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Multi-stage endothelial differentiation and expansion of human pluripotent stem cells

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By

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ABSTRACT OF THESIS

Multi-stage endothelial differentiation and expansion of human pluripotent stem cells

by

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Master of Science in Biological Engineering and Small – scale Technologies

University of California, Merced, 2020

Professor Kara McCloskey, Chair

Human endothelial cells (ECs) have prospects for a wide range of clinical applications including cell-based therapies and tissue engineering and, hold tremendous potential for research in the fields of vascular development, drug discovery and disease modelling. Efficient and robust induction of ECs from human pluripotent stem cells (hPSCs) will serve as a renewable and indefinite source. However, distinct embryonic stem cell (hESC) and induced pluripotent stem (iPSC) cell lines respond differentially to the same microenvironmental signals. Developing an optimized differentiation methodology robust across multiple hPSC lines, including hiPSC lines derived from autologous patient specific cells remains a challenge in the field. We demonstrate a chemically defined multi-stage EC differentiation process across multiple hPSC lines. This method can generate highly purified populations of actively proliferating VE-Cadherin+ functional ECs in 30 days. There are a few published methods for efficient derivation large number of endothelial progenitor cells (EPC) within a week, but their maturation to definitive EC is tough, taking longer and requiring additional purification.

Chapter 1

Abstract

Stem cell therapy utilizing both human embryonic stem cells (hESCs) and human induced pluripotent stem cells (hiPSCs) holds vast potential for treatment of numerous disease conditions, promising great advances in medical field with regenerative therapy (1). The differentiation capacity of human pluripotent stem cells can provide with all cell types in the human body, thus creating an infinite pool of autologous cells that can be used for reconstruction and recovery of impaired tissue systems, for modelling developmental pathways of individual organ systems, for drug discovery and, for disease modeling (Figure 1.1) (2). The McCloskey laboratory is particularly interested in differentiation of stem cells to mature endothelial cells and strives to further direct and characterize populations of cells during vascular differentiation.

A complete working vasculature is critical to the functioning of the vascular system, circulating blood and transporting essential materials to and from different parts of the body. The lumen of blood vessels is lined with endothelial cells that contribute to vascularization. Endothelial cells can be used to make tissue engineered vascular grafts (3), vascularized scaffold materials for patching impaired cardiac tissues during myocardial infarcts, promoting cardiac resuscitation (4,5) and, to generate larger in vitro tissue engineered organoids requiring a functional vasculature to form networks with the host vascular system (6,7). Furthermore, vascular abnormalities in multiple conditions such as cancer, atherosclerosis and ischemia can be investigated using endothelial cells thus advancing research and treatment strategies in the field.

The aim of this project was to optimize a protocol for robust and efficient differentiation of functional ECs from multiple human iPSC lines. Since patient derived human induced pluripotent stem cells can be used for modelling disease and in drug development platforms, including the endothelial function in the aging brain, we used human induced pluripotent stem cells derived from Alzheimer's patients for our experiments. The stem

cell-derived ECs were then thoroughly characterized with multiple functional assays and expanded to create a bank of iPSC derived ECs.

1.1 Background

1.1.1 Vascular Development in the Embryo

During the early embryonic development, cells of the mesoderm layer express the vascular specific marker- vascular endothelial growth factor-2 (VEGFR-2). These cells can be differentiated into either hematopoietic, cardiac or vascular cells (32, 33), which in turn self-organize into a plexus of endothelial cells surrounding hematopoietic precursors, called blood islands. In the extraembryonic mesodermal layer of the yolk sac, the blood islands begin to self-assemble establishing a functional transport system to carry essential nutrients from the extraembryonic compartment into the embryo. Blood islands also self-assemble inside the embryo along the mesodermal layer initially forming the endocardium. The blood islands connect to form a network which is called the primary vascular plexus. De novo blood vessel formation in the embryo in this manner is an example of vasculogenesis. The vascular plexus further develops by a process called angiogenesis where the endothelial precursor cells (EPC) or angioblasts branch from existing blood vessels, establishing a complete working vasculature, transporting essential nutrients and oxygen to the growing embryo. The vasculature is the first organ system in the developing embryo. As the vasculature expands, supporting cells such as smooth muscle cells and pericytes are recruited to further maintain the integrity of the system and helping with contractile motion of the blood vessels. This is followed by development of the coronary vasculature to establish a functioning transport system for the growing heart. The vascular progenitor cells are differentiated to endothelial and smooth muscle cell fate forming a network of capillaries, arteries, and veins that connect to form the coronary vascular system. (8-12)

Many studies confirmed that VEGFR2 is essential for vascular development, associating with various signaling pathways during embryonic growth. VEGFR2 knockout mice cannot form blood vessel network in the yolk sac and have abnormal vascular plexus formation in the intraembryonic compartment causing the embryo to die within the uterus. It is hypothesized that VEGFR2 regulate signals that determine the differentiation of

hemagioblasts with in the mesodermal layer to angioblasts leading to blood vessel formation (15, 16).

1.1.2 Vascular progenitor cells

Vascular progenitor cells are multipotent vascular precursor cells that can be terminally differentiated to either endothelial cells or the mural cell types- pericytes and vascular smooth muscle cells (Figure 1.2). The main differences between pericytes and vascular smooth muscle cells is in their physiological location and appearance. Larger blood vessels such as arteries are mostly surrounded by vascular smooth muscle cells whereas smaller vessels like the capillaries are associated with pericytes.

Nishikawa et al conducted single cell studies of partially differentiated VEGFR2+ cells which then terminally differentiated to hematopoietic cells and endothelial cells. In similar single cell studies done by Yamashita et al. his group derived smooth muscle cells(α-SMA+) and endothelial cells (PECAM-1+) from the same single VEGFR2+ precursor cells (13,14).

1.1.3 Commonly used endothelial cell sources

Human dermal microvascular endothelial cells (HDMEC), human umbilical vein endothelial cells (HUVEC), aortic endothelial cells, microvascular endothelial cells, endothelial progenitor cells (EPCs), endothelial colony-forming cells (ECFCs) and, lung endothelial cells are widely used primary endothelial cell sources for EC research, as these cells can be easily collected from different tissues in the body, cultured and expanded in-vitro. Primary endothelial cells can maintain their endothelial cell phenotype for a few passages, forming perfusable vascular like structures, but these cells lose the EC-specific phenotype over time. Moreover, these primary cell types may not be obtainable in large numbers for all individuals, limiting their potential as cellular based therapies. Another source of endothelial cells are immortalized EC lines, these cells also tend to lose the EC specific phenotype acquiring tumorigenic properties, and therefore, cannot be used for in vivo applications. Thus, alternative sources of patient-specific ECs (26) remain desirable.

1.1.4 Applications of human pluripotent stem cell derived endothelial cells

Endothelial cells derived from human pluripotent stem cells can be an infinite autologous cell source for various applications in regenerative medicine and tissue engineering, for treating conditions such as acute and chronic heart failure, stroke peripheral artery disease, delayed wound healing and, so on. A very straightforward potential therapeutic application of stem cell derived endothelial cells would be to use these cells to restore the function of pathological or nonfunctional endothelial cells present in the vascular system. Previous studies reported that human pluripotent stem cell derived endothelial cell incorporation into 3D gel matrix (56- 69) or zebrafish embryos (70, 71) establishes working blood vessel network, implying that these cells may be capable of treating CVD, by generating blood vessels and promoting blood perfusion. Many studies observed that injecting stem cell derived endothelial cells directly to the site of injury improved wound healing and injury recovery in model animals and increased incorporation of the donor cells into the recipient vascular system prior to transplantation (53, 58, 59, 72- 77). Another approach was to make transplantable tissue grafts by mixing stem cell derived endothelial cells with other cell types. The endothelial cell generated blood vessels established a network between the transplanted graft and the host vasculature (78- 81). Stem cell derived endothelial cells were also incorporated into organ-on a chip models of larger organs such as the cornea, retina, liver and, kidney to establish a functional vasculature (82- 86). These miniature organs can be used in research and development for studying diseases, screening of drugs and cellular therapy. Particularly, patient specific human induced pluripotent stem cell derived endothelial cells can be used to study disease pathophysiology and in high-throughput screening of molecules that regulate signaling cascades leading to endothelial cell abnormalities, thus creating a personalized therapeutic approach, in a patient and disease-specific manner (25).

1.1.5 Biochemical Signaling for Endothelial Cell Induction

Because serum- containing medium cannot be fully specified, can differ from one lot to another. and the unknown serum components can support the growth of contaminating cell

populations, our medium formulations for endothelial induction fully eliminated use of serum. Knockout Serum Replacement (Invitrogen) and Nutridoma-CS (Roche) were used to compose our chemically-defined endothelial cell differentiation mediums, so that the cell niche can be closely regulated and cellular activity in response to growth factors can be assessed. Differentiation of vascular progenitor cells to endothelial cell fate is induced and regulated by chemical signaling pathways involving growth factors (soluble proteins that determine cellular fate). Growth factors control operations such as cell division, survival, differentiation, and cellular movements. In absence of growth factors apoptotic signaling cascades are activated leading to cell death. One of the most reported growth factors initiating and controlling vascular development is VEGF (Figure 1.3) (13- 15. 17-20). By binding to the VEGFR2 receptor, VEGF switches on the P13 kinase/Akt pathway followed by activation of the Ras pathway and prevention of caspase activity, resulting in confirmed vascular commitment and maintenance of endothelial cell survival. VEGFR-2 complex phosphorylates Tyr-1173 and triggers the PLCγ-PKC-MEK-MAPK signaling cascade which induces cell growth and activation of the PLCγ-PKC-eNOS signal (Figure 1.3). Growth factors such as fibroblast growth factor (FGF), the Wnt ligand, and bone morphogenetic protein-4 (BMP-4) are key soluble signals determining mesodermal lineage of cells, distinct by the expression of VEGFR-2 on cell surface. BMP4 plays a key role in the differentiation of pluripotent embryonic stem cells towards VEGFR2+ vascular progenitor cells of the later mesoderm layer and prevents differentiation of cells to a neuronal fate. The soluble molecule also phosphorylates Tie-2 and VEGFR2 markers and promotes endothelial cell migration (21,22). In combination with VEGF, basic FGF (bFGF) supports vascular development and angiogenesis (23).

For induction of mural cells platelet derived growth factor (PDGF), secreted by endothelial cells (9), and transforming growth factor beta (TGF-β) play a role in recruiting and differentiation of mural cell types such as pericytes and vascular smooth muscle cells during blood vessel formation (24). Vascular smooth muscle cells can also aid in angiogenesis. The presence of VEGF growth factor forming a positive feedback loop. In smooth muscle cells VEGF binds to VEGF-receptor 1/ fms like tyrosine kinase 1/Flt-1 which in turn increases the concentration of matrix metalloproteinase-9 that breaks down extracellular matrix to support angiogenesis.

1.1.6 Methods for endothelial cell differentiation from pluripotent stem cells

Endothelial cells were successfully derived from human pluripotent stem cells by several independent research groups. The first protocols published were based on three-dimensional (3D) embryoid body (EB) cultures (25), followed by 2D cultures supported by mouse embryonic fibroblasts feeder cells. Vascular induction using these methods resulted in very low efficiencies of EC differentiation (1±3%) and often were impossible to reproduce since many of the conditions were not chemically defined, utilizing serum such as FBS and feeder cells. More recently, fully defined non-serum containing mediums have been used for the efficient differentiation of large number of ECs using feeder-free monolayer cultures of human ESC (26, 27) and, one labor-intensive intensive protocol published of sprouting endothelial progenitor cells (EPC) into 3D fibrin scaffolds (34). Methods of differentiation of ECs with co-differentiation with pericytes was also developed (28, 29), that temporally activates the Wnt pathway by using a Wnt agonist, GSK3β inhibitor (CHIR-99021), generating cultures containing 50% CD31+/CD34+ EPC and15±25% CD31+/CD34+ EPC, respectively and up to 50% PDGFRβ mesenchymal cells in 10 days. However, generation of mature ECs still requires longer periods of time (27, 35). Moreover, the Wnt pathway plays a complex role in either suppression or induction of both cardiomyocyte and hematopoitic lineage depending on the timing of the induction or inhibition (87). Therefore, differentiation protocols utilizing a Wnt agonist may not be robustly reproduced across multiple hPSC lines. Although there are a few published protocols for differentiating human induced pluripotent stem cells to endothelial cells, were not yet extensively expanded in vitro culture systems in comparison to endothelial cells derived from human embryonic stem cells. (30). Most importantly, currently published endothelial cell differentiation protocols from human stem cells were developed for only a one or two human pluripotent stem cell and are not readily applicable across different stem cells lines (27, 31).

1.1.7 Endothelial cell function

Vascular endothelial cell identity and purity can be determined through analysis of cell surface proteins. For example, some of the commonly analyzed endothelial cell markers are the following:

VEGFR2 (Flk1 in mouse/ KDR in humans)- it is an early marker indicating vascular and hematopoietic lineage commitment [35,36] and activates multiple biochemical signaling cascades that determines cell survival, proliferation and, migration (37-40), *PECAM 1 (CD 31)*- Platelet endothelial cell adhesion molecule is found in cell-cell junctions (41, 42), *VE-cadherin*- found in cell-cell junctions and allows vascular assembly and also plays a role in cell survival in conjunction with VEGFR2 by activating PI3kinase/AKT signaling cascade (43, 44), *Tie-1*- is a protein expressed in matured endothelial cells (47, 48), it is specific to venous phenotype, and it was reported that Tie-1 and Ang-1 double knockouts had disturbed venous development in the right side (45, 46), *Tie-2*- is a protein expressed in matured endothelial cells (47, 48), it *binds to* angiopoietin-1 and coordinates between endothelial cells and smooth muscle cells (49), *von Willebrand factor* – is an essential clotting factor for blood vessels (50) and is localized in vascular basement membrane (51), *EphB4*- endothelial cell marker specific to venous phenotype (52) and, *ephrinB2*- endothelial cell marker specific to arterial phenotype (52).

Multiple reported endothelial markers are utilized to identify the specific stages towards endothelial differentiation of stem cells, generating a map to reproducibly derive endothelial cells for clinical applications. Specific proteins are expressed at different stages of differentiation and these proteins serve as an indicator of the level of maturity of the derived endothelial cells. For example, vascular progenitors are positive for the markers VEGFR2 and PECAM1 and, negative for Tie-1 (54). In our studies we utilized the markers VE- Cadherin and PECAM1 as indicator of the mature endothelial phenotype. Angiogenesis and vasculogenesis are important phenomenon during vascular development. We utilized two-dimensional Matrigel tube formation assay as a vasculogenic invitro assay to study the endothelial phenotype of our derived endothelial cells (55).

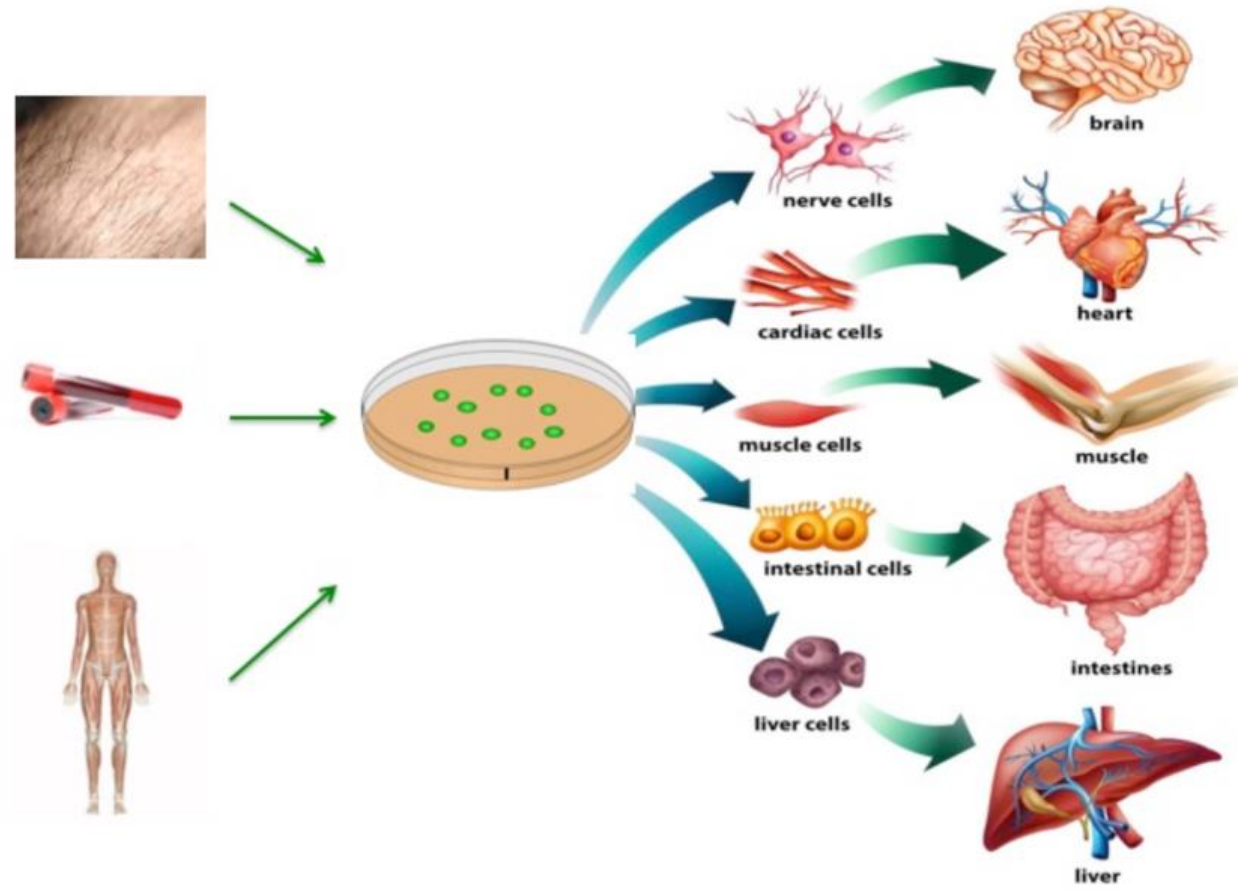

Figure 1.1. Power of Stem Cells

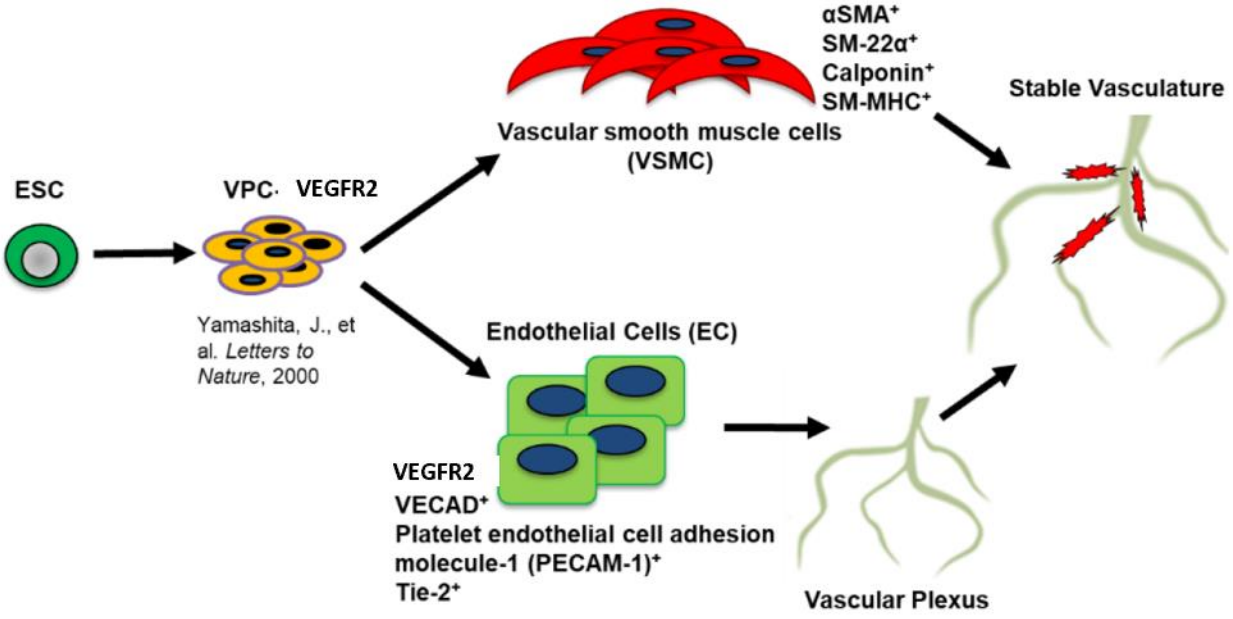

Figure 1.2. Development of the vasculature. EC and VSMC arise from a common progenitor that are positive for Flk-1 expression. The propagation of EC undergoes vasculogenesis that gives rise to a primitive vascular plexus. This primitive plexus stabilizes through VSMC and pruning through angiogenesis which is the process of new blood vessel formation from pre-existing ones to form a mature vascular structure.

