Role of HIF1α in human embryonic stem cell differentiation

A thesis submitted in partial fulfillment of the requirements

For the degree of Master of Science in Biology,

Department of Biology

By

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Date

Date

Date
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Ectopic expression of non-degradable HIF1α in normoxia promotes induction of developmental genes.

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References
ABSTRACT

Role of HIF1α in human embryonic stem cell differentiation

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Hypoxia stimulates human embryonic stem cell (hESC) self-renewal. It has been shown that hypoxia inducible factor 2α (HIF2α) up regulates OCT4 (POU5F1) transcription, a pluripotency marker gene. Hypoxia also increases the efficiency of reprogramming of differentiated cells to a pluripotent-like state. Combined, these results suggest that hypoxia, or more specifically HIF, would impair the purposeful differentiation of pluripotent stem cells. Here, we unexpectedly show that HIF expression also promotes hESC differentiation. shRNA knockdown of HIF1α or its binding partner, aryl hydrocarbon receptor nuclear translocator (ARNT), inhibits the early induction of developmental genes GATA2, GATA3, GATA6, HAND1 and HOXB3 during retinoic acid-induced differentiation. shRNA knockdown of HIF1α or ARNT also impaired embryoid body formation, one of the commonly used in vitro differentiation assays in the human embryonic stem cell world. Conversely, ectopic expression of a stabilized HIF1α mutant, HIF1α (P402A/P564A), in normoxia leads to enhanced developmental genes GATA2, GATA3, GATA6, HAND1 and HOXB3 induction during retinoic acid-induced differentiation. Ectopic expression of HIF1α (P402A/P564A) also increased the
chromatin modifier gene \textit{JMJD3} induction during retinoic acid induced differentiation in normoxia. Combined, the data show a previously unanticipated role for HIF in promoting early hESC differentiation in hypoxia.
Chapter 1

Introduction:

1.1 Stem Cells

Human embryonic stem cells (hESCs) have generated enormous interest because of their developmental plasticity (pluripotency) and their potential for patient-specific applications in regenerative medicine. To be defined as a pluripotent stem cell three essential criteria must be met. First, the cell cannot be specialized for any particular function. Second, the cell must have the ability to differentiate into any specialized cell type. And third, the cell must have the ability to reproduce indefinitely. There are two major categories of stem cells: adult stem cells and embryonic stem cells (ESCs).

The earliest entity of life is the fertilized egg. Up until the 8 cell stage (~3 days after fertilization), the cells are totipotent; they have the potential to give rise to an entire organism. Human embryonic stem cells are not totipotent as they are derived from the inner cell mass of a blastocyst. A blastocyst forms approximately a day after fertilization and is composed of an undifferentiated inner cell mass (ICM) (Figure 1) surrounded by differentiated trophectoderm. The trophectoderm give rise trophoblast cells, while the inner cell mass gives rise to all three germ layers (endoderm, ectoderm and mesoderm) of the body. hESCs are pluripotent because they are uncommitted, can differentiate into all three germ layers, and can eventually give rise to any cell in the body.
Figure 1. Stem Cell Hierarchy.

The earliest entity of life is the fertilized egg known as a zygote. A zygote contains totipotent cells that can give rise to an entire organism. After several divisions, a zygote becomes a ball of cells known as a morula. The morula becomes a blastocyst that contains an inner cell mass (ICM) surrounded with trophectoderm. Human embryonic stem cells are derived from the ICM of a blastocyst and these cells can give rise to all three germ layers of the organism (Endoderm, Ectoderm, Mesoderm)\(^3\). Endoderm, ectoderm, mesoderm further differentiate into germ layer-specific progenitor cells and
eventually organ-specific tissue types.

hESCs can be used to form stem cell lines to circumvent the need for a constant supply of embryos. Currently there are more than 300 established human embryonic stem cell lines worldwide\(^4\). These lines were derived from embryos generated for *in vitro* fertilization (IVF). Not all embryos fertilized for IVF treatment are suitable for implantation. Under tissue banking regulations, defective embryos are destroyed or donated for research with informed consent\(^1\). To derive human embryonic stem cell lines, trophoblast cells are separated from ICM cells by immunosurgery and mechanical dissection. ICM cells are then plated on a feeder cell layer (usually mouse embryonic fibroblast cells) that provide soluble factors and contact mediated support for hESC proliferation\(^4\). In general, healthy hESC cells form compact colonies (Figure 2) and these cells are often tested for expression of commonly used embryonic genes known as embryonic markers OCT-4, NANOG, and SOX-9\(^{1-3}\).
Adult organisms contain a small subpopulation of cells capable of self renewal that are multipotent. Multipotent cells are committed to differentiate into specific specialized cell types, such as hair, skin, bone marrow, and others (Figure 1). Of the adult stem cells, hematopoietic stem cells (HSCs) have generated the most interest due to the ease in obtaining them and their therapeutic potential for human diseases. The adult niche
that supports the HSC is the bone marrow \(^{(3)}\). The universal marker (gene that is expressed in a specific cell type) currently being used to identify HSC is CD34. The expression of CD34 decreases as HSC differentiate into all the different types of blood cells\(^{(3)}\).

**Figure 2.1 The Hematopoietic Stem Cell (HSC) differentiation lineage.**

The curved arrow indicates self-renewal. HSCs give rise to two lineages: myeloid and lymphoid progenitors. Myeloid progenitors give rise to erythrocytes, eosinophils, basophils, neutrophils and macrophages. Lymphoid progenitor gives rise to natural killer cells, T cells and B cells\(^{(46)}\).
1.2 iPSCs

For a long time biologists believed that cells are unidirectional both physiologically and in the laboratory, meaning that a cell from a zygote eventually becomes terminally differentiated, reaches senescence, and cannot dedifferentiate to a former cell stage. A paradigm-shifting discovery was made by Takahashi and Yamanaka in 2006 when they reprogrammed terminally differentiated fibroblasts back into cells resembling human embryonic stem cells\(^{(2-3)}\). To rewire fibroblasts into embryonic stem cell-like cells, four retroviruses encoding specifically selected transcription factors (MYC, SOX2, KLF4, OCT4) were infected into fibroblast cells and stably expressed in fibroblast cells\(^{(3)}\). These reprogrammed cells were named induced pluripotent stem cells (iPSCs). iPSCs have generated great interest amongst stem cell and developmental biologists because they generate a system for studying the commitment and reversion of lineage specification. iPSCs have also generated great interest in medicine and in the general public because their use can circumvent the ethical issues surrounding the derivation of human embryonic stem cells from early human embryos and their potential to generate patient-specific, immune system compatible replacement parts in the emerging field of regenerative medicine\(^{(3-4)}\). One of the biggest disadvantages of iPSCs are unintended virus integrations in to the genome because they are generated using retroviruses which can lead to complications in the patient receiving these cells. In order to overcome this disadvantage, scientists are currently investigating methods to derive iPSCs without retroviral vectors.
Figure 3. Schematic showing a potential application of iPSCs.
Fibroblast cells obtained from a spinal muscular atrophy (SMA) patient are converted to iPSCs with retroviral introduction of the factors cMYC, Klf-4, Oct-4 and Sox-2. The nonfunctional genes in these diseased iPSCs can be repaired by homologues recombination. Subsequently, the corrected iPSCs can be differentiated into healthy motor neurons and transplanted into the patient without the need for immune suppressing drugs. Alternatively, the cells can also be used to carry out customized drug screens *in vitro*\(^4\). Figure adapted from Wobus wt al 2005\(^4\).
1.3 Hypoxia and Stem Cells

