i) and (B) (i) 20% O₂ (A) (ii) and (B) (ii) 2% O₂. All scale bars = 400 µm. Images were taken at 100X ocular lens magnification using Evos® XL.
Figure 5.4 Phase contrast images of differentiated exhibiting sub-population morphology.

(i) star shaped, (ii) striated, (iii) spindle shaped, (iv) Endoderm-like cells (cobble stone shaped), (v) MSC-like cells, (vi) primitive streak-like and (vii) MSC progenitors-like cells. All scale bars = 200 µm.
Images were taken at 100X ocular lens magnification using Evos® XL.
Figure 5.5 Phase contrast images of differentiated hiPSCs on Cytosoft.

TCP and four Cytosoft materials (64 kPa, 16 kPa, 2 kPa and 0.2 kPa) at time point day 2. For each Young’s modulus condition (i) 20% O₂ and (ii) 2% O₂. All scale bars = 200 µm. Images were taken at 100X ocular lens magnification using Evos® XL.
5.3.3 Expression of Pluripotency and Differentiated Markers Using RT-qPCR of 2D Monolayered Spontaneous Differentiation of Human Induced Pluripotent Stem Cells (hiPSCs)

RT-qPCR was carried out on a panel of markers to determine the gene expression of the cell sample for each condition. The genes used for RT-qPCR analysis can be seen in Table 5.2, β-actin was used as a housekeeping gene. Cells were harvested at day 4 and day 8 time points from both 20%O$_2$ and 2%O$_2$. Raw Ct values were normalised with β-actin and corrected using undifferentiated hiPSCs as control. Statistical analysis 2-way ANOVA was applied to these results where significant difference *p<0.05, very significant difference **P<0.01, highly significant ***P,0.001 and extremely significant difference ****P<0.0001), significant differences are reported based on the differences to TCP 20% O$_2$ control unless otherwise stated.

At day 4 there are no significant differences in the mesodermal marker _BRACHYURY_; however, there were minor differences in conditions grown in 2% O$_2$ there the softer the substrate the less _BRACHYURY_ gene expression were present. and ectodermal marker βIII TUBULIN (Figure 5.6-5.8). However, there was a high significance between control compared to all other conditions due to a high SOX17 expression in TCP (Figure 5.6).

Table 5.2 List of markers used in this chapter for RT-qPCR

<table>
<thead>
<tr>
<th>Marker</th>
<th>Day 4</th>
<th>Day 8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ectoderm</td>
<td>βIII TUBULIN</td>
<td>βIII TUBULIN and PAX6</td>
</tr>
<tr>
<td>Endoderm</td>
<td>SOX17</td>
<td>SOX17, FOXA2 and AFP</td>
</tr>
<tr>
<td>Mesoderm</td>
<td>BRACHYURY</td>
<td>BRACHYURY and MSX1</td>
</tr>
</tbody>
</table>
Figure 5.6 RT-qPCR analysis showing mean value of gene expressions for SOX17 at day 4.

Figure shows RT-qPCR results for endoderm marker Sox17 for hiPSCs spontaneously differentiated on TCP and Cytosoft at day 4 under 20%O_2 and 2%O_2. Statistical analysis carried out with 2-way ANOVA and where appropriate with post hoc testing using Tukey method using GraphPad Prism 7.03, n = 3 (biological triplicates), error bars represents standard deviation from mean. Significance difference between conditions are indicated, where *p<0.05, **p<0.01, ***p<0.001 and ****P<0.0001. Expression was normalised with β-actin (housekeeping gene) and then corrected with undifferentiated hiPSCs using 2^\(-\Delta\Delta C_T\).
Figure 5.7 RT-qPCR analysis showing mean value of gene expressions for βIII TUBULIN at day 4.

Figure shows RT-qPCR results for ectoderm marker βIII TUBULIN for hiPSCs spontaneously differentiated on TCP and Cytosoft at day 4 under 20%O₂ and 2%O₂. Statistical analysis carried out with 2-way ANOVA and where appropriate with post hoc testing using Tukey method using GraphPad Prism 7.03, n = 3 (biological triplicates), error bars represents standard deviation from mean. Significance difference between conditions are indicated, where *p<0.05, **p<0.01, ***p<0.001 and ****P<0.0001. Expression was normalised with β-actin (housekeeping gene) and then corrected with undifferentiated hiPSCs using 2−ΔΔCt.
Figure 5.8 RT-qPCR analysis showing mean value of gene expressions for **BRACHYURY** at day 4.

Figure shows RT-qPCR results for mesoderm: **BRACHYURY**; on TCP and four Cytosoft materials (64 kPa, 16 kPa, 2 kPa and 0.2 kPa) for spontaneously differentiated hiPSCs at day 4 under 20%O₂ and 2%O₂.

Statistical analysis carried out with 2-way ANOVA and where appropriate with post hoc testing using Tukey method using GraphPad Prism 7.03, n = 3 (biological triplicates), error bars represents standard deviation from mean. Significance difference between conditions are indicated, where *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001. Expression was normalised with β-actin (housekeeping gene) and then corrected with undifferentiated hiPSCs using $2^{\Delta\Delta Ct}$. 

*Day 4 BRACHYURY*
At day 8 it was decided to expand the markers panel by having more than 1 marker per germ layer, 2 markers were used for each of the 3 germ layers (Table 5.2). For ectoderm markers, there was low expression of both βIII TUBULIN and PAX6 (Figure 5.9-5.10). In addition, there were no significant differences between the conditions.

For mesoderm gene expression markers, BRACHYURY and MSX1 were used (Figure 5.11-5.12). An increase in BRACHYURY’s expression was observed in conditions cultured at 2% O₂ where the softer the substrate was, the higher the expression (Figure 5.12). In the conditions cultured at 20% O₂, the conditions cultured on Cytosoft plates had a higher gene expression than TCP. However, there were no significant differences between the conditions. MSX1 showed the same trend where there was a higher expression in all 2% O₂ conditions and in 20% O₂ conditions there was a high expression in samples grew on 0.2kPa with statistics showing no significant differences between the conditions (Figure 5.11).

SOX17, FOXA2 and AFP were used as endoderm markers (Figure 5.13-5.14). For SOX17 (Figure 5.13), cells cultured in 20% O₂ had higher expression than cells cultured in 2% O₂ and there were no significant differences between the conditions.
Figure 5.9 RT-qPCR analysis showing mean value of gene expressions **PAX6 at day 8**.

Figure shows RT-qPCR results for ectoderm marker **PAX6** for hiPSCs spontaneously differentiated on TCP and Cytosoft at day 8 under 20%O\textsubscript{2} and 2%O\textsubscript{2}. Statistical analysis carried out with 2-way ANOVA and where appropriate with post hoc testing using Tukey method using GraphPad Prism 7.03, n = 3 (biological triplicates), error bars represents standard deviation from mean. Significance difference between conditions are indicated, where *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001. Expression was normalised with β-actin (housekeeping gene) and then corrected with undifferentiated hiPSCs using 2^{\text{-ΔΔCt}}.
Figure 5.10 RT-qPCR analysis showing mean value of gene expressions for βIII TUBULIN at day 8.

Figure shows RT-qPCR results for ectoderm marker βIII TUBULIN for hiPSCs spontaneously differentiated on TCP and Cytosoft at day 4 under 20%O₂ and 2%O₂. Statistical analysis carried out with 2-way ANOVA and where appropriate with post hoc testing using Tukey method using GraphPad Prism 7.03, n = 3 (biological triplicates), error bars represents standard deviation from mean. Significance difference between conditions are indicated, where *p<0.05, **p<0.01, ***p<0.001 and ****P<0.0001. Expression was normalised with β-actin (housekeeping gene) and then corrected with undifferentiated hiPSCs using 2⁻ⁿΔΔCt.
Figure 5.11 RT-qPCR analysis showing mean value of gene expressions for MSX1 at day 8.

Figure shows RT-qPCR results for mesoderm marker MSX1 on TCP and four Cytosoft materials (64 kPa, 16 kPa, 2 kPa and 0.2 kPa) for spontaneously differentiated hiPSCs at day 8 under 20% O2 and 2% O2. Statistical analysis carried out with 2-way ANOVA and where appropriate with post hoc testing using Tukey method using GraphPad Prism 7.03, n = 3 (biological triplicates), error bars represents standard deviation from mean. Significance difference between conditions are indicated, where *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001. Expression was normalised with β-actin (housekeeping gene) and then corrected with undifferentiated hiPSCs using $2^{-\Delta\Delta C_t}$.
Figure 5.12 RT-qPCR analysis showing mean value of gene expressions for BRACHYURY at day 8

Figure shows RT-qPCR results for mesoderm marker BRACHYURY on TCP and four Cytosoft materials (64 kPa, 16 kPa, 2 kPa and 0.2 kPa) for spontaneously differentiated hiPSCs at day 8 under 20%O₂ and 2%O₂.

Statistical analysis carried out with 2-way ANOVA and where appropriate with post hoc testing using Tukey method using GraphPad Prism 7.03, n = 3 (biological triplicates), error bars represents standard deviation from mean. Significance difference between conditions are indicated, where *p<0.05, **p<0.01, ***p<0.001 and ****P<0.0001. Expression was normalised with β-actin (housekeeping gene) and then corrected with undifferentiated hiPSCs using 2^{-ΔΔCt}. 
Figure 5.13 RT-qPCR analysis showing mean value of gene expressions for SOX17 at day 8.

Figure shows RT-qPCR results for endoderm marker SOX17 on TCP and four Cytosoft materials (64 kPa, 16 kPa, 2 kPa and 0.2 kPa) for spontaneously differentiated hiPSCs at day 8 under 20%O₂ and 2%O₂.

Statistical analysis carried out with 2-way ANOVA and where appropriate with post hoc testing using Tukey method using GraphPad Prism 7.03, n = 3 (biological triplicates), error bars represents standard deviation from mean. Significance difference between conditions are indicated, where *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001. Expression was normalised with β-actin (housekeeping gene) and then corrected with undifferentiated hiPSCs using 2^{-ΔΔCt}. 

Relative gene expression normalised to iPSCs

Day 8 SOX17

Conditions

20% Oxygen TCP
20% Oxygen 64kPa
20% Oxygen 16kPa
20% Oxygen 2kPa
20% Oxygen 0.2kPa
20% Oxygen TCP
2% Oxygen 64kPa
2% Oxygen 16kPa
2% Oxygen 2kPa
2% Oxygen 0.2kPa

0
1
2
3
4
5
6
7
8
9
10
Relative gene expression normalised to iPSCs
Figure 5.14 RT-qPCR analysis showing mean value of gene expressions for AFP at day 8.

Figure shows RT-qPCR results for endoderm marker AFP on TCP and four Cytosoft materials (64 kPa, 16 kPa, 2 kPa and 0.2 kPa) for spontaneously differentiated hiPSCs at day 8 under 20%O\textsubscript{2} and 2%O\textsubscript{2}. Statistical analysis carried out with 2-way ANOVA and where appropriate with post hoc testing using Tukey method using GraphPad Prism 7.03, n = 3 (biological triplicates), error bars represents standard deviation from mean. Significance difference between conditions are indicated, where *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001. Expression was normalised with β-actin (housekeeping gene) and then corrected with undifferentiated hiPSCs using $2^{-\Delta\Delta C_t}$. 

Relative gene expression normalised to iPSCs (log 10)
Figure 5.15 RT-qPCR analysis showing mean value of gene expressions for FOXA2 at day 8.

Figure shows RT-qPCR results for endoderm marker FOXA2 on TCP and four Cytosoft materials (64 kPa, 16 kPa, 2 kPa and 0.2 kPa) for spontaneously differentiated hiPSCs at day 8 under 20%O₂ and 2%O₂. Statistical analysis carried out with 2-way ANOVA and where appropriate with post hoc testing using Tukey method using GraphPad Prism 7.03, n = 3 (biological triplicates), error bars represents standard deviation from mean. Significance difference between conditions are indicated, where *p<0.05, **p<0.01, ***p<0.001 and ****P<0.0001. Expression was normalised with β-actin (housekeeping gene) and then corrected with undifferentiated hiPSCs using 2-ΔΔCt.
Through this experiment, it implied that the combination of Young’s modulus and low oxygen tension does influence cell differentiation in that differentiated genes were less expressed in 2% O₂ than conditions cultured in 20% O₂. This can be due to us missing the “window of opportunity” as some markers are only expressed during early stages or stages of cell differentiation. It is shown that AFP and FOXA2, a later endoderm differentiation marker showed highly significant up regulation expression (Figure 5.14-5.15) where there was an 858-fold increase in AFP expression for 0.2kPa 2% O₂ compared to control (Figure 5.14). Moreover, there was also a significant difference between 0.2kPa 20% O₂ and 0.2kPa in 2% O₂. This trend was also seen in FOXA2, where there is a P<0.0001 value between 0.2kPa 2% O₂ compared to control and 0.2kPa in 20% O₂ (Figure 5.15). The high gene expression of AFP and FOXA2 implies that the combination of 0.2kPa and 2% O₂ affects endodermal differentiation outcome.

