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FACULTY OF MEDICINE

Centre for Human Development, Stem Cells and Regeneration

Role of environmental oxygen in the regulation of human embryonic stem cells

by

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To Mario

The love of my life

Abstract

Human embryonic stem (hES) cells derived from the inner cell mass of the blastocyst propagate by self-renewal and can give rise to all cells of the body. However, they have a tendency to spontaneously differentiate *in vitro*, an effect which can be abrogated by culture at low, 5% oxygen tensions. This response is mediated by hypoxia inducible factors (HIFs) and in particular HIF-2α. However, the mechanism of this regulation is still unknown. Chromatin immunoprecipitation (ChIP) analysis showed that HIF-2α directly binds to a predicted hypoxia response element (HRE) in the proximal promoter of OCT4, NANOG, SOX2, GLUT1 and eNOS under hypoxic conditions. An increased level of enrichment ($P<0.01$) was observed in hES cells cultured at 5% oxygen whereas no significant binding was observed in cells maintained at 20% oxygen. Interestingly, HIF-2α induced an array of histone modifications that are associated with gene transcription within the predicted HRE site for all the genes analysed. ChIP assays showed that the chromatin state is more accessible and transcriptionally active in hES cells cultured under hypoxic conditions. In contrast, a heterochromatin state exists in the HRE of OCT4, SOX2, NANOG, GLUT1 and eNOS in hES cells cultured under 20% oxygen tension. This was also confirmed using pyrosequence analysis which revealed a significant hypomethylation pattern ($P<0.01$) in the HRE site within the OCT4 proximal promoter in hES cells cultured at 5% oxygen compared to those maintained at 20% oxygen. hES cells were then used as a model to investigate the mechanisms acquired by resident stem cell populations to maintain an undifferentiate and proliferative state at the site of injury which are characterized by hypoxia and oxidative stress. Interestingly, an enhanced euchromatic state was found when hES cells were exposed to hypoxia/reoxygenation for 72 hours. Surprisingly, this was sustained by HIF-2α which was found significantly enriched within the HRE of all core pluripotency genes but particularly within the NANOG gene promoter. Furthermore, HIF-2α was found responsible of the establishment of a multiprotein complex thereby allowing interaction with an oct-sox *cis*-regulatory element in the NANOG promoter and sustaining self-renewal. Thus, these data have uncovered a novel role of HIF-2α as a direct regulator of key transcription factors controlling self-renewal and epigenetic modifications which enhances the regenerative potential of hES cells exposed to hypoxia and reoxygenation.

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Declaration Of Authorship

I, Raffaella Petruzzelli,

declare that this thesis entitled:

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Abbreviations

ACF: chromatin-assembly factor

ADA-SCID: Adenosine Deaminase Deficiency-related severe combined immunodeficiency

AKT: protein Kinase

ALS: Amyotrophic Lateral Sclerosis

AP1: Activator Protein 1

ARNT: Aryl hydrocarbon Receptor Nuclear Translocator

ASCs: Adult SCs

ATF1/CREB: Activating Transcription Factor 1/ cAMPResponse Element Binding

ATP: Adenosine Triphosphate

BAF: Brg/Brahama-associated factors

b-FGF: basic fibroblast growth factor

bHLH: beta helix-loop-helix

Bcl-2: B cell lymphoma 2

Bcl-X: B cell extra-large lymphoma

BM: Bone Marrow

BMP4: bone morphogenetic protein

BNPI3: BCL2/adenovirus

BRG1: Brahma related gene 1

BSA: Bovine Serum Albumin

CBP: CREB-binding protein

CcO: Cytocrome c Oxidase

CERF: CECR2(cat eye syndrome critical region protein)-containing remodelling factor

CHD 1-9: tandem chromodomain of human family of ATPase

cGMP: Cyclic guanosine monophosphate

ChIP: chromatin Immunoprecipitation Assay

CHRAC: chromatin accessibility complex

Cdc 53/34: Cell division cycle 53/34

cDNA: complementary DNA

cMyc: myelocytomatosis viral homologue oncogene

CpG: C-phosphate-G

CR 1-4: Conserved Region number 1 to 4

CREB-1: cAMP response element-binding protein 1

CSP: cardiac stem progenitor cells

CTCF: 11-zinc finger protein or CCCTC-binding factor

Cul-2: Cullin 2

CVD: cardiovascular disease

CX3CR1-7: C-X-3C chemokine receptor 1 to 7

3C: Chromosome Conformation Capture

4C: Circular Chromosome Conformation Capture

Dax1: dosage-sensitive sex reversal, adrenal hypoplasia critical region, on chromosome X, gene 1

DEPC: diethyl dicarbonate

ddCt: delta delta cycle threshold

DMSO: Dimethyl sulfoxide

DNMT3A, B, 1: DNA methyltransferases A, B, 1

DS trysomy 21: Down Syndrome

DTT: Dithiothreitol

E3: ubiquitin ligase

EC cells: Embryonal Carcinoma cells

EDTA: Ethylenediaminetetraacetic acid

EG cells: Embryonic Germ cells

EHS: Engelbreth-Holm-Swarm mouse sarcoma

eNOS: endothelial Nitric Oxide Synthase

EPAS1: Endothelial PAS domain 1

EPCs : endothelial progenitor cells

EpISCs : epiblast SCs

EPO : erythropoietin

epSPC cells: ependymal progenitor SCs

ERK : Extracellular signal-regulated kinases

ES cells : embryonic SCs

esBAF: embryonic SCs Brg/Brahama-associated factors

ESR1-2 : estrogen receptor 1-2

Esrrb : Estrogen related receptor beta

FGF4-5: Fibroblast Growth Factor 4 and 5

FIH: Factor Inhibiting HIF

FOXP3: Forkhead Box P3

GATA6: transcription factor that bind the sequence GATA

GCMT-2: GDP Mannose Transporters 2

GFP: Green Fluorescent Protein

GLUT1/3: Glucose Transporters 1/3

GSK3β: glycogen synthase kinase 3 beta

G9a: histone-lysine 9 N-methyltransferase

HDAC1-7: Histone Deacetylase Activity 1 to 7

HDMs: histone demethylases

H_2DCFDA: 2', 7'-dichlorodihidrofluorescin diacetate

HeLa: Henrietta Lacks cells

hES cells/ hESC: human Embryonic SCs

HEK-293: human embryonic kidney cells

HIFs: Hypoxia Inducible Factors

HIF-1, 2, 3 α: Hypoxia Inducible factors 1, 2, 3 alpha

HMEC: human dermal microvascular endothelial cells

HMT transferase: histone lysine methyltranspherase

HKF4: Hepatic nuclear Factor 4

HRE: Hypoxia Responsive Elements

H1/3: histone 1/3

H3Ac: histone 3 acetylated

HAT: histone acetyltransferase

H2AZ: histone 2AZ

H3K4me2/3: histone 3 bi/tri methylated at Lysine (K) 4 residue

H3K9me2/3: histone 3 bi/tri methylated at Lysine (K) 9 residue

H3K27me2/3: histone 3 bi/tri methylated at Lysine (K) 27 residue

H3K36me2/3: histone 3 bi/tri methylated at Lysine (K) 36 residue

HMEC: Primary Human Mammalian Epithelial cells

HMG: high mobility group

HO-1: heme oxygenase

HPC: hypoxia preconditioning

HPLC: High-performance liquid chromatography

HS: human serum

HUVEC: Primary Human Umbilical Vein and Endothelial cells

ICM: Inner Cell Mass

ID: Inhibitory Domain

IGF: Insulin growth factor

IL-8: interleukin 8

iNOS: inducible Nitric Oxide Synthase

IPC: ischaemic preconditioning

iPS: induced pluripotent SCs

ISWI: Imitation Switch protein

IVS: in vitro fertilization

JARID2/1A: Jumonji /ARID domain containing protein 2/ 1A

JHDM: Jumonji histone demethylases

JmjC/d: Jumonji contain histone demethylases

JDM: Juvenile-onset type 1 Diabetes Mellitus disease

JMJD1A: JmjC histone demethylases protein 1

K_{ATP} Channel: Potassium channel

KDR: kinase insert domain receptor

KI: Knock-In

KLF4: Kruppel-like factor 4

Laminin-511: Laminin protein that contains an α5-chain, a β1-chain and a γ1 chain

LIF-STAT3: leukemia inhibitory factor-signal transducer and activator of transcription 3 signalling

lncRNA: long non-coding RNA

LSD1: Lysine-specific demethylase-1

MAT2A: Methionine adenosyltransferase 2 A

MAPK: Mitogen-Activated Protein Kinase

MBD3: methyl CpG binding domain proteins 3

MCM: minichromosome maintenance

Mdm2: (ES ubiquitin-ligase): Murine double minute 2

MEFs: Mouse Embryonic Fibroblasts cells

Mi-2: CHD chromodomain elicase DNA binding protein 2

miRNA : micro-RNA

mitoK$_{ATP}$: Mitochondrial adenosine triphosphate-dependent potassium channels

MMLV : murine leukemia viruses

MSCs : mesenchymal SCs

MTA1,2,3: metastasis-associated proteins 1,2,3

Nac1 : Nucleus accumbens-associated protein 1

NaOAc : Sodium acetate

NCX-1 : sodium calcium exchangers-1

NADPH: Nicotinamide Adenine Dinucleotide Phosphate

NANOG : Tir nan Og land of the ever-young

nNOS: neuronal Nitric Oxide Synthase

NODE: Nanog and Oct4 associated deacetylase

NOS: nitric oxide synthase

NoRC: nucleolar remodelling complex

NP40: nonyl phenoxypolyethoxylethanol

NTAD: N-terminal activation domain

NT2: Human neuron-committed teratocarcinoma

NuRD: Nucleosome Remodeling Deacetylase

NURF: nucleosome remodeling factor

OCT4: Octamer Binding Transcription factor 4

ODD: Oxygen-Dependent Degradation Domain

PAS: **Per** – period circadian protein; **Arnt** – aryl hydrocarbon receptor nuclear translocator protein; **Sim** – single-minded protein

PBS: Phosphate buffered saline

PcG: Polycomb Group

PD: Parkinson disease

PHD: Prolyl-4-Hydroxylase proteins

PIPES: piperazine-N,N′-bis(2-ethanesulfonic acid)

PgK: phosphoglycerate kinase

PGKI: protein kinase, cGMP-dependent, type I

PI3K: Phosphatidylinositol 3 kinase

PKCε: protein kinase C epsilon

Pol II: DNA polymerase II

Pou5f: POU domain, class 5, transcription factor 1

PRC2: Polycomb Repressive Complex 2

P53: tumor protein 53

P300/CBP: E1A binding protein 300 / cAMP response element Binding Protein

RbAp 46/48: Retinoblastoma associated protein 46/48

RbX 1: Ring box protein 1

RCC: Renal Clear Carcinoma

RDS: Respiratory Distress Syndrome

Rex1: reduced expression-1, also known as Zfp42

RFP: Red Fluorescent Protein

RIPA: radioimmunoprecipitation assay buffer

RIPC: remote ischemic preconditioning

RNasin: Ribonuclease Inhibitor

RT-qPCR: Real Time quantitative polymerase chain reaction

ROS: Reactive Oxygen Species

Sall4: Sal-like protein 4

SCA1: stem cell antigen 1

SCF: Skp, Cullin, F-box containing complex

SCs: SCs

SCNT: Somatic nuclear transfer

SDS: sodium dodecyl sulfate

S.E.M: Standard Error Mean

SetDB: histone-lysine N-methyltransferase

SET-domain: Site- and state-specific lysine methylation of histones

siRNA: small interference RNA

SKp1: S-phase Kinase associated protein 1

SMAD 2/3: Mothers against decapentaplegic homolog 2/3

SNF2H/2L: human family of the SWI/SNF superfamily

SNAP: S-nitroso-N-acetyl-penicillamine

SNOs: NO donors or S-nitrosothiols

SNOAC: S-nitroso-N-acetylcysteine

SOX2: SRY (Sex determining Region Y)-box 2

SRC-1: steroid receptor co-activator 1

SRR1-SRR2: SOX regulatory region 1 and 2

SSEA-1, 3, 4: Stage-Specific Embryonic Antigen 1,3,4

SUV39h: histone-lysine N-methyltransferase

SWI/SNF: SWItch/Sucrose Non Fermentable

TAD: Transactivation Domain

TAD: topological association domain

Taq Polymerase: Thermus aquaticus Polymerase

Tcfcp2l1: transcription factor CP2-like 1

Tcf4: T cell factor 4

TEMED: Tetramethylethylenediamine

TGFα: Transforming Growth factor alpha

TGFβ1: transforming growth factor beta 1

TIF2: transcriptional intermediary factor 2

Tip60-p400: histone acetyltransferase/histone exchange complex

TRA: Trafalgar

Tris base: tris(hydroxymethyl)aminomethane

TrxG: Thritorax Group

TSA: Tricostatin A

UBC: Ubiquitin C

UHRF1: Ubiquitin-like containing PHD and ring finger domain 1

UTF: Undifferentiate Transcription Factor

VEGF: Vascular Endothelial Growth Factor

VSMCs: vascular smooth muscle cells

VHL/pVHL: Von Hippel-Lindau / Von Hippel-Lindau protein

WICH: WSTF (Williams Syndrome Transcription Factor Protein) ISWI chromatin remodelling

XCI: X chromosome inactivation

Zfp281: zinc finger protein 281

Chapter 1

Introduction

1.1 Stem Cells

Stem Cells (SCs) are unspecialized cells that are able to proliferate by self-renewal and differentiate into one or more cell types. Traditionally, SCs are classified into different groups based on their differentiation potential or potency (Behr et al., 2010) (Figure 1.1):

- totipotent SCs, such as the zygote have the ability to differentiate into embryonic and extraembryonic cell types and thus are able to develop into the entire organism
- pluripotent SCs, such as embryonic stem cells (ES cells) have the potential to differentiate into all the three germ layers: endoderm, mesoderm and ectoderm.
- multipotent SCs are more lineage restricted than pluripotent SCs. Multipotent SCs are able to differentiate into any cell of a specific lineage but not into cells of other lineages. An example of a multipotent SC is the haematopoietic SC which can give rise to all blood cells.
- unipotent SCs are the most lineage restricted being able to produce only one type of cell. An example of a unipotent SC is the spermatogonial SC.

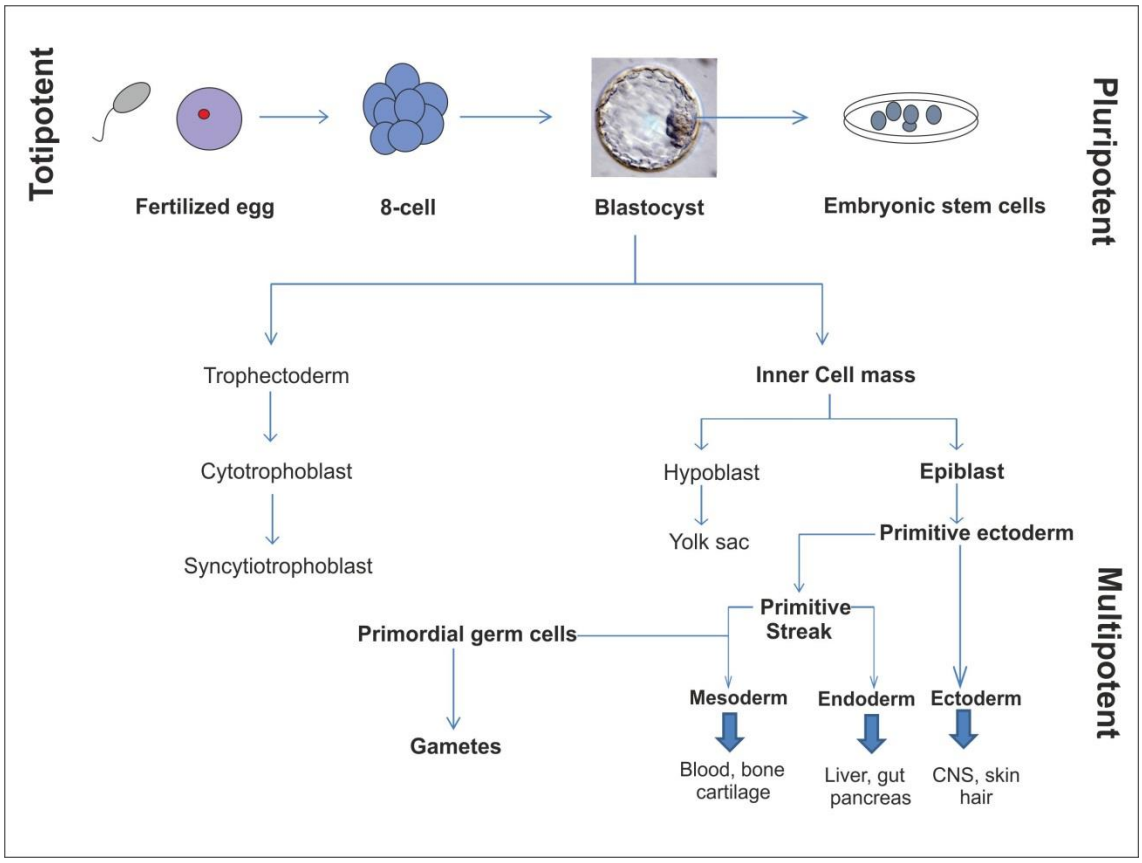

Figure 1.1 Stem Cell potency

Schematic representation of the stem cell lineage potential. Adapted from Pauwelyn and Verfaille et al. (2006).

1.1.1 Pluripotent SCs

Pluripotency is the ability of cells to self-renew and differentiate into all the cells of the body. The property of "self-renewal" means that it is possible to maintain an unlimited number of genetically identical SCs in culture (Thomson and Marshall, 1998). Due to this capacity, pluripotent SCs provide an excellent model to investigate early human development, to study genetic disease and as an *in vitro* system for toxicology testing. Furthermore, hES cells have the potential to treat degenerative disorders such as Alzhaimer's disease and Type 1 diabetes.

Different types of pluripotent SCs have been characterized: embryonic carcinoma cells, embryonic germ cells and induced pluripotent SCs.

1.1.1.2 Embryonic carcinoma (EC) cells

Mouse embryonic carcinoma (EC) cells are derived from gonadal tumours, teratocarcinomas and are considered the malignant counterpart of pluripotent ES cells (Martin, 1981; Thomson et al., 1998). Teratocarcinomas are tumours that arise from the gonads of inbred strains and are characterized by the presence of pluripotent cells as well as of cells differentiated from all the three germ layers (endoderm, mesoderm and ectoderm) (Martin and Evans, 1975). Mouse EC cells show similarities in morphology and developmental capacity to SCs, however, they are usually aneuploid and thus often possess genetic mutations and abnormal karyotypes (Martin, 1981). For this reason it is difficult to differentiate these cells into a specific cell type. Nevertheless, the use of mouse EC lines like P19, provided insight in the understanding of embryonic development and differentiation of ES cells (McBurney and Rogers, 1982). Numerous human EC cell lines have now been established with the aim of investigating mechanisms of human embryonic cell differentiation. Among the human EC lines, NTERA2 cells is one of the most commonly used (Andrews et al., 1984b; Damjanov et al., 1994; Pera et al., 1989).

1.1.1.3 Embryonic germ cells (EG cells)

Human Embryonic germ (EG) cells are derived from primordial germ cells (PGCs) isolated from the gonadal ridge and mesenteries of 5-9 week post-fertilization human embryos (Shamblott et al., 1998). These cells are precursors of gametes (sperm and egg) which retain the ability to generate pluripotent SCs. Human EG cells share some characteristics in common with mouse ES and EG cells such as the property of unlimited self-renewal and the ability to differentiate into all three germ layers (Matsui et al., 1992; Resnick et al., 1992; Shamblott et al., 1998). Unlike the EC cells, EG cells maintain a more stable karyotype and undergo epigenetic modifications such as genome demethylation and X chromosome inactivation and do not form teratomas *in vivo* which allows these cells to be a useful tool to investigate human development (Turnpenny et al., 2003). However, human EG cells are difficult to maintain *in vitro* due to a tendency to undergo spontaneous differentiation and therefore further characterization is required to fully elucidate their potential. Recently, a rare population of germ SCs have been isolated from adult mice and human ovaries (White et al., 2012). This exciting discovery has generated controversy in the field (Telfer and Albertini, 2012; Telfer et al., 2005; Tilly et al., 2009), but offers great potential for the treatment of infertility due to premature menopause or ovarian failure.

1.1.2 Embryonic Stem cells

1.1.2.1 Preimplantation development

Pluripotent SCs are transiently expressed in the blastocyst, the final stage of preimplantation development. A blastocyst develops 5-6 days after fertilization in the human and is composed of 2 distinct cell types: the trophectoderm and the inner cell mass (ICM) (Figure 1.2).

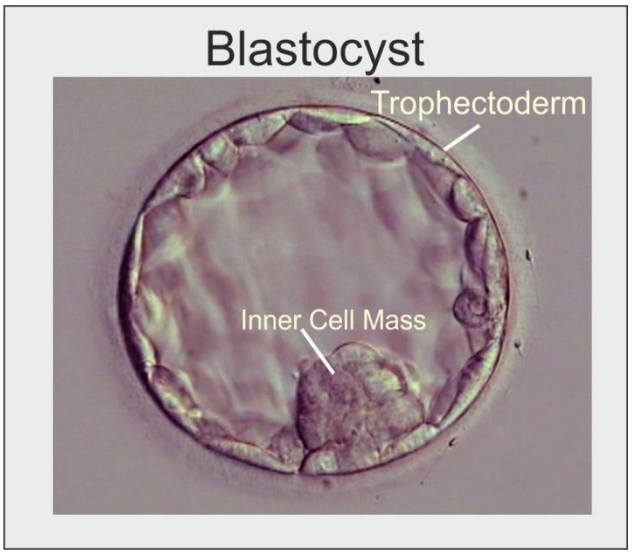

Figure 1.2 Blastocyst stage embryo

As development progresses, the cells of the blastocyst continue to divide and the blastocoel cavity expands. The ICM cells segregate into the hypoblast and the epiblast (Coucouvanis and Martin, 1999). During gastrulation, the epiblast cells give rise to the three germ layers: endoderm, mesoderm and ectoderm (Figure 1.1). All three germ layers will form specific organs such as lung, liver, pancreas and intestine from the endoderm, heart, skeletal muscle and blood from the mesoderm, and skin and neurons from the ectoderm (Aplin, 1996; Murry and Keller, 2008).

1.1.2.2 Mouse embryonic and epiblast SCs

ES cells are derived from the ICM of a blastocyst, these pluripotent cells proliferate by self-renewal and have the capacity of differentiate into all three germ layers (Martin, 1981). Mouse ES cells were discovered independently by two groups (Evans and

Kaufman, 1981; Martin, 1981) and were found to express the same markers and pattern of differentiation as mouse EC cells.

Pluripotent cells also can be derived from the epiblast of the early postimplantation mouse embryo (Tesar et al., 2007). Although EpiSCs are pluripotent, they display a heterogeneous expression of lineage commitment markers and therefore have a more limited developmental potential than mouse ES cells. Hence, the ICM-like state of mouse ES cells have been designated as "naïve" whereas EpiSCs have been termed "primed" (Nichols and Smith, 2009).

1.1.2.3 Human ES (hES) cell discovery

In 1998, James Thomson described the first ES cell line derived from the ICM of a human blastocyst generated through In vitro fertilization (IVF) (Thomson et al., 1998). This was a significant milestone in developmental biology due to the potential of hES cells to be used to treat degenerative disorders. However, despite the potential of these cells for regenerative medicine, their application raises several ethical issues regarding the destruction of embryos. In an attempt to overcome some of these concerns, methodologies have emerged where hES cell lines have been derived from either single blastomeres, or poor quality embryos unsuitable for transfer (Klimanskaya et al., 2006; Zhang et al., 2006). However, for many, ethical issues remain about the use of any form of human embryo, regardless of perceived quality and developmental potential.

It is thought that over 300 hES cell lines have now been derived (Candan and Kahraman, 2010). However, among the cell lines there are some differences in terms of lineage markers (Abeyta et al., 2004; Rao et al., 2004), X chromosome inactivation and chromosome stability (Baker et al., 2007; Enver et al., 2005). Furthermore, genes involved in metabolism and epigenetics have also been found to be differentially expressed across the cell lines (Tang et al., 2010a). The reasons for these differences are still unknown but seem to be the effect of long-term culture.

1.1.2.4 Induced Pluripotent Stem (iPS) cells

The cloning of "Dolly" the sheep by Wilmut et al. (1997) demonstrated that is possible to reprogram the nucleus of a mammalian differentiated cell into an undifferentiated state. This discovery was achieved using a technique called somatic cell nuclear transfer (SCNT). In this process, the nucleus derived from a somatic cell is introduced into an enucleated oocyte and the cells are fused by subjecting them to an electrical pulse. This activates the egg allowing it to develop into an embryo (Wakayama et al., 2001; Wilmut et al., 1997). These experiments proved it was possible to clone a mammal and provided the possibility of generating patient specific ES cells for therapeutic purposes (Figure 1.3). However, the application of this technique presents ethical concerns not only since it would be dependent on the availability of donated human embryos, but also the use of SCNT which is extremely inefficient (Yamanaka, 2008). Another approach used to obtain pluripotent cells was the somatic cell fusion which required the fusion of a hES cell with a human fibroblast cell (Cowan et al., 2005). However, the pluripotent cells obtained with this method displayed abnormal karyotype (Yamanaka, 2008). Finally Takahashi and Yamanaka discovered that the ectopic expression of the transcription factors OCT4, SOX2, c-MYC and KLF4 via retroviral vectors were sufficient to reprogramme human dermal fibroblasts into pluripotent SCs and the first human induced pluripotent stem (iPS) cells were produced (Takahashi and Yamanaka, 2006) (Figure 1.3).

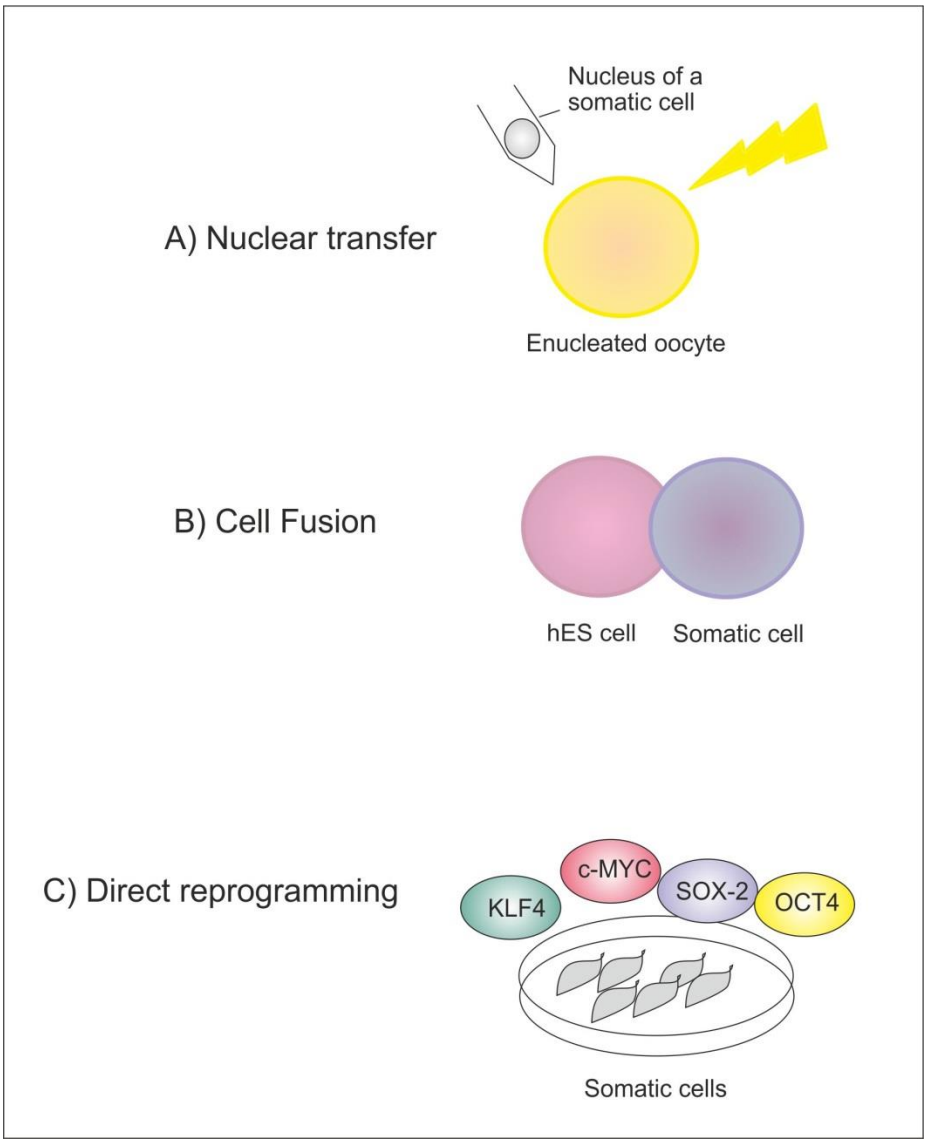

Figure 1.3: Different strategies to convert somatic cells to pluripotent cells using nuclear reprogramming

(A) A somatic cell can be injected into an enucleated oocyte using the technique of the SCNT; (B) Fusion of a hES cell with a somatic cell; (C) ectopic expression of OCT4, SOX2, KLF4 and c-MYC can reprogram somatic cells into induced pluripotent (iPS) cells. Adapted from Amabile et al. (2009).

iPS cells have now been derived from human fetal fibroblasts using lentiviral vectors carrying OCT4 and SOX2 in combination with NANOG and Lin28 homolog (Lin28) instead of c-MYC and KLF4 (Yu et al., 2007). Moreover, pluripotent SCs have also been produced from several cell types using different combinations of factors like

OCT4, SOX2, NANOG and Lin28, that have been recognized to contribute to the reprogramming (Giorgetti et al., 2009; Haase et al., 2009; Yu et al., 2007). In contrast, c-MYC and KLF4 have been found to be important for clonal recovery (Park et al., 2008b).

iPS cell lines have now been generated from patients with specific diseases like Amyotrophic Lateral Sclerosis (ALS) (Dimos et al., 2008), Down syndrome (DS; trisomy 21), Adenosine Deaminase Deficiency-related severe combined immunodeficiency (ADA-SCID), Parkinson disease (PD) and Juvenile-onset, type 1 Diabetes Mellitus disease (JDM) (Park et al., 2008a). The generation of these cell lines will help to produce *in vitro* disease models and lead to new drug discoveries. However, although important advances have been made, the efficiency of iPS cell derivation remains low and the reprogramming process is associated with the acquisition of mutations when the iPS cell are cultured (Gore et al., 2011) limiting the differentiation potential. One of the major concerns that limit the therapeutic use of these cells is that retroviruses can integrate into the genome leading to cell dysfunction and tumorigenesis in patients. To overcome this problem, new approaches have been developed including transient transfections (Okita et al., 2008), adenoviral vectors (Stadtfeld et al., 2008), removable transposon systems (Yusa et al., 2009) and the use of small molecules and soluble factors to mediate the reprogramming (Kim et al., 2009a; Woltjen et al., 2009; Zhou et al., 2009). More recently, iPS cells have been generated using synthetic modified mRNA (Warren et al., 2010) and microRNA (Judson et al., 2009). However, it is still not known which of these methods is suitable for optimal reprogramming and further studies will be required before iPS cells can be used for therapeutic applications.

1.1.3 Multipotent SCs

1.1.3.1 Adult SCs

Adult Stem Cells (ASCs) are undifferentiated cell populations found in the body after development that display limited self-renewal and are able to generate all cell types of the tissue from which they originate (Choumerianou et al., 2008). However, compared to hES cells, ASCs, such as Bone Marrow (BM), are well suited for clinical purposes and pose minimal ethical objection (Choumerianou et al., 2008). In addition, ASCs can be used for autologous treatment of degenerative, traumatic and congenital diseases as they can be isolated from the patient and thus do not initiate an immune response (Choumerianou et al., 2008). BM is one of the best sources of adult SCs. Indeed, BM contains multipotent SCs such as hematopoietic SCs (HSC) and mesenchymal SCs (MSCs). For this reason, BM cells have been used for therapeutic purposes to cure several diseases like lymphomas and leukaemias (Broxmeyer, 2010; Leeb et al., 2010). HSC were isolated for the first time from mouse BM (Spangrude et al., 1988) and have the property to give rise to all cell types of blood (Orkin, 2000). MSCs can be isolated not only from BM but also from postnatal organs and tissues, such as umbilical cord and adipose tissue (da Silva Meirelles et al., 2006; Lindroos et al., 2009) and are able to differentiate osteoblasts, chondroblasts, adipocytes, muscle cells and cardiac cells (Pittenger et al., 1999). Other ASCs are the mammary SCs, intestinal SCs, endothelial and the neuronal SCs. One example of an adult stem cell population which is leading to interesting advances in the treatment of damaged tissue is the cardiac stem cell.

1.1.3.2 Cardiac SCs

Until recently, the heart had been considered a post-mitotic organ which has already reached terminal cell differentiation. However, studies have now revealed that the heart has endogenous regenerative properties and is regulated by stem cell compartments (Leri et al., 2005). Evidence for the regenerative potential of the heart has come from the discovery that cardiac niches are characterized by the presence of cardiac stem/progenitor (CSP) cells (Urbanek, 2006). Once isolated from the heart, these cells display the ability to differentiate into cardiomyocytes and represent a useful tool for cardiac regeneration therapies (Bearzi et al., 2007; Messina et al., 2004; Tateishi et al.,

2007). Indeed, these cells have been found to express typical heart stem cell factors such as c-Kit (Bearzi et al., 2007) or stem cell antigen-1 (Sca-1) (Oh et al., 2003). In addition, it has been possible to isolate from heart a specific progenitor population of cells called "side population cells" capable of differentiating into functional cardiomyocytes and to grow into cardiospheres *in vitro* (Messina et al., 2004; Pfister et al., 2005). In the ischaemic heart, these cells have been found to differentiate in to cardiomyocytes not only through fusion with host cardiomyocytes but also through trans-differentiation (Ieda et al., 2010; Messina et al., 2004; Tateishi et al., 2007). Recent studies have demonstrated that CSP cells can regenerate cardiomyocytes and improve myocardial function in mouse models of ischemia or myocardial infarction (Ellison et al., 2013; Welt et al., 2013). These studies highlight the great potential that CSP cells possess for regenerating damaged heart tissue although further research is required to fully understand the regulatory mechanisms of these cells.

1.1.4 Derivation of hES cells

A hES cell line is derived from the ICM of a blastocyst, the final stage of preimplantation embryo development. To derive a hES cell line, the zona pellucida is first removed from the blastocyst through pronase digestion and the ICM is isolated by immunosurgery. The blastocyst is incubated in an anti-human serum antibody and complement lysis used to lyse trophectoderm cells. The ICM is isolated and placed on mitotically inactive mouse fibroblasts (Reubinoff et al., 2000; Thomson et al., 1998) (Figure 1.5). hES cells can also be derived using the blastocyst outgrowth method in which the blastocyst hatches from its zona pellucida and outgrowths, the ICM is mechanically isolated and transferred to mitotically inactive mouse fibroblasts (Reubinoff et al., 2000).

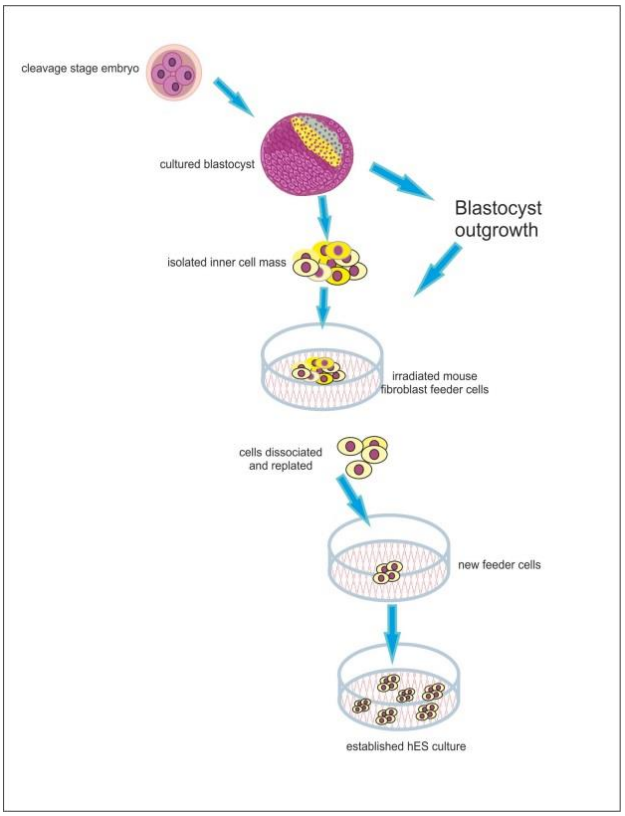

Figure 1.4 Schematic representation of hES cell derivation

1.1.5 Culture of hES cells

hES cells require specific factors that allow cell survival, proliferation and maintainance of self-renewal and are cultured on a feeder layer of extracellular matrix support in an appropriate medium. hES cells tend to grow in colonies maintaining cell to cell contact. Hence, when dissociated into single cells, the cloning efficiency and survival of these cells is limited (Peerani et al., 2009). Furthermore, hES cells tend to spontaneously differentiate in suboptimal culture conditions and form aggregates called embryo bodies (EBs) which contain cells of all three germ layers (Reubinoff et al., 2000).

1.1.5.1 Culture on feeder cell layers

Feeder cell layers are important for the establishment of pluripotent hES cell cultures.

1.1.5.1.1 Mouse and human feeder layers

hES cells require meiotically inactivated mouse embryonic fibroblast (MEFs) feeder cell layer as growth support (Thomson et al., 1998). Furthermore, immortalize MEF cell lines, called STO, can also be used as a feeder layer (Park et al., 2003).

Despite the advantages of the using a MEF feeder layer, the risk of zoonosis transferring animal pathogens limits the applications of hES cells for transplantation. For this reason, several types of human feeder cells were developed such as skin or fetal muscle cells (Amit et al., 2003; Richards et al., 2002), placenta fibroblast cells (Genbacev et al., 2005), bone-marrow derived cells (Cheng et al., 2003) and immortalized fibroblast cells derived from differentiated hES cells (Xu et al., 2004). Thus, these feeders provide an alternative to the use of mouse feeders that are safer for clinical applications.

The use of MEFs or human feeder layers is important to prevent hES cell differentiation but the critical factors produced by the fibroblast feeder layers remain unknown (Eiselleova et al., 2008; Thomson and Odorico, 2000). However, it seems that the presence of soluble factors released by the feeder supports in culture is beneficial for the maintenance of hES cells pluripotency. Indeed, human feeder cells produce basic fibroblast growth factor (bFGF) and Activin A important for the establishment of the

self-renewal state (Eiselleova et al., 2008; Prowse et al., 2007) whereas MEFs do not produce bFGF (Eiselleova et al., 2008). Other important factors secreted by both human and MEF feeders that support the pluripotent and undifferentiated state of hES cells are the transforming growth factor beta 1 (TGFβ1), the bone morphogenetic protein (BMP4) and the Laminin-511 (Eiselleova et al., 2008; Hongisto et al., 2012; Lim and Bodnar, 2002).

1.1.5.1.2 Feeder free culture

In 2001 a method was described to maintain hES cells in feeder free conditions using Matrigel or laminin pre-coated plates in serum free medium conditioned by mouse embryonic fibroblast (MEFs) feeders (Xu et al., 2001). This allowed hES cells, to be maintained, differentiated and also transfected with lentiviral vectors without any interference from MEF cells (Lebkowski et al., 2001). Matrigel is a gelatinous protein mixture extracted from the Engelbreth-Holm-Swarm (EHS) mouse sarcoma a tumour rich in extracellular matrix (Kleinman and Martin, 2005). Matrigel also contains type collagen IV, bFGF, insulin growth factor-1, platelet growth factor (PDGF) and other signalling molecules essential for the self-renewal state (Hughes et al., 2010). Matrigel is one of the most used feeder free substrates for long term culture of pluripotent SCs. However its composition can vary between batches and resulting in problems with SC maintenance. Other feeder free support have been developed using recombinant fibronectin (Amit et al., 2004), recombinant vitronectin (Braam et al., 2008) or laminin (Rodin et al., 2010). These are very important feeder free supports that allow the expansion of hES cells without the risk of zoonosis.

1.1.5.1.3 Cell culture media for pluripotent SCs

Culture media is essential for providing nutrients for the maintenance of pluripotent SCs. Initially, culture of hES cells required the addition of fetal bovine serum (FBS) or human serum (HS) to the culture media with feeder cells as well as in feeder-free conditioned medium. Subsequently, a serum-free alternative, Knockout™ serum-replacement medium was discovered, that allowed more standardized and undifferentiated culture of hES cells (Amit et al., 2004; Richards et al., 2002). Among the soluble factors that sustain hES cell pluripotency there are the fibroblast growth

(FGFs) factors, and in particular FGF2 or bFGF, which have been found important in cell differentiation, proliferation and self-renewal (Amit et al., 2004; Basilico and Moscatelli, 1992; Dvorak et al., 2005; Xu et al., 2005a). The precise mechanism behind the role of FGF2 is still not known. However, it has been found that FGF2 directly regulates OCT4, one of the master genes of pluripotency in hES cells, through the ERK signalling pathway (Brumbaugh et al., 2012). Other factors involved in the regulation of hES cell pluripotency together with FGF2 are the members of the TGFβ/activin and nodal signalling family that are activators of the SMAD2/3 pathway (Vallier et al., 2009). It has been demonstrated that SMAD2/3 interacts with NANOG to increase its expression (Xu et al., 2008). Another member of the TGFβ family is the growth differentiation factor 3 (GDF3) a factor that blocks hES cell differentiation mediated by BMP-4 and maintain pluripotency (Skottman et al., 2006). However, the precise molecular signals that are crucial for maintaining the self-renewal state are still unknown. Recently, several xeno-free media have been produced that can support long term cultures of undifferentiated hES cells (International Stem Cell Initiative et al., 2010; Ludwig et al., 2006; Rajala et al., 2007). One of the most widely used xeno-free media is TeSR1, an animal–free medium that supports the derivation and culture of hES cells (Ludwig et al., 2006). These media contain different cocktails of molecules and proteins like TGFβ and bFGF and have been tested in several culture conditions. However, the use of these media is still limited due to the lack of knowledge regarding the optimal culture conditions for pluripotent SCs and the soluble factors that sustain the undifferentiated state.

1.1.6 Morphological characterization

hES cells have a similar phenotype compared to mouse and human EG cells. They display a high nuclear to cytoplasmic ratio and typical nuclear and chromatin structures (lamina, heterochromatin domain and nuclear speckles) (Meshorer and Misteli, 2006; Reubinoff et al., 2000; Thomson et al., 1998). hES cells display characteristics typical of the ICM cells. In particular, they present large nuclei with a well-developed endoplasmic reticulum, Golgi complexes and immature mitochondria with few christae (Sathananthan et al., 2002).

1.1.6.1 Surface antigens

Human pluripotent SCs, ECs and EpiSCs express similar surface antigens that have been associated with self-renewal. For instance, hES cells express a series of surface antigens like the stage specific embryonic antigens 3 and 4 (SSEA-3,SSEA-4) that are globoseries glycolipids (Andrews et al., 1996; Thomson et al., 1998; Thomson and Marshall, 1998) whereas SSEA-1 is only expressed upon early differentiation (Pera et al., 1988). Another characteristic of hES cells is the expression of antigens that are associated to the pericellular matrix proteoglycan like TRA-1-60, TRA-1-81 and GCMT-2 which detects the keratin sulphate/chondroitin sulphate pericellular matrix proteoglycan (Cooper et al., 1992). hES cells also contain alkaline phosphatase activity (Badcock et al., 1999). The differentiation of EC and ES cells is characterized by the loss of expression of all these markers typical of the undifferentiated state.

1.1.6.2 Molecular Markers of Pluripotency

Pluripotent SCs are also defined by the presence of key transcription factors which have been genetically characterized thereby confirming their importance in embryo development.

OCT4 (or Pou5f) is a POU domain transcription factor which binds the octamer motif ATGC(A/T)AATT found in the regulatory domains of specific genes (Scholer, 1991). Oct4 expression has been extensively study since it has been found to be essential for the pluripotency of ES cells (Yeom et al., 1991). Oct4 is essential for the establishment of pluripotency in cells of the ICM as Oct4-deficient mouse embryos display differentiation along the trophoblast lineage (Nichols et al., 1998). Oct4 has also been found responsible for the bFGF secretion by the ICM thereby providing paracrine signals which are important for the trophectoderm development (Nichols et al., 1998). Furthermore it has been shown that OCT4 is required for the establishment of the pluripotency together with SOX2 (Ambrosetti et al., 2000; Yuan et al., 1995) and NANOG (Chambers et al., 2003; Mitsui et al., 2003). Interestingly, a recent study has shown that Oct4 overexpression led to the up-regulation of differentiation markers of all three germ layers (Radzisheuskaya et al., 2013). This interesting finding reveals that

Oct4 not only is important for the establishment of self-renewal, but also for mouse ES cell differentiation.

SOX2 belongs to the SOX (Sry-related HMG box) a group of proteins which bind an amino acid high mobility group (HMG) domain in the minor groove of the DNA helix. This process is energetically expensive for the cell leading to an increase in DNA bending, thus, SOX2 binds the DNA with a high dissociation constant (Nishimoto et al., 1999). SOX2 is co-expressed with OCT4 in human ES, EG and EC cells regulating the expression of several target genes like FGF4 and undifferentiated embryonic cell transcription factor (UTF) and cooperatively binds to DNA, with the low affinity POU-specific domain and HMG domain adjacent to each other (Ambrosetti et al., 2000).

NANOG is the third partner of this core of pluripotency factors, playing a central role for the maintenance of a robust pluripotent state acting together with OCT4 in hES cells (Chambers and Smith, 2004). The name Nanog is derived from the Celtic name "Tir nan Og" land of the ever-young. NANOG is expressed *in vivo* in the ICM of blastocysts and when over-expressed is able to support prolonged culture of mouse ES cells independently of the LIF (leukemia inhibitory factor) and STAT3 signalling which are required for the mantainance of mouse ES cells (Mitsui et al., 2003). Several studies report that Nanog biochemically interacts with Oct4 (Xie et al., 2006; Zhang et al., 2007) and that this cooperation is important for the pluripotency state in mouse epiblasts and ES cells (Mitsui et al., 2003). Interestingly, Nanog is not homogeneously expressed in murine ES cells; a highly expressing Nanog population is pluripotent while a low expressing Nanog population possess Gata6, an endodermal marker (Singh et al., 2007). Therefore, it is possible that Nanog could play a critical role in balancing pluripotency and differentiation in ES cells (MacArthur et al., 2012). The expression of these transcription factors is fundamental to ensure hES cell identity, indeed, silencing of Oct4 and Sox2 expression has been found to induce differentiation of ES cells into trophectoderm cells (Masui et al., 2007; Nichols et al., 1998) while loss of Nanog induced formation of extraembryonic endoderm cells (Mitsui et al., 2003).

1.1.7 The oct-sox cis regulatory element

The POU and the HMG domain contained by Oct4 and Sox2 respectively are two elements important for the maintenance of a pluripotent cell state (Avilion et al., 2003; Nichols et al., 1998). The POU/HMG ternary complex bound to composite oct-sox elements characterized by two enhancer elements separated each other by 3bp or 0bp which mediate specific protein-protein and DNA-protein interactions (Remenyi et al., 2003; Williams et al., 2004b). Previous studies have characterized the regulatory regions important for Oct4 expression in the early mouse embryo and found that a distal enhancer in the Oct4 proximal promoter is important for the expression of this transcription factor within the morula, ICM and ES cells (Yeom et al., 1996). Alignment between the upstream proximal promoter of Oct4 revealed the presence of 4 conserved regions (CR1, CR2, CR3 and CR4) which display a high degree of homology across species (Nordhoff et al., 2001) and, interestingly, the CR4 overlaps with the distal enhancer containing the composite oct-sox element (Chew et al., 2005). For the Sox2 gene, two regulatory regions called SRR1 and SRR2 (Sox regulatory region 1 and 2) are known to regulate Sox2 expression in ES cells (Tomioka et al., 2002). These two regulatory regions are also bound by Oct4 and Sox2 in both human and mouse ES cells (Trott et al., 2012). The Oct4-Sox2 interaction forms a transcriptional autoregulatory loop in which Oct4 and Sox2 proteins bind their own regulatory element (Figure 1.5 A and B) leading to increased expression and self-renewal properties (Chew et al., 2005).

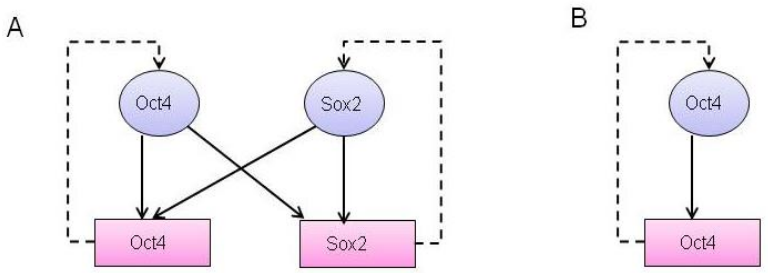

Figure 1.5: Synergic regulation of Oct4 and Sox2 through the oct-sox element

Schematic representation of the interconnection between Oct4 and Sox2 and their respective genes (A) and the autoregulation motif within the Oct4 gene is represented in B. The transcription factors are represented by the blue ovals while the regulatory elements are represented by the pink rectangles. The solid arrow indicates the direct binding to the *cis* regulatory element and the dashed arrow represents the protein synthesis by the respective genes. Figure adapted from Chew et al. (2005).

Oct4 may also bind the regulatory motif of Sox2 in a closed loop, termed the "multicomponent loop motif" thought to allow the switch between self-renewal and differentiation in ES cells (Figure 1.6) (Chew et al., 2005).

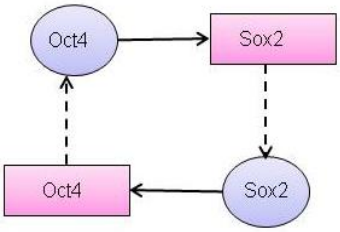

Figure 1.6: Multicomponent loop motif

Schematic representation of the multicomponent loop motif displayed by Oct4 and Sox2 showing the interconnection between the two genes. The transcription factors are represented by the blue ovals while the regulatory elements are represented by the pink rectangles. The solid arrow indicates the direct binding to the cis regulatory element and the dashed arrow represents the protein synthesed by the respective genes. Figure adapted from Chew et al. (2005).

As described above, both Oct4 and Sox2 interact with each other and with Nanog in the core network of pluripotency circuitry (Boyer et al., 2005). It has been found that Fgf4, Utf1 and Fbx15 as well as Nanog, Sox2 and Oct4 are targets of the Oct4 and Sox2 since each of these target genes possesses the composite oct-sox *cis* element (Chew et al., 2005; Nishimoto et al., 1999; Okumura-Nakanishi et al., 2005; Rodda et al., 2005; Tokuzawa et al., 2003; Tomioka et al., 2002; Yuan et al., 1995). In particular, it has been demonstrated that Oct4 and Sox2 interact with a composite oct-sox *cis* regulatory module in the Nanog proximal promoter and that this regulatory element is necessary for pluripotency expression in mouse and in human ES cells (Kuroda et al., 2005; Rodda et al., 2005). Within the Nanog promoter the oct-sox composite element acts as an enhancer, is transcriptionally active in both sense and non-sense orientation and is highly conserved among species (Rodda et al., 2005). This highlights the presence of a high degree of interaction among transcription factors required for the self-renewal of ES cells.

1.1.8 The core regulatory circuitry

OCT4, SOX2 and NANOG form a core and act together to regulate their own promoters, through the establishment of an autoregulatory loop which activates the expression of protein coding and miRNA genes important for the ES cell state (Young, 2011). This regulatory circuit maintains the pluripotent state of hES cells through a positive-feedback-control that takes place on their own promoters and regulatory genes. Indeed, through ChIP assays coupled with DNA microarrays it was found that OCT4, SOX2 and NANOG co-occupy the same DNA regions in hES cells (Figure 1.7) (Boyer et al., 2005; Kim et al., 2008). Almost 352 genes are bound simultaneously by OCT4, SOX2 and NANOG in undifferentiated hES cells (Boyer et al., 2005). Furthermore, OCT4, SOX2 and NANOG are also bound to their own promoters in an autoregulatory loop that is important to maintain ES cells identity (Boyer et al., 2005). This mechanism of regulation sustains ES cell self-renewal until they differentiate into a specific cell type under particular stimuli or developmental cues (Chambers and Smith, 2004). The functions of these key transcription factors are tightly linked so that any change in the circutry would produce changes in gene expression and cell fate (Boyer et al., 2005).

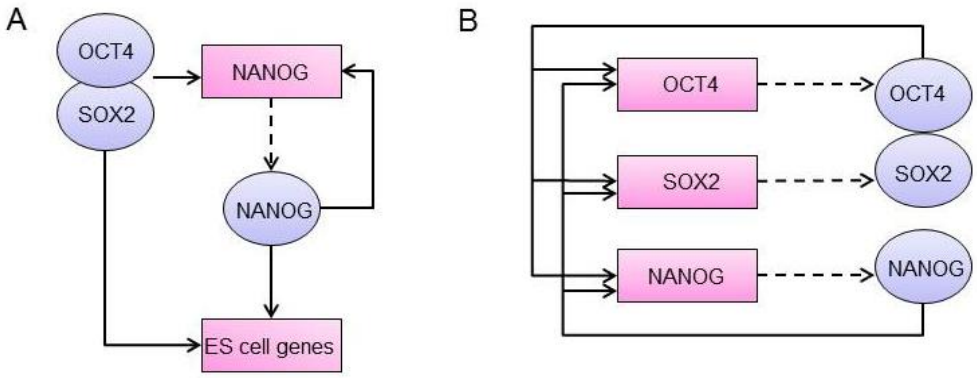

Figure 1.7: Core regulatory circuitry in ES cells

Schematic representation (A) of the feedforward transcriptional regulatory circuitry and (B) the autoregulatory loop formed by OCT4, SOX2 and NANOG. Regulator factors are represented in blue, gene promoters in pink. Adapted from Boyer et al.(2005).

Other studies have found that the core regulatory circuitry interacts also with other transcription factors such as Klf4 (Kruppel-like factor 4), Esrrb (Estrogen receptor-related β) and Tcf4 (T cell factor 4) which help to maintain the expression state important for ES cell pluripotency and self-renewal. At the same time, this core contributes to repression of lineage-specific genes preventing exit from the pluripotent state (Boyer et al., 2005; Johnson and Nasr-Esfahani, 1994). Further characterization of the transcriptional activity and protein network of the core pluripotency factors in ES cells has uncovered complex pathways controlling self-renewal (Kim et al., 2008; MacArthur et al., 2012). In particular, it has been found that almost 28 distinct feedback loops are responsible for regulating the pluripotency network in ES cells. Nanog has been shown to be the central element in this network as loss of Nanog expression was associated with a loss of pluripotency and an increased differentiation of ES cells that was rescued when Nanog was re-introduced (Chambers et al., 2007; MacArthur et al., 2012). Recent data has also found a relationship between the activity of Nanog and the fibroblast growth factor 5 (Fgf5) whose differential expression defines the presence of several sub-networks in ES cells that reveal the existence of heterogeneity in the expression of key regulators of pluripotency (Trott et al., 2012). These fluctuations in the expression of pluripotency genes could affect the long term culture of ES cells and

warrants further investigations (Chambers et al., 2007; Macfarlan et al., 2012). The identification of targets regulating the core pluripotency network including additional transcription factors and chromatin regulators in different environmental conditions will allow of a more comprehensive and detailed map of the transcriptional regulation of ES cells to be discovered.

1.1.9 Additional levels of pluripotency regulation

The transcription factors Oct4, Sox2 and Nanog form a multiprotein complex composed by a wide range of protein-protein interactions. A proteomic study of ES cells using epitope-tagged Nanog, Oct4 and Sox2 discovered the presence of a "mini interactome" in which Nanog, Oct4, Sox2 and other factors such as Dax1, Nac1, Sall4, Tcfcp211, Zfp281 and Rex1 are associated with each other but also with other transcription factors (Ding et al., 2012; Gao et al., 2012; Wang et al., 2006). This high degree of protein interconnection is required for the self-renewal of ES cells and small changes in the transcription factors landscape is likely to block the self-renewal of ES cells and induce differentiation (Gao et al., 2012). Other studies have been performed to better define the core circuitry of pluripotency. A genome wide study revealed that the genomic distribution of the histone modifications (H3K4me3 and H3K27me3) that characterize the bivalent chromatin state in ES cells are associated with the core pluripotency circuitry (Kim et al., 2008). Another study examined the genome wide binding of the core pluripotency genes to the co-activator p300 and the transcription factor insulator CCCTC (CTCF) showing that the presence of co-activators is also necessary for the establishment of the core of pluripotency (Chen et al., 2008). These studies demonstrated that the core transcriptional circuitry is formed by transcription factors that work closely together and used a specific algorithm to define a further 600 genes bound by Oct4, Sox2 and Nanog in ES cells (Chen et al., 2008). Interestingly, it was found that Oct4 was responsible for a *cis* loop within the Nanog promoter (Levasseur et al., 2008). Furthermore, recent work from Apostolou et al (2013) documented a Nanog specific chromatin "interactome" in mouse ES cells and in iPS cells which suggest a role for chromatin structure in controlling pluripotency and self-renewal (Apostolou et al., 2013). Further analysis also revealed the presence of a third level of integration among the core pluripotency and other cellular processes like DNA repair, replication

and transcriptional elongation (Sikorski et al., 2011; Wang et al., 2012). Given this high degree of integration among the core of pluripotency genes, signal transduction and chromatin organization it is possible to assume that a 3D signalling crosstalk allows mouse ES cells to coordinate the response to environmental cues and to maintain their pluripotency. However, it is still not known whether these interactions will also take place in hES cells and the role of other transcription factors in the transcriptional network of pluripotency.

1.2. Hypoxic Regulation of Human Embryonic Stem Cells

1.2.1 Effect of environmental oxygen on preimplantation embryo development

hES cells are difficult to maintain in culture due to their tendency to spontaneously differentiate, an effect likely due to a suboptimal culture environment (Reubinoff et al., 2000; Thomson et al., 1998; Thomson and Marshall, 1998). This effect could be prevented by culturing these cells under low oxygen tension. In *vivo*, pluripotent ES develop from the inner cell mass of the blastocyst which in hamster, rabbit and rhesus monkey, have been shown to reside in a hypoxic environment (Fischer and Bavister, 1993). Indeed, a substantial decrease from 10% to 1% oxygen tension has been found as the embryo moves from oviduct to uterus (Fischer and Bavister, 1993). The functional significance of this reduced oxygen tension in the reproductive tract could be to protect the blastocyst from reactive oxygen species. In fact, a decreased reactive oxygen species formation has been observed in mouse embryo cultured at low oxygen tension compared to those cultured at 20% oxygen (Goto et al., 1993). This finding is really important as reactive oxygen species are involved in the retardation of early embryonic development (Guerin et al., 2001; Johnson and Nasr-Esfahani, 1994; Tarin, 1996) and DNA damage (Takahashi et al., 2000). Several studies demonstrated that culturing preimplantation embryos at lower oxygen concentration (5-7%) increases blastocysts formation (Batt et al., 1991; Bernardi et al., 1996; Dumoulin et al., 1999; Farrell and Foote, 1995; Pabon et al., 1989; Umaoka et al., 1992) and, also embryo quality (Thompson et al., 2000). Moreover bovine blastocysts produced under low oxygen contained more inner cell mass cells than those under 20% oxygen tension (Harvey et al., 2004). Furthermore, reduced oxygen tension has also been found to regulate cytotrophoblast proliferation and differentiation in humans and mice (Adelman et al., 2000; Genbacev et al., 1996; Genbacev et al., 1997). Similar findings have also been confirmed in other studies where embryos cultured at a reduced oxygen tension displayed an increased pregnancy and live birth rate after embryo transfer (Kasterstein et al., 2013; Meintjes et al., 2009) compared to those cultured at atmospheric oxygen

tension. Furthermore, the global gene expression pattern of mouse embryos cultured under hypoxic conditions (5% oxygen tension) was similar to that of *in vivo* derived embryos (Rinaudo et al., 2006).

1.2.1.1 Effect of environmental oxygen tension on hES cells maintenance

Studies described above highlighted the benefit of culturing embryo under hypoxia and were fundamental to better understand the maintenance and the appropriate culture conditions for hES cells. To date, atmospheric oxygen remains the culture environment routinely used in laboratories. However, it has been demonstrated that hES cells cultured under hypoxic conditions, display less differentiation than normoxic colonies (Ezashi et al., 2005). This feature was demonstrated by analyzing the gene and protein expression profile of pluripotency markers OCT4, SOX2 and NANOG which was found increased in hES cells cultured under low oxygen tension (Forristal et al., 2013; Forristal et al., 2010; Westfall et al., 2008). In contrast, culture of hES cells at 20% oxygen displayed smaller colonies, a decreased proliferation and a significantly reduced expression of all self-renewal markers (Forristal et al., 2010). Recently, Lengner et al. (2010) described for the first time the establishment of a hES cell line derived and cultured at low oxygen tension. Using these cells they demonstrated that hES cells cultured at 5% oxygen not only displayed a more immature state and a decreased differentiation but prevented X chromosome inactivation (XCI) (Lengner et al., 2010). These data further indicate that high oxygen concentrations induce XCI. Low oxygen was found also to reduce chromosome instability and aberrations compared to hES cells cultured at atmospheric oxygen tension (Forsyth et al., 2006). However, despite these interesting findings, controversy remains over the benefit of culturing hES cells under hypoxic conditions.

1.2.2 Hypoxia Inducible factors: the transcriptional regulators of the hypoxia response

Under conditions of low oxygen tension cells mount a physiological response to ensure adequate levels of ATP production (Wenger, 2002). Many processes involved in oxygen homeostasis are mediated by the Hypoxia Inducible Factors (HIF). HIFs activate the expression of several oxygen responsive genes involved in energy metabolism, vasculogenesis, cellular proliferation and apoptosis (Carmeliet et al., 1998; Goda et al., 2003; Iyer et al., 1998; Tacchini et al., 1999). HIFs were discovered through the identification of the minimal hypoxic response element (HRE) (A/G)CGTG in the 3' enhancer of the erythropoietin gene (Semenza and Wang, 1992). HIF-α is a phoshorylation-dependent protein which is able to bind the major groove of the DNA under hypoxic conditions (Wang and Semenza, 1993). The first HIF-1 protein discovered is an heterodimeric complex, formed of an HIF-1α and a constitutive expressed HIF-1β (also called ARNT aryl hydrocarbon receptor nuclear translocator) subunit (Keith et al., 2001; Wang and Semenza, 1993, 1995). Either HIF-1α and HIF-1β belong to the basic helix-loop-helix (bHLH)-Per/Arnt/Sim domains (PAS) family, which present several conserved domains including the bHLH region for DNA binding and 2 PAS domain for the dimerization and gene targeting (Wang et al., 1995), moreover ARNT is also able to bind transcriptional coactivators such as p300/CBP (Arany et al., 1996). Subsequently, two other HIFα subunits were discovered that revealed a more restricted tissue expression compared to HIF-1α and their roles remain to be fully characterized. These two factors were called HIF-2α or endothelial PAS (EPAS) (Flamme et al., 1997; Tian et al., 1997), and HIF-3α (Gu et al., 1998). To date, less is still known about HIF-2α and HIF-3α and their roles remain to be fully characterized.

HIF-2α is known to target HREs the hypoxia responsive genes but in specific cell types like vascular endothelial cells, kidney fibroblasts, hepatocytes and in many tumours associated with von-Hippel-Lindau (VHL) diseases (renal clear cell carcinomas and hemangiomas) (Hu et al., 2006), a mechanism independent of HIF-1α. In renal clear cell carcinoma (RCC) cells, isolated from VHL patients, only HIF-2α was expressed and not

HIF-1α, suggesting that HIF-2α plays a specific role during tumorigenesis (Maxwell et al., 1999). HIF-3α is the least characterised HIFα subunit but is expressed in specific cell lines like lung, cerebral cortex, and hippocampus (Heidbreder et al., 2003; Yoshida et al., 2001). Moreover, HIF-3α has been implicated in the hypoxic response of alveolar epithelial cells, but the mechanism is not fully elucidated (Li et al., 2006).

Both HIF-2α present a great homology with HIF-1α, and also heterodimerise with the ARNT at the HRE in the promoter of oxygen sensitive genes. They also have the same functional domain structure (Figure 1.8), in addition to the bHLH and the PAS domain they present two transactivation domains (N-TAD and C-TAD), separated by an inhibitory domain (ID), that is important for the normoxic repression of the TAD domain, and an oxygen-dependent degradation domain (ODD) that is responsible of the normoxic instability of the HIFα proteins (Jiang et al., 1996; Jiang et al., 1997; Pugh et al., 1997). HIF-3α differs from HIF-1α and HIF-2α due to the lack of the TAD domain in the C-terminal, thus HIF-3α is unable to recruit transcriptional regulators. The TADs are important for the recruitment of co-activators like CBP/p300, SRC-1 (steroid or nuclear receptor co-activator) and TIF2 (transcriptional intermediatory factor 2) (Ema et al., 1999; Gu et al., 1998). These coactivators act as histone acetyltransferases that connect HIF to the transcriptosome and perform chromatin remodelling required for the transcription. C-TAD is hydroxylated in an oxygen dependent manner and inhibits the HIF binding to CBP/p300 during normoxia (Sang et al., 2002). The ID is localized between the two TADs and function as a repressor of the transcriptional activity of N-TAD and C-TAD under normoxia (Jiang et al., 1997), while the ODD is important for the oxygen-dependent degradation of HIFs through the ubiquitin-proteasome pathway and serves as a starting point for the hypoxic signalling pathway (Huang et al., 1998). The combinations of these domains allow HIF stabilization and transcriptional activation under hypoxic conditions.

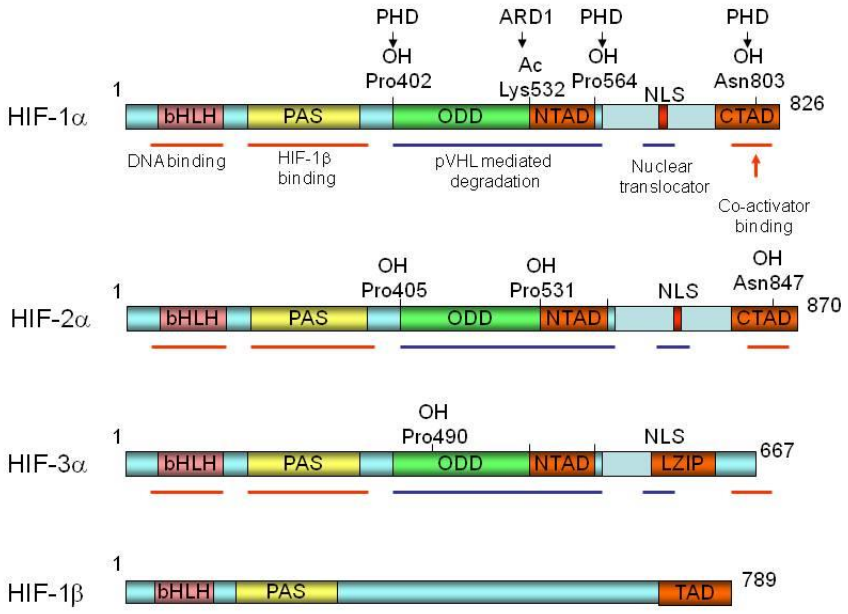

Figure 1.8: Schematic diagram of the HIF-α and HIF-1β proteins

Schematic representation of HIF-1α, HIF-2α, HIF-3α and HIF-1β showing different functional domains (coloured boxes). The modifications of specific residues are noted above each, and the proteins that perform those modifications are indicated. Coloured bars below each protein delineate particular interaction regions within HIF proteins that are important for their function. Adapted from Rocha et al. 2007.

1.2.3 Non-redundancy of HIFs

Despite HIF-1α, and HIF-2α sharing a high degree of homology, experiments on mice null for HIF-1α and HIF-2α loci showed distinct embryonic lethal phenotypes therefore they seem to have non-redundant functions. HIF-1α -/- embryos dies around the 11^{th} day (E11) of gestation with vascular defects, cardiovascular malformations and failure of neural tube closure due to mesenchymal cell death (Iyer et al., 1998; Kotch et al., 1999). Mice heterozygous for HIF-1α display normal development but present right ventricular hypertrophy, aberrant vascular remodelling and pulmonary hypertension (Kline et al., 2002; Yu et al., 1999). In contrast, mice null for HIF-2α -/- are embryonic lethal by E12.5-E16.5 due to bradycardia (Tian et al., 1998), or in some cases mice die shortly after birth from respiratory distress syndrome (RDS) due to failure of alveolar type II cells in the lung (Compernolle et al., 2002). HIF-1β null mice are embryonic lethal at E10.5 and display yolk sac and placental deficiency and a reduced rate of haematopoietic progenitors (Nishi et al., 2004).

1.2.4 Regulation of HIFs

The ODD has an important role for the normoxic turnover of the HIFs as it interacts with the oxygen sensing/transduction pathways (Huang et al., 1998). In the presence of oxygen, the prolyl-4-hydroxylase proteins (PHDs) hydroxylate two specific proline residues (Pro402 and Pro564) of the HIF-α subunit leading to protein inactivation (Flomenberg et al., 2005; Ivan et al., 2001) (Figure 1.6). Ratcliffe and his group identified three prolyl hydroxylase domains (PHDs) 1, 2 and 3 in *C.elegans* (Epstein et al., 2001; Ratcliffe, 2007). The PHDs are dyoxygenase that require oxygen, iron and 2-oxoglutarate as substrate and are able to transfer one oxygen atom in the proline residue of the HIF-α subunits while the second oxygen reacts with 2-oxoglutarate leading to the formation of succinate and carbon dioxide (Wenger, 2002). Therefore PHDs are the sensors that directly modulate the response of HIFs to physiological oxygen concentration. Hydroxylated HIF-α is recognized by the von-Hippel-Lindau (pVHL) protein, an E3 ubiquitin ligase complex, which is rapidly ubiquitinated and degraded in the proteosome (Huang et al., 1998; Ivan et al., 2001; Jaakkola et al., 2001). For

proteosomal degradation of HIF-α the pVHL first interacts with several proteins (Figure 1.8):

- **Elongins B and C**

Elongins B and C form a binary complex that is able to activate the transcriptional elongation activity of elongin A (Aso et al., 1995). This elongin complex increases the RNA polymerase II elongation by suppressing the basal DNA transcription machinery. pVHL binds the elongin B-C complex through the C-terminal residue 157-172, which has a high similarity to the elongin A (Aso et al., 1996).

- **Cullin 2**

pVHL binds to cullin 2 (Cul-2), a member of the cullin family (Lonergan et al., 1998; Pause et al., 1997). Cullins are a family of proteins first identified in *Caenorabditis elegans* (Kipreos et al., 1996) which function as negative regulators of the cell cycle. Furthermore, they display significant sequence similarity to yeast Cdc53 which is responsible for protein ubiquitination and degradation (Peters, 1998).

Cdc53 bind to Cdc34, which is an E2 ubiquitin-conjugating enzyme, to Skp1 (S-phase kinase-associated protein 1) which is an assembly factor, and an F-box protein (an homolog of the cyclin F) that confer target specificity (Peters, 1998). This multi-factor complex is called SCF (Skp1, Cdc53 and F box) (Skowyra et al., 1997) that is responsible of an ubiquitin-mediated proteolysis.

The elongin-SIII complex is similar to the SCF complex, and in particular, pVHL interacts with Cul-2 via elongins B and C in a similar manner to the SKp1 of the SCF complex binds Cdc53 to the F-box protein (Lonergan et al., 1998).

- **Rbx 1**

Rbx1 is an evolutionarily conserved protein that contains a RING-H2 fingerlike motif which complexes with pVHL, Cul-2 and elongins B and C leading to degradation by the cellular ubiquitination machinery. The homolog of Rbx1 in yeast is an activator of the Cdc53-containing SCF ubiquitin complex (Kamura et al., 1999) (Figure 1.8).

Thus, through the interaction with these proteins, the pVHL is able to target HIF-α for proteosomal degradation by the ubiquitin complex.

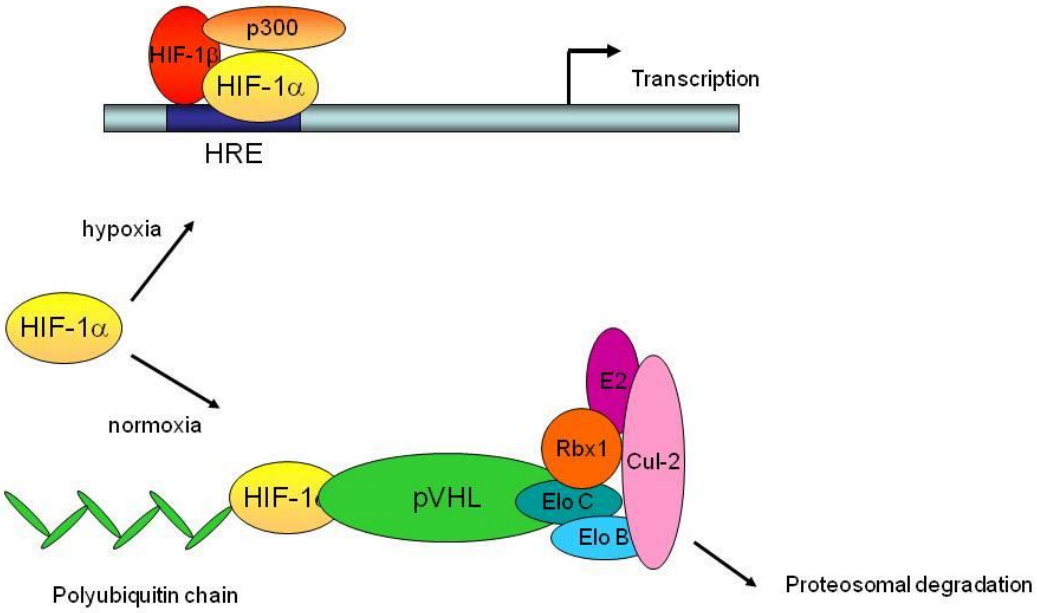

Figure 1.9: pVHL target HIF-1α for the proteosomal degradation by the ubiquitin complex

During hypoxia HIF-α is stable dimerizes with HIF-β and binds to the HRE of target genes to enhance transcription. In normoxia HIF-α is recognized by the pVHL targeting it for proteosomal degradation by the ubiquitin complex. Adapted from Mole at al 2001.

In normoxia pVHL binds to amino-acids 557-571 and 380-417 in HIF-1α and to aminoacids 517-534 and 383-418 in HIF-2α, while with the C-terminal domain binds elongins. Ubiquitin is then transferred to a HIF residue marking it for degradation by the proteosome (Cockman et al., 2000; Iliopoulos et al., 1996; Lisztwan et al., 1999; Ohh et al., 2000; Tanimoto et al., 2000). Under hypoxic conditions, the PHDs and pVHL binding, are inactivated due to oxygen deficiency, preventing the hydroxylation of the proline residues of the HIF (Jewell et al., 2001). This leads to stabilization of the HIF-α protein subunits which then are able to translocate from the cytoplasm to the nucleus where they can bind with HIF1-β, forming an active transcriptional complex to activate target genes (Jewell et al., 2001) (Figure 1.10).

The regulation by pVHL is the major mechanism by which HIFs are degraded, however there is also an additional mechanism of regulation that involves the p53 tumour suppressor gene (An et al., 1998). p53 encodes for a transcription factor that regulates cellular response to several stimuli including hypoxia. Under normoxic conditions, p53 interacts with Mdm2, an E3 ubiquitin-ligase binding protein that also mediate HIF degradation by the proteasome. Under hypoxia, HIF-α protein is stabilized and can recruit target genes (Ravi et al., 2000). In addition, a negative regulator of HIF-α functions is the factor inhibiting HIF (FIH) (Lando et al., 2002). FIH mediates HIF hydroxylation through the C-terminal transactivation domain and inhibits the association between HIFs and protein co-activators like p300-CBP (CREB binding protein) (Ruas et al., 2005).

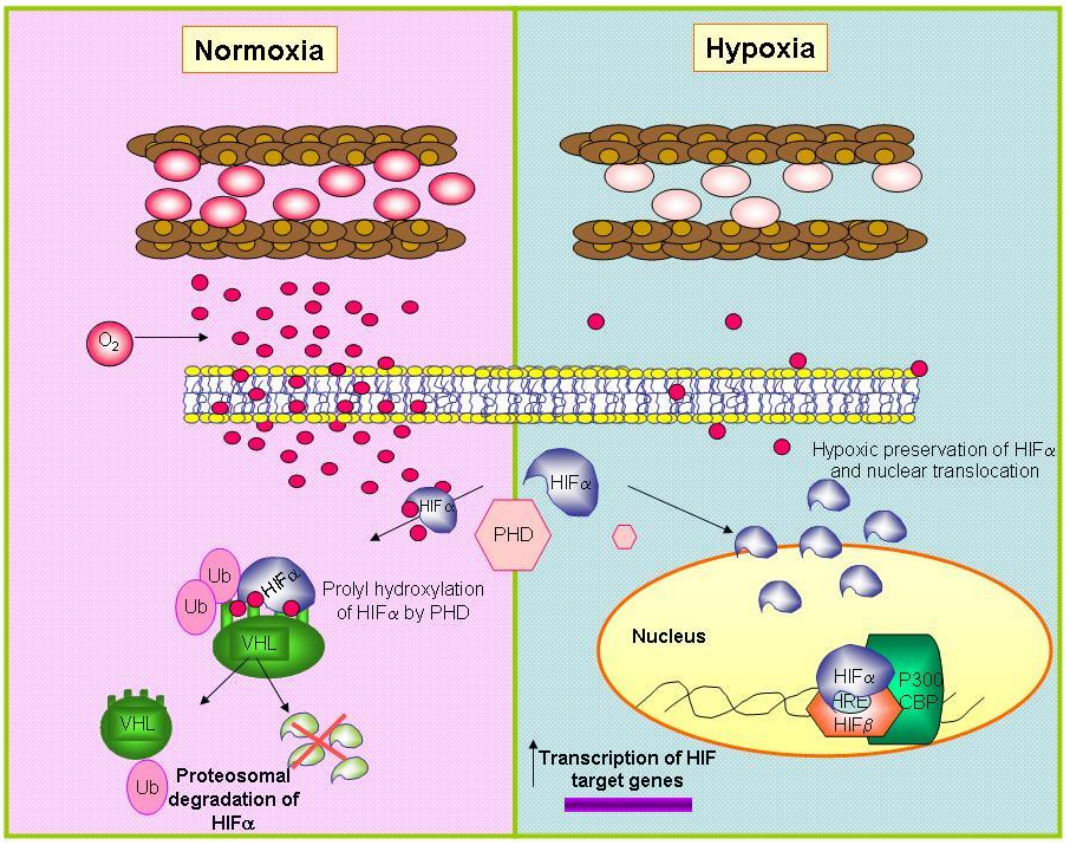

Figure 1.10: HIF activity under hypoxic and normoxic conditions.

In normoxia, hydroxylation promotes HIF-α association with pVHL and HIF-α destruction via the ubiquitin/proteasome. In hypoxia, these processes are inhibited, allowing HIF-α subunits (both HIF-1α and HIF-2α) to escape proteolysis, dimerize with HIF-1β and activate transcription via HREs. Adapted from Ratcliffe et al. (2007).

1.2.4.1 Alternative mechanisms of HIF regulation

There are alternative mechanisms by which the HIF-α subunits are regulated under normoxic conditions. Some of these mechanisms involve cytokines, insulin-like growth factor1 and 2, epidermal growth factor, interleukin 1β, platelet-derived growth factor (Feldser et al., 1999; Gorlach et al., 2001; Jung et al., 1999; Stiehl et al., 2002; Zelzer et al., 1998). How these factors might act is not fully understood but there may be common kinase pathways that increase the stability of the HIFs factors and activate cell-type specific receptors. Two important mechanisms involved in HIF-α regulation, involve nitric oxide (NO) and reactive oxygen species production (ROS):

1.2.4.1.1 Nitric Oxide

NO is a free radical which is an important signalling molecule but high levels can be toxic (Moncada and Palmer, 1991). NO is synthesized from L-arginine by the NO synthases (NOSs) (Ignarro, 1990). Three isoforms of NOS have been characterized: the constitutively expressed neuronal NOS (nNOS), the endothelial NOS (eNOS) and the inducible NOS (iNOS) (Kobzik et al., 1995) each displaying a specific pattern of expression. NO is important for signal transduction and takes part in several pathways like the glycogen synthase kinase 3 beta (GSK3β) signal transduction pattern in mitochondrial respiration (Ignarro, 1991; Waldman and Murad, 1987). Moreover NO and excessive production of NO is associated with infections and acute and chronic inflammation (Beckman and Koppenol, 1996). In mouse ES cells, NO signalling seems to play an important role in the control of differentiation by improving self-renewal (Tejedo et al., 2010). NO also affects HIF-α activation via several concentration dependent mechanisms including, NO metabolites and oxygen availability. In particular, in human embryonic kidney (HEK-293) cells, low NO concentrations have been shown to induce HIF-1α degradation, while high levels stabilize HIF-1α during normoxia mimicking the hypoxic response (Mateo et al., 2003).

1.2.4.1.2 Regulation of HIF-1α by NO during hypoxia

During hypoxic conditions, low oxygen levels reduce cytocrome c oxidase (CcO), an enzyme of the mitochondrial electron transport chain (Xu et al., 2005b). NO competes with oxygen for binding CcO leading to a reduction in oxygen consumption and inducing a condition called "metabolic hypoxia" in which, oxygen consumption in mitochondria is prevented by NO (Moncada and Erusalimsky, 2002). This leads to an increase in the amount of oxygen available for prolyl hydroxylase that can hydroxylate HIF-1α and generate a condition in which cells fail to register hypoxia (Hagen et al., 2003; Moncada and Erusalimsky, 2002). NO has also been found to modulate and decrease HIF-1α accumulation and DNA binding by PHD activity due to inhibition of CcO (Hagen et al., 2003) (Figure1.11).

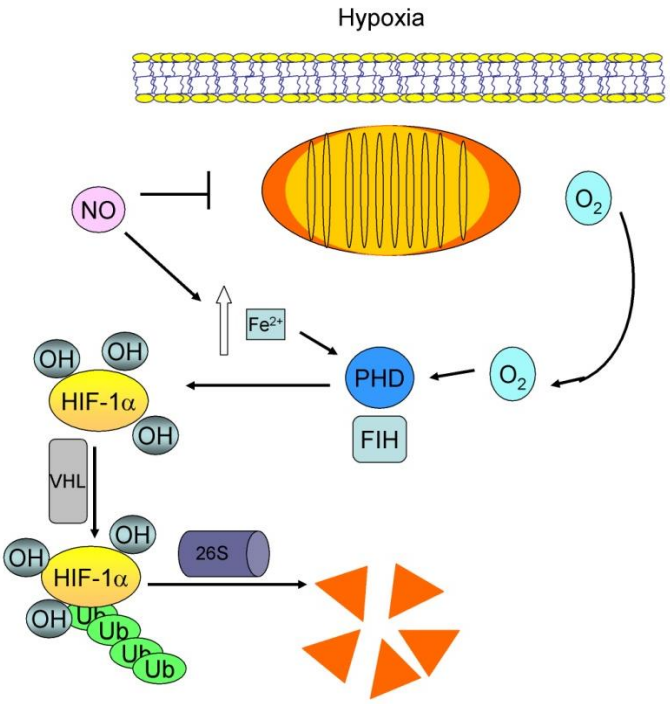

Figure 1.11: Schematic representation of the HIF-1α regulation by NO during hypoxia.

Competition for oxygen binding between NO and cytocrome-c oxidase to replace PHD activity. NO can raise intracellular free iron that increases PHD activity. FIH (Factor inhibiting HIF interacts with PHD in order to hydroxylate HIF and lead to its degradation trough the proteasome. Adapted from Olson and van der Vliet (2011).

1.2.4.1.3 Regulation of HIF-1α by NO during normoxia

NO has been shown to stabilise HIF-1α in normoxia leading to its accumulation and activity (Sandau et al., 2001). In particular, NO interacts with the non-heme $Fe^{2+}$and inhibits PHD and FIH, preventing the hydroxylation of the proline residues that target HIF-1α for degradation through the proteosome. Also, NO and NO donors or S-nitrosothiols (SNOs) like S-nitroso-N-acetylcysteine (SNOAC) (Palmer et al., 2007) or S-nitroso-N-acetyl-penicillamine (SNAP) lead to HIF-1α accumulation and stabilization through PI3K/AKT and MAPK pathway activation by a process called S-nitrosylation (Schleicher et al., 2009). Through this mechanism, SNOs block pVHL recruitment and consequently leads to HIF-1α stabilization. Furthermore, SNOs inhibits FIH (factor inhibiting HIF) and enhance the HIF-C-TAD activity (Figure1.12).

