Epigenetic and Environmental Determinants of Undifferentiated Human Embryonic Stem Cell Renewal

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Dedication

This thesis is dedicated to the two most important people in my life, my parents Charalampos and Eleni Koutsouraki for their unconditional love and support, infinite patience and unwavering faith in my dreams.

I will be forever indebted.
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Abstract

Embryonic stem cells are derived from the inner cell mass of a blastocyst-stage embryo and are characterized by the ability to self-renew and differentiate into all cell types of an adult organism, as demonstrated by their transplantation into embryos in the mouse. Isolation of cells with similar properties from human embryos has permitted the study of human cell differentiation in vitro as might occur during development. As such, human ES cells may be useful to assess and predict the developmental toxicity of environmental compounds capable of epigenetic alterations of the genome and its expression.

The first objective of my research was to validate the functional significance to maintenance of an undifferentiated human ES cell state of expressed genes whose epigenetic modification is conserved across diverse lines and/or likely to be deterministic of an embryo stem cell associated epigenetic state. The second goal was to determine the sensitivity and relationship of the expression of these genes to environmental factors known to perturb the epigenome, specifically subcytotoxic exposure to diverse organic and metallic compounds and the availability of atmospheric oxygen.
siRNA-mediated knockdown of genes previously identified on the basis of the conserved methylation status of gene associated Cytosine-Guanine islands (i.e. GLIS2, HMGA1, PFDN5, TET1 and JMJD2C) and two related family members (TET2 & 3) resulted in induction of cell differentiation in two independent human ES cell lines (RH1 and H9). Differentiation was reflected by morphological changes, reduction or loss of pluripotency associated markers, qualitative and quantitative reduction in genomic 5-hmC and upregulation of diverse germinal lineage markers.

Subcytotoxic exposure of the same human ES cell lines to diverse compounds known to alter the epigenome (i.e. 5-azacytidine, sodium arsenite, cadmium chloride and valproic acid) generally induced downregulation of the aforementioned genes, loss of genomic hydroxymethylation and differentiation when applied under normoxia (20% O₂), the exception being valproic acid. The same treatment applied under hypoxia (0.5% O₂), did not induce differentiation, with the exception of cadmium chloride. Hypoxia is a general feature of developing embryos prior to the establishment of a maternal/fetal placental interface and fetal cardiovasculature. The protective effect of hypoxia was associated with elevation of ROS, expression of the dioxygenases TET1 and JMJD2C, and genomic hydroxymethylation.
This research has demonstrated that genes identified on the basis of a conserved pattern of epigenetic modification function in the maintenance of an undifferentiated human ES cell phenotype. Furthermore, a human ES cell-based toxicology test system has been developed with which one can assess the subcytotoxic effects of compounds known to disrupt the epigenome and affect development by assessing their impact on maintenance of an undifferentiated human ES cell state. This is reflected by alterations in pluripotency markers, epigenetically-defined biomarkers and changes in global 5-hmC levels and the expression of genes responsible for this epigenetic modification (TET1-3). The epigenetically-defined biomarkers of pluripotent human ES cell identity (GLIS2, HMGA1, PFDN5, JMJD2C and TET1) could serve as biomarkers for screenings of compounds at an epigenetic level as their expression has been shown to be altered upon compound exposure along with monitoring the expression of 5-hmC.
Declaration

I declare that this thesis has been composed by myself and is the result of my own unaided work, except where otherwise acknowledged. The work that has been presented in this thesis has not been submitted for any other degree or professional qualification.

Eirini Koutsouraki

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<tr>
<td>2-OG</td>
<td>2-oxoglutarate</td>
</tr>
<tr>
<td>5-fC</td>
<td>5-formylcytosine</td>
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<tr>
<td>5-hmC</td>
<td>5-hydroxymethylcytosine</td>
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<td>5-mC</td>
<td>5-methylcytosine</td>
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<td>8-OHdG</td>
<td>8-Hydroxy-2-deoxyguanosine</td>
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<tr>
<td>AFP</td>
<td>Alpha-fetoprotein</td>
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<td>ALDA</td>
<td>Aldolase A</td>
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<tr>
<td>ANOVA</td>
<td>Analysis of Variance</td>
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<tr>
<td>AP</td>
<td>Apicidin</td>
</tr>
<tr>
<td>ARNT</td>
<td>Arylhydrocarbon Receptor Nuclear Translocator</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine Triphosphate</td>
</tr>
<tr>
<td>bFGF</td>
<td>Basic Fibroblast Growth Factor</td>
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<tr>
<td>BMP</td>
<td>Bone Morphogenic Protein</td>
</tr>
<tr>
<td>bp</td>
<td>Base pairs</td>
</tr>
<tr>
<td>BSO</td>
<td>Buthionine sulfoximine</td>
</tr>
<tr>
<td>C</td>
<td>Cytosine</td>
</tr>
<tr>
<td>caC</td>
<td>Carboxylcytosine</td>
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<tr>
<td>CAC</td>
<td>Citric Acid Cycle</td>
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<td>CAT</td>
<td>Catalase</td>
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<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
</tr>
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<td>Abbreviation</td>
<td>Full Form</td>
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</tr>
<tr>
<td>CFU</td>
<td>Colony Forming Unit</td>
</tr>
<tr>
<td>CGa</td>
<td>Chorionic gonadotropin alpha</td>
</tr>
<tr>
<td>CGH</td>
<td>Comparative Genomic Hybridization</td>
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<tr>
<td>CGI</td>
<td>Cytosine Guanine Islands</td>
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<td>ChIP</td>
<td>Chromatin Immunoprecipitation</td>
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<tr>
<td>CM-H$_2$DCFDA</td>
<td>Chloromethyl derivative of H$_2$DCFDA</td>
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<tr>
<td>c-Myc</td>
<td>Myelocytomatosis oncogene</td>
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<tr>
<td>CNS</td>
<td>Central Nervous System</td>
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<td>CO$_2$</td>
<td>Carbon Dioxide</td>
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<td>CoCl$_2$</td>
<td>Cobalt Chloride</td>
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<td>CpG</td>
<td>Cytosine Phosphate Guanine</td>
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<td>Ct</td>
<td>Cycle Threshold</td>
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<tr>
<td>DAPI</td>
<td>4',6-diamidino-2-phenylindole</td>
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<td>DCF</td>
<td>2',7'-Dichlorofluorescein</td>
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<td>DCF-DA</td>
<td>2', 7'-Dichlorofluorescein diacetate</td>
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<td>CD45</td>
<td>Cluster of Differentiation 45</td>
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<td>DFO</td>
<td>Desferrioxamine</td>
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<td>Dihydrorhodamine 123</td>
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<tr>
<td>DM</td>
<td>Defined Medium</td>
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<tr>
<td>DMOG</td>
<td>Dimethyloxalylglycine</td>
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<td>DMSO</td>
<td>Dimethyl Sulfoxide</td>
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<td>DNA</td>
<td>Deoxyribonucleic Acid</td>
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<td>DNMT</td>
<td>DNA methyltransferase</td>
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<tr>
<td>dsDNA</td>
<td>Double Stranded DNA</td>
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<td>EB</td>
<td>Embryoid Body</td>
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<td>EDTA</td>
<td>Ethylenediaminetetraacetic Acid</td>
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<td>ELISA</td>
<td>Enzyme-Linked Immunosorbent Assay</td>
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<td>Em</td>
<td>Emission</td>
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<td>EPO</td>
<td>Erythropoietin</td>
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<tr>
<td>ES cell</td>
<td>Embryonic Stem Cell</td>
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<td>ESCs</td>
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<td>Ex</td>
<td>Excitation</td>
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<td>FACS</td>
<td>Fluorescence Activated Cell Sorting</td>
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<td>Fe</td>
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<td>FGF</td>
<td>Fibroblast Growth Factor</td>
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<td>FIH</td>
<td>Factor Inhibiting HIF</td>
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<td>FISH</td>
<td>Fluorescent In Situ Hybridization</td>
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<td>GAPDH</td>
<td>Glyceraldehyde 3-phosphate dehydrogenase</td>
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<tr>
<td>GATA2</td>
<td>GATA-binding protein 2</td>
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<td>GFP</td>
<td>Green Fluorescent Protein</td>
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<tr>
<td>GLIS2</td>
<td>GLIS family zinc finger 2</td>
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<td>GLUT-1</td>
<td>Glucose Transporter-1</td>
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<td>GPX</td>
<td>Glutathione peroxidase</td>
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<td>Glutathione</td>
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<tr>
<td>H2AX</td>
<td>H2A Histone, Member X</td>
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<td>H2DCF</td>
<td>2',7'-Dichlorofluorescein</td>
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H$_2$DCF-DA 2’,7’-Dihydrodichlorofluorescein Diacetate
H$_2$O  Water
H$_2$O$_2$  Hydrogen Peroxide
HAND1  Heart- and neural crest derivatives-expressed protein 1
HCl  HydroChloric Acid
HDAC  Histone Deacetylase Inhibitor
HDF  Human Dermal Fibroblast
HDF-CM  Human Dermal Fibroblast - Conditioned Medium
HDF-CM+  Human Dermal Fibroblast - Conditioned Medium (HDF-CM) supplemented with 4 ng/ml of bFGF
HEK  Human Embryonic Kidney
hESC  Human Embryonic Stem Cell
HIF  Hypoxia-Inducible Factor
HMEC  Human Microvascular Endothelial Cell
HMGA1  High Mobility Group A1
HNF4a  Hepatocyte nuclear factor 4 alpha
HO-1  Heme-oxygenase-1
HRE  Hypoxia Response Element
HRP  Horseradish Peroxidase
hrs  Hours
HSC  Hematopoietic Stem Cell
ICM  Inner Cell Mass
IDS  Iduronate sulfatase
iPSCs  Induced Pluripotent Stem Cells
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<thead>
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<th>Abbreviation</th>
<th>Full Form</th>
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<tr>
<td>JMJD2C</td>
<td>Lysine-specific demethylase 4C</td>
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<td>KD</td>
<td>Knockdown</td>
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<td>KLF4</td>
<td>Kruppel-like factor 4</td>
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<td>LDH</td>
<td>Lactate Dehydrogenase</td>
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<td>Log</td>
<td>Logarithm</td>
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<td>Lysine-Specific Demethylase 1</td>
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<td>MBD</td>
<td>Methyl Binding Domain</td>
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<td>mESC</td>
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<td>min</td>
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<td>Mitochondrial Membrane Potential</td>
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<td>mRNA</td>
<td>Messenger Ribonucleic Acid</td>
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<td>Mesenchymal Stem Cell</td>
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<td>Nitrogen</td>
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<td>NAC</td>
<td>N-acetyl-cysteine</td>
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<td>NF</td>
<td>Neurofilament</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric Oxide</td>
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<tr>
<td>NO₂</td>
<td>Nitrogen Dioxide</td>
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<td>NRS</td>
<td>Normal Rabbit Serum</td>
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<td>NSC</td>
<td>Neural Stem Cell</td>
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<td>NS</td>
<td>Nutrient Supplements</td>
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<td>OCT4</td>
<td>Octamer-Binding Transcription Factor 4</td>
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<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>OD</td>
<td>Optical Density</td>
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<td>O$_2^+$</td>
<td>Superoxide Anion</td>
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<td>O$_2$</td>
<td>Oxygen</td>
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<td>OH$^-$</td>
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<td>ONOO$^-$</td>
<td>Peroxynitrite</td>
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<td>PAX6</td>
<td>Paired box protein Pax-6</td>
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<td>PBS</td>
<td>Phosphate Buffered Saline</td>
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<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
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<td>PFDN5</td>
<td>Prefoldin subunit 5</td>
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<td>PHD</td>
<td>Prolyl Hydroxylase Domain</td>
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<td>PHLF</td>
<td>Primary Human Lung Fibroblast</td>
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<td>PL-1</td>
<td>Placental lactogen-1</td>
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<td>PSC</td>
<td>Pluripotent Stem Cell</td>
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<td>qRT-PCR</td>
<td>Quantitative Real-Time Polymerase Chain Reaction</td>
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<td>R123</td>
<td>Rhodamine 123</td>
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<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
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<tr>
<td>RNAi</td>
<td>RNA Interference</td>
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<tr>
<td>roGFP</td>
<td>Reduction-Oxidation Green Fluorescent Protein</td>
</tr>
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<td>ROS</td>
<td>Reactive Oxygen Species</td>
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<tr>
<td>rpm</td>
<td>Revolutions Per Minute</td>
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<td>RT-PCR</td>
<td>Real-Time Polymerase Chain Reaction</td>
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<td>RUNX2</td>
<td>Runt-related transcription factor 2</td>
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<td>SAH</td>
<td>S-Adenosyl-L-Homocysteine</td>
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</tr>
<tr>
<td>SAM</td>
<td>S-Adenosyl Methionine</td>
</tr>
<tr>
<td>SB</td>
<td>Sodium Butyrate</td>
</tr>
<tr>
<td>SCID</td>
<td>Severe Combined Immunodeficiency</td>
</tr>
<tr>
<td>SD</td>
<td>Standard Deviation</td>
</tr>
<tr>
<td>sec</td>
<td>Seconds</td>
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<tr>
<td>SERS</td>
<td>Surface-Enhanced Raman Spectroscopy</td>
</tr>
<tr>
<td>shRNA</td>
<td>Short hairpin RNA</td>
</tr>
<tr>
<td>siRNA</td>
<td>Small Interfering RNA</td>
</tr>
<tr>
<td>SOD</td>
<td>Superoxide dismutase</td>
</tr>
<tr>
<td>SOX2</td>
<td>SRY (sex determining region Y)-box 2</td>
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<tr>
<td>SR</td>
<td>Serum Replacement</td>
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<tr>
<td>SSEA</td>
<td>Stage-Specific Embryonic Antigen</td>
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<td>STAT3</td>
<td>Signal transducer and activator of transcription 3</td>
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<tr>
<td>Succ</td>
<td>Succinate</td>
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<tr>
<td>TET</td>
<td>Ten-Eleven Translocation</td>
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<tr>
<td>TFAM</td>
<td>Transcription Factor A Mitochondrial</td>
</tr>
<tr>
<td>Trx</td>
<td>Thioredoxin</td>
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<tr>
<td>TSA</td>
<td>Trichostatin</td>
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<tr>
<td>VEGF</td>
<td>Vascular Endothelial Growth Factor</td>
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<td>VHL</td>
<td>Von Hippel-Lindau complex</td>
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<tr>
<td>VPA</td>
<td>Valproic Acid</td>
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<tr>
<td>YAP1</td>
<td>Yes-associated protein 1</td>
</tr>
<tr>
<td>WNT</td>
<td>Wingless-type MMTV integration site family</td>
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Chapter 1

General Introduction
1.1 Human Embryonic Stem Cells

1.1.1 Derivation of Human Embryonic Stem Cells

Embryonic stem cells are pluripotent stem cells derived from the inner cell mass (ICM) of mammalian blastocysts, that have the capacity to self-renew and differentiate into cell types of all three germ layers (Evans and Kaufman, 1981; Martin, 1981) (Figure 1.1).

Figure 1.1. Initial stages of human development. The zygote constitutes the first stage of human development and it divides into the 16-cell stage morula, which following further cellular divisions develop into the blastocyst. The blastocyst consists of
the inner cell mass (ICM) and the trophoblast, an outer layer of cells surrounding the ICM and the blastocyst cavity. The ICM can be isolated and grown in culture to produce pluripotent stem cells (ES cells), which retain the capability to differentiate into all three germ layers (mesoderm, endoderm, and ectoderm), which eventually give rise to various cell types of the adult human body (i.e. blood, heart, muscle, liver, pancreas, intestine, CNS, skin) (Gilbert, 2000).

Thomson’s group was the first to successfully derive human embryonic stem cells (hESCs) from inner cell masses of human blastocysts which were in vitro fertilised and 14 of them were cultured on irradiated mouse embryonic fibroblasts (Thomson, et al., 1998; reviewed in Moon, et al., 2006). Five human ES cell lines (H1, H7, H9, H13 and H14) were established with normal karyotype, expressing high levels of telomerase activity and cell-surface markers SSEA-3, SSEA-4, Tra-1-60 and alkaline phosphatase. The human ES cell lines were capable of self-renewal and maintained the potential to differentiate into derivatives of all three embryonic germ layers (endoderm, mesoderm and ectoderm) (Thomson, et al., 1998). Since then a number of derivation methods have been utilized and hundreds of human ES cells have been established in various research laboratories all over the world (reviewed in Allegrucci and Young, 2007). Also, human ES cell lines have been established from parthenogenic derived blastocysts (Revazova, et al., 2007) and by somatic cell nuclear transfer (Hwang, et al., 2005; Tachibana, et al., 2013).
1.1.2 Culture of Human Embryonic Stem Cells

Since the first established human ES cell lines, extensive research has been employed in creating the optimal culture environment (reviewed in Spagnoli and Hemmati-Brivanlou, 2006). Initially, human ES cells were cultured on mitotically inactivated mouse embryonic fibroblasts (MEFs), which not only offered an attachment matrix but were also able to secrete unknown growth factors into the culture medium necessary for maintaining the undifferentiated growth of human ES cells (Ilic, 2006; Améen et al., 2008; Vazin, et al., 2010). The variability among cell batches and xeno-contamination led to the use of human fibroblast-like cell layers (human foreskin feeders), in order to establish a xeno-free culture environment for the expansion of human ES cells (Amit, et al., 2003). Variability between batches and the limitation in supply, led investigators in the search for optimising further the culture conditions. Next, researchers moved towards the use of synthetic products for attachment of human ES cells such as Matrigel™, vitronectin and laminin (Xu, et al., 2001; Braam, et al., 2008; Rodin, et al., 2010) and conditioned media were replaced by various serum-free and defined media formulations (Fletcher, et al., 2006; Ellerström, et al., 2006; Ludwig, et al., 2006; Chin, et al., 2007; Chin, et al., 2010; Rajala, et al., 2010; the International Stem Cell Initiative Consortium, et al., 2010; Miyazaki, et al., 2012; Zhang, et al., 2013). Differences among culture conditions have been reported between various laboratories and that has been reflected in observed variations in gene expression profiles between human ES cell lines (reviewed in Allegrucci and
Young, 2007). Lastly, differences in culture regimes such as culture media, growth factors, passage methods, passage number, and cell densities can accommodate for gene expression variation within human ES cell lines (reviewed in Allegrucci and Young, 2007).

### 1.1.3 Characterization of Human Embryonic Stem Cells

For purposes of human ES cell characterization and quality control, it is nowadays common practise to follow certain criteria. Human embryonic stem cells can be assessed phenotypically by their cell morphology as they are small, densely packed and have large nucleoli, and a high nucleus to cytoplasm ratio (reviewed in Améen et al., 2008). Human ES cells, when they spontaneously differentiate, start to lose their colony morphology with defined tight border, while the cells in the center of the colony begin to flatten and enlarge (reviewed in Améen et al., 2008). Further, cell surface markers characteristic of undifferentiated human ES cells include the SSEA-3, SSEA-4, Tra-1-60, Tra-1-81 (Trounson, 2006), which have been shown to be downregulated upon cellular differentiation. Moreover, undifferentiated human ES cells display telomerase activity and alkaline phosphatase (reviewed in Pera et al., 2000). Another important aspect of undifferentiated human ES cells is their genetic and epigenetic stability in vitro. For the former, cytogenetic assessment is performed via the commonly used methodologies such as karyotyping, CGH and FISH.
(reviewed in Améen et al., 2008). Regarding the latter, epigenetic changes during human ES cell culture can have serious implications for their differentiation capacity and therefore, it is essential to have clear knowledge of the human epigenome (Allegrucci and Young, 2007). Finally, a vital requirement of a human ES cell line is to be pluripotent and to give rise to all derivatives of the three germ layers (mesoderm, endoderm and ectoderm). A widespread method to test this is the in vivo xenotransplantation of undifferentiated human ES cells into SCID mice, where the transplanted human ES cells give rise to teratomas, which in turn contain various tissue types representing all the three germ layers (reviewed in Améen et al., 2008).

1.1.4 Applications of Human Embryonic Stem Cells

One of the potential applications of human ES cells is the generation of functional cell types for the purposes of cell-based therapies, in order to repair and/or replace damaged tissues and organs (reviewed in Améen et al., 2008). Also, until the generation of induced-pluripotent stem cells, human ES cells were considered as an unlimited source of interest for the potential production of patient-specific stem cells (reviewed in Trounson, 2006). The establishment of disease-specific models from patients with neurodegenerative disorders and cancers is important in order to develop differentiated cell populations in the
laboratory that will express the disease phenotype with the intention of screening for molecules interfering with the disease phenotype and discover candidate pharmaceutical agents for these patients (reviewed in Trounson, 2006).

Further, in various biomedical disciplines the lack of functional human cell models makes the research development inadequate and occasionally leads to imprecise results. Hence, access to undifferentiated human embryonic stem cells and their derivatives offers a great opportunity for use, starting from early target identification via cellular screening to the final utilization of functional human cells, in terms of prediction of toxicity response and assessment that would be biologically more relevant than any other animal toxicity model system used up to date (West et al., 2010; Liu, et al., 2013). The ability of pluripotent stem cells to self-renew and differentiate into diverse cell lineages in the body, offer an unlimited source of cells for developmental toxicity assays. Some of the benefits of using a human stem cell based model in predictive toxicology, are the reduced use of animals, acquisition of detailed knowledge on dose-responses directly from human models, better biological relevance, linking of in vitro to in vivo studies, insight into damage-related mechanisms and high throughput screening of various toxicants (Liu, et al., 2013).
Human ES cells provide an alternative cell system for predicting toxicity in humans and discovering biomarkers of toxicant exposure for various pharmaceutical candidate compounds and environmental toxicants. Nowadays there is an accumulating number of studies that are utilizing human ES cells to identify the effects of various environmental and chemical compounds (Balmer, et al., 2012; Krug, et al., 2013; Leist, et al., 2013). Further, the studies of West et al. (2010) and Kleinstreuer et al. (2011) have utilised human ES cells and metabolomics as a developmental toxicity testing model (West et al., 2010; Kleinstreuer et al., 2011). The former study involved the treatment of human ES cells with 18 drugs of known teratogenicity and reported the successful identification of biomarkers for predicting chemical toxicity during early human development, by analyzing metabolites secreted in the media by drug-treated human ES cells versus the untreated group. The latter study managed to profile secreted small molecule metabolites that were perturbed upon exposure to different concentrations of 11 environmental compounds (Kleinstreuer et al., 2011).

### 1.2 Embryonic Stem Cell Pluripotency

Pluripotency is termed as the ability of a single cell to produce all cell lineages of a developing and adult organism (reviewed in Young, 2011). Preservation of pluripotency requires maintenance of self-renewal and inhibition of
differentiation. The discovery of molecular mechanisms and factors controlling pluripotency is important in order to have a better understanding of development.

In embryonic stem cells, pluripotency is mostly governed by the core transcription factors OCT4, NANOG and SOX2, which have been shown to be significant regulators both in vitro and in vivo (Nichols, et al., 1998; Chambers, et al., 2003; Chambers, et al., 2004; Chambers, et al., 2007; Niwa, 2007; Silva and Smith, 2008; Chambers and Tomlinson, 2009; Saunders, et al., 2013). Several studies have reported that all three regulators are highly expressed in human ES cells (Hay, et al., 2004; Hyslop, et al., 2005; Fong, et al., 2008). Depletion of stem cell-specific transcription factors OCT4, NANOG and SOX2 have been shown to result in loss of pluripotency, morphological changes and induction of differentiation (Hay, et al., 2004; Hyslop, et al., 2005; Fong, et al., 2008). The same studies have demonstrated that upon siRNA of any of these factors leads to the induction of trophectoderm and endoderm associate lineage markers (Hay, et al., 2004; Hyslop, et al., 2005; Fong, et al., 2008).

Apart from the fact that OCT4, NANOG and SOX2 are defined as the core pluripotency regulators and they control each others expression, they also co-occupy more than three hundred gene promoters maintaining the stem cell
specific gene expression (Boyer, et al., 2005). Additionally, genome-wide transcriptome analysis of human ES cells and several RNA interference (RNAi) screens have indentified new candidate genes essential in the regulation of self-renewal and pluripotency in embryonic stem cells (Xu, et al., 2002; Sato, et al., 2003; Lim, et al., 2007; Ding, et al., 2009; Chia, et al., 2010; Shah, et al., 2012).

1.3 Induced Pluripotency

Induced pluripotent stem cells (iPSCs) are an important advance in stem cell research (Okita, et al., 2011), as they offer researchers the opportunity to obtain pluripotent stem cells without the use of human blastocysts. iPSCs are derived from somatic cells by overexpression of pluripotency-related transcription factors.

The ability to reprogram somatic cells to iPSCs has opened new avenues for researchers to generate patient-specific cell lines, which will contribute to model human diseases, investigate their mechanisms and causes and elucidate developmental processes. Such cell lines are valuable tools for drug discovery, toxicology and cell-based replacement therapies. This novel technology offers the opportunity to generate cell types that are otherwise difficult to obtain such as neurons and cardiomyocytes, and they are of immense importance for
regenerative medicine as they offer the opportunity to investigate various diseases *in vitro*. Numerous disease-specific iPSCs have already been produced, such as Parkinson’s disease (Soldner, *et al.*, 2009), Huntington’s disease (Zhang, *et al.*, 2010), Alzheimer’s disease (Israel, *et al.* 2012) and Rett syndrome (Marchetto, *et al.*, 2010).

Yamanaka and colleagues in 2006 and 2007 generated the first iPSCs by transduction of retroviral vectors containing the four transcription factors OCT4, SOX2, KLF4 and c-Myc into mouse and human fibroblasts (Takahashi and Yamanaka, 2006; Takahashi, *et al.*, 2007) (Figure 1.2). This cocktail of reprogramming factors was narrowed down from an initial screen of 24 transcription factors known to be involved in the maintenance of pluripotency in ES cells and since then it has been demonstrated to induce pluripotency within various mouse cell types, human cells, rhesus monkey and pig cells (Liu, *et al.*, 2008; Li, *et al.*, 2013). Characterisation of those cells showed that they were similar to ES cells in terms of morphology, marker expression and teratoma formation.
Figure 1.2. Reprogramming of somatic cells into induced pluripotent stem cells (iPSCs). The generation of iPSCs was firstly achieved by the ectopic expression of genes that are involved in pluripotent cell identity (OCT4, SOX2, KLF4, c-Myc). A small number of the transfected cells (grey) become iPSCs (red) and generate ES cell-like colonies.

Several initial studies have demonstrated that iPSCs are similar to pluripotent ESCs in many aspects, including cell morphology, expression of pluripotent markers, embryoid body formation, epigenetic patterns, teratoma formation, viable chimera formation and differentiation potency (Oh, et al., 2012). Recent reports indicate differences between them in terms of epigenomic alterations (Lister, et al., 2009; Doi, et al., 2009; Kim, et al., 2010) and differences in differentiation potential (Hu, et al., 2010).
1.4 Epigenetic Modifications

Epigenetics refers to heritable alterations in gene expression that take place without changes in DNA sequence. The mechanisms by which these can be achieved involve DNA methylation, histone modifications and microRNA expression (reviewed in Weichenhan and Plass, 2013). For many years researchers were focused on studying DNA methylation and the critical role that it plays in development, while just starting to learn about new modifications of the DNA such as 5-hydroxymethylcytosine (reviewed in Weichenhan and Plass, 2013).

1.4.1 DNA Methylation

DNA methylation is the most studied epigenetic modification in mammalian genomes (Koh and Rao, 2013) and it involves the addition of a methyl group at the 5-position of cytosine bases within cytosine-phosphate-guanine dinucleotides (CpG) (Smith and Meissner, 2013). In mammalian cells, 60-70% of the C residues located within the CpG dinucleotides are methylated (Ng and Bird, 1999). DNA methylation is a major epigenetic mechanism affecting gene expression and chromatin organisation and is important for development (Morgan, et al., 2005; Siedlecki and Zielenkiewicz, 2006; Reik, et al., 2007). Mammalian DNA methylation has also an essential role in genomic imprinting,
X-chromosome inactivation and maintenance of pluripotency (reviewed in Bestor, 2000; Bird, 2002; Denis, et al., 2011).

DNA methylation is enzymatically catalysed by DNA methyltransferases (DNMTs) (reviewed by Bestor, 2000; Li, 2002). There are three DNA methyltransferases (DNMT1, DNMT3A and DNMT3B) identified in mammalian cells and the DNMT-related protein (DNMT3L) (Tost, 2010). DNMT1, DNMT3A and DNMT3b catalyse the transfer of a methyl group from S-adenosyl methionine (SAM) to the cytosine base (Tost, 2010). DNMT1 acts as maintenance methyltransferase and is active on hemimethylated DNA, DNMT3A and DNMT3B are required for de novo methylation, and DNMT3L shows no DNA methyltransferase activity and stimulates the activity of DNMT3A and DNMT3B (Suatake, et al., 2004; Klose and Bird, 2006; Tost, 2010). DNMTs are vital for embryonic viability as mutations of DNMT1, DNMT3A and DNMT3B in mice lead to extensive demethylation and embryonic lethality (Tost, 2010; Jurkowska, et al., 2011). Also, dysregulation of DNMTs has been described in human cancers such as breast, colorectal and prostate cancers (Denis, et al., 2011). Lastly, a recent study showed that DNMT3A and DNMT3B but not DNMT1 act as redox-dependent DNA dehydroxymethylases (Chen, et al., 2013).
DNA methylation plays a vital role in the self-renewal and pluripotency of mammalian cells (Fouse, et al., 2008). Human ES cells have a unique DNA methylation signature when compared to differentiated cells (Bibikova, et al., 2006; Meissner, et al., 2008; Lister, et al., 2009), which supports the idea that a specific pattern of DNA methylation may be essential to pluripotency (Altun, et al., 2010). In addition, methylation profiles have been identified that distinguish human ES cells from differentiated cells (Bibikova, et al., 2006) and when compared DNA methylation at CpG sites of human ES cells, iPS cells and fibroblasts, pluripotent cells have found to have slightly higher overall methylation levels than those of fibroblasts (Deng, et al., 2009).

1.4.2 Histone Modifications

Chromatin is the physiological substrate for all genetic processes in the nuclei of cells and changes in its organisation are appearing as regulators of genomic function (reviewed in Fischle, et al., 2003). To establish a global chromatin environment, modifications facilitate to separate the genome into different domains, such as euchromatin and heterochromatin, where DNA is kept accessible for transcription and where chromatin is inaccessible for transcription, respectively; hence modifications coordinate the unravelling of the chromatin in order to assist the execution of DNA-based functions (Kouzarides, 2002). Modifications of histones such as methylation and acetylation are associated
with the conformational status of the chromatin and transcriptional state of genes (Fischle, et al., 2003; Cosgrove and Wolberger, 2005; Margueron, et al., 2005, Pan, et al., 2007). Histone methylation has been recognised as a key player in gene regulation as it has been implicated in propagation of a repressed state via DNA methylation and heterochromatin repression (Kouzarides, 2002). Histone acetylation has been widely studied and it is recognised that hyperacetylated histones are mainly linked to activate genomic regions, while deacetylated lead in repression and silencing (Kouzarides, 2002; reviewed in Fischle, 2003).

Embryonic stem cells have euchromatic nuclei and high amounts of histone modifications linked with open chromatin, compared to differentiated cells where chromatin is more dense and has compacted chromatin blocks as shown via microscopic observations and biochemical analyses (Meshorer, et al., 2006; Pan, et al., 2007; Zhou, et al., 2011). In undifferentiated ES cells, pluripotent genes tend to be in a transcriptionally active state (euchromatic) contrary to differentiation-related genes, which are transcriptionally silent (heterochromatic) (Zhou, et al., 2011). Also, evidence derived from a study where knockdown of the chromatin remodeler Chd1 in mESCs resulted in the accumulation of heterochromatin and distorted differentiation advocating an “open” chromatin in embryonic stem cells (reviewed in Fisher and Fisher, 2011). Further, the lysine-specific demethylase 1 (LSD1) has been recognized as a histone modifier that is
involved in maintaining pluripotency, as depletion of it in human ES cells was found to downregulate pluripotency-associated markers (OCT4, NANOG, SOX2) and induce differentiation, as seen by the upregulation of germinal lineage-associated markers (HNF4, PAX6, TUBB3) (Adamo, *et al.*, 2011).

### 1.4.3 5-Hydroxymethylcytosine

#### 1.4.3.1 Historic overview of 5-hydroxymethylcytosine

5-hydroxymethylcytosine was initially identified in T-even bacteriophages and detected in genomic DNA extracted from brain tissues of mice, rats and frogs during the 1970s, but due to the fact that the findings not being reproducible, this modification received little attention until 2009, when it was described again in different studies (Dahl, *et al.*, 2011; Kinney and Pradhan, 2013). Kriaucionis and Heintz (2009), and Tahiliani, *et al.* (2009) found 5-hmC into mammalian genomes. In the former study, 5-hmC was detected in cerebellar Purkinje neurons and the brain, using thin-layer chromatography assay and it was found that 5-hmC constituted 0.6% and 0.2% of total nucleotides in Purkinje and granule cells, respectively (Kriaucionis and Heintz, 2009). In the latter study, 5-hmC was detected in nuclear DNA from mouse embryonic stem cells, constituting 0.03% of total nucleotides (Tahiliani, *et al.*, 2009). Subsequent studies have detected 5-hmC in mouse tissues (CNS, kidney, heart, lung, liver, spleen, bladder, skeletal muscle) (Globisch, *et al.*, 2010), HeLa and human
embryonic kidney cells (Song, et al. 2011), and human pluripotent stem cells (Ruzov, et al., 2011).

An important milestone was the identification of TET1 as the first enzyme responsible for the conversion of 5-mC to 5-hmC. The authors demonstrated that upon ectopic expression of TET1 in cells that lacked TET1, the 5-hmC was augmented contrary to when ES cells were depleted of TET1 upon RNAi treatment (Tahiliani, et al., 2009). It was the study of Ito, et al. (2010) that showed all three TET proteins (TET1-3) can convert 5-mC to 5-hmC (Ito, et al., 2010; Dahl, et al., 2011). Figure 1.3 illustrates the chemical structure of 5-mC and its oxidation product 5-hmC.

Figure 1.3. Conversion of cytosine to 5-hydroxymethylcytosine. DNMTs convert cytosine to 5-methylcytosine which subsequently is converted to 5-hydroxymethylcytosine by the TET proteins (Modified from Mohr, et al., 2011).
1.4.3.2 Ten-Eleven-Translocation (TET) enzyme family

A major breakthrough in 2009 revealed that the ten-eleven-translocation (TET) family of proteins were responsible for the conversion of 5-methylcytosine (5-mC) to 5-hydroxymethylcytosine (5-hmC) (Kriaucionis and Heintz, 2009; Tahiliani, et al., 2009). Later on, these enzymes were found to further oxidize 5-hmC to 5-formylcytosine (5fC) and 5-carboxylcytosine (caC) (He, et al., 2011; Ito, et al., 2011; Pfaffeneder, et al., 2011). TET proteins belong to the 2-oxoglutarate (2OG)- and Fe(II)-dependent dioxygenase superfamily (Zhao and Chen, 2013), members of which have been reported to be involved in varied cellular processes, such as histone demethylation and hypoxia sensing (Mohr, et al., 2011).

TET proteins have been reported to be involved in cancer development (Dahl, et al., 2011). TET1 has been recognized as a fusion partner in a rare translocation in leukemia, while TET2 mutations have been seen in myelodysplastic malignancies, acute and myeloid leukemia and secondary acute myeloid leukemia (Ko, et al., 2010; Dahl, et al., 2011). Also, TET2 knockdown has been shown to affect myelopoiesis on early hematopoietic progenitors (Ko, et al., 2010). A number of studies using mouse TET2-null model showed that, although the animals are viable and develop normally with age, eventually they start dying due to hematopoietic malignancies (Branco, et al., 2012).
It has been shown that TET1, TET2 and TET3 exhibit different expression patterns. TET1 and TET2 have been shown to be highly expressed in the inner cell mass of mouse blastocysts and ES cells, while TET3 is highly expressed in mouse oocytes and zygotes (Ito, et al., 2010; Iqbal, et al., 2011; Wossidlo, et al., 2011; Zhao and Chen, 2013). TET2 and TET3 are expressed at various levels in adult tissues (Ito, et al., 2010). TET1 and TET2 have shown to be downregulated and TET3 to be upregulated upon differentiation of mouse ES cells (Tahiliani, et al., 2009; Ito, et al., 2010; Koh, et al., 2011).

