

The Effect of the Nutritional Microenvironment on Stem Cell Differentiation

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by

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Declaration

I, Rana Mohamad Khalife, confirm that the work presented in this thesis is my own. This work was carried out between May 2014 and May 2017 and has not been submitted, either in whole or in part, for another degree or another qualification to any university. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Print: Rana Khalife

Date: May 2018

In memory of Khadijeh Khalife

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Abstract

Pluripotent stem cells (PSCs) are considered to be one of the most promising sources of cells for a wide range of regenerative cell therapies. One major challenge remains the efficient induction of differentiation.

Initial studies on the effect of low oxygen on pluripotency maintenance was performed on different cell lines. Culturing stem cells at low oxygen helped in the maintenance of pluripotency in most of the cell lines except feeder-free human induced pluripotent stem cells (hiPSCs; BJ). These findings show for the first time that the maintenance of pluripotency at low oxygen tensions is cell line dependent response and may be due to one or more of the many associated micro-environmental cues. Moreover, we hypothesised that high glucose, pyruvate and oxygen concentrations of typical growth media could be inhibiting the differentiation of certain lineages because they are so different from the environment experienced by developing embryos *in-vivo*. A design of experiments (DoE) was used to investigate the interplay between each three during the spontaneous differentiation of hiPSCs (cord blood). Based on this initial screen we discovered that low oxygen and glucose enhanced the mesodermal and ectodermal lineages. While glucose deprivation was a potent inducer of endodermal lineage formation as 72.73% of the hiPSCs (cord blood) were apoptotic. It was found that apoptosis triggered by staurosporine committed the cells into endodermal lineage. Western blotting analysis revealed that the pathway by which glucose removal enhanced endodermal lineage could be time dependent. Thus, the manipulation of energy levels triggered apoptosis which in return enriched the endodermal germ layer selection. The manipulation of the nutritional environment especially glucose deprivation, is a potential approach for the enhancement of endodermal precursors.

Impact Statement

The implementation of human pluripotent stem cells in cell therapy is facing many challenges especially in the cost of clinical trial due to the usage of growth factors. Thus the need to establish a flexible, controllable platform to direct the differentiation of pluripotent stem cells into germ layers in a defined nutritional microenvironment. There is a gap between the *in vivo* and *in vitro* nutritional conditions during the expansion and differentiation of stem cells. Thus we aimed to mimic the *in-vivo* nutritional environmental levels of three key factors during the differentiation of hiPSCs (Cord blood).

Our findings could present a potential and novel approach for efficient directed endodermal differentiation of pluripotent stem cells into pancreatic or hepatic cells through the manipulation of media components whilst simultaneously limiting the usage of growth factors. For example, a combination of directed pancreatic protocol along with our finding could be studied. Moreover, we provided an enhancement for the formation of ectodermal and mesodermal which could be applied for the generation of lineage 'precursors.

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List of Abbreviations

ADP-Adenosine diphosphate

AFP- Alpha Fetoprotein

ANOVA- Analysis of variance

ATP- Adenosine triphosphate

bFGF- Basic fibroblast growth factor

cDNA- Complementary deoxyribose nucleic acid

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

EB- Embryoid bodies or stem cell aggregate

EDTA- Ethylenediaminetetraacetic acid

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

DMEM- Dulbecco's Modified Eagle Medium

DMSO- Dimethyl sulfoxide

DNA- Deoxyribonucleic acid

FOXA2- Forkhead box A2

GLUT1- Glucose transporter 1

GLUT3- Glucose transporter 3

hESC- Human embryonic stem cell

Hif- Hypoxia inducible factor

hiPSC- Human induced pluripotent stem cells

hMSC -Human mesenchymal stem cell.

ICM- inner cell mass

KOSR- Knockout serum replacement

MEF- Mouse embryonic fibroblast

NEAA- non-essential amino acids

OCT-4- Octamer-binding transcription factor 4

Pax 6- Paired box protein 6 gene

PBS- Phosphate buffered saline

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

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

TRA 1-60- Tumour resistance antigen 1-60

TRA1-81- Tumour resistance antigen 1-81

TSCs- Totipotent stem cells

TUJ1- Beta-III tubulin

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1 Chapter 1: Introduction

1.1 Stem cell and regenerative medicine

The basic idea of stem cells or '*stamzelle*' (German translation of stem cells) was established by the zoologist Ernst Haeckel in the 18th century (Haeckel, 1868). "Stamzelle" was used as a word to describe that all multicellular organisms had evolved from a common unicellular organism (Haeckel, 1868, 1874). Few years after, the term stem cell was more defined and described by two models: the unicellular organism ancestor and the fertilized egg (Haeckel, 1877). Subsequently, differentiated cells and cells with stemness characteristic were fully described. Pluripotent stem cells (PSCs) are distinct by their differentiation potential into any cell lineage precursors, self-renewal and teratoma's formation once injected into immunocompromised mouse. The capability to form teratoma is considered as 'gold standard' for pluripotency assessment as teratoma are benign tumors which are able to form all three germ layers (Till and McCulloch, 1980; Evans and Kaufman, 1981; Martin, 1981; Potten and Loeffler, 1990; J. a Thomson *et al.*, 1998; Amit *et al.*, 2000). Human embryonic stem cells (hESCs) and induced pluripotent stem cells (hiPSCs) have the ability to form embryonic chimeras validating its pluripotency (Mascetti and Pedersen, 2016). The ability of PSCs to differentiate into any cell type is valuable for novel cell therapy and gene therapy, disease modelling for drug discovery, and organogenesis/ development models and pharmaceutical applications (Daley, Goodell and Snyder, 2003; Singh *et al.*, 2005). Current differentiation protocols are dependent on growth factors to direct the differentiation of pluripotent stem cells into a specific cell lineage, nevertheless this approach is expensive. Thus, the need for an efficient and cost-effective methods for differentiating PSCs into therapeutically relevant cells. This approach is a key challenge in the rapidly developing field of regenerative medicine.

1.2 Human embryonic stem cells

The first mouse embryonic stem cells (mESCs) were isolated in 1981 from inner mass of blastocysts and cultured on inactivated mouse fibroblast (Evans and Kaufman, 1981; Martin, 1981). Later in 1998, the first human embryonic stem cells (hESCs) had been derived from an *in vitro* fertilized embryo using an immunosurgical microdrop culture system (Bongso *et al.*, 1994; J. A. Thomson *et al.*, 1998). The first hESCs were cultured on inactivated mouse embryonic fibroblasts, using anti-human serum or guinea pig complement (Bosma, Custer and Bosma, 1983; Reubinoff *et al.*, 2000; Cowan *et al.*, 2004; Ellerström *et al.*, 2006; Aflatoonian *et al.*, 2010). However current methods for isolations are mechanical or enzymatic isolation of the inner cell mass from the trophectoderm to prevent any animal contamination (Genbacev *et al.*, 2005; Liu *et al.*, 2009; Storm *et al.*, 2010). Due to the implantation and progression of hESCs in cell-based therapies, appropriate culture condition should be developed to avoid any animal source contaminant and produce a stable cell population. It has been shown that culturing hESCs in a serum free (Amit *et al.*, 2000) and feeder layer-free condition (Amit, 2003) maintained all the pluripotency characteristics of embryonic stem cells (Amit *et al.*, 2000, 2003).

Differences between human and mouse embryonic stem cells

Unlike human embryonic stem cells, mouse embryonic stem cells cannot form trophoectoderm (Deming, 1982; J. a Thomson *et al.*, 1998; Niwa, Miyazaki and Smith, 2000; Odorico, Kaufman and Thomson, 2001).

The proliferation and self-renewal of mESCs is dependent on leukemia inhibitory factors (LIF), thus mESCs could be cultured without feeders with the presence of LIF (Smith and Hooper, 1987; Williams *et al.*, 1988; Heyner *et al.*, 1989) or LIF cytokines (Conover *et al.*, 1993; Rose *et al.*, 1994; Wolf *et al.*, 1994; Pennica *et al.*, 1995). A

difference of cytokines, pluripotency markers and molecular pathways gene expression of was detected upon apoptosis (Sato *et al.*, 2003; Ginis *et al.*, 2004). For instance, specific embryonic antigen 1 (SSEA1) is considered as a pluripotency marker for mESCs, whereas it is a differentiation marker for hESCs (Krupnick *et al.*, 1994; Draper *et al.*, 2002).

1.3 Induced pluripotent stem cells

1.3.1 The streams that have led to induced pluripotent stem cells (iPSCs) discovery

Three main scientific approaches have led to the emergence of the induced pluripotent stem cells discovery. The first stream was when Professor John Gurdon in 1962, generated tadpoles by combining the nucleus of intestinal cells of adult frogs with unfertilized eggs (Gurdon, 1962). The idea of nuclear transfer was applied in mammals with the first mammal cloning of Dolly (Campbell *et al.*, 1996; Wilmut *et al.*, 1997). These approaches confirmed that differentiated cells contain all the genetic information for appropriate development of the entire organism, and particularly, that the reprogramming of somatic cell nuclei could be achieved by factors present in the oocytes (Wilmut *et al.*, 1997).

The second stream was the discovery of two transcriptional factors in *Drosophila* and mammals. Some of the transcriptional factors are considered as a 'master regulator' that can direct the commitment of cell by regulating its gene expression (Davis, Weintraub and Lassar, 1987; Schneuwly, Klemenz and Gehring, 1987; Yamanaka and Blau, 2010).

The third stream was the isolation of mESCs (Evans and Kaufman, 1981; Martin, 1981) and the identification of growth factors that help in pluripotency maintenance (Heyner *et al.*, 1989; J. A. Thomson *et al.*, 1998).

All of these steams along with the need to overcome ESCs's ethical issues had risen to hESCs gene profiling investigations. Yamanaka and his colleagues were the first to reprogram mouse induced pluripotent stem cells (miPSCs) from adult mouse fibroblasts via the insertion of 4 key transcription factors (Yamanaka's factors) : octamer-binding transcription factor 4 (OCT-4), sex determining region Y box 2 (SOX2), myelocytomatosis viral oncogene homologue (c-MYC) and Kruppel-like factor 4 (KLF4) (Takahashi & Yamanaka, 2006). After one year, human induced pluripotent stem cell (hiPSCs) was also generated by the addition of integrating retroviral vectors expressing the same genes (Takahashi & Yamanaka, 2007). The discovery of hiPSCs has led to the elimination of ethical usage of hESCs (Meyer, 2008) and the development of autogenic cell therapy. A lot of progress has been achieved upon the usage of iPSCs in regenerative medicine for the treatment of Parkinson's disease (Wernig *et al.*, 2008; Kriks *et al.*, 2011), spinal cord injury (Tsuji *et al.*, 2010; Nori *et al.*, 2011), platelet deficiency (Takayama *et al.*, 2010) and retinal and macular degeneration (Okamoto and Takahashi, 2011). Moreover, patient-specific iPSCs were used as a useful model to understand several diseases like amyotrophic lateral sclerosis (ALS) (Dimos *et al.*, 2008), Parkinson's disease (Devine *et al.*, 2011), Alzheimer's disease (Yagi *et al.*, 2011; Yahata *et al.*, 2011), and schizophrenia (Brennand *et al.*, 2011). Other iPSCs models have been generated for the treatment of adenosine deaminase deficiency-related severe combined immunodeficiency, Shwachman-Bodian-Diamond syndrome, Gaucher disease type II, Duchenne and Becker muscular dystrophy, Parkinson disease, Huntington disease, juvenile-onset type 1 diabetes mellitus, Down syndrome, and Lesch-Nyhan syndrome (Park *et al.*, 2008).

1.3.2 Induced pluripotent stem cells reprogramming

The generation of induced pluripotent stem cells (iPSCs) has provided a good *in vitro* model to study in depth the reprogramming mechanisms (Brambrink *et al.*, 2008; Stadtfeld *et al.*, 2008) and it has been integrated in cell-based therapy to treat several disease in mouse models (Hanna *et al.*, 2007; Wernig *et al.*, 2008). iPSCs derived from post-natal somatic cells like mouse (Maherali *et al.*, 2007; Meissner, Wernig and Jaenisch, 2007; Okita, Ichisaka and Yamanaka, 2007; Wernig *et al.*, 2007), murine (Lowry *et al.*, 2008) or human fibroblasts (Takahashi *et al.*, 2007; Hu *et al.*, 2011) were firstly generated using retroviruses and lentiviruses through the insertion of the “Yamanaka factors” (OCT-4, SOX2, c-MYC, KLF4). Although the usage of lentivirus is a powerful tool for reprogramming, it can cause footprint by mutagenesis insertional (Sommer *et al.*, 2009) which was seen in previous attempts in gene therapy (Hacein-Bey-Abina *et al.*, 2003), moreover retrovirus-reprogrammed iPSCs generated using can cause immunogenic response (Kim *et al.*, 2011). Minimal footprint methods were developed to minimise the integration of reprogramming factors. These reprogramming methods which left no trace of transgene in the genome of the reprogrammed iPSCs include: sendai virus (Fusaki *et al.*, 2009; Seki *et al.*, 2010; Ban *et al.*, 2011), adenovirus (Okita *et al.*, 2008; Stadtfeld *et al.*, 2008; Zhou and Freed, 2009), episomal vectors (Junying *et al.*, 2009; Hu *et al.*, 2011), piggyback (Kaji *et al.*, 2009; Woltjen *et al.*, 2009), direct miRNA transfection (Wang *et al.*, 2007, 2008; Greer Card *et al.*, 2008; Judson *et al.*, 2009; Melton, Judson and Blelloch, 2010; Anokye-Danso *et al.*, 2011; Li *et al.*, 2011; Subramanyam *et al.*, 2011), minicircles vectors (Jia *et al.*, 2010; Narsinh *et al.*, 2011), and the overexpression of mRNA (Warren *et al.*, 2010) or recombinant cell-penetrating reprogramming protein (Junying *et al.*, 2009; Zhou and Freed, 2009). Upon the comparison of all the reprogramming methods, it was found

that episomal plasmids and Sendai virus had higher efficiencies in iPSCs production with the absence of footprint. Though a lot of studies have developed many reprogramming methods to produce a translation-grade iPSCs, the urge to have less complex, safer and easier approach with high efficiency was needed. Thus, the combination of chemical, environmental factors and gene approach reprogramming showed a promising tactic to improve the generation of iPSCs (Huangfu *et al.*, 2008; Shi *et al.*, 2008; Yoshida *et al.*, 2009; H. Zhou *et al.*, 2009; Mali *et al.*, 2010).

1.3.3 The difference between human induced pluripotent stem cells and human embryonic stem cells

After the novel discovery of induced pluripotent stem cells, it was important to characterize these stem like cells and compare it with embryonic stem cells. Therefore, the degree of similarities and differences between iPSCs and hESCs was investigated. Although reprogramming of human and mouse iPSCs didn't alter the cell genome, a difference of genomic expression profile was seen between iPSCs and ESCs: micro RNA (miRNA) profiling revealed a novel gene expression signature of iPSCs thus categorising iPSCs in a unique subtype of pluripotent cells (Chin *et al.*, 2009). iPSCs showed a memory retention and a persistent cell gene expression (Marchetto *et al.*, 2009; Ghosh *et al.*, 2010; Kim *et al.*, 2011; Lister *et al.*, 2011; Ohi *et al.*, 2011). Further dissimilarities like cytosine methylation (Ariffin *et al.*, 2009) and teratoma formation was detected (Miura *et al.*, 2009). Nevertheless, disagreeing with previous investigations, some studies reported that iPSCs shouldn't be categorised separately from ESCs even if iPSCs and ESCs have few differences in chromatin structure and DNA methylation (Guenther *et al.*, 2010; Bock *et al.*, 2011). This variation could be due to lab-specific genetic signatures caused by microenvironment features (Newman and

Cooper, 2010). The differentiation of ESCs varies between ESCs clones (Ward, Barrow and Stern, 2004; Osafune *et al.*, 2008) and it has been reported that iPSCs had lower efficiency upon differentiation (Hu *et al.*, 2010). Other studies confirmed an equal differentiation process between the iPSCs and ESCs clones (Bock *et al.*, 2011).

1.4 Early embryo development and germ layer formation

The fertilisation of the oocyte and the sperm occurs during the secretory phase of the menstrual cycle in the ampulla region of the fallopian tube. After 3 days of fertilisation, the zygote undergoes a series of cleavages resulting in the formation of blastocyst which is made up of 12-32 blastomeres (Ducibella and Anderson, 1975; Graham and Deussen, 1978; Johnson and Ziomek, 1981; Moore and Persaud, 2004). Further compaction and cavitation of blastocyst occurs to form trophoblast and embryoblast (inner cell mass). After the implantation, the trophoblast and embryoblast differentiate into further populations as shown in Figures 1.1. Embryoblast is considered as an important cell population as it will give rise to most of the embryo parts, embryoblast will further divide into hypoblast and epiblast (Figure 1.2) (Copp, 1979; Graham and Lehtonen, 1979; Johnson and Ziomek, 1981; Pedersen, Wu and Bałakier, 1986; Pierce *et al.*, 1988). The germ layers formation starts once the embryo is implanted in the endometrial wall at day 7-8. The hypoblast will later form the endoderm and the epiblast will develop into the embryonic ectoderm and primitive streak (Figure 1.3). Once the embryonic disk is established at day 17, the central nervous and cardiovascular system start to develop (Ducibella and Anderson, 1975; Ducibella *et al.*, 1977; Lawson and Hage, 1994; Moore and Persaud, 2004).

(a)

Day	1	2	3	4	5	6	7
Stage of Development in-vivo	Fertilization	Cleavage Compaction	Establishment of Morula	Establishment of Blastocyst	Maturation of Blastocyst	Implantation Begins (bilaminar embryonic disc)	Peri-Implantation
Location	Fallopian tube: Ampulla	Fallopian Tube: Isthmus	End of fallopian tube/ Entrance of Uterine Cavity	Uterine Cavity		Uterine Cavity/ Endometrial Wall	
Corresponding stage at in-vitro culture	Derivation		Derivation/Expansion		Expansion	Expansion/Differentiation	
Cell Population	Blastomere		Trophoblast		Embryoblast		Syncytiotrophoblast Cytotrophoblast Hypoblast Epiblast

(b)

Day	1	2	3	4	5	6	7
Embryo Morphology	Zygote	2/4/8/16 Cell	Morula	Early Blastocyst	Late Blastocyst	Bilaminar Embryonic Disc	

Figure 1.1 The development of embryo during the first week after conception (third week gestation) and their corresponding derivation *in vitro* protocols. (a) The key events during in vivo embryonic development is summarized with the respective locations where these embryos are found. (b) The schematic drawing of developing embryo with respect to the developmental days. Abbreviations indicate the following: a – zona pellucida; b – blastomeres (embryoblast); c – inner cell mass; d – degenerating zona pellucida; e – trophoblast; f – endometrial epithelium (Based on Moore et al., 2004).

(a)

Day	8	9	10	11	12	13	14
Stage of Development in-vivo	Further progress in implantation	Further progress in implantation	Completion of implantation	Lacunar network establishment begins	Further development in lacunar network	Primary chorionic villi appears	Chorion and connecting stalk gets established
Location	Uterine Cavity/ Endometrial Wall		Endometrial Wall				
Corresponding stage at in-vitro culture	Early Differentiation						
Cell Population	Hypoblast Epiblast	Endoderm of umbilical vesicle Ectoderm of amnion	Extraembryonic mesoderm				Primary Chorionic villi

(b)

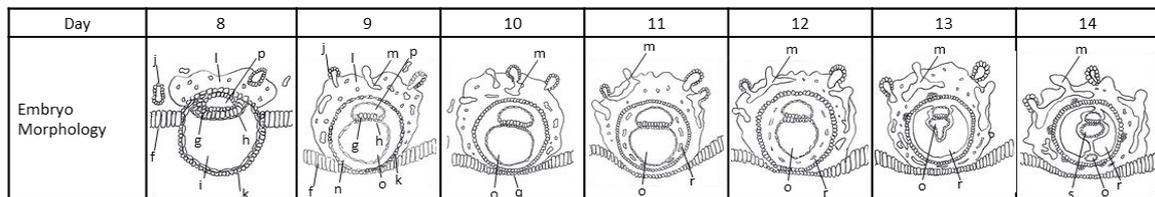


Figure 1.2 The summary of developing embryo during the second week of development after conception (fourth week gestation) and their corresponding derivation in vitro protocols. (a) The key events during in vivo embryonic development is summarized with the respective locations where these embryos are found. (b)The schematic drawing of developing embryo with respect to the developmental days. Abbreviations indicate the following: f – endometrial epithelium; g – epiblast; h – hypoblast; i – exocoelomic cavity; j – uterine gland; k – cytotrophoblast; l – syncytiotrophoblast; m – lacunar network; n – extraembryonic mesoderm; o – primary umbilical vesicle (yolk sac); p – amniotic cavity; q – closing plug; r – extraembryonic coelomic space; s – secondary umbilical vesicle (yolk sac) (Based on Moore et al.,2004).

Day	15	16	17	18	19	20	21
Stage of Development in-vivo	Gastrulation begins (Primitive Streak)	Movement of mesenchyme to establish trilaminar embryonic disc	Establishment of trilaminar embryonic disc	Beginning of neural plate formation, vaculogenesis/ angiogenesis	First Appearance of somite	Further progress in neurulation and vascular network formation	Neural tube formation Circulation of blood and heart begins to pump
Location	Endometrial Wall						
Corresponding stage at in-vitro culture	Germ Layer Formation			Early CNS Differentiation Early Cardiovascular Differentiation		Neural Tube Formation Functional Heart Formation	
Cell Population							

Figure 1.3 The summary of developing embryo during the second week of development after conception (fifth week gestation) and their corresponding derivation in vitro protocols.(a)The key events during in vivo embryonic development is summarized with the respective locations where these embryos are found (Based on Moore et al., 2004).

1.5 The nutritional environment during human embryo development

The aim of this thesis was to investigate the effect of nutrients upon pluripotent stem cell differentiation. Thus, it is important to determine the nutrients 'level during development and compare it to laboratory conditions. Only three factors were selected in this study as it had impact on stem cell differentiation and metabolism. Understanding the shift in metabolism from pluripotent stem cells to differentiated cells via the manipulation of nutrient could lead to new approaches to enhance germ layer formation, directed differentiation of stem cells or even reprogramming into iPSCs (Folmes *et al.*, 2011). This section summarises a review of glucose, pyruvate and oxygen levels during early embryo development.

First week of development: From pre-implantation to peri-implantation

Carbohydrates

Metabolism plays a key role particularly early in embryo development as the viability of the embryo has been associated with carbohydrate metabolism (Leese and Barton, 1984). Upon nutrient limitation, the metabolism has been shown to favour some lineage selection. Culturing human hematopoietic stem cells (HSCs) in low glutamine and glucose stimulated their differentiation into erythroid lineage (Oburoglu *et al.*, 2014). On the other hand, a change in nutrient demand can trigger cell differentiation into a desired lineage (Bracha *et al.*, 2010). In comparison to the strategy of adding multiple recombinant proteins to control cell signalling (Cheng *et al.*, 2012; Sneddon, Borowiak and Melton, 2012), the option of controlling nutrient supply is less cost effective and straightforward.

The level of nutrients during the pre-compaction stage differs from the post-compaction stage and it is dependent on the level of biosynthesis (Epstein and Smith, 1973;

Houghton *et al.*, 1996). Before implantation, the embryos rely more on oxidative metabolism (Wales and Whittingham, 1970). As a result, higher pyruvate consumption is required (Epstein and Smith, 1973). Pyruvate is metabolized via tricarboxylic acid cycle and oxidative phosphorylation to produce ATP and may also be important for: intracellular pH regulation, acting as free radical scavengers, and for reducing the build-up of toxic ammonium ions (Wales and Whittingham, 1970; Tay *et al.*, 1997; Dale *et al.*, 1998; Morales *et al.*, 1999).

During late stage of implantation the glucose is metabolized via glycolysis to provide energy (Wales and Whittingham, 1970; Hardy *et al.*, 1989). Glucose plays an important role for macromolecule synthesis (Brinster, 1969). However, despite its importance, *in-vitro* studies have shown that embryo development undergoes arrest and the formation of reactive oxygen species is augmented in the presence of media containing high glucose levels (Sviderskaya *et al.*, 1996; Iwata *et al.*, 1998). Many studies examined the measurement of carbohydrates' concentration in human fallopian tubal fluid or uterine fluid, either by vascular perfusion (Dickens *et al.*, 1995; Tay *et al.*, 1997) or via direct *in-vivo* measurements (Gardner *et al.*, 1996). For example, glucose is transported to the embryo by facilitated diffusion (Gardner and Leese, 1988). Lactate may be utilized as an energy source when it is the only available energy substrate (Barbehenn, Wales and Lowry, 1978; Gott *et al.*, 1990). The levels of carbohydrates that the embryo is exposed to is dependent on the location of the embryo. For example, during the first 3 days the embryo is exposed to the physiological concentration of the fallopian tube then after day 4 the embryo is exposed to the physiological concentration of the uterus (Table 1.1).

Developmental Days	1	2	3	4	5	6	7	Reference
Pyruvate (mM)		0.17 ± 0.02						Dickens et al., 1995
		0.15 ± 0.03 (luteal)						
Pyruvate (mM)		0.14 ± 0.04						Tay et al., 1997
		0.25 ± 0.09 (secretory)						
Glucose (mM)		0.16 ± 0.06 (secretory)			0.10 ± 0.05			Gardner et al., 1996
		0.53 ± 0.12						Dickens et al., 1995
Glucose (mM)		0.35 ± 0.10 (luteal)						
		1.11 ± 0.14						Tay et al., 1997
Lactate (mM)		1.13 ± 0.21 (secretory)						
		6.19 ± 2.00 (secretory)			3.15 ± 0.31			Gardner et al., 1996
Lactate (mM)		8.58 ± 1.38						Dickens et al., 1995
		8.60 ± 4.20 (luteal)						
Lactate (mM)		5.37 ± 0.53						Tay et al., 1997
		7.58 ± 0.86 (secretory)						
Lactate (mM)		2.32 ± 0.54 (secretory)			5.87 ± 1.19			Gardner et al., 1996

Table 1.1 Concentration of carbohydrates in oviduct (day 1-3) and in uterus (day 4-7). The location matches to where the developing embryo is typically on the developmental days. Day 1 corresponds to the fertilization.

Oxygen

During the preimplantation, the oxygen consumption is low as the energy production relies on oxidative phosphorylation (Brinster, 1969). Reactive oxygen species generated by the oxygen can cause a high-energy electrons during the oxidative phosphorylation process (Catt and Henman, 2000). The *in-vivo* oxygen levels in the human reproductive tracts has not been fully investigated because of the invasive method and ethical concerns. However, oxygen was measured in several animal species such as mice (Auerbach and Brinster, 1968; Quinn and Harlow, 1978; Pabon, Findley and Gibbons, 1989; Umaoka *et al.*, 1992), cattle (Tervit, Whittingham and Rowson, 1972; Thompson *et al.*, 1990), rabbits (Danie, 1968; Li and Foote, 1993; Lindenau and Fischer, 1994) and on hamsters (McKiernan and Bavister, 1990) and in the oviduct and uterus of some mammalian species (rhesus monkeys, hamsters and rabbits) (Fischer and Bavister, 1993).

The rate at which the oxygen reaches the embryo by passive diffusion, is dependent on the oxygen concentration in the gas phase, the solubility of oxygen in the surrounding fluid and the boundary layers around the embryo (Catt and Henman, 2000). It is known that the oxygen levels in the uterus of most mammals varies between 1.5 to 8.7% O₂ (Fischer and Bavister, 1993). The average of oxygen levels in the human uterine surface changes from 2.4% at week 7-10 up to 7-8 % after the maternal blood flows to the foetus in week 11 (Rodesch *et al.*, 1992). The maternal blood flow is not established until week 4 of gestation, thus the embryo is exposed to the nutrients available in the surrounding fluid in the uterus (Hustin and Schaaps, 1987; Foidart *et al.*, 1992; Jauniaux, Gulbis and Burton, 2003). The exocoelomic cavity is then established and the coelomic fluid plays an important role for nutrient transfer for the embryo (Jauniaux *et al.*, 1998).

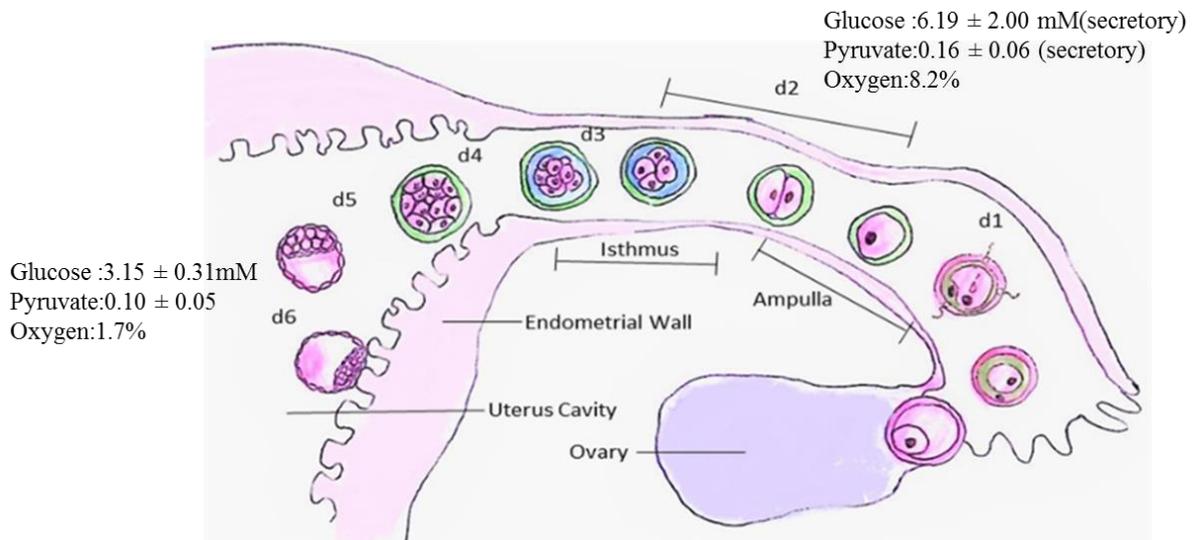


Figure 1.4 Schematic drawing of the human reproductive tract and the relative location of developing embryo with respect to time along with the concentration of glucose, pyruvate and oxygen. Day 1 (d1) corresponds to the first day of conception. From day 2-3 the embryo undergoes cleavage process to establish from 2-cell stage to 10-16 blastomeres. Early blastocyst from day 5 develops into mature blastocyst, which coincides with the beginning of implantation process on day 6 (Based on Moore et al., 2004).

1.6 Project Aims and objectives

Overall Aims

Due to the implementation of pluripotent stem cells in personalised and regenerative medicine, there is a need for a low cost technology that delivers high purity of germ layer precursors. A novel approach through mimicking natural environment during early embryo development could be used to enhance the germ layer formation. This thesis aims to enhance the germ layer formation of human pluripotent stem cells by controlling three environmental factors (glucose, oxygen and pyruvate) to create 3 distinct nutritional microenvironments for ectodermal, mesodermal and endodermal lineages. The initial aim was to determine the effect of lower oxygen tension on the maintenance or differentiation of different pluripotent stem cell lines (hiPSCs and hESCs). Moreover, three important nutritional factors have been selected as the *in vitro* concentration of the factors are different from *in vivo* measurement. We hypothesised that lowering the concentration of these factors could have an impact on germ layer formation. Using design of experiments, 27 different conditions were tested for germ layer gene expression and we were able to select the best desirable condition for each germ. Moreover, we investigated the effect of glucose deprivation on the formation of endodermal lineage and its molecular pathway.

Objectives

Chapter 3: Characterisation of human pluripotent stem cells

The objective of this chapter was to characterize the pluripotency of different stem cell lines (hiPSCs and hESCs) by immunostaining, flow cytometer and their capability to form embryoid bodies.

Chapter 4: The effect of low oxygen tension on the pluripotency of stem cell

Many studies showed that low oxygen tension can maintain the pluripotency of stem cell, thus the effect of culturing pluripotent stem cell at 2% O₂ was assessed by immunostaining.

Chapter 5: The enhancement of human pluripotent stem cells differentiation media using Design of Experiments.

The objective of this chapter was to test the influence of concentration's change of glucose, pyruvate and oxygen levels on germ layer formation. In addition to study the interaction between the different concentrations of the three selected factors. Using design of experiments, 27 different conditions were tested for germ layer gene expressing during 4 days of differentiation. A predicted model for the best condition of each of the germ layer was generated.

Chapter 6: Glucose Deprivation Selects Endodermal Lineages During Human Pluripotent Stem Cell Differentiation.

Having studied the impact of changing the concentration of each of the factors on the germ layer formation, this chapter aimed to study in depth the impact of glucose removal on the endodermal lineage formation. We hypothesised that glucose deprivation will increase the endodermal germ layer in hESCs and hiPSCs. Moreover, we investigated the pathway by which glucose deprivation triggered the upregulation of endodermal cells.

2 Chapter 2: Material and Methods

2.1 Stem Cell culture maintenance

In this study, one human embryonic stem cells line (hESCs) and 3 human induced pluripotent stem cells lines (hiPSCs) were cultured (Table 2.1). These cells lines were cultured on feeder and feeder-free conditions.

Cell Line	Provenance	Reprogramming Method	Passage number used
hiPSCs(BJ) Feeder Free	Fibroblast	Episomal vectors	P 45-55
hiPSCs(BJ) On Feeder	Fibroblast	Episomal vectors	P48-49
hiPSCs(MSU001) Feeder Free	Fibroblast	Lentivirus vector	P70-72
hiPSCs(MSU001) On Feeder	Fibroblast	Lentivirus vector	P60-67
hESCs (Shef 3) Feeder Free	Inner mass of Blastocyst	-	P39-42
hESCs (Shef 3) On Feeder	Inner mass of Blastocyst	-	P29-32
hiPSCs(Cord blood)	cord blood-derived CD34+ progenitors	Episomal vectors	P 41-49

Table 2.1 A summary of all cell lines used, its provenance, reprogramming methods of iPSCs and the passage number.

2.1.1 The maintenance of feeder-dependent stem cell

hESCs (Shef 3) (Draper et al. 2004) were purchased from UK stem cell bank after its derivation from the blastocyst using a microdrop culture system (Aflatoonian et al. 2010). Feeder-dependent hiPSCs (MSU001) which was derived from fibroblasts by Professor Jose B. Cibelli's laboratory (Michigan State University) was acquired from the Spanish stem cell bank, this iPSCs was generated through the overexpression of Oct-4, SOX2, Nanog and Lin28 using lentivirus vector. hESCs (Shef 3) and hiPSCs (MSU001) were cultured on inactivated mouse embryonic fibroblasts (MEFs) with

hESCs media: knockOut Dulbecco's modified Eagle's medium (KnockOut DMEM) (Gibco, catalogue number 10829018), supplemented with 20 % knockout serum replacement (KOSR) (Gibco, catalogue number 10828028), 1% minimum essential medium non-essential amino acid (MEM NEAA) (Gibco, catalogue number 11140035), 0.1mM β -mercaptoethanol (Gibco, catalogue number 31350010), 2 mM L-glutamine (Gibco, catalogue number 25030081), 10 ng/mL bFGF for hiPSCs and 8 ng/mL for hESCs (Millipore, catalogue number SCM075). Cells were grown in gelatine coated T25 flasks (NUNC, Thermo-Fisher Scientific, catalogue number 15217915) with 2.5×10^5 feeder cells and the media was changed daily except the day after passaging.

2.1.1.1 The preparation of inactivated mouse embryonic fibroblast

Feeder layer was prepared by adding mitomycin-C (Sigma-Aldrich catalogue number M4287) for 2 hours on mouse embryonic fibroblast (MEF). MEFs was isolated from CF1 mouse embryo at day 13 by removing the heads and internal organs using sterile scissors. The remaining tissues were washed with PBS (Lonza, catalogue number 17-516F) and tissues 'dissociation was done by incubating the tissues with Trypsin/EDTA (Gibco, catalogue number 25300062) for 5-10 minutes at 37° C. In order to deactivate the effect of Trypsin/EDTA, MEF media containing Dulbecco's modified Eagle's medium (Gibco, catalogue number 11965084), 10% foetal bovine serum (Gibco, catalogue number 10270106), 1% non-essential amino acid (Gibco) was added after while grinding the tissues. After allowing the mixture to settle down, the supernatant was transferred into a centrifuge tube and further MEF media was added before the centrifugation (Centrifuge 5810 R, Eppendorf) at 1000 rpm for 5 minutes. The pellet was re-suspended in MEF medium and transferred into T75 flask (NUNC, Thermo-Fisher Scientific, catalogue number 15227915) and incubated for 2 passages before labelling it as passage zero (P0). A stock of P0 MEF was established by freezing the harvested

MEF with freezing media containing 40% MEF medium, 50% FBS and 10% DMSO(Sigma, catalogue number 276855) and storing it in liquid nitrogen.

P1-P6 MEFs were used for the preparation of feeders after thawing from liquid nitrogen. Frozen vials were incubated at 37° C for 2-3 minutes then transferred into a centrifuge tube containing MEF media. The pellet was then re-suspended after 1200 rpm centrifugation for 3 minutes and plated in T75 flask. For MEFs passaging, cells were trypsinised using Trypsin/EDTA and split at 1:5-1:3 ratio.

Feeders were prepared by adding mitomycin-C (1 mg/mL) on MEFs for 2 hours one day before passaging pluripotent stem cells. Cells were then detached using Trypsin/EDTA and centrifuged at 1200 rpm for 3 minutes. Feeders were then counted and seeded in a T25 flask coated with 0.1% porcine gelatine (Sigma, catalogue number G9391) at a density of 2.5×10^5 cells per flask. 0.1% gelatine was prepared by dissolving it in water. On the day of pluripotent stem cells passaging, MEF medium was replaced with hESCs or hiPSCs media for half an hour.

2.1.1.2 Passaging of feeder-dependent human pluripotent stem cell

When hESCs or hiPSCs reached 70-80% confluence, 7 mL of fresh hiPSCs/hESCs medium was added for at least one hour prior to passage. Using a dissecting microscope, pluripotent and undifferentiated pluripotent colonies were mechanically scraped using sterile Pasteur pipettes (Starlabs, catalogue number E1415-0700). The cells were then transferred into T25 feeder flask previously coated with feeders at a ratio 1:3.

2.1.2 Adaptation of feeder cell line to feeder free and feeder free to feeders

hESCs (Shef 3) and hiPSCs (MSU001) were adapted to feeder free culture. E8 media was added to the feeder dependent cells and mechanical scrapping was performed on the undifferentiated stem cells. These clumps were then transferred into a flask coated with vitronectin. Cells adapted to feeder free were mechanically scraped upon the first 5 passages then chemically passaged with EDTA. hiPSCs (BJ) were adapted to feeder culture. hiPSCs media was added to the flask and the clumps of cells from EDTA passaging were transferred to vitronectin coated T25 flask.

2.1.3 The maintenance of feeder-free pluripotent stem cell lines

Human iPSCs (BJ) was provided by Dr. Amanda Carr (UCL, Institute of Ophthalmology), these cells were reprogrammed using episomal vectors and an amaxa nucleofactor.

Human Episomal hiPSC (Cord blood) Line was pursued from Thermo-Fisher Scientific (catalogue number A18945) and generated using cord blood-derived CD34+ progenitors with seven episomally expressed factors (Oct4, Sox2, Klf4, Myc, Nanog, Lin28, and SV40 T). This line was first grown on mouse feeders and then adapted to feeder free system.

These cell lines were cultured in Essential 8 medium (Gibco, catalogue number A1517001) on vitronectin VTN-N (Thermo-Fisher Scientific, catalogue number A31804) coated flasks.

2.1.3.1 The passage of feeder free pluripotent stem cell lines

Cells were grown in monolayer (70-80% confluence) before passaging. E8 media was removed and discarded, cells were washed with PBS without Calcium and Magnesium before adding 2 mL of 0.5 mM EDTA (UltraPure™ 0.5 M EDTA, pH 8.0, Thermo-Fisher Scientific, catalogue number 15575-038). The flask was then left at room temperature for around 5-10 minutes and checked under the microscope to see if the cells around the edges of the colonies were dislodged from each other. EDTA was then removed and the cells were washed with E8 media and transferred into vitronectin previously coated T25 flask (room temperature for one hour) at a ratio of 1:3. Using chemically defined environment has been showed to improve the derivation of iPSCs along with culturing it on vitronectin coated flask with E8 medium (Chen et al., 2011).

2.1.4 Freezing and thawing pluripotent cells

mFreSR™ (Stemcell technologies, catalogue number 05854) was used in order to freeze down all feeder free cell lines. Cells were detached using 0.5 mM EDTA, centrifuged at 1200 rpm for 3 minutes and then re-suspended with freezing media. Pluripotent stem cells growing on feeders were scrapped under the dissecting microscope, centrifuged at 1200 rpm for 3 minutes then the pellet was re-suspended with 50 % complete hiPSCs or hESCs media, 40% KOSR and 10% dimethyl sulfoxide (DMSO) .1mL aliquots of cryogenic vials (Thermo-Fisher Scientific, catalogue number 11740573) were placed in Mr.Frosty™ (Thermo-Fisher Scientific, catalogue number 11315674) for two days in -80 °C then moved to liquid nitrogen for longer storage.

Upon thawing, vials were taken from liquid nitrogen and incubated in the water bath at 37°C for few minutes, the cells were transferred into a centrifuge tube containing 9 mL of pre-warmed media and centrifuged for 3 minutes at 1200 rpm. The supernatant was

removed and the pellet was re-suspended with media. The feeder free pluripotent stem cells were re-suspended in E8 medium and transferred to a vitronectin coated T25 flask. The mouse dependant feeder stem cells were put on a T25 flask coated with 2.5×10^5 feeder cells.

In early experiments, the effect of low oxygen tension on pluripotency was studied. Lower oxygen level was achieved by supplying the chamber by pumping 2% O₂, 5% CO₂ from gas cylinder (British Oxygen Company, BOC) for 10 minutes, sensors (OXY-4 mini, Presens) were attached to the chamber for 24 hours in order to detect if there were any leakage. Then for further experiments, lower oxygen levels (0 and 2%) was established by supplementing the incubators by nitrogen. The incubators were set at specific oxygen tension and was opened only when the experiment ended.

2.2 Differentiation protocols

2.2.1 Embryoid bodies formation

Embryoid body' formation (EBs) was performed using AggreWell™ 400Ex plates (Stemcell Technologies, catalogue number 34421) to get a uniform sized of stem cell aggregates. hiPSCs (BJ, MSU001 and cord blood iPSCs) and hESCs (Shef 3) were washed with PBS and were dissociated from the flask upon the addition of tryple (Thermo-Fisher Scientific, catalogue number 12604021) for 5-8 minutes at 37° C. iP-SCs medium was added to deactivate the effect of tryple and the cells were transferred into a centrifuge tube through a 40 µm cell strainer (Scientific Laboratory Supplies, catalogue number 352340) then centrifuged at 1250 rpm for 5 minutes. The pellet was re-suspended in 2 mL of EB medium containing 1 ng/mL (10 µM) of Rho Kinase Inhibitor (ROKi) (Millipore, catalogue number SCM075) and 10 µl of cell suspension was mixed with 10 µl of 0.4% trypan blue (Sigma, catalogue number T8154) in order to assess cell count and cell viability using haemocytometer (Reichert Bright-Line). 4.7×10^6 cells were seeded in each well in order to get 1000 cell/EB, media was topped up to 2 mL and the plate was incubated for 24 hours at 37 °C and 5 % CO₂.

The next day the EBs were harvested by gentle pipetting then transferred into a centrifuge tube to allow to settle. Once the EBs were settled, they were resuspended in EB medium without ROCKi. The EB medium was composed of DMEM-F12 (Gibco, catalogue number 11320033), supplemented with 20% knockout serum replacement media, 1% Glutamax, 1% non-essential amino acids and 0.1% β-mercaptoethanol . For the suspended culture, EBs were transferred to low attachment 3 cm bacterial grade dishes. For adherent culture, EBs were transferred to 6 well tissue culture plate

(NUNC, Thermo-Fisher Scientific, catalogue number 10396482) pre-coated with Matrigel (BD Bioscience, catalogue number 734-1440) for 2 hours at room temperature. After removing the Matrigel, 1 mL of EB medium was added and around 6-8 EBs were transferred to each well using the dissecting microscope.

