Characterization of progenitors of endothelial cells (PECs)

Elmira Jalilian

UCL
Institute of Ophthalmology

Thesis submitted in fulfilment of the requirements for the degree of

Doctor of Philosophy

Supervised by Dr Marcus Fruttiger

2017

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

Acknowledgments

First of all I would like to pay special thanks to Dr Marcus Fruttiger for his endless support and enthusiasm throughout my project. It has been a privilege and honour working under his supervision and I am thankful for his inspiration and guidance. Additionally, I am greatly indebted to Professor Pete Coffey for being my second supervisor. I would also like to thank our collaborator Monte Radeke, (University of California, Santa Barbara) for his help and advices on the RNA sequencing data analysis.

Additionally, I would like to thank Dr. Jenny Mckenzie and Dr. Michael Powner for their assistance and advices during my lab work. Thanks to Dr Amanda Carr and Dr Lili Chen for providing us with low passage BJ iPSCs for expansion and primers for qPCR analysis. Thanks to Ali Hadian for his assistance in all statistical analysis. I would also like to thank all other members of the Fruttiger lab who provided assistance or advice with this project.

Last but not least, I am indebted to my family especially my parents, who have always been there for me. They have thought me through inspiration. They have continuously showered upon me love, guidance, unwavering patience and support.

Abstract

There are a number of different stem cell sources that have the potential to be used as therapeutics in vascular degenerative diseases such as diabetic retinopathy. On the one hand, there are so called endothelial progenitor cells (EPCs), which are typically derived from adult blood. They carry the marker CD34, but the true nature and definition of EPCs is still controversial. On the other hand, there are embryonic precursors of endothelial cells (PECs), which also express CD34, and which can be differentiated from embryonic stem cells (ESCs) or induced pluripotent stem cells (iPSCs) in vitro. Furthermore, a subpopulation of human umbilical cord endothelial cells (HUVECs) has also been shown to express CD34. In this study, It was aimed to compare these three different CD34 positive cell populations by full genome transcriptional profiling (RNAseq). To this end I firstly optimised a PEC differentiation protocol and found that VEGF is critical for the transition from mesodermal precursors to PECs. Secondly, I found signalling pathways that regulate CD34 expression in HUVECs and showed a close correlation between CD34 expression and the endothelial tip/stalk cell phenotypes. Thirdly, principal component analysis (PCA) of RNAseq data showed that blood-derived EPCs are fundamentally different from iPS-derived PECs. Lastly, I also identified from RNAseq data number of potentially novel PEC markers. Once validated such novel markers of PECs and EPCs will be useful to better define these cell populations, facilitating the translation of regenerative approaches in this field as well as providing potentially novel diagnostic tools.

List of Abbreviations Used

ADS- Adult stem cell

BMP- Bone Morphogenetic Protein

BIO-Bromoindirubin-3'-oxime

CB- Cord blood

cDNA- complementary DeoxyriboNucleic Acid

DMSO-Dimmethylsulfoxide

DR-Diabetic Retinopathy

ECM- Extracellular matrix

EPC-Endothelial Progenitor Cell

ES- Embryonic stem cell

ESC- Embryonic stem cell

EDTA: EthyleneDiamineTetraacetic Acid

hESC- Human embryonic stem cell

EC-Endothelial Cells

EPC-Endothelial Progenitor Cells

EGF- Epidermal growth factor

FACS- Fluorescent activated cell sorting

FITC- Flourescein isothiocyanate

FGF- Fibroblast growth factor

b-FGF- basic- Fibroblast growth factor

a-FGF- acidic- Fibroblast growth factor

FLK1-Fetal liver kinase (VEGFR2)

G-CSF- Granulocyte- colony stimulating factor

gDNA: genomic DeoxyriboNucleic Acid

HLA- Human leukocyte antigen

HSC-Hematopoietic stem cells

HSC- Haematopoietic stem cell

HUVECs-Human Umbilical Vein Endothelia Cells

ICM- Inner cell mass

iPSC-Induced Pluripotent Stem Cells

kD- Kilo Dalton

IL- Interleukin

Lin- Lineage

LTC-IC- Long-term culture-initiating cell

IgSF- Immunoglobulin Super Family

MNC-Mononuclear cell

NRP-Neuropilin

Mono- Monocyte

PCR: Polymerase Chain Reaction

qPCR: quantitative Polymerase Chain Reaction

PB- peripheral blood

PS-Primitive Streak

PBS-Phosphate buffered saline

PBT-PBS and TritonX100

PFA-Paraformaldehyde

PECAM- Platelet-endothelial cell adhesion molecule

PEC-Progenitor of Endothelial Cells

PPEC-Precursors of Progenitor of Endothelial Cells

MSC- Mesenchymal stem cell

SCF- Stem cell factor

β- Transforming growth factor

UCB- Umbilical cord blood

PCAM- Platelet endothelial cell adhesion molecule

VEGF- Vascular endothelial growth factor

2-D- 2- dimensional

3-D- 3-Dimensional

Contents

List of Figures

List of Table

1 Introduction

1.1 Development of the vascular system during embryogenesis

1.1.1 Mesoderm induction and its derivatives

After fertilization, the zygote divides mitotically and generates a ball of cells, called morula in vertebrates (termed blastula in invertebrates). After the first series of cell divisions, the blastula/molura undergoes a spatial reorganisation called gastrulation. This process starts with an infolding (primitive streak) of the single layered blastula, eventually leading to three distinct germ layers (Gibert, 2000). These three germ layers are known as ectoderm (outer layer which produces cells of the epidermis and nervous systems), endoderm (inner layer which produces most of the internal organs) and mesoderm (middle layer which gives rise to muscles, the heart, the vasculature, bone and other structures) (Lu et al., 2001).

Mesoderm induction occurs through different signalling pathways. It is been shown that Nodal and Activin A (members of the transforming growth factor TGF-ß superfamily) are involved at the beginning of mesoderm induction (Rottinger et al., 2015). For example, studies in mice and chick have demonstrated that gradual expression of Nodal inhibitors away from the primitive streak creates a gradient of Nodal activity inside the streak itself, which induces mesoderm induction (Skromne and Stern, 2002, Kimelman, 2006). Furthermore, there are some studies suggesting that the small amount of Activin A, BMP2, BMP4, and basic FGF mRNA in zygotes is sufficient to start mesoderm formation (Kimelman and Kirschner, 1987, Kelly et al., 1995, Szeto and Kimelman, 2004). Moreover, other studies have shown that wingless-type MMTV integration site family members (WNTs) and FGFs are important in sustaining the mesodermal state (Wittler et al., 2007).

Mesoderm is divided into three different regions, proximal mesoderm, intermediate mesoderm and lateral plate mesoderm each of which differentiates into specific types of tissues. The lateral plate mesoderm gives rise to blood vessels and blood cells as well as the mesodermal component of the limbs (Gibert, 2000). It has been shown that BMPs play important roles in patterning of the mesoderm. Simultaneous expression of BMPs and BMP inhibitors in both dorsal and ventral sides of the embryo suggest that the regulation of mesodermal patterning by BMPs is very complex (De Robertis and Kuroda, 2004). Different groups have also shown the involvement of Nodal in mesodermal patterning (Gritsman et al., 2000, Birsoy et al., 2006).

The circulatory system, as one of the lateral mesodermal derivatives appearing in the third week of embryonic development in humans. The development starts with the formation of blood islets in the yolk sac and hemangioblasts (bipotent cells giving rise to both hematopoietic and angioblastic cells) in the head mesenchyme and posterior lateral plate mesoderm. The emergence of scattered of precursors of endothelial cells (so called angioblasts) through the mesoderm results in the formation of clumps and then cords which consequently differentiate into endothelial cells and functional vessels (Bautch and Caron, 2015) **Figure 1.** The term "endothelial progenitor cells" (EPCs) is often used to refer to all types of vascular progenitor cells. However, given the controversial nature of the "EPC-field" and to avoid misconceptions it was decided to use the term precursors of endothelial cells (PECs) in the context of the embryonic EC lineage and to use the term "EPC" only for adult bone marrow, adult peripheral blood and cord blood derived cells.

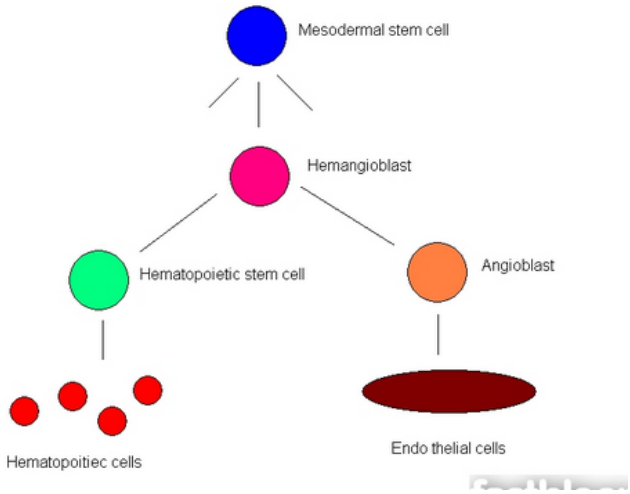


Figure 1: Differentiation of mesodermal stem cells into hematopoietic and endothelial cells

1.1.2 Haematopoietic lineage

The haematopoietic lineage is also derived from the mesoderm and has been extensively studied. In fact, much of our current knowledge about stem cells comes from haematology. Haematopoiesis (generation of blood cells) is a dynamic process, able to respond to haemorrhage, infection or hypoxia by the rapid proliferation of hematopoietic stem cells (HSCs) (Weissman et al., 2001). HSCs sustain blood production during life and have an important role in medicine as they are the functional units of bone marrow transplants. In the adults, HSCs reside in the bone marrow (Kanji et al., 2011), whereas, during development, haematopoiesis occurs in several distinct anatomical sites (Tavassoli, 1991).

In mammals, developmental hematopoiesis occurs in successive waves that take place in different regions. The first, transient wave is primitive haematopoiesis, also called the "mesoblastic phase", and starts in the extra-embryonic yolk sac blood islands. These blood islands originate from posterior lateral plate mesoderm and give rise to transient myeloid and erythrocytes lineages (Palis et al., 1999). Primitive haematopoiesis cannot produce the full set of blood cells found in adults. Some studies have suggested that primitive haematopoietic cells and endothelial cells may have a common precursor, called "hemangioblast" (Choi, 1998), and share common surface markers (CD34, VEGFR-2 and CD133)(Lugus et al., 2005, Wood et al., 1997). Furthermore, Chio also showed that endothelial and hematopoietic cells can originate from the same bi-potential cell type in vitro, called colony forming blast cells (Choi, 1998).

In contrast to primitive haematopoiesis, definite haematopoiesis starts later in development and is characterised by life-long generation of all hematopoietic lineages (Medvinsky et al., 2011). It appears to occur primarily in the aorta-gonad-mesonephephros (AGM) region in vertebrates (**Figure 2)** (Cumano et al., 1993), where HSCs arise from the hemogenic endothelium in the ventral part of the dorsal aorta (Bertrand et al., 2010). In the adult, HSCs are located in the red bone marrow, but can also be found in peripheral blood.

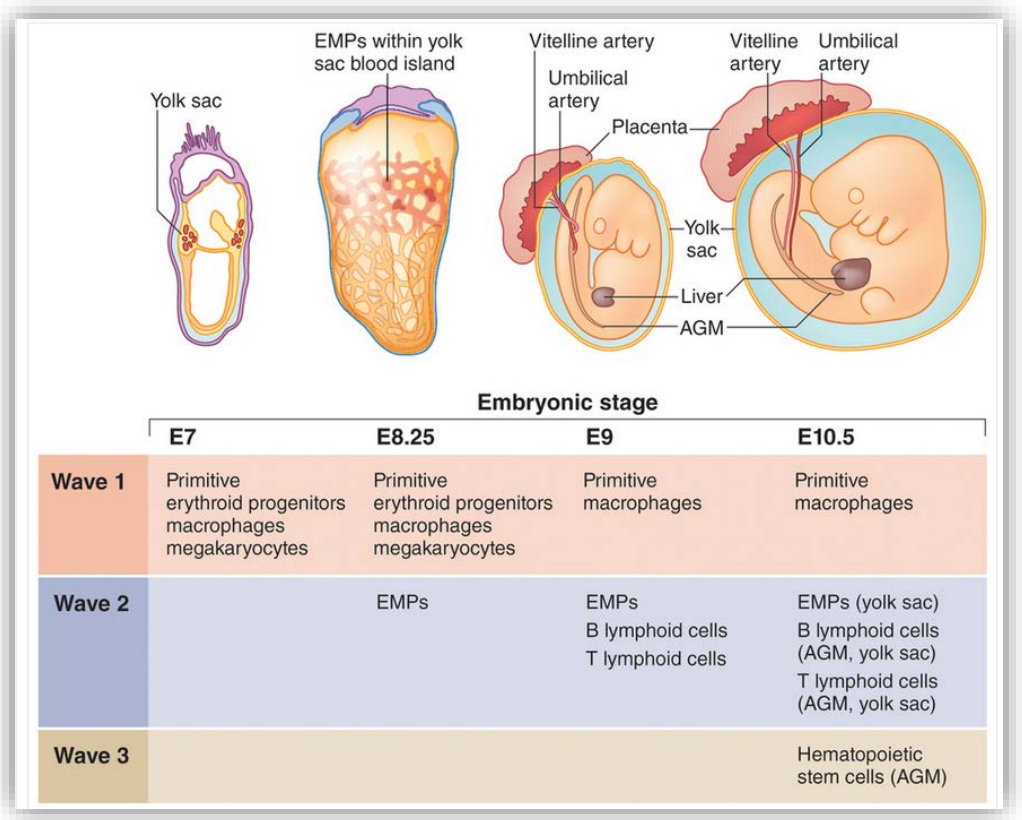


Figure 2: Hematopoiesis develops in three phases.
Early events emerge in the yolk sac and in the AGM area. In the beginning, primitive erythroid progenitors, megakaryocytes and embryonic macrophages appear in the yolk sac. In the second phase, erythromyeloid progenitors originate from hemogenic endothelial cells in the blood island areas in the yolk sac. This phase remains till E9, when B and T lymphoid progenitors arise from hemogenic endothelial cells in the yolk sac and the developing AGM region. Phase 3, initiates on E10.5 when the first HSCs emerging from hemogenic endothelium in the AGM area. At the same time, generation of EMPs in phase 2 and lymphoid progenitors continues in the yolk sac and AGM. Further than E10.5, HSCs are generated in the vitelline, umbilical, and cranial arteries and in the yolk sac and placenta. HSCs from all these sites seed the fetal liver. Up until late in gestation, liver is the main site of hematopoiesis when HSCs are released into the bloodstream and localize into the bone marrow to inaugurate medullary hematopoiesis. Illustration from (Yoder, 2014).

Classic studies in mice describe two populations of HSCs. Long-term HSCs have self-renewal capacity, whereas short-term HSCs (also termed precursors) differentiate to different types of blood cells **(Figure 3)** (Wagers et al., 2002). HSCs differentiate into one of around 10 different hematopoietic lineages (Watt and Contreras, 2005). In in vitro, they are usually described as blast cells, with a large nucleus that is surrounded by a thin cytoplasm (McGuckin et al., 2003). It is important to mention that an increasing body of evidence suggests plasticity in HSCs, which comprise a range of stem cell types (Alison et al., 2003). These cells have shown to be

"plastic" in their proliferative and differentiation capacities, blurring the lines between haematopoietic and non-haematopoietic lineages (Orkin and Zon, 2002).

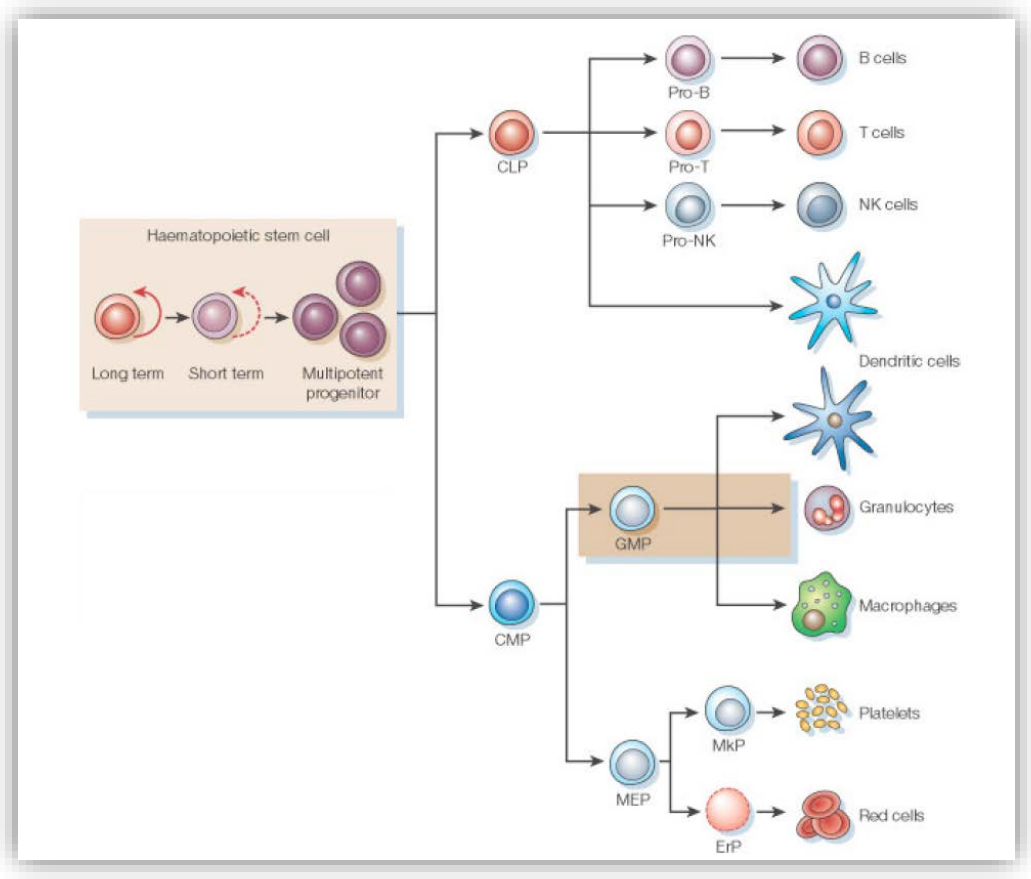


Figure 3: Schematic representation of haematopoietic stem cell.
A multipotential haemopoietic stem cells (HSC) balances between self-renewal (long term HSCs), lineage commitment and differentiation (short term HSCs). They give rise to common lymphoid progenitors which are also called CLP the progenitors of all lymphoid cells, Common myeloid progenitors which are called CMP the precursors of myeloid cells. Both CMPs/GMPs (Granulocyte macrophage precursors) and CLPs can give rise to all known dendritic cells. Illustration from (Reya et al., 2001)

1.1.3 Hemogenic Endothelium

There are several studies that have introduced the concept of a rare population of vascular ECs that can differentiate into hematopoietic stem and progenitor cells during embryogenesis (Zovein et al., 2008, Li et al., 2012). These cells are called hemogenic endothelial cells and are found in restricted anatomical sites (Antas et al., 2013). Specific markers to isolate hemogenic endothelial cells have not been clearly identified. However, lineage-specific markers for ECs such as VE-Cadherin in combination with hematopoietic-specific antigens such as CD45 and Ter119 have been used to isolate these cells (Fraser et al., 2002). Identification of an intermediate stage between integrated ECs and free-floating blood cells is characterised by up-regulation of CD41, c-kit and CD45 (Mizuochi et al., 2012). Furthermore, different signalling pathways have been shown to be involved in the development of hemogenic endothelial cells. Among them are the TGF-ß family member BMP4, Indian hedgehog (IHH) proteins, FGF, VEGF and Notch ligands pathways (Kaimakis et al., 2013, Robert-Moreno et al., 2008). It has also been illustrated that the transcription factor Rux1 is critical for hemogenic endothelial cell development, and conditional deletion of this transcription factor resulted in a failure of ECs to precede the endothelial-hematopoietic transition (Chen et al., 2009). The main function of hemogenic endothelium is to generate definitive hematopoietic precursors.

1.1.4 Endothelial cell lineage

The first step of vascular differentiation in the mesoderm occurs in opposition to the visceral endoderm, suggesting that endoderm-derived signals such as IHH signalling play an essential role in angioblast commitment (Belaoussoff et al., 1998). Different studies have shown the importance of BMP4 and FGF2 in the differentiation of mesoderm into endothelial and hematopoietic cell fates (Lam et al., 2014, Marom et al., 2005, Yamaguchi et al., 1994). However, experiments using mouse stem cells have shown that BMP4 acts upstream of FGF signalling to regulate the specification of angioblasts (Pearson et al., 2008, Winnier et al., 1995), whereas studying human stem cells illustrated that FGF is only required for survival and growth of undifferentiated stem cells and is not playing a role in the commitment of mesodermal cells to the endothelial lineage. Instead, BMP4 is the main regulator functioning downstream of IHH to induce endothelial cell differentiation (Kelly and Hirschi, 2009).

Another endoderm-derived factor that plays an essential role in endothelial cell differentiation is VEGF-A which will be discussed next.

1.1.4.1 Vascular Endothelial Growth Factor (VEGF)

VEGF is transcribed from a single gene that is alternatively processed in different isoforms. It has longer, less soluble isoforms (VEGF-A165, VEGF-A189) and shorter isoforms (VEGFA-121, VEGFA-165) that are freely diffusible (Klagsbrun and D'Amore, 1996). The crucial role of VEGF in vasculogenesis has been clearly established and mice with heterozygous mutation were not viable because of a complete lack of endothelial and blood cell development (Ferrara et al., 1996, Carmeliet et al., 1996). VEGF-A stimulates endothelial cells to proliferate and migrate via its main receptor VEGFR2 (KDR or FLK1). VEGF-A expression is highly regulated by hypoxia and induces blood vessel formation (Carmeliet and Collen, 1998). One of the important properties of VEGF-A is its ability to increase vascular permeability through the rapid development of capillary fenestration (Roberts and Palade, 1995). It is been shown that VEGF-A is also involved in vessel formation through recruitment of macrophages, which can generate different kinds of angiogenic factors. This process is very important during inflammation or myocardial angiogenesis (Clauss et al., 1990). Other roles that can be mentioned for VEGF-A is it affects differentiation of osteoblasts and haematopoietic cells and regulates insulin generation in pancreatic ß-cells (Katoh et al., 1995). For simplicity “VEGF” (instead of VEGF-A) will be used for the rest of the thesis.

During embryogenesis, the expression pattern of VEGF and its receptors suggests an important role in vascular development. It has been shown that VEGF is produced by cells that are in close proximity to developing endothelial cells, such as visceral endoderm cells in the yolk sac (Breier et al., 1995). Endothelial cells need a threshold level of VEGF to continue the differentiation process and to create a vessel lumen. Furthermore, VEGF plays a role in sprouting angiogenesis, remodelling of emerging vessels into an interconnected network and maturation of vessel sprouts through periendothelial cell recruitment (Stone et al., 1995).

1.1.5 Vasculogenesis and Angiogenesis

Blood vessels develop by a combination of vasculogenesis and angiogenesis. Vasculogenesis describes the *de novo* formation of blood vessels and is responsible for the formation of primary vessels such as the dorsal aorta during early embryonic development. It relies on the local

differentiation of mesoderm-derived precursors of endothelial cells (PECs) into ECs that coalesce into primitive networks (**Figure 4).** Angiogenesis is the expansion of a pre-existing vessel network through a combination of sprouting, proliferation, and remodelling processes (Roca and Adams, 2007). In adult life, angiogenesis occurs only during inflammation, wound healing, the female menstrual cycle and in numerous pathological disorders, such as retinopathies, rheumatoid arthritis and tumour growth (Carmeliet, 2005).

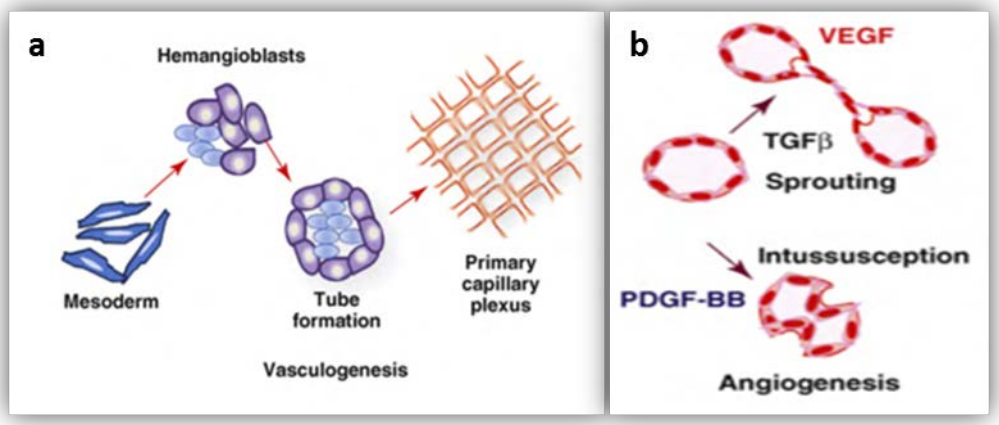


Figure 4: Vascular system development:
(a) Angiogenesis is the generation of new blood vessels from pre-existing ones either by intussusceptive vascular growth or by sprouting angiogenesis and by regulation of important factors such as VEGF, PDGF, TGF-β illustrated from (b) hemangioblasts (mesodermal precursors) differentiation into ECs during embryonic development which generates primary vascular plexus. Illustration from (Pardali et al., 2010)

Angiogenesis can be divided into two phases: activation and resolution **(Figure 5)**. During the activation phase, the basement membrane and extracellular matrix are degraded and endothelial cells start to migrate into the extracellular space, proliferate and form capillary sprouts, resulting in tubular structures **Figure 5b.** During the resolution phase (**Figure 5c)** endothelial cells stop proliferation and migration. In this phase, smooth muscle cells (SMCs) and pericytes are engaged to the newly formed sprouts, and basement membrane (BM) is reconstructed to stabilize the new vessels **(Figure 5c).** In the final step, blood vessels become quiescent. **(Figure 5d)** (Betsholtz et al., 2005).

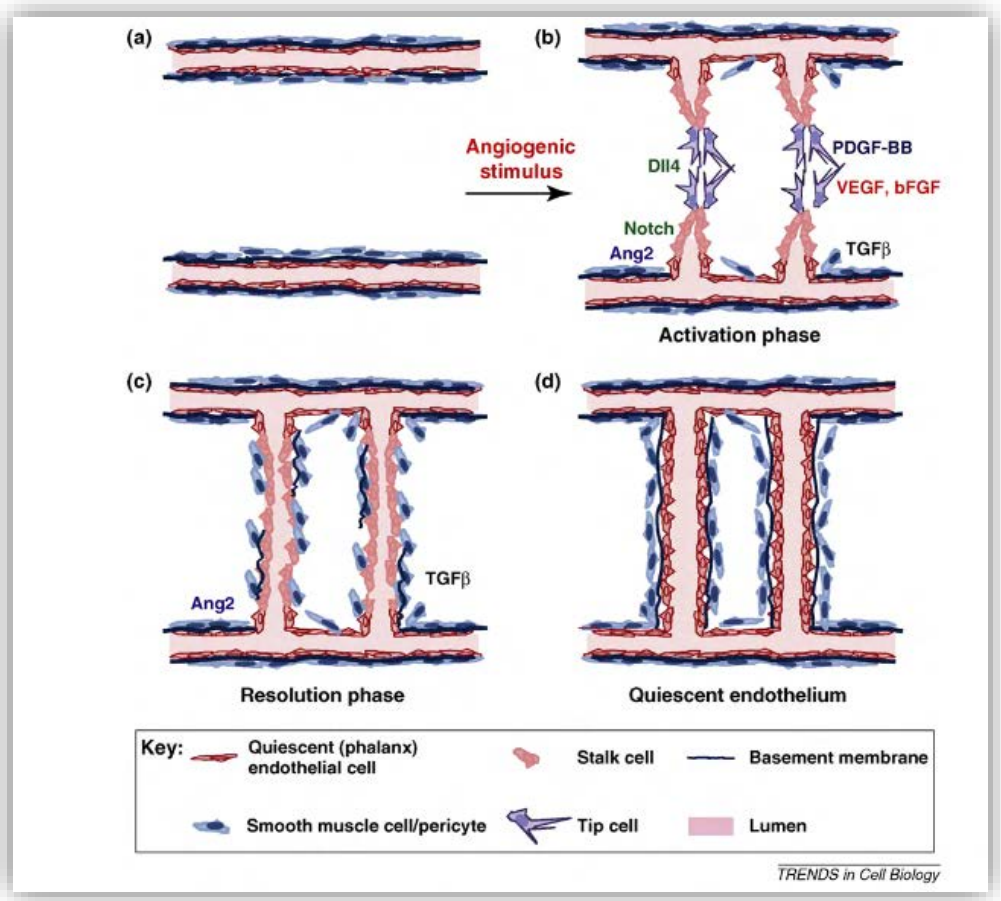


Figure 5: Angiogenesis regulation.
Angiogenesis includes two phases. In the activation phase, basement membrane is degraded by the angiogenic stimulus (VEGF, bFGF, TGF-ß) (figure a) and tip cells in the front position of sprouting vessels invade the tissue by extending filopodia (figure b). Stalk cells proliferate and extend, and the new branches join over tip-cell-tip-cell fusion (figure b). Lastly, in the resolution phase, ECs stop to proliferate and mature by re-formation of basement membrane (figure c) and obtain a quiescent phenotype, which is called phalanx EC (figure d). Illustration from (Pardali et al., 2010).

1.1.5.1 Sprouting angiogenesis: selection of tip versus stalk ECs

The tip cell and stalk cell phenotype are two distinct states ECs can be in, based on their gene expression profiles and their functional specification. The tip cell is the leading cell that is selected to spearhead the emerging sprout. Tip cells are migratory and polarised. They extend filopodia that scan the environment for guidance signals and steer the new vessel in certain directions. (De Smet et al., 2009) **(Figure 6)**. Behind the tip cells are stalk cells that follow. They proliferate during the sprout extension and form the lumen in nascent vessels Figure 6. When stalk cells are lumenised and blood flow has been established, the endothelial cells stop

proliferation and migration and adopt a quiescent phenotype. Quiescent endothelial cells are called phalanx cells. They do not migrate or proliferate and contribute to vessel stabilization by depositing basement membrane. All these EC phenotypes have their specific gene transcriptional profile. Genes that are enriched in tip cells are VEGFR2, platelet derived growth factor B (PDGFB), notch ligand DLL4, netrin receptor unc-5 homolog B (UNC5B), peptide ligand apelin (APLN), EC-specific molecule 1 (ESM1) and the matrix metalloprotease 14 (MMP14) (Strasser et al., 2010, Gerhardt et al., 2003, Hellstrom et al., 2007). Furthermore, there are also some genes that are enriched in stalk cells such as JAG1, FLT1 and IGFBP3, HES1, VEGFR1, HEY1 and Id1/3 (del Toro et al., 2010). Both, the proliferative stalk cells and the quiescent phalanx cells are covered by smooth muscle cells and pericytes (so-called "mural cells") **(Figure 6)**. Mural and endothelial cell interactions play an important role in vessel maturation and differentiation. However, it is not completely understood when and how the activated endothelium transits to the quiescence state (Geudens and Gerhardt, 2011). Each EC has the capacity to take on the tip cell, stalk cell or phalanx cell phenotype in a context-dependent manner. Moreover, the specification of tip and stalk cell identities among ECs is a dynamic process and they can actively take over the position of the other (Arima et al., 2011). In sprouting angiogenesis, the interplay between VEGF and Notch signalling pathways is mainly responsible for tip versus stalk cell specification which will be discussed in next chapter (Marcelo et al., 2013).

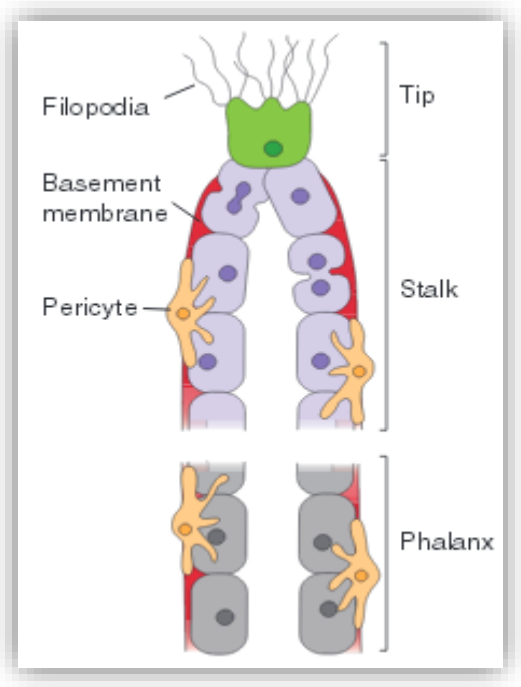


Figure 6: Schematic illustration of a growing vessel.
The sprout is guided by a tip cell (green) that is using filopodia to scan the environment. Stalk cells (purple) form a lumen by proliferation and deposit a basement membrane (red) and attract pericytes (orange). Both tip and stalk cells are a kind of endothelial cells that are activated. In contrast, phalanx ECs (grey) are quiescence endothelial cells that do not proliferate. Illustration from (Geudens and Gerhardt, 2011).

1.1.5.2 Important signalling pathways in angiogenesis

1.1.5.2.1 Notch signalling

Notch signalling is an evolutionally conserved signalling system that controls decision-making processes, such as cell-fate determination and cell differentiation. In endothelium, Notch signalling has roles in artery/vein differentiation, tip and stalk cell dynamics during sprouting angiogenesis and control of blood vessel quiescence. Tip and stalk cell interactions are particularly well studied. Upon induction of angiogenic sprouting, endothelial cells of the tip of the angiogenic sprout are exposed to particularly high levels of VEGF, which induce DLL4 expression in tip cells. This then activates Notch signalling in adjacent stalk cells, which reduces their ability to respond to VEGF-stimulation via a Notch-induced down-regulation of VEGFR2 and 3 and up-regulation of VGEFR1 **(Figure 7)** (Jakobsson et al., 2010). Therefore, DLL4 suppresses tip cell fate in adjacent stalk cells. This mechanism balances tip versus stalk cells selection and limits the number of outgrowth sprouts. Impairment of DLL4-Notch

signalling results in increased tip cell numbers and hyperbranching (Phng and Gerhardt, 2009). For instance, Dll4 heterozygous mutant mice display increased angiogenic sprouting as a result of increased tip cell formation (Suchting et al., 2007). Furthermore, it has been shown that inhibition of notch signalling by DAPT (γ-secretase inhibitor) or endothelial-specific genetic deletion of Notch1 results in increased sprouting and increased expression of tip cell-related genes (Hellstrom et al., 2007). Therefore, Notch signalling plays a key role in the regulation of vascular branching.

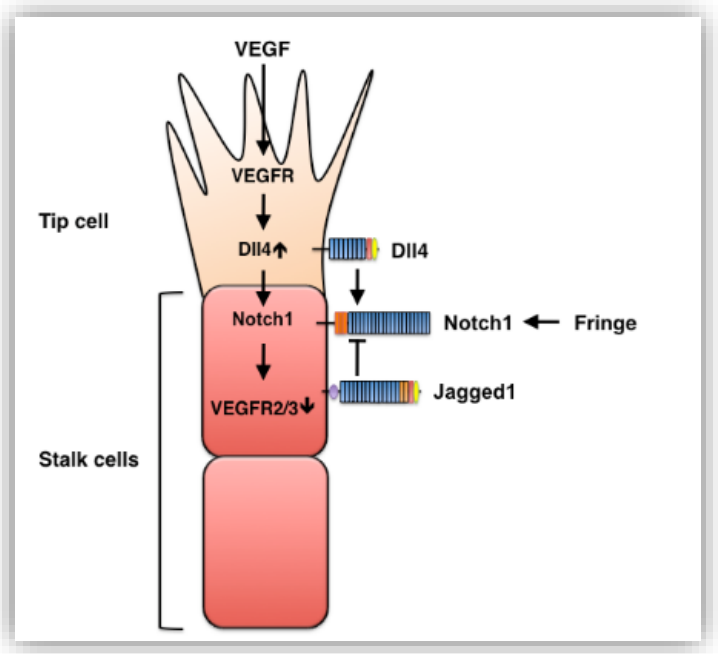


Figure 7: Sprouting angiogenesis.
VEGF signalling induces Dll4 expression in tip cells and consecutively, Dll4 activates Notch signalling in stalk cells. This results in reducing stalk-cell sensitivity to VEGF stimulation, which in turn suppresses the tip cell phenotype. Illustration from (Kume, 2009)

1.1.5.2.2 TGF-β superfamily signalling pathway

The TGF-β superfamily contains a large number of around 30 cytokines including TGF-β1, TGF-β2, TGF-β3, bone morphogenetic proteins (BMPs), Activins, Nodal and the growth and differentiation factors (GDFs), all of which are abundantly involved in many cellular processes both in the adult organism and the developing embryo. They play roles in proliferation, differentiation, migration, apoptosis and survival of different cell types such as fibroblasts, immune, epithelial, perivascular and endothelial cells. In general, signalling initiates with

ligand-induced oligomerisation of the receptors. TGF-β binding to the constitutively active type-II (TβRII) serine/threonine kinase receptors, induces transphosphorylation of type-I, activin receptor-like kinases (ALKs) (TβRI). This leads to signal propagation by phosphorylation of regulatory SMAD proteins (R-Smad). When R-Smads get activated, they complex with the common signalling transducer Smad4 (Co-Smad) and translocate to the nucleus, which results in the transcription of specific genes including Smad 6 and 7 which have negative feedback effects. Activation of specific R-Smad signalling depends on which receptors are participating. In endothelial cells, there are mainly two different type I receptors (Alk1 and Alk5). Alk1 activation results in phosphorylation of Smad1/5/8, whereas activation of Alk5 results in phosphorylation of Smad2/3 **(Figure 8).** The TGF-β superfamily ligands exert their biological activity through specific heteromeric cell surface complexes formed by type-I (TβRI) and type-II (TβRII) serine/threonine kinase receptors. Up to now, seven type-I, and five type-II receptors have been identified in mammals (Hawinkels et al., 2013).

TGF-β and BMPs are two important ligand classes that play important roles in maintaining blood vessel morphogenesis and integrity. The importance of TGF-β signalling in vascular development has been shown in different studies. In murine mouse models, knockout of TGF-β superfamily components usually results in embryonic lethality and severe vascular defects (ten Dijke and Arthur, 2007). For instance, mice having genetically deleted TGF-β1 are, embryonically lethal due to defects in the yolk sac vasculature (Goumans and Mummery, 2000). Furthermore, EC-specific deletion of type II TGF-β receptor (Tgfbr2) after birth in mice causes impaired retinal plexus development. In these animals, Smad 2/3 phosphorylation was reduced and EC clumped together and formed glomeruloid tufts instead of angiogenic sprouts (Liu et al., 2011).

ALK1 the receptor (ACVRL1) is mainly expressed in ECs (Seki et al., 2003) and is responsible for regulation of EC migration and proliferation in vitro (Goumans et al., 2002) and angiogenesis in vivo (Urness et al., 2000). Different studies have shown the important roles of ALK1 during different stages of vascular development either in early postnatal life or in adult mice. In neonatal mouse retinas, it was shown that lose of endothelial ALK-1 causes reduced endothelial pSmad1/5/8, venous enlargement, vascular hyper-branching and arteriovenous malformations (Tual-Chalot et al., 2014). Furthermore, ALK1 deficient mice have been shown to have impaired vessel remodelling, deficient differentiation and defective smooth muscle recruitment (Oh et al., 2000). Further evidence for the importance of ALK1 is derived from an autosomal dominant vascular disorder called “hereditary haemorrhagic telangiectasia” (HHT),

which is characterised by arteriovenous malformations in liver, lung and brain (McAllister et al., 1994).

Before the identification of BMPs, TGF-ß was the only described functional ligand for ALK1(Oh et al., 2000). However, in recent decades, many in vitro and in vivo studies have shown the importance of BMPs signalling in angiogenesis and vasculogenesis. BMP2/4 have been shown to play important roles in blood vessel development (Langenfeld and Langenfeld, 2004). Similarly, BMP2/4 has been shown to induce proliferation and tube formation in bovine aortic endothelial cells (Yao et al., 2009). Previously, it was believed that the angiogenesis promoting the effect of BMPs is due to their influence on VEGF and Id genes only. But recently, two novel genes, Cox 2 (which enhances EC proliferation, migration and assembly) and MyoX (which induces filopodia formation, cell alignment and migration) were also shown to be induced by BMP6 (Isabel et al 2012). Overexpression of BMP4 in retinal pigment epithelial cells in transgenic mice, results in increased Smad1/5 phosphorylation and down-regulation of VEGF and matrix metalloprotease 9 (MMP9) expression, which is associated with reduced angiogenesis (Xu et al., 2012). Although BMPs have generally been shown to stimulate angiogenesis, there is also some evidence that they are anti-angiogenic. (Mathura et al., 2000).

BMP9, another member of the BMP family, is expressed in the liver and plays important roles in cell growth, differentitation, and apoptosis (Hogan, 1996). Circulating BMP9 was indentified as a specific ligand with high affinity for ALK1, inducing vascular quiescence in adult blood vessels by activating Smad 1/5/8 (David et al., 2008). In vitro and in vivo studies have shown that BMP9-Alk1 interaction in mouse embryonic stem cells induces the expression of VEGF receptor VEGFR2 and the angiopoietic receptor Tie2, and results in EC proliferation and tube formation (Suzuki et al., 2010). Furthermore, HUVECs treated with BMP9 have shown increased tube formation (Nolan-Stevaux et al., 2012). However, there is still controversy regarding the pro- and anti-angiogenic effects of BMP9. Some studies have shown that BMP9 significantly promotes vasculogenesis and increases proliferation through ALK1 (Suzuki et al., 2010), whereas other studies showed an inhibitory effect of BMP9 on proliferation and migration (Scharpfenecker et al., 2007, David et al., 2007). Moreover, BMP9-Alk1 interaction has been shown to inhibit migration and proliferation in dermal microvascular endothelial cells (Upton et al., 2009, Nolan-Stevaux et al., 2012). It, therefore, appears that the effects of BMP9 in vitro are context dependent but the components that determine whether BMP9 induces or inhibits EC proliferation are not yet identified.

Recent findings have also introduced BMP10, (structually close to BMP9) as a specific ligand for ALK1, cooperating with BMP9 (David et al., 2007). In previous studies, the involvement of BMP10 has been shown in embryogenesis and in heart development (Chen et al., 2004). However, recent studies have also shown the involvement of this factor on postnatal retinal vascularization. It has been shown that blockade of either BMP9 or BMP10 could slightly increase the retinal vascular density in newborn mice (David et al., 2007). However, blockade of both ligands together by injecting BMP10 neutralizing antibody in BMP9-KO pups signigficantly increased the retinal vascular density (Ricard et al., 2012) similar to ALK1 KO-pups (Tual-Chalot et al., 2014). This shows that BMP9 and BMP10 are two important ALK1 ligands that can substitute for each other.

Recent *in vivo* studies have shown a synergistic effect between ALK1 and Notch signalling. It was shown that ALK1 signals through phosphorylation of SMAD 1/5/8 in stalk cells together with Notch intracellular domain (NICD) to induce expression of the notch target genes HEY1 and HEY2 to repress tip cell formation. Additionally, in vitro studies showed that stimulation of ECs with BMP9 alone directly induced the expression of Notch target genes, HES, HEY1 and HEY2 on top of expressing the ALK1 target gene Id1, which maintains cell quiescence. This describes the anti-sprouting effect of BMP9 seen in mouse retina (Larrivee et al., 2012, Moya et al., 2012).

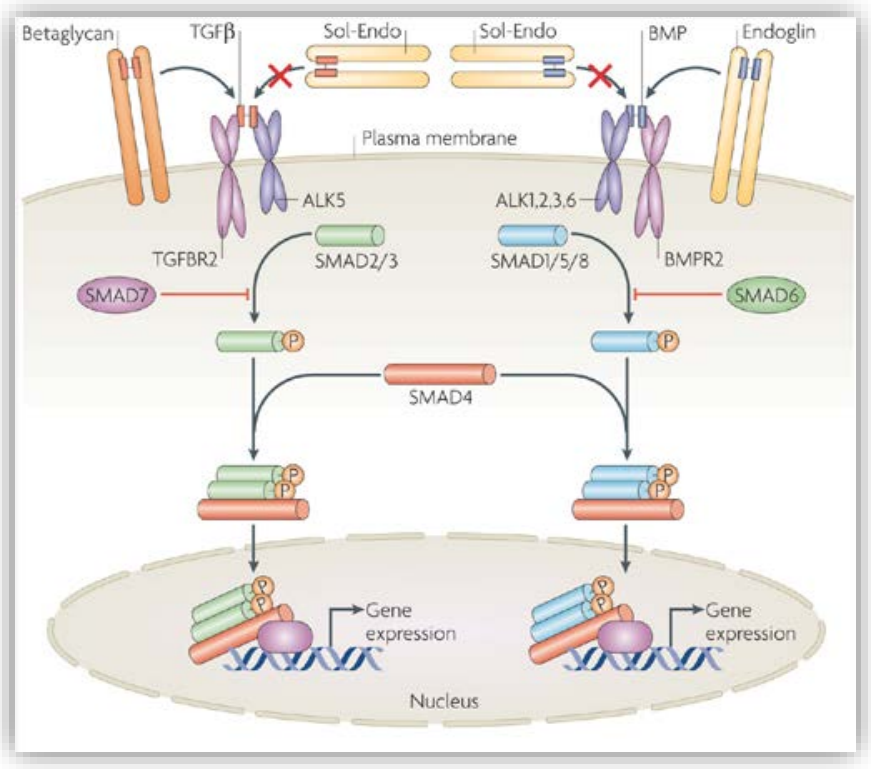


Figure 8: TGF-β Signalling pathway.
Canonical signalling by TGF-ß superfamily members is divided in two intracellular pathways according to the SMAD mediators; SMAD2/3 or SMAD1/5/8. Illustration from (ten Dijke and Arthur, 2007).

1.2 Diabetic Retinopathy (DR)

1.2.1 Prevalence of DR

Diabetes mellitus is a chronic metabolic disease characterised by sustained hyperglycemia that leads to macro and microvascular complications (Henriques et al., 2015). Diabetes is the leading cause of blindness among adults aged between 20 and 74 years old. Recent surveys have predicted that by 2025, the number of patients with diabetes will increase to 380 million worldwide. This global disease will lead to increasing incidence of two major types of late complications: macrovascular and microvascular, which lead to morbidity and premature death. Cerebrovascular, cardiovascular and peripheral vascular diseases are examples of macrovascular disorders in which large vessels are affected. In contrast, microvascular complications affect small vessels and include nephropathy, neuropathy and retinopathy. Retinopathy is one of the most common ischaemic disorders of the retina and the main cause

of blindness in the working age population. It is responsible for 12,000 to 24,000 new cases of blindness each year worldwide (Chistiakov, 2011, Stitt et al., 2011, Willard and Herman, 2012).

Diabetic retinopathy (DR) is the most common ischaemic disorder in the eye and presents as a broad spectrum of manifestations, particularly at the level of the retinal vasculature. DR is responsible for 4.8% of the 37million cases of blindness in the world, according to the World Health Organization (WHO). The main risk factors for DR are high blood pressure, hyperglycaemia and the duration of diabetes. Studies show that there is a pathogenic link between hyperglycemia and the onset and progression of DR. Tight control of blood glucose can delay DR onset and progression. The duration of diabetes is another main risk factor for DR. Although type 1 and type 2 diabetes have some different phenotypic variations, in both patient groups, the prevalence of diabetic retinopathy after 10 years is approximately 75% which increases to 90- 95% after 20 years. Some of the other DR risk factors are gender, age at onset of the disease, ethnicity, cataract extraction and hyperlipidemia (Chistiakov, 2011).

1.2.2 Vascular insufficiency and inner retinal ischemia

Ischemia is characterised by the restriction of blood supply to tissue and organs, which cause a shortage of oxygen and glucose, needed for cellular metabolism, and reduced removal of metabolites (Stitt et al., 2011). Ischemia-related pathologies are central to many diseases and pose a challenge for healthcare systems worldwide. Angina, myocardial infarction, stroke and ischaemic retinopathies are some of the most common ischemia-related diseases which represent a major cause of morbidity and mortality worldwide (Joggerst and Hatzopoulos, 2009).

Vaso-degenerative retinopathies, such as DR, can result in variable degrees of retinal vascular insufficiency and lead to a profound loss of vision. Beyond the significant risk of depriving delicate neural networks of oxygen and nutrients, hypoxia can increase the expression of some growth factors and cytokines. This can result in vascular leakage in the surviving vasculature and/or pre-retinal and papillary neovascularization. If these complications are left untreated, the responses to vascular stasis, ischaemia or hypoxia can result in fibro-vascular scar formation or retinal oedema and blindness (Prisco and Marcucci, 2002, Stitt et al., 2011).

1.2.3 Clinical signs and diagnosis

Many diabetic patients may not experience any noticeable symptoms in the early stage of the disease. However, early detection of DR can help to prevent severe loss of vision and blindness. Different clinical signs of retinopathy include dot and blot retinal haemorrhage, the formation of microaneurysms, cotton wool spots, hard exudates, venous abnormalities and growth of new blood vessels. There are also anatomical changes during DR that have been well documented and include the formation of acellular capillaries, early thickening of the basement membrane, formation of microaneurysms, loss of pericytes and endothelial cells and retinal neovascularization (Durham and Herman, 2011). DR diagnosis involves visual acuity testing, fundus examination (direct and indirect ophthalmoscopy) and retinal photography. Optical coherence topography (OCT) is widely used to examine the major layers of the retina and the various reflectance of visible light (Ikram et al., 2013). By using this technique it is possible to localize retinal lesions in relation to different retinal layers and to quantify the retinal thickness. Furthermore, OCT is also used to measure retinal blood flow and diagnose retinal edema (Chistiakov, 2011)

1.2.4 Classification and treatments

DR can be classified by the clinical presentation either as non-proliferative DR (NPDR) or as proliferative DR (PDR). The first change observed in DR patients is a reduction in the retinal blood flow, which is followed by a loss of pericytes resulting in the development of micro-aneurysms, which may be associated with the appearance of retinal haemorrhages and hard exudates **Figure 9.** These changes are collectively referred to as NPDR. Basement membrane thickening and leakage results in the first noticeable abnormality of NDPR. As the vascular damage progress and a wider area of ischemia develop, neovascularization may become evident in the retina, and over the optic nerve. VEGF is released to develop a new nutrient supply by constructing capillary tubes. This is the stage where DR becomes PDR. These new blood vessels are fragile and tend to bleed and cause scarring on the surface of the retina. This is the most advanced and serious form of diabetic retinopathy (Willard and Herman, 2012, Giuliari, 2012) **(Figure 9)**.

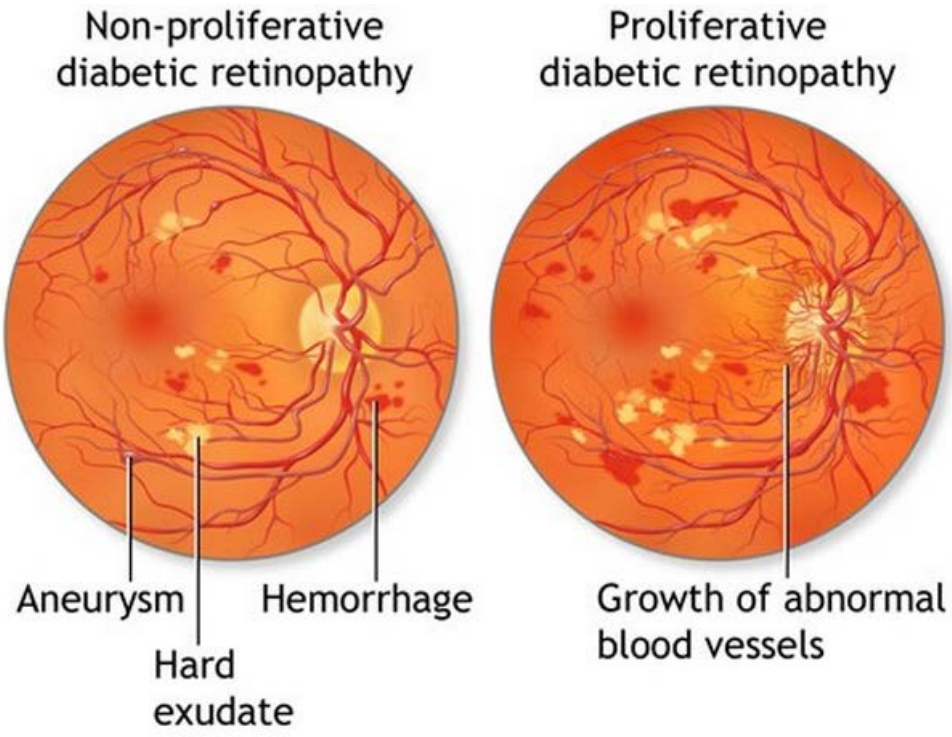


Figure 9: Illustrative pictures of NPDR (left) and PDR (right).
In NPDR damaged blood vessels begin to leak extra fluid and small amounts of blood into the eye which occurs at the earliest stage of DR. In PDR, many retinal blood vessels are closed, which disrupts the blood flow. In response to hypoxia, new blood vessels are generated (neovascularisation), which are abnormal and ineffective. Illustration from https://maxivisioneyehospital.wordpress.com.

At any stage of the disease, DR can be associated with diabetic macular edema (DME). DME is defined as retinal thickening caused by vascular leakage and build-up of fluid and proteins within two disc diameters of the macular region and is the major cause of severe visual impairment in diabetic patients. Diabetic macular ischemia (DMI) occurs when small blood vessels close completely over time, resulting in poor blood flow. Macular ischemia causes the death of nerve cells in the macula responsible for fine vision. This process is irreversible and causes a permanent untreatable central blind spots and decreases central vision (Manousaridis and Talks, 2012).

NPDR and DME are considered the most sight-threatening ocular complication. Studies show that prevention and modification of associated systemic risk factors are the critical steps for the treatment of diabetic retinopathy. Several factors such as the control of blood pressure, blood glucose and the glycosylated haemoglobin levels and lipid levels have been associated with the reduction of the long-term risk of developing sight-threatening ocular complications. Much research has been carried out worldwide and has led to various novel therapeutic targets. For

instance, the Early Treatment Diabetic Retinopathy Study (ETDRS) established pan-retinal and macular laser as the gold standard treatment for these complications (Giuliari, 2012). Laser photocoagulation and vitreoretinal surgery (vitrectomy) are the current surgical therapies that are effective in reducing the loss of vision and are useful for the late stage disease of retinopathy but carry significant sight-threatening side effects. Although laser photocoagulation, and pars plana vitrectomy, have been shown to be useful in the treatment of severe visual loss in DR patients, visual loss continues to develop after therapy (Chistiakov, 2011). More recently, discovering vascular endothelial growth factor (VEGF) and its important role in angiogenesis has opened up new opportunities for therapeutic approaches.

Clinical studies have shown the important role of VEGF in the pathogenesis of DME, and exudative AMD. Damage to the retinal microvasculature results in the in elevation of intraocular levels of VEGF, which has been shown to be an important pathophysiologic mediator in PDR and DME (Virgili et al., 2014). VEGF has also been shown to be associated with a break-down of the blood-retina barrier, causing increased vascular permeability, which results in vascular edema. High levels of VEGF were found in ocular fluids of patients with PDR and DME (Ishida et al., 2003). This has led to the application of anti-VEGF drugs to treat PDR and DME in combination with other techniques such as laser. Currently, four VEGF-binding drugs including Pegaptanib, Ranibizumab, Bevacizumab and Aflibercept **Table 1** have received U.S. Food and Drug Administration (FDA) approval for different diseases and are currently trailed for the treatment of diabetic retinopathy.

Name	Trade-name	Description	Clinical Trials
Pegaptanib	Macugen - Eyetech New York	High affinity to the heparin binding site of VEGF-A isoforms	FDA-approved for AMD but because of disappointment visual results, only used sparingly.
Ranibizumab	Lucentis - Genentech, S. Sam Francisco	Recombinant humanised anti-body fragment (Fab) that binds all isoforms of VEGF	FDA-approved for AMD, macular edema & DME
Bevacizumab	Avastin – Genentech S. Sam Francisco	Recombinant full-length humanised monoclonal anti-body that also binds all VGEF isoforms	FDA-approved for rectal carcinoma, ovarian carcinoma, glioblastoma but is off set for use in ocular diseases (AMD, DME & vein occlusion)
Aflibercept	Elyea – Regeneron , Tarrytown, NY	Recombinant fusion protein with native VEGFR ligand-binding sequences attached to the Fc segment of human IgG1. Binds all isoforms of VEGF-A, VEGF-B and placental growth factor.	FDA-approved for AMD & macular edema and the systemic formulation Zaltrap for colorectal carcinoma.

Table 1: Summary of four different anti-VEGF drugs that are used treatment of diabetic retinopathy.

However, despite promising results with anti-VEGF therapy, some important issues should be considered. First, the requirement of multiple intra-vitreal injections can cause side effects, such as cataracts, uveitis and retinal detachment. Furthermore, it has been reported that some patients with DR respond poorly to VEGF inhibition and in some cases therapy could even be associated with a poor visual outcome. Secondly, current therapies are only applicable for

proliferative disease and DME. They just help to moderate the results of the pathogenic process without affecting the underlying cause. Thirdly, in a subgroup of patients with pure DMI, where small blood vessels close off completely and the retina slowly degenerates, there is absolutely no indication of using anti-VEGF drugs. In this instance, regenerative medicine might introduce an alternative way to regenerate areas of vasodegeneration and might reverse ischemia by regenerating blood vessels. Fourthly, VEGF has also been shown to influence neuronal growth, differentiation, and survival. In vitro studies have shown the effect of VEGF on axonal outgrowth, improvement of cervical and dorsal route ganglion neurons and in total in neuronal development and maintenance within the central nervous system (Jin et al., 2000). In the retina, VEGF exposure resulted in a dose-dependent reduction in neuron apoptosis in ischemia-reperfusion models (Ogata et al., 1998). VEGF receptor-2 (VEGFR2) expression was detected in several neuronal cell layers of the retina, and functional analyses showed the involvement of VEGFR2 in retinal neuroprotection (Foxton et al., 2013). The Nishijima study has also shown the involvement of VEGF in response to retinal ischemia, reducing the number of apoptotic retinal cells, which was shown to be reversed after adding VEGF-inhibitor (Nishijima et al., 2007). Therefore, using anti-VEGF therapy to inhibit unwanted angiogenesis, might inadvertently inhibit adult neurogenesis and neuroprotection (Mackenzie and Ruhrberg, 2012).

1.3 Stem cell-based therapy for ischemic disease

The potential of stem cells to regenerate terminally differentiated organs makes them an ideal source for cell-therapy approaches. Stem cell therapy has introduced a novel way to reverse ischemia for the treatment of micro and macrovascular complications in diseases such as diabetes (Shaw et al., 2011). Stem cells can be isolated from blastocysts before implantation (embryonic stem cells), or from reprogrammed somatic cells (iPSCs) (Harris and Rogers, 2007). Some adult stem cells have also been identified in different organs (hepstic, muscular, neural and hematopoietic). Among them, hematopoietic stem cells in the bone marrow are the most characterized population, with wide-ranging clinical usage for transplantation. However, within the vasculature, stem cells have only been postulated recently.

1.3.1 Different types of stem cells

Stem cells may be sourced from blastocysts before implantation from day 5-7 of the embryo (embryonic stem cells) **(Figure 10),** or after six weeks from foetus, which are considered less pluripotent stem cells (foetal stem cells). Other types of stem cells can be derived from blood or other tissues postnatally (adult stem cells). Each of these stem cells play a unique role in stem cell research and therapeutic applications **(Figure 10)** (Watt and Contreras, 2005)**.** More recently, stem cells can also be generated from somatic cells by re-programming strategies and are called induced pluripotent stem cells (iPSCs)**.** In the following paragraphs, first the definition of stem cells and different types of stem cells will be explained. Then, the potential use of stem cells for vascular regeneration and the advantages and disadvantages of each type will be discussed (Leeper et al., 2010).

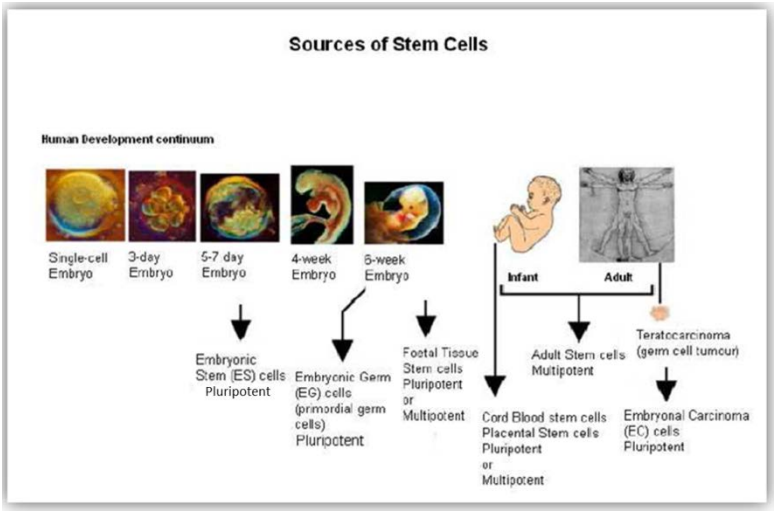


Figure 10: Various sources of stem cells.
ES cell are isolated from the inner cell mass (ICM) of the blastocyst and are considered as pluripotent. Primordial germ cells are derived from embryonic germ cells and are pluripotent. Foetal stem cells are derived from the developing foetus and are pluripotent or multipotent. All stem cells derived after births are known as adult stem cells (ADS). These cells have limited potential and are usually multipotent. Cord blood derived stem cells occupy a niche between ES cell and ADS. These have been categorized to be pluripotent and display some ES cell-like properties. Illustration from www.stemcellresearch.org/testimony/images/20040929prentice.htm.

1.3.2 Definition of stem cells

The concept of the stem cell arose from pioneering studies by McCullogh and Till on haematopoietic stem cells and those of Leblond on intestinal crypt and spermatogenesis (Handbook of Stem Cells, volume1- Embryonic Stem Cells, Lanza 2004). Stem cells can be defined as single cells that are clonal precursors of further identical stem cells and with a defined set of differentiated progeny (Weissman et al., 2001). There are some parameters that play a central role in defining 'stemness', including self-renewal (replication capacity), potency and clonality. Most somatic cells plated in vitro demonstrate a limited number of population doubling, less than 80, before undergoing replication arrest. This is different to the unrestricted proliferation capacity of stem cells in culture. Therefore, it has been defined that if a cell without tumour transformation can undergo more than twice this number of population doublings (160), it may be considered as 'capable of extensive proliferation' (Weissman et al., 2001). Stem cells are defined as clonal entities: single cells that are able to produce more stem cells. This phenomenon is crucial for any definitive characterisation of self-renewal, potential and lineage (Weissman et al., 2001). Stem cells can also be categorized by their potential to differentiate into different cell types, which is defined as potency. A lineage hierarchy classification of stem cells based on potency is as follows.