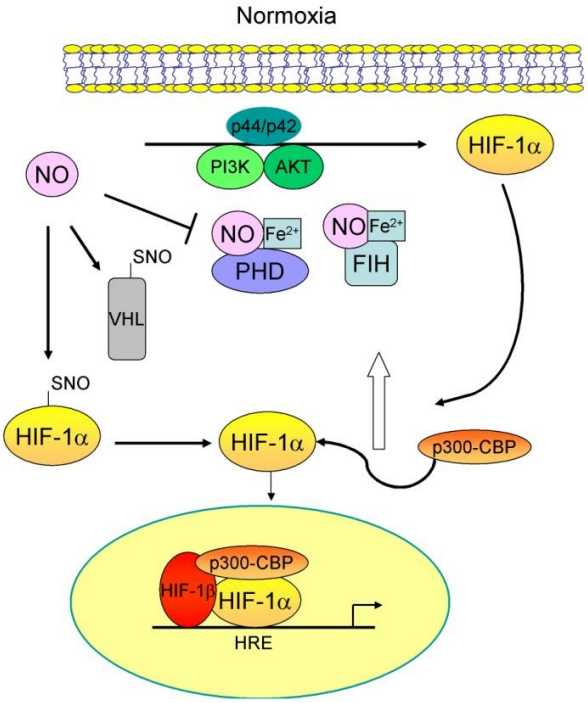

Figure 1.12: Schematic representation of the HIF-1α regulation by NO during normoxia.

NO inhibits PHD and FIH interacting with Fe^{2+} preventing HIF degradation. NO also increases HIF-1α levels through activation of the MAPK pathway which stabilizes HIF-1α by S-nitrosylation. S-nitrosylation of VHL protein prevents HIF-1α degradation. Adapted from Olson and van der Vliet (2011).

1.2.4.1.4 ROS

Recent evidence indicates reactive oxygen species (ROS) as important signalling molecules that mediate changes in oxygen tension and in hormone responses, growth factors and mechanical stress. Moreover ROS appear to have a role in both hypoxic and normoxic HIF-α regulation (Haddad, 2002; Haddad and Land, 2001). In particular, under normoxia, the increased levels of ROS upregulate HIF-α modulating signalling pathways like hydroxylases or kinases and phosphatases. Under hypoxic conditions NADPH oxidases, cytochrome b NAD(P)H oxidoreductases (Goldberg et al., 1988; Zhu et al., 1999) and mitochondria produce ROS and in particular hydrogen peroxide (H_2O_2) which is a reactive oxygen species. H_2O_2 is responsible for proline hydroxylase activity and induces HIF-α proteosomal degradation (Fandrey et al., 1994).

1.2.5 HIF target genes

Cells and organs need to adapt to changes in oxygen supply through the activity of HIFs. HIFs bind a *cis*-acting core consensus HRE in the promoter and enhancer of over 200 genes with varying functions (Semenza et al., 1991). The interaction between HIF-α /β complex and HRE is necessary but not sufficient for the activation of hypoxic genes since a functional HRE often contains additional binding sites for transcription factors that amplify the hypoxic response (Wenger, 2002). HIFs may thus interact with adjacent proteins and transcription factors to form multiprotein complexes which are different in every hypoxia inducible gene. For instance, HIF-α interacts with ATF-1 (Activating Transcription Factor 1) and CREB-1 (cAMP-Responsive Element-Binding-1) in the lactate dehydrogenase A gene (Ebert and Bunn, 1998), with AP-1 (Activator Protein-1) in the VEGF (Vascular Endothelial Growth Factor) gene (Damert et al., 1997) and with the hepatic nuclear factor 4 (HKF4) in the erythropoietin gene (Galson et al., 1995). All these factors cooperate with CBP/p300 (CREB binding protein and p300 are two transcriptional factors with acetyltransferase activity) and are responsible for the fully functional transcription complex on the HRE (Figure 1.13). Often it has been observed that HREs multidimerize with other HREs (Rolfs et al., 1997), as observed in the transferrin HRE and the glucose transporter 1 (GLUT1) (Wenger, 2000).

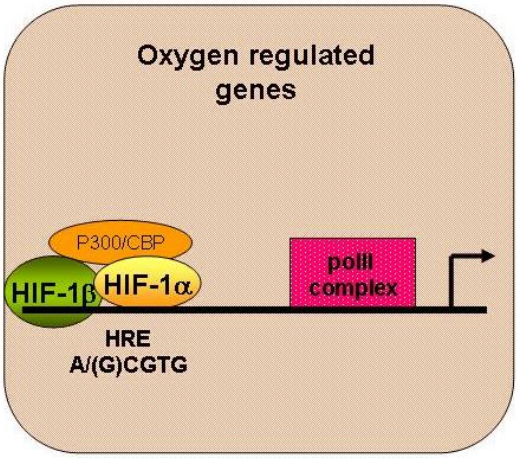

Figure1.13: Schematic representation of HIF-1α binding on HRE of specific target genes.

The diagram shows the HIF-1α/p300/CBP complex on the HRE site and the transcriptional activation of HIF-1α target genes driven by the RNA polymerase II complex. Adapted from Wenger at al. (2000).

1.2.5.1 Glucose Metabolism

Several genes involved in glucose uptake and glycolysis are identified as target genes of HIF-1α (Hu et al., 2003). For example, during the early stages of implantation, the embryo is exposed to an anoxic environment, cellular ATP is formed through the anaerobic glycolysis through the up-regulation of specific glycolytic enzymes and glucose transporters (Rodesch et al., 1992; Sakata et al., 1995). HIF-1α has been found as a modulator of the enzymes in the glycolytic pathway and specifically of the glucose transportes 1 and 3 (GLUT1 and GLUT3) (Chen et al., 2001) whose enhanced activity is associated with increased glucose utilization in hypoxia.

1.2.6 HIF-2α and hypoxic regulation of hES cells

HIF-α subunits have been found to activate genes that contain the HRE sequence located in the promoter region of hypoxia responsive genes (Wenger, 2002) and HIF-2α is able to target this sequence independently to HIF-1α, suggesting that they have different roles (Forristal et al., 2010). Indeed HIF-2α has a unique function in specific tissues and tumours, such as tumours associated with VHL disease or renal clear carcinoma (Harris, 2002; Maxwell et al., 1999). Moreover, it has been found that some hypoxia inducible genes are specifically targeted by HIF-2α rather than HIF-1α (Jiao et al., 2006). In mouse ES cells, HIF-2α has been found as a direct upstream regulator of OCT4, binding to HRE sequences situated in specific Conserved Regions (CR3 and CR4) in the 5' promoter sequence of this gene. It has been found that all the CR (1, 2, 3 and 4) are identical in human, bovine and mouse (Nordhoff et al., 2001), but HIF-2α only recognises CR3 and CR4 in the human OCT4 promoter in RCC cells (Covello et al., 2006). This interaction indicates a potential role of HIF-2α in the regulation of hES cell maintenance. Interestingly, using siRNA HIF-2α was found to regulate the expression of OCT4, SOX2 and NANOG during hypoxic culture, and thus have an important role as a modulator of pluripotency in hES cells (Forristal et al., 2010). This highlights the importance of culturing hES cells under hypoxic conditions. Moreover, it has been found that when HIF-2α is silenced, cells display a decrease in number and colony size (Forristal et al., 2010). However, it remains to be determined whether there is a direct interaction between HIF-2α and the promoter region of pluripotency genes in hES cells. Also, in hES cells the mechanism of hypoxic regulation is different since HIF-1α is only transiently expressed for ~48h of hypoxic culture and HIF-2α seems not to be degraded in normoxic conditions (Forristal et al., 2010), suggesting a potential different mechanism of regulation. Taken together, this information suggest that among the HIFs, HIF-1α is responsible for the adaptive response to hypoxia while HIF-2α is responsible for the long term response to environmental oxygen by regulating hES cell cells pluripotency and proliferation and differentiation (Forristal et al., 2010).

1.3. Epigenetic remodelling and Stem Cells

Epigenetics may be defined as "the study of changes in gene function that are mitotically and/or meiotically heritable and that do not entail a change in DNA sequence"(Wu and Morris, 2001). In fact, epigenetics include all changes in chromatin structure, including DNA repair, replication and recombination, DNA methylation, histone chemical modifications and miRNA expression.

1.3.1 Chromatin regulatory mechanisms in SCs

DNA in the nucleus of eukaryotic cells is packaged by a core of histones into chromatin (Kornberg, 1974). The nucleosome is the repeating unit of chromatin and is formed by 146bp of DNA wrapped in 1.7 superhelical turns around an octamer of hisones (H2A, H2B, H3 and H4 and the linker histone H1). There are three levels of chromatin organization (Figure 1.14):

- The first level of chromatin organization is the "beads-on-a-string" fiber with 11nm diameter (euchromatin which is rich in active gene transcription)
- The interaction between DNA and the linker histone (H1) organizes the nucleosome in a more compact fiber of 30nm (heterochromatin which is inactive)
- The high-order chromatin compaction of the 30nm fiber in the metaphase chromosomes up to 1000nm in thickness

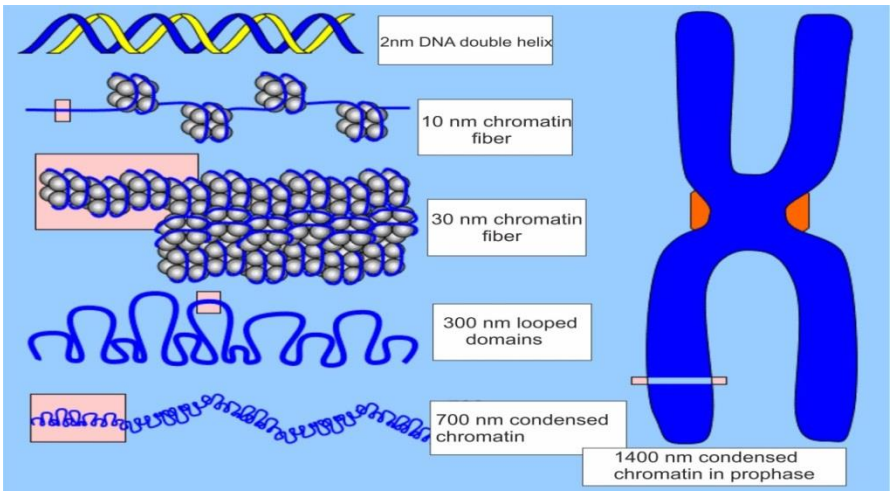

Figure 1.14: Different levels of chromatin compactions

Adapted from Freeman W.H et. al (2005).

Much speculation has occurred regarding the stable and heritable epigenetic mechanisms present in ES cells. In fact the pluripotency and self-renewal, typical characteristics of these cells, are conferred by an unique transcriptional regulation that is the consequence of a balance between euchromatin and heterochromatin dynamic structure (Meshorer and Misteli, 2006). The chromatin of hES cell is euchromatic and transcriptionally active due to the presence of acetylated histones that increase nuclease accessibility (Levings et al., 2006). In contrast, when hES cells start to differentiate, they display a condensed chromatin which is more compact and repressive due to the dynamic incorporation of specific histone variants and structural proteins (Dai and Rasmussen, 2007; Meshorer et al., 2006). The plasticity of hES cell chromatin is fundamental for the rapid transcriptional changes which occur during differentiation and lineage commitment.

OCT4, SOX2 and NANOG are key transcription factors in the maintenance of ES pluripotency. These factors form a "core-regulatory circuitry" (Boiani and Scholer, 2005; Boyer et al., 2005) through feedback mechanisms that ensure the pluripotency state in ES cells. Interactions between the core pluripotency network and chromatin specific remodelling enzymes are essential to stabilize the chromatin in an open structure accessible to gene transcription and to preserve plasticity and pluripotency.

1.3.1.1 Chromatin-Remodelling Complexes

Differentiation of ES cells into specific cell lineages is a process that involves global epigenetic changes in chromatin structure, gene expression and leads to the silencing of stem cell genes and the activation of specific genes. These changes occur at the chromatin level and include regulatory mechanisms such as histone modification, DNA methylation and ATP-dependent chromatin remodelling.

1.3.1.1.1 ATP-dependent chromatin remodelling

ATP-dependent chromatin remodelling enzymes are important regulators of the pluripotent state. There are almost 30 genes that encode for the ATP remodelling enzymes that have been grouped in families based on the ATPase domain. One of the most studied, is the complex SWI/SNF (SWItch/Sucrose Non Fermentable), a yeast nucleosome remodelling complex, composed of several proteins which are highly conserved among different species like flies (Brahama-associated proteins), worms and mammals (Ho et al., 2009). The mammalian homologues the mSWI/SNF or BAF (Brg/Brahama-associated factors) has an ATPase subunit that is necessary for development and differentiation and colony morphology in ES cells (Fazzio et al., 2008; Hansis et al., 2004; Schaniel et al., 2009; Singhal et al., 2010). Studies in mouse ES cells have shown that the BAF subunit undergoes several changes when cells start to differentiate into neuronal progenitors and that mice deficient in these ATPase subunits die at the 2-cell embryo (Bultman et al., 2006). These studies demonstrated that there is a specialized BAF complex, called esBAF (Ho et al., 2009), that is important in maintaining the self-renewal and pluripotency in mouse ES cells (Gao et al., 2008; Ho et al., 2009; Yan et al., 2008). esBAF present specific subunits that are typical for ES cells: an ATPase Brg (Brahama-related gene), two factors BAF155 and BAF60a (Ho et al., 2009) and a protein structural domain or chromodomain which is important for chromatin remodelling (Ho et al., 2009). The eBAF complex interacts directly with the enhancers and promoters of the core pluripotency network, OCT4, SOX2 and NANOG (Figure1.15) (Ho et al., 2009) repressing the transcription of differentiation genes. esBAF also has a role in allowing LIF-STAT3 (leukemia inhibitory factor-signal

transducer and activator of transcription 3 signalling) signalling pathway which is important for self-renewal of mouse ES cell (Ho et al., 2011).

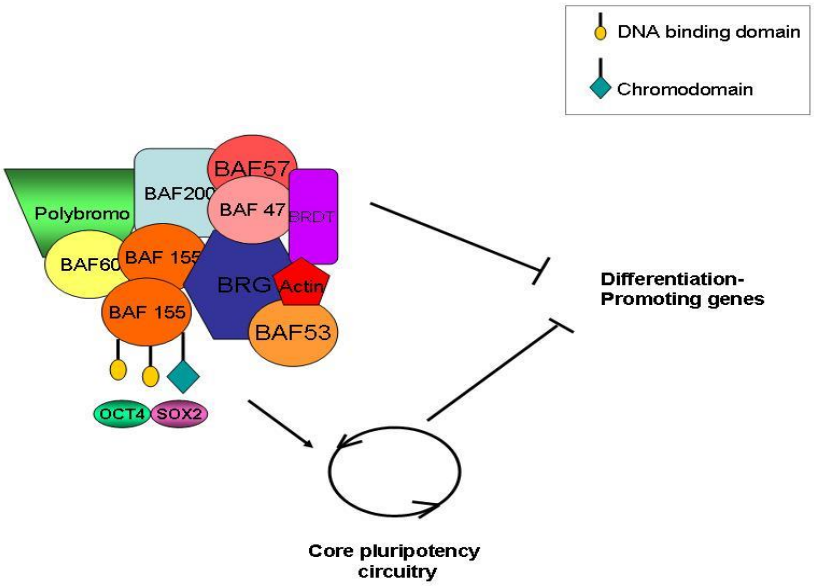

Figure 1.15: Schematic representation of the esBAF complex and its interaction with the core pluripotency transcriptional circuitry.

Diagram showing the esBAF complex and its interaction with the pluripotency transcriptional circuitry to block differentiation genes and maintain pluripotency (colored boxes are the BAF subunits: homodimer BAF155, Brg, BAF47, BAF53, BAF 200, BAF 60, BAF57, polybromo subunit, actin molecule). Domains that allow the subunits to interact with DNA and the protein structural domain (Chromodomain) are explained in the key (Lessard and Crabtree, 2010).

1.3.1.1.2 NuRD complexes

The mammalian NuRD (Nucleosome Remodeling Deacetylase) complex contains several subunits with both ATP-dependent chromatin remodelling and HDAC (Histone Deacetylase Activity) activities (Wade et al., 1998). The complex requires a SNF2/SWI2-related chromatin remodelling ATPase (Mi-2), a member of the MBD family of methyl CpG binding domain proteins (MBD3), histone deacetylases (HDAC1 and HDAC2), a histone binding protein (RbAp46/p48), a protein of unknown function (known as p66), and a subunit encoding the metastasis-associated proteins (MTA1, MTA2, or MTA3) (Wade et al., 1999) (Figure 1.17). These complexes possess either transcriptional repressive and activating functions and have been extensively studied in hematopoietic stem cell self-renewal (Wade et al., 1999; Williams et al., 2004a; Yoshida et al., 2008) and also in ES cell pluripotency and differentiation (Yoshida et al., 2008). Indeed, NuRD physically interacts with LSD1 (lys-specific demethylase 1) leading to silencing of active gene enhancers that are critical for ES cell differentiation (Whyte et al., 2012) suggesting a role for LSD1 in the transition of ES cells into a different developmental stage.

A sub-family of the NuRD complexes called NODE (Nanog and Oct4 associated deacetylase) is able to interact with NANOG and OCT4 and the core of pluripotency circuitry to maintain pluripotency. The NODE complexes have yet to be fully characterized and it is still not clear whether they could be responsible for the regulation of self-renewal or developmental progression (Liang et al., 2008; Wade et al., 1999).

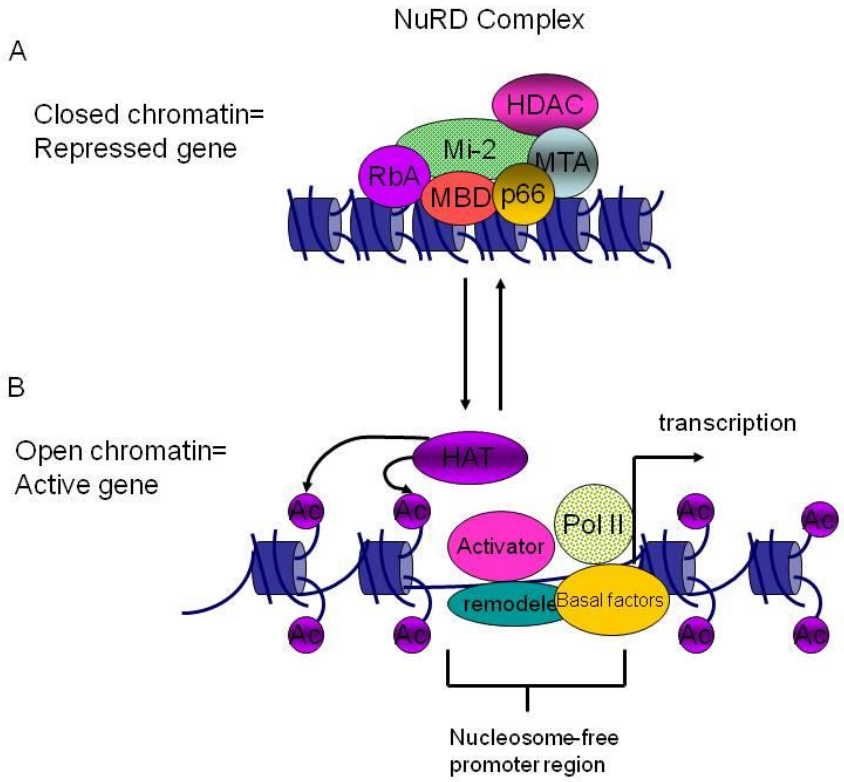

Figure 1.16: Schematic representation of the NURD complex.

Schematic representation of the nucleosome compacted chromatin of a repressed gene (A) and the relaxed chromatin of an actively transcribed gene (B). The Mi-2/NuRD complex has a role in modifying the chromatin structure to initiate and maintain gene repression. HAT= histone acetylases; Pol II=polymerase type II; Basal factors: transcription of machinery. Adapted from Denslow and Wade 2007.

1.3.1.1.3 ISWI complexes

The ISWI (Imitation Switch protein) is a SWI-like ATP-dependent chromatin remodeling factor that was identified in *D. melanogaster* (Eberharter and Becker, 2004). This ATPase is formed by different complexes: NURF (nucleosome remodeling factor), ACF (chromatin-assembly factor) and CHRAC (chromatin accessibility complex) (Dirscherl and Krebs, 2004). In mammals, the ISWI ATPase is called SNF2H or SNF2L, two functionally distinctive subunits. For instance, SNF2L is present in the NURF complex and in the CERF (CECR2-containing remodelling factor), while SNF2H is present in the NoRC (nucleolar remodelling complex), in the WICH (WSTF ISWI chromatin remodelling) complex, ACF and CHRAC complexes (Dirscherl and Krebs, 2004). Among these complexes, NURF and NoRC are important for transcriptional activation and repression, while ACF, CHRAC and WICH are involved in the regulation of nucleosome assembly, in DNA replication and in chromosome segregation but their specific functions in embryonic and ASCs is still unclear (Bozhenok et al., 2002; Eberharter et al., 2001; Hamiche et al., 1999; Langst et al., 1999; Strohner et al., 2001).

1.3.1.1.4 Tip60-p400 complexes

The Tip60-p400 complexes have either histone acetyltransferase or chromatin-remodelling activities so they can be activator or repressors of transcription (Cai et al., 2003a; Ikura et al., 2000). The principal activity of these complexes is to mediate the incorporation and acetylation of histone variant H2AZ in the nucleosome (Sapountzi et al., 2006). It has been observed that mice null for these complexes die before implantation (Gorrini et al., 2007) and that depletion of Tip60-p400 subunits can block self-renewal in ES cells and influence cell morphology (Fazzio et al., 2008). Tip60-p400 also mediates histone acetylation in both active and repressed genes in ES cells (Fazzio et al., 2008).

1.3.1.1.5 CHD1 complexes

The CDH (tandem chromodomain of human) family of ATPase, comprises nine chromodomain-containing members that are classified in three subfamilies based on their principal domain: subfamily I (CHD1 and CHD2), subfamily II (CHD3 and CHD4), subfamily III (CHD5, CHD6, CHD7, CH8 and CHD9) (Hall and Georgel, 2007). Among these factors, CDH1 has been extensively studied as being responsible for the maintenance of chromatin in an open and hyperdynamic conformation in undifferentiated ES cells, preserving lineage plasticity (Gaspar-Maia et al., 2009).

Overall, the hyperdynamic structure of hES cell chromatin is compacted when cells exit the pluripotent state and start differentiating. Chromatin remodellers are fundamental to prevent chromatin compaction (CHD1 complexes) and to repress lineage specific genes (esBAF, Tip60-p400). Transition from self-renewal to specific lineage commitment changes chromatin structure lead to the silencing of pluripotency genes (esBAF and NURD complexes). At the same time, esBAF complexes are able to reverse cell differentiation and reactivate pluripotency genes like OCT4, a process that occurs during nuclear reprogramming observed in *Xenopus laevis* oocytes (Gaspar-Maia et al., 2009) (Figure1.17).

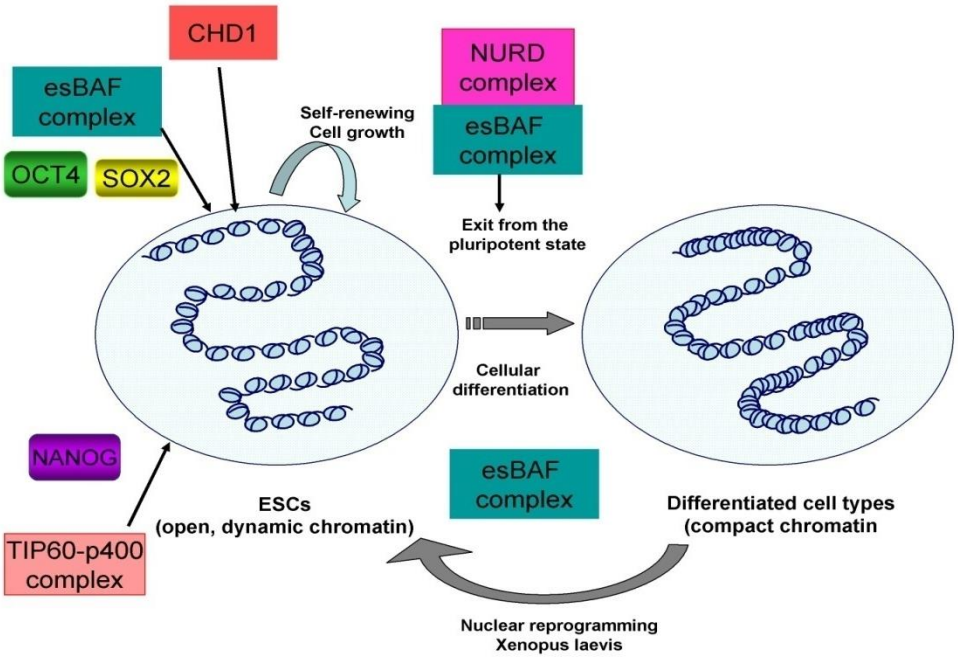

Figure 1.17: Schematic representation of the chromatin remodelling complexes involved in the maintenance of pluripotency.

Adapted from Ho and Crabtree, 2010.

1.3.1.1.6 Polycomb and Trithorax group proteins

Polycomb Group (PcG) and Thritorax Group (TrxG) are multiprotein complexes that maintain chromatin in a silent or active state and have been implicated in stem cell maintenance (Boyer et al., 2006; McMahon et al., 2007; Pasini et al., 2007). There are two important PcG with distinctive and non-overlapping repressive functions: PRC1 is an E3 ubiquitin ligase responsible for chromatin condensation and gene silencing (de Napoles et al., 2004; Francis et al., 2004; Francis et al., 2001; Morey et al., 2012) and PRC2 catalyses the tri-methylation of histone H3 lysine 27 (H3K27me3) (Cao et al., 2002; Cao and Zhang, 2004). The Thritorax group is also a multiprotein complex responsible for transcriptional activation catalysing the H3K4me3 histone modification and stabilization of transcription on the start site. Together, PcG and TrxG form a bivalent and transcriptional chromatin as they can switch the transcription from inactive to active (Boyer et al., 2006; Lee et al., 2006; Mikkelsen et al., 2008). In particular PRC1 and PRC2 have been found to be regulators of lineage commitment in ES cells by silencing genes causing inappropriate differentiation (Pasini et al., 2007). Genome wide analysis has also indicated that OCT4, SOX2 and NANOG are target genes of PcG (Bernstein et al., 2006; Boyer et al., 2006; Lee et al., 2006) and that another factor, JARID2, member of the JumonjiC (JmjC) domain, forms a complex with PcG and promotes the recruitment of the pluripotency genes (Pasini et al., 2010; Peng et al., 2009). These interactions are not fully characterized but seem to regulate the balance between self-renewal and differentiation in ES cells.

1.3.2 DNA Methylation and pluripotency

DNA methylation is a covalent modification of the DNA that involves the addition of a methyl group on the cytosine C5 of CpG island dinucleotides. The reaction is catalyzed by the DNA methyltransferases (DNMTs) and usually occurs on 70-80% of the cytosines that precede a guanosine in the DNA sequence. These particular sequences are called CpG dinucleotides (Bird, 2002). In some genome regions it is possible to find dense clusters of CpGs, usually of 300-3000 base pairs called CpG islands (Gardiner-Garden and Frommer, 1987). It has been estimated that CpG islands are associated with 70% of human promoters and also with developmental genes (Davuluri et al., 2001; Saxonov et al., 2006). The methylation status of all genes with CpG island rich promoters, is inversely related to gene expression (Li et al., 1993). This is thought to be due to interference with transcription factor binding or recruitment of repressor factors (Bogdanovic and Veenstra, 2009) and has been implicated in cell differentiation, X chromosome inactivation and tissue specific gene expression (Farthing et al., 2008). There are three DNA methyltransferases: DNMT3A, DNMT3B and DNMT1 and one regulatory protein DNMT3L that shows no methyltransferase activity but is responsible for *de novo* methylation in germ cells (Jia et al., 2007). Among these enzymes, DNMT3A and DNMT3B cause DNA methylation by targeting unmethylated CpG sites, while DNMT1 is important for methyltransferase maintenance during cell division and preferentially hemi-methylates CpG islands through interaction with UHRF1 (Ubiquitin like, containing PHD and RING finger domain 1) (Avvakumov et al., 2008; Sharif et al., 2007). It has been demonstrated that in mouse ES cells, DNMT3L together with DNMT3A and DNMT3B interact with histones, in particular with the unmethylated Lysine 4 of H3 (H3K4) leading to DNA methylation. These DNA methyltransferases are also responsible for the DNA methylation state of the blastocyst (Okano et al., 1999; Otani et al., 2009; Zhang et al., 2010). Interestingly, the DNMTs are highly expressed in ES cells and defects in expression have been associated with hypomethylation and reduced differentiation capacity (Chen et al., 2003). DNMTs, can methylate histones and being responsible for gene transcription. In embryonic and somatic SCs there is an inverse relationship between DNA methylation and histone H3K4 methylation (Hodges et al., 2009; Meissner et al., 2008), while other histone modifications like H3K36me3,

H3K9me3 and H3K27me3 have been involved in DNA methylation of specific chromatin regions (Dhayalan et al., 2010) (Figure 1.18).

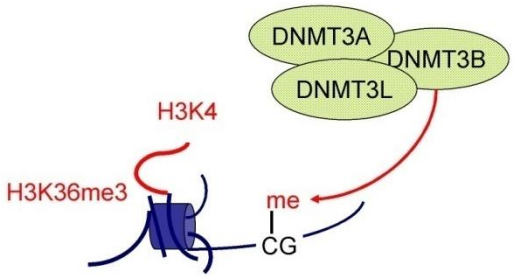

Figure 1.18: Schematic representation of the interaction between DNA methyltransferases and the unmodified Lys4 of Histone H3

Adapted from Denis et al. (2011).

Silencing of gene expression is associated with DNA methylation through the interaction with methyl-CpG binding proteins or in association with HDAC (Histone deacetylase) or by blocking transcription factors (Robertson et al., 2000). CpG islands of actively expressed genes are unmethylated, while silent genes tend to be methylated (Razin and Cedar, 1991), so promoters of pluripotency genes like OCT4, SOX2 and NANOG in ES cells, are unmethylated and became methylated when cells differentiate (Meissner et al., 2008). However, the precise epigenetic signature of hES cells is still not completely known therefore several studies are needed. In particular, studies on cancer cells have allowed a better understanding of the epigenetic mechanisms that occur in hES cells. In fact, it has been found that hES cell and cancer cells have some common characteristics such as their ability to survive in long term culture, for this reason they could have a similar epigenetic pathway that is currently being studied (Altun et al., 2010; Bibikova et al., 2008). One of the most important studies to determine whether it is possible to identify a pluripotent ES cell from a differentiated cell was the discovery that hES cells have a unique DNA methylation signature (Bibikova et al., 2006). ES cells possess high methylation levels in 23 genes encoding proteins involved in cell signalling, stress and apoptosis, cell cycle progression and HLA locus, compared to cancer cells. Also DNA methylation decreases during early

embryonic development in the zygote as the paternal genome is demethylated while the maternal genome displays low methylation until the eight cell stage where the methylation pattern is then reactivated (Reik et al., 2001). Sequencing-based methods of high-throughtput molecular analysis developed in recent years have shown that the methylation profile of hES cell vary enormously when cells are going to differentiate and also that the majority of unmethylated genes correspond to housekeeping and pluripotency genes, while a set of methylated genes with low CpG sites were associated with cell differentiation (Fouse et al., 2008).

1.3.3 Histone modifications

Post-translational modifications of histone residues are associated with transcriptional activation or repression (Kouzarides, 2007; Vermeulen et al., 2010). Histones are subject to several modifications like acetylation, methylation, phoshorylation, ubiquitination. For instance lysine acetylation is associated with gene repression, while lysine methylation is related to both activation and repression based on the particular residue modified (Wang et al., 2009). Also, histones in a lysine residue can be mono-,di-or tri-methylated and be responsible for gene expression or silencing (Wang et al., 2009).

1.3.3.1 Modifications at active promoters (H3K4me3 and H3/H4Ac)

Histone H3/H3Ac, H3K4me3 or H4K4me2 marks are associated with transcriptional activation (Santos-Rosa et al., 2002) and have been found in the promoters of all transcribed genes. Other modifications like H3K36me3 and H3K79me3 were found only in actively transcribed regions (Edmunds et al., 2008). A balance between histone methylation and demethylation is important for gene activation and silencing. For instance, trimethylation of H3K4 is catalysed by lysine methyltransferase and in particular by the SET-domain of the Trithorax group while its deregulation is mediated by histone demethylases such as the Jumonji domain containing (Jmjd) family (Klose and Zhang, 2007). It has been recently discovered that the demethylation of H3K4me2/3, H3K27me2/3 or H3K29me2/3 is particularly important for hES cell self-renewal, pluripotency, differentiation (Klose and Zhang, 2007; Peng et al., 2009;

Yamane et al., 2006). Acetylation of H3 and H4 is also associated with gene transcription. This modification is catalyzed by HAT (histone acetyltransferase) and HDAC (histone deacetylase) enzymes (Xu et al., 2007) that interact with remodelling proteins allowing chromatin to be in an open conformation accessible to transcription factors.

1.3.3.2 Modifications at silenced promoters (H3K27me3 and H3K9me3)

Methylation at specific histone residues like H3K9, H3K27 and H4K20 is associated with gene repression and usually occurs in transposons, repetitive sequences or pericentromeres (Mikkelsen et al., 2007). It is still unclear which enzymes are responsible for these modifications but it is known, for example that H3K9me3 is catalyzed by several lysine methyltransferase (HMT) like SUV39h, SetDB and G9a which are able to methylate DNA through the interaction with MBD proteins (methyl binding domain proteins) and HDACs to form a higher-order chromatin structure that is inaccessible to transcription (Agarwal et al., 2007; Fujita et al., 2003). H3K27me3 is methylated by subunits of the Polycomb group and is a marker for silenced genes and, in ES cells is important for lineage commitment (Lee et al., 2007). The level of chromatin compaction in ES cells seems to be an epigenetic barrier for somatic reprogramming as it has been demonstrated that G9a or JHDM2A knock down increases the efficiency of iPS cells (Ma et al., 2008).

1.3.4 High-order chromatin structures

Recent studies have reported that, beyond the "nucleosomes-on-a string" structure, there is a higher order chromatin organization that has important consequences on gene regulation and pluripotency of ES cells. The technique of Chromatin conformation capture (3C) to study spatial genomic organization has allowed researchers to demonstrate that Oct4 is responsible for a high order chromatin structure within the Nanog promoter in ES cells (Levasseur et al., 2008). The presence of long range interactions in the Nanog promoter is responsible for bringing together distant regulatory elements to enhance Nanog expression (Levasseur et al., 2008). These high order chromatin interactions are mediated by the insulator protein CTCF, cohesin and Mediator chromatin co-activators which co-occupy several loci within the enhancers and core promoter of pluripotency genes (Kagey et al., 2010; Tutter et al., 2009). Indeed, silencing of Mediator and cohesin has been found to induce differentiation in ES cells as a consequence of the loss of chromatin looping formation (Kagey et al., 2010). Furthermore, a recent study showed the presence of a particular network of chromatin interactions between promoters and enhancers called "topologically associating domains" (TAD) which regulates not only gene expression, but also nuclear lamina organization (Dixon et al., 2012). The boundaries of the TAD are enriched for CTCF insulator binding proteins which also modulate TAD function (Dixon et al., 2012). However, it is not known which other proteins are involved in these interactions and how chromatin organization changes during ES cell differentiation. A recent study documented the genome-wide chromatin interactions of Nanog in mouse ES or human iPS cells and showed that key pluripotency factors are able to bring together the Nanog interacting genes to enhance its transcriptional activation (Apostolou et al., 2013). These interactions were also mediated by Mediator and cohesin components which have been found responsible for a 3D chromatin organization within Nanog in ES and iPS cells (Apostolou et al., 2013). More recently, it has been found that Polycomb complexes are responsible of the long-range chromatin interactions in mouse ES cells in which pluripotency genes occupy distinct nuclear spaces that relates to the open/closed chromatin state (Denholtz et al., 2013). Finally, non-coding RNAs (lncRNAs) have also been found involved in ES differentiation through the formation of chromatin loops or through binding to chromatin modifiers (Wang et al., 2011). Although, the *in vivo*

significance of these findings is still unclear they highlight the importance of high order chromatin structure in pluripotency and differentiation of ES cells.

1.3.5 MicroRNAs (miRNA) and epigenetics

miRNA are small non-coding RNAs that are involved in target gene silencing either through translational repression or degradation (Lee et al., 1993). In the nucleus miRNA are transcribed by RNA polymerase II (Pol II) into long segments of non-coding RNA called primary miRNAs or pri-miRNA (Lee et al., 2004). Pri-miRNA are then processed by a complex containing RNase III Drosha and the binding protein DiGeorge syndrome critical region gene 8 (DGCR8) into the precursor miRNAs or pre-miRNA (Kim et al., 2009c). Pre-miRNAs are exported into the cytoplasm by Exportin-5 and processed by another RNase III, Dicer into a functional mature final miRNA (Lee et al., 2002). More than 1000 miRNAs have been found in the human and are important for modulating gene expression. Interestingly, miRNAs might control both epigenetic mechanisms or induce epigenetic modifications within a target gene (Chuang and Jones, 2007). In particular, several miRNAs have been found to have a role in the control of chromatin structure by regulating histone modifier molecules, such as the polycomb complex and histone deacetylase (HDAC) (Godlewski et al., 2008; Noonan et al., 2009). For instance, in prostate cancer, downregulation of miR-449a, causes overexpression of HDAC-1 leading to an increased cancer invasiveness (Noonan et al., 2009). In mouse ES cell differentiation, miRNAs seem to have a role in the establishment of the *de novo* DNA methylation. Indeed, mutations in Dicer block the activity of the ES-specific miR-290 and impair Oct4 DNA methylation and subsequent mouse ES cell differentiation. (Sinkkonen et al., 2008). Furthermore, several miRNAs such as miRNA-1 and 133, are involved in the control of mouse ES cell commitment by promoting mesoderm differentiation (Ivey et al., 2008) This mechanism of miRNA regulation remains still unknown but it has been found that the expression of some miRNAs is transcriptionally repressed in undifferentiated ES cells and then activated when cells start to differentiate (Singh et al., 2008). However, further studies using targeted silencing of the miRNAs pathways will be needed to fully elucidate their role on gene expression, epigenetics and ES cell fate.

1.3.6 Chromatin Modifications in response to Hypoxia

Although HIF regulation is well described in literature, little is known about the epigenetic changes that are associated with the hypoxic response. However, there is increasing evidence that adaption to hypoxia, influences chromatin remodelling through several epigenetic mechanisms:

- Epigenetic regulation of VHL and PHD in HIF stabilization (Hatzimichael et al., 2010; Hatzimichael et al., 2009)
- Open chromatin conformation around the HIF binding sites and interaction between HIF and co-factors (Kallio et al., 1998)
- Histones demethylase enzymes play an important role in gene transcription during hypoxia (Beyer et al., 2008)
- Hypoxia changes histone modifications and DNA methylation pattern (Johnson et al., 2008)

1.3.6.1 Role of HIF co-factors in the hypoxic response

HIF co-activators like histone acetyltransferases (HATs), histone deacetylases (HDACs), p300-CREB binding protein (CBP), and the SWI/SNF chromatin remodelling complex, play an important role in modifying the chromatin and making it more accessible to the transcription factors (Wang et al., 2010). HATs are enzymes associated with transcriptional activation and, through their ability to acetylate histones and recruit remodelling factors, release the chromatin in an open conformation (Peterson and Laniel, 2004; Shogren-Knaak et al., 2006). p300-CBP are important HIF co-factors as they have HAT activity and interact with the transcription machinery elements increasing gene transcription (Arany et al., 1996; Kalkhoven, 2004). These co-factors are regulated by hypoxia and usually interact with the 2 trans-activation domains (NTAD and CTAD) of the HIF protein (Dames et al., 2002; Ruas et al., 2010). During normoxia, Factor Inhibiting HIF (FIH-1) hydroxylates a conserved asparagine residue within the CTAD domain of HIF, blocking the interaction between HIF and p300/CBP, while under hypoxia, FIH-1 is inhibited and HIF can interact with p300/CBP.

Recently, a kinase linked to glucose metabolism, pyruvate kinase M2 (PKM2) has been found to interact with HIF-1α in MEFs and Hela cells (Luo et al., 2011). This protein

seems to interact also with prolyl hydroxylase 3 (PHD3) forming a feedback loop that is able to enhance HIF-1α activity (Luo et al., 2011). Among the HIF-1α co-activators, minichromosome maintenance (MCM) proteins have also been found important in regulating HIF-1α transcriptional activity in human embryonic kidney (293T) cells (Hubbi et al., 2011). MCM proteins are down-regulated under hypoxia but present an increased expression in normoxia where they enhance HIF-1α degradation (Hubbi et al., 2011). Furthermore, it is well established that hypoxia can increase or decrease specific histone modifications in hypoxia-responsive genes leading to their activation or repression as is summarized in table (Table 1.1).

Gene	Event	Reference
Hypoxia-induced genes:		
VEGF (Vascular Endothelial Growth Factor)	↑H3ac ↑H3K4me3 ↓H3K27me3	(Johnson et al., 2008) (Jung et al., 2005)
EPO (Erythropoietin)	↑H3ac ↑H4ac	(Wang et al., 2010) (Wang et al., 2004)
EGR1 (Early growth response protein-1)	↑ H3ac ↑ H3K4me3 ↓ H3K9/27me2 ↓ H3K27me3	(Johnson et al., 2008)
ADM (Adrenomedullin) GDF15 (differentiation factor 15)	↑ H3K9me2	(Krieg et al., 2010)
HMOX1 (Heme oxygenase) DAF (Insulin-like receptor)	↑ H3K4me3	(Zhou et al., 2010)

Table 1.1: Hypoxia induced histone modifications in the promoter regions of hypoxia inducible genes.

Table showing histone modifications that occur at the promoter region of the hypoxia inducible genes. Events associated with transcriptional activation are highlighted in green while those associated with transcriptional repression are highlighted in red. Up and down arrows indicate the increase or decrease of histone modifications respectively. Adapted from Perez-Perri et al. (2011).

Another mechanism of chromatin regulation is mediated by the HDACs (histone deacetylases) (Yang and Seto, 2003) which negatively regulate gene transcription (Chen and Sang, 2011) but are able to modulate HIF transcription either positively or negatively. In particular while HDAC1 seems to exert a negative effect on HIF target genes (Lee et al., 2010a; Lee et al., 2010b), HDAC4, HDAC5 and HDAC7 promote activation on the HIF target genes (Kato et al., 2004; Seo et al., 2009). Furthermore, to enhance gene expression, HIF interacts with p300/CBP co-activator to form a multiprotein complex with HDAC4, HDAC5 and HDAC7 on HIF target genes promoter (Figure 1.19).

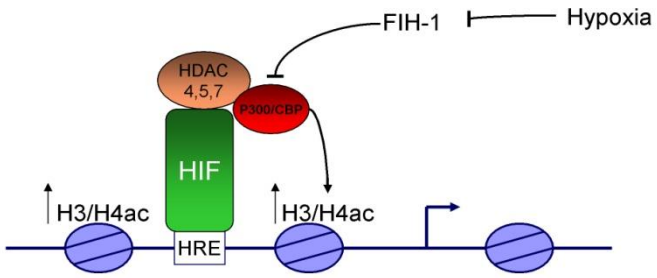

Figure 1.19: HIF target gene induction through p300/CBP and HDAC4, 5, 7 cooperation during hypoxia.

p300/CBP interacts with HIF and acetylates histones in HIF target promoter. Interaction between HIF and p300/CBP is mediated in hypoxia through FIH-1 inhibition. HDAC4, 5, 7 form a complex with HIF and p300/CBP leading to an increase in HIF transcriptional activity. Adapted from Perez-Perri et al. (2011).

1.3.6.2 Role of Chromatin remodelling factors during hypoxia

One of the most important and best characterised chromatin remodelling factor is the SWI/SNF complex that increase the access of transcription factors to the DNA using the energy derived from ATP hydrolysis (Tang et al., 2010b). During hypoxia, the SWI/SNF complex is involved in the transcriptional activation of HIF and its target genes (Kenneth et al., 2009; Wang et al., 2004) through changes in the enhancer and promoter chromatin structure (Figure 1.20). Another chromatin modifier complex, ISWI, an actin dependent chromatin regulator, is involved in the cellular response to hypoxia (Melvin et al., 2011). In particular, ISWI is required for FIH expression and its deletion has been associated with an increase in apoptosis under hypoxia revealing a potential role of ISWI as a survival factor (Melvin et al., 2011).

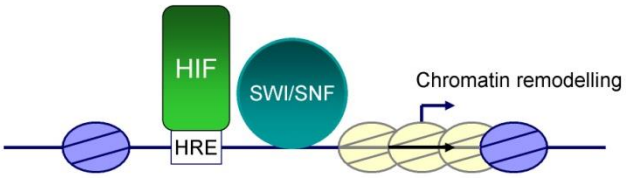

Figure 1.20: Schematic representation of the SWI/SNF chromatin remodelling complex and its activity on the promoter of HIF target genes.

Schematic representation of the SWI/SNF chromatin remodelling complex which activates the expression HIF target genes. Adapted from Perez-Perri et al. (2011).

1.3.6.3 Role of the Jumonji-Domain under hypoxia

Histone methylation at lysine (K) or arginine residues is an important modification that regulates gene expression leading either to an enhancement or repression of gene transcription. Usually, histone H3 methylated at lysine residue 4, 36 and 79 is associated with gene transcription, while histone H3 methylated at lysine residue 9 and 27 and histone H4 methylated at lysine 20 are the markers of gene suppression and chromatin inactivation (Jenuwein and Allis, 2001; Peterson and Laniel, 2004; Shi and Whetstine, 2007). Over recent years it has been demonstrated that methylation is a process that involves either histone methyltransferases or demethylases (HDMs) (Shi and Whetstine, 2007). Among the histone demethylase family, the Jumonji C (JmjC)-domain containing histone demethylase (JHDM) is the best characterized to have a role in hypoxia (Krieg et al., 2010). In fact, some members of the JHDM family seem to be expressed during hypoxia in order to restore the histone demethylase activity that is blocked under hypoxic conditions. In this way the JHDM family play an important role in histone homeostasis (Xia et al., 2009).

In particular, JMJD1A (JmjC histone demethylases protein 1), is necessary for the hypoxic activation of several hypoxia inducible genes that are targets of HIFs and, moreover, JMJD1A is a HIF target gene that may serve to maintain the chromatin in an open conformation accessible to the activation of hypoxic genes (Krieg et al., 2010). In contrast, inhibition of the JARID1A (Jumonji/ARID domain containing protein 2) histone demethylase seems to promote H3K4me3 activity during hypoxia (Zhou et al., 2010) (Figure 1.21).

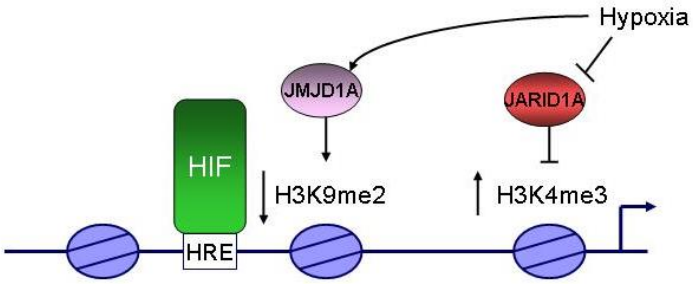

Figure 1.21: Schematic representation of the JHDM during hypoxia.

Low oxygen concentration inhibits JARID1Aand increases JMJD1A leading to an increase in H3K4me3 and a decrease in H3K9me2 in the target promoter. Adapted from Perez-Perri et al. (2011).

1.3.6.4 Other proteins mediating histone modifications in hypoxia

Specific histone modifications are associated with the presence of histone modifiers that have an important role under hypoxia. Indeed, hypoxia enhances the levels of the N-methyltransferase 2 (G9a) which increases the expression of H4K9me2 and induces the repression of several hypoxia inducible genes (Chen et al., 2006). G9a is also responsible of the methylation of Reptin, a subunit of the Tip60 lysine acetyltransferase 5, in mouse embryo and MEFs cells (Lee et al., 2010a). Reptin is a negative regulator of tumor growth and invasion as, through the recruitment of HDAC1, it regulates the expression of several hypoxia inducible genes such as VEGF, BNPI3 and PGKI (Lee et al., 2010a). In contrast, methylation of Pontin, a chromatin remodelling factor, by G9a is responsible of tumor invasiveness under hypoxia (Lee et al., 2011). Finally, the histone variant H2AX seems to be required for the neovascularisation process in Huvec cells and mice under hypoxic conditions (Economopoulou et al., 2009). These studies provide further evidence that the hypoxic response is mediated not only by histone modifications but also by histone modifiers.

1.3.7 DNA methylation under hypoxia

DNA methylation and chromatin modifications are interconnected by the interactions that occur between DNMTs and HMTs. Indeed, G9a has been found to induce the *de novo* DNA methylation within the Oct3/4 gene through the interaction with DNMT3A and DNMT3B (Gidekel and Bergman, 2002). This mechanism occurs during gastrulation when the embryo starts to divide into the 3 germ layers and led to Oct3/4 inactivation through an increase of local heterochromatin formation (Feldman et al., 2006).

One of the first evidence of the influence of hypoxia on DNA demethylation has been observed in hepatoma cells where HIF-1α was able to modulate the expression of MAT2A (Methyonine adenosyltransferase 2A), a marker of genomic methylation (Liu et al., 2011). These data correlated with increased tumor size and invasiveness.

DNMT1 and DNMT3A down-regulation under hypoxia contribute to the tumor-associated CpG demethylation which affects tumour progression in human colorectal cancer (Skowronski et al., 2010). Finally, previous data in neural progenitor cells showed that DNA methylation regulates the activity of HIF-1α hypoxia-regulated microRNAs, such as miR-210, independently of its expression (Xiong et al., 2012). However, more epigenetic mechanisms that are responsible of HIFs activation under hypoxia remain still to be elucidated.

Although chromatin and DNA modifications play an important role in gene regulation under hypoxia, very little is still known regarding the hypoxic regulation of hES cells. Hence, more research is required in order to understand the epigenetic modifications that occur under hypoxia and their biological role. The characterization of these factors will help to elucidate the transcriptional regulation of pluripotency genes in hES cells cultured under hypoxic conditions.

1.4 Hypoxia Preconditioning in Stem Cell therapy

Ischaemic preconditioning (IPC) was first observed by Murry et al. (1986) who demonstrated that exposure to short cycles of ischemia (hypoxia) with intermittent reperfusion of oxygen allowed the heart to be more resistant to a future lethal episode (Murry et al., 1986). To date, IPC represents one of the most powerful mechanisms of protection against ischaemic injury. The IPC induces two distinct windows of protection, the "first window of protection" occurs immediately following the IPC stimulus and lasts 3 to 4 h. After this step the protective effect wanes and re-appears after 12 to 24 h up to 72h which is called the "second window of protection" or delayed IPC (Hausenloy et al., 2010; Kuzuya et al., 1993). However, translated into clinical practice, this effect is limited by the difficulties in manipulating an injured heart. Indeed, several studies are now focused on discovering the mechanisms which underly the protective effect of IPC. It is believed that IPC involves multiple signalling pathways including growth factors (VEGF, IGF), mitochondrial K_{ATP} channels, cytokines and nitric oxide (Huffmyer and Raphael, 2009) therefore, current pre-clinical studies are based on non-genetic strategies to improve the survival and function of SCs and progenitor cells (Mahaffey et al., 1999; Ross et al., 2005).

1.4.1 *In vitro* and *ex vivo* ischaemic and hypoxic preconditioning in SCs

Conditions developed *in vitro* to mimic IPC are usually based on the use of hypoxia or anoxia followed by reoxygenation, termed hypoxic preconditioning (HPC) (Li et al., 2009; Sharma et al., 2008). HPC protocols are usually based on multiple cycles of brief hypoxia with intermitted reoxygenation (Williams and Benjamin, 2000) or a single long-term exposure to hypoxia followed by reoxygenation (Cai et al., 2003b). Such HPC protocols have been shown to induce cytoprotection in stem and progenitor cells *in vitro* through multiple signalling molecules such as NOS2 (Xi et al., 2002), HIF-1α, VEGF, EPO (erythropoietin) and chemokines C-X-C chemokine receptor type 1 (CX3CR1) and 4 (CXCR4) (Hu et al., 2008; Hung et al., 2007). However, one of the major problems to overcome is the poor survival and retention of transplanted stem and

progenitor cells in the ischaemic environment which, in the ischemic myocardium, has been found due to necrosis, ROS, inflammatory response and apoptosis (Goussetis et al., 2006; Terrovitis et al., 2010). These limitations reduce the clinical application of preconditioning to a few cases such as remote ischemic preconditioning (RIPC) that uses ischemic insult applied to limbs or legs to protect the CNS (Przyklenk and Whittaker, 2011). However, HPC has been found to increase the survival and cytoprotection of SCs and progenitor cells also in *in vivo* studies using clinical ischemic models in which HPC improved the functional recovery of the ischaemic tissue (He et al., 2009; Kubo et al., 2008; Uemura et al., 2006; Volkmer et al., 2010). Using this approach, transplantation of hypoxic preconditioned MSCs into mice ischaemic myocardium was able to reduce the infarct size, increase angiogenesis and improve the functionality of the heart tissue (Hu et al., 2008). These affects are attributed to the increase of survival signals including signal transduction pathways that up-regulate proteins such as growth factor VEGF (Potier et al., 2007), anti-apoptotic proteins like Bcl-2 (Francis and Wei, 2010; Theus et al., 2008), antioxidants like heme oxygenase-1, catalase and superoxide dismutase (Peterson et al., 2011), transcription factors like HIF-1α (Liu et al., 2010) which activates other signal transduction pathways like PI3K/Akt (Francis and Wei, 2010), CXCR7 anti-apoptotic proteins (Liu et al., 2010) and miR-210 (Kim et al., 2009b).

1.4.2 Effect of preconditioning on the differentiation and engraftment of SCs

Several studies are now focusing on the functional improvement of HPC to promote an efficient differentiation of SCs in the target tissues after transplant (Theus et al., 2008). However, the role of HPC in SC differentiation is still controversial. Numerous studies have demonstrated that hypoxia is able to induce rat MSC differentiation compared to the normoxic cultures, through the stabilization of HIF-1α (Lennon et al., 2001; Ren et al., 2006). In contrast, other studies have demonstrated that hypoxia inhibits differentiation of human bone marrow derived MSC (Potier et al., 2007; Volkmer et al., 2010) and adipose derived mesenchymal stem cells (Malladi et al., 2006; Wang et al., 2005). Similar controversial results were also been observed in studies on mouse and human ES cells where HPC was found to increase stem cell neural differentiation after transplantation (Francis and Wei, 2010; Theus et al., 2008) while in another study the

short term exposure of human MSCs to hypoxia inhibited differentiation (Potier et al., 2007). It is likely that differences in species and cell type used, together with variation in the duration of hypoxia may explain these differing results. In terms of engraftment functionality, an *in vitro* study demonstrated that HPC increases the gap-junctional intercellular comunication between the neural SCs subjected to preconditioning and the host cells (Jaderstad et al., 2010). Furthermore, it has been found that HPC induces the secretion of soluble factors which serve for the differentiation of MSC cardiomyocytes (Xie et al., 2006). However, it is still not known whether the effect of HPC in promoting cell differentiation and engraftment can be maintained when cells are transferred into adult tissues. Early studies on mouse and pig MSCs and peripheral blood mononuclear cells showed that HPC increases survival, angiogenesis and resistance against oxidative stress when these cells were transplanted into a mouse ischemic tissues (Jaussaud et al., 2013; Kubo et al., 2008; Wei et al., 2012). These works are encouraging and demonstrate that HPC is able to enhance cell survival and could be a useful approach for application of SC based therapies for tissue repair.

1.4.2.1 Stem cell migration and proliferation

In SC therapies it is important to consider the ability of the transplanted stem or progenitor cells to migrate to the injured tissue and enhance repair and regeneration. Exposure to hypoxia and reoxygenation has been shown to enhance cell migration of stem and progenitor cells through the activity of chemokines such as CXC3R1 and CXCR4 (Hung et al., 2007; Rosova et al., 2008) and MSC adhesion through the up-regulation of CXCR4 and CXCR7 (Liu et al., 2010). The induction of these chemokine receptors has been shown to be driven by HIF-1α (Schaniel et al., 2009; Tang et al., 2009). In addition, HPC enhances the migratory function of preconditioned stem progenitor cells through the up-regulation of cytokine signalling, KDR/VEGF expression and tyrosine kinase receptor (Rosova et al., 2008). Since SCs reside in physiological niches where the level of oxygen is about 2-7% (Levesque and Winkler, 2011), it is important to consider the endogenous microenvironment in which these cells are derived in order to maintain their characteristics and to evaluate how these cells respond to a hypoxic environment in injured tissues. Studies investigating the effect of hypoxia on SC proliferation have shown that, compared to the routine normoxic culture condition of 20% oxygen, human MSCs cultured at 1% oxygen reduce their

proliferative potential (Hung et al., 2007). In contrary, bone marrow derived MSC cultured under 2% oxygen showed an increased proliferation (D'Ippolito et al., 2006). However, this might be due to the differences in the hypoxic conditions, cell type and culture (Das et al., 2010). Importantly, short term exposure to hypoxia did not affected the proliferative state of hES cells and human MSCs which is important for the therapeutic applications of HPC (Francis and Wei, 2010; Rosova et al., 2008).

1.4.3 Effect of hypoxic preconditioning on endogenous stem and progenitor cell mobilization

Endogenous stem and progenitor cells may be mobilized from the specific niche(s) where they reside to various organs and tissues via the bloodstream. This property of SCs is called “homing” and has been extensively studied as it could serve to recruit endogenous SCs or progenitor cells to regenerate the injured tissue (Krankel et al., 2011). Beside homing via the bloodstream, a clonogenic population of proliferative SCs may also be recruited to an injured tissue for the healing processes, a condition that is also under investigation for therapeutic purposes (Discher et al., 2009). In particular, it has been found that HPC is able to mobilize rat MSCs from the niches and to protect the infarcted tissue from damage (Rosova et al., 2008). Indeed, subsequent studies found that endothelial progenitor cells and endogenous MSCs are mobilised to the heart of rats when exposed to HPC (Gyongyosi et al., 2010; Rochefort et al., 2006). Moreover in response to the HPC, endothelial progenitor cells produced an array of cardio-protective cytokines and signalling molecules such as eNOS, EPO, VEGF and colony stimulating factors (Gyongyosi et al., 2010; Ii et al., 2005). Furthermore, another study demonstrated that rats preconditioned with hypoxia displayed an increase in CD34, a hematopoietic stem cell marker, and the chemokine CXCR4 in the infarcted hearts which induced to a reduction of the infarct injury (Lin et al., 2008). Although the homing effect induced by ischemic/hypoxic preconditioning on endogenous stem and progenitor cells is intriguing, the major limitation for this approach is that SC mobilization is required to start a few day before the myocardial infarction to exert its regenerative effects (Askari et al., 2003). Furthermore, it is important to better characterize which population of progenitor cells is functionally competent to be mobilised and recruited to a target tissue in order to allow a significant repair (Krankel

et al., 2011). This is particularly important in elderly patients as endogenous SC populations are known to decline with age.

1.4.4 Role of HIFs in Ischemic/hypoxic preconditioning

HIFs play a central role in ischemic/hypoxic preconditioning and neuroprotection against ischemic injuries (Semenza, 2011). When HIF-α subunits translocate into the nucleus, following the hypoxic stimuli, they activate several downstream pathways such as VEGF, EPO, sodium-calcium exchangers-1 (NCX-1), pyruvate dehydrogenase kinase-1 and uncoupling protein-2 which act as survival signals and help maintain cellular homeostasis and the balance between oxidative stress and glycolytic metabolism (Dehne and Brune, 2009; Luo and Semenza, 2011; Semenza, 2011). Furthermore, VEGF and EPO stimulate angiogenesis and vasculogenesis which are important for wound healing and functional repair of brain and heart (Hausenloy et al., 2007; Lee et al., 2000; Li et al., 2011). Beside hypoxia, HIF-1α has been found involved in inflammatory responses mediated by ROS, NO and antioxidant genes involved in the regulation of cell fate (Dehne and Brune, 2009). ROS has been found to be a critical mediator of IPC as it could mimic its protective effect through the opening of the ATP-sensitive mitochondrial potassium ($MitoK_{ATP}$) channels (Andrukhiv et al., 2006; Murry et al., 1986). In the heart, ROS stabilize HIF-1α, which then mediated IPC with a mechanism that is still under further investigation (Bell et al., 2007). Other mechanisms of IPC protection are activated by the HIFs, for example, mice with a germline deletion of HIF-2α survive to birth but display multiple organ dysfunctions which are associated with a decreased expression of antioxidants (Scortegagna et al., 2003). This suggests that HIF-2α is important for cell survival during IPC through the regulation of antioxidants enzymes like heme-oxygenase-1, cyclooxygenase-2 and iNOS which have been associated with an increased resistance to ischemia in mice (Bolli et al., 2002; Mahfoudh-Boussaid et al., 2012).

1.4.4.1 Role of HIF-1α on iNOS in ischemia-reperfusion

There is emerging evidence that HIF-1α displays a protective effect against ischemia/reperfusion injuries through the activation of the iNOS pathway. iNOS is a target of HIF-1α which has been shown to be cardioprotective in chronic and acute heart diseases (Bulhak et al., 2006). Previous studies in cardiac myocytes found high expression levels of iNOS in hearts exposed to chronic hypoxia and a substantial decrease of iNOS when the HIF-1α HRE was mutated (Jung et al., 2000). Furthermore, in another study, it was found that HIF-1α and eNOS overexpression in a transgenic mouse strain attenuated the myocardial infarct size and was able to improve cardiac functionality when hearts were subjected to ischemia/reperfusion (Kido et al., 2005). Finally, it was demonstrated that HIF-1α directly interacts with iNOS in preconditioned hearts. Hence, treatment of rats with cadmium induces HIF-1α degradation and consequently iNOS inactivation thereby blocking the cardioprotective effect of a late IPC (Belaidi et al., 2008). Although the mechanism regulating the effect of eNOS has yet to be fully elucidated, the resulting NO produced is able to activate guanylate cyclase which results in the expression of cGMP that activates protein kinase G leading to the opening of K_{ATP} channels in mitochondria (Natarajan et al., 2006; Sasaki et al., 2000). This signalling cascade seems to promote cardioprotection by inhibiting ATP depletion or limiting cell death through the Bcl2/Bclx pathway (Natarajan et al., 2006).

These studies highlight the importance of iNOS and eNOS as a target genes in HIF-1α mediated cardioprotection and implicate HIF-1α as a therapeutic target in myocardial ischemia. The HIF signalling pathway is an important therapeutic target for protecting cells, organs and tissues against ischemia. How HIFs affect the regenerative potential of SCs or progenitor cells following HPC is still not known. Increased characterization of these transcription factors might help to elucidate the signal transduction pathways in which stem/progenitor cells are protected against oxidative stress.

1.4.5 Epigenetic modifications in hypoxia following reoxygenation

Recent studies suggest that hypoxia is responsible for deacetylation and changes in histone methylation within the promoters of hypoxia inducible genes (Johnson et al., 2008). Alterations in the methylation status of DNA and histones following hypoxia-ischemia remain unknown but may have a profound effect in regulating transcription and in the healing process.

Epigenetic modifications in brain ischemia

The impact of histone acetylation and methylation in brain ischemia was analysed by Gao et al. (2006). Using a rat model of traumatic brain injury a significant decrease of histone H3 acetylation and methylation after 72h of injury was observed suggesting a role for histone modification within the first hours to days after brain injury (Gao et al., 2006). Another study demonstrated that enhancing histone acetylation through hystone deacethylases (HDACs) inhibition resulted in an increased histone methylation which exerts a neuroprotective effect following injury in the nervous system (Rivieccio et al., 2009). A recent study, focused on the DNA methylation level in immature rat brain in response to hypoxia/reperfusion and showed that on day 2 after injury there was a global decrease of DNA methylation compared to the controls (Kumral et al., 2013). This resulted in an increased gene expression after the hypoxic/ischemic insult which was different from previous studies on adult rat brains. However, in the same study, it was found that ischemia/reperfusion did not increase the level of histone H3K4me3 and H3K36me3 which was a characteristic of the immature rat brain (Kumral et al., 2013). Clearly, these preliminary studies demonstrated that hypoxia/reoxygenation have an effect on epigenetics, however, this might vary within different promoters and cell culture conditions and therefore requires further characterization.

Epigenetic modifications in cardiovascular diseases

DNA methylation seems to be associated with cardiovascular conditions such as atherosclerosis and vascular inflammation. Defects in DNA methylation during embryonic growth has been found to led congenital heart diseases and risk of cardiovascular diseases (CVD) as a result of increased methylation of pluripotent or tissue specific genes (Hobbs et al., 2005). Indeed, animal studies demonstrated that maternal hypoxia increases the risk of ischemia and reperfusion injury in the heart of male offspring (Heijmans et al., 2008). This has been found associated with a modification of the DNA methylation signature of cardioprotective genes such as protein kinase C epsilon (PKCε) *in utero* (Patterson et al., 2010). Furthermore, in the vascular smooth muscle cell ESR1 and ESR2, the atheroprotective estrogen receptors genes, have been shown to undergo DNA hypermethylation in human atherosclerosis which decreases the expression of these genes leading to an increased risk of vascular damage (Post et al., 1999). However, these studies are still in infancy and the association between CVD and DNA methylation will require further studies.

Histone modification, also have been found implicated in CVD. The majority of the studies in literature have been focused on eNOS whose differential expression may be regulated through epigenetics (Ignarro, 1990; Palmer et al., 1987). In fact, eNOS is expressed in vascular endothelial cells while is repressed in the vascular smooth muscle cell (VSMC) which reside in the artheries. This differential regulation is mediated by acetylation of H3K9 and H3K12 and methylation of H3K4 at the proximal promoter of eNOS in ECs and subsequently transcription activation by RNA Polymerase II (Charles et al., 2010). In VSMCs lack of these specific histone modification blocks eNOS expression. Interestingly, it has been found that exposure to short-term hypoxia decreases the expression of eNOS in ECs, a condition that seems to play a role in the induction of hypoxia inducible genes involved in angiogenesis (Fish et al., 2010). This condition is reverted following reoxygenation in hypoxic cells where a chromatin remodelling complex, BRG1, has been found to re-activate the transcriptional active state of eNOS (Fish et al., 2010). HDAC enzymes also seem to play a role in the severity of myocardial ischemia and reperfusion. Indeed, it has been found that

repression of HDACs in ES cells using trycostastin A (TSA), an HDAC inhibitor, stimulates myogenesis and angiogenesis and improved functional myocardial recovery after infarcts (Zhang et al., 2012).

Although epigenetic modifications seem to play an important role of HPC in the ischemic brain and heart, further studies are needed to understand the epigenetic control of hypoxia/reoxygenation in stem and progenitor cell populations. Given the important role of HIFs in regulating the regenerative potential of SC populations following ischemia/reperfusion, an improved knowledge of the epigenetic modifications associated with the HREs of the hypoxia inducible genes may lead to the identification of a specific epigenetic signature which promotes angiogenesis and tissue repair.

1.5 Hypothesis and aims

The hypothesis of this study is that HIF-2α is central for the genetic and epigenetic regulation of hES cells cultured under hypoxia and reoxygenation.

The specific aims of my thesis are:

- To investigate the effect of environmental oxygen tension on hES cell pluripotency.
- To investigate whether HIF-2α binds directly to genes implicated in the hypoxic regulation of hES cells using Chromatin Immuno Precipitation (ChIP) assays of hES cells cultured at either 5% or 20% oxygen tension.
- To perform luciferase reporter assays in NT2 cells to investigate whether a potential HRE present in the proximal promoter of the NANOG gene is functionally active.
- To determine the effect of environmental oxygen tension on the DNA methylation of the OCT4 gene in hES cells cultured at 5% or 20% oxygen.
- To analyze whether hypoxia alters the epigenetic state of hES cells cultured at either 5% or 20% oxygen using ChIP assays.
- To investigate the effect of hypoxia followed by reoxygenation on the expression of OCT4, SOX2 and NANOG in hES cells.
- To investigate the effect of hypoxia followed reoxygenation on the chromatin state within the HRE sites of OCT4, SOX2 and NANOG in hES cells.
- To analyse the role of HIF-2α in hES cells exposed to reoxygenation using RT-qPCR and ChIP assays.
- To investigate whether HIF-2α interacts with an oct-sox *cis* regulatory element within the NANOG promoter using ChIP assays in hES cells exposed to hypoxia and reoxygenation.

Chapter 2

Materials and Methods

2.1 Cell Culture

2.1.1 Derivation of Mouse Embryonic Fibroblasts (MEFs)

MF1 mice at 12.5 days pregnancy were killed by cervical dislocation and the uterine horns removed and placed in a petri dish containing PBS (w/o $Ca^{2+}$and Mg^{2+}). Embryos were isolated from the yolk sac, decapitated, eviscerated and macerated in PBS (w/o $Ca^{2+}$and Mg^{2+}). Dissected embryos were washed 3 times with PBS (w/o $Ca^{2+}$and Mg^{2+}), transferred to a 15ml Falcon tube with 2ml of 0.05% Trypsin: EDTA (Life Technologies) and incubated at 37°C in a water bath for 20mins. 5ml of MEF medium (Table 2.1) was added to the sample and pipetted vigorously, any large pieces of tissue were allowed to settle by gravity while the supernatant was transferred to T75 flasks (using a ratio of 3 embryos/flasks) and 19ml MEF medium was added and incubated at 37°C. The following day, the medium was replaced and the cells cultured to 90% confluency before freezing. Cells were also tested for mycoplasma contamination (see Appendix 1).

Table 2.1 Composition of MEF medium

MEFs medium
90% high glucose Dulbecco`s Modified Eagle Medium (DMEM) (Life Technologies)
10% FCS (Life Technologies)
1% Penicillin/ Streptomycin (Life Technologies)

2.1.2 Irradiation of MEF cells

Non-irradiated MEFs were cultured until 90% confluency. MEFs were washed with PBS and dislodged with 5ml 0.05% Trypsin EDTA. Cells were collected in 5ml MEF medium centrifuged for 4 mins at 1500 rpm, resuspended in 40ml MEF medium and irradiated for 23.6min (50G). After irradiating, cells were centrifuged at 1500rpm for 5min and resuspended in 1ml of freezing medium for each T175 flasks (Table 2.2). The cells were transferred to cryovials contained in isopropanol cryotub and stored at -80°C for one day before transferring to liquid nitrogen.

Table 2.2 Composition of freezing medium

Freezing medium
90% Fetal Calf Serum (FCS) (Life Technologies)
10% DMSO (Sigma)

2.1.3 Thawing and plating MEFs

The appropriate number of vials (1 vial per 2 x 6 well plates) of irradiated MEFs were thawed at 37°C, resuspended in MEF medium and centrifuged for 4min at 1500 rpm. The cell pellet was then resuspended in 1ml MEF medium for each well. Irradiated MEFs were plated in 6-well plates pre-treated with 1ml of 0.1% gelatin (Sigma) for 30min at room temperature and cultured in a humidified atmosphere containing 5% CO_2 and 20% O_2. Plates of irradiated MEFs were used to either passage hES cells or to condition medium for feeder-free hES cells culture. When used to condition medium for hES cells, 2ml of hES medium (Table 2.3) was added to each well containing irradiated MEFs and cultured in a humidified atmosphere of 5% CO_2 and 20% O_2. After 24hours the conditioned medium was removed and stored at 4°C and replaced with fresh hES cell medium. Plated for conditioned medium were used for a maximum of 2 weeks.

Table 2.3 Composition of hES cell medium

hES cell medium
knockout DMEM (Life Technologies)
15% knockout serum replacement (Life Technologies),
1% Glutamax 100x (Life Technologies)
0.05mM β-mercaptoethanol
1% non essential amino acids (Life Technologies)
10ng/ml FGF2 (Peprotech Ld)
100μg/ml Penicillin/Streptomycin (Life Technologies)
42μl of 7.5% BSA (Sigma)

2.1.4 Culture of hES cells

Hues-7 hES cells were cultured in 6 well plates on either MEF feeder layers or feeder-free on Matrigel coated plates in a humidified atmosphere containing 5% CO_2 and either 20% O_2 or 5% O_2.

2.1.4.1 Passaging hES cells onto MEF feeder layers

Before passaging hES cells, the MEF medium was removed from the MEF plates (See Method section 2.1.3) and replaced with 1ml of hES cell medium (Table 2.3). Hues-7 hES cells were normally passaged every 3 days by removing the medium from the hES plates and adding 1ml of filtered Collagenase (Table 2.4). Plates are incubated at 37°C for 4mins in the incubator. The amount of hES medium (Table 2.3) added per well varied depending on hES cell density. Cells were then gently scraped to avoid single cell formation, evenly distributed onto previously prepared MEF plates and placed in a humidified atmosphere of either 20% O_2 or 5% O_2 and 5% CO_2. hES cells were cultured for a minimum of 3 passages under hypoxic cultures on MEFs before using.

Table 2.4 Composition of Collagenase

Collagenase 1mg/ml
50mg collagenase (Life Technologies)
50ml Knockout DMEM (Life Technologies)

2.1.4.2 Feeder-free culture of hES cells onto Matrigel coated plates

To culture hES cells under feeder free conditions hES cells cultured on MEFs were passaged onto Matrigel coated plates. Matrigel (BD Bioscience) stocks were made to a concentration of 6mg/ml of protein and stored at -20°C. The Matrigel stock was then diluted to a working solution of 0.4mg/ml in ice cold knockout DMEM. Matrigel coated plates were prepared in advance using 1ml of the working solution for each well of a 6 well plate and incubated at 4°C overnight. hES cells were passaged using 1ml Collagenase (Table 2.4) per well and incubated for 4min at 37°C. Cells were gently scraped from the bottom of the plate and resuspended in conditioned medium (See Method section 2.1.3). The amount of conditioned medium added per well depended on the density of the hES cells. Matrigel was removed from the fresh pre-coated plates and 1ml of cells and 1ml of conditioned media were added. hES cells were cultured for a minimum of three passages on Matrigel at both 5% or 20% oxygen before using in any experiments.

2.2 RNA Extraction

RNA was isolated from hES cells cultured under feeder free conditions on Matrigel coated plates at either 20% or 5% oxygen tension on a 6 well plate of Matrigel on day 3 post-passage using Trizol reagent (Invitrogen). Cells were scraped and harvested in 1ml of Trizol per well. 200µl of chloroform was added to each 1ml of Trizol, allowed to stand at room temperature for 15mins and centrifuged at 13000g for 15mins at 4°C. The upper colourless layer, containing the RNA, was transferred to a clean tube and 0.5ml isopropanol added, allowed to stand for 10 mins at room temperature and centrifuged for 15mins at 13000g at 4°C. The supernatant was removed and the pellet was washed with 1ml of 70% ethanol, centrifuged at 7500g for 10mins and allowed to semi-dry in the air. The pellet was resuspended in 30µl of DEPC water and 1µl of RNAsin (Promega) was added. The concentration of RNA was determined using the Nanodrop. A 260/280 ratio of 1.8-2.0 OD was used in experiments.

2.3 DNAse treatment and cDNA preparation

RNA samples were treated with DNAse to avoid any genomic DNA contamination. 1µg of RNA was incubated in a 10µl total volume containing 10x Reaction buffer, 1µl DNAse (Invitrogen) and DEPC water for 15 mins at room temperature. Samples were incubated for 10mins at 65°C with 1µl 25mM EDTA and immediately placed on ice.

RNA was reverse transcribed into cDNA using Moloney murine leukaemia virus (MMLV) reverse transcriptase (Promega). 1µg RNA was incubated with 1µl Oligo dT primers (500 mg/ml) at 70°C for 10mins, 5x M-MLV reaction buffer, 10mM dNTPs, 1µl M-MLV RT and DEPC water was added to give a total volume of 40µl and incubated at 42°C for 60mins. cDNA samples were analyzed with a PCR reaction to test the presence of genomic DNA contamination. The reaction was performed in 50µl of 10mM dNTPs, 1x Go Taq buffer, 5µM of each primer (OAZ1 fw GGCGAGGGAATAGTCAGAGG; OAZ1 rev GGACTGGACGTTGAGAATCC) and 0.75 µl of Go Taq Polymerase (Promega) in a G-Storm thermocycler. The following cycling parameters were used: 95°C for 5mins followed by 30 cycles at 94°C for 1mins, 58°C for 1min, 72°C for 1 min and 1 cycle at 72°C for 10mins. An amplicon of 224bp was expected for cDNA while genomic DNA produces a 373bp product.

2.4 Quantitative Real Time PCR (RT-qPCR)

hES cells cultured at either 20% or 5% oxygen tension were obtained on day 3 post-passage and the effect of oxygen tension on the expression of hypoxia inducible genes was analyzed through a quantitative Real Time PCR using Taqman Gene expression Assays Probes (Applied Biosystem). Real time PCR analysis was performed using Applied Biosystem reagents in 20μl reactions containing 2x Taqman Universal PCR Master Mix (Applied Biosystems), 1μl of each probe, 1μg cDNA and DEPC water using a 7500 Real-Time PCR System. The following cycling parameters were used: 50C for 2mins, 95°C for 10mins, 45 cycles at 95°C for 15s and 60°C for 1min. UBC was used as housekeeping control gene and all target transcripts were analyzed in duplicate and normalized to UBC (Table 2.5).

Table 2.5: TaqMan Gene expression Assay probes (Applied Biosystems) used for the RT-qPCR

Gene	**TaqMan Gene expression Assay**
OCT4	Hs 01895061_u1
SOX2	Hs 00602736_s1
NANOG	Hs 02387400_g1
UBC	Hs 00824723_m1
HIF-2α	Hs 01026142_m1
JMJD2B	Hs 00943636_m1
JMJD2C	Hs 00909579_m1
JMJD1A	Hs 00218331_m1

2.5 Immunocytochemistry

hES cells cultured on MEFs at 5% and 20% oxygen tension were washed twice in PBS, fixed in 4% paraformaldehyde for 20min at room temperature and washed in PBS. Cells were blocked in 3% donkey serum for 1h for surface molecules and with 3% donkey serum containing 0.1% tritonX-100 for 1h for intracellular proteins. Primary antibodies (Table 2.6) diluted in appropriate block were incubated over-night in a humidified chamber a 4°C. The cells were washed 3 times over 30 mins in PBS before incubating in the appropriate secondary antibody (Table 2.6) for 1h in a humidified chamber in the dark. Cells were washed 4 times over 30mins with PBS and mounted in vectashield containing DAPI (Vecta Laboratories, Peterborough, UK).

Table 2.6: Primary and secondary antibodies used for the Immunocytochemistry with appropriate dilutions.

Primary Antibodies	*Dilutions*	*Secondary Antibodies*	**Dilutions**
mouse IgM anti-TRA-1-60 (Santa Cruz)	1:100	anti-mouse IgM conjugated –FITC (Sigma)	1:200
mouse IgM anti-SSEA1 (Santa Cruz)	1:100	anti-mouse IgM conjugated –FITC (Sigma)	1:200
mouse IgG anti-OCT4 (Santa Cruz)	1:100	anti-mouse IgG conjugated-FITC (Sigma)	1:100
rabbit polyclonal anti-SOX2 (Millipore)	1:500	goat anti-rabbit Alexa 488 (Molecular Probes)	1:700
mouse IgG anti-NANOG (AbCam)	1:50	anti-mouse IgG conjugated-FITC (Sigma)	1:100

2.6 Chromatin Immuno Precipitation (ChIP) analysis

2.6.1 Cross-linking and nuclei preparation from cell cultures

Chromatin from Hues-7 hES cells was isolated from 18 (6 well) plates on day 3 post-passage. Proteins were crosslinked to DNA by adding 1% formaldehyde (Sigma) to cell culture medium for 10min and blocked with 0.125M glycine for 5min at room temperature. Cells were scraped into 4ml of cold PBS supplemented with 0.5mM PMSF (Sigma) and collected by centrifugation at 4°C for 10 min at 1000 rpm. Nuclei were lysed twice with 5 volumes of lysis buffer (5mM PIPES pH 8, 85mM KCl, 0.5% NP40), supplemented with protease inhibitors (Sigma), incubated on ice for 10min and sonicated in 100µl of sonication buffer (1% SDS, 10mM EDTA, 50mM Tris HCl pH8) for each sample to immunoprecipitate.

2.6.2 Sonication

Hues-7 hES cell chromatin was sonicated on ice using a microultrasonic cell disruptor (Soniprep 150 Ultrasonic, Sanyo) to shear DNA to an average fragment size of about 400-1000 bp. Nuclei were centrifuged at 14000 rpm for few seconds and supernatant transferred to a new tube. To check the fragment size, 2µl of the total sonicated chromatin was loaded on a 0.8% agarose gel. Once the correct fragment size was obtained, the chromatin was centrifuged at 14000 rpm for 10mins at 4°C and the supernatantant transferred to a new tube and stored at -80C for several months.

2.6.3 Chromatin IP

Sonicated chromatin was diluted 1:200 and quantified using the Nanodrop (Nanodrop ND-1000, Labtech). 20 to 100µg of chromatin for each sample was precleared with 20µl of protein A/G plus agarose beads (Santa Cruz) for 1h at 4°C on a rotating platform. Samples were centrifuged at 14000 rpm for 5min and the supernatant diluted 1:10 with 0.01% SDS, 10mM EDTA, 50mM Tris HCl pH8 supplemented with protease inhibitors and divided equally among samples. ChIP assays were performed using 10µg of the following antibodies: HIF-2α (Novus Biologicals), normal rabbit IgG (Santa Cruz). For histone modifications analysis the following antibodies were used: H3K9me3 (Abcam), H3K4me3 (Abcam), H3K36 me3 (Abcam). Samples were immuno-precipitated over night with gentle rotation at 4°C. Saturated protein A/G plus

agarose, was prepared using 20µl of protein A/G plus agarose beads (Santa Cruz) and 1µg/µl of sonicated Salmon Sperm DNA (Sigma) and BSA and incubated with gentle rotation at 4°C. The day after, saturated protein A/G plus agarose, was to each IP sample and incubated on a rotating platform for 1-2 h at 4°C.

2.6.4 Chromatin IP and washing

The control IgG was centrifuged for few seconds at 14000 rpm and the supernatant was collected as input sample. The immuno-complexes were washed in ice as followed:

10 times with 600µl buffer A (0.1% SDS, 2mM EDTA, 20mM Tris HCl pH8, 1% Triton X-100, 500mM NaCl), 8 times with 600 µl buffer B (0.1% SDS, 2mM EDTA, 20 mM Tris HCl pH8, 1% Triton X-100, 1 M NaCl), 3 times with 600µl TE (10mM Tris HCl H8, 1mM EDTA) buffer. For each wash protein A/G plus agarose beads were centrifuged for a few seconds, the supernatant removed and 600µl of washing buffer added, mixed at room temperature and incubated on a rotating platform for 5min at 4°C, before being centrifuged.

2.6.5 Elution

After the last wash, every sample was centrifuged again at 14000 rpm for few seconds to remove any traces of buffer. Antibody/protein/DNA complexes were eluted from the beads by adding 2 x 200µl of elution buffer (1% SDS, 100mM NaHCO3), incubated 15min with gently rotation at 4°C. Both eluates were collected in the same tube.

2.6.6 Reverse Formaldehyde crosslinks

Samples from the previous steps were centrifuged at 14000 rpm for 5mins to remove any trace of beads. To each supernatant 200mM NaCl was added and incubated over night at 65°C to reverse formaldehyde crosslinks.

The following day all the samples were incubated for 2h at 42°C with a mixture of 1 µl RNAse A (20µg/ml), 1µl of proteinase K (20µg/ml) (Life Technology), 10mM EDTA, 40mM Tris HCl pH6.5. DNA extraction was performed with an equal amount of phenol: chloroform: isoamyl alcohol (25:24:1) (Life Technology) for each sample, mixed at room temperature and kept on ice for 30min. Samples were centrifuged for 20min at 14000 rpm at 4°C and the supernatants precipitated with 1/10 volume of 3M

Sodium Acetate pH5, 10µg of glycogen (Life Technology) as carrier, 2.5 volumes of 100% ethanol overnight at -20°C. Precipitated samples were washed with 500µl of 70% ethanol and the pellets were allowed to dry completely in air before being resuspended in 30µl of H_2O for each IP and 100µl of H_2O for the input sample (Figure 2.1)

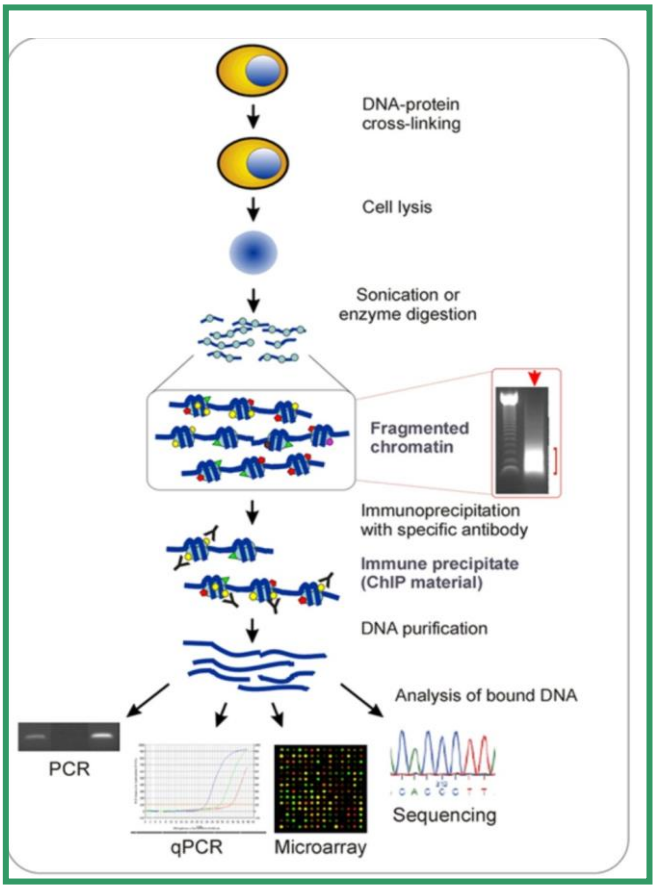

Figure 2.1: Schematic representation of ChIP assay procedure

2.7 Hypoxia Responsive Elements (HRE) amplification

2.7.1 Primer Design

All primers sequences, that overlap the HRE in the proximal promoter region of the genes of interest, were designed using Primer 3 and analyzed through bioinformatics software like BLAST and Electronic PCR to test their specificity (Table 2.7).

