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# Metabolic regulation of differentiation and maturation: from induced pluripotent stem cell to endothelial cell

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Chapter 1

# General Introduction & Outline of This Thesis

## Reversed regulation: How metabolism regulates the cell

This Thesis

Gesa L. Tiemeier

# General introduction

Treatment of chronic kidney disease (CKD) is an unmet medical need. To date, kidney transplantation is the only available cure for CKD patients with renal failure, but suffers from a shortage of high quality transplantable donor kidneys, primarily due to ischemia/reperfusion (I/R) injury during donor kidney graft preservation and transplantation.[1] The group of Professor Rabelink uses two novel approaches to develop regenerative medicine for CKD, based on their expertise in the field of vascular biology and transplantation medicine: (i) Ex vivo regeneration of deceased donor kidneys to improve their transplantation potential, currently limited by endothelial cell (EC) damage due to I/R injury; (ii) Personalized kidney regenerative medicine via generation of kidney organoids (KORs) (derived from patient-derived induced pluripotent stem cells (iPSCs)), offering unprecedented therapeutic opportunities including autologous kidney tissue transplantation[2-4].

## The plasticity of induced pluripotent stem cells

This thesis will focus on the extensive opportunities of regenerative medicine via human-derived induced pluripotent stem cells (hiPSCs). Human pluripotent stem cells (hPSCs) have the ability to give rise to any cell type of the human body. Their capacity to self-renew indefinitely presents an unlimited source of all these cell types. At the blastocyst stage of embryonic development, the pluripotency of embryonic stem cells (ESCs) in the inner cell mass allows the embryo to form all three germ layers and further develop in a fully functional human body. In 2007, human adult somatic cells were reprogrammed into a pluripotent state by overexpression of four transcription factors – OCT3/4, SOX2, KLF4 and MYC – resulting in so-called induced pluripotent stem cells (iPSCs).[5] This discovery resulted in an unlimited source of patient-derived human cell types to examine disease mechanisms, discover new drugs and develop regenerative medicine therapies.

However, maturation and development of IPSC derived tissues such as the KORs are currently limited by inadequate organotrophic vascularization and lack of functional hiPSC-derived endothelial cells (iPSC-EC). To enable vascularization, the functionality following maturation of human iPSC derived endothelial cells (hiPSC-EC) must be carefully assessed before their scientific and therapeutic potential can be realized[6].

## Stem cell differentiation into endothelial cells

To date, multiple protocols have described hiPSC-ECs differentiation [7-13], to steer iPSC towards mesoderm and eventually to the vascular lineage. Various factors can affect the outcome of differentiation: the presence of feeder cells, fetal bovine serum, bovine serum albumin and the quality of stromal cell lines and the addition of growth factors.[14-18] The earliest protocols were mostly based on serum-supplemented medium or stromal cell lines and most of these early methodologies were based on aggregate- or embryoid body formation resulting in very low efficiencies of EC differentiation (< 1.5% ECs).[19] Currently, five aggregate and monolayer protocols are widely used and robustly reproduced in different labs; the protocols of Wimmer *et al.* [7, 20], Gu *et al.* [21], Patsch *et al.* [22] and Orlova *et al.* [4]. All these differentiation protocols first initiate mesoderm induction, using CHIR-99021 (CHIR) and morphogenetic protein 4 (BMP4) with an addition of either Activin A or small molecule Y-27632 (Figure 1). Endothelial cell lineage is then induced by vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF), however the concentration varies for each protocol. Furthermore, some protocols have an EC selection step using either platelet endothelial cell adhesion molecule (CD31) or VE-cadherin (CD144) at the end of the protocol (Figure 1). The slight differences timing and concentration of growth factors, influences the efficiency of the differentiation, measured by the percentage of CD31 and/or CD144 positive cells at the end of differentiation. The quality of differentiation is measured by testing endothelial cell functions, such as sprout formation, barrier formation or expressing endothelial specific proteins. Important, but sometimes neglected are the possibilities to passage and freeze the cells or test the proliferation capacity [4].