Totipotent stem cells are able to differentiate into any cell type in the body, including extra embryonic tissue such as placenta. Therefore, by this definition, a fertilized egg (zygote) is the only totipotent stem cell. Embryonic stem cells (ESCs) are derived from the inner cell mass (ICM) of the blastocyst and are pluripotent. Thus, they are capable of generating tissues of all three germ layers: ectoderm, mesoderm and endoderm; but cannot produce the extra-embryonic tissues (De Felici et al., 2009). Further examples of pluripotent stem cells are embryonic germ cells and cord and cord blood-derived stem cells (Harris and Rogers, 2007). Multipotent stem cells are descendants of pluripotent stem cells and are the precursors of specialized cells in specific tissues. Adult stem cells are a type of multipotent stem cell with a more restricted differentiation potential and ability for self-renewal. Neural stem cells that differentiate into nerve cells (neurons) and neural support cells (oligodendrocytes and astrocytes) and haematopoietic stem cells (HSCs) which give rise to all blood cells, are examples of multipotent stem cells (Harris and Rogers, 2007). Unipotent cells, also known as progenitor cells, have a very restricted differentiation capacity and can only produce one cell

type. For example, erythroid progenitor cells differentiate into red blood cells (Harris and Rogers, 2007).

1.3.3 Induced Pluripotent Stem Cells (iPSCs)

iPSC cells are type of pluripotent stem cells that can be prepared by re-programming adult somatic cells into stem cells, which was shown by Yamanaka in 2006. iPSC technology allows us to derive patient-specific cells in personalized medicine, avoiding some of the ethical concerns surrounding ESCs, allograft rejection and immunogenicity. Furthermore, using iPSC cells allow us to scale up production of a desired cell lineage and hence offering new prospects for regenerative medicine. iPSC generation was first defined in experiments using retroviral gene transfer of 4 transcription factors (Oct3/4, Sox2, c-Myc, and Klf4) in adult mouse dermal fibroblast (Takahashi and Yamanaka, 2006).

Later it was possible to generate human iPSCs only with the transcription factors Oct4 and Sox2 (Takahashi et al., 2007). Nanog and LIN28 also have been shown to affect the efficiency of reprogramming. Interestingly, KLF4 has been shown to be dispensable in generation of human iPSC cells. For the first generation of iPSC cells from re-programmed mouse and human dermal fibroblast, retrovirus was used to deliver the transcription factors. More, recent approaches are using drug inducible lentiviruses to induce temporal control over transgene factors, which has resulted in an increase of more than 100% efficiency in iPSC production. Development of non-integrating lentiviral vectors addresses the concerns regarding tumourigenesis. Therefore, iPSC cells have a great potential as a cell source in regenerative medicine. These cells have been shown to be almost completely identical to embryonic stem cells in terms of their differentiation properties (Narazaki et al., 2008), surface markers and gene expression profiles (Huangfu et al., 2008). iPSCs can also be used as novel evaluation platform to assess drug efficacy. They can be used for primary screening in vitro and reduce costly animal and human trails (Takebe and Taniguchi, 2014).

1.3.4 Adult stem cells in vascular regeneration

Using adult stem cells to regenerate blood vessels was first introduced by Asahara in 1997. It was believed that postnatal neovascularization was exclusively based on fully differentiated ECs, derived from pre-existing blood vessels. However, Asahara (Asahara et al., 1997) showed that putative hematopoietic precursors cells (CD34+, Flk-1+/KDR+) from human adult circulating blood cells can differentiate to ECs in vitro. These cells were named endothelial progenitor cells (EPCs), and were later shown to be present in adult bone marrow and peripheral blood. They are believed to be progenitor cells and able to remain in their immature state. It has been suggested that these cells can be further stimulated to migrate, proliferate or differentiate into a more mature lineage and are able to either directly contribute or indirectly support, vascular regeneration (Alev et al., 2011). Because of their characteristic, progressive differentiation, they are usually described by cellular origin, their isolation methods and their surface markers (Balaji et al., 2013).

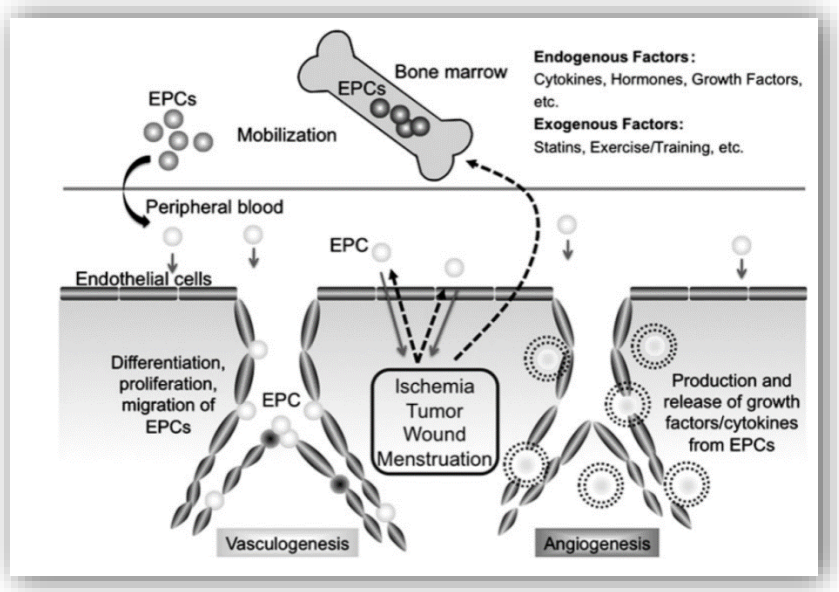


Figure 11: Role of EPCs in angio-/vasculogenesis.
The image shows the concept of angiogenesis, indicating that in response to different endogenous and exogenous factors, pre-existing ECs are stimulated to proliferate and migrate and consequently regenerate new blood vessels. In contrast, a variety of factors that are released from injured tissues, stimulate and mobilize EPCs from BM to the site of the injury, to participate in vascular regeneration. Illustration from (Alev et al., 2011)

1.3.5 Endothelial Progenitor Cells (EPC)

Many studies have shown that in animal models of ischemia, EPCs could incorporate into the site of active angiogenesis and contribute to tissue vascularization after ischemic events in limbs, retina and myocardium (Kawamoto et al., 2001, Kawamoto et al., 2002, Yang et al., 2011). Other studies have also been carried out to establish the stem cell character of these cells. (Rafii et al., 2002, Dome et al., 2008, Miller-Kasprzak and Jagodzinski, 2007).

Bone marrow is one of the main sources for EPCs (Rafii and Lyden, 2003). It was shown that autologous bone marrow cell transplantation remarkably improved coronary perfusion in ischemic heart disease (Hamano et al., 2001). Moreover, circulating BM-derived EPCs were able to cover implanted Dacron vessel grafts (Gill et al., 2001). In another study it was demonstrated that in a rat model of myocardial infarction (athymic nude rats), CD34 (a marker for EPCs) positive cells donated from humans could be observed in newly formed capillaries (Kocher et al., 2001).With regard to DR, transplantation of CD34+ cells derived from hematopoietic stem cells (HSCs) has been shown to repair ischemic retinal vasculature in mice (Dorrell et al., 2004) .

Thus, it appears that EPCs may be a promising source for the maintenance and repair of the retinal vasculature (Shaw et al., 2011), but the detailed mechanism of how EPCs enhance vascular regeneration is not clearly defined yet. Preclinical evidence for the ability of these cells to stimulate vascular regeneration is controversial and some studies have failed to demonstrate any beneficial outcome from stem cell therapy (Shaw et al., 2011). For instance, it was shown that intracoronary injection of autologous mononuclear bone marrow cells did not have any beneficial effect on left ventricular function in patients with myocardial infarction (Lunde et al., 2006). A reason for variable results in EPC-based studies might be different fractionation and isolation methods of bone-marrow-derived cells in different studies. Therefore, some researchers have supported the use of a heterogeneous population as a more physiological strategy for replacement of injured endothelium (Lee et al., 2013).

It is been suggested that the beneficial effects of transplanted bone marrow derived cells may be due to paracrine effects of myeloid cells, secreting pro-angiogenic factors, rather than precursors cells integrating into the endothelium (Chen et al., 2008). These cells known as circulating angiogenic cells (CACs), have also been shown to have beneficial effects in models of ischemia. For example, intravitreal injection of CD44+ cells (characterised as myeloid

progenitors) could promote vascular repair in the retina of a mouse model of oxygen-induced retinopathy. These cells were shown to differentiate to microglia (Ritter et al., 2006) and significantly improved vascular regeneration. Moreover, studies have shown that peripheral blood mononuclear cells can be isolated and enriched by culturing them in the presence of FGF-2, VEGF, IGF and EGF, and that they stimulate the formation of new blood vessels in the ischemic hind limb (Kalka et al., 2000). Since, cells isolated from this macrophages/monocytes fraction could secrete angiogenic growth factors, it was assumed that they may be involved in angiogenesis via the release of inflammatory mediators (Rehman et al., 2003). However, in the context of human pathology and cell therapy, using CACs might be risky. For instance, in ischemic DR they might exacerbate the pre-existing pathology (Shaw et al., 2011).

As mentioned above, different studies have shown benefits as well as lacking benefits of EPCs in vascular repair. The variation may be due to differences in the target tissues studied genetic variability in the animal strains and, perhaps most importantly, due to heterogeneity of EPCs based on their isolation and culture methods (Urbich and Dimmeler, 2004). In vitro studies suggested that there are at least two different types of EPCs; early and late outgrowth EPCs. Although both of these populations showed the capacity to promote vessel regeneration in different animal models, they illustrated different capability to differentiate into ECs and to physically contribute into new blood vessels formation.

“Early EPCs”, also called non-colony forming EPCs have myeloid/hematopoietic characteristics and they share lineage traits with immune cells, specifically macrophages and monocytes. They are isolated from adult peripheral blood mononuclear cells (PB-MNCs) or human cord blood mononuclear cells (CB-MNCs), which are plated on Fibronectin-coated dishes for 48hrs to deplete the adherent macrophages and mature ECs. Then non-adherent cells are removed and re-plated on Fibronectin-coated plates and VEGF containing medium. After 4-7 days so called “early EPCs” can be obtained **Figure 12**. These cells are probably related to CACs mentioned earlier (Balaji et al., 2013). They have myeloid characteristics and they usually do not form colonies under conventional endothelial differentiation conditions (Sharpe et al., 2006).

In contrast, when collagen-coated plates are used, after 2-4 weeks of culturing, late out growth EPCs (OECs) emerge **Figure 12**. These cells have been shown to have all the typical markers and functional characteristics of mature endothelial cells (Medina et al., 2010a). Other groups refer to these cells as endothelial colony forming cells (ECFCs) (Yoder et al., 2007). They have

a cobblestone appearance, high proliferation capacity, can differentiate to EC and they have been shown to physically contribute to new vessel formation in vitro and in vivo (Yoder et al., 2007, Prasain et al., 2014, Lee et al., 2013). Different studies have shown that the ability of late EPCs to form colonies is donor dependent such as age and diseases states (Sen et al., 2011, Celermajer et al., 1993, Chowienczyk et al., 1992). Although different phenotypes have been defined for early versus late EPCs, the possibility that late EPCs originate from a rare population of cells within the early EPCs should be considered since some cultured cells have shown both activities (Balaji et al., 2013) .

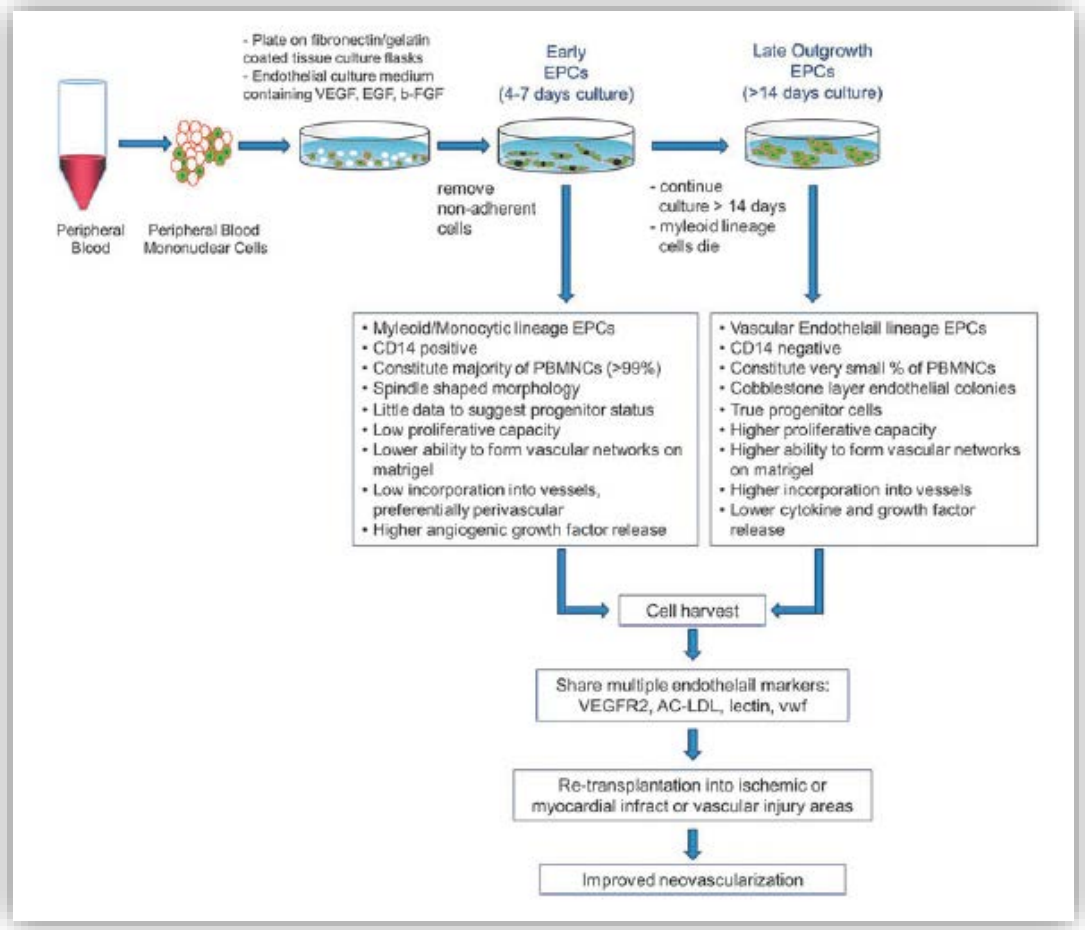


Figure 12: In vitro culture of EPCs; early vs late out growth EPCs.
EPCs are grown from whole peripheral blood mononuclear cells. Early EPCs obtained from short term culturing (4-7 days) on Fibronectin. Small population of early EPCs when plated for > 14 days show an increased capacity for proliferation and assembling the blood vessel formation. This population is called late-outgrowth EPC (or ECFC). Illustration from (Balaji et al., 2013).

The mobilization of EPCs from the bone-marrow is influenced by different entities such as the status of the endothelium, different cytokines and the bone marrow micro-environment. Among these factors, mature ECs are key players in the process of initiating EPC mediated vasculogenesis (Rabelink et al., 2004). In vascular occlusion, ECs can sense altered shear stress and consequently increase the expression of pro-oxidant enzymes. At the same time, the expression of anti-oxidant enzyme is reduced, which consequently results in locally increased redox signalling. NF-kB is activated and causes the release of chemotactic factors and consequently expression of adhesion molecules on ECs (Rabelink et al., 2004). Also hypoxia can be sensed by ECs, leading to an increase of the main hypoxia sensory molecule HIF1-α (Hoenig et al., 2008). Consequently, many growth factors and cytokines such as G-CSF, FGF-2, GM-CSF, VEGF, as well as angiopoietins are released and induce EPC mobilization from bone marrow (Luttun et al., 2002, Kleinman et al., 2007). EPCs are released into the circulation and home to tissue repair sites under the guidance of these signals where they differentiate into mature endothelial cells or regulate pre-existing ECs through paracrine or juxtacrine signals (Fox et al., 2008).

Mobilisation	VEGF	Vascular Endothelial Growth Factor
	SDF-1	Stromal Derived Factor-1
	Ang-1	Angiopoietin-1
	FGF-2	Fibroblast Growth Factor-2
	GM-CSF	Granulocyte Macrophage–Colony Stimulating Factor
	G-CSF	Granulocyte–Colony Stimulating Factor
	EPO	Erythropoietin
	CXCL 12	(C-X-C motif) Ligand-12
	estrogens	
Released by	VEGF	Vascular Endothelial Growth Factor
	HGF	Hepatocyte Growth Factor
	IGF-1	Insulin-like Growth Factor-1
	SDF-1	Stromal Derived Factor-1

Table 2: The most relevant chemokines that are attracted and released by Endothelial Progenitor Cells. Illustrated from (Resch et al., 2012).

There are advantages and disadvantages of using EPCs. Cells harvested from patients do not require immunosuppression when delivered to the patients and there are none of the ethical concerns surrounding the use of embryonic stem cells. However, difficulties around the use of adult stem cells are also important. For instance, autologous delivery of these cells adds a delay in treatments, as the cells needed to be collected, isolated and expanded *ex-vivo* and then

returned back into the patients. Furthermore, because of a lack of unique markers, a mixed population of cells is collected. Finally, cells collected from different individuals, can have different effects in therapies. For instance, EPCs isolated from older patients are less capable of proliferation and incorporation into damaged vasculature.

1.3.6 EPC Markers

There is no unique marker to identify EPCs and there is considerable debate about the true nature of EPCs. Nevertheless, there are some surface markers that have been used frequently: CD34, CD133, and VEGFR2 (Also known as Flk-1 or KDR). These markers represent a link between precursor and mature cells because they have been shown to be expressed on hematopoietic stem cells, along with mature ECs (Hristov et al., 2003, Peichev et al., 2000).

1.3.6.1 CD34

CD34 is a single trans-membrane protein which is expressed on approximately 1-4% of nucleated cells in human bone marrow and <0.1 % of nucleated cells in human peripheral blood (Civin et al., 1990). It has a molecular weight of approximately 115kDa and has an extracellular domain that is heavily sialylated, O-linked glycosylated and has some N-linked glycosylation sites (Nielsen and McNagny, 2008). The protein encoded by this gene mediates the attachment of stem cells to the bone marrow extracellular matrix or directly to stromal cells.

CD34 is an important adhesion molecule that is needed for T cells to enter lymph nodes. CD34 also plays an important role in mediating the vascular lumen formation (Strilic et al., 2009). Furthermore, CD34 has been shown to play a role in cell migration and proliferation and blocks differentiation of progenitor cells (Nielsen and McNagny, 2008). It has also been shown that CD34 can promote the adhesion of lymphocytes to specialised vascular endothelium in lymphatic tissues (Larrucea et al., 2008)

CD34 was the first marker that was identified on primitive hematopoietic cells in 1984 and is still the most commonly used marker for investigative research purposes (Wognum et al., 2003). Although hematopoietic stem cells can be isolated on the basis of their CD34 antigen expression, it is important to note that not all CD34-expressing cells are stem cells. Cells that express the CD34 antigen are often considered to be multipotent. CD34+ cells from non-human primate models have illustrated that they are capable of producing multi-lineage haematopoietic engraftment in myeloablated recipients (Vogel et al., 2000). It is important to mention that even in highly purified CD34+ cells from human bone marrow the frequency of

cells that possess progenitor activity (colony forming unit potential) is <20% (Holyoake et al., 1999).

CD34 is also widely expressing in vascular endothelial progenitor cells. There are some bone-marrow derived CD34-positive cells that are circulating in the peripheral blood and have been used for pro-angiogenic therapies (Hristov and Weber, 2008). Furthermore, CD34-positive cells have been shown to be located within the smaller blood vessels while it has been shown that endothelial cells within arteries and large vein are CD34-negative.

The CD34+ cell population can be categorized into three groups: HSCs, primitive progenitor cells and lineage committed progenitor cells. The difference in their expression pattern of other markers on these cells allows them to be distinguished from one another. CD34-positive cells induce in a broad range of cell types including hematopoietic, epithelial cells, endothelial cells and multipotent mesenchymal stem cells, corneal stroma cells, intestinal cells and fibrocytes shown in **Table 3** (Sidney et al., 2014).

Table 1. Summary of different $CD34^+$ cell types

$CD34^+$ cell type	Associated markers	Differentiation potential	Properties
HSC and progenitors	HLA-DR, CD38, CD117 (c-kit), CD45, CD133	Hematopoietic cells, cardiomyocytes, hepatocytes	Large nucleus, little cytoplasm, high proliferative capacity
MSC	Stro-1, CD73, CD90, CD105, CD146, CD29, CD44, CD271	Adipogenic, osteogenic, chondrogenic, myogenic, angiogenic	$CD34^+$ MSC form a higher proportion of CFU-f colonies than $CD34^-$. $CD34^+$ MSC exhibit a high proliferative capacity. Fibroblastic cells
Muscle satellite cells	CD56, Myf5, Desmin, M-cadherin, CD90, CD106, Flk-1, VEGFR, MyoD, CD146	Myogenic, adipogenic, osteogenic, chondrogenic	The $CD56^+CD34^+$ population may represent a more primitive or pluripotent stem cell. In vivo, $CD34^+$ cells are located near the basal lamina. Small and round
Keratocytes	CD34, CD133, L-selectin, keratocan, ALDH	Fibroblastic, myofibroblastic, adipogenic, osteogenic, chondrogenic, corneal epithelial, corneal endothelial	Dendritic morphology. In vitro population acquires an MSC phenotype
Interstitial cells	CD117, vimentin, Desmin, Connexin-43, PDGFRβ	Not yet fully elucidated	Triangular or spindle-shaped with large nucleus and long cytoplasmic processes. $CD34^+$ population may have a stem cell/progenitor role in the bladder, intestine, and reproductive organs
Fibrocyte	CD45, CD80, CD86, MHC class I and II	Fibroblastic, myofibroblastic, adipogenic, osteogenic, chondrogenic	Small spindle shape. CD34 is lost in culture and upon maturation
Epithelial progenitors	CD49f, CD10, CD146, CD71, S100a4, Dkk3, CD133, CD117, ALDH, CD90	Dermal epithelial cells, neural mesenchymal	Predominantly described in HF niche in skin
Endothelial cells	CD146, VE-cadherin, CD133, CD117, CD14, CD31	Angiogenesis	Elongated with filopodia. Lack tight junctions. CD34 is present on luminal membrane processes and is expressed on filopodia during in vivo angiogenesis. Quiescent in vivo/low proliferation activity

Table 3: Different cell types of CD34-positive cells illustrated from (Sidney et al., 2014).

1.3.6.2 CD133

CD133 is the human homologue of mouse prominin-1. It is a five-transmembrane domain glycoprotein that is localized to cellular protrusions and is found in about 0.75% of peripheral blood derived cells (Gordon et al., 2003). It was first found on neuroepithelial stem cells in mice (Weigmann et al., 1997). CD133 has been used to identify normal and cancer stem cells in different tissues. Expression of CD133 has also been observed in adult stem cells such as undifferentiated epithelium. Furthermore, CD133 neurosphere cells have been isolated from brain and it has been shown that they can differentiate into both neurons and glial cells which suggested that CD133 could be used as specific marker for different stem and progenitor cells (Meregalli et al., 2010). Studies have shown that circulating CD133+ cells differentiated into myogenic cells and could be used for stem cell therapy for muscular dystrophy (Torrente et al., 2004). Furthermore, transplanted bone marrow-derived CD133+ cells improved the function of infarcted myocardium probably as a result of amelioration in blood vessel formation (Stamm et al., 2003). Expression of CD133 has also been shown in leukaemia (Vercauteren and Sutherland, 2001), neural (Uchida et al., 2000) brain tumour (Singh et al., 2004) and kidney cancer (Florek et al., 2005) cells.

1.3.6.3 VEGFR2 (KDR/Flk-1)

The VEGF receptor family consists of three members: VEGFR1 (FLT1), VEGFR2 (KDR, FLK1) and VEGFR3 (FLT4). VEGFR-2 is a type III receptor tyrosine kinase. VEGFR-2 appears to mediate almost all of the known cellular responses to VEGF such as proliferation, migration, survival and tubular morphogenesis and sprouting (Bautch, 2012, Ferrara et al., 2003). The key role of VEGFR-2 in developmental angiogenesis is well established by a lack of vasculogenesis and failure to organise blood vessels in VEGFR2-null mice, which resulted in death in utero between days 8.5 and 9.5 (Shalaby et al., 1995). VEGFR-2 has also been used as EPC marker in combination with other markers such as CD34 and CD133. VEGFR-2 is more commonly found in in vitro expanded late EPC cells that are more committed to the endothelial lineage. Furthermore, it has been shown that VEGF up-regulation has not effect on EPC proliferation, but significantly enhanced EPC migration (Smadja et al., 2007).

1.3.7 ESCs and iPSCs in vascular regeneration

Because of several disadvantages of adult stem cells (mentioned earlier), there was an increase to use other sources of stem cells such as ESCs or iPSCs. These cells can differentiate into ECs or their precursors in vitro. Such stem cell-derived vascular cells have also been used in experiments aiming to regenerate damaged vessels. For instance, different animal investigations have shown the participation and incorporation of ESC-derived ECs into damaged vasculature of ischemic limb and myocardium (Caspi et al., 2007, Laflamme et al., 2007). Tracking the fate and function of ESC-ECs in mice, showed the persistence of these cells up to 8 weeks after injection and improvement in systolic heart function (Huang et al., 2010). Preclinical trials of ESCs for vascular regeneration are at a very early stage and have not yet transitioned from bench to bed side. However, the FDA has now approved the first trial of human ESCs therapy for spinal cord injury, which is a dramatic step in stem cell-based therapies and the future of regenerative medicine. The advantage of ESCs is their pluripotentiality and great capacity for proliferation. However, many concerns remain surrounding ethical issues and immunological barriers. Moreover, these cells have high regenerative potentials, unintentional proliferation may result in undirected growth and increased risk of teratomas after transplantation (Blum and Benvenisty, 2008). Therefore, more investigations are required to show long-term safety of these cells so they can be used in human trial. Some of the limitations of ESC based regenerative medicine approaches can be overcome by using iPSCs. They can be produced patient specific, so do not have the immunologic barriers compared to ESCs. Furthermore, iPSCs are easily accessible from different sources such as skin, hair or blood. Furthermore, there are no ethical concerns surrounding these cells, as they can be derived from adult subjects (Hanna et al., 2007, Park et al., 2008).

Studies on iPSC cells have shown their potential to differentiate into all cardiovascular compartments, such as pericytes, smooth muscle cells, ECs and cardiomyocytes (Mauritz et al., 2008, Narazaki et al., 2008). It has been shown that iPSC-derived ECs are able to incorporate into damaged vasculature of ischemic tissue and improves function (Li et al., 2007, Yamahara et al., 2008). However, for the use iPSCs for vascular regeneration in human several concerns remain. Markers and reproducible protocol are required to differentiate iPSC into the vascular lineage. As with ESCs, it is crucial to efficiently exclude pluripotent cells to avoid teratoma formation. Regarding this, a recent study showed that injected allogenic iPSCs in a

murine model of myocardial ischemia could differentiate to cardiac, smooth muscle cells and ECs and no teratomas occurred (Nelson et al., 2009).

However, the main concern about using iPSCs, is the clinical development of these cells. Genetic manipulation of these cells (using retrovirus or lentivirus) results in the integration of viral DNA into the chromosome that can increase the risk of silencing indispensable genes or inducing ontogenesis (Okita et al., 2008). Although using adenoviruses or plasmids has reduced these risks in part, even episomal vectors carry risks of DNA integration (Sommer et al., 2009). Another concern in using iPSCs is their genetic and acquired abnormalities that could be transmitted from the patients' cells into their iPSC cells, which will reduce the regenerative capacity of these cells and might contribute to vascular inflammation (Leeper et al., 2010).

1.4 In vitro generation of ECs from stem cells

Optimizing the in vitro differentiation of vascular precursors from stem cells is key for future cell therapy approaches. Because of the limited expansion ability of blood derived EPCs, using ESCs or iPSCs as a source of generating sufficient cell numbers, might be more practical for therapeutic angiogenesis (Yamahara and Itoh, 2009). Therefore, many groups have started using ESCs/iPSC to differentiate them into vascular endothelial cells (Chaudhury et al., 2012). The Two most common techniques that are used to induce vascular endothelial cells from ESCs are: embryoid body (EB) formation (Levenberg et al., 2002) and co-culture on monolayers of OP9 cells (murine bone marrow stromal cells) (Vodyanik et al., 2005). In the EB formation technique, ESCs can differentiate spontaneously into all three germ layers and usually contain cellular networks that express vascular markers (such as VE-cadherin and CD31), consistent with primordial endothelial cells. These cells can be isolated by fluorescent activated cell sorting (FACS) techniques and then plated onto Fibronectin or other extracellular matrices that promote endothelial cell differentiation and proliferation (Levenberg et al., 2002).

It has also been shown that co-culture of ESCs on OP9 feeders can promote robust generation of vascular endothelial cells. It was shown that endothelial specific markers appear between day 10 and 14 of co-culture, followed by the up-regulation of hematopoietic markers by day 21, indicating sequential differentiation of endothelial and hematopoietic cells (Kelly and Hirschi, 2009). One of the main problems of the two techniques mentioned is the presence of undefined serum and other cell types (stromal cells) that can contribute to vascular

differentiation, which introduces complications for scaling up EC production (Li et al., 2011, Choi et al., 2009) (Rufaihah et al., 2011, Costa et al., 2013).

There are other studies that have used feeder free monolayer cultures to orderly differentiate human iPSCs to specific cell lineages such as hematopoietic cells. Manipulating cytokine combinations made it possible to commit the human stem cells to the specific lineages of functional blood cells with high reproducibility (Niwa et al., 2011). These days there are several studies being conducted to find more reliable protocols to culture ECs or their precursors (iPSC and ESCs) and to develop humanized larger scale culture systems to use these cells (Balaji et al., 2013). To show the full potential of iPS-derived EC precursors, it is important to first optimize the culture system and select specific markers for efficient expansion of these cells and then to study the functional characteristics and vasculogenic capacity of these cells *in vivo*. However, selecting suitable markers to isolate iPS-derived EC precursors and differentiating them into EC is still problematic. For instance, Rekha and colleagues (Samuel et al., 2013) have shown that CD34 as an EPC marker was in their hands not sufficient to select and expand vascular precursor populations. Instead a combination of three markers CD34, KDR and NRP1 was required to derive large numbers of endothelial cells that had the potential to generate functional blood vessels in vivo.

Recently two publications have shown the generation of ECs using a feeder-free culture system in in two dimensional (2D) environments (Prasain et al., 2014, Orlova et al., 2014). The protocol by (Prasain et al., 2014) does not require embryonic body formation, serum, or TGF-β inhibition. As is shown in **Figure 13** cells were first stimulated with Activin A, BMP4, bFGF and VEGF at day zero to stimulate mesoderm formation, which was followed by withdrawing Activin A from day two. Cells were harvested at day 12 by sorting NRP1+ CD31+ cells, which were able to give rise to stable endothelial precursor colonies (ECFCs). Sorted NRP1+ CD31+ cells showed 60% more endothelial colonies compared to NRP1- CD31+ cells. Generated ECFCs were proliferated and expanded for 4 weeks and 1 trillion ECFCs could be harvested at day 61 of differentiation **Figure 13.** This study also showed the isolated ECFCs capacity to generate vessels and repair vessels in the ischemic mouse retina and hind limb.

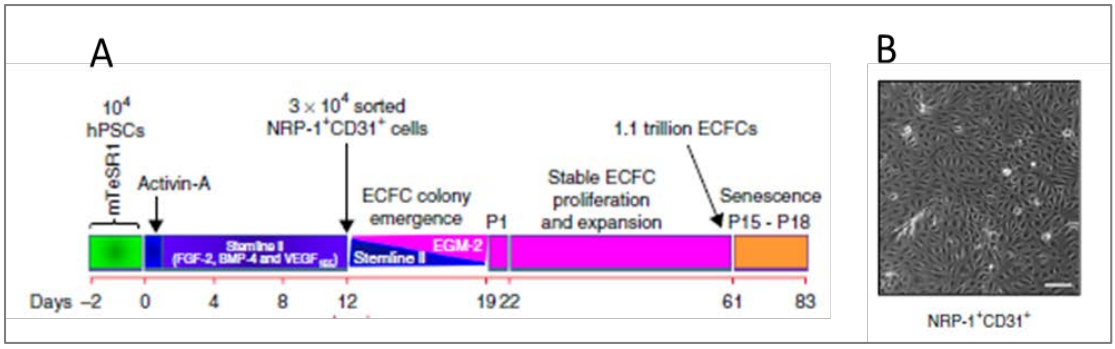


Figure 13: Schematic representation of simple one-step protocol from (Prasain et al., 2014). The 2D, serum endothelial lineage differentiation protocol (A) and representative of ECFC colony obtained from NPR1+CD31+ cells (B)

The second protocol by (Orlova et al., 2014) also used iPSCs line in feeder-free culture condition (mTeSR1 medium on Matrigel substrates). In this protocol simultaneous derivation of ECs and pericytes could be shown. The authors used Activin A, BMP4, CHIR and VEGF to stimulate the mesodermal lineage for 3 days and then VEGF and SB 431542 for 4 days and then harvested the cells at day 10 by selecting CD31+ cells **Figure 14**. Furthermore, the authors also demonstrated that the iPS-derived ECs exhibited an embryonic-like phenotype and showed great functionality in multiple in vitro assays and were able to integrate into host vasculature in zebrafish xenograft model.

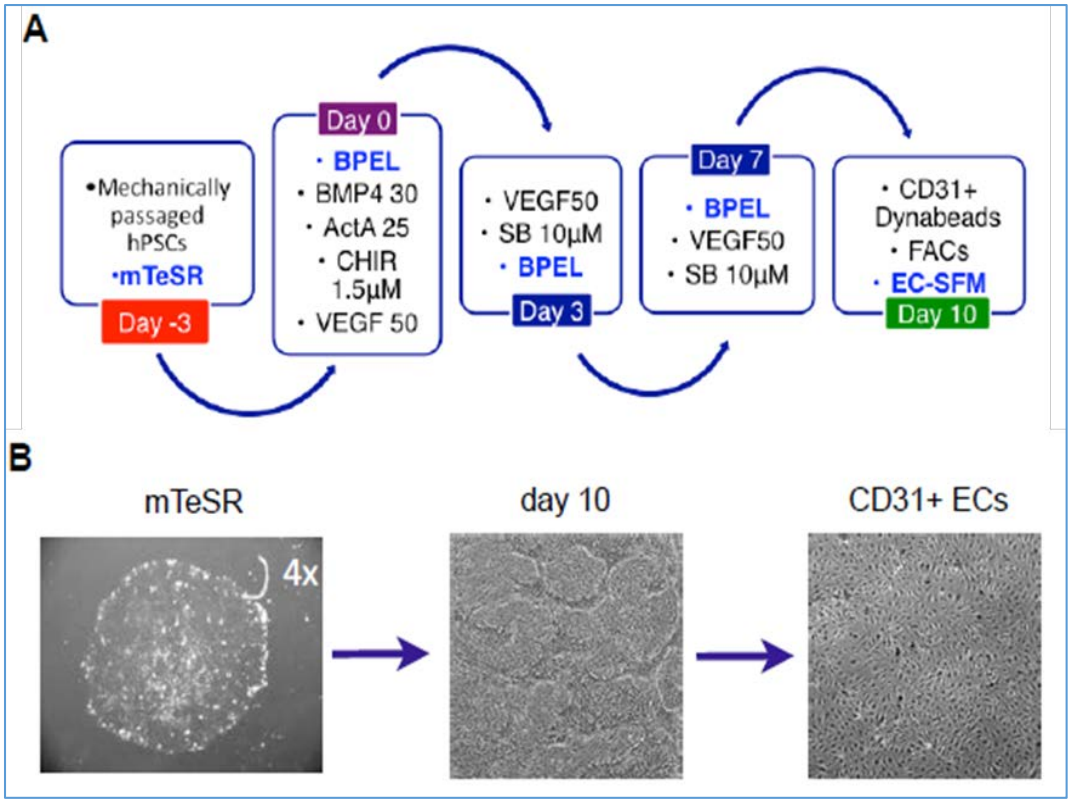


Figure 14: Schematic representation of workflow of EC differentiation in Christin Mummery's protocol (A).
Bright field images of undifferentiated hPSCs in mTeSR1, Endothelial islands at day 10 and isolated endothelial CD31+ cells (Orlova et al., 2014).

1.5 CD34 as a marker for broad range of cells and controversy about the identity of these cells

Progenitors of endothelial cells are known to be a promising stem cell source for vascular regeneration. They are typically derived from adult stem cells including peripheral blood (PB), umbilical cord blood (CB) and bone marrow (BM). However, the controversy over the origin, differentiation and cellular identity of these cells remains a potential issue. CD34 is the main marker of these cells. However, CD34 has also been shown in several other types of cells. CD34 was first known as hematopoietic stem cells marker, and then was established as a marker of several other non-hematopoitic cell types, including corneal keratocytes, intestinal cells, muscle satellite cells, epithelial progenitors and vascular endothelial progenitors (Fina et al., 1990, Blanpain et al., 2004, Nielsen and McNagny, 2008). Despite a high number of

studies for the clinical usefulness of CD34+ cells, it is still not completely understood what exactly these cells are and what are the exact role of this population outside of each individual specificity. On one hand, a small population of circulating cells in adult peripheral blood stream has been shown to generate CD34+ cells which can integrate themselves into damages endothelium and facilitate vascular regeneration. However, it is not clearly defined whether these “healing” properties are based on true EPCs or whether other bone marrow derived cells, such as macrophages, may play a more important role. On the other hand, during embryonic development, endothelial cells develop from a precursor’s population. These “true” embryonic progenitor cells also express CD34, which are likely to be very different from adult peripheral blood CD34+ cells. To date, it is not well understood what these differences are.

Furthermore, there are other studies showing that CD34 is also expressed in a subpopulation of HUVECs in vitro. The migratory CD34 expressing cells is shown to have similar gene expression profile as tip cells which are the specialised type of endothelial cells present in the leading edge of sprouting angiogenesis. (Siemerink et al., 2012). To date it is not well understood what the differences between all these CD34 populations are. Furthermore, a few markers are associated with these precursor cell populations. Therefore, comparative studies of different CD34+ cells populations based on their broad range of molecular characteristics may contribute to understanding the difference between all these populations and would offer an insight into overlapping properties of the cells that express CD34 and would help to define potential novel biomarker for them.

Thesis Aim

The aim of this project was to use gene expression profiling to better characterise the identity of circualting adult EPCs and human embryonic/iPS derived PECs and possibly find novel biomarker for these cells. CD34 was used as the main marker to isolate these cell populations from different sources. To this end, the following objectives were set;

- It was first aimed to establish an efficient protocol to generate iPSC-deived CD34+ cells in sufficient quantity in vitro (first chapter of this thesis). Adult CD34+ cells isolated from cord blood and peripheral blood, and CD14 (as a classic monocytes) were purchased in purified form (Stemcell Technologies).
- Furthermore, since it was shown that HUVECs have a subpopulation of CD34 expressing cells, despite the initial aim to use them as totally differentitated endothelial cells as a positive control, it was intended to explore the mechanisms that regulate the expression of CD34+ cells in HUVECs. Therefore, the second chapter of this thesis is dedicated to study the mechanism and signalling pathways that control the HUVECs phenotype. This could lead towards establishing a protocol to control CD34 expression in HUVECs and therefore be able to clearly separate two disticnt CD34+ / CD34- populations for further gene expresion profiling analysis.
- Finally, RNA was isolated from different cell types (CD34+ cell from iPS, peripheral blood, cord blood, VEGF-treated HUVECs and also CD34- cells from BMP9-treated HUVECs and CD14+ monocytes). In order to isolate the cells of sufficient purity, flow cytemetry was used. To find unique gene expression profiles, TruSeq illumina RNA sequencing platform was used to compare gene expression profile from all different types of cells. The data was then analysed for differences in gene expression and biochemical pathways and to identify potential markers.

2 Methods and Materials

2.1 Media and coating plates

2.1.1 MEF medium

500ml MEF media consist of 450ml Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen), 50ml FBS (Invitrogen) 5ml L-glutamine (Sigma) and 300ul gentamycin (Invitrogen).

2.1.2 Preparation of human ES medium

To make 500ml hES medium: 2.5ml of Glutamine (Sigma, G7513) were added to 2.5ml of 1X Dulbecco's Phosphate Buffered Saline (DPBS), without Ca & Mg (PAA laboratories). Then 3.5ul of 2-mercapthoethanole (Invitrogen, 31350-010) were added. These 5ml then added to 390mls of Knockout DMEM (Gibco-Invitrogen, 41965-039). In addition, 100ml of Knockout SR (Gibco-Invitrogen, 10828-028) serum replacement for ES cells, 5 ml MEM non-essential amino acid (NEAA) (Invitrogen, 11140-035), 300ul gentamicin (Gibco-Invitrogen, 15750-037) (aminoglycoside against gram-negative and gram-positive organism antibiotic) were also added. Then the medium were filtered in a 500ml 0.22µm, filtered unit (Corning Incorporated) to create a sterile solution. At the end bFGF (Peprotech, 13256-029) were added to the bottle to make the end concentration of 4ng/ml and 8ng/ml.

Final Concentration	Stock Concentration	Cat #
80% Knockout DMEM		Gibco 10829-018
20% GIBCO knockout SR		Gibco 10828-028
1% Non-essential amino acid solution	100X MEM non-essential amino acid solution	Gibco 11140-035
1mM L-glutamine	0.146g in 10ml PBS	Gibco 21051-016
0.1mM β-mercaptoethanol	14.3M β-mercaptoethanol	Sigma M-7154
4ng/ml human bFGF	2µg/ml in PBS (w/o Ca^{+}, Mg^{++}) with 0.1% BSA	Gibco 13256-029

Table 4: Summary of all concentrations of solution needed for hES medium.

2.1.3 EGM-2 medium

EGM-2 medium consist of EBM-2 Basal Medium 500 ml that is supplemented with EGM-2 BulletKit. Bullet Kit contains basal medium and SingleQuots* Kit: it does not contain BBE (Bovine Brain Extract). Kits includes hEGF, Hydrocortisone, GA-1000 (Gentamicin, Amphotericin-B), FBS (Fetal Bovine Serum) 10 ml, VEGF, hFGF-B, R3-IGF-1, Ascorbic Acid,Heparin. Final serum concentration is 2%. EGM-2 is optimized for the proliferation of large vessel endothelial cell.

2.1.4 mTeSR1 medium

mTeSR1 medium (STEMCELL Technologies) is a serum-free, defined formulation used for the feeder-free expansion and maintenance iPS and hES cells. mTeSR1 contains Basal medium and 5X supplement. Supplement contains recombinant human basic fibroblast growth factor (rh bFGF) and recombinant human transforming growth factor β (rh TGFβ). mTeSR1 should be used by either Matrigel or Vitronectin as a culture matrix.

2.1.5 Matrigel-coated plates

I used basement membrane Matrix growth factor-reduced Matrigel (354277, BD Bioscience) for the experiments. To prepare Matrigel aliquots, Matrigel were slowly thawed at 4°C to avoid the formation of gel. Then 10ml of cold Knockout DMEM were added to the bottle containing 10ml Matrigel. Mixture should be kept on ice and mix well with pipette and then it can be aliquot 1-2ml into pre-chilled tube and stored at -20°C. To prepare the Matrigel-coated plates, Matrigel aliquots should be thawed slowly at 4°C and then diluted 1:15 in cold Knockout DMEM (to make the final concentration of 1:30). For each 6 well plate, 1ml of Matrigel solution is needed to cover the plates. Incubation for 1-2 hours at room temperature and overnight incubation at 4°C is enough to make it ready to use. Before use, Matrigel should be removed immediately.

2.1.6 Fibronectin-coated plates

We used Fibronectin (Sigma, F1141) for the experiments. Fibronectin was kept at 4°C. Before coating, it was diluted it 1:20 with 1X PBS and incubated in room temperature for 30 minutes and just remove immediately before using.

2.1.7 Culture condition

Cells were cultured in Heraeus HERAcell 240 CO2 incubator (Thermo Scientific) at 37°C under 5% CO2 and 20% O2 (Normoxia) or 5% O2 (Hypoxia).

2.2 MEFs preparation

2.2.1 Derivation of mouse embryonic fibroblast (MEFs)

Female mice MF1 in E15 and E17 of pregnancy were used to isolate the fibroblasts. Abdomen was liberally covered with 70% ethanol. The skin and peritoneum were cut and uterine horns were removed and placed in a petri dish containing PBS. Embryos were removed from the embryonic sac and then placenta and membrane were dissected out and discarded. Embryos were decapitated and were washed three times with PBS. Carcasses were placed on cleaned petri dish and then minced with scalpel blade as much as possible. 5ml Trypsin/EDTA (Invitrogen, 15090-046) and DNAase were added and were incubated at 37°C for 30mintunes. Then 50ml DMEM/FCS were added to the petri dish and all were transferred to 50ml tube vigorously re-suspended. Large chunks were allowed to settle by gravity and then supernatant were transferred to one T175 flask and were put into the incubator at 37°C for 2 days. After two days they were passaged in 12 big T175. (Each 1:6)(Using 1X Trypsin/EDTA and then they can be passaged when they are 90% confluent.

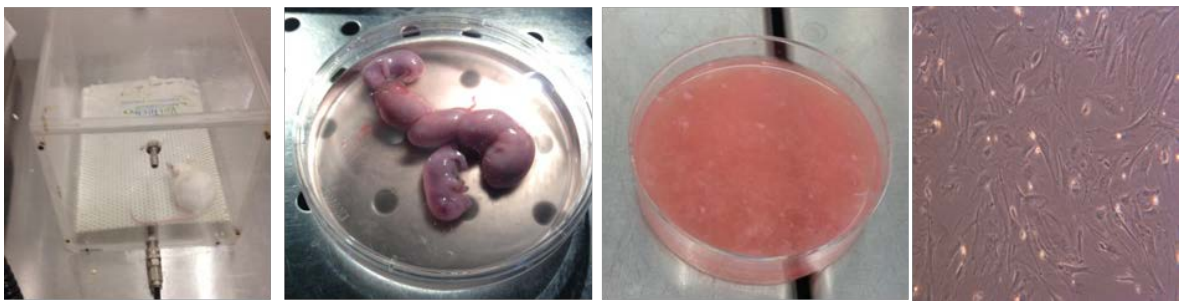


Figure 15: Different stages of MEFs cells preparation

2.2.2 Freezing down the MEFs

Cells were washed once with 1X PBS. Then, 10ml Trypsin/EDTA were added to each T75 flask and cells were incubated for 5 minutes at 37°C. Then, detached cells were re-suspended in 20 ml MEF medium and transferred to 50ml tube. Cells were then centrifuged at 1200 RCF for 5min at 20°C. Then, cells were re-suspended in 3ml freezing buffer. [90%FBS + 10%

Dimethyl Sulfoxide (DMSO)] and each 1ml were put into 1 Cryovial. Cryovials were transferred to a Nalge Nune Cryo 1°C (Mr Frosty) freezing container (Nalgene) that controls freezing at constant rate of -1°C/minute and allows to go down to -80°C overnight. The next day tubes were transferred to liquid nitrogen (LN2) for long time storage.

2.2.3 Defrosting MEFs

MEFs were kept frozen in 90% Fetal Calf Serum (FBS) (Invitrogen) and 10% DMSO (Sigma) buffer. 1 tube of MEFs was removed from liquid nitrogen and by slightly loosens the cap, trapped nitrogen could escape. By immersing the button of the tube in 37°C in water bath, I let the cells to defrost. The next step was to gently add the content of the cryovial to 15 ml tube containing 10ml MEF medium. Then cells were mixed very gently and spin down at 1200 RCF at 20°C for 5minutes. After spinning, supernatant was removed and cells were re-suspend in 10ml MEF medium, mixed properly and divided (2.5 ml) in 4 T75 flasks each contained 7.5 ml MEF medium and left in 37°C incubator for 2days.

2.2.4 Passage of the MEFs

First step was to aspirate the medium and washing the cells once with 10ml PBS. Then 3ml trypsin was added to each flask and cells were incubated for 5mintutes in 37°C. After 5 minutes, by tapping the flask I let the cells to dislodge. Then, 9ml of MEF medium was added and cells were mixed completely and transferred into 15ml tube. Then cells were spin down at 1200 RCF for 5min at 20°C. Supernatant were then removed and cells were re-suspended in 10ml of MEF medium. Cells were split 1:3 ratios in total of 10ml MEF medium in each T75 flask and placed in 37°C incubator. MEFs should be passaged 1 or 2 days before inactivation.

2.2.5 Inactivation of Mouse Embryonic Fibroblast (MEF) cells by using Mitomycin C

MEFs feeder layer was one of the feeders that were used in this project. Before inactivating the MEFs (stop cell division), cells were cultured in T75 flask and when they were confluent enough (about 70%), they were proceeded for inactivation. Medium was aspirated from 1 T75 tube and cells were washed once with PBS. Then 10-50 µg/ml pre-heated Mitomycine C from Streptomyces caespitosus (Sigma Aldrich, M4287) were added. Then cells were incubated in

37°C for 2-3 hours. At the same time enough number of flasks coated with 0.1% gelatin were prepared and incubated at room temperature for 3 hours.

After 2 to 3 hours, cells were washed three times with BPS. Then 2ml Trypsin/EDTA (Invitrogen, 15090-046) were added and cells were incubated for 3 minutes to generate cell suspension. Cell detachment was confirmed by examination under the microscope. Cell suspension were diluted with 8ml MEF medium and transferred into 15ml tube (BD Biosciences) and was centrifuged in 1200 RCF at 20°C for 5 minutes. Then cells were re-suspended in 10ml MEF medium and then counted with Haemocytometer. Approximately 200,000 to 300,000 cells were plated in each T25 flasks with 5ml of MEFs medium. MEFs could be used after 6-7 hrs up until 10days and MEFs cannot be used after passage 5.

Note: 10X Collagenase Type IV (Invitrogen, 17104-019) were made by adding 1mg/ml of collagenase in DMEM/F12 and sterilise with 0.2 micron filter and were allocated and frozen in 6ml tube in -20°C. 10X Collagenase should be diluted with DMEM/F12 and be used in 1X dilution.

2.3 hES preparation

2.3.1 Freezing down hES cells

Shef3 and Shef6 hES cell lines were provided by Sheffield University. Cells were grown on mitotically inactive mouse embryonic fibroblast feeder and hES medium (Sheffield university protocol) supplemented by 4ng/ml recombinant human basic fibroblast growth factor (bFGF) (Invitrogen). Freezing buffer consisting of 90% Knock Out Serum replacement and 10% Dimethyl Sulfoxide (DMSO) (Sigma-Aldrich) was used to freeze down the hES cells. Medium were aspirated from flask and washed once with PBS. After that 3ml 1x Collagenase IV were added to each T25 flask and cells were incubated for 6 minutes till the edges of the colonies start to curl. Then 7ml of hES medium were added and cells scraped gently by scraper. In this stage, cells were spin down at 50g for 3min at 20°C. Then supernatant was aspirated and cells were re-suspended in 1ml freezing medium. Cryovials should be kept on ice. Cells are transferred to cryovial. (Each T25 flask goes to 1tube). Cryovials are transferred to a Nalge Nune Cryo 1°C (Mr Frosty) freezing container (Nalgene) that controls freezing at constant rate of -1°C/minute and allows to go down to -80°C overnight. The next day tubes transferred to liquid nitrogen (LN2) for long time storage.

2.3.2 Defrosting hES cells:

Vial was removed from liquid nitrogen and slightly loosens the cap to allow trapped nitrogen to escape. By immersing the button of the cap in 37°C in water bath for 1min I let the cells to defrost. During the thawing process DMSO should be removed properly as it is toxic for the cells, therefore, thawing process should be very quick and precise. Two ways were used to plate the cells; First, cells were added very gently (drop by drop) to 24ml hES medium containing Y276332, mixed properly and transferred to a T25 flask of inactivated MEFs. Cells were incubated in 37°C for 24 hours and the day after medium was refreshed with 8ml fresh medium every day. Differentiated colonies cells should be removed every few days to keep the cells in more undifferentiated state. In the second processing way, cells were added very gently (drop by drop) to 10ml hES medium and then cells were spin down in 50g for 3 minutes at 20°C to remove DMSO properly. Supernatant was aspirated and cells were re-suspended in 10ml fresh hES medium. Cells were put in the incubation for 48 hours. Then, medium should be changed every day and the levels of bFGF should be contained high as it prevents spontaneous differentiation on the stem cell colonies.

2.3.3 Passage of human ES cells

Routine culture maintenance of hES cells was performed by Sheffield University protocol. When hES cells became very confluent they could be split on pre-prepared inactivated MEFs. The medium was aspirated from T25 flask. Cells were washed once with PBS. Then 3ml of collagenase IV was added per T25 and was incubated for 6 minutes in 37°C until the edges of the colonies start to curl. Then cells were washed once with PBS. Consequently, 6ml of hES medium was added and then scraper was used to gently scrape the colonies from the bottom of the flask. Then, MEFs medium was aspirated from T25 flasks and 4ml of hES medium was added to each flask. Then, cells were split 1:3 ratio and cells were returned in to the incubator. Medium was refreshed every day.

2.4 iPSCs preparation

2.4.1 Freezing down the iPSC cells

Cells scraped gently by scraper and then spin down at 50g for 3min at 20 °C. Then supernatant were aspirated and cells were re-suspended in 1ml of freezing medium Bambanker (Anachem, BB03). Cryovials should be kept on ice. Cells are transferred to cryovial. (Each T25 flask goes to 1tube). Cryovials are transferred to a Nalge Nune Cryo 1°C (Mr Frosty) freezing container (Nalgene) that controls freezing at constant rate of -1°C/minute and allows to go down to -80°C overnight. The next day tubes transferred to liquid nitrogen (LN2) for long time storage.

2.4.2 Defrosting iPSC cells,

Vial was removed from liquid nitrogen and slightly loosens the cap to allow trapped nitrogen to escape. By immersing the button of the cap in 37°C in water bath for 1min we let the cells to defrost. During the thawing process DMSO should be removed properly as it is toxic for the cells, therefore, thawing process should be very quick and precise.

Cells were added very gently (drop by drop) to 10ml mTeSR1 (05850, STEMCELL Technologies) and then spin down in 50g for 3 min at 20°C to remove DMSO properly. Then supernatant was aspirated and cells were re-suspended in 10ml fresh mTeSR1 medium in combination with Y27632 Rock inhibitor (688000-1MG, Calbiochem). Then cells were put into the incubation for 24hours. Medium should be changed every day to prevent spontaneous differentiation.

2.4.3 Passaging iPSC cells

iPSC cell (BJ iPSc) were provided by Dr Amanda Carr and Dr Lili Chen at UCL institute of ophthalmology. Cells were grown on Matrigel coated plates ((hES-qualified, 354277, BD Bioscience) in mTeSR1 medium (05850, STEMCELL Technologies). Passage number for these cells was between 10 to 25. Routine culture maintenance for BJ iPSC was prepared when cells were over 80% confluent. The medium was aspirated from T25 flasks. 3ml of Cell Dissociation Reagent (07174, Stem cell technologies) was added and left for 2 to 4 minutes in room temperature. Cells were checked every minute under the microscope until the edge of the colonies start to curl. Then Reagent was aspirated and 6ml mTeSR1 medium was added into the flask. Then scraper was used to gently scrape the colonies from the bottom of the flask.

Matrigel was aspirated after 1hr coating in 37°C from flasks. Cells were split 1:3 ratios in total of 6ml mTeSR1 medium. Then flasks were return into the incubator. Media needs to be changed every day with 6ml fresh mTeSR1 medium.

2.5 Differentiation protocol to generate iPS-derived CD34+ cells

iPSC cells were routinely mainlined in Matrigel coated plates (1:30 final ratio). Confluent iPSC were then detached by gentle enzymatic treatment for 7 minutes at RT with 1X TrypLE (Life technology, 12604-013). Then 1X TrypLE containing cells were diluted with mTeSR1 medium and centrifuged at 800g, for 3mintues at 25°C. Then supernatant was removed and cells were re-suspended in 5ml fresh mTeSR1 medium. At this stage Rock inhibitor (Y276332 (Calbiochem, 688000-1MG) were added when the cells were single suspended to prevent them from going apoptosis. Y-276332 is a cell-permeable, highly potent and selective inhibitor of Rho-associated protein kinase which enhance the survival of stem cells when they are dissociated to single cells (Ungrin et al., 2008). Then cells were counted with haemocytometer and seeded onto Matrigel coated 96 plates at 4x104 cells/well. Cells were then left in incubator to settle for 48 hrs. Differentiation was induced two days after passaging by replacing mTeSR1 medium with differentiation media (DF) (DMEM/F12 + B2 + N27) and timed addition of the following growth factors: 25ng/ml Activin A (Peprotech, 120-14), 30ng/ml (BMP)4 (Peprotech, AF-120-05) and BIO (TOCRIS, 3194) with extra Matrigel 1:80 final ratio). This medium was refreshed at day 1 with the same factors. Then factors were replaced to VEGF-165 (Peprotech, 100-20) and SB 431542 (TOCRIS, 1614) at day 3 up to day 5. Then cells were fixed at day 5 and immunocytochemistry for CD34 was used to identify the cells of interest.

To optimise differentiation protocol to generate iPS-derived CD34+ cells, in each set of experiments different conditions (including different factor composition and concentrations) were investigated (each in triplicate and inducted with two independent people). Then data were analysed by immunostaining method (explained in more details below). Outcome from the first set of experiments were used to change the design (factor compositions and concentrations) for the next set of experiment which again were inducted with two independent people and on triplicate. Conditions with more cell death and no CD34 expression were excluded. Better conditions were selected to repeat in the next experiments in addition to new conditions. In total eight sets of experiments could clearly establish a reliable protocol to generate iPS-derived CD34+ cells. In the first experiment, it was intended to define the optimum medium for differentiation which was conducted with specific factor composition.

Six different media were tested. Cells were treated with different combinations of factors in these six-different media in 96 well plates (all details about media are explained in section 4.1. DMEM/F12+N2+B27 medium which was called Differentiated Medium (DF) a defined serum-free medium was chosen as the most suitable medium and used for the reminder of the experiments.

Moreover, key parameters such as factors and substrates were also modified in further continuous experiments to optimise the best conditions in which to grow PECs. Over the first, second and third experiment, the length of the experiment (reduced to 6 days) and concentration of BIO and SB 431542 were reduced and optimised to 0.15uM and 2uM respectively. The concentrations for Activin A 25ng/ml, VEGF 50ng/ml and bFGF 25ng/ml were not changed over continuous optimisation procedure. However, because not enough CD34 enrichment was observed, an attempt was made to optimise the protocols by modifying different parameters such as substrate and different combinations of factors in further experiments. Through the optimisation process, it was shown that 3D environment by adding extra Matrigel on top pf the media could remarkably increase the number of CD34 positive cells. In further experiments, best factor composition and concentration was optimised by excluding or including of different conditions.

2.6 HUVECs preparation

2.6.1 Isolating Human Umbilical Vein Endothelial cells (HUVECs) from umbilical cords

In these experiments, HUVECs were used from different sources; (1) HUVECs pooled from multiple donors (Lonza) or (2) it was isolated from umbilical veins by collagenase digestion according to a published protocol (van der Schaft et al., 2000). Isolation of HUVEC was possessed as follow: First a T25 flask was coated with 0.1% gelatin and incubated for half an hour in an incubator or in a hood at room temperature for 1 hour. Then, cord was taken from 1× PBS and kept in a petri dish and cleaned with ethanol. By using razor blade, two ends were cut of to clearly distinguish the big vein from arteries. Blood flow was cleaned by taping the ends to the ethanol-sprayed tissue. The vein should be washed inside with 1× PBS. I used BD venflon (391453) by firmly pushing it into the veins in both the sides after taking out the needles and checked for punctures. Then a tie rips in both the ends was used (shown in the figure) to fix the venflon firmly to the vein. Then the lid of the venflon was opened from both

the sides. By using a syringe, 1× PBS was flushed into the vein from one end. 1× PBS moved gently through the vein and came out through the other end. This washing step should be repeated twice. Then a mixture of 1× collagenase II and 1× collagenase IV (17104-019, Invitrogen) was injected into the vein from one side, and the other side was blocked using a clip. In this stage the vein was massaged by fingers for 1 minute and then the whole cord was left in a 50-ml tube at room temperature for 40 minutes instead of 20 minutes (**Figure 16**). After 40 minutes, it was needed to flush out everything with endothelial cell growth media-2 (EGM-2) twice and collect the cells in the tube and then centrifuged the cells for 5 minutes in 12,000 RCF. After aspirating the supernatant, the pellet was re-suspended in EGM-2. Finally, cells were placed in a T25 flask coated with 0.1% gelatin and were left at 37°C in an incubator for 2 days. Growing human umbilical vein endothelial cells (HUVECs) generated a confluent monolayer in 4 days.

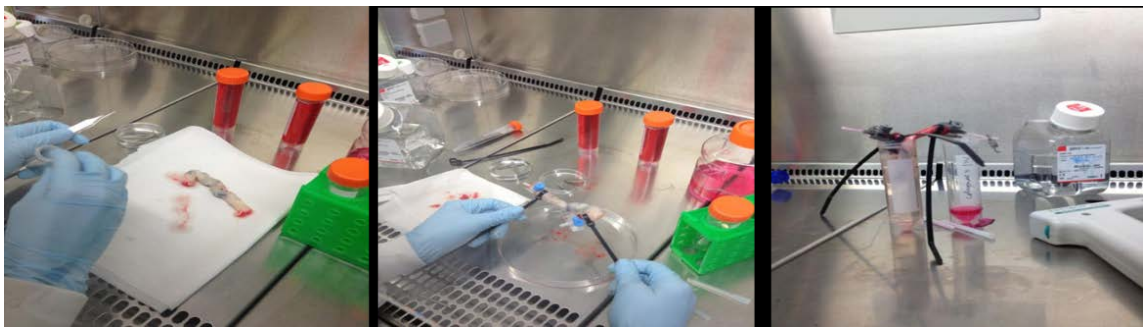


Figure 16: Process of isolating human umbilical vein endothelial cells (HUVECs).

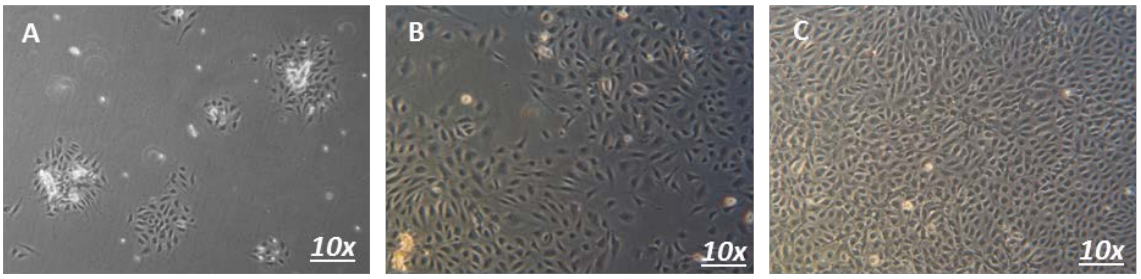


Figure 17: Light microscopy images of the growing HUVECs.
HUVECs after isolation and plating on gelatin coated T25 flask; after 1 day (A), 4 days (B) and 6 days (C).

2.6.2 HUVECs Culture

HUVECs were routinely grown on EGM-2 (Lonza) on plates coated with 0.1% gelatine at 37°C in 5% CO_2. The medium was changed every other day, and HUVECs were used up to passage 8. For assessing the effect of different factors, HUVECs were plated in 24 well plates in the EGM-2 medium. After reaching 70% confluency, the cells were switched to the LVEM+ medium for 24 hours. HUVECs were then starved overnight in large vessel endothelial media (LVEM) (2253, Cellworks). This medium contains sterile HEPES and bicarbonate, and the buffered medium contains essential and non-essential amino acids and vitamins, but no proteins or hormones. The supplement contains PBS heparin, FGF-2, EGF and hydrocortisone. Therefore, this medium does not have FBS and was used as a medium in different stimulation experiments to assess the effect of different growth factors. Cells were treated with different factors mentioned in **Table 5** for specific time courses according to the experimental plan. Then the cells were fixed with 4% paraformaldehyde and were preceded for staining. In the case of RNA extraction, Trizol was added to each well, and the cells were preceded for RNA extraction.

