

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

Es - Tiemeier, G.L. van

Citation

Es - Tiemeier, G. L. van. (2021, September 15). *Metabolic regulation of differentiation and maturation: from induced pluripotent stem cell to endothelial cell*. Retrieved from https://hdl.handle.net/1887/3210399

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Author: Es - Tiemeier, G.L. van Title: Metabolic regulation of differentiation and maturation: from induced pluripotent stem cell to endothelial cell Issue Date: 2021-09-15

Chapter 6

Discussion & Concluding Remarks

Cell type specific metabolic plasticity is the hallmark of maturity

This Thesis

Gesa L. Tiemeier, Rosalie J.G Rietjens, Cathelijne W. van den Berg, Bernard M. van den Berg, Ton J. Rabelink

In the previous 10 years there has been a gradual shift in the methods of characterization of cells and tissues for their phenotype and functionality. First it was argued that in addition to transcriptomics, proteomics, give a more reliable result to determine the phenotype of cells. Consequently, iPSC-derived tissues and cells have been extensively studied and characterized on specific protein signatures and the cell type was determined by positivity for certain protein markers.[1-5] Only in recent years, cell metabolism has gained more interest as an indicator of functionality and maturity. Theoretically, metabolomics is closer to actual functioning of the cell compared to transcriptomics and proteomics, however technical limitations were minimizing the attention to this field. Recent developments in Mass Spectrometry (MS), Nuclear Magnetic Resonance (NMR) spectroscopy and Mass Spectrometry Imaging (MSI) techniques lead to robust and innovative results. In addition, single cell approaches in all "–omic" fields, are further enhancing our knowledge and we are extending the level of detailed understanding.[6, 7] Eventually, integrating these single cell approaches will give the most reliable and relevant outcomes.[8, 9]

Metabolism in somatic cell reprogramming

Reprogramming somatic cells into iPSCs makes the cells exhibit morphological and proliferative properties of embryonic stem cells (ESCs) and express similar levels of pluripotency marker genes.[10] Subsequently, changes in the metabolism of iPSCs are observed (Figure 1). The vast majority of somatic adult cells prefer oxidative phosphorylation (OxPhos) to produce their necessary energy and therefore the most important metabolic change during reprogramming to the iPSC state, is a shift from OxPhos towards glycolysis-dependency (Figure 1).[11, 12]

This metabolic shift is paradoxically preceded by a burst of OxPhos activity in the early stage of reprogramming, which results in a temporal peak of hypoxia inducible factor α (HIF- α) activity, causing the upregulation of glycolysis (Figure 1).[13, 14], [15, 16] Considering the important role of HIF α in reprogramming, it figures that hypoxic conditions enhance reprogramming efficiency.[17, 18]

Similarly, somatic cells with already a higher activity of glycolysis seem to be more easily reprogrammed because of the smaller metabolic contrast between the somatic baseline and the pluripotent state.[19] These metabolic changes in iPSCs can also be linked to changes in their epigenetic profile.[20] For example, shortterm opening of the mitochondrial permeability transition pore (mPTP) is associated with demethylation of activating histone marks at pluripotency genes.[21] Opening of the mPTP alters ROS production resulting in increased cellular ROS levels. [22] Subsequently, mitochondrial ROS signaling upregulates expression of plant



Figure 1. Induced pluripotent stem cells (iPSC) differ in their metabolic profile compared to their adult somatic cell and differentiated counterparts. Adult somatic cells rely on oxidative phosphorylation to provide in energy demand, which results in a high level of reactive oxygen species. Upon overexpression of the four reprogramming factors, the metabolic profile shifts to more glycolysis-dependency. This metabolic switch is reinforced by mitochondrial fission and HIF α expression. iPSCs have a high glycolytic flux, resulting in high cellular levels of pyruvate and lactate. Intermediates of the glycolysis can be used for antioxidant, amino acid, lipid and nucleotide synthesis. Compared to somatic cells, iPSCs have an open mitochondrial permeability transition pore (mPTP) and higher expression of monocarboxylate transporter 1 (MCT1). 6

homeodomain finger protein 8 (*PHF8*), a histone demethylase specific for methyl groups on histone 3 at lysine 9 and 27 (H3K9me3 and H3K27me3), both regarded as repressive histone marks.[21]