There are several contradicting reports on whether knockdown or knockout of TET genes changes pluripotency and differentiation of ESCs (Kinney and Pradhan, 2013). Two studies demonstrated that knockdown of TET1 in mESCs resulted in changes in morphology, decreased expression of pluripotency-associated genes and induction of differentiation (Ito, et al., 2010; Freudengerg, et al., 2011), contrary to the work of other groups (Dawlaty, et al., 2011; Koh, et al., 2011; Williams, et al., 2011), where did not show any morphological changes and sustained normal levels of key pluripotency markers when TET1 was depleted. Further, it has been demonstrated that TET1/2 double knockdown in mESCs led to the downregulation of pluripotency-related genes but not OCT4, NANOG and SOX2 (Ficz, et al., 2011).
Following the work in ESCs, various research groups have explored members of the TET family as reprogramming factors to generate iPSCs. The study of Gao and colleagues (Gao, et al., 2013) has revealed that TET1 can replace exogenous OCT4 and commence somatic cell reprogramming in combination with SOX2, KLF4 and c-Myc, in order to generate fully pluripotent iPSCs (Gao, et al., 2013). The authors have also demonstrated the significant reduction of iPSCs colonies via shRNA-mediated TET1 knockdown. Further, Costa et al. (2013) has established that TET1 and TET2, along with the key pluripotent marker NANOG, improve the efficiency of somatic reprogramming (Costa, et al., 2013). Another study has demonstrated that depletion of TET1 but not TET2 and TET3 via siRNA significantly reduces 5-hmC in human iPSCs (Wang, et al., 2013).

1.4.3.3 5-Hydroxymethylcytosine can serve as a biomarker of toxicity in predictive epigenetic toxicology

Traditional toxicity testing had focused on phenotypical and behavioural changes in animals, tumour formation and genetic changes, such as mutations and chromosomal abnormalities (Thomson, et al., 2014). In recent years, the potential contribution of epigenomic analyses in toxicity studies and drug safety assessments has been in the spotlight (Arita and Costa, 2009; Baccarelli and Bollati, 2009; Cheng, et al., 2011; Smirnova, et al., 2012; Thomson, et al., 2014).
Nowadays, with advanced development in the field of epigenetics and the continuous highly developed technologies, there is an immense opportunity to dissect the effect of xenobiotics at epigenetic levels and produce valuable knowledge on the short and long-term effects of toxicants (Smirnova, et al., 2012; Thomson, et al., 2014).

Modifications in 5-hydroxymethylcytosine patterns upon exposure to xenobiotics have been reported by Thomson, et al. (2012), where mice treated with phenobarbital revealed that 5-mC patterns had changed over long-term treatment with the compound, contrary to the remarkable changes in 5-hmC patterns within one day of treatment (Thomson, et al., 2012; Thomson, et al., 2014). Also, the authors reported at a later stage that the epigenetic signature was dependent on the length of the compound dosage as well (Thomson, et al., 2014). Results from this study provided information of distinctive epigenetic signature differences between the untreated and treated groups demonstrating that it is possible to consider 5-hmC patterns as a distinct signature of xenobiotic exposure, in terms of predictive toxicology (Thomson, et al., 2014).
1.5 Oxygen Tension and Development

Oxygen is a vital element of complex living organisms and throughout evolution they have been required to acclimatize to changes in atmospheric concentrations (reviewed in Brahimi-Horn and Pouysségur, 2007). To maintain oxygen homeostasis is fundamental for the higher eukaryotes and therefore they developed specialized mechanisms to improve oxygen uptake and distribution (Bruick, 2003). Particularly, mammals have developed complex circulatory systems to ensure every cell receives adequate oxygen for normal function, along with mechanisms to sense and respond to any possible hypoxic occurrence (Iyer, et al., 1998) due to pathophysiological conditions, such as neurodegenerative disorders, stroke, tumours and tissue ischemia, resulting in cellular redox imbalances (Liu, et al., 2008). What is considered as physiologically normoxic conditions differs for embryonic or adult cells, but generally falls within the 2-9% oxygen range with the exceptions of bone marrow niches, thymus and kidney medulla (1% O₂ or lower) (reviewed in Simon and Keith, 2008). Hypoxia, except from being the pathophysiological component of various disorders, also occurs in the early normal developing embryo. Exposure of mammalian cells to low-oxygen tension surroundings is known to elicit a hypoxic response by implicating the transcription factor hypoxia-inducible factor (HIF) (reviewed in Simon and Keith, 2008).
1.5.1 Hypoxia-inducible factors (HIFs)

Mammalian cells exposed to decreasing oxygen levels respond quickly by activating transcription factors named hypoxia-inducible factors (HIFs) before reaching an anoxic state and compromise their survival (Figure 1.4). The HIF family consists of three members named HIF-1, HIF-2 and HIF-3. Virtually all cells of the body express HIF-1α, while HIF-2α and HIF-3α are selectively expressed in certain tissue. HIFs activate more than one hundred genes known to allow cells to adapt and survive in hypoxic environments (Klimova and Chandel, 2008; Majmundar, et al., 2010). Despite their role in response to oxygen shortage, HIFs are implicated in diverse biological processes, such as lipid metabolism, angiogenesis, inflammation, cancer (Majmundar, et al., 2010).

**Figure 1.4. Mammalian cellular response to hypoxia.** Mammalian cells respond immediately to decreasing oxygen levels by initiating a stress signal response which activates hypoxia-inducible factors 1 and/or 2 for cellular adaptation and survival (adapted from Klimova and Chandel, 2008).
The hypoxia-inducible factor (HIF) is known to be an oxygen-sensitive transcription factor and the master regulator of adaptation for cells to low oxygen environments, and has been shown to play an essential role in embryonic development, as it is required for embryonic cell survival. Evidence originated from gene knockout of HIFs in mice results in embryonic lethality. HIF-1α/− mice die around embryonic day 10.5-11.0, attributable to lack of blood vessel formation and cardiovascular malformation. The HIF-1β knockout mice died by embryonic day 10.5 due to defective blood vessel formation and angiogenesis of the yolk sac and branchial arches. It has also been reported that HIF-1β/− cells failed to activate genes that normally respond to hypoxia. Further, mice lacking HIF-2α, die between embryonic day 12.5 and 16.5 as a result of inadequate blood vessel fusion and impaired fetal lung maturation (Harvey, et al., 2002; Ke and Costa, 2006).

To establish a role for HIF-1, Iyer and colleagues generated HIF-1α-deficient ES cells and mice that lack HIF-1α expression. Exposure of HIF-1α/− ES cells to hypoxia (1% O₂) showed reduced cell proliferation (Iyer, et al., 1998). Analysis of the HIF-1α-deficient mice embryos revealed that HIF-1α is required for normal cardiovascular development, as they showed several defects, such as pericardia effusion and disorganised cardiac morphogenesis (Iyer, et al., 1998). In addition, HIF-1α/− ES cells show decreased expression of hypoxic responsive
genes such as VEGF, GLUT-1, lactate dehydrogenase (LDH) and Aldolase A (ALDA), indicating the importance of HIF-1α for their activation (Ryan, *et al.*, 1998).

Further, a recent study has demonstrated that HIF induce human ES cell markers in cancer. It has been found that in 11 cancer cell lines tested; hypoxia via HIF induced a human ES cell-like transcriptional program, including the iPSC inducers (OCT4, NANOG, SOX2, c-Myc and microRNA-302) as well as generating iPSC-like colonies when the iPSC inducers combined with nondegradable forms of HIF-α. This data supports that HIF targets may act as inducers of a stemness state in pathological conditions (Mathieu, *et al.*, 2011).

HIF-1α was the first HIF identified and the study by Greg Semenza provided the field with valuable insight into the processes that regulate hypoxic adaptation and survival for cells (Semenza and Wang, 1992; Mohyeldin, *et al.*, 2010). HIF-1α is firmly controlled by the cellular oxygen levels and is produced continuously and accumulates in hypoxic cells, while it is rapidly degraded in the presence of sufficient oxygen levels (Smith, *et al.*, 2008). Several studies have shown that HIF-1 activates the expression of numerous genes involved in the adaptation and survival of cells to low oxygen tensions, such as vascular endothelial growth
factor (VEGF), erythropoietin (EPO), glucose transporters 1 and 3 (GLUT-1, -3) and other glycolytic enzymes (Harvey, et al., 2002).

**Figure 1.5. Stabilization of HIF-1α.** Under normoxic atmospheric conditions PHDs hydroxylate proline residues of hypoxia-inducible factors (HIFα). The von Hippel-Lindau (VHL) complex recognises and marks hydroxylated HIF-1α for degradation. Upon oxygen level decline (hypoxia), HIF-1α accumulates and interacts with HIF-1β subunit forming a dimer. The resulting heterodimer binds to hypoxia response elements (HRE) in the genome, triggers hypoxic response and promotes the transcription of downstream HIF target genes (Modified from Smith, et al., 2008).
Several studies have demonstrated that hypoxia itself is not the only signal that can result in HIF-1α stabilization (Lee, et al., 2007). Reactive oxygen species (ROS) produced by the mitochondria, mainly via complex III, have been reported to play a role in the stabilisation of HIF-1α (Chandel, et al., 2000; López-Lázaro, 2006; Lee, et al., 2007; Ma, 2010; Hamanaka and Chandel, 2009; Hamanaka and Chandel, 2010; Zepeda, et al., 2013). It has been suggested that when cells experience oxidative stress, the activity of PhDs responsible for the HIF-1α degradation is disrupted, ensuring the stabilization of HIF-1α (Lee, et al., 2007).

Further, various chemicals, environmental contaminants or pharmaceutical agents have been reported to experimentally stabilize HIF-1α. Examples of such chemicals are the desferrioxamine (DFO) and dimethyloxalylglycine (DMOG) (Lee, et al., 2007; Pollard, et al., 2008), the environmental contaminants nickel, cobalt, manganese, arsenite, cadmium and zinc (Salnikow, et al., 2000; Salnikow, et al., 2002; Salnikow, et al., 2004; Vengellur and LaPres, 2004; Lee, et al., 2007; Park, et al., 2007), and chemotherapeutic agent 5-azacytidine (Arany, et al., 2011; Tian, et al., 2013). DFO, DMOG, nickel, cobalt and manganese are known to induce HIF-1α by inhibiting the PHD activity (Lee, et al., 2007). On the contrary, histone deacetylase inhibitors (HDACIs), such as valproic acid (VPA), trichostatin (TSA), sodium butyrate (SB) and apicidin (AP) have been shown to attenuate HIF-1α when used on human and mouse tumour
cell lines under 1% hypoxia (Kim, et al., 2007). Further, VPA, TSA, SB and AP were shown to inhibit HIF-1α in mESCs exposed under 1% hypoxia, with VPA being the most efficient HDAC inhibitor (Lee and Kim, 2012).

1.5.2 Oxygen Tension Influence in Stem Cell Biology

Stem cells reside in defined microenvironments, known as stem cell niches (Simon and Keith, 2008; Zhang and Li, 2008). A vital element of such niches appears to be the different oxygen tensions that can affect diverse cellular responses, which results in changes to the properties of the cells. Several studies have demonstrated that low oxygen tensions might influence stem cell niches, by promoting the undifferentiated state in various stem and progenitor cell types, or through inhibition of differentiation of specific stem cell types (Simon and Keith, 2008; Szablowska-Gadomska, et al., 2011).

There are various types of stem cells that exist under low oxygen conditions. Hypoxia maintains the undifferentiated states of neural, hematopoietic, mesenchymal and embryonic stem cells (Mohyeldin, et al., 2010). Embryonic stem cells are derived from embryos, which exist in 3-5% oxygen tension (Covello, et al., 2006). Hematopoietic stem cells in adult mammals are located in a low-oxygen environment in the bone marrow. Neural stem cells of the
mammalian CNS reside in relative hypoxic in the hippocampus and subventricular zone (Szablowska-Gadomska, et al., 2011).

1.5.2.1 The Role of Oxygen in the Regulation of Neural Stem Cells

In the human brain, oxygen tension varies approximately 3-4% (reviewed in Mohyeldin et al., 2010). Oxygen has an important role in the mammalian central nervous system in controlling growth, self-renewal and differentiation processes of neural stem cells and neuronal precursor cells. A number of reports have provided evidence of the beneficial properties of low oxygen tension on neural stem cells and their derivatives (Studer, et al., 2000; Storch, 2001; Li, et al., 2005; Pistollato, et al., 2007; Clarke, et al., 2009; Stacpoole, et al., 2011).

A low oxygen tension between 2.5% and 5% promotes neural stem cell self-renewal and have been shown to support engraftment of in vitro-expanded NSCs that have been transplanted into the brain of experimental animals (De Filippis and Delia, 2011). Recent studies have shown that low oxygen concentration alters differentiation, cell survival, proliferation rate and apoptosis rate. Mild hypoxia (2.5-5% O₂) augmented human NSC proliferation and significantly increased the neuronal and oligodendroglial cell differentiation
(Santilli, et al., 2010). Culture of rat neural crest stem cells under hypoxic conditions resulted in improvement of proliferation and formation of multipotential colonies compared to those of normoxic culture (Morrison, et al., 2000).

Transplantation of human ES cell-derived neural progenitors provide a potential therapeutic strategy, but poor graft survival is a major obstacle as grafted cells show 30-90% cell death within a short period of time after transplantation (Francis and Wei, 2010). In order to resolve the problem, hypoxic preconditioning has been applied to improve ES cell transplantation efficiency. The work of Francis and Wei (2010) has demonstrated that brief hypoxic preconditioning at 0.1% O₂ for 12 hrs reduced cell death compared to the normoxic conditions (21% O₂) and increased human ES cell neuronal differentiation. Also, stabilisation of HIF-1α and HIF-2α and an increase of key neuroprotective genes suggest that they might play a role in the cytoprotection of human ES cell-derived neural cells (Francis and Wei, 2010). Hence, hypoxia could play an important role in improving human neural precursor transplantations and advance (ES) stem-cell based therapies.
1.5.2.2 Hypoxia and Hematopoietic Stem Cells

Hematopoietic stem cells (HSCs) exist inside the bone marrow niche, which has been suggested to be relatively hypoxic compared to other tissues (reviewed in Mohyeldin, et al., 2010). Studies have reported that hypoxic culture of HSCs preserves and/or promotes their colony-forming ability and long-term reconstituting ability (Danet, et al., 2003; Eliasson, et al., 2010; Suda, et al., 2011). Adelman, et al., (1999) has reported significantly higher numbers of CFUs under hypoxia and that hypoxia stimulated the expansion of hematopoietic progenitors in an ARNT-dependent way (Adelman, et al., 1999). Oxygen tension has been demonstrated to affect differently various hematopoietic progenitors as they exhibit varying degree of sensitivity to hypoxia (Cipolleschi, et al., 1993). Further, Takubo, et al., (2010) has shown that HSCs are hypoxic in the bone marrow and express HIF-1α in vivo. The authors demonstrated that HIF-1α was expressed in LT-HSCs and upon its loss the intracellular oxygenation state of HSCs is affected (Takubo, et al., 2010; Pollard and Kranc, 2010). HIF-1α has been also reported to be elevated in adult HSCs and augmented the expression of hypoxia-inducible genes (Simsek, et al., 2010; Pollard and Kranc, 2010).
1.5.2.3 Low Oxygen Tension and Mesenchymal Stem Cells

Another stem cell type where oxygen tension is a critical parameter is the mesenchymal stem cell (MSC). MSCs are multipotent stem cells able to differentiate towards adipogenic, chondrogenic and osteogenic lineages (reviewed in Mohyeldin, et al., 2010) and can be found in bone marrow, adipose tissue and other adult tissues (Rosová, et al., 2008). Generally, MSCs are maintained under normoxic (21% O\textsubscript{2}) culture conditions but their original physiological environment, such as in bone marrow is at lower oxygen tension (Rosová, et al., 2008; reviewed in Mohyeldin, et al., 2010). Also, long-term hypoxia has been reported to maintain the cells in an undifferentiated state and promote plasticity (Basciano, et al., 2011). Further, hypoxic preconditioning of MSCs has demonstrated better migratory phenotype and faster tissue repair (Rosová, et al., 2008), and shown to prevent proliferation senescence and increase the differentiation efficiency (Tsai, et al., 2011). Additionally, hypoxic isolation and expansion of human bone marrow MSCs led to a more efficient \textit{in vitro} chondrogenic differentiation compared to normoxic conditions (Adesida, et al., 2012).

Further, there have been studies where hypoxia has been shown to have negative effects on the proliferation and differentiation potential of MSCs. It has been reported that hypoxia inhibits osteogenic differentiation in MSCs via direct
regulation of RUNX2 by TWIST (Yang, et al., 2011). In this study, hypoxia (1% \( O_2 \)) inhibited the expression of type 1 RUNX2 and its downstream target genes, such as osteocalcin, osteopontin, bone sialoprotein and collagen type 1 alpha. Also, hypoxia inhibited the functional mineralization of MSCs at days 14 and 21 of osteogenic differentiation (Yang, et al., 2011). Additionally, the study of Holzwarth, et al. (2010) showed that low physiological oxygen tension during in vitro culture of human MSCs resulted in an evident reduction of differentiation towards adipose and bone tissue. Contrary to other published data sets, this study also demonstrated that low oxygen tensions led to decreased levels of MSC proliferation (Holzwarth, et al., 2010).

Ultimately, due to the differences in the MSC isolation, the use of MSCs from different species, culture conditions and experimental protocols have made it complicated to conclude the exact role of hypoxia in MSC biology (reviewed in Mohyeldin, et al., 2010; Holzwarth, et al., 2010).

### 1.5.3 Oxygen Tensions and Embryonic Stem Cell Pluripotency

The normal physiologic environment of ES cells is of low oxygen concentration starting at implantation and continuing through fetal development. The environment is hypoxic due to the lack of access to maternal circulation during
implantation of the embryo. Even when the embryo establishes connection to the maternal vasculature the placental oxygen tension is not higher than 8% O\textsubscript{2} (Abdollahi, et al., 2009).

Besides the extensive work that has highlighted the importance of oxygen concentration in the culture and manipulation of various ES stem cell lines, the majority of laboratories continue their daily tissue culture under atmospheric oxygen conditions. Numerous studies have demonstrated the beneficial effects of maintaining ES cells under low oxygen conditions resembling the \textit{in vivo} embryonic development.

Increasing evidence illustrates that culturing ES cells under low oxygen tensions improves morphology, diminishes levels of spontaneous differentiation, increases proliferation rate, enhances expression of pluripotency markers and reduces chromosomal abnormalities (Ezashi \textit{et al.}, 2005; Forsyth \textit{et al.}, 2006; Westfall, \textit{et al.}, 2008; Forristal, \textit{et al.}, 2010; Rajala, \textit{et al.}, 2011).

Zachar, \textit{et al.} (2010) investigated for the first time the long-term effects of maintaining human ES cell lines under 5% oxygen. Human ES cells were maintained under an uninterrupted low oxygen concentration for over 18
months. Prolonged exposure to hypoxia maintained normal colony morphology with uniform undifferentiated appearance. Quantitative histomorphometric examination demonstrated that 98% of the colony retained its undifferentiated state under hypoxic culture conditions, contrary to 56% of that of normoxic cultures. The markers OCT4 (self-renewal) and SSEA-1 (differentiation) further confirmed the undifferentiated state of the hypoxia-maintained cells. Further, both normoxic and hypoxic human ES cell cultures demonstrated the ability to differentiate in vitro into all three germ layer lineages. This study provided valuable insight into the long-term preservation of human ES cells and the improvement of the undifferentiated phenotype at low oxygen concentration in an in vitro environment (Zachar, et al., 2010).

An equally important aspect of research along with the beneficial effects of low oxygen tensions on ES cell pluripotency was to investigate the functional significance of hypoxia inducible factors in regulating the hypoxic responses of ES cells. Studies have shown HIF induction upon exposure to hypoxia in order to regulate and ensure the cellular adaptation to low environmental oxygen tensions.

In agreement with previous studies, Forristal, et al. (2010), observed that hypoxia (5% O₂) alters the morphology of human ES cells as colonies emerge.
more compact and with clearly defined borders, larger colony diameter and higher cell number, compared with those cultured at 20% O\textsubscript{2} where cultures show peripheral spontaneous differentiation, smaller diameter and lower cell number. In addition, there was a significant decrease of the mRNA expression of pluripotency markers OCT4, NANOG and SOX2 under normoxia. The HIFs (HIF-1\textalpha, HIF-1\beta, HIF-2\alpha and HIF-3\alpha) were all upregulated at an mRNA level under hypoxic conditions, with the exemption of HIF-1\alpha at a protein level. Following silencing of HIF-1\alpha, HIF-2\alpha and HIF-3\alpha, the authors observed that there was no change in human ES cells morphology after HIF-1\alpha and HIF-3\alpha knockdowns under 5% oxygen for 48 hrs, contrary to HIF-2\alpha knockdown where colonies appear to have large areas of differentiation. Upon double-knockdown of HIF-1\alpha and either HIF-2\alpha or HIF-3\alpha the cells failed to form colonies and possessed large differentiation areas. In the case of HIF-2\alpha and HIF-3\alpha double-knockdown, cells were capable of forming colonies with large areas of differentiation. Further, the cell number and colony size were significantly reduced when HIF-2\alpha was silenced and upon the HIF-2\alpha and HIF-3\alpha double-knockdown. Additionally, knockdowns of HIF-2\alpha and HIF-3\alpha showed significant decrease of OCT4, NANOG and SOX2 while the HIF-1\alpha knockdown did not affect the pluripotency of human ES cells (Forristal, et al., 2010). Hence, this study not only confirmed observations of earlier studies but provided additional evidence regarding the response of human ES cells upon low oxygen tension, since HIF-1\alpha was shown to be responsible for the initial adaptation of cells to
hypoxic conditions. HIF-2α regulated the long-term response to hypoxia by controlling proliferation and pluripotency, and HIF-3α controlled the expression of both HIF-α and HIF-2α. Further, HIF-2α has been shown to activate OCT4, one of the core regulators of pluripotency (Simon and Keith, 2008). Covello and colleagues have identified OCT4 as a novel HIF-2α target and reported that expanded HIF-2α expression is sufficient to induce OCT4 and VEGF in the context of tumour progression and embryonic development (Covello, et al., 2006). Additionally, a recent study using different human ES cell lines has demonstrated that hypoxia enhances SSEA-3 and MYC, a surface marker known to decline in response to differentiation, and a marker essential for maintaining pluripotency and when overexpressed known to induce pluripotency, respectively (Närvää, et al., 2013). The authors have shown that prolonged hypoxic culture (7 days at 4% O2) is beneficial for human ES cells in maintaining an enhanced pluripotent state and preventing differentiation, thereby confirming previous reports. An interesting finding described in this study and for the first time in terms of human ES cells was the fact that HIF prolyl hydroxylases PHD1-3 were expressed under hypoxia (7-day time point). PHD2 and PH3 were expressed significantly higher after 24 hrs hypoxic exposure. Both genes were transcriptionally active under hypoxia in all tested human ES cell lines (Närvää, et al., 2013), whereas up to date PHDs were shown to be oxygen-dependent in other cell systems. Collectively, these studies offered a
better insight into the functional role of hypoxia and HIFs in the regulation of human ES cell pluripotency.

### 1.5.4 Low Oxygen Tensions and Induced Pluripotency

Somatic cells reside in specialised microenvironments and environmental factors such as temperature, stromal cell contacts and oxygen concentration can affect stem cell function (Yoshida, et al., 2009). In view of the fact that numerous studies have demonstrated hypoxia to promote the survival of different stem cell types and prevent the differentiation of human ES cells, Yamanaka’s group investigated the possibility that hypoxic treatment might endorse reprogramming and consequently generation of induced pluripotent stem cells (Yoshida, et al., 2009). Results from this study revealed successful generation of iPSCs from mouse embryonic fibroblasts (MEFs) and human dermal fibroblasts (HDFs) under mild hypoxia (5% O$_2$). Analysis of the human iPS cell lines generated under hypoxic culture conditions, showed successful differentiation potential (cells were positive for endoderm, mesoderm and ectoderm markers) and teratomas differentiated into tissues that represented all three germ layers. Furthermore, analysis of transduced MEFs maintained under hypoxia displayed a 73.2% upregulation of ESC-specific genes and 85.8% downregulation of MEF-specific genes, an enhanced proliferative effect and increased the expression of pluripotency markers OCT4 and NANOG (Yoshida, et al., 2009).
In Shimada, et al. study (2012), adipose-derived stem cells were transduced with the four factors OCT4, SOX2, KLF and L-Myc and a cocktail of chemical inhibitors (A8301, CHIR99021, PD0325901, sodium butyrate, Y-27632) under physiological hypoxia (5% O₂). Within six days, human ES cell-like colonies emerged. At the end of the reprogramming experiment the established human iPS cell lines were positive for OCT4, NANOG, SSEA-4, Tra-1-81 and alkaline phosphatase. The iPS cells were capable of differentiating in vitro into all three germ layers, form teratomas and maintain a normal karyotype. Upon microarray and genome-wide methylation analysis, the human iPS cells showed similar gene expression patterns and epigenetic states to those of H9 human ES cells. The authors were able to develop a method for rapid generation of human iPS cells using a combination of chemical inhibitors and physiological hypoxia (Shimada, et al., 2012).

In addition, the effects of hypoxia on pluripotency and the role of hypoxia inducible factors were investigated in mouse iPS cells. Sugimoto, et al. (2013) retrovirally transduced murine fibroblasts using four transcription factors (OCT4, SOX2, KLF4 and c-Myc) and three factors (OCT4, SOX2 and KLF4) under normoxic (20% O₂) and hypoxic (5% O₂) culture conditions. Differences in cell morphology and number were observed as iPS cells maintained under hypoxia had an oval shape with a smooth surface, contrary to the normoxic controls where differentiated cells appeared at the periphery of cells. The transduced
cells at 5% O₂ were grown faster than at 20% O₂ but started to decrease after seven days of culture. Under hypoxic conditions, the pluripotency markers OCT4, NANOG and SOX2 were significantly elevated. Further, HIF-1α, HIF-2α and HIF-3α were silenced to examine the effects of HIFs on pluripotency. Data revealed that HIF-2α knockdown led to a decrease in colony size while silencing of HIF-1α and HIF-3α had no effect in cell morphology. A significant decrease in OCT4, NANOG and SOX2 mRNA and protein levels was observed in HIF-2α and HIF3-α knockdown but not HIF-1α. Collectively, these results demonstrated that HIF-2α is the most influential HIF for the maintenance of murine iPS cell pluripotency (Sugimoto, et al., 2013).

Apart from dermal fibroblasts, hypoxia has been used to investigate the potential reprogramming of human dental pulp cells (Iida, et al., 2013). Previous work has shown that hypoxia (3% O₂) has been beneficial for the proliferation and inhibition of differentiation of human dental pulp cells and that the oxygen tension of rat pulp tissue is lower than that of air (Iida, et al., 2013). Based on this knowledge, human dental pulp cells were reprogrammed using the four classical transcription factors (OCT4, SOX2, KLF4, c-Myc) and cells were cultured under normoxia and hypoxia (21% and 3% O₂, respectively). Exposure of dental pulp cells under hypoxia for the first six days resulted in significant increase of NANOG expression, the number of alkaline phosphatase positive
cells and ES cell-like colonies. The cells maintained under hypoxia for two days, showed augmented levels of reactive oxygen species, which reduced upon treatment with antioxidants such as vitamins C and E. Characterisation of the generated iPSC colonies showed ES cell-like morphology, alkaline phosphatase positive activity, expression of OCT4, SSEA-4, Tra-1-60 and Tra-1-81, comparable NANOG expression to that of human ES cells and formation of teratomas, indicating that hypoxia enhanced the reprogramming of dental pulp cells (Iida, et al., 2013).

1.5.5 Low Oxygen Tensions and “De-differentiation”

Based on the fact that hypoxia has been shown to maintain the undifferentiated state of various stem cell types and enhances the efficiency of generating iPS cells, scientists were urged to determine whether hypoxia could change cell fate and reverse early committed cells back to the human ES cell state. Mathieu, et al. (2013) used two human ES and one iPS cell lines and serum forced undirected differentiation. Subsequently, the lines were maintained under normoxic (20% O$_2$) and hypoxic (2% O$_2$) culture conditions. Human ES cell-like colonies emerged in cultures under hypoxia within 1-2 weeks (“de-differentiated” cells) and had similar morphology to that of normoxia under self-renewing culture conditions, contrary to the normoxic control cultures that continued to differentiate. The expression of ES stem cell markers OCT4, NANOG and SOX2
was significantly downregulated in the cultures under 20% O$_2$ and several early differentiation markers were upregulated, while cultures at 2% O$_2$ showed similar levels of expression for the ES stem cell markers to the human ES cell control condition and downregulation of the markers of early differentiation. Genome wide expression analysis of the “de-differentiated” cells revealed similar expression profiles to human ES cells. The expression profiles also revealed that at an oxygen concentration of 2% O$_2$, the “de-differentiated” cells showed HIF stabilisation as there was a highly significant increase of HIF-1$\alpha$ and HIF-2$\alpha$ target genes. This study presented data suggesting that HIF activation, due to hypoxic conditions, may direct stem cells that drift inappropriately towards differentiation back to a more embryonic state (Mathieu, et al., 2013).

1.6 Oxidative Stress

Oxidative stress is the general term to describe the imbalance between the production of reactive oxygen species (ROS) and a biological system’s ability to detoxify the reactive intermediates (Pizzimenti, et al., 2010). It can be induced by various environmental factors including radiation, UV stress, chemotherapeutic compounds, environmental toxins and oxygen shortage (hypoxia and anoxia) (Pizzimenti, et al., 2010). The effect of oxidative stress on cellular processes depends on cell, tissue and organ types, cellular location and
concentration of ROS, species differences, cellular antioxidant defence systems and the duration and strength of the exposure.

1.6.1 Generation of Reactive Oxygen Species (ROS)

Reactive oxygen species are chemical species that contain oxygen and can oxidise other molecules. They are reactive towards DNA, proteins and lipids. Most ROS are derived from superoxide, which is generated by the one electron reduction of oxygen.

ROS are constantly produced and eliminated in a biological system via various scavenging systems that cells possess under normal physiological conditions to minimise potential damage to the cellular environment. Once believed to be harmful by-products of the metabolism, recent studies now recognise ROS as physiological messengers involved in the control of adaptive cell biology (Finkel, 2011). The harmful effects of ROS are counteracted by various mechanisms, permitting an effective antioxidant defence system. Under physiological conditions, cellular antioxidant capacity neutralises the generation of ROS. On the contrary, when there is an excess of ROS production and/or an inefficient antioxidant defence network, an oxidative stress condition is created, leading to the deleterious effects of ROS (Batandier, et al., 2002). Except for a role as
damaging agents, reactive oxygen species function as intracellular signaling molecules. Various studies have demonstrated that ROS have a role in cell signalling, gene expression, apoptosis, host cell defence mechanisms, pathological conditions, stem cell biology, epigenetic status of cells and hypoxia (Figure 1.6) (Thannickal and Fanburg, 2000; Ji, et al., 2010; Klaunig, et al., 2010; Pizzimenti, et al., 2010; Vieira, et al., 2011).

Figure 1.6. Illustration of the diverse roles of reactive oxygen species.

Whether there is a decrease or increase in oxidative stress and ROS production during hypoxia, has been an object of debate, mainly based on the behaviour of existing oxidant-sensitive probes (Guzy, et al., 2005) and due to conflicting study
conclusions that might have contributed to the differences in the actual oxygen tension (1-5%) used and the possible changes of oxygen content during cell handling, which might result in re-oxygenation and increase in ROS production (Saretzki, 2011).

The main reactive oxygen species include superoxide (O$_2^*$), hydrogen peroxide (H$_2$O$_2$) and hydroxyl radical (OH*). Reactive oxygen species differ from each other in terms of their reactivity. Hydrogen peroxide, superoxide and hydroxyl radical can be categorised as moderately, intermediately and extremely reactive, respectively (Yechiel, 2005).

Significant levels of ROS result from endogenous sources such as the normal by-products of essential cellular metabolic processes. Historically, mitochondria have been mainly considered as major consumers of oxygen in cells, as they utilise the great amount of 85-90% to allow oxidative phosphorylation, which is the primary metabolic pathway for ATP production (Solaini, et al., 2010). In addition to energy production via the mitochondrial electron transport chain, the mitochondria have been characterised as an important source of ROS within mammalian cells. During mitochondrial respiration, under normal oxygen environments, molecular oxygen is chemically reduced to water and the resulting energy change is conserved in the form of ATP. It has been estimated
that 2-3% of the oxygen consumed by the mitochondria is incompletely reduced, resulting in the formation of superoxide ($O_2^-$) (Tormos and Chandel, 2010). Superoxide is the proximal ROS generated by mitochondria and is converted to hydrogen peroxide ($H_2O_2$) which arises from the dismutation of the superoxide (Murphy, 2009). Hydrogen peroxide is then degraded to water by catalase or by glutathione peroxidase (Chandel, et al., 1998).

The model of mitochondria being a source of ROS generation in mammalian cells has been a controversial issue in the scientific community, along with dispute about whether hypoxia results in a decrease or increase of ROS production from mitochondria. This is due to the fact that initial studies relied on pharmacological tools, technical concerns regarding the ROS measurements in living cells and conflicting studies in the literature (Guzy, et al., 2005). A potential reason for the contradictory data is that some studies investigated the stabilization of HIF-1α under conditions of 1-2% $O_2$ and others close to anoxia (0% $O_2$) (Brunelle, et al., 2005).

It might be expected that hypoxia would decrease ROS generation due to low oxygen levels and to the reduced mitochondrial respiration, but paradoxically ROS levels increased. As oxygen levels decrease, cells respond by activating hypoxia-inducible factor (HIF) dependent gene transcription to assist adaptation
to hypoxia. Over a decade ago, evidence provided by the studies of Chandel, et al., (1998, 2000) showed that mitochondrial ROS activate hypoxia-induced transcription and that ROS generated at mitochondrial Complex III stabilize HIF-1α during hypoxia. Cells devoid mitochondrial DNA and cells that had been treated with mitochondrial electron transport inhibitors failed to activate HIF under hypoxic conditions, due to lack of mitochondrially generated ROS (Chandel, et al., 1998; Chandel, et al., 2000).

Duranteau and colleagues (1998) employed fluorescence imaging of the dye 2',7'-dichlorofluorescein to assess mitochondrial ROS generation in embryonic cardiomyocytes, during different levels of hypoxia. They demonstrated that mitochondrial ROS generation increased as the oxygen concentration decreased during hypoxia. A progressive increase of DCF fluorescence was observed at 5%, 3% and 1% O₂, with the highest level of oxidation recorded at the lowest oxygen concentration, compared to the normoxic conditions at 15% O₂. Administration of two antioxidant compounds at 3% O₂ resulted in a significant reduction of the cell fluorescent signal within two hours, indicating that the oxidant signal was responsible for the increase of DCF fluorescence. They further confirmed that mitochondria was the source of ROS generation by loading the cells with 2',7'-dichlorofluorescein during hypoxia under the exposure of two mitochondrial electron transport inhibitors which resulted in the rapid attenuation in DCF fluorescence (Duranteau, et al., 1998).
Another study in the search of whether hypoxia induces mitochondrial ROS generation revealed that Hep3B cells increase ROS generation when exposed at hypoxic conditions of 1.5% O$_2$. Wild-type and respiration-deficient Hep3B cells exposed to different levels of hypoxia assessed for ROS generation using 2',7'-dichlorofluorescein (Chandel, et al., 1998). Data revealed that there was a graded increase in fluorescence during 5%, 3% and 1% O$_2$, with the higher increase observed at the lower oxygen level. Further, respiration-deficient Hep3B cells exposed to hypoxia (1% O$_2$) with 2',7'-dichlorofluorescein showed no increase in DCF fluorescence indicating that ROS generation derived from functional mitochondria. In addition, the authors tested the ROS generation in wild-type cells via the use of mitochondrial inhibitors (which suppress the formation of ubisemiquinone at complex III) during 2% O$_2$ and found that all the inhibitors individually abolished the increase of DCF fluorescence. Administration of antimycin A resulted in elevation of DCF fluorescence in wild-type cells during hypoxia. Convincing evidence that mitochondria are important in the hypoxic response was demonstrated by the respiration-deficient cells lacking the ability to activate HIF-1 during hypoxia. The results of this study clearly indicate that mitochondrial complex III acts as the main site of ROS generation during hypoxic conditions (Chandel, et al., 1998).

Following studies have shown that mitochondria increase the levels of cytosolic ROS to activate HIF during hypoxic exposure of the cells (Bell, et al., 2007) and
others confirmed that mitochondria-produced ROS are required for hypoxic stabilisation of HIF (Brunelle, et al., 2005; Guzy, et al., 2005; Mansfield, et al., 2005). RNA interference against a mitochondrial complex III subunit prevented the hypoxic stabilization of HIF-1α, as shown by Brunelle, et al., (2005). Cells transfected with siRNA against the Rieske iron-sulfur protein (a component of complex III) resulted in failure to stabilise the HIF-1α under hypoxia. Further, to establish which ROS type is responsible for the hypoxic stabilization of HIF-1α, cells were infected with an adenovirus containing SOD1, SOD2, GPX1 or catalase (21% O₂ normoxia and 1.5% O₂ hypoxia) and it was found that GPX1 or catalase reduced HIF-1α stabilisation while SOD1 and SOD2 failed to modify it. Hence, the hypoxic stabilisation of HIF-1α protein required hydrogen peroxide and not superoxide (Brunelle, et al., 2005).