Hypoxia occurs when there is 1-5% oxygen available as opposed to the normal 21% oxygen in ambient air \(^{(5)}\). Hypoxia constitutes a stress for mammalian cells in many pathophysiological conditions such as ischemia (shortage of oxygen caused by restriction of blood supply), cardiovascular and pulmonary diseases, cancer, and sleep apnea. As mentioned earlier, human embryos are exposed to hypoxia at a very early stage in life. The blastocyst survives in a hypoxic microenvironment estimated at 1.5 – 5.3% in mammalian reproductive tract until implantation step\(^{(5-9)}\) (Figure 4). Upon establishing the connection between the mother and the early trophoblast cells that form the placenta, the embryo is exposed to increased oxygen concentrations. Consequently, angiogenesis is stimulated and the embryo begins to form its own circulatory system and conditions to normoxia.
Figure 4. Fate of the human embryo post fertilization.
The embryo survives in a strictly hypoxic environment until 4-5 days post fertilization. Note: the inner cell mass is already surrounded by differentiated trophoblast cells at preimplantation stage. Adapted from Medical Embryology Langman 5th edition Chapter 2, Pg 32, Figure 2-11.

hESC have a different metabolic profile compared to most differentiated cells. Pluripotent embryonic stem cells require high flux of glucose uptake and produce lactate, the normal method of metabolism in an anaerobic or hypoxic state. In contrast, differentiated cells have ample access to oxygen and synthesize their ATP via oxidative phosphorylation (Oxphos). Previously it was believed that human embryonic stem cells had underdeveloped mitochondria that are incapable of producing ATP by Oxphos. In the Teitell lab at UCLA, Zhang J. have shown that stem cells have fully functional mitochondria even in the hypoxic blastocyst inner cell mass (ICM) cells. hESCs
express the protein UCP2 (Uncoupling protein 2) that blocks pyruvate from entering the Krebs cycle (Figure 5). hESCs grown under hypoxia self-renew with reduced levels of spontaneous differentiation compared to hESCs grown in ambient oxygen. Hypoxia also improves the efficiency of Yamanaka factor-induced cellular reprogramming to a pluripotent-like state. Combined, these studies show the importance of low O2 tension in supporting stem cell self-renewal and in suppressing spontaneous, typically unwanted hESC differentiation. Even though many studies have predicted molecular mechanisms underlying hypoxia and stemness (the ability to remain a stem cell and not differentiate), the relationship between hypoxia and differentiation is yet to be determined.

**Figure 5. Dissociation of respiration from ATP synthesis in hPSCs.**

hPSCs predominantly use glycolysis to produce ATP. hPSCs express Uncoupling protein 2 (UCP2), which blocks pyruvate becoming AcetyleCoA then entering the Krebs cycle. Differentiated cells do not express UCP2, and as a result pyruvate becomes Acetyle CoA and enters the Krebs cycle and ATP is synthesized via ATP synthase and oxidative
phosphorylation\(^{(9)}\). Zhang et al showed knocking down UCP2 in hPSCs blocked cellular differentiation, indicating a critical role UCP2 plays in stem cell pluripotency\(^{(13)}\).

1.4 Hypoxia and HIF

Stem cells reside in a specific niche where oxygen concentration is a critical component of the microenvironment\(^{(10)}\). Aerobic organisms require oxygen to produce energy and maintain cellular homeostasis. In mitochondrial Oxphos, oxygen is the final electron acceptor\(^{(12-13)}\). As electrons pass through complex I-IV, protons are transported from the mitochondrial matrix to the intermembrane space (Figure 6) \(^{(14-15)}\). The resulting electrochemical gradient (also known as the proton motive force) powers ATP synthase. Although reduction of oxygen by cytochrome c is precisely orchestrated, it is inevitable that occasionally oxygen is only partially reduced and results in unpaired oxygen (\(e^- + O_2 \Rightarrow O_2- \cdot\))\(^{(14-16)}\). Accumulations of unpaired oxygen molecules, called reactive oxygen species (ROS) are a particularly potent releaser of free radicals. Evolution has produced enzymes, which we call antioxidant enzymes (a major one being superoxide dismutase) to prevent the harmful effects of free radicals\(^{(16)}\).
Figure 6. Electron transport chain and reactive oxygen species.
Electrons from reduced coenzymes NADH and FADH$_2$ pass through a series of redox centers in the electron transport chain before reducing oxygen. During the electron transfer process, protons are translocated from the matrix to the intermembrane space to form an electrochemical gradient. This free energy drives the ATP synthase, and ATP is synthesized by combining inorganic phosphate (Pi) with ADP$^{(15)}$.

Other than the dismutase activity, auxiliary adaptive responses are also exerted to balance cellular homeostasis to match the metabolic and energy demands of the cells$^{(14-16)}$. Such responses include cell cycle arrest, less energy consumption, and secretion of survival and proangiogenic factors like fibroblast growth factor (FGF), vascular endothelial growth factor (VEGF), etc$^{(15)}$. Hypoxia Inducible Factors (HIFs) are the major regulators of cellular responses to hypoxia$^{(4-7)}$. They form a small family of transcription factors whose protein levels are controlled by oxygen-dependent
degradation (Figure 7a). In the presence of oxygen, prolines 402 and 564 (Figure 7a and b) of HIF1α are hydroxylated by the prolyl hydroxylases (PHDs) and factor inhibiting HIF (FIHs). Hydroxylation allows HIFs to be recognized by an E3 ubiquitin ligase, VHL (Von Hippel-Lindau), ultimately leading to degradation in the proteosome\(^{8-10}\). Under hypoxic conditions, these prolines are not hydroxylated and the HIF1α protein is stabilized and can dimerize with ARNT (HIF1β) in the nucleus to transactivate specific target genes through binding to hypoxia responsive elements (HRE). The HRE consensus sequence is RCGTG, where R is an A or a G) (Figure 7c and d). ROS created in the mitochondria inactivate PHDs and FIHs in the cytosol, stabilizing HIFs and upregulating the auxiliary adaptive responses mentioned earlier. HIFs also upregulate many glycolysis genes such as PGK1, HK 1 and Cox-1 \(^{8-10}\).

Figure 7a. Oxygen dependent hydroxylation of HIFs.
Oxygen is required by PHDs and FIH to be active. When PHDs and FIHs are active, HIFs are subjected to proteosomal degradation by pVHL resulting in HIF inactivity. As
the oxygen level is decreased, PHDs and FIHs are inactivated, and inversely HIFs are activated\textsuperscript{(16)}.

**Figure 7b. Schematic of HIF1α protein.**
HIF1α contains a basic helix-loop-helix domain. This domain allows HIF1α to dimerize and then bind to DNA in order to upregulate its down stream target genes. HIF1α also contains two PAS domains A and B. Per Arnt Sim domains play important roles in sensitivity stimuli such as oxygen, redox potential or light intensity\textsuperscript{(10)}.

**Figure 7c. Schematic of HIF1α downstream targets.**
Note: HIF1α enters the nucleus under hypoxic conditions and dimerizes with HIF1β to upregulate angiogenesis and glucose metabolism-related genes.⁹

Figure 7d. Schematic showing ROS activation of HIF.

Reactive oxygen species are created in mitochondria and inhibit the prolyl hydroxylases (PHDs) and factor inhibiting HIF (FIHs). As a result, HIF1α is stabilized. HIF1α then enters the nucleus to dimerize with HIF1β and allows the dimerized unit to bind to the hypoxic regulatory element (HRE) on the promoter of the vascular endothelial growth factor (VEGF) gene. VEGF is a pro-angiogenic factor secreted in response to cellular stress caused by ROS.¹⁴
1.5 HIF and demethylases

According to the central dogma in biology, information flows from DNA to RNA to protein\(^{16}\). DNA carries the information to make proteins and DNA binding proteins provide structure and package DNA into chromatin. Many nuclear processes require DNA to be unraveled from its compact chromatin structure because chromosome condensation affects the rates of transcription. The basic structural unit of chromatin is the nucleosome. A nucleosome is composed of DNA binding proteins together called a histone octamer and the associated DNA. Two copies of each H2A, H2B, H3 and H4 histone proteins make up a histone octamer and 146 base pairs of DNA wraps around each histone octamer to form a nucleosome\(^{18-22}\). Each histone protein has an amino terminal (N terminal) polypeptide tail that can undergo several protein modifications including methylation, acetylation, ubiquitination, SUMOylation and phosphorylation\(^{15}\) (Figure 8a). Modification of histone protein tails by chromatin remodeling enzyme complexes results in compaction or relaxation of chromatin, depending on the modifications induced.