5.3.4 Cell Count and Cell Viability

After carrying out the RT-qPCR panel with 10 conditions, it was decided to narrow down the conditions to reduce costs. The conditions were narrowed down from 10 conditions (TCP, 64, 16, 2 and 0.2kPa all in 20% O₂ and 2%O₂) to 6 conditions mentioned above to cover the significant differences ranges which was TCP is control, 64kPa and 0.2kPa.

Figure 5.16 shows the cell count at day 1 and day 8, at day 1 there are no significant differences between all the conditions as equal amounts of cells (3 x 10⁵ cells) were seeded in each condition. At day 8, there are significantly lower cell count in 64kPa and 0.2kPa than TCP at both 20% O₂ and 2% O₂ with 2kPa in 2%
O2 with the lowest cell count at $3.98 \times 10^8$ cells/mL compared to TCP in 20% O$_2$ with $6.53 \times 10^8$ cells/mL.

Cell viability was calculated by the total cell number and cell viability which was recorded daily (data not shown) during the 8-day duration using the Vi-cell machine (Materials and Methods Section 2.2). Figure 5.17 exhibits cell viability of each condition TCP, 64kPa and 0.2kPa both in 20% O$_2$ and 2% O$_2$.

Data showed at day 1 that there are significant differences between TCP 20% O$_2$ to 64kPa in 2% O$_2$ and 0.2kPa in 2%O$_2$. This decrease indicated that the combination of Young’s modulus and low oxygen may have affected cell proliferation rates. At time point day 8, condition 0.2kPa in 2% O$_2$ oxygen had the lowest viability of cells. The same trend was seen on day 1 with no significant change. At both time points conditions in 2% O$_2$ have a lower viability percentage than the same substrate condition in 20% O$_2$. This data indicates that softer substrates and low O$_2$ conditions in combination as well as independently leads to lower viability in cell culture.
Figure 5.16 Total cells count of cell culture at day 1 and day 8 for conditions TCP, 64kPa and 0.2kPa in 20% O$_2$ and 2% O$_2$.

Total cells count of cell culture at day 1 and day 8 for conditions TCP, 64kPa and 0.2kPa in 20% O$_2$ and 2% O$_2$. Statistical analysis was performed using Two-Way Anova method using GraphPad Prism 7.03, n = 3 (biological triplicates), error bars represents standard deviation from mean. Significance difference between conditions are indicated, where *p<0.05, **p<0.01, ***p<0.001 and ****P<0.0001.
Figure 5.17 Viability of cell culture at day 1 and day 8 for conditions TCP, 64kPa and 0.2kPa in 20% O₂ and 2% O₂.

Relative cell viability = [\frac{\text{Absorbance}}{\text{Absorbance of control}}] \times 100. Statistical analysis was performed using Two-Way Anova method using GraphPad Prism 7.03, n = 3 (biological triplicates), error bars represents standard deviation from mean. Significance difference between conditions are indicated, where *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001.
5.3.5 Flow Cytometry of 2D Monolayered Spontaneous Differentiation

Human Induced Pluripotent Stem Cells (hiPSCs)

As day 4 RT-qPCR results mainly showed no significant differences between the conditions it was decided to carry out quantitative analysis using Flow cytometry with only day 8 samples. Flow cytometry (Figure 5.18) was carried out using pluripotency marker OCT4, BRACHYURY and AFP with isotype control used for gating (see Appendix I). The data showed that in the condition of 0.2kPa with 2% O₂ there was the highest percentage of AFP positive cells at 85% which correlates with RT-qPCR. Moreover, there were no positive populations of mesoderm and undifferentiated hiPSCs (data not shown). Single repeats of flow cytometry were carried out as supporting data to use in correlation with the RT-qPCR and immunocytochemistry results. Ectoderm was not a marker used in this flow cytometry assay as it was unavailable at the time the assay was carried out.
Figure 5.18 Comparing flow cytometry analysis showing positive percentage of hiPSCs monolayer spontaneous differentiation for OCT4, BRACHYURY and AFP.
5.3.6 Immunocytochemistry of 2D Monolayered Spontaneous Differentiation of Human Induced Pluripotent Stem Cells (hiPSCs)

Single repeats immunocytochemistry was carried out on the cell cultures as an additional assay to assess the population of the 3 germ layers visually, it was carried out as a confirmation assay to be used in evaluation alongside RT-qPCR and flow cytometry and was used to show special differentiation. Following on from RT-qPCR and flow cytometry, it was decided that six conditions would be investigated consisting of TCP (as control), 64kPa (as hard) and 0.2kPa (as soft) in 20% O\textsubscript{2} and 2% O\textsubscript{2}. Conditions were reduced to six as the RT-qPCR results showed that 16kPa and 2kPa did not have any significant differences. Cells were fixed and stained with differentiated antibodies for further characterization (Materials and Methods Section 2.5). The cells were stained positive for all the three germ layer markers, it is believed that this is due to their natural ability to form the three different germ layers. Here, we will be investigating to see the higher yield or gene expression of a germ layer.

The markers used for immunocytochemistry were \textit{AFP} (endoderm), $\beta$III \textit{TUBULIN} (ectoderm) and $\alpha$ – \textit{SMA} (mesoderm). Figures 5.19-5.21 exhibit all the staining, $\beta$III \textit{TUBULIN} and $\alpha$ – \textit{SMA} can be seen in all conditions. However, for \textit{AFP}, there is a higher expression of \textit{AFP} in 0.2kPa 20% O\textsubscript{2} and 0.2kPa in 2%O\textsubscript{2}. This \textit{AFP} positive results correlate with flow cytometry data and RT-qPCR.
Figure 5.19 Immunocytochemistry showing positive staining for endoderm marker *AFP*.

For conditions 20% O$_2$ (A) TCP, (B) 20% O$_2$ 64kPa, (C) 20% O$_2$ 0.2kPa, (D) 2% O$_2$ TCP, (E) 2% O$_2$ 64kPa and (F) 20% O$_2$
0.2kPa. In each condition there are phase contrast, *AFP* and DAPI images. (G) Secondary only staining was carried out as control: (i) secondary only mouse IgG (ii) secondary only mouse IgM All scale bar = 200um. Images were taken at 100X
ocular lens magnification using Evos® XL.
Figure 5.20 Immunocytochemistry showing positive staining for ectoderm marker βIII TUBULIN.

For conditions 20% O₂ (A) TCP, (B) 20% O₂ 64kPa, (C) 20% O₂ 0.2kPa, (D) 2% O₂ TCP, (E) 2% O₂ 64kPa and (F) 20% O₂ 0.2kPa. In each condition there are phase contrast, βIII TUBULIN and DAPI images. (G) Secondary only staining was carried out as control: (i) secondary only mouse IgG (ii) secondary only mouse IgM All scale bar = 200μm. Images were taken at 100X ocular lens magnification using Evos® XL.
Figure 5.21 Immunocytochemistry showing positive staining for mesoderm marker α-SMA.

For conditions 20% O$_2$ (A) TCP, (B) 20% O$_2$ 64kPa, (C) 20% O$_2$ 0.2kPa, (D) 2% O$_2$ TCP, (E) 2% O$_2$ 64kPa and (F) 20% O$_2$ 0.2kPa. In each condition there are phase contrast, α-SMA and DAPI images. (G) Secondary only staining was carried out as control: (i) secondary only mouse IgG (ii) secondary only mouse IgM. All scale bar = 200um. Images were taken at 100X ocular lens magnification using Evos® XL.
5.4 Discussion

Creating a higher yield of desired differentiated cells is a major hurdle for the successful translation of stem cell research into clinical applications. Creating a higher yield by manipulating the ECM without growth factors would result in a reduced overall cost as growth factors are expensive.

As mentioned, prior, ECM has been shown to influence cell behaviour. In this chapter, we looked at the combined effects of Young’s modulus and low oxygen tension on monolayer spontaneous differentiation of hiPSCs. Oxygen tension in cell culture and substrate stiffness are two factors that have been widely investigated independently. As far as we know, the experiments carried out in this chapter were the first study on the combined effects of Young’s modulus and low oxygen on hiPSCs. We first investigated on hiPSCs spontaneous differentiation in a monolayer culture. Monolayer culture was chosen so that the majority of cells were exposed to the substrate surface and the same oxygen tension during culture.

The data shows that Young’s modulus and low oxygen tension individually as well as in combination both have effects on the cell’s morphology in culture. Firstly, Sub-population morphology of cells was observed. This included cobblestone appearance, definite endoderm-like cells (Siller et al., 2016), star-shaped cells (Docheva et al., 2008; Haasters et al., 2009), mesenchymal progenitor cells (Moslem et al., 2013), MSC-like cells (Muraglia, Cancedda and Quarto, 2000), spindle-shaped cells, striated cells and primitive streak cells (Turner et al., 2014; Siller et al., 2016; Suchorska et al., 2017; Sierra et al., 2018). As hiPSCs have the ability to differentiate into the three-germ layers it is expected that they will be differentiated into all of them, especially as the protocol is a spontaneous
differentiation protocol. Moreover, it was found that cell colonies on the softer substrate did not spread as much as cells seeded on the harder substrates. This has also been reported in the literature (Lo et al., 2000; Yeung et al., 2005). Other noticeable differences were that the cell morphology of cells grown on the same Young’s modulus value but in different oxygen tensions. Generally, cells grown in 20% O₂ were bigger in size, elongated with accentuated membranes compared to cells grown at 2% O₂ (Figure 5.5). This has also been seen in literature low oxygen tension results in smaller cells and less accentuated membrane in hESCs and hiPSCs (Närvä et al., 2013). A similar morphological profile was also recorded with MSCs (Grayson et al., 2007; Estrada et al., 2012) and human dental pulp stem cells (DPSCs) (Ahmed et al., 2016). The morphological images alone do not indicate the existence of the cell type as further analysis would be required. These images may also represent different stages of cells and that individually or in combination Young’s modulus and low oxygen tension may be the inducer of cell morphology.

Viability data showed lower viability of cell population on soft substrate of 2kPa and 0.2kPa, although the mechanism behind how the ECM influences the cell proliferation and viability remains unclear, the trend was also reported in other studies (Peyton and Putnam, 2005; Subramanyam et al., 2011; Sun et al., 2018). The viability of all samples grown in 2% O₂ was lower than their counterpart grown in 20% O₂.

As this experiment has never been carried out before, we decided to have two-time points on day 4 and day 8 rather than one time point to collect more data and try to obtain a better understanding. As mentioned in Chapter 1, It is known that ECM can regulate stem cell fate. However, it is unknown how the combination
of Young’s modulus and low oxygen tension will affect the cell differentiation. At
day 4 gene expression results showed no significant differences between the
conditions except SOX17 (Figure 5.6) where there was a significant down
regulation of SOX17 between all Young’s modulus and low oxygen conditions
compared to TCP. This could be due to a missed “window of opportunity” as
SOX17 is an early endodermal marker with expression peaking at day 2-3
(Takayama et al., 2011). Moreover, RT-qPCR data at day 4 showed no significant
differences for βIII TUBULIN and BRACHYURY. However, day 8 showed no
significant differences between the control and the conditions for ectoderm and
mesoderm markers. For endoderm markers AFP and FOXA2, it was found that
there was a P<0.0001, highly significant difference between condition 0.2kPa 2%
O₂ compared to TCP and 0.2kPa 20% O₂ (Figure 5.14 – 5.15). As there were no
significant differences 0.2kPa or 2% O₂ independent conditions, it can be concluded
that the combined effects of Young’s modulus at 0.2 kPa and low oxygen tension
at 2% O₂ caused this gene up regulation. A summary of the RT-qPCR results can
be seen in Table 5.3 and Table 5.4.

Flow cytometry data allows us to quantitatively analyse the cell population.
It was found that the endoderm population was highest in 0.2kPa in 2%O₂ with 85%
of the population being positive for AFP compared to all other conditions at 80%
positive. This positive presence of AFP is used in conjunction with RT-qPCR data
to support the of the presence of AFP.

