SCALABLE ENGINEERING OF IPSC-DERIVED LIVING BLOOD VESSELS

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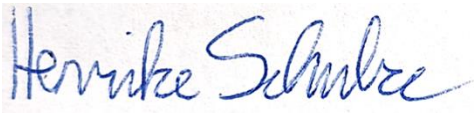
Signature: 

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Abstract

Atherosclerosis and subsequent coronary artery disease are a major cause of death in westernized society. The most frequent treatment for this prevalent disease is the implantation of venous bypass grafts. But some patients lack suitable veins, and the implanted veins are prone to develop thrombosis, occlusion, and aneurysm. Therefore, new, and improved treatment approaches are in demand. Hence, there is a huge market for artificial living blood vessels.

This research project aims at scaling up the production of functional living blood vessels from human induced pluripotent stem cells (hiPSC). Thus, we focused on producing and characterizing the required building blocks for the blood vessels. Vascular smooth muscle cells (vSMC) and endothelia cells (EC) were differentiated from hiPSC, an unlimited, renewable cell source.

A particular focus was put on the EC differentiation, purification, and characterization. Two differentiation protocols were compared and one of them was optimized regarding differentiation efficacy, cost, and time. During optimization, we were able to increase the yield from 1% to 20%, halve the cell loss during purification and decrease the production costs by nearly 50%. The EC were characterized via CD31+, CD144+ staining and a tubing assay. Using an optimized protocol, one million pure EC can be obtained from one million hiPSC within 8 days. With the vSMC differentiation protocol, eight million vSMC (CD140b+ and aSMA+) can be produced from 1 million hiPSC within 6 days.

Although further characterization of both EC and especially vSMC are required, we are able to provide sufficient cells to meet the demand for living blood vessel production.

Key Words: endothelial cell differentiation, vascular smooth muscle cell differentiation, protocol optimization

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List of Abbreviations

Abbreviation	Full Form
aSMA	alpha Smooth Muscle Actin
bFGF	basic Fibroblast Growth Factor
BMP4	Bone Morphogenetic Protein 4
CDM	Caldesmon
CNN	Calponin
EB	Embryoid Body
EC	Endothelial Cells
ECM	Extracellular Matrix
EPO	Erythropoietin
FACS	Fluorescence-Activated Cell Sorting
FB	Fibroblasts
FBS	Fetal Bovine Serum
FGF-2	Fibroblast growth factor
hiPSC	human induced Pluripotent Stem Cells
HLA	Human Leukocyte Antigens
IF	Immune Fluorescence
IL	Interleukin
MACS	Magnetic-activated Cell Sorting
MYH11	Myosin Heavy Chain 11
OCT-4	Octamer-binding transcription factor 4
PBS-	Phosphate-Buffered Saline without calcium and magnesium
PBS+	Phosphate-Buffered Saline with calcium and magnesium
PDGF-BB	Platelet-Derived Growth Factor - BB
PDGFRB	Platelet-derived growth factor receptor beta
PD-L1	Programmed Death-Ligand 1
PECAM-1	Platelet Endothelial Cell Adhesion Molecule
PenStrep	Penicillin Streptomycin
PFA	Paraformaldehyde
ROCK inhibitor	Rho-kinase Inhibitors
SCF	Stem Cell Factor
SM22α	Smooth Muscle 22α

SMNT	Smoothelin
SSEA-4	Stage-specific embryonic antigen 4
TEVG	Tissue Engineered Vascular Grafts
TGF- β	Tumor Necrosis Factor beta
TPO	Thrombopoietin
TRA-1-60	T cell Receptor Alpha locus
VE-cadherin	Vascular Endothelial cadherin
VEGF	Vascular Endothelial Growth Factor
vSMC	vascular Smooth Muscle Cells

1 Introduction

1.1 Atherosclerosis

Atherosclerosis, the “hardening” of arteries, is a major cause for death worldwide. It is the primary root of coronary or peripheral artery disease which leads to myocardial infarction, stroke, and angina pectoris. Atherosclerosis generally starts when a person is young and worsens over time. Typically, it begins with plaque build ups inside arteries. The plaques consist of macrophages, fat, cholesterol, and calcium. Over time, plaques harden and narrow the arteries. This limits the flow of oxygen-rich blood to organs and other parts of the body. Risk factors include abnormal cholesterol levels, high blood pressure, diabetes, smoking, obesity, lack of physical activity, family history and an unhealthy diet (1, 2). Treatments for atherosclerosis can include lifestyle changes, medication, and medical procedures/surgery.

The most effective form of treatment is coronary artery bypass grafting. It is a surgery, which uses mostly veins, from other areas in the body, to bypass narrowed coronary arteries (2). Even though venous grafts are most widely used, some patients lack suitable veins for transplantation as a result of age or disease. Furthermore, venous grafts are still prone to thrombosis, occlusion, and aneurysm. On the other hand, arteries can be used. Their downside is that primary arterial endothelial cells have limited expansion potential and undergo dedifferentiation in culture (3). The final option is to implant tissue engineered blood vessels, also called tissue engineered vascular grafts (TEVG)/ artificial blood vessels. They should ideally resemble the native blood vessels as closely as possible in composition and distribution of extracellular matrix (ECM) and cells.

1.2 Blood Vessels

Blood vessels carry blood from the heart to the rest of the body, thus supplying organs and tissues with the oxygen and nutrients they need to work. They are built to withstand the forces applied by blood flow and pressure, and the surrounding tissues.

A blood vessel consists of different cell types embedded in ECM. ECM is a complex, 3D networks of proteins, proteoglycans and glycosaminoglycans. The ECM composition determines the biomechanical properties of tissue, such as the compliance, stiffness, and burst

strength of blood vessels, and contributes to cellular phenotype. The ECM provides a scaffold on which cells adhere to and migrate on and anchor many proteins such as growth factors and enzymes. In blood vessels collagen and elastin are the most abundant ECM proteins (4).

The exact composition of a vessel depends on its type and size, but in general blood vessels consist of three layers:
tunica intima, tunica media, and tunica adventitia as seen in *figure 1*.

The innermost layer is composed of endothelial cells (EC). EC are essential in controlling vessel patency by providing a potent antithrombotic barrier between blood and tissue. Furthermore, they play a key role in regulating immune responses and inflammation. EC direct inflammatory cells to foreign materials, sites of infection and areas in need of repair. And finally, they control the vascular tone through signaling to the vSMC.

The tunica media is composed of aligned vascular smooth muscle cells (vSMC), elastin, and collagen fibers. The vSMC contract (vasoconstriction) or relax (vasodilatation) the vessel and thus maintain the proper blood pressure.

The outer layer is comprised of fibroblasts (FB), some elastin, but mainly collagen fibers oriented longitudinally as wavy bundles (4-6).

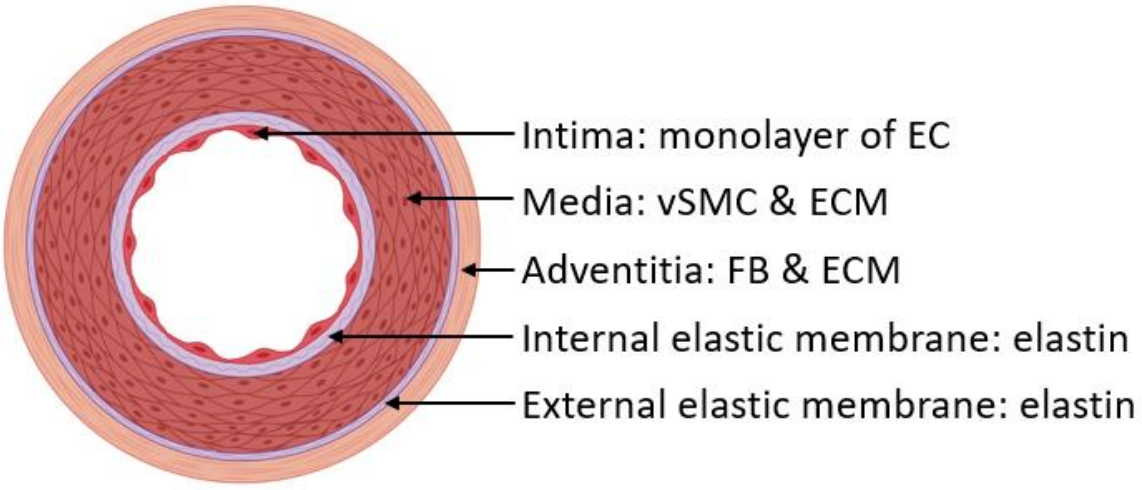

Figure 1: *Cross-section of a blood vessel: tunica intima, tunica media, and tunica adventitia, internal elastic membrane and external elastic membrane (4).*

1.3 Tissue Engineered Vascular Grafts

1.3.1 The Scaffold

A scaffold provides stability and a three-dimensional structure on which cells can grow. The ideal scaffold creates a microenvironment that promotes cell adhesion and differentiation and permits deposition of ECM. When deciding on a scaffold biocompatibility, mechanical properties, and biodegradability must be considered (4).

1.3.1.1 Scaffold Material

On the one hand there are scaffolds made up by naturally occurring materials, include collagen, gelatin, Matrigel, hyaluronate, glycosaminoglycan, alginate, silk, fibrin, chitosan, dextran or even decellularized ECM. On the other hand, there are scaffolds produced from synthetic polymers like polyethylene glycol, polyglycolic acid, polylactic acid, polylactic-co-glycolic acid, poly-L-lactic acid, poly-ε-caprolactone, polyvinyl alcohol, polypropylene fumarate, and polyacrylic acid ... (4). Each of these materials comes with its unique set of advantages and challenges.

1.3.1.2 Scaffold Fabrication

There are countless techniques to fabricate them into a scaffold such as electrospinning, particulate or porogen leaching, freeze-drying (lyophilization), solvent casting, melt molding, foaming, phase separation, fiber mesh, fiber bonding, self-assembly, rapid prototyping , membrane lamination and solid-state drawing … (7).

1.3.2 The Cells

When deciding on cells for the TEVG two major cell sources / types are available:

1.3.2.1 Primary Cells

One the one hand primary cells can be used. They robustly deposit collagen and have a mechanical strength similar to native tissue. On the downside, they are very limited in their expansion potential and accessibility. Furthermore, there is broad variability from donor to donor, depending on the donor's age and health. This results in large batch-to-batch variations in regard to cellular functions (e.g., ECM deposition and mechanical strength).

Additionally, all allogeneic cells are immunogenic to the recipient and are therefore prone to rejection. In contrast, when employing a patient's own cells, only a limited number of cells is available. Additionally, they may frequently be in suboptimal condition, due to a patients' compromised health status. Moreover, their use is impracticably time wise, since the process to obtain, expand and apply EC for reendothelialization takes about 23 days. This makes their use not feasible from a commercial standpoint (8).

1.3.2.2 Stem Cell-derived building blocks

On the other hand, cells derived from stem cells like hiPSC can be used. They provide a robust, scalable source of cells with comparably little batch-to-batch variation. hiPSC-derived vSMC or EC can be pre-generated, expanded, and cryopreserved at large scales.

Autologous hiPSC can be directly derived from patient's somatic cells, but this still would be impractical for clinical use due to the long patient-wait-time. Alternatively, hypoimmunogenic hiPSC cell lines could be generated (7). Common approaches include the elimination of Human Leukocyte Antigens (HLA) and expressing immune modulatory factors, such as CD47(integrin associated protein), PD-L1 (programmed death-ligand 1) (9). Thus, the allogeneic immunogenicity can be minimized or completely suppressed. These hypoimmunogenic cells are immunocompatible with a large population of patients and are ideal for widespread, commercial, of-the-shelf products.

The downside to hiPSC-derived cells is their maturity, or rather the lack thereof. Their contractile function, ECM deposition and mechanical strength is weaker than in primary cells. But there are already several maturation techniques available to mitigate this problem (8).

And finally, with all hiPSC-derived cells there is a substantial biological concern regarding potential tumorigenicity and stability in terms of lineage commitment during differentiation (7).

1.3.3 Cell Seeding and Endothelialization

There are also numerous techniques for cell seeding. Ranging from passive seeding (static and gravitational seeding), dynamic seeding (rotational and vacuum seeding), electrostatic and magnetic cell seeding to sheet-based cell seeding … . (7). Passive seeding is the most common technique and by far the simplest. It works by directly applying the cells onto the scaffold and then relays on their own ability to attach (10).

One special type of cell seeding is endothelialization. Endothelialization is the formation of endothelial tissue on the inner surface of engineered blood vessels. It is essential for a proper functioning of the blood vessels. A lack of complete endothelialization is a major reason for vascular graft failure (11).

1.3.4 Example of Living Blood Vessel Fabrication

The fabrication protocol used in the Chaikof laboratory utilizes hiPSC derived vSMC and EC for their engineered living blood vessels:

First, an ultrathin collagen sheet is extruded into a buffer bath by using a microfluidic bi-oprinter. This sheet is seeded with hiPSC derive vSMC and cultured for 2 days (sheet-based seeding). Then, the sheet is rolled onto a teflon-coated mandrel and cultured for seven days on the mandrel. The resulting tube is infused with hiPSC-derived EC in a way to allow them to settle (passive seeding). The tube is rotated every 18 hours to ensure an equal coating of the tubes from inside. In the end, a small blood vessel with an internal diameter of 1.5 mm, wall thickness of 250 µm and a length of 15 mm is produced.

1.4 Differentiations

Methods to differentiate EC from hiPSC can be divided into 3 general categories:

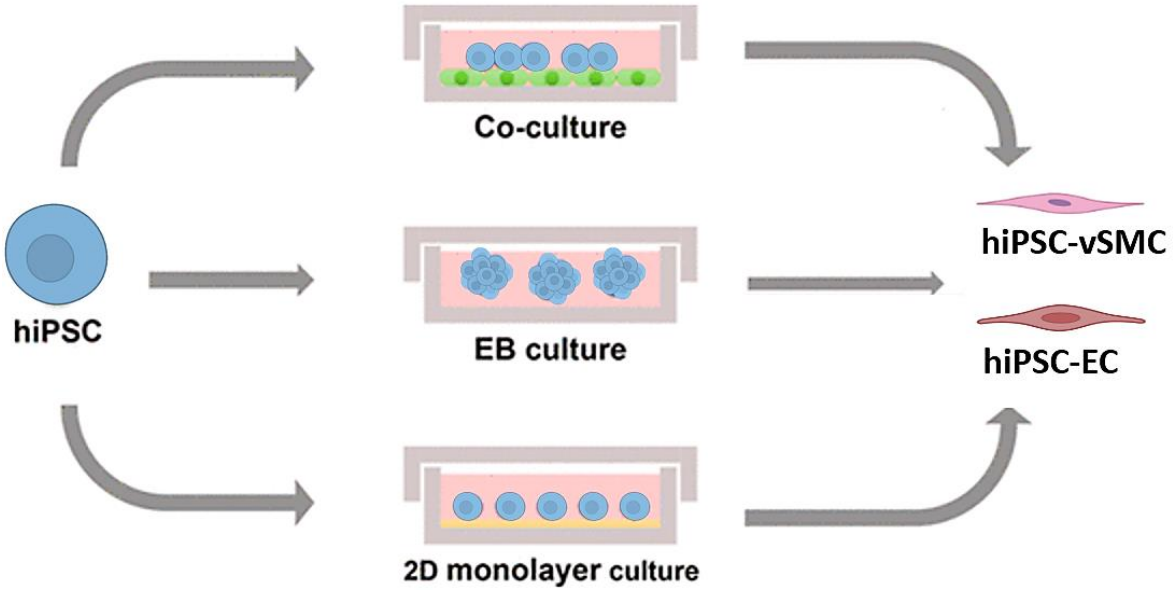

Figure 2: *Three approaches to differentiate EC from hiPSC, co-culture, embryoid body culture and 2D-mononlyer culture (12).*

1.4.1 Endothelial Cells

The method of co-culture with stromal cells was mainly used in earlier studies. It is an undirected differentiation strategy with generally low differentiation efficacy. Furthermore, the produced EC were often mixed with other cell types (hematopoietic cells, smooth muscle cells and inevitably stromal cells). Thus, this method is not ideal for the generation of a large number of EC (12).