Maintenance of pluripotency requires a high glycolytic state

To meet the high demand for energy and biosynthetic precursors iPSCs rely on glycolysis to sustain their proliferative nature [11, 23]. Another advantage of relying on glycolysis, is that ROS levels in the cells are reduced, resulting in lower oxidative stress and thus diminishing the need to detoxify reactive intermediates.[24]

The glycolytic preference of iPSCs is tightly regulated by several cellular signals, in which the core pluripotency transcription factors (CPTF) directly affect metabolism, with OCT4 binding to the genes encoding the key glycolytic enzymes hexokinase II (HK2) and pyruvate kinase isozyme 2 (PKM2).[11, 25] Secondly, proteins involved in the tricarboxylic acid (TCA) cycle and OxPhos contribute to aerobic glycolysis, independent of the glycolytic proteins. In iPSCs, uncoupling protein 2 (UCP2) is known to augment FA and glutamine oxidation, and to prevent glucose oxidation in the mitochondria by reducing the mitochondrial membrane potential (Figure 1). UCP2 shunts pyruvate away from mitochondrial oxidation, instead steering it towards the pentose phosphate pathway (PPP). Doing so, it controls the substrate access for mitochondria and thereby helps regulating iPSC bioenergetics.[26]

Another set of mitochondrial proteins, the pyruvate dehydrogenase (PDH) complex, is inactive in iPSCs[11], limiting the conversion of pyruvate into acetyl-CoA for the TCA cycle. Lastly, mitochondrial morphology plays a role in promoting glycolysis. Due to mitochondrial fission, mitochondria in iPSCs have a punctate appearance with immature inner membrane cristae .[11] These immature, round mitochondria are not able to provide the cells with sufficient energy and they help sustain CPTF expression, thereby promoting glycolysis in iPSCs.[27] Another demonstration of mitochondrial morphology importance in regulating pluripotency is given by the inhibition of the mitochondrial fusion proteins mitofusin 1 and 2 (MFN1/2). [28] MFN1/2 depletion and subsequent reduced mitochondrial fusion facilitates upregulation of glycolysis through activation of HIF1α signaling.[28]

Switching of metabolism is germ layer specific

Since the discovery of reprogramming, a tremendous number of differentiation protocols have been developed in which embryogenic cues are faithfully mimicked to drive a targeted differentiation pathway by adding growth factors such as BMP4, Wnt, TGF β and Activin A. Cellular metabolism has been recently proposed to be a driver rather than a consequence of differentiation, as was demonstrated by iPSC differentiation into iPSC-derived hepatocytes through the metabolites in conditioned fetal hepatocyte medium.[29]

As specification into different cell types requires a variety of metabolic cues and phenotypic changes, it excludes the idea of a unique metabolic process occurring during differentiation. Instead, metabolic requirements differ for the different germ layers: endoderm, mesoderm and ectoderm (Figure 1).[30]

Metabolic switching from high glycolysis back to an OxPhos-dependent metabolism is restricted to mesoderm and endoderm in early differentiation, whereas ectoderm keeps utilizing its maximal glycolytic capacity. During differentiation, the *MYC* : *MYCN* ratio seems to play a crucial role in controlling metabolic flux. MYC signaling affects many metabolic regulators and enzymes, such as lactate dehydrogenase A (LDHA) and pyruvate dehydrogenase (PDH), which alter the cellular levels of pyruvate, lactate and acetyl-CoA and thus steer the flux through glycolysis and OxPhos.[31, 32]