2.2.2 Single cell suspension

Another method of iPSCs differentiation was used. Single cell suspension was performed to expose all the cells to the same nutritional environment. The analysis of germ layer expression of EBs and single cell suspension was done to compare which method had the highest expression. hiPSCs (episomal) and hESCs (Shef 3) were washed with PBS before adding tryple. After 5 minutes of incubation at 37 °C, differentiation media was added and the cell clumps were removed by allowing the cells to go through a 40 µm cell strainer then centrifuged at 1250 rpm for 5 minutes. After counting, cells were seeded as single cell in a 12-well plate pre-coated with Matrigel for 2 hours at a density of 0.8×10^5 cells/mL. Differentiation was induced by treating hiPSCs and hESCs with DMEM-F12 and customised DMEM-F12 (Gibco, catalogue number 11320033) for the deprivation studies. Customised media was deprived from glucose, pyruvate and glutamine. Customised media was ordered through the manufacture and was tested for osmolality, The media was supplemented with 20% knock-out serum replacement media, 1% Glutamax, 1% non-essential amino acids and 0.1 β-mercaptoethanol and 1 ng/mL of Rho Kinase Inhibitor (ROKi) . Cells were left for 4 days to differentiate.

To assess the effect of apoptosis on germ layer formation staurosporine (1µM) (Abcam, catalogue number ab120056) was added to the single cell suspension and left for 2, 4, 9 and 24 hours. Staurosporine stock solution (10 µg/mL) was prepared by dissolving 100µg of staurosporine powder in 10 mL of DMSO, 2 mL aliquots were

stored in -20 °C. hiPSCs (Cord blood) were seeded at 0.8×10^5 cells/mL in 12 well plates. After 24 hours, the cells were treated with staurosporine (1 μ M) by diluting the stock solution with differentiation media. After 2, 4, 9 and 24 hours treatment, cells were collected to check germ layer expression and apoptosis.

2.3 Immunocytochemistry

Pluripotent stem cells plated in 24-well tissue culture plate and single cell suspension plated in 12-well tissue culture were fixed with 4 % paraformaldehyde (PFA, Sigma-Aldrich, catalogue number P6148) at room temperature for 30 minutes. 40 g of paraformaldehyde powder was added to 800 mL of 1X PBS and left on a stir plate in ventilated hood.

PFA was removed and cells were washed 3 times with 1x PBS. For intracellular markers cells were permeabilized with 0.25% Triton X-100 (Millipore, catalogue number 1086431000) for 30 minutes at 37 °C. After 3 washes with 1x PBS, cells were treated with blocking solution (1x PBS with 5% foetal bovine serum) and covered in foil for 30 minutes at room temperature. Cells were stained with primary antibodies (with a dilution of 1/200 in blocking solution) for one hour at 37°C (Table 2.2). Cells were washed again 3 times with 1x PBS before adding the secondary antibodies for one hour at room temperature (with a dilution of 1/300) (Table 2.3). Nuclear staining was performed by diluting DAPI (4',6-diamidino-2-phenylindole) (1mg/mL) (Thermo-Fisher Scientific, catalogue number 62248) with 1x PBS to a final concentration of 0.1 μ g/mL (dilution 1/10000). After 5 minutes, the cells were washed with 1x PBS and stored with 1x PBS at 4° C. Cells were stained with isotype control to check if there was any non-specific binding of the secondary antibodies. Pictures of the results were taking using multichannel fluorescence imaging system of EvosTM FL microscope (Thermo-

Fisher Scientific, catalogue number AMF4300) and confocal microscopy (LSM 770, software Zen 2009, Carl Zeiss).

Primary Antibody	Host	Antibody	Dilution	Company	Code
Pluripotency Markers					
Oct 3/4	Mouse	IgG mono-clonal	1:200	Santa Cruz Biotechnology	Sc-5279
TRA-160	Mouse	IgM mono-clonal	1:200	Millipore	MAB4360C3
TRA-181	Mouse	IgM mono-clonal	1:200	Millipore	MAB4381A4
SSEA3	Mouse	IgM mono-clonal	1:200	Millipore	MAB4303
SSEA4	Mouse	IgG3 mono-clonal	1:200	Millipore	MAB4304
SSEA1	Mouse	IgM mono-clonal	1:50	P. Andrews, Sheffield University, UK	
Differentiation Markers					
TUJ	Rabbit	IgG mono-clonal	1:500	Life Technologies	A25532
SMA	Mouse	IgG2a mono-clonal	1:100	Life Technologies	A25531
AFP	Mouse	IgG1 mono-clonal	1:500	Life Technologies	A25530
PAX6	Mouse	IgG mono-clonal	1:200	Millipore	MAB5552
Brachyury	Mouse	IgM κ mono-clonal	1:200	Santa Cruz Biotechnology	Sc-166962
Sox17	Mouse	IgG1 mono-clonal	1:200	Abcam	Ab84990
PDX1	Mouse	IgG1 mono-clonal	1:200	Abcam	Ab84987

Table 2.2 List of primary antibodies used to confirm the pluripotency and differentiation of pluripotent stem cells.

Secondary Anti-body	Anti-	Dilution	Company	Code
Alexa Fluor® 488 Goat Anti-Mouse		1:300	Thermo-Fisher Scientific	A11001
Alexa Fluor® 647 donkey anti-rabbit; for use with anti-TUJ1		1:250	Thermo-Fisher Scientific	A25537
Alexa Fluor® 488 goat anti-mouse IgG1; for use with anti-AFP		1:250	Thermo-Fisher Scientific	A25536
Alexa Fluor® 594 goat anti-mouse IgG2a; for use with anti-SMA		1:250	Thermo-Fisher Scientific	A25534

Table 2.3 List of secondary antibodies used against primary antibodies for the assessment of the pluripotency and differentiation of pluripotent stem cells.

2.4 Flow Cytometry

2.4.1 Flow Cytometry Staining

Human iPSCs Cells (BJ, MSU001 and episomal) and human ESCs (Shef 3) were incubated at 20 % O₂ in 12-well tissue culture plate. After 24 hours, cells were detached using tryple , centrifuged at 1500 rpm for 5 minutes. The pellet was re-suspended and fixed with 4 % paraformaldehyde for 20 minutes at room temperature. Cells were then washed, centrifuged and incubated with stemness markers conjugated antibodies (SSEA1, SSEA4; BD Bioscience, Catalogue number 560477) overnight at 4° C at 1:200 ratio. For intracellular marker, cells were permeabilised with 0.25% Triton X-100 for half an hour prior staining with primary conjugated antibody (OCT-4; BD Bioscience, Catalogue number 560477). Cells were then washed and run on the C6 flow BD ACCURI C6 (BD Bioscience).

2.4.2 Detection of Apoptosis by Annexin V/PI.

Apoptosis and necrosis were detected using Annexin V-PI staining (Annexin V-FITC Apoptosis Detectin Kit, Abcam, catalogue number ab14085). In each condition, cells were collected, centrifuged at 1500 rpm at 4°C for five minutes. 500 µL of suspension Buffer was used to re-suspend the pellet before the addition of 5 µL of Annexin and 5 µL Propidium Iodide and immediately run on the flow cytometer (BD-C6 Accuri). Upon apoptosis, phosphatidylserine will translocate from cytoplasmic fraction to the outer leaflet of cell membrane, thus it will be exposed for its binding to Annexin V. Live cells are PI impermeable. Cells that are stained negative for Annexin V-PI are considered living cells. Cells that are stained positive for both Annexin V and PI are considered at the end stage of apoptosis, cells that are Annexin negative and PI positive are considered to be necrotic.

2.5 Gene expression by Real Time Polymerase Chain Reaction

2.5.1 RNA extraction

In order to study the gene expression, RNA was extracted using RNeasy Micro Kit (Qiagen, catalogue number 74004). Cells were washed with 1x PBS then scrapped, centrifuged, the supernatant was then removed and the pellet was stored at -80 °C. On the day of extraction, RLT buffer was added and the suspension was transferred into Qias shredder Columns (Qiagen, catalogue number 79654). Then the RNA was precipitated in RNeasy columns and further steps including DNA digesting step was performed in order to remove any DNA contaminant. At the end of the protocol, 40 µl of RNA samples diluted in RNase free water were collected and stored in -80°C. Quantification was performed using spectrophotometer (NanoDrop ND-1000, Thermo-Fisher Scientific) by measuring the absorbance at 260/280 nm.

2.5.2 cDNA synthesis :Reverse transcriptase polymerase chain reaction

cDNA synthesis was done using QuantiTect Reverse Transcription Kit (Qiagen, catalogue number 205310). The synthesis of complementary deoxyribonucleic acid (cDNA) was performed by first eliminating any genomic DNA contaminant by the addition of gDNA Wipeout buffer, which was added to up to 1µg of mRNA then topped up to a final volume of 14 µl with RNase free water. The mixture was incubated at 42°C for 2 minutes in a thermocycler. A mastermix of Primer Mix Quantiscript, RT buffer and Quantiscript Reverse Transcriptase were added to the template RNA (total volume of 20 µl). The samples were run at 42°C for 15 minutes then at 95°C for 3 minutes and store at -20°C.

2.5.3 Quantitative Real Time Polymerase Chain Reaction (RT-PCR)

The cDNA synthesised from previous step was mixed with Quantitect SYBR Green PCR Kits (Qiagen, catalogue number 204145), Quantitect pre-validated primer assay (Table 2.4) and RNase free water were added to the cDNA to a total volume of 25 μ l and were loaded in triplicate in Hard-Shell Low Profile Thin-Wall 96 Well Skirted PCR plates (Biorad, catalogue number HSP9601) and run on Biorad CFX 96 Connect Real-Time PCR machine.

Polymerase chain reaction was performed screening for differentiation and pluripotent gene along with house-keeping gene (β -Actin), standard controls as no reverse transcriptase control (NoRT) and no template control (NTCs) in order to check if there was any contaminants or primer dimer formation.

The C_t values were taken from the Biorad CFX Manager 3.0 and fold change was calculated using the normalized relative expression ($\Delta\Delta C_t$) using the formula below (K. Livak, PE-ABI; sequence Detector User Bulletin 2):

$$\text{Fold change} = 2^{-\Delta\Delta C_t}$$

Where $2^{-\Delta\Delta C_t} = [(C_t \text{ gene of interest} - C_t \text{ internal control})_{\text{sample A}} - (C_t \text{ gene of interest} - C_t \text{ internal control})_{\text{sample B}}]$

Biomarker	Description	QuantiTect Assay	Primer	Catalog Number	Company
β-Actin	House Keeping gene	Hs_ACTB_2_SG		QT01680476	Qiagen
OCT 4	Pluripotency	Hs_POU5F1_1_SG		QT00210840	Qiagen
PAX6	Ectodermal marker	Hs_PAX6_1_SG		QT00071169	Qiagen
Tubulin 3	Ectodermal marker	Hs_TUBB3_1_SG		QT00083713	Qiagen
Nestin	Ectodermal marker	Hs_NES_2_SG		QT01015301	Qiagen
SOX17	Endodermal marker	Hs_SOX17_1_SG		QT00204099	Qiagen
FOXA2	Endodermal marker	Hs_FOXA2_1_SG		QT00212786	Qiagen
CXCR4	Endodermal marker	Hs_CXCR4_2_SG		QT02311841	Qiagen
Brachyury	Mesoderm marker	Hs_T_1_SG		QT00062314	Qiagen
KIFA	Mesoderm marker	Hs_KIF1A_1_SG		QT00072604	Qiagen

Table 2.4 The gene expression for germ layer formation and pluripotency was evaluated using these primers.

2.6 Western Blot

Single cell suspension were plated in normal and deprived conditions for 24, 32, 40 and 48 hours. The cells were scraped and centrifuged at 1500 rpm at 4°C. Protein extraction was done by the addition of Laemmli buffer (Bio-rad, catalogue number 161-0737) containing 5% β -mercaptoethanol (Bio-rad) to the cell pellet, then denatured at 95 °C for 9 minutes and centrifuged at 13000 xg for 5 minutes and stored at -20°C.

Using SDS-PAGE proteins were separated on 4-20% mini-protein TGX Gels (Bio-Rad, catalogue number 4561094) and transferred to PVDF membranes (Bio-Rad, catalogue number 1620174) at 0.25 mA for 90 minutes. The membranes were then blocked with 5% blotting-grade blocker (nonfat dry milk, Bio-Rad, catalogue number 1706404) dissolved in 0.5x TBS (Bio-Rad, catalogue number 1706435), 0.05% Tween-20 (Sigma, catalogue number P1379) for 1 hour at 4°C. Membranes were then incubated with primary antibody for mouse monoclonal IgG anti- β -Actin (Abcam), anti-mTOR (Cell signalling technology), anti-p-MTOR(Ser2448) (Cell signalling technology), anti-p-AMPK α 1/2(Thr 183/172)(Santa Cruz Biotechnology), anti-AMPK α 1(71.54) (Santa Cruz Biotechnology), anti-phospho-Tuberin/TSC2(Thr1462) (Cell signalling technology), anti-Tuberin/TSC2(D93F12) (Cell signalling technology), p53(DO-1) (Santa Cruz Biotechnology) at a concentration of 1:1000 overnight at 4°C on a shaker. The membranes were then washed with 1X TBS with 0.1% Tween-20 for one hour. After the washing step, the membranes were incubated with secondary antibody (Anti-mouse and anti-rabbit IgG HRP-conjugated secondary antibodies (Abcam) at a concentration of 1:2000 for 1 hour at 4°C (Table 2.5). The membranes were then washed and the development was done using SuperSignal West femto maximum sensitivity substrate (Thermo-Fisher Scientific, catalogue number 34095).

Reactivity	Host	Antibody	Dilution	Company	Code
β-Actin	Mouse	IgG mon- oclonal	1:1000	Abcam	Ab8226
mTOR	Rabbit	IgG poly- clonal	1:1000	Cell signal- ling technol- ogy	2972
p-MTOR(Ser2448)	Rabbit	IgG poly- clonal	1:1000	Cell signal- ling technol- ogy	2971
p-AMPKα1/2(Thr 183/172)	Rabbit	IgG poly- clonal	1:1000	Santa Cruz Biotechnol- ogy	sc- 101630
AMPKα1(71.54)	Mouse	IgG Mon- oclonal	1:1000	Santa Cruz Biotechnol- ogy	sc- 130394
phospho-Tu- berin/TSC2(Thr1462)	Rabbit	IgG poly- clonal	1:1000	Cell signal- ling technol- ogy	3611
Tu- berin/TSC2(D93F12)	Rabbit	IgG Mon- oclonal	1:1000	Cell signal- ling technol- ogy	4308
p53(DO-1)	Mouse	IgG Mon- oclonal	1:1000	Santa Cruz Biotechnol- ogy	sc-126
Mouse IgG (H+L)	Goat	IgG-HRP	1:2000	Abcam	Ab6789
Rabbit IgG (H+L)	Goat	IgG-HRP	1:2000	Abcam	Ab6721

Table 2.5 List of primary and secondary antibodies used for western blot.

2.7 Mycoplasma Testing and Karyotype

All cell lines were tested for mycoplasma (Surrey Diagnostics, Cranleigh, UK). hiPSCs (Cord blood) was tested for karyotype abnormalities using G banding at early passage (P40) and late passage number (P63) and cells were sent to Cell Guidance System (Babraham Research Campus, Cambridge, UK).

2.8 Cell count

Two techniques were used for cell counting and viability. First method used was trypan blue exclusion method by manual counting: 10 μ l of cell suspension was mixed with 10 μ l of 0.4% of trypan blue and then half of the mixture was transferred to haemocytometer (Reichert Bright-Line), the average of four quadrant was used to calculate the cell count by multiplying the average with 10^4 and any dilution factor used. The second method was by taking 500 μ l of the sample and run it on a Vi-Cell counter (Beckman Coulter). Around 50 images were taken to determine cell concentration and viability.

2.9 Cell Proliferation Kit (WST-1)

WST-1 (Sigma-Aldrich, catalogue number 05015944001) assay was used to measure if the cells were metabolically active. The cleavage of the tetrazolium salt to a soluble formazan occurred in the presence of NAD(P)H. Cells were seeded into 12-well plates at a density of 0.8×10^5 Cells/mL. 100 μ l of WST-1 was added to each well as a dilution 1/10 and the plates were incubated in the dark for 4 hours in a humidified atmosphere (37°C, 5% CO₂). The solubilized formazan product was spectrophotometrically measured using an ELISA reader (Tecan) at 450 nm.

2.10 Design of Experiments

A total of 27 different combinations of three factors: oxygen, pyruvate and glucose were tested for the germ layer gene expression of hiPSCs (Cord blood) along with the cell counts. The experimental design space was generated using the design expert software (Statistic made easy, Stat-Ease). A full factorial design was used in order to allow the estimation of all possible interactions. Thus, as each of the 3 factors have 3 levels, a total of 27 different conditions was generated as shown in Table 2.6. Each condition was performed in triplicate and the data generated from the RT-PCR during the 4 days of differentiation process was fed back into the design expert software. Models for each response factor (i.e. gene expression maker) was created using the fit model tool in design expert which creates the best fitting model based on the data set provided. For all response factors in this study, the quadratic model was shown to have the best fit and hence this was selected over others. Using the gene expression for each of the germ layer and viable cell count a predictable mathematical model was generated to determine the best condition (with highest desirability) for the enhancement of each germ layer.

2.11 Statistics

The experiments were carried out in biological triplicate or more and all values shown represent the mean with standard deviation as error bars. Analysis of data was done using two-way ANOVA for glucose deprivation and control and staurosporine treatment. t-test analysis was used to compare the gene expression before and after normal media switch. p values less than 0.05 were considered significant.

Condition	Oxygen (%)	Glucose(mM)	Pyruvate(mM)
1	0	0	0
2	0	0	0.14
3	0	0	0.5
4	0	3.14	0
5	0	3.14	0.14
6	0	3.14	0.5
7	0	17.5	0
8	0	17.5	0.14
9	0	17.5	0.5
10	2	0	0
11	2	0	0.14
12	2	0	0.5
13	2	3.14	0
14	2	3.14	0.14
15	2	3.14	0.5
16	2	17.5	0
17	2	17.5	0.14
18	2	17.5	0.5
19	20	0	0
20	20	0	0.14
21	20	0	0.5
22	20	3.14	0
23	20	3.14	0.14
24	20	3.14	0.5
25	20	17.5	0
26	20	17.5	0.14
27	20	17.5	0.5

Table 2.6 Summary of the combination of different levels of the factors (oxygen, glucose and pyruvate) in each of the 27 combination assessed by Design of experiment.

3 Chapter 3: Characterisation of Human Pluripotent Stem Cells

3.1 Introduction

During the early derivation of embryonic stem cells (ESCs), cells were cultured and passaged on inactivated mouse fibroblasts (MEFs) (Evans and Kaufman, 1981; Martin, 1981; J. A. Thomson *et al.*, 1998). Since inactivated MEFs do not proliferate, feeders are used as an extracellular matrix and for the secretion of growth factors such as bFGF which maintains the pluripotency of stem cells (Martin and Evans, 1975; Martin, Wiley and Damjanov, 1977; Ludovic Vallier, Morgan Alexander, 2005; Wang *et al.*, 2005; Malladi *et al.*, 2006; Ström *et al.*, 2007; Park *et al.*, 2011). However, culturing pluripotent stem cells on feeders has raised a number of concerns associated with the usage of animal derived material such as batch-to-batch variation and presence of contaminants such as mycoplasma (Akopian *et al.*, 2010). Thus, many protocols had moved toward a standard xeno-free platform for the derivation and expansion of pluripotent stem cells (Nakagawa *et al.*, 2014). Good Manufacture Practice (GMP) compliant platform for clinical trials could be done by either using human feeder (Richards *et al.*, 2002; Simón *et al.*, 2005; Ellerström *et al.*, 2006; Fong and Bongso, 2006) or feeder-free culture (Klimanskaya *et al.*, 2005; Sjögren-Jansson *et al.*, 2005; Ludwig *et al.*, 2006; Steiner *et al.*, 2010). It was been shown that feeder free culture helped in the maintenance of different pluripotent stem cell lines, and decreased the risk of contamination with animal product (Nasonkin and Koliatsos, 2006; Tannenbaum *et al.*, 2012).

It's recommended by the International Stem Cell Banking Initiative to carry several test to demonstrate the pluripotency of the cells used. These tests include: checking for

pluripotency marker; differentiation; DNA fingerprinting; genome analysis and contaminants testing (Crook *et al.*, 2010). The generation of iPSCs provided new possibilities for regenerative medicine as it could serve as raw materials for cell-based therapy. However, pluripotent stem cells should be tested to confirm its suitability as raw material to ensure quality, efficacy and safety. Thus, it was important to establish a comparability of 'clinical-grade' induced pluripotent stem cells which is dependent on an agreement on the critical quality attributes. The quality and safety of the product used in clinical trials should be assessed based on an appropriate range of the physical, chemical and biological properties of iPSCs (Sullivan *et al.*, 2018)

In this thesis, feeder-dependent and feeder-free human embryonic stem cells (hESCs) and human induced pluripotent stem cells (hiPSCs) were cultured and feeder-dependent cells lines were adapted to feeder-free. The characterisation of pluripotent stem cells cultured or adapted to new conditions is an important step before starting any differentiation protocol in order to assess the pluripotency of the cell line used.

3.2 Chapters Aims and Objectives

The aims of this chapter were to characterise the pluripotency of the stem cell lines used and to adapt feeder-dependent cell lines to feeder-free. The specific objectives were:

- To adapt the feeder-dependent hESCs (Shef 3) and hiPSCs (MSU001) cell lines to feeder-free pluripotent stem cell lines.
- To assess pluripotency marker's expression of hESCs (Shef 3) and hiPSCs (BJ; MSU001 and cord blood) by immunochemistry and flow cytometry.
- To evaluate the ability of the stem cell lines to form embryoid bodies (EB).

3.3 Results and Discussion

3.3.1 Adaptation of feeder-dependent hiPSCs and hESCs to feeder-free cell lines.

Feeder-dependent hiPSCs (MSUH001) was adapted for more than 10 passages to feeder-free system by manual scrapping (Wagner and Welch, 2010). Cells were then transferred to vitronectin coated T-25 flask and cultured with Essential 8 media. Numerous differentiated cells were detected at early passages, thus mechanical scrapping was applied during these passages. After approximately 10 passages, the morphology of the hiPSCs (MSU001) showed a negligible amount of differentiated cells, so the cells were adapted to chemical passaging using EDTA (Figure 3.1).

Once the feeder-dependent hESCs (Shef 3) were transferred into feeder-free system a number of morphological changes such as fibroblast-like structure and embryoid bodies were observed. Manual scrapping was performed for the early 10 passages, then chemical passaging was used. The feeder-free hESCs (Shef3) were compacted and formed defined colonies (Figure 3.2). After the adaptation process was completed, pluripotent markers were assessed to check the pluripotency of hESCs (Shef 3) and hiPSCs (MSUH001) .

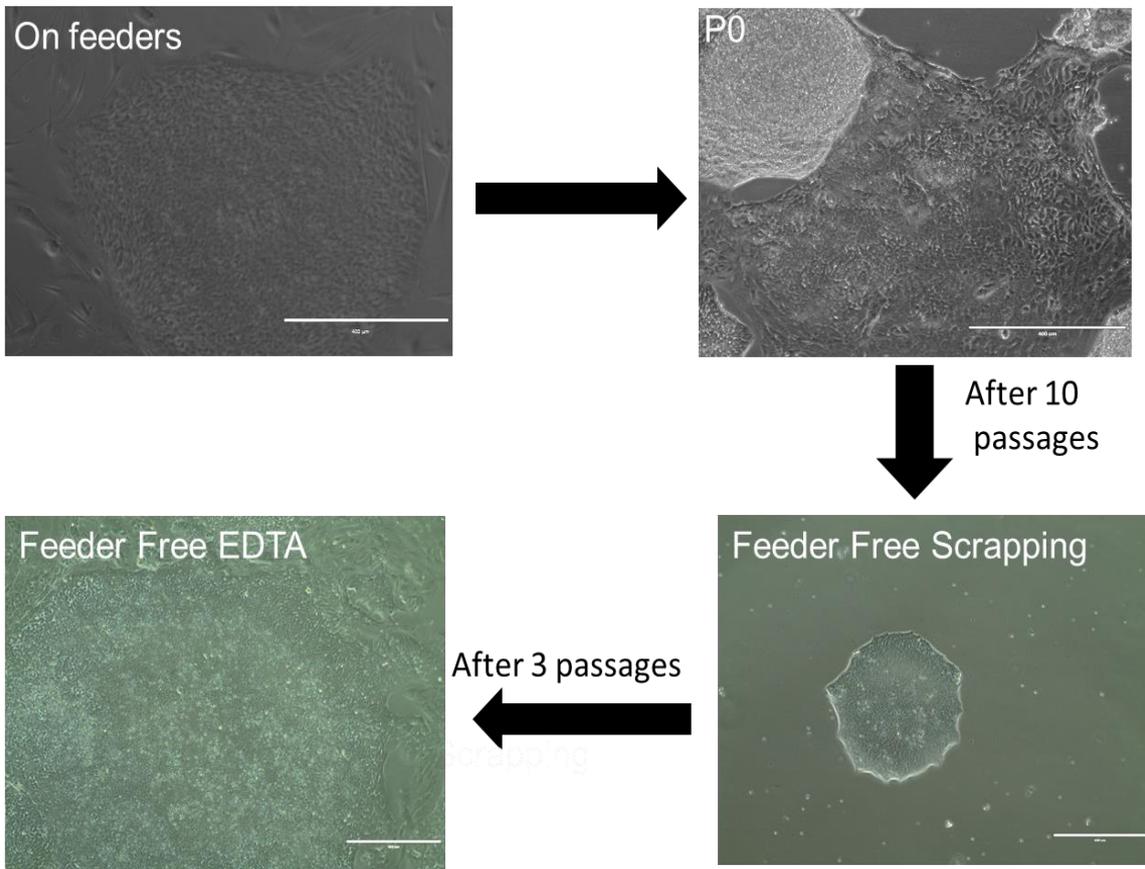


Figure 3.1 Adaptation of hiPSCs (MSUH001) to feeder-free system. Cells were adapted to feeder-free system by manual scrapping for more than 10 passages, then adapted to chemical passaging using EDTA. Images were taken at 100X ocular lens magnification using Evos[®] XL. Scale bars=400μm.

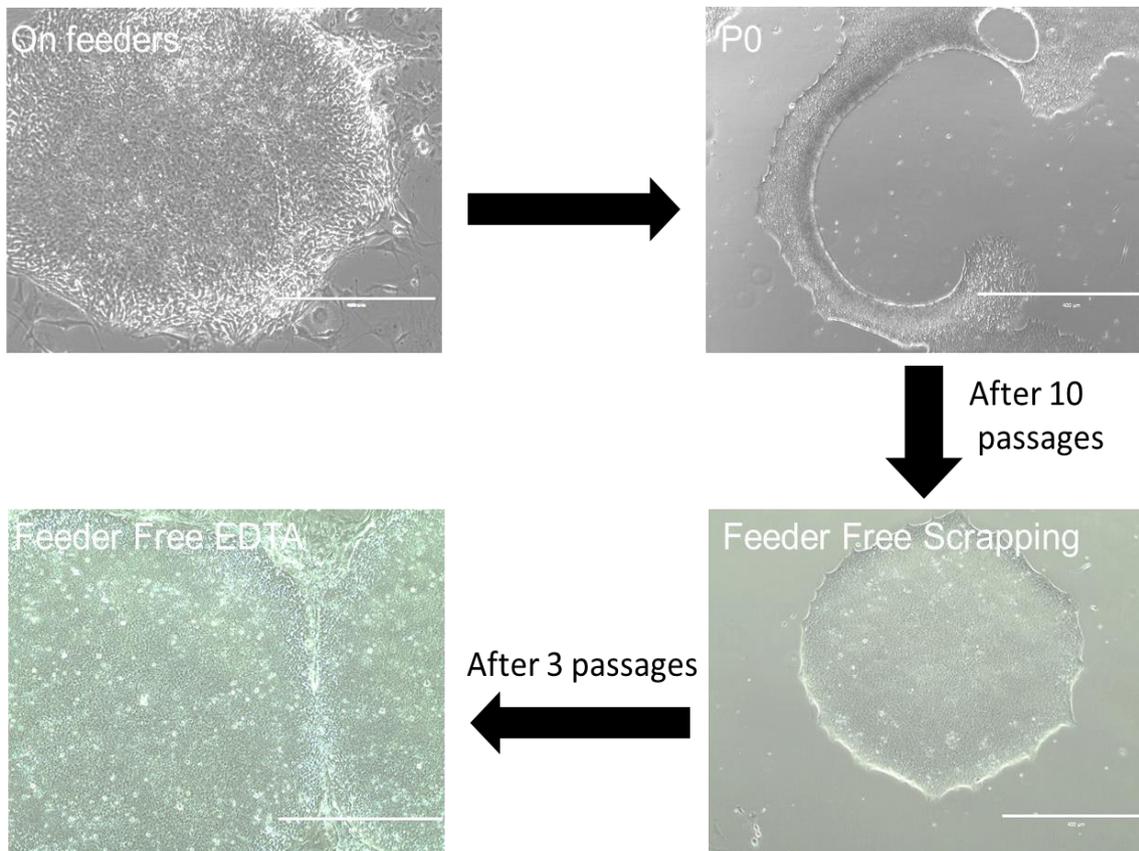


Figure 3.2 Adaptation of hESCs (Shef 3) to feeder-free system. Cells were adapted to feeder-free system by manual scrapping for more than 10 passages, then adapted to chemical passaging using EDTA. Images were taken at 100X ocular lens magnification using Evos® XL. Scale bars=400µm.

3.3.2 Characterisation of feeder-free and feeder-dependent hiPSCs and hESCs by immunocytochemistry

hiPSCs (BJ, feeder-free)

Immunocytochemistry is one of the most common techniques to assess the pluripotency of stem cells. It has been shown that pluripotent stem cells were positive for SSEA4, TRA 1-60 and OCT-4 (Okamoto *et al.*, 1990; Schöler *et al.*, 1990; J. A. Thomson *et al.*, 1998; Reubinoff *et al.*, 2000; PAN *et al.*, 2002; Rosler *et al.*, 2004). hiPSCs (BJ, feeder-free) were stained to check if the cells expressed pluripotency markers. Feeder-free hiPSCs (BJ) cultured at normoxia were positively stained for extracellular pluripotency markers: SSEA4 and TRA 1-60 and intracellular marker OCT-4 (Figure 3.3).

hiPSCs (BJ, feeder-dependent)

After the preliminary results of hiPSCs (BJ, feeder-free) exposed to lower oxygen levels (§Chapter 4), it was necessary to study the same effect of low oxygen levels on the same cell line but with the presence of feeders to assess whether the extracellular matrix could interfere with the effect of low oxygen levels. Thus hiPSCs (BJ) were adapted to feeder system for more than 3 passages, hiPSCs (BJ, on feeders) were still pluripotent and expressing all extracellular and intracellular pluripotent markers (Figure 3.4).

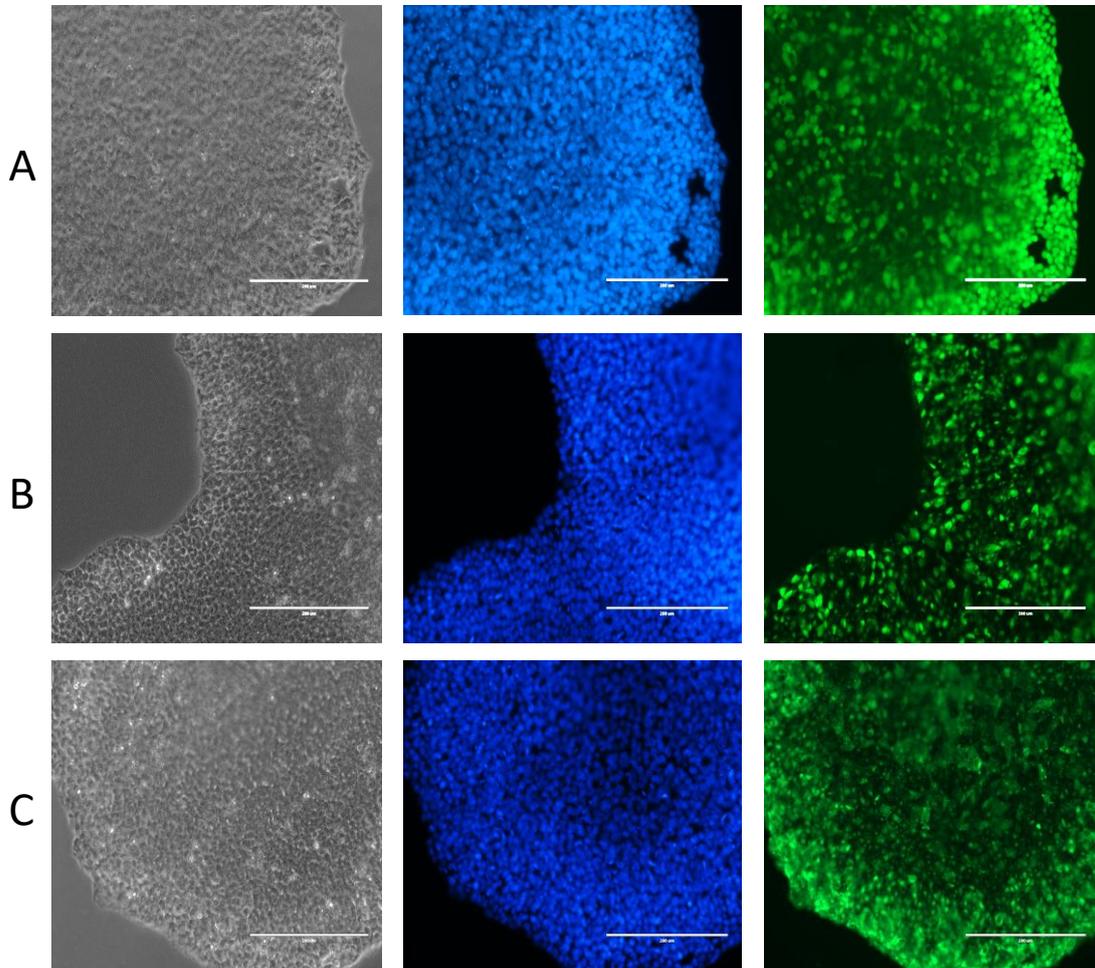


Figure 3.3 Characterization of hiPSCs (BJ, feeder free) by immunostaining. Cells were plated into 12 well plate and stained the next day for pluripotency marker such as OCT- 4, TRA-160 and SSEA 4 (A, B, C). hiPSCs were positively stained for all the pluripotency markers with green fluorescence and DAPI stain was used as nuclear marker. Images were taken at 200X ocular lens magnification using Evos[®] FL. Scale bars=200 μ m.

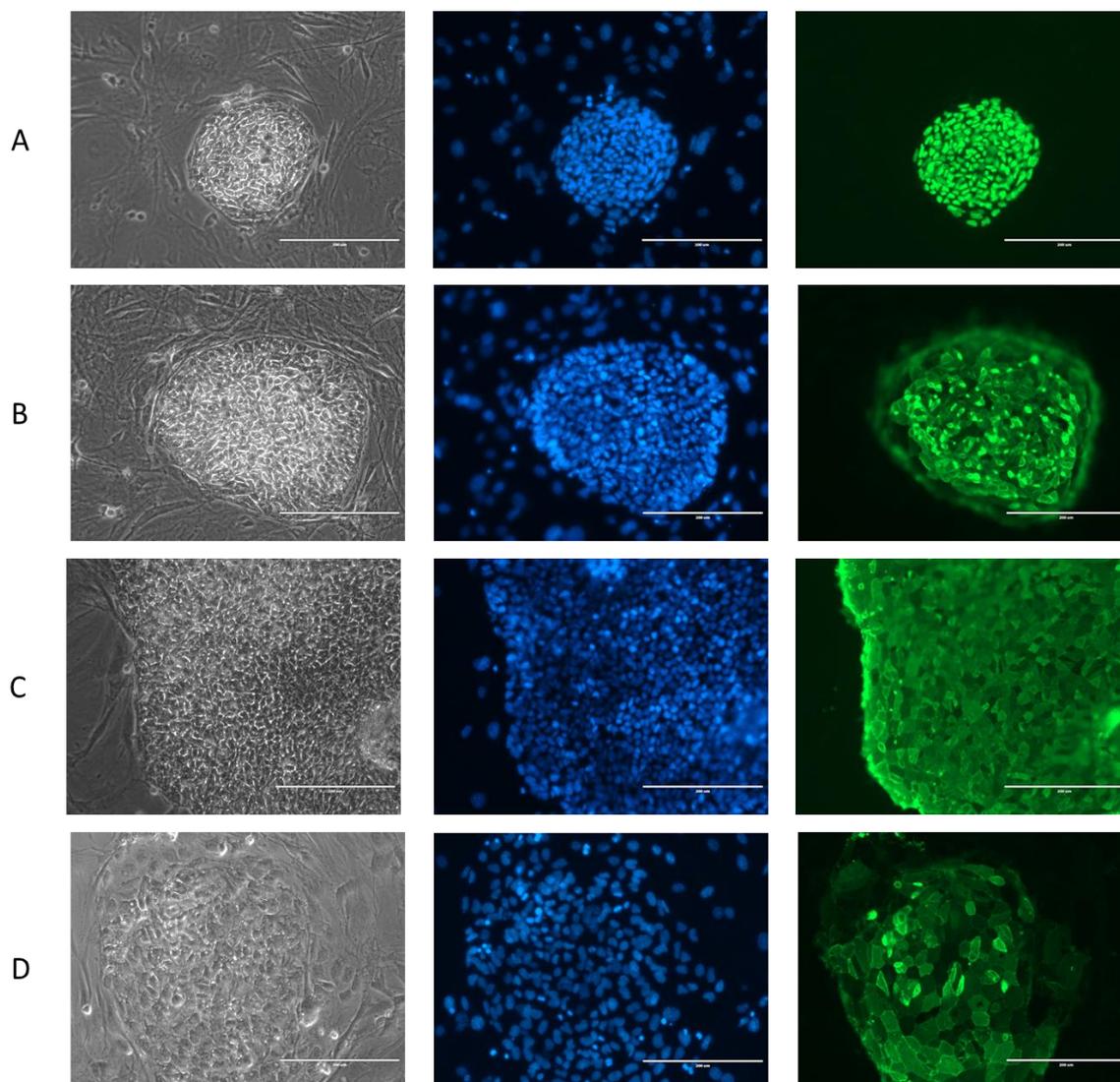


Figure 3.4 Immunostaining of hiPSCs (BJ, on feeders). Cells were adapted to feeder system and then plated into 12 well plate and stained the next day for pluripotency marker such as OCT-4 (A), TRA 1-60(B), SSEA 4(C) and TRA1-81(D). Cells were counterstained with DAPI as a nuclear marker. Images were taken at 200X ocular lens magnification using Evos[®] FL. Scale bars=200 μ m.

hiPSCs (MSU001, feeder-dependent and feeder-free)

hiPSCs (MSUH001) cultured on feeders was adapted for more than 10 passages to feeder-free system. Feeder-dependent hiPSCs (MSUH001) were positively stained for the pluripotent markers (Figure 3.5, I), similar findings were observed by Bae et al. (2012). Even after adaptation to feeder-free system, these cells still expressed pluripotency markers OCT-4 (A), TRA 1-60(B), SSEA 4(C) and TRA1-81(D) (Figure 3.5, II).

hESCs (Shef 3, feeder-dependent and feeder-free)

Feeder-dependent hESCs (Shef 3) was expanded and cultured. Immunostaining for pluripotency markers showed positive staining for OCT-4 (A), TRA 1-60(B), SSEA 4(C) and TRA1-81(D) (Figure 3.6).

After the adaptation of hESCs for more than 10 passages to feeder-free system by mechanical scrapping then chemical passaging (EDTA), the immunostaining results showed that these cells expressed extracellular pluripotency markers such as TRA1-60, TRA1-81, SSEA4 and intracellular marker OCT-4 (Figure 3.7). Studies showed that hESCs (Shef 3, Shef 6) can maintain their pluripotency even after adaptation to feeder-free system (Aflatoonian *et al.*, 2010; Bae *et al.*, 2012).

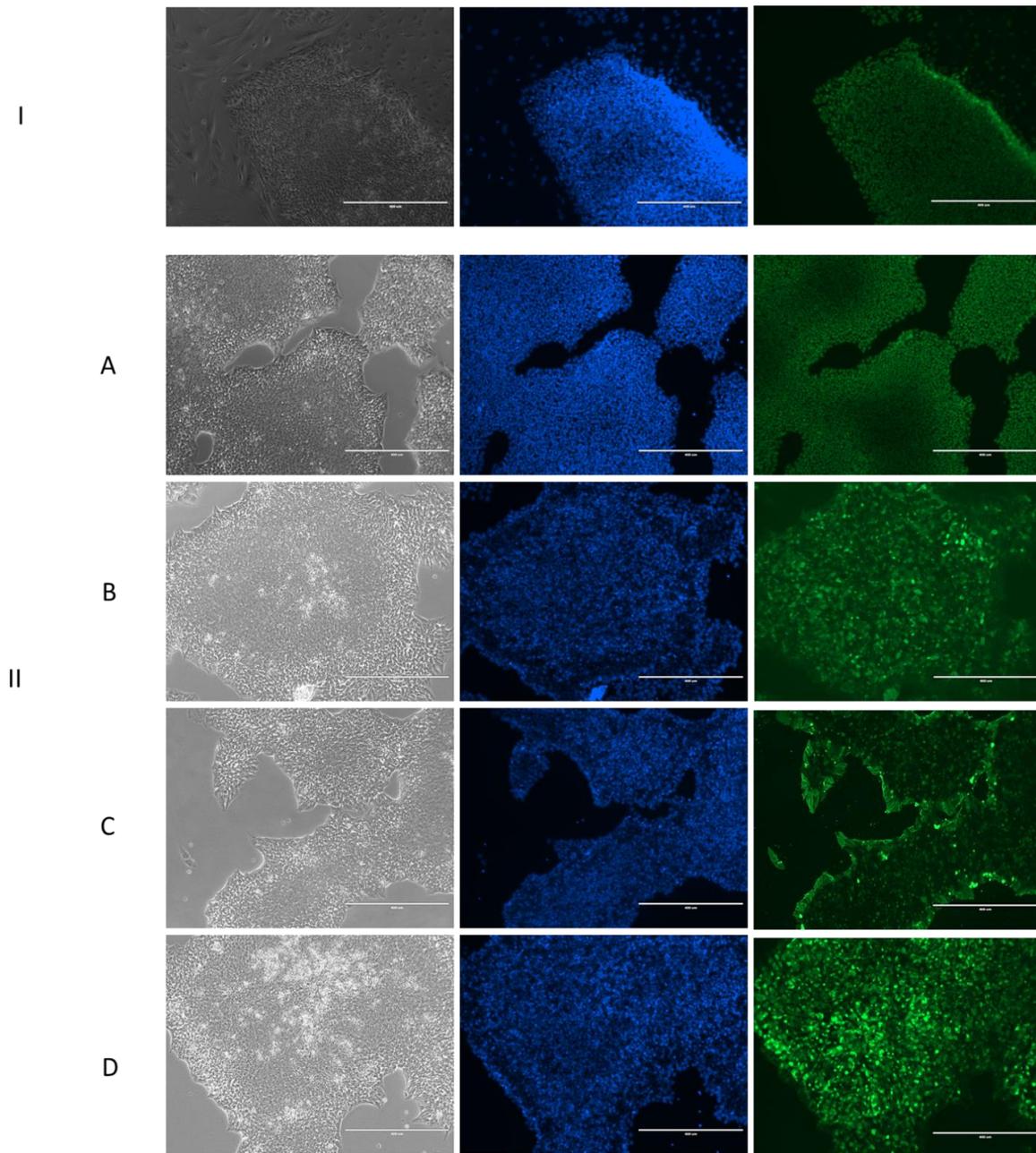


Figure 3.5 Characterization of hiPSCs (MSU001) on feeders (I) and feeder free (II). Feeder-dependent iPSCs was positive for OCT-4 (I), feeder free cells were pluripotent even after adaptation: OCT-4 (A), TRA 1-60(B), SSEA 4(C) and TRA1-81(D). Cells were counterstained with DAPI as a nuclear marker. Images were taken at 100X ocular lens magnification using Evos[®] FL. Scale bars=400 μ m.