Bell, et al., (2007) demonstrated that hypoxia (1.5% O₂) increased the replicative life span of primary human lung fibroblasts (PHLFs), compared to normoxic culture conditions. The cells maintained under hypoxic conditions showed an increase in the proliferation rate and a significant elevation in mitochondria-generated reactive oxygen species (H₂O₂) at 1.5% O₂ and 3% O₂, as determined by Amplex Red. When the culture of PHLFs was supplemented with MitoQ, a mitochondrion-targeted antioxidant, the increase of ROS observed under hypoxia was abolished. This data revealed that an increase in
mitochondrial ROS generation during hypoxic exposure was associated with extended replicative life span (Bell, et al., 2007). Further, another assay was utilized for the measurement of ROS generation under normoxic (21% O$_2$) and hypoxic (1.5% O$_2$) culture conditions via the infection of PHLFs with an adenovirus expressing a biological target of ROS in living cells, roGFP protein (redox-sensitive GFP protein that contains GFP mutations with two surface-exposed cysteine residues). When an oxidant is present, a disulfide bond forms between the two surface-exposed cysteines and increase the excitation at 400 nm. The cells exposed to hypoxia showed a significant elevation in ROS levels compared to the normoxic controls, signifying that the ROS produced during hypoxia are adequate to react with protein targets in living cells. Additionally, the authors showed that ROS generated under hypoxic culture conditions (1.5% and 3% O$_2$) did not result in DNA damage, as shown by the lack of phosphorylation of H2AX (marker of DNA double-stranded breaks), contrary to the treatment with staurosporine (DNA damaging agent). This result indicated that ROS generated under hypoxia are not acting as DNA damaging factors but rather like signalling molecules. Similar to previous studies, the authors were able to demonstrate that hypoxia-induced generation of mitochondrial ROS triggered HIF-1$\alpha$ activation in PHLFs. Treatment with the mitochondrion-targeted antioxidant MitoQ, eliminated the stabilisation of HIF-1$\alpha$ protein under hypoxic conditions, suggesting that cells required hypoxic induction of mitochondrial ROS for the activation of HIF-1$\alpha$ (Bell, et al., 2007).
Another study in the same year from Bell, et al., (2007) provided data indicating that mitochondrial electron transport has a significant role in the stabilisation of HIF-1α during hypoxia (1.5% O₂) and that complex III is the primary site of ROS generation. Additionally, it was revealed that HIF-1α stabilises under normoxic conditions due to exogenous ROS production, when cells were treated with glucose oxidase, an enzyme that generates H₂O₂. Glucose oxidase was found to increase ROS levels under normal oxygen conditions, similar to those determined under hypoxic conditions. Upon treatment with the antioxidant catalase, inhibition of HIF-1α was achieved, indicating that the generation of H₂O₂ was responsible for the stabilisation of HIF-1α protein. Further evidence from the study showed that cells treated with shRNA against the mitochondrial transcription factor A (TFAM), which is required for proper transcription and replication of mitochondrial DNA, had diminished their capacity to stabilise HIF-1α under hypoxia. The authors were able to demonstrate that the Qₒ site of the mitochondrial complex III is necessary to increase cytosolic ROS generation, which leads to the stabilisation of the HIF-1α protein during hypoxia (Bell, et al., 2007).

The mitochondrial formation of ROS has been implicated in various human pathological conditions. Studies have demonstrated that mitochondria produce ROS at higher rates than their scavenging capacity. There is strong evidence
that mitochondria are associated in the pathogenesis of various neurodegenerative diseases due to oxidative damage and has reinforced the links among mitochondrial dysfunction and elevated ROS production (Johri and Beal, 2012). Research in the pathogenesis of Alzheimer’s disease (Mecocci, et al., 1994; Sultana, et al., 2011), Amyotrophic Lateral Sclerosis (Warita, et al., 2001; Menzies, et al., 2002; Cozzolino, et al., 2012), Huntington’s disease (Browne, et al., 1997; Johri and Beal, 2012; Ayala-Peña, 2013) and Parkinson’s disease (Cassarino, et al., 1997; Merad-Boudia, et al., 1998; Motherwell and Zuo, 2013) have revealed oxidative damage events.

1.6.2 Determination and Quantification of ROS

There are several direct and indirect methods available to assess the production of reactive oxygen species in vivo and in vitro. It is of major importance that reliable methods are used to detect ROS taking into consideration the chemical properties of the probe, variability in sensitivity and specificity of fluorescent probes, physical properties, such as absorption and emission spectra, and solubility of the culture medium, and the biological tissue under investigation (Wardman, 2007).
It is of critical importance to distinguish between the measurements of specific ROS and assess the damage that these cause. There is a variety of oxidative damage makers used to identify oxidative damage in cellular systems, such as breakdown products of damaged DNA and lipid peroxidation products. Nevertheless, the accumulation of a biomarker for oxidative injury does not provide a direct link with the generation of a particular ROS. The improvement of specific biomarkers of oxidative damage is essential in order to accurately validate oxidative damage in different cellular systems, since it is unlikely that a single biomarker will give a complete assessment of damage, as different types of oxidative injury to proteins, lipids or the genome will result in different patterns of biomarker accumulation, an accumulation can further be influenced by the interference of antioxidants (Murphy, et al., 2011).

ROS exist mainly from low to very low concentrations ($10^{-4}$-$10^{-9}$ M) and this requires the usage of highly sensitive analytical methods. Various techniques have been developed, over the years, for the detection and quantification of reactive oxygen species including spectrophotometric and fluorometric methods (Batandier, et al., 2002).

A widespread method to measure the oxidant levels in living cells is the CM-H$_2$DCFDA (5-(and-6) -chloromethyl- 2’, 7’-dichlorohydrofluorescein diacetate). It
is rapidly taken up by cells. Intracellularly, it is converted into non-fluorescent CM-H₂DCF by esterase action and in the presence of intracellular oxidants, it oxidizes into highly fluorescent CM-DCF. The lack of specificity of CM-DCF formation makes it difficult to identify the nature of the oxidants detected by the probe and, for this reason, the formation of CM-DCF cannot be used as a direct reporter to identify specific ROS, but should be utilised as a general indicator to measure overall cellular redox status in cellular systems (Forkink, et al., 2010). Another marker for measuring overall oxidant levels is dihydrorhodamine 123 (DHR123). It is a reduced rhodamine that is not reactive towards superoxide and hydrogen peroxide in the absence of catalysts. Fluorescent R123 is formed in the presence of reactive oxygen and reactive nitrogen species, such as OH⁺, NO₂⁻ and ONOO⁻ (Forkink, et al., 2010).

A more advantageous method to detect reactive oxygen species is the use of protein-based ROS reporter molecules. These molecules have the advantage that they can particularly be expressed in different cellular compartments (e.g. nucleus, mitochondrial matrix, Golgi membrane) via N- or C-terminal fusion to specific targeting sequences. Further, protein-based ROS reporters can be fused to specific ROS detoxifying or ROS generating proteins of interest, permitting local detection (Forkink, et al., 2010).
1.6.3 Antioxidant Cellular Systems and Antioxidant-related Enzymes

Molecules or substances that prevent and/or eliminate oxidative damaging of cells are considered as antioxidants. Mammalian cells have antioxidant cellular systems, such as glutathione, superoxide dismutases, peroxiredoxins, catalase and thioredoxin, to ensure the maintenance of redox homeostasis (Pizzimenti, et al., 2010; Finkel, 2011).

1.6.3.1 Glutathione (GSH) System

Glutathione is found in all mammalian tissues. Cells have three main reservoirs of glutathione, having a small percentage in the endoplasmic reticulum, 10% in the mitochondria and approximately 90% present in the cytosol. Cellular GSH is a multifunctional molecule with diverse roles in important cellular processes, including scavenging of reactive oxygen species, detoxifying xenobiotics, maintaining essential thiol status of proteins, providing a reservoir for cysteine, promoting cell growth and implicating in the modulation of cell death (Lu, 2009).

GSH is a vital antioxidant system for mammalian cells, as they are exposed to a certain levels of physiological oxidative stress, derived from the mitochondrial respiration. Mitochondrial GSH is vital in the defence against the physiological...
and pathological production of reactive oxygen species, such as superoxide and hydrogen peroxide, that are formed and can lead to cell damage. The endogenously hydrogen peroxide formed is metabolised to water by GSH in the presence of GSH peroxidase in the mitochondria and by catalase in the peroxisome (Lu, 2009).

Glutathione maintains a balanced intracellular redox status and is essential for the epigenetic maintenance of mammalian cells. When cells are exposed to conditions of excess oxidative stress, GSH pools begin to deplete. The cellular demand for GSH increases, leading to rapidly reduced availability of cysteine, which is the vital substrate for GSH biosynthesis. Depletion of this important factor perturbs the methionine pool, resulting in deficiencies of SAM (S-adenosylmethionine (SAM) and SAH (S-adenosylhomocysteine). Reduction of the SAM/SAH ratio can have significant effects on the epigenetic regulation of cells, as it affects various methylation reactions and can ultimately alter gene expression (Cyr and Domann, 2011).

1.6.3.2 Endogenous Antioxidant Defence Enzymes

Cellular antioxidant defence enzymes against oxidative stress include the superoxide dismutases, glutathione peroxidases and catalase (Pizzimenti, et al.,
One of the fundamental antioxidant enzymes is superoxide dismutase (SOD) which catalyses the reaction of superoxide to hydrogen peroxide. There are three forms to this enzyme, SOD1, a copper/zinc isoform present in the mitochondrial intermembrane and cytosol; SOD2, a manganese isoform present in the mitochondrial matrix; and SOD3, a copper/zinc isoform present in the extracellular matrix (Batandier, et al., 2002; Magder, 2006). Knockout of SOD2 in mice is lethal in the first week of life (Li, et al., 1995; Melov, et al., 1999) while deficiencies in SOD1 and SOD3 are not lethal, but lead to less tolerance of neuronal injury (Reaume, et al., 1996). Further, another family of antioxidant enzymes are the GSH peroxidises, which play an essential part in the removal of oxidants from the cells, by catalyzing the reduction of hydrogen peroxide (Allen and Ballin, 1989). Evidence support the claim that GPXs increase during development in mammalian tissues and their changes are tissue specific (Allen and Ballin, 1989). There are at least six mammalian isoforms which catalyze the reduction of hydrogen peroxide (Finkel, 2011) and are localised in mitochondria, cytoplasm and nucleus (Batandier, et al., 2002). Lastly, catalase is known to augment in many tissues, but not all, during development and its activity has been shown to be affected by iron availability (Allen and Venkatraj, 1992). Catalase is concentrated in peroxisomes and its function is to react with hydrogen peroxide to form water and oxygen (Allen, 1991).
1.6.3.3 Exogenous Administration of Antioxidants

A widespread compound characterised as an antioxidant in biological conditions is N-acetylcysteine (NAC). NAC is a membrane-permeable precursor of glutathione and has been shown, in several studies, to decrease oxidative stress. Animal and human studies propose the effective use of NAC for the treatment of diseases considered to be mediated by oxidative stress (Zhang, et al., 2011). Evidence shows that the antioxidant NAC protects the human kidney proximal tubule epithelial cells against ROS-induced cytotoxicity, inhibiting mitochondrial membrane potential collapse and ROS generation and diminished DNA damage (Zhang, et al., 2011).

Qian and Yang (2009), investigated the effects of NAC on neuronal differentiation of mouse ES cells induced by retinoic acid. Retinoic acid has been shown to generate reactive oxygen species in mouse embryonic stem cell culture, leading to cell death (Castro-Obregón and Covarrubias, 1996). Data revealed that NAC enhanced the number of neurons and suppressed cell death caused by retinoic acid during differentiation (Qian and Yang, 2009).

Further, during in vitro culture conditions, the cells are exposed to various environmental factors that can induce alteration in cellular performance. One
key underlying mechanism of such impact is oxidative stress. A recent study has investigated the changes of cellular properties during hematopoietic differentiation of induced pluripotent stem cells and utilised NAC as a potential antioxidant and cytoprotective agent. Results showed that over the course of hematopoietic differentiation there was a significant elevation of ROS that was reduced upon NAC treatment. In addition, NAC decreased apoptosis, prevented cellular senescence and significantly attenuated mitochondrial membrane potential collapse in differentiating iPSCs. Further investigation revealed that treatment with NAC increased the efficiency of hematopoietic differentiation of iPSCs, as seen by the increase in expression of hematopoietic markers CD34 and CD45. These results demonstrated that treatment with the antioxidant NAC was beneficial for the culture and improved the hematopoietic differentiation of iPSCs (Berniakovich, et al., 2012).

1.7 Epigenetic Responses to Hypoxia

Oxygen is a vital component for various cellular processes in many organisms and, any variations in its availability, can cause significant changes at cellular levels (Melvin and Rocha, 2012). There are a number of dioxygenases involved in oxygen sensing, such as PHDs and FIH that interact with HIF, which is the master regulator of cellular adaptation to hypoxia and one of the main cofactors they require to function is oxygen (Hitchler and Domann, 2007; Melvin and
Rocha, 2012). Since the discovery of these enzymes, many others have been found to play a role in response to low oxygen tensions, such as the Jumonji demethylases. These function similar to PHD and FIH as they utilise the same cofactors (oxygen, Fe(II), α-ketoglutarate) (Hitchler and Domann, 2007). Hence, oxygen might be a critical determinant in various epigenetic modifications and drive developmental processes via cell type-specific gene expression (Hitchler and Domann, 2007; Cyr and Domann, 2011; Perez-Perri, et al., 2011; Melvin and Rocha, 2012).

1.7.1. Hypoxia and DNA Methylation

Hypoxia has been reported to inhibit the activity of DNMTs and prevent DNA methylation (Melvin and Rocha, 2012). A recent study has shown that short-term (24 hrs) hypoxic (1% O₂) exposure of primary neuronal cells, led to changes in the DNA methylation status. The authors reported that the level of CpG methylation revealed a greater level of hypomethylation in the hypoxic cells, compared to the control group and that it remained long after the hypoxic stress was eliminated. This data indicated that even a short-term strong hypoxia leads to long-term changes in DNA methylation (Hartley, et al., 2013). The study of Showronski, et al. (2010) demonstrated that upon hypoxic exposure of human colorectal cancer cells, there was a reduction of DNMTs at mRNA and protein levels (Showronski, et al., 2010). Also, DNA hypomethylation was observed in
colorectal cancer, melanoma cell lines and normal human fibroblasts exposed to short-term severe hypoxia (< 0.1% O₂). In this study, the reduction of 5-mC was greater in the normal fibroblasts than the cancer cell lines (Shahrzad, et al., 2007). Further, it has been found that hypoxia leads to DNA demethylation via the activation of HIF-1α in hematoma cells (Liu, et al., 2011). On the contrary, there are studies that demonstrated chronic hypoxia leads to increases in DNA methylation (Whatson, et al., 2009; Melvin and Rocha, 2012).

1.7.2. Hypoxia and Histone Modifications

Although there are only a few studies on how hypoxia affects histone modifications (histone acetylation and methylation), researchers have shown evidence that histone modifications take place upon exposure to reduced O₂ availability, under diverse cellular models, oxygen tensions and exposure duration (Melvin and Rocha, 2012). Johnson and colleagues (2008) have reported that treatment of tumour cells in vitro, under hypoxic conditions, induced histone modifications classically associated with transcriptional activation or repression (Johnson, et al., 2008). Also, it has been found that hypoxia (and hypoxia mimetics) increases global H3K9me2 in various mammalian cell lines and this induction correlated with elevated G9a methyltransferase activity (Chen, et al., 2006). A different study showed that
changes in histone methylation, in mouse macrophage cells exposed to short-term hypoxia, were observed under 3% O₂ conditions (Melvin and Rocha, 2012).

1.7.3. Hypoxia and Jumonji-Domain Containing Histone Demethylases

Most Jumonji-domain containing histone demethylases are dioxygenases and their activity requires factors such as α-ketoglutarate, Fe(II) and oxygen, hence their enzymatic activity could be affected by oxygen tension. JMJDC domain proteins promote transcriptional activation or repression and influence various processes, such as stem cell renewal, differentiation, germ cell development and proliferation (Beyer, et al., 2008).

There is evidence that hypoxia and HIF induce the expression of JMJD1A and JMJD2B genes and that their demethylating activity is still present even under severe hypoxic conditions. The HIF-mediated induction of their expression could provide a compensatory mechanism to maintain regulation of H3K9 methylation under low oxygen atmosphere (Beyer, et al., 2008). As all dioxygenases, JMJD1A and JMJD2B require oxygen to be active and hypoxia could be a factor that might limit their function. This study has demonstrated that under 0.5% hypoxic conditions, there was a significant induction of JMJD1A, JMJD2B and
JMJD2C, when compared to the normoxic 20% O\(_2\) conditions. Even under severe hypoxia (0.2% O\(_2\)), JMJD1A and JMJD2B retained histone demethylase activity, indicating that both of them can tolerate moderately low oxygen tensions (Beyer, et al., 2008; Yang, et al., 2009). Further, upon suppression of HIF-1\(\alpha\) by siRNA it was found that JMJD1A and JMJD2B were significantly downregulated (Beyer, et al., 2008).

Animal studies have revealed that hypoxia increases the expression of JMJD1A \textit{in vivo} and \textit{in vitro}, following 6-hour hypoxic exposure of rats. Tissues such as brain, heart, kidney and lungs showed increased levels of JMJD1A. The same investigators have demonstrated the hypoxic induction of JMJD1A \textit{in vitro}, using human embryonic kidney cells (HEK-293) and human microvascular endothelial cells (HMEC-1). In addition, they have shown that overexpression of HIF-1\(\alpha\) increased significantly JMJD1A under normal atmospheric conditions (Wellmann, et al., 2008).

Further, bioinformatics analyses have predicted that there are around sixty 2-OG-dependent dioxygenases encoded in the human genome (Pollard, et al., 2008). Pollard and colleagues (Pollard, et al., 2008), have studied the responses of human 2-OG-dependent dioxygenases to hypoxia using various human cancer cell lines. Their study demonstrated that several of the selected 2-OG-
dependent dioxygenases were upregulated by hypoxia, especially that of JMJD1A and JMJD2B. The authors performed further experiments on a subset of 2-OG-dependent dioxygenases (JMJD1-C and JMJD2A-D) and showed that JMJD1A, JMJD2B and JMJD2C are upregulated during hypoxia (0.5% O₂). Upon treatment of cells with HIF-1α-directed siRNA, JMJD1A, JMJD2B and JMJD2C were significantly downregulated, indicating that their hypoxic regulation is HIF-1α-dependent (Pollard, et al., 2008). In addition, cells treated with either DFO or DMOG (both agents upregulating HIF) led to the significant induction of JMJD1A, JMJD2B and JMJD2C, confirming that all three of them are HIF target genes.

Recent studies have provided additional evidence demonstrating that HIFs influence gene expression in an indirect way under hypoxia, at the level of histone methylation (Krieg, et al., 2010). Krieg et al. (2010) has confirmed previous studies by demonstrating induction of JMJD1A and JMJD2B under hypoxia (0.5% O₂) and that both Jumonji-domain containing histone demethylases are direct HIF target genes. This study indicates that the induction of JMJD1A by HIF-1α can act as an epigenetic amplifier to augment cellular responses to hypoxia (Krieg, et al., 2010). Further, using CHIP sequencing, it was reported that 17 out of 22 Jumonji family members were upregulated due to hypoxia. Three of them were the JMJD1A, JMJD2B and JMJD2C and found to
be direct HIF-1 targets (Yang, et al., 2009). Lastly, JMJD2C has recently been shown to selectively interact with HIF-1α acting as a HIF-1 coactivator required for cancer progression (Luo, et al., 2012).

1.8 Environmental Epigenetics

Hypoxia and oxidative stress have been known to induce epigenetic modifications. As oxygen is an important cofactor in the activity of dioxygenases, any changes in its availability can affect histone methylation and subsequently gene expression (Chernova and Costa, 2012). Apart from changes in oxygen tension that has been shown to alter gene expression via epigenetic mechanisms, environmental toxicants, especially metals, have been reported to induce oxidative stress, affect the antioxidant cellular defence system and alter epigenetic marks and/or gene expression (Zhao, et al., 1997; Ercal, et al., 2001; Takiguchi, et al., 2003; Fowler, et al., 2004; Reichard, et al., 2007; Baccarelli and Bollati, 2009; Cheng, et al., 2011; Martinez-Zamudio and Ha, 2011; Chernova and Costa, 2012). There is accumulating evidence of the environmentally-induced epigenetic alterations caused by metals, such as cadmium and arsenic and developmental and/or the pharmaceutical compounds 5-azacytidine and valproic acid.
1.8.1. Cadmium

Cadmium is a transition metal that has been classified as a human carcinogen and is found in water, food, tobacco products, battery production and metal industries (Ercal, et al., 2001; Arita and Costa, 2009; Martinez-Zamudio and Ha, 2011; Cheng, et al., 2011; Jomova and Valko, 2011). It has been associated with various forms of cancer and it is a great hazard to human health, as it has a long half-life (10-30 years), cannot go through metabolic degradation and is poorly excreted (Ercal, et al., 2001; Arita and Costa, 2009; Jomova and Valko, 2011).

Exposure to cadmium leads to GSH changes and increase of oxidative stress, as shown by inhibition of cellular antioxidant enzymes SOD and GPX, increase in HO-1 and MT-1 levels, augmentation of lipid peroxidation and loss of mitochondrial membrane potential (Beyersmann and Hechtenberg, 1997; Yang, et al., 1997; Ercal, et al., 2001; Wang, et al., 2004; Pathak and Khandelwal, 2006; Wright and Baccarelli, 2007; Im, et al., 2006; Jomova and Valko, 2011; Martinez-Zamudio and Ha, 2011, Cheng, et al., 2011). Further, cadmium is shown to be toxic in human placenta and inhibits trophoblast cell proliferation in Jar choriocarcinoma cells (Powlin, et al., 1997), while in animals using cadmium chloride on rabbit blastocysts revealed significant changes in lysosomes of inner cell mass and endodermal cells (Abraham, et al., 1984).
Studies have reported changes in DNA methylation (Arita and Costa, 2009; Martinez-Zamudio and Ha, 2011) upon exposure to cadmium, either by inducing hypomethylation (Takiguchi, et al., 2003; Huang, et al., 2008) or hypermethylation (Takiguchi, et al., 2003; Benbrahim-Talla, et al., 2007; Jiang, et al., 2008). Further cadmium exposure could result in changes of chromatin structure for transcriptional activation (Martinez-Zamudio and Ha, 2011).

1.8.2. Arsenic

Arsenic is an environmental contaminant found in water, soil and airborne particles and classified as a human carcinogen (Arita and Costa, 2009; Martinez-Zamudio and Ha, 2011; Jomova and Valko, 2011) which has been linked with various diseases such as hypertension, diabetes and liver, bladder, kidney, lung and skin cancers (Ercal, et al., 2001; Martinez-Zamudio and Ha, 2011). Exposure to arsenic occurs in the form of either arsenite or arsenate (Arita and Costa, 2009). Further, arsenic has been reported to affect several cellular processes, such as redox status, cellular antioxidant defence systems and epigenetic mechanisms (Ercal, et al., 2001; Liu, et al., 2005; Martinez-Zamudio and Ha, 2011).
Several studies have documented evidence that arsenic increases ROS and affects the cellular antioxidant defences via increasing or reducing the activity of GPXs, SODs and CAT, depending on the treated cell types (Ercal, et al., 2001; Hei and Filipic, 2004; Wright and Baccarelli, 2007; Wang, et al., 2009; Lii, et al., 2011; Liu, et al., 2011). ROS are elevated even under noncytotoxic concentrations of arsenite (Barchowsky, 1999) and have shown to increase up to 90th cell generations post arsenite exposure when compared to the untreated parental group (Sciandrello, et al., 2011). Arsenite exposure results in loss of the mitochondrial membrane potential along with the increase of ROS (Hei and Filipic, 2004; Wang, et al., 2009).

Arsenic induces heme oxygenase and metallothionein, proteins involved with oxidative stress, in cells and animals (Ercal, et al., 2001; Hughes, 2009). Another marker of oxidative stress that has been shown to be affected, upon exposure to arsenic, is GSH. It has been shown that arsenic is responsible for decreased levels of GSH (Jomova and Valko, 2011). Further, 8-hydroxy-2-deoxyguanosine (8-OHdG), a known ROS-induced DNA damage biomarker, has been reported upon treatment with arsenite (Ercal, et al., 2001; Kessel, et al., 2002; Xu, et al., 2008; Kojima, et al., 2009; Liu, et al., 2011).
Numerous studies have shown that arsenite disrupts DNA methylation, either by inducing hypomethylation or hypermethylation (Arita and Costa, 2009). Exposure to arsenic has been found to induce gene-specific DNA hypermethylation in humans (Martinez-Zamudio and Ha, 2011). Short and chronic and even low concentrations of arsenite are sufficient to inhibit DNMTs, deplete the SAM pool and result in global DNA hypomethylation (Zhao, et al., 1997; Reichard, et al., 2007; Martinez-Zamudio and Ha, 2011). In addition, increasing evidence indicates that in utero and early-life human exposure to arsenic, leads to changes in DNA methylation status and is associated with congenital defects and risk of cancer mortality during early adulthood (Intarasunanont, et al., 2012). Also, animal in utero studies demonstrated the formation of tumours in offspring, upon reaching adulthood, due to arsenic exposure (Intarasunanont, et al., 2012). Further, effects of arsenic exposure on maternal and fetal health have been demonstrated, including arsenic accumulation in cord blood, indicating that arsenic can pass into the placenta, impairing fetal growth in mothers exposed to arsenic, during pregnancy, via drinking water and increased cancer incidents in early-life (Vahter, 2009). In addition to changes in DNA methylation, arsenic has been documented to cause changes in histone methylation (Zhou, et al., 2008; Arita and Costa, 2009; Martinez-Zamudio and Ha, 2011) and histone acetylation (Ramirez, et al., 2008; Jensen, et al., 2008).
1.8.3. 5-Azagacytidine

5-azagacytidine is a cytosine analog, a hypomethylating agent and it is used as a chemotherapeutic compound (Tsuji-Takayama, et al., 2004, Braiteh, et al., 2008). It induces hypomethylation in vitro and in vivo and attenuates cellular DNMT activity (Tsuji-Takayama, et al., 2004, Braiteh, et al., 2008). Additionally, epigenetic modifier drugs, such as 5-azagacytidine, are known to increase mitochondrial ROS production (Arany, et al., 2011; Tian, et al., 2013).

Treatment with 5-azagacytidine has been reported to be a more effective method for the derivation of mouse and bovine embryonic stem cells, compared to already established protocols (Lim, et al., 2011). Further, 5-azagacytidine has shown to reverse differentiation of mouse embryonic stem cells and increase the expression of pluripotency markers OCT4, NANOG and SOX2, along with alkaline phosphatase activity and SSEA-1 surface expression (Tsuji-Takayama, et al., 2004). On the contrary, studies have utilized 5-azagacytidine, as an agent, to successfully induce cardiac differentiation of P19 embryonic carcinoma cells (Choi, et al. 2004; Yang, et al., 2009) and human embryonic stem cells (Yoon, et al., 2006).
1.8.4. Valproic Acid

Valproic acid is an anticonvulsant and mood-stabilizing compound used for the treatment of epilepsy, bipolar and other neurological disorders (Na, et al., 2003; Braiteh, et al., 2008; Chen, et al., 2012). Studies have shown that exposure to valproic acid during pregnancy can cause heart, limb, skeletal and neural tube defects (Defoort, et al., 2006; Tung and Winn, 2011). Further, valproic acid is well documented for its ability to elevate intracellular ROS (Na, et al., 2003; Tong, et al., 2005; Defoort, et al., 2006; Zhang, et al., 2010; Tung and Winn, 2011; Pourahmad, et al., 2012). It has also been shown to deplete GSH and reduce mitochondrial membrane potential (Tong, et al., 2005; Pourahmad, et al., 2012).

Valproic acid is also a histone deacetylase inhibitor and it has been demonstrated to induce histone H3K9 acetylation in mouse ES cells, advocating that H3K9 acetylation level is correlated with pluripotency (Hezroni, et al., 2011). Further, valproic acid has been utilized as a small molecule for the reprogramming of primary human fibroblasts to iPSCs, without the addition of KLF4 and c-Myc (Huangfu, et al., 2008). Recently, amniotic fluid stem cells have been treated with valproic acid in human ES cell culture medium on Matrigel, without the addition of ectopic factors and reprogrammed to pluripotency. The generated cells were reported to share 82% transcriptome identity with human
ES cells, were able to form EB and teratomas, maintained genetic stability and the ability to differentiate into all three germinal layer lineages (Moschidou, et al., 2012). Lastly, valproic acid affects epigenetic marks such as 5-hmC and TET expression in a mitochondrial DNA context (Chen, et al., 2012). This study demonstrated that TET2 and TET3 mRNA levels were significantly increased following 24 hrs and 3-day exposure to valproic acid, while TET1 was considerably elevated after 24 hrs, but significantly reduced after 3 days. The TET1 expression pattern at day 3 of treatment, corresponds with the expression of 5-hmC in mitochondrial DNA, where it was significantly attenuated compared to day 1, which remained unchanged. At the same time, at days 1 and 3 of exposure to valproic acid, 5-hmC in nuclear DNA was not affected (Chen, et al., 2012).
1.9 Objectives of the thesis

The aim of this thesis was to establish a novel human ES cell-based toxicology test system with which to evaluate the subcytotoxic effects of compounds on epigenetic determinants of undifferentiated human embryonic stem cell phenotype.

The specific objectives of this thesis were:

1. To confirm the functional significance of selected human ES cell epigenetic biomarkers, genes that possess a conserved and unique pattern of epigenetic modification and expression in undifferentiated human embryonic stem cells.

2. To evaluate the sensitivity of selected human ES cell epigenetic biomarker expression to subcytotoxic concentrations of compounds (5-azacytidine, cadmium chloride, sodium arsenite and valproic acid) known to disrupt epigenetic determinants and animal development.

3. To assess the effect of subcytotoxic exposure to test compounds under hypoxic culture.

4. To investigate the potential mechanism by which subcytotoxic exposure to test compounds induced differentiation and how hypoxia exerts a protective effect.
2.1 Mammalian Cell Culture

2.1.1 Culture of human embryonic stem cells

RH1 (Fletcher, et al., 2006) and H9 (Thomson, et al., 1998) human embryonic stem cell lines (passage 45 – 70) were cultured on Matrigel™ (BD Biosciences, Cat. No. 354230)-coated 6-well plates and maintained in mTeSR™1 defined medium (StemCell Technologies, Cat. No. 05850) or in a human dermal fibroblast conditioned medium (HDF-CM) supplemented with 4 ng/ml of human bFGF (Peprotech, Cat. No. 100-18B) under an antibiotic-free and feeder-free humidified environment at 37°C, 5% CO₂ and 20% O₂ unless otherwise stated. Fresh medium was supplied daily.

2.1.2 Microscopy of human embryonic stem cells

The morphology of human ES cells was observed and captured with an Axiovert 40 CFL (ZEISS) phase contrast microscope using the x4 objective (unless otherwise stated) and a Canon PowerShot A650 IS camera.

2.1.3 Passaging of human embryonic stem cells

Human ES cells were passaged once they reached a confluence of 80%. Cells were washed once with PBS and incubated with 1 ml of Collagenase IV (Life
Technologies, Cat. No. 17104-019) diluted in KO-DMEM (Life Technologies, Cat. No. 10829-018) at a concentration of 200 U/ml for 3 min in a 37°C incubator. The collagenase was aspirated when the colonies started to lift around the edges and the cells were washed gently once with PBS. The colonies were scraped with a cell scraper, disaggregated by gentle pipetting and then plated onto freshly prepared Matrigel™ coated plates. The cells were usually subcultured 1:2 (RH1 hESCs) and 1:4 (H9 hESCs).

2.1.4 Freezing of human embryonic stem cells

Cells were detached from the tissue culture dishes, as previously described, centrifuged at 1,000 rpm for 5 min and resuspended in 1 mL of cold Cryostor CS10 freezing medium (Sigma-Aldrich, C2874). The cell suspensions were aliquoted into 1.5 ml CryoTube vials, placed into a Nalgene® - “Mr. Frosty” freezing container (Thermo Scientific, Cat. No. 5100-0001) and left at 4°C for 10 min prior to transferring at a -80°C freezer for overnight storage. The cryovials were transferred to liquid nitrogen the next day for long-term storage.

2.1.5 Thawing of human embryonic stem cells

The cryovials were removed from the liquid N₂ and kept on dry ice until thawed by immersing their bottom half in a 37°C water bath for 2 min. The cell
suspension was aspirated with a 5 ml pipette containing warm culture medium and transferred slowly to a 15 ml conical tube and centrifuged at 1,000 rpm for 5 min. The medium was removed and the cells were resuspended in 2 ml fresh culture medium and plated onto freshly prepared Matrigel™ coated dishes.

2.1.6 Hypoxic induction of human embryonic stem cells

For hypoxic induction, human ES cells were cultured in a multigas incubator where nitrogen gas was supplied to maintain the controlled reduced percentage of 0.5% O₂. Cell culture media and PBS were all pre-equilibrated by placing into the incubator with the hypoxic atmosphere overnight prior to every medium change in order to minimize the exposure of cells to atmospheric oxygen levels (20% O₂). Culture medium changes were performed every two days and the process was performed within 2 min in order to reduce exposure to atmospheric oxygen as much as possible, unless otherwise stated.

2.1.7 Culture and passaging of Human Dermal-Fibroblasts

Human Dermal-Fibroblasts (HDFs) were maintained in Medium 106 (Life Technologies, Cat. No. M-106-500) in a humidified atmosphere of 5% CO₂ in air. HDFs were passaged using 0.01% Trypsin/EDTA (Life Technologies, Cat. No. 15090-046) and mechanical dissociation.
2.2 Study Test Compounds

The compounds used in this thesis were 5-azacytidine (Sigma-Aldrich, Cat. No. A2385), cadmium chloride (Acros Organics, Cat. No. AC296330050), sodium arsenite (Fisher Scientific, Cat. No. S225l-100), valproic acid (Sigma-Aldrich, Cat. No. P4543), N-acetyl-cysteine (Sigma Aldrich, Cat. No. A9165), buthionine sulfoximine (Sigma-Aldrich, Cat. No. B2515), cobalt chloride (Sigma-Aldrich, Cat. No. 232696) and desferrioxamine (Sigma-Aldrich, Cat. No. D9533). All compounds were stored according to suppliers’ instructions. The compounds were dissolved in \( \text{H}_2\text{O} \) to make the stock solutions prior to each experimental run. All solutions were stored at 4\(^{\circ}\)C during the experiment.

2.2.1 Cytotoxicity range finder study

The cytotoxic range finder experiment was performed in order to determine a rank of concentrations in which a test compound will be toxic to undifferentiated human ES cell cultures. Initially the IC\(_{10}\) concentration of a compound (concentration where a compound has a 10\% reduction on cell viability) compared to the control one was defined.

The cytotoxic range finder experiment was performed using undifferentiated human ES cells cultured with HDF-CM+ culture medium. At day 0, human ES
cells were seeded at a density of $1 \times 10^4$/well in 96-well tissue culture plates in 0.1 ml of HDF-CM+ medium for 24 hours prior to any compound exposure. Untreated control cultures were added the same volume of HDF-CM+ media in the absence of any test compounds. All cultures were incubated at 37°C, 5% CO$_2$ for 24 hours. The following day, the culture media were removed and 0.1 ml of each test compound dissolved in HDF-CM+ medium was added to the cultures. All cultures were incubated as previously described. Media were removed and replaced with 0.1 ml of test compound media every 48 hours until the completion of a culture period of seven days. At this end-point, the CellTiter-Blue® Cell Viability Assay (Promega, Cat. No. G8080) was used to determine the cytotoxic effects of the test compounds on human ES cells. All test compounds were evaluated in triplicates for each of the compound concentrations investigated and for the untreated cultures.

2.2.2 Cell viability assay

The CellTiter-Blue® Cell Viability Assay was performed according to manufacturer’s instructions. Briefly, 20 µl of CellTiter-Blue Reagent was added directly to 100 µl of cell culture medium per well. Following two hours incubation at 37°C, 5% CO$_2$ the fluorescent signal (560$_{Ex}$/590$_{Em}$) of each well was measured using a multi-detection microplate reader POLARstar OPTIMA (BMG LABTECH).
2.2.3 Subcytotoxic treatment of compounds on undifferentiated human ES cells

Undifferentiated human ES cells (RH1 and H9) were seeded at 5x10^4 cells/well on Matrigel™-coated 12-well plates in 1 ml of HDF-CM+ medium for 24 hours prior to treatment. The next day, media were removed and 1 ml of the test compounds dissolved in HDF-CM+ medium (at indicated concentrations) was added to the cells. All cultures were incubated at 37°C, 5% CO₂. The media were removed and replaced with fresh media every two days for a total culture period of seven days.