Factors	Concentrations
VEGF	25ng/ml
Recombinant Human GDF2 (BMP9)	20ng/ml
Γ-Secretase inhibitor (DAPT)	2/10 uM
Recombinant Human TGF- β1	2ng/ml
Alk1-Inhibior	20uM

Table 5: Different factors and combinations used in HUVECs experiments.

Results were analysed by ImageJ. To quantify the number of CD34+ cells in all experiments and compare them between different conditions, five pictures were taken from each stained coverslip. Each picture was score blinded using the following scale: 1, very faint CD34 staining; 2, clear expression of CD34; 3, very strong expression of CD34. Scores were summed up for each picture and the average score was determined for each slide. Then, this number was

divided with the total cell number (nuclei were stained using Hoechst 33342). The average of the five images was considered as one experiment. All experiments were repeated four to six times in different sets of experiments.

2.7 Migration assay

Confluent monolayer of HUVECs plated on 0.1% gelatine coated 24 well plates stimulated with different factors were scratched with 1,000ul pipette tip. Images were collected at 10X at time zero and 8hrs. The scratched area was quantified using ImageJ (NIH) and the area covered by migration of the cells was calculated by subtracting the area at t= 8 h from the area at t=0.

2.8 BrdU

2.8.1 Labelling in vitro

Culture cells were labelled in S phase by adding 10uM BrdU (diluted in LVEM) into each well and left in incubator 37°C for 2 hours. Cells were then washed with 1X PBS and fixed with 4% (w/v) paraformaldehyde for 10 minutes before performing immunocytochemistry.

2.8.2 BrdU staining in combination with CD34 staining

For CD34 staining, cells on coverslips were blocked and permeabilised in blocking buffer (1% BSA, 0.1% Triton, 0.01% tween 20/PBS.) for 30min. after that, conjugated primary antibodies (CD34-FITC- human, Miltenyi Biotec Ltd) were diluted 1/100 into blocking buffer and 50ul was added per coverslips. Primary anti-body were applied to the cells for 1hour at room temperature. Then cells were washed three times with PBT (1X PBS PH 7.0, 0.001% Tritonx-100) for 5min. secondary antibody IgG (Alexa Fluor 488 Goat anti-mouse, Invitrogen) were diluted 1/200 with blocking buffer and then 50ul added to each coverslip. The incubation time for secondary staining is about 45min to 1hr. after this time; coverslips were washed with PBS for 10min.

Cells were then post fixed with 4% paraformaldehyde for 15 minutes to fix the antibodies from the previous step. After that, cells were incubated for 15 min in 70% ethanol in room temperature and then were permeabilised in 1% triton-X-100 in PBS for 15min. Next step was acid wash: cells were incubated in 6M HCL/1 %(v/v) Triton-X-100 in PBS for 20-30 min. Then cells were washed three times with 1X PBS for 10, 30 and 15 minutes so then pH of the PBS was measured by pH test strips to check they are back to neutral level pH=7. Then again

blocking buffer was applied for 20 minutes and after that cells were incubated overnight at 4°C in anti-BrdU (hybridoma supernatant BU209; Magaud et al., 1989), diluted in 1:4 in 0.1% (v/v) Triton X-100 in PBS. Cell were washed in 1X PBS and then incubated in goat-anti mouse IgG (Pierce; 1:100 in 0.1% Triton X-100 in PBS) for 30minutes. After the final wash with 1X PBS for 10 minutes, coverslips were mounted onto microscope slides in anti-fade reagent (Citifluor) mowiol and sealed around the edges using nail vanish. For each condition, at least 6 coverslips were prepared and from each coverslips, 5 random pictures were taken and BrdU labelling index was calculated at the proportion of the cells that were expressing BrdU by considering the whole number of cells in the coverslip.

2.9 Immunocytochemistry

Medium was aspirated and cells were washed once with 1X PBS. Cells were then fixed with 4% paraformaldehyde (PFA) for 10-15 minutes in room temperature. Then cells were washed two times with 1X PBS and then incubated with blocking buffer (blocking buffer: 1% BSA, 0.1% Triton, 0.01% tween 20/PBS.) for 30min. after that, primary antibodies were diluted into blocking buffer and were added per well plates. Primary anti-bodies were incubated for 1hour at room temperature. Then cells were washed three times with 1X PBS (5 minutes each) to ensured that all excess unbound antibodies were washed off. Then secondary antibodies IgG (Alexa Fluor 488, 594 Goat anti-mouse, Invitrogen), (Alexa Fluor 488, Donkey anti-mouse, Invitrogen) and (Alexa Fluor, Donkey anti-goat 594 Invitrogen) all were diluted 1/200 in blocking buffer and were added to the wells. Incubation time for secondary antibodies was 1 hr. After incubation time, cells were washed once with 1X PBS for 5 minutes to get rid of any unbound secondary antibody. Then 1ug/ml dilution of Hoechst was added for nuclei staining only for 30 seconds. Then cells were washed for another 5 minutes with PBS and then were examined under the florescent microscope.

Antibody	Immunoglobulin Subtype	Fluorochrome	Source
CD34	Human IgG1	FITC-conjugated	Miltenyi Biotec Ltd.
CD34	Human IgG1	PE	Miltenyi Biotec Ltd.
VE-Cadherin	Human IgG2B	Allophycocyanin	SantaCruz Biotechnology
PECAM-1 (CD31)	IgG2a		BD Biosciences
CD133	Human IgG1	PE	Miltenyi Biotec Ltd.
CD309	Human IgG1	APC	Miltenyi Biotec Ltd.
CD31	Human IgG1	FITC	Miltenyi Biotec Ltd.

Table 6: List of the antibodies used for immune-staining, MACS and FACS.

Antibody	Immunoglobulin Subtype	Host and clonality	Source
Serpine E2/PN1	Human IgG1	Mouse monoclonal	Novus
Glu3	Human	Rabbit polyclonal	Novus
EGFL7 (VE-Statin)	Human	Rabbit polyclonal	Novus
GNG11	Human	Rabbit polyclonal	Novus
Integrin beta1 (CD29)	Human, Mouse, Rat IgG	Rabbit monoclonal	Novus

Table 7: List of the antibodies use for immunostainings

2.10 Microscopy

2.10.1 Light microscopy

All staining were examined and photographed using photomicroscope (Zeicc Axiophot) attached to a CCD camera (ORCA-ER (Hamatsu). For each staining, different pictures (at least 4 pictures from each plate) were taken with different magnification in an attempt to represent most faithfully. The magnification is shown in each picture.

2.10.2 Fluorescence microscope

Up right Axioscope and inverted S100 fluorescence microscopies (Carl Zeiss) were used to analysis the immune-staining results.

2.11 Magnetic Cell Sorting (MACS)

The MACS CD34 MicroBead Kit (Miltenyi Biotec) was used to purify the population of CD34 expressing cells. For this experiment cells were kept at law temperatures 4C (on ice). MACS buffer made up of phosphate-buffered saline (PBS), PH 7.2, 0, 5% bovine serum albumin (BSA) and 2mM EDTA. Buffer was degassed by centrifugation at 3000g for 3 minutes as air bubbles could block the column. Solutions were pre-cooled to avert the capping of the antibodies on the cell surface and non-specific cell labelling. Cells were washed three times with PBS. Single cell suspension was derived by adding 5ml TrypLE to each T25 flask and incubated for 10-15 minutes. After incubation, cells were diluted with 5ml of EGM-2 medium and then were filtered by Pre-separation filters, 30 µm (Miltenyi Biotec) to prevent cell clumps from clogging the magnetic column. Then cells were centrifuge at 1200 RCF in 20°C degree for 5min. after spinning, cells were re-suspended in 300ul MACS buffer and 10uM of Y-27632 were added to keep the cells alive as most of them are single in this stage.

In this stage cell were counted. If the cells were less than 10^7 cells, just 100ul of FCR was enough. Then 100ul MicroBeads conjugated to monoclonal mouse anti-human CD34 antibodies (isotype: mouse IgG1) (Miltenyi Biotec Ltd, 130-081-001), was added, completely mixed and were incubated in 4°C for 30 minutes. Then cells were transferred to 10ml MACS buffer and centrifuged in 1200 RCF for 10 minutes. After spinning, and aspirating the supernatant, cells were re-suspended in 500ul of MACS buffer and 10uM of Y-27632 and preceded to magnetic separation. For this stage magnetic separation with MS columns:

(Miltenyi Biotec) was used. It was important to pre-moisten the strainer with buffer to minimize the cell loss.

Column should be placed in the extremely strong magnetic field (MiniMACS separator: Miltenyi Biotec) mounted on the MACS MultiStand (Miltenyi Biotec). Column was rinsed by 500ul of MACS buffer. Then, cell sample conjugated with CD34 MicroBeads was loaded to the reservoir of the column. The unlabelled cells flow-through the column collected in a 15ml centrifuge tube. The column was washed 3 times with 500ul MACS buffer. Finally, The MS magnetic column was removed from the magnetic field, 500ul of MACS buffer was loaded in the column reservoir and magnetically labelled cells were flushed out the column by pushing the plunger steadfastly into the reservoir of the column. Magnetically labelled cells were collected in 1.7ml graduated micro tube.

Then both labelled and un-labelled cells were centrifuged at 0.8 to 1 rpm for 5 minutes and then re-suspended into appropriate medium (EGM-2 or hES media, DF) and plated into different surfaces such as Fibronectin, Matrigel, inactivated MEFs or ECCM. Cell were plated either 1 to 4 days according to the experimental plan.

2.12Fluorescence-activated cell sorting (FACS)

A FACSCalibur (Becton-Dickinson Biosciences) flow cytometer was used for data acquirement. This mentioned cytometer is equipped with an organ ion laser emitting at 636nm and 488nm red laser and can detect SSC, FSC and up to 4 fluorescence channels at the same time. Experiments were prepared with analysis of 10^6 cells per run. FlowJo software was used to analyse the acquired data. Traditional gating strategies were used to generate density plots and histograms. MoFlo XDP (Beckman Coulter) was used to sort different unfixed samples of hES or iPSC cells. Cell samples derived from human embryonic stem cells or iPSC cells were labelled with fluorescence conjugated antibodies.

FACS buffer is composed of PBS (pH 7.2), 1% BSA and 2mM EDTA. The whole process was done on ice. A single cell suspension derived by adding 5ml TryPle (1x) (Invitrogen) and incubation at 37°C for 10 minutes. Then 5ml of suitable medium (EGM-2, mTeSR1) was added to the flask and then cells were counted. In this step, cells were transferred to 15ml tube and were centrifuged at 1200 for 5mintes and 20°C. Pellet was re-suspended in 90ul FACS buffer plus 10ul of FcR-blocking reagent (Miltenyi Biotec) and incubated at 4°C for 10min. (adding the amount of FcR-blocking reagent depends on number of cells, if the cell number is less than

10^7 10ul of FcR-blocking reagent (Miltenyi Biotec) is added). To sort CD34+ cells, three tubes were needed: same amount of cells were added to each tube. The first tube is the control sample with no staining and contained just $1x10^6$ cells and 100ul of FACS buffer. The second tube was for Isotype control-FITC (IgG2a) (90ul FACS buffer +10ul of IgG2a) and the last tube was for CD34+-FITC (90ul of FACS buffer+ 10ul of CD34-FITC) followed by 10min incubation at 4°C. After that, cells were centrifuged in 1200 RCF for 5min and then re-suspended in 200ul of FACS buffer. Then cells were transferred on ice to sort on FACS machine. Cells were either sorted directly into the Trizol or suitable medium for RNA extraction or plating respectively.

2.13Molecular Analysis

2.13.1 Extraction of RNA for CD34+ and CD34- cells

During FACS, Cells were directly sorted into 1ml of Trizol (Sigma-Aldrich) and then were transferred into 1.5ml Eppendorfs and then were rotated in shaker for 10 minutes in room temperature to allow lysis to start. Then 200ul Chloroform (VWR, UK) was added to each Eppendorf, mixed completely and were left at room temperature for 10 minutes. After that, tubes were centrifuged at 12000 RCF for 15min at 4°C. The top aqueous phase including RNA was removed and placed into the new tube and organic layer including proteins and phenol (pink) were discarded. 1ul of RNase-free glycogen (Ambion, UK) and 500ul of Isopropanol (VWR, UK) were added to precipitate the RNA. Tubes were inverted to mix and were incubated at room temperature for 10minutes and then were centrifuged at 12000 RCF at 4°C for 10min. After centrifuge, small line pellet appears. The supernatant were poured off and 1ml of 70% Ethanol was added and completely mixed with vortex and centrifuged at 4°C at 7400 RCF for 5min. after the last centrifuge, supernatant was pour off and the pellet was left to air-dry (about 20 to 30min). Then pellet was re-suspended in 21ul of RNAssecure (Ambion, UK) and were heated to 50°C for 10 min.

2.13.2RNA quantification- Nanodrop

To evaluate the purity and the integrity of the mRNA produced the samples were tested on a NanoDrop spectrophotometer ND-1000 and NanoDrop 1000 v3.7.1 software (LbTech International, An A260/A280 ratio of 1.9-2.1 was considered as pure RNA. UK). RNA was kept in -80 degree for RNA quality and was sent for RNA-sequencing later. For qPCR analysis, mRNA was treated for cDNA synthesis immediately after extraction.

2.13.3 cDNA preparation (RT reaction)

cDNA was generated using QuantiTect reverse transcription kit (Qiagen, UK) reverse. This kit comprises: 100 µl 7x gDNA Wipeout Buffer, 10 µl Quantiscript Reverse Transcriptase, 200 µl 5x Quantiscript RT Buffer, 50 µl RT Primer Mix, 1.9 ml RNase-Free Water. Samples were kept on ice during the whole process of making cDNA. First step was to eliminate genomic DNA which was done by adding 2ul of gDNA without buffer (7X) (Qiagen, UK), 1000ng template RNA and the rest was RNase-free water to reach the final volume of 14ul. Reaction starts at 4C for 5 Minutes. Then 1ul of Quantiscript reverse transcriptase, 4ul Quantriscript RT buffer (5X) and 1ul RT primer mix (primers and dNTPs) (Qiagen, UK) were added to the reactions to reach the total volume of 20ul. Then reaction was incubated at 42 C for 15minutues at 95 C for 3 minutes. The resulting cDNA were then stored at -20°C for further Quantitative real time polymerase chain reaction (qPCR).

2.13.4 Primer design

A list of the primers used during the course of this study is explained in **Table 8**. All Primers were designed using PubMed. Primers were reconstituted from powder to concentrated stock solutions by adding DEPC-treated water. To make 100uM of stock solution, 5ul of primers (Reverse and forward) of target genes were diluted with 190ul of DEPC H2O. Primers were tested for their functionality.

Primers	Length	Tm	%GC	Sequence
HPRT-F	24	69.1	50	TTGAGCACACAGAGGGCTACAATG
HPRT-R	24	69.2	50	ATGGACAGGACTGAACGTCTTGCT
HEY1-F	21	71.6	48	GCTGCTACCCCAGCCAGTGTC
HEY1-R	21	71.5	52	TTCTCCAGCTTGGAATGCCGC
Jagged-1-F	24	67.3	50	AGTGGTCTTTCAGGTGTGAGCAGT
Jagged-1-R	24	71.4	50	TTGTGAGCCTAATCCCTGCCAGAA
Tie2-F	24	63.8	41.6	GACTTTGAAGCCTTAATGAACCAG
Tie-2-R	20	63.5	55	CGTATCCTGATTGCCTCTCC
SMAD7-F	22	59.55	40.9	CCACACTTCAAACTACTTTGCT
SMAD7-R	21	59.47	42.8	AAACAGAACACAAACGAGGAC
Apelin-F	20	72.7	65	GAATCTGCGGCTCTGCGTGC
Apelin-R	20	76.3	65	TCGGGAAGCGGCATCAGGGA
DLL4-F	24	67.1	50	ACTGGGAGAAGAAAGTGGACAGGT
DLL4-R	24	68.6	50	AGTTCACAGTAGGTGCCCGTGAAT
KLF4-F	21	65.0	47.6	CATTACCAAGAGCTCATGCCA
KLF4-R	23	62.7	47.8	GAGATGGGAACTCTTTGTGTAGG
Nanog-F	21	63.5	47.6	AACTGTGTTCTCTTCCACCCA
Nanog-R	22	62.6	50	GGTCTTCACCTGTTTGTAGCTG
Oct4-F	19	64.7	52.6	**CTTCGCAAGCCCTCATTTC**
Oct4-R	19	63.7	66.6	GAGAAGGCGAAATCCGAAG
LYNE-1-F	23	62.5	30.4	TGAAAATAAAGCAGCATTCAAGA
LYNE-1-R	21	63.3	57.1	GGAGAGCAAGCACTAGCAGAG
VE-Cadherin-F	20	63_8	50	AAGCCTCTGATTGGCACAGT

VE-Cadherin-R	18	64.0	61.1	CTGGCCCTTGTCACTGGT
CD34-F	21	63.4	47.6	GTGAAATTGACTCAGGGCATC
CD34-R	21	62.7	52.3	CCCCTGTCCTTCTTAAACTCC
VEGFR2-F	22	63.4	45.4	GGTTGCATTACTGTACCCATCA
VEGFR2-R	20	64.4	50	TTTTAGGTGTCGGCCACTGT
Tie2-F	24	63.8	41.6	GACTTTGAAG CTTAATGAACCAG
Tie2-R	20	63.5	55	CGTATCCTGATTGCCTCTCC
CD133-F	24	62.6	37.5	**TCCACAGAAATTTACCTACATTG G**
CD133-R	20	64.6	55	CAGCAGAGAGCAGATGACCA

Table 8: List of the all Primers used for qPCR analysis

2.13.5 Quantitative Real-time PCR (real-time)

Real time PCR, allowed us to detect and quantify the nucleic acid sequences using ABI PRISM ©7900 HT instrument (Applied Biosystems). CXR (Carboxy-X-rhodamine) dye is a fluorescent molecule that binds all double-stranded DNA but has a very weak florescence signal in the absence of double strand DNA and Florescent signal is monitored at the end of each PCR cycle and can be plotted as an amplification graph **Figure 18.** The increase in fluorescence intensity correlates with amplification of the existing cDNA during the reaction. Baseline is identified as PCR cycles but is under the limits of detection of the instrument. Threshold: the signals that are identified above the threshold are considered a real signal which could be used to detect the threshold cycle (Ct) for a sample. Ct: is described as the fractional PCR cycle number in which the reporter fluorescence is greater than the threshold. Ct is the main principle of the qRT-PCR which is necessary for producing accurate and reproducible data.

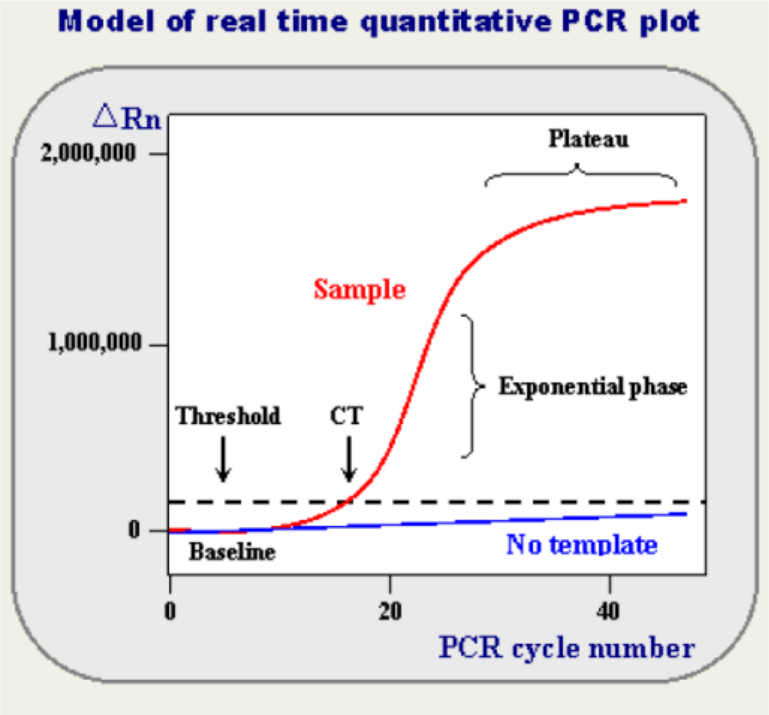


Figure 18: Nomenclature used in qRT-PCR

qRT-PCR was performed to measure the expression changes in selected genes (table 1) using Promega Kit. It was performed in 25 ul reaction volumes (Table 4) in 96-well plates and samples were assayed in triplicate. Negative controls were run in triplicate with RNase-free water instead of cDNA and included in each PCR assay (Table 2). Each plate was sealed with an adhesive film and centrifuged. A foam plate sealed was used to prevent the samples from evaporating whilst in the PCR machine. Samples in 96 well plates were centrifuged at low speed for 20 seconds to bring all reaction components together and remove air bubbles. Results were analysed using data analysis for Real-time PCR (DART PCR). Beta Actin (ACTB) used as an endogenous baseline control for each sample run.

Temperature	Time	Purpose
Stage 1: Single cycle		
95C	10 Minutes	Activate Enzyme
Stage 2:repeated 40 cycles		
95	15 Seconds	Denatures double strands
60 C	1 Minutes	Annealing and Elangation
Data Collection		
Stage 3: (dissociation curve) single cycle		
95 C	15 seconds	Denature
60 C	15 seconds	Annealing
Slow ramp to 95 C		Records deaturation temperature (s)
95 C	15 seconds	Full denaturing of double strands
Data Collection		

Table 9: QRT-PCR cycling parameters.

Reagent	Volume per well
SYBR Green Master Mix (Applied Biosystems)	12.75ul
Primer (forward and reverse mix)	1.5ul
RNase-Free Water	8.92ul
cDNA (2.5-10ng)	0.33ul
Total volume	25ul

Table 10: PCR reaction mix for each primer.

2.13.6 Analysis of qRT-PCR data

The data produced can be visualized in a two-dimensional plot of log of relative cycle number (x-axis) vs florescence intensity (y-axis). This is PCR amplification curve which is used to identify the Ct (cycle threshold) values. Ct is identified as the number of cycles required for fluorescence signal to cross the threshold. The Ct values are inversely proportional (smaller Cts mean higher expression of a gene) to the total amount of target nucleic acid in the sample that can be used to identify the amount of template in each sample. The mean Ct of each triplicate was used to create R0 values while outliers were excluded from the analysis. The expression of Actin (reference gene) was used as internal control for the normalization of the data. The plots were designed in Microsoft Office Excel. Results were analysed using Data Analysing for Real-Time PCR (DART PCR) (Peirson et al., 2003)

2.13.7 RNA-sequencing

RNA sequencing is a new evolutionary technique to revolutionise the manner in which transcriptomes are analysed. This technique has replaced the gene expression microarrays. This novel high-throughput DNA sequencing method provides a new way for both mapping and quantifying transcriptomes and has a clear advantage over other existing approaches. In this technique, a population of RNA (total or fractionated, such as poly-A^+) is converted to a library of cDNA fragments with adaptors attached to one or both ends. Each molecule is then sequenced in a high-throughput manner to obtain short sequences from one or both ends; the readings are usually 30–400bp. In our project, Illumina TruSeq RNA v2 (Wang et al., 2009) RNA sequencing protocol was used. As a starting material, approximately 250 ng of Trizol-extracted RNA was sent to the sequencing facility of UCL Institute of Child Health.

The protocol starts with two rounds of poly-A RNA selection using dT beads. Later metal hydrolysis is used to fragment the RNA to 200bp and is then reverse transcribed to cDNA using random hexameric primers. Then double-stranded RNA is generated after RNA template is removed. Then polymerase and exonuclease are used to convert the overhang fragments to blunt ends. Then the ends of the cDNAs are re-paired, and poly-A tails are generated to allow the ligation of the Illumina TruSeq index adaptors. The final step is the amplification of samples by 15 cycles of PCR which amplifies those RNA fragments that have adaptor molecules on both the ends. This step is processed before quality control, library quantification qPCR (optimum cluster densities through each lane of each flow cell) and normalisation to

10nM equimolar pooling. Sequencing is then developed in one lane of the Illumina HiSeq 2000, generating >180 m 100bp paired end readings from the pool. Finally, readings are de-multiplexed using Illumina CASAVA 1.8.2 software, which generates 36 million paired end readings per sample in FASTAQ format.

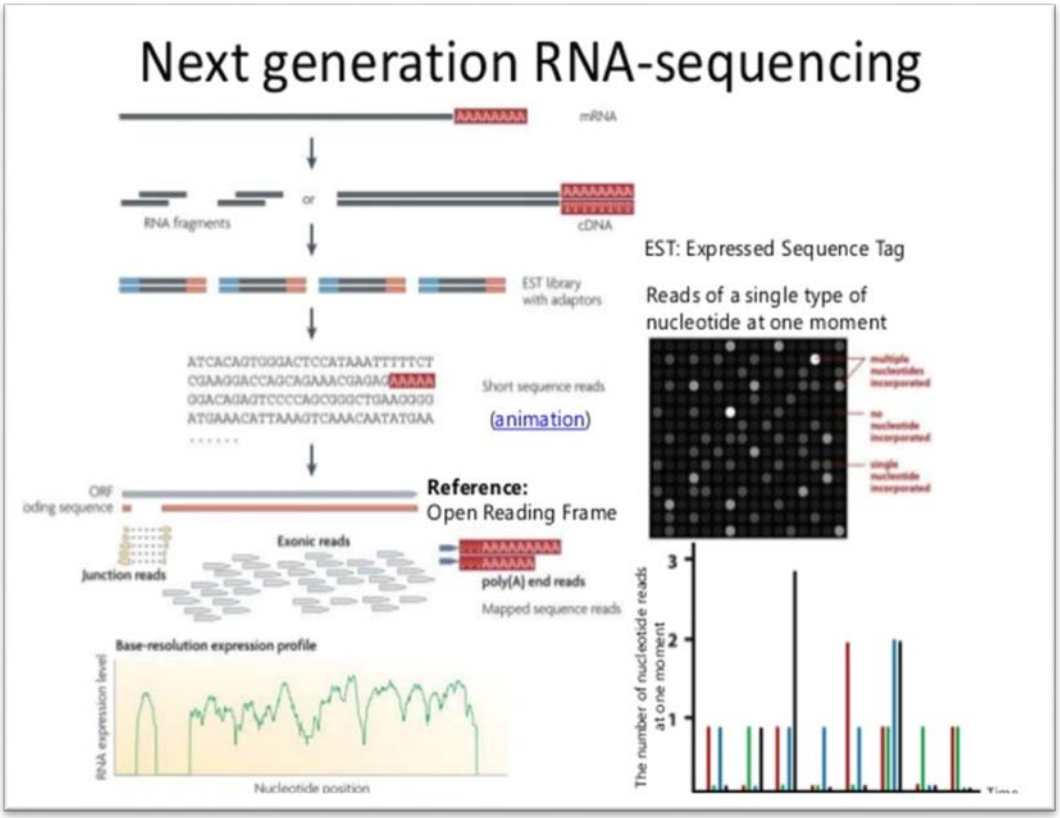


Figure 19: Different stages of RNAsequencing technique.

FASTAQ files generated were then sent to Monte Radeke, a collaborators in Neuroscience Research Institute (NRI) at University of California Santa Barbara (UCSB) for alignment. TopHat 2 was used to align the sequencing data to the Jan 2013 RefSeq transcriptome and the Hg19v37.2 genome build and then total number of readings per genome was obtained. The principle of TopHat is to align RNA-seq readings to mammalian-sized genome by applying high throughput short read aligner (Bowtie) and then study the mapping results, which helps to determine the splice junctions between exons.

3 Results

3.1 In vitro generation of PECs

3.1.1 In vitro derivation of progenitors of endothelial cells (PECs) from embryonic stem cells (ESCs)

3.1.1.1 Different culture systems to derive hES-derived PECs

In order to study PECs, it was first needed to establish an efficient protocol to derive these cells from pluripotent cells in vitro. A simple technique to generate PECs is based on embryonic bodies (EBs) (Levenberg et al., 2002). In this method, hES cells are aggregated into small clumps that then spontaneously differentiate into random tissues, which contain blood vessels. In previous work in the lab, it has been shown (by Jenny Mckenzie) that CD34+ PECs generated by this method express endothelial surface markers (such as VE-Cadherin and CD31) **(Figure 20)**. However, the problem with EB formation is that PECs are hard to isolate out of the densely aggregated EBs. Furthermore, the generation of PECs in EBs is unpredictable, unreliable and did not seem to create a lot of PECs. Therefore, an attempt was made to establish 2D culture conditions from which it might be easier and more efficient to isolate PECs.

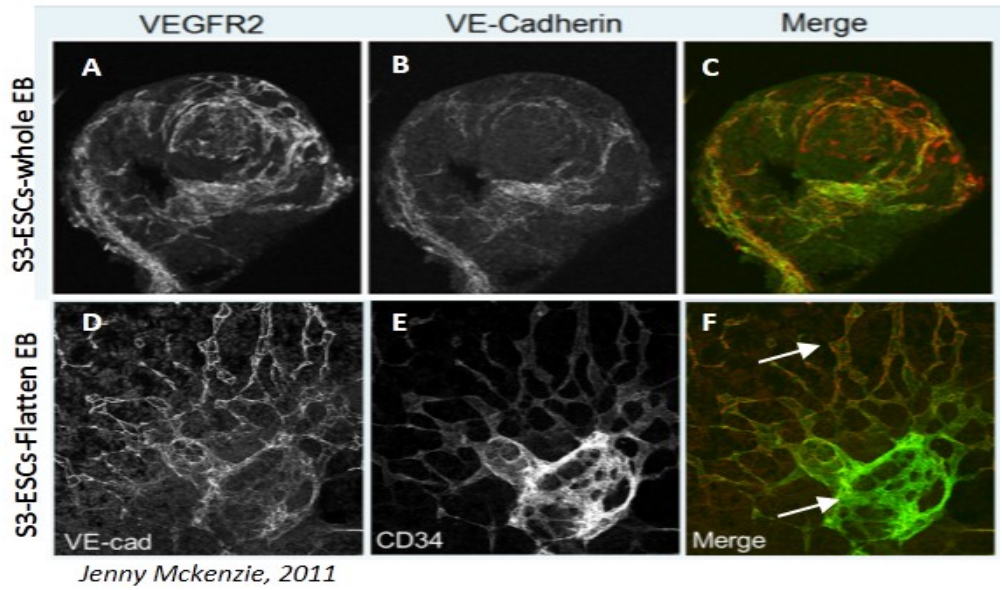


Figure 20: Generation of PECs from EBs.
The first row (A, B & C) depicts whole EB. The second row (D, E & F) is a section of flatten EB. Vascular cells express CD34 (green) and as they migrate out and differentiate they stop expressing CD34 and start expressing VE-Cadherin (red) (F). (Image from Jenny Mckenzie)

The strategy for the production of hESC-derived PECs under 2D culture conditions was as follows: hESCs (Shef3) were propagated on an inactivated mouse embryonic fibroblast (MEF) feeder layer for 5-7 days in hES/bFGF medium, until they became sufficiently confluent for passaging. Then cells were passaged with Collagenase and re-plated on the same feeder and medium. After around 7 days CD34+ cells started to appear. However, this 2D method, produced only low number of PECs (<1% of the all cells). I therefore, tried to find a culture conditions that produce higher numbers of PECs. Firstly, I tested PECs generation on three different substrates (Inactivated MEFs, Matrigel & Fibronectin). Each set of experiments was run for three different time courses of 7, 14 and 20 days **(Figure 21)**. To assess the impact of different culture conditions, the number of CD34 expressing cells was analysed by immunocytochemistry. This did not show any noticeable difference between different culture conditions. All of them had very low expression of CD34, which was hardly detectable **Figure 22**. Therefore, these substrates do not appear to improve the number of CD34 positive cells.

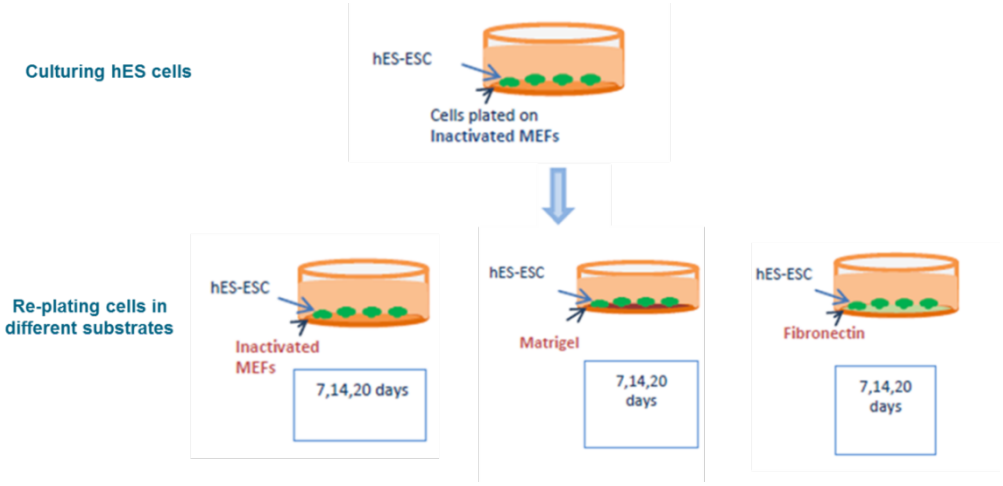


Figure 21: Schematic diagram for testing different substrates for PECs generation. Cells were plated in three different substrates (MEF feeder cells, Matrigel and Fibronectin) for different time courses (7, 14 & 20 days) and then the expression of CD34+ was assessed by immunocytochemistry.

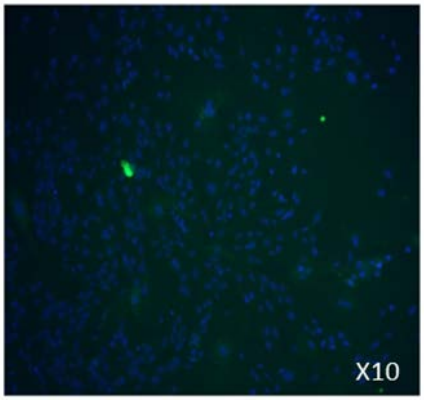


Figure 22: Representative picture of cells plated on hES medium and MEFs feeders. Immunocytochemistry did not show any noticeable CD34 expression.

3.1.1.2 Using FACS to analyse effect of media on hESC-derived PECs

In the next step, it was intended to see the effect of different media on the differentiation of CD34 cells. In order to achieve this, cells were grown on two different media (hES and EGM-2) for 14 days (on MEFs feeder cells). Then cells were stained with CD31 antibody conjugated to FITC and CD34 antibody conjugated to PE and further analysed by flow cytometry. To properly define the positive stained cells and separate them from background autoflorescence for gate inclusion, FMO strategy were used. FMO controls are samples that include all the conjugated antibodies but one and the channel for the missing conjugated antibody is that of the FMO gating control. This is important since FMO controls are essential for setting thresholds in cell populations that expected to express continuous spectrum of different number of receptors. Analysis here done by Dawn Sim (PhD student in the lab). To remove all auto fluorescence and non-specific binding of antibodies and present the most pure CD34 cell population, she selected smaller areas PE (2) in both conditions, with 2.31% and 3.29% of CD34+ expression out of the whole area of CD34 expressing cells PE (14.4% and 17.1%) to present. Here, I present that the PE cell population (14.4% and 17.1%) were purified for qPCR analysis. Considering both PE and PE (2) and comparing hES with EGM-2 medium, showed a slight increase of CD34+ cells from 2.3% to 3.29% in PE (2) or (14.4% to 17.1% in PE). Population of CD31+ cells remained similar (0.286% and 0.256%). All this data suggested that the EGM-2 medium is slightly better than hES medium to increase the number of CD34 positive cells. **Figure 23.**

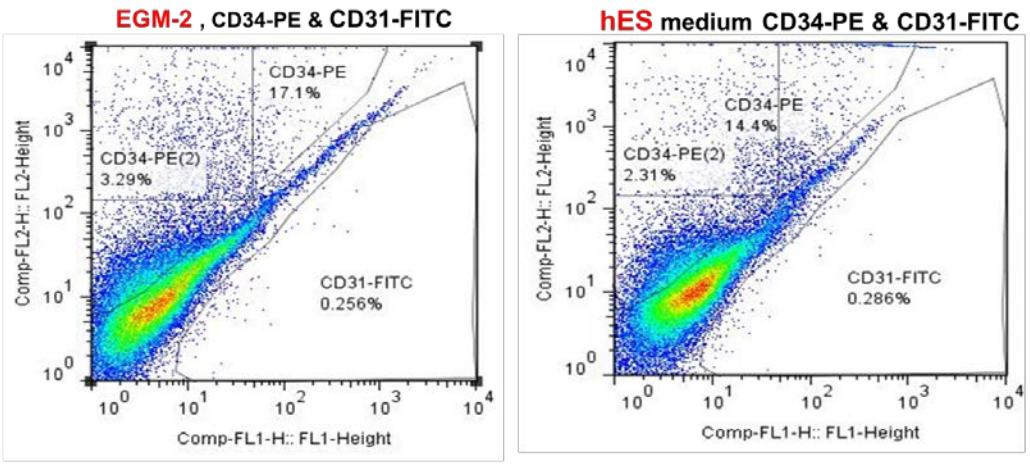


Figure 23: FACS results for hESCs cells cultured in hES and EGM-2 media.
FACS gating strategy applied for identification of CD34 and CD31 sub-populations derived from human embryonic stem cells that were grown in two different Media (hES and EGM-2). FlowJo software was used to analyse the percentage of CD34+ and CD31+ cells. Cells were labeled with CD34-PE and CD31-FITC. FMO control samples used to determine correct gating of CD34 and CD31 population identification. (Data were analyzed by Dawn Sim, PhD student in the lab). In order to remove all auto fluorescence and non-specific binding of antibodies and demonstrate the most pure CD34 cell population, she selected smaller area PE (2) in both conditions, (with 2.31% and 3.29% in hES and EGM-2 media) respectively to present. However, it should be mentioned that the PE area with 14.4% (hES) and 17.1% (EGM-2) of CD34 expressing cells were purified for qPCR analysis. Furthermore, 0.286% and 0.256% of cells were expressing CD31 in both hES and EGM-2 respectively.

In the next experiment RNA was extracted from both positive (PE) and negative populations mentioned above and qPCR was applied to confirm that the CD34+ cells generated in culture conditions are vascular progenitor cells. qPCR results were analysed by DARTPCR sheet and normalised to the housekeeping gene Actin. qPCR results were analysed by DARTPCR sheet and normalised to the housekeeping gene Actin. The expression of CD133 was compared between the CD34+ and CD34- populations and it was found that expression of CD133 as an early marker of endothelial progenitor cells was remarkably higher in CD34+ populations **Figure 24**. These results demonstrate that the CD34+ cells appearing in our culture system are indeed PECs.

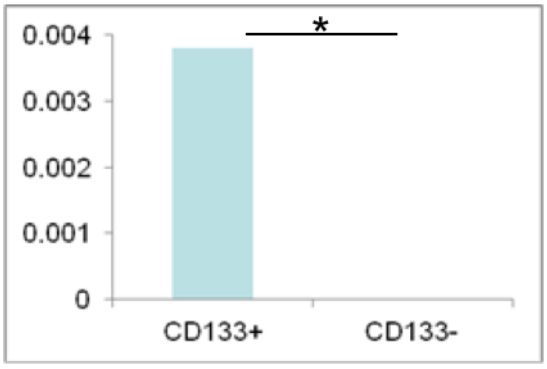


Figure 24: Real time PCR results.
qPCR for CD133 genes in isolated CD34+ vs CD34- cells sorted by FACS. The Ratio of the expression of CD133 was significantly higher in CD34+ cells compared to CD34- populations. (P<0.005).

3.1.2 Re-programming the Shef3 cell lines using 3iL and 4iL media

It is important to mention that in this study, experiments using hES cells often failed and showed considerable variability despite our best efforts to maintain the cells in good condition. Although it is generally assumed that the stem cells have potential to divide unlimited, in practical terms, stem cells beyond passage number 50 often accumulate epigenetic changes and they are no more useful for experiments (personal communication from Yoshiki Sasai). Because Shef3 cell line used in this study had very high passage numbers (> 70), it was considered to use a combination of small molecules that have been reported to reprogram the embryonic stem cells from a more "prime or epiblast-like" state back to a more "Naïve or ground" state (Gafni et al., 2015).

To this end, two recently published papers were followed. The first one used 3iL medium containing mTeSR, including a high concentration of bFGF, in combination with LIF, MEK1/2 inhibitor (PD0325901), GSK3-β inhibitor (WNT signalling activator) and Dorsomorphin (BMP signalling inhibitor) (Chen et al., 2010). hESCs cells plated in this medium increased the expression of Nanog and other genes such as Klf4 and Tbx3 that are commonly expressed in epiblast cells (Chan et al., 2013). In the second study using "4iL medium", which was 3iL medium in combination with a fourth component (P53 MAP kinase and Jun kinase inhibitor) has been reported. In these study, it was shown that ESCs were push towards a more naïve state in which Nanog and Dusp were up regulated, whereas lineage committed genes (Zic1, Sox6 and Sox11) were down regulated (Gafni et al., 2013).

Therefore, according to these papers, the current work investigated whether the partially differentiated cells that were in a more "primed state" could be dedifferentiated - turned back

into more naive state by using these factors and inhibitors. If successful, then the highly passaged hESC cells might be rejuvenated. To briefly explain, high passage hESC cells (P70) were cut off from a feeder surface and re-plated in a feeder-free surface. Naïve human stem cell medium (NHSM) and hESC medium were used as control conditions. Media were changed every day and because of a slow proliferation rate, cells were passaged every 7-9 days. Pictures were taken after each passage to monitor morphologic changes. Total RNA was isolated with Trizol after the second and third passages from all conditions (hESC, NHSM, 3iL, 4iL). HUVEC and iPSC cells were also used as negative (undifferentiated) and positive (totally differentiated) controls accordingly. The morphology of the colonies after each passage was a black, circular appearing mound, which was more indicative of differentiation of the cells to embryoid bodies rather than induction to a naïve epiblast-like state **(Figure 25).**

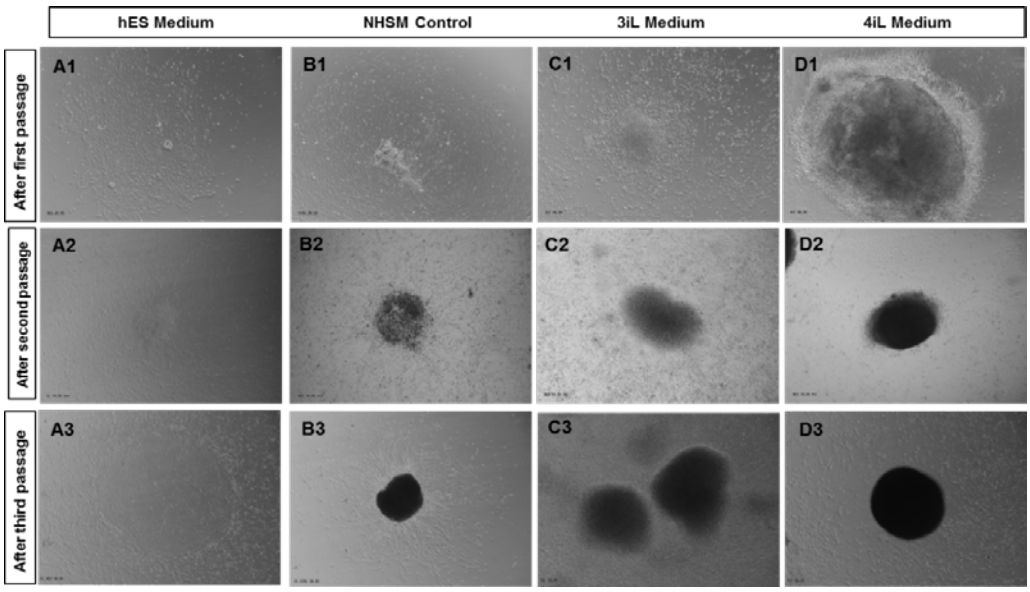


Figure 25: Re-programming hES cells in different media.
Light microscopy images of hESC cells (passage >70) cultured on Matrigel coated plates over three passages and in four different media. hES medium (A1,A2 &A3), NHSM medium (B1, B2 & B3), 3iL (C1, C2 & C3) and 4iL (D1, D2 & D3). Results showed more differentiation of cells to embryonic bodies in three conditions rather than induction to a naïve epiblast-like state.

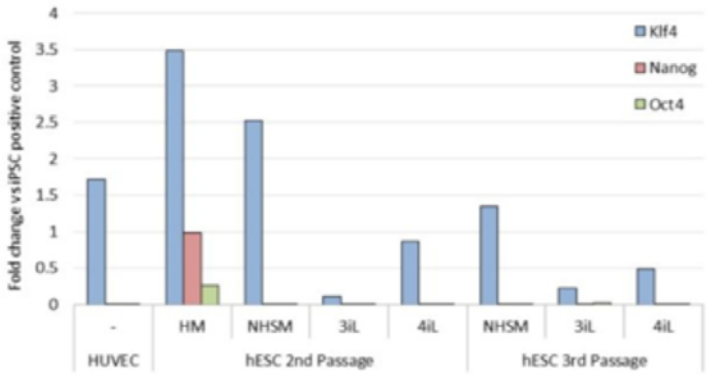


Figure 26: qPCR analysis for hES cells growing on different re-programing media.
The expression of relative levels of pluripotency markers Klf4, Oct4 and Nanog after the second and third passages. Expression levels are normalised against iPSC positive controls. qPCR results did not show any up-regulation of pluripotent genes after plating cells in 3iL and 4iL media (analysis from Willian Raimes).

Furthermore, qPCR analysis did not show any up-regulation in the pluripotency genes, Nanog and Klf4 (Gafni et al., 2013). With the exception of hES cells that were plated in hES medium, all other hES cells that were plated in 3iL, 4iL and NHSM medium differentiated and qPCR results showed no expression of Oct4 or Nanog. Furthermore, high expression of Klf4 observed in these cells could suggest a drift towards an endodermal lineage (Cao et al., 2012), as it has been shown that Klf4 is expressed in differentiated tissues such as intestinal wall endogenously (Lee et al., 2014). High expression of Klf4 was particularly obvious in hES cells cultured in hES and NHSM media. HUVEC cells also showed high expression of Klf4 which was consistent with previous studies and hence it was another positive control for this marker (Ho et al., 2010).

There are a number of possible explanations for the failure to reproduce induction to a naïve-like state. In the original paper, hES cells were used in a passage number (<40) which was much lower than used here (>70). Our later experiments also showed that these high passage cells have much less potential to differentiate, which is consistent with previous studies (Albrecht et al., 2006), showing that long term culture conditions can cause gene alteration in high passage cell lines (P70-80). Therefore, it could be concluded that these high passage number cells are not only unresponsive to differentiation signals, but are also not influenced by ground state reprograming. I therefore, decided to proceed by using iPSC cell lines that could be obtained with low passage numbers.

3.1.3 In vitro differentiation of PECs from human induced pluripotent stem cells (iPSCs)

3.1.3.1 Background

During embryonic development, the formation of progenitors of endothelial cells requires a series of inductive signals through the mesodermal lineage so that they can eventually differentiate into an endothelial lineage and generate endothelial cells. Asahara showed this process in three phases (Yang et al., 2011). In phase one, pluripotent stem cells differentiate into mesodermal precursors. They then commit to endothelial progenitor cells in phase two and finally, in phase three, they differentiate to ECs.

Previous studies have shown a number of signalling pathways that are important in mesodermal induction. Among them are Nodal/Activin A, BMP, Wnt and FGF and VEGF (Zhang et al., 2008, Hansson et al., 2009, Sumi et al., 2008). Although the contribution of these signalling pathways is conserved among many species, their specific effect that can be either inductive or repressive, or their time window of activity, could be different in different species, which might explain the contrasting findings in different papers. Recently, many studies have been carried out to understand the key factors in a very early phase of mesodermal induction and also later in endothelial differentiation.

Activin A is a member of the TGFß family. It is been shown that primitive streak (PS) formation is the crucial step before the generation of mesoderm and endoderm derivatives in vivo and the Nodal/Activin A pathway is very important in induction of PS formation. In in vitro studies, it was shown that Nodal/Activin A induces anterior and posterior PS in a dose-dependent manner in embryoid bodies (Gadue et al., 2006) and adherent cultures (Hansson et al., 2009). Furthermore, high doses of Activin A (10-100 ng/ml) has shown to induce the expression of Gsc (mesendoderm marker) in cells plated in a monolayer (Tada et al., 2005)

BMPs apply their diverse functions via integrating signal inputs from different intrinsic and extrinsic factors. It has been shown that in human stem cells, strong BMP signals induce differentiation and a low BMP signal is necessary to maintain the self-renewal of stem cells (Li and Chen, 2013). It was also shown that inhibition of BMP signalling, by using its antagonist Noggin, reduced SMAD 1/5/8 phosphorylation and at the same time, sustained long term self-renewal of human stem cells (Wang et al., 2005). Many studies have shown the importance of BMP signalling in the differentiation of stem cell-derived mesendoderm to multiple cell lineages, including cardiac, hematopoietic, pancreatic and liver lineages. It was

shown that BMP4 supplementation in EB cultures resulted in the formation of posterior PS and mesodermal cells (Nostro et al., 2008), which is indicative of its involvement in mesoderm differentiation during gastrulation. Takei has shown that short-term exposure of hESCs to BMP4 generates mesoderm progenitor cells that can further differentiate into hematopoietic and cardiac lineages (Takei et al., 2009), and temporary stimulation of hESCs with BMP4 in combination with VEGF, Activin A and FGF2 for one day could stimulate mesoderm and mesendoderm induction (Evseenko et al., 2010).

Furthermore, various studies have shown that WNT signalling is important for PS differentiation. The absence of this signalling in Wnt3-/- mutant mouse embryos led to defects in anterior-posterior alignment, PS and mesoderm induction (Barrow et al., 2007). Accordingly, inhibition of Wnt signalling by Dkk1 was shown to reduce the PS formation, which was shown by low expression of Mixl1 and Sox17 (Hansson et al., 2009). It appears that Wnt signalling, similar to BMP4, is needed for PS formation (Kemp et al., 2007).

Furthermore, Different studies have shown the diverse effects of bFGF signalling during embryonic development (Sui et al., 2013(Cleary et al., 2015). Active bFGF signalling is known to be important in keeping the hESC cells in a pluripotent state. However, a recent study has shown that inhibition of MARK, a downstream effector of bFGF, does not affect self-renewal of hESCs but prevents mesendodermal differentiation (Ding et al., 2010). The role of bFGF in germ layer specification depends on the context (Cha et al., 2008). But a few studies have shown the importance of bFGF in mesoderm derivation from hESCs cells. For instance, using bFGF antagonism can severely reduce mesendoderm induction (Sumi et al., 2008).

Many studies have shown the role of TGF-ß signalling during vasculogenesis and also the double-edged effect of this pathway on hematopoietic and endothelial lineage specification (Cha et al., 2008). Early activation of this signalling is needed for mesodermal progenitor differentiation and generation of CD31+, CD34+, VE-Cadherin+ cells and the addition of SB 431542 (Alk5 inhibitor) before mesoderm induction reduces the expression of mesodermal markers and decreases the number of CD31+, CD34+, and VE-Cadherin+ progenitor cells (Bai et al., 2013). However, the addition of SB 431542 after mesodermal induction and vascular commitment increases endothelial identity and expression of CD31+, CD34+, and VE-Cadherin (James et al., 2010). Furthermore, it has been shown that administration of SB 431542 to hESC cells could improve endothelial cell differentiation when it was combined with VEGF from day 7-14 (James et al., 2010).

Finally, VEGF signalling is shown to be crucial for angioblast expansion and formation of primary blood vessels during embryogenesis. It is later needed for endothelial cell sprouting and migration (Bautch, 2012). Genetic data also suggests that regulated spatial VEGF presentation is important for proper vessel morphogenesis, whereas overall VEGF levels are required for endothelial proliferation (Stalmans et al., 2002). Endothelial cells also express low levels of VEGF themselves, which is necessary for vessel homeostasis, and possibly for sprouting migration through integrin regulation of VEGFR-2 (Lee et al., 2008).

In order to establish a protocol to generate EPC in vitro, sequential experiments were developed. Each experiment provided continuous feedback which was used for the further optimization process.

In this study, it was first intended to reproduce two recent published protocols that were shown to generate a high yield of endothelial progenitor cells with high efficiency (Orlova et al., 2014, Prasain et al., 2014) and choose the most suitable medium. Schematic diagrams protocols are shown in **Figure 27**. Both protocols use Activin A, BMP4 and VEGF in the first phase of treatment with slightly different concentrations. These factors have been shown to be crucial for mesodermal induction. However, the Orlova protocol uses CHIR (WNT signalling activator) and SB431542 (TGF-ß inhibitor) in BPEL medium, whereas the Prasain protocol uses bFGF in combination with other factors and the protocol is based on using a transient gradual of the medium. Here I used iPSC lines provided by Amanda Carr and Lili Chen at the UCL institute of Ophthalmology.

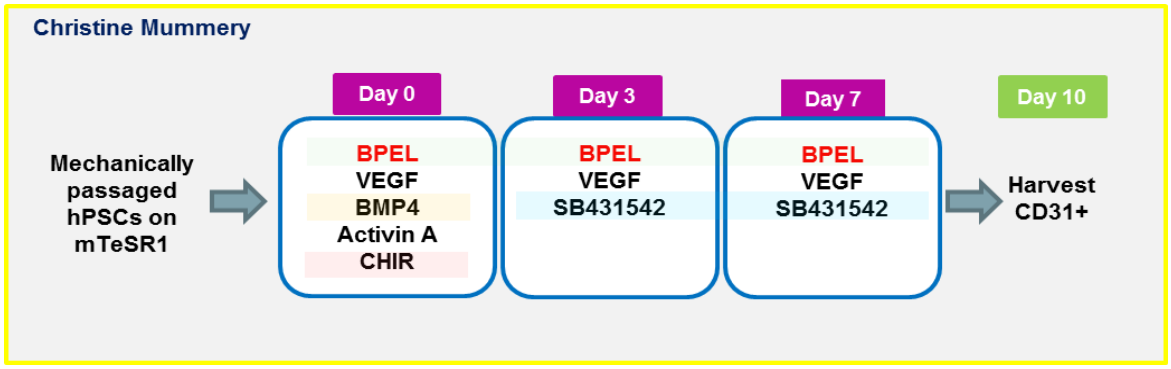


Figure 26: Schematic diagram of growth factors, combinations and timing to differentiate iPSC to endothelial cell lineage in Christine Mummery's protocol.

iPS cells were mechanically passaged and propagated in the mTeSR1 medium. At day 0, the medium was changed to BPEL medium (Ng et al., 2008) and cells were induced to differentiate by a sequential application of growth factors. Medium from day 0 to 3 contained 25ng/ml Activin A (Miltenyi, 130-095-547), 30ng/ml BMP4 (Miltenyi, 130-095-549), 1.5 µM GSK-3 kinase inhibitor CHIR99021 (CHIR) (Tocris, 4423) and 50 ng/ml VEGF (R&D Systems, 293-VE). At Day 3 of differentiation, the medium was changed to VEGF in combination with SB (SB431542; 10µmol/L) (Tocris, 1614). The highest percentage of mature CD31+ endothelial cells were observed at day 10.

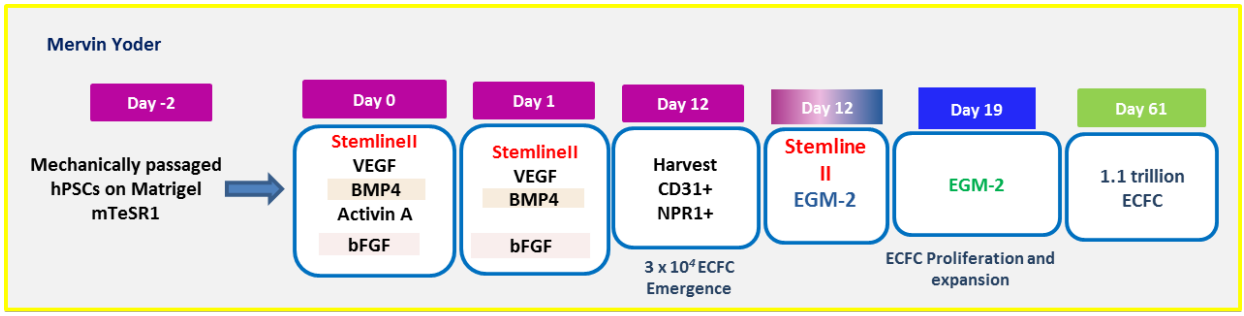


Figure 27: Schematic diagram of growth factors, combinations and timing to differentiate iPSC to endothelial cell lineage in Mervin Yoder's protocol (according to his presentation).
Cells were mechanically passaged and maintained in the mTeSR1 medium. At day 0, different growth factors, including bFGF, BMP4, Activin A (all 10ng/ul) and VEGF (50ng/ml), were added to the mTeSR1 medium. At day 1, Activin A was removed and at day 12, cells were harvested by sorting NRP1+ CD31+ cells. The medium was gradually changed from Stemline II to EGM-2 between days 12 to day 19 and subsequently changed to EGM-2 after day 22. Generated endothelial precursors were proliferated and expanded for 4 weeks. One trillion endothelial cells could be harvested at day 61 which showed homologues differentiation into endothelial cells.

3.1.4 Optimizing a protocol to derive PECs from hiPS cells

First experiment; Optimizing the Medium (Serum versus serum-free culture system)

Different research groups have tried different types of media; however, the major limitation in many published protocols is the use of undefined culture systems that contain animal serum. In current work first step of optimization was to find the most suitable medium. Six different media Table 11 were tested. Cells were treated with different combinations of factors in these six different media in 96 well plates which **Table 11.** Under the conditions "MTeSR1/EGM-2", the media gradually changed from MTeSR1 to EGM-2. DMEM/F12+N2+B27 medium which was called Differentiated Medium (DF) was a defined serum-free medium. N-2 and B-27 supplements were chosen as serum replacements. It has previously been shown that N-2 and B-27 were suitable for long-term cultures of primary embryonic hippocampal cells (Brewer, 1995), differentiation of umbilical cord blood stem cells towards neural cells (Ali et al., 2009), or differentiation of endothelial cells from pluripotent stem cells (Orlova et al., 2014). Both of these supplements were applied to the medium (DMEM/F12). Details of each of supplements are shown in **Table 2**. mTeSR1 and hESC media are routine stem cell media and were described in more details in method section. Over the optimization procedures the concentrations of Activin A 25ng/ml, VEGF 50ng/ml and bFGF 25ng/ml were not changed. In current experiment, BIO and SB431512 were used in the final concentration of 10uM. At the end of day 8 cells were fixed with 4% PFA and then processed for immune-staining to assess the CD34 expression. To optimise differentiation protocol to generate iPS-derived CD34+ cells, in each set of experiments different conditions (including different factor composition and concentrations) were investigated (each in triplicate and inducted with two independent people). Then data were analysed by immunostaining method (explained in more details below). Outcome from the first set of experiments were used to change the design (factor compositions and concentrations) for the next set of experiment which again were inducted with two independent people and on triplicate. Conditions with more cell death and no CD34 expression were excluded. Better conditions were selected to repeat in the next experiments in addition to new conditions. In total eight sets of experiments could clearly establish a reliable protocol to generate iPS-derived CD34+ cells.

Basal media	Factors added
MTeSR1	-
EGM-2	-
EGM-2	FBS (2%)
MTeSR/EGM-2	-
DMEM/F12	N2-B27
hESC medium	-

Table 11. **Different media used for optimization of best culture conditions.**

Composition of B-27 Supplement

Components	Final mg/dl
Vitamins	
Biotin	0.10
Hormones	
Progesterone	0.0063
Insulin	4.0
Corticosterone	0.02
Retinol, acetate	0.1
T3 (triodo-L-thyronine)	0.002
Antioxidants	
Catalase	2.5
Glutathione (reduced)	1.0
D, L-α-Tocopherol (Vit.E)	1.0
D, L-α-Tocopherol acetate	1.0
Superoxide dismutase	2.5
Others	
Putrescine	16.1
Selenium	0.016
L-Carnitine	2.0
Ethanolamine	1.0
Transferrin	5.0
Albumin, bovine	2500.0
Linolenic acid	1.0
Linoleic acid	1.0

Composition of N2 Supplement

Component	Final mg/L
Insulin (bovine)	5mg/L
Na selenite	30nM
Transferrin (human)	100 mg/L
Putrescine	100uM
Progesterone	20nM

Table 12. Composition of N2 and B-27 Supplement (A Richardson 2001).

In all experiments each treatment condition was performed in triplicate. Most of the experiments were repeated two times with two independent people (myself and William Raimas the MSc student in the group). Therefore, n=2 biological replicate and n=6 of technical replicate was considered for each treatment condition. Relative expression of CD34 was analysed in a semi-quantitative score method. Relative CD34+ cell yield (number sign), cell detachment (low/ medium/ high) and CD34+ coverage (disperse/ aggregated / extensive) were qualitatively assessed for each treatment condition by two independent observers.

Regarding the score method using "number sign" conditions with 1 or 2 small clusters or few dispersed cells were scored with 1 number sign (#), whereas conditions with 2 to 5 small clusters and/or a few numbers of dispersed CD34 positive cells covering around 5-10% of the surface were scored with 2 number sign (# #). Conditions having between 5 to 10 clusters and/or quite high number of dispersed cells covering around 15-20% of the surface were scored with 3 number sign (# # #). Conditions having 10 to 20 small and medium clusters with high number of dispersed CD34 expressing cells covering between 20-30 % of the surface were scored with 4 number sign (# # # #). Furthermore, conditions with considerable amount of small/medium clusters and high number of dispersed cells covering between 30-40% of surface were scored with 5 number sign (# # # # #). Similar conditions with comparatively more coverage of CD34 expressing cells between 40-50% of surface were scored with 6 number sign (# # # # # #). Conditions having very strong expression of CD34 covering between 50-60% of the surface containing big clusters of CD34 expression and very high number of dispersed cells were scored with 7 number sign (# # # # # # #) whereas comparatively higher percentage coverage between 60-70% were scored with 8 number sign (# # # # # # # #).

Since in some conditions, a big region of cells were dead and detached from the surface, cell detachment was also scored in a semi-quantitative way according to three low, medium and high levels of detachment. Furthermore, it was found that CD34 expressing cells in different conditions had different morphologies. Some conditions had more cluster-like structures whereas others were dispersed cells expressing CD34. Therefore, conditions were also categorised either dispersed, aggregated or in the case of having strong expression of both were categorised as extensive. All experiments with no expression of CD34 were shown by minus sign (-).

First experiment was repeated two times and similar results were observed form both experiments. (n=2 biological replicate and n=6 of technical replicate). Relative expression of CD34 was analysed in a semi-quantitative score method as mentioned above. Immune-staining results from first experiment showed no enrichment of CD34 positive cells with any of methods

used by (Orlova et al., 2014 and Prasain et al., 2014). However, among all the media tested, DMEM/F12 in combination with B2+ N27 was the best medium to induce the most CD34 positive cells in two separate conditions (conditions 6 and 10 in Figure 13). Thus, DMEM/F12 + B2+ N27 medium was selected for the remainder of the experiments**.** Furthermore, conditions having BIO (1.5uM) (conditions 4, 5, 7 and 9) had a big area of cell death presumably because of the high concentration of BIO (10uM). Therefore, the concentration of BIO was reduced to 0.5uM for the next experiment. Moreover, key parameters such as factors and substrates were also modified in further continuous experiments to optimise the best conditions in which to grow PECs.

