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Pluripotent stem cell-derived endothelial cells for vascular regeneration

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Declaration

I declare that this thesis is the result of my own work, and includes nothing that is the outcome of work done in collaboration, except where indicated in the text.

The work in this thesis has not been submitted for any other degree or professional qualification.

Elizabeth M Skinner

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Abstract

Background:

Vascular endothelial dysfunction plays a major role in the pathogenesis of atherosclerosis. As such, the study of endothelial cells has sought to identify causal pathways and novel therapeutic approaches to promote vascular repair. Induced pluripotent stem (iPS) cell technology may be a particularly useful tool, and could be used to derive endothelial cells and their progenitors from individuals with endothelial dysfunction to explore these pathways and develop novel strategies for vascular regeneration. Whilst iPS cells are conventionally obtained from the reprogramming of dermal fibroblasts, it was hypothesised that endothelial cells could also be reprogrammed, and that these pluripotent cells would have enhanced capacity for endothelial differentiation and vascular regeneration.

Objectives:

To generate iPS cells from human fibroblasts and endothelial cells and to assess their potential for endothelial differentiation and vascular regeneration.

Methods and Results:

A) Reprogramming: Dermal fibroblasts and endothelial outgrowth cells from blood were obtained from healthy donors (n=5) and transfected with episomal vectors containing six reprogramming factors: Sox2, Klf4, Oct3/4, L-Myc, Lin28 and Shp53. Successfully reprogrammed fibroblast-derived iPS (fiPS) and endothelial cell-derived iPS (eiPS) arose as colonies, and were isolated and expanded. Reprogrammed cells expressed pluripotency markers SSEA3, SSEA4, TRA 1 60, Oct3/4 and NANOG, and developed into all three germ layers following embryoid body formation. *B) Endothelial differentiation:* iPS and ES cell lines were aggregated into embryoid bodies in stem cell growth media containing mesoderm-inducing cytokines. Embryoid bodies were then disaggregated and cultured in endothelial medium supplemented with VEGF. After seven days, a population of $CD31^+$ cells was isolated and further cultured. Mature endothelial cell antigen

expression was confirmed by flow cytometry. $CD31^+$ cells were similar to mature endothelial cells in functional assays of proliferation, migration, nitric oxide production and angiogenesis. *C) Comparison of fiPS versus eiPS:* eiPS differentiated into endothelial cells with greater efficiency than fiPS (21±3% *versus* 3±2%, $P<0.05$). fiPS-derived endothelial cells and eiPS-derived endothelial cells expressed similar levels of endothelial markers CD146, CD31, VEFGR2 and CD34 compared to control endothelial cells. When grown on Matrigel, they formed tubule-like structures with a similar number of vessel connections. *In vivo,* endothelial cells derived from fiPS and eiPS increased neovasculogenesis in a nude mouse model: vessel density was increased after implantation of endothelial cells from fiPS and eiPS by 3.50 vessel counts ($P\leq0.001$) and 3.47 vessel counts ($P\leq0.001$) respectively, when compared to controls. By comparison control endothelial cells did not increase vessel density compared to control ($P>0.05$).

Conclusions:

Endothelial cells can be isolated from blood and reprogrammed to form pluripotent stem cells with enhanced capacity to differentiate into endothelial cells than those derived from dermal fibroblasts. Endothelial cells derived from both sources promote angiogenesis *in vivo,* and have major potential for therapeutic applications in vascular regeneration.

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List of abbreviations

ºC	Degree Celsius
AAD	Amino Actinomycin D
ANOVA	Analysis Of Variance
APC	Allophycocyanin
bFGF	basic Fibroblast Growth Factor
BMP	Bone Morphogenetic Protein
cDNA	Complementary DNA
CO_2	Carbon Dioxide
DAPI	4', 6-diamidino-2-phenylindole
dH_2O	deionised water
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic acid
dNTP	2'-deoxyribonucleoside 5'-triphosphate
EB	Embryoid Body
EBNA	Epstein–Barr virus Nuclear Antigen
EC	Endothelial Cell
EDTA	Ethylenediaminetetraacetic acid
EGM	Endothelial Growth Medium
EHS	Engelbreth-Holm-Schwann
eiPS	Endothelial-induced pluripotent stem (cell)
eNOS	endothelial Nitric Oxide Synthase
EOC	Endothelial Outgrowth Cells
ES	Embryonic Stem
FACS	Fluorescence-Activated Cell Sorting
FBS	Fetal Bovine Serum
FGF	Fibroblast Growth Factor
fiPS	Fibroblast-induced pluripotent stem (cell)
FITC	Fluorescein isothiocyanate
GMP	Good Manufacturing Practise

GFP	Green Fluorescent Protein
GFR	Growth Factor Reduced
GSK-3β	Glycogen synthase kinase-3β
H&E	Haematoxylin and Eosin
H_2O	Water
HCl	Hydrochloric acid
HUVEC	Human Umbilical Vein Endothelial Cells
IgG	Immunoglobulin G
iPS	Induced Pluripotent Stem
Klf	Krüppel-like factor
KO-DMEM	Knock Out Dulbecco's Modified Eagle Medium
KOSR	Knock Out Serum replacement
LDL	Low-Density Lipoprotein
MEF	Mouse Embryonic Fibroblasts
MEM	Minimal Essential Medium
MNC	Mononuclear Cells
NaOH	Sodium Hydroxide
NEAA	Non-Essential Amino Acids
NGS	Normal Goat Serum
NGS-PBST	Normal Goat Serum- Phosphate buffered saline- Triton X
NSG	Non-Obese Diabetic/ Severe Combined Immunodeficiency/ Gamma
NIH	National Institutes of Health
Oct3/4	Octamer-binding transcription factor-3/4
PBS	Phosphate buffered saline
PBST	PBS-Triton X
PCR	Polymerase chain reaction
PDGF	Platelet Derived Growth Factor
PE	Phycoerythrin
PFA	Paraformaldehyde

RNA	Ribonucleic acid
RPM	Revolutions per minute
RT-PCR	Reverse Transcriptase - Polymerase Chain Reaction
SEM	Standard Error of the Mean
Sox2	SRY-related-high-mobility-group (HMG)-box protein-2
SMA	Smooth Muscle Actin
SNP	Single nucleotide polymorphism
SSEA	Stage Specific Embryonic Antigen
TBE	Tris borate buffer with EDTA
TGF-β	Transforming growth factor beta
TE	Tris buffer with EDTA
Tris	Tris[hydroxymethyl]aminomethane
TSP	Thrombospondin
UV	Ultraviolet light
V	Volts
VE-Cadherin	Vascular Endothelial-Cadherin
VEGF	Vascular Endothelial Growth Factor
VEGFR2	Vascular Endothelial Growth Factor Receptor 2
vWF	von Willebrand Factor
Y4	Yamanaka factors

Chapter 1

Introduction

1.1 Overview

The genetic factors predisposing individuals to atherosclerosis, a major cause of coronary heart disease, are poorly understood. Deficiencies in endothelial cell function play a role in the genetic basis of coronary artery disease. Studies of the endothelium from patients with premature atherosclerosis (occurring before age is considered a risk factor: men under 45 and women under 55; National Heart Lung and Blood Institute) include 'non-invasive clinical investigations', for example flow-mediated dilation of the brachial artery in response to endothelium-dependent vasoactive factors, or detection of biomarkers in peripheral blood (Wong, 2012). However, whilst informative, these techniques give little insight into the molecular and cellular mechanisms behind endothelial dysfunction. Studies of endothelial cells *in vitro* allow more in depth phenotypic, genomic and functional analyses. The scope of *in vitro* endothelial cell research has been expanded by the generation of endothelial cells from pluripotent stem cells (both embryonic and induced), which provide a long-term, renewable, expandable and bankable source of endothelial cells (Reed *et al.,* 2013). Induced pluripotent stem cell technology has the potential to generate patient-specific endothelial cells for *in vitro* disease modelling, with the hope of elucidating genetic causes of endothelial cell dysfunction. Moreover, it represents a population of cells with the potential for novel vascular regenerative therapies.

1.2 The role of endothelial dysfunction in atherosclerosis

The most common cause of premature death in the UK is coronary heart disease, responsible for 74,000 deaths per year (British Heart Foundation). In the majority of cases, coronary heart disease is preceded by atherosclerosis, a complex multi-factorial condition involving inflammation and plaque formation in arterial blood vessels (Hansson, 2005). Multiple environmental risk factors for atherosclerosis are well characterised, including smoking, hypertension, exposure to pollution, and a cholesterol-rich diet. However, individuals not exposed to these factors, but with a first degree relative who has coronary artery disease are also at risk of developing the condition, indicating a hereditary component (Mayer *et al.,* 2007). Though this is less well understood than the environmental risk factors, genome wide association studies suggest that pathogenesis of the disease could be potentiated in part by defects in endothelial cell function (reviewed by Damani and Topol, 2007).

1.2.1 Vascular endothelium

The endothelium, a monolayer of cells lining blood vessels, plays a critical role in vascular health. It was initially considered an interface between the blood and surrounding connective tissue (Florey, 1966), but its importance in the regulation and maintenance of vascular homeostasis has since been realised. The endothelium releases vasoactive factors for dilation or constriction of the vessel in response to changes in blood flow, shear stress or circulating humoral factors (Cines *et al*, 1998). A key endothelial vasodilator is nitric oxide, which has multiple 'anti-atherogenic' effects. Nitric oxide (NO) regulates vessel wall permeability, inhibits platelet aggregation and adhesion of leukocytes to the vessel wall, and prevents smooth muscle cell proliferation (Kawashima, 2004). The endothelium also regulates inflammatory processes (e.g., the expression of surface adhesion molecules on activated endothelial cells attract leukocytes) and thrombogenesis (the production of prostaglandins to prevent platelet aggregation and clot formation) (Deanfield *et al.,* 2007). The post-natal endothelium is typically quiescent in mature adults, with little

cell turnover (Cines *et al.*, 1998), although it retains an ability to undergo remodelling in response to changing functional demand, through the formation of new vessels (neovasculogenesis), and the growth of new vessels from pre-existing ones (angiogenesis) (Davis *et al.*, 2011).

Various factors threaten endothelial health, for example exposure to free radicals caused by cigarette smoking, elevated circulating low-density lipoprotein (LDL) or plasma homocysteine concentrations, hypertension, and/or genetic alterations (Ross, 1999). The integrity of the endothelium depends, not only on the extent of injury, but also on its capacity for regeneration and repair. Persistent endothelial cell injury can lead to increased expression of adhesion molecules and synthesis of pro-inflammatory or pro-thrombotic factors; this disrupts endothelial homeostasis and can ultimately manifest as vascular disease (Widlansky *et al.*, 2003).

1.2.2 Pathophysiology of atherosclerosis

It is widely accepted that endothelial cell dysfunction following chronic endothelial injury is one of the earliest events in the pathogenesis of atherosclerosis (Davignon *et al.*, 2004; Celermajer *et al*., 1997). A major effect of endothelial dysfunction is loss of endothelium-dependent dilation, due in part to decreased nitric oxide synthesis (Sitia *et al.*, 2010). Due to its diverse physiological functions, a decrease in NO bioavailability has widespread effects. For example, one of the consequences of reduced NO bioavailability is increased permeability of the vessel wall, an attenuation of NO's actions to decrease permeability of the endothelium (John *et al*, 2001; Wever *et al*., 1998). It has been theorised that this allows infiltration of low-density lipoprotein (LDL) particles into the vessel intima (Cardona-Sanclemente and Born, 1995). These particles become oxidised by factors released from the endothelium, prompting a local inflammatory response (Hansson and Libby, 2006). Platelets adhere to 'activated' endothelial cells, which attract circulating monocytes that diffuse into the intima. The latter develop into macrophages which digest oxidised LDL and are gradually converted into large lipid-rich foam cells (Choudhury *et al.*, 2005). Meanwhile, smooth muscle cells proliferate and migrate

from the media to the intima in response to cytokines secreted by the endothelium, where they form a fibrous cap over the fibro-fatty atheroma, made up of lipid debris and migrated inflammatory cells (Gomez and Owens, 2012). The inflammatory response is propagated by apoptosis of foam cells, which can form a necrotic core. If plaque growth continues over time, the artery becomes occluded, and can be prone to rupture depending of the stability of the fibrous cap (Newby and Zaltsman, 1999). This can lead to complete arterial occlusion, preventing blood flow to the areas of the heart supplied by that vessel, leading to myocardial ischaemia and infarction.

1.2.3 Genetic basis of atherosclerosis

Atherosclerosis can develop in the absence of environmental risk factors, suggesting there is likely to be a hereditary component to the disease. Indeed, a history of premature coronary artery disease in a first degree relative is an independent risk factor for coronary heart disease (Shea *et al*., 1984; Myers *et al.,* 1990; Grech *et al.,* 1992). A group of individuals whose only major cardiovascular risk factor was family history were found to have dysfunctional endothelium (indicated by impaired endothelium-dependent flow mediated dilation) in a study by Clarkson *et al.* (1997). These patients likely had genetic risk factors predisposing them to subsequent arterial disease, and may have an inherited abnormality of the vessel wall, or increased vulnerability to circulating risk factors.

Genome wide association studies, which identify DNA sequence variations associated with risk of disease development, show that atherosclerosis is a polygenic condition, as there are multiple candidate genes that contribute to disease susceptibility (Lusis *et al.,* 2012). Though the genetic causes of atherosclerosis are poorly understood, these candidate genes provide insight into the molecular pathways which, if dysfunctional, lead to the pathogenesis of atherosclerosis. Some of these pathways are those involved in lipid metabolism, vascular homeostasis, coagulation of inflammatory factors, thrombolysis and endothelial cell function (Alavantic and Djuric, 2006; Damani and Topol, 2007). Genetic influence can also be subject to significant modulation by environmental factors, complicating an

individual's atherogenic predisposition. Defective genes which lead to endothelial cell dysfunction (one of the earliest events in the onset of atherosclerosis) include those involved in NO production and release, interactions between inflammatory and endothelial cells, and permeability of the endothelium permitting infiltration of plasma constituents (Damani and Topol, 2007).

A key pathogenic feature of atherosclerosis is impaired endothelium-dependent relaxation, often caused by disturbances in NO production. NO is constitutively produced by the endothelium from the conversion of L-arginine to L-citrulline, a reaction driven by the enzymatic activity of endothelial nitric oxide synthase (eNOS) (Cannon, 1998). Therefore, defective eNOS, leading to a reduction in NO levels, can result in impairment of endothelium-dependent relaxation and acceleration of lesion formation in atherosclerotic vessels. Meta-analyses have sought to define the association between polymorphisms in the eNOS gene with occurrence of cardiovascular disease. A polymorphism in the eNOS gene on chromosome 7, G894T, has been identified and results in a non-synonymous Glu298Asp substitution which causes decreased NO plasma levels, and therefore a reduction in NO's beneficial anti-atherosclerotic effects (Casas *et al.,* 2006). Individuals homozygous for this mutation have a slightly increased risk of ischaemic heart disease. Other studies report an association of this polymorphism with coronary artery disease (Zhang *et al*., 2012a) and with myocardial infarction (Luo *et al.,* 2014); although this association only manifests in the Asian population, indicating the role of ethnicity in this association. Further large-scale studies are needed to elucidate this association.

Given the variety of functions of the endothelium, and the multiple genes which orchestrate these functions, genome-wide association studies have identified candidate genes in pathways other than eNOS which if mutated have the potential to cause endothelial cell dysfunction. For example, defects in the thrombospondin (TSP) family of matrix proteins are linked to cardiovascular disease (Topol *et al.,* 2001; Wessel *et al*., 2004). A base pair substitution in TSP 4 variant (A387P) alters the tertiary structure of the protein and disrupts the calcium binding site; this is associated with a significant reduction in the proliferative capacity of the endothelium, thus severely decreasing the endothelium's capacity for repair and

regeneration following minor injury. This polymorphism correlates with an increased risk of premature myocardial infarction (Stenina *et al.,* 2003).

Another cause of endothelial cell dysfunction is thought to be due to a defect in the gene encoding for connexin 37 (Yamada *et al.,* 2002), specifically the C1019T single nucleotide polymorphism (SNP; Guo *et al.,* 2014). In its normal state, connexin 37 is an endothelial gap junction protein, which aids the integration of endothelial cell and smooth muscle cell function, and is involved in endothelium-dependent hyperpolarising factor (EDHF)-mediated vasodilation (Söhl & Willecke, 2004). In general, EDHF-mediated reactions increase intracellular calcium levels, leading to the opening of calcium-activated potassium channels of small and intermediate conductance, and the hyperpolarisation of endothelial cells. This prompts hyperpolarisation and relaxation of the smooth muscle cells (Feletou and Vanhoutte, 2006), causing vasodilation. When mutated, connexin 37 has an abnormal tertiary structure and, therefore, formation of endothelial-smooth muscle cell gap junctions is altered and normal endothelial functions are compromised. Dysfunctional connexin 37 has also been associated with an increased risk of myocardial infarction (Yamada *et al.,* 2002).

The polygenic nature of endothelial cell dysfunction, in combination with its variability between affected individuals, indicates the complexity of the hereditary component of atherosclerosis. Further research is required to elucidate these mutations and the mechanisms by which they cause endothelial cell dysfunction on an individual basis.

1.2.4 Assessment of endothelial cell function

Endothelial dysfunction in the brachial artery is closely correlated with dysfunction in the coronary circulation (Anderson *et al.,* 1995), demonstrating the systemic nature of endothelial dysfunction. Thus the vessels of the forearm are used for clinical assessment of endothelial function. A popular clinical assessment of endothelial function is flow-mediated vasodilation (measured by ultrasound or

angiography), which is predictive of cardiovascular morbidity and mortality (Wong *et al*., 2012). Acetylcholine infusion of the brachial artery causes endothelium-dependent vasodilation in healthy vessels, whilst in unhealthy vessels, smooth muscle-mediated vasoconstriction occurs (Langrish *et al.,* 2012). This finding is based on the pivotal studies by Furchgott (Furchgott and Zawadzki, 1980), in which rabbit aorta strips denuded of endothelial cells constricted in response to acetylcholine, whilst healthy (non-denuded) vessels demonstrated acetylcholine-induced relaxation. Further investigations confirmed that this phenomenon is due to stimulation of muscarinic acetylcholine receptors, triggering NO synthesis by eNOS (Kamimura *et al.,* 2003). Venous occlusion plethysmography is another method used to measure vasomotor responses of the brachial artery to vasoactive factors. Endothelial function can also be measured by quantification of levels of circulating biomarkers - for example soluble adhesion molecules, which are increased due to vascular inflammation or endothelial activation, or endothelial progenitor cells, which are reduced in patients with cardiovascular risk factors (Burger *et al.,* 2012). Endothelial cell biopsies have also been used for immunohistochemical and genetic single cell analyses (Lehle *et al.,* 2010). However, there are limitations to these approaches, either due to limited numbers of endothelial cells or the invasive nature of the techniques, and they have yet to provide novel molecular insight into endothelial cell biology.

The primary culture of endothelial cells *in vitro* was developed over forty years ago (Jaffe *et al.*, 1973) and has greatly increased understanding of endothelial cell biology through phenotypic, genomic and functional studies. However, primary cell lines have limited capacity to expand *in vitro,* thus the range of these studies have been enhanced by use of endothelial cells derived from pluripotent stem cells, both embryonic (ES) and induced (iPS). These immortal cell lines are capable of differentiating into all types of cardiovascular lineages: endothelial cells, vascular mural cells, smooth muscle cells and cardiomyocytes (Narazaki *et al*., 2008; Yoshida and Yamanaka, 2010), opening up new avenues for cardiac and vascular regeneration and to study the genetic and molecular basis of endothelial cell dysfunction.

1.3 Pluripotent stem cells

Human embryonic stem (ES) cells are derived from the inner cell mass of the pre-implantation blastocyst, and were first derived in James Thomson's laboratory in the University of Wisconsin in 1998 (Thomson *et al.,* 1998). Donated human embryos produced by *in vitro* fertilisation were cultured for approximately five days until they became blastocysts, consisting of the outer trophoectoderm (the cells providing nutrients to the embryo) and the inner cell mass (the cells from which the fetus develops). The inner cell masses were isolated, and from them ES cells were derived as described by Thomson *et al.,* 1995. In culture, these cells cluster together as colonies- they are small and rounded and have a high nucleus-to-cytoplasm ratio. Activity of telomerase is high in ES cells (Thomson *et al.,* 1998). Telomerase is a ribonucleoprotein that maintains chromosome length by adding telomere repeats to the ends of chromosomes and has a significant effect on cell life span (Shay and Wright, 2005). This accounts for the ability of ES cells to proliferate and remain undifferentiated for long periods in culture (Rosler *et al.,* 2004). ES cells express many markers associated with pluripotency- for example the stage specific embryonic antigens 3 and 4 (SSEA3 and SSEA4) and stem cell specific markers Tra-1-60 and Tra-1-81. In addition, ES cells form teratomas when injected into immunocompromised mice, which contain derivative cells from all three primary germ layers: mesoderm, ectoderm and endoderm, demonstrating their pluripotent potential (Zhang *et al.,* 2012b).

Human ES cells can be differentiated into endothelial cells *in vitro* (Vittet *et al.,* 1996; Yamashita *et al.,* 2000; Levenberg *et al.,* 2002). Though they represent a useful source for the derivation of many cell types, ethical and immunological issues have hampered their use in research. These issues may be avoided by the use of an alternative stem cell type: induced pluripotent stem cells.

1.3.1 Induced pluripotent stem cells

Induced pluripotent stem (iPS) cells were first produced in 2006, by Yamanaka and Takahashi, at Kyoto University (Takahashi *et al*., 2006). Pluripotency was induced in

murine somatic fibroblasts, by ectopic expression of four transcription factors: octamer-binding transcription factor-3/4 (Oct 3/4), SRY-related-high-mobility-group (HMG)-box protein-2 (Sox2), Kruppel-like factor 4 (Klf4) and c-myc, a process called cellular reprogramming. These four transcription factors act in concert in cellular reprogramming. Firstly, c-myc acetylates specific histones, leading to chromatin remodelling. This allows greater access of Oct3/4 and Sox2 (which both target oncogenes) to their respective binding sites, promoting cellular proliferation. Usually, this would induce apoptosis through tumour suppression mechanisms, however the presence of Klf4 (another oncogene) results in suppression of p53 and upregulation of Nanog, the result of which inhibits cell death. Transcription factors Oct and Sox also activate other critical embryonic genes and recruit chromatin-remodelling complexes that promote reprogramming (Buganim *et al*., 2013). A year after the generation of murine iPS cells, this process was replicated using human fibroblasts, generating the first human iPS cells (Takahashi *et al*., 2007; Yu *et al.,* 2007). Human iPS cells are strikingly similar to human ES cells: they replicate via mitotic division whilst maintaining an undifferentiated state (in theory, indefinitely), and they have the potential to differentiate into all somatic cell types when provided with the necessary stimuli. Through directed differentiation systems, hepatocytes (Sullivan *et al*, 2010), adipocytes (Tashiro *et al*, 2009), motor neurons (Dimos *et al,* 2008), osteoblasts (Bilousova *et al* 2011), and pancreatic insulin-producing cells (Zhang *et al.*, 2009) have all been derived from iPS cells, to name a few examples. iPS cells can also differentiate to all lineages of the cardiovascular system: endothelial cells, vascular mural cells, smooth muscle cells and cardiomyocytes (Narazaki *et al*., 2008; Tulloch *et al*., 2008).

Avoiding the ethical and immunological issues that typically hamper ES cell research, the arrival of iPS cell technology marked the start of an exciting new dimension of stem cell research, expanding the potential of regenerative cell medicine. iPS cells are patient-specific and can be used for the modelling of monogenic and polygenic human disease phenotypes, *in vitro* and *in vivo.* They could also be applied to the screening of novel drugs and toxicology studies. In time, iPS cell technology may prove to be a reliable and renewable source of cells for autologous cellular therapy. In the case of polygenic diseases, such as atherosclerosis, in which the genetic

predisposition may vary between individuals, patient-specific iPS cell technology can offer personalised strategies for the study of endothelial cell dysfunction for elucidation of genetic causes.

1.3.2 Cellular reprogramming methods

Cellular reprogramming methods can be categorised based upon whether or not the delivery systems cause genetic modifications to the donor cell. Cellular transfection with viral vectors, transposons, or transfer of linear DNA involves the integration of exogenous genetic material, whilst transfection with transient episomal plasmids, RNA or protein does not. Initially, retroviral vectors were used to deliver the reprogramming transcription factors (Takahashi *et al*., 2007). Whilst effective, via this method the retroviral vector backbone and transgenes are permanently and randomly integrated into the genome, and therefore have the potential to cause mutations. Transgene expression can also influence specific lineage differentiation (Yu *et al.,* 2007), or may result in tumourigenesis (Okita *et al.,* 2007). Aside from this, viral vectors pose risks to the user, especially when containing the oncogene c-myc. Proper facilities and equipment are required for handling viruses, and they are not appropriate for the production of clinical grade cells. Cre/LoxP recombination methods were developed as an attempt to excise integrated transgenes (Soldner *et al.,* 2009), however these can still leave behind residual vector sequences. As an alternative to viral approaches to iPS cell generation, non-integrating methods of delivery of reprogramming factors have been promoted. Episomal plasmids are only present in the cell transiently; once pluripotency is established, the expression of extra-genomic transcription factors is not required for its maintenance (Abujarour and Ding, 2009). This integration-free method therefore generates cells which are karyotypically normal, identical to their donor cell and suitable for clinical applications.

1.3.3 Somatic cell source

Initially, pluripotency was induced in fibroblasts with retroviral vectors, but since then the field of iPS cell technology has grown rapidly, with many aspects modified and optimised. As well as the method of reprogramming, the optimal somatic donor cell for reprogramming has been investigated. Fully pluripotent iPS cells have been derived from keratinocytes (Aasen *et al*., 2008), peripheral blood cells (Loh *et al*., 2009) and neural progenitor cells (Eminli *et al*., 2008), to name a few examples. In comparison with traditionally used fibroblasts, many of these studies report enhanced reprogramming efficiencies. For example, human scalp keratinocytes are reprogrammed with 100-fold more efficiency than human fibroblasts (Aasen *et al.,* 2008). Furthermore, it appears in a number of cases that iPS cells differentiate preferentially along the cell lineage of their donor cell type (Bar-Nur *et al.*, 2011). For example, along a blood differentiation protocol, blood-derived iPS cells formed more hematopoietic colonies than fibroblast-derived iPS cells. Conversely, differentiation to osteoblasts (bone progenitor cells derived from mesenchymal stem cells) was more successful in fibroblast-derived iPS cells than blood-derived iPS cells, demonstrated by formation of osteogenic colonies, and high expression of osteoblast-associated genes (Kim *et al.,* 2010). Many of these reports suggest that variable reprogramming efficiency of somatic cells and subsequent differentiation bias of the derived iPS cells is due to their epigenetic profiles: it is likely that cells which are reprogrammed with relative ease have an epigenetic profile amenable to reprogramming. For example, Chou *et al.* (2011) reported that mononuclear cells derived from peripheral and cord blood had epigenetic features and gene expression profiles closer to pluripotent stem cells than age-matched fibroblasts have to pluripotent stem cells. They reported that these cell types reprogrammed with a higher efficiency than fibroblasts. The reprogramming process involves resetting of the epigenome, meaning the erasure of characteristic chemical modifications of DNA and chromatin structures of the donor cell that dictate gene activation and repression. If this erasure is incomplete, some of these epigenetic characteristics remain on the iPS cells, which are relayed into donor gene expression patterns. Reports suggest that epigenetic marks at centromeres and telomeres are particularly resistant to

reprogramming (Lister *et al.,* 2011). This can result in iPS cells which, whilst pluripotent, can be more efficient in differentiation back to their original donor cell type.

Advances have been made recently in identifying hematopoietic and endothelial cell alternatives to fibroblasts as more attractive cell types for reprogramming. Geti *et al.* (2012) isolated endothelial outgrowth cells (EOC) from peripheral blood and generated iPS cells with normal karyotypes through viral reprogramming. Reprogramming kinetics and efficiency was higher for EOCs than fibroblasts, and was performed in a 96 well format, which is promising for translation to high throughput platforms. Additionally, 80% of derived iPS cell lines did not acquire any copy number variations during reprogramming. Also derived from mobilised human peripheral blood, cells expressing the hematopoietic and endothelial marker CD34 are amenable to reprogramming via retroviral transduction of the Yamanaka factors (Loh *et al.*, 2009). The resulting iPS cells were described as indistinguishable from embryonic stem cells and were able to differentiate *in vitro* and in teratomas. In comparison to fibroblasts, $CD34^+$ peripheral blood cells had increased reprogramming efficiency. A key benefit to using this cell type is the ease with which they are obtained: a blood sample is a less invasive cell source than a skin biopsy (required with fibroblasts). In addition, Loh *et al.* report that blood-derived cells only required a short time in culture prior to reprogramming. Haase *et al.* (2009) report successful reprogramming of cells obtained from cord blood. They found that cord blood-derived endothelial cells were more efficiently reprogrammed than primary lung fibroblasts, adult peripheral blood mononuclear cells or monocytes. The cord blood endothelial cells had the added benefit of being free from any nuclear or mitochondrial mutations that accumulate as organisms progress to and through adulthood. HUVECs have also been used as a source of iPS cells. Lagarkova *et al.* (2009) report that HUVECs can be successfully reprogrammed using retroviral delivery of Yamanaka's transcription factors, to generate *bone fide* iPS cells with ES cell-like properties. They demonstrated that these cells could be differentiated back to endothelial cells *in vitro.* Furthermore, HUVEC-derived iPS cells were found to have an epigenetic profile, which had been 'reset' to resemble that of ES cells. Promoter elements of endothelial specific genes had been silenced during

reprogramming, whilst promoters of pluripotency-associated genes were activated, at similar levels to ES cells. Relatively efficient and rapid HUVEC retroviral reprogramming has also been demonstrated by Panopoulous *et al.*, 2011, who report that iPS cell colonies appeared six days following HUVEC reprogramming, compared with the standard ~three weeks.

These studies described in this section give credibility to the hypothesis that endothelial cells can be expected to reprogram with higher efficiency than fibroblasts, and thus may be an optimal somatic cell substrate for the generation of iPS cells.

1.3.4 Comparison between ES and iPS cells

When human iPS cells were initially derived in 2007, they were found to be strikingly similar to ES cells in their ability to self-renew and differentiate when provided with the right stimuli. However, as the field progressed, reports of differences between the two cell types emerged. Genomic variations occur between many stem cell lines, and collectively, some iPS cell lines have been shown to deviate from ES cells in terms of expression of hundreds of genes (Chin *et al.,* 2009) and DNA methylation patterns (Doi *et al.,* 2009). It has been reported that the epigenome of the two cell types may vary considerably, in particular as some iPS cell lines have similar gene expression patterns to the donor cells from which they were derived (Ghosh *et al.*, 2010).

There are however other studies that find ES and iPS cells indistinguishable, and argue that reported differences can be attributed to variations in reprogramming method and culture conditions between laboratories. In a review by Yamanaka (Cell Stem Cell, 2012), it is noted that the studies which report significant differences between the two cell types often compared low numbers of clones; whereas those which argue that it is difficult to distinguish between them typically have compared higher numbers of ES and iPS clones. This suggests that whilst some cell lines are notably different in their gene expression patterns, variation in gene expression arises to some extent between all stem cell lines and types. It also raises the point that iPS

and ES cell lines can be of variable quality, either in their induction, or in their continued culture, and stresses that continued assessment of pluripotency is required throughout the passaging of the cells to ascertain that they retain their characteristics *in vitro*.

Differentiation efficiencies of ES and iPS cells can be compared when the two cell types are subjected to the same protocol in the same laboratory, to eliminate variable factors. There have been reports that the two cell types differentiate in the same way: Zhang *et al*. (2009) reported comparable efficiencies of differentiation of ES and iPS cells to pancreatic insulin-producing cells. Chambers *et al.* (2009) also reported similarities in neural differentiation by ES and iPS cells. Conflicting publications, however, conclude that there are differences between ES and iPS cell differentiation. Takayama *et al.* (2013) reported variability in iPS cell differentiation to hepatocytes; whilst some lines were comparable to ES differentiation, some were not. Grigoriadis *et al.* (2010) suggested that intrinsic differences exist between all pluripotent stem cell types, after studies showed variations in hematopoietic differentiation efficiency between all stem cell lines tested, which were not specific to ES or iPS cell types. iPS cell lines can have variable differentiation propensities; this can be an inconsistent factor in the comparison of different ES cell lines as well (Osafune *et al*., 2008). These differences should be taken into account when selecting pluripotent stem cell lines for cellular applications. More robust differentiation protocols may, however, overcome this variation. Through describing variation in differentiation abilities of their ES and iPS cell lines, Kim *et al*. (2010) described a small molecule approach to inhibiting BMP and Activin/Nodal signalling for differentiation to neural progenitor cells, which is efficient in all lines regardless of any initial differentiation propensity.

In terms of endothelial differentiation, it is often difficult to compare ES and iPS differentiation if they are not carried out in the same laboratory following the same protocol. Taura *et al.* (2009) reported that ES and iPS cell differentiation kinetics were comparable; between 1.5-2.2% of their ES cell lines and 1.3-4.4% of their iPS cell lines expressed endothelial markers vascular endothelial growth factor receptor 2 (VEGFR2) and Vascular Endothelial (VE) Cadherin after 10 days of endothelial differentiation. Also, Yamashita (2000) reported that comparable levels of

cardiovascular cells (all derived from $VEGFR2^+$ cells) could be induced from ES and iPS cells.

1.3.5 Risks and limitations of iPS cells

Whilst iPS cell technology offers many benefits in terms of generation of large numbers of cells for the study of disease conditions, there are risks and limitations associated with its use.

Teratoma formation could occur *in vivo* from inadvertent administration of an undifferentiated iPS cell. Moreover, iPS-derived cells are genetically manipulated and thus pose safety and regulatory concerns; even episomal plasmids carry a risk of genomic integration. The original paper describing their use reported an iPS clone, which contained copies of EBNA-1 DNA, likely the result of inadvertent plasmid integration (Okita *et al.*, 2011). For therapeutic use, rigorous screening for pure populations of pluripotent cell-derived endothelial cells would have to be carried out prior to their administration.

A limitation of iPS cell research is the lack of standardised differentiation protocols, hence the difficulties in comparing iPS cells and their derivatives between laboratories. This is evident by comparison of published studies in this field, which vary in terms of differentiation protocols, time points for characterisation and markers used for validation. A more standardised approach is required to minimise variation and allow direct comparison of different iPS cell lines.

1.4 Induced pluripotent stem cells for the study of endothelial biology

As discussed in Chapter 1.2.4, clinical assessments of vascular cell function are informative, but provide limited insight into endothelial cell biology. Culture of endothelial cells - from a primary or commercial source - has improved understanding of their biology, but there are issues that need to be overcome. For

example, the derivation of primary endothelial cell cultures often require invasive procedures, such as biopsy of the superficial forearm vein. Furthermore, often these cells are isolated from vascular beds that do not develop atherosclerosis (e.g. umbilical vein). Also, primary cell cultures have a limited lifespan and begin to senesce after a number of population doublings (unpublished observations), meaning that researchers have finite period of time in which to analyse these cells. Therapeutic strategies require large numbers of cells, which may not be obtained in sufficient quantity from blood or biopsy samples. As an alternative, pluripotent stem cell-derived endothelial cells have many advantages over primary cultures. In the future, patient-specific stem cells could be generated that are renewable and expandable, thus representing an infinite source from which endothelial cells can be derived. Also, iPS cells could be differentiated into other patient specific cardiovascular cell types, for example cardiomyocytes, smooth muscle cells and pericytes, as it is likely that cardiovascular regenerative therapies will require a multi-cellular approach.

iPS cell technology lends itself to the study of diseases which are suspected to have a strong genetic component, either monogenic or polygenic. Cells from these patients can be cultured, reprogrammed and differentiated to the disease-affected cell type, which is likely to display the disease phenotype when compared to healthy controls. In comparison, in patients with diseases that have arisen as a consequence of environmental or epigenetic factors, derived cells are unlikely to carry the disease phenotype (Figure 1.1). The study of cells from patient specific iPS cells can assist in the study of novel pathways or drug therapies, which could alter disease progression.

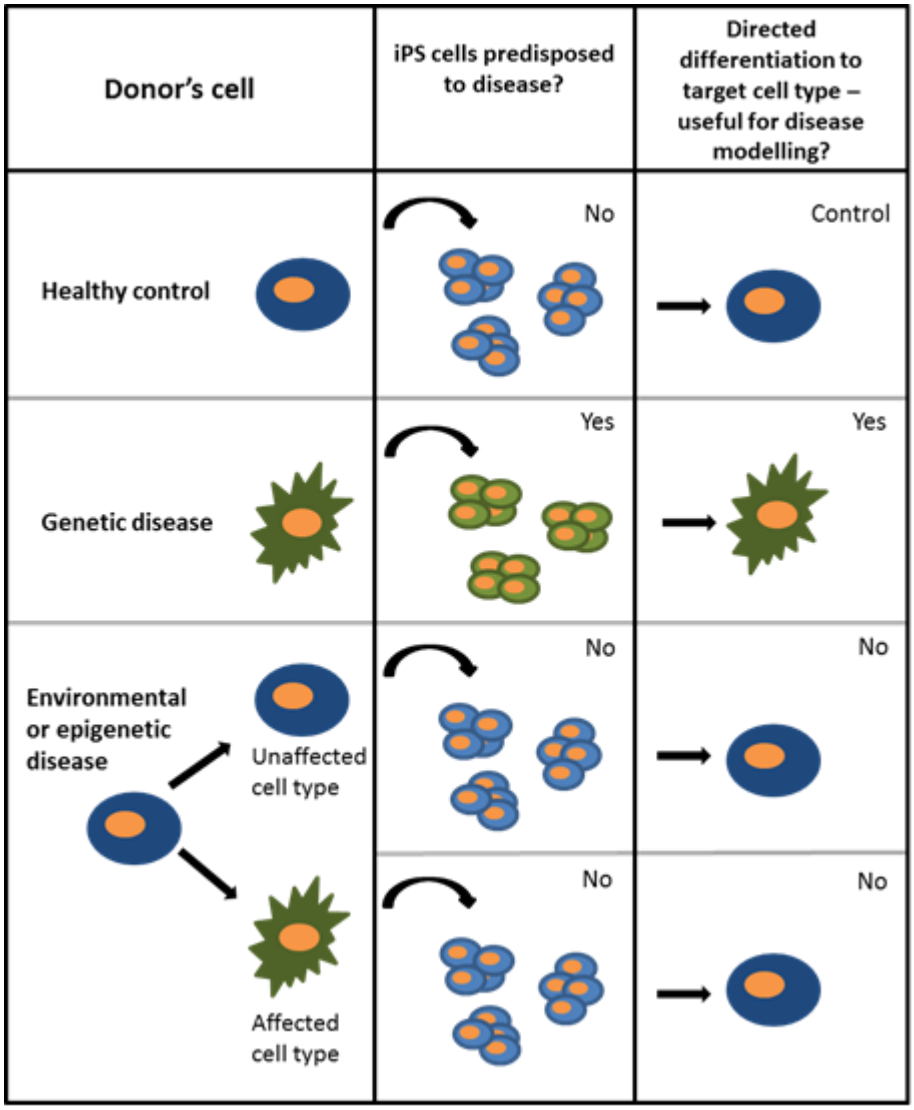

Figure 1.1: Generation of patient-specific iPS cells for disease modelling. Diseases with a genetic component lend themselves to iPS cell generation for directed differentiation and disease modelling. Figure adapted from Cherry & Daley (2012), with permission.

Many recent studies have shown persistence of a disease phenotype in iPS-derived cells following cellular reprogramming and differentiation. This was first reported in cells derived from a patient with spinal muscular atrophy (Ebert *et al.*, 2009). Fibroblast-derived iPS cells were generated from a spinal muscular atrophy patient using lentiviral constructs containing Oct3/4, Sox2, Nanog and Lin28 transcription factors. These patient-specific cells were then differentiated to motor neurones,

which displayed selective deficits in comparison with healthy controls, and therefore may reflect disease processes. *In vitro* disease modelling has also been carried out with iPS cells derived from patients with amylotrophic lateral sclerosis (Dimos *et al.*, 2008). iPS cells were generated from the fibroblasts of an elderly patient with the condition, through retroviral reprogramming with transgenes encoding the 4 Yamanaka factors, Klf4, Sox2, Oct3/4 and c-myc. Small molecules were used to direct differentiation to generate neuron-like outgrowths, and the data suggested that iPS-derived motor neurons arose from progenitors similar to those found in the developing spinal cord, indicating the resemblance between the *in vitro* conditions and *in vivo* processes. IPS cells have also been derived retrovirally from the fibroblasts of patients with LEOPARD syndrome (Carvajal-Vergara *et al.*, 2010), a disease phenotype, which includes hypertrophic cardiomyopathy. *In vitro* derived cardiomyocytes from these patients were found to be bigger and have other nuclear features synonymous with diseased cardiomyocytes, in comparison with healthy controls. The patient derived iPS-cardiomyocytes were also used to investigate the signalling pathways, which contribute to the disease phenotype. Overall, these reports show that the generation of iPS cells from patients with genetic disorders can be used in the elucidation of the causes and mechanisms by which complex diseases develop.

To date, endothelial cells have not been derived from patients with a genetic predisposition to endothelial dysfunction. A strong genetic component is suspected in patients with premature atherosclerosis in the absence of environmental risk factors (Damani and Topol, 2007), and it is likely that iPS derived-endothelial cells from these patients will have a phenotype representative of their endothelial cells *in vivo*. The studies in this thesis form the foundation for investigations into the functions of iPS-derived endothelial cells from patients with premature atherosclerosis, to give insight into genetic and molecular mechanisms of endothelial cell dysfunction.

iPS cell technology lends itself to the development of personalised cellular therapies for vascular regeneration - the restoration of functional vascular networks. After correction of suspected intrinsic genetic defects, patient derived endothelial cells could be administered to aid in reparative mechanisms and reendothelialisation of damaged vessels. They could also be involved in promotion of angiogenesis and

neovasculogenesis for the recovery of ischaemic tissues. Cells could be administered by injection to the site of injury or used in the *ex vivo* engineering of stents or vessel grafts. For example, a report by Ott *et al.* (2008) claimed that de-cellularised rat hearts reseeded with endothelial and cardiac cells were restored with basic heart function in response to physiological load and electrical stimulation after 8 days.

1.5 Endothelial differentiation of pluripotent stem cells

Traditional techniques for the study of endothelial biology (outlined in Chapter 1.2.4), whilst informative, have limitations. Thus, *in vitro* studies provide greater insight into the molecular biology of endothelial cells, and the scope of this research has been greatly expanded by derivation of large numbers of endothelial cells from ES and iPS cells. Cells derived from iPS cells have the same molecular and genetic characteristics of the individual from whom they originated (Park *et al.*, 2008) and, therefore, can be used in the study of the effects of genetic or epigenetic alterations on endothelial function (Wu and Hochedlinger, 2011).

1.5.1 Derivation of endothelial cells from human embryonic stem cells

Since embryonic stem cells were derived in culture by Thomson *et al.* in 1998, attempts have been made to optimise directed differentiation protocols, to generate pure populations of somatic cell types. Many publications document successful endothelial cell differentiation from embryonic stem cells, a process often governed by exposure to soluble growth factors, endothelial cell-inducing cytokines, and the extracellular matrix to which the differentiating cells adhere.

A logical approach to the development of mesodermal cells is the formation of stem cell aggregates known as embryoid bodies (EBs), which mimic the conditions of embryonic development (Baker, 2008). EBs are grown in suspension culture, and within them spontaneous differentiation occurs. Over time, the three primary germ layers (endoderm, mesoderm and ectoderm) appear. In the case of endothelial cell

differentiation, initial embryoid body formation is a method employed by many groups by which to obtain mesodermal cells, from which endothelial cells can be derived (Vittet *et al*., 1996; James *et al*., 2010). Use of embryoid bodies for the derivation of endothelial cells from human embryonic stem cells was first used by the laboratory of Robert Langer (Levenberg *et al.,* 2002). After 13 days, they identified a population of cells expressing endothelial cell markers: CD31, VE-cadherin and von Willebrand Factor (vWF), organised as vascular-like tubules. When isolated from embryoid bodies and transplanted subcutaneously into Non-Obese Diabetic/ Severe Combined Immunodeficiency/ Gamma (NSG) mice, these cells formed perfusing microvessels. Vittet *et al*. (1996) also utilised the close parallels between embryonic vasculogenesis and differentiation of ES cells within EBs. Through detection of endothelial cell markers VEGF-R2, CD31, Tie2 and VE-cadherin they identified primitive vascular-like structures in the embryoid bodies, and found that their formation was encouraged by addition of vascular endothelial growth factor (VEGF). Ferreira *et al*. (2007) reported that they could spontaneously differentiate embryonic stem cells into vascular progenitor cells after 10 days of embryoid body formation. Following this, cells expressing CD34 were differentiated into either endothelial or smooth muscle-like cells, when supplemented with angiogenic factor VEGF or platelet derived growth factor (PDGF), respectively.

Another approach to directed endothelial differentiation of embryonic stem cells is via 2-dimensional culture conditions, in which cells are grown either on feeder layers that provide soluble factors supporting growth, or on a substrate (for example collagen or gelatin) that mimics connective tissue adhesions that occur *in vivo.* Step-wise differentiation can be induced by the addition of growth factors, (such as basic fibroblast growth factor (bFGF) and VEGF) and/or mesoderm-inducing cytokines, (such as bone morphogenic protein (BMP) 4 and Activin A). Endothelial cell differentiation *in vitro* in various culture conditions has been demonstrated in multiple studies. Sone *et al*. (2007) differentiated human ES cells on OP9 feeder cells (a murine stromal cell line which releases soluble factors such as VEGF-C and Ang1; Kono *et al.,* 2006). They found that over the first 8 days a distinct population of $TRA160^{-}VEGFR2^{+}$ cells emerged. This population was isolated, and upon further culture in medium supplemented with VEGF, a $CD34^{+}$VE-cadherin$^{+}CD31^{+}eNOS^{+}$

population of cells emerged which were similar to mature endothelial cells in terms of their morphology and angiogenic capacity. Yamashita *et al*. (2000) also derived vascular cells from embryonic stem cells. $VEGFR2^+$ cells were induced from culture of undifferentiated human ES cells as a monolayer on collagen IV in media containing 10% serum. On sorting the $VEGFR2^+$ cells and adding VEGF to the culture medium, endothelial cells were derived- identified based on morphology, expression of endothelial markers VE-cadherin, CD105 and CD34, and ability to organise into 3-dimensional tubule-like structures *in vitro.* It was concluded that $VEGFR2^+$ cells from ES cells have the potential to serve as common vascular progenitor cells. Kane *et al.* (2010) differentiated human embryonic stem cell lines as a monolayer in endothelial differentiation media: large-vessel endothelial growth media supplemented with hydrocortisone, epidermal growth factor, basic fibroblast growth factor and heparin. Over 21 days, expression of pluripotency associated genes (Oct3/4, Sox2 and Nanog) was decreased whilst those associated with an endothelial phenotype (CD34, CD31 and VE-cadherin) increased. At this stage, the cells formed a cobblestone monolayer, were producing nitric oxide, and responded to pharmacological stimulation similarly to mature endothelial cells. *In vivo*, ES-derived endothelial cells were found to contribute to neovascularisation and recovery of blood flow via engraftment into the vasculature, demonstrated by the hind limb murine model of ischemia (Sone *et al.* 2007).

In addition to biochemical stimuli, the effect of shear stress has been investigated *in vitro* on the endothelial differentiation of mouse ES cells (Yamamoto *et al.,* 2005), as shear stress is thought to influence epigenetics in embryonic development (Illi *et al*., 2003). ES cells were cultured in Minimal Essential Medium (MEM) containing FBS and β-mercaptoethanol. Supplementation of 50ng/ml VEGF induced $VEGFR2^+$ cells, which were isolated and exposed to controlled levels of shear stress. In comparison to cells in static conditions, $VEGFR2^+$ cells exposed to some degree of shear stress had enhanced expression of endothelial markers (CD31 and VE-cadherin) measured at both the protein and mRNA level. An *in vitro* angiogenesis assay on collagen gel (formation of tubule connections between cells) also showed angiogenesis in shear stress-exposed cells. Increased endothelial characteristics after shear stress exposure have been attributed to two phenomena: increased transport of bioactive factors to

the cells (speeding up the differentiation process) and/or the exertion of a mechanical force on the cell, thought to influence endothelial morphology, alignment and function.

Though these studies demonstrate effective derivation of endothelial cells from embryonic stem cells, iPS cells may represent a better source for endothelial cell derivation. The ethical issues surrounding embryonic stem cells continue to hamper their use in research, limiting the commercial availability of ES cell lines. Practical considerations involved in obtaining embryonic tissues for the isolation of embryonic stem cells is another limitation to their large scale production. iPS cells offer a non-controversial alternative, which are more likely to secure funding and support. Somatic cell sources of iPS cells are more readily available, and there are abundant options of somatic cell lineages, which can be reprogrammed. These are relatively easily obtained, in comparison with embryonic tissue, and often relatively low numbers of cells ($0.1\text{-}0.5x10^6$) are required for each reprogramming attempt. Somatic cells can be expanded in culture and banked for multiple transfection experiments. Clinically, large scale production of stem cells is useful for their use in *in vitro* differentiation protocols to generate cells for the elucidation of disease mechanisms, drug screening, toxicology studies and development of cellular therapies. There are safety issues concerning the immunogenicity of allogenic embryonic stem cell derivatives. Their use in cellular therapies would likely involve co-administration of immunosuppressant drugs, adding complexity. In comparison, iPS cells are patient-specific, therefore suited to autologous cell therapy, avoiding the risks of immunorejection. Another major advantage of iPS cells is that they can express a certain disease phenotype for both Mendelian and complex genetic disorders, for the modelling of disease processes *in vitro*. In comparison, ES cell derivatives would have to be exposed to disease-inducing conditions, which are less likely to accurately reflect the phenotype of the condition.

1.5.2 Derivation of endothelial cells from human induced pluripotent stem cells

Endothelial differentiation from iPS cells has been attempted by several groups, but not to the same extent as ES cell differentiation. In the initial publication detailing the induction of pluripotency of human fibroblasts (Takahashi *et al.,* 2007), iPS cells were differentiated to neuronal and cardiac lineages, and two years later the same group differentiated iPS cells to endothelial cells (Taura *et al.,* 2009). Though generally the approaches used for ES cell differentiation can be applied to the differentiation of iPS cells, there is not yet a well-established endothelial differentiation protocol for iPS cells.

Embryoid body (EB) formation has been used for endothelial differentiation of human iPS cells. Rufaihah *et al.,* (2011) formed EBs from iPS cells derived retrovirally from human foreskin fibroblasts. EBs were suspended for 4 days in differentiation media, (α-Minimum Eagle's Medium, fetal bovine serum (FBS), β-mercaptoethanol and non-essential amino acids) supplemented with VEGF and BMP4. These were then disaggregated and the cells seeded onto gelatin-coated plates for 10 days, from which a population of $CD31^+$ cells was isolated. As well as possessing phenotypic similarities to endothelial cells, these cells were capable of forming vascular networks and increasing blood flow to ischemic hind limbs of NSG mice. Wong *et al*. (2012) used a very similar endothelial differentiation protocol via EB formation for 4 days in the presence of VEGF and BMP4. Again, disaggregated EBs were seeded on gelatin-coated plates, though differentiation continued in the absence of BMP4. A population of $CD31^+$ VE-$cadherin^+$ cells was isolated which could be expanded and maintained in endothelial growth medium.