Table 2.7: PCR primers designed within predicted HREs for the indicated genes.

Gene	*Primer Forward* *5'* *3'*	*Primer Reverse* *5'* *3'*	*Amplicon size*
CR3OCT4	GCCTGCAATGAGAAGCCTTA	GCCTGTCTGTGAGGGATGAT	191bp
CR4OCT4	TGGCAGACAGCAGAGAGATG	GTCTGCCGGAGGTCTACAA	206bp
SOX2	AGATGTGGCTGGGGCTAAG	GACCCAAACCTCTGTCCTCA	196bp
NANOG	AGGTCTCACTGTGCCTCCTC	AAGACCAGCCTGACCAACAT	201bp
GLUT1	CCTCCCTTCCAAGGGTAACT	CCAGCATAGGCTAGGACCAC	249bp

2.7.2 HRE PCR Analysis

PCR detection of the proximal gene target promoter region was performed in 20µl reaction mix containing 1X Go Taq buffer (Promega), 10mM dNTPs, 10µM of each primer and 1/10 volume of DNA and nuclease free water on an G-Storm thermocycler instrument. The thermal profile consisted of 1 cycle at 95°C for 5mins followed by 30 cycles at 94°C for 2mins, 58°C for 45sec, 72°C for 45secand 1 cycle at 72°C for 5mins. Amplified products were separated on a 1.5% agarose gel.

2.7.3 HRE Real time PCR Analysis

Reverse cross-linked DNA was analysed through a quantitative Real Time PCR in 5µl reaction containing 1/10 ChIP DNA, 2.5µl 2x Taqman Universal PCR Master Mix (Applied Biosystems), 0.25µl Custom Probes designed using the Primer Express 3.0 design tool (Applied Biosystem) (Table 2.8, 2.9 and 2.10) and the qPCR was performed using an ABI 7900 HT Fast Real Time System (Applied Biosystems) in a 384 wells plate. Percentage of input was calculated for each sample compared three independent ChiP assays on chromatin of hES cells cultured at either 20% oxygen , 5% oxygen or after 72h post hypoxia.

Table 2.8: TaqMan Custom probes designed to cover the predicted HREs sites in OCT4, NANOG, SOX2A and SO2G

OCT4 CR3	*Sequence (5'→ 3')*	*Amplicon size*
Forward	TGAGAAGCCTTACTTAAGTCGACAGA	96bp
Reverse	TCAGCGTGCCCAGTC	
Probe	TTCGAAGCTGTGGGGAGC	

NANOG	*Sequence (5'→ 3')*	*Amplicon size*
Forward	TGGAAACGTGGTGAACCTAGAA	83bp
Reverse	AACCGAGCAACAGAACCTGAA	
Probe	TATTTGTTGCTGGGTTTGT	

SOX2 G	*Sequence (5'→ 3')*	*Amplicon size*
Forward	CGGCCACCACAATGGAAA	96bp
Reverse	TCCCTCCCACGCAGAGTTC	
Probe	AGGCTGGTTCTGCT	

SOX2 A	*Sequence (5'→ 3')*	*Amplicon size*
Forward	AACGGACGTGCTGCCATT	85bp
Reverse	TGTCCCGACGTAAAGATTTCAA	
Probe	CCCTCCGCATTGAG	

Table 2.9: TaqMan Custom probes designed to cover the predicted HREs sites in eNOS, GLUT1. TaqMan probe was also designed to cover a region between 2 HREs in the FOXP3 gene.

eNOS	*Sequence (5'→ 3')*	*Amplicon size*
Forward	CGTGTACGTGTGTATGTGAAGATACCT	77bp
Reverse	CTGAGCCTTCTTTG	
Probe	TCTGTGCAGGAAGCAGGGA	

GLUT1	*Sequence (5'→ 3')*	*Amplicon size*
Forward	CAAATGTGTGGATGTGAGTTGC	51bp
Reverse	CCATCACGGTCCTTCTTCATG	
Probe	AGGCTGAGCGTGTAAA	

FOXP3	*Sequence (5'→ 3')*	*Amplicon size*
Forward	CCCCAGAGACCCTCAAATATCC	56bp
Reverse	CCCGAGGCAGGCAGAGA	
Probe	CTCACTCACAGAATGGT	

Table 2.10: TaqMan Custom probes designed to cover the predicted oct-sox *cis*-regulatory element sites in the NANOG and OCT4 proximal promoter and in SOX2 distal intron and the control probe to detect the intermediate region in the NANOG proximal promoter

NANOG oct-sox element	*Sequence (5'→ 3')*	*Amplicon size*
Forward	CGGTTTTCTAGTTCCCCACCTA	56bp
Reverse	CCAAGGCCATTGTAATGCAA	
Probe	TCTGGGTTACTCTGCAGCT	

OCT4 oct-sox element	*Sequence (5'→ 3')*	*Amplicon size*
Forward	GCCGTCTTCTTGGCAGACA	64bp
Reverse	CCCCAGGACAGAACCATCAC	
Probe	AGAGAGATGCATGACAAAG	

SOX2 oct-sox element	*Sequence (5'→ 3')*	*Amplicon size*
Forward	GGCCAGCCATTGTAATGCATAT	66bp
Reverse	GAGCAAGAACTGGCGAATGTG	
Probe	CGGATTATTCACGTGGTAAT	

NANOG intermediate region	*Sequence (5'→ 3')*	*Amplicon size*
Forward	GGGTTTGTCTTCAGGTTCTGTTG	65bp
Reverse	GCTGCAGAGTAACCCAGACTAGGT	
Probe	CGGTTTTCTAGTTCCC	

2.8 Statistical analysis

An Anderson-Darling normality test was performed to determine whether data were normally distributed. Relative gene expression differences between cells cultured at 5% and 20% oxygen tension were analysed using a 1-sample *t*-test. Percentage of Input (non-immunoprecipitate chromatin) was calculated as $100x2^{[Ct\ (Input)-Ct\ (IP)]}$ for each sample. Differences in chromatin relative enrichment between cells cultured at 5% and 20% oxygen tension were analysed using a Student's *t*-test. A value of $P<0.05$ was considered significant. Differences between HIF-2α binding to the oct-sox element or intermediate region were determined using an Anova test followed by a Fisher's test. All data are presented as a mean ± SEM

Chapter 3

Mechanisms by which HIF-2α regulates hES cell pluripotency under hypoxic conditions

3.1 Introduction

3.1.1 hES cells morphology

hES cells are derived from the inner cell mass of the blastocyst and are defined as pluripotent and self-renewing cells (Thomson et al., 1998) making them a useful tool for tissue replacement and regenerative medicine. However, hES cells are difficult to maintain in culture as they tend to spontaneously differentiate *in vitro* so several criteria have been established to assess and validate hES cells cultures. In particular, studies on long-term stability of hES cells, have indicated that the expression of characteristic markers remain sustained following prolonged periods of cultures on MEFs feeder layers (Richards et al., 2002) or on Matrigel with conditioned media (Rosler et al., 2004).

Among surface markers, hES cells lines exhibit the expression of TRA1-60, TRA1-81 and SSEA-4, that allow the characterization of several glycolipids and glycoproteins originally identified in human preimplantation embryos (Andrews et al., 1984a). In contrast, SSEA-1 is only expressed upon hES cell differentiation (Pera et al., 1988).

Among the nuclear self-renewal transcription factors hES cells express OCT4 (Rosner et al., 1990; Yeom et al., 1991), NANOG (Chambers et al., 2003) and SOX2(Yuan et al., 1995). Recent studies suggest that another criterion for characterizing hES cells is the analysis of the epigenetic profile in order to demonstrate that hES cells derived and maintained in several culture conditions may respond differently to environmental stimuli (Hawkins et al., 2011; Hoffman and Carpenter, 2005). In fact, emerging evidence showed that the environment in which these cells are derived is particularly important in order to improve the hES cells cultures (Lengner et al., 2010). These observations take into account that *in vivo*, preimplantation embryos develop in the uterine tract which is characterized by low oxygen tension (Fischer and Bavister, 1993) a condition known to improve human embryo development (Calzi et al., 2012). Moreover, culture of hES cells under low oxygen concentrations has been found beneficial for their maintenance in terms of increased self-renewal and pluripotency

(Forristal et al., 2013; Forristal et al., 2010; Westfall et al., 2008) and reducing chromosomal aberrations (Forsyth et al., 2006). Indeed, previous published data demonstrated that hES cells cultured at 5% oxygen maintained a more pluripotent phenotype compared to cells cultured at 20% oxygen which displayed spontaneous differentiation (Forristal et al., 2010). Furthermore hES cells cultured under hypoxia displayed more rounded and defined colonies while those maintained at atmospheric oxygen tension were enlarged and diffuse (Forristal et al., 2010).

3.1.2 Hypoxia Inducible Factors

3.1.2.1 Effect of Hypoxia Inducible Factors on pluripotency genes

Many processes involved in oxygen homeostasis are mediated by the hypoxia inducible factors (HIFs). These are heterodimers formed of a constitutively expressed HIF-1β (also called ARNT aryl hydrocarbon receptor nuclear translocator) subunit and one of the three different HIF-α subunits (HIF-1α, HIF-2α, HIF-3α) (Keith et al., 2001; Wang and Semenza, 1993, 1995). All three HIF-α subunits bind a canonical recognition sequence (A/G)CGTG termed a hypoxic response element (HRE), in the proximal enhancer or promoter of HIF target genes (Semenza and Wang, 1992). It is widely recognized that HIFs activate the expression of several oxygen responsive genes including those involved in energy metabolism, vasculogenesis, cellular proliferation and apoptosis (Carmeliet et al., 1998; Goda et al., 2003; Iyer et al., 1998; Tacchini et al., 1999). Although HIF-1α and HIF-2α display many similarities, they have different target genes. HIF-1α has been found to be ubiquitously expressed and has a main role in the hypoxic response (Semenza and Wang, 1992). HIF-2α has been found in specific cell types like vascular endothelial cells, kidney fibroblasts, hepatocytes and in many tumors associated with VHL diseases (renal clear cell carcinomas and hemangiomas) (Hu et al., 2006) and seems to play a role during tumorigenesis (Maxwell et al., 1999). Some hypoxia inducible genes are specifically targeted by HIF-1α such as the phosphoglycerate kinase (PgK), the pro-apoptotic gene BCL2 and the heme oxygenase-1 (HO-1) and IL-8 (Loboda et al., 2009; Raval et al., 2005) whereas others are induced by HIF-2α such as the erythropoietin gene and transforming growth factor α (TGFα) (Rankin et al., 2007; Raval et al., 2005). Moreover, in mouse ES cells, HIF-2α has been found as a direct upstream regulator of OCT4, binding to HRE sequences situated in specific Conserved Regions (CR3 and CR4) in the 5' promoter sequence of this gene. It has been found that all the CR (1, 2, 3 and 4) are identical in human, bovine and mouse (Nordhoff et al., 2001), but in RCC (Renal Clear Carcinoma) cells HIF-2α has been found to recognise only CR3 and CR4 in the human OCT4 promoter (Covello et al., 2006). Recently, it has been also documented a role for HIF-2α in the long term adaptation to hypoxia through the control of pluripotency in hES cells (Forristal et al., 2010). Indeed, this study revealed that HIF-2α regulates the expression of OCT4, SOX2 and NANOG during hypoxic culture (Forristal et al., 2010). However, it remains to be

determined whether there is a direct interaction between HIF-2α and the promoter region of genes regulating self-renewal in hES cells.

3.1.2.2 Effect of hypoxia on glucose metabolism

Cellular response to hypoxia is a mechanism that allows cells to adapt to environmental cues. The response to oxygen deprivation involves the activation of several hypoxia-inducible genes that leads to cell adaptation (Hu et al., 2003). Metabolic genes and in particular glucose transporters such as GLUT1 are extensively regulated by HIFs under environmental oxygen tension (Iyer et al., 1998). Glucose transporter expression increase the amount of glucose uptake by the cellular metabolic system (Flier et al., 1987). In particular, GLUT-1 seems to be an important regulator of glucose metabolism in mouse ES cells, placenta, brain and retina (Flier et al., 1987; Froehner et al., 1988; Kahn and Flier, 1990). Shifting from oxidative phosphorylation to the oxygen independent glycolysis is one the most conserved mechanism of cellular response to hypoxia (Hu et al., 2003). Similar mechanisms also occur in ES cells and iPS cells which display a glycolytic metabolism (Folmes et al., 2011; Varum et al., 2011). Interestingly, a recent study showed an increase of glucose uptake together with high levels of GLUT-1 expression in hES cells cultured at 5% oxygen suggesting that environmental oxygen regulates energy metabolism and self-renewal of hES cells (Forristal et al., 2013). Importantly, GLUT-1 is a hypoxia inducible gene which is regulated by HIF-1α in RCC, endothelial cells (Hu et al., 2003) and also in trophoblast-derived cell lines (Hayashi et al., 2004). Less is still known about the role of HIF-2α in the regulation of glucose metabolism. However, it is likely that in hES cells the hypoxic regulation of energy metabolism is different since HIF-1α is degraded after 48 hours of exposure to hypoxia while HIF-2α is stabilized and translocates to the nucleus (Forristal et al., 2010). This suggests that the glycolytic metabolism in which hES cells rely might be regulated by HIF-2α through a direct interaction with glucose transporters genes like GLUT1 and suggest a new mechanism by which environmental oxygen regulates glucose utilization in hES cells.

3.1.2.3 Role of HIFs on eNOS

Nitric oxide (NO) is a signalling molecule that is responsible for several biological effects such as cell survival and proliferation in different types of tissues (Li et al., 2002; Tejedo et al., 2004). NO has different isoforms (iNOS, eNOS and nNOS) each displaying a specific pattern of expression (Kobzik et al., 1995). Among them eNOS, the endothelial form of NO, is expressed in endothelial and tumour cells and seems involved in the pathway of Nestin-and Notch which regulate tumour invasiveness (Charles et al., 2010). Since the solid tumour microenvironment is hypoxic, HIFs and in particular HIF-2α, has been found to regulate signalling pathways in cancer SCs (Li et al., 2009) and has been associated to highly aggressive tumour phenotype. Interestingly, HIF-2α modulates eNOS expression under hypoxia in human endothelial cells (Coulet et al., 2003) which suggests the presence of an autocrine loop that regulates cancer SCs in hypoxic conditions. However, this mechanism has not been elucidated but could have several implications on the role of signalling molecules not only in different SCs niches but also in stem cell self-renewal. Indeed, NO was found to modulate OCT4 expression in mouse bone marrow SCs (Chu et al., 2008) suggesting a role for NO and its isoforms in the regulation of stem cell pluripotency. Furthermore, several studies have documented a role for eNOS in the regulation of hES differentiation and self-renewal (Mujoo et al., 2006; Tejedo et al., 2010). However, the mechanisms involved in the regulation of these signalling pathways are still unknown. As hypoxia regulates hES cell pluripotency (Forristal et al., 2010), the aim of this work will be to investigate whether HIF-2α regulates eNOS expression.

3.1.3 Study Aim

The aim of this chapter is to analyse the role of hypoxia in hES cell maintenance. Specific aims of this chapter were:

- To characterize hES cell pluripotency under normoxia (20% oxygen) or hypoxia (5% oxygen) using immunocytochemistry and RT-qPCR analysis.
- To determine whether endogenous HIF-2α interacts directly with predicted HRE sites in the proximal promoter of OCT4, SOX2, NANOG, GLUT1 and eNOS in hES cells cultured under low oxygen tension.

3.2 Materials and Methods

RT-qPCR was used to analyse the effect of environmental oxygen tension on the expression of nuclear transcription factors OCT4, NANOG and SOX2 using Taqman probes (See Methods section 2.4).

3.2.1 Validation of Taqman probes used for RT-qPCR

The efficiency of Taqman probes is guarantee to be >90% but to verify this, the amplification efficiency of OCT4 and UBC was calculated.

To determine whether the ddCt method could be used to quantify gene expression, the efficiency of each probe was determined. PCR amplification efficiency is the amount of amplicon produced in every reaction and for every molecule of the target gene, and is expressed as a percentage value. When the amplicon doubles in quantity during amplification then the PCR assay has 100% efficiency and the slope of the standard curve will be -3.32. Standard curves with a slope between -3.1 and -3.6 are considered acceptable. The amplification efficiency is calculated from the slope of each graph using the equation:

$$\% \text{ Efficiency} = (10^{(-1/\text{slope})}-1) \times 100$$

This method assumes that the efficiency between two genes tested is close to 100%, and vary between 90-110%. The Pearson value (R^2) showed on the standard curve represented the precision of all the replicates and generally, when the data are perfectly aligned, the R^2 is between 0 and 1.

A 4-log dilution range was obtained using 10-fold serial dilution of cDNA. Each dilution was amplified in triplicate with OCT4 and UBC Taqman probes and a plot of Ct against log cDNA concentration was used to determine efficiency. The 4-log measurements showed a Pearson coefficient (R^2) value of 0.99 or 99% and a slope of -0.3 for both UBC and OCT4 (Figure 3.7). This result confirmed the purity of the Taqman probes and the replicate experiments indicated that the amplification efficiency increased when using a broader range of dilutions for the cDNA template.

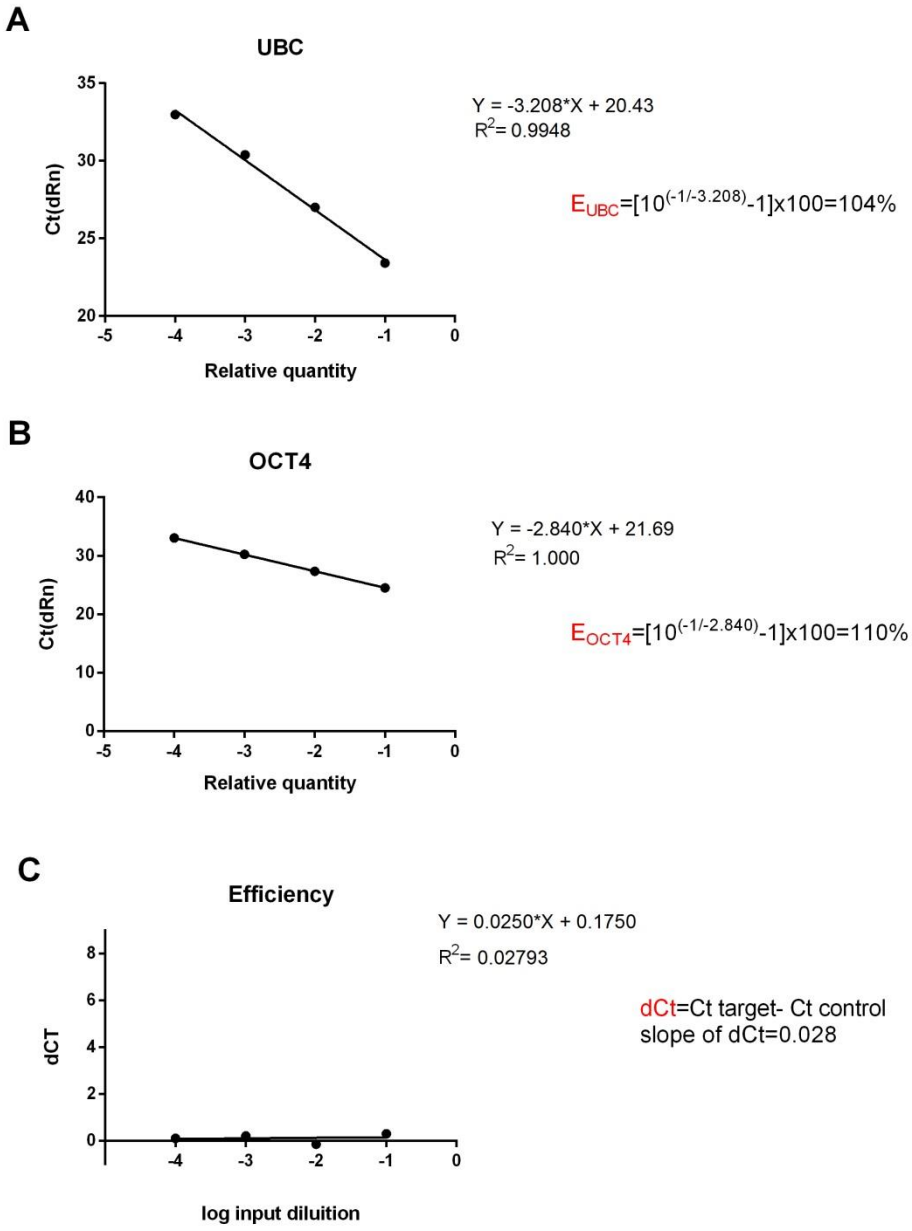

Figure 3.1: OCT4 probe displays 99% efficiency

Efficiency of UBC (A) and OCT4 (B) primers is calculated from the gradient generated from 4-log diluitions. Difference in Ct value between OCT4 and UBC are represented in C.

3.2.2 Culture of Ntera-2 cells

Ntera-2 (NT2) cells, a pluripotent human testicular embryonic carcinoma cell line, were cultured in DMEM (Invitrogen) supplemented with 10% FBS (Invitrogen), 1% Penicillin/Streptomycin (Invitrogen) in 75cm^2 tissue culture flasks (Greiner Bio One, Glos, UK) at 37°C with 5% CO_2 in a humidified atmosphere. Cells at 90% confluency were washed in PBS and trypsinized with 1ml 0.05% trypsin-EDTA (Invitrogen) and incubated for 5min in incubator to detach cells from the flask. Cells were suspended in 5ml of DMEM to neutralise trypsin and diluted 1:4 with fresh growth medium.

3.2.3 Western Blot Analysis

3.2.3.1 Protein extraction and quantification

Proteins were isolated from Hues-7 hES cells cultured either at 20% or 5% oxygen from 3 wells of a 6 well plate. 80µl of RIPA buffer (37.5 mM Tris buffer, 0.25% sodium deoxycholate, 1% NP40, 0.75 mM sodium orthovanadate, 1mM sodium fluoride, 1mM phenylmethylsulfonyl fluoride and protease inhibitor (Roche) was used to lyse cells. Samples were incubated for 20mins on ice prior to being sonicated for 30 sec and centrifuged for 10mins at 4°C. A Bradford assay was performed to determine the total protein concentration. A series of 5 dilution of BSA (bovine serum albumin) between 2 and 20µg were made in 1ml of H_2O. A volume of 180 µl of each serial dilution was mixed with 20µl of Bradford reagent (Biorad) and used to generate a standard curve of 595 nm absorbance. The concentration of protein in each collected sample, after dilution 1/1000 was calculated from measurements of the absorbance at 595 nm (Figure 3.2).

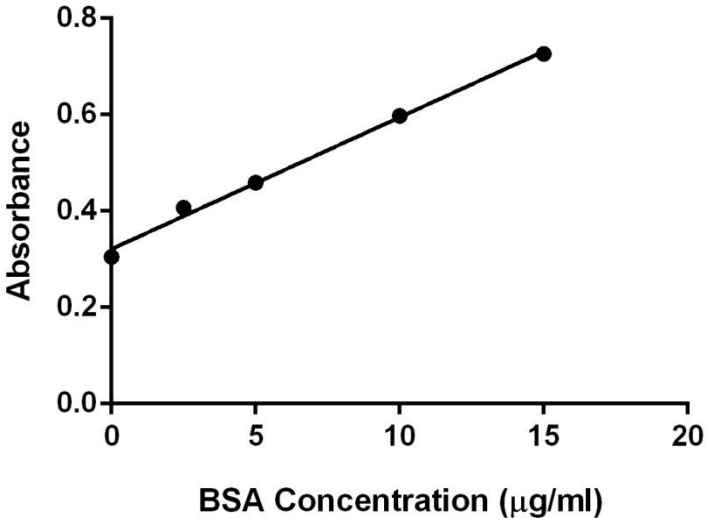

Figure 3.2: Representative BSA standard curve for protein quantification

3.2.3.2 Western Blot

Cell extracts (75µg) were mixed with 1M DTT (Sigma), 4x sample buffer (Invitrogen) and RIPA buffer to make a total volume of 45µl. Protein samples were denatured by boiling for 5mins prior loading on a 12% SDS polyacrylamide running gel (2.5ml 1.5M Tris pH 8.8, 100µl 10% SDS, 3ml acrylamide, 5ml H_2O, 100µl 10% ammonium persulphate, 5µl TEMED) with a 4% stacking gel (695µl 0.9M Tris pH 6.8, 50µl 10% SDS, 625µl acrylamide, 3.63ml H_2O, 50µl 10% ammomium persuphate, 5µl TEMED). Gel electrophoresis was performed at 55mA for approximately 70mins in 1X Running Buffer diluted from a 5X stock solution (15.1g 25mM Tris pH 8.3, 94g 250mM glycine, 900ml H_2O, 50ml 10% SDS for a total volume of 1L). Proteins were transferred to a nitrocellulose membrane (Amersham Hybond-ECL) using the Mini Trans-Blot Electrophoresis Transfer cell (Biorad) which was run at a constant 250mA for 2h in 1X Transfer buffer (2.93g glycine, 32ml 1.5M Tris pH 8.3, 200ml methanol to a total volume of 1L). Membrane was blocked for 1.5h with 1X Western Wash buffer diluted from a 10X stock solution (24.2 g Tris 80g NaCl, Tween-20 to 1% pH 7.6) containing 5% non-fat dry milk (NFDM) before being incubated with a rabbit anti-HIF-2α

antibody (Novus) diluted 1:5000 in 1X Western Wash buffer over night at 4 °C. The membrane was then washed with 1X Western Wash buffer for 35 mins at room temperature (1 X 15 mins, 2 X 10 mins washes) before being incubated with an HRP conjugated anti-Rabbit IgG antibody (Amersham) diluted 1:50000 in 5% NFDM for 1h at room temperature. After washing for 35 mins at room temperature, the membrane was drained and treated with ECL kit (Lumigen). Solution A and B provide by the ECL kit (Lumigen) were mixed in equal volumes, spread on the membrane and left for 1min in the dark. Protein signal was detected by film impression in dark room. The membrane was either exposed to autoradiography film and Chemidoc XRS system (Bio-Rad) for protein visualization. An autoradiography film was exposed to the membrane for a time variable depending on the signal intensity. After exposition, the film was incubated for 2-5 minutes in the developer solution (Kodak), rinsed in water, incubated s for 5 minutes in fixing solution (Kodak) and rinsed in water before being air-dried. Images of dried films were acquired by scanning. After detection of the primary antibody, the membrane was washed in 1X Western Wash buffer and incubated with a β-actin-HRP tagged antibody diluted 1:50000 in 5% milk for 1h. After the incubation, the membrane was washed in 1X Western Wash buffer as described previously and then exposed to ECL and autoradiography as described above.

3.2.4 DNA extraction from hES cells

hES cells cultured at 5 and 20% oxygen tension on a 6 well plate of Matrigel were collected on day 3 post passage in 600µl RLT Lysis Buffer (Qiagen). The lysate was passed 5 times through a 20-gauge needle (0.9 mm diameter) fitted to a syringe, transferred to an AllPrep DNA (Qiagen) spin column and centrifuged for 30s at 13000 rpm. 500µl Buffer AW1(Qiagen) was added to the column and centrifuged for 15s at 10.000 rpm to wash the spin column membrane, after this step, 500µl of Buffer AW2 (Qiagen) was added to the column and centrifuged for 2mins at full speed. DNA samples were eluted with 50µl of Buffer EB (Qiagen), incubated at room temperature and centrifuged at 13000 rpm

3.2.5 NANOG promoter amplification

A 630 bp region of from -512 to + 207 from the transcription start site of NANOG promoter was amplified with specific primers that contain XhoI and HINDIII restriction sites (NanogXho fw: CTCGAGCGGCTGGTTTCAAACTCCTGA; NanogHINDIII rev: TTCGAACCGGATGCTTCAAAGC) (Figure 3.3). A tail with extra bases has been added to the 5' of each primer to stabilize the sequence (highlighted in red). A PCR reaction was performed in 20µl reaction mix containing 1X Go Taq buffer (Promega), 10mM dNTPs, 10µM of each primer, 100ng of DNA isolated from hES cells cultured at 5% oxygen tension (Method Section 3.2.4) and nuclease free water on an G-Storm thermocycler. The thermal profile consisted of 1 cycle at 95°C for 5mins followed by 40 cycles at 94°C for 2mins, 58°C for 45sec, 72°C for 45sec and 1 cycle at 72°C for 5mins. Amplified products were separated on a 1% agarose gel.

Figure 3.3: NANOG promoter sequence showing the location of the -437bp to +207bp amplicon

Diagram showing the location of the NANOG PCR amplicon and respective primers at -437 bp to +207 bp from the transcription site (+1) highlighted in red. Forward primer is shown in pink and reverse primer in blue. The HRE site at -301 bp is highlighted in pink while the HINDIII restriction site is highlighted in yellow.

3.2.6 TOPO vector cloning

The PCR product was cloned directly into the TOPO cloning vector kit (Invitrogen) (Figure 3.4) in 6µl reaction containing 4µl of 10X PCR Buffer (100mM Tris-HCl, pH 8.3, 500mM KCl, 25mM $MgCl_2$, 0.01% gelatin), 1µl of Salt Solution (1.2M NaCl, 0.06M $MgCl_2$) and 1µl (10ng/µl) of TOPO vector. After 5mins incubation at room temperature, 6µl of ligation were incubated with the One Shot TOP10 competent bacteria strain (Invitrogen) on ice for 30mins. The reaction was heat shocked at 42°C for 30sec, incubated on ice for 2mins before 250µl of SOC medium (2% Tryptone, 0.5 % Yeast Extract, 10mM NaCl, 2.5mM KCl, 10mM $MgCl_2$, 10mM MgSO4, 20mM glucose) was added. The mixture was incubated at 37°C for 1h in a shacking incubator and then spread on a LB (Luria Bertani 10g/l Tryptone; 5g/l Yeast Extract; 5g/l NaCl; 15g/l Agar) with 100µg/ml Ampicillin plate and incubated over-night at 37°C. Selected colonies were inoculated in 3ml of LB media and incubated over-night at 37°C before plasmid DNA was purified using a QIAprep Miniprep Kit (Qiagen Crawley, UK) (See Appendix 2).

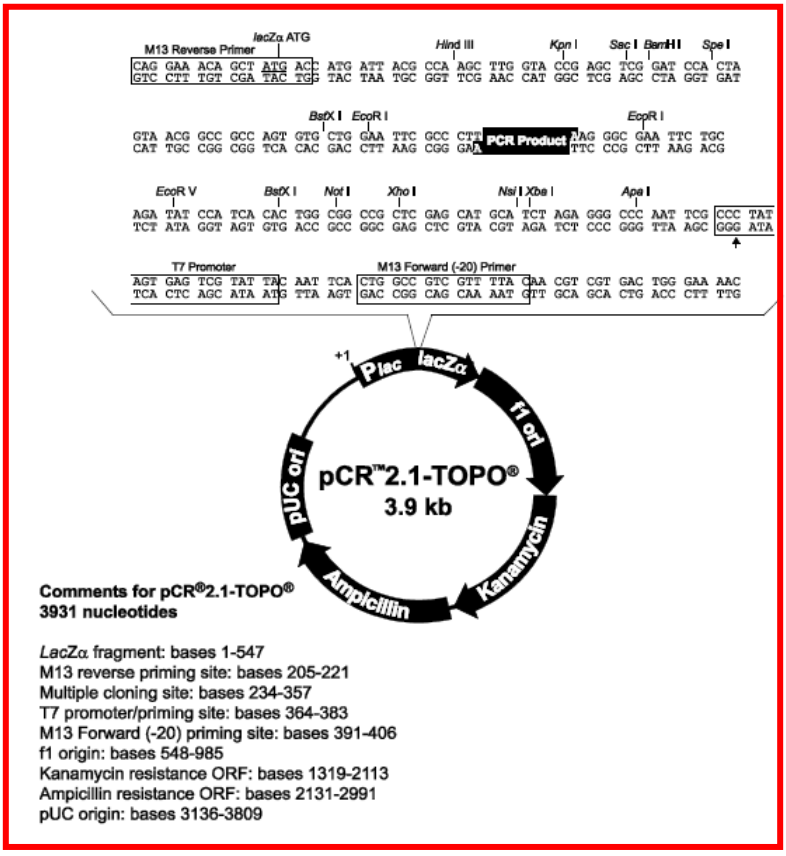

Figure 3.4: Schematic representation of the TOPO vector (Invitrogen)

3.2.7 Restriction analysis

To evaluate if selected colonies were successfully cloned into the TOPO vector, a restriction analysis using XhoI and HINDIII enzymes was performed in a 20µl reaction containing 1X NEB reaction buffer 2 (New England Biolabs), 10X BSA, 20u/µl of each enzyme and 5µl of DNA. The reaction was incubated at 37°C for 3h and then loaded on a Nancy (Sigma) stained 1% agarose gel. A specific band of the expected 630 bp product was cut from the gel and purified using the QIAquick Gel Extraction kit (Qiagen, Crawley, UK) (See Appendix 3), eluted in 30µl of sterile water and concentrated in the SpeedVacuum until desired volume of 6 µl was obtained. The correct identity of the product was determined by sequence analysis using primers M13FW (TGTAAAACGACGGCCAGT) and M13Rev (CAGGAAACAGCTATGAC). PCR product was sequenced by Geneservice - Source BioScience plc. using 1 ng/µl for every 100 bp of PCR product and 3.2 pmol/µl of sequencing primers.

3.2.8 Cloning in the pGL3 basic control vector

The pGL3 control vector (Promega) contains a gene for firefly luciferase that catalyzes a bioluminescent reaction in which the luciferin is converted in oxyluciferine, producing light (Figure 3.5). The pGL3 control vector also presents a functional promoter which is lost after the digestion with XhoI/HINDIII used to generate compatible ends in order to clone the specific NANOG insert. The vector displays the presence of the Amp gene to allow the identification of transformant colonies. 5µg of the pGL3 control vector was digested with 25U each of either XhoI and HINDIII in 50µl final volume at 37°C for 3h. To ensure the vector was digested, it was ran on an agarose gel and the expected product extracted using the QIAquick Gel Extraction kit (Qiagen, Crawley, UK) (See Appendix 3). DNA was quantified using the NanoDrop and concentrated in the SpeedVacuum until the volume of 6µl. Half of the digested vector was dephosphorylated to avoid self-ligation of the PGL3 vector. The dephosphorylation reaction was performed in a total volume of 25µl by mixing the digested DNA with 5U of Antarctic phosphatase (NEB) in the restriction enzyme buffer and incubating at 37°C for 1h. After this time the reaction was heat-inactivated by incubating at 65°C for 5min. Digested pGL3 control vector was ligated with the digested NANOG insert in 10µl

reaction containing 6µl of NANOG digested promoter fragment, 2µl digested pGL3 control vector, 1µl Ligase Buffer (New England Biolabs) and 1µl ligase (New England Biolabs). The ligation was transformed in One Shot TOP10 competent bacteria strain (Invitrogen) on LB agar plates supplemented with Ampicillin. Individual colonies were picked and grown in 3ml LB with 100µg/µl Ampicillin at 37°C over-night. Small scale plasmid DNA preparation was performed using the QIAprep Miniprep Kit (Qiagen Crawley, UK) (See Appendix 2). To confirm the presence of the NANOG promoter insert in the pGL3 control vector, 1µg of all DNA preparations was digested with 20U/l of HINDIII for 1h and then XhoI was added to each sample and digested at 37°C for 2h. Plasmids were purified using Genopure Plasmid Maxi kit (Roche) following manufacturer's instructions (See Appendix 5).

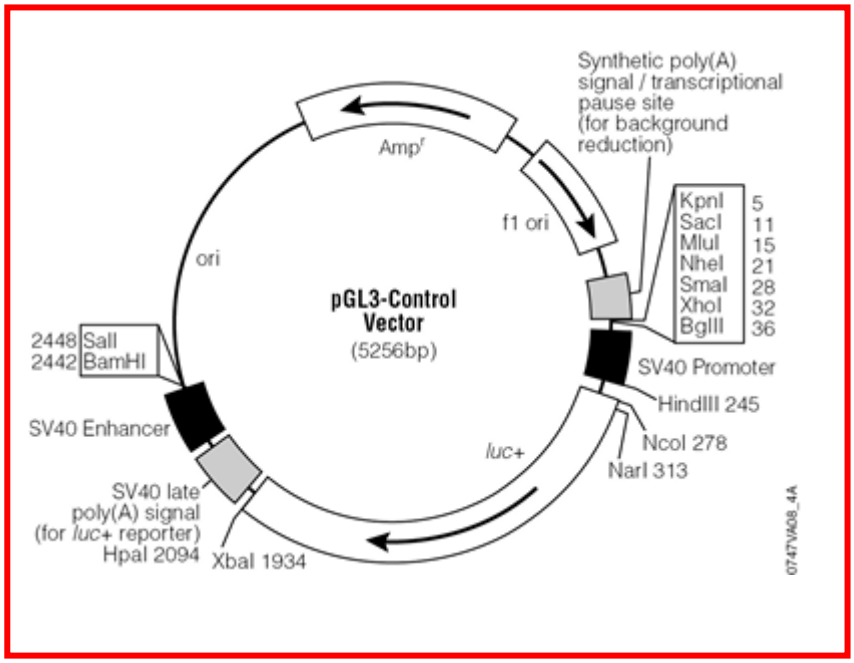

Figure 3.5: Schematic representation of the pGL3 control vector (Promega)

3.2.9 Production of pGL3-NANOG HRE mutant

pGL3-NANOG HRE mutant construct was generated using the same procedure of the pGL3-NANOG construct with the exception that the pGL3-NANOG construct was used as PCR template for the mutagenesis. Mutagenic primers were designed to change the HRE sequence (acgtg) into an XhoI restriction site (acTCgAG) (Table 3.1). Primers were designed using a bionformatic web site: http://bioinformatics.org/primerx and following the protocol of the QuickChange Manual (Stratagene, Texas, USA). 4ng of the pGL3-NANOG construct was amplified using 0.2µl of Pfu DNA polymerase with 2.5 pmoles/µl of forward and reverse primers, 10mM of dNTPs, 0.6µl of DMSO and 10x PfuUltra Buffer (Stratagene Texas, USA) in a total volume of 20µl. PCR was performed on a G-Storm thermocycler using the following cycling conditions: 1 cycle at 98°C for 30sec followed by 18 cycles at 98°C for 10sec, 55°C for 30sec, 72°C for 30sec and 1 cycle at 72°C for 5mins. Resulting PCR products were digested with DpnI enzyme at 37°C for 1h. DpnI only cleaves at methylated sites found on the parental strand of the plasmid but not in the PCR product (Figure 3.6). 2µl of digested PCR product was used to transform TOP10 supercompetent cells (Invitrogen). Colonies were picked from the plates and grown in 3ml LB supplemented with 100µg/µl Ampicillin at 37°C over-night. Small scale plasmid DNA preparation was performed using the QIAprep Miniprep Kit (Qiagen Crawley, UK) (See Appendix 2). To confirm mutation of the HRE site, 1µg of all DNA preparations was digested with 20U of XhoI at 37°C for 2h. As the pGL3-NANOG does not contain an XhoI site, this should not cut with the restriction enzyme, while the XhoI positive mutants should. Plasmids were purified using Genopure Plasmid Maxi kit (Roche) (See Appendix 5) following the manufacturer's instructions.

Table 3.1 Mutagenesis primers

pGL3-NANOG Mut Fw
CTGATTTAAAAGTTGGAAACTCGAGGAACCTAGAAGTATTTGTTG
pGL3-NANOG Mut Rev
CAACAAATACTTCTAGGTTCCTCGAGTTTCCAACTTTTAAATCAG

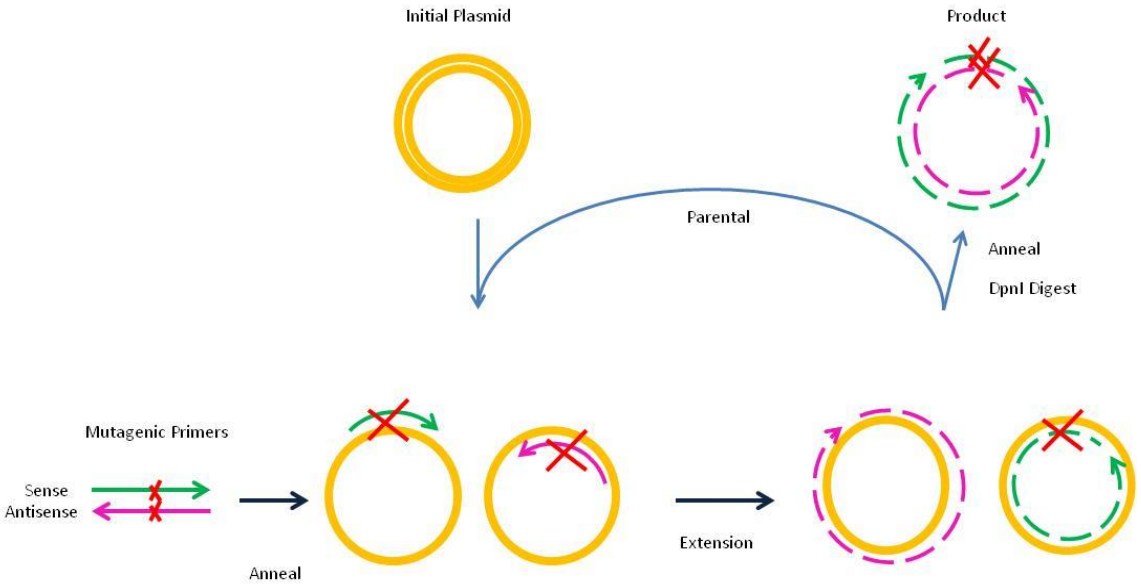

Figure 3.6: Site Directed mutagenesis

Schematic representation of the site-directed mutagenesis procedure. Mutagenic primers (green and violet arrows) will anneal to the plasmid template in a regular PCR reaction. Resulting PCR products are digested with DpnI enzyme that cleaves at methylated sites found on the parental strand of the plasmid but not in the PCR product. The resulting product will contain the mutated sequence introduced with the specific mutagenic primers.

3.2.10 Transfection of NT2 cells

NT2 cells were plated the day before transfection onto 24 well culture plates at a density of $8x10^4$ cells/well in 500μl of DMEM growth medium and incubated at 37°C at 20% oxygen tension. To evaluate the effect of HIF-2α on the NANOG HRE, cells were co-transfected with 100ng of pcDNA-HIF-2α expression plasmid (kindly provided by Prof. David Russell from the Southwestern Medical Centre (Texas, USA) and 100ng of each promoter-pGL3 construct. The pGL3-null and pcDNA3 null empty vectors (100ng) were used as negative controls whereas the pRL-SV40 Renilla luciferase vector (1ng) (Promega) was used to normalize the luciferase assay. The transfection reaction was performed with DMEM containing no serum, proteins or antibiotics to a total volume of 75μl. Samples were mixed briefly and then incubated with 7.5μl of SuperFect transfection reagent (Quiagen) for 10mins at room temperature. After incubation transfection complexes were added drop wise to the cells. Cells were incubated for 3 hours either at 20% or 5% oxygen then washed in PBS and supplemented with DMEM containing serum and antibiotics and incubated for 24 hours.

3.2.11 Luciferase assay

Luciferase assay was performed using the Dual-Luciferase Reporter Assay System (Promega, Southampton, UK). This is an in vitro tool system that allows study of gene expression by a quantitative measure of the reporter activity through an enzymatic reaction. Renilla and Firefly luciferase are two different reporter genes used as reporter and normalizer respectively in the assay. These genes encode for enzymes that convert luciferase in a luminescent substrate, luciferine, with emission of light. Luciferase assays were performed on NT2 cells transfected with promoter-pGL3 constructs as described above. Transfected cells were washed in PBS and incubated with 100μl of Passive Lysis Buffer (Promega Southampton, UK) and mixed for 20mins at room temperature. Cells were centrifuged at 13.000 rpm for 5mins to pellet cell debris and the supernatant was used for the assay. To measure samples luminescence, 20μl of samples were added to 40μl of Luciferase Assay Reagent (LAR) in a tube placed in the luminometer (TD-20/20 Turner Design) that was set to 100% sensitivity with a 2 sec delay before reading and 10 sec reading of luciferase activity.

3.3 Results

3.3.1 hES cells morphology

To characterise and investigate the effect of environmental oxygen on hES cell morphology, hES cells were maintained at both 5% and 20% oxygen tension on MEFs and under feeder free conditions on Matrigel. At both oxygen tensions a typical hES cell colony morphology was observed on MEFs and Matrigel (Figure 3.7). On MEFs, hES cell colonies presented well defined round edges and high level of compaction at both oxygen tensions (Figure 3.7). Under feeder free conditions, colonies cultured at 5% oxygen appeared compact with distinct boarder, whereas at 20% oxygen, a more diffuse morphology was observed (Figure 3.7).

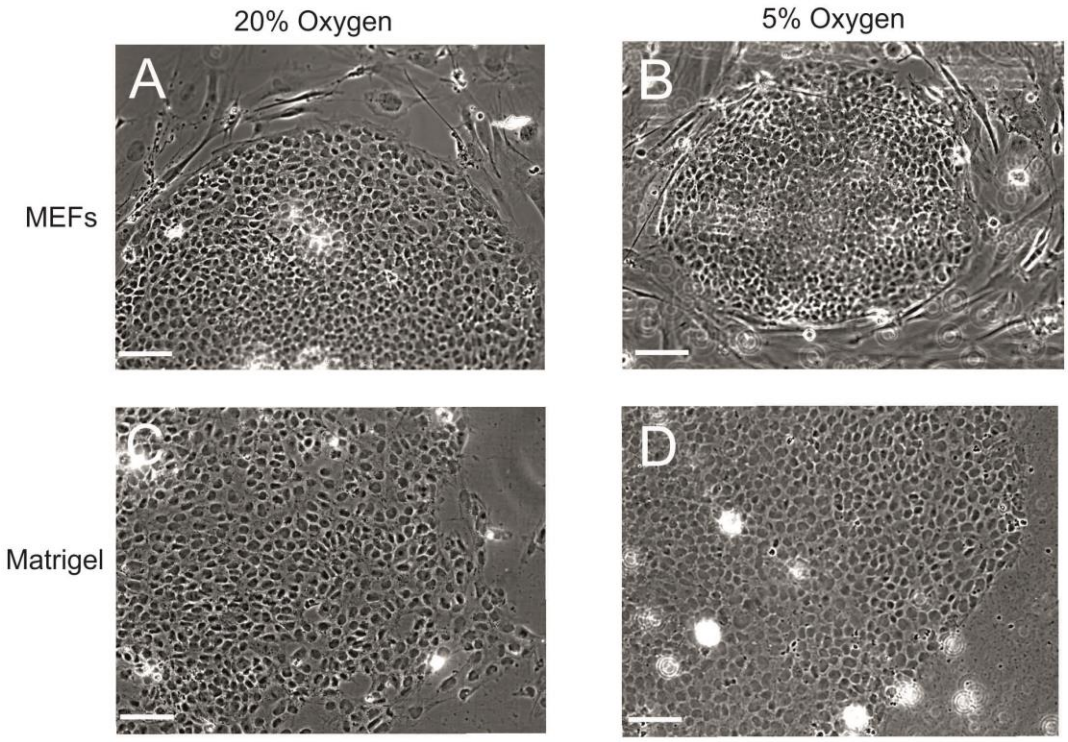

Figure 3.7: hES cell colony morphology on MEFs and on Matrigel

Representative phase contrast images of Hues 7 hES cell colonies cultured on MEFs (A and B) and Matrigel (C and D) at 20% oxygen (A and C) or 5% oxygen (B and D) on day 3 post passage. Scale bar = 100μm.

To investigate the effect of oxygen tension on pluripotency marker expression immunocytochemistry was performed on hES cells cultured at 20% or 5% oxygen tension. Cells maintained at 20% oxygen tension expressed OCT4, SOX2, NANOG as well as the surface marker TRA-1-60. Interesting, some hES cells colonies maintained at 20% oxygen were also found to be positive for the early differentiation surface marker SSEA-1 at the edge but also in the centre of the colony (Figure 3.8).

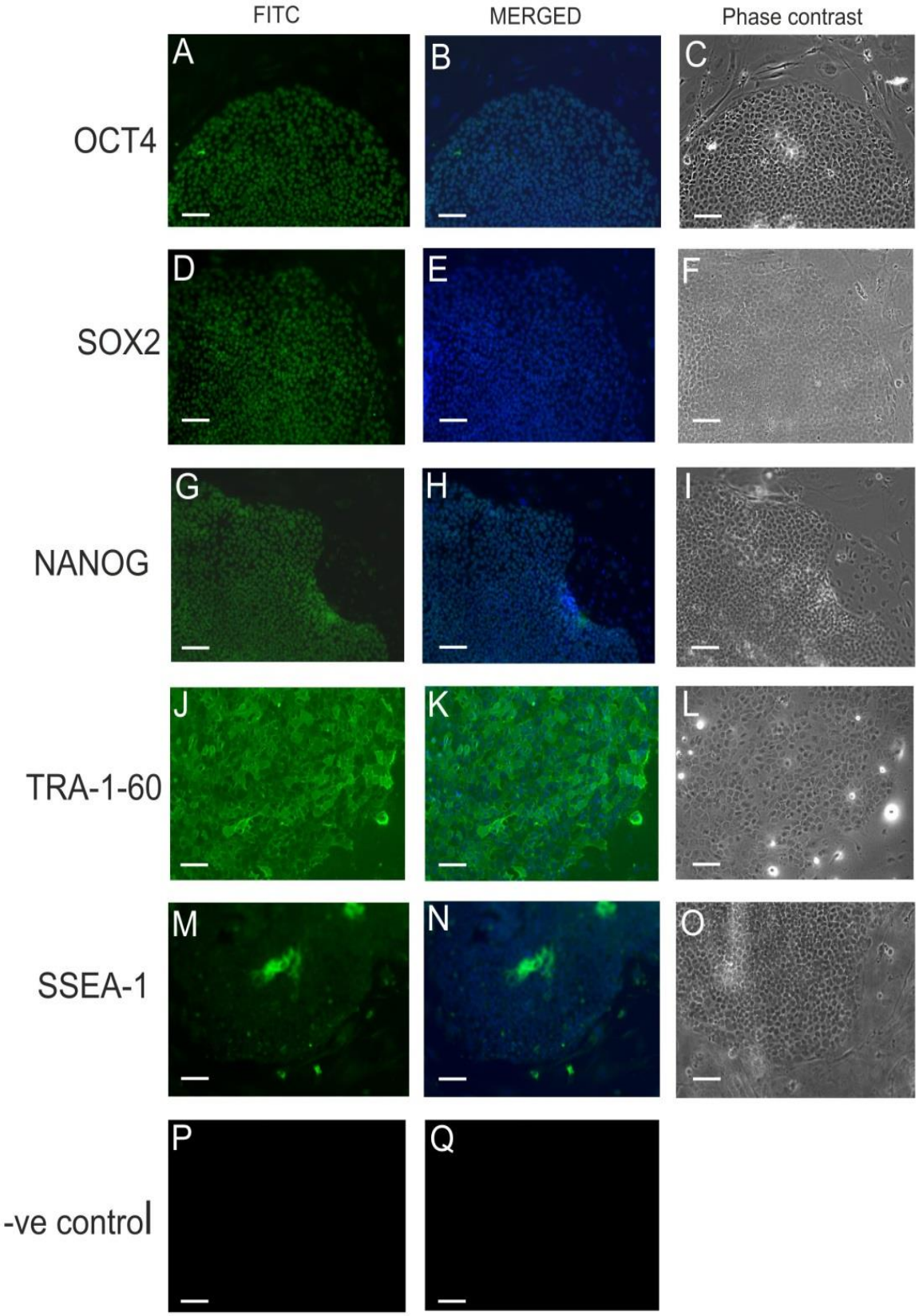
FITC
MERGED
Phase contrast
OCT4
SOX2
NANOG
TRA-1-60
SSEA-1
-ve control
A
B
C
D
E
F
G
H
I
J
K
L
M
N
O
P
Q

Figure 3.8: Characterization of hES cells cultured at 20% oxygen tension.

Representative immunocytochemistry labelling of OCT4 (A-B), merged with DAPI (B), SOX2 (D-E), merged with DAPI (E), NANOG (G-H), merged with DAPI (H), TRA-1-60 (J-K), merged with DAPI (K), SSEA-1 (M-N), merged with DAPI (N), secondary antibody only negative controls (P and Q), Phase contrast images of colonies (C-F-I-L-O) of hES cells cultured on MEFs at 20% oxygen tension. Scale bar 100μm.

Using higher power magnification the nuclear localization of OCT4, SOX2 and NANOG was clearly observed as well as the surface markers TRA-1-60 and SSEA-1 in hES cells cultured at 20% oxygen (Figure 3.9). Interestingly the early differentiation marker SSEA-1 was observed predominantly towards the edge of the colonies.

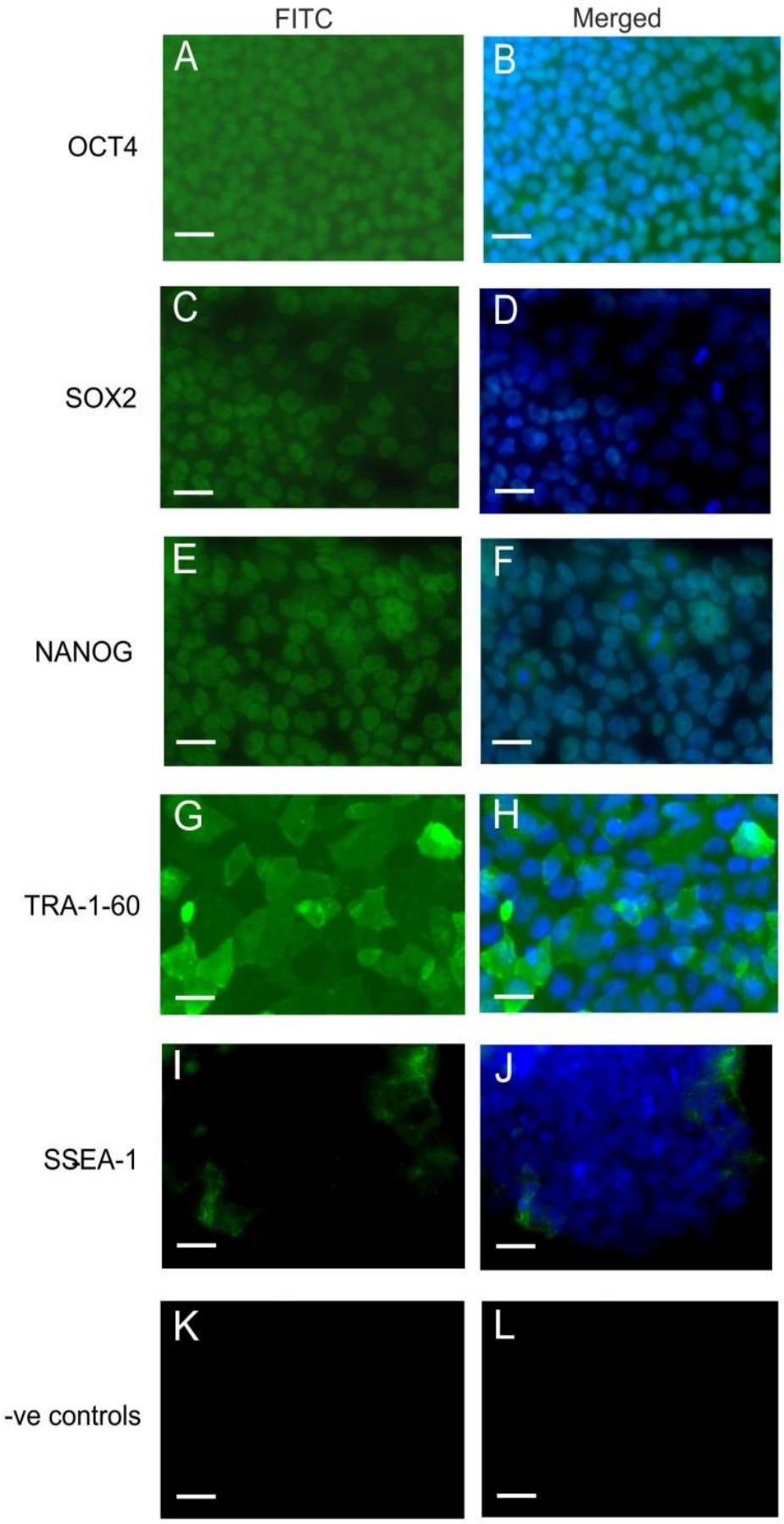
FITC
Merged
A
B
OCT4
C
D
SOX2
E
F
NANOG
G
H
TRA-1-60
I
J
SSEA-1
K
L
-ve controls

Figure 3.9: Expression of pluripotency markers OCT4, SOX2 and NANOG and the differentiation marker SSEA-1 by immunocytochemistry in hES cells cultured at 20% oxygen

Representative immunocytochemistry images of OCT4 (A-B), merged with DAPI (B), SOX2 (C-D), merged with DAPI (D), NANOG (E-F), merged with DAPI (F), TRA-1-60 (G-H), merged with DAPI (H), SSEA-1 (I,-J), merged with DAPI (J), secondary antibody only negative controls (K and L) of hES cells cultured on MEFs at 20% oxygen. Scale bar 25μm.

hES cells cultured under hypoxic conditions were also positive for OCT4, SOX2, NANOG and for the surface marker TRA-1-60. It is was noticed that SSEA-1 staining appeared less in hES cells maintained at 5% oxygen compared to those cultured at 20% oxygen (Figure 3.10).

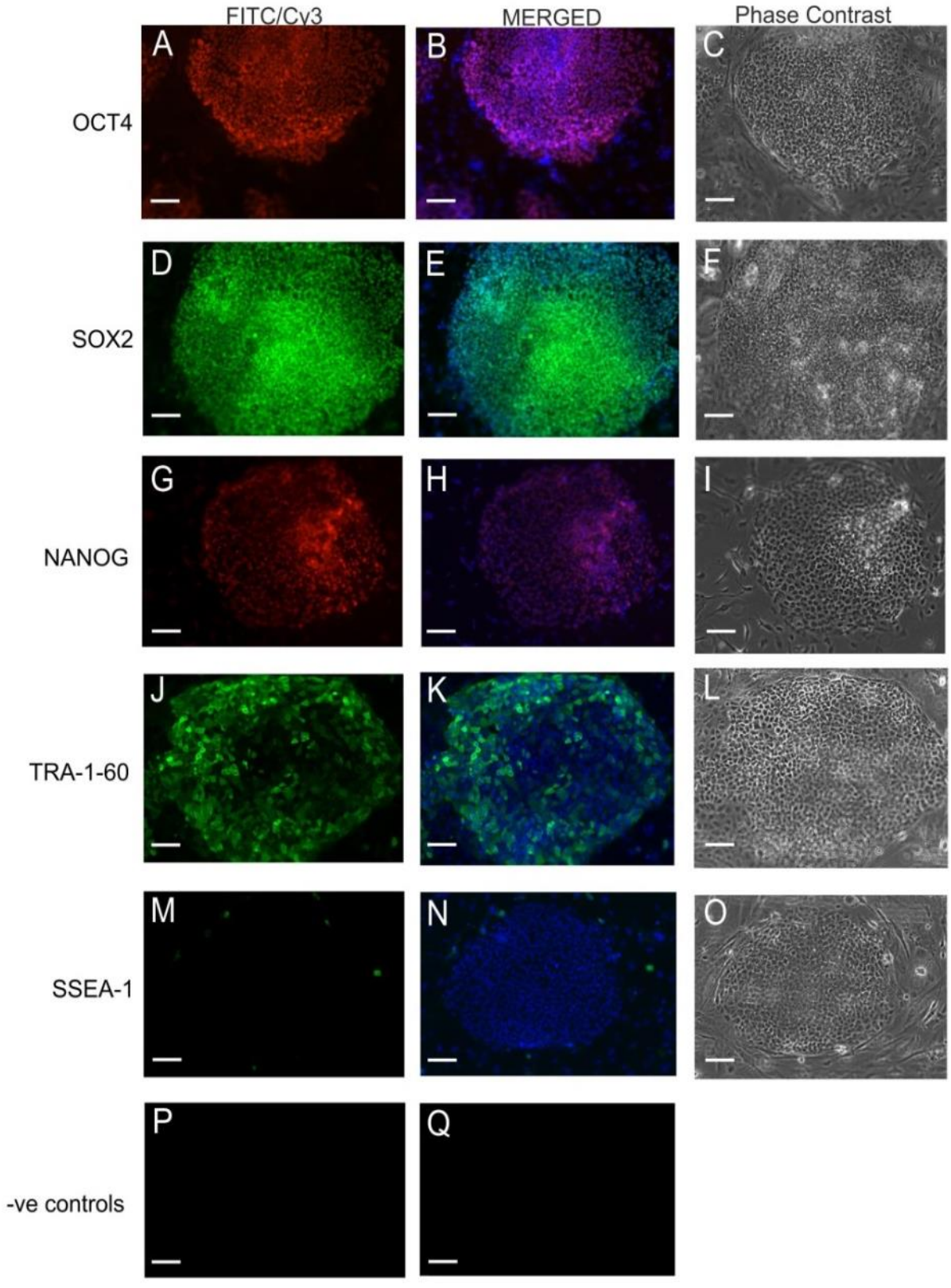
FITC/Cy3
MERGED
Phase Contrast
OCT4
SOX2
NANOG
TRA-1-60
SSEA-1
-ve controls
A
B
C
D
E
F
G
H
I
J
K
L
M
N
O
P
Q

Figure 3.10: Characterization of hES cells cultured at 5% oxygen tension

Representative immunocytochemistry labelling OCT4 (A-C), merged with DAPI (B), SOX2 (D-F), merged with DAPI (E), NANOG (G-I), merged with DAPI (H), TRA-1-60 (J-L), merged with DAPI (K), SSEA-1 (M-O), merged with DAPI (N), secondary antibody only negative controls (P and Q) and Phase contrast images of colonies (C-O) of hES cells cultured on MEFs at 5% oxygen tension after 3 days post passage. Scale bar 100μm.

Higher magnification of 5% colonies allow to better appreciate the positivity for the pluripotency markers OCT4, SOX2, NANOG and TRA 1-60 and the low level of the differentiation marker SEEA-1 as displayed in Figure 3.11.

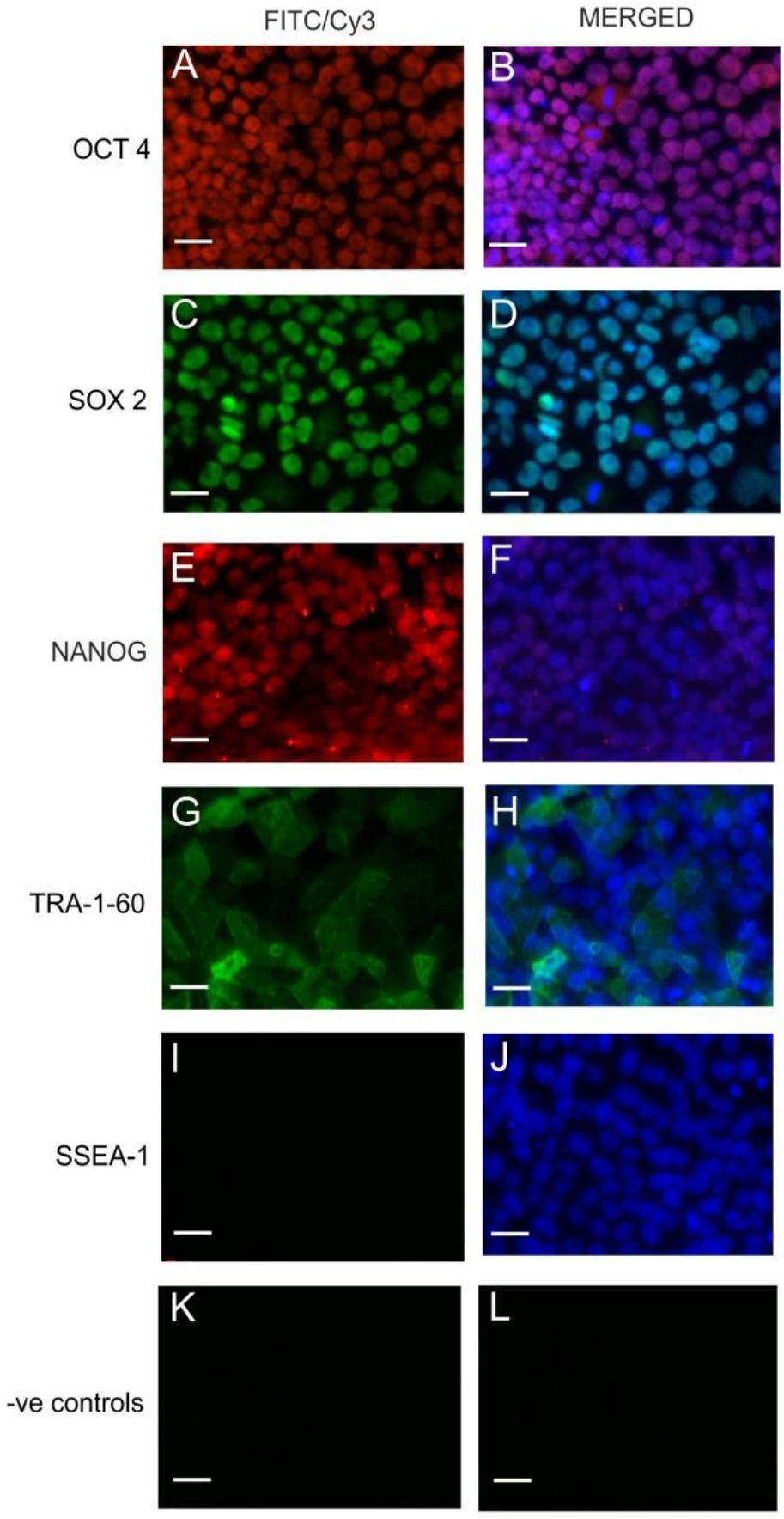
FITC/Cy3
MERGED
A
B
OCT 4
C
D
SOX 2
E
F
NANOG
G
H
TRA-1-60
I
J
SSEA-1
K
L
-ve controls

Figure 3.11: Expression of pluripotency markers OCT4, SOX2 and NANOG and the differentiation marker SSEA-1 by immunocytochemistry in hES cells cultured at 5% oxygen

Immunocytochemistry of OCT4 (A-B), merged with DAPI (B), SOX2 (C-D), merged with DAPI (D), NANOG (E-F), merged with DAPI (F), TRA-1-60 (G-H), merged with DAPI (H), SSEA-1 (I-J), merged with DAPI (J), secondary antibody only negative controls (K and L) of hES cells cultured on MEFs at 5% oxygen tension after 3 days post passage. Scale bar 25μm.

3.3.2 cDNA validation prior to RT-qPCR

Prior to performing qPCR, cDNA generated from hES cells cultured at 5% or 20% oxygen was tested to ensure it was free from genomic contamination. This was performed by determining the expression of OAZ1 gene using intron spanning primers (See Methods 2.3). A cDNA product was expected to generate a fragment of 122bp while a 373bp amplicon would be produced from genomic DNA.To quantify the mRNA expression in hES cells, RT-qPCR was performed (Figure 3.12).

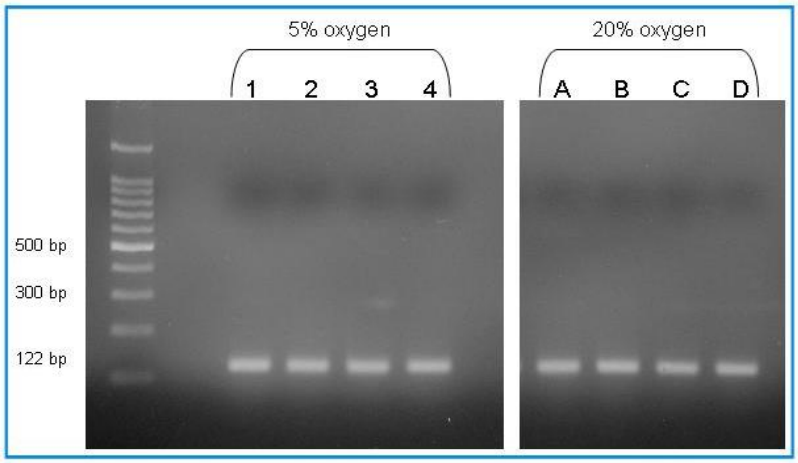

Figure 3.12: cDNA from hES cells cultured at 5% and 20% oxygen tension are free from genomic contamination

An ethidium bromide stained 1% agarose gel showing amplified PCR products for OAZ1. Lane 1-4 cDNA from Hues 7 hES cells cultured at 5% oxygen tension; lane A-D cDNA from Hues 7 hES cells cultured at 20% oxygen tension. Expected cDNA product size = 122 bp; expected genomic DNA product = 373 bp.

3.3.3 Effect of oxygen tension on hES cells pluripotency

To investigate the expression of OCT4, SOX2 and NANOG in hES cells cultured under hypoxia or normoxia, RT-qPCR analysis was performed.

There was a significant reduction in the mRNA expression of all 3 pluripotency markers in hES cells cultured at 20% oxygen compared to those maintained at 5% oxygen. hES cells cultured at 20% oxygen displayed an approximate 50% reduction in *OCT4* ($P<0.05$), *NANOG* ($P<0.05$) and *SOX2* ($P<0.05$) expression compared to cells cultured at 5% oxygen (Figure 3.13).

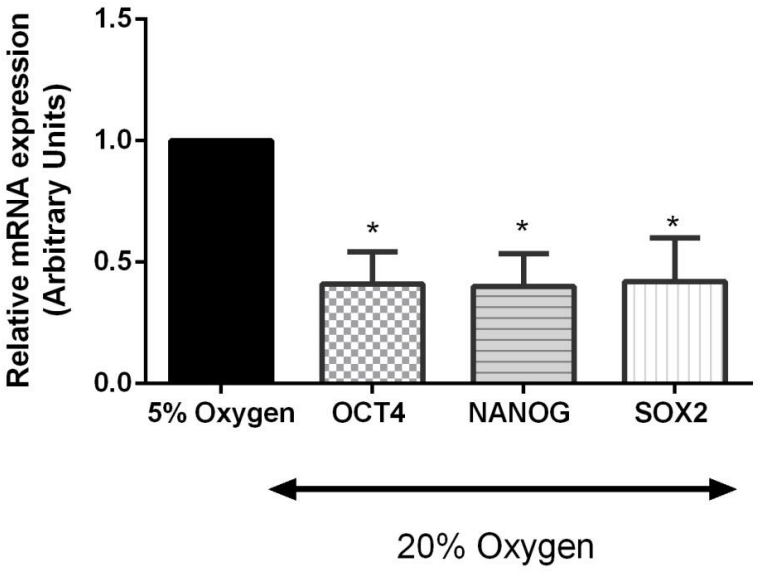

Figure 3.13: Pluripotency markers are reduced in hES cells mantained at 20% oxygen compared to those cultured at 5% oxygen

RT-qPCR analysis of *OCT4*, *SOX2* and *NANOG* in hES cells cultured at 5% or 20% oxygen. All data have been normalized to *UBC* and to 1 for 5% oxygen. Values are mean of 4 independent experiments ± SEM (*$P<0.05$). The greatest error for cells cultured at 5% oxygen was obtained for NANOG which was 1±0.4.

These results show, in agreement with previous published data, showing that hES cells maintained at 5% oxygen display a more pluripotent phenotype than those cultured under atmospheric oxygen tension.

3.3.4 Effect of oxygen tension on the expression of HIF-2α

3.3.4.1 HIF-2α mRNA expression in hypoxia and normoxia

To analyze the effect of hypoxia on the mRNA expression levels of HIF-2α, RT-qPCR was performed. *HIF-2α* was expressed both in hES cells cultured at 20% oxygen and at 5% oxygen (Figure 3.14). No significant difference in the mRNA expression level was found when hES cells cultured at 5% oxygen were compared to those maintained at 20% oxygen (Figure 3.14).

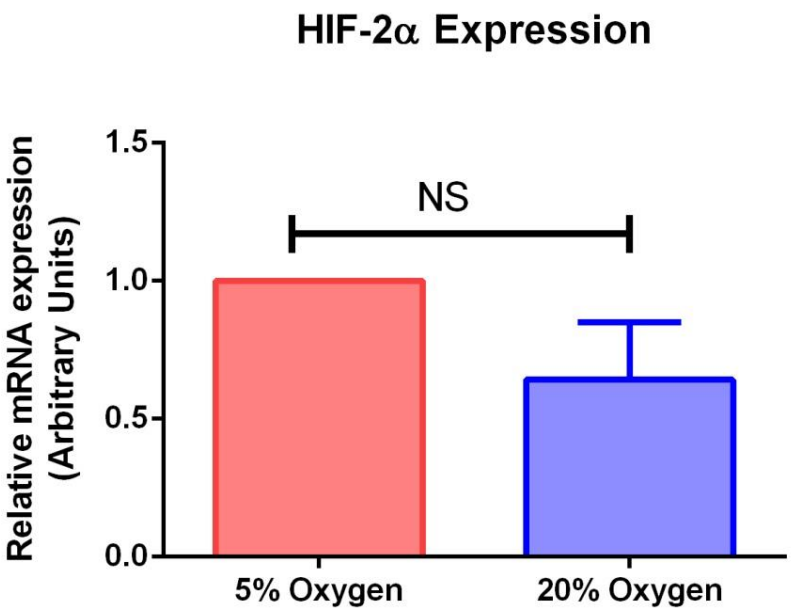

Figure 3.14: *HIF-2α* mRNA quantification in hES cells cultured at 5% or 20% oxygen tension

Representative RT-qPCR of *HIF-2α* mRNA expression in hES cells cultured at 5% and 20% oxygen. Data have been normalized to *UBC* and to 1 for 5% oxygen. An average of 3 independent experiments is shown. The error for ***HIF-2α*** at 5% oxygen was 1±0.01.

3.3.4.3 HIF-2α protein expression

Western Blot analysis was performed to ensure the HIF-2α antibody recognised a protein of the correct size and thus was suitable to use in further experiments. It was noticed that HIF-2α protein was less expressed in hES cells cultured at 20% compared to hES cells cultured at 5% oxygen (Figure 3.15). These data are in agreement with previous published data showing that HIF-2α protein is significantly up-regulated under 5% oxygen (Forristal et al., 2010).

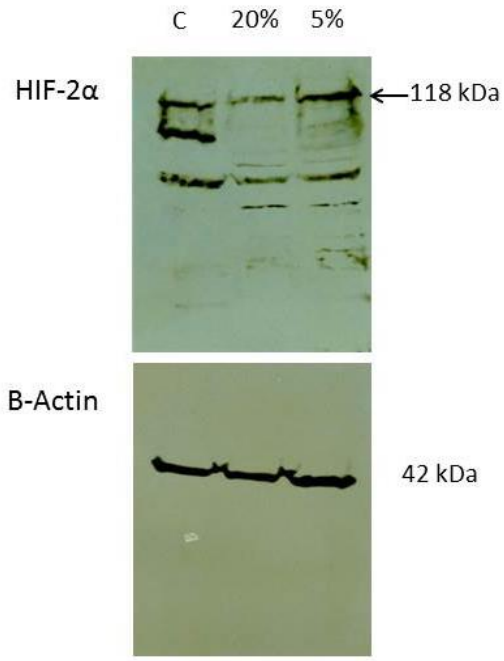

Figure 3.15: Expression of HIF-2α protein using Western Blot analysis in hES cells cultured at 20% and 5% oxygen

Representative Western Blot of HIF-2α and β-Actin expression: C: NT2 protein lysate used as positive control; 20%: hES cells cultured at 20% oxygen; 5%: hES cells cultured at 5% oxygen. Data represent n=1 experiment normalized to β-Actin.

3.3.5 Chromatin Immuno-Precipitation Assay (ChIP) to examine genes regulated by HIF-2α

To determine whether HIF-2α directly interacts *in vivo* with HREs (Hypoxia Responsive Elements) in the promoter region of OCT4, NANOG, SOX2, GLUT1 and eNOS genes, ChIP assays were performed on hES cells cultured either at 5% or 20% oxygen tension on Matrigel coated plates. Chromatin isolated from hES cells was sonicated to obtain fragments of approximatively of 0.5-1kb in length (Figure 3.16) and immunoprecipitated with anti-HIF-2α antibody. A rabbit Immunoglobulin G antibody was used as a negative control. It was noticed that in general, chromatin was less concentrated and easier to sonicate when derived from hESCs cultured at 20% oxygen compared to 5% oxygen.

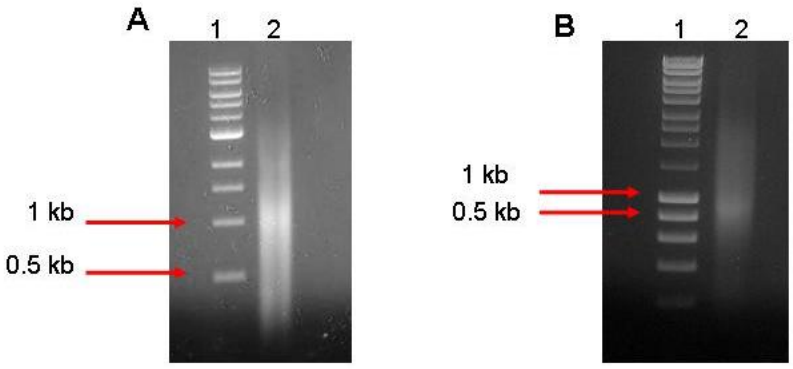

Figure 3.16: Comparison between sonicated chromatin from hES cells cultured at 5% or 20% oxygen tension

Representative sonicated chromatin isolated from hES cells cultured at 5% oxygen (A) and 20% oxygen (B). Lane 1: Hyperladder; lane 2: fragmented chromatin.

Initial attention was focused on OCT4 due to the extensive sequence analysis of both the mouse and human OCT4 promoter and enhancer region (Nordhoff et al., 2001) which revealed several putative HRE sites. To verify that HIF-2α was capable of binding to the OCT4 proximal promoter, chromatin from Hues 7 hES cells cultured at 5% oxygen for at least 3 passages was immunoprecipitated with anti-HIF-2α antibody.

Using primers specific for either the Conserved Regions 3 a strong enrichment was observed (Figure 3.17) (See Methods section 2.7).

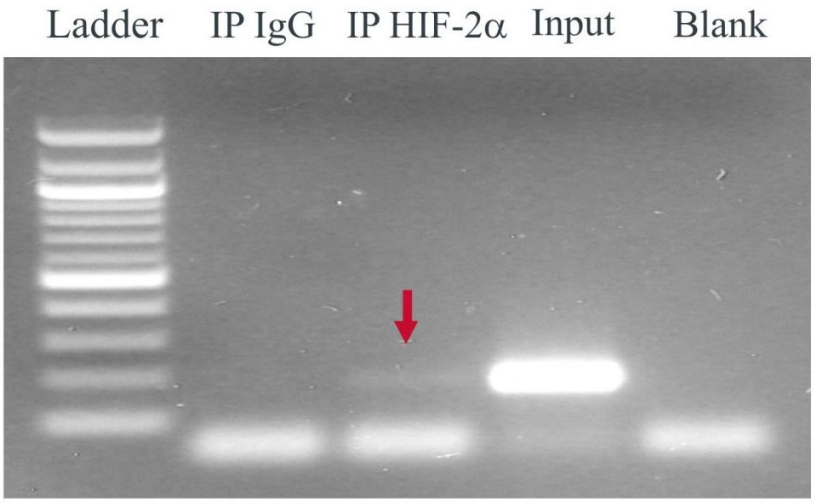

Figure 3.17: HIF-2α binds *in vivo* to the CR3 OCT4 proximal promoter region in hES cells cultured at 5% oxygen

Representative ethidium bromide-stained 1.5% agarose gel-electrophoresis of CR3 OCT4 promoter amplicons, respectively from hES cells immunoprecipitated with anti HIF-2α (IP HIF2α) or IgG (IP IgG) antibodies. The arrow indicate the specific enrichment in hESCs maintained at 5% oxygen sample immunoprecipitated with the anti HIF-2α antibody (band of 200 bp was observed). Input sample is the non-precipitated chromatin and represent the positive control, the blank sample is the negative water control for the PCR reaction.

To verify binding within the HRE sites of NANOG and SOX2 proximal promoters PCR analysis was performed. Moreover, specific primers were designed to amplify the HRE sites in the proximal promoter of GLUT1 known to be a hypoxia-inducible gene (Wenger, 2002). However, due to the poor sensitivity of the method, no interaction was found. As the experiment was performed to test the *in vivo* binding of the endogenous HIF-2α on the HRE sites of its target genes, it is likely that a regular PCR was not sensitive enough to highlight the chromatin interaction.

3.3.6 ChIP analysis using qPCR

To better identify and quantify the interactions between HIF-2α and its target genes, a more sensitive technique employing qPCR and Taqman probes was performed.

All probes were specifically designed to cover the HRE of interest for each gene of interest using the Applied Biosystem Software Design.

Interestingly, qPCR showed a significant level of enrichment in HIF-2α immunoprecipitated chromatin from hES cells cultured at 5% oxygen for the selected HRE in the OCT4, SOX2, NANOG, GLUT1 and eNOS proximal promoter, compared to the IgG negative control immunoprecipitated chromatin. No significant binding was found when the chromatin of 20% oxygen cells was immunoprecipitated with the HIF-2α antibody and the enrichment obtained was similar to that observed in the IgG negative control. Comparison between immunoprecipitated samples for HIF-2α in 5% oxygen cells for all of the genes of interest, showed a significant enrichment ($P<0.05$) compared with both the 5% IgG control and with 20% chromatin immunoprecipitated with HIF-2α antibody, meaning that HIF-2α is binding only in hypoxic conditions.

Specific binding of HIF-2α was observed at the HRE at -1956 bp corresponding to CR3 in the proximal OCT4 promoter. ChIP analysis revealed a 4-fold increase of enrichment in hES cells cultured under hypoxic conditions compared to the IgG control ($P<0.01$). There was no significant difference in the level of OCT4 enrichment between cells maintained at 20% oxygen when compared to the IgG control antibody (Figure 3.18). However, HIF-2α was found to significantly bind to the HRE in OCT4 promoter in cells cultured under hypoxia relatively to normoxia and the IgG negative control (Figure 3.18). This reveals that HIF-2α is binding to the proximal OCT4 promoter only in hypoxic conditions.