Upon stimulation by upstream signals, the transcription of most genes in the nucleus is regulated by the coordinate action of transcription factors (genetic mechanisms) and chromatin remodeling complexes (epigenetic mechanisms). In the context of epigenetics, Histone 3 (H3), methylation occurs on N terminal arginine or lysine residues (Figure 8b). Each lysine residue can undergo three stages of methylation,
resulting one (mono), two (di), or three (tri) methyl groups on the lysine side chain\(^{(21)}\). Arginine can only be mono or dimethylated. Depending on the location of the lysine residue methylation it can either activate or repress transcription\(^{(23-26)}\). For example, when H3 lysine residues 79 (H3K79me), 36 (H3K36me) and 4 (H3K4me) are methylated, chromatin relaxes, and transcriptional activity increases with a “open” chromatin state. (Figure 8b). Methylation on lysine residues 27 (H3K27me) and 4 (H3K9me) causes compaction of the chromatin, reducing transcriptional activity, and resulting in a “closed” chromatin state\(^{(25)}\).

(a)

**Figure 8. Nucleosome and Histone 3 (H3) methylation residues.**
(a) Schematic of a nucleosome. A nucleosome is composed of a histone octamer. Two of the following histones H2A, H2B, H3 and H4 are wrapped around by 146 bp of DNA.
(b) H3 Methylation on the N terminal lysine residues. In this schematic, when K residues methylated marked by red suppresses transcription and green activates transcription (25). Methylation of H3K9, H3K27, and H4K20 are associated with transcriptional repression,
whereas methylation at H3K4, H3K36, and H3K79 results in transcriptional activation.

Histone demethylase is an enzymes that remove methyl groups from lysine residues. Lysine specific demethylase 1 (LSD1) was the first demethylase identified and removes mono- (me) or dimethyl (me2) groups from H3K4 or H3K9. Subsequently, two Jumonji C (JmjC) domain containing proteins named ubiquitously transcribed tetratricopeptide repeat X chromosome (UTX) and JmjC domain-containing histone demethylase 3 (JMJD3) were identified as histone demethylases that remove tri-methyl groups (22-25). This family of enzymes catalyzes the removal of methylation by a hydroxylation reaction and requires iron and α-ketoglutarate as cofactors (18-19).

Histone methylation on lysine residues plays an important role in generating specific molecular structures in chromatin, thus causing a dynamic balance between transcriptionally active and inactive states. In Pan et al 2007, from Thomson’s Lab (38) (University of Wisconsin) showed that early developmental genes contain both
H3K4me3 and H3K4me3. H3K4me3 and H3K4me3 enrichment is termed “bivalent domains”. Chromatin immunoprecipitation (ChIP) combined quantitative real time PCR (QPCR) demonstrated that hESC genes encoding key developmental transcription factors are enriched with bivalent domains (Figure 9). Several of the genes that were highlighted in this paper are pluripotent genes NANOG, POU5F1, SOX2, SOX1, and developmental genes PAX6 (neural ectoderm marker gene), HAND1 (mes mesoderm maker gene); GATA4, GATA6 (endoderm maker genes); and GATA2, GATA3, CDX2 (trophectoderm marker genes).

**Figure 9. Bivalent domains of pluripotent and key developmental genes.**
Note: H3K4me3 marks a transcriptional active state. Pluripotent genes are enriched with H3K4me3 (purple) in ES cells. Developmental genes on the other hand have equal amounts of H3K4me3 (purple) and H3K27me3 (yellow) (38).
When hESCs line HSF1 were cultured under hypoxic conditions, quantitative real-time PCR showed an increase of the demethylase JMJD3, a putative HIF1α target gene. JMJD3 demethylase regulates the expression and silencing of many developmental genes. Therefore, we predict that HIF1α may have a direct role in epigenetic gene regulation by increasing the levels and potentially the activity of this histone demethylase in hypoxic conditions to push cells towards terminal differentiation.
Chapter 2
Materials and Methods

**hESC culture**
HSF1 cells were obtained from the UCLA Stem Cell Core facility. Initially, cells were co-cultured with mouse embryonic fibroblast cells (MEFs) and passaged onto matrigel™-coated plates (BD Biosciences, MA) prior to experiments. Cells were cultured in Dulbecco’s Modified Eagle Medium (DMEM) F-12 + GlutaMAX™ (GIBCO, USA) supplemented with StemPro® hESC SFM growth supplement (GIBCO, USA), 1.8% BSA (GIBCO, USA), 10µg/mL fibroblast growth factor (bFGF) and 55mM 2-Mercaptoethanol. Differentiation was induced using 1µM retinoic acid for either 2 or 3 days depending on the experiment. For passaging, hESC colonies were treated with 1mg/mL collagenase type IV or 1mg/mL Dispase (GIBCO, USA), washed three times with phosphate saline buffer (1X PBS), and harvested by manual scraping.

**RNA Extraction and First Strand cDNA Synthesis**
Total RNA extraction was performed using Trizol® reagent (Invitrogen). On the day of harvesting, cells were washed with 1X PBS 2 -3 times and 1 mL of trizol reagent was added (Invitrogen). The 1mL trizol cell solution was transferred to a 1.5mL eppendorf tube and 200ul of pheno/chloroform solution was added to the tube, mixed vigorously, and centrifuged at 4°C for 20 minutes. The post centrifugation upper phase was transferred to a clean RNAse free tube and RNA was precipitated by ethanol
precipitation. A total of 5ug RNA was used for cDNA synthesis using SuperScript® III First strand synthesis kit (Invitrogen) using the following parameters:

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Incubate 65°C for 5 min, place on ice for at least 5 min

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|               |             | 2 min 30 S
|               |             | 5min
|               |             | 35 Cycles
|               |             | 7min
cDNA amplification of HIF1α

The nucleotide sequence of the HIF1α coding region was obtained from the USCIS Genome browser (NM_001530).

Forward primer 1 5’ ATGGAGGGCGCCGCGGCGGAACG 3’ and Reverse primer 2 5’ TCAGTTAACTTGATCCAAAGCTCTG 3’ were designed to amplify the 2481bp HIF1α coding region. PCR amplification was performed on cDNA made from RNA extracted from HEK293T cells (Invitrogen, detailed protocol under RNA extraction and cDNA synthesis). PCR amplification was performed with the following PCR mix set up:

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<tr>
<td>Reverse Primer</td>
<td>1ul</td>
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<tr>
<td>Proofreading Polymerase</td>
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<tr>
<td>10X Buffer (with MgCl₂)</td>
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<tr>
<td>dNTPs</td>
<td>1ul</td>
</tr>
<tr>
<td>H2O</td>
<td>Upto 20ul</td>
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Amplified PCR product was confirmed for the correct size by gel electrophoresis. The 2.5kb band was excised and gel purified using Zymoclean gel recovery kit (Zymo research).