The mechanism on how substrate stiffness and low oxygen tension affects
cell behaviour is still unknown. However, it was established in this article that the
substrate stiffness affected mesodermal differentiation via the Wnt/β-catenin
pathway (Przybyla et al., 2016). It is also widely known that HIF-1α regulates Wnt/β-catenin pathway and improves mesodermal yield in hypoxic conditions (Medley et al., 2013). Although those publications are on Wnt/β-catenin pathway and mesoderm differentiation, it is known that Wnt/β-catenin pathway is essential for primitive streak formation, which gives rise to both mesoderm and endoderm germ layers (Ginsburg, Snow and McLaren, 1990; Tam et al., 2004; Gadue et al., 2006; Hay et al., 2008)

Immunostaining images show a positive expression for all three germ layer markers of α - SMA (mesoderm) and SOX17 (Ectoderm) in all conditions. AFP was positively expressed in conditions 0.2 kPa in 20% O₂ and TCP, 64 and 0.2 kPa in 2% O₂. Despite positive staining for all three-germ layer markers, there is a higher expression of AFP in the condition of 0.2kPa in 20% and 2% O₂. This AFP data correlates with flow cytometry and RT-qPCR data of high AFP in 0.2kPa 2%O₂ conditions.

The chapter’s results indicated that the combination of extracellular environment condition of Young’s modulus and low oxygen tension does affect the hiPSCs monolayer spontaneous differentiation. We were able to show high AFP expression in RT-qPCR and support it with flow cytometry and immunocytochemistry. However, further experiments would be required as more endodermal markers should be investigated. Moving on, we decided in our next chapter to investigate the effects of Young’s modulus and low oxygen tension on embryoid bodies as multiple differentiation protocols use embryoid bodies.
Table 5.3 Summary table of day 4 RT-qPCR results

<table>
<thead>
<tr>
<th>Tissue Culture Plastic (TCP)</th>
<th>Control Condition</th>
<th>Endoderm</th>
<th>Mesoderm</th>
<th>Ectoderm</th>
<th>Endoderm</th>
<th>Mesoderm</th>
<th>Ectoderm</th>
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<tbody>
<tr>
<td>20% Oxygen</td>
<td>2% Oxygen</td>
<td></td>
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<td>BRACHYURY</td>
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<tr>
<td>ß-III TUBULIN</td>
<td>ß-III TUBULIN</td>
<td>ß-III TUBULIN</td>
<td>ß-III TUBULIN</td>
<td>ß-III TUBULIN</td>
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<td>Increased</td>
</tr>
<tr>
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<td>Decreased</td>
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</table>
Table 5.4 Summary table of day 8 RT-qPCR results

<table>
<thead>
<tr>
<th>Tissue Culture Plastic (TCP)</th>
<th>20% Oxygen</th>
<th>Control Condition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Endoderm</td>
<td>Mesoderm</td>
</tr>
<tr>
<td>SOX17</td>
<td>Increased</td>
<td>Increased</td>
</tr>
<tr>
<td>FOXA2</td>
<td>Decreased</td>
<td>Increased</td>
</tr>
<tr>
<td>AFP</td>
<td>Decreased</td>
<td>Increased</td>
</tr>
<tr>
<td>BRACHYURY</td>
<td>Increased</td>
<td>Increased</td>
</tr>
<tr>
<td>MSX1</td>
<td>Decreased</td>
<td>Increased</td>
</tr>
<tr>
<td>β-III TUBULIN</td>
<td>Increased</td>
<td>Increased</td>
</tr>
<tr>
<td>PAX6</td>
<td>Decreased</td>
<td>Increased</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Tissue Culture Plastic (TCP)</th>
<th>2% Oxygen</th>
<th>Day 8</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Endoderm</td>
<td>Mesoderm</td>
</tr>
<tr>
<td>SOX17</td>
<td>Decreased</td>
<td>Increased</td>
</tr>
<tr>
<td>FOXA2</td>
<td>Decreased</td>
<td>Increased</td>
</tr>
<tr>
<td>AFP</td>
<td>Decreased</td>
<td>Increased</td>
</tr>
<tr>
<td>BRACHYURY</td>
<td>Decreased</td>
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<td>β-III TUBULIN</td>
<td>Decreased</td>
<td>Increased</td>
</tr>
<tr>
<td>PAX6</td>
<td>Decreased</td>
<td>Increased</td>
</tr>
</tbody>
</table>

**Significant increase: ** to TCP control and 20% 0.2kPa.

***Significant increase: **** to TCP control and 20% 0.2kPa.
6 Chapter 6: The Effects of Young’s Modulus and Low Oxygen Tension on 3D Aggregates on Spontaneous Differentiation of Human Induced Pluripotent Stem Cells (hiPSCs)

6.1 Introduction

There have been independent studies on the effect of low oxygen tension or Young’s modulus on stem cells as previously stated in Chapter 1. To the best of our knowledge, this is the first study on the combined effects of these two factors. In Chapter 5, we investigated the combined effects of Young’s modulus and low oxygen tension on the spontaneous differentiation of monolayer cultures. As there are two ways to differentiate cells, monolayer and embryoid bodies (EBs), here we will study the combined effects of Young’s modulus and low oxygen tension on EBs culture.

EB culture is a subject of interest, as multiple directed differentiation protocols of hiPSCs are based on EBs. EBs is an effective way to differentiate cells as it mimics embryogenesis (Itskovitz-Eldor et al., 2000). Moreover, 2D monolayer differentiation is not feasible for large scale production and EBs are the feasible way for large scale production, of cells for the industry (Sasaki et al., 2009; Zweigerdt, 2009; Tavassoli et al., 2018). As EBs differentiate into the three germ layers they are widely used as a differentiation method as it mimics early embryonic development. Examples include cardiomyocytes (Müller et al., 2000; Kehat et al.,
osteoblasts (Sottile, Thomson and McWhir, 2003; Bielby et al., 2005), hepatocytes (Rambhatla et al., 2003) and neurons (Carpenter et al., 2001; Schuldiner et al., 2001; Zhang et al., 2001).

As previously mentioned in Chapter 1, low oxygen has been shown to facilitate stem cell culturing. Along with stem cell maintenance (Ivanovic et al., 2000; Studer et al., 2000; Simon and Keith, 2008), and monolayer differentiation (Khan, Adesida and Hardingham, 2007), it also improves EBs differentiation capabilities (Correia et al., 2014) EBs cultured in low oxygen (< 20% O₂) have also been widely investigated, and have been shown to enhance the differentiated outcome of endothelial cells when cultured in 5% O₂ (Kusuma et al., 2014), cardiomyocytes at 4% O₂ (Correia et al., 2014), neurons and higher expression of ectodermal gene expression in hiPSCs preconditioned at 2% O₂ (Fynes et al., 2014).

Another factor in this study is Young’s modulus of the substrate. Along with monolayer differentiation, studies have been carried out with EBs differentiation on various Young’s modulus. Studies include the effect of Young’s modulus on EBs differentiation of osteogenic cells (Evans et al., 2009), neuronal (Macri-Pellizzeri et al., 2015), cardiomyocytes (Chung et al., 2012; Shkumatov, Baek and Kong, 2014) and endodermal lineages (Jaramillo et al., 2012).

Cells are affected by substrate through mechanotransduction. As these cells are adherent cells, they attach themselves to surfaces through proteins called integrins. The cell’s integrin continuously applies forces to the substrate and effects how the cell actin cytoskeletal is structured (Discher, Janmey and Wang, 2005). Cytoskeleton have been known to regulate various pathways including Rho-A (McBeath et al., 2004; Keung et al., 2011) and Wnt (Hoppler and Nakamura, 2014).
which are both key regulators of the cytoskeleton (Hall, 1998; Kaibuchi, Kuroda and Amano, 1999; Jaffe and Hall, 2005; Komiya and Habas, 2008). For example, hESCs grown on soft 60kPa substrates underwent mesoderm differentiation through the Wnt pathway (Przybyla et al., 2016) and hESCs and hiPSCs have been grown on 0.1kPa resulted in ectodermal lineages once Rho-A was inhibited (Kueng et al., 2012).

In the previous Chapter 5, we investigated the effects of Young’s modulus and low oxygen tension on monolayer spontaneous differentiation, here we will explore the effects of Young’s modulus and low oxygen (2% O$_2$) tension on EBs spontaneous and directed differentiation. The difference between 2D monolayer culture and 3D EBs culture is that with the 3D culture there are natural occurring cell-cell interactions (Konno et al., 2005; Koike et al., 2007; Kurosawa, 2007; Bratt-Leal, Carpenedo and McDevitt, 2009; Gothard et al., 2009). The reason for directed differentiation studies is because it will constitute to another step towards mimicking in vivo conditions by attempting to complete the extracellular environment myriad by introducing exogenous factors into the cell culture media.

To the best of our knowledge, there have not been any research carried out on the combined effect of Young’s modulus with low oxygen. We hypothesized that the effect of Young’s modulus and low oxygen tension will have a significant positive effect on hiPSCs EBs differentiation due to more cell-cell interaction, in particular the soft substrates would differentiate cells towards ectodermal and endodermal lineages and harder substrates towards the mesodermal lineages. Germ layer differentiation would depend on the stiffness of the microenvironment of the cell’s in vivo conditions with respect to the microenvironment.
6.2 Aims and Objectives

The aim of this chapter was to determine if the combination of Young’s modulus and low oxygen tension had an effect on EBs spontaneous and EBs directed differentiation of hiPSCs. The markers used are what were available at the time when the assays were carried out.

The chapter’s objectives were:

- To establish and characterise EB size
- To study the impact of Young’s modulus (64kPa, 16kPa, 2kPa and 0.2kPa) and low oxygen tension (2% O₂) on EBs spreading on substrates using ImageJ.
- To study the impact of Young’s modulus (64kPa, 16kPa, 2kPa and 0.2kPa) and low oxygen tension (2% O₂) on spontaneous differentiation of EBs using analytical methods of:
  - Using qRT-PCR panel of differentiation markers
    - endoderm: AFP, SOX17, GATA4
    - mesoderm: BRACHYURY, MiXL1
    - ectoderm: β III TUBULIN, PAX6
  - Immunocytochemistry of differentiation markers of the three germ layers: SOX17, β III TUBULIN and BRACHYURY.
  - Flow cytometry using differentiation markers of the three germ layers: SOX17, β III TUBULIN and BRACHYURY.
Study the impact of Young’s modulus (64kPa, 16kPa, 2kPa and 0.2kPa) and low oxygen tension (2% O₂) on directed differentiation of EBs using analytical methods of:

- RT-qPCR panel of differentiation markers
  - endoderm: AFP, SOX17, FOXA2, CXCR4 and GATA4
- Immunocytochemistry of differentiation markers of endoderm marker: SOX17

6.3 Results

6.3.1 Experimental Plan on The Effects of Young’s Modulus and Low Oxygen Tension on 3D Aggregates on Spontaneous Differentiation of Human Induced Pluripotent Stem Cells (hiPSCs)

EBs were produced using hiPSCs via forced aggregation using the Aggrewell™ plates. Single hiPSCs were cultured on Aggrewell™ plates for 24 hours before being harvested. The harvested EBs were then cultured on Matrigel-coated TCP and Cytosoft 6-well plates of various Young’s modulus value (64, 16, 2 and 0.2kPa) and cultured in 20% O₂ or 2% O₂. Cells were allowed to spontaneously differentiate for 8 days (consistent time point as Chapter 5 protocol) and then collected for analysis (Figure .6.1) (Materials and Methods Section 2.4).
Figure 6.1 3D EBs spontaneous differentiation protocol experimental setup.

i) Experimental plan: EBs were produced using Aggrewell™ plates and seeded on Matrigel coated TCP, 64, 16, 2 and 0.2kPa 6-well plates and incubated for 8 days in 20% O₂ and 2% O₂. ii) Diagram showing EBs spread on different Young's modulus substrates.
6.3.2 Embryoid Bodies (EBs) size

It was of utmost importance that all EBs were of similar size, as it is known that different size EBs can affect the outcome of differentiation (Ng et al., 2005; Bauwens et al., 2008; Hwang et al., 2009; Niebruegge et al., 2009; Choi et al., 2010; Mohr et al., 2010; Yanai et al., 2013). For the experiments, it was decided that the EBs size would be 400µm, this was based on literature as it was found that 400µm sized EBs influences endoderm commitment (Niebruegge et al., 2009; Cha et al., 2015). Moreover, large EBs size >800µm were not chosen due to mass transfer limitations which may lead to necrosis (Dang et al., 2004; Van Winkle, Gates and Kallos, 2012). As the aim of this thesis was to improve the bioprocessing platform of cell culture, we also had to consider the ease of the EBs size for large scale culture. Figure 6.2 shows phase contrast of hiPSCs seeded onto AggreWells™ at day 0 and day 1 when collection occurs. Figure 6.3i shows how the EBs were measured using ImageJ and Figure 6.3ii shows size distribution graph from measurements collected from 130 EBs. The data indicated that the average EB size used was 400µm and there were no significant differences between the samples.
Figure 6.2 Phase contrast images of EBs production.