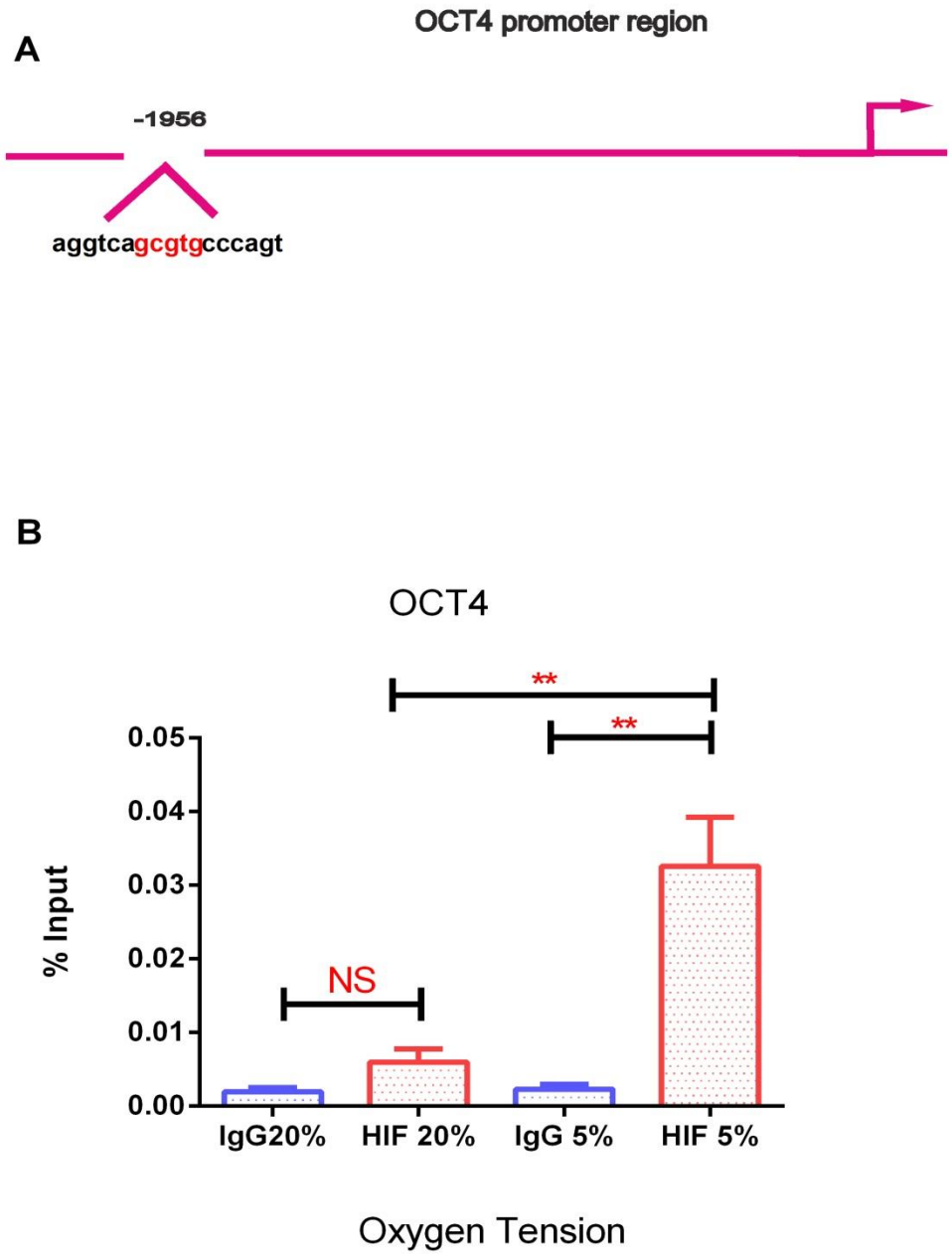

Figure 3.18: HIF-2α binds OCT4 proximal promoter in hypoxia

(A) Schematic representation of the OCT4 proximal promoter and the putative HRE located at -1956 from the start site. ChIP assays were performed with anti-HIF-2α (HIF) or IgG control antibodies on chromatin isolated from hESCs cultured either at 20% or 5% oxygen tension. Comparison of OCT4 enrichment at 5% oxygen compared to 20% oxygen (B). DNA enrichment is expressed as a percentage of input (non-immunoprecipitated chromatin). An average of 3 independent experiments is represented (**P<0.01; NS: no significant difference).

Amplification of a putative HRE at -301 bp in the NANOG proximal promoter revealed a significant 4-fold enrichment in cells cultured at low oxygen tension. A similar experiment was performed on hES cells cultured at 20% oxygen tension and no significant enrichment was seen when compared to IgG negative control. A significant enrichment of HIF-2α binding was found in hES cells cultured at 5% oxygen when compared to hES cells cultured at 20% oxygen. No significant binding was found in hES cells cultured at 20% oxygen (Figure 3.19). This is the first demonstration of a direct interaction of HIF-2α with the NANOG promoter under hypoxic conditions (Figure 3.19).

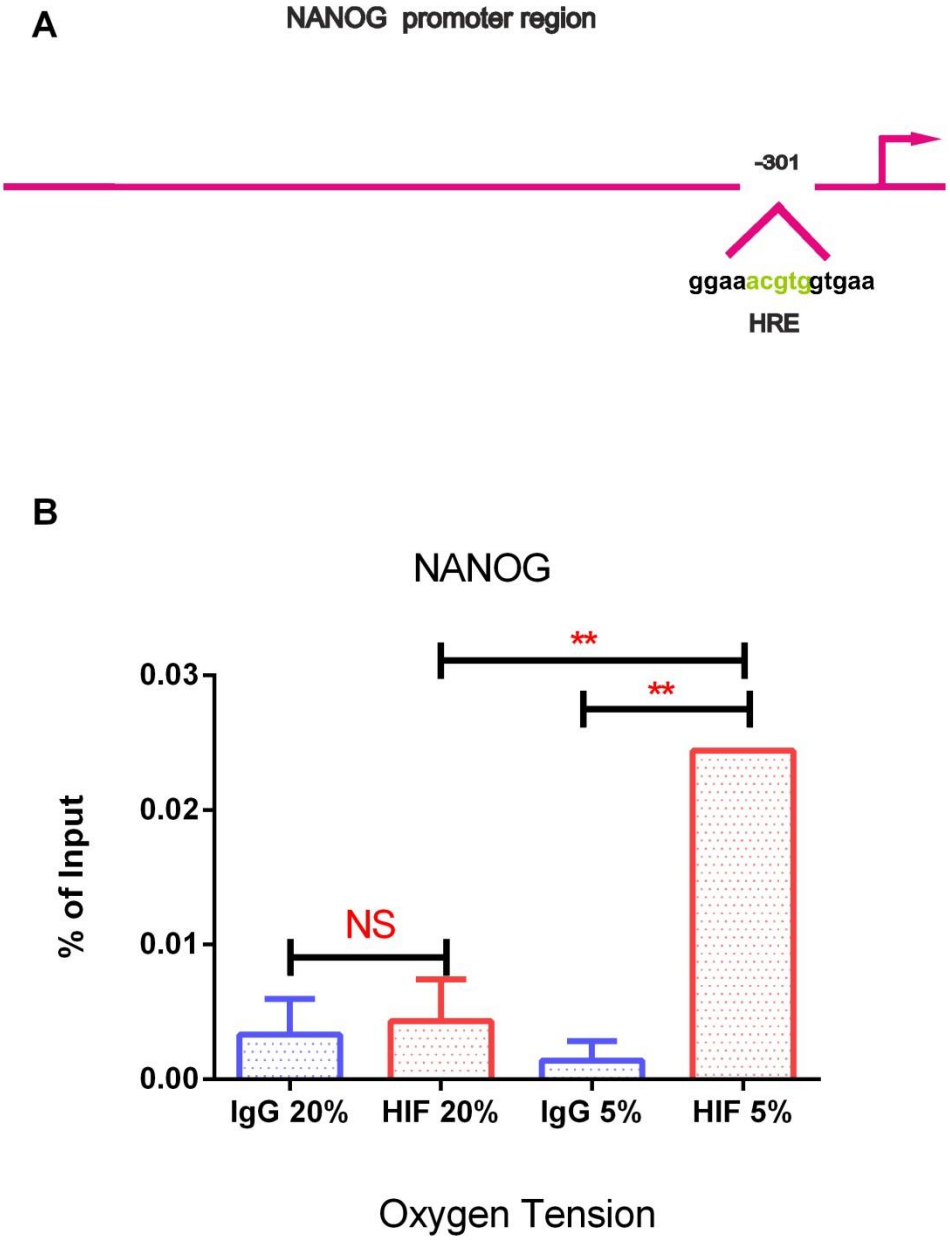

Figure 3.19: HIF-2α binds NANOG proximal promoter in hypoxia

(A) Schematic representation of the NANOG proximal promoter and the putative HRE site located at -301 bp from the start site. ChIP assays were performed with either an anti-HIF-2α (HIF) or IgG control antibodies on chromatin isolated from hESCs cultured either at 20% or 5% oxygen tension. Comparison of NANOG enrichment at 5% oxygen compared to 20% oxygen (B). DNA enrichment is expressed as a percentage of input (non-immunoprecipitated chromatin). An average of 3 independent experiments is represented (**P<0.01; NS: no significant difference).

For the SOX2 proximal promoter 2 different HREs situated at -1450 bp and -1100 bp from the start site were analyzed. These HREs differ for the presence of the A nucleotide (A)CGTG (SOX2A) or a G nucleotide (G)CGTG (SOX2G). ChIP samples of cells cultured at 5% oxygen tension were significantly enriched for both SOX2A (Figure 3.20) and SOX2G (Figure 3.21) compared to those maintained at 20% oxygen. However, the level of enrichment varied displaying a 6-fold and a 3 fold increase in the SOX2A and SOX2G proximal promoter respectively. No significant binding was found to the HREs in the chromatin of hES cells cultured at 20% oxygen tension and compared to the IgG or to hES cells cultured at 5% oxygen (Figure 3.20 and 3.21).

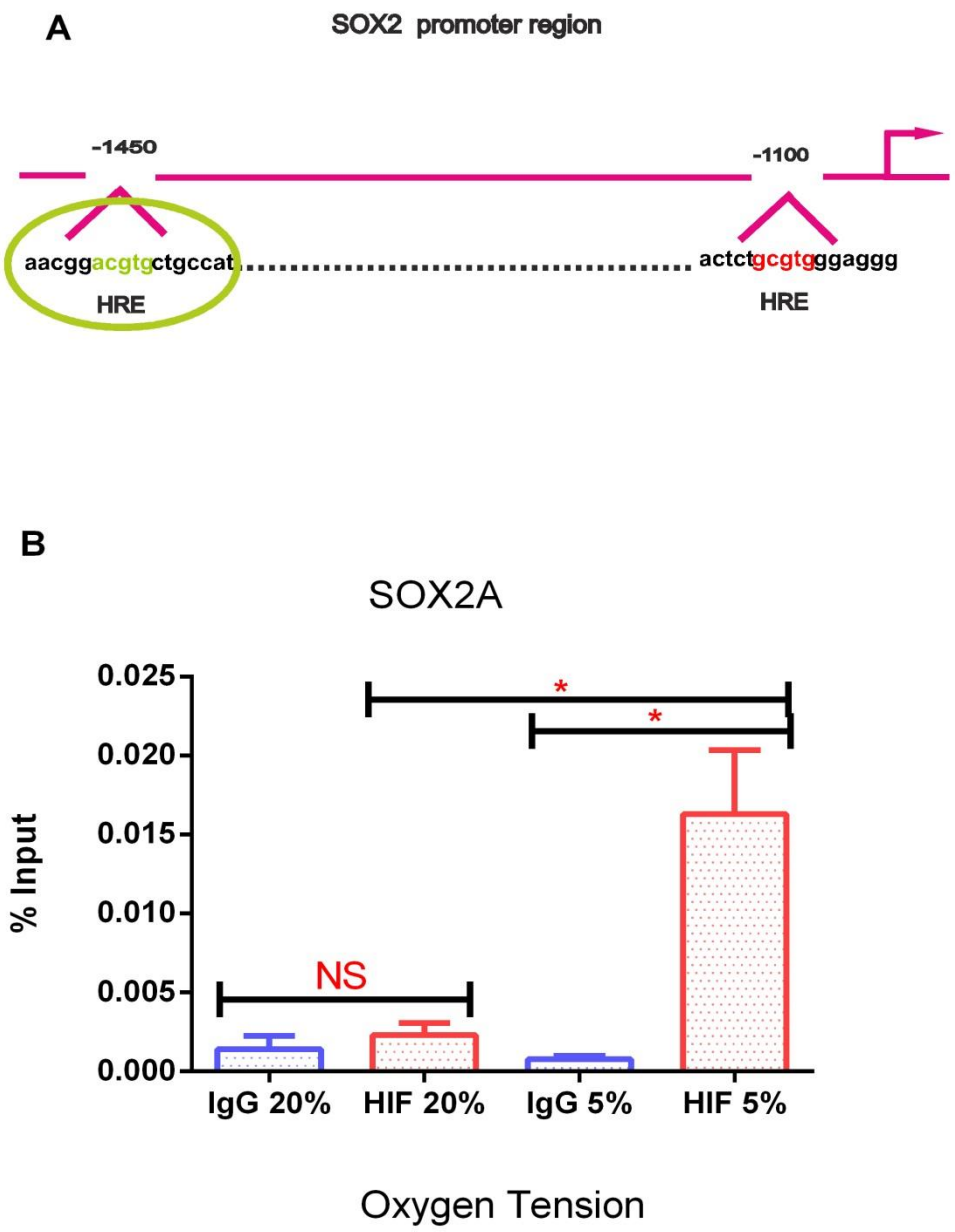

Figure 3.20: HIF-2α binds SOX2A proximal promoter in hypoxia

(A) Schematic representation of 2 potential HRE sites at -1450 (SOX2A) and -1100 (SOX2G). ChIP analysis of HIF-2α binding to the proximal promoter of SOX2 at the HRE located at -1450 from the start site. ChIP assays were performed with either an anti-HIF-2α (HIF) or IgG control antibodies on chromatin isolated from hESCs cultured either at 20% or 5% oxygen tension. Comparison of SOX2A enrichment at 5% oxygen compared to 20% oxygen (B). DNA enrichment is expressed as a percentage of input (non-immunoprecipitated chromatin). An average of 3 independent experiments is represented (*P<0.05; NS: no significant difference).

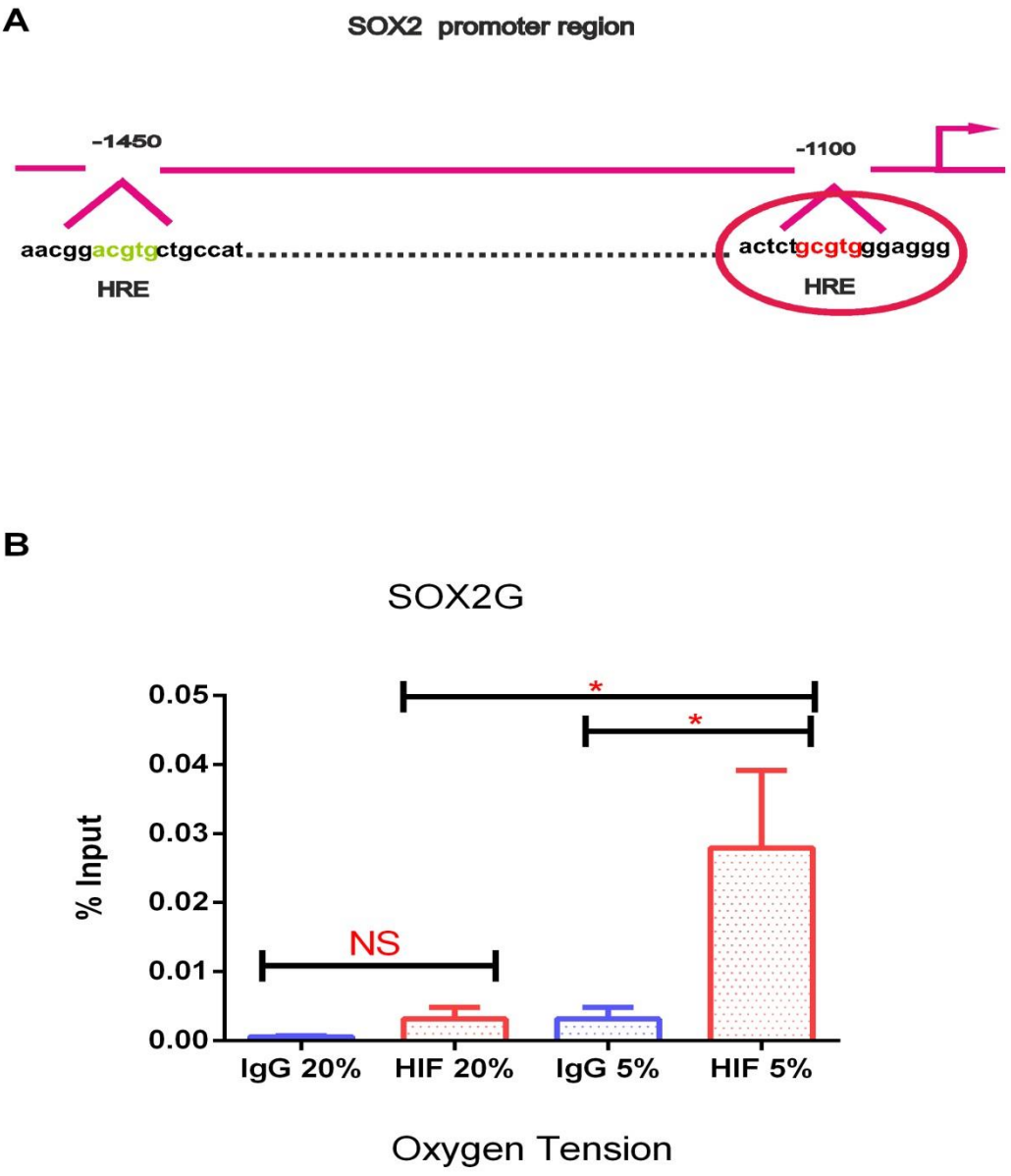

Figure 3.21: HIF-2α binds SOX2G proximal promoter in hypoxia

(A) Schematic representation of 2 potential HRE sites at -1450 (SOX2A) and -1100 (SOX2G). ChIP analysis of HIF-2α binding to the proximal promoter of SOX2 at HRE located at -1100 from the start site. ChIP assays were performed with either an anti-HIF-2α (HIF) or IgG control antibodies on chromatin isolated from hESCs cultured either at 20% or 5% oxygen tension. Comparison of SOX2G enrichment at 5% oxygen compared to 20% oxygen (B). DNA enrichment is expressed as a percentage of input (non-immunoprecipitated chromatin). An average of 3 independent experiments is represented (*P<0.05; NS: no significant difference).

A significant enrichment (13-fold increase) in HIF-2α binding was found in hES cells maintained under hypoxia compared to the IgG negative control (Figure 3.22). The HRE amplified was -2644 bp from the start site and is formed by two contiguous HREs (GTGACGTG). No significant enrichment was found in hES cells maintained under normoxia compared to the IgG control. HIF-2α binding to the eNOS proximal romoter was significantly enriched only in hES cells cultured at 5% oxygen compared to those maintained at 20% oxygen (Figure 3.22). This data highlights a significant interaction of HIF-2α on the eNOS proximal promoter only in hypoxic conditions.

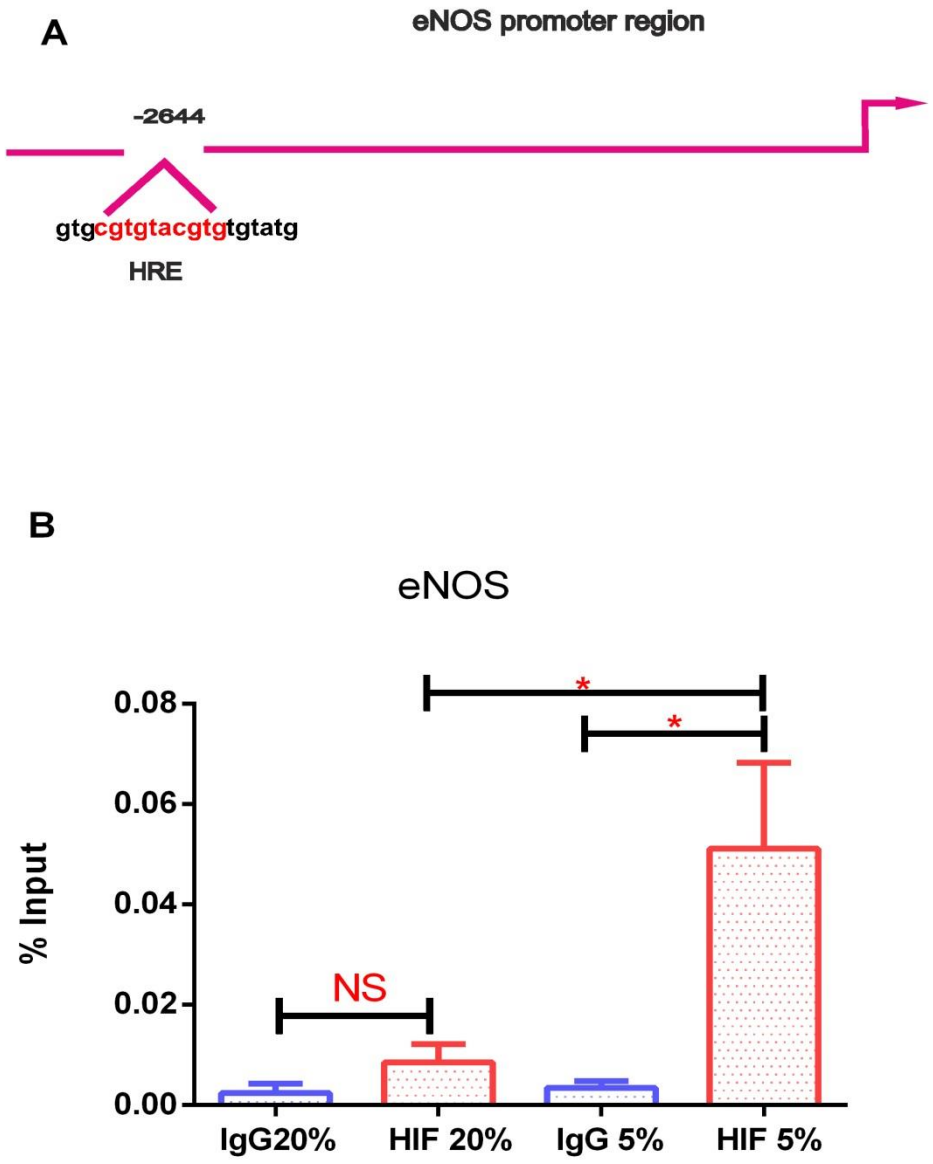

Figure 3.22: HIF-2α binds eNOS proximal promoter in hypoxia

A) Schematic representation of the proximal promoter of eNOS and the putative HRE located at -2644 from the start site. ChIP assays were performed with either an anti-HIF-2α (HIF) or IgG control antibodies on chromatin isolated from hESCs cultured either at 20% or 5% oxygen tension. Comparison of eNOS enrichment at 5% oxygen compared to 20% oxygen (B). DNA enrichment is expressed as a percentage of input (non-immunoprecipitated chromatin). An average of 3 independent experiments is represented (*P<0.05; NS: no significant difference).

Binding of HIF-2α to an HRE at -1691 bp upstream of the transcription start site in the proximal promoter of GLUT1 was investigated. ChIP assays from hES cells under hypoxia revealed a strong 4-fold enrichment over the IgG control (Figure 3.23). No significant binding of HIF-2α in hES cells cultured under normoxia was found when compared to the IgG control. hES cells cultured at 5% oxygen displayed a significantly increased binding compared to those maintained at 20% oxygen (Figure 3.23). This data reveals a specific HIF-2α interaction with the GLUT1 proximal promoter in hES cells only when cultured in a hypoxic environment.

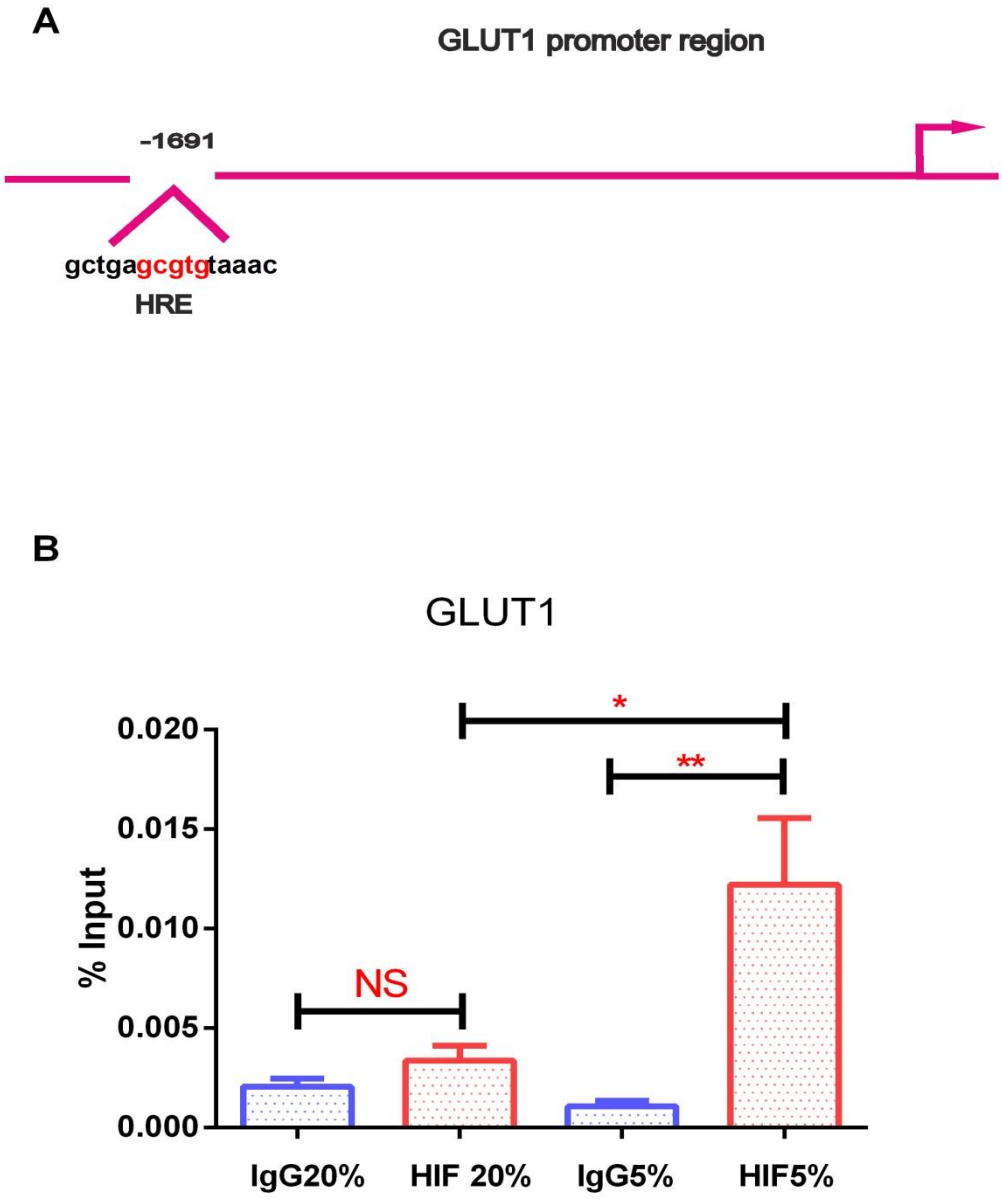

Figure 3.23: HIF-2α binds GLUT1 proximal promoter in hypoxia

(A) Schematic representation of the proximal promoter of GLUT1 and the putative HRE located at -1691 from the start site. ChIP assays were performed with either an anti-HIF-2α (HIF) or an IgG control antibodies on chromatin isolated from hESCs cultured either at 20% or 5% oxygen tension. Comparison of GLUT1 enrichment at 5% oxygen compared to 20% oxygen (B). DNA enrichment is expressed as a percentage of input (non-immunoprecipitated chromatin). An average of 6 independent experiments is represented (**P<0.01; *P<0.05; NS: no significant difference).

To further verify the specificity of HIF-2α binding a negative control probe specific for the FOXP3 promoter was used. This probe was designed to amplify a region in the proximal promoter which does not contain an HRE but instead was situated between 2 HREs at -670 and +104 respectively from the transcription start site (Figure 3.24). qPCR on three different ChIP experiments on chromatin derived from hES cells cultured at either 5% or 20% oxygen tension revealed no significant enrichment for HIF-2α in this FOXP3 promoter region ($P>0.05$). This control verifies the specificity of the interactions observed previously.

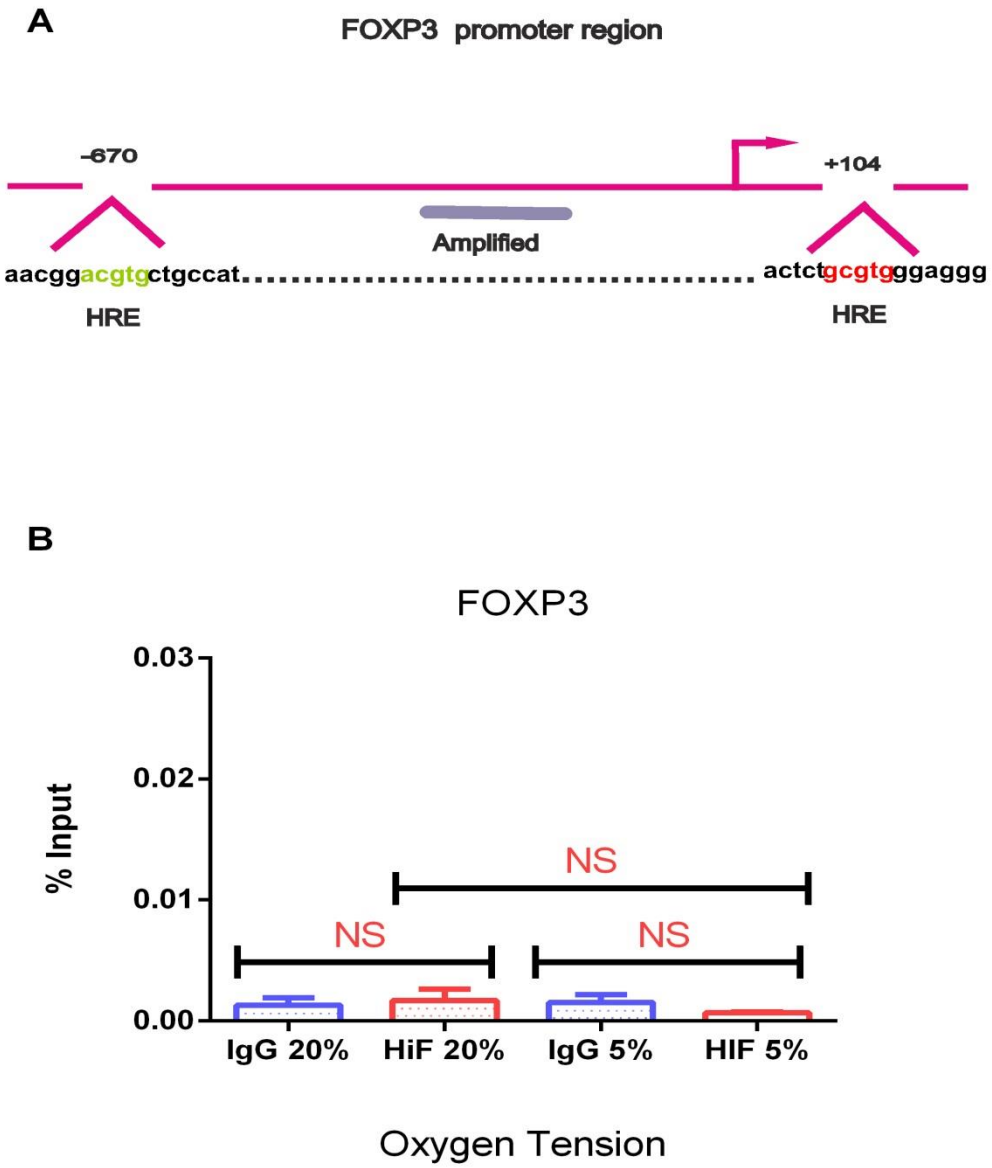

Figure 3.24: HIF-2α binds FOXP3 proximal promoter in hypoxia

(A) Schematic representation of the proximal promoter of FOXP3 (note that the amplified region is between 2 HRE at -670 and +104 from the start site). ChIP assays were performed with either an anti-HIF-2α (HIF) or IgG control antibodies on chromatin isolated from hESCs cultured either at 20% or 5% oxygen tension. Comparison of FOXP3 enrichment at 5% oxygen compared to 20% oxygen (B). DNA enrichment is expressed as a percentage of input (non-immunoprecipitated chromatin). An average of 3 independent experiments is represented (NS: no significant difference).

3.3.7 Luciferase Reporter assay analysis of NANOG proximal promoter

To further characterize the role of HIF-2α as a modulator of NANOG gene expression, a reporter gene assay was performed.

3.3.7.1 Generation of NANOG promoter constructs

To subclone the portion of the NANOG promoter, harbouring the HRE site, into the TOPO vector a fragment of 630 bp from -512 bp to + 207 bp from the transcription start site of NANOG promoter was amplified from DNA of hES cells cultured at 5% oxygen with specific primers that contain XhoI and HINDIII restrictions sites using a regular PCR (See Methods section 3.2.5).

The PCR product was resolved on a 1% agarose gel and expected 630bp product was observed (Figure 3.25).

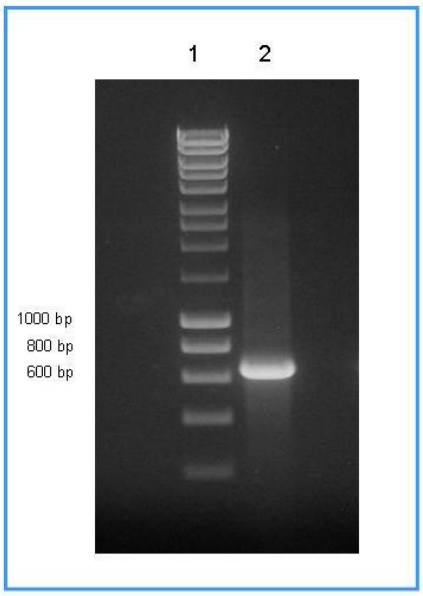

Figure 3.25: Specific PCR product for a fragment of the NANOG promoter in DNA of hES cells cultured at 5% oxygen tension

An ethidium bromide stained 1% agarose gel showing the amplified PCR product for the fragment of the NANOG promoter. Lane 1: Hyperladder; Lane 2: PCR amplicon. Expected DNA product size =630 bp.

The PCR product containing single overhanging 3'deoxythymidine (T) was ligated into a TOPO vector and transformed into competent TOP10 bacteria and plated on LB agar plates (See Methods section 3.2.6). DNA from individual colonies were purified and digested with XhoI and HINDIII to confirm the presence of the expected insert of 630 bp (Figure 3.26).

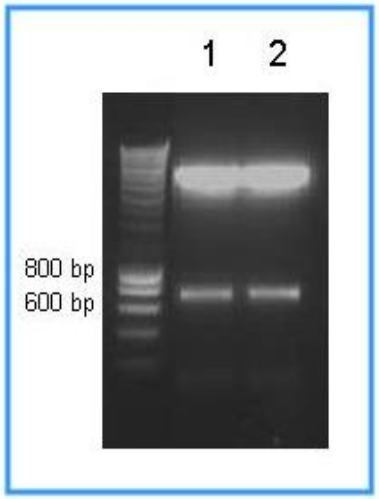

Figure 3.26: Digests of NANOG-TOPO vector clones with XhoI and HINDIII

A NANOG-TOPO vector clones (Lanes 1 and 2) were digested with XhoI and HINDIII generating an expected band of 630 bp.

To further verify that selected colonies were successfully cloned into the TOPO vector, a clone was sent off to Oxford to the Geneservice - Source BioScience plc. for sequencing using M13 Forward and Reverse primers (see Methods section 3.2.7) (Figure 3.27).

```
clone           ATGGATATCTGCAGAATTCGCCCTTCTCGAGCGGCTGGTTTCAAACTCCTGACTTCAGGT
NANOG           GTAGAGA---GGGGGTTTCGCCATGTTGGCCAGGCTGGTTTCAAACTCCTGACTTCAGGT
                 * ** *   *  *  ****** *  * *   ****************************

clone           GATCCGCCTGCCACGGCCTCCCAACTTACTGGGATTACAGGGGTGGACCACCGCGCCCGG
NANOG           GATCCGCCTGCCACGGCCTCCCAATTTACTGGGATTACAGGGGTGGGCCACCGCGCCCGG
                ********************** ******************** **************

clone           CCTTTTTCTTAATTTTTAAAAATATTAAAGTTTTATCCCATTCCTGTTGAACCATATTCC
NANOG           CCTTTTTCTTAATTTTTAAAAATATTAAAGTTTTATCCCATTCCTGTTGAACCATATTCC
                ************************************************************

clone           TGATTTAAAAGTTGGAAACGTGGTGAACCTAGAAGTATTTGTTGCTGGGTTTGTCTTCAG
NANOG           TGATTTAAAAGTTGGAAACGTGGTGAACCTAGAAGTATTTGTTGCTGGGTTTGTCTTCAG
                ************************************************************

clone           GTTCTGTTGCTCGGTTTTCTAGTTCCCCACCTAGTCTGGGTTACTCTGCAGCTACTTTTG
NANOG           GTTCTGTTGCTCGGTTTTCTAGTTCCCCACCTAGTCTGGGTTACTCTGCAGCTACTTTTG
                ************************************************************

clone           CATTACAATGGCCTTGGTGAGACTGGTAGACGGGATTAACTGAGAATTCACAAGGGTGGG
NANOG           CATTACAATGGCCTTGGTGAGACTGGTAGACGGGATTAACTGAGAATTCACAAGGGTGGG
                ************************************************************

clone           TCAGTAGGGGGTGTGCCCGCCAGGAGGGGTGGGTCTAAGGTGATAGAGCCTTCATTATAA
NANOG           TCAGTAGGGGGTGTGCCCGCCAGGAGGGGTGGGTCTAAGGTGATAGAGCCTTCATTATAA
                ************************************************************

clone           ATCTAGAGACTCCAGGATTTTAACGTTCTGCTGGACTGAGCTGGTTGCCTCATGTTATTA
NANOG           ATCTAGAGACTCCAGGATTTTAACGTTCTGCTGGACTGAGCTGGTTGCCTCATGTTATTA
                ************************************************************

clone           TGCAGGCAACTCACTTTATCCCAATTTCTTGATACTTTTCCTTCTGGAGGTCCTATTTCT
NANOG           TGCAGGCAACTCACTTTATCCCAATTTCTTGATACTTTTCCTTCTGGAGGTCCTATTTCT
                ************************************************************

clone           CTAACATCTTCCAGAAAAGTCTTAAAGCTGCCTTAACCTTTTTTCCAGTCCACCTCTTAA
NANOG           CTAACATCTTCCAGAAAAGTCTTAAAGCTGCCTTAACCTTTTTTCCAGTCCACCTCTTAA
                ************************************************************

clone           ATTTTTTCCTCCTCTTCCTCTATACTAACATGAGTGTGGATCCAGCTTGTCCCCAAAGCT
NANOG           ATTTTTTCCTCCTCTTCCTCTATACTAACATGAGTGTGGATCCAGCTTGTCCCCAAAGCT
                ************************************************************

clone           TGCCTTGCTTTGAAGCATCCGGTTAAGGGCGAATTCCAGCACACTGGCGGCCGTTACTAG
NANOG           TGCCTTGCTTTGAAGCATCCGACTGTAAA-GAATCTT----CACCTATGCCTGTGATTTG
                ********************  *      ****       ***    * * ** * * *
```

Figure 3.27: Sequence analysis of a clone showed a complete homology with the NANOG sequence.

Sequence analysis obtained from one of the NANOG-TOPO clones with the M13Fw primer and analysed through the Clustalw 2.1 programe. Multiple sequence alignment between NANOG promoter sequence (highlighted in green) and one of the NANOG-TOPO clones showing complete homologies. The HRE site at -301 bp is highlighted in pink while the translational start codon ATG is highlighted in yellow.

In order to perform luciferase assays experiments, the NANOG-TOPO clone was cloned into a PGL3 control vector (See Methods section 3.2.8). The NANOG-TOPO clone was digested with XhoI and HINDIII restriction enzymes. The digested fragments were cut out from the gel, purified and cloned into the pGL3 control expression plasmid vector which was digested with XhoI and HINDIII to allow cloning of the purified insert (Figure 3.28).

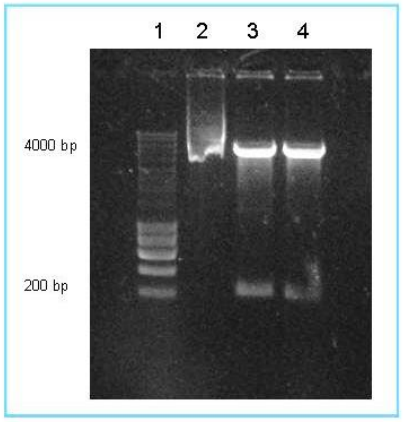

Figure 3.28: pGL3 control vector digested with XhoI and HINDIII

A Nancy stained 1% agarose gel showing pGL3 promoter vector digested with XhoI and HINDIII. Lane 1: Ladder; Lane 2: non digested pGL3 control vector; Lane 3 and 4: Digested and linearized pGL3 control vector digested displaying the released 200bp product corresponding to the excised SV40 promoter.

The digested and linearised pGL3 control vector was purified from the gel and ligated with the digested NANOG promoter fragment. In addition, a ligation with a phosphatased pGL3 vector was performed to minimize self-ligated vector during the transformation procedure (See Methods section 3.2.8). DNA from individual colonies was purified and a sequential digest performed with HINDIII followed by XhoI to confirm the presence of the expected insert of 630 bp (Figure 3.29). Both the ligation reactions generated positive colonies and released the expected 630 bp NANOG promoter band.

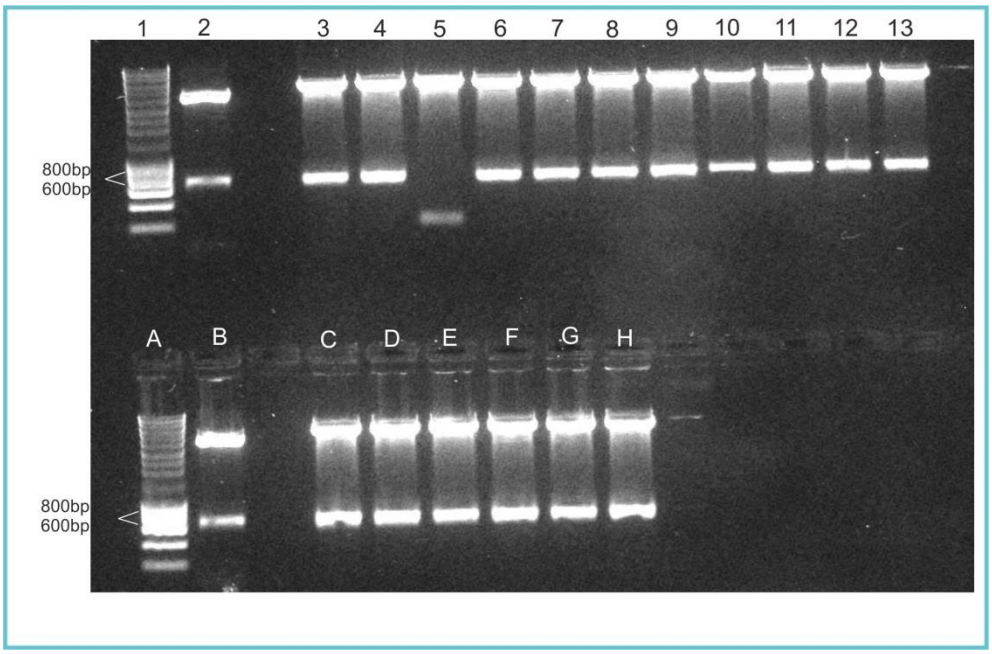

Figure 3.29: Digests of pGL3-NANOG clones with HINDIII and XhoI

A selection of clones from a phosphatased pGL3-NANOG digested with HINDIII and XhoI and generating the appropriate 630 bp insert. Lane 1 and A: Ladder; Lane 2 and B: digested TOPO-NANOG vector used as a positive control; Lane 3 to 13: digested pGL3-NANOG clones released the expected product except for the clone in lane 5 which was negative; Lane C to H: clones derived from a non phosphatased pGL3 vector. Digested pGL3-NANOG clones released the expected sized product.

3.3.7.2 Mutagenesis of the HRE in the NANOG proximal promoter

To characterize the functional role of HIF-2α in the modulation of NANOG gene expression, mutagenesis of the HRE at -301 from the transcription start site was performed. A pGL3-NANOG HRE mutant construct was generated using the same procedure as the pGL3-NANOG construct with the exception that the pGL3-NANOG construct was used as a template PCR for the mutagenesis using specific primers designed to change the HRE sequence (acgtg) into an XhoI restriction site (acTCgAG). PCR products were digested with DpnI enzyme in order to cleave the parental strand and used for transformation (See Method section 3.2.9). DNA from individual colonies were purified and digested with XhoI enzyme. As the pGL3-NANOG does not contain a XhoI site, this should not cut with the restriction enzyme, while the XhoI positive mutant clones released the expected 140 bp fragment (Figure 3.30).

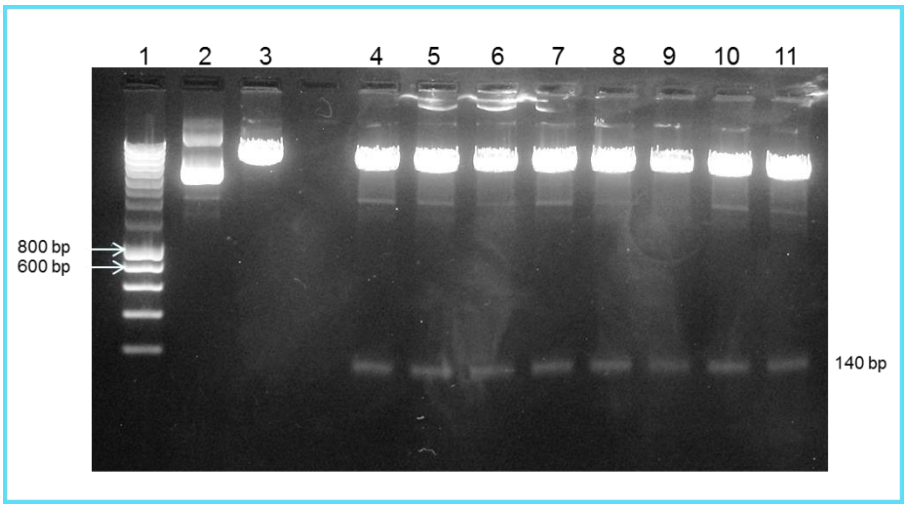

Figure 3.30: Digests of pGL3-NANOG HRE mutant clones with XhoI

pGL3-NANOG HRE mutant clones were digested with XhoI generating the appropriate 140 bp insert. Lane 1: Hyperladder; Lane 2: undigested pGL3-NANOG vector; Lane 3: digested pGL3-NANOG vector used as negative control; loaded in Lane 4 to 11: digested clones released the expected 140 bp product.

3.3.7.3 Functional Analysis of the HRE in the NANOG proximal promoter

To verify the novel direct binding of endogenous HIF-2α in the HRE (-301 bp) in the NANOG proximal promoter, luciferase reporter assays were performed (See Method section 3.2.11). Either the pGL3-NANOG or the pGL3-NANOG HRE mutant vector (PGL3 Mut-NANOG) and a pcDNA-HIF-2α expression plasmid were transiently co-transfected into NT2 cells. In addition, the pGL3-null and pcDNA3 null empty vectors were used as negative controls whereas the pRL-SV40 Renilla luciferase vector was used to normalize the luciferase assay. There was a significant increase in luciferase activity when the pGL3-NANOG vector was co-transfected with the pcDNA-HIF-2α expression vector (**P<0.01) compared to the negative control pGL3-null (Figure 3.31). This result shows that HIF-2α directly binds the HRE in the NANOG promoter and is responsible for the transcription of NANOG. In contrast, when NT2 cells were co-transfected with the pGL3 Mut-NANOG vector and the pcDNA-HIF-2α expression plasmid there was a significant decrease in the luciferase activity compared to cells transfected with the pGL3-NANOG vector (**P<0.01) (Figure 3.31). This confirms that a disruption of the HRE site in the NANOG promoter abolishes HIF-2α binding (Figure 3.31).

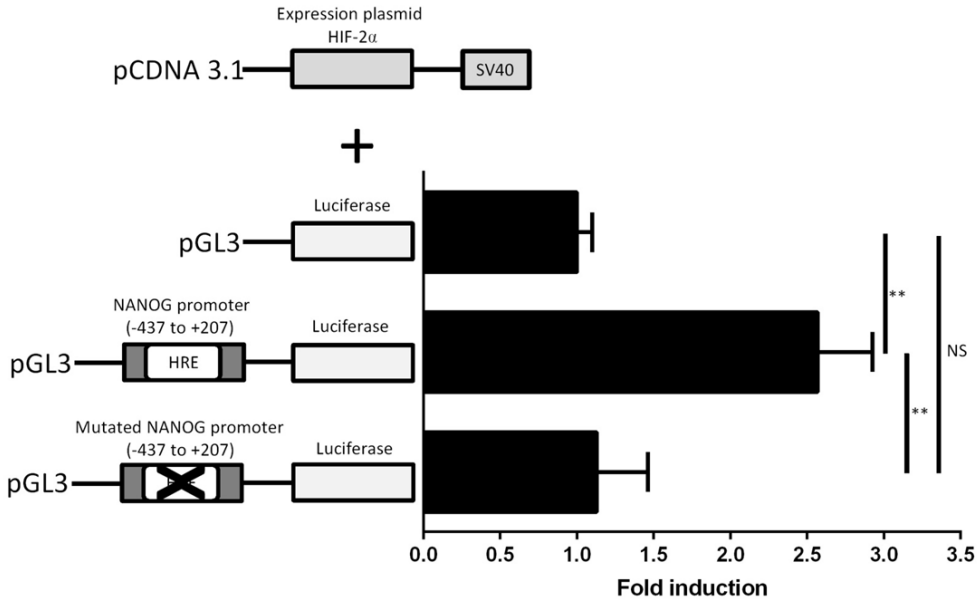

Figure 3.31: The *novel* HRE site in the NANOG promoter is a functional HIF-2α binding site

Schematic representation of the HIF-2α expression vector (pcDNA3-HIF-2α top panel). NT2 cells cultured at 5% oxygen were transfected with pGL3-NANOG and either an empty vector or the pcDNA-HIF-2α expression vector (PcDNA3-HIF-2α). Luciferase Assays showed a significant increase (**P<0.01) in luciferase activity when cells were co-transfected with pGL3-NANOG and PcDNA3-HIF-2α plasmid constructs compared to the control. NT2 cells were also co-transfected with a pGL3 Mut-NANOG vector and with either an empty vector or the HIF-2α expression vector (PcDNA3-HIF-2α). A significant decrease in the luciferase activity (**P<0.01) was determined when cells were co-transfected with pGL3Mut-NANOG plasmid compared to the PcDNA3-HIF-2α plasmid. An average of four independent experiments is shown.

3.4 Discussion

3.4.1 hES cells characterization

Environmental oxygen tension has an important role for hES cell maintenance and pluripotency. It has been demonstrated that hES colonies under hypoxic conditions display less differentiation than under normoxic conditions (Ezashi et al., 2005). However, characterization of hES cells cultured at both oxygen concentrations is necessary to assess and validate the quality of colonies. As well as morphology, this should also include the expression of characteristic pluripotency markers to ensure consistency across experiments.

In agreement with Forristal et al. (2010), data showed in this chapter confirmed that hES cells cultured at 5% oxygen tension displayed an increased expression of OCT4, SOX2 and NANOG compared to those maintained at 20% oxygen. To investigate the effect of hypoxia on hES cell morphology, hES cells were cultured on MEFs as well as feeder-free on Matrigel. However, on day 3 post-passages, hES cells cultured at either atmospheric or 5% oxygen displayed a similar morphology.

RT-qPCR analysis showed a significant decrease of the RNA expression levels of OCT4, NANOG and SOX2 in cells cultured under normoxia compared to cells cultured under hypoxia. These results demonstrate that cells cultured under normoxic conditions gradually loose pluripotency, prior to overt morphological differences, compared to cells maintained under hypoxia. This data is in agreement with previous studies (Ezashi et al., 2005; Westfall et al., 2008) which demonstrated that a low oxygen tension was beneficial for preventing spontaneous differentiation and increasing the self-renewal of hES cells.

Using immunocytochemisty, it was not possible to observe any difference in the expression of the nuclear pluripotency markers OCT4, SOX2, NANOG due probably to the sensitivity of technique. This is in agreement with Forristal et al. (2010) who found that OCT4 protein was significantly decreased in hES cells cultured under normoxic compared to hypoxic conditions using Western Blot. More recently, a similar increase

in NANOG and SOX2 protein has also been observed in hES cells culture at 5% compared to atmospheric oxygen (Forristal et al., 2013). Interestingly, colonies cultured under normoxic conditions displayed slight positivity for the early differentiation marker SSEA-1. Less positivity to the early differentiation marker SSEA-1 was observed in hES cells cultured under hypoxic conditions.

The current work highlights the importance of hypoxic conditions for the maintenance of hES cell morphology and pluripotency

3.4.2 ChIP Analysis

HIF-2α, an important regulator of the hypoxic response, has been found to have a potential role in the modulation of hES cell maintenance under hypoxic conditions. Previous published data showed that silencing HIF-2α caused an overall decrease in OCT4, SOX2 and NANOG expression (Forristal et al., 2010) but the molecular mechanisms that underlie this beneficial effect have not been characterised. Hence, it is not known whether HIF-2α acts directly on key pluripotency genes or whether the effect is indirect.

ChIP assays were performed to determine whether endogenous HIF-2α interacts *in vivo* with the proximal promoter region of pluripotency genes OCT4, NANOG, SOX2 but also on the proximal promoter region of eNOS and GLUT1 genes that are up-regulated under hypoxic conditions (Chen et al., 2001; Coulet et al., 2003). ChIP assays were assessed on hES cells cultured at 20% oxygen, or those that had be maintained at 5% oxygen for a minimum of 3 passages. Interestingly it was noticed that the chromatin isolated from hES cells cultured under normoxia required less sonication to shear. This may relate to the morphology of the colonies which were more diffuse under normoxic conditions, whereas those maintained under hypoxic conditions were densely packed.

Initially, regular PCR analysis was performed on OCT4 ChIP samples. However, as the IP was performed by immunoprecipitating endogenous HIF-2α, this technique was not sensitive enough to quantify binding. Thus TaqMan probes and qPCR analysis were instead used to quantify HIF-2α binding.

ChIP results showed that endogenous HIF-2α binds the CR3 in the OCT4 proximal promoter under hypoxic conditions. This result was similar to that observed in hypoxic human RCC cells, which express only HIF-2α but not HIF-1α and where it was found that HIF-2α but not HIF-1α interacted with the HRE in CR3 and in the HIF-2α-Knock-In mouse model (Covello et al., 2006). ChIP experiments also revealed no significant interaction between HIF-2α and the HRE in CR3 in normoxic conditions and this could further explain why hES cells cultured at 20% oxygen tension display a decrease in the

expression of pluripotency markers and have a tendency differentiate (Figure 3.18). These data suggest that the microenviroment can influence the dynamics and accessibility of transcription factors to the chromatin determining the differentiation of hES cells (Covello et al., 2006; Watson et al., 2010). Furthermore, it is interesting in terms of cancer biology where OCT4 may contribute to the tumour promoting activity of HIF-2α. This is evidenced by data where HIF-2α has been shown to promote cell proliferation in hypoxia and enhance cancer tumour and (ES)-derived tumour progression through activation of OCT4, c-MYC, Cyclin D and TGF-α (Alt et al., 2000; Covello et al., 2006; Gidekel et al., 2003; Gordan et al., 2007).

It is widely demonstrated that NANOG not only has a central role in hES cell pluripotency and self-renewal, but also in cell cycle progression (Chambers et al., 2003; MacArthur et al., 2008; Zhang et al., 2009). In particular, previous work showed that silencing of HIF-2α leads to a decrease of NANOG expression and a concomitant significant decrease in hES cell proliferation (Forristal et al., 2010). As NANOG has been shown to interact with two cell cycle regulators, CDK6 and CDC25A it may be responsible for the delay in cell cycle progression (Zhang et al., 2009). Interestingly, in this study, ChIP assays demonstrated that HIF-2α interacts directly with a *novel* HRE site within the proximal promoter of NANOG in hES cells cultured at 5% oxygen tension while no significant binding was observed in hES cells cultured at 20% oxygen tension (Figure 3.19). Furthermore, data presented in this chapter indicate for the first time, a direct involvement of HIF-2α in NANOG gene expression. To further characterize the functional activity of HIF-2α on the proximal promoter of NANOG gene, luciferase reporter assays were performed. The proximal NANOG promoter that includes the HRE site for HIF-2α showed a promoter-driven increased transcription only when NT2 cells were co-transfected with a HIF-2α expression vector whereas a significant decrease of NANOG transcription was observed when a mutant Renilla plasmid vector was co-transfected with HIF-2α. These results suggest that this HRE site is functionally required for NANOG transcription (Figure 3.31). Indeed, the environmental oxygen level could be a potential parameter to consider during the development of new strategies for the expansion and culture of undifferentiated population of hES cells. Furthermore, this data suggests that HIF-2α takes part in

regulating the core circuitry of pluripotency under prolonged hypoxia allowing the maintenance of hES cell self renewal.

ChIP data, showed a significant interaction of HIF-2α with 2 HREs in the proximal promoter region of the SOX2 gene in hES cells cultured at 5% oxygen tension while no binding was observed in normoxia. These results reinforce the role of HIF-2α in hES cell maintenance, under hypoxic conditions, through the regulation of pluripotency genes like SOX2. Although previous studies described binding of HIF-2α to two HRE sites in the proximal promoter region of SOX2 in rat ependymal progenitor SCs (epSPC) (Moreno-Manzano et al., 2010), this thesis provides the first evidence in the human. Hence, in the current study it has been shown that HIF-2α strongly interacts with two HREs which differ by a single nucleotide change in their sequence (SOX2A and SOX2G) and that HIF-2α preferentially binds the SOX2A compared to the SOX2G (Figure 3.20 and 3.21). The possible explanation for this difference in HIF-2α binding could be related either to a difference in the occupancy of other transcription factors that cooperate with HIF-2α in the regulation of SOX2 expression or to the chromatin conformation around the HRE and warrants further investigation.

A significant enrichment in HIF-2α binding to the eNOS proximal promoter was observed in hES cells maintained under hypoxia suggesting that endogenous HIF-2α modulates the *in vivo* the expression of eNOS in hypoxic conditions. These data are in agreement with previous studies in which HIF-2α has been found to interact with two HREs in the proximal promoter of the human eNOS gene (Coulet et al., 2003) leading to an increase of eNOS expression in HMEC (Primary Human Mammalian Epithelial cells) and HUVEC (Primary Human Umbilical Vein and Endothelial cells) cell lines. However, this is the first demonstration of a direct interaction of HIF-2α with the eNOS proximal promoter in hES cells cultured under hypoxic conditions (Figure 3.22). The role of eNOS has been little studied in hES cells but culture in the presence of an NO donor increased the expression of pluripotency markers in mouse bone marrow multipotent progenitor cells (Chu et al., 2008). Interesting published data showed that NO donors stimulate hES cell differentiation toward the cardiac lineage (Mujoo et al., 2006) whereas low NO concentrations were able to promote self-renewal by inhibiting

apoptosis (Tejedo et al., 2010). Although, the effect of hypoxia in the regulation of eNOS in hES cells is still poorly understood, data presented in this chapter suggest that eNOS could have a role in pluripotency marker expression through HIF-2α activation and will need further analysis.

It is widely recognised that HIF-1α can regulate glucose transporters 1 and 3 (GLUT1 and GLUT3) (Chen et al., 2001; Vannucci et al., 1998) whose enhanced expression is associated with increased up-regulation of glycolytic enzymes and glucose transporters (Wenger, 2002). Under hypoxic conditions, HIFs contribute to the switch in energy substrate utilization from being reliant on the TCA (oxygen-dependent tricarboxylic acid) to the oxygen-independent glycolysis providing enough ATP for energetic reactions. Indeed, recent published data showed that hES cells cultured at 5% oxygen expressed more GLUT1 and consumed more glucose than those maintained under physiological oxygen tension (Forristal et al., 2013). The expression of glucose transporters increase the level of glucose metabolized by the cells, a mechanism common to placenta, brain and also to solid tumours progression (Chen et al., 2001; Dang and Semenza, 1999; Flier et al., 1987; Froehner et al., 1988; Kahn and Flier, 1990; Maxwell et al., 1997). ChIP data obtained in this thesis, reported that HIF-2α is able to bind directly to an HRE in the proximal promoter of GLUT1 only in hES cells cultured under hypoxic conditions (Figure 3.23). These results may clarify the role of HIF-2α as a modulator of glucose metabolism through direct interaction with one of the glucose transporters and suggest the presence of an adaptive response that increases glucose uptake under hypoxia.

Taken all together, the ChIP results are particularly important as they have revealed for the first time a specific *in vivo* interaction between endogenous HIF-2α and the proximal promoter region of OCT4, SOX2, NANOG, eNOS and GLUT1 genes only in hES cells cultured at 5% oxygen. This confirms a role for HIF-2α as a direct modulator of the hypoxic response (Figure 3.32).

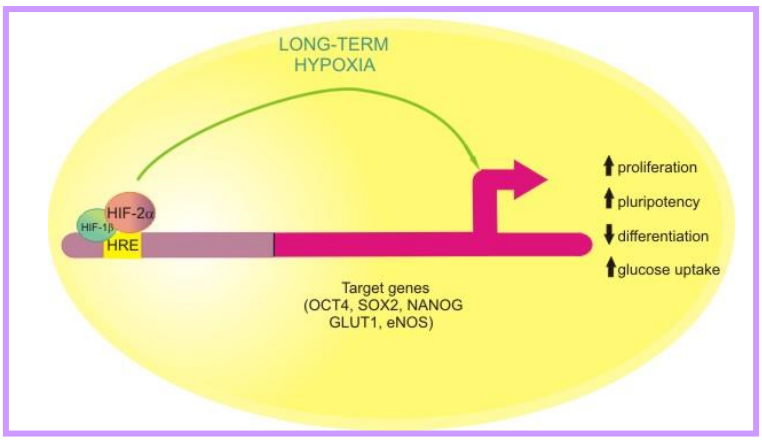

Figure 3.32: Schematic representation of the HIF-2α target genes and the possible effect on hES cells.

The work completed in this chapter provides evidence of the molecular mechanisms that underlie the transcriptional control of the core network of pluripotency regulated by HIF-2α. This study demonstrates that HIF-2α is a trans-acting transcription factor of genes involved in the hES cell self-renewal and thus contributes to maintaining the hypoxic cellular response. Furthermore, this work suggests a role for HIF-2α in the regulation of both glucose uptake and the signalling molecule, eNOS in hES cells cultured under hypoxic conditions. These data implicate HIF-2α as a hypoxic metabolic regulator in stem cell biology and may help to elucidate the complex mechanisms that allow hES cells to respond to environmental cues and maintain self-renewal.

Chapter 4

Effect of environmental oxygen tension on the epigenetic regulation of hES cells

4.1 Introduction

4.1.2 Chromatin modifications

Epigenetic processes that include DNA methylation, chromatin modifications and miRNA expression are being intensely studied in hES cell biology in order to understand the regulatory mechanisms which maintain genes poised for transcription in undifferentiated cells (Lister et al., 2009; Meshorer et al., 2006). Indeed hES cells are widely characterized to have a "unique" epigenetic signature compared to other cell lines (Bibikova et al., 2006) that contributes to their stemness state.

Since the epigenetic regulation of hES cells plays an important role in the regulation of pluripotency and differentiation, the role of chromatin methylation in the control of gene expression has been intensively studied (Bernstein et al., 2006; Meshorer and Misteli, 2006). Histone modification mapping revealed that the genome in hES cells contains a "bivalent" chromatin state which is characterized by the presence of both histone modifications associated with gene activation (H3K4me3) and silencing (H3K27me3) (Bernstein et al., 2006; Gifford et al., 2013; Mikkelsen et al., 2007). This epigenetic profile is a marker for genes and transcription factors that are normally repressed in pluripotent hES cells but "poised" to be expressed upon differentiation (Zhao et al., 2007). However, how transcription factors and epigenetics cooperate to create multiple regulatory mechanisms in the switch from hES cell pluripotency to differentiation is still not known.

hES cells as well as other multipotent progenitor cells reside in a complex microenvironment characterized by low oxygen levels that promotes their survival (Ezashi et al., 2005). Increasing evidence suggests that the activity of the HIFs, as well as the long term adaptation to hypoxia is mediated by epigenetic changes that modulate DNA methylation and histone modifications (Johnson et al., 2008). One of the first studies that proved that the hypoxic response is mediated by epigenetic was found by studying the co-activation complex, in hypoxia inducible genes, where chromatin remodelling enzymes, such as CBP/p300, have been found associated and interacting with HIF-1α (Kallio et al., 1998). Later, further studies showed in pancreatic cancer

cells that also the HRE site needs to be in an active chromatin conformation in order to allow HIF-1α binding (Okami et al., 2004).

Only a few studies have investigated the effect of hypoxia on histone methylation revealing dramatic changes in both active and repressive markers as a consequence of either an increase in histone methyltransferase activity or a decrease in histone demethylase function (Johnson et al., 2008; Zhou et al., 2010). Interestingly, hypoxia has been found to increase the expression of H3K4me3 and H3K9me2 in activated and repressed hypoxia inducible genes suggesting that chromatin is more flexible under hypoxic conditions and able to rapidly react to environmental changes (Chen et al., 2006; Xia et al., 2009). Furthermore, Jumanji histone demethylases (JHDMs) have been found to be important regulators of the plasticity of ES chromatin through the removal of methyl groups from specific histone modifications (Loh et al., 2007; Tsukada et al., 2006). In particular, Jmjd1a and jmjd2c seem to have a role in the regulation of Oct4 and Nanog in mouse ES cells (Loh et al., 2007), while jmjd1a and jmjd2b have been found to specifically target HIF-1α (Beyer et al., 2008). However, although chromatin modifications play an important role in gene regulation under hypoxia, very little is still known on the hypoxic regulation of hES cells and how histone modifications can change the expression of genes important for the maintenance of hES cells in response to environmental oxygen changes.

4.1.3 DNA methylation

Development from a zygote to an adult organism requires specific cellular processes in which DNA methylation and histone modifications have a critical role in the maintenance of chromatin structures and gene expression (Feng et al., 2010; Morgan et al., 2005). DNA methylation, in fact, represents one of the major epigenetic regulations that occur during embryonic development (Gill et al., 2012; Jacob and Moley, 2005).

In hES cells the CpG islands in the promoters of pluripotency genes such as OCT4 and NANOG remain demethylated allowing their active expression to maintain the self-renewal phenotype (Meissner et al., 2008). An increased CpG methylation occurs when cells start to differentiate into a specific cell type thereby allowing the expression of specific development genes (Lister et al., 2009). This signature is a characteristic that is sufficient to distinguish hES cells from adult cells, somatic and tumour cell populations (Bibikova et al., 2006). However, since hES cells originate in a hypoxic environment, these findings do not take into account the role of hypoxia in the DNA methylation of self-renewal genes.

Studies on cancer cell lines have shown that in the absence of HIF-1α, the DNA methylation state is globally increased as a consequence of the expression of the DNA methyltransferase 3b enzyme (DNMT3b) (Watson et al., 2009). The mechanism which regulates this effect is not known but implicates HIFs in the regulation of DNA methyltransferases. Recent published data on ovarian carcinoma cells showed that also the DNA methylation of the HRE site is important for HIF binding to a S100 Calcium binding protein A4 (S100A4) gene which is associated with tumour invasiveness (Horiuchi et al., 2012). Indeed, it was found that the DNA within the HRE site is methylation free and that a degree of methylation may influence the binding of HIF-1α (Horiuchi et al., 2012). This may be related to either the inhibition of DNMT (Skowronski et al., 2010) or by induction of the methionine adenosyltransferase MAT2A (Liu et al., 2011). However, it remains to be clarified whether it is the hypoxic microenvironment, or HIF binding to the HRE that influences the methylation state. However, in neural progenitor cells DNA methylation was shown to regulate the activity of HIF-1α hypoxia-regulated microRNAs, such as miR-210, independently of

its expression (Xiong et al., 2012). Cell type specific analysis will be required to properly define the role of hypoxia in the control of DNA methylation.

With regard to hES cells, previous published data highlighted the role of hypoxia and, in particular of HIF-2α, not only in hES cells pluripotency (Covello et al., 2006; Forristal et al., 2010) but also in the regulation of GLUT1 glucose transporter (Forristal et al., 2013). Recently published data showed that hypoxia is able to revert the methylation status of the OCT4 promoter of differentiated hES cells back to a stem cell-like state (Mathieu et al., 2013). However, the specific role of HIF-2α in the regulation of the CpGs methylation within the promoter of self-renewal genes is not fully understood. Since HIF-2α has been found to modulate OCT4 expression under hypoxia (Forristal et al., 2010) it will be interesting to investigate whether this transcription factor regulates the DNA methylation state within the OCT4 proximal promoter.

4.1.4 Study Aims

The aim of this chapter was to investigate the effect of environmental oxygen tension on the epigenetic regulation of hES cells.

The specific aims were:

- To investigate the histone modification pattern at the HRE of the selected genes using ChIP assays
- To analyse the expression of the Jumonji histone demethylases proteins in hES cells cultured under normoxia and hypoxia using RT-qPCR
- To investigate the global OCT4 DNA methylation profile and the CpG site within the HRE in the CR3 region of the OCT4 promoter in hES cells cultured at either 20% or 5% oxygen using Pyrosequence analysis.

4.2 Materials and Methods

4.2.1 DNA extraction for Pyrosequencing Analysis

hES cells cultured at 5% and 20% oxygen tension on a 6 well plate of Matrigel were collected on day 3 post passage in 600μl RLT Lysis Buffer (Qiagen). The lysate was passed 5 times through a 20-gauge needle (0.9 mm diameter) fitted to a syringe, transferred to an AllPrep DNA (Qiagen) spin column and centrifuged for 30s at 13.000 rpm. 500μl Buffer AW1 (Qiagen) was added to the column and centrifuged for 15s at 10000 rpm to wash the spin column membrane. Buffer AW2 (Qiagen) (500μl) was added to the column and centrifuged for 2mins at full speed. DNA samples were eluted with 50μl of Buffer EB (Qiagen), incubated for 1min at room temperature and centrifuged at 13000 rpm.

4.2.3 Primer Design

All primers for Pyrosequencing were designed to amplify as many as the CpG islands in the OCT4 proximal promoter and first exon spanning from -237 bp up to +219 from the start site (Set 2 from +40 bp to + 219 bp, Set 3 from -89 bp to +72 bp and Set 4 from -242 bp to -90 bp from the start site) (Kingham et al., 2013) (Figure 4.1).

Primers were also designed which covered two CpG islands around HRE sites of interest for OCT4 CR3 proximal promoter (-2016 bp and -1956 bp from the start site) (Figure 4.2). All primers were designed using the PSQ Assay Design Software version 1.0.6 (Biotage AB, Kungsgatan, Sweden) (Table 4.1) and have a score around 90% which indicates that there are no similar annealing sites in the amplification product and that the quality is stringent for bisulfite-treated DNA. The size of the amplification product was restricted to 200bp to avoid secondary structures such as loops that can inhibit the sequencing reaction or increase the background signal due to the extension of the 3'-terminus. Reverse primers were 5'-biotinylated and HPLC-purified.

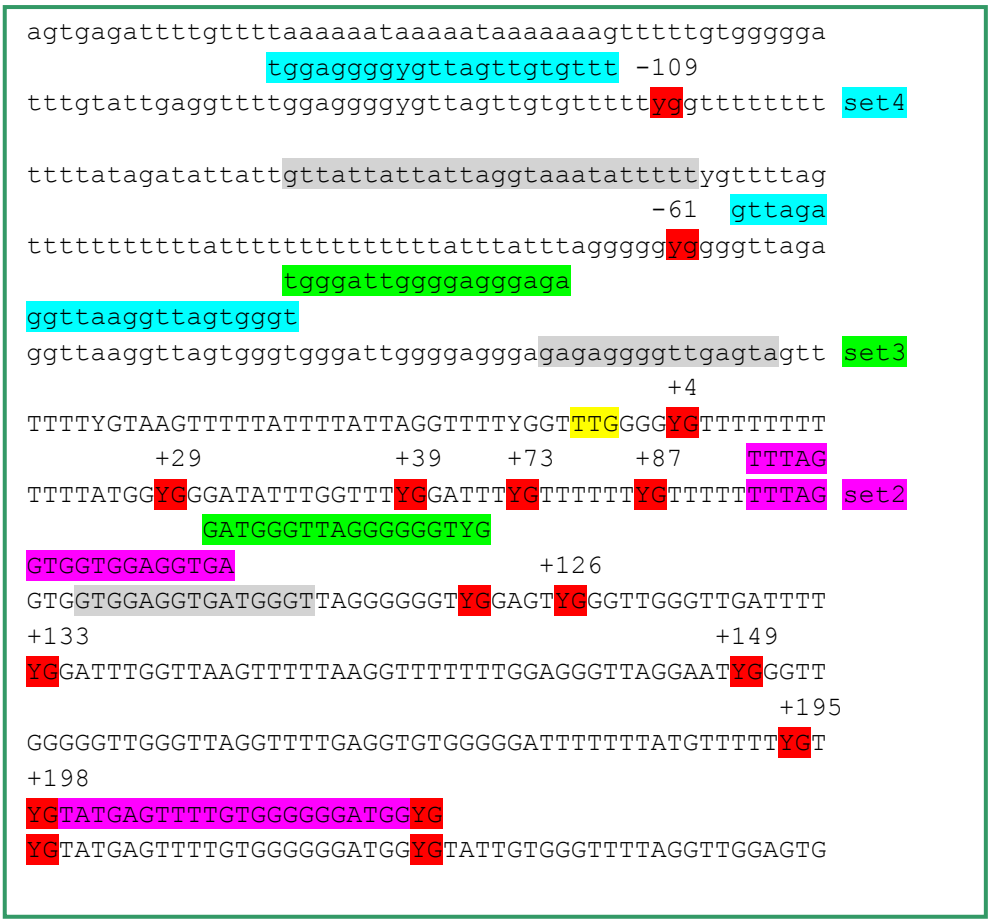

Figure 4.1: Oct4 upstream promoter and exon sequence showing the location of the CpGs from -109 to +219 from the transcriptional start site.

Diagram shows the location of OCT4 pyrosequencing amplicons and sequencing primers on bisulfite converted DNA. Set 2 forward and reverse primers are highlighted in pink and sequencing primers highlighted in yellow. Set 3 forward and reverse primers are highlighted in green and sequencing primers highlighted in grey. Set 4 forward and reverse primers are shown highlighted in blue and sequencing primer in grey highlighted. CpG islands are highlighted in red as YG and numbering of the CpGs is shown above the sequence. The transcriptional initiation site TTG is highlighted in yellow.

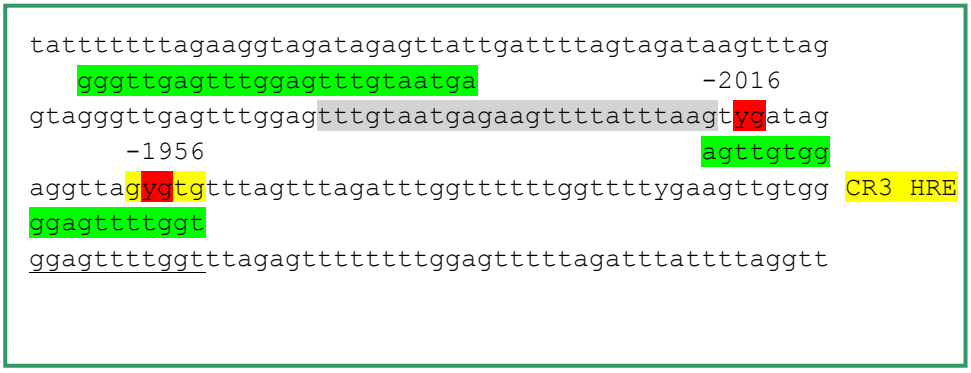

Figure 4.2: Oct4 upstream promoter and exon sequence showing the location of the -2107 bp to -1960 bp pyrosequencing amplicons from the transcriptional start site.

Diagram shows the location of OCT4 pyrosequencing amplicons and sequencing primers on bisulfite converted DNA. OCT4 CR3 forward and reverse primers are highlighted in green and sequencing primer highlighted in grey. The CR3 HRE is highlighted in yellow.

Table 4.1: OCT4 Pyrosequencing and sequencing primers

Set 2 OCT4 (+40 bp to + 219 bp)	*Sequence (5'→ 3')*	Annealing Temperature (°C)
Forward	TTTAGGTGGTGGAGGTGAT	58
Reverse (5'Biotin)	CATCCCCCCACAAAACTCATAC	
Sequencing	GTGGAGGTGATGGGT	

Set 3 OCT4 (-89 bp to +72 bp)	*Sequence (5'→ 3')*	Annealing Temperature (°C)
Forward	TGGGATTGGGGAGGGAGA	58
Reverse (5'Biotin)	ACCCCCCTAACCCATCAC	
Sequencing	AGAGGGGTTGAGTA	

Set 4 OCT4 (-242 bp to -90 bp)	*Sequence (5'→ 3')*	Annealing Temperature (°C)
Forward	GTTTTGGAGGGGAGTTAGTTAGTTGTGTTT	58
Reverse (5'Biotin)	ACCCACTAACCTTAACCTCTAAC	
Sequencing	GTTATTATTATTAGGTAAATATTTT	

Primer OCT4 CR3 (-2123 bp to -2015 bp)	*Sequence (5'→ 3')*	Annealing Temperature (°C)
Forward	GGGTTGAGTTTGGAGTTTGTAATGA	57
Reverse (5'Biotin)	ACCAAAACTCCCCACAACT	
Sequencing	TTTGTAATGAGAAGTTTTATTTAAG	

4.2.4 DNA denaturation and Bisulfite conversion

DNA denaturation and bisulfite conversion of hES cells cultured at 5% and 20% oxygen were performed using the EZ DNA Methylation Kit (Zymo Research) using 20µl of 500ng DNA. Each DNA sample was mixed with 130µl of CT Conversion Reagent and placed in a thermocycler and incubated as follows: 98°C for 10mins, 64°C for 2.5h and stored at 4°C. The samples were mixed with 600µl of M-Binding Buffer on a Zymo-Spin IC column and centrifuged for 30sec at full speed. DNA was desulphonated by adding 200µl M-Desulphonation Buffer to the column and incubated for 20min at room temperature. 200µl M-Washing Buffer was added to the column and centrifuge at full speed. This step was repeated twice. Elution was performed by adding 10µl M-Elution Buffer pre-wormed to 65°C, directly to the column matrix.

4.2.5 PCR for Pyrosequencing

Bisulfite DNA samples were analysed using a qualitative PCR to observe product specificity. The PCR was performed in 40µl reaction mix containing 1X Super Mix High Fidelity (Invitrogen), 20µM of each primer and 3.2µl DNA and nuclease free water on a G-Storm thermocycler. The thermal profile consisted of 1 cycle at 95°C for 5mins followed by 45 cycles at 95°C for 15sec, 58°C for 30sec, 72°C for 30sec and 1 cycle at 72C for 5mins. Amplified products were separated on a 1% agarose gel.

4.2.6 Pyrosequencing

4.2.6.1 Immobilization of PCR Product to Beads

The optimized PCR reaction was added in a 96-well plate and made up to 40µl with high-purity water. Samples were mixed with a master mix of 38µl of Binding Buffer (Qiagen) and 2µl of Streptavidin coated sepharose beads (Qiagen). The plate was sealed and incubated for 5-10mins at room temperature using a vibrating shaker (Orbis).

4.2.6.2 Strand Separation

The single-stranded DNA template was prepared using immobilization on streptavidin-coated sepharose beads and subsequent alkali treatment was used to remove salts that could inhibit subsequent enzymatic reactions. After template preparation, the sequencing primers were hybridized to the template several bases 5' to the site of interest for the sequencing. 0.5µl of each specific sequencing primer (10ng/µl) was mixed with 11.5µl of Annealing Buffer (Qiagen) and dispensed into each well in a PSQ HS 96 plate (Biotage AB, Kungsgatan, Sweden) and placed in the holding bay of a Vacuum Prep Worktable (Biotage AB, Kungsgatan, Sweden). The vacuum preparation tool (Vacuum Prep Tool) was important to capture the beads and hold them during the different purification steps, whereas the solution passes through the filters. The Vacuum Prep Tool was filled with: 70% ethanol, denaturation solution (0.2M NaOH), washing buffer and high-purity water following the manufacturers instructions. Once the vacuum was open the beads were slowly lowered into the Vacuum Prep Tool into the PCR plate for a minimum of 3mins, then the Vacuum Prep Tool was moved first into 70% ethanol for 5sec, then in to the denaturation solution for 5sec and finally in to the washing buffer for 5sec. After these steps the vacuum was closed and the beads released into the PSQ plate by shaking the Vacuum Prep Tool while allowing the probes to rest on the bottom of the wells.

4.2.6.3 Primer Annealing and Running

The PSQ plate from the previous step was then incubated at 80°C for 2mins with a "touch down" heating device and then cooled down for 5mins before Pyrosequencing. All the Pyrosequencing assays were performed using a Pyromark MD Pyrosequencer (Biotage AB, Kungsgatan, Sweden) and the correct amount of enzyme/substrate/nucleotides needed for assay (Qiagen) was determined by importing an assay file made in Assay Design Software. The retrieved amount of enzyme, substrate and nucleotides was settled in correspondent dispensing 'NDT' (Qiagen) tips and settled in the cartridge avoiding the production of bubbles in the dispensing tips. A running test of all the 6 tips and the samples run was performed using the manufacturer's instruction software. Percentage of methylation was analysed by the Pyromark MD Pyrosequencer Software (Biotage AB, Kungsgatan, Sweden).

4.3 Results

4.3.1 Histone modifications within the predicted HRE sites in OCT4, SOX2 NANOG, eNOS and GLUT1

To investigate the effect of hypoxia on histone modifications within predicted HRE sites of hypoxia inducible genes, ChIP analysis was performed. Chromatin from hES cells cultured at either 5% or 20% oxygen was sonicated and immunoprecipitated with specific histone modification antibodies H3K4me3 and H3K36me3, markers of transcriptional activation, and H3K9me3 marker of gene silencing while immunoprecipitation with a non-specific antibody, IgG, was used as a negative control. ChIP direct interactions were analyzed using qPCR and specific Taqman Custom probes designed to cover the HRE sites in the OCT4, SOX2, NANOG, eNOS and GLUT1 proximal promoter. Differences in relative chromatin enrichment between hES cells cultured at 5% and 20% oxygen were analysed using Student's *t*-test and displayed in bar charts. In contrast, pie charts were used to represent the percentage Input precipitated by each modified histone as a proportion of the total, for each gene of interest. While the bar charts represent a comparison of individual epigenetic marks, the pie charts provide a more global indication of the status "open" versus "closed" of the chromatin.

Using probes designed to cover the predicted HRE sites at -1956 bp in the OCT4 proximal promoter, a substantial increase of the H3K36me3 histone marker was found in hES cells cultured under hypoxic conditions compared to the IgG control and relative to normoxia (Figure 4.3). In contrast, H3K4me3 and H3K9me3 represented only a small proportion of the histone modifications investigated in hES cells cultured under hypoxia suggesting the presence of a bivalent chromatin state (Figure 4.4).

A similar combination of histone modifications was observed within the HREs of NANOG and SOX2. Indeed, a significant increase of H3K4me3 and H3K36me3 histone modification markers were found in hES cells cultured at 5% oxygen compared to 20% oxygen (Figure 4.3). H3K9me3 was found not statistically different in hES cells cultured at 20% oxygen compared to those cultured at 5% oxygen (Figure 4.3).

However, pie charts showed an increase in the proportion of H3K9me3 bound in hES cells cultured under normoxia compared to hypoxia (Figure 4.4). These data confirm that the chromatin state in hES cells cultured at 20% oxygen tension is more heterochromatic and inaccessible to transcription factors or chromatin remodelling proteins.

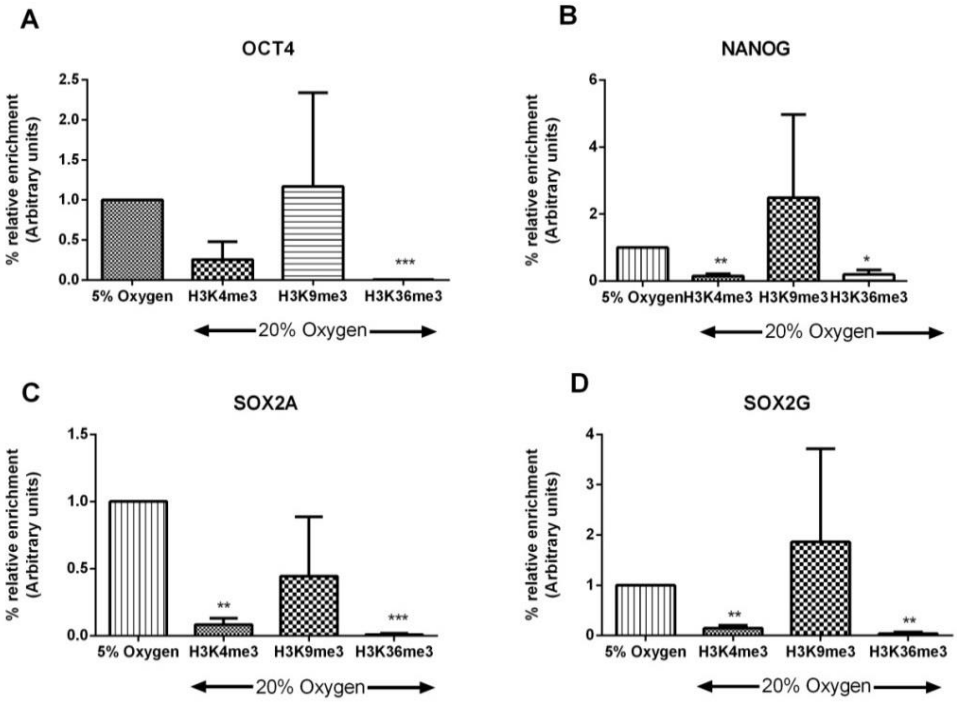

Figure 4.3: Hypoxia induces post-translational histone modifications within the HRE of OCT4, NANOG and SOX2 genes in hES cells cultured at either 5% or 20% oxygen

ChIP assays were performed with 2µg of anti-H3K4me3, H3K9me3 or H3K36me3 or IgG control antibodies on chromatin isolated from hES cells cultured either at 20% or 5% oxygen tension. DNA enrichment is expressed as a percentage of Input (non-immunoprecipitated chromatin) minus the background IgG. All data have been normalized to 1 for 5% oxygen. An average of 3 to 4 independent experiments is represented (*P<0.05, **P<0.01; ***P<0.001). The greatest error for cells cultured at 5% oxygen was obtained for H3K36me3 in SOX2A which was 1±0.9.