2.3 Determination of reactive oxygen species (ROS) production

The generation of ROS in human ES cell cultures was evaluated using the 2', 7'-dichlorofluorescein diacetate (DCF-DA) method. Human ES cells were seeded at 2x10^5 cells/well in 6-well Matrigel™ pre-coated plates and exposed to each compound for seven days under normoxic (20% O₂) and hypoxic (0.5% O₂) culture environments. At the end-point, cells were harvested by scraping in cold PBS, pelleted by centrifugation (1,000 rpm, 5 min) and resuspended in DCF-DA (Sigma, Cat. No. D6883) dissolved in DMSO (Sigma-Aldrich, Cat. No. D2650) at a final concentration of 2 µM. Cells were incubated for 40 min in a 37°C water
bath in darkness. Following incubation, the cells were washed twice in cold PBS and the fluorescence was measured with a FACSCalibur (BD Biosciences) Flow Cytometer with an excitation and an emission wavelength of 488 nm and 525 nm, respectively. Fluorescence intensity of at least $1 \times 10^4$ cells was measured for each sample and experiments performed in quadruplicate ($n=4$ per experimental condition). Data was analysed using the FCS Express 4 Image Cytometry software (De Novo Software).

### 2.4 Determination of mitochondrial membrane potential (MMP)

The mitochondrial membrane potential in human ES cells was measured using Rhodamine 123 (Sigma-Aldrich, Cat. No. R8004), a cell permeable fluorescence dye which preferentially enters into mitochondria. Depolarisation of the mitochondrial membrane results in the loss of Rhodamine 123 from mitochondria and reduced intracellular fluorescence (Ruiz-Ramos, *et al.*, 2009). Briefly, $1 \times 10^4$ cells/well were cultured in 96-well plates and treated with different compounds under normoxic (20% O$_2$) and hypoxic (0.5% O$_2$) atmospheres for seven days. At day 7, cells were washed once with PBS and incubated with Rhodamine 123 (at a final concentration of 2 μM) for 40 min at 37°C in darkness. Following incubation, the fluorescence was measured using a microplate reader POLARstar OPTIMA (BMG LABTECH). The fluorescence
intensity was measured for quadruplicate biological samples and experiments performed in triplicate for each human ES cell line.

2.5 Immunocytochemistry

2.5.1 OCT4 and NANOG

Human ES cells were cultured on Matrigel™ in 12-well plates (Costar, Corning Inc.). At the end-point of all treatments, cells were washed three times with PBS and fixed in 4% PFA/PBS for 20 minutes at room temperature. Cells were washed three times with PBS and permeabilised with 0.2% IGEPAL CA-630 (Sigma-Aldrich, Cat. No. I3021) in PBS for 20 minutes. Non-specific antibody binding was blocked with 10% NRS (Sigma-Aldrich, Cat. No. R9133) for one hour. Primary antibody staining was performed with anti-OCT4 (C-10 peptide, Santa Cruz Biotech, Cat. No. SC-5279) and NANOG (R and D Systems, Cat. No. AF1997) overnight at 4°C, followed by three washes with PBS the next day. Secondary antibodies used were Alexa Fluor® 488 rabbit anti-mouse IgG (Life Technologies, Cat. No. A-11059) and Alexa Fluor® 555 donkey anti-goat IgG (Life Technologies, A21432) for one hour in the dark at room temperature. Nuclei were labelled with DAPI (1:1000, Life Technologies, Cat. No. D1306). Images were captured using a Zeiss Observer fluorescence microscope and
prepared using the Zeiss AxioVision 4.8.2 software. Details of the antibodies and dilutions are listed in Appendix I.

2.5.2 5-Hydroxymethylcytosine (5-hmC) and 5-methylcytosine (5-mC)

Immunohistochemistry for 5-hmC and 5-mC was performed with cells fixed for 20 min in 4% PFA/PBS (Sigma-Aldrich, Cat. No. 158127) at room temperature. The cells were permeabilised with 0.5% Triton X-100 (Promega, Cat. No. H15141) in PBS for 30 min. DNA was denatured with 4M HCl followed by three washes with 0.1% Tween<sup>®</sup>20/PBS (Sigma-Aldrich, Cat. No. P5927). Cells were treated with 10% goat serum at room temperature for one hour. The applied primary antibodies for 5-hmC (Active Motif, Cat. No. 39769) and 5-mC (Clone 33D3, Active Motif, Cat. No. 39649) were incubated at room temperature for two hours. Following three washes with 0.1% Tween<sup>®</sup>20/PBS, appropriate secondary antibodies were applied in the dark at room temperature for one hour. Nuclei were labelled with DAPI (1:1000, Life Technologies, Cat. No. D1306) stain and images were captured using the Zeiss Observer fluorescence microscope. Images were prepared using the Zeiss AxioVision 4.8.2 software. All antibodies used are listed in Appendix I.
2.6 Molecular Techniques

2.6.1 RNA Isolation and Quantification

Total RNA isolation was performed using TRIZol® Reagent (Life Technologies, Cat. No. 15596-018), according to the manufacturer’s protocol under RNAse-free conditions. Briefly, at the end-point of each experiment, cultured cells were lysed directly in the culture dish by adding 1 ml of TRIZol Reagent for 5 min at room temperature and pipetting the cell lysate up and down. Samples were collected in 1.5 ml Eppendorf tubes and 0.2 ml of chloroform per 1 ml of TRIZol was added. The samples were vigorously shaken for 15-20 sec and incubated at room temperature for 2-3 min. Following centrifugation at 12,000 rpm for 15 min at 4°C, the upper aqueous phase was carefully removed and transferred into a new tube and 0.5 ml of isopropyl alcohol was added. Samples were incubated at room temperature for 10 min and then centrifuged at 12,000 rpm for 10 min at 4°C. The supernatant was removed and the RNA pellet was mixed with 1 ml of 75% ethanol. After mixing by vortexing and centrifugation at 7,500 rpm for 5 min at 4°C, the RNA pellet was air-dried and dissolved in RNase-free water. For long-term storage the samples were placed at -80°C.

The RNA quality and concentration were assessed using a NanoDrop™ ND-1000 spectrophotometer. 1 µl of purified RNA was used for the quantification.
RNA purity was evaluated by analysing the $A_{260}/A_{280}$ ratio, for which a value of 1.9-2.0 was considered optimal. RNA was treated with RQ1 RNase-Free DNase (Promega, Cat. No. M6101) in order to remove any possible DNA contamination prior to RT-PCR according to manufacturer’s protocol. In brief, reactions were set up using 1 unit of RQ1 RNase-Free DNase and 1 µl of RNase-Free DNase 10X Reaction Buffer per microgram of RNA and incubated at 37°C for 30 min. To terminate the reactions, 1 µl of DNase Stop Solution was used and incubated at 65°C for 10 min for the DNase to be inactivated.

2.6.2. Complementary DNA (cDNA) Synthesis

cDNA was synthesized using the SuperScript III First-Strand Synthesis System for RT-PCR Kit (Life Technologies, Cat. No. 18080-051) according to manufacturer’s instructions using oligo(dT) primers. 1 µg of RNA per sample was used as a template. cDNA was diluted 1:20 in sterile water and used for qRT-PCR.

2.6.3 Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR)

Quantitative RT-PCR was performed in triplicates with a DyNAmo Flash SYBR Green qPCR Kit (Thermo Scientific, Cat. No. F-415L) using the StepOne
Plus apparatus (Applied Biosystems). SYBR Green molecules bind to double stranded DNA (dsDNA) and produces fluorescence when bound. Thus, the fluorescence intensity reflects the amount of dsDNA in each reaction. A master mix for each primer pair was prepared for n+1 reactions. The master mix was aliquoted into 96-well reaction plates (STAR LAB, Cat. No. E1403-7700) to which cDNA was added directly and gentle mixed. The cycling protocol and primer sequences are presented in Appendices II and III.

The Applied Biosystems StepOne™ Software v2.1 automatically calculated the cycle threshold (Ct) values from the amplification curves generated. Technical triplicate reactions of quadruplicate biological samples were performed (unless otherwise stated) and the Ct values were averaged prior to calculating the fold difference of gene expression. If data from the parallel reactions varied more than 0.5 cycles, the reactions were repeated or excluded from the data analysis.

The qPCR data in this thesis were analysed using the Comparative Ct ($2^{-\Delta\Delta Ct}$) method. The comparative Ct method calculates changes in gene expression as a relative fold difference between an experimental and calibrator sample.
Initially, the $\Delta Ct$ value for each sample was determined by calculating the difference between the $Ct$ value of the target gene of interest (e.g. OCT4, NANOG, SOX2) and the $Ct$ value of the reference gene (e.g. GAPDH or $\beta$-Actin). The $\Delta Ct$ values were calculated for each treated sample and the calibrator (i.e. untreated control) by the following equations:

**Equation 1:** $\Delta Ct$ (treated sample) = $Ct$ (target gene) – $Ct$ (reference gene) and

**Equation 2:** $\Delta Ct$ (calibrator) = $Ct$ (target gene) – $Ct$ (reference gene).

Subsequently, the $\Delta \Delta Ct$ value for each sample was calculated by subtracting the $\Delta Ct$ values of the calibrator from the $\Delta Ct$ value of the treated sample by using the formula:

**Formula 1:** $\Delta \Delta Ct = \Delta Ct$ (treated sample) - $\Delta Ct$ (calibrator).

Further, equations 1 and 2 were applied to formula 1. Therefore,

$\Delta \Delta Ct = \left[ Ct \ (target \ gene, \ treated \ sample) - Ct \ (reference \ gene, \ treated \ sample) \right]$

$\quad - \left[ Ct \ (target \ gene, \ calibrator) - Ct \ (reference \ gene, \ calibrator) \right]$ where

$Ct$ (target gene, treated sample) = $Ct$ value of gene of interest in the treated sample,
Ct (reference gene, treated sample) = Ct value of reference gene in the treated sample,

Ct (target gene, calibrator) = Ct value of gene of interest in the calibrator and

Ct (reference gene, calibrator) = Ct value of reference gene in the calibrator.

The ratio of the target gene in the treated sample relative to the calibrator was calculated by using the formula $2^{-\Delta \Delta Ct}$ (Information adapted from the instruction manual DyNAmo™ Flash SYBR® Green qPCR Kit; FINNZYMES, Cat Nos. F-415 & F-416).

It has to be noted that the $\Delta \Delta Ct$ of the calibrator equals to zero and $2^0$ equals one, so that the fold change in gene expression relative to the untreated control equals one, by definition.

An example of calculations is presented below:

<table>
<thead>
<tr>
<th></th>
<th>Ct value of calibrator (untreated control)</th>
<th>Ct value of treated sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reference Gene</td>
<td>22.2</td>
<td>21.0</td>
</tr>
<tr>
<td>Target Gene</td>
<td>27.5</td>
<td>25.0</td>
</tr>
</tbody>
</table>
\[ \Delta \Delta Ct = [Ct \text{ (target, calibrator)} - Ct \text{ (reference, calibrator)}] - [Ct \text{ (target, treated sample)} - Ct \text{ (reference, treated sample)}] = (27.5 - 22.2) - (25.0 - 21.0) = 5.3 - 4 = 1.3. \] 

Thus, \[ 2^{\Delta \Delta Ct} = 2^{-1.3} = 2.462. \] The transcript level of the gene of interest has changed by 2.462 times in the treated sample compared to the calibrator (i.e. untreated control).

The resulted fold differences of gene expression presented throughout this thesis were plotted on a logarithmic scale \((\log_{10})\). Work presented was based on that fact that due to the exponential nature of PCR, in every cycle of PCR the amount of DNA is approximately duplicated and the Threshold Cycle (Ct) values are in the logarithmic scale. Also, a logarithmic scale was used due to the broad range in fold change across various experimental treatments. This way, the data sets are presented in a graphical way allowing easier visualisation, interpretation and understanding of the obtained small changes in gene expression. The statistical analysis performed on the qPCR data sets is described in Section 2.9 of the thesis.

2.6.4 DNA Purification

Total DNA purification was performed using the DNeasy (Qiagen, Cat. No. 69504), according to manufacturer’s protocol “Purification of Total DNA from
Animal Blood or Cells” (Qiagen, DNeasy Blood and Tissue Handbook, July 2006, p 25-27). Following the purification protocol, the DNA quality and concentration were assessed using a NanoDrop™ ND-1000 spectrophotometer. 1 µl of purified DNA sample was used for the quantification. DNA purity was evaluated by analysing the $A_{260}/A_{280}$ ratio, for a value of 1.8-1.9 was deemed optimal.

2.7 Enzyme-linked immunosorbent assay (ELISA) of global DNA 5-hmC content

The 5-hmC content of extracted genomic DNA from untreated and treated human ES cells was measured with the hydroxymethylated DNA quantification kit (Quest 5-hmC™ DNA ELISA Kit, ZYMO Research, Cat. No. D5426) according to manufacturer’s protocol. In brief, 100 ng of denatured genomic DNA was bound to a 96-well ELISA plate pre-coated with anti-5-hmC polyclonal antibody. The single stranded 5-hmC-containing DNA bound to the anti-5-hmC polyclonal antibody is recognized by an anti-DNA-HRP antibody and the addition of the HRP Developer (enzyme substrate) produces a greenish-blue colour in the wells containing hydroxymethylated DNA. Positive controls were performed concurrently. The quantification of the 5-hmC containing DNA was performed by
reading the absorbance at 450 nm in a POLARstar OPTIMA microplate reader (BMG LABTECH).

The percentage of 5-hmC in experimental samples was calculated based on a standard curve generated using the Control DNA Set which was provided in the Quest 5-hmC™ DNA ELISA Kit. The Control DNA Set consisted of five double-stranded genomic DNA controls containing a specified percentage of 5-hmC (Table 2.1).

<table>
<thead>
<tr>
<th>Control DNA Sample Set</th>
<th>Percentage of 5-hmC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control A (negative)</td>
<td>0%</td>
</tr>
<tr>
<td>Control B</td>
<td>0.03%</td>
</tr>
<tr>
<td>Control C</td>
<td>0.12%</td>
</tr>
<tr>
<td>Control D</td>
<td>0.23%</td>
</tr>
<tr>
<td>Control E (positive)</td>
<td>0.55%</td>
</tr>
</tbody>
</table>

Table 2.1. Control DNA samples A-E and corresponding percentage of 5-hydroxymethylcytosine. (Adapted from the instruction manual Quest 5-hmC™ DNA ELISA Kit v1.5.0; Zymo Research Corp, Cat Nos. D5425 & D5426).
The standard curve was generated based on measuring the absorbance at 405 nm of all five control DNA samples in duplicates as instructed by the manufacturer. Following measurements at 405 nm, the control data were plotted as absorbance (Y-axis) versus the percentage of 5-hmC (X-axis) (Instruction manual Quest 5-hmC™ DNA ELISA Kit v1.5.0; Zymo Research Corp, Cat Nos. D5425 & D5426).

In this thesis, all DNA controls (A-E) provided absorbance values including the negative DNA control A which provided an absorbance value in the absence of hydroxymethylated DNA content verifying the kit’s standard curve. Furthermore, visual observation of all the ELISA assay plates provided confirmation of the obtained data as the wells loaded with the negative DNA control A produced no colour and those containing different percentages of hydroxymethylated DNA (B-E, 0.03-0.55%) produced a greenish-blue colour in the wells.

The lineage regression equation \( y = a + bx \) where \( x \) and \( y \) are the variables, \( a \): the intercept point of the regression line and the y axis, and \( b \): the slope of the regression line was used. Hence, the percentage of 5-hmC in each DNA sample was determined as \( \% \text{ 5-hmC} = \frac{\text{absorbance} - \text{y-intercept}}{\text{Slope}} \). The controls were always included together with the samples for every ELISA assay.
Finally, the Quest 5-hmC™ DNA ELISA Kit has been tested by the manufacturer for its sensitivity to detect quantities of hydroxymethylated DNA in samples of human ES cells, the cell type of investigation in this thesis.

2.8 Small Interfering RNA (siRNA) knockdown

Small interfering RNA knockdown experiments for the genes GLIS2, HMGA1, PFDN5, JMJD2C, TET1, TET2 and TET3 were performed using two human ES cell lines (RH1 and H9). Anti-IDS-null (a derivative of an siRNA directed at IDS carrying 4 mutations which results in an siRNA directed against no transcript in the human genome; negative control), anti-YAP1 (YAP1 knockdown has been reported not to result in the reduction of OCT4 expression in three human ES cell lines (Chia, et al., 2010)) and anti-OCT4 (positive control) were used as controls. Human ES cells were seeded (1x10⁵/well) on Matrigel™ in 12-well plates with 0.5 ml mTeSR™1 culture medium. The cells were transfected the following day using siRNA at a concentration of 20 nM along with 1% Lipofectamine®RNAiMAX (Life Technologies, Cat. No. 13778030) as the transfection reagent. After 24 hrs of exposure, the medium was removed and a second transfection with the siRNA-Lipofectamine complex was performed. In the case of triple knockdowns, 20 nM of siRNA along with 1% Lipofectamine were mixed into 1.5 ml of medium and added into individual wells. Following 24 hrs of the second transfection, fresh mTeSR™1 culture medium was added.
Cells were harvested at 24, 48 or 72 hours post-second siRNA treatment for qRT-PCR, immunocytochemistry and ELISA analysis. The knockdown efficiency was assessed using qRT-PCR. The knockdown sequences used are listed in Appendix IV. Where one knockdown control was performed many times, these data were combined (Appendix V).

2.9 Statistical analysis

Statistical analyses were performed using GraphPad Prism software version 5 for Windows (GraphPad Software, San Diego, California USA). For each analysis, triplicate or quadruplicate biological samples for each experimental condition were assessed. Values were expressed as the mean ± standard deviation (SD). Differences between the various conditions were assessed by t-test, one-way ANOVA with Dunnett’s post test or two-way ANOVA unless otherwise specified. The values were considered statistically significant at \( p < 0.05 \).

In particular, for the qPCR data sets, different experimental treatment groups (e.g. treated sample vs. untreated control) were tested for their statistical significant difference and \( p \) values were calculated using the Student’s t-test. At first, the Ct values of the technical replicates for both the target gene of interest
(e.g. OCT4, NANOG, SOX2) and the reference (GAPDH or β-actin) gene for each biological sample (for both the treated and calibrator samples) were averaged. Next, the ΔCt value for each biological sample was calculated (as described in Section 2.6.3) per experimental condition (i.e. treated sample and calibrator sample). Based on these ΔCt values, the Student’s t-test was performed and the p values were determined. The data were plotted at a logarithmic scale (Log$_{10}$) due to the aforementioned reasons (Section 2.6.3) and no error bars were calculated and presented on the graphs as it is incorrect to include those on fold change values as these are not normally distributed and will likely be misleading (http://blog.mcbryan.co.uk/2013/06/qpcr-normalisation.html).

Consequently, along with the statistical significance of a p value that was performed for the qPCR data sets, the effect size was calculated as well in order to further determine and reveal the magnitude of differences found between treated samples (e.g. OCT4 siRNA) and the calibrator (e.g. IDS-null siRNA) (Sullivan and Feinn, 2012). For this purpose, the effect size was calculated for all experimental conditions as described in this section. Since the effect size quantifies the size of the difference among two groups (e.g. treated sample vs. untreated control), it is therefore thought to be a true measurement of the
significance of the difference even when there is an extremely small sample size 
\( N \leq 5 \) (Coe, 2002; Winter, 2013).

The effect size was measured by the standardised difference of mean (group 1) 
– mean (group 2) / standard deviation. In this study, the size of the experimental 
effects was calculated using Cohen’s standardised mean difference \( d \) (Cohen, 
1988; Coe, 2002; Ellis, 2009). Cohen’s \( d \) is defined as the difference between 
two means divided by the pooled standard deviation for the data:

\[
d = \frac{\text{mean}_1 - \text{mean}_2}{\text{SD}_{\text{pooled}}}
\]

where mean 1 is the mean of the treatment group, mean 2 is the control group 
and \( \text{SD}_{\text{pooled}} \) is the pooled (or average) of the two groups’ standard deviations. 
The formula for the pooled standard deviation is:

\[
\text{SD}_{\text{pooled}} = \sqrt{\frac{\text{SD}_1^2 - \text{SD}_2^2}{2}}
\]

Based on Cohen’s criteria, an effect size can be categorized as small, medium 
and large (Ellis, 2009; Sullivan and Feinn, 2012). The thresholds for interpreting 
effect size are presented in Table 2.2.
<table>
<thead>
<tr>
<th>Value of $d$</th>
<th>Effect Size Threshold</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.20</td>
<td>Small</td>
</tr>
<tr>
<td>0.50</td>
<td>Medium</td>
</tr>
<tr>
<td>0.80</td>
<td>Large</td>
</tr>
<tr>
<td>1.3</td>
<td>Very Large</td>
</tr>
</tbody>
</table>

**Table 2.2. The thresholds for interpreting effect size.** (Adapted from Ellis, 2009).

Further, supplementing Cohen’s original small, medium and large effect size classification, Rosenthal has added a classification of very large (Ellis, 2009; Sullivan and Feinn, 2012). For example a $d = 1.3$ means that the two groups differ by a 1.3 standard deviations and that the difference between them is very large and consistent to be really significant.
Chapter 3

The Functional Significance of Epigenetically-Defined Biomarkers of the Undifferentiated Human Embryonic Stem Cell Phenotype
3.1 Introduction

Embryonic stem cells have the unlimited power to self-renew and maintain their undifferentiated phenotype over a prolonged period of time under self-renewing culture conditions while harbouring the capacity to generate any cell type of the human body upon differentiation (Thomson, et al., 1998; Odorico, et al., 2001; Wobus and Boheler, 2005). Human ES cells are a highly valuable tool for investigating early human developmental events and understanding the mechanisms of pluripotency and differentiation.

Pluripotency is known to be governed by a complex interaction between genetic and epigenetic cues (Loh, et al., 2008). Evidence illustrated that apart from transcription factors and signalling pathways, a defined epigenetic status is fundamental for the maintenance of human ES cell identity (Bibikova, et al., 2006; Fazzio, et al., 2008; Ding, et al., 2009; Christophersen, et al., 2010). A detailed molecular understanding of how pluripotency is established and maintained in human ES cells and how genetic and epigenetic factors influence the balance between the pluripotent state and differentiation is vital in order to exploit the full potential of human ES cells for aspects of basic and clinical research.
Stem cell-specific transcription factors OCT4, NANOG and SOX2 are key regulator factors of the transcriptional network of pluripotency and have been shown to be vital for early development and maintenance of the undifferentiated state of human ES cells (Adachi, et al., 2010). Extensive work exemplified the loss of pluripotency via depletion of these factors, highlighting their importance in the maintenance of human ES cell pluripotency (Hay, et al., 2004; Matin, et al., 2004; Zaehres, et al., 2005; Hyslop, et al., 2005, Babaie, et al., 2007; Lim, et al., 2007; Fong, et al., 2008; Hohenstein, et al., 2008; Adachi, et al., 2010; Ma, et al., 2010). Apart from the transcription factors OCT4, NANOG and SOX2 several RNA interference (RNAi) screens have identified additional genes essential for the regulation of self-renewal and pluripotency in ES cells (Lim, et al., 2007; Ding, et al., 2009; Chia, et al., 2010; Shah, et al., 2012).

Moreover, a plethora of studies have demonstrated the importance of epigenetic mechanisms as fundamental factors that further control the maintenance of pluripotency and lineage specification of human ES cells (Tanasijevic, et al., 2009; Meshorer and Misteli, 2006; Brunner, et al., 2009). The epigenetic state of undifferentiated ES cells has been widely studied and revealed that ES cells possess a unique DNA methylation signature compared to differentiated cell populations (Altun, et al., 2010; Bibikova, et al., 2006; Fouse, et al., 2008; Meissner, et al., 2008). While established ES cell lines in use nowadays display comparable colony morphology and expression profiles regarding cell-surface
antigens and developmentally-regulated pluripotency genes there is a significant amount of evidence that describes variability in epigenetics states of different human ES cell lines (Tanasijevic, et al., 2009). Indeed, extensive studies have shown that human ES cells undergo genetic and epigenetic perturbations *in vitro* (Maitra, et al., 2005; Shen, et al., 2006; Rugg-Gunn, et al., 2007; Tanasijevic, et al., 2009). Understanding the functional significance of genes which preserve a conserved pattern of epigenetic modification against environmentally induced variation may lead to a better understanding of the mechanisms which cover stem cell renewal and lineage potency, leading to improved safety and efficacy in the use of these cells for basic and applied purposes in discovery, screening and therapy.

In our laboratory, colleagues have assessed human ES cell lines (RH1, RH3 and RCM1) for variable and conserved patterns of Cytosine-Guanine Island (CGI) methylation by means of hybridisation of methyl-binding domain column-purified genomic DNA to a human CGI array representing 17,387 biologically-defined CGIs (Figure 3.1; Pells, et al., unpublished). The derived CGI methylation patterns were then compared to patterns from differentiated tissues such as female and male blood, brain, muscle and spleen (Illingworth, et al., 2008) and to human ES cell mRNA expression data sets obtained for the human ES cell lines RH1, RH3 and RCM1.
This comparison identified limited sets of CGIs with conserved human ES cell-specific methylation patterns. Gene ontology analysis of these gene data sets showed that expressed genes with associated CGIs that were unmethylated or methylated specifically in human ES cells were significantly enriched for transcriptional activators and repressors, respectively (see Appendix VI).

(A)

<table>
<thead>
<tr>
<th>Human ES Cell Line</th>
<th>Sex</th>
<th>Embryo Origin</th>
<th>Passage</th>
<th>Substrate</th>
<th>Medium</th>
</tr>
</thead>
<tbody>
<tr>
<td>RH1</td>
<td>Female</td>
<td>Cleavage stage</td>
<td>76</td>
<td>Matrigel</td>
<td>HDF-CM + bFGF/SR/NS</td>
</tr>
<tr>
<td>RH3</td>
<td>Female</td>
<td>Cleavage stage</td>
<td>57</td>
<td>Laminin</td>
<td>DM + bFGF/NS</td>
</tr>
<tr>
<td>RCM1</td>
<td>Female</td>
<td>Failed egg</td>
<td>50</td>
<td>Matrigel</td>
<td>HDF-CM + bFGF/SR/NS</td>
</tr>
</tbody>
</table>
Figure 3.1. Experimental strategy for the identification of epigenetic biomarkers of human ES cells. (A) Summary of the human ES cell lines employed to assess Cytosine-Guanine Island methylation. These cell lines were isolated from blastocyst stage embryos as previously described in Fletcher et al., (2006) and De Sousa et al.,
The lines were cultured on HDF feeder cells for up to 10 passages in either HDF-CM or X-Vivo10 DM, supplemented with bFGF, serum replacement (SR) and/or nutrient supplements (Non-essential amino acids, Glutamine, β-mercaptoethanol; NS) followed by expansion on maintenance of feeders on either Matrigel™ or human Laminin. At the passage number indicated, genomic DNA or mRNA was analysed by hybridisation of replicate sample sets to human CGI and Affymetrix U133plus2 gene chip arrays, respectively. (B) Evaluation of methylation status of CGIs in human ES cells using a human CGI array set which represents 17,387 CGIs. Genomic human ES cell DNA was hybridised to a methylated DNA-binding column. MBD-purified and total input were subsequently hybridised to the human CGI array (Pells, et al., unpublished).

Abbreviations/Acronyms: HDF, Human Dermal Fibroblast; HDF-CM: Human Dermal Fibroblast-Conditioned Medium; DM, defined medium; bFGF, basic Fibroblast Growth Factor; NS, nutrient supplements; CGI: Cytosine-Guanine Island, MBD, methyl-CpG binding domain.

CGIs whose methylation state was determined to be unique to human ES cells using the above method enabled us to define putative biomarkers of the human ES cell pluripotent state at an epigenetic level. These included transcriptional activators GLIS2 and HMGA1, and transcriptional repressor PFDN5. We decided to further investigate a potential functional role of these three markers for various reasons, GLIS2 encodes a zinc finger transcription factor which interacts with the p120 catenin, a member of the pluripotency-associated WNT pathway (Pells, et al., unpublished). GLIS2 has been reported to be important in embryonic development (endoderm and ectoderm) and is expressed in kidney, cranial and dorsal ganglia, neural tube and intermediate zones of the hindbrain (Zhang, et al., 2002; Kim, et al., 2008). Exogenous expression of GLIS2 has
been shown to promote differentiation of neuronal precursors (Lamar, et al., 2001; Kim, et al., 2008). Further, another member of the GLIS protein family, GLIS1, has recently been discovered to enhance the generation of iPSCs from both human and mouse fibroblasts (Maekawa, et al., 2011; Maekawa and Yamanaka, 2011) and has been shown to promote multiple pro-reprogramming pathways such as NANOG, WNT, Myc, Essrb and Lin28 (Maekawa, et al., 2011).

Recent studies have identified the HMGA1 (high mobility group A1) gene as a highly enriched factor in human ES cells, adult stem cells and poorly differentiated stem-like cancer cells (Shah, et al., 2012; Shah, et al., 2013). Lack of HMGA1 expression in ES cells induces changes in hematopoietic differentiation due to a reduction in macrophage and monocyte populations, enhanced numbers of megakaryocyte precursors, reduced capacity to generate embryoid bodies (EBs) and increased globin gene expression in vitro and in vivo (Battista, et al., 2003). To further explore the role of HMGA1 in pluripotent stem cells, Shah, et al. (2012) demonstrated for the first time that HMGA1 enhances cellular reprogramming of adult somatic cells to fully pluripotent stem cells (iPSCs), promotes pluripotency by inducing stem cell transcriptional networks and maintains the undifferentiated state of human ES cells (Shah, et al., 2012).
The transcription factor PFDN5 (Prefoldin 5) was selected because of its high expression in human ES cells and interaction with WNT and MYC, two genes with essential roles in the self-renewal and growth of ES cells (Pells, et al., unpublished). PFDN5 is essential for ectodermal development as it is required for a normal sensory and neuronal phenotype (Lee, et al., 2011). A study using a mouse model demonstrated that a mutation in PFDN5 resulted in photoreceptor degeneration, central nervous system defects and male infertility (Lee, et al., 2011).

In this chapter, small interfering RNA (siRNA) targeting of the selected putative biomarkers GLIS2, HMGA1 and PFDN5 identified in our epigenetic screening, was employed in two human ES cell lines (H9 and RH1) to investigate the functional significance of these biomarkers in the human pluripotent ES cell identity.
3.2 Results

3.2.1 siRNA-mediated gene knockdown of GLIS2, HMGA1 and PFDN5 reduces the expression of thereof in H9 and RH1 human ES cells

To demonstrate the functional significance of our selected transcription factors for maintenance of an undifferentiated human ES cell phenotype in human ES cells, small interference RNA (siRNA)-mediated gene knockdown of GLIS2, HMGA1 and PFDN5 was employed in H9 and RH1 human ES cell lines (Figure 3.2).

Figure 3.2. Schematic illustration of siRNA-mediated knockdown experiments. H9 and RH1 human ES cells were transfected twice at a 24 hour interval with siRNA directed against the mRNA of GLIS2, HMGA1, PFDN5 and appropriate positive and negative controls. All cultures were maintained under feeder-free conditions in mTeSR™1. Samples were collected for qRT-PCR, immunostaining and ELISA. Abbreviation/Acronym: 12-w, 12-well; siRNA, small interfering RNA.
Quadruplicate H9 and RH1 cultures were transfected with GLIS2, HMGA1 and PFDN5 siRNA oligonucleotides using RNAiMAX Lipofectamine. For control purposes, human ES cells were transfected with control IDS-null, YAP1 or OCT4 siRNA oligonucleotides (IDS-null is a mutant oligonucleotide which does not target any transcript in the human genome, while YAP1 has been reported to not be required for the maintenance of OCT4 expression when knocked-down in three human ES cell lines (Chia, et al., 2010) and OCT4 served as a positive control). Transfected cells were cultured in mTeSR™1 medium which maintains the self-renewal of human ES cells in vitro and media was changed every 24 hrs. Samples for analysis were collected at 24 and 72 hrs after the final siRNA treatment and the knockdown efficiency was assessed by qRT-PCR.

The knockdown samples of GLIS2, HMGA1, PFDN5, YAP1 and OCT4 were all compared to the negative control IDS-null for all analyses. In quantitative analysis such as the qRT-PCR, the difference between each knockdown treatment and the negative control IDS-null was calculated using Student’s t-test to determine if it was statistically significant. This statistical test was chosen for this study as it is designed for small sample size (Winter, 2013). Published work suggests that is feasible to apply the Student’s t-test on small sample size studies (even a sample size as small as two) and that small size samples can be used as long as the effect size in the population is expected to be large (Janušonis, 2009; Winter, 2013).
Most commonly, the statistical significance of the difference between two groups is determined by calculating the $p$ value and the difference is judged to be statistically significant when the $p$ value is below a predefined limit ($p = 0.05$ or lower); this way, small $p$ values correspond to strong evidence (du Prel, et al., 2009). It can be argued that the concept of $p$ values alone do not allow any direct statement about the size of the difference between different groups (Coe, 2002; de Prel, et al., 2009; Sun, et al., 2010). Hence, aside from reporting $p$ values, a measurement of the effect size (or effect strength) in the studied population is important in order to determine the magnitude of the significance of the difference between two groups even when there is an extremely small sample size (Coe, 2002; Winter, 2013). The effect size, as proposed by Cohen, is defined as the difference between two means divided by the pooled standard deviation of the two groups (Cohen, 1988). As it has been described in Section 2.9, an effect value of 0.8 or higher represents a large or very large difference between two groups (Maher, et al., 2013).

In this study, both the $p$ value and the effect size were calculated and reported not only because they complement each other but also to further check whether a discrepancy between $p$ value and effect size exists (Sun, et al., 2010). The effect size for both significant and non-significant $p$ values are presented as it enables a clear understanding of the size and the meaning of the effect (Sun, et al., 2010).
Expression of all three putative biomarkers and control genes was significantly downregulated following siRNA treatment in both H9 and RH1 cells compared to their expression levels in the negative control cells. In H9 cells, siRNA-mediated knockdown of GLIS2 and PFDN5 was very efficient, with statistical significance \( p < 0.0001 \) and very large size effects of \( d > 1.3 \) (Figure 3.3 and Table 3.1). HMGA1 knockdown efficiency was lower in comparison (\( p < 0.001 \); \( d = 5.09 \), very large effect size), and expression quickly returned to, and then significantly (\( p < 0.001 \); \( d = 4.99 \), very large effect size) exceeded transcript levels seen in IDS-null siRNA treated ES cells at 72 hrs post knockdown (Figure 3.3 and Table 3.1). Taken together, our qRT-PCR data indicate that knockdown was efficient and that we generated cells lacking GLIS2, HMGA1 or PFDN5, providing us cellular tool to assess roles of these markers in human ES cell pluripotency.
Figure 3.3. siRNA-mediated knockdown of GLIS2, HMGA1 and PFDN5 in H9 human ES cells. H9 cells were transfected with siRNA (twice, 24 hrs apart) directed against GLIS2, HMGA1 or PFDN5, and controls IDS-null, YAP1 and OCT4. RNA was harvested at 24 (grey bars) and 72 hrs (black bars) following siRNA treatment and transcript levels were assayed by qRT-PCR. The expression level of each gene was normalised to the Glyceraldehyde-3-Phosphatase Dehydrogenase (GAPDH) gene and expressed compared to the negative control IDS-null, which is set as 1.0. Asterisks indicate levels of statistical significance (**p<0.001, ****p<0.0001) as calculated by Student's t-test. × denotes that no target transcript was detectable.
Table 3.1. Effect size of differences in gene expression following YAP1, OCT4, GLIS2, HMGA1 and PFDN5 knockdowns in H9 human ES cells. Cohen’s $d$ effect size was calculated based on the formula described in Section 2.9 for each of the knockdowns and the measurement of effect of each knockdown compared to the IDS-null knockdown (negative control). Based on Cohen’s criteria (Table 2.2), a value of $d > 1.3$ equals very large effect size. All knockdown treatments had a very large effect with $d$ values of 4.99 or greater.

For RH1 ES cells, HMGA1 and PFDN5 transcript levels were significantly decreased 72 hours after the second siRNA treatment with statistical significance $p < 0.001$ and $p < 0.0001$, respectively (Figure 3.4). GLIS2 knockdown efficiency was significantly reduced 24 hours after the final siRNA treatment ($p < 0.0001$) and expression quickly returned to transcript levels close to baseline at 72 hrs post-second knockdown with statistical significance $p < 0.0001$ (Figure 3.4). All knockdown treatments demonstrated a very large effect when
compared to the negative control IDS-null knockdown with the exception of GLIS2 knockdown at 72 hrs post-second siRNA treatment (Table 3.2).

Figure 3.4. siRNA-mediated knockdown of GLIS2, HMGA1 and PFDN5 in RH1 human ES cells. RH1 cells were transfected with siRNA (twice, 24 hrs apart) directed against GLIS2, HMGA1 or PFDN5, and controls IDS-null, YAP1 and OCT4. RNA was harvested at 24 (grey bars) and 72 hrs (black bars) following siRNA treatment and transcript levels were assayed by qRT-PCR. The expression level of each gene was normalised to GAPDH and compared to the negative control IDS-null, which is set as 1.0. Asterisks indicate levels of statistical significance (**p<0.001, ****p<0.0001) as calculated by Student's t-test. × denotes that no target transcript was detectable.
<table>
<thead>
<tr>
<th>KD</th>
<th>Effect Size (d value)</th>
<th>Effect Size Measure</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>24 hrs</td>
<td>72 hrs</td>
</tr>
<tr>
<td>YAP1</td>
<td>10.59</td>
<td>1.68</td>
</tr>
<tr>
<td>OCT4</td>
<td>37.56</td>
<td>16.08</td>
</tr>
<tr>
<td>GLIS2</td>
<td>12.96</td>
<td>0.36</td>
</tr>
<tr>
<td>HMGA1</td>
<td>10.89</td>
<td>6.91</td>
</tr>
<tr>
<td>PFDN5</td>
<td>18.58</td>
<td>13.75</td>
</tr>
</tbody>
</table>

Table 3.2. Effect size of differences in gene expression following YAP1, OCT4, GLIS2, HMGA1 and PFDN5 knockdowns in RH1 human ES cells. Cohen’s $d$ effect size was calculated based on the formula described in Section 2.9 for each of the knockdowns and the measurement of effect of each knockdown compared to the IDS-null knockdown (negative control). Based on Cohen’s criteria (Table 2.2), a value of $d > 1.3$ equals very large effect size. All knockdown treatments had a very large effect with $d$ values of 1.68 or greater, except for GLIS2 at the time point of 72 hours.

