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Regulation of stemness in humans: the role of Hypoxia Inducible Factors and Tie2

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A dissertation

submitted in partial fulfillment of the

requirements for the degree of

Doctor of Philosophy

University of Washington

2017

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Program Authorized to Offer Degree:

Biochemistry

University of Washington

Abstract

Regulation of stemness in humans: the role of Hypoxia Inducible Factors and Tie2

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Pluripotent stem cells hold great promise for the future of regenerative medicine. Though many studies have examined the genetic determinants of pluripotency, the role of metabolism in stemness acquisition and maintenance remains an active area of investigation. Due to its role in the acquisition of different metabolic states, hypoxia, via action of the Hypoxia Inducible Factors (HIFs), is also suspected to regulate pluripotency. Indeed this low oxygen level is a key feature of a variety of stem cell niches. We thus decided to study the roles of HIFs in the acquisition and maintenance of pluripotency. First, we tested the role of HIFs in stemness acquisition by reprogramming human fibroblasts into induced pluripotent stem cells (iPSCs). We found that even though both HIF1α and HIF2α are needed for iPSC formation, HIF2α overexpression towards the later stages of reprogramming inhibits colony formation through TRAIL activation.

Then, in the human embryonic stem cell paradigm we showed that HIF1α is required for the transition from the naïve to the primed hESC, two separate pluripotent states characterized by distinct metabolic profiles. Together these results underline the crucial role of hypoxia and HIFs in the regulation of stemness and metabolism.

Another area in which metabolism can act to regulate pluripotency is through the regulation of epigenetics. Previous studies showed that energy metabolism and the availability of certain metabolites can influence stem cells and their stemness capacities. The Polycomb repressive complex 2 (PRC2) has methyltransferase activity on histones, primarily adding the repressive trimethylation mark on histone 3 lysine 27 (H3K27me3). We investigated the role of JARID2, a key protein of this complex, in the maintenance of pluripotency of hESCs. Interestingly, the loss of JARID2 in naïve hESC (2iL-I-F) induces a concomitant loss of H3K27me3 and pluripotency markers.

A variety of solid tumors contain a subpopulation of cancer stem cells (CSCs), cancer cells characterized by their ability to self-renew, initiate tumors and lead to metastasis. CSCs share several features with normal adult stem cells in terms of metabolic profile. Clarifying the role of metabolism in stemness acquisition could have interesting applications for cancer stem cells. Interestingly, both primed stem cells and cancer stem cells share a similar glycolytic metabolism. Adult stem cells in the model organism *Drosophila melanogaster* have been recently used as a model to study cancer stem cells. In vivo studies allow for considerations of the interactions between stem cells and the stem cell niche. These interactions have been shown to be critical in the resistance of stem cells to apoptosis following exposure to ionizing radiation. Germline stem cells are able to survive IR damage through Pvf-1 mediated Tie-2 receptor activation. The human homologs Angiopoietin1 (Ang1) and Tie2 are therefore interesting targets for inhibitors and

activators in human stem cells. A combination of *in silico* designed scaffolds with the binding domain of Ang1, F-domain was used to activate the Tie2 receptor.

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ACKNOWLEDGEMENTS

The road towards my graduate studies has been a challenging but extremely gratifying one. In all these years, many people have been instrumental to the completion of the PhD. I would like to extend my profound and sincere gratitude and appreciation to the following people:

My advisor Dr. Hannele Ruohola-Baker, who gave me the opportunity to be part of her lab. Professor Hannele Ruohola-Baker is always willing to educate and give everyone the chance to expand his or her purpose through research. Thank you Hannele for teaching me how to bombard and making me stronger in the process.

My PhD committee members: Jim Hurley, Carol Ware, Randy Moon, Gabriele Varani, David Morris, Susan Brokerhoff, and David Baker, who all gave freely their time and their knowledge. I would like to express particular gratitude to Dr. Carol Ware for kindly and patiently reading through my thesis.

I most definitely would not have completed my PhD without Dr. Julie Mathieu. Thank you Julie for guiding me with kindness through out all these years. You are an inspiration and an amazing role model.

I further extend my gratitude Damien Detraux, Filippo Artoni, Henrik Sperber, Sonia Sidhu, and Shiri Levy, who gifted me with their friendship and support. You guys have always been there for me and I really appreciate your company during the good and the bad times. I know

that although we won't be working on the same space anymore, we will always remain close. I considered you to be part of my best friends.

Thank you colleagues in the Ruohola-Baker lab and the ISCRM members for their valuable discussions and support during my time at the lab. Especially to Jenn Hesson and Chris Cavanaugh for always cheering me up when I was working at the core.

Thank you to my undergraduate mentor Laura Serbus, who taught me how to pipet and how to follow a protocol.

Finally, I thank my family for their unconditional love, support, and encouragement. I am especially lucky to have my mother Ana Maria Warfel and my partner Jon Wellnitz who always encourage me and care for me.

Thank you!

Amy A. Ferreccio

DEDICATION

I dedicate this work to my mother, Ana Maria Warfel, for all her love and support.

Chapter 1. INTRODUCTION

As the aging population rises, the need for regenerative therapies and organ repair increases. With their capacity for self-renewal and differentiation into different cell types, stem cells are the key to lengthen the health and improve the quality of the aging population. If well understood and controlled, the capacity to differentiate into multiple cell types while maintaining a constant pool of undifferentiated cells would permit therapies involving tissue growth, or even the growth of a whole organ. These can be used for transplantation and/or repair of damaged organs. The control of differentiation while maintaining a stem cell pool is fundamental to maintain a correct tissue homeostasis. In case of improper regulation these features could lead to catastrophic consequences in the body. Similar to stem cells, cancer stem cells have self-renewal and differentiation capacities however, unlike stem cells, these properties are chaotic and not well regulated. These similarities allow us to use stem cells as a model for cancer stem cell research.

It is thus extremely important to understand what regulates stem cell properties and understand how to maintain a correct tissue homeostasis in the stem cell niche. For my Ph.D. studies, I focused on how stem cells properties are regulated, particularly by how metabolism and the epigenetic landscape influence cell fate. Also, I have used protein design and genome editing to create tools that help us understand these properties.

1.1 POTENCY

It is possible to distinguish different types of stem cells depending on their capacity for regeneration; different levels of potency exist within cells during the developmental process. On top of the potency hierarchy are the totipotent stem cells arising from the fertilized egg. Indeed,

shortly after fertilization, and for a limited amount of time, each cell of the developing embryo is able to give rise to both placental and embryonic tissues. However, these cells rapidly lose this feature when they form the inner cell mass (ICM) at the blastocyst stage. At this point, the cells lose their ability to become extra-embryonic tissues and become pluripotent stem cells. In adults, stem cells still exist under the form of multipotent cells with a restricted potential (such as mesenchymal stem cells) or to unipotent stem cells commited to a particular lineage. Several stages have been stabilized *in vitro* that represent different levels of pluripotency [1, 2].

1.2 Different *in vitro* Types of HESC

First, in mice, then later in human, two different types of pluripotent stem cells have been described and isolated in-vitro: the naive ESC, found in the inner cell mass (ICM) of the pre-implantation embryo; and the primed ESC, representing cells from the epiblast at the post-implantation stage. Naïve stem cells have higher pluripotency as seen in their ability to contribute to chimeras in mice. When injected into the blastocoel of a host blastocyst, pluripotent cells integrate into the inner cell mass to participate in development. Contribution to the germ line is considered a stringent test for cellular pluripotency [3]. The primed state represents the epiblast cells shortly after embryo implantation and cannot commonly contribute to chimeras. These two different developmental stages exhibit distinct metabolic profiles and epigenetic landscapes, however the precise mechanism that underlies these differences in developmental potentials remains unknown. It might be the intrinsic differences in these cells that give them different pluripotency capacity.

Another type of pluripotent stem cells are induced pluripotent stem cells (iPSCs). These cells were first described by Takahashi and Yamanaka[4], who are the first team to induce the direct

reprogramming of somatic cells into iPSCs. By adding 4 reprogramming factors: Oct4, Sox2, cMyc,and Klf4, differentiated cells are able dedifferentiate into a pluripotent state. This technology allows pluripotent cells to be obtained from adult somatic cells without having to destroy embryos. This revolutionary technology could allow regenerative therapies to be performed with an autologous graft, which would minimize the risk of an autoimmune response or rejection of the transplanted organs.

Very recently, Extended Pluripotent Stem Cells (EPSCs) cells have been pushed into a state which remarkably is able to contribute to both embryonic and extra embryonic tissues, as well as greatly contribute to mouse chimeras when compared to other hESCs. It is not quite clear yet what kind of *in vivo* cells EPS represent [5]. In order to be able to benefit from stem cell technologies, it is important that we understand how different stabilized cell lines represent different *in vivo* stages as well as what properties they have and how they can be regulated.

Even though these cells represent really close stages in embryo development, they exhibit dramatic differences in terms of developmental potential, X-chromosome inactivation pattern, metabolic activity, and epigenetic landscapes.

1.3 Epigenetic Differences in Naïve and Primed

Epigenetics studies the changes that influence gene expression that are not modulated by the DNA sequence [6]. There are four main types of epigenetic modifications: post-translational modification of histone proteins, chromatin accessibility, DNA modifications, and non-coding regulatory RNA[7]. Histones are proteins, which the DNA sequence is wrapped around. Modification of the histones such as methylation, can result in the tightening or relaxation of the packing of DNA, affecting its accessibility for transcription[8]. Genomic DNA of naïve cells is

generally hypomethylated, whereas primed is hypermethylated. Histone modification patterns are also different between naïve and primed cells however, it is difficult to pinpoint which of these differences are critical [9]. Primed cells exhibit increased presence of histone 3 trimethylation on Lysine27 (H3K27me3) repressive marks. H3K27me3 is considered one of the bivalent marks that poise the cells for rapid gene upregulation upon differentiation. By contrast, naïve cells have relatively few H3K27me3 and open chromatin [10].