At (i) Day 0 seeded on AggreWells™, (ii) Day 1 on Aggrewells™ and (iii) Day 1 after harvested from Aggrewells™ and seeded onto 6-well plates. All scale bars = 400 µm. Images were taken at 100X ocular lens magnification using Evos® XL.
Figure 6.3 EB size distribution.

(i) Phase contrast images of EBs diameters measurements taken as averages of 3 measurements per EB (dorso-ventral, horizontal and diagonal. All scale bars = 400 µm. Images were taken at 100X ocular lens magnification using Evos® XL. (ii) EB size distribution plot for n=130. Statistical analysis was carried out with 2-way ANOVA with post hoc using Tukey method using GraphPad Prism 7.03, error bars represents the standard deviation from the 3 measurements per EB. No statistical significance was found between the samples.
6.3.3 Morphology of Spontaneous Differentiation

Figure 6.4-6.5 shows the morphology of the EBs on each of the condition on day 2, 4 and 8. Similarly, in the previous chapter, there was a mixture of various sub-population exhibited in all the conditions. The morphology that is highly expressed in the 0.2kPa, 2% O\textsubscript{2} condition are endoderm-like cells with hexagonal cobblestone shapes (Siller \textit{et al.}, 2016) and are hepatocyte-like cells (Cai \textit{et al.}, 2012). Figure 6.6 highlights sub-populations seen in the cultures which included elongated striated cells (Docheva \textit{et al.}, 2008; Haasters \textit{et al.}, 2009), mesenchymal-like cells (Muraglia, Cancetta and Quarto, 2000; Moslem \textit{et al.}, 2013), endodermal-like cells and primitive streak-like cells (Turner \textit{et al.}, 2014; Siller \textit{et al.}, 2016; Suchorska \textit{et al.}, 2017; Sierra \textit{et al.}, 2018). Morphology of sub-populations were seen throughout all the conditions. However, Figure 6.6 shows examples of sub-populations of neural progenitors-like cells in cultures grown in 2kPa at 2% O\textsubscript{2} (Figure 6.6i), spindle shaped-like cells found in conditions grown on 16kPa at 2% O\textsubscript{2} (Figure 6.6ii), MSC-like progenitors found in 64kPa at 20% O\textsubscript{2} (Figure 6.6iii), primitive streak-like cells on 16kPa at 2% O\textsubscript{2} (Figure 6.6iv), endoderm-like cells (Figure 6.6v) and immature hepatocyte-like cells present in 0.2kPa at 2% O\textsubscript{2} (Figure 6.6vi).
Figure 6.4 Images of EBs differentiated hiPSCs on Cytosoft at 20% O₂.

Images of EBs on TCP and four Cytosoft materials (64 kPa, 16 kPa, 2 kPa and 0.2 kPa) in 20% O₂ at time point day 2, 4 and day 8, for each Young’s modulus condition. All scale bars = 400 µm. Images were taken at 100X ocular lens magnification using Evos® XL.
Figure 6.5 Images of EBs differentiated hiPSCs on Cytosoft at 2% O$_2$.

Images of EBs on TCP and four Cytosoft materials (64 kPa, 16 kPa, 2 kPa and 0.2 kPa) in 2% O$_2$ at time point day 2, 4 and day 8, for each Young's modulus condition. All scale bars = 400 µm. Images were taken at 100X ocular lens magnification using Evos® XL.
Figure 6.6 Phase contrast images of differentiated exhibiting sub-population morphology.

Images of (i) neural progenitors-like cells, (ii) striated spindle shaped, (iii) MSC progenitors, (iv) primitive streak-like cells (v) endoderm-like cells, (vi) immature hepatocyte-like cells (vii) All scale bars = 200 µm. Images were taken at 100X ocular lens magnification using Evos® XL.
6.3.4 Embryoid Bodies (EBs) Spread on Different Young’s Modulus

Once we established the EB size, the EBs spread in different conditions were monitored. The EBs were seeded onto Matrigel-coated TCP and Cytosoft 6 well plates (Materials and Methods Section 2.4). Phase contrast images were taken daily, and measurements were carried out using the ImageJ software (Figure 6.8). The EBs spread was measured using the ImageJ software where 3 diameters measurements were taken (dorso-ventral, horizontal and diagonal) on each EB and an average of those 3 measurements were calculated (Figure 6.8). Figure 6.7 exhibit EBs has grown on TCP, 64kPa and 0.2kPa in 20% O\textsubscript{2} and 2% O\textsubscript{2} between day 1 and 4. These two time points were chosen as beyond day 4 the EBs were spread too wide for the microscope to capture in its frame.

Figure 6.8 shows significant differences between the size of the EBs between each Young’s modulus condition (64 and 0.2kPa in both 20% O\textsubscript{2} and 2% O\textsubscript{2}) to TCP at 20% O\textsubscript{2} (p<0.05 and P<0.01 respectively) at day 1 and highly significant day 4 (p<0.001 and p<0.0001 respectively), as well as a significant difference between the cultures grown on Young’s modulus value 64kPa at 20% O\textsubscript{2} and 2% O\textsubscript{2} at day 1 (p<0.01). However, there were no significant differences between 64kPa at day 4 between the 2 oxygen concentrations and 0.2kPa at 20% O\textsubscript{2} and 2% O\textsubscript{2}. This signifies that oxygen tension and Young’s modulus may both independently effect EBs spread, nevertheless, this data signifies that Young’s modulus influences the EBs spread more than the oxygen tension as there are higher significant between cultures grown on different Young’s modulus on the same oxygen concentration to TCP control than cells grown on the same Young’s modulus condition in different oxygen concentration.
Figure 6.7 Phase contrast images of EB spread Cytosoft at day 1, 4 and 8.

Images of EBs on TCP and Cytosoft (64 kPa, 16 kPa, 2 kPa and 0.2 kPa) in 20% O₂ at time point day 2, 4 and day 8, for each Young’s modulus condition. All scale bars = 400 µm. Images were taken at 100X ocular lens magnification using Evos® XL.
Figure 6.8 EB spreading distribution.

(i) Phase contrast images of EBs diameters measurements taken as averages of 3 measurements per EB (dorso-ventral, horizontal and diagonal). All scale bars = 400 µm. Images were taken at 100X ocular lens magnification using Evos® XL. (ii) EB spread distribution plot between day 1 and day 4 for n= 20. Statistical analysis carried out with 2-way ANOVA and where appropriate with post hoc testing using Tukey method using GraphPad Prism 7.03, n = 3 (biological triplicates), error bars represents standard deviation from mean. Significance difference between conditions are indicated, where *p<0.05, **p<0.01, ***p<0.001 and ****P<0.0001.
6.3.5 Quantitative Polymerase Chain Reaction (RT-qPCR) of Spontaneous Differentiation

Similarly to the spontaneous differentiation experiments on 2D monolayer culture, the same protocol was carried out on EBs. However, it was decided to have only one time point at day 8 to allow the cells to be differentiated (Materials and Methods Section 2.4). RT-qPCR was carried out using multiple markers for the three germ layers (Table 6.1).

The result demonstrates that EBs differentiation leans towards endodermal lineages as there was significant up-regulation in all the five endodermal markers used in response to cells grown on 0.2kPa in 2% O\(_2\) compared to TCP control. AFP results (Figure 6.9i) shows highly significant differences between cells grown on 0.2kPa 20% O\(_2\) (1,810,000-fold increase) and 0.2kPa 2% O\(_2\) (4,190,000-fold increase) in relative to the control, this high AFP expression is usually expressed once endoderm specific cells start to differentiate to hepatic progenitor cells (hepatoblast) (Freyer et al., 2016; Yu et al., 2012). There was also a significant increase in SOX17 expression in 0.2kPa 20% O\(_2\) (512-fold increase) and 2% O\(_2\) (385-fold increase) compared to control (Figure 6.10). Regarding FOXA2 there were significantly high 121-fold increase in gene expression between control to 0.2kPa in 2% O\(_2\), but no significant differences between control and 0.2 kPa in 20% O\(_2\) (Figure 6.11). For endodermal marker GATA4, as well as having a significant difference between 0.2kPa in 20% O\(_2\) (59-fold increase) and 2% O\(_2\) (48-fold increase) to control (Figure 6.12). There were also significant differences between cells grown on 64kPa in 20% O\(_2\) and 2%O\(_2\) (Figure 6.12). In contrast to the other
endodermal lineage marker, CXCR4 had significant differences between control and all other conditions except 0.2kPa in 20% O₂ and 2%O₂ (Figure 6.13). These endodermal markers demonstrate that low Young’s modulus alone and the combination of Young’s modulus and low oxygen had an influence on cell differentiation as samples cultured on 0.2kPa in both 20% O₂ and 2% O₂ had an increase in endodermal expression. However, cells cultured on TCP at 2% O₂ did not.

The differentiation into mesoderm lineage was also induced, however, high gene expression was only seen in cultures in 20% O₂ and gene expression was suppressed in cultures in 2% O₂. With BRACHYURY, there was a significant increase in the expression between all conditions grown in 2% O₂ and control, moreover, there were also significant differences between each Young’s modulus and their counterpart in 2% O₂. In 20% O₂, there was a significant up-regulation between 64kPa and control (Figure 6.14). Figure 6.15 shows gene expression of another mesodermal lineage MiXL1. The data displays a similar trend to BRACHYURY results where there was a suppression of gene expression in samples cultured in 2% O₂. Statistical analysis indicated significant differences between the control and all the 9 other samples. Likewise, there were also significant differences between each Young’s modulus and their counterpart in 2% O₂. Mesodermal linages gene expression implied that Young’s modulus independently influences mesodermal lineages and low oxygen tension suppresses mesodermal gene expression, there were no influences caused by the combined effects of Young’s modulus and low oxygen tension.
Figure 6.16-6.17 shows the ectodermal gene expression. Figure 6.16 displays \( \beta III TUBULIN \) gene expression and revealed a significant increase between 2kPa and 0.2kPa in 2% \( O_2 \) in relative to control. \( PAX6 \) displayed a significant increase in gene expression between 0.2kPa in 2% \( O_2 \) to control. There were also significant differences between EBs cultured on 2kPa and 0.2kPa in 20% \( O_2 \) to their counterpart in 2% \( O_2 \) (Figure 6.17). This data suggests that the combination of Young’s modulus and low oxygen influences the increase in ectodermal expression.

These data imply that germ layers can be influenced by Young’s modulus or low oxygen independently but by combining the two extracellular environments, it can also significantly induced lineage gene expression, especially for endodermal lineages. As seen in cells grown on 0.2kPa in 20% \( O_2 \) and 2% \( O_2 \), with an increased endoderm gene expression \( AFP, SOX17, FOXA2 \) and \( GATA4 \) where 0.2kPa in 2% \( O_2 \) where highly significant compared to TCP control.

<table>
<thead>
<tr>
<th>Germ layer</th>
<th>marker</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ectoderm</td>
<td>( AFP, SOX17, GATA4, CXCR4 ) and ( FOXA2 )</td>
</tr>
<tr>
<td>Endoderm</td>
<td>( PAX6 ) and ( \beta III Tubulin )</td>
</tr>
<tr>
<td>Mesoderm</td>
<td>( BRACHYURY ) and ( MiXL1 )</td>
</tr>
</tbody>
</table>
Figure 6.9 RT-qPCR analysis showing mean value of gene expressions for endoderm marker AFP and SOX17 for spontaneously differentiated EBs. Figure showing RT-qPCR data of endoderm marker AFP on TCP and four Cytosoft materials (64 kPa, 16 kPa, 2 kPa and 0.2 kPa) for spontaneously differentiated EBs at day 8 under 20%O\textsubscript{2} and 2%O\textsubscript{2}. Statistical analysis carried out with 2-way ANOVA and where appropriate with post hoc testing using Tukey method using GraphPad Prism 7.03, n = 3 (biological triplicates), error bars represents standard deviation from mean. Significance difference between conditions are indicated where *p<0.05, **p<0.01 and ***p<0.001. Expression was normalized with β-actin (housekeeping gene) and then corrected with undifferentiated hiPSCs using 2\textsuperscript{-ΔΔCt}. 

![Day 8 AFP graph](image-url)
Figure 6.10 RT-qPCR analysis showing mean value of gene expressions for endoderm marker SOX17 for spontaneously differentiated EBs.