Choi *et al*. (2009) describe hematopoietic and endothelial differentiation of human iPS cells. They plated iPS cells on an OP9 feeder layer, in differentiation medium (consisting of α-MEM, FBS and monothioglycerol) and after 8 days and were able to select $CD34^+CD31^+$ cells. These were further cultured in medium supplemented with endothelial cell growth factor, and appeared to develop endothelial characteristics: expression of endothelial markers VEGFR2, CD146 and VE-cadherin, and formation

of connections on Matrigel, an indication of angiogenic potential. Taura *et al.* (2009) also differentiated human iPS cells into endothelial cells by co-culture with an OP9 feeder layer. After 8 days, they isolated and further cultured a Tra-1-60^{-}VEGFR2^{+} population, which expressed endothelial cell markers: CD31, CD34, VE-cadherin and eNOS. The properties of iPS cell endothelial differentiation via this protocol were very similar to ES cell endothelial differentiation under the same conditions (Sone *et al.*, 2007).

1.6 Scope of studies in this thesis

Within this thesis, protocols were developed for the generation of iPS cells from cellular reprogramming of fibroblasts and endothelial cells. The endothelial differentiation protocols used herein were developed from assessment of the literature, preliminary studies carried out in our own laboratory and following correspondence with Professor Andrew Baker at the University of Glasgow. Extensive phenotypic and functional characterisation of pluripotent stem cell derived endothelial cells was carried out *in vitro* and *in vivo*. All studies were performed on healthy cells obtained from human donors.

1.7 Hypothesis

This thesis addresses the hypothesis that endothelial cells can be derived from human embryonic and induced pluripotent stem cells, which are functional and able to contribute to neovasculogenesis *in vivo*. Furthermore, it is hypothesised that iPS cells generated from endothelial cells will differentiate back to endothelium with greater efficiency than iPS cells generated from other somatic cell sources.

1.8 Aims

- To induce pluripotency in human fibroblasts and endothelial cells via episomal plasmid transfection.
- To derive functional endothelial cells from human embryonic and induced pluripotent stem cells.
- To characterise the morphology and phenotype of endothelial cells derived from embryonic and induced pluripotent stem cells.
- To characterise the functionality of endothelial cells derived from embryonic and induced pluripotent stem cells *in vitro* and *in vivo.*

Chapter 2

Materials and Methods

2.1 General solutions

4% Paraformaldehyde (PFA): 4g PFA (Sigma, UK) was added to 100ml PBS (Gibco, UK) and heated to 60°C. 1M NaOH (Sigma, UK) was added dropwise until the solution cleared; pH was adjusted to 7.4 with 1M HCl (Sigma, UK).

5X Tris-Borate EDTA (TBE): 54g Tris base (Sigma, UK) and 27.5g Boric acid (Sigma, UK) were added to approximately 900ml dH_2O. 20ml 0.5M EDTA (Invitrogen, UK) was added and the final volume adjusted to 1 litre. The solution was diluted to 1X (working concentration) with dH_2O.

Induced pluripotent stem (iPS) cell culture medium: 400ml KO-DMEM, 100ml KOSR, 5ml NEAA, 1ml β -mercaptoethanol, 1ml L-glutamine (all supplied by Gibco, UK).

Modified iPS culture medium: 400ml KO-DMEM, 100ml FCS (Gibco, UK), 5ml NEAA, 1ml β -mercaptoethanol, 1ml L-glutamine.

Endothelial medium: 450ml Endothelial Basal Medium (Lonza, UK), with supplements: 0.2ml hydrocortisone, 2ml hFGF-B, 0.5ml VEGF, 0.5ml R3-IGF-1, 0.5ml ascorbic acid, 0.5ml heparin, hEGF, 0.5ml GA-1000 (all supplied by Lonza, UK, provided as EGM-2 bullet kit supplements), 50ml Hyclone-FBS (Gibco, UK).

Wash medium, PBST: PBS, 0.5% (v/v) Tween (Sigma, UK).

Differentiation medium: 450ml Alpha-Minimal Essential Medium, 50ml FCS, 1ml β -mercaptoethanol

2.2 Cell culture

2.2.1 Ethics

These studies were performed with the approval of the local research ethics committee, in accordance with the Declaration of Helsinki. The written consent of each participant was obtained before entry into these studies.

2.2.2 Endothelial cell lines

All endothelial cell lines were maintained either on collagen I coated plates (BD Biosciences, UK) or on tissue culture treated plastic in endothelial medium (Chapter 2.1). Upon reaching 70-80% confluence, endothelial cell lines were passaged enzymatically. Firstly, wells were washed with phosphate buffered saline (Gibco, UK; approximately 1ml per well of a 6 well plate), then incubated for 5-7 minutes in TrypLE Select (Gibco, UK; approx. 1ml per well of a 6 well plate) at 37°C, after which cells could be observed rounding up and detaching from the plate. Two times the volume of endothelial medium was added (i.e. 2ml per well of a 6 well plate), to stop the TrypLE Select activity, and at this point the cells could be collected and counted with a haemocytometer. Cells were centrifuged for 5 minutes at 10,000 RPM. The supernatant was discarded and the pellet resuspended in the required volume of endothelial medium, either for experimental use or for further culture. Seeding density of subculture was kept consistent throughout endothelial cell culture. Cells were seeded at approximately the following densities:

	Surface Area (mm^2)	Seeding density ($x10^5$)
Plates (data per well)		
6 well	960	0.48
12 well	400	0.2
24 well	200	0.1
Flasks		
T-25	2500	1.25
T-75	7500	3.75

Table 2.1: Typical seeding densities of endothelial cells cultured in various sized culture vessels

Endothelial cell lines could typically be used up to passage 9 or 10, at which point signs of senescence appeared: for example 'stringy' morphology and increased population doubling times (Wagner *et al.,* 2001).

2.2.2.1 Human Umbilical Vein Endothelial Cells

As a representative population of mature endothelial cells, commercially-supplied human umbilical vein endothelial cells (HUVEC) were used (Lonza, UK). HUVEC were cultured on tissue culture treated plastic flasks or plates in endothelial medium at 37°C / 5% CO_2 / 95% relative humidity, for up to 10 passages.

2.2.2.2 Donors

Five healthy volunteers were used in the study, from which late outgrowth endothelial cells from blood, endothelial cells from the vessel wall and fibroblasts from skin biopsy were obtained.

Donor reference	Age	Sex	Type	Endothelial cells		Fibroblasts
				Blood	Vessel wall	
D1	30	M	British South Asian	✓	✓	✓
D2	30	F	Caucasian	✓*		
D3	29	F	Caucasian	✓	✓	✓
D4	31	M	Caucasian	✓	✓	
D5	65	M	Caucasian	✓	✓	✓

Table 2.2: Details of five healthy donors, whose somatic cells were used for reprogramming. Endothelial cells were derived either from blood (*cord blood) or the vessel wall. Fibroblasts were derived from skin biopsy.

2.2.2.3 Late outgrowth endothelial cells

Late outgrowth endothelial cell (EOC) culture was an established technique in the laboratory, performed as described by Tura *et al*. (2007). EOC were obtained and cultured by Olga Tura and Mairi Brittan. Briefly, 100ml peripheral or cord blood was collected into EDTA (Gibco, UK), from which mononuclear cells were isolated by density gradient centrifugation, using Ficoll-Paque PLUS (GE Healthcare, UK), according to the manufacturer's protocol. The cells were seeded in endothelial medium, plated onto collagen I coated plates (Becton Dickinson, UK) and incubated at 37°C / 5% CO2 / 95% relative humidity for 3-4 weeks. Medium was changed every 2 days for 7 days and then twice a week until first passage. Colonies, resembling a typical cobblestone endothelial monolayer, appeared within 1-3 weeks (Figure 2.1)

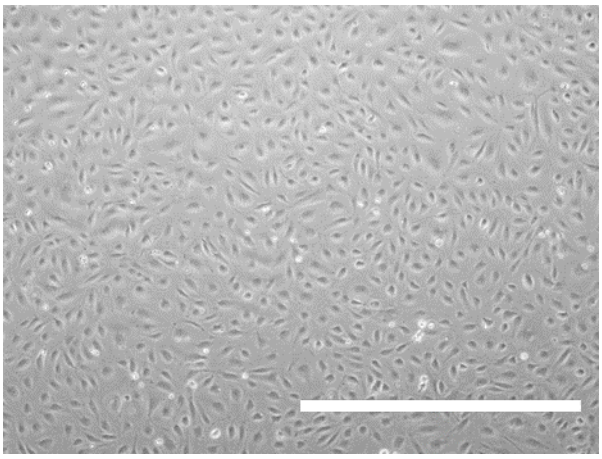

Figure 2.1: EOC in culture have a rounded 'cobblestone' morphology, typical of endothelial cells. Bar = 1mm.

2.2.2.4 Vessel wall-derived endothelial cells

Vessel wall-derived endothelial cells were obtained and cultured by Mairi Brittan. Endothelial cells were derived via intimal wire biopsies of the superficial forearm vein, as previously described (Colombo *et al.*, 2002). A J-shaped wire (0.018-inch diameter) was inserted into the superficial forearm vein through an 18-gauge angiocatheter. Following a number of passes, the distal end of the wire was removed and transferred to a 50ml falcon tube containing EGM2. Cells were washed with phosphate buffered saline (PBS), recovered by centrifugation (5 minutes at 10,000 RPM), and seeded on collagen I-coated 6 well plates.

2.2.2.5 Dermal fibroblasts

Dermal fibroblasts were obtained and cultured by Mairi Brittan. Using aseptic technique and local anaesthesia (lidocaine 2%), a 2-3mm punch skin biopsy was obtained from the lower back. The skin was stretched taut and the punch biopsy instrument applied firmly downwards to obtain a full thickness skin biopsy. The samples were cut into small pieces and plated down onto plastic underneath a 13mm coverslip in DMEM (BD, UK) supplemented with 10% (v/v) Hyclone-FBS and 0.1%

(v/v) penicillin-streptomycin. The cultures were incubated at 37°C / 5% CO2 / 95% relative humidity, media was changed every 2-3 days. After 2-3 weeks fibroblast explant cultures became visible and could be passaged with 0.25% trypsin-EDTA.

2.2.3 Pluripotent stem cell lines

2.2.3.1 Embryonic stem cell lines: H1 and H9

Human embryonic stem cell lines H1 and H9 were supplied by Wicell, USA. Both lines were derived by the Thomson laboratory at the University of Wisconsin (Thomson *et al.*, 1998). They had normal karyotypes and were NIH registry approved. H1 cells were derived from a male with blood type O+; H9 cells were derived from a female with blood type A+. They were used between passages 25-80.

2.2.3.2 Induced pluripotent stem cell lines: 33D6, 33D9 and 34D6

Stock iPS cell lines, 33D6, 33D9 and 34D6 were kindly provided by G Sullivan, University of Edinburgh (Sullivan *et al.*, 2010). They were derived retrovirally from healthy donors, as detailed by Sullivan *et al.* (2010). Briefly, fibroblasts were isolated and reprogrammed via infection with 4 retroviral plasmids (PMXs). Each contained a single reprogramming factor, either Oct3/4, Sox2, Klf4 or c-myc, referred to as the 'Yamanaka factors', or 'Y4'. Three days post infection the cells were plated into 10cm plates on a feeder layer of irradiated mouse embryonic feeder cells (MEFs) and cultured under ES cell conditions (Chapter 2.2.2.5). Successfully transfected fibroblasts started to proliferate and colonies were generated after 2 to 3 weeks. These colonies were isolated with a wide-tipped pipette tip, and cultured separately in ES cell conditions. Successfully established colonies proliferated and were analysed for signs of pluripotency, based on phenotype (Chapter 2.3) and genotype (Chapter 2.4). They could be used for differentiation between passages 25-80. Passage 25 was assumed to be enough time in culture for these lines to be stable, established and ready to be experimented with, whilst by passage 80 is was possible

that the cells were sub-optimal for use given the negative effects of extended culture, for example development of karyotypic abnormalities (Buzzard *et al.*, 2004).

2.2.3.3 Fibroblast-derived induced pluripotent stem cell line, fiPS

Fibroblasts were derived from a 30 year old male, by skin biopsy and explant culture as detailed in Chapter 2.2.1.5. Upon confluence, the cells were reprogrammed by transfection with episomal plasmids containing the Yamanaka factors (Takahashi *et al.*, 2007), Oct3/4, Sox2, Klf4 and L-myc, in combination with Lin28 and Shp53, using the Amaxa system (Lonza; detailed in Chapter 2.8.2). Successfully transfected cells arose as colonies and were cultured in iPS cell conditions (Chapter 2.2.2.5). Established lines were characterised as iPS cells based on phenotype and genotype (Chapter 2.3 and 2.4, respectively).

2.2.3.4 Endothelial-derived induced pluripotent stem cell line, eiPS

Cord blood late endothelial outgrowth cells were derived as detailed in Chapter 2.2.1.3. Cord blood EOC were established in culture and, upon confluence, were reprogrammed by transfection with episomal plasmids containing the Y4 transcription factors with Lin28 and Shp53, using the Neon system (Invitrogen), see Chapter 2.8.3. Successfully transfected cells arose as colonies and were cultured in iPS cell conditions (Chapter 2.2.2.5). Established lines were characterised as iPS cells based on phenotype and genotype (Chapter 2.3 and 2.4, respectively).

Detailed characteristics of all donors from which iPS cell lines were derived are given in Table 2.3.

iPS cell line	Somatic cell type	Gender	Age	Type	Reprogramming method
33D6	Fibroblast	M	56	Caucasian	Retroviral
33D9	Fibroblast				Retroviral
34D6	Fibroblast	F	46	Caucasian	Retroviral
fiPS	Fibroblast	M	30	British South Asian	Episomal
eiPS	Cord blood derived EOC	F	30 [age of mother]	Caucasian	Episomal

Table 2.3: Characteristics of the somatic cell lines from which the iPS cell lines have been derived.

2.2.3.5 Maintenance of pluripotent stem cell lines

All ES and iPS cell lines were maintained long term on an irradiated mouse embryonic fibroblast (MEF) (VHBio, UK) feeder layer in 6 well Matrigel-coated plates with iPS culture medium (Chapter 2.1), at 37°C / 5% CO_2 / 95% relative humidity. Media was replenished every 24 hours. When 70-80% confluent, cells were passaged using enzymatic and mechanical methods. Firstly, the wells were washed with PBS, then incubated with collagenase IV (1ml per well of a 6 well plate; Gibco, UK) for approximately 5 minutes at 37°C. Then the wells were observed under the microscope – the colonies could be seen 'rounding up' at the edges. They were once again washed with PBS, and fresh media was added, at the appropriate volume for the split ratio. A cell scraper (Corning, UK) was used to detach the cells from the plate: firm but gentle pressure was applied across the plate until most of the colonies had detached, but were not broken up too small. The colonies were collected and replated on fresh Matrigel-coated plates, atop a MEF feeder layer. The new plates were shaken gently in the incubator to allow colonies to settle over the whole well area. Wells were observed every day for signs of growth. Areas of spontaneous differentiation could be removed by direct aspiration or by brief incubation in collagenase IV for 1 minute, followed by PBS wash and media replenishment.

Shortly prior to differentiation, if required, cells were transitioned to feeder free conditions. They were passaged onto growth factor-reduced (GFR) Matrigel (BD Biosciences, UK)-coated plates and maintained either in MEF-conditioned media (R&D Systems, UK) supplemented with bFGF (10µg/ml; Peprotech, UK) or in mTeSR-E8 (Stem cell technologies, UK). Towards the second half of this PhD, both ES and iPS cell lines were maintained long term, feeder free on Matrigel-coated plates in mTeSR-E8. iPS cell culture technology is continuously developing, and this more cost-effective method of maintenance was advised from another laboratory (Megaw R, University of Edinburgh). Cell lines had the same characteristics when maintained in either MEF or feeder free conditions: morphology was the same, as was expression of pluripotency markers and differentiation potential (unpublished observations).

Characterisation of the cell lines for pluripotency was performed by flow cytometry, immunocytochemistry, embryoid body assay, polymerase chain reaction and genomic DNA analysis via SNP array (descriptions of these techniques to follow). Cells were also routinely tested for mycoplasma infection. Supernatant of a confluent layer of cells was collected, at least 24 hours after feeding, and analysed via the MycoAlert^{TM} Mycoplasma Detection Kit (Lonza, UK).

2.3 Phenotypic analysis

2.3.1 Flow cytometry

2.3.1.1 Immunofluorescent staining of cell samples

Cells were trypsinised as previously described, counted and resuspended in their original culture medium (100µl per $1x10^5$ cells). They were pipetted into the required number of FACS tubes (determined by the number of antibody panels + blank control) and were incubated with the appropriate pre-conjugated mouse anti-human monoclonal antibody/ies (Table 2.4) for 30 minutes in the dark at room temperature. Blank controls were left unstained. Cells that required permeabilisation for intracellular staining were first incubated for 15 minutes on ice with Cytofix/cytoperm (BD Biosciences, UK; 400µl per 100µl cell solution). They were washed twice with 1X PermWash solution (BD Biosciences, UK), pelleted by centrifugation (5 minutes 10,000 RPM) and resuspended in 1X PermWash, to which the primary antibody/ies were added. All subsequent wash steps were performed with 1X PermWash to maintain the cells in a permeabilised state. After antibody incubation, cells were washed with PBS (1ml per FACS tube), centrifuged for 5 min at 10,000 RPM and resuspended in 10% Cell Fix solution (300µl per FACS tube; BD Biosciences, UK). Cells could be analysed immediately or stored for up to a week at 4°C in the dark until analysis.

Monoclonal antibody	Flurochrome	Clone	Host species; isotype	Manufacturer	Laser filter	Band pass
CD31	FITC	WM59	Mouse IgG1 κ	BD Biosciences	Blue	530/30
CD45	V450	HI30	Mouse IgG1, κ	BD Biosciences	Violet	450/50
VEGFR2	PE	89106	Mouse IgG1	R&D Systems	Yellow/ Green	582/15
CD146	PE/Cy7	SHM-57	Mouse IgG2a, κ	Biolegend	Yellow/ Green	780/60
CD34	APC/Cy7	581	Mouse IgG1, κ	Biolegend	Red	780/60
CD133	APC	AC133	Mouse IgG1	Miltenyi Biotec	Red	670/30
CD105	APC	166707	Mouse IgG1	R&D Systems	Red	670/30
TRA 1 60	FITC	TRA-1-60	Ms IgM, κ	BD Biosciences	Blue	530/30
SSEA3	FITC	MC-631	Rat IgM, κ	Biolegend	Blue	530/30
SSEA4	PE	MC813-70	Ms IgG_3	BD Biosciences	Yellow/ Green	582/15
OCT3/4*	PerCp-Cy5.5	40/Oct-3	Mouse IgG1, κ	BD Biosciences	Blue	695/40
NANOG*	APC	N31-355	Mouse IgG1, κ	BD Biosciences	Red	670/30

Table 2.4: Mouse anti-human antibodies used in flow cytometry for characterisation of surface antigens of a particular cell line. Antibodies typically used to stain endothelial cells; antibodies typically used to stain stem cells. * required permeabilisation prior to staining.

Cells were analysed with the BD LSRFortessa Flow Cytometer using BD FACS Diva software. A gating strategy based on unstained controls (to select live single cells of interest) is displayed in Figure 2.2. Initially, the cell population of interest was gated based on cell size and granularity, which excluded dead cells and debris. From this population, single cells were then selected, based on their height to area ratio. Finally, negative gates were drawn around the populations, which would

distinguish between stained and unstained cells when applied to the sample. A minimum of ten thousand events per sample was collected; data were analysed with FlowJo (Treestar Inc., USA).

Experimental design of multicolour flow cytometry included the use of single-stain compensation beads (BDTM CompBeads, BD Biosciences, UK) to remove 'spill over' of fluorescence emission between fluorophores. Separate positive and negative peaks were observed for each bead set, and compensation automated by the FACSDiva software, which was applied to all analysis experiments.

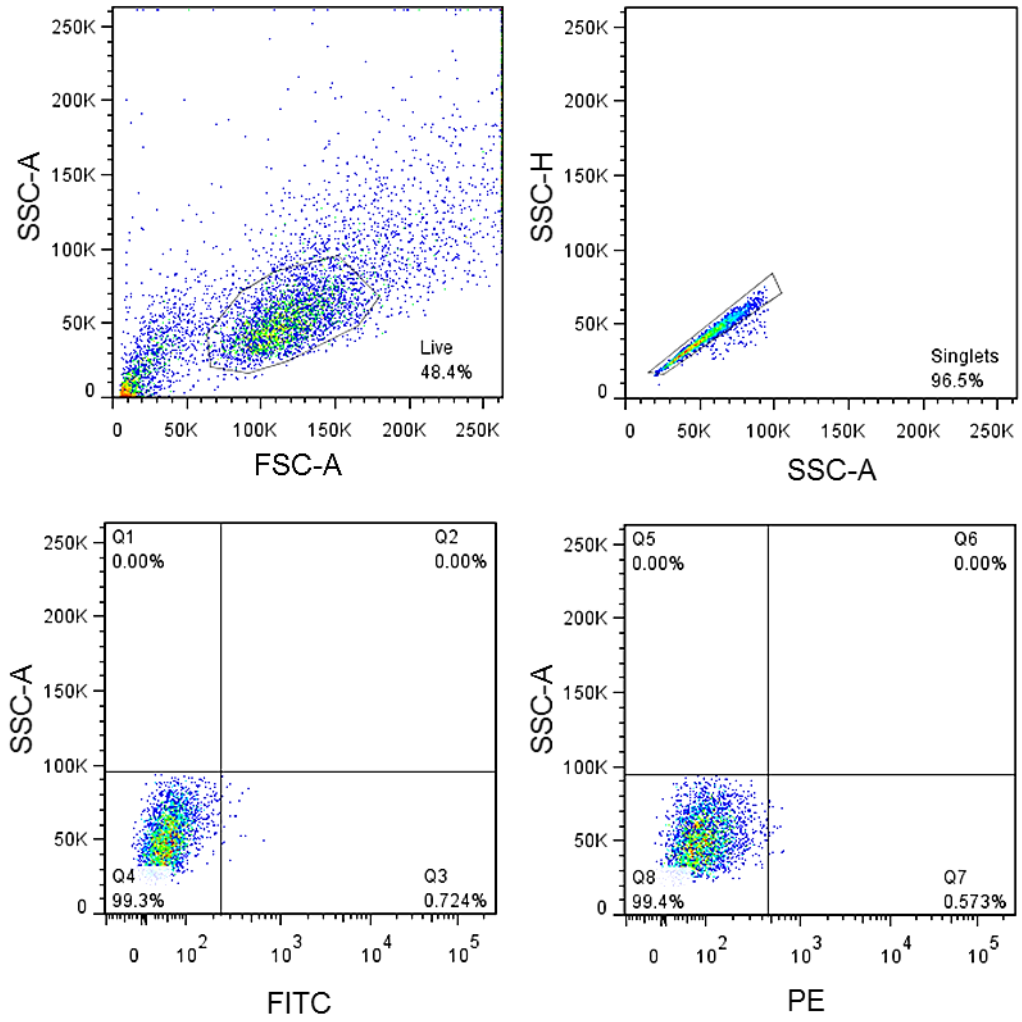

Figure 2.2: Gating strategy applied to the phenotypic analysis of cells by flow cytometry. FSC-A vs SSC-A: live population of cells based on size and granularity. SSC-A vs SSC-H: singlet population, excluding doublets or aggregates of cells. SSC-A vs FITC: FITC-stained positive cells; SSC-A vs PE: PE-stained positive cells.

2.3.1.2 Fluorescence-activated cell sorting (FACS)

Cells were harvested as detailed in Chapter 2.2.1, carefully filtered through a 5ml cell strainer-capped tube (BD Biosciences, UK), counted with a haemocytometer and resuspended at $5x10^6$ cells per ml. $1x10^5$ cells were removed and kept as the unstained control sample, while the rest were incubated with the appropriate antibody (10µl per $5x10^6$ cells) for 30 minutes at room temperature. The stained and unstained samples were washed in PBS, centrifuged for 5 minutes at 10,000 RPM and resuspended in culture medium adjusted to 2% serum. 10% serum culture medium was prepared for collection of cells after sorting. Immediately prior to sorting, the cells were stained with 7-AAD, a viability stain, which can enter dead cells through disrupted membranes and bind to DNA. Cells were sorted through a BD FACSAria II machine. Gating strategy of the cell populations based on unstained controls was similar to that used for analytical flow cytometry, with an extra stain (7AAD) to ensure elimination of dead cells prior to sorting (Figure 2.3).

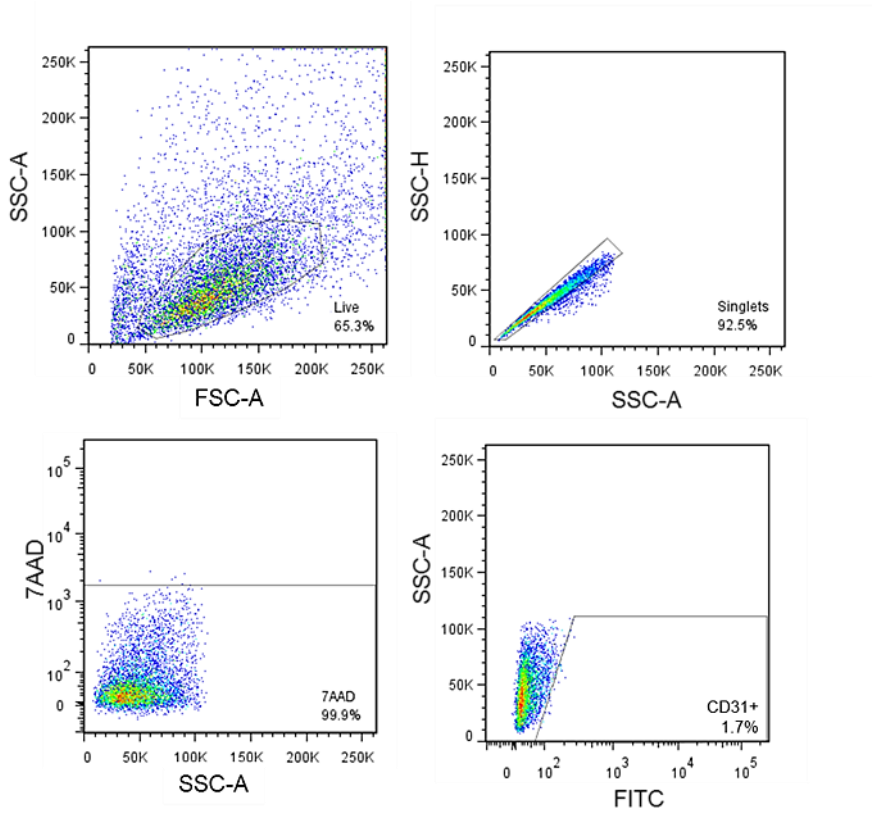

Figure 2.3: Gating strategy (based on unstained control) applied for FACS isolation of CD31+ cells. FSC-A vs SSC-A: live population of cells based on size and granularity. SSC-A vs SSC-H: singlet population, excluding doublets or aggregates of cells. SSC-A vs 7AAD: 7AAD negative cells (live). SSC-A vs FITC: CD31 positive cells.

Upon collection, cells were centrifuged for 3 minutes at 8,000 RPM and resuspended in culture medium at a volume in accordance with cell count and plate size. Media was changed after 24 hours and then every 2-3 days.

2.3.2 Immunocytochemistry

Cells were fixed by washing twice with PBS and then incubating with 4% PFA for 10 minutes at 4°C. They were again PBS washed twice and left in PBS at 4°C; the plate was sealed with Parafilm® (Bemis Flexible Packaging, USA) for long term storage. If permeabilisation was required (for intracellular staining), the cells were permeabilised with 0.1% Triton X (Sigma, UK) for 5 minutes on a rocking platform. To prevent non-specific antibody binding they were then blocked with 10% Normal Goat Serum/ Phosphate buffered saline/ Triton X (NGS-PBST) for 30 minutes. Following three PBST washes, samples were incubated with the appropriate primary anti-human antibodies overnight at 4°C, or for 1 hour at room temperature (Table 2.5). One well per 'raised in' species was used as a negative control - instead of the primary antibody, samples were incubated with either isotype control for mouse primary antibody (Invitrogen, UK) or isotype control for rabbit primary antibody (Invitrogen, UK).

Antibody	Manufacturer	Isotype	Raised in	Dilution
eNOS	Abcam	IgG	Rabbit	1:200
vWF	BD Biosciences	Ms $IgG_{1, K}$	Mouse	1:250
CD31	Dako	$IgG_{1, K}$	Mouse	1:100
TRA160	Millipore	IgM	Mouse	1:200
NANOG	Abcam	IgG	Rabbit	1:200
OCT3/4	Santa Cruz	IgG_{2b}	Mouse	1:400

Table 2.5: Primary mouse and rabbit anti-human antibodies used in immunocytochemistry. Antibodies typically used to stain endothelial cells; antibodies typically used to stain stem cells.

After primary antibody incubation, cells were washed with PBST and stained with the appropriate secondary fluorophore-conjugated antibody, either AF488 Goat Anti-Mouse IgG (Invitrogen, UK) to detect antibodies raised in mouse, or AF568 Goat Anti-Rabbit IgG (Invitrogen, UK), to detect antibodies raised in rabbit. Samples were then incubated at room temperature for one hour, after which they were washed with

PBST, followed by dH_2O, to remove any saline, which may crystallise. A droplet of Prolong gold anti-fade DAPI (Life Technologies, UK) was applied to the cells as a nuclear stain, then they were cover-slipped and stored at 4°C in the dark until imaging on Zeiss Observer microscope with Axiovision software 4.8 (Carl Zeiss microscopy).

2.3.3 Embryoid body assay

A widely used method for pluripotency assessment *in vitro* is the formation of embryoid bodies (EBs) from stem cells. EBs are three-dimensional aggregates of stem cells, in which spontaneous differentiation gives rise to cells derived from all three primary germ layers (Sheridan *et al.*, 2012).

Wells of confluent ES and iPS cells were aggregated into embryoid bodies, using cell scrapers, and resuspended on ultra-low adherent plates. The cells were maintained for seven days in modified iPS culture media (Chapter 2.1). Then they were seeded down on 0.1% gelatin-coated plates in the same media and maintained for 10 days. After this period, the cells were fixed and immunostained with markers of all three germ layers: mesodermal (smooth muscle actin), ectodermal (beta-tubulin) and endodermal (alpha-fetoprotein) markers, as described in Chapter 2.3.2.

2.4 Genotypic analysis

2.4.1 Reverse transcriptase polymerase chain reaction

2.4.1.1 RNA extraction

Cultured cells were washed twice with PBS and lysed by treatment with Trizol (Life technologies, UK; 1ml/well of a 6 well plate). Immediately after Trizol application, the trizol/cell mixture was collected in a 1.5ml Eppendorf tube; at this point samples could be stored long term at -80°C if required. For phase separation, 200µl chloroform was added per 1ml of trizol sample and the tubes vortexed for 15 seconds. Samples were incubated on ice for 10 minutes and then centrifuged for 15 minutes at 12000 RPM at 4°C. The upper aqueous phase containing the RNA extract was transferred to a new tube and isopropanol was added at a 1:1 volume ratio to precipitate the RNA from the aqueous phase. This mixture was incubated on ice for 30 minutes and then centrifuged at 12000 RPM for 15 minutes at 4°C to pellet the RNA. The supernatant was discarded and the RNA pellet washed with 70% ethanol. The sample centrifuged again at 7500 RPM for 5 minutes at 4°C. The ethanol was then carefully pipetted off and the pellet air-dried for 10 minutes. 20µl RNase-free H_2O was then added, and the pellet resuspended. After 5-10 minutes RNA concentration was measured with a NanoVue spectrophotometer (GE Lifesciences, UK). Extracted RNA was treated to remove potential contaminants. For this, the SV Total RNA Isolation System was used (Promega, UK). The following reaction mix was set up on ice: 1µg extracted RNA, 1µl Promega buffer, 2µl Promega DNase, made up to 10 µl with Nuclease free H_2O. The reaction was incubated at 37°C for one hour, then 1µl DNase stop reagent was added and the sample incubated at 65°C for 10 minutes. The quality of the RNA was assessed by measuring the ratio of absorbance at 260 versus 280nm using a NanoVue spectrophotometer (GE Lifesciences, UK). RNA values greater than 1.8 were acceptably pure to use for cDNA synthesis, as advised by GE Lifesciences; lower values indicate the presence of protein, phenol or other contaminants.

2.4.1.2 cDNA synthesis

RNA was reverse transcribed into cDNA with the High Capacity cDNA transcription kit (Applied Biosystems, Roche, UK). The following reaction was set up in an 0.5ml PCR tube on ice: 0.5µg RNA, 2µl 10X RT buffer, 2µl 10X RT Random Primers, 0.8µl 25X dNTP Mix (100mM), 1µl Multiscribe Reverse Transcriptase, made up to 20µl with Nuclease free water. In parallel, tubes were set up for each sample without reverse transcriptase (multiscript) as a negative control. The tubes were transferred to the Hybaid express PCR thermal cycler for the following incubation: 25°C for 10 minutes, 37°C for 120 minutes, 85°C for 5 minutes, held at 4°C. The reaction product, cDNA, was stored at -20°C or used immediately for PCR amplification. Again, cDNA quality was assessed using a NanoVue spectrophotometer. cDNA 260 versus 280nm ratios which were greater than 1.8 were acceptable to use for PCR. cDNA samples were diluted 1:1 with nuclease free water before PCR amplification.

2.4.1.3 Polymerase Chain Reaction

To each PCR tube, the following were added: 10µl AmpliTaq Gold PCR master mix (Applied Biosystems, Roche, UK), 2µl forward primers, 2µl reverse primers (Table 2.6), and 1µl template cDNA. The reaction mix was made up to 20µl per tube with nuclease free H_2O. The forward and reverse primer sequences for each gene are displayed in Table 2.6. The samples were loaded into the Hybaid PCR express thermal cycler. Standard PCR cycle parameters were as follows: initiation (required for hot start polymerases to be heat activated) at 95ºC for 5 minutes, denaturation at 95ºC for 15 seconds, annealing at 60ºC for 15 seconds, extension at 72ºC for 65 seconds, final elongation at 72ºC for 7 minutes and final hold at 4ºC. Denaturation, annealing and extension steps were repeated for 30 cycles. Taq DNA polymerase acts by extending the annealed oligonucleotides to synthesise DNA from the 3‘end of the oligonucleotide. This process of denaturation, annealing and synthesis is repeated numerous times, with each newly synthesised DNA acting as a template for the next

reaction. Agarose gel electrophoresis (Chapter 2.4.1.4) was used to ensure that the PCR product was the correct size.

Plasmid-spanning primer sequences: **exogenous**			Product size
OCT 3/4	Forward	CATTCAAACTGAGGTAAGGG	134bp
	Reverse	TAGCGTAAAAGGAGCAACATAG	
KLF4	Forward	CCACCTCGCCTTACACATGAAGA	282bp
	Reverse	TAGCGTAAAAGGAGCAACATAG	
SOX2	Forward	TTCACATGTCCCAGCACTACCAGA	160bp
	Reverse	TTTGTTTGACAGGAGCGACAAT	
L-MYC	Forward	GGCTGAGAAGAGGATGGCTAC	258bp
	Reverse	TTTGTTTGACAGGAGCGACAA T	
Genome-spanning primer sequences: **endogenous**			
OCT 3/4	Forward	CCCGCCGTATGAGTTCTGTG	130bp
	Reverse	CATCGGAGTTGCTCTCCACC	
KLF4	Forward	AGCATCGTGGCCCCGGAAAAGGACC	152bp
	Reverse	TGATTGTAGTGCTTTCTGGCTGGGCTCC	
SOX2	Forward	AGTCCGAGGCCAGCTCCA	119bp
	Reverse	TAGTGCTGGGACATGTGAAGTC	
L-MYC	Forward	TACCCTCTCAACGACAGCAG	136bp
	Reverse	GTCTCCTCATGGAGCACCAGG	

Table 2.6: Primer sequences, and their PCR product sizes, for the detection of episomal plasmid (exogenous) or genomic (endogenous) Oct3/4, Klf4, Sox2 and L-myc. Bp= base pair.

2.4.1.4 Agarose gel electrophoresis

Agarose gel electrophoresis was used for separation of different sized DNA fragments within a sample. A 1.5% agarose gel solution was prepared in 1X Tris/Borate/EDTA (TBE) buffer (Chapter 2.1). Heating was required to dissolve the agarose; once cooled, 3X GelRed (Biotium, UK) was added and the mixture poured

into a gel tray containing a gel comb to create the wells. This was allowed to set, then 10X loading dye (Thermo Scientific, UK) was added to the DNA samples (3.2µl per 20µl sample). The samples were loaded into the gel wells, along with a 1kb GeneRuler marker (Fermentas, UK). DNA gels were typically run at 120 V in 1X TBE buffer. Bands were visualised using a UV light source (Sygene), and imaged with GeneSys software.

2.4.2 Detection of genetic variations via single nucleotide polymorphism array

In order to verify that there were no aberrations in genomic DNA following reprogramming, iPS karyotypes were analysed for duplications, deletions and areas of homozygosity, via SNP array.

DNA extraction was performed using MasterPure Complete DNA and RNA Purification Kit (Epicentre, UK). First of all, the cell samples were lysed. Approximately 0.5-1.0x10^6 cells were collected per well and pelleted by centrifugation. The supernatant was discarded and the pellet resuspended in Tissue and Cell Lysis solution containing Proteinase K at a dilution of 1:300. This was mixed thoroughly and incubated at 65°C for 15 minutes; the sample was vortexed every 5 minutes. 1µl of 5mg/ml RNase A was added per sample and the samples incubated for 30 minutes at 37°C. After this, the samples were placed on ice and 150µl MPC Protein Precipitation Reagent added. They were vortexed for 10 seconds, then the debris pelleted by centrifugation at 4°C for 10 minutes at 10 000G. The supernatant was transferred to a clean tube and the pellet (containing protein and other contaminants) discarded. 500µl isopropanol was added to each sample of recovered supernatant and the tubes inverted 30-40 times. The DNA was pelleted by centrifugation at 4°C for 10 minutes. The DNA pellet was rinsed twice with 70% ethanol and resuspended in 35µl TE buffer. DNA concentration and purity was measured using a NanoVue spectrophotometer. The samples were processed by AROS (Denmark) who analysed the samples with the HumanCytoSNP-12 DNA Analysis BeadChip Kit (Illumina, UK).

2.5 *In vitro* functional analysis

2.5.1 Vascular tubule formation

A key feature of endothelial cells is their capacity for angiogenesis, the sprouting of new blood vessels from pre-existing ones, which occurs in both physiological and pathological processes (Lamalice *et al.,* 2007). Angiogenic potential of endothelial cells can be tested *in vitro.* When cultured on Matrigel (a laminin-based substrate; BD biosciences, UK), endothelial cells form connections that resemble tubules. The movement of endothelial cells and the connections they form with each other is an indication of their ability to form new vessels through angiogenesis *in vivo*. As this assay is fairly simple and quick to perform, it has become a prominent in the *in vitro* study of angiogenesis (Grant *et al*., 1989).

Phenol free, GFR Matrigel was thawed at 4°C until it reached a gel state. 150µl Matrigel was plated out per well of a 48 well plate. The surrounding wells were filled with PBS. The plate was incubated at 37°C for 1 hour. Meanwhile, cells were harvested, filtered through a 20µm cell strainer and counted. 20 000 cells were suspended in 250µl medium and applied to the Matrigel plates. After 24 hours, the plates were imaged with the Zeiss Observer microscope, connected to an AxioCam-mRM camera (Carl Zeiss microscopy, Germany) and processed via AxioVision 4.8 software (Carl Zeiss microscopy, Germany). The whole well was photographed using the Axiovision Mosaic tool. Images were quantified by ImageJ to count the number of tubules formed, and the number of junctions between tubules.

2.5.2 Wound healing

A scratch assay was used for *in vitro* modelling of the processes of cell migration and proliferation that occur during wound healing. In this assay, the endothelial monolayer is scratched; the cells at the edges of the scratch are observed over time as they move towards each other, until the wounded area is recovered and cell-cell

connections are re-established. This assay mimics the processes of migration and proliferation that occur *in vivo* in response to endothelial denudation of blood vessels (Haudenschild and Schwartz, 1979).

Cells were grown to confluence in 6 well plates. A P1000 pipette tip was used to mark a vertical line down the middle of the well. The wounded area was washed and maintained in Endothelial Medium at 37°C in a humidified 5% CO_2 incubator. The width of the wounded area was imaged at time points: 0hr, 6hr and 24hr, using the Zeiss Observer microscope. The Axiovision Mosaic tool was used to image the whole well. Data were quantified as percentage area of cell coverage over time, calculated by ImageJ software (National Institutes of Health, USA).

2.5.3 Nitric oxide release

A key function of endothelium is the release of vasodilator Nitric Oxide (NO). In order to assess NO release *in vitro*, the 'Total NO/Nitrate/Nitrite Assay' kit was used (R&D Systems, UK). Confluent layers of cells were stimulated with ACh (10µM) (Miochol-E; Bausch + Lomb, UK). Supernatant was collected after 24 hours, centrifuged to remove debris and stored at -20°C until the assay was run.

Due to the short half-life of NO, the R&D kit measures the concentrations of its metabolites: nitrite and nitrate, in the cell supernatant. The assay utilises the Griess reaction- the measurement of organic nitrite compounds within a sample. Thus an initial step of enzymatic conversion of nitrate to nitrite is required, by use of nitrate reductase. Firstly the concentration of endogenous nitrite was measured, then endogenous nitrate was converted to nitrite, so that total nitrite could be measured. Endogenous and converted nitrite were detected colourimetrically as azo dye products (which absorb light at 540-570nm) of the Griess reaction, a test widely used for the detection of organic nitrite compounds (Eid *et al.*, 2007; Kuklinska *et al.*, 2009). Standard dilutions of both nitrite and nitrate (provided with the kit) were run in parallel with the samples, ranging from 0 to 200µmol/L. Negative controls were also included: blank wells and media-only controls. Standard curves were plotted

along a log of concentration verses log corrected optical density. The equation of the line of best fit was used to calculate the concentrations of the samples.

2.6 *In vivo* functional analysis

2.6.1 Sponge implantation

To assess the function of derived endothelial cells *in vivo*, the murine subcutaneous sponge implantation model was used. This model is widely used to assess the influence of compounds (Hague *et al.*, 2002; Small *et al.*, 2005) or cells (Barclay *et al.*, 2012) on spontaneous vascularisation of subcutaneous sponges. The protocols described here are derived from Barclay *et al.* (2012). All animal experiments were carried out in accordance with the British Home Office Animals (Scientific Procedures) Act 1986.

Cells to be implanted were harvested and counted. 1 x 10^5 cells per sponge were suspended in 100µl 1:1 EGM2/ phenol free GFR Matrigel (BD Biosciences, UK). Sponges were compressed into this suspension in 0.5ml eppendorf tubes, and incubated at 37°C for 30 minutes to allow the Matrigel to reach a gel-like state. Matrigel-only sponges were prepared as matched controls. Male NSG mice aged 10-12 weeks (Charles River, UK) were anaesthetised intraperitoneally with Domitor (containing Medetomidine; Orion Pharma, UK) and Vetalar (containing Ketamine; Pfizer, UK); both were administered at 0.1ml per 10g body weight. Upon sedation, an analgesic, Vetergesic (containing buprenorphine; Alstoe Animal Health, UK), was administered subcutaneously at 0.1mg per kg body weight. The site of surgery was shaved and wiped with anti-bacterial surgical swab. The skin was 'nicked' with fine scissors, and subcutaneous pouches were created with curved forceps and scissors. Matrigel-only sponges were implanted into the left flank and cell-embedded sponges in the right. The sides of the wound were apposed with forceps and closed using skin staples (9mm Autoclips, Harvard Apparatus, USA). After surgery, a reversal agent, Antisedan (containing Atipamezole; Orion Pharma, UK) was administered subcutaneously at 0.05ml per 10g body weight and the mice observed on a heat mat until recovery. The wound was checked every day for 3 days post-surgery and after 7 days the staples were removed. After 21 days, mice were sacrificed by cervical

dislocation and the sponges removed. Sponges were fixed in 4% PFA overnight, then transferred to 70% IMS and stored at 4°C.

2.6.2 Sponge processing

Fixed sponges were processed by the histology service, QMRI, University of Edinburgh. Briefly, sponges were embedded in paraffin wax, and 4µm transverse sections were cut and adhered onto poly-l-lysine coated slides. Slides were oven dried overnight at 37°C, then dewaxed by xylene and rehydrated through a series of 5 minute emersions in ethanol, at 99%, 90% and 70%.

Sections to be assessed for vessel density (Chapter 2.6.3) were stained with haematoxylin and eosin (H&E) for five minutes, rinsed in distilled water, dipped in acid alcohol and rinsed in distilled water for a further 15 minutes. Slides were then immersed in eosin for one minute and then washed with distilled water. Stained slides were dehydrated prior to cover slipping, by emersion for one minute in 95% ethanol, followed by one minute in 100% ethanol, then five minutes in xylene.

2.6.3 Vessel density counts

H&E stained sponge sections were scored by means of a Chalkley count, to determine blood vessel density (Fox *et al.,* 1995; Hague *et al.,* 2002). A 25-point Chalkley eyepiece graticule was used to examine the sponge sections at x40 magnification. Three non-overlapping counts were performed in the areas with the highest vascularity, subjectively identified under low magnification (x10); slides were examined blinded to whether the sponge contained cells or was a Matrigel control. The eyepiece was rotated to the point where the maximum number of vessels was overlaid by dots. The mean of these triplicate counts was taken on two slide sections per sponge to give the overall Chalkley score.

2.6.4 Immunocytochemistry

Paraffin sectioned sponges were also analysed by immunocytochemistry, to determine whether the cells lining the vessels were of mouse or human origin.

Vessels were identified by staining with either SMA or CD31 antibodies (raised in rabbit) reactive to both mouse and human cells. These were paired with human specific antibodies (CD31 and CD146, respectively), raised in mouse (Table 2.7), for the detection of human cells within the sponges.

Paraffin embedded sponge sections were first dewaxed in 100% xylene (10 min) and rehydrated by immersion (5 min in each) in a graded sequence of alcohols: 100% - 90% - 80% - 70% ethanol, and dH_2O. Heat-induced epitope retrieval was then performed: the slides were placed in simmering 1X EDTA solution for 20 minutes in a water bath. They were cooled in running tap water, then transferred to a Sequenza staining rack. 3 drops of Image-It-Fx signal enhancer were applied to each slide, then slides were blocked with PBST-10% NGS for 30 minutes. The slides were then washed three times with PBST (wash medium) and the primary antibodies added at the appropriate dilution (Table 2.7).

Antibody pairing	Primary antibody	Raised in:	Reactive to:	Manufacturer	Dilution	Secondary antibody stain
Set 1	Isotype matched IgG control to mouse or rabbit primary antibodies; negative control					AF488, green and AF568, red
Set 2	Smooth Muscle Actin	Rabbit	Mouse/ Human	Abcam	1:100	AF488, green
	CD31	Mouse	Human	Dako	1:200	AF568, red
Set 3	CD31	Rabbit	Mouse/ Human	Epitomics	1:100	AF488, green
	CD146	Mouse	Human	Abcam	1:100	AF568, red

Table 2.7: Details of the primary and secondary antibodies used for immunocytostaining of paraffin embedded sponge sections. Each set comprised an antibody cross reactive to mouse and human (stained green), and an antibody specific to human cells (stained red). Antibodies were diluted with PBST-2% NGS.

200µl of each antibody set was added per slide, and they were incubated either overnight at 4°C, or for 1 hour at room temperature. The slides were then washed

five times with wash medium, and stained with secondary antibodies (fluorescent conjugates). AF568 Goat Anti-Mouse IgG (Invitrogen, UK) and AF488 Goat Anti-rabbit IgG (Invitrogen, UK) were prepared in PBST-2% NGS at dilutions of 1:200; 200µl added to each slide. They were incubated at 37°C for 3 hours, then washed five times with wash medium. The slides were removed one at a time from the Sequenza rack and washed in tap water, blotted, and Prolong Gold Antifade Reagent with DAPI applied. Slides were cover-slipped and stored at 4°C in the dark until analysis using light microscopy. Stained sponges were visualised using the Zeiss microscope (Axiovision software) or the Olympus BX61 microscope (Velocity software) for higher resolution. The Matrigel-only sponges were used as positive mouse controls, and human fibroid tissue as positive human controls.

2.7 Endothelial differentiation of ES and iPS cell lines

Optimal endothelial differentiation of ES and iPS cell lines was developed through comparison of four different protocols. These protocols were based on preliminary investigations, published protocols and collaboration with Professor Andrew Baker's laboratory (University of Glasgow).

Many protocols begin with transferring stem cell populations to standard Differentiation Medium (αMEM, FCS, β-mercaptoethanol; Sone *et al.*, 2007; Narazaki *et al.*, 2008; Choi *et al.*, 2009), and this medium was found to produce the highest endothelial differentiation efficiency from previous studies in the laboratory (E Skinner, MSc in Cardiovascular Biology, 2010). Cells were isolated using FACS based on their expression of CD31 or CD34, 7 or 14 days after plating.

In endothelial differentiation protocol A (Figure 2.4), stem cells were transferred to a single-cell monolayer culture system, on collagen I-coated plates in differentiation medium. At day seven, cells negative for SSEA3 and SSEA4 pluripotency markers were isolated via FACS sorting and further cultured in endothelial medium on collagen I. At day fourteen, $CD31^+$ cells were sorted and further cultured on collagen I-coated plates in endothelial medium.

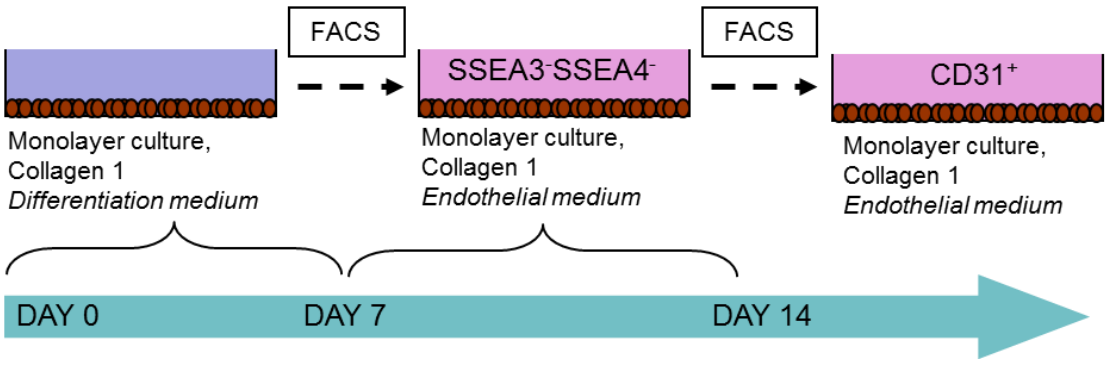

Figure 2.4: Endothelial differentiation protocol A. Stem cells were plated onto collagen I in differentiation medium. After seven days, SSEA3-SSEA4- cells were isolated by Fluorescence-Activated Cell Sorting (FACS) and replated on collagen I in endothelial medium. Cells expressing CD31 were isolated by FACS on day 14 and culture continued on collagen I in endothelial medium.