1.4 Polycomb Repressive Complex2

The polycomb repressive complex 2 (PRC2) is a histone methyl-transferase, which regulates key biological processes like maintaining cell identity and cell differentiation. PRC2's primary role is adding repressive tri-methyl marks on lysine 27 of histone 3 (H3K27me3). The core components or the PRC2 are: Enhancer of Zeste Homolog (EZH1/EZH2), Embryonic Early Development (EED), and Suppressor of Zeste 12 (SUZ12). The auxiliary components are Jumanji and AT-Rich Interaction Domain containing 2 (JARID2), Adipocyte Enhancer Binding Protein 2 (AEBP2) and RbAP46/48. JARID2 is crucial in the recruitment of the PRC2 with a bias for GC-rich regions of the genome [11]. The trimethylation of Histone 3 at lysine 27 is important for the regulation of metabolic pathways in naïve to primed transition [12].

1.5 Difference in Metabolism in Naïve and Primed

Another key difference in naïve and primed cells is metabolism [12, 13]. Naïve stem cells are bivalent in energy production and can switch between glycolysis and mitochondrial respiration. Primed cells are highly glycolytic, a metabolic feature which they share with many cancer cells,

also known as the Warburg effect. When the cells differentiate further from the primed stage, they switch their metabolism back to using their mitochondria and become metabolically bivalent. It is still not understood why primed cells enter this highly glycolytic, cancer-like metabolic state, and how they leave it after differentiation. Differences in the metabolic pathways included the tryptophan degradation pathways in which the enzyme IDO1 is unregulated, resulting in an increase in the tryptophan breakdown product: Kynurenine. Additionally, the primed state has an accumulation of lipids, highly unsaturated lipids in particular. Epigenetic modifications are further linked to metabolic changes by the down-regulation of naïve state. NNMT catalyzes the methylation of nicotinamide to 1-methyl nicotinamide, making less methylation substrate available for histone modifications in the naïve state [12]. Another key difference is the stabilization of transcription factor HIF1α in primed hESC stage. In mice, it has been shown that the stabilization of HIF1α forwards the transition of naïve mouse cells towards the primed stage [13]

1.6 Hypoxia Inducible Factors

Cells respond to hypoxia through the activation of oxygen sensitive transcription factors HIFs (Hypoxia Inducible Factors). HIFs are dimeric transcription factors that target genes involved in the survival and adaptation of low oxygen levels. HIFα is constitutively transcribed, in the presence of oxygen. In normoxic conditions HIFα is hydroxilated and consecutively polyubiquitinated and marked towards proteosomal degradation. In low oxygen conditions, HIFα are not degraded. HIFα accumulates in the cytosol and then travels to the nucleus to form a transcription complex with HIF beta and other proteins that control the transcription of hypoxia responses such as angiogenesis, proliferation, and glucose metabolism.

1.7 CANCER STEM CELLS

Like normal stem cells, cancer stem cells (CSCs), or tumor initiating cells (TIC), have the ability to self-renew and give rise to multiple cell types. However, while stem cells' self-renewal and differentiation are tightly regulated, cancer stem cells' division is often misregulated to the detriment of the organism [14]. When using *Drosophila melanogaster* as a model organism to study cancer stem cells in the niche environment, it has been shown that there is a community effect from the daughter cells in the niche acting on the stem cells. After irradiation, the Tie receptor is activated by Pvf1 ligand, which is secreted from the dying cells in the niche. This ligand stops the stem cells from going through apoptosis and signals them to become quiescent. [15]. This might be the same mechanism used by cancer stem cells after chemogenic and irradiation therapies. CSCs might be the reason why often time cancer patients relapse after treatment since most treatments target the fast dividing cells rather than targeting the quiescent cancer stem cells [16]. Pvf1/TIE ligand/receptor have a human homolog Angiopoietin1 (Ang1)/Tie2 that are being studied for their connection to cancer.

1.8 TIE2 AND ANGIOPOIETIN1

The tyrosine Kinase receptor TIE2 and its ligand Angiopoietin1 (Ang1) play a central role in regulation of angiogenesis and lymphatic vessel formation, both in normal development and tumor formation. Overexpression of the Tie2 receptor has been observed in breast, ovarian, hepatocellular, and glioblastoma tumors [17] [18] [19]. Therefore, Tie2 receptor inhibitors are an interesting target for cancer therapeutics. Ang1 contains 3 main domains: N terminus, coil-coil

domain, and a Fibrinogen-like region (F-domain). It is the F-domain region that modulates the binding to the Tie2 receptor, bundled in close proximity by the coil-coil domain [20].

1.10 CRISPR

In order to study the role of our proteins of interest in the stem cell system, we used CRISPR-Cas9 genome editing [21]. The CRISPR-Cas9 system utilizes a guide RNA (gRNA) that is incorporated into a protein effector nuclease (Cas9) to target and cut DNA. Targeting the desired mutation site, the gRNA is a chimeric guide RNA composed of a ~20nt 'protospacer' sequence which is used for target recognition and a structural RNA specific for Cas9 interaction, required for gRNA-Cas9 complex formation. In addition, the DNA cleavage requires an appropriate protospacer adjacent motif (PAM) at the 3' end of the protosequence in the targeted gene (NGG, where N is any nucleotide). Cas9 promotes gene editing, stimulated by triggering a DNA double-strand break (DSB) about 3 bp upstream of the PAM via two catalytic nuclease domains (HNH and RuvC-like), which is repaired by the error-prone non-homologous end joining (NHEJ) or the higher fidelity homology-directed repair (HDR) pathway. NHEJ repaired gRNA-Cas9 targeting leaves small insertion/deletion (indel) mutations, which, when occurring in an exon, cause frame shifts and premature stop codons in the target gene.

Chapter 2. HYPOXIA INDUCIBLE FACTORS IN THE ACQUISITION OF PLURIPOTENCY

2.1 HIFs IN REPROGRAMMING

Pluripotent stem cells have distinct metabolic requirements than differentiated cells. In the process of reprogramming, cells need to shift from an oxidative to a glycolytic metabolism. Hypoxia Inducible factors have been shown to play an important role in this metabolic transition[13]. We used the reprogramming assay as a model for stemness acquisition and used it to study the role of HIFs in the acquisition of stemness. We reprogram human fibroblasts (IMR90 and MRC5) in normoxia conditions by infecting them with lentivirus inducing transcription factors: Oct4, Sox2, Lin28, and Nanog.

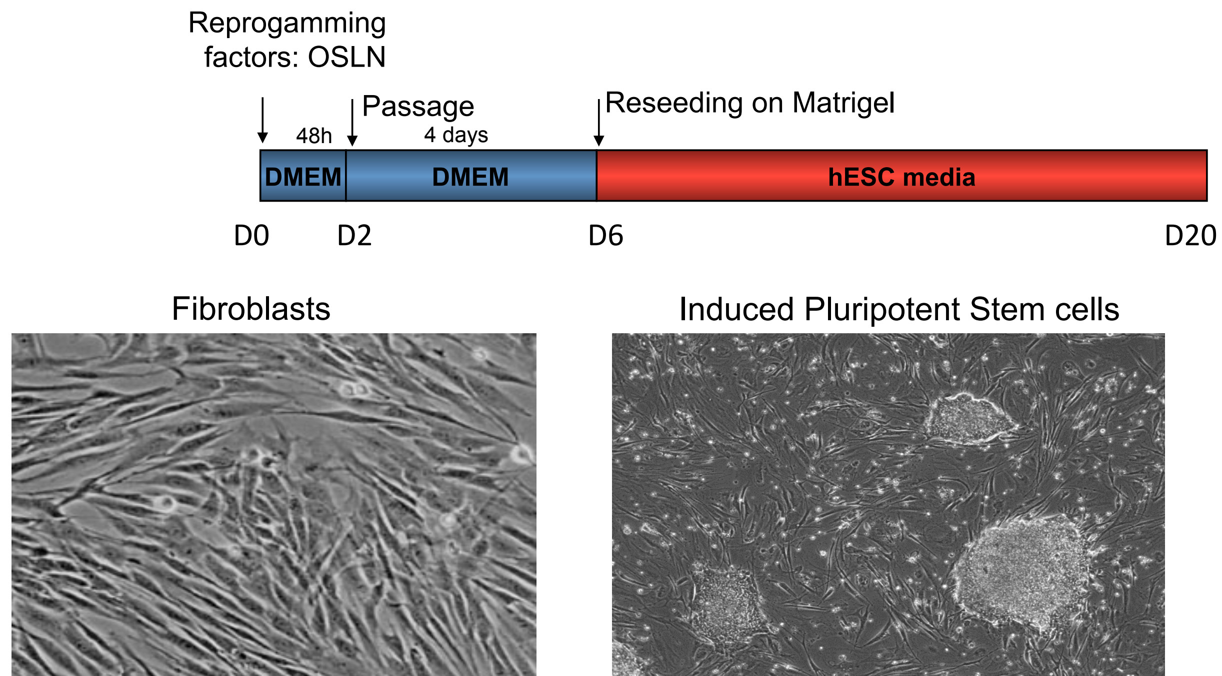

Figure 2.1. Reprograming assay used to study the role of HIFs in iPSCs induction.