In Embryoid body (EB) differentiation, the EB recapitulate the progression of early embryonic development. Published EB methods rely on the spontaneous differentiation of aggregated hiPSC in the context of a self-assembled 3D structure. The differentiation of EBs is also not a fully controlled process. Furthermore, even longer 10–12-day protocols usually only give rise to a low number of EC (12)

To improve the differentiation efficacy and decrease the time to 3-6 days, growth factors that promote mesoderm and/or are endothelial lineage specification are added. This can include but is not limited to: Bone Morphogenetic Protein 4 (BMP4), Activin A, basic Fibroblast Growth Factor (bFGF), and Vascular endothelial growth factor (VEGF). This improved protocol is able to robustly induce mesoderm and immature endothelial cells. Afterwards, an enrichment to separate the EC from miscellaneous other cell types is necessary. For example, fluorescence-activated cell sorting (FACS) or magnetic-activated cell sorting (MACS), which both use monoclonal antibodies that identify the expression of mesodermal and/or endothelial cell surface markers, can be used. The EC are then usually further expanded in monolayer culture conditions that favors the growth of EC (12).

The final strategy (feeder-free monolayer differentiation) starts out with a monolayer of hiPSC on a matrix coated culture plate and treats them with different growth factors and/or small molecules. These protocols over all are able to achieve some of the highest EC yields.

Feeder-free monolayer differentiation can be divided into a mesoderm differentiation phase and an endothelial differentiation phase. The culture medium and growth factors are usually changed between those two stages. Most protocols do not disturb the cells between the stages and simply switch out the media, but a purification step is possible.

Most protocols first manipulate the signaling pathways for mesoderm induction. Thus, growth factors like Activin A, BMP4, and bFGF are used. Glycogen synthase-Kinase 3 (GSK-3) inhibitors like CHIR99021, CP21 and BIO are also frequently added to promote

the canonical Wnt signaling pathway which stimulates the differentiation of the hiPSC into mesoderm (11-15).

In the second phase, cells are further driven to the endothelial lineage and expanded. VEGF is used in nearly all protocols. Furthermore, inhibitors of Tumor necrosis factor beta (TGF-β) signaling pathway, like SB431542, have been reported to promote the endothelial specification from mesoderm cells and the maintenance of EC (to avoid endothelial to mesenchymal transition) (11-13, 16).

1.4.2 Vascular Smooth Muscle Cell

Once again vSMC differentiation with stromal co-culture or via the formation of embryoid bodies (EB) have been reported. But these methods suffer from the same down sides as mentioned in the EC differentiation (17).

Most commonly employed is the feeder-free monolayer differentiation. Here three lineage-specific differentiation can be separated. vSMC can be obtained from neural crest, lateral plate mesoderm or paraxial mesoderm (17).

When deriving vSMC from mesoderm, the mesoderm induction is congruent to the EC protocols. Then a vSMC linage is induced. The vast majority of protocols uses platelet-derived growth factor subunit B (PDGF-B) and TGF-β1. In some cases, VEGF, FGF-2, Activin A, heparin, CDM … are added (11, 17-19).

The hiPSC derived vSMC frequently resemble a rather fetal, synthetic, fast dividing state, with lower maximum contractile force, slower upstroke velocity and immature mitochondrial function. Therefore, it is important to facilitate their maturation. Small molecules and growth factors such as Torin-1, TGF-β1 and RepSox have been proven to be effective in previous experiments (1, 20, 21).

Additionally, mechanical stretching can increase the expression of vSMC markers and extracellular matrix (ECM) as well as formation of filamentous actin bundles with a preferred alignment perpendicular to the direction of stretching as described by Luo et. al. (21).

1.5 Research Question

The overarching aim of this research project is to scale up the production of functional living blood vessels using cellular building blocks derived from human induced pluripotent stem cells (hiPSC). This paper in particular focuses on the production of endothelial cells (EC)

and vascular smooth muscle cells (vSMC). Therefore, the aim is to establish scalable protocols for both vSMC and EC differentiation and optimize them with regards to differentiation efficacy, required time and costs. A particular focus was put on the EC differentiation and expansion since their differentiation efficiency was found to be well below the yield of vSMC.

2 Materials and Methods

2.1 Cell Culture

2.1.1 Human Induced Pluripotent Stem Cell Culture

Materials:

- Geltrex (Gibco, A14132- 02) LDEV-Free
- DMEM (Gibco, 11995-065)
- mTeSR™ Plus Basal Medium (StemCell Technologies, 100-0274)
    - mTeSR™ Plus 5X Supplement (StemCell Technologies, 100-0275)
- StemFlex (Thermo Scientific™, 3349401)
- Plasmocin® - Mycoplasma Elimination Reagent (Invivo Gen, ant-mpp)
- Penicillin Streptomycin Solution (100x) (Corning, 30-002-Cl)
- Accutase (Innovative Cell Technologies, AT-104)
- PBS- = DPBS/Modified (cytiva, SH30028.02)
- ROCK Inhibitor Y-27632 dihydrochloride (ChemCruz, sc-281642A)
- KNOCKOUT™ SR (gibco, 10828-028)
- DMSO = Dimethyl sulfoxide (Millipore Sigma, D8418-50ML)
- mFreSR™ (StemCell Technologies, 05855)

The hiPSC line UCSD142i-86-1 was used for all experiments. It was derived from fibroblast of a female donor with the universal blood type O in Kelly Frazer's laboratory at UCSD (22).

The hiPSC were maintained at 37°C in a humidified incubator containing 5% CO2. They were cultured on plates coated with Geltrex, reduced Growth factor basement membrane matrix. As a medium either mTeSR™ Plus or StemFlex with added PenStrep (1:100) and Plasmocin was used. The medium was changed every 2 days.

Before reaching confluency, the hiPSC were split either 1:10 or 1:4: Thus, the cells were first washed with PBS- and then detached using Accutase diluted PBS- 1:4. The cell suspension was collected in a capture tube filled with DMEM, spun down at 1300 rpm for 4 min. The supernatant was removed, the cells were resuspended in fresh media with 10 µM ROCK Inhibitor and transferred to a new coated plate.

2.1.1.1 Thawing and Freezing

The cells within the cryovial were thawed in a water bath, until only a tiny piece of ice was left in the tube. Then the suspension was transferred into a DMEM fill falcon tube, spun down at 1300 rpm for 4min. The supernatant was removed, the pellet was resuspended in fresh media with ROCK inhibitor and transferred into the plate.
To freeze the cells, a dry cell pellet was obtained and resuspended in either in mFreSR or in KO (knock out) media with 10% DMSO & 10 µM ROCK inhibitor. Per cryovial 1-0.5 ml with 0.5-3 mil cells were frozen. The vials were first frozen at –80°C and later on transferred into the liquid nitrogen tank.

2.1.2 Endothelial Cell Culture

Materials:

- Fibronectin (Sigma-Aldrich, 10838039001)
- EGM-2 MV SingleQuots (Lonza; CC-4147)
    - EGM-2 SingleQuots supplement (Lonza, CC-4147))
- Freezing Medium Cryo-SFM (Promo Cell, C-29912)

EC were maintained at 37°C in a humidified incubator containing 5% CO2. They were cultured on human fibronectin-coated plates in EGM-2 MV SingleQuots with its supplement and PenStrep (1:100). The medium was changed every 2 days.
Before reaching confluency, the EC were split 1:4 to 1:6: The cells were first washed with PBS- and then resuspended in Accutase. After 5 minutes the cells started teaching and the plate was tabbed to speed up this process. After an additional 2 min the cell suspension was collected in a capture tube filled with DMEM, spun down at 2400 rpm for 4 min. The supernatant was removed, the pellet was resuspended in fresh medium and transferred to a new plate.
The Cryo-SFM with 10 µM ROCK Inhibitor was used for freezing.

2.1.3 Vascular Smooth Muscle Cell culture

Materials:

- 0.1% Gelatin (StemCell Technologies, 07903)
- DMEM with SmGM-2 SingleQuots (Lonza; CC-41499)
- N2B27 with PDGF-BB

- 0.25% Trypsin, 1X (Corning, 25.050-CI)

The vSMC were maintained at 37°C in a humidified incubator containing 5% CO2. They were cultured on gelatin-coated plates in DMEM with SmGM-2 SingleQuots supplement and P/S (1:100) or in N2B27 with 10ng/ml PDGF-BB. The medium was changed every 2 days.

Before reaching confluency the vSMC were split either 1:4 or 1:8: The cells were first washed with PBS- and then suspended in 0.01% Trypsin 1:5 in PBS-). After 5 minutes the cells started detaching and the plate was tapped to speed up this process. After an additional 2 min the cell suspension was collected in a capture tube filled with DMEM + 10% HI FBS (gibco, 1862872), spun down at 2400 rpm for 4 min. Subsequently, the supernatant was removed, the pellet was resuspended in fresh media and transferred to the plate.

For freezing down cells, Cryo-SFM with 10 µM ROCK Inhibitor was used.

2.2 Differentiations

2.2.1 Endothelial Cell Differentiation Protocol by the Schrepfer Laboratory (16)

Materials:

- **Differentiation Media:**
  - RPMI-1640 (Gibco, 11875093)
  - B-27 minus insulin (Gibco, A1895601)
  - 5 ml Penicillin Streptomycin Solution (100x) (Corning, 30-002-Cl)
- **Expansion media:**
  - 500 ml EBM-2 (Lonza, CC-3156)
  - 1x EGM-2 SingleQuots (Lonza, CC-4147)
  - 5 ml Penicillin Streptomycin Solution
  - 50 ml HI FBS (gibco, 1862872)
- **Cytokines & Small Molecules:**
  - CHIR-99032 (Cayman Chemicals,13122)
  - Human VEGF165 (PeproTech, 100-20-100UG)
  - FGFb (R&D Systems, 233-FB-010/CF)
  - Y-27632 (Sigma-Aldrich, 32160801)
  - SB 431542 (Sigma-Aldrich, 301836-41-9)

- PluriSln-1 (StemCell Technologies, 72822)

Protocol

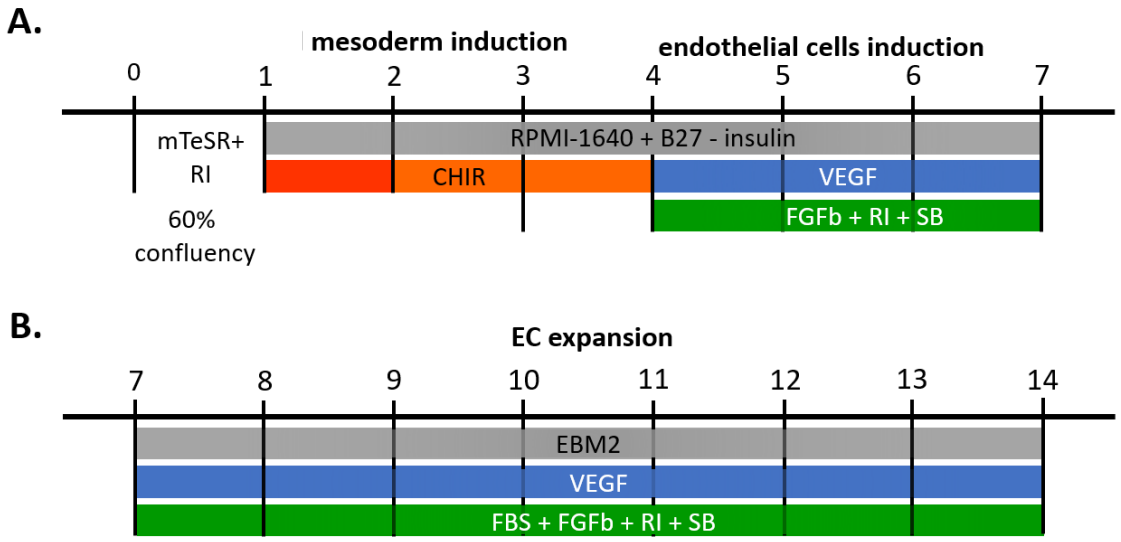

Figure 3:*Timeline EC differentiation by the Schrepfer laboratory protocol.*

On day 0 the hiPSC were dissociated using Accutase and plated on growth-factor-reduced Matrigel (Geltrex) at a density of 70.000 - 90.000 cells/cm^2 in mTeSR Plus with 10 µM ROCK inhibitor.

After 24 h the medium was changed to RPMI-1640 + 5 µM CHIR. On day 2 and 3 the medium was switched to RPMI-1640 + 2 µM CHIR.

On day four the medium was changed to RPMI-1640 + 50 ng ml−1 VEGF + 10 ng ml−1 FGFb + 10 µM Y-27632 (RI) + 1 µM SB 431542 and cultured with daily media changes.

On day 7 the medium was switched to EGM-2 SingleQuots media + 10% FCS + 25 ng ml−1 VEGF + 2 ng ml−1 FGFb + 10 µM Y-27632 + 1 µM SB 431542 and cultured with daily media changes.

On the last 2 days 20 µM PluriSln-1 was added to the media for cell purification.

The differentiation was concluded on day 14 and cells were detached and replated.