This protocol was adapted to form endothelial differentiation protocol B (Figure 2.5), eliminating the initial pluripotent sort. Stem cells were transferred to a single-cell monolayer culture system, on collagen I-coated plates in differentiation medium. At day seven, $CD31^+$ cells were isolated via FACS sorting and further cultured in endothelial medium on collagen I-coated plates.

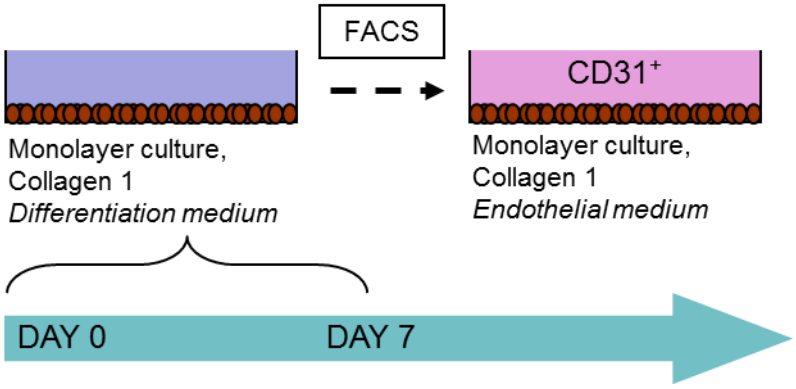

Figure 2.5: Endothelial differentiation protocol B. Stem cells were plated onto collagen I in differentiation medium. After seven days, cells expressing CD31 were isolated by Fluorescence-Activated Cell Sorting (FACS) and replated on collagen I in endothelial medium.

Some published protocols use the addition of mesoderm-inducing cytokines (for example, BMP4, Activin A, VEGF, FGF) for endothelial differentiation (James *et al.*, 2010; Goldman *et al.*, 2009; Ferreira *et al.*, 2007). Protocol C (Figure 2.6) was developed based on the studies of Ferreira *et al.* (2007). Stem cells were transferred to a single-cell monolayer culture system, on collagen I-coated plates in differentiation medium. At day seven, $CD34^+$ cells were sorted and further cultured on collagen I coated plates in endothelial medium, with or without 50ng/ml VEGF supplementation.

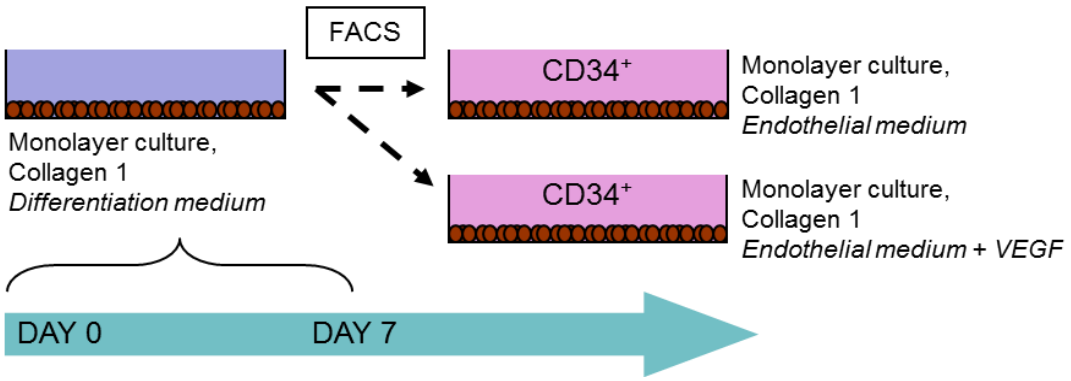

Figure 2.6: Endothelial differentiation protocol C. Stem cells were plated onto collagen I in differentiation medium. After seven days, cells expressing CD34 were isolated by Fluorescence-Activated Cell Sorting (FACS) and replated on collagen I in endothelial medium, with or without VEGF supplementation.

After collaboration with the University of Glasgow, unpublished protocol D (Figure 2.7) was adopted. Stem cells were aggregated into EBs in suspension with mesoderm-inducing cytokines: 10 ng/ml BMP4, 10 ng/ml Wnt3a, 10 ng/ml VEGF, 5 ng/ml Activin A and 400 nM BIO. At day two, the following cytokines were added to the suspension: 20 ng/ml BMP4, 10 ng/ml Wnt3a, 10 ng/ml VEGF, 5 ng/ml Activin A, and 10 ng/ml FGFa, along with 400nM BIO (a selective ROCK1 inhibitor; ROCK 1 inhibition is thought to contribute to a reduction in vascular inflammation through upregulation and activation of eNOS, Noma *et al*., 2006). At day three, EBs were disaggregated and the cells transferred to a single-cell monolayer culture system, on 0.1% gelatin in endothelial medium supplemented with 50ng/ml VEGF. At day seven, $CD31^+$ cells were sorted and further cultured on 0.1% gelatin coated plates in endothelial medium with 50ng/ml VEGF supplementation. This protocol was supplied by the University of Glasgow- the decision of which cytokines to use and their concentrations had been previously optimised on ES cell lines H1 and H9. In the interest of time, no further optimisation was carried out.

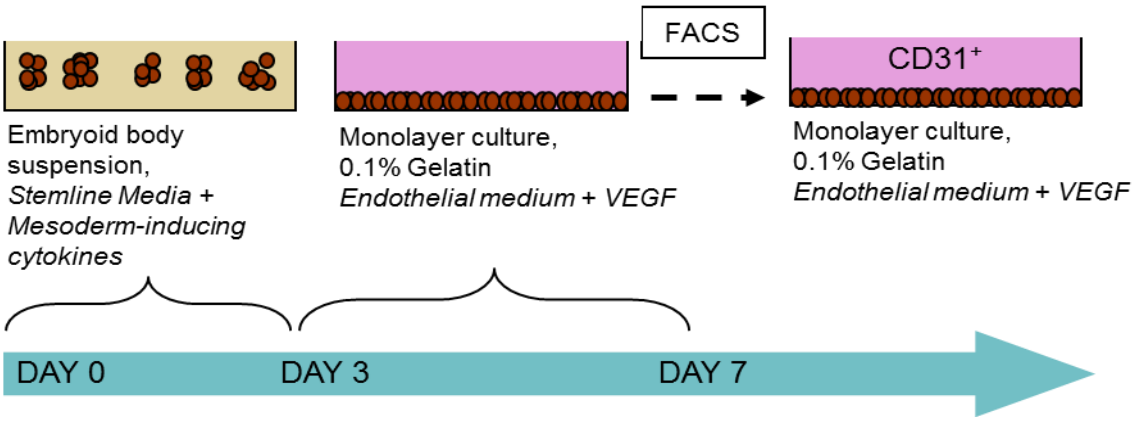

Figure 2.7: Endothelial differentiation protocol D. Stem cells were aggregated into embryoid bodies, and suspended in Stemline media supplemented with mesoderm-inducing cytokines. At day three, embryoid bodies were disaggregated and plated as a monolayer on 0.1% gelatin in endothelium media supplemented with VEGF. At day seven, cells expressing CD31 were isolated by Fluorescence-Activated Cell Sorting (FACS) and further cultured on 0.1% Gelatin in Endothelial medium supplemented with VEGF.

2.8 Cellular reprogramming of fibroblasts and endothelial cells

2.8.1 Episomal vectors

Episomal vectors are circular plasmids which are replicated in the nucleus of eukaryotic cells (Van Craenenbroeck *et al.*, 2000). They were used as a delivery system of the cellular reprogramming factors, a method which was first described by Okita *et al.* (2011). Episomal vectors do not integrate into the host genome and therefore avoid the risk of mutagenesis, a considerable concern associated with traditional retroviral reprogramming methods (Yamanaka, 2009). Three episomal plasmids were supplied by Addgene (Accession numbers: pCXLE-hOCT3/4-Shp53: 27077; pCXLE-hSK: 27078; pCXLE-hUL: 27080; Cambridge, MA, USA; Okita *et al*., 2011). Between them, they contained the four Yamanaka factors (Y4) required for reprogramming: Oct3/4, Sox2, Klf4 and L-Myc, and two factors which increase reprogramming efficiency: Lin28 and Shp53. In parallel, cells were transfected with a single GFP plasmid, as a positive control and an indication of electroporation efficiency (Accession number: 27082; Addgene, Cambridge, MA, USA; Figure 2.8).

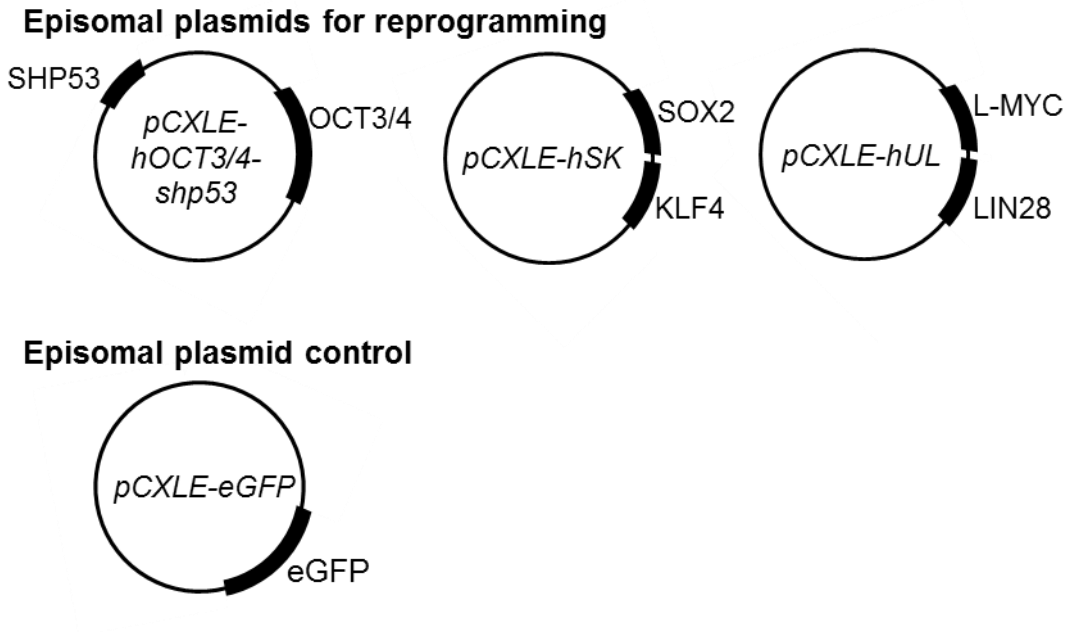

Figure 2.8: Three episomal expression plasmids, with the pCXLE backbone, were used to deliver the reprogramming factors to cells. They contained the Yamanaka factors: Octamer-binding transcription factor-3/4 (OCT3/4), SRY-related-high-mobility-group (HMG)-box protein-2 (SOX2), Krupple-like factor 4 (KLF4), L-MYC, and two factors to enhance reprogramming efficiency: short hairpin RNA against p53 (SHP53) and LIN28. To give an indication of successful transfection, a GFP episomal plasmid control was transfected in parallel.

These plasmids were prepared by maxi-preparation, the large-scale method used to extract and purify plasmid DNA, which was carried out by Olga Tura and Mairi Brittan, using the Genopure Plasmid Maxi Kit (Roche).

2.8.2 Amaxa system: reprogramming of fibroblasts

Fibroblast reprogramming experiments were carried out on the Amaxa system (Lonza, UK), used in conjunction with the Amaxa ® NDHF Nucleofector® Kit (Lonza, UK). $0.5x10^6$ cells were used for each electroporation, and two electroporations were carried out for each cell line: one with the episomal plasmids containing the reprogramming factors, and one with the GFP control plasmids. Cells

were harvested and counted with a haemocytometer. Each $0.5x10^6$ cells were able to take up 5µg DNA: this is enough for efficient reprogramming of the cells whilst maintaining viability. $0.5x10^6$ cells were resuspended in 100µl buffer + supplement (provided by Amaxa kit) and, 5µg of the three plasmids added. The cell/ plasmid/ buffer mixture was carefully pipetted into the electroporation cuvette, and was electroporated with the Amaxa machine, at the 'U-023 NHDF high efficiency' setting (the Nucleofector® program recommended for dermal fibroblast transfection). Cells were plated on 0.1% gelatin in 10% DMEM; antibiotics were omitted from the media for the first 24 hours. Images of the GFP control were taken after 24 hours, to give an indication of successful electroporation and plasmid transfection.

2.8.3 Neon system: reprogramming of endothelial cells

The Neon system provides 24 different nucleofection settings, which vary in terms of pulse number, length and amplitude. From previous experiments in the laboratory carried out by Olga Tura, setting 18 (2 pulses of 950mV for 30ms) appeared to yield the most effective transfection of episomal plasmids into endothelial cells (unpublished data). In later experiments, settings 16 to 20 were also used in an attempt to further optimise endothelial cell reprogramming conditions.

Neon Setting	Number of pulses	mV	Duration of pulses (ms)
16	2	1400	20
17	2	850	30
18	2	950	30
19	2	1050	30
20	2	1150	30

Table 2.8: Neon system electroporation settings 16-20 used for transfection of somatic cells with episomal plasmids containing reprogramming factors.

Cells were harvested, counted and centrifuged into $0.1x10^6$ cell pellets. As before, for each cell line to be transfected, two electroporations were carried out: one with the episomal plasmids containing the reprogramming factors, and one with the GFP control plasmid. Each $0.1x10^6$ cell pellet was resuspended in 10µl Neon buffer, in a V-bottomed tube and plasmids were added (1µg total DNA per $0.1x10^6$ cell pellet). The cell/plasmid solution was carefully drawn up into the 10µl gold tip of the Neon pipette, and electroporated by the Neon system, at the appropriate setting. Electroporated cells were seeded onto pre-warmed collagen I coated plates in endothelial medium; antibiotics were omitted from the media for the first 24 hours. GFP transfected cells were imaged after 24 hours.

In the laboratory, the Amaxa system had previously been used successfully for the reprogramming of fibroblasts, using its fibroblast-specific settings for high efficiency. As there were no endothelial-specific settings for electroporation of endothelial cells, the Neon system was used instead. This had the added benefit of multiple electroporation settings which were required in later experiments.

2.8.4 Culture of transfected cells

Electroporation causes transient permeabilisation of cell membranes, allowing entry of the plasmids. Following transfection, cells were cultured in their original media for seven days (the first 24 hours of which were without antibiotics to prevent them entering the still-permeabilised cell membranes), which was replenished every 2-3 days. After 7 days, the cells were transferred to iPS cell culture conditions in a 9cm culture dish: either on a MEF feeder layer in iPS cell culture medium (Chapter 2.1), or on Matrigel-coated plates with E8 media. Media was replenished every 2-3 days (Figure 2.9). Successfully transfected cells proliferated into clonogenic colonies and could be detected by eye three to four weeks after electroporation. Individual colonies were manually isolated with a wide-tipped P20 pipette tip, and cultured separately in iPS cell conditions. Each colony represented its own cell line.

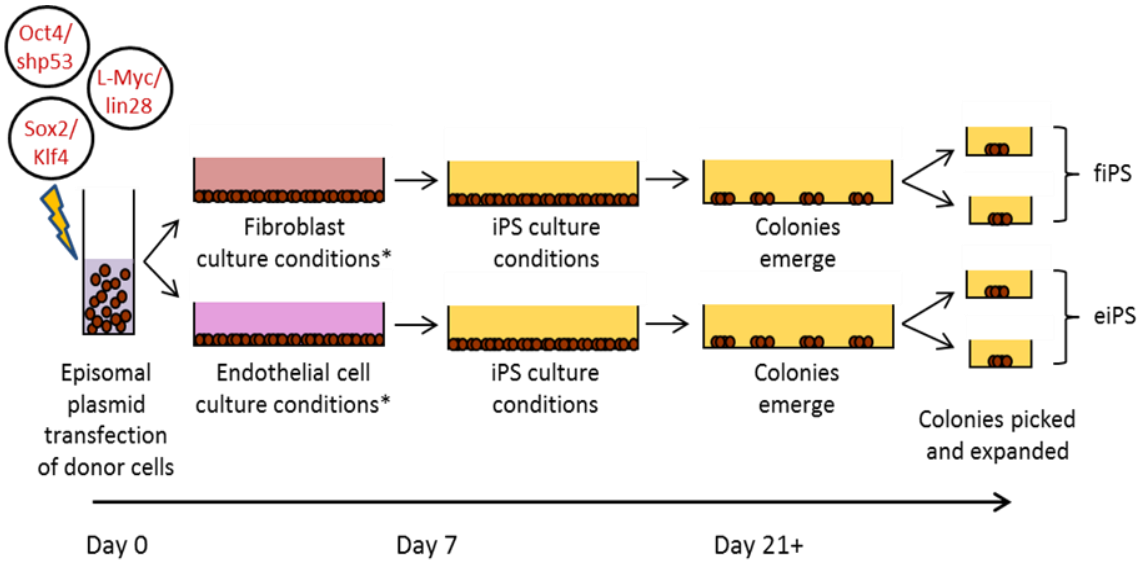

Figure 2.9: Episomal plasmids and protocol for the reprogramming of fibroblasts or endothelial cells, to generate fibroblast-iPS (fiPS) or endothelial-iPS (eiPS) cells. * First 24 hours without antibiotics

2.8.5 Characterisation of reprogrammed colonies

Once the colonies had been established and could be expanded into 6 well plates, they were characterised to verify their pluripotency. Their phenotype was assessed based on detection of pluripotency markers SSEA3, SSEA4, TRA 1 60, Nanog and Oct3/4 by flow cytometry (Chapter 2.3.1) and immunocytochemistry (Chapter 2.3.2). Reverse transcriptase PCR was carried out to identify the presence of endogenous expression of Oct3/4, Sox2, Klf4, L-Myc, and to make sure that there was no longer expression of these genes exogenously, within any remaining episomal plasmids (Chapter 2.4.1). Cells were also used to form embryoid bodies to test whether they could spontaneously differentiate to all three germ layers: an indication of their pluripotency (Chapter 2.3.3). Genomic DNA was extracted to be assessed for any aberrations or alterations following transfection (Chapter 2.4.2). Successfully transfected clones expressed all pluripotency markers, had endogenous rather than exogenous expression of the Y4 genes, could form all three germ layers and had 'normal' genomic DNA, i.e. were free from significant deletions or duplications.

2.9 Data analysis and statistics

All statistical analyses were carried out using Microsoft Excel and Graphpad Prism (GraphPad Software, USA). Quantitative data are shown as mean ± standard error of the mean (SEM) unless otherwise indicated. Student's t tests (paired or unpaired), one-way and two-way ANOVA were used to determine statistical significance of differences between data sets, followed by Bonferroni *post hoc* corrections. Differences were regarded significant when $P<0.05$.

Chapter 3

Induction of pluripotent stem cells from fibroblasts and endothelial cells

Abstract

Introduction: Induced pluripotent stem cell technology primarily focussed on the reprogramming of fibroblasts to an embryonic-stem cell like state. As the field has developed, the importance of cell source has been studied to enhance reprogramming and differentiation efficiencies. There are reports that iPS cell lines, whilst fulfilling the criteria for pluripotency, have enhanced differentiation potential towards their cell type of origin (Kim *et al.*, 2010). Endothelial cells can be obtained from blood outgrowth and would be a convenient source of cells for reprogramming. Furthermore, pluripotent stem cells derived from endothelial cells may have enhanced capacity to reform endothelium, and therefore may have applications in therapeutic vascular regeneration.

Methods and Results: Fibroblasts and endothelial cells (from cord or peripheral blood, and the vessel wall) were obtained from five healthy donors. They were reprogrammed via transfection of episomal plasmids, for delivery of 'Yamanaka' transcription factors Oct3/4, Sox2, Klf4 and L-myc, as well as Lin28 and Shp53. Two out of three fibroblast lines and one endothelial cell line were successfully reprogrammed. Reprogramming efficiency - taken as the percentage of cells from the starting population from which colonies were derived - was low for fibroblasts (0.0002%) and endothelial cells (0.003%). Reprogramming was unsuccessful in all endothelial cell lines derived from vasculature or from peripheral blood. This was despite GFP positive controls run in parallel, demonstrating successful electroporation and plasmid transfection. Fibroblast-induced pluripotent stem (fiPS) cells and endothelial-induced pluripotent stem (eiPS) cells were successfully expanded in culture for multiple passages. Expression of Oct3/4, Sox2, Klf4 and L-myc was apparent at passages 31-35 and appeared endogenous, suggesting plasmids had been shed and cells were inherently pluripotent. Apart from a minor deletion on chromosome 6 of fiPS, both lines were karyotypically normal. Flow cytometry analysis showed high expression of pluripotency markers SSEA3 (fiPS, 79%±6%; eiPS, 79%±5%) and SSEA4 (fiPS, 91%±4%; eiPS, 96%±1%) in late passage cells, whilst immunocytochemistry demonstrated expression of Nanog, Tra 1 60 and Oct3/4 in both lines. Spontaneous differentiation within embryoid bodies (stem cell

aggregates) demonstrated that both fiPS and eiPS could differentiate to all three germ layers, confirming their pluripotent nature.

Conclusions: Pluripotency can be induced in fibroblasts and endothelial cells through episomal plasmid transfection of Oct3/4, Sox2, Klf4, L-myc, Lin28 and Shp53. Reprogramming efficiency, though low, was marginally higher for endothelial cells isolated from cord blood.

3.1. Introduction

Pluripotent stem cells may be used to generate endothelial cells for therapeutic use in the treatment of patients with coronary heart disease or peripheral vascular disease. Induced pluripotent stem (iPS) cell technology offers many benefits: patient-derived stem cells can be stored for long periods of time, are readily expandable, have unlimited capacity for renewal, and would avoid immune-rejection if administered back to the same patient (Yamanaka, 2012; Cherry and Daley, 2013).

Initially, iPS cell technology focussed on the reprogramming of fibroblasts to an embryonic stem cell-like state (Takahashi *et al.*, 2006; 2007). However, as the field has progressed over the past decade, other somatic cell types have been reprogrammed. For example, iPS cells have been derived from pancreatic islet beta cells (Bar-Nur *et al.*, 2011), keratinocytes (Aasen *et al.*, 2008), peripheral blood cells (Loh *et al.*, 2009) and neural progenitor cells (Eminli *et al.*, 2008). These studies suggest that somatic tissues have variable reprogramming efficiencies, many of which may be greater than fibroblasts. For example, induction of pluripotency has been reported as 100 fold more efficient in keratinocytes than in fibroblasts (Aasen *et al.*, 2008). In addition, differentiation efficiency along lineages related to the donor cell may be enhanced (Kim *et al.*, 2010), thought to be a consequence of epigenetic memory.

For these reasons, it could be suggested that iPS cells derived from endothelial cells represent the ideal population for generation of patient-specific endothelium, and are likely to offer benefits over fibroblast-derived iPS cells. Another benefit is that endothelial outgrowth cells are more readily accessible as they are obtained from a blood sample, a minor well-tolerated procedure, rather than fibroblasts which require a skin biopsy. Therefore, endothelial cells obtained from vascular biopsies and endothelial outgrowth from blood (EOC), as well as fibroblasts, were reprogrammed.

3.2. Hypothesis

Endothelial cells can be reprogrammed to pluripotency with greater efficiency than fibroblasts, and, therefore, represent an optimal starting somatic cell population for the induction of pluripotent stem cells.

3.3. Aims

- To compare the reprogramming efficiency of dermal fibroblasts to that of endothelial cells.
- To characterise fibroblast- and endothelial cell-derived iPS cells, and compare them to embryonic stem cell controls.

3.4. Method development

3.4.1. Cellular reprogramming

Typically, somatic cells have been reprogrammed by delivery of transgenes within retroviral vectors (Takahashi *et al*., 2007). However, these integrate into the genome, and, therefore, have the potential to create mutations which may affect differentiation potential, thus limiting the utility of these cells in both research and clinical applications (Varas *et al*., 2009). For these reasons it was decided to use an episomal plasmid system of reprogramming, described by Okita *et al*. (2011) as a viral- and integration-free method which avoids risk of genomic abnormalities (Chapter 2.8).

Somatic cell populations to be reprogrammed were either fibroblasts or endothelial cells, harvested from healthy volunteers as detailed in Chapter 2.2.1. Five healthy donors were used to obtain endothelial cells from the superficial forearm vein, peripheral blood or cord blood. Three of these donors were also used to obtain fibroblasts from skin biopsy (Table 3.1).

Donor reference	Age	Sex	Type	Endothelial cells		Fibroblasts
				Blood	Vessel wall	
D1	30	M	British South Asian	✓	✓	✓
D2	30	F	Caucasian	✓*		
D3	29	F	Caucasian	✓	✓	✓
D4	31	M	Caucasian	✓	✓	
D5	65	M	Caucasian	✓	✓	✓

Table 3.1: Details of five healthy donors, whose somatic cells were used for reprogramming. Endothelial cells were derived either from blood (*cord blood) or the vessel wall. Fibroblasts were derived from skin biopsy.

Cells were reprogrammed as detailed in Chapter 2.8. Of the multiple endothelial lines (four from human peripheral blood and four from vascular biopsies) that were transfected with the reprogramming factors, no colonies formed (Table 3.2). This

indicates that reprogramming efficiency is low. In contrast, a cord blood endothelial cell line (from donor 2) was successfully reprogrammed, as were 2 out of 3 fibroblast lines (from donors 1 and 5). Attempts to improve reprogramming efficiency in peripheral blood and vessel wall endothelial cells were made by altering electroporation parameters. Endothelial cells derived from donors 1, 2, 3 and 4 were electroporated with Neon system setting 18; however, the majority of these lines did not produce colonies. Therefore, settings 16-20 were used for D5 but this did not lead to colony formation (Table 3.2).

Donor reference	**Endothelial cells**		**Fibroblasts**
	Blood	**Vessel wall**	
D1	X^P	X	✓
D2	✓^{C}		
D3	X^P	X	X
D4	X^P	X	
D5	X^P	X*	✓

Table 3.2: Attempted reprogramming of endothelial cells from blood and the vessel wall, and fibroblasts. ✓= colonies arose following Y4 plasmid transfection. X= no colonies arose following Y4 plasmid transfection. All endothelial lines were reprogrammed with the Neon system setting 18. Endothelial cell lines marked * were also reprogrammed with Neon system settings 16, 17, 19 and 20. P= endothelial cells derived from peripheral blood; C= endothelial cells derived from cord blood.

To verify plasmid uptake, a GFP control plasmid was transfected into cells in parallel to reprogramming plasmid transfection. The presence of GFP positive cells indicated successful transfection (Figure 3.1).

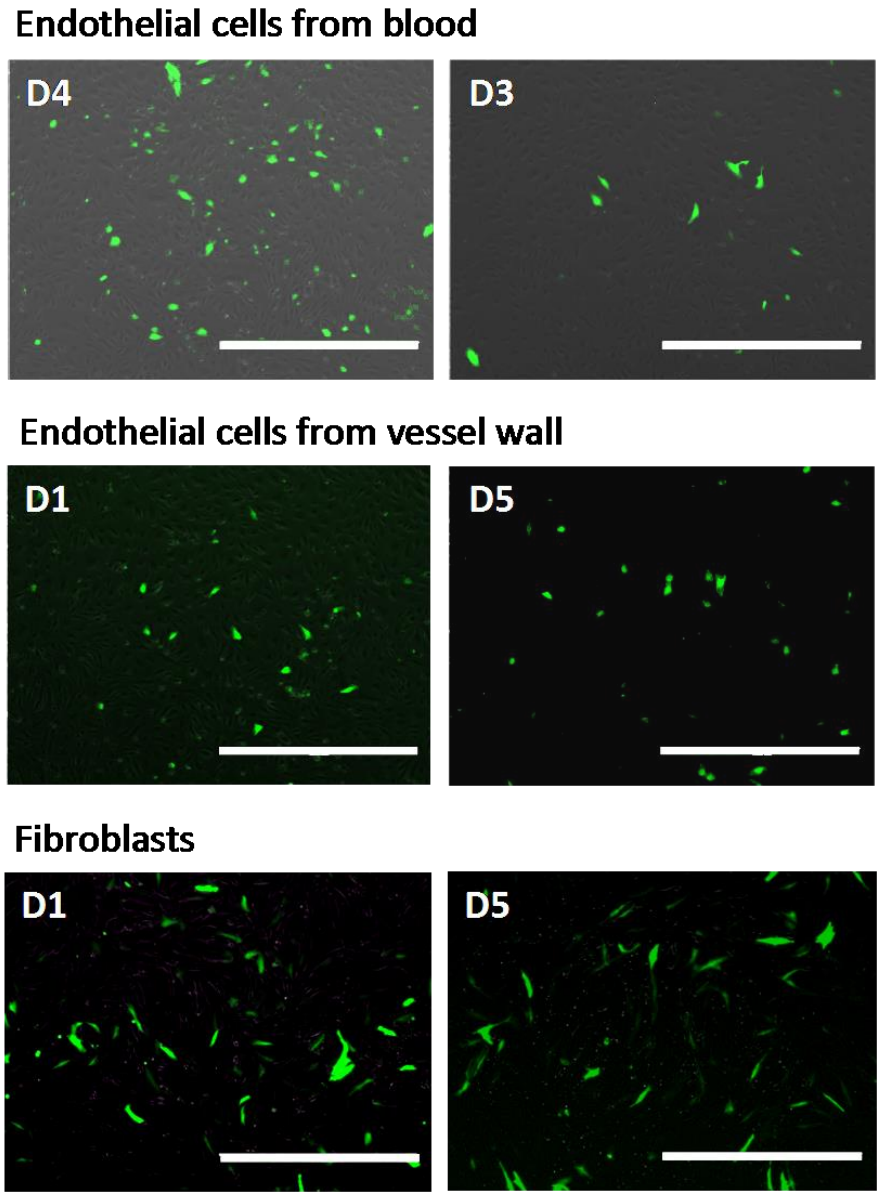

Figure 3.1: Representative examples of expression of GFP 24 hours after electroporation of cells with pCXLE-eGFP, in endothelial cells derived from blood, vessel wall, and fibroblasts derived from healthy donors. Bar: 1mm.

Successfully reprogrammed fibroblast populations exhibited morphological changes approximately 2 weeks after transfection, prior to the emergence of colonies. The cell layer, which once comprised solely long spindle-like fibroblasts (Figure 3.2A), developed areas of small rounded cells (Figure 3.2B). These were the first stages of

development of pluripotent stem cell colonies. Similar morphological changes occurred in successfully transfected cord blood derived endothelial outgrowth cells.

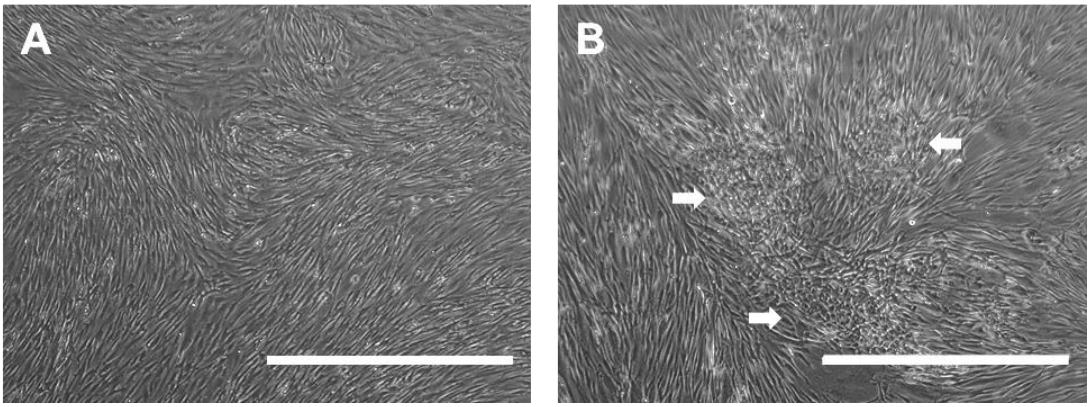

Figure 3.2: Morphological changes occur in culture following reprogramming. A) Monolayer of fibroblasts 5 days after transfection with reprogramming factors, B) Morphological changes occur in the monolayer after approximately 12 days- some cells became small and rounded (arrows). Bar = 1mm

With further time in iPS culture conditions, many of these morphologically distinct areas developed into 'emerging colonies' (Figure 3.3). The small, rounded cells had a high nucleus-to-cytoplasm ratio (determined by eye, not quantitatively verified), typical of embryonic stem cells (Thomson *et al*., 1998). As they grew, they became visible by eye. Each was isolated with a wide-tipped pipette and cultured separately.

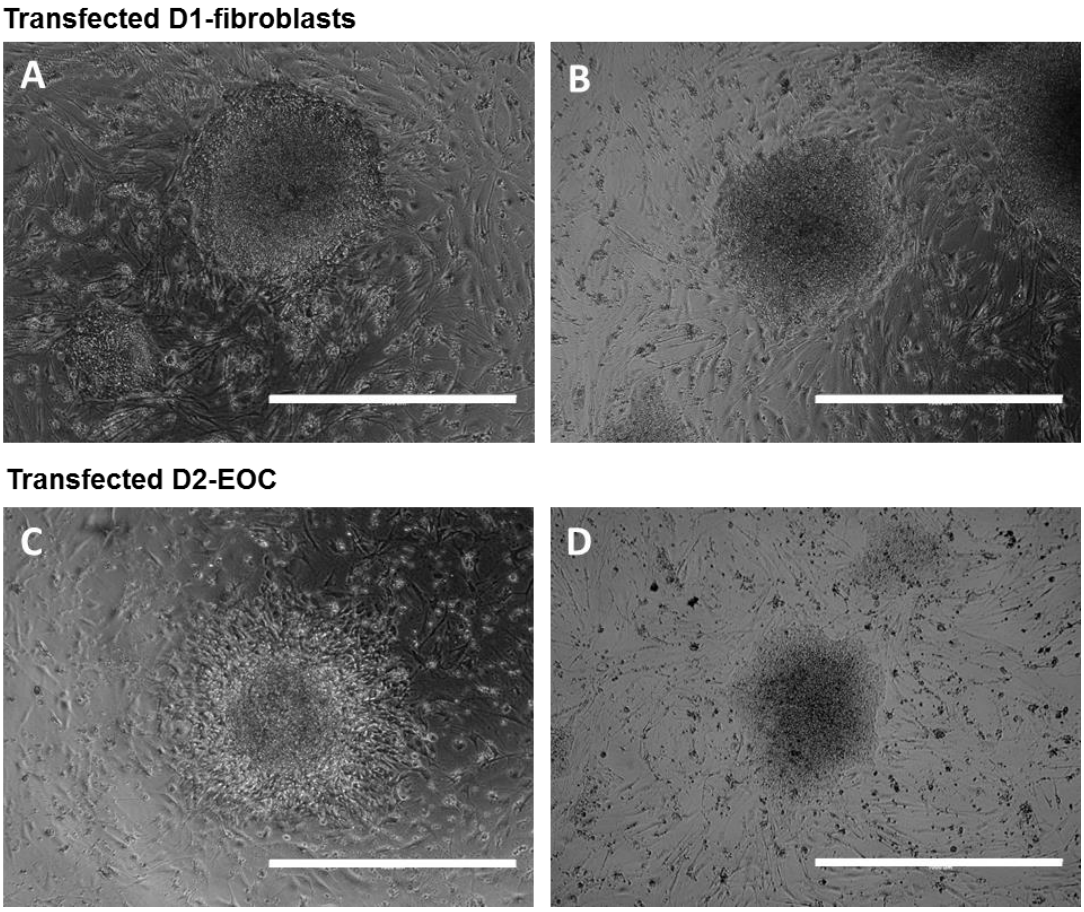

Figure 3.3: Colonies emerge in iPS culture conditions, prior to isolation. The morphology of successfully transfected fibroblasts and EOC changed considerably, to resemble embryonic stem cells. A&B = examples of colonies from D1 fibroblasts, C&D = examples of colonies from D2 cord blood derived endothelial outgrowth cells. Bar = 1mm.

At the early stages (before passage 10) of expanding colonies in culture, passaging and sub-culture was a delicate procedure as the colonies were very small and highly sensitive to cell culture conditions. Consequently, despite labour intensive maintenance (daily observation, aspiration of differentiated areas within the well and media replenishment), some colonies differentiated and were lost over time. This accounts for the difference seen between number of colonies isolated versus number of colonies expanded (Table 3.3).

Reprogramming efficiency was determined by calculating the percentage of cells from the starting population which were successfully reprogrammed and formed established iPS cell lines: number of established colonies generated / number of cells in starting population transfected with reprogramming factors * 100. Reprogramming efficiency was low for all donors and cell types. It was highest in endothelial outgrowth from cord blood (Table 3.3).

Donor reference	**Somatic cell of origin**	**System**	**Starting population**	**Number of colonies isolated**	**Number of colonies expanded**	**Reprogramming efficiency (%)**
D1	Fibroblast	Amaxa	500 000	76	1	0.0002
D5	Fibroblast	Amaxa	500 000	35	3	0.0006
D2	CB-EOC	Neon	100 000	11	3	0.003

Table 3.3: Two fibroblast lines (from donors 1 and 5) and one cord blood-derived endothelial cell line (from donor 2) were successfully reprogrammed, via the Amaxa and Neon systems, respectively. In all cases, the majority of isolated colonies were lost and did not expand. Reprogramming efficiency, calculated as the percentage of cells, which were reprogrammed from the starting population, was low for all lines, but highest in cord blood-derived endothelial cells. CB-EOC: Cord blood-derived endothelial outgrowth cells.

Of the colonies, which expanded and became established lines, the D1-fibroblast line, referred to as 'fiPS' and D2 cord blood EOC line, clone 1 of 3, referred to as 'eiPS', were fully characterised and used for further studies. D1 was was selected over D5 for practical reasons: this line was the first to be reprogrammed and therefore was the first to expand to the required population size.

As the fiPS and eiPS cell lines expanded they became more resilient in culture – the colonies reached approximately 0.5-1.5mm in size and had clearly defined edges with no signs of differentiation (Figure 3.4). The cells were small, round and tightly compacted into colonies – typical of embryonic stem cell-like morphology (Thomson *et al.,* 1998). This appearance was consistent over extended passaging (at least up to passage 35).

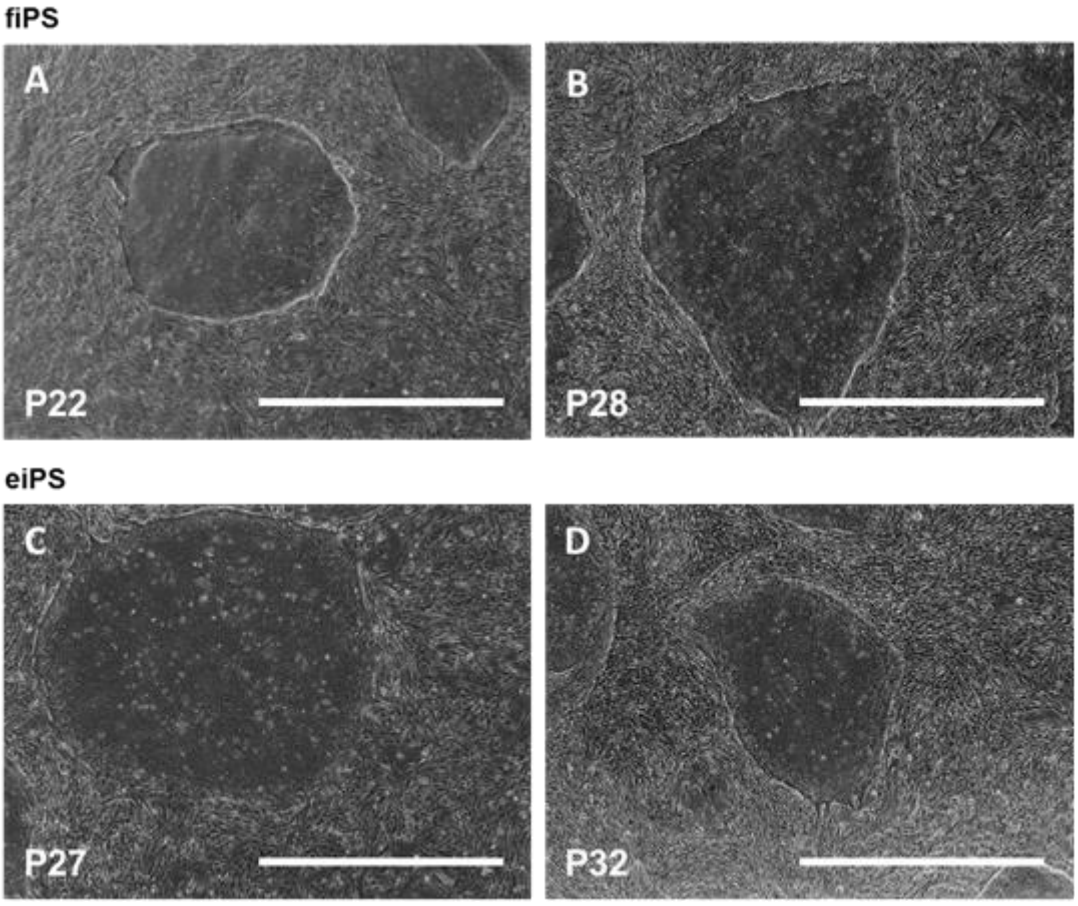

Figure 3.4: After multiple passaging, the morphology of established cell lines fiPS and eiPS resembles embryonic stem cells. Cells are small with a high nucleus-to-cytoplasm ratio and are compacted into distinct colonies. P= passage number. A&B= examples of colonies from D1 fibroblasts, C&D= examples of colonies from D2 cord blood derived endothelial outgrowth cells. Bar=1mm.

The properties of these two lines are shown in the following figures, detailing experiments used to assess pluripotency.

Episomal plasmids replicate once per cell cycle, and are lost at a rate of ~5% per cell cycle due to defects in vector synthesis and partitioning (Yu *et al.,* 2009). Reprogramming factors are required for induction of pluripotency rather than maintenance (Okita *et al.,* 2007), so in *bone fide* iPS cells, over time expression of the Yamanaka factors changes from exogenous (from remaining plasmids) to endogenous (inherent to the properties of the cell). Reverse Transcriptase-

Polymerase Chain Reaction (RT-PCR) was carried out with plasmid or genome specific primers to distinguish whether expression of the reprogramming factors was exogenous or endogenous (Chapter 2.4.1). At passage 35 (fiPS) and passage 31 (eiPS), expression of Oct3/4, Sox2 and L-Myc was endogenous. Klf4 too was detected endogenously, though this was faint in the eiPS, and there appeared to be residual exogenous expression in the fiPS (Figure 3.5). These results indicate that pluripotency is largely inherent to the fiPS and eiPS cell lines, and is not a consequence of residual plasmid expression.

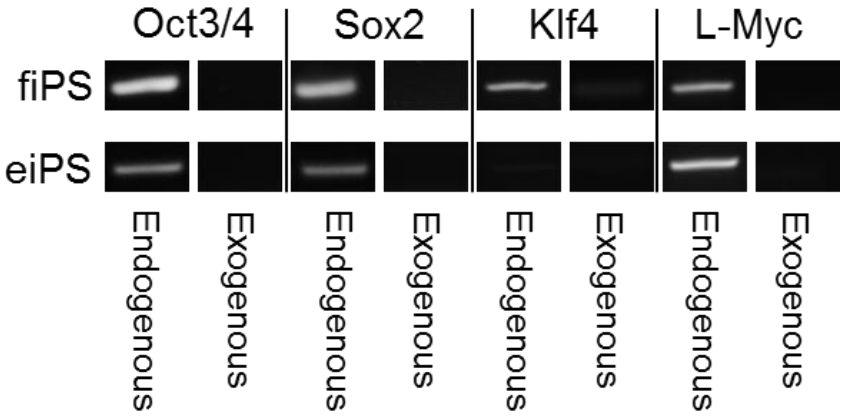

Figure 3.5: Endogenous or exogenous expression of the Y4 factors octamer-binding transcription factor-3/4 (Oct3/4), SRY-related-high-mobility-group (HMG)-box protein-2 (Sox2), Krupple-like factor 4 (Klf4) and L-Myc by fiPS and eiPS, assessed with plasmid (exogenous) and genome (endogenous) specific primers via Reverse Transcriptase Polymerase Chain Reaction (RT-PCR). fiPS cells were analysed at passage 35, eiPS cells were analysed at passage 31. Product sizes were as follows: Endogenous Oct3/4: 130bp (basepairs), Sox2: 119bp, Klf4: 152bp, L-Myc: 136bp; Exogenous Oct3/4: 134bp, Sox2: 160bp, Klf4: 282bp, L-Myc: 258bp.

Though episomal vectors are non–integrating, it was important to confirm that the derived cells were free from genomic defects, as a normal karyotype is desirable for clinical translation of iPS cell lines (Ben-David *et al.*, 2011). Karyotypes, and genetic abnormalities of the derived cells were analysed via highly sensitive single nucleotide polymorphism (SNP) arrays (HumanCytoSNP-12 DNA Analysis BeadChip Kit; Illumina, UK; analysis carried out using GenomeStudio software; see Chapter 2.4.2). Briefly, the test sample DNA was amplified, fragmented, and hybridised to complementary 50-mer probes on the SNP array. From enzymatic single-base extension with fluorescently labelled nucleotides, hybridisation signal

intensities for each probe were used by specialised software to generate the Log R Ratio of test/normal signals. These corresponded to copy number variations. Beta allele frequency for each SNP was calculated by B/(A+B), where allele varients A=0 and B=1. There was a microdeletion in chromosome 6 of the fiPS line, which spans PARK2 gene isoforms (Figure 3.6). eiPS were karyotypically normal. Full analysis is displayed in Appendix Figure 1 and Figure 2.[1]

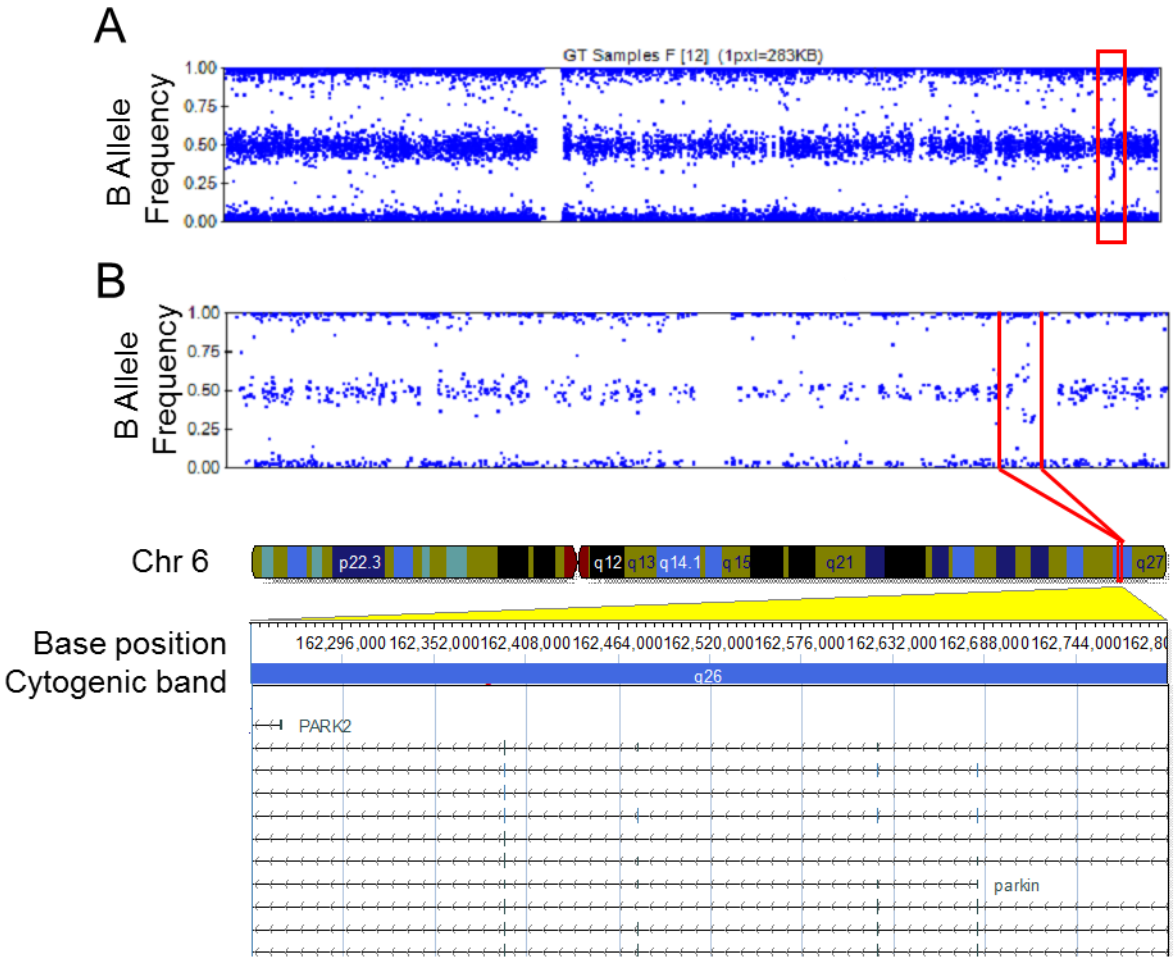

Figure 3.6 A) Genome-wide SNP array analysis of fiPS, displayed as beta allele frequency, identified a microdeletion at the end of chromosome 6, identified in red box, expanded in B. **B)** Closer analysis of this region showed that the microdeletion region was defined as chromosome 6: 162,242,404 to 162,790,478, spanning isoforms of PARK2/parkin.

[1] Non-reprogrammed donor cells were also supplied for analysis and determined to be normal [data not shown], meaning that they could be represented by the standardised probes. This eliminates the possibility that the microdeletion was already present in the donor, and suggests that it has come about as a result of the reprogramming process.

In summary, the iPS cell lines produced had the following characteristics:

iPS cell line	Origin	Sex	Age	Cell substrate	Reprogramming method		Endogenous Y4 expression?	Karyotyping
					System	Vectors		
D1; fiPS	Healthy volunteer	M	30	Fibroblast	Amaxa, setting: 'NHDF high efficiency'	Episomal	Yes: Oct3/4, Sox2, Klf4, L-myc	1 microdeletion; chromosome 6
D2; eiPS	Healthy volunteer	F	30 [Age of mother]	EOC from cord blood	Neon, setting: 18	Episomal	Yes: Oct3/4, Sox2, L-myc	Normal

Table 3.4: Summary of established iPS cell lines, fibroblast-derived induced pluripotent stem cells (fiPS) and endothelial-derived induced pluripotent stem cells (eiPS), detailing characteristics of starting somatic cell population, method of reprogramming, expression of Yamanaka transcription factors (Y4) and karyotypic features.