Another important target of MYC-driven glycolysis is *SLC16A1*, encoding the monocarboxylate transporter 1 (MCT1). MCT1 transports lactate and pyruvate in a proton-linked bidirectional manner across the cell membrane and high *MCT1* expression is associated with the glycolysis-dependent pluripotent state of hPSC. [33, 34] Blocking of *MCT1* expression results in reduced pluripotent markers and increased differentiation. In contrast, in **chapter 3** we describe that high glycolysis in primary endothelial cells was also associated with a high expression of MCT1, whereas the more immature iPSC-derived endothelial cells were found to have a reduced glycolysis and reduced MCT1 expression, independent of the expression of MYC.[35]

iPSC fate decisions through regulation of MYC

Physiologically, high expression of *MYC* and *MYCN* maintains the transcriptional profile that is required for high levels of aerobic glycolysis. For endo- and mesoderm differentiation, both factors are lost during early differentiation, whereas only *MYC* expression is lost during ectoderm differentiation. In the late stages of ectoderm differentiation, *MYCN* expression decreases which shows that ectoderm also undergoes metabolic switching, but only in a later stage than the other two germ layers.

These observations were supported by Lees et al. who showed that in early neural differentiation through the ectodermal layer, glycolytic metabolism first peaks before

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the overall metabolism declines.[36] Consequently, the mitochondrial metabolic proportion increases relative to the glycolytic proportion. Endo- and mesoderm layers, however, switch rapidly upon differentiation, displaying an upregulation of TCA flux and OxPhos with low lactate levels, accompanied with lower expression of *LDHA* and *PKM2*.[30]

Besides mitochondrial oxidation / activity, BMP4-induced mesoderm differentiation requires pyruvate production for cell-fate determination.[37] This mesodermal lineage specification can be potentiated by adding exogenous pyruvate, which acts through the modulation of the MAP kinase (AMPK) and mammalian target of rapamycin (mTOR) pathways (Figure 1).

In addition, inhibition of the glycolysis-associated proteins HIF1a and LDHA and supplementation of fatty acids were all shown to promote a metabolic shift from aerobic glycolysis to OxPhos, resulting in improved differentiation and maturation of the iPSC-derived cardiomyocytes.[38-40] Furthermore, in both iPSC-derived cardiomyocytes and endothelial cells, closure of the mPTP with cyclosporine A led to improved differentiation and maturation, again indicating that metabolism can be a driving force in differentiation. **(chapter 2)**[22, 41, 42]

iPSC derived endothelial cells require a third metabolic switch

Although endothelial cells derive from the mesoderm, in which both MYC and MYCN expression is lost, mature endothelial cells mainly rely on glycolysis for their energy needs. Mitochondrial capacity is maintained, however mitochondria are mainly fueled by glutamine and fatty acids to produce necessary precursors for biosynthesis [43].

Compared to other mesodermal cells, such as cardiomyocytes, endothelial cells therefore have a low number of mitochondria and increased MYC expression (**chapters 2 and 3**). This indicates that endothelial cells undergo a <u>third metabolic switch</u>, after reprogramming and differentiation to the mesodermal lineage. These recurrent changes in metabolism and consequently changes in metabolite availability directly influence the epigenetic landscape of the cells and might alter long term functionality. We therefore argue that metabolic fine-tuning of endothelial cells to obtain fully mature, functional and adaptive cells for prolonged *in vitro* culture is particularly challenging.

Blood circulation is the limiting step in organogenesis

To date, the only iPSC derived endothelial cells that are described to be fully mature are EC within transplanted 3D organoid structures. In 2019 Wimmer et al. described

the transplantation of 3D human blood vessel organoids from hiPSC that exhibit morphological, functional and molecular features of human microvasculature consisting of arterioles, capillaries and venules [44]. ECs in these vascular organoids produced a glycocalyx and generated Weibel-Palade bodies, indicating (metabolic) maturation (**chapter 2 and 3**). Similarly, van den Berg et al. showed maturation of nephrons, including glomeruli, in kidney organoids transplanted under the kidney capsule of mice [45]. Interestingly, both studies show extensive connections between human and mouse vasculature resulting in vessels perfused with (host) blood.



