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Thesis title:

"Vascular Engineering: Generation of Endothelial Cells From human Pluripotent Stem Cells"

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ABSTRACT

Tissue engineering restores impaired function of tissues or organs by generating constructs harbouring cells, often stem cells, that upon implantation in sites of injury or degeneration can regenerate the tissue. The potential of cell survival and viability is correlated with the vascularisation of the implantable construct, and thus, generating blood vessels and their components, perivascular cells (PCs) and endothelial cells (ECs), *in vitro* allows for studies to further comprehend their function and contribution to the vascularisation process.

As described in the current thesis, the host laboratory has developed a method for the differentiation of hESCs/hiPSCs to ECs (Tsolis et al. 2016) using chemically defined conditions (APEL medium) supplemented with growth factors. Whereas the efficiency of the protocol is overall satisfactory (around 25%) there is room for improvement, additionally, the generated ECs exhibit low proliferative capacity and require sorting of the acquired mixed population. In this thesis two different protocols of differentiation to ECs were tested alongside the already established protocol to further improve the generation of ECs. The first protocol tested, published by Patch and coworkers (Patch, C., et al., 2015), was selected due to the high differentiation efficiency (66 – 88 %) and the highly proliferative ECs produced, whereas the second protocol (Harding et al., 2017) exhibited high differentiation efficiency (73 – 83%), on day 8 of differentiation, with no requirement for cell sorting or magnetic purification to yield a very pure population.

The Patch et al. protocol involved testing the effect of the initial cell density of the hPSCs and the concentration of the GSK3 inhibitor used in the differentiation towards the mesodermal state. Performing the protocol on H1 hESCs and testing various seeding densities of the starting population and a range of GSK3i concentrations revealed that the highest seeding density and the highest GSK3i concentration led to the optimal differentiation efficiency (38.3 % of $CD34^+$ and 27.2% of $CD31^+$ cells). However, the differentiation efficiency that was not equivalent to the efficiency suggested by the authors (66 – 88%) and did not improve the efficiency compared to the differentiation protocol already established in the host laboratory (~25 %) (Tsolis et al., 2016).

The Harding et al. protocol tested the effect of i) method of cell dissociation during subculturing, ii) the use of various culturing substrates, iii) composition of culture media and serum concentration.

The optimal protocol involved passaging H1 hESCs with dispase, seeding VPCs on fibronectin and were culturing the hESC-ECs in EGM-2 medium supplemented with 50 ng/ml VEGF and serum up to 5%. The efficiency of differentiation reached 57.4% $CD31^+$, more than double than the efficiency obtained with the protocol of the host laboratory (~25 %) (Tsolis et al., 2016).

While the current thesis focuses more on the phenotypical characterization of the derived ECs, future plans involve the functional characterization of the population, with assays such as LDL uptake, in vitro angiogenesis and tube formation that are typical in a population of ECs. Finally, further improving the efficiency and the desired conditions of the Harding et al. protocol could reveal a protocol that can be used in totally serum free conditions, which is ideal in certain types of experiments, such as experiments dissecting molecular pathways.

ΠΕΡΙΛΗΨΗ

Η μηχανική ιστών, που αποτελεί τη βάση της αναγεννητικής ιατρικής, υπόσχεται να αποκαταστήσει την μειωμένη λειτουργία ιστών ή οργάνων με την δημιουργία κατασκευών με χρήση ικριωμάτων, συμβατών με κύτταρα, κατά κύριο λόγο βλαστικά κύτταρα, τα οποία, κατά την εμφύτευση σε θέσεις που έχουν υποστεί τραυματισμό ή εκφυλισμό κυττάρων, να επάγουν την αναγέννηση του ιστού. Η βιωσιμότητα των κυττάρων στα εμφυτεύματα συσχετίζεται με την αγγειοποίηση του εμφυτεύματος και, επομένως, ο *in vitro* σχηματισμός των αιμοφόρων αγγείων και των δομικών συστατικών τους, δηλαδή των περιαγγειακών κυττάρων (perivascular cells, PCs) και των ενδοθηλιακών κυττάρων (ECs), δύναται να επιτρέψει την διεξαγωγή μελετών για την περαιτέρω κατανόηση της λειτουργίας και της συνεισφοράς αυτών στην διαδικασία της αγγειογένεσης.

Όπως περιγράφεται στην παρούσα μεταπτυχιακή διατριβή, το εργαστήριο υποδοχής έχει αναπτύξει μία μέθοδο για τη διαφοροποίηση των ανθρώπινων εμβρυϊκών βλαστικών κυττάρων (hESCs) και των ανθρώπινων επαγόμενων πολυδύναμων βλαστικών κυττάρων (hiPSCs) σε ECs (Tsolis et al. 2016), χρησιμοποιώντας χημικά καθορισμένες συνθήκες (θρεπτικό μέσο καλλιέργειας APEL) εμπλουτισμένες με αυξητικούς παράγοντες. Ενώ η απόδοση του πρωτοκόλλου είναι γενικά ικανοποιητική (περίπου 25%), υπάρχει περιθώριο για βελτίωση, ενώ παράλληλα τα παραγόμενα ECs παρουσιάζουν χαμηλή πολλαπλασιαστική ικανότητα και απαιτούν διαλογή του μεικτού πληθυσμού που προκύπτει. Στην παρούσα διατριβή εξετάστηκαν δύο διαφορετικά πρωτόκολλα διαφοροποίησης προς ECs, σε συνδυασμό με το ήδη καθιερωμένο πρωτόκολλο διαφοροποίησης, προκειμένου να βελτιωθεί περαιτέρω η παραγωγή των ECs. Το πρώτο πρωτόκολλο που εξετάστηκε, δημοσιευμένο από τους Patch et al. (Patch, C., et al., 2015), επιλέχθηκε λόγω της υψηλής απόδοσης διαφοροποίησης (66 – 88%) και της υψηλής πολλαπλασιαστικής ικανότητας των παραγόμενων ECs, ενώ το δεύτερο πρωτόκολλο (Harding et al., 2017) παρουσίασε υψηλή απόδοση διαφοροποίησης (73 – 83%) την 8η ημέρα της διαφοροποίησης, χωρίς να απαιτεί διαλογή των κυττάρων για την απόκτηση ενός καθαρού πληθυσμού.

Το πρωτόκολλο των Patch et al. περιελάμβανε την αξιολόγηση της επίδρασης της αρχικής κυτταρικής πυκνότητας των hPSCs και της συγκέντρωσης του αναστολέα GSK3 που χρησιμοποιείται κατά την διαφοροποίηση προς το μεσόδερμα. Η εφαρμογή του πρωτοκόλλου σε H1 hESCs και η δοκιμή διαφόρων πυκνοτήτων του αρχικού πληθυσμού και μιας σειράς συγκεντρώσεων του αναστολέα GSK3 έδειξαν ότι η υψηλότερη αρχική

κυτταρική πυκνότητα και η υψηλότερη συγκέντρωση του αναστολέα GSK3 οδήγησαν στην βέλτιστη απόδοση διαφοροποίησης (38,3% $CD34^+$ και 27,2% $CD31^+$ κυττάρων). Ωστόσο, η απόδοση της διαφοροποίησης δεν ήταν αντίστοιχη με την απόδοση που προτείναν οι συγγραφείς (66 – 88%) και δεν βελτίωσε την απόδοση σε σύγκριση με το πρωτόκολλο διαφοροποίησης που είχε ήδη καθιερωθεί στο εργαστήριο (~25%) (Tsolis et al., 2016).

Το πρωτόκολλο των Harding et al. εξέταζε την επίδραση: i) της μεθόδου αποκόλλησης των κυττάρων κατά την ανακαλλιέργεια, ii) της χρήσης διαφόρων υποστρωμάτων καλλιέργειας, iii) της σύνθεσης των μέσων καλλιέργειας και της συγκέντρωσης ορού. Η βέλτιστη εκτέλεση του πρωτοκόλλου περιελάμβανε την αποκόλληση των H1 ανθρώπινων εμβρυϊκών βλαστικών κυττάρων (hESCs) με δισπάση, την προσκόλληση των προγονικών αγγειακών κυττάρων (VPCs) σε υπόστρωμα φιμπρονεκτίνης και την καλλιέργεια των hESC-ECs σε θρεπτικό μέσο καλλιέργειας EGM-2 εμπλουτισμένο με 50 ng/ml VEGF και ορό μέχρι 5%. Η απόδοση της διαφοροποίησης έφτασε το 57,4% σε $CD31^+$ κύτταρα, δηλαδή πάνω από διπλάσια από την απόδοση που επιτεύχθηκε με το πρωτόκολλο του εργαστηρίου (~25%) (Tsolis et al., 2016).

Παρότι η παρούσα μεταπτυχιακή διατριβή εστιάζει στον φαινοτυπικό χαρακτηρισμό των επαγόμενων ενδοθηλιακών κυττάρων (ECs), στα μελλοντικά πλάνα περιλαμβάνεται ο λειτουργικός χαρακτηρισμός του πληθυσμού, με δοκιμές όπως πρόσληψη του LDL και in vitro αγγειογένεση που είναι τυπικά χαρακτηριστικά του πληθυσμού των ECs. Τέλος, περαιτέρω βελτίωση της απόδοσης και των επιθυμητών συνθηκών του πρωτοκόλλου που προτάθηκε από τους Harding et al., θα μπορούσε να αποκαλύψει ένα πρωτόκολλο που μπορεί να χρησιμοποιηθεί υπό συνθήκες χωρίς ορό, που αποτελεί ιδανική συνθήκη για ορισμένα είδη πειραμάτων, όπως πειράματα που εξετάζουν μοριακά μονοπάτια.

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ABBREVIATIONS

A

ACE – Angiotensin Converting Enzyme

ACE2 – Angiotensin Converting Enzyme 2

Ac-LDL – Acetylated Low-Density Lipoproteins

ALK5 – Activin Receptor-Like Kinase 5

Ang1 – Angiopoietin 1

Ang2 – Angiopoietin 2

ASCs – Adult Stem Cells

αSMA – alpha Smooth Muscle Actin

B

bFGF – Basic Fibroblast Growth Factor

BM – Basement Membrane

BMP-4 – Bone Morphogenic Protein 4

C

CAM – Cell Adhesion Molecules

cGMP – Cyclic Guanosine 3',5'-cyclic Monophosphate

c-Myc – c Cellular Myelocytomatosis Oncogene

cvSMCs – Contractile Vascular Smooth Muscle Cells

E

ECs – Endothelial Cells

ECM – Extracellular Matrix

ESCs – Embryonic Stem Cells

ET-1 – Endothelil 1

ETS – E-Twenty-Six

ETS-1 – E-Twenty-Six 1

Etv-2 – ETS Variant 2

F

FACS – Fluorescence-Activated Cell Sorting

FBS – Fetal Bovine Serum

FCS – Fetal Calf Serum

FGF – Fibroblast Growth Factor

FGF-2 – Fibroblast Growth Factor 2

FLI-1 – Friend Leukemia Integration 1 Transcription Factor

Flk-1 – Fetal Liver Kinase 1

FVIII – Factor VIII

G

GSK3i – Glycogen Synthase Kinase 3 Inhibitor

H

hESCs – Human Embryonic Stem Cells

hiPSCs – Human induced Pluripotent Stem Cells

HUVECs – Human Umbilical Vein Endothelial Cells

I

ICAM-1 / CD54 – Intramolecular Cell Adhesion Molecule 1

ICAM-2 – Intramolecular Cell Adhesion Molecule 2

ICM – Inner Cell Mass

iPSCs – Induced Pluripotent Stem Cells

K

Klf4 – Kruppel-like Factor 4

L

LFA-1 – Leukocyte Function-Associated Antigen-1

Lin28 – Cell Lineage Abnormal 28 RNA-binding Protein

LRRK2 – Leucine-Rich Repeat Kinase 2

M

MEF – Mouse Embryonic Fibroblasts

MHC Class I - Major Histocompatibility Complex

MMPs – Matrix Metalloproteinases

MSC – Mesenchymal Stem Cell

N

NANOG – Homeobox Protein NANOG

NG2 – Neuron-Glial 2

NOS – Nitric Oxide Synthase

O

OCT3/4 – Octamer Binding Transcription Factor 3/4

OCT4 - Octamer Binding Transcription Factor 4

P

PAF – Platelet Activating Factor

PBS – Phosphate-Buffered Saline

PCs – Perivascular Cells

Pcs – Pericytes

PDGF – Platelet-Derived Growth Factor

PDGF-AA – Platelet Derived Growth Factor Subunit A

PDGF-BB – Platelet Derived Growth Factor Subunit B

PDGFB – Platelet-Derived Growth Factor B

PDGFR – Platelet-Derived Growth Factor Receptor

PDGFRβ – Platelet-Derived Growth Factor Receptor β

PECAM-1/CD31 – Platelet Endothelial Cell Adhesion Molecule 1

PGI-2 – Prostacyclin 2

PI-2 – Prostaglandin I-2

PSC – Pluripotent Stem Cells

R

RAS – Renin-Angiotensin System

S

SCs – Stem Cells

SM22α – Smooth Muscle 22 alpha

SMαA – Smooth Muscle α-Actin

SMCs – Smooth Muscle Cells

SMMHC - Smooth Muscle Myosin Heavy Chain

SOX2 – Sex Determining Region Y-Box

SSEA-1 – Stage-Specific Embryonic Antigen-1

SSEA-3 – Stage-Specific Embryonic Antigen-3

SSEA-4 – Stage-Specific Embryonic Antigen-4

svSMCs – Synthetic vascular Smooth Muscle Cells

T

TE – Trophectoderm

TGF-β – Transforming Growth Factor β

TGFβR1 – Transforming Growth Factor Beta Receptor I

TIE2 – Tyrosine Kinase With Immunoglobulin Like And EGF Like Domains 2
t-PA – Tissue-Type Plasminogen Activator
TRA Antigens – T-cell Receptor Alpha Antigens

V

vCAM – Vascular Cell Adhesion Molecule
vCAM-1/CD106 – Vascular Cell Adhesion Molecule 1
VEGF – Vascular Endothelial Growth Factor
VEGF-A – Vascular Endothelial Growth Factor A
VEGF-R1 – Vascular Endothelial Growth Factor Receptor 1
VEGF-R2 – Vascular Endothelial Growth Factor Receptor 2
VLA-4 – Very Late Antigen - 4
VPCs – Vascular Progenitor Cells
vSMCs – vascular Smooth Muscle Cells
vWF – Von Willebrand Factor

W

WPB – Weibel-Palade Bodies

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1. INTRODUCTION

1.1 Regenerative Medicine

Regenerative medicine is an emerging field with potential to restore the function of tissues and organs that injuries or diseases have inflicted damage to. The importance of this field comes from transplantation demands and the inability of innate repair mechanisms to restore extended function loss due to severe trauma, requiring a different approach to conventional medicine.

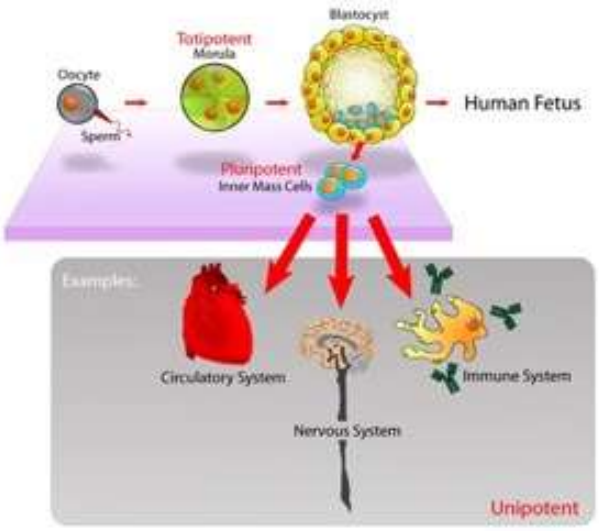

Figure 1.1. *Embryonic stem cells are pluripotent and originate from the inner cell layer of the blastocyst, called inner cell mass (ICM). These stem cells can become any tissue in the body, except placenta (modified from Stupar et al., 2013).*

A key goal of regenerative medicine is to repair, replace, or regenerate tissues and organs that are malfunctioning or damaged by injury through cell therapy. Thus, it uses this knowledge to prevent and treat chronic degenerative diseases (eg diabetes, osteoarthritis, degenerative diseases of the heart and nervous system) and repair traumatic injuries. Stem cells are a key component of regenerative medicine. Indeed, embryonic stem cells can practically regenerate all body tissues (Figure 1.1), while adult stem cells have a narrower range of differentiation. It has been written that stem cell-based therapy is going to be the third therapeutic principle in the future, after surgery and pharmaceutical therapy. The use of embryonic stem cells (ES-cells) and adult stem cells (adult stem cells) in Therapy has been widely discussed and has recently begun to be tested (the American FDA approved the first clinical trials with human ES cells in January of 2009).

1.2 Stem Cells (SCs)

Stem cells are found in both embryos and adults and can be defined on the basis of their origin and potency into Adult Stem Cells (ASCs) and Embryonic Stem Cells (ESCs). Stem cells possess the ability to either self-renew indefinitely or to differentiate into more mature cells with specialized functions. Considering their differentiation potency, stem cells have several steps of specialization, with each step defined by a reduced differentiation and developmental potency and are thus classified from lowest to highest differentiation capacity into: unipotent, oligopotent, multipotent, pluripotent, and totipotent stem cells. (Zakrzewski W., et al., 2019).

Unipotent stem cells are adult stem cells characterized by the narrowest differentiation capabilities of all stem cell categories associated with the property of dividing repeatedly. The latter feature distinguishes them from non-stem cells and the former means that these cells are committed to one specific lineage, having the ability to form only one cell type, for example the dermatocytes.

Oligopotent stem cells can differentiate into only a few cell types and include myeloblast stem cells, which can divide into three types of white blood cells (eosinophils, neutrophils, and basophils) but not red blood cells.

Multipotent stem cells have a wider spectrum of differentiation than uni- and oligopotent stem cells and specialize in discrete cells of specific cell lineages. For example, haematopoietic stem cells first differentiate and become oligopotent stem cells, which are restricted to a specific cell lineage and can then develop into several types of blood cells.

Pluripotent stem cells (PSCs) give rise to all three germ layers but not extraembryonic structures, such as the placenta. Examples of PSCs are embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs), with the former derived from the inner cell mass of the blastocyst of preimplantation embryos and the latter from the epiblast layer of implanted embryos. Their pluripotency exists for only a specific time period of pre-implantation development in the cells forming Inner Cell Mass (ICM). As the cells differentiate into other cell lineages, their self-renewing potential decreases due to various epigenetic changes which leads to the loss of pluripotency and thus to the formation of less potent cells, such as multi-, oligo- and unipotent cells.

Totipotent stem cells have the highest differentiation potential and can differentiate into embryonic and extra-embryonic structures, such as the placenta, giving rise to cells of the whole organism. One example of a totipotent cell is a zygote, which is formed after sperm fertilizes an egg. These cells can later develop either into any of the three germ layers or form a placenta (Zakrzewski W., et al., 2019).

1.2.1 Embryonic Stem Cells (ESCs)

In embryonic development, during the preimplantation stage, which is the first five to six days after fertilization and prior to implantation, the human zygote develops into a blastocyst that is composed of two distinct cell types: an outer layer of trophectoderm (TE), which encloses the inner layer of inner cell mass cells (ICM) found in the blastocyst cavity. The TE forms extraembryonic structures needed for support of the embryo, such as the placenta, and the ICM contains two types of cells, the epiblast and the hypoblast, with the former contributing to cells and tissues of the embryo and the latter forming an epithelial layer on the epiblast. As TE cells differentiate to extraembryonic membranes, ICM cells retain their undifferentiated, fully pluripotent state, allowing them to form any cell of the whole organism. (Rossant J., et al.., 2022).

Human embryonic stem cells (hESCs) are pluripotent stem cells derived from the pre-implantation blastocyst and specifically from the ICM of a human blastocyst stage embryo and can form three distinct cell aggregates named germ layers, which differentiate to different cell types and tissues in the embryo: ectoderm, mesoderm and endoderm. This differentiation occurs after implantation of the blastocyst and after that hESCs have limited pluripotency and become multipotent stem cells, giving rise to cells of the specific embryonic layer. (Rossant J., et al.., 2022).

Human Embryonic Stem Cells (hESCs), as pluripotent stem cells, are defined by pluripotent transcription factors such as OCT4, SOX2 and NANOG, and, in their undifferentiated state, they are characterized by expression of a number of cell surface markers and transcription factors including stage-specific embryonic antigen-4 (SSEA-4), SSEA-3, TRA antigens, Oct3/4, Nanog and the absence of hESC negative markers, such as SSEA-1. (Vazin T., et al.., 2010).

1.2.2 Challenges Attributed to the Use of ESCs

However, the use of embryonic stem cells runs into two problems. The generation of embryonic stem cells from human embryos or in vitro fertilization material is not ethically acceptable. Furthermore, the use of such embryonic stem cell lines will, in some patients, fail due to immune rejection. As shown by a series of pioneering studies, these problems are circumvented by the use of reprogrammed stem cells (induced pluripotent stem cells-iPS cells), where terminally differentiated cells (fibroblasts, keratinocytes) from a patient are transformed ex vivo into stem cells with transient expression of specific transcription factors (e.g, Oct4, Sox2, Nanog, Klf4 (see below). A series of studies have shown that iPS cells have the properties of embryonic stem cells differentiating to all tissues of the body, although this point is still being examined. Thus , embryonic stem cells can replace regenerative medicine approaches. This system, discovered by Yamanaka and colleagues and developed by leaps and bounds in recent years (Takahashi and Yamanaka, 2006), is free of the usual limitations that accompany the use of embryonic stem cells (immune rejection, bioethical problems) and has very good prospects in terms of the development of cell models that reproduce the pathogenesis of specific diseases (disease-specific models), facilitate the testing of new drugs and support the invention of personalized regimens cell therapy.

The infinite proliferative capacity of ESCs serves as both a blessing and a curse, as these cells can lead to the formation of tumors after transplantation, in the form of teratomas. Tumorigenicity can be attributed to genetic alterations, from single nucleotide mutations to copy number variations, that occurred during the in vitro culture of the undifferentiated ESCs, and can deem a cell line inappropriate for further use in medical applications (Yamanaka S., 2020).

Another critical issue regarding the use of ESCs in cell therapy and tissue transplantation is immune rejection. The recipient’s immune system deems ESC-derived transplanted cells or tissues as allogenic because the lack of or mismatch of class I MHC expression on ESC-derived grafts is recognized by the adaptive immune response (Boyd A. S., et al.., 2012). Traditionally, the problem of immune rejection in organ transplantation has been overcome by the life-long use of immuno-suppressive drugs for long-term immunosuppression, however, the use of these drugs is associated with numerous complications, either immediate, such as infections, or delayed, such as secondary lymphomas. Thus, the option

of immunosuppressive treatment is not optimal for the incorporation of ESC-derived drafts into the damaged tissue or organ (Yamanaka S., 2020).

Additionally, different ESC cell lines are characterized by heterogeneity in gene expression, which is correlated with the differences in the genetic background and the epigenetic variations of the various cell lines. This heterogeneity can become a problem when these cells are needed for medical applications, such as cell therapies (Yamanaka S., 2020).

1.2.3 Human Induced Pluripotent Stem Cells (hiPSCs)

The turning point for stem cell research was the discovery of the possibility of reprograming multipotent adult stem cells to their pluripotent state by Shinya Yamanaka and Kazutoshi Takahashi in 2006 (Takahashi K., et al.., 2006). The reprogramming to induced pluripotent stem cells (iPSCs) was conducted with retrovirus transduction of first of mouse fibroblasts and then of human fibroblasts with four transcription factors (Oct-3/4, Sox2, KLF4, and c-Myc). This method opened a new field in stem cell research with a generation of patient specific iPSC lines bypassing the limitations of ESCs (Zakrzewski W., et al.., 2019). The generation of autologous iPSCs helped overcome various hurdles posed by the use of allogenous ESCs in medical applications.

Human induced pluripotent stem cells (hiPSCs) can be reprogrammed from fibroblasts, as performed by Yamanaka in 2007 (Takahashi K., et al., 2007). But, because a biopsy is needed to acquire these cells, other more accessible cell types can be used, such as peripheral blood cells, keratinocytes or renal epithelial cells. Although, alternative stem cell sources are available for the generation of iPSCs, the best stem cell source appears to be the fibroblasts, because of a faster and better controlled stimulation (Zakrzewski W., et al.., 2019).