**Figure 2.1 Coding region of HIFα extracted from UCSC genome browser.**

Primers 1 and 2 were designed with GC content close to 50%. Primer 1 and 2 were used for the amplification of the coding region. PCR fragment was sequenced at UCLA sequencing facility to confirm the correct sequence.
**Gel Purification:**

To purify the PCR product, 3 volumes of ADB buffer was added to the excised agarose fragment in an eppendorf tube. (Example 100ul agarose 300ul buffer ADB). This mix was then incubated at 55°C for 5-10 minutes. The melted agarose was then transferred to Zymo-Spin™ column in a collection tube. The column was then spun down at maximum speed for 30-60 seconds. The column was washed two times with 200ul of washing buffer and spun at max speed for 30-60 seconds. Transfer the spin column to a new eppendorf tube. DNA was eluted in 20ul of elution buffer. Post gel purification PCR products were cloned into Fermentas CloneJET™ PCR cloning kit (Catalog K1232).

**CloneJET™ PCR product cloning.**

The blunting reaction is set up as follows:

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<td>PCR product</td>
<td>2ul</td>
</tr>
<tr>
<td>Water, nuclease free</td>
<td>5ul</td>
</tr>
<tr>
<td>DNA blunting enzyme</td>
<td>1ul</td>
</tr>
<tr>
<td>Total volume</td>
<td>Upto 18ul</td>
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The above components were briefly vortexed and centrifuged for 3-5minutes. The mixture was incubated at 70°C for 5min. The tubes were chilled on ice for several seconds. Post ligation, the following components were added to the reaction mixture. From this 1 ul of pJET1.2/blunt cloning vector (50ng/ul) and 1ul of T4 DNA ligase. The reaction was incubated at room temperature for 5-20minutes. Transformed chemically competent cells with 2.5ul of the ligation mixture.
Transformation of CloneJET ligation reactions:
Chemically competent DH5α cells (70ul) mixed with 2.5µl of ligation mixture was incubated on ice for 2 minutes, heat shocked at 42°C for 1 minute and then transferred back to ice for 2 minutes. To this 300µl of LB broth was added to the same tube and incubated at 37°C for 30-45 minutes. Tubes were centrifuged and resuspended with 150µl of LB broth containing ampicillin at (50mg/mL). Sample was plated on LB ampicillin plates and incubated at 37°C for 16 hours. Colonies were tested for transformants by colony PCR.

Colony PCR.
From the above transformation colonies were picked into 50uL of LB ampicillin media in PCR tubes and incubate at 37°C for 1 hour. From this sample 2ul was used for the PCR set up as below.

<table>
<thead>
<tr>
<th>Colony</th>
<th>2ul</th>
</tr>
</thead>
<tbody>
<tr>
<td>pJET Forward Primer</td>
<td>1ul</td>
</tr>
<tr>
<td>pJET Reverse Primer</td>
<td>1ul</td>
</tr>
<tr>
<td>Polymerase</td>
<td>1ul</td>
</tr>
<tr>
<td>10X Buffer (with MgCl₂)</td>
<td>2ul</td>
</tr>
<tr>
<td>dNTPs</td>
<td>1ul</td>
</tr>
<tr>
<td>H2O</td>
<td>Upto 20ul</td>
</tr>
</tbody>
</table>

Post PCR checked for 2.5kb fragment by electrophoresis in a 1% TBE gel.

<p>| | | |</p>
<table>
<thead>
<tr>
<th></th>
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<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Denature</td>
<td>94°C</td>
<td>3min</td>
</tr>
<tr>
<td>Denature</td>
<td>93°C</td>
<td>1min</td>
</tr>
<tr>
<td>Annealing</td>
<td>55°C</td>
<td>2 min 30 S</td>
</tr>
<tr>
<td>Extension</td>
<td>72°C</td>
<td>5min</td>
</tr>
<tr>
<td></td>
<td></td>
<td>35 Cycles</td>
</tr>
<tr>
<td>Extension</td>
<td>72°C</td>
<td>7min</td>
</tr>
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</table>
Figure 2.2 HIF1α in pJET1.2

Schematic showing the insertion of HIF1α amplified coding region (green) into the pJET 1.2 (Grey). Software used for the map creation Genious v5.4.
Creating double mutant HIF1α

Site directed mutagenesis was employed to substitute P402A and PA564.

<table>
<thead>
<tr>
<th></th>
<th>402 (Primers 3/4)</th>
<th>564 (Primers 4/5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proline</td>
<td>CCA</td>
<td>CCC</td>
</tr>
<tr>
<td>Alanine</td>
<td>GCA</td>
<td>GCC</td>
</tr>
</tbody>
</table>

Primer 3 CTTTAACTTTGTGGCGCCGAGCCGCTGGAGAC
Primer 4 GTCTCCACGGCTGCGGCCAGCAGAAGTTAAAG
Primer 5 GACTTGGAGATGTTAGCTGCTATATCCCAATGG
Primer 6 CCATTGGGATATAGGCAAGCTAACATCTCCAAGTC

Mutagenic primers were created with mutation in both primers, and included 10bp of complementarity on each side of the point mutation. Melting temperature of the primers were equal or greater than 78°C.

PCR reaction was set up as follows:

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration/Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasmid 50ng/ul</td>
<td>1ul</td>
</tr>
<tr>
<td>Forward Primer 125ng/ul</td>
<td>1ul</td>
</tr>
<tr>
<td>Reverse Primer 125ng/ul</td>
<td>1ul</td>
</tr>
<tr>
<td>Pfu turbo DNA polymerase</td>
<td>1ul</td>
</tr>
<tr>
<td>10X Reaction Buffer (with MgCl₂)</td>
<td>5ul</td>
</tr>
<tr>
<td>dNTPs</td>
<td>1ul</td>
</tr>
<tr>
<td>H2O</td>
<td>Upto 50ul</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Step</th>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Denature 1</td>
<td>95°C</td>
<td>30S</td>
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<tr>
<td>Denature 2</td>
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<td>30S</td>
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<tr>
<td>Annealing</td>
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<tr>
<td>Extension 1</td>
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<td>5 min</td>
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<tr>
<td>Extension 2</td>
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<td>12min</td>
</tr>
<tr>
<td>12 Cycles</td>
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<td></td>
</tr>
</tbody>
</table>


After the PCR was complete, 1μl of the enzyme Dpn-1 (10U/μl) was added and incubated at 37°C for 1 hour. This step digested the methylated parental plasmid and subsequent transformation only gave mutant colonies. A control PCR with no primers was preformed and transformed to determine the Dpn-1 enzyme digestion efficiency. Post transformation HIF1α double mutant containing pJET1.2 vectors were sequenced to ensure the mutations were incorporated correctly.

**Figure 2.3 Amino acid alignment between HIF1α and double mutant HIF1α.**

Note: Prolines (yellow) 402 and 564 indicated with green box have been changed to alanines (grey).
Figure 2.4 Schematic of double mutant HIF1α inserted into pNUS vector.

HIF1α is constitutively expressed and is degraded in normoxia\(^{(10)}\). This protein contains two proline residues at positions 402 and 564. To over express HIF1α in normoxia we created a double mutant that contains alanines at the residues 402 and 564. The protein was over expressed under EF1 – Elongation factor alpha promoter. Green bars 402 and 564 indicate the amino acid changes (proline to alanine). IRES – Internal ribosomal entry site. 3xNLS – Nuclear localizing signal.

To insert the double mutant HIF1α sequence to the above vector primers were created with restriction enzyme sites on both ends. On the 5’ side a EcoRI (CCC GGG ) and on the 3’ side Not1 (CGC GGC ) sites were created and cloned into a vector containing EF1 promoter, IRES, GFP and NLS sequence (Figure 2.4). In frame cloning was not required because of the IRES site in-between the coding region of HIF1α and GFP (IRES GFP is a bi-cistronic vector). PCR conditions used were the same as above for amplification of HIF1α from cDNA.