## Current limitations of iPSC derived endothelial cells

For the studies in this thesis, we have used the protocol of Orlova *et al*.[4], and to address the protocol differences we have compared RNA sequencing results of our cells with data of the Gu, Wimmer and Patsch protocols.[7, 20, 22, 23] We observed no significant differences comparing our protocol to that of Gu and Wimmer, however, these data showed that the Patsch ECs seem to cluster closer to primary endothelial cells (ECs). (Figure 2) Although different in gene expression, an extensive comparison between the Wimmer and Patsch protocol by the Wimmer group[22] could not find any significant functional differences. Although batch correction has been used, it has to be taken into account that comparing RNA sequencing results from different studies has more technical limitations then performing the RNA sequencing at the same time. Due to very high costs and limited benefits, these tests have not been done for multiple protocols to date.

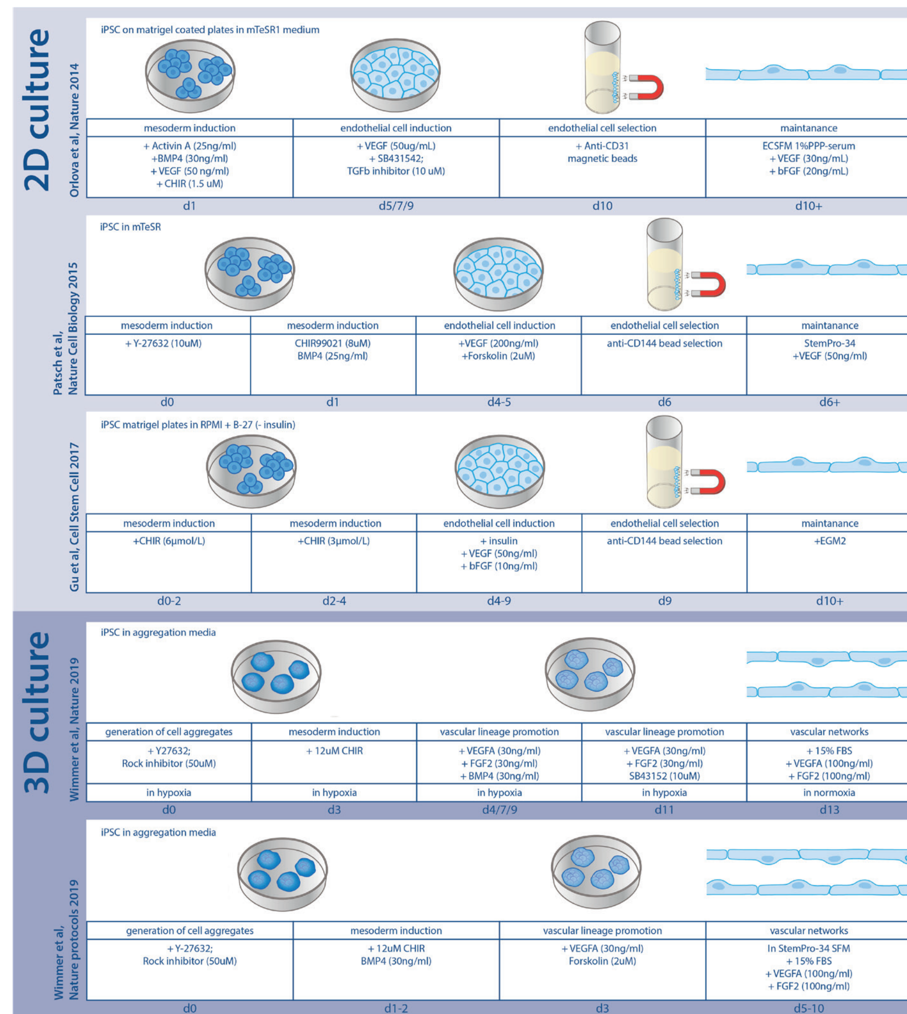


**Figure 1. Protocols to differentiate hiPSC into hiPSC-EC**. Illustration of 5 widely used differentiation protocols of hiPSC-EC, including the most recent protocols of Wimmer. All protocols first induce mesoderm and then induce vascular lineage by adding growth factors and small molecules. Orlova, Patsch and Gu use a monolayer approach followed by magnetic bead selection against CD31 or CD144 at the end of differentiation. Wimmer has developed a protocol for endothelial cells starting from iPSC aggregates which are differentiated into mesoderm aggregates and further cultured to form vascular organoids (3D culture). Endothelial cells can be either cultured in 3D in association with pericytes or isolated for further use. Only upon transplantation into mice, these vascular organoids fully mature and form arteries, veins and capillaries. The first protocol of Wimmer cultured the cells during differentiation in hypoxic conditions. However, this was not essential as shown in his newer protocol without hypoxic culture.

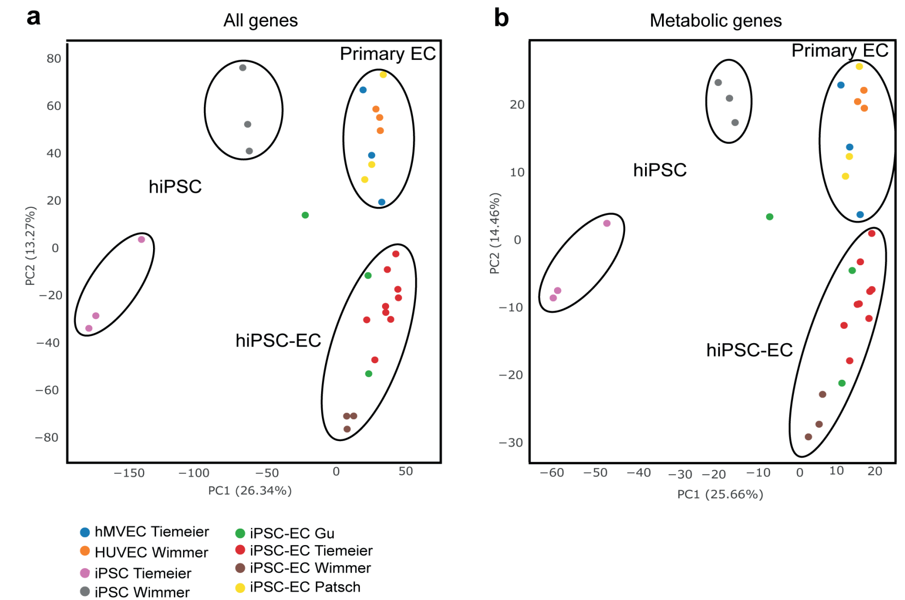