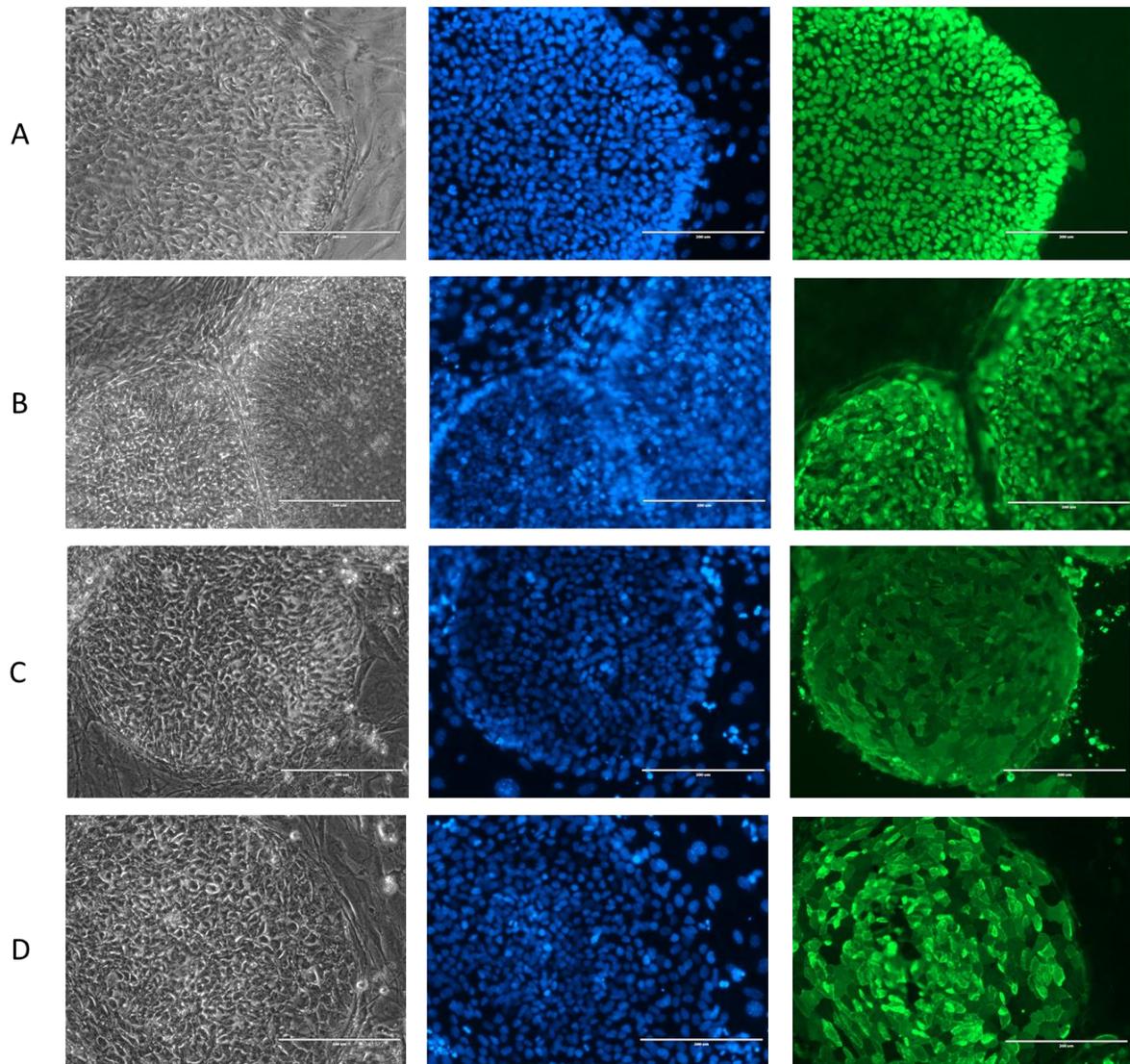


Figure 3.6 Characterization of hESCs (Shef 3, on feeders). Shef 3 were stained positive for pluripotency marker such as OCT-4 (A), TRA 1-60(B), SSEA4 (C) and TRA 1-81(D). Cells were counterstained with DAPI as a nuclear marker. Images were taken at 200X ocular lens magnification using Evos[®] FL. Scale bars=200 μ m.

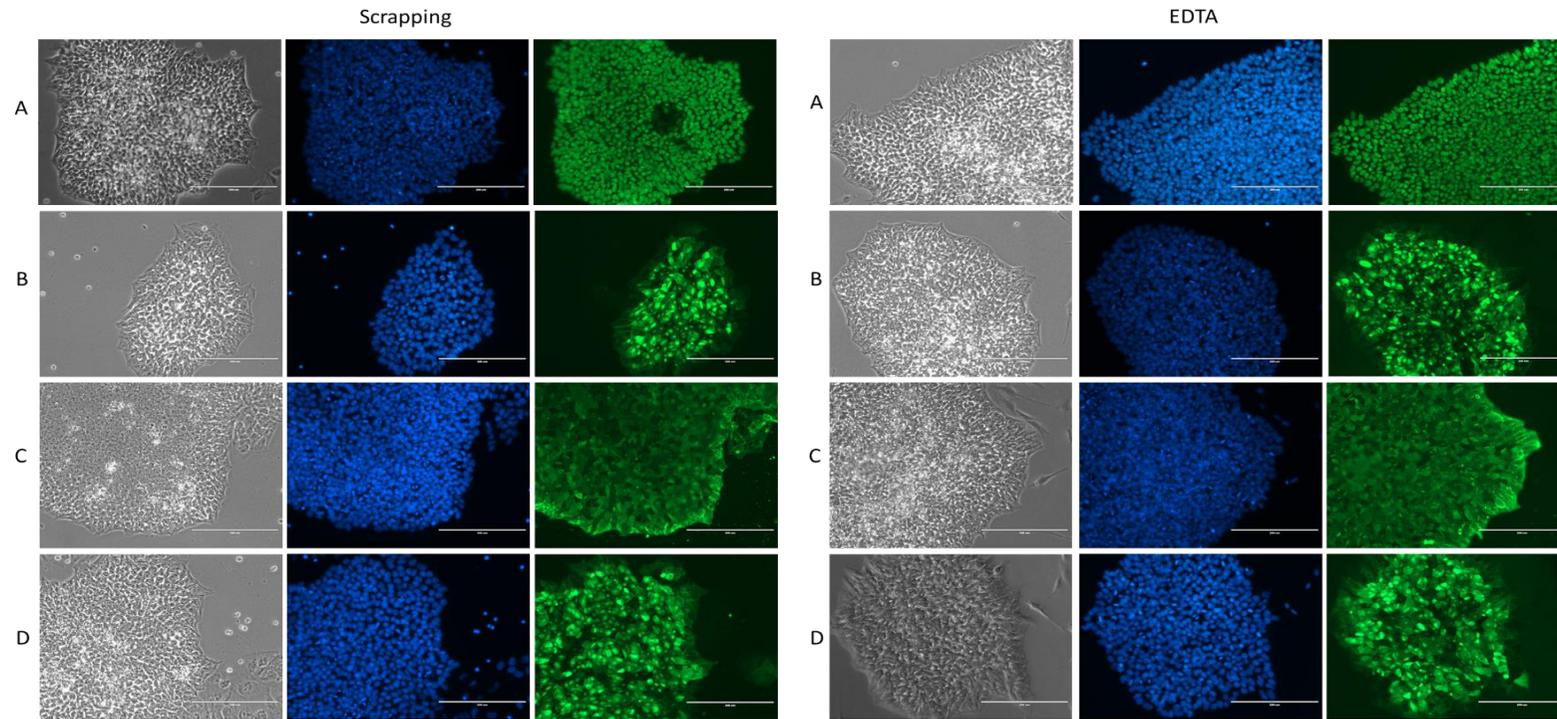


Figure 3.7 Adaptation of hESCs (Shef 3) to feeder free system. Shef 3 passage with scrapping and EDTA were stained positive for pluripotency marker: OCT-4 (A), TRA 1-60(B), SSEA4 (C) and TRA 1-81(D). hESCs were counterstained with DAPI as a nuclear marker. Images were taken at 200X ocular lens magnification using Evos[®] FL. Scale bars=200 μ m.

hiPSCs (Cord blood, feeder free)

Episomal hiPSCs derived from cord blood was purchased from Thermo-Fischer Scientific. This cell line was derived on feeders then adapted by the manufacturer to feeder-free. After thawing and expanding the new cell line, the cells expressed pluripotency markers such as OCT-4, SSEA4, TRA1-60 and TRA1-81 shown by immunocytochemistry (Figure 3.8). Our results confirmed the results obtained from BurrIDGE *et.al*, in 2011 where these hiPSCs were also positive for OCT-4, nanog, TRA1-81 and SSEA4 (BurrIDGE *et al.*, 2011).

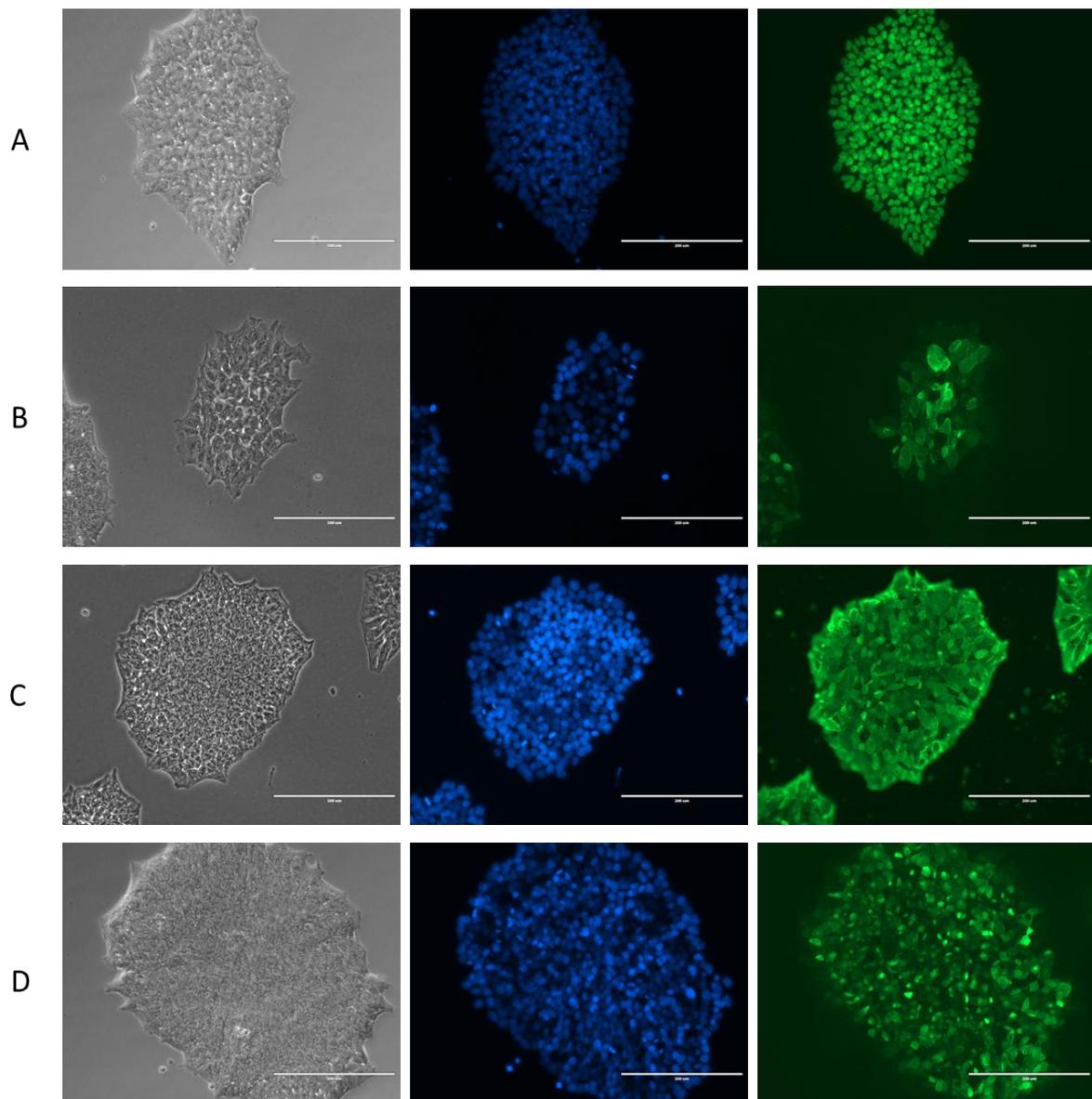


Figure 3.8 Immunostaining of human episomal feeder-free iPSCs (Cord blood). hiPSCs were positively stained for DAPI and pluripotency marker such as OCT-4 (A), TRA 1-60(B), SSEA4 (C) and TRA 1-81(D). Images were taken at 200X ocular lens magnification using Evos® FL. Scale bars=200µm.

3.3.3 Characterisation of pluripotency by flow cytometry

A quantitative method to assess the pluripotency of feeder-free hiPSCs (MSU001 and cord blood) and hESCs (Shef 3) was by evaluating the expression of the extracellular and intracellular pluripotency markers by flow cytometry. SSEA1 and 4 known as Stage-Specific Embryonic Antigen 1 and 4, are surface markers used to assess for stem cell differentiation and pluripotency. SSEA1 is considered as an early differentiation marker for human pluripotent stem cells (Draper *et al.*, 2002; Henderson *et al.*, 2002). The flow cytometry results along the previous staining results confirmed that hESCs (Shef 3) cultured on feeders and feeder-free were pluripotent: 0.39% of feeder-dependant hESCs were stained for SSEA1 and 94.34% were positive for OCT-4 and SSEA4 (Figure 3.9, A). 99.18% of feeder-free hESCs were positive for OCT-4 and SSEA4 (Figure 3.9, B). Moreover, 99.84% of feeder-free hiPSCs (MSU001) expressed the pluripotency markers (SSEA4, OCT-4) (Figure 3.9, C). Similar results were seen for hiPSCs (cord blood) (Figure 3.9, D).

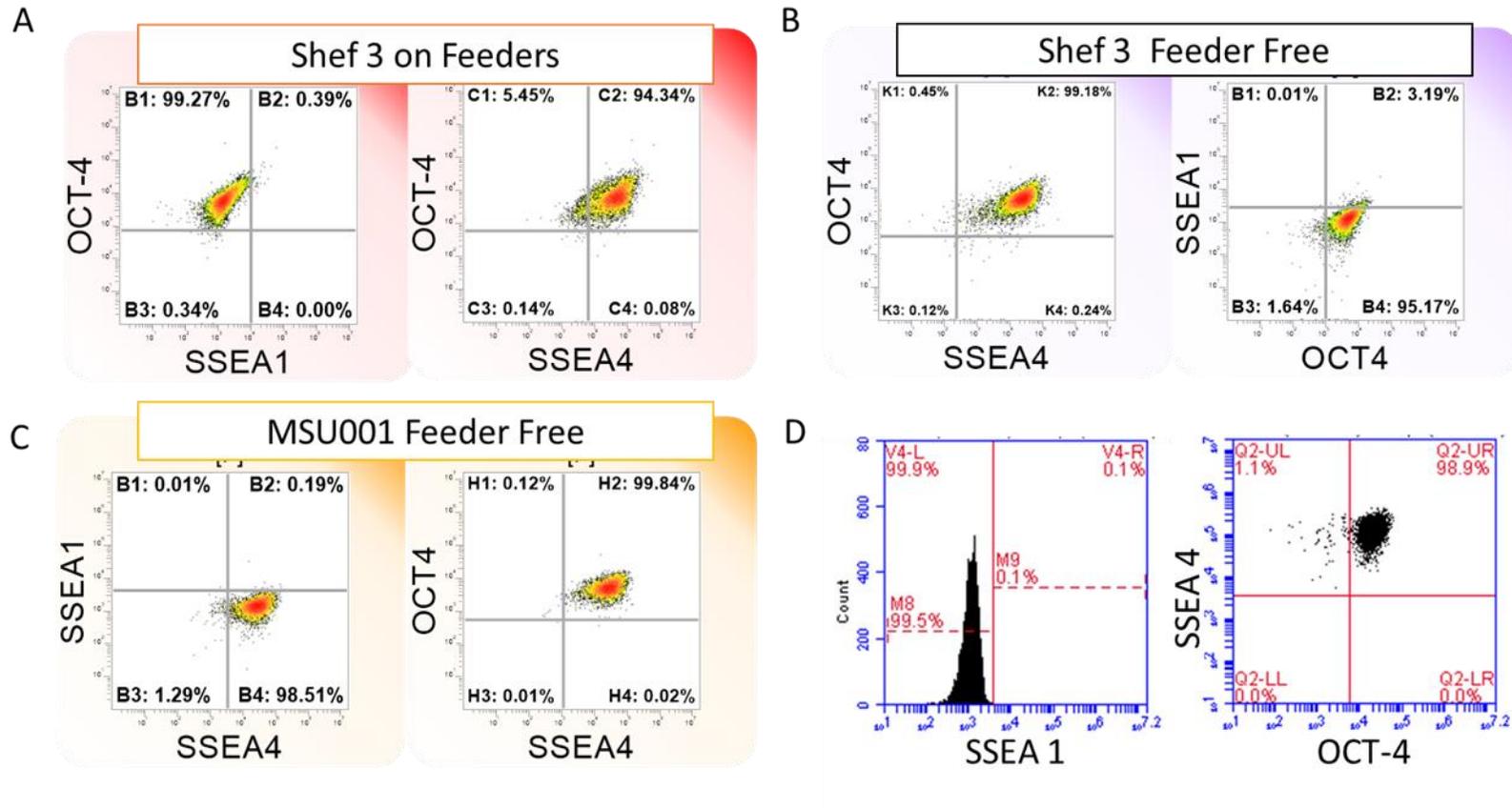


Figure 3.9 Flow cytometer results of hiPSCs and hESCs. Cells were fixed and stained for pluripotency markers, all cell lines (A: Shef 3 on feeders, B: Shef 3 feeder-free, C: MSU001 feeder-free and D: hiPSCs (cord blood)) expressed all pluripotency marker (SSEA4 and OCT4) and were negative for differentiation marker (SSEA1).

3.3.4 Embryoid Body Formation

Another characteristic of pluripotent stem cells is their ability to differentiate and form embryoid bodies (EBs). EBs imitate the early embryonic development giving rise to precursors of germ layers (Desbaillets et al. 2000; Thomson et al. 1998). Feeder-free hiPSCs (BJ, Episomal and MSU001) and hESCs (Shef 3) cultured at 20% O₂ were able to form EBs using AggreWell™ 400Ex plates (Stem Cell Technologies). AgreeWell plate was used to form EBs as it generates uniform controlled and reproducible size EBs (1000 cells/EB). hiPSCs (BJ,MSU001) and hESCs (Shef3) formed same size EBs, whereas hiPSCs (Episomal)'s EBs showed larger size at day 3. These cell lines formed EB confirming their pluripotency and their ability to form EBs (Figure 3.10).

3.3.5 Karyotype Analysis

Standard Giemsa banding (G-banding) karyotype analysis of fixed episomal iPSCs (Cord blood) (P40 and P63) showed a normal diploid karyotype (46, XX) with no visual chromosomal aberrations in a sample of 20 cells examined (Figure 3.11).

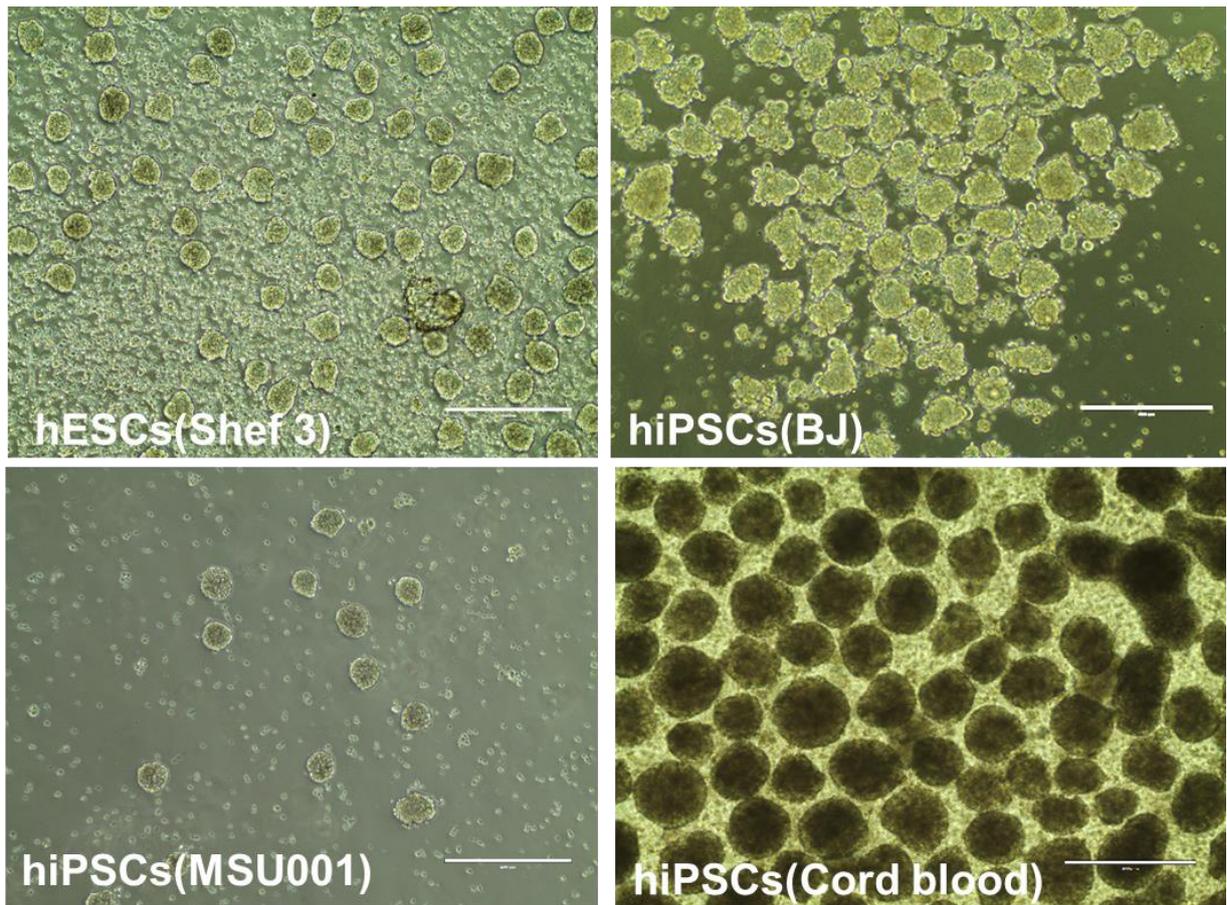


Figure 3.10 Feeder free hiPSCs (BJ, Cord blood and MSU001) and hESC (Shef 3) formed embryoid bodies using AggreWell™ 400 plates. Images were taken at 100X ocular lens magnification using Evos® XL. Scale bars=400µm.

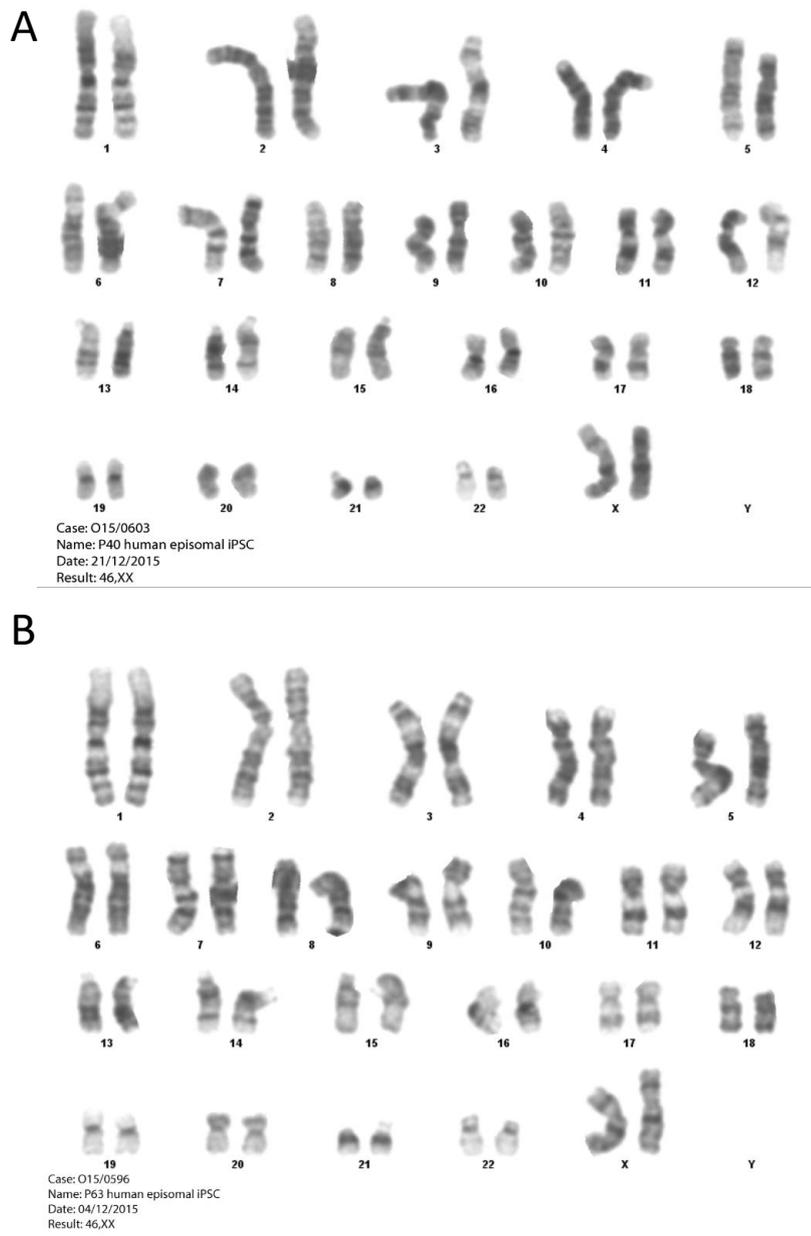


Figure 3.11 Human iPSCs (cord blood) at early (A) and late (B) passage number indicated normal karyotype. Normal G banded chromosomes for human iPSCs (cord blood) at both passages showing 46, XX karyotype.

3.3.6 Discussion

Different techniques has been developed to adapt feeder-dependent cell lines to feeder-free using chemically defined media or xeno-free in combination with human feeder (Richards *et al.*, 2002; Simón *et al.*, 2005; Ellerström *et al.*, 2006; Fong and Bongso, 2006), Accutase® (enzymatic passaging on matrigel) (Stover and Schwartz, 2011), matrix free culture system (Bigdeli *et al.*, 2008) or in flask freshly coated with laminin (Nakagawa *et al.*, 2014). The technique used in this thesis to adapt the pluripotent cell lines to feeder-free was by mechanically scrapping while culturing in flasks pre-coated with Vitronectin with E8 media (Rowland *et al.*, 2010; Chen *et al.*, 2011; Badenes *et al.*, 2016; Kaini *et al.*, 2016).

Feeder-free and feeder-dependant hiPSCs (MSU001, cord blood, BJ) showed typical pluripotent stem cell morphology: small, compacted cells with flat and large nucleus (Takahashi *et al.*, 2007; Yu *et al.*, 2007). While the morphology of hESCs (Shef3) revealed small, rounded compacted cells with large nuclei (J. A. Thomson *et al.*, 1998; Amit and Itshkovitz-Eldor, 2012).

Pluripotency characterisation of the cell lines used was an important step to unequivocally define the hESCs or iPSCs population before testing any condition during the differentiation of these cells line.

As a summary, all hiPSCs and hESCs cultured on feeders or feeder-free expressed all the pluripotency markers (Table 3.1) and had the ability to differentiate and form EBs.

Cell Line	OCT-4	TRA-160	SSEA 4	TRA1-81
hiPSCs(BJ) Feeder Free	Positive	Positive	Positive	-
hiPSCs(BJ) On Feeder	Positive	Positive	Positive	Positive
hiPSCs(MSU001) Feeder Free	Positive	Positive	Positive	Positive
hiPSCs(MSU001) On Feeder	Positive	-	-	-
hESCs (Shef 3) Feeder Free	Positive	Positive	Positive	Positive
hESCs (Shef 3) On Feeder	Positive	Positive	Positive	Positive
hiPSCs(Cord blood)	Positive	Positive	Positive	Positive

Table 3.1 A summary of the immunostaining results reviewing the pluripotency expression of all of the cell lines used.

4 Chapter 4: The Effects of Low Oxygen Tension on The Pluripotency of Stem Cells

4.1 Introduction

The oxygen tension in the uterus of a mammal varies between 1.7 to 8.2% (Fischer and Bavister, 1993). It has been shown that during week 7 and 11 of pregnancy, the average oxygen level in the human uterine surface changes from 2.4% to 7-8% (Rodesch *et al.*, 1992) (§Chapter 1). During early development and before implantation, the embryo is enclosed by the amniotic fluid, thus oxygen molecule reaches the embryo through passive diffusion (Catt and Henman, 2000). After implementation, nutrients and oxygen will be transported to the embryo via vascular perfusion of the fallopian tube (Dickens *et al.*, 1995; Gardner *et al.*, 1996; Tay *et al.*, 1997). Low oxygen niche is necessary to maintain stem cells (Cipolleschi, Dello Sbarba and Olivotto, 1993; Braun *et al.*, 2001; Erecińska and Silver, 2001). Therefore, there has been precedent literature that investigated the effect of mimicking the uterus niche environment especially during early embryo development (Karagenc *et al.*, 2004; Y. Yang *et al.*, 2016).

It has been reported that hypoxia promoted the maintenance of adult stem cells and precursors (Cipolleschi, Dello Sbarba and Olivotto, 1993; Lennon, Edmison and Caplan, 2001; Rehman *et al.*, 2004; Grayson *et al.*, 2006; Lin, Lee and Yun, 2006; Malladi *et al.*, 2006; Thangarajah *et al.*, 2009; Eliasson and Jönsson, 2010).

Furthermore, lower oxygen tensions maintained the pluripotency of hESCs (Toshihiko Ezashi, 2005; Chen *et al.*, 2009; Prasad *et al.*, 2009; Silván *et al.*, 2009; Fernandes *et al.*, 2010; Forristal *et al.*, 2010) and iPSCs (Abaci *et al.*, 2010; Guo *et al.*, 2013; Zhi *et al.*, 2018). For instant, an increase in the pluripotency marker SSEA-3 expression has been detected upon culturing human embryonic stem cells (hESCs) at lower oxygen

concentration (4% O₂) for several passages whilst the expression of other pluripotency markers, such as TRA 1-60, TRA 1-80 or SSEA-4, was unaffected (Närvä *et al.*, 2013). Moreover, culturing cells at lower oxygen tensions during differentiation enhanced stem cell differentiation. Culturing hESCs at 3% O₂ upon differentiation stimulated the formation of oligodendrocyte and chondrogenic precursor cells (Khan, Adesida and Hardingham, 2007; Koay and Athanasiou, 2008; Stacpoole *et al.*, 2013). The exposure of embryonic stem cells to low oxygen levels during differentiation resulted in an increase in neuronal markers (Mondragon-Teran, Lye and Veraitch, 2009; Mondragon-Teran *et al.*, 2011), endothelium formation (Prado-Lopez *et al.*, 2010) and lung progenitor (Garreta *et al.*, 2014), cardiomyocytes (Niebruegge *et al.*, 2009).

Thus the effect of mimicking the uterus's oxygen level (2 % O₂) on the pluripotency maintenance of different human pluripotent stem cells lines was studied in this chapter.

4.2 Chapter Aims and Objectives

The aim of this chapter was to test the influence of low oxygen tension (2 %) on the pluripotency of feeder-free and feeder-dependent human pluripotent stem cells. Lowering oxygen tension may differentiate or aid in pluripotency maintenance; therefore it is necessary to confirm these possible effects before differentiating the cells at low oxygen tension. The detailed objectives are:

- To assess the effect of lower oxygen tension (2% O₂) on feeder-free and feeder-dependent human embryonic stem cells (hESCs: Shef 3) and induced pluripotent stem cells (hiPSCs: BJ, MSU001, cord blood)
- To evaluate the effect of the presence of feeder on the pluripotency maintenance of hiPSCs (BJ) at 2% O₂.

4.3 Results and Discussion

4.3.1 The spontaneous differentiation of hiPSCs (BJ, Feeder-free) exposed to 2 % oxygen tension

Feeder-free hiPSCs (BJ) were cultured for 10 passages at low oxygen concentration (2 % O₂). The proliferation rate of hiPSCs (BJ) cultured at 2 % O₂ was lower than hiPSCs (BJ) exposed to 20 % O₂. hiPSCs (BJ) exposed to 2 % O₂ showed spontaneous differentiation from day 3. The cells failed to form defined colonies, moreover absence of large nuclei and compacted cells were detected (Figure 4.1, B). hiPSCs (BJ) showed some fibroblastic like morphology during the culture for 10 passages at 2 % O₂ (Figure 4.1, C-E). The persistence of differentiated morphologies was not seen when the cells were cultured on feeders as shown in Figure 4.1, F.

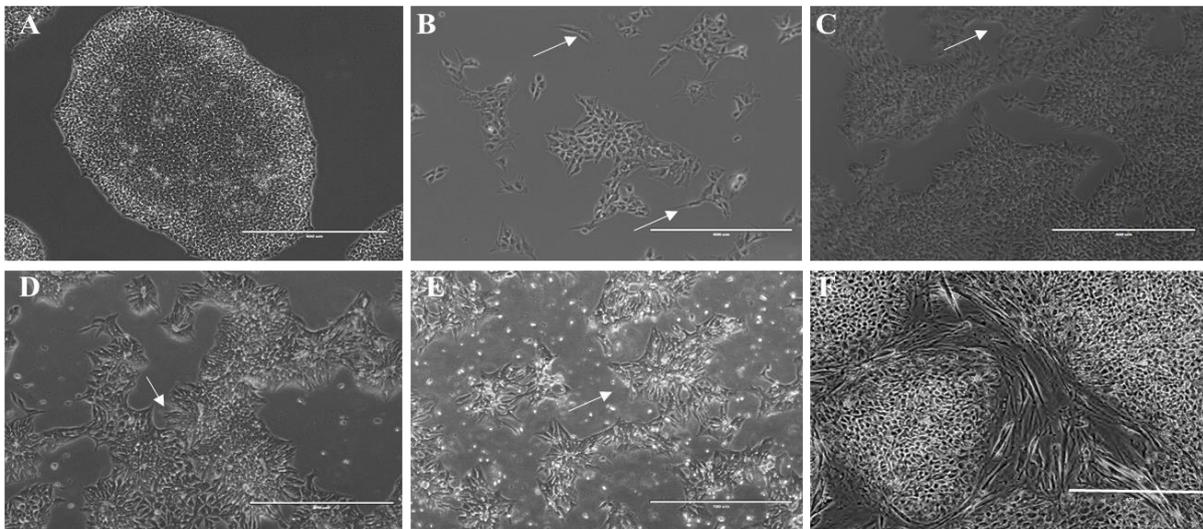


Figure 4.1 Morphology of hiPSC (BJ) cultured at 20 % and 2 % O₂. A: iPSCs cultured at 20% O₂ at day 3, B: iPSCs after 3 days at 2% O₂, C: iPSCs after one passage at 2% O₂, D: iPSCs after 5 passages at 2% O₂, E: iPSCs after 10 passages at 2% O₂, F: iPSCs cultured on feeders at 2% O₂. Images were taken at 100X ocular lens magnification using Evos[®] XL. Scale bars=400µm.

The characterisation of pluripotency was assessed by immunostaining through the detection of expression of differentiation and pluripotency markers. hiPSCs (BJ) incubated for 3 days and for 2 passages at 2 % O₂ were positively stained for early differentiation markers like mesodermal marker (Brachyury) and ectodermal marker (Nestin), moreover the expression of the pluripotency marker OCT-4 was still observed. Spontaneous differentiation caused by the exposure to 2 % O₂ was inhibited upon culturing the cells on feeders. hiPSCs (BJ) cultured on feeders at 2 % O₂ did not express the differentiation markers (Brachyury and Nestin) as shown in in Figure 4.2.

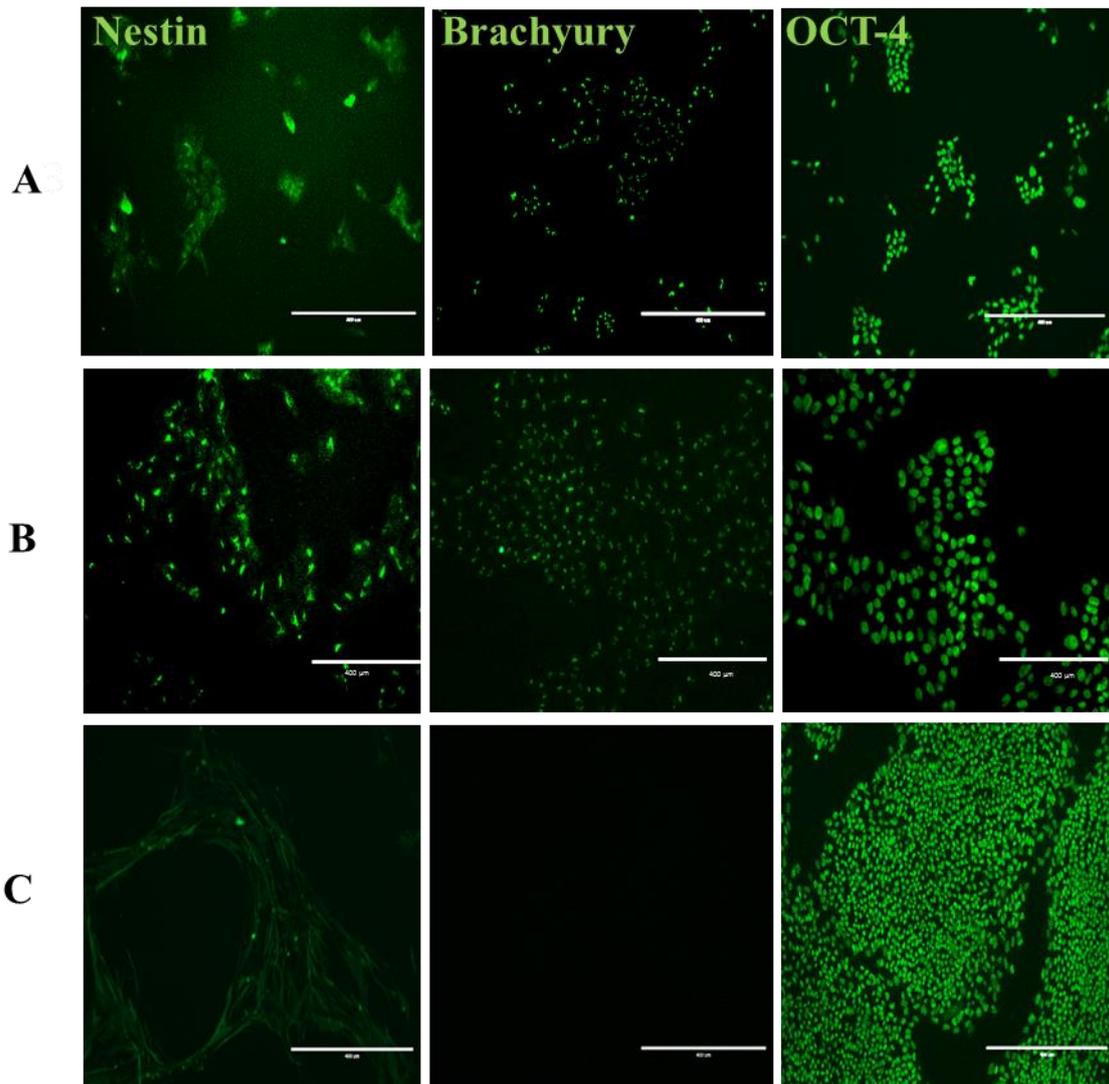


Figure 4.2 Immunostaining for pluripotency (OCT-4) and early differentiation markers (Nestin, Brachyury) of hiPSCs (BJ) at 2 % O₂. hiPSCs (BJ) were incubated for 3 days (A), 2 passages (B) and on feeders (C) at lower oxygen tension. Only the hiPSCs on feeders were stained negatively for differentiation markers. Images were taken at 100X ocular lens magnification using Evos[®] FL. Scale bars=400µm.

The pluripotent and differentiation gene expression of hiPSCs (BJ) cultured at multiple passages at 2% O₂ was assessed through Real time polymerase chain reaction (RT-PCR) (Figure 4.3). A significant increase in mesodermal marker (Brachyury) was seen when hiPSCs (BJ) were cultured for 3 days and 5 passages at 2% O₂. OCT-4 expression did not change significantly, Fgf5 which is an ectodermal marker, was upregulated after 5 passages (p<0.001). The ectodermal marker Nestin was expressed equally in all hypoxic passages.

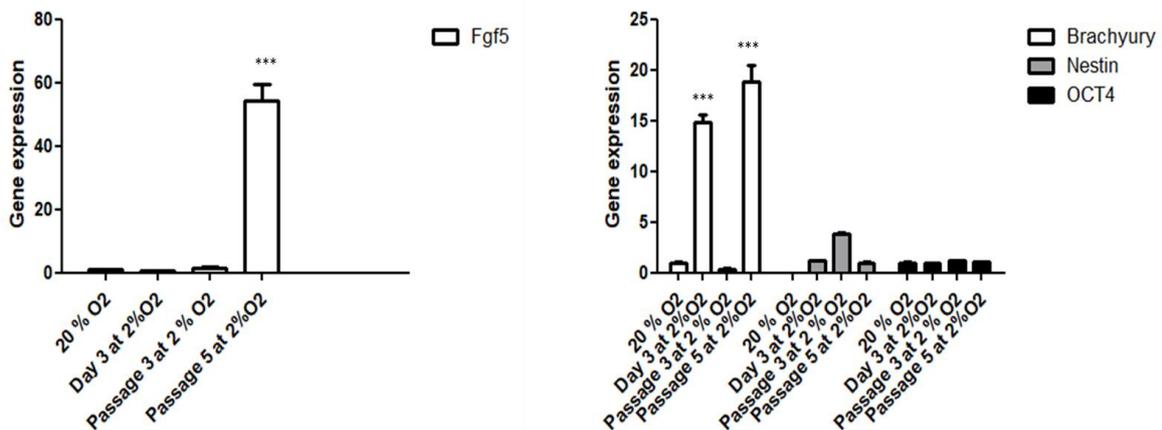


Figure 4.3 Quantitative RT-PCR analysis of hiPSCs (BJ) cultured at 20 and 2 % O₂. Cells were incubated for 3 days, 3 and 5 passages at lower oxygen tension. Gene expression was normalised to housekeeping gene (β -Actin). Statistical analysis was performed using Two-Way Anova and data are reported as mean \pm S.E.M of n=3, asterisks indicate the statistical significance: *** P<0.001, **P<0.01, *P<0.05). Data are reported as mean \pm S.E.M of n=3, p<0.05 was considered as significant).

The ability of hiPSCs (BJ) cultured for 3 days at 2 % O₂ to form embryoid bodies (EBs) was assessed using AggreWell™ 400Ex plates. Some of these EBs were transferred to laboratory conditions (20% O₂), while the others remained at 2 % O₂ for 10 days. hiPSCs (BJ) previously cultured at 2 % O₂ for 3 days failed to form EBs even after transferring these EBs to 20% O₂ (Figure 4.4). The absence of EB formation indicates that hiPSCs (BJ) cultured at 2 % O₂ were potentially differentiated before transferring it to AggreWell.

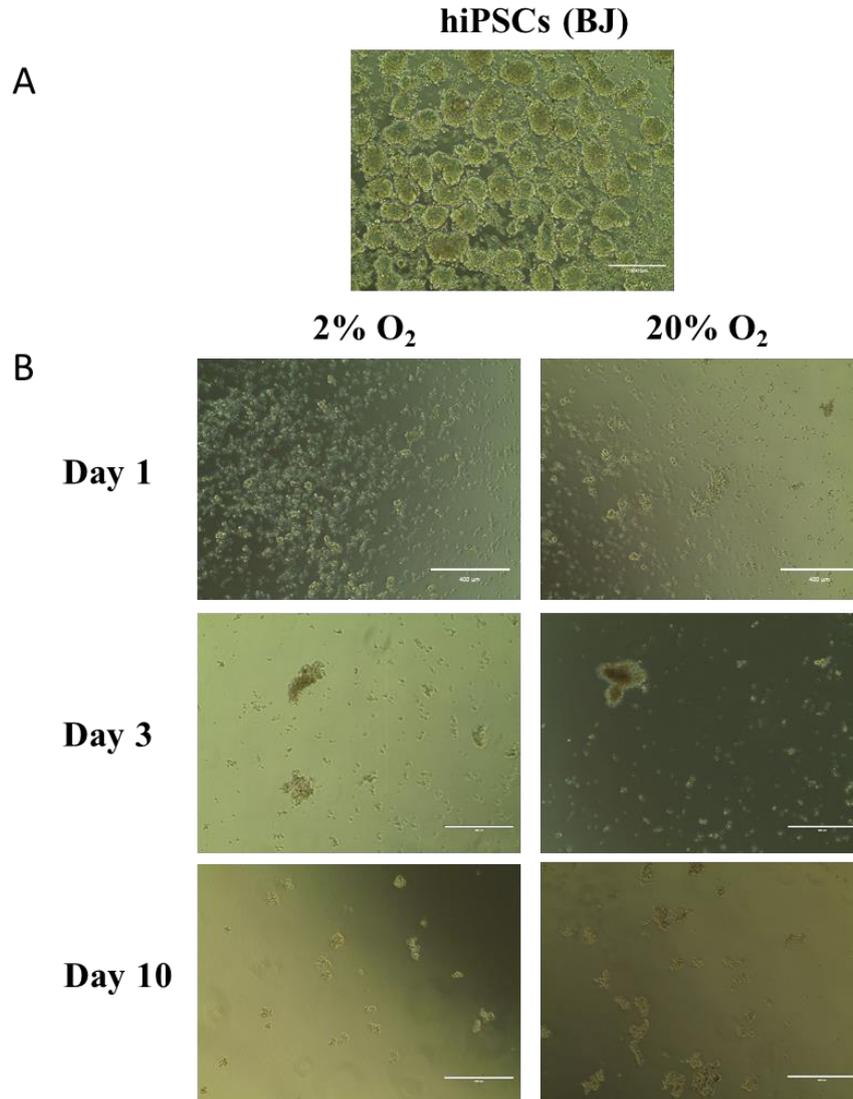


Figure 4.4 Formation of hiPSCs (BJ) embryoid bodies after 3 passages at 2% O₂ using AggreWell™ 400Ex plates. A: hiPSCs (BJ) cultured at 20 % O₂ formed EBs. B: 2 % O₂ adapted cells failed to form any EBs even after culturing these at normal oxygen tension. Images were taken at 100X ocular lens magnification using Evos® XL. Scale bars=400µm.