Treatment No	Experiment 1 (iPSC) on Monolayer				Relative CD34+	Cell Detachment	Coverage
	Day 1	Day 2	Day 4	Day 6			
1. 1	mT(ActA/B4/V/F)	mT(B4/V/F)	mT/EGM2(B4/V/F)	EGM2(B4/V/F)	–	medium	dispersed
1. 2	mT(ActA/B4/V/F)	mT(B4/V/F)	mT/EGM2+(B4/V/F)	EGM2+(B4/V/F)	–	low	dispersed
1. 3	mT(ActA/B4/V/F)	mT(B4/V/F)	mT/EGM2(V/S)	EGM2(V/S)	–	medium	N/A
1. 4	mT(ActA/B4/V/BIO)	mT(ActA/B4/V/BIO)	mT/EGM2(V/S)	EGM2(V/S)	–	high	N/A
1. 5	mT(ActA/B4/V/BIO)	mT(ActA/B4/V/BIO)	mT/EGM2+(V/S)	EGM2+(V/S)	–	high	N/A
1. 6	DF(ActA/B4/V/F)	DF(B4/V/F)	DF(B4/V/F)	DF(B4/V/F)	###	low	aggregated
1. 7	DF(ActA/B4/V/BIO)	DF(ActA/B4/V/BIO)	DF(V/S)	DF(V/S)	–	high	N/A
1. 8	DF	DF	DF	DF	–	low	N/A
1. 9	DF(ActA/B4/V/BIO)	DF(B4/V/F)	DF(B4/V/F)	DF(B4/V/F)	–	high	N/A
1. 10	DF(B4)	DF(V/F)	DF(V/F)	DF(V/F)	#	low	dispersed
1. 11	hES(B4)	hES(V/F)	hES(V/F)	hES(V/F)	–	low	N/A
1. 12	mT(B4)	mT(V/F)	mT/EGM2(V/F)	EGM2(V/F)	–	low	N/A
1. 13	EGM2	EGM2	EGM2	EGM2	–	low	N/A
1. 14	EGM2+	EGM2+	EGM2+	EGM2+	–	low	N/A
1. 15	EGM2+	EGM2+	EGM2+(V/S)	EGM2+(V/S)	–	low	N/A

Table 13 Different treatment conditions in experiment 1. Data with n=2 biological replicate and n=6 of technical replicates are shown. The experiment was repeated 2 times with similar results. (n=2 biological replicates). Cells were seeded onto Matrigel-coated 96-well plates in a monolayer at 4 x 10^4 cells/well. Cells were left to settle for 48hrs. Experimental timelines ran 8 days long and consisted of growth factors addition in fresh medium on day 1, 2, 4 and 6. Cells were fixed and stained on day 8. Immunocytochemistry data were analysed in a semi-quantitative score method. Relative CD34+ cell yield (number sign), cell detachment (low/ medium/ high) and CD34+ coverage (disperse/ aggregated / extensive) were qualitatively assessed for each treatment condition by two independent observers. iPSC: induced pluripotent stem cell; mT: mTeSR1 medium; EGM2:Endothelial Cell Growth Medium2; EGM2+: endothelial growth media2 + 2% FBS; DF: DMEM/F12+B27+N2; ActA: Activin A 25ng/ml; B4: Bone Morphogenetic Protein 4, 30ng/ml; V: Vascular Endothelial Growth Factor (VEGF) 50ng/ml; F: basic Fibroblast Growth Factor 25ng/ml ; BIO: 6-Bromoindirubin-3'-oxime (Wnt pathway activator) 1.5 µM; S: SB431542 (inhibitor of ALK5, ALK4 and ALK7) 10 µM

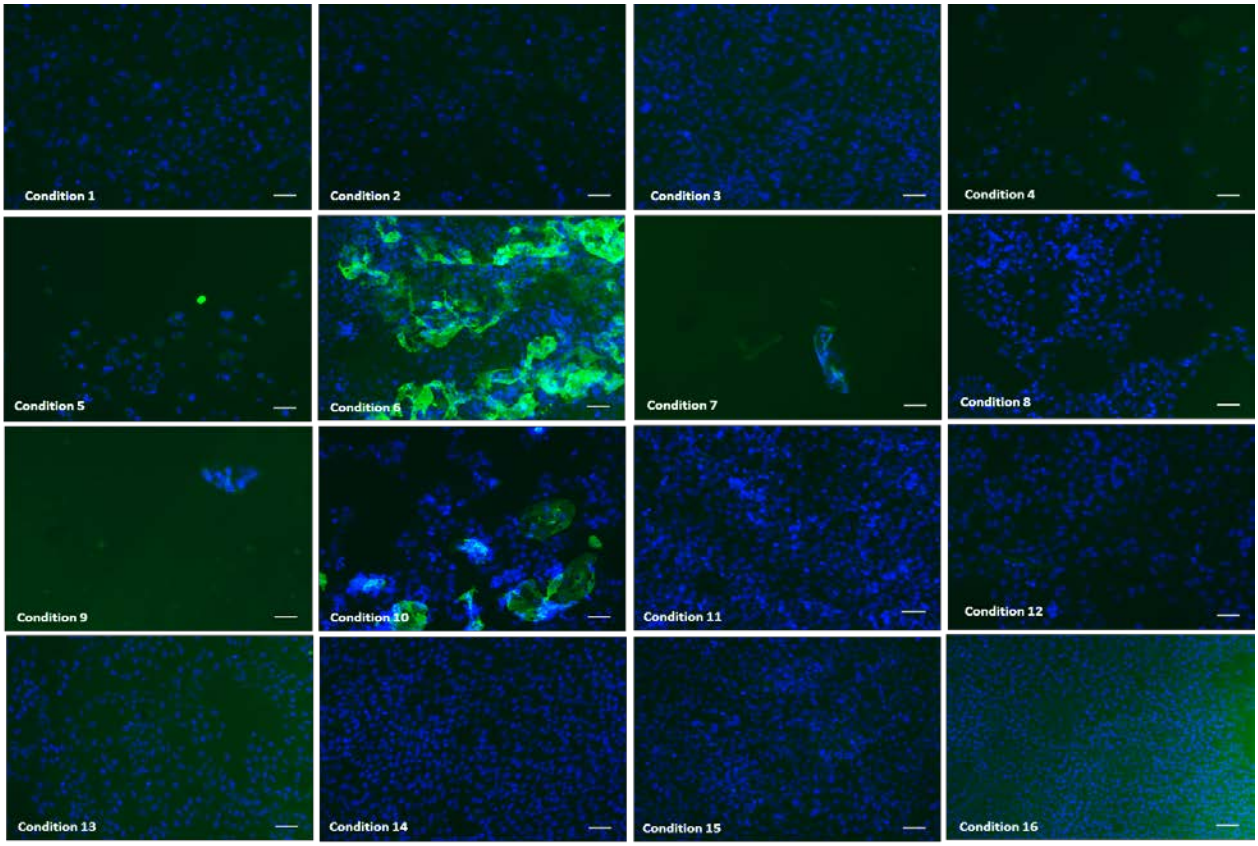


Figure 28. Immunofluorescent staining of the expression of PECs marker CD34 (green) for experiment 1.
Among sixteen treatment conditions testing different media (Table 11), conditions 6 and 10 treated with DMEM/F12+N2+B27 illustrated the expression of CD34. Rest of the conditions did not show any CD34 expression. Conditions 7 and 9 had a big area of cell death due to suspected cytotoxicity concentrations of BIO And SB431542. Nuclei are stained with DAPI in blue. Scale bar, 64 µm.

In the second experiment, only differentiated medium (DMEM/F12+N2+B27) was used for all treatment conditions. Each treatment condition was performed in triplicate. This experiment was repeated two times and similar results were observed form both experiments. (n=2 biological replicate and n=6 technical replicate). Relative expression of CD34 was analysed in a semi-quantitative score method. Cell was plated on monolayer Matrigel-coated 96 well plates. Two conditions that worked in experiment 1 were repeated. Additionally, various growth factor combinations were tested to further optimise the protocol which is summarised in **Table 14**.

Treatment No	Experiment 2 (iPSC, DM + monolayer)				Relative CD34+Yeild	Cell Detachment	Coverage
	Day 1	Day 2	Day 4	Day 6			
2.1	ActA/B4/V/BIO*	A/B/V/BIO*	V/S	Fixed	–	high	N/A
2.2	ActA/B4/V/F	B4/V/F	B4/V/F	Fixed	–	low	dispersed
2.3	ActA/B4/V/F	B4/V/F/S	B4/V/F/S	Fixed	–	high	N/A
2.4	ActA/B4/V/F	V/F	V/F	Fixed	–	low	dispersed
2.5	ActA/B4/V/F	V/F/S	V/F/S	Fixed	–	high	N/A
2.6	ActA/B4/V/F	V/S	V/S	Fixed	–	high	N/A
2.7	ActA/B4/V	V	V	Fixed	–	low	aggregated
2.8	B4	V/F	V/F	Fixed	–	low	N/A
2.9	B4	B/V/F	B4/V/F	Fixed	–	low	N/A
2.10	B4	V/F	V/F/S	Fixed	–	high	dispersed
2.11	DF	DF	DF	Fixed	–	low	N/A

Table 14. Treatment condition in experiment 1.
In this experiment the timeline treatment was reduced to 6 days instead of 8 days. As mention before, because of high cell death observed in conditions containing BIO 1.5 uM the concentration of BIO was reduced to 0.5uM. However, immunostaining results did not show any expression of CD34 even for conditions that worked in experiment 1 (condition 2 and 8 in Figure 30). Up to this point, not consistent results using bFGF in treatments observed in repeated experiments. Moreover, high amount of cell death observed in the condition containing BIO (condition 1) and also cells containing SB 431542 from day 2 (conditions 3, 5, 6 and 10). Therefore, in further experiments, the concentration of BIO was further reduced to 0.15uM and the concentration of SB 431542 was reduced to 2uM.

Treatment No	Experiment 2 (iPSC, DM + monolayer)				Relative CD34+ Yeild	Cell Detachment	Coverage
	Day 1	Day 2	Day 4	Day 6			
2.1	ActA/B4/V/BIO*	A/B/V/BIO*	V/S	Fixed	–	high	N/A
2.2	ActA/B4/V/F	B4/V/F	B4/V/F	Fixed	–	low	dispersed
2.3	ActA/B4/V/F	B4/V/F/S	B4/V/F/S	Fixed	–	high	N/A
2.4	ActA/B4/V/F	V/F	V/F	Fixed	–	low	dispersed
2.5	ActA/B4/V/F	V/F/S	V/F/S	Fixed	–	high	N/A
2.6	ActA/B4/V/F	V/S	V/S	Fixed	–	high	N/A
2.7	ActA/B4/V	V	V	Fixed	–	low	aggregated
2.8	B4	V/F	V/F	Fixed	–	low	N/A
2.9	B4	B/V/F	B4/V/F	Fixed	–	low	N/A
2.10	B4	V/F	V/F/S	Fixed	–	high	dispersed
2.11	DF	DF	DF	Fixed	–	low	N/A

Table 14. Different treatment conditions in experiment 2.
Data with n=2 biological replicate and n=6 of technical replicates are shown. Cells were seeded in triplicate onto Matrigel-coated 96-well plates in monolayer at 4 x 10^4 cells/well. Cells were left to settle for 48hrs. Experimental timelines reduced to 6 days long and consisted of growth factors addition in fresh medium on day 1, 2 and 4. Cells were fixed and stained on day 6. Immunocytochemistry data were analysed in a semi-quantitative score method. Relative CD34+ cell yield (number sign), cell detachment (low/ medium/ high) and CD34+ coverage (disperse/ aggregated / extensive) were qualitatively assessed for each treatment condition by two independent observers. iPSC: induced pluripotent stem cell; mT: mTeSR1 medium; EGM2: Endothelial Cell Growth Medium2; EGM2+: endothelial growth media2 + 2% FBS; DF: DMEM/F12+B27+N2; ActA: Activin A 25ng/ml; B4: Bone Morphogenetic Protein 4, 30ng/ml; V: Vascular Endothelial Growth Factor (VEGF) 50ng/ml; F: basic Fibroblast Growth Factor 25ng/ml ; BIO: 6-Bromoindirubin-3'-oxime (Wnt pathway activator) 0.5 µM; S: SB431542 (inhibitor of ALK5, ALK4 and ALK7) 10 µM.

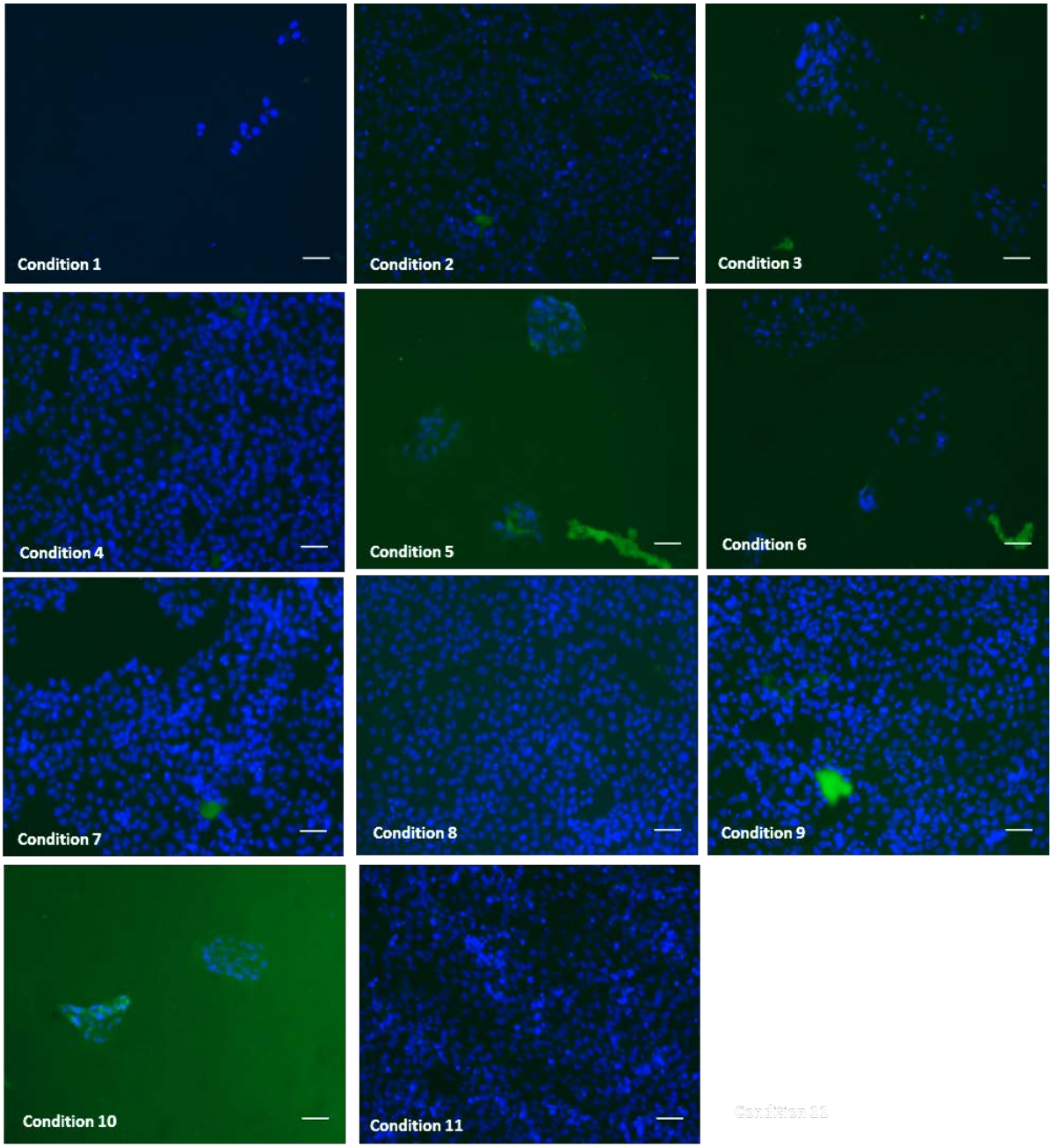


Figure 29. Immunofluorescent staining of the expression of PECs marker CD34 (green) for experiment 2.
Among eleven treatment conditions no expression of D34 was observed in any of treatment conditions. Conditions 1, 3, 5, 6 and 7 had big area of cell death due to suspected cytotoxicity concentrations of BIO And SB431542. Nuclei are stained with DAPI in blue. Scale bar, 64 µm.

The third experiment was a repeat of the second experiment. The only difference between the second and the third experiment was just the concentration of BIO and SB 431542, which were reduced to 0.15uM and 2uM respectively. Each treatment condition was performed in triplicate. This experiment was repeated two times and similar results were observed form both experiments. (n=2 biological replicate and n=6 technical replicate). Relative expression of CD34 was analysed in a semi-quantitative score method. Observations showed considerable expression of CD34 in condition 1. Moreover, in all conditions containing BIO, there was an extensive reduction in cell death, which was shown by the very small area of cell detachment. This could suggest the optimal concentrations of 0.15uM and 2uM for both BIO and SB 431542. However, some conditions having SB 431542 from day 2 (conditions 3 and 6) still showed some areas of cell detachment, which could be an indicative of toxicity of this factor for a longer period. Furthermore, condition 8 comprising of bFGF from day two had a considerable amount of cell detachment.

Treatment No	Experiment 3 (iPSC, DM + monolayer)				Relative CD34+Yeild	Cell Detachment	Coverage
	Day 1	Day 2	Day 4	Day 6			
3. 1	ActA/B4/V/BIO**	ActA/B4/V/BIO**	V/S	Fixed	##	low	aggregated
3. 2	ActA/B4/V/F	B4/V/F	B4/V/F	Fixed	–	low	N/A
3. 3	ActA/B4/V/F	B4/V/F/S	B4/V/F/S	Fixed	–	medium	N/A
3. 4	ActA/B4/V/F	V/F	V/F	Fixed	–	low	N/A
3. 5	ActA/B4/V/F	V/F/S	V/F/S	Fixed	–	low	N/A
3. 6	ActA/B4/V/F	V/S	V/S	Fixed	–	medium	N/A
3. 7	ActA/B4/V	V	V	Fixed	–	low	N/A
3. 8	B4	V/F	V/F	Fixed	–	high	N/A
3. 9	B4	B/V/F	B4/V/F	Fixed	–	low	N/A
3. 10	B4	V/F	V/F/S	Fixed	–	low	N/A
3. 11	DF	DF	DF	Fixed	–	low	N/A

Table 15. Different treatment conditions in experiment 3.
Data with n=2 biological replicate and n=6 of technical replicates are shown. The experiment was repeated 2 times with similar results. (n=2 biological replicates). Cells were seeded onto Matrigel-coated 96-well plates in a monolayer at 4 x 104 cells/well. Cells were left to settle for 48hrs. Experimental timelines ran 6 days long and consisted of growth factors addition in fresh medium on day 1, 2 and 4. Cells were fixed and stained on day 6. Immunocytochemistry data were analysed in a semi-quantitative score method. Relative CD34+ cell yield (number sign), cell detachment (low/ medium/ high) and CD34+ coverage (disperse/ aggregated / extensive) were qualitatively assessed for each treatment condition by two independent observers. iPSC: induced pluripotent stem cell; ActA: Activin A 25ng/ml; B4: Bone Morphogenetic Protein 4, 30ng/ml; V: Vascular Endothelial Growth Factor (VEGF) 50ng/ml; F: basic Fibroblast Growth Factor 25ng/ml ; BIO: 6-Bromoindirubin-3'-oxime (Wnt pathway activator) 0.5 µM; S: SB431542 (inhibitor of ALK5, ALK4 and ALK7) 10 µM.

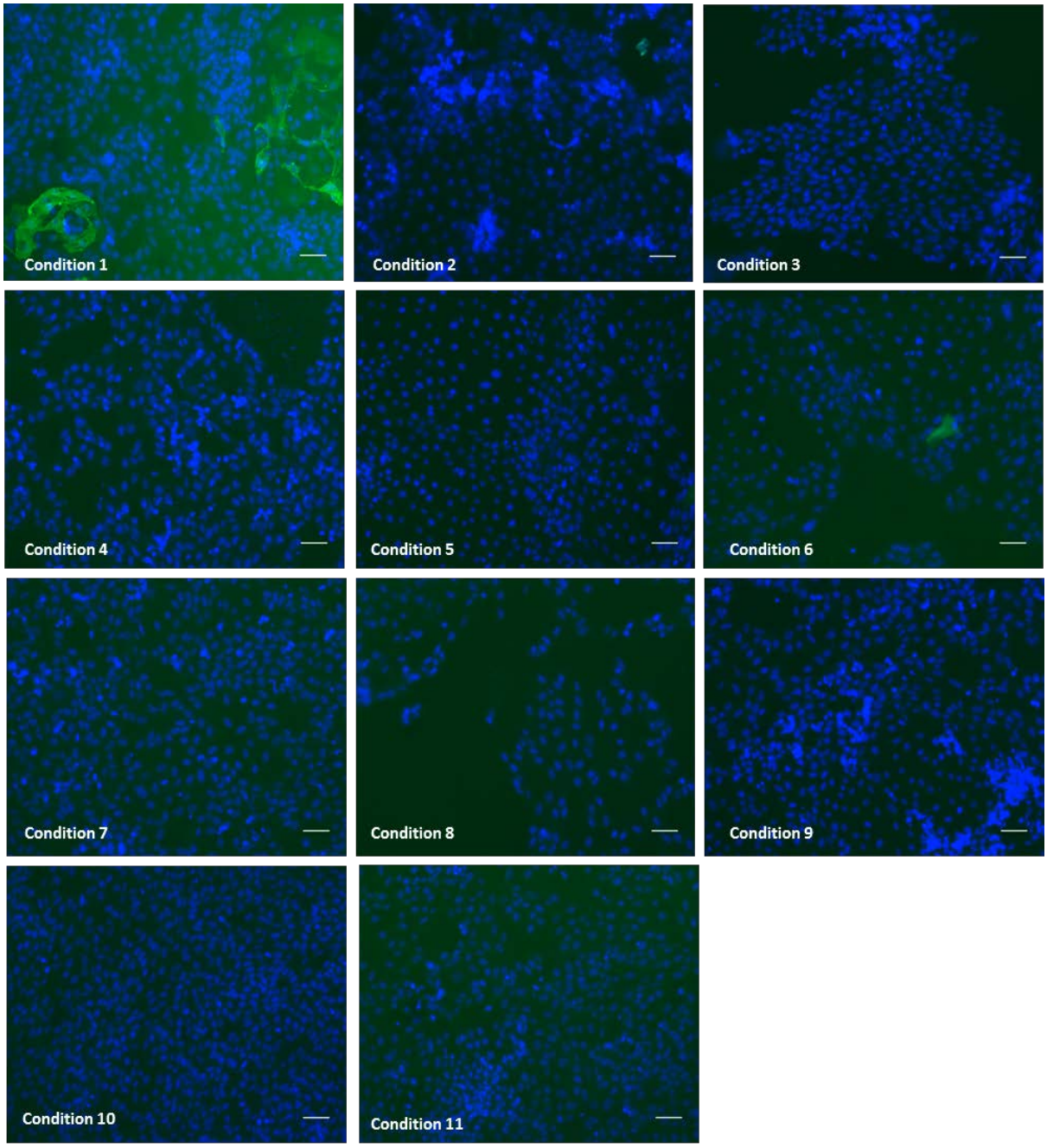


Figure 30. Immunofluorescent staining of the expression of PECs marker CD34 (green) for experiment 3.
Among eleven treatment conditions condition 1 containing reduced concentration of BIO and SB431542 (0.15uM and 2uM respectively) illustrated the expression of CD34 marker. Rest of the conditions did not show any CD34 expression. Conditions 3 and 6 had some area of cell detachment presumably due to the long-term cytotoxic effect of SB431542. Condition 8 comprising of bFGF from day two had a considerable amount of cell detachment. Nuclei are stained with DAPI in blue. Scale bar, 64 µm.

Therefore, in the first three experiments the best medium and the best concentrations of BIO and SB were optimised. However, because not enough CD34 enrichment was observed an attempt was made to optimise the protocols by modifying different parameters such as substrate and different combinations of factors. Furthermore, since variable results were observed over repeated experiments up to this point, it was assumed that the number of cells initially plated might also be an effective factor in the induction of CD34+ cells. Therefore, in the next experiment substrate was modified in parallel with three different cell numbers (low, medium and high) to find out its influence to enhance CD34-derived iPSCs.

3.1.4.1.1 Effect of substrate and cell number on deriving CD34-positive cells

The substrate was an important parameter assessed for the optimization. Two-dimensional (2D) cell culture has been widely used in in vitro research because of the benefits in terms of high speed and low cost. However, the major limitations of 2D culture systems are the unnatural geometric and mechanical restrictions that are imposed upon the cells. Monolayer systems cannot realistically mimic the three-dimensional morphologies of the cells in tissues and 3D culture systems are better at mimicking a normal physiological environment (Albrecht et al., 2006).

Here, the intention was to mimic 3D conditions by adding extra Matrigel on top of the medium and then compared it with 2D culture condition. This experiment were performed once and in triplicate. (n=1 biological replicate and n=3 technical replicate). However, the accuracy of the data and the advantage of the 3D culture conditions compared to 2D culture systems was further confirmed in the next experiments**.** Four different conditions that illustrated the most CD34 expression in previous experiments were used. iPSC cells were passaged and plated in high density (about 40,000 cells), middle density (25000 cells) and low density (13000 cells) in 96 well plates coated with Matrigel **Table 16**. Cells were left for 48 hours to settle. The medium was changed to Differentiated Medium (DF) on the first day of treatment in combination with specific factors plus additional 2.5% Matrigel (1:80 final working ratios), which was mixed into the medium and added to the plates. This concentration of Matrigel was added every time the medium was changed. Similar conditions were used for 2D culture conditions (no added Matrigel on top of the medium). Cells were then fixed on day five with 4% Paraformaldehyde and processed for immunostaining.

Results obtained from immune-staining analysis showed that adding extra Matrigel (2.5%) on top of the medium considerably increased the CD34 yield in all conditions. Therefore, the addition of extra Matrigel was used for all subsequent experiments **Table 16 and Figure 32.** Furthermore, different treatments had slightly different expression levels in different conditions and also in different densities. For instance, conditions 1 and 2 had the highest expression of CD34 in middle and high density in 3D culture system whereas conditions 3 and 4 had the highest expression in low and middle density. Condition 3 and 4 was another repeat of condition 6 and 10 in experiment 1 respectively which did not show any CD34 expression in 2D culture system. As a concluded outcome, consistent results using bFGF in the treatments were not observed in repeated experiments. Since CD34 expression was observed in conditions without bFGF, this factor was excluded from later experiments.

Treatment No		Experiment 4 (iPSC, DM + extra Matrigel 2.5%)				Relative CD34+ Yeild	Cell Detachment	CD34+ Coverage
		Day 1	Day 2	Day 4	Day 6			
Low density , With 2.5% Matrigel	1	ActA/B4/V/BIO**	ActA/B4/V/BIO**	V/S	Fixed	# # #	medium	dispersed
	2	ActA/B4/V/BIO**	B4/V/BIO**	V/S	Fixed	# # #	medium	aggragated
	3	ActA/ B4/ V/ F	F/ B4/ V	F/ B4 / V	Fixed	# # # #	low	aggragated
	4	B4	F / V	F / V	Fixed	#	low	aggragated
Low density, without Matrigel	1	ActA/B4/V/BIO**	ActA/B4/V/BIO**	V/S	Fixed	#	low	dispersed
	2	ActA/B4/V/BIO**	B4/V/BIO**	V/S	Fixed	_	low	N/A
	3	ActA/ B4/ V/ F	F/ B4/ V	F/ B4 / V	Fixed	_	medium	N/A
	4	B4	F / V	F / V	Fixed	_	medium	N/A
Middle density , with 2.5% Matrigel	1	ActA/B4/V/BIO**	ActA/B4/V/BIO**	V/S	Fixed	# # # # # # #	low	extensive
	2	ActA/B4/V/BIO**	B4/V/BIO**	V/S	Fixed	# # # # # # #	low	extensive
	3	ActA/ B4/ V/ F	F/ B4/ V	F/ B4 / V	Fixed	# # # # #	low	aggragated
	4	B4	F / V	F / V	Fixed	_	low	N/A
Middle density , without Matrigel	1	ActA/B4/V/BIO**	ActA/B4/V/BIO**	V/S	Fixed	# #	low	aggragated
	2	ActA/B4/V/BIO**	B4/V/BIO**	V/S	Fixed	# #	medium	aggragated
	3	ActA/ B4/ V/ F	F/ B4/ V	F/ B4/ V	Fixed	_	medium	N/A
	4	B4	F / V	F / V	Fixed	_	medium	N/A
High density , with 2.5% Matrigel	1	ActA/B4/V/BIO**	ActA/B4/V/BIO**	V/S	Fixed	# # # # # #	low	extensive
	2	ActA/B4/V/BIO**	B4/V/BIO**	V/S	Fixed	# # # # # # #	low	extensive
	3	ActA/ B4/ V/ F	F/ B4/ V	F/ B4 / V	Fixed	# # #	low	aggragated
	4	B4	F / V	F / V	Fixed	_	low	N/A
High density , without Matrigel	1	ActA/B4/V/BIO**	ActA/B4/V/BIO**	V/S	Fixed	# # #	low	aggragated
	2	ActA/B4/V/BIO**	B4/V/BIO**	V/S	Fixed	# # #	low	aggragated
	3	ActA/ B4/ V/ F	F/ B4 / V	F/ B4 / V	Fixed	_	medium	N/A
	4	B4	F / V	F / V	Fixed	_	medium	N/A

Table 16. Different treatment conditions in experiment 4.
Data with n=2 biological replicate and n=6 of technical replicates are shown. The experiment was performed once (n=1 biological replicates). Cells were seeded onto Matrigel-coated 96-well plates at 1.3 x 10^4 (low density), 2.5 x 10^4 (medium density) and 4 x 10^4 (high density) cells/well. Four different conditions were used. 3D culture conditions (2.5% Matrigel was added on top of the medium) were compared to 2D culture conditions (no Matrigel on top). Experimental timelines ran 6 days long and consisted of growth factors addition in fresh medium on day 1, 2 and 4. Cells were fixed and stained on day 6. Immunocytochemistry data were analysed in a semi-quantitative score method. Relative CD34+ cell yield (number sign), cell detachment (low/ medium/ high) and CD34+ coverage (disperse/ aggregated / extensive) were qualitatively assessed for each treatment condition by two independent observers. iPSC: induced pluripotent stem cell; ActA: Activin A 25ng/ml; B4: Bone Morphogenetic Protein 4, 30ng/ml; V: Vascular Endothelial Growth Factor (VEGF) 50ng/ml; F: basic Fibroblast Growth Factor 25ng/ml ; BIO: 6-Bromoindirubin-3'-oxime (Wnt pathway activator) 0.15 µM; S: SB431542 (inhibitor of ALK5, ALK4 and ALK7) 2 µM.**

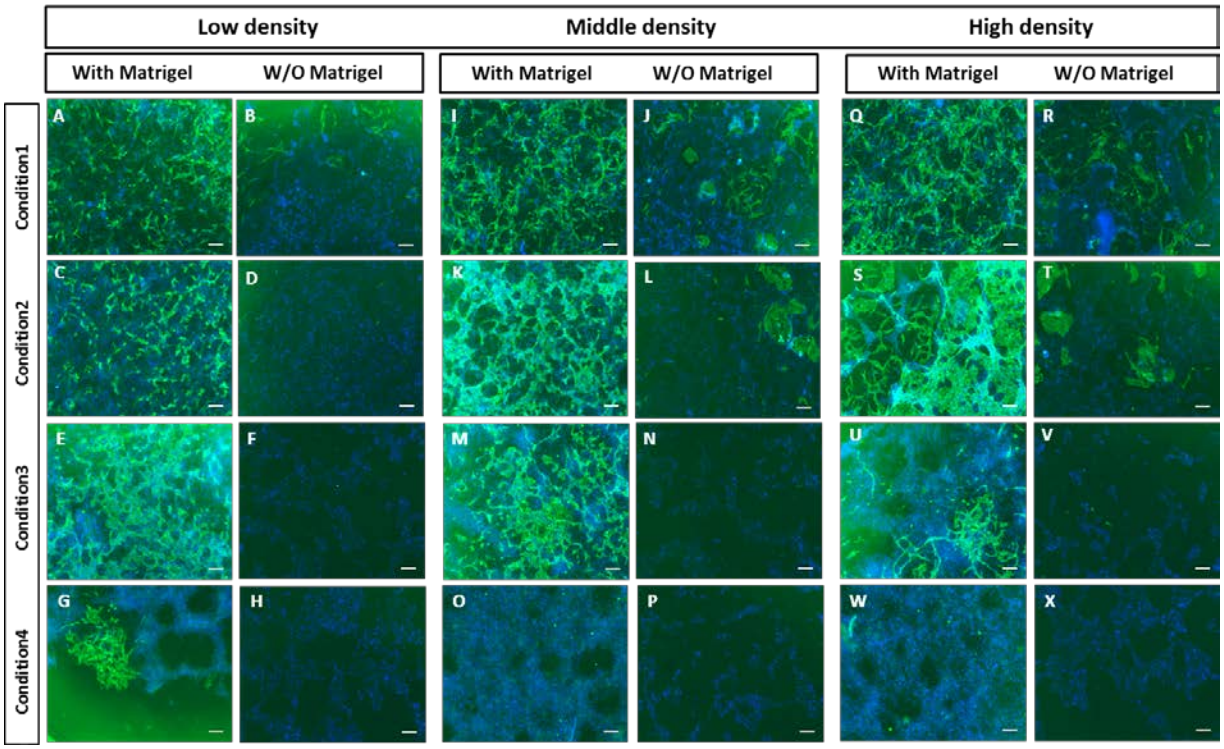


Figure 31 Immunofluorescent staining of the expression of PECs marker CD34 (green) for experiment 4.

Four treatment conditions were used to compare 3D versus 2D environments in three different cell densities (low/medium/high). Conditions having extra Matrigel on top of the medium as a multi-layer environment had remarkable expression of CD34 (A, C, E, I, K, M, Q, S & U) compared to 2D culture conditions (B, D, F, J, L, N, R, T and V) in all treatment conditions except condition 4 that did not show any considerable expression between two different culture systems (G, O, W vs. H, P, X). Furthermore, conditions 1 and 2 showed increasing trend in CD34 expression from low density to high density in both 3D and 2D culture systems whereas conditions 4 and 5 had decreasing trend in CD34 expression from high density to low density in both 3D and 2D culture systems (E, F, M, N, U, V and G, H, Q, P, W, X). Nuclei are stained with DAPI in blue. Scale bar, 164 µm. Each condition is shown in bigger magnification in appendix.

3.1.4.1.2 Effect of plating time before starting the treatment

Removal of cells for passaging and plating on different surfaces requires enzymatic or mechanical methods that change the morphological appearance of the harvested cells. For example, enzymatic digestion creates disaggregated cells with a round appearance, and these morphological changes are associated with cellular membrane disruptions. Cells are in a very close connection with the extra cellular matrix (ECM) and any damage to the cells can also damage the ECM underlying these cells, which can consequently affect the cell culture conditions (Canavan et al., 2005).

The first few experiments yielded inconsistent results. The cells were dissociated to single cells (using 1X TrypLE) and re-plated according to different time courses (between two to five days)

before starting the differentiation protocol. Hence, it was hypothesized that starting the differentiation treatment shortly after disaggregation of the cells did not allow sufficient time for recovery of appropriate connections between cells and the ECM. However, it was also presumed that the ECM or the seeded cells might secrete certain factors if left too long, which might also have negatively affected further treatments. Therefore, in the current experiment, cells were plated for different time courses after dissociation and before treatment as follows

1) High density cell plating with treatment starting two days after seeding
2) Low density cell plating with treatment starting six days after seeding
3) Intermediate density cell plating, followed by the addition of further cells after two days, treatment starting six days after the starting point.

Immunocytochemistry results did not reveal any negative effect of long term culturing (six days) before treatment. CD34 expression was slightly higher in long-term plating (six days) compared to the other two conditions **(**Figure 32**)**. However, since a sufficiently high quantity of CD34 expression was observed by plating the cells for only two days, and in order to keep the whole experimental period as short as possible, it was decided to keep the plated cells for only two days before differentiation treatment.

Treatment No	Experiment 5 (iPSC, Extra 2.5% Matrigel)				Relative CD34+ Yeild	Cell Detachment	Coverage
	Day 1	Day 2	Day 4	Day 6			
8. 1	ActA / B4 / V / BIO** (2 days)	ActA / B4 / V / BIO**	V / S	Fixed	######	low	extensive
8. 2	ActA / B4 / V / BIO** (2 days mixed with 6 days)	ActA / B4 / V / BIO**	V / S	Fixed	#######	low	extensive
8. 3	ActA / B4 / V / BIO** (6 days)	ActA / B4 / V / BIO**	V / S	Fixed	#######	low	extensive

Table 13. Treatment conditions to assess the effect of time delay after seeding cells before starting the differentiation treatment.
Data with n=2 biological replicate and n=6 of technical replicates are shown. The experiment was repeated 2 times with similar results. (n=2 biological replicates). Cells were seeded onto Matrigel-coated 96-well plates in 3D culture condition at 4 x 10^4 cells/well. Cells were left to settle for three different time courses before starting the differentiating treatment (2days / 6 days and a mixture of 6 days and 2 days cells). One condition was used. Experimental timelines ran 6 days long and consisted of growth factors addition in fresh medium on day 1, 2 and 4. Cells were fixed and stained on day 6. Immunocytochemistry data were analysed in a semi-quantitative score method. Relative CD34+ cell yield (number sign), cell detachment (low/ medium/ high) and CD34+ coverage (disperse/ aggregated / extensive) were qualitatively assessed for each treatment condition. No remarkable difference was observed within different treatments. Cells seeded for 7 days before treatments (8.1) appeared to have more CD34 yield than the other 2 conditions but it was not remarkable. iPSC: induced pluripotent stem cell; ActA: Activin A 25ng/ml; B4: Bone Morphogenetic Protein 4, 30ng/ml; V: Vascular Endothelial Growth Factor (VEGF) 50ng/ml; BIO: 6-Bromoindirubin-3'-oxime (Wnt pathway activator) 0.15 µM; S: SB431542 (inhibitor of ALK5, ALK4 and ALK7) 2uM.

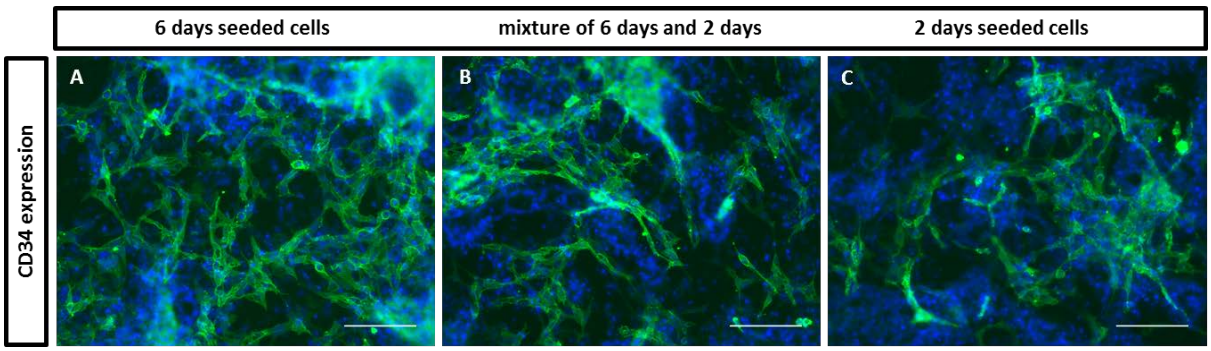


Figure 32. Immunofluorescent staining of the expression of PECs marker CD34 (green) for experiment 5.
Cells were plated for different time points before staring the differentiating treatment. Comparatively more CD34 expression was observed in condition A (six days seeding), compared to other conditions (B & C). However, this difference was not major and condition C was chosen for the remaining experiments. Nuclei are stained with DAPI in blue. Scale bar, 64 µm.

Up to this point, the important role of some factors for induction of progenitors of PECs is completely clarified. Notably, addition of Activin A on day 0 to 3 resulted in more robust and reproducible differentiation among a range of hiPSC lines. Cells treated without Activin A did not have any expression of CD34 and all conditions with one or three days of Activin A had a remarkably higher expression of CD34. Therefore, it was concluded that Activin A (25 ng/ml) is necessary for the early phase of treatment. However, conditions without Activin A (condition 10 in experiment 1 and condition 4 in experiment 4) had also low expression level of CD34. However, due to low efficiency and not consistency in CD34 expression in conditions without Activin A, including Activin A was considered in the further experiments. Moreover, important effect and optimised concentration of BIO in the beginning and SB 431542 in the late phase of treatment was shown. However, the effect of these factors in different treatment phase or for the longer time period was still to be evaluated in further experiments. Regarding VEGF (50 ng/ml) comparing different conditions with or without VEGF showed that VEGF is the key factor for generation of CD34+ cells and no expression was detected without VEGF. Furthermore, it was concluded seeding cells in high density (40000 cells/well) and 3D culture condition is sufficient to induce the most CD34 positive cells. Furthermore, 48hrs is sufficiently enough after passaging and seeding cells to settle for treatment.

3.1.4.1.3 Effect of BMP4 and SB 431542 in induction of CD34 positive cells

In the next experiment **Table 18**, the effect of BMP4 alone (condition 10) or in combination with different factors was assessed for different time points and it was compared with the condition without BMP4 (condition 8 **Table 18**). It was intended to find out if BMP4 would be sufficient enough to induce the high amount of CD34 or adding other factors to the cocktail is important to induce the CD34 expression. Condition with only VEGF treatment was also considered (condition 11 **Table 18**) to assess if VEGF alone would be enough to induce CD34 expression. Previous experiments could clearly illustrate that low concentration of SB 431542 (2uM) had much lower cell death compared to high concentrations (10uM). This experiment was repeated two times and similar results were observed form both experiments. (n=2 biological replicate and n=6 technical replicate). Relative expression of CD34 was analysed in a semi-quantitative score method. In current experiment SB 431542 (2uM) was used in low concentration but at different time points to find out its effect in increasing the CD34 expression and comparing this to conditions without SB 431542. Furthermore, the effect of BIO for the first 1 or 3 days was also assessed in combination with other factors. Differentiated Medium (DF) was used as a negative control which contains only the Basel medium without factors. Therefore, the Cells were seeded in triplicate onto Matrigel-coated 96-well plates in 3D culture system at 4 x 10^4 cells/well. Cells were left to settle for 48hrs. Experimental timelines ran 6 days long and consisted of growth factors addition in fresh medium on day 1, 2 and 4. Cells were fixed and stained on day 6.

Immunocytochemistry results showed that BMP4 for three days is more effective than one day in inducing CD34 expression. However, having BMP4 alone (condition 10 **Table 18**) is not effective and BMP4 should be applied in combination with specific factors. From current experiment, it could be concluded that BIO is a critical factor to be considered with BMP4 in the cocktail. Conditions containing BMP4 but not having BIO had no or very low expression of CD34 (conditions 1, 2, 3, 10 and 11). The presence of BIO either for one or three days seems to have high influence in inducing the CD34 positive cells (conditions 5, 6 and 7). However, it is not clear how would be the influence of BIO if it would be kept longer up to day 5. Therefore, this hypothesis was assessed in the next experiment. Furthermore, SB 431542 was another important factor to be considered albeit in combination with specific factors and specific time period. Conditions without SB 431542 had very low or no expression of CD34 (conditions 1, 2, 10 and 11 **Table 18**) which could show the importance of this factor in CD34 induction. Moreover, conditions with the highest expression of CD34 had SB 431542 only for two days.

Conditions containing SB 431542 for more than 2 days (from day 2 to 6) had a big area of cell death and no or very low expression of CD34 (conditions 3, 4, 8 and 9 **Table 18**). This observation could suggest that SB 431542 only for the last two days is sufficient enough to induce the most CD34 positive cells. This was consistent with previous findings showing that inhibition of TGF-ß before mesodermal induction results in a reduction of CD34 expression (Bai et al., 2013). Therefore, SB 431542 was considered to apply for only two days during the late phase of treatment (day three to five). Moreover, conditions containing BMP4 from day 1 to 5 alone (conditions 10 **Table 18**) or in combination with other factors (condition 1 **Table 18**) did not show any expression of CD34 which could indicate that BMP4 is a critical signaling molecule required for inducing the mesodermal lineage only in the very early phase of mesodermal induction. Additionally, having VEGF (50ng/ml) alone was not enough to induce the CD34 expression (condition 11 **Table 18**). Current experiment could show the importance of BIO and BMP4 in CD34 induction. Since this experiment could show the importance of BIO in CD34 induction, next experiment was designed to further investigate the longer effect of BIO and its effect in combination with or without BMP4 and SB 431542.

Treatment No	Experiment 6 (iPSC, DM, Extra 2.5% Matrigel)				Relative CD34+ Yeild	Cell Detachment	CD34+ Coverage
	Day 1	Day 2	Day 4	Day 6			
6. 1	ActA / B4 / V	B4 / V	B4 / V	Fixed	#	low	aggragated
6. 2	ActA / B4 / V	V	V	Fixed	#	low	aggragated
6. 3	ActA / B4 / V	V / S	V / S	Fixed	–	medium	N/A
6. 4	ActA / B4 / V / BIO**	V / S	V / S	Fixed	#	medium	disperesed
6. 5	ActA / B4 / V / BIO**	B4 / V	V / S	Fixed	#######	low	extensive
6. 6	ActA / B4 / V / BIO**	B4 / V / BIO**	V / S	Fixed	#######	low	extensive
6. 7	ActA / B4 / V / BIO**	ActA / B4 / V / BIO**	V / S	Fixed	########	low	extensive
6. 8	ActA / V / BIO**	V / S	V / S	Fixed	#	medium	dispersed
6. 9	ActA / B4 / V / BIO**	B4 / V / S	B4 / V / S	Fixed	–	medium	N/A
6. 10	B4	B4	B4	Fixed	–	low	N/A
6. 11	V	V	V	Fixed	–	low	N/A
6. 12	DF	DF	DF	Fixed	–	low	N/A

Table 18. Effect of SB 431542 and BMP4 for different time points.
Data with n=2 biological replicate and n=6 of technical replicates are shown. The experiment was repeated 2 times with similar results. (n=2 biological replicates). Cells were seeded onto Matrigel-coated 96-well plates in 3D culture system at 4 x 10^4 cells/well. Cells were left to settle for 48hrs. Experimental timelines ran 6 days long and consisted of growth factors addition in fresh medium on day 1, 2 and 4. Cells were fixed and stained on day 6. Immunocytochemistry data were analysed in a semi-quantitative score method. Relative CD34+ cell yield (number sign), cell detachment (low/ medium/ high) and CD34+ coverage (disperse/ aggregated / extensive) were qualitatively assessed for each treatment condition with two observers. High CD34 expression was observed in conditions 5, 6 & 7 containing BMP4 for the first three days and BIO for either 1 or 3 days and SB 431542 for 2 days only. These cells had low cell detachment and cell coverage was categorised as aggregated. Conditions containing BMP4 without BIO had very low expression of CD34 (conditions 1, 2, 3 and 10). Conditions containing SB 431542 from day 2 to 5 had a comparatively high number of cell detachment and very low CD34 expression (conditions 3, 4, 8 & 9). BMP4 and VEGF alone was not sufficient to induce CD34 positive cells (conditions 10 & 11). iPSC: induced pluripotent stem cell; ActA: Activin A 25ng/ml; B4: Bone Morphogenetic Protein 4, 30ng/ml; V: Vascular Endothelial Growth Factor (VEGF) 50ng/ml; BIO: 6-Bromoindirubin-3'-oxime (Wnt pathway activator) 0.15 µM; S: SB431542 (inhibitor of ALK5, ALK4 and ALK7) 2 µM.**

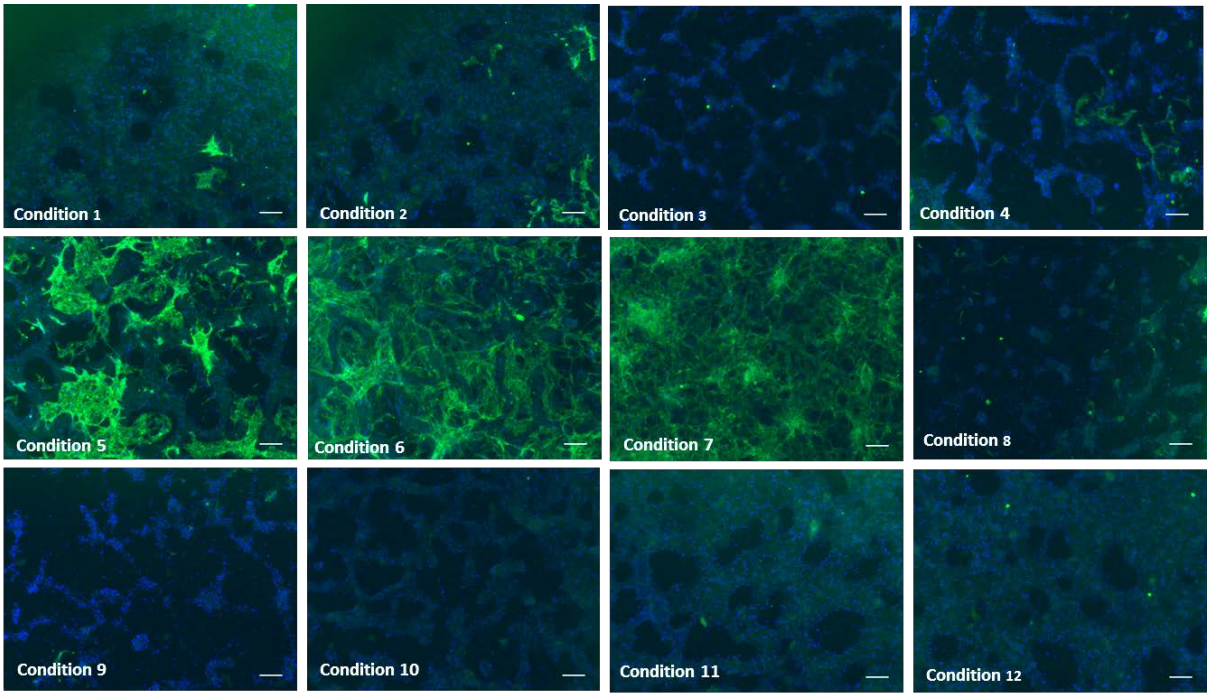


Figure 33. Immunofluorescent staining of the expression of PECs marker CD34 (green) for experiment 5.

Eleven treatment conditions were used to assess the effect of BMP4, BIO and SB 431542 for different time points. Activin A and VEGF are present in all the conditions except 10 and 11. Conditions 1 and 2 containing BMP4 but not BIO and SB 431542 had very low CD34 expression. Conditions 3 and 4 containing SB 431542 for 4 days had a big area of cell death and no or very low expression of CD34. Conditions 5, 6 & 7 containing BMP4 and BIO and also only 2 days SB 431542 showed an extensive amount of CD34 coverage and very low cell detachment area. Conditions 8 & 9 containing SB 431542 from day 2 to 5 had a considerable amount of cell death and no or very low expression of CD34. Conditions 10 and 11 with BMP4 only and VEGF only treatment respectively does not show any CD34 expression. Condition 12 is the negative control (DF only) condition. Nuclei are stained with DAPI in blue. Scale bar, 164 µm.

3.1.4.1.4 Long term effect of BIO in induction of CD34 positive cells

In the current experiment, as a result of previous experiments SB 431542 was only considered for the last two days of treatment. From the fact that applying BIO either for 1 or 3 days was shown to be very effective in inducing the high number of CD34, current experiment was designed to find out the longer effect of BIO in CD34 induction. Furthermore, since the effect of BIO was found to be very effective in induction of CD34 positive cells current experiment was designed to find out longer effect of this factor in the absence or presentence of SB 431542 or BMP4. Considering all these findings, in current experiment BIO was considered for all conditions for either 1, 2 or 5 days. Furthermore, BMP4 was also considered for either 1 or 3 days which was combined with SB 431542 in some of the conditions to compare the different combinations of factors. Furthermore, some conditions evaluated the effect of BIO in absence of BMP4 with or without SB 431542. This experiment was repeated two times and similar results were observed form both experiments. (n=2 biological replicate and n=6 technical replicate). Relative expression of CD34 was analysed in a semi-quantitative score method. **Immunocytochemistry** results revealed that conditions adding BIO to the medium for longer time period (day zero to five) suppressed the expression of CD34 positive cells (condition 3 and 4 **Table 19**). BIO is necessary only for the early phase of treatment but becomes detrimental at later stages. Furthermore, the combination of this factor with SB 431542 (condition 3 **Table 19**) from day 3 to 5 had relatively bigger area of cell detachment compared to condition without SB 431542. Conditions having BIO and BMP4 for 3 days and then VEGF + SB 431542 for 2 days had comparatively more CD34 expression compared to similar condition without SB 431542. (Condition 5 vs. 6 Table 19). Furthermore, it was observed that conditions having BIO for 3 days but excluding BMP4 from day 2 (conditions 5 & 6) still had high expression of CD34 and it was comparatively more if SB 431542 was added to the cocktail in the last 2 days. However, if BIO excluded from day 2 and instead BMP4 gets included up to day 3 (condition 7 and 8 Table 19), less CD34 was observed compared to 3 days stimulation with BIO. Similarly, having SB 431542 had slightly higher CD34 coverage compared to VEGF only condition in the last 2 days of treatment. In condition 9 and 10 BMP4 is excluded from day 1 and is only included in condition 10 from day 2 to 5. Comparison of the two condition shows that including BMP4 is effective in increasing the number of CD34 positive cells. Comparisons of conditions 9 and 11 Table 19 which are similar and the only difference is the presence or absence of SB 431542 emphasise the positive effect of SB 431542 in inducing of the CD34 positive cells. Furthermore, excluding all factors except VEGF from day 2 does not

seem to be effective in CD34 induction (condition 12). Condition without any of these factors does not show any CD34 expression condition 13 Table 19. Comparing all possible factor compositions shows that including BIO and BMP4 together for three days is more effective than having each of them separately for 3 days or including one of them for 1 day and the other for 3 days. Furthermore, including SB 431542 for the last two days is effective for increasing the number of CD34 positive cell. Moreover, in all current experiment Activin A was removed from day 2. Comparing immune-staining results from this experiment with previous experiment Table 18 which contained Activin A for three days reveals that presence of Activin A for three days is more effective than 1 day. Therefore, combining Activin A, BIO, BMP4 and VEGF for the first day and then SB 431542 and VEGF only for two days seems to be very effective to induce the most number of CD34 positive cells.

Treatment No	Experiment 7 (iPSC, DM, Extra 2.5% Matrigel)				Relative CD34+Yeild	Cell Detachment	CCD34+ overage
	Day 1	Day 2	Day 4	Day 6			
7.1	ActA/B4/V/BIO**	B4/V/BIO**	V/S	Fixed	#####	low	dispersed/ aggragated
7.2	ActA/B4/V/BIO**	B4/V/BIO**	V	Fixed	####	low	dispersed
7.3	ActA/B4/V/BIO**	B4/V/BIO**	BIO**/V/S	Fixed	#	medium	dispersed
7.4	ActA/B4/V/BIO**	B4/V/BIO**	V/BIO**	Fixed	#	low	dispersed
7.5	ActA/B4/V/BIO**	V/BIO**	V/S	Fixed	#####	low	aggragated
7.6	ActA/B4/V/BIO**	V/BIO**	V	Fixed	####	low	aggragated
7.7	ActA/B4/V/BIO**	B4/V	V	Fixed	###	low	aggragated
7.8	ActA/B4/V/BIO**	B4/V	V/S	Fixed	####	low	aggragated
7.9	ActA/V/BIO**	V/BIO**	V/S	Fixed	#####	low	dispersed
7.10	ActA/V/BIO**	B4/V/BIO**	V/S	Fixed	######	low	dispersed/aggragated
7.11	ActA/V/BIO**	V/BIO**	V	Fixed	###	low	dispersed
7.12	ActA/V/BIO**	V	V	Fixed	##	low	aggragated
6.13	DF	DF	DF	Fixed	–	low	N/A

Table 19. Effect of BIO for different time points in absence or presence of BMP4 and SB 431542. Data with n=2 biological replicate and n=6 of technical replicates are shown. The experiment was repeated 2 times with similar results. (n=2 biological replicates). Cells were seeded onto Matrigel-coated 96-well plates in 3D culture system at 4 x 10^4 cells/well. Cells were left to settle for 48hrs. Experimental timelines ran 6 days long and consisted of growth factors addition in fresh medium on day 1, 2 and 4. Cells were fixed and stained on day 6. Relative CD34+ cell yield (number sign), cell detachment (low/ medium/ high) and CD34+ coverage (disperse/ aggregated / extensive) were qualitatively assessed for each treatment condition. Comparatively high number of CD34+ cells was observed in conditions having BIO or BMP4 or both for the first 3 days (conditions 1, 2, 5, 6, 7, 8, 9. 11 & 12). However, having BIO for 5 days (conditions 3 & 4) resulted in very low expression of CD34 and also bigger area of cell detachment when BIO and SB 431542 were used together (condition 3 vs.4). Furthermore, adding SB 431542 seems to be more effective in inducing CD34 positive cells compared to VEGF only conditions. (Conditions 1, 5, 8, 9 & 10 vs. 2, 6, 7, 8, 11 & 12). iPSC: induced pluripotent stem cell; ActA: Activin A 25ng/ml; B4: Bone Morphogenetic Protein 4, 30ng/ml; V: Vascular Endothelial Growth Factor (VEGF) 50ng/ml; BIO: 6-Bromoindirubin-3'-oxime (Wnt pathway activator) 0.15 µM; S: SB431542 (inhibitor of ALK5, ALK4 and ALK7) 2 µM.**

.

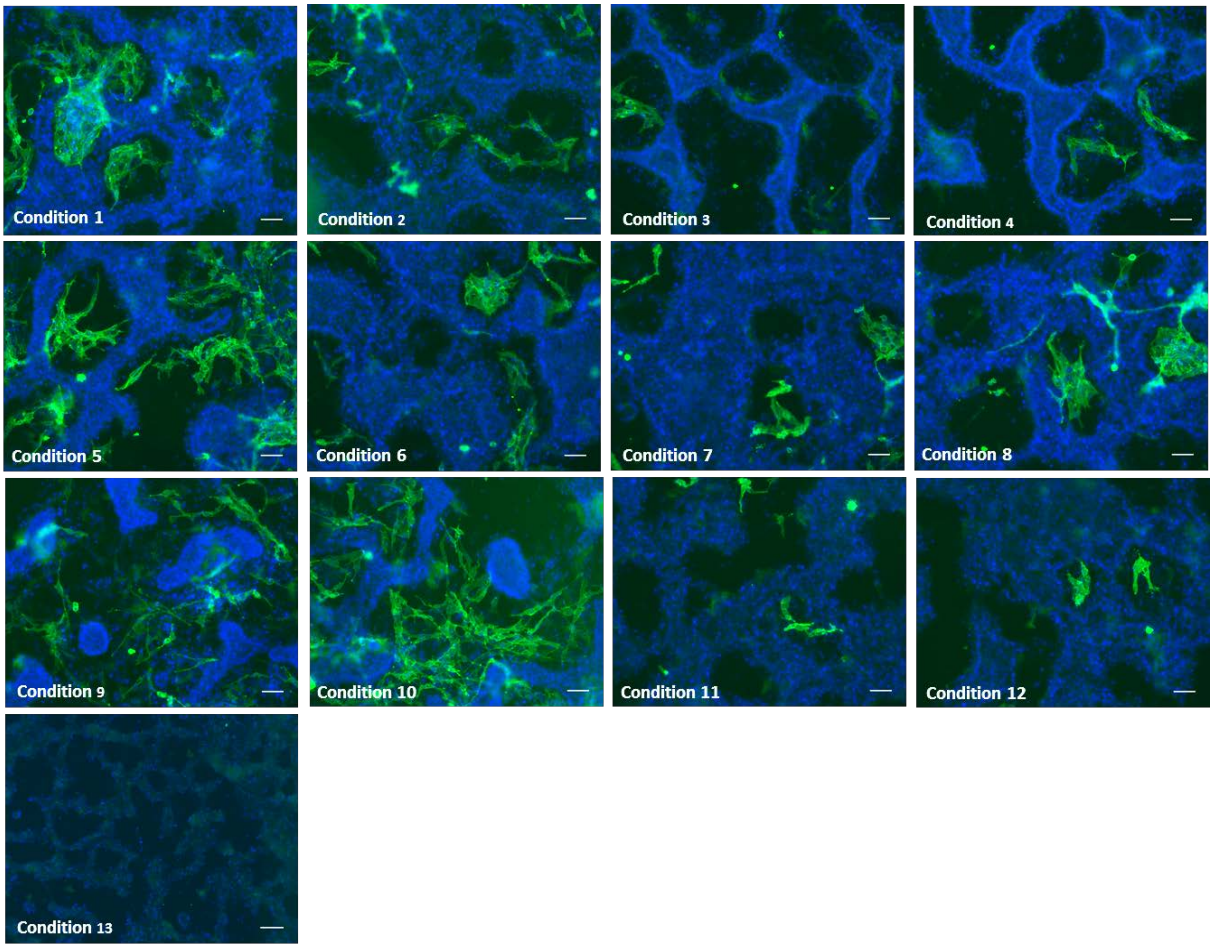


Figure 34. . Immunofluorescent staining of the expression of PECs marker CD34 (green) for experiment 5.

Twelve treatment conditions were used to assess the effect of BIO for different time points in presence or absence of SB 431542 and BMP4. Activin A and VEGF are present in all the conditions except condition 13 as the control. Conditions 1 and 2 containing BMP4 and BIO for 3 days and the difference between the two conditions is SB 431542 which is not included in condition 2 and it shows relatively less CD34 expression. Conditions 3 and 4 have BIO for 5 days which clearly shows considerable reduction in CD34 expression. Furthermore, condition 3 has, more area of cell detachment compared to condition 4 which is most probably due to having both SB 431542 and BIO together for the last 2 days. Condition 5 containing BIO for 3 days also SB 431542 for the last 2 days had comparatively higher CD34 expression compared to similar condition without SB 431542 (condition 6). Furthermore, conditions having BIO for 3 days (conditions 1, 5, 6, 9 & 10) have more CD34 expression in comparison with conditions having BIO only for 1 day (condition 7 & 8). This is become more evident when using SB 431542 for the last 2 days (conditions 2 vs 10). Condition 13 is the negative control (DF only) condition. Nuclei are stained with DAPI in blue. Scale bar, 64 µm.

3.1.4.1.5 Effect of VEGF different time points

VEGF signalling is necessary for angioblast expansion and formation of primary blood vessels during embryogenesis. It is later needed for endothelial cell sprouting and migration (Bautch, 2012). Genetic data also suggests that regulated spatial VEGF presentation is important for proper vessel morphogenesis, whereas overall VEGF levels are required for endothelial proliferation (Stalmans et al., 2002). Endothelial cells also express low levels of VEGF themselves, which is necessary for vessel homeostasis, and possibly for sprouting migration through integrin regulation of VEGFR-2 (Lee et al., 2008).

Because VEGF-A has been identified as an essential factor for endothelial differentiation, it was initially used VEGF (50 ng/ml) from day 0-5. Comparing different conditions with or without VEGF over different experiments showed that VEGF is the key factor for generation of CD34+ cells and no expression was detected without VEGF. (Experiment 1 conditions 13, 14 and experiment 6 condition 11, 13). However, it is important to mention that not all conditions containing VEGF expressed CD34 expression (Conditions 2, 3, 4, 5, 6, 7 8, 9, 10 in experiment 3 and condition 4 in experiment 5).