2.2.2 Endothelial Cell Differentiation Protocol by the Cowan Laboratory (11)

Materials:

- **N2B27 Medium**: ~ 1L
  - 500 ml Neurobasal Medium (gibco, 21103-049)
  - 500 ml DMEM / F12 + GlutaMAXTM-I (1x) (gibco, 10565-018)
  - 20 ml B-27 Supplement (50x) (gibco, 17504 044) (1.94%)
  - 10 ml N-2 Supplement (100x) (gibco, 17502-048) (0.97%)
  - 1 ml 2-β-Mercaptoethanol (gibco,21985-023) (0.097%)
  - 10 ml Penicillin Streptomycin Solution (100x) (Corning, 30-002-Cl)
- **StemPro-34 Medium**: ~0.5L
  - 500 ml StemPRO-34 SFM (1x) medium (gibco, 2312586)
  - 13 ml StemPRO-34 Nutrient Supplement (gibco, 2337511)
  - 5 ml GlutaMAXTM-I (100x) (gibco, 35050-061)
  - 5 ml Penicillin Streptomycin Solution (100x) (Corning, 30-002-Cl)
- **Cytokines & Small Molecules:**
  - CHIR-99032 (Cayman Chemicals,13122)
  - Human BMP-4 (PeproTech, 120-05-100UG)
  - Human VEGF165 (PeproTech, 100-20-100UG)
  - Forskolin, Adenylyl cyclase activator (abcam, ab120058)

Protocol:

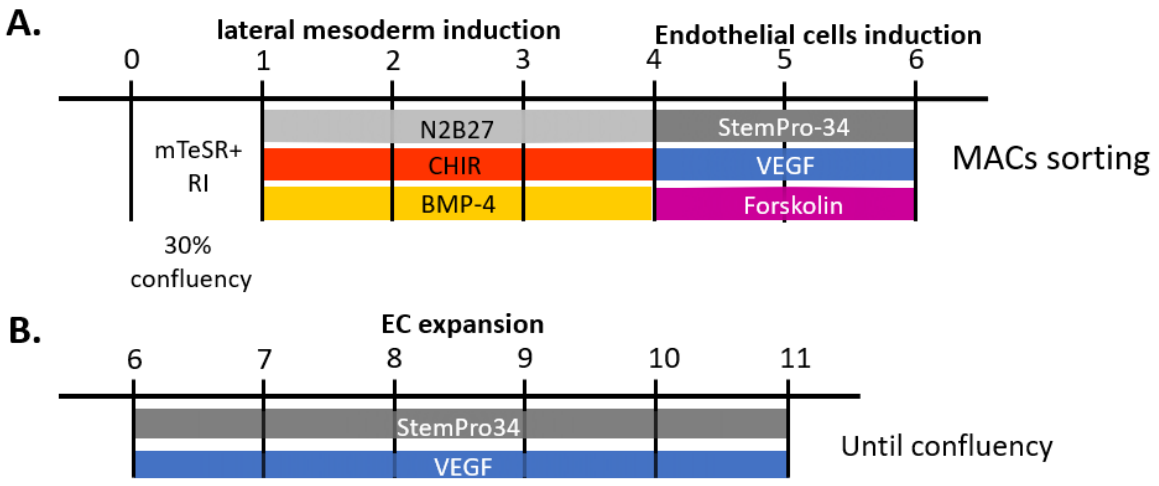

Figure 4: *EC differentiation (A.) and expansion (B) by the Cowan laboratory protocol.*

On day 0 the hiPSC were dissociated using Accutase and plated on growth-factor-reduced Matrigel (Geltrex™) at a density of 37.000-47.000 cells/cm^2 (~2 mil cell per 10 cm plate or 6 well plate) in mTeSR Plus with 10 µM ROCK inhibitor.

After 24 h the medium was changed to N2B27 medium + 8 µM CHIR + 25 ng/m BMP4 and the cells were kept in this media with daily media changes.

On day four the medium was switched to StemPro-34 SFM medium + 100 ng/ml VEGF + 2 µM forskolin and cultured with daily media changes.

On day 6 the differentiation was completed, and the cells were detached with Trypsin, and subsequently MACS sorted, replated and expanded.

Notes:

Every time when switching between two different media the cells were washed with PBS-.

Media changes were consistently done every 24h (+/- 2h), especially when switching between media.

2.2.3 vSMC Differentiation (11)

Materials:

- **N2B27 Medium**: ~ 1L
- **vSMC medium**
    - 500 ml DMEM (1X) (gibco, 11995-065)
    - 5 ml Penicillin Streptomycin Solution (100x) (Corning, 30-002-Cl)
    - 1x SmGM-2 SingleQuots (Lonza, CC-4149)
- **Cytokines:**
    - CHIR-99032 (Cayman Chemicals,13122)
    - Human BMP-4 (PeproTech, 120-05-100UG)
    - PDGF-BB (PeproTech, 100-14B-10UG)
    - Human/murine/rat Activin A (PeproTech, 120-14P-10UP)

Protocol:

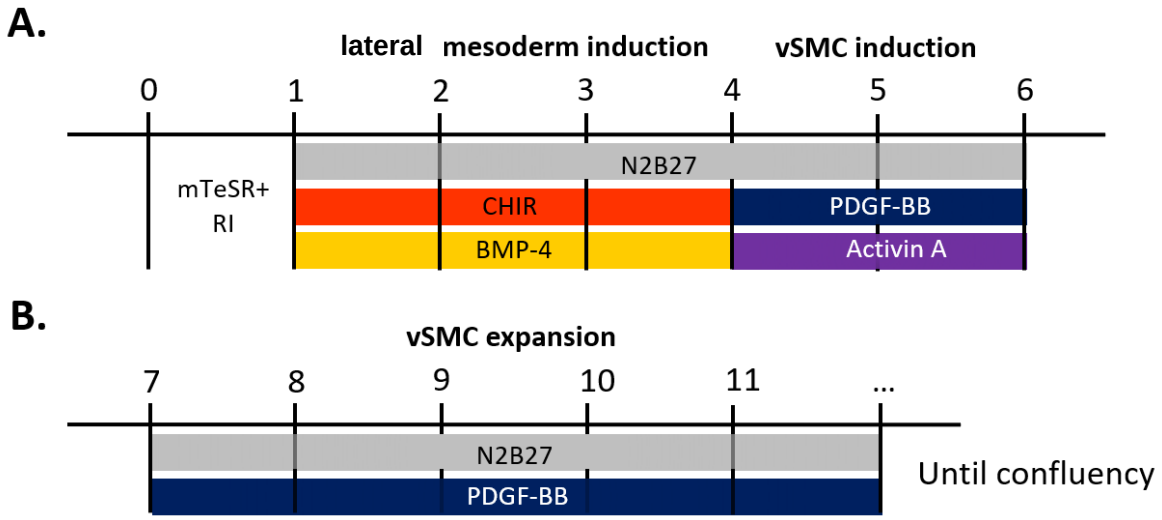

Figure 5: *vSMC differentiation (A) and expansion (B) by Cowan laboratory protocol.*

On day 0 the hiPSC were dissociated using Accutase and plated on growth-factor-reduced Matrigel (Geltrex™) at a density of 37.000-47.000 cells/cm^2 (~2 mil cell per 10 cm plate or 6 well plate) in mTeSR Plus with 10 µM ROCK inhibitor.

After 24 h the medium was changed to N2B27 medium + 8 µM CHIR + 25 ng/m BMP4 and the cells were kept in this media with daily media changes.

On day four the medium was switched to N2B27 medium + 10ng/ml PDGF-BB + 2ng/ml activin A. On day 6 the differentiation was competed.

The cells were washed twice with PBS-, detached with 0.25% Trypsin. The Trypsin was deactivated with DMEM + 10% FBS and the cells were replated on gelatin coted plates on a at least 4times bigger area.

Notes:

Every time when switching between two different media the cells were washed with DPBS.

Media changes were consistently done every 24h (+/- 2h), especially when switching between media.

2.3 Magnetic-activated Cell Sorting

Materials:

- DNase (Worthington, LS006331)
- MACS separation kit
- CD144 (vascular endothelial cadherin (VE-cadherin)) MicroBeads, human 2ml (Miltenyi Biotec, 130-097-857)
- MidiMACS™ Separator (Miltenyi Biotec, 130-042-302)
- MACS® MultiStand (Miltenyi Biotec, 130-042-303)
- LS Columns (Miltenyi Biotec, 130-042-401)

- MACS running buffer
  - 500 ml DPBS (1x) (gibco, 2393832)
  - 5 ml KNOCKOUT™ SR (gibco, 10828-028)

Protocol:

After completion of the EC differentiation, cells were thoroughly washed with PBS-, detached with 0.25% Trypsin and counted. From this point onward the protocol was performed as fast as possible, and both the cells and reagents were kept cold. The cells were washed with PBS- spun down at 2400 rpm for 4 min and resuspended in appropriate volume of cold MACS running buffer and CD144-microbeads. For each 10 mil cells 80 µl cold MACS running buffer and 20 µl CD144-microbeads were used. They were incubated for 20 min at 4°C in the fridge and occasionally mixed during this incubation time. Unbound beads were removed by adding 1-2 ml cold MACS running buffer per 10 mil cells, mixing and spinning down at 2400 rpm for 4 min. Then the cells were resuspended in MACS running buffer. Next, they were filtered through a 30 µm mesh into tubes. This step was crucial to avoid clogging of the columns later on. The most efficient filtration was achieved by placing the pipette tips at an exactly 90° angle to filter and continuously moving the pipette tip over the filter to avoid clogging of the filter. If the filter still clogged it was exchanged for a new one. To avoid cell loss, tube and filter were rinsed with 0.5 ml MACS running buffer.

The Magnet (=MACS separator) was placed on the metallic backboard (MACS Multi-Stand) and the LS column was inserted into the magnet. A 15 ml falcon tube was placed below as a capture tube. The column was pre wetted with 3 ml of MACS running buffer and the filtered cell suspension (1 ml) was applied to the column. The column was washed three times with 3 ml of MACS running buffer. Each time the new buffer was only added after the reservoir on top of the column was empty. To obtain the CD144 positive

cells, the column was removed from the magnet and placed into an empty 15 ml flacon tube. 5 ml MACS running buffer was added and the cells were eluted by pressing the buffer through the column in one continuous motion with a plunger. Then the cells were seeded on gelatin- or fibronectin-coated plates in EGM-2 MV SingleQuots.

2.4 Fluorescence-activated Cell Sorting

Materials:

- Blocking Buffer:
  - 4% FBS in PBS-
- Washing Buffer:
  - 1% FBS in PBS-
- Fixing solution:
  - 1% Paraformaldehyde (PFA) in PBS-
- Antibodies
  - CD31 (Platelet endothelial cell adhesion molecule (PECAM-1))
    CD31 (BioLegend, 303115) APC - 1µl per sample
  - CD144 (BD Pharmingen, 560410) PE - 3 µl per sample
  - CD140b (Platelet-derived growth factor receptor beta (PDGFRB))
    CD140b (BD Pharmingen, 558821) PE - 3µl per sample
  - TRA-1-60 (T cell receptor alpha locus)
    TRA-1-60 (Bioxcinece,12-8863-80) PE - 1µl per sample
  - SSEA-4 (Stage-specific embryonic antigen 4)
    SSEA-4 (BioLegend, 330417) APC - 1µl per sample

Protocol:

The detached cells were resuspended in bocking buffer (150-200µl per Epi) for 20-30 min. A master mix (50µl of washing buffer + appropriate antibodies per sample) was prepared and added to each sample. The samples were regularly vortexed and kept in the dark for 45min – 1h.

Afterwards they were washed by adding 700 µl washing buffer -> vortexed -> spun down -> aspirate -> resuspended pellet by vortexing -> + 700 µl washing buffer -> resuspend by vortexing -> spun down -> resuspended by vortexing. Then the cells were fixed by adding 250 - 300 µl fixing solution, vortexed once again and stored in the dark at RT until samples could be read with the CytoFLEX Flow Cytometer (Beckman Coulter).

The obtained data was analyzed with the Software FlowJo.

2.5 Immunohistochemistry

Materials:

- **primary Antibodies**
  - Anti-Oct4 antibody – IgG rabbit (abcam, ab19857)
  - Anti-Nanog antibody – IgG rabbit (abcam, ab21624)
  - Anti-TRA-1-60 antibody – IgM mouse (Millipore sigma, MAB4360)
  - Anti-TRA-1-81 antibody – IgM mouse (Stemgent, 9-00119)
- **secondary Antibodies**
  - Alexa Fluor 488 donkey anti rabbit IgG (Life technologies, A21206)
  - Alexa Fluor 594 donkey anti rabbit IgG (Life technologies, A11062)
  - Alexa Fluor 488 goat anti mouse IgM (Life technologies, A21042)
- DAPI (Sigma-Aldrich, D9542)

Protocol:

For the IF staining the cells were first washed with PBS+ than fixed with 4% PFA for 10 min. After 2 additional washing steps the cells were permeabilized and blocked at the same time with 5% Goat serum + 0.1% Triton for 30 min, while maintaining a continues gentle shaking. Then the cells were washed twice with PBS+, and subsequently, 1% Goat serum with the primary antibody (1:100) was added and kept overnight at 4°C with gentle shaking.
The next day the cells were washed thoroughly 3 times and 1% goat serum in PBS+ with the secondary antibody (1:500) was added for 45 min. After one quick rinse with PBS+, DAPI in PBS+ (1:5000) was added for 5 min. Once again, the cells were washed 3 time with PBS+ and kept in PBS+ until imaging with AMG EVOS FL Fluorescence Imaging Microscope.

2.6 Karyotyping

Cytogenetic analysis was performed on 20 G-banded metaphase cells by Cell Line Genetics (Wisconsin).

3 Results

3.1 Human induced Pluripotent Stem Cell

3.1.1 Pluripotency

First, the pluripotency of the used hiPSC was reconfirmed by immune fluorescence (IF) staining:

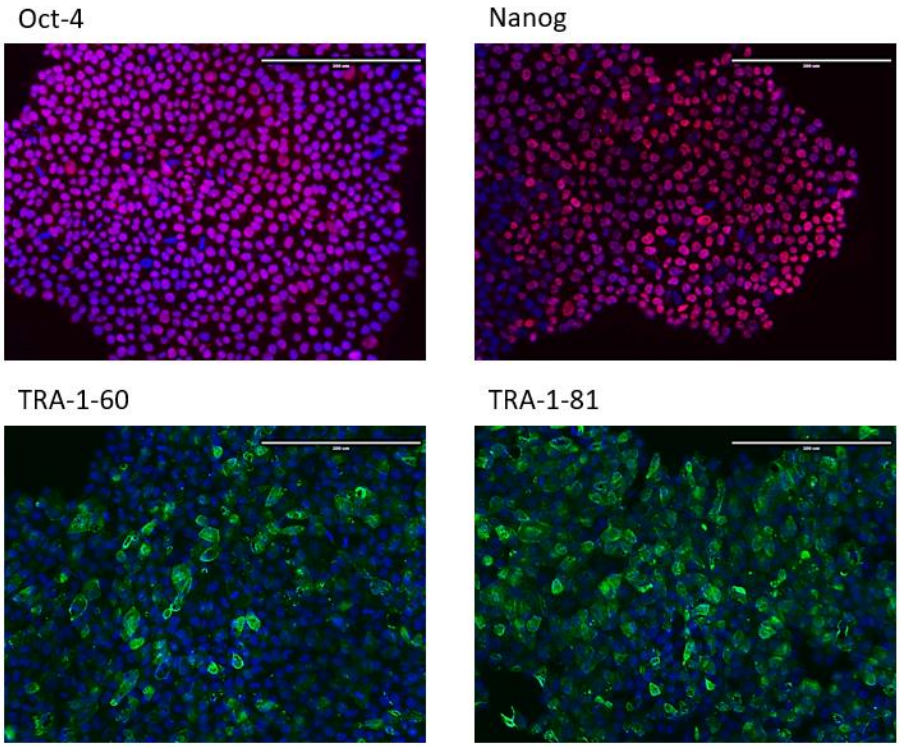

Figure 6: *IF pluripotency staining of hiPSC with Oct-4 (red), Nanog (red), TRA-1-60 (green), TRA-1-81 (green) and DAPI (blue).*

As seen in *figure 6*, the cells stained positive for both the nuclear (Oct-4 (Octamer-binding transcription factor 4) and Nanog) and the surface (TRA-1-60 and TRA-1-81) markers indicative of pluripotency. In the case of Oct-4 and Nanog, the red staining is isolated to the nucleus. Together with the blue DAPI staining of the nuclei, the overlay of the two images produces a purple-pink color. All nuclei have this purple tint; thus, all cells stain positive for these makers. On the other hand, TRA-1-60 and TRA-1-81 are surface makers, so they are visible all over the cell. Especially with TRA-1-81, a bright staining can be seen. TRA-1-60 expression and SSEA-4 was also confirmed by FACS (*sup. figure 1*). Since the cells stained

positive for all four makers, it can be assumed they are still pluripotent and are able to differentiate into all desired cell types.