Taken together, these results demonstrate successful siRNA-mediated knockdown of our putative biomarkers GLIS2, HMGA1 and PFDN5, and control genes YAP1 and OCT4.
Previous work has demonstrated that GLIS2, HMGA1 and PFDN5 have OCT4 binding sites in their promoter regions. We therefore anticipated that OCT4 knockdown would impact on the expression of the three markers. Indeed, siRNA-mediated knockdown of OCT4 affected the expression of all three putative biomarkers in human ES cells, in that (i) knockdown of OCT4 resulted in a significant downregulation of GLIS2 in both H9 and RH1 cells, and (ii) OCT4 knockdown resulted in the significant upregulation of HMGA1 and PFDN5 in both H9 and RH1 cells (Figure 3.5). The effect size of all these changes in expression were found to be very large (Table 3.3), concurring the statistical significance of the data based on p values.

![Figure 3.5. OCT4 knockdown downregulates GLIS2 expression and upregulates HMAG1 and PFDN5 expression in human ES cells. siRNA-mediated knockdown of](image-url)
OCT4 in H9 (black bars) and RH1 (golden bars) human ES cells perturbs transcript levels of all three markers compared to the negative control IDS-null where expression levels have been assigned a value of 1.0. Asterisks indicate levels of statistical significance as calculated by Student’s t-test (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001).

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Table 3.3. Effect size of OCT4 knockdown on GLIS2, HMGA1 and PFDN5 in H9 and RH1 human ES cells. Cohen’s d effect size calculations based on the formula described in Section 2.9 show that OCT4 knockdown has a very large effect on the expression of GLIS2, HMGA1 and PFDN5 in both H9 and RH1 ES cells, with values of 2.23 or greater.

To assess whether knockdown of GLIS2, HMGA1 and PFDN5 had any biological effect, cell morphology was examined 72 hours post-second siRNA treatment. Gene knockdown of all three markers resulted in apparent morphological changes in both H9 and RH1 cell lines reminiscent of differentiation (Figure 3.6 A and B). Consistent with previous studies (Hay, et al., 2004; Matin, et al., 2004; Babaie, et al., 2007) both lines exhibited markedly
altered morphology following knockdown of OCT4, specifically loss of the characteristic undifferentiated cell morphology and the induction of cell differentiation (Figure 3.6). Knockdown of IDS-null and YAP1 failed to induce any morphological changes in H9 and RH1 cells (Figure 3.6).
Figure 3.6. Knockdown of GLIS2, HMGA1 and PFDN5 affects ES cell phenotype. Knockdown of GLIS2, HMGA1, PFDN5 and OCT4 but not IDS-null and YAP1 induces cell morphological changes and differentiation in cultures of H9 (A) and RH1 (B) human
ES cells post siRNA treatment. The morphology was observed and captured with an Axiovert 40 CFL microscope and a Canon PowerShot A650 IS camera. Scale bar represents 100 μm. Abbreviation: KD, knockdown.

### 3.2.2 Knockdown of GLIS2, HMGA1 and PFDN5 affects mRNA expression of pluripotency markers OCT4, NANOG and SOX2

To determine whether our putative biomarkers for human ES cell state could modulate the expression of the key pluripotency factors mRNA expression of OCT4, NANOG and SOX2 was assessed in H9 and RH1 cells by qRT-PCR, 24 and 72 hours following knockdown treatment of GLIS2, HMGA1 and PFDN5.

In H9 human ES cells, GLIS2, HMGA1 and PFDN5 knockdowns did not result in any evident changes in the steady expression of mRNA levels for the three pluripotent markers, except from the significant decrease in NANOG transcript levels after GLIS2 knockdown at 24 hrs (p<0.05) (Figure 3.7). In RH1, however, OCT4, NANOG and SOX2 were all significantly repressed within 24 hrs following the second siRNA-mediated knockdown of HMGA1 and PFDN5 (Figure 3.8). GLIS2 knockdown resulted only in a significantly reduced expression of SOX2 at this time point (p<0.0001) (Figure 3.8). At the second
time point (48 hrs post-second siRNA transfection), in both H9 (Figure 3.7) and RH1 (Figure 3.8) cells, OCT4, NANOG and SOX2 were all significantly downregulated or completely repressed following knockdown of GLIS2, HMGA1 or PFDN5.

As previously, the effect size of these findings was assessed using Cohen’s standardised mean difference $d$ calculations. The effect sizes of mRNA expression of pluripotency markers following knockdown of our three biomarkers in H9 cells showed evidence of a significant difference when compared to the negative control IDS-null as indicated by the high $d$ values at 24 hours, confirming the observations of statistical significance ($p$ values) as shown in Table 3.4. Exceptions to the former are the expression of OCT4 following YAP1 knockdown, expression of SOX2 following GLIS2 knockdown, OCT4 and SOX2 expression following HMGA1 knockdown, and SOX2 expression following PFDN5 knockdown. Similarly, at 72 hours (48 hrs post-second siRNA transfection), the effect sizes of all three biomarker knockdowns for H9 cells corroborate the results of the statistical significance regarding the magnitude of difference compared to the negative control IDS-null knockdown, except NANOG expression following YAP1 knockdown. For RH1 cells, at 24 hours, the effect size is classified as high, indicating a strong effect of knockdown of all three biomarkers, with the only exception the effect of GLIS2 knockdown on the expression of OCT4 and NANOG. At 72 hrs, the high $d$ values indicate a very
large effect of GLIS2, HMGA1 and PFDN5 knockdown on the expression of pluripotency markers OCT4, NANOG and SOX2 in RH1, which affirms the observations of the statistical significance (Table 3.5).
Figure 3.7. siRNA-mediated knockdown of GLIS2, HMGA1 and PFDN5 affects expression of pluripotency genes in H9 cells. Quadruplicate cultures of H9 human
ES cells were treated twice (24 hrs apart) with siRNA directed against the GLIS2, HMGA1, PFDN5 and controls IDS-null, YAP1 and OCT4. Cells were harvested at 24 and 72 hrs post-second siRNA transfection for qRT-PCR analysis of the pluripotency genes OCT4, NANOG and SOX2. Observed changes are relative to GAPDH expression and normalised to the negative control IDS-null which has a value of 1.0. Asterisks indicate levels of statistical difference (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001) as calculated by Student's t-test. × denotes that no target transcript was detectable.

The transcript levels of OCT4, NANOG and SOX2 in YAP1 knockdown in H9 and RH1 cells (Figures 3.7 and 3.8) are close to those of the control cells, with the only exception of a significant reduction in OCT4 and SOX2 at 72 hours in H9 cells (Figure 3.7). Further, the positive control OCT4 siRNA knockdown was accompanied by a significant decrease (p<0.0001) of all three pluripotent genes in both human ES cell lines at 24 and 72 hours, with the exception of SOX2 at 24 hours in RH1 cells which was maintained at control mRNA levels (Figures 3.7 and 3.8). Effect size calculations based on Cohen's criteria (Table 2.2), showed a very large effect on the expression of OCT4, NANOG and SOX2 following the OCT4 knockdown in both H9 and RH1 cells, with the exception of SOX2 expression at 24 hours in RH1 cells following OCT4 knockdown (medium effect) (Tables 3.4 and 3.5).
Table 3.4. Effect size of impact of YAP1, OCT4, GLIS2, HMGA1 and PFDN5 knockdowns on the expression of pluripotency markers in H9 human ES cells. Quadruplicate cultures of H9 cells were treated twice (24 hrs apart) with siRNA directed against the controls IDS-null, YAP1 and OCT4, and selected biomarkers GLIS2, HMGA1 and PFDN5. Samples were analysed at 24 and 72 hrs using qRT-PCR to measure the expression of OCT4, NANOG and SOX2 mRNA. To define the magnitude
of the difference in expression between each knockdown and the negative control IDS-null knockdown Cohen’s $d$ effect size was calculated based on the formula described in Section 2.9 indicating that the effect size varies from no effect to very large for 24 hrs samples, while at 72 hrs all the knockdown treatments showed large and very large size effects.
Figure 3.8. siRNA-mediated knockdown of GLIS2, HMGA1 and PFDN5 affects expression of pluripotency genes in RH1 cells. Quadruplicate cultures of RH1 cells were transfected twice (24 hrs apart) with siRNA directed against the biomarkers.
GLIS2, HMGA1, PFDN5 and controls IDS-null, YAP1 and OCT4. Samples were collected at 24 and 48 hrs post-second siRNA transfection for qRT-PCR analysis of the pluripotency genes OCT4, NANOG and SOX2. Observed changes are relative to GAPDH expression and normalised to the negative control IDS-null which has a value of 1.0. Asterisks indicate levels of statistical significance (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001) as calculated by Student's t-test. × denotes that no target transcript was detectable.

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Table 3.5. Effect size of impact of YAP1, OCT4, GLIS2, HMGA1 and PFDN5 knockdowns on the expression of pluripotency markers in RH1 human ES cells.
Quadruplicate cultures of RH1 cells were treated twice (24 hrs apart) with siRNA directed against GLIS2, HMGA1 and PFDN5, and controls IDS-null, YAP1 and OCT4. Samples were analysed at 24 and 48 hrs post-second siRNA transfection using qRT-PCR to measure the expression of OCT4, NANOG and SOX2 mRNA. To define the magnitude of the difference in expression between each knockdown and the negative control IDS-null knockdown Cohen’s $d$ effect size was calculated based on the formula described in Section 2.9 indicating that the effect size varies from small to very large at 24 hrs, while at 72 hrs the knockdown treatments showed mainly very large size effects.

To assess whether in addition to changes in mRNA expression of pluripotency markers OCT4 and NANOG, also protein expression was affected by knockdown of GLIS2, HMGA1 or PFDN5, immunocytochemistry was performed for OCT4 and NANOG in H9 and RH1 cells. As shown in figures 3.9 and 3.10 OCT4 and NANOG could be readily detected in both cell lines treated with the controls IDS-null and YAP1. However, as expected, OCT4 and NANOG expression could not be detected in cells treated with the positive control OCT4 siRNA, consistent with the qRT-PCR data presented in figures 3.7 and 3.8. For both H9 and RH1 cells, knockdown of GLIS2, HMGA1 or PFDN5 resulted in loss of protein expression for both OCT4 and NANOG, while NANOG was still expressed in H9 and RH1 cells following PFDN5 knockdown, though at lower levels compared to those of the IDS-null and YAP1 controls (Figures 3.7 and 3.8).
Collectively, the qRT-PCR and immunocytochemistry data demonstrate that knockdown of GLIS2, HMGA1 and PFDN5 along with the positive control OCT4 significantly affected the expression of the pluripotency markers OCT4 and NANOG at mRNA as well as protein level, in both human ES cell lines.
Figure 3.9. Epigenetically-defined human ES cell biomarkers play a role in maintenance of pluripotency of H9 cells. H9 human ES cells were treated twice (24 hrs apart) with siRNAs directed against the mRNA of GLIS2, HMGA1 or PFDN5 and controls IDS-null, YAP1 and OCT4. Samples were fixed with 4% PFA/PBS 48 hrs after the second siRNA transfection. Representative images of immunohistochemical analysis for the pluripotency markers OCT4 (green) and NANOG (red) shows significant reduction in protein levels of OCT4 and NANOG following knockdown of the three biomarkers. DAPI (blue) was used as a nuclear staining. Images were captured using a
Figure 3.10. Epigenetically-defined human ES cell biomarkers play a role in maintenance of pluripotency of RH1 cells. RH1 human ES cells were transfected twice (24 hrs apart) with siRNA directed against GLIS2, HMGA1 or PFDN5 and controls IDS-null, YAP1 and OCT4. Samples were fixed with 4% PFA/PBS 48 hrs post-second siRNA transfection. Representative images of immunohistochemical analysis for the
human ES cell pluripotency markers OCT4 (green) and NANOG (red) shows that protein levels of OCT4 and NANOG are downregulated following knockdown of the three biomarkers. DAPI (blue) was used as a nuclear staining. These images were captured using a Zeiss Observer fluorescence microscope and prepared using the Zeiss AxioVision 4.8.2 software. The scale bar represents 100 μm. Abbreviation: KD, knockdown.

3.2.3 Knockdown of GLIS2, HMGA1 and PFDN5 induces differentiation of human ES cells

The observed loss of an undifferentiated human ES cell phenotype and induced morphological differentiation following knockdown of GLIS2, HMGA1 and PFDN5 in both H9 and RH1 cells (Section 3.2.1) did not provide much detail as to the extent of cell differentiation. Hence, to establish whether the knockdown of GLIS2, HMGA1 and PFDN5 led to a pronounced lineage specification or more general/undirected differentiation, qRT-PCR analysis for lineage-associated markers was performed. Quadruplicate biological samples for GLIS2, HMGA1, PFDN5 and OCT4 knockdowns were assessed for a panel of markers representative of lineage-associated ES cell differentiation including CDX2, CGα, PL1 (trophoblast), Brachyury, VEGF, BMP2, GATA2 (mesoderm), GATA4, AFP, Albumin, HNF4α, GATA6 (endoderm), PAX6, Nestin, Tubulin III, NF-200 (ectoderm) and HAND1 (early lineage). The gene expression of the indicated
lineage markers for all siRNA knockdowns were compared to those of the negative control of IDS-null.

siRNA-mediated knockdown of GLIS2 in H9 cells resulted in a significant upregulation of the trophoblast markers CDX2, $\mathrm{CG}_\alpha$, PL1 and endodermal markers GATA4, AFP, Albumin and GATA6 (Figure 3.11A). In addition, the mesodermal markers Brachyury, GATA2 and ectodermal markers Tubulin III and NF-200 were upregulated as well. A mixed pattern was observed in HMGA1 knockdown as only specific markers of the trophoblast (CDX2, $\mathrm{CG}_\alpha$), endoderm (GATA4, AFP) and ectoderm (Tubulin III, NF-200) lineages were significantly induced while the remaining markers did not show noticeable changes or were significantly downregulated (Figure 3.11B).

Knockdown of PFDN5 did not lead to a defined lineage specification either since it altered the overall lineage expression but not in a uniform manner, with the only exemption of the ectodermal markers, none of which were notably affected by the knockdown (Figure 3.11C). For OCT4 knockdown, there was a significant induction of specific endodermal (GATA4, AFP) and ectodermal (PAX6, Nestin, Tubulin III) markers. Expression of GATA4 and Nestin is consistent with
previous work following depletion of OCT4 by siRNA in human ES cells (Hay, et al., 2004).

(A) GLIS2 KD
(B) HMGA1 KD

(C) PFDN5 KD
Figure 3.11. Embryonic lineage marker expression in H9 human ES cells following knockdown of GLIS2, HMGA1 and PFDN5. Quadruplicate cultures of H9 cells were treated twice (24 hrs apart) with siRNA directed against (A) GLIS2, (B) HMGA1, (C) PFDN5 and (D) OCT4 mRNAs. Samples were collected at 72 hrs (48 hrs after the final siRNA treatment) for qRT-PCR analysis for trophoblast (CDX2, CGα, PL1), mesoderm (Brachyury, VEGF, BMP2, GATA2), endoderm (GATA4, AFP, Albumin, HNF4α, GATA6), ectoderm (PAX6, Nestin, Tubulin III, NF-200) and early lineage (HAND1) markers. Observed changes are relative to GAPDH expression and normalised to the negative control IDS-null, which has a value of 1.0, and plotted on a log_{10} scale. Asterisks indicate levels of statistical significance (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001) as calculated by Student’s t-test. × denotes that no target transcript was detectable.
Similar to findings in H9 cells, RH1 cells treated with GLIS2 siRNA (Figure 3.12A) demonstrated a significant increase in trophoblast (CDX2, CGα) and endodermal (AFP, Albumin, HNF4α) markers. There was variability in the expression of mesodermal lineage markers, with only Brachyury and GATA2 being upregulated, while the ectodermal markers were mostly unchanged. Following HMGA1 knockdown (Figure 3.12B), there was a significant downregulation of the trophoblast markers CDX2 and PL1 while CGα was upregulated and mesodermal and endodermal markers were downregulated, with the exception of AFP which showed significant increase. At the same time, all the ectodermal markers and HAND1 (marker expressed in early lineages) showed no important expression changes following HMGA1 knockdown.

PFDN5 knockdown in RH1 cells (Figure 3.12C) resulted in a mixed expression pattern for trophoblast markers, with CDX2 and CGα significantly increased and decreased, respectively, whilst PL1 remained unchanged. Mesodermal and ectodermal markers showed expression close to that of the baseline of the negative control IDS-null following PFDN5 knockdown, with the exception of Brachyury, which was significantly induced. In terms of endodermal marker expression, only AFP was considerably increased compared to the rest of the set, which were mainly downregulated. Lastly, OCT4 knockdown resulted in downregulation of CGα and PL1, significant upregulation of Brachyury and
GATA2, whereas the endodermal and ectodermal markers showed slightly decreased expression with the exception of NF-200, which was upregulated.

(A) GLIS2 KD
Figure 3.12. Embryonic lineage marker expression in RH1 human ES cells following knockdown of GLIS2, HMGA1 and PFDN5. Quadruplicate cell cultures of RH1 cells were transfected twice (24 hrs apart) with siRNA directed against (A) GLIS2, (B) HMGA1, (C) PFDN5 and (D) OCT4. Samples were collected at 72 hrs (48 hrs after the final siRNA treatment) for qRT-PCR analysis for trophoblast (CDX2, CGα, PL1), mesoderm (Brachyury, VEGF, BMP2, GATA2), endoderm (GATA4, AFP, Albumin, HNF4α, GATA6), ectoderm (PAX6, Nestin, Tubulin III, NF-200) and early lineage (HAND1) markers. Observed changes are relative to GAPDH expression and normalised to the negative control IDS-null, which has a value of 1.0, and plotted on a log₁₀ scale. Asterisks indicate levels of statistical significance (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001) as calculated by Student’s t-test. × denotes that no target transcript was detectable.
In summary, we here show that ablation of GLIS2 expression consistently resulted in a significant upregulation of multiple trophoblast and endodermal markers in H9 and RH1 cells. These results suggest that GLIS2 may have a role preventing the trophoblast and endodermal specification in human ES cells. Further, there were similarities between the two human ES cell lines when treated with the HMGA1 knockdown which led to the upregulation of CGα and downregulation of PL1 (trophoblast). Concurrently, most of the endodermal markers were significantly downregulated in both cell lines (Albumin, HNF4α, GATA6) except for AFP, which was upregulated. Likewise, in the PFDN5 knockdown of both cell lines, AFP was significantly upregulated while the majority of the same endodermal markers shown a decrease. In contrast, the response of the ES cells to OCT4 knockdown with regards to lineage marker expression was not consistent between the two cell lines.

3.2.4 The impact of knockdown of GLIS2, HMGA1 and PFDN5 on the expression of epigenetic markers in human ES cells

Knockdown of our putative pluripotency biomarkers GLIS2, HMGA1 and PFDN5 demonstrated loss of the undifferentiated human ES cell phenotype and induction of differentiation at the level of cell morphology (Figure 3.6), mRNA
(Figures 3.7 and 3.8) and protein (Figures 3.9 and 3.10) pluripotency marker expression and cell lineage marker expression (Figures 3.11 and 3.12). To further explore a potential functional significance of these three markers on the human ES cell identity, I sought to study their effects on the epigenetic status of human ES cells.

Studies in ES cells have revealed evidence that the epigenetic marker 5-hydroxymethylcytosine (5-hmC) plays an essential role in embryonic stem cell pluripotency, with TET protein family (TET1-3) being responsible for the conversion of 5-methylcytosine (5-mC) to 5-hmC (Tahiliani, et al., 2009; Ito, et al., 2010; Koh, et al., 2011). Following these studies in ES cells, members of the TET family and 5-hmC have been proven to be important in the process of somatic cell reprogramming. Recent work has identified TET1 and TET2 as interaction partners of NANOG in enhancing the efficiency of somatic cell reprogramming and increased levels of 5-hmC were detected in the case of TET1 (Costa, et al., 2013). At the same time, another study demonstrated that TET1 can replace exogenous OCT4 to generate fully pluripotent iPSCs in combination with SOX2, KLF4 and c-Myc while 5-hmC should be acquired and reset cells to an ESC-like state (Gao, et al., 2013).
In order to examine the effects of GLIS2, HMGA1 and PFDN5 depletion on the epigenome of H9 and RH1 cells, the expression of 5-mC, 5-hmC and TET1-3 genes were evaluated by three methods, namely (1) immunocytochemistry, (2) qRT-PCR and (3) ELISA. Immunocytochemical assessment of 5-mC and 5-hmC at a nuclear level in control knockdowns IDS-null and YAP1 revealed this knockdown did not impair their detection in both H9 and RH1 cells (Figures 3.13 and 3.14).

In H9 cells, the levels of 5-mC were readily detectable in the cultures treated with the HMGA1 and PFDN5 siRNA and similar to those of the OCT4 but with lower intensity compared to the IDS-null and YAP1 controls. The GLIS2 knockdown cultures showed pronounced qualitative reduction in 5-mC compared to its expression in HMGA1, PFDN5 and OCT4 knockdowns. Expression of 5-hmC, however, was reduced in GLIS2, HMGA1, PFDN5 and OCT4 (Figure 3.13).

Compared to H9 cells, expression of 5-mC in RH1 cells was more apparent in all three biomarker and OCT4 knockdown cultures. Expression of 5-hmC was assessed as lower in GLIS2, HMGA1, PFDN5 and OCT4 knockdown as compared to levels in IDS-null and YAP1 knockdowns (Figure 3.14).
Figure 3.13. Knockdown of GLIS2, HMGA1 and PFDN5 in H9 human ES cells perturbs expression of epigenetic markers. H9 cells were transfected twice (24 hrs apart) with siRNA against IDS-null, YAP1, GLIS2, HMGA1, PFDN5 and OCT4. Samples were fixed with 4% PFA/PBS 48 hrs post-second siRNA transfection and stained for the epigenetic markers 5-methylcytosine (5-mC, green) and 5-hydroxymethylcytosine (5-hmC, red). H9 cells show decreased levels of 5-mC and 5-hmC after knockdown of GLIS2, HMGA1 and PFDN5. DAPI (blue) represents nuclear staining. Staining was assessed using a Zeiss Observer fluorescence microscope and
images captured and prepared using the Zeiss AxioVision 4.8.2 software. The scale bar represents 100 μm. Abbreviation: KD, knockdown.

Figure 3.14. Knockdown of GLIS2, HMGA1 and PFDN5 in RH1 human ES cells perturbs expression of epigenetic markers. RH1 cells were transfected twice (24 hrs apart) with siRNA against IDS-null, YAP1, GLIS2, HMGA1, PFDN5 and OCT4. Samples were fixed with 4% PFA/PBS 48 hrs post-second siRNA transfection and stained for the epigenetic markers 5-methylcytosine (5-mC, green) and 5-hydroxymethylcytosine (5-hmC, red). RH1 cells show reduced levels of 5-mC and 5-
hmC after knockdown of GLIS2, HMGA1 and PFDN5. DAPI (blue) represents nuclear staining. Staining was assessed using a Zeiss Observer fluorescence microscope and images captured and prepared using the Zeiss AxioVision 4.8.2 software. The scale bar represents 100 μm. Abbreviation: KD, knockdown.

To confirm these qualitative immunocytochemistry results, the effects of GLIS2, HMGA1 and PFDN5 knockdown on the human ES cell epigenome were assessed in a quantitative manner using a DNA-based ELISA assay for detection of 5-hmC. This Quest 5-hmC™ DNA ELISA kit includes a control DNA set which has been calibrated to accurately measure the percentage of 5-hmC in DNA sample by the use of a standard curve. This control DNA set was included in every measurement in order to accurately determine the percentage of 5-hmC (see procedure description in Section 2.7). DNA samples of GLIS2, HMGA1 and PFDN5 knockdown were compared to the IDS-null and YAP1 controls, and the positive control OCT4, as well as to DNA from untreated H9 and RH1 cells.

DNA samples collected at 48 hrs post the second-treatment of GLIS2, HMGA1 and PFDN5, and IDS-null, YAP1 and OCT4 knockdown, along with the additional DNA samples isolated from untreated H9 and RH1 cells. All the experimental samples were loaded in one ELISA 96-well plate at equal DNA
concentrations. Control DNA samples were loaded in duplicate, DNA samples from untreated H9 cells and H9 cells which were treated with the IDS-null, YAP1 or OCT4 siRNA were loaded in triplicates and DNA from GLIS2, HMGA1 and PFDN5 siRNA treated H9 cells in quadruplicate (i.e. four biological replicates). In turn, each biological sample was represented by technical triplicates. ELISA buffer alone was loaded in order to subtract background absorbance (Figure 3.15).

As can be seen in Figures 3.15 and 3.18, for both the H9 and RH1 sample plates, the duplicate control DNA samples supplied by the kit, loaded as sets 1 (A-E, negative to positive) to generate the standard curve for OD readout clearly show a dose-related increase in colour intensity. While wells containing DNA from untreated human ES cells (consistent with published work (Ruzov, et al., 2011)), as well as from control siRNA IDS-null and YAP1 clearly show 5-hmC expression, samples from GLIS2, HMGA, PFDN5 and OCT4 H9 and RH1 knockdowns demonstrate decreased 5-hmC levels, confirming the immunocytochemical observations described earlier (Figures 3.13 and 3.14).
Figure 3.15. DNA ELISA shows downregulation of 5-hydroxymethylcytosine in H9 human ES cells following GLIS2, HMGA1 and PFDN5 knockdowns. H9 cells were assessed for 5-hmC expression using a Quest 5-hmC™ DNA ELISA kit, following treatment with siRNA against GLIS2, HMGA1 and PFDN5 mRNAs, and controls against IDS-null, YAP1 and OCT4. Untreated H9 cells served as additional positive control. Darker well colour corresponds with higher 5-hmC content. Comparing colour intensity of various sets indicate that samples derived from H9 with GLIS2, HMGA1 and PFDN5 knockdown express lower levels of 5-hmC than any of the control sets. Abbreviation: KD, knockdown; OD: Optical Density.

To assess expression more quantitatively, absorbance was measured at 405 nm using a fully automated multi-detection microplate reader, and results plotted (Figures 3.16 and 3.19). For both H9 and RH1 experiment these graphs show that GLIS2, HMGA1 and PFDN5 knockdowns, like the positive control OCT4,
have markedly and significantly reduced absorbance compared to cells treated with IDS-null siRNA, verifying the observations by eye based on colour intensity (Figures 3.15 and 3.18).

**Figure 3.16. Absorbance following DNA ELISA show downregulation of 5-hydroxymethylcytosine in H9 human ES cells following GLIS2, HMGA1 and PFDN5 knockdowns.** H9 cells were assessed by DNA ELISA for 5-hmC expression following treatment with siRNA against GLIS2, HMGA1 and PFDN5 (quadruplicate biological samples, plated in triplicate) mRNAs and control IDS-null, YAP1 and OCT4 (triplicate biological samples, loaded in triplicate) knockdowns. Untreated H9 cells (triplicate biological samples, loaded in triplicate) served as additional positive control (first bar; H9 hESCs). Absorbance was measured at 405 nm using a multi-detection microplate reader. A standard curve using supplied DNA samples served as technical control (see Appendix VII). Data are shown as mean values ± S.D. and the asterisks
indicate levels of statistical significance (***p<0.001) as calculated by ANOVA with Dunnett’s post-hoc test.

Applying a linear regression equation (Appendices VII and VIII), to the provided DNA to generate a dose-response curve, allowed calculation of the percentage of 5-hmC. As indicated in Figures 3.17 and 3.20, the IDS-null and YAP1 controls maintain levels of 5-hmC similar to the untreated H9 and RH1 cell samples, indicating that the knockdown of these genes does not influence the expression of 5-hmC, as expected. In contrast, the level of 5-hmC for the positive control OCT4 knockdown was significantly decreased (p<0.001) in both cell lines reinforcing that 5-hmC is an epigenetic marker of pluripotency. Similarly, compared to the IDS-null negative control, siRNA knockdown of GLIS2, HMGA1 and PFDN5 resulted in significant reduction of 5-hmC levels in H9 cells (p<0.001 for all genes) and RH1 cells (p<0.001 for all genes).
Figure 3.17. GLIS2, HMGA1, PFDN5 and OCT4 knockdown reduces expression of 5-hydroxymethylcytosine in H9 human ES cells. H9 cells were assessed by DNA ELISA for 5-hmC expression following treatment with siRNA against GLIS2, HMGA1 and PFDN5 (quadruplicate biological samples, plated in triplicate) mRNAs and control IDS-null, YAP1 and OCT4 (triplicate biological samples, loaded in triplicate) knockdowns. Untreated H9 cells (triplicate biological samples, loaded in triplicate) served as additional positive control (first bar; H9 hESCs). Absorbance was measured at 405 nm using a multi-detection microplate reader. Applying a linear regression equation on a dose-response curve (see Appendix VII) obtained from supplied DNA samples allowed percentage calculations of expression. Data are shown as mean values ± S.D. and the asterisks indicate levels of statistical significance (***p<0.001) as calculated by ANOVA with Dunnett’s post-hoc test.
Figure 3.18. DNA ELISA shows downregulation of 5-hydroxymethylcytosine in RH1 human ES cells following GLIS2, HMGA1 and PFDN5 knockdowns. RH1 cells were assessed for 5-hmC expression using a Quest 5-hmC™ DNA ELISA kit, following treatment with siRNA against GLIS2, HMGA1 and PFDN5 mRNAs, and controls against IDS-null, YAP1 and OCT4. Untreated RH1 cells served as additional positive control. Darker well colour corresponds with higher 5-hmC content. Comparing colour intensity of various sets indicate that samples derived from RH1 with GLIS2, HMGA1 and PFDN5 knockdown express lower levels of 5-hmC than any of the control sets. Abbreviation: KD, knockdown; OD: Optical Density.
Figure 3.19. Absorbance following DNA ELISA show downregulation of 5-hydroxymethylcytosine in RH1 human ES cells following GLIS2, HMGA1 and PFDN5 knockdowns. RH1 cells were assessed by DNA ELISA for 5-hmC expression following treatment with siRNA against GLIS2, HMGA1 and PFDN5 (quadruplicate biological samples, plated in triplicate) mRNAs and control IDS-null, YAP1 and OCT4 (triplicate biological samples, loaded in triplicate) knockdowns. Untreated RH1 cells (triplicate biological samples, loaded in triplicate) served as additional positive control (first bar; RH1 hESCs). Absorbance was measured at 405 nm using a multi-detection microplate reader. A standard curve using supplied DNA samples served as technical control (see Appendix VIII). Data are shown as mean values ± S.D. and the asterisks indicate levels of statistical significance (**p<0.001) as calculated by ANOVA with Dunnett's post-hoc test.
Figure 3.20. GLIS2, HMGA1, PFDN5 and OCT4 knockdown reduces expression of 5-hydroxymethylcytosine in RH1 human ES cells. RH1 cells were assessed by DNA ELISA for 5-hmC expression following treatment with siRNA against GLIS2, HMGA1 and PFDN5 (quadruplicate biological samples, plated in triplicate) mRNAs and control IDS-null, YAP1 and OCT4 (triplicate biological samples, loaded in triplicate) knockdowns. Untreated RH1 cells (triplicate biological samples, loaded in triplicate) served as additional positive control (first bar; RH1 hESCs). Absorbance was measured at 405 nm using a multi-detection microplate reader. Applying a linear regression equation on a dose-response curve (see Appendix VIII) obtained from supplied DNA samples allowed percentage calculations of expression. Data are shown as mean values ± S.D. and the asterisks indicate levels of statistical significance (**p<0.001) as calculated by ANOVA with Dunnett’s post-hoc test.
Given that quantification of 5-hmC levels by ELISA showed significant ablation in the cultures treated with OCT4, GLIS2, HMGA1 and PFDN5 siRNA, I next examined whether the expression of TET genes was altered as well as previous studies have demonstrated that the TET enzymes TET1, TET2 and TET3 alter the methylation status of DNA as they are responsible for the conversion of 5-mC to 5-hmC and play important role in ES cell pluripotency (Ito, et al., 2010; Koh, et al., 2011). TET mRNA expression was measured after siRNA-mediated knockdown of the positive control OCT4 and our putative biomarkers GLIS2, HMAG1 and PFDN5 in H9 and RH1 cells by qRT-PCR.

Figures 3.21 and 3.22 illustrate the effects of GLIS2, HMAG1 and PFDN5, and OCT4 knockdown on TET expression in H9 and RH1 cells, respectively. Knockdown of OCT4 resulted in changes in TET mRNA levels in that TET3 but not TET1 or TET2 increased considerably (p<0.01) in H9 cells (Figure 3.21) while only TET2 was affected in RH1 cells, showing a significant reduction (p<0.01) (Figure 3.22).

Knockdown of GLIS2 had no significant effect on TET transcript levels in either cell line (Figures 3.21 and 3.22). Most evident changes in TET expression were observed in H9 cells following HMGA1 or PFDN5 knockdown (less than 1.5-fold; Figure 3.21). In the case of HMGA1 knockdown, H9 cells showed significant
p perturbation in all three TET mRNA levels while HMGA1 knockdown in RH1
cells did not induce noticeable changes (Figure 3.22). Lastly, in response to
PFDN5 knockdown, both cell lines showed moderate elevation of TET1
expression (p<0.05). While there was a significant increase in only TET3
expression (p<0.001) in H9 cells, there were no changes in TET2 and TET3 in
RH1 cells following PFDN5 knockdown.

Figure 3.21. Knockdown of GLIS2, HMGA1 and PFDN5 affects expression of TET
genes in H9 ES cells. H9 cells were transfected twice (24 hrs apart) with siRNA
directed against the mRNA of GLIS2, HMGA1 and PFDN5, and positive control OCT4,
and mRNA levels for TET1, TET2 and TET3 assessed by qRT-PCR. Results are
presented as fold difference in expression of the three TET genes relative to GAPDH
expression and normalised to the negative control IDS-null which has a value of 1.0.
Asterisks indicate levels of statistical significance (*p<0.05, **p<0.01, ***p<0.001,
****p<0.0001) as calculated by ANOVA with Dunnett’s post-hoc test.
Figure 3.22. Knockdown of GLIS2, HMGA1 and PFDN5 affects expression of TET genes in RH1 ES cells. RH1 cells were transfected twice (24 hrs apart) with siRNA directed against the mRNA of GLIS2, HMGA1 and PFDN5, and positive control OCT4, and mRNA levels for TET1, TET2 and TET3 assessed by qRT-PCR. Results are presented as fold difference in expression of the three TET genes relative to GAPDH expression and normalised to the negative control IDS-null which has a value of 1.0. Asterisks indicate levels of statistical significance (*p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001) as calculated by ANOVA with Dunnett’s post-hoc test.

To summarise, H9 and RH1 cells responded similarly to siRNA-mediated knockdown of GLIS2, HMGA1 and PFDN5, in that both lines (1) their undifferentiated human ES cell phenotype and pluripotency was lost, (2) reduced expression of 5-hmC and (3) TET mRNA expression affected.
Regarding the induction of differentiation, both cell lines showed significant upregulation of CDX2, CGα (trophoblast), Brachyury, GATA2 (mesoderm), AFP, Albumin (endoderm), NF-200 (ectoderm) markers following GLIS2 knockdown. In both lines, knockdown of HMGA1 led to the significant induction of CGα and AFP genes, while knockdown of PFDN5 resulted in the significant increase of CDX2 and AFP.

### 3.3 Discussion

The key pluripotency factors OCT4, NANOG and SOX2 are known to be essential for early developmental events and maintenance of the undifferentiated state of ES cells. Extensive work has demonstrated that depletion of these factors compromise ES cell pluripotency and leads to differentiation. In addition to the genetic elements, the maintenance of pluripotency and lineage specification of ES cells is controlled by epigenetic mechanisms. Studies on the epigenetic status of undifferentiated ES cells have revealed that ES cells possess a unique DNA methylation identity compared to differentiated cell populations. Hence, it is of major importance to identify new transcription factors that may control pluripotency and extend the current perception of ES cell transcriptional network beyond the three pluripotency genes OCT4, NANOG and SOX2 along with dissecting the epigenetics of these
cells and provide a better insight in the establishment and maintenance of the ESC pluripotent and lineage-specific differentiation processes.