We used western blot analysis to analyze HIF1α and HIF2α protein levels during the reprogramming process in normoxia. Surprisingly, HIF1α levels are stabilized during the different days of reprograming, whereas HIF2α levels decrease during the second half of the process. (Figure 2.2)

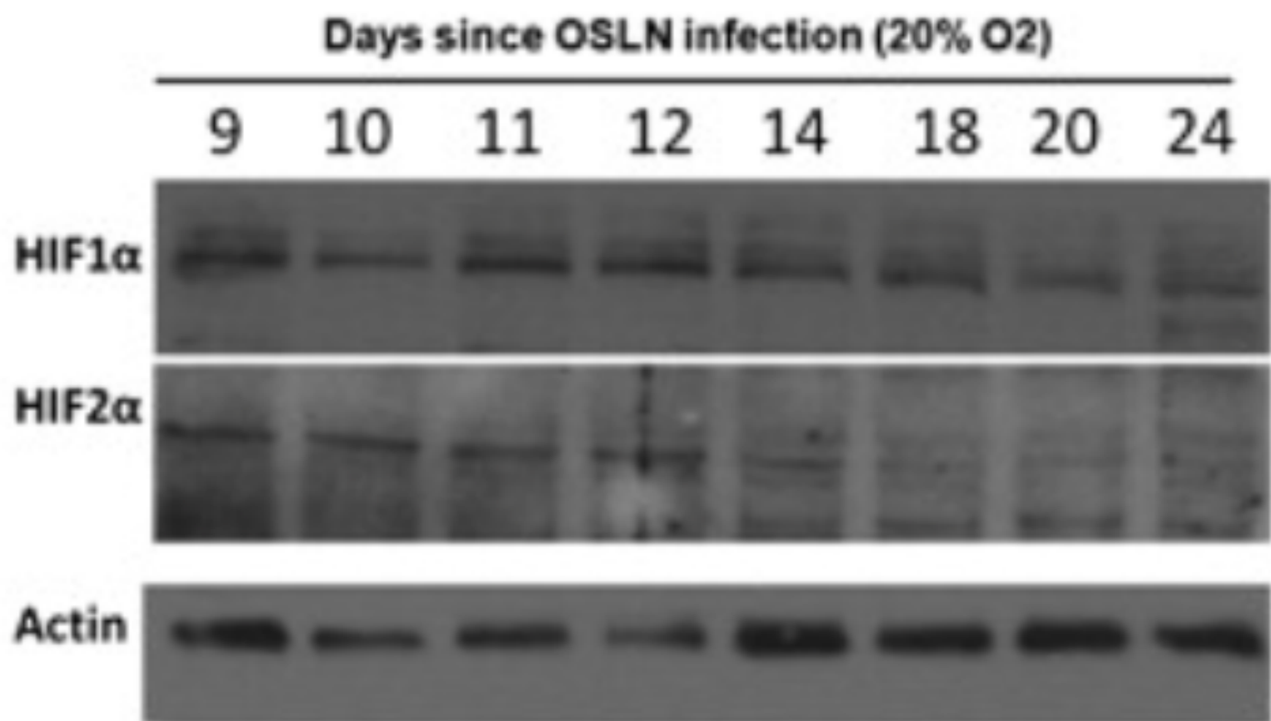

Figure 2.2. HIF1α and HIF2α levels during reprogramming in normoxia

To test if the role of HIF1α and HIF2α is required for reprogramming, we used small hairpin RNA (shRNA) against both HIF1α and HIF2α. Both constructs resulted in a significant reduction of protein levels. When we reduced the levels of HIF1α or HIF2α from the initiation of the reprogramming process we observed a significantly reduced number of iPSC both in normoxia and hypoxia conditions. (Figure 2.3)

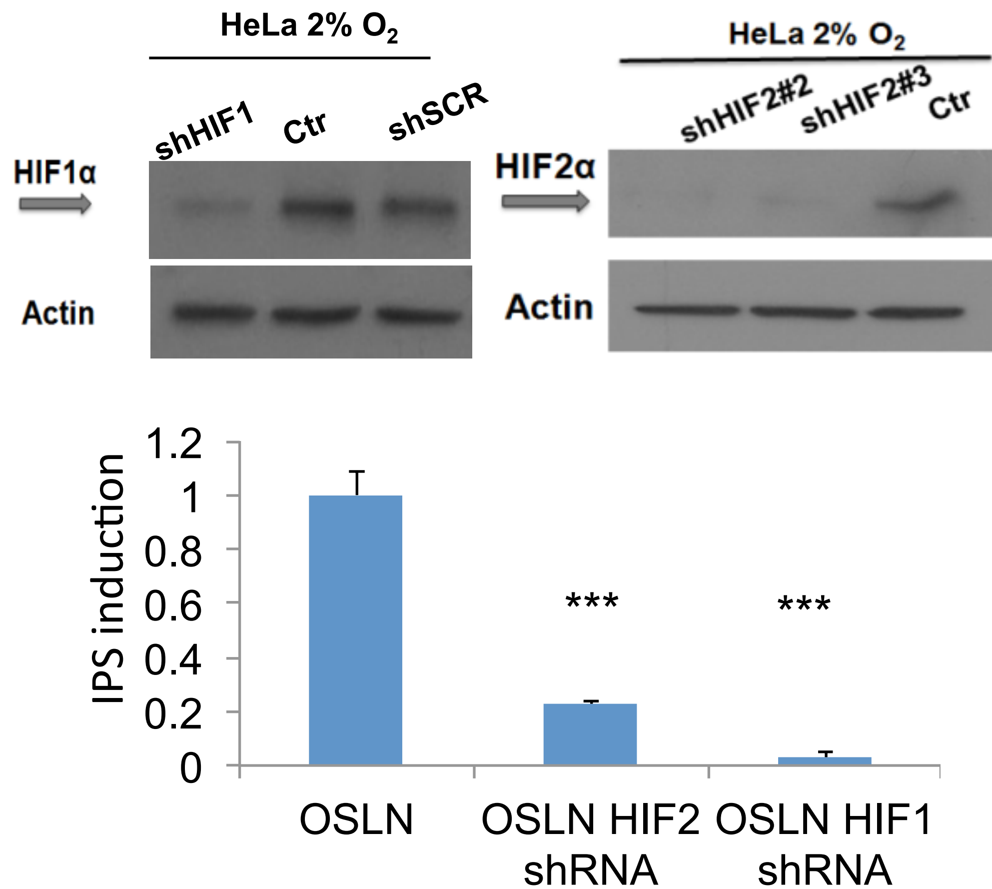

Figure 2.3.shRNA against HIFs show reduced protein levels and inhibition of iPSCs.

To characterize the metabolic profile of the cells during reprogramming, we measured their Oxygen Consumption Rate (OCR) by Seahorse Extracellular Flux Analyzer technology. During this assay, cells are first treated with oligomycin, an ATP synthase inhibitor, followed by FCCP, which forces maximal respiration in the mitochondria. This allows the mitochondrial reserve capacity to be calculated by subtracting the basal respiration from the maximal respiration

capacity. Fibroblasts showed significantly higher levels of OCR compared to reprogrammed cells and primed hESC. However when shRNA against HIFs were used, reprogrammed cells were not able to shift to a glycolytic metabolism, which resulted in low iPS induction (Figure 2.4). These results show that both HIF1α and HIF2α are required for reprograming.

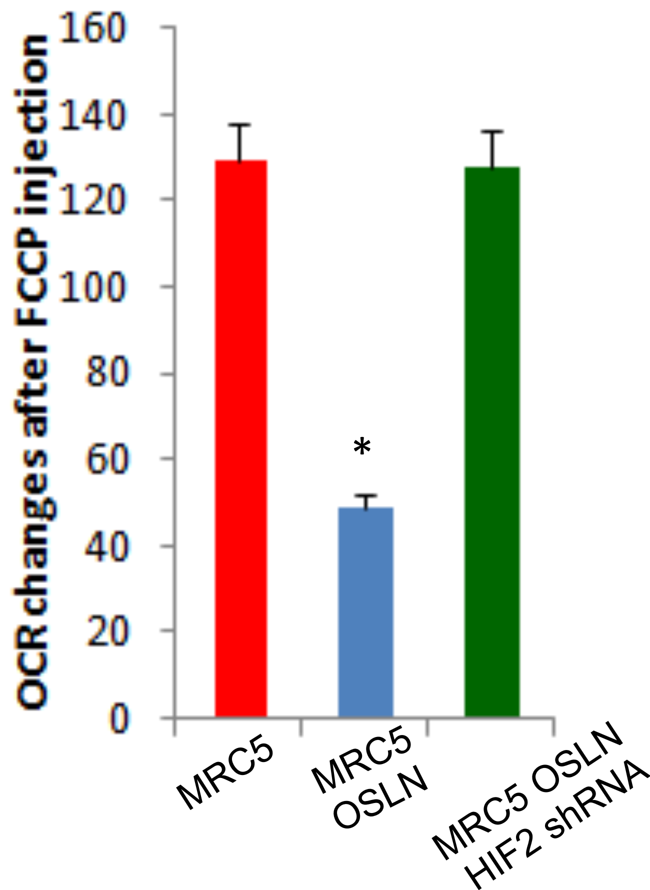

Figure 2.4. OCR levels do not shift during reprograming if HIFs are silenced

Since Hypoxia is beneficial for reprogramming [22], we tested the overexpression of a non-degradable version of HIF1α and HIF2α during the reprograming process. These constructs have mutations on the oxygen sensing Prolines and will not be recognized by VHL, and therefore

HIFs are constitutively expressed. We hypothesized that non-degradable HIF overexpression might enhance the production of iPSC colonies. As expected, HIF1α overexpression increased the number of colonies obtained after the reprogramming process both in normoxia and hypoxia. Interestingly, prolonged stabilization of HIF2α repressed reprogramming even when overexpressing with a combination of HIF1α and inhibited colony formation during reprograming (Figure 2.5).