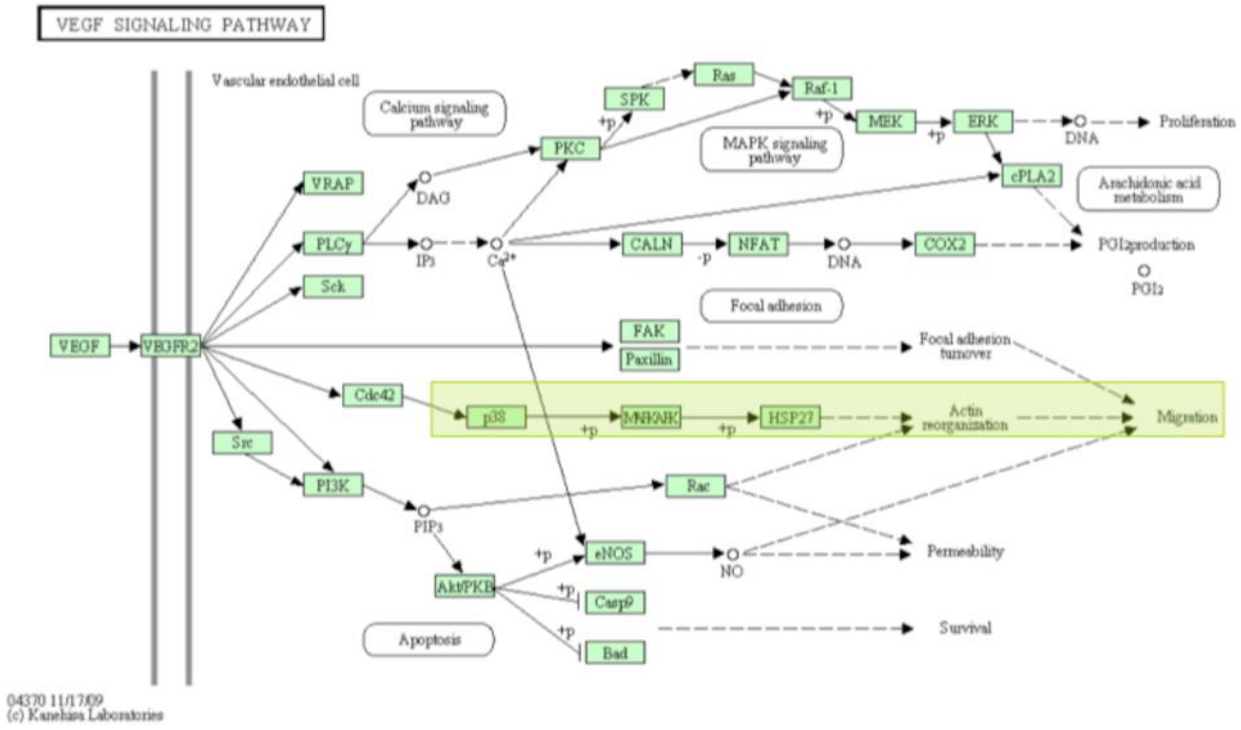

Figure 1.3. VEGF signaling modulates endothelial behavior and function. Proliferation, migration, and survival of endothelial cells are all in response to VEGF. In order to determine the mechanism behind in vitro angiogenic behavior, we examined HSP27 phosphorylation, an important factor for EC migration (pathway highlighted in green). Modified from Kanehisa Laboratories (http://www.genome.jp/kegg/pathway/hsa/hsa04370.html)

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Chapter 2

Induced pluripotent stem cell culture: method for maintenance, expansion and, cell banking

Abstract

Induced pluripotent stem cells can differentiate into cells from the three different embryonic germ layers: ectoderm, endoderm, and mesoderm (1). These cells are optimal for applications in patient-specific cellular therapy, drug discovery and "disease-in-a-dish" cellular research (2). The pluripotent cell types can be differentiated into different cell types of the body using cell specific differentiation strategies.

We were able to culture several induced pluripotent stem cell lines derived from the neurodegenerative Alzheimer's disease patients and from heathy donors on feeder- free culture systems utilizing completely chemically defined medium. The induced pluripotent stem cell lines retained their ability to self-renew and differentiate to other cell types of the body when subjected to corresponding differentiation strategies up to twenty passages. The human pluripotent stem cells were also differentiated to cardiomyocytes by regulating Wnt/ β-Catenin pathways as partial evidence the cells retained their differentiation capacity.

2.1 Introduction

Embryonic stem cells originate from the inner cell mass of mammalian blastula on day five of embryonic development and are associated with various ethical concerns as the process of generating a new cell line usually destroys the viable embryos. Additionally, embryonic stem cells do not come from individual patients and are not autologous. Yamanaka's group was able to generate embryonic stem cell-like cells from terminally differentiated adult cells of the body by switching on expression of four pluripotency genes typically expressed during early embryonic development (1, 4). The stem cell-like cells were termed induced pluripotent stem cells (iPSCs) and could self-renew and differentiate into cells from the three different embryonic germ layers: ectoderm, endoderm, and mesoderm. Stem cell therapy and research utilizing induced pluripotent stem cells bypasses the ethical concerns of embryo. The cells can also be used to make a patient/disease specific cell bank.

Despite advances in the field of stem cell research, in vitro stem cell culture to maintain, expand and, preserve stem cell culture systems in an undifferentiated condition remains challenging. Culture techniques may vary between different stem cell lines and can be optimized based on factors such as cell morphology, growth kinetics, metabolism, cell clumping, attachment, death and so on. Another important consideration is the source and type of pluripotent stem cells, for example mouse embryonic stem cells (mESC) require leukemia inhibitory factor (LIF) (6, 7, 8) in their culture media whereas human pluripotent stem cells require fibroblast growth factor (FGF) (9, 10). In addition, during human pluripotent stem cell culture the media is changed every day and the cell colonies are regularly checked for any contaminated differentiated cells. Any differentiated cell colony must be manually removed from the culture system as it will affect the pluripotency of the cells lowering differentiation ability to other cell types. Although induced pluripotent stem cells are positive for pluripotency markers and resemble embryonic stem cells, embryonic stem cells can be differentiated into other cell types more robustly and effectively compared to induced pluripotent stem cells (10, 11). Moreover, induced pluripotent stem cell lines and cells derived from them exhibit greater variability compared with ESCs (3).

Here, we describe the step-wise protocol we used to culture multiple patient-derived human induced pluripotent stem cell lines and human embryonic stem cells on feeder-free

Matrigel matrix, using a chemically defined TeSR™-E8™ and mTeSR™ medium, respectively (13- 16). This protocol has been successful in routine cell maintenance, passaging, expansion, and, cryopreservation. Robust across several human stem cell lines, this protocol is an effective and reliable technique for human stem cell culture systems. The human pluripotent stem cells were also differentiated to cardiomyocytes by regulating Wnt/ β-Catenin pathways as an evidence the cells retained their pluripotency.

2.1.1 Stem Cell Culture in Feeder- free Systems

Extensive research has led to development of feeder-free culture systems for human pluripotent stem cells meaning the cells can attach for growth on a biological extracellular matrix surface instead of feeder cell layers. Induced pluripotent stem cells inherently tend to differentiate losing their pluripotency if not cultured in optimal conditions. Therefore, induced pluripotent stem cell culture methods differ from culturing other cell types such as primary cells, adult stem cells or continuous cell lines. The primary purpose of using a feeder free technique is to obtain fine tune between supporting exponential growth of pluripotent stem cells and avoiding random differentiation of cell colonies by chemically defining all the components used for cell culture. Some of the advantages of eliminating the feeder layer from the cell culture system are as follows- 1) culture techniques are easier requiring fewer steps, 2) the same experimental conditions can be repeated more precisely, 3) feeder-free culture systems can be scaled-up more easily, and 4) there is no risk contaminating stem cell cultures with the feeder fibroblast population.

2.2 Reagents and Equipment set up

Preparation of TeSR-E8 complete stem cell medium. TeSR-E8 was prepared by adding TeSR-E8 basal media to the TeSR-E8 supplement kit. We aliquoted 40 ml of the medium into 50 ml centrifuge tubes and stored them at −20 °C for up to 1 year. When the aliquots were thawed and stored at 4 °C we used them for up to 2 weeks.

Aliquoting Matrigel for long-term storage. Matrigel hESC-qualified matrix was thawed on ice and aliquoted onto microfuge tubes. The labware (microfuge tubes, tips and, pipettor) were all precooled in -20 °C freezer. We aliquoted 270uL of Matrigel into each tube to achieve a final concentration of 2mg/tube however the volume may vary depending on the concentration of protein and is specific to each lot. The aliquots were stored in -20ºC freezer.

Note: The goal is to keep it cold when handling Matrigel. At temperatures above 15°C Matrigel solidifies in 15-30 minutes and cannot be worked with, so labware is constantly kept on ice to stop premature gel formation.

Aliquoting ROCK Inhibitor Y27632 for long-term storage. We reconstituted 1 mg of ROCK Inhibitor Y27632 into 624 µl of PBS to make a 10 mM stock solution. We made aliquots of 20 µl to avoid repeated freeze-thaw cycles and stored the aliquots at −20°C for up to 6 months.

Preparation of Matrigel coated cell culture dishes. To prepare Matrigel coated cell culture dishes we thawed one 270 ul Matrigel aliquot on ice. To a precooled 50-ml tube we added cold 24 ml DMEM/F12 and then quickly added the Matrigel to it using cold tips and mixed by pipetting. We coated cell culture plates by using 1 ml Matrigel solution for each well of a six-well plate and, 4 ml for a 100 mm dish. We then sealed the coated plates and stored at 4 °C. The plates were used up to 3 weeks.

Preparation of RPMI/B-27 -insulin (510 ml). We added 10 ml of B-27 -insulin to 500 ml of RPMI. The media can be stored for up to 1 month at 4 °C.

Preparation of RPMI/B-27 without insulin + 12 mM CHIR99021 (24 ml). We added 8 µl of 36 mM CHIR99021 into 24 ml of RPMI/B-27 – insulin.

Aliquoting IWP2 (5 mM) for long term storage. We reconstituted 10 mg of IWP2 into 4.28 ml of DMSO and incubated for 10 minutes at 37 °C to solubilize IWP2. to make a 5 mM stock solution. We made aliquots of 100 µl to avoid repeated freeze-thaw cycles and stored the aliquots at −20°C for up to 1 year.

Aliquoting CHIR99021 (36 mM) for long term storage. We reconstituted 25 mg of CHIR99021 into 1.49 ml of DMSO. The aliquots can be stored at −20°C for up to 1 year.

2.3 Methods

2.3.1 Thawing cryopreserved induced pluripotent stem cell lines

TeSR-E8, was prewarmed in a 37°C bead bath and Matrigel plates were warmed at room temperature about 30 minutes prior to use. The medium was not warmed any longer as the process may degrade the growth factors and only required volume was warmed to avoid repeated freeze-thaw cycles. In order to thaw, cell cryovial was safely removed from the nitrogen tank, placed in the bead bath for 3- 5 minutes and taken out as soon as there were only a few ice crystals in the vial. The cell suspension was gently transferred from the cryovial to the bottom of a 15 ml tube containing 4 ml TeSR-E8. The cell suspension was centrifuged at 200 x g for 5 minutes at room temperature. The supernatant then aspirated and discarded. To the cell pellet we gently added 1 ml of TeSR-E8 and carefully resuspended with a 1 mL tip gently pipetting up and down 2 or 3 times. We were careful not to over pipette and singularize the cell clumps, as it is crucial to maintain cells clumps for maximum cell viability. Cells from each cryovial ($\sim 2.5 \times 10^5$) were plated onto 2 Matrigel™-coated wells of a 6-well plate with 2 ml of TeSR-E8. 10 nM of ROCK inhibitor Y27632 was added to the medium of each well. The total number of plates seeded were altered depending on the cell density in the cryovial. Generally, a stem cell cryovial stores approximately 1×10^5 to 5×10^5 cells. Typically, larger density of cell colonies was required to be seeded when thawing cells in comparison to regular passaging. The cell culture dish was incubated at 37°C in a humidified 5% CO_2/95% air incubator, the cell morphology was observed, and medium was completely changed every day starting from day 2 of culture. After day 2, the medium was no longer supplemented with ROCKI, which induces spindle-like morphology, but the morphology goes back to normal after ROCKI is removed.

2.3.2 Passaging induced pluripotent stem cell lines

We passed and split the iPSC cultures when they reached about 80%, before the colonies could touch other colonies, this was typically between 4-7 days of culture depending on the proliferation rate of the cell line and the starting cell seeding number. We scaled our stem cell dissociation and passaging technique based on one- well of a six- well cell culture plate and the reagent volumes were scaled up or down as needed based on the size of the

cell culture dish. TeSR-E8, was prewarmed in a 37°C bead bath and Matrigel plates were warmed at room temperature about 30 minutes prior to use. Spent medium was gently aspirated out of the plate and the cells were washed twice with 2 ml of PBS per well, followed by addition of 1 ml of Versene for lifting the cells, then incubated at room temperature (RT) for 3- 6 minutes. The incubation time is crucial and was optimized for each cell line cultured, some cell types tend to dissociate more easily than others, so we were careful to not break down the colonies into single cells. The cells are dissociated sufficiently when outsides of the colonies start to loosen and detach from the dish and this can be confirmed by looking through the microscope starting at 3 minutes. We aspirated the Versene adding 1 ml of warm TeSR-E8 and gently used a cell scraper to detach the cell colonies from the plate. The cell suspension was transferred to a 15 ml conical tube and the aggregates were broken down to smaller pieces by carefully pipetting the cell suspension a few times until the aggregate size was approximately in the range of 100–500 μm. Cell clumping is inconsistent between the different iPSC lines, some singularize more easily that the others so pipetting to break the clumps was done carefully. When plating stem cells colony size is crucial since single cells tend to die whereas too big colonies may limit differentiation ability of the culture system. The cells were split such that cell colonies from one well could be cultured on 6-10 wells of a precoated Matrigel plate. The splitting ratio was determined based on the proliferation rate of the specific cell line. The ROCK inhibitor Y27632 was not added to the media during routine passaging as it interferes with several unknown cellular pathways and was also observed to affect cell morphology. The cell culture dish was incubated at 37°C in a humidified 5% CO_2/95% air incubator, the cell morphology was observed, and medium was completely changed every day.

2.3.3 Cryopreserving induced pluripotent stem cell lines

We froze down cells when the plates reached about 80% confluency. We developed our stem cell cryopreservation technique based on a six- well cell culture plate and the reagent volumes were scaled up or down as needed based on the growth area of the cell culture dish. The stem cells were dissociated using the same technique as described during passaging. During freezing after the Versene was aspirated, 0.5 ml of freezing medium

Bambanker was added to each well of a 6-well plate. The cells were gently lifted from the plate while retaining small colonies as the cells can withstand the harsh freeze-thaw process more effectively when frozen down in colonies. We transferred the cell suspension from 2 well of a 6- well plate to 1 cryovial. Generally, a stem cell cryovial stores approximately 1×10^5 to 5×10^5 cells. To have an approximate count of the cell numbers stored per cryovial we also counted the cell density per ml of freezing medium using a cell cytometer. The cells were slowly cooled down at -80°C in Mr. Frosty containers. The cells were not kept in the -80°C freezer for more than 48hrs after which the cryovials were moved to the vapor phase of liquid nitrogen tank.

2.3.4 Differentiation of human pluripotent stem cells to cardiomyocytes

We adapted our cardiomyocyte differentiation protocol based (5) on a 6- well stem cell culture plate and 12- well induction plate; the reagent volumes were scaled up or down as needed based on the size of the cell culture dish. The stem cells were lifted using Versene, cells from 2-4 wells of a 6 well plate (~ 0.5- 1 million cells) were passed onto each well of a Matrigel precoated 12-well plate, adding 2mL of stem cell media to each well of a12-well plate. This time is represented as DAY-4 (Figure 2.7). The following three days the stem cell media was replaced with 2 mL fresh media daily. On the fifth day represented as DAY 0 (Figure 2.7) stem cell media was aspirated and the cells in each well were fed with 2mL RPMI/B27-insulin with CHIR99021. After 24 hours RPMI/B27-insulin with CHIR99021 was aspirated and 2mL RPMI/B27 -insulin was added to each well. After 48 hours represented as DAY 3 half of the spent media was carefully aspirated from each well and 1mL of fresh RPMI/B27 –insulin media with IWP2 was added combining old media with fresh media. After 48 hours represented as DAY 5, old medium was completely aspirated from each well and replaced with fresh 2mL RPMI/B27 -insulin. After 48 hours represented as DAY 7 and every three days then on spent medium was completely aspirated from each well and replaced with fresh 2mL RPMI/B27.