Primer 7’ CCC GGG ATC CGC CGC CAT GG 3’
Primer 8’ CGC GGC GGC CGC TCA GTT ACC3’
**Transient Transfection**

One day prior to transfection HSF1 cells were trypsinized and plated to obtain $2 \times 10^6$ cells/well in a 6 well tissue culture plate. Media was supplemented with Rho Kinase (ROCK) inhibitor (10uM) to minimize cell death (Note: Stem cells are healthy when they grow in colonies. Trypsinizing cells digests the cell-cell interactions; when stem cells are trypsinized and plated without ROCK inhibitor it increases cell death. Stem cells do not transfect well when they are in colonies therefore they must be trypsinized prior to transfections). On the day of the transfection antibiotics were not included in the media to maximize transfection efficiency. HSF1 cells were transiently transfected using the FugeneHD® kit (Roche). The following components were added to an eppendorf tube and incubated at room temperature for 20 minutes prior to transfection. Post-transfection GFP expression was observed under the microscope to measure transfection efficiency. If 80% of the cells displayed green florescence, the cells were harvested for RNA extractions (All of the RNA extractions were performed using Invitrogen Trizol method as explained above).

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Media to a final volume</td>
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</tr>
<tr>
<td>DNA</td>
<td>2ug</td>
</tr>
<tr>
<td>Fugene HD ® Transfection reagent</td>
<td>7ul</td>
</tr>
</tbody>
</table>
**Immunoblotting**

Two days post transfection cells were washed with 1X PBS and harvested by manual cell scraping. Cells were lysed in lysis buffer (50mM Tris-HCL, pH 7.4, 150mM NaCl, 1mM EDTA and 1% Triton X-100) containing 1X protease inhibitor cocktail (Sigma). Protein concentration was measured using the Bradford assay. Protein (50 µg/lane) was resolved by 8-15% SDS-PAGE, transferred to nitrocellulose membranes, incubated for 1h with 5% milk TBS-T, then incubated overnight with primary antibodies in 5% BSA. Antibodies included OCT4 (Santa Cruz), TOM40, TIM23, GAPDH (Abcam) HIF1β (Cell Signaling) and HIF1α (Active Motif). ECL plus (G.E. Healthcare) was used for chemiluminescent detection.

<table>
<thead>
<tr>
<th>Acrylamide concentration</th>
<th>Stacking Gel (4 mL)</th>
<th>Separating gel (10 mL)</th>
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<tbody>
<tr>
<td>MW Range (kDal):</td>
<td>-</td>
<td>60-200</td>
</tr>
<tr>
<td>30% Acrylamide mix</td>
<td>-</td>
<td>60-200</td>
</tr>
<tr>
<td>(29:1 acrylamide:bis-</td>
<td>0.67 mL</td>
<td>16-70</td>
</tr>
<tr>
<td>acrylamide)</td>
<td>1.7 mL</td>
<td>14-60</td>
</tr>
<tr>
<td>1.5M Tris pH8.8</td>
<td>2.5 mL</td>
<td>12-45</td>
</tr>
<tr>
<td>1M Tris pH6.8</td>
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<td>-</td>
</tr>
<tr>
<td>H2O</td>
<td>2.4 mL</td>
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</tr>
<tr>
<td>10% SDS</td>
<td>40 µL</td>
<td>-</td>
</tr>
<tr>
<td>10% ammonium persulfate</td>
<td>30 µL</td>
<td>-</td>
</tr>
<tr>
<td>TEMED</td>
<td>3 µL</td>
<td>-</td>
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</table>
HIF1α, and ARNT knock down.

ARNT, HIF1α and scramble shRNA in the lentiviral vector of pLKO were purchased from Sigma. The shRNA against ARNT and HIF1α are

TRCN0000003819

**Target Sequence:** GAGAAGTCAGATGGTTTATTT

**Hairpin Sequence:** 5'-CCGGGAGAAGTCAGATGGTTTATTT-CTCGAG AAATAAACCATCTGACTTCTC-TTTTT-3')

and TRC0000003810

**Target Sequence:** GTGATGAAAGAATTACCGAAT

**Hairpin Sequence:** 5'-CCGG-GTGATGAAAGAATTACCGAAT-CTCGAG-ATTCGGTAATTCTTTCATCAC-TTTTT-3')

Lentivirus was made by transfecting 2x10⁶ HEK293T cells with 10 µg lentiviral vector, 6.5 µg pCMV-ΔR8.2 packaging vector, and 3.5 µg pCMV-VSV-G envelope vector using Fugene reagent (Roche). Two days later, medium was collected, filtered and virus was concentrated using the Lenti-X concentrator (Clontech). Concentrated virus was added to HSF1 followed by mixing for 2 h at 37°C in the presence of 8µg/ µl polybrene, then plating onto Matrigel Plates in Stem Pro SFM medium.

Embryoid Body formation.

Embryoid Body Formation assay was performed using Aggre well 400 plates (Stemcell Technologies) according to the manufactures instructions using ~10⁶ cells plated onto AggreWell400. Media used for this assay was EB formation medium (AggreWell™), ACCUTASE™ (provided with Kit) and DMEM/F-12 in room temperature. We supplement EB formation medium with ROCK inhibitor to a final concentration of 10uM. Stem cells that were used for this assay was rinsed cells with 2mL of DMEM/F-12. Cells were strained using 40um cell strainer (catalog #27215) to remove any clumps. Cells were then centrifuged at 300gx5min at room temperature. A viability count was
performed prior to adding cells into AggreWell™ plate and centrifuged the AggreWell™ 400 times at 100g x 3 minutes to capture the cells in microwells. Cells were then incubated at 37°C, 5% CO₂ and 95% humidity for 24 hours. Embryoid bodies were harvested after 24 h and further cultured in IMDM media supplemented with 20% FBS.

**Real-time PCR**

Real time PCR was performed to detect mRNA levels of *NANOG, OCT4, GATA2, GATA3, GATA6, and HAND1* using SYBR green real time PCR kit (Diagenode). qRT-PCR was performed using Roche LightCycler ® 480 system using the following parameters: denaturation 94°C 15s, annealing 60°C 30S, extension 72°C 45S for 40 cycles.

**Gene Primer Sequences**

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<tr>
<th>Gene</th>
<th>Upper</th>
<th>Lower</th>
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</thead>
<tbody>
<tr>
<td>Nanog</td>
<td>ttggaagctgtggggaag</td>
<td>gtgggaggagggagaga</td>
</tr>
<tr>
<td>Oct4</td>
<td>cagtcgccgaaacccac</td>
<td>ggacaccagacagcctcaaa</td>
</tr>
<tr>
<td>Gata2</td>
<td>gtcactgaagggagcatga</td>
<td>gccttgaacaggagag</td>
</tr>
<tr>
<td>Gata3</td>
<td>gtcactgaagggagcatga</td>
<td>gccttgaacaggagag</td>
</tr>
<tr>
<td>Handl</td>
<td>aaaggccctactcagcag</td>
<td>tgcccttgttaatgtc</td>
</tr>
<tr>
<td>Gata6</td>
<td>gccaactgtcacacac</td>
<td>tggagctggaatgggaat</td>
</tr>
</tbody>
</table>
**Cell Cycle Analysis**

Cells were stained in hypotonic buffer containing 0.1g/L propidium iodide, 1g/L sodium citrate, 0.3% Triton X-100, and 0.02g/L ribonuclease A for 5min, and analyzed on a BD FACS caliber flow cytometer.
Chapter 3

Results

Endogeneous HIF is required for optimal hESC developmental gene induction during early differentiation in hypoxia.

ARNT protein is constitutively expressed in cells regardless of hypoxia (5% O$_2$) or normoxia (21% O$_2$). ARNT and HIF1$\alpha$ knockdown HSF1 cells were generated by $shRNA$. In hypoxia, knockdown of ARNT is shown in Figure 3.1A. $shARNT$ cells had significantly reduced ARNT protein levels compared to control HSF1 transduced with scramble $shRNA$ cells. HIF1$\alpha$ protein is also constitutively expressed but is degraded in normoxia$^{(4)}$. HIF1$\alpha$ is not or is scarcely detectable by western blot in normoxia (See Figure 3.3A). However, in hypoxia, HIF1$\alpha$ protein is stabilized as shown in Figure 1B.