3.1.2 Karyotype

Next, karyotyping was performed, to analyze the genetic stability of the hiPSC line.

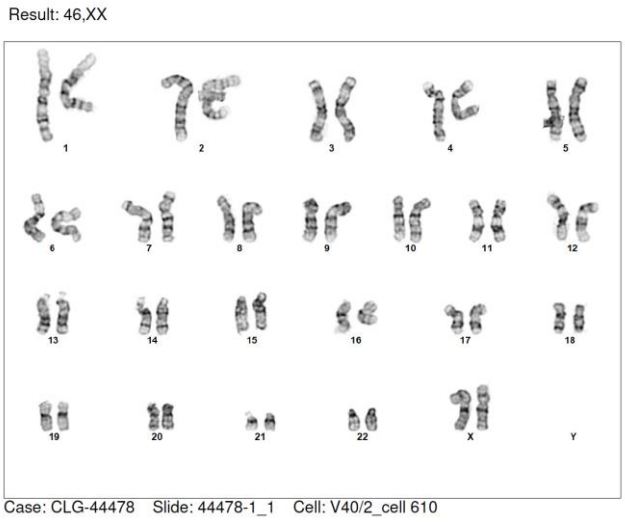

Figure 7: *Karyotype of used hiPSC cell line.*

In *figure 7*, the regular set of two times 23 autosomes and two X-chromosomes, since the donor was female, are displayed. Moreover, no translocations or amplifications can be observed. Therefore, the cells present a healthy, normal karyotype and their genetic integrity is maintained.

3.2 Endothelial Cells

3.2.1 Schrepfer Laboratory Protocol Differentiation

EC were differentiated following the protocol developed in the Schrepfer laboratory (UCSF). Their protocol takes in total 14 days. During our first trial (*figure 8* #1), the cells were detached at day 7 and samples were taken to assess the differentiation efficacy. The remaining cells were replated on fibronectin coated plates. During the last 2 days of the differentiation, PluriSln-1 was added. PluriSln-1 is a stearoyl-CoA desaturase 1 inhibitor. It is used to selectively eliminate undifferentiated hiPSC, by inducing endoplasmic reticulum stress, attenuating protein synthesis and finally inducing apoptosis (23). To see the effect of PluriSln-1 a sample was also acquired before addition at day 12.

Additionally, the protocol was also conducted in one continuous go, without detaching cells at day 7, as seen in *figure 8 #2*.

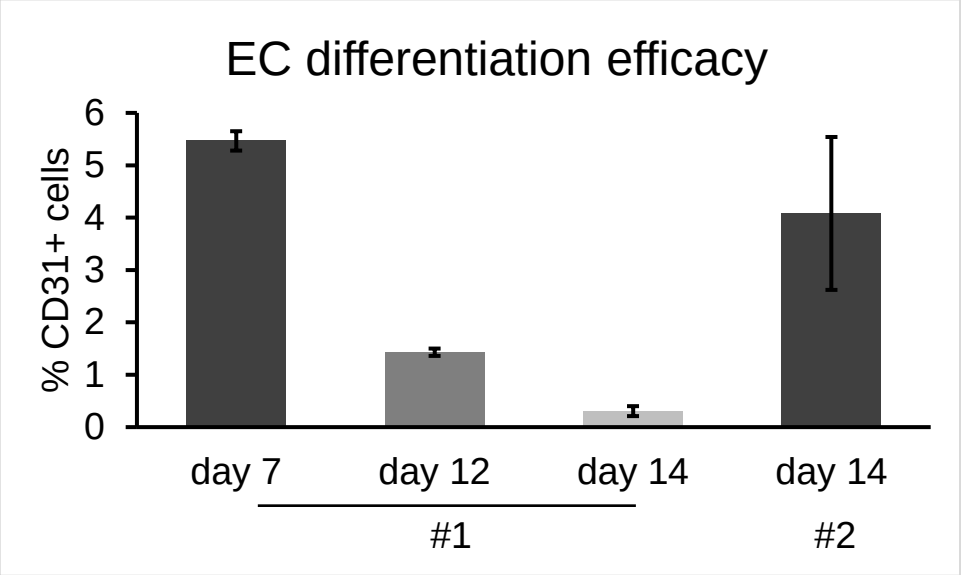

Figure 8: *EC differentiation efficacy (by FACS CD31 (PECAM-1) staining): disrupted protocol (#1) with sample acquisition at day 7, 12 and 14 vs continuous protocol (#2) with sample acquisition only on day 14.*

As seen in *figure 8* at day 7, we were able to obtain at least around 5% EC but, the percentage of EC dropped to below 1% during expansion until day 14. The 14-day continuous differentiation also only produced around 4% of endothelial cells.

Furthermore, the addition of PluriSln-1 did not lead to an increase in EC, rather a further decline in EC number was visible as seen by the drop between day 12 and day 14 *in figure 8 #1*, which correspond to the PluriSln-1 treatment. The potency of PluriSln-1 was checked on undifferentiated, confluent hiPSC. Here the same concentration of PluriSL1 was used and the PluriSln-1 was able to kill all hiPSC (data not shown).

3.2.2 Cowan Laboratory Protocol

3.2.2.1 Endothelial Cell Differentiation

Additionally, EC were differentiated using the protocol developed in the Cowan laboratory (Harvard University).

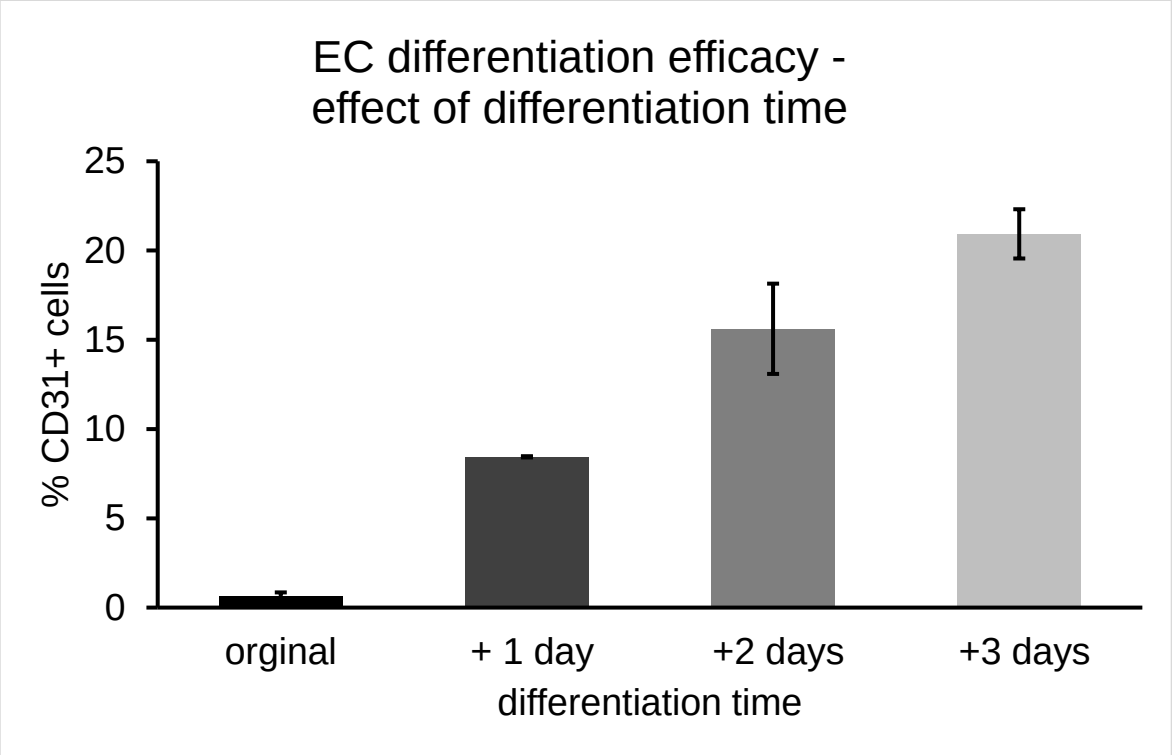

Figure 9: *EC differentiation efficiency (by FACS CD31 staining): Comparison between the original protocol and extended protocol (+1,2,3 days); n≥2, seeding density 38-44k cells/cm2*

As seen in *figure 9*, EC were differentiated following the Cowan laboratory protocol. Yet only less than 1% EC were obtained by strictly following the original protocol. The EC yield was significantly improved by simply extending the protocol. The protocol was extended by adding 1 to 3 days with the EC induction media at the end of the protocol. The differentiation efficacy was improved to ~ 8% (+ 1 day), ~16% (+ 2 days) and ~21% (3 days).

Secondly, after observing the persistence of large hiPSC clusters during the differentiation, the seeding density was reduced and the time between seeding and start of the differentiation was kept below 24 h (ideally around 20 h).

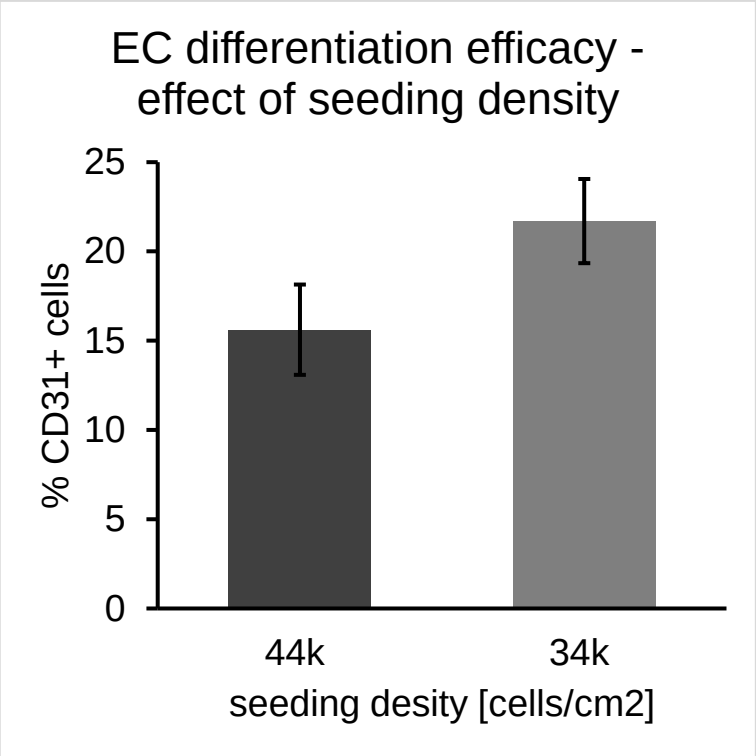

Figure 10: *EC differentiation efficacy (by FACS CD31 staining): Effect of the seeding density; n≥2, extended protocol + 2 days.*

As seen in *figure 10*, we were able to obtain ~22% EC using a reduced seeding density of 34k/cm^2 combined with an extended protocol of 2-days. This is equal to the percentage of EC obtained by day 3 (*figure 9*).

Thus, we settled on an 8-day differentiation protocol with reduced seeding density as seen in *figure 11*.

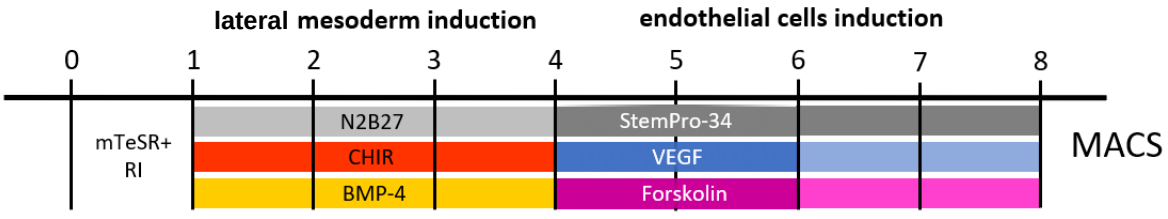

Figure 11: *EC differentiation timeline - extended by 2 days.*

Furthermore, the EC were imaged during the differentiation and some representative images for each day are shown in *figure 12:*

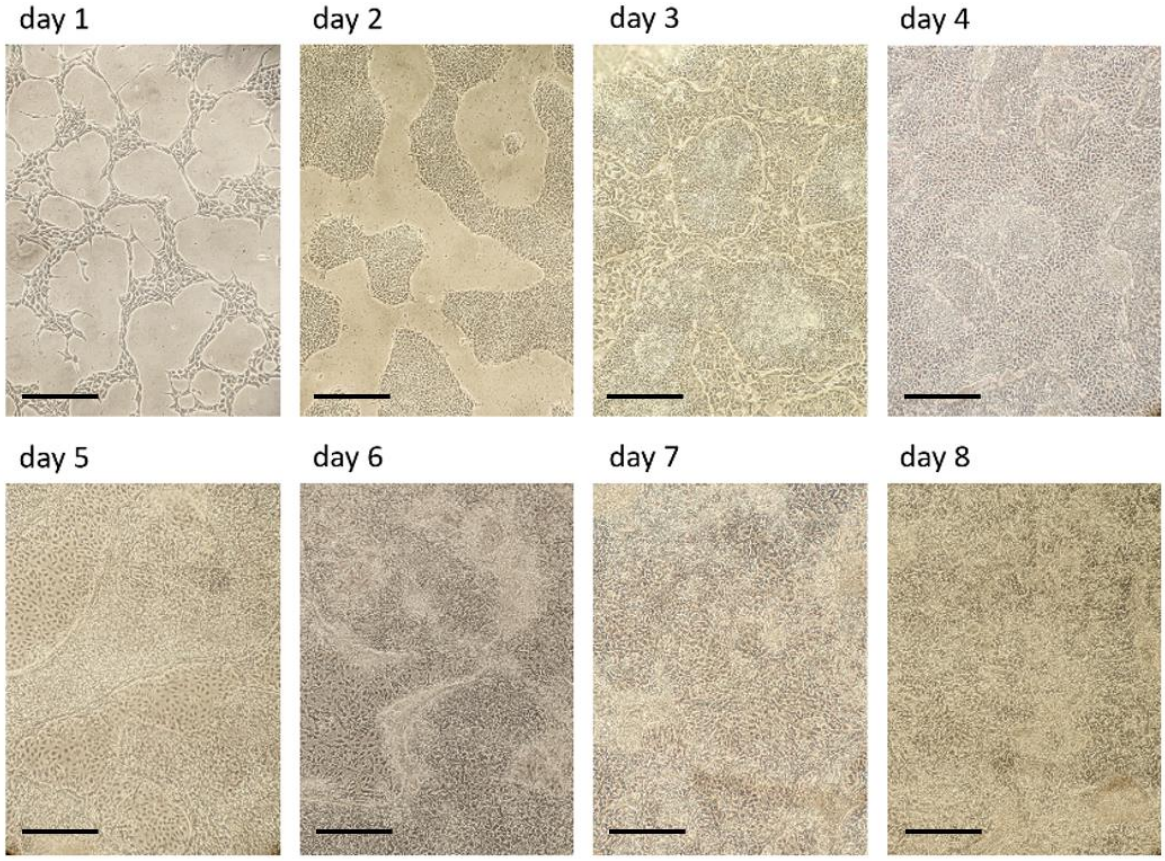

Figure 12: *EC Differentiation – adjusted seeding density and protocol extension by 2 days - daily images to assays the cell phenotype.*

As seen in *figure 12* on day 1 and day 2, the cells still look like stem cells and no/barely any mesodermal cells are visible. While by day 3 the cell number explodes, and a lot of mesoderm cells (phenotype: lager cobblestone like) are visible, but stem cell clusters (phenotype: brighter, smaller in tightly packed clusters) also remain. Over the rest of the differentiation, these stem cell like clusters disappear until no or barely any stem cells were left on day 8. This was tested through the addition of PluriSln-1, a compound that kills stem cell. With the addition of PluriSln-1 neither cell death out of the ordinary, nor an improved differentiation efficacy was visible (data not shown).