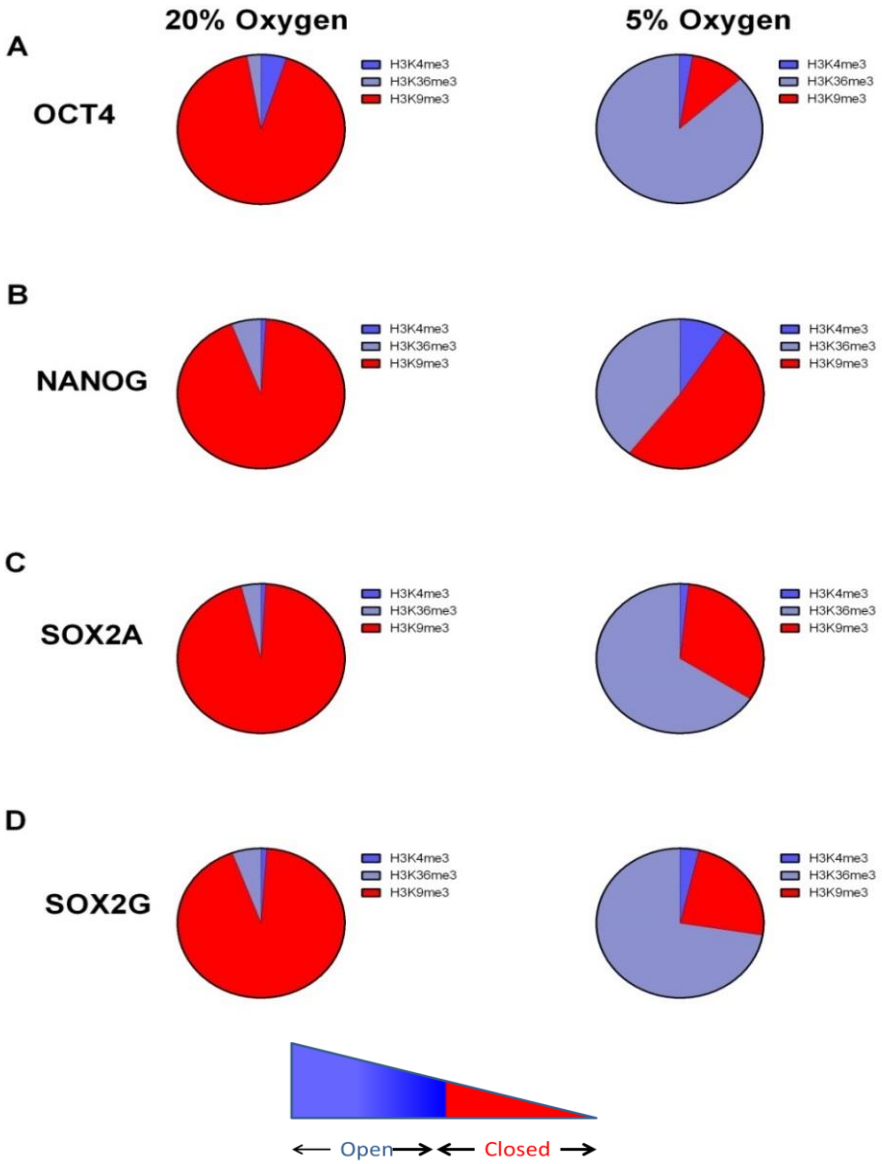

Figure 4.4: Hypoxia is responsible for an "open" chromatin conformation within the HRE of OCT4, NANOG and SOX2 genes in hES cells cultured 5% oxygen tension.

Histone modifications associated with transcriptional activation and repression in the predicted HRE site of the OCT4 (-1956) (A), NANOG (-301 bp) (B) SOX2 A (-1450 bp) (C) and SOX2 G (-1100 bp) (D) proximal promoter. Pie charts show a comparison of enrichment at 5% oxygen compared to 20% oxygen for each gene analysed. DNA enrichment is expressed as a percentage of Input (non-immunoprecipitated chromatin). An average of 3 to 4 independent experiments is represented.

Increased levels of H3K36me3 were also found within the HRE of eNOS and GLUT1 in hES cells cultured under hypoxic conditions in agreement with the chromatin state of the pluripotency markers (Figure 4.5 and 4.6). However, the proportion of H3K9me3 histone modification bound to the chromatin of hES cells cultured at 20% was higher than hES cells cultured at 5% oxygen (Figure 4.6).

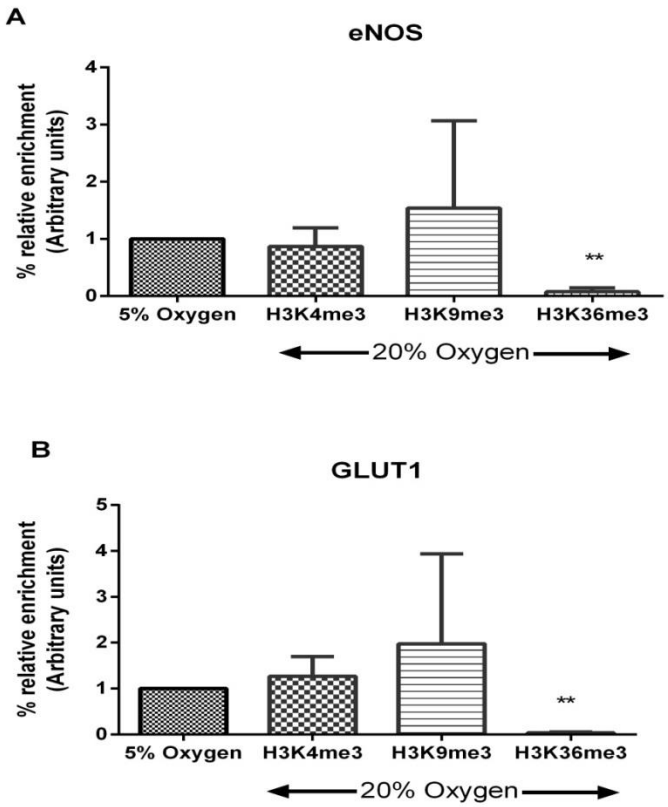

Figure 4.5: ChIP of histone modifications induced within the HRE of eNOS and GLUT1 genes in hES cells cultured at either 5% or 20% oxygen

ChIP assays were performed with 2μg of anti-H3K4me3, H3K9me3 or H3K36me3 or IgG control antibodies on chromatin isolated from hES cells cultured either at 20% or 5% oxygen tension. DNA enrichment is expressed as a percentage of Input (non-immunoprecipitated chromatin) minus the background IgG. All data have been normalized to 1 for 5% oxygen. An average of 3 independent experiments is represented (**P<0.01). The greatest error for cells cultured at 5% oxygen was obtained for H3K9me3 in all the genes of interest which was 1±0.99.

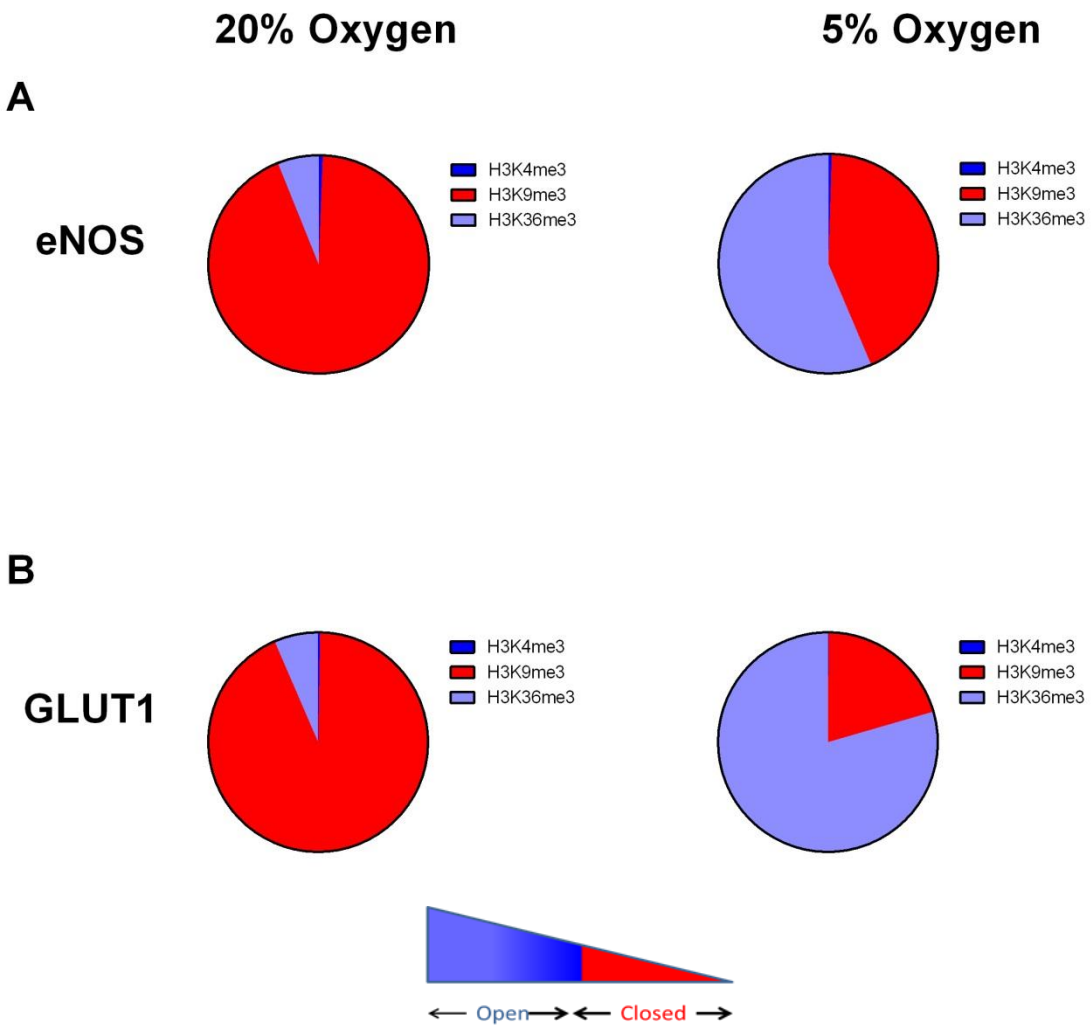

Figure 4.6: Hypoxia induces an "open" chromatin conformation within the HRE of eNOS and GLUT1 genes in hES cells cultured at either 20% or 5% oxygen tension. Histone modifications associated with transcriptional activation and repression in the predicted HRE site of the eNOS (-2644) (A) and GLUT1 (-1691) (B) proximal promoter. Pie charts show a comparison of enrichment at 5% oxygen compared to 20% oxygen for each gene analysed. DNA enrichment is expressed as a percentage of Input (non-immunoprecipitated chromatin). An average of 3 independent experiments is represented.

Overall, the data presented in this chapter demonstrated that hypoxia increases H3K4me3 and H3K36me3 expression level in the proximal promoters of the pluripotency genes compared to normoxia (Figure 4.3). These findings are consistent with a more open chromatin conformation of hES cells cultured under low levels of oxygen and correlate with transcriptional activity. As predicted, under hypoxia there was also a small proportion of H3K9me3 binding in all genes analysed in this study which may be due, in part, to an increased activity of the G9a methyltransferase that does not alter the transcriptional activation under hypoxic conditions (Chen et al., 2006). The expression of H3K36me3 and H3K9me3 under hypoxic conditions reflects the dynamic state of the chromatin enabling it to react rapidly in response to environmental changes while small changes in H3K4me3 are associated with genes poised for activation. Of the chromatin modifications investigated H3K9me3 was found consistently higher in the proximal promoter of OCT4, SOX2, NANOG, eNOS and GLUT1 in hES cells cultured under normoxic conditions, indicating a transcriptional repressing activity. This is consistent with cells at 20% oxygen having a more compacted chromatin state compared to those cultured at 5% oxygen. Furthermore, a dramatic reduction in the proportion of H3K4me3 and H3K36me3 levels was observed in all genes analysed when hES cells were cultured under normoxia compared to those maintained under hypoxia (Figure 4.4 and 4.6).

4.3.2 Histone demethylase expression in hES cells under normoxia or hypoxia

In attempt to evaluate whether normoxia and hypoxia have an effect on the regulation of the JmjC domain-containing histone demethylases proteins in hES cells, RT-qPCR analysis was performed.

When hES cells were cultured at 20% oxygen, there was a significant decrease in the *JMJD1a* mRNA expression compared to hES cells maintained at 5% oxygen (See Methods section 2.4 and Table 2.5 for jumanji and UBC probes used) (Figure 4.7). No significant difference was found in the mRNA expression of *JMJD2b* and *JMJD2c* in hES cells cultured at 20% oxygen compared to 5% oxygen. The reduction of *JMJD1a* in normoxia, observed in this study, is in agreement with data from HeLa and HEK293 cells in which a robust expression of this histone demethylases was found in hypoxia where it specifically demethylates H3K9me3 (Beyer et al., 2008).

Nevertheless, a great variability was observed across the samples analysed which might suggest that an increase in the "n" number is needed to better define the mechanisms of regulation of the JmjC proteins in hES cells.

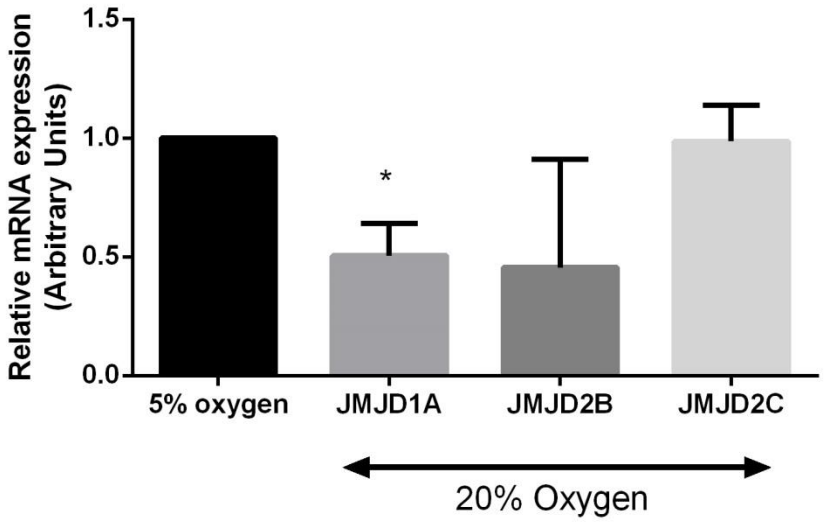

Figure 4.7: Jumanji histone demethylases expression is reduced in hES cells mantained at 20% oxygen compared to those cultured at 5% oxygen condition

RT-qPCR analysis of *JMJD1a*, *JMJD2b* and *JMJD2c* in hES cells cultured at 5% or 20% oxygen. All data have been normalized to *UBC* and to 1 for 5% oxygen. Values are mean of 3 independent experiments ± SEM. The greatest error for cells cultured at 5% oxygen was obtained for *JMJD1a* which was 1±0.08.

4.3.3 Pyrosequence analysis of 13 CpG sites within the exon and upstream promoter region of the OCT4 gene

To determine whether the methylation status of CpG islands in the OCT4 proximal promoter and first exon were modified in hES cells cultured either under hypoxic or normoxic conditions, pyrosequencing analysis was performed using genomic DNA isolated from hES cells cultured at either 5% and 20% oxygen tension. Genomic DNA template for the reaction was bisulfite converted and amplified using validated primers in order to amplify CpG islands in the OCT4 promoter spanning from -90bp up to +219bp from the start site (See Methods section 4.2, Table 4.1) (Figure 4.8).

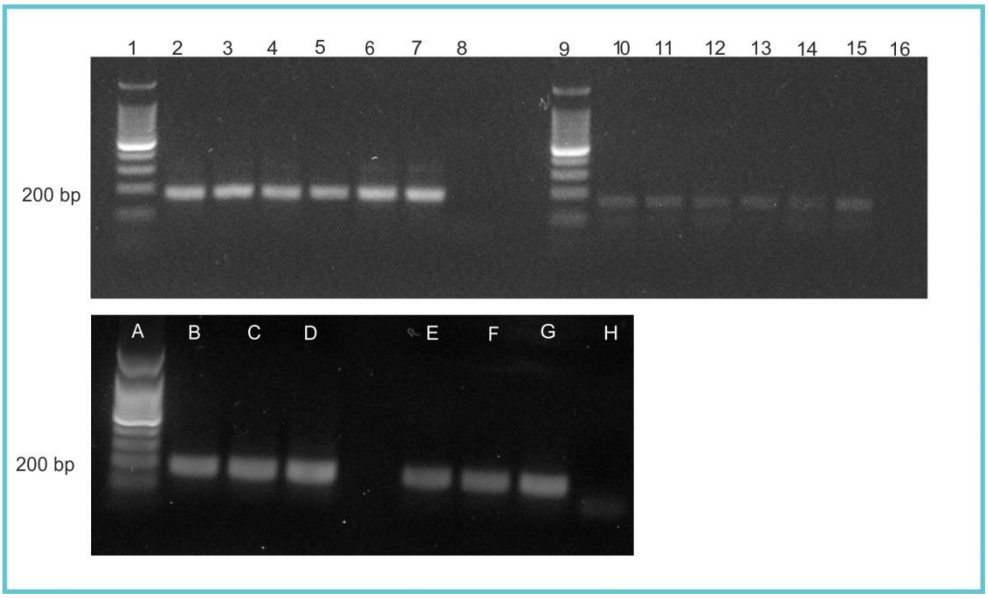

Figure 4.8: PCR analysis of the CpGs of the OCT4 promoter region from -90 bp to +219 bp from the first codon

An Ethidium stained 1% agarose gel showing amplicons for the CpG sites in the OCT4 proximal promoter. PCR analysis of the OCT4 proximal promoter CpG from +40 bp to +219 bp. Lane 1, 9, A: 1Kb Ladder; DNA of hES cells cultured at 5% oxygen (Lane 2-4) or 20% oxygen (Lane 5-7). Expected product =179 bp. PCR analysis of the OCT4 proximal promoter CpG from -89 bp to +72 bp, DNA of hES cells cultured at 5% oxygen (Lane 10-12) or 20% oxygen (Lane 13-15). Expected product = 161 bp. PCR analysis of the OCT4 proximal promoter CpG from -242 bp to -90 bp. DNA of hES cells cultured at 5% oxygen (Lane B-D) or 20% oxygen (Lane E-G). Expected product =152 bp. Lane 8, 16 and H water.

In addition, 2 CpG sites in the OCT4 proximal promoter situated around the HRE in the CR3 region from -1956 bp and -2016 bp from the start site were analysed. These CpGs were amplified with specific primers (See Method section 4.2, Table 4.1) that gave the expected product of 108bp (See Methods section 4.2) (Figure 4.9).

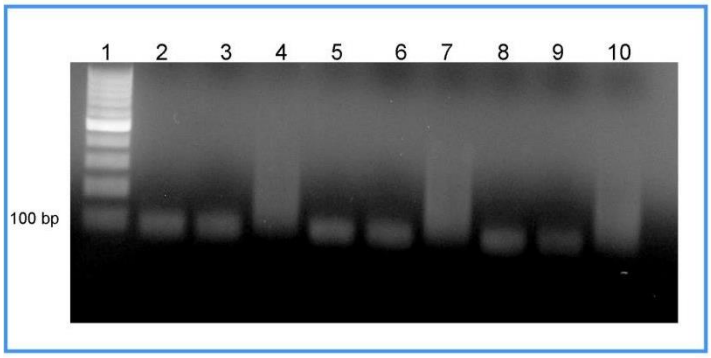

Figure 4.9: PCR analysis of the CpGs of the CR3 OCT4 proximal promoter region
An Ethidium stained 1% agarose gel showing amplicons for the CpG sites in the CR3 OCT4 proximal promoter. Lane 1: Ladder; Lane 2, 5, 8 DNA of hES cells cultured at 5% oxygen tension; Lane 3, 6, 9 DNA of hES cells cultured at 20% oxygen tension; Lane 4, 7, 10 water. Expected product= 108bp.

The PCR products were used for the pyrosequencing analysis using validated sequencing primers for each primer set. Bisulfite treated controls were added in the experiment which ensures that any cytosine not followed by a guanine was 100% converted to thymine. The percentage of unconverted bisulfite DNA was set at the standard of 4.5% so that any CpGs with a lower rate of methylation were not measured.

Analysis of the CpG sites in the OCT4 promoter region and first exon, in hES cells cultured at 20% oxygen tension revealed that CpGs (CpGs -109, -61, +4, +29, +39, +73, +79, +87) were between 10% and 20% methylated with CpGs -109, +39, +73 and + 79 being less than 10% methylated. In comparison, a higher percentage of methylation (up to 60%) was observed in CpGs +126, +133, +149, +195, +198 (Figure 4.10).

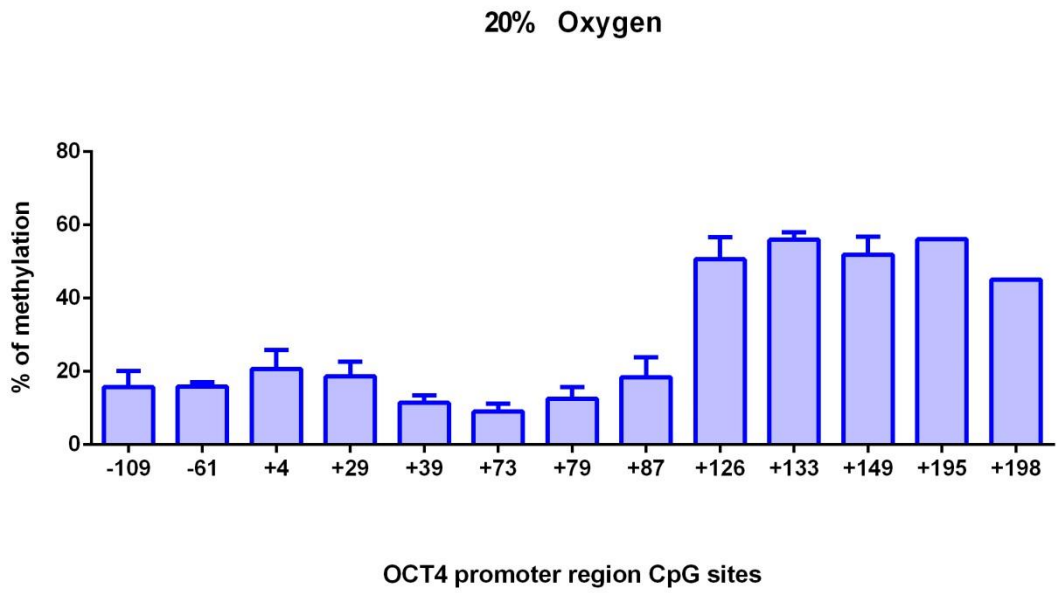

Figure 4.10: Pyrosequence analysis of CpGs within the OCT4 proximal promoter and first exon in hES cells cultured at 20% oxygen tension

Pyrosequencing data showing the mean of percentage of methylation in the OCT4 proximal promoter in hES cells cultured at 20% oxygen tension. Data with no error bars indicate that only 1 CpG was detected. Values are mean of 3 independent experiments ± SEM.

Pyrosequencing analysis of CpG sites in the OCT4 proximal promoter region and first exon, in hES cells cultured at 5% oxygen tension showed that CpGs (CpGs -109, -61) possess a level of methylation below the 4.5% bisulfite conversion threshold and therefore were undetectable. CpGs +4, +29, +39, +73, +79 and +87 displayed a percentage of methylation below 10% while CpGs +126, +133, +149, +195, +198 displayed a greatly enhanced level of methylation (Figure 4.11).

Overall, there was a non-significant trend of CpG methylation towards a lower methylation profile observed in hES cells cultured at 5% oxygen (Figure 4.12).

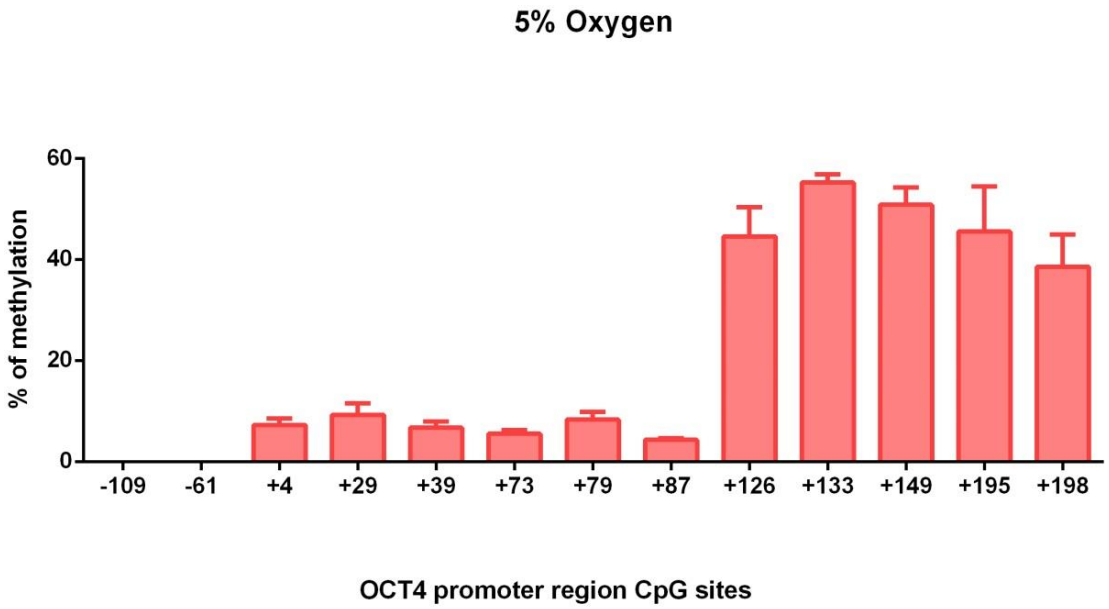

Figure 4.11: Pyrosequence analysis of CpGs within the OCT4 proximal promoter and first exon in hES cells cultured at 5% oxygen tension

Pyrosequencing data showing the mean percentage of methylation in the OCT4 proximal promoter in hES cells cultured at 5% oxygen tension. Values are mean of 3 independent experiments ± SEM.

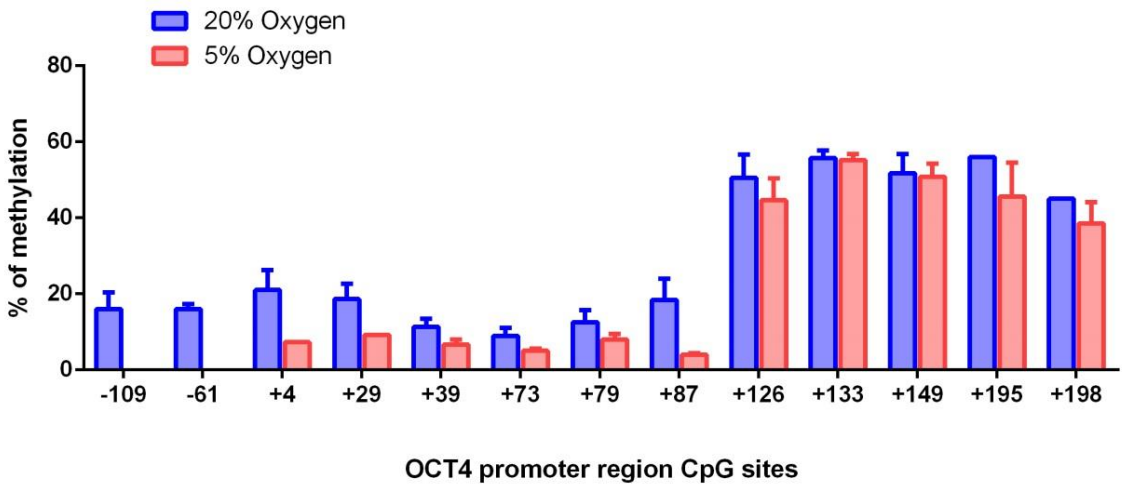

Figure 4.12: Pyrosequence analysis of CpGs within the OCT4 proximal promoter and first exon in hES cells cultured at either 5% or 20% oxygen

The data displays an overall lower rate of methylation in hES cells cultured in hypoxic conditions. Pyrosequencing data showing the mean percentage of methylation in the OCT4 proximal promoter in hES cells cultured under 5% and 20% oxygen tension. Data with no error bars indicate that only 1 CpG was detected. Values are mean of 3 independent experiments ± SEM.

Analysis of 2 CpGs in the CR3 region of the OCT4 proximal promoter was performed. Pyrosequencing analysis in hES cells cultured either at 5% or 20% oxygen tension showed that CpGs (CpGs -2016 and -1956) possess a level of methylation below the 4.5% bisulfite conversion threshold and consequently were deemed undetectable. For this reason, although the level of methylation at CpG -1956 appeared to be significantly affected by hypoxia (P<0.01) the value obtained for hES cells cultured at 5% oxygen was below the bisulfite threshold and so unreliable (Figure 4.13).

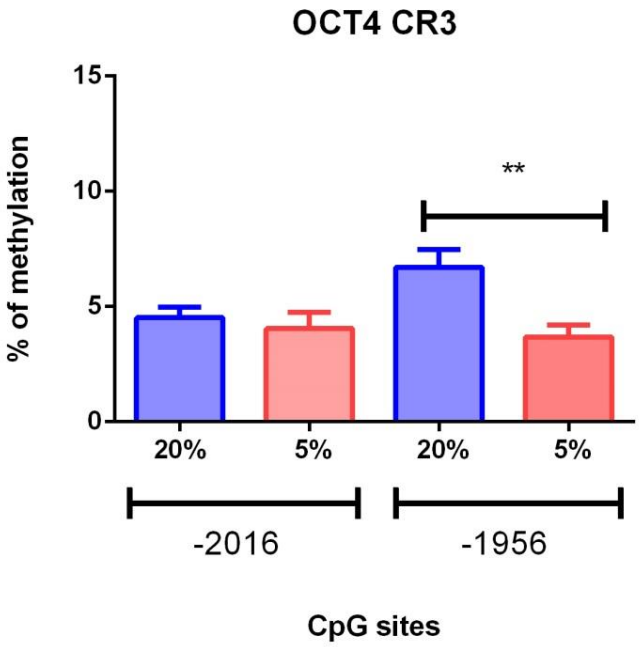

Figure 4.13: Pyrosequence analysis of CpGs within the CR3 OCT4 proximal promoter region in hES cells cultured at 5% and 20%oxygen tension.

Pyrosequencing data showing the mean percentage methylation in hES cells cultured at either 5% or 20% oxygen tension. Values are mean of 3 independent experiments ± SEM (** P<0.01).

4.3.4 Pyrosequencing analysis of hES cells cultured in absence of FGF2

In order to perform pyrosequencing analysis, genomic DNA samples collected after 24 hour in the absence of FGF2 were bisulfite converted and amplified using validated primers in order to amplify CpG islands in the OCT4 promoter spanning from -90 bp up to +219 from the start site (Figure 4.14) or the CR3 OCT4 proximal promoter (See Methods section 4.2) (Figure 4.15).

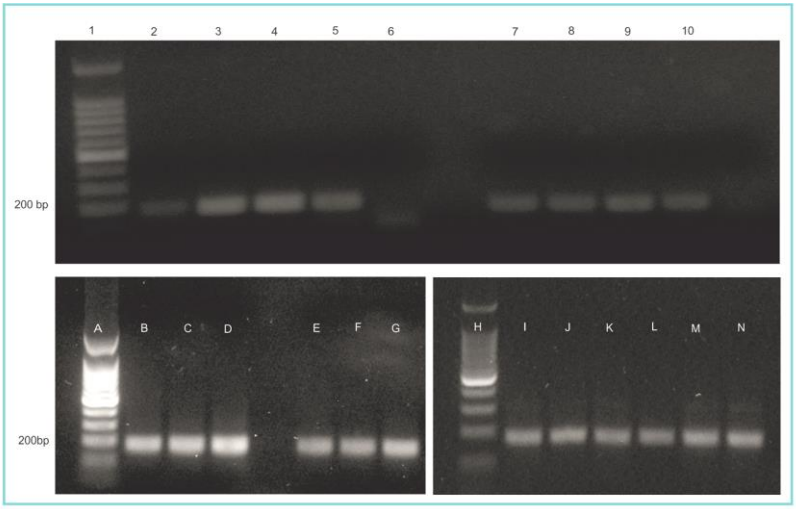

Figure 4.14: PCR analysis of the CpGs of the OCT4 promoter region from -90 bp to +219 bp from the first codon in hES cells cultured without FGF2 for 24h

An Ethidium stained 1% agarose gel showing amplicons for the CpG sites in the OCT4 proximal promoter. PCR analysis of the OCT4 proximal promoter CpG from +40 bp to + 219 bp. Lane 1, A, H: Ladder; DNA of hES cells cultured at 5% oxygen (Lane 2-5) or at 20% oxygen (Lane 7-10). Expected product =179 bp. PCR analysis of the OCT4 proximal promoter CpG from -89 bp to +72 bp, DNA of hES cells cultured at 5% oxygen (Lane B-D) or 20% oxygen (Lane E-G). Expected product = 161 bp. PCR analysis of the OCT4 proximal promoter CpG from -242 bp to -90 bp. DNA of hES cells cultured at 5% oxygen (Lane I-K) or at 20% oxygen (Lane L-N). Expected product =152 bp. Lane 6 water.

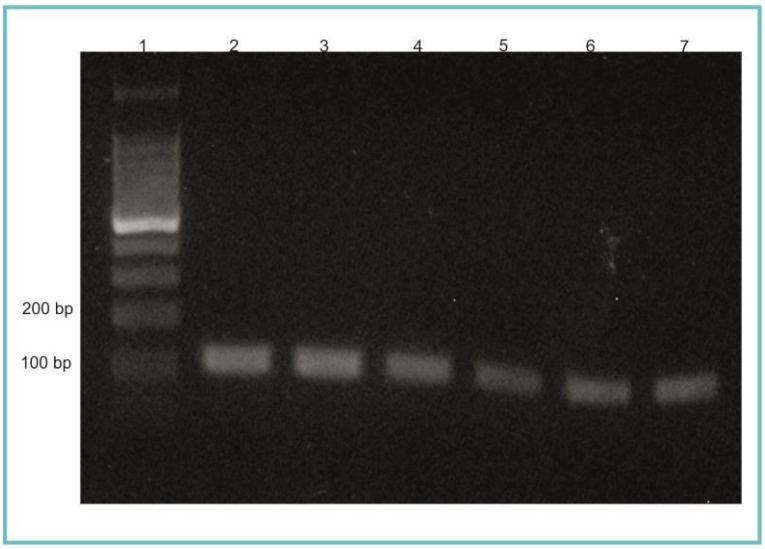

Figure 4.15: PCR analysis of the CpGs of the CR3 OCT4 proximal promoter region in hESC cultured at 20% or 5% oxygen without FGF2 for 24h

An Ethidium stained 1% agarose gel showing amplicons for the CpG sites in the CR3 OCT4 proximal promoter. Lane 1: Ladder; Lane 2, 3, 4 DNA of hES cells cultured at 5% oxygen; Lane 5, 6, 7 DNA of hES cells cultured at 20% oxygen tension; Expected product= 108bp.

Analysis of the CpG sites in the OCT4 promoter region, first exon and CR3 in hES cells cultured at 20% oxygen with or without FGF2 revealed a similar level of methylation in all CpGs analysed with the exception of CpGs +39, +4 and -1956 being less than 10% methylated in the –FGF2 samples (Figure 4.16). Samples were not statistically significant.

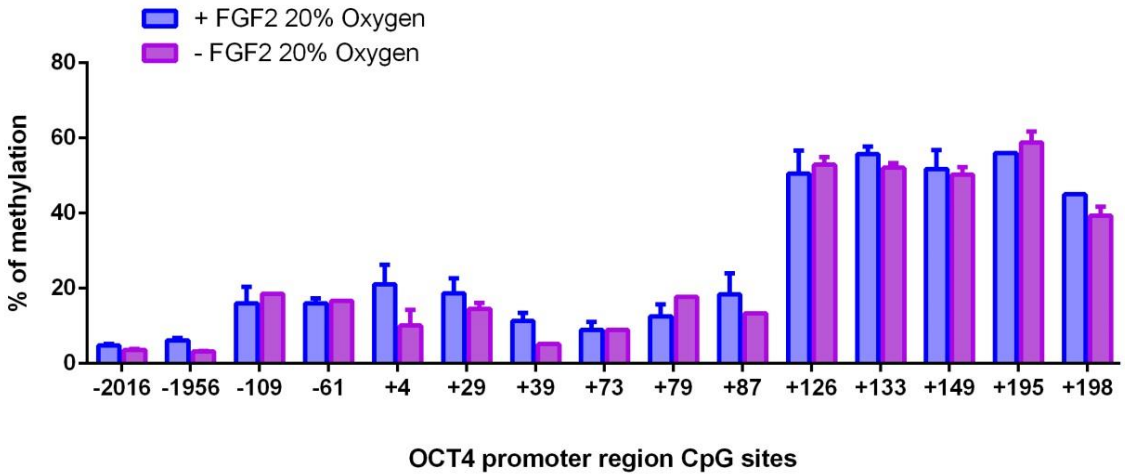

Figure 4.16: Pyrosequence analysis of CpGs within the OCT4 proximal promoter, first exon and CR3 in hES cells cultured at 20% oxygen tension in the presence or absence of FGF2.

The data displays similar level of methylation among hES cells cultured at either 20% oxygen supplemented with FGF or at 20% oxygen without FGF2. Data with no error bars indicate that only 1 CpG was detected. Values are mean of 6 independent experiments ± SEM.

Comparison of CpG sites in the OCT4 promoter, first exon and CR3 in hES cells cultured at 5% oxygen with or without FGF revealed a slight increase of the level of methylation of CpGs +195, +126, +79,+73, +39 cultured without FGF2 samples compared to samples cultured in presence of FGF2. This could be may related to an increase of differentiation in the –FGF hES cells. However, samples were not statistically significant. CpGs -109 and -61 revealed a level of methylation below 4.5% bisulfite conversion threshold and consequently were undetectable. No difference in the level of methylation within the CR3 region was observed in hES cells cultured without FGF2 compared to hES cells supplemented with FGF2 (Figure 4.17).

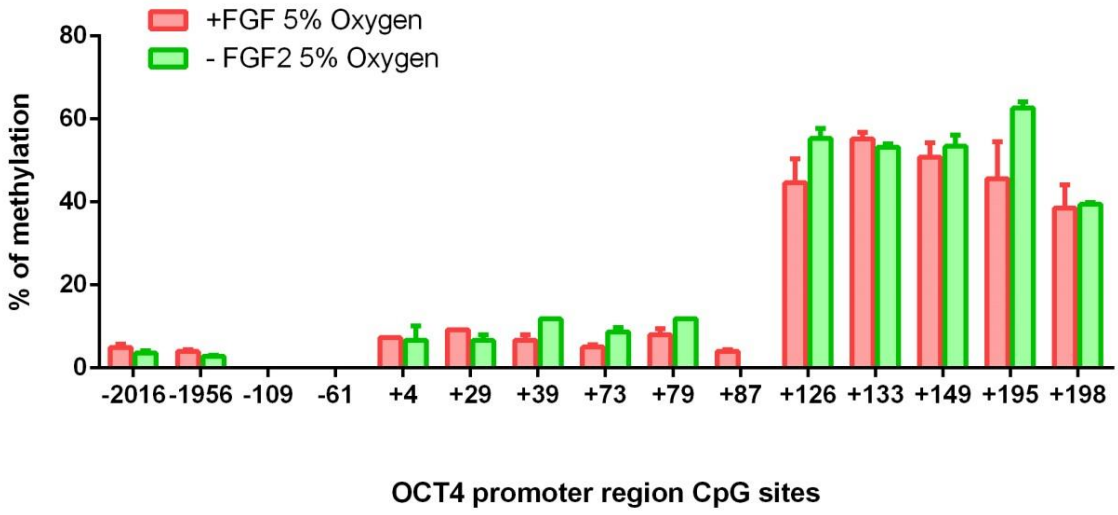

Figure 4.17: Pyrosequence analysis of CpGs within the OCT4 proximal promoter, first exon and CR3 in hES cells cultured at 5% oxygen in the presence or in absence of FGF2.

Graph shows a comparison between hES cells cultured at 5% oxygen with or without FGF2. Data with no error bars indicate that only 1 CpG was detected. Values are mean of 3 independent experiments ± SEM.

No significant differences in the level of methylation in CpGs +195, +149, +139 and +39 were observed in hES cells cultured at 5% oxygen without FGF2 compared to cells cultured at 20% oxygen without FGF2. These data suggest that FGF does not have an influence on the overall level of CpG methylation in hES cells cultured at either normoxic or hypoxic conditions (Figure 4.18).

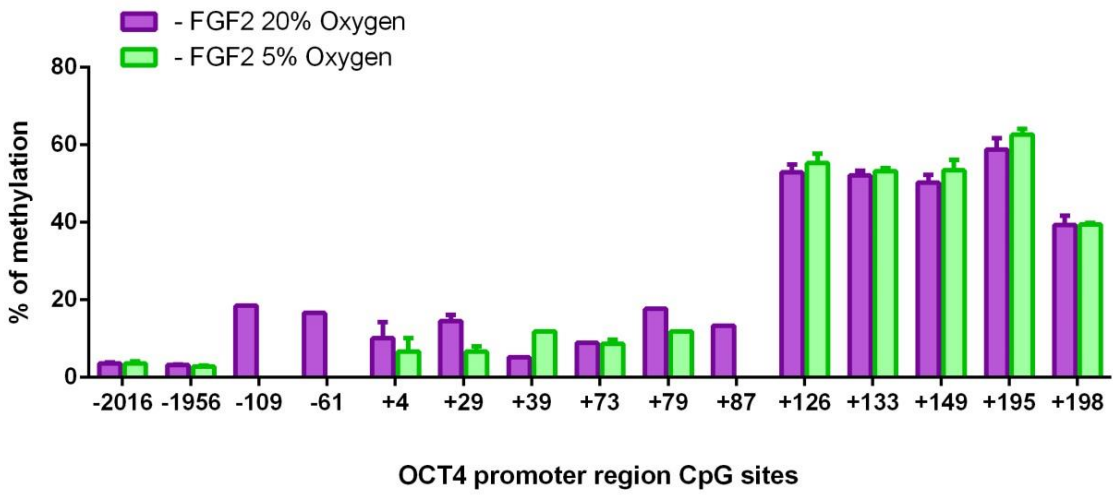

Figure 4.18: Pyrosequencing analysis of CpGs within the OCT4 proximal promoter, first exon and CR3 in hES cells cultured at 5% and 20% oxygen tension without FGF2.

The data displays an overall similar rate of methylation in hES cells cultured at either normoxic or hypoxic conditions without FGF2. Pyrosequencing data showing the mean methylation percentage between 2 to 3 different DNA samples of hES cells cultured under 5% and 20% oxygen tension without FGF. Data with no error bars indicate that only 1 CpG was detected. Values are mean of 3 independent experiments ± SEM.

4.3.4.1 OCT4 mRNA expression in –FGF2 hES cells

In terms of mRNA expression, when FGF2 was removed for 24 hours from the culture medium of hES cells cultured at either 5% or 20% oxygen, there was no significant reduction in *OCT4* mRNA expression compared to hES cells maintained at 5% oxygen (See Methods section 2.4 for OCT4 and UBC probes used) (Figure 4.19). Furthermore, no difference was observed in *OCT4* mRNA expression in hES cells cultured at 20% oxygen in the absence of FGF2 when compared to those cultured at 20% oxygen supplemented with FGF2 (Figure 4.20)

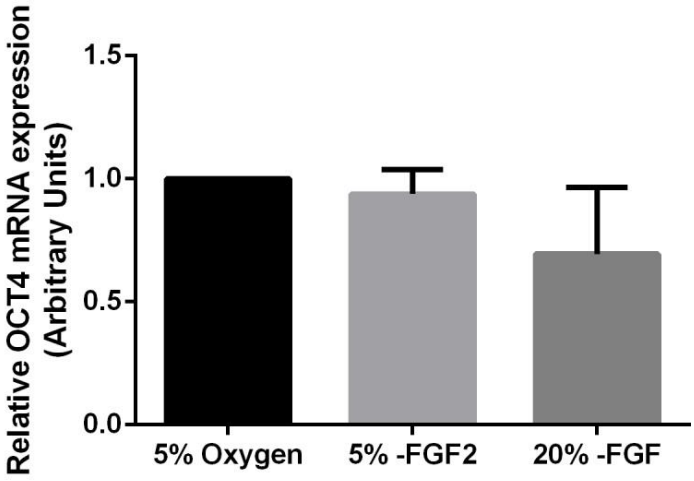

Figure 4.19: *OCT4* expression was not affected by the removal of FGF2 from hES cells cultured at either 5% or 20% oxygen

RT-qPCR analysis of *OCT4* mRNA in hES cells cultured at 5% or 20% oxygen without FGF2 for 24h. All data have been normalized to *UBC* and to 1 for 5% oxygen. Values are mean of 3 independent experiments ± SEM. The error for *OCT4* at 5% oxygen was 1 for all the conditions analyzed.

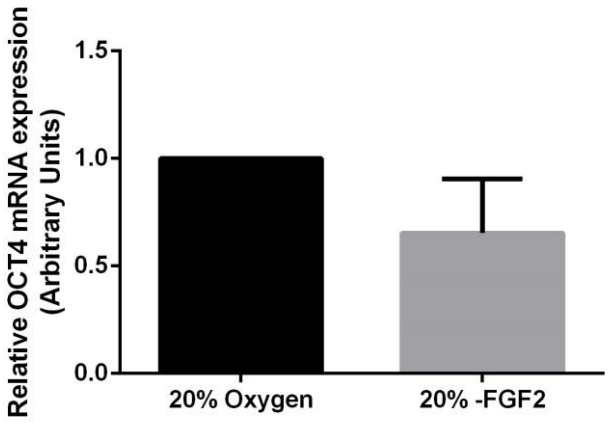

Figure 4.20: *OCT4* expression was not affected by FGF2 removal in hES cells cultured at 20% oxygen

RT-qPCR analysis of *OCT4* mRNA in hES cells cultured at 20% oxygen without FGF2 for 24h. All data have been normalized to *UBC* and to 1 for 20% oxygen. Values are mean of 3 independent experiments ± SEM. The greatest error for *OCT4* at 5% oxygen was 1 for all the conditions analyzed.

These data were different from the results obtained by Forristal et al. (2013) which found a significant decrease in OCT4 expression in hES cells cultured without FGF for 16h and might explain the pyroseqencing results described in this chapter. However, since Kingham et al. (2013) showed a significant increase of the methylation profile in OCT4 gene after culturing hES cells for 72h without FGF2 this might suggest that a 72h period of FGF2 removal are likely to be ideal for the pyrosequence analysis.

4.4 Discussion

4.4.1 Chromatin Modification Analysis under hypoxia

It is well known that chromatin is formed by DNA wrapped around an octamer of histones and other related proteins. This DNA-histone structure is dynamic and important for many biological processes like DNA replication, repair and transcription (Kornberg, 1974). However, how chromatin structure changes in response to low oxygen tension is currently unknown. The identification of HIFs allowed the characterization of several cellular processes that require binding to DNA target sequence in order to maintain oxygen homeostasis (Semenza et al., 1991). Binding of HIF to its target genes occurs in the context of complex chromatin structures that require the interaction between HIFs and epigenetic modifying enzymes (Watson et al., 2009). It has been demonstrated that during the cellular response to hypoxia, HIFs interact with chromatin remodelling factors such as CBP/p300, HATs and HDAC (Kalkhoven, 2004; Ruas et al., 2010) and that these interactions can be disrupted by FIH (Factor inhibiting HIF) or by inhibiting the binding to the VHL complex, resulting in repressed expression of HIF target genes (Fath et al., 2006; Mahon et al., 2001). These interactions change the histone acetylation and methylation status in the promoter of hypoxia inducible genes and allow their repression or active transcription. However, the dynamics of these chromatin changes is not completely understood therefore, the aim of this chapter is to investigate whether hypoxia leads to epigenetic modifications within the proximal promoter of the hypoxia inducible genes.

Data in this thesis have demonstrated that HIF-2α physically interacts not only with the proximal promoter of pluripotency genes but also with eNOS and GLUT1. Indeed it is possible to assume that these interactions could also be influenced by chromatin modifications. Hypoxia, in fact, might contribute to maintain the chromatin within the HRE sites in an open conformation which allows the transcription of its target genes by interacting with chromatin remodelling factors or histone demethylases.

In an attempt to determine whether HIF-2α could be responsible for these epigenetic changes, in hES cells cultured either under hypoxia or normoxia, ChIP assays with antibodies that recognize H3K4me3 and H3K36me3, markers of transcriptional activation, and H3K9me3, a marker of transcriptional silencing in the HRE of all selected genes were performed.

Hypoxia induced a bivalent chromatin state at the core promoters of all the selected genes characterized by the presence of both H3K9me3 and H3K36me3, markers of gene silencing and activation respectively compared to atmospheric conditions (Figure 4.4 and 4.6). This finding was unexpected as the "bivalent chromatin" signature in hES cells usually refers to the specific modification pattern consisting of H3K4 and H3K27 methylation (Bernstein et al., 2006; Johnson et al., 2008). However, these results are supported by other studies in different cell lines under hypoxic conditions (Tausendschon et al., 2011; Xia et al., 2009). For example, an increased level of H3K9me3 may reflect, in part, the hypoxic induction of G9a methyltransferase (Chen et al., 2006) or maybe a consequence of the HIF-2α binding and the recruitment of co-factors to allow gene transcription. Furthermore, recent published data demonstrated that the presence of histone demethylases, such as JMJD1a, demethylate H3K9me3 to allow an enhanced GLUT3 expression by a cooperative interaction with HIF-1α in endothelial SCs and the establishment of a high order chromatin structure (Mimura et al., 2012). These data represent the first demonstration of specific histone modifications within the GLUT1 promoter which may suggest that in hES cells, HIF-2α is responsible for a dynamic chromatin conformation within the HRE site which may involve the presence of histone demethylases. Increased expression of H3K9me3 was found also within the HRE of eNOS proximal promoter in hES cells cultured at low oxygen tension (Figure 4.6) and it is possible that the upregulation of histone demethylases may serve as a compensatory mechanism for minimizing the effect of an increased methylation in H3K9me3. As tumour angiogenesis is a process characterized by high levels of eNOS expression (Li et al., 2009), the presence of an epigenetic pathway activated specifically by HIF-2α that enhance eNOS transcription might have relevant implications in tumour invasiveness.

The H3K4me3 histone modification has been associated either with genes actively transcribed (Bernstein et al., 2006) or inactive (Guenther et al., 2007). Although OCT4, NANOG and SOX2 genes are actively transcribed in hES cells, only a small increase of this marker within the predicted HRE sites in hES cells cultured under hypoxic condition was found. No H3K4me3 was observed within eNOS and GLUT1 HRE under the same conditions. This could be related to the epigenetic homeostasis driven by a family of jumonji-domain histone demethylases. It is widely recognised that HIF-1α and HIF-2α transactivate JARID1B histone demethylase which plays an important role for maintaining H3K4 methylation levels under low oxygen tension (Xia et al., 2009).

The H3K36me3 modification is associated with gene transcription elongation (Bannister et al., 2005) therefore hES cells may respond rapidly to oxygen changes by accumulating OCT4, NANOG, SOX2 and GLUT1 transcripts. This is in agreement with data presented in Chapter 3 and also with Forristal et al. (2013) which demonstrated that the mRNA expression level of GLUT1, as well as the protein level of pluripotency markers was highly expressed in hES cells cultured under low oxygen tension. Moreover, high levels of H3K36me3 has been found associated with high level of H3K4me3 in macrophages cultured under hypoxia (Tausendschon et al., 2011) and seems to be related to a JMJD2C histone demethylase activity.

When the chromatin state within the HRE in hES cells cultured under normoxic conditions was assessed, a marked decrease of H3K4me3 and H3K36 with a significantly increased methylation of H3K9me3 was found in all the genes analysed. This is related to a more heterochromatic state which may prevents HIF-2α binding and account for the decreased transcription of OCT4, NANOG, SOX2, eNOS and GLUT1 transcription factors (Figure 4.4 and 4.6).

RT-qPCR analysis of the jmjC histone demethylases showed a significant increase in the mRNA expression of JMJD1a in hES cells cultured at 5% compared to cells cultured at 20% oxygen. The reduction observed in JMJD1a in hES cells cultured at 20% oxygen is sustained by previous published data in other cell lines (Beyer et al., 2008) and will need further investigation. Surprisingly, JMJD2b was not found up-

regulated under hypoxia which is in contrast with previous published data showing a direct role of HIF-1α in the regulation of this histone demethylase in other cell lines (Pollard et al., 2008). However, data presented in this thesis have analysed the effect of long term exposure to hypoxia on hES cells where HIF-1α has been found not expressed (Forristal et al., 2010). To date, this is the first characterization of the role of the JmjC histone demethylases in hES cells under hypoxia and normoxia, hence further studies will need to further understand their specific role. Furthermore, it would be interestingly to analyse the activity of HIF-2α in the regulation of JMJD1a and JMJD2c in hES cells, since previous work described a role for these Jumanji in the modulation of Oct4 and Nanog in mouse ES cells (Loh et al., 2007). Overall, the effect of hypoxia on the global chromatin methylation in hES cells is still poorly understood and future genome mapping of histone demethylases and histone density will help to elucidate the specific hypoxia-epigenetic signatures of the hypoxia inducible genes.

Overall the results presented in this chapter demonstrated that environmental oxygen tension is able to create a pool of histone modifications within all the HREs analyzed suggesting that an epigenetic signature plays an important role in gene regulation under long term exposure to hypoxia. This could have important implications in cancer stem cell self-renewal and proliferation as HIF-2α is involved in tumour initiation and progression (Regan Anderson et al., 2013) but also in regulating hES cells self-renewal (Forristal et al., 2010). Given the importance of HIF-2α in regulating self-renewal in both development and disease, it is conceivable to hypothesize that this transcription factor could also have also implications for iPS cell biology. Indeed, it has been found that iPS cells generated and cultured under hypoxic conditions increased the rate of reprogramming with a mechanism that is still not completely understood (Mathieu et al., 2013; Yoshida et al., 2009). Therefore, a better comprehension of the hypoxia epigenetic signature might allow the discovery of new pathways of regulation mediated by HIF-2α that could be manipulated in order reprogramme differentiated somatic cells to iPS cells.

4.4.2 DNA Methylation Analysis

To analyse whether hypoxia has an effect not only on the histone modification profile but also on the DNA methylation status of hES cells, pyrosequencing analysis was performed. Previously published data reported that low oxygen concentrations can induce modification of the methylation status of gene sequences through the alteration not only of the normoxic histone codes but also by modulation of DNA methylation (Watson et al., 2009). hES cells usually present a low level of DNA methylation that allow the transcription of SCs markers, like OCT4, in order to maintain the pluripotency and self-renewal (Yeo et al., 2007). When cells start to differentiate, DNA methylation occurs in order to gradually repress SC marker expression (Bibikova et al., 2006).

Pyrosequence results presented in this chapter showed that overall CpG sites displayed a low level of methylation as expected but this level is slightly higher in cells cultured under normoxic conditions. In particular CpG sites in the OCT4 promoter region and first exon, in hES cells cultured at 20% oxygen tension were found to be almost 10% and 20% of CpG methylated with the exception of 4 CpGs sites (-109, +39, +73 and + 79) being less than 10% methylated. This is in agreement with previous published data which found similar percentage of methylation in the OCT4 promoter and distal enhancer respectively (Kingham et al., 2013; Yeo et al., 2007). Higher percentage of methylation around 60% was observed in CpGs sites spanning the first exon (Figure 4.10). Data reported in the literature showed that the high methylation pattern observed in the first exon of OCT4 arises as a consequence of DNA methyltransferases activities on transcriptionally inactive sites (Yeo et al., 2007).

Methylation profile of OCT4 CpG sites in hES cells cultured at 5% oxygen tension displayed for almost all CpG sites a level of methylation below the 4.5% bisulfite conversion threshold and therefore were undetectable. Since the sensitivity of the pyrosequencer is 4.5%, the interpretation of this data is difficult and should be treated with caution (Figure 4.11). However, these data agree with the very hypomethylated DNA status in hES cells and this is particularly true for hES cells cultured under

hypoxic conditions, as they present a more pluripotent population compared to hES cells cultured under normoxia.

Our study was also focused on the analysis of the methylation status in the CR3 region in the OCT4 proximal promoter. The canonical sequence of an HRE itself contains a CpG site and previous studies in erythropoietin and beta-tubulin genes demonstrated that HIF-1α binding is influenced by the level of methylation of the CpG within the consensus HRE (Raspaglio et al., 2008; Rossler et al., 2004). In fact, high levels of oxygen can modulate the methylation level of the CpG within the HRE of a target gene leading to a decrease of the HIF-dependent transcription. In this study we found that, despite the very low methylation profile within the OCT4 gene, there is a significant hypomethylation in the HRE in the CR3 of the OCT4 gene promoter in hES cells cultured under low oxygen tension compared to normoxic conditions. Having confirmed in hES cells that HIF-2α directly binds to the HRE in the CR3 of the OCT4 promoter, we can assume that the low level of methylation (below 10%) observed particularly at 5% oxygen, is part of the epigenetic landscape that relies on HIF-2α rather than HIF-1α activity. These data are in agreement with Forristal et al. (2010) who demonstrated that only HIF-2α is expressed in hES cells cultured under 5% oxygen tension. However, the data were still below the bisulfite threshold and therefore should be treated with caution (Figure 4.13).

With the attempt of further analyse the methylation pattern of OCT4 gene, FGF2, required for the maintenance of hES cells, was removed for 24h from the medium either used to culture cells under normoxia or hypoxia. However, the global methylation of hES cell cultured at 5% or 20% oxygen following FGF2 removal was not statistically different to hES cells cultured at either 5% or 20% oxygen and supplemented with FGF2. Indeed, the OCT4 mRNA expression was similarly not found to be significantly different between these experimental conditions. This was surprisingly as recent published data reported an enhanced methylation state within the same CpGs in the OCT4 promoter of hES cells cultured at 20% oxygen following 72h of FGF2 removal (Kingham et al., 2013). However, this suggests that 24h of FGF2 removal from the media that were not sufficient to increase hES cell differentiation. Furthermore, no

differences in the level of methylation within the HRE of the OCT4 promoter was observed among hES cells cultured with or without FGF2 in the media and suggest that the chromatin state rather than DNA methylation plays a major role in the hypoxic cellular response.

Many studies in cancer cells have demonstrated that hypermethylation blocks HIF binding to the HRE (Horiuchi et al., 2012; Huang et al., 2010; Place et al., 2011), however, this has not been demonstrated in hES cells and will need further analysis. Moreover, it will be very interesting to study the role of DNA methyltrasferases or MAT2A in hES cells cultured under hypoxia as several studied showed that hypoxia prevents DNA methylation through the inhibition of DNA methyltransferases (Skowronski et al., 2010) or the induction of MAT2A (Liu et al., 2011). Given the crosstalk between DNA and histone methylation in hES cells (Meissner et al., 2008), data presented in this chapter showed that the effect of hypoxia is more evident at the chromatin level rather than at the DNA level. However, this could be related to the fact the OCT4 promoter is already hypomethylated in hES cells therefore, the effect of hypoxia on the DNA state was not evident.

Overall, data presented in this chapter highlight the transcriptional role of HIF-2α on its target genes and establish a new pattern of epigenetics marks specific for the hypoxia response that allows hES cells to maintain self-renewal, glucose metabolism and signalling in the hypoxic environment.

Chapter 5

Effect of reoxygenation on hES cell self-renewal

5.1 Introduction

5.1.2 SCs and hypoxic preconditioning

Stem cell transplantation therapy for ischemia, stroke, heart attack, kidney failure and wound healing is a rapidly developing tool in regenerative medicine. SCs or resident progenitor SC populations have the potential to promote tissue repair supporting cell replacement and provide increasing expectation for the use of SC therapies in a variety of disorders (Alper, 2009; Malliaras and Marban, 2011). Although recent advances in stem cell medicine have highlighted the potential of stem cell therapies, some critical issues, such as, the right choice of cells to use for tissue repair and regeneration and the poor survival of transplanted cells (engraft) remain to overcome (Pagani et al., 2003).

One of the endogenous protective mechanisms that were discovered to improve stem cell therapies was hypoxic or ischemic preconditioning (Murry et al., 1986; Pong, 2004). In the heart, it has been observed that exposure to short cycles of hypoxia followed by intermitted reperfusion, allowed cells to be more resistant to the ischemic insult (Murry et al., 1986). The cytoprotective effect of the hypoxic/ischemic preconditioning occurs in two phases: Phase I is an acute protection that lasts for a few hours after the insult, while Phase II is a delayed or "late window" protection that occurs many hours after hypoxia and can lasts for days or even weeks (Pong, 2004). The "late window" of protection requires the transcriptional activation of HIFs which result in the activation of several pathways that promote glycolysis, inhibit apoptosis and ROS and increase VEGF and EPO (Pong, 2004; Semenza, 2000a). In particular VEGF and EPO stimulate angiogenesis and neurogenesis which are important in wound healing and functional repair of brain and heart (Keogh et al., 2007; Li et al., 2008). Indeed, HIF activation seems to have a cytoprotective effect as it has been demonstrated that HIFs regulate several pathways such as Heme Oxygenase-1 (HO-1) which may promote cell survival through the increase of cellular antioxidant capacity (Semenza, 2000b), or iNOS and VEGF which have been found to increase resistance to ischemia in mice and enhance vascular endothelial supply (Natarajan et al., 2006; Semenza, 2000b). Furthermore, HIFs seem to up-regulate the transcription and translation of CXCR7 (Liu et al., 2010) and Bcl-2 (Francis and Wei, 2010) which have an anti-apoptotic effect in

SCs and progenitor cells. However, these specific mechanisms are not fully understood and their activation may be cell-type or tissue specific.

As progenitor SC populations reside in specialized niche and are exposed to hypoxia and oxidative stress, several studies have analysed their gene expression profile to define a characteristic signature that provides resistance against environmental stress and to regenerate a damaged tissue (Ivanova et al., 2002; Liadaki et al., 2005). This is true also for hES cells that when cultured at low oxygen tension, which is comparable with the levels observed in the mammalian reproductive tract and in the brain, exert significant cell proliferation, pluripotency and chromosomal stability (Ezashi et al., 2005; Forristal et al., 2010; Lengner et al., 2010). Therefore, on the basis of these findings, hES cells should be able to acquire enhanced tolerance to injuries and might represent a model to investigate the regenerative potential of these cells in conditions of oxidative stress followed by hypoxia. Moreover, in hES cells, HIF-2α has been found to promote self-renewal genes expression and reduce cell differentiation under hypoxia (Forristal et al., 2010) and to have a cytoprotective effect on mice kidneys and heart (Kojima et al., 2007; Martin et al., 2008; Scortegagna et al., 2003). These findings highlight the importance of HIFs and hypoxia reoxygenation in maintaining the regenerative potential of hES cells and progenitor cells during the repair process. However, the hypoxic and ischemic preconditioning response of hES cells is not known, neither is the mechanism which regulates the maintenance of a high proliferative stem cell state in the area of injury. A better understanding of the transcriptional regulation and stabilization of HIFs may highlights new insights in the field of therapeutic medicine and provide a reoxygenation model to protect organs against oxidative stress.

5.1.3 Effect of hypoxic preconditioning on epigenetics

Epigenetic pathways are dynamic processes that allow cells to respond to environmental cues, and endogenous or exogenous stimuli (Jaenisch and Bird, 2003). Emerging evidence suggests that epigenetic marks allow the establishment of a cellular memory of the environment in which cells were exposed in early life and that this memory is maintained throughout the life leading to changes in gene transcription that can cause disease in later life (Heijmans et al., 2008). Hypoxia is a microenvironmental factor that plays critical role in several biological processes such as development, metabolism, inflammation, tumour progression and stemness (Semenza, 2012). In hypoxia, chromatin modifications allow cells to adapt to the hypoxic stress. Indeed, the histone code has been implicated not only in regulating gene expression, but also in DNA damage response, meiosis and apoptosis (Pusarla and Bhargava, 2005). However, little is known about the role of hypoxia and reoxygenation on the epigenetic modifications. Cardiovascular disease pathways such as atherosclerosis, ischemia-reperfusion damage and cardiovascular response to hypoxia and reperfusion are now becoming extensively studied in terms of epigenetic regulation (Shirodkar and Marsden, 2011). The most well characterized endothelial gene that has cardiovascular implications is eNOS. In vascular endothelial cells the differential regulation of eNOS was attributed to the acetylation and methylation of H3K9, H4K12 and H3K4 (Charles et al., 2010). Interestingly, HDAC also appears to have a role in the severity of myocardial ischemia and reperfusion (Zhang et al., 2012). Indeed, HDAC inhibition was found to enhance the formation of myocytes in the heart and to stimulate angiogenesis after a myocardial infarction (Zhang et al., 2012). Both eNOS and HDAC have been shown to affect cell survival in hypoxia followed by reoxygenation and are regulated by HIFs (Coulet et al., 2003; Kim et al., 2007) which have been shown to modulate the expression of chromatin remodelling factors such as CBP/p300 (Ruas et al., 2010) and to induce epigenetic modifications (Johnson et al., 2008). Since multiple cycles of hypoxia and reoxygenation have been shown to activate HIFs and several survival pathways it might possible that this fluctuation also affects the epigenetic regulation. Therefore, it will be interesting to investigate the role of HIFs in cell survival against oxidative insults not only at the cellular but also at the chromatin level. Given the importance of HIFs in

regulating cell survival and function of transplanted cells or progenitor cells (Francis and Wei, 2010) this could help to discover new potential hES cell treatments for ischemic tissue injury by manipulating HIFs. Indeed, the establishment of environmental signals driven by HIFs and their effects on chromatin structure, gene expression and protein function will lead to the identification of specifically tailored epigenetically active drugs that might promote angiogenesis and tissue repair.

5.1.4 Study Aims

The aim of this study is to determine the effect of hypoxia followed reoxygenation on the regulation of hES cell self-renewal.

The specific aims were:

- To analyse the effect of reoxygenation on the mRNA expression levels OCT4, SOX2 and NANOG in hES cells RT-qPCR will be performed
- To investigate the role of HIF-2α in the regulation of pluripotency genes in hypoxia/reperfusion conditions RT-qPCR and ChIP assays will be performed
- To analyze the epigenetic signature of the HRE site within OCT4, SOX2 and NANOG proximal promoter in hES cells subjected to 72 hours reoxygenation, ChIP assays will be performed.

5.2 Material and Methods

Hues-7 hES cells were cultured following the procedure described in Materials and Methods section 2.1.4. For reoxygenation experiments, hES cells were cultured on Matrigel plates and maintained for a minimum of three passages at 5% oxygen before being transferred to 20% oxygen and cultured for an additional 24, 48 or 72 hours.

See Methods Section 2.4 for Jumanji probes (Table 2.5) and Method Section 2.7.3 for oct-sox probes (Table 2.10).

5.3 Results

5.3.1 Hypoxia followed by Reoxygenation affects hES cell pluripotency

To analyze the effect of hypoxia followed by reoxygenation, hES cells were maintained at 5% oxygen on Matrigel for a minimum of three passages and then transferred to 20% oxygen for either 24, 48 or 72 hours respectively.

5.3.1.1 Morphological characterization

After 24 hours of culture under reoxygenation conditions, hES cells formed sparse and loose colonies with irregular borders (Figure 5.1). This is potentially due to the rapid exposure to the oxidative stress. After 48 hours of reoxygenation, colonies appear more compact and larger with some colonies displaying spontaneous differentiation that resemble the feature of hES colonies cultured at 20% oxygen (Figure 5.1). When hES cells were exposed to 72 hours of reoxygenation, colonies were larger and highly compact (Figure 5.1). Overall, hES cells cultured under hypoxia followed by reoxygenation present different morphology when compared to hES cells cultured at 5% oxygen which are characterized by highly compact colonies with clearly defined borders and highly cells compaction (Figure 5.1).

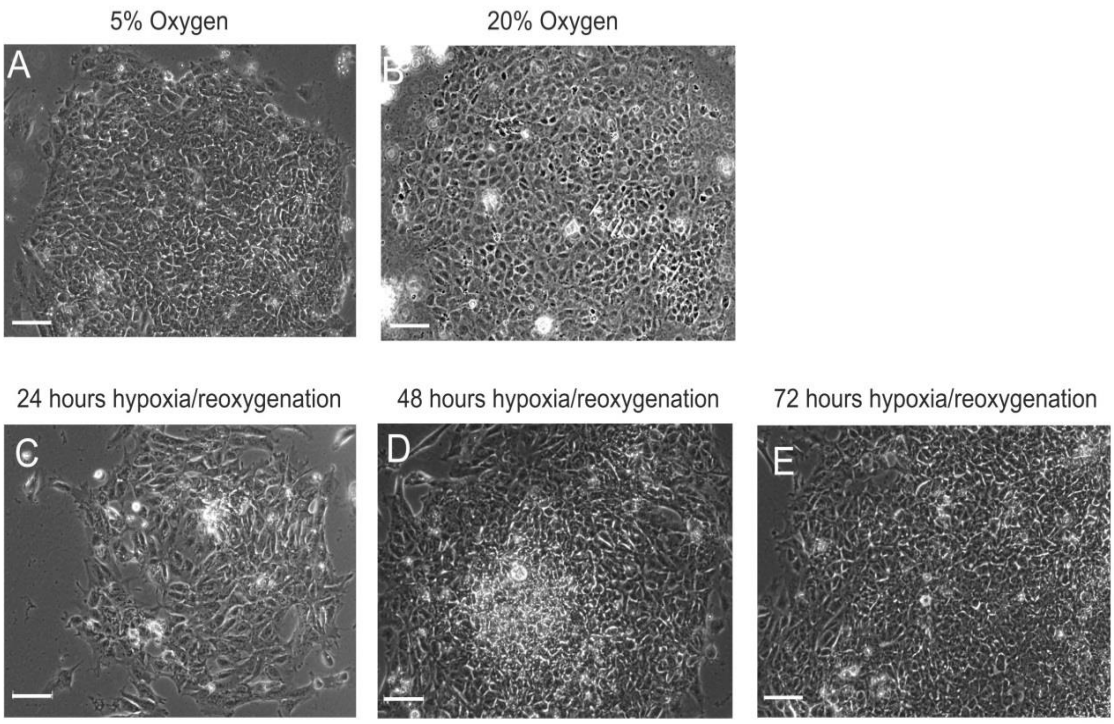

Figure 5.1: hES cell colony morphology of hES cells exposed to hypoxia followed reoxygenation on matrigel coated plates

Representative phase contrast images of hES cell colonies cultured at (A) 5% oxygen (B) 20% oxygen. hES cells maintained at 5% oxygen for a minimum of 3 passages before being cultured at 20% oxygen for 24h (C), 48 (D), and 72 (E). Scale bar = 100μm.

5.3.1.2 Pluripotency marker expression

To investigate whether hypoxia following reoxygenation affected OCT4, SOX2 and NANOG expression, RT-qPCR was performed.

There was a significant 5-fold increase ($P<0.01$) in *NANOG* mRNA expression after 24 h of reoxygenation compared to hES cells cultured at 20% oxygen (Figure 5.2 A). This significant increase was maintained at 7-fold ($P<0.01$) and 8-fold ($P<0.001$) followed by 48h and 72h of reoxygenation respectively (Figure 5.2 B and C) and compared to hES cells cultured at 20% oxygen. *OCT4* mRNA showed a more gradual but significant increase in expression throughout the reoxygenation exposure (Figure 5.2 A, B, C). In particular, there was a significant 2-fold increase ($P<0.001$) of *OCT4* expression after 24h of reoxygenation and compared to hES cells cultured at 20% oxygen (Figure 5.2 A). This expression was sustained at a 3-fold ($P<0.001$) and 4-fold ($P<0.01$) increase after 48h and 72h of reoxygenation respectively (Figure 5.2 B and C) compared to hES cells cultured at 20% oxygen. In contrast, there was no significant difference in *SOX2* expression in cells exposed to hypoxia and reoxygenation compared to those maintained at 20% oxygen (Figure 5.2 A, B, C). Taken together, these results revealed that hES cells exposed to hypoxia followed by reoxygenation maintain an enhanced stemness state through the expression of *OCT4* and *NANOG*. Therefore, these data might suggest that the exposure to an oxidative insult triggers the establishment of an epigenetic memory that sustains pluripotency marker expression when hES cells are transferred from a hypoxic to a normoxic environment.

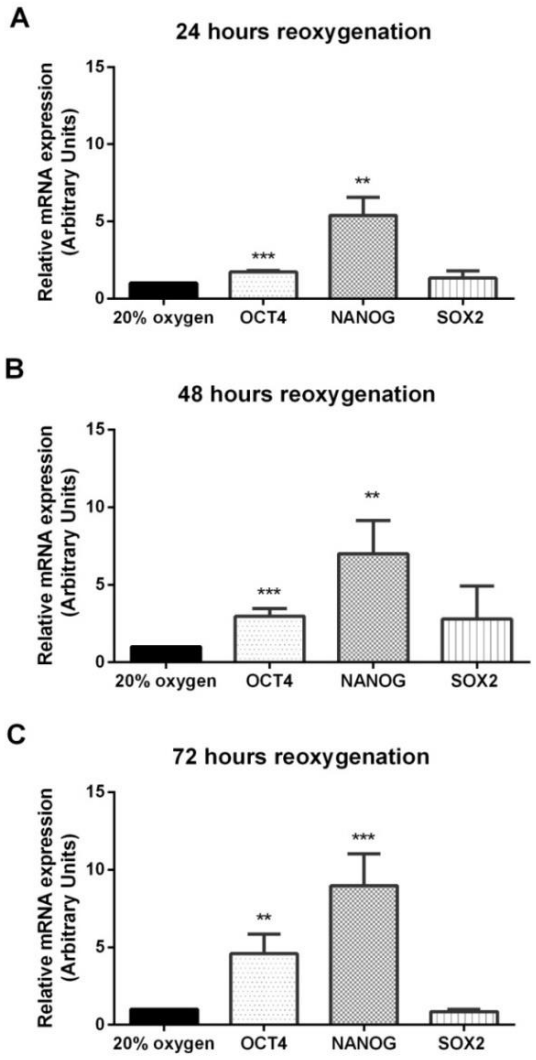

Figure 5.2: Hypoxia and reoxygenation sustain pluripotency gene expression

RT-qPCR analysis of *OCT4*, *NANOG* and *SOX2* mRNA in hES cells maintained at 5% oxygen for a minimum of 3 passages before being cultured at 5% oxygen for (A) 24, (B) 48 and (C) 72 hours compared to those maintained at 20% oxygen. All data have been normalized to *UBC* and to 1 for hES cells maintained at 20% oxygen. Values are mean of 5 independent experiments ± SEM (**P<0.01, ***P<0.001 significantly different from 20% oxygen). The greates error for cells cultured at 20% oxygen was obtained for NANOG which was 1± 0.35.

5.3.2 Exposure to hypoxia/reoxygenation affects histone modifications within the HREs of pluripotency genes

Since exposure to hypoxia followed by reoxygenation induced an increased expression of OCT4 and NANOG, it was considered that the epigenetic status of the cells may also be affected. Therefore, ChIP assays were performed to analyse the histone modification profile within the HRE sites of OCT4, SOX2 and NANOG.

5.3.2.1 Histone Modification analysis

Chromatin of hES cells cultured under hypoxic conditions followed by 72h of reoxygenation was amplified using specific TaqMan probes designed to cover the predicted HRE sites analysed previously in Chapter 3.3.6. Differences in relative chromatin enrichment between hES cells cultured at 5% oxygen followed 72h of reoxygenation were compared to those cultured at either 5% or 20% oxygen. Data were analysed using a Student's *t*-test and presented in bar charts. In contrast, pie charts were used to represent the percentage input precipitated by each modified histone as a proportion of the total, for each gene of interest. The bar charts represent a comparison of individual epigenetic marks while the pie charts depict a more global picture of the chromatin status.

Among the histone modification markers associated with gene activation, reoxygenation did not affected the expression of H3K36me3 and H3K4me3 within the HRE of OCT4 proximal promoter compared to chromatin of hES cells cultured at 20% oxygen (Figure 5.3). However, the proportion of H3K36me3 and H3K4me3 binding in hES cells exposed to reoxygenation was higher than hES cells cultured at 20% oxygen (Figure 5.5). Surprisingly, a significant decrease of H3K9me3, associated with gene silencing, was found within the proximal promoter of OCT4 in hES cells subjected to reoxygenation compared to hES cells cultured at both 20% and 5% oxygen (Figure 5.3 and 5.4). This suggests that cells exposed to hypoxia followed by reoxygenation present a more euchromatic state. When the histone modification markers in hES cells exposed to reoxygenation were compared to those cultured at 5% oxygen, no significant difference in H3K4me3 and H3K36me3 was observed (Figure 5.4). In contrast, a significant reduction of H3K9me3 was found in the OCT4 and SOX2A HREs in hES

cells subjected to reoxygenation compared to those cultured at 5% oxygen (Figure 5.4). This data revealed that the chromatin conformation within the CR3 under reoxygenation is more open relative to normoxia and similar to that observed under hypoxia (Figure 5.5). This may be due to an increased demethylation of H3K9me3 in hypoxia followed by reoxygenation.

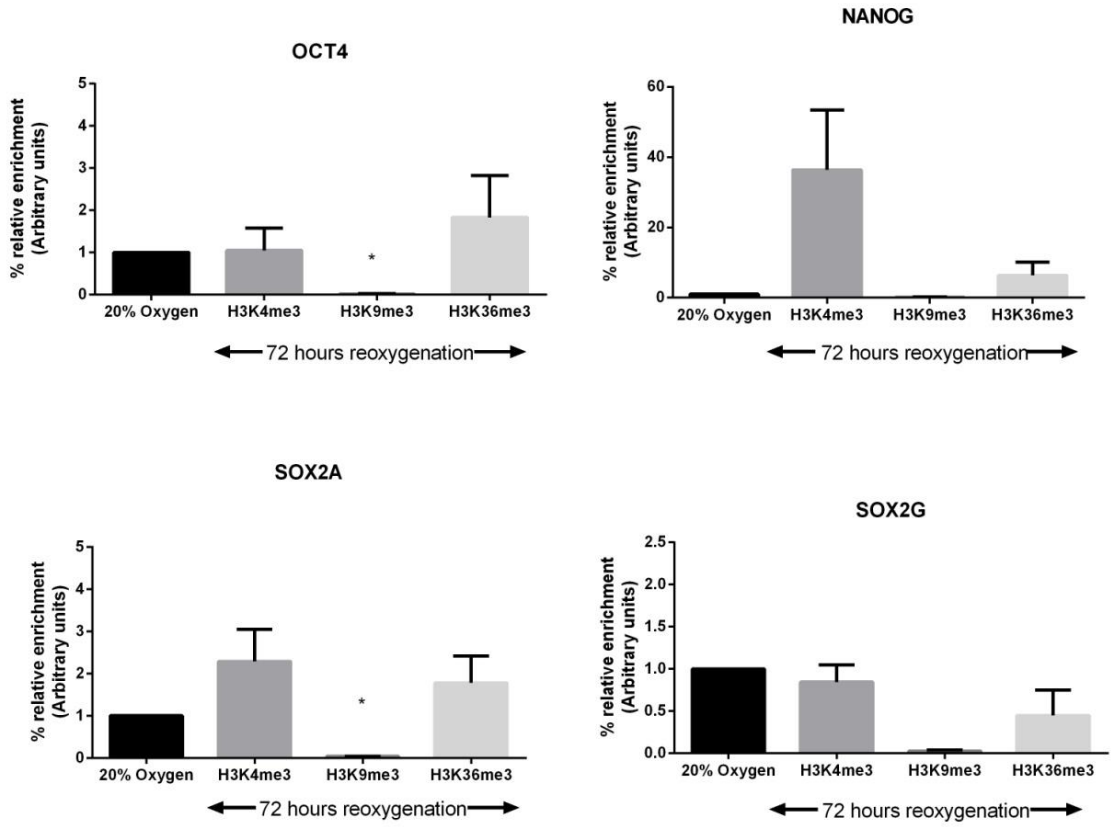

Figure 5.3: Histone modifications induced within the HRE of OCT4, NANOG and SOX2 genes in hES cells cultured in hypoxia and following reoxygenation

ChIP assays of H3K4me3, H3K9me3 or H3K36me3 histone modification markers on chromatin isolated from hES cells maintained at 5% oxygen for a minimum of 3 passages before being cultured for 72 hours at 20% oxygen. Data have been normalized to hES cells cultured at 20% oxygen. DNA enrichment is expressed as a percentage of input minus the background IgG. An average of 3 to 4 independent experiments is represented ± SEM (*P<0.05). The greatest error for cells cultured at 20% oxygen was obtained for H3K9me3 in all the genes of interest which was 1±0.99.

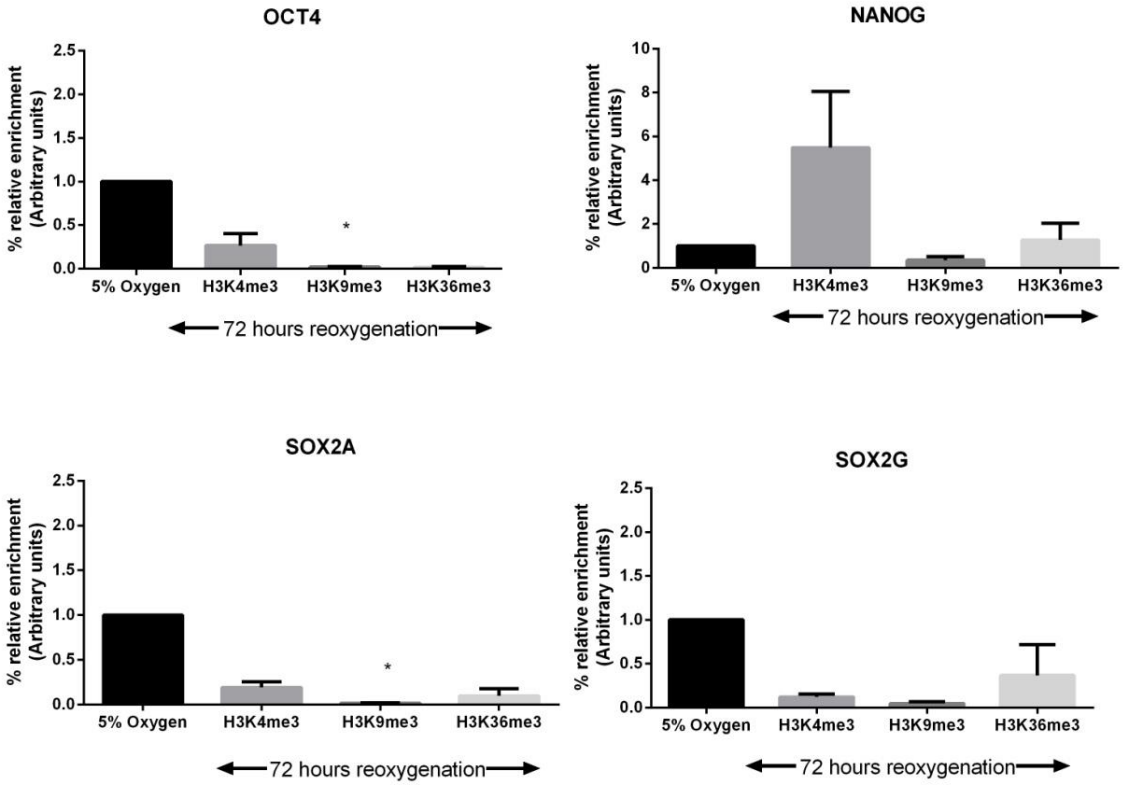

Figure 5.4: Histone modification analysis of hES cells cultured in hypoxia and following reoxygenation

ChIP assays of H3K4me3, H3K9me3 or H3K36me3 histone modification markers on chromatin isolated from hES cells maintained at 5% oxygen for a minimum of 3 passages before being cultured for 72 hours at 20% oxygen. Data have been normalized to hES cells cultured at 5% oxygen. DNA enrichment is expressed as a percentage of input minus the background IgG. An average of 3 to 4 independent experiments is represented ± SEM (*P<0.05). The greatest error for cells cultured at 5% oxygen was obtained for H3K36me3 in SOX2A which was 1±0.9.