We also examined the mRNA levels of typical downstream target genes of HIF signaling pathway which includes glycolysis genes PGK1 (Phosphoglycerate kinase 1), HK 1 (Hexokinase 1) and GLUT1 (Glucose transporter 1). If the ARNT and HIF1$\alpha$ knockdown was successful a reduction of the above genes were expected because HIF1$\alpha$ transcriptionally up regulate the above genes. In Figure 3.1C both ARNT and HIF1$\alpha$ knockdown cells had reduced levels of HIF target genes PGK1 (first panel), HK1 (second panel), and GLUT1 (third panel) expression detected by mRNA levels, confirming the functional knock down of HIF1$\alpha$.

We hypothesized that HIF signaling is required to up regulate developmental genes in hypoxia. Figure 3.1D supports our hypothesis in that knocking down HIF signaling greatly reduces the mRNA levels of the developmental genes GATA2, GATA6,
MSX2, and HAND1 during early differentiation initiated by retinoic acid (RA) treatment.

In Figure 3.2D, comparison of mRNA levels at 0 days (black) not treated with retinoic acid whereas 2 Days (gray) represents post treatment with retinoic acid (Wild type – Scr, shHIF1α – HIF1α knockdown cells, shARNT – ARNT knockdown HSF1 cells). All of the developmental genes we tested showed strong induction after 2 days of RA-induced differentiation (control), however in knockdown cells, induction was significantly reduced (Fig. 3.1D, n=3, p<0.05). Interestingly, ARNT knockdown had a greater repressive effect than HIF1α (Fig. 3.1D). Notably, for GATA2, GATA6 and HAND1, low amounts of ARNT completely blocked induction of developmental genes at the 2nd day of differentiation (Fig. 3.1A).

We also tested for two demethylases, JMJD3 and JMJD1A demethylases that removes methyl groups from histone lysine 27 and lysine 9 residues. JMJD3 (Figure 3.1E left panel) and JMJD1A (Figure 3.1E right panel) levels in shARNT and shHIF1α cells. In normal cells JMJD3 and JMJD1A are up regulated only when cells are differentiating. In HSF1 undifferentiated cells these genes are not expressed (data not shown). When ARNT and HIF1α knockdown cells were induced for differentiation with retinoic acid for 2 days, Scr (control) showed higher levels of JMJD3 and JMJD1A mRNA compared to shARNT and shHIF1α. To determine whether knocking down ARNT and HIF1α would affect global methylation levels, we performed a western blot analysis on whole cell protein lysates collected from shARNT and shHIF1α cells RA treated for 2 days. Strikingly, knocking down HIF1α showed increased amounts of H3K9me3 (Figure 3.1F top panel) and H3K27me3(Figure 3.1F bottom panel) levels compared to the scramble d shRNA as expected.
Figure 3.1

A

<table>
<thead>
<tr>
<th></th>
<th>scr</th>
<th>shARNT</th>
</tr>
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<tr>
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B

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<td>GAPDH</td>
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</table>

C

C

D

D

E

E

F

F
Figure 3.1 Endogeneous HIF is required for optimal hESC developmental gene induction during early differentiation in hypoxia.

(A, B) Western blot of shARNT and shHIF1α in HSF1 cells in hypoxia (5% oxygen). (C) mRNA levels of HIF target genes PGK1 (left panel), HK1 (middle panel) and GLUT1 (right panel) in RNAi-mediated scramble (control), shARNT and shHIF1α knock down HSF1 in hypoxia. (D) mRNA levels of developmental genes GATA2 (top left panel), GATA6 (top right panel), MSX2 (bottom left panel), and HAND1 (bottom right panel), genes in RNAi-mediated scramble (control), shARNT and shHIF1α knock down HSF1 in hypoxia. (E) mRNA levels of JMJD3 (left panel) and JMJD1A (right panel) genes in RNAi-mediated scramble (control), shARNT and shHIF1α knock down HSF1 in hypoxia. (F) Western blot showing global H3K9 (top panel) and H3K27 (bottom panel) tri methylation in scramble (control) and HIF1α knock down cells in hypoxia. Tubulin is shown as a loading control. (C-E: mRNA levels were detected by real time PCR and Mean SD, n=3, p<0.05). Relative mRNA levels were normalized to housekeeping gene 36B4.
Ectopic expression of stabilized HIF1α in normoxia augments developmental gene expression during early differentiation.

If HIF signaling is required for efficient early differentiation of HSF1 in hypoxia, then ectopic expression of HIFs in normoxia should accelerate differentiation. To test this hypothesis, a double mutant HIF1α that will not undergo degradation in normoxia was engineered. To stabilize HIF1α in HSF1 cells, site-directed mutagenesis was employed to change proline 402 and 564 to alanines (Figure 3.2A). These alanine substitutions prevent ubiquitination and subsequent proteosome-dependent degradation of HIF1α(12). The double mutant HIF1α is resistant to degradation in HSF1 cells in normoxic conditions as seen by western blot in Figure 2B. To test the transcriptional functionality of this double mutant, downstream target gene COX4-2 mRNA levels were detected by real time PCR (Figure 3.2C). Compared to day 0, day 2 expressed more COX4-2 in the presence of over expressed HIF1α. Further examination of the same developmental genes GATA2, GATA6, MSX2, and HAND1 showed that ectopic-expression of HIF1α also increased expression of the developmental genes (Figure 3.2D, panels). We also looked at the expression of histone 3 lysine 27 modifier gene, JMJD3, that increases during cell differentiation. Stabilization of HIF1α only increased JMJD3 at day 2 and day 4 of differentiation (Figure 3.2E).
Figure 3.2

A

[Diagram showing genetic elements: EF1 promoter, HIF1α, IRES, hrGFP, 3xNLS]

B

[Images of protein gels labeled EV and HIF1α* showing HIF1α and TUBULIN]

C

[Bar graphs showing relative COX4-2 expression for EV and HIF1α* across RA Day 0 and RA Day 2]

D

[Bar graphs showing relative GATA2 expression for EV and HIF1α* across RA Day 0 and RA Day 4]

E

[Bar graphs showing relative MSX2 and HAND1 expression for EV and HIF1α* across RA Day 0 and RA Day 4]
Figure 3.2 Ectopic expression of stabilized HIF1α in normoxia augments developmental gene expression during early differentiation.

(A) Schematic of double mutant HIF1α created by site directed mutagenesis. Prolines 402 and 564 were changed to alanines. (B) Over expression of stabilized HIF1α double mutant in HSF1 cells in normoxia (21% oxygen) shown by western blot. Tubulin is shown as a loading control. (C) HIF1α target gene COX4-2 expression revealed by mRNA levels detected using real time PCR. (D) Differentiation gene expression revealed by mRNA levels detected using real time PCR GATA2 (top left panel), GATA6 (top right panel), MSX2 (bottom left panel) and HAND1 (bottom right panel). (E) JMJ3 expression shown by real time PCR with RA induced differentiation. (C-E : mRNA levels were detected by real time PCR and Mean ±SD, n=3, p<0.05)
Endogeneous HIF is required for optimal induction of developmental genes during early differentiation as assayed by EB formation