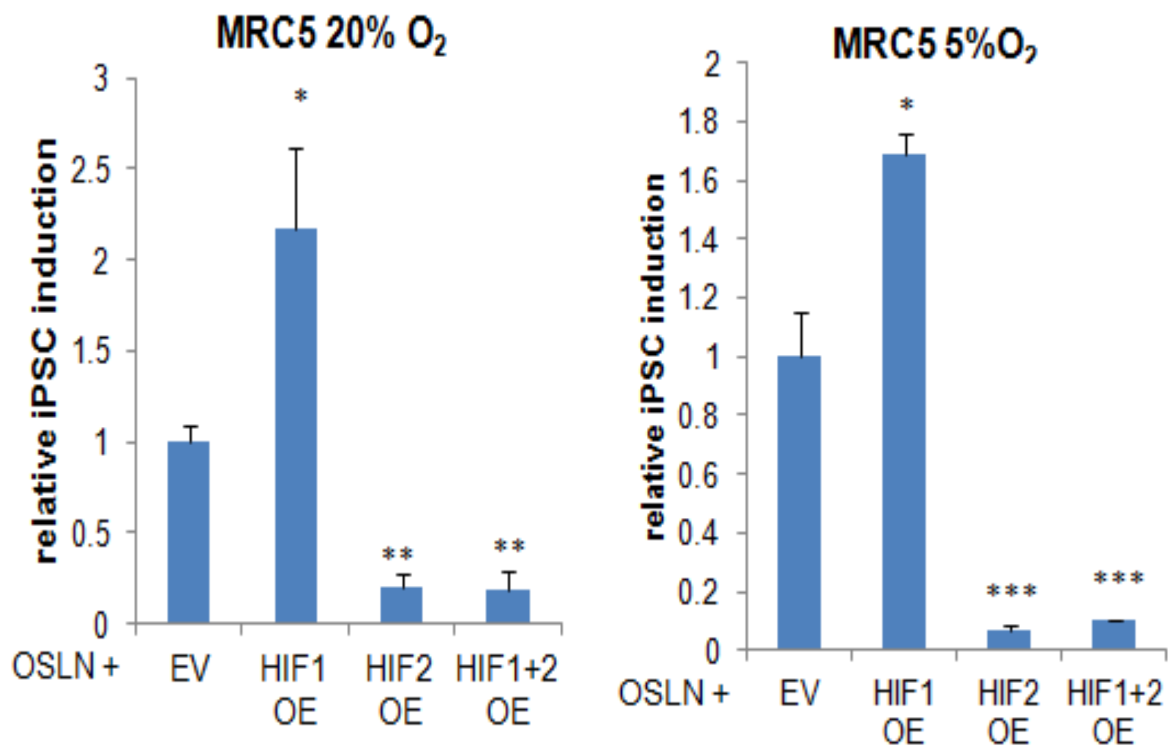

Figure 2.5 Colony formation after HIFs overexpresion

We saw that the expression of HIF2α is needed during reprograming but in a time regulated way since it is essential for colony formation in the first days of reprogramming but detrimental if activated during the last days. We then did a microarray analysis to investigate gene expression during the reprograming assay with and without HIF2α stabilization. When we examined the gene expression profiles of HIF2α overexpression reprogrammed cells compared to control

reprogrammed cells, we identified TNF-related apoptosis inducing ligand (TRAIL) as the most stable and HIF2 α relevant up regulated gene (Figure 2.6). TRAIL inhibits iPSC generation by repressing apoptotic Caspase 3 activity [23]. It has been shown that apoptosis is needed for reprogramming [24] [25]. We were able to counter-act the HIF2α overexpression phenotype by addition of TRAIL neutralizing antibody, which rescues the formation of colonies during reprogramming even with HIF2α overexpression.

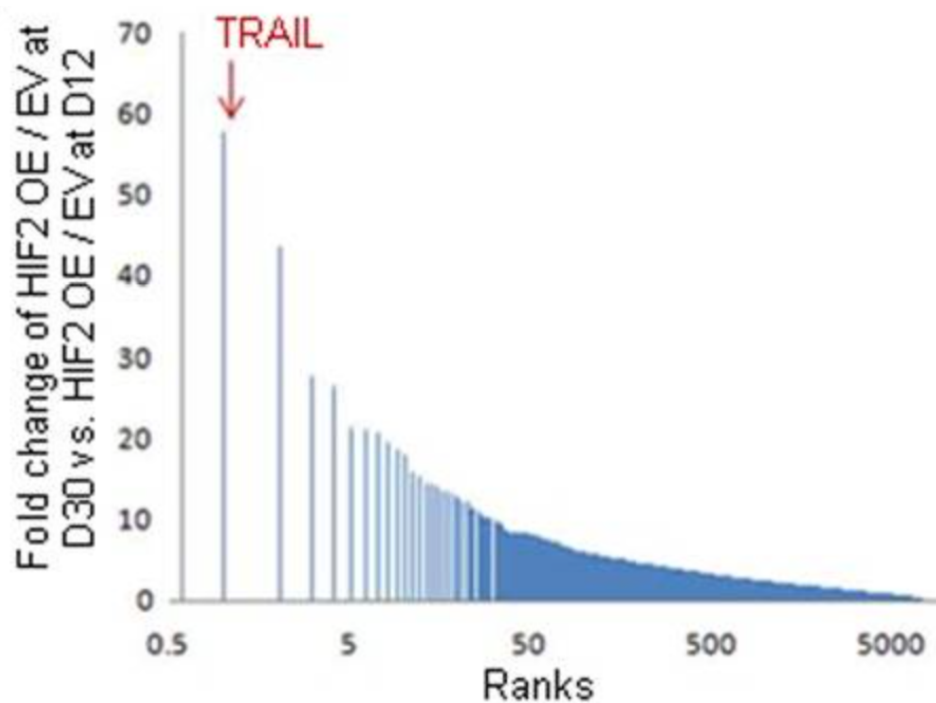

Figure 2.6 Gene expression in reprogrammed cells with stable HIF2α levels

2.2 HYPOXIA IN THE TRANSITION FROM NAÏVE TO PRIMED

Pluripotency does not represent a defined state. *In vivo*, during development, pluripotency is a process that is constantly changing. *In vitro*, we can observe different states of pluripotency with

different gene expression, epigenetics, and metabolic landscapes. Naïve and ground state hESCs have been derived to represent preimplantation. Primed hESCs represent cells inside an implanted blastocyst. A dramatic metabolic switch from bivalent to glycolytic state has to occur for cells to transition from naïve to primed [13]. When measured by Seahorse Extraflux Analyzer, naïve cells and cells that have transitioned from primed to naïve show higher OCR response, indicating that primed cells have lower mitochondrial respiration capacity. Although mitochondrial morphology is significantly different in naïve and primed cells, the higher mitochondrial capacity in naïve cells is not due to more mature mitochondria nor the deterioration of the mitochondrial genome [26].

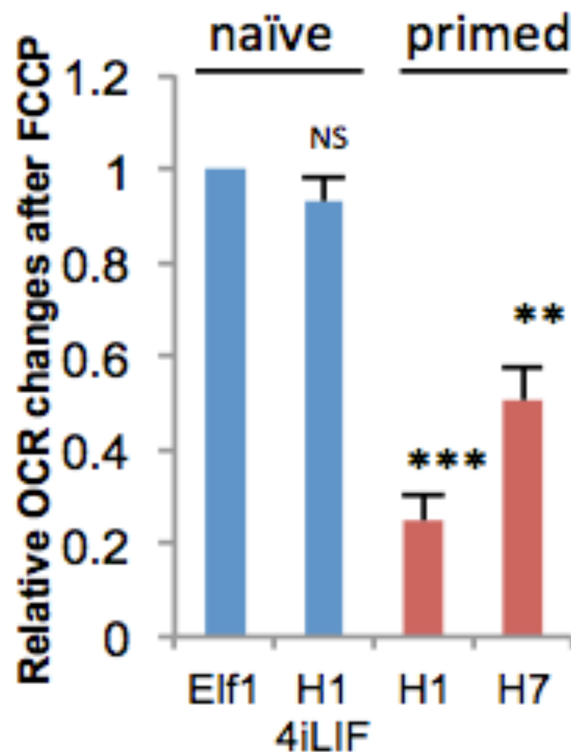

Figure 2.7. Naïve and Primed cells show a dramatic difference in their OCR levels

HIF1α has been shown to have a crucial role in the transition of naïve mouse cells to epiblast [13]. We analyzed HIF1α levels in human naïve cells and showed that HIF1α is stabilized in primed stage but not in the naïve. Interestingly, HIF2α is stabilized in primed cells and at a reduced level in naïve cells (Figure 2.8). The stabilization of HIF1α in primed cells but not in naïve cells is consistent and correlates to the change of EGLN1 expression, a key regulator of HIF1α [27] [28].