Figure showing RT-qPCR data of endoderm marker SOX17 on TCP and four Cytosoft materials (64 kPa, 16 kPa, 2 kPa and 0.2 kPa) for spontaneously differentiated EBs at day 8 under 20%O₂ and 2%O₂. Statistical analysis carried out with 2-way ANOVA and where appropriate with post hoc testing using Tukey method using GraphPad Prism 7.03, n = 3 (biological triplicates), error bars represents standard deviation from mean. Significance difference between conditions are indicated where *p<0.05, **p<0.01 and ***p<0.001. Expression was normalized with β-actin (housekeeping gene) and then corrected with undifferentiated hiPSCs using 2^(-∆∆Ct).
Figure 6.11 RT-qPCR analysis showing mean value of gene expressions for endoderm marker *FOXA2* for spontaneously differentiated EBs.

Figure showing RT-qPCR data of endoderm marker *FOXA2* on TCP and four Cytosoft materials (64 kPa, 16 kPa, 2 kPa and 0.2 kPa) for spontaneously differentiated EBs at day 8 under 20%O\textsubscript{2} and 2%O\textsubscript{2}. Statistical analysis carried out with 2-way ANOVA and where appropriate with post hoc testing using Tukey method using GraphPad Prism 7.03, n = 3 (biological triplicates), error bars represents standard deviation from mean. Significance difference between conditions are indicated where *p<0.05, **p<0.01 and ***p<0.001.

Expression was normalized with β-actin (housekeeping gene) and then corrected with undifferentiated hiPSCs using \(2^{-\Delta\Delta\text{Ct}}\).
Figure 6.12 RT-qPCR analysis showing mean value of gene expressions for endoderm marker *GATA4* for spontaneously differentiated EBs.

Figure showing RT-qPCR data of endoderm marker *GATA4* on TCP and four Cytosoft materials (64 kPa, 16 kPa, 2 kPa and 0.2 kPa) for spontaneously differentiated EBs at day 8 under 20%O<sub>2</sub> and 2%O<sub>2</sub>. Statistical analysis carried out with 2-way ANOVA and where appropriate with post hoc testing using Tukey method using GraphPad Prism 7.03, n = 3 (biological triplicates), error bars represents standard deviation from mean. Significance difference between conditions are indicated where *p*<0.05, **p*<0.01 and ***p*<0.001. Expression was normalized with β-actin (housekeeping gene) and then corrected with undifferentiated hiPSCs using 2<sup>-ΔΔCt</sup>. 
Figure 6.13 RT-qPCR analysis showing mean value of gene expressions for endoderm marker CXCR4 for spontaneously differentiated EBs.

Figure showing RT-qPCR data of endoderm marker CXCR4 on TCP and four Cytosoft materials (64 kPa, 16 kPa, 2 kPa and 0.2 kPa) for spontaneously differentiated EBs at day 8 under 20%O_2 and 2%O_2. Statistical analysis carried out with 2-way ANOVA and where appropriate with post hoc testing using Tukey method using GraphPad Prism 7.03, n = 3 (biological triplicates), error bars represents standard deviation from mean. Significance difference between conditions are indicated where *p<0.05, **p<0.01 and ***p<0.001. Expression was normalized with β-actin (housekeeping gene) and then corrected with undifferentiated hiPSCs using 2^{-ΔΔCt}. 
Figure 6.14 qPCR analysis showing mean value of gene expressions for mesoderm marker *BRACHYURY* for spontaneously differentiated EBs.

Figure showing RT-qPCR data of mesoderm marker BRACHYURY on TCP and four Cytosoft materials (64 kPa, 16 kPa, 2 kPa and 0.2 kPa) for spontaneously differentiated EBs at day 8 under 20%O$_2$ and 2%O$_2$. Statistical analysis carried out with 2-way ANOVA and where appropriate with post hoc testing using Tukey method using GraphPad Prism 7.03, n = 3 (biological triplicates), error bars represents standard deviation from mean. Significance difference between conditions are indicated where *p<0.05, **p<0.01 and ***p<0.001. Expression was normalized with β-actin (housekeeping gene) and then corrected with undifferentiated hiPSCs using $2^{-\Delta\Delta Ct}$.
Figure 6.15 RT-qPCR analysis showing mean value of gene expressions for mesoderm marker *MiXL1* for spontaneously differentiated EBs.

Figure showing RT-qPCR data of mesoderm marker *MiXL1* on TCP and four Cytosoft materials (64 kPa, 16 kPa, 2 kPa and 0.2 kPa) for spontaneously differentiated EBs at day 8 under 20%O₂ and 2%O₂. Statistical analysis carried out with 2-way ANOVA and where appropriate with post hoc testing using Tukey method using GraphPad Prism 7.03, n = 3 (biological triplicates), error bars represents standard deviation from mean. Significance difference between conditions are indicated where *p<0.05, **p<0.01 and ***p<0.001.

Expression was normalized with β-actin (housekeeping gene) and then corrected with undifferentiated hiPSCs using 2^{-ΔΔCt}. 
Figure 6.16 RT-qPCR analysis showing mean value of gene expressions for ectoderm marker βIII TUBULIN for spontaneously differentiated EBs.

Figure showing RT-qPCR data of mesoderm marker βIII TUBULIN on TCP and four Cytosoft materials (64 kPa, 16 kPa, 2 kPa and 0.2 kPa) for spontaneously differentiated EBs at day 8 under 20%O₂ and 2%O₂.

Statistical analysis carried out with 2-way ANOVA and where appropriate with post hoc testing using Tukey method using GraphPad Prism 7.03, n = 3 (biological triplicates), error bars represents standard deviation from mean. Significance difference between conditions are indicated where *p<0.05, **p<0.01 and ***p<0.001. Expression was normalized with β-actin (housekeeping gene) and then corrected with undifferentiated hiPSCs using 2^{−ΔΔCt}.
Figure 6.17 Figure 6.14 RT-qPCR analysis showing mean value of gene expressions for ectoderm marker PAX6 for spontaneously differentiated EBs.

Figure showing RT-qPCR data of ectoderm marker PAX6 on TCP and four Cytosoft materials (64 kPa, 16 kPa, 2 kPa and 0.2 kPa) for spontaneously differentiated EBs at day 8 under 20%O\textsubscript{2} and 2%O\textsubscript{2}. Statistical analysis carried out with 2-way ANOVA and where appropriate with post hoc testing using Tukey method using GraphPad Prism 7.03, n = 3 (biological triplicates), error bars represents standard deviation from mean. Significance difference between conditions are indicated where *p<0.05, **p<0.01 and ***p<0.001. Expression was normalized with β-actin (housekeeping gene) and then corrected with undifferentiated hiPSCs using 2^{∆∆Ct}. 
6.3.6 Immunocytochemistry of Spontaneous Differentiation

After carrying out RT-qPCR all the 10 conditions, we decided to narrow it down to 2 conditions, where TCP represents control and 0.2kPa as the soft substrate to study the protein expression via immunostaining. The reason behind this decision was because 0.2kPa in 2%O₂ showed significant results. Figure 6.18 and 6.19 show positive staining for endoderm marker AFP and SOX17 on cells grown in condition 0.2kPa in 2% O₂ compared to negative staining in TCP in 20% O₂. This immunocytochemistry provides evidence to support the RT-qPCR data.
Figure 6.18 Immunocytochemistry showing positive staining for endoderm marker *AFP*.

Where (A) 20% O₂ TCP and (B) 2% O₂ 0.2kPa. In each condition where i) phase contrast, ii) *AFP* and iii) *DAPI*. (C) Secondary only staining was carried out as control: (i) secondary only mouse IgG (ii) secondary only mouse IgM. All scale bar = 200μm. Images were taken at 100X ocular lens magnification using Evos® XL.
Figure 6.19 Immunocytochemistry showing positive staining for endoderm marker SOX17.

Where (A) 20% O₂ TCP and (B) 2% O₂ 0.2kPa. In each condition where i) phase contrast, ii) SOX17 and iii) DAPI. (C) Secondary only staining was carried out as control: (i) secondary only mouse IgG (ii) secondary only mouse IgM. All scale bar = 200um. Images were taken at 100X ocular lens magnification using Evos® XL.
6.3.7 Flow Cytometry of Spontaneous differentiation

To further support the RT-qPCR and immunocytochemistry data, quantitative flow cytometry analysis was performed (Figure 6.20). In this instance four conditions were used for flow cytometry analysis, whereby three were used as controls TCP 20% O$_2$, TCP 2% O$_2$ and 0.2kPa 20% O$_2$ in relation to the condition in question which was 0.2kPa in 2% O$_2$. Cells were stained with conjugated endoderm marker AFP. As can be seen in Figure 6.17 condition 0.2kPa in 2% O$_2$ had the highest positive population of AFP positive cells at 77.6% compared to 9.22% TCP and 35.9% 0.2kPa 20% O$_2$. This supports the immunocytochemistry and RT-qPCR data.
Figure 6.20 Flow cytometry analysis showing EBs spontaneous differentiation staining of *AFP*.

Where (i) is isotype control, (ii) TCP 20% O2, (iii) 0.2kPa 20% O2, (iv) TCP 2% O2 and (v) 0.2kPa 2% O2.
6.3.8 The Effects of Young’s Modulus and Low Oxygen Tension on 3D Aggregates on Spontaneous Differentiation of Human Induced Pluripotent Stem Cells (hiPSCs) Cell Line: MSU001

In order to ensure that these results were not cell line-specific, we carried out spontaneous differentiation experiments on EBs made from another hiPSCs cell line, MSU001 (characterised in Chapter 3). RT-qPCR of *AFP* was carried out on hiPSCs (MSU001) grown on TCP, 64 and 0.2 kPa grown in 2% O2 compared to TCP grown in 20% O2. Much like hiPSCs (cord blood) data, Figure 6.21 shows RT-qPCR data of endoderm marker *AFP* on hiPSCs (MSU001) and it shows the same trend of an increase in gene expression of *AFP*. Data shows highly significant differences (P<0.0001) in *AFP* expression between 0.2kPa 2% O2 in relation to control TCP. This data gives evidence that the combined effects of soft 0.2kPa substrate and 2% O2 seen with hiPSCs (cord blood) produced in this thesis is not cell line specific.
Figure 6.21 RT-qPCR analysis showing mean value of gene expressions for endoderm marker *AFP* for spontaneously differentiated hiPSCs (MSU001) cell line.

hiPSCs MSU0001 cell line on TCP and 2 Cytosoft materials (64 kPa and 0.2 kPa) for spontaneously differentiated hiPSCs at day 8 under 20% O\textsubscript{2} and 2% O\textsubscript{2}. Statistical analysis carried out with 2-way ANOVA and where appropriate with post hoc testing using Tukey method using GraphPad Prism 7.03, n = 3 (biological triplicates), error bars represents standard deviation from mean. Significance difference between condition are indicated where *p<0.05, **p<0.01, ***p<0.001 and ****P<0.0001 showing Expression was normalized with β-actin (housekeeping gene) and then corrected with undifferentiated hiPSCs using 2^{-ΔΔCt}. 
6.3.9 The Effects of Young’s Modulus and Low Oxygen Tension on 3D Aggregates on Directed Differentiation of Human Induced Pluripotent Stem Cells

After studying the effect of Young’s modulus ranges and low oxygen tension on spontaneous differentiation of EBs on the three germ layers formation. The results suggested that the combined effect of Young’s modulus and low oxygen could influence the fate of stem cell differentiation. It was shown earlier in this chapter that cells were grown on Young’s modulus 0.2kPa in 2% O₂ influenced cells to differentiate towards the endoderm lineage based on increased gene expression of AFP, SOX17, FOXA2, GATA4 and CXCR4. Due to the previous data on spontaneous differentiation, we then wanted to investigate using directed differentiation protocols to complete the extracellular environment myriad and determine if the combination of Young’s modulus, low oxygen tension and endoderm driving growth factors would further increase the cell differentiation outcome.

6.3.10 Experimental Plan Directed differentiation

We have shown that the combination of Young’s modulus and low oxygen tension had a positive effect on endoderm differentiation of EBs when allowed to spontaneously differentiate. For directed differentiation into endoderm germ layer 100ng/ml of ACTIVIN A was used as in the majority of endoderm directed differentiated protocols 100 ng/ml ACTIVIN A was used (McLean et al., 2007; Hay et al., 2008; Basma et al., 2009; Cai, 2014; Guo et al., 2014; Carpentier et al., 2016; Ang et al., 2018), and therefore based on the literature, we decided to adapt it and only use ACTIVIN A in our directed differentiation protocol. ACTIVIN A is a growth
factor in the transforming growth factor beta (TGF-β) family of proteins (Ling et al., 1986; Piek, Heldin and Dijke, 1999; Pangas and Woodruff, 2000). Adding ACTIVIN A to the culture media is an important step in order to complete the extracellular environment (Figure 6.22i), as in vivo cells are exposed to growth factors that drive their differentiation outcome. We hypothesised that with the addition of ACTIVIN A to Young’s modulus and low oxygen conditions, there would be a higher gene expression of the endodermal lineages.