**Figure 2. Comparison of hiPSC-EC differentiation protocols by RNA-sequencing** Principle component analysis of all genes **(a)** and metabolic genes **(b)** acquired from RNA-sequencing results of hiPSC (Wimmer: pink, Tiemeier: grey), primary mature ECs (HUVEC: orange, hMVECs: blue) and hiPSC-ECs derived by several differentiation protocols (Wimmer: brown, Gu: green, Patsch: yellow, Tiemeier: red) in static 2D conditions. Tiemeier *et al*. used three hiPSC lines to create hiPSC-ECs: NCRM1, L72, L99.

Using various protocols in previous studies, researchers have shown that iPSC-EC have an endothelial phenotype, however these ECs did not recapitulate all features of their adult (primary) counterparts [24-27] and remain in an immature stage of development. Although the phenotype of hiPSC-EC is not thoroughly studied, initial research shows no barrier response to histamine and a reduced barrier response to thrombin in hiPSC-EC, questioning the maturity of the endothelial barrier and hemostatic capacity[25]. In addition, several studies have identified the reduced expression of von Willebrand Factor (VWF) and limited amount of Weibel Palade Bodies (WPB) in hiPSC-EC.[7, 20, 25] Since Weibel Palade Bodies play a crucial role in the inflammation, hemostasis, regulation of vascular tone and angiogenesis [28], the proper function and maturation of WPB is limiting for use of hiPSC-EC in various applications. Interestingly in the most recent work of 3D models of iPSC-EC the expression of VWF in both 2D and 3D is very low compared to HUVECs with only a minor increase in 3D culture, even though maturation of iPSC-ECs and differentiation into different types of ECs (venule, capillary, arteriole and artery) is achieved.[7, 20]