1.2.4 Applications in Medicine

The discovery of iPSCs has led to studies for disease modeling, drug discovery and regenerative medicine. The advancement of iPSCs technology can be utilized to give rise to patient-specific cell lines derived from patients to study the underlying mechanisms of disease and the potential therapies, but also to generate tissue-specific cells for cell-replacement therapy and transplantation. Furthermore, iPSCs bypass the immunorejection issue caused by heterologous cells of ECs, due to the generation of iPSCs from autologous cells. Additionally, many medical conditions, such as birth defects, cancer, spinal cord

injuries, retinal degeneration and heart failure, remain untreatable and can be attributed to improper differentiation and thus iPSCs technology can shed some light to stem cell physiology and potential stem cell therapies (Wu S. M., et al.., 2011, Zakrzewski W., et al.., 2019).

iPSCs have a wide variety of applications in research and clinical studies such as disease modeling, regenerative medicine and drug cytotoxicity studies.

In disease modeling patient-specific and disease-specific iPSCs can be generated from somatic cells of patients suffering from a disease with a known or suspected etiology to investigate the evolution of the disease and the potential therapeutic applications. The solution to finding a treatment for many diseases lies in the understanding of the underlying mechanisms regarding disease progression, which can be achieved by using disease models. Many disease models have been used, such as rat, mice, primates, however, the variability in the genetic makeup of these animals and the differences with the human genetic background as well as the differences in the cellular environment and metabolism of different species, led to the identification of iPSCs as a better alternative model. Human iPSCs, with their unlimited differentiation potential self-renewal capabilities and the possibility to combine a 3D culture with extracellular matrix proteins, can mimic the in-vivo human microenvironment.

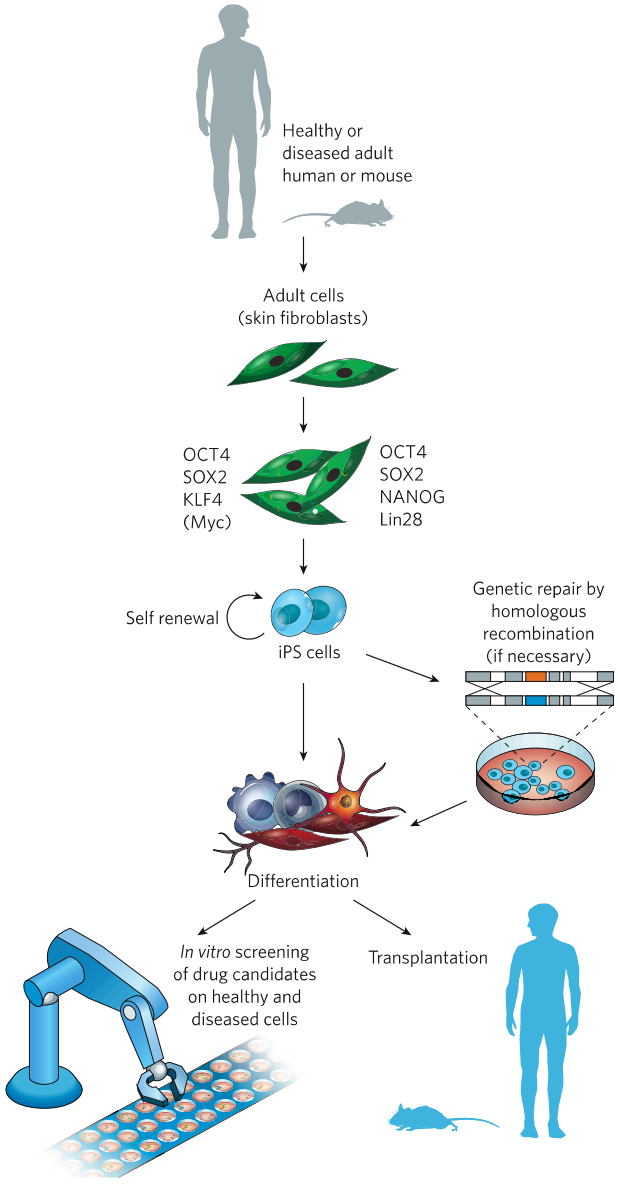

Figure 1.2. *In 2007 human induced pluripotent stem (iPS) cells were generated from adult skin fibroblasts, by the overexpression of OCT4 and SOX2, in combination with two other proteins, NANOG and Lin28. The generated iPSCs showed the essential characteristics of ESCs in terms of*

morphology, cell-surface markers, gene-expression profiles and telomerase activity, could be maintained in culture for several months and could be induced to differentiate into all three embryonic germ layers. Reactivation of Myc increased tumorigenicity in chimeric mice derived from mouse iPS cells, and a modified protocol was developed that did not require activation of Myc in either mouse or human cells. Thus, it became feasible to generate iPS cells from fibroblast cultures from patients (with genetic defects corrected if necessary), and these cells could then, in principle, be induced to differentiate into a variety of patient-specific cell types, allowing transplantation without the risk of immune rejection. The more immediate applications of human iPS cells could be the creation of human models of human disease in vitro for studying the underlying molecular mechanisms of disease, for screening drug candidates, and for assessing drug safety and toxicity (modified from Passier R., et al., 2008).

In regenerative medicine, the generation of iPSCs has raised the potential of stem cell therapies for various conditions, such as macular degenerations, strokes, osteoarthritis and neurodegenerative diseases. Differentiation of iPSCs to these specific cell types or cell types that have acquired an injury in a damaged tissue can lead to the generation of tissues and organs that can be transplanted to the site of injury or degeneration and reverse the effects of such conditions. Since the somatic cells used for the generation of iPSCs will be derived from the patient's own body, a number of problems regarding transplantations can be overcome, such as non-availability of donor tissues and organs and immunorejection due to different physiological profile of donor and patients.

For pharmacological testing and drug discovery, iPSCs can be utilized in clinical and research studies for drug screenings to examine the toxicity of molecules potentially used as therapeutic agents for certain diseases. The use of animals as testing systems for predictions of drug toxicity has limitations regarding the differences between the physiological conditions in different species which can lead to unwanted side-effects or no positive outcome when the drugs are tested on humans. Because newly discovered drugs and therapies must be tested on human cells to be extensively used it is important to utilize a system that closely resembles the human organism. For this purpose, the generation of iPSCs from specific somatic cells, healthy or diseased and the subsequent pharmacological testing performed on these cells can help directly identify potentially harmful effects of a drug composition and the changes needed in order to render a drug effective, with minimal side-effects (Singh V. K., et al., 2015, Robinton D. A., et al., 2012).

1.3 Blood Vessels

1.3.1 Vascular Cells and Blood Vessel Formation

Vascular tissue engineering is an emerging field that establishes the regeneration of blood vessels and the restoration of blood circulation for vascular disease treatment and modelling or for the incorporation of healthy vascular tissue at sites of injury (Wanjare M., et.al, 2013).

Blood vessels are the first organ to develop during embryogenesis, are derived from the mesoderm and involve different cell types that offer a variety of properties, with the basic components being perivascular cells (PCs) and endothelial cells (ECs). Differences in cellular composition and vessel size vary, which leads to different vascular functions (Figure 1.3). Small blood vessels, such as capillaries, which are the most abundant vessels in the human body, consist of ECs enveloped by basal lamina and a single layer of pericytes (Figure 1.3, b). Larger blood vessels consist of three layers (from innermost to outermost layer) (Figure 1.3, d): the intima, media and adventitia. The intima is comprised mostly of one cell type – the endothelium – which lines the luminal surface, with intimal SMCs found to a lesser extent. The media is composed of multiple layers of SMCs and is separated from the intima by an internal elastic lamina. The outermost adventitial layer consists of loose connective tissue and contains smaller blood vessels and nerves (Karen K., et al., 1996). Similarly, veins are irregularly covered by smooth-muscle cells and pericytes and have valves to prevent the backflow of blood (Figure 1.3, c), whereas arteries have strong, elastic vessel walls with dense populations of concentrically formed smooth-muscle cells to withstand the higher blood pressures (Figure 1.3, c) (Bergers G., et al., 2005).

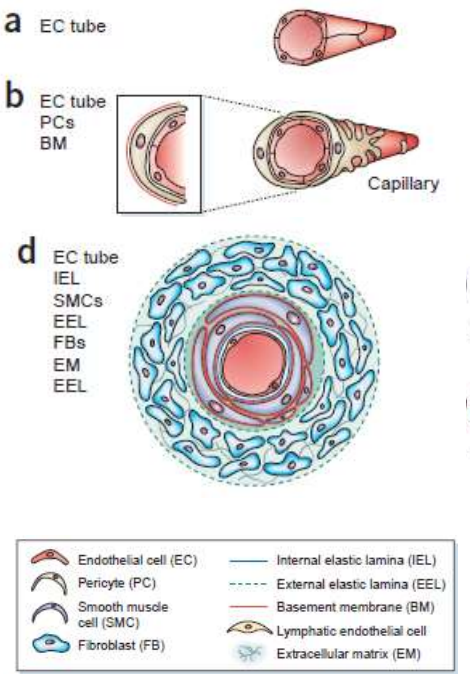

Figure 1.3. *Wall composition of nascent and mature vessels. (a) Nascent vessels consist of a tube of ECs, which mature into the specialized structure of capillaries, arteries and veins. (b) Capillaries consist of ECs surrounded by basement membrane (BM) and pericytes (Pcs) embedded within the BM. (c) Arterioles and venules have an increased coverage of mural cells compared to capillaries. (d) The walls of larger vessels consists of three specialized layers: an intima composed of ECs, a media of SMCs and an adventitia of fibroblasts with matrix and elastic laminae (modified from Jain R. K., et al., 2003).*

Blood vessel formation occurs via vasculogenesis or angiogenesis (Figure 1.4). Vasculogenesis refers to the de novo formation of blood vessels typically occurring during embryonic development, which is critical for embryonic survival and later organogenesis, but can occur in adults by circulating progenitor stem cells of a specific tissue (endothelial progenitors, hematopoietic stem cells or stromal stem cells). Angiogenesis is the formation of blood vessels from preexisting structures, which occurs in both adults and embryos (Wanjare M., et.al, 2013).

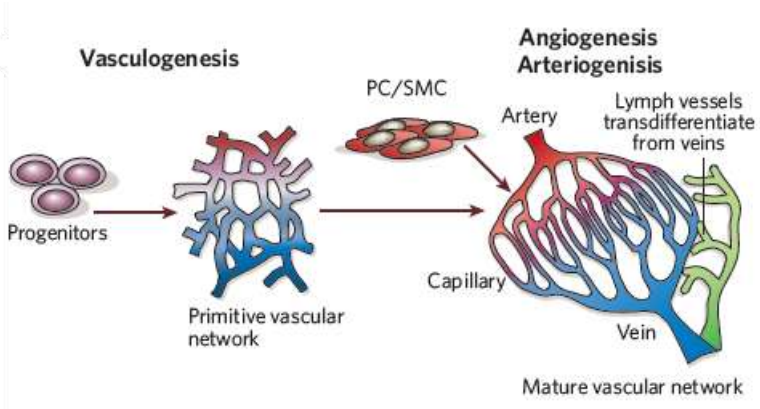

Figure 1.4. *Development of the vascular systems: during vasculogenesis, endothelial progenitors give rise to a primitive vascular labyrinth of arteries and veins; during subsequent angiogenesis, the network expands, pericytes (PCs) and smooth muscle cells (SMCs) cover nascent endothelial channels, and an organized vascular network emerges (modified from Carmeliet P., 2005).*

During vasculogenesis blood vessels assembly by clustering primitive vascular cells or hemangioblasts into blood islands which give rise to tube-like endothelial structures that define the pattern of the vasculature. These blood islands are composed of two cell types: angioblasts that are found primarily in embryonic mesoderm and form the outer layer of ECs encasing the blood islands, and hematopoietic stem cells, in the inner cluster, which give rise to the first embryonic blood cells (Risau W., 1997). Fibroblast growth factors (FGF) and vascular endothelial growth factor (VEGF) are important for the formation of angioblasts and

haematopoietic cells from mesoderm. For vasculogenesis, expression of VEGF receptors and sufficient production of VEGF are both necessary. VEGF is produced by the endoderm, while its receptors are expressed by mesoderm-derived angioblasts, and acts in a paracrine

manner. Induction of VEFG-R2 is thought to initiate angioblast differentiation, while quantity and activity of VEGF determines angioblast survival (Risau W., 1997).

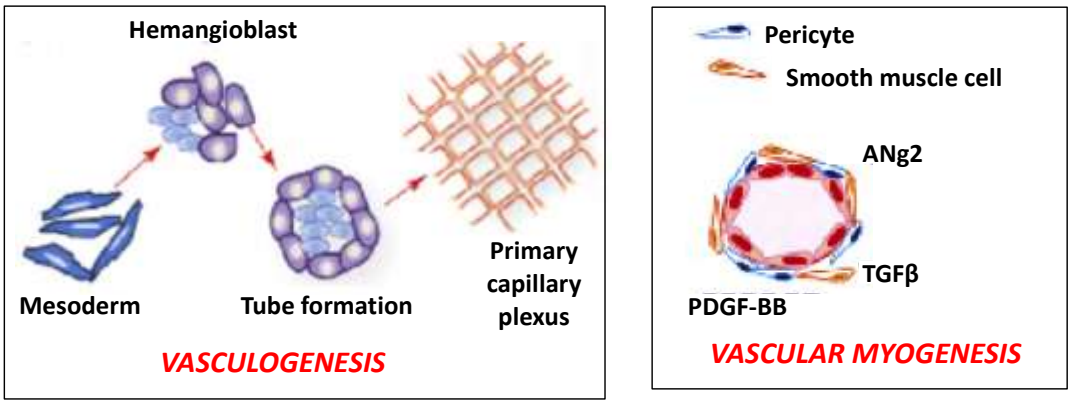

Figure 1.5. *Development of the vascular system. (a) During vasculogenesis, mesodermal precursors, the hemangioblasts, differentiate into ECs and form a primary vascular plexus. (c) Maturation and stabilization of the nascent plexus relies on the recruitment of pericytes and SMCs and deposition of extracellular matrix under the control of the coordinated action of PDGF, Ang2 (angiopoietin 2) and TGF-b signaling (modified from Pardali E., et al., 2010*).

Once the main vessels have been produced, branching and remodeling of such structures, a process known as angiogenesis, leads to the formation of a primitive vascular network, which occurs in both adults and embryos (Figure 1.6).

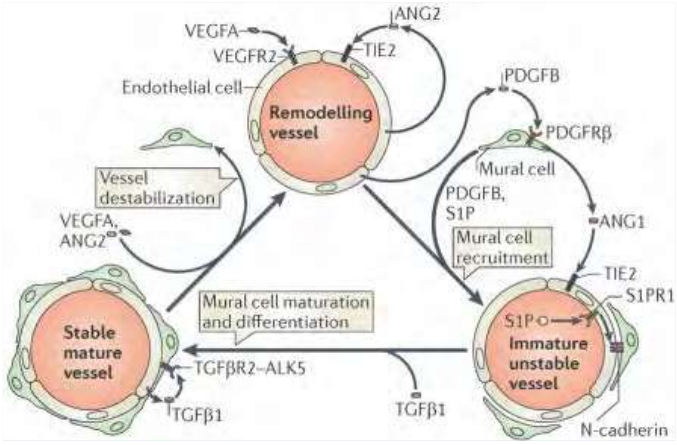

Figure 1.6. *The recruitment of perivascular cells (pericytes and vascular smooth muscle cells (vSMCs)) to nascent blood vessels plays an essential part in the stabilization and maturation of new vascular networks. Whereas pericytes primarily associate with small-caliber capillaries, vSMCs ensheathe larger arteries and veins. Initially, platelet-derived growth factor B (PDGFB) is released from endothelial cells (ECs) undergoing angiogenic remodeling. Recruited pericytes are incorporated into the wall of immature vessels and establish direct cell–cell contacts with ECs. Furthermore, angiopoietin 1 (ANG1) released from perivascular cells activates the TIE2 receptor in*

ECs, promoting EC survival and cell attachment. Activation of activin receptor like kinase 5 (ALK5; also known as TGFβR1) by TGFβ1 in perivascular cells may then promote vSMC differentiation to generate vSMC-ensheathed quiescent mature vessels. Importantly, this whole process can be reversed in response to pro angiogenic signals, such as vascular endothelial growth factor A (VEGFA) or the TIE2 antagonist ANG2, which promote mural cell detachment and vessel destabilization to allow further rounds of vascular remodeling (modified from Herbert S. P., et 2011).

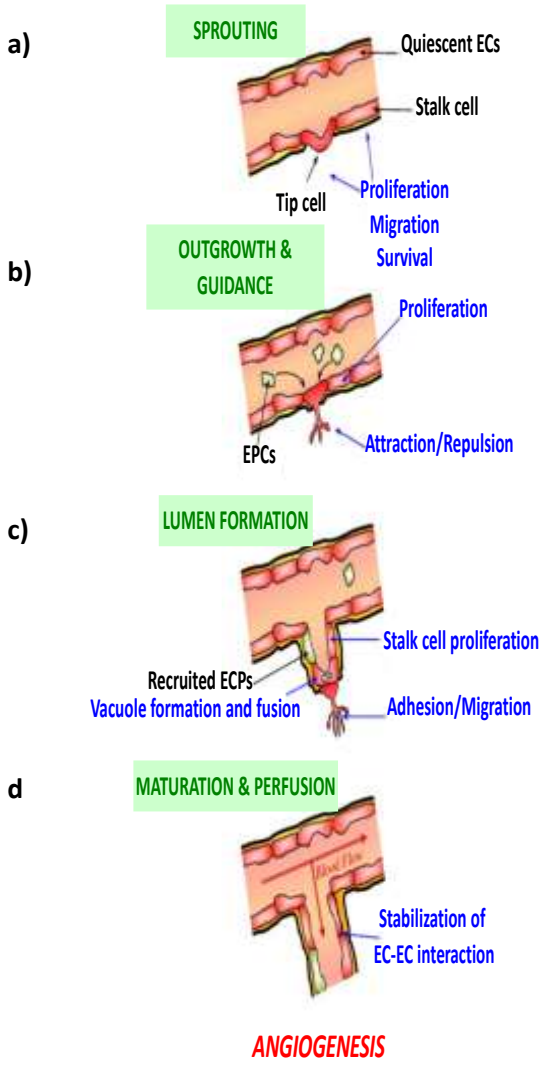

Figure 1.7. *Main steps of physiological angiogenesis. (a) Formation of new blood vessels from resident endothelial cells (ECs, in red) is controlled upon pro-migratory, proliferative and survival signals, such as vascular endothelial growth factor (VEGF) and delta like-4 (Dll4). (b) Vessel outgrowth is conducted by the effects of angiogenic factors and molecules, such as VEGF, and the contribution of endothelial circulating progenitors (ECPs, in green). (c) Fusion of EC vacuoles induces lumen formation in stalk ECs. Growth of new vessels is controlled by adhesive interactions on the migrating tip EC. (d) Once the new vessel is formed, EC-EC interactions are stabilized upon VEGF and basic fibroblast growth factor (bFGF) control (modified from Galan M. E. M. et al., 2009).*

he first step towards the maturation of the blood vessels is the formation of new capillaries by sprouting or splitting from the vessel of origin (Figure 1.7, a). Sprouting angiogenesis is induced by VEGF and occurs in the yolk sac and in the embryo. The process includes the degradation of the extracellular matrix, the migration and the proliferation of the endothelial cells and the maturation of the endothelium. One of the factors that induce angiogenesis is VEGF. Non-sprouting angiogenesis includes either the proliferation of endothelial cells inside a vessel, which produce a wide lumen that can be split by transcapillary pillars, or

fusion and splitting of capillaries. This process is defined by the expression of endothelial receptor tyrosine kinase TIE-2 or TEK. TIE-2 regulates VEGF activity and induces endothelial cells to narrow their lumina for sprouting or splitting.

The vascular system is then submitted to pruning (Figure 1.7, c), which is the remodeling of the formed vessels to form a mature system of larger and smaller vessels, and finally maturation (Figure 1.7, d), which leads to the modification of the basal lamina and the differentiation of pericytes and smooth muscle cells and the connection of the vessels with the tissue or organ they supply or regression. At some point during angiogenesis, survival of endothelial cells becomes independent of VEGF and maturation is affected by intracellular and extracellular interactions of endothelial cells. This process is also affected by circulation, where shear stress affects interactions of endothelial cells and expression of grown factors. For example, PDGF-β is upregulated and activates its receptors on perivascular cells, promoting their attachment to the endothelium and activating TGF-β, which alternates the composition of extracellular matrix, stabilizes the phenotypical characteristics of endothelial cells and inhibits their proliferation. For maturation, tissue factor, a procoagulant receptor expressed by the endothelium, recruits perivascular cells and induces the expression of TGF-β, which inhibits endothelial cell proliferation (Risau W., 1997).

1.3.2 Perivascular Cells (PCs)

Perivascular cells (PCs) consist of pericytes and vascular smooth muscle cells (vSMCs) and surround the inner endothelial lining, conferring support and stabilization. During vessel development, ECs recruit both pericytes and vSMCs in the newly formed vasculature to promote stabilization by wrapping around the blood vessels. The cell composition of blood vessels is directly correlated with the location of the vessel: mature vSMCs circumferentially wrap around the inner layers of larger arteries and veins including the aorta, carotid artery, and the saphenous vein, while pericytes surround smaller blood vessels or microvasculature, such as capillaries, in which a single EC makes up the inner perimeter of the blood vessel, precapillary arterioles, and postcapillary venules (Wanjare M., et.al, 2013).

1.3.2.1 Vascular Smooth Muscle Cells (vSMCs)

Vascular smooth muscle cells (vSMCs), which originate from mesodermal lineages, are found primarily in larger vessels and to a lesser extent in small vessels, such as capillaries, and they provide support to the endothelial tube of the vessels by regulating intravascular pressure. vSMCs are separated from the endothelium with the intervention of the basement membrane and the elastic lamina containing extracellular matrix (ECM), distinguishing them from pericytes which are connected with ECs with the basement membrane. These are characterized by plasticity, which is important for the completion of different functions of the vascular smooth muscle cells, such as contraction, proliferation and synthesis of extracellular matrix.

Dyring embryogenesis, vSMCs, with their phenotypic plasticity, play a crucial role in the maturation of the vessel following organization of endothelial cells into primary vascular plexus. In adults, a different set of conditions can cause a phenotypic switch in vSMCs by regulating the expression of smooth muscle cell markers. In wound healing and reparation of injuries implicated on the vascular wall, dedifferentiated synthetic SMCs are recruited at the site of the injury and form the neointima, the innermost part of larger blood vessels, consisting primarily of endothelial cells. This is accomplished by the decrease in the expression of contractile proteins, leading to the phenotypic switch in smooth muscle cells. Similarly, in some cardiovascular pathologies, such as atherosclerosis, restenosis and aortic aneurysm disease, these recruited SMCs can possess a synthetic phenotype while pathological lesions are formed (Wang G., et al., 2015).