Using the EB protocol, hiPSCs (cord blood) were plated on Matrigel-coated TCP and Cytosoft 6-well plates at conditions TCP and 0.2kPa 20% O₂ and 2% O₂. ACTIVIN A was added to the spontaneous differentiation medium at a concentration of 100ng/mL. Cells were collected at day 3, 5 and 8 for RT-qPCR analysis for of endoderm markers (SOX17, AFP and GATA4) (Materials and Methods Section 2.41) (Figure 6.22ii). Timepoints day 8 was selected for these directed differentiation experiments because the spontaneous differentiation experiments were carried out for 8 days and therefore, we wanted to have the same timepoint to be able to compare the results. Timepoint day 3 and 5 was selected as commercial endoderm directed differentiated media protocols such as Thermo Fisher Scientific’s PSC Definative Endoderm Induction Kit (catalogue number A3062601) and Merck’s Definite Endoderm Induction Medium (catalogue number SCM302) are 3 days long. Additionally, STEMdiff™ Definitive Endoderm Kit by Stemcell Technologies (catalogue number 05110) is day 5 long and therefore we wanted to investigate if the combination of Young’s modulus, 2% oxygen tension and ACTIVIN A would lead to cell populations expressing endoderm marker in the same timeframes as these protocols.
Figure 6.22 Directed differentiation experimental plan.

(i) Extracellular environment – combing Young’s modulus, oxygen tension and growth factors to mimic in vivo conditions. (ii) 3D directed differentiation with an addition of ACTIVIN A protocol experimental setup. EBs were produced using Aggrewell™ plates and seeded on Matrigel coated TCP, 64, 16, 2 and 0.2kPa 6-well plates and incubated for 8 days in 20% O₂ and 2% O₂.
6.3.11 Morphology of Directed Differentiation using Activin A

Morphology evaluation of cells of EBs on day 3, 5 and 8 of DMEM (spontaneous differentiation media Section 2.3.1) and ACTIVIN A (Section 2.4.4) cultures are shown in Figure 6.23-6.27, where different morphology can be the indication of different cell type. Overall, phase contrast images show a more homogenous population of cell morphology in ACTIVIN A cultures. The morphology of the cells shown of sub-populations are definite endoderm-like (Siller et al., 2016), hepatoblast-like cells (Inamura et al., 2011; Cai, 2014; AU - Wang et al., 2017; Li et al., 2017; Lucendo-Villarin et al., 2017), elongated striated cells mesenchymal-like cells (Muraglia, Cancetta and Quarto, 2000; Moslem et al., 2013), primitive streak-like cells (Cai, 2014; Diekmann and Naujok, 2015) (Figure 6.27). Morphology of sub-populations were seen throughout all the conditions. However, examples of sub-populations are shown in Figure 6.27 where primitive streak-like cells (Figure 6.27i), striated spindle shaped (Figure 6.27i) and MSC progenitors (Figure 6.27iv) were seen in directed differentiation ACTIVIN A 64kPa in 2% O₂. Additionally, neural progenitors-like cells (Figure 6.27i), endoderm-like cells, (Figure 6.27v) and immature hepatocyte-like cells (Figure 6.27vi) were seen in condition directed differentiation ACTIVIN A 0.2kPa in 2% O₂.
Figure 6.23 Phase contrast images of EBs on TCP 20% O₂ at time point day 3, 5 and 8 for directed differentiation.

Cells cultured in spontaneous differentiation DMEM and directed differentiation ACTIVIN A. All scale bars = 400µm. Images were taken at 100X ocular lens magnification using EVOS® XL.
Figure 6.24 Phase contrast images of EBs on TCP 2% O₂ at time point day 3, 5 and 8 for directed differentiation.

Cells cultured in spontaneous differentiation DMEM and directed differentiation ACTIVIN A. All scale bars = 400µm. Images were taken at 100X ocular lens magnification using EVOS® XL.
Figure 6.25 Phase contrast images of EBs on 64 kPa 2% O2 at time point day 3, 5 and 8 for directed differentiation.

Cells cultured in spontaneous differentiation DMEM and directed differentiation ACTIVIN A. All scale bars = 400µm. Images were taken at 100X ocular lens magnification using EVOS® XL.
Figure 6.26 Phase contrast images of EBs on 0.2 kPa 2% O₂ at time point day 3, 5 and 8 for directed differentiation.

Cells cultured in spontaneous differentiation DMEM and directed differentiation ACTIVIN A. All scale bars = 400µm. Images were taken at 100X ocular lens magnification using EVOS® XL.
Figure 6.27 Phase contrast images of differentiated exhibiting sub-population morphology in ACTIVIN A cultures.

Images showing subpopulations of (i) primitive streak-like cells, (ii) neural progenitors-like cells, (iii) striated spindle shaped, (iv) MSC progenitors, (v) endoderm-like cells, (vi) immature hepatocyte-like cells. All scale bars = 200 µm. Images were taken at 100X ocular lens magnification using Evos® XL.
6.3.12 Quantitative Polymerase Chain Reaction (RT-qPCR) of Directed Differentiation using Activin A

RT-qPCR was carried out using endoderm markers SOX17, FOXA2, CXCR4 and AFP. Samples were collected at day 3, 5 and 8. Three-time points were used to see the gene expression differences between those time points. Results show that these four endoderm markers have significant differences between TCP control and 0.2kPa at 2%O₂. Although it is known that ACTIVIN A can influence mesoderm differentiation (Murata et al., 1988; Cornell and Kimelman, 1994; Cerdan et al., 2012), only endoderm markers were used in this experiment. This was because we wanted to compare the endoderm gene expression of cell-cultured on Young’s modulus, low oxygen tension with ACTIVIN A or without and see the effect ACTIVIN A have on these conditions.

In this chapter spontaneous differentiation refers to cells cultured in normal spontaneous differentiation media whereas ACTIVIN A refers to cells cultured with an addition of 100ng/ml ACTIVIN A in the spontaneous differentiation media and TCP 20% O₂ as control.

SOX17 showed significant differences at day 3 and 5 between to 0.2kPa 2% O₂ ACTIVIN A relative to control spontaneous differentiation and control ACTIVIN A (p<0.001 and p<0.0001). Moreover, there were significant differences in the up regulation of the expression between 0.2kpa 2% O₂ spontaneous differentiation relative to 0.2kpa 2% O₂ ACTIVIN A at day 3 (p<0.05). However, at day 8 ACTIVIN A cultures were down regulated compared to spontaneous differentiation cultures (Figure 6.28). Interestingly the same trend occurred for FOXA2 where there was significant increase into 0.2kPa 2% O₂ ACTIVIN A relative to control spontaneous
differentiation (p<0.001), control ACTIVIN A (p<0.05) and 0.2kPa 2% O₂ spontaneous differentiation (p<0.01) at day 3 and 5 (p<0.001) but a downregulation on ACTIVIN A conditions at day 8 (Figure 6.29). AFP expression was increased at day 3 (p<0.05) for 0.2kPa 2% O₂ ACTIVIN A and then it was down-regulated whereas 0.2kPa 2% O₂ spontaneous differentiation expression became up-regulated at day 5 (p<0.01) and 8 (p<0.0001) (Figure 6.30). Figure 6.31 displays an up-regulation of CXCR4 expression to 0.2kPa 2% O₂ ACTIVIN A relative to spontaneous differentiation (p<0.0001), control ACTIVIN A (p<0.01) and 0.2kPa 2% O₂ spontaneous differentiation (p<0.001) at day 5 and 8 (p<0.0001 and p<0.05 respectively).

These data demonstrate that ACTIVIN A alone does have an influence on cell differentiation proven by differences in the gene expression, but it has a bigger impact when used in combination with Young’s modulus and low oxygen. This signifies that ACTIVIN A in combination with soft Young’s modulus and low oxygen tension results in a higher expression of endoderm markers and that the use of three different extracellular environment factors does increase the gene expression of endoderm lineages more than using one or two factors.
Figure 6.28 RT-qPCR analysis showing mean value of gene expressions for endoderm marker SOX17 for directed differentiated EBs.

Figure shows RT-qPCR at time points (i) day 3, (ii) day 5 and (iii) day 8 on TCP and 2 Cytosoft materials (64 kPa and 0.2 kPa) for directed differentiated EBs under 20%O$_2$ and 2%O$_2$. Statistical analysis carried out with 2-way ANOVA and where appropriate with post hoc testing using Tukey method using GraphPad Prism 7.03, n = 3 (biological triplicates), error bars represents standard deviation from mean. Significance difference between condition are indicated where *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001. Expression was normalized with β-actin (housekeeping gene) and then corrected with undifferentiated hiPSCs using $2^{ΔΔCt}$. 
Figure 6.29 RT-qPCR analysis showing mean value of gene expressions for endoderm marker FOXA2 for directed differentiated EBs.

(i) day 3, (ii) day 5 and (iii) day 8 on TCP and 2 Cytosoft materials (64 kPa and 0.2 kPa) for directed differentiated EBs under 20%O\textsubscript{2} and 2%O\textsubscript{2}. Statistical analysis carried out with 2-way ANOVA and where appropriate with post hoc testing using Tukey method using GraphPad Prism 7.03, n = 3 (biological triplicates), error bars represents standard deviation from mean. Significance difference between condition are indicated where *p<0.05, **p<0.01, ***p<0.001 and ****P<0.0001. Expression was normalized with β-actin (housekeeping gene) and then corrected with undifferentiated hiPSCs using 2^{-ΔΔCt}.
Figure 6.30 RT-qPCR analysis showing mean value of gene expressions for endoderm marker AFP for directed differentiated EBs.

(i) day 3, (ii) day 5 and (iii) day 8 on TCP and 2 Cytosoft materials (64 kPa and 0.2 kPa) for directed differentiated EBs under 20%O\textsubscript{2} and 2%O\textsubscript{2}. Statistical analysis carried out with 2-way ANOVA and where appropriate with post hoc testing using Tukey method using GraphPad Prism 7.03, n = 3 (biological triplicates), error bars represents standard deviation from mean. Significance difference between condition are indicated where *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001. Expression was normalized with β-actin (housekeeping gene) and then corrected with undifferentiated hiPSCs using 2\textsuperscript{-ΔΔCt}
Figure 6.31 RT-qPCR analysis showing mean value of gene expressions for endoderm marker CXCR4 for directed differentiated EBs.

(i) day 3, (ii) day 5 and (iii) day 8 on TCP and 2 Cytosoft materials (64 kPa and 0.2 kPa) for directed differentiated EBs under 20%O2 and 2%O2. Statistical analysis carried out with 2-way ANOVA and where appropriate with post hoc testing using Tukey method using GraphPad Prism 7.03, n = 3 (biological triplicates), error bars represents standard deviation from mean. Significance difference between condition are indicated where *p<0.05, **p<0.01, ***p<0.001 and ****P<0.0001. Expression was normalized with β-actin (housekeeping gene) and then corrected with undifferentiated hiPSCs using 2-ΔΔCt.
6.3.13 Immunocytochemistry of Directed Differentiation using Activin A

To support the RT-qPCR data, immunocytochemistry of SOX17 was carried out on TCP 20% O$_2$ and 0.2kPa 2% O$_2$ spontaneous differentiation both as controls and 0.2kPa 2% O$_2$ ACTIVIN A. Figure 6.32 shows positive SOX17 expression in 0.2kPa 2% O$_2$ ACTIVIN A and negative expression in the two controls. Only SOX17 staining was carried out as a mean of support data to confirm our RT-qPCR result.
Figure 6.32 Immunocytochemistry of directed differentiation showing positive staining for endoderm marker **SOX17**.