3.2.2.2 Magnetic-activated Cell Sorting

The EC were detached and purified by MACS with the help of CD144 (VE-cadherin) magnetic beads after each differentiation. The percentage of EC was assessed by FACS with CD31 staining before and after MACS.

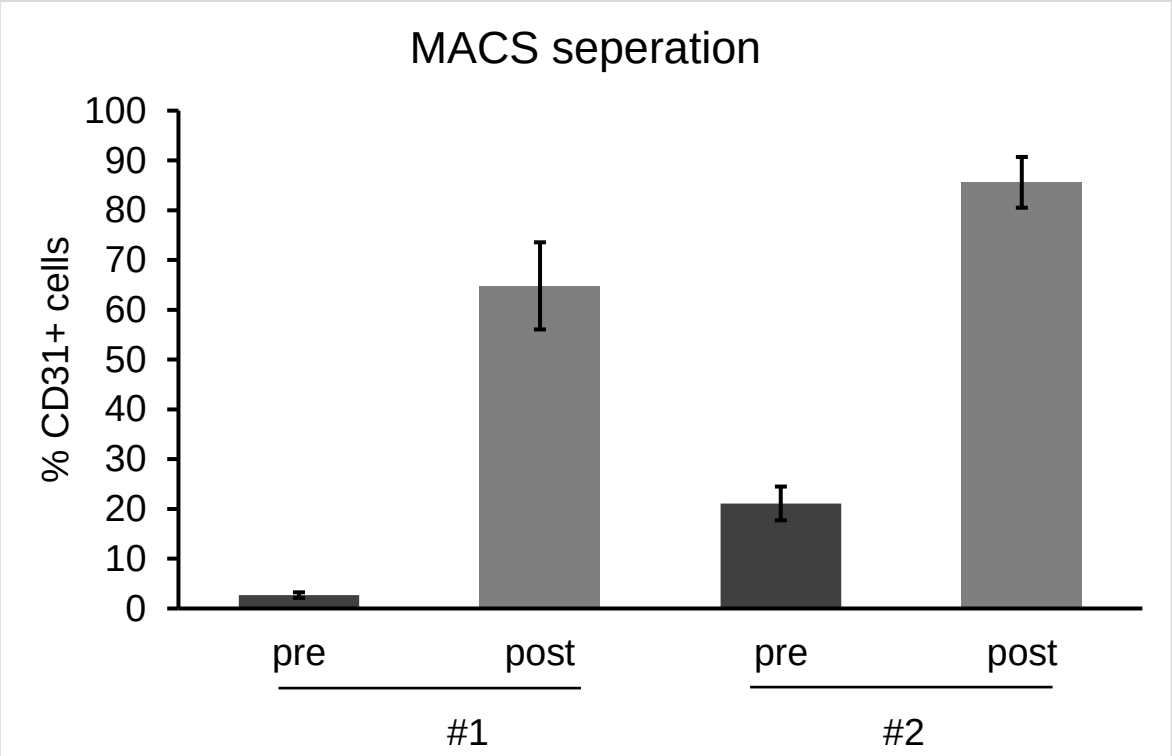

Figure 13: *Effect of MACS on EC purity (by FACS CD31 staining): Purification through MACS and the effect of different starting purities (pre) on the final purity (post).*

As seen in *figure 13*, the obtained purity after MACS separation depended strongly on the purity before the separation. Here two representative data sets: In #1 with a starting purity of ~3% EC only a purity of 65% EC could be obtained. With a starting purity of ~21% EC, a purity of ~85% could be obtained. Thus, a consistent purity between 80-95% could be obtained with our optimized protocol.

One downside to the MACS sorting was the substantial cell loss during the procedure. All the extracellular matrix produced during the differentiation and the DNA from dead cells led to a very sticky and clumpy cell suspension. The cell suspension had to be filtered to avoid clogging of the MACS columns. During this filtering process a lot of the cells got stuck and lost. Thus a 1h 40 U/ml DNase treatment step was implemented to digest all DNA, before cell detachment

Table 1: *Cell numbers pre and Post MACS: In particular the number of all cells, percentage of EC and thus calculated number of EC pre-MACS, and cell number post MACS showing the effect of DNase treatment.*

pre-MACS			**post MACS**	
number of all cells	Percentage of EC	number of EC	regular	+ DNase treatment
			number of cells	number of cells
23.3 ± 2.5 mio	20.0 ± 3.1 %	4.5 ± 0.8 mio	0.9 ± 0.3 mio	2.0 ± 0.3 mio

Table 1 shows the cornet cell number from one differentiation, which started with 2 million hiPSC and took 8 days. About ~ 23 mil cell could be obtained. Assuming a differentiation efficacy of 20%, about 4.5 ± 0.8 mil EC were produced. But only ~1 mil of those cells could be separated after MACS sorting, which amounts to only ~ 23% of all available EC.

After including a one-hour DNase treatment to digest any sticky DNA, ~ 2 mil of all EC could be obtained, which represents ~ 42% of all produced EC (*figure 14*).

These calculation works under the assumption, that all cells after MACS were EC, even though in realty the MACS separation efficiency ranged from 80-95%. Thus, the real number of EC and percentage of EC is even below the shown values.

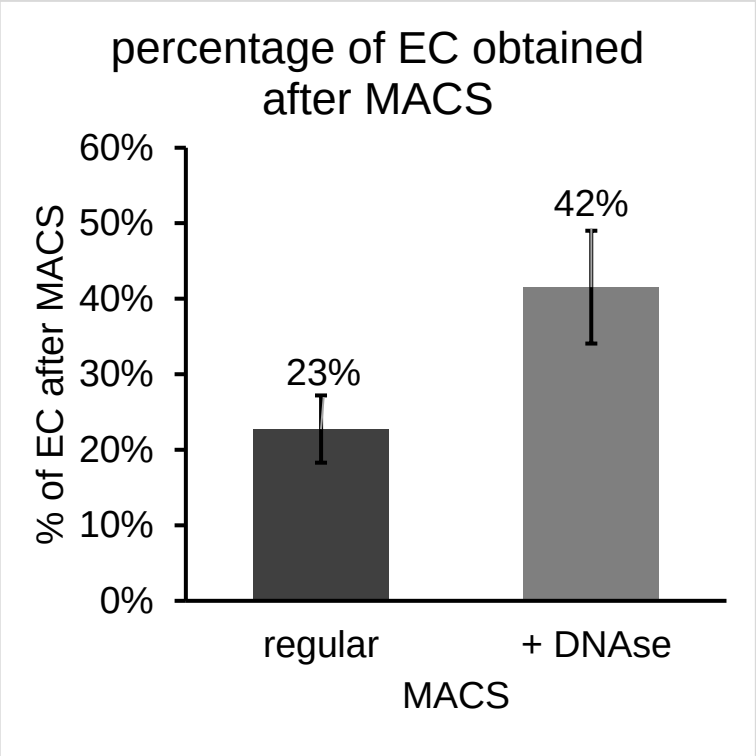

Figure 14: *Percentage of EC obtained after MACS with and without DNase treatment before MACS. The total available number of EC was assumed as 100%.*

3.2.2.3 Endothelial Cell Expansion

After the differentiation and the MACS, the EC were plated on fibronectin-coated plates to expand them. First StemPro 34 + VEGF, as described in the Cowan laboratory protocol was used, but barely any cell division and expansion was visible. Thus, the regular EC culture (EGM-2 MV SingleQuots) medium was used instead. Here a nice expansion could be obtained. Later on, an additionally 10% FBS was added to the EGM-2 MV SingleQuots, and this resulted in even better expansion.

Two representative images of an EC culture are shown in *figure 15*, first at a lower density in just EGM-2 MV SingleQuots medium and second at higher density in EGM-2 MV SingleQuots + 10% FBS. Both densities display that EC are very social cells, that wind and twist around each other. The EC divided very well until passage 3. The cells were usually split 1:4 at confluency. Afterwards it took them approximately 1 week to reach confluency. At passage 5 the cell division rate started to slow down, and if impurities were present, they became noticeable.

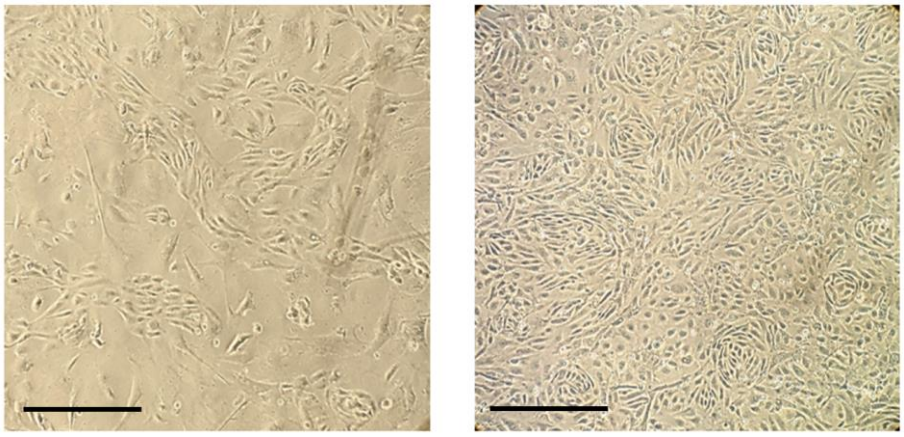

Figure 15: *EC at p1 and low and high density.*

3.2.2.4 Endothelial Cell Characterization by Tubing Assay

A tubing assay was performed to confirm EC cell identity and further characterize the cells. The assay relays on the EC innate ability to independently assemble into tubes, when cultured on a soft matrix. They self-assembled into tubes within 24h after plating the EC on Geltrex is seen in *figure 16*. This once again confirms that the differentiated cells are truly EC.

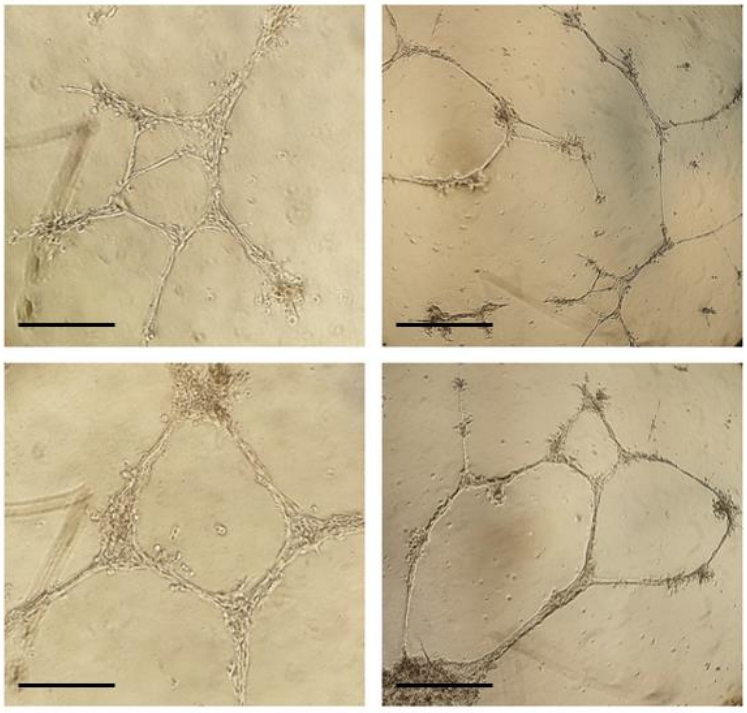

Figure 16: *Confocal microscopic images EC (p3) tube formation 24 h after seeding on Geltrex.*

3.2.2.5 Endothelial Cell Culture Stability

It is not only important to obtain pure EC, but also to maintain the purity of the culture. Thus, the dedifferentiation of EC or the overgrowth of other cell types could pose a problem. Other cell types frequently have a faster division rate and are thus able to overgrow EC over time. Therefore, the percentage of EC during continuous culture was observed. The percentage of EC dropped from around 80% to 60% after about 2 weeks of culture at passage 3.

3.2.2.6 Endothelial Cell Differentiation Protocol Costs

The long-term goal, behind producing EC, is to use them in engineering artificial blood vessels. Thus, we require a protocol that quickly and cost effectively, produces large quantities of pure EC. Purity, time, quantity, and differentiation efficacy have been addressed in detail above, but what about the production cost? The costs for producing 4.5 mil EC are around $70 for the 2-day extended protocol and with adjusted seeding density. A differentiation efficacy of at least 20% EC and a cell number of 23 mil were assumed, which results in 4.5 mil EC. The costs only consider media and retargets reagents used during the differentiation, as listed in *table 2*.

The price could be further reduced by ordering cytokines (the usually ordered amount was for 15-50 differentiation) in in bulk / larger quantities, but this would only result in minor savings.

When looking at all reagents, StemPro 34 SFM stands out by making up over 50% of the costs. To reduce the production cost, DMEM was used instead of StemPro 34 SFM as the EC induction media. The use of DMEM nearly cut the price in half to $38 instead of $70 (saves $32 per differentiation).

Table 2: *Cost of 1 EC differentiating in a 10 cm plate with the extended 2-day extended protocol, to obtain 4.5 mil EC. All values rounded up. Contrasting the price difference StemPro™-34 SFM vs DMEM would make.*

Differentiation cost for 4.5 mil EC				
media	reagents	cost	reagents	cost
Day 1 - 3	N2B27	$12	N2B27	$12
	+ BMP4	$14	+ BMP4	$14
	+ CHIR	$8	+ CHIR	$8
Day 4 - 8	StemPro™-34 SFM	$36	DMEM	$4
	+ VEGF	$4	+ VEGF	$4
	+ Forskolin	$1	+ Forskolin	$1
	sum	**$70**	**sum**	**$38**

To see whether DMEM is a viable alternative, it was used during one differentiation. As seen in *figure 17*, regular DMEM with the adjusted seeding density and the 2-day extended protocol also produces 19% EC, which is slightly below the previously obtained ~ 21% EC obtained with StemPro™-34 SFM, but considering the standard deviation there is not significant difference.