HIF1α protein is constitutively expressed and degraded in normoxia. When the oxygen concentration is above 5%, prolyl hydroxylases (Phd1–3) hydroxylate two proline residues (402 and 564) of HIF1α enabling the binding of the von Hippel-Lindau tumor suppressor protein (Vhl) to HIF1α. Vhl is the recognition component of an E3 ubiquitin-protein ligase that targets HIF1α for ubiquitylation and proteasomal degradation. Here we show scarce levels of HIF1α protein in normoxia compared to hypoxia (Figure 3.3A). Usually hESCs are grown at ~80% confluence to obtain sufficient cells for analysis and we predict this could create local hypoxic regions allowing HIF1α to be stable. To test this we cultured HIF1α and ARNT knockdown cells in normoxia and detected reduced expression of HIF target genes HK1, PGK1, and GLUT1 expression (Figure 3C, 1st panel, 2nd panel, and 3rd panel). Embryonic stem cells (ESCs) display a very unusual cell cycle structure, in that they enter a short G1 phase and a long S-phase marked for proliferation. To discern if the knockdown cells cultured in normoxia were differentiating, possibly caused by local hypoxic regions, we performed a viability test and counted for cells that were in S phase by propidium iodide staining. These cells had a suppressed cell cycle as indicated by S phase accumulation (Figure 3.3D) without increasing in cell death (Figure 3.3D and E). When the cells were induced for differentiation with retinoic acid as in our hypoxic experiment, normoxic knockdown showed reduced levels of developmental genes GATA2, HAND1 and HOX B3 (Figure 3.3F, top left panel, top right panel and the bottom right panel). Combined, our data shows that HIF is required for the early
induction of differentiation genes, even when hESCs are cultured in routine normoxic conditions.
Figure

3.3

A

Hyp.

Nor. 1h 3h 8h 24h 72h

HIF1α

ACTIN

B

Rel. HIF1α mRNA Exp.

scrmRNA Exp.

shHIF1α

shARNT

C

Rel. HIF1 Exp. Rel. PK1 Exp. Rel. GLUT1 Exp.

scr shHIF1α shARNT shHIF1α shARNT shHIF1α shARNT

D

% S phase

scr shHIF1α shARNT shHIF1α shARNT shHIF1α shARNT

F

Rel. GATA2 Exp. Rel. HAND1 Exp.

scr shHIF1α shARNT shHIF1α shARNT shHIF1α shARNT

E

% Viability Rel. HOX83 Exp.

scr shHIF1α shARNT shHIF1α shARNT shHIF1α shARNT

scr shHIF1α shARNT

shHIF1α

shARNT

Day 0

Day 2

Day 3

Day 4
Figure 3.3 Endogeneous HIF is required for optimal induction of developmental gene during early differentiation assayed by EB formation.

(A) HIF1α protein in normoxia and hypoxia shown by western blot. (B) mRNA expression of HIF1α in scramble (control) and knock down HIF1α. (C) Expression of HIF1α target genes in normoxia of shARNT and shHIF1α knockdown HSF1 cells (HK1 – first panel, PGK1-second panel and HK1- third panel in the figure). (D) Percentage of HSF1 in S phase of the cell cycle in normoxia, with and without HIF or ARNT knockdown. (E) Percentage of viable HSF1 determined by propidium iodide staining and FACs analysis in normoxia. (F) mRNA expression of developmental genes GATA2, HAND1 and MSX2 from HSF1 in normoxia with and without HIF knockdown induced to differentiation with retinoic acid (GATA2 – Top left, HAND1-top right and MSX2 bottom left panel) (F: mRNA levels were detected by real time PCR and Mean ±SD, n=3, p<0.05).
**HIF is required for proper EB formation and trophectoderm gene expression**

To understand the role of HIF in differentiation we performed embryoid body formation assay using *shHIF1α* and *shARNT* knockdown HSF1 cells. In hypoxia, *shHIF1α* and *shARNT* knockdown cells showed smaller and abnormally-shaped EBs with distorted trophectoderm at 3 days post formation of EBs, compared to control HSF1 (Figure 3.4A). *HAND1* gene is currently being used as a universal marker for trophectoderm\(^{(51)}\). When we tested for the *HAND1* mRNA levels in the EBs, *shHIF1α* and *shARNT* knockdown cells showed significantly reduced *HAND1* levels compared to control (Figure 3.4B). Strikingly, HIF1α and ARNT knockdown cells in normoxic condition also developed abnormal embryoid bodies starting from day 5 (Fig. 3.4C). Combined, this data revealed a critical role of HIF1 signaling in EB formation, and early differentiation in a range of oxygen contents from 5% to 21%.
Figure 3.4

A  

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<td></td>
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<td>20x</td>
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</tr>
<tr>
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Normoxia

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Hypoxia
Figure 3.4 HIF is required for proper EB formation and trophectoderm gene expression.

(A) EB formation of scramble (control) -1st column, shHIF1α - 2nd column, and shARNT -3rd column in HSF1 cells in normoxia (21% O2) for 5 days. (B) EB formation of scramble (control), shHIF1α, and shARNT in HSF1 cells in normoxia hypoxia (5% oxygen) for 3 days. (C) mRNA expression of trophectoderm differentiation gene HAND1 in scramble and HIF knock down HSF1 cells. (C): mRNA levels were detected by real time PCR and Mean SD, n=3, p<0.05)
Chapter 4.

Discussion

Human embryonic stem cells are derived from an inner cell mass of a blastocyst that survives a hypoxic environment\(^{(31)}\). Therefore, theoretically, hypoxic conditions are more physiological to the growth of human embryonic stem cells. Many studies have addressed the role of hypoxia in promoting pluripotency. To date, hypoxia-inducible factor 2α (HIF2α) and HIF3α have been shown to transcriptionally up regulate the universal pluripotency marker \(OCT4\) in hESCs\(^{(27)}\). These findings are consistent with HIF2α activating \(OCT4\) expression in mouse ESCs\(^{(33)}\). Since HIF expression favors self-renewal, it might be expected that HIF expression would also inhibit hESC differentiation, although this idea has not been tested. Studies have shown that hypoxia enhances embryoid body (EB) formation and endothelial and cardiomyocyte differentiation\(^{(29, 34-36)}\). \(Hif1a\), \(Hif2a\), and \(Arnt\) knockout mice that are deficient in HIF activity are impaired in placental development\(^{(37)}\), heart development \(^{(28-29)}\), and endochondrial bone formation during early embryogenesis \(^{(30-31)}\). However, mechanisms responsible for many of the complex morphogenetic changes remain unclear. In early human development, the role of hypoxia and HIF transactivation on differentiation has not been established. To begin addressing this issue, we specifically evaluated HIF transactivation in early hESC differentiation using loss and gain-of-function approaches.
We report that HIF is required for induction of germ layer and trophoblast genes, and that ectopic HIF1α expression, even though insufficient to initiate spontaneous differentiation, promotes induced differentiation. Our studies reveal that HIFs could have dual roles in preventing the differentiation of self-renewing hESCs and in stimulating purposeful differentiation of induced hESCs.

**Endogenous HIF signaling is required for induction of developmental genes at early differentiation in hypoxia**

HIF1α stimulates glycolytic energy production by transactivating genes involved in extracellular glucose import such as GLUT1 and enzymes responsible for glycolytic breakdown of glycolysis such as PGK1 and HK1\(^{(20)}\). Even when hESCs grow in normoxic conditions they require high glucose uptake to produce ATP via glycolysis\(^{(12)}\). Data from our first set of experiments (Figure 1) shows that knocking down HIF signaling via shARNT and shHIF1α decreases the levels of the glycolysis genes PGK1, HK1 and GLUT1 in hypoxia. This data supports the already accepted notion that HIF1α is required for human embryonic stem cell survival in hypoxia\(^{(20)}\). Furthermore, we also show a reduction in the developmental genes GATA2, GATA6, MSX2 and HAND1 using the same knockdown cell lines in hypoxia. GATA2, GATA6, MSX2 and HAND1 are genes that are turned on during early differentiation process and marks the germ layers mesoderm, endoderm, ectoderm, and trophectoderm. Knockdown cells shARNT and shHIF1α induced for differentiation with RA showed a reduction of all four genes indicating that hypoxic differentiation requires HIF signaling respectively. Knocking down HIF signaling also reduces the demethylases JMJD3 and JMJD1A. These
demethylases are required to remove methyl groups that transcriptionally silence developmental genes *GATA2*, *GATA6*, *MSX2* and *HAND1*. Knocking down HIF signaling also increased the levels of H3K9me3 and the amounts of H3K27me3\(^{(21)}\). Combined, these data suggest a novel role of HIF signaling in hypoxia where endogenous HIF signaling is required for induction of the demethylases *JMJD3/JMJD1A* which consequently activate developmental genes *GATA2*, *GATA6*, *MSX2* and *HAND1* during early differentiation.