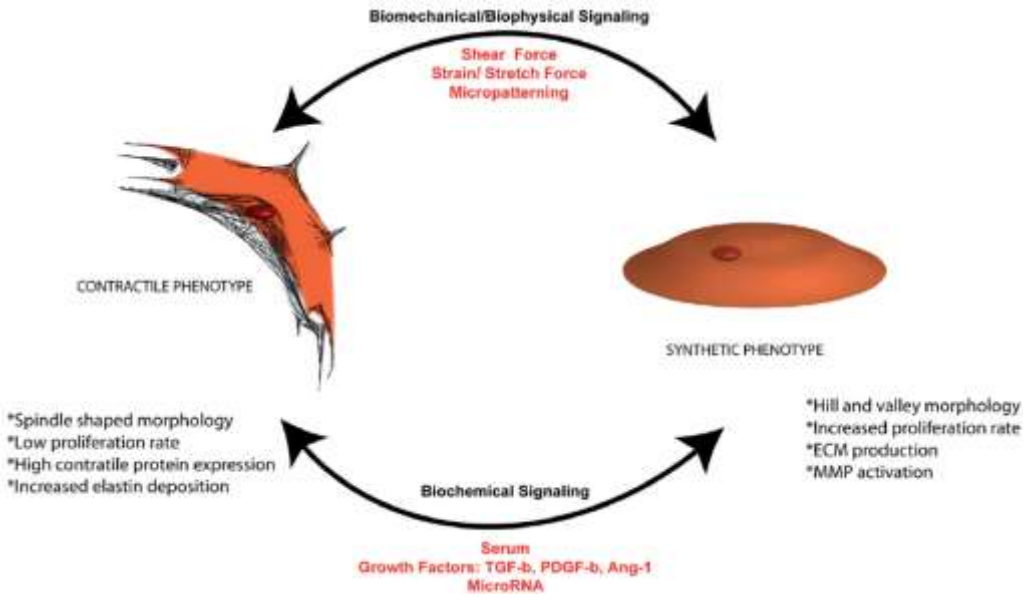

Figure 1.8. *Phenotypic plasticity of vSMCs. Characteristics of the synthetic and contractile phenotypes – including morphology, proliferation, ECM and contractile protein expression, and phenotypic switch – are regulated by various biochemical and biomechanical cues (from Wanjare M., et al., 2013).*

vSCMs can acquire a contractile or synthetic phenotype, with the former referred to as differentiated SMCs and found in healthy adult blood vessels where they play a role in contraction and the later referred to as dedifferentiated SMCs with proliferating capacity found in the embryo during the neovascularization or in injured adult blood vessels (Wanjare M., et.al, 2013). These two phenotypes are distinguished by a different set of smooth muscle cell markers expressed: the contractile phenotype expresses markers for cytoskeleton and contractile proteins, such as smooth muscle α-actin (SMαA), smooth muscle myosin heavy chain (SMMHC), calponin and SM22α, which are down-regulated in the synthetic phenotype (Wang G., et al., 2015).

Contractile vSMCs:

Contractile SMCs are considered to be the mature SMCs in the vessel under normal physiological conditions, wrapping circumferentially around the inner layers of larger arteries and veins (Wanjare M., et.al, 2013).

Contractile vSMCs are characterized by a spindle-like morphology, with low proliferation rate and by wrapping circumferentially around the vasculature, they promote stabilization and contraction of the vessel wall. Some markers that define and promote contractile vSMCs phenotype are FGF9 (Fibroblast Growth Factor 9), which induces the wrapping and stabilization of the vasculature, elastin, which offers elasticity and resilience, and smooth muscle myosin heavy chain (SMMHC), which powers the contraction. At different developmental stages, a variety of markers are expressed: alpha smooth muscle actin (αSMA) early on, SM22α, calponin, caldesmon heavy chain and smoothelin intermediately and SMMHC at later mature stages. Also, TGF-β1 plays an important role in vessel formation, by promoting differentiation of vascular progenitor cells into pericytes and vSMCs and by increasing the expression of contractile proteins (Wanjare M., et.al, 2013).

Mature SMCs are sensitive to growth factors, mitogens, inflammatory mediators and mechanical forces, which can promote functional and morphological changes, referred to as

phenotypic switch. During this process, a number of contractile protein markers are downregulated and thus SMCs lose their contraction properties, and instead they migrate, proliferate and accumulate in the intima, the innermost layer of a larger blood vessel consisting of ECs. Thus, mature SMCs dedifferentiate to the synthetic phenotype (Wang G., et al., 2015).

Synthetic vSMCs:

Synthetic vSMCs are prevalent in vessels that have undergone remodeling or that have endured injuries. The cells are defined by hill and valley morphology, with higher proliferation rate and, after enduring injury in the vessel wall, they promote thickening of the internal vascular tube and migration by producing ECM proteins, such as fibronectin and collagen and matrix metalloproteinases (MMPs) respectively.

During vessel remodeling, interactions with ECs affect the synthetic phenotype, by the secretion of PDGF-B, that recruits vSMCs containing PDGFR-β receptor. PDGFB acts by repressing the contractile phenotype and downregulating the smooth muscle cell markers they express, such as αSMA, SMMHC, and SM22α. This effect is exacerbated by the activation of KLF4 factor. Synthetic vSMCs are identified by caldesmon light chain, vimentin, non-smooth muscle myosin heavy chain B (SMemb)., tropomyosin 4, and cellular retinol binding protein 1 (Wanjare M., et.al, 2013).

1.3.2.2 Pericytes (PCs)

Pericytes – Morphological Characteristics:

Pericytes, as the name suggests, along with vascular smooth muscle cells (vSMCs), are found adjacent to capillaries in a variety of tissues and surround the inner endothelial lining, conferring support and stabilization. During vessel development, both pericytes and vSMCs are recruited to stabilize newly formed vasculature (Karen K., et al., 2013). These cells are distinctively shaped, with elongated morphology and polymorphic structures, that possess many cytoplasmic processes that protrude from the cell body and encircle endothelial cells in the microvasculature (Shepro D., et al., 1993).

The shape, size and distribution of pericytes is related to the type of vessels they decorate. They usually surround smaller blood vessels, microvasculature, such as capillaries, in which

ECs form an inner layer of the blood vessel, precapillary arterioles and postcapillary venules (Wanjare M., et al., 2013).

Phenotypically pericytes possess characteristics that closely resemble microvascular smooth muscle cells. Unlike smooth muscle cells, pericytes are covered by the same basement membrane of endothelial cells except where two mural cells are in contact. Pericytes also exhibit a number of characteristics consistent with muscle-cell activity and express contractile smooth-muscle actin (Karel K., et al., 1996).

Pericytes – Functions:

These morphological differences are intertwined with the vessel- and tissue-specific roles pericytes possess: regulation of capillary blood flow, phagocytosis and regulation of new capillary growth (Karen K., et al., 1996).

Pericytes possess a number of characteristics that resemble the vascular smooth muscle cell phenotype, the most important being the presence of contractile proteins, such as SMA-(actin), myosin and tropomyosin and adhesive protein fibronectin, which support the regulation of the contraction of the underlying endothelium. This controls the blood flow by the regulation of "vessel tone", via the expression of vasodilator vasoconstrictor endothelin 1 and angiotensin II receptors, that recognize the vasoactive substances produced by ECs, which in turn relax the contracted SMC via the cGMP-dependent mechanism. Additionally, pericytes possess cholinergic and adrenergic receptors: the -adrenergic response in pericytes leads to relaxation, whereas the cholinergic response is antagonistic and produces contraction. (Bergers G., et al., 2005, Karen K., et al., 1996).

The interaction between pericytes and the endothelial cells offers an advantage in the regulation of capillary growth, as pericytes inhibit the ECs proliferation in newly formed vessels during wound healing. At the same time, since there appears to be tight control of ECs and perivascular cells in the vasculature, this control must be regulated at multiple sites, which include soluble paracrine or autocrine factors, mechanical forces produced by blood flow and pressure and homotypic (EC-EC, mural-mural cell) and heterotypic (EC-mural) interactions (Bergers G., et al., 2005, Karen K., et al., 1996).

Injuries imposed on the vessel wall cause inflammation which in turn promotes the phenotypic switch of pericytes, which stop surrounding the vessels and acquire migratory

characteristics, including the loss of connection with the basement membrane and loss of stress fibers (Bergers G., et al., 2005, Karen K., et al., 1996).

Pericytes – Molecular Markers:

Due to the broad range of the phenotypical characteristics pericytes possess and their varying location in the body, there are no general pan-pericyte molecular markers that can be used for their identification. The most common identification is a CD146+PDGFRβ+CD34–CD31– population, but a number of markers can be used depending on the tissue they accommodate.

A number of growth factors majorly affect the phenotypical characteristics of pericytes. In culture, pericytes are positive for NG2, αSMA, CD44, CD146, platelet-derived growth factor β (PDGFRβ), and nestin and negative for CD56, CD34, CD31, and von Willebrand factor. Additionally, pericytes have mesenchymal stem cell (MSC) markers, such as CD44, CD73, CD90, and CD105. Transmembrane chondroitin sulfate proteoglycan neuron-glial 2 (NG2 or cspg4) and αSMA are used interchangeably to identify pericytes in different types of vessels: pericytes in capillaries are NG2+αSMA–, of the venules are NG2–αSMA+, and of the arterioles are NG2+αSMA+ (Bergers G., et al., 2005).

Cell-surface proteins found in pericytes are neuron-glial 2 (NG2), a chondroitin sulfate proteoglycan, proteoglycan, and platelet-derived growth factor receptor beta (PDGFR), a tyrosine-kinase receptor. Neuron-glial 2 (NG2) chondroitin sulfate proteoglycan is considered a characteristic of immature neural cells capable of differentiating into either glia or neurons. It is expressed on the surface of pericytes during the formation of new vessels and acts by binding with high affinity to basic fibroblast growth factor (bFGF), PDGF-AA, and plasminogen and angiostatin. PDGF-BB promotes the recruitment of pericytes which in turn offer stabilization to the vessel walls, VEGF stimulates pericyte migration in injured vessels, TGF- β1 increases the expression of αSMA and regulates the contractile phenotype (Karen K., et al., 1996).

1.3.2.3 Endothelial Cells

The vascular system is, as mentioned above, composed of a variety of cells that interact to form intact wall vessels. Vascular endothelium consists of approximately $1x10^{13}$ endothelial cells and forms a monolayer that covers the entire vascular system by being anchored to the blood vessel wall through the basement membrane and is thus considered the largest organ

in the human body. This monolayer acts as the first selective permeable barrier for all molecules, cells or pathogens circulating in the bloodstream, while offering various synthetic and metabolic properties, such as regulation of thrombosis and thrombolysis, platelet adherence, modulation of blood flow and regulation of immune and inflammatory responses. The discontinuity of the endothelium is correlated with cardiovascular pathologies, such as atherosclerosis (Hennigs J., et al., 2021)

Hemangioblast precursor cells, which are differentiated from mesenchymal cells (from the splanchnopleuric mesoderm) give rise to an intermediate pre-endothelial cell which then differentiates into either the hematopoietic cell line or endothelial cells. Endothelial cells in turn possess the ability to transdifferentiate into mesenchymal cells and intimal smooth muscle cells. Since endothelial cells cover different parts of the vascular tree, either arterial or venous cells, they do not show phenotypical changes depending on their location (Hennigs J., et al., 2021).

The mesodermal specification as well as the formation of hematopoietic and endothelial cell lines are regulated by two key signaling components: fibroblast growth factor 2 (FGF2 or bFGF) and bone morphogenetic protein 4 (BMP4). FGF2 activates FGF receptor type I and induces differentiation to mesoderm, while BMP4 acts by activating the pathway downstream of Indian hedgehog (IHH) and induces differentiation of endothelial cells. The formation of both endothelial and hematopoietic cells is regulated by the secretion of IHH, which acts as an inductive in vivo signal. Along with these factors, vascular endothelial growth factor (VEGF) is known to play a role in vasculogenesis, by regulating the survival or propagation of endothelial cells, from the early embryonic stages, as it is expressed by the extraembryonic visceral endoderm and follows the formation of blood islands in the yolk sac. VEGF-A is recognized by its main receptors VEGFR1 (fms-related tyrosine kinase-1 [Flt-1]) and VEGFR2 (fetal liver kinase-1 [Flk-1]), and also interacts with the coreceptors neuropilin-1 and -2. Even though Flk-1 has lower affinity for VEGF-A, its activation is usually connected to the responses of endothelial cells (Marcelo K., et al., 2013).

Endothelial cell development is also defined by the expression of E-twenty-six (ETS) transcription factors (such as Ets1, Erg, Fli-1, Etv2), that mostly act through transcriptional activation of endothelial specific genes. Etv2 expression is initially observed to be more widespread within the primitive streak mesoderm, but is soon restricted to developing vascular endothelial cells (Marcelo K., et al., 2013).

Endothelial cells exhibit a variety of biosynthetic pathways that can be used for their identification that are either specific for the endothelial cell line or general but used in combination with other specific cellular markers. Angiotensin converting enzyme (ACE) catalyzes the conversion of angiotensin I (AngI) in the vasoactive peptide angiotensin II (AngII) that catabolizes bradykinin (BK). Endothelial cells produce endothelin-1 (ET-1), which acts as an endogenous vasoconstrictor, prostaglandin I-2 (PI-2), which acts as a vasodilator, and express nitric oxide synthase (NOS), which, along with PI-2, maintains homeostasis of blood vessels. Additionally, endothelial cells express lipoprotein receptors to incorporate acetylated low-density lipoproteins (Ac-LDL) and remove them from circulation, where they can cause vascular inflammation (Marcelo K., et al., 2013).

1.3.2.3.1 Endothelial Cells – Functions

Vascular endothelium is both morphologically and functionally heterogeneous and endothelial cells exhibit specialized functions depending on the tissue they are located in.

The structure of the endothelium and the continuity of the endothelial cells are important for the maintenance of the structure of the vessel wall and the blood circulation. The endothelium is semi-permeable for small molecules and acts as a barrier, responsible for receiving and translating signals from the blood. Changes in the circulating blood, such as mechanical stress (elongation and wall shear stress) and changes in the concentrations of metabolic factors, are detected by endothelial cells, which serve paracrine and endocrine functions and transduce these signals to the underlying layers of the vascular wall, such as smooth muscle cells. To exert these paracrine and endocrine actions, endothelial cells express distinct receptors to respond to growth factors, such as the VEGF or basic fibroblast growth factor (bFGF), hormones and cytokines, such as interleukins or to bacterial toxins (Hennigs J., et al., 2021).

Regarding regulation of blood circulation, endothelial cells regulate the vascular tone by producing and secreting vasoactive factors, such as nitric oxide (NO) and prostacyclin (PGI2), that inhibit platelet aggregation and cause vasodilation of the vessels, and vasoconstrictive factors, such as angiotensin and endothelin-1 and -2 (Hennigs J., et al., 2021).

Endothelial cells act by balancing coagulation and fibrinolysis. Concerning coagulation, endothelial cells synthesize platelet activating factor (PAF), which adheres platelets and

neutrophils to endothelium via P-selectin. Activated platelets express CD154, which binds to CD40 on endothelial cells and induces the expression of leukocyte adhesion molecules on the surface of the endothelial cells. Another important factor in coagulation is Von Willebrand factor (vWF). Although, platelets contain vWF, the majority is synthesized from endothelial cells, in two forms: the vWF dimers secreted into the plasma and subendothelial matrix and granular vWF multimers stored in endothelial storage granules called Weibel–Palade bodies, for rapid mobilization in response to activating molecules, such as thrombin. When vascular wall is injured, vWF is released from the Weibel-Palade bodies, binds to the damaged area and to the platelets, causing platelet aggregation and in turn formation of blood clots, preventing hemorrhage. When the healing of the injured blood vessel is completed, the thrombus is removed from the site of injury through the action of plasmin and the accumulation of fibrin is prevented through a process referred to as fibrinolysis. Endothelial cells contain heparin like glycosaminoglycan receptors on their surface, which are targeted by antithrombin and thrombomodulin for the inactivation of thrombin and produce tissue type plasminogen activator (t-PA), which releases plasmin (Hennigs J., et al., 2021).

Endothelial cells also play important roles in immune and inflammatory responses by dilating arterioles, capillaries and venules, increasing permeability, blood flow and lymphocyte and leukocyte movement into tissues. Lymphocytes express integrins, such as leukocyte function-associated antigen-1 (LFA-1) or very late antigen-4 (VLA-4), and interact with endothelial cells through adhesion molecules, such as intramolecular cell adhesion molecules 1 and 2 (ICAM-1 and ICAM-2), and vascular cell adhesion molecule (VCAM). ICAM-2 is constitutively expressed on resting endothelial cells, while ICAM-1 and VCAM are expressed in response to infection (Hennigs J., et al., 2021).

Endothelial cells also express growth factor receptors, such as VEGFR2, on their surface and initiate angiogenesis by the recognition of VEGF.

1.3.2.3.2 Endothelial Cells – Molecular Markers

Cell surface markers are proteins expressed on the surface of cells that often serve as markers of specific cell types. Von Willebrand factor (VWF), together with the Weibel–Palade bodies (WPB), angiotensin-converting enzyme (ACE, CD143), and the cobblestone morphology specific for monolayer cultures, was previously referred to as a few obligate criteria to

confirm the authenticity and the purity of endothelial cell culture. A modern list of endothelial markers with their characteristics, in short, is analyzed below (Goncharov, et al., 2017).

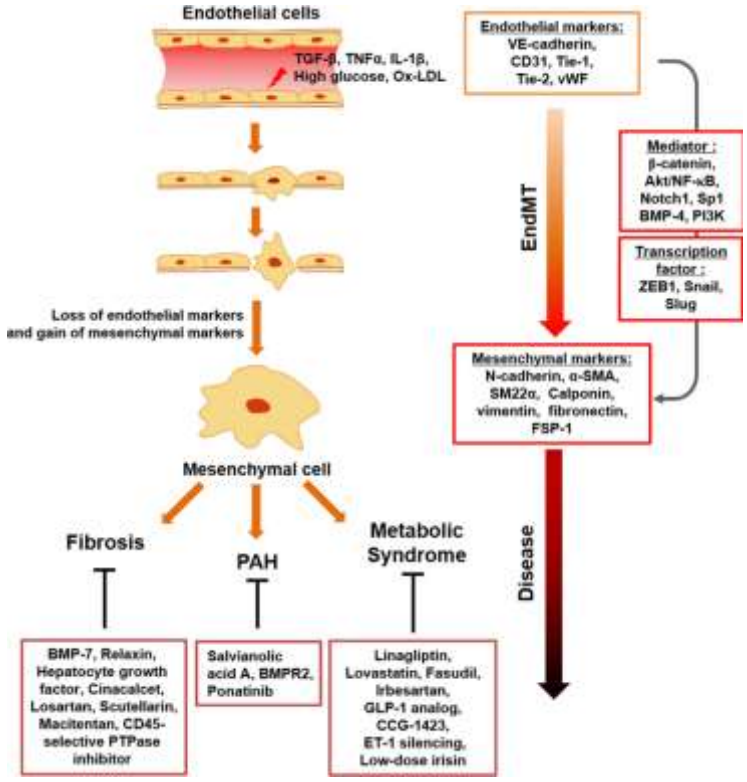

Figure 1.9. Schematic representation of endothelial to mesenchymal transition (EndMT) in response to inflammatory stimuli and metabolic dysfunction. Upon chronic inflammatory conditions, involving tumor necrosis factor-α (TNF-α), transforming growth factor beta (TGFβ), IL-1β, and endotoxin and metabolic dysfunction, such as increased serum LDL, glucose, diverse ECs undergo activation, which results in loss of endothelial cell markers and acquisition of mesenchymal-cell markers. EndMT contributes to endothelial dysfunction under inflammatory conditions and metabolic dysfunction, with EndMT mediators identified. This process can cause a variety of postnatal diseases, such as fibrosis, PAH, and metabolic syndrome (from Cho J. G., et al., 2018).

The Weibel–Palade bodies are specific endothelial organelles containing VWF, P-selectin (CD62P), and angiopoietin-2 (Ang2), participating in platelet binding, leukocyte recruitment, and modulation of inflammation, respectively. VWF, produced in megakaryocytes and ECs, is a glycoprotein participating in blood coagulation, exhibits a binding site for factor VIII (FVIII) and also for heparin. VWF size and function are regulated by protease ADAMTS-13, and disturbance of this function can lead to thrombotic thrombocytopenic purpura. VWF was found in about 80% of the HUVEC cell population in culture. Interestingly, endothelial VWF can be involved in angiogenesis. Additionally, a loss

of VWF in ECs results in enhanced and dysfunctional angiogenesis, which is consistent with the clinical observations that in some patients with VWF disease vascular malformations can cause severe gastrointestinal bleeding.

Angiotensin-converting enzyme (ACE, EC 3.4.15.1), one of the principal members of renin-angiotensin system (RAS), is a COOH-terminal dipeptidyl carboxypeptidase I, converting angiotensin I to vasoconstrictor angiotensin II, degrading bradykinin and amyloid beta-protein. On average, only 20% of capillary ECs in each organ stains for ACE, with the exception of the lung and kidney. In the lung, all capillary ECs express ACE, whereas in the kidney, all the vasculature is devoid of ACE. Angiotensin-converting enzyme 2 (ACE2) is a relatively new member of the RAS. Normal levels of ACE2 in the lung are necessary for the host to combat inflammatory lung disease. ACE and ACE2 maintain blood pressure homeostasis and fluid salt balance, mainly due to generation of angiotensin II and inactivation of bradykinin. Also, ACE activities play roles in immunity, reproduction, and neuropeptide regulation. The main active peptides of the RAS include angiotensin II (Ang II), Ang III, Ang IV, and angiotensin-(1-7) (Ang-(1-7)), among which Ang II and Ang-(1-7) are the most important in health and disease. Functional effects of Ang-(1-7) are different from those of AT(1) receptor stimulation and include vasodilatation, natriuresis, antiproliferation, and an increase in the bradykinin-NO (nitric oxide) system. It has also been suggested that the Mas oncogene may function as a receptor for Ang-(1-7) and thus the ACE2/Ang-(1-7)/Mas axis is a pathway that acts against the detrimental effects of the renin-angiotensin system, with several factors such as Akt phosphorylation, PKC activation, and MAP kinase inhibition involved in this signaling pathway. Cofilin-1, which is a widely distributed intracellular actin-modulating protein that binds and depolymerizes filamentous F-actin and inhibits the polymerization of monomeric G-actin, is involved in the translocation of actin-cofilin complex from cytoplasm to nucleus and plays a dominant role in Ang-(1-7)-induced G0/G1 arrest and autophagy in human aortic ECs.

VEGF receptors 1–3 contain an extracellular segment with seven immunoglobulin-like domains, a transmembrane segment, a juxta membrane segment, a protein kinase domain with an insert of about 70 amino acid residues, and a C-terminal tail. VEGF-A stimulates the activation of preformed VEGFR2 dimers by the autophosphorylation of activation segment tyrosines followed by the phosphorylation of additional protein-tyrosines that recruit phospho tyrosine-binding proteins thereby leading to signaling by the ERK1/2, AKT, Src,

and p38 MAP kinase pathways. Blood vessel formation is primarily achieved by angiogenesis—EC sprouting from pre-existing vessels. Vessel networks expand when sprouts form new connections, and vessel anastomosis is spatially regulated by VEGFR1 (Flt1), a VEGF-A receptor that acts as a decoy receptor. VEGFR1 modulates the activity of VEGFR2, which is the chief pathway in vasculogenesis and angiogenesis. Oxidized low-density lipoprotein (ox-LDL) impairs angiogenesis via VEGFR2 degradation and markedly suppresses HUVEC tube formation, along with induced apoptosis. VEGFR3 and its ligands (VEGF-C and VEGF-D) are involved primarily in lymphangiogenesis.

In addition to the VEGF receptor pathway, the angiopoietin (Angpt)-Tie is another EC-specific ligand-receptor signaling pathway necessary for embryonic cardiovascular and lymphatic development. The Angpt-Tie system also controls postnatal angiogenesis, vascular remodeling, and permeability to maintain vascular homeostasis in adults. This pathway is involved in many diseases where the vasculature plays a significant role, such as in cancer, sepsis, diabetes, atherosclerosis, and so forth. Mutations in the TIE2 signaling affect vascular morphogenesis, resulting in venous malformations and primary congenital glaucoma. ECs specifically express Tie-2, its paralog Tie-1, the tyrosine phosphatase VE-PTP, and its ligand Angpt-2, while Angpt-1 is secreted by pericytes. In the quiescent vasculature, Tie-2 is phosphorylated at tyrosine residues in its intracellular domain, thus promoting barrier function and anti-inflammation.

Cell adhesion molecules (CAM) make up a significant group (at least a couple of dozen) of endothelial markers, which are involved in homo- or heterophilic binding with other cells or with the extracellular matrix. All representatives of the four principal protein families (immunoglobulins, integrins, cadherins, and selectins) are expressed on the surface of ECs, including IgGs. Platelet endothelial cell adhesion molecule 1 (PECAM-1, CD31) is widely distributed on endothelium and hematopoietic-derived cells. It maintains the integrity of the blood vessels and therefore is involved in leukocyte-endothelium interaction and in leukocyte-transendothelial migration during inflammation. As ECs are often present at inflammation sites, the cells of the BBB are involved in development and/or manifestation of Alzheimer's disease, Parkinson's disease, multiple sclerosis, some cases of bacterial meningitis, trauma, and tumor-associated ischemia. PECAM-1 and its soluble form (sPECAM-1) are potential markers and possible targets for therapies.