3.4.2. Characterisation of pluripotent stem cell lines

Detailed characterisation was carried out to assess pluripotency of established fiPS and eiPS cell lines. Flow cytometry was used to detect levels of SSEA3 and SSEA4 expression (Chapter 2.3.1), and immunocytochemistry to detect Nanog, Tra 1 60 and Oct3/4 (Chapter 2.3.2). An *in vitro* 'embryoid body' assay was carried out to allow spontaneous differentiation of the iPS lines, after which differentiated derivatives of the three primary germ layers were detected through immunocytochemical staining of α-fetoprotein (AFP), β-tubulin and smooth muscle actin (SMA), which are markers of endoderm, ectoderm and mesoderm, respectively (Chapter 2.3.3).

fiPS and eiPS cell lines expressed pluripotency markers SSEA3 and SSEA4, assessed by flow cytometry (Figure 3.7). This analysis was split into early passage (P13-18) and later passage (>P22), to determine whether surface antigen expression was stable over time. eiPS had consistently high SSEA3 and SSEA4 levels, whereas fiPS gained this high level of expression over time, as early passage levels were significantly lower, $P=0.042$. Expression level of both pluripotency markers in fiPS and eiPS at later passages were similar to ES cells.

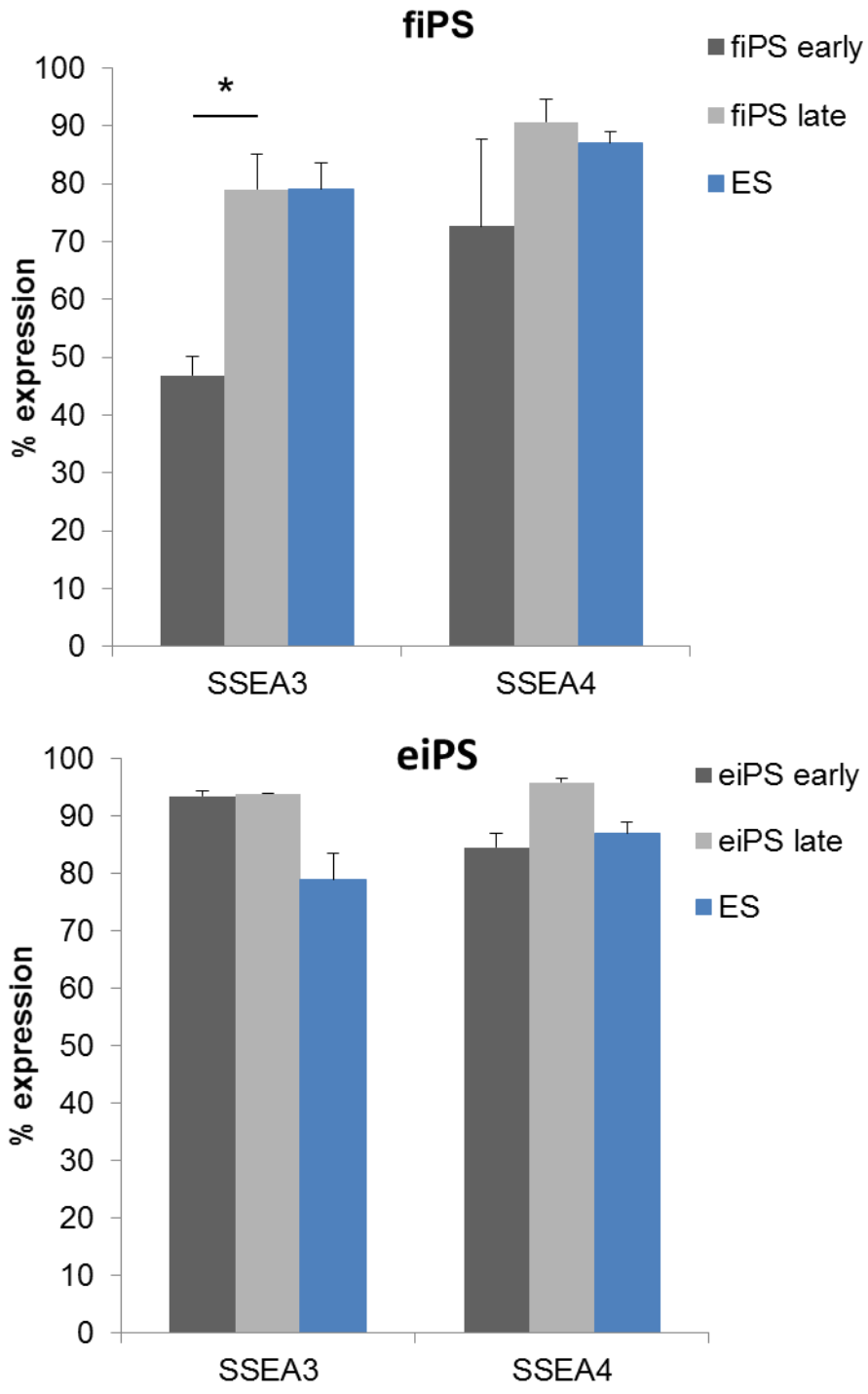

Figure 3.7: SSEA3 and SSEA4 expression on fibroblast-derived induced pluripotent stem cells (fiPS) and endothelial-derived induced pluripotent stem cells (eiPS) at early and late passage, in comparison with embryonic stem (ES) cell lines. Data are expressed as mean ± SEM, and were analysed by unpaired Student's t-test, *= $P<0.05$, N=3.

In combination with expression of the pluripotency markers SSEA3 and SSEA4, eiPS were analysed for loss of their endothelial phenotype. Levels of CD31, CD105 and eNOS, which were high in cord blood EOC, were negligible following reprogramming. VEGFR2, which was highly expressed in cord blood EOC, was still apparent in a proportion of reprogrammed cells (Figure 3.8). The EOCs used in this experiment were passage 6. Previous experiments carried out by Tura *et al.* 2013 in the laboratory demonstrated that expression of these markers was stable in EOCs through passaging up until at least passage 9.

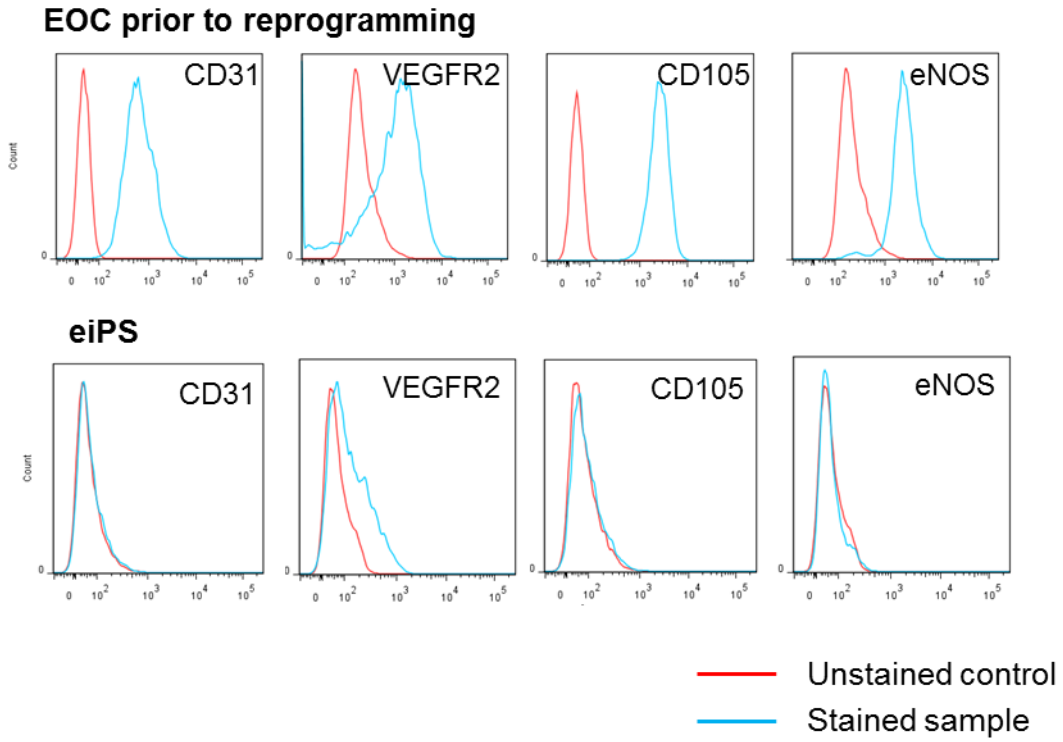

Figure 3.8: Expression of all endothelial markers was decreased following reprogramming. Representative histograms show expression of surface markers associated with endothelial cells (CD31, VEGFR2, CD105 and eNOS), on EOC and eiPS, assessed by flow cytometry. VEGFR2= vascular endothelial growth factor receptor 2, eNOS= endothelial nitric oxide synthase, EOC= endothelial outgrowth cells, eiPS = endothelial-derived induced pluripotent stem cells.

To further examine expression of pluripotency markers, immunocytochemistry was performed to detect Nanog, Oct3/4 and Tra 1 60. All were strongly expressed by the derived cell lines (Figures 3.9 and 3.10). All staining appeared specific to the colonies apart from Nanog, which was also detected on surrounding cells. This is likely because MEFs have been known to express pluripotency markers, including Nanog (Yusuf *et al.*, 2013)

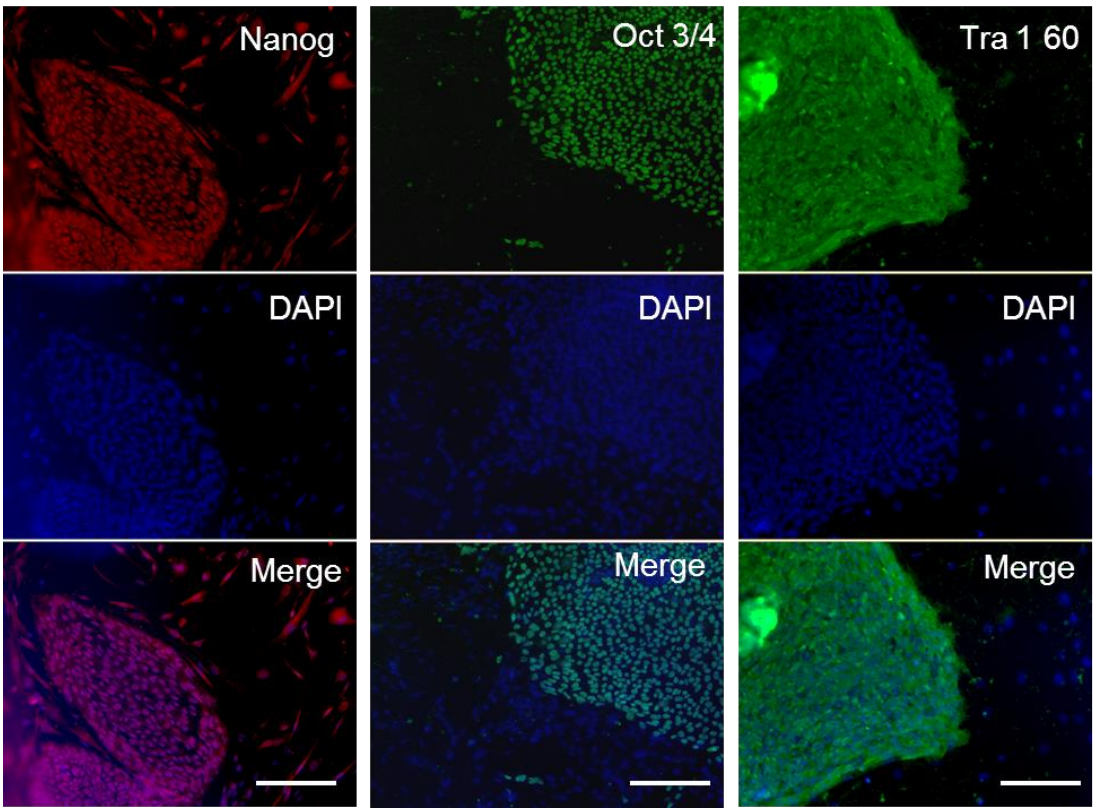

Figure 3.9: fiPS colonies were positively stained by antibodies against pluripotency markers Nanog (red), Oct3/4 (green) and Tra 1 60 (green). Nuclei were stained by DAPI (blue). Bar= 200µm.

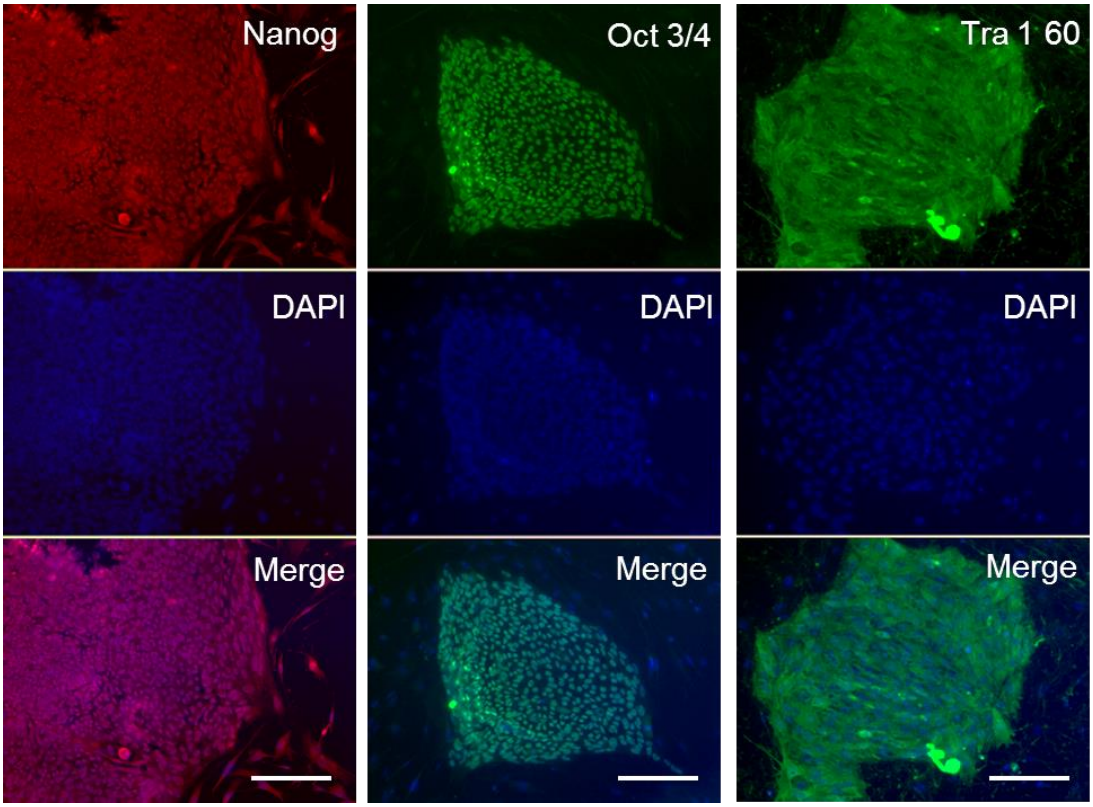

Figure 3.10: eiPS colonies were positively stained by antibodies against pluripotency markers Nanog (red), Oct3/4 (green) and Tra 1 60 (green). Nuclei were stained by DAPI (blue). Bar= 200µm.

After spontaneous differentiation in embryoid bodies (the equivalent to the teratoma assay *in vivo;* Takahashi *et al.*, 2006), AFP, β-tubulin and SMA were detected (Figure 3.11). This indicates that both fiPS and eiPS have the ability to differentiate into derivatives of all three primary germ layers: mesoderm, ectoderm and endoderm. This is a key feature of iPS and ES cells, and confirms their pluripotency

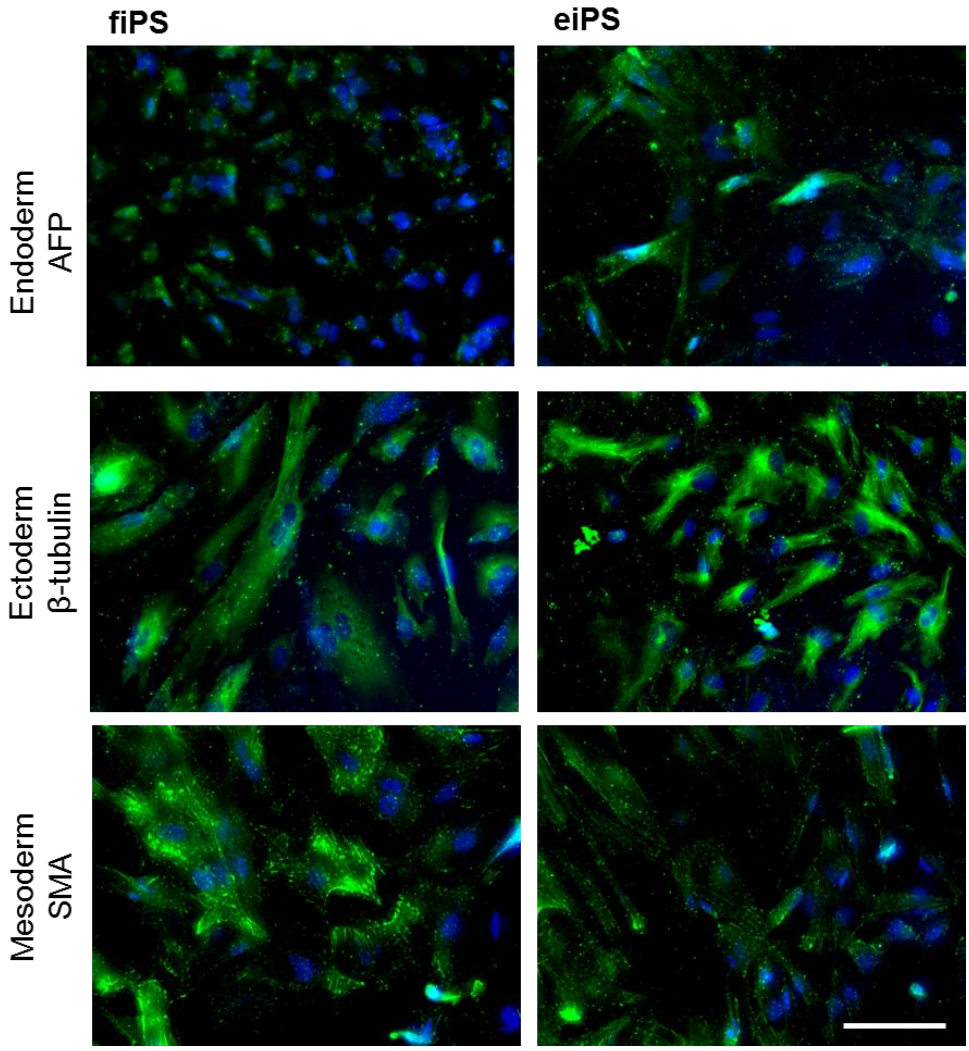

Figure 3.11: Following spontaneous differentiation of fiPS and eiPS in embryoid bodies (stem cell aggregates in suspension), derivatives of all three germ layers- endoderm, ectoderm and mesoderm- were detected by immunocytochemistry, demonstrating pluripotency. Alpha fetoprotein (AFP), β-tubulin and smooth muscle actin (SMA) are stained green, representing endoderm, ectoderm and mesoderm, respectively. Nuclei are stained blue by DAPI. Bar= 100μm. Lack of staining on undifferentiated colonies by these three primary antibodies was run as a negative control [images not shown].

3.5. Discussion

The work described in this chapter tested the hypothesis that endothelial cells reprogram to pluripotency with greater efficiency than fibroblasts. This was investigated by reprogramming both cell types via transfection of episomal plasmids containing all the factors required to induce pluripotency. The overall finding was that endothelial cells derived from cord blood reprogram with marginally higher efficiency than fibroblasts (though efficiency of both was low). Reprogramming of endothelial cells derived from peripheral blood was unsuccessful, despite multiple attempts with different electroporation settings. iPS cell lines derived from fibroblasts and cord blood-derived endothelial cells both appear similar to embryonic stem cells, in terms of their expression of pluripotency markers, and ability to spontaneously differentiate to cell types from all three primary germ layers.

3.5.1. Fibroblasts and endothelial cells can be reprogrammed to pluripotency using a viral- and integration-free episomal plasmid system.

Through episomal plasmid transfection, fibroblasts and endothelial cells can be induced into pluripotency by a process which does not require genomic integration or the continued presence of exogenous reprogramming factors. Whilst mature umbilical vein endothelial cells and endothelial outgrowth cells from peripheral blood have been reprogrammed before using retroviruses (Panopoulos *et al.*, 2011; Chang *et al.*, 2013), to date, this is the first time that endothelial outgrowth cells from cord blood have been reprogrammed. The studies in this thesis form the basis for the generation of endothelium from patient-specific iPS cells, for disease modelling and potential therapeutic use.

3.5.2. The process of reprogramming is inefficient and only a minority of transfected cells progress to pluripotent stem cells.

Though technically straightforward, reprogramming is widely reported to be inefficient; a finding independent of reprogramming method or somatic cell type (Yamanaka, 2012). To some extent this is unsurprising, and indicates the complexity of the mechanisms involved in the resetting of an adult somatic nucleus back to a pluripotent state. Reprogramming efficiency of fibroblasts by Y4 episomal plasmid transfection is reported to be 0.003% by Yu *et al.* (2009), and 0.005% by Okita *et al.* (2011). Of the fibroblast lines transfected here, two out of three were successful, with lower efficiencies (D1: 0.0002%, D5: 0.0006%) than reported in the literature. Possible reasons for the low efficiencies seen here could be variation in induction technique and subsequent laboratory or culture conditions. Interestingly, reprogramming efficiency of endothelial outgrowth cells from cord blood (0.003%) was higher than for fibroblasts, supporting the finding that somatic cells other than fibroblasts have the potential to become iPS cells (González *et al.*, 2011).

Episomal plasmids were used for delivery of reprogramming factors for a variety of reasons. Human iPS cell derivation previously required retroviral vectors, which apart from logistical obstacles (the risks involved with viral work, and the need for proper facilities and equipment for their use) are not ideal as they integrate into the genome, which can create mutations and limit their use for clinical application (Ji *et al.*, 2012). Thus the use of an episomal plasmid was desirable as a non-integrating, single transfection alternative (Okita *et al*., 2011). With time in culture, the episomal plasmids are shed, resulting in iPS cell lines free from vector or transgene sequences, thus removing one obstacle from the clinical application of iPS cells (Argyros *et al*., 2008). Episomal plasmids were first described by the laboratory of James Thomson (Yu *et al*., 2009), and initially comprised pEP4-based plasmids containing the Y4 reprogramming factors. Since then much work has been done to determine the optimal combination of transcription factors, and the plasmid backbones to use. The approach described here used pCXLE plasmids, described by Okita *et al.* (2011), as they were found to be more efficient than pEP4 based plasmids. As well as the

original Y4 factors, of which c-Myc was replaced by the less oncogenic L-Myc, the optimal combination of reprogramming factors included Lin28 and short hairpin RNA against p53, which were both found to enhance iPS cell generation (Yu *et al*., 2007; Hong *et al.,* 2009). The episomal plasmid system is routinely used by many laboratories for reprogramming, and is favoured for its simplicity and reproducibility. In addition, a systematic comparison of viral, episomal and mRNA reprogramming methods found that the episomal plasmid method was the most efficient and robust for reprogramming primary human fibroblasts, and described it as the most suitable for the production of GMP grade iPS cells (Goh *et al.*, 2013).

In terms of translating this to clinical situations, low efficiency could be overcome by scaling up cell populations to guarantee generation of a certain number of iPS colonies. A more pressing issue is that certain donor cell types did not reprogram. GFP positive cells were present 24 hours after electroporation with pCXLE-eGFP, indicating that the settings used for electroporation were suitable for entry of Y4 plasmids (a similar size to the GFP plasmid). Genetic differences between donor may influence the ability of their cells to reprogram, a finding highlighted by Kajiwara *et al.* (2012). This study indicated that the major determinant of reprogramming efficiency is the donor's genetic background, and that this is more important than cell type or reprogramming method. Further experiments are required to determine possible genetic features of a cell, which may predispose it to reprogramming. Further experiments could also investigate other mechanisms of reprogramming to determine the conditions which could most effectively reprogram these 'resistant' populations.

3.5.3. Reprogramming efficiency of different somatic cell types

Based on the literature, it was predicted that endothelial cells would reprogram with greater efficiency than fibroblasts, as this has been reported before (Geti *et al.,* 2012; Chang *et al.,* 2013). This hypothesis was confirmed in EOC derived from cord blood, but not those derived from peripheral blood.

Of all the cellular substrates, cord blood derived endothelial outgrowth cells were most efficiently reprogrammed, followed by fibroblasts. The greater efficiency of reprogramming of cells derived from cord blood has been described before, in comparison with fibroblasts or other mature cell types. For example, mononuclear cells (MNC) derived from cord blood have a reprogramming efficiency 100 fold greater than fibroblasts, with colonies which appeared 1-3 weeks earlier (Hu *et al.*, 2011). MNC from cord blood are also reported to have gene expression and epigenetic profiles which are closer to embryonic stem cells than they are to fibroblasts, which may explain their readiness over mature cells to revert to a pluripotent state (Chou *et al*., 2011). EOC from cord blood are derived from MNC (Tura *et al.,* 2013; Ingram *et al.,* 2004), so these reports may help to explain why they too are more efficiently reprogrammed. Though the reprogramming of defined endothelial progenitor cell populations from cord blood has not been reported before, $CD34^+$ (Okita *et al.,* 2013) and $CD133^+$ (Giorgetti *et al.,* 2009) cells derived from cord blood have been successfully reprogrammed to pluripotency. Interestingly, Giorgetti *et al.* report that $CD133^+$ cells only require two (Oct3/4 and Sox2) of the four traditional reprogramming factors to generate iPS cells, and suggest that the progenitor status of cord blood-derived cells predisposes them to more efficient reprogramming.

There may be differences between cord blood EOC and peripheral blood EOC which could explain the former's more successful reprogramming. Ingram *et al.,* (2004), described a hierarchy of endothelial progenitor cells, reporting that EOC colonies derived from cord blood arose earlier, were larger, and could undergo more population doublings than those derived from peripheral blood. They also had greater levels of telomerase activity. This description could explain the increased affinity of cord blood EOC to revert to a pluripotent state (as they are closer to their multipotent mesodermal/endothelial precursor), rather than peripheral blood EOC. It is also possible that the faster cell kinetics associated with cord blood EOC over peripheral blood EOC may account for their increased reprogramming efficiency. Recent reports suggest that actively dividing cells more successfully reprogram, compared to non-adherent, slow cycling cells (Haase *et al.,* 2009). The replication of EBNA1-

based episomal plasmids (including pCXLE plasmids) is synchronised with host cell division, therefore host cell proliferation is important for the maintenance of the episomal plasmids and the expression of the reprogramming factors they contain (Okita *et al*., 2013; Hanna *et al.,* 2009).

There are however contradictions to the concept that differences in cord and peripheral blood EOC account for variation in their reprogramming efficiencies. It has been found that EOC from blood (both cord and peripheral) are indistinguishable in culture to mature HUVEC (Tura *et al.,* 2013), in terms of their phenotypic profile, proliferative capacity and functional abilities. They may not represent a true 'progenitor' population *in vitro* and thus it is unclear if the increased reprogramming efficiency of cord blood EOC reported here is due to their progenitor or endothelial properties.

Despite repeated attempts of the reprogramming of cells from multiple donors, and variation of electroporation settings for transfection, reprogramming of peripheral blood-derived EOC and vessel wall-derived endothelial cells was unsuccessful. This was unexpected, as was the higher reprogramming efficiency of fibroblasts in comparison, a finding most apparent when these three cell types were reprogrammed from the same individuals, (D1 and D5). This contradicts reports in the literature, many of which detail higher reprogramming efficiencies of umbilical vein endothelial cells (Panopoulos *et al*., 2011) and peripheral blood endothelial cells (Geti *et al.,* 2012) compared with fibroblasts. Geti *et al,* (2012) describe higher reprogramming kinetics and efficiencies of $CD34^+$ EOC derived from peripheral blood in comparison with fibroblasts, and report fewer karyotypic differences between iPS and donor cells with reprogrammed endothelial cells rather than fibroblasts. Chang *et al.* (2013) also reprogrammed late outgrowth endothelial progenitor cells from peripheral blood, by retroviral delivery of the Y4 factors. In the characterisation process, they analysed the transcriptome and found gene expression profiles similar to those of embryonic stem cell controls. Though less research has been carried out on the reprogramming of mature endothelial cells, retroviral reprogramming of HUVEC (Panopoulous *et al.,* 2011), had increased efficiency in

comparison with fibroblasts, and that iPS like colonies arose earlier. Panopoulous *et al.,* reported that HUVEC-derived iPS were indistinguishable from embryonic stem cells. However, all of these referenced studies used viral delivery systems, and to the best of my knowledge, reprogramming of endothelial cells with episomal plasmids has not been published before. This difference in reprogramming technique may account for the lack of success reported in this thesis. Optimisation of the episomal reprogramming technique, or experimentation with alternative reprogramming methods, is required for induction of pluripotency in the peripheral blood and vessel wall endothelial cells.

3.5.4. Established fiPS and eiPS cell lines are pluripotent and have similar characteristics to ES cells

High expression levels of pluripotency markers SSEA3 and SSEA4 (in cells expanded beyond passage 22), as well as Nanog, Tra 1 60 and Oct3/4 indicate that these cells are pluripotent and similar to embryonic stem cells. Nanog was however also detected in cells surrounding the iPS colonies, as it is not specific to undifferentiated cells (Ambady *et al.,* 2010). Differentiation to derivatives of all three primary germ layers is additional proof that these cells are pluripotent and able to differentiate to complex tissues.

There were however minor differences between fiPS and eiPS lines. eiPS expression of endogenous Klf4 was very faint - it may be possible that these cells require more time in culture for full endogenous expression to be more apparent. There also appears to be residual exogenous Klf4 expression in fiPS. This again suggests that more time in culture may be required for complete shedding of the episomal plasmids, and the switch from exogenous to endogenous expression of all of these factors. SSEA3 expression was lower in early passage fiPS compared with early passage eiPS, which may indicate that they take longer to fully reprogram, or that there is a degree of heterogeneity in the population that is lost as the cells reach later passages.

Abnormalities of reprogrammed cells or differentiation bias can manifest due to abnormal karyotypes (Robinton and Daley, 2012). SNP arrays were used as a more informative method of genomic analysis than traditional Giemsa staining of the karyotype, as the readout detects the presence of deletions, duplications or areas of homozygosity (detailed in Devine *et al.*, 2011). The microdeletion in the fiPS cell line came within the gene PARK2, which encodes the protein parkin. Loss of function mutations of this gene lead to dopaminergic cell death in autosomal recessive juvenile Parkinson's disease (Hattori *et al.*, 2004). This is, therefore, unlikely to have an effect on stem cell function, or on performance in mesodermal differentiation protocols. The reprogramming process may have caused this abnormality, and whilst it does not appear to have affected the cell type or function, it emphasises the need to analyse the karyotype of all derived pluripotent stem cells prior to their use in differentiation protocols. Though subtle, these minor differences may indicate that cord blood EOC more quickly adopt pluripotency following reprogramming than fibroblasts, which would be consistent with enhanced reprogramming efficiencies of cord blood-derived cells (Okita *et al.* (2013); Giorgetti *et al.* (2009)). There is an indication of residual VEGFR2 expression following EOC reprogramming. VEGFR2 is a marker found on endothelial cells as well as on hematopoietic stem cells. Its expression on the eiPS suggests that the resulting cells, whilst pluripotent, may still have mesodermal features remaining. It is possible that this may influence future endothelial differentiation potential.

3.5.5. Limitations and future studies

The reprogramming efficiency was low for all lines, with multiple unsuccessful attempts at peripheral blood EOC and vessel wall-derived endothelial cell reprogramming. Though it was exciting and interesting that cord blood EOC were successfully reprogrammed, it was disappointing that peripheral blood EOC were not. Peripheral blood would have been the optimal source for the derivation of iPS cells from patients, requiring only a blood sample, whereas cord blood EOC are unlikely to be applicable for generation of patient iPS cell lines. The optimisation of reprogramming of peripheral blood endothelial cells would, therefore, be worthwhile future work. In the meantime, reprogramming experiments could be scaled up to counteract low efficiency. In addition, plasmid design may be optimised. It is likely that the reprogramming factors transfect a higher proportion of cells than those successfully reprogrammed, and that in the majority of transfected cells incomplete reprogramming takes place. In these cells, the reprogramming process may not be completed due to variable plasmid uptake by the cell, and, therefore, variable dosages of reprogramming factors. This could call for optimised episomal plasmid design – for example development of a single plasmid containing all the required plasmids to ensure delivery of a complete set of reprogramming factors to every transfected cell. An issue for consideration is large plasmid sizes (above 15kb), which typically have low transfection efficiencies, and can compromise cell viability, so may be a limiting factor in plasmid design. Single transfection reprogramming methods have been used before- Chou *et al.,* (2011) described reprogramming using a single OriP/EBNA plasmid, which contained 5 reprogramming factors (Y4 and Lin28) and was able to replicate extra-chromosomally as a circular episome in many types of primate cell. Through this method, they reported 0.001% iPS-like colonies arising from fibroblast transfection; still low efficiency but higher than that reported in this thesis. Kaji *et al*., 2009 also report somatic cell reprogramming using a single plasmid, containing the Y4 factors linked with 2A peptides. Again, this approach was designed to ensure that all electroporated cells received an equal dose of all transcription factors. This method could be considered for increasing reprogramming

efficiency of future reprogramming attempts. The “three-plasmid” method described in this thesis was adopted based on laboratory experience and available resources.

Reproducing these results with more healthy volunteers is required, with further attempts to reprogram all cell types from a single volunteer, so that reprogramming efficiency of somatic cell source could be investigated without genetic background as a variable factor. Genetic background of the donor is thought to have an impact on reprogramming efficiency: iPS cell induction is reported to vary between donors, independent of age or gender, (Okita *et al.*, 2013), and it has been reported that this is a major determinant of efficiency rather than reprogramming method or starting cell type (Kajiwara *et al.*, 2012). Repeating these experiments with more healthy volunteers would lead to greater understanding of the characteristics of starting cell populations which influence successful reprogramming, and may improve the approach to development of patient derived iPS cell lines.

3.6. Conclusions

Fibroblasts and cord blood-derived endothelial cells can be induced into pluripotency through transfection with episomal plasmids containing reprogramming factors. These lines express multiple pluripotency markers and can differentiate into all three primary germ layers. This process of reprogramming is in general fairly inefficient, but consistent with current literature.

Though more cellular reprogramming work is required, the work presented in this chapter suggests that endothelial outgrowth cells from healthy cord blood reprogram with greater efficiency than those from healthy peripheral blood or fibroblasts. It is unclear if this is due to their progenitor or endothelial phenotype.

Whether iPS cells derived from endothelial cells have a greater potential for differentiation towards a cardiovascular lineage is unknown and will be tested in Chapter 5.

Chapter 4

Endothelial differentiation of ES and iPS cells

Abstract

Introduction: The study of endothelial cells *in vitro* may elucidate the mechanisms of endothelial dysfunction in patients with atherosclerosis. Functional tests and culture of primary endothelial cell lines can offer insight, but understanding of endothelial biology could be extended by studying the differentiation of endothelial cells from patient-specific pluripotent stem cells. The aim of this chapter was to compare the efficiency of endothelial differentiation protocols from human embryonic stem cells and iPS cells.

Results: The first three endothelial differentiation protocols tested (adapted from preliminary studies in the laboratory and published protocols) were unsuccessful, with very low yields of endothelial cells that were unable to expand in culture. Endothelial differentiation through a protocol involving embryoid body formation and exposure to mesoderm-inducing cytokines, followed by culture in endothelial cell conditions (supplemented with VEGF) was successful. Differentiation efficiency was determined by the percentage of cells expressing endothelial cell marker CD31 at day 7. Although this was a low yield (ES: 2.5%±0.5; iPS: 1.0% ± 0.3%), isolated $CD31^+$ populations were maintained and expanded in culture. $CD31^+$ cells isolated from differentiating ES and iPS cells had a cobblestone morphology, typical of endothelial cells, and expressed high levels of endothelial cell markers over long term culture, in particular CD31 (ES-EC: 84±8%; iPS-EC: 97±2%), CD146 (ES-EC: 67±8%; iPS-EC: 83±13%) and CD105 (ES-EC: 73±14%; iPS-EC: 98±2%). Comparatively, expression of hematopoietic and pluripotency markers was low (all <5%). vWF and eNOS were also detected in these populations by immunocytochemistry. *In vitro* functional assays demonstrated similar properties of ES-EC and iPS-EC to endothelial cell controls, in terms of vascular tubule formation and wound healing. In a subcutaneous sponge implant model of neovascularisation, ES-EC and iPS-EC significantly increased vessel density when compared to controls (by 1.83 vessel counts, $P \leq 0.01$ and 3.50 vessel counts, $P \leq 0.001$ for both), whilst control endothelial cells did not.

Conclusions: Differentiation of ES and iPS cell lines produced $CD31^+$ cells that could be isolated and expanded with endothelial-like morphology, phenotype and

function *in vitro*. These derived endothelial cells were pro-angiogenic, increasing vessel density in a subcutaneous sponge model of neovasculogenesis. This approach has major potential to generate endothelial cells for future therapeutic application in patients with ischemia.

4.1. Introduction

Atherosclerosis, the primary cause of coronary heart disease, is the commonest cause of premature death in the UK (British Heart Foundation). There are patients who present with premature atherosclerosis (symptoms occurring under the age of 50) in the absence of typical environmental factors (Jousilahti *et al.,* 1996); in this patient population genome-wide association studies have identified that defects in endothelial function may be responsible for the disease (Damani and Topol *et al.,* 2004). The study of endothelial cells derived from this patient population is, therefore, important to assess their molecular characteristics and functionality and to possibly identify causative mutations and to develop novel therapeutic approaches.

Aspects of endothelial dysfunction can be investigated with functional tests - for example forearm venous occlusion plethysmography (Newby *et al.,* 1999) - but the molecular characteristics of dysfunctional endothelium are still unclear and are complicated by endothelial heterogeneity in the cardiovascular tree (Hendrickx *et al.,* 2004). For example, biomechanical forces can influence endothelial phenotype, and determine if they are susceptible to atherosclerosis (atheroprone) or not (atheroprotective) (Berk, 2008). The extraction of endothelial cells from patients suffering clinical symptoms is difficult, therefore the study of patient endothelial cells has been coupled with induced pluripotent stem (iPS) cell technology. It is envisioned that iPS cells derived from patients can be differentiated to produce large quantities of endothelial cells with the same molecular characteristics of the patients from which they were derived. Genetic defects that may predispose these patients to endothelial dysfunction may persist through reprogramming and differentiation processes. This has previously been observed in motor neurons in iPS cells derived from patients with Parkinson's disease (Sanchez-Danes *et al.,* 2012), allowing disease modelling *in vitro,* with the potential for genetic correction and clinical translation.

Although there are various published protocols to generate endothelial cells from stem cells (for example: Levenberg *et al.,* 2002; Taura *et al.,* 2009; Narazaki *et al.,* 2008), there is not a universal protocol. Published endothelial differentiation

protocols differ in terms of culture conditions, time point(s) at which cells are analysed, definition of an endothelial cell, the species in which these protocols have been developed, whether or not they have been carried out with ES or iPS cells, and the functionality of the differentiated cells.

Though the cells derived from these protocols may appear endothelial-like, based on morphology and phenotype, many studies do not isolate a definitive endothelial cell population, meaning there is likely a degree of heterogeneity in the resultant population. This could pose a problem if cells are to be used therapeutically, as there is a risk that multi/pluripotent cell types may be present. There is therefore a need to optimise and validate an endothelial differentiation protocol in order to determine a robust, reproducible system to generate pure populations of functional endothelial cells from human pluripotent stem cells.

4.2. Hypothesis

Directed differentiation of human embryonic and induced pluripotent stem cells generates populations of cells with similar characteristics to endothelial cell lines.

4.3. Aims

- To compare endothelial cell differentiation from healthy human ES and iPS cell lines, using published and unpublished protocols.
- To use phenotypic and functional assays (*in vitro* and *in vivo*) to characterise endothelial-like cells derived from stem cells, and compare them with mature endothelial cell lines.

4.4. Experimental design

4.4.1. Endothelial differentiation of ES and iPS cells

As detailed in Chapter 2.7, ES and iPS cells were subjected to four endothelial differentiation protocols. These protocols varied in terms of cell culture system (2D or 3D), cell culture conditions and the time point at which endothelial-like cells were isolated by FACS. The number of replicates of each differentiation protocol is given in Table 4.1.

Endothelial Differentiation Protocol	Reference	Chapter 2 figure reference	Cell line	N
A	Adapted from Choi *et al.,* 2009 and Sone *et al.,* 2007	Figure 2.4	ES	1 H9; 2 H1
			iPS	2 34D6
B	Adaptation of protocol A	Figure 2.5	ES	1 H9; 2 H1
C	Ferreira *et al.,* 2007	Figure 2.6	ES	2 H9; 2 H1
			iPS	2 34D6; 2 33D9
D	Unpublished; correspondence with University of Glasgow	Figure 2.7	ES	5 H9; 7 H1
			iPS	3 34D6; 4 33D9

Table 4.1 Embryonic stem (ES) cells and induced pluripotent stem (iPS) cells were differentiated via four different protocols.

4.4.2. Characterisation of CD31$^+$ derived cells

In vitro analysis of the CD31$^+$ cell populations was carried out for phenotypic and functional characterisation between passages 2-5 post-sort, as detailed (Chapter 2.3 & 2.5). Phenotype was characterised by flow cytometry and immunocytochemistry; function by their ability to form connections when cultured on Matrigel, reparative mechanisms following a scratch healing assay, and nitric oxide production following

stimulation with acetylcholine. The derived cells were compared to control endothelial cell lines, HUVEC and EOC (derivation of which is detailed in Chapter 2.2.1.3). *In vivo* analysis of the $CD31^+$ cell populations was carried out using the subcutaneous sponge implant murine model of vascularisation (see Chapter 2.6). 10-12 week old NSG mice, which are inbred for immunodeficiency, were used. This meant that implantation of material containing human cells did not elicit an immune response. Mice were grouped according to sponge treatments (Table 4.2). EOC conditioned media was used as a negative control, to detect any effects media has on vascularisation. Cell treated sponges were grouped as: EOC control, embryonic stem cell-derived endothelial cells (ES-EC) and induced pluripotent cell-derived endothelial cells (iPS-EC).

Treated sponge contents	Number of mice per group
EOC conditioned media	4
EOC	6
ES-EC	6
iPS-EC	6

Table 4.2 Sponge implants and group sizes for the murine subcutaneous sponge angiogenesis assay. EOC= late endothelial outgrowth cells; ES-EC: embryonic stem cell-derived endothelial cells; iPS-EC: induced pluripotent cell-derived endothelial cells.

Species specific antibodies were used to identify cells (Chapter 2.6.4). Two sets of antibodies were used for immunocytochemistry, as detailed in Table 2.7.

4.5. Results: summary

The proportions of $CD31^+$ or $CD34^+$ endothelial cells derived via protocols A, B and C were low (0.1-0.9%, 0-0.6%, 0-0.9%, respectively; Table 4.3). As such, there were not sufficient numbers of $CD31^+$ or $CD34^+$ cells for characterisation. $CD31^+$ cells derived via protocol D were maintained and expanded in culture for comparison with endothelial controls.

Protocol	Reference	Key features of the protocol	ES/iPS cell line	Efficiency of differentiation (range)	Further culture?	Comments
A	Choi *et al.*, 2009 Sone *et al.*, 2007	Pluripotency sort day 7; CD31 sort day 14	ES	0.1-0.9%	No	Low yield, did not survive
			iPS	0.3-0.8%	No	
B	Adaptation of protocol A	CD31 sort day 7	ES	0-0.5%	No	$CD31^+$ expression lost
C	Ferreira *et al.*, 2007	CD34 sort day 7; VEGF supplementation	ES	0-0.6%	No	$CD34^+$ expression lost; VEGF supplementation did not prompt CD31 expression
			iPS	0.2-0.9%	No	
D	Unpublished; correspondence with University of Glasgow	EB formation; CD31 sort day 7	ES	0.3-6.2% $CD31^+$ day 7	Yes	Despite low yields, $CD31^+$ expression was maintained and the cells proliferated well in culture
			iPS	0.3-2.0% $CD31^+$ day 7	Yes	

Table 4.3: Overview of the key features and efficiencies of embryonic stem (ES) and induced pluripotent stem (iPS) cell endothelial differentiation, via protocols A, B, C and D. EB: embryoid body

The following results detail the characteristics of $CD31^+$ cells isolated from differentiation of ES and iPS cells via endothelial differentiation protocol D. This protocol was selected as the most successful due to the relative ease of cell culture following isolation of cells expressing CD31.

4.5.1. *In vitro* analysis of endothelial-like cells derived from ES and iPS cells via differentiation protocol D

Seven days of endothelial differentiation of ES and iPS cells generated a mixed population of cells, expressing various endothelial (CD31, CD34, VEGFR2, CD146 and CD105), hematopoietic (CD133 and CD45) and pluripotent (SSEA3 and SSEA4) markers (Figure 4.1). Morphology was varied: there were some areas of cells which appeared endothelial-like, and some which remained small and rounded, characteristic of stem cells.

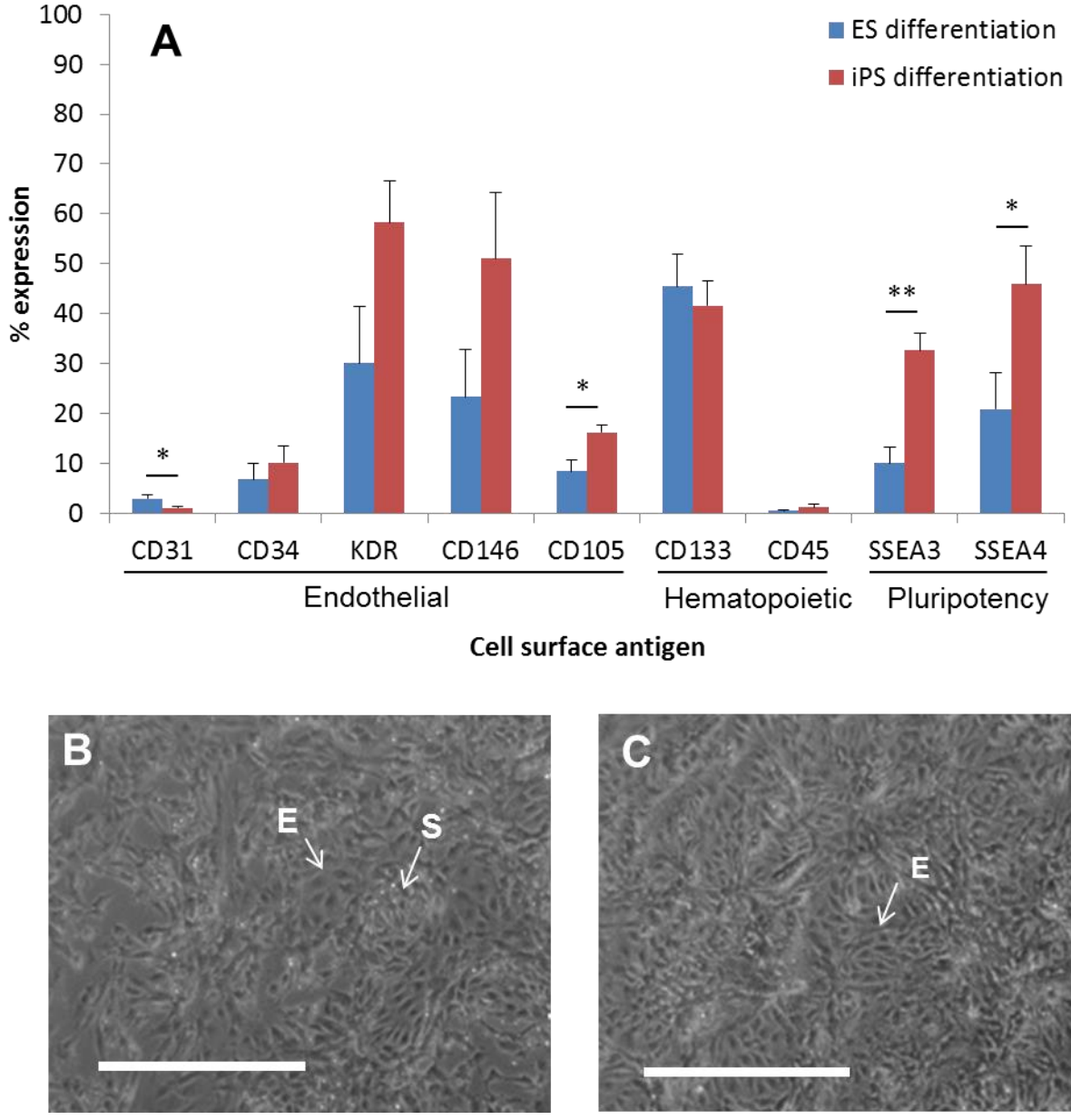

Figure 4.1: After seven days of endothelial differentiation, embryonic stem (ES) and induced pluripotent stem (iPS) cells formed a mixed population. A) Phenotype (expression of endothelial, hematopoietic and pluripotency markers) was assessed by flow cytometry; data are expressed as mean ± SEM and were analysed via unpaired t-test; *P≤0.05, **P≤0.01; N=7-12. B) & C) Morphology of differentiating ES and iPS cells, respectively, is endothelial-like ('E' arrows) and stem cell-like ('S' arrows). Bar= 0.5mm.

The percentage of $CD31^+$ cells at day 7 was low for both differentiating ES and iPS cell lines. This was used to quantify the differentiation efficiency of each cell line. Differentiation efficiency was significantly higher in ES cells compared to iPS cells (Figure 4.2). $CD31^+$ cells are referred to as 'ES-EC' (Embryonic Stem cell-derived endothelial cells) or 'iPS-EC' (induced pluripotent stem cell-derived endothelial cells).

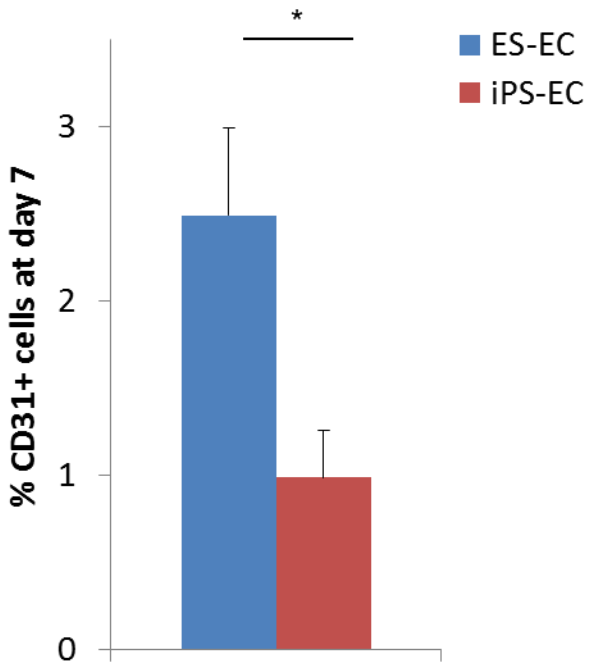

Figure 4.2: % of cells expressing CD31 after seven days of embryonic stem (ES) and induced pluripotent stem (iPS) cell endothelial differentiation. Data are expressed as mean ± SEM and were analysed via unpaired t-test; $P \leq 0.05$=*; ES, n=12; iPS, N=7.