In current experiment, it was intended to find out if VEGF is needed from day zero up to day five. Or if excluded from different time points during the treatment, whether it would still affect the induction of endothelial progenitor cells. This experiment was repeated three times and similar results were observed form both experiments. (n=3 biological replicate and n=9 technical replicate). Relative expression of CD34 was analysed in a semi-quantitative score method. It was found that adding VEGF only during the late phase of treatment and for only two days was sufficient to induce the expression of CD34 positive cells. Interestingly, Immunocytochemistry results of cells just before adding VEGF at day three, showed faint CD34 expression (arrows in **Figure 36** No VEGF condition) These weakly CD34 positive cells might be “precursors of progenitors of endothelial cells”, here designated “PPECs”.

Treatment No	Experiment 8 (iPSC, Extra 2.5% Matrigel)				Relative CD34+ Yeild	Cell Detachment	Coverage
	Day 1	Day 2	Day 4	Day 6			
8. 1	ActA / B4 / V / BIO**	ActA / B4 / V / BIO**	V / S	Fixed	#######	low	extensive
8. 2	ActA / B4 / BIO**	ActA / B4 / V / BIO**	V / S	Fixed	########	low	extensive
8. 3	ActA / B4 / BIO**	ActA / B4 / BIO**	V / S	Fixed	########	low	extensive
8. 4	ActA / B4 / BIO**	ActA / B4 / BIO**	S	Fixed	–	low	N/A

Table 14. Treatment conditions to assess the effect of VEGF **for different time points.**
Data with n=2 biological replicate and n=6 of technical replicates are shown. The experiment was repeated 3 times with similar results. (n=3 biological replicates). Cells were seeded onto Matrigel-coated 96-well plates in 3D culture condition at 4 x 10^4 cells/well. Experimental timelines ran 6 days long and consisted of growth factors addition in fresh medium on day 1, 2 and 4. Cells were fixed and stained on day 6. Relative CD34+ cell yield (number sign), cell detachment (low/ medium/ high) and CD34+ coverage (disperse/ aggregated / extensive) were qualitatively assessed for each treatment condition. Condition 1 was a repeat of condition 8 in experiment 6 which also showed high expression of CD34. Condition 2 had VEGF from day 2 to 5 which had relatively high expression of CD34 compared to condition 1. Interestingly, condition 3 containing VEGF from day 3 to 5 had high expression of CD34 which was comparatively more than condition 1. Condition 4 with no VEGF did not show any expression of CD34. Very low cell detachment was observed in all conditions. iPSC: induced pluripotent stem cell; ActA: Activin A 25ng/ml; B4: Bone Morphogenetic Protein 4, 30ng/ml; V: Vascular Endothelial Growth Factor (VEGF) 50ng/ml; BIO: 6-Bromoindirubin-3'-oxime (Wnt pathway activator) 0.15 µM; S: SB431542 (inhibitor of ALK5, ALK4 and ALK7) 2uM.

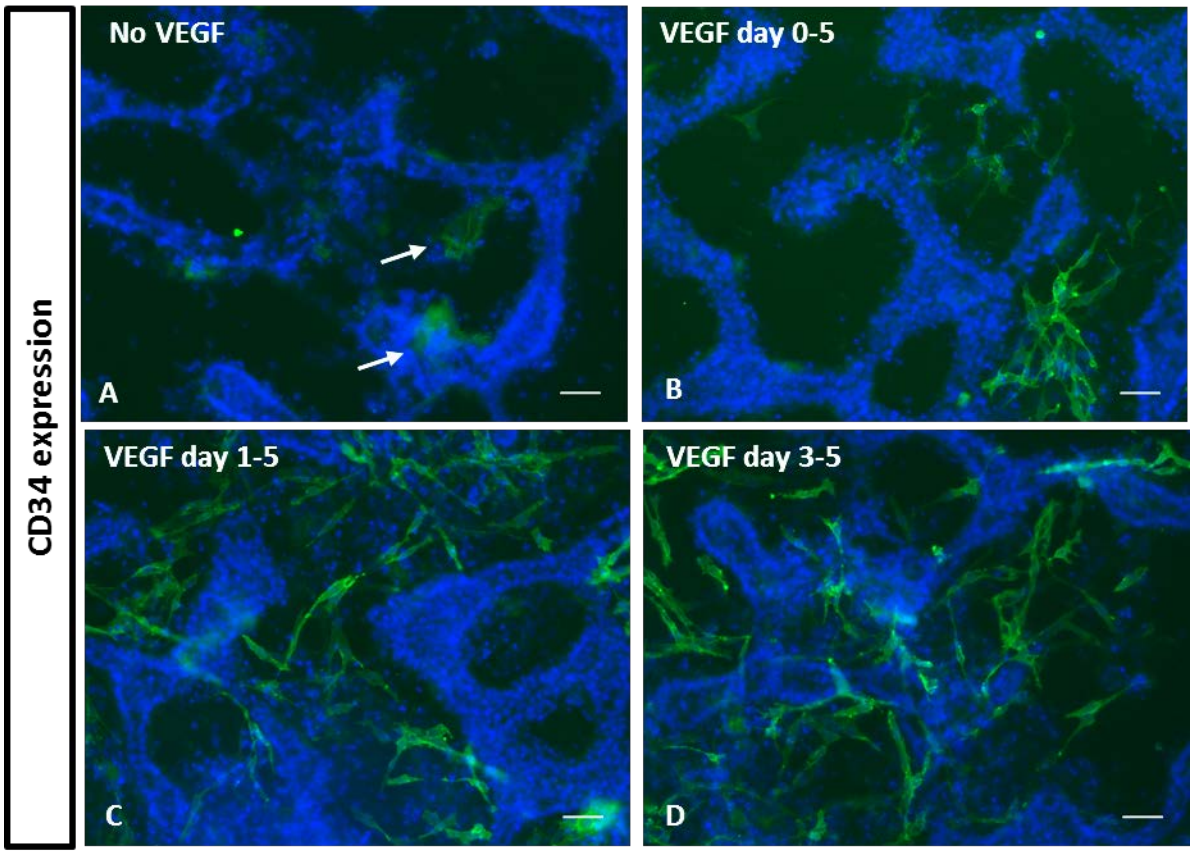


Figure 35. Immunofluorescent staining of the expression of PECs marker CD34 (green) for different time point effect of VEGF.

Four treatment conditions were used to assess the different time point effect of VEGF. The only difference between conditions is the VEGF that was added in different time points. No clear CD34 expression was observed in condition with no VEGF. Very low expression (faint staining) of CD34 was observed in the absence of VEGF (Arrows in A). These cells were called precursors of progenitors of endothelial cells "PPECs". Other conditions with different time points of VEGF had clear expression of CD34 which shows the importance of VEGF in induction of PECs. Interestingly, condition with only 2 days VGEF stimulation (D) had relatively higher CD34 expression compared to 4 and 5 days VEGF stimulation (B & C). Nuclei are stained with DAPI in blue. Scale bar, 64 µm.

However, in second set of experiments, using different iPS cell batch (generated by Lili Chen) strongly CD34 expressing cells appeared in condition containing only SB 431542 and no VEGF in three repeated experiments (n=3 biological replicates and n=6 technical replicates) **Figure 37.** Further observations revealed that against similar cell number seeded in the first place the cell density in the second set of experiments (iPS cells generated by Lili Chen) was much higher after five-day treatment compared to first set of iPS cells (generated by Amanda Carr). Therefore, it was assumed that because of high confluency and a lack of oxygen, cells

might have generated their own VEGF at this stage to compensate for their environmental conditions.

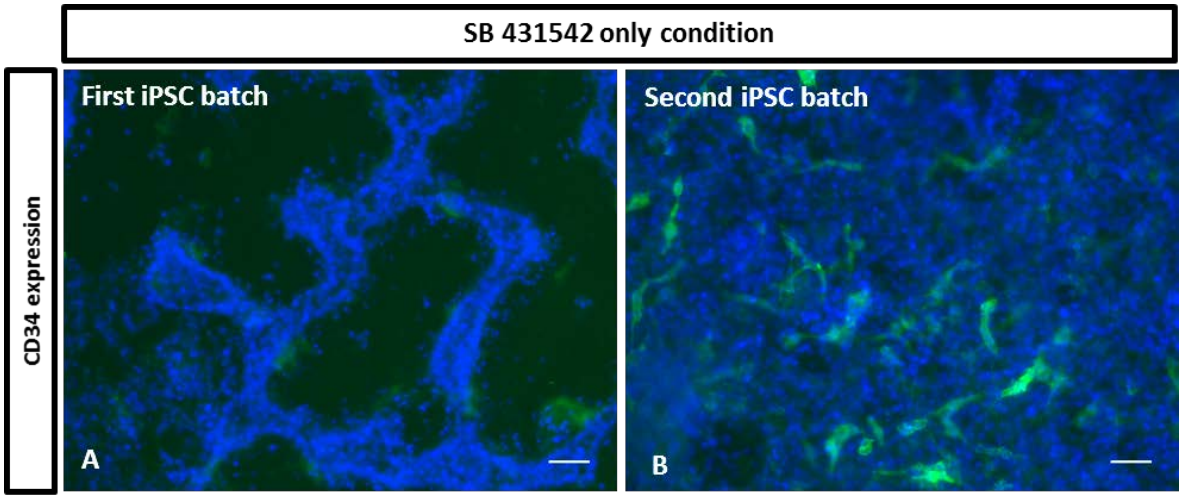


Figure 36. Illustrative picture of CD34 expression under similar conditions (SB 4311542 only). Data from two iPSC batches shows comparatively different expression patterns. The second batch of iPSC cells (B) expressed more CD34 compared to the first batch of CD34 at day five of treatment. (n=3 biological replicate and n=6 technical replicate). Scale bar, 64 μm.

To test for this, I used aflibercept (Eylea), vascular endothelial growth factor (VEGF) inhibitor (1:100 dilutions) which is an anti-VEGF drug (explained in introduction). It binds to circulating VEGF and acts as a "VEGF trap" for treatment of wet macular degeneration. In the current experiment, cells were treated based on the protocol established earlier. At day three of differentiation, Elyea was added in combination with SB 431542 and was maintained until day five. Then cells were fixed at day five for further immunostaining analysis. Experiment was repeated 2 times and each time in triplicate (n=2 biological replicates and n=3 technical replicates). Immunostaining results illustrated that in presence of Eylea, the expression of CD34 were highly suppressed (very dim expression) **(Figure 38)**. Using Eylea resulted in the generation of a homologous population of cells with no indication of CD34 expression.

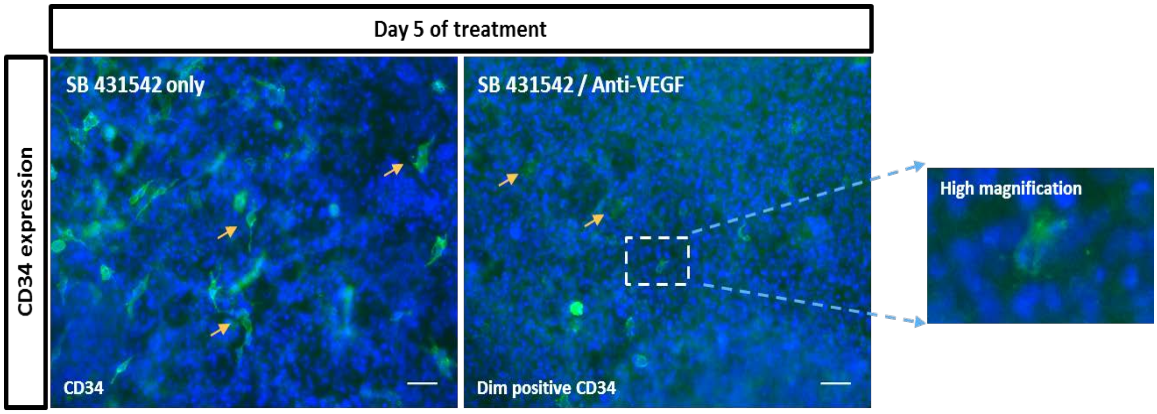
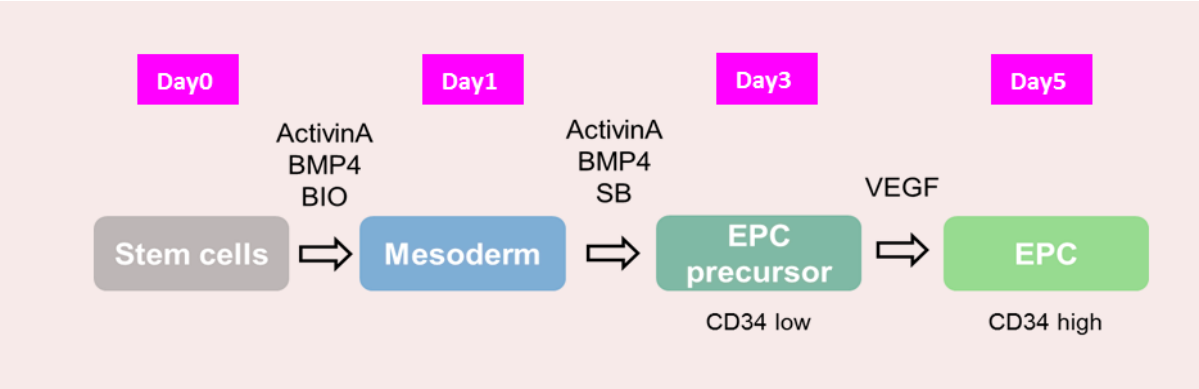
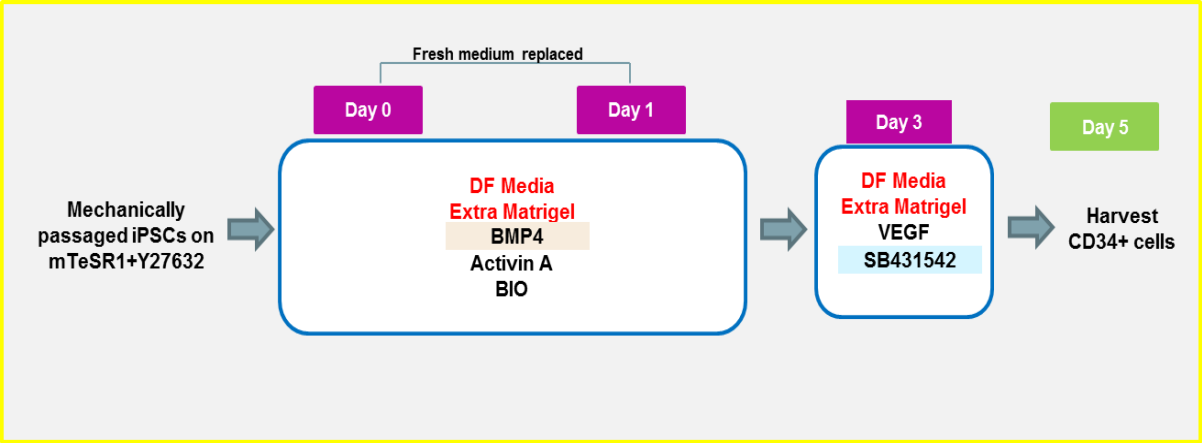


Figure 37. immunostaining of iPSC-derived PECs in the presence of SB 431512 only.
Data for five days shows relatively high expression of CD34 positive cells (A). However, using SB 431512 in combination with Eylea (VEGF inhibitor) remarkably suppressed the expression of CD34 positive cells, only some dimly positive cells could be observed (B). (n=2 biological replicates and n=3 technical replicate). Scale bar, 64 μm.

3.1.4.2 Summary of optimized protocol

I found that administration of BMP4, Activin A and BIO (Wnt signalling activator) at an early phase induce the cells through the mesoderm lineage. Further stimulation with VEGF and SB431542 (TGFβ-receptor type one inhibitor) during the second phase of treatment leads to efficient differentiation of PECs from human iPSCs within five days.





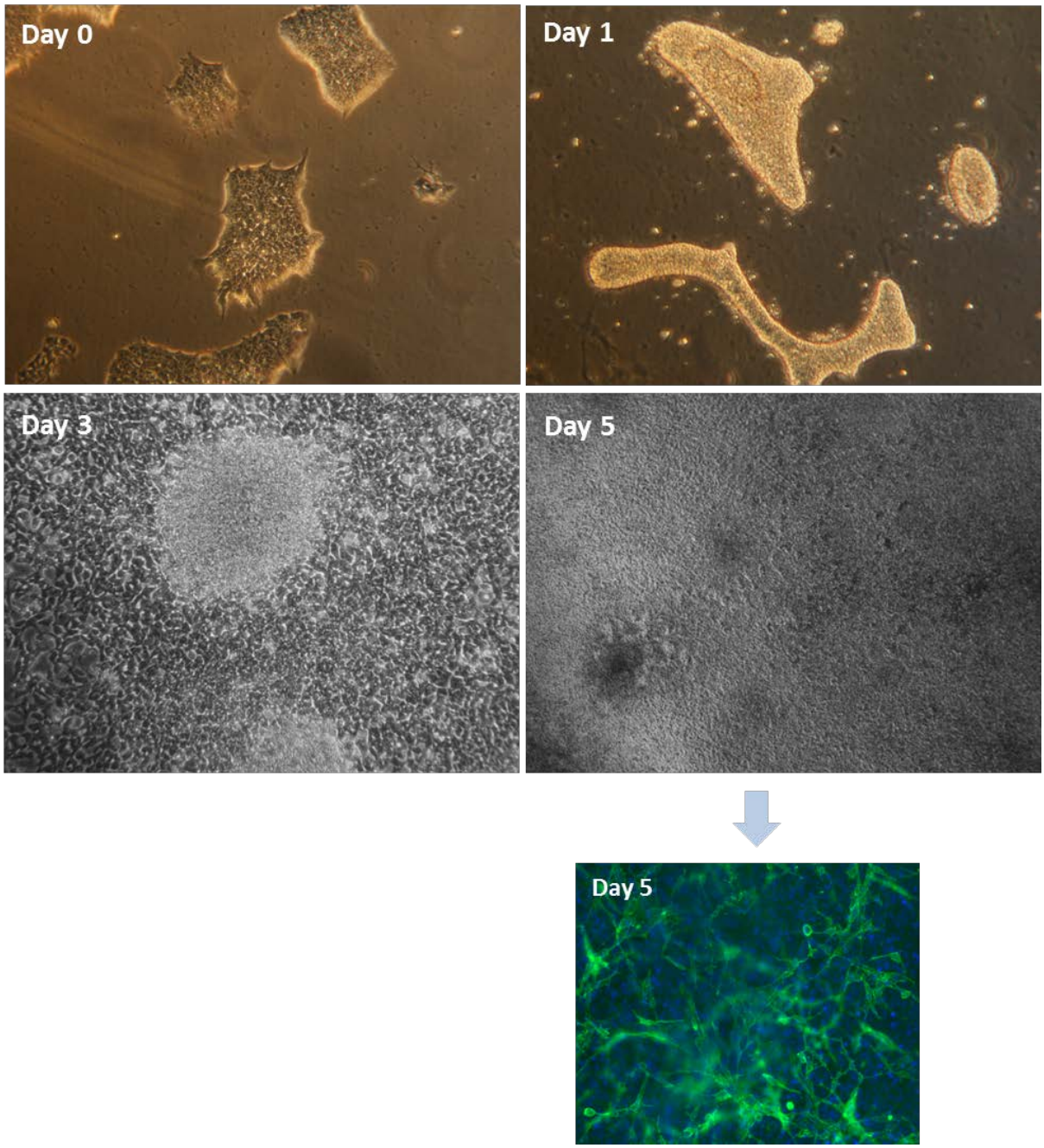


Figure 38. Figure 39. Schematic workflow of PEC differentiation from iPSC cells.
Bright-field microscopy shows the morphology of the cells at different stages. Undifferentiated iPSC cells in mTeSR1 before treatment at day zero (A), after one day of treatment (B) and after three and five days of treatment, respectively (B & C). Scale bar, 164µm.

3.1.4.3 Validation of iPSC-derived PECs

iPSC-derived PECs were purified with CD34-labeled beads using MACS technique at day 5 of differentiation. This experiment was performed two times in triplicate. CD34+ cells were plated on Fibronectin-coated plates in EGM-2+ 25% serum containing VEGF 165 (50ng/ml) and SB 431542 (2uM) for 4 days. Then they were fixed with 4% PFA and were assessed for the expression of the vascular endothelial markers PE-CAM and VE-Cadherin by immunocytochemistry. PE-CAM (CD31) and CD34 appear to be co-localised suggesting that they are really vascular endothelial cells. Furthermore, VE-Cadherin expression was also observed. However, CD34 and VE-Cadherin expression is inversely related. This suggests that CD34 expressing cells generated with our protocol are more at immature stage of progenitor cells and has not completely differentiated to ECs and are indeed PECs **(Figure 41)**

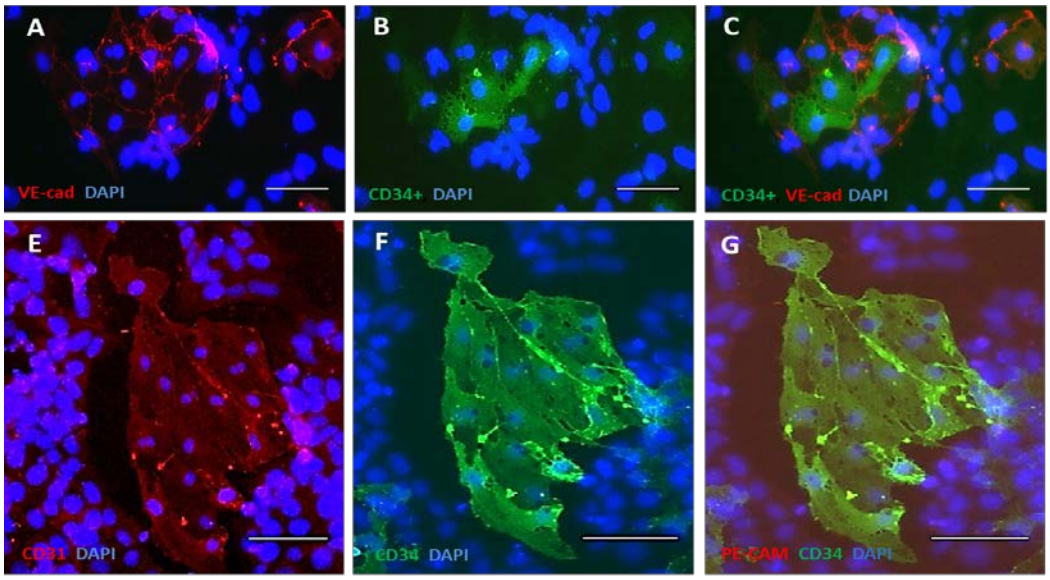


Figure 40. Representative immunofluorescence pictures of iPSC-derived PECs after 4 days plating on fibronectin.

iPSC-derived PECs were purified with CD34-labeled beads at day 5 of differentiation and plated on Fibronectin-coated plates for 4 days. First row, VE-cadherin (red); CD34 (green). Second row, CD31 / PE-CAM (red), CD34 (green). Nucleus (DAPI, blue). Experiments were performed two times and in triplicate. CD34 positive cells were negative for VE-Cadherin (A, B and C) which suggests that CD34 expressing cells are more at immature stage of progenitor cells and has not completely differentiated to ECs. Therefore, it could prove that cells are differentiating through the endothelial lineage. Furthermore, CD31 is co-expressed with CD34 at this stage (E, F, and G). Scale bar, 50 µm.

3.1.4.4 Isolation and expansion of iPSC-derived PECs

Next, iPSC-derived PECs were isolated by magnetic activated cell soring (MACS) after five days of treatment and were reseeded under different conditions to further investigate their differentiation towards an endothelial lineage. “MACSed” CD34+ and CD34- fractions were re-plated on two different substrates; Fibronectin and Matrigel. Both populations were cultured in EGM-2 medium for four days. Media were supplemented either with 1% or 25% FBS in combination with different factors (SB 431512, VEGF, BMP9) in order to assess proliferation. The morphology of the cells and their expansion was observed after four days; cells were then fixed for immunostaining analysis.

3.1.4.5 Morphology of the EC-derived PECs

Morphology of the cells after MACSing and reseeding for four days were accessed according to different parameters as follows;

A. Different serum concentration (1 % vs 25% FBS)
B. Different substrates (Matrigel vs Fibronectin)
C. Different factors (SB 431512, VEGF, and BMP9)

A) Different serum concentration (1 % vs 25% FBS)

Previous data on growing CD34-positive and CD34-negative cells after MACS showed a very low proliferation rate even after 10 days of plating in EGM-2 medium **(Figure 42C).** Therefore, the current experiment sought to establish the effect of adding extra serum. Two concentrations of serum (1% and 25% FBS) were used in this study. It was observed that CD34+ cells just four days after MACSing and plating on Fibronectin, were more confluent in **EGM-2+25% serum** (**Figure 42A)** compared to **EGM-2+1% serum (Figure 42B).** However, proliferation was considerably affected by other parameters (substrate and factors).

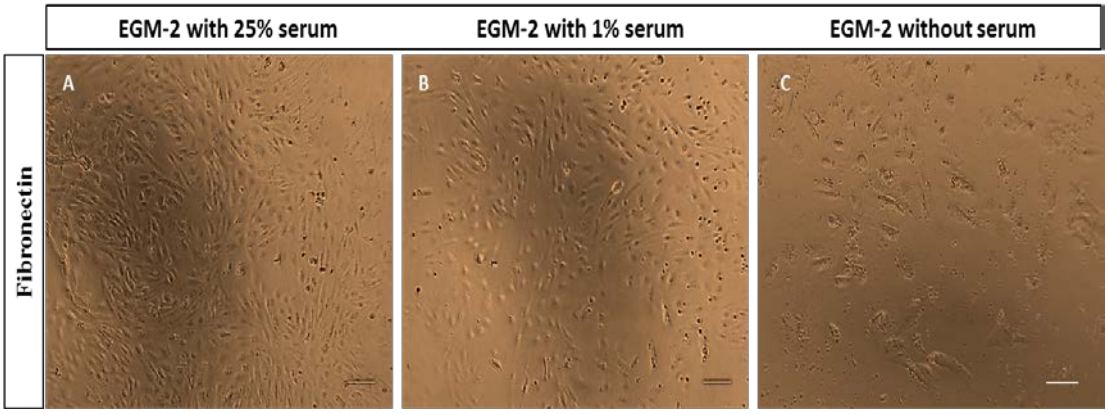


Figure 41: Bright-field microscopy images of isolated PECs after 4 days in different serum concentrations.

PECs were generated using the optimized protocol and were MACSed sorted. CD34+ cells were plated on Fibronectin for four days in EGM-2 medium in combination with 25% (A) or 1% (B) FBS concentrations. PECs plated in EGM-2 without FBS (C) was also provided to compare with other conditions. Cells proliferated noticeably higher in 25% serum (A) compared to 1% (B) or no serum culture condition (C). Scale bar, 100 µm.

B) Different substrates (Matrigel vs Fibronectin)

Two substrates (Fibronectin and Matrigel) were chosen to find which one was more effective for final differentiation of endothelial cells. Light microscopy observations showed proliferation was remarkably high on Fibronectin coated plates compared to Matrigel-coated plates **(Figure 43)**.

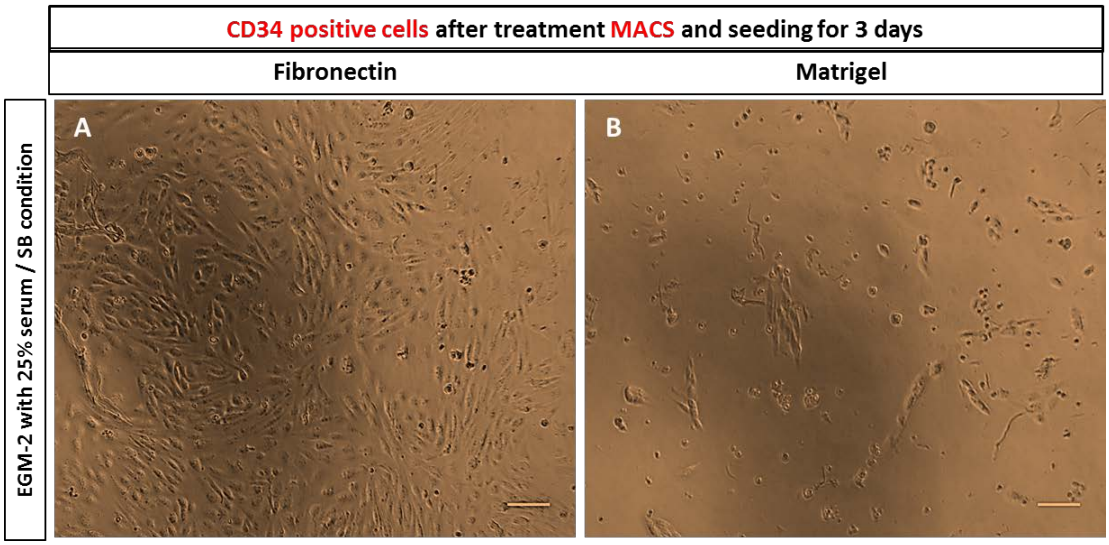


Figure 42: Bright-field microscopy images of isolated PECs after 4 days in different substrates. PECs were generated using the optimized protocol and were MACSed sorted. CD34+ cells were seeded on two different substrates; Fibronectin (A) and Matrigel (B) in EGM-2 + 25% FBS for 3 days. High cell proliferation was found on Fibronectin-coated plates (A), compared to very low proliferation on Matrigel-coated plates (B). Scale bar, 100 µm.

C) Different factors (SB 431512, VEGF, and BMP9)

Since factors such as VEGF and SB 431215 were used only at the late phase of treatment for two days in the our five day differentiation protocol, the effects of these factors in more homogenous population of PECs and for a longer period of time were sought. Furthermore, because of the effect of BMP9 on proliferation, it was chosen as another factor. Good compatibility of VEGF was observed with both substrates whereas SB431542 and BMP9 had optimal proliferation only on Fibronectin-coated plates. Furthermore, the morphology of the cells in conditions containing SB 431542 (SB only and SB 431542 +VEGF) was different from other conditions. Cell shapes were more spindle-like, while those grown in other conditions (VEGF and VEGF+BMP9) were more cobblestones shaped. Therefore, it seems that SB 431542 stimulates endothelial cell differentiation **(Figure 44).**

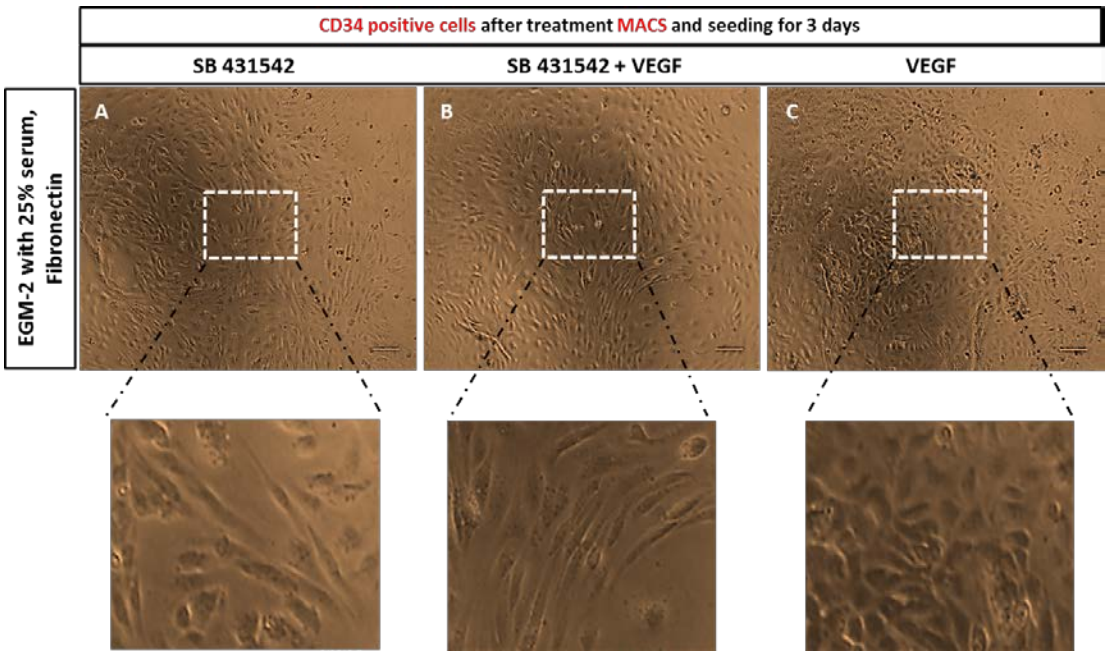


Figure 43**: Bright-field microscopy images of isolated PECs after 4 days in different conditions. PECs were generated using the optimized protocol and were MACSed sorted. CD34+ cells were plated on Fibronectin for three days in EGM-2 medium + 25% FBS in combination with different factors SB 431542 (A), SB 431542 + VEGF (B) and VEGF only (C). Cells were more spindle-like shape in the presence of SB431542 (A & B), which confirms endothelial nature of these cells compared to VEGF condition with more cobblestone-like shape (C). Scale bar, 100 µm.**

In the other two conditions (BMP9 alone, BMP9 + VEGF), a considerable reduction was observed in the proliferation on Matrigel-coated plates compared to Fibronectin-coated plates. This is consistent with some unpublished data suggesting that some substrates attract more angiogenic stimuli than others and cells do not behave in the same way on different substrates regardless of similar factors **(Figure 45)**

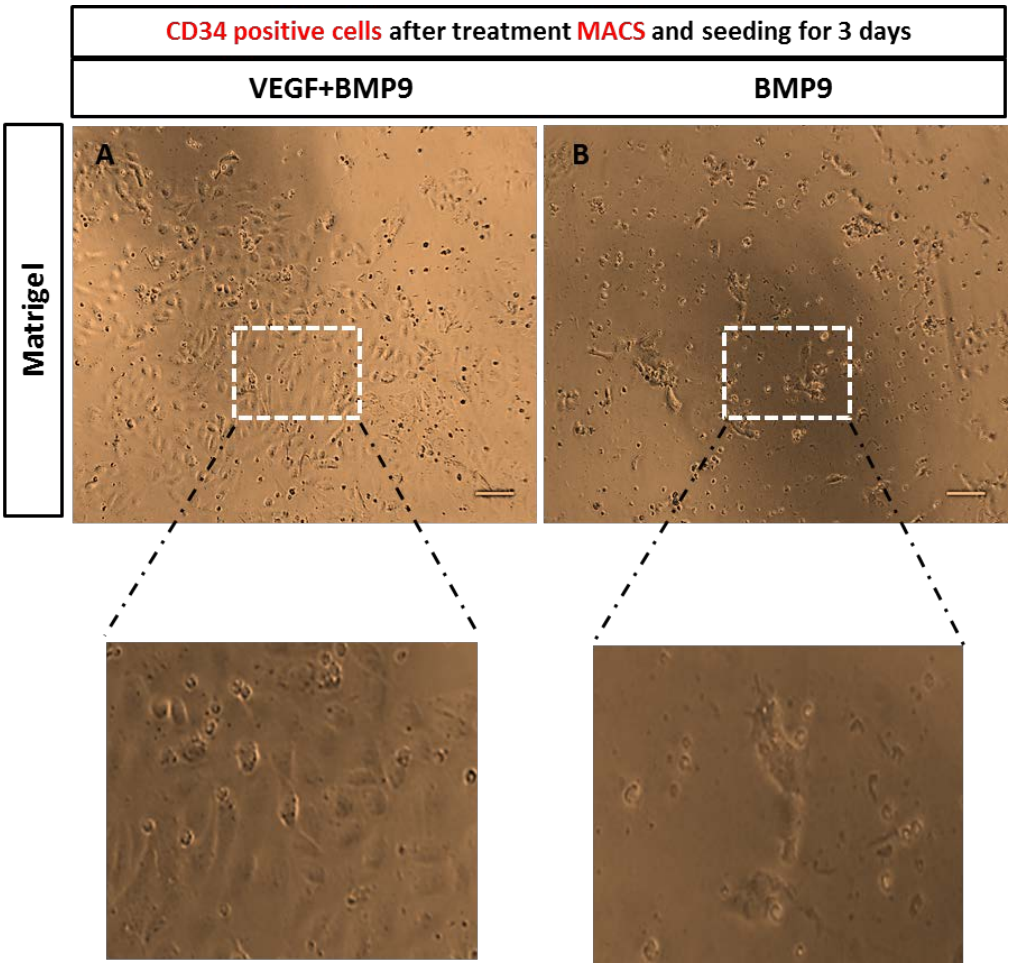


Figure 44**: Bright-field microscopy images of isolated PECs after 4 days on Matrigel on two conditions. PECs were generated using the optimized protocol and were MACSed sorted. CD34+ cells were plated on Matrigel for three days in EGM-2 medium + 25% FBS in combination with different. Cells are proliferating more in VEGF+BMP9 (A) than BMP9 only (B) condition. Scale bar, 100 µm**

3.1.4.6 Immunostaining of CD34+/CD34- after MASCing and plating for four days

MACSed iPSC-derived PECs plated on Fibronectin and Matrigel were fixed after four days and immunostained to assess the expression of endothelial marker (VE-Cadherin) also CD34. Immunostaining analysis showed that CD34+ cells plated on Fibronectin lose the expression of CD34 after four days and differentiate into more mature endothelial cells **(Figure 46).** Nevertheless, PECs plated on Matrigel under similar conditions did not show much proliferation and did not differentiate into endothelial cells after four days. Instead, cells were still highly expressing CD34 **(Figure 47).** Immunostaining results of CD34-negative cells did not show any expression of VE-Cadherin on either substrate **(Figure 48).** These data suggests that choosing a suitable substrate in one of the crucial factors in detecting the cells into specific lineage albeit in combination with suitable factors.

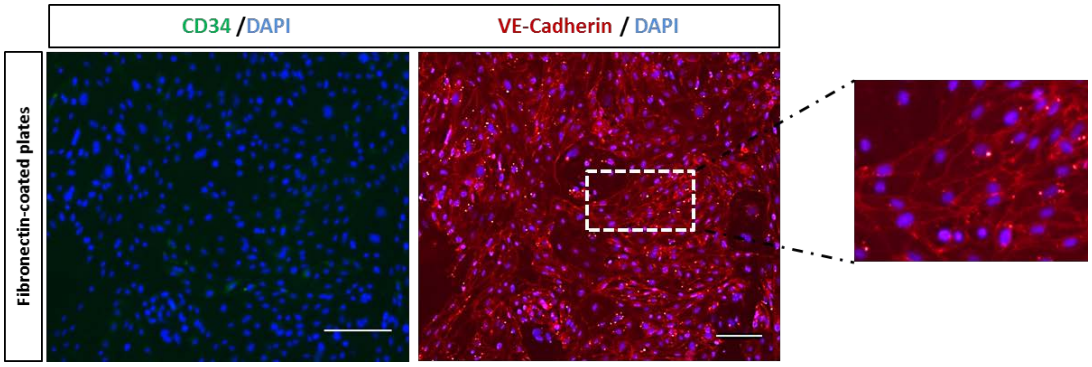


Figure 45: VE-Cadherin and CD34 immunocytochemistry.
On isolated PECs grown for 4 days on Fibronectin shows that the vast majority of the isolated cells express the endothelial cell marker VE-Cadherin (B), whereas CD34 has been strongly reduced (A). Nuclei are stained with DAPI in blue. Scale bar, 50 µm.

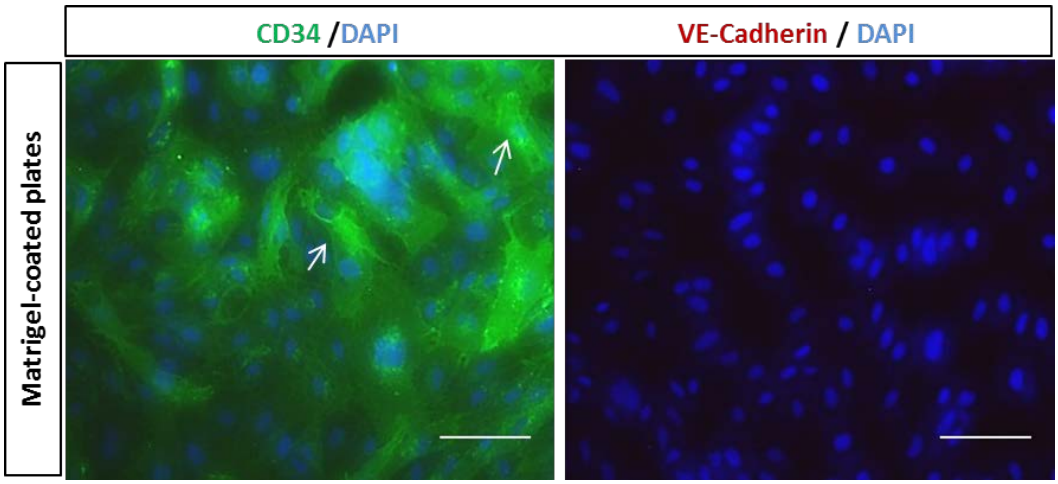


Figure 46: VE-Cadherin and CD34 immunocytochemistry.
On isolated PECs grown for 4 days on Matrigel shows that cell are still expressing CD34 (A) and there is no expression of VE-Cadherin after four days (B). Nuclei are stained with DAPI in blue. Scale bar, 50 µm.

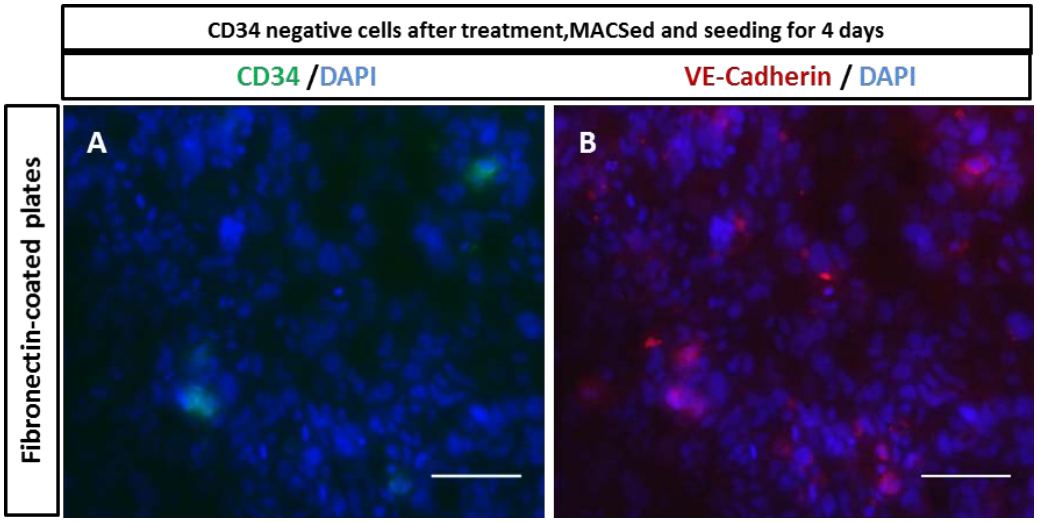


Figure 47: VE-Cadherin and CD34 immunocytochemistry.
On CD34- cells on Fibronectin-coated plates after four days. No expression of VE-Cadherin (red) or CD34 (green) was observed in either substrate (A & B). Nuclei are stained with DAPI in blue. Scale bar, 50 µm.

3.1.5 Discussion

hES cells

The aim of this section was to use hESC as potential sources to derive PECs. However, I failed to improve the derivation of PECs from hESCs on the bases of the protocols that already existed in the lab. This was most likely due to the very high passage numbers of our hESCs and I therefore abandoned all works with hESCs and switched the attention to iPSCs because I had access to iPSC lines with low passage number (<20). In contrast to the hESCs, iPSCs line yielded much more reliable and consistent results and my PEC derivation protocol could be rapidly improved.

iPS cells

The protocol I eventually developed consists of these steps:

A) Plating cells at high density and leaving them for two days to settle down in mTeSR1 medium

B) Exposure to mesodermal inducing factors

C) Stimulation of endothelial lineage to induce PECs

Initial mesodermal commitment was achieved using a cocktail of BMP4, Activin A and BIO in serum-free culture conditions for three days, EC lineage commitment was achieved with VEGF and SB431542 (Alk5 inhibitor) for two days. This led to efficient differentiation of CD34 positive cells from hiPSCs within five days.

Studies have shown the importance of BIO (WNT signalling activator) in the presence of Activin A (as a part of TGF-ß) in a very early phase of mesodermal induction (Hansson et al., 2009). The current research also found the presence of these two factors to be crucial in the early phase. Although, BMP4 has been shown to play a significant role in differentiation of stem cells towards a mesodermal lineage (Nostro et al., 2008, Ying et al., 2003), in our hands administration of BIO and Activin A with or without BMP4 resulted in high expression of CD34. This suggests that BMP4 is not mandatory for the early phase of mesodermal induction. However, the cluster-like morphology of CD34 expressing cells in the presence of BMP4 (versus singly dispersed cells in the absence of BMP4) BMP4 might influence migration and proliferation of already

committed precursors rather than their determination. In contrast, the crucial role of VEGF in endothelial lineage commitment is well-known. However, our finding that VEGF is not needed during the early phase of differentiation is less well known. In fact, most investigations aiming to derive ECs or PECs from stem cells add VEGF right from the beginning of their differentiation protocols.

Another major finding in our study was the discovery that adding extra Matrigel on top of the cells could dramatically enhance the numbers of CD34+ cells in our cultures. This might be caused by an improvement of the 3D matrix around cells, mimicking a more natural microenvironment.

Overall, this protocol was shown to be a very efficient method, which was time effective, involved fewer steps compared to other published protocols, required less cell manipulation and was reproducible over repeated experiments. It was demonstrated that at the end of this protocol, over 40% of cells were expressing the CD34 marker. Generated PECs exhibited strong expression of CD34 after five days of differentiation. Isolation of the CD34+ PECs (by MACS) clearly demonstrated that these cells are committed to the EC lineage as they expressed several EC markers.

Two iPS cell batches used in this work were generated by two different lab members (Amanda Carr and Lili Chen) but from one donor (BJ iPS cell line). However, beside of the similarity of the protocol and the source of somatic cell line (BJ skin fibroblast) that was used to generate the iPS cells, it appeared that iPS cells behaved slightly different from batch to batch in their proliferation rate and further response to specific factors such as “SB 431542 only condition” during the differentiation protocol. This could demonstrate the sensitivity of each iPS batch and indicates that how the confluency of the cells can affect their response to various growth factors which is essential for directing the differentiation process from early mesendoderm via mesoderm towards a more differentiated PECs. The sensitivity of iPS cells in response to stimulus factors was also evident within one cell line but different passage numbers. It was observed that as the passage number was increasing the capacity of iPS cell to differentiate into PECs were reducing and this was noticeably evident after passage number above 22. The limitations of the cell lines at high passage number should be taken into consideration especially in the level of cell therapy products for therapeutic applications. Routine cell line monitoring including cell morphology checks, growth

rate or protein expression levels needs to be established to maintain consistent cell performance.

Furthermore, normal maturation/ differentiation process within the developing organ in vivo in the body from very blastocyst stage to differentiated EC takes more than three weeks during embryogenesis. However, the established protocol in this study take the advantage of 3D culture system and different stimulus factors to reduce the different process to 5 days. This could raise this question that how similar are the generated cells to in vivo generated cells.

However, it is important to consider that although by using the established protocol, remarkably high number of CD34 was generated, more detail studies are needed to define whether this is heterogeneous cell population or a single cell type that can differentiate into more mature endothelial cells. Furthermore, it would be interesting to test whether these cells can be injected into mouse retina or into diabetic retinopathy mouse models to generate functional vessels.

Moreover, concerns regarding the use of iPS cells in further therapeutic application should be considered. Reports on differences in gene expression suggest that reprogramming in iPSC is not complete (Saric and Hescheler, 2008).

Furthermore, this differentiation protocol similar to other differentiation protocols rely on multistep and time-sensitive protocols which require subjecting cells to administration of timed growth factor supplementation. However, these types of protocols might not be efficiently compatible within the in vivo culture condition. Thus, the ability to dynamically regulate the expression of bioactive factors and temporal control of them is a major step to translate these systems into clinically relevant 3D approaches (Leijten and Khademhosseini, 2016).

I also looked at the factors that influence PEC to EC conversion. Using PECs isolated with MACS I found that high serum concentration (25%) dramatically improves proliferation and differentiation into endothelial cells (EC). It is likely that specific factors in serum are responsible for this, but their identity has yet to be established. Furthermore, we also tested the effect of substrate by comparing Matrigel versus Fibronectin.

Morphologically, the expanded cells on Fibronectin-coated plates formed a more confluent monolayer of endothelial cells compared to Matrigel coated plates. Moreover, PECs grown on Fibronectin showed low CD34 expression and high expression of VE-Cadherin, suggesting further differentiation towards a mature EC phenotype. In contrast, PECs grown on Matrigel still expressed CD34 after four days of culture. This data could suggest that Fibronectin favours the differentiation of PECs into EC.

Furthermore, it was observed that not all factors were compatible with all substrates. Our data demonstrated that cells grown on Matrigel, even in high concentrations of serum, had a very low proliferation rate in the medium containing BMP9, whereas cells in similar conditions but different factors (BMP9 + VEGF) showed noticeably higher proliferation. This suggests that cells do not behave in the same way on different substrates, regardless of factors applied.

Another interesting observation was the effect of SB 431542 (Alk5 inhibitor) on MACSed cells after four days. No big difference was found on cells grown on BMP9 and VEGF (on Fibronectin). In both conditions, cells had cobblestone-like appearances. However, in the SB 431542-containing conditions cell proliferation was high and the morphology of the cells was spindle-like shape. Previous studies have shown that embryonic cells in their immature stage are more sensitive to TGFß-mediated growth inhibition than their mature stage (James et al., 2010). Thus the noticeable changes in the morphology of SB 431542 treated cells could be explained by the more immure phenotype of these cells.

In summary, our protocol could be used as a platform to develop EPCs into a more reliable therapeutic product. However, one of the main issues that should be addressed before translation of these cells is the limitation of using Matrigel in clinical applications. Matrigel is a blend of extracellular matrix proteins derived from a murine tumour that contains many basement membrane and ECM components (such as laminin and collagen IV), as well as matrix degrading enzymes and different growth factors (Kohen et al., 2009). Matrigel is xenogeneic in origin, contains undefined components and can be immunogenic. It has relatively high production costs, is hard to scale up for wide-spread in clinical use, exhibits lot-to-lot variability, has an inherent risk of pathogenic contamination (Carlson Scholz et al., 2011) and difficulties with sterilization limit the scalability potential (Martin et al., 2005). Therefore, replacement with a

completely synthetic environment that is amenable with current good manufacturing practice (cGMP), would offer significant benefits toward a source of clinically usable stem cells (Enam and Jin, 2015). Generating suitable 3D environments is likely to be key for the success of such approaches.

3.2 Human Umbilical Vein Endothelial Cells (HUVECs)

3.2.1 Introduction

This thesis is focused on CD34+ PECs. Apart from generating them from iPSCs in culture (previous chapter), I am also interested in comparing these cells and their properties to other cells. In this context, it was decided to use HUVECs as a reference, representing fully differentiated ECs. In my previous experiment, I found that differentiating PECs down-regulated CD34 expression as they matured towards an EC phenotype. Therefore, initially it was assumed that HUVECs would not express CD34. However, recently it was demonstrated that a subset of migratory CD34+ exists in HUVEC monolayers. These cells have gene expression profiles similar to tip cells, whereas CD34- HUVECs were enriched for genes related to proliferation and possibly stalk cells (Siemerink et al., 2012). The precise mechanisms that regulate the expression of CD34 in HUVEC cultures are not understood. I, therefore, decided to investigate these mechanisms before using HUVECs as reference cells in our PEC study. This will enable us to better control HUVEC phenotypes, but it might also provide some insight into tip and stalk cell biology.

3.2.2 Choosing a serum-free medium

The first step was to choose a suitable serum-free medium for HUVEC culture so that I could investigate the effects of different growth factors. Human Large Vessel Endothelial Cell Growth Medium (LVEM) (Cellworks ZHM-2953) was chosen which is defined medium contains Heparin, bFGF, EGF, and hydrocortisone. In the first instance, the intention was to find out if it is possible to exclude bFGF2 or EGF to reduce potential cross effects of these factors while keeping the cells in optimal growing conditions.

To this end, HUVECs were cultured in LVEM containing each of the factors alone or in combination (EGF only, bFGF only, or EGF + bFGF) (each 10ng/ml). The EGM-2 and LVEM+ supplements were used as positive controls while LVEM (without supplement) was used as a negative control. Cells were left in these conditions for four days while a fresh medium was provided every other day. Finally, the density of the cells was observed by bright field microscopy **Figure 49**.

This showed over 90% cell death in LVEM without the supplemented medium (Figure 1A), which indicated that cells needed supplement containing factors (EGF and bFGF) to survive. Cells in conditions containing EGF alone and bFGF alone were sparser. Comparing these two conditions showed that cells were proliferating relatively better in bFGF than in EGF, which could suggest that bFGF is more important than EGF for HUVEC proliferation (Figure 1D, E). The condition containing both factors (EGF + bFGF) was similar to condition B (LVEM+ supplement), which points out that both factors were needed to keep the cells in the optimal growing condition. Therefore, LVEM+ supplements were chosen to investigate the effect of other factors in later experiments.

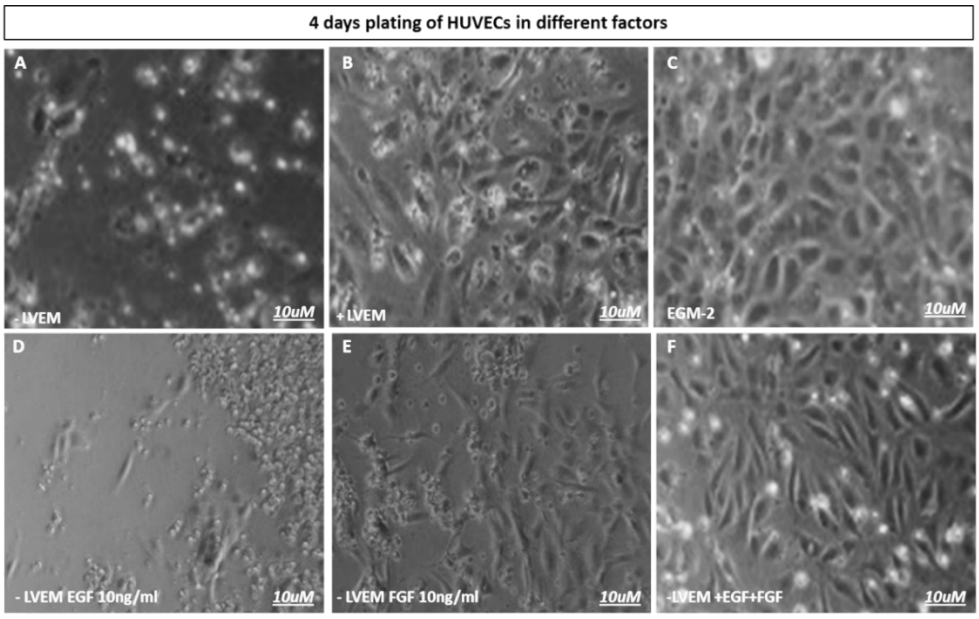


Figure 48: Effects of factors (EGF & bFGF) in LVEM supplement on HUVEC survival. Light microscope images illustrated that cells in Large Vessel Endothelium Medium (LVEM) without supplement could not survive after four days (A). Cells in LVEM+ supplement and EGM-2 as positive controls survived after four days as expected (B & C). However, cells in LVEM to which each factor was added separately (EGF and bFGF) (10ng/ml) had low proliferation rate (D & E). The condition containing both factors together showed high proliferation rate (F). Scale bar, 64 µm.

3.2.3 Effect of VEGF on CD34 expression and tip-stalk phenotypes in HUVECs

VEGF has been demonstrated to be a key factor in inducing tip cell phenotype during angiogenesis (Gerhardt et al., 2003). Since CD34+ HUVECs expressed tip cell markers, I wanted to see whether VEGF can influence the expression of CD34 in HUVECs.

HUVECs were plated on 24 well plates in EGM-2 (2% FBS) medium. When they reached 80% confluency, the medium was switched to LVEM+ for 24 hours. Then, the cells were stimulated with VEGF (25ng/ml) for 24, 48, and 120 hours. After each time course, cells were fixed with 4% PFA, and the expression of CD34 was assessed by immunocytochemistry and fluorescent microscopy and quantified using ImageJ. To quantify the number of CD34+ cells in all experiments and compare them between different conditions, five random images were taken from each stained coverslip. Each picture was scored blinded using the following scale: 1, for each faint CD34 expression; 2, for each clear CD34 expression; 3, for each very high CD34 expression. Scores were summed up for each picture, and this number was divided by the total cell number (nuclei were stained using Hoechst 33342) for each picture. The average score from five images was considered as 1 technical replicate. Each set of experiment had 2 to 3 replicates and each experiment design were repeated three to six times in different time course (considered as biological replicate). All statistical analysis was done in SPSS version 24 with the assistance of statistician (Ali Hadian).

Immunostaining results from current experiment showed that the expression of CD34 increased over time. Five days of VEGF stimulation considerably increased the expression of CD34-positive cells and statistical analyses showed a significant difference in CD34 expression after 48 and 120hrs respectively **Figure 50.**

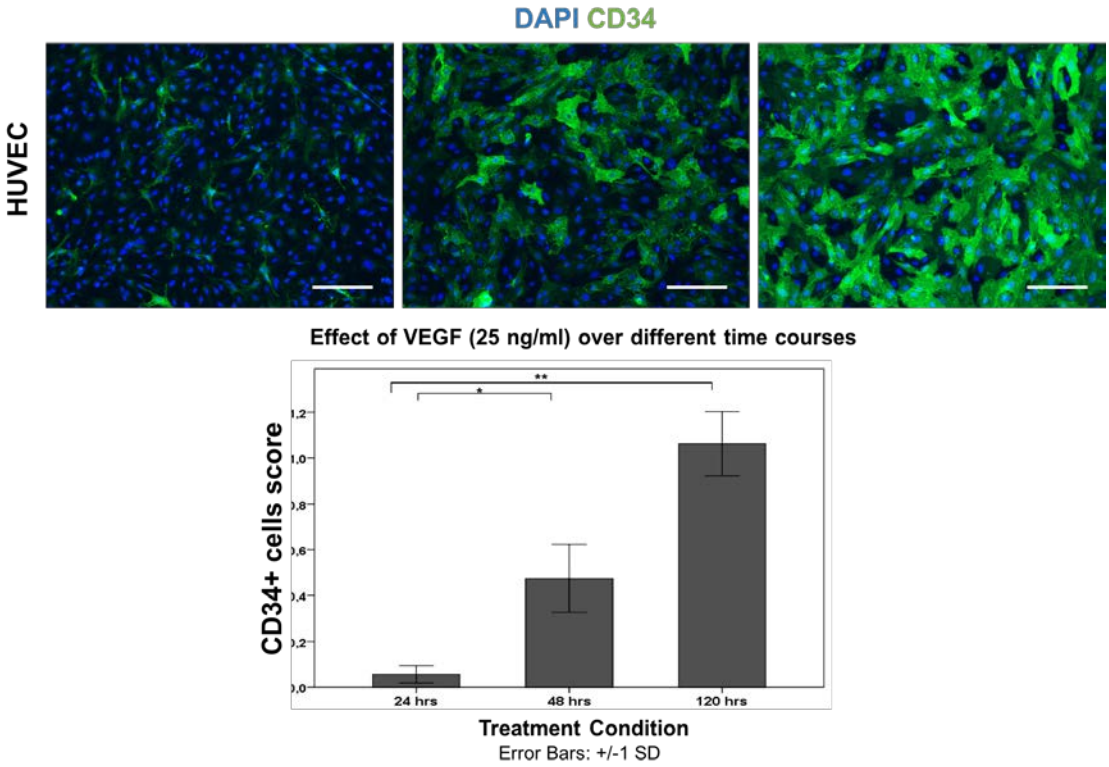


Figure 49: Effect of VEGF (25ng/ml) for different time courses on the expression of CD34+ cells on HUVECs.
(a) Immunostaining of the cells showed significantly high expression of CD34-positive cells (green) after 120 hours compared to 24 and 48 hours. (n=3 biological replicates & n=6 technical replicates) (b) To quantify the CD34 + cells a one-way repeated measures ANOVA was conducted to determine whether there were statistically significant differences in CD34+ score over the three time courses. There were no outliers and the data was normally distributed, as assessed by boxplot and Shapiro-Wilk test ($p > .05$), respectively. The assumption of sphericity was met, as assessed by Mauchly's test of sphericity, $\chi2(2) = 2.246$, $p = .325$. The VEGF effect elicited statistically significant changes in CD34+ score over time, $F(2, 6) = 58.573$, $p < .0005$, partial $\eta2 = 0.951$, with CD34+ score increasing from 24hrs (M = 0.057, SD = 0.038) to 48hrs (M = 0.475, SD = 0.148) to 120hrs (M = 1.063, SD = 0.140). Post hoc analysis with a Bonferroni adjustment revealed that CD34+ score was statistically significantly increased from 24hrs to 48hrs (M = 0.418, 95% CI [0.14, 0.7], p = .017), and from 24hrs to 120hrs (M = 1.006, 95% CI [0.6, 1.42], p = .004), but not from 48hrs to 120hrs (M = 0.588, 95% CI [-0.017, 1.192], p = .054). Scale bar, 64 µm.

3.2.4 Interaction of Notch and VEGF signalling in differentiation of CD34+ cells in HUVECs

Since the interconnection of VEGF signalling with Notch signalling plays a crucial role in specifying the tip/stalk cell phenotype in vivo (Jakobsson et al., 2009), it was intended to find out whether Notch signalling could also be involved in the regulation of CD34 expression in HUVEC monolayer. To this end, I used the Notch inhibitor, DAPT, which in vivo increases the number of tip cells. I designed an experiment considering the combination of VEGF 25ng/ml and different concentrations of DAPT to detect the effect of Notch inhibition on the differentiation of CD34+ cells. HUVECs were plated in 24 well-plates in the EGM-2 medium. After reaching 70% confluency, the cells were switched to the LVEM+ medium for 24 hours. Then, the Notch signalling pathway was inhibited by adding two concentrations of DAPT (2 and 10uM) to HUVEC cell containing VEGF (25ng/ml) for 24 hours. Cells were then fixed with 4% PFA, and the frequency of CD34+ cells was assessed by immunocytochemistry and fluorescent microscopy and quantified using ImageJ. Immunostaining data showed considerable increase in CD34 expression in cells containing VEGF + DAPT 2uM and VEGF + DAPT 10uM respectively compared to VEGF (25ng/ml) only condition. Therefore, this

could mirror the behaviour of tip cells in vivo exposed to high levels of VEGF at the tip of sprouting vessels.