SOX2A showed no differences in the expression of H3K4me3 and H3K36me while a significant decrease of H3K9me3 histone modification marker was found when compared to hES cells cultured at 20% oxygen (Figure 5.3). In contrast, SOX2G showed no significant differences in the levels of H3K4me3, H3K36me3 and H3K9me3 in hES cells exposed to reoxygenation when compared to normoxia (Figure 5.3). However, a trend towards a reduction of H3K9me3 was found within SOX2G which indicated a more open chromatin conformation compared to cells exposed to normoxia (Figure 5.3). When the chromatin state within SOX2G was compared to hES cells cultured at 5% oxygen, no differences were found in all the histone modifications markers of hES cells followed by 72h of reoxygenation (Figure 5.4). However, due to the lowest relative proportion of H3K9me3 in hES cells cultured in hypoxia followed by reoxygenation, the chromatin state was more open when compared to hES cells cultured at either 5% or 20% oxygen (Figure 5.5). This was particularly evident for the HRE within the NANOG promoter, where the percentage relative enrichment of H3K4me3 and H3K36me3 displayed a 37 and 7 fold increase respectively compared with hES cells cultured at 20% oxygen (Figure 5.3). A trend towards an increased enrichment in H3K4me3 histone modification was also observed when reoxygenated cells were compared to hES cells cultured at 5% oxygen, while H3K36me3 was unchanged (Figure 5.4). A dramatic decrease of H3K9me3 was found within the HRE of the NANOG promoter when compared to either chromatin of hES cells cultured at 20% oxygen or at 5% oxygen (Figure 5.3 and 5.4). This indicated that the chromatin state within the NANOG HRE in hypoxia followed by reoxygenation possessed a more open conformation compared with hES cells cultured at either 20% or 5% oxygen (Figure 5.5).

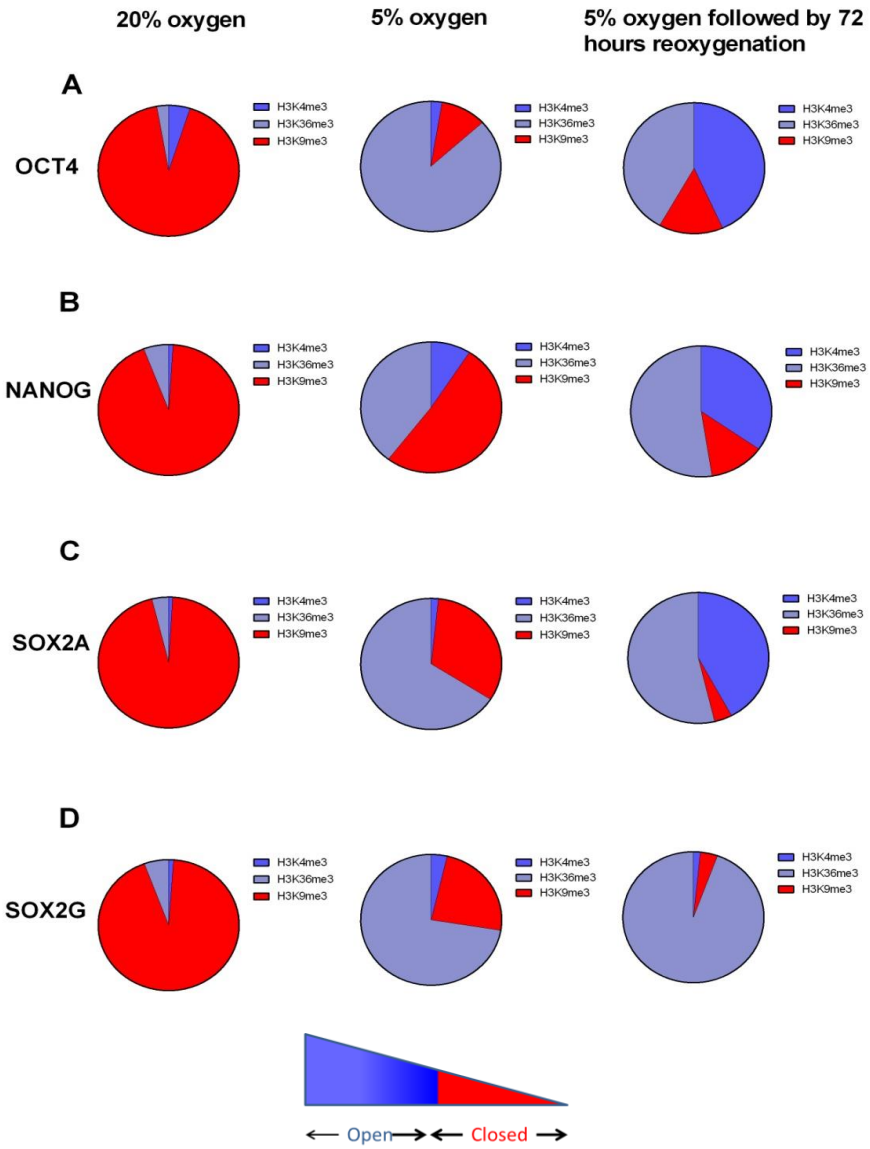

Figure 5.5: Hypoxia and reoxygenation enhance the expression of pluripotency genes through a euchromatic chromatin conformation within the HRE site

Pie charts showing ChIP analysis of histone modification markers H3K4me3, H3K36me3 and H3K9me3 binding a predicted HRE site in the proximal promoter of OCT4 **(**A), NANOG (B), SOX2A (C) and SOX2G (D) in hES cells cultured at either 5% oxygen, 20% oxygen or 5% oxygen followed by 72hours at 20% oxygen respectively. DNA enrichment is expressed as a percentage of input (non-immunoprecipitated chromatin). An average of 3 to 4 independent experiments is represented.

Taken together, these data highlight the presence of a more open chromatin conformation when hES cells are exposed to hypoxia/reoxygenation for 72 hours compared to hypoxia or normoxia. This is the first demonstration of a specific "reoxygenation signature" in hES cells which is characterized by a prevalence of the active markers H3K4me3 and H3K36me3 and a lower expression of the silencing marker H3K9me3 (Figure 5.5). These data suggest that cycles of hypoxia followed by reoxygenation mediate chromatin modifications which result in enhanced gene expression and sustained pluripotency.

5.3.2.2 Histone demethylase expression in hES cells following hypoxia-reperfusion

Histone methylation is a modification that could be influenced by environmental changes such as oxygen fluctuations thereby affecting gene expression. To evaluate whether long term exposure to hypoxia followed reoxygenation have an effect on the regulation of the JmjC domain proteins in hES cells, RT-qPCR analysis was performed.

It was found that *JMJD1a* mRNA expression was constantly low throughout the reoxygenation period and significantly reduced ($P<0.001$) after 24h and after 72h ($P<0.01$) of reoxygenation when compared to 5% hES cells (Figure 5.6). There was no significant difference in *JMJD2b* expression after 24h and 48h of reoxygenation but, was significantly decreased after exposure to 72h of reoxygenation compared to hES cells cultured at 5% oxygen (Figure 5.7). These data suggest that *JMJD1a* and *JMJD2b* expression are affected by the time post reoxygenation. The only Jumanji that displayed a trend towards an increase after 24h, of 48h and 72h of reoxygenation when compared to hES cells cultured at 5% oxygen was *JMJD2c* (Figure 5.6). However, since oxygen fluctuations and cell type specificity might have an effect on the expression of different demethylases, this data should be considered with caution and will require further investigations.

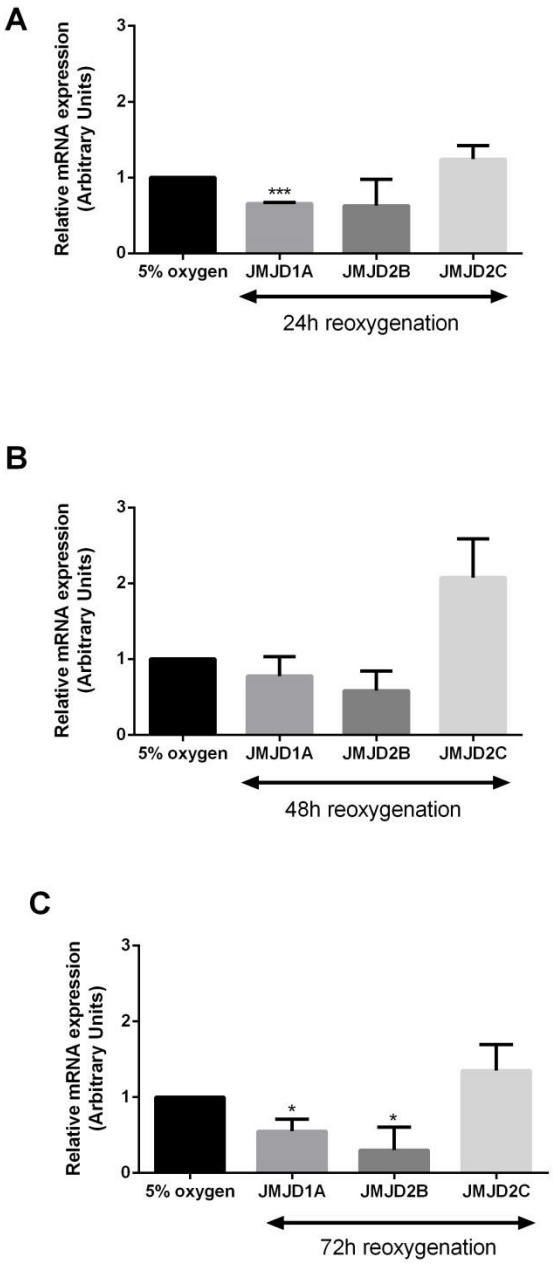

Figure 5.6: Jumanji histone demethylase expression in hES cells exposed to hypoxia and reoxygenation

RT-qPCR analysis of *JMJD1a*, *JMJD2b* and *JMJD2c* in hES cells maintained at 5% oxygen for a minimum of 3 passages before being cultured for 24h (A), 48h (B) and 72h (C) at 20% oxygen. All data have been normalized to *UBC* and to 1 for 5% oxygen. Values are mean of 3 to 4 independent experiments ± SEM. The greatest error for cells cultured at 5% oxygen was obtained for *JMJD1a* which was 1±0.1.

5.3.3 Role of HIF-2α in the regulation of OCT4, SOX2 and NANOG in hypoxia followed by reoxygenation

ChIP results presented in this chapter were intriguing and demonstrated that exposure to hypoxia followed by reoxygenation influence the chromatin state within the HRE site of OCT4, SOX2 and NANOG. Since data presented in Chapter 3 revealed that HIF-2α binds to the HREs within the proximal promoter of pluripotency genes, it was considered that this transcription factor might also be involved in the epigenetic changes observed when hES cells were exposed to reoxygenation.

5.3.3.1 HIF-2α mRNA expression in hES cells cultured under hypoxia and followed by reoxygenation

RT-qPCR analysis was performed in order to quantify the mRNA expression of *HIF-2α* in hES cells cultured after 24, 48 and 72 hours of hypoxia followed reoxygenation respectively. *HIF-2α* mRNA displayed a significant 4-fold ($P<0.05$) increase in expression after 24h of reoxygenation compared to hES cells cultured at 20% oxygen (Figure 5.7). A similar, 5-fold ($P<0.05$) and 4-fold ($P<0.05$) increase was observed following 48h or 72h post-reoxygenation respectively compared to hES cells cultured at 20% oxygen (Figure 5.7).

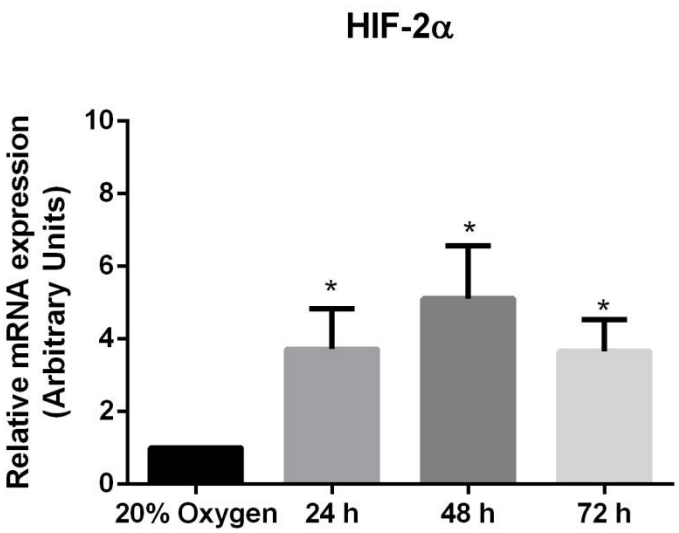

Figure 5.7: *HIF-2α* mRNA expression is increased in hES cells cultured in hypoxia followed by reoxygenation

RT-PCR of *HIF-2α* mRNA expression in hES cells maintained at 5% oxygen for a minimum of 3 passages following by either 24, 48 or 72 hours at 20% oxygen. All data have been normalized to *UBC* and to 1 for hES cells cultured at 20% oxygen. Values are mean of 6 independent experiments ± SEM (*P<0.05). The error for *HIF-2α* at 20% oxygen was 1±0.05.

Interestingly, when the expression of *HIF-2α* following reoxygenation was compared to that expressed in hES cells maintained at 5% oxygen there was no significant difference (Figure 5.8). These data were intriguing and may suggest that HIF-2α is post-transcriptionally regulated in reoxygenation conditions. Potentially, a post transcriptional regulation might sustain high HIF-2α protein levels following ischaemia preconditioning in order to maintain the expression of self-renewal markers.

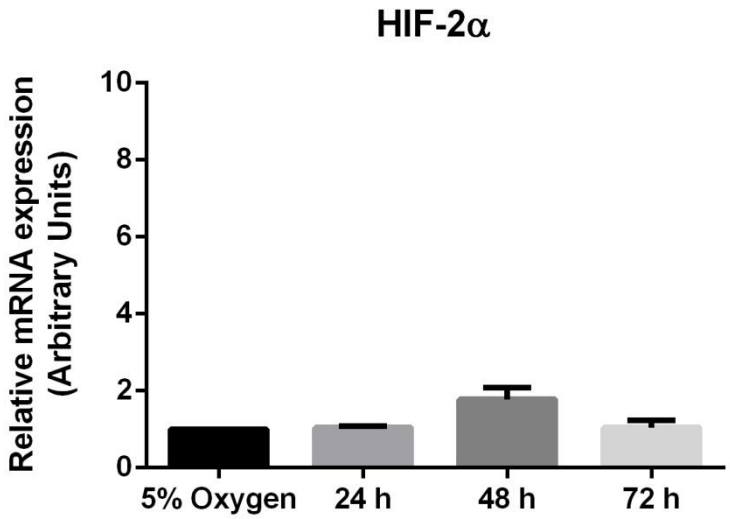

Figure 5.8: *HIF-2α* mRNA expression remains unaltered in hES cells cultured in hypoxia followed by reoxygenation

RT-PCR of *HIF-2α* mRNA expression in hES cells maintained at 5% oxygen for a minimum of 3 passages followed by culture at for 24, 48 or 72 hours at 20% oxygen. All data have been normalized to *UBC* and to 1 for hES cells cultured at 5% oxygen. Values are mean of 3 independent experiments ± SEM (*P<0.05). The error for *HIF-2α* at 5% oxygen was 1±0.06.

5.3.3.2 HIF-2α ChIP on hES cells subjected to reoxygenation

It was next considered that nuclear HIF-2α may not be degraded following reoxygenation but instead immunoprecipitate the chromatin within the HRE of all core pluripotency genes and could be responsible for these epigenetic changes. To determine whether HIF-2α protein directly interacts with OCT4, SOX2 and NANOG HREs in hES cells cultured at 5% oxygen followed by 72h at 20% oxygen, ChIP analysis was performed.

Among all the HIF-2α immunoprecipitated samples, a significantly enrichment ($P<0.001$) was found in the HRE within the NANOG promoter compared to the chromatin of hES cells cultured at 20% oxygen (Figure 5.9 A). This significant increase was observed also within the HRE of OCT4 and SOX2A ($P<0.05$) and SOX2G ($P<0.01$) when compared with chromatin of hES cells cultured at 20% oxygen and immunoprecipitated with HIF-2α antibody (Figure 5.9 A). This was an interesting finding since it revealed that HIF-2α protein was not degraded when hES cells were cultured at 5% oxygen followed by 72h at 20% oxygen. When enrichment of reoxygenated samples was compared to hES cells cultured at 5% oxygen, only HIF-2α binding to the NANOG promoter was significant ($P<0.001$) (Figure 5.9 B). There was no significant binding of HIF-2α to the HRE of OCT4 and SOX2 promoter (Figure 5.9 B). These data revealed that HIF-2α is not degraded after exposure to reoxygenation, but is able to sustain higher NANOG transcriptional activation together with similar levels of OCT4 and SOX2 after 3 days reoxygenation. Therefore, HIF-2α may be responsible for a form of cell protection that enhances NANOG expression and induces a euchromatic state when hES cells are exposed to hypoxia followed by an oxidative insult.

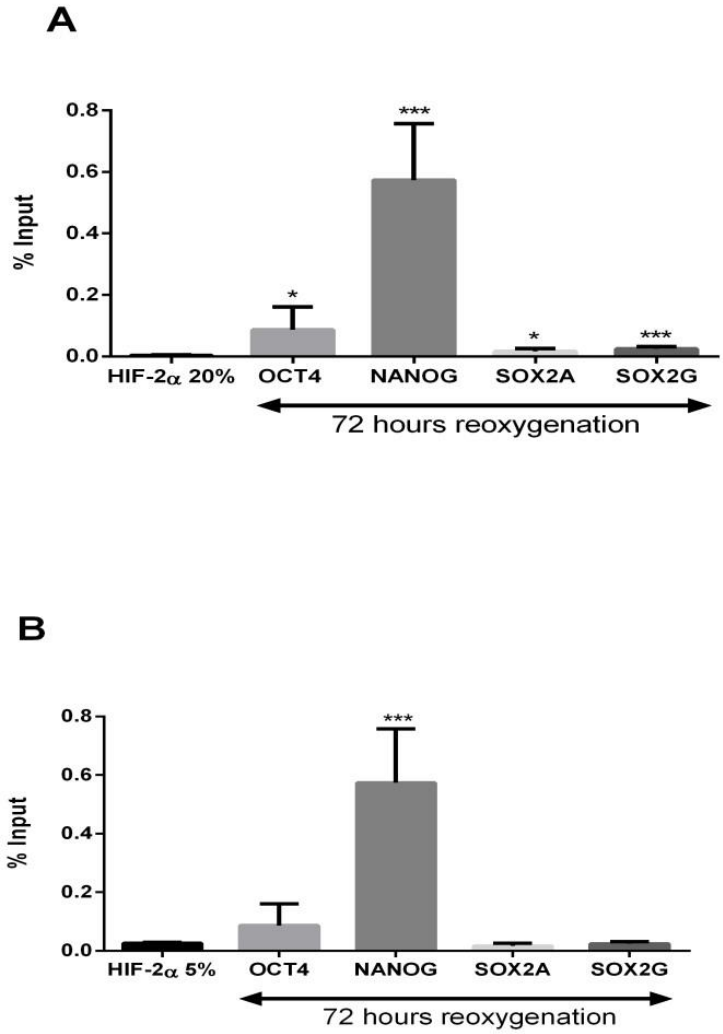

Figure 5.9: HIF-2α sustain the expression of NANOG following hypoxia/reoxygenation

ChIP analysis of HIF-2α binding a predicted HRE site in the proximal promoter of OCT4, NANOG, SOX2A and SOX2G genes on chromatin isolated from hES cells maintained at 5% oxygen for a minimum of 3 passages before being cultured for 72 hours at 20% oxygen. Graphs show HIF-2α binding to the HRE of pluripotency genes at 20% oxygen (A) or 5% oxygen (B) compared to those obtained following reoxygenation. DNA enrichment is expressed as a percentage of input (non-immunoprecipitated chromatin). Values are mean of 3 independent experiments ± SEM (*P<0.05, ***P<0.001).

5.3.4 HIF-2α interacts with an oct-sox *cis* regulatory element within the NANOG promoter

The positive correlation with the active histone marks H3K4me3 and H3K36me3 and the significant binding of HIF-2α to the HRE of NANOG in hES cells exposed to reoxygenation was intrigue and suggested a possible role for HIF-2α in regulating short-range chromatin conformational loops within the NANOG locus.

Using a bioinformatic approach (MatlInspector) to analyze the NANOG proximal promoter, an oct-sox *cis*-regulatory element at -208 bp (TTTGCATTACAATG) from the transcription start site was identified. This element has already been described as being important for the pluripotent stem cell regulatory network (Rodda et al., 2005) and is situated in close proximity (93bp) to the HRE in the NANOG promoter.

Therefore it was speculated that HIF-2α might function as an enhancer by binding to the oct-soc *cis* regulatory element and forming a multiprotein complex that would allow sustained NANOG expression throughout the reoxygenation period. To obtain *in vivo* evidence of this potential *novel* role for HIF-2α, ChIP assays were performed using a specific TaqMan probe that covers the oct-sox element. ChIP analysis revealed no interaction of HIF-2α with the oct-sox element of the NANOG promoter in hES cells cultured at either 5% or 20% oxygen (Figure 5.10 A and B).

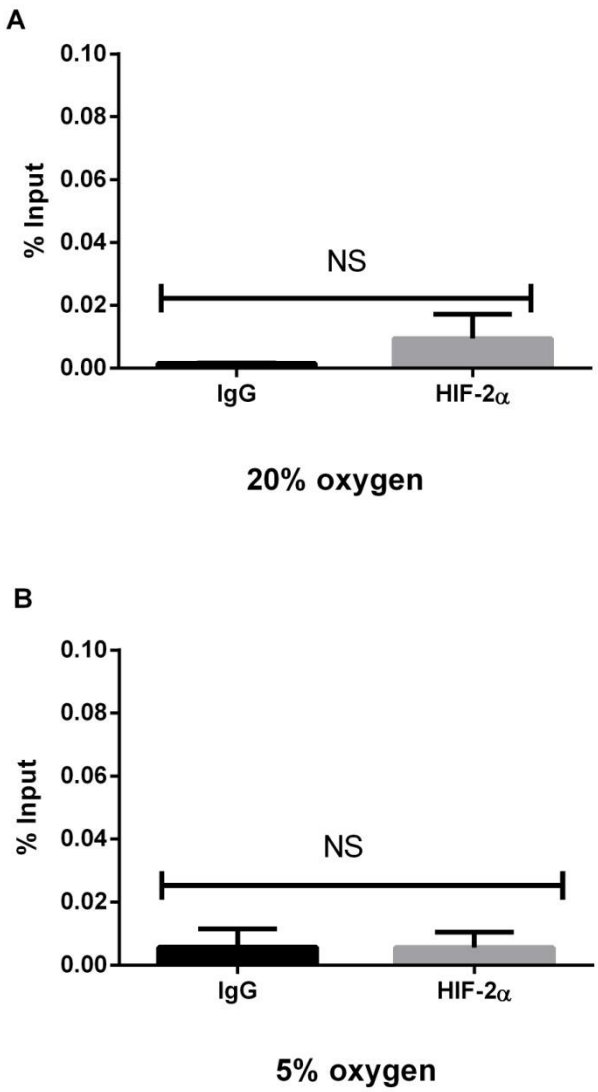

Figure 5.10: HIF-2α does not interacts to the oct-sox *cis* element in NANOG promoter in hES cells cultured either at 20% or 5% oxygen

ChIP analysis of HIF-2α binding the oct-sox *cis*-regulatory element in the NANOG proximal promoter on chromatin isolated from hES cells cultured at either 20% oxygen (A) or at 5% oxygen (B). ChIP assays were performed with an anti HIF-2α or IgG control antibodies. DNA enrichment is expressed as a percentage of Input. Values represent mean of 3 independent experiments is represented ± SEM.

Surprisingly, when hES cells were subjected to hypoxia followed by 72 hours reoxygenation HIF-2α was found to interact significantly with the *cis* oct-sox regulatory element in the proximal NANOG promoter when compared to immunoprecipitation with an IgG control antibody (Figure 5.11 A and B).

Moreover, to further confirm the loop formation and prove that the physical interaction with HIF-2α was not due to an intermediate region binding, a control probe that covers the 93bp sequence between the HRE site and the oct-sox element in the NANOG promoter was designed (Figure 5.12 A). As expected, qPCR on four independent ChIP experiments on chromatin derived from hES cells subjected to hypoxia followed by reoxygenation for 72 hours and immunoprecipitated with HIF-2α revealed a significant 3 fold increase ($P<0.05$) of HIF-2α binding within the oct-sox element compared to the intermediate region and a significant enrichment ($P<0.01$) when compared to the IgG control (Figure 5.11 B). Furthermore, no significant binding of HIF-2α to the intermediate region compared to the IgG control was found (Figure 5.11 B).

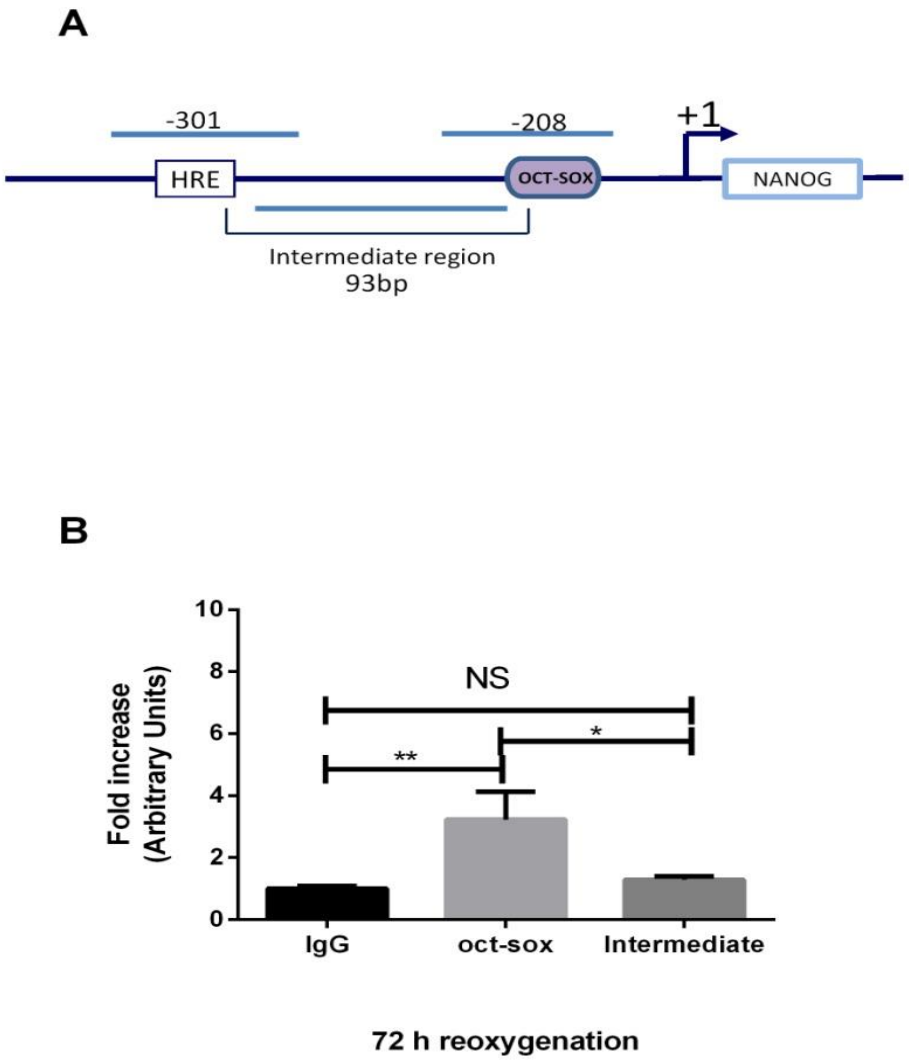

Figure 5.11: Hypoxia/reoxygenation induces HIF-2α interaction to the oct-sox *cis* element in NANOG promoter

Schematic representation of the probes designed to cover the HRE, oct-sox *cis* element and intermediate region on the NANOG proximal promoter (A).

ChIP analysis of HIF-2α binding to the oct-sox-*cis* element and the intermediate region in hES cells maintained at 5% oxygen for a minimum of 3 passages before being cultured for 72 hours at 20% oxygen (B). Values are mean of 4 independent experiments ± SEM (*P<0.05, **P<0.01).

The oct-sox element is a regulatory sequence important for the circuitry of pluripotency interaction (Rodda et al., 2005) and is also present at -2340bp in the OCT4 proximal promoter (Chew et al., 2005; Okumura-Nakanishi et al., 2005) and SOX2 down-stream intron (Tomioka et al., 2002). To investigate whether HIF-2α was also able to interacts with the oct-sox element within OCT4 and SOX2 genes, ChIP assays were performed. Using specific TaqMan probes to cover the oct-sox regulatory element within the OCT4 and SOX2, no interaction was found (Figure 5.12 A and B).

These results confirmed the specificity of HIF-2α binding only in the NANOG proximal promoter.

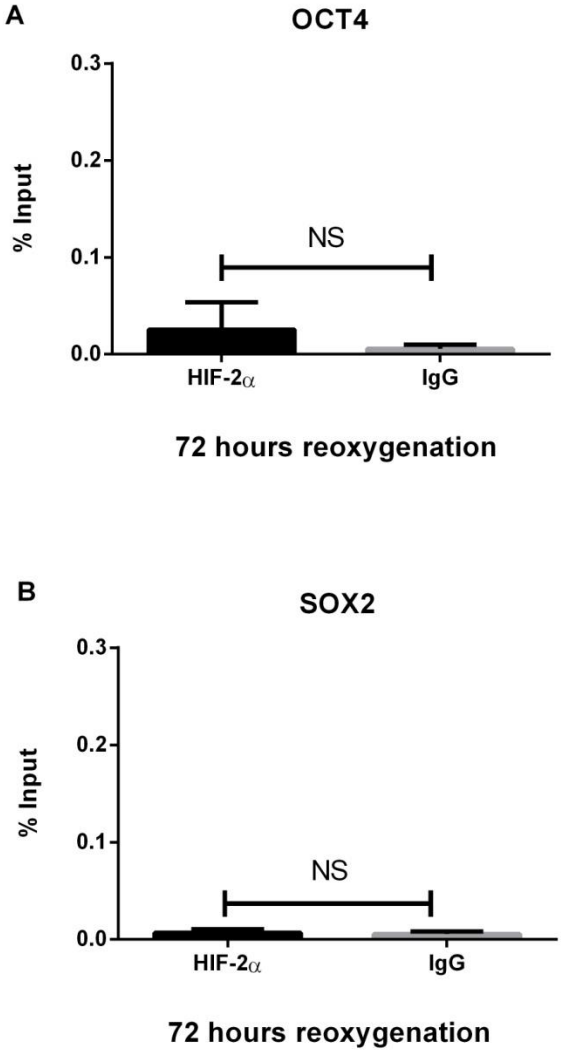

Figure 5.12: HIF-2α does not interacts with the oct-sox element within OCT4 and SOX2

ChIP analysis of HIF-2α binding the oct-sox *cis*-regulatory element in OCT4 proximal promoter (A) and SOX2 intron (B) on chromatin isolated from hES cells maintained at 5% oxygen for a minimum of 3 passages before being cultured for 72 hours at 20% oxygen. ChIP assays were performed with an anti-HIF-2α or IgG control antibody. DNA enrichment is expressed as a percentage of input (non-immunoprecipitated chromatin). Values are mean of 3 independent experiments ± SEM.

These results revealed for the first time that both the HRE and oct/sox binding sites are in physical contact with HIF-2α when hES cells are exposed to reoxygenation. This suggests the formation of a multiprotein complex that might allow the formation of a high order chromatin conformational loop in the NANOG promoter where HIF-2α may function as an enhancer to increase the expression of NANOG under conditions of reoxygenation.

5.4 Discussion

Increasing evidence suggests that resident stem/progenitor cell populations are able to promote survival in response to oxidative stress and repopulate damaged tissues. However the precise "stemness" feature which promotes stem cell survival and regeneration is not fully understood. Data in this thesis highlights that hES cells exposed to hypoxia and reoxygenation maintain their stemness through a *novel* mechanism of epigenetic regulation whereby HIF-2α enhances NANOG expression to protect cells from oxidative stress.

5.4.1 Effect of reoxygenation on hES cell self-renewal

To mimic the effect of ischemia-reperfusion, hES cells were subjected to 72 hours of reoxygenation post hypoxia. After 24 hours of hypoxia/reoxygenation it was possible to observe changes in hES cell morphology. As a consequence of the oxidative stress exposure after being at 5% oxygen for more than 3 passages, cells appeared sparse and irregular but gradually acquired the typical feature of a hES colony cultured at 20% oxygen (Forristal et al., 2010). Indeed, after 48 hours colonies were more compact but presented some areas of differentiation whereas after 72 hours of reoxygenation hES cells were large with distinct boarders and some small areas of differentiation. Overall it was noticed that hES cells exposed to hypoxia reoxygenation appeared more confluent on the plate and seemed to proliferate faster than hES cells cultured at 20% oxygen although measurement of cell proliferation using Ki67 labelling would be required to validate this observation. However, the apparent increase in proliferation may be due to the significant increase in NANOG mRNA expression when hES cells were exposed to hypoxia followed reoxygenation since NANOG has been found to upregulate cell cycle factors such as CDK6 and CDC25A (Zhang et al., 2009). OCT4 expression levels were also found significantly higher when hES cells were exposed to reoxygenation compared to normoxia and suggest that in the reoxygenation environment hES cells maintain pluripotency and increase proliferation. These results are in agreement with recent data which suggests that intermittent hypoxia enhances the stem-like

characteristics in neuroblastoma tumors resulting in an immature-neural crest phenotype and decreased differentiation (Bhaskara et al., 2012).

In contrast, SOX2 was found constantly decreased when hES cells were moved from hypoxia to normoxia compared to hES cells cultured at 20% oxygen suggesting that the oxidative insult does not affect the expression of this pluripotency marker.

5.4.2 Effect of reoxygenation on hES cell chromatin modifications

Having demonstrated that the chromatin of hES cells is able to act as an oxygen sensor and to maintain a euchromatic state under hypoxia and a heterochromatic state under normoxia (Chapter 4), it was interesting to determine whether hypoxia followed by reoxygenation was able to affect the chromatin state within the HRE of the OCT4, SOX2 and NANOG proximal promoter. Histone modification analysis showed an increased expression of H3K4me3 and H3K36me3 histone modifications associated with gene activation in hES cells exposed to reoxygenation for 72 hours when compared to hES cells cultured at 20% oxygen while a dramatic reduction of H3K9me3, a marker associated with gene silencing, was found within the OCT4 and SOX2 gene promoter. Reoxygenation induced also an increased expression of H3K4me3 and H3K36me3 when compared to hypoxia. In particular, the HRE in the NANOG proximal promoter was enriched for H3K4me3 when compared to both 20% and 5% oxygen and indicates that the NANOG gene is more active compared to OCT4 and SOX2. When compared to hES cells cultured at 5% oxygen, H3K9me3 was found dramatically decreased in all pluripotency genes analyzed and reflect a more active chromatin state. This was intriguing as it suggests that reoxygenation may inhibit the activity of G9a methyltransferases or induce an elevation of Pol II complex recruitment to the transcribed regions. Indeed, in Human vein endothelial cells (HUVEC) the proximal promoters and enhancers of eNOS were characterized by differentially modified histones and the Pol II complex is highly enriched and correlates with enhanced transcriptional activity (Fish et al., 2005). Therefore, data presented in this thesis are the first demonstration of a specific epigenetic signature in hES cells subjected to reoxygenation and indicate that exposure to oxidative stress following hypoxia allows chromatin to be in a more open conformational state sustaining hES cell self-renewal.

5.4.3 Effect of reoxygenation on histone demethylases

Modifications of the N-terminal tails of the histones by methyltransferases is a post-transcriptional regulation that affect chromatin structure as well as transcriptional activation and repression (Shi and Whetstine, 2007). The Jumonji domain proteins rely on the presence of 2-oxoglutarate and oxygen to mediate changes in histone methylation (Tsukada et al., 2006). Previous studies have found that Jmjd1a and Jmjd2c are targets of Oct4 in mouse ES cells (Loh et al., 2007). In particular, Jmjd2c has also been found to be involved in regulating the euchromatic state of the Nanog gene in order to maintain self-renewal during normoxia (Loh et al., 2007). However, the influence of hypoxia and reoxygenation on the pluripotent state of hES cells is still not known but the fluctuation in oxygen availability may affect the Jumanji proteins. Results presented in this chapter demonstrated that reoxygenation effects JMJD1a and JMJD2b as the expression of these demethylases were found significantly decreased, after 72h of reoxygenation. This implies that the length of reoxygenation time affects Jumanji expression. In contrast, JMJD2c showed a trend towards an increased expression throughout the reoxygenation period. Previous data on mouse ES cells showed a specific role for Jmjd2c in the maintenance of Nanog demethylation in normoxia (Loh et al., 2007). However, since JMJD2c was not significantly expressed after 72h reoxygenation this could indicate the presence of other mechanisms of chromatin demethylation downstream from JMJD2c in the manteinance of the NANOG euchromatic state. Overall, these data are difficult to interpret as the biological effects of the Jumonji domain proteins are not fully understood but it is possible to speculate that exposure to hypoxia followed reoxygenation might inhibit the functionality of these demethylases since they are regulated by HIFs in different cell types at 1% oxygen (Luo et al., 2012; Pollard et al., 2008; Tsukada et al., 2006). Therefore, the specific expression of the Jumonji domain containing demethylases might be critically affected by the ischemic insult, the target gene, or the cell type.

5.4.3 Effect of HIF-2α on hES cells following hypoxia-reoxygenation

It is widely recognized that HIFs affect the cellular and adaptive responses to hypoxia and ischemia (Semenza, 2000a), so it was considered that HIF-2α might be implicated for the epigenetic changes observed within the HRE of OCT4, SOX2 and NANOG. Interestingly, HIF-2α mRNA was found to be significantly increased in hES cells exposed to hypoxia followed by 24, 48 and 72h of reoxygenation when compared to cells cultured at 20% oxygen. This was intriguing and suggested that HIF-2α is regulated also at the transcriptional level which is in contrast to previous published data that demonstrated post transcriptional regulation (Wenger et al., 1997). Since it is known that the long term exposure to hypoxia in hES cells is mediated by HIF-2α (Forristal et al., 2010) data presented in this thesis could also highlight a new role of HIF-2α in the adaptation to reoxygenation.

To investigate whether endogenous HIF-2α was able to bind *in vivo* the proximal promoter of OCT4, SOX2 and NANOG in hES cells exposed to hypoxia followed by 72 hours of reoxygenation ChIP assays were performed. Interestingly, HIF-2α was found to be significantly enriched within the HRE of all core pluripotency genes but particularly within the NANOG gene promoter. This finding was intriguing since HIF-2α was not degraded following reoxygenation and is contrary to previously published data where HIF-1α was lost after only 1 hour of reoxygenation (D'Angelo et al., 2003; Jewell et al., 2001). The current data suggests that HIF-2α may be stabilized due to either a different mechanism of degradation, post-transcriptional modifications or altered activities of PHDs.

Recently low p53 and high HIF-2α levels have been associated with increased NANOG expression upon reoxygenation and with an enhanced stemness state characterized by high levels of Glutathione (GSH) which is associated with cytoprotection (Das et al., 2012). However, the results presented in this chapter extend this finding and implicate epigenetic modifications driven by HIF-2α in the promotion of NANOG activity, a mechanism which likely protects cells from oxidative stress. Interestingly, we suggest that HIF-2α contributes to a novel chromatin conformational change during

reoxygenation through the interaction with an oct-sox *cis*-regulatory element in the NANOG proximal promoter. The current data shows for the first time that the HRE and the oct-sox element of the NANOG promoter are in close proximity only when hES cells are subjected to hypoxia followed by reoxygenation. This interaction may lead the chromatin to form a tight loop on the HRE site bringing together HIF-2α with OCT4 and SOX2 on the NANOG promoter and enhancing NANOG expression when cells were exposed to hypoxia and oxidative stress. In contrast, this chromatin conformation is not present under hypoxic or normoxic conditions. Nevertheless, it could also be possible that another HIF-2α molecule interacts with the oct-sox element through the presence of ancillary factors thereby leading to an increased transcription of NANOG only under reoxygenation. However, since HIF-2α is not degraded under reoxygenation, a tight loop conformation may protect HIF-2α from degradation maintaining its nuclear localization and sustaining NANOG expression.

The oct-sox element is functionally important for NANOG expression and establishing a pluripotent phenotype in SCs (Kuroda et al., 2005; Rodda et al., 2005) but the presence of OCT4 and SOX2 on this element are not the only signals that allow the pluripotent-specific expression of NANOG. Data in this chapter demonstrated that HIF-2α is recruited as a co-activator to the oct-sox element under reoxygenation, subsequently changing the chromatin conformation suggesting a possible role for HIF-2α in the core circuitry of pluripotency.

This finding might include HIF-2α in the pluripotency-specific chromatin "interactome" that has recently been discovered in Nanog in ES cells and iPSCs (Apostolou et al., 2013). This interactome seems to sustain the pluripotency state and will help to establish a role for HIF-2α-induced chromatin modifications and the regulation of high order chromatin structure in reoxygenation. This 3-D conformation might also function to resist differentiation in the reoxygenation environment to protect hES cells from oxidative stress and acquire a high degree of stemness that could increase the regenerative potential.

Therefore, data presented in this thesis propose a novel mechanism of epigenetic regulation in hES cells whereby HIF-2α forms a multiprotein complex together with OCT4 and SOX2 and other chromatin remodelling factors to enhance NANOG

expression. This finding is likely to be of therapeutic potential and may explain the higher degree of stemness observed not only in hES cells but also in cardiac progenitor cells following hypoxia/reoxygenation (Das et al., 2012; Ivanova et al., 2002; Martin et al., 2008; Ramalho-Santos et al., 2002). Based on these findings, a hypoxic environment is preferential for the stimulation of an HIF-2α-HRE system that maintains a population of highly pluripotent hES cells that can enhance the degree of stemness when cells are exposed to hypoxia followed by oxidative stress.

The epigenetic mechanisms observed in this thesis may form part of a molecular program characteristic of resident stem/progenitor cell populations that repopulate and promote survival during the post-injury stress or in a wide range of diseases associated with ischemia and reoxygenation.

Chapter 6

Discussion and Future work

6.1 Discussion

The work completed in this thesis provides genetic and biochemical evidence that endogenous HIF-2α binds directly to HREs in the proximal promoter of OCT4, NANOG, SOX2, GLUT1 and eNOS in hES cells cultured under long term exposure to hypoxia but not at atmospheric oxygen tensions. These findings have an important impact on hES cell maintenance in terms of cell self-renewal, glucose metabolism and NO signalling and suggest that a hypoxic environment is beneficial for the culture of a highly pluripotent population of cells.

It has also been shown that changes in gene expression upon hypoxia are associated with specific histone modifications, increased histone demethylase activity and decreased DNA methylation. The interplay between these modifications and HIF-2α influence the chromatin architecture leading to an enhanced expression of HIF-2α target genes only under hypoxic conditions (Figure 6.1). These mechanisms allow hES cells to adapt to the hypoxic environment and to acquire increased resistance to oxidative stress. Indeed, the identification of environmental signals that allow chromatin regions to be "active" or "inactive" during hES cell commitment will lead to a better understanding of the self-renewal state and thus improved regenerative potential.

The presence of a "hypoxia signature" driven by HIF-2α in hES cells could have important implications not only in cancer stem cell self-renewal and proliferation but also for iPS cell biology. Indeed, this chromatin signature via HIF-2α could be manipulated to increase the expression of stem cell markers used to generate iPS cells from differentiated somatic cells. A better understanding of the epigenetic modifications and molecular mechanisms driven by the hypoxic environment and HIF-2α in hES cells, could also be used to manipulate cell fate, differentiation and lineage reprogramming or transdifferentiation which could have useful applications for gene therapy and regenerative medicine.

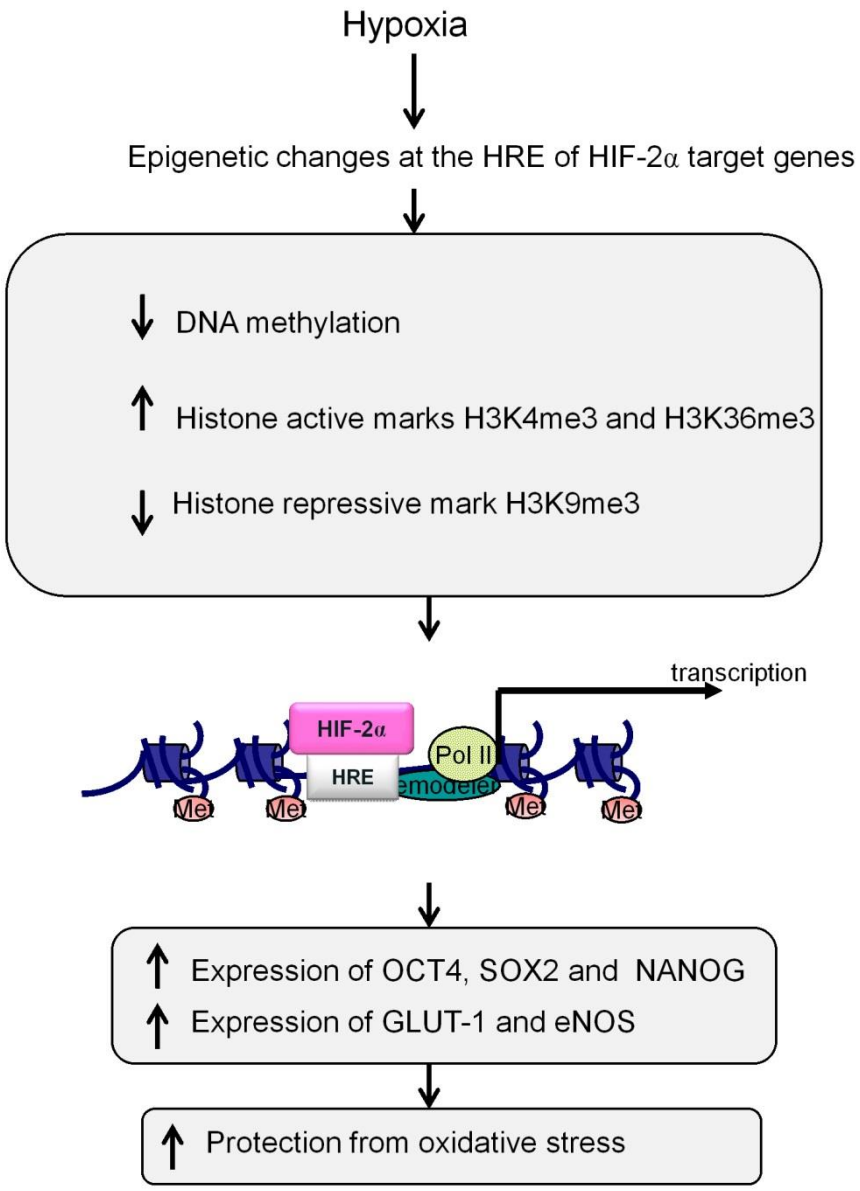

Figure 6.1: Schematic representation of the epigenetic changes that occur at the HRE of HIF-2α target genes which lead to alterations in gene expression

This thesis was also focused on the effect of hypoxia followed by reoxygenation in the regulation of hES cell pluripotency. The aim of this study was to provide a model for understanding how SCs are regulated in the site of injury which is characterized by hypoxia and ROS production. Indeed, a recent study demonstrated that after exposure to hypoxia early committed SCs are prone to assume a phenotype that is similar to hES cells, with an increased capacity for proliferation, differentiation and ability to de-differentiate (Mathieu et al., 2013). These findings suggest that exposure to hypoxia could be used to stimulate SCs to improve their proliferation and capacity for regeneration. Nevertheless, injury sites are subjected to cycles of hypoxia followed by reoxgenation which seem to protect organs against ischemia and improve SC proliferation and differentiation. Therefore, hES cells represent a useful model to investigate the mechanisms acquired to maintain an undifferentiated and proliferative state at the site of injury.

Data presented in this thesis have demonstrated that hES cells exposed to hypoxia and reoxygenation display an increased expression of all core pluripotency genes but, in particular NANOG. This allows hES cells to sustain a greater self-renewal phenotype that resists differentiation. Furthermore, chromatin of hES cells exposed to reoxygenation was found enriched in H3K4me3 and H3K36me3 and low in H3K9me3 indicating a rapid reaction to environmental changes which was correlated with an enhanced transcriptional activation. This is the first demonstration of a "reoxygenation signature" within the HRE site of pluripotency genes and correlates with the enhanced stemness state demonstrated in this thesis. Interestingly, HIF-2α was found to increase the expression of NANOG through the direct binding to the NANOG HRE and thus might be responsible for these epigenetic changes.

Why HIF-2α was not degraded following reoxygenation is unknown but it might be stabilized independently of PHDs, possibly through other post-transcriptional modifications, or via ROS production which is known to inhibit PHD activity. This might be the case for HIF-2α stabilization. However, in this study, hES cells were exposed up to 72h reoxygenation suggesting that in such stress conditions HIF-2α could escape proteasome degradation and might activate other pathways like mTOR, which

will lead to a long term stabilization of HIF-2α. This will promote cell survival and increased glucose uptake similar to solid tumours. Overall, further work is required to investigate the mechanisms which regulate HIF-2α following hypoxia and reoxygenation. Since HIFs are able to confer cytoprotection against ischemic insult, a better understanding of the mechanisms regulating HIF-2α stabilization under reoxygenaton could have therapeutic applications. Indeed, the pharmacological stabilization of HIF-2α could be used to precondition cells prior to transplant and might confer protection against insult at a cellular, tissue and organ level.

One of the major findings of this thesis is that HIF-2α is recruited as a co-activator to the oct-sox element in the NANOG promoter only under reoxygenation which suggests a possible role for this transcription factor in the core circuitry of pluripotency. This thesis proposes that HIF-2α brings into physical proximity the HRE and the oct-sox element within the NANOG promoter and it might be responsible for a 3D chromatin loop that enhances NANOG expression. This was interesting as HIF-2α does not interact with the oct-sox element under hypoxia. Hence, it is possible to speculate that under reoxygenation hES cells activate an alternative mechanism of regulation in which HIF-2α activity is central to the maintenance of self-renewal through the interaction with an oct-sox element. This alternative regulation could be a form of "survival" mechanism that hES cells activate as a protection against the oxidative insult. Nevertheless, an alternative interpretation of this data is that an additional HIF-2α molecule may bind the oct-sox element through the presence of ancillary proteins. However, a tight loop conformation is more likely since it may act to protect HIF-2α from degradation maintaining its nuclear localization upon reoxygenation.

There is increasing evidence that chromatin conformational changes affect hES cell self-renewal (Apostolou et al., 2013; Levasseur et al., 2008). Hence, this finding might include HIF-2α in the maintenance of the *cis* DNA loop within the NANOG promoter. Oct4 has previously been found to be responsible for a similar chromatin loop formation in Nanog (Levasseur et al., 2008) and hence it is proposed that HIF-2α may be implicated in the establishment of a high order chromatin structure following hypoxia and reoxygenation. Indeed, HIF-2α could form part of the pluripotency-specific

chromatin “interactome” recently discovered in Nanog in ES cells and iPSCs (Apostolou et al., 2013). However, it is likely that HIF-2α will cooperate not only with OCT4 and SOX2 proteins but also with chromatin remodeling factors such as the members of the Mediator and cohesin families which have been found to be responsible for the protein-protein interactions that occur in ES cells. Therefore, it is possible to hypothesize that these cooperators form a “bridge” that connect the HRE to the oct-sox element thereby allowing the HIF-2α interaction. Since this chromatin conformation has been found specifically associated with reoxygenation, this might function to protect resident stem/progenitor cell populations from oxidative stress and acquire a high degree of stemness that promote survival during the post-injury stress or in diseases associated with ischemia and reoxygenation.

Overall, data presented in this thesis propose three different mechanisms regulating pluripotency genes, and in particular NANOG, in the mantainance of hES cells at different oxygen conditions (Figure 6.2). Under normoxic conditions, HIF-2α is degraded leading to a heterochromatic state within the HRE site with increased expression of histone modification marker H3K9me3 associated with gene silencing, and inhibition of NANOG gene expression. Under hypoxia, HIF-2α binding to the HRE is associated with an increased expression of active histone modification markers H3K4me3 and H3K36me3 and a decrease of H3K9me3, a marker of gene silencing. This euchromatic conformation facilitates NANOG expression. Under reoxygenation, the histone modification markers H3K4me3 and H3K36me3 are significantly activated. HIF-2α binds the NANOG HRE site and interacts with an oct-sox regulatory element via the cooperation with co-activators to form a 3D chromatin conformation loop (Figure 6.2 C). This loop would bring the HRE structurally close to the oct-sox *cis*-regulatory element which facilitates a robust up-regulation of NANOG expression. Alternatively, it is possible that an additional HIF-2α molecule binds to co-activators present on the oct-sox regulatory element thereby leading to an enhanced NANOG transcription (Figure 6.2 D).

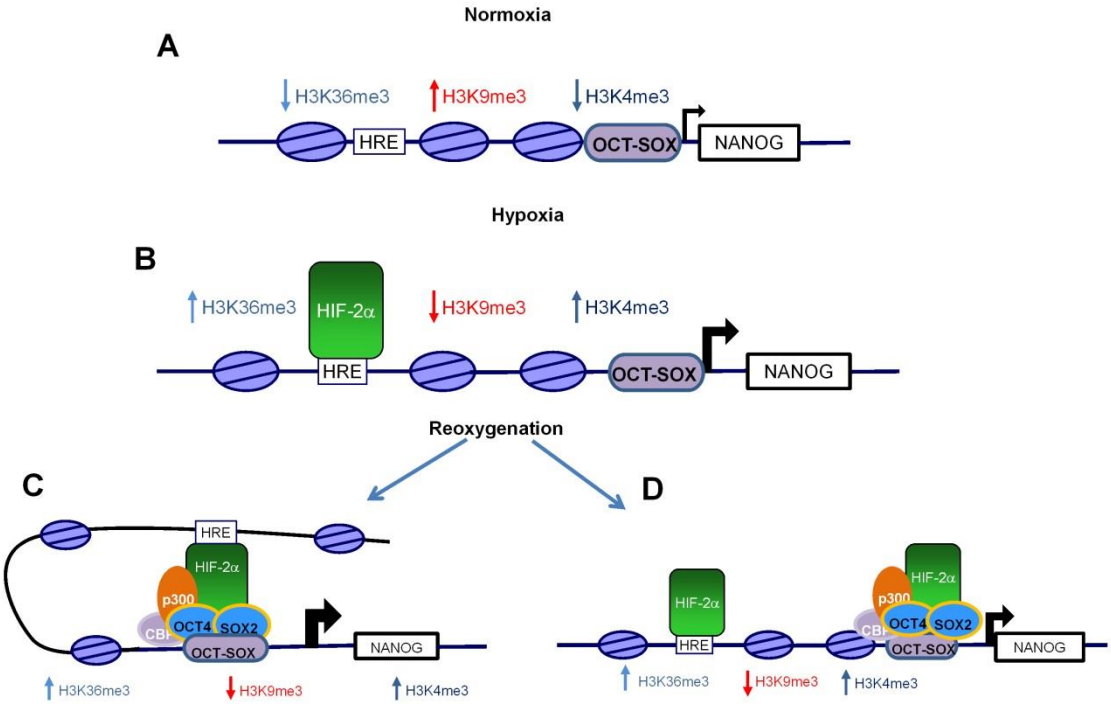

Figure 6.2: Schematic representation of NANOG promoter regulation in hES cells under conditions of A) normoxia and B) hypoxia. (C and D) Two different potential mechanisms of NANOG promoter regulation in hES cells after hypoxia followed by 72h reoxygenation.

Given the importance of SCs in tissue replacement and regenerative medicine, these data provide evidence that hypoxia is preferential for culturing a pure population of hES cells. Indeed, the hypoxic pre-treatment of SCs is able to produce beneficial effects by improving viability and proliferation in the injured tissue. Data in this thesis also suggests that treating SCs or hES cells with hypoxia followed by reoxygenation might improve the survival and regeneration of grafted cells following ischaemia and enhance the efficacy of clinical trials or therapeutic treatments. Hence, in the future, culture of SCs or hES cells on biomaterials releasing HPC mimetics such as HIF-2α, could prolong cell proliferation after engraftment and improve tissue regeneration.

6.2 Future work

To further elucidate the role of HIF-2α in the regulation of hES cells under hypoxia and reoxygenation it would be interesting to conduct the following experiments:

- To determine whether HIF-2α binding to the oct-sox element occurs through a loop conformation that blocks HIF-2α degradation, siRNA could be performed in hES cells exposed to reoxygenation.

  hES cells exposed to hypoxia followed by 72h of reoxygenation could be transfected with either HIF-2α siRNA, or a non targeting negative control siRNA and ChIP performed using a HIF-2α antibody. HIF-2α binding to the NANOG HRE will be investigated using qPCR.
- To investigate whether the binding of HIF-2α to the NANOG oct-sox *cis* regulatory element is functional, luciferase reporter assays could be performed. NT2 cells or hES cells exposed to hypoxia followed by reoxygenation could be co-transfected with a PGL3 vector harboring a mutated oct-sox *cis* regulatory element and also a HIF-2α expression plasmid.
- To confirm the presence of a 3D conformational loop within the NANOG proximal promoter in hES cells exposed to hypoxia followed by reoxygenation Chromatin Conformation Capture (3C) could be performed.

  Furthermore, high resolution circular chromosome conformation capture (4C) assays coupled with sequencing will allow the discovery of genome wide NANOG interactions in hES cells exposed to normoxia, hypoxia and reoxygenation. This experiment will provide a detailed chromatin-interaction map within the NANOG locus.
- To determine whether HIF-2α binding to the oct-sox *cis* element within the NANOG promoter could have an effect also on the chromatin state within this regulatory sequence, ChIP assays using H3K4me3, H3K36me3 and H3K8me3 could be performed.
- To evaluate whether HIF-2α is able to form a multiprotein complex within the oct-sox *cis* regulatory element when hES cells are exposed to hypoxia followed by reoxygenation co-immunoprecipitation experiments could be performed. By using a specific HIF-2α antibody, it would be possible to identify potential binding partners such as OCT4 and SOX2. This could also be performed using

Fluorescent Resonance Energy Transfer (FRET) in hES cells exposed to normoxia, hypoxia or hypoxia followed by reoxygenation.

Furthermore, unknown HIF-2α binding partners on the oct-sox *cis* element could be investigated by using co-immunoprecipitation and Mass Spectrometry for hES cells cultured at 5% oxygen and following hypoxia and reoxygenation.

- To analyse the expression levels of PHD1, 2 and 3 in hES cells exposed to normoxia, hypoxia and reoxygenation RT-qPCR and Western Blot analysis could be performed. These experiments would elucidate which PHDs are more likely to be involved in the regulation of HIF-2α under different oxygen conditions. The use of specific antibodies that could detect the level of hydroxylation of the ODD domain in HIF-2α could also provide information on the specific proline residues that are more involved in the stabilization of this specific HIF following by reoxygenation. Moreover, to analyze whether the ubiquitin pathway is altered after hypoxia and reoxygenation, ubiquitination assays could be performed. This would require HIF-2α protein to be synthesized *in vitro* and then mixed with the ubiquitination complexes and resolved on SDS-PAGE followed by autoradiography.
- To further investigate the mechanism of HIF-2α stabilization in hES cells exposed to reoxygenation, the mTOR pathway could be analysed using RT-qPCR and Western Blotting.
- To determine whether reactive oxygen species have a role in the stabilization of HIF-2α in hES cells upon hypoxia and reoxygenation an intracellular ROS assay could be performed. This test would measure the intracellular ROS level using a cell permeant probe such as H_2DCFDA (2', 7'-dichlorodihydrofluorescein diacetate) through cleavage by intracellular esterases, the probe is retained within the cells and upon oxidation by ROS generate a fluorescent signal which can be measured using a fluorescence plate reader.

Appendices

Appendix 1

Mycoplasma test

Mycoplasma test was performed on MEFs cells using the e-Myco™ Mycoplasma Detection Kit (iNtRON Biotechnology). A small number of MEF cells were scraped from a T75 flask and transferred into a 15ml Falcon tube. Cells were centrifuged for 1min at 1500rpm and the pellet was resuspended in 1ml of PBS, transferred into a 1.5ml epperndorf tube and centrifuged for 1min at 1500rpm. After the washing, the pellet was resuspended in 100µl of sterile PBS, heated for 10min, vortex for 5-10sec and centrifuged for 2min at 13000rpm at room temperature. 10µl of the supernatant was used added to each tube of e-Myco™ Mycoplasma PCR Detection kit and resuspended after adding 10µl of sterile water for a 20µl PCR reaction volume. A positive control for the reaction was performed by adding 1 µl of control DNA to a tube of e-Myco™ Mycoplasma PCR Detection kit and 19µl of sterile water for a final volume of 20 µl PCR reaction. A negative control was perfomed by adding 20 µl of steril water to one tube of the e-Myco™ Mycoplasma PCR Detection kit.

PCR was performed using the following conditions: initial denaturation at 94°C for 1min followed by 35 cycles of 94°C for 30sec, 60°C for 20sec and 72°C for 1min anf final extension at 72°C for 5min. 7µl of PCR product were loaded on a 1.5% agarose gel.

Appendix 2

Miniprep purification

To perform miniprep purification the QIAprep Spin Miniprep Kit (Qiagen Crawley, UK) was employed. After DNA transformation a single colony from the LB agar plate (See Method section 3.2.5) was grown over night in 3 ml of LB containing 100µg/ml Ampicillin. The culture was centrifuged at 13000 rpm, 4°C for 2min and the resultant pellet was resuspended in 250µl of Buffer P1 supplemented with RNAse A and transferred to a 1.5ml tube. Bacteria were lysed in 250µl of Buffer P2 and gently inverted for 4-6 times to shear genomic DNA. 300µl of Buffer N3 were then added to the solution and centrifuged for 10min at 13000 rpm. The supernatant containing plasmid DNA was added to a QIAprep spin column and centrifuged at 13000 rpm for 1min. The DNA bound to the column was washed first by adding 500µl of Buffer PB and centrifuged 1min at 13000 rpm then with 750µl of Buffer PE and centrifuged 1min at 13000 rpm. An additional centrifuge of 1min was added to remove the residual washing buffer. After this step, the QIAprep column was placed in a 1.5ml tube. To elute DNA, 50µl of water were added to the centre of the column and let stand 1 min before centrifuge for another 1min at 13000 rpm.

Appendix 3

Gel extraction

To obtain clean DNA products, digested DNA was separated by electrophoresis (See Methods 3.2.6) and the specific band was cut from the agarose gels and purified using the Qiagen QIAquick Gel Extraction kit (Qiagen).

100µl of Buffer QG were added for every 100mg of sliced gel and incubated at 50°C for 10min and mixed every 2-3min until the gel slice has completely dissolved. 100µl of isopropanol was added for every 100mg of agarose gel slice and the solution was transferred to a QIAquick spin column and centrifuged at 13000rpm for 1min. The column was then filled with 500µl of Buffer QG centrifuged at 13000rpm for 1min. To wash the column, 750µl of Buffer PE (containing ethanol) were added to the column and centrifuged at 13000 rpm for 1min. An additional centrifuge at 13000 rpm for 1min was performed to remove residual ethanol from the membrane column which can interfere with the future analysis. The DNA was then eluted by adding to the column 30µl of water to the centre of the membrane, let the column stand for 1min and then centrifuged for 1min at 13000 rpm.

Appendix 4

Maxiprep purification

To perform Maxiprep purification the Genopure Plasmid Maxi kit (Roche) was used. After DNA transformation a single colony from the LB agar plate (See Method section 3.2.5) was grown over night in 3 ml of LB containing 100µg/ml Ampicillin. The culture was diluted into 200ml of LB containing the appropriate selective antibiotic and grown overnight. The culture was centrifuged at 4000g, 4°C for 15min and the resultant pellet was resuspended in 12ml of Suspension Buffer supplemented with RNase. 12ml of Lysis Buffer was added to the suspension, mixed by inverting the tube 6-8 times and incubated 2-3min at room temperature. To neutralise the Lysis Buffer and to precipitate bacterial proteins and genomic DNA, 12ml of cold Neutralization Buffer were added to the suspension gently by inverting the tube 6-8 times until an homogenous suspension is formed and incubated 5min on ice. The cloudy and flocculent solution is then filtered using a folded filter placed into a funnel that has been inserted into a 50ml plastic tube. Before adding the lysate, the filter was moistened with few drops of Equilibration Buffer. The resulting supernatant was added to a column already equilibrated with 6ml of Equilibration Buffer and passed through the column by gravity. To wash the DNA, 16ml of Wash Buffer was added to the column and the buffer was passed through twice. The DNA was eluted in 15ml of prewarmed Elution Buffer and precipitated by adding 11ml of isopropanol and immediately centrifuged at 7500g, 4°C for 1h. The resultant pellet was washed in 1ml of RNAse free water and divided into 3 aliquots in 1.5ml tubes. To precipitate the DNA, 1/10 of NaAc and 750µl of 100% ethanol was added and samples mixed to allow DNA precipitation into a white pellet. DNA samples were centrifuged at 13000rpm at 4°C for 15min and each pellet was resuspended in 500µl of water and quantified by measuring the absorbance at 260nm using a UV spectrophotometry.

Appendix 5

Publication based on the work of this thesis

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PLOS ONE

Environmental Oxygen Tension Regulates the Energy Metabolism and Self-Renewal of Human Embryonic Stem Cells

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Abstract

Energy metabolism is intrinsic to cell viability but surprisingly has been little studied in human embryonic stem cells (hESCs). The current study aims to investigate the effect of environmental O_2 tension on carbohydrate utilisation of hESCs. Highly pluripotent hESCs cultured at 5% O_2 consumed significantly more glucose, less pyruvate and produced more lactate compared to those maintained at 20% O_2. Moreover, hESCs cultured at atmospheric O_2 levels expressed significantly less OCT4, SOX2 and NANOG than those maintained at 5% O_2. To determine whether this difference in metabolism was a reflection of the pluripotent state, hESCs were cultured at 5% O_2 in the absence of FGF2 for 16 hours leading to a significant reduction in the expression of SOX2. In addition, these cells consumed less glucose and produced significantly less lactate compared to those cultured in the presence of FGF2. hESCs maintained at 5% O_2 were found to consume significantly less O_2 than those cultured in the absence of FGF2, or at 20% O_2. GLUT1 expression correlated with glucose consumption and using siRNA and chromatin immunoprecipitation was found to be directly regulated by hypoxia inducible factor (HIF)-2α at 5% O_2. In conclusion, highly pluripotent cells associated with hypoxic culture consume low levels of O_2, high levels of glucose and produce large amounts of lactate, while at atmospheric conditions glucose consumption and lactate production are reduced and there is an increase in oxidative metabolism. These data suggest that environmental O_2 regulates energy metabolism and is intrinsic to the self-renewal of hESCs.

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Introduction

Human embryonic stem cells (hESCs) are pluripotent cells derived from the inner cell mass (ICM) of the blastocyst, the final stage of preimplantation embryo development. They proliferate through self-renewal and provide an excellent model to investigate developmental mechanisms since they have the potential to differentiate into all cells of the body [1]. However, if these cells are to be of therapeutic use it is imperative to ensure that a highly pluripotent population of hESCs are maintained which can then be directed down specific lineage pathways.

hESCs are notoriously difficult to maintain *in vitro* as the colonies have a propensity to spontaneously differentiate suggestive of suboptimal culture conditions; an effect which may be circumvented by the use of low environmental O_2 tensions [2]. Culture under atmospheric O_2 tensions has been found to decrease hESC proliferation and reduce pluripotency marker expression compared to culture under low (2–5%) O_2 tensions [3,4,5], an effect regulated by hypoxia inducible factors (HIFs), specifically HIF-2α [5]. This promotion of an immature, stem cell like phenotype has also been observed in both malignant and non-malignant cells cultured under hypoxic conditions [6]. Despite these encouraging data, many of the biochemical and physiological implications of hypoxic culture on hESCs remain to be elucidated.

Among the ~200 genes regulated by HIFs, metabolic genes feature extensively [7] suggesting that environmental O_2 tensions may also have a significant impact on hESC metabolism. However, the metabolic status of hESCs and the impact of environmental O_2 tension have received remarkably little attention. Significantly, our previous work has shown that metabolic activity is a central regulator of the phenotype and developmental potential of human preimplantation embryos [8,9] and highlights metabolism as a fundamental regulator of cellular function.

Morphologically, hESCs share many characteristics with ICM cells; a high nuclear to cytoplasmic ratio, low mitochondrial number, and expression of the same surface antigens [10,11,12,13]. It is therefore likely that the nutrition of the ICM may inform on the metabolism of hESCs. In terms of glucose utilisation, the murine ICM is wholly glycolytic compared to the differentiated trophectoderm where only 55% of the glucose consumed may be accounted for by lactate production [14]. Moreover, the ICM is also metabolically relatively quiescent in terms of mitochondrial activity, O_2 consumption and ATP production compared to the trophectoderm [15]. Knowledge of the metabolism of hESCs is still in its infancy but it has been

shown that pluripotency may be enhanced by inhibiting the mitochondrial respiratory chain [16]. This data was further supported by confirmation that hESCs and induced pluripotent stem cells rely on glycolysis for their energy requirements [17,18,40].

This study aims to investigate how environmental O_2 tension affects the regulation and energy metabolism of hESCs in terms of O_2, glucose and pyruvate consumption and lactate production. Moreover, the effect of early hESC differentiation as demonstrated by the short term removal of FGF2 on hESC metabolism and pluripotency marker expression has also been investigated. These data suggest that environmental O_2 regulates glucose utilisation and is intrinsic to the energy metabolism and self-renewal of hESCs.

Materials and Methods

hESC Culture

Hues7 (D. Melton, Howard Hughes Medical Institute/Harvard University) [41] and Shef3 (Supplied by the UK Stem Cell Bank) hESCs were cultured at 20% O_2 in Knockout DMEM (Invitrogen) supplemented with 15% knockout serum replacement (Invitrogen), 100 μg/ml penicillin streptomycin (Invitrogen), 1 mM L-glutamine (Invitrogen), 1× non-essential amino acids (Invitrogen), 0.1 mM 2-mercaptoethanol and 10 ng/ml FGF2 (Peprotech) on γ irradiated mouse embryonic fibroblasts (MEFs; a primary source derived in institutional facilities following University of Southampton ethical review committee approval and in accordance with UK Home Office regulations). hESCs were then transferred to Matrigel (BD Biosciences) coated plates and cultured in MEF-conditioned medium at both 20% and 5% O_2. They were maintained for a minimum of 3 passages on Matrigel at both O_2 tensions prior to use.

Measurement of Carbohydrate Utilisation

hESCs were passaged on to 12-well Matrigel coated plates and cultured in MEF conditioned medium. On day 2, 3 and 4 post-passage hESCs were pre-incubated in a defined metabolic medium [8] containing 1 mM glucose, 5 mM lactate, 0.47 mM pyruvate, 0.5% human serum albumin and amino acids [19] for 30 mins. The medium was then replaced with pre-determined quantities (300–500 μl) of defined medium for 1.5–3.5 h. At the end of the incubation period, all but 100 μl of medium was removed from each well and stored at −80°C prior to analysis of carbohydrate content and the number of cells in each well was determined using a haemocytometer. In subsequent experiments, the effect of removing FGF2 for 16 hours on hESC metabolism was monitored. FGF2 was removed from the medium prior to MEF conditioning and used to replace regular, MEF conditioned medium containing FGF2 on day 2 post-passage. Enzyme linked biochemical assays were used to measure the concentration of pyruvate, glucose and lactate in 180 μl of spent medium using a Konelab 20 autoanalyser (Thermo Scientific). The concentration of carbohydrates in cell containing wells was compared to cell-free control wells and the consumption of pyruvate and glucose and the production of lactate by hESCs calculated in pmol/cell/h.

O_2 Assay

A 96-well O_2 biosensor plate (BD Biosciences) containing 3 wells of 200 mM sodium sulphite (0% O_2 control) and 3 wells of a defined metabolic medium (20% O_2 control) was incubated at 37°C in a fluorescence plate reader (BMG Labtech) for 30 mins. Hues7 hESCs on day 3 post-passage were pre-incubated with metabolic medium for 30 mins, harvested into 310 μl of fresh, pre-warmed metabolic medium, added to a well of the O_2 biosensor plate and sealed using an adhesive PCR foil (Thermo Fisher) with care taken to ensure the absence of air bubbles. The fluorescence (excitation 485 nm and emission 612 nm) of each well was recorded every 2 minutes over a two hour period. After the final measurement, the protein content of each well was determined using the Bradford assay. O_2 consumption was calculated as μl O_2/mg protein/h.

RT-qPCR

RNA was isolated from Hues7 hESCs cultured under feeder-free conditions on Matrigel on day 3 post-passage using TriReagent (Sigma) and 2 μg reverse transcribed to cDNA using MMLV-reverse transcriptase (Promega). cDNA (4 μg) was amplified in 20 μl reactions containing 1 μl probes and primer mix (OCT4: Hs01895061_u1; SOX2: Hs00602736_s1; NANOG: Hs02387400_g1; GLUT1: Hs00197884_m1; UBC Hs00824723_m1) and 10 μl 2× Taqman Universal PCR Master Mix (Applied Biosystems) using an ABI 7500 real time PCR system. The conditions used were 2 mins at 50°C, 10 mins at 95°C followed by 45 cycles of 95°C for 15 secs and 60°C for 1 min. Placental cDNA (0–10 ng) was used to produce a standard curve for each gene of interest as well as the endogenous control, UBC and used to quantify gene expression. All genes were analysed in duplicate and normalised to UBC.

siRNA

siRNA was used to silence either *HIF-1α*, *HIF-2α* or *HIF-3α* in Hues7 hESCs cultured on Matrigel coated plates at 5% O_2. The cells were passaged and the following day 50 nM siRNA (HIF-1α: Hs_HIF1A_5; HIF-2α: Hs_EPAS1_5; or HIF-3α: Hs_HIF3A_1), 12 μl HiPerfect transfection reagent (Qiagen) and 200 μl knockout DMEM were mixed, incubated at room temperature for 10 mins and added in a drop wise manner to hESCs. Allstars negative control siRNA (Qiagen) was used as a negative control. The ability of these siRNA to silence individual HIF-α isoforms has previously been validated [5]. The cells were harvested 48 h post-transfection and *GLUT1* mRNA quantified as above.

Western Blotting

Protein was isolated from Hues7 hESCs cultured on Matrigel on day 3 post-passage by incubating in ice cold radio immunoprecipitation assay (RIPA) buffer for 30 mins followed by sonication for 30 secs. Protein (75 μg) was resolved on an 8% SDS bisacrylamide gel, transferred to nitrocellulose membrane and blocked in PBS containing 0.1% Tween-20 and 5% milk for 1 h at room temperature. The membrane was incubated in primary antibody (OCT4 (Santa Cruz) 1:1000; SOX2 (Millipore) 1:1000; NANOG (Abcam) 1:1000) diluted in blocking buffer overnight at 4°C. Membranes were washed and incubated in horse radish peroxidase-conjugated secondary antibodies (anti-mouse (GE Healthcare) 1:100,000 or anti-rabbit (GE Healthcare) 1:50,000) for 1 h at room temperature. The Enhanced chemiluminescence advanced Western blotting detection kit (GE Healthcare) was used to develop the membranes prior to imaging on the Biorad Chemidoc XRS. Protein expression was quantified relative to β-actin (mouse anti-β-actin peroxidise conjugated antibody (Sigma) 1:50,000).

Chromatin Immunoprecipitation (ChIP) Assays

Hues7 hESCs cultured in normoxic (20% O_2), or hypoxic (5% O_2) conditions were cross-linked with 1% formaldehyde for 10 min and the reaction blocked with 0.125 M glycine. ChIP

experiments were performed with HIF-2α (Novus Biologicals) or immunoglobulin G (IgG) antibody (Santa Cruz) as previously described [20,21] except that immuno-complexes were washed using high-salt buffers as followed: 10 times with 600 µl buffer A (0.1% SDS, 2 mM EDTA, 20 mM Tris HCl pH8, 1% Triton X-100, 500 mM NaCl), 8 times with 600 µl buffer B (0.1% SDS, 2 mM EDTA, 20 mM Tris HCl pH8, 1% Triton X-100, 1 M NaCl), 3 times with 600 µl TE (10 mM Tris HCl H8, 1 mM EDTA) buffer. Recovered DNA was amplified with custom Taqman Assays (Applied Biosystems) spanning a predicted hypoxia response element (HRE) site at −1691 bp of the GLUT1 proximal promoter (GLUT1 fwd: CAAATGTGTGGATGT-GAGTTGC; GLUT1 rev: CCATCACGGTCCTTCTTCATG; GLUT1 probe: AGGCTGAGCGTGTAAA). qPCR was performed using an ABI 7900 HT Fast Real Time System (Applied Biosystems) in a 384 well plate.

Statistical Analysis

All data were tested to determine whether they were normally distributed using the Anderson Darling normality test. Any differences in the utilisation of carbohydrates or glycolytic rate with O_2 tension were analysed using a Student's t-test. Differences in mRNA expression were normalised to the endogenous control, UBC and then to 1 for cells cultured at 5% O_2, or to Allstars transfection controls when genes were silenced. Differences in protein expression were normalised to β-actin and then to 1 for cells cultured at 5% O_2. In both cases a one sample t-test was used to determine significance from either 5% O_2 or transfection controls. Differences in O_2 consumption between hESCs maintained at 5%, 20% and 5% O_2 in the absence of FGF2 was determined using a one-way analysis of variance followed by a Fisher's test. Differences in binding of HIF-2α to the GLUT1 promoter with O_2 tension was expressed as a percentage of input (non-immunoprecipitated chromatin) calculated using $100\times2^{[Ct\ (Input)-Ct\ (IP)]}$ for each sample and expressed as box and whisker plots. All data represent at least 3 independent experiments and are presented as mean ± SEM.

Results

Environmental O_2 Tension Regulates Carbohydrate Utilisation

hESCs maintained at 5% O_2 display an increased proliferation and expression of pluripotency markers compared to those cultured at 20% O_2 [5]. The current study aimed to determine the impact of environmental O_2 tension on the energy metabolism of hESCs. Hues7 hESCs were cultured at either 5% or 20% O_2 and the consumption of glucose and pyruvate and the production of lactate were analysed on days two to four post-passage. Similarly, the effect of environmental O_2 tension on the depletion of glucose and production of lactate by Shef3 hESCs on day 3 post-passage was also determined. Under each O_2 tension, glucose was found to be the predominant energy substrate utilised. Interestingly, both cell lines consumed almost twice as much glucose ($P<0.001$) and produced approximately 2–3 times the amount of lactate ($P<0.01$–$P<0.001$) when cultured at 5% O_2 compared to 20% O_2 (Fig. 1A–D). A similar, low level (~0.1 pmol/cell/h) of pyruvate was consumed by Hues7 hESCs on each day post-passage (Fig. 1 A–C).

Environmental O_2 and Short-term Removal of FGF2 Alters hESC Energy Metabolism

To investigate whether the difference in energy substrate utilisation between hESCs cultured at 5% and 20% O_2 was a reflection of the degree of cell pluripotency, FGF2 was removed from the medium used to culture cells at 5% O_2 for 16 hours. Morphologically, there was no overt differentiation observed in any of the treatment groups on day 3 post-passage (Fig. S1). This is in agreement with previous observations of hESCs cultured at 5% or 20% O_2 [5].

In terms of metabolism, removing FGF2 for 16 hours from hESCs cultured at 5% O_2 resulted in a significant reduction in the amount of glucose consumed in Hues7 cells and a near significant reduction in Shef3 cells compared to those maintained in the presence of FGF2 (Fig. 2A and B). However, in the absence of FGF2, both cell lines displayed a significant reduction in the amount of lactate produced. Interestingly, twice as much pyruvate was consumed when Hues7 cells were cultured at 5% O_2 in the absence of FGF2 for 16 hours compared to when FGF2 was present ($P<0.01$; Fig. 2 A). This suggests that even the very early stages of differentiation are associated with an increased reliance on oxidative metabolism.

To determine the global ability of hESCs to produce energy, O_2 consumption was measured. Hues7 hESCs cultured at 20% O_2 were found to consume approximately 4 µl O_2/mg protein/h, which was significantly greater than hESCs cultured at both 5% O_2 ($P<0.001$) and 5% O_2 where FGF2 was removed for 16 hours ($P<0.001$; Fig. 2 C). Interestingly, the removal of FGF2 for 16 hours from hESCs cultured at 5% O_2 significantly increased O_2 consumption compared to those cultured in the presence of FGF2 ($P<0.05$). This suggests that hESCs maintained at 20% O_2 have a greater energy requirement than those cultured at 5% O_2 in the absence of FGF2. hESCs cultured at 5% O_2 in the presence of FGF2 are the quietest metabolically having the lowest rate of O_2 consumption.

Environmental O_2 and Short Term FGF2 Removal Regulates the Self-renewal of hESCs

In agreement with our previous report [5], OCT4 protein expression was significantly decreased in Hues7 hESCs maintained at 20% O_2 compared to those cultured at 5% O_2 ($P<0.05$; Fig. 3 A, B). A similar reduction in SOX2 ($P<0.05$) and NANOG ($P<0.05$) expression was also observed at 20% O_2 (Fig. 3 C–F). Interestingly, when FGF2 was removed for 16 hours from hESCs cultured at 5% O_2, SOX2 protein expression decreased significantly while OCT4 and NANOG expression displayed a non-significant reduction to levels comparable to hESCs cultured under atmospheric O_2 tensions (Fig. 3A–F).

GLUT1 mRNA is Differentially Expressed Under Hypoxic Conditions and Regulated by HIF-2α

To determine whether differences in glucose transport may be responsible for the increased consumption observed by Hues7 hESCs maintained at 5% O_2 compared to 20% O_2, the expression of GLUT1 was investigated. GLUT1 mRNA expression was significantly decreased in cells maintained at 20% O_2 compared to those cultured at 5% O_2 (Fig. 4 A). This suggests a correlation between the mRNA expression of GLUT1 and the uptake of glucose in hESCs.

Since HIFs are important regulators of the hypoxic response, siRNA was used to determine whether any of the HIFα subunits were responsible for the increased GLUT1 expression in hESCs cultured at 5% O_2. GLUT1 mRNA expression was not affected when either HIF-1α or HIF-3α were silenced but was significantly reduced when HIF-2α was knocked down ($P<0.001$; Fig. 4 B). This suggests that HIF-2α is an upstream regulator of GLUT1 in hESCs cultured at 5% O_2.