Inflammation modulates gene expression through the activation of NF-κB and other transcription factors and promotes disassembly of the adherens junction and a loss of intercellular adhesion, creating gaps between the ECs allowing the diffusion of small molecules and transmigration of leukocytes. Adhesion of leukocytes through multiple transmembrane proteins—such as ICAM-1 (CD54), VCAM-1 (CD106), and CD47—promotes activation of small GTPases (Rac1, RhoA, and RhoG) and PTK signaling, such as activation of Src and Pyk2. ICAM-1 is one of the principal adhesion molecules, which determines changes of endothelial permeability and transendothelial leukocyte migration. Expression of ICAM-1 is increased after activation of ECs by proinflammatory stimuli; the effect of which is mediated by signaling pathways involving Akt/PKB, NF-κB, MAP-kinase p38, and ERK1/2. ICAM-1 protein binds with integrins CD11/CD18 and LFA-1 of leukocytes, mainly neutrophils, after that they easily penetrate into tissues (Goncharov, et al., 2017).

1.4 Differentiation Protocols

1.4.1 Differentiation of Pluripotent Stem Cells (PSCs) to Endothelial Cells (ECs)

The differentiation protocols that have been developed for the generation of ECs from iPSCs and ESCs have helped shed some light on the effect ECs have on pathological conditions.

There are three primary methods to generate endothelial cells from ESCs or iPSCs: stromal cell co-culture, feeder-free monolayer differentiation, and three-dimensional embryoid bodies, which utilize a number of growth factors that promote proliferation and differentiation to ECs (Lin, et al., 2017, Wilson, et al., 2014).

When stromal cells are co-cultured with ECs and iPSCs, they enhance differentiation towards endothelial cells. The stromal cells, extensively used in the earlier stages of the field, were usually murine bone marrow-derived cell lines such as OP9 or M10B2. Drawbacks, such as low differentiation efficiency producing a mixed population of ECs with other cell types (haematopoietic cells, smooth muscle cells and murine stromal cells) rendered this strategy inadequate for applications in regenerative medicine.

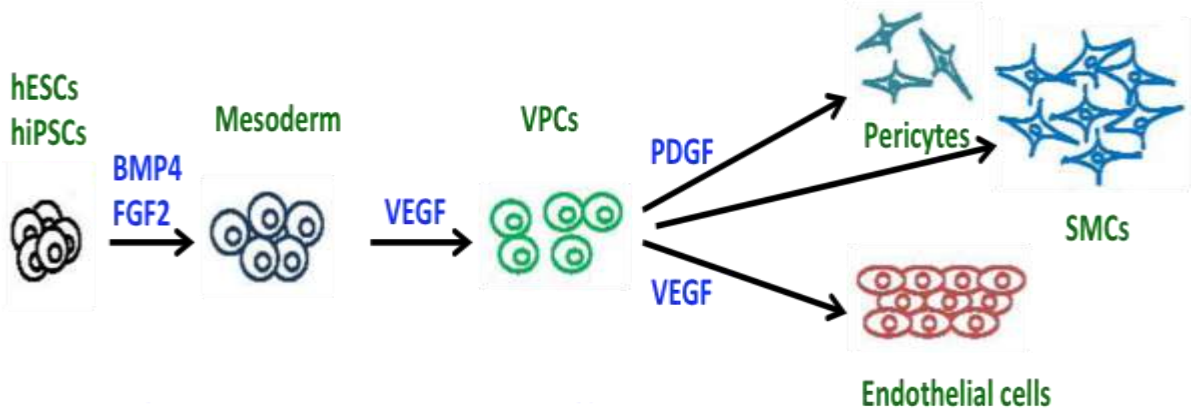

Figure 1.10. *Schematic overview of cell surface marker expression during mesoderm specification and vascular cell derivation from ESCs. During vascular specification, specific markers are modulated: from the pluripotency of ESC, through their mesodermal commitment and VPC lineage, into their derivation into endothelial and mural lineage. A map of the selective markers utilized to define and track the different steps of vascular differentiation can be made (from Descamps B. and Emanueli C., 2012).*

In feeder-free monolayer differentiation protocols ESCs and iPSCs are cultured in two-dimensional culture conditions on tissue culture plates coated with extracellular matrix proteins such as Matrigel, fibronectin and gelatin, in medium containing growth factors that promote and enhance differentiation towards mesoderm and then endothelial cells. The protocols that are based on this method yield to higher yield of ECs.

In three-dimensional embryoid bodies, ECs and iPSCs are cultured in conditions that promote self-aggregation of cells into three-dimensional embryoid bodies, leading to a not fully controlled, spontaneous differentiation. The embryoid bodies are comprised of different lineages of cells which give rise to the three germ layers: endoderm, mesoderm and ectoderm, recapitulating the progression of early embryonic development. The cells found in the mesoderm differentiate to haematopoietic and endothelial lineage cells. This strategy has shown to generate endothelial cells that can self-organize into vascular structures within the embryoid bodies, while endothelial cell differentiation is enhanced by the addition of a variety of growth factors (Lin, et al., 2017, Wilson, et al., 2014).

Table 1.1. *Defects in components of TGF-b signaling pathways lead to vascular abnormalities in human and mouse (modified from Pardali E., et al., 2010).*

Gene (mouse/human)	Animal model	Human disease
Ligands		
Tgfb1/TGFB1	KO: embryonic lethal with vascular defects or postnatal lethality from autoimmune disease	Camurati–Engelmann disease[a]
Tgfb2/TGFB2	KO: aortic arch defects, cardiac septal defects, perinatal lethality	unknown
Tgfb3/TGFB3	KO: cleft palate, delayed lung maturation, die shortly after birth	unknown
Receptors		
Tgfbr2/TGFBR2	KO: embryonic lethal, vascular defects	MFS2[a], LDS[a]
SM22-Cre-Tgfb2$^{fl/fl}$	KO: embryonic lethal, vascular defects	
Tie1-Cre-Tgfbr2$^{fl/fl}$	KO: embryonic lethal, vascular defects	
Tgfbr1 (Alk5)/TGFBR1 (ALK5)	KO: embryonic lethal, angiogenesis defects	LDS
Tie1-Cre-Tgfbr1$^{fl/fl}$	KO: embryonic lethal, angiogenesis defects	
Acvrl1 (Alk1) / ACVRL1 (ALK1)	KO: embryonic lethal, reduced VSMC differentiation, dilated vessels, AVMs.	HHT[b]
Bmpr2/BMPR2	KO: embryonic lethal (pre-angiogenesis) lethality. Transgenic *BMPR2*-mutant allele: pulmonary hypertension	PAH[b]
Accessory receptors		
Eng/ENG (endoglin)	KO: embryonic lethal due to vascular defects, reduced VSMC differentiation, heart defects. Het: vascular lesions similar to HHT	HHT
Soluble endoglin		Pre-eclampsia
Tgfbr3/TGFBR3 (betaglycan)	KO: poorly formed cardiac septa, incomplete compaction of ventricular walls	Unknown
Smads		
Smad1/SMAD1	KO: embryonic lethal due to defects in chorioallantoic circulation	Unknown
Smad4/SMAD4	KO: embryonic lethal	JP[b] and HHT
Smad5/SMAD5	KO: embryonic lethal due to angiogenesis defects	Unknown
Smad6/SMAD6	KO: heart abnormalities, aortic ossification and elevated blood pressure	Unknown
Smad7/SMAD7	KO: embryonic lethal due to cardiovascular defects	Unknown

Table 1.2. *Knockout animals from the VEGFR/VEGF, Tie2/Ang, and Eph/ephrin families have exhibited a variety of embryonic defects in vascular development (from Gale N. W. and Yancopoulos G. D., 1999).*

Gene knockout	Time of death	Stage of vessel development	Causes of lethality
VEGF-A (+/−)	E11.5	vasculogenesis/ (angiogenesis)	reduced red blood cells; defective heart and aorta formation; defective vessel connectivity; defective sprouting
VEGF-A (−/−)*	E10.5	vasculogenesis	absent dorsal aorta; defective endothelial cell development
VEGFR-1	E8.5–E9.5	vasculogenesis	failure of endothelial cell formation
VEGFR-2	E8.5–E9.5	vasculogenesis	excess endothelial cells form abnormal vessel structures entering vessel lumens
VEGFR-3	E10.5–E12	vasculogenesis	defective vessel remodeling and organization; irregular large vessels with defective lumens
Ang1	E10.5	angiogenesis	defective vessel remodeling, organization, and sprouting; heart trabeculation defects
Ang2	E12.5–P1	maturity	poor vessel integrity, edema, and hemorrhage
Tie1	E13.5–P1	maturity	poor vessel integrity, edema, and hemorrhage
Tie2	E10.5	angiogenesis	defective vessel remodeling, organization, and sprouting; heart trabeculation defects
ephrin-B2	E10.5	(vasculogenesis)/ angiogenesis	some defective vessel primordia; defective vessel remodeling, organization, and sprouting; heart trabeculation defects
EphB2/EphB3	E10.5 (~30%)	(vasculogenesis)/ angiogenesis	some defective vessel primordia; defective vessel remodeling, organization, and sprouting; heart trabeculation defects
EphB4	E10.5	?	?
EphA2	viable	—	—

2. AIMS

Regenerative Medicine (RM) is an interdisciplinary field of research and clinical applications, focused on repair, replacement, or regeneration of cells, tissues, or organs to restore impaired function resulting congenital defects, disease, and trauma (Mao & Mooney, PNAS 2015). Development of successful cell-based therapies relies upon tissue engineering for the arrangement of assorted cells into correct spatial organization and the creation of optimal microenvironments for growth and differentiation.

A major requirement for viability and function of the implantable construct is the availability of blood vessels to support its *in vivo* growth. Vascularisation is one of the most important aspects to the success of tissue engineered constructs and constitutes a major hurdle facing the regenerative medicine field (Carmeliet & Jain, Nature 2000). Vascularisation remains a critical obstacle in engineering thicker, metabolically demanding organs, such as the heart muscle, the brain and the liver. Regenerating tissue over 100–200 μm exceeds the capacity of nutrient supply and waste removal by diffusion requiring an intimate supply of vascular networks (Carmeliet & Jain, Nature 2000; Jain, Science 2005). It takes several weeks for a scaffold to become fully vascularized *in vivo* (Nillesen et al.. Biomaterials 2007). Recently, there is great interest in generating tissue-engineered constructs that are already pre-vascularised before implantation to shorten the time needed for implant vascularization and survival.

The host laboratory has developed a method for the differentiation of hESCs/hiPSCs to ECs (Tsolis et al. 2016) using chemically defined conditions (APEL medium) supplemented with growth factors. Whereas the protocol overall functions satisfactorily, the generated ECs exhibit low efficiency of differentiation (around 25 %), low proliferative potential and require sorting.

The aim of the present study is to improve the protocol of the host laboratory for generating differentiated ECs by comparing it to selected appropriate published protocols aiming at adopting steps that will:

- optimise the differentiation protocol to generate ECs exhibiting higher differentiation efficiency.

- facilitate the phenotypical characterisation and functional analysis of the generated ECs using visualisation and quantification by specific expression markers.

3. MATERIALS AND METHODS

3.1 Cell Culture Methods

3.1.1 Cell Culture of hPSCs (hiPSCs, hESCs)

For the purpose of this thesis the differentiation protocols were assessed on hPSCs, both hiPSCs and hESCs. hiPSCs were generated from human fibroblasts as previously described (Kyrkou et al., 2016), and the H1 hESC line was purchased from Wicell Research Institute (Madison, WI, United States). Also, two different pluripotent cell lines were cultured, this time iPSCs derived from patients with Parkinsons' disease, one that contained the **leucine-rich repeat kinase 2 (*LRRK2*)** mutation and one that had the gene corrected, from postdoc Maria Markou.

hiPSCs from human fibroblasts were cultured on six-well tissue culture plates (Corning, 3506), coated with hESC-qualified Matrigel (Corning, 354277), at 37°C and 5% CO_2, in mTeSR1 medium (StemCell Technologies, 05850), which was changed every 1–2 days. GC and IM2 iPSCs were cultured on Matrigel-coated six-well culture plates, at 37°C and 5% CO_2, in StemFit medium (AMSBIO, SFB-503), which was changed every 1–2 days.

The homogeneity of the culture was maintained by removing cells that started to differentiate and presented a different phenotype from the rest of the culture. Those cells were found either in single colonies or at the edges of connected uniform colonies. The removal of these cells was performed during medium change, by marking the areas containing these cells and suctioning them with an aspirator pump in the biological safety cabinet.

hPSCs were recultured when the majority of the colonies had grown in size, had compact centers and the edges had started to connect. Every 4–6 days, hPSCs were passaged enzymatically using 1 mg/ml dispase (Invitrogen, 17105-041) for 2 min at 37°C. hPSC colonies were then harvested, dissociated into small clumps and replated onto Matrigel-coated 6-well plates (ratio 1:6).

For the reculturing of the hPSCs, wells in a 6-well plate were covered with 1ml of 1% Matrigel, diluted in DMEM/F12 (ThermoFisher Scientific, 11320) medium and incubated for 1h at room temperature. Simultaneously, mTeSR medium used for the culturing of the hPSCs, was incubated at room temperature. After the 1h incubation, the well covered with matrigel was washed with 1 ml DMEM/F12, to remove excess Matrigel. In the well of the

6-well plate containing the hPSCs that needed reculturing, the medium was removed using an aspirator pump and the well was washed with 1 ml DMEM/F12 medium. hPSCs were passaged enzymatically using 1 ml of dispase at a concentration of 1 mg/ml (ThermoFisher Scientific, 1710541) and incubating the plate in an incubator at 37°C for 1 min, until the edges of the colony started to detach, without detaching the whole colony from the well. After 1 min incubation, dispase was removed using an aspirator pump and the well was washed two times with 2 ml of DMEM/F12, to remove the excess dispase.

The colonies were gently detached by adding 1 ml of mTeSR medium to the well and scraping with a cell scraper (Corning, 3010). The detached cell aggregates were transferred to a 15 mL conical tube. This was performed twice, to ensure that all colonies were transferred. The 15 ml tube was centrifuged at 1.200 rpm (210 g), for 5 min, at room temperature, the supernatant was aspirated, and the cell pellet was resuspended with 1 ml of mTeSR medium. The cell aggregate mixture was pipetted up and down 2–3 times to break up the aggregates, but not to create a single-cell suspension, and the cell mixture was evenly distributed to the new matrigel-coated wells from a 6-well plate, in a 1:7 analogy. The 6-well plate was shaken mildly and incubated in an incubator, at 37°C and 5% CO_2.

3.1.2 Cell Culture of Human Umbilical Vein ECs (HUVECs)

ECs from umbilical vein (HUVECs) were cultured in M199 full medium, containing M199 medium (Gibco, ThermoFisher Scientific, 11150067) supplemented with 20% heat inactivated fetal calf serum (FCS), 47 mg/ml endothelial cell growth supplement (ECGS), 4.7 m/ml heparin (Sigma) and 1% penicillin-streptomycin as previously described (Bellou et al., 2012), at 37°C and 5% CO_2.

Medium change was performed every 1–2 days and reculturing was performed when 80-90% of the plate area was covered. Before reculturing, plates were covered with 25 mg/ml Type I collagen (Corning, 354236), incubated at 37°C for 20 min and then washed twice with PBS. For the reculturing, the medium was aspirated, and each plate was washed with PBS. Then 0.05% Trypsin – EDTA (Gibco, ThermoFisher Scientific, 25300054) was added to lift the cells off the plate and cover the whole plate, and then immediately removed from the plate and incubated in incubator at 37°C for 1 min. After incubation, M199 full medium was added and cells were transferred in collagen-coated plates, in a 1:3 ratio.

3.2 Isolation Methods

3.2.1 Isolation of ECs from Umbilical Cord Veins (HUVECs, Human Umbilical Vein Endothelial Cells)

Umbilical cords were used to isolate endothelial cells from the veins (HUVECs). In each HUVECs isolation, 2-3 umbilical cords were used.

Before the procedure, PBS (Biosera, LM-S2043) was incubated at room temperature. One single 10 cm^2 culture plate and one single 6 cm^2 culture plate were covered with 25mg/ml type I collagen from rat tail and incubated at 37°C for 20 min. After 20 min, the plates were washed twice with PBS.

The procedure was performed in a biological safety cabinet. The umbilical cords were cleaned to remove the blood and cut with a scalpel to remove any clotted or disfigured parts. The cords were kept in a petri dish containing PBS, for hydration. The vein was found, along with the arteries, and was washed with PBS, using a syringe, until the vein was cleared of the blood it contained. After washing with PBS, a sterile hemostat was secured on each side of the vein of each cord, to control the liquid flow through the veins. PBS was added using a syringe and let flow through each side of the veins to ensure correct application of the hemostats and then air was applied with a syringe to remove the excess PBS from the walls of the cord so as not to dilute the collagenase (Sigma). One side of each vein was closed off using the hemostat and 0.1% collagenase diluted in PBS was added in the vein, until it filled up, and then the hemostat on the other side was closed. The cords were then submerged in PBS and incubated in a waterbath, at 37°C strictly for 12 min, to collect only the endothelial cells found in the inner layer of the veins. During incubation, M199 medium with 5% FBS was prepared using M199 medium (Gibco, ThermoFisher Scientific, 31150-022) supplemented with 5% FBS. After incubation, one hemostat of each cord was opened so the collagenase mixture could flow through the vein into a 50 ml falcon tube. Each vein was washed twice with the prepared M199 medium with 5% FBS, which flowed through the veins into the 50 ml falcon tube. The 50 ml falcon tube was centrifuged for 5 min at 1.200 rpm and the supernatant was aspirated. The cell pellet was resuspended in M199 full medium, which was prepared using M199 medium supplemented with 20% fetal calf serum (FCS), 47 mg/ml endothelial cell growth supplement (ECGS), 4.7 m/ml heparin (Sigma) and

1% penicillin-streptomycin as previously described (Bellou et al., 2012), and distributed in the collagen-coated plates. The cells were incubated in an incubator, at 37°C and 5% CO_2.

Medium change was performed the day after plating and reculturing was performed every 2–3 days, when 80-90% of the plate area was covered.

3.2.2 Isolation of Collagen from Rat Tails

The protocol used for the isolation of collagen from rat tails was performed as suggested by Piatti et al., 2022. Collagen stock preparation was performed in the biological safety cabinet.

Rat tails were stored at -20°C. Each time the collagen isolation protocol was performed, 5-6 tails were thawed, for 24–48 hours at 4°C. The tails were then submerged in 70% EtOH, in a sterile beaker, for 30 min. The tails were kept in PBS to maintain their hydration and one tail at a time was handled. Two techniques were used to remove the collagen fibers, depending on how long the tails were frozen for.

When the tails were frozen for less than one month at -20°C, the first technique was used, as suggested by Piatti et al., 2022. Each tail was cut at the base and the skin was cut longitudinally with a scalpel. The inner part of the tail was held using forceps and the skin was pulled with another forceps, separating the skin from the underlying layers of connective tissue, exposing the collagen fibers. The tip of the tail was held with the forceps using the left hand at the vertebra joint and the base of the tail was held with the other forceps set with the right hand. Both forceps were held at a 45° angle. 45° angle right and left wrist movements were made with the right hand forceps (base of the tail) while holding the tip and pulled with right hand forceps until a string of collagen detached. The collagen strings were transferred immediately into a beaker containing 250 mL of 70% EtOH. The tissue end was cut with a scissors before submerging the collagen tail. This process was repeated from base to tip of the tail until all the collagen was pulled, with the collagen strings getting thicker and shorter.

For tails that were frozen for longer periods of time at -20°C, a different technique was used. The skin of the tail was removed with a set of forceps and scalpel as suggested before. The connective tissue along the collagen strings was cut with a scalpel and then the collagen strings were pulled with forceps.

When collagen strings from all the tails were extracted, the collagen strings were washed 3 times with 70% EtOH, by submerging the collagen strings in 3 different beakers containing 70% EtOH. The collagen strings were then transferred to a sterile Erlenmeyer flask and were weighed. For each gram of strings, 200 mL of 0.1% acetic acid solution (prepared with cell culture H2O) was added. The Erlenmeyer flask was shaken 1-2 times a day and was placed at 4°C for 48 h, until the collagen strings were completely dissolved. After 48 h, the acetic acid solution (with debris) was transferred into sorvall dry spin centrifuge bottles (Sorvall, 06829) and centrifuged at 4°C for 90 min at 11,000 g. The supernatant was transferred into 50-mL tubes and frozen for at least 24 h.

After at least 24 h, the frozen tubes were placed in the lyophilizer/freezer dryer for 5 days at -83°C using a 0.027 Mbar pressure. Then the tubes were taken out of the lyophilizer/freezer dryer, the collagen was weighed and stored frozen.

Five days before using the collagen, the lyophilized collagen was resuspended in the appropriate volume of 0.1% acetic acid solution to a stock concentration of 15 mg/mL and kept at 4°C, while mixing vigorously once per day until the solution became transparent. The day before the experiment, the transparent collagen was spun down at 2000 g at 4°C for 10 min to remove bubbles.

For the hydrogel preparation, the protocol followed was described by Piatti et al., 2022. For a 0.75% w/v (7.5 mg/mL) final collagen solution, the following reagents were needed: Type I collagen stock solution (1.5% w/v—15 mg/mL), 1 N sodium hydroxide (NaOH) solution, M199 10X medium supplement, Endothelial Cell Growth Medium (EGM-2). The volume of the stock collagen solution (V stock) was prepared following the equation: V stock = V final * (C final / C stock). V final is the final volume of gel required, C final is the desired concentration of the gel, and C stock is the concentration of the stock collagen solution. Using a 1-mL syringe, the appropriate volume of stock collagen was transferred to an empty 30-mL conical tube. The volumes of neutralizing reagents (NaOH, M199 10x and EGM-2 medium) were calculated using the equation mentioned above and the reagents were mixed in a 15-mL conical tube and added to the aliquoted collagen. The mixture was stirred using a spatula until the formation of a homogeneous gel, without the introduction of bubbles. The pH was checked using a pH strip and was adjusted accordingly to obtain pH ¼ 7. The final collagen solution was spun down at +4 C, 1900 g for 20 min to remove bubbles.

3.3 Protocols of Differentiation to Endothelial Cells (ECs)

3.3.1 Tsolis et al. Protocol

The protocol of differentiation to endothelial cells already established in the lab was published in 2016. (Tsolis et al., 2016). This protocol involves differentiation of hESCs to CD34+ cells under feeder-free, chemically defined conditions. H1 colonies were first dissociated into small clumps and replated onto Matrigel-coated six-well plates as per normal routine passaging.

After 48 h, mTeSR medium was changed to differentiation medium (APEL), which was synthesized as previously described, (Ng, et al., 2008) supplemented with 5 μM glycogen synthase kinase-3 (GSK-3) inhibitor (CHIR99021, Selleckchem, S2924). After 24 h, the medium was replaced with the same basal medium supplemented with bone morphogenetic protein-4 (BMP-4) (25 ng/mL, Life Technologies, PHC9534) for 48 h and then with VEGF-A (80 ng/mL; Immunotools) for another 48 h.

Cells were cultured for 5 days, when the percentage of $CD34^+$ cells was evaluated with FACS analysis to measure the differentiation efficiency.