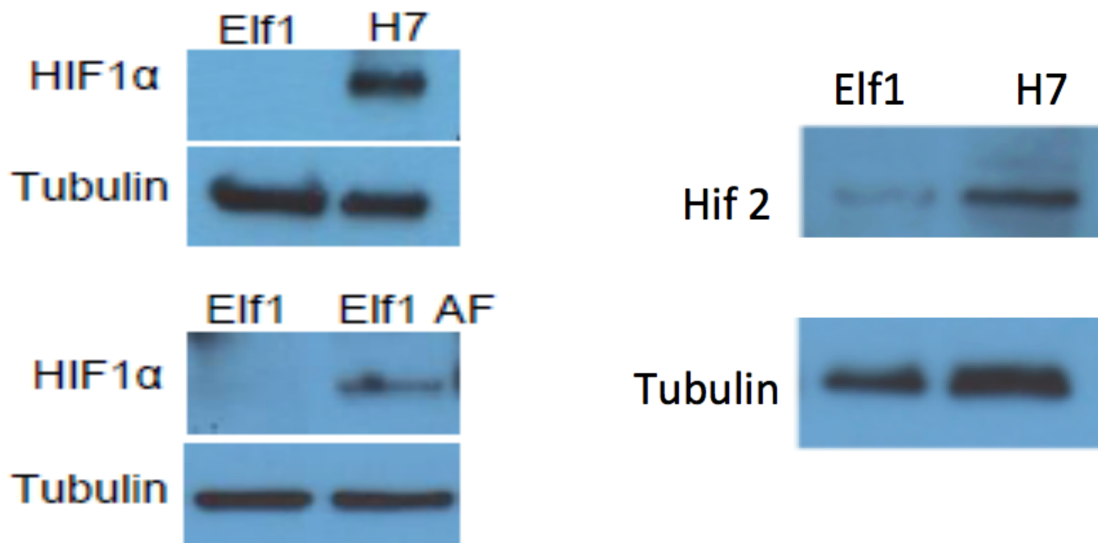

Figure 2.8. HIF1α and HIF2α levels in human naïve and primed cells

To answer the question of whether the role of HIF1α is essential in the naïve to primed transition in humans we designed a naive human HIF1α CRISPR knockout. We generated two HIF1α null clonal lines HIF1α KO 6.3.1 and HIF1α KO 6.2.1. The clones showed no HIF1α protein when cultured in hypoxia nor when pushed to the primed stage by culture in TeSR media. The HIF1α null cells failed to switch their metabolism from OXPHOS metabolism to a highly glycolytic primed metabolism as measured by OCR (Figure 2.9) HIF1α null lines failed to become primed

when pushed with TeSR media and upregulate known primed markers. This data suggests that HIF1α is essential for the metabolic switch between human naïve cells to primed.

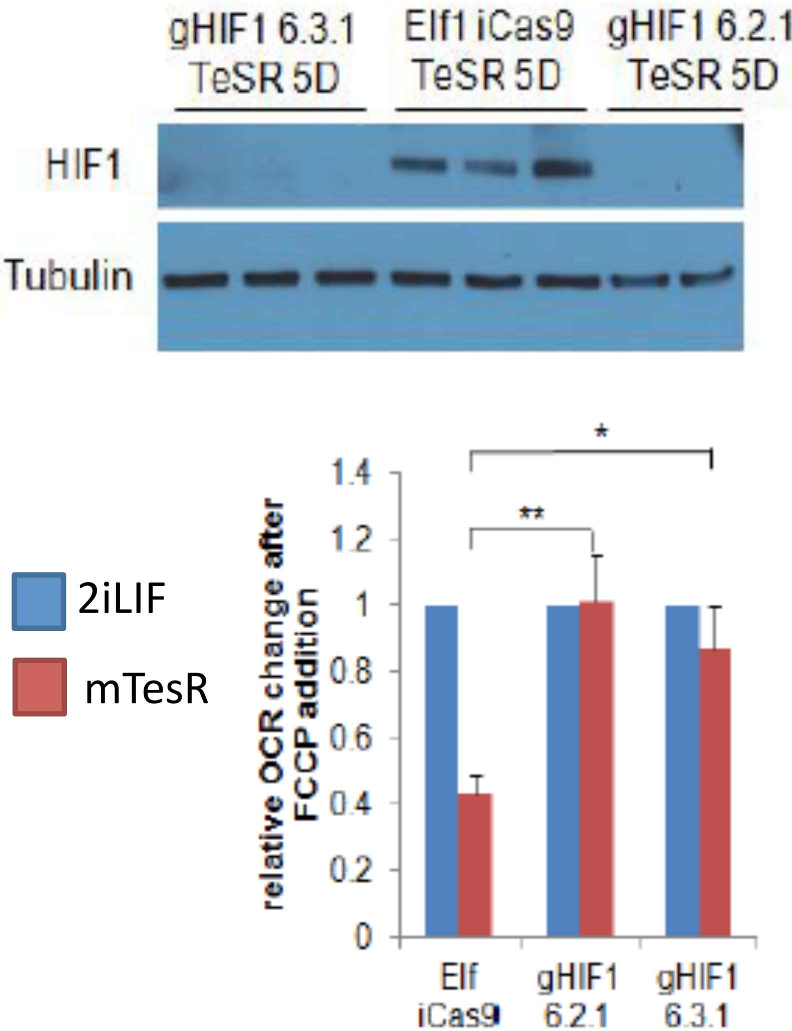

Figure 2.9. HIF1α CRISPR KO shows high mitochondria activity even when cultured in primed conditions

Chapter 3. PRC2 AND JARID2 IN PLURIPOTENCY

The PRC2 complex is regulates the epigenome by adding repressive marks H3K27me3. Although there are currently many efforts to understand how epigenetic modifications affect gene expression, Histone and DNA modifications are regulated in a spatial and temporal manner. It is important to dissect the function of the PRC2 in the different stages of pluripotency that have been derived *in vitro* since stage-specific epigenetic landscapes and PRC2 requirements may give ground, naïve, and primed stages altering biological profiles.

3.1 PRC2 INDUCIBLE DISRUPTION IN NAÏVE TO PRIMED

Knowing the importance of PRC2 activity and H3K27me3 dynamics both in development and reprogramming [29], we aimed to study the requirements of the complex in early human development. Traditionally, the integrity of the PRC2 complex is required for stem cell identity and maintenance. However, mouse naïve embryonic stem cells are able to retain pluripotency upon loss of the complex. So far, this feature has not been explored in humans. Whereas this difference is due to intrinsic difference between species or is an *in vitro* artifact has to be elucidated. To answer this question, we designed a doxycicline inducible EED-binder protein (EB22.2) to disrupt the PRC2 complex activity in a conditional manner. With a higher affinity for EED than the endogenous EZH2, the EED-Binder efficiently competes for the formation of a complex, thus disrupting the formation of a functional PRC2. Induction of this synthetic protein expectedly induced differentiation of primed or naïve (2i-L-I-F) hESC, as shown by the loss of the colony morphology and the loss of pluripotency gene expression such as Oct4, Sox2 and

NANOG. Surprisingly, this phenotype was not observed in ground state hESC (5iLA). (Figure 3.1)

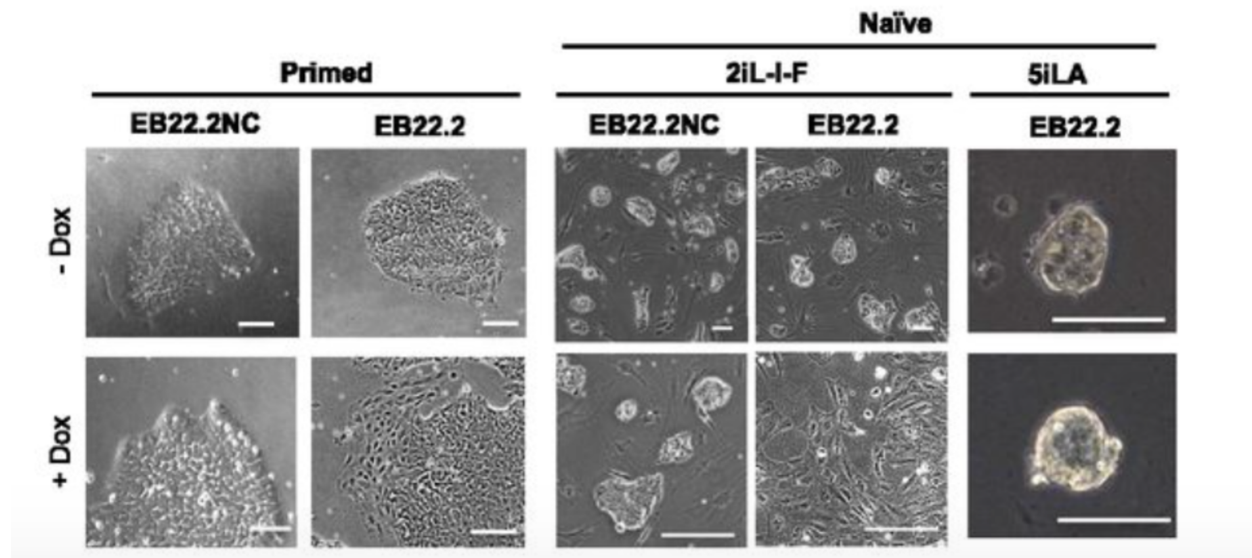

Figure 3.1. EED binder (EB22.2) and negative control (EB22.2NC) induction in different human pluripotent cells

3.2 JARID2 KNOCK OUT IN PLURIPOTENT CELLS

JARID2 protein is one of the DNA binding components of PRC2. The JARID2 protein contains 5 main features: an Ubiquitin Interactive Motif (UIM), a transcription repressive domain (TRD), two inactive histone demethylase domains (JmjN, JmjC), two DNA binding domains (ARID, ZnF), and the 'GSGFP' Suz12 binding motif [42–44].