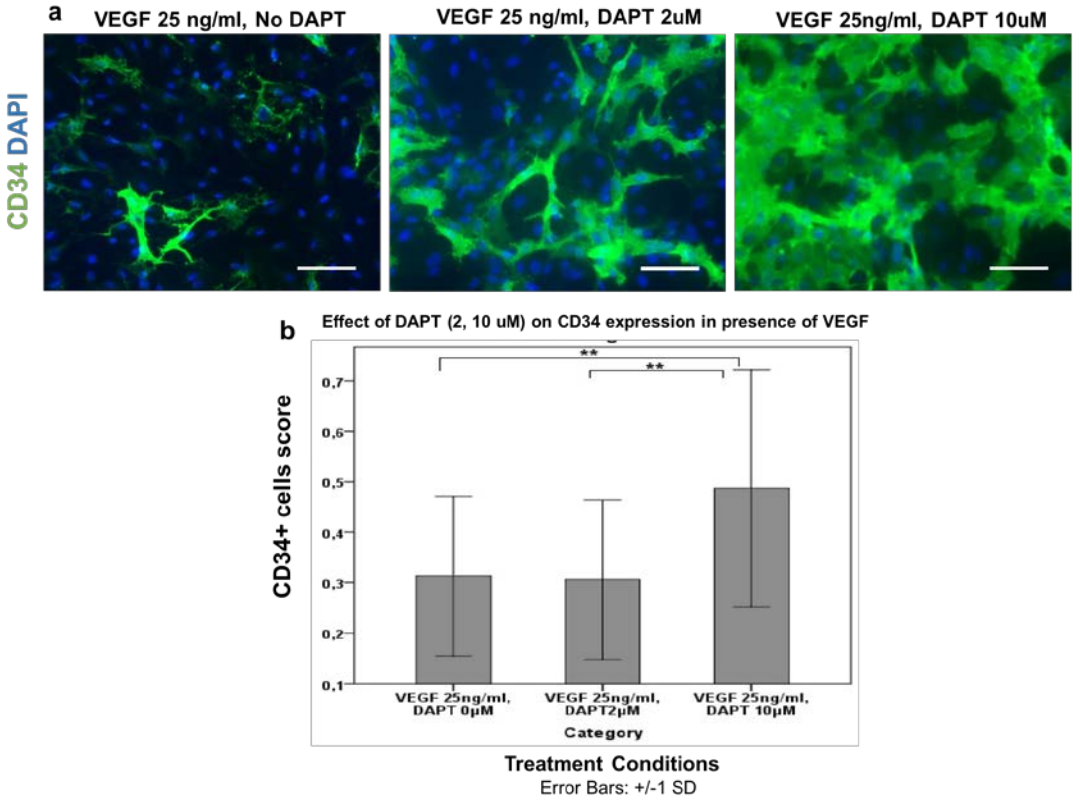


Figure 50: Immunostaining and statistical data from the effect of DAPT (2 & 10uM) in the presence of VEGF (25ng/ml).
(a) Representative pictures of HUVECs expressing CD34 after stimulation with VEGF 25ng/ul in combination with DAPT (2 &10 uM). Immunostaining results showed considerably higher expression of CD34 expression in the presence of DAPT (2 & 10 uM) respectively compared to VEGF only conditions (n=3 biological replicate & n=7 technical replicate). (b)To quantify the CD34 + cells, A one-way Welch ANOVA was conducted to determine whether there were statistically significant differences in CD34+ score in the presence of DAPT with different concentrations (2 & 10uM). There were no outliers, and the data was normally distributed for each group, as assessed by boxplot and Shapiro-Wilk test ($p < .05$), respectively. Homogeneity of variances was violated, as determined by Levene's Test of Homogeneity of Variance ($p = .045$). CD34 score was statistically significantly different between the various DAPT groups, Welch's $F(2, 62.489) = 7.776$, $p < .005$. CD34 score increased from the VEGF25 DAPT2 group (0.306 ± 0.158) to the VEGF25 DAPT0 (0.313 ± 0.158), and VEGF25 DAPT10 (0.487 ± 0.235) group, in that order. Games-Howell post hoc analysis revealed that the increase from VEGF25DAPT2 to VEGF25DAPT10 (0.181, 95% CI (0.063 to 0.299) was statistically significant ($p < .0005$), as well as the increase from VEGF25DAPT0 to VEGF25DAPT10 (0.174, 95% CI (0.056 to 0.292), $p = .002$). Scale bar, 32 µm.

3.2.5 ALK1 and its effect on CD34 differentiation

Because it has been shown that blockade of ALK1 enhances tip cell formation in vivo (Larrivee et al., 2012), I wanted to see whether blocking ALK1 in HUVECs increases CD34 expression similar to DAPT (as shown in previous section).

In this experiment, HUVECs were plated in 24 well plates in the EGM-2 medium. After reaching 70% confluency, cells were switched to the LVEM+ medium to starve for 24 hours. Then, the cells were treated with different concentrations of VEGF (1, 5 and 25ng/ml) in combination with constant concentration of ALK1-inhibitor for 24 hours. Cells were then fixed with 4% PFA and the frequency of CD34+ cells was assessed by immunocytochemistry and fluorescent microscopy and quantified. Immunostaining data showed that the inhibition of ALK1 increased the CD34 expression, which was relatively notable at the high concentration of VEGF (25ng/ml). However, statistical analysis did not show significant difference between any VEGF-only groups vs VEGF + Alk1-Inhibitor. **Figure 52**.

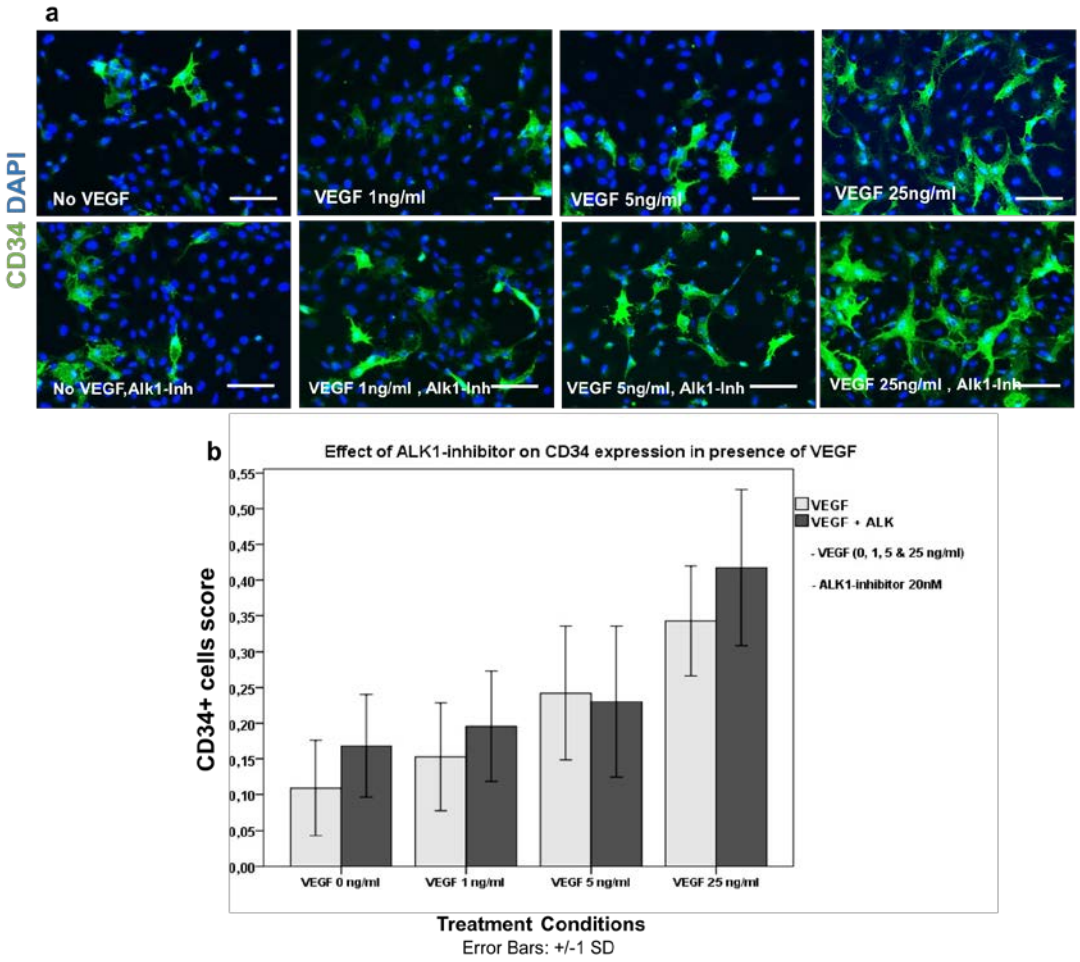


Figure 51: Immunostaining and statistical data from the effect of ALK1-inhibitor in the presence of VEGF.
(a) Representative pictures of HUVECs expressing CD34 after stimulation with different concentrations of VEGF alone or in combination with the constant concentration of ALK1-inhibitor. Immunostaining results showed comparatively high expression of CD34 expression in the presence of Alk1-Inhinitor compared to VEGF only conditions. (n=2 biological replicates & n=4 technical replicates) (b) To statically analyze the CD34 expression one-way ANOVA was conducted to determine if CD34+ score was different for groups with different VEGF/Alk1-Inhibitor levels. Samples were classified into eight groups: four groups with VEGF only and four in combination with ALK1-Inhibitor. There were no outliers, as assessed by boxplot; data was normally distributed for each group, as assessed by Shapiro-Wilk test ($p > .05$); and there was homogeneity of variances, as assessed by Levene's test of homogeneity of variances ($p = .198$). Data is presented as mean ± standard deviation. Tukey post hoc analysis revealed that the increase from VEGF0 to VEGF25 (0.233, 95% CI (0.15 to 0.32) was statistically significant ($p < .0005$), but no significant difference was found between any VEGF only groups vs VEGF + Alk1-Inhibitor. Scale bar, 32 µm.

3.2.6 Blocking ALK1 and Notch signalling

Several studies have shown that ALK1 synergises with Notch in tip cell suppression during angiogenic sprouting (Kerr et al., 2015, Larrivee et al., 2012). Therefore, in this experiment, I investigated the inhibitory effects of both ALK1 and Notch signalling together. Immunostaining results showed that the expression of CD34+ cells was significantly increased in the VEGF + DAPT + ALK1-inhibitor condition compared to the VEGF + DAPT condition which was consistent with previous in vivo findings (Larrivee et al., 2012). Statistical analysis also confirmed the significant difference between VEGF + DAPT versus the VEGF + DAPT + ALK1-inhibitor condition **Figure 53**.

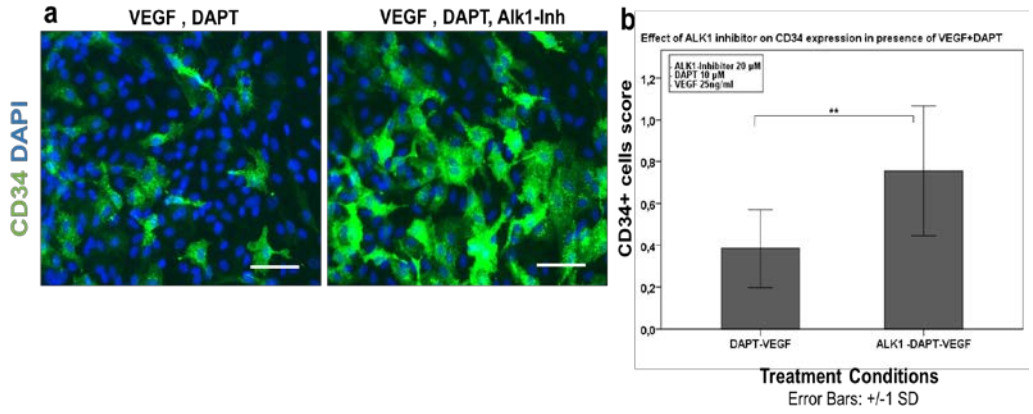


Figure 52: Immunostaining and statistical data from the effect of ALK1-inhibitor in the presence of VEGF / DAPT.
(a)Representative pictures of HUVECs expressing CD34 after stimulation with VEGF + DAPT vs. VEGF + DAPT + ALK1-inhibitor for 24 hours which shows higher expression of CD34 in the presence of Alk1-Inhibitor (n=2 biological replicates and n=6 technical replicates). (b) To statically analyze the CD34 expression an independent-samples t-test was run to determine if there were differences in CD34+ score between DAPT-VEGF and DAPT-VEGF-Alk1-Inhibitor. There were no outliers in the data, as assessed by inspection of a boxplot. CD34+ scores for each group were normally distributed, as assessed by Shapiro-Wilk's test (p > .05), but the assumption of homogeneity of variances was violated, as assessed by Levene's test for equality of variances (p = .018). Data is presented as mean ± standard deviation. The CD34+ score was higher in DAPT-VEGF-Alk1-Inhibitor (0.756 ± 0.310) than in DAPT-VEGF (0.384 ± 0.187), a statistically significant difference of 0.372 (95% CI, 0.24 to 0.50), t (47.670) = -5.619, p < .0005. Scale bar, 32 µm.

3.2.7 Blocking of Notch signalling in presence of FBS and its effect on CD34 differentiation

Fetal bovine serum (FBS) contains high levels of BMP9, which is needed for cells to survive, grow, and divide. In current experiments, it was assumed that adding FBS would decrease the expression of CD34 similar to the effect of BMP9. Therefore, in this experiment, HUVECs were stimulated for 24 hours with VEGF + DAPT in the presence of 10% FBS. Immunostaining of these cells showed a considerable reduction in CD34 expression. Furthermore, statistical analysis showed a significant decrease in the numbers of CD34+ cells in VEGF +DAPT + FBS compared to the VEGF + DAPT condition. **Figure 54**.

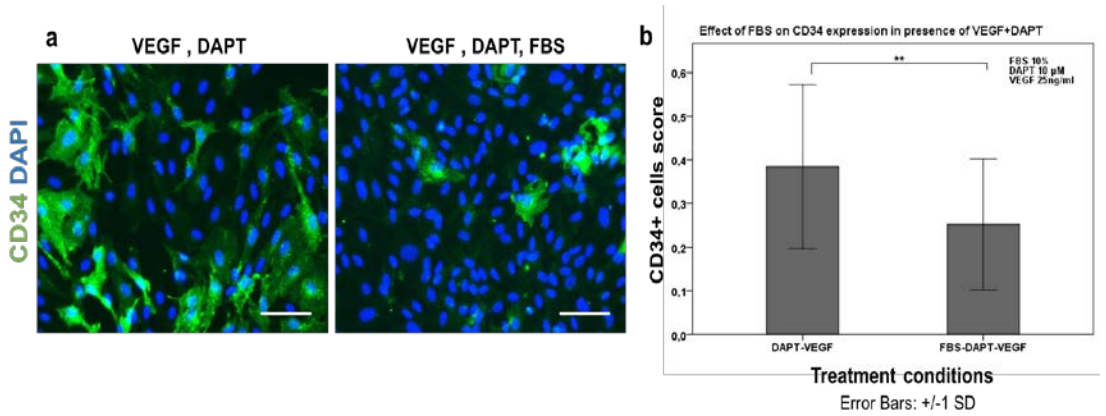


Figure 53: Immunostaining and statistical data from the effect of ALK1-inhibitor in presence of VEGF / DAPT.
(a) Representative pictures of HUVECs expressing CD34 after stimulation with VEGF + DAPT vs. VEGF + DAPT 10% FBS for 24 hours which shows reduced expression of CD34 in presence of 10% FBS. (n=3 biological replicates and n=6 technical replicates) (b) To statically analyze the CD34 expression an independent-samples t-test was run to determine if there were differences in CD34+ score between DAPT-VEGF and FBS-DAPT-VEGF. There were no outliers in the data, as assessed by inspection of a boxplot. CD34+ scores for each group were normally distributed, as assessed by Shapiro-Wilk's test (p > .05), and there was homogeneity of variances, as assessed by Levene's test for equality of variances (p = .094). Data is presented as mean ± standard deviation. The CD34+ score was higher in DAPT-VEGF (0.384 ± 0.187) than in FBS-DAPT-VEGF (0.252 ± 0.150), a statistically significant difference of 0.132 (95% CI, 0.22 to 0.45), t(58) = 3.020, p = .004. Scale bar, 32 µm.

3.2.8 Effect of BMP9 in presence of VEGF on differentiation of CD34

BMP9 is a circulating factor in human plasma (5ng/ml) that is produced largely in the liver. In the tip/stalk cell context, BMP9 has an antagonistic effect on tip cell formation (via Alk1) and promotes the stalk cell phenotype. Therefore, it has also been called “vascular quiescence factor”. I, therefore, wanted to study the effect of BMP9 in our HUVEC culture model. Since BMP9 stimulates Alk1 I anticipate here an opposite response to the previous experiments where Alk1 was blocked. Immunostaining results showed a considerable reduction in CD34 expression after adding BMP9 (20ng/ml) in all concentrations of VEGF (1, 5 and 25ng/ml). Furthermore, statistical data showed a significant reduction in CD34 expression between VEGF-only and VEGF + BMP9 conditions. Figure 55.

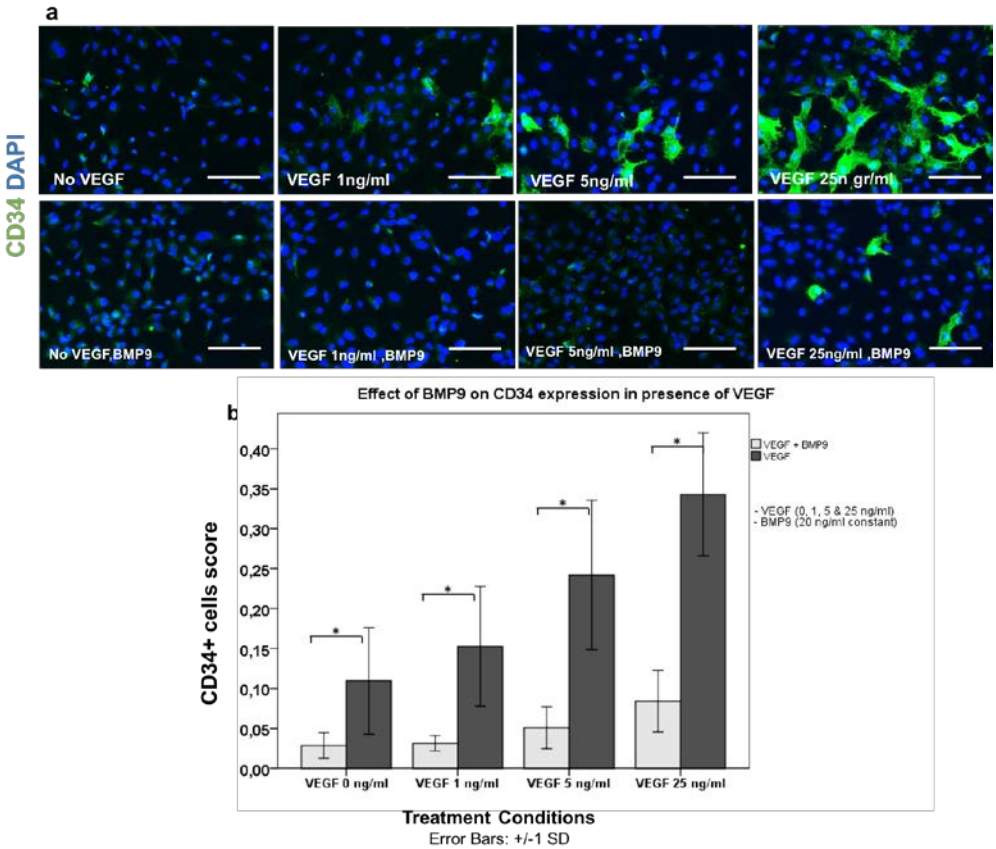


Figure 54: Immunostaining and statistical data from the effect of BMP9 in presence of VEGF.

(a)Representative Pictures of HUVEC cells expressing CD34+ after stimulation with different concentrations of VEGF only (0, 1, 5, and 25ng/ml) and in combination with constant concentration of BMP9 (20ng/ml) for 24 hours. Immunostaining results showed that BMP9 significantly reduced the expression of CD34 even in a high concentration of VEGF (n=3 biological replicates & n=6 technical replicates). (b) To quantify the CD34 + cells, a one-way Welch ANOVA was conducted to determine if the CD34+ cell scores were different for groups with different VEGF levels and VEGF/BMP9 levels. Groups were divided into four VEGFs and four combinations with BMP9. There were no outliers and the data was normally distributed for each group, as assessed by boxplot and Shapiro-Wilk test ($p < .05$), respectively. Homogeneity of variances was violated, as assessed by Levene's Test of Homogeneity of Variance ($p < .0005$). CD34+ score was statistically significantly different between the various VEGF only sample groups and VEGF + BMP9 groups, Welch's $F(7, 61.941) = 70.487$, $p < .0005$. CD34+ score increased from VEGF0 BMP9 (0.029 ± 0.016) to VEGF1 BMP9 (0.032 ± 0.010), VEGF5 BMP9 (0.051 ± 0.026), VEGF25 BMP9 (0.084 ± 0.039), VEGF0 (0.110 ± 0.067), VEGF1 (0.153 ± 0.075), VEGF5 (0.242 ± 0.093), and VEGF25 (0.343 ± 0.077) groups, in that order. Games-Howell post hoc analysis revealed that the increase from VEGF25 BMP9 to VEGF25 (mean increase of 0.258, 95% CI (0.19 to 0.32) was statistically significant ($p < .0005$). Scale bar, 32 µm.

Our previous data showed that exposing HUVECs to VEGF for longer periods (5 days) can dramatically increase the number of CD34-positive cells. To find out whether the inhibitory effect of BMP9 persists even in 5-day culture, I plated HUVECs in VEGF-only (25ng/ml) and VEGF (20ng/ml) + BMP9 (20ng/ml). Immunostaining and statistical observations showed that the expression of CD34 was strongly suppressed after five days in the VEGF + BMP9 condition. Quantitative real-time (qPCR) also confirmed the significant difference in CD34 gene expression between VEGF-treated and VEGF + BMP9-treated HUVECs. These data confirmed the strong effect of BMP9 signalling in inhibition of tip cell-like phenotype in in vitro systems.

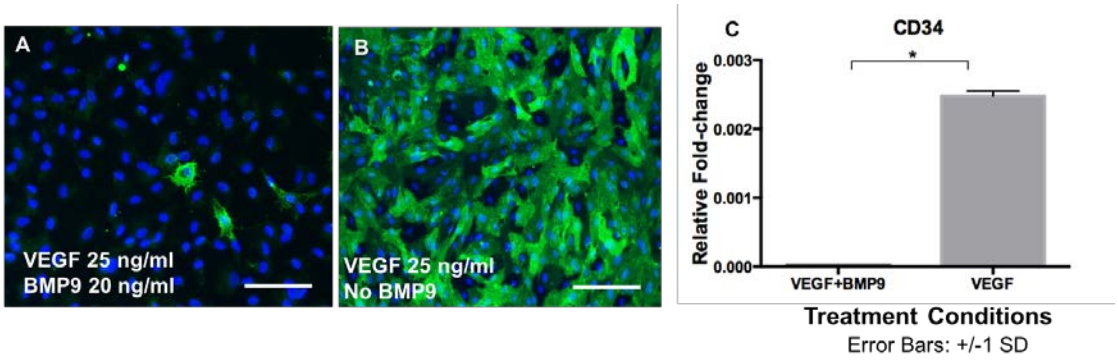


Figure 55: Representative pictures of HUVEC treated with VEGF + BMP9 (A) compared to VEGF alone (B).
Results showed a significant reduction in expression of CD34-positive cells. qPCR analysis also showed significant reduction of CD34 expression in VEGF + BMP9-treated HUVEC compared to those treated with VEGF only (C). Scale bar, 32 µm.

3.2.9 Effect of BMP9 and VEGF on tip and stalk cell gene expression in HUVECs

In the previous experiment manipulation of VEGF/Notch signalling affected CD34 expression in HUVECs in a way that is consistent with effects on tip and stalk cell phenotypes in vivo. I, therefore, wanted to determine whether VEGF and BMP9 influence the expression of tip/stalk cell genes in HUVECs. To do this, HUVECs were treated with VEGF (20ng/ml) and VEGF + BMP9 (each 20ng/ml) for 48 hours; then, RNA was extracted for qPCR analysis to assess the expression of the genes listed in Table 2.

Notch & BMP9 target genes
SMAD7
HEY1
Stalk cells Marker
Jagged 1
Tie2
Tip cells Marker
CD34
DLL4
Apelin 1

Table 15: Different genes used for qPCR

As expected, SMAD7 and HEY1 expression (downstream targets of Notch and BMP9 signalling) were up-regulated by BMP + VEGF compared to VEGF-only. This is consistent with the recent findings that the BMP9 through SMAD 1, 5, 8 directly activates the HEY1, HEY2, and JAG1 promoters (Morikawa et al., 2011). VEGF + BMP9 also induced the expression of JAG1 and Tie2 expression (stalk cell markers) and significantly inhibited the expression of tip the cell markers CD34, Dll4, and Apelin1. These data are consistent with previous findings on the antagonistic effect of BMP9 on tip cell formation (David et al., 2007). Altogether, these qPCR data suggest that the effects of BMP9 and VEGF on CD34 expression in HUVEC monolayer represent many general shifts towards tip and stalk cells phenotypes in ECs.

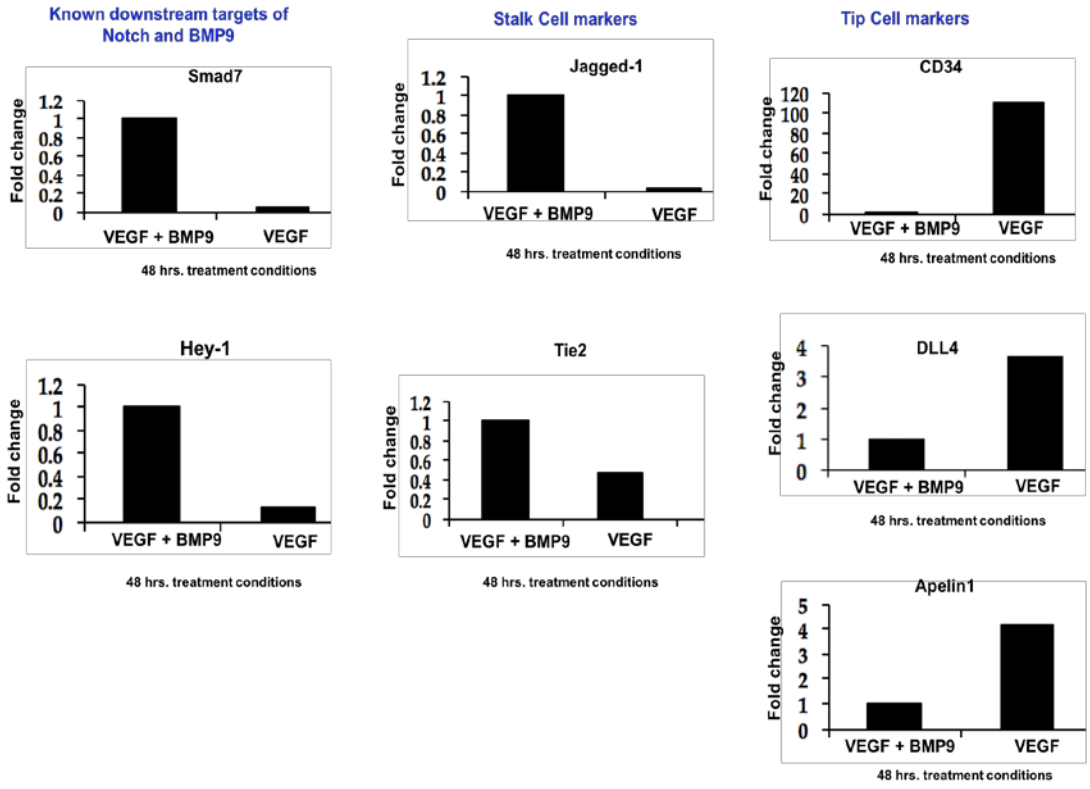


Figure 56: qPCR analysis of genes in HUVECs treated with VEGF + BMP9 compared to the VEGF-only condition.
Results showed a remarkable increase in downstream targets of Notch and BMP9 (SMAD7 & HEY1) and stalk cell markers (Jagged1 & Tie2), whereas notable reduction was observed in tip cell markers (CD34, DLL4, and Apelin1). Genes were normalised to Actin as a reference gene. (biological replicate=1 and technical replicate=3).

3.2.10 Effect of VEGF and BMP9 on migration of EC

The tip cell phenotype is dominated by the migratory behaviour of endothelial cells and low proliferation. In contrast, stalk cells proliferate more and migrate less. I, therefore, aimed to test whether VEGF and BMP9 also affect these functional aspects of the tip and stalk cell phenotypes.

Among different types of migration assays, I used in vitro wound healing (scratch assay) to study the migration of HUVECs on 2D surfaces. The confluent monolayers of HUVECs were scratched with a 1,000ul pipette tip (Corning). Images were taken at 10X at 0 and 8 hours after scratching. The area covered by migration was calculated by subtracting the area at t=8h from the area at t=0 (using ImageJ). Results showed that migration of HUVECs comparatively increased in response to BMP9 versus VEGF-

only or VEGF + BMP9 conditions; however, the increase is not significant. LVEM and EGM-2 were used as negative and positive control respectively. Since the accuracy of starch assay is low, and because the size of the scratch is variable in different experiments, I decided not to pool the data for further analysis. Instead, I used data from one single dish in each experiment and tried to measure the same position of the scratched area at different time points. This experiment was repeated 3 times and each time similar effect of factors observed. (e.g. condition treated with BMP9 had the most migration compared to other conditions).

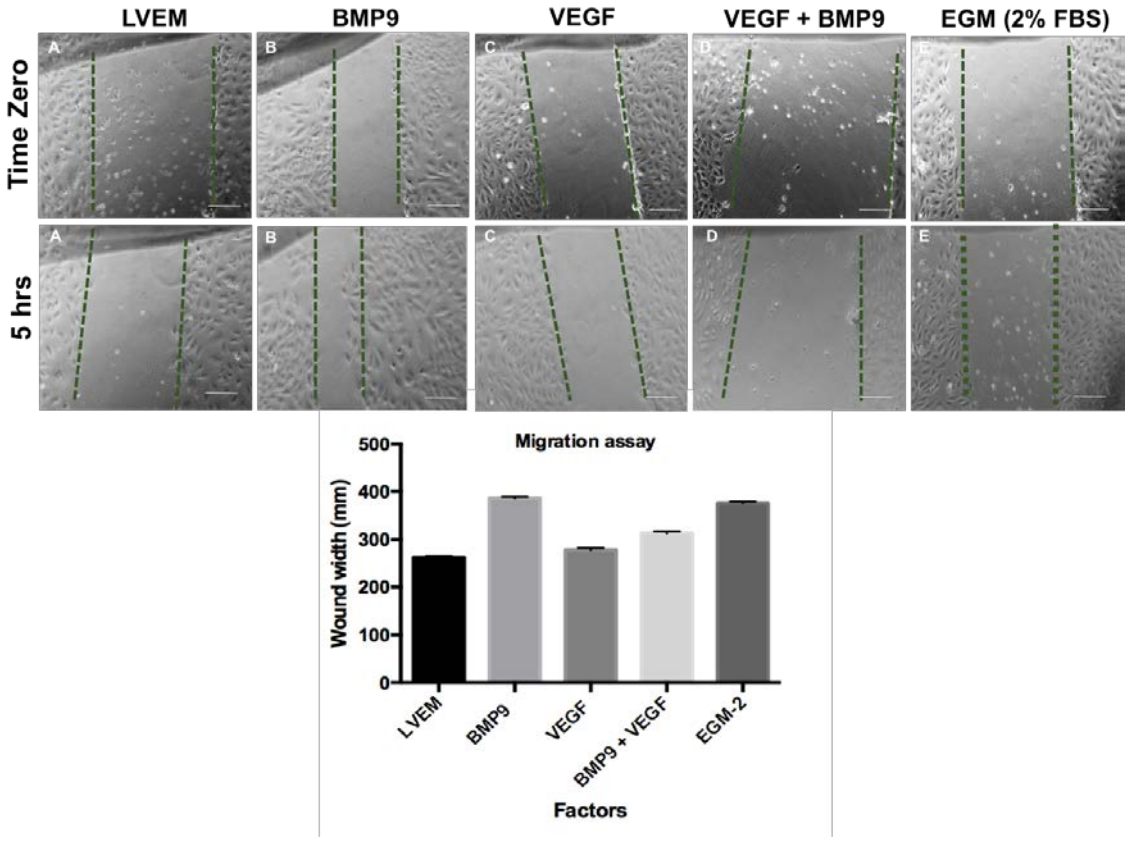


Figure 57: Migration assay on HUVECs in response to BMP9 (B) and VEGF (C) and their combination (D).

LVEM and EGM-2 were used as negative and positive control respectively (A & E). Confluent monolayers of HUVECs were scratched with a 1,000ul pipette tip. Cells were photographed at time 0 and after 5 hours. Scale bar, 164 µm. Determination of HUVEC migration in the presence of different factors. HUVECs in the presence of BMP9 showed increased migration compared to other conditions (n=4).

3.2.11 Effect of VEGF and BMP9 on proliferation of ECs

To further evaluate the effect of different factors on HUVEC proliferation, I tested the influence of factors BMP9 and VEGF on BrdU (5-bromo-2'-deoxyuridine) uptake. In this experiment, HUVECs were exposed to the same conditions as in the previous section. 2 hours before fixation 10uM BrdU was added to each well. Proliferation rates were calculated by the ratio of BrdU to the total number of cells using ImageJ. Quantification of BrdU demonstrated that treating HUVECs with BMP9 could relatively enhance the number of proliferating cells **(Figure 59 .A, B, C, D, E, F)**, and statistical results showed notable difference between BMP9 vs VEGF and BMP9 vs VEGF + BMP9. Furthermore, only a small percentage of the cells were proliferating, for example, 3.28% in the case of VEGF-treated cells or 13.36% in the case of the EGM-2 medium Figure 59. Furthermore, none of the cells seemed positive for both CD34 and BrdU at the same time. CD34-positive cells were negative for BrdU staining and vice versa. Furthermore, the percentage of the cells that were either proliferating or expressing CD34 was just a very small percentage of the whole population. For example, in VEGF-treated HUVECs, 16.07% of cells were expressing CD34 and 3.28% were proliferating; around 80% of cells were neither proliferating nor expressing CD34. It shows that HUVECs are a heterogeneous population and each subpopulation has their own specific characteristics **Figure 59.**

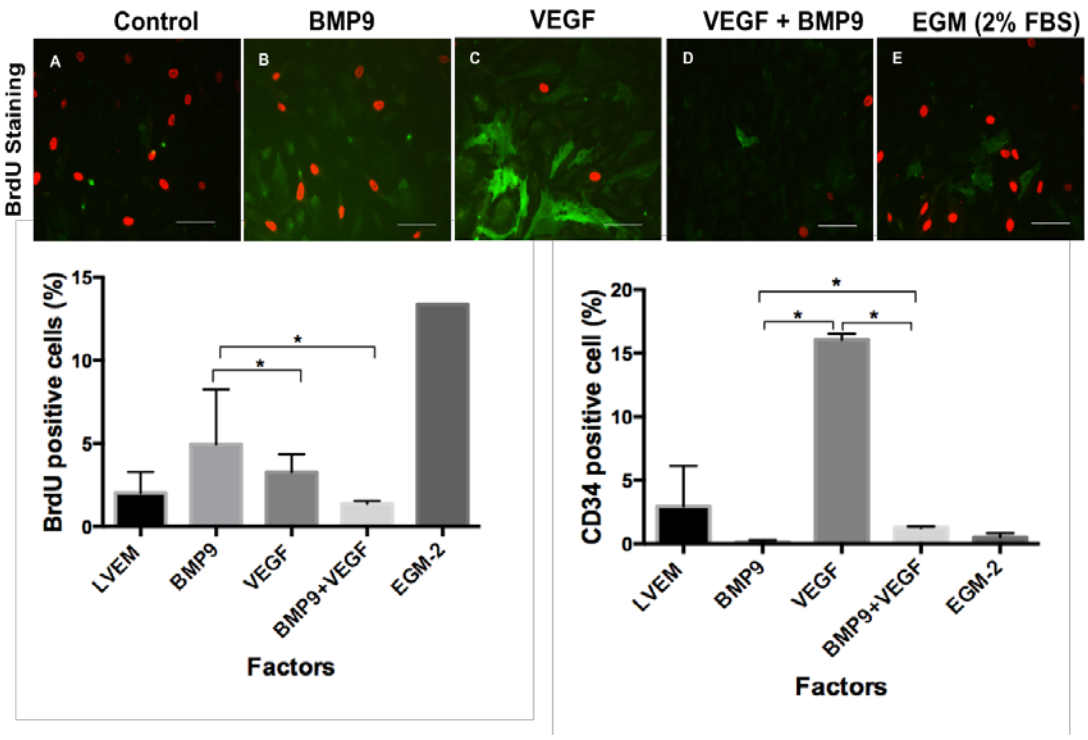


Figure 58**: Effect of different factors in proliferation and CD34 expression on HUVECs.**

Immune-staining results showed noticeably high proliferation in BMP9 (B) compared to VEGF and VEGF + BMP9 respectively (C & D). CD34 expression was remarkably high in VEGF (C) compared to other conditions. To statically analyse the proliferation and CD34 expression one-way ANOVA was conducted to determine if BrdU and CD34+ scores were different between different treated groups. Samples were classified into five groups with different factors/controls: Data is presented as mean ± standard deviation. Brown-Forsythe test revealed that the there was a significant reduction in cell proliferation from BMP9 to VEGF and BMP9 + VEGF respectively. (p <0.0005). Also, CD34 expression was shown to be significantly high in VEGF treated samples compared to BMP9 and BMP9 + VEGF conditions. (p<0.0005). (Biological replicate=3 & technical replicate 10). BrdU (red) and CD34 (Green). Scale bar, 32 µm.

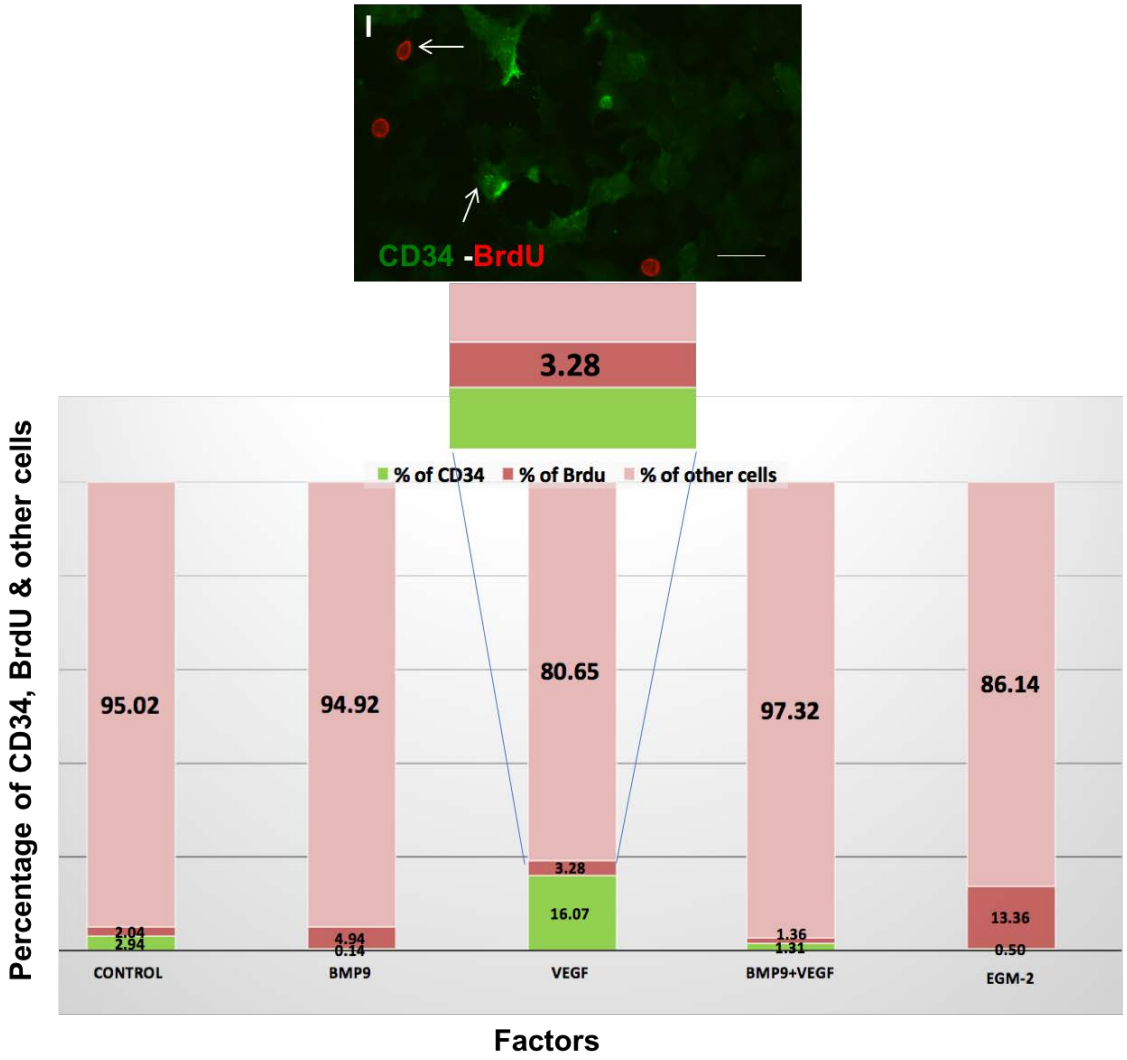


Figure 59: Comparison between proliferated, CD34 expressing cells and other cells in presence of different factors on HUVECs.

Immuno-staining results showed that cells that are positive for CD34 are negative for BrdU (I). Statistical data showed that only small populations of the cells are either CD34 positive or BrdU-positive. However, a high percentage of the cells are negative for both staining. Scale bar, 32 µm.

In the next step, the intention was to see the effect of factors when cells were pre-treated in particular factors. To evaluate this, HUVECs were stimulated with either VEGF (20ng/ml) or BMP9 (20ng/ml) separately for 48 hours. On day three, confluent monolayers of HUVECs were scratched with a 1,000ul pipette tip (Corning) and media were replaced with media containing specific factors: BMP9 (20ng/ml) and BMP9 + DAPT. Images were taken at time 0 and after 9 hours and the area covered by migration was calculated. Statistical results showed that cells pre-treated with BMP9 migrate more than cells pre-treated with VEGF.

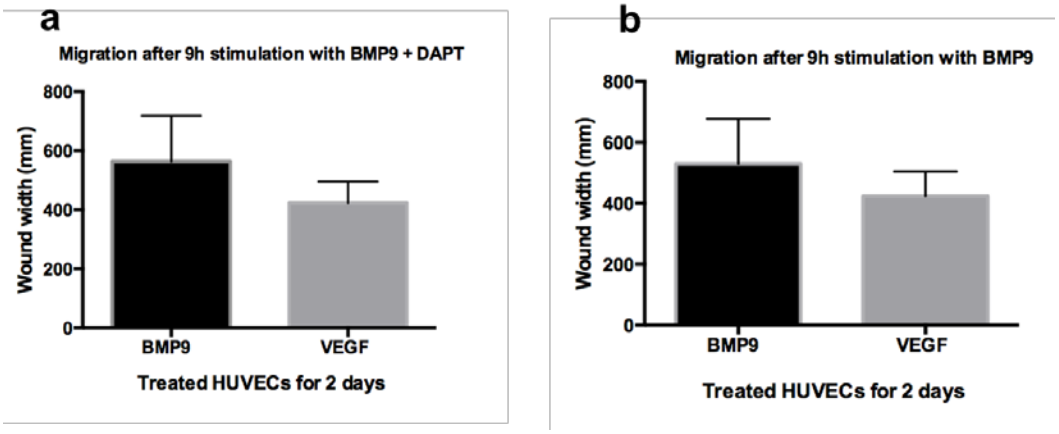


Figure 60: Statistical analysis from the scratch assay.
HUVECs were subjected to in vitro scratch assay with images captured at 0 and 9 h after stimulation with factors shown. The rate of migration was measured by quantifying the total distance that HUVEC cells moved from the edge of the scratch toward the centre of the scratch. t test nonparametric data showed that migration increased in BMP9 treated cells compared to VEGF-treated ones. But it was not shown to be significant. (n=4 biological data) and P-value was (P= 0.6571).

3.2.12 Discussion

The importance of VEGF gradient in angiogenesis has been shown in many in vivo studies, and a clear example is the retina, where the VEGF gradient is needed for generation and polarisation of tip cell filopodia (Gerhardt et al., 2003). However, in vitro studies using HUVECs have failed to show the tip/stalk cell behaviour in monolayer culture conditions, as they do not typically form angiogenic sprouts under monolayer culture conditions (Koizumi et al., 2003). However, the data presented in our study suggests that HUVEC monolayer indeed can display elementary forms of tip and stalk cell phenotypes and so could be used as an in vitro model to study the molecular mechanisms of tip cell biology.

In contrast, in a recent study on HUVECs (Siemerink et al., 2012), it could not been shown that inhibition of Notch signalling can increase the number of CD34+ cells. This difference might be explained by higher and more prolonged VEGF expose in our study. This may have mirrored the micro environment of tip cells in vivo, which are exposed to high levels of VEGF.

Furthermore, inhibition of ALK1 and Notch signalling in the presence of VEGF could further increase the expression of CD34+ cells. Interactions between ALK1 and Notch signalling have also been shown in previous in vivo studies (Fu et al., 2009). Studies have also shown that the blockade of ALK1 or Notch signalling separately could increase the tube formation in vivo (Carvalho et al., 2004). Furthermore, the blockade of both types of signalling together further enhanced hypervascularization in the retina of DAPT + ALK1Fc-injected pups (Larrivee et al., 2012) or in mice with genetic deletion of DLL4 or other Notch signalling components (Lobov et al., 2007, Suchting et al., 2007, Hellstrom et al., 2007).

However, it is important to note that the effects of ALK1 and Notch blocking were absolutely dependent on the presence of VEGF in our experiments, and the inhibition of ALK1 had a limited effect in increasing the CD34 expression in the absence of VEGF. This could suggest that in in vivo conditions, ALK1 and Notch inhibition only regulate tip/stalk cell phenotypes in the presence of angiogenic stimuli. This is consistent with studies showing that ALK1 inhibition did not have any effect on mature established vessels in the skin (Larrivee et al., 2012).

I further showed an antagonistic effect of BMP9 on CD34 expression in HUVECs that significantly suppressing the tip cell-like phenotype, most probably through ALK1 and SMAD 1/5 signalling.

Results of the present study also indicated that migration and proliferation were increased in the presence of BMP9. Therefore, compared to some studies (Suzuki et al., 2010), I could also show the proliferative effect of BMP9 on ECs against other contradictory studies showing the inhibitory effect of BMP9 on proliferation and migration (Scharpfenecker et al., 2007, David et al., 2007). However, the proliferative and migratory effect of BMP9 was strongly dependent on pre-treated conditions; also, the factors that cells were stimulated in in combination with BMP9 again show the importance of signal contractions with each other. Our results showed a comparatively higher proliferative rate in HUVECs pre-treated with BMP9 compared to VEGF in most conditions. However, within each pre-treated group, stimulation with different factors had slightly different effects on proliferation and migration. In HUVECs pre-treated with BMP9 and then stimulated with BMP9, DAPT, and then BMP9 + DAPT, a reduction in proliferation and migration after stimulation with DAPT was observed. However, when they were stimulated with a combination of BMP9 and DAPT, migration increased where proliferation considerably dropped. Studies have shown that BMP9 promotes EC migration through Id1 (Itoh et al., 2004) along with proliferation (Suzuki et al., 2010), which is the reason for high proliferation and migration in BMP9 conditions. Furthermore, it has been shown that inhibition of Notch signalling alters the expression level of Notch signalling target genes that might participate in the regulation of migration and proliferation (Liu et al., 2013). It also has been shown that synergistic up-regulation of the Herp2 expression by Notch and BMP9 activation antagonises BMP9-induced EC migration (Itoh et al., 2004). Therefore, the inhibition of Notch signalling results in Herp2 down-regulation, which in turn induces EC migration. This could explain why migration increased when BMP9 and DAPT were used together. Furthermore, significant reduction in proliferation in the VEGF + DAPT condition suggests that the increased rate to cover the scratched area was due to the migratory effect and not proliferation. However, increased migration in VEGF pre-treated HUVECs in DAPT-stimulated conditions could be explained by the stimulatory effect of VEGF, which has been shown to increase the migration. All these data suggest that

the dynamic oscillatory network between BMP9 and Notch signalling strongly regulates EC behaviour in specific conditions.

Another notable observation was that none of the cells were positive for both CD34 and BrdU at the same time. Cells positive for CD34 were negative for BrdU and vice versa. It could indicate that CD34-positive cells have more migratory characteristics than proliferative characteristics, which was consistent with previously published data (Siemerink et al., 2012). Furthermore, the percentage of the cells that were either proliferating or expressing CD34 was just a very small percentage of the whole population. It shows that HUVECs are a heterogeneous population and each subpopulation has their own specific characteristics. More investigations need to be done to understand more about these different populations.

In summary, vascular morphogenesis is a series of connected events (migration, proliferation, extending filopodia, or becoming quiescent), which requires strict temporal and spatial regulation of different cell signalling pathways for proper assembly of growing vessels. Therefore, ECs need to behave differently at different points of time to form a lumen, and it is very important to realise that all signalling pathways involved in angiogenesis (VEGF, Notch, and TGF-β) will only be understood when they are studied at the temporal and systems level.

One of the limitations of this study that caused high degree of variability for some experiments which made us to repeat some experiments over six times to reduce the standard deviation was due to using different passage numbers of HUVECs. Unlike cell lines, primary HUVECs do change with passages which cause considerable effects on their response to treatments. Furthermore, HUVECs are limited to 15-16 population doubling and after that they start to senesce and stop growing. In fact, the number of population doublings (which reduces over several passages) is the main factor that affects the proliferation and thus the consistency of the results over long term culturing of HUVECs. Effects on the proliferation rate influence the confluency of the cells during the experimental procedure. Difference in confluency can have huge effect in outcome results. Proliferating and contact inhibited cells affect cells' response to treatment which has been shown in previous studies (Browne et al., 2006).

Furthermore, in many HUEVCs studies serum-containing media have been used as the basal media. However, the presence of BMP9 in the serum, which has considerable effect on directing the cells to stalk cells phenotype, has not been really considered in these studies. Our experiments showed that adding FBS could significantly reduce the number of CD34+ cells. Thus, this shows the importance of using defined medium. Furthermore, the time cells need to change their behaviour and respond to specific stimuli is important. In fact, our time period experiments showed that having cells for several days in BMP9 increased the cells response to VEGF and BMP9. This is something that should be taken into account when working with HUVECs.

3.3 Transcriptional profiling

3.3.1 Introduction

So far three different types of CD34+ endothelial lineage related cells were discussed in this thesis: adult “EPCs” (introduction), iPSC-derived PECs (chapter 4.1) and VEGF treated HUVECs (chapter 4.2). I next wanted to establish how related these different CD34+ populations were to each other by establishing their genome wide transcriptional profile. To this end, Truseq illumine RNA sequencing platform was used. RNA-sequencing is a newly emerging method for transcriptional profiling which is based on recent advances in deep sequencing. In this method mRNA is first broken into short (around 100-200bp) fragments and then converted to corresponding cDNA fragments. A library is then made from these fragments and sequenced which results in several million sequences. High abundant transcripts will be sequenced many times whereas rare transcripts will be identified less often providing the possibility to quantify gene expression.

For the adult EPC population in this study, I used commercially obtained CD34+ cells that have been purified from adult peripheral blood and from cord blood. A sample of CD14+ monocytes (purified from adult blood) as a monocyte reference population was also purchased. For the PEC population I used my protocol (chapter 4) to differentiate iPSC towards the EC lineage in the presence of VEGF or a VEGF blocker. FACS was then used to purify the CD34+ cells from the cultures that contained VEGF. In the case of the cultures that contained the VEGF blocker, all cells were used without purification. For the HUVEC samples also all cells were used in cultures that contained BMP9, whereas HUVECs that were treated with VEGF were split into a CD34+ and CD34- fraction using FACS.

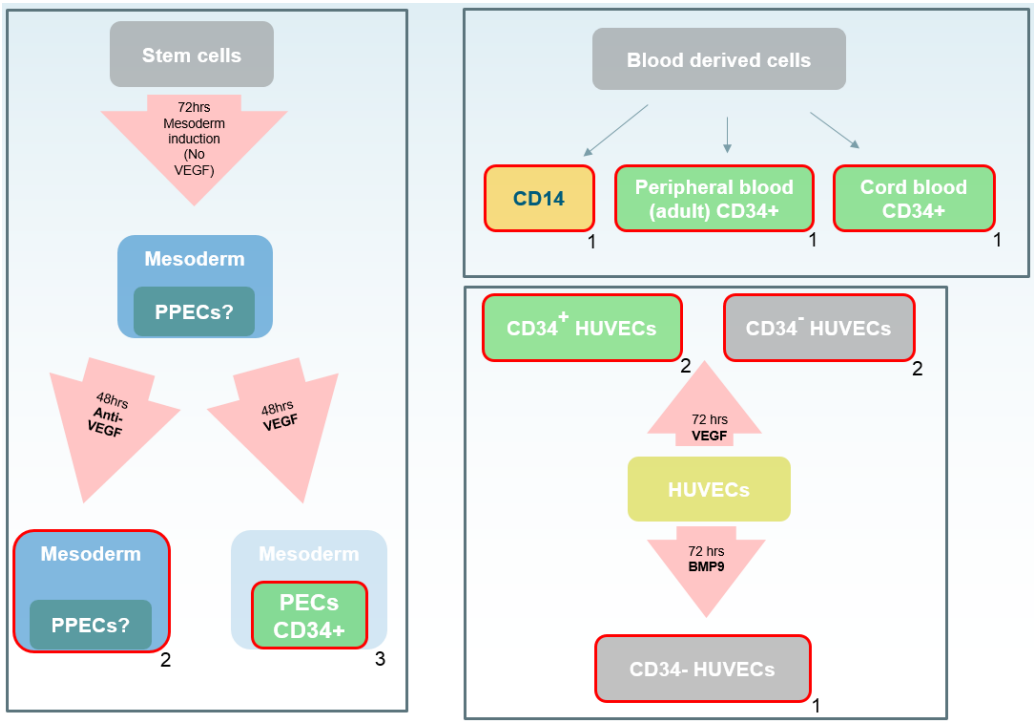
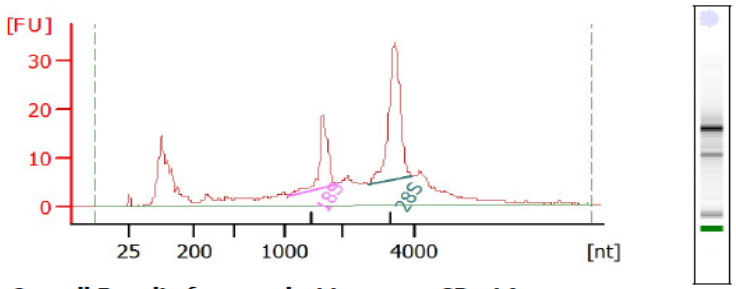


Figure 61: Different CD34+ and CD34- cell populations in the endothelial related lineage. Samples used for the RNAseq analysis are framed in red. Numbers next to red boxes indicate the number of replicates analysed.

RNA was then isolated from all eight sample populations (from about 10,000-500,000 cells per sample). Trizol extracted RNA from all samples was then sent to the sequencing facility of UCL Institute of Child Health.

Before RNA can be processed for RNA-sequencing, it must be tested for quality (von Ahlfen et al., 2007). Many researches use the Agilent RNA Integrity Number (RIN) to determine RNA quality for RNA sequencing analysis. The Agilent Bioanalyzer RNA assay analyses the quality of RNA using only 1ul of sample. An RNA Integrity Number (RIN score) is generated for each sample on a scale of 1-10 (1=lowest; 10=highest) as an indication of RNA quality. The 18s/28s ratio and an estimation of concentration are also produced (Kuschel, et al 2013). **Figure 63** shows one example of RNA Integrity numbers (RINs) used in the current study to assess the RNA quality control. After quality control, RNA samples were further proceeded for library preparation and sequencing on the Illumina TruSeq RNA v2 platform.



Overall Results for sample 11 : CD+14

RNA Area:	407.3
RNA Concentration:	520 ng/µl
rRNA Ratio [28s / 18s]:	2.0
RNA Integrity Number (RIN):	8.3 (B.02.08)
Result Flagging Color:	
Result Flagging Label:	RIN: 8.30

Fragment table for sample 11 : CD+14

Name	Start Size [nt]	End Size [nt]	Area	% of total Area
18S	1,047	1,907	37.3	9.1
28S	2,738	3,946	76.3	18.7

Figure 62: Example quality control for RNA isolated from CD14+ cells. RNA Integrity numbers (RINs) were calculated from the Bioanalyzer traces.

3.3.2 Normalization of RNAseq data

The raw data that RNAseq analysis returns is a large number of short sequences (around 45bp long). These sequences have to be matched against specific genes and have then to be counted. Because RNAseq is still a relatively new technique, there is so far no simple software for the alignment process and I enlisted the help of a collaborator with bioinformatics expertise (Monte Radeke, UCSB, Santa Barbara, USA). He also normalised the different libraries (samples) against each other using software called "edgeR".

edgeR operates on a table of integer read counts. In this table, genes are listed in rows and the different samples are in columns. The counts represent the total number of reads aligning to each gene (Chen et al., 2014). Normalization issues arise only to the extent that technical factors have sample-specific effects. This commonly becomes important when a small number of genes are very highly expressed in one sample, but not in another. The highly expressed genes can consume a considerable proportion of the total library size, causing the remaining genes to be under-sampled in that sample. Unless this RNA *composition* effect is adjusted for, the remaining genes may falsely appear to

be down-regulated in that sample (Robinson and Smyth, 2007, Robinson and Smyth, 2008). In edgeR, normalization takes the form of correction factors that take the form of scaling factors for the different libraries.

The default method for computing these scale factors uses a trimmed mean of values (TMM) between each pair of samples (Chen et al., 2014). TMM normalization factors across several samples can be calculated for each lane, considering one lane as a reference sample and the others as test samples. For each test sample, TMM is computed as the weighted mean of ratios between this test and the reference, after exclusion of the most expressed genes and the genes with the largest log ratios. In TMM normalization for RNAseq there is no need to modify the data, and the estimated normalization factors can directly be used in statistical models, and since data have not modified, they can be used in further applications such as comparing expression between genes.

Our RNAseq data was normalised using the TMM method that was applied in the edgeR Bioconductor package (version 2.4.0). The number of counts from RNAseq data were adjusted to reads per million gene alignments per 1kb transcript length (CPMK) to facilitate transparent comparison of transcript levels both within and between samples (Mortazavi et al., 2008).

3.3.3 Validation of RNAseq data

Once the RNAseq raw data was processed and turned into an excel spreadsheet with 13 columns (for each sample) and around 16000 lines (for different genes) (by Monte Radeke), I continued the analysis of the data. Firstly I checked that the different sample populations express specific genes I expected them to express. For instance, since CD14 expression was the selection criteria for CD14+ monocytes I anticipated that gene to be highly expressed in this population. The same applies to CD34 in CD34+ cell populations.

As shown in **Table 22** CD14 was strongly expressed in CD14 monocytes while its expression was very low in the rest of the cell populations. Furthermore, CD34 expression was high in all CD34+ populations and low in CD34- samples, confirming the efficiency of the cell sorting used to purify some of these samples.

Genes	Mesoderm PPECs (n= 2)	iPS-derived PEC a (n=1)	iPS-derived PEC b (n=1)	iPS-derived PEC c (n=1)	HUVEC VEGF CD34+ (n=2)	HUVEC VEGF CD34- (n=2)	HUVEC BMP9 (n=1)	Cord Blood CD34+ (n=1)	Adult Blood CD34+ (n=1)	Adult monocyt CD14+ (n=1)
CD14	11	14	77	43	21	33	168	110	99	87106
CD34	104	9262	38166	74732	189574	3215	4296	38347	36999	1

Table 16: Confirmation of selection criteria by choosing two important genes. CD34 & CD14 were expected to be high in all CD34+ cell population and in CD14 monocytes respectively.

Next I checked whether certain genes that are known to be specifically expressed by certain cells match our RNAseq data. For instance, the monocyte population is anticipated to strongly express monocyte markers such as CD11b (IT6AM), CD68 and others (Medina et al., 2010b, Rossetto et al., 2013, Beyer et al., 2012). As shown in **Table 23** these genes were highly expressed in the CD14 population, which was another confirmation for the validity of the RNAseq data.

Genes	Mesoderm PPECs (n= 2)	iPS-derived PEC a (n=1)	iPS-derived PEC b (n=1)	iPS-derived PEC c (n=1)	HUVEC VEGF CD34+ (n=2)	HUVEC VEGF CD34- (n=2)	HUVEC BMP9 (n=1)	Cord Blood CD34+ (n=1)	Adult Blood CD34+ (n=1)	Adult monocyt CD14+ (n=1)
S100A9	2	2	57	507	3	1	1	1180	433	549647
CD68	121	349	868	1585	392	1382	1072	926	1170	121669
LYZ	101	174	659	294	64	149	149	6661	2437	1164957
TYROBP	5	2	1	34	3	1	1	696	422	118169
FGR	6	13	32	26	6	4	6	701	921	131242
ITGAM	12	13	11	1	6	4	1	742	1286	40184
CD4	365	361	164	293	5	4	6	1151	473	60230
CD48	4	10	6	127	3	8	1	5318	454	46945
CD36	22	33	149	76	445	71	543	1021	1346	50088
HLA-DRA	13	7	1	76	5	1	1	67417	44272	376139

Table 17: Representative genes that are strongly expressed in CD14 monocytes and not in the rest of the cell populations. Another confirmation of selection criteria used for monocyte population.

Furthermore, classic markers of the myeloid lineage (MPO, CD38 and KCNK17) were highly expressed in blood derived CD34+ cells **(Table 24).** Similarly, all HUVEC samples strongly expressed classic EC marker, such as PECAM1, VWF and others **(Table 25).**

Genes	Mesoderm PPECs (n= 2)	iPS-derived PEC a (n=1)	iPS-derived PEC b (n=1)	iPS-derived PEC c (n=1)	HUVEC VEGF CD34+ (n=2)	HUVEC VEGF CD34- (n=2)	HUVEC BMP9 (n=1)	Cord Blood CD34+ (n=1)	Adult Blood CD34+ (n=1)	Adult monocyt CD14+ (n=1)
MPO	5	2	83	9	9	3	11	76962	23009	850
CD38	5	5	83	18	12	3	1	1459	1535	242
KCNK17	28	16	409	1	1	3	1	1507	3346	3

Table 18: Blood-derived CD34+ cells are highly expressing classic myeloid markers (MPO, CD38 and KCNK17) compared to the rest of the cell populations.

Genes	Mesoderm PPECs (n= 2)	iPS-derived PEC a (n=1)	iPS-derived PEC b (n=1)	iPS-derived PEC c (n=1)	HUVEC VEGF CD34+ (n=2)	HUVEC VEGF CD34- (n=2)	HUVEC BMP9 (n=1)	Cord Blood CD34+ (n=1)	Adult Blood CD34+ (n=1)	Adult monocyt CD14+ (n=1)
PECAM1	59	1248	16540	4520	505854	147645	133111	9250	11563	60876
VWF	809	1959	2509	5506	1370749	236023	810720	541	2423	168
ENG	105	517	6448	3602	190485	108868	351735	4106	4012	5826
CDH5	30	4921	28740	38608	226474	140643	146044	23	8	25

Table 19: High expression of EC markers (PECAM1 & VWF) in all HUVEC populations. Another confirmation of selection criteria used for monocyte population.