**Ectopic expression of non-degradable HIF1α in normoxia promotes induction of developmental genes**

Since knocking down HIF signaling reduces differentiation gene expression and demethylase expression we hypothesised that over expressing HIF1α would increase the levels of differentiation and demethylase gene expression. To test this hypothesis we created a non-degradable double mutant of HIF1α. As described earlier, HIF1αP402P564 protein is constitutively on and is degraded in normoxia\(^{(8\text{-}10)}\). HIFs are subjected to proteosomal degradation by ubiquitinase, a prerequisite for von Hippel-Lindau (Pvhl) E3 ubiquitin ligase complex which interact with hydroxylated proteins\(^{(8\text{-}10)}\). To test whether over expression of HIF1α increases the levels of differentiation and demethylase gene expression in normoxia, we created a double mutant HIF1α. This double mutant contained two alanines at residues 402 and 564 instead of prolines that would normally undergo degradation in normoxia. As anticipated from our knock-down studies in hypoxia (Figure 3.1), augmentation of the differentiation genes *GATA2*, *GATA6*, *MSX2*, and *HAND1* in normoxia.
Over the past several years, particular interest in identifying the transcriptional circuitry of pluripotent cells with specific interest in gene regulation by chromatin modifications has occurred. Histone methylation at lysine tails of distinct core histones H3 and H4 plays a dominant role in human ES gene transcription\(^{(19)}\). Promoters of developmentally regulated genes are repressed at the pluripotent state as they are enriched with Histone H3 lysine 27 tri-methylation (H3K27me3)\(^{(19,24)}\). The demethylase JMJD3 catalyzes the removal of methyl groups on H3K27 is observed by days 2 and 4 of RA induced differentiation with concomitant over expression of HIF1\(\alpha\) compared to HSF1 cells without treatment. Together this shows that when differentiation is induced with RA, HIF signaling augments the differentiation genes \textit{GATA2, GATA6, MSX2, HAND1} and the demethylase \textit{JMJD3}. However, HIF alone is insufficient to initiate differentiation. For a long time people believed that HIF signaling was limited to hypoxia and HIF1\(\alpha\) did not have a role in normoxia. This work demonstrates that HIF1\(\alpha\) plays a critical role in early differentiation in normoxia as well as hypoxic condition.

**Baseline level of HIF signaling exists in normoxia and is required for induction of developmental genes**

Although HIF1\(\alpha\) is continuously degraded in normoxia\(^{(9)}\), low levels of this protein were detected by western blot (Figure 3.3A) in normoxia in our study. Reduced HIF signaling via \textit{shARNT} and \textit{shHIF1\(\alpha\)} in normoxia also decreased the levels of glycolysis genes \textit{HK1, PGK1 and GLUT1} (Figure 3.3C). Results suggest that basal levels of HIF1\(\alpha\) are required to up regulate metabolic genes. The cell cycle consists of four distinct phases for replication and transmission of genetic material. Synthesis (S) and
mitosis (M) are responsible for chromosome replication and chromosome transmission. Gap 1 (G1) and gap 2 (G2) phases separate the S and M phases and include checkpoints to ensure cells are ready to divide. Unlike differentiated cells, human embryonic stem cells lack a fully formed cell cycle. That is, they lack G1 and G2 phases or they contain very short G1 and G2 phases\(^{(45)}\). ES cells spend 60% of their cell cycle time in S phase, a well known mark of cells in a proliferative state\(^{(45)}\). Interestingly, \(shARNT\) and \(shHIF1\alpha\) cells showed suppressed cell cycle levels indicated by reduced S phase duration, but cell viability was 90% or higher (Figure 3.3D). Reduced duration of the cell cycle by reduction of S phase is indicative of cell death or cell differentiation\(^{(45)}\). We induced knockdown cells for differentiation in normoxia and results demonstrated reduced expression levels \(GATA2\), \(HAND1\) and \(HOXB3\) (Figure 3.3F), suggesting that HIF is required for early induction of differentiation even when cells are cultured in normoxia. When combined, these data suggest that hESCs require HIF signaling to maintain their proliferative state as well as induce differentiation, and additionally that HIF signaling is not limited to hypoxia.

**HIF is required for EB and Trophectoderm formation in hypoxia and normoxia.**

Human embryonic stem cells are derived from a blastocyst that survives in a hypoxic (5% oxygen) in the female reproductive tract\(^{(9-10)}\). A differentiated cell type called the trophectoderm surrounds the blastocyst at four days post fertilization, and is distinct from the inner cell mass \(^{(32)}\). Development of the trophectoderm is the earliest lineage commitment or cellular differentiation event of a premplantation embryo. Trophectodermal cells are the progenitors of the trophoblast cells\(^{(31-33)}\), which establish
the connection between the placenta villi for oxygen and other nutrient exchange between
the mother and the embryo. We assessed embryoid body (EB) formation as a measure of
testing for differentiation potential of our knockdown HSF1 cell lines. In hypoxia and
normoxia, shHIF1α and shARNT knockdown cells showed smaller and abnormally
shaped EBs (Figure A and B). These EBs also had a distorted trophectoderm compared to
the HSF1 control cells. To confirm our observation that defective trophectoderm
formation was due to reduced HIF signaling, we measured the mRNA levels of the
trophectoderm marker gene HAND1. A reduced HAND1 expression level combined with
the defective EB morphology in the absence of HIF suggests a critical role of HIFs in
early differentiation.

In conclusion, our focused study shows that HIF promotes hESCs differentiation
once differentiation is induced. When HIF signaling is reduced, differentiation gene
induction is also reduced. On the other hand, when HIF signaling is increased,
differentiation gene induction is augmented. Attenuation of HIF signals disrupts EB
formation with a reduction of the trophectoderm specific gene HAND1. When
considering the predominantly accepted notion that HIF2α up regulates OCT4 in
hypoxia, our results appear paradoxical\(^{(26-27)}\). However, this contradiction can be resolved
by taking into account the chromatin states of pluripotent and developmental genes
before and after the induction of differentiation\(^{(38-42)}\). In undifferentiated hESCs, the
OCT4 promoter has an open chromatin configuration marked by hyper H3K4me3.
Differentiation marker gene promoters in hESCs cells are marked by both active
H3K4me3 and repressive H3K27me3 (bivalent domain) histone modifications and are
poised for expression\textsuperscript{(38)}. Therefore, it seems reasonable to infer that the predominant hypoxic effect is on HIF2\(\alpha\) induction of OCT4 expression at the pluripotent state (prior to differentiation). When the cells have been induced for differentiation, the OCT4 promoter shows more H3K27me3 repressive chromatin modifications, whereas differentiation genes show increased active chromatin marked by H3K4me3, and these genes become accessible to further transcriptional activation from HIF and from HIF inducible chromatin modifications, as provided by JMJD3 and JMJD1A\textsuperscript{(19,43-44)}. Our studies show the regulatory function of HIF on these chromatin modifiers in hESCs (Figure 1E). We have revealed that HIF1\(\alpha\) changes the global H3K27me3 and H3K9me3 status (Figure 1F), pointing to an unexplored role for HIF regulation of hESC differentiation through epigenetic modifications.

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