To study the role of JARID2 in pluripotent hESCs, we generated a doxycycline inducible CRISPR/Cas9 naïve cell line that can be toggled back into 5iLA conditions as well as pushed into primed stage with TeSR media [21]. We nucleofected four plasmids into the naive ELF1 cells: two containing TALENS targeting the AAVS1 safe harbor site, a constitutive reverse tetracycline transactivator (M2rtTA) expression cassette with neomycin resistance, and one containing the inducible-Cas9 cassette with puromycin resistance. (Figure 3.2)

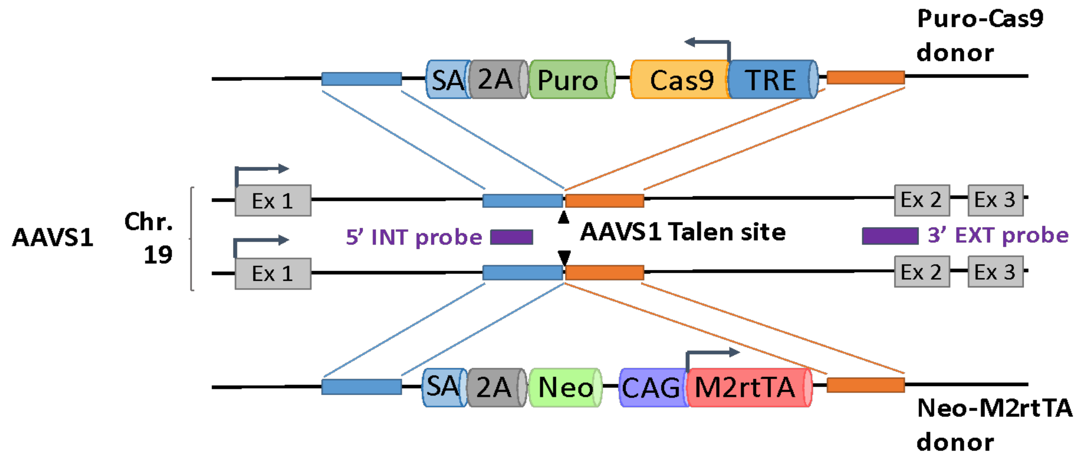

Figure 3.2 Design of the inducible Cas9 construct inserted into the AAVS1 safe-harbor site

To investigate the relationship between JARID2 and Oct4 in different stages of pluripotency of hESC we introduced guides targeting the exon 6 of JARID2 gene and induced Cas9. We analyzed the cellular effect in Oct4 expression in 2iL-I-F and 5iLA conditions. We performed immunofluorescence for JARID2 and OCT4 proteins immediately after induction of mutations in Elf1-iCas9 2iL-I-F and 5iLA lines using the JARID2 guide 6.1. Confocal analysis of these JARID2 knock-out pools of cells revealed that while wild type clones express JARID2 and OCT4, the JARID2 knockout cells lacked OCT4 staining in 2iL-I-F state, but had normal nuclear Oct4 staining in 5iLA state (Figure 3.4, arrows). These data indicates that 2iL-I-F, but not 5iLA hESC that lack JARID2 lose their pluripotency markers, OCT4 and NANOG, consistent with the EED binder data above.

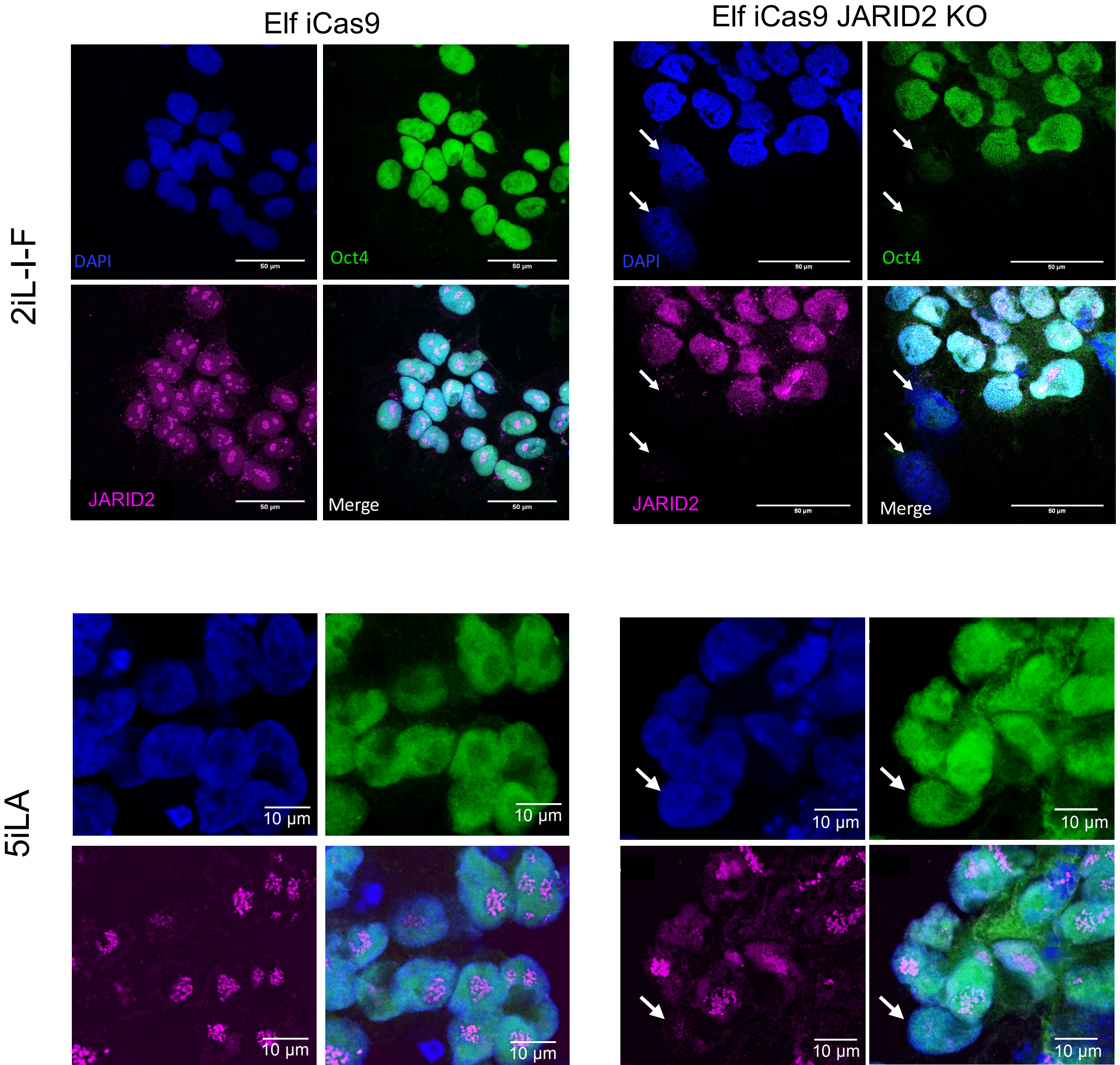

Figure 3.4 Elf1-iCas9 JARID2 Knock-down in decreases Oct-4 levels in naïve (2iL-I-F) but not in naïve-ground (5iLA) conditions

Chapter 4. USING PROTEIN DESIGN FOR TIE2 MODULATION

Angiogenesis is the process by which new blood vessels are formed from pre-existing vessels. The formation of new vessels is of great importance in healthy development since oxygen and

nutrients are needed for growth. In healthy tissues, angiogenesis is essential for early development as well as wound healing, however, it is also needed in the process of tumor metastasis. New tumors require rapid new vascularity, and Tie2 is a key receptor in one of the pathways that controls angiogenesis, this is why Tie2 is an interesting target for cancer therapeutics [30]. We used multiple approaches to design a Tie2 binder: first a small *de novo* designed peptide, PCR mutagenesis of the F-domain, and a combination of different scaffolds with the F-domain.

4.1 *De Novo* Design of a Small Inhibitor Peptide

Tie2 receptor is turned on when in close proximity with other Tie2 receptors and is auto-phosphorylated. Ligand Ang1 super clusters by the coil-coil domain and allows for clustering and activation of the Tie2 pathway. The F-domain is the binding surface of Ang1, which binds to the Ig2 domain of Tie2 [31, 32].

The strategy for the small peptide inhibitor was to create a binding surface with higher affinity to Tie2 than that of the affinity of the F-domain. The small peptide would block Tie2 by preventing Tie2 receptors from clustering and activating. The method used by the Institute for Protein Design (Seattle) to design small proteins is called Netmotif. This method first separates the binding surface of Tie2 into different domains. Then, it utilizes Rosetta [33] to screen for surfaces that would have high affinity to the specific domains. After the best possible binders are found for each independent domain, the surfaces that do not clash with each other are selected. This process creates the best possible binding surface based on data from the crystal structures archived on the Protein Data Bank (PDB). To finalize the design, the best binding surface is added to a protein scaffold from a library of small proteins found in nature. The final peptides are

predicted to have high affinity to the desired surface, while remaining small in size and highly stable. (Figure 4.1. Red: *de novo* designed small peptide, blue: Tie2 binding surface.)

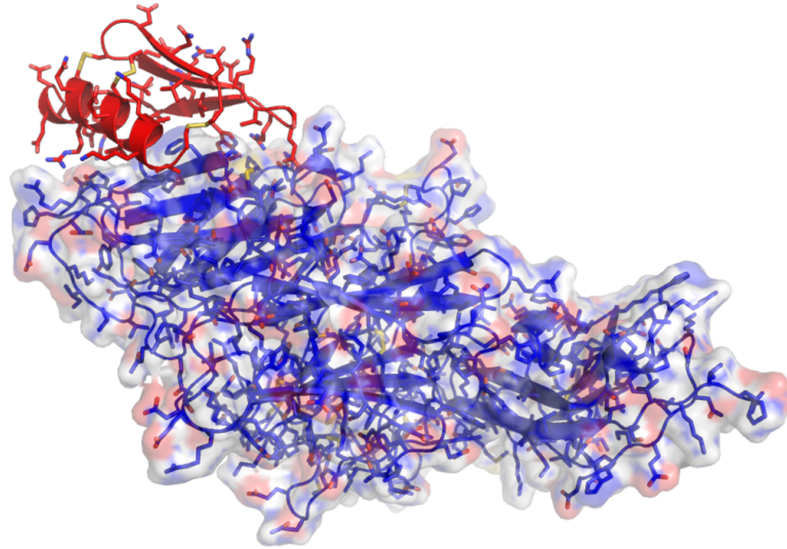

Figure 4.1. Small *de novo* designed peptide binding to Tie2

We screened 3 different libraries, each containing l000 small designs, however our *de novo* designs failed to bind Tie2 with high affinity. This surface proves to be a challenging target since, unlike most usual binding surfaces, does not contain pockets with polar residues. The design methods are fast evolving and rapidly improving.