These studies show only evidence of mature WPB in the vascular networks of their transplanted vascular organoid, which could suggest that full maturation of iPSC-EC depends on connection to a host vasculature and to the host blood flow. Van den Berg *et al*. have found similar results in the maturation of the glomeruli in human kidney organoids, upon transplantation in mice.[29]

## Mechanosensing and the role of the glycocalyx: the hallmark of endothelial cells

As the lining of all blood vessels the endothelium is directly exposed to the forces of blood flow and circumferential wall stress.[30] By sensing the shear stress of blood and transducing this in biochemical signals, the endothelial cell coordinates vascular tone and homeostasis. This regulation between vasodilation and contraction, vascular permeability, inflammation and immune signaling are crucial elements of vascular function. Shear stress directly regulates the crucial endothelial transcription factors Krüppel-like factor 2, and -4, (KLF2 and KLF4), YAP (an effector of the Hippo signaling pathway), and NFkB [31, 32]. Laminar levels of shear stress range from 1 to 70 dyne/cm$^2$, with 10-20 dyne/cm$^2$ in most arteries and 1-6 dyne/cm$^2$ in veins. The mechanosensing machinery consists of the ion channels, the cytoskeleton, composed of microtubules, microfilaments and intermediate filaments, which sense tension via junctions and extracellular matrix.[33]

The extracellular matrix on the apical membrane of the endothelial cell, called the glycocalyx or endothelial surface layer is directly sensing the shear stress through sugar chain shedding or protein scissor-like motions such as found for the proteoglycan, syndecan-4. [34] The main contributors to the negatively charged gel-like glycocalyx are glycosaminoglycans (GAGs), proteoglycans (PG) and glycoproteins. The main long carbohydrate GAG chains present in the endothelial glycocalyx are heparan sulfate (HS), hyaluronan (HA) and chondroitin sulfate (CS). [34, 35] In addition, heparan sulfate covalently attached to membrane bound core-proteins such as syndecans 1 and -4, glypican-1 and versican, constitute the proteoglycans. [35, 36] Proteoglycan core-proteins crossing the membrane, such as syndecans can, similarly to integrin receptors, link cytoskeletal proteins with the extracellular matrix and thereby transduce the sensed forces. [33]

This resultant interconnected matrix of carbohydrates has been proposed to be involved in almost all functions of the endothelial layer, such as inflammation, coagulation, permeability, shear sensing and regulation of perfusion [33, 35, 37, 38]. Binding of growth factors, such as FGF-2 and VEGF, or cytokines (e.g. chemokines) have been shown to be critically dependent on binding to specific sulfated HS moieties, generating gradients over the endothelium to guide cells towards the underlying tissue or concentrate factors near their receptor (e.g. growth factors). [38-40] The molecular composition of the glycocalyx is a dynamic balance between continued biosynthesis and degradation or shedding of glycans depending on micro-environment, cell metabolism and shear stress exposure [41, 42].

## The role of the metabolism in glycocalyx formation

Recent studies discovered that reduction or loss of the glycocalyx causes microvascular destabilization, which results in organ damage, for example seen in renal glomeruli and retinal capillaries in diabetes mellitus.[43] Furthermore, we found that increasing endothelial glycolysis activity, inducing angiogenic cells, impairs the formation of a functional glycocalyx layer through reducing substrate availability necessary for hyaluronan synthesis. On the other hand, lowering EC glycolysis, for example by KLF2 overexpression, induced vessel normalization and tightens the vascular barrier.[44-48] (Figure 3) Studies from the Professor Carmeliet lab revealed that ECs reprogram their metabolism to (re)generate new blood vessels.[49]