As previously mentioned in Figure 4.1, although hiPSCs (BJ) showed spontaneous differentiation when exposed to 2% O₂, the differentiation was inhibited when cells were grown on feeders. To study if the effect of the feeders was because of its secretion or because it served as an extracellular matrix, hiPSCs (BJ) were cultured at 2% O₂ for 3 days with feeder conditioned media (§Chapter 2.1.2). The expression of differentiation markers such as SSEA1, Brachyury and PAX6 was lower when the hiPSCs (BJ) were cultured with conditioned media. Thus, the growth factors secreted by the feeder could play a role in the pluripotency maintenance of hiPSCs (BJ) at 2% O₂ (Figure 4.5).

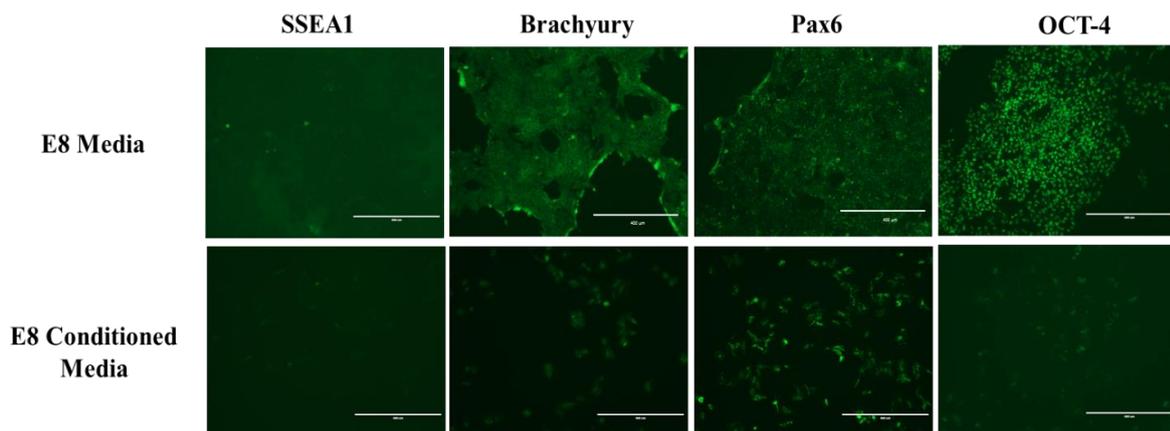


Figure 4.5 Comparison between hiPSCs (BJ) incubated with E8 conditioned media and normal E8 at 2 % O₂. Although in both conditions differentiation was observed after 3 days at 2%O₂, cells cultured with conditioned media expressed less differentiation markers. Images were taken at 100X ocular lens magnification using Evos® FL. Scale bars=400µm.

hiPSCs (BJs) were adapted to a feeder system for more than 3 passages and then cultured at lower oxygen level, no significant differentiation was seen: cells were positive for OCT-4 and negatively stained for differentiation marker SSEA1 (Figure 4.6).

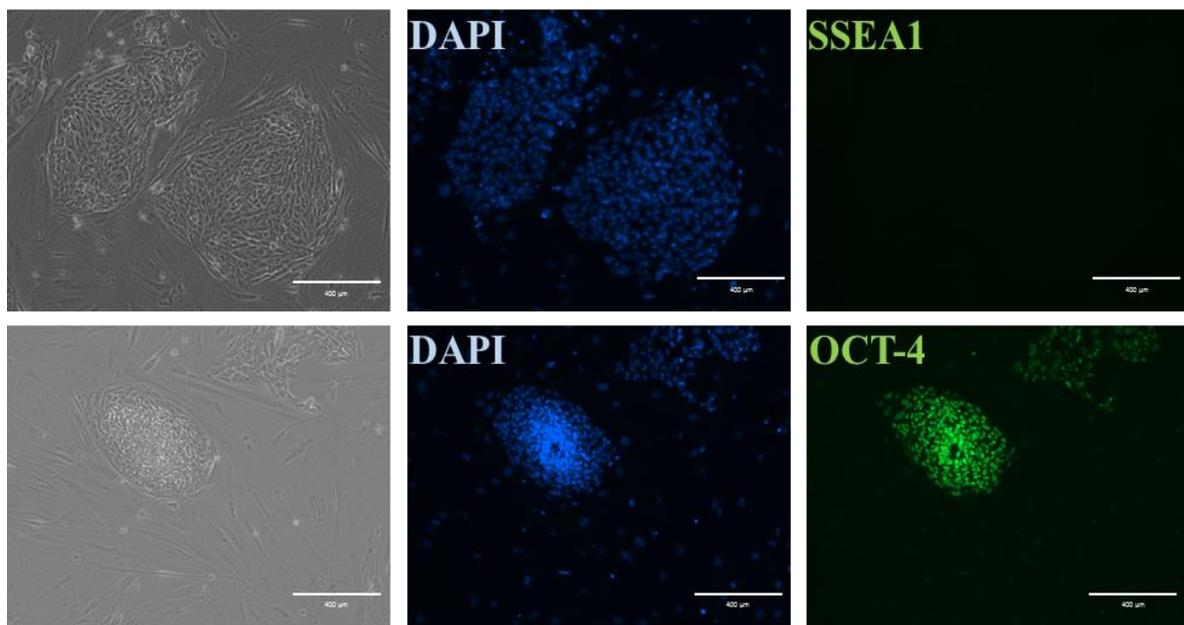


Figure 4.6 Feeder-dependent hiPSCs (BJ) cultured at 2 % O₂. hiPSCs (BJ) adapted for more than 3 passages to a feeder system did not differentiate at lower oxygen concentration. Cells were counterstained with DAPI as a nuclear marker. Images were taken at 100X ocular lens magnification using Evos[®] FL. Scale bars=400μm.

4.3.2 Low oxygen tension maintained the pluripotency of hESCs (Shef 3) and hiPSCs (MSU001, cord blood)

Feeder-dependent and feeder-free hiPSCs (MSU001)

In order to assess if hiPSCs (BJ) differentiation at lower oxygen concentration was cell specific response, hiPSCs (MSU001) derived from fibroblasts were expended. All cell lines including adapted cell lines were passaged at least 3 times before doing any experiments. Feeder-dependent and feeder-free hiPSCs (MSU001) were cultured at 2% O₂ for 3 days. Cells were stained for pluripotency marker (OCT-4) and early differentiation marker (SSEA1) to assess differentiation. Feeder-dependent and feeder-free hiPSCs (MSU001) were stained negatively for early differentiation marker (SSEA1) and expressed OCT-4. The results are shown in Figure 4.7.

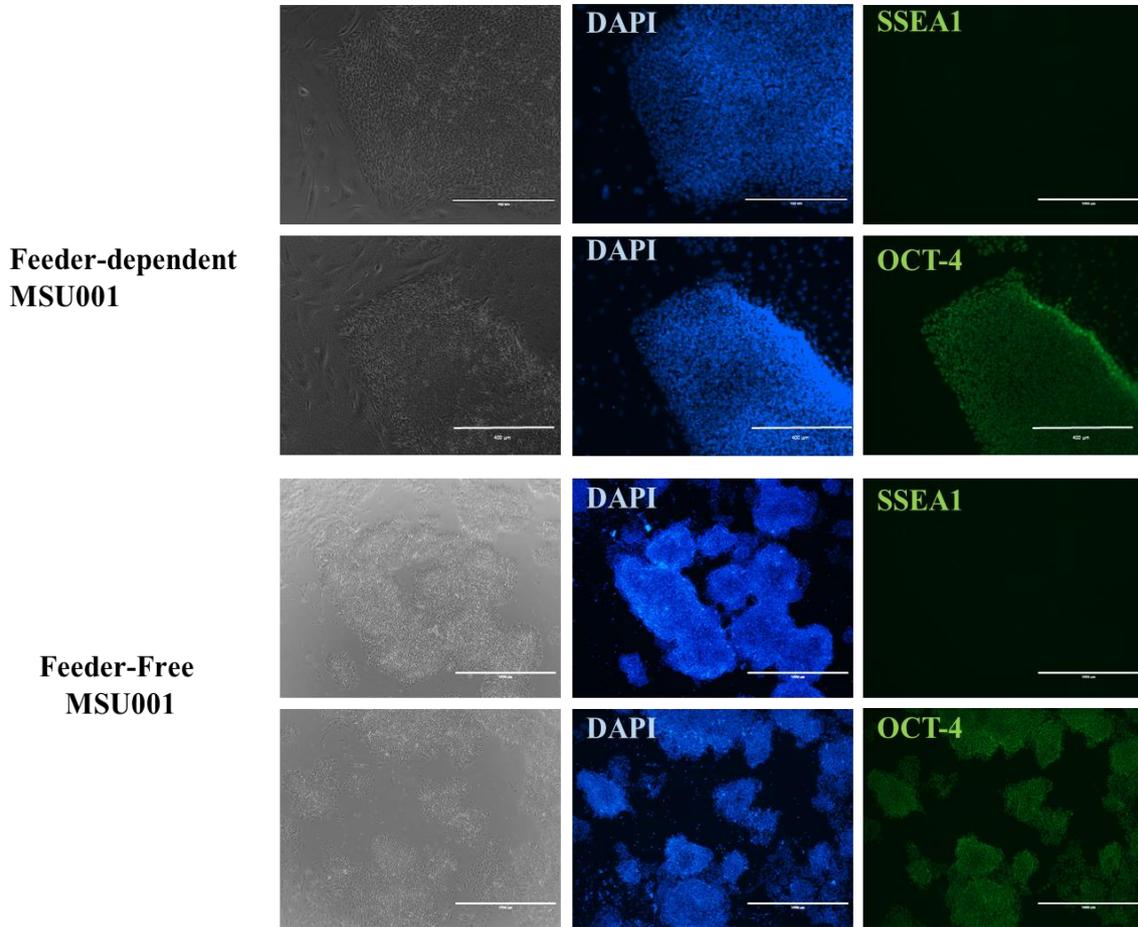


Figure 4.7 Immunostaining of feeder-dependent and feeder-free hiPSCs (MSU001) cultured at lower oxygen concentration. Feeder-free and feeder-dependent hiPSCs (MSU001) maintained its pluripotency at 2 % O₂. Cells were counterstained with DAPI as a nuclear marker. Images were taken at 100X ocular lens magnification using Evos[®] FL. Scale bars=400µm.

Feeder-dependent and feeder-free hESCs (Shef 3)

The influence of low oxygen on human embryonic stem cells was also studied. Thus, feeder-dependent hESCs (Shef 3) were expanded in culture and adapted to feeder-free system. hESCs (Shef 3) grown on feeders didn't differentiate when exposed to 2 % O₂ (Figure 4.8). The morphology of the cells showed compacted well defined pluripotent cells. Feeder-dependent hESCs (Shef 3) were negative for early differentiation marker SSEA1 and positive for pluripotency marker OCT-4.

hESCs (Shef 3) were adapted to feeder-free system (§ Chapter 2.1.3) and after 10 passages, the cells were cultured at 2 % O₂ for 3 days. No differentiation was observed and the hESCs were negatively stained for SSEA1 (Figure 4.8).

Feeder-free hiPSCs (Cord blood)

After testing the effect of low oxygen concentration on feeder-free hiPSCs (BJ, MSU001) and hESCs (Shef 3), an episomal hiPSC (Cord blood) was purchased. This approach was executed to check if the spontaneous differentiation seen upon culturing feeder free hiPSCs (BJ) at 2 % O₂ was dependent on the source of derivation of iPSC, as the BJ and MSU001 were derived from fibroblasts and hiPSCs (Cord blood) was derived from cord blood. Upon culturing human episomal hiPSCs (Cord blood) at 2 % O₂, no differentiation was seen: cells were negatively stained for early differentiation marker (SSEA1) and positively stained for pluripotency marker (OCT-4) (Figure 4.9).

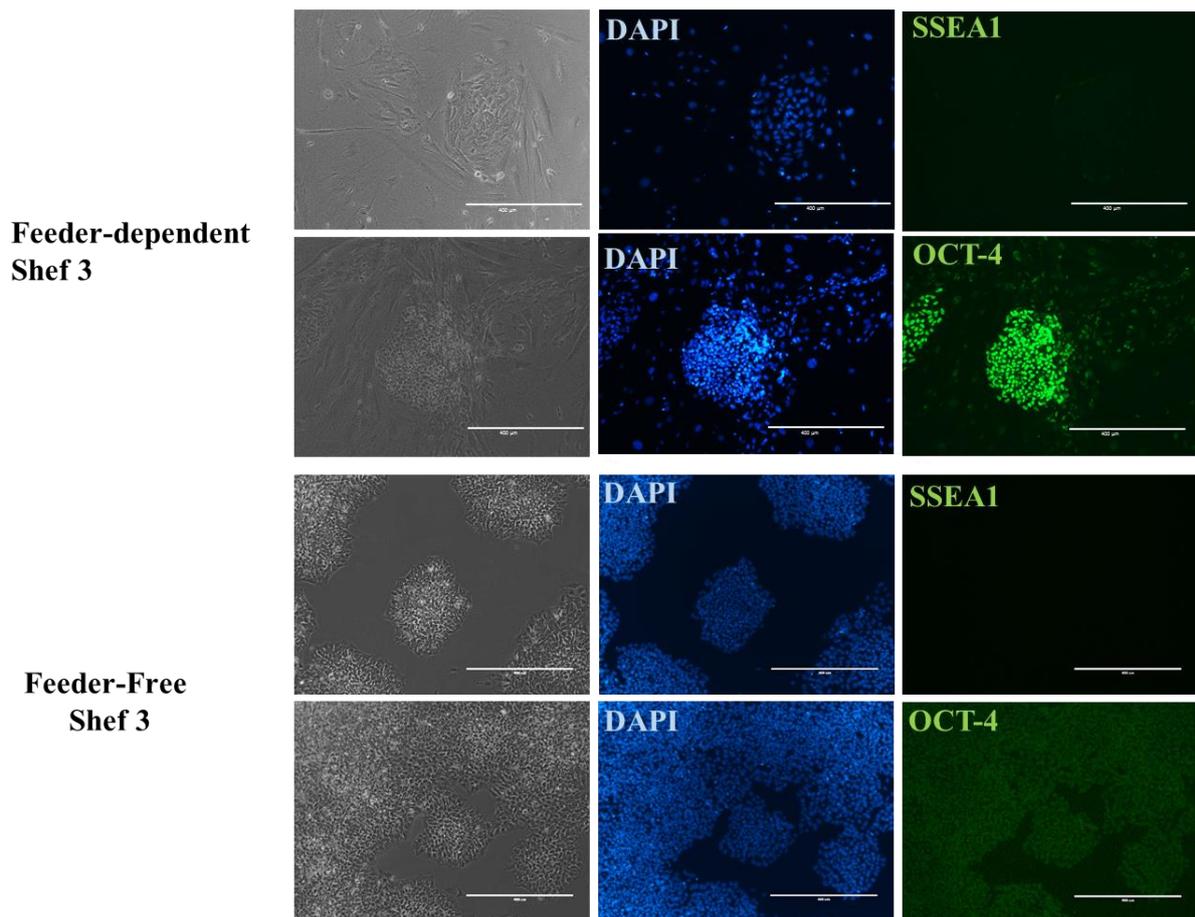


Figure 4.8 Effect of low oxygen tension on hESCs (Shef 3) grown on feeders and feeder-free. No differentiation was seen upon culturing Shef 3 at 2%O₂ for 3 days. In both conditions, Shef 3 were negative for SSEA-1 and positive for OCT-4. Cells were counterstained with DAPI as a nuclear marker. Images were taken at 100X ocular lens magnification using Evos[®] FL. Scale bars=400μm.

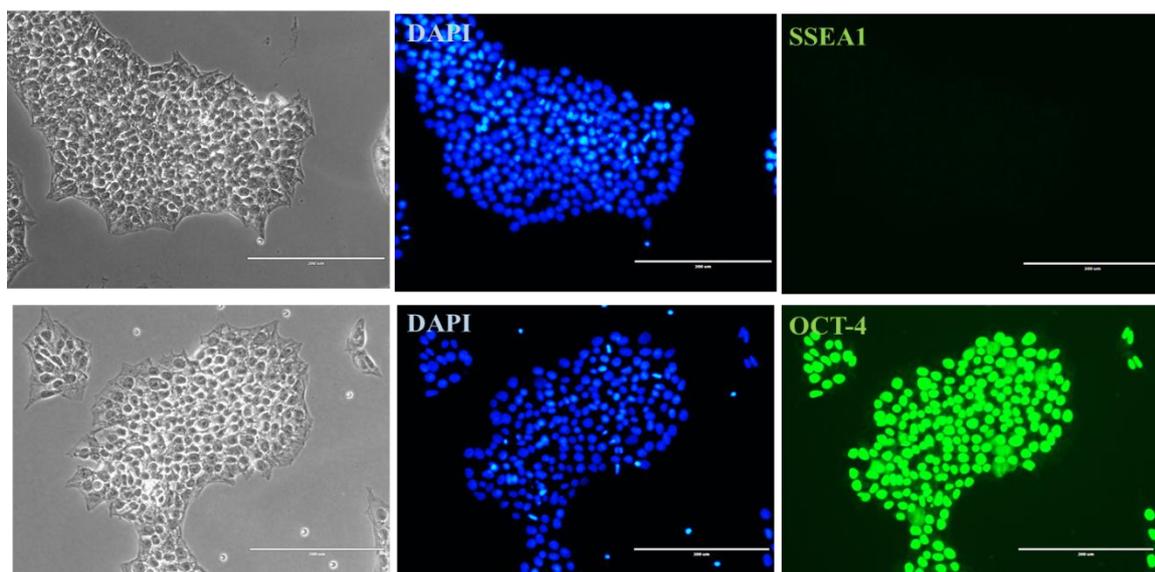


Figure 4.9 Episomal hiPSC (Cord blood) cultured at (2 % O₂). Immunostaining revealed negative stain for SSEA1 and positive stain for pluripotent marker (OCT-4). Cells were counterstained with DAPI as a nuclear marker. Images were taken at 100X ocular lens magnification using Evos[®] FL. Scale bars=400µm.

4.3.3 Discussion

Lowering the oxygen concentration during *in-vitro* studies has several effects on different cell types (Rehman *et al.*, 2004; Grayson *et al.*, 2006; Malladi *et al.*, 2006; Thangarajah *et al.*, 2009; Eliasson and Jönsson, 2010; Abdollahi *et al.*, 2011).

To assess the effect of low oxygen tension on cell lines used, 7 cell lines were cultured at 2 % O₂ for 3 days. Culturing feeder-free and feeder-dependent hESCs (Shef 3) at 2 % O₂ maintained the pluripotency of these cells. Moreover, hESCs (Shef 3) cultured at 2 % O₂ were morphologically similar to hESCs (Shef 3) cultured at 20 % O₂. Similar results were obtained when hESCs were exposed to 5 % O₂, 4 % O₂ and 2 % O₂. Lower oxygen tension maintains the pluripotency of stem cells via of HIF-2alpha (Toshihiko Ezashi, 2005; Chen *et al.*, 2009; Prasad *et al.*, 2009; Fernandes *et al.*, 2010; Forristal *et al.*, 2010; Närvä *et al.*, 2013). Nevertheless, culturing mouse ESCs at lower oxygen concentration can also trigger differentiation (Jeong *et al.*, 2007).

While lowering oxygen concentration maintained the pluripotency of induced pluripotent stem cells (Guo *et al.*, 2013; Zhi *et al.*, 2018) and hiPSCs (MSU001, Cord blood), 2% O₂ induced spontaneous differentiation of feeder-free hiPSCs (BJ). No differentiation was seen when feeder-dependent hiPSCs (BJ) were cultured at 2% O₂, this could be due to the presence of feeders. It has been shown that feeder cells promoted pluripotency, self-renewal and inhibited differentiation of stem cells through the secretion of factors such as fibroblast growth factor, provided an extracellular matrix for cell attachment (J. A. Thomson *et al.*, 1998; Reubinoff *et al.*, 2000; Amit *et al.*, 2003; Eiseleova *et al.*, 2008; Liu *et al.*, 2009; Pekkanen-Mattila *et al.*, 2012; Park *et al.*, 2015; H. Yang *et al.*, 2016). The effect of feeders on the pluripotency maintenance of hiPSCs

(BJ) cultured at low oxygen could be due to cell-cell interaction, providing an extracellular matrix. Thus, changing the extracellular matrix could have an effect on feeder-free hiPSCs (BJ) cultured at low oxygen levels. hiPSCs (BJ) were seeded at 2% O₂, on matrigel and vitronectin. There was no significant change in viable cell count of attached cells in both matrixes. Thus, as culturing the cells in conditioned media had a lower differentiation expression and as changing the extracellular matrix did not have an effect on the differentiation, it could be speculated that the growth factors like fibroblast growth factor helped in the pluripotency maintenance.

The pluripotency maintenance at lower oxygen tension was not dependent on the origin of the cells as hiPSCs (BJ, MSU001) were both derived from fibroblasts. This finding could be applied to enhance the pluripotency of hESCs (Shef 3) or hiPSCs (MSU001, cord blood) at lower oxygen concentration. Moreover, culturing hiPSCs (BJ) at lower oxygen tension could enhance germ layer differentiation.

5 Chapter 5: The Enhancement of Human Pluripotent Stem Cells Differentiation Media Using Design of Experiments.

5.1 Introduction

The metabolism of pluripotent stem cells is different from their differentiated counterparts. Glucose consumption is switched on in blastocyst stage, thus glycolysis is increased. While of the differentiated cells relies on oxidative phosphorylation for energy, pluripotent stem cells utilise glucose through glycolysis over oxidative phosphorylation, this is accompanied with high expression of glycolytic genes, low level expression of mitochondrial respiratory chain protein and enzymes (Varum *et al.*, 2011; Leese and Barton, 1984).

Thus, any perturbation in the energy metabolism could affect self-renewal, differentiation and stem cell fate (Kondoh *et al.*, 2006; Varum *et al.*, 2011; Sperber *et al.*, 2015; Vernardis *et al.*, 2017).

As discussed in chapter 1.5, during early development, the embryo is exposed to nutrients via vascular perfusion of the fallopian tube (Dickens *et al.*, 1995; Gardner *et al.*, 1996; Tay *et al.*, 1997). The embryo resides in a low oxygen niche environment (Fischer and Bavister, 1993) and a switch from oxidative phosphorylation to glycolysis is sensed (Leese and Barton, 1984). However, the *in vivo* level of key nutrients that could have an effect on the metabolism such as oxygen, glucose and pyruvate is much lower than the *in vitro* differentiation conditions (§Figure 1.4) (Fischer and Bavister, 1993; Dickens *et al.*, 1995; Gardner *et al.*, 1996; Tay *et al.*, 1997; Y. Yang *et al.*, 2016). As a result, the aim of this chapter was to investigate whether mimicking natural environment or depriving the oxygen, glucose and pyruvate could enhance germ layer formation.

To allow the investigation of three factors with multiple levels in parallel, a design of experiments (DoE) approach was chosen to provide more information on the experimental space and interactions within factors (Lundstedt *et al.*, 1998; Montgomery, 2012).

The ability of DoE to generate an algorithmic model could help in the optimisation of experimental conditions by improving it or highlighting any interaction that might exist between the factors while reducing the number of experiments (Lundstedt *et al.*, 1998; Montgomery, 2012). DoE has been applied in several fields for example engineering (Thielman and Ge, 2006; Ariffin *et al.*, 2009), in drug optimization (Singh *et al.*, 2005; Martinello *et al.*, 2006), microbiology (Mannall, Titchener-Hooker and Dalby, 2007; Esteban *et al.*, 2014) and in the optimization of the pluripotent stem cell media (Marinho, Chailangkarn and Muotri, 2015). Nevertheless, studies have not been investigated for the effect of nutritional microenvironment on germ layer formation using DoE.

5.2 Chapter Aims and Objectives

The aim of this Chapter was to determine if different levels of glucose, pyruvate, oxygen, or their interactions generate a microenvironment that enhances the differentiation of each of the germ layers. hiPSCs (Cord blood) was the only cell line used in this chapter as 27 different conditions were set to test with time and material limitation. The detailed objectives were as follows:

- To determine whether glucose, pyruvate and oxygen have an effect on each ectoderm, mesoderm and endoderm lineages.
- To determine if interactions between glucose, pyruvate and oxygen have an effect on germ layer formation.
- To identify three distinct microenvironments for ectoderm, mesoderm and endoderm that enhance each germ layer formation.

5.3 Results and Discussion

5.3.1 Design of Experiments

To determine the best nutritional environment for each of the germ layers, a combination of the relative concentrations of glucose, pyruvate and oxygen at laboratory, natural (body) and deprivation environments (as shown in table 5.1) was studied. A full factorial design comprising a set of 27 different conditions was generated in Design-Expert® (Stat-Ease Inc.) (Table 2.5). The initial differentiation protocol was set for 7 days as the formation of germ layers occurs one week after gestation. As preliminary data showed low viability in some conditions while expressing high gene expression, the differentiation time was shortened to 4 days. Glutamine levels were not measured as glutamax was supplemented in the media.

Factor	Level		
	Deprivation	Natural environment	Laboratory conditions
Oxygen (% v/v)	0	2	20
Glucose (mM)	0	3.14	17.5
Pyruvate (mM)	0	0.14	0.5

Table 5.1 Summary of the level of the factors used for the DoE.

Glucose deprivation enhanced endodermal lineage formation

Monolayer single cell differentiated hiPSCs (cord blood) were cultured in 27 different conditions. Germ layer gene expression was assessed during the 4 days of differentiation. Log values of the gene expression were used to create a model using Design-Expert® (Stat-Ease Inc.). The quadratic model was chosen for most of the endodermal markers during the 4 days of differentiation. The models' F-values implied that the models were significantly fitted except for FOXA2 model at day 3 (Table 5.2). No changes in the early differentiation marker Sox17' expression was detected in the 27 conditions during the first day. CXCR4's expression was higher at low oxygen tensions (Figure 5.1). Cell viability increased in low pyruvate conditions (0 mM, 0.14mM) and at 0% O₂ while the glucose was maintained high at day 1 and 2 (Figure 5.1-5.2). However, higher Sox 17'expression was seen at day 2 when cells were exposed to deprived glucose media while maintaining the laboratory oxygen and pyruvate levels (Figure 5.2). The effect of glucose removal on Sox17 expression was seen clearly at day 3 and 4 (Figure 5.3-5.4). The deprivation of glucose only induced an increase in all endodermal genes with a maximum of 2300-fold change of Sox17 at day 3. Increasing the pyruvate concentration in all the days didn't have an impact on Sox17'expression (Figures 5.1-5.4).

Late endodermal marker FOXA2' expression was higher upon the exposure to 0% O₂ or 20% O₂ at day 1 (Figure 5.1). The impact of some condition on FOXA2'expression was seen at day 3 especially when the media was deprived from glucose as shown in Figure 5.3.

CXCR4'expression was improved only at 20% O₂, 0 mM glucose; 0% O₂, 0 mM glucose and 0% O₂, 17.5 mM glucose.

Increasing the oxygen tension to 20 % while keeping the cells deprived from glucose and under 0.14 mM pyruvate seems to increase the endodermal markers'expression even at day 4 (1700 -fold increase of Sox17, 9-fold increase of FoxA2 and 80-fold increase of Cxcr4) more than exposing the cells to same condition with higher pyruvate the pyruvate (0.5mM) (Figure 5.3). The deprivation of all the factors induced endodermal expression with a maximum of 500 fold increase of Sox17 and 40 fold increase of Cxcr4 at day 4 without inducing definitive endodermal marker (FoxA2) (Figure 5.4). Contour plot of the gene expression of endodermal markers are presented in Appendix II, Figures 1-4.

Depriving glucose from media while keeping the natural environment levels of oxygen and pyruvate (2%, 0.14 mM respectively) enhances all endodermal markers from day 2 (Figures 5.2-5.4).

Although higher expression of endodermal markers was seen in glucose deprived media, this condition had the lowest cell viability.

Mesodermal differentiation was enhanced at low oxygen and glucose levels.

The model for all mesodermal markers was significant except for Brachyury at day 1 (Table 5.3). As previous results, deprivation or exposing the cells to pyruvate didn't have an effect in the mesodermal gene expression of Brachyury and KIF11 during the 4 days of differentiation as seen in the contour plot in Appendix II, Figures 5-8. The expression of KIF11 was increased when low oxygen tension was applied (Figures 5.5-5.8). Glucose deprived media had an effect of KIF11's expression only on day 4 (Figure

5.8). Brachyury's expression was increased only on the border of the contouring area: when the oxygen was low or high.

Moreover, lower oxygen tension enhanced the Brachyury and KIF1 in the 4 days of differentiation (Figures 5.5-5.8). The contour area for mesodermal expression wasn't showing that much difference in the gene expression for the 27 condition at day 1 and 2 but clear defined conditions were observed at day 3 and 4 with high expression at low oxygen tension (Appendix II, Figures 5-8).

High oxygen levels decreased ectodermal gene expression

2FI, quadratic and linear models were fitted significantly for ectodermal markers (Table 5.4). As the pyruvate increased from 0 mM to 0.5 mM, the expression of ectodermal markers (Nestin and β -Tubulin) didn't change except at day 3 (Appendix II, Figures 9-12). The expression of Nestin at day 1 and 2 was almost similar with alike gene expression pattern observed along the 27 conditions except at 20% O₂. Moreover, β -Tubulin's expression was higher when the cells were cultured at low oxygen tension during the 4 days (Figures 5.9-5.12). The general observed pattern in the ectodermal expression was that upon increasing the glucose and oxygen levels the level of expression of Nestin and β -Tubulin decreased (Figures 5.9-5.12). Differentiating the hiP-SCs at 0% O₂ and 0 mM glucose induced ectodermal markers with a maximum gene expression at day 3 (Figure 5.11).

The effect of glucose and oxygen levels on cell viability.

Culturing the cells in laboratory conditions led to highest cell counts. Thus, stressing the cells through exposure to deprived condition especially low glucose and oxygen had an impact on cell viability. Although in some conditions higher gene expression for each germ layer was observed a lot of dead cells were detected. Cell viability which

was assessed by trypan blue exclusion method is reported in Figure 5.1-12 and the contour plot in Appendix II, Figure 13. Lower glucose and oxygen levels decreased cell viability. The quadratic model chosen to fit in the viable cell count was significant (Table 5.5).

5.3.2 Germ layer optimisation and model predictability

Taking into consideration that cell viability decreased in the deprivation studies, a model predictability was generated from Design-Expert® to maximize gene expression of each germ layer while maintaining high cell viability. Combining all of the gene expression and the cell viability during the 4 days of differentiation, the best conditions for each germ layer was detected. In almost all the germ layers, the best condition during the 4 days were found to be at the extreme of the contouring plot. The enhancement of endodermal lineage occurred at low glucose levels while low oxygen and glucose enhanced the mesodermal and ectodermal gene expression (Figure 5.13). Details for factors concentration and desirability for each germ layers are summarised in Table 5.6.

Germ layer	Response	Model	Sum of squares	df	Mean Square	F value	p Value
Endoderm	Sox17 Day1	2 FI	8.66	6	1.44	4.87	0.0003
	Sox17 Day2	2 FI	31.90	9	3.54	7.49	< 0.0001
	Sox17 Day3	Quadratic	31.90	9	3.54	7.49	< 0.0001
	Sox17 Day4	Quadratic	20.52	9	2.28	7.32	< 0.0001
	FoxA2 Day1	Quadratic	80.00	9	8.89	5.12	< 0.0001
	FoxA2 Day2	2 FI	21.03	6	3.50	2.45	0.0323
	FoxA2 Day3	2 FI	19.13	6	3.19	1.50	0.1897
	FoxA2 Day4	Quadratic	87.09	9	9.68	7.47	< 0.0001
	Cxcr4 Day 1	Quadratic	5.78	9	0.64	2.99	0.0044
	Cxcr4 Day 2	Quadratic	4.57	9	0.51	9.52	< 0.0001
	Cxcr4 Day 3	Quadratic	9.30	9	1.03	11.22	< 0.0001
	Cxcr4 Day 4	Quadratic	11.43	9	1.27	6.13	< 0.0001

Table 5.2 Analysis of variance (ANOVA) of the regression model for normalised endodermal germ layer. The best fit model was used for each of the endodermal markers during the 4 days of differentiation. “df” is an abbreviation for ‘degrees of freedom’. p-value < 0.05 was considered statistical relevant for the mathematical model.

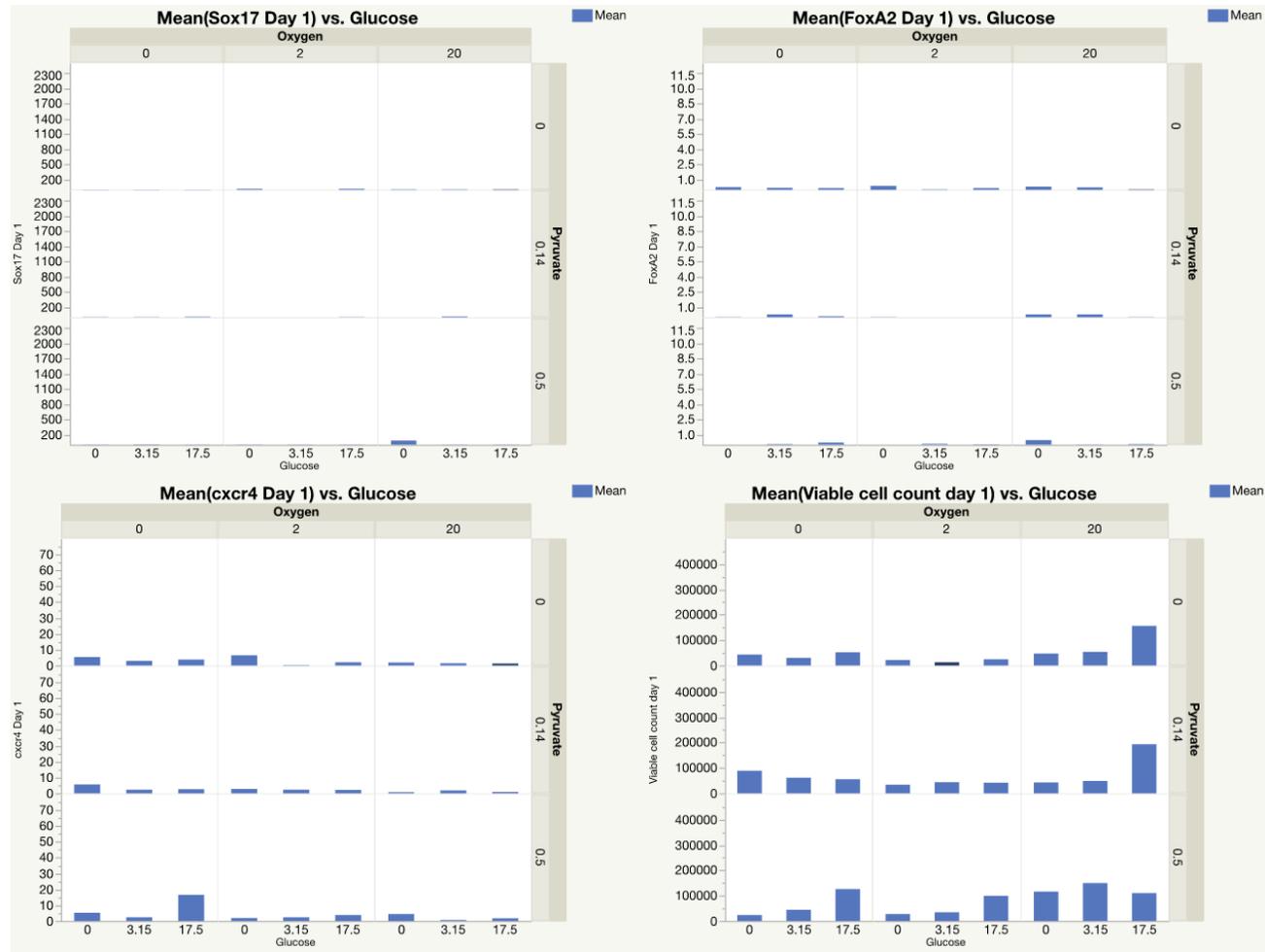


Figure 5.1 Summary of the mean values of the gene expression of endodermal markers (Sox17, FOXA2 and CXCR4) and cell viability for the 27 conditions at day 1. Using JMP software the mean of the raw data was calculated.

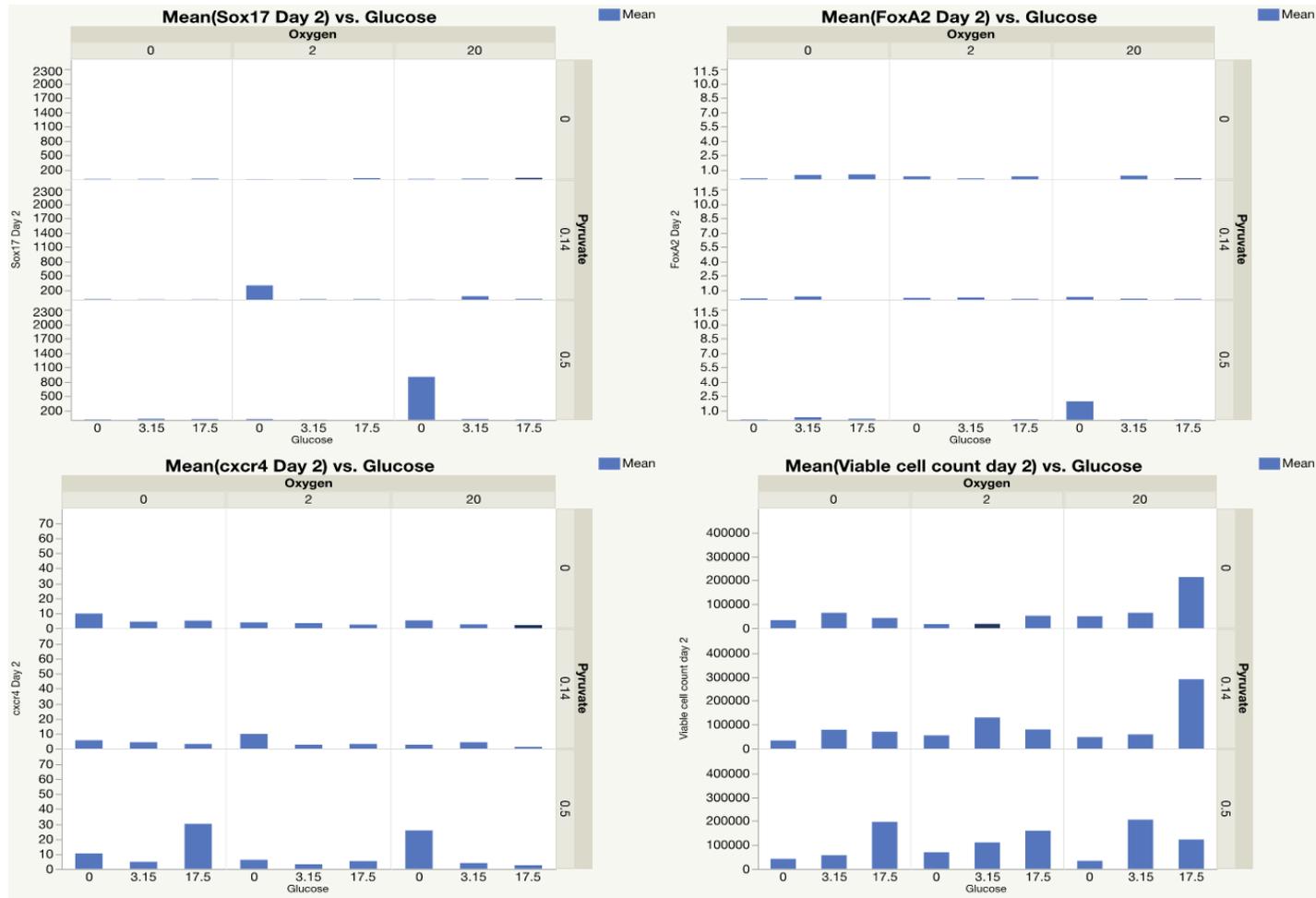


Figure 5.2 Summary of the mean values of the gene expression of endodermal markers (Sox17, FOXA2 and CXCR4) and cell viability for the 27 conditions at day 2. Using JMP software the mean of the raw data was calculated.

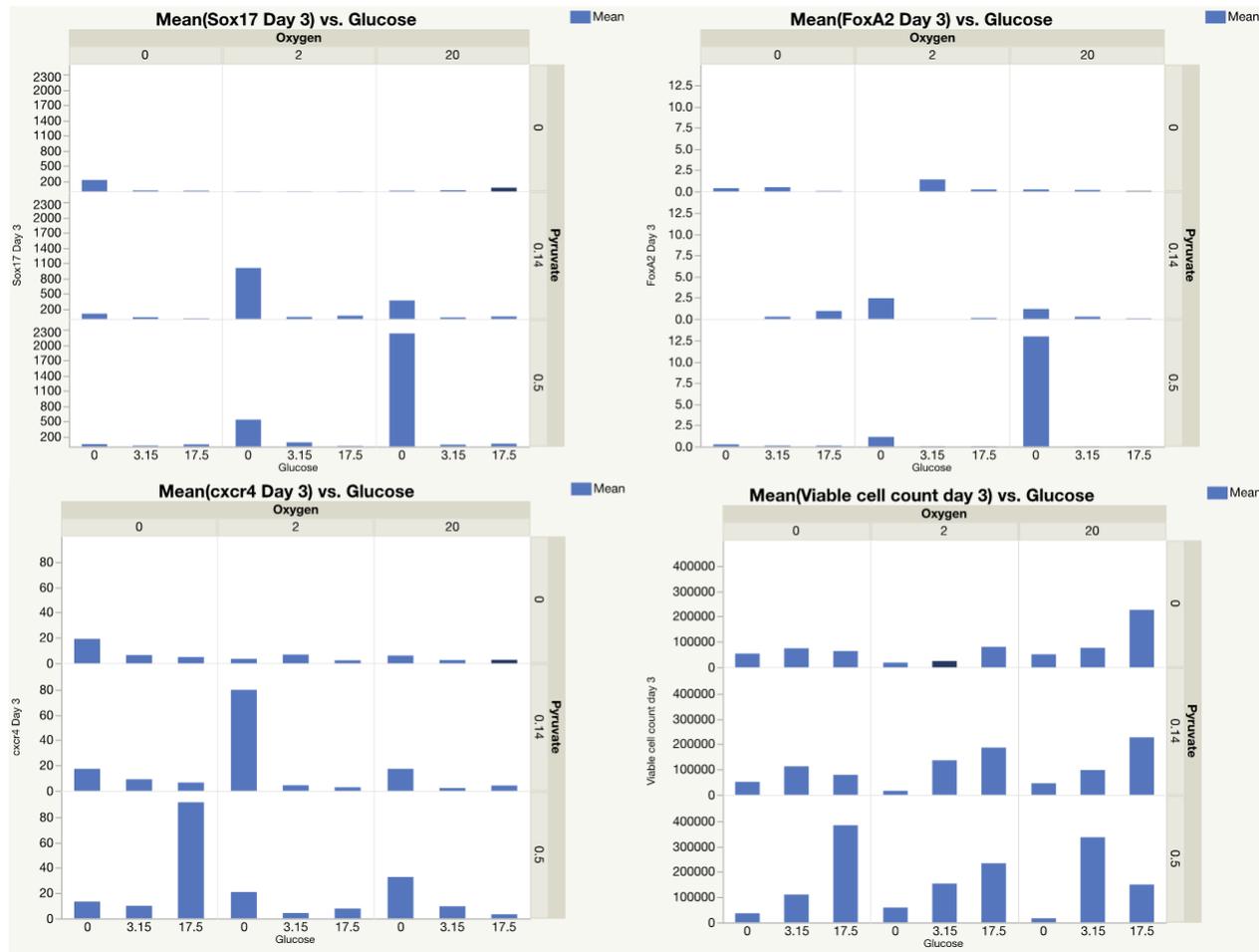


Figure 5.3 Summary of the mean values of the gene expression of endodermal markers (Sox17, FOXA2 and CXCR4) and cell viability for the 27 conditions at day 3. Using JMP software the mean of the raw data was calculated.