3.3.2 Patch et al. Protocol

In the protocol published by Patch, et al., 2015, human pluripotent stem cells were routinely cultured on matrigel in mTeSR1 medium. Cultures were passaged every 3–5 days using Accutase (Stem Cell Technologies, 07920). An 80% confluent 10cm plate was required to start differentiation in one well from a 12-well plate. For the accutase passaging, one well in a 12-well plate was coated with growth factor reduced matrigel by thawing it on ice and diluting it 1:30 and then incubated at room temperature for 1h. The well was then washed once with DMEM-F12 (ThermoFisher Scientific, 11320). From the PSC plate, culture medium was aspirated and rinsed with 1 ml of pre-warmed DMEM-F12. 1.5 ml of accutase was added and incubated at a 37°C incubator for 3-5 minutes, or until most cells were detached. After incubation, 1.5 ml of DMEM/F12 was added in the well to lift the cells twice and transferred in a 15 ml falcon tube. 10 μL of cell mixture were added in a Neubauer cell counting chamber for cell counting. 37.000 to 47.000 cells/cm^2 were added in a new 15 ml falcon tube, optimized according to the different cell lines used. The cell suspension was centrifuged at 1200 rpm \(210g) for 5min. The supernatant was aspirated, and the cell pellet

resuspended in ½ culture volume of pre-warmed mTeSR Medium supplemented with 2 μL of ROCKi (Fasudil, 5 mM stock, 5 μM/μL) to concentration of 10 mM. The cells were transferred in the matrigel-coated well in a 12-well plate and incubated at 37°C, 5% CO2 for 24 h.

For lateral mesoderm induction, after 24 h the medium was replaced with pre-warmed N2B27 Medium supplemented with 6-8 μM CHIR-99021 (Selleckchem, S2924) and 25ng/ml BMP4 \(3 ml/12well), for 3 days without medium change. For the preparation of 1 L of N2B27 Medium**,** 500ml of DMEM/F12 medium and 500ml Neurobasal medium (Life Technologies, 21103049) were supplemented with 20ml B27 \(1.94%) (Minus Vitamin A, Life Technologies, 12587010), 10ml N2 \(0.97%) (100X, Life Technologies, 17502048), 1ml β-Mercaptoethanol 50 mM \(0.097%) (Life Technologies, 21985023) and filtered with 0.22 μm sterile filter.

For endothelial cell induction, after 3 days the media was replaced with StemPro-34 SFM medium (Life Technologies, 10639011) supplemented with 200ng/ml VEGF (Immunotools) and 2μM forskolin (Abcam, ab120058) \(2 ml/12well) and changed every day for 2 days. Cells were cultured for 6 days, when the percentage of $CD31^+$ and $CD34^+$ cells was tested with FACS analysis to measure the differentiation efficiency.

3.3.3 Harding et al. Protocol

In the publication Harding et al. 2017 an additional protocol for the differentiation of hiPSCs and hESCs to ECs, in three stages, was presented. hiPSC or hESCs cells were manually passaged as small clusters onto hESC-qualified Matrigel-coated culture plates in mTeSR medium, as described before, with accutase or dispase and incubated at 37°C, 5% CO_2 for 24 h.

For mesoderm induction, culture medium was changed to APEL medium (Ng, et al., 2008) with 6 μM of CHIR99012 (Selleckchem, S2924) for 48 h.

For endothelial cell induction, the cells were cultured in APEL medium supplemented with 25 ng/ml BMP4, 10 ng/ml FGF2, and 50 ng/ml VEGF for 48 h.

After 4 days, cells were lifted with accutase (e.g. page 33) and seeded onto wells from 12-well plates covered with 0.1% gelatin (Stem Cell Technologies, 07903), collagen or fibronectin (Sigma) at $5 \times 10^3 - 1 \times 10^4$ cells per cm^2 in EC Growth Medium MV2 (ECGM-

MV2, PromoCell) with an additional 50 ng/ml VEGF. The medium was changed every 2 days for 4-6 days to generate ECs.

Cells were cultured for 6-9 days, when the percentage of $CD31^+$ and $CD34^+$ cells was tested with FACS analysis to measure the differentiation efficiency.

1. EGM-2 Medium (LONZA, CC3162) Components

Fetal Calf Serum	0.02 ml / ml
Epidermal Growth Factor (recombinant human)	5 ng / ml
Basic Fibroblast Growth Factor (recombinant human)	10 ng / ml
Insulin-like Growth Factor (Long R3 IGF)	20 ng / ml
Vascular Endothelial Growth Factor 165 (recombinant human)	0.5 ng / ml
Ascorbic Acid	1 µg / ml
Heparin	22.5 µg / ml
Hydrocortisone	0.2 µg / ml

2. ECGM-2 Medium (Promocell) Components

Fetal Calf Serum	0.05 ml / ml
Epidermal Growth Factor (recombinant human)	5 ng / ml
Basic Fibroblast Growth Factor (recombinant human)	10 ng / ml
Insulin-like Growth Factor (Long R3 IGF, recombinant human)	20 ng / ml
Vascular Endothelial Growth Factor 165 (recombinant human)	0.5 ng / ml
Ascorbic Acid	1 µg / ml
Hydrocortisone	0.2 µg / ml

3. APEL Medium Components

1 x Iscove's modified Dulbecco's medium (IMDM)	1x
1 x Ham's F-12 nutrient mixture	1x
Human Serum Albumin	100 mg ml^{-1} (10%)
Polyvinylalcohol (PVA)	5%
Linoleic acid (100 ng ml^{-1})	10,000x
Linolenic acid (100 ng ml^{-1})	10,000x
Cholesterol (Sigma, C3045)	7,200x
α-Monothioglycerol (α-MTG) (3.9 ml per 100 ml; B350–450 mM)	13 ml in 1 ml IMDM
rh Insulin-transferrin-selenium-ethanolamine solution (rhITS-Eth)	100x
Protein-free hybridoma mixture II (PFHMII) (5%)	1x
Ascorbic acid 2 phosphate (50 mg ml^{-1})	5mg ml^{-1}
GlutamaxI (L-alanyl-L-glutamine) (2 mM)	200 mM (100x)

Figure 3.1. *Composition of media. 1 is for EGM-2 medium (LONZA, CC3162) used in the current project, 2 is for the ECGM-2 medium (Promocell) used in the Harding et al. protocol and 3 is for the APEL medium suggested by Harding et.al.*

3.4 Analysis Methods

3.4.1 Immunofluorescence

Immunofluorescence (IF) is a technique that permits visualization of cell components in any given tissue or cell type, through combinations of specific antibodies tagged with fluorophores.

Indirect immunofluorescence on adherent cells was performed using primary and secondary antibodies (Figure 3.2). Cells were cultured on coverslips placed in 24-well plates until confluency, at which time the medium was removed, and the cells were washed with culture volume (0.5 ml) of PBS. For fixation, 0.5 ml of 3% paraformaldehyde (Merck, 104005) was added and incubated at room temperature for 15 min. PFA was removed and cells were washed with 0.5 ml of PBS. To quench the free aldehyde groups, 0.5 ml of 50 mM ammonium chloride (99.5 % NH_4Cl Sigma-Aldrich : SIAL A4514-100G) in PBS was added and incubated at room temperature for 15 min. Ammonium chloride was removed followed by washing with 0.5 ml of PBS. For cell membrane permeabilization, 0.5 ml of 0.1% Triton X-100 (Sigma-Aldrich : Fluka 93418) in PBS was added and incubated at room temperature for strictly 4 min and then removed, followed by washing with 0.5 ml of PBS. The coverslips were then placed in a plastic container padded with Wattman papers, that were washed with PBS and covered with a piece of parafilm. For blocking of non-specific sites, 40 μL of 10% FCS was added and incubated at room temperature for at least 20 min. The 1st antibody was diluted in 10% FCS or PBS, according to the manufacturer's instructions. The 10% FCS was then removed and 50 μL of the 1st antibody were added and incubated at room temperature for 1 h. The coverslips were then transferred to a 24-well plate and washed twice with 0.5 ml of PBS while placed on a shaker, to remove the excess antibody, and transferred onto the parafilm. The 2nd antibody was diluted in 10% FCS or PBS, 50 μL of which were added to the coverslips and incubated at room temperature for 1 h, under dark conditions, to protect the light-sensitive 2nd antibodies. The coverslips were again transferred to a 24-well plate and washed twice with 0.5 ml of PBS while placed on a shaker.

Cell nuclei were stained using propidium iodide-PI (Sigma), samples were mounted in moviol plus dabco, and images were taken on a Leica TCS SP5 confocal microscope using HCX PL APO CS 40 _ 1.25 OIL objective.

3.4.2 Fluorescence-Activated Cell Sorting (FACS) Analysis

Fluorescence-activated cell sorting (FACS) is a specialized type of flow cytometry, that utilizes the specific light scattering and fluorescent characteristics of each cell to count and profile cells in a heterogenous fluid mixture (Figure 3.2).

Cells were enzymatically detached from the culture using 0.05% trypsin, by incubating at 37°C for 30-45 sec, and lifted with a PBS mixture supplemented with 2% FBS and 1 mM

EDTA of the cell mixture was placed on a Neubauer cell counting chamber and counted 200.000 cells were transferred to eppendorf tubes and centrifuged at 1200 rpm \(210g) for 5 min and then resuspended in 40 μL of PBS/2%FBS-EDTA mixture. In each eppendorf tube, 8 μL of FITC-Fluorescein isothiocyanate or PE-Phycoerythrin labeled antibodies were added and incubated on ice and in the dark for 30 min. Then, 400 μL of PBS were added and centrifuged at 1200 rpm \(210g) for 5 min. The supernatant was removed, and the cell pellet was resuspended in 1 ml of PBS and transferred to 1.5 ml FACS tube compatible with the FACS analysis machine CyFlow (Partec, Münster, Germany) used (Department of Biological Applications & Technology, E4 Premises, ground floor). For each sample, 20.000 events were collected, and the analysis was performed with the use of FlowMax Software. All antibodies had previously been titrated. Isotypic control antibodies were used as negative controls.

1. Antibodies for Immunofluorescence	2. Antibodies for FACS Analysis
Primary Antibodies	Mouse IgG1 Isotypic Control FITC (ImmunoTools, 21275513)
CD31 Monoclonal Antibody (AbCam)	Anti-Human CD31 FITC (ImmunoTools, 21270313)
vWF Monoclonal Antibody (AbCam)	Anti-Human CD34 FITC (ImmunoTools, 21270343)
Secondary Antibodies	Mouse IgG1 Isotypic Control PE (ImmunoTools, 21270214)
Alexa Fluor® 488 AffiniPure Donkey Anti-Mouse IgG (H+L) (Jackson ImmunoResearch Laboratories, USA)	Anti-Human CD31 PE (ImmunoTools, 21270314)
Alexa Fluor® 488 AffiniPure Donkey Anti-Rabbit IgG (H+L) (Jackson ImmunoResearch Laboratories, USA)	Anti-Human CD34 PE (ImmunoTools, 21620344)

Figure 3.2. *1) Antibodies used for Immunofluorescence. 2) Antibodies used for FACS Analysis.*

4. RESULTS

The host laboratory has developed and established a method to generate ECs from a starting pluripotent population, as shown in Figure 4.1. hESCs are first differentiated to mesodermal intermediates and then towards CD34+ VPCs under feeder-free, chemically defined conditions. Briefly, H1 colonies are first dissociated into small clumps and replated onto Matrigel coated six-well plates. After 48 h, mTeSR medium is changed to differentiation medium (APEL), supplemented with 5 μM glycogen synthase kinase-3 (GSK-3) inhibitor (CHIR99021, Selleckchem). After 24 h, the medium is replaced with the same basal medium supplemented with bone morphogenetic protein-4 (BMP-4) (25 ng/mL) for 48 h and then with VEGF-A (80 ng/ml) for another 48 h. On day 5, successful commitment to endothelial lineage is confirmed by FACS analysis (Tsolis, K. C., et al., 2016).

With this protocol, each growth factor is added separately, for better control over the differentiation process, offering the possibility to study each step separately, while at the same time acquiring in a fast manner, within 5 days, the population of interest. The differentiation event gives rise to two distinct cellular populations: the CD34+ VPCs and a CD34− population that probably contains mixed cells, which can be further differentiated into other cell types of mesenchymal lineages.

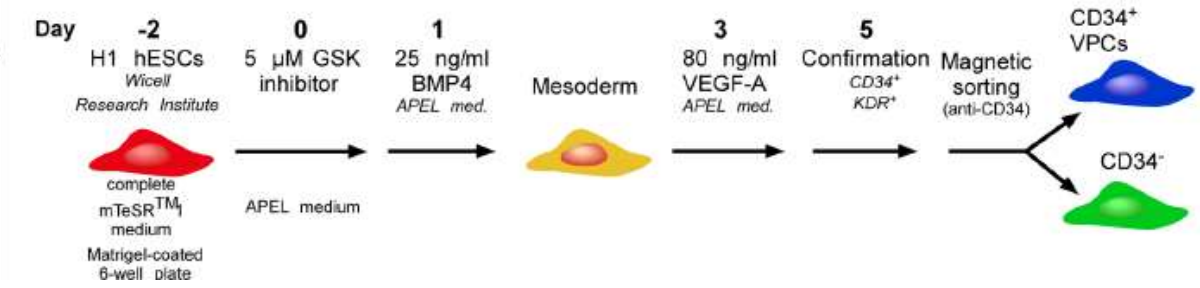

Figure 4.1. *Overview of the established differentiation protocol to ECs used in the laboratory, published by Tsolis K. C. et. l, 2016. The differentiation process, lasting 5 days, is carried out under feeder-free, chemically defined conditions. Starting from a pluripotent population, dissociated in small clumps and replated into matrigel-coated 6-well plates, the cells are successively cultured in culture media that support the differentiation towards ECs on day 5 of differentiation.*

A number of downsides are correlated with the use of the aforementioned differentiation protocol, despite the important advantages mentioned above (such as chemically defined conditions and fast acquisition of population of interest). The aforementioned protocol leads to the generation of low proliferative ECs, with the possibility of keeping the cells up to passage 1-2, has no possibility of cryopreservation. In addition, magnetic sorting is required to sort the mixed population acquired on day five, and it ultimately has a moderate differentiation efficiency (20-30%). For this reason, we selected and tested a number of published differentiation protocols using either serum free or reduced serum conditions.

In order to have a comparison of the differentiation protocols tested in the current thesis with the established protocol in the laboratory (Tsolis K. C., et al., 2016), the established differentiation protocol was performed. Anti-CD34 and CD31 antibodies, used throughout this thesis to determine differentiation efficiency, were first tested on HUVEC cells isolated as described in Materials and Methods (see Supplementary Figure 1). Having established that the antibodies were functional we proceeded with the experiments in stem cells. H1 hESCs were plated in matrigel-coated 12-well plates and the differentiation carried out as shown in Figure 4.1. The population acquired on day five of the differentiation process was stained with CD34 PE antibodies and subjected to FACS analysis compared to isotypic control antibody (Figure 4.2). FACS analysis revealed that the $CD34^+$ endothelial cell population derived from H1 hESCs was 23.5% (Figure 4.2, histogram 2).

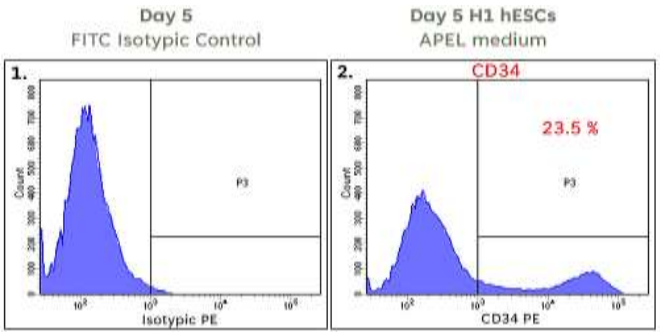

Figure 4.2. FACS analysis performed on the H1-hESC-derived endothelial cell population on day 5 of the differentiation process, stained with anti-CD34 PE or isotypic control antibodies. Histogram 1 shows PE isotypic control and histogram 2 shows the CD34 expression of H1 hESCs-derived ECs. The experiment was performed and kindly shared by Maria Markou, post-doctoral member of the laboratory.

4.1 Comparison to the Differentiation Protocol Published by Patch et al.. 2015

The protocol of differentiation to ECs published by Patch and coworkers (Patch, C., et al.., 2015) was selected due to the following advantages: (1) serum free conditions, allowing the culturing of cells in defined medium, (2) the high differentiation efficiency (66 – 88 % depending on the PSC line used), (3) the high proliferative ECs produced (cultured up to passage 4 – 6), and (4) the possibility of cryopreservation of the ECs, which is essential for consistent reproducible results.

4.1.1 Description of the Patch et al.. Protocol - Parameters Affecting Yield

This protocol, depicted in Figure 4.3, involves culturing hPSCs for 4 days as a monolayer in chemically defined medium supplemented with a GSK3β inhibitor and BMP4 inducing differentiation of the cell population to the mesodermal state. Cells are then cultured for 2 days in a different medium containing VEGF, supplemented with forskolin, which is a potent activator of adenylyl cyclases, modulating angiogenesis (Namkoong, S., et al., 2009). On day six, EC differentiation was evaluated by analyzing the expression of endothelial cell markers (CD31, CD34).

Several parameters affect the final yield of ECs including (1) initial cell density, (2) concentration of GSK3 inhibitor, CHIR and (3) PSC line used. We tested each of these as follows:

1. **Initial cell density:** For the first step of the differentiation process, we plated the hPSCs (H1 hESCs or iPSCs) as single cells at different densities to determine the most efficient starting number of cells (Figure 4.3, Day 0). The suggested cell density was 37.000-47.000 cells/cm^2 (Patch et al. published at Nature Protocol Exchange (doi:10.1038/protex.2015.055), and therefore we tested the lowest and highest cell number from this range for both H1 hESCs and iPSCs.

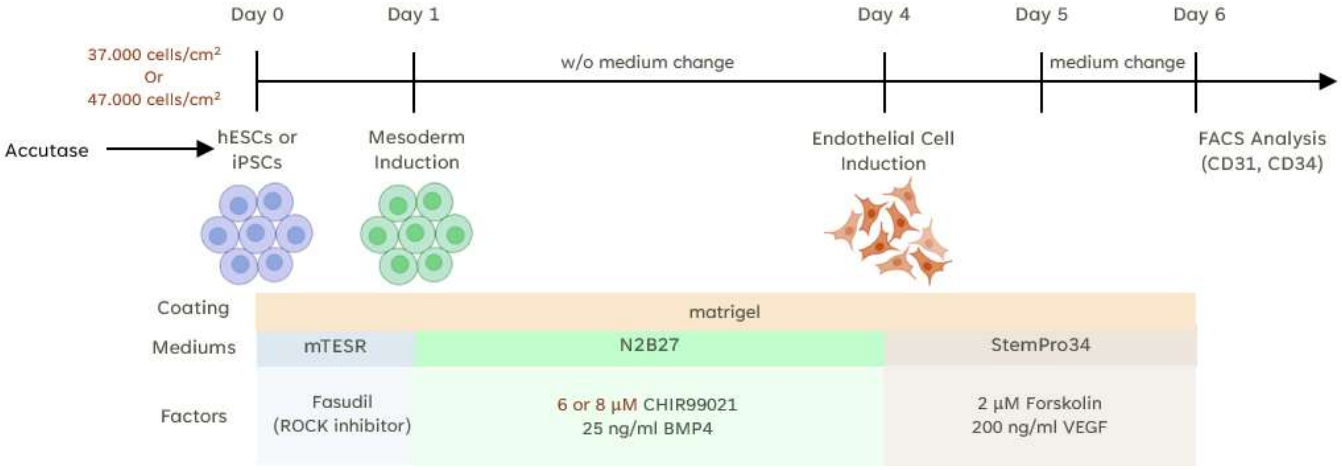

Figure 4.3. *Overview of the differentiation protocol we tested, based on the published protocol by Patch et. l, 2015. The differentiation process, lasting 6 days, starts from a pluripotent population passaged in small clusters enzymatically (accutase) into matrigel-coated 12-well plates, in culture media that successively support the differentiation towards ECs on day 6 of differentiation.*

2. **Concentration of CHIR**: For the second step of the differentiation protocol, cells were differentiated to mesoderm using either CP21 or CHIR and/or BMP4 (Figure 4.3, Day 1). Patch and coworkers suggest that when either GSK3 inhibitor was used at its optimal concentration, the yield of $CD144^+$ cells was equivalent. Based on this, CHIR99021 was used as a GSK3 inhibitor.

   Even though Patch and coworkers suggested that CHIR induced CD144 expression most efficiently at a concentration of 6μM, (Patch, C., et al., 2015), they suggested that some cell lines may require minor changes of the GSK3β inhibitor concentration as well as the seeding density. The recommended CHIR99021 concentration was between 6-8 mM, and thus we tested the lowest and highest concentrations from this range.

3. **PSC line used**: Patch et al. tested this strategy on several hPSC lines, including HUES9, SA001, BJ-RiPS, and a commercially available iPS cell line and promoted the differentiation of hPSCs into ECs with efficiencies between 61.8% and 88.8% as assessed by flow cytometry of $CD144^+$ cells. We tested the differentiation protocol in hiPSCs as well as H1 hESCs.

4.1.2 Highest Seeding Density of H1 hESCs and Highest GSK3i Concentration Lead to the Highest Differentiation Efficiency

The differentiation protocol was performed in matrigel-coated wells of 12-well plates, which have a growth area of 3.8 cm^2. H1 hESCs were passaged with Accutase (Figure 4.3, day 1). The cells were counted in a Neubauer chamber, and 140.600 cells for cell density of 37.000 cells/cm^2 and 178.600 cells for 47.000 cells/cm^2 were transferred to 12-well plates. Then, on day one of differentiation, 6 mM or 8 mM CHIR99021 were added to both 37.000 and 47.000 cells/cm^2 cells and the protocol was followed as described. The ECs acquired on day six of the differentiation process were stained with CD31 PE and CD34 FITC antibodies and subjected to FACS analysis compared to isotypic control antibodies (Figure 4.4, day 6, Induction).

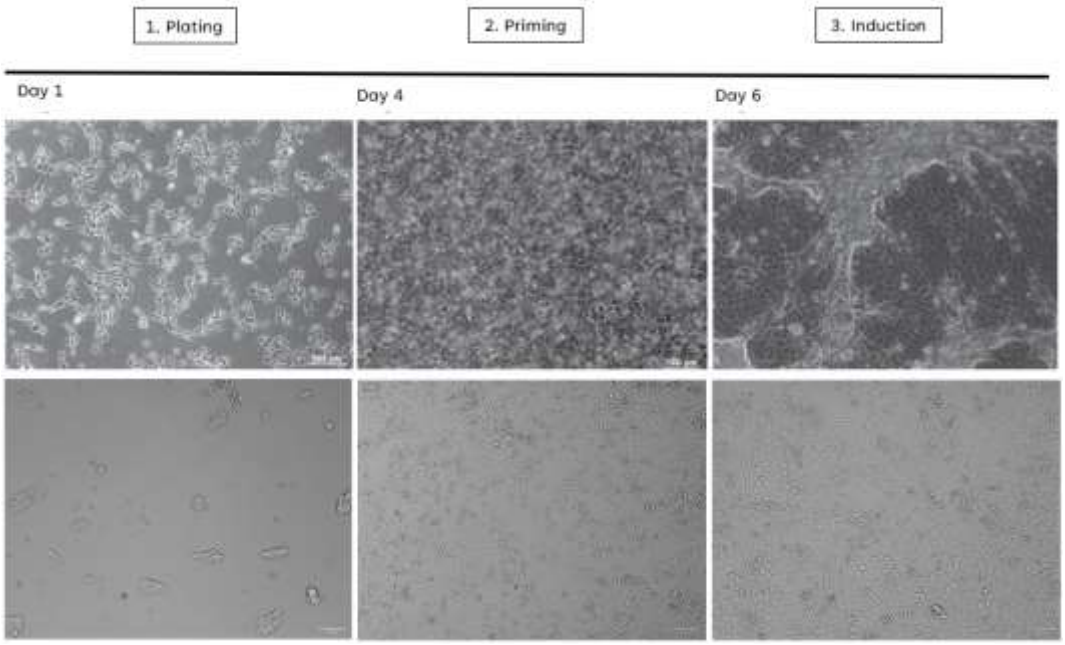

Figure 4.4. *TIRF microscopy imaging depicting the different stages the cells undergo from pluripotent state (iPSCs) to endothelial cell differentiation during the 6 days of the differentiation process for the Patch et al. protocol. The upper panel shows the imaging from the Patch et al. protocol with iPSCs as a starting population and the lower panel shows the imaging acquired in this project with a starting population of H1 hESCs. Plating occurs on day 1, when pluripotent cells are passaged and plated on matrigel-coated plates. Priming occurs on day 4 when the pluripotent population is exposed to factors leading to mesodermal population. Induction refers to the induction of ECs, which are acquired on day 6. Upper panel scale bars: 200 mm, Lower panel scale bars: 100 mm. Representative images of 2 different experiments.*

FACS analysis revealed that with a cell density of 37.000 cells/cm^2 and CHIR99021 concentration of 6 mM, the CD34+ endothelial cell population was 23.8% and the CD31+ was 14.4% (Figure 4.5A, histograms 2 and 4), while with 8 mM CHIR99021 concentration CD34+ cells were at 36.8% and CD31+ cells at 22.1% (Figure 4.5B, histograms 2 and 4). With a cell density of 47.000 cells/cm^2 and a CHIR99021 concentration of 6 mM, the CD34+ cells were 30.7% and the CD31+ cells 18.9% (Figure 4.5C, histograms 2 and 4), while with 8 mM CHIR99021 concentration, the CD34+ cells were 38.3% and CD31+ cells were 27.2% (Figure 4.5D, histograms 2 and 4). These results suggest that the highest differentiation efficiency is achieved with the highest seeding density (47.000 cells/cm^2) and the highest concentration of CHIR99021 GSK3 inhibitor (8 mM) (Figure 4.5D).