4.2 PCR Mutagenesis to Increase the Affinity of F-Domain

We used PCR mutagenesis to create a library of modified F-domain binders with the goal of increasing the affinity of the F-domain to Tie2. Our target for making the library was to make a mutation of one amino acid swap per design. We enriched the concentration of high affinity designs by sorting and collecting the top 1% binders using yeast surface display, followed by, fluorescence activated cell sorting (FACS). We progressively decreased the concentration of our

target, Tie2, from 100nM, 20nM, 10nM, and 5nM, while enriching for the top 1% binders. (Figure 4.2).

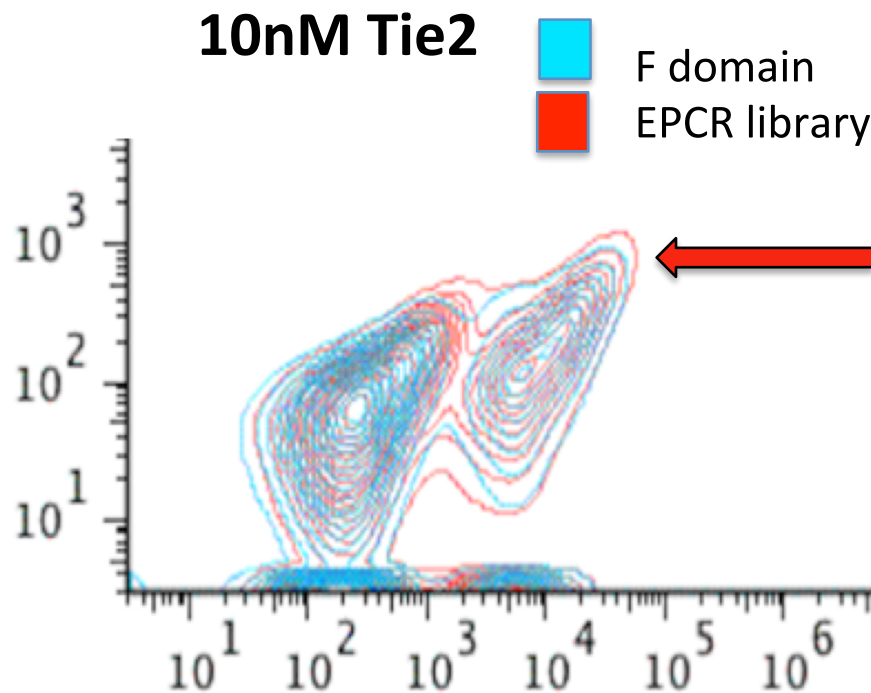

Figure 4.2 Error prone PCR (EPCR) library compared to F-domain binding 10nM of target Tie2

4.3 *De Novo* Designed Scaffold spy-tagged to F-domain

We clustered multiple F-domains on a scaffold to create a high affinity Tie2 binder. The *de novo* designed two-component cage is symmetrical, self-assembling, and highly stable. We used the "Spy Catcher" system, which creates an irreversibly covalent bond between the spy-tag and the spy-catcher components [34]. This allows for the cluster of 120 F-domain on the same scaffold when fully loaded. We tested our super activator binder on HUVEC cells and measured Phosphorylated Akt protein levels, which are the downstream target of Tie2 (Figure 4.3).

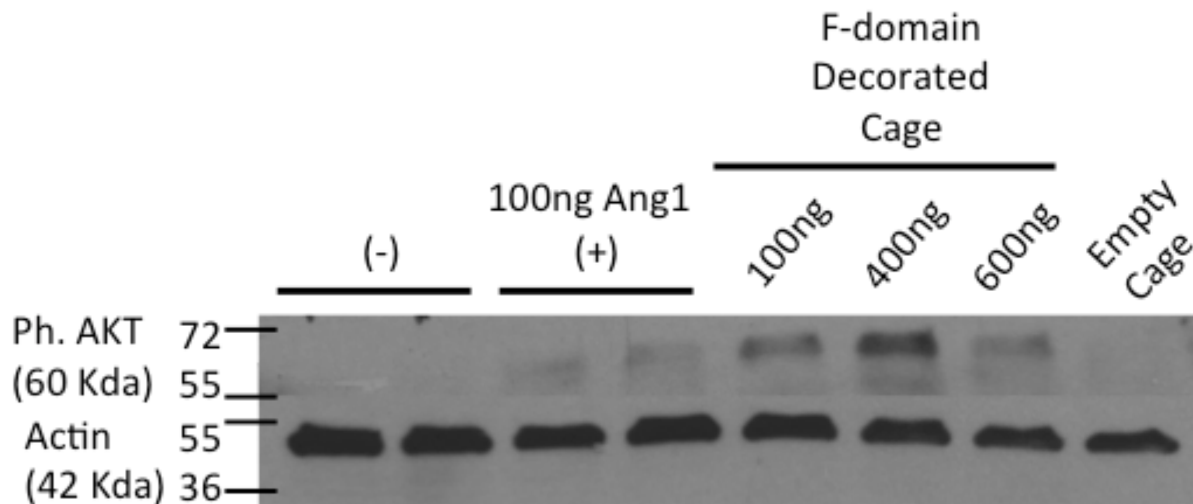

Figure 4.3. HUVEC cells treated with decorated cage show high levels of phosphorylated Akt

Chapter 5. MATERIALS AND METHODS

Cell culture

Naïve hESC [Elf-1(NIH_hESC Registry #0156), were cultured as previously described [12]. All 4 hESC lines have a normal, diploid karyotype. For 2iL-I-F conditions the cells were grown on a feeder layer of irradiated primary mouse embryonic fibroblasts in hESC media: DMEM/F-12 media supplemented with 20% knock-out serum replacer (KSR), 0.1mM nonessential amino acids (NEAA), 1 mM sodium pyruvate, and penicillin/streptomycin (all from Invitrogen, Carlsbad, CA) and 0.1 mM β-mercaptoethanol (Sigma-Aldrich, St. Louis, MO). hESC media was supplemented with 1µM GSK3 inhibitor (CHIR99021, Selleckchem), 1µM of MEK inhibitor (PD0325901, Selleckchem), 10ng/mL human LIF (Chemicon), 5ng/mL IGF1 (Peprotech) and 10ng/mL bFGF. For 5iLA naïve conditions [24] cells were grown in base medium containing: 120 ml DMEM/F12 (Invitrogen; 11320), 120 ml Neurobasal (Invitrogen;

21103), 2.5 ml N2 supplement (Invitrogen; 17502048), 5 ml B27 supplement (Invitrogen; 17504044), 1 mM glutamine (Invitrogen), 1% nonessential amino acids (Invitrogen), 0.1 mM b-mercaptoethanol (Sigma), penicillin-streptomycin (Invitrogen), and 50 mg/ml BSA (Sigma), and freshly supplemented with 5i/L/A: BRAF (0.5μM), SRC (1μM), MEKi (1μM), GSK3i (1μM), ROCKi (10μM), recombinant human LIF (20ng/mL), and Activin A (10ng/mL). For EPS conditions (extended pluripotency conditions)[18], cells were grown in base medium containing 100 mL DMEM/F12, 100 mL Neurobasal, 1 mL N2 supplement, 2 mL B27 supplement, 1% GlutaMAX , 1% NEAA, 0.1 mM β-mercaptoethanol, penicillin-streptomycin and 5% KSR, and freshly supplemented with 10 ng/ml hLIF, GSK3i (1 μM), ROCKi (2 μM), (S)-(+)-Dimethindene maleate (2 μM; Tocris), Minocycline hydrochloride (2 μM; Santa Cruz Biotechnology) and IWR-endo-1 (0.5-1 μM; Selleckchem). Cells were adapted to 5i/L/A or EPS conditions for at least 3 passages before analysis. Elf1-iCas9 cells were pushed toward differentiation using DMEM media supplemented with 20% FBS and retinoic acid (0.1μM RA; Sigma) for 5 days. All cells were cultured at 37 degrees Celsius in 5% CO_2. Elf1-iCas9 were generated in low (5%) O_2 and further expanded in normoxia (20% O_2) for experimental studies. Cells were exposed to 2% O_2 for 4 hours prior to protein extraction for the analysis of HIF2α expression. Experiments in naïve 5iLA hESC and EPS cells were performed in hypoxia (5% O_2).

Insertion of inducible Cas9 into AAVS1 site of Elf1 cells

$10x10^6$ cells of Elf-1p17 were transfected with 5μg AAVS1-TALEN R plasmid (Addgene #59026), 5μg AAVS1-TALEN L plasmid (Addgene #59025), 40μg Puro-Cas9 donor plasmid (Addgene #58409), 40μg Neo-M2rtTA donor plasmid (Addgene #60843) with Amaxa Lonza Human stem cell Kit #2. The cells were then plated with 5μM of ROCK inhibitor (ROCKi) onto

11, 10cm plates onto an irradiated Drug Resistance 4 Mouse embryonic fibroblast feeder cells (DR4). Three days following the nucleofection, the cells were selected for neomycin resistance with Genetecin (50μg/ml) for four days, then selected with Puromycin (0.5μg/ml) for three days. 14 clones survived after the double selection and were isolated and expanded. Of these 14 clones, eight (CL#1,2,4,6,8,11,12,13) clones were plated onto Matrigel with or without doxycycline (2μg/ml) and RNA was extracted in order to analyze the level of Cas9 expression by qPCR. Insertion of iCas9 into the AAVS1 site was confirmed by Southern blot analysis in clones #2, 11 and 12. Clone #2 was sent for G-banded karyotype analysis (Diagnostic Cytogenetics, Seattle) and displayed a normal karyotype.