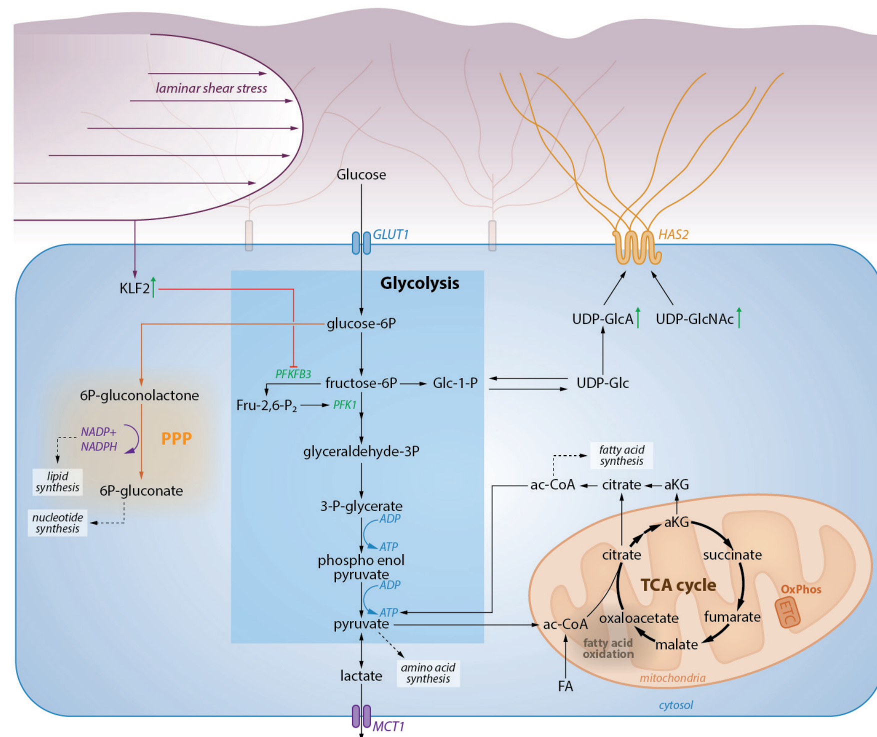


**Figure 3. Endothelial cell metabolism during laminar shear stress**. Schematic overview of endothelial metabolism and its role in glycocalyx production and redox balance hyaluronan biosynthesis regulation. Glucose enters the cell through a glucose transporter (GLUT), after which it is used to fuel the glycolysis. From the glycolysis, several side branches arise like the pentose phosphate pathway (PPP) and Hyaluronan synthase 2 (HAS2) substrate UDP-Glucuronic acid (UDP-GlcA). The availability of UDP-GlcA and UDP-GlcNAc determine HA synthesis by HAS2. At the end of the glycolysis route, pyruvate can be transported into the mitochondria to be converted into acetyl-CoA. This metabolite, together with acetyl-CoA derived from fatty acid oxidation, can subsequently fuel the TCA cycle. All these different metabolic pathways provide the precursors for lipid, nucleotide, fatty acid and amino acid synthesis. Furthermore, reactive oxygen species (ROS) is produced by oxidative phosphorylation (OxPhos) and antioxidants are produced in the PPP. In a quiescent state, laminar shear stress increases KLF2 and thereby reducing the activity of 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3 (PFKB3), which causes a break in the lower part of glycolysis, increasing the production of glycocalyx substrates, antioxidants and reducing mitochondrial oxidation.
aKG = α-ketoglutarate, FA = fatty acid, OxPhos = oxidative phosphorylation, ETC = electron transport chain, PFKFB3 = 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3, PFK1 = phosphofructo kinase 1, UDP-Glc = Uridine diphosphate glucose, UDP-GlcA = Uridine diphospho-glucuronic acid, UDP-GlcNAc = uridine diphosphate*N*-acetylglucosamine, MCT1= monocarboxylate transporter 1, GLUT 1 = Glucose transporter 1, HAS2 = Hyaluroanan synthase 2, KLF2= Krüppel-like factor re, the redox balance is regulated by formation of reactive oxygen species 2, ATP = Adenosine Triphosphate, ADP = Adenosine diphosphate, PPP = Pentose phosphate pathway, TCA cycle = The tricarboxylic acid cycle