Though the percentage of $CD31^+$ cells within these differentiating populations was low, upon staining with a fluorescent-conjugated CD31 antibody, they appear as a distinct population and could be isolated via FACS. A representative example of an ES cell sort is displayed in Figure 4.3.

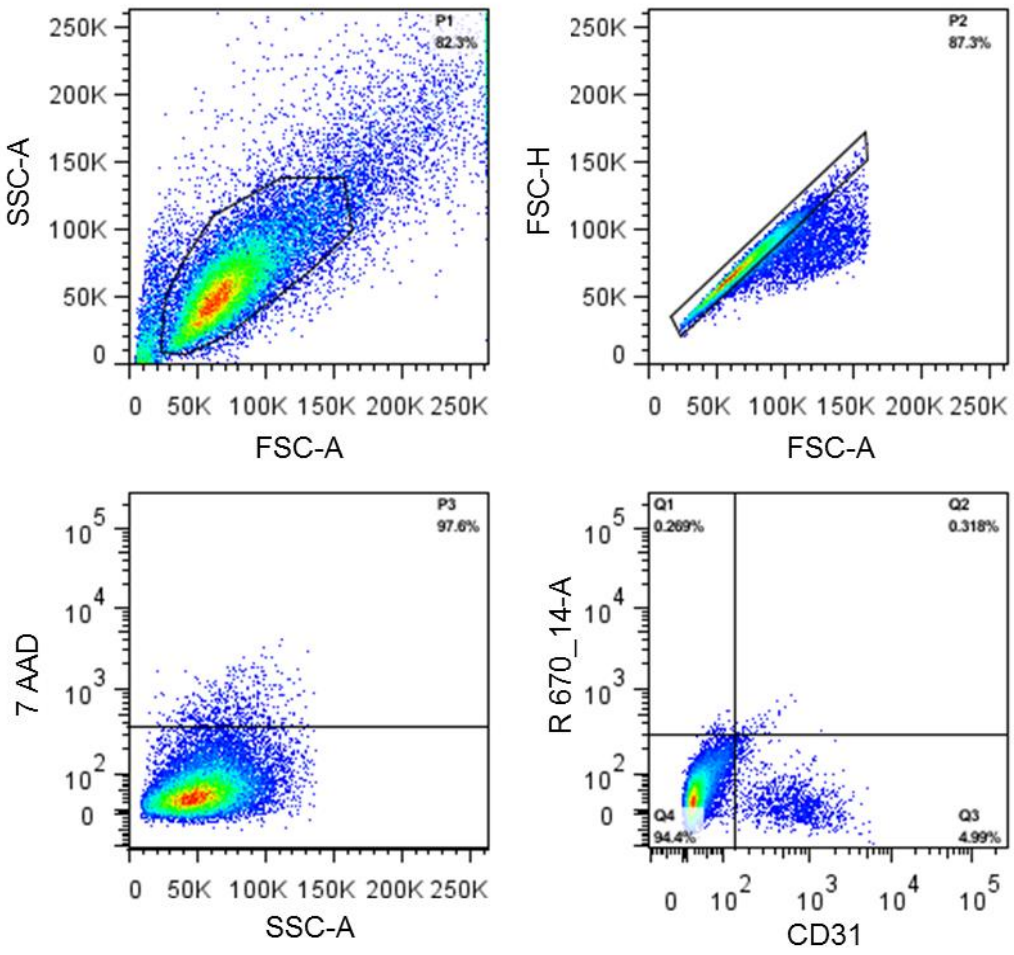

Figure 4.3: Representative example of isolation of $CD31^+$ cells by fluorescence-activated cell sorting (FACS) at day 7 of endothelial differentiation of ES cells. Gates were set by unstained control. P1= live population; P2: singlets; P3: 7AAD negative cells; Q3: $CD31^+$ cells; Q4: $CD31^-$ cells.

Upon reaching confluence in culture, sorted $CD31^+$ cells displayed a 'cobblestone' morphology, characteristic of endothelial cells (Figure 4.4).

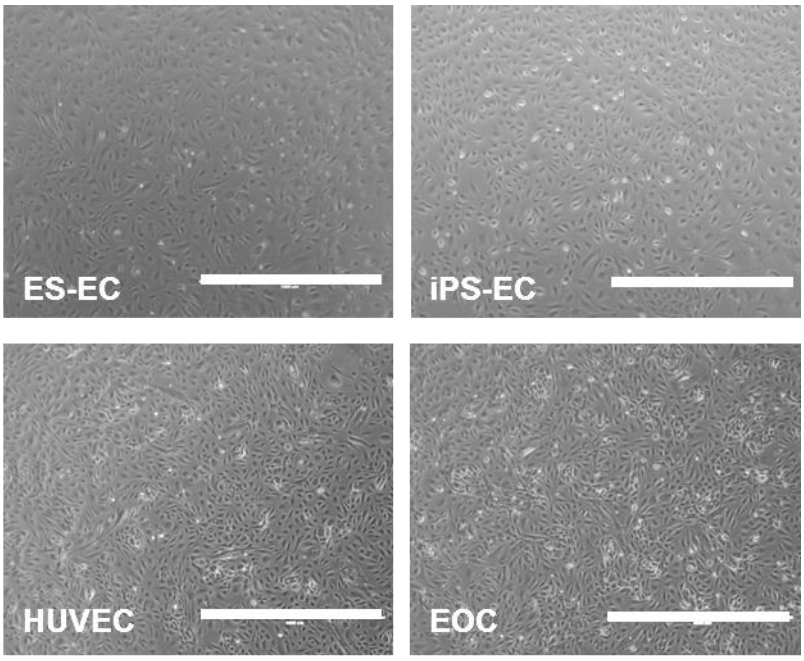

Figure 4.4: Differentiated cells and endothelial control lines display similar morphology. ES-EC: embryonic stem cell derived endothelial cells; iPS-EC: induced pluripotent stem cell derived endothelial cells; HUVEC: Human umbilical vein endothelial cells; EOC: Late outgrowth endothelial cells. Bar = 1mm. ES-EC: passage 4, post sort; iPS-EC: passage 4, post sort; HUVEC: passage 6; EOC: passage 4.

Endothelial cells derived from ES cells and iPS cells were phenotypically analysed via flow cytometry at early (P1-3) and late (P4-6) passage post-sort, to determine the stability of these populations in culture. Expression levels of endothelial markers CD31, CD34, VEGFR2, CD146 and CD105 are shown in comparison with expression levels prior to differentiation (Figure 4.5). With the exception of VEGFR2, both ES and iPS cells displayed very low expression of all endothelial cell surface markers prior to differentiation. Both ES-EC and iPS-EC populations maintained high levels of CD31 expression in culture following sorting. Although there were no significant differences between early and late passage populations, there appeared to be a trend for increased expression of CD34, VEGFR2, CD146 and

CD105 on late passage ES-EC, and CD34 and VEGFR2 on late passage iPS-EC, compared to early passage cells.

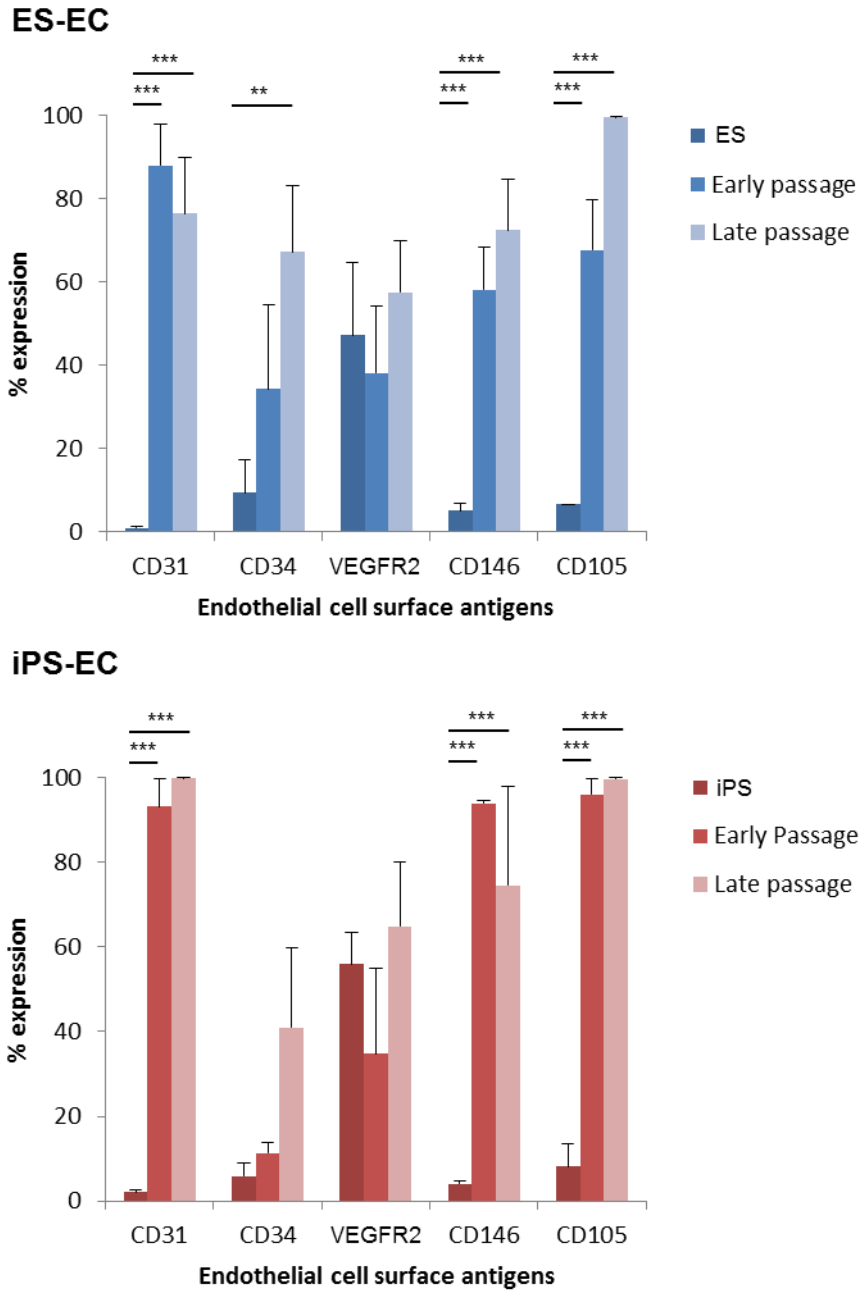

Figure 4.5: Expression of endothelial cell surface antigens on embryonic stem cell-derived endothelial cells (ES-EC), induced pluripotent stem cell-derived endothelial cells (iPS-EC) over time and cells prior to differentiation, assessed by flow cytometry. Early passage: P2-P3, late passage: P4-P5. Data are expressed as mean ± SEM and were analysed via one way ANOVA, P≤0.01=**; P≤0.001=***. N=3-4.

Early and late passage data were combined and compared to endothelial cell control lines, HUVEC and EOC (Figure 4.6). Expression of endothelial cell markers CD31, CD146 and CD105 was high and comparable to HUVEC and EOC control lines. CD34 and VEGFR2 expression was lower but also comparable to HUVEC and EOC control lines. There were no significant differences in expression of any endothelial markers between ES-EC and iPS-EC.

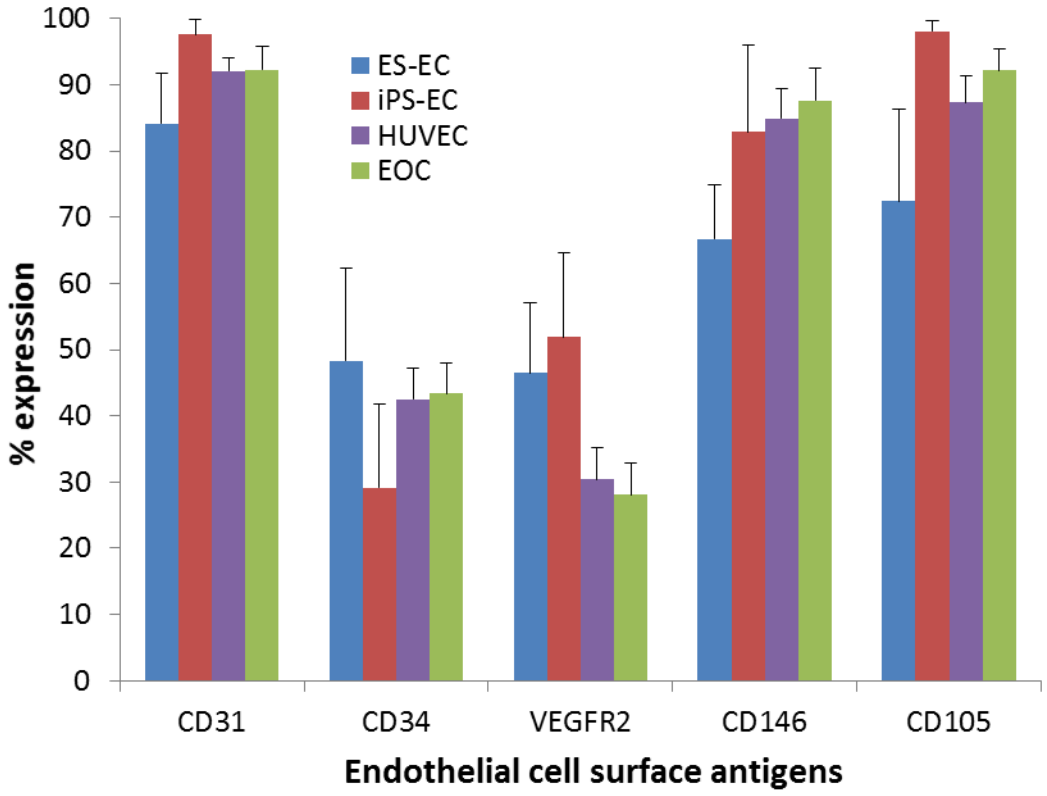

Figure 4.6: Expression of endothelial cell (EC) surface antigens on sorted $CD31^{+}$ cells from embryonic stem cells (ES-EC) and induced pluripotent stem cells (iPS-EC), in comparison with human umbilical vein endothelial cells (HUVEC) and late outgrowth endothelial cell (EOC) control lines. Data are expressed as mean ± SEM and were analysed by one-way ANOVA, $P>0.05$ for all. N=5-7.

Endothelial cells derived from ES and iPS cells were also analysed based on the expression of hematopoietic (CD133 and CD45) and pluripotent (SSEA3 and SSEA4) markers. In comparison with undifferentiated starting cell populations, expression of CD133, SSEA3 and SSEA4 is very low following differentiation (Figure 4.7). Taking into account these expression levels over time (early verses late passage), expression of SSEA3 appears to decrease over time in differentiating ES cells.

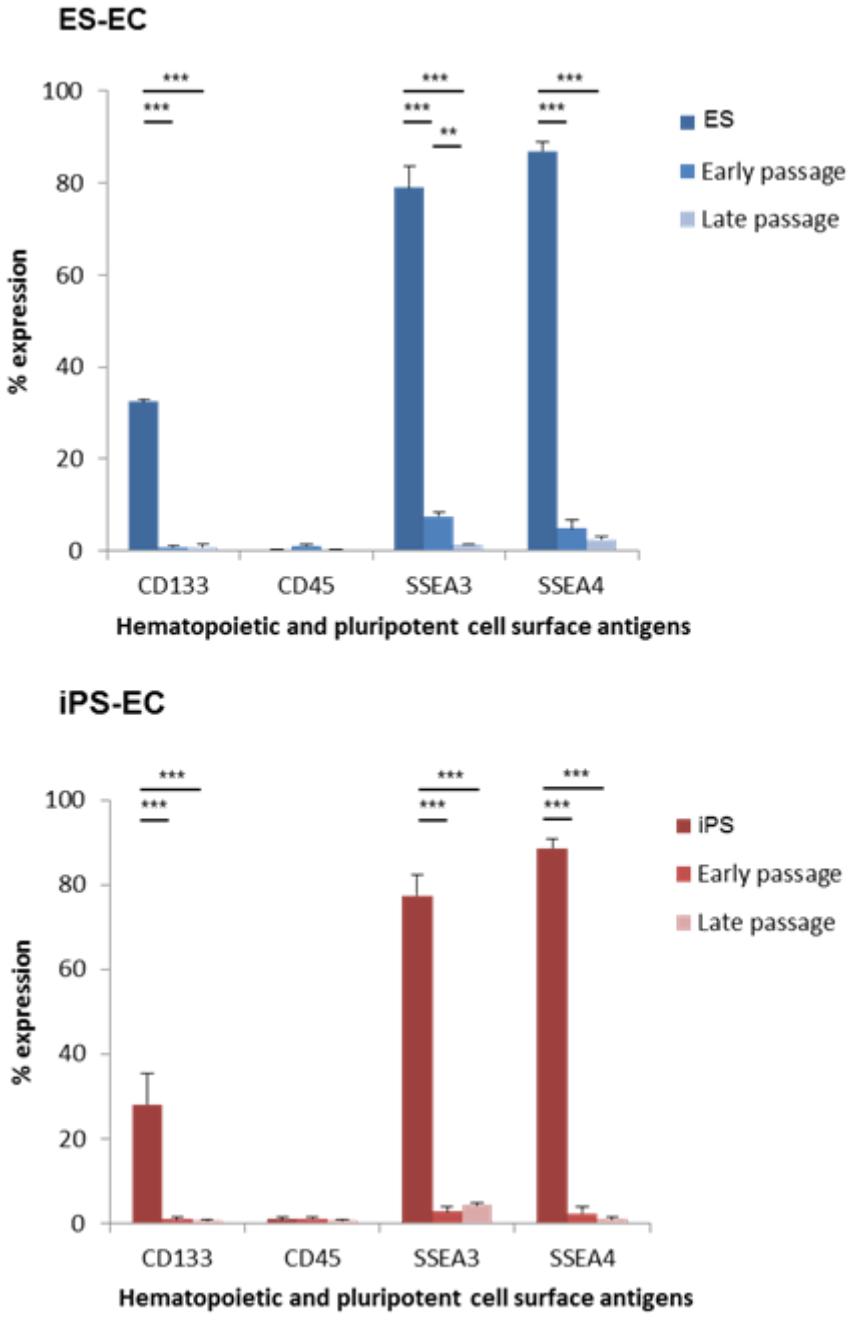

Figure 4.7: Expression of cell surface antigens on embryonic stem cell-derived endothelial cells (ES-EC) and induced pluripotent stem cell-derived endothelial cells (iPS-EC), assessed by flow cytometry. Early passage: P2-P3, late passage: P4-P5. Data are expressed as mean ± SEM and were analysed via one way ANOVA; $P \leq 0.05$=*, $P \leq 0.001$=***. N=3-4.

Early and late passage data were combined and is shown in comparison to endothelial cell control lines, HUVEC and EOC (Figure 4.8). Expression of hematopoietic and pluripotent markers was very low following differentiation and comparable to both control endothelial cell lines.

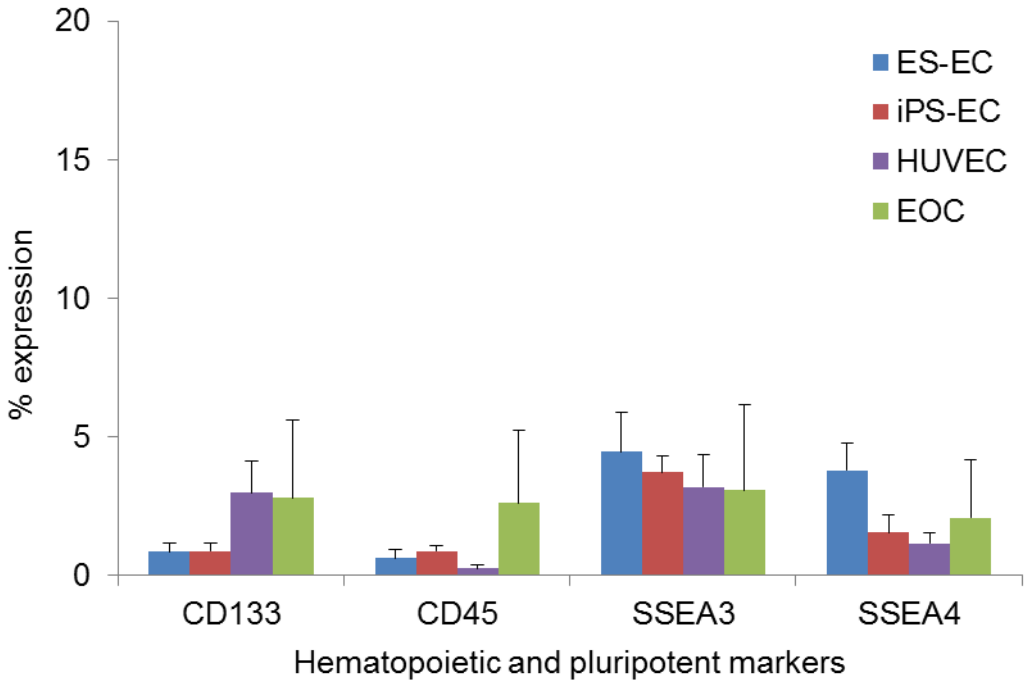

Figure 4.8: Expression of hematopoietic and pluripotent cell surface antigens on sorted $CD31^+$ cells from differentiating embryonic stem (ES) and induced pluripotent stem (iPS) cells, in comparison with human umbilical vein endothelial cells (HUVEC) and late outgrowth endothelial cell (EOC) control lines. Data are expressed as mean ± SEM and were analysed by one-way ANOVA, $P>0.05$ for all. N=5-7.

Phenotypic analysis via immunocytochemistry (Figures 4.9 and 4.10) showed that ES-EC and iPS-EC both express endothelial markers: von Willebrand Factor (vWF) and endothelial Nitric Oxide Synthase (eNOS).

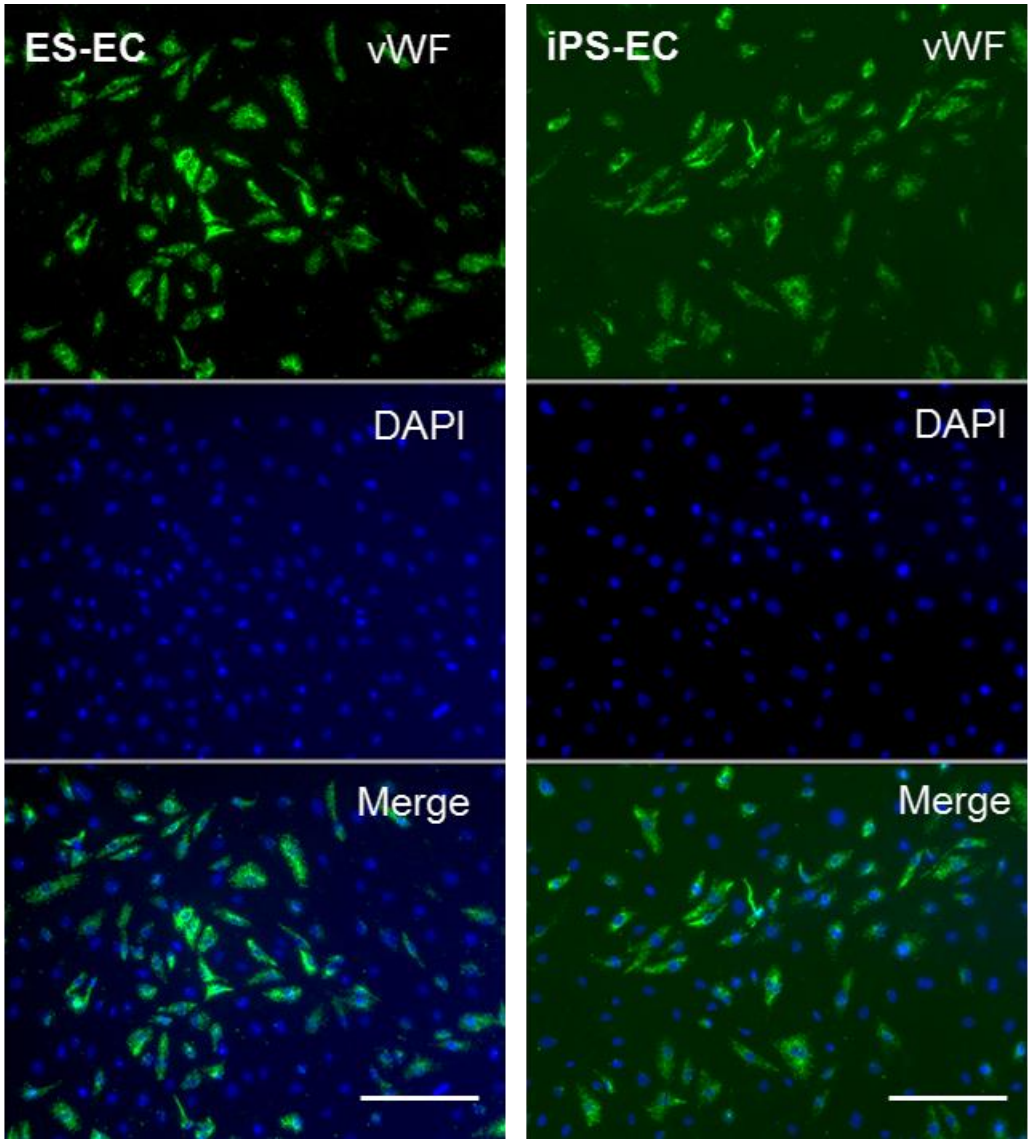

Figure 4.9: Representative images of embryonic stem cell-derived endothelial cells (ES-EC) and induced pluripotent stem cell-derived endothelial cells (iPS-EC), immunostained with antibodies against vWF (green). Nuclei are stained with DAPI (blue). Bar = 200µm.

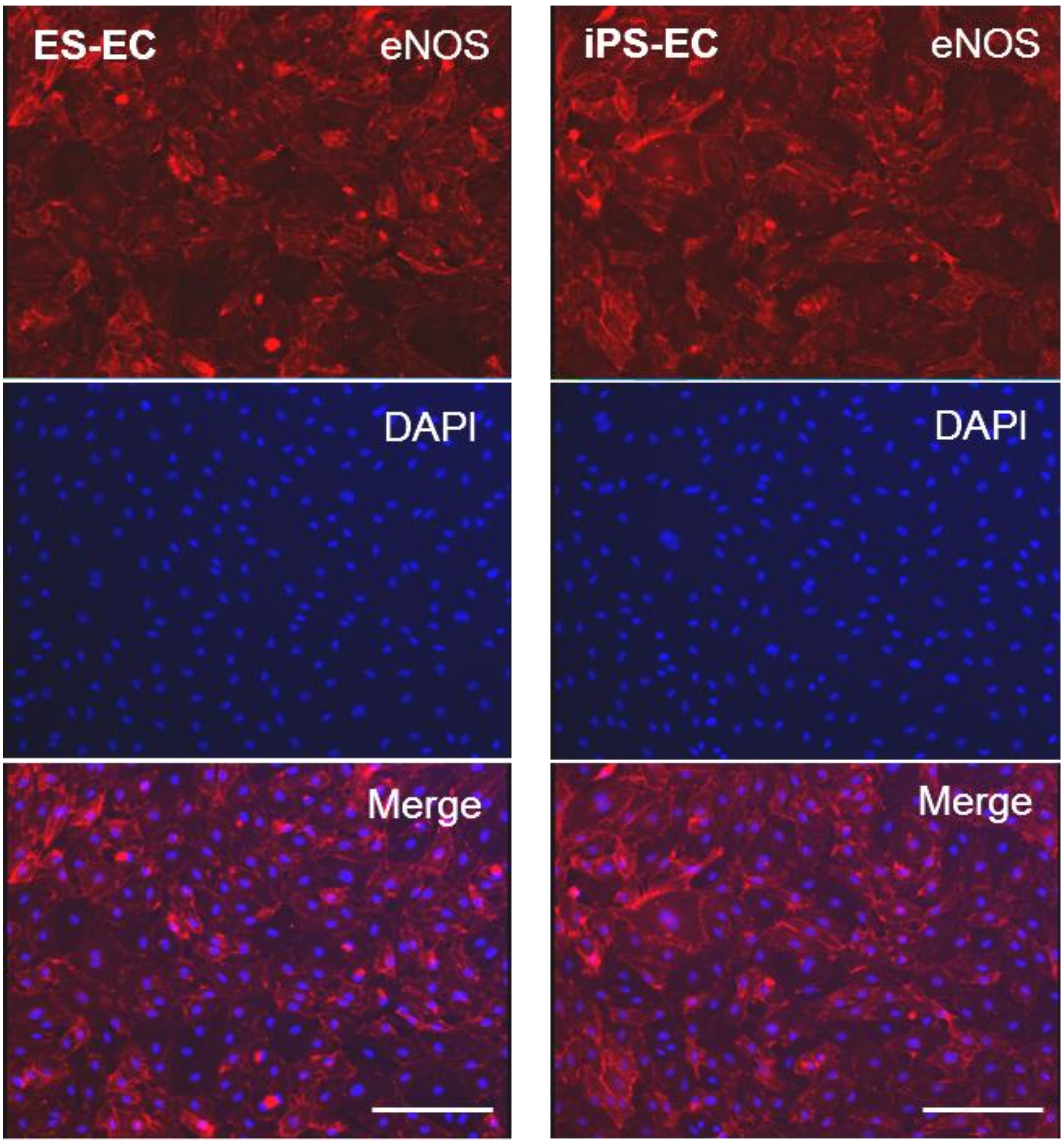

Figure 4.10 Representative images of embryonic stem cell-derived endothelial cells (ES-EC) and induced pluripotent stem cell-derived endothelial cells (iPS-EC), immunostained with antibodies against eNOS (red). Nuclei are stained with DAPI (blue). Bar = 200µm.

ES-EC, iPS-EC and endothelial cell controls perform in a similar way in the *in vitro* Matrigel assay, in which endothelial cells form connections as an indication of their angiogenic capacity (Figure 4.11). By comparison, undifferentiated ES and iPS cells did not form connections.

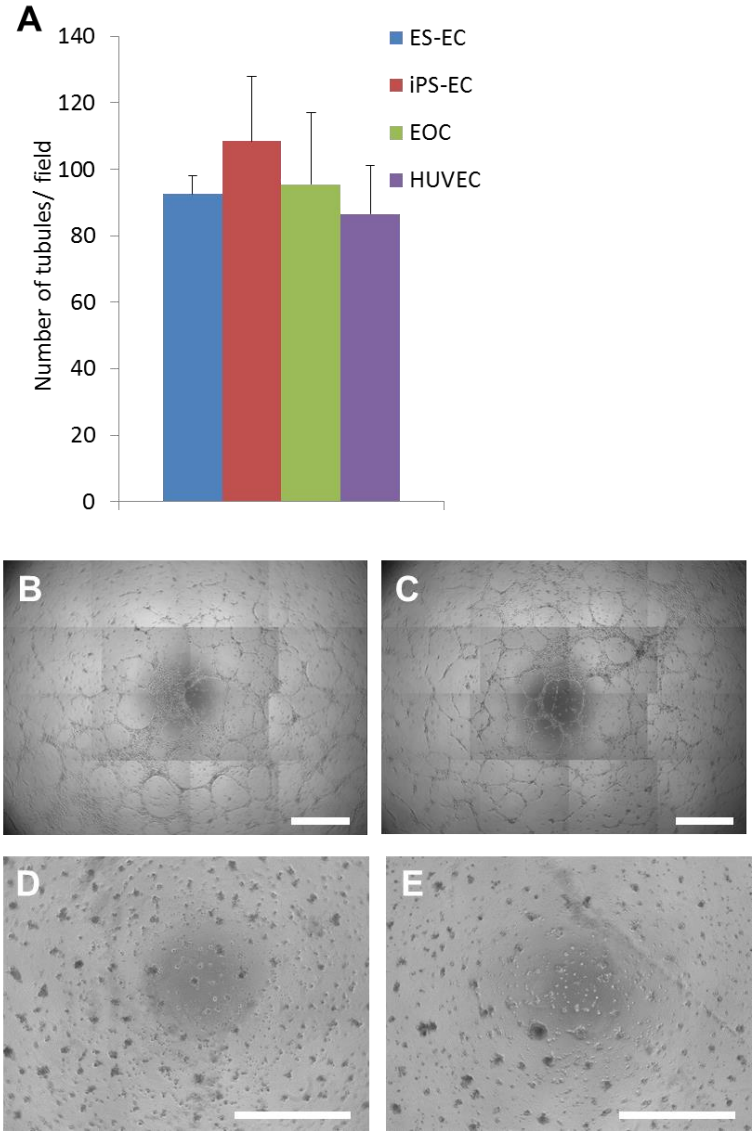

Figure 4.11: Endothelial cells derived from embryonic stem cells (ES-EC) and from induced pluripotent stem cells (iPS-EC) form connections on Matrigel, which may be an indication of angiogenic potential. A) Quantification of cell connections suggests similar angiogenic potential of ES-EC, iPS-EC, HUVEC and EOC. Data are expressed as mean ± SEM and were analysed via one-way ANOVA, $P>0.05$; N=3-5. Representative images of tubule-like structures formed by B) ES-EC and C) iPS-EC. By comparison, both D) ES and E) iPS, did not form tubes when cultured on matrigel. Bar = 1mm.

ES-EC, iPS-EC and EOC performed similarly in the wound healing assay (Figure 4.12). By 24 hours, cell coverage for all lines was 100%.

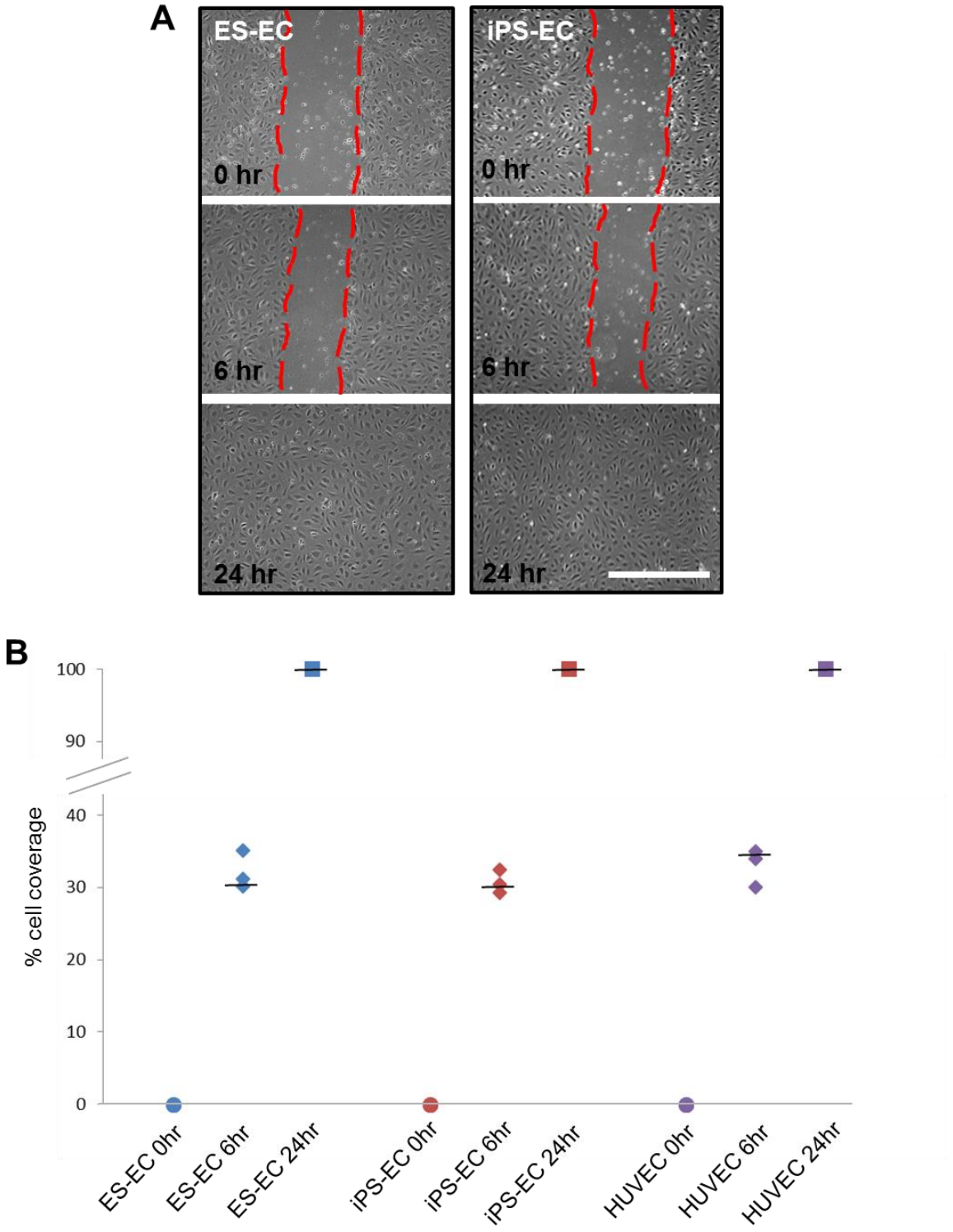

Figure 4.12: ES-EC and iPS-EC have similar migratory and proliferative abilities during recovery from a scratch in the wound healing assay A) Representative images of wound healing of ES-EC and iPS-EC over time. Bar= 500µm B) Quantification of wound healing of ES-EC, iPS-EC and HUVEC. Data were analysed via one-way ANOVA, $P>0.05$, $N=3$.

To mimic NO release *in vitro*, cells were stimulated with 10µM acetylcholine (ACh) for 24 hours. As NO has a short half-life (Hakim *et al*., 1996), in this assay the levels of nitrite and nitrate (its more stable metabolites) were taken as indirect measurements (Chapter 2.5.3). With the exception of ES-EC, unstimulated cells had similar nitrite and nitrate release to those stimulated with ACh (Figure 4.13). ES-EC produced significantly higher levels of nitrite and nitrate when stimulated with ACh.

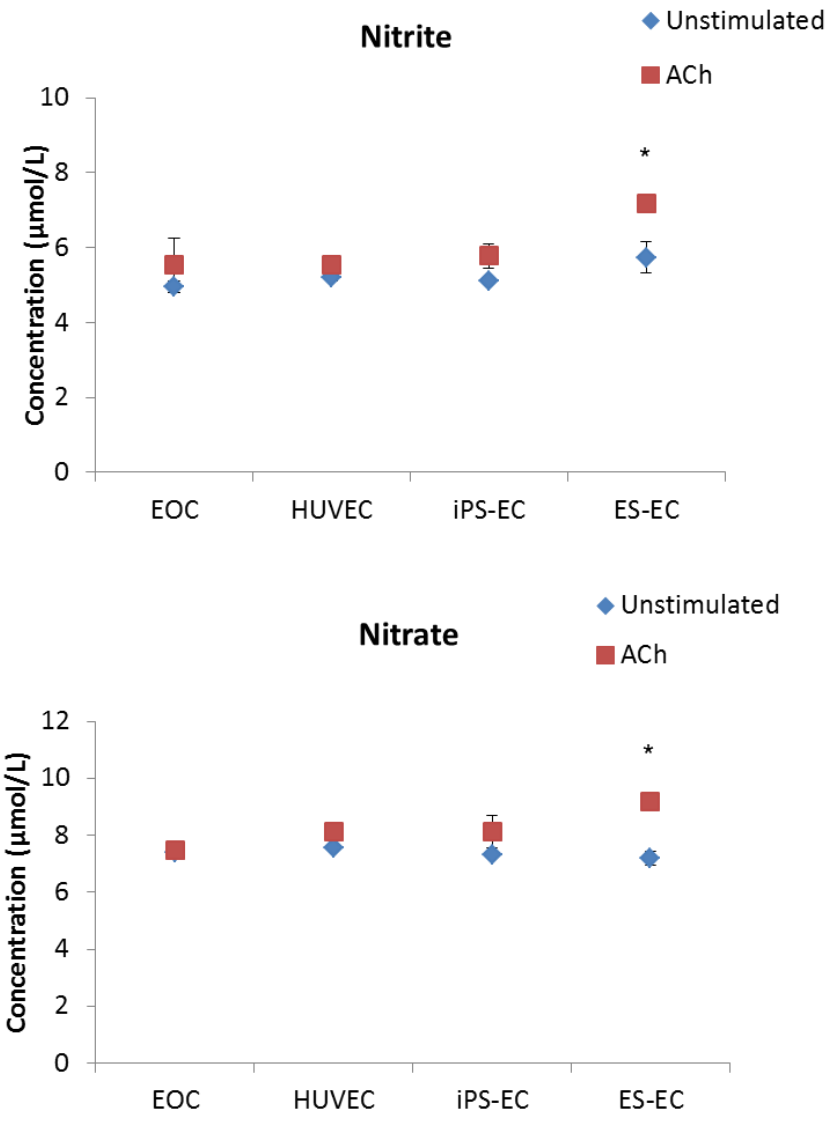

Figure 4.13: Concentrations of nitrite and nitrate detected from endothelial cells derived from embryonic stem cells (ES-EC), those derived from induced pluripotent stem cells (iPS-EC), human umbilical vein endothelial cells (HUVEC) and late endothelial outgrowth cells (EOC) under basal conditions or following stimulation with acetylcholine (ACh; 10µM) over 24 hours. Data are expressed as mean ± SEM and were analysed via Student's paired t test, P≤0.05=*; N=3.

4.5.2. *In vivo* analysis of endothelial–like cells derived from ES and iPS cells via differentiation protocol D

In vitro assays suggested that the differentiated cells have angiogenic capacity (Figure 4.11). To further test angiogenic function of these cells *in vivo*, the murine model of subcutaneous sponge implantation was used (Chapter 2.6). The sponge sections displayed in Figure 4.14 show hematoxylin staining nuclei blue/purple, while eosin stains cytoplasmic structures pink. Blood vessels were identified by the presence of red blood cells, stained red by eosin (arrows indicate perfused vessels). There were a variety of vessel sizes within the sponges: some areas contained very small capillaries, sometimes bigger vessels were apparent.

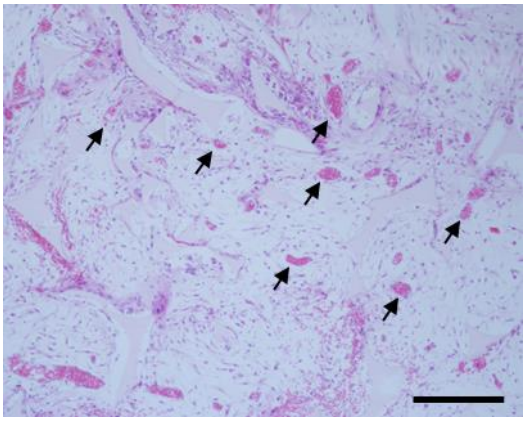

Figure 4.14: Representative images of hematoxylin and eosin stained sections of sponge implants. Blood vessels (arrows) were identified by the presence of red blood cells. Bar = 200µm. Cells in this section other than those of blood vessels are likely to be murine connective tissue cells (such as fibroblasts) which have migrated into the sponge, extracellular matrix or non-incorporated implanted cells.

Vessel density within the sponges was quantified by Chalkley count (Figure 4.15). ES-EC and iPS-EC increased vessel density compared to paired Matrigel controls within the same mouse, (ES-EC: 5.3±0.5 vs 7.1±0.7, P=0.02; iPS-EC: 4.1±0.3 vs 7.6±0.4, P<0.001). Neither EOC nor EOC conditioned media increased vessel counts (EOC: 4.8±0.4 vs 5.1±0.5, P=0.56; EOC-CM: 3.8±0.7 vs 4.7±0.6, P=0.30).

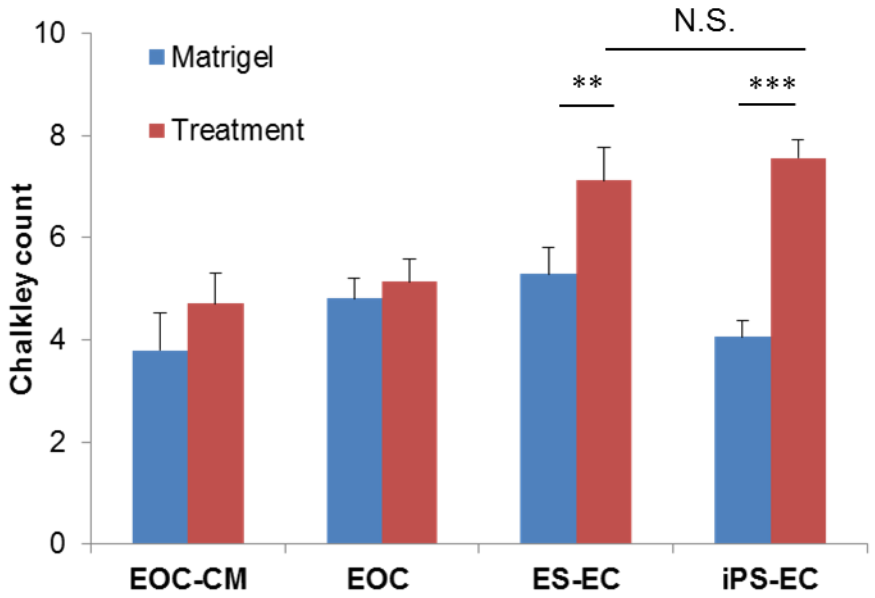

Figure 4.15: Vessel density in implanted sponges was increased by the presence of endothelial cells derived from embryonic stem cells (ES-EC) and those derived from induced pluripotent stem cells (iPS-EC). Sponges contained either endothelial outgrowth cell conditioned medium (EOC-CM; cell medium was collected after 24 hours in culture), EOC, ES-EC or iPS-EC. Vessel density was scored by Chalkley count. Data are expressed as mean ± SEM and were analysed via two-way ANOVA; P≤0.01=**, ***=P≤0.001; N.S.= not significant; N=4-6 mice per group.

Control experiments were carried out to confirm there was no non-specific binding of secondary fluorescent antibodies (Figure 4.16). As expected, only autofluorescence from erythrocytes and sponges was detected from staining of sponge sections with AF488 Goat anti-rabbit IgG (green) and AF568 Goat anti-mouse IgG (red). Most of the vessels examined contained red blood cells, showing that they are functional and perfusing.

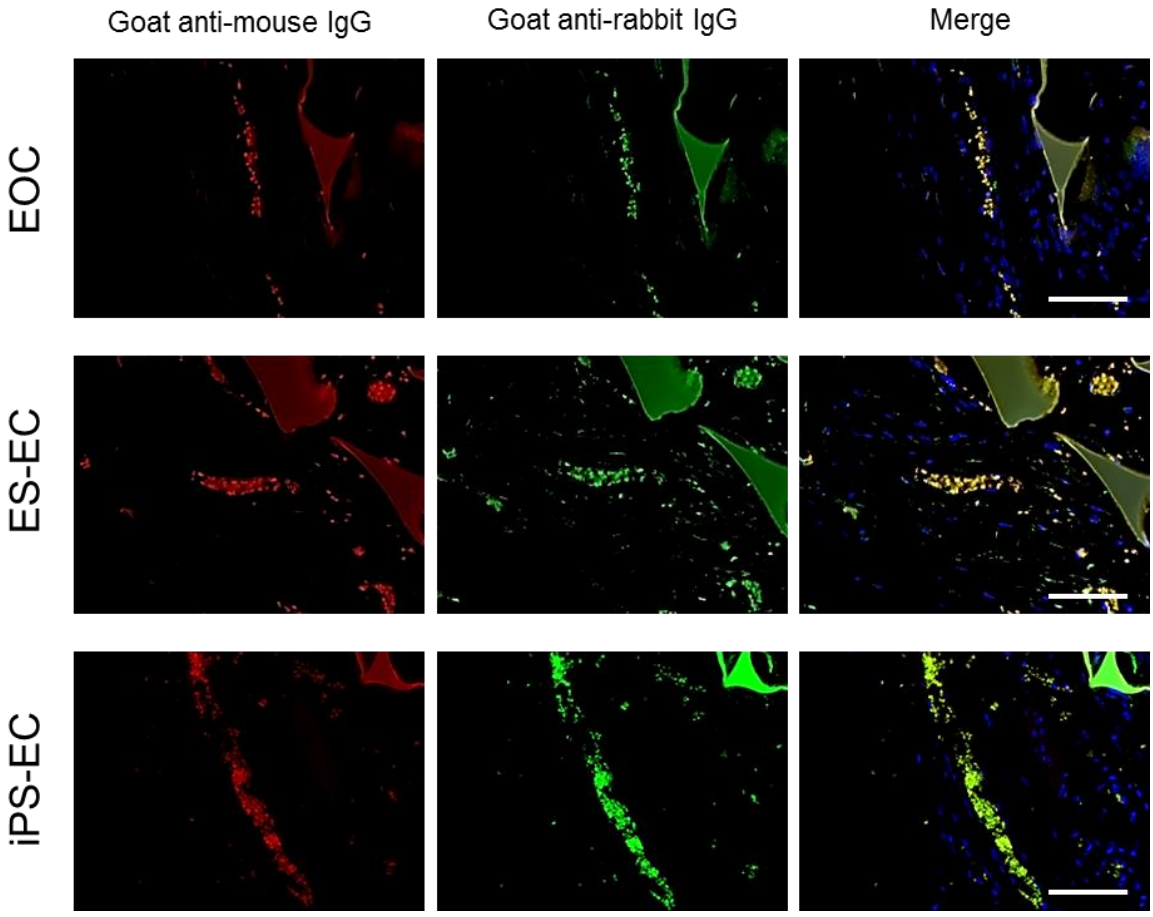

Figure 4.16: Negative control of sponge section immunocytochemistry. Erythrocytes and triangular sponge sections autofluoresce in AF488 (green) and AF568 (red) channels. Nuclei were stained with DAPI (blue). Bar= 100µm.

Figure 4.17 shows representative examples of the control Matrigel-treated sponges, not containing any human cells, stained with paired antibody sets 2 and 3 (Table 2.7). This demonstrates that SMA and CD31 antibodies (from antibody sets 2 and 3, respectively), which are cross reactive to mouse and human cells can be used to identify vasculature. Absence of red fluorescence (apart from erythrocyte and sponge autofluorescence) confirms that the human specific antibodies (CD31 from set 2 and CD146 from set 3) do not stain mouse vasculature.