The purpose of this study was to demonstrate the functional significance of three putative biomarkers (GLIS2, HMGA1 and PFDN5) in the maintainance of human ES cell pluripotency. These markers were selected due to their methylation status being unique in undifferentiated human ES cells and their involvement with pluripotency-associated genes and/or pathways. GLIS2 interacts with p120 catenin which is a member of the pluripotency-associated WNT pathway and that its homologue GLIS1 promotes pro-reprogramming pathways in fibroblasts to iPSCs (Maekawa, et al., 2011). HMGA1 has been shown to interact with molecules influencing the human ES cell phenotype such as Lin28, Myc and STAT3. PFDN5 interacts with WNT which is important in ES cell self-renewal (Yoshida, et al., 2008). Together, these notions support a potential role for these epigenetic genes in the transcriptional network supporting of human ES cell pluripotency.

To validate their role, small interference RNA (siRNA) was employed in two different human ES cell lines (H9 and RH1) and the effects on the subsequent knockdown on human ES cell pluripotency and epigenome observed. In this chapter compelling evidence is presented that highlights the functional
significance of our selected markers GLIS2, HMGA1 and PFDN5 in the maintenance of a human ES cell undifferentiated state. We show that perturbation of GLIS2, HMGA1 and PFDN5 caused loss of human ES stem cell phenotype along with downregulation of the key pluripotency transcription regulators OCT4, NANOG and SOX2 and induction of differentiation towards multiple embryonic lineages, indicating that these factors play a role in controlling human ES cell pluripotency. Moreover, GLIS2, HMGA1 and PFDN5 knockdown resulted in significant epigenetic effects as shown by the ablation of 5-hydroxymethylcytosine, an epigenetic marker associated with the ES cell pluripotency.

Data also suggest that knockdown of all three selected candidate epigenetic biomarkers, GLIS2, HMGA1 and PFDN5, induced various fates in human ES cells. GLIS2 knockdown gave the most consistent response across the two human ES cell lines and was showed to upregulate mesodermal and ectodermal lineage genes in the targeted cells, consistent with work describing the established role of GLIS2 in kidney (mesodermal) development and neurogenesis (ectodermal) (Zhang, et al., 2002). While the work described in this chapter was in progress, HMGA1 knockdown in H9 human ES cells was reported to result in significant downregulation of OCT4 and SOX2, indicating an essential role in the maintainance of human ES cell pluripotency, findings in line with our data. The same group has also shown that HMGA1 promotes the
cellular reprogramming of adult somatic cells to induced pluripotent stem cells (Shah, et al., 2012). Further, similar to siRNA-mediated HMGA1 knockdown, siRNA-mediated knockdown of PFDN5, showed a significant induction of trophoblast and endoderm markers, along with a significant induction of HAND1 (in the case of RH1 cells). Additionally, our reciprocal findings that knockdown of OCT4 perturbed the expression of GLIS2, HMGA1 and PFDN5 and perturbation of the epigenetic genes led to the downregulation of the core pluripotency transcription factors OCT4, NANOG and SOX2 is in line with the fact that all three genes GLIS2, HMGA1 and PFDN5 have OCT4 binding sites in their promoters (Lister, et al., 2009; Kunarso, et al., 2010; Mullen, et al., 2011; Pells, et al., unpublished). Collectively, this suggests that they may interact with the core pluripotency network in human ES cells.

Identification of new factors pertinent to maintainance of the human ES cell identity will critically contribute to our understanding of the mechanisms and/or pathways that govern stem cell pluripotency. Work presented here has identified three novel epigenetically-defined genes that affect the expression of key pluripotent regulators (OCT4, NANOG and SOX2) and epigenetic markers (5-hmC, TET genes), thereby contributing to the inventory of transcription factors required to maintain the human ES cell pluripotent state. Assessment of the methylation status of the CGIs described in this study can be a valuable criterion for confirming the epigenomic state of human ES cell lines as well as for
ascertaining the degree to which somatic cell-derived induced pluripotent stem cells have been able to reinstate a true ES cell epigenomic status. Building on these findings and approaches future experiments may enable us to identify additional novel regulators required for maintaining human ES cell state and as such may hold the potential to advancing future human somatic cell reprogramming.
Chapter 4

The Role of Dioxygenases TET1/2/3 in Human Embryonic Stem Cell Pluripotency
4.1 Introduction

DNA cytosine methylation is the most studied epigenetic modification in mammalian cells. DNA methylation is associated with the repression of gene expression as it can prevent transcription factor binding and result to changes in chromatin structure (Prokhortchouk and Defossez, 2008). As such, it regulates several cellular processes such as tissue specific gene expression, maintenance of epigenetic memory, genomic imprinting and X-chromosome inactivation (Wu and Zhang, 2011). It occurs at most CpG dinucleotides in the mammalian genome, catalysed by a family of DNA methyltransferases (DNMTs), and is essential for embryonic viability (Klose and Bird, 2006).

DNA methylation plays an essential role in the regulation of self-renewal and pluripotency of cells. Human ES cells possess a unique DNA methylation signature compared to differentiated cell populations and cancer cells (Bibikova, et al., 2006; Fouse, et al., 2008; Meissner, et al., 2008; Ball, et al.2009; Deng, et al.2009; Altun, et al., 2010). Promoter methylation contributes to suppression of the core pluripotency genes OCT4 and NANOG (Koh and Rao, 2013). OCT4 and NANOG are largely unmethylated in ES cells and iPSCs but methylated in differentiated cells (Mitsui, et al., 2003; Okita, et al., 2007; Altun, et al., 2010).
In 2009, a novel epigenetic modification known as 5-hydroxymethylation came into focus and attracted tremendous attention when two different research groups demonstrated a way in which cytosines in mammalian cells can be hydroxylated (Kriaucionis and Heintz, 2009; Tahiliani, et al., 2009). 5-hmC is now considered to be the sixth base of the genome and is found highly expressed in the adult central nervous system and pluripotent embryonic stem cells (Mukherjee and Hsieh, 2013; Rudenko, et al., 2013; Zhang, et al., 2013; Ruzov, et al., 2011) and at lower levels in blood, kidney, lung and muscle cells (Bhutani, et al., 2011). The conversion of 5-mC to 5-hmC is executed by three mammalian 5-mC dioxygenases, TET1/2/3 (Tahiliani, et al., 2009). These proteins belong to the ten-eleven-translocation (TET) enzymes (Kinney and Pradhan, 2013) and they are members of the human Fe(II) and 2-oxoglutarate (2OG) oxygenases family (Tahiliani, et al., 2009; Loenarz and Schofield, 2009). The three TETs show different expression patterns with TET1 and TET2 being highly expressed in the ICM of mouse blastocysts and ES cells, while TET3 is highly expressed in mouse oocytes and zygotes (Ito, et al., 2010; Iqbal, et al., 2011; Wossidlo, et al., 2011; Zhao and Chen, 2013). Moreover, TET1 and TET2 expression is downregulated, but TET3 is upregulated upon differentiation of mESCs (Tahiliani, et al., 2009; Ito, et al., 2010; Koh, et al., 2011).

Several recent studies have looked at the roles 5-hmC and the TET proteins play in the pluripotent state of ES cells producing a wealth of epigenomic data.
However, these studies have produced conflicting results on whether knockdown/knockout of the TET genes alters pluripotency and differentiation of ES cells (Wu and Zhang, 2011; Kinney and Pradhan, 2013). The studies by Ito, et al. (2010) and Freudengerg, et al. (2011) showed that knockdown of TET1 in mouse ES cells led to cellular morphological changes, reduced ES cell growth rate, decrease of pluripotency-associated gene expressions and induction of cell differentiation (Ito, et al., 2010; Freudengerg, et al., 2011). In contrast, other studies (Dawlaty, et al., 2011; Koh, et al., 2011; Williams, et al., 2011) did not find any changes in morphology and showed maintenance of normal levels of key pluripotency genes following TET1 depletion. Moreover, Ficz and colleagues (2011) demonstrated that TET1/2 double knockdown in mouse ES resulted in the downregulation of pluripotency-related genes but not OCT4, NANOG and SOX2 (Ficz, et al., 2011). These inconsistencies between these in vitro studies could be due to differences in culturing conditions, mouse ES cell background and/or off target effects of shRNAs (Wu and Zhang, 2011).

As stated in chapter 3 of this thesis, our laboratory has assessed different human ES cell lines (RH1, RH3, RCM1) for variable and conserved patterns of Cytosine-Guanine Island (CGI) methylation, followed by comparisons of the derived CGI methylation patterns obtained from differentiated tissues (Illingworth, et al., 2008) and to human embryonic stem cell mRNA expression data (Pells, et al., unpublished). This work has identified defined sets of CGIs
with unique human ES cell-specific methylation patterns which facilitated
definition of genes that were methylated and expressed in human ES cells but
not the somatic tissues. One of the genes included in this data set is TET1. As
TET1 has been shown to be an important factor of murine ES cell pluripotency
and able to replace OCT4 in somatic cell reprogramming and generate iPSCs
(Gao, et al., 2013), I investigated the functional role of TET1, along with other
members of the TET family, TET2 and TET3, in maintenance of human
pluripotent embryonic stem cell state.

Small interfering RNA (siRNA) targeting of the TET family members TET1, TET2
and TET3 were employed in H9 and RH1 human ES cell lines to assess their
function, utilising the same methodologies as in chapter 3.
4.2 Results

4.2.1 siRNA-mediated gene knockdown of TET1, TET2 and TET3 reduces the expression of thereof in H9 and RH1 human ES cells

To investigate the functional role of TET1/2/3 in the maintenance of an undifferentiated human ES cell phenotype, siRNA-mediated gene knockdown of TET1, TET2 and TET3 was employed in two different human ES cell lines (H9 and RH1; Figure 4.1).

Figure 4.1. Schematic illustration of the siRNA-mediated knockdown experiments. H9 and RH1 human ES cells were transfected twice at a 24 hour interval with siRNA directed against the mRNA of TET1, TET2, TET3 and all three TETs (TET1-3), and appropriate negative and positive controls. All cultures were maintained under feeder-free conditions in mTeSR™1. Samples were collected for qRT-PCR,
Quadruplicate cultures of human ES cells were transfected with siRNA oligonucleotides directed against each of the TET genes TET1, TET2, TET3 individually and all TETs together (TET1-3, triple knockdown). For control purposes, cells were transfected with IDS-null, YAP1 or OCT4 siRNA oligonucleotides ((IDS-null is a mutant oligonucleotide which does not target any transcript in the human genome, while YAP1 has been reported to not be required for the maintenance of OCT4 expression in human ES cells (Chia, et al., 2010) and OCT4 served as a positive control)). Transfected cells were cultured in mTeSR™1 medium which maintains the self-renewal of human ES cells in vitro and media was changed every 24 hours. Samples for analysis were collected at 24 hrs post-second siRNA treatment and the knockdown efficiency was assessed by qRT-PCR.

As shown in Figure 4.2, in H9 cells, the expression of the oxygenases TET1/2/3 was significantly reduced following the TET siRNA-mediated knockdown of individual or combined TETs 24 hrs after the second transfection treatment when compared to their expression in the IDS-null knockdown negative control samples, indicating that the treatment was effective. Similarly, expression of both controls YAP1 and OCT4 was significantly downregulated following their
knockdown. RH1 cells (Figure 4.3) also showed ablation in the transcript levels of TET1, TET2 and TET3, as well as the controls YAP1 and OCT4, after siRNA treatment, though the reduction of TET3 did not reach statistical significance.

**Figure 4.2.** siRNA-mediated knockdown of TET1, TET2, TET3 and TET1-3 in H9 human ES cells. H9 cells were transfected with siRNA (twice, 24 hrs apart) directed against the oxygenases TET1, TET2, TET3 and TET1-3 (triple knockdown), and controls IDS-null, YAP1 and OCT4. RNA was harvested 24 hrs post second-siRNA transfection and transcript levels were assayed by qRT-PCR. The expression level of each gene was normalised to GAPDH and compared to the negative control IDS-null, which is set as 1.0. Asterisks indicate levels of statistical significance (*p<0.05, **p<0.001, ***p<0.0001) as calculated by Student’s t-test.
Figure 4.3. siRNA-mediated knockdown of TET1, TET2, TET3 and TET1-3 in RH1 human ES cells. RH1 cells were transfected with siRNA (twice, 24 hrs apart) directed against the oxygenases TET1, TET2, TET3 and TET1-3 (triple knockdown), and controls IDS-null, YAP1 and OCT4. RNA was harvested 24 hrs post second-siRNA transfection and transcript levels were assayed by qRT-PCR. The expression level of each gene was normalised to GAPDH and compared to the negative control IDS-null, which is set as 1.0. Asterisks indicate levels of statistical significance (**p<0.01, ***p<0.001, ****p<0.0001) as calculated by Student's t-test.

For our study, both the p values and the effect size were calculated for all knockdowns. The latter, was performed to further establish the magnitude of the significance of the difference between compared groups. The effect size, as proposed by Cohen (Cohen, 1988) has been described in Section 2.9., where it
is described (Table 2.2) that an effect value of $d = 0.8$ or higher represents a significant difference between two groups, and a value of $d > 1.3$ indicates a very large effect when compared to that of the negative control IDS-null.

When the above was applied to the gene expression data in TET and control knockdown samples, the obtained effect sizes, ranging from large to very large, indicated that the degree of difference between each knockdown and the negative control was significant for both H9 and RH1 cells (Tables 4.1 and 4.2). Of note, TET3 knockdown in RH1 cells resulted in gene expression loss that despite not reaching statistical significance did have an effect size classified as very large. Taken together, the statistical significance and the effect size complement each other regarding the magnitude of each knockdown result and support previous conclusions that all TET genes were indeed effectively knocked down in both cell lines.
<table>
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<th>Effect Size Measure</th>
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<td>OCT4</td>
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Table 4.1. Effect size of differences in gene expression following YAP1, OCT4, TET1, TET2, TET3 and TET1-3 in H9 human ES cells. Cohen’s $d$ effect size was calculated based on the formula described in Section 2.9 for each of the knockdowns and the measurement of effect of each knockdown compared to the IDS-null knockdown (negative control). Based on Cohen’s criteria (Table 2.2), a value of $d > 1.3$ equals very large effect size. All knockdown treatments had a very large effect with $d$ values of 3.90 or greater.
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Table 4.2. Effect size of differences in gene expression following YAP1, OCT4, TET1, TET2, TET3 and TET1-3 in RH1 human ES cells. Cohen’s $d$ effect size was calculated based on the formula described in Section 2.9 for each of the knockdowns and the measurement of effect of each knockdown compared to the IDS-null knockdown (negative control). Based on Cohen’s criteria (Table 2.2), values of $d > 0.8$ and $d > 1.3$ equal large and very large effect sizes, respectively. All knockdown treatments had a very large effect.

Taken together, the data indicate that the knockdown approach was efficient and that we generated H9 and RH1 cells lacking TET1, TET2 and TET3, providing us with cellular tool to assess possible roles of these markers in the maintenance of human ES pluripotency.
To determine possible functional consequences of TET1/2/3 knockdown, I assessed cell morphology following gene knockdown using microscopic evaluation. Knockdown of TET1/2/3 individually and combined, and OCT4 (as described previously (Matin, et al., 2004; Hay, et al., 2004; Babaie, et al., 2007)) resulted in morphological changes evident of differentiation in both H9 ad RH1 cells (Figure 4.4), while controls IDS-null and YAP1 H9 and RH1 knockdown cells did not show any morphological changes and maintained the characteristic human ES cell undifferentiated phenotype.
(A, H9 hESCs)
Figure 4.4. Knockdown of TET1, TET2, TET3 and TET1-3 affects ES cell phenotype. (A) H9 and (B) RH1 were transfected with siRNA (twice, 24 hrs apart) directed against TET1, TET2, TET3, TET1-3 (triple knockdown) and controls IDS-null,
YAP1 and OCT4. Knockdown of TET1, TET2, TET3, TET1-3 and OCT4 but not IDS-null and YAP1 induces cell morphological changes and differentiation in cultures of H9 (A) and RH1 (B) human ES cells post siRNA treatment. The morphology was observed and captured with an Axiovert 40 CFL microscope and a Canon PowerShot A650 IS camera. Scale bar represents 100 μm. Abbreviation: KD, knockdown; hESCs, human embryonic stem cells.

4.2.2 Knockdown of TET1/2/3 affects expression of core pluripotency genes in human ES cells

To gain an insight into the mechanism underlying the loss of the undifferentiated human ES cell phenotype (Figure 4.4) following the knockdown of TET1, TET2 and TET3, analysis of the TET knockdown effects on the expression of core pluripotency factors OCT4, NANOG and SOX2 was performed by qRT-PCR on H9 and RH1 cells 24 hours post-second siRNA transfection.

In H9 cells (Figure 4.5), TET1 knockdown resulted in a minor decrease of OCT4 and a significant increase in SOX2 (p<0.05) transcript levels, while in TET2, TET3 and TET1-3 knockdowns OCT4, NANOG and SOX2 were all significantly suppressed. In RH1 cells (Figure 4.6), TET1 knockdown produced similar changes as seen in H9 cells, in that OCT4 and SOX2 expression were
downregulated and upregulated, respectively, with both OCT4 and SOX2 reduction reaching statistical significance (p<0.001). Like for H9 cells, following TET2 knockdown in RH1 cells, OCT4 expression was significantly suppressed, as in H9 cells, whereas NANOG expression remained at baseline levels and SOX2 expression showed an increase (Figure 4.6). Further, TET3 knockdown in RH1 cells caused a decrease of OCT4 only while NANOG and SOX2 expression remained close to baseline. Similar to H9 cells the TET1-3 triple knockdown led to the reduction of all three pluripotency markers, though with only OCT4 and SOX2 reaching statistical significance (p<0.05 and p<0.01, respectively). The transcript levels of pluripotent genes in human ES cells treated with the control YAP1 knockdown remained at baseline levels with the exemption of a significant increase of SOX2 in H9 cells (Figure 4.5). As expected, knockdown of OCT4 resulted in a significant decrease of OCT4 in both H9 and RH1 cells (p<0.001) (Figures 4.5 and 4.6). Finally, knockdown of OCT4 suppressed NANOG expression in both cell lines, though only significantly so in RH1 cells (p<0.001), and suppressed SOX2 only in RH1 cells (p<0.001) (Figures 4.5 and 4.6).

As before, the effect size of the mRNA expression data was assessed using Cohen’s $d$ calculations. The effect sizes of changes in mRNA expression of pluripotency markers following knockdown of the three TET genes in H9 cells
showed evidence of a significant difference when compared to the negative control IDS-null as indicated by the high \( d \) values at 24 hours post-second siRNA treatment, confirming the observations of statistical significance (\( p \) values) as shown in Table 4.3. Exceptions to the former are the expression of NANOG and SOX2 in the OCT4 knockdown cells, OCT4 expression in the TET1 knockdown cells and NANOG expression in the YAP1 knockdown cells. For RH1 cells, the effect size measurements confirm the observations of the statistical significance of gene suppression observed in TET1, TET2 and OCT4 knockdowns, ranging from small to very large (Table 4.4). For TET3 and TET1-3 RH1 knockdowns, the effect sizes are very large demonstrating a large difference in the expression of all three pluripotency markers when compared to the negative control IDS-null. For YAP1 RH1 knockdown, however, the size effect of resultant effects on the pluripotency marker expression showed a large effect, which contradicts the lack of statistical significance (Table 4.4).
Figure 4.5. siRNA-mediated knockdown of TETs affects expression of pluripotency genes in H9 human ES cells. Quadruplicate cultures of H9 cells were transfected twice (24 hrs apart) with siRNA directed against TET1, TET2, TET3, TET1-3 and controls IDS-null, YAP1 and OCT4. Cells were harvested at 24 hrs post-second siRNA transfection for qRT-PCR analysis of the pluripotency genes OCT4, NANOG and SOX2. Observed changes are relative to GAPDH expression and normalised to the
negative control IDS-null which has a value of 1.0. Asterisks indicate levels of statistical
difference (*p<0.05, **p<0.01, ***p<0.001) as calculated by Student’s t-test.

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<th>KD</th>
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<th>Effect Size Measure</th>
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Table 4.3. Effect size of impact of YAP1, OCT4 and TET gene knockdown on the expression of pluripotency markers in H9 human ES cells. Quadruplicate cultures of H9 human ES cells were treated twice (24 hrs apart) with siRNA directed against the controls IDS-null, YAP1 and OCT4, and TET oxidases TET1, TET2, TET3 and TET1-3. Samples were analysed using qRT-PCR to measure the expression of OCT4, NANOG and SOX2 mRNA. To define the magnitude of the difference in expression between each knockdown and the negative control IDS-null knockdown Cohen’s d effect size was calculated based on the formula described in Section 2.9 indicating very large size effects, with the exception of OCT4 and NANOG expressions following YAP1 and TET1 knockdown, respectively.
Figure 4.6. siRNA-mediated knockdown of TETs affects expression of pluripotency genes in RH1 human ES cells. Quadruplicate cultures of RH1 cells were transfected twice (24 hrs apart) with siRNA directed against TET1, TET2, TET3, TET1-3 and controls IDS-null, YAP1 and OCT4. Cells were harvested at 24 hrs post-second siRNA transfection for qRT-PCR analysis of the pluripotency genes OCT4, NANOG and SOX2. Observed changes are relative to GAPDH expression and normalised to the negative control IDS-null which has a value of 1.0. Asterisks indicate...
levels of statistical difference (*p<0.05, **p<0.01, ***p<0.001) as calculated by Student’s t-test.

<table>
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<th>KD</th>
<th>Value of $d$</th>
<th>Effect Size Measure</th>
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Table 4.4. Effect size of impact of YAP1, OCT4 and TET gene knockdown on the expression of pluripotency markers in RH1 human ES cells. Quadruplicate cultures of RH1 human ES cells were treated twice (24 hrs apart) with siRNA directed against the controls IDS-null, YAP1 and OCT4, and TET oxidases TET1, TET2, TET3 and TET1-3. Samples were analysed using qRT-PCR to measure the expression of OCT4, NANO and SOX2 mRNA. To define the magnitude of the difference in expression between each knockdown and the negative control IDS-null knockdown Cohen’s $d$ effect size was calculated based on the formula described in Section 2.9 indicating large and very large size effects, with the exception of NANO expression following TET1 and TET2 knockdown.

To further investigate a potential effect of TET1/2/3 knockdown on human ES cell pluripotency, the expression of selected epigenetic markers related to human ES cell phenotype (GLIS2, HMGA1 and PFDN5; described in chapter 3)
was examined. GLIS2, HMGA1 and PFDN5 mRNA levels were measured at 48 hrs post siRNA-mediated knockdown of TET1, TET2 and TET3 in H9 and RH1 cells and compared to the negative control IDS-null. H9 cells treated with TET1, TET2, TET3 and TET1-3 siRNAs showed no apparent changes in the expression of GLIS2, HMGA1 and PFDN5 except in the TET1 knockdown which significantly increased HMGA1 expression (p<0.001) (Figure 4.7 A). Similarly, in RH1 cells knockdown of TET2 did not affect levels of GLIS2, HMGA1 and PFDN5 expression. In contrast to H9 cells, knockdown of TET1 in RH1 cells led to the considerable downregulation of HMGA1 and PFDN5 (p<0.001), while TET3 knockdown resulted in increased expression of GLIS2 and HMGA1 but only the latter was significant (p<0.01). Lastly, combined downregulation of TET1/2/3 significantly suppressed the expression of all three biomarkers contrary to H9 cells where their expression was unaffected by the triple knockdown (Figure 4.7 B).

Cohen’s d effect size calculations for these observations are presented in Table 4.5 and show that in H9 human ES cells, based on the d values, TET gene knockdown had a variable effect on GLIS2 and PFDN5 expression (ranging from small to large) and a significant effect regarding expression changes in HMGA1. For RH1 human ES cells, the effect size of TET knockdowns on GLIS2 expression was small for TET1 and TET2 but very large for TET3 and TET1-3. All TET knockdowns had a very large effect on the HMGA1 expression, whilst
effect sizes for changes in expression of PFDN5 ranged from small to very large (Table 4.5).
Figure 4.7. siRNA-mediated knockdown of TET genes affects expression of epigenetic biomarkers in H9 and RH1 human ES cells. (A) H9 and (B) RH1 human ES cells were treated twice (24 hrs apart) with siRNA directed against TET1, TET2, TET3 and TET1-3 (triple knockdown). Cells were harvested at 24 hrs post-second siRNA transfection and assessed for GLIS2, HMGA1 and PFDN5 expression using qRT-PCR. Changes are relative to GAPDH expression and data are presented as log$_{10}$ fold difference normalised against the negative control siRNA IDS-null which has a value of 1.0. Asterisks indicate levels of statistical difference (*p<0.05, **p<0.01, ***p<0.001) based on quadruplicate biological samples as calculated by Student’s t-test. Abbreviation: KD, knockdown; hESCs, human embryonic stem cells.
Table 4.5. Effect size of impact of TET gene knockdown on the expression of GLIS2, HMGA1 and PFDN5 in human ES cells. Quadruplicate cultures of H9 and RH1 human ES cells were treated twice (24 hrs apart) with siRNA directed against TET1, TET2, TET3 and TET1-3, and controls IDS-null, YAP1 and OCT4. Samples were analysed at 24 hrs using quantitative RT-PCR to measure the expression of GLIS2, HMAG1 and PFDN5 mRNA. To measure the magnitude of difference between each knockdown and the negative control IDS-null knockdown Cohen’s $d$ effect size was calculated based on the formula described in Section 2.9 indicating that the effect size varies from small to very large. For H9 cells, based on the $d$ values, the effect of TET knockdowns had a varied effect on GLIS2 and PFDN5 expression and mainly significant regarding HMGA1 expression. The size effect of TET knockdowns on GLIS2 expression in RH1 cells was small for TET1 and TET2 but significant for TET3 and TET1-3. All TET knockdowns had a very large effect on the HMGA1 expression while there was a mixed response of effect on the expression of PFDN5.
Using qRT-PCR to assess pluripotency marker expression following knockdown of TET genes we found that OCT4 and NANOG were downregulated (Figures 4.5 and 4.6). To determine whether knockdown also affected OCT4 and NANOG expression at protein level, I next performed immunocytochemistry on H9 and RH1 cells following TET1/2/3 knockdown. While OCT4 and NANOG were expressed in H9 and RH1 cells treated with controls IDS-null and YAP1 siRNA, their expression was decreased following TET1, TET2, TET3 and TET1-3 knockdown in both cell lines. As expected, knockdown of OCT4 in both H9 and RH1 cells resulted in reduced expression of OCT4 and NANOG (Figures 4.8 and 4.9).
Figure 4.8. Knockdown of TET1/2/3 proteins suppresses expression of OCT4 and NANOG in H9 human ES cells. H9 human ES cells were treated twice (24 hrs apart) with siRNAs directed against IDS-null, YAP1, OCT4, TET1, TET2, TET3 and TET1-3. Samples were fixed with 4% PFA/PBS 24 hrs after the second siRNA transfection and stained for OCT4 (green) and NANOG (red). Representative images illustrate a
reduction of both proteins following TET1, TET2, TET3, TET1-3 and OCT4. Images were captured using Zeiss Observer fluorescence microscope and prepared using the Zeiss AxioVision 4.8.2 software. The scale bar represents 100 μm. Abbreviation: KD, knockdown.
Figure 4.9. Knockdown of TET1/2/3 proteins suppresses expression of OCT4 and NANOG in RH1 human ES cells. RH1 human ES cells were treated twice (24 hrs apart) with siRNAs directed against IDS-null, YAP1, OCT4, TET1, TET2, TET3 and TET1-3. Samples were fixed with 4% PFA/PBS 24 hrs after the second siRNA transfection and stained for OCT4 (green) and NANOG (red). Representative images illustrate a reduction of both proteins following TET1, TET2, TET3, TET1-3 and OCT4. Images were captured using Zeiss Observer fluorescence microscope and prepared using the Zeiss AxioVision 4.8.2 software. The scale bar represents 100 μm. Abbreviation: KD, knockdown.

4.2.3 Knockdown of TET1/2/3 induces lineage differentiation in human ES cells

As shown in sections 4.2.1 and 4.2.2, the knockdown of TET1/2/3 resulted in the loss of the undifferentiated human ES cell phenotype and induced cell differentiation. To establish whether the knockdown of TET1, TET2, TET3 and TET1-3 led to a directed lineage specification, qRT-PCR analysis for lineage-associated markers was performed. Quadruplicate biological samples for all knockdown conditions were assessed for a panel of markers that represent germinal lineage-associated ES cell differentiation. The panel of lineage markers examined is the same as in chapter 3 and included CDX2, CGα, PL1 (trophoblast), Brachyury, VEGF, BMP2, GATA2 (mesoderm), GATA4, AFP, Albumin, HNF4α, GATA6 (endoderm), PAX6, Nestin, Tubulin III, NF-200
(ectoderm) and HAND1 (early lineage). The transcript levels of the indicated lineage markers for all siRNA knockdowns were compared to those of the negative control of IDS-null.

As shown in Figure 4.10A, knockdown of TET1 in H9 cells caused no significant expression changes in the trophoblast markers. In addition, the TET1 knockdown produced a mixed response regarding mesodermal and endodermal markers as only specific markers (BMP2, HNF4a, GATA6) were significantly upregulated ($p<0.001$, $p<0.05$, $p<0.05$, respectively) while the remaining markers either showed noticeable downregulation (Brachyury, AFP) ($p<0.05$) or no change (VEGF, GATA2, GATA4, Albumin). Interestingly, all the ectodermal markers were significantly upregulated (PAX6, $p<0.001$; Nestin, $p<0.01$; Tubulin III, $p<0.01$; NF-200, $p<0.05$). The early lineage marker HAND1 showed significant downregulation ($p<0.05$).

Further, knockdown of TET2 (Figure 4.10 B) in H9 cells resulted in the significant reduction of CGα trophoblast marker ($p<0.05$). Also, a significant upregulation of specific mesoderm (BMP2, $p<0.01$), endoderm (HNF4a, GATA6, $p<0.05$) and ectoderm (PAX6, $p<0.001$) markers was observed. As in TET1 knockdown, HAND1 was considerably downregulated. Cells treated with TET3
siRNA (Figure 4.10 C), did not show any major expression changes regarding mesodermal and ectodermal markers except the upregulation of BMP2 (p<0.05). As TET2 knockdown, only PAX6 was significantly (p<0.01) upregulated of the ectodermal markers tested.

Cells where all three TET genes were downregulated by siRNA (Figure 4.10 D), showed significant induction of trophoblast (CDX2, CGα, p<0.05) and all ectodermal markers, while markers of mesoderm and endoderm demonstrated a mixed response. Only VEGF and BMP2 (mesoderm) and Albumin and GATA6 (endoderm) were significantly (VEGF, p<0.01; BMP2, p<0.01; Albumin, p<0.05; GATA6, p<0.01) induced. In OCT4 knockdown cells (Figure 4.10 E), a significant increase of CDX2, GATA6, PAX6, Nestin and HAND1 was observed, while CGα and Tubulin III were significantly downregulated.
Figure 4.10. Induction of embryonic lineage preference in H9 human ES cells following knockdown of TET1, TET2, TET3 and TET1-3. Quadruplicate cultures of H9 cells were treated twice (24 hrs apart) with siRNA directed against TET1, TET2, TET3, TET1-3, IDS-null, YAP1 and OCT4 mRNAs. Samples were collected at 24 hrs post-second siRNA treatment for gene expression analysis by qRT-PCR for trophoblast (CDX2, CGα, PL1), mesoderm (Brachyury, VEGF, BMP2, GATA2), endoderm (GATA4, AFP, Albumin, HNF4α, GATA6), ectoderm (PAX6, Nestin, Tubulin III, NF-200) and early lineage (HAND1) markers. The log₁₀ fold difference in expression of the indicated embryonic lineage marker genes is presented for (A) TET1, (B) TET2, (C) TET3, (D) TET1-3 and (E) OCT4 siRNA treatments. Each biological sample was represented by triplicate technical replicates. Observed changes are relative to GAPDH expression and subsequently normalised to the negative control IDS-null which has a value of 1.0. Asterisks indicate levels of statistical significance (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001) as calculated by Student’s t-test.
As it can be seen in Figure 4.11, knockdown of TET1 (Figure 4.11 A) in RH1 human ES cells resulted in significant increases in trophoblast markers (CDX2, PL1) (p<0.01 and p<0.05, respectively) along with endodermal (Albumin, HNF4α, GATA6) (p<0.05, p<0.01, p<0.05, respectively) and all ectodermal (PAX6, Nestin, Tubulin III, NF-200) (p<0.001, p<0.01, p<0.0001, p<0.05, respectively) markers. Similar to TET1 knockdown, TET2 knockdown (Figure 4.11 B) caused significant upregulation of all the ectodermal markers while the rest of the lineage markers showed transcript levels near to the baseline of the negative control IDS-null with the exception of CGα (downregulated) and BMP2 (upregulated). Further, RH1 cells following TET3 knockdown (Figure 4.11 C) showed a significant increase of ectodermal markers and endodermal AFP and GATA6 (p<0.01 and p<0.05, respectively) in conjunction with CDX2, BMP2 and HAND1 (p<0.05, p<0.01, p<0.05, respectively). In response to simultaneous knockdown of the three TETs (TET1-3) (Figure 4.11 D), the mesodermal (VEGF, BMP2, GATA2) (p<0.01, p<0.01, p<0.05, respectively) and ectodermal (PAX6, Tubulin III, NF-200) (p<0.0001, p<0.001, p<0.05, respectively) markers were mainly upregulated at significant levels together with CDX2, AFP, GATA6 and HAND1 (p<0.05, p<0.05, p<0.01, p<0.05, respectively). Lastly OCT4 knockdown (Figure 4.11 E) induced some differentiation but showed a mixed response as to the expression profile of the various markers in that CDX2, GATA2, GATA6, PAX6 and Nestin were significantly (p<0.01, p<0.01, p<0.01, p<0.001, p<0.05,
respectively) upregulated while Brachyury and Albumin were considerably downregulated.
Figure 4.11. Induction of embryonic lineage preference in RH1 human ES cells following knockdown of TET1, TET2, TET3 and TET1-3. Quadruplicate cultures of RH1 cells were treated twice (24 hrs apart) with siRNA directed against TET1, TET2, TET3, TET1-3, IDS-null, YAP1 and OCT4 mRNAs. Samples were collected at 24 hrs post-second siRNA treatment for gene expression analysis by qRT-PCR for trophoblast (CDX2, CGα, PL1), mesoderm (Brachyury, VEGF, BMP2, GATA2), endoderm (GATA4, AFP, Albumin, HNF4α, GATA6), ectoderm (PAX6, Nestin, Tubulin III, NF-200) and early lineage (HAND1) markers. The log10 fold difference in expression of the indicated embryonic lineage marker genes is presented for (A) TET1, (B) TET2, (C) TET3, (D) TET1-3 and (E) OCT4 siRNA treatments. Each biological sample was represented by triplicate technical replicates. Observed changes are relative to GAPDH expression and subsequently normalised to the negative control IDS-null which has a value of 1.0. Asterisks indicate levels of statistical significance (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001) as calculated by Student’s t-test.

4.2.4 Knockdown of TET1/2/3 suppresses the expression of 5-hydroxymethylcytosine in human ES cells

As shown earlier, siRNA-mediated knockdown of TET1, TET2, TET3 and TET1-3 resulted in loss of the undifferentiated human ES cell phenotype and induction of cell differentiation. Given the role of TET1/2/3 in converting 5-mC to 5-hmC, and to further explore the functional significance of the TET oxygenases on human ES cell pluripotent state, I next studied the effects of TET1, TET2, TET3 and TET1-3 knockdown on the epigenetic status of human ES cell by
immunocytochemical assessment of 5-mC and 5-hmC. While 5-hmC was expressed in both the IDS-null and YAP1 knockdown controls, OCT4 knockdown resulted in reduced expression (Figures 4.12 and 4.13). Likewise, H9 and RH1 cells treated with each of the TET1, TET2, TET3 and TET1-3 siRNAs exhibited significant attenuation of 5-hmC (Figures 4.12 and 4.13).
Figure 4.12. Knockdown of TET proteins perturbs 5-hydroxymethylcytosine expression in H9 human ES cells. H9 hESCs were transfected twice (24 hrs apart) with siRNA against IDS-null, YAP1, TET1, TET2, TET3, TET1-3 and OCT4. Samples were fixed with 4% PFA/PBS 24 hrs post-second siRNA transfection and stained for 5-methylcytosine (5-mC, green) and 5-hydroxymethylcytosine (5-hmC, red) and
representative images shown knockdown of TET1, TET2, TET3 and TET1-3 results in decreased levels of 5-hmC expression. DAPI represents nuclear staining. The immunostaining was recorded using a Zeiss Observer fluorescence microscope and images were captured at x20 magnification and prepared using the Zeiss AxioVision 4.8.2 software. The scale bar represents 100 μm.
Figure 4.13. Knockdown of TET proteins perturbs 5-hydroxymethylcytosine expression in RH1 human ES cells. RH1 hESCs were transfected twice (24 hrs apart) with siRNA against IDS-null, YAP1, TET1, TET2, TET3, TET1-3 and OCT4. Samples were fixed with 4% PFA/PBS 24 hrs post-second siRNA transfection and stained for 5-methylcytosine (5-mC, green) and 5-hydroxymethylcytosine (5-hmC, red) and
representative images shown knockdown of TET1, TET2, TET3 and TET1-3 results in decreased levels of 5-hmC expression. DAPI represents nuclear staining. The immunostaining was recorded using a Zeiss Observer fluorescence microscope and images were captured at x20 magnification and prepared using the Zeiss AxioVision 4.8.2 software. The scale bar represents 100 µm.