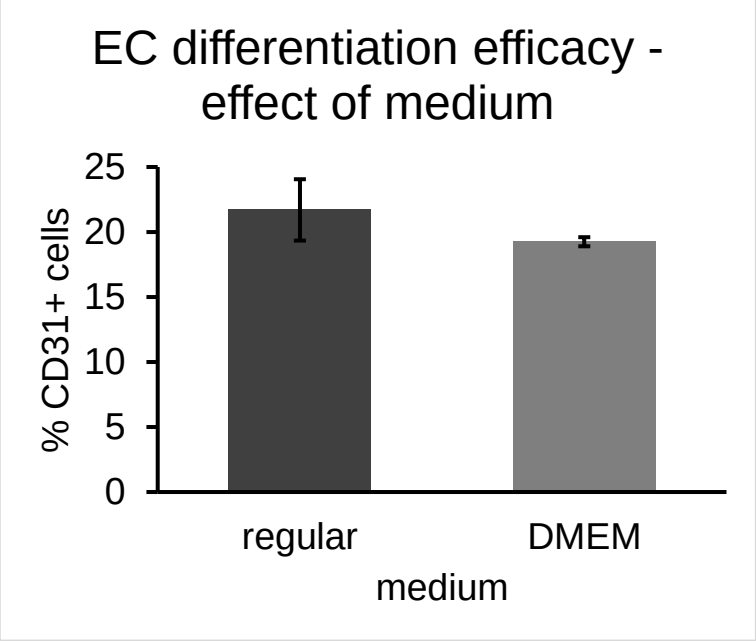

Figure 17: *EC differentiation efficacy (by FACS CD31 staining): effect of media and differentiation efficacy. The regular StemPro 34 SFM media was replaced with DMEM.*

3.3 Vascular Smooth Muscle Cell

3.3.1 Vascular Smooth Muscle Cell differentiation

VSMC were differentiated using a protocol also developed in the Cowan laboratory at Harvard University. The protocol is identical with the EC Protocol during mesoderm induction and only changes on day 4 to a vSMC specific protocol. The daily images taken during differentiation are shown in *figure 18*.

The expansion of cell number and the presence of mesodermal cells can be observed once again on day 3.

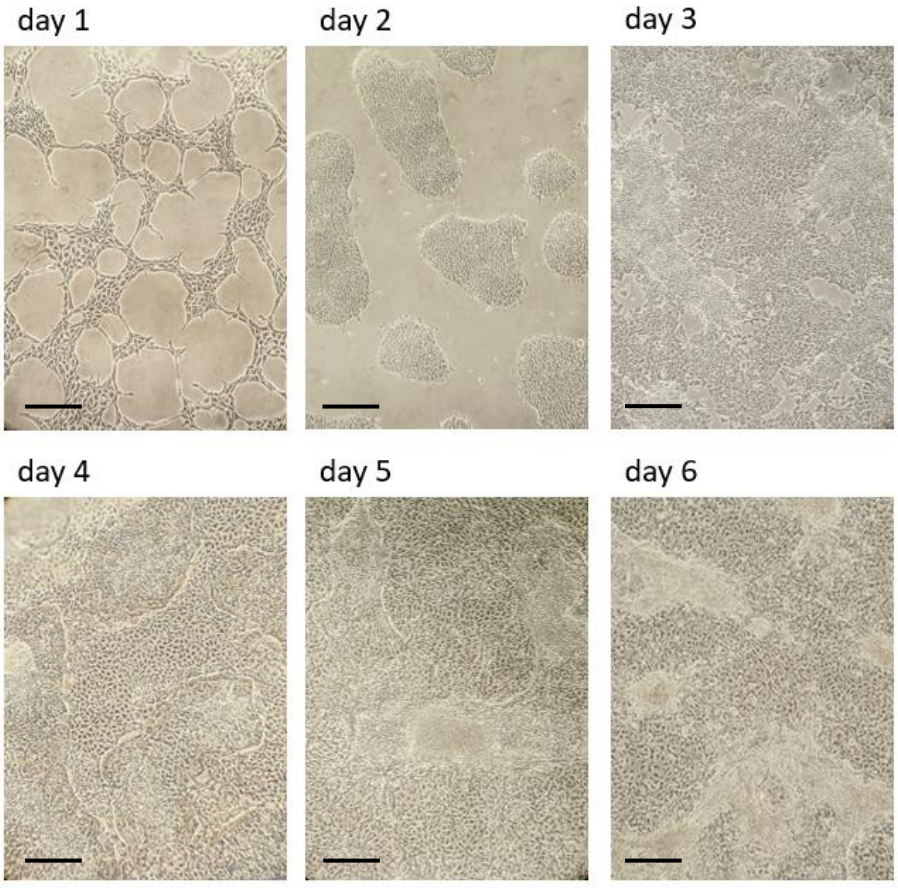

***Figure 18**: vSMC differentiation – daily images to assays the cell phenotype.*

The efficacy of vSMC differentiation was assessed with the marker CD140b (PDGFRB). But unlike the CD31 expression there is no clear separation between CD140b positive and negative cells. Instead, there is a continuous upregulation, as seen in *figure 19*. Therefore, mean fluoresce intensity was considered for some aspects.

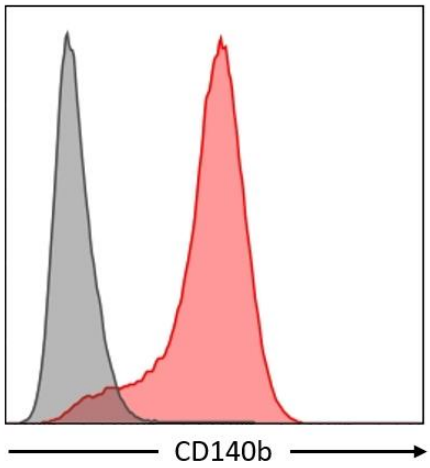

Figure 19: *vSMC differentiating CD140b expression.*

Directly after the differentiation, 90 ± 7% of the cells were CD140b positive. This percentage of CD140b positive cells was maintained during continuous culture as shown in *figure 20*, by representative data from passage 2 and passage 5

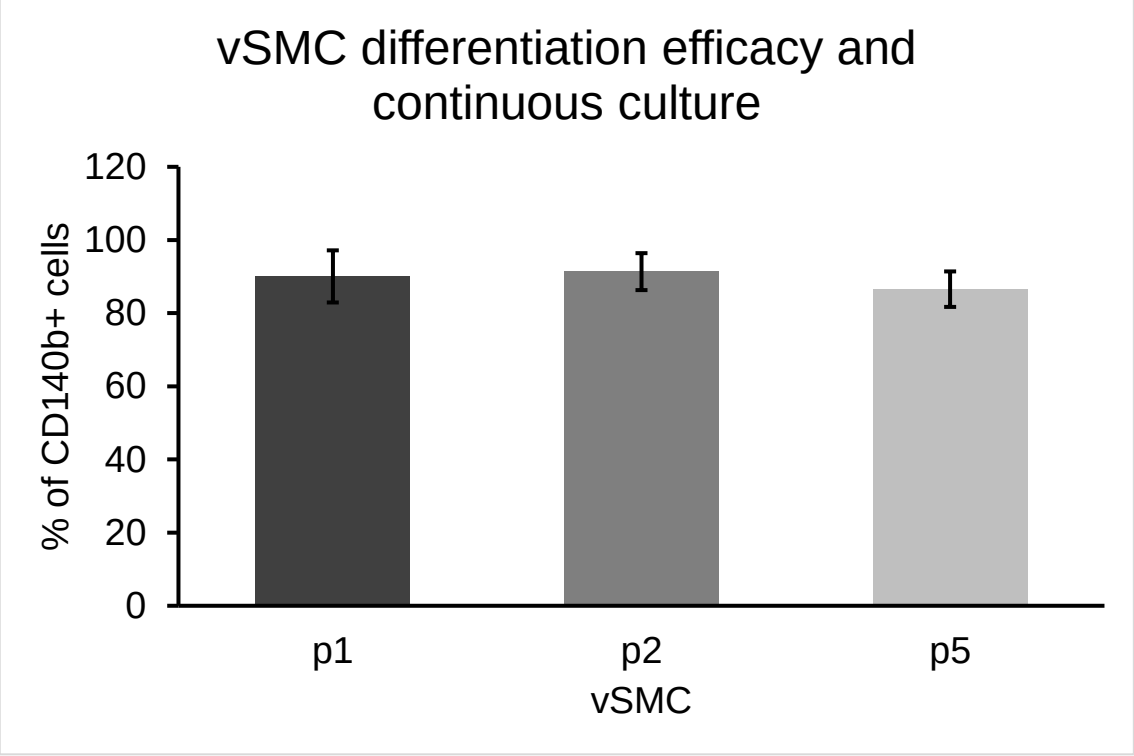

Figure 20: *vSMC differentiation efficacy and maintenance in culture, data from CD140b FACS staining.*

3.3.2 Vascular Smooth Muscle Cell Expansion

Three different media were tested for vSMC expansion: the regular medium N2B27 with 10ng/ml PDGFBB, as described by the Cowan lab, DMEM with the addition of a smooth muscle cell growth media supplement and a mixed version of both DMEM with supplement and PDGF-BB as seen in *figure 21*.

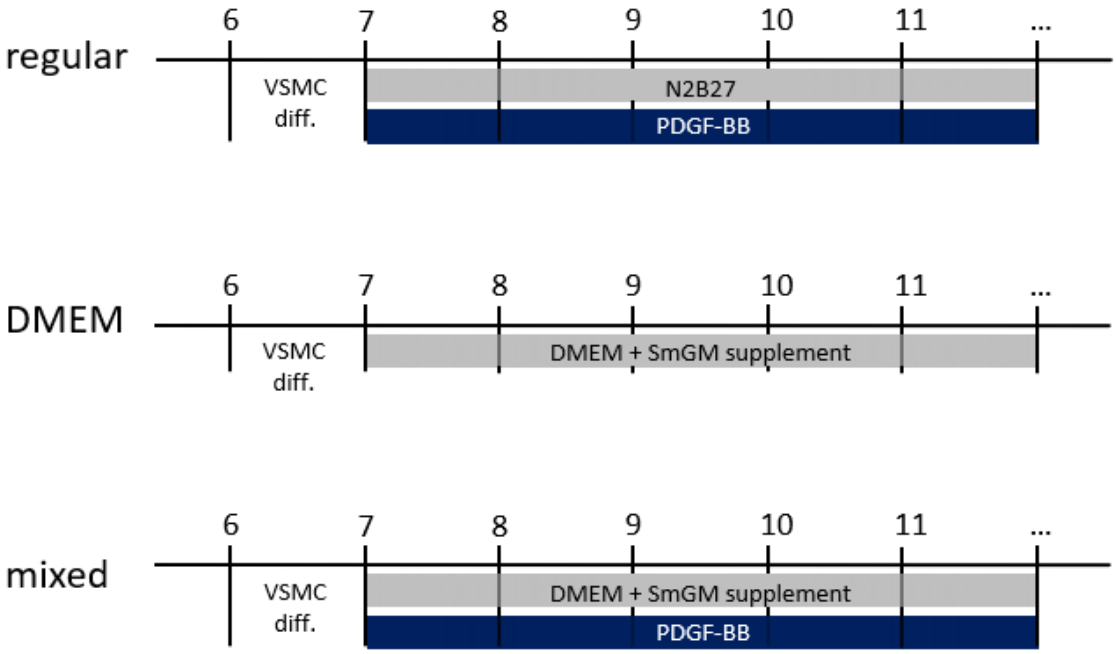

Figure 21: *Timelines for vSMC expansion with the 3 different media.*

As seen in *figure 22*, both the regular medium (94%) and the DMEM + supplement (97%) maintained a high percentage of CD140b positive cells. The mixed medium led to a drastic drop in CD140b positive cell to 45% after 5 days of culture.

Since, as previously mentioned, CD140b displays a rather gradual expression, an even more meaningful difference can be seen when looking at the mean fluorescence intensity (MFI). Here barely any CD140b expression is visible with the mixed media. And the difference between regular medium and DMEM + supplement becomes more distinct, with DMEM + supplement leading to a significantly higher CD140b expression. Thus, this media was used for all further cultures.

It was also used for vSMC expansion. During expansion cells were split as soon as they reached confluency 1:4. After about half a week of culture confluency was once again obtained. Thus, we could expand 1 mil vSMC into 16 mil vSMC by passage 3 in less than 2 weeks.

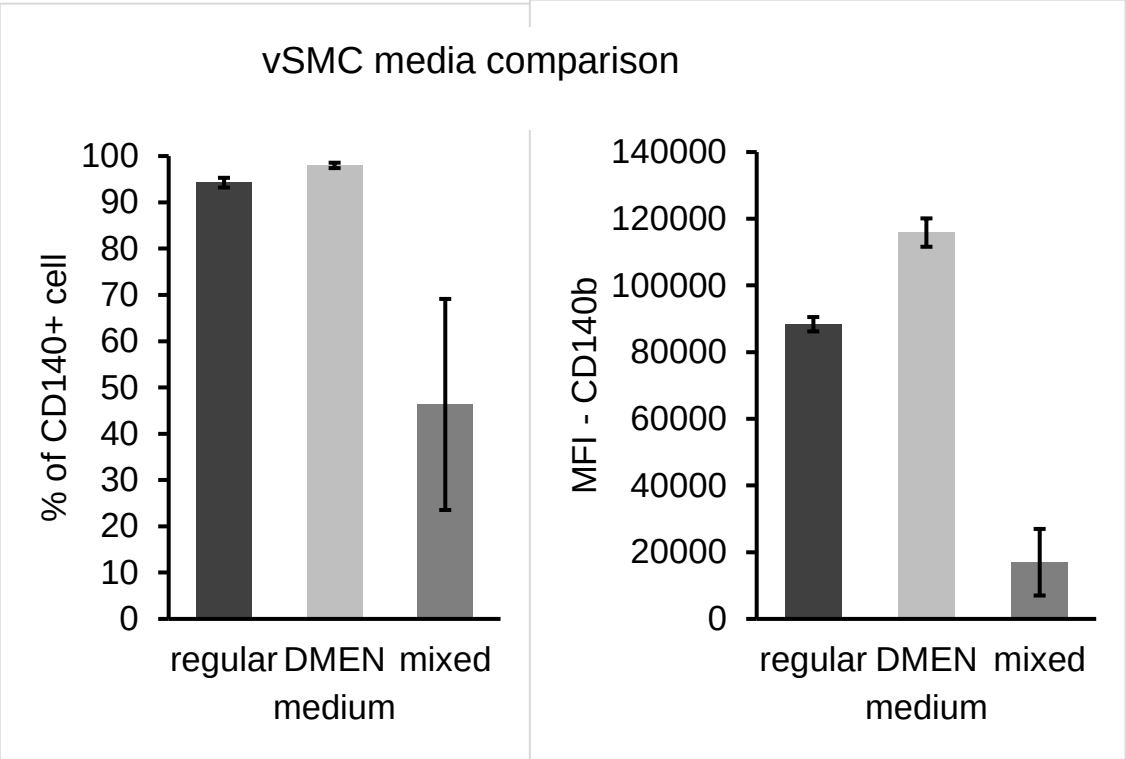

Figure 22: *VSMC media comparison: both, percentage of CD140b positive cells and MFI of CD140b expression after 5 days of culture.*

The difference between the three media is so pronounced that it can even be observed by looking at the cell's phenotype. The DMEM + supplement produced elongated, individually visible cells, while the mixed media showed a very indistinct clustered phenotype. The regular medium produced a phenotype somewhere in between with characteristics from both.