I also looked at difference within the HUVEC samples. I have shown that VEGF-treated HUVECs appear to take on more of tip-cell like phenotype, and BMP9-treated HUVECs appear more stalk cell-like, and indeed the RNAseq analysis showed that the classic tip cell markers APLN, DLL4, CXCR4, UNC5B, ANGPT2 and KDR were highest in the VEGF-treated, CD34+ HUVECs **(Table 26).**

Genes	Mesoderm PPECs (n= 2)	iPS-derived PEC a (n=1)	iPS-derived PEC b (n=1)	iPS-derived PEC c (n=1)	HUVEC VEGF CD34+ (n=2)	HUVEC VEGF CD34- (n=2)	HUVEC BMP9 (n=1)	Cord Blood CD34+ (n=1)	Adult Blood CD34+ (n=1)	Adult monocyt CD14+ (n=1)
DLL4	22	273	6810	3385	19364	2181	1885	91	17	12
CXCR4	1988	2473	404	118	68158	3322	526	21256	3641	23435
ANGPT2	262	59	240	37	64354	4610	14642	271	341	246
KDR	5246	60345	77782	210901	54843	10764	7954	23	41	34

Table 20: VEGF-treated HUVECs show high expression of classic tip cell markers compared to the rest of the cell populations.

However, this data also allows us to find novel candidate markers for tip cells by searching for genes that are especially highly expressed in the CD34+, VEGF-treated HUVECs samples but not in the others. **Table 27** illustrates some of the potentially novel tip cell markers to be further analysed (PCDH12, CHST1, NID2, GPR116 and PGF).

Genes	Mesoderm PPECs (n= 2)	iPS-derived PEC a (n=1)	iPS-derived PEC b (n=1)	iPS-derived PEC c (n=1)	HUVEC VEGF CD34+ (n=2)	HUVEC VEGF CD34- (n=2)	HUVEC BMP9 (n=1)	Cord Blood CD34+ (n=1)	Adult Blood CD34+ (n=1)	Adult monocyt CD14+ (n=1)
PCDH12	82	638	7896	3805	60654	4089	329	269	280	379
CHST1	184	216	852	464	45163	3784	1853	175	153	177
NID2	6021	6713	10159	14776	35955	4235	531	151	259	40
GPR116	14	313	4593	1294	86721	7474	4829	12	31	8
PGF	1329	1024	862	176	170097	21532	18295	28	13	1

Table 21: Genes that are highly expressed in VEGF-treated CD34+ HUVECs and may be considered as novel tip cell markers.

The same comparison was applied for CD34- VEGF-treated and BMP9-treated HUVECs, which I have shown earlier to be stalk-like. As expected, the classic stalk cell markers JAG1 and FLT1 were comparatively highly expressed in this population of HUVECs. Furthermore, SMAD6 (a downstream target of BMP9 signalling) was highly expressed in BMP9-treated HUVECs as expected. However, the expression of SMAD6 was noticeably low in CD34- VEGF-treated HUVECs, which suggests that SMAD6 signalling is only directly influenced by presence of BMP9. Insulin-like growth factor binding protein3 (IGFBP3) was also highly expressed in only BMP9-treated HUVECs and not CD34- HUVECs, which is consistent with previous studies showing the induction of IGFBP3 expression through TGF-β signalling elements (Schedlich et al., 2013) **(Table 28).**

Genes	Mesoderm PPECs (n= 2)	iPS-derived PEC a (n=1)	iPS-derived PEC b (n=1)	iPS-derived PEC c (n=1)	HUVEC VEGF CD34+ (n=2)	HUVEC VEGF CD34- (n=2)	HUVEC BMP9 (n=1)	Cord Blood CD34+ (n=1)	Adult Blood CD34+ (n=1)	Adult monocyt CD14+ (n=1)
JAG1	12270	2739	2474	4019	2727	8297	9863	486	374	537
FLT1	1482	15795	45169	47204	13867	16097	27034	962	235	280
SMAD6	1495	5630	4100	3111	515	774	16195	64	8	181
IGFBP3	192	648	164	76	1768	528	9183	9	10	14

Table 22: BMP9-treated HUVECs show high expression of some classic stalk cell markers (JAG1, FLT) and SMAD6 but not others (TIE1 and TEK).

As above, other investigations through different markers has also revealed potentially novel stalk cell markers (shown in **Table 29**).

Genes	Mesoderm PPECs (n= 2)	iPS-derived PEC a (n=1)	iPS-derived PEC b (n=1)	iPS-derived PEC c (n=1)	HUVEC VEGF CD34+ (n=2)	HUVEC VEGF CD34- (n=2)	HUVEC BMP9 (n=1)	Cord Blood CD34+ (n=1)	Adult Blood CD34+ (n=1)	Adult monocyt CD14+ (n=1)
CD44	363	1264	3773	2428	993	27776	14743	25175	22471	81645
VEGFC	96	66	1	1	583	3851	11392	9	22	3
GDF6	75	86	6	18	2358	5533	6743	23	15	3
LTBP1	5901	4668	2794	5704	7695	27912	59684	367	838	25
FSTL3	750	527	210	168	812	6211	10276	826	440	1772
PLXNA4	150	303	2561	1704	1372	6037	17853	64	80	38
NR2F2	429	410	1007	1957	6729	18928	24431	26	13	12

Table 23: Potential novel markers for Stalk cells. Genes that are highly expressed in CD34-VEGF-treated HUVECs and BMP9-treated HUVECs and may be novel stalk cell markers.

Regarding the embryonic EC lineage, I analysed 3 samples of iPSC-derived CD34+ PECs. Although all these samples were treated the same way (in separate experiments) they expressed different levels of CD34. Other vascular markers, such as ENG, PECAM1, CDH5 and VWF showed a similar distribution, increasing from sample a) to c) **(Table 25).** These genes were even higher expressed in HUVECs. This might be indicative of different EC maturation levels in our PEC samples.

3.3.4 Computational analysis of RNAseq data

To analyse the expression profiles in a more comprehensive way, also bioinformatics approaches were applied to interpret the collected data in the context of biological processes, pathways and networks. Systematic analysis was applied using the different annotation tools, AutoSOME, WebGestalt and DAVID.

3.3.4.1 Analysing RNAseq data using AutoSOME

RNAseq experiments usually yield massive, high-dimensional datasets that require practical methods to find natural clusters. AutoSOME is a computational method for clustering large, multi-dimensional biological sequence datasets without prior knowledge of cluster number or structure (Newman and Cooper, 2010). AutoSOME is purely based on mathematical analysis and creates clusters according to the similarity in expression between different groups (Newman and Cooper, 2010) .

In current RNAseq analysis, AutoSOME identified 72 different clusters in total. Among them, the three blood derived samples formed the largest cluster. Figure 64 shows the heat map for the four largest clusters. The second biggest cluster belonged to genes that were expressed meaningfully higher in the HUVEC populations (both VEGF and BMP9 treated). The third biggest cluster belonged to genes with high expression in iPSC-derived PECs, iPSC+ anti-VEGF cells and HUVECs, compared to blood-derived cells. The fourth biggest cluster contained highly expressed genes in iPSC-derived PECs and iPSC+ anti-VEGF cells **(Figure 64).** These data show that cell populations from the same origin tend to have similar level of gene expressions.

For further analysis of these clusters, DAVID online tools were used to find whether there were any biological processes enriched within each cluster. The DAVID functional annotation cluster analysis is an online suite of bioinformatics tools that are designed to provide functional interpretation of large gene/protein lists derived from high-throughput genomic experiments. For any given gene list, DAVID tools identify enriched biological themes, particularly Gene Ontology (GO) terms (Lee et al., 2014).

David showed the involvement of the first gene cluster in the regulation of immune response, leukocyte and lymphocytes activation. This is not surprising since these cells are all part of the immune system. However, analysing the other clusters showed their

involvement in very different biological pathways. Because AutoSOME, did not show how closely related or how far apart the different cell populations are far from each other, Principle Component Analysis (PCA) were used in the next step.

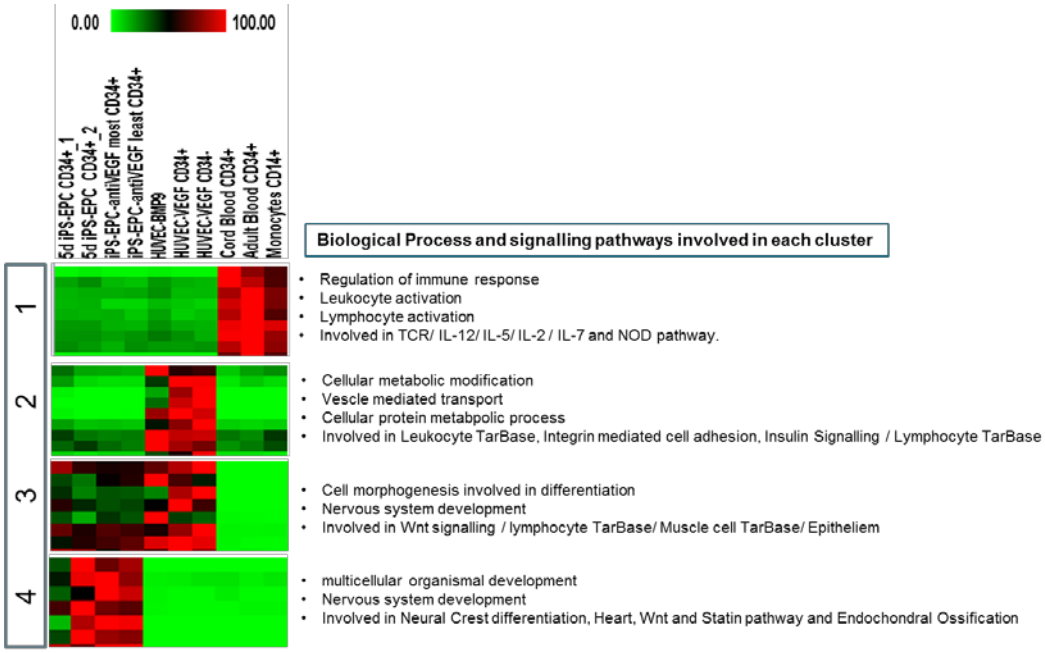


Figure 63: Gene expression heat map showing that the first four largest gene expression clusters.
(Low expression is shown in green, high expression is shown in red). The first four clusters belong to blood-derived, HUVECs, iPSC-derived PECs, iPSC+ anti-VEGF respectively.

3.3.4.2 Principle Component Analysis (PCA)

Current data contains information about the expression levels of around 16,000 genes. Such dimensionality makes it difficult to visualize the data in its entirety. PCA is a mathematical algorithm that reduces the dimensionality of complex data while retaining most of the variation in the data set. It undertakes this reduction by identifying directions called principle components, alongside which the variation in the data is the highest. In this technique, by considering few components, each sample could be signified by a few numbers (typically 2 or 3), instead by values for thousands of variables. Then it is possible to plot the samples spatially, which make it easy to visually evaluate similarities and differences between samples and establish whether samples can be grouped (Ringnér, 2008).

PCA was performed by using an “R” software module. (This was done by Hannah Thompson, a BSc student in our lab). Then, a plot was generated to visualize the specific gene signatures that represent the similarity between the sample populations. The 13 samples were clustered roughly in 3 different groups **(Figure 65)**. HUVEC samples were clustered in 1 group regardless of their treatment conditions which demonstrates different treatment conditions did not remarkably change their gene expression profile. Furthermore, all blood derived cells (cord/adult CD34+ and CD14+ cells) were clustered together (labelled 1, 2 &3) which were clearly segregated from iPSC-derived CD34+ and CD34- cells (labelled 4, 5, 6, 7 &8).

This data surprisingly demonstrates that adult CD34+ cells are fundamentally different cell population from embryonic derived CD34+ cells. This data is also consistent with AutoSOME data which clustered all blood derived cells together and separated from HUVEC and iPS derived cells. This data suggest that origin of derived-cells is more important than expression of specific marker to indicate the similarities between different cell populations

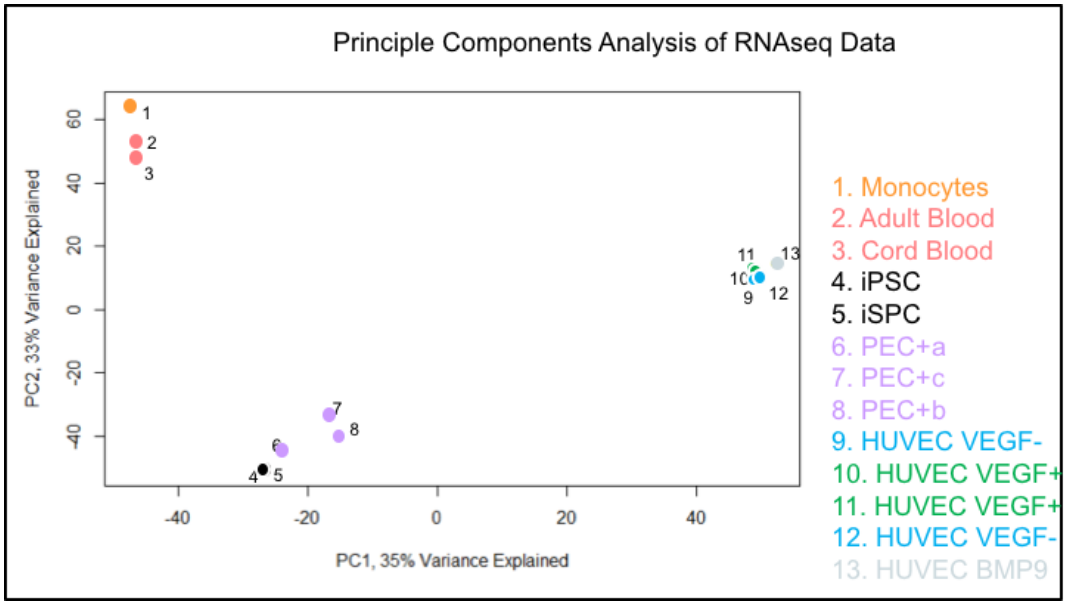


Figure 64: Principle Component Analysis (PCA).
PCA results show the clustering of each cell population and separation of the clusters according to their gene profiling. PCA analysis demonstrated HUVEC, PECs and blood-derived CD34+ cells are fundamentally different cell populations.

3.3.5 Novel markers for PECs

In order to find potential markers for PECs, I searched for genes that were low in the anti-VEGF treated cultures and that had increasing expression levels in the 3 CD34+ PEC samples. Prominent genes with this expression pattern were APLN, CD34, SOX7, MMRN2, THBD and EGFL7 **(Table 30).**

Genes	iPS mesoderm anti-vegf (n=2)	iPS-PEC a CD34+ (n=1)	iPS-PEC b CD34+ (n=1)	iPS-PEC c CD34+ (n=1)	HUVEC VEGF CD34+ (n=2)	HUVEC VEGF CD34- (n=2)	HUVEC BMP9 . (n=1)	Cord Blood CD34+ (n=1)	Adult Blood CD34+ (n=1)	Adult monocytes CD14+ (n=1)
APLN	43	17813	37226	130810	500346	85456	8203	17	27	11
CD34	104	9262	38166	74732	189574	3215	4296	38347	36999	1
SOX7	87	5760	20447	41496	12402	5446	6506	124	176	148
MMRN2	80	3683	22207	30909	42079	34592	29962	9	31	27
THBD	59	2357	10312	14530	15571	3698	5236	257	36	4902
EGFL7	1638	13757	50741	121669	53629	25761	27311	13356	9985	97

Table 24: Potential gene candidates that are highly expressed in PEC1, PEC2 & PEC3 and then increasing or decreasing in HUVECs.

In order to find further potential PEC markers a more systematic approach was applied. Firstly, only genes were considered that showed an average increase from the iPSC mesoderm (with anti-VEGF) to the CD34+ iPSC-PECs of at least 2 fold. Next, only genes with read numbers of 10,000 or more were considered. The cut-off of 10,000 was chosen because this was the roughly expression level in the PEC sample with the lowest CD34 expression (the aim is to find novel markers that are at least as strongly expressed as CD34). This led to a list of 270 genes **(Table 31)**.

These 270 genes were then run into WebGestalt and DAVID to find whether there were any biological processes enriched within these genes. WebGestalt website is an enrichment analysis tool that can plot “directed acyclic graph” (DAG) to reveal the hierarchical relationship of enriched (gene ontology) GO. The enriched DAG visualisation has been applied to the phenotype enrichment results for visualising the hierarchical relationship of enriched phenotype terms. In the outcome results, the red label signifies enriched categories and the black label represents their non-enriched patterns (Wang et al., 2013). Furthermore, the pathway database of KEGG records network interactions between the different molecules in cells and connects them with a

gene list with the aim of finding the cascade of pathways including genes that have shown to have an exclusive function of a unique pathway (Collard and Hinsenkamp, 2015).

1) KDR	55) DCHS1	109)MYL12A	163)EPHA2	217)PALD1
2) IGFBP4	56) TUBB6	110)ADAM15	164)TGOLN2	218)PPP1R15A
3) SERPINH1	57) HSPG2	111)CD99	165)GPR124	219)DUSP6
4) SLC2A3	58) ARHGEF2	112)PIK3C2A	166)CORO1B	220)NR4A1
5) MSN	59) F2RL2	113)GSN	167)ARPC1B	221)CYR61
6) FSCN1	60) HLA-E	114)SHANK3	168)DAB2	222)JAK1
7) APLN	61) PHLDA1	115)NRP1	169)LGALS1	223)ARHGEF12
8) APLNR	62) PLK2	116)TM4SF1	170)TAGLN2	224)ERG
9) EGFL7	63) RASIP1	117)DOCK6	171)FMNL3	225)KCTD20
10) NRP2	64) FLI1	118)TIMP1	172)THBD	226)ZBTB39
11) SERPINE2	65) MMRN2	119)PPP1R18	173)SLC12A7	227)FAM69B
12) ESYT1	66) PEA15	120)TPST2	174)MCAM	228)CRHBP
13) COL1A2	67) AKAP12	121)IDH2	175)ATP6AP1	229)ZNF710
14) PCF11	68) ILK	122)CXorf36	176)AKAP2	230)CCNY
15) SHC1	69) NID1	123)TMC6	177)VWA5A	231)MED15
16) ITGB1	70) PLVAP	124)FAM83H	178)APOA1	232)FXYD5
17) CLIC1	71) COLEC12	125)ST3GAL1	179)TGFB1	233)ELTD1
18) CD34	72) FLT4	126)TFPI	180)JUN	234)TMEM8A
19) COL18A1	73) TXNDC5	127)MYO1C	181)TNFAIP1	235)ALPK3
20) SPTAN1	74) SHISA5	128)TSPAN14	182)FAM65A	236)PPFIBP1
21) PLXND1	75) TRIM24	129)RASGRP3	183)EPCAM	237)GADD45A
22) COL4A1	76) IER2	130)CTSB	184)LMO2	238)VAV3
23) SPTBN1	77) BCAR1	131)AMOT	185)STAT6	239)GATA2
24) ETS1	78) SLC2A14	132)FGFR4	186)FLRT3	240)SH2B3
25) COL5A1	79) HDAC7	133)ARAP3	187)RALB	241)CDC42SE1
26) PMP22	80) ASH2L	134)PPP1R16B	188)ABHD17A	242)CLIP2
27) DYSF	81) THY1	135)SORBS1	189)ARHGAP29	243)SOCS3
28) HAPLN1	82) RALA	136)IDS	190)AES	244)PCDH10
29) TPM3	83) EPHB4	137)BIN1	191)FGD5	245)SP6
30) FKBP1A	84) LEPRE1	138)F2R	192)FBLN2	246)SPG20
31) FLT1	85) NTS	139)YES1	193)ATP11A	247)ARL4D
32) COL6A2	86) AFAP1L1	140)MICAL2	194)CMIP	248)TMEM214
33) ARHGDIA	87) NOS3	141)PRCP	195)MYOF	249)CDK17
34) TIE1	88) RAB1B	142)TMBIM1	196)IFITM2	250)SH3GLB1
35) TP53I11	89) KIAA1462	143)SAMSN1	197)S100A10	251)RGL2
36) CCND1	90) MMP2	144)LIFR	198)KIAA1551	252)NECAP2
37) RGS5	91) EHD2	145)GRAP2	199)TIMP3	253)TAX1BP3
38) ITGA5	92) RHOC	146)PECAM1	200)TEK	254)ICAM2
39) MMP14	93) S100A11	147)STX3	201)SLC44A1	255)C1GALT1
40) CD81	94) CAPN2	148)FAM129B	202)SMTN	256)HHEX
41) GPX1	95) PNP	149)APOB	203)SYTL5	257)FAM222A
42) SOX7	96) TAL1	150)CD93	204)MAP3K3	258)ARHGAP31
43) AP1B1	97) SEC13	151)PXN	205)SEC24D	259)ARHGAP23
44) CORO1C	98) FURIN	152)COL9A2	206)SNX3	260)ALDH2
45) SLC20A1	99) JAM3	153)IL6ST	207)LHX1	261)CMTM3
46) GNG11	100)IFI16	154)DAP	208)GDPD5	262)EFCAB14
47) COL5A2	101)ZYX	155)SH3BGRL3	209)ADAMTS9	263)B4GALT1
48) CDH5	102)MAP3K11	156)MESDC1	210)CA2	264)GATA4
49) TLN1	103)PROCR	157)PCDH17	211)TM4SF18	265)RGL1
50) PTRF	104)GAD1	158)BCL6B	212)PPP1R9B	266)WSCD1
51) HK1	105)ARF4	159)CFLAR	213)PTP4A3	267)ZCCHC24
52) PPM1F	106)MYADM	160)ESAM	214)PIEZO1	268)ACVRL1
53) COL4A2	107)SV2A	161)BAMBI	215)SNPH	269)SEMA7A
54) MBOAT7	108)VASP	162)STARD8	216)SLC9A3R2	270)SULF2

Table 25: Genes with an expression level of at least 10,000 and at least 2 fold enrichment from mesoderm to CD34+ PEC.

In the first exploration step, the 270 selected genes were analysed in WebGestalt. Enriched gene groups are shown in red in **Figure 66.** A large group (63 genes out of 270) fell into the cardiovascular development group and 44 of those belonged to angiogenesis. This outcome is likely due to the effects of the blocking and the adding of VEGF in the two samples, which were otherwise treated fairly similarly.

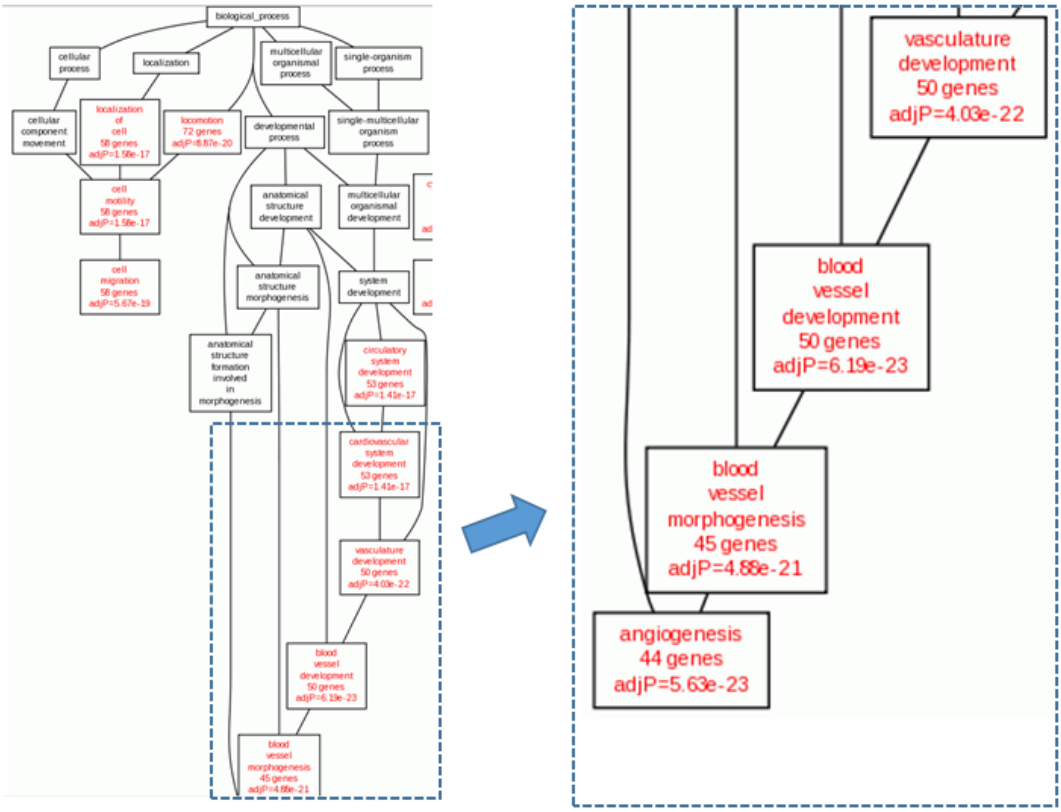


Figure 65: WebGestalt outcomes.

'Biological processes obtained from WebGestalt for a set of 270 genes that were differentially expressed in our RNAseq data and selected according to 2 criteria: expression level > 10,000 and fold change >2x. Categories in red are enriched, while those in black are non-enriched parents. Listed in the boxes are the name of the GO category, the number of genes in the category and the P-value indicates that enrichment is significant.

3.3.5.1 Pathways involved in differentially expressed genes

KEGG pathway embedded in WebGestalt was used to perform enrichment analysis based on KEGG pathway databases to detect the signaling pathways in which the differentially expressed genes were involved. “KEGG (Kyoto Encyclopedia of Genes and Genomes) is a bioinformatics resource which records networks of molecular interactions in cells for the better understanding of high-level functions of the biological system from large-scale molecular datasets (ref: Kanehisa M et al., 2004, Yu Y et al., 2015). Enrichment analysis based on KEGG pathway database illustrated that differentially expressed genes (270 selected genes) were involved in 6 pathways Table 11. Of these six signalling pathways, four of them (Focal adhesion (23 genes), ECM-receptor interaction (10 genes), Regulation of actin cytoskeleton (12 genes) and Cell adhesion molecules (9 genes)) are involved in some form of cytoskeletal interactions with the extracellular space, relating to cell adhesion and cell migration processes. Current results suggest a meaningful association of these pathways with VEGF-related pathway and need further investigations. Based on KEGG pathway database, the Focal adhesion pathway was found to involve the maximum number of genes (23 genes) and selected to be explored in more details.

PathwayName	#Gene	EntrezGene	Statistics
Focal adhesion	23	10451 5829 1292 1290 1284 7094 3791 3678 3611 9564 10627 595 3725 824 7791 7408 6464 2324 1289 2321 3688 1282 1278	C=200;O=23;E=1.23;R=18.65;rawP=1.88e-22;adjP=1.41e-20
Leukocyte transendothelial migration	14	4267 7070 10451 90952 5829 7408 1003 4313 3688 5175 4478 9564 10627 83700	C=116;O=14;E=0.72;R=19.57;rawP=1.89e-14;adjP=7.09e-13
ECM-receptor interaction	10	1292 1290 1284 3339 9900 1289 3688 3678 1282 1278	C=85;O=10;E=0.52;R=19.07;rawP=1.40e-10;adjP=3.50e-09
Protein digestion and absorption	9	1292 1290 1284 5547 1289 80781 1282 1278 1298	C=81;O=9;E=0.50;R=18.01;rawP=2.00e-09;adjP=3.75e-08
Regulation of actin cytoskeleton	12	2264 10451 2934 5829 10095 3688 3678 23365 4478 9564 2149 10627	C=213;O=12;E=1.31;R=9.13;rawP=1.05e-08;adjP=1.58e-07
Cell adhesion molecules (CAMs)	9	4267 90952 1003 3133 3688 947 5175 3384 83700	C=133;O=9;E=0.82;R=10.97;rawP=1.58e-07;adjP=1.97e-06

Table 26: **Functional gene enrichment of the KEGG focal adhesion pathway. Genes of interest are involved in six different signalling pathways. The row lists the following statistics: C: the number of reference genes in the category; O: the number of genes in the gene set and also in the category; E: the expected number in the category; R: ratio of enrichment; raw P: p value from hypergeometric test; P: p value adjusted by the multiple test adjustment.**

Focal adhesion was one of the pathways that was shown to be enriched in KEGG pathway database which diagram was further investigated in Figure 6. KEGG pathway diagram represents the role of involvement of the specific genes at a particular location in the Focal Adhesion pathway. For instance, 23 genes (ECM, ITGA, ITGB, RTK, Talin, Paxillin, etc.) among 270 selected genes are involved in Focal adhesion pathway. Focal adhesion is a critical signaling pathway at the interface between cells and the ECM (Xin L et al., 2016, Parsons JT et al., 2010, Zaidel-Bar R et al., 2007) and direct numerous signaling proteins at sites of integrin binding and clustering. Focal adhesion pathway is playing essential roles in cell proliferation, differentiation, motility and regulation of gene expression.

KEGG diagram shows upregulation of some intracellular focal adhesion turnover signaling such as (Talin, Paxillin, Calpain, ILK, VASP) or some genes involved in ECM-receptor interactions such as (ITGA, ITGB, and RTK). Association of Focal adhesion pathway with angiogenesis in embryonic development has been shown in previous studies on conditional Focal adhesion kinases (FAK) knockout mouse models (Zhao X et al., 2011). Current enriched pathway also indicates the high association of Focal adhesion pathway with angiogenesis and need further investigation.

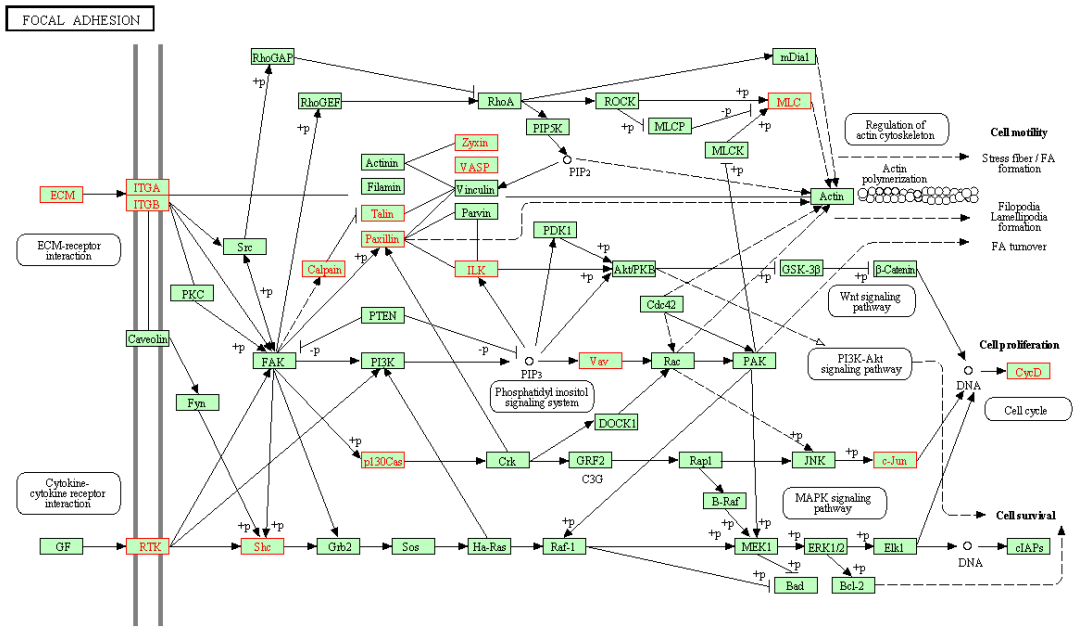
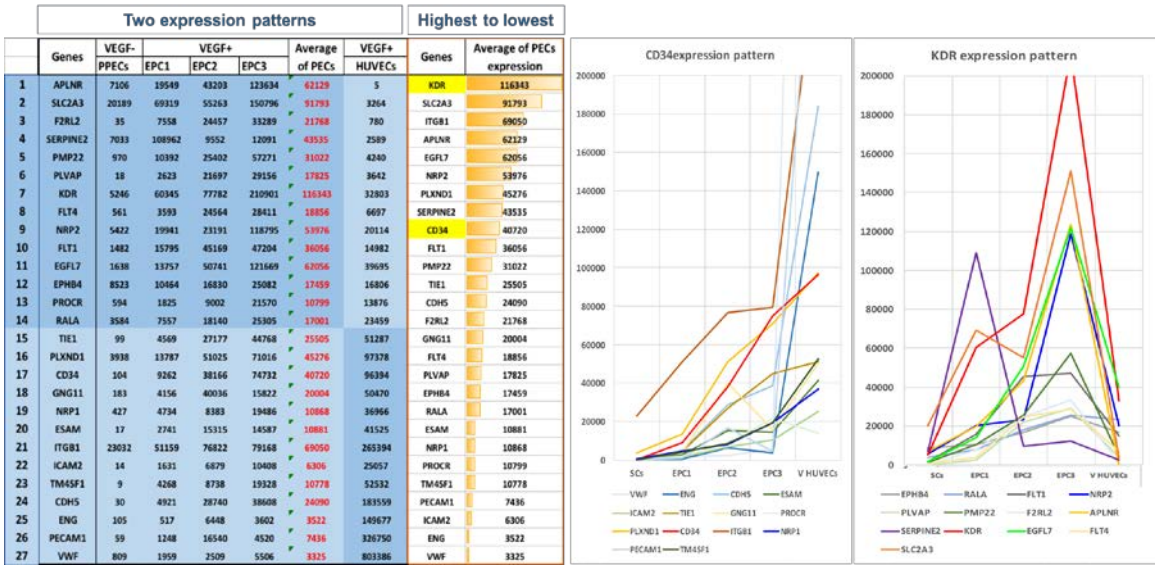


Figure 66: : KEGG Focal Adhesion diagram. KEGG pathway analysis figure represents the role of involvement of the specific genes at a particular location in the Focal Adhesion pathway. Red labelled genes are among the differentially expressed genes in the RNA-seq data.

3.3.6 Validation of potentially novel PEC markers by immune-staining

Since potential novel markers of PECs would be most useful if they can be detected with antibodies on living cells, 24 candidates based on previous selection criteria (> than 2-fold change between PECs and PPECs and having reads more 10.000) with cell surface localization were selected for further analysis. Furthermore, as a reference, the endothelial genes VWF, ENG and PECAM1 were also included. The selected 24 genes fell into two groups. The first group followed the expression pattern of CD34 (ESAM, ICAM2, TIE1, GNG11, PROCP, PLXND1, CD34, ITGB1, NPR1, PCAM1 and TM4SF1), with low levels in mesoderm and a continuous rise from PEC a) to PEC c) and then HUVECs (left graph in from number 15 to 27 in Figure 7). The endothelial genes are also shown in this panel, with a low level in mesoderm and PEC, and a strong increase in HUVECs (number 25, 26 &27 in Figure 7).

The second category followed the expression pattern of KDR (EPHB4, RALA, FLT1, NRP2, PLVAP, PMP22, F2RL2, APLNR, KDR, EGFL7, FLT4, SLC2A3), with low levels in mesoderm and a continuous rise from PEC a) to PEC c) and a drop in HUVECs (numbers 1 to 14 in left graph in Figure 7). The only exception is SERPINE2, which already drops in the PEC b) sample. This category was chosen because genes with specifically high expression only in PECs with following decrease in HUVEC are potentially novel markers for progenitors of endothelial cells. From all these 14 potential markers, four of them with the highest expression in the average of PECs (1, two &3) were selected for further validation steps by immunocytochemistry (APLR, SLC2A3, EGFL7, NPR1). Furthermore, among markers with similar expression pattern as CD34+ cells, ITGB1 was selected as it has higher expression level than CD34+ cells in PECs. These markers were selected based on the availability of suitable antibody in the market.



	Genes	VEGF-	VEGF+			Average of PECs	VEGF+
		PPECs	EPC1	EPC2	EPC3		HUVECs
1	APLNR	7106	19549	43203	123634	62129	5
2	SLC2A3	20189	69319	55263	150796	91793	3264
3	F2RL2	35	7558	24457	33289	21768	780
4	SERPINE2	7033	108962	9552	12091	43535	2589
5	PMP22	970	10392	25402	57271	31022	4240
6	PLVAP	18	2623	21697	29156	17825	3642
7	KDR	5246	60345	77782	210901	116343	32803
8	FLT4	561	3593	24564	28411	18856	6697
9	NRP2	5422	19941	23191	118795	53976	20114
10	FLT1	1482	15795	45169	47204	36056	14982
11	EGFL7	1638	13757	50741	121669	62056	39695
12	EPHB4	8523	10464	16830	25082	17459	16806
13	PROCR	594	1825	9002	21570	10799	13876
14	RALA	3584	7557	18140	25305	17001	23459
15	TIE1	99	4569	27177	44768	25505	51287
16	PLXND1	3938	13787	51025	71016	45276	97378
17	CD34	104	9262	38166	74732	40720	96394
18	GNG11	183	4156	40036	15822	20004	50470
19	NRP1	427	4734	8383	19486	10868	36966
20	ESAM	17	2741	15315	14587	10881	41525
21	ITGB1	23032	51159	76822	79168	69050	265394
22	ICAM2	14	1631	6879	10408	6306	25057
23	TM4SF1	9	4268	8738	19328	10778	52532
24	CDH5	30	4921	28740	38608	24090	183559
25	ENG	105	517	6448	3602	3522	149677
26	PECAM1	59	1248	16540	4520	7436	326750
27	VWF	809	1959	2509	5506	3325	803386

Genes	Average of PECs expression
KDR	116343
SLC2A3	91793
ITGB1	69050
APLNR	62129
EGFL7	62056
NRP2	53976
PLXND1	45276
SERPINE2	43535
CD34	40720
FLT1	36056
PMP22	31022
TIE1	25505
CDH5	24090
F2RL2	21768
GNG11	20004
FLT4	18856
PLVAP	17825
EPHB4	17459
RALA	17001
ESAM	10881
NRP1	10868
PROCR	10799
TM4SF1	10778
PECAM1	7436
ICAM2	6306
ENG	3522
VWF	3325

Figure 67**: Representative genes (number = 24) selected according to their high expression level and strong fold change and two expression pattern; The first group followed the expression pattern of KDR from number 1 to 14 with low levels in mesoderm PPECs and a continuous rise from PEC 1 to PEC 3 (shown by darker blue) and a drop in HUVECs (shown by lighter blue). The second group followed the expression pattern of CD34 from number 15 to 27 with low expression levels in mesoderm PPECs and a continuous rise from PEC a) to PEC c) (lighter blue) and then HUVECs (darker blue). VWF, ENG, and PECAM1 were chosen as reference genes. The right table illustrates the average of PECs expression pattern that is sorted from highest to lowest. Right diagrams also show the expression pattern of each group separately.**

A panel of five antibodies was selected to confirm the RNA-sequencing data. (APLR, SLC2A3, EGFL7, NPR1 and ITGB1). It should be mentioned that antibodies were used for four different types of cells as follows; iPSC-derived PECs which were expected to show high expression of these markers, mesoderm PPECs (iPSC+ Anti-VEGF) which were iPSCs in more immature state (mesoderm) and were expected to show very low/ no expression of these markers, iPSC-derived endothelial cells that were purified-CD34 PECs (MACS-sorted cells) and plated in EGM-2 media and totally differentiated to endothelial cells after 4 days (showed high expression of VE-cadherin and no expression of CD34). iPSC-derived endothelial cells similar to HUVECs were expected to show very low/ no expression of APLR, SLC2A3 (GLU3), EGFL7, NPR1 (KDR expression pattern). However, ITGB1 antibody were selected and were expected to show low expression in PPECs, and high expression in PECs, iPSC-derived EC and HUVECs respectively (CD34 expression pattern).

Among five different antibodies that were used to confirm the RNA-sequencing data, only two worked for immunocytochemistry (Those that were not worked are not shown here). However, immunocytochemistry turned out to be more problematic than anticipated. The two antibodies (against SLC2A3, and EGFL7) appeared to give a specific looking labeling. Immuno-staining showed strong staining of anti-SLC2A3 on both, mesoderm PPECs and CD34+ PECs, which conflicts with our RNAseq data which showed no (or low) expression in mesoderm PPECs. Furthermore, since SLC2A3 should be expressed on PEC population, immunostaining data were expected to show co-localisation of this marker with CD34. However, the staining did not show any co-localisation of this marker with CD34+ labeled cells (Figure 8). iPSC-derived ECs did not show any SLC2A3 expression consistent with RNA-seq data. However, HUVECs showed some expression of SLC2A3 which was again inconsistent with RNA-sequencing data.

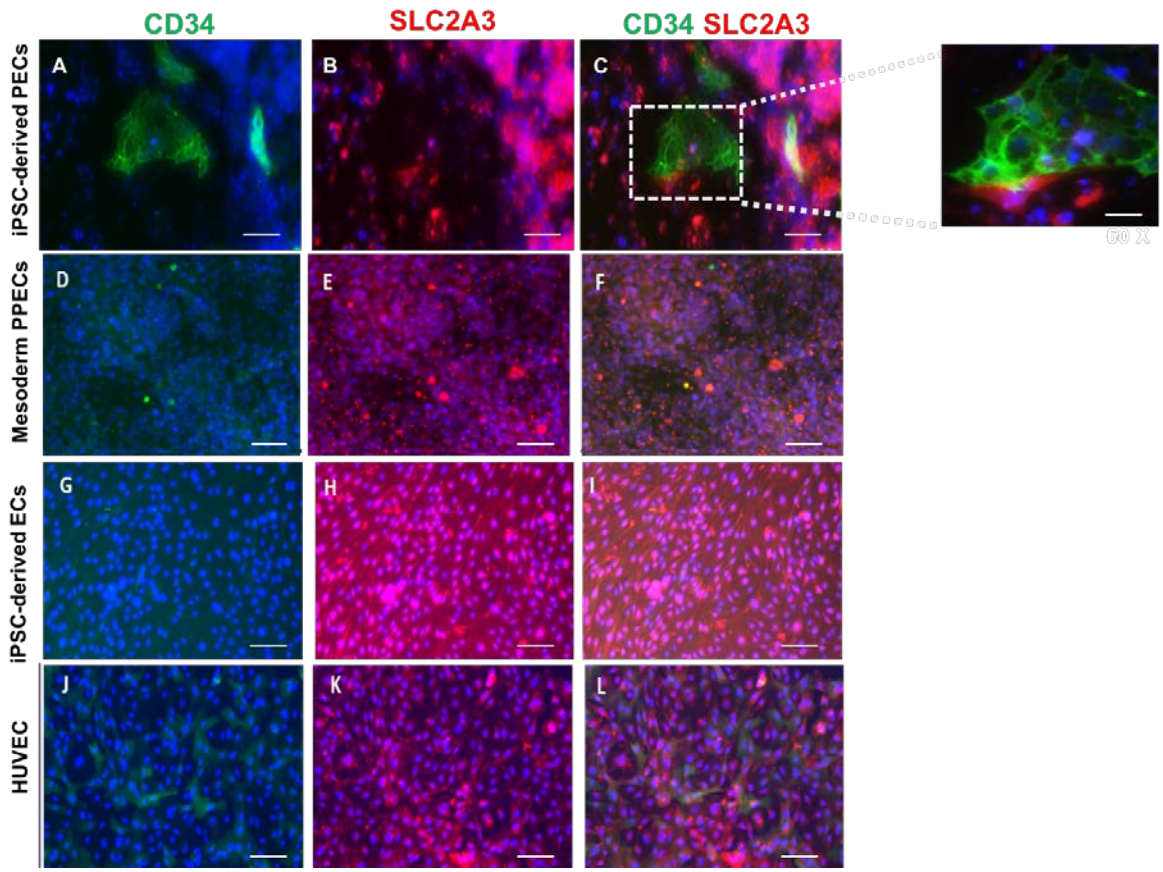


Figure 68: SLC2A3 and CD34 immunostaining results.
Representative pictures of iPSC-derived PECs (A, B & C) and mesoderm PPECs (D, E & F) showed strong staining of anti-SLC2A3. However, higher magnification of staining did not show any co-localization of CD34 and SLC2A3. No expression of CD34 and SLC2A3 observed in iPSC-derived EC (G, H &I). HUVECs showed some expression of CD34 and SLC2A3 (J, K &L). (No other high magnification of other cells available). Nuclei are stained with DAPI in blue. Scale bar, 164 µm.

EGFL7 immunostaining looked more promising as it was low on mesoderm PPECs consistent with the RNA-seq data and high expression in iPSC-derived PECs. However, similar to SLC2A3 staining, EGFL7 was not co-expressed with CD34 positive cells Figure 70. Furthermore, EGFL7 showed some degree of expression in both iPSC-derived ECs and HUVECs which was inconsistent with RNA-sequencing results. Because of the discrepancy with the RNA-seq data, it was concluded that both of these antibodies gave non-specific staining. Because the immunostaining data did not show clear staining, no more images were taken from different magnifications. Due to problems that might happen with antibodies, it was decided to screen the expression of markers using FACS in further experiments.

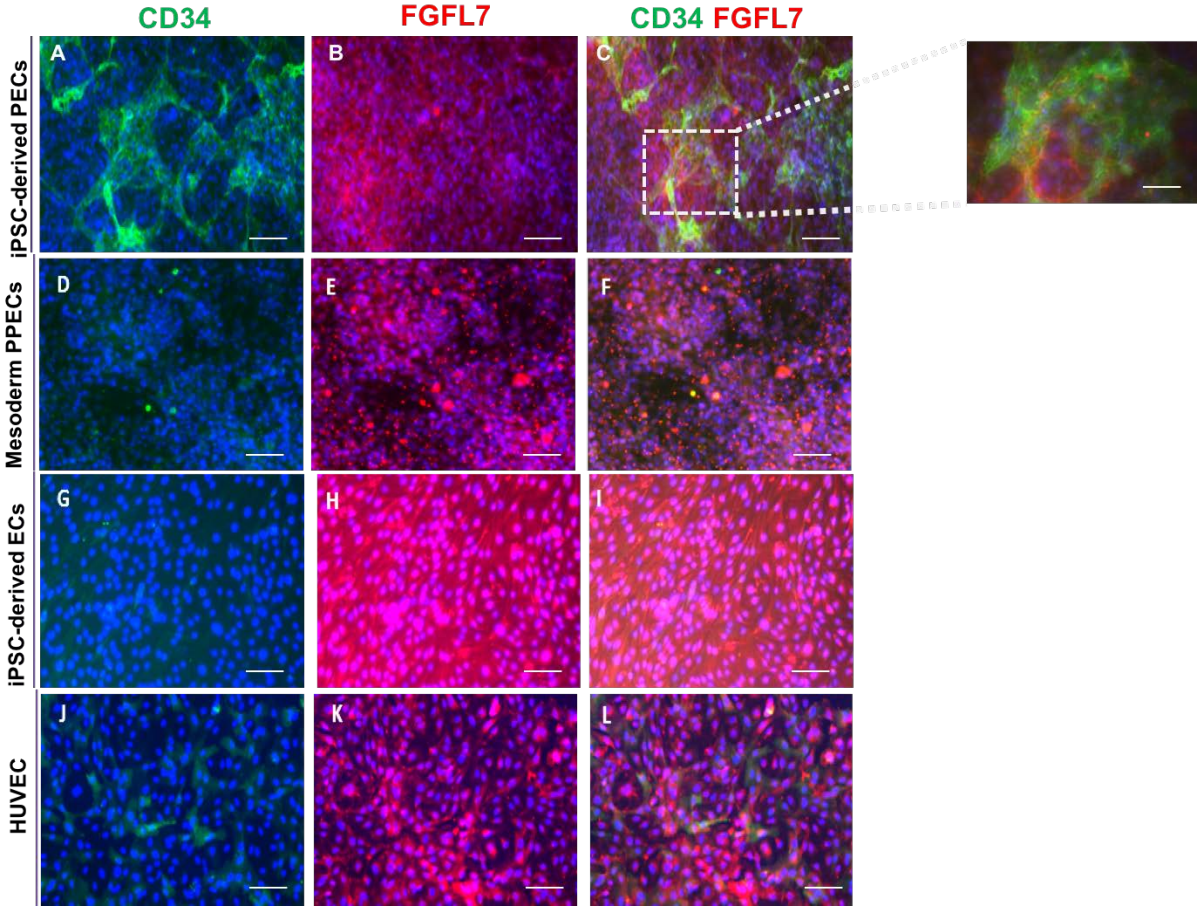


Figure 69: FGFL7and CD34 immunostaining results.

Representative pictures of iPSC-derived PECs (A, B & C) illustrated high expression of CD34 and FGFL7, however, higher magnification of staining did not show any co-localization of CD34 and FGFL7. No expression of either CD34 or FGFL7 observed in mesoderm PPECs (D, E & F). iPSC-derived EC (G, H &I) and HUVECs (J, K & l) showed some degree of FGFL7 expression. (No other high magnification of other cells available). Nuclei are stained with DAPI in blue. Scale bar, 164 µm.

3.3.7 Discussion

RNAseq is a novel approach for transcriptome profiling that provides more precise measurement of levels of transcripts, as compared to other methods. This method's main advantage over other techniques, such as qPCR, is that it is not restricted to particular primers that are individually designed, and it is also not limited to only genes we are aware of, but can also be used to identify every single gene that is expressed in each type of cell.

After the validation of RNAseq data, by choosing selection criteria, genes were further investigated within the groups to get more insight into each specific group. Our investigations showed high expression levels of monocyte/macrophage markers in CD14+ cells which is not surprising as is know these cells are a part of the immune system.

However, some of these genes (TYROBP, FCER1G, HLA-DRA, S100A9, and ITGB2), were also shown to be expressed in so-called "early EPCs" (Medina et al., 2010b). Our investigations displayed the high expression levels of these genes in the CD14+ cell population. Therefore, this data confirms the validity of previous studies regarding the characteristics of early EPCs, and that they share lineage traits with immune cells, specifically macrophages and monocytes.

Additionally, gene transcriptional profiling of different HUVEC populations were in accordance with previous studies, showing that CD34+ and CD34- cells in HUVECs have similar gene expression profiles to tip and stalk cells respectively (Siemerink et al., 2012, Blanco and Gerhardt, 2013, Jakobsson et al., 2009). All known tip cell markers such as DLL4, CXCR4, UNC5B were expressed remarkably high (>3 fold change) in CD34+ cells; whereas stalk cell specific markers such as JAG1 and FLT1 were highly expressed in the CD34- population (both VEGF and BMP9-treated HUVECs).

Our previous in vitro analysis demonstrated the generation of these phenotypes at the cellular level, which was shown by a high expression of CD34 in VEGF-treated HUVECs versus a very low expression of CD34 in BMP9-treated HUVECs. These results were further confirmed by our RNAseq data.

Furthermore, comparing highly expressed tip cell markers through all populations, revealed that some genes such as APLN had increasing expression from PEC a) to PEC c). Also, the expression level in CD34- VEGF-treated HUVECs was high (>10 fold change) compared to BMP9 CD34- cells. However, other genes such as IGF2 did not show any increased from PEC1 to PEC3, while much higher expression levels were observed in BMP9 treated HUVECs (>7) compared to the VEGF-treated ones. These observations could suggest that genes with similar expression pattern as APLN play a role in the differentiation of stem cells towards progenitors of endothelial cells.

Further investigation on CD34- HUVECs (VEGF versus BMP9 treated) showed reasonably high similarity between these two populations. One exception was expression of SMAD6 (shown to block the signal transduction of BMPs), was found to be meaningfully higher (>22-fold change), in BMP9 vs. VEGF CD34- cells. Taking into account previous studies, showing that over-expression of SMAD6 inhibits SMAD 1/5/8 phosphorylation (Horiki et al., 2004), it could be suggested that SMAD6 and BMP9 were directly affecting each other, and over-expression of BMP9 resulted in up-regulation of SMAD6, to inhibit stimulatory effect of BMP9 in BMP9-treated HUVECs. Further investigations could also introduce novel markers for tip (GPR116 and PCDH12) and stalk (FSTL3 and GDF6) cells.

Finally, comparison of the three different PEC samples showed different expression levels of CD34 and other EC markers, which may be indicative of different maturation levels in these populations. In order to find potential markers for PECs I searched for genes that were low in the anti-VEGF treated cultures and that had increasing expression levels in the 3 CD34+ PEC samples. This yielded a list of 22 promising candidates but so far I have been unsuccessful in our attempts to validate these makers by immunocytochemistry. Most likely, the antibodies I purchased were not specific. Hence, further work is required.

PCA analysis to decompose the overall variability of gene expression data indicated that iPSC-derived PECs and iPSC mesoderm (with anti-VEGF) had the greatest similarity in their gene expression levels. This might be due to the fact that these samples came from the same cell sources (+/- VEGF for two days). However, it was surprising to find that the cord and peripheral blood derived CD34+ cells were clustered so close to the iPSCs. This could suggests that blood derived CD34+ cells are closer to

mesodermal type of cells than expected, and, therefore, may have some “stem” properties,

PCA analysis also placed the HUVECs at quite some distance to our PEC, which might be due to a higher maturation level of HUVECs. Furthermore, comparing VEGF versus BMP9-treated HUVECs, suggests that adding this factor is sufficient to change the gene expression profile that could be completely distinguishable in an overall gene expression analysis. This could also confirm our in vitro experiments which showed that the phenotype of HUVECs could be remarkably changed by exposing them to specific factors.

Overall, RNAseq data revealing a distinct transcriptome profile in different CD34+ / CD34- populations, suggested that a five-day differentiation protocol profoundly affected iPSC cells, to differentiate them into endothelial lineage which was reflected in their gene expression levels.

It is important to consider that the quality of RNA has important effect on gene expression profiles. Some technical factors such as sample collection, transportation and storage conditions as well as RNA isolation techniques can considerably affect the overall gene expression(Thach et al., 2003). In our study, RNA extraction procedures were applied in different days and although it was attempt to maintain the cells in similar conditions, some factors were out of our control. For instance, during FACS procedure, cells were sorted directly into the Trizol to capture the cells straight away after treatment and avoid RNA degradation that might affect the gene expression profile. However, in some experiments, the ratio of sort volume to Trizol (which was recommended to be 1:10) was exceeded. This may have prevent Trizol from working properly.

4 General Discussion

In this thesis, the main aim was to conduct a gene expression analysis to better understand the nature of EPCs and PECs and to find novel markers for these cells. In order to achieve this CD34 was used as marker for EPCs. This marker has been found both in adult circulating EPCs and embryonic PECs. However, because only a small population of PECs was generated from our stem cells, I first needed to develop an efficient protocol to derive PECs in larger quantities. Therefore, in the first part of the project, I optimised a protocol to reproducibly differentiate iPS cells into PECs, which enabled us to isolate these cells in sufficient quantities.

Although some previous studies suggested that VEGF likely regulates the survival or propagation of PECs, and not necessarily their differentiation, our studies could clearly show that VEGF plays a key role in the transition of precursors to PECs. Generated PECs exhibited strong expression of CD34+ cells after 5 days of differentiation treatment. CD34 and VE-Cadherin expression were inversely related at this stage, which indicates our CD34+ cells are progenitors of ECs. Additionally, further culture of purified PECs on fibronectin, differentiated them into completely mature ECs after 4 days. These cells formed a homologous monolayer with cobblestone appearance that exhibited strong expression of VE-Cadherin and confirmed the endothelial nature of our isolated cells.

Furthermore, since CD34+ HUVECs have been shown to be similar to tip cells in their gene expression profile, and because VEGF, Notch and TGF-β signalling are known to regulate the tip/stalk cell phenotype, I hypothesised that these signalling pathways may also regulate the differentiation of CD34+ HUVECs. I could confirm this hypothesis by demonstrating effects of VEGF, BMP9, DAPT and ALK1 blocker on CD34 expression in HUVECs. Furthermore, proliferation and migration of HUVECs was also affected by these factors consistent with accepted theories on the behaviour of endothelial tip and stalk cells during angiogenic sprouting. It therefore, appears that HUVECs even in monolayer culture are able to display elementary forms of tip and stalk cell phenotypes in vitro and therefore, suggesting HUVECs as a possible in vitro model to study the molecular mechanisms of tip cell biology.

Finally, our transcriptional profiling of different CD34+ and CD34- cell populations had two main outcomes. Firstly, PCA analysis grouped, against our expectation, cord and blood derived CD34+ cells close to iPS-derived CD34+ cells. This could suggest that blood derived CD34+ cells are closer related to mesodermal cell types than expected and therefore, may have some stemness properties. Furthermore, our gene expression profile also showed that CD34+ PECs were more closely related to mesodermal cells than differentiated HUVECs. This might not be too surprising because our PECs were exposed to VEGF for only two days and otherwise had a very similar history to the mesodermal cells, whereas the HUVECs were derived from a much more differentiated source.

The second outcome of the RNAseq results was to the identification of potential PEC markers. But unfortunately our attempts to validate by immunocytochemistry some of these potential PEC markers have so for been not successful because I have not identified specific antibodies yet that are suitable for this. Although I expected to see the co-localisation of our antibodies against novel PEC markers with CD34 (based on the RNAseq data from isolated CD34+ PECs) this did not happen. Therefore as one of the important future plans, I need to further test the suitability of these markers by finding better antibodies.

Further characterisation of our PECs could be done by applying different in vitro assays. For example, co-culture of these cells with pericytes will be useful to see if they can form blood vessel like structures in culture. Furthermore, I can use the spheroid angiogenesis assay to test their capability to generating blood vessels in 3-dimensional environments. Moreover, I could also conduct vivo experiments for functional analysis. For example, injecting of PECs into the developing mouse retina or into diabetic retinopathy mouse models will be useful to see whether these cells can integrate into vasculature and differentiate to endothelial cells.

Next, it would also be interesting to see whether selected markers are expressed in the in the blood (possibly by circulating EPCs) and also, if the expression of these markers is changing in patients with specific diseases. Such markers may not only be of diagnostic value in ischemic diseases such as diabetic retinopathy, but they could also provide a much need platform to develop EPCs into a more reliable therapeutic product.

Thus I believe that this work could facilitate the translation of the promise that EPCs currently hold into useful therapy for diabetic retinopathy.

5 References

ALEV, C., II, M. & ASAHARA, T. 2011. Endothelial progenitor cells: a novel tool for the therapy of ischemic diseases. *Antioxid Redox Signal,* 15**,** 949-65.

ALISON, M. R., POULSOM, R., OTTO, W. R., VIG, P., BRITTAN, M., DIREKZE, N. C., PRESTON, S. L. & WRIGHT, N. A. 2003. Plastic adult stem cells: will they graduate from the school of hard knocks? *J Cell Sci,* 116**,** 599-603.

ANTAS, V. I., AL-DREES, M. A., PRUDENCE, A. J., SUGIYAMA, D. & FRASER, S. T. 2013. Hemogenic endothelium: a vessel for blood production. *Int J Biochem Cell Biol,* 45**,** 692-5.

ARIMA, S., NISHIYAMA, K., KO, T., ARIMA, Y., HAKOZAKI, Y., SUGIHARA, K., KOSEKI, H., UCHIJIMA, Y., KURIHARA, Y. & KURIHARA, H. 2011. Angiogenic morphogenesis driven by dynamic and heterogeneous collective endothelial cell movement. *Development,* 138**,** 4763-76.

ASAHARA, T., MUROHARA, T., SULLIVAN, A., SILVER, M., VAN DER ZEE, R., LI, T., WITZENBICHLER, B., SCHATTEMAN, G. & ISNER, J. M. 1997. Isolation of putative progenitor endothelial cells for angiogenesis. *Science,* 275**,** 964-7.

BALAJI, S., KING, A., CROMBLEHOLME, T. M. & KESWANI, S. G. 2013. The Role of Endothelial Progenitor Cells in Postnatal Vasculogenesis: Implications for Therapeutic Neovascularization and Wound Healing. *Adv Wound Care (New Rochelle),* 2**,** 283-295.

BAUTCH, V. L. 2012. VEGF-directed blood vessel patterning: from cells to organism. *Cold Spring Harb Perspect Med,* 2**,** a006452.

BAUTCH, V. L. & CARON, K. M. 2015. Blood and lymphatic vessel formation. *Cold Spring Harb Perspect Biol,* 7**,** a008268.

BELAOUSSOFF, M., FARRINGTON, S. M. & BARON, M. H. 1998. Hematopoietic induction and respecification of A-P identity by visceral endoderm signaling in the mouse embryo. *Development,* 125**,** 5009-18.

BERTRAND, J. Y., CHI, N. C., SANTOSO, B., TENG, S., STAINIER, D. Y. & TRAVER, D. 2010. Haematopoietic stem cells derive directly from aortic endothelium during development. *Nature,* 464**,** 108-11.

BETSHOLTZ, C., LINDBLOM, P. & GERHARDT, H. 2005. Role of pericytes in vascular morphogenesis. *EXS***,** 115-25.

BEYER, M., MALLMANN, M. R., XUE, J., STARATSCHEK-JOX, A., VORHOLT, D., KREBS, W., SOMMER, D., SANDER, J., MERTENS, C., NINO-CASTRO, A., SCHMIDT, S. V. & SCHULTZE, J. L. 2012. High-resolution transcriptome of human macrophages. *PLoS One,* 7**,** e45466.

BIRSOY, B., KOFRON, M., SCHAIBLE, K., WYLIE, C. & HEASMAN, J. 2006. Vg 1 is an essential signaling molecule in Xenopus development. *Development,* 133**,** 15-20.

BLANCO, R. & GERHARDT, H. 2013. VEGF and Notch in tip and stalk cell selection. *Cold Spring Harb Perspect Med,* 3**,** a006569.

BLANPAIN, C., LOWRY, W. E., GEOGHEGAN, A., POLAK, L. & FUCHS, E. 2004. Self-renewal, multipotency, and the existence of two cell populations within an epithelial stem cell niche. *Cell,* 118**,** 635-48.

BLUM, B. & BENVENISTY, N. 2008. The tumorigenicity of human embryonic stem cells. *Adv Cancer Res,* 100**,** 133-58.

BREIER, G., CLAUSS, M. & RISAU, W. 1995. Coordinate expression of vascular endothelial growth factor receptor-1 (flt-1) and its ligand suggests a paracrine regulation of murine vascular development. *Dev Dyn,* 204**,** 228-39.

BROWNE, C. D., HINDMARSH, E. J. & SMITH, J. W. 2006. Inhibition of endothelial cell proliferation and angiogenesis by orlistat, a fatty acid synthase inhibitor. *Faseb j,* 20**,** 2027-35.

CARMELIET, P. 2005. Angiogenesis in life, disease and medicine. *Nature,* 438**,** 932-6.

CARMELIET, P. & COLLEN, D. 1998. Vascular development and disorders: molecular analysis and pathogenic insights. *Kidney Int,* 53**,** 1519-49.

CARMELIET, P., FERREIRA, V., BREIER, G., POLLEFEYT, S., KIECKENS, L., GERTSENSTEIN, M., FAHRIG, M., VANDENHOECK, A., HARPAL, K., EBERHARDT, C., DECLERCQ, C., PAWLING, J., MOONS, L., COLLEN, D., RISAU, W. & NAGY, A. 1996. Abnormal blood vessel development and lethality in embryos lacking a single VEGF allele. *Nature,* 380**,** 435-9.

CARVALHO, R. L., JONKER, L., GOUMANS, M. J., LARSSON, J., BOUWMAN, P., KARLSSON, S., DIJKE, P. T., ARTHUR, H. M. & MUMMERY, C. L. 2004. Defective paracrine signalling by TGFbeta in yolk sac vasculature of endoglin mutant mice: a paradigm for hereditary haemorrhagic telangiectasia. *Development,* 131**,** 6237-47.

CASPI, O., HUBER, I., KEHAT, I., HABIB, M., ARBEL, G., GEPSTEIN, A., YANKELSON, L., ARONSON, D., BEYAR, R. & GEPSTEIN, L. 2007. Transplantation of human embryonic stem cell-derived cardiomyocytes improves myocardial performance in infarcted rat hearts. *J Am Coll Cardiol,* 50**,** 1884-93.

CELERMAJER, D. S., SORENSEN, K. E., GEORGAKOPOULOS, D., BULL, C., THOMAS, O., ROBINSON, J. & DEANFIELD, J. E. 1993. Cigarette smoking is associated with dose-related and potentially reversible impairment of endothelium-dependent dilation in healthy young adults. *Circulation,* 88**,** 2149-55.