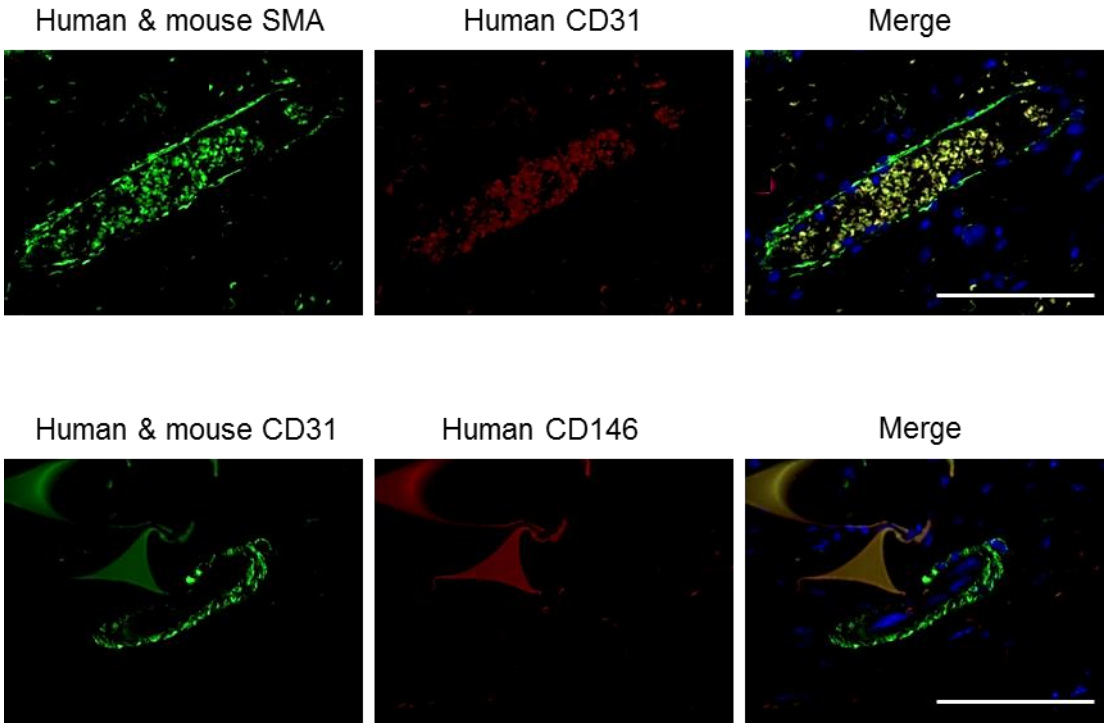

Figure 4.17: As a positive control, murine vessels were detected by staining with SMA and CD31 antibodies (left panels). In comparison, murine vessels were not stained by human specific antibodies CD31 or CD146 (middle panels). Nuclei were stained with DAPI (blue). Erythrocytes autofluoresced in both AF488 and AF568 channels. The triangular structures are sponge matrix. Bar= 100µm.

As vascularised tissue, human fibroid tissue was used as a positive control to test human specific antibodies CD31 and CD146 (Figure 4.18). Positive single stains of each of these antibodies showed that they could both be used to detect vasculature containing cells of human origin.

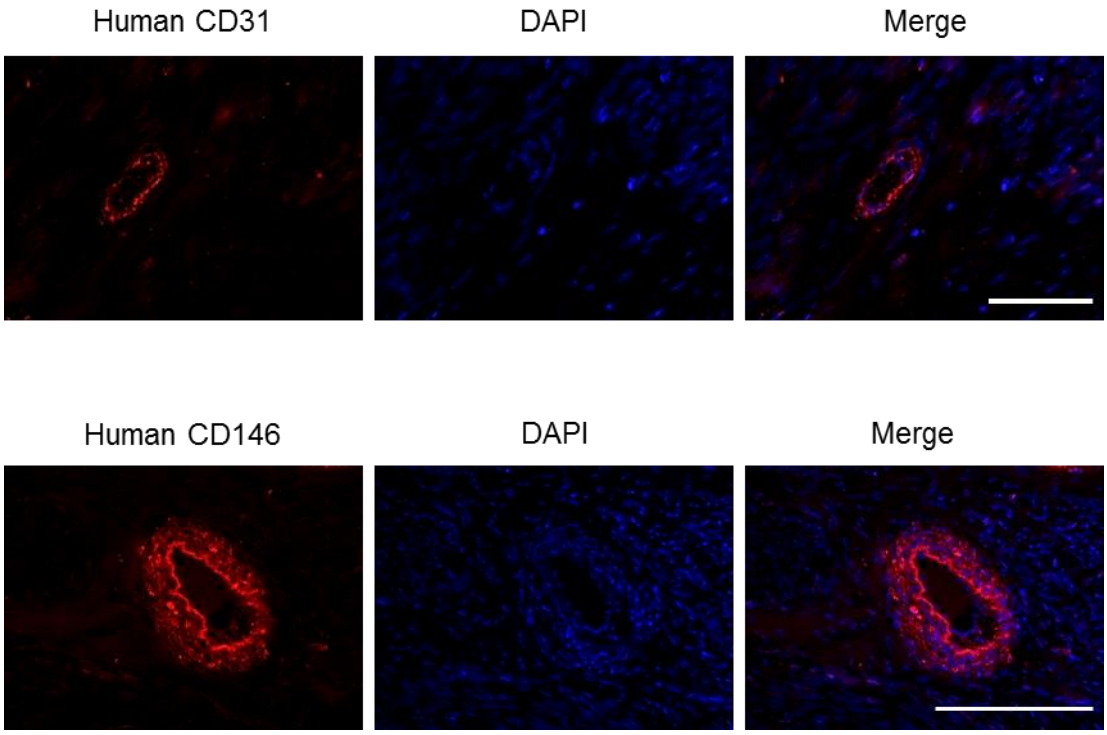

Figure 4.18: Human fibroid tissue was stained by human specific antibodies against CD31 and CD146, as a positive control. Nuclei were stained with DAPI (blue). Bar= 100µm.

Within the following figures, immunostained sponge sections containing EOC (Figure 4.19), ES-EC (Figure 4.20) and iPS-EC (Figure 4.21) are displayed. Vessels could be detected by the green fluorescence generated from binding of either smooth muscle actin or CD31 antibodies, cross reactive to human and murine cells. Red fluorescence, generated from binding of human specific antibodies CD31 or CD146 indicated the presence of human cells. Red fluorescence within a vessel wall therefore indicated that implanted human cells had incorporated into the vasculature. Two examples of incorporation are shown in EOC-treated sponges (Figure 4.19). No incorporation of human cells into vasculature was detected in ES-EC (Figure 4.20) or iPS-EC (Figure 4.21) treated sponges.

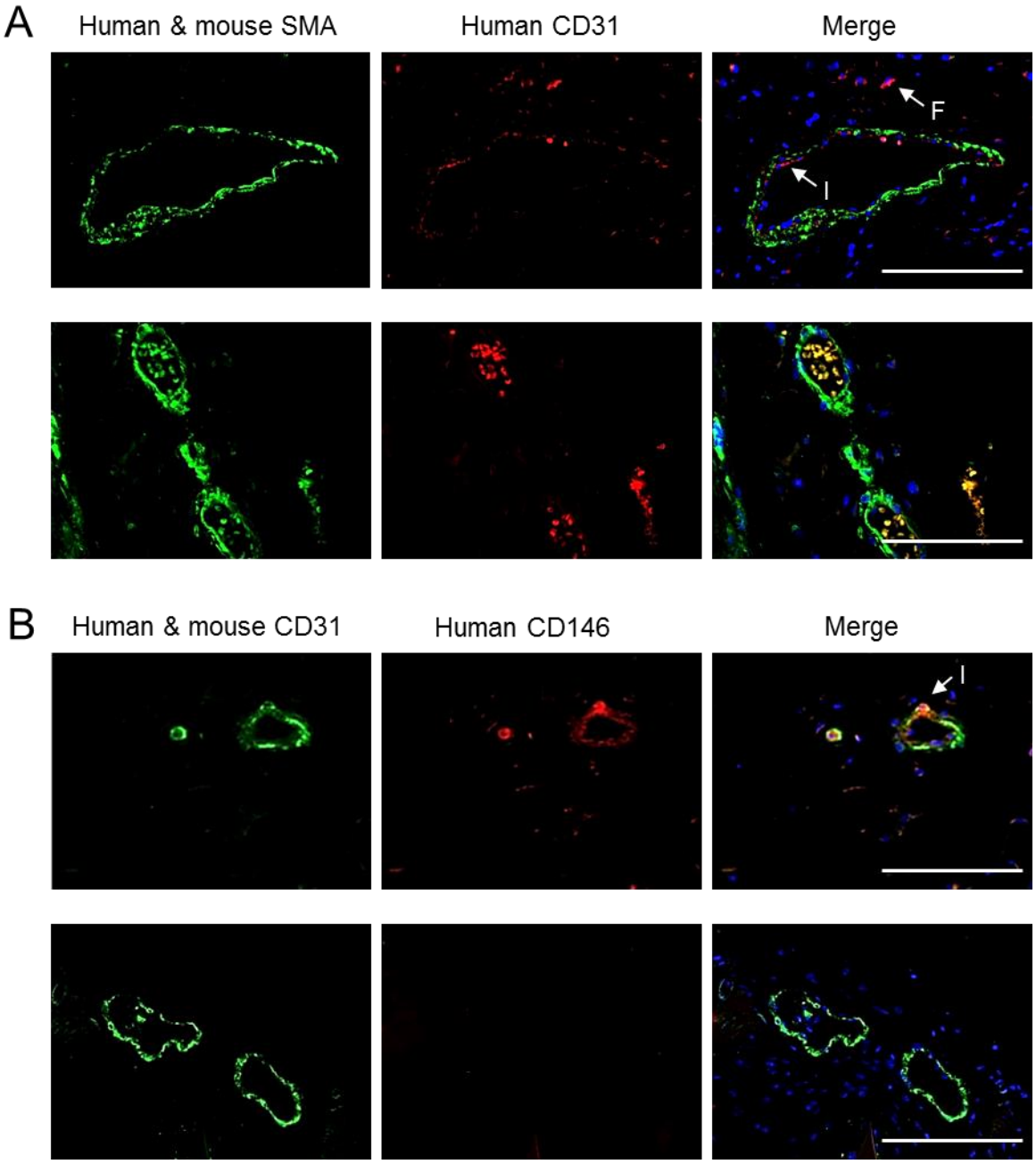

Figure 4.19 Neovasculature detected in sponge sections containing EOC. A) Some vascular structures stained positive for smooth muscle actin antibody (green) and human specific CD31 antibody (red), indicating incorporation of human EOC into the walls of murine vasculature ('I' arrows). Human EOC could also be detected outwith vascular structures ('F' arrows) B) Vascular structures could also be found which stained positive for CD31 (green) and human specific CD146 antibody (red). DAPI stained nuclei blue. Erythrocytes autofluoresced in both AF488 and AF568 channels. Bar = 100µm.

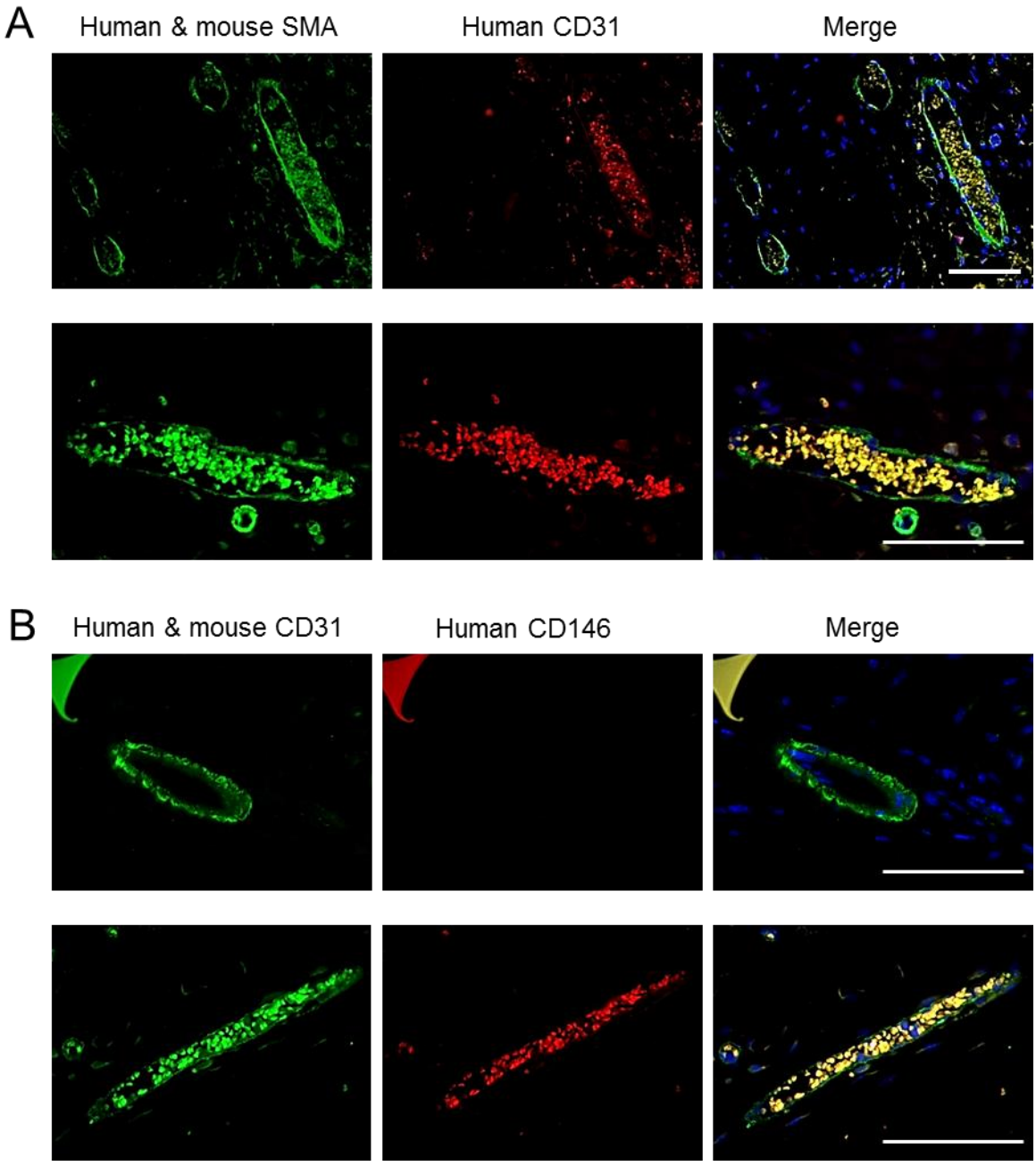

Figure 4.20 Neovasculature detected in sponge sections containing ES-derived endothelial cells. A) Vascular structures stained positive for smooth muscle actin antibody (green), but not human specific CD31 antibody (red). B) Vascular structures stained positive for CD31 (green), but not human specific CD146 antibody (red). DAPI stained nuclei blue. Erythrocytes autofluoresced in both AF488 and AF568 channels. Bar = 100µm.

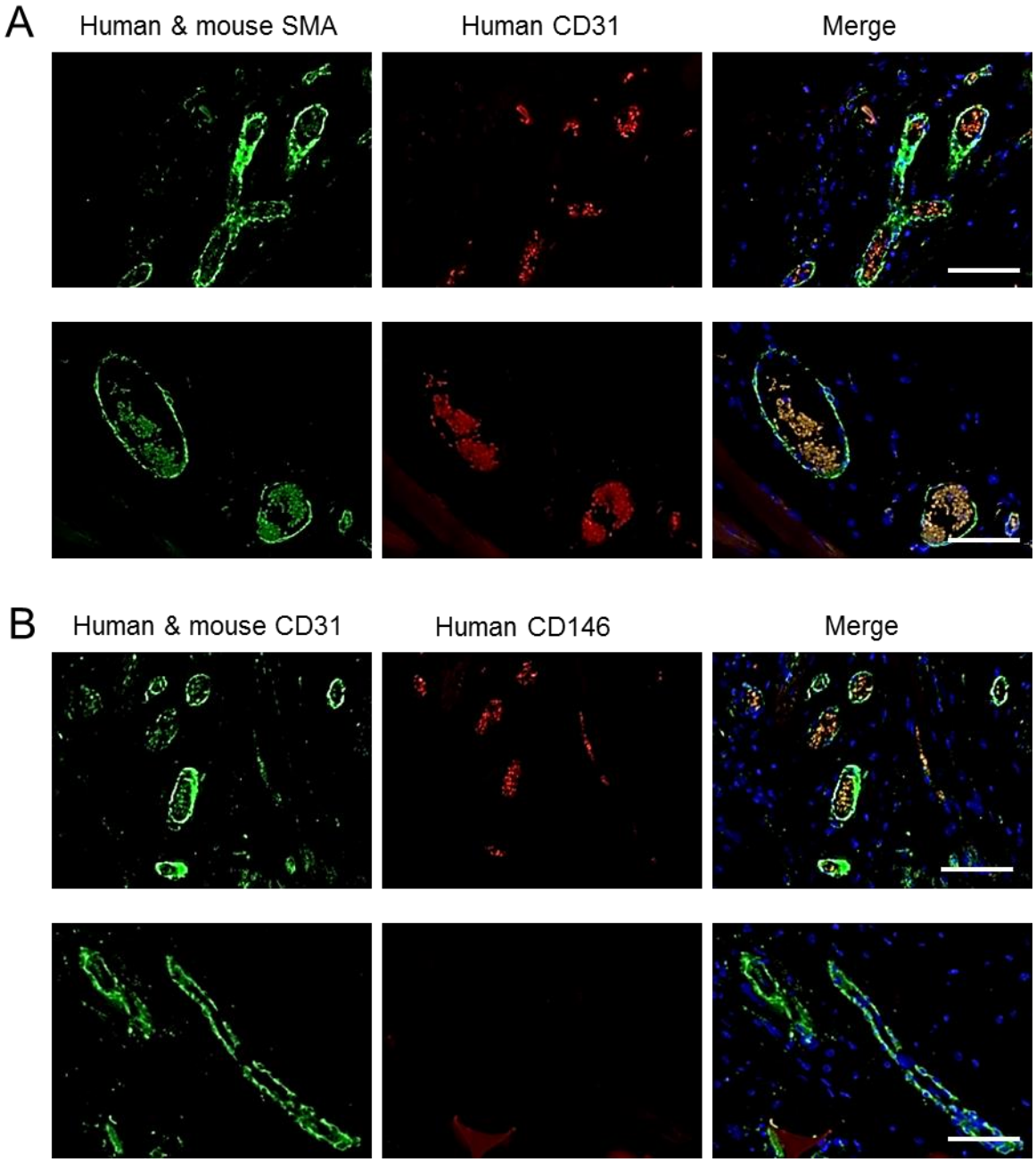

Figure 4.21: Neovasculature detected in sponge sections containing iPS-derived endothelial cells. A) Vascular structures stained positive for smooth muscle actin antibody (green), but not human specific CD31 antibody (red). B) Vascular structures stained positive for CD31 (green), but not human specific CD146 antibody (red). DAPI stained nuclei blue. Erythrocytes autofluoresced in both AF488 and AF568 channels. Bar = 100µm.

In all sponges, human cells outwith vessels were detected by human specific CD31 staining (Figures 4.22).

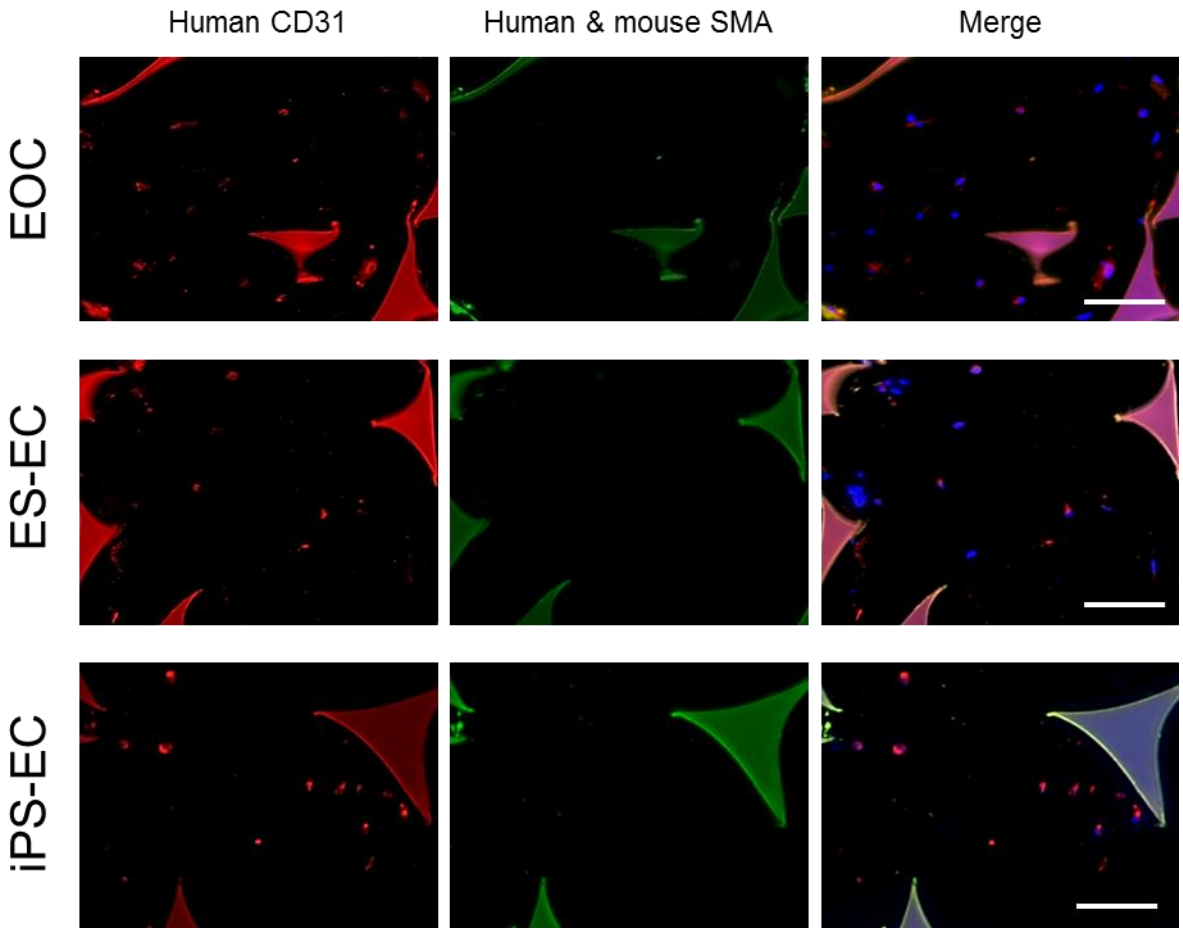

Figure 4.22: Human cells, stained red with CD31 antibody, were identified outwith vascular structures, in sponges containing EOC, ES-ECs and iPS-ECs. DAPI stained nuclei blue. The large triangular structures are sponge matrix. Bar = 100µm.

Implantation of EOC-CM or EOC did not significantly increase new vessel formation in subcutaneous sponges, as measured by the difference in vessel density between treated and untreated paired sponges (Table 4.4). This was despite evidence of incorporation of EOC into newly formed vessels. Conversely, vessel density of sponges was considerably increased by implantation of endothelial cells derived from both ES or iPS cells, though these cells could not be detected in vascular structures.

Cell type implanted	Vessel density (number of blood vessels per 0.5mm^2 section)				Immunocytochemical vessel staining			
	Untreated sponge	Treated sponge	Difference	P value	# vessels analysed	Evidence of incorporation into vessels?	# vessels with human cells incorporated	Detection of human cells out with vessels?
EOC-CM	3.79 ± 0.73	4.71 ± 0.6	0.92	0.301	-	-	-	-
EOC	4.81 ± 0.4	5.14 ± 0.45	0.33	0.557	180	Yes	2	Yes
ES-EC	5.29 ± 0.51	7.13 ± 0.65	1.84	0.025 *	180	No	-	Yes
iPS-EC	4.06 ± 0.32	7.56 ± 0.35	3.50	<0.001 ***	180	No	-	Yes

Table 4.4: Performance of endothelial outgrowth cell conditioned medium (EOC-CM), EOC, endothelial cells derived from induced pluripotent stem cells (iPS-EC) and endothelial cells derived from embryonic stem cells (ES-EC) in a subcutaneous sponge murine model of vascularisation. Neither EOC-CM nor EOC increase vessel density, though there is evidence of EOC incorporation into murine neovasculature. iPS-EC and ES-EC significantly increase vessel density within implanted sponges, though do not incorporate into murine neovasculature. Data are expressed as mean ± SEM. Probability was determined by one-way ANOVA.

4.6. Discussion

This chapter tested the hypothesis that endothelial cells can be generated from human embryonic and induced pluripotent stem cells through a directed differentiation protocol. Though efficiency was low, endothelial cells were successfully generated from pluripotent stem cells through a differentiation protocol involving embryoid body formation, exposure to mesoderm-inducing cytokines, and further culture in endothelial cell conditions supplemented with VEGF. Differentiated ES and iPS cells displayed similar properties to endothelial cell control lines, in terms of their phenotype and functionality *in vitro.* In an *in vivo* model of vascularisation, they appear pro-angiogenic in comparison with endothelial cell controls.

4.6.1. Endothelial differentiation of ES and iPS cells involving embryoid body formation in the presence of mesoderm-inducing cytokines is successful

Of the four differentiation protocols tested, sorted cell populations were consistently low: $CD31^+$ isolated from protocols A, B and D; $CD34^+$ isolated from Protocol C. Protocols A and B were adapted from Choi *et al.,* (2009) and Sone *et al.,* (2007), and used differentiation media, common in a lot of endothelial differentiation protocols (Gerecht-Nir *et al.,* 2003; Yamamoto *et al.,* 2005). Choi *et al.* (2009) report up to 4.5% $CD31^+$ cells from iPS cells and 7.2% $CD31^+$ cells from ES cells after eight days differentiation in this media. Differentiation media is fairly basic and not specific to the culture of endothelial – or even mesodermal – cells; the rationale for its use was to allow general differentiation away from pluripotency, followed by selection of mesodermal/ endothelial cells via FACS. Protocols A and B were unsuccessful, as the low yields of sorted cells did not survive in culture. Some published protocols, which have used differentiation media have co-cultured stem cells with OP9 cells, a murine stromal cell line, described as a 'feeder' layer (Taura *et al.,* 2009; Sone *et al.,* 2007). These cells release soluble factors, such as VEGF-C

and Ang1, which are thought to influence endothelial differentiation (Kono *et al.*, 2006), and have also been used in hematopoietic differentiation protocols (Nakano, 2003). This suggests that whilst differentiation medium provides a 'base' culture condition, further cytokines/growth factors are likely required for effective endothelial differentiation. Consistent with this concept, studies have attempted to develop more sophisticated differentiation protocols with the use of defined factors thought to induce mesodermal and endothelial differentiation (James *et al.,* 2010; Goldman *et al.,* 2009). Protocol C was based on studies by Ferreira *et al.* (2007) who demonstrated that culturing embryonic stem cells for seven days in differentiation media generated a population of $CD34^+$ cells, of which 94% express CD31 upon further culture with VEGF. However, unexpectedly, in our laboratory this result could not be reproduced and addition of VEGF supplement did not alter CD31 expression in the $CD34^+$ sorted cell population derived from either ES or iPS cells. The low yields of cells from protocols A, B and C indicated that optimisation of the protocol was required. Being unable to reproduce published findings may be explained by differences in the starting stem cell populations and their culture conditions prior to differentiation, often a variable factor between laboratories. Newman *et al.,* (2010) showed that culture conditions and laboratory environments can have an effect on gene expression patterns in stem cell lines, which may affect their differentiation potential. This leads on to the suggestion that these protocols are not robust enough for their results to be translated across different ES and iPS cell lines. The most success came from unpublished endothelial differentiation protocol D, from which distinct $CD31^+$ cell populations were generated from ES and iPS cells following exposure to a host of mesoderm-inducing cytokines. Signalling of BMP, Wnt and Activin are involved in directing embryonic stem cell developmental pathways to hematopoietic (Nostro *et al.,* 2008) and cardiovascular (Kattman *et al.,* 2011) lineages. This indicates the complexity of endothelial differentiation, underestimated in protocols A and B. Success with multiple cytokines may indicate that VEGF alone (protocol C) was insufficient for endothelial differentiation. $CD31^+$ populations generated from protocol D could be isolated, maintained and expanded in culture, and CD31 expression was retained over time. Though higher yields of cells from protocol D would be desirable (and optimisation may be required to

increase differentiation efficiency), for the purpose of characterisation, sizes of starting cell populations were increased to compensate for low cell yields. Expression of CD31 was low in this mixed population of differentiating ES and iPS cells, relative to other endothelial cell markers. Of the endothelial markers analysed, CD31 could be considered the most specific for endothelial cells: it is expressed on endothelial cells and haematopoietic cells, whereas other cell markers are expressed on a wider variety of cell populations. For example, CD105 is found on smooth muscle cells and monocytes (Rohde *et al*., 2006); CD146 is expressed by mesenchymal stem cells and pericytes (Espagnolle N, *et al.*, 2014; Blocki *et al.*, 2013) as well as endothelial cells. This was the rationale behind using CD31 as a marker of differentiating endothelial cells. The expression levels of endothelial haematopoietic and pluripotency markers indicate a heterogenous cell population, of which it is likely only a small subset of cells are CD31 positive and truly endothelial in nature at this stage.

With the exception of VEGFR2, both ES and iPS cells displayed very low expression of all endothelial cell surface markers prior to differentiation. This was unexpected, as these cells had been shown to be pluripotent (able to differentiate to derivatives of all three germ layers), whereas VEGFR2 expression is associated with stem cells in the early stages of commitment to a mesodermal cell lineage (Kubo and Alitalo, 2003). Indeed, it is well known that $VEGFR2^{+}$ cells represent a common precursor for haematopoietic stem cells and endothelail cells (Choi et al 1998). Expression seen here may represent a subpopulation of cells, which are tending towards an endothelial phenotype in culture.

Based on CD31 expression, ES cells appear to have a higher differentiation efficiency than iPS cells. This may be because protocol D had been optimised for ES cell differentiation. That iPS are less efficient may indicate that there are underlying differences between ES and iPS cells. Though both cell types are pluripotent, in the literature there is controversy about how similar these two cell types are, both in terms of their characteristics when undifferentiated, and their differentiation potential. For example, a study by Bock *et al.* (2011) involving 20 ES and 12 iPS cell lines

found that, despite many common features, gene expression and epigenetic patterns varied between different iPS cell lines, between different ES cell lines and between iPS and ES cell lines. Chng *et al.* (2010) have carried out comprehensive studies using multiple iPS cell lines (which vary in terms of reprogramming method and somatic cell source) showing that lines can be categorised based on their differentiation bias towards a certain germ layer. It would be interesting to compare the endothelial differentiation potential of other iPS cell lines, generated by other laboratories, to identify to what extent this variability may affect endothelial differentiation. It would also be interesting to differentiate these iPS lines to other lineages, of ectoderm or endodermal origin. This would indicate any propensity they may have for directed differentiation to a particular lineage. Another consideration is that studies have shown directed differentiation of iPS cells can be influenced by residual epigenetic memory of the somatic cells from which iPS cells were derived. Methylation patterns of the original somatic cell could potentially remain and influence gene expression or differentiation efficiency (Kim *et al.,* 2010; Sullivan *et al.,* 2010). This concept is discussed further in Chapter 5.

4.6.2. $CD31^+$ cells isolated from differentiating ES and iPS cells have an endothelial-like phenotype.

A mixed population of cells was generated after seven days of endothelial differentiation through protocol D. The protocol used specified isolation of $CD31^+$ cells, as this is a key endothelial marker, often used in the characterisation of endothelial cells (Choi et al., 2009; Kane et al., 2010). However, there are also sub-populations of cells after seven days of differentiation, which express endothelial markers CD34, VEGFR2, CD146 and CD105. Though none of these markers are specific to endothelial cells, it is possible that these populations also have the potential to develop into endothelial cells, and may acquire CD31 expression at a later stage. It would be interesting to test this potential in further experiments. As previously discussed, though CD31 is not a definitive marker of endothelial cells, it is one of the most specific ones (only being expressed on haematopoietic cells as well as endothelial). Sorted $CD31^+$ cells were phenotypically characterised by flow

cytometry and immunocytochemistry. High levels of CD31 expression in ES-EC and iPS-EC were comparable to control endothelial cell lines and indicated that this marker is retained following sorting. Although there were no significant changes in expression of these endothelial markers on ES-EC or iPS-EC over time, expression of CD34, VEGFR2 and CD105 appeared heightened in later passage cells. This may indicate that these cells retain plasticity in culture and that differentiation towards a fully mature endothelial cell is a step-wise process, as has been suggested by Fujimoto *et al.* (2001). Negligible expression of hematopoietic markers (CD133 and CD45) indicated that ES-EC and iPS-EC have diverged from mesodermal cells and committed to an endothelial, rather than a hematopoietic, lineage.

Interestingly, SSEA3 and SSEA4 expression appeared lower in later passage populations, a finding which reached significance in terms of SSEA3 expression of ES-EC. This supports the suggestion that differentiation is occurring in stages and that these cells display plasticity in culture until they reach a stage of maturity towards later passages, with minimal pluripotency marker expression and maximal endothelial marker expression. Later passage populations have expression levels of SSEA3 and SSEA4 between 2-4%, similar to levels in control endothelial HUVEC and EOC populations. These cells may be a distinct population. Studies have shown that $SSEA3^+$ cells within a somatic cell culture of fibroblasts (which co-express CD105) have properties similar to bone marrow- and fat derived mesenchymal stem cells, which proliferate in response to cell injury and may play a role in regeneration. (Crespo *et al*, 2012). It is possible that endothelial cells may too retain plasticity *in vitro* and that the $SSEA3^+$ populations seen here may have progenitor-like potential. Wakao *et al*, 2011 also describe a $SSEA3^+CD105^+$ population of cells, named multilineage-differentiating stress enduring (MUSE) cells, which have enhanced reprogramming efficiency compared to non-MUSE cells. It would be interesting to isolate the $SSEA3^+$ populations seen here to ascertain if these cells too display enhanced reprogramming properties.

Whilst as a panel, the markers analysed by flow cytometry give an indication of an endothelial phenotype, none are completely specific to the endothelial cell. For

example, CD146 (melanoma cell adhesion molecule) can also be detected on lymphocytes and pericytes, whilst CD105 (endoglin) can be detected on monocytes and vascular smooth muscle cells. To complete the phenotypic profile of cells, immunocytochemistry was performed to detect factors associated with endothelial cells: von Willebrand Factor (vWF) and endothelial Nitric Oxide Synthase (eNOS). vWF is a multimeric glycoprotein involved in hemostasis and has a characteristic granular staining pattern in endothelial cells. It is stored in, and released from, Weibel-Palade bodies in endothelial cells (Wagner *et al.,* 1982), consistent with the granular cytoplasmic pattern of staining seen in ES-EC and iPS-EC. eNOS, the endothelial-specific form of nitric oxide synthase, plays a key role in the regulation of vascular function and maintenance of hemostasis (Lamas *et al.,* 1992), and could also be detected in the membranes and cytoplasmic regions of stem cell-derived endothelial cells. This is consistent with localisation of eNOS within the endothelial cell, where it is targeted to cholesterol-rich domains of the plasma membrane and to the Golgi complex (Fulton *et al.,* 2004).

Combined, these results showed that $CD31^+$ cells sorted from differentiating ES and iPS cells had a similar phenotype to control endothelial cell lines, suggesting that this is an effective protocol for the production of endothelial cells.

4.6.3. ES and iPS derived endothelial-like cells are functional and may have potential for therapeutic use

4.6.3.1. *In vitro* analysis

A series of *in vitro* functional assays demonstrated that the ES- and iPS-derived endothelial cells were functional, with similar capabilities as control endothelial cell lines. ES-EC and iPS-EC formed connections *in vitro* when applied to Matrigel, in a similar way to control endothelial lines. This suggests they may be able to form tubules or contribute to capillary growth *in vivo*. Matrigel is prepared from Engelbreth-Holm-Schwann (EHS) tumour cells- its primary component is laminin, a biologically active protein of the basement membrane (Aumailley *et al.,* 1998). Upon

contact with endothelial cells, laminins promote adhesion, migration and differentiation (Grant *et al.,* 1989), and they also play a key role in the organisation and establishment of the basement membrane matrix (Davis and Senger, 2005). With Matrigel as a scaffold, endothelial cells characteristically form connections, sometime described as tube-like structures (Logie *et al.,* 2010); this assay has been in use since 1988 (Kubota *et al.,* 1988), and is used to demonstrate the cell-cell communication that occurs in angiogenesis. Whilst angiogenesis is a complex process, this *in vitro* assay is a rapid, quantitative way to determine the potential of endothelial cells for the migration and differentiation stages of the angiogenic cascade, and is a useful assay to precede *in vivo* angiogenesis experiments. Though this assay is commonly used in the analysis of endothelial cells, other cell types have been found to form connections on Matrigel. For example, primary human fibroblasts and U87-MG (a glioblastoma cell line) form tubules (Donovan *et al.,* 2001). It is, nonetheless, still an indication of angiogenic potential of endothelial cells, and there is a distinction in the morphology of the tubules formed between endothelial cells and connections formed between other cell types (Arnaoutova *et al.,* 2009). More frequent imaging of the tubules, or tracking their formation with time lapse microscopy would give more insight into the rate (and possibly mechanism) of formation of these tubule-like structures (Logie *et al.,* 2010). Further experiments could also include testing functional responses to angiogenic factors (for example, VEGF, demonstrated by Donovan *et al.,* 2001). Elucidation of how these cells might react to pro-angiogenic factors or compounds could be included in the design of cellular therapeutic approaches for repair of atherosclerotic vessels or revascularisation of ischaemic tissue.

The ability of cells to migrate and proliferate to undergo wound healing *in vitro* was quantified by a scratch assay with ES-EC, iPS-EC and endothelial control line HUVEC. This assay looks at the totality of cell behaviour following wounding; through both migration and proliferation of recovering cells (adapted from Liang *et al.,* 2007). This assay mimics the process of wound healing *in vivo*: disruption of the endothelial layer induces migration and proliferation of nearby endothelial cells to repair the area (Alberts *et al.,* 2002). The wounding assay is usually used as

measurement of a cytokinesis response. However, in this case, no growth factors or cytokines were added to the media, therefore these experiments represent the migrational response and movement of cells post-wounding. These experiments could be developed further to assess responses to VEGF, for example, to demonstrate cytokinesis of endothelial cells. In all cell lines used in these experiments, the cell layer had completely recovered after 24 hours; and there was no significant difference in wounding after 6 hours, suggesting that rates of cell migration are the same for all cell lines, another indication of the endothelial nature of the ES- and iPS- derived cells.

The production of nitric oxide (NO), a potent vasodilator catalysed by eNOS, is a key function of the endothelium (Ignarro et al., 1987). As eNOS was present on ES-EC and iPS-EC (detected by immunocytochemistry), the NO assay would have confirmed it's functionality in response to stimulation by acetylcholine. However, as neither nitrite nor nitrate could be detected from endothelial cell control lines, this assay may not be best suited to the detection of NO release *in vitro*. It is therefore unlikely that the increases in nitrite and nitrate concentrations in the supernatant of ES-EC are real. Though experimentally, ACh has been shown to stimulate NO release (Amezcua *et al.*, 1988; Joannides *et al*., 1995), this is usually an immediate reaction. For example, in isolated arteries, acetylcholine causes muscarinic receptor-mediated release of NO within seconds (Cohen and Vanhoutte, 1995). Further studies need to be carried out to optimise the concentration of acetylcholine used for stimulation, and the length of time before supernatant collection. Also, as an alternative to ACh, LPS could be used in future experiments as a positive control for stimulation of NO release (Cendan et al., 1994). Whilst the colorimetric Griess assay is widely used for the detection of organic nitrite compounds (Granger et al., 1996; Schulz et al., 1999), there are limitations associated with it. Failure to detect nitrite produced from reduction of nitrate may indicate that this reaction is incomplete (Sharma *et al.*, 2008). Incomplete nitrate reduction could be due to the presence of, for example, azide, ascorbic acid or sulphydryl-containing compounds within the sample, which could interfere with nitrate reductase, lowering the conversion of nitrate to nitrite and therefore giving rise to lower estimates of NO. (Yao *et al*., 2004). Ascorbic acid is present in the culture media (EGM-2). In repeating this experiment,

a different culture media could be used, for example Minimal Essential Medium, which does not contain ascorbic acid. The Griess reaction itself is inhibited by the presence of pyridine nucleotides (NADH and NADPH) (Grisham *et al*. 1995); the former is involved in the nitrate reductase process. Unreacted levels of NADH prior to colour detection may account for a lack of signal from the Griess reaction. An alternative method of nitric oxide detection is via a Chemiluminescence NO analyser, in which nitric oxide, upon reaction with ozone, produces luminescence directly proportional to the levels of NO in the conditioned media (Bussolati *et al.*, 2001). As this measures NO directly, rather than its metabolites, it may be more suited to analysis of production from endothelial cells, and is something to consider in the design of future experiments.

In summary, *in vitro* assays confirmed that there are many similarities between stem-cell derived $CD31^+$ cells and endothelial cells. In combination with phenotypic analysis, and in comparison with endothelial cell controls, it is clear that endothelial-like cell populations have been derived from differentiation of stem cells.

4.6.3.2. *In vivo* analysis

In vitro assays have a limited capacity to inform about the functional properties of endothelial cells, due to the 2 dimensional and artificial nature of cell culture. Flat monolayers of cells in culture do not take into account interactions with other cell types, or exposure to circulating cytokines or shear stress that occurs within the tissues microenvironment. Greater insight into the functions of these cells can be provided by *in vivo* studies.

Though there were no differences between stem cell derived cells and endothelial controls in the *in vitro* angiogenesis assay, ES-EC and iPS-EC displayed increased angiogenic potential *in vivo* in comparison to EOC or EOC-conditioned media. This was in the form of increased vessel density, and suggests that they are a pro-angiogenic cell type in comparison with endothelial cell controls. The disparity

between *in vitro* and *in vivo* assays likely reflects the sheer complexity of angiogenesis, many factors of which are not considered in the *in vitro* assay.

Many *in vivo* studies concerning endothelial cells have been published, to assess their functional potential, ranging from attempts to induce neovasculogenesis (Hague *et al.,* 2002), to assistance in the processes of reendothelialisation following injury (Xiao *et al.,* 2006). In the design of these experiments it is important to consider the optimal cell type to promote angiogenesis. Whilst there is confusion around the precise definition of an 'endothelial progenitor cell' (Yoder *et al.,* 2007), cellular outgrowth from mononuclear cells in blood which express an endothelial cell phenotype are thought to have a heightened capacity for migration and proliferation to aid in repair and regeneration of the endothelium (Dimmeler *et al.,* 2004). These blood outgrowth cells have been widely used in *in vivo* models of vasculogenesis and angiogenesis (Barclay *et al.,* 2012). Endothelial outgrowth cells from cord or peripheral blood are thought to be the best example of an endothelial progenitor cell, and have demonstrated potential to form blood vessels *in vivo*. For example, Au *et al.* (2007), implanted EOC in a cranial window model of angiogenesis to test their stability and function over time *in vivo*, and found that both cord blood and peripheral blood-derived EOC formed vessels. Ott *et al.* (2005) found that $CD34^{+}$-derived EOC form vascular structures in a model of ischaemic myocardium, and are able to improve left ventricular function in a model of myocardial infarction. Though the EOC used as a control cell line in this thesis did not have any effect on vessel density, this may be because they had gone through 5 passages. Barclay *et al*., 2012 commented that EOC of P0/P1 did incorporate into vasculature, whereas later passage cells did not. This period of time in culture may be enough to alter cell characteristics, reducing their angiogenic potential, as has been reported by Melero-Martin *et al* 2008. Melero-Martin *et al.* (2008) reported that maturation of EOC lines *in vitro* decreased their ability to vascularise Matrigel plugs *in vivo*.

Immuno-analysis by species-specific panels of antibodies was robust, as shown by clear positive and negative controls. All the vessels analysed from the sponges treated with ES-EC and iPS-EC appear to be of mouse origin, demonstrated by the

absence of human specific antibody staining in the vessel walls. Apart from one example of incorporation, this was also the case for implanted EOC. From antibody set 2, green SMA staining was bright in most cases, and appeared to stain the endothelial lumen, which was useful for the detection of vessels. Other studies have shown SMA staining of endothelial cells (Azuma et al, 2009; Lu *et al.*, 2004), though it is also known to stain mesenchymal cells (Melero-Martin *et al.*, 2008). Set 3 staining of CD31 is distinctive, indicating that it is an appropriate marker of endothelial cells in vessel walls. Some of the fields imaged contained 'free' $CD31^+$ human cells, not contained within vascular structures, confirming that the implanted human cells were able to survive the process of implantation for 21 days, even if they were unable to incorporate into vascular structures. A substantial proportion of the sponge was sectioned for analysis, therefore I am confident that human cell incorporation is not being missed due to methodological reasons.

In addition to lack of incorporation, a common feature of all sponges is that the vessels are mostly, if not all, found towards the periphery of the sponge, suggesting that the vessels which form are predominantly mouse vessels growing in to the sponge from outside (angiogenesis), rather than the embedded cells sprouting vessels from within the sponge (neovasculogenesis). As ES-EC and iPS-EC increase vessel density without incorporating into vascular structures, it is likely that they are secreting paracrine factors that stimulate migration and differentiation of nearby mouse endothelial cells, enhancing angiogenesis. *In vivo* studies into paracrine effects on vessel growth have been investigated before and suggest that a number of factors (including IL-8, MCP1, HGF, bFGF, VEGFa) secreted by implanted cells can be pro-angiogenic (Krenning *et al*. 2013). Further experiments are required to ascertain the mechanism by which these cells are exerting their pro-angiogenic effects. For example *in vitro* analysis of migration kinetics of murine endothelial cells in response to ES-EC or iPS-EC conditioned media. Metabolomic analysis of this conditioned media may also offer insight into pro-angiogenic paracrine factor secretion.

4.6.4. Limitations and future studies

In some cases, variable differentiation efficiencies exist between iPS cell lines that have originated from different laboratories, possibly due to variations in cell type of origin or reprogramming technique (Bock *et al.*, 2010). A clearer assessment of the suitability of the endothelial differentiation protocol would be gained from testing it on a wider panel of iPS cell lines. Experiments were limited to three iPS cell lines that were available within the institute, due to the time-consuming maintenance demands of stem cell culture. Further studies could include iPS cells derived from other reprogramming methods or from other cell types of origin, to scrutinise the efficiency of the endothelial differentiation protocol and assess its suitability for application to multiple ES and iPS cell lines.

There may be advantages in implanting a non-purified population of differentiating cells for *in vivo* angiogenic assessment, as systemic factors may facilitate the differentiation and maturation of endothelial cells; as such it would be interesting to further study cells recovered from sponges, to assess effects of exposure to systemic *in vivo* conditions. Conversly, implantation of immature cells, or populations still containing pluripotent cells may lead to their differentiation down non-endothelial pathways, or tumour formation.

The *in vivo* murine model of subcutaneous sponge implantation of endothelial cells is more suited to assessment of angiogenic potential than *in vitro* assays, though it too has its limitations. The cells used in the murine subcutaneous sponge implant model were tested under non-stressed physiological conditions (the mice were healthy and immuno-compromised), and therefore pathological factors associated with atherosclerosis which influence angiogenesis do not feature in these experiments. For example, in atherosclerosis, inflammation contributes to the stimulation of angiogenesis (Carmeliet and Jain, 2000). Inflammatory cells surrounding the lesion- monocytes, macrophages and other leukocytes- produce soluble factors including VEGF, Ang1, and bFGF (Seljelid *et al.*, 1999) which stimulate angiogenesis. Also, angiogenesis can be stimulated by hypoxia, which can arise from the disruption of

blood flow due to atherosclerotic plaques (Felmeden *et al.,* 2002). In this case, hypoxic inducible transcription factors are activated, which function downstream to induce expression of various angiogenic factors, for example VEGF, eNOS, PDGF and Ang2 (Semenza, 1998). As the overarching aim of this study is the generation of endothelial cells that have the potential to repair ischaemic tissues following myocardial injury, a progression of these studies could be *in vivo* assessment of differentiated cells in a pathological model of angiogenesis. This could determine how they respond to pro-angiogenic factors, (which include VEGF, Ang1, bFGF, TGF-β1, PDGF, TNF-α, among many others; Carmeliet and Jain, 2000), released from stromal cells and inflammatory cells. Additionally, assessment of differentiated endothelial cells in hypoxic conditions could be carried out in the murine ischaemic hindlimb model. Rufiahah *et al.* (2011) report that implanted iPS-ECs secrete angiogenic cytokines (Ang-1, VEGF-A, VEGF-C and PDGF). Testing these cells *in vitro* showed that these secretions are upregulated in hypoxic conditions, compared with normoxic conditions. Further studies may also help to elucidate any differences between the processes of angiogenesis in physiological and pathological conditions.

It would also be interesting to further understand the effect of EOC on neovascularisation of an implanted sponge by using a fully murine model with murine EOC (obviating the need for NSG mice). Murine EOC have been found to act as endothelial progenitors, and have demonstrated persistant seeding in liver, lung, spleen and bone marrow of recipient mice (Somani *et al*, 2007).

4.7. Conclusions

The work described in this chapter demonstrated that endothelial cells can be generated from both human embryonic and induced pluripotent stem cells. ES-EC and iPS-EC had a typical endothelial phenotype, similar to that of EOC and HUVEC, and, despite their low differentiation efficiency, could be expanded and maintained in culture. *In vitro* functional assays confirm that endothelial cells derived in this way were functional within the tested parameters. *In vivo* studies suggested that implanted ES-EC and iPS-EC, in a murine sponge implant model, were pro-angiogenic, and

likely increasing vessel density through paracrine mechanisms. Therefore, this is a suitable method for generating populations of endothelial cells for *in vitro* and *in vivo* functional studies. In the future, iPS cell technology could be applied to generate patient specific cell lines to elucidate the mechanisms of endothelial dysfunction in patients with premature cardiovascular disease.

Chapter 5

Differentiation of fibroblast- and endothelium-derived iPS cells into mature endothelial cells.

Abstract

Introduction: Endothelial differentiation of human pluripotent stem cells is challenging, with low yields of functional endothelial cells. Reports suggest that iPS cells derived from sources other than fibroblasts may have enhanced differentiation potential towards their cell type of origin. This chapter hypothesised that that pluripotent stem cells derived from endothelial cells (eiPS) would have enhanced capacity for endothelial differentiation compared to those derived from dermal fibroblasts (fiPS).

Results: Differentiation of eiPS generated more $CD31^+$ endothelial cells after 7 days compared to differentiation of fiPS (21±3% *versus* 3±2% respectively; $P<0.05$). Endothelial cells from eiPS cells (eiPS-EC) have many similar properties to endothelial cell controls, with high expression of CD31 (75%±7%), CD146 (86%±6%), CD105 (91%±3%), vWF and eNOS. Endothelial cells from fiPS (fiPS-EC) were similar, with the exception of CD105 expression, which at 30%±12% was significantly reduced compared to control endothelial cells. In addition, fiPS-$CD31^+$ retained some expression of pluripotency markers SSEA3 (16%±7%) and SSEA4 (23%±5%) and appeared to have diminished migratory and proliferative properties in the wound healing assay. In comparison to endothelial cell controls, the angiogenic capacity of fiPS-EC and eiPS-EC was similar *in vitro*, though enhanced *in vivo* when compared to endothelial cell controls. In a murine subcutaneous sponge implant model of neovasculogenesis, fiPS-EC and eiPS-EC cells increased vessel density (by 3.50 and 3.47 vessel counts respectively, $P\leq0.001$ for both), whereas endothelial cell controls did not ($P>0.05$).

Conclusions: Endothelial differentiation efficiency is enhanced using induced pluripotent stem cells derived from somatic cells of endothelial lineage, in comparison to iPS cells from dermal fibroblasts. The mechanisms that underpin this are unclear, but it seems likely that these cells retain epigenetic factors that increase efficiency. Endothelial cells derived from eiPS are phenotypically closer to control endothelial cells than fiPS-EC, suggesting they may be the ideal source for generating iPS cells for therapeutic vascular regeneration.