2.4 Results and Discussion

The following human pluripotent stem cell lines were expanded in culture and banked- hESC-H9, 6160-7ADRC iPSCs-12 Clone 1119-7, 10196-10ADRC iPSCs-75 Clone 5, 7329-6ADRC iPSCs-35 Clone 4 and, 7306 -6 ADRC iPSCs-23 Clone 1, 7469-10ADRC iPSCs-34 Clone 2, 7527-4ADRC iPSCs-27 Clone 14, 10312-10ADRC iPSCs-21 Clone 1, 6160-7ADRC iPSCs-12 Clone 2 (Figure 2.1) . The passage cycle of the cell lines was between 3- 7 days depending on the growth kinetics. For example, the stem cell line 10196-10ADRC iPSCs-75 Clone 5 was observed to have a high proliferation rate and a passage cycle of 3 days (Figure 2.5), whereas the stem cell line 7527-4ADRC iPSCs-27 Clone 14 had a slower proliferation rate and a passage cycle of 7 days. The cultures were passaged when they reached > 80% confluence and the cells were rounded and compacted (Figure 2.2- 2.6). Medium was replaced with fresh medium daily, however some the cultures contained spontaneously differentiated colonies (Figure 2.7). Spontaneous differentiation was identified when the colonies did not have defined edges, the cells of the colonies were not compacted, and the cells may also appear flattened or fibroblastic. Some differentiation around the edges may be tolerated however if amount of differentiation was > 25% we removed the differentiated colonies by gently scraping the colonies with a pipette tip. The research hIPCs line was differentiated to cardiomyocytes and spontaneous beating was observed on Day 10 of differentiation (Figure 2.9).

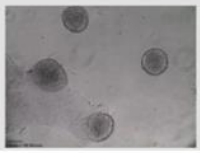
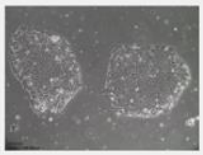
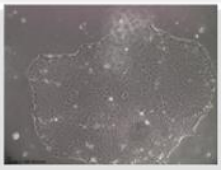
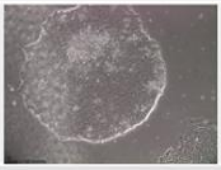
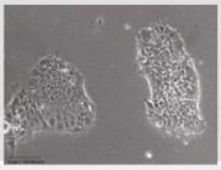
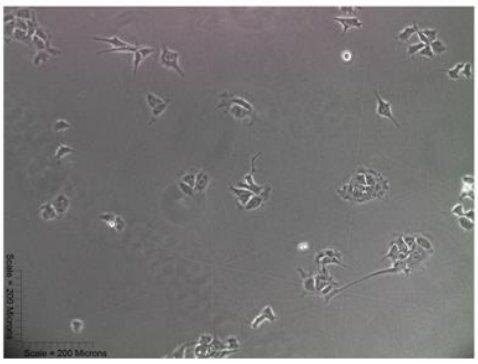
Sample ID	IPSC Name	IPSC ID	IPSC Clone	
10196	10 ADRC iPSC	75	Clone 5	
7469	10 ADRC iPSC	34	Clone 2	
7527	4 ADRC iPSC	27	Clone 14	
7306	6 ADRC iPSC	23	Clone 1	
7329	6 ADRC iPSC	35	Clone 4	
10312	10 ADRC iPSC	21	Clone 1	
6160	7 ADRC iPSC	12	Clone 1119-7	
6160	7 ADRC iPSC	12	Clone 2	

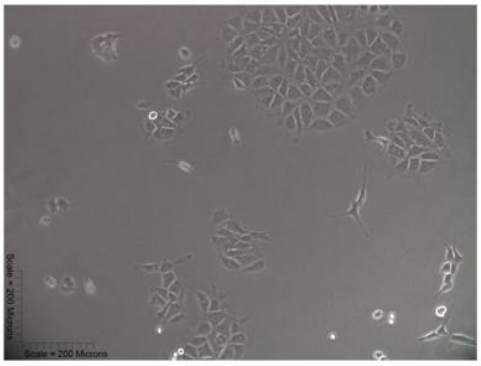
Figure 2.1. List of human induced pluripotent stem cells in culture.

a)



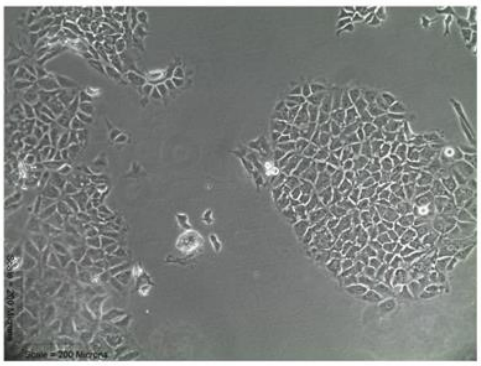
DAY 1- Post thaw
Colonies of 4-8 cells have attached to the plate.

b)



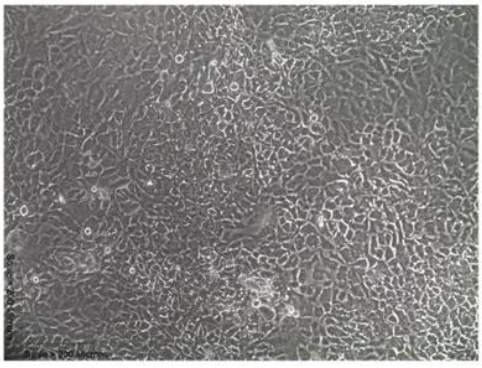
DAY 2
Colony sizes are small and overall confluency is <25%.

c)



DAY 3
Colony sizes are medium, cell densities within colonies are higher than Day 2 between 50- 60% and, overall confluency is between 30-40%

d)

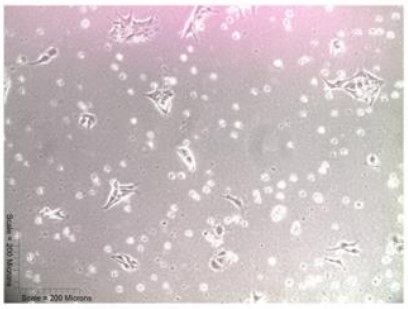


DAY 4
Colonies started touching neighboring colonies, cell density within colonies is high>75%, overall confluency is >75% and, amount of differentiation <25%. Cells are ready for passaging.

Figure 2.2. Phase-contrast images of research human induced pluripotent stem cell line in culture. (a) Small colony the day after thawing. (c, d) Colony sizes and overall confluency on Day 2 and 3 (d) hPSC colony before passaging.

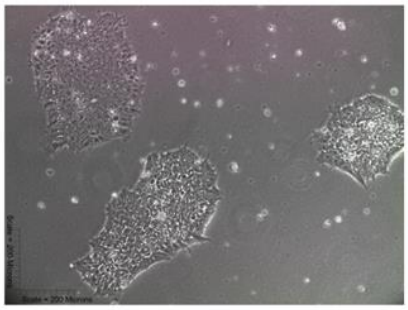
6160 7 ADRC iPS 12 Clone 1119-7

a)



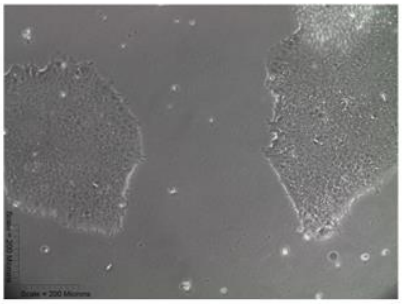
DAY 1- Post thaw
Colonies of 3-8 cells have attached to the plate. Floating dead cells that did not attach were observed.

b)



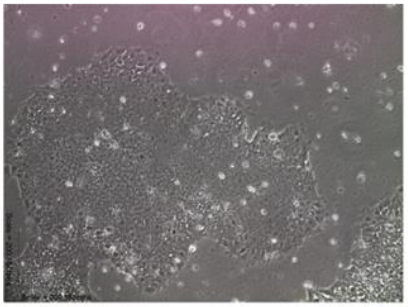
DAY 2
Colony sizes are small and overall confluency is < 30%.

c)



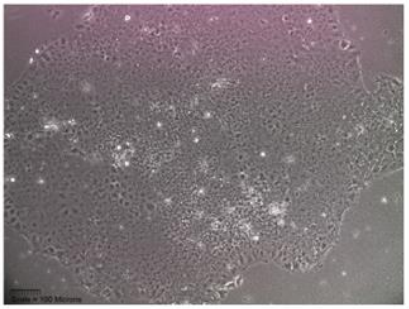
DAY 3
Colony sizes are medium, cell densities within colonies are between 50- 60% and, overall confluency is between 40-50%

d)



DAY 4
Colonies sizes are large, cell density within colonies is high>75%, overall confluency is between 60-70% and, amount of differentiation <25%. Cells can be passaged on Day 4.

e)

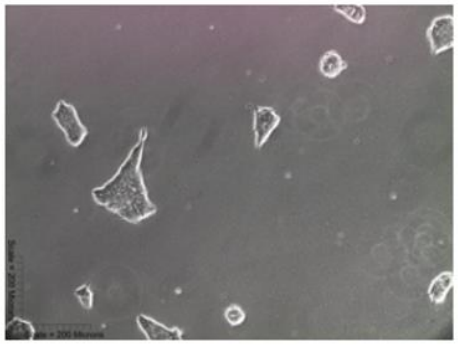


DAY 5
Colonies started touching neighboring colonies, cell density within colonies is high>75%, overall confluency is >85% and, amount of differentiation <25%. Cells are ready for passaging.

Figure 2.3. Phase-contrast images of 6160 7 ADRC iPS 12 Clone 1119-7 in culture. (a) Small colony the day after thawing. (c, d & e) Colony sizes and overall confluency on Day 2, 3 and, 4 (d) hPSC colony before passaging.

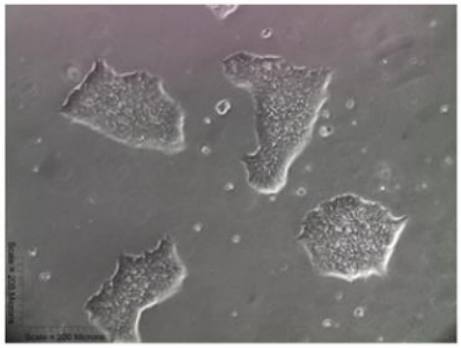
hESC- H9

a)



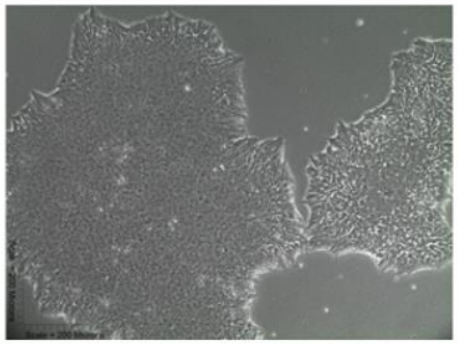
DAY 1- Post thaw
Tiny cell attached to the plate.

b)



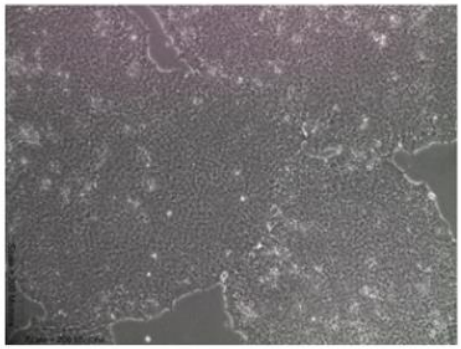
DAY 2
Colony sizes are small and overall confluency is <25%.

c)



DAY 3
Colony sizes are medium to large, cell densities within colonies is high and, overall confluency is between 60-70%.

d)



DAY 4
Colonies started touching neighboring colonies, cell density within colonies is high>75%, overall confluency is >85% and, amount of differentiation is <25%. Cells are ready for passaging.

Figure 2.4. Phase-contrast images of research human embryonic stem cell line H9. (a) Small colony the day after thawing. (c, d) Colony sizes and overall confluency on Day 2 and 3 (d) hPSC colony before passaging.

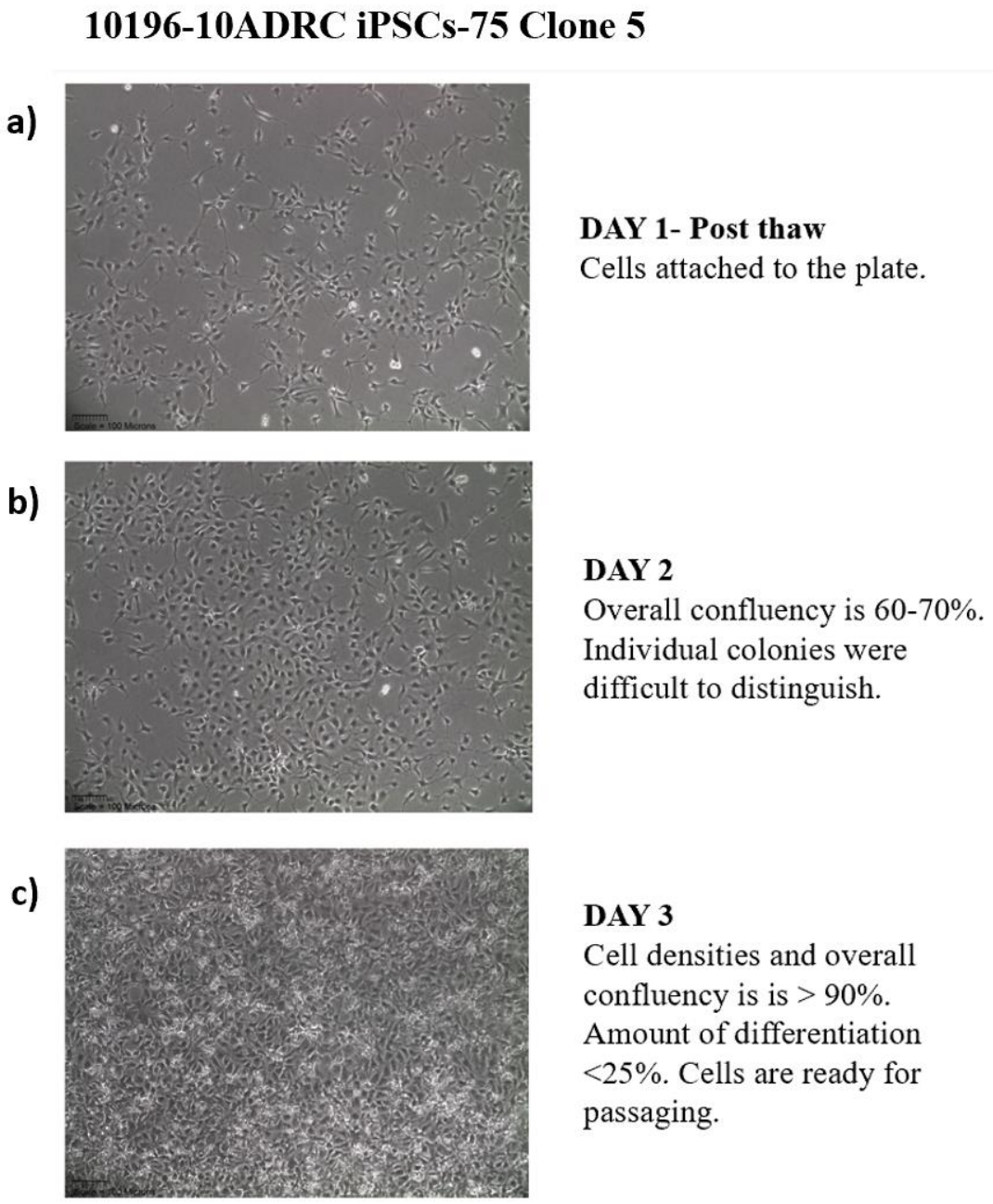


Figure 2.5. Phase-contrast images of 10196-10ADRC iPSCs-75 Clone 5 (a) Small colony the day after thawing. (c) Colony sizes and overall confluency on Day 2 (c) hPSC colony before passaging.