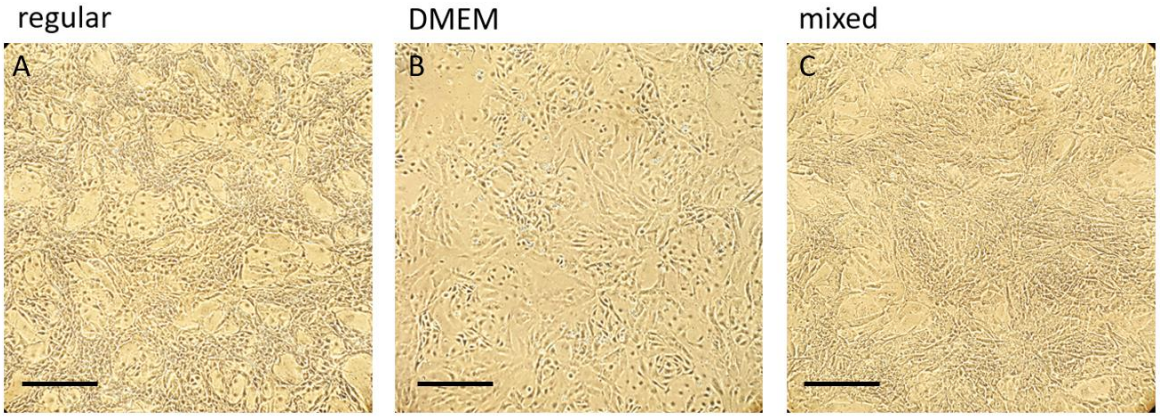

Figure 23: *Brightfield images of vSMC (p1) cultured in 3 different media at day 5.*

3.3.3 Vascular Smooth Muscle Cell Characterization

Finally, an IF staining for alpha smooth muscle actin (aSMA) was performed to further characterize the vSMC. All cells express SMA but to varying degrees as seen in *figure 24* in 20x magnification. This is in agreement with the FACS data about CD140b expression confirming there are cells of different levels of maturity in the culture. Furthermore, *figure 24* also shows the close up of three particularly bright vSMC cells.

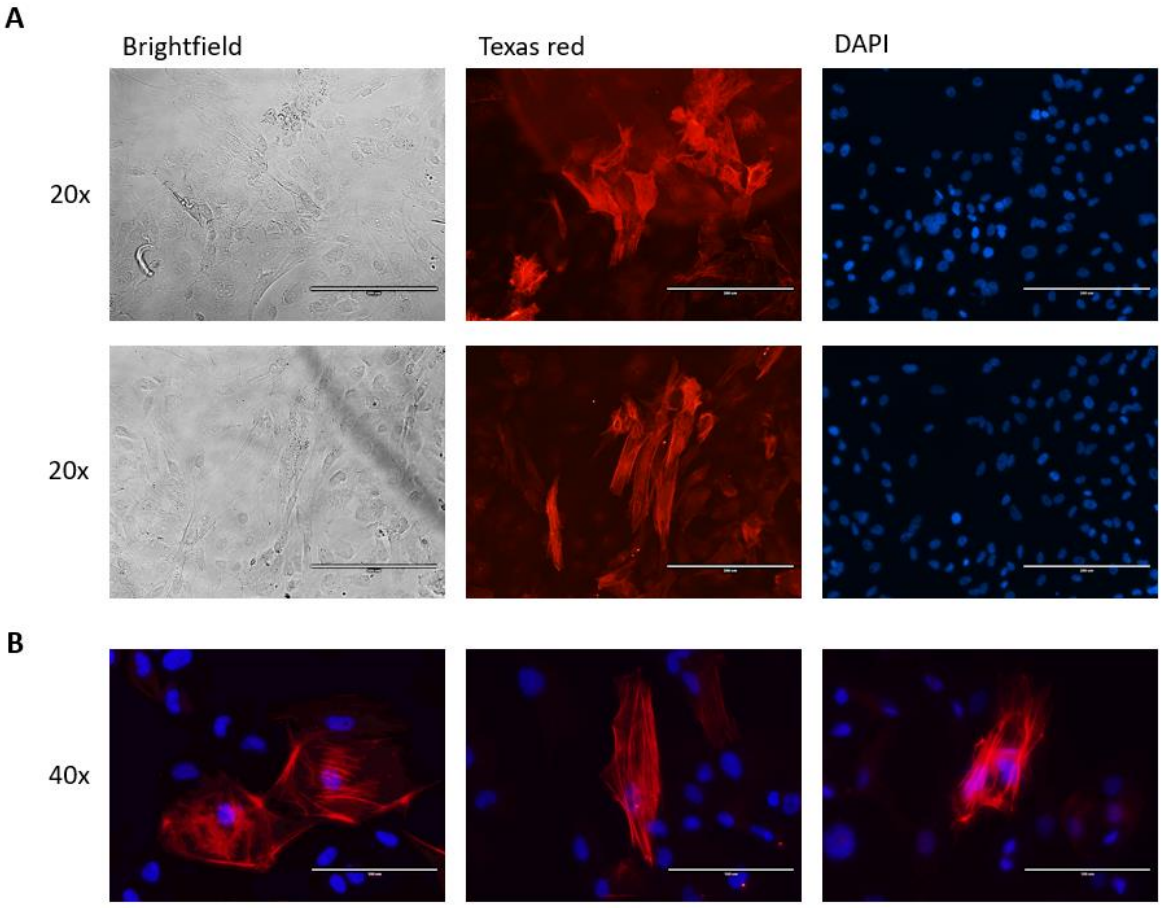

Figure 24: *IF staining of vascular smooth muscle cells. A) In 20x pictures of brightfield, aSMA (red) and nuclei (blue) next to each other. B) In 40x magnification of cells of interest, overly of aSMA and DAPI channel.*

4 Discussion

4.1 Endothelial Cell Production

We successfully differentiated EC from our pluripotent, karyotypically normal hiPSC. CD31 (Platelet Endothelial Cell Adhesion Molecule (PECAM-1)) was used as an essential marker to identify EC in FACS. CD31 is expressed mainly on EC and platelets and to a lesser extend also on some leucocytes (24). It makes up a substantial portion of EC intercellular junctions. Even though hematopoietic progenitor (origin of platelets and leucocytes) also express CD31 and their differentiation is especially in the first steps quite similar to the EC differentiation, it is very unlikely, that any hematopoietic cells were produced. Both differentiations first induce mesoderm and therefore use cytokines like BMP4, VEGF and bFGF. But afterwards all hematopoietic protocol requires, cytokines specific for their lineage like SCF, TPO, EPO, IL-3, IL-11 … (25, 26), which are of cause not used for the EC differentiation.

In addition, a CD144 staining was performed hand in hand with the CD31 staining. CD144, also called cadherin 5 or vascular endothelial cadherin (VE-cadherin), is a calcium-dependent cell-cell adhesion protein. It is essential in endothelial adherence junction assembly and maintenance and is only expressed in the endothelium, thus specific to EC (27, 28). This double staining was performed for about 1/3 of the assays (*sup. figure 2*). Nearly all cells expressing CD31 also expressed CD144 with CD31 usually being the more stringent marker. Thus, it is safe to assume that all CD31 positive cells are truly EC. Furthermore, CD144 is also used as the antibody target during the MACS separation, so all cells not expressing CD144 are separated out any way. And finally, during the tubing assay, the cells successfully assembled into tubes, which is an attribute exclusive to EC. Thus, the EC cell identity is confirmed without a doubt.

For the EC differentiation 2 different protocols were used and compared:

Both protocols started with a quite different cell density from the get-go. While in the Schrepfer protocol a seeding density of 60% was used, the Cowan protocol aimed for a confluency of 30%. But these different densities make sense when comparing the two media used in detail as seen in *sup. table 1.* The Cowan N2B27 medium is a richer medium. The biggest difference is created because of the N2 supplement, which contains growth promoting progesterone and insulin. In comparison, the Schrepfer protocol lacks any insulin, which is common in cardiomyocyte differentiations, because it inhibits cardiac mesoderm formation,

but not mesoderm formation in general. Especially during the first steps, the Schrepfer protocol is quite similar to the cardiomyocyte differentiation protocol, as described by Lian et al. (29, 30).

Both protocols reach confluency at day 3 and show the appearance of mesodermal cells (phenotype: lager, daker, cobblestone like). This is not surprising since both use the GSK3beta inhibitor CHIR, a Wnt agonist, to induce mesoderm. The Cowan laboratory protocol in addition, adds BMP4, which drives the mesoderm towards lateral plate mesoderm (11).

The Cowan protocol only relies on VEGF as an instructive cue to induce EC. Forskolin is added only to enhance VEGF-induced EC differentiation: Forskolin activates adenylate cyclase, which leads to an increase of cyclic adenosine monophosphate, which in turn activates the protein kinase A and this increases the cells' sensitivity to VEGF (11, 31).

In comparison, the Schrepfer protocol also uses VEGF, but at a lower concentration. Additional it adds bFGF, ROCK inhibitor and SB-431542. ROCK inhibitor and bFGF mainly promote growth. SB-431542 inhibits the kinase activity of the receptors for TGF-β and activin, thus facilitating proliferation and EC sheet formation (32). Moreover, it up-regulates the growth and integrity of EC and suppresses endothelial to mesenchymal transition (12).

5 ± 0.2% of EC could be obtained with the Schrepfer protocol after 7 days. After replating the cells and expanding them, the EC percentage dropped below 1% after a total of 14 days of culture. This is not even comparable with the above 95% EC the Schrepfer group claims to achieve in their paper (16). We hypothesized, that the detaching at day 7 needlessly stressed the cells, thus a continuous 14-day differentiation was performed, which also produced only 4 ± 1.5% of EC.

Furthermore, the addition of PluriSln-1, a stearoyl-CoA desaturase 1 inhibitor, which eliminate undifferentiated cells, did not lead to an increase in the number of EC, rather a further decline of EC was observed. The potency of PluriSln-1 was checked on hiPSC and PluriSln-1 successfully killed them. Thus, it was confirmed that it works and is used in the appropriate concentration. Additionally, no cell death out of the ordinary was visible during the 2 days PluriSln-1 was added. So, no stem cells must have been left in the culture. The decrease between day 12 and 14 is probably not caused by PluriSln-1, most likely the EC just continued to be overgrown by other cells in culture. This is the most likely reason for the drop in EC between day 7 and day 12.

It is not clear why the Schrepfer protocol did not produce EC in a percentage remotely close to their reported yield, further investigation would be needed to assess, why and optimize the protocol. Furthermore, I might help to contact the Schrepfer laboratory directly. This just shows how difficult it is to transfer a protocol form one laboratory to the other, as minor details and variation can have a large - sometimes cumulative - impact on differentiation efficacies. This is especially pronounced in protocols with wany many variables, especially if the protocol is not robust enough.

Because the protocols from the Schrepfer laboratory were not very promising the remaining focus was put on the Cowan laboratory protocol:

The first result of the Cowan laboratory protocol was not ideal: Only 1 ± 0.2% of EC could be obtained. But by simply optimizing the time and seeding density, 21 ± 2.3% of EC were obtained in 3 consecutive differentiations. The 2-day extended protocol with adjusted seeding density was chosen for all future differentiations, since it strikes a good balance between increasing the efficiency and speed. But even with 21 ± 2.3% EC we are still well below their reported 61.8% to 88.8% (11).

Olmer et al. (33) also used the Cowan laboratory protocol: They adapted it for aggregate suspension culture. They started with floating hiPSC aggregates instead of our monolayer and also extended the protocol by 2 days. They were able to obtain 27.9% ± 4.2% of CD31 positive cells. This result is still above our 21 ± 2.3% but still in the comparable range. After optimization of aggregate size, the reached between 50-65% EC in three different cell lines. Notably they increased their VEGF concentration to 260ng/m in comparison to the 100ng/ml we used. Therefore, it might be promising to increase the VEGF concentration in future differentiation.

A purity of 80-95% EC was obtained via MACS separation, which is comparable to the values in the Cowan laboratory paper (11). The only downside to the MACS was the cell loss during this procedure. The majority of the cells were probably lost during the filtration step. Since during the differentiation quite a considerable amount of ECM was produced, which makes the cell pellet and suspension after detachment quite clumpy and sticky. Additionally, as a natural part of any differentiation unwanted cells die. These dead cells than release DNA and DNA is innately sticky, and thus adheres to both live cells and ECM. Thereby, further contributing to the cell suspensions stickiness. As a result, a lot of cells, together with the clumps of ECM, get stuck in the filter during the filtration step. As a relieve a 1h DNase treatment (suspension of cells for 1h in serum free medium with 40 U/ml DNase

before detachment) was included, to degrade all free DNA. Afterwards, the number of obtained EC nearly doubled, but still only a yield of 42% could be achieved.

In future MACS sorting, it might help to include Collagenase to digest collagen, one of the major ECM components. This has been done with great success in cardiomyocyte differentiation as described by Schneider et al. (34).

The expansion of the EC worked well. The EC grew quite well in EGM-2 MV SingleQuots media, a media optimized for the culture of micro vessels, and even better in the EGM-2 MV SingleQuots with added 10% FBS. Moreover, they mostly remained EC, since after passage 3 there were still 60% EC left. In cultures with very low densities, long culture times and lower initial EC purities, overgrowth by different cell types could be observed. Thus, it is advisable to not seed cells at too low densities (don't split below 1:4) and not culture them for too long.

In a first step towards making the protocol more feasible from a commercial standpoint, StemPro 34 SFM was replaced with regular DMEM. This nearly cuts the differentiation cost in half. And the differentiation efficacy remained at 20 ± 0.4%. Thus, DMEM is a viable alternative. It might be worth exploring advanced DMEM which has a reduced FBS contend in comparison to regular DMEM. Since StemPro 34 SFM is a serum free medium, advanced DMEM would match it closer and is in the same price range as regular DMEM.

Next steps for the EC should include the test of the barrier function, trans-endothelial electrical resistance (TEER), capacity of lipid uptake and the effects of proinflammatory cytokines (TNF-α and IL-1β). Which would provide valuable information for the tissue engineering later on (11).