5.1. Introduction

The genetic risk factors predisposing individuals to atherosclerosis (the major cause of coronary heart disease) are poorly understood. Impaired capacity of the endothelium to repair and regenerate following injury is one of the earliest events in the pathogenesis of atherosclerosis. Genome wide association studies indicate that premature atherosclerosis occurs in some instances due to inherited deficiencies in endothelial integrity (as reviewed by Damani and Topol, 2007). To elucidate the mechanisms of endothelial dysfunction, induced pluripotent stem (iPS) cell technology may be used to study the function of endothelial cells from patients with atherosclerosis. Theoretically, large quantities of cells can be generated this way, and the study of their functional properties *in vitro* and *in vivo* can give insight into the mechanisms underlying endothelial dysfunction. It is also envisioned that patient-specific endothelial cells could also be used for drug development and possibly for the identification of causative mutations for future genetic therapies.

iPS cells were initially generated from fibroblasts (Takahashi *et al*., 2007), but as the field has developed, other somatic cell types have been reprogrammed. In some cases iPS cells possess residual epigenetic memory of their former cell type, in the form of characteristic methylation or histone marks. For example, Ohi *et al.* (2011) report that iPS cell lines with the same genetic background but derived from somatic cells representative of all three embryonic germ layers have transcriptional memory associated with their original somatic cell. Furthermore, this can lead to preferential differentiation back towards their cell lineage of origin (Kim *et al.*, 2011), a finding that can be exploited to produce somatic cell types that have been otherwise difficult to obtain from ES or fibroblast-iPS cell lines. As demonstrated in chapter four, the differentiation of endothelial cells from healthy pluripotent stem cell lines is challenging, with limited efficiency (from embryonic stem (ES) and iPS cells, 2.5%± 0.5%, 1.0% ± 0.3% endothelial cells are generated, respectively). Therefore, in an attempt to increase endothelial differentiation efficiency, iPS cells were generated from an endothelial cell type (Chapter 3), and were expected to have enhanced capacity for endothelial differentiation in comparison with fibroblast-derived iPS

cells. This could lead to generation of large numbers of endothelial cells for phenotypic and functional analysis, and will form the basis for generation of patient-specific endothelial cells for disease modelling.

5.2. Hypothesis

Pluripotent stem cells induced from endothelial cells have enhanced capacity to form endothelium compared to those induced from fibroblasts.

5.3. Aims

- To compare the efficiency of endothelial cell differentiation from fibroblast- and endothelial-derived induced pluripotent stem cells.
- To compare the phenotype and function of endothelial cells derived from pluripotent stem cells with mature endothelial cell controls.

5.4. Experimental design

5.4.1. Cell lines

iPS cell lines were derived *in house* from fibroblasts or endothelial outgrowth cells from cord blood, fiPS and eiPS, via transfection with episomal plasmids containing Oct3/4, Sox2, Klf4, L-myc, Lin28 and Shp53, as detailed in Chapter 3.4.1. Two endothelial control lines were used. HUVEC were supplied by Lonza, UK, and EOC were derived from healthy volunteers, as detailed in 2.2.1.3.

5.4.2. Derivation of endothelial cells from fiPS and eiPS

Differentiation into endothelial cells was carried out using protocol D detailed in Chapter 2.7. Briefly, stem cells were aggregated into embryoid bodies in suspension with mesoderm-inducing cytokines. After three days, embryoid bodies were disaggregated and the cells transferred to a single cell monolayer culture system, on 0.1% gelatin-coated plates, in endothelial medium supplemented with 50ng/ml VEGF. After seven days, $CD31^+$ cells were isolated by FACS and further cultured on 0.1%-coated gelatin plates in endothelial medium with 50ng/ml VEGF supplementation.

5.4.3. Characterisation of fiPS-EC and eiPS-EC: *in vitro*

Following expansion in culture, sorted $CD31^+$ cells were analysed *in vitro* for phenotypic and functional characterisation, as detailed in Chapter 2.3 and 2.5. For phenotypic characterisation, flow cytometry was carried out to assess expression levels of a panel of endothelial, hematopoietic and pluripotency cell surface antigens (Chapter 2.3.1). Immunocytochemistry was performed to assess expression of endothelial markers von Willebrand Factor (vWF) and endothelial Nitric Oxide Synthase (eNOS) (Chapter 2.3.2). For functional characterisation, the cells were assessed on their ability to form connections when cultured on Matrigel, as an indication of angiogenic capacity (Auerbach *et al.*, 2003) (Chapter 2.5.1). They were

also subjected to the wound healing assay, in which their ability to recover from a scratch was assessed, indicating migratory and proliferative properties (Chapter 2.5.2). Finally, the cells were assessed for production of nitrite and nitrate metabolites (indirect measurements of nitric oxide release) following 24 hours stimulation with acetylcholine (Chapter 2.5.3). All parameters were compared to two mature endothelial control lines: HUVEC and EOC.

5.4.4. Characterisation of fiPS-EC and eiPS-EC: *in vivo*

In vivo analysis of the $CD31^+$ cell populations was carried out using the murine model of subcutaneous sponge vascularisation, detailed in Chapter 2.6.1. NSG mice between 10-12 weeks of age were used, and grouped as shown in Table 5.1:

Sponge treatment	**Number of mice per group**
Conditioned media from Endothelial Outgrowth Cells	4
Endothelial outgrowth cells	6
Endothelial cells differentiated from fibroblast-derived induced pluripotent stem cells	6
Endothelial cells differentiated from endothelial- derived induced pluripotent stem cells	6

Table 5.1: Sponge implants and group sizes of the murine subcutaneous angiogenesis assay. Control groups (Conditioned media from Endothelial Outgrowth Cells and Endothelial Outgrowth Cells) were the same as those used in chapter 4 [pg 118].

After 21 days, sponges were removed and fixed in 70% IMS overnight, then transferred to 4% PFA until sectioning. They were analysed in two ways. Firstly they were sectioned and stained with H&E, for detection of erythrocyte-perfused vessels. From these sections, vessel density was quantified by Chalkley counts, using a 25 point graticule (Hague *et al.,* 2002). Three areas were scored and a mean count taken to give the final score. All analysis was carried out blinded to cell type. The second

phase of *in vivo* analysis was to determine the location of implanted cells, to assess whether or not they incorporated into vessels. Paraffin embedded sections were prepared and immunostained as detailed in Chapter 2.6.3, with the specifes specific antibody sets outlined in Table 2.7.

Vessels were detected around the periphery of the sponge, and scored for red and/or green fluorescence to give an indication of incorporation of the implanted human cells. Non vascularised sections were also imaged for detection of human $CD31^{+}$ red fluorescence (antibody set 3), to show the presence of implanted cells outwith vascular structures. Full details of these techniques can be found in Chapter 2.6.

5.5. Results

5.5.1. Endothelium-derived induced pluripotent stem cells differentiate to endothelial cells with greater efficiency than embryonic stem cells or fibroblast-derived induced pluripotent stem cells.

iPS cell lines derived from either fibroblasts (fiPS) or endothelial cells (eiPS) were subjected to endothelial differentiation. A greater proportion of $CD31^+$ cells were generated from eiPS than fiPS, shown by representative examples of flow cytometry dot plots from day 7 FACS sorts (Figure 5.1).

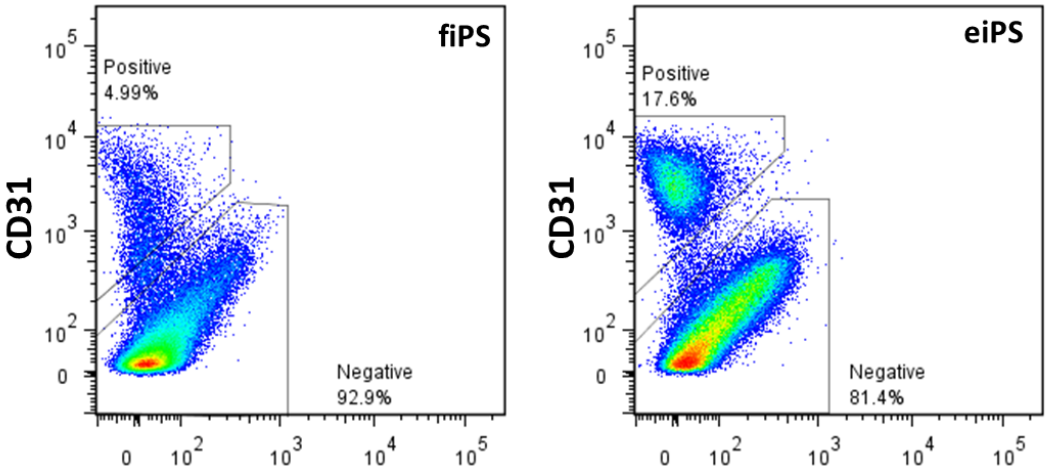

Figure 5.1: Distinct populations of $CD31^+$ cells are present following endothelial differentiation of fibroblast-derived iPS cells (fiPS) and endothelial derived iPS cells (eiPS); this population is larger following eiPS differentiation. These populations were isolated by FACS sorting. Gates were set by unstained controls.

Differentiation efficiency (the percentage of cells expressing the endothelial cell marker CD31 at day 7) of fiPS and eiPS was quantified from multiple differentiations, and compared against ES and stock iPS cell lines. Differentiation efficiency for fiPS was low (3.0 ± 2.2%, N=3) and similar to the ES and stock iPS cells previously differentiated (Figure 5.2). Endothelial differentiation efficiency of eiPS was significantly higher than all other lines (20.8 ± 3.1%, N=3, P=0.0059).

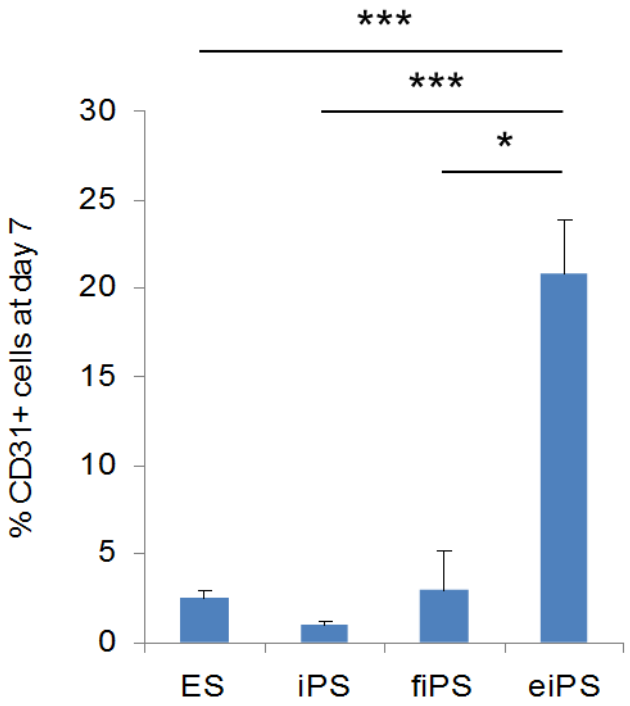

Figure 5.2: The endothelial differentiation efficiency of the endothelial-induced pluripotent stem (eiPS) cell line was higher than that of embryonic stem (ES) cell, induced pluripotent stem (iPS) cell, or fibroblast-induced pluripotent stem (fiPS) cell lines. Data are expressed as mean ± SEM and were analysed by one-way ANOVA; *=P≤0.05, ***=P≤0.001; ES, n=12; iPS, n=7; fiPS, n=3; eiPS, n=3.

5.5.2. Characterisation of fiPS-EC and eiPS-EC *in vitro*

Over time, $CD31^+$ cells derived from fiPS and eiPS (referred to as fiPS-EC and eiPS-EC, respectively) expanded in culture. Both lines appeared rounded with 'cobblestone' morphology, typical of endothelial cells and similar to HUVEC and EOC control lines (Figure 5.3).

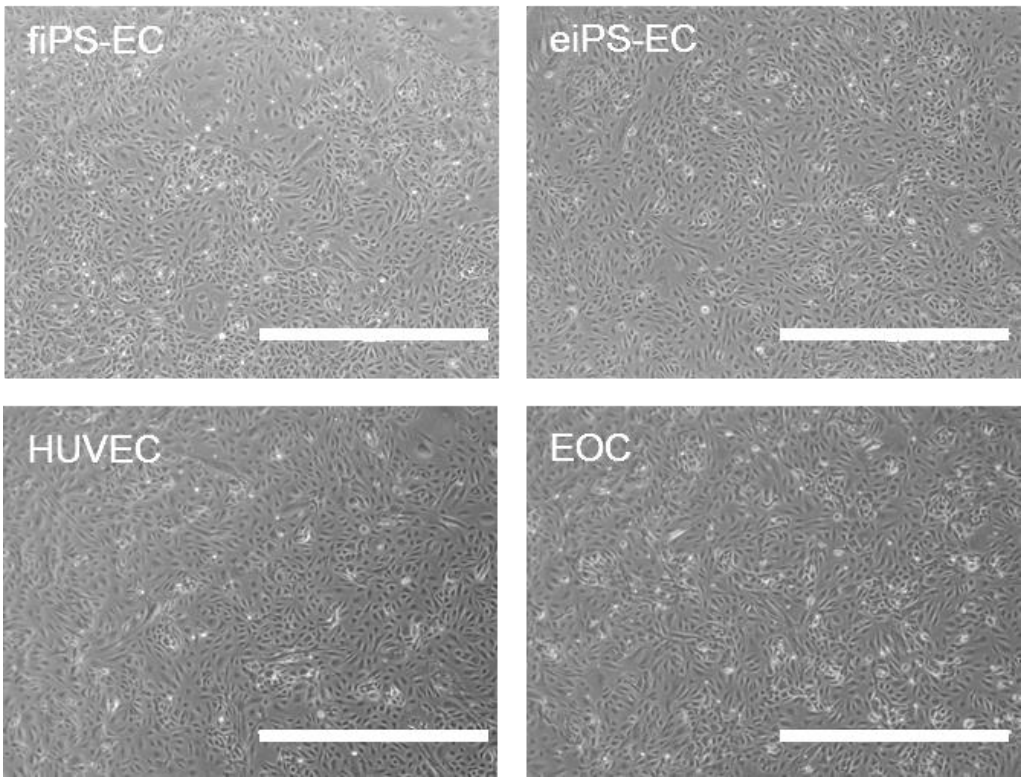

Figure 5.3: Endothelial cells derived from fibroblast-induced pluripotent stem cells (fiPS-EC) and from endothelium-induced pluripotent stem cells (eiPS-EC) display typical endothelial morphology, similar to human umbilical vein endothelial cells (HUVEC) and late endothelial outgrowth cells (EOC). Bar = 1mm.

Phenotypic analysis of fiPS-EC and eiPS-EC by flow cytometry showed that they have some similar characteristics to control endothelial lines (Figure 5.4.). CD31 expression was maintained in fiPS-EC and eiPS-EC in culture and, at 85±14% and 75±7% respectively, was similar to control lines. Expression of CD146 was also high at 89±9% for fiPS-EC and 86±6% for eiPS-EC, and comparable to control lines (CD146: 85±5%, 88±5% for HUVEC and EOC, respectively). Expression levels of

CD34 and VEGFR2 were lower (CD34: 17±1% and 19±8%; VEGFR2: 61±7% and 44±10% for fiPS-EC and eiPS-EC, respectively). These levels appear to vary slightly from control lines (CD34: 43±5%, 43±5%; VEGFR2: 30±5%, 28±5% for HUVEC and EOC, respectively), however, do not reach statistical significance. fiPS-EC differed from eiPS-EC and the control lines in terms of expression of CD105, which at 30±12% was significantly lower than CD105 expression of eiPS-EC (91±3), HUVEC (87±4) and EOC (92±3) (Figure 5.4).

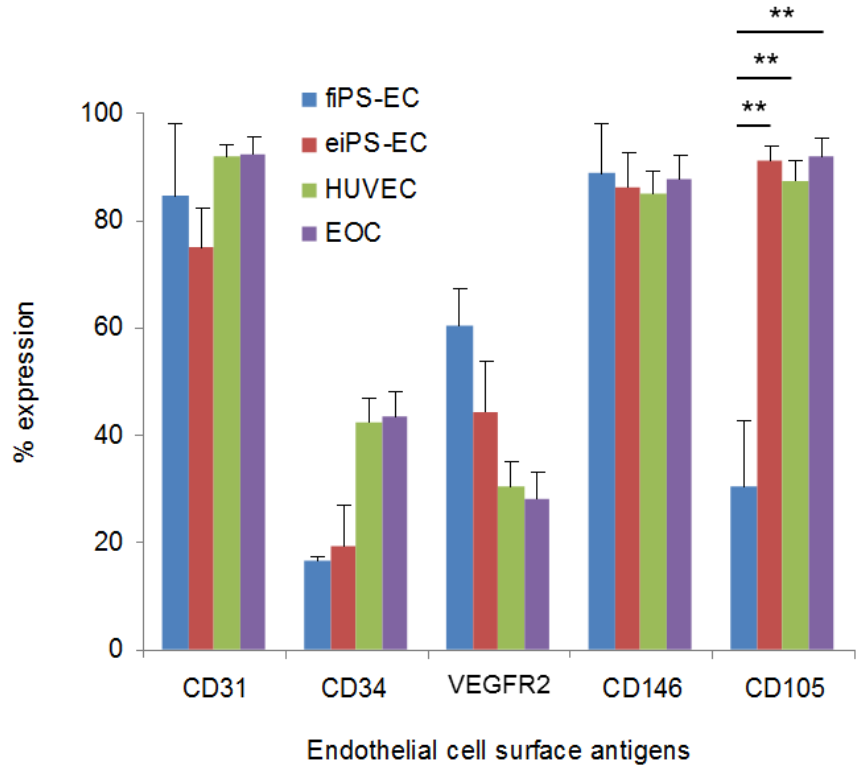

Figure 5.4: Endothelial cells derived from fibroblast-induced pluripotent stem cells (fiPS-EC) and from endothelium-induced pluripotent stem cells (eiPS-EC) have similar phenotypes to human umbilical vein endothelial cell (HUVEC) and late endothelial outgrowth cell (EOC) control lines, in terms of expression of endothelial cell surface antigens. Data are expressed as mean ± SEM and were analysed by one-way ANOVA, **= P≤0.01; N=3-8.

Hematopoietic cells are closely linked to endothelial cells - both are derived from the mesoderm and there are theories that they originate from a common precursor, the hemangioblast (Choi, 2002). In order to confirm that differentiation was leading to development of endothelial, rather than hematopoietic cells, fiPS-EC and eiPS-EC

were assessed for expression of the hematopoietic markers CD133 and CD45. As expected, fiPS-EC and eiPS-EC both had low expression levels of CD45 (fiPS-EC: 2.0±0.6%; eiPS-EC: 0.8±0.3%; Figure 5.5), though expression of CD133 was significantly higher on fiPS-EC (10.6±1.6%) than eiPS-EC (2.0±0.2%). In anticipation of the loss of pluripotency, which accompanies differentiation, expression of SSEA3 and SSEA4 markers was also assessed (Figure 5.5). Within the fiPS-EC population, 15.8±6.9% cells were SSEA3^{+}, and 23.4±4.5% were SSEA4^{+}, higher levels than those seen in the eiPS-EC and control lines.

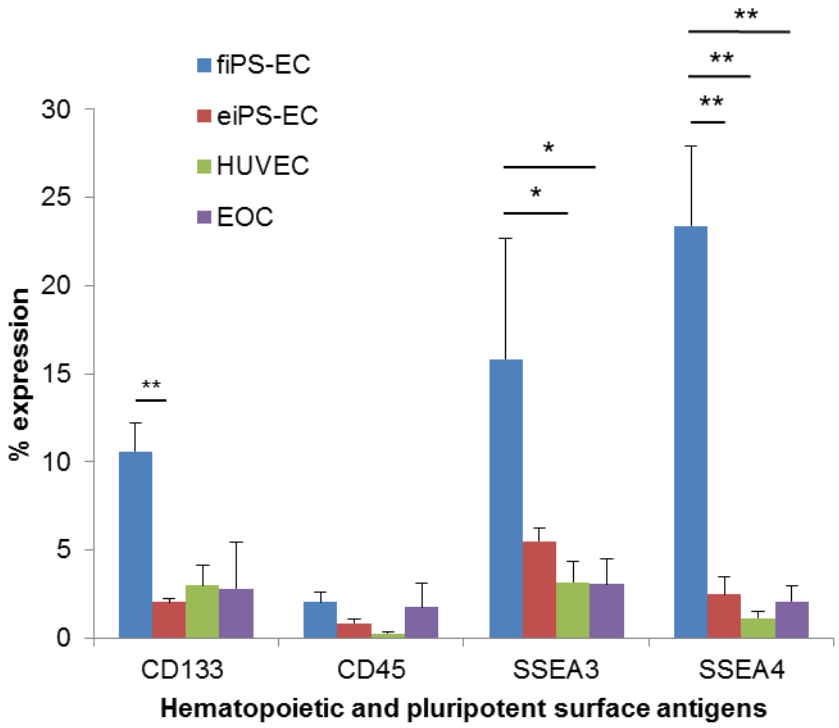

Figure 5.5: A higher proportion of endothelial cells derived from fibroblast-induced pluripotent stem cells (fiPS-EC) express CD133, SSEA3 and SSEA4, in contrast to endothelial cells derived from endothelium-induced pluripotent stem cells (eiPS-EC) and control endothelial cell lines. Data are expressed as mean ± SEM and were analysed by one-way ANOVA, *= P≤0.05, **= P≤0.01; N=3-8.

To add to this phenotypic profiling of fiPS-EC and eiPS-EC, immunocytochemical analysis showed that endothelial cell markers vWF and eNOS were expressed in the majority of cells within fiPS-EC and eiPS-EC populations (Figures 5.6 and 5.7).

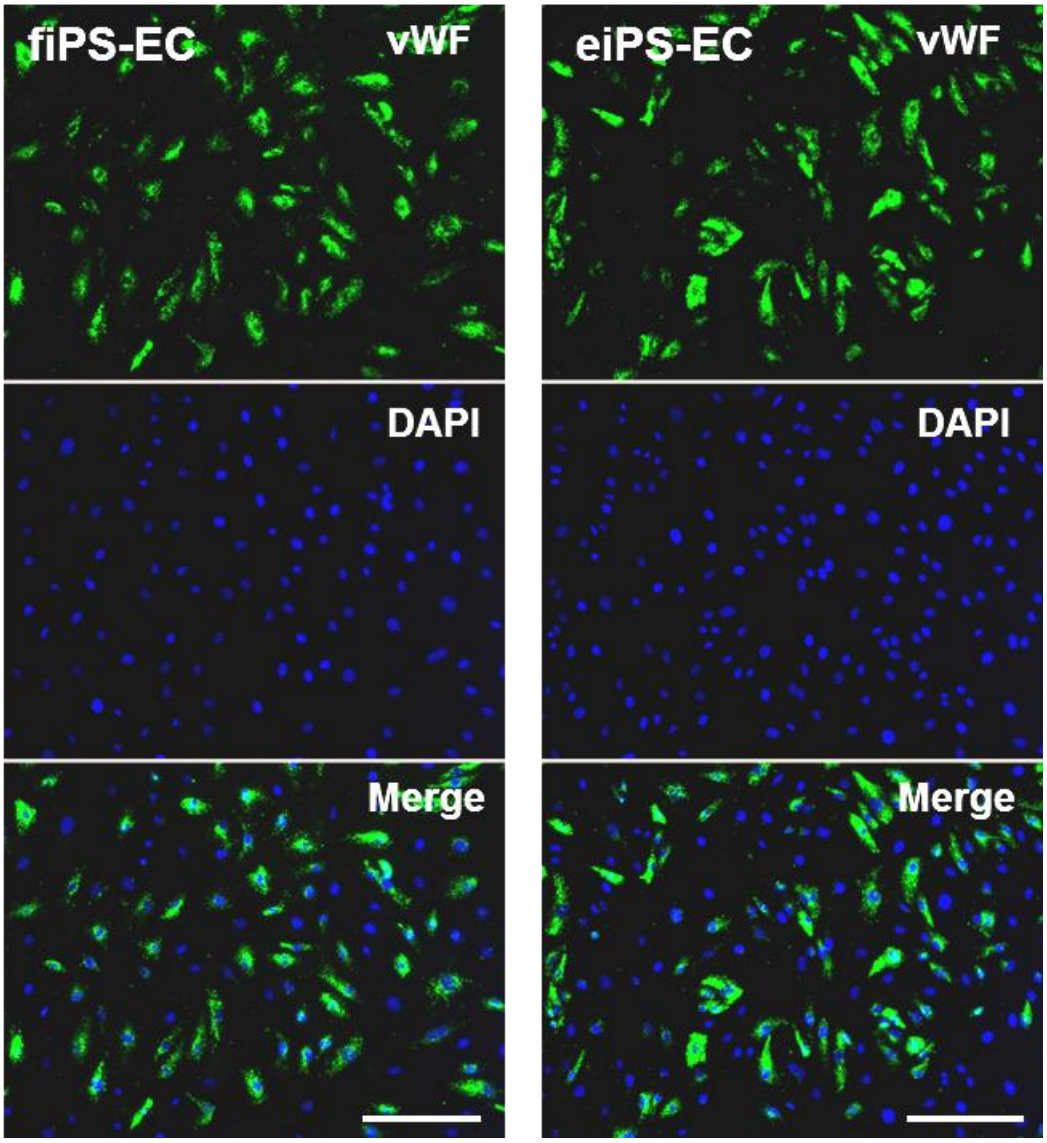

Figure 5.6 Endothelial cells derived from fibroblast-induced pluripotent stem cells (fiPS-EC) and those derived from endothelium-induced pluripotent stem cells (eiPS-EC) were immunostained with antibodies raised against von Willebrand Factor (vWF) (green) and fluorescent nuclear stain DAPI (blue). Bar = 200µm. The majority of cells display cytoplasmic granular staining of vWF, typical of endothelial cells.

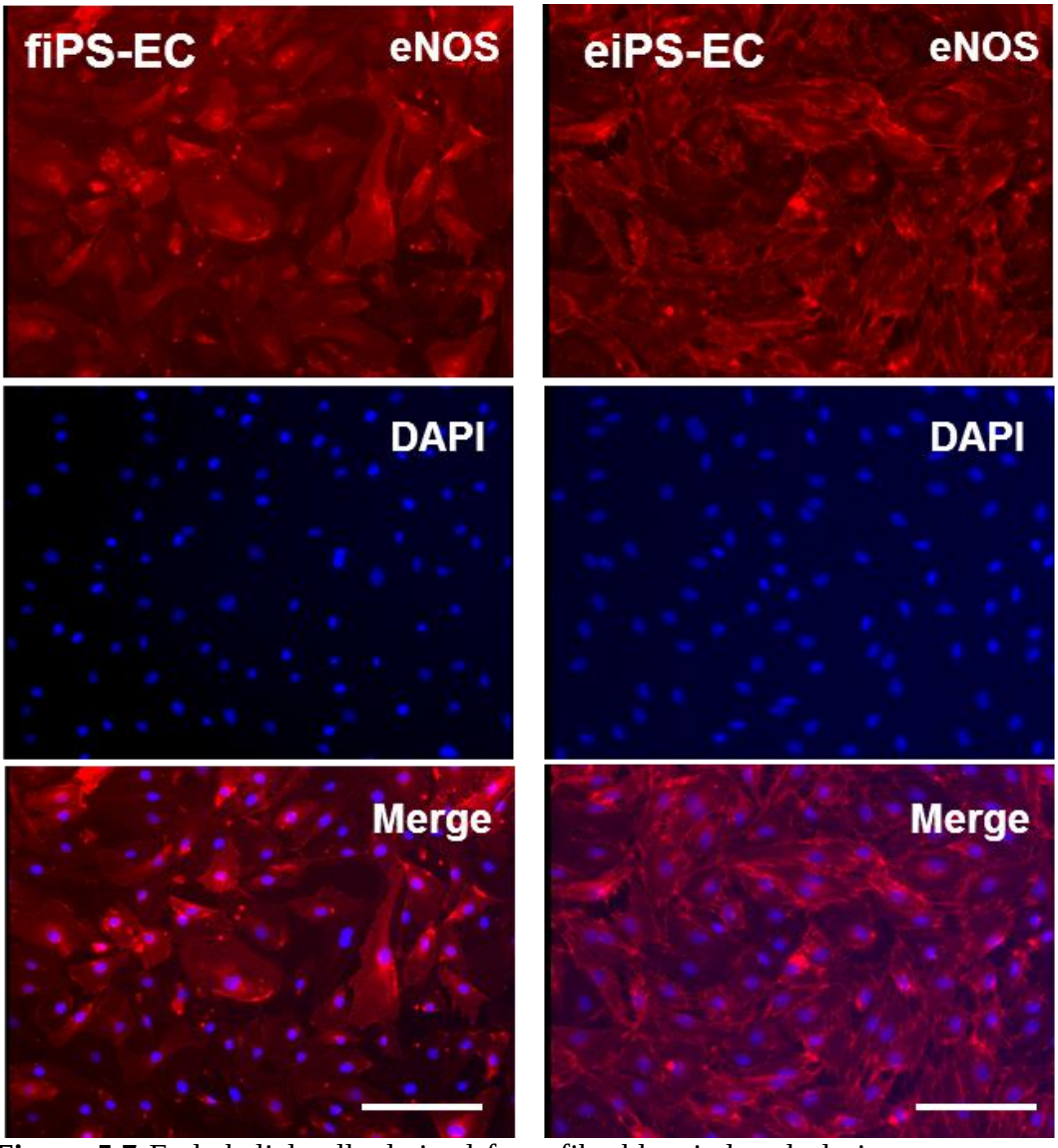

Figure 5.7 Endothelial cells derived from fibroblast-induced pluripotent stem cells (fiPS-EC) and those derived from endothelium-induced pluripotent stem cells (eiPS-EC) were immunostained with antibodies raised against endothelial Nitric Oxide Synthase (eNOS) (red), and fluorescent nuclear stain DAPI (blue). Bar = 200µm.

fiPS-EC and eiPS-EC have the capacity to form connections on Matrigel, a characteristic of endothelial cells thought to indicate their angiogenic potential (Figure 5.8). Quantification of this assay showed that both lines have similar angiogenic capacity, comparable to that of HUVEC and EOC.

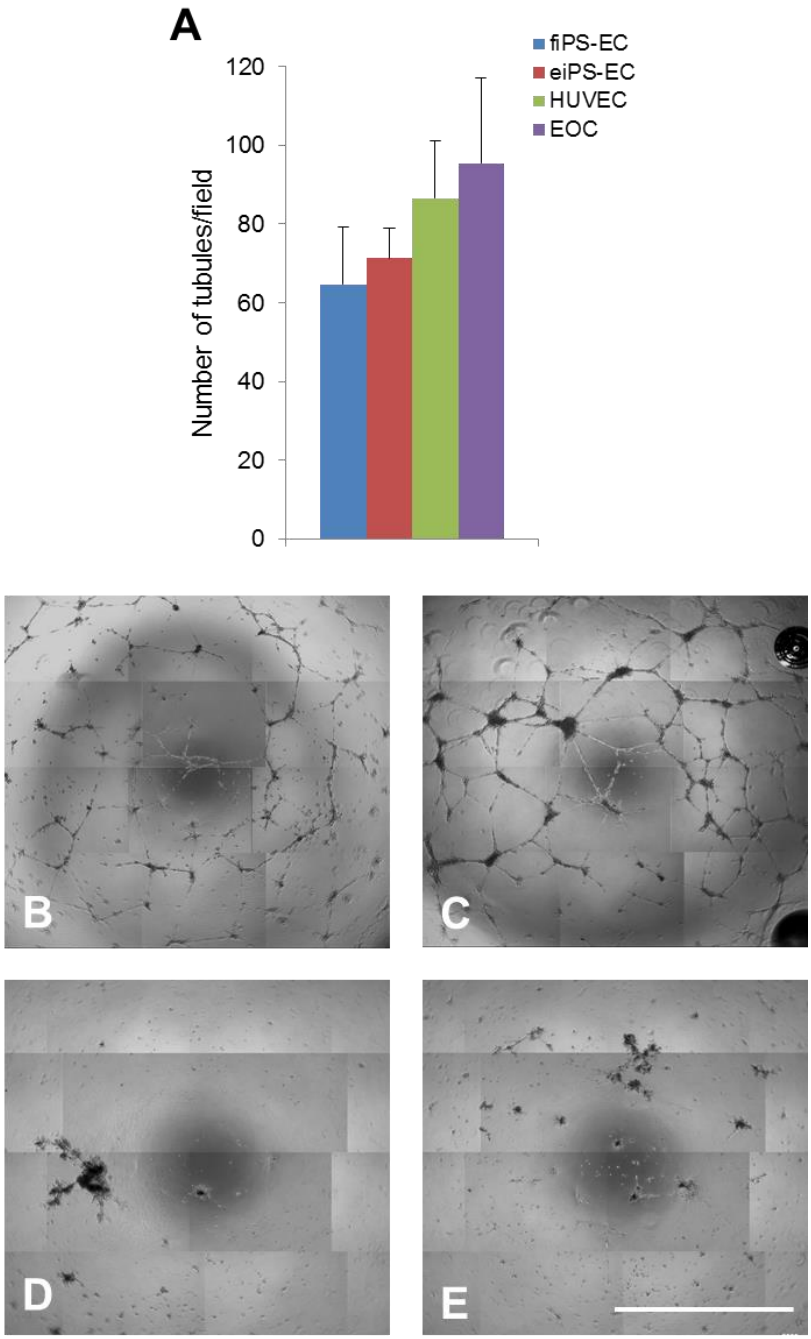

Figure 5.8 Endothelial cells derived from fibroblast-induced pluripotent stem cells (fiPS-EC) and those derived from endothelium-induced pluripotent stem cells (eiPS-EC) form connections on Matrigel. A) Quantification of cell connections suggests similar angiogenic potential of fiPS-EC, eiPS-EC, HUVEC and EOC. Data are expressed as mean ± SEM and were analysed via one-way ANOVA, $P>0.05$; N=3-5. Representative images of tubule-like structures formed by B) fiPS-EC and C) eiPS-EC. By comparison, both D) undifferentiated fiPS and E) undifferentiated eiPS, did not form tubes when cultured on matrigel. Bar = 200µm.

A wound healing assay was used to assess the ability of fiPS-EC and eiPS-EC to migrate and proliferate over time to recover from a scratch (Figure 5.9). After 24 hours, eiPS-EC had always fully recovered (cell coverage was 100%, comparable to HUVEC control). In contrast, in the recovering fiPS-EC monolayer, a scratched area remained after 24 hours, suggesting that the proliferative and migratory properties of fiPS-EC are diminished.

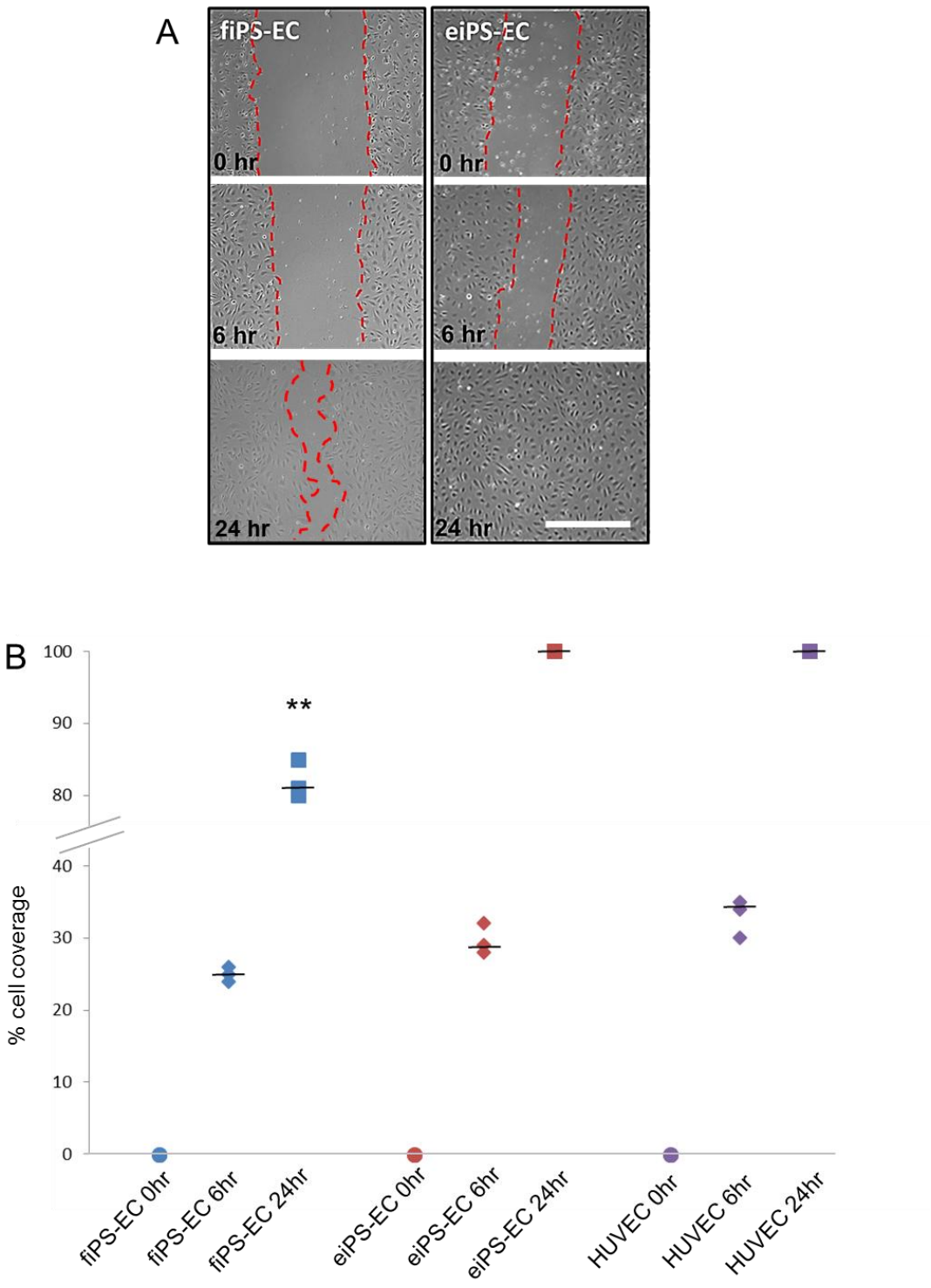

Figure 5.9 fiPS-EC and eiPS-EC have similar migratory and proliferative abilities during recovery from a scratch in the wound healing assay A) Representative images of wound healing of fiPS-EC and eiPS-EC over time. Bar= 500µm B) Quantification of wound healing of fiPS-EC, eiPS-EC and HUVEC. Data were analysed via one-way ANOVA, **=P≤0.01, N=3.

Nitric oxide release was measured indirectly to complete the *in vitro* characterisation of fiPS-EC and eiPS-EC. Nitrite and nitrate levels were detected colorimetrically following stimulation with 10µM ACh. Unstimulated cells had similar nitrite and nitrate release to those stimulated with ACh, and levels released from HUVEC and EOC were in the same region as those released from fiPS-EC and eiPS-EC (Figure 5.10).

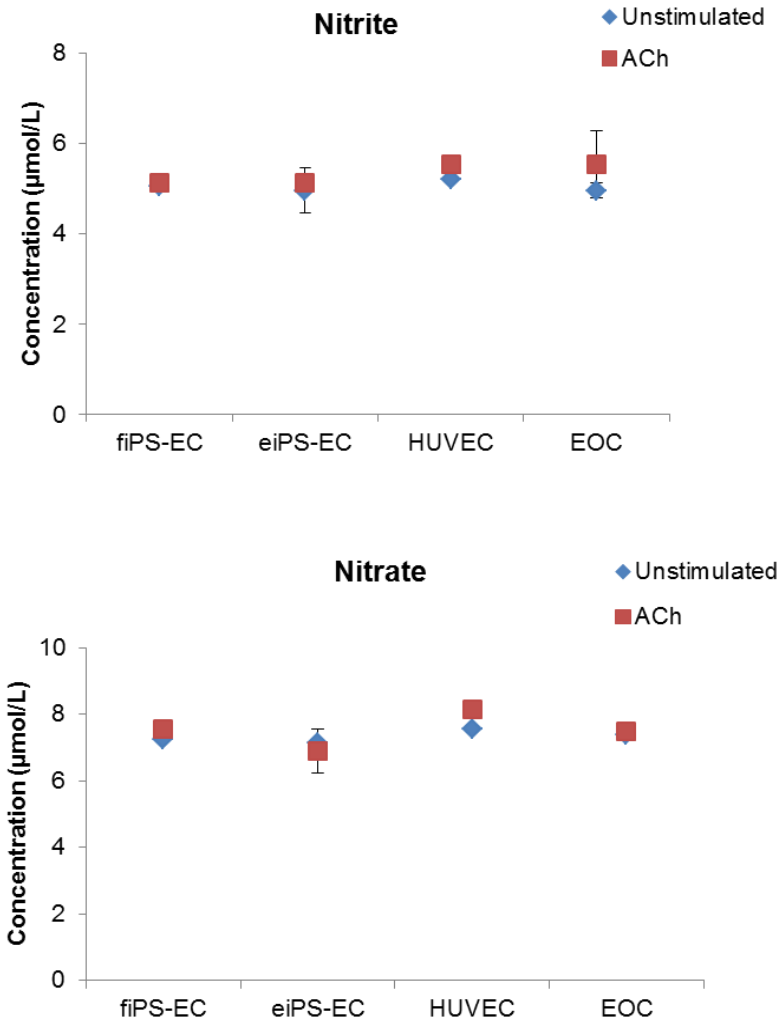

Figure 5.10 Similar concentrations of nitrite and nitrate are detected from endothelial cells derived from fibroblast-induced pluripotent stem cells (fiPS-EC) and those derived from endothelium-induced pluripotent stem cells (eiPS-EC) human umbilical vein endothelial cells (HUVEC) and late endothelial outgrowth cells (EOC) under basal conditions or following stimulation with acetylcholine (ACh; 10µM) over 24 hours. Data are expressed as mean ± SEM and were analysed via Student's paired t test, P>0.05; N=3.

5.5.3. Characterisation of fiPS-EC and eiPS-EC *in vivo*

The murine subcutaneous sponge assay, an *in vivo* model of vascularisation, was used to further characterise the differentiated cells and ascertain their angiogenic response to systemic stimuli over a period of 21 days, as detailed in Chapter 2.6 and Chapter 5.4.4.

Sponges were treated with EOC conditioned medium, EOC, fiPS-EC or eiPS-EC and implanted into the right flank; a Matrigel-treated sponge was implanted into the left flank as a paired control. After 21 days, the sponges were removed and sectioned for analysis of vessel density, determined by Chalkley counts (Figure 5.11). fiPS-EC and eiPS-EC increased vessel density compared to paired Matrigel controls (fiPS-EC: 4.1±0.3 vessels vs 7.6±0.4 vessels, P≤0.001; eiPS-EC: 4.8±0.4 vessels vs 8.3±0.4 vessels, P≤0.001). Vessel density was increased to the same extent after implantation of fiPS-EC and eiPS-EC. Neither EOC conditioned medium nor EOC increased vessel density.

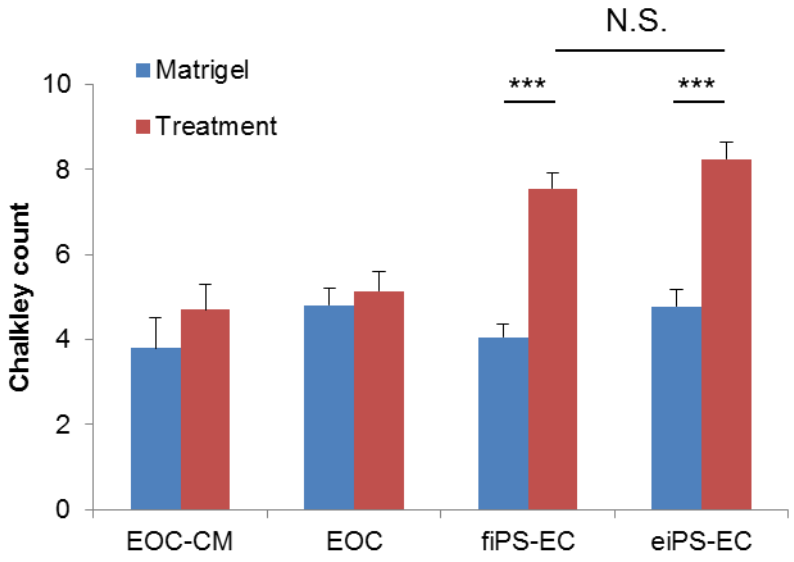

Figure 5.11 Vessel density in implanted sponges was increased by the presence of endothelial cells derived from fibroblast-induced pluripotent stem cells (fiPS-EC) and those derived from endothelium-induced pluripotent stem cells (eiPS-EC). Sponges contained endothelial outgrowth cell conditioned medium (EOC-CM), EOC, fiPS-EC or eiPS-EC; vessel density was scored by Chalkley count. Data are expressed as mean ± SEM and were analysed via two-way ANOVA, ***=P≤0.001; N.S.= not significant; N=4-6 mice per group. EOC were used at passage 5.

Sponge sections containing fiPS-EC and eiPS-EC were analysed via immunocytochemistry, with paired antibody sets detailed in Table 2.7. Sections of control Matrigel sponges and human fibroid tissue were used as positive controls to test all antibodies (Chapter 4.5.2, Figures 4.17 and 4.18). Within the following figures, vessels could be detected by the green fluorescence generated from binding of either smooth muscle actin or CD31 antibodies, cross reactive to human and murine cells. Red fluorescence, generated from the binding of human specific antibodies CD31 or CD146 indicated the presence of human cells. Red fluorescence within a vessel wall therefore indicated that implanted human cells incorporated into the vasculature. No incorporation of human cells into vasculature was detected in fiPS-EC (Figure 5.12) or eiPS-EC (Figure 5.13) treated sponges, though human CD31 positive cells were present in other non-vascularised areas of the sponge (Figure 5.14).

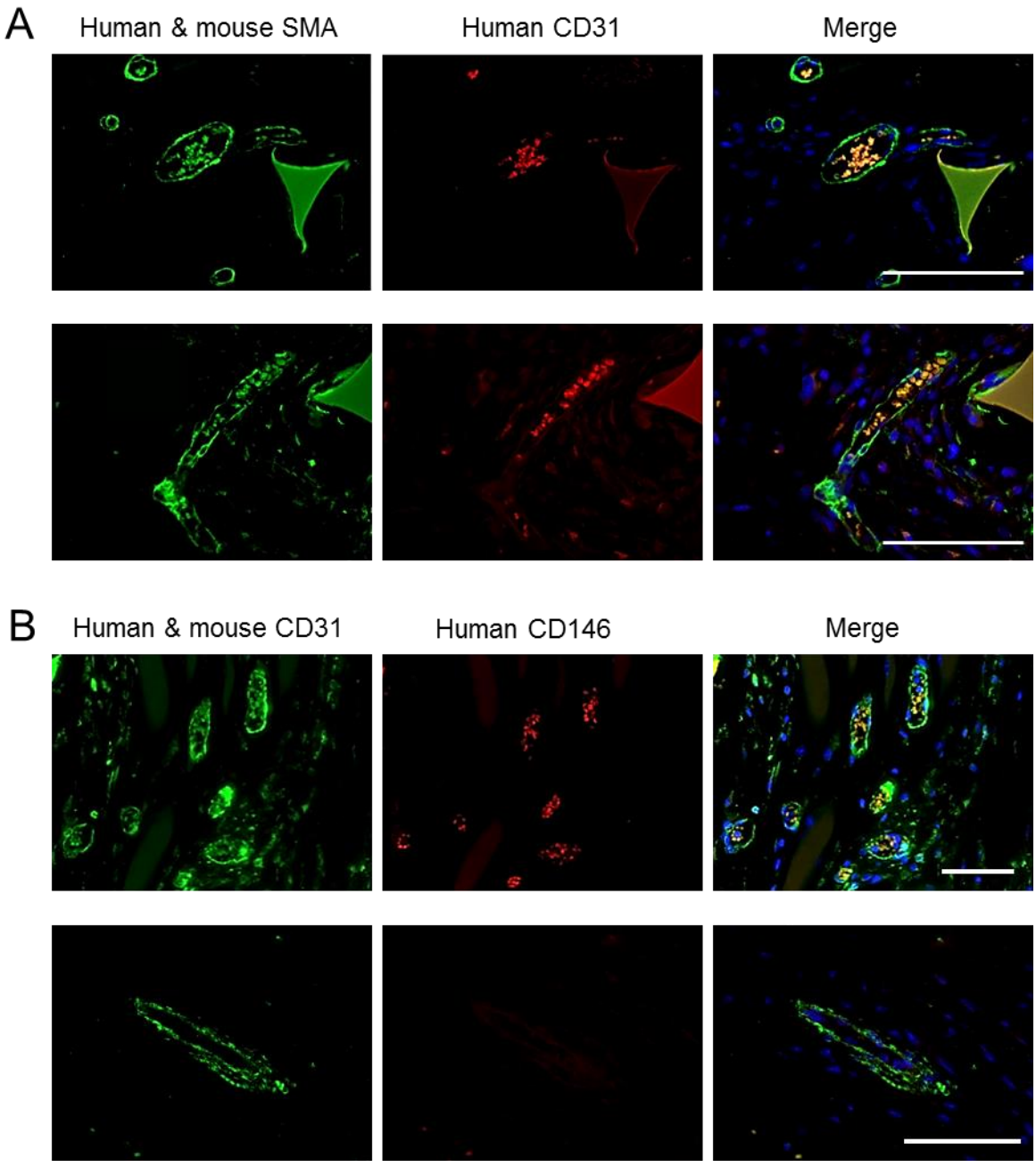

Figure 5.12 Neovasculature detected in sponge sections containing fibroblast induced pluripotent stem cell-derived endothelial cells. A) Vascular structures stained positive for smooth muscle actin antibody (green), but not human specific CD31 antibody (red). B) Vascular structures stain positive for CD31 (green), but not human specific CD146 antibody (red). DAPI stained nuclei blue. Erythrocytes autofluoresced in both AF488 and AF568 channels. Bar = 100µm.