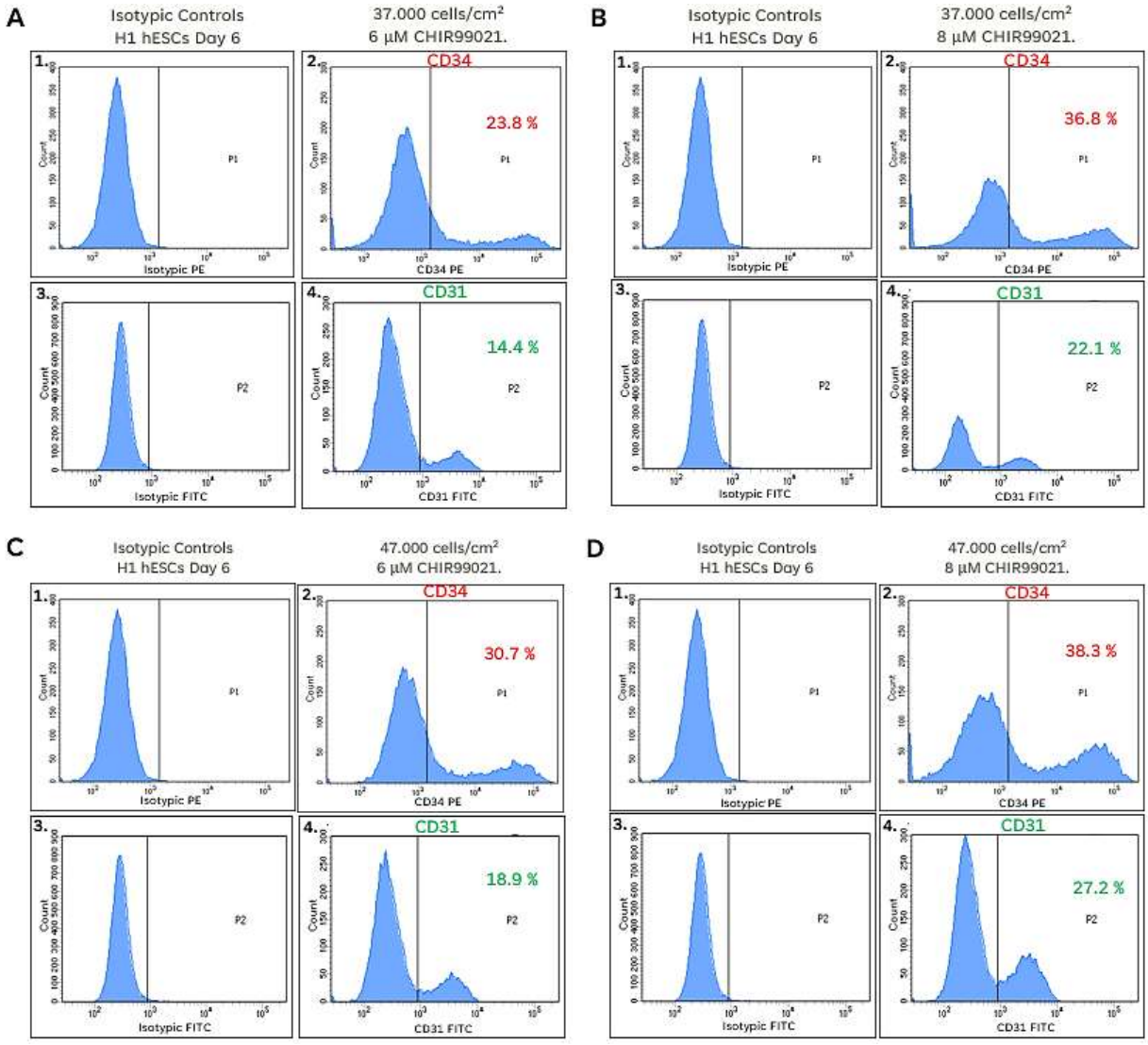

Figure 4.5. *Graphs from FACS analysis performed on the H1-hESC-derived endothelial cell population on day 6 of the differentiation process, stained with anti-CD31-FITC and anti-CD34 PE or isotypic control antibodies. (A) 37.000 cells/cm^2 cell density of H1 hESCs starting population and*

6 mM CHIR99021 concentration, (B) 37.000 cells/cm^2 H1 hESCs density and 8 mM CHIR99021 concentration, (C) 47.000 cells/cm^2 H1 hESCs density and 6 mM CHIR99021 concentration, (D) 47.000 cells/cm^2 and 8 mM CHIR99021 concentration. The first column of each graph shows histograms 1 and 3 for the isotypic PE and FITC controls and the second column of each graph shows histograms 2 and 4 for the CD34 and CD31 expression, respectively. of the 37.000 cells/cm^2 with 6 mM CHIR99021. Representative graphs are shown of 2 experiments for 2 different wells per condition.

From the results presented in Figure 4.5D we concluded that the most efficient conditions for the H1 hESCs were a seeding density of 47.000 cells/cm^2 and CHIR99021 concentration of 8 mM, yielding the highest levels of expression of both CD31 and CD34 (Figure 4.5D, histograms 3 and 6). The protocol was repeated on different batches of H1 hESCs to verify the differentiation efficiency. 47.000 cells/cm^2 and CHIR99021 (8 mM) were used. The results on day six of differentiation were CD31$^+$ 20.8% and CD34$^+$ 28.2% (Figure 4.6, histograms 2 and 3), which was similar to the experiment shown in Figure 4.5D (histograms 3 and 6).

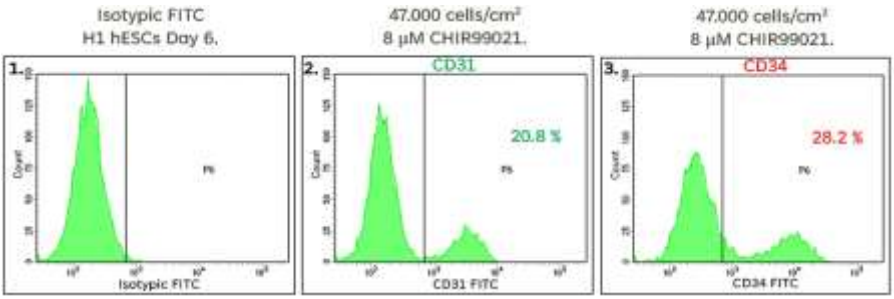

Figure 4.6. *FACS analysis performed on the H1-hESC-derived endothelial cell population on day 6 of the differentiation process. H1 cells were seeded at a density at 47.000 cells/cm^2 and 8 mM CHIR99021 concentration used. ECs on day 6 were stained with CD31 FITC, CD34 FITC and isotypic FITC antibodies and FACS analysis was performed. Histogram 1 shows the isotypic control strained H1 hESC-derived ECs, histogram 2 shows the CD31 expression for the 47.000 cells/cm^2 with 8 mM CHIR99021 and histogram 3 shows the CD34 expression for the 47.000 cells/cm^2 with 8 mM CHIR99021 for CD34 expression. Representative graphs are shown of 1 experiment for 3 different wells.*

4.1.3 Highest Seeding Density of iPSCs and Highest GSK3i Concentration Lead to a Low Differentiation Efficiency

Since the differentiation efficiency we acquired following this protocol was not equivalent to the differentiation efficiency suggested by Patch et al (66 – 88%, depending on the cell

line), the protocol was tested on a different cell line, iPSCs, which were derived from patients with **leucine-rich repeat kinase 2 (*LRRK2*) mutation** Parkinson's Syndrome (iPSC IM2). For this, the highest seeding density (47.000 cells/cm^2) was tested to begin the differentiation process in matrigel-coated 12-well plates, as well as the highest concentration of GSK3 inhibitor CHIR99021 (8 μM) on day one of differentiation. The ECs acquired on day six of differentiation were stained with CD31 and CD34 FITC antibodies and subjected to FACS analysis. The results revealed that with a cell density of 47.000 cells/cm^2 and 8 μM the CD31$^+$ cells were at 16.3% and the CD34$^+$ cells were at 21.8% (Figure 4.7, histograms 2 and 3).

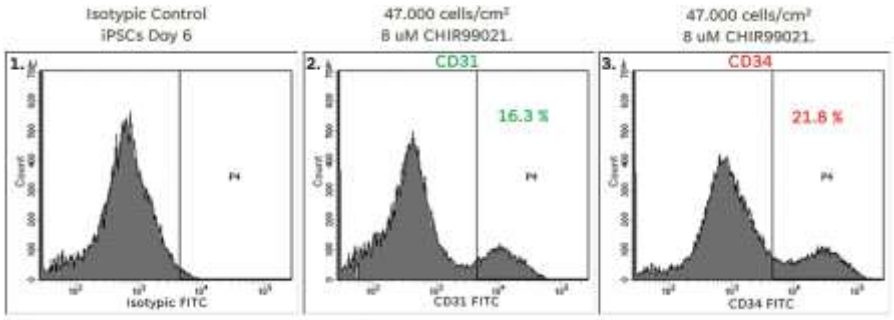

Figure 4.7. *FACS analysis performed on the iPSC-derived endothelial cell population on day 6 of the differentiation process. iPSCs were seeded at a density at 47.000 cells/cm^2 and 8 mM CHIR99021 concentration used. ECs on day 6 were stained with CD31 FITC, CD34 FITC and isotypic FITC antibodies and FACS analysis was performed. Histogram 1 shows the isotypic control strained iPSC-derived ECs, histogram 2 shows the CD31 expression for the 47.000 cells/cm^2 with 8 mM CHIR99021 and histogram 3 shows the CD34 expression for the 47.000 cells/cm^2 with 8 mM CHIR99021 for CD34 expression. Representative graphs are shown of 3 independent experiments for 2 different wells.*

Conclusion: The differentiation efficiency we acquired following the Patch protocol was between 28-38% of CD34$^+$ cells using H1 hESCs and at around 21.8% of CD34$^+$ using iPSCs, which are not equivalent to the differentiation efficiency suggested by Patch et al (66 – 88%). Additionally, compared to the already established protocol of differentiation to ECs, there was not much of an improvement in the differentiation efficiency (Figure 4.2, 23.5% of CD34$^+$).

These results suggest that this differentiation protocol is not ideal for the laboratory, since it is not as efficient as the suggested protocol in two of the cell lines used and not an improvement to the currently used protocol.

4.2 Comparison to the Differentiation Protocol Published by Harding et al.. 2017

This protocol of differentiation to ECs was published by Harding and coworkers (Harding et al., 2017). The protocol has a high differentiation efficiency (73 – 83% on day 8 of differentiation, depending on the cell line), with no requirement for cell sorting or magnetic purification to yield a very pure population.

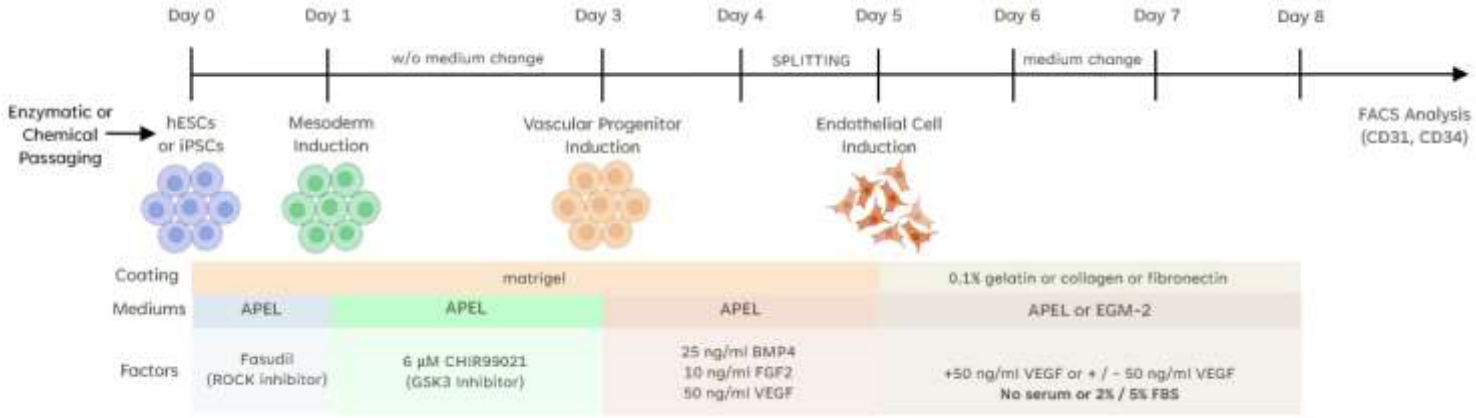

Figure 4.8. *Overview of the differentiation protocol published by Harding et. l, 2017. The differentiation process, lasting 8 days, starts from a pluripotent population passaged in small clusters enzymatically (accutase or dispase) or chemically (versene) into matrigel-coated 12-well plates, in culture mediums that successively support the differentiation towards vascular progenitor cells on day 5. The cells are then detached enzymatically (accutase) and placed on different coated plates (0.1% gelatin, collagen or fibronectin) with different mediums (APEL or EGM-2) supporting the differentiation to ECs on day 8 of the differentiation process*.

4.2.1 Description of the Harding et al. Protocol

Differentiation of ECs from hiPSCs and hESCs was progressively induced in three stages, see Figure 4.8, for an overview of the differentiation protocol. Briefly, hiPSC or hESCs cells were manually passaged as small clusters onto Matrigel-coated culture plates in Mouse Embryonic Fibroblast (MEF) conditioned hESC medium with an additional 10 ng/ml of FGF2. After 1 day, the medium was changed to StemDiff APEL medium (STEMCELL Technologies, Cambridge, MA) with 6 μM of CHIR99021 for 2 days to acquire the mesodermal population.

Harding et al. then cultured the derived mesodermal cells in APEL medium supplemented with 25 ng/ml BMP4, 10 ng/ml FGF2 and 50 ng/ml VEGF for 2 days to acquire the VPCs. Cells were then passaged using accutase on day 4 and seeded onto p100 culture dishes at 5

$\times 10^3 - 1 \times 10^4$ cells per cm^2 in EC Growth Medium MV2 (ECGM-MV2, PromoCell) with an additional 50 ng/ml VEGF, or APEL medium, and the medium was changed every 2 days for 4-6 days to generate ECs.

Harding et al. showed that the hiPSC or hESC-derived ECs (hiPSC-ECs or hESC-ECs) on day 8 of differentiation, expressed endothelial lineage markers, CD31/PECAM1 at 94-97%, from which 73%-81% cells were $CD34^+$ and 78%-83% were $CD144^+$/VE-Cadherin. These cells also expressed a mature EC marker, Von Willebrand factor (vWF), as revealed by immunostaining. Passaging the population of ECs, led to the loss of CD34 expression, but yielded a nearly pure population of ECs (99.7% of $CD31^+$ and 96.8% $CD144^+$).

4.2.2 H1 hESCs Passaged with Dispase and Vascular Progenitor Cells Seeded on 0.1% Gelatin – Testing of Different Endothelial Cell Culture Mediums

To follow the differentiation protocol outlined above we needed to clarify several issues: (1) Harding et al. did not describe the type of manual passaging preferred for the generation of small clusters of hiPSC or hESC cells to start the differentiation protocol, so we tested dispase and versine and (2) The type of coating was not specified at the stage when the vascular progenitors were acquired, but rather we suggested 3 options, gelatin, collagen or fibronectin. Papers implementing this protocol on their differentiation process suggested either the use of 0.5 – 1% gelatin (Lee I.W., et al., 2023, Deinsberger, J., et al., 2023), or the use of collagen type IV (Karagiannis et al., 2024). Vascular and endothelial progenitor cells can be cultured on fibronectin-coated plates, with fibronectin promoting the appearance of endothelial cell forming colonies earlier than collagen (Colombo E., et al., 2013). Therefore, we decided to test also fibronectin.

We first tested the Harding et al. protocol on H1 hESCs. Starting the differentiation process, the pluripotent population of H1 hESCs was passaged enzymatically with dispase and small clusters were plated onto matrigel-coated 12-well plates in a splitting ratio of 1:7 (Figure 4.8, day 1 of differentiation protocol). The protocol was then followed as suggested, and, on day 5 of differentiation, the vascular progenitors (Figure 4.9, days 2 and 4 of differentiation protocol) were passaged with accutase and seeded onto 12-well plates coated with 0.1% gelatin, at a cell density of 38.000 cells/cm^2. Two types of media were used on day 5 of

differentiation (Figure 4.8, day 5 of differentiation protocol), EGM-2 medium or APEL medium (Figure 3.1, materials and methods, composition of media).

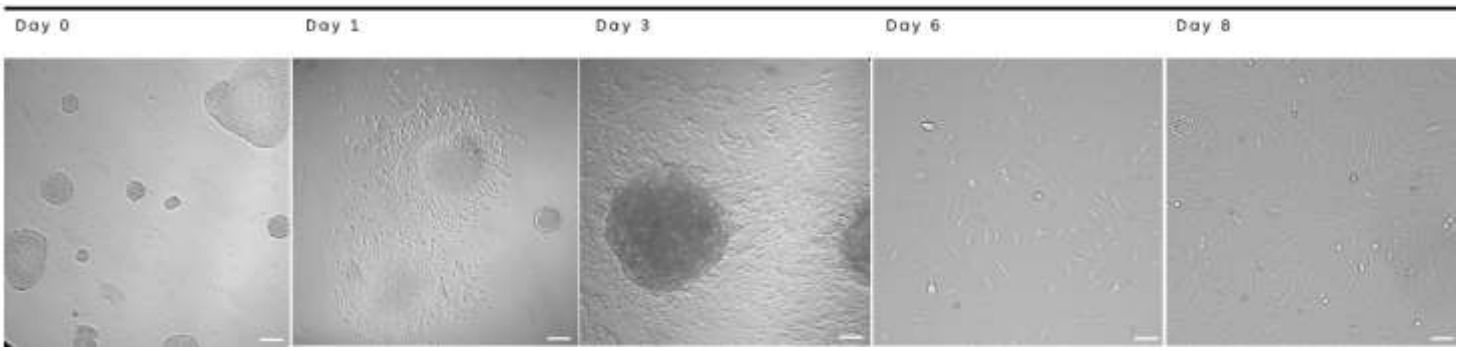

Figure 4.9. *TIRF microscopy imaging depicting the different stages the cells undergo from pluripotent state to endothelial cell differentiation during the 8 days of the differentiation process for the Harding et al. protocol. On Day 0 the pluripotent cells were plated on a matrigel-coated plate in small clusters. Day 1 shows the effect GSK3 inhibitor CHIR99021 has on the cell clusters, with single cells translocating towards the periphery of the center. Day 3 shows the increasing number of cells in the periphery, which are the cells that need to be dissociated and replated onto new plates. Days 6 and 8 show the endothelial cell population. Scale bars: 100 mm. Representative images are shown of 2 independent experiments.*

4.2.3 Culturing hESCs-ECs in APEL Medium Serum Free Conditions Leads to a High Differentiation Efficiency with Major Drawbacks

First, to achieve serum-free conditions throughout the differentiation process and since the serum is added on day 5 of differentiation, cells were cultured on day 5 onwards on APEL medium, a serum free medium. Since APEL medium's composition does not include VEGF (Figure 3.1, materials and methods, composition of mediums) in the first test APEL medium was used supplemented with 50 ng/ml VEGF and ECs were cultured until day 8 (Figure 4.8 and Figure 4.9, day 8 of differentiation protocol), when the cells were stained with CD31 FITC and CD34 PE antibodies and a FACS analysis was performed. The results indicated that ECs from H1 hESCs passaged with dispase, cultured on 0.1% gelatin-coated plates and in APEL medium supplemented with 50 ng/ml VEGF were $CD31^+$ at 45.3% and $CD34^+$ at 69.1% respectively (Figure 4.10, histograms 2 and 4).

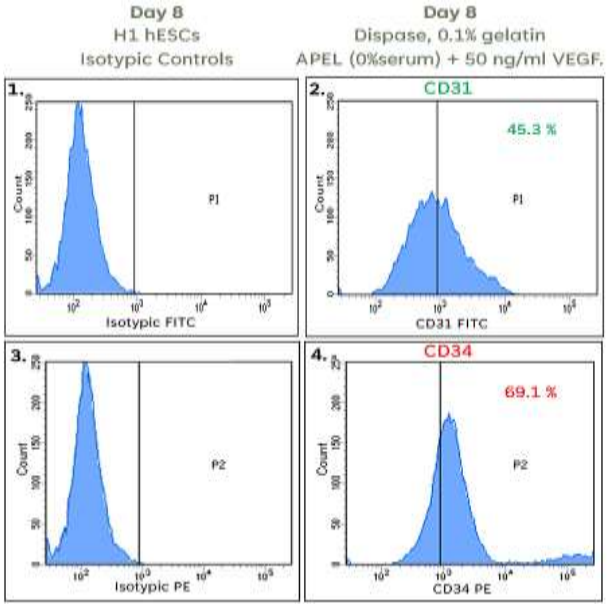

Figure 4.10. *Histograms from FACS analysis performed on the H1-hESC-derived endothelial cell population on day 8 of the differentiation process. In this experiment, the starting pluripotent population was passaged enzymatically with dispase, and the vascular progenitor cells were plated on 0.1% gelatin-coated plates. Endothelial cell culture medium (APEL) and with the addition of 50 ng/ml VEGF (APEL with VEGF) was used to culture the cells. ECs on day 8 were stained with CD31 FITC and CD34 PE antibodies and FACS analysis was performed. The first row shows histograms for the CD31 FITC antibody, and the second row shows histograms for the CD34 PE antibody. The first column (histograms 1 and 3) is for the isotypic FITC (histogram 1) and for the isotypic PE (histogram 3) H1 hESCs controls. The second column (histograms 2 and 4) is for the APEL medium supplemented with 50 ng/ml VEGF and the expressions of CD31 (histogram 2) and CD34 (histogram 4). Representative graphs shown from 1 experiment for 2 different wells.*

Although ECs from H1 hESCs passaged with dispase, cultured on 0.1% gelatin-coated plates and APEL medium supplemented with 50 ng/ml VEGF yielded a high percentage of CD31 and CD34 expression (Figure 4.10, histograms 2 and 4), a problem arose regarding the cells' viability. The cells were low proliferative after passage 1 and couldn't survive in the culture medium, thus limiting the use of these cells for further experiments, which require passages up to 3 and 4. For this reason, the APEL medium supplemented with VEGF was not further used for the culture of ECs from day 5 onwards.

4.2.4 Culturing hESCs-ECs in EGM-2 Medium Without Additional VEGF for Low Serum Conditions Leads to a Low Differentiation Efficiency

Based on the aforementioned result regarding the APEL medium, EGM-2 medium, an endothelial cell growth medium, similar in composition to ECGM-2 medium suggested by the protocol, with 2% serum instead of 5% contained in ECGM-2 (Figure 3.1, materials and methods, compositions of mediums) was used. EGM-2 medium was first tested without the addition of VEGF, since the medium already contains VEGF (0.5 ng/ml) (Figure 3.1, materials and methods, composition of media). ECs were cultured until day 8 (Figure 4.8 and Figure 4.9, day 8 of differentiation protocol), when the cells were stained with CD31 FITC and CD34 PE antibodies and a FACS analysis was performed.