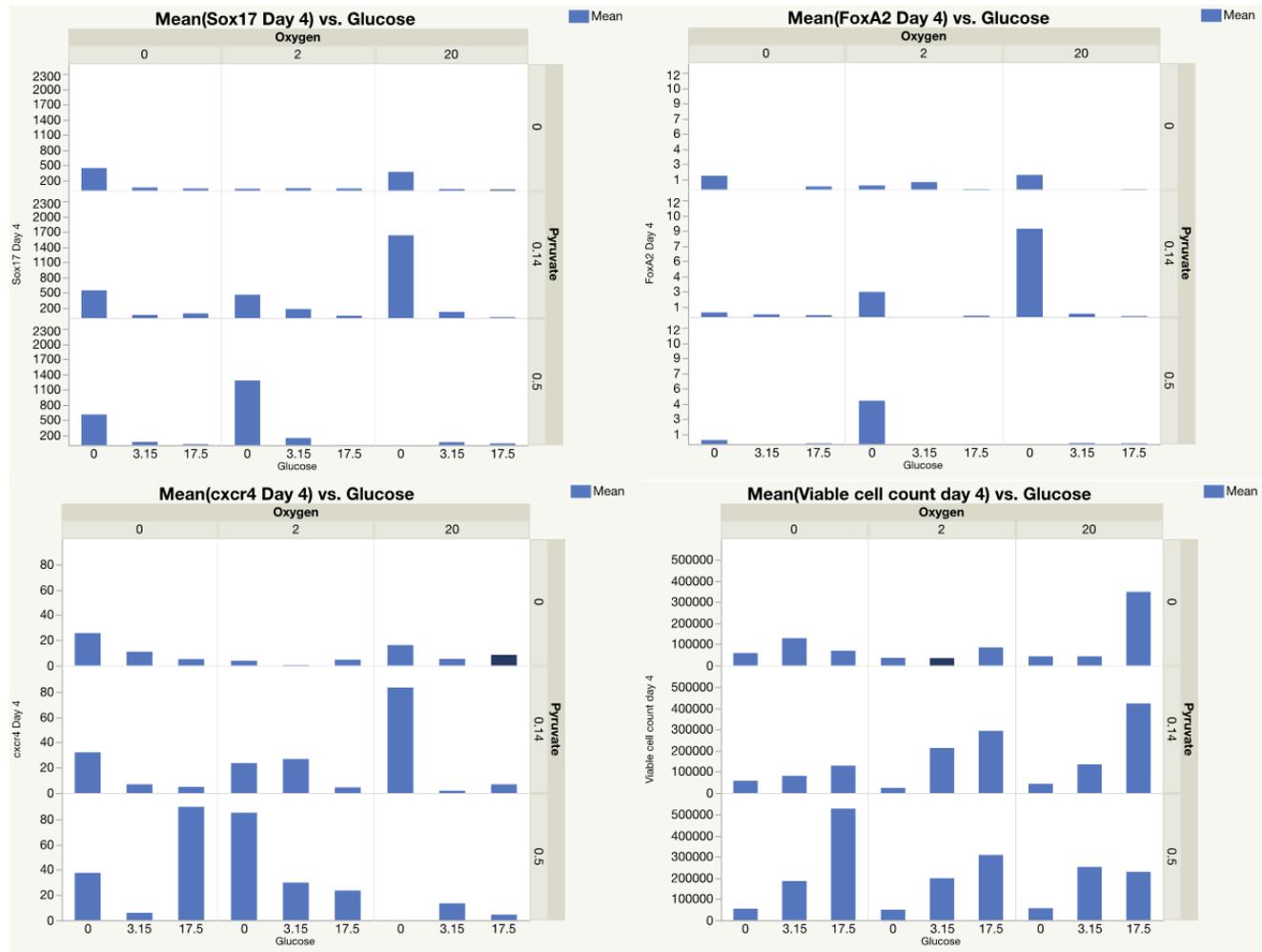


Figure 5.4 Summary of the mean values of the gene expression of endodermal markers (Sox17, FOXA2 and CXCR4) and cell viability for the 27 conditions at day 4. Using JMP software the mean of the raw data was calculated.

	Response	Model	Sum of squares	df	Mean Square	F value	p Value
Mesoderm	Brachyury Day1	Quadratic	9.81	9	1.09	1.13	0.3521
	Brachyury Day2	Quadratic	4.83	9	0.54	2.76	0.0082
	Brachyury Day3	Quadratic	23.81	9	2.65	4.10	0.0003
	Brachyury Day4	Quadratic	9.70	9	1.08	8.50	< 0.0001
	KIF11 Day1	Quadratic	5.19	9	0.58	9.06	< 0.0001
	KIF11 Day2	Quadratic	5.33	9	0.59	7.34	< 0.0001
	KIF11Day3	Quadratic	9.36	9	1.04	17.50	< 0.0001
	KIF11 Day4	Quadratic	22.47	9	2.50	37.69	< 0.0001

Table 5.3 Analysis of variance (ANOVA) of the regression model for normalised mesodermal germ layer. The best fit model was used for each of the mesodermal markers during the 4 days of differentiation. “df” is an abbreviation for ‘degrees of freedom’. p-value < 0.05 was considered statistical relevant for the mathematical model.

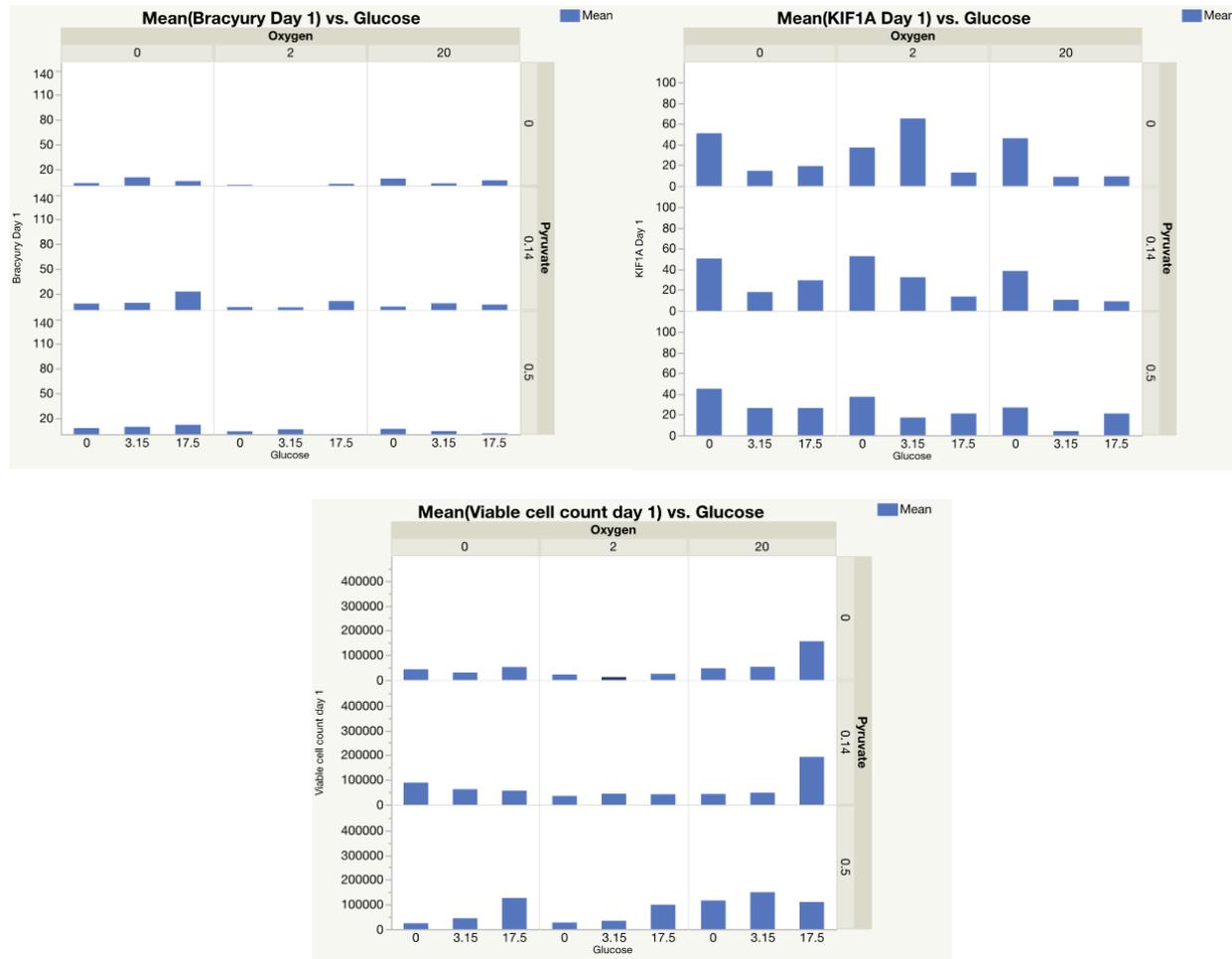


Figure 5.5 Summary of the mean values of the gene expression of mesodermal markers (Brachyury, KIF1A) and cell viability for the 27 conditions at day 1. Using JMP software the mean of the raw data was calculated.

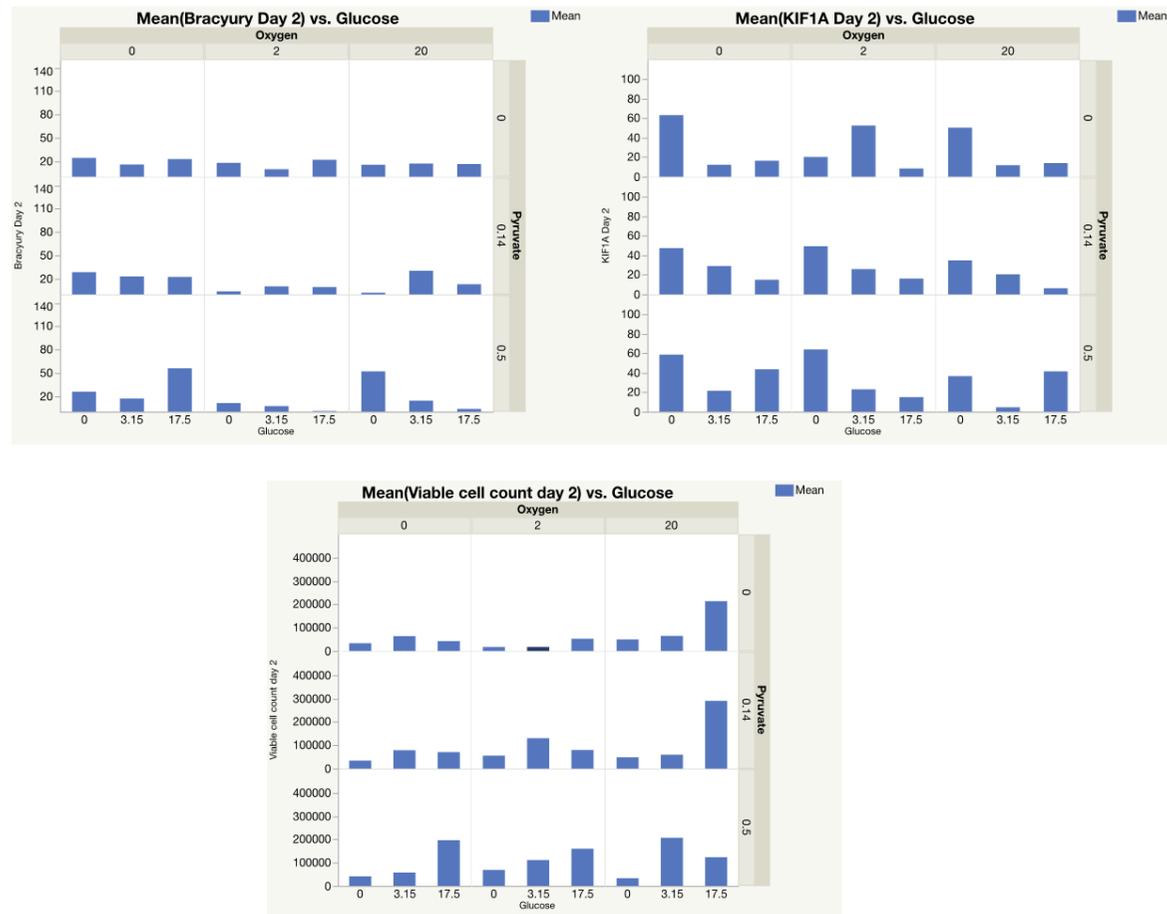


Figure 5.6 Summary of the mean values of the gene expression of mesodermal markers (Brachyury, KIF1A) and cell viability for the 27 conditions at day 2. Using JMP software the mean of the raw data was calculated.

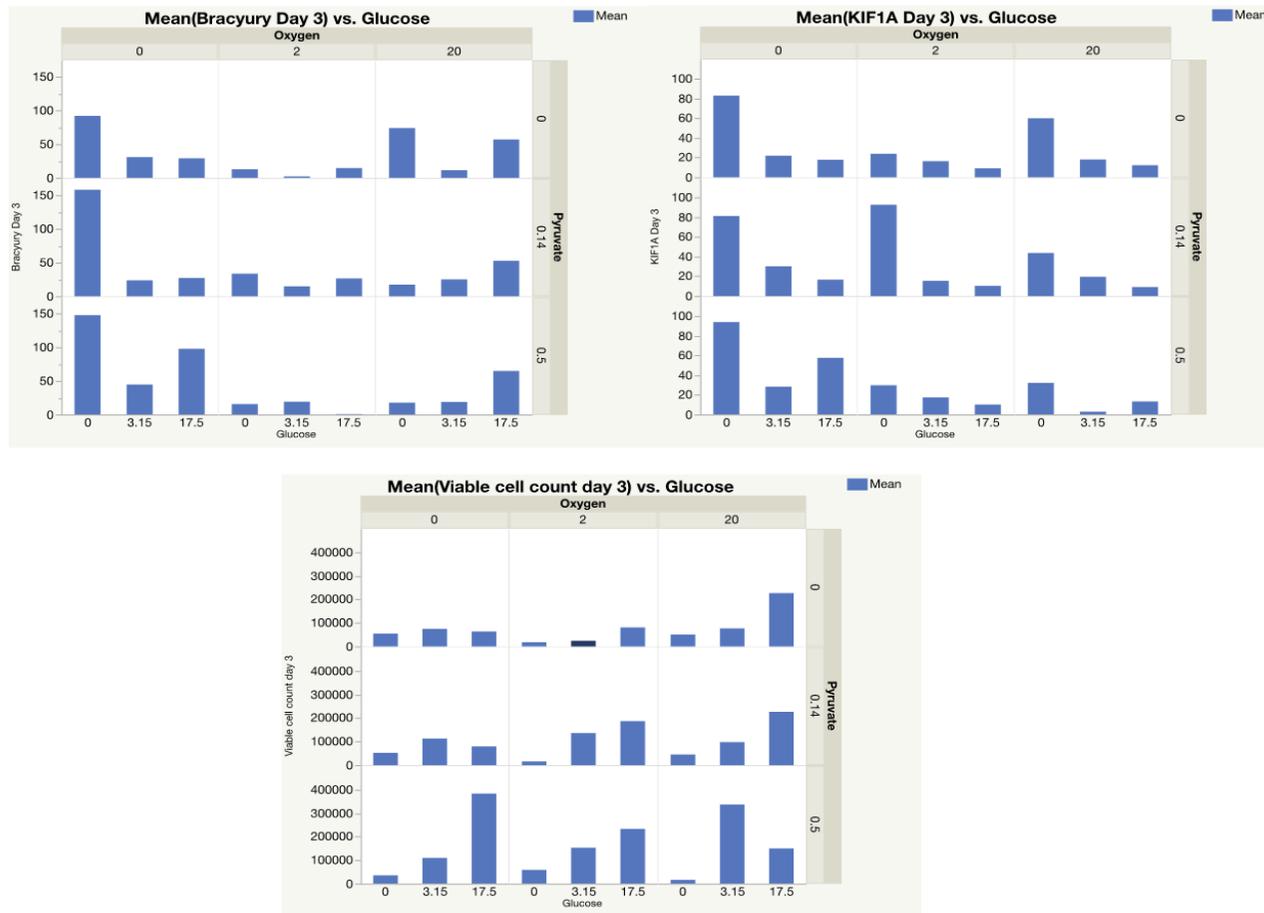


Figure 5.7 Summary of the mean values of the gene expression of mesodermal markers (Brachyury, KIF1A) and cell viability for the 27 conditions at day 3. Using JMP software the mean of the raw data was calculated.

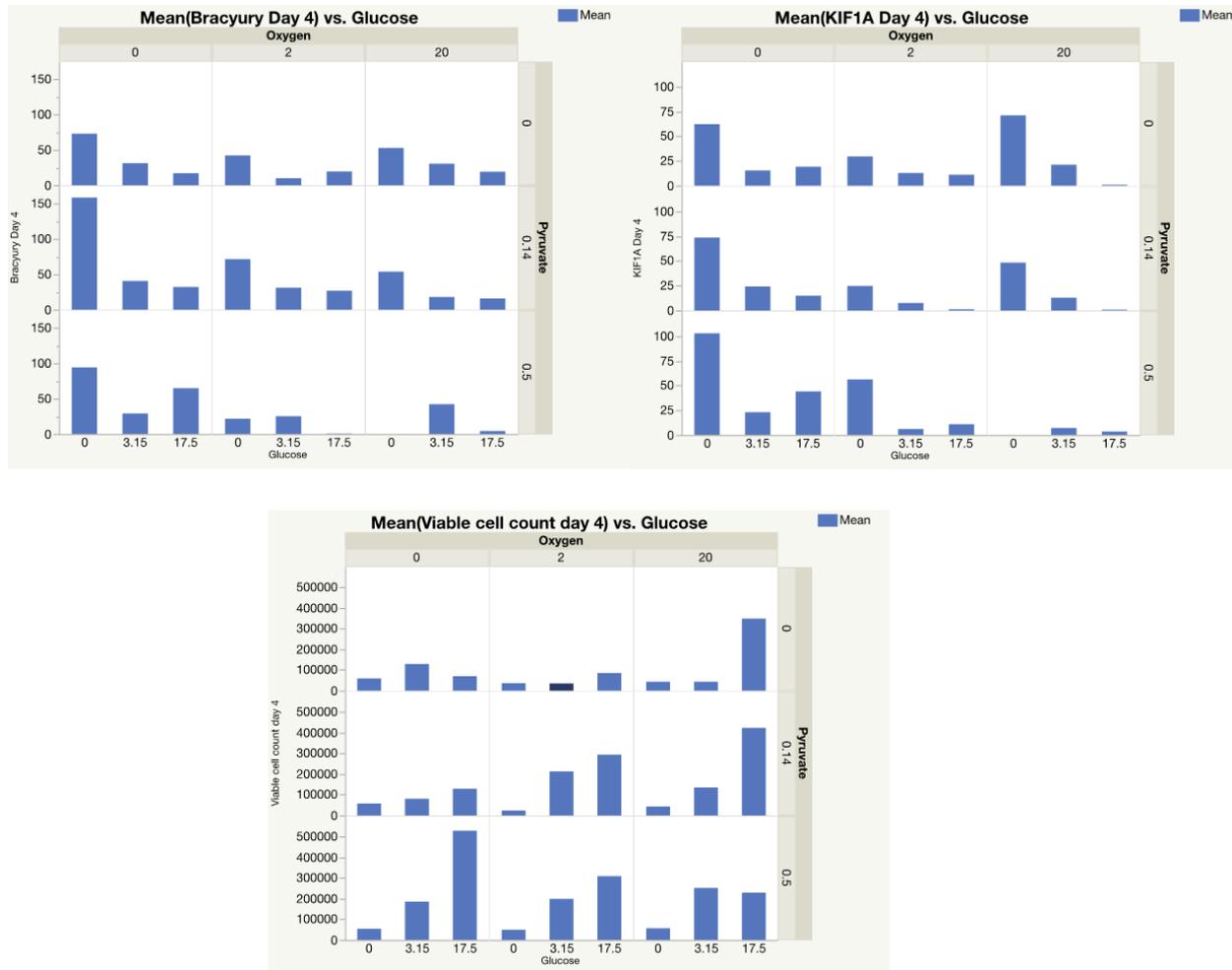


Figure 5.8 Summary of the mean values of the gene expression of mesodermal markers (Brachyury, KIF1A) and cell viability for the 27 conditions at day 4. Using JMP software the mean of the raw data was calculated.

	Response	Model	Sum of squares	df	Mean Square	F value	p Value
Ectoderm	Nestin Day 1	2 FI	10.89	6	1.82	12.46	< 0.0001
	Nestin Day 2	2 FI	9.97	6	1.66	10.53	< 0.0001
	Nestin Day 3	Quadratic	19.27	9	2.14	12.42	< 0.0001
	Nestin Day 4	Quadratic	13.65	9	1.52	8.02	< 0.0001
	β -Tubulin Day 1	Quadratic	6.08	9	0.68	8.37	< 0.0001
	β -Tubulin Day 2	Quadratic	4.45	9	0.49	7.95	< 0.0001
	β -Tubulin Day 3	Quadratic	9.25	9	1.03	15.28	< 0.0001
	β -Tubulin Day 4	Quadratic	4.76	9	0.53	11.22	< 0.0001

Table 5.4 Analysis of variance (ANOVA) of the regression model for normalised ectoderm germ layer. The best fit model was used for each of the ectodermal markers during the 4 days of differentiation. “df” is an abbreviation for ‘degrees of freedom’. p-value < 0.05 was considered statistical relevant for the mathematical model.

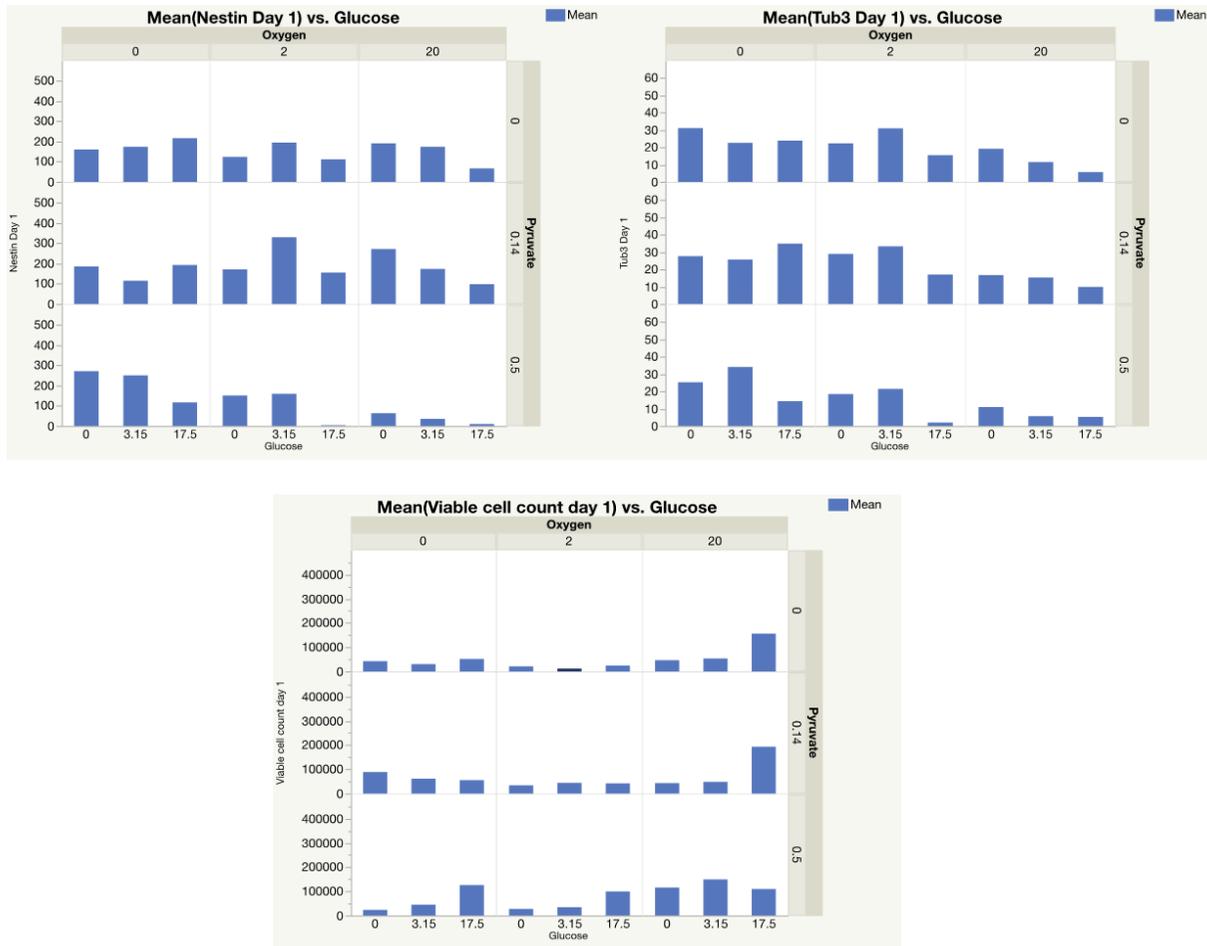


Figure 5.9 Summary of the mean values of the gene expression of ectodermal markers (Nestin and β -tubulin) and cell viability for the 27 conditions at day 1. Using JMP software the mean of the raw data was calculated.

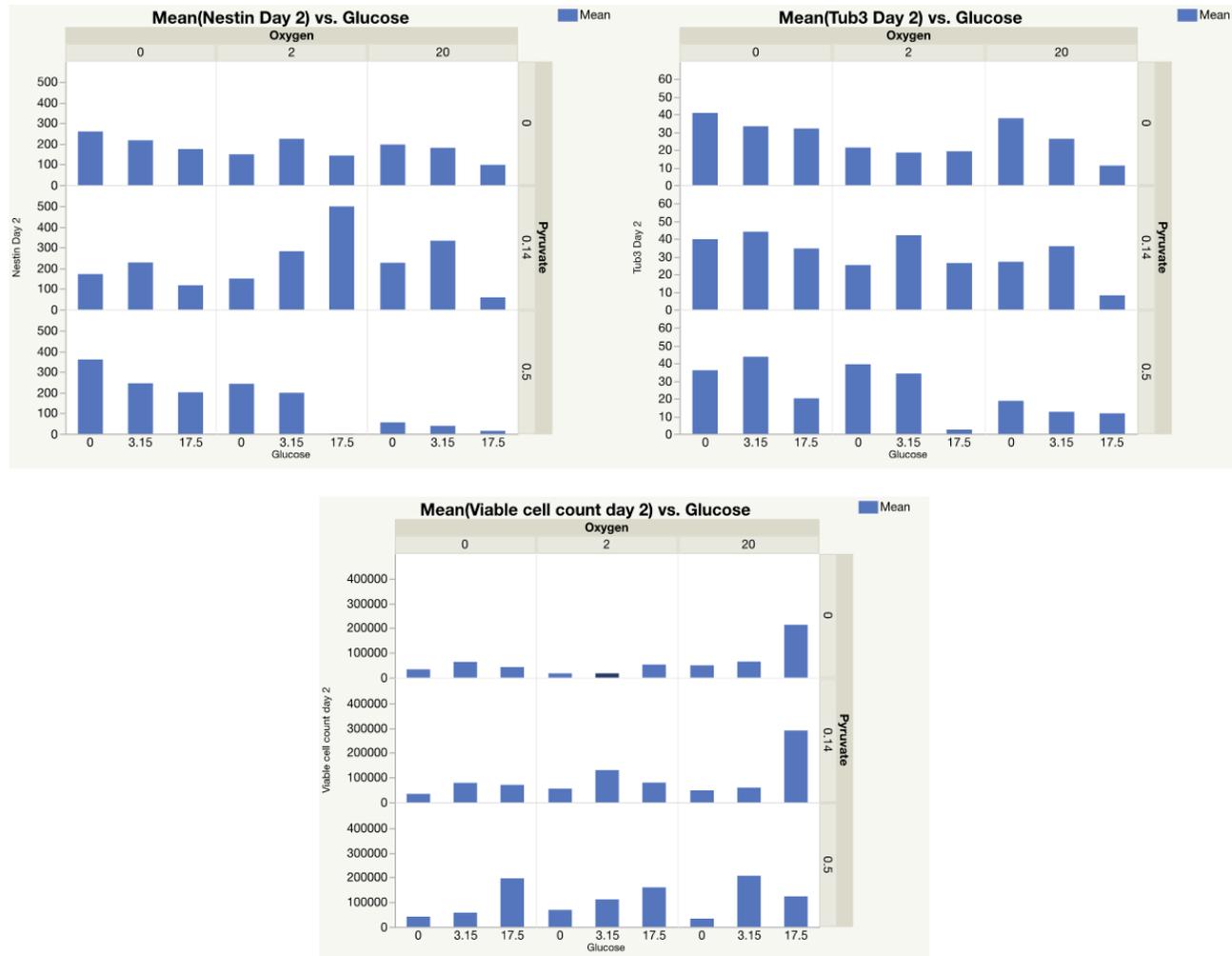


Figure 5.10 Summary of the mean values of the gene expression of ectodermal markers (Nestin and β -tubulin) and cell viability for the 27 conditions at day 2. Using JMP software the mean of the raw data was calculated.

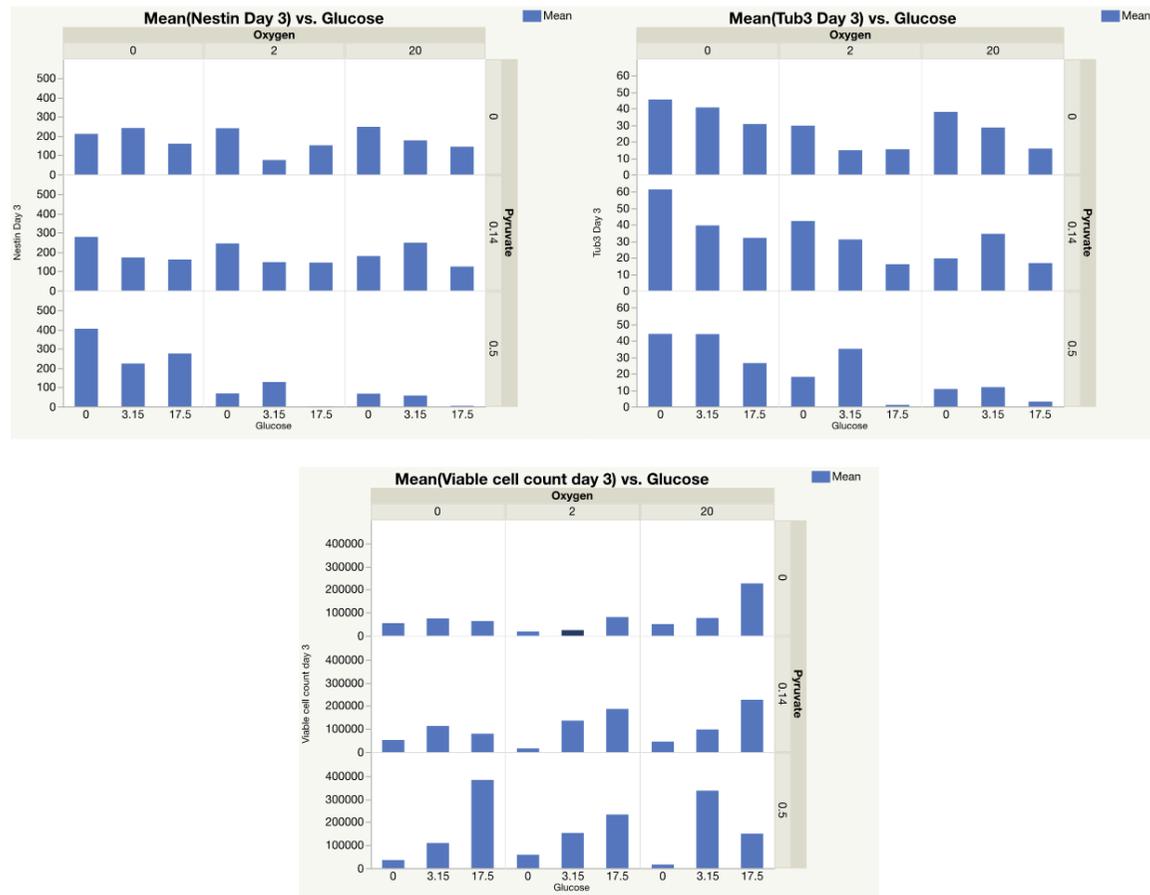


Figure 5.11 Summary of the mean values of the gene expression of ectodermal markers (Nestin and β -tubulin) and cell viability for the 27 conditions at day 3. Using JMP software the mean of the raw data was calculated.

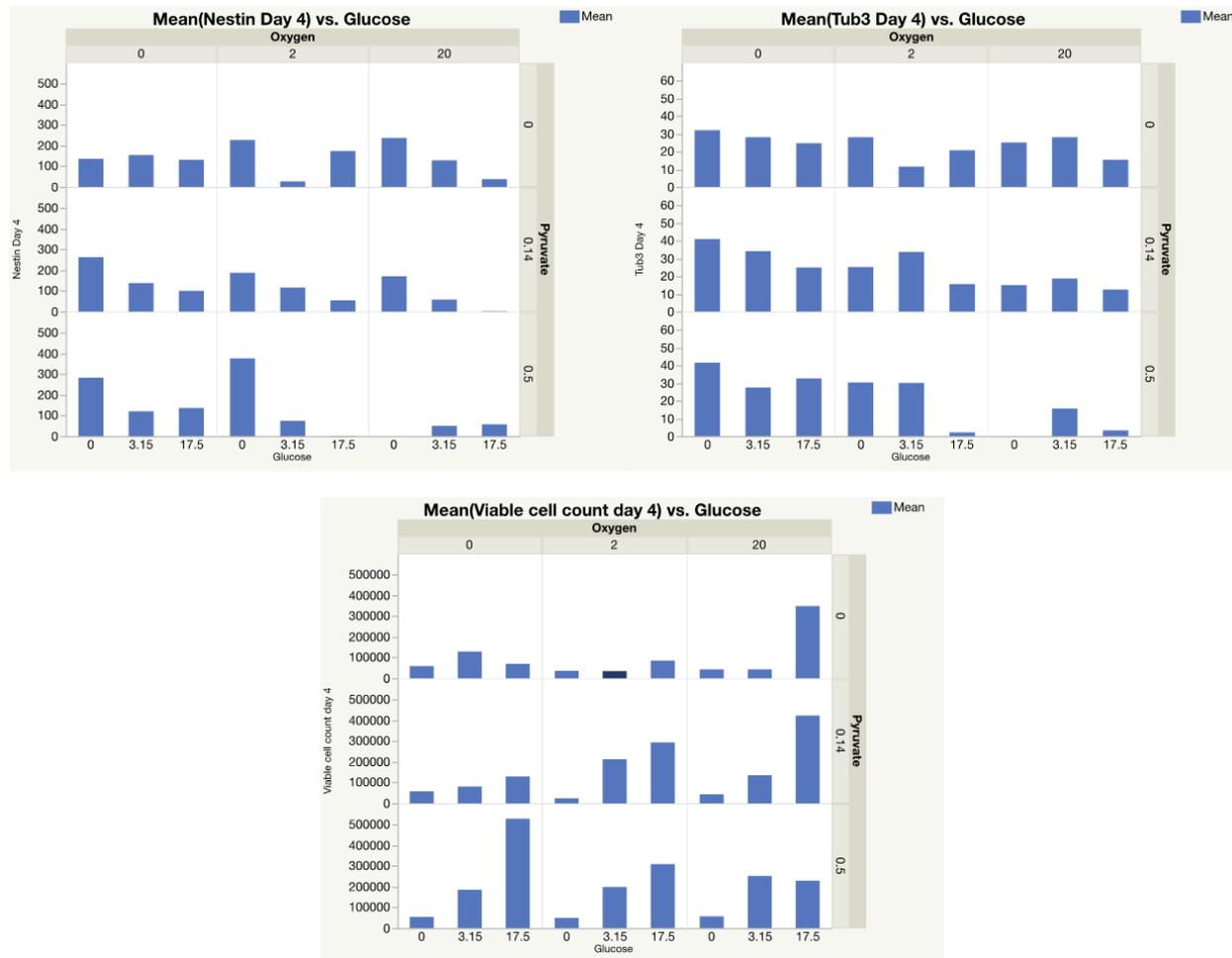


Figure 5.12 Summary of the mean values of the gene expression of ectodermal markers (Nestin and β -tubulin) and cell viability for the 27 conditions at day 4. Using JMP software the mean of the raw data was calculated.

	Response	Model	Sum of squares	df	Mean Square	F value	p Value
	Day1	Quadratic	1.190E+011	9	1.322E+010	14.20	< 0.0001
Viable cell count (Cells/mL)	Day2	Quadratic	2.408E+011	9	2.675E+010	13.20	< 0.0001
	Day3	Quadratic	4.424E+011	9	4.916E+010	11.25	< 0.0001
	Day4	Quadratic	9.964E+011	9	1.107E+011	18.23	< 0.0001

Table 5.5 Analysis of variance (ANOVA) of the regression model for normalised viable cell count. The best fit model was used for cell viability during the 4 days of differentiation. “df” is an abbreviation for ‘degrees of freedom’. p-value < 0.05 was considered statistical relevant for the mathematical model.

A	Oxygen	Glucose	Pyruvate	LogSox17	LogFoxA2	LogCxcr4	Viable cell count	Desirability	Day
	20.000	0.000	0.000	0.645	-0.194	0.293	50593.580	0.711	1
	20.000	0.000	0.500	2.022	-1.227	1.112	45906.256	0.755	2
	20.000	0.000	0.475	2.822	-0.221	1.242	66585.702	0.756	3
	20.000	0.000	0.380	3.310	1.550	1.592	53076.899	0.917	4
B	Oxygen	Glucose	Pyruvate	LogBrachyury	LogKIF1	Viable cell count	Desirability	Day	
	0.000	0.000	0.143	0.769	1.755	54011.907	0.694	1	
	0.000	0.000	0.500	1.453	1.697	68054.373	0.848	2	
	0.000	0.000	0.263	2.291	2.011	45381.687	0.982	3	
	0.000	0.000	0.500	2.005	1.952	110145.083	0.911	4	
C	Oxygen	Glucose	Pyruvate	LogNestin	Logb-Tubulin	Viable cell count	Desirability	Day	
	0.000	0.000	0.202	2.353	1.626	58548.281	0.741	1	
	0.000	0.000	0.291	2.384	1.722	63602.704	0.865	2	
	0.000	0.000	0.294	2.719	1.883	49972.747	0.994	3	
	0.000	0.000	0.500	2.572	1.693	110145.083	0.928	4	

Table 5.6 The detailed conditions obtained from the predictive model for germ layer enhancement (A: endoderm, B: mesoderm, C: ectoderm) during the 4 days of differentiation.

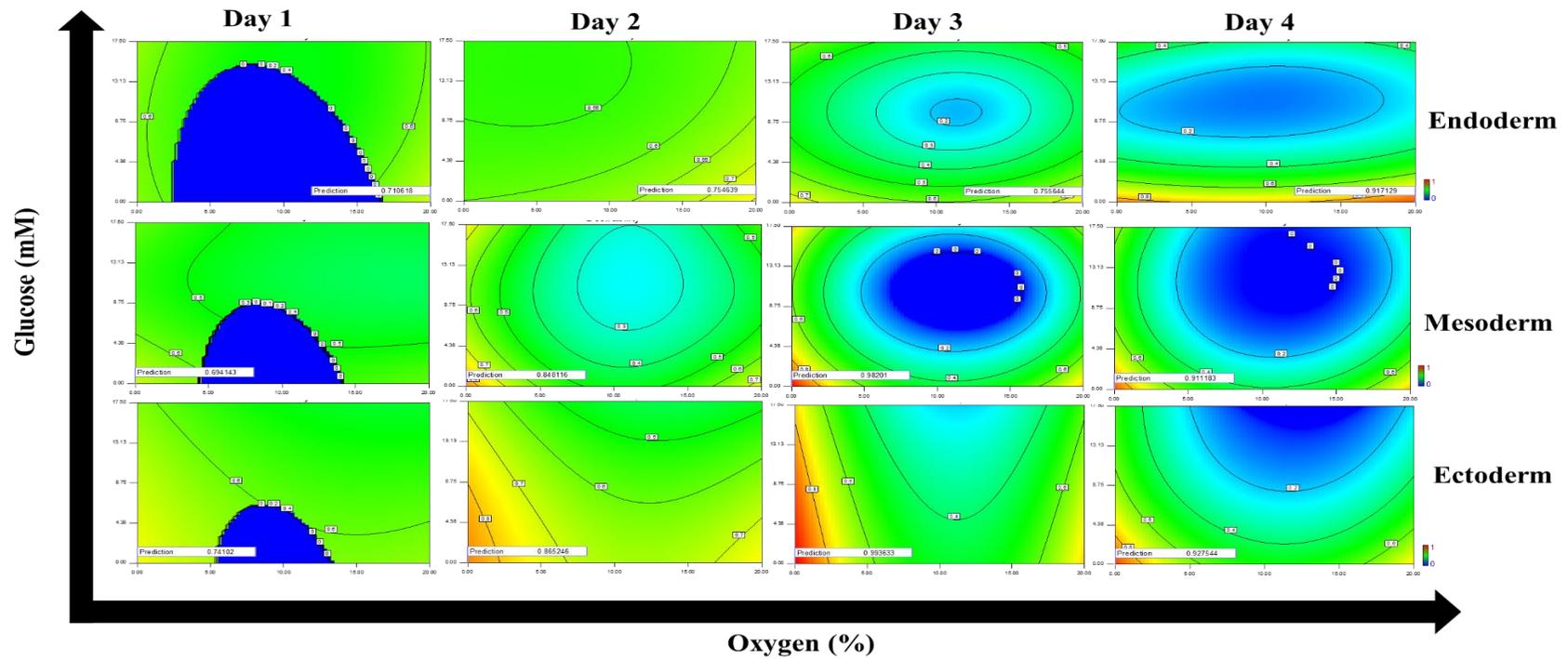


Figure 5.13 Predictive model. All the gene expression for each germ layer was added up for this model along with viable cell count. Colours ranging from dark blue (0) to dark red (1) represent the lowest and highest value for desirability. The dashed area in each model represents the optimum range predicted by the model to maximize the readout.

5.3.3 Discussion

As mentioned before, the differentiation of stem cells into any lineage is hindered by several bioprocess challenges. Thus, the urge to study in depth the early days of embryo development to optimise the differentiation process. During early embryo development, the level of oxygen and metabolites is less than laboratory conditions (§Chapter 1), thus the initial aim was to investigate the effect of oxygen and nutrients as carbohydrates, lipids and proteins. Nevertheless, oxygen and carbohydrates were taken forward for initial studies as it showed an impact on stem cells proliferation and differentiation. For instance, many studies investigated the effect of glucose concentration on the proliferation of mouse embryonic stem cells (Kim, Heo and Han, 2006), human bone marrow-derived MSC (Li *et al.*, 2007), human hematopoietic stem cell differentiation (Oburoglu *et al.*, 2014) and mesenchymal stem cells (MSC) (Stolzing, Coleman and Scutt, 2006; Dhanasekaran *et al.*, 2013), yet no studies investigated the effect of oxygen, glucose and pyruvate or their interaction on germ layer formation.

It was found that oxygen and glucose deprivation enhanced the ectodermal and mesodermal germ layers formation from day 2. Culturing differentiated hESCs or mESCs at lower oxygen tension stimulated the formation of mesodermal (Khan, Adesida and Hardingham, 2007; Koay and Athanasiou, 2008; Stacpoole *et al.*, 2013) and ectodermal precursors (Mondragon-Teran, Lye and Veraitch, 2009; Mondragon-Teran *et al.*, 2011).

Moreover, culturing the cells in glucose-deprived media enhanced the endodermal gene expression while maintaining normal oxygen and pyruvate levels. Previous studies reported that low glucose has been shown to stimulate the commitment of human

hematopoietic stem cells into the erythroid lineage (Oburoglu *et al.*, 2014) and maturation of MSCs (Farrell *et al.*, 2015).

Our results suggested that depriving the cells from glucose during the first 3 days of differentiation can upregulate the endodermal gene expression up to 2300 fold increase of Sox17. Meanwhile lower oxygen and glucose levels were needed for the enhancement of mesodermal and ectodermal lineage. Using design expert, desirable conditions for the formation of the germ layers were detected and suggested using the formulas fed into the predictability model (Appendix II).

As the glucose removal had a major impact on endodermal gene expression and cell viability, this condition (20% O₂, 0.5 mM pyruvate, 0 mM glucose) was studied in depth in chapter 6.