4.2 Vascular Smooth Muscle Cell Production

We successfully differentiated vSMC from our pluripotent, karyotypically normal hiPSC and were able to obtain about 90 ± 7% CD140b positive cells. Thus, our results are very similar to the 95.4% CD140b psitive cells described by the Cowan laboratory (11).

CD140b, also known as platelet-derived growth factor receptor beta, plays an essential role in blood vessel development. Additionally, it is also involved in vSMC migration and the formation of neointima at vascular injury sites (35). CD140b is highly expressed in vSMC but not exclusive to them. For example, it can also be expressed in mesenchymal cells, pericytes and in fibroblasts (36). Thus, on its own, it is not a reliable maker for vSMC identity.

Therefore, an additional aSMA staining was performed. aSMA is important for vascular contractility, but is also not exclusive to vSMC: It is expressed in fibroblast and myofibroblasts as well (37, 38). All our cells expressed aSMA, but most at low levels. We also observed some extremely bright cells. The aSMA was integrated into actin stress fibers of the bright cells. Especially if you compare the 3 pictures taken at 40x magnification in *figure 24 B* with the depictions of stress fibers as seen in Tojkander et al. (39). The presence of stress fibers could indicate a shift towards a more contractile phenotype, as described by Wanjare et al. (40). Comparing our results with the aSMA staining by the Cowan lab, there are similar levels of heterogenicity in the staining intensity visible (11).

Next steps would include the use of additional vSMC makers, to further confirm cell identity: Interesting makers could be smooth muscle 22α/taglin (SM22α/taglin), myosin heavy chain 11 (MYH11), calponin 2 & 1 (CNN2 & 1) and Smoothelin (SMNT). Furthermore, these makers could help in evaluating vSMC maturity and phenotype. aSMA and SM22 are highly expressed in early, synthetic vSMC, while CNN1, MYH11 and SMNT are strongly expressed in later, contractive vSMC (17, 41). Heparin or Activin A could be used to induce a more mature contractile phenotype.

Furthermore, the mechanical properties of the vSMC could be assessed by a three-dimensional collagen contractility assay.

5 Conclusion

Our optimized Cowan laboratory EC differentiation protocol produces the best results, with regards to differentiation efficacy, total number of obtained EC, cost, and time. It utilizes a seeding density of 34,000 cells/cm^2 with first exposure to N2B27 medium + CHIR + BMP4 for 72 h followed by 96 h DMEM + VEGF + forskolin. Finally, a 1 h 40 U/ml DNase treatment before detaching the cells is requires. Expansion was optimal in EGM-2 MV SingleQuots +10% FBS and cell identity was undoubtedly confirmed by CD144, CD31 FACS staining, and a tube formation assays.

Regarding the vSMC differentiation, already 90 ± 7% vSMC could be obtained in line with the original Cowan laboratory protocol. They were identified by CD140b and aSMA staining, but additional vSMC specific makers are required to confirm cell state and identify whether the vSMC express a more contractile or synthetic phenotype.

To conclude, we are able to produce one million EC and eight million vSMC from one million hiPSC. Therefore, we will be able to match the demand set by hiPSC-derived living blood vessel production after having followed a proper cell expansion.

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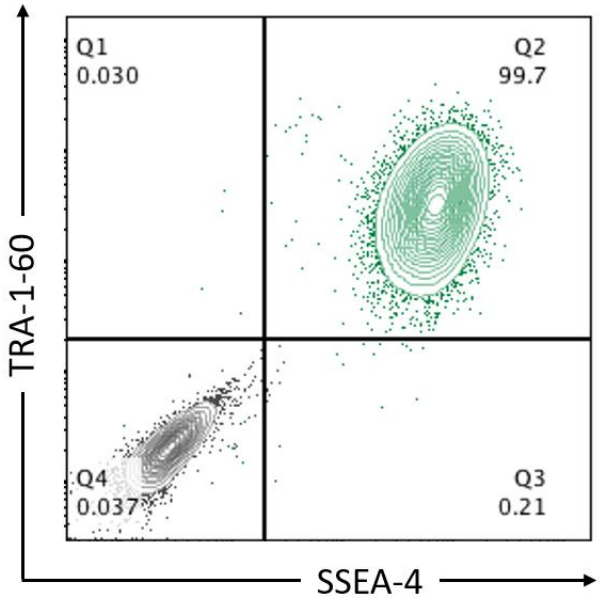
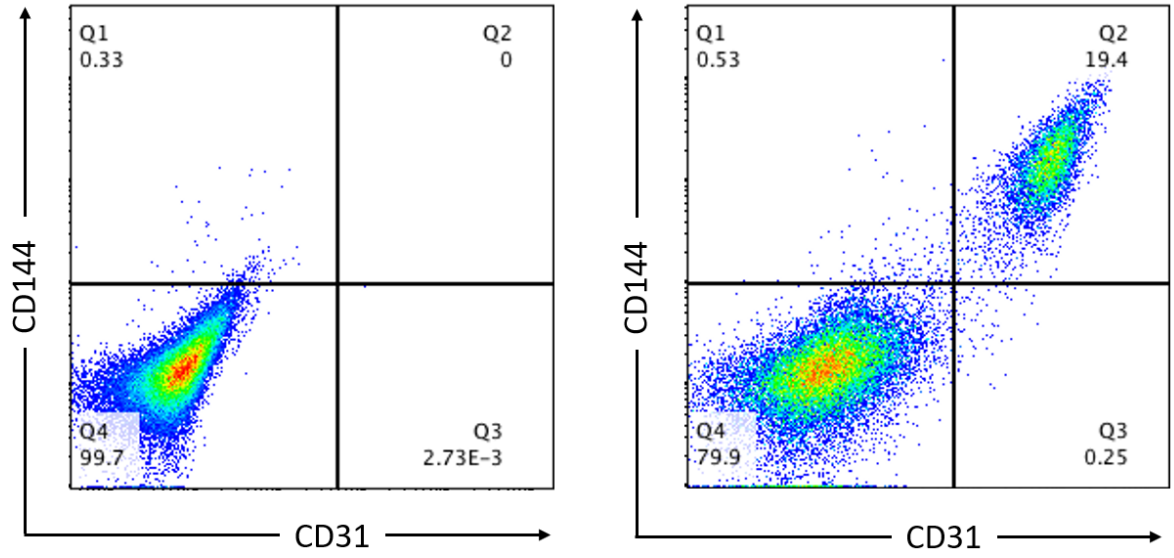
Supplement

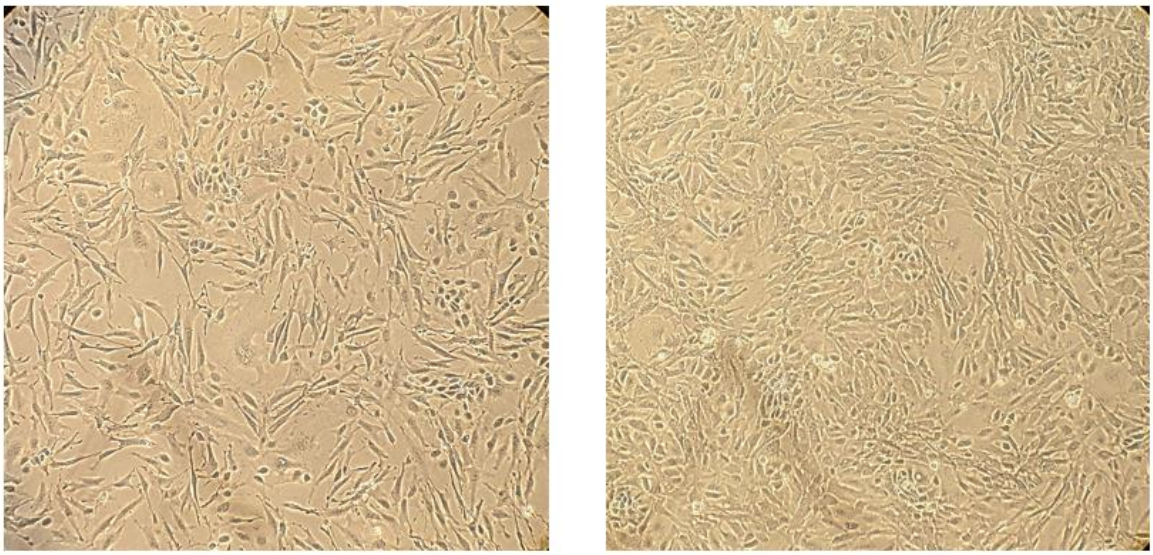


Sup. Figure 1: *FACS Pluripotency staining of TRA-1-60 and SSEA-4.*



Sup. Figure 2: *FACS unstained control and CD144 and CD31 double staining.*

Phenotype of a typical culture of vSMC culture at low and high density in DMEM + supplement can be seen I *sup. Figure 3*.



Sup. Figure 3: *vSMC – p3 at low and high density.*

Sup. Table 1: *Comparison of media used in the Schrepfer and Cowan differentiation protocol media. All minor differences in yellow and al major differences marked in red.*

N2B27 medium + additions for EC Cowan	**RPMI-1640 + additives for EC Schrepfer**
N-2 Supplement	
Human Transferrin	
Insulin Recombinant Full Chain	
Progesterone	
Putrescine	
Selenite	
B27	**B-27 minus insulin**
Vitamins	**Vitamins**
Biotin	Biotin
DL Alpha Tocopherol Acetate	DL Alpha Tocopherol Acetate
DL Alpha-Tocopherol	DL Alpha-Tocopherol
Vitamin A (acetate)	Vitamin A (acetate)
Proteins	**Proteins**

BSA, fatty acid free Fraction V
Catalase
Human Recombinant Insulin
Human Transferrin
Superoxide Dismutase
Other Components
Corticosterone
D-Galactose
Ethanolamine HCl
Glutathione (reduced)
L-Carnitine HCl
Linoleic Acid
Linolenic Acid
Progesterone
Putrescine 2HCl
Sodium Selenite
T3 (triodo-I-thyronine)

BSA, fatty acid free Fraction V
Catalase
Human Transferrin
Superoxide Dismutase
Other Components
Corticosterone
D-Galactose
Ethanolamine HCl
Glutathione (reduced)
L-Carnitine HCl
Linoleic Acid
Linolenic Acid
Progesterone
Putrescine 2HCl
Sodium Selenite
T3 (triodo-I-thyronine)

DMEM/F-12, GlutaMAX™ supplement

Amino Acids
Glycine
L-Alanine
L-Alanyl-L-Glutamine
L-Arginine hydrochloride
L-Asparagine-H2O
L-Aspartic acid
L-Cysteine hydrochloride-H2O
L-Cystine 2HCl
L-Glutamic Acid
L-Histidine hydrochloride-H2O
L-Isoleucine
L-Leucine
L-Lysine hydrochloride

RPMI-1640 + 1% glutamate

Amino Acids
Glycine
L-Glutamine
L-Arginine (free base)
L-Asparagine (anhyd)
L-Aspartic Acid
L-Cystine•2HCl
L-Glutamic Acid
L-Histidine (free base)
L-Isoleucine
L-Leucine
L-Lysine•HCl
L-Methionine
L-Phenylalanine

L-Methionine	L-Proline & Hydroxy-L-Proline
L-Phenylalanine	L-Serine
L-Proline	L-Threonine
L-Serine	L-Tryptophan
L-Threonine	L-Tyrosine•2Na•2H2O
L-Tryptophan	L-Valine
L-Tyrosine disodium salt dihydrate	**Vitamins**
L-Valine	D-Biotin
Vitamins	Choline Chloride
Biotin	D-Pantothenic Acid•½Ca
Choline chloride	Folic Acid
D-Calcium pantothenate	Niacinamide
Folic Acid	Pyridoxine•HCl
Niacinamide	Riboflavin
Pyridoxine hydrochloride	Thiamine•HCl
Riboflavin	Vitamin B-12
Thiamine hydrochloride	myo-Inositol
Vitamin B12	p-Amino Benzoic Acid
i-Inositol	**Inorganic Salts**
Inorganic Salts	
Calcium Chloride (CaCl2) (anhyd.)	
Cupric sulfate (CuSO4-5H2O)	
Ferric Nitrate (Fe (NO3)3"9H2O)	
Ferric sulfate (FeSO4-7H2O)	MgSO4 (anhyd)
Magnesium Chloride (anhydrous)	KCl
Magnesium Sulfate (MgSO4) (anhyd.)	NaHCO3
Potassium Chloride (KCl)	NaCl
Sodium Bicarbonate (NaHCO3)	Na2HPO4 (Anhyd)
Sodium Chloride (NaCl)	
Sodium Phosphate dibasic (Na2HPO4) anhydrous	
Sodium Phosphate monobasic (NaH2PO4-H2O)	Ca(NO3)2•4H2O 0

Zinc sulfate (ZnSO4-7H2O)	**Other Components**
	D-Glucose
Other Components	
D-Glucose (Dextrose)	
Hypoxanthine Na	
Linoleic Acid	Phenol Red•Na
Lipoic Acid	
Phenol Red	
Putrescine 2HCl	
Sodium Pyruvate	Glutathione (reduced)
Thymidine	

Neurobasal

Amino Acids
Glycine
L-Alanine
L-Arginine hydrochloride
L-Asparagine-H2O
L-Cysteine
L-Histidine hydrochloride-H2O
L-Isoleucine
L-Leucine
L-Lysine hydrochloride
L-Methionine
L-Phenylalanine
L-Proline
L-Serine
L-Threonine
L-Tryptophan
L-Tyrosine
L-Valine
Vitamins
Choline chloride
D-Calcium pantothenate

RPMI-1640

Amino Acids
Glycine
L-Arginine (free base)
L-Asparagine (anhyd)
L-Aspartic Acid
L-Cystine•2HCl
L-Histidine (free base)
L-Isoleucine
L-Leucine
L-Lysine•HCl
L-Methionine
L-Phenylalanine
L-Proline & Hydroxy-L-Proline
L-Serine
L-Threonine
L-Tryptophan
L-Tyrosine•2Na•2H2O
L-Valine
Vitamins
Choline Chloride
D-Pantothenic Acid•½Ca

Folic Acid	Folic Acid
Niacinamide	Niacinamide
Pyridoxal hydrochloride	Pyridoxine•HCl
Riboflavin	Riboflavin
Thiamine hydrochloride	Thiamine•HCl
Vitamin B12	Vitamin B-12
i-Inositol	myo-Inositol
	p-Amino Benzoic Acid
Salts	**Inorganic Salts**
Calcium Chloride (CaCl2) (anhyd.)	
Ferric Nitrate (Fe(NO3)3"9H2O)	
Magnesium Chloride (anhydrous)	MgSO4 (anhyd)
Potassium Chloride (KCl)	KCl
Sodium Bicarbonate (NaHCO3)	NaHCO3
Sodium Chloride (NaCl)	NaCl
Sodium Phosphate monobasic (NaH2PO4-H2O)	Na2HPO4 (Anhyd)
Zinc sulfate (ZnSO4-7H2O)	
	Ca(NO3)2•4H2O 0
Other Components	**Other Components**
D-Glucose (Dextrose)	D-Glucose
HEPES	
Phenol Red	Phenol Red•Na
Sodium Pyruvate	
	Glutathione (reduced)