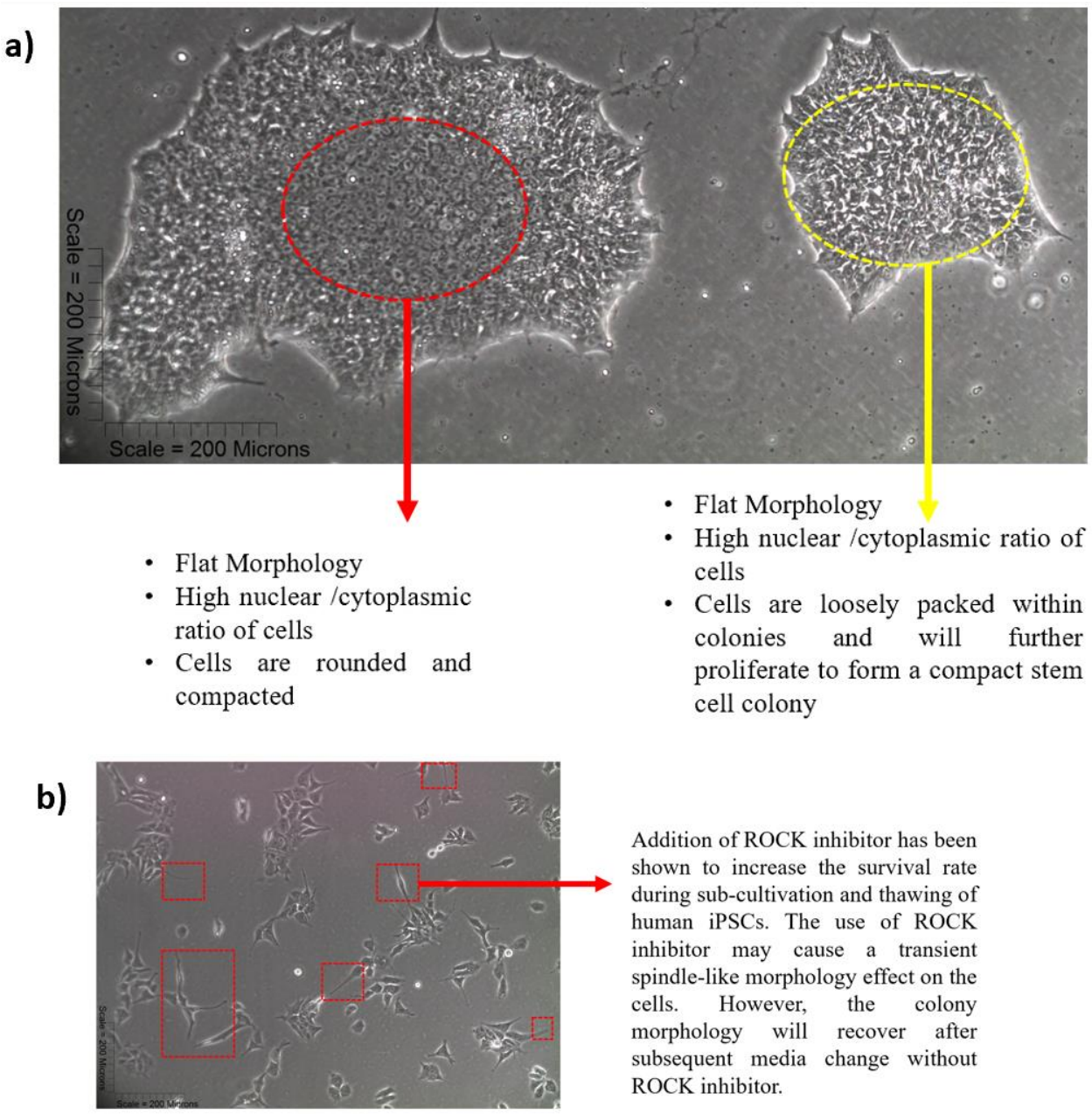
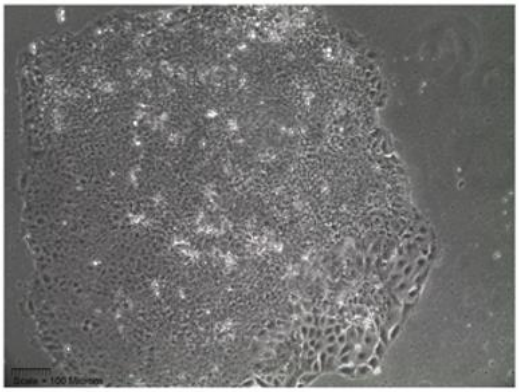
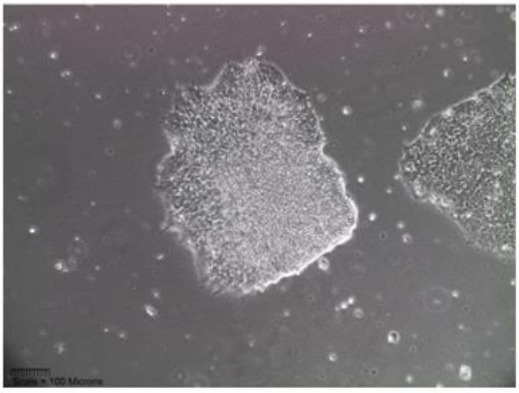


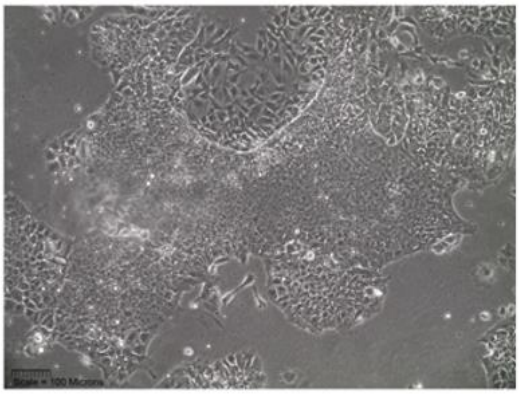
Figure 2.6. Morphology and confluency of human pluripotent stem cell lines.



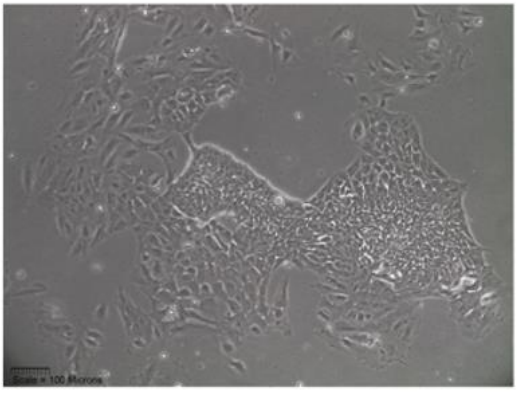
Acceptable iPS cell colonies with medium level of differentiation around the edges in culture.



Non uniform morphology across colonies lacking obvious iPS morphology



High level of differentiated iPS cells in culture



High level of differentiated iPS cells in culture

Figure 2.7. Spontaneous differentiation of human pluripotent stem cell colonies.

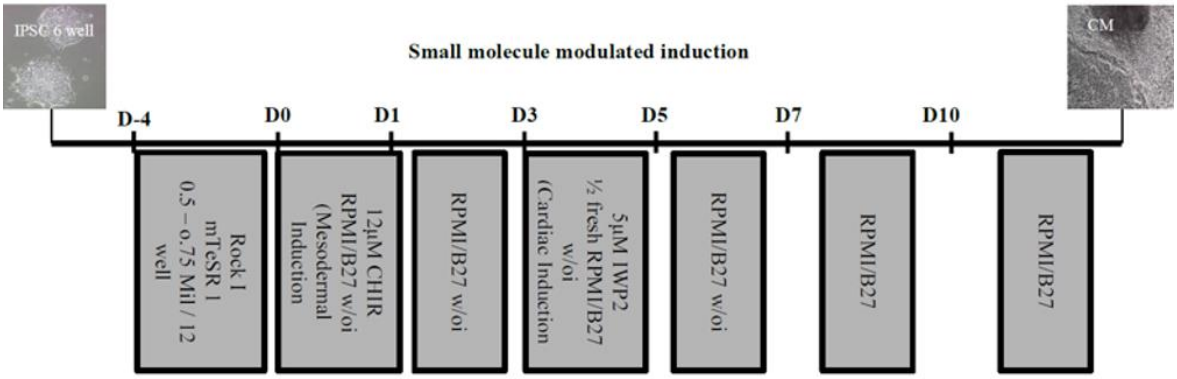


Figure 2.7 *Timeline and description of small molecule modulated induction. CM differentiation from hPSC by modulating Wnt/ β-Catenin signaling using small molecules.*

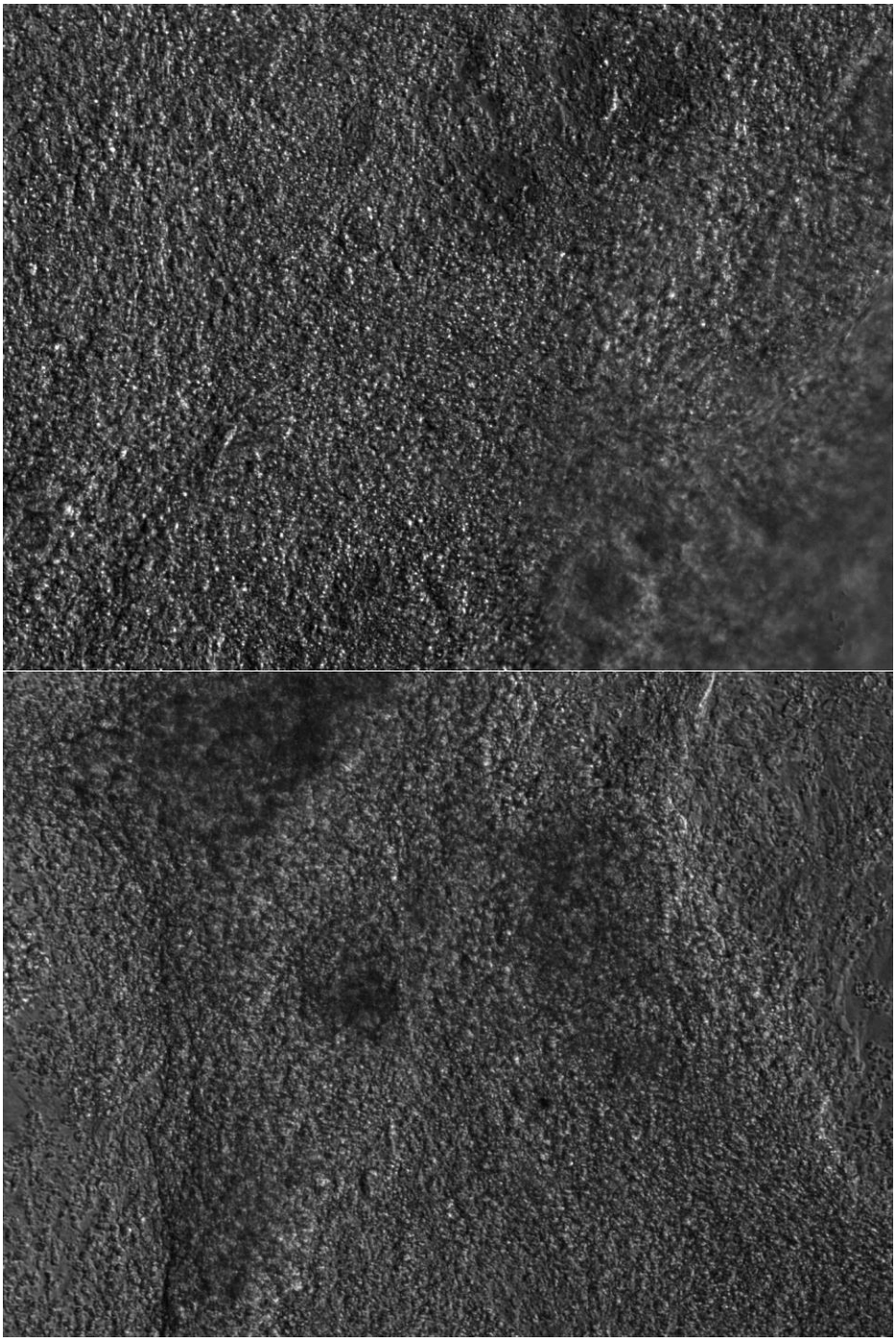


Figure 2.9. Morphology and beating motion of human pluripotent stem cell derived cardiomyocytes on DAY 12 of differentiation.

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Chapter 3

Pre-optimization of KDR+ expression kinetics across multiple human induced pluripotent stem cell lines

Abstract

Human endothelial cells (ECs) are crucial to the progress of research and development in advanced therapies, drug development, regenerative medicine, modelling pathophysiology of diseases, studying vascular development mechanisms such as angiogenesis and vasculogenesis, and tissue engineering of small scale organoids to larger multicellular organ systems. During embryonic development, endothelial cells are generated from the mesodermal germ layer. Cells of the mesoderm layer are initially induced to VEGFR2+ vascular progenitor cells which are multipotent vascular precursor cells that can be terminally differentiated to either endothelial cells or the mural cell types- pericytes and vascular smooth muscle cells. Here, we have developed a multi-stage protocol for endothelial cell generation from human pluripotent stem cells closely mimicking the process of endothelial cell generation during embryogenesis. In the first stage of differentiation the pluripotent stem cells were induced towards a mesodermal lineage, followed by a purification step using FACs sorting for KDR+ vascular progenitor cells. Each embryonic stem cell and induced pluripotent stem cell line is unique and can behave very distinctly to similar physical, chemical and environmental signals. We explain and report an optimization technique for generating maximum number of KDR+ vascular progenitor cells (VPC) across multiple human pluripotent stem cell lines by examining KDR+ expression kinetics between day 6 to day 13.

3.1 Introduction

3.1.1 Differentiation of mesodermal cells during embryonic development and mesoderm specific markers

During early embryonic development the internal cell compartment (ICM) in the blastocyst produces the primitive epiblast and endoderm consisting of ectoderm, mesoderm and endoderm, which are the three primary germ layers giving rise to all types of cells in the body (Figure 3.1). The mesoderm generates endothelial cells and vascular smooth muscle cells. For in vitro differentiation of pluripotent stem cells to mesodermal cells and vascular cells, it is important to understand the process of mesodermal cell generation and differentiation during embryonic development, and identify the specific proteins expressed in the mesodermal layer (2, 3). At approximately gestation, day 6.5, the mesoderm layer starts to form with the start of gastrulation. Early mesodermal cells move from the epiblast to the primitive streak and then transfer between endoderm and ectoderm to form the mesodermal germ layer which in turn is divided into axial, paraxial, intermediate, and lateral mesoderm based on their distance from the primitive streak (4, (5,6,7). The cardiovascular system consisting of the heart and the blood vessels originate from the lateral mesoderm. Specific cellular markers can be used to characterize and map mesodermal germ layer generation and differentiation in vitro. Mesodermal commitment involves a cascade of events through networking of several molecular signals bringing about induction, patterning, and specification of mesodermal lineage (Figure 3.2). During very early mesoderm formation the cells are positive for (BMP)2 followed by expression of the Brachyury marker (8). Brachyury subsequently down regulates as the germ layer differentiates towards specific tissues such as vascular and cardiac cells (9). While hematopoietic precursors, are positive for the homeobox gene MIXL1, cells of the mesodermal lineage are additionally positive for markers like N-Cadherin, CD34, Sca-1, CD31 and Nodal. Platelet-derived growth factor receptor (PDGFR)-α and vascular endothelial growth factor receptor (VEGFR)-2 are two markers that are expressed on the cell surface during mesoderm formation and these proteins can be used for segregating mesodermal cells (1, 10, 11, 12, 13).

3.1.2 Vascular progenitor cell purification

Vascular progenitor cells are multipotent vascular precursor cells that can be terminally differentiated to either endothelial cells or the mural cells. Vascular progenitor cells express the surface marker VEGFR2 (Flk1 in mice and KDR in human). VEGFR2+ cells are terminally differentiated to hematopoietic cells and endothelial cells. We selected the KDR surface protein to derive a purified population of vascular progenitor cells since it has been reported as one of the earliest markers determining lineage commitment of the mesodermal cells towards hematopoietic or vascular cell fate [14, 15]. The KDR molecule is an extracellular protein, so it has the potential to be used as a target to isolate vascular progenitor cells from a heterogenous cell population using flow activated cell sorting (FACS) without having to kill the cells. In comparison to magnetic-activated cell separation (MACS) FACs yields cells of superior purity, therefore we chose to purify the cell populations using FACS. We could also use the MACS technique for our purposes, however in that case the cells would need to be sorted a few times to completely isolate the KDR+ cells and discard any other cell type.

3.2 Reagents and Equipment set up

1. Human Embryonic and Induced Pluripotent Stem Cell Lines: hESCH9,10196-10 ADRC iPSCs-75-Clone 5, 6160-7 ADRC iPSCs-12-Clone 1119-7, 7329-6 ADRC iPSCs-35-Clone 4, 7306-6 ADRC iPSCs-23-Clone 1
2. Versene Solution (Gibco™ catalogue # 15040066)
3. TrypLE Express Enzyme (1X), No Phenol Red (Gibco™ catalogue #12604021)
4. Dulbecco's Modified Eagle Medium: High glucose DMEM (Gibco™catalogue # 11965092)
5. Minimum Essential Medium, Alpha 1X with Earl's salts, ribonucleosides, deoxyribonucleosides and L-glutamine (CORNING, catalogue# 10-022-CV)
6. Penicillin/Streptomycin (5000 U/ml) (100-ml; Gibco™ catalogue # 15070−063).
7. L-Glutamine (200 mM) (100-ml; Gibco™ catalogue # 25030−081)
8. MEM Non-Essential Amino Acids Solution (100X) (Gibco™ catalogue#11140050)

9. Knockout Serum Replacement (500-ml; Gibco™ catalogue # 10828−028)

10. Bovine Serum Albumin powder, BioXtra (10 G, Sigma-Aldrich, catalogue # A3311).

Preparation of BSA, 0.5% (wt/vol) in PBS. We solubilized 1 g of bovine albumin in 200 ml PBS. The solution was sterilized by filtering through a 0.22-μm membrane filter. The solution could be stored 4°C for up to 4 weeks.

11. Y-27632- RHO/ROCK pathway inhibitor; Inhibits ROCK1 and ROCK2 (StemCell Technologies, catalogue # 72304).

Aliquoting ROCK Inhibitor Y27632 for long-term storage. We reconstituted 1 mg of ROCK Inhibitor Y27632 into 624 μl of PBS to make a 10 mM stock solution. We made aliquots of 20 μl to avoid repeated freeze-thaw cycles and stored the aliquots at −20°C for up to 6 months.

12. Recombinant Human Bone Morphogenic Protein-4 (BMP4, (50 μg, Peprotech, catalogue # 120–05).

Preparation of BMP4 (50 μg ml^{1} stock solution). The contents of the vial were reconstituted to a final concentration of 50 μg ml^{1} in PBS containing 1% (wt/vol) BSA. We made aliquots of 100 μl to avoid repeated freeze-thaw cycles and stored the aliquots at −80°C for up to 1 year.

13. Recombinant Human Vascular Endothelial Growth Factor (VEGF165) (100 μg, Peprotech, catalogue # 100-20).

Preparation of VEGF (50 μg ml^{1} stock solution). The contents of the vial were reconstituted to a final concentration of 50 μg ml^{1} in PBS containing 1% (wt/vol) BSA. We made aliquots of 50 μl to avoid repeated freeze-thaw cycles and stored the aliquots at −80°C for up to 1 year.

14. β-Mercaptoethanol (25ML; Sigma- Aldrich, catalogue # M3148).

Dilution of β-Mercaptoethanol (100 mmol stock solution). We added 6.6 ml dH20 to 1 ml of 14.3 M β -ME.

15. Fetal Bovine Serum (FBS)-Heat Inactivated (500-ml; Gibco™ catalogue # 10082-147)

16. Human Fibronectin, 5 mg (Corning® catalogue # 356008)

Aliquoting Human Fibronectin (1 mg ml^{1}). We reconstituted 5 mg of fibronectin into 5 ml of PBS (pH 7.2) to have a stock concentration of 1 mg ml^{1}. We made aliquots of 1 ml to avoid repeated freeze-thaw cycles and stored the aliquots at −20°C for up to 2 weeks.

Preparation of fibronectin coated cell culture dishes. 1 ml of PBS was added to 10 µl or 50 µl of fibronectin (1 mg ml^{1}), to have a final concentration of 10 µg *ml^{1}*and 50 µg *ml^{1}*, respectively. To every 100-mm culture plate, 4 ml of fibronectin solution was added (67 µl ***cm^{2}*** per culture dish). The plate was incubated with fibronectin for at least 1 h at 37°C for a minimum of 1 h prior to use. The plates were stored for use for up to 2 weeks at 37°C.