To confirm these qualitative immunocytochemical results, the effects of TET1/2/3 knockdown on 5-hmC expression were assessed in a quantitative manner using a Quest 5-hmC™ DNA ELISA kit (described in Section 2.7). The kit includes a control DNA set which has been calibrated to accurately measure the percentage of 5-hmC in DNA samples by using a standard curve. This control DNA set was included in every measurement in order to accurately determine the percentage of 5-hmC. DNA samples of TET1/2/3 knockdown cultures were compared to the IDS-null, YAP1 and OCT4 controls, as well as to DNA from untreated H9 and RH1 human ES cells.

DNA samples were collected 24 hours post the second-treatment of TET1/2/3, and IDS-null, YAP1 and OCT4 knockdown H9 and RH1 cells, along with the additional DNA samples isolated from untreated H9 and RH1 cells. Experimental samples were loaded in ELISA 96-well plates (one for H9 cells and one for RH1 cells) at equal DNA concentrations. Control DNA samples were loaded in duplicate, DNA samples from untreated cells and cells which were treated with
the IDS-null, YAP1 or OCT4 siRNA were loaded in triplicates and DNA from TET1, TET2, TET3 and TET1-3 siRNA treated human ES cells were loaded in triplicates (i.e. three biological replicates). In turn, each biological sample was represented by three technical replicates. ELISA buffer alone was loaded in order to subtract background absorbance (Figures 4.14 and 4.15).

Figure 4.14. DNA ELISA assay showing reduction of 5-Hydroxymethylcytosine following TET1, TET2, TET3 and TET1-3 knockdown in H9 human ES cells. H9 cells assessed for 5-hmC expression 24 hrs following transfection with siRNA against IDS-null, YAP1, OCT4, TET1, TET2, TET3 and TET1-3 mRNAs. Untreated H9 cells (known to express 5-hmC) were used as positive control, while ELISA buffer alone was loaded to allow for background absorbance subtraction. Colour intensity corresponds to DNA concentration, with darker colours indicating higher levels of 5-hmC. There is an
apparent difference in 5-hmC levels as inferred from the colour intensity of wells containing IDS-null and YAP1 controls versus the TET knockdown cells, and the positive control OCT4 knockdown cells. Abbreviation: KD, knockdown.

Figure 4.15. DNA ELISA assay showing reduction of 5-Hydroxymethylcytosine following TET1, TET2, TET3 and TET1-3 knockdown in RH1 human ES cells. RH1 cells assessed for 5-hmC expression 24 hrs following transfection with siRNA against IDS-null, YAP1, OCT4, TET1, TET2, TET3 and TET1-3 mRNAs. Untreated RH1 cells (known to express 5-hmC) were used as positive control, while ELISA buffer alone was loaded to allow for background absorbance subtraction. Colour intensity corresponds to DNA concentration, with darker colours indicating higher levels of 5-hmC. There is an apparent difference in 5-hmC levels as inferred from the colour intensity of wells
containing IDS-null and YAP1 controls versus the TET knockdown cells, and the positive control OCT4 knockdown cells. Abbreviation: KD, knockdown.

Expression levels of 5-hmC in H9 cells (Figure 4.14) and RH1 cells (Figure 4.15) treated with TET1, TET2, TET3 and TET1-3 siRNAs were found to be reduced compared to levels seen in untreated H9 and RH1 cells and in the control IDS-null and YAP1 knockdown H9 and RH1 cells. To allow more quantitative analysis, absorbance was measured at 405 nm using a fully automated multi-detection microplate reader, and results plotted, in Figures 4.16 and 4.17. These graphs show that for both H9 and RH1 cells, TET1, TET2, TET3 and TET1-3 knockdowns, like the positive control OCT4, have markedly and significantly reduced absorbance compared to untreated cells, and cells treated with IDS or YAP1 siRNA, verifying the observations by eye inferred from colour intensity (Figures 4.14 and 4.15).
Figure 4.16. Absorbance data of DNA ELISA assay following TET1, TET2, TET3 and TET1-3 knockdown in H9 human ES cells. H9 cells were treated twice (24 hrs apart) with siRNA against TET1, TET2, TET3 and TET1-3, and controls IDS-null, YAP1 and OCT4. Untreated H9 cells were used as a positive control. After the ELISA reaction, the absorbance was measured using a POLARstar OPTIMA microplate reader. The OD in wells containing TET1, TET2, TET3, TET1-3 and OCT4 knockdown samples show significantly lower absorbance than wells containing controls IDS-null and YAP1. Data are shown as mean values ± S.D. of triplicate biological samples, each measured in triplicate. Asterisks indicate levels of statistical significance (**p<0.01, ***p<0.001) as calculated by ANOVA with Dunnett’s post-hoc test.
Figure 4.17. Absorbance data of DNA ELISA assay following TET1, TET2, TET3 and TET1-3 knockdown in RH1 human ES cells. RH1 cells were treated twice (24 hrs apart) with siRNA against TET1, TET2, TET3 and TET1-3, and controls IDS-null, YAP1 and OCT4. Untreated RH1 cells were used as a positive control. After the ELISA reaction, the absorbance was measured using a POLARstar OPTIMA microplate reader. The OD in wells containing TET1, TET2, TET3, TET1-3 and OCT4 knockdown samples show significantly lower absorbance than wells containing controls IDS-null and YAP1. Data are shown as mean values ± S.D. of triplicate biological samples, each measured in triplicate. Asterisks indicate levels of statistical significance (**p<0.001) as calculated by ANOVA with Dunnett’s post-hoc test.

Applying a linear regression equation (Appendices IX and X) to the provided DNA to generate a dose-response curve allowed calculation of the percentage of 5-hmC expressed by the samples presented in Figures 4.16 and 4.17, enabling further quantification (Figures 4.18 and 4.19). Confirming data
presented in Figures 4.12 and 4.13, the IDS-null and YAP1 controls maintain levels of 5-hmC similar to the untreated H9 and RH1 cell samples, indicating that the knockdown of these genes does not influence the expression of 5-hmC in either cell line, as expected (Figures 4.18 and 4.19). In contrast, the level of 5-hmC for the positive control OCT4 knockdown was significantly decreased for both H9 (p<0.01) and RH1 (p<0.001) cells, reinforcing that 5-hmC is an epigenetic marker of pluripotency. Similarly, compared to the IDS-null negative control, siRNA knockdown of TET1, TET2, TET3 and TET1-3 resulted in significant reduction of 5-hmC levels in H9 cells (TET1, p<0.001; TET2, p<0.01; TET3, p<0.001; TET1-3, p<0.001) and RH1 cells (p<0.001 for all knockdowns) (Figures 4.18 and 4.19).
Figure 4.18. TET1, TET2, TET3, TET1-3 and OCT4 knockdown reduces expression of 5-hydroxymethylcytosine in H9 human ES cells. H9 cells were transfected twice (24 hrs apart) with siRNA against TET1, TET2, TET3, TET1-3, and controls IDS-null, YAP1 and OCT4 (triplicate biological samples, plated in triplicate). Untreated H9 cells served as a positive control. Absorbance was measured at 405 nm using a multi-detection microplate reader. Applying a linear regression equation (Appendix IX) on a dose-response curve obtained from supplied DNA samples allowed percentage calculations on expression. TET1, TET2, TET3, TET1-3 and OCT4 knockdowns resulted in a significant reduction of 5-hmC expression. Data are shown as mean values ± S.D. and the asterisks indicate levels of statistical difference (**p<0.01, ***p<0.001) as calculated by ANOVA with Dunnett’s post-hoc test.

Figure 4.19. TET1, TET2, TET3, TET1-3 and OCT4 knockdown reduces expression of 5-hydroxymethylcytosine in RH1 human ES cells. RH1 cells were transfected twice (24 hrs apart) with siRNA against TET1, TET2, TET3, TET1-3, and controls IDS-null, YAP1 and OCT4 (triplicate biological samples, plated in triplicate). Untreated RH1 cells served as a positive control. Absorbance was measured at 405 nm using a multi-
detection microplate reader. Applying a linear regression equation (Appendix X) on a dose-response curve obtained from supplied DNA samples allowed percentage calculations on expression. TET1, TET2, TET3, TET1-3 and OCT4 knockdowns resulted in a significant reduction of 5-hmC expression. Data are shown as mean values ± S.D. and the asterisks indicate levels of statistical difference (***p<0.001) as calculated by ANOVA with Dunnett’s post-hoc test.

Given that quantification of 5-hmC levels by ELISA demonstrated significant reduction of its expression in H9 and RH1 cells following TET1/2/3 and TET1-3 knockdown, I then examined whether the expression of TET genes themselves was altered following individual and triple TET knockdowns in H9 and RH1 cells, by measuring TET mRNA expression by qRT-PCR.

Figures 4.20 and 4.21 illustrate the effects of TET1/2/3, TET1-3 and OCT4 knockdown on TET expression in H9 and RH1 cells. In H9 cells (Figure 4.20), knockdown of YAP1 did not affect TET mRNA expression as it was equal to levels of the negative control IDS-null (which were set at 1.0) while OCT4 and TET1 knockdowns resulted in the significant (p<0.01, p<0.05) reduction of TET1 only. Following knockdown on TET2, TET3 and TET1-3, however, there was a significant (p<0.001) decrease in all three TET genes in H9 cells. Knockdown of TETs and OCT4 in RH1 human ES cells (Figure 4.21), elicited different responses compared to H9 cells in that RH1 cells showed significant reduction of mRNA levels of all three TET transcript levels following OCT4 knockdown in
contrast to H9 cells where only TET1 mRNA was suppressed. Following TET1 knockdown, TET2 mRNA was significantly downregulated (p<0.05), while TET2 knockdown did not induce noticeable changes in TET1 and TET3 mRNA expression in RH1 cells (Figure 4.21). Lastly, TET3 and TET1-3 knockdown had similar effects on TET expression in RH1 cells as H9 cells, as both knockdowns led to the significant decrease of TET1 mRNA.

To further quantify the strength of the effect each knockdown (TET1, TET2, TET3 and TET1-3) and controls (YAP1 and OCT4), have on the mRNA expression of TET1, TET2 and TET3 in H9 human ES cells, Cohen’s effect sizes were calculated (as previously described in Section 2.9) for the data presented in Figure 4.20 and presented in Table 4.6. For H9 cells, the d values showed a significant difference between each knockdown and the negative control IDS-null except in the case of YAP1 knockdown for the expression of all three TET oxidases and the expression of TET2 following OCT4 and TET1 knockdowns. Overall, the effect size measurement results complement the statistically significant p values for differences in mRNA expression between genes of interest and controls, with the only exception of TET3 expression following OCT4 and TET1 knockdowns where the effect size reveals a large effect.
For RH1 cells (Figure 4.7), the effect size measurements revealed a significant difference between each knockdown and the negative control IDS-null with the exception of TET3 mRNA expression following TET1 and TET2 knockdowns. Observations via effect size analysis have revealed strong differences between the negative control IDS-null and TET2 knockdown in terms of TET1 mRNA expression, and TET2 and TET3 mRNA expression following TET3 and TET1-3 knockdowns. These differences were not identified using statistical significance analysis; hence revealing the added value of measuring the Cohen’s effect size for such data.
Figure 4.20. Knockdown of TET1, TET2 and TET3 genes perturbs TET mRNA expression in H9 human ES cells. H9 cells were treated twice (24 hrs apart) with siRNA directed against IDS-null, YAP1, OCT4, TET1, TET2, TET3 and TET1-3. Results are presented as log_{10} fold difference in mRNA expression of the three TET genes. Observed changes are relative to GAPDH expression and normalised to the negative
control IDS-null which has a value of 1.0. Asterisks indicate levels of statistical difference (*p<0.05, **p<0.01, ***p<0.001) as calculated by Student’s t-test.

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Table 4.6. Effect size of differences in TET mRNA expression following TET1, TET2, TET3 and TET1-3 knockdown in H9 human ES cells. Quadruplicate cultures of H9 cells were treated twice (24 hrs apart) with siRNA directed against IDS-null, YAP1 and OCT4 (controls), and TET1, TET2, TET3 and TET1-3. Samples were analysed at 24 hrs post-second siRNA transfection using qRT-PCR to measure the expression of TET1, TET2 and TET3. To define the magnitude of difference between each knockdown and the negative control IDS-null knockdown on the expression of TETs, Cohen’s $d$ effect size was calculated based on the formula described in Section 2.9. Based on Cohen’s criteria (Table 2.2), a pronounced difference was observed between each TET1/2/3 and OCT4 knockdown and the negative control IDS-null with the exemption of YAP1 knockdown for the mRNA expression of all three TETs and the expression of TET2 mRNA following OCT4 and TET1 knockdowns.
Figure 4.21. Knockdown of TET1, TET2 and TET3 genes perturbs TET mRNA expression in RH1 human ES cells. RH1 cells were treated twice (24 hrs apart) with siRNA directed against IDS-null, YAP1, OCT4, TET1, TET2, TET3 and TET1-3. Results are presented as log$_{10}$ fold difference in mRNA expression of the three TET genes. Observed changes are relative to GAPDH expression and normalised to the negative control IDS-null which has a value of 1.0. Asterisks indicate levels of statistical difference (*p<0.05, **p<0.01, ***p<0.001) as calculated by Student’s t-test.
Table 4.7. Effect size of differences in TET mRNA expression following TET1, TET2, TET3 and TET1-3 knockdown in RH1 human ES cells. Quadruplicate cultures of RH1 cells were treated twice (24 hrs apart) with siRNA directed against IDS-null, YAP1 and OCT4 (controls), and TET1, TET2, TET3 and TET1-3. Samples were analysed at 24 hrs post-second siRNA transfection using qRT-PCR to measure the expression of TET1, TET2 and TET3. To define the magnitude of difference between each knockdown and the negative control IDS-null knockdown on the expression of TETs, Cohen’s $d$ effect size was calculated based on the formula described in Section 2.9. Based on Cohen’s criteria, the effect size revealed a significant difference between each TET1/2/3, YAP1 and OCT4 knockdown and the negative control IDS-null, except from the expression of TET3 mRNA following TET1 and TET2 knockdowns, where the difference is small and medium, respectively.

In summary, both H9 and RH1 cell lines lost their undifferentiated human ES cell morphology and pluripotency upon knockdown of TET1, TET2, TET3 and TET1-
3. Further, there was an overlap of induced lineage-associated markers and there was a significant reduction of 5-hmC expression in both cell lines. Regarding the expression of the epigenetic biomarkers and TETs there were differences among H9 and RH1 cells.

4.3 Discussion

Methylation of cytosines is a major epigenetic modification in mammalian genomes, often associated with transcriptional repression (Pfeifer, et al., 2013; Zhao and Chen, 2013). 5-methylcytosine was long thought to be the only modified base in mammalian cells (Pfeifer, et al., 2013) until 2009 when 5-hydroxymethylcytosine was re-discovered in DNA derived from mouse Purkinje neurons (Kriaucionis and Heintz, 2009) and TET1 was identified as the enzyme able to catalyse the conversion of 5-mC to 5-hmC (Tahiliani, et al., 2009). 5-hmC is an oxidative product of 5-mC and various studies have provided evidence that 5-hmC and TET differ considerably between tissues and cellular types (Ito, et al., 2010; Song, et al., 2011; Iqbal, et al., 2011; Wossidlo, et al., 2011; Nestor, et al., 2012; Koh and Rao, 2013; Pfeifer, et al., 2013; Zhao and Chen, 2013). Further, TET1 and TET2 are expressed in the ICM and mouse ES cells (Wu and Zhang, 2011). TET1 and TET2 have demonstrated to be
decreased while TET3 is induced upon differentiation of mouse ES cells (Tahiliani, et al., 2009; Ito, et al., 2010; Koh, et al., 2011).

In this chapter, I describe the role of dioxygenases TET1/2/3 in human embryonic stem cell pluripotency. siRNA-mediated knockdown of TET1, TET2, TET3 alone or all three TETs combined (TET1-3) in two human ES cell lines, led to cellular morphological changes, induction of differentiation, attenuation of key pluripotency-associated genes and reduced 5-hmC expression levels. To elucidate the role of TET1/2/3 in embryonic stem cells, several other groups have performed knockdown or knockout of these genes in mouse ES cells but conflicting results have been reported as to whether knockdown/knockout of the TET genes alters pluripotency and differentiation of ES cells (Wu and Zhang, 2011; Kinney and Pradhan, 2013). The work of Ito, et al. (2010) and Freudengerg, et al. (2011) revealed that knockdown of TET1 in mouse ES cells led to changes in morphology, reduced expression of pluripotency genes and induced cell differentiation (Ito, et al., 2010; Freudengerg, et al., 2011). Our findings using two human ES cell lines confirm these observations and furthermore identified similar trends with regards to TET knockdown-induced lineage-associated markers. Both the Ito and Freudengerg labs reported upregulation of CDX2 (trophoblast marker), GATA4 and GATA6 (endoderm), Nestin (ectoderm) and HAND1 (expressed by multiple early lineages) upon TET1 knockdown, while we show that in human ES cells following TET1
knockdown, CDX2 was upregulated in both H9 and RH1 cells, though only significantly in RH1 cells. GATA4 expression was detected at baseline levels while GATA6 was significantly induced in both H9 and RH1 cells. Nestin was significantly induced in both cells lines and HAND1 was modestly upregulated in RH1 cells only. In contrast, work of others (Dawlaty, et al., 2011; Koh, et al., 2011; Williams, et al., 2011) did not demonstrate any morphological changes and indicate maintained steady state levels of pluripotency markers following depletion of TET1. Koh, et al., (2011) have reported significant upregulation of CDX2 upon TET1 knockdown and PAX6 upon TET2 knockdown, observations which we confirm in human ES cells. In addition to the selective induction of certain lineage-associated markers, knockdown of TET genes in H9 and RH1 human ES cells has revealed the significant upregulation of BMP2 (mesoderm) and PAX6 (ectoderm) in both cell lines under all TET knockdown conditions (i.e. TET1, TET2, TET3, TET1-3 KD) indicating that TET knockdown favours embryonic cell specification more towards the mesoderm and ectoderm lineages.

Reported studies on mouse ES cells have furthermore shown that upon TET knockdown, 5-hmC is reduced, an observation that I confirm in this chapter. Specifically, Koh, et al. (2011) have shown that individual knockdown of TET1 or TET2 led to a moderate decrease of 5-hmC expression while combined depletion of both TETs resulted in 75%-80% reduction in 5-hmC (Koh, et al.,
The study of Ficz, et al., (2011) has revealed that declining levels of TETs during differentiation correlated with reduced 5-hmC. In my study, both H9 and RH1 cell lines showed significant decline of 5-hmC expression following knockdown of all TETs (individual and combined knockdown).

To date, this is the first study that investigates the role of TET1/2/3 in human ES cells. Using two different human ES cell lines I have confirmed reports describing a loss of human ES cell undifferentiated morphology, downregulation of key pluripotency-associated genes, induction of differentiation and reduced 5-hmC levels.
Investigation of the Functional Significance of Histone Demethylase JMJD2C in Undifferentiated Human Embryonic Stem Cells
5.1 Introduction

A layer of epigenetic regulation in mammalian cells and especially in ES cells is histone modifications associated with chromatin silencing and gene repression (Lunyak and Rosenfeld, 2008; Gaspar-Maia, et al., 2011). Until recently, histone methylation was considered a stable modification but this dogma changed when several histone demethylase families were identified (Agger, et al., 2008; Cloos, et al., 2008). Demethylases are known to play a role in hormone signaling, organogenesis, neuronal development, cancer, and ES cell pluripotency (Cloos, et al., 2006; Nottke, et al., 2009; Luo, et al., 2012).

An important family of histone demethylases is the Jumonji (JMJC)-domain containing proteins which have been studied in different species. They play essential part in multiple developmental processes. There are 27 different JMJC domain proteins in the human genome, essential for oxidative demethylation reactions (Agger, et al., 2008) and can function as both transcriptional corepressors and coactivators (Cloos, et al., 2008). The Jumonji-domain-containing proteins belong to the dioxygenase superfamily and its demethylation mechanism requires multiple co-factors such as oxygen, Fe(II) and α-ketoglutarate (Nottke, et al., 2009; Cascella and Mirica, 2012).
Interestingly, although JMJC domain proteins are members of the human 2-oxoglutarate- and oxygen-dependent oxygenases, it has been reported that they are upregulated under hypoxia conditions and regulated by the hypoxia-inducible factor (HIF) system (Pollard, et al., 2008). siRNA-mediated knockdown of HIF-1α in hypoxic cells resulted in significant downregulation of JMJD2C expression along with that of JMJD1A and JMJD2B. Additionally, cells treated with desferrioxamine (DFO) and dimethylloxalylglycine (DMOG), chemical agents that upregulate HIF (Groenman, et al., 2007) under normoxic culture conditions, displayed elevated levels of JMJD2C expression alongside that of other JMJD-domain demethylases (Pollard, et al., 2008).

Both human and mouse JMJD2C have been shown to be preferentially expressed in undifferentiated ES cells, have been implicated in the epigenetic reprogramming during early embryogenesis and are suggested to be an evolutionary target of NANOG (Katoh and Katoh, 2007). Further, JMJD2C has been demonstrated to be stage-specifically expressed in preimplantation mouse embryos from the two- to eight-cell stage and essential for early embryo development (Wang, et al., 2010). Also, in early mouse embryos, depletion of JMJD2C led to a significant decrease in the pluripotency genes OCT4, NANOG, SOX2 along with downregulation of the proliferation-related genes KLF4 and Myc (Wang, et al., 2010). Recent work has provided evidence that JHDM1B promotes OCT4 reprogramming via its histone demethylase activity and that
vitamin C enhances iPSCs generation by facilitating the function of JHDM1A/1B histone demethylases (Wang, et al., 2011).

Studies of different demethylases propose that they facilitate modulation of the progression of pluripotent cells into differentiated cell populations during development (Nottke, et al., 2009). The work of Loh et al. demonstrated that histone demethylases JMJD1a and JMJD2C regulate self-renewal of mouse ES cells. Depletion of JMJD1a or JMJD2C by RNA interference resulted in the decrease of ES cell specific genes and induction of differentiation (Loh, et al., 2007). In the same study, JMJD2C was shown to regulate the expression of NANOG, which encodes a core transcription factor for ES cell self-renewal. Also, OCT4 has been shown to regulate JMJD1a and JMJD2C as depletion of it by RNAi led to the decreased expression of both demethylases (Loh, et al., 2007; Lunyak and Rosenfeld, 2008).

As described in chapters 3 and 4 of this thesis, in our laboratory colleagues have assessed different human ES cell lines for variable and conserved patterns of Cytosine-Guanine Island methylation. The derived CGI methylation patterns were compared to those from differentiated tissues (Illingworth, et al., 2008) and to human ES cell mRNA expression data (Pells, et al., unpublished). This effort has identified sets of CGIs with unique human ES cell-specific methylation
patterns and therefore enabled definition of genes that were methylated and expressed in human ES cells but not the somatic tissues. Among the epigenetically-defined candidate genes in this data set, JMJD2C was selected for further investigation due to the fact that belongs to the dioxygenase superfamily as TET1/2/3 described in the previous chapter and it has been shown to be associated with other pluripotency transcription factors such as OCT4 and NANOG in mouse ES cells. Our human ES cell mRNA data showed that JMJD2C is expressed in human ES cells examined by us. As JMJD2C has been demonstrated to be an important factor of ES cell self-renewal, regulated by OCT4 while in turn it regulates NANOG expression (Loh, et al., 2007), I here studied its functional role in human pluripotent embryonic stem cell state.

Small interfering RNA (siRNA)-mediated knockdown of JMJD2C was employed in H9 and RH1 human ES cell lines, utilising the same methodologies as in preceding chapters, in order to study its functional role in human pluripotent ES cell state.
5.2 Results

5.2.1 Knockdown of JMJD2C fails to maintain human ES cell pluripotency

To examine the functional role of the histone demethylase JMJD2C in the maintenance of an undifferentiated human ES cell phenotype, small interference RNA (siRNA)-mediated gene knockdown of JMJD2C was employed in H9 and RH1 human ES cell lines.

Figure 5.1. Schematic illustration of siRNA-mediated knockdown experiments. H9 and RH1 human ES cells were transfected twice (24 hours apart) with siRNA directed against the mRNA of histone demethylase JMJD2C and appropriate negative and positive controls. Cells were maintained under self-renewing conditions in mTeSR™1 medium. Samples were collected for qRT-PCR, immunostaining for pluripotent and
epigenetic markers, and ELISA analysis 24 hours post-second transfection. Abbreviation/Acronym: 12-w, 12-well; siRNA, small interfering RNA.

Quadruplicate cultures of human ES cells were transfected twice (24 hrs apart) with JMJD2C, IDS-null (negative control), YAP1 and OCT4 (positive control). Transfected cultures were maintained in mTeSR™1 medium and media were changed every day. Samples for analysis were collected 24 hrs post-second siRNA treatment and the knockdown efficiency was confirmed through qRT-PCR analysis.

Figure 5.2 displays the knockdown levels of JMJD2C in H9 and RH1 human ES cells. Following 48 hrs of siRNA treatment, JMJD2C expression was significantly reduced in both cell lines, with RH1 cells demonstrating higher levels of downregulation than H9. Knockdown of the pluripotency transcription factor OCT4, resulted in the considerable suppression of JMJD2C which corroborates previously published work showing that OCT4 regulates JMJD2C (Loh, et al., 2007). As expected, siRNA against the control YAP1 had no effect on JMJD2C transcript levels.
**Figure 5.2. siRNA-mediated knockdown of JMJD2C in human ES cells.** H9 and RH1 cells were transfected with two subsequent treatments of siRNA (24 hrs apart) directed against the mRNA of the histone demethylase JMJD2C or controls IDS-null, YAP1 and OCT4. RNA was isolated 24 hrs post second-siRNA transfection and mRNA levels for JMJD2C were assayed by qRT-PCR. Data shown are normalised against GAPDH and compared to the negative control IDS-null, which has a value of 1.0. Asterisks indicate levels of statistical significance (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001) as calculated by Student's t-test.

Furthermore, both the *p* values and the effect size were calculated for all three knockdowns. The latter, was performed to further establish the magnitude of the significance of the difference between compared groups. The effect size has been described in Section 2.9 (Table 2.2) and an effect value of $d = 0.8$ or higher represents a significant difference between two groups, while a value of $d$
> 1.3 indicates a very large effect when compared to that of the negative control.

Based on Cohen’s criteria, the knockdowns of JMJD2C and OCT4 had a very large effect size in both cell lines, while the control YAP1 knockdown showed a large effect in H9 cells and no effect in RH1 cells (Table 5.1).

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Table 5.1. Effect size of differences in JMJD2C expression following YAP1, OCT4 and JMJD2C knockdowns in H9 and RH1 human ES cells. Cohen’s $d$ effect size was calculated based on the formula described in Section 2.9 for each of the knockdowns. The measurement of effect of each knockdown compared to the negative control IDS-null knockdown in H9 and RH1 ES cells is presented. Based on Cohen’s criteria, a value of $d > 1.3$ suggests that the JMJD2C knockdown had a very large effect size in both cell lines. Similarly, the positive control OCT4 knockdown showed a very large effect size, while the control YAP1 knockdown showed a large effect in H9 cells and no effect in RH1 cells. Abbreviation: KD, knockdown.

Downregulation of JMJD2C resulted in differentiation as observed by the apparent morphological changes in H9 and RH1 human ES cells (Figure 5.3). In the case of the OCT4 knockdown, both cell lines showed differentiation when
compared to the controls IDS-null and YAP1 which failed to have any effect on morphology.

**Figure 5.3. Knockdown of JMJD2C affects ES cell phenotype.** H9 and RH1 human ES cells were transfected with two subsequent treatments of siRNA (24 hrs apart)
directed against JMJD2C or controls IDS-null, YAP1 and OCT4. Knockdown of JMJD2C and OCT4 but not IDS-null and YAP1 induces cell morphological changes and differentiation in cultures of H9 and RH1 cells 24 hrs post-second transfection. The morphology was observed and captured with an Axiovert 40 CFL microscope and a Canon PowerShot A650 IS camera. Scale bar represents 100 μm. Abbreviation: KD, knockdown.

5.2.2 Expression of JMJD2C is associated with human ES cell pluripotency

To gain an insight into the mechanism underlying the loss of the undifferentiated human ES cell phenotype following the knockdown of histone demethylase JMJD2C, analysis of the JMJD2C knockdown effect on the expression of core pluripotency transcription factors was performed. The expression of pluripotency markers OCT4, NANOG and SOX2 was assessed in both H9 and RH1 human ES cells by qRT-PCR at 24 hrs following the second siRNA transfection.

JMJD2C knockdown in human ES cells (Figure 5.4), resulted in a significant downregulation of the pluripotency markers OCT4, NANOG and SOX2 in both human ES cell lines, with the exception of SOX2 in H9 cells where expression remained at baseline. siRNA-mediated knockdown of OCT4 caused significant
suppression of all three pluripotency markers in H9, and OCT4 and NANOG in RH1 cells. The mRNA levels of pluripotent genes in human ES cells treated with the control YAP1 siRNA were maintained at control IDS-null mRNA levels with the exception of a small but significant increase of SOX2 (p<0.05) in H9 cells and a small but significant decrease of OCT4 (p<0.01) in RH1 cells.
Figure 5.4. siRNA-mediated knockdown of JMJD2C affects expression of pluripotency genes in H9 and RH1 cells. Quadruplicate cultures of H9 and RH1 human ES cells were transfected twice (24 hrs apart) with siRNA directed against JMJD2C or controls IDS-null, YAP1 and OCT4. Cells were harvested at 24 hrs post-second siRNA transfection for qRT-PCR analysis of the core pluripotency genes OCT4, NANOG and SOX2. Observed expression changes are relative to GAPDH and normalised to the negative control siRNA IDS-null which is set as 1.0. Asterisks indicate levels of statistical significance (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001) as calculated by Student’s t-test. Abbreviation: KD, knockdown.

Additionally, in order to assess the size of difference between JMJD2C and the negative control IDS-null knockdowns on the expression of the pluripotency markers OCT4, NANOG and SOX2, Cohen’s d effect size was calculated (using the formula described in Section 2.9) for H9 and RH1 human ES cells. Based on Cohen’s criteria, in H9 cells, there was a very large effect on the expression of SOX2 following the YAP1 knockdown and a significant effect on all three pluripotency markers in both OCT4 and JMJD2C knockdowns (Table 5.2). In RH1 cells, the outcome of effect size measurements ranged from small to very large in the case of YAP1 knockdown. The OCT4 knockdown showed a very large effect size on the expression of OCT4 and NANOG but not of SOX2. The knockdown of histone demethylase JMJD2C in RH1 cells demonstrated a very large effect size for all three pluripotency markers OCT4, NANOG and SOX2 (Table 5.2). In this part of the study, it is noticeable that the statistical significance and the effect size results mainly complement each other regarding
the level of the observed knockdown effects with the exception of SOX2 expression following JMJD2C and YAP1 knockdowns in H9 and RH1 cells, respectively.

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Table 5.2. Effect size of differences in gene expression of pluripotency genes following YAP1, OCT4 and JMJD2C knockdown in H9 and RH1 cells. Quadruplicate cultures of H9 and RH1 human ES cells were treated twice (24 hrs apart) with siRNA directed against IDS-null, YAP1, OCT4 or JMJD2C. Samples were analysed using qRT-PCR to measure the expression of the pluripotency markers OCT4, NANOΓ and SOX2. To define the size of difference between each knockdown and the negative control IDS-null knockdown on the expression of the pluripotency markers, Cohen’s $d$ effect size was calculated based on the formula described in Section 2.9 indicating that
the effect size varies from medium to very large in H9 cells, while in RH1 cells the knockdown treatments showed small to very large size effects. Abbreviation: KD, knockdown.

To further assess the effect of JMJD2C downregulation by siRNA on human ES cell pluripotency, the expression of epigenetically-defined biomarkers associated with a human ES cell phenotype, GLIS2, HMGA1 and PFDN5 were assessed (Figure 5.5). The transcript levels of all three markers were measured 48 hrs after siRNA treatment with JMJD2C and compared to the negative control IDS-null. We found that H9 and RH1 cells responded in a similar way as they both showed significant upregulation of GLIS2, HMGA1 and PFDN5 expression except in the case of H9 cells where HMGA1 expression did not change significantly.
Figure 5.5. siRNA-mediated knockdown of JMJD2C affects the expression of GLIS2, HMGA1 and PFDN5 in H9 and RH1 cells. H9 and RH1 human ES cells were transfected with siRNA directed against JMJD2C. Samples were collected 24 hrs post-second siRNA treatment and assessed for GLIS2, HMGA1 and PFDN5 expression by qRT-PCR. Changes are relative to GAPDH expression and data are presented as log_{10} fold difference normalised to the expression of the markers in the negative control siRNA IDS-null which are set at 1.0. Asterisks indicate levels of statistical significance (**p<0.01, ***p<0.001, ****p<0.001) based on quadruplicate biological samples as calculated by Student's t-test. Knockdown of JMJD2C resulted in significant upregulation of the three markers in both human ES cell lines with the exception of HMGA1 in H9 cells where upregulation did not reach statistical significance.

To further study the effect of JMJD2C knockdown compared to the negative control IDS-null on expression of selected genes GLIS2, HMGA1 and PFDN5, the magnitude of the effect was measured in H9 and RH1 human ES cells with
Cohen’s $d$ effect size approach as described in Section 2.9. In both H9 and RH1 human ES cell lines (Table 5.3), the effect of JMJD2C knockdown on all three biomarkers GLIS2, HMAG1 and PFDN5 was found to be very large.

<table>
<thead>
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<th>KD</th>
<th>Value of $d$</th>
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<tr>
<td>PFDN5</td>
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Table 5.3. Effect size of JMJD2C knockdown on the expression of GLIS2, HMGA1 and PFDN5 in H9 and RH1 human ES cells. Quadruplicate cultures of H9 and RH1 human ES cells were treated twice (24 hrs apart) with siRNA directed against the mRNAs of the control IDS-null and JMJD2C. Samples were analysed using qRT-PCR to measure the expression of GLIS2, HMGA1 and PFDN5. To define the size of difference between JMJD2C knockdown and the negative control IDS-null knockdown on the expression of the three genes, Cohen’s $d$ effect size was calculated based on the formula described in Section 2.9 indicating that the knockdown of JMJD2C had a significant effect on the expression of all three genes in both human ES cell lines. Abbreviation: KD, knockdown.

In order to establish if there were changes in pluripotent protein levels of human ES cells treated with siRNA directed against IDS-null, YAP1, JMJD2C and
OCT4, immunocytochemistry for the pluripotent markers OCT4 and NANOG was performed in H9 and RH1 human ES cells.

Figures 5.6 and 5.7 demonstrate that the expression of OCT4 and NANOG was readily detectable in both human ES cell lines treated with the controls IDS-null and YAP1 siRNAs. Cells transfected with the positive control OCT4 siRNA showed a modest reduction in the expression of OCT4 and NANOG in H9 and RH1 cells. In contrast, cells where JMJD2C was knocked-down OCT4 and NANOG expression was downregulated compared to those of the controls IDS-null and YAP1. Collectively, the immunocytochemical analysis of H9 and RH1 human ES cell cultures where JMJD2C or OCT4 was knocked-down showed downregulation of the expression of the pluripotency markers OCT4 and NANOG at a protein level.
Figure 5.6. Knockdown of JMJD2C reduces protein expression of OCT4 and NANOG in H9 cells. H9 human ES cells were treated twice (24 hrs apart) with siRNAs directed against the mRNA of IDS-null, YAP1, JMJD2C and OCT4. Samples were fixed with 4% PFA/PBS 24 hrs after the second siRNA transfection. Representative images of the immunohistochemical analysis for the pluripotency markers OCT4 (green) and NANOG (red) show reduction in protein expression levels of OCT4 and NANOG following the knockdown of JMJD2C while expression was not altered in the two controls IDS-null and YAP1. DAPI (blue) was used as a nuclear staining. Images were captured using a Zeiss Observer fluorescence microscope and prepared using the Zeiss AxioVision 4.8.2 software. The scale bar represents 100 μm. Abbreviation: KD, knockdown.
Figure 5.7. Knockdown of JMJD2C reduces protein expression of OCT4 and NANOG in RH1 cells. RH1 human ES cells were treated twice (24 hrs apart) with siRNAs directed against the mRNA of IDS-null, YAP1, JMJD2C and OCT4. Samples were fixed with 4% PFA/PBS 24 hrs after the second siRNA transfection. Representative images of the immunohistochemical analysis for the pluripotency markers OCT4 (green) and NANOG (red) show reduction in protein expression levels of OCT4 and NANOG following the knockdown of JMJD2C while expression was not altered in the two controls IDS-null and YAP1. DAPI (blue) was used as a nuclear staining. Images were captured using a Zeiss Observer fluorescence microscope and prepared using the Zeiss AxioVision 4.8.2 software. The scale bar represents 100 μm. Abbreviation: KD, knockdown.
5.2.3 JMJD2C knockdown induces differentiation of human ES cells

Knockdown of histone demethylase JMJD2C resulted in an apparent loss of the characteristic undifferentiated human ES cell phenotype and induced morphological changes in both H9 and RH1 cells, as shown in section 5.2.1. To ascertain whether the depletion of JMJD2C led to a biased lineage specification or not, qRT-PCR analysis for lineage-associated markers was carried out. Quadruplicate biological samples for JMJD2C and OCT4 knockdowns were assessed for a panel of markers that represent all germ lineages, which, like before comprised the trophoblast markers CDX2, CGα, PL1, mesodermal Brachyury, VEGF, BMP2, GATA2, endodermal GATA4, AFP, Albumin, HNF4α, GATA6, ectodermal PAX6, Nestin, Tubulin III, NF-200 and early lineage HAND1. The transcript levels of these markers for JMJD2C and OCT4 siRNA knockdowns were compared to those in the negative control IDS-null siRNA.