Figure 2. Host blood flow through transplanted kidney organoids results in maturation. Representative cross-sectional confocal images stained for human VWF (green) and human CD31 (red) in (a) iPSC derived kidney organoids and (b) iPSC derived kidney organoids transplanted in a chicken embryo. The experiments were conducted in parallel using iPSC cellijn LUMC72. (c) TEM image of a mature WPB (green arrow) in a kidney organoid transplanted in a chicken embryo transfused with chick derived blood (erythrocyte with nucleus). 6

These results were again confirmed in our lab by transplantation of kidney organoids in chicken embryo's (M. Koning, C.W. van den Berg, G. Wang, G.L. Tiemeier; unpublished data). Detailed investigation of endothelial cells in un-transplanted and transplanted kidney organoids by Transmission Electron Microscopy (TEM) and fluorescent staining revealed only full maturation of endothelial cells (indicated by presence of mature Weibel Palade Bodies) when host blood from the chicken embryo perfused the hiPSC-derived blood vessels (Figure 1a-c)

Further study has to reveal if blood flow is the limiting factor in hiPSC-EC maturation *in vitro*. The influence of blood flow remains difficult to study *in vitro* and we have shown that both shear stress and pericyte co-culture are insufficient to achieve full maturation (**chapters 2 and 3**). Another explanation of formation of mature blood vessels in transplanted organoids could be the substitution of hiPSC-EC by more mature host endothelial cells (either mouse or chick derived), such as endothelial colony forming cells (ECFCs) [46-48].

Importance of angiocrine and inter-cell signaling for vascular development

In zebrafish, a commonly used model to study vascular development, as well as in other vertebrates, endothelial and hematopoietic cells in embryos arise in close association with one another, and are thought to be derived from a common precursor. In mammals, endothelial and hematopoietic cells develop in extraembryonic yolk sac blood islands. Single-cell resolution fate mapping in zebrafish embryos through gastrulation showed that a minor proportion of endothelial and hematopoietic cells share a common bipotential precursor [49], although it is unclear to what extent this finding reflects a common early mesodermal hemangioblast precursor to both lineages or so-called "hemangiogenic endothelium" capable of giving rise to definitive hematopoietic precursors [50].

Interestingly, during the differentiation of vascular organoids both pericytes, endothelium, mesenchymal stem-like cells and hematopoietic cells are simultaneously formed, possibly improving the maturation by inter-cell signaling. [44, 51]

Limitations in maturation of cellular metabolism in iPSC differentiation

The studies in this thesis repeatedly show the differences in metabolism of iPSC-ECs compared to primary endothelial cells (**chapters 2, 3 and 4**). We found that hiPSC-EC have a reduced mitochondrial capacity for oxidative phosphorylation, despite higher overall mitochondrial content in the hiPSC-ECs (**chapter 2**). Furthermore,

ultrastructural analysis showed immature, round mitochondria that lacked mature cristae development and this was associated with increased reactive oxygen species (ROS) leakage. Together these data indicated an open mitochondrial permeability transition pore (mPTP), which is characteristic of mitochondrial immaturity.[52] Furthermore, we show that formation of mature Weibel Palade Bodies (WPB) in hiPSC-ECs is limited by increased intracellular pH (pHi) around the Golgi apparatus and Trans-Golgi Network (TGN). This increase in pHi is caused by reduced intracellular lactate accompanied by reduced H⁺, as a result of reduced glycolytic flux and reduced cellular uptake of lactate and H⁺ via the Monocarboxylate transporter 1 (MCT1) transporter (**chapter 3**).[53]

Although glycolysis and mitochondrial respiration were reduced, similar levels of Adenosine triphosphate (ATP) were found in hiPSC-EC. We found that hiPSC-EC fuel their mitochondria with free fatty acids instead of glucose. This increased reliance on free fatty acid oxidation (FFO) was independent of the peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1 α) expression. This FFO is necessary to sustain the tricarboxylic acid cycle (TCA cycle) by supplying acetyl-CoA and thereby enabling the regeneration of NADPH from NADP+ in order to maintain the redox balance of the cell.[54] Despite the efforts of maintaining the redox balance, the reduced amounts of glutatthione in hiPSC-EC we observed, the main ROS scavenger, could be an additional driver of the alternative redox balancing.

Advantages of cell immaturity for clinical application

Bioengineered tissues have the potential to address the worldwide allograft shortage, however perfusion of organs is one of the greatest challenges in tissue regeneration [55].