17. Fc Receptor Binding Inhibitor Polyclonal Antibody 1: 1000 (eBioscience, catalogue # 14-9161-73)
18. Fixable Viability Dye eFluor 780, 1: 1000 (eBioscience, catalogue # 65-0865-14)
19. Anti-human CD309 (VEGFR2) Antibody (PE) 1:100 (BioLegend, catalogue # 359903)

3.3 Methods

3.3.1 Mesodermal induction of human pluripotent stem cells

Human stem cells were lifted from a 6-well plate when the culture was about 80% confluent, by using 1 ml of Versene for each well followed by incubation between 3- 6 minutes depending on the specific stem cell line, and then aspirating the Versene. 1 ml of culture medium was added to each well. This followed physical dissociation by gently scraping the surface of the dish pipetting the culture medium up and down with a pipette. Cells detached from the surface of the culture dish to small aggregates of 4 to 8 cells, as viewed under the microscope. To begin the induction process, we replated the stem cells from Matrigel matrix to fibronectin-coated 100-mm plates. The ratio of plating was such that cell colonies from 2 wells of a 6-well plate were transferred to a 100 mm tissue culture plate and the cell count was higher during induction in comparison to regular passaging. When making fibronectin coated plates we used a 50 µg/ml solution of fibronectin. To each 100-mm plate 6 ml of stage 1 Induction Medium (Table 3.1) containing the growth factors VEGF (15 ng/ml) and BMP4 (5 ng/ml) was added. The medium contained 20% KSR as

serum replacement. We added 10 nM of Y27632 to the medium to help cells survive through the shock of the induction conditions which were different from the stem cell culture conditions. The cells were incubated at 37°C and 5% CO_2 and the medium of the induction plate was not changed in the starting 3 days. When changing medium on day 3 the spent medium was removed, cells were washed with 6 ml of PBS two times and fresh prewarmed stage 1 induction medium was added to the cell culture dish. The medium was no longer supplemented with Y27632. Following day 3 the cells were checked for their morphology, confluency and color of spent medium daily and the medium was completely changed every other day. We set experiments to FACs sort for KDR+ vascular progenitor cells between day 6 to day 13 for the multiple stem cell lines cultured and optimized the day when the cell population expressed maximum percentage of KDR+ cells.

3.3.2 Purification of KDR+ vascular progenitor cells

The culture medium was aspirated, and the cells were washed with 6 ml of PBS per 100-mm tissue culture dish. To each dish 6 ml of TrypLE was added the cells were placed at 37°C in a humidified 5% CO_2/95% air incubator for 3 minutes. Starting at 3 minutes the cells were viewed under the microscope. As soon as most of the cells detached and were floating, we neutralized the enzyme solution. The incubation time typically between 3- 5 minutes was crucial and was optimized for each cell line cultured, some cell types tend to dissociate more easily than others, so we were careful not to incubate the cells with the enzyme solution for too long as this would kill the cells. For neutralizing TrypLE 6 ml of DMEM to each 100-mm plate and the solution was pipetted up and down several times. The solution was then washed over the edge and bottom of the plate so all cells could be removed. A cell scraper was gently scraped over the dish surface to remove any remaining cells adhering to the surface. The culture plate was viewed under the microscope to ensure the cells were broken down into single cells. Some stem cell lines have greater adhesive forces than others and they tend to stick to each other and the surface of the dish more. For flow cytometry purposes it is very important to dislodge the cell clumps into single cells. The cell suspension was transferred to a 15-ml centrifuge tube and centrifuged at 200×g room temperature, for 5 minutes. Each ~ 80% confluent 100-mm cell culture dish yields

approximately 1.5–2 × 10^6 cells. The supernatant is aspirated from the cell pellet followed by resuspension of the cell pellet DMEM containing 0.5% wt/vol BSA. Cells from each 100-mm plate was suspended in 200 µl of DMEM and this ratio was scaled up based the starting number of induction plates. Human BD Fc Block was then added to the cell suspension at 0.5 µg/ml concentration and incubated at room temperature for 15 minutes. Fixable Viability Dye eFluor 780 was then added to the cell suspension at 1 µl/ml concentration and incubated at room temperature in the dark for 10 minutes. A cell suspension aliquot of 50 µl was transferred into another 15-ml centrifuge tube and the tube was labelled as "cells only". Another A cell suspension aliquot of 50 µl was transferred into another 15-ml centrifuge tube and the tube was labelled as "isotype control". The initial centrifuge tube was labelled as "KDR+ cells". To the tube labelled "KDR+ cells" we added anti-human CD309/VEGFR2 PE monoclonal (Biolegend) at concentration of 1.33 µl/10^6 cells or 1.33 µl/100 µl cell suspension and then vortexed the cell suspension. To the tube labelled "isotype control" we added PE Mouse IgG1, κ Isotype Ctrl (FC) Antibody (Biolegend) at concentration of 1.33 µl/10^6 cells or 1.33 µl/100 µl cell suspension and then vortexed the cell suspension. All three tubes were placed in the dark on ice or at 4°C and incubated for 30 min. After antibody incubation 4 ml BSA buffer solution was added to all tubes and the cell suspensions were centrifuge at 200×*g* at 4°C for 4–5 minutes. The supernatant was removed, and the cell pellet was washed additional two times using BSA buffer solution in the same manner. Washing was necessary to remove any unbound free-floating antibodies that would give false positive results during FACS sorting. The cell suspension was resuspended in DMEM after the last was at concentration of 1 × 10^6 cells/100 µl (the approximate cell density was determined from the initial number of 100-mm induction plates used). The cell suspension was pipetted up and down ensure the cells were evenly dispersed in the suspension. Any cell population that was observed to be clumpy and adhesive was filtered through a strainer prior to cell sorting so that the clumps do not clog up the sorter. The cell suspensions were transferred 5-ml round bottomed polystyrene FACS tube. A 15-ml centrifuge tube was filled with Endothelial Specification Medium and this tube was used to collect the sorted cell populations. The cells undergo severe mechanical and chemical stress during the sorting process and collecting the cells in serum enriched medium like FBS can help the cells survive through the shock.

Generally during the purification step of KDR+ cells a distinct subpopulation of KDR+ expressing cells were observed at any time point the cells were sorted. However, KDR+ cell population was the highest on different days for different stem cell lines. The day when the maximum percentage of KDR+ vascular progenitor cells were expressed was optimized for each stem cell line and the brightest cell population was sorted and collected in the collection tube containing cold media.

3.4 Results and Discussion

The following human pluripotent stem cell lines- hESC-H9, 6160-7ADRC iPSCs-12 Clone 1119-7, 10196-10ADRC iPSCs-75 Clone 5, 7329-6ADRC iPSCs-35 Clone 4 and, 7306-6 ADRC iPSCs-23 Clone 1 were differentiated towards a mesodermal lineage to generate KDR+ vascular progenitor cells using complete chemically defined Stage 1: Induction Medium (Table 3.1) [15 - 20]. The Stage 1: Induction Medium was supplemented with the growth factors BMP-4 and VEGF which supports development of the ventral mesoderm and promote growth of hematopoietic precursor cells, while arresting growth of cells towards a neuronal fate [21- 23]. On the other hand, during the mesoderm induction phase when generating KDR+ vascular progenitor cells, the medium was not supplemented with basic fibroblast growth factor (bFGF) as it is a promoter of angiogenesis and was included at a later stage when cells were induced towards specific endothelial cell fate [24]. Each cell line was cultured on fibronectin substrate, between 6 to 13 days the cell population was analyzed and FACS sorted for cells expressing the vascular progenitor marker KDR. For the human pluripotent stem cell lines, it was not possible to analyze the cells for KDR+ expression any earlier than day 6 since we were not able to collect enough cells for flow cytometry analysis before this time. The cell line10196-10ADRC iPSCs-75 Clone 5 was observed to have a very high proliferation rate during the induction phase, so at day 8 the cells were passed and split into 2 100-plates. From routine imaging of the cell lines, it was observed that when the cells start to differentiate towards a mesodermal lineage, they also undergo morphological changes. The three-dimensional stem cell colonies transform to a two-dimensional cobblestone like monolayer of cells (Figure 3.5). The day of maximum

KDR+ cell population was distinct for each cell line (Figure 3.3). For example, for the cell lines 6160-7ADRC iPSCs-12 Clone 1119-7 and 10196-10ADRC iPSCs-75 Clone 5 the percentage of KDR+ cells were the highest on day 12 (~70- 80%), while constantly remaining significantly lower in earlier days of culture. For the human embryonic stem cell line hESC-H9 the percentage of KDR+ cells constantly remained significantly lower on days 6 and 8 in comparison to day10 when it peaked to the highest (~90%), followed by a significant drop of KDR+ cells on day 12. The cell line 7329-6ADRC iPSCs-35 had very low percentage of KDR+ cells between days 6-10, with the KDR+ cell population being greatest on day 13 (~70%). For the cell line 7306-6 ADRC iPSCs-23 Clone 1 the percentage of KDR+ cells peaked in day 9 (~60%) followed by a steady drop-in day 10, 11 and, 12 (Figure 3.4). We optimized the day of maximum KDR expression for the abovementioned human pluripotent stem cell lines and observed that it was between day 8 to day 14 of the mesodermal induction stage. Around this point in induction, the stem cell cultures contained a mixed population of vascular progenitor cells, with a considerable number of cells expressing the KDR surface protein. Few of the cells are also positive for the endothelial specific marker VE-cadherin. We used HUVECs as positive control which exhibited similar levels of KDR expression (Figure 3.3).

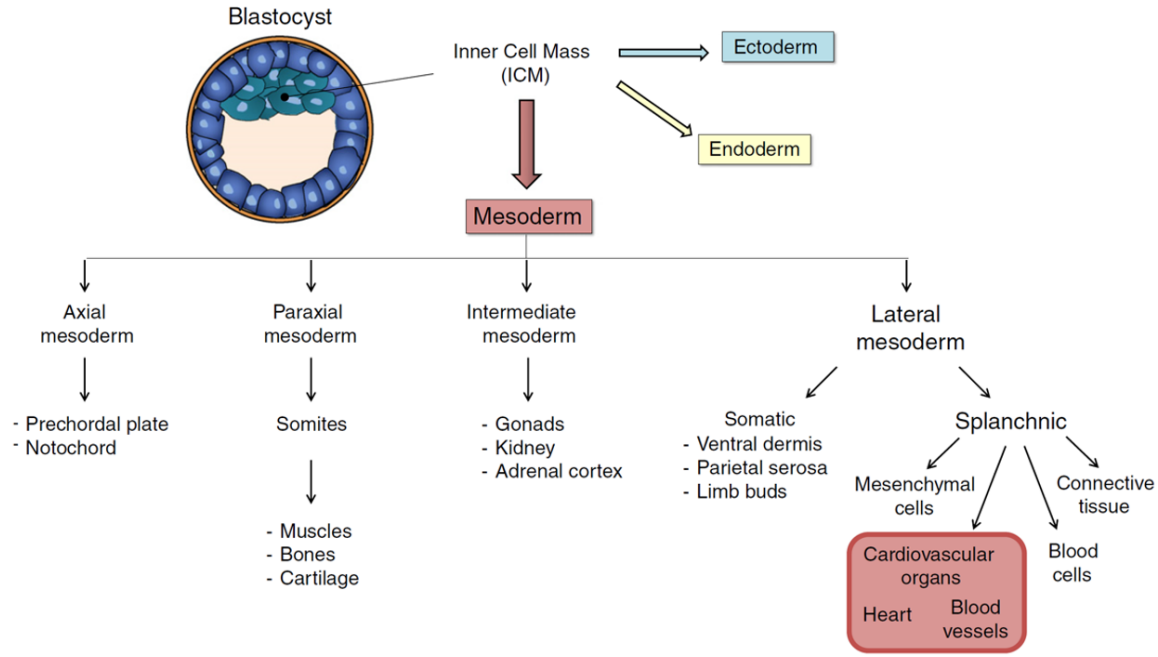
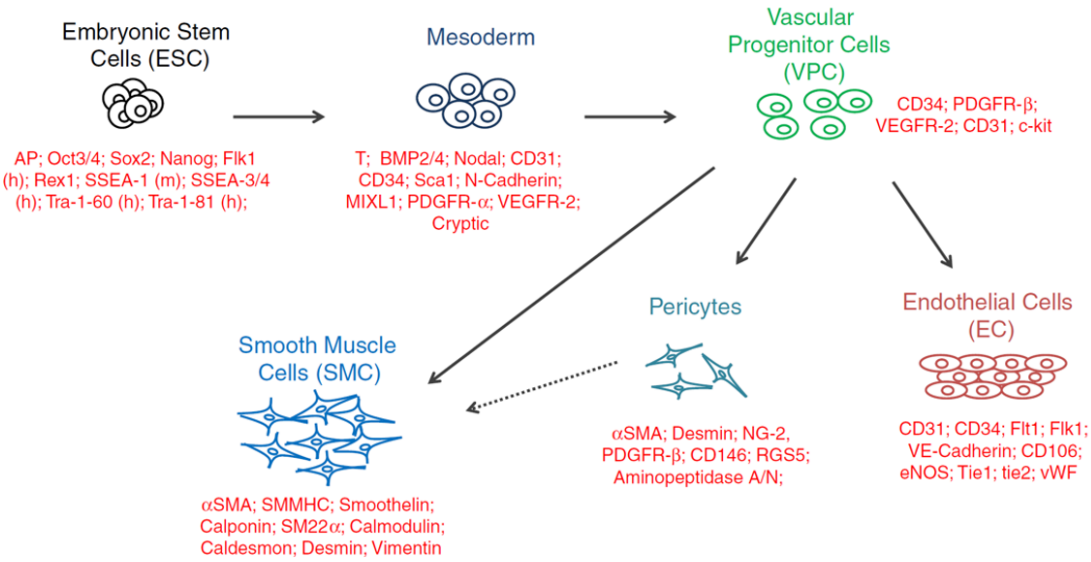


Figure 3.1. Schematic overview of the development of different organs from the mesoderm. During early vertebrae embryogenesis, the ICM gives rise to the three primary germ layers: ectoderm, mesoderm and endoderm, from which all organ tissues are developed. Mesoderm layer forms distinct developmental fates: axial, paraxial, intermediate, and lateral mesoderm, in order of their proximity to the primitive streak. The lateral mesoderm gives rise to the somatic and splanchnic mesoderm. Somatic mesoderm forms ventral dermis, parietal serosa and limb buds. Splanchnic mesoderm forms mesenchymal cells and connective tissue, and cardiovascular organs and blood cells.

(Descamps, B.et al., 2012)



AP, Alkaline Phosphatase ;
(m), murin ESC expression only;
(h), human ESC expression only

Figure 3.2. Schematic overview of cell surface marker expression during mesoderm specification and vascular cell derivation from embryonic stem cell. During vascular specification, specific markers are modulated: from the pluripotency of ESC, though their mesodermal commitment and VPC lineage, 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.

Descamps, B.et al., 2012

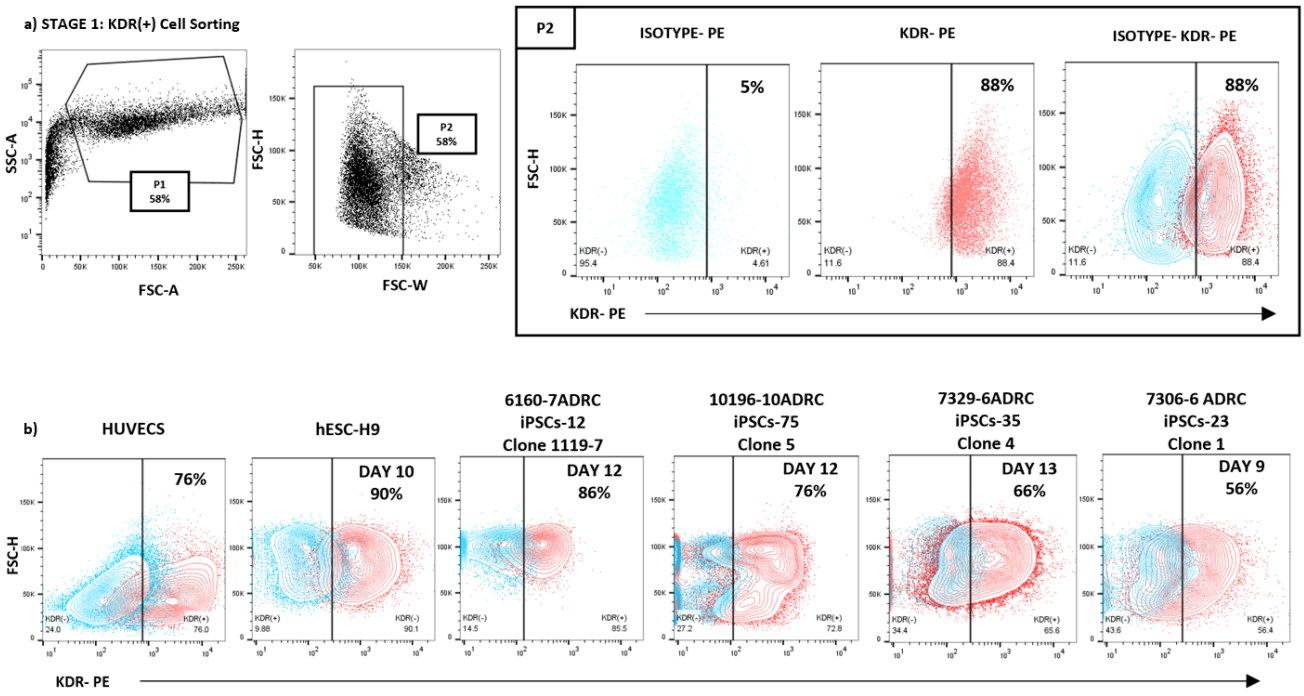


Figure 3.3 . Flow cytometric analysis of KDR+ cell population during mesodermal induction phase, of multiple hPSC lines. (a) Representative Isotype-PE and KDR-PE samples; gated from the live cell population (P1: FSC, forward scatter; SSC, side scatter) and single cell population (P2: FSC-W, forward scatter width; FSC-H, forward scatter height). ***(b)*** *Percentage of KDR(+) cells, respectively* of multiple hPSC lines. Percentages of positive cells were calculated based on the isotype controls (4%- 5%; blue scatter plot).