6 Chapter 6: Glucose Deprivation Selects Endodermal Lineages During Human Pluripotent Stem Cell Differentiation.

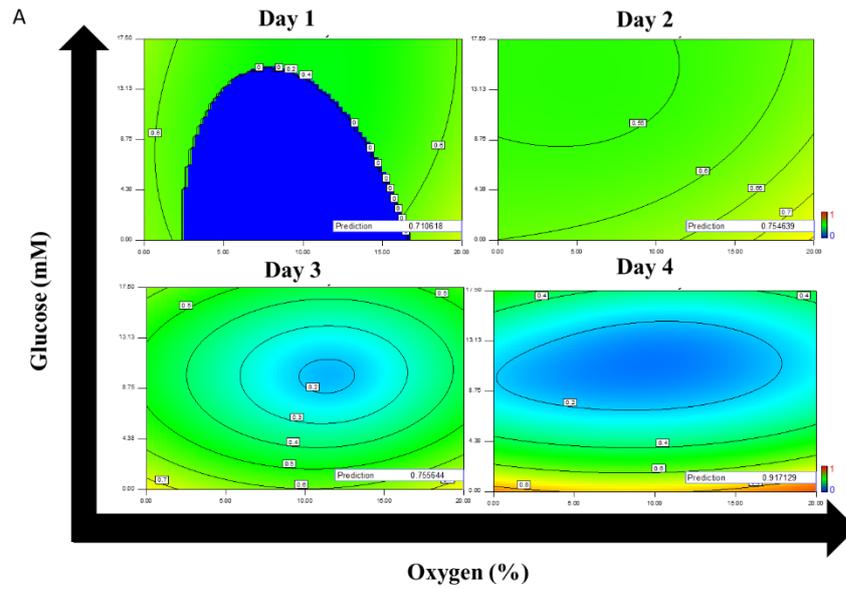
6.1 Introduction

ESCs and iPSCs have a wide range of biomedical applications including cell and gene therapy, disease modelling for drug discovery and organogenesis/development models (Singh *et al.*, 2005). Efficient and cost-effective methods for differentiating PSCs into therapeutically relevant cells is a key challenge in the rapidly developing field of regenerative medicine.

Using the mathematical model generated from the previous design of experiments (§Chapter 5), the optimum condition for endodermal germ layer generation was when the cells were deprived of glucose whilst maintaining atmospheric oxygen and pyruvate levels (Figure 6.1). This finding could be associated by the fact the embryo is exposed to low glucose concentration during early days of development (Dickens *et al.*, 1995; Gardner *et al.*, 1996; Tay *et al.*, 1997) (§ Chapter 1.5). Thus glucose concentration could be considered as a key factor for germ layer formation.

Moreover, glucose is needed for macromolecules synthesis which are needed for embryo development (Brinster, 1969). Glucose is metabolized via glycolysis to provide energy especially in the late stage preimplantation development (Wales and Whittingham, 1970; Hardy *et al.*, 1989; Houghton *et al.*, 1996; Houghton and Leese, 2004). Despite the importance of glucose, some *in-vitro* studies have shown that embryo development underwent arrest and the formation of reactive oxygen species was increased in the presence of media containing high glucose levels (Sviderskaya *et al.*, 1996; Iwata *et al.*, 1998). Culturing cells at 0 mM glucose, 20% O₂, 0.5 mM pyruvate, 0 mM glucose resulted in high endodermal gene expression, thus the main objective

of this chapter was to investigate the effect of lowering glucose concentration on endodermal formation and to study how glucose deprivation induced cell detachment and apoptosis. To study if glucose deprivation triggered apoptosis thus enhancing endoderm formation, staurosporine was used to check if it could influence lineage commitment. Moreover, few studies considered manipulating other pathways to trigger pluripotent stem cell differentiation. For example. It has been shown that the inhibition of cell:cell contacts can promoted mesendoderm formation while its maintenance promoted neuroectoderm (Hughes *et al.*, 2009). Staurosporine enhanced the mesendoderm and mesoderm population in differentiated mouse primitive ectoderm-like cells while inducing primitive streak marker gene expression in human embryonic stem cells (Hughes *et al.*, 2014). Thus it is important to study the effect of triggering apoptosis on cell lineage commitment.



B

Number	Oxygen	Glucose	Pyruvate	LogSox17 Day 1	LogFoxA2 Day 1	LogCxcr4 Day 1	Viable cell count Day 1	Desirability	Days
1	20.000	0.000	0.000	0.645	-0.194	0.293	50593.580	0.711	Day 1
2	20.000	0.000	0.003	0.646	-0.237	0.292	51176.809	0.710	
3	19.924	0.000	0.000	0.643	-0.236	0.283	49543.757	0.708	
1	20.000	0.000	0.500	2.022	-1.227	1.112	45906.256	0.755	Day 2
2	19.998	0.000	0.498	2.016	-1.228	1.109	46317.614	0.754	
3	20.000	0.084	0.500	2.018	-1.225	1.097	47639.235	0.753	
1	20.000	0.000	0.475	2.822	-0.221	1.242	66585.702	0.756	Day 3
2	20.000	0.000	0.481	2.816	-0.209	1.241	66453.374	0.756	
3	20.000	0.000	0.467	2.828	-0.236	1.244	66746.292	0.756	
1	20.000	0.000	0.380	3.310	1.550	1.592	53076.899	0.917	Day 4
2	20.000	0.000	0.378	3.309	1.550	1.592	53527.203	0.917	
3	20.000	0.000	0.384	3.311	1.549	1.591	52440.313	0.917	

Figure 6.1 Predictable model for endodermal germ layer formation. The gene expression of Sox17, Cxcr4 and FoxA2 along with cell viability data were used for the model predictability. Colours ranging from dark blue (0) to dark red (1) represent the lowest and highest value for desirability for each day (A). A detailed summary of the best conditions with the highest desirability was generated by the mathematical model (B).

6.2 Chapter Aims and Objectives

The aim of this chapter was to investigate the effect of glucose deprivation on endodermal germ layer formation of differentiated human pluripotent stem cells hiPSCs (cord blood) and hESCs (Shef 3). The detailed objectives are:

- Study the impact of glucose removal on the germ layer gene expression of monolayer differentiated hiPSCs (Cord blood) and hESCs (Shef3) while maintaining laboratory oxygen and pyruvate conditions
- Evaluate if apoptosis played role in the germ layer selection by the induction of apoptosis via staurosporine.
- Identify the molecular pathway by which glucose deprivation triggered endodermal lineage formation.
- Compare glucose-deprived media approach with direct endodermal induction by definitive endodermal kit.

6.3 Results and Discussion

6.3.1 The effect of glucose deprivation on monolayer differentiation of human induced pluripotent stem cells (iPSCs, cord blood)

To examine the effect of glucose deprivation on hiPSCs, monolayer differentiated cells were cultured for 3 days in normal DMEM-F12 media and custom made media devoid of glucose. During the first day, the cells in both conditions had almost the same morphology, cells were growing as single fibroblastic like morphology cells (Figure 6.2, A). After day 1, numerous detached cells were observed in the supernatant of glucose-deprived cells, while cells were proliferating in the normal condition (Figure 6.2, A). Deprived cells showed a time dependant cell detachment (Figure 6.2 A). We studied the effect of adding glucose back to media after 3 days for deprivation. Cells showed a recovery from day 6 and proliferated (Figure 6.2 B).

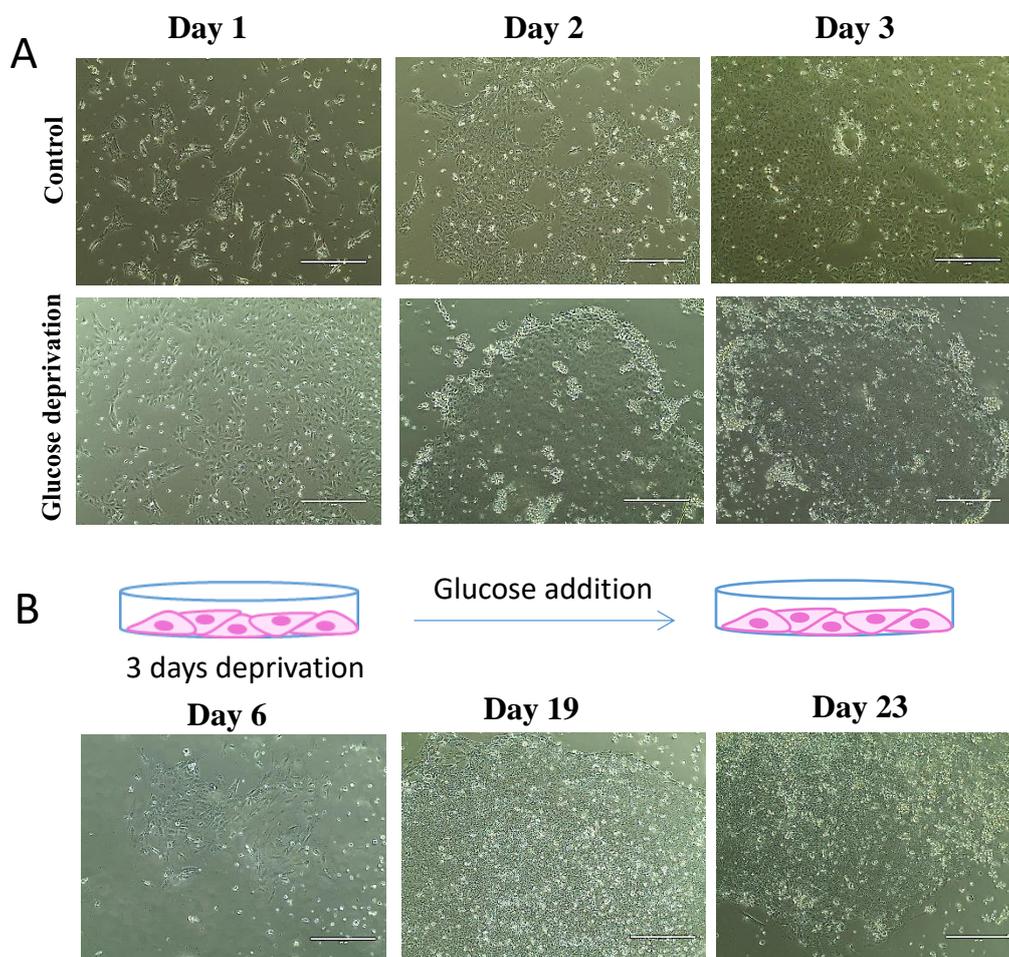


Figure 6.2 The morphology of differentiated hiPSCs (Cord blood) cultured with normal and deprived media. (A) Phase contrast images of single cell differentiated hiPSCs at day 1, 2 and 3 in normal and deprived conditions. (B) Schematic presentation of the protocol during glucose switch. The effect of switching to normal media after 3 days of deprivation on cell morphology was detected using Evos[®] XL. Images were taken at 100X ocular lens magnification. Scale bars=400 μ m.

Cell viability was measured by trypan blue exclusion method and WST-1 assay. Cultures subject to glucose deprivation exhibited progressive cell death with significant reductions in cell viability (Figure 6.3 A, B). To determine the extent to which the decrease in cell viability was influenced by apoptosis or necrosis, annexin V/PI staining was performed. Under glucose deprived conditions, 61.06 % of the cells were Annexin V-PI-positive (considered late apoptotic) at day 2 and 72.73 % at day 3, as seen in Figure 6.3 C. These results confirmed that the detached cells detected in Figure 6.2 were going through apoptosis.

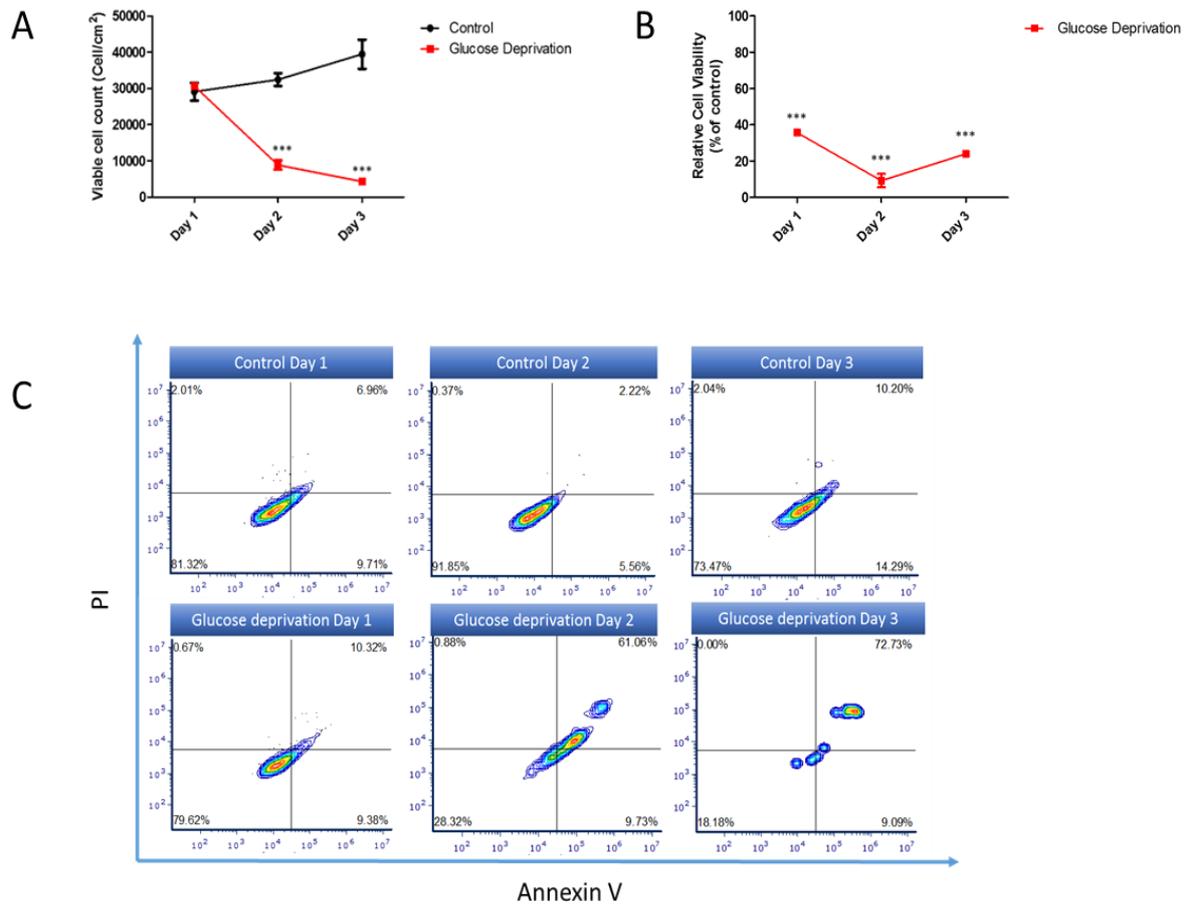


Figure 6.3 The effect of glucose deprivation on apoptosis and cell viability. (A, B) Cell viability of differentiated hiPSCs was detected by trypan blue exclusion method and WST-1 assay respectively. Relative cell viability=[(Absorbance)/Absorbance of control]*100 (C) Cells were fixed and immunostained with FITC-conjugated annexin V antibody and PI and analysed by flow cytometry. Cells negative for Annexin V and PI were considered viable cells whereas Annexin V-positive-PI-positive were considered late apoptotic, annexin V-negative-PI positive cells were necrotic and annexin V-positive-PI-positive cells were selected as early apoptotic. Statistical analysis was performed using Two-Way Anova and data are reported as mean±S.E.M of n=3, asterisks indicate the statistical significance: *** P<0.001, **P<0.01, *P<0.05). Data are reported as mean±S.E.M of n=3, p<0.05 was considered as significant).

To determine the cell phenotype of the hiPSCs in both conditions, staining for germ layer marker was performed. Differentiated monolayer hiPSCs cultured in normal media expressed higher ectodermal (TUJ, PAX 6) and mesodermal (SMA, brachyury) than the cells grown in deprived conditions (Figure 6.4). While glucose deprivation especially in day 3 showed the positive expression of endodermal markers (Sox17, AFP and PDX1) in the absence of glucose from the media as seen in Figure 6.4. As the cells cultured in deprived conditions were positively stained for the pancreatic marker PDX1 (Fagerberg et al., 2014; Ahlgren et al., 1998; Guz et al., 1995; Ahlgren et al., 1996; Jonsson et al., 1994) and definitive endodermal marker Sox17 (Séguin et al., 2008; Borowiak et al., 2009), therefore, the cells were differentiated towards definitive endoderm.

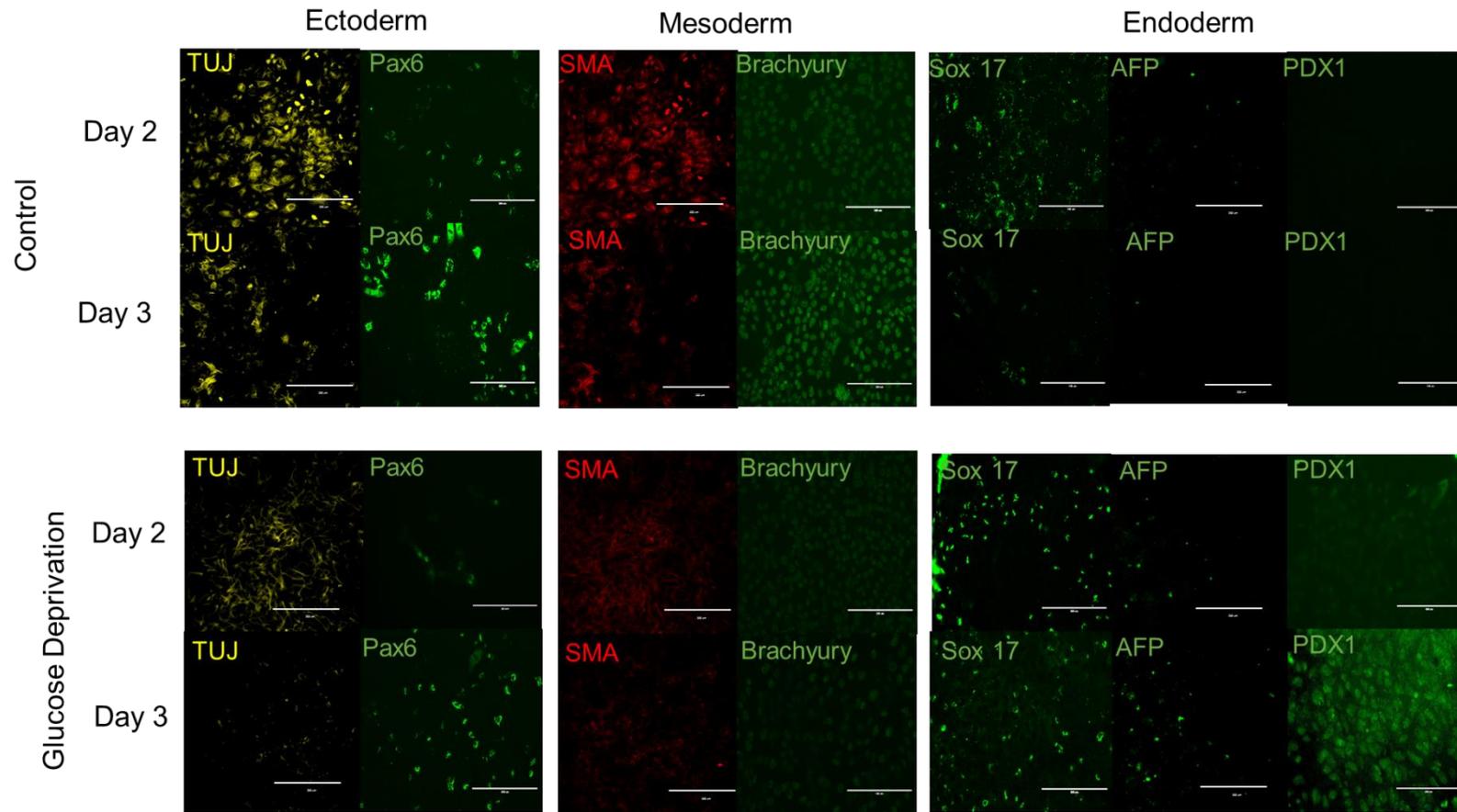


Figure 6.4 Immunofluorescence of differentiated hiPSCs at day 2 and 3. Cells were stained for ectodermal (TUJ, Pax6), mesodermal (SMA, Brachyury) and Endodermal (Sox17, AFP and PDX1) markers. Cells cultured with glucose deprivation showed an increase in endodermal markers. Images were taken using confocal microscope and Evos[®] FL. Images were taken at 200X ocular lens magnification. Scale bars=200 μ m.

Relative mRNA expression levels of a panel of common germ layer markers were assessed by Real time polymerase chain reaction (RT-PCR) (Chapter 2.5) over 3 days of differentiation. There was an upregulation of all endodermal markers (FoxA2, Cxcr4 and Sox 17) compared to the cells cultured in normal media : a steady increase in the expression of Cxcr4 for the deprived cells was observed, moreover up to 2233-fold change increase of Sox 17 at day 3 ($p < 0.001$) was observed when the glucose was removed from the media. Cells cultured with deprived media showed a slight significant increase in the expression of tubulin 3 at day 2 and 3 ($p < 0.05$) and brachyury at day 2 ($p < 0.05$) between the control and glucose deprived cells (Figure 6.5). The single differentiated hiPSCs (cord blood) showed an endodermal phenotype.

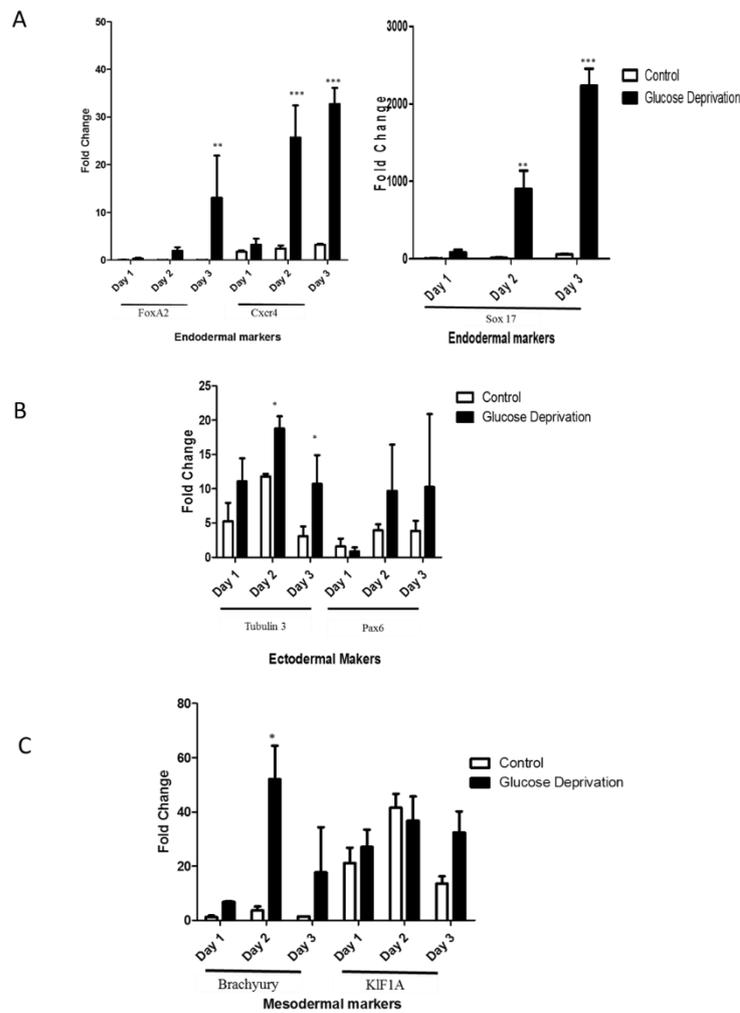


Figure 6.5 Endodermal lineage expression of differentiated human episomal iPSCs (cord blood) was enhanced by the removal of glucose from media. Real time PCR was performed in order to assess the fold gene expression of each germ layer: (A) Endoderm (Sox 17, FOXA2, CXCR4), (B) Ectoderm (Nestin, Tubulin 3, Pax6) and (C) mesoderm (Brachyury, KIF1A). Statistical analysis was performed using Two-Way Anova and data are reported as mean \pm S.E.M of n=3, asterisks indicate the statistical significance: *** P<0.001, **P<0.01, *P<0.05). Data are reported as mean \pm S.E.M of n=3, p<0.05 was considered as significant).

To establish whether glucose deprived cells could proliferate and retain their high endodermal gene expression after the 3 day starvation period, media containing glucose was added to maintain the cells from day 4 onwards. The glucose deprived cells were able to proliferate and maintain high endodermal gene expression even 23 days post starvation (Figure 6.6, D). The endodermal gene expression of Sox 17 and Cxcr4 decreased significantly after the glucose addition while the expression of FOXA2 remained unchanged (Figure 6.6, A). While there was no significant change in the ectodermal (Tubulin and Pax6) and mesodermal (Brachyury and KIFIA) expression as seen in Figure 6.6, B-C. The addition of glucose helped in the proliferation of the differentiated cells while maintaining its phenotype.

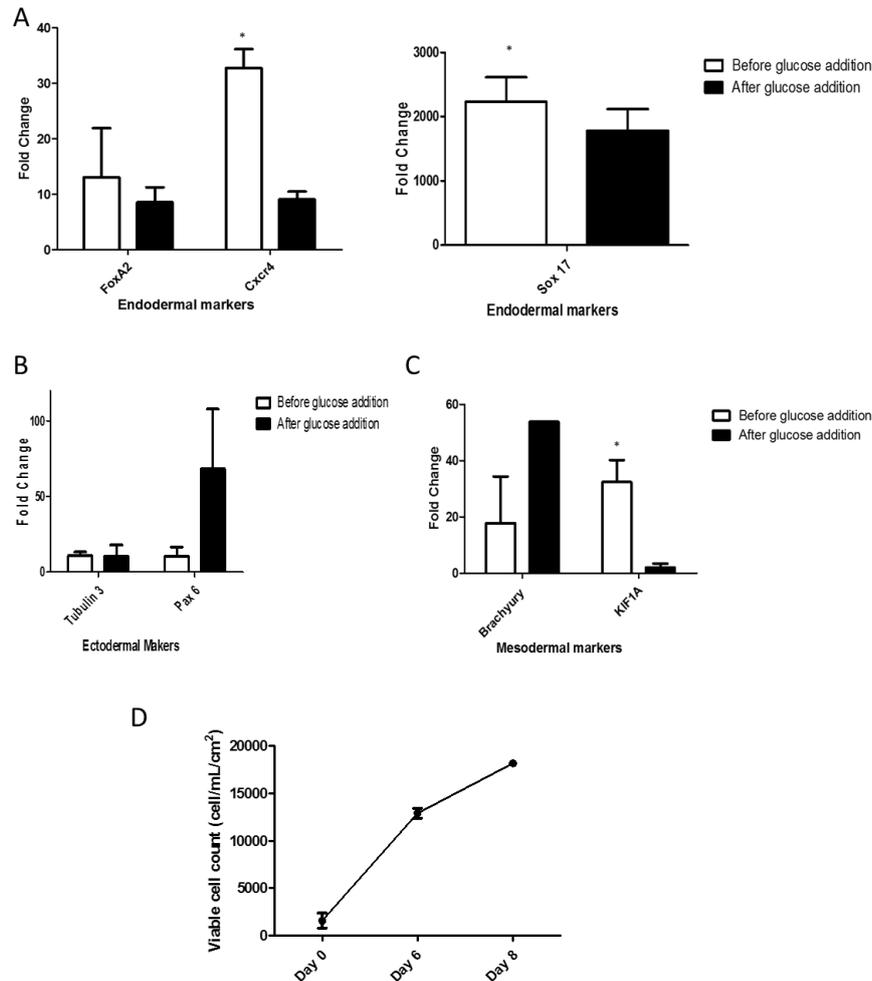


Figure 6.6 Adding glucose to the media retained the endodermal lineage expression of differentiated human episomal iPSCs (Cord blood). Real time PCR was performed in order to assess the fold gene expression of each germ layer: (A) Endoderm (Sox 17, FOXA2, CXCR4), (B) Ectoderm (Nestin, Tubulin 3, Pax6) and (C) mesoderm (Brachyury, KIF1A) for normal and deprived conditions. (D) cell viability was assessed by trypan blue exclusion method. Statistical analysis was performed using Two-Way Anova and data are reported as mean \pm S.E.M of n=3, asterisks indicate the statistical significance: *** P<0.001, **P<0.01, *P<0.05). Data are reported as mean \pm S.E.M of n=3, p<0.05 was considered as significant).

6.3.2 The role of glucose removal from media on human embryonic stem cells (hESCs)

In order to determine if the effect of glucose deprivation was cell line specific, hESCs (Shef 3) were differentiated in the normal and glucose deprived media for 4 days. Cells grown in normal media proliferated as expected whereas the cells in the glucose free media detached and underwent apoptosis (Figure 6.7 A). mRNA was extracted and RT-PCR was performed to characterise the cells based on expression of selected germ layer markers. Much like the glucose deprived hiPSCs cultures, hESCs expressed higher endodermal markers than the cells growing in normal media: Day 2 onwards Sox 17 gene expression increased 58.64-fold while differentiated cells grown normal condition expressed up to 3.017 fold change. Cxcr4 's expression was upregulated in the glucose deprivation condition. The expression levels of mesodermal (Brachyury) and ectodermal (Pax6) markers were increased in cells cultured in normal media (Figure 6.7 B). Hence, an enhancement of endodermal marker was observed upon the removal of glucose from the differentiated media.

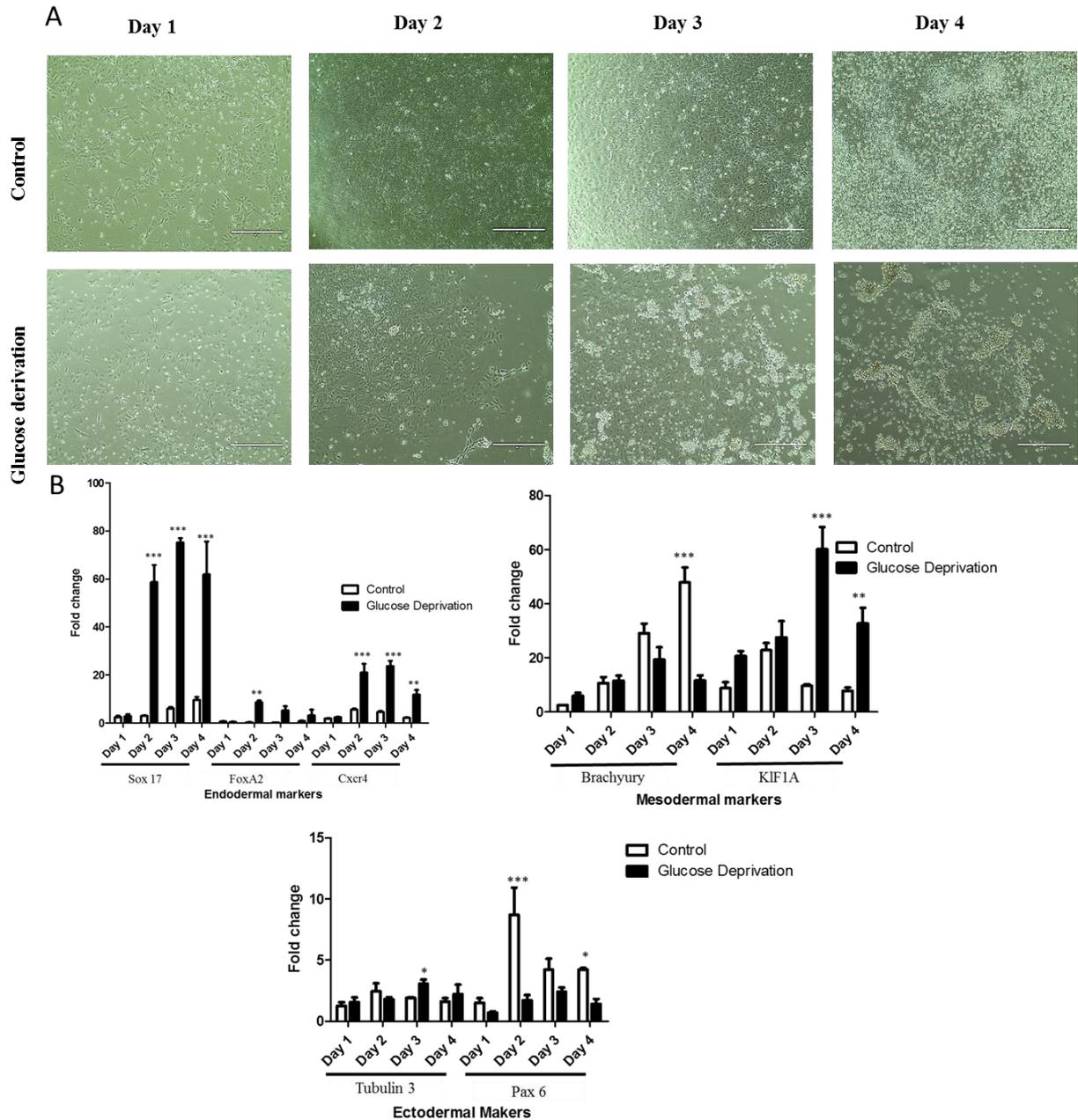


Figure 6.7 Glucose deprivation caused an increase in endodermal lineage expression of differentiated hESCs (Shef 3). (A) Representative images of the phase contrast of single cell differentiated hESCs at day 1, 2, 3 and 4 in normal and deprived conditions. Images were taken at 100X ocular lens magnification using Evos® XL. Scale bars=400µm. (B) Fold gene expression of each germ layer: Endoderm (Sox 17, FOXA2, CXCR4), Ectoderm (Nestin, Tubulin 3, Pax6) and mesoderm (Brachyury,

KIF1A) was performed by Real time PCR for normal and deprived conditions. Statistical analysis was performed using Two-Way Anova and data are reported as mean±S.E.M of n=3, asterisks indicate the statistical significance: *** P<0.001, **P<0.01, *P<0.05). Data are reported as mean±S.E.M of n=3, p<0.05 was considered as significant).

6.3.3 The effect of staurosporine on the germ layer formation.

Staurosporine, which is isolated from the bacterium *Streptomyces* (Omura et al., 1977), was used to induce apoptosis and to study its effect on germ layer formation. After 2 hours, the morphology of single hiPSCs treated with staurosporine changed, in the images shown in Figure 6.8(B), the cytoplasm of the cells was decreased and the cells had neuron-like phenotype. After adding back the glucose, the cells regained their normal morphology (Figure 6.8, C). Hence staurosporine treatment caused cell detachment and morphology change of the differentiated hiPSCs.

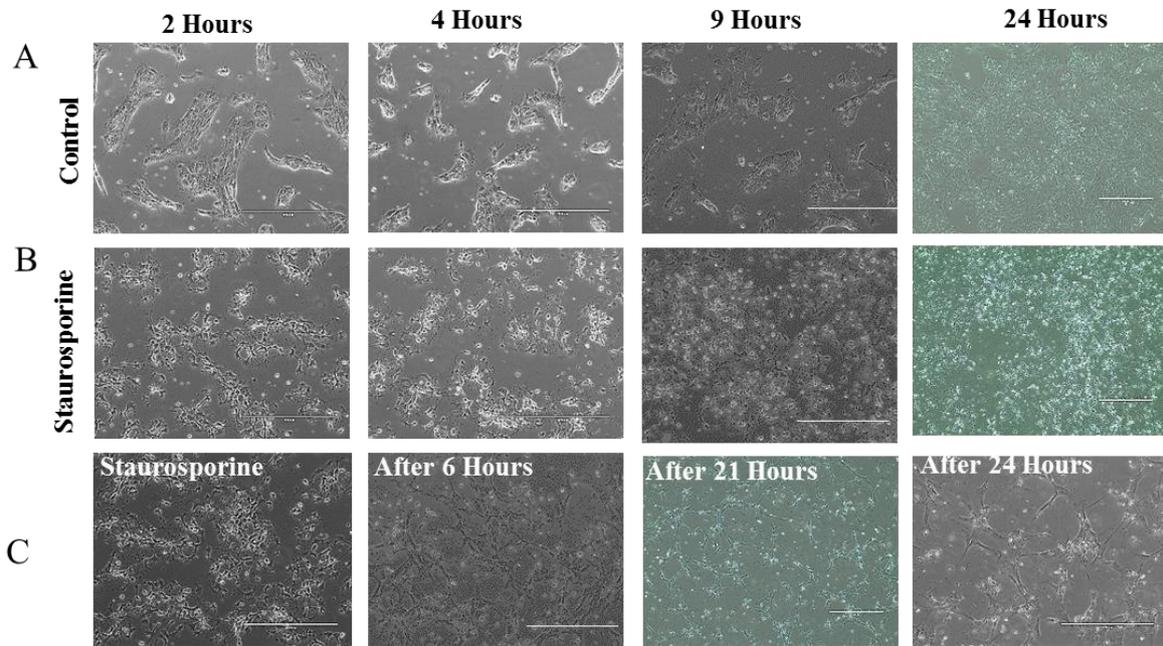


Figure 6.8 The effect of staurosporine treatment on the morphology of single cell hiP-SCs (cord blood). Images of the control (A) and treated (B) cells were taken after 2, 4, 9 and 24 hours. (C) Glucose was re-added after 3 hours of treatment. . Images were taken at 100X ocular magnification using Evos® XL. Scale bars=400µm.

The viable cell count for differentiated hiPSCs treated with staurosporine showed a decrease in cell viability as presented in Figure 6.9 (A). Cells that were incubated for 3 hours with staurosporine and then treated with normal media showed an increase in cell viability (Figure 6.9, B). Annexin V-PI staining showed that staurosporine induced apoptosis just after 2 hours of incubation (20.2% of cells were apoptotic) then there was an increase in apoptosis up to 72.14 % after 24 hours (Figure 6.9, C). Staurosporine was shown to induce apoptosis of human embryonic stem cells over the 24 hours of exposure (Hughes *et al.*, 2014).Staurosporine triggered apoptosis of the hiPSCs.

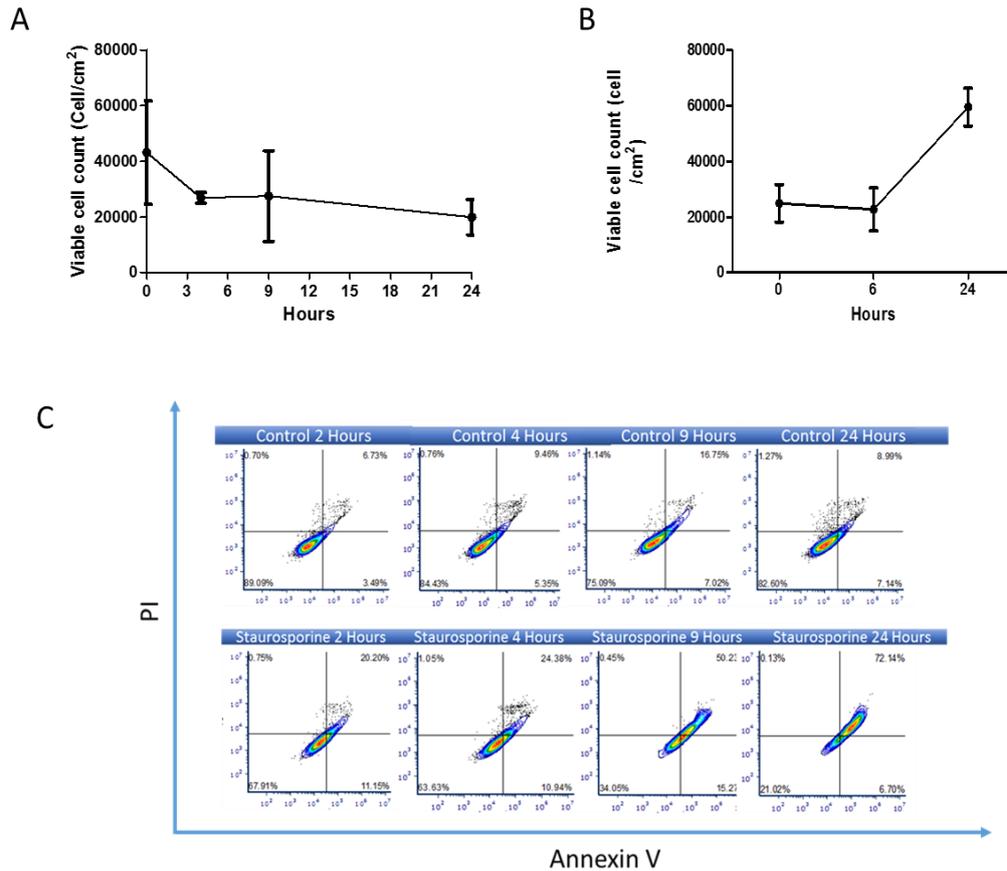


Figure 6.9 Staurosporine treatment triggered apoptosis of pre-differentiated human iPSCs. (A) Cell viability decreased when cells were treated with staurosporine. (B) The cell viability of pre-treated cells with staurosporine increased when switching to normal media. (C) After staurosporine treatment, cells were fixed and immunostained with FITC-conjugated annexin V antibody and PI and run on the flow cytometer. Cells negative for Annexin V and PI were considered viable cells whereas Annexin V-positive-PI-positive were considered late apoptotic, annexin V-negative-PI positive cells were necrotic and annexin V-positive-PI-positive cells were selected as early apoptotic. Statistical analysis was performed using Two-Way Anova and data are reported as mean±S.E.M of n=3, asterisks indicate the statistical significance: *** P<0.001, **P<0.01, *P<0.05). Data are reported as mean±S.E.M of n=3, p<0.05 was considered as significant).

To determine the effects of this treatment on germ layer differentiation, mRNA was collected at multiple time points (2, 4, 9 and 24 hours) and RT-PCR was performed to investigate which cell lineages were present. A significant increase of endodermal markers (Sox 17 and FoxA2) was seen upon at 24 hours. In addition there was a decrease in ectodermal makers (Nestin, Tublin3) and mesodermal markers (Brachyury, KIF1A) (Figure 6.10). Our results showed that staurosporine treatment selected the endodermal lineage formation of iPSCs (cord blood).

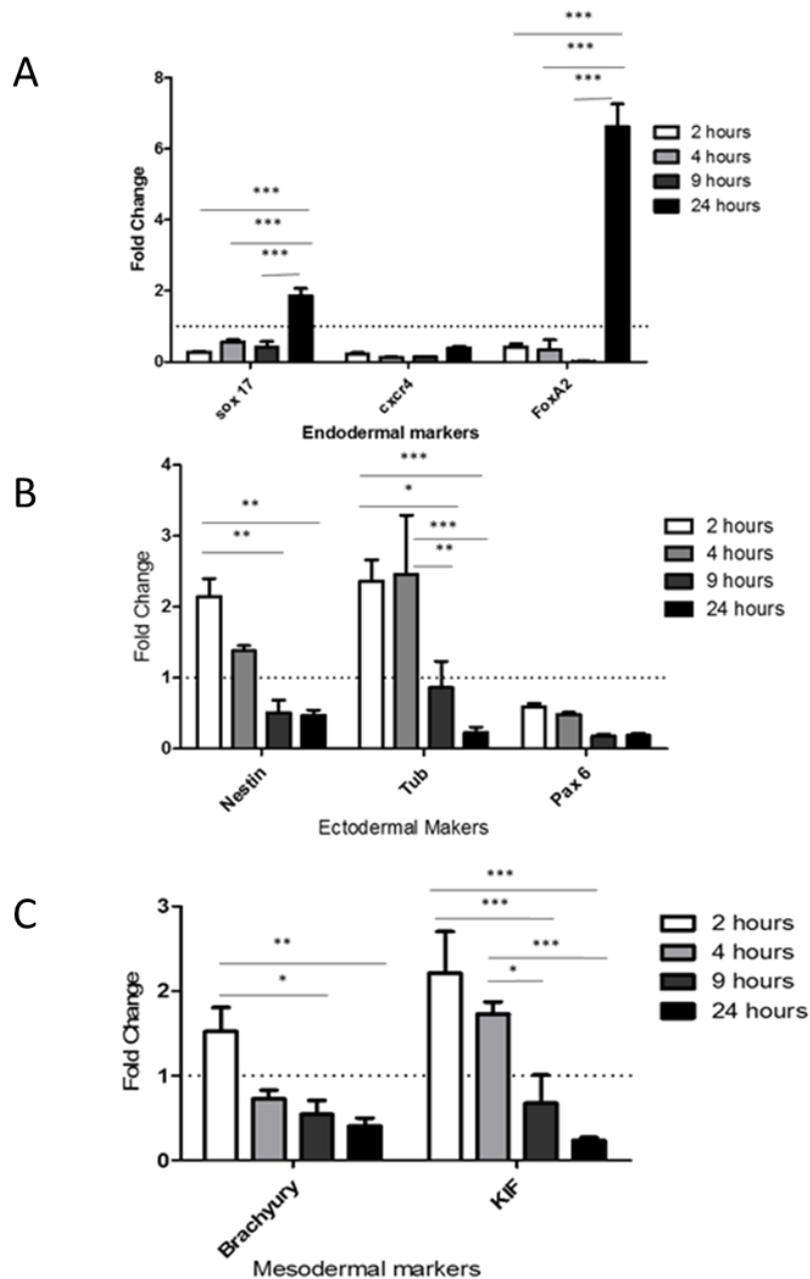


Figure 6.10 Staurosporine treatment enhanced endodermal lineage formation. Gene expression of each germ layer: (A) Endoderm (Sox 17, FOXA2, CXCR4), (B) Ectoderm (Nestin, Tubulin 3, Pax6) and (C) mesoderm (Brachyury, KIF1A) was assessed by real time PCR. Statistical analysis was performed using Two-Way Anova and data are reported as mean±S.E.M of n=3, asterisks indicate the statistical significance: *** P<0.001, **P<0.01, *P<0.05). Data are reported as mean±S.E.M of n=3, p<0.05 was considered as significant).