### iPSC derived endothelial cell metabolism

Induced pluripotent stem cells (iPSC) are known to undergo considerable changes in their metabolism upon reprogramming from adult somatic cells to their pluripotent state. The most important change is a shift from oxidative phosphorylation (OxPhos)-dependency towards glycolysis-dependency. The glycolytic intermediates fuel the synthesis of nucleotides and lipids, pyruvate can be used to synthesize nonessential amino acids, and acetyl-CoA is a precursor for the synthesis of fatty acids (FA), all of which are crucial for highly proliferative iPSCs. During differentiation, iPSCs once again undergo metabolic switching, which depends on the requirements for the different germ layers. The mesodermal and endodermal lineages switch back to an OxPhos-dependent metabolism, whereas the ectodermal lineage keeps utilizing its maximal glycolytic capacity. Interestingly the vascular lineage seems to undergo further metabolic switch, going van oxidative mesoderm back to highly glycolytic endothelial cells. Although an increasing number of studies display the tight connection between metabolism and cell-fate consequences, the underlying mechanism that links these two is not fully understood. This thesis discusses the role of metabolism on endothelial cell functions in the wider context of iPSCs and iPSC-derived cells. Thereby, we aim to provide more insight into the interplay between these processes and speculate how this knowledge could be used to improve differentiation and maturation of iPSCs and iPSC-derived cells.

## Outline of this thesis

### Research questions

Although many EC differentiation protocols have been published, the question about the level of maturity and functionality remains not fully answered. To further drive maturation and use the hiPSC-EC in more complex experimental setups or even transplantation, we questioned how the hiPSC-EC would respond to prolonged shear stress and pericyte co-culture. Both have been shown to improve endothelial cell function and induce a more quiescent state. Furthermore, we were interested in studying the endothelial glycocalyx of hiPSC-EC, since the glycocalyx is involved in most functions of the EC. Since recent studies showed the involvement of the cellular metabolism in differentiation and maturation of iPSC derived tissues, it drove us to explore this cellular metabolism in more detail.

## Hypothesis: Reversed regulation; how metabolism regulates the functionally of the cell

The metabolism of iPSCs has long been thought to be a consequence of their state, but recently it has been proposed that metabolism has an important and active role in various cellular processes rather than solely providing the energy the cell needs. Notably, cellular metabolism has been marked as driving force in reprogramming, pluripotency maintenance and differentiation.[50, 51] During these processes, changes in key metabolic pathways contribute to alterations in the availability of metabolites such as acetyl-CoA, Nicotinamide-adenine-dinucleotide$^{+}$ ($NAD^{+}$), lactate and fatty acids, which have been shown to have crucial signaling roles.[52]

Although Patsch *et al.*, showed that hiPSC-EC are more similar to primary EC then stem cells when assessing the global transcriptomics and metabolomics, the functional metabolic activities of hiPSC-EC have not yet been studied.[22] By understanding and influencing the cellular metabolism we aimed to improve functionality and maturation of hiPSC-ECs. Not only is cellular metabolism providing the building blocks for a cell, such as nucleotides, lipids, amino-acid and glycocalyx substrate, it also regulates the cellular redox-homeostasis.(Figure 3) Loss of equilibrium between pro- and antioxidant signaling produces oxidative stress, the latter arises from redox signals such as ROS from mitochondria ($O_2^{-\cdot}$) and reactive nitrogen species [RNS such as NO, peroxynitrite (ONOO−), nitrous oxide ($^{\cdot}NO_2$), $N_2O_3$] as well as the activation of pro-inflammatory or proangiogenic pathways. This loss of equilibrium in the redox state is the center of endothelial dysfunction, for example by activation of transcription factors such as NFkB, AP-1 and hypoxia inducing factor 1α (HIF-1α) and directly causing damage the endothelial glycocalyx. [33, 53-55]

Due to the multiple metabolic switches during the differentiation from stem cells to endothelial cells, regulating ROS signaling is supposed to be particularly challenging.[56] Recent evidence from both pluripotent embryonic and adult stem cell studies suggests that the balance between self-renewal and differentiation is crucial for stem cell function during both early development and tissue homeostasis throughout life and this balance is partly regulated by ROS produced during metabolic processes.[57-59]