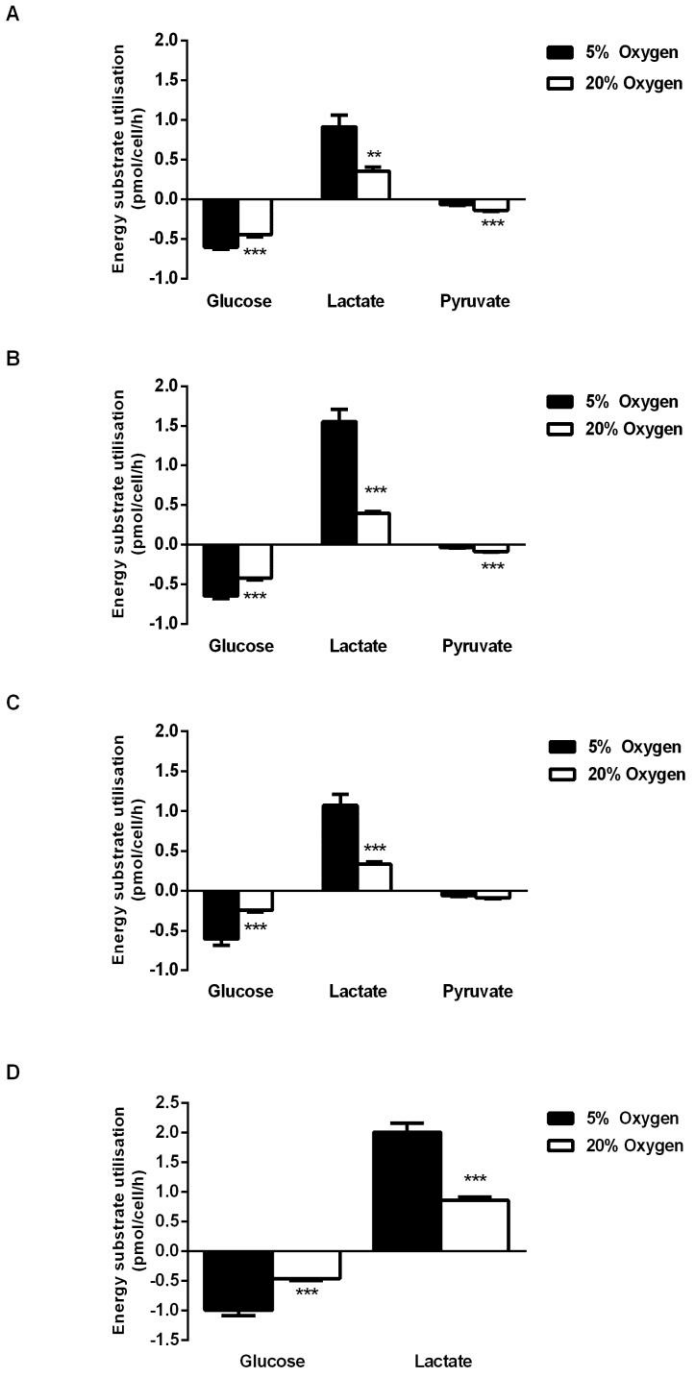

Figure 1. Hypoxic culture promotes glucose uptake and lactate production in hESCs. Glucose, pyruvate and lactate utilisation were non-invasively measured in a defined hESC medium. More glucose was consumed and lactate produced by Hues7 hESCs cultured at 5% O_2 than at 20% O_2 on (A) day 2 (B) day 3 and (C) day 4 post-passage. In contrast, less pyruvate was consumed by hESCs at 5% O_2 compared to 20% O_2. The rate of glucose consumption and lactate production was also greater on day 3 post-passage in Shef3 hESCs cultured at 5% O_2 compared to those maintained at 20% O_2 (D). **P<0.01, ***P<0.001 significantly different to 5% O_2 (n = 12–23).
doi:10.1371/journal.pone.0062507.g001

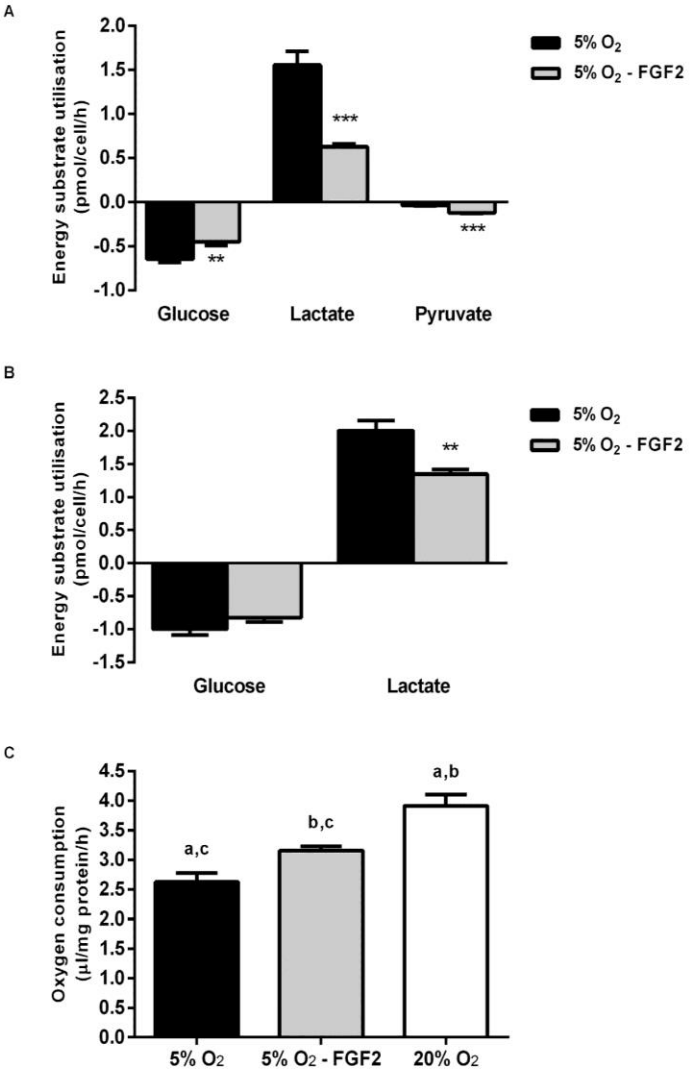

Figure 2. Short term removal of FGF2 at 5% O_2 alters hESC metabolism and promotes O_2 consumption. Removal of FGF2 for 16 hours from Hues7 hESCs cultured at 5% O_2 (5% O_2– FGF2) resulted in a reduction of glucose consumption and lactate production, whereas pyruvate consumption dramatically increased (A). Shef3 hESCs cultured at 5% O_2– FGF2 displayed a significant reduction in lactate production (B). **P<0.01, ***P<0.001 significantly different to 5% O_2+FGF2 (n = 10–18). Hues7 hESCs cultured at 5% O_2 consumed less O_2 than when FGF2 was removed for 16 hours (C). hESCs maintained at 20% O_2 consumed the greatest amount of O_2. Bars with the same superscript are significantly different; a, b, P<0.001, c, P<0.05 (n = 7–8).
doi:10.1371/journal.pone.0062507.g002

HIF-2α Binds Directly to the GLUT1 Promoter

To determine whether HIF-2α binds directly to a potential HRE in the proximal promoter of GLUT1 ChIP assays were performed on Hues7 hESCs. We compared the enrichment, using qPCR, of the sequence corresponding to the GLUT1 proximal promoter when precipitating with an antibody specific for HIF-2α, compared to the IgG isotype control. A 4-fold enrichment over the IgG control was observed in the chromatin isolated from hESCs maintained at 5% O_2 (Fig. 4C). In contrast, no significant binding of HIF-2α was observed in chromatin isolated from hESCs maintained at 20% O_2. This data reveals a specific HIF-2α interaction with the GLUT1 proximal promoter only in hESCs cultured under hypoxic conditions.

Discussion

hESC metabolism has received little attention, despite being intrinsic to cellular function. Several studies have highlighted beneficial effects of culturing hESCs at low O_2 tensions including improved morphology, increased expression of pluripotency markers, a reduction in chromosomal abnormalities and a higher

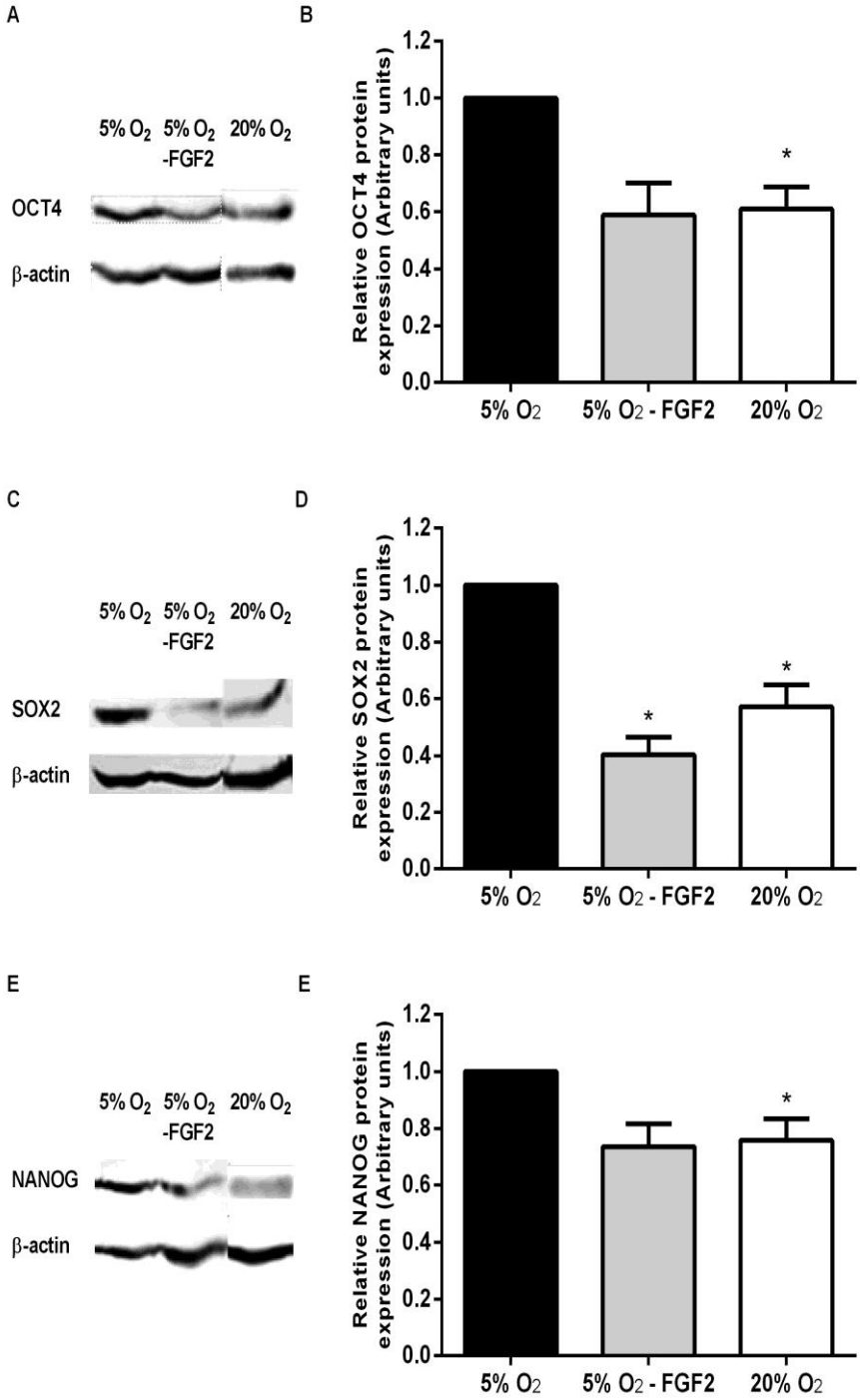

Figure 3. hESCs maintained at atmospheric O_2 levels express reduced levels of pluripotency markers compared to those cultured at 5% O_2. Hues7 hESCs were cultured at either 5% O_2, 5% O_2 with FGF2 removed for 16 hours (5% O_2– FGF2) or 20% O_2. Protein was isolated and OCT4 (A and B), SOX2 (C and D) and NANOG (E and F) quantified using Western blotting. All data has been normalised to β-actin and to 1 for 5% O_2. *P<0.05, **P<0.01, ***P<0.001 significantly different from 5% O_2 (n = 3–4).
doi:10.1371/journal.pone.0062507.g003

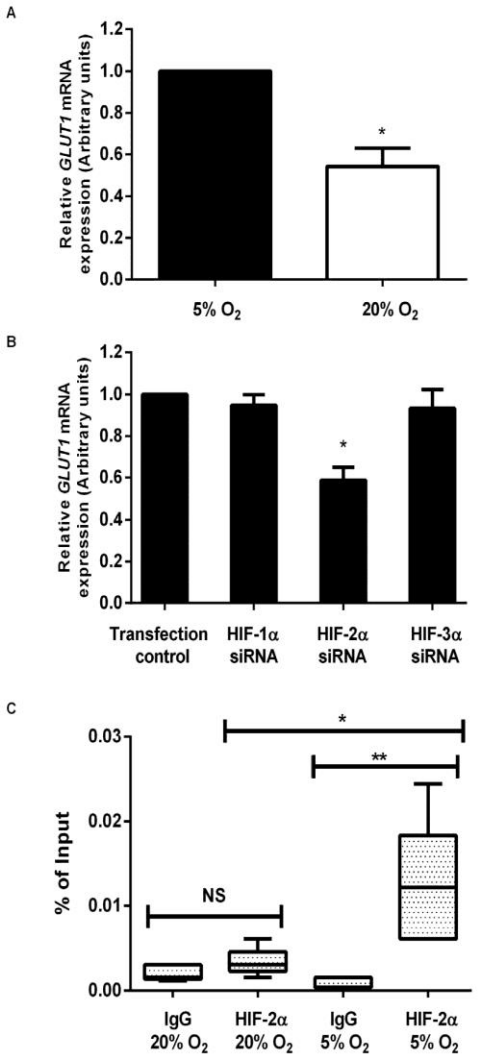

Figure 4. GLUT1 expression parallels glucose utilisation and is directly regulated by HIF-2α under hypoxic conditions. RT-qPCR was used to quantify *GLUT1* mRNA expression in Hues7 hESCs cultured at either 5% O_2, or 20% O_2 on day three post-passage (A). All data has been normalised to *UBC* and to 1 for 5% O_2. *$P<0.05$ significantly different to 5% O_2 ($n=3$). Using siRNA to silence HIF-α subunits in Hues7 hESCs cultured at 5% O_2, *GLUT1* mRNA was found to be regulated by HIF-2α (B). All data has been normalised to *UBC* and to 1 for the transfection control. *$P<0.05$ significantly different to transfection control ($n=6$). Using ChIP HIF-2α was found to bind to the proximal promoter of GLUT1 only in hESCs cultured at 5% O_2. ChIP assays were performed with either a HIF-2α or IgG control antibody on chromatin isolated from Hues7 hESCs cultured at either 20% O_2 or 5% O_2. DNA enrichment is expressed as a percentage of input (non-immunoprecipitated chromatin). *$P<0.05$, **$P<0.01$, NS indicates no significant difference ($n=5$).
doi:10.1371/journal.pone.0062507.g004

rate of proliferation [3,4,5,22] but the impact on cellular metabolism is unknown. Thus, this study sought to investigate the influence of environmental O_2 on the carbohydrate utilisation, energy metabolism and self-renewal of hESCs.

Independent of O_2 tension, glucose was found to be the predominant substrate utilised by hESCs. However, highly pluripotent hESCs cultured under hypoxic conditions were found to deplete significantly more glucose and produce higher levels of lactate than cells maintained at atmospheric O_2 tensions. This was intriguing and suggested a correlation between metabolism and self-renewal. To investigate this further, FGF2, a factor required to sustain self-renewal and support growth of undifferentiated hESCs [23,24], was removed for just 16 hours from highly pluripotent cells cultured at 5% O_2. The removal of FGF2 resulted in a reduced utilisation of glucose and significant decrease in the amount of lactate produced. This was intriguing and highlights the ability of hESC metabolism to adapt to changes in environmental conditions. Moreover, the resultant rates of glucose utilisation and lactate production observed in hESCs cultured in the absence of FGF2 for 16 hours were similar to cells cultured at 20% O_2. This was interesting since cells maintained at 20% O_2 expressed significantly less OCT4, SOX2 and NANOG than those cultured under hypoxic conditions. A similar trend was also mirrored by hESCs cultured at 5% O_2 in the absence of FGF2. These data suggest that energy metabolism may represent a novel parameter to quantify the self-renewal potential of hESC cultures.

The mechanism of how FGF2 regulates hESC energy metabolism under hypoxic conditions is unknown but data from adipocytes implicates the involvement of HIF-1α [25]. These investigators found that the culture of adipocytes under hypoxic conditions in the presence of FGF2 caused an increase in both GLUT1 expression and lactate production through the induction of HIF-1α. In hESCs HIF-1α is degraded after ~48 h of hypoxic culture after which HIF-2α is stabilised [5]. Thus, it could be speculated that the reduced amount of glucose consumed and lactate produced by hESCs cultured at 5% O_2 in the absence of FGF2 may be due to the destabilisation/degradation of HIF-2α and the resultant decrease in expression of hypoxia responsive genes.

Hues7 hESCs cultured at 5% O_2 consumed significantly lower levels of O_2 and pyruvate than those maintained at either 20% O_2 or 5% O_2 in the absence of FGF2. Since O_2 consumption provides the best global indication of the ability of a cell to produce energy, this suggests that hESCs cultured at 5% O_2 are more metabolically quiescent than those cultured at 5% O_2 in the absence of FGF2, or at 20% O_2. As OCT4, SOX2 and NANOG expression were also significantly reduced in hESCs cultured at 20% O_2 compared to 5% O_2, this suggests that as differentiation occurs a more active metabolism ensues. These results are comparable to that in the mouse blastocyst where the ICM was found to be metabolically relatively quiescent consuming low levels of O_2 compared to the differentiated trophectoderm [15].

Our data also suggest that hESCs display a glycolytic metabolism consuming glucose and producing lactate. This is in agreement with mouse ES cells and mesenchymal stem cells which utilise glycolysis as a primary source of ATP production in the undifferentiated state and switch to oxidative phosphorylation upon differentiation [26,27]. Similarly, nuclear reprogramming associated with induced pluripotent stem cells has been shown to be associated with a shift from an oxidative metabolism to one dependent on glycolysis [18,39]. Together with the current data, this highlights the importance of glycolysis for maintaining the pluripotent state.

hESCs cultured at 5% O_2 expressed significantly more *GLUT1* than those maintained at 20% O_2. Glucose transporter expression is known to increase the amount of glucose taken up by cells and

GLUT1 is thought to be the predominant transporter in many cell types including mouse ESCs, brain, placenta, and retina [28,29,30]. Our finding of an increase in GLUT1 expression under hypoxic conditions is in agreement with that observed in mouse ESCs [31]. As a HRE is present in the promoter region of the GLUT1 gene [32,33] we were interested to determine whether any of the 3 regulated HIF-α subunits were responsible for this increased expression. Using siRNA, HIF-α subunits were silenced individually and the effect on GLUT1 expression determined. GLUT1 mRNA was down-regulated only when HIF-2α was silenced, suggesting that HIF-2α is an upstream regulator of GLUT1. The HIF family of transcription factors have also been found to mediate the expression of GLUT1 in mouse ESCs, MEFs and cardiomyocytes [34,35,36]. However, in these cell types, it was HIF-1α, not HIF-2α which regulated GLUT1 expression. This represents a fundamental difference in the regulation of GLUT1 between mouse and human ESCs.

Using ChIP, HIF-2α was found to bind directly to the region containing a putative HRE in the proximal promoter of the GLUT1 gene only in hESCs cultured at 5% O_2. This was an exciting finding since although GLUT1 has been extensively studied as a hypoxia inducible gene, to the best of our knowledge this is the first report of HIF-2α binding directly to GLUT1. It is therefore possible that the increased expression of GLUT1 observed may be responsible for the greater uptake of glucose into hESCs cultured at 5% O_2. However, since many of the genes involved in glucose metabolism including hexokinase, phosphofructokinase, glyceraldehyde-3-phosphate dehydrogenase, enolase, pyruvate kinase and lactate dehydrogenase have also been shown to be regulated by environmental O_2, alternative mechanisms of regulation remain a possibility [34,37,38].

Summary

These studies demonstrate that hESCs utilise glucose as a predominant source of energy. Highly pluripotent hESCs cultured at 5% O_2 have a low level of O_2 consumption, consume high levels of glucose and produce large amounts of lactate. The onset of early differentiation, through the removal of FGF2 for 16 hours in hESCs cultured at 5% O_2, or by maintaining cells at 20% O_2 leads to a more oxidative metabolism, demonstrated by an increased consumption of O_2 and a decreased uptake of glucose and production of lactate. The rise in glucose uptake observed under hypoxic conditions corresponds to an increased expression of *GLUT1* which is directly regulated by HIF-2α. This data provides further metabolic support for maintaining hESCs under hypoxic conditions, rather than culturing at atmospheric levels of O_2. Finally, our data highlights the intrinsic importance of energy metabolism for hESC maintenance and may provide a novel method for the assessment of self-renewal.

Supporting Information

Figure S1 Typical morphology of Shef3 hESCs on day 3 post-passage cultured at 5% O_2 (A), 5% O_2 in the absence of FGF2 for 16 hours (5% O_2– FGF2; B) and 20% O_2. Scale bar = 100 μm.
(TIF)

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Author Contributions

Technical support: KLP. Conceived and designed the experiments: FDH CEF. Performed the experiments: CEF DRC FEC RP. Analyzed the data: CEF DRC FEC RP. Wrote the paper: FDH CEF TS RP DRC.

References

1. Thomson JA, Itskovitz-Eldor J, Shapiro SS, Waknitz MA, Swiergiel JJ, et al. (1998) Embryonic stem cell lines derived from human blastocysts. Science 282: 1145 1147.
2. Ludwig TE, Levenstein ME, Jones JM, Berggren WT, Mitchen ER, et al. (2006) Derivation of human embryonic stem cells in defined conditions. Nat Biotechnol 24: 185 187.
3. Ezashi T, Das P, Roberts RM (2005) Low O2 tensions and the prevention of differentiation of hES cells. Proceedings of the National Academy of Sciences of the United States of America 102: 4783 4788.
4. Westfall SD, Sachdev S, Das P, Hearne LB, Hannink M, et al. (2008) Identification of oxygen-sensitive transcriptional programs in human embryonic stem cells. Stem cells and development 17: 869 881.
5. Forristal CE, Wright KL, Hanley NA, Oreffo RO, Houghton FD (2010) Hypoxia inducible factors regulate pluripotency and proliferation in human embryonic stem cells cultured at reduced oxygen tensions. Reproduction 139: 85 97.
6. Lofstedt T, Fredlund E, Holmquist-Mengelbier L, Pietras A, Ovenberger M, et al. (2007) Hypoxia inducible factor-2alpha in cancer. Cell Cycle 6: 919 926.
7. Hu CJ, Wang LY, Chodosh LA, Keith B, Simon MC (2003) Differential roles of hypoxia-inducible factor 1alpha (HIF-1alpha) and HIF-2alpha in hypoxic gene regulation. Mol Cell Biol 23: 9361 9374.
8. Houghton FD, Hawkhead JA, Humpherson PG, Hogg JE, Balen AH, et al. (2002) Non-invasive amino acid turnover predicts human embryo developmental capacity. Hum Reprod 17: 999 1005.
9. Brison DR, Houghton FD, Falconer D, Roberts SA, Hawkhead J, et al. (2004) Identification of viable embryos in IVF by non-invasive measurement of amino acid turnover. Hum Reprod 19: 2319 2324.
10. Sathananthan H, Pera M, Trounson A (2002) The fine structure of human embryonic stem cells. Reproductive biomedicine online 4: 56 61.
11. Van Blerkom J, Davis P, Mathwig V, Alexander S (2002) Domains of high-polarized and low-polarized mitochondria may occur in mouse and human oocytes and early embryos. Hum Reprod 17: 393 406.
12. Henderson JK, Draper JS, Baillie HS, Fishel S, Thomson JA, et al. (2002) Preimplantation human embryos and embryonic stem cells show comparable expression of stage-specific embryonic antigens. Stem Cells 20: 329 337.
13. Draper JS, Fox V (2003) Human embryonic stem cells: multilineage differentiation and mechanisms of self-renewal. Arch Med Res 34: 558 564.
14. Hewitson LC, Leese HJ (1993) Energy metabolism of the trophectoderm and inner cell mass of the mouse blastocyst. J Exp Zool 267: 337 343.
15. Houghton FD (2006) Energy metabolism of the inner cell mass and trophectoderm of the mouse blastocyst. Differentiation 74: 11 18.
16. Varum S, Momcilovic O, Castro C, Ben-Yehudah A, Ramalho-Santos J, et al. (2009) Enhancement of human embryonic stem cell pluripotency through inhibition of the mitochondrial respiratory chain. Stem cell research 3: 142 156.
17. Varum S, Rodrigues AS, Moura MB, Momcilovic O, Easley CA, et al. (2011) Energy metabolism in human pluripotent stem cells and their differentiated counterparts. PLoS One 6: e20914.
18. Folmes CD, Nelson TJ, Martinez-Fernandez A, Arrell DK, Lindor JZ, et al. (2011) Somatic oxidative bioenergetics transitions into pluripotency-dependent glycolysis to facilitate nuclear reprogramming. Cell Metab 14: 264 271.
19. Tay JI, Rutherford AJ, Killick SR, Maguiness SD, Partridge RJ, et al. (1997) Human tubal fluid: production, nutrient composition and response to adrenergic agents. Hum Reprod 12: 2451 2456.
20. Petruzzelli R, Gaudino S, Amendola G, Sessa R, Puzone S, et al. (2010) Role of the cold shock domain protein A in the transcriptional regulation of HBG expression. Br J Haematol 150: 689 699.
21. Weinmann AS, Farnham PJ (2002) Identification of unknown target genes of human transcription factors using chromatin immunoprecipitation. Methods 26: 37 47.
22. Forsyth NR, Musio A, Vezzoni P, Simpson AH, Noble BS, et al. (2006) Physiologic oxygen enhances human embryonic stem cell clonal recovery and reduces chromosomal abnormalities. Cloning Stem Cells 8: 16 23.
23. Amit M, Margulets V, Segev H, Shariki K, Laevsky I, et al. (2003) Human feeder layers for human embryonic stem cells. Biology of reproduction 68: 2150 2156.
24. Xu C, Inokuma MS, Denham J, Golds K, Kundu P, et al. (2001) Feeder-free growth of undifferentiated human embryonic stem cells. Nature biotechnology 19: 971 974.
25. Kihira Y, Yamano N, Izawa-Ishizawa Y, Ishizawa K, Ikeda Y, et al. (2011) Basic fibroblast growth factor regulates glucose metabolism through glucose

transporter 1 induced by hypoxia-inducible factor-1alpha in adipocytes. Int J Biochem Cell Biol 43: 1602 1611.
26. Kondoh H, Lleonart ME, Nakashima Y, Yokode M, Tanaka M, et al. (2007) A high glycolytic flux supports the proliferative potential of murine embryonic stem cells. Antioxid Redox Signal 9: 293 299.
27. Chen CT, Shih YR, Kuo TK, Lee OK, Wei YH (2008) Coordinated changes of mitochondrial biogenesis and antioxidant enzymes during osteogenic differentiation of human mesenchymal stem cells. Stem Cells 26: 960 968.
28. Flier JS, Mueckler M, McCall AL, Lodish HF (1987) Distribution of glucose transporter messenger RNA transcripts in tissues of rat and man. J Clin Invest 79: 657 661.
29. Froehner SC, Davies A, Baldwin SA, Lienhard GE (1988) The blood-nerve barrier is rich in glucose transporter. J Neurocytol 17: 173 178.
30. Kahn BB, Flier JS (1990) Regulation of glucose-transporter gene expression in vitro and in vivo. Diabetes Care 13: 548 564.
31. Lee SH, Heo JS, Han HJ (2007) Effect of hypoxia on 2-deoxyglucose uptake and cell cycle regulatory protein expression of mouse embryonic stem cells: involvement of Ca^{2+}/PKC, MAPKs and HIF-1alpha. Cell Physiol Biochem 19: 269 282.
32. Hayashi M, Sakata M, Takeda T, Yamamoto T, Okamoto Y, et al. (2004) Induction of glucose transporter 1 expression through hypoxia-inducible factor 1alpha under hypoxic conditions in trophoblast-derived cells. J Endocrinol 183: 145 154.
33. Zelzer E, Levy Y, Kahana C, Shilo BZ, Rubinstein M, et al. (1998) Insulin induces transcription of target genes through the hypoxia-inducible factor HIF-1alpha/ARNT. The EMBO journal 17: 5085 5094.
34. Iyer NV, Kotch LE, Agani F, Leung SW, Laughner E, et al. (1998) Cellular and developmental control of O_2 homeostasis by hypoxia-inducible factor 1 alpha. Genes Dev 12: 149 162.
35. Seagroves TN, Ryan HE, Lu H, Wouters BG, Knapp M, et al. (2001) Transcription factor HIF-1 is a necessary mediator of the pasteur effect in mammalian cells. Mol Cell Biol 21: 3436 3444.
36. Wright G, Higgin JJ, Raines RT, Steenbergen C, Murphy E (2003) Activation of the prolyl hydroxylase oxygen-sensor results in induction of GLUT1, heme oxygenase-1, and nitric-oxide synthase proteins and confers protection from metabolic inhibition to cardiomyocytes. J Biol Chem 278: 20235 20239.
37. Ryan HE, Lo J, Johnson RS (1998) HIF-1 alpha is required for solid tumor formation and embryonic vascularization. EMBO J 17: 3005 3015.
38. Semenza GL, Jiang BH, Leung SW, Passantino R, Concordet JP, et al. (1996) Hypoxia response elements in the aldolase A, enolase 1, and lactate dehydrogenase A gene promoters contain essential binding sites for hypoxia-inducible factor 1. J Biol Chem 271: 32529 32537.
39. Panopoulos AD, Yanes O, Ruiz S, Kida YS, Diep D, et al. (2012) The metabolome of induced pluripotent stem cells reveals metabolic changes occurring in somatic cell reprogramming. Cell Res 22: 168 177.
40. Prigione A, Fauler B, Lurz R, Lehrach H, Adjaye J (2010) The senescence-related mitochondrial/oxidative stress pathway is repressed in human induced pluripotent stem cells. Stem Cells 28: 721 733.
41. Cowan CA, Klimanskaya I, McMahon J, Atienza J, Witmyer J, et al. (2004) Derivation of embryonic stem-cell lines from human blastocysts. N Engl J Med 350: 1353 1356.

Reference List

Abeyta, M.J., Clark, A.T., Rodriguez, R.T., Bodnar, M.S., Pera, R.A., and Firpo, M.T. (2004). Unique gene expression signatures of independently-derived human embryonic stem cell lines. Hum Mol Genet *13*, 601-608.

Adelman, D.M., Gertsenstein, M., Nagy, A., Simon, M.C., and Maltepe, E. (2000). Placental cell fates are regulated in vivo by HIF-mediated hypoxia responses. Genes Dev *14*, 3191-3203.

Agarwal, N., Hardt, T., Brero, A., Nowak, D., Rothbauer, U., Becker, A., Leonhardt, H., and Cardoso, M.C. (2007). MeCP2 interacts with HP1 and modulates its heterochromatin association during myogenic differentiation. Nucleic Acids Res *35*, 5402-5408.

Alper, J. (2009). Geron gets green light for human trial of ES cell-derived product. Nat Biotechnol *27*, 213-214.

Alt, J.R., Cleveland, J.L., Hannink, M., and Diehl, J.A. (2000). Phosphorylation-dependent regulation of cyclin D1 nuclear export and cyclin D1-dependent cellular transformation. Genes Dev *14*, 3102-3114.

Altun, G., Loring, J.F., and Laurent, L.C. (2010). DNA methylation in embryonic stem cells. J Cell Biochem *109*, 1-6.

Ambrosetti, D.C., Scholer, H.R., Dailey, L., and Basilico, C. (2000). Modulation of the activity of multiple transcriptional activation domains by the DNA binding domains mediates the synergistic action of Sox2 and Oct-3 on the fibroblast growth factor-4 enhancer. J Biol Chem *275*, 23387-23397.

Amit, M., Margulets, V., Segev, H., Shariki, K., Laevsky, I., Coleman, R., and Itskovitz-Eldor, J. (2003). Human feeder layers for human embryonic stem cells. Biol Reprod *68*, 2150-2156.

Amit, M., Shariki, C., Margulets, V., and Itskovitz-Eldor, J. (2004). Feeder layer- and serum-free culture of human embryonic stem cells. Biol Reprod *70*, 837-845.

An, W.G., Kanekal, M., Simon, M.C., Maltepe, E., Blagosklonny, M.V., and Neckers, L.M. (1998). Stabilization of wild-type p53 by hypoxia-inducible factor 1alpha. Nature *392*, 405-408.

Andrews, P.W., Banting, G., Damjanov, I., Arnaud, D., and Avner, P. (1984a). Three monoclonal antibodies defining distinct differentiation antigens associated with different high molecular weight polypeptides on the surface of human embryonal carcinoma cells. Hybridoma *3*, 347-361.

Andrews, P.W., Casper, J., Damjanov, I., Duggan-Keen, M., Giwercman, A., Hata, J., von Keitz, A., Looijenga, L.H., Millan, J.L., Oosterhuis, J.W., *et al.* (1996). Comparative analysis of cell surface antigens expressed by cell lines derived from human germ cell tumours. Int J Cancer *66*, 806-816.

Andrews, P.W., Damjanov, I., Simon, D., Banting, G.S., Carlin, C., Dracopoli, N.C., and Fogh, J. (1984b). Pluripotent embryonal carcinoma clones derived from the human teratocarcinoma cell line Tera-2. Differentiation in vivo and in vitro. Laboratory investigation; a journal of technical methods and pathology *50*, 147-162.

Andrukhiv, A., Costa, A.D., West, I.C., and Garlid, K.D. (2006). Opening mitoKATP increases superoxide generation from complex I of the electron transport chain. Am J Physiol Heart Circ Physiol *291*, H2067-2074.

Aplin, J.D. (1996). The cell biology of human implantation. Placenta *17*, 269-275.

Apostolou, E., Ferrari, F., Walsh, R.M., Bar-Nur, O., Stadtfeld, M., Cheloufi, S., Stuart, H.T., Polo, J.M., Ohsumi, T.K., Borowsky, M.L., *et al.* (2013). Genome-wide chromatin interactions of the Nanog locus in pluripotency, differentiation, and reprogramming. Cell Stem Cell *12*, 699-712.

Arany, Z., Huang, L.E., Eckner, R., Bhattacharya, S., Jiang, C., Goldberg, M.A., Bunn, H.F., and Livingston, D.M. (1996). An essential role for p300/CBP in the cellular response to hypoxia. Proc Natl Acad Sci U S A *93*, 12969-12973.

Askari, A.T., Unzek, S., Popovic, Z.B., Goldman, C.K., Forudi, F., Kiedrowski, M., Rovner, A., Ellis, S.G., Thomas, J.D., DiCorleto, P.E., *et al.* (2003). Effect of stromal-cell-derived factor 1 on stem-cell homing and tissue regeneration in ischaemic cardiomyopathy. Lancet *362*, 697-703.

Aso, T., Haque, D., Barstead, R.J., Conaway, R.C., and Conaway, J.W. (1996). The inducible elongin A elongation activation domain: structure, function and interaction with the elongin BC complex. Embo J *15*, 5557-5566.

Aso, T., Lane, W.S., Conaway, J.W., and Conaway, R.C. (1995). Elongin (SIII): a multisubunit regulator of elongation by RNA polymerase II. Science *269*, 1439-1443.

Avilion, A.A., Nicolis, S.K., Pevny, L.H., Perez, L., Vivian, N., and Lovell-Badge, R. (2003). Multipotent cell lineages in early mouse development depend on SOX2 function. Genes Dev *17*, 126-140.

Avvakumov, G.V., Walker, J.R., Xue, S., Li, Y., Duan, S., Bronner, C., Arrowsmith, C.H., and Dhe-Paganon, S. (2008). Structural basis for recognition of hemi-methylated DNA by the SRA domain of human UHRF1. Nature *455*, 822-825.

Badcock, G., Pigott, C., Goepel, J., and Andrews, P.W. (1999). The human embryonal carcinoma marker antigen TRA-1-60 is a sialylated keratan sulfate proteoglycan. Cancer Res *59*, 4715-4719.

Baker, D.E.C., Harrison, N.J., Maltby, E., Smith, K., Moore, H.D., Shaw, P.J., Heath, P.R., Holden, H., and Andrews, P.W. (2007). Adaptation to culture of human embryonic stem cells and oncogenesis in vivo. Nat Biotechnol *25*, 207-215.

Bannister, A.J., Schneider, R., Myers, F.A., Thorne, A.W., Crane-Robinson, C., and Kouzarides, T. (2005). Spatial distribution of di- and tri-methyl lysine 36 of histone H3 at active genes. J Biol Chem *280*, 17732-17736.

Basilico, C., and Moscatelli, D. (1992). The FGF family of growth factors and oncogenes. Advances in cancer research *59*, 115-165.

Batt, P.A., Gardner, D.K., and Cameron, A.W. (1991). Oxygen concentration and protein source affect the development of preimplantation goat embryos in vitro. Reprod Fertil Dev *3*, 601-607.

Bearzi, C., Rota, M., Hosoda, T., Tillmanns, J., Nascirnbene, A., De Angelis, A., Yasuzawa-Amano, S., Trofimova, I., Siggins, R.W., LeCapitaine, N., *et al.* (2007). Human cardiac stem cells. Proc Natl Acad Sci U S A *104*, 14068-14073.

Beckman, J.S., and Koppenol, W.H. (1996). Nitric oxide, superoxide, and peroxynitrite: the good, the bad, and ugly. Am J Physiol *271*, C1424-1437.

Behr, B., Ko, S.H., Wong, V.W., Gurtner, G.C., and Longaker, M.T. (2010). Stem cells. Plastic and reconstructive surgery *126*, 1163-1171.

Belaidi, E., Beguin, P.C., Levy, P., Ribuot, C., and Godin-Ribuot, D. (2008). Prevention of HIF-1 activation and iNOS gene targeting by low-dose cadmium results in loss of myocardial hypoxic preconditioning in the rat. Am J Physiol Heart Circ Physiol *294*, H901-908.

Bell, E.L., Klimova, T.A., Eisenbart, J., Moraes, C.T., Murphy, M.P., Budinger, G.R., and Chandel, N.S. (2007). The Qo site of the mitochondrial complex III is required for the transduction of hypoxic signaling via reactive oxygen species production. J Cell Biol *177*, 1029-1036.

Bernardi, M.L., Flechon, J.E., and Delouis, C. (1996). Influence of culture system and oxygen tension on the development of ovine zygotes matured and fertilized in vitro. J Reprod Fertil *106*, 161-167.

Bernstein, B.E., Mikkelsen, T.S., Xie, X., Kamal, M., Huebert, D.J., Cuff, J., Fry, B., Meissner, A., Wernig, M., Plath, K., *et al.* (2006). A bivalent chromatin structure marks key developmental genes in embryonic stem cells. Cell *125*, 315-326.

Beyer, S., Kristensen, M.M., Jensen, K.S., Johansen, J.V., and Staller, P. (2008). The histone demethylases JMJD1A and JMJD2B are transcriptional targets of hypoxia-inducible factor HIF. J Biol Chem *283*, 36542-36552.

Bhaskara, V.K., Mohanam, I., Rao, J.S., and Mohanam, S. (2012). Intermittent hypoxia regulates stem-like characteristics and differentiation of neuroblastoma cells. PLoS One *7*, e30905.

Bibikova, M., Chudin, E., Wu, B., Zhou, L., Garcia, E.W., Liu, Y., Shin, S., Plaia, T.W., Auerbach, J.M., Arking, D.E., *et al.* (2006). Human embryonic stem cells have a unique epigenetic signature. Genome Res *16*, 1075-1083.

Bibikova, M., Laurent, L.C., Ren, B., Loring, J.F., and Fan, J.B. (2008). Unraveling epigenetic regulation in embryonic stem cells. Cell Stem Cell *2*, 123-134.

Bird, A. (2002). DNA methylation patterns and epigenetic memory. Genes Dev *16*, 6-21.

Bogdanovic, O., and Veenstra, G.J. (2009). DNA methylation and methyl-CpG binding proteins: developmental requirements and function. Chromosoma *118*, 549-565.

Boiani, M., and Scholer, H.R. (2005). Regulatory networks in embryo-derived pluripotent stem cells. Nat Rev Mol Cell Biol *6*, 872-884.

Bolli, R., Shinmura, K., Tang, X.L., Kodani, E., Xuan, Y.T., Guo, Y., and Dawn, B. (2002). Discovery of a new function of cyclooxygenase (COX)-2: COX-2 is a cardioprotective protein that alleviates ischemia/reperfusion injury and mediates the late phase of preconditioning. Cardiovascular research *55*, 506-519.

Boyer, L.A., Lee, T.I., Cole, M.F., Johnstone, S.E., Levine, S.S., Zucker, J.P., Guenther, M.G., Kumar, R.M., Murray, H.L., Jenner, R.G., *et al.* (2005). Core transcriptional regulatory circuitry in human embryonic stem cells. Cell *122*, 947-956.

Boyer, L.A., Plath, K., Zeitlinger, J., Brambrink, T., Medeiros, L.A., Lee, T.I., Levine, S.S., Wernig, M., Tajonar, A., Ray, M.K., *et al.* (2006). Polycomb complexes repress developmental regulators in murine embryonic stem cells. Nature *441*, 349-353.

Bozhenok, L., Wade, P.A., and Varga-Weisz, P. (2002). WSTF-ISWI chromatin remodeling complex targets heterochromatic replication foci. Embo J *21*, 2231-2241.

Braam, S.R., Zeinstra, L., Litjens, S., Ward-van Oostwaard, D., van den Brink, S., van Laake, L., Lebrin, F., Kats, P., Hochstenbach, R., Passier, R., *et al.* (2008). Recombinant vitronectin is a functionally defined substrate that supports human embryonic stem cell self-renewal via alphavbeta5 integrin. Stem Cells *26*, 2257-2265.

Broxmeyer, H.E. (2010). Umbilical cord transplantation: epilogue. Semin Hematol *47*, 97-103.

Brumbaugh, J., Hou, Z., Russell, J.D., Howden, S.E., Yu, P., Ledvina, A.R., Coon, J.J., and Thomson, J.A. (2012). Phosphorylation regulates human OCT4. Proc Natl Acad Sci U S A *109*, 7162-7168.

Bulhak, A.A., Sjoquist, P.O., Xu, C.B., Edvinsson, L., and Pernow, J. (2006). Protection against myocardial ischaemia/reperfusion injury by PPAR-alpha activation is related to production of nitric oxide and endothelin-1. Basic research in cardiology *101*, 244-252.

Bultman, S.J., Gebuhr, T.C., Pan, H., Svoboda, P., Schultz, R.M., and Magnuson, T. (2006). Maternal BRG1 regulates zygotic genome activation in the mouse. Genes Dev *20*, 1744-1754.

Cai, Y., Jin, J., Tomomori-Sato, C., Sato, S., Sorokina, I., Parmely, T.J., Conaway, R.C., and Conaway, J.W. (2003a). Identification of new subunits of the multiprotein

mammalian TRRAP/TIP60-containing histone acetyltransferase complex. J Biol Chem *278*, 42733-42736.

Cai, Z., Manalo, D.J., Wei, G., Rodriguez, E.R., Fox-Talbot, K., Lu, H., Zweier, J.L., and Semenza, G.L. (2003b). Hearts from rodents exposed to intermittent hypoxia or erythropoietin are protected against ischemia-reperfusion injury. Circulation *108*, 79-85.

Calzi, F., Papaleo, E., Rabellotti, E., Ottolina, J., Vailati, S., Vigano, P., and Candiani, M. (2012). Exposure of embryos to oxygen at low concentration in a cleavage stage transfer program: reproductive outcomes in a time-series analysis. Clinical laboratory *58*, 997-1003.

Candan, Z.N., and Kahraman, S. (2010). Establishment and characterization of human embryonic stem cell lines, Turkey perspectives. In Vitro Cell Dev-An *46*, 345-355.

Cao, R., Wang, L., Wang, H., Xia, L., Erdjument-Bromage, H., Tempst, P., Jones, R.S., and Zhang, Y. (2002). Role of histone H3 lysine 27 methylation in Polycomb-group silencing. Science *298*, 1039-1043.

Cao, R., and Zhang, Y. (2004). SUZ12 is required for both the histone methyltransferase activity and the silencing function of the EED-EZH2 complex. Mol Cell *15*, 57-67.

Carmeliet, P., Dor, Y., Herbert, J.M., Fukumura, D., Brusselmans, K., Dewerchin, M., Neeman, M., Bono, F., Abramovitch, R., Maxwell, P., *et al.* (1998). Role of HIF-1alpha in hypoxia-mediated apoptosis, cell proliferation and tumour angiogenesis. Nature *394*, 485-490.

Chambers, I., Colby, D., Robertson, M., Nichols, J., Lee, S., Tweedie, S., and Smith, A. (2003). Functional expression cloning of Nanog, a pluripotency sustaining factor in embryonic stem cells. Cell *113*, 643-655.

Chambers, I., Silva, J., Colby, D., Nichols, J., Nijmeijer, B., Robertson, M., Vrana, J., Jones, K., Grotewold, L., and Smith, A. (2007). Nanog safeguards pluripotency and mediates germline development. Nature *450*, 1230-1234.

Chambers, I., and Smith, A. (2004). Self-renewal of teratocarcinoma and embryonic stem cells. Oncogene *23*, 7150-7160.

Charles, N., Ozawa, T., Squatrito, M., Bleau, A.M., Brennan, C.W., Hambardzumyan, D., and Holland, E.C. (2010). Perivascular nitric oxide activates notch signaling and promotes stem-like character in PDGF-induced glioma cells. Cell Stem Cell *6*, 141-152.

Chen, C., Pore, N., Behrooz, A., Ismail-Beigi, F., and Maity, A. (2001). Regulation of glut1 mRNA by hypoxia-inducible factor-1. Interaction between H-ras and hypoxia. J Biol Chem *276*, 9519-9525.

Chen, H., Yan, Y., Davidson, T.L., Shinkai, Y., and Costa, M. (2006). Hypoxic stress induces dimethylated histone H3 lysine 9 through histone methyltransferase G9a in mammalian cells. Cancer Res *66*, 9009-9016.

Chen, S., and Sang, N. (2011). Histone deacetylase inhibitors: the epigenetic therapeutics that repress hypoxia-inducible factors. J Biomed Biotechnol *2011*, 197946.

Chen, T., Ueda, Y., Dodge, J.E., Wang, Z., and Li, E. (2003). Establishment and maintenance of genomic methylation patterns in mouse embryonic stem cells by Dnmt3a and Dnmt3b. Mol Cell Biol *23*, 5594-5605.

Chen, X., Xu, H., Yuan, P., Fang, F., Huss, M., Vega, V.B., Wong, E., Orlov, Y.L., Zhang, W.W., Jiang, J.M., *et al.* (2008). Integration of external signaling pathways with the core transcriptional network in embryonic stem cells. Cell *133*, 1106-1117.

Cheng, L., Hammond, H., Ye, Z., Zhan, X., and Dravid, G. (2003). Human adult marrow cells support prolonged expansion of human embryonic stem cells in culture. Stem Cells *21*, 131-142.

Chew, J.L., Loh, Y.H., Zhang, W., Chen, X., Tam, W.L., Yeap, L.S., Li, P., Ang, Y.S., Lim, B., Robson, P., *et al.* (2005). Reciprocal transcriptional regulation of Pou5f1 and Sox2 via the Oct4/Sox2 complex in embryonic stem cells. Mol Cell Biol *25*, 6031-6046.

Choumerianou, D.M., Dimitriou, H., Perdikogianni, C., Martimianaki, G., Riminucci, M., and Kalmanti, M. (2008). Study of oncogenic transformation in ex vivo expanded mesenchymal cells, from paediatric bone marrow. Cell proliferation *41*, 909-922.

Chu, L., Jiang, Y., Hao, H., Xia, Y., Xu, J., Liu, Z., Verfaillie, C.M., and Zweier, J.L. (2008). Nitric oxide enhances Oct-4 expression in bone marrow stem cells and promotes endothelial differentiation. Eur J Pharmacol *591*, 59-65.

Chuang, J.C., and Jones, P.A. (2007). Epigenetics and microRNAs. Pediatr Res *61*, 24R-29R.

Cockman, M.E., Masson, N., Mole, D.R., Jaakkola, P., Chang, G.W., Clifford, S.C., Maher, E.R., Pugh, C.W., Ratcliffe, P.J., and Maxwell, P.H. (2000). Hypoxia inducible factor-alpha binding and ubiquitylation by the von Hippel-Lindau tumor suppressor protein. J Biol Chem *275*, 25733-25741.

Compernolle, V., Brusselmans, K., Acker, T., Hoet, P., Tjwa, M., Beck, H., Plaisance, S., Dor, Y., Keshet, E., Lupu, F., *et al.* (2002). Loss of HIF-2alpha and inhibition of VEGF impair fetal lung maturation, whereas treatment with VEGF prevents fatal respiratory distress in premature mice. Nat Med *8*, 702-710.

Cooper, S., Pera, M.F., Bennett, W., and Finch, J.T. (1992). A novel keratan sulphate proteoglycan from a human embryonal carcinoma cell line. Biochem J *286 (Pt 3)*, 959-966.

Coucouvanis, E., and Martin, G.R. (1999). BMP signaling plays a role in visceral endoderm differentiation and cavitation in the early mouse embryo. Development *126*, 535-546.

Coulet, F., Nadaud, S., Agrapart, M., and Soubrier, F. (2003). Identification of hypoxia-response element in the human endothelial nitric-oxide synthase gene promoter. J Biol Chem *278*, 46230-46240.

Covello, K.L., Kehler, J., Yu, H., Gordan, J.D., Arsham, A.M., Hu, C.J., Labosky, P.A., Simon, M.C., and Keith, B. (2006). HIF-2alpha regulates Oct-4: effects of hypoxia on stem cell function, embryonic development, and tumor growth. Genes Dev *20*, 557-570.

Cowan, C.A., Atienza, J., Melton, D.A., and Eggan, K. (2005). Nuclear reprogramming of somatic cells after fusion with human embryonic stem cells. Science *309*, 1369-1373.

D'Angelo, G., Duplan, E., Boyer, N., Vigne, P., and Frelin, C. (2003). Hypoxia up-regulates prolyl hydroxylase activity: a feedback mechanism that limits HIF-1 responses during reoxygenation. J Biol Chem *278*, 38183-38187.

D'Ippolito, G., Diabira, S., Howard, G.A., Roos, B.A., and Schiller, P.C. (2006). Low oxygen tension inhibits osteogenic differentiation and enhances stemness of human MIAMI cells. Bone *39*, 513-522.

da Silva Meirelles, L., Chagastelles, P.C., and Nardi, N.B. (2006). Mesenchymal stem cells reside in virtually all post-natal organs and tissues. J Cell Sci *119*, 2204-2213.

Dai, B., and Rasmussen, T.P. (2007). Global epiproteomic signatures distinguish embryonic stem cells from differentiated cells. Stem Cells *25*, 2567-2574.

Damert, A., Ikeda, E., and Risau, W. (1997). Activator-protein-1 binding potentiates the hypoxia-inducible factor-1-mediated hypoxia-induced transcriptional activation of vascular-endothelial growth factor expression in C6 glioma cells. Biochem J *327 (Pt 2)*, 419-423.

Dames, S.A., Martinez-Yamout, M., De Guzman, R.N., Dyson, H.J., and Wright, P.E. (2002). Structural basis for Hif-1 alpha /CBP recognition in the cellular hypoxic response. Proc Natl Acad Sci U S A *99*, 5271-5276.

Damjanov, I., Zhu, Z.M., Andrews, P.W., and Fenderson, B.A. (1994). Embryonal carcinoma cells differentiate into parietal endoderm via an intermediate stage corresponding to primitive endoderm. In vivo *8*, 967-973.

Dang, C.V., and Semenza, G.L. (1999). Oncogenic alterations of metabolism. Trends Biochem Sci *24*, 68-72.

Das, B., Bayat-Mokhtari, R., Tsui, M., Lotfi, S., Tsuchida, R., Felsher, D.W., and Yeger, H. (2012). HIF-2alpha suppresses p53 to enhance the stemness and regenerative potential of human embryonic stem cells. Stem Cells *30*, 1685-1695.

Das, R., Jahr, H., van Osch, G.J., and Farrell, E. (2010). The role of hypoxia in bone marrow-derived mesenchymal stem cells: considerations for regenerative medicine approaches. Tissue engineering Part B, Reviews *16*, 159-168.

Davuluri, R.V., Grosse, I., and Zhang, M.Q. (2001). Computational identification of promoters and first exons in the human genome. Nat Genet *29*, 412-417.

de Napoles, M., Mermoud, J.E., Wakao, R., Tang, Y.A., Endoh, M., Appanah, R., Nesterova, T.B., Silva, J., Otte, A.P., Vidal, M., *et al.* (2004). Polycomb group proteins Ring1A/B link ubiquitylation of histone H2A to heritable gene silencing and X inactivation. Dev Cell *7*, 663-676.

Dehne, N., and Brune, B. (2009). HIF-1 in the inflammatory microenvironment. Experimental cell research *315*, 1791-1797.

Denholtz, M., Bonora, G., Chronis, C., Splinter, E., de Laat, W., Ernst, J., Pellegrini, M., and Plath, K. (2013). Long-Range Chromatin Contacts in Embryonic Stem Cells Reveal a Role for Pluripotency Factors and Polycomb Proteins in Genome Organization. Cell Stem Cell.

Dhayalan, A., Rajavelu, A., Rathert, P., Tamas, R., Jurkowska, R.Z., Ragozin, S., and Jeltsch, A. (2010). The Dnmt3a PWWP domain reads histone 3 lysine 36 trimethylation and guides DNA methylation. J Biol Chem *285*, 26114-26120.

Dimos, J.T., Rodolfa, K.T., Niakan, K.K., Weisenthal, L.M., Mitsumoto, H., Chung, W., Croft, G.F., Saphier, G., Leibel, R., Goland, R., *et al.* (2008). Induced pluripotent stem cells generated from patients with ALS can be differentiated into motor neurons. Science *321*, 1218-1221.

Ding, J.J., Xu, H.L., Faiola, F., Ma'ayan, A., and Wang, J.L. (2012). Oct4 links multiple epigenetic pathways to the pluripotency network. Cell Res *22*, 155-167.

Dirscherl, S.S., and Krebs, J.E. (2004). Functional diversity of ISWI complexes. Biochem Cell Biol *82*, 482-489.

Discher, D.E., Mooney, D.J., and Zandstra, P.W. (2009). Growth factors, matrices, and forces combine and control stem cells. Science *324*, 1673-1677.

Dixon, J.R., Selvaraj, S., Yue, F., Kim, A., Li, Y., Shen, Y., Hu, M., Liu, J.S., and Ren, B. (2012). Topological domains in mammalian genomes identified by analysis of chromatin interactions. Nature *485*, 376-380.

Dumoulin, J.C., Meijers, C.J., Bras, M., Coonen, E., Geraedts, J.P., and Evers, J.L. (1999). Effect of oxygen concentration on human in-vitro fertilization and embryo culture. Hum Reprod *14*, 465-469.

Dvorak, P., Dvorakova, D., Koskova, S., Vodinska, M., Najvirtova, M., Krekac, D., and Hampl, A. (2005). Expression and potential role of fibroblast growth factor 2 and its receptors in human embryonic stem cells. Stem Cells *23*, 1200-1211.

Eberharter, A., and Becker, P.B. (2004). ATP-dependent nucleosome remodelling: factors and functions. J Cell Sci *117*, 3707-3711.

Eberharter, A., Ferrari, S., Langst, G., Straub, T., Imhof, A., Varga-Weisz, P., Wilm, M., and Becker, P.B. (2001). Acf1, the largest subunit of CHRAC, regulates ISWI-induced nucleosome remodelling. Embo J *20*, 3781-3788.

Ebert, B.L., and Bunn, H.F. (1998). Regulation of transcription by hypoxia requires a multiprotein complex that includes hypoxia-inducible factor 1, an adjacent transcription factor, and p300/CREB binding protein. Mol Cell Biol *18*, 4089-4096.

Economopoulou, M., Langer, H.F., Celeste, A., Orlova, V.V., Choi, E.Y., Ma, M., Vassilopoulos, A., Callen, E., Deng, C., Bassing, C.H., *et al.* (2009). Histone H2AX is integral to hypoxia-driven neovascularization. Nat Med *15*, 553-558.

Edmunds, J.W., Mahadevan, L.C., and Clayton, A.L. (2008). Dynamic histone H3 methylation during gene induction: HYPB/Setd2 mediates all H3K36 trimethylation. Embo J *27*, 406-420.

Eiselleova, L., Peterkova, I., Neradil, J., Slaninova, I., Hampl, A., and Dvorak, P. (2008). Comparative study of mouse and human feeder cells for human embryonic stem cells. The International journal of developmental biology *52*, 353-363.

Ellison, G.M., Vicinanza, C., Smith, A.J., Aquila, I., Leone, A., Waring, C.D., Henning, B.J., Stirparo, G.G., Papait, R., Scarfo, M., *et al.* (2013). Adult c-kit(pos) Cardiac Stem Cells Are Necessary and Sufficient for Functional Cardiac Regeneration and Repair. Cell *154*, 827-842.

Ema, M., Hirota, K., Mimura, J., Abe, H., Yodoi, J., Sogawa, K., Poellinger, L., and Fujii-Kuriyama, Y. (1999). Molecular mechanisms of transcription activation by HLF and HIF1alpha in response to hypoxia: their stabilization and redox signal-induced interaction with CBP/p300. Embo J *18*, 1905-1914.

Enver, T., Soneji, S., Joshi, C., Brown, J., Iborra, F., Orntoft, T., Thykjaer, T., Maltby, E., Smith, K., Abu Dawud, R., *et al.* (2005). Cellular differentiation hierarchies in normal and culture-adapted human embryonic stem cells. Hum Mol Genet *14*, 3129-3140.

Epstein, A.C., Gleadle, J.M., McNeill, L.A., Hewitson, K.S., O'Rourke, J., Mole, D.R., Mukherji, M., Metzen, E., Wilson, M.I., Dhanda, A., *et al.* (2001). C. elegans EGL-9

and mammalian homologs define a family of dioxygenases that regulate HIF by prolyl hydroxylation. Cell *107*, 43-54.

Evans, M.J., and Kaufman, M.H. (1981). Establishment in culture of pluripotential cells from mouse embryos. Nature *292*, 154-156.

Ezashi, T., Das, P., and Roberts, R.M. (2005). Low O2 tensions and the prevention of differentiation of hES cells. Proc Natl Acad Sci U S A *102*, 4783-4788.

Fandrey, J., Frede, S., and Jelkmann, W. (1994). Role of hydrogen peroxide in hypoxia-induced erythropoietin production. Biochem J *303 (Pt 2)*, 507-510.

Farrell, P.B., and Foote, R.H. (1995). Beneficial effects of culturing rabbit zygotes to blastocysts in 5% oxygen and 10% carbon dioxide. J Reprod Fertil *103*, 127-130.

Farthing, C.R., Ficz, G., Ng, R.K., Chan, C.F., Andrews, S., Dean, W., Hemberger, M., and Reik, W. (2008). Global mapping of DNA methylation in mouse promoters reveals epigenetic reprogramming of pluripotency genes. PLoS Genet *4*, e1000116.

Fath, D.M., Kong, X., Liang, D., Lin, Z., Chou, A., Jiang, Y., Fang, J., Caro, J., and Sang, N. (2006). Histone deacetylase inhibitors repress the transactivation potential of hypoxia-inducible factors independently of direct acetylation of HIF-alpha. J Biol Chem *281*, 13612-13619.

Fazzio, T.G., Huff, J.T., and Panning, B. (2008). An RNAi screen of chromatin proteins identifies Tip60-p400 as a regulator of embryonic stem cell identity. Cell *134*, 162-174.

Feldman, N., Gerson, A., Fang, J., Li, E., Zhang, Y., Shinkai, Y., Cedar, H., and Bergman, Y. (2006). G9a-mediated irreversible epigenetic inactivation of Oct-3/4 during early embryogenesis. Nat Cell Biol *8*, 188-194.

Feldser, D., Agani, F., Iyer, N.V., Pak, B., Ferreira, G., and Semenza, G.L. (1999). Reciprocal positive regulation of hypoxia-inducible factor 1alpha and insulin-like growth factor 2. Cancer Res *59*, 3915-3918.

Feng, S., Jacobsen, S.E., and Reik, W. (2010). Epigenetic reprogramming in plant and animal development. Science *330*, 622-627.

Fischer, B., and Bavister, B.D. (1993). Oxygen tension in the oviduct and uterus of rhesus monkeys, hamsters and rabbits. J Reprod Fertil *99*, 673-679.

Fish, J.E., Matouk, C.C., Rachlis, A., Lin, S., Tai, S.C., D'Abreo, C., and Marsden, P.A. (2005). The expression of endothelial nitric-oxide synthase is controlled by a cell-specific histone code. J Biol Chem *280*, 24824-24838.

Fish, J.E., Yan, M.S., Matouk, C.C., St Bernard, R., Ho, J.J., Gavryushova, A., Srivastava, D., and Marsden, P.A. (2010). Hypoxic repression of endothelial nitric-oxide synthase transcription is coupled with eviction of promoter histones. J Biol Chem *285*, 810-826.

Flamme, I., Frohlich, T., von Reutern, M., Kappel, A., Damert, A., and Risau, W. (1997). HRF, a putative basic helix-loop-helix-PAS-domain transcription factor is closely related to hypoxia-inducible factor-1 alpha and developmentally expressed in blood vessels. Mech Dev *63*, 51-60.

Flier, J.S., Mueckler, M., McCall, A.L., and Lodish, H.F. (1987). Distribution of glucose transporter messenger RNA transcripts in tissues of rat and man. J Clin Invest *79*, 657-661.

Flomenberg, N., Devine, S.M., Dipersio, J.F., Liesveld, J.L., McCarty, J.M., Rowley, S.D., Vesole, D.H., Badel, K., and Calandra, G. (2005). The use of AMD3100 plus G-CSF for autologous hematopoietic progenitor cell mobilization is superior to G-CSF alone. Blood *106*, 1867-1874.

Folmes, C.D., Nelson, T.J., Martinez-Fernandez, A., Arrell, D.K., Lindor, J.Z., Dzeja, P.P., Ikeda, Y., Perez-Terzic, C., and Terzic, A. (2011). Somatic oxidative bioenergetics transitions into pluripotency-dependent glycolysis to facilitate nuclear reprogramming. Cell Metab *14*, 264-271.

Forristal, C.E., Christensen, D.R., Chinnery, F.E., Petruzzelli, R., Parry, K.L., Sanchez-Elsner, T., and Houghton, F.D. (2013). Environmental oxygen tension regulates the energy metabolism and self-renewal of human embryonic stem cells. PLoS One *8*, e62507.

Forristal, C.E., Wright, K.L., Hanley, N.A., Oreffo, R.O., and Houghton, F.D. (2010). Hypoxia inducible factors regulate pluripotency and proliferation in human embryonic stem cells cultured at reduced oxygen tensions. Reproduction *139*, 85-97.

Forsyth, N.R., Musio, A., Vezzoni, P., Simpson, A.H., Noble, B.S., and McWhir, J. (2006). Physiologic oxygen enhances human embryonic stem cell clonal recovery and reduces chromosomal abnormalities. Cloning Stem Cells *8*, 16-23.

Fouse, S.D., Shen, Y., Pellegrini, M., Cole, S., Meissner, A., Van Neste, L., Jaenisch, R., and Fan, G. (2008). Promoter CpG methylation contributes to ES cell gene regulation in parallel with Oct4/Nanog, PcG complex, and histone H3 K4/K27 trimethylation. Cell Stem Cell *2*, 160-169.

Francis, K.R., and Wei, L. (2010). Human embryonic stem cell neural differentiation and enhanced cell survival promoted by hypoxic preconditioning. Cell Death Dis *1*, e22.

Francis, N.J., Kingston, R.E., and Woodcock, C.L. (2004). Chromatin compaction by a polycomb group protein complex. Science *306*, 1574-1577.

Francis, N.J., Saurin, A.J., Shao, Z., and Kingston, R.E. (2001). Reconstitution of a functional core polycomb repressive complex. Mol Cell *8*, 545-556.

Froehner, S.C., Davies, A., Baldwin, S.A., and Lienhard, G.E. (1988). The blood-nerve barrier is rich in glucose transporter. J Neurocytol *17*, 173-178.

Fujita, N., Watanabe, S., Ichimura, T., Tsuruzoe, S., Shinkai, Y., Tachibana, M., Chiba, T., and Nakao, M. (2003). Methyl-CpG binding domain 1 (MBD1) interacts with the Suv39h1-HP1 heterochromatic complex for DNA methylation-based transcriptional repression. J Biol Chem *278*, 24132-24138.

Galson, D.L., Tsuchiya, T., Tendler, D.S., Huang, L.E., Ren, Y., Ogura, T., and Bunn, H.F. (1995). The orphan receptor hepatic nuclear factor 4 functions as a transcriptional activator for tissue-specific and hypoxia-specific erythropoietin gene expression and is antagonized by EAR3/COUP-TF1. Mol Cell Biol *15*, 2135-2144.

Gao, W.M., Chadha, M.S., Kline, A.E., Clark, R.S., Kochanek, P.M., Dixon, C.E., and Jenkins, L.W. (2006). Immunohistochemical analysis of histone H3 acetylation and methylation--evidence for altered epigenetic signaling following traumatic brain injury in immature rats. Brain Res *1070*, 31-34.

Gao, X., Tate, P., Hu, P., Tjian, R., Skarnes, W.C., and Wang, Z. (2008). ES cell pluripotency and germ-layer formation require the SWI/SNF chromatin remodeling component BAF250a. Proc Natl Acad Sci U S A *105*, 6656-6661.

Gao, Z.H., Cox, J.L., Gilmore, J.M., Ormsbee, B.D., Mallanna, S.K., Washburn, M.P., and Rizzino, A. (2012). Determination of Protein Interactome of Transcription Factor Sox2 in Embryonic Stem Cells Engineered for Inducible Expression of Four Reprogramming Factors. Journal of Biological Chemistry *287*, 11384-11397.

Gardiner-Garden, M., and Frommer, M. (1987). CpG islands in vertebrate genomes. J Mol Biol *196*, 261-282.

Gaspar-Maia, A., Alajem, A., Polesso, F., Sridharan, R., Mason, M.J., Heidersbach, A., Ramalho-Santos, J., McManus, M.T., Plath, K., Meshorer, E., *et al.* (2009). Chd1 regulates open chromatin and pluripotency of embryonic stem cells. Nature *460*, 863-868.

Genbacev, O., Joslin, R., Damsky, C.H., Polliotti, B.M., and Fisher, S.J. (1996). Hypoxia alters early gestation human cytotrophoblast differentiation/invasion in vitro and models the placental defects that occur in preeclampsia. J Clin Invest *97*, 540-550.

Genbacev, O., Krtolica, A., Zdravkovic, T., Brunette, E., Powell, S., Nath, A., Caceres, E., McMaster, M., McDonagh, S., Li, Y., *et al.* (2005). Serum-free derivation of human

embryonic stem cell lines on human placental fibroblast feeders. Fertil Steril *83*, 1517-1529.

Genbacev, O., Zhou, Y., Ludlow, J.W., and Fisher, S.J. (1997). Regulation of human placental development by oxygen tension. Science *277*, 1669-1672.

Gidekel, S., and Bergman, Y. (2002). A unique developmental pattern of Oct-3/4 DNA methylation is controlled by a cis-demodification element. J Biol Chem *277*, 34521-34530.

Gidekel, S., Pizov, G., Bergman, Y., and Pikarsky, E. (2003). Oct-3/4 is a dose-dependent oncogenic fate determinant. Cancer Cell *4*, 361-370.

Gifford, C.A., Ziller, M.J., Gu, H., Trapnell, C., Donaghey, J., Tsankov, A., Shalek, A.K., Kelley, D.R., Shishkin, A.A., Issner, R., *et al.* (2013). Transcriptional and epigenetic dynamics during specification of human embryonic stem cells. Cell *153*, 1149-1163.

Gill, M.E., Erkek, S., and Peters, A.H. (2012). Parental epigenetic control of embryogenesis: a balance between inheritance and reprogramming? Curr Opin Cell Biol *24*, 387-396.

Giorgetti, A., Montserrat, N., Aasen, T., Gonzalez, F., Rodriguez-Piza, I., Vassena, R., Raya, A., Boue, S., Barrero, M.J., Corbella, B.A., *et al.* (2009). Generation of induced pluripotent stem cells from human cord blood using OCT4 and SOX2. Cell Stem Cell *5*, 353-357.

Goda, N., Ryan, H.E., Khadivi, B., McNulty, W., Rickert, R.C., and Johnson, R.S. (2003). Hypoxia-inducible factor 1alpha is essential for cell cycle arrest during hypoxia. Mol Cell Biol *23*, 359-369.

Godlewski, J., Nowicki, M.O., Bronisz, A., Williams, S., Otsuki, A., Nuovo, G., Raychaudhury, A., Newton, H.B., Chiocca, E.A., and Lawler, S. (2008). Targeting of

the Bmi-1 oncogene/stem cell renewal factor by microRNA-128 inhibits glioma proliferation and self-renewal. Cancer Res *68*, 9125-9130.

Goldberg, M.A., Dunning, S.P., and Bunn, H.F. (1988). Regulation of the erythropoietin gene: evidence that the oxygen sensor is a heme protein. Science *242*, 1412-1415.

Gordan, J.D., Bertout, J.A., Hu, C.J., Diehl, J.A., and Simon, M.C. (2007). HIF-2alpha promotes hypoxic cell proliferation by enhancing c-myc transcriptional activity. Cancer Cell *11*, 335-347.

Gore, A., Li, Z., Fung, H.L., Young, J.E., Agarwal, S., Antosiewicz-Bourget, J., Canto, I., Giorgetti, A., Israel, M.A., Kiskinis, E., *et al.* (2011). Somatic coding mutations in human induced pluripotent stem cells. Nature *471*, 63-67.

Gorlach, A., Diebold, I., Schini-Kerth, V.B., Berchner-Pfannschmidt, U., Roth, U., Brandes, R.P., Kietzmann, T., and Busse, R. (2001). Thrombin activates the hypoxia-inducible factor-1 signaling pathway in vascular smooth muscle cells: Role of the p22(phox)-containing NADPH oxidase. Circ Res *89*, 47-54.

Gorrini, C., Squatrito, M., Luise, C., Syed, N., Perna, D., Wark, L., Martinato, F., Sardella, D., Verrecchia, A., Bennett, S., *et al.* (2007). Tip60 is a haplo-insufficient tumour suppressor required for an oncogene-induced DNA damage response. Nature *448*, 1063-1067.

Goto, Y., Noda, Y., Mori, T., and Nakano, M. (1993). Increased generation of reactive oxygen species in embryos cultured in vitro. Free Radic Biol Med *15*, 69-75.

Goussetis, E., Manginas, A., Koutelou, M., Peristeri, I., Theodosaki, M., Kollaros, N., Leontiadis, E., Theodorakos, A., Paterakis, G., Karatasakis, G., *et al.* (2006). Intracoronary infusion of CD133+ and CD133-CD34+ selected autologous bone marrow progenitor cells in patients with chronic ischemic cardiomyopathy: cell isolation, adherence to the infarcted area, and body distribution. Stem Cells *24*, 2279-2283.

Gu, Y.Z., Moran, S.M., Hogenesch, J.B., Wartman, L., and Bradfield, C.A. (1998). Molecular characterization and chromosomal localization of a third alpha-class hypoxia inducible factor subunit, HIF3alpha. Gene Expr *7*, 205-213.

Guenther, M.G., Levine, S.S., Boyer, L.A., Jaenisch, R., and Young, R.A. (2007). A chromatin landmark and transcription initiation at most promoters in human cells. Cell *130*, 77-88.

Guerin, P., El Mouatassim, S., and Menezo, Y. (2001). Oxidative stress and protection against reactive oxygen species in the pre-implantation embryo and its surroundings. Human reproduction update *7*, 175-189.

Gyongyosi, M., Posa, A., Pavo, N., Hemetsberger, R., Kvakan, H., Steiner-Boker, S., Petrasi, Z., Manczur, F., Pavo, I.J., Edes, I.F., *et al.* (2010). Differential effect of ischaemic preconditioning on mobilisation and recruitment of haematopoietic and mesenchymal stem cells in porcine myocardial ischaemia-reperfusion. Thrombosis and haemostasis *104*, 376-384.

Haase, A., Olmer, R., Schwanke, K., Wunderlich, S., Merkert, S., Hess, C., Zweigerdt, R., Gruh, I., Meyer, J., Wagner, S., *et al.* (2009). Generation of induced pluripotent stem cells from human cord blood. Cell Stem Cell *5*, 434-441.

Haddad, J.J. (2002). Recombinant human interleukin (IL)-1 beta-mediated regulation of hypoxia-inducible factor-1 alpha (HIF-1 alpha) stabilization, nuclear translocation and activation requires an antioxidant/reactive oxygen species (ROS)-sensitive mechanism. Eur Cytokine Netw *13*, 250-260.

Haddad, J.J., and Land, S.C. (2001). A non-hypoxic, ROS-sensitive pathway mediates TNF-alpha-dependent regulation of HIF-1alpha. FEBS Lett *505*, 269-274.

Hagen, T., Taylor, C.T., Lam, F., and Moncada, S. (2003). Redistribution of intracellular oxygen in hypoxia by nitric oxide: effect on HIF1alpha. Science *302*, 1975-1978.

Hall, J.A., and Georgel, P.T. (2007). CHD proteins: a diverse family with strong ties. Biochem Cell Biol *85*, 463-476.

Hamiche, A., Sandaltzopoulos, R., Gdula, D.A., and Wu, C. (1999). ATP-dependent histone octamer sliding mediated by the chromatin remodeling complex NURF. Cell *97*, 833-842.

Hansis, C., Barreto, G., Maltry, N., and Niehrs, C. (2004). Nuclear reprogramming of human somatic cells by xenopus egg extract requires BRG1. Curr Biol *14*, 1475-1480.

Harris, A.L. (2002). Hypoxia--a key regulatory factor in tumour growth. Nat Rev Cancer *2*, 38-47.

Harvey, A.J., Kind, K.L., Pantaleon, M., Armstrong, D.T., and Thompson, J.G. (2004). Oxygen-regulated gene expression in bovine blastocysts. Biol Reprod *71*, 1108-1119.

Hatzimichael, E., Dasoula, A., Shah, R., Syed, N., Papoudou-Bai, A., Coley, H.M., Dranitsaris, G., Bourantas, K.L., Stebbing, J., and Crook, T. (2010). The prolyl-hydroxylase EGLN3 and not EGLN1 is inactivated by methylation in plasma cell neoplasia. Eur J Haematol *84*, 47-51.

Hatzimichael, E., Dranitsaris, G., Dasoula, A., Benetatos, L., Stebbing, J., Crook, T., and Bourantas, K.L. (2009). Von Hippel-Lindau methylation status in patients with multiple myeloma: a potential predictive factor for the development of bone disease. Clin Lymphoma Myeloma *9*, 239-242.

Hausenloy, D.J., Lim, S.Y., Ong, S.G., Davidson, S.M., and Yellon, D.M. (2010). Mitochondrial cyclophilin-D as a critical mediator of ischaemic preconditioning. Cardiovascular research *88*, 67-74.

Hausenloy, D.J., Mwamure, P.K., Venugopal, V., Harris, J., Barnard, M., Grundy, E., Ashley, E., Vichare, S., Di Salvo, C., Kolvekar, S., *et al.* (2007). Effect of remote ischaemic preconditioning on myocardial injury in patients undergoing coronary artery bypass graft surgery: a randomised controlled trial. Lancet *370*, 575-579.

Hawkins, R.D., Hon, G.C., Yang, C., Antosiewicz-Bourget, J.E., Lee, L.K., Ngo, Q.M., Klugman, S., Ching, K.A., Edsall, L.E., Ye, Z., *et al.* (2011). Dynamic chromatin states in human ES cells reveal potential regulatory sequences and genes involved in pluripotency. Cell Res *21*, 1393-1409.

Hayashi, M., Sakata, M., Takeda, T., Yamamoto, T., Okamoto, Y., Sawada, K., Kimura, A., Minekawa, R., Tahara, M., Tasaka, K., *et al.* (2004). Induction of glucose transporter 1 expression through hypoxia-inducible factor 1alpha under hypoxic conditions in trophoblast-derived cells. J Endocrinol *183*, 145-154.

He, A., Jiang, Y., Gui, C., Sun, Y., Li, J., and Wang, J.A. (2009). The antiapoptotic effect of mesenchymal stem cell transplantation on ischemic myocardium is enhanced by anoxic preconditioning. The Canadian journal of cardiology *25*, 353-358.

Heidbreder, M., Frohlich, F., Johren, O., Dendorfer, A., Qadri, F., and Dominiak, P. (2003). Hypoxia rapidly activates HIF-3alpha mRNA expression. Faseb J *17*, 1541-1543.

Heijmans, B.T., Tobi, E.W., Stein, A.D., Putter, H., Blauw, G.J., Susser, E.S., Slagboom, P.E., and Lumey, L.H. (2008). Persistent epigenetic differences associated with prenatal exposure to famine in humans. Proc Natl Acad Sci U S A *105*, 17046-17049.

Ho, L., Jothi, R., Ronan, J.L., Cui, K., Zhao, K., and Crabtree, G.R. (2009). An embryonic stem cell chromatin remodeling complex, esBAF, is an essential component of the core pluripotency transcriptional network. Proc Natl Acad Sci U S A *106*, 5187-5191.

Ho, L., Miller, E.L., Ronan, J.L., Ho, W.Q., Jothi, R., and Crabtree, G.R. (2011). esBAF facilitates pluripotency by conditioning the genome for LIF/STAT3 signalling and by regulating polycomb function. Nat Cell Biol *13*, 903-913.

Hobbs, C.A., Cleves, M.A., Melnyk, S., Zhao, W., and James, S.J. (2005). Congenital heart defects and abnormal maternal biomarkers of methionine and homocysteine metabolism. The American journal of clinical nutrition *81*, 147-153.

Hodges, E., Smith, A.D., Kendall, J., Xuan, Z., Ravi, K., Rooks, M., Zhang, M.Q., Ye, K., Bhattacharjee, A., Brizuela, L., *et al.* (2009). High definition profiling of mammalian DNA methylation by array capture and single molecule bisulfite sequencing. Genome Res *19*, 1593-1605.

Hoffman, L.M., and Carpenter, M.K. (2005). Characterization and culture of human embryonic stem cells. Nat Biotechnol *23*, 699-708.

Hongisto, H., Vuoristo, S., Mikhailova, A., Suuronen, R., Virtanen, I., Otonkoski, T., and Skottman, H. (2012). Laminin-511 expression is associated with the functionality of feeder cells in human embryonic stem cell culture. Stem Cell Res *8*, 97-108.

Horiuchi, A., Hayashi, T., Kikuchi, N., Hayashi, A., Fuseya, C., Shiozawa, T., and Konishi, I. (2012). Hypoxia upregulates ovarian cancer invasiveness via the binding of HIF-1alpha to a hypoxia-induced, methylation-free hypoxia response element of S100A4 gene. Int J Cancer *131*, 1755-1767.

Hu, C.J., Iyer, S., Sataur, A., Covello, K.L., Chodosh, L.A., and Simon, M.C. (2006). Differential regulation of the transcriptional activities of hypoxia-inducible factor 1 alpha (HIF-1alpha) and HIF-2alpha in stem cells. Mol Cell Biol *26*, 3514-3526.

Hu, C.J., Wang, L.Y., Chodosh, L.A., Keith, B., and Simon, M.C. (2003). Differential roles of hypoxia-inducible factor 1alpha (HIF-1alpha) and HIF-2alpha in hypoxic gene regulation. Mol Cell Biol *23*, 9361-9374.

Hu, X., Yu, S.P., Fraser, J.L., Lu, Z., Ogle, M.E., Wang, J.A., and Wei, L. (2008). Transplantation of hypoxia-preconditioned mesenchymal stem cells improves infarcted heart function via enhanced survival of implanted cells and angiogenesis. The Journal of thoracic and cardiovascular surgery *135*, 799-808.

Huang, K.T., Mikeska, T., Dobrovic, A., and Fox, S.B. (2010). DNA methylation analysis of the HIF-1alpha prolyl hydroxylase domain genes PHD1, PHD2, PHD3 and the factor inhibiting HIF gene FIH in invasive breast carcinomas. Histopathology *57*, 451-460.

Huang, L.E., Gu, J., Schau, M., and Bunn, H.F. (1998). Regulation of hypoxia-inducible factor 1alpha is mediated by an O2-dependent degradation domain via the ubiquitin-proteasome pathway. Proc Natl Acad Sci U S A *95*, 7987-7992.

Hubbi, M.E., Luo, W., Baek, J.H., and Semenza, G.L. (2011). MCM proteins are negative regulators of hypoxia-inducible factor 1. Mol Cell *42*, 700-712.

Huffmyer, J., and Raphael, J. (2009). Physiology and pharmacology of myocardial preconditioning and postconditioning. Semin Cardiothorac Vasc Anesth *13*, 5-18.

Hughes, C.S., Postovit, L.M., and Lajoie, G.A. (2010). Matrigel: a complex protein mixture required for optimal growth of cell culture. Proteomics *10*, 1886-1890.

Hung, S.C., Pochampally, R.R., Hsu, S.C., Sanchez, C., Chen, S.C., Spees, J., and Prockop, D.J. (2007). Short-term exposure of multipotent stromal cells to low oxygen increases their expression of CX3CR1 and CXCR4 and their engraftment in vivo. PLoS One *2*, e416.

Ieda, M., Fu, J.D., Delgado-Olguin, P., Vedantham, V., Hayashi, Y., Bruneau, B.G., and Srivastava, D. (2010). Direct reprogramming of fibroblasts into functional cardiomyocytes by defined factors. Cell *142*, 375-386.

Ignarro, L.J. (1990). Biosynthesis and metabolism of endothelium-derived nitric oxide. Annu Rev Pharmacol Toxicol *30*, 535-560.

Ignarro, L.J. (1991). Signal transduction mechanisms involving nitric oxide. Biochem Pharmacol *41*, 485-490.

Ii, M., Nishimura, H., Iwakura, A., Wecker, A., Eaton, E., Asahara, T., and Losordo, D.W. (2005). Endothelial progenitor cells are rapidly recruited to myocardium and mediate protective effect of ischemic preconditioning via "imported" nitric oxide synthase activity. Circulation *111*, 1114-1120.

Ikura, T., Ogryzko, V.V., Grigoriev, M., Groisman, R., Wang, J., Horikoshi, M., Scully, R., Qin, J., and Nakatani, Y. (2000). Involvement of the TIP60 histone acetylase complex in DNA repair and apoptosis. Cell *102*, 463-473.

Iliopoulos, O., Levy, A.P., Jiang, C., Kaelin, W.G., Jr., and Goldberg, M.A. (1996). Negative regulation of hypoxia-inducible genes by the von Hippel-Lindau protein. Proc Natl Acad Sci U S A *93*, 10595-10599.

International Stem Cell Initiative, C., Akopian, V., Andrews, P.W., Beil, S., Benvenisty, N., Brehm, J., Christie, M., Ford, A., Fox, V., Gokhale, P.J., *et al.* (2010). Comparison of defined culture systems for feeder cell free propagation of human embryonic stem cells. In vitro cellular & developmental biology Animal *46*, 247-258.

Ivan, M., Kondo, K., Yang, H., Kim, W., Valiando, J., Ohh, M., Salic, A., Asara, J.M., Lane, W.S., and Kaelin, W.G., Jr. (2001). HIFalpha targeted for VHL-mediated destruction by proline hydroxylation: implications for O2 sensing. Science *292*, 464-468.

Ivanova, N.B., Dimos, J.T., Schaniel, C., Hackney, J.A., Moore, K.A., and Lemischka, I.R. (2002). A stem cell molecular signature. Science *298*, 601-604.

Ivey, K.N., Muth, A., Arnold, J., King, F.W., Yeh, R.F., Fish, J.E., Hsiao, E.C., Schwartz, R.J., Conklin, B.R., Bernstein, H.S., *et al.* (2008). MicroRNA regulation of cell lineages in mouse and human embryonic stem cells. Cell Stem Cell *2*, 219-229.

Iyer, N.V., Kotch, L.E., Agani, F., Leung, S.W., Laughner, E., Wenger, R.H., Gassmann, M., Gearhart, J.D., Lawler, A.M., Yu, A.Y., *et al.* (1998). Cellular and developmental control of O2 homeostasis by hypoxia-inducible factor 1 alpha. Genes Dev *12*, 149-162.

Jaakkola, P., Mole, D.R., Tian, Y.M., Wilson, M.I., Gielbert, J., Gaskell, S.J., Kriegsheim, A., Hebestreit, H.F., Mukherji, M., Schofield, C.J., *et al.* (2001). Targeting of HIF-alpha to the von Hippel-Lindau ubiquitylation complex by O2-regulated prolyl hydroxylation. Science *292*, 468-472.

Jacob, S., and Moley, K.H. (2005). Gametes and embryo epigenetic reprogramming affect developmental outcome: implication for assisted reproductive technologies. Pediatr Res *58*, 437-446.

Jaderstad, J., Brismar, H., and Herlenius, E. (2010). Hypoxic preconditioning increases gap-junctional graft and host communication. Neuroreport *21*, 1126-1132.

Jaenisch, R., and Bird, A. (2003). Epigenetic regulation of gene expression: how the genome integrates intrinsic and environmental signals. Nat Genet *33 Suppl*, 245-254.

Jaussaud, J., Biais, M., Calderon, J., Chevaleyre, J., Duchez, P., Ivanovic, Z., Couffinhal, T., and Barandon, L. (2013). Hypoxia-preconditioned mesenchymal stromal cells improve cardiac function in a swine model of chronic myocardial ischaemia. European journal of cardio-thoracic surgery : official journal of the European Association for Cardio-thoracic Surgery *43*, 1050-1057.

Jenuwein, T., and Allis, C.D. (2001). Translating the histone code. Science *293*, 1074-1080.

Jewell, U.R., Kvietikova, I., Scheid, A., Bauer, C., Wenger, R.H., and Gassmann, M. (2001). Induction of HIF-1alpha in response to hypoxia is instantaneous. Faseb J *15*, 1312-1314.

Jia, D., Jurkowska, R.Z., Zhang, X., Jeltsch, A., and Cheng, X. (2007). Structure of Dnmt3a bound to Dnmt3L suggests a model for de novo DNA methylation. Nature *449*, 248-251.

Jiang, B.H., Rue, E., Wang, G.L., Roe, R., and Semenza, G.L. (1996). Dimerization, DNA binding, and transactivation properties of hypoxia-inducible factor 1. J Biol Chem *271*, 17771-17778.

Jiang, B.H., Zheng, J.Z., Leung, S.W., Roe, R., and Semenza, G.L. (1997). Transactivation and inhibitory domains of hypoxia-inducible factor 1alpha. Modulation of transcriptional activity by oxygen tension. J Biol Chem *272*, 19253-19260.

Jiao, B., Huang, X., Chan, C.B., Zhang, L., Wang, D., and Cheng, C.H. (2006). The co-existence of two growth hormone receptors in teleost fish and their differential signal transduction, tissue distribution and hormonal regulation of expression in seabream. J Mol Endocrinol *36*, 23-40.

Johnson, A.B., Denko, N., and Barton, M.C. (2008). Hypoxia induces a novel signature of chromatin modifications and global repression of transcription. Mutat Res *640*, 174-179.

Johnson, M.H., and Nasr-Esfahani, M.H. (1994). Radical solutions and cultural problems: could free oxygen radicals be responsible for the impaired development of preimplantation mammalian embryos in vitro? Bioessays *16*, 31-38.

Judson, R.L., Babiarz, J.E., Venere, M., and Blelloch, R. (2009). Embryonic stem cell-specific microRNAs promote induced pluripotency. Nat Biotechnol *27*, 459-461.