CHA, S. W., LEE, J. W., HWANG, Y. S., CHAE, J. P., PARK, K. M., CHO, H. J., KIM, D. S., BAE, Y. C. & PARK, M. J. 2008. Spatiotemporal regulation of fibroblast growth factor signal blocking for endoderm formation in Xenopus laevis. *Exp Mol Med,* 40**,** 550-7.

CHAUDHURY, H., RABORN, E., GOLDIE, L. C. & HIRSCHI, K. K. 2012. Stem cell-derived vascular endothelial cells and their potential application in regenerative medicine. *Cells Tissues Organs,* 195**,** 41-7.

CHEN, H., SHI, S., ACOSTA, L., LI, W., LU, J., BAO, S., CHEN, Z., YANG, Z., SCHNEIDER, M. D., CHIEN, K. R., CONWAY, S. J., YODER, M. C., HANELINE, L. S., FRANCO, D. & SHOU, W. 2004. BMP10 is essential for maintaining cardiac growth during murine cardiogenesis. *Development,* 131**,** 2219-31.

CHEN, L., TREDGET, E. E., WU, P. Y. & WU, Y. 2008. Paracrine factors of mesenchymal stem cells recruit macrophages and endothelial lineage cells and enhance wound healing. *PLoS One,* 3**,** e1886.

CHEN, M. J., YOKOMIZO, T., ZEIGLER, B. M., DZIERZAK, E. & SPECK, N. A. 2009. Runx1 is required for the endothelial to haematopoietic cell transition but not thereafter. *Nature,* 457**,** 887-91.

CHEN, Y., MCCARTHY, D., ROBINSON, M. & SMYTH, G. K. 2014. *edgeR: dierential expression analysis of digital gene expression data [User's Guide]*

[Online]. Available: http://www.bioconductor.org/packages/release/bioc/vignettes/edgeR/inst/doc/edgeRUsersGuide.pdf [Accessed 19/03 2015].
CHISTIAKOV, D. A. 2011. Diabetic retinopathy: pathogenic mechanisms and current treatments. *Diabetes Metab Syndr,* 5**,** 165-72.
CHOI, K. 1998. Hemangioblast development and regulation. *Biochem Cell Biol,* 76**,** 947-56.
CHOI, K. D., YU, J., SMUGA-OTTO, K., SALVAGIOTTO, G., REHRAUER, W., VODYANIK, M., THOMSON, J. & SLUKVIN, I. 2009. Hematopoietic and endothelial differentiation of human induced pluripotent stem cells. *Stem Cells,* 27**,** 559-67.
CHOWIENCZYK, P. J., WATTS, G. F., COCKCROFT, J. R. & RITTER, J. M. 1992. Impaired endothelium-dependent vasodilation of forearm resistance vessels in hypercholesterolaemia. *Lancet,* 340**,** 1430-2.
CIVIN, C. I., STRAUSS, L. C., FACKLER, M. J., TRISCHMANN, T. M., WILEY, J. M. & LOKEN, M. R. 1990. Positive stem cell selection--basic science. *Prog Clin Biol Res,* 333**,** 387-401; discussion 402.
CLAUSS, M., GERLACH, M., GERLACH, H., BRETT, J., WANG, F., FAMILLETTI, P. C., PAN, Y. C., OLANDER, J. V., CONNOLLY, D. T. & STERN, D. 1990. Vascular permeability factor: a tumor-derived polypeptide that induces endothelial cell and monocyte procoagulant activity, and promotes monocyte migration. *J Exp Med,* 172**,** 1535-45.
CLEARY, M. A., VAN OSCH, G. J., BRAMA, P. A., HELLINGMAN, C. A. & NARCISI, R. 2015. FGF, TGFbeta and Wnt crosstalk: embryonic to in vitro cartilage development from mesenchymal stem cells. *J Tissue Eng Regen Med,* 9**,** 332-42.
COLLARD, J. F. & HINSENKAMP, M. 2015. Cellular processes involved in human epidermal cells exposed to extremely low frequency electric fields. *Cell Signal,* 27**,** 889-98.
COSTA, M., SOURRIS, K., LIM, S. M., YU, Q. C., HIRST, C. E., PARKINGTON, H. C., JOKUBAITIS, V. J., DEAR, A. E., LIU, H. B., MICALLEF, S. J., KOUTSIS, K., ELEFANTY, A. G. & STANLEY, E. G. 2013. Derivation of endothelial cells from human embryonic stem cells in fully defined medium enables identification of lysophosphatidic acid and platelet activating factor as regulators of eNOS localization. *Stem Cell Res,* 10**,** 103-17.
CUMANO, A., FURLONGER, C. & PAIGE, C. J. 1993. Differentiation and characterization of B-cell precursors detected in the yolk sac and embryo body of embryos beginning at the 10- to 12-somite stage. *Proc Natl Acad Sci U S A,* 90**,** 6429-33.
DAVID, L., MALLET, C., KERAMIDAS, M., LAMANDE, N., GASC, J. M., DUPUIS-GIROD, S., PLAUCHU, H., FEIGE, J. J. & BAILLY, S. 2008. Bone morphogenetic protein-9 is a circulating vascular quiescence factor. *Circ Res,* 102**,** 914-22.
DAVID, L., MALLET, C., MAZERBOURG, S., FEIGE, J. J. & BAILLY, S. 2007. Identification of BMP9 and BMP10 as functional activators of the orphan activin receptor-like kinase 1 (ALK1) in endothelial cells. *Blood,* 109**,** 1953-61.
DE FELICI, M., FARINI, D. & DOLCI, S. 2009. In or out stemness: comparing growth factor signalling in mouse embryonic stem cells and primordial germ cells. *Curr Stem Cell Res Ther,* 4**,** 87-97.

DE ROBERTIS, E. M. & KURODA, H. 2004. Dorsal-ventral patterning and neural induction in Xenopus embryos. *Annu Rev Cell Dev Biol,* 20**,** 285-308.

DE SMET, F., SEGURA, I., DE BOCK, K., HOHENSINNER, P. J. & CARMELIET, P. 2009. Mechanisms of vessel branching: filopodia on endothelial tip cells lead the way. *Arterioscler Thromb Vasc Biol,* 29**,** 639-49.

DEL TORO, R., PRAHST, C., MATHIVET, T., SIEGFRIED, G., KAMINKER, J. S., LARRIVEE, B., BREANT, C., DUARTE, A., TAKAKURA, N., FUKAMIZU, A., PENNINGER, J. & EICHMANN, A. 2010. Identification and functional analysis of endothelial tip cell-enriched genes. *Blood,* 116**,** 4025-33.

DOME, B., DOBOS, J., TOVARI, J., PAKU, S., KOVACS, G., OSTOROS, G. & TIMAR, J. 2008. Circulating bone marrow-derived endothelial progenitor cells: characterization, mobilization, and therapeutic considerations in malignant disease. *Cytometry A,* 73**,** 186-93.

DORRELL, M. I., OTANI, A., AGUILAR, E., MORENO, S. K. & FRIEDLANDER, M. 2004. Adult bone marrow-derived stem cells use R-cadherin to target sites of neovascularization in the developing retina. *Blood,* 103**,** 3420-7.

DURHAM, J. T. & HERMAN, I. M. 2011. Microvascular modifications in diabetic retinopathy. *Curr Diab Rep,* 11**,** 253-64.

FERRARA, N., CARVER-MOORE, K., CHEN, H., DOWD, M., LU, L., O'SHEA, K. S., POWELL-BRAXTON, L., HILLAN, K. J. & MOORE, M. W. 1996. Heterozygous embryonic lethality induced by targeted inactivation of the VEGF gene. *Nature,* 380**,** 439-42.

FERRARA, N., GERBER, H. P. & LECOUTER, J. 2003. The biology of VEGF and its receptors. *Nat Med,* 9**,** 669-76.

FINA, L., MOLGAARD, H. V., ROBERTSON, D., BRADLEY, N. J., MONAGHAN, P., DELIA, D., SUTHERLAND, D. R., BAKER, M. A. & GREAVES, M. F. 1990. Expression of the CD34 gene in vascular endothelial cells. *Blood,* 75**,** 2417-26.

FLOREK, M., HAASE, M., MARZESCO, A. M., FREUND, D., EHNINGER, G., HUTTNER, W. B. & CORBEIL, D. 2005. Prominin-1/CD133, a neural and hematopoietic stem cell marker, is expressed in adult human differentiated cells and certain types of kidney cancer. *Cell Tissue Res,* 319**,** 15-26.

FOX, A., SMYTHE, J., FISHER, N., TYLER, M. P., MCGROUTHER, D. A., WATT, S. M. & HARRIS, A. L. 2008. Mobilization of endothelial progenitor cells into the circulation in burned patients. *Br J Surg,* 95**,** 244-51.

FOXTON, R. H., FINKELSTEIN, A., VIJAY, S., DAHLMANN-NOOR, A., KHAW, P. T., MORGAN, J. E., SHIMA, D. T. & NG, Y. S. 2013. VEGF-A is necessary and sufficient for retinal neuroprotection in models of experimental glaucoma. *Am J Pathol,* 182**,** 1379-90.

FRASER, S. T., OGAWA, M., YU, R. T., NISHIKAWA, S., YODER, M. C. & NISHIKAWA, S. 2002. Definitive hematopoietic commitment within the embryonic vascular endothelial-cadherin(+) population. *Exp Hematol,* 30**,** 1070-8.

FU, Y., CHANG, A., CHANG, L., NIESSEN, K., EAPEN, S., SETIADI, A. & KARSAN, A. 2009. Differential regulation of transforming growth factor beta signaling pathways by Notch in human endothelial cells. *J Biol Chem,* 284**,** 19452-62.

GAFNI, O., WEINBERGER, L., MANSOUR, A. A., MANOR, Y. S., CHOMSKY, E., BEN-YOSEF, D., KALMA, Y., VIUKOV, S., MAZA, I., ZVIRAN, A., RAIS, Y., SHIPONY, Z., MUKAMEL, Z., KRUPALNIK, V., ZERBIB, M., GEULA,

S., CASPI, I., SCHNEIR, D., SHWARTZ, T., GILAD, S., AMANN-ZALCENSTEIN, D., BENJAMIN, S., AMIT, I., TANAY, A., MASSARWA, R., NOVERSHTERN, N. & HANNA, J. H. 2015. Corrigendum: Derivation of novel human ground state naive pluripotent stem cells. *Nature,* 520**,** 710.

GERHARDT, H., GOLDING, M., FRUTTIGER, M., RUHRBERG, C., LUNDKVIST, A., ABRAMSSON, A., JELTSCH, M., MITCHELL, C., ALITALO, K., SHIMA, D. & BETSHOLTZ, C. 2003. VEGF guides angiogenic sprouting utilizing endothelial tip cell filopodia. *J Cell Biol,* 161**,** 1163-77.

GEUDENS, I. & GERHARDT, H. 2011. Coordinating cell behaviour during blood vessel formation. *Development,* 138**,** 4569-83.

GIBERT, S. F. 2000. *Developmental Biology*

GILL, M., DIAS, S., HATTORI, K., RIVERA, M. L., HICKLIN, D., WITTE, L., GIRARDI, L., YURT, R., HIMEL, H. & RAFII, S. 2001. Vascular trauma induces rapid but transient mobilization of VEGFR2(+)AC133(+) endothelial precursor cells. *Circ Res,* 88**,** 167-74.

GIULIARI, G. P. 2012. Diabetic retinopathy: current and new treatment options. *Curr Diabetes Rev,* 8**,** 32-41.

GORDON, P. R., LEIMIG, T., BABARIN-DORNER, A., HOUSTON, J., HOLLADAY, M., MUELLER, I., GEIGER, T. & HANDGRETINGER, R. 2003. Large-scale isolation of CD133+ progenitor cells from G-CSF mobilized peripheral blood stem cells. *Bone Marrow Transplant,* 31**,** 17-22.

GOUMANS, M. J. & MUMMERY, C. 2000. Functional analysis of the TGFbeta receptor/Smad pathway through gene ablation in mice. *Int J Dev Biol,* 44**,** 253-65.

GOUMANS, M. J., VALDIMARSDOTTIR, G., ITOH, S., ROSENDAHL, A., SIDERAS, P. & TEN DIJKE, P. 2002. Balancing the activation state of the endothelium via two distinct TGF-beta type I receptors. *Embo j,* 21**,** 1743-53.

GRITSMAN, K., TALBOT, W. S. & SCHIER, A. F. 2000. Nodal signaling patterns the organizer. *Development,* 127**,** 921-32.

HAMANO, K., NISHIDA, M., HIRATA, K., MIKAMO, A., LI, T. S., HARADA, M., MIURA, T., MATSUZAKI, M. & ESATO, K. 2001. Local implantation of autologous bone marrow cells for therapeutic angiogenesis in patients with ischemic heart disease: clinical trial and preliminary results. *Jpn Circ J,* 65**,** 845-7.

HANNA, J., WERNIG, M., MARKOULAKI, S., SUN, C. W., MEISSNER, A., CASSADY, J. P., BEARD, C., BRAMBRINK, T., WU, L. C., TOWNES, T. M. & JAENISCH, R. 2007. Treatment of sickle cell anemia mouse model with iPS cells generated from autologous skin. *Science,* 318**,** 1920-3.

HARRIS, D. T. & ROGERS, I. 2007. Umbilical cord blood: a unique source of pluripotent stem cells for regenerative medicine. *Curr Stem Cell Res Ther,* 2**,** 301-9.

HAWINKELS, L. J., GARCIA DE VINUESA, A. & TEN DIJKE, P. 2013. Activin receptor-like kinase 1 as a target for anti-angiogenesis therapy. *Expert Opin Investig Drugs,* 22**,** 1371-83.

HELLSTROM, M., PHNG, L. K., HOFMANN, J. J., WALLGARD, E., COULTAS, L., LINDBLOM, P., ALVA, J., NILSSON, A. K., KARLSSON, L., GAIANO, N., YOON, K., ROSSANT, J., IRUELA-ARISPE, M. L., KALEN, M., GERHARDT, H. & BETSHOLTZ, C. 2007. Dll4 signalling through Notch1 regulates formation of tip cells during angiogenesis. *Nature,* 445**,** 776-80.

HENRIQUES, J., VAZ-PEREIRA, S., NASCIMENTO, J. & ROSA, P. C. 2015. [Diabetic Eye Disease]. *Acta Med Port,* 28**,** 107-113.

HOENIG, M. R., BIANCHI, C. & SELLKE, F. W. 2008. Hypoxia inducible factor-1 alpha, endothelial progenitor cells, monocytes, cardiovascular risk, wound healing, cobalt and hydralazine: a unifying hypothesis. *Curr Drug Targets,* 9**,** 422-35.

HOGAN, B. L. 1996. Bone morphogenetic proteins in development. *Curr Opin Genet Dev,* 6**,** 432-8.

HOLYOAKE, T. L., NICOLINI, F. E. & EAVES, C. J. 1999. Functional differences between transplantable human hematopoietic stem cells from fetal liver, cord blood, and adult marrow. *Exp Hematol,* 27**,** 1418-27.

HORIKI, M., IMAMURA, T., OKAMOTO, M., HAYASHI, M., MURAI, J., MYOUI, A., OCHI, T., MIYAZONO, K., YOSHIKAWA, H. & TSUMAKI, N. 2004. Smad6/Smurf1 overexpression in cartilage delays chondrocyte hypertrophy and causes dwarfism with osteopenia. *J Cell Biol,* 165**,** 433-45.

HRISTOV, M., ERL, W. & WEBER, P. C. 2003. Endothelial progenitor cells: mobilization, differentiation, and homing. *Arterioscler Thromb Vasc Biol,* 23**,** 1185-9.

HRISTOV, M. & WEBER, C. 2008. Endothelial progenitor cells in vascular repair and remodeling. *Pharmacol Res,* 58**,** 148-51.

HUANG, N. F., NIIYAMA, H., PETER, C., DE, A., NATKUNAM, Y., FLEISSNER, F., LI, Z., ROLLINS, M. D., WU, J. C., GAMBHIR, S. S. & COOKE, J. P. 2010. Embryonic stem cell-derived endothelial cells engraft into the ischemic hindlimb and restore perfusion. *Arterioscler Thromb Vasc Biol,* 30**,** 984-91.

HUANGFU, D., OSAFUNE, K., MAEHR, R., GUO, W., EIJKELENBOOM, A., CHEN, S., MUHLESTEIN, W. & MELTON, D. A. 2008. Induction of pluripotent stem cells from primary human fibroblasts with only Oct4 and Sox2. *Nat Biotechnol,* 26**,** 1269-75.

IKRAM, M. K., ONG, Y. T., CHEUNG, C. Y. & WONG, T. Y. 2013. Retinal vascular caliber measurements: clinical significance, current knowledge and future perspectives. *Ophthalmologica,* 229**,** 125-36.

ISHIDA, S., USUI, T., YAMASHIRO, K., KAJI, Y., AHMED, E., CARRASQUILLO, K. G., AMANO, S., HIDA, T., OGUCHI, Y. & ADAMIS, A. P. 2003. VEGF164 is proinflammatory in the diabetic retina. *Invest Ophthalmol Vis Sci,* 44**,** 2155-62.

ITOH, F., ITOH, S., GOUMANS, M. J., VALDIMARSDOTTIR, G., ISO, T., DOTTO, G. P., HAMAMORI, Y., KEDES, L., KATO, M. & TEN DIJKE PT, P. 2004. Synergy and antagonism between Notch and BMP receptor signaling pathways in endothelial cells. *EMBO J,* 23**,** 541-51.

JAKOBSSON, L., BENTLEY, K. & GERHARDT, H. 2009. VEGFRs and Notch: a dynamic collaboration in vascular patterning. *Biochem Soc Trans,* 37**,** 1233-6.

JAKOBSSON, L., FRANCO, C. A., BENTLEY, K., COLLINS, R. T., PONSIOEN, B., ASPALTER, I. M., ROSEWELL, I., BUSSE, M., THURSTON, G., MEDVINSKY, A., SCHULTE-MERKER, S. & GERHARDT, H. 2010. Endothelial cells dynamically compete for the tip cell position during angiogenic sprouting. *Nat Cell Biol,* 12**,** 943-53.

JIN, K. L., MAO, X. O. & GREENBERG, D. A. 2000. Vascular endothelial growth factor: direct neuroprotective effect in in vitro ischemia. *Proc Natl Acad Sci U S A,* 97**,** 10242-7.

JOGGERST, S. J. & HATZOPOULOS, A. K. 2009. Stem cell therapy for cardiac repair: benefits and barriers. *Expert Rev Mol Med,* 11**,** e20.

KAIMAKIS, P., CRISAN, M. & DZIERZAK, E. 2013. The biochemistry of hematopoietic stem cell development. *Biochim Biophys Acta,* 1830**,** 2395-403.

KALKA, C., MASUDA, H., TAKAHASHI, T., KALKA-MOLL, W. M., SILVER, M., KEARNEY, M., LI, T., ISNER, J. M. & ASAHARA, T. 2000. Transplantation of ex vivo expanded endothelial progenitor cells for therapeutic neovascularization. *Proc Natl Acad Sci U S A,* 97**,** 3422-7.

KANJI, S., POMPILI, V. J. & DAS, H. 2011. Plasticity and maintenance of hematopoietic stem cells during development. *Recent Pat Biotechnol,* 5**,** 40-53.

KATOH, O., TAUCHI, H., KAWAISHI, K., KIMURA, A. & SATOW, Y. 1995. Expression of the vascular endothelial growth factor (VEGF) receptor gene, KDR, in hematopoietic cells and inhibitory effect of VEGF on apoptotic cell death caused by ionizing radiation. *Cancer Res,* 55**,** 5687-92.

KAWAMOTO, A., ASAHARA, T. & LOSORDO, D. W. 2002. Transplantation of endothelial progenitor cells for therapeutic neovascularization. *Cardiovasc Radiat Med,* 3**,** 221-5.

KAWAMOTO, A., GWON, H. C., IWAGURO, H., YAMAGUCHI, J. I., UCHIDA, S., MASUDA, H., SILVER, M., MA, H., KEARNEY, M., ISNER, J. M. & ASAHARA, T. 2001. Therapeutic potential of ex vivo expanded endothelial progenitor cells for myocardial ischemia. *Circulation,* 103**,** 634-7.

KELLY, G. M., GREENSTEIN, P., EREZYILMAZ, D. F. & MOON, R. T. 1995. Zebrafish wnt8 and wnt8b share a common activity but are involved in distinct developmental pathways. *Development,* 121**,** 1787-99.

KELLY, M. A. & HIRSCHI, K. K. 2009. Signaling hierarchy regulating human endothelial cell development. *Arterioscler Thromb Vasc Biol,* 29**,** 718-24.

KERR, G., SHELDON, H., CHAIKUAD, A., ALFANO, I., VON DELFT, F., BULLOCK, A. N. & HARRIS, A. L. 2015. A small molecule targeting ALK1 prevents Notch cooperativity and inhibits functional angiogenesis. *Angiogenesis*.

KIMELMAN, D. 2006. Mesoderm induction: from caps to chips. *Nat Rev Genet,* 7**,** 360-72.

KIMELMAN, D. & KIRSCHNER, M. 1987. Synergistic induction of mesoderm by FGF and TGF-beta and the identification of an mRNA coding for FGF in the early Xenopus embryo. *Cell,* 51**,** 869-77.

KLAGSBRUN, M. & D'AMORE, P. A. 1996. Vascular endothelial growth factor and its receptors. *Cytokine Growth Factor Rev,* 7**,** 259-70.

KLEINMAN, M. E., GREIVES, M. R., CHURGIN, S. S., BLECHMAN, K. M., CHANG, E. I., CERADINI, D. J., TEPPER, O. M. & GURTNER, G. C. 2007. Hypoxia-induced mediators of stem/progenitor cell trafficking are increased in children with hemangioma. *Arterioscler Thromb Vasc Biol,* 27**,** 2664-70.

KOCHER, A. A., SCHUSTER, M. D., SZABOLCS, M. J., TAKUMA, S., BURKHOFF, D., WANG, J., HOMMA, S., EDWARDS, N. M. & ITESCU, S. 2001. Neovascularization of ischemic myocardium by human bone-marrow-derived angioblasts prevents cardiomyocyte apoptosis, reduces remodeling and improves cardiac function. *Nat Med,* 7**,** 430-6.

KOIZUMI, K., TSUTSUMI, Y., KAMADA, H., YOSHIOKA, Y., WATANABE, M., YAMAMOTO, Y., OKAMOTO, T., MUKAI, Y., NAKAGAWA, S., TANI, Y. & MAYUMI, T. 2003. Incorporation of adult organ-derived endothelial cells into tumor blood vessel. *Biochem Biophys Res Commun,* 306**,** 219-24.

KUME, T. 2009. Novel insights into the differential functions of Notch ligands in vascular formation. *J Angiogenes Res,* 1**,** 8.

LAFLAMME, M. A., CHEN, K. Y., NAUMOVA, A. V., MUSKHELI, V., FUGATE, J. A., DUPRAS, S. K., REINECKE, H., XU, C., HASSANIPOUR, M., POLICE, S., O'SULLIVAN, C., COLLINS, L., CHEN, Y., MINAMI, E., GILL, E. A., UENO, S., YUAN, C., GOLD, J. & MURRY, C. E. 2007. Cardiomyocytes derived from human embryonic stem cells in pro-survival factors enhance function of infarcted rat hearts. *Nat Biotechnol,* 25**,** 1015-24.

LAM, A. Q., FREEDMAN, B. S., MORIZANE, R., LEROU, P. H., VALERIUS, M. T. & BONVENTRE, J. V. 2014. Rapid and efficient differentiation of human pluripotent stem cells into intermediate mesoderm that forms tubules expressing kidney proximal tubular markers. *J Am Soc Nephrol,* 25**,** 1211-25.

LANGENFELD, E. M. & LANGENFELD, J. 2004. Bone morphogenetic protein-2 stimulates angiogenesis in developing tumors. *Mol Cancer Res,* 2**,** 141-9.

LARRIVEE, B., PRAHST, C., GORDON, E., DEL TORO, R., MATHIVET, T., DUARTE, A., SIMONS, M. & EICHMANN, A. 2012. ALK1 signaling inhibits angiogenesis by cooperating with the Notch pathway. *Dev Cell,* 22**,** 489-500.

LARRUCEA, S., BUTTA, N., ARIAS-SALGADO, E. G., ALONSO-MARTIN, S., AYUSO, M. S. & PARRILLA, R. 2008. Expression of podocalyxin enhances the adherence, migration, and intercellular communication of cells. *Exp Cell Res,* 314**,** 2004-15.

LEE, E. J., MALIK, A., POKHAREL, S., AHMAD, S., MIR, B. A., CHO, K. H., KIM, J., KONG, J. C., LEE, D. M., CHUNG, K. Y., KIM, S. H. & CHOI, I. 2014. Identification of genes differentially expressed in myogenin knock-down bovine muscle satellite cells during differentiation through RNA sequencing analysis. *PLoS One,* 9**,** e92447.

LEE, J. H., LEE, S. H., YOO, S. Y., ASAHARA, T. & KWON, S. M. 2013. CD34 hybrid cells promote endothelial colony-forming cell bioactivity and therapeutic potential for ischemic diseases. *Arterioscler Thromb Vasc Biol,* 33**,** 1622-34.

LEEPER, N. J., HUNTER, A. L. & COOKE, J. P. 2010. Stem cell therapy for vascular regeneration: adult, embryonic, and induced pluripotent stem cells. *Circulation,* 122**,** 517-26.

LEIJTEN, J. & KHADEMHOSSEINI, A. 2016. From Nano to Macro: Multiscale Materials for Improved Stem Cell Culturing and Analysis. *Cell Stem Cell,* 18**,** 20-4.

LEVENBERG, S., GOLUB, J. S., AMIT, M., ITSKOVITZ-ELDOR, J. & LANGER, R. 2002. Endothelial cells derived from human embryonic stem cells. *Proc Natl Acad Sci U S A,* 99**,** 4391-6.

LI, Z., HU, S., GHOSH, Z., HAN, Z. & WU, J. C. 2011. Functional characterization and expression profiling of human induced pluripotent stem cell- and embryonic stem cell-derived endothelial cells. *Stem Cells Dev,* 20**,** 1701-10.

LI, Z., LAN, Y., HE, W., CHEN, D., WANG, J., ZHOU, F., WANG, Y., SUN, H., CHEN, X., XU, C., LI, S., PANG, Y., ZHANG, G., YANG, L., ZHU, L., FAN, M., SHANG, A., JU, Z., LUO, L., DING, Y., GUO, W., YUAN, W., YANG, X. & LIU, B. 2012. Mouse embryonic head as a site for hematopoietic stem cell development. *Cell Stem Cell,* 11**,** 663-75.

LI, Z., WU, J. C., SHEIKH, A. Y., KRAFT, D., CAO, F., XIE, X., PATEL, M., GAMBHIR, S. S., ROBBINS, R. C., COOKE, J. P. & WU, J. C. 2007. Differentiation, survival, and function of embryonic stem cell derived endothelial cells for ischemic heart disease. *Circulation,* 116**,** I46-54.

LIU, L., NAM, S., TIAN, Y., YANG, F., WU, J., WANG, Y., SCUTO, A., POLYCHRONOPOULOS, P., MAGIATIS, P., SKALTSOUNIS, L. & JOVE, R. 2011. 6-Bromoindirubin-3'-oxime inhibits JAK/STAT3 signaling and induces apoptosis of human melanoma cells. *Cancer Res,* 71**,** 3972-9.

LIU, W., JIN, G., LONG, C., ZHOU, X., TANG, Y., HUANG, S., KUANG, X., WU, L., ZHANG, Q. & SHEN, H. 2013. Blockage of Notch signaling inhibits the migration and proliferation of retinal pigment epithelial cells. *ScientificWorldJournal,* 2013**,** 178708.

LOBOV, I. B., RENARD, R. A., PAPADOPOULOS, N., GALE, N. W., THURSTON, G., YANCOPOULOS, G. D. & WIEGAND, S. J. 2007. Delta-like ligand 4 (Dll4) is induced by VEGF as a negative regulator of angiogenic sprouting. *Proc Natl Acad Sci U S A,* 104**,** 3219-24.

LU, C. C., BRENNAN, J. & ROBERTSON, E. J. 2001. From fertilization to gastrulation: axis formation in the mouse embryo. *Curr Opin Genet Dev,* 11**,** 384-92.

LUGUS, J. J., PARK, C. & CHOI, K. 2005. Developmental relationship between hematopoietic and endothelial cells. *Immunol Res,* 32**,** 57-74.

LUNDE, K., SOLHEIM, S., AAKHUS, S., ARNESEN, H., ABDELNOOR, M., EGELAND, T., ENDRESEN, K., ILEBEKK, A., MANGSCHAU, A., FJELD, J. G., SMITH, H. J., TARALDSRUD, E., GROGAARD, H. K., BJORNERHEIM, R., BREKKE, M., MULLER, C., HOPP, E., RAGNARSSON, A., BRINCHMANN, J. E. & FORFANG, K. 2006. Intracoronary injection of mononuclear bone marrow cells in acute myocardial infarction. *N Engl J Med,* 355**,** 1199-209.

LUTTUN, A., CARMELIET, G. & CARMELIET, P. 2002. Vascular progenitors: from biology to treatment. *Trends Cardiovasc Med,* 12**,** 88-96.

MACKENZIE, F. & RUHRBERG, C. 2012. Diverse roles for VEGF-A in the nervous system. *Development,* 139**,** 1371-80.

MANOUSARIDIS, K. & TALKS, J. 2012. Macular ischaemia: a contraindication for anti-VEGF treatment in retinal vascular disease? *Br J Ophthalmol,* 96**,** 179-84.

MARCELO, K. L., GOLDIE, L. C. & HIRSCHI, K. K. 2013. Regulation of endothelial cell differentiation and specification. *Circ Res,* 112**,** 1272-87.

MAROM, K., LEVY, V., PILLEMER, G. & FAINSOD, A. 2005. Temporal analysis of the early BMP functions identifies distinct anti-organizer and mesoderm patterning phases. *Dev Biol,* 282**,** 442-54.

MATHURA, J. R., JR., JAFARI, N., CHANG, J. T., HACKETT, S. F., WAHLIN, K. J., DELLA, N. G., OKAMOTO, N., ZACK, D. J. & CAMPOCHIARO, P. A. 2000. Bone morphogenetic proteins-2 and -4: negative growth regulators in adult retinal pigmented epithelium. *Invest Ophthalmol Vis Sci,* 41**,** 592-600.

MAURITZ, C., SCHWANKE, K., REPPEL, M., NEEF, S., KATSIRNTAKI, K., MAIER, L. S., NGUEMO, F., MENKE, S., HAUSTEIN, M., HESCHELER, J., HASENFUSS, G. & MARTIN, U. 2008. Generation of functional murine cardiac myocytes from induced pluripotent stem cells. *Circulation,* 118**,** 507-17.

MCALLISTER, K. A., GROGG, K. M., JOHNSON, D. W., GALLIONE, C. J., BALDWIN, M. A., JACKSON, C. E., HELMBOLD, E. A., MARKEL, D. S., MCKINNON, W. C., MURRELL, J. & ET AL. 1994. Endoglin, a TGF-beta binding protein of endothelial cells, is the gene for hereditary haemorrhagic telangiectasia type 1. *Nat Genet,* 8**,** 345-51.

MCGUCKIN, C. P., PEARCE, D., FORRAZ, N., TOOZE, J. A., WATT, S. M. & PETTENGELL, R. 2003. Multiparametric analysis of immature cell populations in umbilical cord blood and bone marrow. *Eur J Haematol,* 71**,** 341-50.

MEDINA, R. J., O'NEILL, C. L., HUMPHREYS, M. W., GARDINER, T. A. & STITT, A. W. 2010a. Outgrowth endothelial cells: characterization and their potential for reversing ischemic retinopathy. *Invest Ophthalmol Vis Sci,* 51**,** 5906-13.

MEDINA, R. J., O'NEILL, C. L., SWEENEY, M., GUDURIC-FUCHS, J., GARDINER, T. A., SIMPSON, D. A. & STITT, A. W. 2010b. Molecular analysis of endothelial progenitor cell (EPC) subtypes reveals two distinct cell populations with different identities. *BMC Med Genomics,* 3**,** 18.

MEDVINSKY, A., RYBTSOV, S. & TAOUDI, S. 2011. Embryonic origin of the adult hematopoietic system: advances and questions. *Development,* 138**,** 1017-31.

MILLER-KASPRZAK, E. & JAGODZINSKI, P. P. 2007. Endothelial progenitor cells as a new agent contributing to vascular repair. *Arch Immunol Ther Exp (Warsz),* 55**,** 247-59.

MIZUOCHI, C., FRASER, S. T., BIASCH, K., HORIO, Y., KIKUSHIGE, Y., TANI, K., AKASHI, K., TAVIAN, M. & SUGIYAMA, D. 2012. Intra-aortic clusters undergo endothelial to hematopoietic phenotypic transition during early embryogenesis. *PLoS One,* 7**,** e35763.

MORIKAWA, M., KOINUMA, D., TSUTSUMI, S., VASILAKI, E., KANKI, Y., HELDIN, C. H., ABURATANI, H. & MIYAZONO, K. 2011. ChIP-seq reveals cell type-specific binding patterns of BMP-specific Smads and a novel binding motif. *Nucleic Acids Res,* 39**,** 8712-27.

MORTAZAVI, A., WILLIAMS, B. A., MCCUE, K., SCHAEFFER, L. & WOLD, B. 2008. Mapping and quantifying mammalian transcriptomes by RNA-Seq. *Nat Methods,* 5**,** 621-8.

MOYA, I. M., UMANS, L., MAAS, E., PEREIRA, P. N., BEETS, K., FRANCIS, A., SENTS, W., ROBERTSON, E. J., MUMMERY, C. L., HUYLEBROECK, D. & ZWIJSEN, A. 2012. Stalk cell phenotype depends on integration of Notch and Smad1/5 signaling cascades. *Dev Cell,* 22**,** 501-14.

NARAZAKI, G., UOSAKI, H., TERANISHI, M., OKITA, K., KIM, B., MATSUOKA, S., YAMANAKA, S. & YAMASHITA, J. K. 2008. Directed and systematic differentiation of cardiovascular cells from mouse induced pluripotent stem cells. *Circulation,* 118**,** 498-506.

NELSON, T. J., MARTINEZ-FERNANDEZ, A., YAMADA, S., PEREZ-TERZIC, C., IKEDA, Y. & TERZIC, A. 2009. Repair of acute myocardial infarction by human stemness factors induced pluripotent stem cells. *Circulation,* 120**,** 408-16.

NEWMAN, A. M. & COOPER, J. B. 2010. AutoSOME: a clustering method for identifying gene expression modules without prior knowledge of cluster number. *BMC Bioinformatics,* 11**,** 117.

NIELSEN, J. S. & MCNAGNY, K. M. 2008. Novel functions of the CD34 family. *J Cell Sci,* 121**,** 3683-92.

NISHIJIMA, K., NG, Y. S., ZHONG, L., BRADLEY, J., SCHUBERT, W., JO, N., AKITA, J., SAMUELSSON, S. J., ROBINSON, G. S., ADAMIS, A. P. & SHIMA, D. T. 2007. Vascular endothelial growth factor-A is a survival factor for retinal neurons and a critical neuroprotectant during the adaptive response to ischemic injury. *Am J Pathol,* 171**,** 53-67.

NIWA, A., HEIKE, T., UMEDA, K., OSHIMA, K., KATO, I., SAKAI, H., SUEMORI, H., NAKAHATA, T. & SAITO, M. K. 2011. A novel serum-free monolayer

culture for orderly hematopoietic differentiation of human pluripotent cells via mesodermal progenitors. *PLoS One,* 6**,** e22261.

NOLAN-STEVAUX, O., ZHONG, W., CULP, S., SHAFFER, K., HOOVER, J., WICKRAMASINGHE, D. & RUEFLI-BRASSE, A. 2012. Endoglin requirement for BMP9 signaling in endothelial cells reveals new mechanism of action for selective anti-endoglin antibodies. *PLoS One,* 7**,** e50920.

OGATA, N., YAMANAKA, R., YAMAMOTO, C., MIYASHIRO, M., KIMOTO, T., TAKAHASHI, K., MARUYAMA, K. & UYAMA, M. 1998. Expression of vascular endothelial growth factor and its receptor, KDR, following retinal ischemia-reperfusion injury in the rat. *Curr Eye Res,* 17**,** 1087-96.

OH, S. P., SEKI, T., GOSS, K. A., IMAMURA, T., YI, Y., DONAHOE, P. K., LI, L., MIYAZONO, K., TEN DIJKE, P., KIM, S. & LI, E. 2000. Activin receptor-like kinase 1 modulates transforming growth factor-beta 1 signaling in the regulation of angiogenesis. *Proc Natl Acad Sci U S A,* 97**,** 2626-31.

OKITA, K., NAKAGAWA, M., HYENJONG, H., ICHISAKA, T. & YAMANAKA, S. 2008. Generation of mouse induced pluripotent stem cells without viral vectors. *Science,* 322**,** 949-53.

ORKIN, S. H. & ZON, L. I. 2002. Hematopoiesis and stem cells: plasticity versus developmental heterogeneity. *Nat Immunol,* 3**,** 323-8.

ORLOVA, V. V., DRABSCH, Y., FREUND, C., PETRUS-REURER, S., VAN DEN HIL, F. E., MUENTHAISONG, S., DIJKE, P. T. & MUMMERY, C. L. 2014. Functionality of endothelial cells and pericytes from human pluripotent stem cells demonstrated in cultured vascular plexus and zebrafish xenografts. *Arterioscler Thromb Vasc Biol,* 34**,** 177-86.

PALIS, J., ROBERTSON, S., KENNEDY, M., WALL, C. & KELLER, G. 1999. Development of erythroid and myeloid progenitors in the yolk sac and embryo proper of the mouse. *Development,* 126**,** 5073-84.

PARDALI, E., GOUMANS, M. J. & TEN DIJKE, P. 2010. Signaling by members of the TGF-beta family in vascular morphogenesis and disease. *Trends Cell Biol,* 20**,** 556-67.

PARK, I. H., ARORA, N., HUO, H., MAHERALI, N., AHFELDT, T., SHIMAMURA, A., LENSCH, M. W., COWAN, C., HOCHEDLINGER, K. & DALEY, G. Q. 2008. Disease-specific induced pluripotent stem cells. *Cell,* 134**,** 877-86.

PEARSON, S., SROCZYNSKA, P., LACAUD, G. & KOUSKOFF, V. 2008. The stepwise specification of embryonic stem cells to hematopoietic fate is driven by sequential exposure to Bmp4, activin A, bFGF and VEGF. *Development,* 135**,** 1525-35.

PEICHEV, M., NAIYER, A. J., PEREIRA, D., ZHU, Z., LANE, W. J., WILLIAMS, M., OZ, M. C., HICKLIN, D. J., WITTE, L., MOORE, M. A. & RAFII, S. 2000. Expression of VEGFR-2 and AC133 by circulating human CD34(+) cells identifies a population of functional endothelial precursors. *Blood,* 95**,** 952-8.

PEIRSON, S. N., BUTLER, J. N. & FOSTER, R. G. 2003. Experimental validation of novel and conventional approaches to quantitative real-time PCR data analysis. *Nucleic Acids Res,* 31**,** e73.

PHNG, L. K. & GERHARDT, H. 2009. Angiogenesis: a team effort coordinated by notch. *Dev Cell,* 16**,** 196-208.

PRASAIN, N., LEE, M. R., VEMULA, S., MEADOR, J. L., YOSHIMOTO, M., FERKOWICZ, M. J., FETT, A., GUPTA, M., RAPP, B. M., SAADATZADEH, M. R., GINSBERG, M., ELEMENTO, O., LEE, Y., VOYTIK-HARBIN, S. L., CHUNG, H. M., HONG, K. S., REID, E., O'NEILL, C. L., MEDINA, R. J.,

STITT, A. W., MURPHY, M. P., RAFII, S., BROXMEYER, H. E. & YODER, M. C. 2014. Differentiation of human pluripotent stem cells to cells similar to cord-blood endothelial colony-forming cells. *Nat Biotechnol,* 32**,** 1151-7.

PRISCO, D. & MARCUCCI, R. 2002. Retinal vein thrombosis: risk factors, pathogenesis and therapeutic approach. *Pathophysiol Haemost Thromb,* 32**,** 308-11.

RABELINK, T. J., DE BOER, H. C., DE KONING, E. J. & VAN ZONNEVELD, A. J. 2004. Endothelial progenitor cells: more than an inflammatory response? *Arterioscler Thromb Vasc Biol,* 24**,** 834-8.

RAFII, S. & LYDEN, D. 2003. Therapeutic stem and progenitor cell transplantation for organ vascularization and regeneration. *Nat Med,* 9**,** 702-12.

RAFII, S., LYDEN, D., BENEZRA, R., HATTORI, K. & HEISSIG, B. 2002. Vascular and haematopoietic stem cells: novel targets for anti-angiogenesis therapy? *Nat Rev Cancer,* 2**,** 826-35.

REHMAN, J., LI, J., ORSCHELL, C. M. & MARCH, K. L. 2003. Peripheral blood "endothelial progenitor cells" are derived from monocyte/macrophages and secrete angiogenic growth factors. *Circulation,* 107**,** 1164-9.

RESCH, T., PIRCHER, A., KAHLER, C. M., PRATSCHKE, J. & HILBE, W. 2012. Endothelial progenitor cells: current issues on characterization and challenging clinical applications. *Stem Cell Rev,* 8**,** 926-39.

REYA, T., MORRISON, S. J., CLARKE, M. F. & WEISSMAN, I. L. 2001. Stem cells, cancer, and cancer stem cells. *Nature,* 414**,** 105-11.

RICARD, N., CIAIS, D., LEVET, S., SUBILEAU, M., MALLET, C., ZIMMERS, T. A., LEE, S. J., BIDART, M., FEIGE, J. J. & BAILLY, S. 2012. BMP9 and BMP10 are critical for postnatal retinal vascular remodeling. *Blood,* 119**,** 6162-71.

RINGNÉR, M. 2008. What is principal component analysis? *Nature Biotechnology,* 26**,** 303 - 304.

RITTER, M. R., BANIN, E., MORENO, S. K., AGUILAR, E., DORRELL, M. I. & FRIEDLANDER, M. 2006. Myeloid progenitors differentiate into microglia and promote vascular repair in a model of ischemic retinopathy. *J Clin Invest,* 116**,** 3266-76.

ROBERT-MORENO, A., GUIU, J., RUIZ-HERGUIDO, C., LOPEZ, M. E., INGLES-ESTEVE, J., RIERA, L., TIPPING, A., ENVER, T., DZIERZAK, E., GRIDLEY, T., ESPINOSA, L. & BIGAS, A. 2008. Impaired embryonic haematopoiesis yet normal arterial development in the absence of the Notch ligand Jagged1. *Embo j,* 27**,** 1886-95.

ROBERTS, W. G. & PALADE, G. E. 1995. Increased microvascular permeability and endothelial fenestration induced by vascular endothelial growth factor. *J Cell Sci,* 108 (Pt 6)**,** 2369-79.

ROBINSON, M. D. & SMYTH, G. K. 2007. Moderated statistical tests for assessing differences in tag abundance. *Bioinformatics,* 23**,** 2881-7.

ROBINSON, M. D. & SMYTH, G. K. 2008. Small-sample estimation of negative binomial dispersion, with applications to SAGE data. *Biostatistics,* 9**,** 321-32.

ROCA, C. & ADAMS, R. H. 2007. Regulation of vascular morphogenesis by Notch signaling. *Genes Dev,* 21**,** 2511-24.

ROSSETTO, C. C., TARRANT-ELORZA, M. & PARI, G. S. 2013. Cis and trans acting factors involved in human cytomegalovirus experimental and natural latent infection of CD14 (+) monocytes and CD34 (+) cells. *PLoS Pathog,* 9**,** e1003366.

ROTTINGER, E., DUBUC, T. Q., AMIEL, A. R. & MARTINDALE, M. Q. 2015. Nodal signaling is required for mesodermal and ventral but not for dorsal fates in the indirect developing hemichordate, Ptychodera flava. *Biol Open*.

RUFAIHAH, A. J., HUANG, N. F., JAME, S., LEE, J. C., NGUYEN, H. N., BYERS, B., DE, A., OKOGBAA, J., ROLLINS, M., REIJO-PERA, R., GAMBHIR, S. S. & COOKE, J. P. 2011. Endothelial cells derived from human iPSCS increase capillary density and improve perfusion in a mouse model of peripheral arterial disease. *Arterioscler Thromb Vasc Biol,* 31**,** e72-9.

SAMUEL, R., DAHERON, L., LIAO, S., VARDAM, T., KAMOUN, W. S., BATISTA, A., BUECKER, C., SCHAFER, R., HAN, X., AU, P., SCADDEN, D. T., DUDA, D. G., FUKUMURA, D. & JAIN, R. K. 2013. Generation of functionally competent and durable engineered blood vessels from human induced pluripotent stem cells. *Proc Natl Acad Sci U S A,* 110**,** 12774-9.

SARIC, T. & HESCHELER, J. 2008. Stem cells and nuclear reprogramming. *Minim Invasive Ther Allied Technol,* 17**,** 64-78.

SCHARPFENECKER, M., VAN DINTHER, M., LIU, Z., VAN BEZOOIJEN, R. L., ZHAO, Q., PUKAC, L., LOWIK, C. W. & TEN DIJKE, P. 2007. BMP-9 signals via ALK1 and inhibits bFGF-induced endothelial cell proliferation and VEGF-stimulated angiogenesis. *J Cell Sci,* 120**,** 964-72.

SCHEDLICH, L. J., YENSON, V. M. & BAXTER, R. C. 2013. TGF-beta-induced expression of IGFBP-3 regulates IGF1R signaling in human osteosarcoma cells. *Mol Cell Endocrinol,* 377**,** 56-64.

SEKI, T., YUN, J. & OH, S. P. 2003. Arterial endothelium-specific activin receptor-like kinase 1 expression suggests its role in arterialization and vascular remodeling. *Circ Res,* 93**,** 682-9.

SEN, S., MCDONALD, S. P., COATES, P. T. & BONDER, C. S. 2011. Endothelial progenitor cells: novel biomarker and promising cell therapy for cardiovascular disease. *Clin Sci (Lond),* 120**,** 263-83.

SHALABY, F., ROSSANT, J., YAMAGUCHI, T. P., GERTSENSTEIN, M., WU, X. F., BREITMAN, M. L. & SCHUH, A. C. 1995. Failure of blood-island formation and vasculogenesis in Flk-1-deficient mice. *Nature,* 376**,** 62-6.

SHARPE, E. E., 3RD, TELERON, A. A., LI, B., PRICE, J., SANDS, M. S., ALFORD, K. & YOUNG, P. P. 2006. The origin and in vivo significance of murine and human culture-expanded endothelial progenitor cells. *Am J Pathol,* 168**,** 1710-21.

SHAW, L. C., NEU, M. B. & GRANT, M. B. 2011. Cell-based therapies for diabetic retinopathy. *Curr Diab Rep,* 11**,** 265-74.

SIDNEY, L. E., BRANCH, M. J., DUNPHY, S. E., DUA, H. S. & HOPKINSON, A. 2014. Concise review: evidence for CD34 as a common marker for diverse progenitors. *Stem Cells,* 32**,** 1380-9.

SIEMERINK, M. J., KLAASSEN, I., VOGELS, I. M., GRIFFIOEN, A. W., VAN NOORDEN, C. J. & SCHLINGEMANN, R. O. 2012. CD34 marks angiogenic tip cells in human vascular endothelial cell cultures. *Angiogenesis,* 15**,** 151-63.

SINGH, S. K., HAWKINS, C., CLARKE, I. D., SQUIRE, J. A., BAYANI, J., HIDE, T., HENKELMAN, R. M., CUSIMANO, M. D. & DIRKS, P. B. 2004. Identification of human brain tumour initiating cells. *Nature,* 432**,** 396-401.

SKROMNE, I. & STERN, C. D. 2002. A hierarchy of gene expression accompanying induction of the primitive streak by Vg1 in the chick embryo. *Mech Dev,* 114**,** 115-8.

SMADJA, D. M., BIECHE, I., HELLEY, D., LAURENDEAU, I., SIMONIN, G., MULLER, L., AIACH, M. & GAUSSEM, P. 2007. Increased VEGFR2 expression during human late endothelial progenitor cells expansion enhances in vitro angiogenesis with up-regulation of integrin alpha(6). *J Cell Mol Med,* 11**,** 1149-61.

SOMMER, C. A., STADTFELD, M., MURPHY, G. J., HOCHEDLINGER, K., KOTTON, D. N. & MOSTOSLAVSKY, G. 2009. Induced pluripotent stem cell generation using a single lentiviral stem cell cassette. *Stem Cells,* 27**,** 543-9.

STAMM, C., WESTPHAL, B., KLEINE, H. D., PETZSCH, M., KITTNER, C., KLINGE, H., SCHUMICHEN, C., NIENABER, C. A., FREUND, M. & STEINHOFF, G. 2003. Autologous bone-marrow stem-cell transplantation for myocardial regeneration. *Lancet,* 361**,** 45-6.

STITT, A. W., O'NEILL, C. L., O'DOHERTY, M. T., ARCHER, D. B., GARDINER, T. A. & MEDINA, R. J. 2011. Vascular stem cells and ischaemic retinopathies. *Prog Retin Eye Res,* 30**,** 149-66.

STONE, J., ITIN, A., ALON, T., PE'ER, J., GNESSIN, H., CHAN-LING, T. & KESHET, E. 1995. Development of retinal vasculature is mediated by hypoxia-induced vascular endothelial growth factor (VEGF) expression by neuroglia. *J Neurosci,* 15**,** 4738-47.

STRASSER, G. A., KAMINKER, J. S. & TESSIER-LAVIGNE, M. 2010. Microarray analysis of retinal endothelial tip cells identifies CXCR4 as a mediator of tip cell morphology and branching. *Blood,* 115**,** 5102-10.

STRILIC, B., KUCERA, T., EGLINGER, J., HUGHES, M. R., MCNAGNY, K. M., TSUKITA, S., DEJANA, E., FERRARA, N. & LAMMERT, E. 2009. The molecular basis of vascular lumen formation in the developing mouse aorta. *Dev Cell,* 17**,** 505-15.

SUCHTING, S., FREITAS, C., LE NOBLE, F., BENEDITO, R., BREANT, C., DUARTE, A. & EICHMANN, A. 2007. The Notch ligand Delta-like 4 negatively regulates endothelial tip cell formation and vessel branching. *Proc Natl Acad Sci U S A,* 104**,** 3225-30.

SUZUKI, Y., OHGA, N., MORISHITA, Y., HIDA, K., MIYAZONO, K. & WATABE, T. 2010. BMP-9 induces proliferation of multiple types of endothelial cells in vitro and in vivo. *J Cell Sci,* 123**,** 1684-92.

SZETO, D. P. & KIMELMAN, D. 2004. Combinatorial gene regulation by Bmp and Wnt in zebrafish posterior mesoderm formation. *Development,* 131**,** 3751-60.

TAKAHASHI, K., TANABE, K., OHNUKI, M., NARITA, M., ICHISAKA, T., TOMODA, K. & YAMANAKA, S. 2007. Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell,* 131**,** 861-72.

TAKAHASHI, K. & YAMANAKA, S. 2006. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell,* 126**,** 663-76.

TAKEBE, T. & TANIGUCHI, H. 2014. Human iPSC-derived miniature organs: a tool for drug studies. *Clin Pharmacol Ther,* 96**,** 310-3.

TAVASSOLI, M. 1991. Embryonic and fetal hemopoiesis: an overview. *Blood Cells,* 17**,** 269-81; discussion 282-6.

TEN DIJKE, P. & ARTHUR, H. M. 2007. Extracellular control of TGFbeta signalling in vascular development and disease. *Nat Rev Mol Cell Biol,* 8**,** 857-69.

THACH, D. C., LIN, B., WALTER, E., KRUZELOCK, R., ROWLEY, R. K., TIBBETTS, C. & STENGER, D. A. 2003. Assessment of two methods for handling blood in collection tubes with RNA stabilizing agent for surveillance

of gene expression profiles with high density microarrays. *J Immunol Methods,* 283**,** 269-79.

TORRENTE, Y., BELICCHI, M., SAMPAOLESI, M., PISATI, F., MEREGALLI, M., D'ANTONA, G., TONLORENZI, R., PORRETTI, L., GAVINA, M., MAMCHAOUI, K., PELLEGRINO, M. A., FURLING, D., MOULY, V., BUTLER-BROWNE, G. S., BOTTINELLI, R., COSSU, G. & BRESOLIN, N. 2004. Human circulating AC133(+) stem cells restore dystrophin expression and ameliorate function in dystrophic skeletal muscle. *J Clin Invest,* 114**,** 182-95.

TUAL-CHALOT, S., MAHMOUD, M., ALLINSON, K. R., REDGRAVE, R. E., ZHAI, Z., OH, S. P., FRUTTIGER, M. & ARTHUR, H. M. 2014. Endothelial depletion of Acvrl1 in mice leads to arteriovenous malformations associated with reduced endoglin expression. *PLoS One,* 9**,** e98646.

UCHIDA, N., BUCK, D. W., HE, D., REITSMA, M. J., MASEK, M., PHAN, T. V., TSUKAMOTO, A. S., GAGE, F. H. & WEISSMAN, I. L. 2000. Direct isolation of human central nervous system stem cells. *Proc Natl Acad Sci U S A,* 97**,** 14720-5.

UNGRIN, M. D., JOSHI, C., NICA, A., BAUWENS, C. & ZANDSTRA, P. W. 2008. Reproducible, ultra high-throughput formation of multicellular organization from single cell suspension-derived human embryonic stem cell aggregates. *PLoS One,* 3**,** e1565.

UPTON, P. D., DAVIES, R. J., TREMBATH, R. C. & MORRELL, N. W. 2009. Bone morphogenetic protein (BMP) and activin type II receptors balance BMP9 signals mediated by activin receptor-like kinase-1 in human pulmonary artery endothelial cells. *J Biol Chem,* 284**,** 15794-804.

URBICH, C. & DIMMELER, S. 2004. Endothelial progenitor cells: characterization and role in vascular biology. *Circ Res,* 95**,** 343-53.

URNESS, L. D., SORENSEN, L. K. & LI, D. Y. 2000. Arteriovenous malformations in mice lacking activin receptor-like kinase-1. *Nat Genet,* 26**,** 328-31.

VAN DER SCHAFT, D. W., TOEBES, E. A., HASEMAN, J. R., MAYO, K. H. & GRIFFIOEN, A. W. 2000. Bactericidal/permeability-increasing protein (BPI) inhibits angiogenesis via induction of apoptosis in vascular endothelial cells. *Blood,* 96**,** 176-81.

VERCAUTEREN, S. M. & SUTHERLAND, H. J. 2001. CD133 (AC133) expression on AML cells and progenitors. *Cytotherapy,* 3**,** 449-59.

VIRGILI, G., PARRAVANO, M., MENCHINI, F. & EVANS, J. R. 2014. Anti-vascular endothelial growth factor for diabetic macular oedema. *Cochrane Database Syst Rev,* 10**,** CD007419.

VODYANIK, M. A., BORK, J. A., THOMSON, J. A. & SLUKVIN, II 2005. Human embryonic stem cell-derived CD34+ cells: efficient production in the coculture with OP9 stromal cells and analysis of lymphohematopoietic potential. *Blood,* 105**,** 617-26.

VOGEL, W., SCHEDING, S., KANZ, L. & BRUGGER, W. 2000. Clinical applications of CD34(+) peripheral blood progenitor cells (PBPC). *Stem Cells,* 18**,** 87-92.

VON AHLFEN, S., MISSEL, A., BENDRAT, K. & SCHLUMPBERGER, M. 2007. Determinants of RNA quality from FFPE samples. *PLoS One,* 2**,** e1261.

WAGERS, A. J., SHERWOOD, R. I., CHRISTENSEN, J. L. & WEISSMAN, I. L. 2002. Little evidence for developmental plasticity of adult hematopoietic stem cells. *Science,* 297**,** 2256-9.

WANG, J., DUNCAN, D., SHI, Z. & ZHANG, B. 2013. WEB-based GEne SeT AnaLysis Toolkit (WebGestalt): update 2013. *Nucleic Acids Res,* 41**,** W77-83.

WANG, Z., GERSTEIN, M. & SNYDER, M. 2009. RNA-Seq: a revolutionary tool for transcriptomics. *Nat Rev Genet,* 10**,** 57-63.

WATT, S. M. & CONTRERAS, M. 2005. Stem cell medicine: umbilical cord blood and its stem cell potential. *Semin Fetal Neonatal Med,* 10**,** 209-20.

WEIGMANN, A., CORBEIL, D., HELLWIG, A. & HUTTNER, W. B. 1997. Prominin, a novel microvilli-specific polytopic membrane protein of the apical surface of epithelial cells, is targeted to plasmalemmal protrusions of non-epithelial cells. *Proc Natl Acad Sci U S A,* 94**,** 12425-30.

WEISSMAN, I. L., ANDERSON, D. J. & GAGE, F. 2001. Stem and progenitor cells: origins, phenotypes, lineage commitments, and transdifferentiations. *Annu Rev Cell Dev Biol,* 17**,** 387-403.

WILLARD, A. L. & HERMAN, I. M. 2012. Vascular complications and diabetes: current therapies and future challenges. *J Ophthalmol,* 2012**,** 209538.

WINNIER, G., BLESSING, M., LABOSKY, P. A. & HOGAN, B. L. 1995. Bone morphogenetic protein-4 is required for mesoderm formation and patterning in the mouse. *Genes Dev,* 9**,** 2105-16.

WITTLER, L., SHIN, E. H., GROTE, P., KISPERT, A., BECKERS, A., GOSSLER, A., WERBER, M. & HERRMANN, B. G. 2007. Expression of Msgn1 in the presomitic mesoderm is controlled by synergism of WNT signalling and Tbx6. *EMBO Rep,* 8**,** 784-9.

WOGNUM, A. W., EAVES, A. C. & THOMAS, T. E. 2003. Identification and isolation of hematopoietic stem cells. *Arch Med Res,* 34**,** 461-75.

WOOD, H. B., MAY, G., HEALY, L., ENVER, T. & MORRISS-KAY, G. M. 1997. CD34 expression patterns during early mouse development are related to modes of blood vessel formation and reveal additional sites of hematopoiesis. *Blood,* 90**,** 2300-11.

XU, J., ZHU, D., SONODA, S., HE, S., SPEE, C., RYAN, S. J. & HINTON, D. R. 2012. Over-expression of BMP4 inhibits experimental choroidal neovascularization by modulating VEGF and MMP-9. *Angiogenesis,* 15**,** 213-27.

YAMAGUCHI, T. P., HARPAL, K., HENKEMEYER, M. & ROSSANT, J. 1994. fgfr-1 is required for embryonic growth and mesodermal patterning during mouse gastrulation. *Genes Dev,* 8**,** 3032-44.

YAMAHARA, K. & ITOH, H. 2009. Potential use of endothelial progenitor cells for regeneration of the vasculature. *Ther Adv Cardiovasc Dis,* 3**,** 17-27.

YAMAHARA, K., SONE, M., ITOH, H., YAMASHITA, J. K., YURUGI-KOBAYASHI, T., HOMMA, K., CHAO, T. H., MIYASHITA, K., PARK, K., OYAMADA, N., SAWADA, N., TAURA, D., FUKUNAGA, Y., TAMURA, N. & NAKAO, K. 2008. Augmentation of neovascularization [corrected] in hindlimb ischemia by combined transplantation of human embryonic stem cells-derived endothelial and mural cells. *PLoS One,* 3**,** e1666.

YANG, J., II, M., KAMEI, N., ALEV, C., KWON, S. M., KAWAMOTO, A., AKIMARU, H., MASUDA, H., SAWA, Y. & ASAHARA, T. 2011. CD34+ cells represent highly functional endothelial progenitor cells in murine bone marrow. *PLoS One,* 6**,** e20219.

YAO, Y., WATSON, A. D., JI, S. & BOSTROM, K. I. 2009. Heat shock protein 70 enhances vascular bone morphogenetic protein-4 signaling by binding matrix Gla protein. *Circ Res,* 105**,** 575-84.

YODER, M. C. 2014. Inducing definitive hematopoiesis in a dish. *Nat Biotechnol,* 32**,** 539-41.

YODER, M. C., MEAD, L. E., PRATER, D., KRIER, T. R., MROUEH, K. N., LI, F., KRASICH, R., TEMM, C. J., PRCHAL, J. T. & INGRAM, D. A. 2007. Redefining endothelial progenitor cells via clonal analysis and hematopoietic stem/progenitor cell principals. *Blood,* 109**,** 1801-9.

ZOVEIN, A. C., HOFMANN, J. J., LYNCH, M., FRENCH, W. J., TURLO, K. A., YANG, Y., BECKER, M. S., ZANETTA, L., DEJANA, E., GASSON, J. C., TALLQUIST, M. D. & IRUELA-ARISPE, M. L. 2008. Fate tracing reveals the endothelial origin of hematopoietic stem cells. *Cell Stem Cell,* 3**,** 625-36.

YU Y1, QIN J, CHEN D, WANG H, WANG J, YU Y. 2015. Chronic cardiovascular disease-associated gene network analysis in human umbilical vein endothelial cells exposed to 2,3,7,8-tetrachlorodibenzo-p-dioxin. *Cardiovasc Toxicol*.15(2):157-71.

KANEHISA M1, GOTO S, KAWASHIMA S, OKUNO Y, HATTORI M. 2004. The KEGG resource for deciphering the *genome. Nucleic Acid Res*. D277-80.

XIN L1, XU B2, MA L1, HOU Q1, YE M1, MENG S2, DING X1, GE W2. 2016. Proteomics study reveals that the dysregulation of focal adhesion and ribosome contribute to early pregnancy loss. *Proteomics Clin Appl*. 10(5):554-63

ZAIDEL-BAR R1, ITZKOVITZ S, MA'AYAN A, IYENGAR R, GEIGER B. 2007. Functional atlas of the integrin adhesome. *Nat Cell Biol*. ;9(8):858-67.

PARSONS JT1, HORWITZ AR, SCHWARTZ MA. 2010. Cell adhesion: integrating cytoskeletal dynamics and cellular tension. *Nat Rev Mol Cell Biol*. 11(9):633-43.

ZHAO X1, GUAN JL. 2011. Focal adhesion kinase and its signaling pathways in cell migration and angiogenesis. *Adv Drug Deliv Rev*. 18;63(8):610-5.

YU Y1, QIN J, CHEN D, WANG H, WANG J, YU Y. 2015. Chronic cardiovascular disease-associated gene network analysis in human umbilical vein endothelial cells exposed to 2,3,7,8-tetrachlorodibenzo-p-dioxin. *Cardiovasc Toxicol*.15(2):157-71.

KANEHISA M1, GOTO S, KAWASHIMA S, OKUNO Y, HATTORI M. 2004. The KEGG resource for deciphering the *genome. Nucleic Acid Res*. D277-80.

XIN L1, XU B2, MA L1, HOU Q1, YE M1, MENG S2, DING X1, GE W2. 2016. Proteomics study reveals that the dysregulation of focal adhesion and ribosome contribute to early pregnancy loss. *Proteomics Clin Appl*. 10(5):554-63

ZAIDEL-BAR R1, ITZKOVITZ S, MA'AYAN A, IYENGAR R, GEIGER B. 2007. Functional atlas of the integrin adhesome. *Nat Cell Biol*. ;9(8):858-67.

PARSONS JT1, HORWITZ AR, SCHWARTZ MA. 2010. Cell adhesion: integrating cytoskeletal dynamics and cellular tension. *Nat Rev Mol Cell Biol*. 11(9):633-43.

ZHAO X1, GUAN JL. 2011. Focal adhesion kinase and its signaling pathways in cell migration and angiogenesis. *Adv Drug Deliv Rev*. 18;63(8):610-5.