The results indicated that ECs from H1 hESCs passaged with dispase, cultured on 0.1% gelatin and EGM-2 medium without the addition of extra VEGF, were $CD31^+$ at 23.1% and $CD34^+$ at 26.2%, (Figure 4.11, histograms 2 and 4).

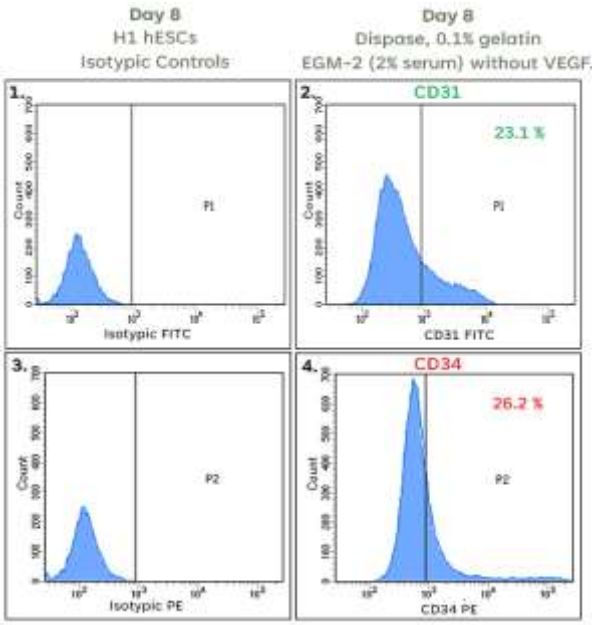

Figure 4.11. *Histograms from FACS analysis performed on the endothelial cell population on day 8 of the differentiation process. The fluorescent marker FITC was used to detect CD31 expression levels and PE was used to detect CD34 expression levels. In this experiment, the starting pluripotent population was passaged enzymatically with dispase, and the vascular progenitor cells were plated on 0.1% gelatin-coated plates. Here one of the three different conditions were tested for two parameters: endothelial cell culture medium (EGM-2, 2% serum) and without the addition of 50 ng/ml VEGF (EGM-2, 2% serum, without VEGF). ECs on day 8 were stained with CD31 FITC and CD34 PE antibodies and FACS analysis was performed. The first row shows histograms for the CD31*

FITC antibody, and the second row shows histograms for the CD34 PE antibody. The first column (histograms 1 and 3) is for the isotypic FITC (histogram 1) and for the isotypic PE (histogram 3) H1 hESCs controls. The second column (histograms 2 and 4) is for the EGM-2 medium, 2% serum, without VEGF and the expressions of CD31 (histogram 2) and CD34 (histogram 4). Representative graphs are shown of 1 experiment for 2 different wells.

ECs derived from H1 hESCs cultured on 0.1% gelatin-coated plates, with EGM-2 medium (2% serum) without 50 ng/ml VEGF were cultured up to passage 1, and subjected to immunofluorescent staining for CD31, VE-Cadherin and vWF. These stainings revealed that the H1 hESC-ECs cultured on EGM-2 medium (2% serum) without extra VEGF had very low expression of CD31 marker, which was seen in very few, clustered areas of ECs (Figure 4.12, images 2 and 5, 3 and 6, Figure 4.13 images 3 and 7, 4 and 8). This immunofluorescent staining is in agreement with the FACS results (Figure 4.11, histogram 2). As well as having a low expression of CD31 marker, these cells exhibited very low signal of vWF expression factor (Figure 4.13, images 2 and 6, 4 and 8).

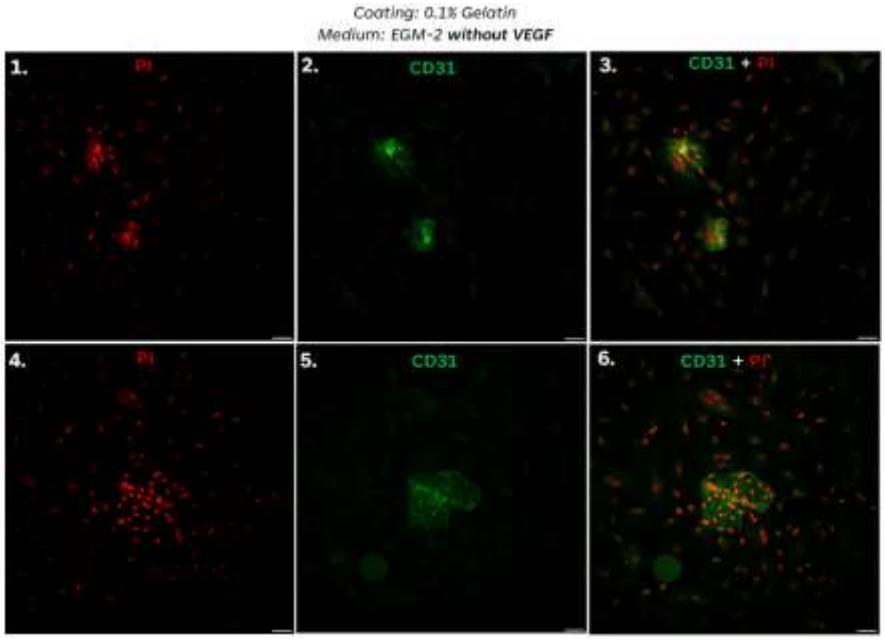

Figure 4.12. *Immunostaining of EC-specific markers on H1 hESC derived-ECs. Images 1 and 4 are for PI staining, images 2 and 5 are for CD31 staining and images 3 and 6 are for merge images for H1-hESC-ECs cultured on 0.1% gelatin-coated plates with EGM-2 medium (2% serum) without VEGF. Scale bars: 100 μM. Representative images are shown of 1 experiment.*

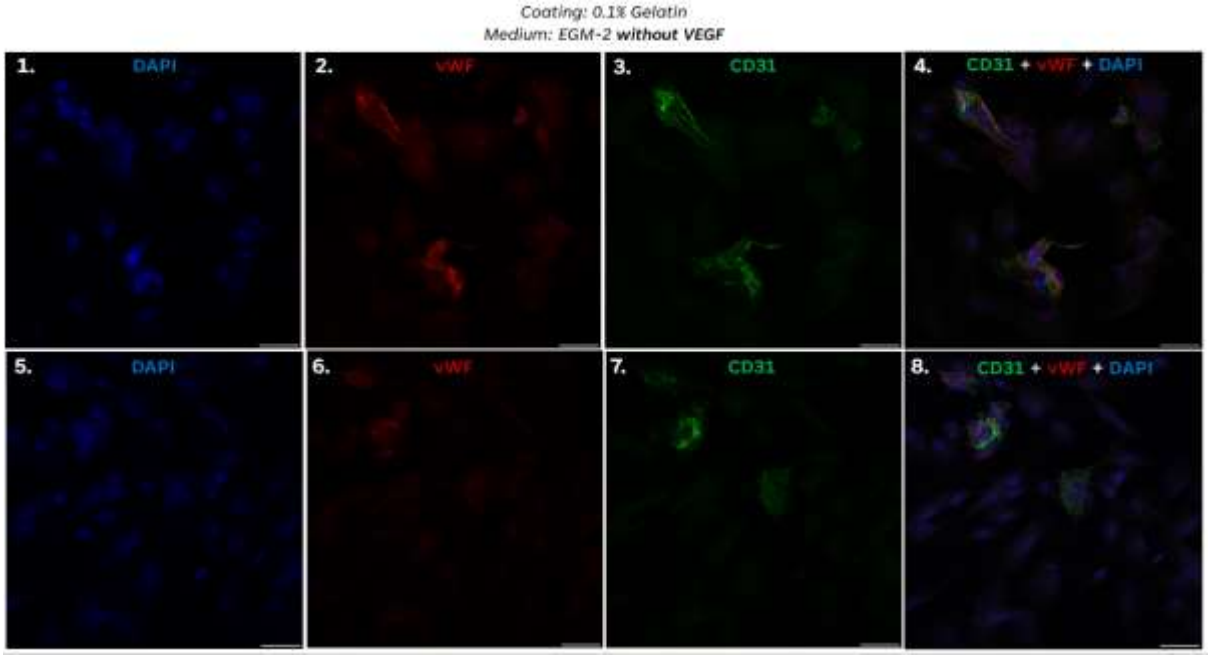

Figure 4.13. *Immunostaining of EC-specific markers on H1 hESC derived-ECs. Cells were fixed and processed for indirect immunofluorescence as outlined in materials and Methods. Images 1 and 2 are stained with DAPI, images 2 and 6 show vWF staining, images 3 and 4 show CD31 staining and images 4 and 8 are merged images for H1-hESC-ECs cultured on 0.1% gelatin-coated plates with EGM-2 medium (2% serum) without extra VEGF. Scale bars: 100 μM. Representative images are shown of 1 experiment.*

4.2.5 Culturing hESCs-ECs in EGM-2 Medium With extra VEGF for Low Serum Conditions Leads to a High Differentiation Efficiency

Since the addition of VEGF improves the differentiation efficiency and its external addition to the endothelial cell culture medium is suggested according to the protocol on day 5 of the differentiation process, the EGM-2 medium with 2% serum was tested with the addition of 50 ng/ml VEGF. ECs were cultured until day 8 (Figure 4.8 and Figure 4.9, day 8 of differentiation protocol), when the cells were stained with CD31 FITC and CD34 PE antibodies and a FACS analysis was performed.

The results indicated that ECs from H1 hESCs passaged with dispase, cultured on 0.1% gelatin-coated plates and EGM-2 medium supplemented with 50 ng/ml VEGF were $CD31^+$ at 31.3% and $CD34^+$ at 53% respectively (Figure 4.14, histograms 2 and 4). Thus, we confirmed that the addition of 50 ng/ml VEGF led to an increase in the expression of markers CD31 from 23.1% (Figure 4.11, histogram 2) to 31.9% (Figure 4.14, histogram 2).

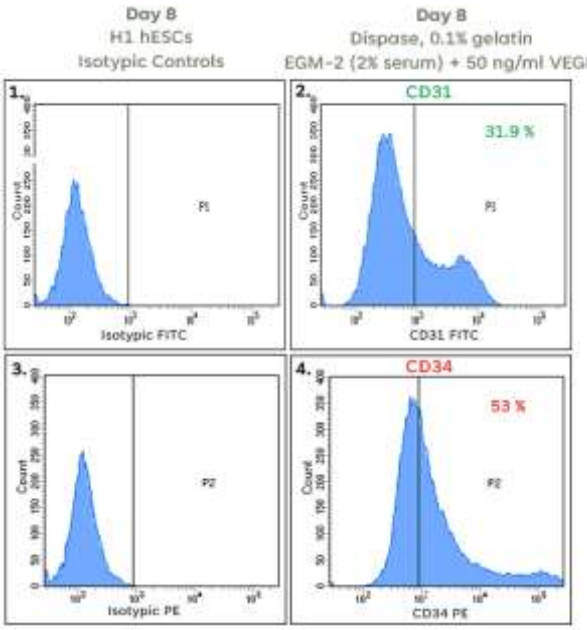

Figure 4.14. *Histograms from FACS analysis performed on the endothelial cell population on day 8 of the differentiation process. In this experiment, the starting pluripotent population was passaged enzymatically with dispase, and the vascular progenitor cells were plated on 0.1% gelatin-coated plates. EGM-2, 2% serum plus the addition of 50 ng/ml VEGF (EGM-2, 2% serum, with 50 ng/ml VEGF) was used. ECs on day 8 were stained with CD31 FITC and CD34 PE antibodies and FACS analysis was performed. The first row shows histograms for the CD31 FITC antibody, and the second row shows histograms for the CD34 PE antibody. The first column (histograms 1 and 3) is for the isotypic FITC (histogram 1) and for the isotypic PE (histogram 3) H1 hESCs controls. The second column (histograms 2 and 4) is for the EGM-2 medium, 2% serum, supplemented with 50 ng/ml VEGF and the expressions of CD31 (histogram 2) and CD34 (histogram 4). Representative graphs are shown of 2 different experiments for 2 different wells per condition.*

ECs derived from H1 hESCs cultured on 0.1% gelatin-coated plates, with EGM-2 medium (2% serum) with the addition of 50 ng/ml VEGF were cultured up to passage 1, and subjected to immunofluorescent staining for CD31, VE-Cadherin and vWF. These stainings revealed that the H1 hESC-ECs cultured on EGM-2 medium (2% serum) supplemented with 50 ng/ml VEGF were positive for CD31 marker of ECs (Figure 4.15, images 2 and 4, 3 and 6) in a manner that coincided with the FACS analysis results (Figure 4.14, histogram 2), At the same time it was observed that H1 hESC-ECs positive for CD31 also expressed vWF (Figure 4.16, images 2 and 4, 6 and 8).

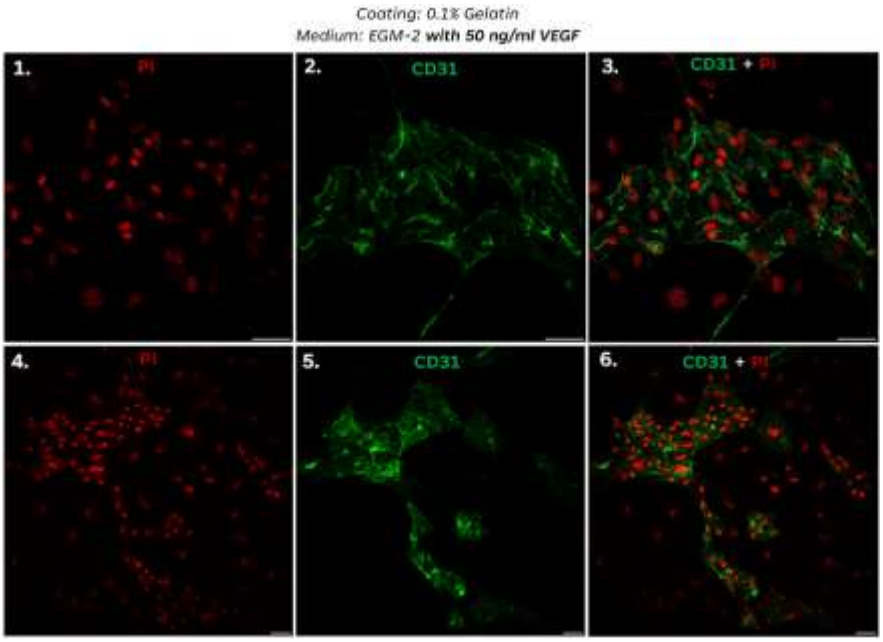

Figure 4.15. *Immunostaining of EC-specific markers on H1 hESC derived-ECs. Cells were fixed and processed for indirect immunofluorescence as outlined in materials and Methods. Images 1 and 2 are for PI staining, images 2 and 5 are for CD31 staining and images 3 and 6 are for merge images for H1-hESC-ECs cultured on 0.1% gelatin-coated plates with EGM-2 medium (2% serum) with 50 ng/ml VEGF. Scale bars: 100 μM. Representative images are shown of 1 experiment.*

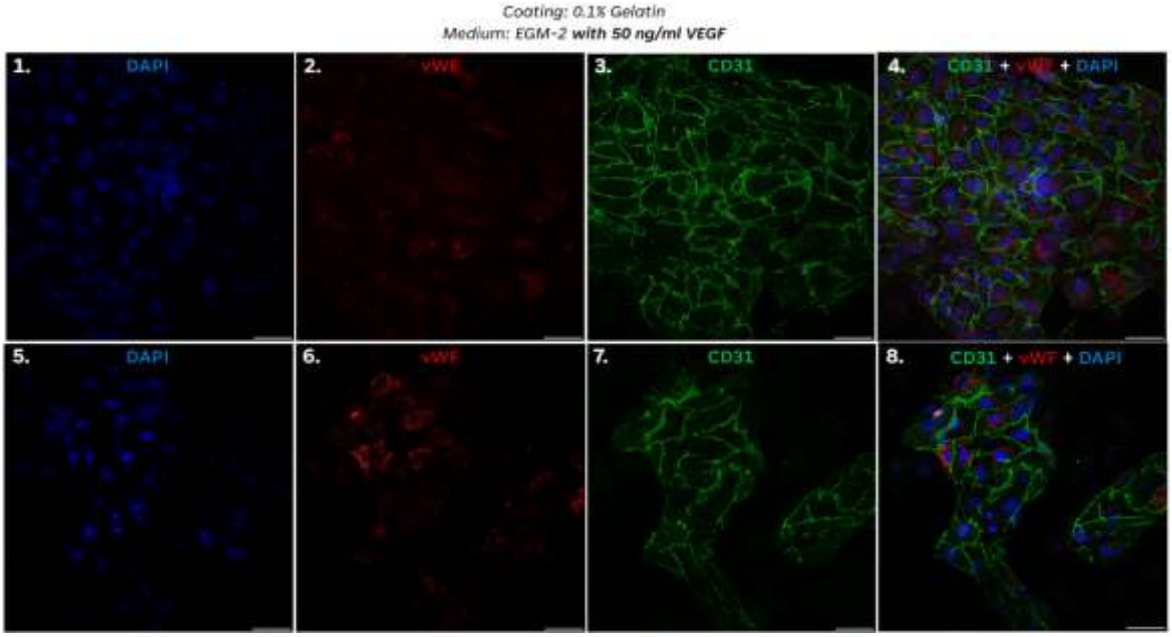

Figure 4.16. *Immunostaining of EC-specific markers on H1 hESC derived-ECs; vWF=Von Willebrand factor. Cells were fixed and processed for indirect immunofluorescence as outlined in materials and Methods. Images 1 and 5 are DAPI staining, images 2 and 6 are vWF staining and images 3 and 7 are CD31 staining and images 4 and 8 are for merge images for H1-hESC-ECs cultured on 0.1% gelatin-coated plates with EGM-2 medium (2% serum) with 50 ng/ml VEGF. Scale bars: 100 μM. Representative images are shown of 1 experiment.*

4.2.6 Culturing hESCs-ECs in EGM-2 Medium with VEGF and Addition of Serum Further Increases the Differentiation Efficiency

Since the Harding et al. protocol uses ECGM-2 endothelial growth medium, which contains 5% serum, as opposed to 2% serum contained in EGM-2 medium used so far for the differentiation in this project, another test was performed to evaluate the effect the additional serum had on the differentiation efficiency and survival of the acquired endothelial cell population. For this purpose, the differentiation protocol was performed on a starting population of H1 hESCs, which were passaged enzymatically with dispase as small clusters onto matrigel-coated 12-well plates. The protocol was followed as suggested and, on day 5 of differentiation, the vascular progenitors were dissociated with accutase and seeded onto 12-well plates coated with fibronectin, at a cell density of 38.000 cells/cm^2, with EGM-2 medium, supplemented with 50 ng/ml VEGF and additional FBS serum, for a total of 5% serum. ECs were cultured until day 8, when the cells were stained with CD31 FITC antibody and a FACS analysis was performed. The results indicated that ECs from H1 hESCs passaged with dispase, cultured on fibronectin and EGM-2 medium supplemented with 50 ng/ml VEGF and 3% serum (for a total of 5% serum) were CD31$^+$ at 57.4% (Figure 4.17, histogram 3).

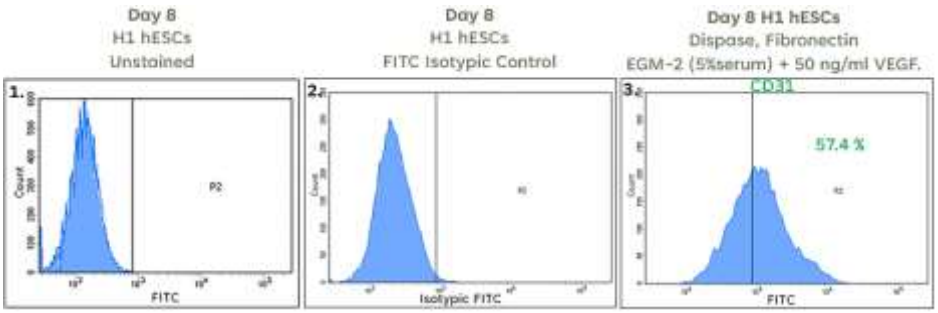

Figure 4.17. *Histograms from FACS analysis performed on the endothelial cell population on day 8 of the differentiation process. The fluorescent marker FITC was used to detect CD31 expression levels. In this experiment, performed on H1 hESCs, one condition was tested for three parameters: starting population passaged enzymatically (dispase), ECs cultured on plates coated with fibronectin and EGM-2 medium supplemented with serum (5% total serum) and 50 ng/ml VEGF. ECs on day 8 were stained with CD31 FITC and FACS analysis was performed. Histogram 1 is for the unstained H1 hESCs used as a control, histogram 2 for the H1 hESCs FITC isotypic control and histogram 3 for the CD31 expression. Representative graphs are shown of 1 experiment for 2 different wells.*

4.2.7 Culturing hiPSCs-ECs in EGM-2 Medium with VEGF and Addition of Serum Leads to a Very High Differentiation Efficiency

Since these conditions further improved the differentiation efficiency from 31.9% to 57.4% for $CD31^+$, this differentiation protocol was performed on two different pluripotent cell lines, this time iPSCs derived from patients with Parkinsons' disease, one that contained the **leucine-rich repeat kinase 2 (*LRRK2*)** mutation and one that had the gene corrected, from postdoc Maria Markou. The starting pluripotent population of iPSC IM2 and iPSC GC were passaged enzymatically with dispase as small clusters onto matrigel-coated 12-well plates. On day 5 of differentiation, the vascular progenitors were dissociated with accutase and seeded onto 12-well plates coated with fibronectin, at a cell density of 38.000 cells/cm^2, with EGM-2 medium, supplemented with 50 ng/ml VEGF and additional FBS serum, for a total of 5% serum. ECs were cultured until day 8, when the cells were stained with CD31 FITC antibody and a FACS analysis was performed. The results indicated that ECs from iPSC GC and iPSC IM-2 passaged with dispase, cultured on fibronectin and EGM-2 medium supplemented with 50 ng/ml VEGF and 3% serum (for a total of 5% serum) were $CD31^+$ at 55.8 % and 73.2% respectively (Figure 4.18, histograms 3 and 4).

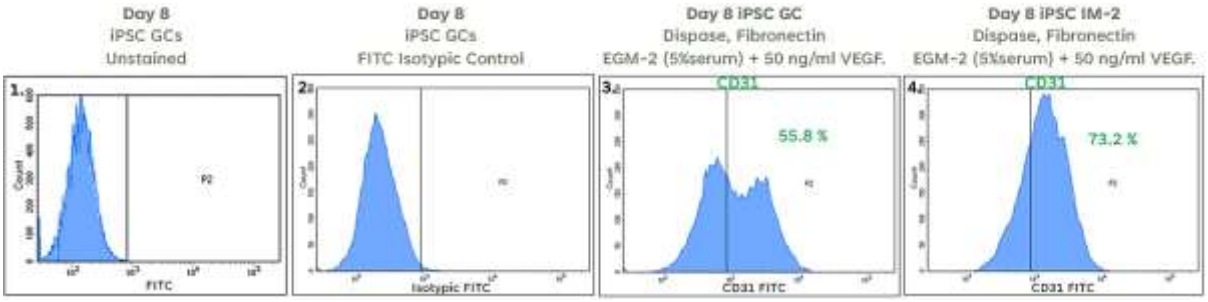

Figure 4.18. *Histograms from FACS analysis performed on the endothelial cell population on day 8 of the differentiation process. In this experiment, performed on iPSC cell lines (GC and IM-2 cell lines), the starting population was passaged with dispase, ECs cultured on plates coated with fibronectin and EGM-2 medium supplemented with serum (5% total serum) and 50 ng/ml VEGF. ECs on day 8 were stained with CD31 FITC and FACS analysis was performed. Histogram 1 is for the unstained iPSC GC cells used as a control, histogram 2 for the iPSC GC cells with FITC isotypic control, histogram 3 for the CD31 expression in iPSC GC cells and histogram 4 for the CD31 expression in iPSC IM-2 cells. Representative graphs are shown of 1 experiment for 2 different wells.*

Testing the differentiation protocol suggested by Harding and his colleagues (Harding, et al., 2017) on different cell lines, such as H1 hESCs (Figure 4.17), iPSCs derived from patients with **leucine-rich repeat kinase 2 (*LRRK2*) mutation** Parkinson's Syndrome (iPSC IM2) or the iPSCs derived from patients with the gene corrected **leucine-rich repeat kinase 2 (*LRRK2*) mutation** Parkinson's Syndrome (iPSC GC) (Figure 4.18), revealed that there is a difference between different cell lines in terms of efficiency. The differentiation efficiency we acquired following this protocol, 57.4% $CD31^+$ for H1 hESC-derived ECs (Figure 4.17, histogram 3), 55.8% $CD31^+$ for iPSC GC-derived ECs (Figure 4.18, histogram 3) and 73.2% $CD31^+$ for iPSC IM2-derived ECs (Figure 4.18, histogram 4) was higher than the differentiation efficiency acquired with the established protocol by Tsolis and his colleagues (Tsolis et al., 2016) used in the laboratory, which was 23.5% $CD34^+$ for H1 hESC-derived ECs (Figure 4.2). These results suggest that this differentiation protocol is ideal for the laboratory, since it is more efficient than the suggested protocol in the H1 hESC line and is efficient in all three of the cell lines tested in the current thesis, H1 hESC, iPSC GC and iPSC IM2.