Where (A) 2% O$_2$ 0.2kPa with spontaneous differentiation DMEM and (B) 2% O$_2$ 0.2kPa with directed differentiation ACTIVIN A. In each condition where i) phase contrast, ii) **AFP** and iii) **DAPI**. (C) Secondary only staining was carried out as control: (i) secondary only mouse IgG (ii) secondary only mouse IgM. All scale bar = 200um. Images were taken at 100X ocular lens magnification using Evos® XL.
6.3.14 Discussion

In the studies carried out in this chapter, we compared the effects of spontaneous differentiation and directed differentiation on the combination of Young’s modulus and low oxygen tension on hiPSCs EBs. The Young’s modulus values 64, 16, 2 and 0.2kPa and 2% O₂ tension was chosen to mimic *in vivo* conditions. Other than Young’s modulus values and low oxygen tension, many parameters such as EBs number, days of expansion and media formulation were kept identical to allow for comparison between each condition. As mention earlier and in Section 1.1.1, substrates are coated with protein in which the cell’s integrin attach to. Despite the coating, the cells can sense the Young’s modulus of the substrate it has been shown by Yeh *et al.*, (Yeh *et al.*, 2017) that soft substrate effects the mouse mammary gland epithelial cells regardless of the substrate being coated with collagen, poly-L-lysine, fibronectin or Matrigel. Additionally, Trappmann *et al.*, (Trappmann *et al.*, 2012) reported that human keratinocytes grown on fibronectin and collagen coated polyacrylamide gels had the same results. Based on the experiments carried out, the data presented in this chapter demonstrated that despite the coating, Young’s modulus value of the substrate in combination with low oxygen tension does have an effect on hiPSCs EBs culture, specifically for the enhancement of endodermal lineage gene expression. Moreover, the effects of these extracellular environment factors on EBs are in agreement with monolayer results in Chapter 5, it was found that EBs are more significant than the effects on monolayer cell culture as reflected in the significantly higher gene expression.

Firstly, the manipulation of the two extracellular environment factors (Young’s modulus and low oxygen tension) induces morphological differences
during the 8 days culture period. This includes the EBs spreading differences between each substrate, it is well known that cells spread less on soft substrates (Yeung et al., 2005) and the data we have supports these studies in the literature. On top of EB spreading, the morphology of cells is also different. Similarly, to monolayer cultured, the morphology contained various sub-population characteristics. This included cobblestone definite endoderm-like cells (Siller et al., 2016), star-shaped cells (Docheva et al., 2008; Haasters et al., 2009), mesenchymal progenitor cells (Moslem et al., 2013), MSC-like cells (Muraglia, Cancetta and Quarto, 2000), spindle-shaped cells, striated cells, and primitive streak cells (Siller et al., 2016). There were also hexagonal-shaped cells we suspect are hepatoblasts-like morphology (Cai et al., 2014). Studies have determined that the actin adhesions between cell and substrates affect the cytoskeleton changes of cell shape and in turn affect the cell differentiation outcome (Meyers, Craig and Odde, 2006; Neves et al., 2008; Discher, Mooney and Zandstra, 2009).

The RT-qPCR results of spontaneous differentiation of EBs in combined conditions of Young’s modulus and low oxygen tension showed that the cells were differentiated into a mixture of cells in the three germ layers. However, there was a significantly higher expression of endodermal lineages on soft 0.2kPa in 2% O\(_2\) of AFP, SOX17, CXCR4 and FOXA2. This aligned with literature where soft substrate had a higher expression of SOX17, AFP and HNF4A which are endoderm lineages (Jaramillo et al., 2012). The flow cytometry and immunostaining images also confirm the presence of endoderm markers in the cell culture. In literature, it has also been studied that low oxygen tension increased endodermal expression, specifically in hepatocytes differentiation (Si-Tayeb et al., 2010; Cai, 2014). Due to
the high $\text{AFP}$ expression and hexagonal shaped cell morphology, we believe a large population of our differentiated cells may be hepatoblasts, as $\text{AFP}$ is a hepatoblast marker, which are progenitor cells of hepatocytes (Shiojiri, 1981; Schmid and Schulz, 1990; Cascio and Zaret, 1991; Nava et al., 2005; Terentiev and Moldogazieva, 2013; Agarwal, Holton and Lanza, 2008; Cai, 2014; Blackford et al., 2019). This data correlates with literature where Young’s modulus had an increase effect on endoderm differentiation. In a study, it was found that 20kPa substrate resulted in an 8-fold yield increase of pancreatic endodermal cells (Maldonado et al., 2017), another study grew cells on 0.013kPa and had a higher fold change in endodermal marker: $\text{SOX17}$ and $\text{HNF4A}$ expression (Jaramillo et al., 2015), as well as improving hepatocyte differentiation when grown on soft 0.4kPa substrates (Cozzolino et al., 2016). As well as low oxygen of 4% $\text{O}_2$ (Cai et al., 2012) and 5% $\text{O}_2$ (van Wenum et al., 2018) resulted in higher yield of hepatocytes. Table 6.2-6.3 shows a summary of the EBs spontaneous differentiation results.

As mentioned in the previous chapter, the mechanism on how the Young’s modulus and low oxygen tension effect cell behaviour is not yet known. However, we believe that the soft substrates activate the Activin/WNT pathway which directs the cell into an endo-mesodermal linage as it was established in Przybyla’s study. In the study, substrate stiffness affected mesodermal differentiation via the Wnt/β-catenin pathway (Przybyla et al., 2016). It is also widely known that HIF-1α regulates Wnt/β-catenin pathway and improves mesodermal yield in hypoxic conditions (Medley et al., 2013). Although those publications are on Wnt/β-catenin pathway and mesoderm differentiation, it is known that Wnt/β-catenin pathway is
essential for primitive streak formation, which gives rise to both mesoderm and endoderm germ layers (Ginsburg et al., 1990; Tam and Loebel, 2007; Tam et al., 2004; Gadue et al., 2006; Hay et al., 2008). After the endo-mesoderm phase, the cells then either become mesoderm or endoderm. In our case, we suspect that the combinations of the two extracellular environment factors conditions have influenced the cells to identify in the endoderm lineage.

To ensure that the effect of Young’s modulus and oxygen is not cell line-specific, spontaneous differentiation of EBs were carried out with another cell line - hiPSCs (MSU001). RT-qPCR results show the same trend in the gene expression of AFP where AFP gene expression was significantly higher in 2kPa, 2% O\textsubscript{2} compared to control TCP. This therefore give evidence that the results are not cell line specific.

The next experiments carried out were directed differentiation with the combination of Young’s modulus and low oxygen tension. Directed differentiation in the form of ACTIVIN A was added to the spontaneous media to investigate how it will affect EBs differentiation. As ACTIVIN A is a common growth factor added in endodermal directed differentiation protocol (Hay et al., 2008; Basma et al., 2009; Cai, 2014; Guo et al., 2014). Our data showed that by combining a soft substrate with low Young’s modulus, low oxygen tension and ACTIVIN A led to a higher expression of endoderm markers at day 3, 5 and 8. Specifically in day 3 and 5 where RT-qPCR data showed highly significant p<0.0001 for markers SOX17 and FOXA2 between cells grown in 0.2kPa, 2% O\textsubscript{2} ACTIVIN A in relation to control. Moreover, hepatoblast markers AFP and CXCR4 were highly expressed at day 5 and 8 with highest expression found in cells grown in 0.2kPa, 2% O\textsubscript{2} with ACTIVIN
A. The expression of SOX17 and FOXA2 at day 3 and 5 are expected as they are early endodermal markers (McLean et al., 2007; Takayama et al., 2011). In addition, the hepatoblast markers: AFP (Mansuroglu et al., 2009; Li et al., 2010; Nakamae et al., 2018) and CXCR4 (Hay et al., 2008; Kido et al., 2015) had high expression at day 8 as expected. On top of RT-qPCR results, immunocytochemistry also shows positive expression for SOX17.

The results shown in this chapter demonstrated for the first time that a combination of low Young’s modulus and low oxygen tension induced the endoderm differentiation of EBs. To conclude, it was found that when EBs where allowed to spontaneous differentiation on soft 0.2 kPa substrates and low oxygen tension of 2%, they had a high gene expression of endoderm lineages when compared to conventional cell culturing conditions of TCP in 20% O₂. Moreover, we also demonstrated that EBs culture has a significantly higher gene expression than monolayer which signifies that cell-cell interaction plays a critical role in the differentiation (Konno et al., 2005; Koike et al., 2007; Kurosawa, 2007; Bratt-Leal, Carpenedo and McDevitt, 2009; Gothard et al., 2009). We also further attempt to complete the extracellular environment factors myriad by introducing only 1 exogenous factor (ACTIVIN A) into the cell culture with soft 0.2 kPa substrate in 2% O₂, resulting in three factors that were involved in the cell culture. By mimicking in vivo conditions, we have demonstrated an increase in endodermal gene expression which can be used as an improvement in the bioprocessing platform. Establishing the optimum conditions for cell culturing and differentiation of each germ layer is critical for the efficiency of stem cell culture in regenerative medicine therapies as well as in vitro research of drug and disease modelling studies. The
data suggest 0.2kPa in 2% O₂ have a tendency for endoderm differentiation and possibly further into immature hepatocytes. This is an important find as this may help improve the current bioprocessing platform by providing an alternative differentiation protocol which could possibly provide a more efficient differentiation yield environment for desired cell type. On top of improving the bioprocessing platform, using these factors will benefit cell manufacturing due to the low cost of goods compared to large amount of expensive growth factors currently required for directed differentiation. To accomplish this goal, it is important to fully understand the underlying mechanisms regulating cell culture differentiation fate by each extracellular environment factors combination.
Table 6.2 Summary table of day 8 EBs spontaneous differentiation RTq-PCR results for endoderm markers

<table>
<thead>
<tr>
<th>Tissue Culture Plastic (TCP)</th>
<th>20% Oxygen</th>
<th>2% Oxygen</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SOX17</td>
<td>FOXA2</td>
</tr>
<tr>
<td>Control Condition</td>
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<td>Decreased</td>
</tr>
<tr>
<td>64kPa</td>
<td>Decreased</td>
<td>Decreased</td>
</tr>
<tr>
<td>16kPa</td>
<td>Decreased</td>
<td>Decreased</td>
</tr>
<tr>
<td>2kPa</td>
<td>Decreased</td>
<td>Decreased</td>
</tr>
<tr>
<td>0.2kPa</td>
<td>Significant increase **</td>
<td>Increase</td>
</tr>
</tbody>
</table>
Table 6.3 Summary table of day 8 EBs spontaneous differentiation RTq-PCR results for ectoderm and mesoderm markers

<table>
<thead>
<tr>
<th>Day 8</th>
<th>20% Oxygen</th>
<th>2% Oxygen</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><strong>Mesoderm</strong></td>
<td><strong>Ectoderm</strong></td>
</tr>
<tr>
<td>Tissue Culture Plastic (TCP)</td>
<td>Control Condition</td>
<td></td>
</tr>
<tr>
<td>Plastic (TCP)</td>
<td><strong>BRACHYURY</strong></td>
<td><strong>MiXL1</strong></td>
</tr>
<tr>
<td>64kPa</td>
<td>Significant increase *</td>
<td>Significant increase ****</td>
</tr>
<tr>
<td>16kPa</td>
<td>Increase</td>
<td>Significant increase ***</td>
</tr>
<tr>
<td>2kPa</td>
<td>Increase</td>
<td>Significant increase ****</td>
</tr>
<tr>
<td>0.2kPa</td>
<td>Increase</td>
<td>Significant increase ****</td>
</tr>
</tbody>
</table>
Chapter 7: Conclusions and Future Work

7.1 Conclusions and Key Findings

Human pluripotent stem cells are a classification of cells capable of becoming any cell in the human body (Till and McCulloch, 1980; Evans and Kaufman, 1981; Martin, 1981; Potten and Loeffler, 1990; Thomson et al., 1998). In humans, there are two types of pluripotent stem cells: human embryonic stem cells (hESCs) (Evans and Kaufman, 1981; Martin, 1981; Thomson et al., 1998) and human induced pluripotent stem cells (hiPSCs) (Takahashi and Yamanaka, 2006; Takahashi et al., 2007). In this thesis, we decided to focus on induced pluripotent stem cells as we wanted to initially focus on one type of pluripotent stem cell and found hiPSCs more favourable due to ethical reasons. In 2007, hiPSCs were discovered by Yamanaka and his team (Takahashi and Yamanaka, 2006; Takahashi et al., 2007). His discovery showed that hiPSCs were able to be reprogrammed from somatic cells. Studies have confirmed hiPSCs have similar properties to hESCs cells and are able to differentiate into cell types just like embryonic stem cells (Bock et al., 2011). The advantage of hiPSCs includes no ethical concerns regarding these cell origins as they are not derived from human embryos (Meyer, 2000). Moreover, hiPSCs have been used as a model for drug modelling and modelling biology development, as well as disease-specific hiPSCs culture, this model has been beneficial to the medical and pharmaceutical industry.