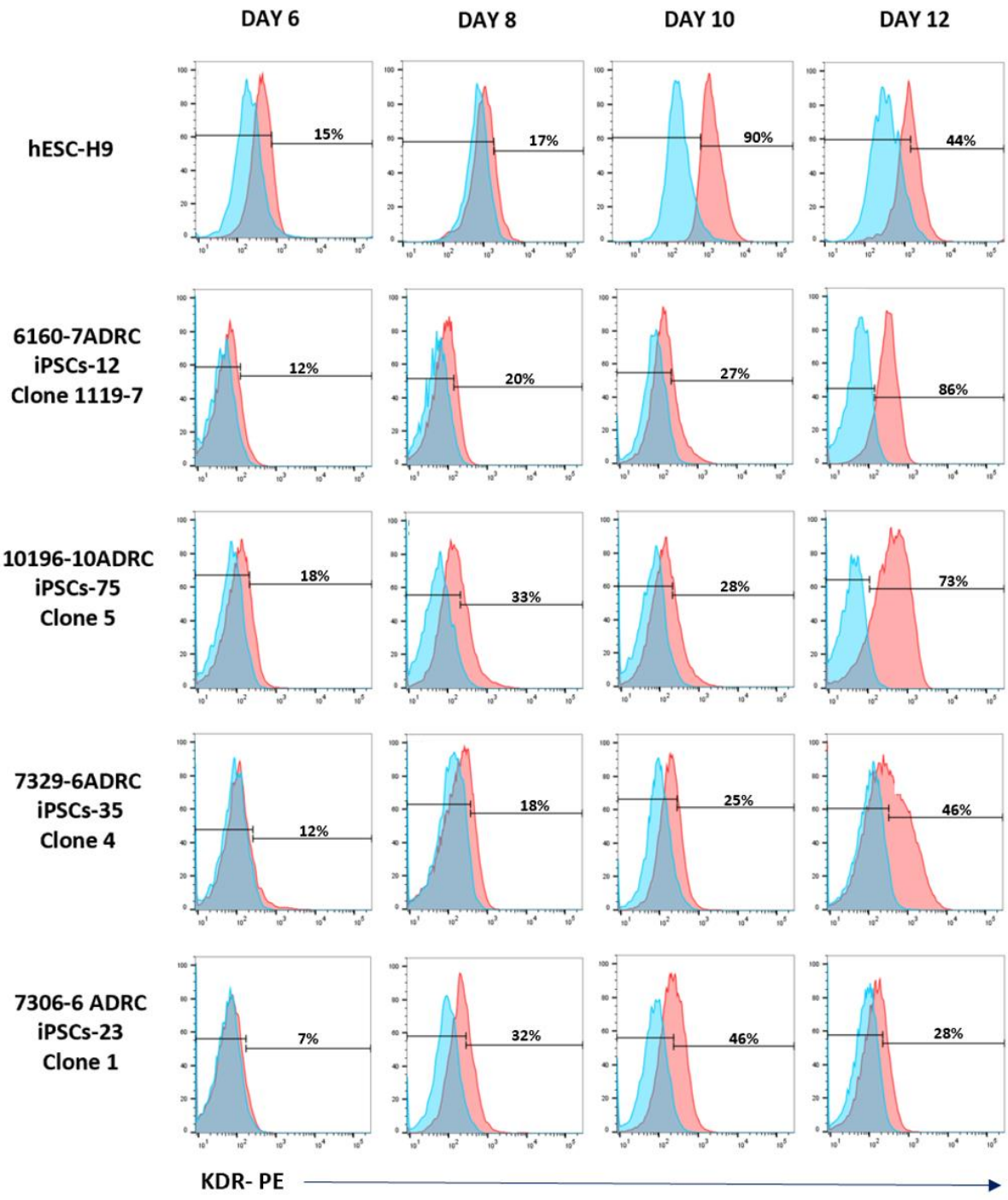


Figure 3.4. Representative histograms of the KDR(+) expression of hESC-H9, 6160-7ADRC iPSCs-12 Clone 1119-7, 10196-10ADRC iPSCs-75 Clone 5, 7329-6ADRC iPSCs-35 Clone 4 and, 7306-6 ADRC iPSCs-23 Clone as they change over time.

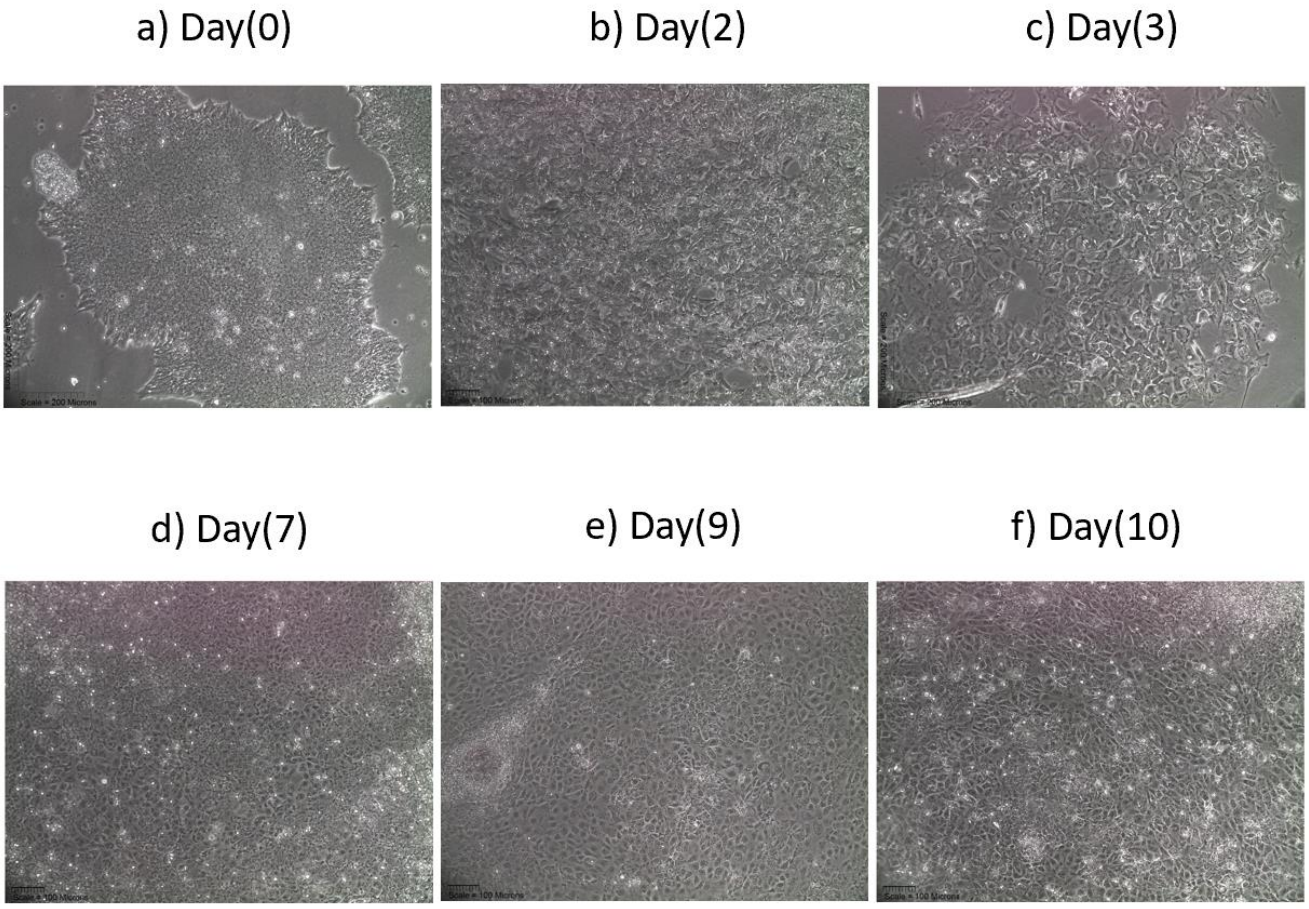


Figure 3.5 . Phase-contrast images of hPSCs at different stages of differentiation.

Table 3.1. Medium components for Induction medium

Stage 1: Induction Medium

Composition	Volume (100 ml)	Final Concentration
Alpha MEM	77 ml	
20% KSR	20 ml	20%
Non- Essential Amino Acids	1 ml	1%
Penicillin- Streptomycin	1 ml	1%
L- glutamine (2 mM)	1 ml	1%
dBME (100 mM)	50 µl	0.05 mM
VEGF (50 µg/ml)	30 µl	15 ng/ml
BMP4 (50 µg/ml)	10 µl	5 ng/ml

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Chapter 4

Generation, expansion and functional characterization of human induced pluripotent stem cell derived endothelial cells

Abstract

Human endothelial cells (ECs) are crucial to the progress of research and development in areas of, advanced therapies, drug development, regenerative medicine, modelling pathophysiology of diseases, studying vascular development mechanisms such as angiogenesis and vasculogenesis and, tissue engineering of small scale organoids to larger multicellular organ systems. Human pluripotent stem cells have been differentiated to endothelial cells in vitro by genetic manipulation and immortalization, using embryoid bodies or by monolayer cultures. However, most derivation protocols have been developed for only a specific stem cell line or were highly inefficient in generating homogenous cultures and large number of mature endothelial cells required for clinical applications. We set forward to design a differentiation protocol that was robust across different human pluripotent stem cell lines. We previously optimized the time point for KDR+ cell sorting for multiple human pluripotent stem cell lines. Here the KDR+ vascular progenitor cells were further directed towards a mature functional VE-Cadherin+ endothelial phenotype generating a homogenous culture of highly proliferative endothelial cells over a period of 30 days. In comparison to other reports VE-cadherin positive peaks were seen considerably later and took significantly more time than generating intermediate populations of vascular progenitor cells. This three-stage endothelial differentiation protocol utilizes complete chemically defined reagents eliminating use of any animal derived products such as bovine serum or calf components therefore limiting any batch to batch variability and closely regulating differentiation signals.

4.1 Introduction

All organ systems in the human body require large volumes of functioning blood vessels running through tissues. The lumen of blood vessels is lined by endothelial cells that has crucial functions in studying vascular development mechanisms and, tissue engineering of small-scale organoids to larger multicellular organ systems. A commonly available and widely researched endothelial cell source are human umbilical vein endothelial cells (HUVECs). Some other commonly used primary endothelial cells include microvascular endothelial cells, aortic endothelial cells, colony-forming cells (ECFCs) or endothelial progenitor cells (EPCs) collected from blood, and blood brain barrier endothelial cells isolated from brain tissue. Major disadvantages associated with using primary endothelial cells include collection from human tissues and the tendency of primary cells to lose their endothelial specific phenotype after a few passages. The immense differentiation capacity of human pluripotent stem cells can provide with all cell types in the human body, thus creating an infinite pool of stem cells. Endothelial cells have been successfully derived from hPSCs by several independent research groups. The first protocols published were based on three-dimensional (3D) embryoid body (EB) cultures (Levenberg et al., 2002), followed by 2D cultures supported by mouse embryonic fibroblasts feeder cells. Vascular induction using these methods resulted in very low efficiencies of EC differentiation (1±3%) and were often difficult to reproduce. More recently, fully defined reagents have been used for the efficient differentiation of large number of ECs using feeder-free monolayer cultures of human ESC (Kane et al., 2010; Glaser et al., 2016) and, sprouting endothelial progenitor cells (EPC) into 3D fibrin scaffolds(Zhang et al., 2014). Methods of co-differentiation of ECs with pericytes have also been developed (Orlova et al., 2014; Orlova et al., 2014), that temporally activates the Wnt pathway by using a Wnt agonist, GSK3β inhibitor (CHIR-99021), generating cultures containing 50% CD31+/CD34+ EPC and15±25% CD31+/CD34+ EPC, respectively and up to 50% PDGFRβ mesenchymal cells in 10 days (Lian et al., 2014). It still requires longer periods of time to derive mature VE-cadherin+ ECs. It has been reported that in comparison to human embryonic stem cell0derived endothelial cells, human induced pluripotent stem cell-derived endothelial cells display limited ability to proliferate in in vitro culture systems (Li et al., 2011).

Another drawback in the field is that the existing differentiation strategies have only been optimized for specific stem cell lines and cannot be readily used with other cell lines.

Here, we present a differentiation methodology specifically designed for robustness across multiple human iPSC lines producing actively proliferating large number of endothelial cells. We developed a three-stage protocol with 2 stages of cell sorting: early purification of KDR+ vascular progenitor cells, as well as, later VE-cadherin+ cell sorting of ECs. We report the differentiation of vascular progenitor cells to mature functional endothelial cells positive for various endothelial specific markers followed by cryopreservation and expansion while maintaining endothelial specific phenotype for functional applications for up to 8–10 passages.

4.2 Reagents and equipment setup

Reagents used in chapter 2 and 3 are not listed here.

1. Dulbecco's Modified Eagle Medium: High glucose DMEM (Gibco™catalogue # 11965092)
2. Nutridoma™-CS **(**Roche catalogue # 11363743001**)**
3. Recombinant Human Fibroblast Growth Factor (FGF-basic 146 a.a.) (50 μg, Peprotech, catalogue # 100–18 C)

Preparation of* bFGF (25 *μg ml*^{1} *stock solution). The contents of the vial were reconstituted to a final concentration of 25 μg ml^{1} in PBS containing 1% (wt/vol) BSA. We made aliquots of 50 μl to avoid repeated freeze-thaw cycles and stored the aliquots at −80°C for up to 1 year.

4. TGF-β RI Kinase Inhibitor VI, SB431542- CAS 301836-41-9 – Calbiochem (5MG, Sigma-Aldrich, catalogue # 616461).

Aliquoting* SB431542 *for long-term storage. We reconstituted 5 mg of SB431542 into 1.3 ml of DMSO to make a 10 mM stock solution. We made aliquots of 100 μl to avoid repeated freeze-thaw cycles and stored the aliquots at −20°C indefinitely.

5. Fetal Bovine Serum (FBS)-Heat Inactivated (500-ml; Gibco™ catalogue # 10082-147)

6. Endothelial Cell Growth Medium BulletKit EGM™ (500-ml; LONZA catalogue #: CC-3124)
7. Dimethyl sulfoxide (DMSO) (100ML; Sigma-Aldrich, catalogue # 276855)
8. Triton X-100 (50ML; Sigma-Aldrich, catalogue # T8787).

0.5% Triton solution. 5 ml Triton was reconstituted in 1 L PBS and mixed for 10 minutes.

9. Paraformaldehyde (PFA) (1 KG Millipore Sigma catalogue # 1040051000**).**

4% aqueous PFA. 4 g PFA was reconstituted in 100 ml PBS. Store for up to 1 month in the dark at 4°C.

10. DAPI nucleic acid stain (10 MG; Invitrogen, catalogue # D1306)
11. Anti-human CD144 (VE-Cadherin) Antibody (PE/Cy7) 1:100 (BioLegend, catalogue # 34851)
12. Anti–human von Willebrand Factor Domain (vWF)-A2 Domain Antibody 1:50 (R&D Systems, cat. no. RB01)
13. Anti–human ephrin-B4 (PE) 1:200 (LifeSpan BioSciences, Inc. catalogue # LS-C486174-25)
14. Anti–human ephrin-B2 (FITC) 1:100 (SANTA CRUZ BIOTECHNOLOGY, catalogue # sc-398735 FITC)
15. Anti–human CD184 (CXCR4) (PE) 1:100 (BioLegend, catalogue # 306505)
16. Anti–human CD31(PECAM-1)-allophycocyanin (APC) 1:100 (eBioscience, catalogue # 17-0319)
17. Image-iT FX Signal Enhancer (Invitrogen catalogue # I36933)
18. Low Density Lipoprotein, Human Plasma, Acetylated, Alexa Fluor™Conjugate (Alexa Fluor™ 488 AcLDL) 1: 100 (Invitrogen, catalogue# L23380)

4.3 Methods

4.3.1 Differentiation of vascular progenitor cells to endothelial cells

Vascular progenitor cells were generated and purified for each cell line as described in Chapter 3. After sorting the VPCs were collected and centrifuged at 200×g at room temperature for 5 minutes. The supernatant was removed and the VPC pellet was

resuspended in 1 mL of Endothelial Specification Medium (Table 4.2). The cells were re-plated in 50 μg/ml fibronectin precoated 100-mm culture plates. The cells were plated at density of 20,000–30,000 cells/cm^2 and the total number of cells collected was determined from the sorting events in the FACS cell sorter. To each 100-mm culture dish 8 ml of Endothelial Specification Medium was added, and the dish was placed for the next 4 days in a 37°C incubator. Four days after cell sorting, spent media is replaced with new medium and cultured further at 37°C. Seven days after cell sorting, the Endothelial Specification Medium was supplemented with 10 μmol/ml SB431542 (Table 4.3). From then onwards full medium changes were done every 72 hours with Endothelial Specification Medium supplemented with 10 μmol/ml of SB431542. During culture if the cells reached 80% confluence the cells were passaged at a density of 10,000 cells/cm^2 by enzymatic dissociation technique using TrypLE.

4.3.2 Purification of VE-Cadherin+ endothelial cells

Between days 24 to 26, after start of induction the VE-cadherin+ endothelial cells are purified using FACS sorting. The culture medium was aspirated, and the cells were washed with 6 ml of PBS per 100-mm tissue culture dish. To each dish 6 ml of TrypLE was added the cells were placed at 37°C in a humidified 5% CO_2/95% air incubator for 5 minutes. The cells were then viewed under the microscope. As soon as most of the cells detached and were floating, we neutralized the enzyme solution. The incubation time is approximately 5 minutes. For neutralizing TrypLE 6 ml of DMEM to each 100-mm plate and the solution was pipetted up and down several times. The solution was then washed over the edge and bottom of the plate so all cells could be removed. For flow cytometry purposes it is very important to dislodge the cell clumps into single cells. The cell suspension was transferred to a 15-ml centrifuge tube and centrifuged at 200×g room temperature, for 5 minutes. Each ~ 80% confluent 100-mm cell culture dish yields approximately 1.5–2 × 10^6 cells. The supernatant is aspirated from the cell pellet followed by resuspension of the cell pellet DMEM containing 0.5% wt/vol BSA. Cells from each 100-mm plate was suspended in 200 μl of DMEM and this ratio was scaled up based the starting number of induction plates. Human BD Fc Block was then added to the cell suspension at 0.5 μg/ml concentration and incubated at room temperature for 15 minutes. Fixable Viability Dye eFluor 780 was then

added to the cell suspension at 1 µl/ml concentration and incubated at room temperature in the dark for 10 minutes. A cell suspension aliquot of 50 µl was transferred into another 15-ml centrifuge tube and the tube was labelled as "cells only". Another A cell suspension aliquot of 50 µl was transferred into another 15-ml centrifuge tube and the tube was labelled as "isotype control". The initial centrifuge tube was labelled as "VE-cadherin+ cell". To the tube labelled "VE-cadherin+ cell" we added anti-human CD144/VE-cadherin PE-Cyanine7 monoclonal (eBioscience) at a concentration of 1 µl/10^6 cell or 1 µl /100 µl cell suspension and then vortexed the cell suspension. To the tube labelled "isotype control" we added PE-Cyanine7 Mouse IgG1, k Isotype Ctrl (eBioscience) at concentration of 1 µl/10^6 cell or 1 µl/100 µl cell suspension and then vortexed the cell suspension. All three tubes were placed in the dark on ice or at 4°C and incubated for 30 min. After antibody incubation 4 ml BSA buffer solution was added to all tubes and the cell suspensions were centrifuge at 200×*g* at 4°C for 4–5 minutes. The supernatant was removed, and the cell pellet was washed additional two times using BSA buffer solution in the same manner. Washing was necessary to remove any unbound free-floating antibodies that would give false positive results during FACS sorting. The cell suspension was resuspended in DMEM after the last was at concentration of 1 × 10^6 cells/100 µl (the approximate cell density was determined from the initial number of 100-mm induction plates used). The cell suspension was pipetted up and down ensure the cells were evenly dispersed in the suspension. Any cell population that was observed to be clumpy and adhesive was filtered through a strainer prior to cell sorting so that the clumps do not clog up the sorter. The cell suspensions were transferred 5-ml round bottomed polystyrene FACS tube. A 15-ml centrifuge tube was filled with Endothelial Expansion Medium (Table 4.4) and this tube was used to collect the sorted cell populations. The cells undergo severe mechanical and chemical stress during the sorting process and collecting the cells in serum enriched medium like FBS can help the cells survive through the shock. However, we did not use serum at all because our goal was to develop a completely chemically defined differentiation protocol. Generally during the purification step of VE-cadherin+ cells a distinct subpopulation of VE-cadherin+ expressing cells 70−80% of the total cell population is observed. The brightest VE-cadherin+ cell population was sorted and collected in the collection tube containing cold Endothelial Expansion Medium.