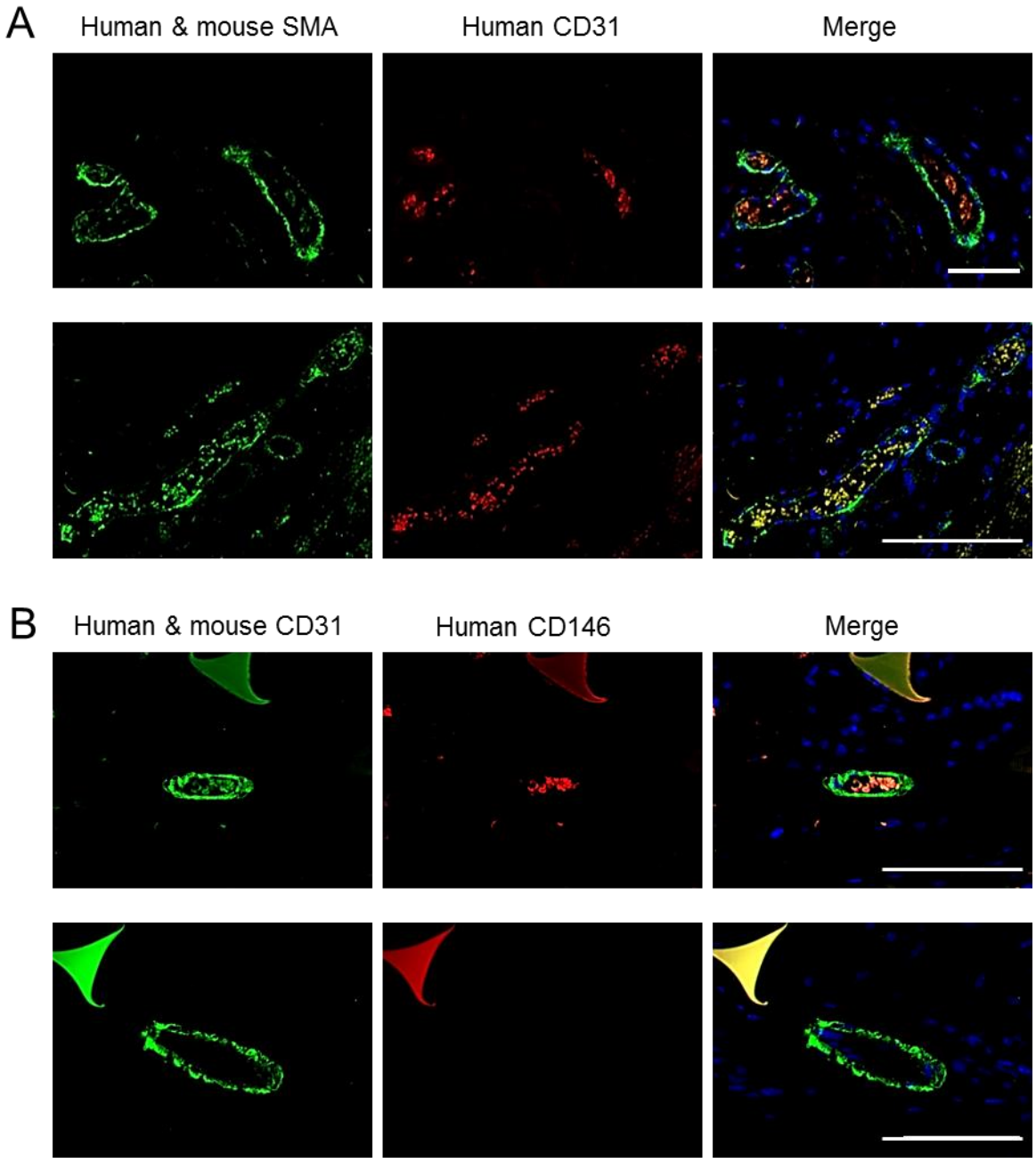

Figure 5.13 Neovasculature detected in sponge sections containing endothelial-induced pluripotent stem cell-derived endothelial cells. A) Vascular structures stained positive for smooth muscle actin antibody (green), but not human specific CD31 antibody (red). B) Vascular structures stain positive for CD31 (green), but not human specific CD146 antibody (red). DAPI stained nuclei blue. Erythrocytes autofluoresced in both AF488 and AF568 channels. Bar = 100µm.

In all sponges, human cells outwith vascular structures were detected with human specific antibody against CD31 (Figure 5.14).

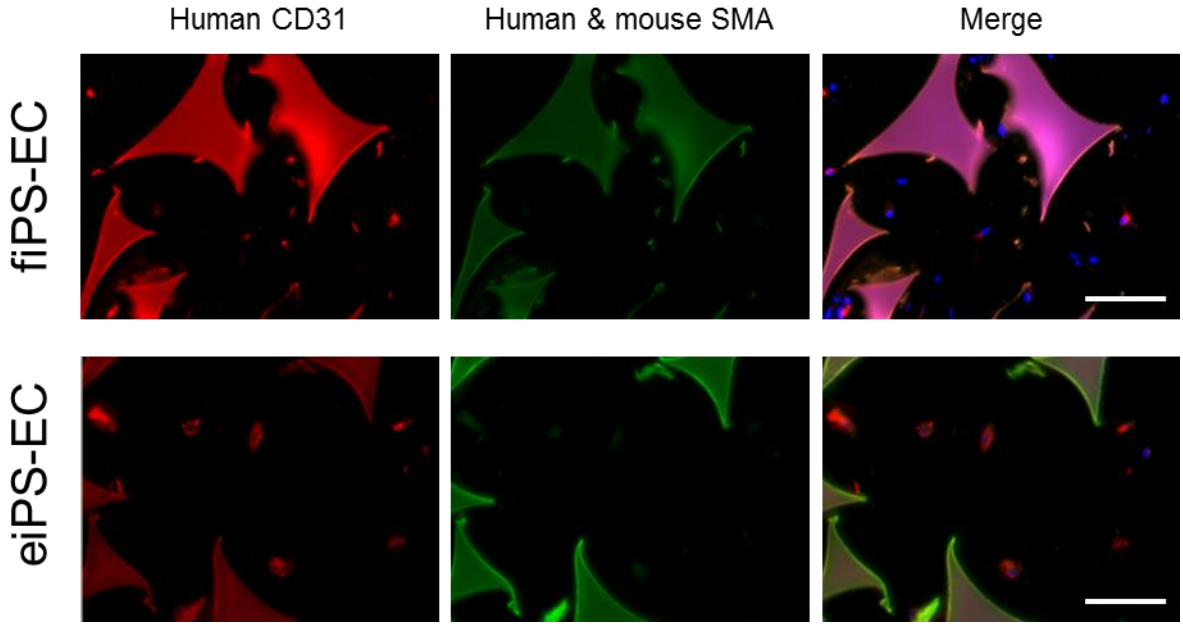

Figure 5.14: Human cells, stained red with CD31 antibody, were identified outwith vascular structures in sponges containing endothelial cells derived from fibroblast- and endothelial- induced pluripotent stem cells. DAPI stained nuclei blue. The large triangular structures are sponge matrix. Bar = 100µm.

Vessel density was significantly increased (to the same extent) by implantation of fiPS-EC and eiPS-EC, whereas implanted EOC or conditioned media from EOC did not increase vessel density (Figure 5.11). This indicates enhanced angiogenic potential of iPS-derived cells over a control endothelial line. However, immunocytochemical analysis of vascular structures did not identify incorporation of any of these cells. *In vivo* data are summarised in Table 5.2.

Sponge treatment	Vessel density				Immunocytochemical vessel staining			
	Untreated sponge	**Treated sponge**	**Difference**	**P value**	**Number of vessels analysed**	**Incorporation of human cells into vessels**	**Number of vessels with human cells incorporated**	**Detection of human cells outwith vessels**
EOC-CM*	3.8 ± 0.7	4.7 ± 0.6	0.9	0.301	-	-	-	-
EOC*	4.8 ± 0.4	5.1 ± 0.5	0.3	0.557	180	Yes	1	Yes
fiPS-EC	4.1 ± 0.3	7.6 ± 0.4	3.5	≤0.001, ***	180	No	-	Yes
eiPS-EC	4.8 ± 0.4	8.3 ± 0.4	3.5	≤0.001, ***	180	No	-	Yes

Table 5.2: Performance of endothelial outgrowth cell conditioned medium (EOC-CM), EOC, endothelial cells derived from fibroblast-induced pluripotent stem cells (fiPS-EC) and endothelial cells derived from endothelium-induced pluripotent stem cells (eiPS-EC) in a subcutaneous sponge murine model of vascularisation. fiPS-EC and eiPS-EC significantly increase vessel density within implanted sponges, though do not incorporate into murine neovasculature. *Neither EOC-CM nor EOC increase vessel density, though there is evidence of EOC incorporation into murine neovasculature (data displayed in Chapter 4; Figures 4.19 and 4.23). Data are expressed as mean ± SEM. Probability was determined by one-way ANOVA.

5.6. Discussion

This chapter confirmed the hypothesis that pluripotent stem cells derived from an endothelial cell have enhanced capability to differentiate into endothelial cells compared to fibroblasts. These observations raise fundamental questions about the use of different somatic cells for reprogramming and suggest that there may be advantages in reprogramming cardiovascular cells for vascular regeneration. Differentiated endothelial cells from fibroblast- and endothelial-derived iPS cells appeared pro-angiogenic in comparison to endothelial cell controls, and may have potential for vascular regeneration.

5.6.1. Endothelial differentiation efficiency is considerably higher in eiPS than fiPS.

That eiPS cells more readily differentiate to endothelial cells, in comparison with ES and fiPS cell lines, indicates that whilst they meet all the criteria to be defined as pluripotent, there are underlying characteristics which distinguish them from other pluripotent cell types. This phenomenon has been described before in iPS cells derived from a non-fibroblast cell type which preferentially differentiate back towards their cell type of origin. For example, Kim *et al.* (2010) report that blood-derived iPS cells form more hematopoietic colonies than fibroblast-derived iPS cells when both are subjected to a hematopoietic differentiation protocol. Many studies have concluded that this is likely due to residual epigenetic memory. Tissue specific differences are brought about by epigenetics (variations in chromatin structure and chemical modifications of DNA that influence gene expression). The reprogramming process reverts the vast majority of these epigenetic features to those of an ES-like state (Lister *et al*., 2011; Doi *et al*., 2009). However, this epigenetic 'resetting' is not always complete. For example, Lister *et al*. (2011) report that certain chromosomal regions near telomeres and centromeres are resistant to epigenetic resetting, and that donor cell DNA methylation patterns in these regions may persist through reprogramming and differentiation processes. Incomplete reversal of the original somatic cell's epigenome is referred to as 'residual epigenetic memory'. It would

therefore be interesting to analyse global gene expression profile (by microarray) of eiPS (and fiPS) cell lines in their differentiated and undifferentiated states. DNA methylation arrays (Sandoval *et al.*, 2011) could also be used to identify distinctive DNA methylation marks or histone modifications on eiPS, which may help to elucidate enhanced endothelial differentiation potential. There is, however, some disagreement regarding the impact of residual epigenetic memory. Some reports indicate that the gene expression and differentiation efficiency variations detected in early passage iPS cells are attenuated by long term passaging, implying that epigenetic memory is transient and that reprogramming is a gradual process (Polo *et al*., 2010). This suggests that a detectable state of pluripotency in early passage cells may be acquired before complete resetting of the epigenome. The cells used for differentiation in this chapter were at a fairly early passage (eiPS: between passage 15-19, fiPS 15-21), whereas all ES and stock iPS cell lines used were passage 42 and above. Study of late passage eiPS and fiPS would be interesting, to determine whether the enhanced endothelial differentiation potential of eiPS is lost with increasing passage. If this was found to be the case, it could be exploited by using early passage iPS cells for efficient endothelial cell generation, and would be a key consideration in the design of future therapeutic applications.

There are many similarities between the differentiated stem cells and endothelial cell line controls, from which it can be concluded that the differentiation protocol generates a mature and functional endothelial cell. Many other endothelial differentiation protocols do not include an isolation step (for example, Rufaihah *et al.*2013), and instead characterise the levels of endothelial markers within the entire differentiating population. Whilst this may generate larger yields of 'endothelial-like' cells, there is likely to be a high degree of heterogeneity within the population, which could confuse analyses or limit use in therapeutic applications. By isolating early populations of cells expressing a fairly specific endothelial marker, a much more homogeneous population of cells is generated, within which all cells are at the same stage of differentiation. As discussed in chapter 4, CD31 was selected as it is considered to be one of the most specific endothelial cell markers. Also known as platelet endothelial cell adhesion molecule (PECAM-1), it is a major constituent of

the endothelial cell intercellular junction (Dejana, 2004) and is required for leukocyte transendothelial migration under most inflammatory conditions (Muller *et al.*, 1993). It is also present on the surface of various hematopoietic cells, including circulating platelets, monocytes, neutrophils and selected T cell subsets (Newman, 1997). Expression of CD45 (a molecule that is expressed on the surface of all nucleated hematopoietic cells and their precursors) distinguishes between hematopoietic and endothelial cell types. As the isolated cells were confirmed to be $CD31^+$ and $CD45^-$, they could be placed in the endothelial subset. One of the benefits of this protocol is the generation of a proliferative, pure population of endothelial cells after a fairly short period of time.

Day seven was the time point optimised for peak expression of CD31 by the University of Glasgow's laboratory from which the protocol was derived (unpublished data). As it has been demonstrated that there are variations in differentiation kinetics between iPS cell lines (Bock *et al.*, 2011), these experiments could be further developed to examine whether emergence of $CD31^+$ cells differs between iPS cell lines. For example, it may be that ES and fiPS take longer to differentiate than eiPS; as such the differentiation protocol could be altered to accommodate this. Assessment of the levels of other endothelial markers over time would also be interesting, to determine at which point they are expressed, to give insight into the molecular mechanisms behind the differentiation process. This could also add to the understanding of possible epigenetic processes, which may be affecting eiPS differentiation.

The data in this chapter suggests that there are advantages of eiPS over fiPS for the generation of endothelial cells to model disease or develop novel therapeutic approaches for vascular regeneration. Larger cell numbers can be generated through more effective differentiation, therefore shortening the time in culture required to derived large numbers of cells required for analysis or, in the future, therapeutic application. In general, less time in culture is desirable, as there are negative effects of extended periods of cell culture. Alterations can occur which can mean cells are no longer representative of their phenotype *in vivo;* these can include changes in

karyotype, cell surface antigen expression or growth properties (Auerbach *et al.*, 2000; Staton *et al.*, 2004).

5.6.2. Endothelial cells derived from fiPS and eiPS have similar phenotypes and functions

Multiple phenotypic and functional assays indicate that fiPS-EC and eiPS-EC have many similar properties, to each other and to the endothelial cell control lines. As well as expression of CD31, both lines highly express CD146, an endothelial intracellular adhesion molecule, which facilitates the formation of a confluent layer of endothelial cells (Bardin *et al.*, 2001). There were no significant differences between expression levels of CD34 and endothelial cell controls, a marker also expressed on hematopoietic stem and progenitor cells; thought to be up-regulated on endothelial cells in angiogenesis (Siemerink *et al*., 2012). Also similar to endothelial cell controls are the levels of VEGFR2 on fiPS-EC and eiPS-EC, a Tyrosine Kinase-linked receptor, which binds the angiogenic molecule VEGF (Ferrara *et al.*, 2003). The presence of vWF, with a granular staining pattern characteristic of endothelial cells, and eNOS, the endothelium-specific constitutively produced enzyme which catalyses nitric oxide production (Fleming and Busse, 2003), indicates that these differentiated cells have properties characteristic of mature endothelial cells.

In the *in vitro* angiogenesis assay, fiPS-EC and eiPS-EC appear to form similar numbers of connections, to each other and to the endothelial cell controls. By comparison, undifferentiated fiPS and eiPS did not form connections on Matrigel. This is a quick assay, is easy to set up and reproducible, however it is not necessarily sensitive enough to detect differences in function between different types of endothelial cell (Staton *et al.*, 2004). Heterogeneity in endothelial cells arises due to tissue specific functions, and the response of endothelial cells to growth factors and cytokines varies depending on their tissue of origin (Staton *et al.*, 2004; Garlanda *et al.*, 1997). Therefore, some endothelial cell populations may have greater angiogenic capacity than others. Whilst this assay is a visual indication of the functionality of differentiated cells, similar to endothelial cell controls, these limitations meant that

angiogenic potential was also assessed *in vivo* to allow stronger conclusions to be drawn regarding functionality.

The scratch assay was used to assess cell migration and proliferation, which occurs during wound healing. This technique was adequate for the purposes of this thesis; however higher throughput and reproducibility could have been increased by automation, for example use of robotic techniques (Simpson *et al.*, 2008). Though here there is no distinction between migration and proliferation, two processes integral to physiological wound healing, this allows assessment of the totality of cellular behaviour in response to injury. From their performance in the wound healing assay the fiPS-EC do not appear to reach confluence 24 hours after the scratch wound, a finding which reached statistical significance once quantified. This could suggest that they have less proliferative and migratory capacities, and would support the suggestion that they less readily reach an endothelial cell type than eiPS.

The production of nitric oxide (catalysed by eNOS) is a key function of the endothelium. Despite the presence of eNOS on fiPS-EC and eiPS-EC, detected by immunocytochemistry, stimulation with acetylcholine did not significantly increase production of nitrite or nitrate for either of these cell lines, or the endothelial controls. As discussed (Chapter 4.6.3.1), the lack of response was unexpected, as acetylcholine has a direct physiological effect on endothelial cell activity by increasing nitric oxide production (Amezcua *et al*., 1988; Joannides *et al*., 1995). Further studies need to be carried out to optimise the concentration of acetylcholine used for stimulation, and the length of time before supernatant collection.

Some differences exist between the fiPS-EC and eiPS-EC populations, suggesting that some $CD31^+$ cells derived from fiPS lack the full endothelial cell profile. For example, significantly higher levels of CD133, a stem cell marker, and the SSEA3 and 4 pluripotency markers in the fiPS-EC population (compared with levels in eiPS-EC, HUVEC and EOC), suggest that there may still be a degree of pluripotency within the population, which may indicate that these cells are plastic in culture, and can express CD31 and SSEA3/4 simultaneously. As pluripotency marker levels in

eiPS-EC cells are significantly lower, this finding may suggest that differentiated fiPS cells may take longer to form a complete endothelial cell profile, contributing to the suggestion that eiPS are a better source for endothelial cell generation.

CD105, also known as endoglin, is a constituent of the transforming growth factor-beta receptor and is thought to be limited to actively proliferating endothelial cells (Nassiri *et al.,* 2011), as well as smooth muscle cells and macrophages. Lower expression levels of CD105 on fiPS-EC may indicate that they are less proliferative in culture than eiPS-EC or control cell lines. This possibility could be investigated by population doubling studies.

5.6.3. fiPS-EC and eiPS-EC increase vessel density *in vivo,* though they appear to have similar angiogenic capacity to EOC *in vitro*.

In a similar way to the ES-EC analysed in Chapter 4.5.2, fiPS-EC and eiPS-EC increased vessel density *in vivo,* adding to the suggestion that pluripotent stem cell-derived endothelial cells are pro-angiogenic in comparison with control endothelial cell lines. However, neither fiPS-EC nor eiPS-EC appeared to incorporate into neovasculature, as no human specific cells could be detected in the vessels after 21 days. It seems likely, therefore, that that the implanted cells are releasing angiogenic factors, thus influencing neovasculature growth in a paracrine manner. Further experiments are required to elucidate these paracrine pro-angiogenic mechanisms. For example, it is possible that factors are released which stimulate the migration of nearby murine endothelial cells, a phenomenon which could be studied by assessing the chemotaxis (Schor *et al.,* 2001) of murine endothelial cells across a Boyden chamber membrane in response to fiPS-EC or eiPS-EC conditioned medium. Implantation of sponges containing fiPS-EC or eiPS-EC conditioned medium may also demonstrate paracrine effects on angiogenesis. Increased vessel density could also be a result of enhanced proliferation of these cells *in vivo;* to investigate this it would be interesting to study the effect of systemic murine factors on fiPS-EC and eiPS-EC proliferation rates.

The *in vivo* sponge assay was well suited to the study of angiogenesis, as vessels were visible around the periphery of sectioned sponges, with erythrocytes often still present within them once the sponge had been sectioned and stained. Angiogenesis is a complex process *in vivo*, the intricacies of which are not fully replicated by single cell *in vitro* assays. For example, although endothelial cells are central to the angiogenic process, they are supported by other cell types- smooth muscle cells, pericytes and fibroblasts (Carmeliet, 2000)- not taken into account in single cell *in vitro* assays. Previous studies within our laboratory used the murine sponge implant model of vascularisation *in vivo*, with extensive research into the optimal combinations of human specific and human/mouse cross-reactive antibodies (Barclay *et al.,* 2012, including extensive supplementary information). Confidence in this method of human cell detection can be drawn from using the same antibodies as Barclay *et al.,* and from distinct positive controls (as shown in Chapter 4, Figures 4.17 and 4.18). Though relatively sparsely spread throughout the sponge, implanted fiPS-EC and eiPS-EC were identified by human specific $CD31^+$ staining, showing that they were able to survive the process of implantation. Another benefit of this study was the demonstration that implantation of both fiPS-EC and eiPS-EC did not result in teratoma formation. Typically, subcutaneous injection of mice with human ES cells will result in teratomas formed of cells derived from all three germ layers. Usually, tumour formation is assessed after 6-12 weeks, Wesselschmidt *et al*., 2011. However, Gropp *et al*., 2012 report that tumours can be detected 3-5 weeks after injection of $1x10^5$ human ES cells. This was the same number of cells used in the *in vivo* experiments reported here, so it is likely that tumours would have been detected had any undifferentiated stem cells been injected. A major obstacle anticipated in the clinical translation of this work is the teratoma formation associated with implantation of (formerly) pluripotent stem cells (Miura *et al.,* 2009). Theoretically this could occur from implantation of only one undifferentiated cell, or from cells not yet committed to the endothelial lineage. This would be a key feature of safety trials prior to therapeutic translation (Hentze *et al.,* 2009).

There are weaknesses to the *in vivo* murine sponge assay. Species-specific differences between human and mouse must also be taken into account, as implanted

cells may respond differently upon exposure to murine systemic factors compared with human systemic factors. For example, human endothelial cells bind UEA-1 (Ulex europaeus lectin-1, a plant lectin used as a non-specific marker of human endothelial cells; Holthofer *et al.,* 1982) whereas murine endothelial cells do not (Staton *et al.,* 2004). The inherent variability between animals must also be considered in interpretation of the results. It was fairly difficult to identify human cells in the immuno-analysis, as they were sparsely spread throughout the sponge. The murine sponge assay was followed as described in the literature (Barclay *et al.*, 2012, Tura *et al*., 2013), except for the later passage of implanted cells. As previously discussed, this may be a contributing factor to explain why the experiment was unsuccessful in terms of vascular incorporation. The experiments could be adapted for the sponges to contain more cells. 10^5 is a relatively small population to put in a 0.5cm x 1cm sponge, and this may have been why it was difficult to identify human cells in the immuno-analysis. In addition, due to time constraints, it was not possible to analyse the entirety of each sponge by immunocytochemistry, however non-consecutive sections were stained to give an overview of approximately a third of each sponge, therefore I can say with confidence that this is representative of most of the sponge. Experimenting with larger cell numbers may allow detection of human cells in vascularised regions of the sponge. As an alternative, tagging the implanted cells with lentiviral vectors, or GFP reporter vectors would ease their identification once sectioned. The assay could also be developed with the use of tracking techniques (Magnetic Resonance Imaging or Positron Emission Tomography, for example), to determine if the cells stay in the same place, or if they move and interact with each other or with murine cells.

To support immunoanalysis, additional studies could be done using fluorescent *in situ* hybridisation, for identification of sex mismatched mouse and human cells (Han *et al.,* 2012). This would complement the immunostaining results and ease the identification of implanted human cells. Pilot investigations were performed using this method but were not completed due to time constraints. In addition, study of endothelial cell function *in vivo* could be expanded by using other models to give further insight in to the potential of these cells for vascular regeneration. For example,

re-endothelialisation of vessels could be investigated following artery denudation in the balloon-injured rat carotid artery model (Tulis, 2007).

5.6.4. Limitations and future studies

The fiPS and eiPS cell lines were not from the same donor, and therefore genetic background is a variable factor to consider in their comparison. It has been reported that genetic background of donor cells is a major factor in determining iPS cell differentiation propensity, and that this may have more of an influence on differentiation potential than somatic cell type or epigenetic memory (Kajiwara *et al*. 2012, Ghosh *et al*,. 2010). Ludovic Vallier's group report that human iPS cell lines appear to strongly vary in their capacity to differentiate, for uncertain reasons. They found that iPS cell lines can be grouped into two categories, those which have high capacity to differentiate to mesoendoderm and those which have high capacity to differentiate to neuroectoderm, and that iPS cell lines derived from one individual all belonged in the same category, independent of tissue of origin, passage number or reprogramming method (as reviewed by Brack and Hochedlinger, 2013). Further studies with iPS cells derived from different somatic cell types from the same donor would be extremely important for the elimination of genetic background as a variable factor.

The eiPS were derived from cord blood outgrowth rather than peripheral blood outgrowth, which limits their potential for clinical application, as cord blood is not a feasible approach to generating personalised iPS cell lines for regeneration. iPS cells derived from endothelial cell outgrowth of peripheral blood would be a more therapeutically applicable donor cell type (as this is a more accessible source than cord blood), and therefore reprogramming methods must be worked upon to achieve this. That an endothelium-based iPS cell can efficiently differentiate back to endothelium may suggest that other endothelial cell types (i.e. those from peripheral blood outgrowth) would do the same. It is hoped that reprogramming methods will be developed to induce pluripotency in endothelial cells derived from the vessel wall, and from peripheral blood.

The decreased expression of pluripotent cell surface antigens on eiPS after differentiation indicates that they are committing to an endothelial cell lineage. It may be interesting to exploit the enhanced differentiation potential of eiPS to see if they also differentiate more effectively into other mesodermal linages, for example cardiomyocytes. This would increase the disease modelling potential and therapeutic application of these cells.

This work was carried out in healthy volunteer lines, to establish the reprogramming and differentiation protocols before the production of patient-specific stem and endothelial cell lines. Further studies on patient specific cells would enable *in vitro* disease modelling to aid in the elucidation of mechanisms of endothelial dysfunction. Prior to therapeutic application, all procedures would have to be made GMP (Good Manufacturing Practise) grade, meaning that all media used would be fully defined and free from animal products.

5.7. Conclusions

Differentiation of human pluripotent stem cells into endothelial cells is an emerging technology, one that has proven challenging with consistently low yields of endothelial cells generated from ES and iPS cells. However, populations of functional endothelial cells can be differentiated with relatively high efficiency from endothelium-derived iPS cells of healthy individuals. Though the mechanisms that underpin this are unclear, it seems likely that these cells retain epigenetic factors that increase efficiency; therefore an endothelium-based iPS cell may be the ideal source to generate large numbers of endothelial cells for therapeutic use.

Chapter 6

Discussion and future perspectives

iPS cell technology can be used to generate large numbers of endothelial cells for the study of their function *in vitro* and *in vivo.* This thesis examined the hypothesis that healthy human embryonic and induced pluripotent stem cells can be used as a source for the derivation of large populations of fully functional endothelial cells. It was also hypothesised that the generation of endothelial cells is more efficient from iPS cells which had been generated from endothelial cells, in comparison to fibroblasts. The aims of this research were to induce pluripotency in various somatic cell types, and to optimise and validate an efficient endothelial differentiation protocol. Furthermore, the functional properties of the resulting endothelial cells were analysed *in vitro* and *in vivo.*

6.1. Fibroblasts and endothelial cells can be induced into pluripotency through transfection with episomal plasmids containing specific reprogramming factors

As an integration-free, viral-free and therefore safer method of nuclear reprogramming (compared to retroviral vectors), episomal plasmids were used as a delivery system for the reprogramming of somatic cells. They contained transcription factors Oct3/4, Sox2, Klf4, L-myc, Lin28 and Shp53 (a combination optimised by Okita *et al*., 2011), to induce pluripotency in fibroblasts and late outgrowth endothelial cells from cord blood. A myriad of studies have sought to identify the combination of transcription factors for optimal reprogramming, with a variation of conclusions. As few as two factors (Sox and Oct) have been claimed to be sufficient for reprogramming in some cases (Rizzino *et al.,* 2009). The 'Okita combination' was decided upon as this report has influenced a large number of studies since its high impact publication. As expected, low efficiency of reprogramming was reported in all cell lines. It was marginally higher for cord blood-derived late endothelial outgrowth cells in comparison to fibroblasts. Though reprogramming efficiencies were low, this is a common finding in the field, indicating the complexity of the molecular changes which occur during the reprogramming process. All derived iPS cells were found to express cell surface pluripotency markers SSEA3 and SSEA4;

colonies were also stained positive for Oct3, Nanog and Sox2. As a test of true pluripotency, they could spontaneously differentiate into derivatives of all three primary germ layers, and at later passages were found to be mostly free from exogenous episomal plasmids. As previously reported, these are only required to induce pluripotency, not maintain it (Okita *et al.,* 2011). Nuclear reprogramming was, however, unsuccessful in endothelial cells derived from peripheral blood or from vascular biopsies. This was unexpected, as there are reports in the literature that endothelial cells derived from blood can be reprogrammed, often with higher efficiencies than fibroblasts (Geti *et al.,* 2012). The inability to reprogram peripheral blood EOCs, in comparison to cord blood-EOCs may indicate innate differences between the two cells types. Faster cell kinetics are associated with cord blood derived EOCs, and they typically survive a greater number of population doublings in culture (Ingram *et al.* 2004). It is possible that these characteristics render them more amenable to reprogramming than peripheral blood EOCs.

Despite claims that episomal plasmids are the optimal vector for delivery of the transcription factors required for reprogramming (Hu, 2014), alternative methods of reprogramming endothelial cells from adult/peripheral blood and vasculature could be investigated in further experiments. For example, Warren *et al.* (2010) report relatively high efficiency of reprogramming (4.4%) using a synthetic mRNA cocktail containing *Oct3/4*, *Sox2*, *Klf4*, *cMyc* and *Lin28*, in the reprogramming of fibroblasts and keratinocytes. This is amongst the highest reprogramming efficiencies reported in the literature, so may be an interesting alternative to explore in the reprogramming of peripheral blood-derived endothelial cells.

Though more replicates of these cellular reprogramming experiments are required to confirm the findings presented in this chapter, this work suggests that endothelial outgrowth cells from healthy cord blood reprogram with marginally greater efficiency than those from healthy peripheral blood or fibroblasts. Though it is unclear if this phenomenon is due to their progenitor or endothelial phenotype; it is likely that it may be the result of their progenitor cell type, as it has been reported that progenitor cells may be more amenable to reprogramming than mature cells (Eminli *et al.,* 2008; Giorgetti *et al.,* 2009). The fact that the more mature endothelial

cell types (derived from adult peripheral blood and vasculature) did not successfully reprogram supports this argument, that 'younger' endothelial cells derived from cord blood, which haven't had time to accumulate DNA mutations (Bauman, 2009), are more easily reprogrammed.

It is unfortunate that endothelial cells from sources other than cord blood were not reprogrammed, as this would be the ideal source for the routine generation of iPS cells of patients, a strategy for their study *in vitro* envisioned for the future. A simple blood sample is more easily obtained than a skin biopsy (from which fibroblasts are obtained), and not many people have cord blood stored. However, the relatively higher efficiency of reprogramming of endothelial cells derived from cord blood offers opportunities to study the properties of endothelial-derived iPS cells.

6.2. Pro-angiogenic endothelial cells can be differentiated from human embryonic and induced pluripotent stem cells

Endothelial differentiation from human embryonic and induced pluripotent stem cells is challenging with consistently low yields of functional endothelial cells generated from a range of differentiation protocols. Protocols which involved a 2D monolayer differentiation system with 'differentiation medium' did not yield big enough populations of cells expressing endothelial cell markers which were feasible for further culture following sorting. Despite reports of VEGF addition to differentiation media significantly enhancing CD31 expression (Ferreira *et al.*, 2007), this did not enhance differentiation efficiency. The most efficient method of endothelial differentiation was a 3-dimensional embryoid body system, in which stem cell aggregates were exposed to a series of mesoderm-inducing cytokines and growth factors- members of the Wnt, TGF-beta and FGF families. Though the molecular mechanisms that control endothelial differentiation during embryogenesis are not completely understood, certain signalling pathways & their cytokines have been elucidated. The BMP and Wnt signal transduction pathways are known mediators of endothelial differentiation and function, interacting in such a way to target endothelial-specific genes for the induction of angiogenesis *in vivo* (La Bras *et al.*,

2010). Extensive optimisation of the cytokines used in this endothelial differentiation protocol was carried out on ES cell lines H1 and H9 by the Univeristy of Glasgow. The ES and iPS cell lines used in these studies were indistinguishable in terms of their pluripotent characteristics, thus it was assumed that a protocol optimised for ES cells would work in a similar way for iPS cells. This was found to be the case. Upon disaggregation of the embryoid bodies and further culture, distinct populations of $CD31^+$ cells were isolated from both ES and iPS differentiating populations. These appeared to have many similar features to control endothelial cell lines, ascertained through immunophenotyping experiments. In addition, the functions of these cells were investigated *in vitro*, which indicated that their angiogenic, proliferative and migratory properties were comparable to endothelial control lines. However, the stem cell derived endothelial cells differed from controls in a murine model of vascularisation *in vivo*, where they appeared pro-angiogenic. Whilst they increased vessel density, they did not incorporate into murine vasculature, suggesting that they are stimulating angiogenic processes through paracrine mechanisms. The morphological, phenotypic and functional endothelial characteristics of all stem cell derived $CD31^+$ cells confirm that endothelial differentiation through this embryoid-body protocol is successful, despite low efficiencies. The pro-angiogenic properties of these derived cells *in vivo* is an exciting finding, and it would be interesting to investigate further the mechanisms by which they appear to increase vessel density. In the future, this differentiation protocol could be applied for the study of endothelial cells from patient populations to elucidate mechanisms of endothelial dysfunction.

There is not a great distinction between the phenotypes of progenitor and mature endothelial cell types. Indeed, the definition of an 'endothelial progenitor cell' is not clear, and whilst phenotypes have been suggested, there remains uncertainty regarding the identity and source of these cells (Ingram *et al.,* 2005). Experiments within the laboratory (Tura *et al.,* 2013) indicate that endothelial outgrowth cells from blood have very similar properties to human umbilical vein endothelial cells, a cell line widely used in cell culture research. However it is unclear if these cell types are inherently the same, or if their similar properties are a consequence of the

uniformity of cell culture conditions. The endothelium is widespread throughout the body, due to its functions which are fundamental to the survival of all tissues and organs. These functions vary to some extent depending on location and local tissue-specific requirements, therefore subsets of endothelial cells vary slightly in phenotype. For example, fenestrated endothelium (containing pores 60-80nm in diameter) lines capillaries of organs that are involved in filtration or secretion of small molecules and proteins, such as the kidney (Satchell and Braet, 2009). In comparison, an uninterrupted lining is provided by a continuous layer of endothelial cells in arteries and veins; here the cells are held together by tight junctions which only allow diffusion of smaller molecules (Aird, 2007). However, the source of endothelial cell line is not often taken into account in their culture, with most laboratories often culturing endothelial cells in endothelial growth medium, or endothelial medium, as in this thesis. Whilst cells in culture are kept in physiological conditions in terms of temperature and gaseous exposure, in the absence of their physiological micro-environment and exposure to biochemical and mechanical stimuli, the characteristics which define sub-sets of endothelial cells may be altered or lost. This is an issue which must be considered in general when working with cells in culture. Therefore, though HUVEC and EOC provide a standard endothelial cell control, they may not completely represent endothelial cells in physiological conditions, as the subtleties in different phenotypes that exist between different endothelial populations may be lost in cell culture. Though this is a limitation of the system of cell culture itself, further experiments could look into the differentiation of specific types of endothelial cell. Rufaihah *et al.* (2013) demonstrate that endothelial cells derived from an embryoid body based differentiation protocol and purified based on their expression of CD31 can be differentiated to arterial endothelial cells when supplemented with 8-bromoadenosine-3’:5’-cyclic monophosphate sodium salt (8Br-cAMP); whereas without this supplement venous endothelial cells are derived (Rufaihah *et al*., 2013). In the presence of 8Br-cAMP higher levels of Ephrin B2 were expressed, typical of arterial endothelial cells and involved in arteriogenesis (Korff *et al.,* 2008). This heterogeneity may be something to consider for the refinement of the differentiation protocol in the future.

Exposure to shear stress is likely to more effectively mimic physiological conditions, and may increase efficiency of differentiation (Yamamoto *et al.,* 2005). Reports indicate that cell culture of ES cells in standard 'differentiation medium' under shear stress (generated by fluid flow) induces expression of vascular endothelial cell markers VEGFR2, VE-cadherin and CD31 (Yamamoto *et al.,* 2005). This was significantly greater than expression in cells in static conditions. Furthermore, shear stress-exposed cells were able to form tube-like structures in collagen gel at a faster rate than cells not exposed to shear stress. The apparatus to study this was being established as this PhD work was coming to an end, so would be interesting to investigate in the near future.

The most recognisable difference between stem cell derived endothelial cells and endothelial cell controls was their performance in the *in vivo* subcutaneous sponge assay, designed to assess the angiogenic effects of implanted cells. Upon quantification of this assay, it was found that implanted EC-ES and EC-iPS enhanced murine vessel density, whilst EOC or EOC conditioned media did not. Although stem cell derived endothelial cells did not incorporate into the neovasculature, the increase in vessel density suggests that they may be releasing factors to influence angiogenesis or vasculogenesis in a paracrine manner.

6.3. Pro-angiogenic endothelial cells are generated more efficiently from iPS cells which derived from endothelial cells rather than fibroblasts

Low efficiencies of endothelial differentiation from ES and fibroblast-derived iPS cells are overcome when carried out in iPS cells generated from late endothelial outgrowth cells: efficiency increases to 20.8%. This suggests that whilst different types of stem cell line can appear to be fully pluripotent, there may be underlying differences that cause differentiation bias: the propensity to differentiate towards a particular cell type. The mechanisms that underpin this phenomenon require elucidation; from comparison with other studies of iPS cell differentiation it appears likely that it is the result of residual epigenetic memory.

Being able to generate large numbers of cells in a short space of time (from efficient endothelial differentiation) means that cells are not in culture as long before use in analysis experiments or before cellular therapies. There are limitations of having to expand cells in culture: significant changes can occur such as alterations of activated state, karyotype, expression of cell surface antigens and growth kinetics (Staton *et al.*, 2004). Over time, endothelial cells become quiescent and senesce after a number of population doublings (for example, HUVEC can be kept in culture for up to 10 passages). The generation of large numbers of endothelial cells from endothelial-derived iPS cells will therefore shorten the time in culture required to expand the population to a reasonable size for analysis and in the future, therapeutic use.

It would be interesting to determine if endothelial-derived iPS cells also efficiently differentiate into other mesodermal or cardiovascular lineages. Similarly, if their differentiation to cells types of other lineages (ectoderm and endoderm) is less efficient.

6.4. Future directions

6.4.1. Reproducibility

Additional experiments would help confirm the reproducibility of the findings and conclusions suggested in this thesis. The reprogramming work would benefit from being reproduced in different cell types from one individual, to confirm or deny the suggestion that iPS cells are most efficiently generated from EOC from cord blood, over those from other sources, and over fibroblasts. This would give insight into the influence of genetic background on reprogramming efficiency.

6.4.2. Epigenetics

It will be important to elucidate the proposed epigenetic mechanisms that underpin the enhanced endothelial differentiation of eiPS over fiPS, to more fully understand the variations in reprogramming and differentiation that occur between different somatic cell types. DNA microarrays could be used to study gene expression and

chromatin structure of the cells at each stage of the process: before reprogramming, once they are iPS cells and once they are differentiated. Sample cDNA strands, stained with fluorescent nucleotides hybridise to synthetic DNA on a microarray plate, which have known sequences. From this, fluorescent dots correspond to the specific DNA sequences, from which the sample DNA can be sequenced. Methylated DNA immunoprecipitation can also give insight into the chromatin structure of the sample, by isolating and enriching methylated DNA sequences. These DNA sequences are then purified and correspond to methylated genes..If it were found to be the case that certain endothelial phenotype-associated genes remain active throughout the process, predisposing them to endothelial differentiation, it would be interesting to repeat differentiation with later passage eiPS. There are reports that extended passaging of iPS cells may ameliorate the effect of epigenetic memory (Chin *et al.*, 2009), likely because pluripotency may be detected before complete resetting of the epigenome. In fact, Chin *et al. (*2009) describe differences between the gene expression profiles of early passage iPS cells and ES cells. ES signature genes were found to be expressed at lower levels in early passage iPS cells, but increase over time in culture to match ES cell levels.

6.4.3. Patient studies

The use of personalised iPS cell technology opens many avenues of research, for example the elucidation of causative mutations and the modelling of disease processes. The work presented in this thesis provides the foundation for the generation of iPS cells from patients with premature atherosclerosis due to a suspected hereditary component. As multiple genes could be the cause of atherosclerosis, iPS cell technology lends itself to a personalised approach for the study of endothelial dysfunction mechanisms, which could vary patient to patient. In the absence of typical environmental risk factors, it can be difficult to ascertain the cause of the disease in these patients due to the complex nature of the condition and multiple candidate genes. In a subpopulation of these patients, endothelial dysfunction may be predisposing them to the condition, decreasing the ability of their vessels to repair and regenerate following injury. Endothelial dysfunction is well established as one of the earliest events in the pathogenesis of atherosclerosis

(Davignon, 2004). iPS cells derived from premature atherosclerosis patients would provide novel opportunities for the study of disease mechanisms. Patient endothelial-iPS could be compared to healthy age matched controls, to ascertain any differences in functionality. For example, wound healing ability, cytokinetic response to angiogenic factors or ability to align to shear stress could be tested.

If a patients' atherosclerosis has a hereditary component, this will likely be transferred to their iPS cells and derivatives. Envisioned for the future is the large scale, long term storage of patient specific iPS cells which could be genetically corrected, differentiated to endothelial cells and administered back to the patient to assist in the repair and regeneration of damaged endothelium. The use of iPS-endotheilal cells in tissue engineering could be investigated as a therapeutic application. For example, experiments have shown that microvessels can be generated from the seeding of endothelial progenitor cells on biodegradable scaffolds (Wu *et al.,* 2004). This group discovered that in combination with smooth muscle cells, seeding of endothelial progenitor cells formed capillary like structures throughout a biopolymeric construct. Though tissue engineering is beyond the scope of this thesis, this is an interesting concept for consideration of the use of iPS-derived endothelial cells for future experiments.

These cells could also be used for the modelling of disease pathways and for the screening of novel therapeutics. The next step towards the translation of these cells to the clinic would be to make the reprogramming and differentiation processes Good Manufacturing Practise (GMP)-standard, by removing and replacing animal and non-human products with synthetic alternatives. This would avoid potential immune responses of the host to animal protein.

6.5. Conclusions

The benefits of stem cell technology for the study of endothelial cell function are gradually being realised. Endothelial differentiation from embryonic and induced pluripotent stem cells is challenging, however, a distinct population of cells with endothelial properties can be generated from a differentiation protocol involving embryoid body formation in media containing mesoderm-inducing cytokines. Resulting endothelial cells were found to highly express CD31, CD146, CD105, vWF, and eNOS, demonstrated angiogenic capacity in vitro and recovered from a scratch assay in a similar way to endothelial cell controls. Low differentiation efficiencies found in both ES and iPS differentiation to an endothelial phenotype can be enhanced by using induced pluripotent stem cells derived from somatic cells of an endothelial lineage. The mechanisms that underpin this are unclear but it seems likely that whilst these cells can be reprogrammed to pluripotency, and have similar characteristics to ES cells, they may retain epigenetic factors that increase endothelial differentiation efficiency. Endothelial cells derived from both fibroblasts and endothelial sourced iPS cells promote angiogenesis in vivo, observed through increased vessel density of subcutaneous sponges in the murine model of vasculogenesis. These cells do not, however, incorporate into murine vasculature, unlike early passage endothelial cell controls. It is likely therefore, that stem cell derived endothelial cells act to increase vessel density through paracrine mechanisms. I propose that an endothelial-based iPS cell is the ideal source for the generation of endothelial cells for disease modelling and possibly vascular regenerative therapies in the future. It is hoped that applying iPS cell technology to the study of endothelial cells from patients with premature atherosclerosis will clarify the molecular mechanisms of endothelial dysfunction in its pathogenesis. iPS-derived endothelial cells have major potential for future clinical applications in vascular repair and regeneration.

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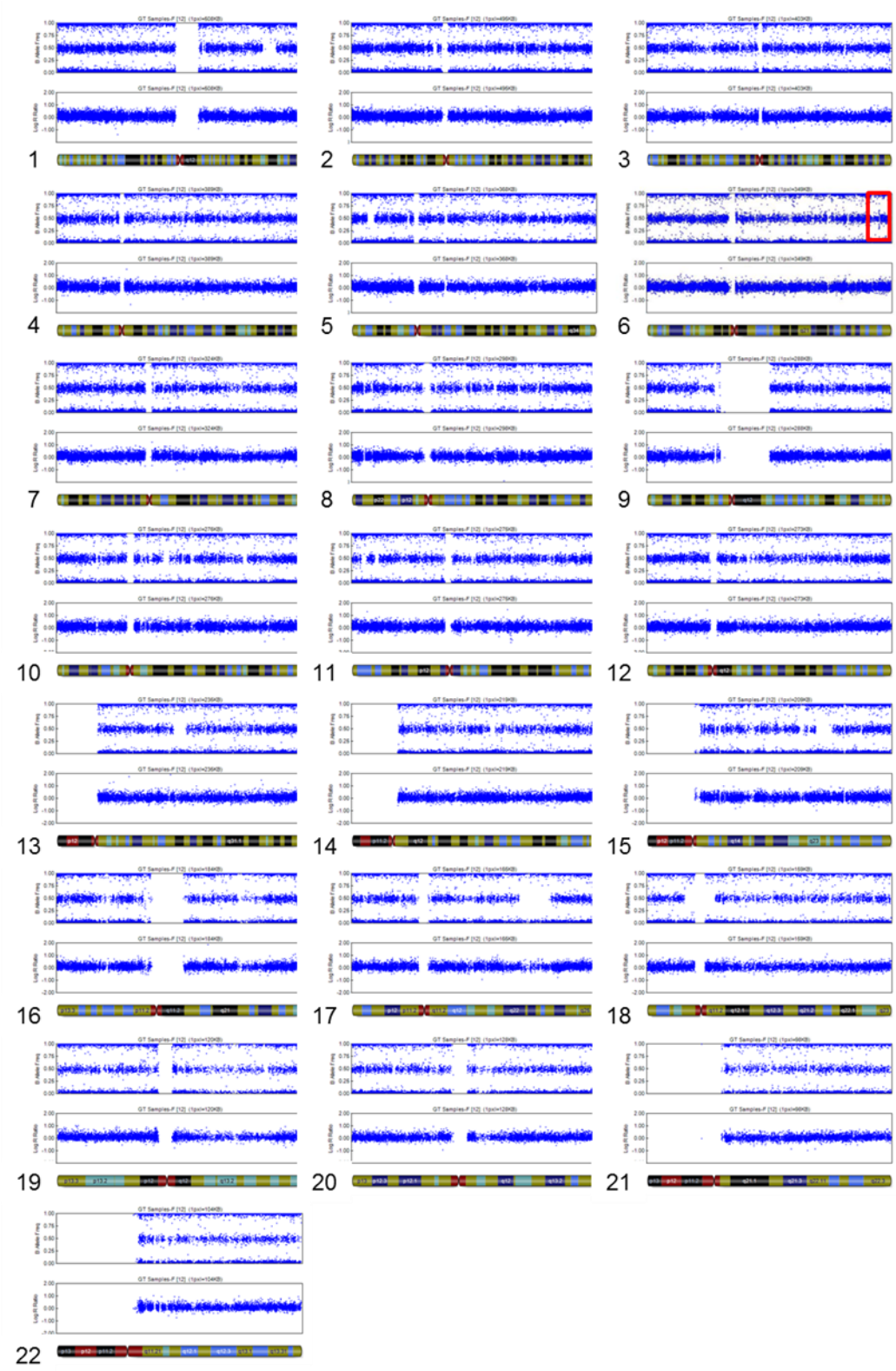
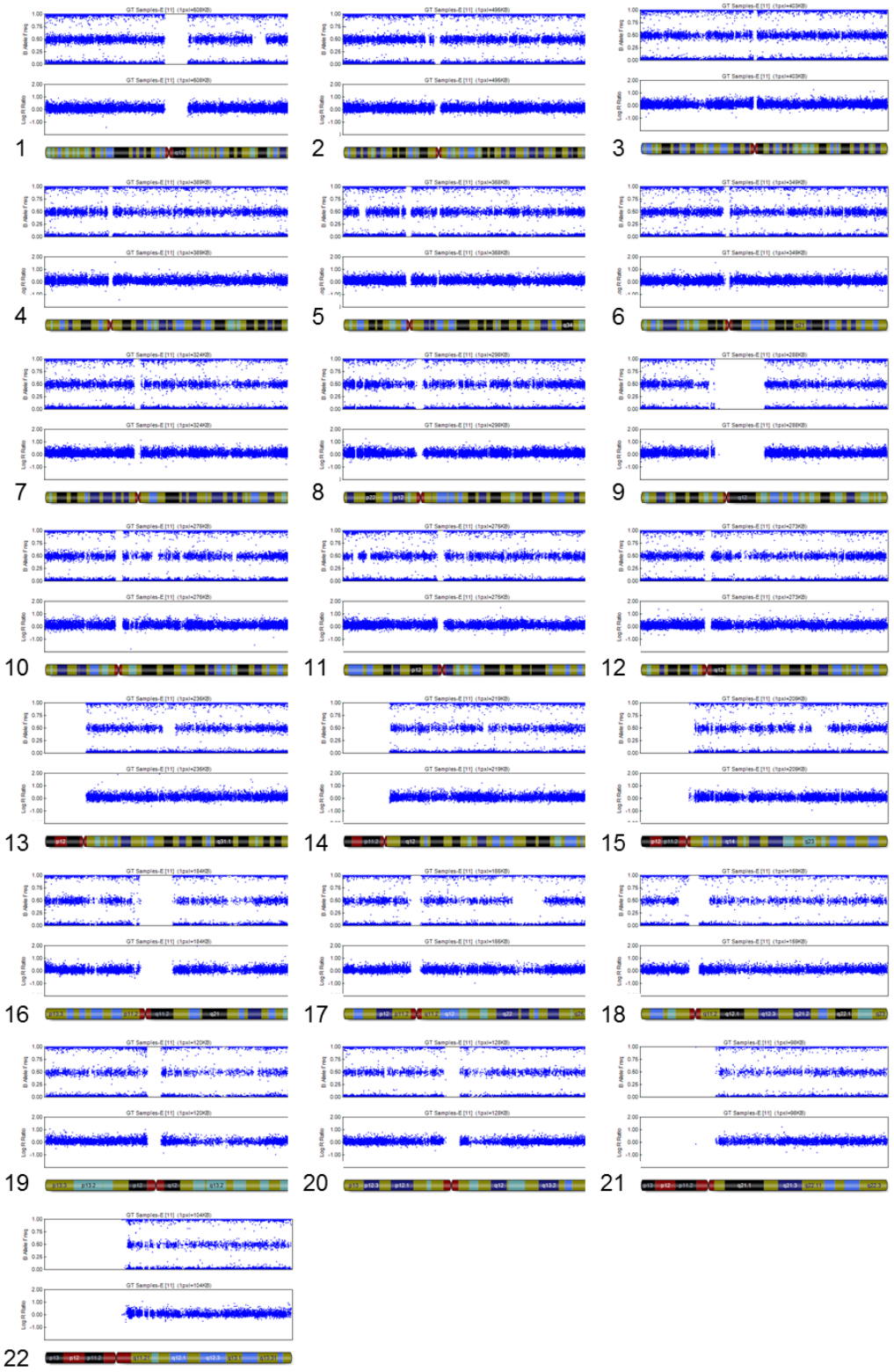
Appendix



Appendix Figure 1: Complete genomic data set following SNP array of fiPS; one microdeletion on chromosome 6 (red box).



Appendix Figure 2: Complete genomic data set following SNP array of eiPS; normal karyotype.