In addition to vascular necessity for further iPCS maturation, immunologic incompatibility could still be detrimental issue. Initially, iPSCs were generally assumed to be immune-privileged, but several studies have indicated otherwise.

In **chapter 5** we characterized for the immunogenicity of hiPSC-ECs by assessing surface expression of histocompatibility markers, such as the human leukocyte antigens (HLA), and of complement inhibiting factors. At a functional level, we tested differences in an allogeneic PBMC activation assay.

We found higher HLA-A,B,C expression on hiPSC-ECs in an inflammatory environment, which could potentially have a negative effect on graft rejection rates through several mechanisms. Next to CD8⁺ T cell activation, HLA-A,B,C

plays an important role in T cell recruitment by endothelial cells. Moreover, higher HLA-A,B,C expression could make hiPSC-ECs more vulnerable to donor specific antibody (DSA) deposition, potentially leading to higher rates of antibody mediated rejection (AMR), a major cause of allograft failure[56-58]. However the proportion of HLA-DR positive cells, after stimulation with IFN- γ and IL-1 β , was much lower in hiPSC-ECs than primary hECs, indicating that hiPSC-ECs have not a fully matured expression of HLA-DR, which might reduce the activation of allogenic CD4⁺T cell activation.

The role of metabolism in immunomodulation

The allogeneic PBMC co-cultures suggested that hiPSC-ECs were less potent to activate both CD4⁺ and CD8⁺ T cells compared to primary hEC, again indicating a partly immature phenotype of hiPSC-EC. Whether these differences in hiPSC-ECs result in a better protection from allogeneic rejection, needs further *in vivo* research. Research on auto-immune and (chronic) inflammatory diseases has shown the cellular metabolism of endothelial cells is highly involved in that immunomodulation. For example, tight regulation of nitric oxide influences the expression of receptors involved in immune response, such as ICAM1 and PDL1. Furthermore, lactate produced by glycolysis in endothelial cells is directly taken up by CD8 T-cells and thereby regulates the production of cytokines. Ongoing research has to reveal the role of endothelial cell metabolism is inflammatory responses.

Concluding remarks

Although tinkering with the intracellular metabolism as shown of hiPSC-EC **in chapter 2-4**, such as closing the mPTP with CsA or reducing the pHi with acetate, lead to more maturation and functionality, recent work suggests that we lack fundamental knowledge on the role of metabolism in development and the metabolic processes involved in maturation.[52, 53] To generate mature and functional cells and tissues from iPSCs we have to gain more insight into the metabolic processes driving differentiation, maturation and (epi)genetic modulation.

Future directions

Recent studies have illustrated how metabolites, such as acetyl-CoA or aKG, impact epigenetic editing enzymes via substrate availability, thereby directly regulate genes, for example by histone methylation or acetylation. We argue that the repeated metabolic switching that occurs during both reprogramming and differentiation as described above, strongly influences epigenetic modulation by changes in availability of metabolites. This emerging field of metaboloepigenetics is enabled by new state-of-the art technologies, such as Mass Spectrometry Imaging,

which allow researchers to study the metabolism and epigenetics at the same time, bringing these fields closer together.

Furthermore, single cell technologies will drastically change the field of endothelial biology. A recent single cell sequencing study of mouse kidneys by Dumas et al, revealed 24 different endothelial phenotypes, indicating the heterogeneity and specialization of endothelial cells.[59] Although for many organoid models single cell sequencing has been performed [60-63], only limited studies have found a cluster of endothelial cells. [64] When comparing the human kidney to the kidney organoid, the discrepancy between the number of different types of endothelial cells is striking. To better understand the development of the vasculature and maturation of iPSC-ECs, single cell lineage tracing and cell fate tracing techniques could be used. Thereby, single cell RNA-sequencing combined with single cell metabolomics could reveal the instructiveness of surrounding cells and the development of tissue specific endothelial cells.

In our ongoing work we are comparing the adult and fetal kidney to un-transplanted and transplanted organoids, providing us insight in the functional development of the organoids. As described in this thesis, these results will provide targets of regulation of metabolism, which in turn will regulate differentiation, maturation and cellular function. [65]

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