4.3.2 In vitro derived endothelial cell culture and expansion

After sorting the VE-cadherin+ cells were collected in a collection tube which was centrifuged at 200×g at room temperature for 5 minutes. The supernatant was removed, and the cell pellet was resuspended in 1 mL of Endothelial Expansion Medium (Table 4.4). The cells were re-plated in 50 μg/ml fibronectin precoated T-75 flasks. The cells were plated at density of 10,000 cells cells/cm^2 and the total number of cells collected was determined from the sorting events in the FACS cell sorter. To each 100-mm culture dish 12 ml of Endothelial Expansion Medium was added, and the dish was placed in a 37°C incubator. Spent medium was replaced with fresh medium every 3 days. The cells were passaged when the culture reached 70–80% confluence in approximately every 4-6 days. During passaging the total cell number was counted and the cells were seeded at a density of 5000 cells/cm^2 onto 10 μg/ml fibronectin pre-coated flasks. During endothelial cell expansion the cells were either frozen or plated onto subsequently larger and larger containers (T-75 cm^2 flask, then T-175 cm^2 flask, and then multiple T-175 cm^2 flasks). In order to verify that the cells retained their endothelial specific phenotype during expansion the cultures were regularly tested for expression of endothelial markers such as VE-Cadherin and PECAM1 following every 3-4 passages.

4.3.3 Fluorescent Imaging

On to 10 μg/ml fibronectin pre-coated glass coverslips we seeded 40,000 cells/cm^2 endothelial cells and fed with Endothelial Expansion Medium. The cells were cultured until the culture reached confluence. Then the medium was aspirated, and the coverslips were washed with PBS. The cells were fixed by adding 4% PFA solution to the culture at room temperature and incubated for 5–10 minutes. The coverslips were then washed with PBS two times. If necessary, the fixed cells in the coverslip samples could be preserved for as long as 1 week at 4°C until the cells were stained with antibodies. For permeabilizing the cell membrane 0.5% Triton was added to fixed cells and incubated for 3–5 min at room temperature. Then for blocking the cells to prevent unspecific binding of conjugated secondary antibodies the samples were incubated with 5–10% normal donkey serum in

0.5% BSA buffer at room temperature for 1 hour. The staining solutions were prepared by diluting the primary antibodies with 0.5% BSA buffer at desired concentrations (specific to each antibody and is listed in the reagents section). Staining solutions were added to the coverslips in volume enough to cover the cell surface, and the coverslips were incubated at 4°C overnight. The next day the coverslips were rinsed with PBS thrice. The secondary antibody solution was then added to the coverslips. The secondary antibody solution was prepared by diluting at a ratio of 1:200 in 0.5% BSA. Negative controls were prepared by adding only secondary and no primary antibody to coverslips with cells. After adding the secondary antibody, the coverslip samples were incubated for 30 min at room temperature and then rinsed with PBS thrice. To counterstain cell nuclei DAPI was added to the coverslips at a dilution of 1:1000 in PBS. In order to preserve the coverslip samples for longer periods of time we attached them to glass slides using fluorescent mounting media. In this way the stained coverslips could be stored at 4 °C in the dark for up to 1 year.

4.3.4 Matrigel tube-formation assay for vascular cells

To each well of a 96 well culture plate 100 µl of Corning Matrigel™ Matrix was very carefully added to prevent formation of any trapped bubbles. If there were any trapped air bubbles the plate was centrifuged in a 4°C precooled centrifuge for 10 min at 300x*g*. All the tips and culture plates were precooled in ice before adding Matrigel. The plate was then placed in an incubator for 30-60 minutes, so the Matrigel could form a gel layer. The remaining liquid supernatant was then gently aspirated out of the plate. To each well we added 200 µl of Endothelial Expansion Medium and seeded 30,000 human pluripotent stem cell derived endothelial cells. The plates were placed in a 37°C incubator overnight. The plates were imaged in the next 24 and 48 hours.

4.3.5 Low density lipoprotein (LDL)-uptake

Endothelial cells were plated at a density of 40,000 cells/cm^2 onto 24 well plate pre-coated with 10 µg/ml fibronectin using in 500 µl Endothelial Expansion Medium per well. The cells were cultured until the cell confluency reached ~80%. The spent medium was

aspirated, and the cells were washed with PBS. Alexa Fluor 488 acetylated low- density lipoprotein (LDL; Invitrogen) was diluted at a ratio of 1:100 in DMEM with high glucose (Invitrogen) and added to the cell culture. The cell culture plate was incubated for 5 hours in a 37°C incubator. The cells were then rinsed with PBS thrice. The cell nucleus was counterstained with DAPI by diluting DAPI at a concentration of 1:1000 in PBS, and the plate was incubated at room temperature for 10 minutes. The cells were washed with PBS twice and were imaged using a fluorescent microscope.

4.3.6 Freezing and thawing of human pluripotent stem cell derived endothelial cells

The endothelial cells were cryopreserved using the following freezing medium: 40% Endothelial Specification Medium, 50% FBS, 10% DMSO (Table 4.5). Cryopreservation retains cell banks until the cells are used again. The cells were frozen down at a concentration of 1–2 × 10^6 cells per ml in freezing medium. When the cells reached ~80% confluence the spent medium was aspirated from the cell culture flask and the flask washed with PBS. TrpLE was added to the culture for cell dissociation. After 5 minutes DMEM was added to in equal volumes to stop the enzymatic reaction and the cell suspension was centrifuged for 5 minutes. The supernatant was aspirated out and the cell pellet was re-suspended in freezing medium. Each cryovial was filled with 1 mL of cell suspension in freezing medium. For the next 24 hours the cryovials were stored in −80°C. For long term storage the cryovials were moved to a liquid N2 tank. The frozen cryovials were thawed carefully in a bead bath at 37°C. A 15-ml tube was filled with 5 ml cold Endothelial Expansion Medium and the thawed cell suspension was gently transferred to the tube. The cell suspension was centrifuged at room temperature for 5 minutes. The supernatant was aspirated out and the cell pellet was re-suspended in Endothelial Expansion Medium. Cells from one cryovial was plated onto one 10 μg/ml fibronectin pre-coated T-75 flask.

4.4 Results and Discussion

The following human pluripotent stem cell lines- hESC-H9, 6160-7ADRC iPSCs-12 Clone 1119-7, 10196-10ADRC iPSCs-75 Clone 5, 7329-6ADRC iPSCs-35 Clone 4 and, 7306-6 ADRC iPSCs-23 Clone 1 were differentiated towards a mesodermal lineage and the

optimal time point when the culture should be purified to generate KDR+ vascular progenitor cells was determined. Adding an initial KDR purification stage appears to be a tradeoff of process complexity vs. efficiency whereas existing protocols require only one sorting stage, generally on an EC marker at the end of differentiation. However, we observed that when the cells were not purified for KDR+ cells in Stage1, VE-Cadherin expression remained very low in Day 25, undifferentiated stem cells and contamination cell populations may have interfered with endothelial cell differentiation. When the cells were then sorted for VE-Cadherin on Day 25 in Stage 1, the cells did not exhibit endothelial specific phenotype and continued to express very low levels of VE-Cadherin after further expansion in culture (Figure 4.6a) After FACS purification step the KDR+ cells were plated in Endothelial Specification Medium (Table 4.3). Sorting induces stress to the cells and can cause cell death, therefore the cells were seeded in higher numbers 20,000–30,000 cells/cm^2 in comparison to routine passaging. Additionally, in the first 4 days after sorting the plate was not moved and medium change was not performed allowing cells to recover and attach to the tissue culture plate. Endothelial Specification Medium was supplemented with 10 μmol/ml SB431542 one week after culturing the vascular progenitor cells. SB431542 is a small-molecule inhibitor that interferes with transforming growth factor-β (TGF-β) activity by inhibiting activin receptor–like kinase-4, activin receptor– like kinase-5 and activin receptor–like kinase-7(ALK-4, ALK-5 and ALK-7, respectively) signaling pathways and thus supports endothelial specification and proliferation. When the culture was ~80% confluent the cells were passaged and plated at a density of 10,000 cells/cm^2. The vascular progenitor cells were directed towards mature endothelial phenotype and most of the cell population was positive for endothelial specific VE-Cadherin surface marker. However, the culture may still be populated by a small number of contaminating cell types such as undifferentiated stem cells or smooth muscle cells and these cells can interfere with endothelial cell differentiation and proliferation. If the endothelial cells were not purified the contaminating cell types outnumbered the endothelial cells and dominated the culture. Between days 24 to 26 of total differentiation ~70- 90% of the total cells was positive for the VE-Cadherin surface protein and this marker was used as a target to derive a purified population of mature endothelial cells by flow activated cell sorting (FACS) technique. The purification step can be stressful to cells and can lead to cell death however

the derived endothelial cells could not tolerate heterogeneous cell types and therefore this step was required to obtain an enriched endothelial cell population. The VE-cadherin+ endothelial cells were highly proliferative and were positive for endothelial specific markers such as CD31 (PECAM-1), VE-cadherin and, Von Willebrand Factor in levels similar to the primary endothelial cell HUVECs. The negative control cells HuAoSMC were negative for the endothelial specific markers (Figure 4.4 a). The derived endothelial cells formed vessel-like tubes on Matrigel and took up low density lipoprotein (Figure 4.4 b & c). During derived endothelial cell culture and maintenance, the cells were passaged before reaching > 70- 80% confluence. The cells were seeded in a density of 5000 cells/cm^2. When the cells reached 100% confluence it was observed that there was a fall in endothelial specific marker expression this may be because of lack of space for cell proliferation within the tissue culture flask may cause selection pressure to induce phenotypic alterations or cell dedifferentiation. It was observed that the growth kinetics of the specific stem cell line was correlated to the yield of the derived endothelial cells. For example the highly proliferative stem cell lines hESC-H9, 6160-7ADRC iPSCs-12 Clone 1119-7 and, 10196-10ADRC iPSCs-75 Clone 5 yielded ~2.5 X 10^6, 1.5 X 10^6 and, 3×10^6 ECs/ 100-mm plate, respectively, whereas the stem cell lines 7329-6ADRC iPSCs-35 Clone 4 and 7306-6 ADRC iPSCs-23 Clone 1 had a lower proliferation rate and yielded ~6.5 X 10^5 and 6×10^5 ECs/ 100-mm plate , respectively (Table 4.1). The derived endothelial cells retained endothelial specific phenotype for as long as 8–10 population doublings (Figure 4.6 b).

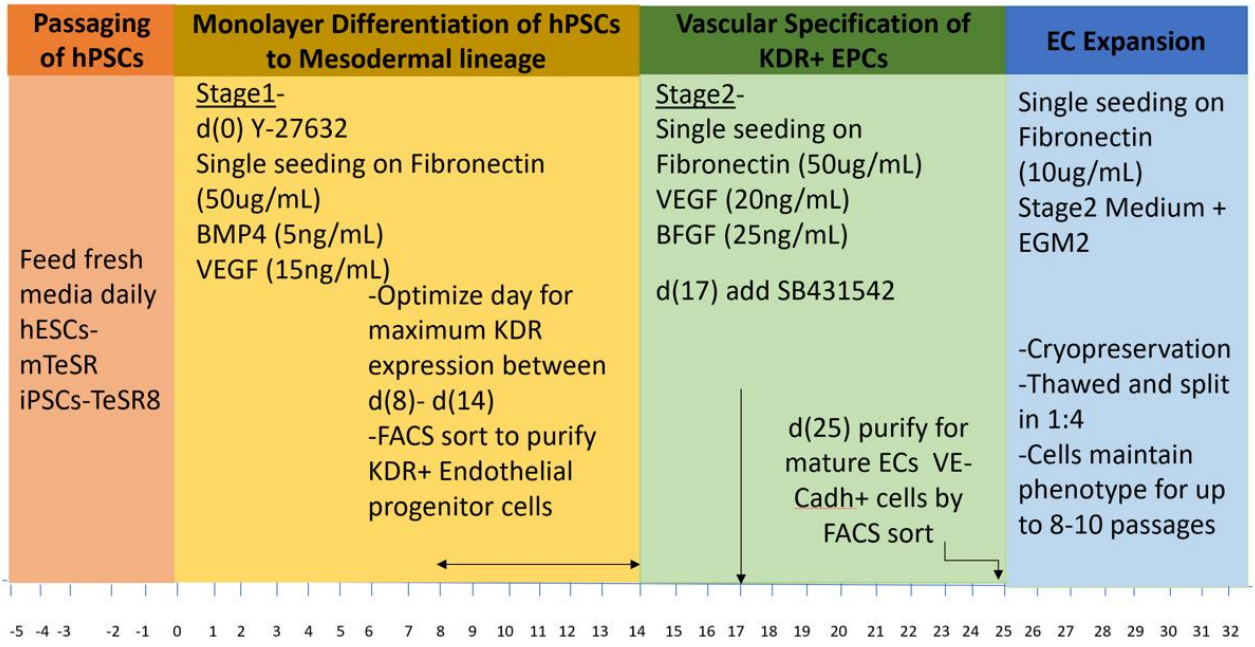

Figure 4.1. Timeline of Protocol procedures.

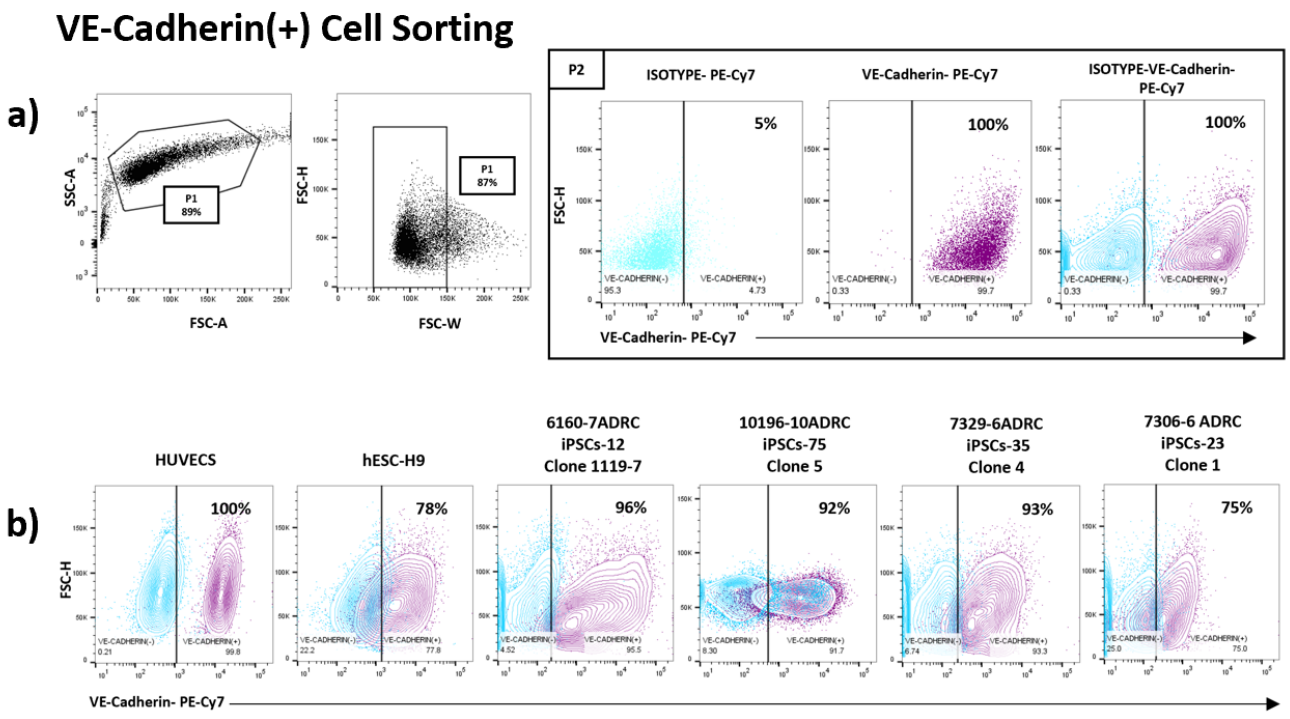

Figure 4.2. Flow cytometric analysis of KDR and VE-Cadherin expression at Stage 2 of differentiation, of multiple hPSC lines. (a) Representative Isotype-PE-Cy7 and VE-Cadherin-PE-C7 samples; gated from the live cell population (P1: FSC, forward scatter; SSC, side scatter) and single cell population (P2: FSC-W, forward scatter width; FSC-H, forward scatter height). ***(b)*** *Percentage of VE-Cadherin(+) cells* of multiple hPSC lines. Percentages of positive cells were calculated based on the isotype controls (4%- 5%; blue scatter plot).

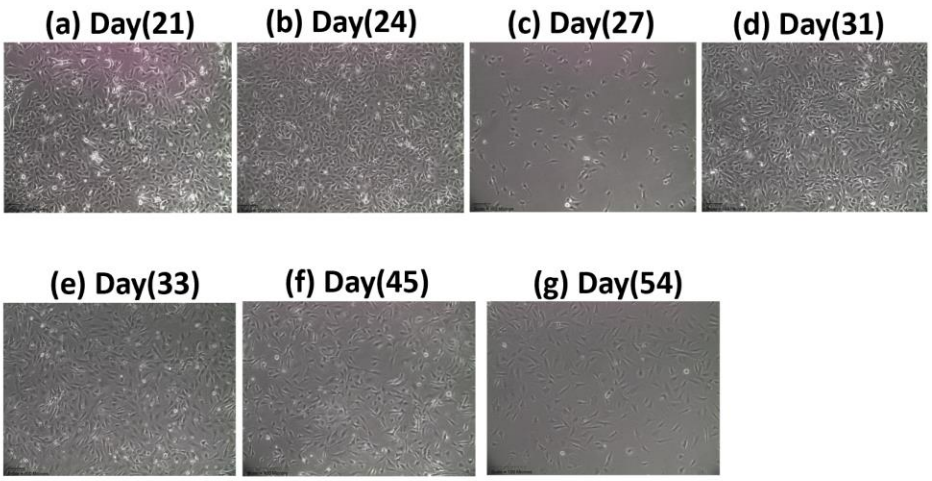

Figure 4.3. Phase-contrast images of hPSCs derived endothelial cells. (a & b) derived endothelial c
before VE-Cadherin(+) purification at different stages of differentiation (c, d & e) derived endothelia
cells in culture after VE-Cadherin(+) purification (f & g) derived endothelial cells in culture after
cryopreservation and thawing.