DNA extraction and sequencing

Genomic DNA was collected using DNAzol reagent (Invitrogen) according to manufacturer's instructions and quantified using Nanodrop ND-1000. Genomic regions flanking the CRISPR target sites were PCR amplified with the designed primers, purified by PCR Purification Kit (Invitrogen) and sent to Genewiz for sequencing. Alternatively, following GoTaq PCR amplification of the targeted region, the JARID2 mutations were cloned using the CloneJET PCR Cloning Kit (Thermo Fisher Scientific) following the manufacturer's guidelines. The ligated plasmid was then transformed into DH5α competent cells (Invitrogen), colonies were tested for insert and inserts sent for sequencing.

RNA extraction and RT-qPCR analysis

RNA was extracted using Trizol (Life Technologies) according to manufacturer's instructions. RNA samples were treated with Turbo DNase (ThermoFischer) and quantified using Nanodrop

ND-1000. Reverse transcription was performed using Random Hexamers (Invitrogen) and Omniscript reverse transcription kit (Qiagen). 10ng of cDNA was used to perform qRT-PCR using SYBR Green, with suitable primers (Suppl.Table 1) on an Applied Biosystems 7300 real time PCR system with PCR conditions as stage 1 50°C for 2mins, stage 2 as 95°C for 10mis, 95°C for 15sec, 60°C for 1min(40 Cycles). ß-actin was used as an endogenous control.

Guide RNA design, synthesis and transfection

The gRNAs targeting the HIF1α and JARID2 genes were designed using the CHOPCHOP or CRISPRscan web tools[26, 27] and ordered as T7-gRNA primers. A dsDNA fragment was synthesized from these primers by self annealing PCR to a complementary scaffold primer, which is used to attach the guide to Cas9. The dsDNA fragment was followed by Q5 High Fidelity-based PCR (New England Biolabs). This 120 bp strand served as template for IVT (MAXIscript T7 kit, applied Biosystems). The RNA was then purified using Pellet Paint® Co-Precipitant (Novagen). Elf1-iCas9 cells were treated with doxycycline (2μg/ml) for 2 to 3 days before and during transfection. For transfection, cells were dissociated with trypsin, transfected in suspension with gRNAs using Lipofectamine RNAiMAX (Life Technologies) and re-plated onto matrigel-coated plates for NHEJ generated knockouts or onto irradiated MEF plates to generate PSEN2 mutations. gRNA was added at a 40 nM final concentration when added alone and at 15nM when added with 15nM of Ultramer repair template DNA. A second transfection was performed after 24 h. Two days after the last gRNA transfection, Elf1-iCas9 cells were dissociated into single cells and replated onto MEF-coated plates or collected for DNA analysis. In the next passage single colonies from MEF-coated plates were randomly selected and amplified.

Protein extraction and Western blot analysis

Cells were lysed directly on the plate with lysis buffer containing 20mM Tris-HCl pH 7.5, 150mM NaCl, 15% Glycerol, 1% Triton x-100, 1M *β*-Glycerolphosphate, 0.5M NaF, 0.1M Sodium Pyrophosphate, Orthovanadate, PMSF and 2% SDS. 25 U of Benzonase® Nuclease (EMD Chemicals, Gibbstown, NJ) was added to the lysis buffer right before use. Proteins were quantified by Bradford assay (Bio-rad), using BSA (Bovine Serum Albumin) as Standard using the EnWallac Vision. The protein samples were combined with the 4x Laemli sample buffer (900 µl of sample buffer and 100 µl β-Mercaptoethanol), heated (95°C, 5mins) and run on SDS-PAGE (protean TGX pre-casted gradient gel, 4%-20%, Bio-rad) and transferred to the Nitro-Cellulose membrane (Bio-Rad) by semi-dry transfer (Bio-Rad). Membrane were blocked for 1hr with 5% milk, and incubated in the primary antibodies overnight in 4°C. The antibodies used for western blot were β-Tubulin III (Promega G7121, 1:1000), JARID2 (Cell Signaling D6M9X, 1:1000), Oct-4 (Santa Cruz sc-5279, 1:1000, Novus Biologicals NB110-90606, 1:500), Nanog (Karol Bomsztyk, University of Washington, 1:1000), H3K27me3 (Active Motive 39155 1:1000), EZH2 (Cell Signaling D2C9, 1:1000) and HIF2α (Abcam, 1:1000). The membranes were then incubated with secondary antibodies (1:10000, goat anti-rabbit or goat anti-mouse IgG HRP conjugate(Bio-Rad) for 1hr and the detection was performed using the immobilon-luminol reagent assay (EMP Millipore).

OCR and ECAR measurement using Seahorse cellular flux assays.

Naive and primed ESCs were seeded onto 96-well Seahorse plates pre-coated with Matrigel at 25 $\times 10^4$ or 40 $\times 10^4$ cells per well. Culture media were exchanged for base media (unbuffered

DMEM, Sigma D5030) supplemented with sodium pyruvate (Gibco, 1 mM) and with 25 mM glucose (for the MitoStress assay), 25 mM glucose and 50 µM carnitine (for palmitate assay), or 2 mM glutamine (for glucose stress assay) 1 h before the assay. Substrates and selective inhibitors were injected during the measurements to achieve final concentrations of glucose (2.5 mM), 4-(trifluoromethoxy)phenylhydrazone (FCCP, 300 nM–500 nM), oligomycin (2.5 µM), antimycin (2.5 µM), rotenone (2.5 µM), palmitate (50 µM in BSA), BSA and ETO (50 µM). The OCR and ECAR values were normalized to the number of cells present in each well, quantified by the Hoechst staining (HO33342; Sigma-Aldrich). Changes in OCR and ECAR in response to the addition of substrates and inhibitors were defined as the maximal change after the chemical injection compared with the last OCR value before the injection.

Immunostaining and confocal imaging

Cells were fixed in 4% paraformaldehyde in PBS for 15 min, permeabilized for 10 min in 0.1% Triton X-100 and blocked for 1h in 2% BSA. The cells were then incubated in primary antibody overnight, washed with PBS (3x5min), incubated with the secondary antibody in 2% BSA for 1hr, washed (4x10mins, adding 1µg/ml DAPI in 2nd wash), mounted (2% of n-Propyl Gallate in 90% Glycerol and 10% PBS) and stored in the 4°C. Analysis was done on a Leica TCS-SPE Confocal microscope using a 40x objective and Leica Software. The antibodies for immunostaining were anti-JARID2 (Cell Signaling, 1:200 for 2iL-I-F, 1:100 for 5iLA), anti-Oct-4 (Novus Biologicals, 1:150), anti-Nanog (R&D, 1:200); and Alexa 488- or Alexa 647-conjugated secondary antibodies (Molecular Probes).

HUVEC assays for cage analysis

Cells were grown up to 70% confluency on 0.1% gelatin coated plates in EGM2 media. Cells where vigorously washed with PBS, then starved for 16 hours in starvation media: low glucose DMEM, 0.1% FBS after two. Cells were treated with different concentration of Ang1 and cages for 30 minutes and harvested immediately after with lysis buffer containing phosphatase inhibitor.

Chapter 6. DISCUSSION AND CONCLUSION

Stem cells need to be tightly regulated since their aberrant proliferation and differentiation could lead to catastrophic consequences. Because of their impact on gene regulation, epigenetics and metabolism must be strictly coordinated to reach a correct homeostasis of stem cell features. Repressive methylation marks such as H3K27me3, regulated by the PRC2 complex, can be at the intersection between stemness and metabolism. For example during early development repressive marks on the promoter region of ENGL1, will regulate the metabolic state through the stabilization of HIFs. Since HIF a transcription factor involved in the metabolic switch between naïve and primed ESC we are thus able to link these two pathways in stem cells.

The acquisition of a glycolytic metabolism during implantation of the early embryo could be an evolutionary mechanism. Indeed, though the reduction of mitochondrial OXPHOS activity, stem cells also reduce the production of reactive oxygen species (ROS). With the ability to transfer electrons to proteins or DNA, ROS have the potential to induce harmful mutations in either the mitochondrial or the nuclear DNA. By reducing the protection of these entities, primed stem cells also reduce the risk of inducing these DNA damages. This is important at this stage of implantation since stem cells chose the fate to primordial germ cells (PGCs) giving rise further to

gametes. One could easily understand the protective mechanism that such a protection of PGCs and thus protection of the progeny [35].

As I have shown previously, HIFs respond to key changes in the oxygen content of the environment and have an important role in the acquisitions if the glycolytic metabolism that is needed in the naïve to primed hESC transition, as well as in the reprogramming of iPSC. Understanding this metabolic shift and its regulation is imperative because of the similarity to the metabolism of cancer cells, previously described as the Warburg effect. HIFs have also been shown to enhance reprogramming however prolonged stabilization of HIF2α inhibits iPSC by upregulation of TRAIL. The opposite effect of HIF1 and HIF2 during reprogramming is unexpected and highlights the non-redundant roles of these two forms of HIF [23].

Next, we have showed for the first time that there is very early stage of naïve hESC that is completely independent from the PRC2 complex. This data is similar to mouse naïve ground ESC data [36, 37]. We have also shown that Jarid2, a key component of the PRC2 is required for pluripotency maintenance in early embryonic development but not in the very early naïve ground stage of hESC [38]. The epigenetics field is a rapidly growing field that still has many unanswered questions. It is still not clear why those very first stages are PRC2 independent.

It might be possible that the low oxygen levels that the blastocyst has to travel through before implantation in the uterus promote apoptosis in some surrounding cells. HIF stabilization can activate apoptosis pathways as well as angiogenesis. Hypothetically, dying daughter cells around the embryo might be able to signal the blastocyst to promote and express the survival mechanisms, maybe even through the angiopoietin1/Tie2 pathway. Although it is known that HIFs and epigenetic changes are key for the change in cell fate during development and reprogramming, many questions remain unanswered on how this changes precisely occur and

how the precise mechanism of cell fate is regulated. Additional work with newly developed tools, such as the Elf1-iCas9 in genome editing and the Tie2 decorated cage in protein design will help further studies to elucidate these important questions.

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