After 3 hours of staurosporine treatment, normal media was added to the differentiated hiPSCs and cells were collected after 6 and 24 hours. After 6 hours of recovery, the cells expressed higher endodermal markers in comparison with ectodermal and mesodermal markers (Figure 6.11). There was a significant increase in endodermal marker (FOXA2) and decrease in ectodermal (Nestin, Tubulin III) and mesodermal (Brachyury) marker. Cells retained their endodermal phenotype even after growing in normal media, RT-PCR results are shown in Figure 6.11.

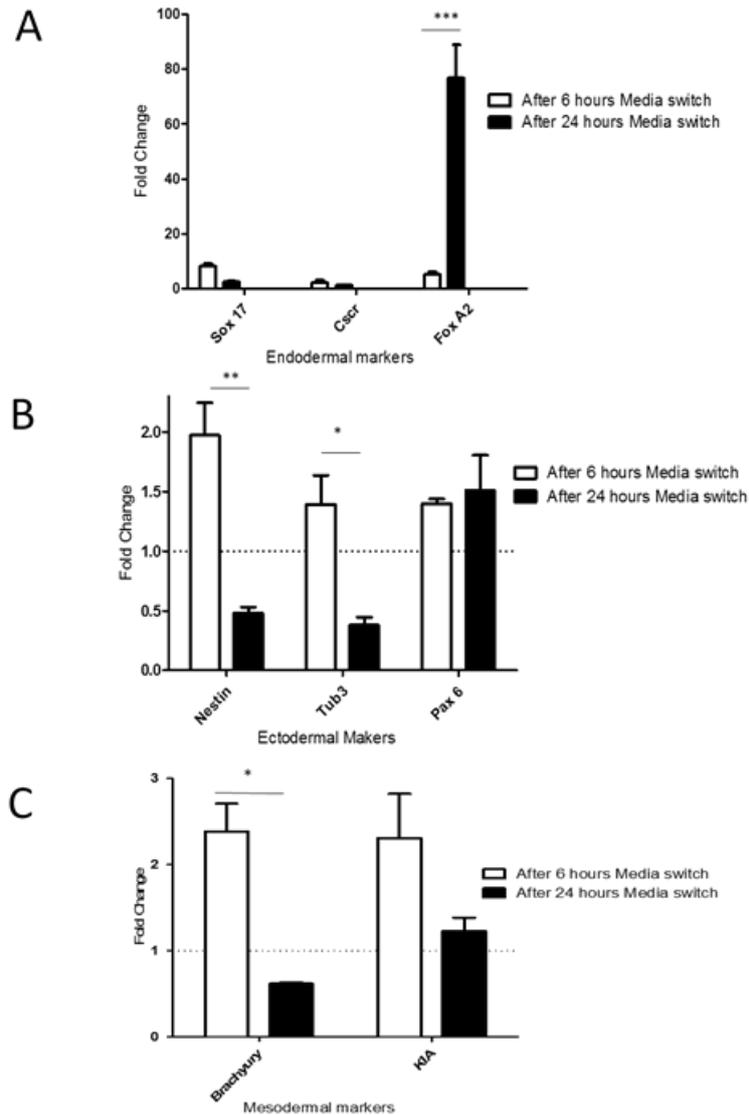


Figure 6.11 Endodermal phenotype was maintained after the addition of normal media on the staurosporine treated cells. Gene expression of each germ layer: (A) Endoderm (Sox 17, FOXA2, CXCR4), (B) Ectoderm (Nestin, Tubulin 3, Pax6) and (C) mesoderm (Brachyury, KIF1A) was assessed by real time PCR. Statistical analysis was performed using Two-Way Anova and data are reported as mean±S.E.M of n=3, asterisks indicate the statistical significance: *** P<0.001, **P<0.01, *P<0.05). Data are reported as mean±S.E.M of n=3, p<0.05 was considered as significant).

6.3.4 The effect of glucose deprivation on the activation of endodermal pathways.

The mammalian Target of Rapamycin (mTOR) is one of the serine/threonine kinases which is expressed in all eukaryotic cells. It is formed of two complex: mTOR complex 1 and 2, these two complexes have different sensitivities to rapamycin (Brown *et al.*, 1994; Kunz *et al.*, 1993; Sabers *et al.*, 1995; Sebatini *et al.*, 1994). It was reported that the inhibition of mTOR resulted in the downregulation of pluripotent genes and increased endodermal and mesodermal lineage gene expression (J. Zhou *et al.*, 2009). Thus, mTOR pathway was selected to investigate if lowering ATP/AMP levels through deprivation could activate the TSC-2 leading to mTOR inhibition. mTOR and p-mTOR expression levels steadily decreased over time in glucose deprived cells, the expression of these proteins was lower in the controls (Figure 6.12). There wasn't any change in the mTOR's upstream regulator, TSC2 and p-TSC2 expression in the differentiated cells cultured in normal media. TSC2 and p-TSC2 expression was higher in cells cultured in glucose-deprived media with an upregulation in the first 32 hours then a down-regulation. Both p53 and p-AMPK were downregulated within the first 40 hours of deprivation (Figure 6.12, A). The cells were collected within the first 48 hours due to rapid decrease in viable cell counts of the deprived conditions over time (Figure 4, B). Glucose deprivation activated mTOR pathway in time-dependant manner.

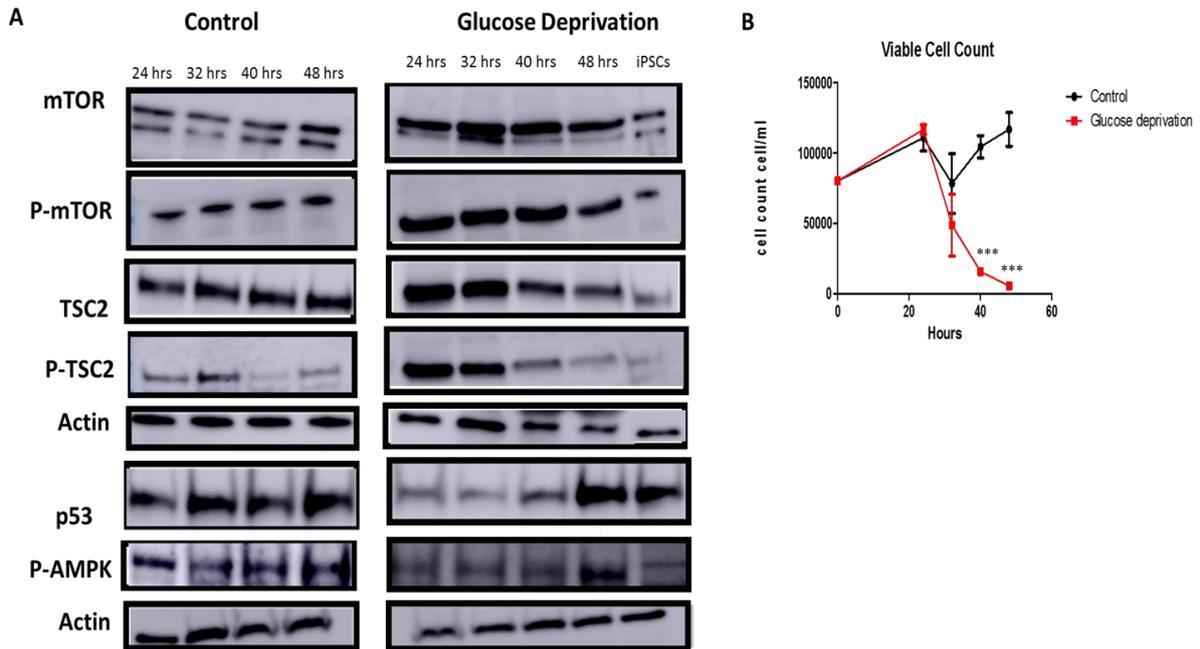


Figure 6.12 The effect of glucose deprivation on the endodermal signalling pathway. (A) Cells were collected at 24, 32, 40 and 48 hours. The protein expression of mTOR, p-mTOR, TSC2, p-TSC2, p53, p-AMPK and actin was assessed by western blotting. Each result shown is representative of three independent experiments. (B) Cells were collected at interval of time and viability was measure by trypan blue exclusion method (data are reported as mean±S.E.M of n=3, p<0.05 was considered as significant).

6.3.5 Direct endoderm differentiation

Various approaches were developed to direct the differentiation of hESCs into a definitive endoderm through the manipulation of different factors including Wnt3a, Activin A (D'Amour *et al.*, 2005; Kroon *et al.*, 2008; Touboul *et al.*, 2010; Schulz *et al.*, 2012; Brafman *et al.*, 2013). PSC Definitive endoderm induction kit was used (ThermoFisher Scientific) to compare the endodermal induction of glucose-deprived media approach with definitive endoderm kit. Upon culturing hiPSCs with Media A, the morphology of the cells changed into anterior primitive streak like cells, then after 2 days, definitive endoderm induction was established (Figure 6.13, A). Immunostaining shown in Figure 6.13 (B), revealed the differentiation of hiPSCs into the three germ layer after 2 days of incubation with media A and B. Thus the cells still expressed ectodermal (Nestin) and mesodermal markers (Brachyury). In comparison with our results (Figure 6.4), glucose deprived differentiated hiPSCs (Cord blood) expressed higher PDX1 and AFP. Whilst, gene expression of endodermal marker Sox17, cxcr4 and FoxA2 was upregulated up to 100,000, 200 and 600 fold change respectively upon the usage of the definitive endodermal kit as shown in Figure 6.12(C). Nevertheless, the differentiated cells still expressed mesodermal (Brachyury, KIF1A) and ectodermal (Nestin, Tubulin III and PAX6) genes confirming the immunostaining results. There was an upregulation of endodermal genes upon the differentiation of hiPSCs (Cord blood) using PSC Definitive endoderm induction kit.

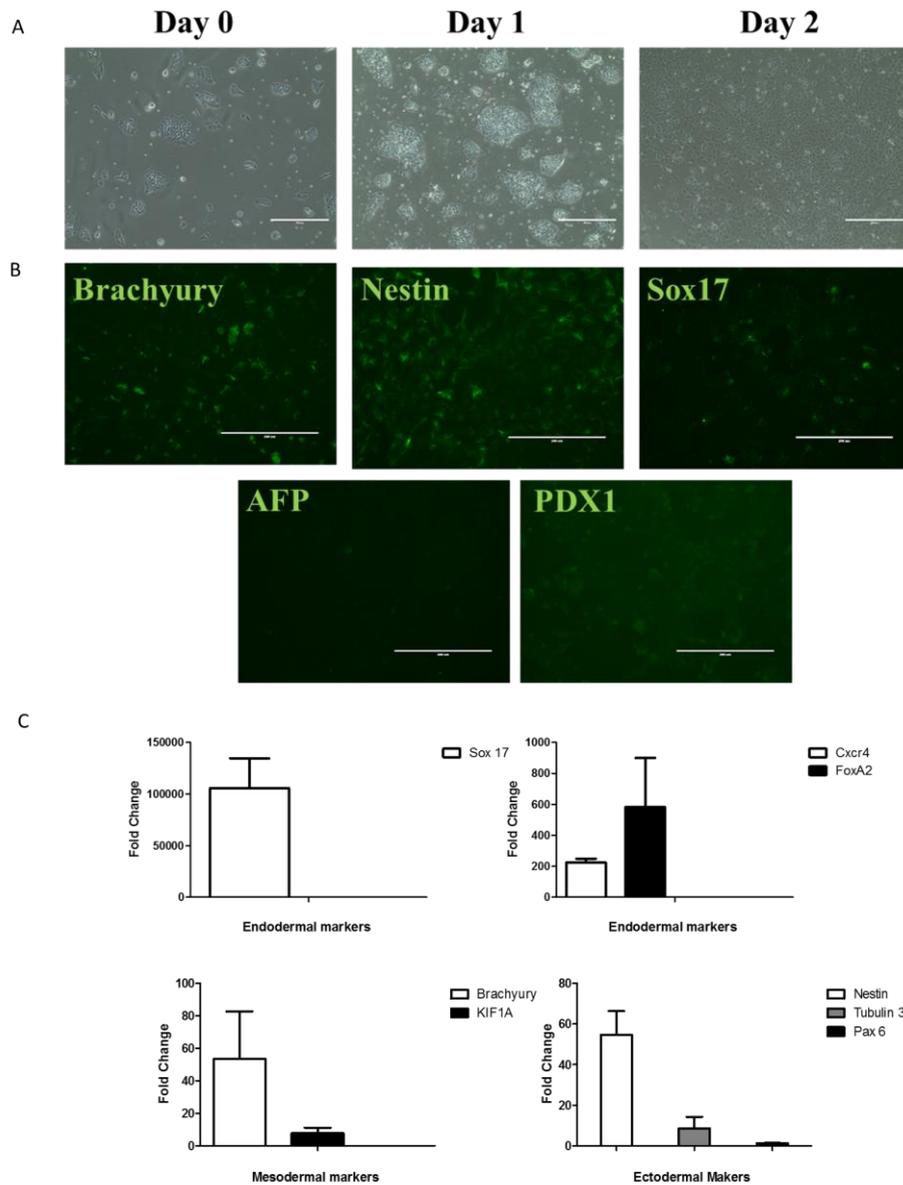


Figure 6.13 Direct differentiation of hiPSCs (Cord blood) using PSC Definitive endoderm induction kit.(A) Phase contrast of hiPSCs (Cord blood) using Evos® Fluorescence Microscope .Scale bars=400µm. (B) Immunostaining of differentiated cells at day 2. Scale bars=200µm. (C) Fold gene expression of each germ layer: Endoderm (Sox17, FOXA2, CXCR4), Ectoderm (Nestin, Tubulin 3, Pax6) and mesoderm (Brachyury, KIF1A) was performed by Real time PCR.

6.3.6 Discussion

Stem cells are at the forefront of cell and gene therapy and disease modelling for drug discovery (Singh *et al.*, 2005). Yet the differentiation process into specific germ layers is hindered by several bioprocessing challenges like the cost of the process... Thus, the importance of studying the effects of varying nutritional micro-environmental conditions during stem cell differentiation.

The effects of the key nutrient glucose on stem cells has not been studied in depth, however it has been shown that increasing glucose concentration enhanced the proliferation of mouse embryonic stem cells (Cho *et al.*, 2006), while it had no effect on human bone marrow-derived mesenchymal stem cells (MSCs) (Li *et al.*, 2007; Dhana-sekaran *et al.*, 2013). Interestingly, decreased glucose concentrations enhanced MSC proliferation and decreased apoptosis (Stolzing, Coleman and Scutt, 2006; Bastianelli *et al.*, 2014) and its purification (Tohyama *et al.*, 2013).

In this study, both hiPSCs and hESCs had the same response to the decrease of glucose concentration. Our data showed for the first time that glucose deprivation triggered a reaction in which the endodermal lineage was prevalent through apoptosis, suggesting a pre-selection of subpopulations. Similar findings were seen with the glucose deprivation of 3D MSCs. The maturation of MSCs was limited by glucose with limited cell survival suggesting that each population responded differently (Farrell *et al.*, 2015). Lineage commitment upon variation of glucose and glutamine concentrations through a metabolic switching mechanism was also observed in HSCs (Oburoglu *et al.*, 2014).

The response of glucose deprivation and enhancement of differentiation could be due to the changes in metabolism. It has been reported that the metabolism of pluripotent stem cells is different from the differentiated cells. Hence, understanding the shift in metabolism could lead to new approaches to enhance germ layer formation, directed differentiation of stem cells or even reprogramming into iPSCs (Folmes *et al.*, 2011). During early embryo development, high glycolytic rate leads to high lactate production and the oxidative phosphorylation is deactivated (Leese and Barton, 1984; Panteleon and Kaye, 1998) (Figure 6.14, A). Upon differentiation, the glycolytic flux decreases while oxidative phosphorylation increases leading to low lactate production due to high conversion of pyruvate to acetyl-coenzyme A (Cho *et al.*, 2006; Chung *et al.*, 2007; Facucho-Oliveira *et al.*, 2007) (Figure 6.14, B). Mitochondrial DNA and mitochondrial mass were found to be high upon the commitment of ESCs to a specific lineage (Cho *et al.*, 2006; Facucho-Oliveira *et al.*, 2007).

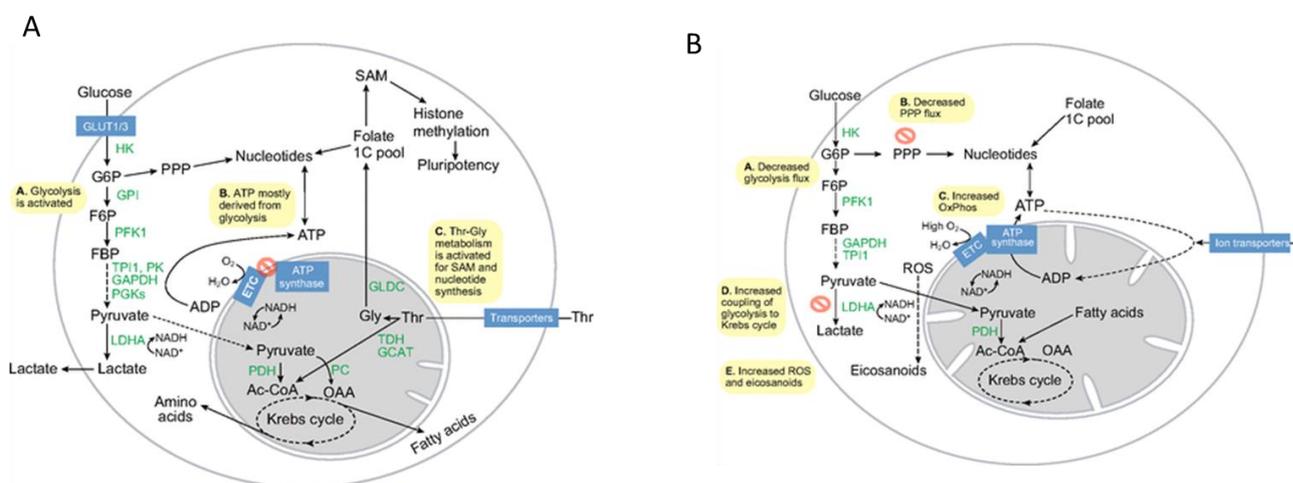


Figure 6.14 Metabolism in pluripotent (A) and differentiating (B) embryonic stem cells. Ac-CoA: acetyl coenzyme A; F6P: fructose-6-phosphate; FBP: fructose-1,6-bisphosphate; G6P: glucose-6-phosphate; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; GPI: glucose-6-phosphate isomerase; LDHA: lactate dehydrogenase A; OAA:

oxaloacetate; PC: pyruvate carboxylase; PDH: pyruvate dehydrogenase complex; PK: pyruvate kinase; TPI1: triosephosphate isomerase (Chang *et al.*, 2013).

To investigate the molecular pathway by which glucose deprivation promoted differentiation towards the endodermal lineage, western blot analysis was performed. According to Zhou *et al.* mTOR controls the pluripotency and differentiation of hESCs. The inhibition of mTOR resulted in the downregulation of pluripotent genes and increased endodermal and mesodermal lineage gene expression (J. Zhou *et al.*, 2009). Moreover, the activity of mTOR could be affected by the bioenergetic state of the cell, for instance, low-nutrient conditions triggered the downregulation of mTOR (Fingar and Blenis, 2004). This could be explained by the fact that AMP-activated protein kinase (AMPK) was activated once the ATP:ADP ratio decreases (Carling, 2004). Thus, the rise of AMPK activity could lead to the inhibition of mTOR through TSC2 (Inoki, Zhu and Guan, 2003; Hahn-Windgassen *et al.*, 2005). Once phosphorylated by Akt, TSC-2 inhibited the activity of mTOR (Bjornsti and Houghton, 2004).

Our results suggested that during the 40 hours in glucose deprived media a downregulation of P-AMPK and p53 along with the activation of TSC-2 and P-TSC-2 was detected. This suggests that the pathway by which glucose deprivation induced endodermal lineage formation could be time dependent. During the first 32 hours of deprivation, Akt inhibited TSC-2 through its phosphorylation, thus the inhibition of TSC-2 activated mTOR. Moreover, activated mTOR downregulated p53 (Figure 6.15, A). After 40 hours of glucose deprivation the inhibition of TSC-2 was decreased by the downregulation of its phosphorylation leading to a decrease in the expression of mTOR (Figure 6.15, B).

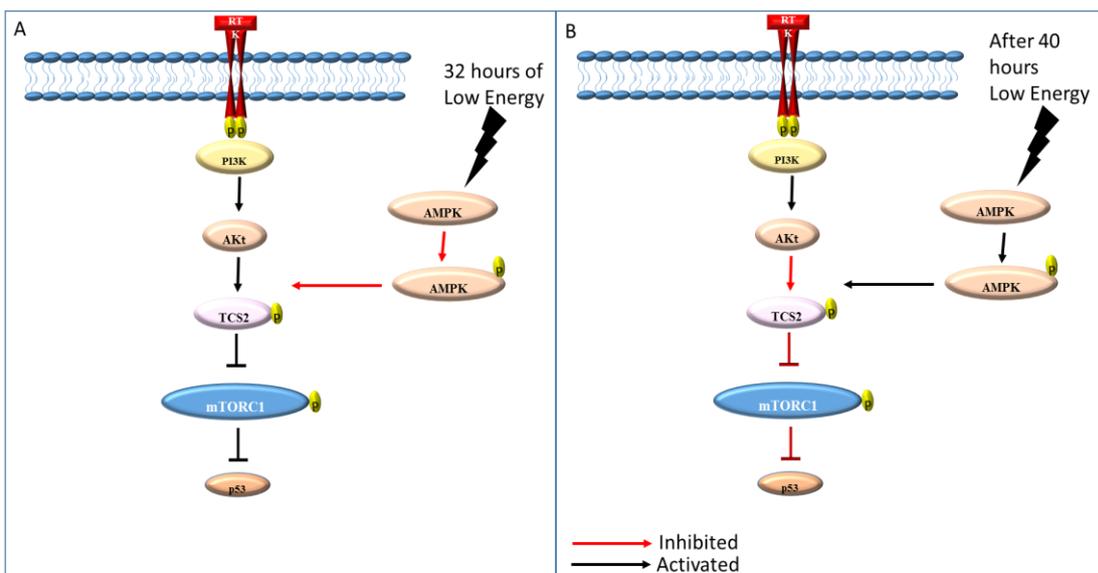


Figure 6.15 The effect of low energy on the activation of mTOR pathway. Once Akt is activated by PI3K, it will phosphorylate TSC-2 on Thr1462. The phosphorylation of TSC-2 will inhibit its downregulation of mTOR. Low energy level occurs when ATP/AMP level changes leading to the activation of AMPK. Once AMPK is phosphorylated it will activate TSC-2 leading to mTOR inhibition.

Alternatively, glucose deprivation triggered mitochondrial apoptosis of cardiac myocytes (Bialik *et al.*, 1999). Thus, hiPSCs were treated with staurosporine to study the effect of apoptosis on germ layer. Staurosporine triggered differentiation of neuronal precursors (Morioka *et al.*, 1985; Hashimoto and Hagino, 1989; Shea and Beermann, 1991; Lombet *et al.*, 2001), whereas it inhibited the formation of neurectoderm of differentiated early primitive ectoderm by gastrulation induction (Hughes *et al.*, 2014). Whilst it induced ectodermal derivatives in murine ESCs (Schumacher *et al.*, 2003), staurosporine was found to induce mesendoderm formation of human embryonic stem cells by regulating the cell adherens junctions' integrity (Hughes *et al.*, 2014).

These findings could present a potential and novel approach for efficient directed endodermal differentiation of pluripotent stem cells into pancreatic or hepatic cells through the manipulation of media components whilst simultaneously limiting the usage of growth factors.

7 Chapter 7: Conclusion and future work

7.1 Key Findings

Despite the rapid growth of phase I/II clinical trials of pluripotent stem cells (Shizuru *et al.*, 1996; Keller, 2005; Dimos *et al.*, 2008; Stover and Schwartz, 2011; Schwartz *et al.*, 2012; Rennie *et al.*, 2013; Shroff, Gupta and Barthakur, 2014), many bioprocessing challenges must be solved to enable its wider applications in cell therapy. The development of the stem cell field is hindered by challenges in various aspects due to a lack of fundamental biological understanding.

Although the discovery of hiPSCs overcame ethical concerns regarding hESCs and favoured the development of autologous cell therapy (Meyer, 2008). Recent approach led by Nobel winner Yamanaka at Kyoto University was to develop a universal hiPSCs-derived cell line for cell therapy. Generating a universal iPSCs could help overcoming the cost of autologous and scale up to lower the cost of manufacturing. Currently they were able to generate an iPSCs which is compatible to 20 % of the Japanese population (Okita *et al.*, 2011). iPSCs has been used for the treatment of various diseases like parkinson's, spinal cord injuries and amyotrophic lateral sclerosis (Dimos *et al.*, 2008; Wernig *et al.*, 2008; Tsuji *et al.*, 2010; Kriks *et al.*, 2011; Nori *et al.*, 2011) and disease model to understand Alzheimer's, schizophrenia, Parkinson's diseases (Hanna *et al.*, 2007; Brennand *et al.*, 2011; Devine *et al.*, 2011; Yagi *et al.*, 2011; Yahata *et al.*, 2011).

Differentiation protocols mainly depend on growth factors and extracellular matrix to direct the differentiation. It was reported that extracellular matrix favoured the commitment of the cells into one lineage upon differentiation. For example, extracellular matrix rich in collagen IV and fibronectin enhanced endothelial differentiation (Wijelath *et*

al., 2004; Yamashita *et al.*, 2000). In addition, a combinatorial extracellular matrix such as collagen IV+heparan sulfate+laminin or gelatin enhanced the differentiation of mESCs into endothelial cells (Hou *et al.*, 2017).

Few studies investigated the effect of mimicking the physiological factors levels during differentiation. Thus, the aim of this thesis was to investigate the effect of three major environmental factors: glucose, pyruvate and oxygen on germ layer formation.

Initial results showed that lowering oxygen levels had maintained the pluripotency of the feeder-dependent and feeder-free hESCs (Shef 3) and hiPSCs (MSU001 and cord blood) except for feeder-free hiPSCs (BJ). This showed that feeders may have a particular supportive role in pluripotency maintenance at low oxygen levels by providing extracellular matrix or secreting growth factors that help in pluripotency maintenance.

DoE is a powerful screening tool for investigating how multiple cues work in concert to drive differentiation. Using design of experiments (DoE) approach, a predictable model was generated to determine the best condition for each germ layer. Deprivation of both oxygen and glucose enhanced ectodermal and mesodermal lineages, whereas only glucose deprivation enhanced endodermal lineage of hiPSCs (cord blood). DoE generated the best nutritional environment for each of the germ layers. Thus, these new favoured conditions could be used to generate a new protocol for endodermal, mesodermal and ectodermal differentiation.

Studies have shown that stimulating certain metabolic pathways by limiting nutrient supply can lead to efficient directed differentiation. Culturing human hematopoietic stem cells (HSCs) in low glutamine and glucose stimulated their differentiation into erythroid lineage (Oburoglu *et al.*, 2014). A change in nutrient demand via activating or inhibiting metabolic enzyme can trigger cell differentiation into a desired lineage

(Bracha *et al.*, 2010). In comparison to the traditional strategy of adding multiple expensive recombinant proteins to control cell signalling (Cheng *et al.*, 2012; Sneddon, Borowiak and Melton, 2012) controlling nutrient supply is a less costly approach. Thus similar approach was done in this thesis, an enhancement of endodermal gene expression was detected when hiPSCs (Cord blood) and hESCs (Shef 3) were differentiated when glucose was depleted. This mechanism was through germ layer selection of endodermal cells in the presence of a severe apoptosis-inducing environment. Further investigation on the effect of apoptosis on germ layer selection was studied. Staurosporine was shown to enhance the mesendoderm and mesoderm population in differentiated mouse primitive ectoderm-like (Hughes *et al.*, 2014). The addition of normal media to the deprived cells helped in cell recovery while maintaining the endodermal phenotype. Thus this could be used for definitive endodermal differentiation protocol. Treatment of differentiated hiPSCs with the apoptotic reagent staurosporine favoured the endodermal lineage.

7.2 Future Work

Depriving the cells from glucose was a key finding for endodermal lineage formation especially pancreatic precursor as PDX1 was upregulated (Fagerberg *et al.*, 2014). Furthermore, endodermal precursors especially pancreatic have been studied intensively due to their wide treatment application. One of the main disease that affects nearly 35000 cases a year in USA is type 1 diabetes (Hering B, 1999). Although transplantation has been used for many years, the main obstacle is the lack of suitable compatible pancreatic tissues (Shapiro *et al.*, 2000). Moreover, transplantation of pan-

creatic islet cells is considered as a promising treatment of diabetes, and several studies have demonstrated the formation of hepatic cells that expressed insulin from stem cells (Abe *et al.*, 1996; Ramiya *et al.*, 2000; Hamazaki *et al.*, 2001; Lumelsky *et al.*, 2001; Hori *et al.*, 2002; Jones *et al.*, 2002; Yamada, Yoshikawa, Kanda, *et al.*, 2002; Yamada, Yoshikawa, Takaki, *et al.*, 2002; Atsushi Kubo, Katsunori Shinozaki, John M. Shannon, Valerie Kouskoff, Marion Kennedy, Savio Woo, Hans Joerg Fehling, 2004), human embryonic stem cells (Assady *et al.*, 2001; Hamazaki *et al.*, 2001; D'Amour *et al.*, 2005; Touboul *et al.*, 2010) and iPSCs (Loh *et al.*, 2014; Rajaei *et al.*, 2017). Nevertheless, the directed pancreatic differentiation using growth factor is costly and multi-step protocol (D'Amour *et al.*, 2005; Touboul *et al.*, 2010; Bernardo *et al.*, 2011). Many suggestions for future lines of investigation arise from this work. Thus, to address the effect of glucose deprivation on endodermal lineage formation, glucose-free media could be applied in the media used for direct pancreatic or any endodermal lineage formation.

There are no *in vivo* measurement of the nutrients levels to which the ICM and epiblast tissues are exposed to due to invasive methods and sample size. Thus, a mass transfer model could be studied to detect the exact concentration of nutrients and the substrate uptake.

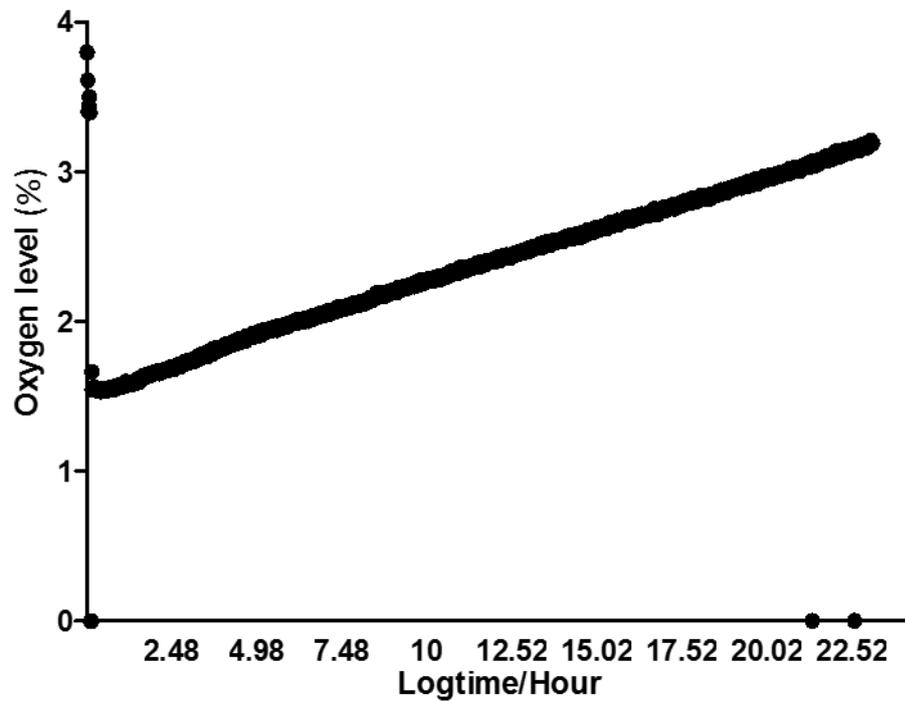
Only three key factors were studied in this thesis, nevertheless, many other factors such as extra-cellular matrix type and stiffness, lipids and proteins may have an effect on germ layer optimisation. Moreover the model created by DoE needs more experiments to investigate certain combinations in more detail or adding new factors to the model. To validate the results obtained, new iPSCs cell lines could be used to confirm the effect of certain combinations on each of the germ layer to build a robust predicted model.

7.3 Final Thesis Conclusion

Directed differentiation of pluripotent stem cells is hindered by many challenges.

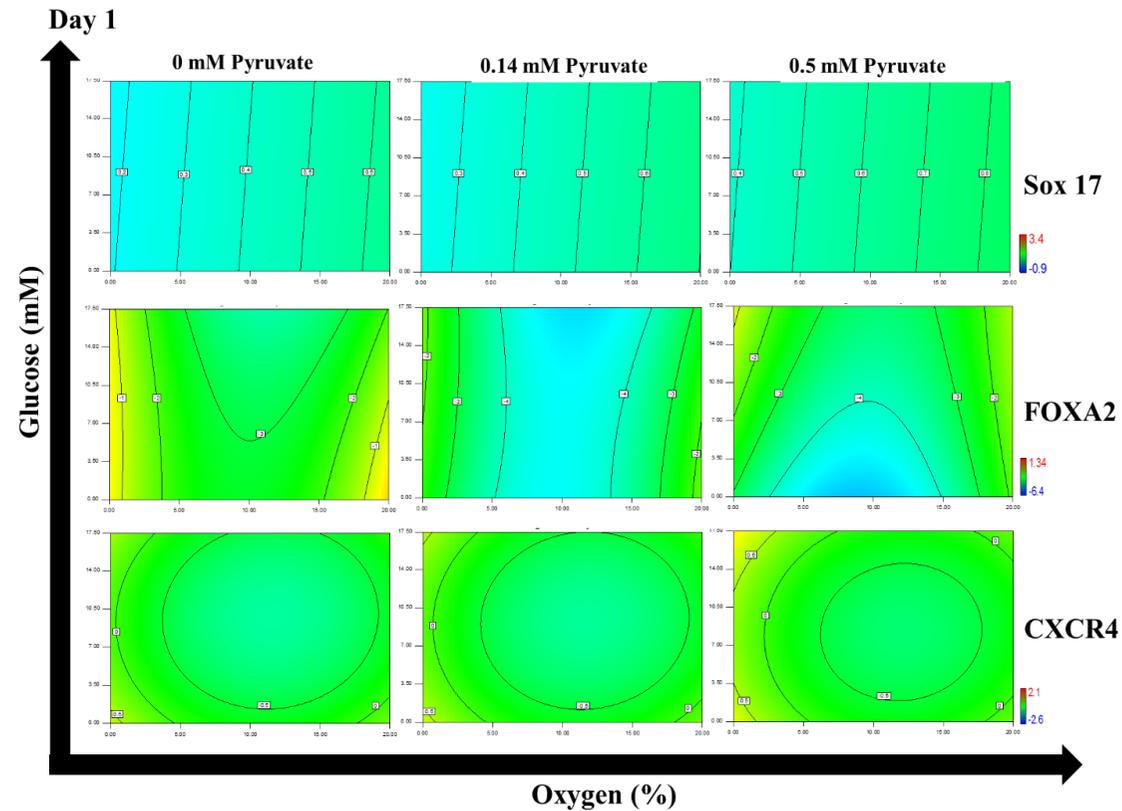
This thesis has shown that the manipulation of key factors in the nutritional environment could improve germ layer formation. Changing or depriving one of the three factors (oxygen, glucose and pyruvate) in the differentiation protocol could help in understanding the effect of the presence or absence of some nutritional factors during early embryo development.

Appendix I

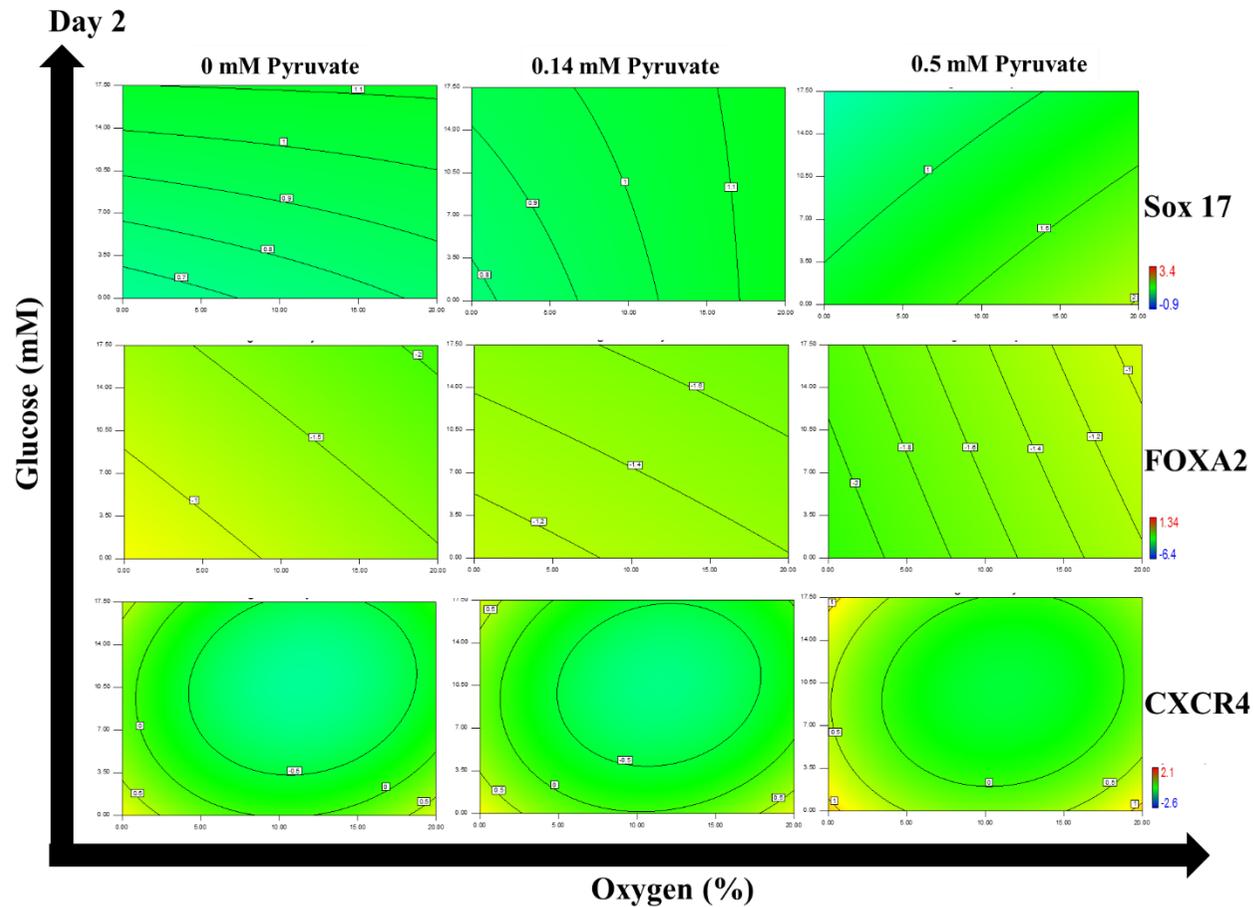


Appendix I. Figure 1 The measurement of oxygen levels (%) inside the incubator using PreSens. Sensors were attached to the bottom of the 12 well plate and the oxygen was monitored for 22-24 hours .

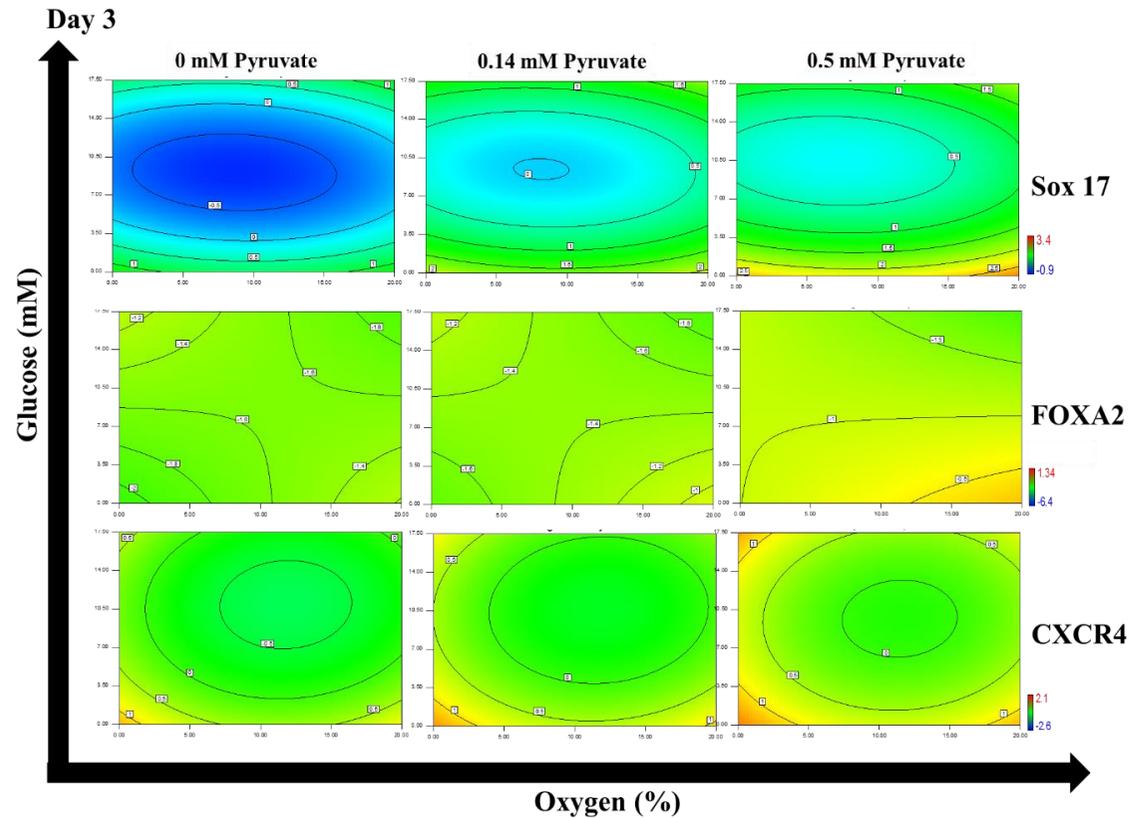
Appendix II



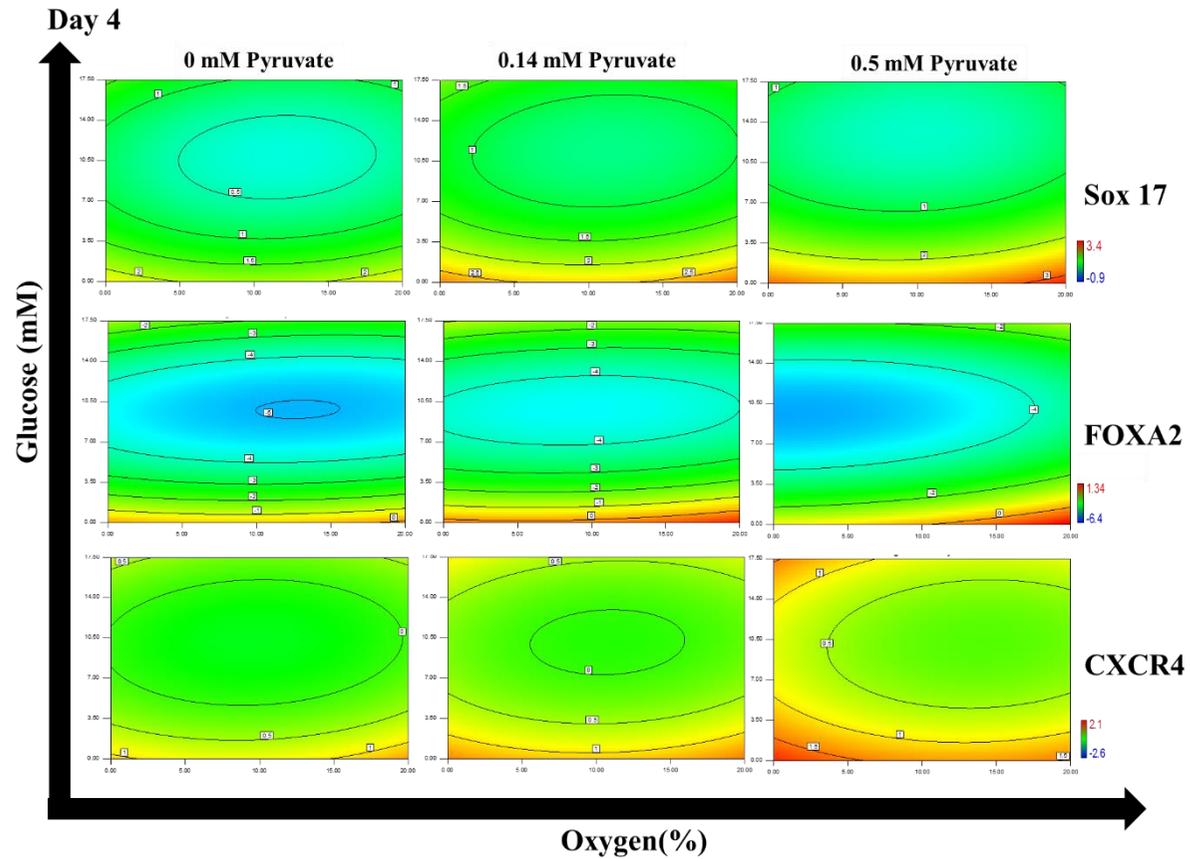
Appendix II Figure 1 Contour maps of a quadratic and 2 FI mathematical model generated for endodermal gene expression at day 1. Differentiated hiPSCs were cultured in 27 different conditions for four days. Gene expression was assessed by RT-PCR. Colours ranging from dark blue to dark red represent the lowest and highest log value for endodermal gene expression of Sox17, FOXA2 and CXCR4.