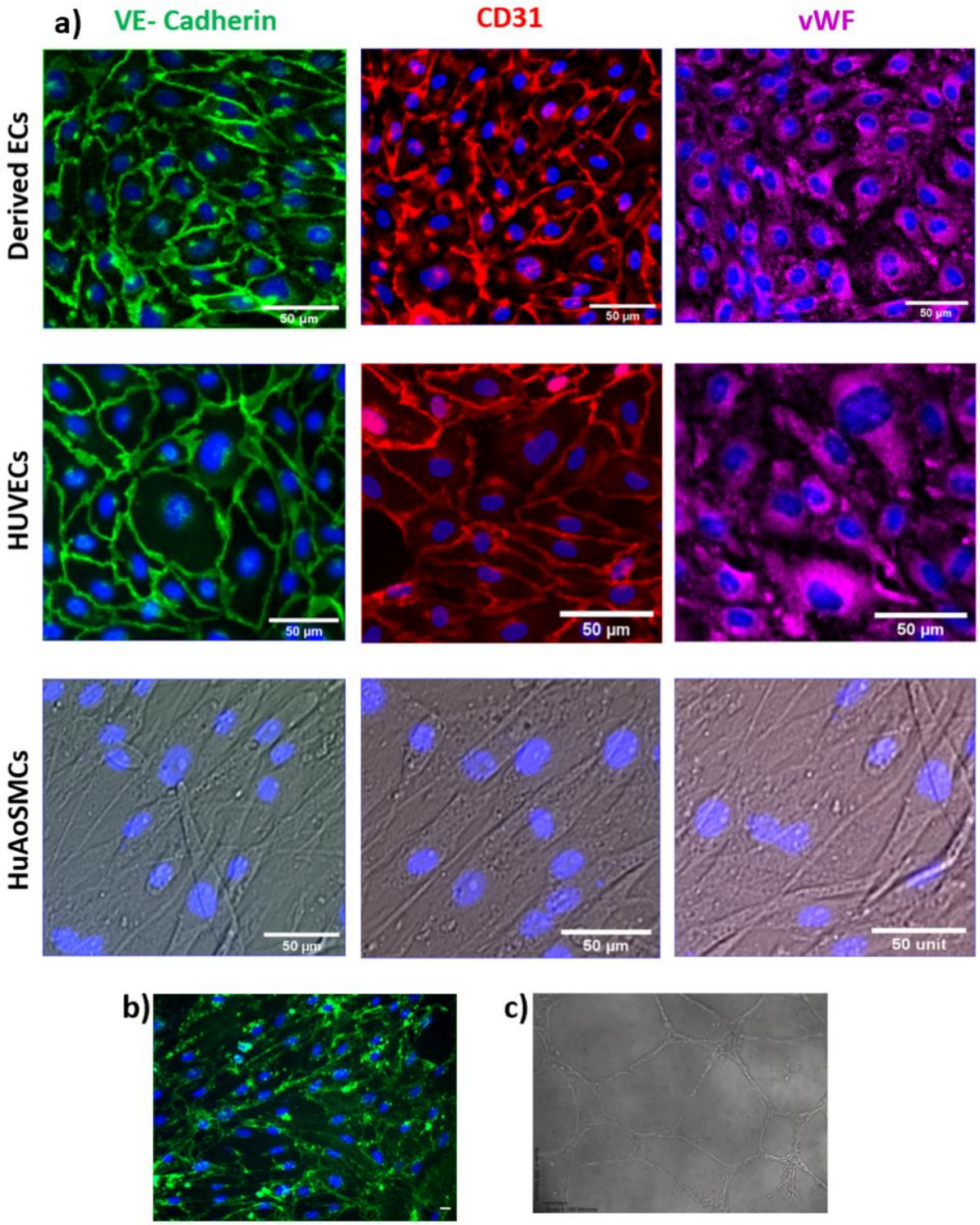

Figure 4.4. Characterization of ECs. (a) Representative confocal images of VE-cadherin, (in green), CD31 (in red), Von Willebrand (in magenta) and DAPI (in blue) of hPSC-derived ECs, HUVECs, and HuAoSMCs. ***(b, c)*** *Derived ECs* ***(b)*** took up acetylated LDL. Scale bars, 50 µm. ***(c)*** formed vascular-like structures in Matrigel™ within 24.

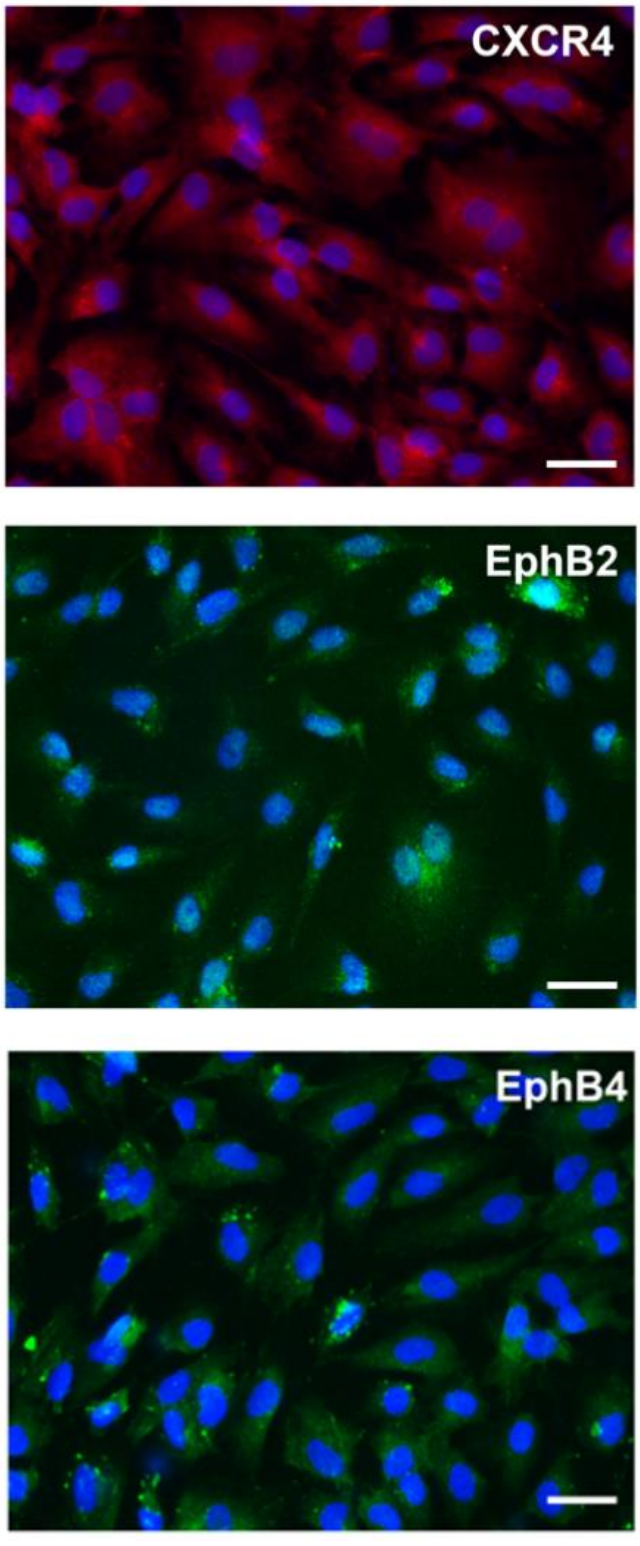

Figure 4.5. Characterization of ECs. Representative confocal images of, EphrinB2, EphrinB4 and, VE-cadherin, (in green), CXCR4 (in red) and DAPI (in blue) in hPSC-derived ECs. Scale bars, 50 µm

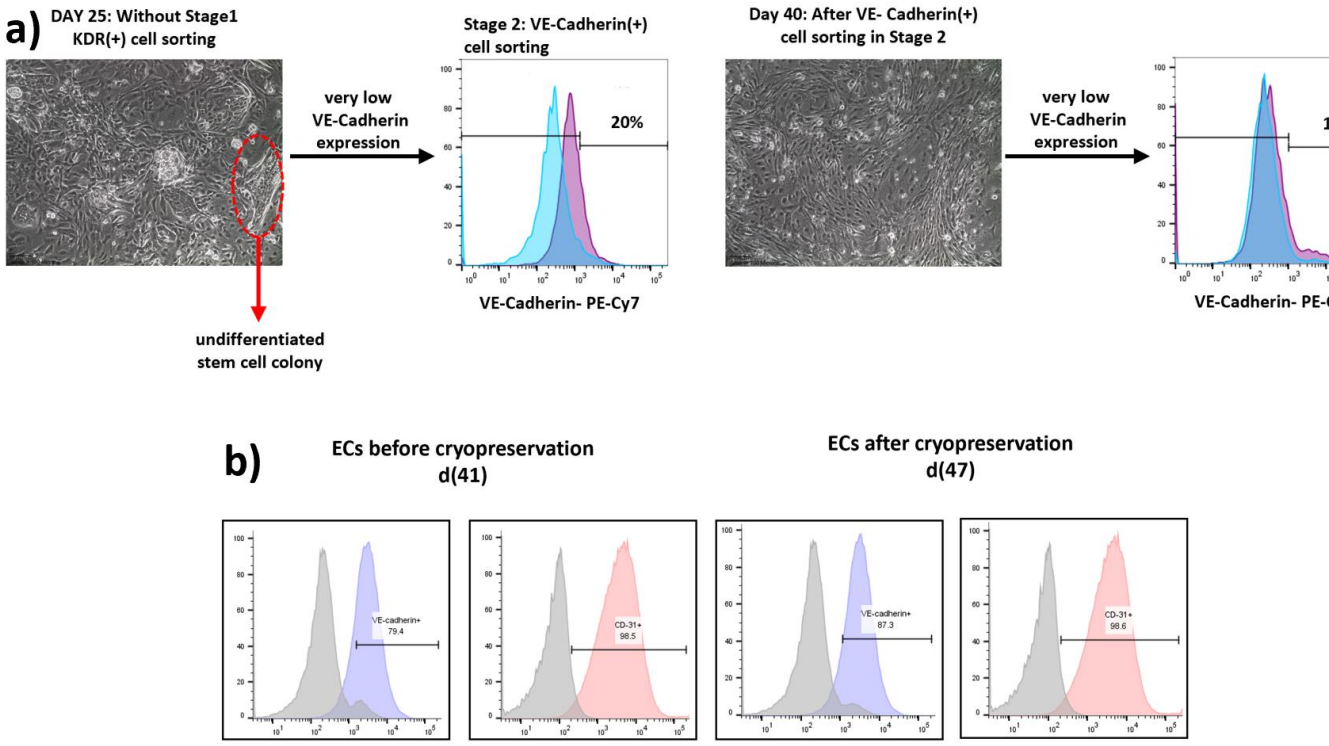

Figure 4.6. (a) hPSCs could not be differentiated to EC phenotype without the initial KDR sorting stage, V Cadherin expression in Day 40 remains very low after sorting for EC marker in Day 25. ***(b)*** Flow cytomet analysis of derived ECs, showing histograms of VE-Cadherin and CD31 expression, before and af cryopreservation on day 41 day 47, respectively.

hPSC line	Stem Cell Passage Time/ Days	Seeding Density Stage1/ cm^2	Percentage of KDR+ cells	Percentage of VE-Cadherin+ cells Day 25	EC Yield
hESC-H9	4	20,000	Day 10 ~80- 90%	~70- 80%	~2.5 X 10^6
6160-7ADRC iPSCs-12 Clone 1119-7	5	20,000	Day 12 ~80- 90%	~85- 95%	~1.5 X 10^6
10196-10ADRC iPSCs-75 Clone 5	3	20,000	Day 12 ~70- 80%	~85- 95%	~3.0 X 10^6
7329-6ADRC iPSCs-35 Clone 4	7	20,000	Day 13 ~60- 70%	~85- 95%	~6.5 X 10^5
7306-6 ADRC iPSCs-23 Clone 1	7	20,000	Day 9 ~50- 60%	~75- 85%	~6.0 X 10^5

Table 4.1

Summary of Stem Cell Line-Specific Endothelial Differentiations. The table lists the specific stem cell expansion conditions, initial seeding density, pre-optimized time point and percentage of the presorted KDR+ VPCs, the final VE-cadherin+ EC purity and yield for each of the human pluripotent stem cell lines.

Table 4.2. Medium components for Vascular Specification Medium

Stage 2: Vascular Specification Medium

Composition	Volume (100 ml)	Final Concentration
Alpha MEM	64 ml	
High Glucose DMEM	28 ml	
Nutridoma	4 ml	4%
Non- Essential Amino Acids	1 ml	1%
L- glutamine (2 mM)	1 ml	1%
Penicillin- Streptomycin	1 ml	1%
dBME (100 mM)	50 µl	0.05 mM
VEGF (50 µg/ml)	40 µl	20 ng/ml
bFGF (25 µg/ml)	100 µl	25 ng/ml

Table 4.3. Medium components for Vascular Specification Medium

Vascular Specification Medium SB431542 substituted

Composition	Volume (100 ml)	Final Concentration
Alpha MEM	64 ml	
High Glucose DMEM	28 ml	
Nutridoma	4 ml	4%
Non- Essential Amino Acids	1 ml	1%
L- glutamine (2 mM)	1 ml	1%
Penicillin- Streptomycin	1 ml	1%
dBME (100 mM)	50 µl	0.05 µM
VEGF (50 µg/ml)	40 µl	20 ng/ml
bFGF (25 µg/ml)	100 µl	25 ng/ml
SB431542 (10 mM)	200 µl	20 µM

Table 4.4. Endothelial Expansion Medium

Endothelial Expansion Medium

Composition	Volume (100 ml)	Final Concentration
Vascular Specification Medium SB431542 substituted	50 ml	50%
EGM2 w/o FBS	50 ml	50%

Chapter 5

Conclusion

Tissue engineering aims to make functional tissue that supports tissue regeneration in the body and meet the growing needs of organ transplantation [18]. A major challenge in developing tissue engineered organs is the lack of a functional vasculature to retain viability of the constructs and to allow integration with the host vasculature after transplantation. Blood vessels are lined by endothelial cells and supporting cell types. Therefore, EC research will help to advance development of invitro derived organs. Human pluripotent stem cells can provide with all cell types in the human body, serving as an infinite pool of autologous cells for patients. Additionally, Patient derived iPSCs are optimal for applications in patient specific cellular therapy, drug discovery and "disease-in-a-dish" cellular research. The aim of this project was to optimize a protocol for robust and efficient differentiation of functional ECs from multiple human iPSC lines and create a bank of iPSC derived ECs.

The McCloskey laboratory has extensive experience in differentiation of stem cells to mature endothelial cells and strives to further characterize vascular differentiation. Blancas et al. differentiated the murine mESC line R1 to functional endothelial cells using chemically defined medium (1). Drew et al. worked with single factors affecting differentiation of stem cells towards endothelial fate, using the mESC lines R1 and A3, and hESC lines H7 and H9. However, the yield of hESC derived ECs were low (2). My work focused on optimizing our existing differentiation protocol to generate ECs from human pluripotent stem cells robustly and efficiently.

In this present work, multiple Alzheimer's patient derived hIPSCs were expanded in culture. The following human pluripotent stem cell lines- hESC-H9, 6160-7ADRC iPSCs-12 Clone 1119-7, 10196-10ADRC iPSCs-75 Clone 5, 7329-6ADRC iPSCs-35 Clone 4 and, 7306-6 ADRC iPSCs-23, were successfully differentiated towards a reasonably pure culture of highly proliferating mature endothelial cells over a period of 30 days. Generally, the growth factors, medium and differentiation strategy were the same for all the hESC and iPSC cell lines, however since the growth kinetics was moderately different for each of the

cell line, we needed to optimize the time form KDR+ VPC purification in Stage1.The hPSCs were initially cultured in fibronectin coated plates using, Stage 1 Mesodermal Induction medium substituted with the growth factors BMP4 and VEGF. The time for sorting KDR+ cells was optimized for all the hPSC lines between 6-12 days of differentiation. The purified VPC populations were cultured in fibronectin coated plates using, Stage 2 Vascular Specification medium substituted with the growth factors bFGF and VEGF. After one week the medium was substituted with SB431542. Between days 24-25 of differentiation, cultures were purified for VE-cadherin+ cells and ~70- 90% of the total cells was positive for the cell-cell junction protein. The derived ECs expressed the surface markers VE-cadherin (CD144), CD31 (PECAM-1), and Von Willebrand Factor like HUVECs. The derived ECS also took up low density lipoprotein and formed tubes on Matrigel. The yield of derived ECs was in the range of 6×10^5 and 3×10^6 (per 100mm plate) and the derived cells retained EC specific phenotype for as long as 8–10 population doublings.

Challenges

Optimizing endothelial cell derivation from hPSC lines is very challenging. The derivation technique and the derived ECs must be handled with great care requiring specific training for culturing, passaging and maintaining cell viability during process steps such as the purification steps. Alterations to process steps and handling techniques including change in operator can affect cell survival and cell fate. For example, it was best to use prepared medium within 1 week of preparation to prevent the growth factors from degrading. The differentiation methodology consists of two rounds of FACs sorting and this step is very stressful to the cells. It was crucial to minimize time during antibody staining and sorting so the cells can be plated. During antibody incubation steps all solutions were placed in cold medium and ice to stop reactions within the cell suspension. For sorting purposes, the cells were broken down into single cells and this can be difficult to achieve since some cell lines were very clumpy and breaking them into single cells increased cell date. After FACs sorting the cells were plated and were not moved from the incubator to allow cells to attach to the plate. Additionally, the confluency of the cells were closely monitored throughout all the stages of differentiation, if cell confluency is too high this induces selection pressure

and alterations in cell surface protein expression and cell specific phenotype. For some of the cell lines the proliferation rate was very high and the cells needed to be passaged during Stage1 or Stage 2, this step also was optimized to prevent phenotypic alterations from overcrowding. In order to maintain reproducibility in cell surface protein expression between batches of induction plates and between different hPSC lines we started induction with 2- 100 mm plates during each induction. We observed that if we used smaller plates surface protein expression was inconsistent and did not sort enough cells that could be recovered after plating. Another major challenge was optimizing the antibodies to be used that did not give false positive or negative. Since the differentiation process was 30 days followed by expansion and freezing also including prior stem cell culture, the process was very time consuming and strenuous. Minor variations lead to inconsistency in derived EC populations therefore strict operation procedures were followed.

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