5. DISCUSSION

Tissue engineering, first used as a term in 1985 by Y.C. Fung, a pioneer in field of biomechanics and bioengineering, is considered an interdisciplinary field that employs aspects of cell biology and transplantation, materials science, and biomedical engineering to develop biological substitutes that can restore and maintain the normal function of damaged tissues and organs. These techniques can include the two most basic components of tissue engineering: functional cells injected into a nonfunctional site to stimulate regeneration, promote vascularisation, and/or supplement the production of hormones and growth factors and/or the use of biocompatible materials, which include both natural and synthetic matrices (commonly called “scaffolds”), to create new tissues and organs.

A major requirement for viability and function of the implantable construct is the availability of blood vessels to support its *in vivo* growth and vascularisation poses an obstacle in engineering thicker, metabolically demanding organs, such as the heart muscle, the brain and the liver, since these engineered tissues exceed the capacity of nutrient supply and waste removal by diffusion requiring an intimate supply of vascular networks (Carmeliet & Jain, Nature 2000; Jain, Science 2005). Constructing pre-vascularised tissue-engineered constructs has been found to overcome the struggles of *in vivo* vascularisation of scaffolds, which takes several weeks to complete, and thus generating blood vessels and their components, perivascular cells (PCs) and endothelial cells (ECs), *in vitro* allows for studies to further comprehend their function and contribution to the vascularisation process.

As described in the current thesis, the host laboratory has developed a method for the differentiation of hESCs/hiPSCs to ECs (Tsolis et al. 2016) using chemically defined conditions (APEL medium) supplemented with growth factors. Whereas the efficiency of the protocol is overall satisfactory, the generated ECs exhibit low efficiency of differentiation (around 25 %), low proliferative capacity and require sorting of the acquired mixed population.

Through this thesis, two different protocols of differentiation to ECs were tested alongside the already established protocol to further improve the generation of ECs. The first protocol tested was published by Patch and coworkers (Patch, C., et al.., 2015) and was selected due to advantages, such as serum free culturing conditions, allowing the culturing of cells in defined medium, high differentiation efficiency (66 – 88 %), high proliferative ECs produced (cultured up to passage 4 – 6), and the possibility of cryopreservation of the ECs, which is

essential for consistent reproducible results. The second protocol tested was published by Harding and coworkers (Harding et al., 2017), has a high differentiation efficiency (73 – 83% on day 8 of differentiation, depending on the cell line), with no requirement for cell sorting or magnetic purification to yield a very pure population.

Comparison to the Differentiation Protocol Published by Patch et al. 2015

This protocol utilises the information published regarding the effects of a potent activator of adenylyl cyclases, forskolin, on angiogenesis. Forskolin, which is a diterpene extracted from plants, acts by binding to specific cellular receptors known as adenylyl cyclase isoforms, such as AC1 to AC8 except AC9, increasing the cellular cAMP levels, an intracellular second messenger thought to be related to the phosphorylation and activation of multiple selective cellular substrates, including PKA and Epac. Forskolin ultimately induces the activation of Akt, eNOS, CREB, and ERK as well as increases NO production and VEGF expression, which are closely linked to angiogenesis and drastically increased endothelial cell proliferation, migration, and tube formation *in vitro* as well as neovascularisation *in vivo* (Namkoong et al., 2009).

The protocol first involves culturing hPSCs for 4 days as a monolayer in chemically defined medium supplemented with a GSK3β inhibitor and BMP4 differentiating towards the mesodermal state, then cultured for 2 days in a different medium containing VEGF, supplemented with forskolin and on day 6, EC differentiation was evaluated by analysing the expression of endothelial cell markers (CD31, CD34) (Figure 4.3, overview of the protocol). Since the differentiation efficiency is suggested to change based on the PSC line used as a starting population (efficiencies between 61.8% and 88.8% on different cell lines), the optimal conditions must be tested accordingly, on both initial cell density and concentration of GSK3i.

Performing the protocol on H1 hESCs and testing the lowest and highest seeding density of the starting population of H1 hESCs (37.000 cells/cm^2 and 47.000 cells/cm^2) and the lowest and highest GSK3i concentration (6 and 8 mM) (four different conditions, as shown in Figure 4.5) revealed that the protocol performed with the highest seeding density and the highest GSK3i concentration led to the optimal differentiation efficiency we could acquire with the H1 hESC line, which was 38.3 % of CD34$^+$ and 27.2% of CD31$^+$ cells (Figure 5.1,

B for the highest differentiation efficiency and Figure 4.5 for the comparison on the different combinations of seeding density and GSK3i concentration). This result was not equivalent to the differentiation efficiency suggested by the authors (66 – 88%) and didn't improve the differentiation efficiency compared to the differentiation protocol established by Tsolis and his colleagues (~25 %, Tsolis et al., 2016) used in the laboratory (Figure 20A). Thus, these results are deeming this differentiation protocol as not ideal for the laboratory, since it is not as efficient as the suggested protocol in the H1 hESCs. Whether the efficiency is higher in other PSCs remains to be tested, potentially providing promising results on different cell lines.

Comparison to the Differentiation Protocol Published by Harding et al. 2017

This protocol of differentiation to ECs published by Harding and coworkers (Harding et al., 2017) was chosen for the high differentiation efficiency (73-83% on day 8 of differentiation, depending on the cell line), and the generation of a very pure population with no requirement for cell sorting or magnetic purification. Harding et al. showed that the hiPSC-ECs or hESC-ECs on day 8 of differentiation, expressed endothelial lineage markers, CD31/PECAM1 at 94-97%, from which 73-81% cells were $CD34^+$.

In the protocol, hiPSC or hESCs cells were manually passaged as small clusters onto Matrigel-coated culture plates in MEF conditioned hESC medium with an additional FGF2. After 1 day, the medium was changed to APEL with CHIR99021 (GSK3i) for 2 days to acquire the mesodermal population (Figure 4.8), followed by culturing in APEL medium supplemented with BMP4, FGF2 and VEGF for 2 days to acquire the VPCs. Cells were then passaged using accutase on day 4 and seeded onto p100 culture dishes at $5 \times 10^3 – 1 \times 10^4$ cells per cm^2 in EC Growth Medium MV2 with additional VEGF to generate ECs following medium changing every 2 days for 4-6 days. The MEF conditioned medium supports the feeder-free growth of PSCs (both iPSCs and ESCs) and helps maintain their pluripotency by including growth factors required for stem cell growth and pluripotency (Stover, A.E. et al., 2011).

A number of issues not clarified by Harding et al. needed to be addressed in order to evaluate the efficiency of this protocol on the host laboratory's cell lines, such as the manual passaging performed on the starting pluripotent population (day 0 of differentiation), the

type of coating at the stage of the vascular progenitors (day 3 of differentiation) and the medium for the culture of the ECs (day 5 of differentiation).

The manual passaging performed on the starting population of hESC or hiPSC cells was not mentioned, and thus we tested both versine and dispase. Versine, not shown in the results, generated small clusters of cells, but all the differentiations performed led to a low differentiation efficiency and was thus not further tested. Dispase on the other hand led to small clusters of cells and ultimately to varying differentiation efficiencies, depending on other changes we made on the protocol.

Regarding the coating at the VPs stage, papers implementing this protocol on their differentiation process suggested either the use of 0.5 – 1% gelatin (Lee I.W., et al., 2023, Deinsberger, J., et al., 2023) or the use of collagen type IV (Karagiannis et al., 2024). Vascular and endothelial progenitor cells are known to be cultured on fibronectin-coated plates, with fibronectin promoting the appearance of endothelial cell forming colonies earlier than collagen (Colombo E., et al., 2013). Therefore, we decided to test 0.5% gelatin, collagen and fibronectin.

Two types of media were used on day 5 of differentiation, EGM-2 medium or APEL medium, even though the suggested medium was ECGM-2 (Figure 3.1, materials and methods, composition of media). APEL medium is a serum-free medium and was opted to achieve serum free conditions throughout the differentiation protocol. EGM-2 medium, an endothelial cell growth medium, similar in composition to ECGM-2 medium suggested by the protocol, contains 2% serum instead of 5% contained in ECGM-2 and was tested on both 2% and 5% serum to compare with the results Harding et al. acquired.

H1 hESCs passaged with dispase, VPCs seeded on gelatin and hESC-ECs cultured in APEL medium supplemented with VEGF revealed a high differentiation efficiency of 45.3% of $CD31^+$ and 69.1% of $CD34^+$ (Figure 4.10). Culturing the ECs in the APEL medium supplemented with VEGF alone led to cells with viability problems (couldn't be maintained in culture beyond passage 1), however, supplementing the medium with additional FGF2, which is known to promote the proliferation of the ECs (Sahni A., 2004), could help maintain the ECs to higher passages, with the additional benefit of using serum free conditions.

EGM-2 medium was first tested without the addition of VEGF, since it contains 0.5 ng/ml VEGF (Figure 3.1, materials and methods, composition of media). H1 hESCs passaged with

dispase, VPCs seeded on gelatin and hESC-ECs cultured in EGM-2 medium without the addition of VEGF revealed a low differentiation efficiency of 23.1% of $CD31^+$ and 26.2% of $CD34^+$ (Figure 4.11). Supplementing EGM-2 medium with VEGF increased the differentiation efficiency to 31.3% of $CD31^+$ and 53% of $CD34^+$ (Figure 4.14). Adding serum up to the level of 5%, which equals that of the ECGM-2 medium used by Harding et al.., further increased the efficiency of the EC differentiation to 57.4% $CD31^+$.

Testing the last conditions (Harding protocol, passaging with dispase and culturing with EMG-2 medium with 5% serum plus additional VEGF) on iPSCs derived from patients with **leucine-rich repeat kinase 2 (*LRRK2*) mutation** Parkinson's Syndrome (iPSC IM2) or the iPSCs derived from patients with the gene corrected **leucine-rich repeat kinase 2 (*LRRK2*) mutation** Parkinson's Syndrome (iPSC GC) led to high differentiation efficiencies of 55.8% $CD31^+$ for iPSC GC-derived ECs (Figure 19C, histogram 9) and 73.2% $CD31^+$ for iPSC IM2-derived ECs (Figure 19C, histogram 10).

The highest differentiation efficiency of H1 hESCs using the Harding protocol and further optimized as described above, was 57%. This is higher than the differentiation efficiency acquired with the established protocol by Tsolis and his colleagues (Tsolis et al., 2016) used in the laboratory, which was 23.5% $CD34^+$ for H1 hESC-derived ECs (Figure 5.1 for comparisons). These results suggest that the modified Harding differentiation protocol is ideal for the laboratory, since it is more efficient than the suggested protocol in the H1 hESC line and is efficient in all three of the cell lines tested in the current thesis, H1 hESC, iPSC GC and iPSC IM2.

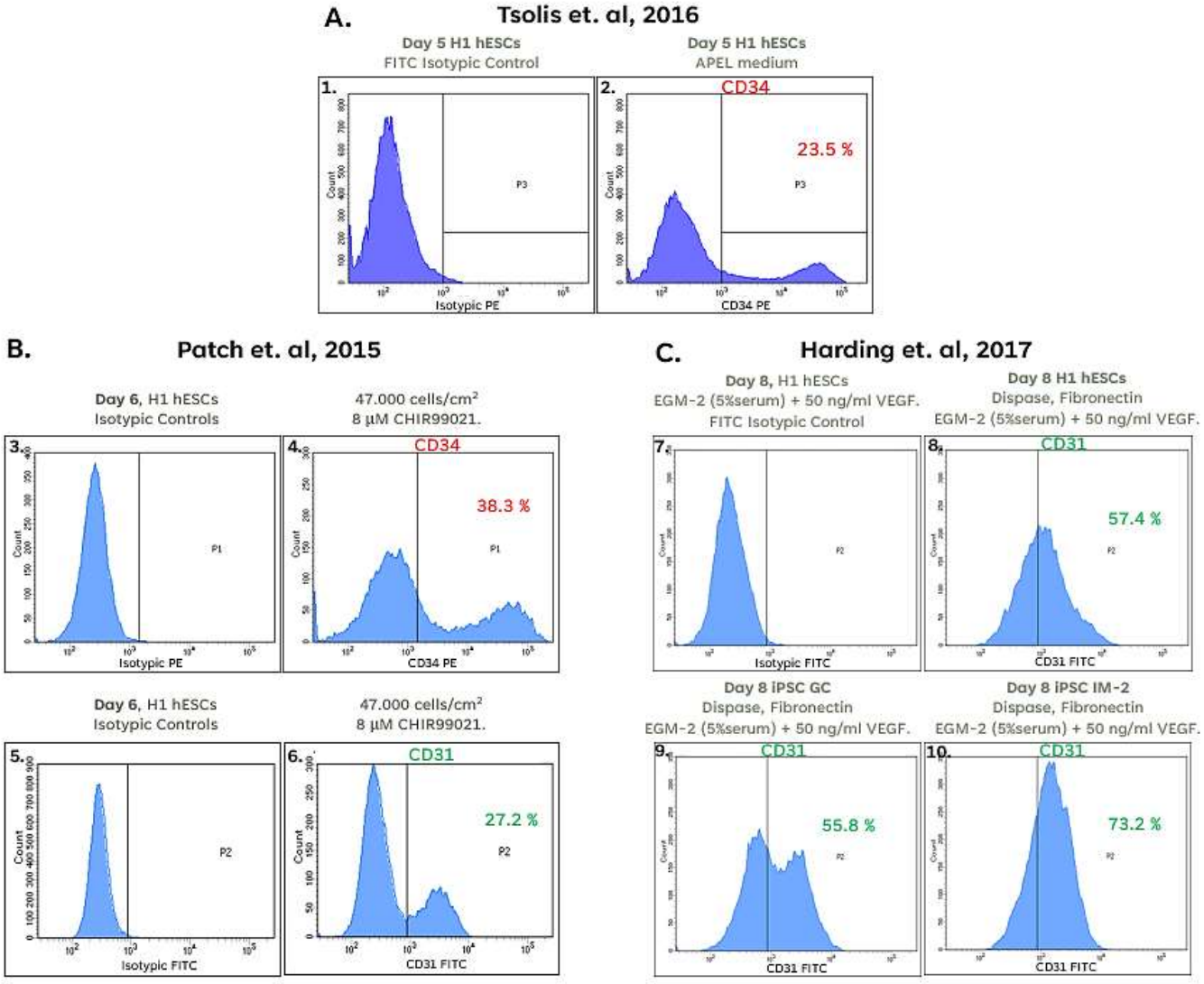

Figure 5.1. *Overview of the differentiation protocols presented in the current thesis with the histograms from FACS analysis performed on the EC population on the last day of each differentiation process.* ***(A)*** *Tsolis et al., 2016 protocol. FACS analysis performed on the H1-hESC-derived EC population on day 5 of the differentiation process, stained with anti-CD34 PE or isotypic control antibodies. Histogram 1 shows PE isotypic control and histogram 2 shows the CD34 expression of H1 hESCs-derived ECs.* ***(B)*** *Patch et al., 2015 protocol. FACS analysis performed on the H1-hESC-derived endothelial cell population on day 6 of the differentiation process, stained with anti-CD31-FITC and anti-CD34 PE or isotypic control antibodies, at 37.000 cells/cm2 cell density of H1 hESCs starting population and 6 mM CHIR99021 concentration. The first column shows histograms 3 and 5 for the isotypic PE and FITC controls and the second column shows histograms 4 and 6 for the CD34 and CD31 expression, respectively.* ***(C)*** *Harding et al., 2017 protocol. FACS analysis performed on the EC population stained with anti-CD31-FITC and isotypic control antibodies on day 8 of the differentiation process. Starting population passaged enzymatically (dispase), ECs cultured on plates coated with fibronectin and EGM-2 medium supplemented with serum (5% total serum) and 50 ng/ml VEGF. Histogram 7 is for the H1 hESCs FITC isotypic control, histogram 8 for the CD31 expression on H1 hESC-derived ECs, histogram 9 for the CD31 expression on iPSC GC-derived ECs and histogram 10 for the CD31 expression of iPSC IM-2-derived ECs.*

FUTURE GOALS

The current and similar RM projects will be greatly benefited by the expansion of our knowledge into several scientific directions that will enable us to develop

i. **Differentiation protocols in chemically defined conditions**: This will avoid using serum allowing faster translation in Clinical applications.

ii. **Assays for functional characterization of the differentiated cells/vessels**: Such assays include LDL uptake assay, in vitro angiogenesis assays, tube formation assays and vascular organoids (Markou M., et al., 2020), but more are needed.

iii. **Prevascularised tissue engineering 3D constructs with vessels as close as possible to the *in vivo* ones**: To achieve this, we require not only ECs but also Mural Cells (MCs), including pericytes (PCs) and/or vascular smooth muscle cells (vSMCs) in order to promote the maturation and stability of nascent vasculature. MCs are primarily responsible for stabilisation, inhibition of regression, contraction of the vessel as well as production and deposition of extracellular matrix (ECM) proteins (Shepro D. et al., 1993, Chistiakov D.A., et al., 2015). Interactions between MCs and ECs are critical in the process of vascular development (Armulik A., 2005, Regan J.N., et al., 2009, Trkov S., et al., 2010). MCs are composed of vSMCs, surrounding larger vessels, such as arteries and veins, and PCs, typically surrounding smaller microvessels and capillaries. Regarding vSMCs, two distinct phenotypes have been identified: synthetic and contractile (Hedin U., et al., 1987, Kusuma S., et al., 2013, Holm A., et al., 2018, Beamish J.A., et al., 2010). Both participate in neovascularization, but synthetic vSMCs predominate in the embryo and in diseased or injured adult vessels, while contractile vSMCs predominate in healthy adult vessels. In this context, understanding distinctions between MCs and the molecular mechanisms underlining their phenotypic stability and plasticity, will enable improved therapeutics in a tissue specific manner. However, although the role of MCs in engineering vascularised constructs for therapeutic applications is unquestionable (Wanjare M., et al., 2013, Dar A & Itskovitz-Eldor J., 2013) their dynamic phenotypic nature has not been extensively studied

mainly due to limitations of isolation/expansion and phenotypic plasticity during *in vitro* culture of primary MCs (Cathery W., et al., 2018). The host lab has developed a robust method for the generation of defined synthetic and contractile vSMC phenotypes from hPSCs (Markou M., et al., 2020) and now the importance of these cells, especially the synthetic vSMCs, in vasculogenesis can be directly addressed.

iv. **Microfluidic perfused 3D prevascularised constructs for evaluating the effect of flow-derived forces**: Success in future aim iii is a prerequisite for successful accomplishment of this goal too Campinho P., 2020, Gaengel K., 2009). Blood flow-derived forces control the growth and shape of both newly formed and established vascular network in collaboration with cellular genetic identity. Shear stress in laminar blood flow is essential for vessel development and along with circumferential and axial stress, define shape and wall thickness of vasculature (Hoefer I. E., 2013). RNA seq experiments on CD31+ hiPSCs-ECs revealed that a laminar shear stress of 15 dyne/cm^2 promotes a more stable, homogenous and quiescent EC phenotype compared to static conditions. Activation and proliferation of the CD31$^+$ hiPSCs-ECs was inhibited under flow conditions, compatible with a more mature, quiescent phenotype. In the same study it has also been shown that, CD31$^+$ hiPSCs-ECs under flow show a tendency to gain a venous phenotype by induced expression of relative markers (Helle E., et al., 2020). However, in another study with different experimental approach, a threshold shear stress level of ~4 dyne/cm^2 was determined as sufficient to promote an arterial phenotype to hiPSC-ECs (Anora S., et al., 2019). Importantly, experiments carried out to date address the role of flow on CD31$^+$ hiPSCs-ECs, without considering the contribution of MCs, even though the interplay between ECs and MCs including growth factor secretion, cell-cell contact and extracellular matrix modulation, is of great significance during early vasculogenesis (Sweeney M., et al., 2018). Therefore, using the PSC derived ECs and MCs and a microfluidic system (available in the host-lab) a more physiological 3D vessel can be generated and studied.

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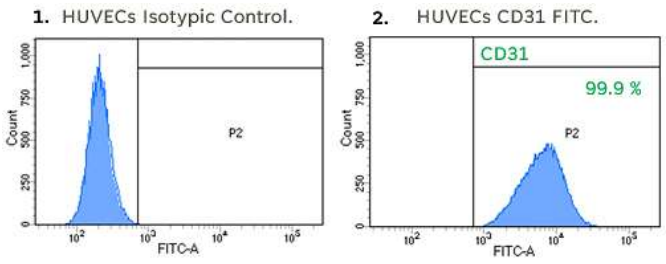
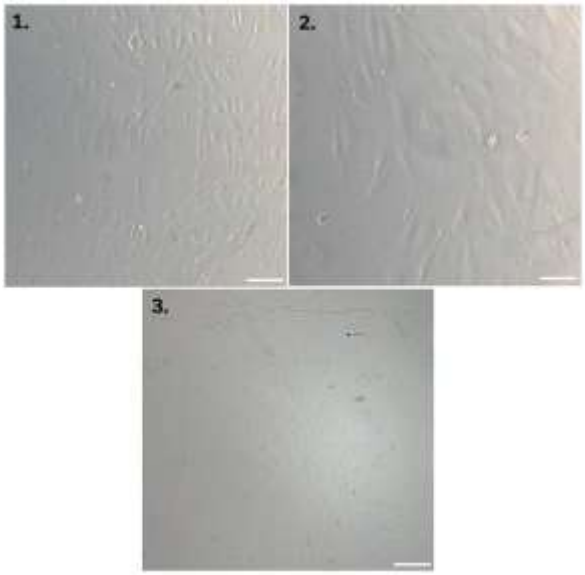
SUPPLEMENTARY FIGURES

The anti-CD31 antibody used throughout the thesis in FACS analysis was first tested on HUVEC cells, isolated as described in Materials and Methods, to establish its functionality and proceed with testing on stem cells. In HUVECs, CD31 expression is 99.9% across the cells in a typical culture, while CD34 is expressed in only a small percentage of the cells, thus not shown in Supplementary Figure 1.



Supplementary Figure 1. *FACS analysis performed on the HUVEC population stained with anti-CD31 FITC or isotypic control antibodies. Histogram 1 shows the FITC isotypic control and histogram 2 shows the CD31 expression of HUVECs. Representative graphs are shown of 1 experiment.*

Collagen isolated from rat tails as described in Materials and Methods was used as a coating for the culture of HUVEC cells. To test the quality of the collagen isolated 12-well plates were coated with collagen at a concentration of 1.5% w/v (15 mg/mL) and HUVECs were seeded and observed 1 day after plating (Supplementary Figure 2). The HUVECs attached well and showed no signs of toxicity.



Supplementary Figure 2. *TIRF microscopy imaging depicting HUVECs cells cultured in M199 Full Medium on collagen-coated plates after one day of plating. The collagen used was isolated as described in Materials and Methods. Upper panel scale bars: 200 mm, Lower panel scale bars: 100 mm. Representative images are shown of 1 experiment for 2 different wells.*