For the studies in this thesis, we have used state-of-the art techniques to measure the functional metabolism, such as Seahorse (measuring lactate excretion and $O_2$ consumption), Nuclear magnetic resonance (NMR)-based metabolomics and ROS and intracellular pH (pHi) assays, to gain deeper understanding in the role of metabolism during development and maturation of IPSC derived cells and tissues. The obtained results and targets can be used to improve vascularisation of KOR, improve hiPSC kidney scaffold re-cellularization, and to further develop a more mature nephron-on-a-chip with a vascularized glomerulus, which will be of unprecedented value for drug screening, disease modelling and regenerative kidney medicine.

## Contents of this thesis

In this thesis, we addressed the role of cellular metabolism in maturation of hiPSC-ECs. In **Chapter 2**, we compare hiPSC derived EC functionality and metabolism with primary human microvascular endothelial cells (hMVEC) determining the presence of a luminal glycocalyx as a main functional target. This chapter presents new insights in mitochondrial dysfunction of hiPSC-EC, limiting their ability to produce sufficient glycocalyx and align to shear stress. Underlying the mitochondrial dysfunction, we found that hiPSC-EC have an open mitochondrial permeability transition pore (mPTP), indicating mitochondrial immaturity. By closing the mPTP during differentiation with Cyclosporin-A (CsA), binding to cyclophilin D of the mPTP, we were able to mature mitochondria and improve functionality of these cells, resulting in a reduction in ROS, increased glycocalyx production and the therefore providing iPSC-ECs the ability to align to shear stress.

**Chapter 3** Continues with the comparison of hiPSC-EC with hMVEC, focusing on von Willebrand Factor (VWF) and the production of Weibel Palade Bodies (WPB). Testing several differentiation protocols and even after addition of CsA, hiPSC-EC were found to lack mature WPBs. We showed that neither shear stress nor co-culture with pericytes could induce WPB formation. By further studying the metabolism with NMR we found that hiPSC-EC have a reduced glycolysis and lactate production and an increased intracellular pH (pHi). This coincides with a reduced expression of the proton coupled monocarboxylate transporter MCT1, which transports $H^{+}$ and lactate into the cell to keep pHi in balance. Reducing the intracellular pH led to increased presence of functional VWF and maturation of WPB in hiPSC-ECs.

In **Chapter 4** we addressed the question how hiPSC-EC maintain their redox-balance and produce enough ATP, since the vast majority of ATP and antioxidants of EC are obtained by glycolysis, which is significantly reduced in iPSC-ECs. We found that hiPSC-EC rely mainly on free fatty acid oxidation and presumably use NADPH do maintain their redox balance. This alternative metabolic state in hiPSC-EC was found to be independent of the high expression of the transcriptional coactivator peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC1α), which regulates fatty acid storage, glucose metabolism, lipid uptake and mitochondrial biogenesis. Since PGC1α is directly activated by ROS and is the master

regulator of the energy metabolism, the high levels of PGC1α expression in hiPSC-EC could also be a consequence instead of the cause of the observed differences in metabolism.

In **Chapter 5**, we studied the immunogenic surface of hiPSC-ECs, since transplantation induced rejection by the host immune system is an essential hurdle in usage of IPSC derived tissue. Previous studies suggested that IPSC derived cell recognition by the host immune system is diminished compared to transplantation of allogenic human alternatives. On the other hand, endothelial surface MHC class 1 and 2 molecules do play an active, 'APC like', role in adult immunity. Therefore, we characterized hiPSC-EC surface immune complexes, unstimulated and after cytokine stimulation. In addition we tested how the observed difference in expression could influence CD8 T-cell activation. Furthermore, we characterized the expression of complement inhibitors on the cell surface, necessary for the protection against unprovoked complement activation in the blood.

**Chapter 6** provides a summary and discussion of the observations in this thesis, including future perspectives on studying metabolism and metabolepigenetics in iPSC derived kidney organoids by mass spectrometry imaging.



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