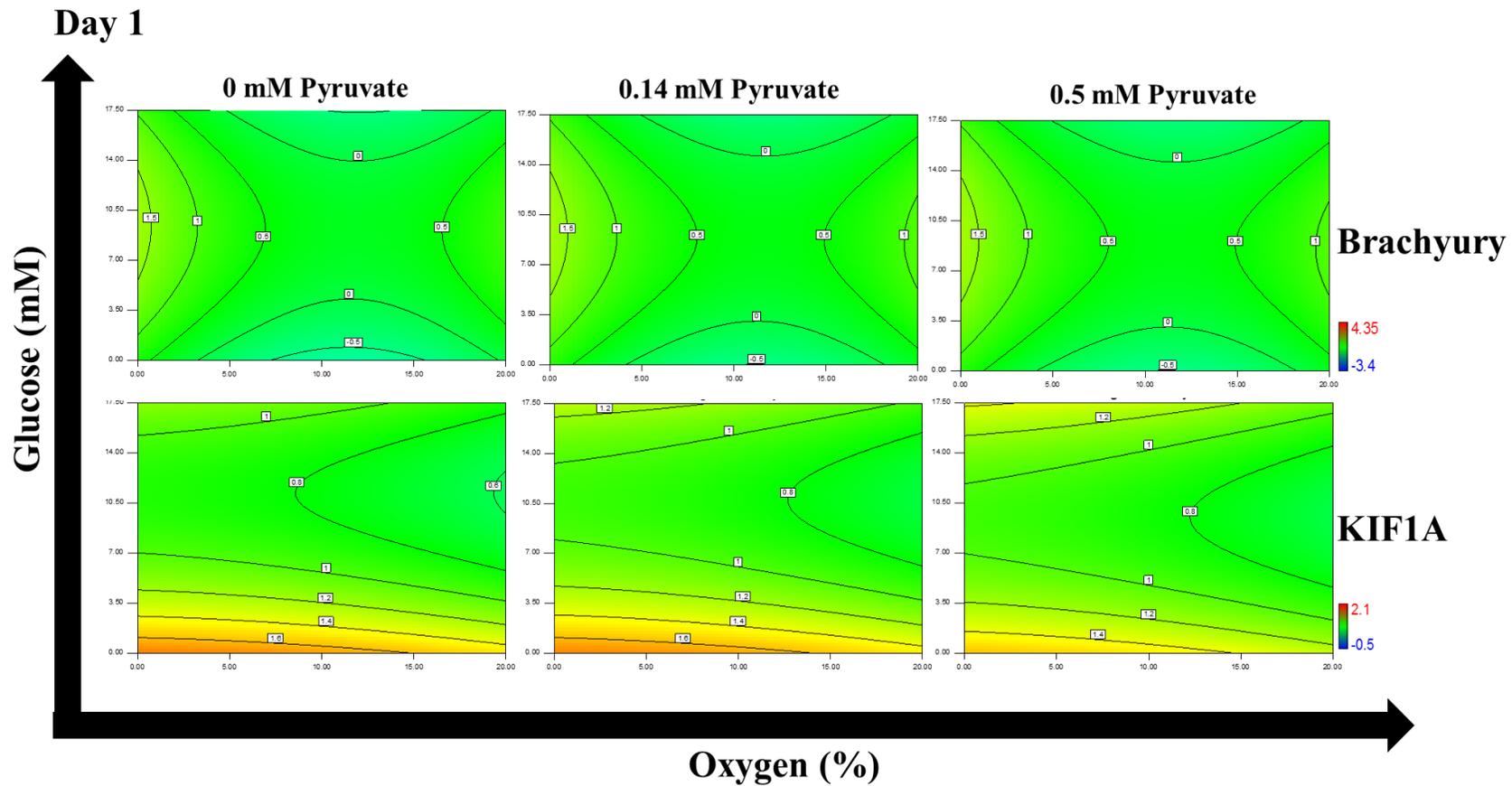
Appendix II Figure 2 Contour maps of a quadratic and 2 FI mathematical model generated for endodermal gene expression at day 2. Differentiated hiPSCs were cultured in 27 different conditions for four days. Gene expression was assessed by RT-PCR. Colours ranging from dark blue to dark red represent the lowest and highest log value for endodermal gene expression of Sox17, FOXA2 and CXCR4.



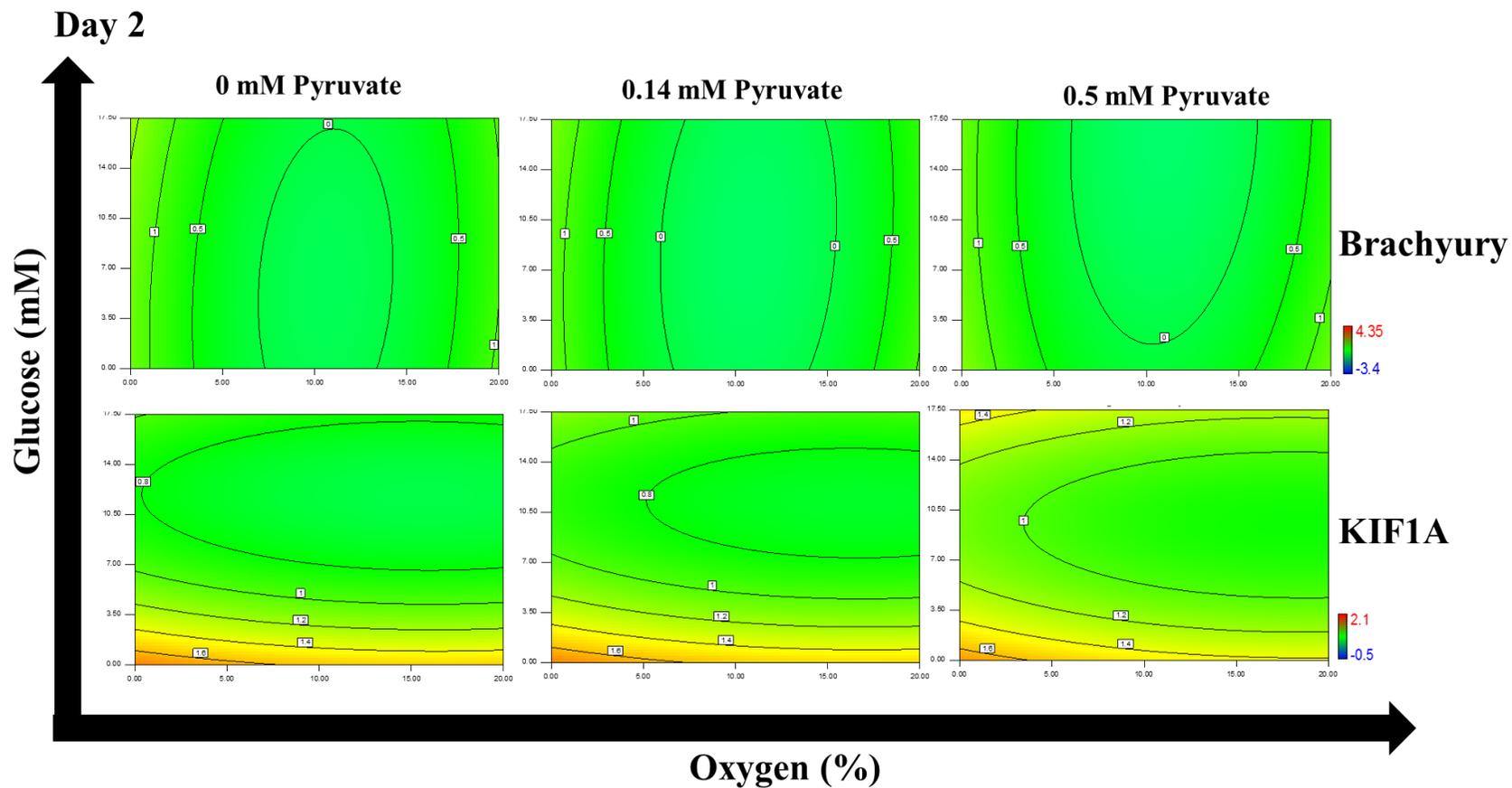
Appendix II Figure 3 Contour maps of a quadratic and 2 FI mathematical model generated for endodermal gene expression at day 3. Differentiated hiPSCs were cultured in 27 different conditions for four days. Gene expression was assessed by RT-PCR. Colours ranging from dark blue to dark red represent the lowest and highest log value for endodermal gene expression of Sox17, FOXA2 and CXCR4.



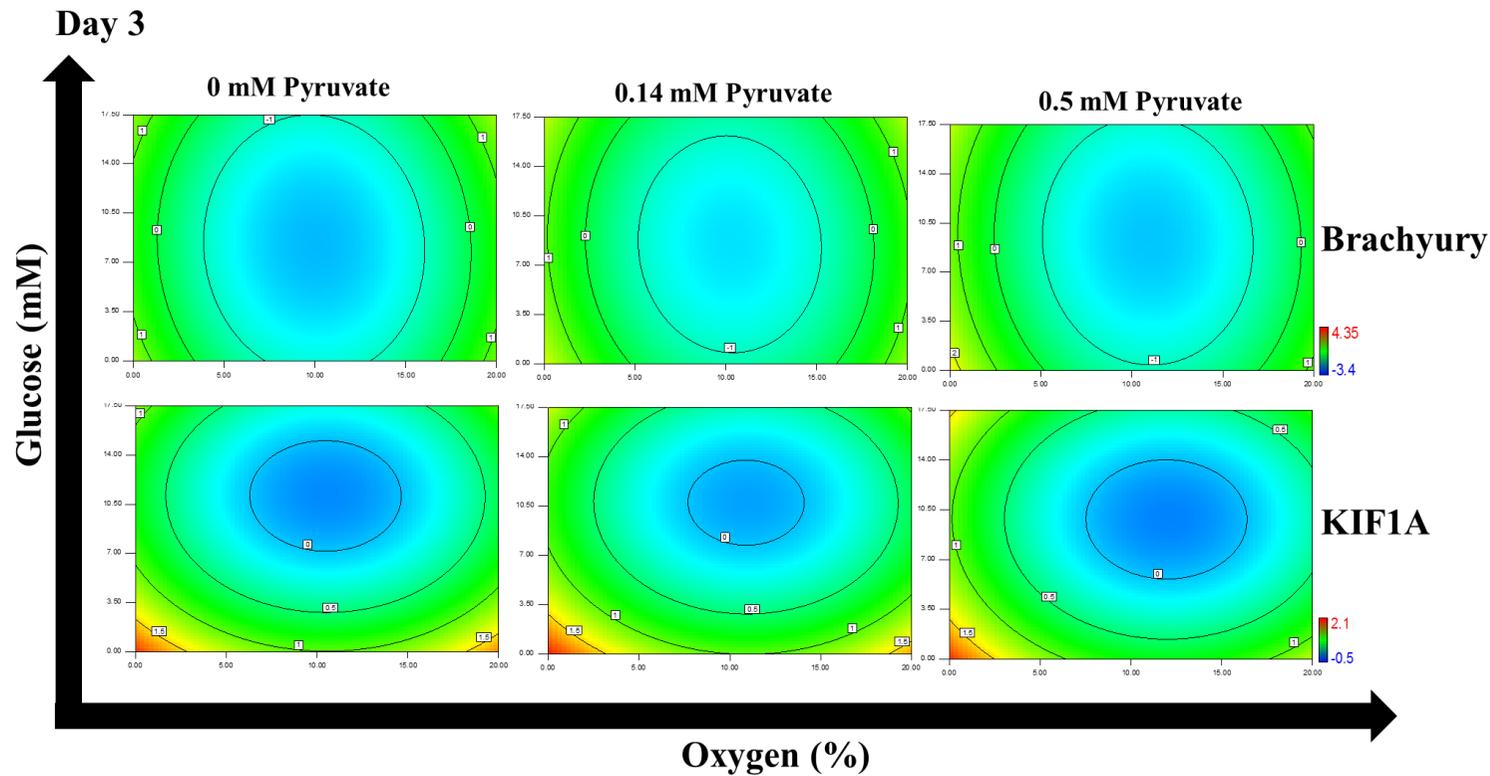
Appendix II Figure 4 Contour maps of a quadratic and mathematical model generated for endodermal gene expression at day 4. Differentiated hiPSCs were cultured in 27 different conditions for four days. Gene expression was assessed by RT-PCR. Colours ranging from dark blue to dark red represent the lowest and highest log value for endodermal gene expression of Sox17, FOXA2 and CXCR4.



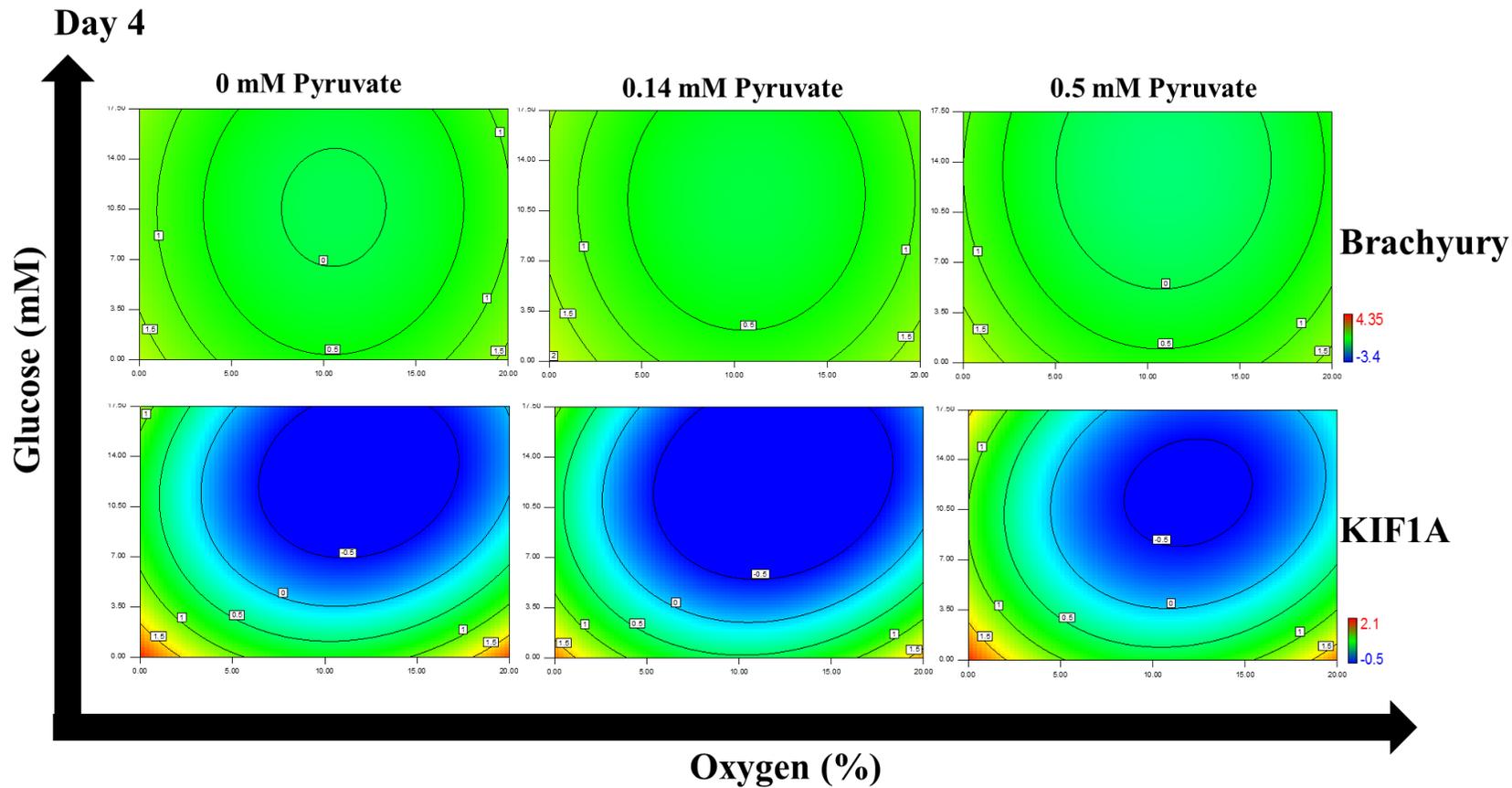
Appendix II Figure 5 Contour plots of the information function of quadratic mathematical model generated for mesoderm gene expression at day 1. Differentiated hiPSCs were cultured in 27 different conditions for four days. Gene expression was assessed by RT-PCR. Colours ranging from dark blue to dark red represent the lowest and highest log value for mesodermal gene expression of Brachyury and KIF1A.



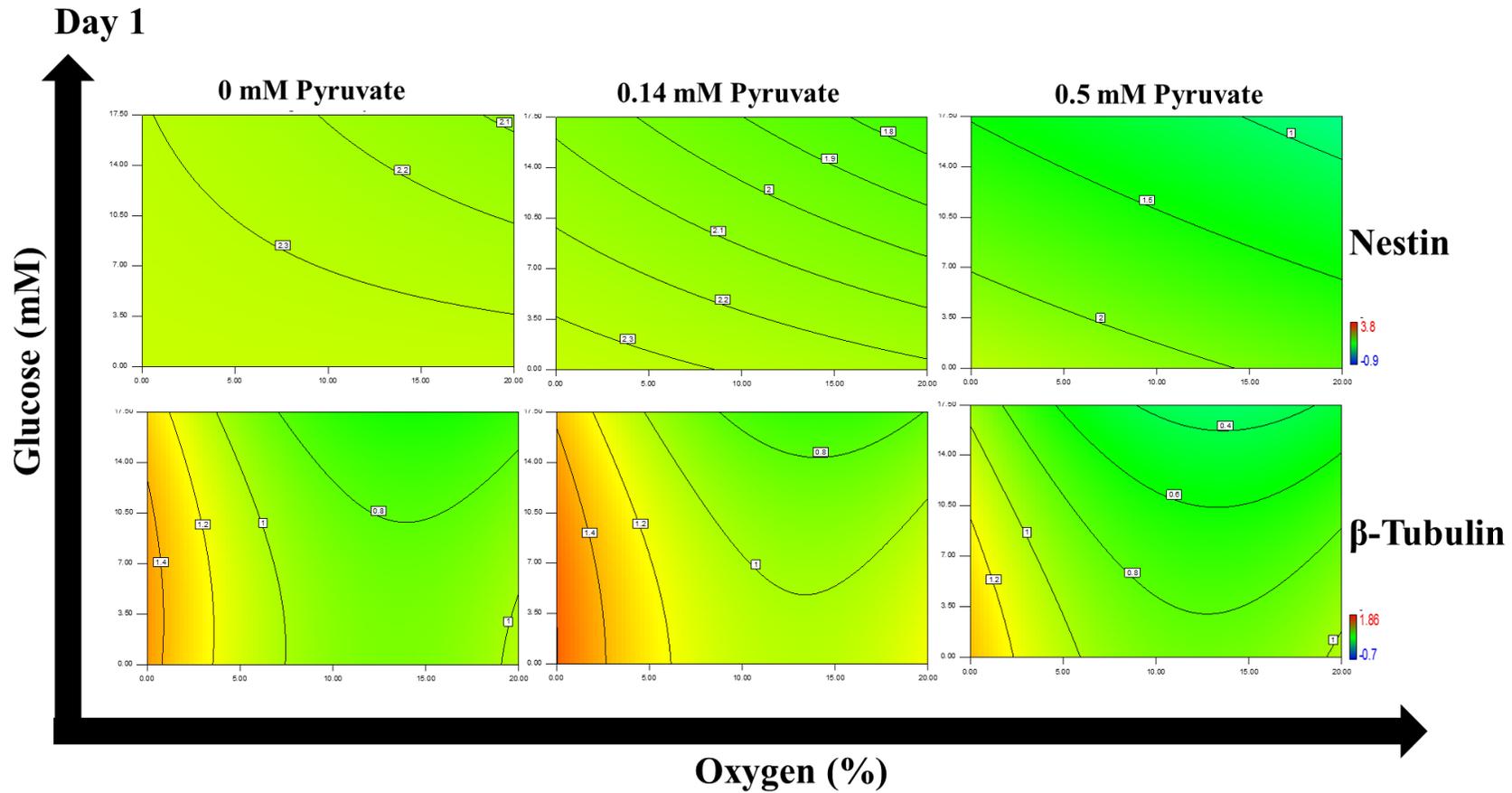
Appendix II Figure 6 Contour plots of the information function of quadratic mathematical model generated for mesoderm gene expression at day 2. Differentiated hiPSCs were cultured in 27 different conditions for four days. Gene expression was assessed by RT-PCR. Colours ranging from dark blue to dark red represent the lowest and highest log value for mesodermal gene expression of Brachyury and KIF1A.



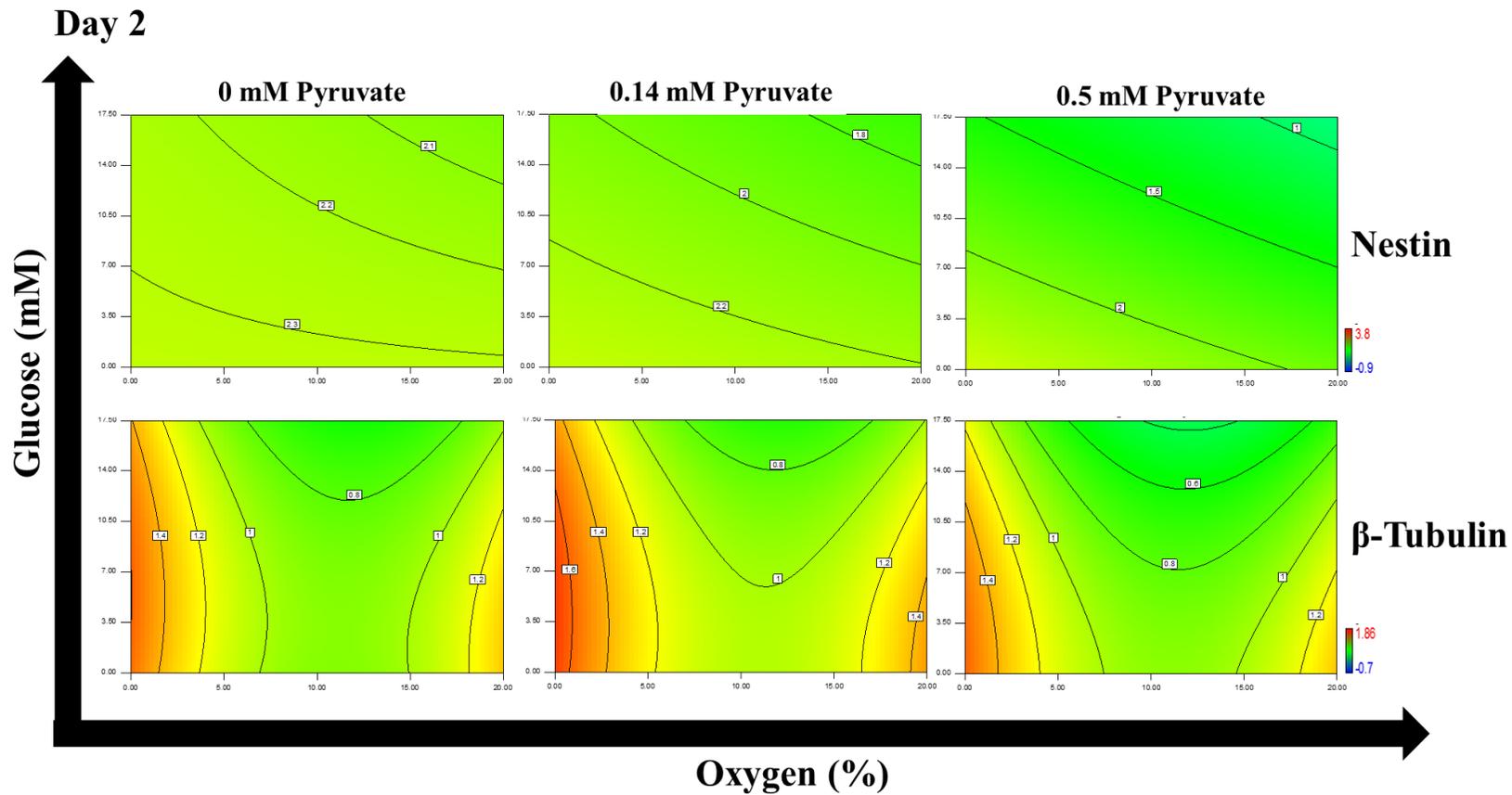
Appendix II Figure 7 Contour plots of the information function of quadratic mathematical model generated for mesoderm gene expression at day 3. Differentiated hiPSCs were cultured in 27 different conditions for four days. Gene expression was assessed by RT-PCR. Colours ranging from dark blue to dark red represent the lowest and highest log value for mesodermal gene expression of Brachyury and KIF1A.



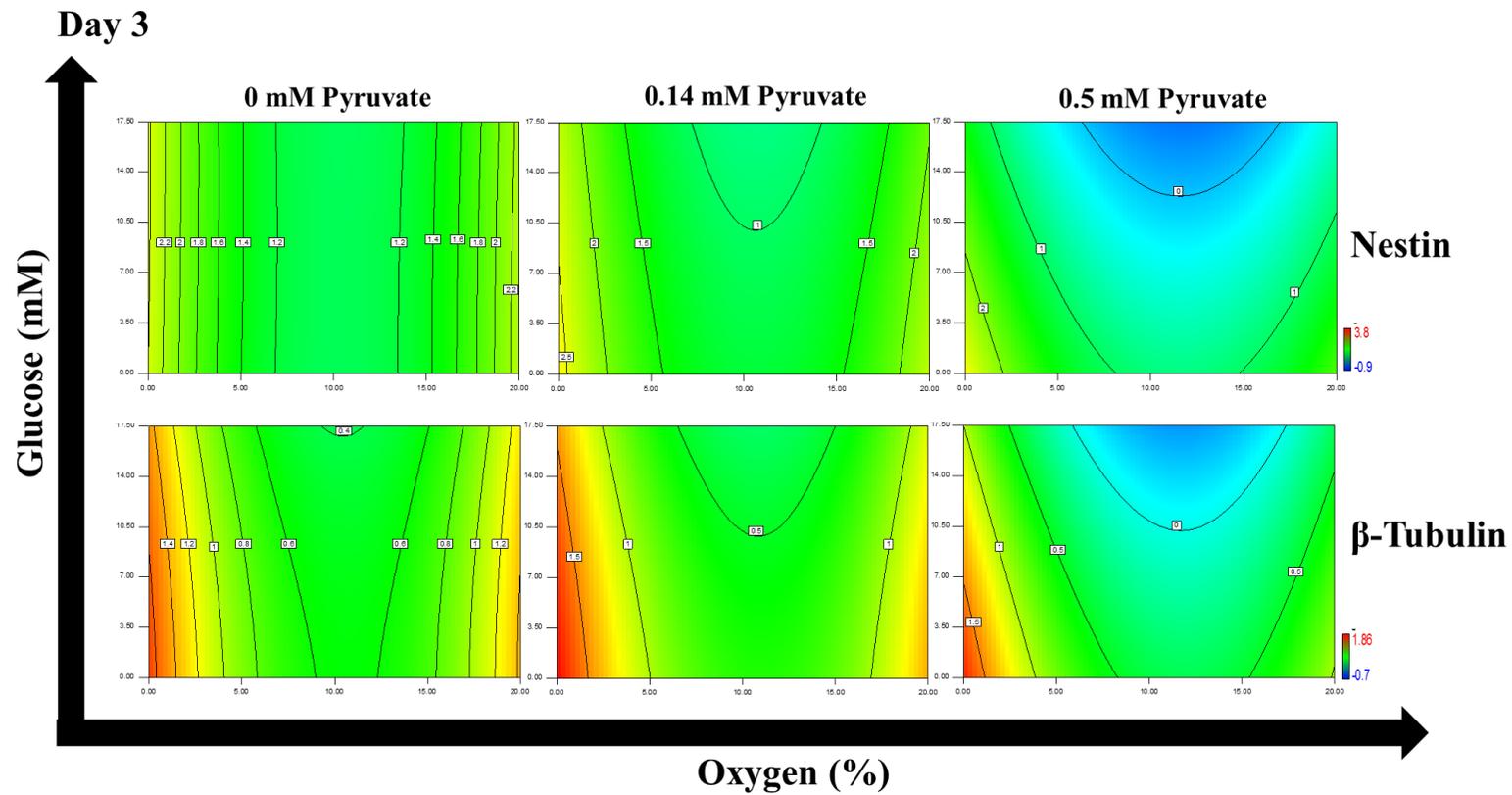
Appendix II Figure 8 Contour plots of the information function of quadratic mathematical model generated for mesoderm gene expression at day 4. Differentiated hiPSCs were cultured in 27 different conditions for four days. Gene expression was assessed by RT-PCR. Colours ranging from dark blue to dark red represent the lowest and highest log value for mesodermal gene expression of Brachyury and KIF1A.



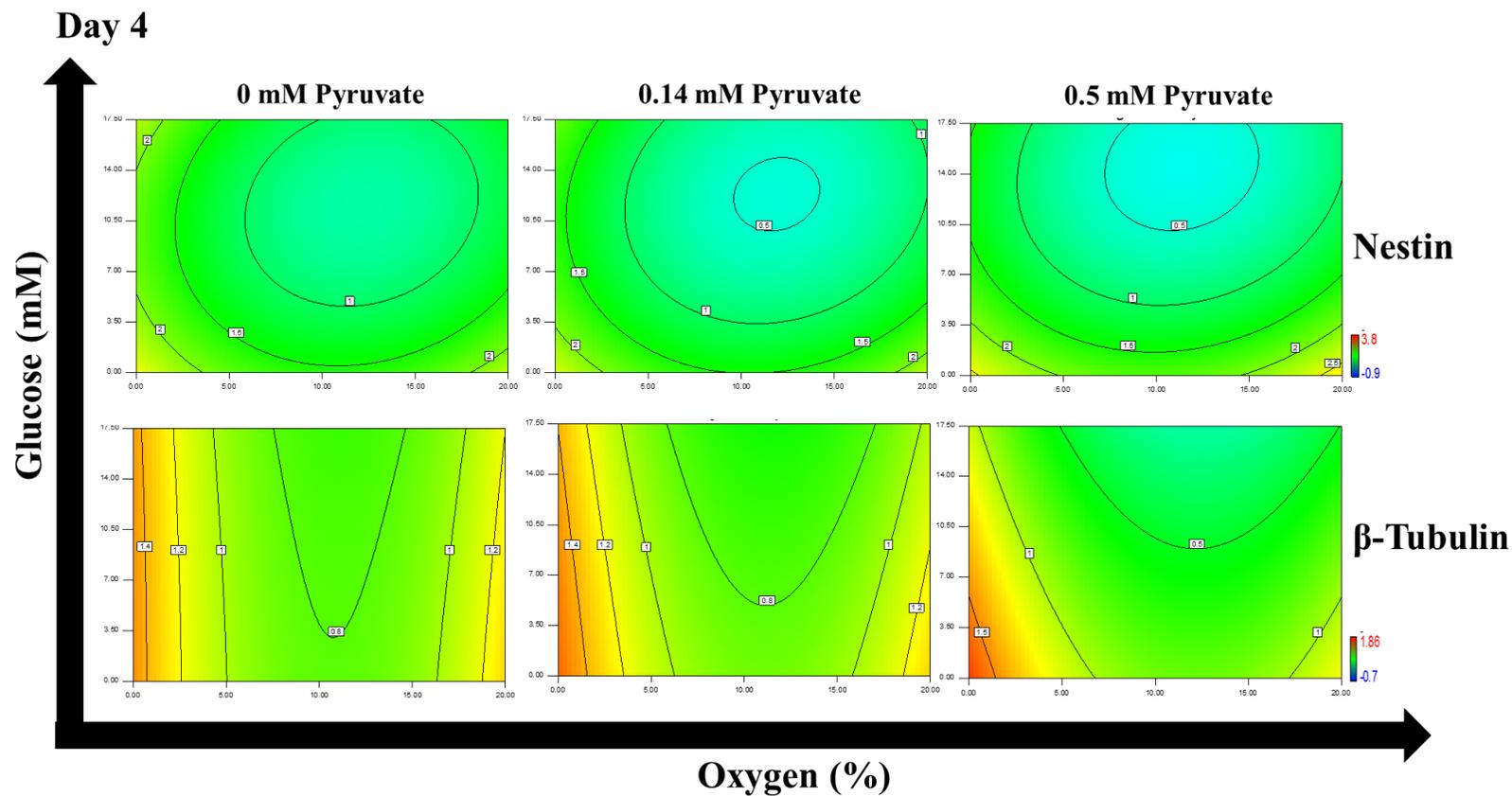
Appendix II Figure 9 Contour plots of a quadratic and 2 FI mathematical model generated for ectodermal gene expression at day 1. Differentiated hiPSCs were cultured in 27 different conditions for four days. Gene expression was assessed by RT-PCR. Colours ranging from dark blue to dark red represent the lowest and highest log value for ectodermal gene expression of Nestin and β -Tubulin.



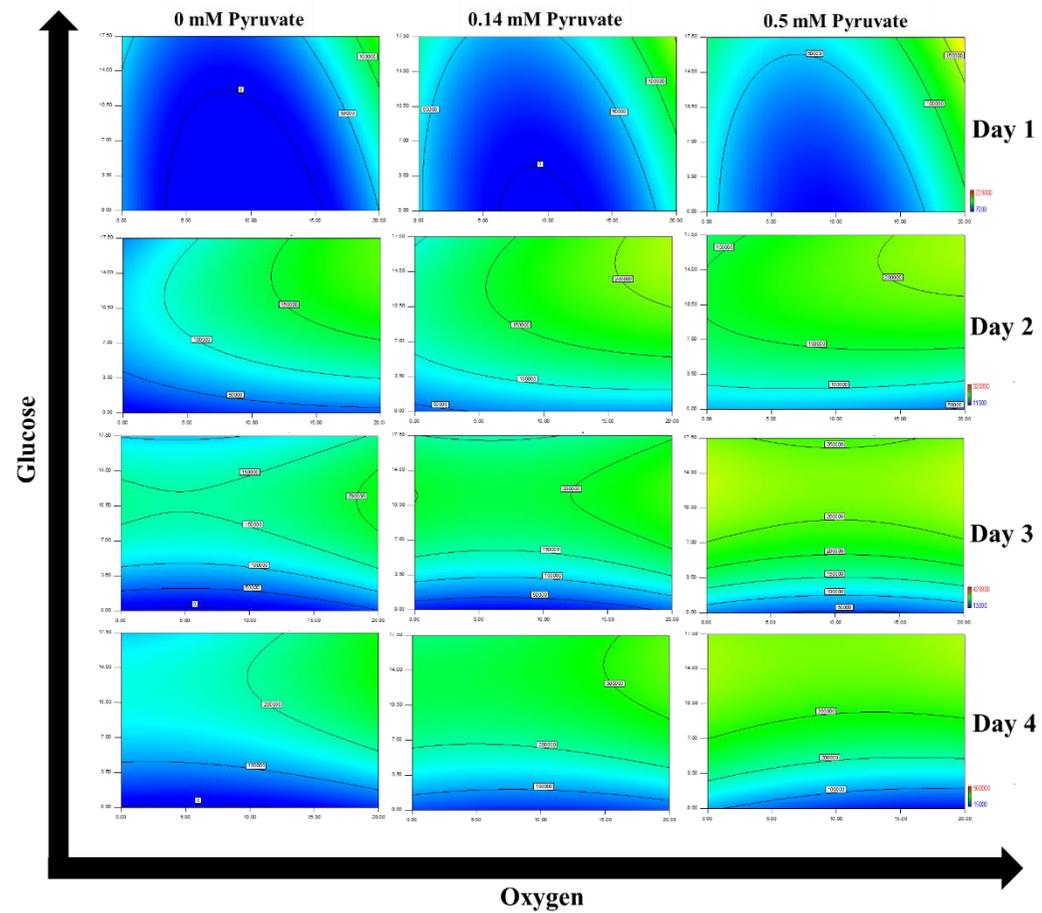
Appendix II Figure 10 Contour plots of a quadratic and 2 FI mathematical model generated for ectodermal gene expression at day 2. Differentiated hiPSCs were cultured in 27 different conditions for four days. Gene expression was assessed by RT-PCR. Colours ranging from dark blue to dark red represent the lowest and highest log value for ectodermal gene expression of Nestin and β -Tubulin.



Appendix II Figure 11 Contour plots of a quadratic mathematical model generated for ectodermal gene expression at day 3. Differentiated hiPSCs were cultured in 27 different conditions for four days. Gene expression was assessed by RT-PCR. Colours ranging from dark blue to dark red represent the lowest and highest log value for ectodermal gene expression of Nestin and β -Tubulin.



Appendix II Figure 12 Contour plots of a quadratic mathematical model generated for ectodermal gene expression at day 4. Differentiated hiPSCs were cultured in 27 different conditions for four days. Gene expression was assessed by RT-PCR. Colours ranging from dark blue to dark red represent the lowest and highest log value for ectodermal gene expression of Nestin and β -Tubulin.



Appendix II Figure 13 A representative contour map of quadratic model of viable cell count of the 27 conditions within the 4 days of differentiation. Cell viability was assessed by trypan blue exclusion method. Colours ranging from dark blue to dark red represent the lowest and highest value for viable cell count.

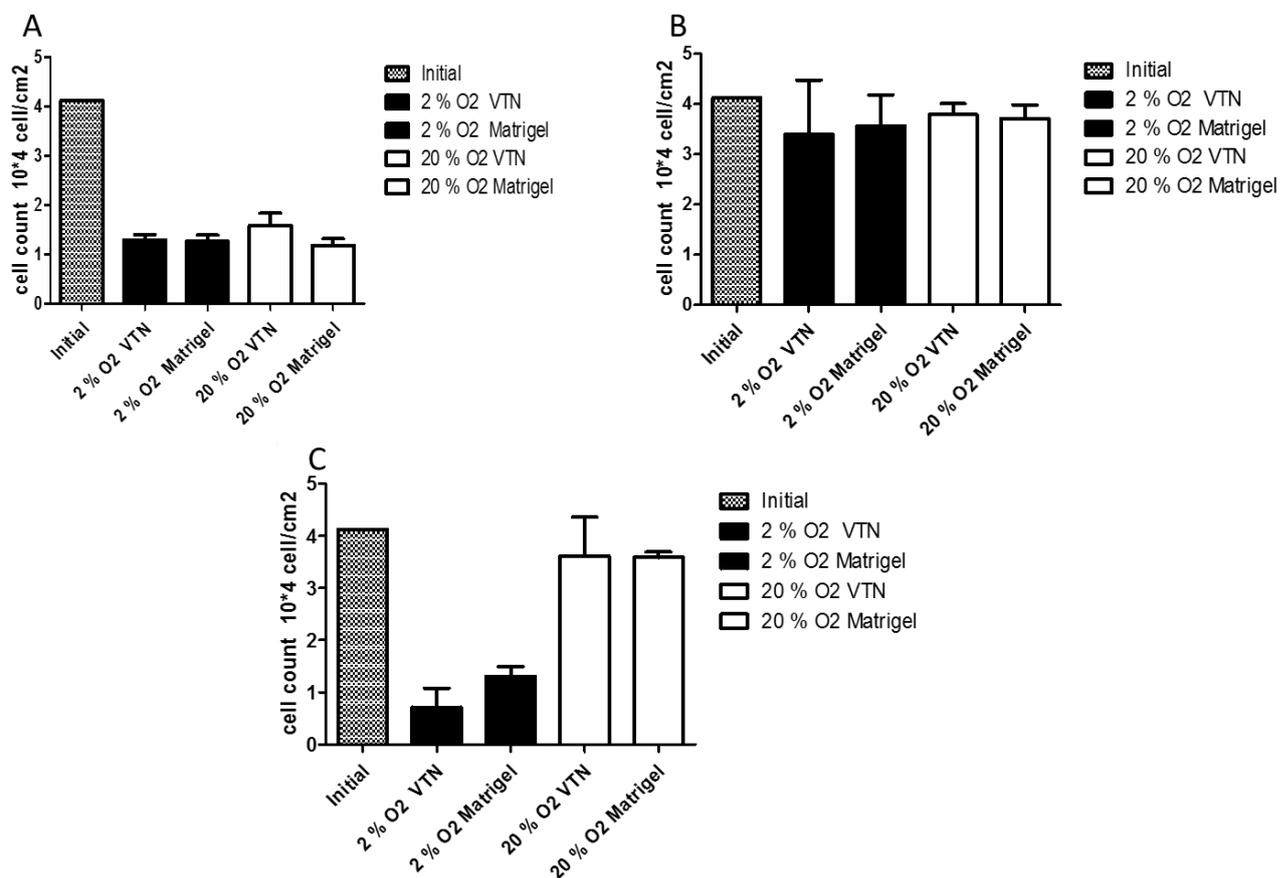
The contour plot of the predictability model was generated for each response using the formula below:

$$\begin{aligned}
 & \textit{Response (Cell viability)} \\
 & = \beta_0 + \beta_1 \textit{ glucose} + \beta_2 \textit{ pyruvate} + \beta_3 \textit{ oxygen} + \beta_4 \textit{ glucose} * \textit{ pyruvate} \\
 & + \beta_5 \textit{ pyruvate} * \textit{ oxygen} + \beta_6 \textit{ glucose} * \textit{ oxygen} \\
 & + \beta_7 \textit{ glucose}^2 \\
 & + \beta_8 \textit{ pyruvate}^2 \\
 & + \beta_9 \textit{ oxygen}^2
 \end{aligned}$$

$$\begin{aligned}
 & \textit{Response (log biomarker 1)} \\
 & = \beta_0 + \beta_1 \textit{ glucose} + \beta_2 \textit{ pyruvate} + \beta_3 \textit{ oxygen} + \beta_4 \textit{ glucose} * \textit{ pyruvate} \\
 & + \beta_5 \textit{ pyruvate} * \textit{ oxygen} + \beta_6 \textit{ glucose} * \textit{ oxygen} \\
 & + \beta_7 \textit{ glucose}^2 \\
 & + \beta_8 \textit{ pyruvate}^2 \\
 & + \beta_9 \textit{ oxygen}^2
 \end{aligned}$$

Where β_{0-9} are the intercept of quadratic interaction coefficient.

Appendix III



Appendix III Figure 1 Viable cell count of hiPSCs (BJ) cultured at 2%O₂. Cell viability of attached cells was measured after plating hiPSCs (BJ) for 6 hours (A), 24 hours (B) and 3 days (C) on matrigel and vitronectin. There was no difference in viable cell count between the two matrixes.

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