Conventional cell culture methods of hiPSCs on tissue culture plastic (TCP) coated with Matrigel at atmospheric oxygen tension does not represent the \textit{in vivo}
environment. Therefore, growing cells at various Young’s modulus and low oxygen tension in the differentiation protocol could help to understand early embryo formation and differentiation development. There are currently multiple challenges in the culturing and differentiation of pluripotent stem cells. Many of these challenges are a result of insufficient of knowledge and understanding of cell behaviour and differentiation. Independent studies on the effect of Young’s modulus or oxygen tension have led us to the hypothesis that such a combination would result in a higher yield of desired differentiated cells by mimicking in vivo conditions.

As previously covered in Chapter 1, multiple independent studies have been carried out on the effect of Young’s modulus on cell differentiation as well as low oxygen on cell differentiation independently. In the literature we have seen how low oxygen affects differentiation (Fink et al., 2004; Bauwens et al., 2005; Ren et al., 2006; Grayson et al., 2007; Xu et al., 2007; Koay et al., 2008; Chen et al., 2010; Ng et al., 2010; Bae et al., 2012; Correia et al., 2014) and how Young’s modulus effects differentiation (Engler et al., 2006; Shih et al., 2011; Kshitiz et al., 2012; Narayanan et al., 2013; Xue et al., 2013; Maia et al., 2014; Przybyla, Lakins and Weaver, 2016). Specifically, the effect of low oxygen on endoderm differentiation(Binó et al., 2016) (Prado-Lopez et al., 2010; Pimton et al., 2014; Gao and Liu, 2017; Ayabe et al., 2018; Kimura et al., 2018; Sambathkumar et al., 2018) as well as the effect of Young’s modulus on endoderm differentiation (Evans et al., 2009; Cai, 2014; Jaramillo et al., 2015; Mittal et al., 2016). However, to the best of our knowledge, this is the first study on the combination of Young’s modulus and low oxygen tension on cell culture.
In Chapter 3, the two cell lines of hiPSCs (cord blood and MSU0001) were characterized. We proved through phase contrast images, immunocytochemistry and flow cytometry that the cells were indeed pluripotent. The pluripotent markers used were well-known pluripotency makers of OCT4, SSEA3/4, TRA181, TRA160 and early differentiation marker of SSEA1. Both cell lines were found to be positive for OCT4, SSEA3/4, TRA181, TRA160 and negative for SSEA1. This gave us enough evidence that the cells were undifferentiated pluripotent stem cells, and they were used for experiments in this thesis.

In Chapter 4, six different substrates were evaluated which included: PDMS, GXG, PƐK, Cytosoft, Softwell and Excellness. The objective was firstly for the in-house substrates production (GXG, PƐK and PDMS) to reach the desired Young’s modulus values of 0.2, 2, 16 and 64kPa to mimic in vivo conditions. In-house substrates’ Young’s modulus was measured using the Atomic Force Microscopy machine and it was found that the in-house produced substrate were unable to reach values as low as 0.2kPa. Therefore, it was decided to use the commercially available substrates. Cytosoft, Softwell and Excellness were three commercially available matrices chosen for examination. The three commercially available substrates were initially characterised to see if hiPSCs cultures were able to grow on them. From the initial experiments, it was decided that subsequent experiments would be carried out using Cytosoft. The hiPSCs grown on Cytosoft were further characterised using immunostaining and flow cytometry of the pluripotent markers.

In Chapter 5, we investigated the combined effects of Young’s modulus and 2% O₂ on spontaneous differentiation of hiPSCs in a 2D monolayer culture. Monolayer cultured was initially chosen so that the majority of cells were exposed
to the substrate surface and the same oxygen tension during culture. Cells were cultured in 0.2, 2, 16, 64 kPa and tissue culture plastic (TCP) coated with Matrigel in 20% O₂ and 2% O₂ and were allowed to spontaneously differentiate for 4 and 8 days. The data shows that Young’s modulus and low oxygen individually as well as in combination both have effects on the cell’s morphology in culture. Day 8 samples showed higher expression of AFP using RT-qPCR and flow cytometry between cells grown on 0.2kPa in 2% O₂ in relative to control. The results did not show highly significant differences and it was decided for Chapter 6 to investigate using Embryoid bodies (EBs) culture to compare the differences. We hypothesised that EBs culture would have a bigger differentiation impact due to cell-cell communication and mimicking embryogenesis (Bratt-Leal, Carpenedo and McDevitt, 2009).

In Chapter 6, we investigated the combined effects of Young’s modulus and 2% O₂ on spontaneous differentiation of hiPSCs in a 3D EBs culture. Cells were allowed to spontaneously differentiate for 8 days on 0.2, 2, 16, 64 kPa and tissue culture plastic (TCP) in 20% O₂ and 2% O₂. At day 8, cells were collected and analysed using RT-qPCR, immunocytochemistry and flow cytometry. It was discovered via RT-qPCR that there was a higher gene expression of endoderm markers SOX17, FOXA2, AFP and GATA4. This was then confirmed using immunocytochemistry and flow cytometry. Due to the high AFP expression (Terentiev and Moldogazieva, 2013) and morphology (Agarwal et al., 2008; Cai et al., 2014), we believe the population of our differentiated cells are hepatoblasts-like cells, which are progenitor cells of hepatocytes (Zhao and Duncan, 2005; Miyajima, Tanaka and Itoh, 2014; Kimura et al., 2018). Our results aligned with literature.
where soft substrate had a higher expression of SOX17, AFP and HNF4 which are endoderm lineages (Jaramillo et al., 2015, 2018; Cozzolino et al., 2016) and low oxygen (Cai et al., 2012; van Wenum et al., 2018).

In order to compare our approach with the protocols used for endodermal differentiation, EBs were then direct differentiated by adding ACTIVIN A to the media. 100ng/mL of ACTIVIN A was added to the spontaneous media solution, based on various endoderm protocols (Hay et al., 2008; Basma et al., 2009; Cai, 2014; Guo et al., 2014). RT-qPCR was carried out using endoderm markers and data showed that EBs grown on soft 0.2kPa substrates in 2% O₂ had higher endoderm gene expression than cells grown on TCP in 20% O₂ and 0.2kPa in 20% O₂. This confirms and concludes that the combined effects of Young’s modulus and low oxygen tension of 2% O₂ do affect hiPSCs differentiation by increasing endoderm gene expression. Moreover, the data demonstrated that the combination of Young’s modulus and 2% O₂ has a significant difference in the differentiation outcome compared to Young’s modulus or low oxygen tension independently.

Overall, this thesis had shown that the combination of Young’s Modulus and low 2% oxygen tension mimicking in vivo conditions does have an effect on hiPSCs differentiation, especially in EBs differentiation. We have given evidence that by having multiple extracellular environment factors to mimic in vivo conditions resulted in a higher yield of endoderm expressing cells and from the high expression of AFP, we have reason to believe that the cells are hepatoblasts-like cells. This was further investigated by the addition of exogenous factor (ACTIVIN A) to attempt to complete the extracellular environment factors by direct differentiation. We have shown that by adjusting extracellular environment factors
in the differentiation protocol influences cell outcome, this could help to understand the effect of the extracellular environment factors myriad in early embryo development and differentiation and contribute to the improvement of the bioprocessing platform in vitro.

7.2 Future Work

Conventional stem cell culturing has multiple manufacturing challenges including biomaterials, optimisation of culturing conditions, optimisation of directed differentiation protocols, scaling up and functionality of the cells. In this thesis, we had explored alternative options to tackle biomaterials, alternative culturing conditions and differentiation protocols. Future work for this research project would be first would be to show reproducibility by repeating all experiments with an additional hiPSCs cell line as well as hESCs. To further prove that the data obtained was not pluripotent cell type specific to hiPSCs. Then the next steps would be to explore directed differentiation into a functional adult cell type. A sensible adult cell type to initially focus on would be functional mature hepatocytes as results in this thesis suggest that the cells are hepatoblasts-like cells (Zhao and Duncan, 2005; Miyajima, Tanaka and Itoh, 2014; Kimura et al., 2018). The work should aim to study the efficiency of direct differentiating EBs into functional mature hepatocytes by using the combination of Young’s Modulus, low oxygen tension and minimal exogenous factors. Functional hepatocytes would be beneficial to the medical and pharmaceutical world as it can be used for in vitro modelling, transplantation models, drug development studies, drug metabolism studies and future potential of clinical transplantations in both cell therapy and tissue engineering for patients with liver disease. As liver disease is the third biggest caused of deaths 18 to 65 years
old and the leading caused of death in people aged 35 to 49 years old in the United Kingdom (British Liver Trust, 2019). Additionally, liver disease also accounts for 3.5% of deaths worldwide resulting in around 2 million deaths globally per year (Asrani et al., 2019). Another future work would also be to carry out Design of Experiment on various Young’s modulus, oxygen tension and exogenous factors to find the “sweet spot” where it is the optimal condition for growth and differentiation of functional hepatocytes. On top of this, studies on the pathway mechanism via methods such as western blot should be carried out on how Young’s modulus and low oxygen are affecting the cell to fully understand cell culture. Following on from that, directed differentiation using a combination of Young’s modulus and low oxygen tension should then be explored for the differentiation of endoderm, mesoderm and ectodermal cell lineages and cell types such as pancreatic cells, neuronal cells and cardiomyocytes. The aim would be to find optimum extracellular environment conditions specific to each functional adult cell type. This procedure would potentially increase efficiency in the bioprocessing platform of differentiation and potentially reduce the cost of unnecessary excess exogenous factors for directed differentiation. Once a protocol is established for each functional cell type, the next step would be to investigate these functional cells for their potential in their medical and clinical applications. Additionally, studies on scaling up using the combined conditions of Young’s modulus, low oxygen and exogenous factors would have to be investigated for the for large scale manufacturing.
8 Chapter 8: Appendix I

Flow cytometry results of spontaneous differentiation of hiPSCs in monolayer (Section 5.3.5). The markers used were OCT4, BRACHYURY and AFP with isotype control used for gating. Ectoderm was not a marker used in this flow cytometry assay as it was unavailable at the time the assay was carried out.

Appendix I Figure 1: Flow cytometry analysis showing hiPSCs spontaneous differentiation on TCP 20% O2. Data shows negative for staining for i) isotype control, ii) pluripotency OCT4, iii) mesoderm BRACHYURY and positive for endoderm marker (iv) AFP.
Appendix I Figure 2: Flow cytometry analysis showing hiPSCs spontaneous differentiation on 64kPa 20% O2. Data shows negative for staining for i) isotype control, ii) pluripotency OCT4, iii) mesoderm BRACHYURY and positive for endoderm marker (iv) AFP.
Appendix I Figure 3: Flow cytometry analysis showing hiPSCs spontaneous differentiation on 16kPa 20% O2. Data shows negative for staining for i) isotype control, ii) pluripotency OCT4, iii) mesoderm BRACHYURY and positive for endoderm marker (iv) AFP.
Appendix I Figure 4: Flow cytometry analysis showing hiPSCs spontaneous differentiation on 2kPa 20% O2. Data shows negative for staining for i) isotype control, ii) pluripotency OCT4, iii) mesoderm BRACHYURY and positive for endoderm marker (iv) AFP.
Appendix I Figure 5: Flow cytometry analysis showing hiPSCs spontaneous differentiation on 0.2kPa 20% O2. Data shows negative for staining for i) isotype control, ii) pluripotency, iii) mesoderm and positive for endoderm marker (iv) AFP.
Appendix I Figure 6: Flow cytometry analysis showing hiPSCs spontaneous differentiation on TCP 2% O2. Data shows negative for staining for i) isotype control, ii) pluripotency OCT4, iii) mesoderm *BRACHYURY* and positive for endoderm marker (iv) *AFP*. 
Appendix I Figure 7: Flow cytometry analysis showing hiPSCs spontaneous differentiation on 64kPa 2% O2. Data shows negative for staining for i) isotype control, ii) pluripotency OCT4, iii) mesoderm BRACHYURY and positive for endoderm marker (iv) AFP.
Appendix I Figure 8: Flow cytometry analysis showing hiPSCs spontaneous differentiation on 16kPa 2% O2. Data shows negative for staining for i) isotype control, ii) pluripotency OCT4, iii) mesoderm BRACHYURY and positive for endoderm marker (iv) AFP.
Appendix I Figure 10: Flow cytometry analysis showing hiPSCs spontaneous differentiation on 2kPa 2% O2. Data shows negative for staining for i) isotype control, ii) pluripotency OCT4, iii) mesoderm BRACHYURY and positive for endoderm marker (iv) AFP.
Appendix I Figure 11: Flow cytometry analysis showing hiPSCs spontaneous differentiation on 0.2kPa 2% O2. Data shows negative for staining for i) isotype control, ii) pluripotency OCT4, iii) mesoderm BRACHYURY and positive for endoderm marker (iv) AFP.
9 Chapter 9: Bibliography


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