Jung, F., Palmer, L.A., Zhou, N., and Johns, R.A. (2000). Hypoxic regulation of inducible nitric oxide synthase via hypoxia inducible factor-1 in cardiac myocytes. Circ Res *86*, 319-325.

Jung, J.E., Lee, H.G., Cho, I.H., Chung, D.H., Yoon, S.H., Yang, Y.M., Lee, J.W., Choi, S., Park, J.W., Ye, S.K., *et al.* (2005). STAT3 is a potential modulator of HIF-1-mediated VEGF expression in human renal carcinoma cells. FASEB journal : official publication of the Federation of American Societies for Experimental Biology *19*, 1296-1298.

Jung, T., Schrader, N., Hellwig, M., Enssle, K.H., and Neumann, C. (1999). Soluble human interleukin-4 receptor is produced by activated T cells under the control of metalloproteinases. Int Arch Allergy Immunol *119*, 23-30.

Kagey, M.H., Newman, J.J., Bilodeau, S., Zhan, Y., Orlando, D.A., van Berkum, N.L., Ebmeier, C.C., Goossens, J., Rahl, P.B., Levine, S.S., *et al.* (2010). Mediator and cohesin connect gene expression and chromatin architecture. Nature *467*, 430-435.

Kahn, B.B., and Flier, J.S. (1990). Regulation of glucose-transporter gene expression in vitro and in vivo. Diabetes Care *13*, 548-564.

Kalkhoven, E. (2004). CBP and p300: HATs for different occasions. Biochem Pharmacol *68*, 1145-1155.

Kallio, P.J., Okamoto, K., O'Brien, S., Carrero, P., Makino, Y., Tanaka, H., and Poellinger, L. (1998). Signal transduction in hypoxic cells: inducible nuclear translocation and recruitment of the CBP/p300 coactivator by the hypoxia-inducible factor-1alpha. Embo J *17*, 6573-6586.

Kamura, T., Koepp, D.M., Conrad, M.N., Skowyra, D., Moreland, R.J., Iliopoulos, O., Lane, W.S., Kaelin, W.G., Jr., Elledge, S.J., Conaway, R.C., *et al.* (1999). Rbx1, a component of the VHL tumor suppressor complex and SCF ubiquitin ligase. Science *284*, 657-661.

Kasterstein, E., Strassburger, D., Komarovsky, D., Bern, O., Komsky, A., Raziel, A., Friedler, S., and Ron-El, R. (2013). The effect of two distinct levels of oxygen concentration on embryo development in a sibling oocyte study. Journal of assisted reproduction and genetics.

Kato, H., Tamamizu-Kato, S., and Shibasaki, F. (2004). Histone deacetylase 7 associates with hypoxia-inducible factor 1alpha and increases transcriptional activity. J Biol Chem *279*, 41966-41974.

Keith, B., Adelman, D.M., and Simon, M.C. (2001). Targeted mutation of the murine arylhydrocarbon receptor nuclear translocator 2 (Arnt2) gene reveals partial redundancy with Arnt. Proc Natl Acad Sci U S A *98*, 6692-6697.

Kenneth, N.S., Mudie, S., van Uden, P., and Rocha, S. (2009). SWI/SNF regulates the cellular response to hypoxia. J Biol Chem *284*, 4123-4131.

Keogh, C.L., Yu, S.P., and Wei, L. (2007). The effect of recombinant human erythropoietin on neurovasculature repair after focal ischemic stroke in neonatal rats. The Journal of pharmacology and experimental therapeutics *322*, 521-528.

Kido, M., Du, L., Sullivan, C.C., Li, X., Deutsch, R., Jamieson, S.W., and Thistlethwaite, P.A. (2005). Hypoxia-inducible factor 1-alpha reduces infarction and attenuates progression of cardiac dysfunction after myocardial infarction in the mouse. Journal of the American College of Cardiology *46*, 2116-2124.

Kim, D., Kim, C.H., Moon, J.I., Chung, Y.G., Chang, M.Y., Han, B.S., Ko, S., Yang, E., Cha, K.Y., Lanza, R., *et al.* (2009a). Generation of human induced pluripotent stem cells by direct delivery of reprogramming proteins. Cell Stem Cell *4*, 472-476.

Kim, H.W., Haider, H.K., Jiang, S., and Ashraf, M. (2009b). Ischemic preconditioning augments survival of stem cells via miR-210 expression by targeting caspase-8-associated protein 2. J Biol Chem *284*, 33161-33168.

Kim, J., Chu, J., Shen, X., Wang, J., and Orkin, S.H. (2008). An extended transcriptional network for pluripotency of embryonic stem cells. Cell *132*, 1049-1061.

Kim, S.H., Jeong, J.W., Park, J.A., Lee, J.W., Seo, J.H., Jung, B.K., Bae, M.K., and Kim, K.W. (2007). Regulation of the HIF-1alpha stability by histone deacetylases. Oncology reports *17*, 647-651.

Kim, V.N., Han, J., and Siomi, M.C. (2009c). Biogenesis of small RNAs in animals. Nat Rev Mol Cell Biol *10*, 126-139.

Kingham, E., White, K., Gadegaard, N., Dalby, M., and Oreffo, R. (2013). Nanotopographical cues augment mesenchymal differentiation of human embryonic stem cells. Small (Weinheim an der Bergstrasse, Germany) *9*, 2140-2151.

Kipreos, E.T., Lander, L.E., Wing, J.P., He, W.W., and Hedgecock, E.M. (1996). cul-1 is required for cell cycle exit in C. elegans and identifies a novel gene family. Cell *85*, 829-839.

Kleinman, H.K., and Martin, G.R. (2005). Matrigel: basement membrane matrix with biological activity. Seminars in cancer biology *15*, 378-386.

Klimanskaya, I., Chung, Y., Becker, S., Lu, S.J., and Lanza, R. (2006). Human embryonic stem cell lines derived from single blastomeres. Nature *444*, 481-485.

Kline, D.D., Peng, Y.J., Manalo, D.J., Semenza, G.L., and Prabhakar, N.R. (2002). Defective carotid body function and impaired ventilatory responses to chronic hypoxia in mice partially deficient for hypoxia-inducible factor 1 alpha. Proc Natl Acad Sci U S A *99*, 821-826.

Klose, R.J., and Zhang, Y. (2007). Regulation of histone methylation by demethylimination and demethylation. Nat Rev Mol Cell Biol *8*, 307-318.

Kobzik, L., Stringer, B., Balligand, J.L., Reid, M.B., and Stamler, J.S. (1995). Endothelial type nitric oxide synthase in skeletal muscle fibers: mitochondrial relationships. Biochem Biophys Res Commun *211*, 375-381.

Kojima, I., Tanaka, T., Inagi, R., Kato, H., Yamashita, T., Sakiyama, A., Ohneda, O., Takeda, N., Sata, M., Miyata, T., *et al.* (2007). Protective role of hypoxia-inducible factor-2alpha against ischemic damage and oxidative stress in the kidney. J Am Soc Nephrol *18*, 1218-1226.

Kornberg, R.D. (1974). Chromatin structure: a repeating unit of histones and DNA. Science *184*, 868-871.

Kotch, L.E., Iyer, N.V., Laughner, E., and Semenza, G.L. (1999). Defective vascularization of HIF-1alpha-null embryos is not associated with VEGF deficiency but with mesenchymal cell death. Dev Biol *209*, 254-267.

Kouzarides, T. (2007). Chromatin modifications and their function. Cell *128*, 693-705.

Krankel, N., Spinetti, G., Amadesi, S., and Madeddu, P. (2011). Targeting stem cell niches and trafficking for cardiovascular therapy. Pharmacology & therapeutics *129*, 62-81.

Krieg, A.J., Rankin, E.B., Chan, D., Razorenova, O., Fernandez, S., and Giaccia, A.J. (2010). Regulation of the histone demethylase JMJD1A by hypoxia-inducible factor 1 alpha enhances hypoxic gene expression and tumor growth. Mol Cell Biol *30*, 344-353.

Kubo, M., Li, T.S., Suzuki, R., Shirasawa, B., Morikage, N., Ohshima, M., Qin, S.L., and Hamano, K. (2008). Hypoxic preconditioning increases survival and angiogenic potency of peripheral blood mononuclear cells via oxidative stress resistance. Am J Physiol Heart Circ Physiol *294*, H590-595.

Kumral, A., Tuzun, F., Tugyan, K., Ozbal, S., Yilmaz, O., Yesilirmak, C.D., Duman, N., and Ozkan, H. (2013). Role of epigenetic regulatory mechanisms in neonatal hypoxic-ischemic brain injury. Early human development *89*, 165-173.

Kuroda, T., Tada, M., Kubota, H., Kimura, H., Hatano, S.Y., Suemori, H., Nakatsuji, N., and Tada, T. (2005). Octamer and Sox elements are required for transcriptional cis regulation of Nanog gene expression. Mol Cell Biol *25*, 2475-2485.

Kuzuya, T., Hoshida, S., Yamashita, N., Fuji, H., Oe, H., Hori, M., Kamada, T., and Tada, M. (1993). Delayed effects of sublethal ischemia on the acquisition of tolerance to ischemia. Circ Res *72*, 1293-1299.

Lando, D., Peet, D.J., Gorman, J.J., Whelan, D.A., Whitelaw, M.L., and Bruick, R.K. (2002). FIH-1 is an asparaginyl hydroxylase enzyme that regulates the transcriptional activity of hypoxia-inducible factor. Genes Dev *16*, 1466-1471.

Langst, G., Bonte, E.J., Corona, D.F., and Becker, P.B. (1999). Nucleosome movement by CHRAC and ISWI without disruption or trans-displacement of the histone octamer. Cell *97*, 843-852.

Lebkowski, J.S., Gold, J., Xu, C., Funk, W., Chiu, C.P., and Carpenter, M.K. (2001). Human embryonic stem cells: culture, differentiation, and genetic modification for regenerative medicine applications. Cancer J *7 Suppl 2*, S83-93.

Lee, J.S., Kim, Y., Bhin, J., Shin, H.J., Nam, H.J., Lee, S.H., Yoon, J.B., Binda, O., Gozani, O., Hwang, D., *et al.* (2011). Hypoxia-induced methylation of a pontin chromatin remodeling factor. Proc Natl Acad Sci U S A *108*, 13510-13515.

Lee, J.S., Kim, Y., Kim, I.S., Kim, B., Choi, H.J., Lee, J.M., Shin, H.J., Kim, J.H., Kim, J.Y., Seo, S.B., *et al.* (2010a). Negative regulation of hypoxic responses via induced Reptin methylation. Mol Cell *39*, 71-85.

Lee, K.J., Lee, K.Y., and Lee, Y.M. (2010b). Downregulation of a tumor suppressor RECK by hypoxia through recruitment of HDAC1 and HIF-1alpha to reverse HRE site in the promoter. Biochim Biophys Acta *1803*, 608-616.

Lee, M.G., Villa, R., Trojer, P., Norman, J., Yan, K.P., Reinberg, D., Di Croce, L., and Shiekhattar, R. (2007). Demethylation of H3K27 regulates polycomb recruitment and H2A ubiquitination. Science *318*, 447-450.

Lee, R.C., Feinbaum, R.L., and Ambros, V. (1993). The C. elegans heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14. Cell *75*, 843-854.

Lee, S.H., Wolf, P.L., Escudero, R., Deutsch, R., Jamieson, S.W., and Thistlethwaite, P.A. (2000). Early expression of angiogenesis factors in acute myocardial ischemia and infarction. N Engl J Med *342*, 626-633.

Lee, T.I., Jenner, R.G., Boyer, L.A., Guenther, M.G., Levine, S.S., Kumar, R.M., Chevalier, B., Johnstone, S.E., Cole, M.F., Isono, K., *et al.* (2006). Control of

developmental regulators by Polycomb in human embryonic stem cells. Cell *125*, 301-313.

Lee, Y., Jeon, K., Lee, J.T., Kim, S., and Kim, V.N. (2002). MicroRNA maturation: stepwise processing and subcellular localization. Embo J *21*, 4663-4670.

Lee, Y., Kim, M., Han, J., Yeom, K.H., Lee, S., Baek, S.H., and Kim, V.N. (2004). MicroRNA genes are transcribed by RNA polymerase II. Embo J *23*, 4051-4060.

Leeb, C., Jurga, M., McGuckin, C., Moriggl, R., and Kenner, L. (2010). Promising new sources for pluripotent stem cells. Stem Cell Rev *6*, 15-26.

Lengner, C.J., Gimelbrant, A.A., Erwin, J.A., Cheng, A.W., Guenther, M.G., Welstead, G.G., Alagappan, R., Frampton, G.M., Xu, P., Muffat, J., *et al.* (2010). Derivation of pre-X inactivation human embryonic stem cells under physiological oxygen concentrations. Cell *141*, 872-883.

Lennon, D.P., Edmison, J.M., and Caplan, A.I. (2001). Cultivation of rat marrow-derived mesenchymal stem cells in reduced oxygen tension: effects on in vitro and in vivo osteochondrogenesis. J Cell Physiol *187*, 345-355.

Leri, A., Kajstura, J., and Anversa, P. (2005). Cardiac stem cells and mechanisms of myocardial regeneration. Physiol Rev *85*, 1373-1416.

Lessard, J.A., and Crabtree, G.R. (2010). Chromatin regulatory mechanisms in pluripotency. Annu Rev Cell Dev Biol *26*, 503-532.

Levasseur, D.N., Wang, J., Dorschner, M.O., Stamatoyannopoulos, J.A., and Orkin, S.H. (2008). Oct4 dependence of chromatin structure within the extended Nanog locus in ES cells. Genes Dev *22*, 575-580.

Levesque, J.P., and Winkler, I.G. (2011). Hierarchy of immature hematopoietic cells related to blood flow and niche. Current opinion in hematology *18*, 220-225.

Levings, P.P., Zhou, Z., Vieira, K.F., Crusselle-Davis, V.J., and Bungert, J. (2006). Recruitment of transcription complexes to the beta-globin locus control region and transcription of hypersensitive site 3 prior to erythroid differentiation of murine embryonic stem cells. Febs J *273*, 746-755.

Li, E., Beard, C., and Jaenisch, R. (1993). Role for DNA methylation in genomic imprinting. Nature *366*, 362-365.

Li, H., Wallerath, T., Munzel, T., and Forstermann, U. (2002). Regulation of endothelial-type NO synthase expression in pathophysiology and in response to drugs. Nitric Oxide *7*, 149-164.

Li, Q.F., Wang, X.R., Yang, Y.W., and Lin, H. (2006). Hypoxia upregulates hypoxia inducible factor (HIF)-3alpha expression in lung epithelial cells: characterization and comparison with HIF-1alpha. Cell Res *16*, 548-558.

Li, W.L., Fraser, J.L., Yu, S.P., Zhu, J., Jiang, Y.J., and Wei, L. (2011). The role of VEGF/VEGFR2 signaling in peripheral stimulation-induced cerebral neurovascular regeneration after ischemic stroke in mice. Experimental brain research Experimentelle Hirnforschung Experimentation cerebrale *214*, 503-513.

Li, W.L., Yu, S.P., Ogle, M.E., Ding, X.S., and Wei, L. (2008). Enhanced neurogenesis and cell migration following focal ischemia and peripheral stimulation in mice. Developmental neurobiology *68*, 1474-1486.

Li, Z., Bao, S., Wu, Q., Wang, H., Eyler, C., Sathornsumetee, S., Shi, Q., Cao, Y., Lathia, J., McLendon, R.E., *et al.* (2009). Hypoxia-inducible factors regulate tumorigenic capacity of glioma stem cells. Cancer Cell *15*, 501-513.

Liadaki, K., Kho, A.T., Sanoudou, D., Schienda, J., Flint, A., Beggs, A.H., Kohane, I.S., and Kunkel, L.M. (2005). Side population cells isolated from different tissues share transcriptome signatures and express tissue-specific markers. Experimental cell research *303*, 360-374.

Liang, J., Wan, M., Zhang, Y., Gu, P., Xin, H., Jung, S.Y., Qin, J., Wong, J., Cooney, A.J., Liu, D., *et al.* (2008). Nanog and Oct4 associate with unique transcriptional repression complexes in embryonic stem cells. Nat Cell Biol *10*, 731-739.

Lim, J.W., and Bodnar, A. (2002). Proteome analysis of conditioned medium from mouse embryonic fibroblast feeder layers which support the growth of human embryonic stem cells. Proteomics *2*, 1187-1203.

Lin, J.S., Chen, Y.S., Chiang, H.S., and Ma, M.C. (2008). Hypoxic preconditioning protects rat hearts against ischaemia-reperfusion injury: role of erythropoietin on progenitor cell mobilization. The Journal of physiology *586*, 5757-5769.

Lindroos, B., Boucher, S., Chase, L., Kuokkanen, H., Huhtala, H., Haataja, R., Vemuri, M., Suuronen, R., and Miettinen, S. (2009). Serum-free, xeno-free culture media maintain the proliferation rate and multipotentiality of adipose stem cells in vitro. Cytotherapy *11*, 958-972.

Lister, R., Pelizzola, M., Dowen, R.H., Hawkins, R.D., Hon, G., Tonti-Filippini, J., Nery, J.R., Lee, L., Ye, Z., Ngo, Q.M., *et al.* (2009). Human DNA methylomes at base resolution show widespread epigenomic differences. Nature *462*, 315-322.

Lisztwan, J., Imbert, G., Wirbelauer, C., Gstaiger, M., and Krek, W. (1999). The von Hippel-Lindau tumor suppressor protein is a component of an E3 ubiquitin-protein ligase activity. Genes Dev *13*, 1822-1833.

Liu, H., Xue, W., Ge, G., Luo, X., Li, Y., Xiang, H., Ding, X., Tian, P., and Tian, X. (2010). Hypoxic preconditioning advances CXCR4 and CXCR7 expression by activating HIF-1alpha in MSCs. Biochem Biophys Res Commun *401*, 509-515.

Liu, Q., Liu, L., Zhao, Y., Zhang, J., Wang, D., Chen, J., He, Y., Wu, J., Zhang, Z., and Liu, Z. (2011). Hypoxia induces genomic DNA demethylation through the activation of HIF-1alpha and transcriptional upregulation of MAT2A in hepatoma cells. Molecular cancer therapeutics *10*, 1113-1123.

Loboda, A., Stachurska, A., Florczyk, U., Rudnicka, D., Jazwa, A., Wegrzyn, J., Kozakowska, M., Stalinska, K., Poellinger, L., Levonen, A.L., *et al.* (2009). HIF-1 induction attenuates Nrf2-dependent IL-8 expression in human endothelial cells. Antioxid Redox Signal *11*, 1501-1517.

Loh, Y.H., Zhang, W., Chen, X., George, J., and Ng, H.H. (2007). Jmjd1a and Jmjd2c histone H3 Lys 9 demethylases regulate self-renewal in embryonic stem cells. Genes Dev *21*, 2545-2557.

Lonergan, K.M., Iliopoulos, O., Ohh, M., Kamura, T., Conaway, R.C., Conaway, J.W., and Kaelin, W.G., Jr. (1998). Regulation of hypoxia-inducible mRNAs by the von Hippel-Lindau tumor suppressor protein requires binding to complexes containing elongins B/C and Cul2. Mol Cell Biol *18*, 732-741.

Ludwig, T.E., Bergendahl, V., Levenstein, M.E., Yu, J., Probasco, M.D., and Thomson, J.A. (2006). Feeder-independent culture of human embryonic stem cells. Nature methods *3*, 637-646.

Luo, W., Chang, R., Zhong, J., Pandey, A., and Semenza, G.L. (2012). Histone demethylase JMJD2C is a coactivator for hypoxia-inducible factor 1 that is required for breast cancer progression. Proc Natl Acad Sci U S A *109*, E3367-3376.

Luo, W., Hu, H., Chang, R., Zhong, J., Knabel, M., O'Meally, R., Cole, R.N., Pandey, A., and Semenza, G.L. (2011). Pyruvate kinase M2 is a PHD3-stimulated coactivator for hypoxia-inducible factor 1. Cell *145*, 732-744.

Luo, W., and Semenza, G.L. (2011). Pyruvate kinase M2 regulates glucose metabolism by functioning as a coactivator for hypoxia-inducible factor 1 in cancer cells. Oncotarget *2*, 551-556.

Ma, D.K., Chiang, C.H., Ponnusamy, K., Ming, G.L., and Song, H. (2008). G9a and Jhdm2a regulate embryonic stem cell fusion-induced reprogramming of adult neural stem cells. Stem Cells *26*, 2131-2141.

MacArthur, B.D., Please, C.P., and Oreffo, R.O. (2008). Stochasticity and the molecular mechanisms of induced pluripotency. PLoS One *3*, e3086.

MacArthur, B.D., Sevilla, A., Lenz, M., Muller, F.J., Schuldt, B.M., Schuppert, A.A., Ridden, S.J., Stumpf, P.S., Fidalgo, M., Ma'ayan, A., *et al.* (2012). Nanog-dependent feedback loops regulate murine embryonic stem cell heterogeneity. Nat Cell Biol *14*, 1139-1147.

Macfarlan, T.S., Gifford, W.D., Driscoll, S., Lettieri, K., Rowe, H.M., Bonanomi, D., Firth, A., Singer, O., Trono, D., and Pfaff, S.L. (2012). Embryonic stem cell potency fluctuates with endogenous retrovirus activity. Nature *487*, 57-63.

Mahaffey, K.W., Puma, J.A., Barbagelata, N.A., DiCarli, M.F., Leesar, M.A., Browne, K.F., Eisenberg, P.R., Bolli, R., Casas, A.C., Molina-Viamonte, V., *et al.* (1999). Adenosine as an adjunct to thrombolytic therapy for acute myocardial infarction: results of a multicenter, randomized, placebo-controlled trial: the Acute Myocardial Infarction STudy of ADenosine (AMISTAD) trial. Journal of the American College of Cardiology *34*, 1711-1720.

Mahfoudh-Boussaid, A., Zaouali, M.A., Hadj-Ayed, K., Miled, A.H., Saidane-Mosbahi, D., Rosello-Catafau, J., and Ben Abdennebi, H. (2012). Ischemic preconditioning reduces endoplasmic reticulum stress and upregulates hypoxia inducible factor-1alpha in ischemic kidney: the role of nitric oxide. Journal of biomedical science *19*, 7.

Mahon, P.C., Hirota, K., and Semenza, G.L. (2001). FIH-1: a novel protein that interacts with HIF-1alpha and VHL to mediate repression of HIF-1 transcriptional activity. Genes Dev *15*, 2675-2686.

Malladi, P., Xu, Y., Chiou, M., Giaccia, A.J., and Longaker, M.T. (2006). Effect of reduced oxygen tension on chondrogenesis and osteogenesis in adipose-derived mesenchymal cells. American journal of physiology Cell physiology *290*, C1139-1146.

Malliaras, K., and Marban, E. (2011). Cardiac cell therapy: where we've been, where we are, and where we should be headed. British medical bulletin *98*, 161-185.

Martin, C.M., Ferdous, A., Gallardo, T., Humphries, C., Sadek, H., Caprioli, A., Garcia, J.A., Szweda, L.I., Garry, M.G., and Garry, D.J. (2008). Hypoxia-inducible factor-2alpha transactivates Abcg2 and promotes cytoprotection in cardiac side population cells. Circ Res *102*, 1075-1081.

Martin, G.R. (1981). Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. Proc Natl Acad Sci U S A *78*, 7634-7638.

Martin, G.R., and Evans, M.J. (1975). Differentiation of clonal lines of teratocarcinoma cells: formation of embryoid bodies in vitro. Proc Natl Acad Sci U S A *72*, 1441-1445.

Masui, S., Nakatake, Y., Toyooka, Y., Shimosato, D., Yagi, R., Takahashi, K., Okochi, H., Okuda, A., Matoba, R., Sharov, A.A., *et al.* (2007). Pluripotency governed by Sox2 via regulation of Oct3/4 expression in mouse embryonic stem cells. Nat Cell Biol *9*, 625-635.

Mateo, J., Garcia-Lecea, M., Cadenas, S., Hernandez, C., and Moncada, S. (2003). Regulation of hypoxia-inducible factor-1alpha by nitric oxide through mitochondria-dependent and -independent pathways. Biochem J *376*, 537-544.

Mathieu, J., Zhang, Z., Nelson, A., Lamba, D.A., Reh, T.A., Ware, C., and Ruohola-Baker, H. (2013). Hypoxia Induces Re-Entry of Committed Cells into Pluripotency. Stem Cells.

Matsui, Y., Zsebo, K., and Hogan, B.L. (1992). Derivation of pluripotential embryonic stem cells from murine primordial germ cells in culture. Cell *70*, 841-847.

Maxwell, P.H., Dachs, G.U., Gleadle, J.M., Nicholls, L.G., Harris, A.L., Stratford, I.J., Hankinson, O., Pugh, C.W., and Ratcliffe, P.J. (1997). Hypoxia-inducible factor-1 modulates gene expression in solid tumors and influences both angiogenesis and tumor growth. Proc Natl Acad Sci U S A *94*, 8104-8109.

Maxwell, P.H., Wiesener, M.S., Chang, G.W., Clifford, S.C., Vaux, E.C., Cockman, M.E., Wykoff, C.C., Pugh, C.W., Maher, E.R., and Ratcliffe, P.J. (1999). The tumour suppressor protein VHL targets hypoxia-inducible factors for oxygen-dependent proteolysis. Nature *399*, 271-275.

McBurney, M.W., and Rogers, B.J. (1982). Isolation of male embryonal carcinoma cells and their chromosome replication patterns. Dev Biol *89*, 503-508.

McMahon, K.A., Hiew, S.Y., Hadjur, S., Veiga-Fernandes, H., Menzel, U., Price, A.J., Kioussis, D., Williams, O., and Brady, H.J. (2007). Mll has a critical role in fetal and adult hematopoietic stem cell self-renewal. Cell Stem Cell *1*, 338-345.

Meintjes, M., Chantilis, S.J., Douglas, J.D., Rodriguez, A.J., Guerami, A.R., Bookout, D.M., Barnett, B.D., and Madden, J.D. (2009). A controlled randomized trial evaluating the effect of lowered incubator oxygen tension on live births in a predominantly blastocyst transfer program. Hum Reprod *24*, 300-307.

Meissner, A., Mikkelsen, T.S., Gu, H., Wernig, M., Hanna, J., Sivachenko, A., Zhang, X., Bernstein, B.E., Nusbaum, C., Jaffe, D.B., *et al.* (2008). Genome-scale DNA methylation maps of pluripotent and differentiated cells. Nature *454*, 766-770.

Melvin, A., Mudie, S., and Rocha, S. (2011). The chromatin remodeler ISWI regulates the cellular response to hypoxia: role of FIH. Molecular biology of the cell *22*, 4171-4181.

Meshorer, E., and Misteli, T. (2006). Chromatin in pluripotent embryonic stem cells and differentiation. Nat Rev Mol Cell Biol *7*, 540-546.

Meshorer, E., Yellajoshula, D., George, E., Scambler, P.J., Brown, D.T., and Misteli, T. (2006). Hyperdynamic plasticity of chromatin proteins in pluripotent embryonic stem cells. Dev Cell *10*, 105-116.

Messina, E., De Angelis, L., Frati, G., Morrone, S., Chimenti, S., Fiordaliso, F., Salio, M., Battaglia, M., Latronico, M.V.G., Coletta, M., *et al.* (2004). Isolation and expansion of adult cardiac stem cells from human and murine heart. Circ Res *95*, 911-921.

Mikkelsen, T.S., Hanna, J., Zhang, X., Ku, M., Wernig, M., Schorderet, P., Bernstein, B.E., Jaenisch, R., Lander, E.S., and Meissner, A. (2008). Dissecting direct reprogramming through integrative genomic analysis. Nature *454*, 49-55.

Mikkelsen, T.S., Ku, M., Jaffe, D.B., Issac, B., Lieberman, E., Giannoukos, G., Alvarez, P., Brockman, W., Kim, T.K., Koche, R.P., *et al.* (2007). Genome-wide maps of chromatin state in pluripotent and lineage-committed cells. Nature *448*, 553-560.

Mimura, I., Nangaku, M., Kanki, Y., Tsutsumi, S., Inoue, T., Kohro, T., Yamamoto, S., Fujita, T., Shimamura, T., Suehiro, J., *et al.* (2012). Dynamic change of chromatin conformation in response to hypoxia enhances the expression of GLUT3 (SLC2A3) by cooperative interaction of hypoxia-inducible factor 1 and KDM3A. Mol Cell Biol *32*, 3018-3032.

Mitsui, K., Tokuzawa, Y., Itoh, H., Segawa, K., Murakami, M., Takahashi, K., Maruyama, M., Maeda, M., and Yamanaka, S. (2003). The homeoprotein Nanog is required for maintenance of pluripotency in mouse epiblast and ES cells. Cell *113*, 631-642.

Moncada, S., and Erusalimsky, J.D. (2002). Does nitric oxide modulate mitochondrial energy generation and apoptosis? Nat Rev Mol Cell Biol *3*, 214-220.

Moncada, S., and Palmer, R.M. (1991). Biosynthesis and actions of nitric oxide. Semin Perinatol *15*, 16-19.

Moreno-Manzano, V., Rodriguez-Jimenez, F.J., Acena-Bonilla, J.L., Fustero-Lardies, S., Erceg, S., Dopazo, J., Montaner, D., Stojkovic, M., and Sanchez-Puelles, J.M. (2010). FM19G11, a new hypoxia-inducible factor (HIF) modulator, affects stem cell differentiation status. J Biol Chem *285*, 1333-1342.

Morey, L., Pascual, G., Cozzuto, L., Roma, G., Wutz, A., Benitah, S.A., and Di Croce, L. (2012). Nonoverlapping functions of the Polycomb group Cbx family of proteins in embryonic stem cells. Cell Stem Cell *10*, 47-62.

Morgan, H.D., Santos, F., Green, K., Dean, W., and Reik, W. (2005). Epigenetic reprogramming in mammals. Hum Mol Genet *14 Spec No 1*, R47-58.

Mujoo, K., Krumenacker, J.S., Wada, Y., and Murad, F. (2006). Differential expression of nitric oxide signaling components in undifferentiated and differentiated human embryonic stem cells. Stem Cells Dev *15*, 779-787.

Murry, C.E., Jennings, R.B., and Reimer, K.A. (1986). Preconditioning with ischemia: a delay of lethal cell injury in ischemic myocardium. Circulation *74*, 1124-1136.

Murry, C.E., and Keller, G. (2008). Differentiation of embryonic stem cells to clinically relevant populations: lessons from embryonic development. Cell *132*, 661-680.

Natarajan, R., Salloum, F.N., Fisher, B.J., Kukreja, R.C., and Fowler, A.A., 3rd (2006). Hypoxia inducible factor-1 activation by prolyl 4-hydroxylase-2 gene silencing attenuates myocardial ischemia reperfusion injury. Circ Res *98*, 133-140.

Nichols, J., and Smith, A. (2009). Naive and primed pluripotent states. Cell Stem Cell *4*, 487-492.

Nichols, J., Zevnik, B., Anastassiadis, K., Niwa, H., Klewe-Nebenius, D., Chambers, I., Scholer, H., and Smith, A. (1998). Formation of pluripotent stem cells in the mammalian embryo depends on the POU transcription factor Oct4. Cell *95*, 379-391.

Nishi, H., Nakada, T., Kyo, S., Inoue, M., Shay, J.W., and Isaka, K. (2004). Hypoxia-inducible factor 1 mediates upregulation of telomerase (hTERT). Mol Cell Biol *24*, 6076-6083.

Nishimoto, M., Fukushima, A., Okuda, A., and Muramatsu, M. (1999). The gene for the embryonic stem cell coactivator UTF1 carries a regulatory element which selectively interacts with a complex composed of Oct-3/4 and Sox-2. Mol Cell Biol *19*, 5453-5465.

Noonan, E.J., Place, R.F., Pookot, D., Basak, S., Whitson, J.M., Hirata, H., Giardina, C., and Dahiya, R. (2009). miR-449a targets HDAC-1 and induces growth arrest in prostate cancer. Oncogene *28*, 1714-1724.

Nordhoff, V., Hubner, K., Bauer, A., Orlova, I., Malapetsa, A., and Scholer, H.R. (2001). Comparative analysis of human, bovine, and murine Oct-4 upstream promoter sequences. Mamm Genome *12*, 309-317.

Oh, H., Bradfute, S.B., Gallardo, T.D., Nakamura, T., Chi, X., Gaussin, V., Mishina, Y., Pocius, J., Michael, L.H., Behringer, R.R., *et al.* (2003). Cardiac progenitor cells from adult myocardium: Differentiation in vitro, in utero, and after homing to the infarct border. Circulation *108*, 144-145.

Ohh, M., Park, C.W., Ivan, M., Hoffman, M.A., Kim, T.Y., Huang, L.E., Pavletich, N., Chau, V., and Kaelin, W.G. (2000). Ubiquitination of hypoxia-inducible factor requires direct binding to the beta-domain of the von Hippel-Lindau protein. Nat Cell Biol *2*, 423-427.

Okami, J., Simeone, D.M., and Logsdon, C.D. (2004). Silencing of the hypoxia-inducible cell death protein BNIP3 in pancreatic cancer. Cancer Res *64*, 5338-5346.

Okano, M., Bell, D.W., Haber, D.A., and Li, E. (1999). DNA methyltransferases Dnmt3a and Dnmt3b are essential for de novo methylation and mammalian development. Cell *99*, 247-257.

Okita, K., Nakagawa, M., Hyenjong, H., Ichisaka, T., and Yamanaka, S. (2008). Generation of mouse induced pluripotent stem cells without viral vectors. Science *322*, 949-953.

Okumura-Nakanishi, S., Saito, M., Niwa, H., and Ishikawa, F. (2005). Oct-3/4 and Sox2 regulate Oct-3/4 gene in embryonic stem cells. J Biol Chem *280*, 5307-5317.

Orkin, S.H. (2000). Diversification of haematopoietic stem cells to specific lineages. Nature reviews Genetics *1*, 57-64.

Otani, J., Nankumo, T., Arita, K., Inamoto, S., Ariyoshi, M., and Shirakawa, M. (2009). Structural basis for recognition of H3K4 methylation status by the DNA methyltransferase 3A ATRX-DNMT3-DNMT3L domain. EMBO Rep *10*, 1235-1241.

Pabon, J.E., Jr., Findley, W.E., and Gibbons, W.E. (1989). The toxic effect of short exposures to the atmospheric oxygen concentration on early mouse embryonic development. Fertil Steril *51*, 896-900.

Pagani, F.D., DerSimonian, H., Zawadzka, A., Wetzel, K., Edge, A.S., Jacoby, D.B., Dinsmore, J.H., Wright, S., Aretz, T.H., Eisen, H.J., *et al.* (2003). Autologous skeletal myoblasts transplanted to ischemia-damaged myocardium in humans. Histological analysis of cell survival and differentiation. Journal of the American College of Cardiology *41*, 879-888.

Palmer, L.A., Doctor, A., Chhabra, P., Sheram, M.L., Laubach, V.E., Karlinsey, M.Z., Forbes, M.S., Macdonald, T., and Gaston, B. (2007). S-nitrosothiols signal hypoxia-mimetic vascular pathology. J Clin Invest *117*, 2592-2601.

Palmer, R.M., Ferrige, A.G., and Moncada, S. (1987). Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. Nature *327*, 524-526.

Park, I.H., Arora, N., Huo, H., Maherali, N., Ahfeldt, T., Shimamura, A., Lensch, M.W., Cowan, C., Hochedlinger, K., and Daley, G.Q. (2008a). Disease-specific induced pluripotent stem cells. Cell *134*, 877-886.

Park, I.H., Lerou, P.H., Zhao, R., Huo, H., and Daley, G.Q. (2008b). Generation of human-induced pluripotent stem cells. Nature protocols *3*, 1180-1186.

Park, J.H., Kim, S.J., Oh, E.J., Moon, S.Y., Roh, S.I., Kim, C.G., and Yoon, H.S. (2003). Establishment and maintenance of human embryonic stem cells on STO, a permanently growing cell line. Biol Reprod *69*, 2007-2014.

Pasini, D., Bracken, A.P., Hansen, J.B., Capillo, M., and Helin, K. (2007). The polycomb group protein Suz12 is required for embryonic stem cell differentiation. Mol Cell Biol *27*, 3769-3779.

Pasini, D., Cloos, P.A., Walfridsson, J., Olsson, L., Bukowski, J.P., Johansen, J.V., Bak, M., Tommerup, N., Rappsilber, J., and Helin, K. (2010). JARID2 regulates binding of the Polycomb repressive complex 2 to target genes in ES cells. Nature *464*, 306-310.

Patterson, A.J., Chen, M., Xue, Q., Xiao, D., and Zhang, L. (2010). Chronic prenatal hypoxia induces epigenetic programming of PKC{epsilon} gene repression in rat hearts. Circ Res *107*, 365-373.

Pause, A., Lee, S., Worrell, R.A., Chen, D.Y., Burgess, W.H., Linehan, W.M., and Klausner, R.D. (1997). The von Hippel-Lindau tumor-suppressor gene product forms a stable complex with human CUL-2, a member of the Cdc53 family of proteins. Proc Natl Acad Sci U S A *94*, 2156-2161.

Peerani, R., Bauwens, C., Kumacheva, E., and Zandstra, P.W. (2009). Patterning mouse and human embryonic stem cells using micro-contact printing. Methods in molecular biology *482*, 21-33.

Peng, J.C., Valouev, A., Swigut, T., Zhang, J., Zhao, Y., Sidow, A., and Wysocka, J. (2009). Jarid2/Jumonji coordinates control of PRC2 enzymatic activity and target gene occupancy in pluripotent cells. Cell *139*, 1290-1302.

Pera, M.F., Blasco-Lafita, M.J., Cooper, S., Mason, M., Mills, J., and Monaghan, P. (1988). Analysis of cell-differentiation lineage in human teratomas using new monoclonal antibodies to cytostructural antigens of embryonal carcinoma cells. Differentiation *39*, 139-149.

Pera, M.F., Cooper, S., Mills, J., and Parrington, J.M. (1989). Isolation and characterization of a multipotent clone of human embryonal carcinoma cells. Differentiation *42*, 10-23.

Peters, J.M. (1998). SCF and APC: the Yin and Yang of cell cycle regulated proteolysis. Curr Opin Cell Biol *10*, 759-768.

Peterson, C.L., and Laniel, M.A. (2004). Histones and histone modifications. Curr Biol *14*, R546-551.

Peterson, K.M., Aly, A., Lerman, A., Lerman, L.O., and Rodriguez-Porcel, M. (2011). Improved survival of mesenchymal stromal cell after hypoxia preconditioning: role of oxidative stress. Life sciences *88*, 65-73.

Pfister, O., Mouquet, F., Jain, M., Summer, R., Helmes, M., Fine, A., Colucci, W.S., and Liao, R. (2005). CD31(-) but not CD31(+) cardiac side population cells exhibit functional cardiomyogenic differentiation. Circ Res *97*, 52-61.

Pittenger, M.F., Mackay, A.M., Beck, S.C., Jaiswal, R.K., Douglas, R., Mosca, J.D., Moorman, M.A., Simonetti, D.W., Craig, S., and Marshak, D.R. (1999). Multilineage potential of adult human mesenchymal stem cells. Science *284*, 143-147.

Place, T.L., Fitzgerald, M.P., Venkataraman, S., Vorrink, S.U., Case, A.J., Teoh, M.L., and Domann, F.E. (2011). Aberrant promoter CpG methylation is a mechanism for impaired PHD3 expression in a diverse set of malignant cells. PLoS One *6*, e14617.

Pollard, P.J., Loenarz, C., Mole, D.R., McDonough, M.A., Gleadle, J.M., Schofield, C.J., and Ratcliffe, P.J. (2008). Regulation of Jumonji-domain-containing histone demethylases by hypoxia-inducible factor (HIF)-1alpha. Biochem J *416*, 387-394.

Pong, K. (2004). Ischaemic preconditioning: therapeutic implications for stroke? Expert opinion on therapeutic targets *8*, 125-139.

Post, W.S., Goldschmidt-Clermont, P.J., Wilhide, C.C., Heldman, A.W., Sussman, M.S., Ouyang, P., Milliken, E.E., and Issa, J.P. (1999). Methylation of the estrogen receptor gene is associated with aging and atherosclerosis in the cardiovascular system. Cardiovascular research *43*, 985-991.

Potier, E., Ferreira, E., Andriamanalijaona, R., Pujol, J.P., Oudina, K., Logeart-Avramoglou, D., and Petite, H. (2007). Hypoxia affects mesenchymal stromal cell osteogenic differentiation and angiogenic factor expression. Bone *40*, 1078-1087.

Prowse, A.B., McQuade, L.R., Bryant, K.J., Marcal, H., and Gray, P.P. (2007). Identification of potential pluripotency determinants for human embryonic stem cells following proteomic analysis of human and mouse fibroblast conditioned media. Journal of proteome research *6*, 3796-3807.

Przyklenk, K., and Whittaker, P. (2011). Remote ischemic preconditioning: current knowledge, unresolved questions, and future priorities. Journal of cardiovascular pharmacology and therapeutics *16*, 255-259.

Pugh, C.W., O'Rourke, J.F., Nagao, M., Gleadle, J.M., and Ratcliffe, P.J. (1997). Activation of hypoxia-inducible factor-1; definition of regulatory domains within the alpha subunit. J Biol Chem *272*, 11205-11214.

Pusarla, R.H., and Bhargava, P. (2005). Histones in functional diversification. Core histone variants. Febs J *272*, 5149-5168.

Radzisheuskaya, A., Chia Gle, B., dos Santos, R.L., Theunissen, T.W., Castro, L.F., Nichols, J., and Silva, J.C. (2013). A defined Oct4 level governs cell state transitions of pluripotency entry and differentiation into all embryonic lineages. Nat Cell Biol *15*, 579-590.

Rajala, K., Hakala, H., Panula, S., Aivio, S., Pihlajamaki, H., Suuronen, R., Hovatta, O., and Skottman, H. (2007). Testing of nine different xeno-free culture media for human embryonic stem cell cultures. Hum Reprod *22*, 1231-1238.

Ramalho-Santos, M., Yoon, S., Matsuzaki, Y., Mulligan, R.C., and Melton, D.A. (2002). "Stemness": transcriptional profiling of embryonic and adult stem cells. Science *298*, 597-600.

Rankin, E.B., Biju, M.P., Liu, Q., Unger, T.L., Rha, J., Johnson, R.S., Simon, M.C., Keith, B., and Haase, V.H. (2007). Hypoxia-inducible factor-2 (HIF-2) regulates hepatic erythropoietin in vivo. J Clin Invest *117*, 1068-1077.

Rao, R.R., Calhoun, J.D., Qin, X., Rekaya, R., Clark, J.K., and Stice, S.L. (2004). Comparative transcriptional profiling of two human embryonic stem cell lines. Biotechnology and bioengineering *88*, 273-286.

Raspaglio, G., Filippetti, F., Prislei, S., Penci, R., De Maria, I., Cicchillitti, L., Mozzetti, S., Scambia, G., and Ferlini, C. (2008). Hypoxia induces class III beta-tubulin gene expression by HIF-1alpha binding to its 3' flanking region. Gene *409*, 100-108.

Ratcliffe, P.J. (2007). HIF-1 and HIF-2: working alone or together in hypoxia? J Clin Invest *117*, 862-865.

Raval, R.R., Lau, K.W., Tran, M.G., Sowter, H.M., Mandriota, S.J., Li, J.L., Pugh, C.W., Maxwell, P.H., Harris, A.L., and Ratcliffe, P.J. (2005). Contrasting properties of hypoxia-inducible factor 1 (HIF-1) and HIF-2 in von Hippel-Lindau-associated renal cell carcinoma. Mol Cell Biol *25*, 5675-5686.

Ravi, R., Mookerjee, B., Bhujwalla, Z.M., Sutter, C.H., Artemov, D., Zeng, Q., Dillehay, L.E., Madan, A., Semenza, G.L., and Bedi, A. (2000). Regulation of tumor angiogenesis by p53-induced degradation of hypoxia-inducible factor 1alpha. Genes Dev *14*, 34-44.

Razin, A., and Cedar, H. (1991). DNA methylation and gene expression. Microbiol Rev *55*, 451-458.

Regan Anderson, T.M., Peacock, D.L., Daniel, A.R., Hubbard, G.K., Lofgren, K.A., Girard, B.J., Schorg, A., Hoogwijs, D., Wenger, R.H., Seagroves, T.N., *et al.* (2013).

Breast tumor kinase (Brk/PTK6) is a mediator of hypoxia-associated breast cancer progression. Cancer Res.

Reik, W., Dean, W., and Walter, J. (2001). Epigenetic reprogramming in mammalian development. Science *293*, 1089-1093.

Remenyi, A., Lins, K., Nissen, L.J., Reinbold, R., Scholer, H.R., and Wilmanns, M. (2003). Crystal structure of a POU/HMG/DNA ternary complex suggests differential assembly of Oct4 and Sox2 on two enhancers. Genes Dev *17*, 2048-2059.

Ren, H., Cao, Y., Zhao, Q., Li, J., Zhou, C., Liao, L., Jia, M., Zhao, Q., Cai, H., Han, Z.C., *et al.* (2006). Proliferation and differentiation of bone marrow stromal cells under hypoxic conditions. Biochem Biophys Res Commun *347*, 12-21.

Resnick, J.L., Bixler, L.S., Cheng, L., and Donovan, P.J. (1992). Long-term proliferation of mouse primordial germ cells in culture. Nature *359*, 550-551.

Reubinoff, B.E., Pera, M.F., Fong, C.Y., Trounson, A., and Bongso, A. (2000). Embryonic stem cell lines from human blastocysts: somatic differentiation in vitro. Nat Biotechnol *18*, 399-404.

Richards, M., Fong, C.Y., Chan, W.K., Wong, P.C., and Bongso, A. (2002). Human feeders support prolonged undifferentiated growth of human inner cell masses and embryonic stem cells. Nat Biotechnol *20*, 933-936.

Rinaudo, P.F., Giritharan, G., Talbi, S., Dobson, A.T., and Schultz, R.M. (2006). Effects of oxygen tension on gene expression in preimplantation mouse embryos. Fertil Steril *86*, 1252-1265, 1265 e1251-1236.

Rivieccio, M.A., Brochier, C., Willis, D.E., Walker, B.A., D'Annibale, M.A., McLaughlin, K., Siddiq, A., Kozikowski, A.P., Jaffrey, S.R., Twiss, J.L., *et al.* (2009). HDAC6 is a target for protection and regeneration following injury in the nervous system. Proc Natl Acad Sci U S A *106*, 19599-19604.

Robertson, K.D., Ait-Si-Ali, S., Yokochi, T., Wade, P.A., Jones, P.L., and Wolffe, A.P. (2000). DNMT1 forms a complex with Rb, E2F1 and HDAC1 and represses transcription from E2F-responsive promoters. Nat Genet *25*, 338-342.

Rochefort, G.Y., Delorme, B., Lopez, A., Herault, O., Bonnet, P., Charbord, P., Eder, V., and Domenech, J. (2006). Multipotential mesenchymal stem cells are mobilized into peripheral blood by hypoxia. Stem Cells *24*, 2202-2208.

Rodda, D.J., Chew, J.L., Lim, L.H., Loh, Y.H., Wang, B., Ng, H.H., and Robson, P. (2005). Transcriptional regulation of nanog by OCT4 and SOX2. J Biol Chem *280*, 24731-24737.

Rodesch, F., Simon, P., Donner, C., and Jauniaux, E. (1992). Oxygen measurements in endometrial and trophoblastic tissues during early pregnancy. Obstet Gynecol *80*, 283-285.

Rodin, S., Domogatskaya, A., Strom, S., Hansson, E.M., Chien, K.R., Inzunza, J., Hovatta, O., and Tryggvason, K. (2010). Long-term self-renewal of human pluripotent stem cells on human recombinant laminin-511. Nat Biotechnol *28*, 611-615.

Rolfs, A., Kvietikova, I., Gassmann, M., and Wenger, R.H. (1997). Oxygen-regulated transferrin expression is mediated by hypoxia-inducible factor-1. J Biol Chem *272*, 20055-20062.

Rosler, E.S., Fisk, G.J., Ares, X., Irving, J., Miura, T., Rao, M.S., and Carpenter, M.K. (2004). Long-term culture of human embryonic stem cells in feeder-free conditions. Dev Dyn *229*, 259-274.

Rosner, M.H., Vigano, M.A., Ozato, K., Timmons, P.M., Poirier, F., Rigby, P.W., and Staudt, L.M. (1990). A POU-domain transcription factor in early stem cells and germ cells of the mammalian embryo. Nature *345*, 686-692.

Rosova, I., Dao, M., Capoccia, B., Link, D., and Nolta, J.A. (2008). Hypoxic preconditioning results in increased motility and improved therapeutic potential of human mesenchymal stem cells. Stem Cells *26*, 2173-2182.

Ross, A.M., Gibbons, R.J., Stone, G.W., Kloner, R.A., Alexander, R.W., and Investigators, A.-I. (2005). A randomized, double-blinded, placebo-controlled multicenter trial of adenosine as an adjunct to reperfusion in the treatment of acute myocardial infarction (AMISTAD-II). Journal of the American College of Cardiology *45*, 1775-1780.

Rossler, J., Stolze, I., Frede, S., Freitag, P., Schweigerer, L., Havers, W., and Fandrey, J. (2004). Hypoxia-induced erythropoietin expression in human neuroblastoma requires a methylation free HIF-1 binding site. J Cell Biochem *93*, 153-161.

Ruas, J.L., Berchner-Pfannschmidt, U., Malik, S., Gradin, K., Fandrey, J., Roeder, R.G., Pereira, T., and Poellinger, L. (2010). Complex regulation of the transactivation function of hypoxia-inducible factor-1 alpha by direct interaction with two distinct domains of the CREB-binding protein/p300. J Biol Chem *285*, 2601-2609.

Ruas, J.L., Poellinger, L., and Pereira, T. (2005). Role of CBP in regulating HIF-1-mediated activation of transcription. J Cell Sci *118*, 301-311.

Sakata, M., Kurachi, H., Imai, T., Tadokoro, C., Yamaguchi, M., Yoshimoto, Y., Oka, Y., and Miyake, A. (1995). Increase in human placental glucose transporter-1 during pregnancy. Eur J Endocrinol *132*, 206-212.

Sandau, K.B., Fandrey, J., and Brune, B. (2001). Accumulation of HIF-1alpha under the influence of nitric oxide. Blood *97*, 1009-1015.

Sang, N., Fang, J., Srinivas, V., Leshchinsky, I., and Caro, J. (2002). Carboxyl-terminal transactivation activity of hypoxia-inducible factor 1 alpha is governed by a von Hippel-Lindau protein-independent, hydroxylation-regulated association with p300/CBP. Mol Cell Biol *22*, 2984-2992.

Santos-Rosa, H., Schneider, R., Bannister, A.J., Sherriff, J., Bernstein, B.E., Emre, N.C., Schreiber, S.L., Mellor, J., and Kouzarides, T. (2002). Active genes are tri-methylated at K4 of histone H3. Nature *419*, 407-411.

Sapountzi, V., Logan, I.R., and Robson, C.N. (2006). Cellular functions of TIP60. Int J Biochem Cell Biol *38*, 1496-1509.

Sasaki, N., Sato, T., Ohler, A., O'Rourke, B., and Marban, E. (2000). Activation of mitochondrial ATP-dependent potassium channels by nitric oxide. Circulation *101*, 439-445.

Sathananthan, H., Pera, M., and Trounson, A. (2002). The fine structure of human embryonic stem cells. Reprod Biomed Online *4*, 56-61.

Saxonov, S., Berg, P., and Brutlag, D.L. (2006). A genome-wide analysis of CpG dinucleotides in the human genome distinguishes two distinct classes of promoters. Proc Natl Acad Sci U S A *103*, 1412-1417.

Schaniel, C., Ang, Y.S., Ratnakumar, K., Cormier, C., James, T., Bernstein, E., Lemischka, I.R., and Paddison, P.J. (2009). Smarcc1/Baf155 couples self-renewal gene repression with changes in chromatin structure in mouse embryonic stem cells. Stem Cells *27*, 2979-2991.

Schleicher, M., Yu, J., Murata, T., Derakhshan, B., Atochin, D., Qian, L., Kashiwagi, S., Di Lorenzo, A., Harrison, K.D., Huang, P.L., *et al.* (2009). The Akt1-eNOS axis illustrates the specificity of kinase-substrate relationships in vivo. Sci Signal *2*, ra41.

Scholer, H.R. (1991). Octamania: the POU factors in murine development. Trends Genet *7*, 323-329.

Scortegagna, M., Ding, K., Oktay, Y., Gaur, A., Thurmond, F., Yan, L.J., Marck, B.T., Matsumoto, A.M., Shelton, J.M., Richardson, J.A., *et al.* (2003). Multiple organ pathology, metabolic abnormalities and impaired homeostasis of reactive oxygen species in Epas1-/- mice. Nat Genet *35*, 331-340.

Semenza, G.L. (2000a). HIF-1: mediator of physiological and pathophysiological responses to hypoxia. Journal of applied physiology *88*, 1474-1480.

Semenza, G.L. (2000b). Surviving ischemia: adaptive responses mediated by hypoxia-inducible factor 1. J Clin Invest *106*, 809-812.

Semenza, G.L. (2011). Hypoxia-inducible factor 1: regulator of mitochondrial metabolism and mediator of ischemic preconditioning. Biochim Biophys Acta *1813*, 1263-1268.

Semenza, G.L. (2012). Hypoxia-inducible factors in physiology and medicine. Cell *148*, 399-408.

Semenza, G.L., Nejfelt, M.K., Chi, S.M., and Antonarakis, S.E. (1991). Hypoxia-inducible nuclear factors bind to an enhancer element located 3' to the human erythropoietin gene. Proc Natl Acad Sci U S A *88*, 5680-5684.

Semenza, G.L., and Wang, G.L. (1992). A nuclear factor induced by hypoxia via de novo protein synthesis binds to the human erythropoietin gene enhancer at a site required for transcriptional activation. Mol Cell Biol *12*, 5447-5454.

Seo, H.W., Kim, E.J., Na, H., and Lee, M.O. (2009). Transcriptional activation of hypoxia-inducible factor-1alpha by HDAC4 and HDAC5 involves differential recruitment of p300 and FIH-1. FEBS Lett *583*, 55-60.

Shamblott, M.J., Axelman, J., Wang, S., Bugg, E.M., Littlefield, J.W., Donovan, P.J., Blumenthal, P.D., Huggins, G.R., and Gearhart, J.D. (1998). Derivation of pluripotent stem cells from cultured human primordial germ cells. Proc Natl Acad Sci U S A *95*, 13726-13731.

Sharif, J., Muto, M., Takebayashi, S., Suetake, I., Iwamatsu, A., Endo, T.A., Shinga, J., Mizutani-Koseki, Y., Toyoda, T., Okamura, K., *et al.* (2007). The SRA protein Np95 mediates epigenetic inheritance by recruiting Dnmt1 to methylated DNA. Nature *450*, 908-912.

Sharma, R.K., Zhou, Q., and Netland, P.A. (2008). Effect of oxidative preconditioning on neural progenitor cells. Brain Res *1243*, 19-26.

Shi, Y., and Whetstine, J.R. (2007). Dynamic regulation of histone lysine methylation by demethylases. Mol Cell *25*, 1-14.

Shirodkar, A.V., and Marsden, P.A. (2011). Epigenetics in cardiovascular disease. Curr Opin Cardiol *26*, 209-215.

Shogren-Knaak, M., Ishii, H., Sun, J.M., Pazin, M.J., Davie, J.R., and Peterson, C.L. (2006). Histone H4-K16 acetylation controls chromatin structure and protein interactions. Science *311*, 844-847.

Sikorski, T.W., Ficarro, S.B., Holik, J., Kim, T., Rando, O.J., Marto, J.A., and Buratowski, S. (2011). Sub1 and RPA Associate with RNA Polymerase II at Different Stages of Transcription. Mol Cell *44*, 397-409.

Singh, A.M., Hamazaki, T., Hankowski, K.E., and Terada, N. (2007). A heterogeneous expression pattern for Nanog in embryonic stem cells. Stem Cells *25*, 2534-2542.

Singh, S.K., Kagalwala, M.N., Parker-Thornburg, J., Adams, H., and Majumder, S. (2008). REST maintains self-renewal and pluripotency of embryonic stem cells. Nature *453*, 223-227.

Singhal, N., Graumann, J., Wu, G., Arauzo-Bravo, M.J., Han, D.W., Greber, B., Gentile, L., Mann, M., and Scholer, H.R. (2010). Chromatin-Remodeling Components of the BAF Complex Facilitate Reprogramming. Cell *141*, 943-955.

Sinkkonen, L., Hugenschmidt, T., Berninger, P., Gaidatzis, D., Mohn, F., Artus-Revel, C.G., Zavolan, M., Svoboda, P., and Filipowicz, W. (2008). MicroRNAs control de novo DNA methylation through regulation of transcriptional repressors in mouse embryonic stem cells. Nature structural & molecular biology *15*, 259-267.

Skottman, H., Stromberg, A.M., Matilainen, E., Inzunza, J., Hovatta, O., and Lahesmaa, R. (2006). Unique gene expression signature by human embryonic stem cells cultured under serum-free conditions correlates with their enhanced and prolonged growth in an undifferentiated stage. Stem Cells *24*, 151-167.

Skowronski, K., Dubey, S., Rodenhiser, D., and Coomber, B. (2010). Ischemia dysregulates DNA methyltransferases and p16INK4a methylation in human colorectal cancer cells. Epigenetics *5*, 547-556.

Skowyra, D., Craig, K.L., Tyers, M., Elledge, S.J., and Harper, J.W. (1997). F-box proteins are receptors that recruit phosphorylated substrates to the SCF ubiquitin-ligase complex. Cell *91*, 209-219.

Spangrude, G.J., Heimfeld, S., and Weissman, I.L. (1988). Purification and characterization of mouse hematopoietic stem cells. Science *241*, 58-62.

Stadtfeld, M., Nagaya, M., Utikal, J., Weir, G., and Hochedlinger, K. (2008). Induced pluripotent stem cells generated without viral integration. Science *322*, 945-949.

Stiehl, D.P., Jelkmann, W., Wenger, R.H., and Hellwig-Burgel, T. (2002). Normoxic induction of the hypoxia-inducible factor 1alpha by insulin and interleukin-1beta involves the phosphatidylinositol 3-kinase pathway. FEBS Lett *512*, 157-162.

Strohner, R., Nemeth, A., Jansa, P., Hofmann-Rohrer, U., Santoro, R., Langst, G., and Grummt, I. (2001). NoRC--a novel member of mammalian ISWI-containing chromatin remodeling machines. Embo J *20*, 4892-4900.

Tacchini, L., Bianchi, L., Bernelli-Zazzera, A., and Cairo, G. (1999). Transferrin receptor induction by hypoxia. HIF-1-mediated transcriptional activation and cell-specific post-transcriptional regulation. J Biol Chem *274*, 24142-24146.

Takahashi, K., and Yamanaka, S. (2006). Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell *126*, 663-676.

Takahashi, M., Keicho, K., Takahashi, H., Ogawa, H., Schultz, R.M., and Okano, A. (2000). Effect of oxidative stress on development and DNA damage in in-vitro cultured bovine embryos by comet assay. Theriogenology *54*, 137-145.

Tang, F., Barbacioru, C., Bao, S., Lee, C., Nordman, E., Wang, X., Lao, K., and Surani, M.A. (2010a). Tracing the derivation of embryonic stem cells from the inner cell mass by single-cell RNA-Seq analysis. Cell Stem Cell *6*, 468-478.

Tang, L., Nogales, E., and Ciferri, C. (2010b). Structure and function of SWI/SNF chromatin remodeling complexes and mechanistic implications for transcription. Prog Biophys Mol Biol *102*, 122-128.

Tang, Y.L., Zhu, W., Cheng, M., Chen, L., Zhang, J., Sun, T., Kishore, R., Phillips, M.I., Losordo, D.W., and Qin, G. (2009). Hypoxic preconditioning enhances the benefit of cardiac progenitor cell therapy for treatment of myocardial infarction by inducing CXCR4 expression. Circ Res *104*, 1209-1216.

Tanimoto, K., Makino, Y., Pereira, T., and Poellinger, L. (2000). Mechanism of regulation of the hypoxia-inducible factor-1 alpha by the von Hippel-Lindau tumor suppressor protein. Embo J *19*, 4298-4309.

Tarin, J.J. (1996). Potential effects of age-associated oxidative stress on mammalian oocytes/embryos. Mol Hum Reprod *2*, 717-724.

Tateishi, K., Ashihara, E., Takehara, N., Nomura, T., Honsho, S., Nakagami, T., Morikawa, S., Takahashi, T., Ueyama, T., Matsubara, H., *et al.* (2007). Clonally amplified cardiac stem cells are regulated by Sca-1 signaling for efficient cardiovascular regeneration. J Cell Sci *120*, 1791-1800.

Tausendschon, M., Dehne, N., and Brune, B. (2011). Hypoxia causes epigenetic gene regulation in macrophages by attenuating Jumonji histone demethylase activity. Cytokine *53*, 256-262.

Tejedo, J.R., Cahuana, G.M., Ramirez, R., Esbert, M., Jimenez, J., Sobrino, F., and Bedoya, F.J. (2004). nitric oxide triggers the phosphatidylinositol 3-kinase/Akt survival pathway in insulin-producing RINm5F cells by arousing Src to activate insulin receptor substrate-1. Endocrinology *145*, 2319-2327.

Tejedo, J.R., Tapia-Limonchi, R., Mora-Castilla, S., Cahuana, G.M., Hmadcha, A., Martin, F., Bedoya, F.J., and Soria, B. (2010). Low concentrations of nitric oxide delay the differentiation of embryonic stem cells and promote their survival. Cell Death Dis *1*, e80.

Telfer, E.E., and Albertini, D.F. (2012). The quest for human ovarian stem cells. Nat Med *18*, 353-354.

Telfer, E.E., Gosden, R.G., Byskov, A.G., Spears, N., Albertini, D., Andersen, C.Y., Anderson, R., Braw-Tal, R., Clarke, H., Gougeon, A., *et al.* (2005). On regenerating the ovary and generating controversy. Cell *122*, 821-822.

Terrovitis, J.V., Smith, R.R., and Marban, E. (2010). Assessment and optimization of cell engraftment after transplantation into the heart. Circ Res *106*, 479-494.

Tesar, P.J., Chenoweth, J.G., Brook, F.A., Davies, T.J., Evans, E.P., Mack, D.L., Gardner, R.L., and McKay, R.D. (2007). New cell lines from mouse epiblast share defining features with human embryonic stem cells. Nature *448*, 196-199.

Theus, M.H., Wei, L., Cui, L., Francis, K., Hu, X., Keogh, C., and Yu, S.P. (2008). In vitro hypoxic preconditioning of embryonic stem cells as a strategy of promoting cell survival and functional benefits after transplantation into the ischemic rat brain. Experimental neurology *210*, 656-670.

Thompson, J.G., McNaughton, C., Gasparrini, B., McGowan, L.T., and Tervit, H.R. (2000). Effect of inhibitors and uncouplers of oxidative phosphorylation during compaction and blastulation of bovine embryos cultured in vitro. J Reprod Fertil *118*, 47-55.

Thomson, J.A., Itskovitz-Eldor, J., Shapiro, S.S., Waknitz, M.A., Swiergiel, J.J., Marshall, V.S., and Jones, J.M. (1998). Embryonic stem cell lines derived from human blastocysts. Science *282*, 1145-1147.

Thomson, J.A., and Marshall, V.S. (1998). Primate embryonic stem cells. Curr Top Dev Biol *38*, 133-165.

Thomson, J.A., and Odorico, J.S. (2000). Human embryonic stem cell and embryonic germ cell lines. Trends Biotechnol *18*, 53-57.

Tian, H., Hammer, R.E., Matsumoto, A.M., Russell, D.W., and McKnight, S.L. (1998). The hypoxia-responsive transcription factor EPAS1 is essential for catecholamine homeostasis and protection against heart failure during embryonic development. Genes Dev *12*, 3320-3324.

Tian, H., McKnight, S.L., and Russell, D.W. (1997). Endothelial PAS domain protein 1 (EPAS1), a transcription factor selectively expressed in endothelial cells. Genes Dev *11*, 72-82.

Tilly, J.L., Niikura, Y., and Rueda, B.R. (2009). The current status of evidence for and against postnatal oogenesis in mammals: a case of ovarian optimism versus pessimism? Biol Reprod *80*, 2-12.

Tokuzawa, Y., Kaiho, E., Maruyama, M., Takahashi, K., Mitsui, K., Maeda, M., Niwa, H., and Yamanaka, S. (2003). Fbx15 is a novel target of Oct3/4 but is dispensable for embryonic stem cell self-renewal and mouse development. Mol Cell Biol *23*, 2699-2708.

Tomioka, M., Nishimoto, M., Miyagi, S., Katayanagi, T., Fukui, N., Niwa, H., Muramatsu, M., and Okuda, A. (2002). Identification of Sox-2 regulatory region which is under the control of Oct-3/4-Sox-2 complex. Nucleic Acids Res *30*, 3202-3213.

Trott, J., Hayashi, K., Surani, A., Babu, M.M., and Martinez-Arias, A. (2012). Dissecting ensemble networks in ES cell populations reveals micro-heterogeneity underlying pluripotency. Molecular bioSystems *8*, 744-752.

Tsukada, Y., Fang, J., Erdjument-Bromage, H., Warren, M.E., Borchers, C.H., Tempst, P., and Zhang, Y. (2006). Histone demethylation by a family of JmjC domain-containing proteins. Nature *439*, 811-816.

Turnpenny, L., Brickwood, S., Spalluto, C.M., Piper, K., Cameron, I.T., Wilson, D.I., and Hanley, N.A. (2003). Derivation of human embryonic germ cells: an alternative source of pluripotent stem cells. Stem Cells *21*, 598-609.

Tutter, A.V., Kowalski, M.P., Baltus, G.A., Iourgenko, V., Labow, M., Li, E., and Kadam, S. (2009). Role for Med12 in regulation of Nanog and Nanog target genes. J Biol Chem *284*, 3709-3718.

Uemura, R., Xu, M., Ahmad, N., and Ashraf, M. (2006). Bone marrow stem cells prevent left ventricular remodeling of ischemic heart through paracrine signaling. Circ Res *98*, 1414-1421.

Umaoka, Y., Noda, Y., Nakayama, T., Narimoto, K., Mori, T., and Iritani, A. (1992). Effect of visual light on in vitro embryonic development in the hamster. Theriogenology *38*, 1043-1054.

Urbanek (2006). Cardiac stem cells possess growth factor-receptor systems that after activation regenerate the infarcted myocardium, improving ventricular function and long-term survival (vol 97, pg 663, 2005). Circ Res *98*, E27-E27.

Vallier, L., Mendjan, S., Brown, S., Chng, Z., Teo, A., Smithers, L.E., Trotter, M.W., Cho, C.H., Martinez, A., Rugg-Gunn, P., *et al.* (2009). Activin/Nodal signalling maintains pluripotency by controlling Nanog expression. Development *136*, 1339-1349.

Vannucci, S.J., Reinhart, R., Maher, F., Bondy, C.A., Lee, W.H., Vannucci, R.C., and Simpson, I.A. (1998). Alterations in GLUT1 and GLUT3 glucose transporter gene

expression following unilateral hypoxia-ischemia in the immature rat brain. Brain Res Dev Brain Res *107*, 255-264.

Varum, S., Rodrigues, A.S., Moura, M.B., Momcilovic, O., Easley, C.A.t., Ramalho-Santos, J., Van Houten, B., and Schatten, G. (2011). Energy metabolism in human pluripotent stem cells and their differentiated counterparts. PLoS One *6*, e20914.

Vermeulen, M., Eberl, H.C., Matarese, F., Marks, H., Denissov, S., Butter, F., Lee, K.K., Olsen, J.V., Hyman, A.A., Stunnenberg, H.G., *et al.* (2010). Quantitative interaction proteomics and genome-wide profiling of epigenetic histone marks and their readers. Cell *142*, 967-980.

Volkmer, E., Kallukalam, B.C., Maertz, J., Otto, S., Drosse, I., Polzer, H., Bocker, W., Stengele, M., Docheva, D., Mutschler, W., *et al.* (2010). Hypoxic preconditioning of human mesenchymal stem cells overcomes hypoxia-induced inhibition of osteogenic differentiation. Tissue engineering Part A *16*, 153-164.

Wade, P.A., Gegonne, A., Jones, P.L., Ballestar, E., Aubry, F., and Wolffe, A.P. (1999). Mi-2 complex couples DNA methylation to chromatin remodelling and histone deacetylation. Nat Genet *23*, 62-66.

Wade, P.A., Jones, P.L., Vermaak, D., and Wolffe, A.P. (1998). A multiple subunit Mi-2 histone deacetylase from Xenopus laevis cofractionates with an associated Snf2 superfamily ATPase. Curr Biol *8*, 843-846.

Wakayama, T., Tabar, V., Rodriguez, I., Perry, A.C., Studer, L., and Mombaerts, P. (2001). Differentiation of embryonic stem cell lines generated from adult somatic cells by nuclear transfer. Science *292*, 740-743.

Waldman, S.A., and Murad, F. (1987). Cyclic GMP synthesis and function. Pharmacol Rev *39*, 163-196.

Wang, D.W., Fermor, B., Gimble, J.M., Awad, H.A., and Guilak, F. (2005). Influence of oxygen on the proliferation and metabolism of adipose derived adult stem cells. J Cell Physiol *204*, 184-191.

Wang, F., Zhang, R., Beischlag, T.V., Muchardt, C., Yaniv, M., and Hankinson, O. (2004). Roles of Brahma and Brahma/SWI2-related gene 1 in hypoxic induction of the erythropoietin gene. J Biol Chem *279*, 46733-46741.

Wang, F., Zhang, R., Wu, X., and Hankinson, O. (2010). Roles of coactivators in hypoxic induction of the erythropoietin gene. PLoS One *5*, e10002.

Wang, G.L., Jiang, B.H., Rue, E.A., and Semenza, G.L. (1995). Hypoxia-inducible factor 1 is a basic-helix-loop-helix-PAS heterodimer regulated by cellular O2 tension. Proc Natl Acad Sci U S A *92*, 5510-5514.

Wang, G.L., and Semenza, G.L. (1993). Characterization of hypoxia-inducible factor 1 and regulation of DNA binding activity by hypoxia. J Biol Chem *268*, 21513-21518.

Wang, G.L., and Semenza, G.L. (1995). Purification and characterization of hypoxia-inducible factor 1. J Biol Chem *270*, 1230-1237.

Wang, J., Zhuang, J.L., Iyer, S., Lin, X.Y., Whitfield, T.W., Greven, M.C., Pierce, B.G., Dong, X.J., Kundaje, A., Cheng, Y., *et al.* (2012). Sequence features and chromatin structure around the genomic regions bound by 119 human transcription factors. Genome Res *22*, 1798-1812.

Wang, J.L., Rao, S., Chu, J.L., Shen, X.H., Levasseur, D.N., Theunissen, T.W., and Orkin, S.H. (2006). A protein interaction network for pluripotency of embryonic stem cells. Nature *444*, 364-368.

Wang, K.C., Yang, Y.W., Liu, B., Sanyal, A., Corces-Zimmerman, R., Chen, Y., Lajoie, B.R., Protacio, A., Flynn, R.A., Gupta, R.A., *et al.* (2011). A long noncoding RNA maintains active chromatin to coordinate homeotic gene expression. Nature *472*, 120-124.

Wang, Z., Zang, C., Cui, K., Schones, D.E., Barski, A., Peng, W., and Zhao, K. (2009). Genome-wide mapping of HATs and HDACs reveals distinct functions in active and inactive genes. Cell *138*, 1019-1031.

Warren, L., Manos, P.D., Ahfeldt, T., Loh, Y.H., Li, H., Lau, F., Ebina, W., Mandal, P.K., Smith, Z.D., Meissner, A., *et al.* (2010). Highly efficient reprogramming to pluripotency and directed differentiation of human cells with synthetic modified mRNA. Cell Stem Cell *7*, 618-630.

Watson, J.A., Watson, C.J., McCann, A., and Baugh, J. (2010). Epigenetics, the epicenter of the hypoxic response. Epigenetics *5*, 293-296.

Watson, J.A., Watson, C.J., McCrohan, A.M., Woodfine, K., Tosetto, M., McDaid, J., Gallagher, E., Betts, D., Baugh, J., O'Sullivan, J., *et al.* (2009). Generation of an epigenetic signature by chronic hypoxia in prostate cells. Hum Mol Genet *18*, 3594-3604.

Wei, L., Fraser, J.L., Lu, Z.Y., Hu, X., and Yu, S.P. (2012). Transplantation of hypoxia preconditioned bone marrow mesenchymal stem cells enhances angiogenesis and neurogenesis after cerebral ischemia in rats. Neurobiology of disease *46*, 635-645.

Welt, F.G., Gallegos, R., Connell, J., Kajstura, J., D'Amario, D., Kwong, R.Y., Coelho-Filho, O., Shah, R., Mitchell, R., Leri, A., *et al.* (2013). Effect of cardiac stem cells on left-ventricular remodeling in a canine model of chronic myocardial infarction. Circulation Heart failure *6*, 99-106.

Wenger, R.H. (2000). Mammalian oxygen sensing, signalling and gene regulation. J Exp Biol *203*, 1253-1263.

Wenger, R.H. (2002). Cellular adaptation to hypoxia: O2-sensing protein hydroxylases, hypoxia-inducible transcription factors, and O2-regulated gene expression. Faseb J *16*, 1151-1162.

Wenger, R.H., Rolfs, A., Kvietikova, I., Spielmann, P., Zimmermann, D.R., and Gassmann, M. (1997). The mouse gene for hypoxia-inducible factor-1alpha--genomic organization, expression and characterization of an alternative first exon and 5' flanking sequence. European journal of biochemistry / FEBS *246*, 155-165.

Westfall, S.D., Sachdev, S., Das, P., Hearne, L.B., Hannink, M., Roberts, R.M., and Ezashi, T. (2008). Identification of oxygen-sensitive transcriptional programs in human embryonic stem cells. Stem Cells Dev *17*, 869-881.

White, Y.A., Woods, D.C., Takai, Y., Ishihara, O., Seki, H., and Tilly, J.L. (2012). Oocyte formation by mitotically active germ cells purified from ovaries of reproductive-age women. Nat Med *18*, 413-421.

Whyte, W.A., Bilodeau, S., Orlando, D.A., Hoke, H.A., Frampton, G.M., Foster, C.T., Cowley, S.M., and Young, R.A. (2012). Enhancer decommissioning by LSD1 during embryonic stem cell differentiation. Nature *482*, 221-225.

Williams, C.J., Naito, T., Arco, P.G., Seavitt, J.R., Cashman, S.M., De Souza, B., Qi, X., Keables, P., Von Andrian, U.H., and Georgopoulos, K. (2004a). The chromatin remodeler Mi-2beta is required for CD4 expression and T cell development. Immunity *20*, 719-733.

Williams, D.C., Cai, M.L., and Clore, G.M. (2004b). Molecular basis for synergistic transcriptional activation by Oct1 and Sox2 revealed from the solution structure of the 42-kDa Oct1 center dot Sox2 center dot Hoxb1-DNA ternary transcription factor complex. Journal of Biological Chemistry *279*, 1449-1457.

Williams, R.S., and Benjamin, I.J. (2000). Protective responses in the ischemic myocardium. J Clin Invest *106*, 813-818.

Wilmut, I., Schnieke, A.E., McWhir, J., Kind, A.J., and Campbell, K.H. (1997). Viable offspring derived from fetal and adult mammalian cells. Nature *385*, 810-813.

Woltjen, K., Michael, I.P., Mohseni, P., Desai, R., Mileikovsky, M., Hamalainen, R., Cowling, R., Wang, W., Liu, P., Gertsenstein, M., *et al.* (2009). piggyBac transposition reprograms fibroblasts to induced pluripotent stem cells. Nature *458*, 766-770.

Wu, C., and Morris, J.R. (2001). Genes, genetics, and epigenetics: a correspondence. Science *293*, 1103-1105.

Xi, L., Tekin, D., Gursoy, E., Salloum, F., Levasseur, J.E., and Kukreja, R.C. (2002). Evidence that NOS2 acts as a trigger and mediator of late preconditioning induced by acute systemic hypoxia. Am J Physiol Heart Circ Physiol *283*, H5-12.

Xia, X., Lemieux, M.E., Li, W., Carroll, J.S., Brown, M., Liu, X.S., and Kung, A.L. (2009). Integrative analysis of HIF binding and transactivation reveals its role in maintaining histone methylation homeostasis. Proc Natl Acad Sci U S A *106*, 4260-4265.

Xie, X.J., Wang, J.A., Cao, J., and Zhang, X. (2006). Differentiation of bone marrow mesenchymal stem cells induced by myocardial medium under hypoxic conditions. Acta pharmacologica Sinica *27*, 1153-1158.

Xiong, L., Wang, F., Huang, X., Liu, Z.H., Zhao, T., Wu, L.Y., Wu, K., Ding, X., Liu, S., Wu, Y., *et al.* (2012). DNA demethylation regulates the expression of miR-210 in neural progenitor cells subjected to hypoxia. Febs J *279*, 4318-4326.

Xu, C., Inokuma, M.S., Denham, J., Golds, K., Kundu, P., Gold, J.D., and Carpenter, M.K. (2001). Feeder-free growth of undifferentiated human embryonic stem cells. Nat Biotechnol *19*, 971-974.

Xu, C., Jiang, J., Sottile, V., McWhir, J., Lebkowski, J., and Carpenter, M.K. (2004). Immortalized fibroblast-like cells derived from human embryonic stem cells support undifferentiated cell growth. Stem Cells *22*, 972-980.

Xu, R.H., Peck, R.M., Li, D.S., Feng, X., Ludwig, T., and Thomson, J.A. (2005a). Basic FGF and suppression of BMP signaling sustain undifferentiated proliferation of human ES cells. Nature methods *2*, 185-190.

Xu, R.H., Sampsell-Barron, T.L., Gu, F., Root, S., Peck, R.M., Pan, G., Yu, J., Antosiewicz-Bourget, J., Tian, S., Stewart, R., *et al.* (2008). NANOG is a direct target of TGFbeta/activin-mediated SMAD signaling in human ESCs. Cell Stem Cell *3*, 196-206.

Xu, W., Charles, I.G., and Moncada, S. (2005b). Nitric oxide: orchestrating hypoxia regulation through mitochondrial respiration and the endoplasmic reticulum stress response. Cell Res *15*, 63-65.

Xu, W.S., Parmigiani, R.B., and Marks, P.A. (2007). Histone deacetylase inhibitors: molecular mechanisms of action. Oncogene *26*, 5541-5552.

Yamanaka, S. (2008). Pluripotency and nuclear reprogramming. Philosophical transactions of the Royal Society of London Series B, Biological sciences *363*, 2079-2087.

Yamane, K., Toumazou, C., Tsukada, Y., Erdjument-Bromage, H., Tempst, P., Wong, J., and Zhang, Y. (2006). JHDM2A, a JmjC-containing H3K9 demethylase, facilitates transcription activation by androgen receptor. Cell *125*, 483-495.

Yan, Z., Wang, Z., Sharova, L., Sharov, A.A., Ling, C., Piao, Y., Aiba, K., Matoba, R., Wang, W., and Ko, M.S. (2008). BAF250B-associated SWI/SNF chromatin-remodeling complex is required to maintain undifferentiated mouse embryonic stem cells. Stem Cells *26*, 1155-1165.

Yang, X.J., and Seto, E. (2003). Collaborative spirit of histone deacetylases in regulating chromatin structure and gene expression. Curr Opin Genet Dev *13*, 143-153.

Yeo, S., Jeong, S., Kim, J., Han, J.S., Han, Y.M., and Kang, Y.K. (2007). Characterization of DNA methylation change in stem cell marker genes during

differentiation of human embryonic stem cells. Biochem Biophys Res Commun *359*, 536-542.

Yeom, Y.I., Fuhrmann, G., Ovitt, C.E., Brehm, A., Ohbo, K., Gross, M., Hubner, K., and Scholer, H.R. (1996). Germline regulatory element of Oct-4 specific for the totipotent cycle of embryonal cells. Development *122*, 881-894.

Yeom, Y.I., Ha, H.S., Balling, R., Scholer, H.R., and Artzt, K. (1991). Structure, expression and chromosomal location of the Oct-4 gene. Mech Dev *35*, 171-179.

Yoshida, T., Hazan, I., Zhang, J., Ng, S.Y., Naito, T., Snippert, H.J., Heller, E.J., Qi, X., Lawton, L.N., Williams, C.J., *et al.* (2008). The role of the chromatin remodeler Mi-2beta in hematopoietic stem cell self-renewal and multilineage differentiation. Genes Dev *22*, 1174-1189.

Yoshida, T., Kuwahara, M., Maita, K., and Harada, T. (2001). Immunohistochemical study on hypoxia in spontaneous polycystic liver and kidney disease in rats. Exp Toxicol Pathol *53*, 123-128.

Yoshida, Y., Takahashi, K., Okita, K., Ichisaka, T., and Yamanaka, S. (2009). Hypoxia enhances the generation of induced pluripotent stem cells. Cell Stem Cell *5*, 237-241.

Young, R.A. (2011). Control of the embryonic stem cell state. Cell *144*, 940-954.

Yu, A.Y., Shimoda, L.A., Iyer, N.V., Huso, D.L., Sun, X., McWilliams, R., Beaty, T., Sham, J.S., Wiener, C.M., Sylvester, J.T., *et al.* (1999). Impaired physiological responses to chronic hypoxia in mice partially deficient for hypoxia-inducible factor 1alpha. J Clin Invest *103*, 691-696.

Yu, J., Vodyanik, M.A., Smuga-Otto, K., Antosiewicz-Bourget, J., Frane, J.L., Tian, S., Nie, J., Jonsdottir, G.A., Ruotti, V., Stewart, R., *et al.* (2007). Induced pluripotent stem cell lines derived from human somatic cells. Science *318*, 1917-1920.

Yuan, H., Corbi, N., Basilico, C., and Dailey, L. (1995). Developmental-specific activity of the FGF-4 enhancer requires the synergistic action of Sox2 and Oct-3. Genes Dev *9*, 2635-2645.

Yusa, K., Rad, R., Takeda, J., and Bradley, A. (2009). Generation of transgene-free induced pluripotent mouse stem cells by the piggyBac transposon. Nature methods *6*, 363-369.

Zelzer, E., Levy, Y., Kahana, C., Shilo, B.Z., Rubinstein, M., and Cohen, B. (1998). Insulin induces transcription of target genes through the hypoxia-inducible factor HIF-1alpha/ARNT. Embo J *17*, 5085-5094.

Zhang, L., Qin, X., Zhao, Y., Fast, L., Zhuang, S., Liu, P., Cheng, G., and Zhao, T.C. (2012). Inhibition of histone deacetylases preserves myocardial performance and prevents cardiac remodeling through stimulation of endogenous angiomyogenesis. The Journal of pharmacology and experimental therapeutics *341*, 285-293.

Zhang, L., Rayner, S., Katoku-Kikyo, N., Romanova, L., and Kikyo, N. (2007). Successful co-immunoprecipitation of Oct4 and Nanog using cross-linking. Biochem Biophys Res Commun *361*, 611-614.

Zhang, X., Neganova, I., Przyborski, S., Yang, C., Cooke, M., Atkinson, S.P., Anyfantis, G., Fenyk, S., Keith, W.N., Hoare, S.F., *et al.* (2009). A role for NANOG in G1 to S transition in human embryonic stem cells through direct binding of CDK6 and CDC25A. J Cell Biol *184*, 67-82.

Zhang, X., Stojkovic, P., Przyborski, S., Cooke, M., Armstrong, L., Lako, M., and Stojkovic, M. (2006). Derivation of human embryonic stem cells from developing and arrested embryos. Stem Cells *24*, 2669-2676.

Zhang, Y., Jurkowska, R., Soeroes, S., Rajavelu, A., Dhayalan, A., Bock, I., Rathert, P., Brandt, O., Reinhardt, R., Fischle, W., *et al.* (2010). Chromatin methylation activity of Dnmt3a and Dnmt3a/3L is guided by interaction of the ADD domain with the histone H3 tail. Nucleic Acids Res *38*, 4246-4253.

Zhao, X.D., Han, X., Chew, J.L., Liu, J., Chiu, K.P., Choo, A., Orlov, Y.L., Sung, W.K., Shahab, A., Kuznetsov, V.A., *et al.* (2007). Whole-genome mapping of histone H3 Lys4 and 27 trimethylations reveals distinct genomic compartments in human embryonic stem cells. Cell Stem Cell *1*, 286-298.

Zhou, H., Wu, S., Joo, J.Y., Zhu, S., Han, D.W., Lin, T., Trauger, S., Bien, G., Yao, S., Zhu, Y., *et al.* (2009). Generation of induced pluripotent stem cells using recombinant proteins. Cell Stem Cell *4*, 381-384.

Zhou, X., Sun, H., Chen, H., Zavadil, J., Kluz, T., Arita, A., and Costa, M. (2010). Hypoxia induces trimethylated H3 lysine 4 by inhibition of JARID1A demethylase. Cancer Res *70*, 4214-4221.

Zhu, H., Qiu, H., Yoon, H.W., Huang, S., and Bunn, H.F. (1999). Identification of a cytochrome b-type NAD(P)H oxidoreductase ubiquitously expressed in human cells. Proc Natl Acad Sci U S A *96*, 14742-14747.