Downregulation of JMJD2C in H9 hESC resulted in a significant upregulation of the trophoblast markers CDX2, CGα, and all examined mesodermal and endodermal markers (Figure 5.8 A). Further, the JMJD2C knockdown produced a mixed response regarding ectodermal markers as PAX6 was significantly
decreased while Nestin was significantly increased, whereas there were no noticeable effects on the expression of Tubulin III and NF-200.

A different response was observed in RH1 human ES cells (Figure 5.8 C) treated with the JMJD2C siRNA. Trophoblast markers CDX2 and PL1 were significantly downregulated as was the expression of the mesoderm markers Brachyury and VEGF. GATA2 however was upregulated, similar to the H9 JMJD2C knockdown cells. The endodermal markers showed a mixed response in RH1 with significant downregulation of AFP and Albumin while GATA4 and GATA6 were considerably upregulated. Finally, the ectodermal markers were expressed in a more uniform manner with Nestin, Tubulin III and NF-200 all notably upregulated while PAX6 remained close to baseline levels.

As far as the OCT4 knockdown is concerned, there was a significant induction mainly of trophoblast and ectodermal markers in H9 cells (Figure 5.8 B), whereas in the case of RH1 cells (Figure 5.8 D) only a few lineage markers showed significant change in expression with CDX2, GATA6, PAX6 and NF-200 levels upregulated while AFP was significantly downregulated.
(A, H9 hESCs, JMJD2C KD)
(B, H9 hESCs, OCT4 KD)
(C, RH1 hESCs, JMJD2C KD)
Figure 5.8 Embryonic lineage marker expression in human H9 and RH1 ES cells following knockdown of the histone demethylase JMJD2C. Quadruplicate cultures of H9 and RH1 human ES cells were treated twice (24 hrs apart) with siRNA directed against the mRNAs of IDS-null, JMJD2C and OCT4. Samples were harvested at 24 hrs post-second siRNA treatment for gene expression analysis by qRT-PCR for trophectoderm (CDX2, CGα, PL1), mesoderm (Brachyury, VEGF, BMP2, GATA2), endoderm (GATA4, AFP, Albumin, HNF4α, GATA6), ectoderm (PAX6, Nestin, Tubulin III, NF-200) and early lineage (HAND1) markers. The fold difference in expression of the indicated embryonic lineage marker genes is presented for (A) JMJD2C and (B) OCT4 in H9, and (C) JMJD2C and (D) OCT4 in RH1 cells. Quadruplicate biological replicates were assayed in triplicate (technical replicates). Observed changes are relative to GAPDH expression and subsequently normalised to the negative control IDS-null which has a value of 1.0. Asterisks indicate levels of statistical significance (*p<0.05, **p<0.01,
***p<0.001, ****p<0.0001) as calculated by Student’s t-test. Abbreviation: KD, knockdown, hESCs, human embryonic stem cells.

5.2.4 Knockdown of histone demethylase JMJD2C perturbs the epigenome of human ES cells

siRNA-mediated knockdown of histone demethylase JMJD2C showed loss of the undifferentiated human ES cell phenotype and induction of differentiation as assessed by qRT-PCR of various germ lineage markers. To further study the functional significance of JMJD2C on the human ES cell state, the effects of JMJD2C depletion on the epigenetic status of human ES cells were next examined.

As mentioned earlier, work in mouse ES cells has shown that the core pluripotency marker OCT4 regulates JMJD2C expression, while JMJD2C regulates the expression of NANOG (Loh, et al., 2007). Given the role of JMJD2C in maintaining an undifferentiated phenotype in mESCs and the fact that JMJD2C belongs to the same superfamily of dioxygenases as the TET family members TET1/2/3 (responsible for the conversion of 5-methylcytosine to 5-hydroxymethylcytosine), knockdown of JMJD2C by siRNA was used to determine if loss of JMJD2C affects genomic 5-hmC in human ES cells.
Expression of 5-mC and 5-hmC was assessed in both H9 and RH1 cells following JMJD2C knockdown and OCT4 knockdown. The analysis was performed by immunocytochemistry. As expected, expression of both 5-mC and 5-hmC was not altered in H9 and RH1 human ES cells following the control knockdown IDS-null and YAP1. In contrast, there was a modest decrease in expression of genomic 5-hmC in the positive control OCT4 siRNA (Figures 5.9 and 5.10). Interestingly, in both cell lines there was a significant decrease of nuclear 5-hmC expression contrary to that of 5-mC which was considerably increased following the knockdown of JMJD2C.
Figure 5.9. Knockdown of JMJD2C affects the expression of the epigenetic markers 5-methylcytosine and 5-hydroxymethylcytosine in H9 cells. H9 human ES cells were transfected twice (24 hrs apart) with siRNA against IDS-null, YAP1, JMJD2C and OCT4. Samples were fixed with 4% PFA/PBS 24 hrs post-second siRNA transfection. Images of immunohistochemical analysis for the epigenetic markers 5-methylcytosine (5-mC, green) and 5-hydroxymethylcytosine (5-hmC, red) showed decreased levels of 5-hmC and increased levels of 5-mC following the JMJD2C knockdown. DAPI (blue) was used as a nuclear staining. Images were captured using a Zeiss Observer fluorescence microscope and prepared using the Zeiss AxioVision 4.8.2 software. The scale bar represents 100 μm. Abbreviation: KD, knockdown.
Figure 5.10. Knockdown of JMJD2C affects the expression of the epigenetic markers 5-methylcytosine and 5-hydroxymethylcytosine in RH1 cells. RH1 human ES cells were transfected twice (24 hrs apart) with siRNA against IDS-null, YAP1, JMJD2C and OCT4. Samples were fixed with 4% PFA/PBS 24 hrs post-second siRNA transfection. Images of immunohistochemical analysis for the epigenetic markers 5-methylcytosine (5-mC, green) and 5-hydroxymethylcytosine (5-hmC, red) showed decreased levels of 5-hmC and increased levels of 5-mC following the JMJD2C knockdown. DAPI (blue) was used as a nuclear staining. Images were captured using a Zeiss Observer fluorescence microscope and prepared using the Zeiss AxioVision 4.8.2 software. The scale bar represents 100 µm. Abbreviation: KD, knockdown.

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<thead>
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To further validate the significant loss of 5-hmC expression following knockdown of JMJD2C in human ES cells as assessed by immunocytochemistry, the effects
of the knockdown on 5-hmC was measured using a quantitative ELISA assay, as described in Chapter 2 of this thesis. Samples from all the various siRNA test groups were harvested 24 hrs post-second transfection and compared to the negative control IDS-null, using the Quest 5-hmC™ DNA ELISA kit (see Materials and Methods). The kit includes a control DNA set which has been calibrated to accurately measure the percentage of 5-hmC in DNA samples by using a standard curve. This control DNA set was included in every measurement in order to accurately determine the percentage of 5-hmC (see procedure description in Section 2.7). DNA samples from triplicate cultures of H9 cells transfected with IDS-null, YAP1, OCT4 or JMJD2C were assayed in triplicate and absorbance was plotted (Figure 5.11).
Figure 5.11. Absorbance following DNA ELISA show downregulation of 5-hydroxymethylcytosine in H9 ES cells following OCT4 and JMJD2C knockdowns. H9 human ES cells were assessed by DNA ELISA for 5-hmC expression following treatment with siRNA against IDS-null, YAP1, OCT4 and JMJD2C mRNAs (triplicate biological samples, plated in triplicate). DNA was extracted from samples 24 hrs post-second siRNA transfection and absorbance was measured at 405 nm using a multi-detection microplate reader. Data are shown as mean values ± S.D. and the asterisks indicate levels of statistical significance (***p<0.001) as calculated by ANOVA with Dunnett’s post-hoc test.

Using the linear regression equation (see Section 2.7), a standard curve was generated (Appendix XI). While the percentage of 5-hmC in the controls IDS-null and YAP1 maintain equal levels to those of the untreated H9 samples, the levels
of 5-hmC for OCT4 and JMJD2C knockdown were considerably reduced (p<0.001; Figure 5.12).

Figure 5.12 JMJD2C and OCT4 knockdown reduces expression of 5-hydroxymethylcytosine in H9 human ES cells. H9 human ES cells were assessed by DNA ELISA for 5-hmC expression following treatment with siRNA against IDS-null, YAP1, OCT4 and JMJD2C mRNAs (triplicate biological samples, plated in triplicate). Untreated H9 cells served as an additional positive control (first bar; H9 hESCs). DNA was extracted from samples 24 hrs post-second siRNA transfection and absorbance was measured at 405 nm using a multi-detection microplate reader. Applying a linear regression equation (Appendix XI) on a dose-response curve obtained from supplied DNA samples allowed percentage calculations on expression. Data are shown as mean values ± S.D. and the asterisks indicate levels of statistical significance (***,p<0.001) as calculated by ANOVA with Dunnett’s post-hoc test.
The same procedure was followed for the quantification of 5-hmC in RH1 human ES cells. The absorbance of all DNA samples was measured at 405 nm and the readings are presented in Figure 5.13.

Figure 5.13. Absorbance following DNA ELISA show downregulation of 5-hydroxymethylcytosine in RH1 ES cells following OCT4 and JMJD2C knockdowns. RH1 human ES cells were assessed by DNA ELISA for 5-hmC expression following treatment with siRNA against IDS-null, YAP1, OCT4 and JMJD2C mRNAs (triplicate biological samples, plated in triplicate). DNA was extracted from samples 24 hrs post-second siRNA transfection and absorbance was measured at 405 nm using a multi-detection microplate reader. Data are shown as mean values ± S.D. and the asterisks indicate levels of statistical significance (**p<0.001) as calculated by ANOVA with Dunnett’s post-hoc test.
As in the case of H9 cells, triplicate RH1 cultures transfected with the IDS-null, YAP1, OCT4 and JMJD2C siRNA were quantified for 5-hmC. As expected, the controls IDS-null and YAP1 in RH1 cells maintain equal levels of 5-hmC with the untreated RH1 cells (Figure 5.14). Compared to the negative control IDS-null, knockdown of the positive control OCT4 and JMJD2C knockdown resulted in a significant decrease \( (p<0.001) \) of 5-hmC.

![Graph showing 5-hmC expression in RH1 human ES cells](image)

**Figure 5.14.** JMJD2C and OCT4 knockdown reduces expression of 5-hydroxymethylcytosine in RH1 human ES cells. RH1 human ES cells were assessed by DNA ELISA for 5-hmC expression following treatment with siRNA against IDS-null, YAP1, OCT4 and JMJD2C mRNAs (triplicate biological samples, plated in triplicate). Untreated RH1 cells served as an additional positive control (first bar; RH1 hESCs). Absorbance was measured at 405 nm using a multi-detection microplate reader. Applying a linear regression equation (Appendix XII) on a dose-response curve obtained from supplied DNA samples allowed percentage calculations on expression.
Data are shown as mean values ± S.D. and the asterisks indicate levels of statistical significance (***p<0.001) as calculated by ANOVA with Dunnett’s post-hoc test.

Given that quantification of expression levels by ELISA illustrated considerable decrease of 5-hmC in human ES cell cultures transfected with JMJD2C siRNA, I then examined how the expression of TET genes was altered in respect to JMJD2C downregulation using qRT-PCR.

Knockdown of JMJD2C in H9 and RH1 human ES cells elicited similar responses as all TETs (TET1/2/3) were significantly downregulated with the exception of TET2 in H9 cells, which was expressed close to the baseline levels (Figure 5.15). In the case of OCT4 knockdown, TET expression altered between the two cell lines in that there were no noticeable changes in H9 cells whereas all three TETs were significantly downregulated in RH1 cells. In human ES cells treated with the control siRNA YAP1 there was mainly no change in TET expression compared to IDS-null except TET2 and TET3 in RH1 and H9 cells, respectively (Figure 5.15).
Figure 5.15. Knockdown of JMJD2C downregulates the expression of TET oxidases in human H9 and RH1 ES cells. H9 and RH1 human ES cells were treated twice (24 hrs apart) with siRNA directed against the mRNA of IDS-null, YAP1, OCT4 and JMJD2C. Results are presented as log₁₀ fold difference in expression of the three oxidases TET1, TET2 and TET3. Observed changes in expression are relative to GAPDH expression and normalised to the negative control IDS-null, set as 1.0. Asterisks indicate levels of statistical significance (*p<0.05, **p<0.01, ****p<0.0001) as calculated by ANOVA with Dunnett’s post-hoc test. Abbreviation: KD, knockdown.

To measure the size of these observed differences between each knockdown (YAP1, OCT4 and JMJD2C) and the negative control IDS-null knockdown on the expression of TET oxidases TET1, TET2 and TET3 in H9 and RH1 human ES cells, effect sizes calculations based on Cohen’s criteria (Table 2.2) were performed and data is presented in Table 5.4. In H9 and RH1 cells, the effect size measurements revealed different effect size for each TET oxidase following YAP1 knockdown. In the case of the OCT4 knockdown, there was no significant effect size observed in H9 cells. In contrast, in RH1 cells there was a significant effect on all three TET oxidases. Following the knockdown of JMJD2C, a significant difference was found when compared to the negative control IDS-null knockdown in both human ES cell lines as indicated by the high $d$ values.
<table>
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<th>Effect Size Measure</th>
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Table 5.4. Effect size of impact of YAP1, OCT4 and JMJD2C knockdowns on the expression of TET oxidases in H9 and RH1 human ES cells. Quadruplicate cultures of H9 and RH1 human ES cells were treated twice (24 hrs apart) with siRNA directed against IDS-null, YAP1 and OCT4 (controls), and histone demethylase JMJD2C. Samples were analysed at 24 hrs post-second siRNA transfection using qRT-PCR to measure the expression of oxidases TET1, TET2 and TET3. To define the size of difference between each knockdown and the negative control IDS-null knockdown on the expression of TET oxidases, Cohen's $d$ effect size was calculated based on the formula described in Section 2.9 indicating a mixed response regarding the YAP1 knockdown in both human ES cell lines ranging from a small to very large effect size. There was no significant size difference in H9 cells treated with siRNA directed against OCT4, contrary to the significant difference observed in RH1 cells. The knockdown of
JMJD2C resulted in a significant difference in H9 and RH1 cells. Abbreviation: KD, knockdown.

To summarise, the two human ES cell lines responded similarly to the knockdown of JMJD2C in terms of loss of undifferentiated human ES cell phenotype and pluripotency, upregulation of biomarkers identified in our lab, expression of OCT4, NANOG, 5-mC and 5-hmC levels, downregulation of the three TETs and loss of 5-hmC expression. With regards to the induction of differentiation, both cell lines showed significant upregulation of CGα (trophoblast), GATA2 (mesoderm), GATA4, GATA6 (endoderm) and Nestin (ectoderm) expression by qRT-PCR.

5.3 Discussion

Jumonji-domain-containing proteins in the human genome are necessary for oxidative demethylation reactions (Agger, et al., 2008) and can act as both transcriptional coactivators and corepressors (Cloos, et al., 2008). They belong to the superfamily of dioxygenases and can demethylate lysine through an oxidative mechanism that requires a-ketoglutarate and Fe(II) as cofactors. The demethylation is thought to occur by direct hydroxylation of the methyl group which results in the formation of an unstable hydroxymethyl product (Klose, et
al., 2006; Nottke, et al., 2009; Cascella and Mirica, 2012). Demethylases have been shown to play an important role in ESC pluripotency (Cloos, et al., 2006; Nottke, et al., 2009; Luo, et al., 2012) as significant decrease in the pluripotency genes OCT4, NANOG and SOX2 upon JMJD2C depletion has been observed in mESCs (Loh, et al., 2007) and early mouse embryos (Wang, et al., 2010).

In this chapter, using siRNA-mediated knockdown in two different human ES cell lines, convincing evidence is presented that underline the functional significance of the epigenetically-defined biomarker JMJD2C in the maintenance of a human ES cell undifferentiated state. Knockdown of JMJD2C resulted in the loss of human ES stem cell phenotype, downregulation of the key pluripotency markers OCT4, NANOG and SOX2, and the induction of lineage-associated developmental genes. Data derived from our siRNA-mediated JMJD2C knockdown in human ES cells underpinning findings by Loh and colleagues (2007) who depleted JMJD2C in mouse ES cells. Similar to the study of Loh et al. (2007), JMJD2C knockdown led to the upregulation of diverse lineage markers indicating that the resultant cell population was composed of multiple differentiated cell types. Among the induced genes in our study we have confirmed JMJD2C knockdown resulted in the induction of endodermal lineage markers GATA4 and GATA6 (in both human ES cell lines), and trophoblast marker CDX2 (only in H9 human ES cells) as in the mESC study. Both human ES cell lines tested in this study appeared to have similar outcomes regarding
loss of undifferentiated human ES cell phenotype and significant decrease of the core pluripotency markers. However, we observed differences in the induction of lineage markers in that H9 showed to be significantly differentiated towards cell types mainly representing trophoblast, mesoderm and endoderm, while RH1 cells were induced mostly towards ectoderm. Further, downregulation of the pluripotency markers OCT4, NANOG and SOX2 in our study is in line with findings by Loh et al. (2007) studying mouse ES cells. Interestingly, JMJD2C knockdown led to the significant upregulation of GLIS2 and PFDN5, in both cell lines and HMGA1 in H9 only. Additionally, knockdown of JMJD2C significantly affected 5-hydroxymethylcytosine and the genes (TET1-3) responsible for this epigenetic modification in both human ES cell lines.

To date, this is the first study demonstrating that JMJD2C in human ES cells is essential for the maintenance of a human ES cell undifferentiated phenotype and its knockdown results in significant attenuation of the epigenetic markers 5-hmC and TET1-3. Jumonji-domain-containing proteins and TETs mediate demethylation of histones and DNA via hydroxylation that is catalysed by a-ketoglutarate and Fe(II), respectively (Tsukada, 2012). There is evidence that both histone and DNA demethylation regulate pluripotency in ESCs (Loh, et al., 2007; Wang, et al., 2010; Ito, et al., 2010; Freudengerg, et al., 2011). Further additional evidence that histone demethylases regulate human ES cell pluripotency comes from the study of Adamo, et al. (2011). The authors showed
that the histone demethylase LSD1 (lysine-specific demethylase 1), which is highly expressed in human ES cells and progressively declines during differentiation, regulates human ES cell pluripotency (Adamo, et al., 2011). Specifically, knockdown of LSD1 results in loss of the undifferentiated human ES cell phenotype and pluripotency, as shown by the downregulation of OCT4, NANOG and SOX2 expression. Also, depletion of LSD1 in human ES cells led to the induction of differentiation, especially towards endodermal and mesodermal lineages (Adamo, et al., 2011). Besides the fact that LSD1 uses a different demethylation mechanisms than the Jumonji-domain containing demethylases (Nottke, et al., 2009), it is evident that histone demethylation plays an essential part in the regulation and maintainance of human ES cell pluripotency.
Chapter 6

Defining the Subcytotoxic Effects of Various Compounds and Oxygen Tensions on Epigenetic Determinants of Human Embryonic Stem Cell Identity

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6.1 Introduction

6.1.1. Human ES cells as a predictive developmental toxicity model

Human ES cells provide a great opportunity to serve as an *in vitro* predictive developmental toxicity model especially as animal models do not truly correlate to human response (West, *et al.*, 2010). Also, human ES cells provide an unlimited source of material as they are of embryonic origin with limited genetic diversity thereby reducing the use of animals (Liu, *et al.*, 2013). In addition, the pluripotency of human ES cells provides a valuable assay to assess toxic compounds that are known to disrupt development (West, *et al.*, 2010). For many years, researchers focused on the genotoxic effects of environmental chemicals and pharmaceutical products that humans are exposed to on a daily basis (Smirnova, *et al.*, 2012). Over the last decade, the interest in identifying the epigenetic mechanisms underlying the toxicological effects of xenobiotics has increased, especially since the study of Fraga, *et al.* (2005) which demonstrated epigenetic differences arising during the lifetime of monozygotic twins in terms of global DNA methylation and histone acetylation (Smirnova, *et al.*, 2012). Numerous studies have documented the effects of environmental chemicals on epigenetic mechanisms, including DNA methylation and histone modifications (Arita and Costa, 2009; Baccarelli and Bollati, 2009; Cheng, *et al.*, 2011).
In this study, four test compounds (5-azacytidine, cadmium chloride, sodium arsenite and valproic acid) were selected based on their effects on germinal lineage determination and epigenetic mechanisms affecting gene expression and cell phenotype.

5-azacytidine is a cytosine nucleotide chemical analogue which can reduce DNA methyltransferase activity in the cells and has shown to reverse differentiation of ES cells (Tsuji-Takayama, et al., 2004). The heavy metal cadmium is an established human carcinogen that has been shown to reduce genome methylation and inhibit DNA methyltransferase activity (Baccarelli and Bollati, 2009). Furthermore, it is toxic in human placentae and has been reported to inhibit trophoblast cell proliferation in Jar choriocarcinoma cells (similar to early human trophoblast cells) (Powlin, et al., 1997). Arsenic is a toxic environmental contaminant which causes epigenetic changes such as global DNA hypomethylation in mammalian cells at low dosages and histone modifications (Reichard, 2007; Smirnova, et al., 2012). Arsenic occurs in two forms, namely arsenite and arsenate (Arita and Costa, 2009). Lastly, valproic acid is a histone deacetylase inhibitor which has been reported to cause heart malformations and neural tube defects (Na, et al., 2003). Treatment of mouse ES cells with low level valproic acid results in increased pluripotency (Hezroni, et al., 2011).
6.1.2. Influence of low oxygen tension on human ES cells

Human embryonic stem cells prior to implantation and vascularisation in vivo are exposed to a hypoxic environment in which they remain pluripotent and resistant to spontaneous differentiation (Ezashi, et al., 2005; Cho, et al., 2006). Several studies have reported that a low oxygen environment plays an essential role in the maintenance of pluripotency and induced pluripotency (iPSCs) (Ezashi, et al., 2005; Yoshida, et al., 2009; Forristal, et al., 2010; Lim, et al., 2011; Szablowska-Gadomska, et al., 2011; Iida, et al., 2013; Mathieu, et al., 2013).

The HIF (Hypoxia-inducible factors) transcription factors respond to hypoxic conditions in cells and mediate oxygen-dependent expression of numerous genes involved in erythropoiesis, angiogenesis and glycolysis (Harvey, et al., 2002; Ma, 2010). They are essential for embryonic development (Harvey, et al., 2002; Ke and Costa, 2006). Apart from hypoxia itself, HIF-1α has been shown to be stabilised via ROS (reactive oxygen species) generation due to hypoxia (Chandel, et al., 2000; López-Lázaro, 2006; Ma, 2010; Hamanaka and Chandel, 2010).
6.1.3. Hypoxia, oxidative stress and epigenetic maintenance

Oxidative stress can elicit changes at an epigenetic level such as alterations in epigenetic enzyme functionality, depletion of GSH which can subsequently lead to an attenuation of SAM and further methyltransferase dysregulation, and the activity of 2-OG and Fe(II)-dependent dioxygenases (Hitchler and Domann, 2007; Cyr and Domann, 2011). Cellular oxidative stress can be induced via the treatment with chemical agents proven to generate ROS such as the test compounds of this study (5-azacytidine, cadmium chloride, sodium arsenite and valproic acid) or changes in oxygen tension (e.g. hypoxia). ROS can influence epigenetic changes as progressive oxidation of 5-mC could result in CpG demethylation (Sardina, et al., 2012) and can generate 5-hmC (Donkena, et al., 2010).

Further, in response to hypoxia, epigenetic enzymes, such as most members of the Jumonji-domain containing histone demethylase family, have been shown to be induced despite the fact that they use oxygen as a co-factor in the demethylation reaction (Perez-Perri, et al., 2011). Members of this family have been reported to be essential for the maintenance of the pluripotency of ES cells (Loh, et al., 2007; Wang, et al., 2010). Interestingly, it has also been suggested that they are transcriptional targets of HIF-1α (Beyer, et al., 2008; Pollard, et al., 2008; Wellmann, et al., 2008; Luo, et al., 2012).
6.2 Results

6.2.1. Treatment of human ES cells with compounds known to disrupt development and the epigenome

The subcytotoxic concentrations of 5-azacytidine, cadmium chloride and sodium arsenite had been previously established in our lab, while I established the concentrations for valproic acid (Appendix XIII). Briefly, H9 and RH1 cells were treated with ten different concentrations of each test compound for seven days and the IC$_{10}$ (a concentration affecting a 10% decrease in cell viability) and a 3-log fold lower than IC$_{10}$ concentrations were determined. The concentrations used in this study for each test compound and human ES cell line are presented in Table 6.1.

<table>
<thead>
<tr>
<th></th>
<th>H9 hESCs</th>
<th></th>
<th>RH1 hESCs</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IC$_{10}$ concentration (Molar)</td>
<td>3 log-fold lower concentration (Molar)</td>
<td>IC$_{10}$ concentration (Molar)</td>
</tr>
<tr>
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<td>1x10$^{-9}$</td>
<td>3.3x10$^{-9}$</td>
</tr>
<tr>
<td>Cadmium Chloride</td>
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<td>1x10$^{-8}$</td>
<td>1x10$^{-6}$</td>
</tr>
<tr>
<td>Sodium Arsenite</td>
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<td>1x10$^{-12}$</td>
<td>3.3x10$^{-11}$</td>
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<tr>
<td>Valproic Acid</td>
<td>1x10$^{-8}$</td>
<td>1x10$^{-11}$</td>
<td>1x10$^{-7}$</td>
</tr>
</tbody>
</table>

Table 6.1. Summary of subcytotoxic concentrations for H9 and RH1 human ES cells. Undifferentiated H9 and RH1 cells were treated with a range of concentrations of...
5-azacytidine, cadmium chloride, sodium arsenite and valproic acid for seven days. At day 7, the CellTiter-Blue® Cell Viability Assay was performed to determine the cytotoxic effects of the test compounds on human ES cells. All test compounds were evaluated in triplicates for each of the compound concentrations. Same applied for the untreated control human ES cell cultures. A concentration affecting a 10% decrease in cell viability (IC$_{10}$ concentration) relative to the untreated controls for 5-azacytidine, cadmium chloride, sodium arsenite and valproic acid, and concentrations derived from a 3-log fold dilution of the IC$_{10}$ were determined for H9 and RH1 cells.

To study the subcytotoxic effects of 5-azacytidine, cadmium chloride, sodium arsenite and valproic acid on epigenetic determinants of human ES cell identity, the two concentrations were tested in H9 and RH1 human ES cells under normoxic (20% O$_2$) and hypoxic (0.5% O$_2$) atmospheric culture conditions (Figure 6.1). Quadruplicate cultures for each experimental condition were maintained in human dermal fibroblast conditioned medium (HDF-CM) supplemented with 4 ng/ml of human basic fibroblast growth factor (bFGF) which maintains human ES cell self-renewal in vitro. Culture media in the presence (compound-treated) or absence (untreated controls) of a compound were changed every two days. Samples for multiple analyses were collected after seven days of culture unless otherwise stated.
Figure 6.1. Schematic illustration of the treatment of human ES cells with 5-azacytidine, cadmium chloride, sodium arsenite and valproic acid at subcytotoxic concentrations. H9 and RH1 cells were treated with 5-azacytidine, cadmium chloride, sodium arsenite and valproic acid at an IC_{10} and a 3-log fold lower than IC_{10} concentration for seven days. Cells were maintained in human dermal fibroblast conditioned medium supplemented with 4 ng/ml of human bFGF which maintains hESC self-renewal in vitro. Culture media change in the presence or absence of a compound was performed at a two-day interval. Samples were collected at day 7 for analysis unless otherwise stated. Abbreviation: 12-w, 12-well.

6.2.1.1 Subcytotoxic effects of 5-azacytidine, cadmium chloride, sodium arsenite and valproic acid on the undifferentiated human ES cell phenotype

Treatment of H9 and RH1 human ES cells with 5-azacytidine, cadmium chloride and sodium arsenite resulted in apparent morphological changes consistent with differentiation (Figure 6.2) under normoxic culture conditions while treatment with valproic acid did not cause any morphological alterations as it failed to
induce differentiation. In contrast, treatment with each of the four compounds under hypoxia resulted in the maintenance of a human ES cell undifferentiated phenotype, except in the case of cadmium chloride where cells remained differentiated. Similar results were observed in both human ES cell lines exposed to both test concentrations.
(A1, H9 hESCs, IC_{10} concentration)

<table>
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<th>HYPOXIA</th>
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</tr>
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<tr>
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(A2, H9 hESCs, 3-log fold lower concentration)

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</table>
(B1, RH1 hESCs, IC_{10} concentration)

<table>
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<td><img src="image" alt="Valproic Acid HYPOXIA" /></td>
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</tbody>
</table>
(B2, RH1 hESCs, 3-log fold lower concentration)
**Figure 6.2. Subcytotoxic effects of 5-azacytidine, cadmium chloride, sodium arsenite and valproic acid on the undifferentiated human ES cell phenotype.** (A1, A2) H9 and (B1, B2) RH1 cells were treated with 5-azacytidine, cadmium chloride, sodium arsenite and valproic acid (1: IC$_{10}$ and 2: 3-log fold lower concentration) for seven days. Cells were maintained in HDF-CM+. A normal undifferentiated hESC phenotype is observed at the untreated controls under normoxia and hypoxia. Differentiation was observed in the compound-treated hESCs under normoxia, the exception being valproic acid, while under hypoxia only cadmium chloride treatment resulted in differentiation. Images were captured using an Axiovert 40 CFL microscope and a Canon PowerShot A650 IS camera. Scale bar represents 100 μm. Abbreviation: KD, knockdown.

### 6.2.1.2 Subcytotoxic effects of 5-azacytidine, cadmium chloride, sodium arsenite and valproic acid on human ES cell pluripotency and epigenome

To determine the effects of the exposure of 5-azacytidine, cadmium chloride, sodium arsenite and valproic acid on human ES cell pluripotency and epigenome, quadruplicate H9 and RH1 cultures were treated with each of the four compounds at the indicated concentrations as shown in Table 6.1. Human ES cells were cultured in HDF-CM+ medium which maintain self-renewal *in vitro* and incubated under normoxic (20% O$_2$) or hypoxic (0.5% O$_2$) atmospheric conditions at 37°C, 5% CO$_2$. Culture media were removed and replaced with fresh with or without the test compounds every two days. Following seven days
of culture, samples were collected for analysis and assessed for the core pluripotency markers OCT4, NANOG, SOX2, epigenetically-defined biomarkers GLIS2, HMGA1, PFDN5, JMJD2C, TET1 and TET related family members (TET2 and TET3) by qRT-PCR. Also, immunocytochemistry for the pluripotency markers OCT4, NANOG and epigenetic markers 5-mC and 5-hmC were performed. Quantitative analysis of 5-hmC was carried out using an ELISA assay as described in previous chapters (Sections 2.7 and 3.2.4).

Treatment at an IC$_{10}$ concentration with 5-azacytidine, cadmium chloride or sodium arsenite caused a significant downregulation in H9 human ES cells of the pluripotency markers OCT4, NANOG, SOX2, epigenetically-defined biomarkers GLIS2, HMGA1, PFDN5, JMJD2C, TET1 and the TET related family members, TET2 and TET3, under normoxic culture conditions, the exception being TET2 in 5-azacytidine-treated cells and NANOG in cadmium chloride-treated cells which was significantly upregulated (Figure 6.3). On the contrary, treatment of H9 cells with valproic acid led to the upregulation and/or the expression at baseline transcript levels of the selected markers, with the exception of NANOG and GLIS2 that were decreased. Treatment of H9 human ES cells under hypoxic conditions showed higher marker expression compared to their normoxic counterparts in the cases of 5-azacytidine, sodium arsenite and valproic acid. Treatment of cells with cadmium chloride under hypoxia led to the downregulation of the markers except TET2 which was significantly
increased.

Further, H9 human ES cells treated with the lower concentration of the test compounds under normoxia showed a similar trend with the IC_{10} concentration as 5-azacytidine and cadmium chloride reduced the expression of the markers, with the exception of NANOG in 5-azacytidine-treated cells and SOX2 in sodium arsenite-treated cells (Figure 6.3). Upon valproic acid treatment, H9 cells did not show a reduction of markers except for the significant reduction of OCT4 transcript levels. Cells maintained under hypoxia at this concentration produced a mixed response in the case of 5-azacytidine with SOX2, GLIS2, HMG1, PFDN5, JMJD2C, TET1 and TET3 being significantly decreased. Similarly to the IC_{10} concentration, treatment with cadmium chloride resulted in the downregulation of all the genes except TET2. At the same time, the sodium arsenite-treated cells showed reduced expression of all markers apart from TET2 and the valproic acid-treated cells expressed the genes at baseline levels except SOX2. These data confirmed the morphological observations where under normoxia all test compounds, except valproic acid, induced differentiation while hypoxia protected against the differentiation inducing effects of 5-azacytidine and sodium arsenite but not cadmium chloride.
(A, IC_{10} concentration)
(B, 3-log fold lower concentration)

5-Azacytidine

![Graph showing log mRNA fold difference for 5-Azacytidine under normoxia and hypoxia conditions.]

![Graph showing log mRNA fold difference for Cadmium Chloride under normoxia and hypoxia conditions.]

- **OCT4**, **NANOG**, **SOX2**, **GLIS2**, **HMGA1**, **PFDM5**, **JMJD2C**, **TET1**, **TET2**, **TET3**

- **NORMOXIA** and **HYPOXIA**

**Key:**
- **OCT4:** 
- **NANOG:** 
- **SOX2:** 
- **GLIS2:** 
- **HMGA1:** 
- **PFDM5:** 
- **JMJD2C:** 
- **TET1:** 
- **TET2:** 
- **TET3:**
**Figure 6.3. Subcytotoxic effects of 5-azacytidine, cadmium chloride, sodium arsenite and valproic acid on pluripotent and epigenetic genes in H9 human ES cells.** H9 cells were treated with 5-azacytidine, cadmium chloride, sodium arsenite and valproic acid at an (A) IC_{10} and (B) a 3-log fold lower than IC_{10} concentrations under normoxia (20% O_2) and hypoxia (0.5% O_2) for seven days. RNA was isolated at day 7 and qRT-PCR was employed to assess the transcript levels of pluripotency markers OCT4, NANOG, SOX2, epigenetically-defined biomarkers GLIS2, HMGA1, PFDN5 and epigenetic markers JMJD2C, TET1, TET2 and TET3. The samples were normalised against β-actin. The untreated controls have a value of 1.0. Asterisks indicate levels of statistical significance (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001) of the compound-treated cultures under normoxia and hypoxia against the untreated controls as calculated by Student’s t-test.

Next, in order to examine how the expression of pluripotency markers OCT4, NANOG, SOX2, epigenetically-defined biomarkers GLIS2, HMGA1, PFDN5, JMJD2C, TET1 and TET related family members (TET2 and TET3) are affected by the two experimental factors (i.e. compound treatment and oxygen tension), a two-way ANOVA test was performed for both concentrations studied. The two-way AVOVA statistical test produces a p value for every gene and for each of the following: (i) the effect of the interaction among compound treatment and oxygen tension in every gene, (ii) the effect of the compound treatment in the expression of every gene and (iii) the effect of oxygen tension in the expression of every gene (Goni, et al., 2009). A summary of p values for each experimental factor and of the interaction (if any) between the two for each gene is presented in Table 6.2. It is evident that the effects of the compound treatment or oxygen tension and the interaction between the two factors are extremely significant.
(**p<0.0001) for all genes in the case of the IC\textsubscript{10} concentration. As far as the 3-log fold lower than IC\textsubscript{10} concentration is concerned, the effects in all three cases ranges from significant (*p<0.05) to extremely significant (****p<0.0001).

<table>
<thead>
<tr>
<th>IC\textsubscript{10} concentration</th>
<th>Oxygen Tension</th>
<th>Compound Treatment</th>
<th>Interaction</th>
</tr>
</thead>
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<td>&lt; 0.0001</td>
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Table 6.2. Two-way ANOVA statistical analysis of the effects of compound treatment and oxygen tension on pluripotency, biomarker and TET genes in H9 human ES cells. H9 cells were treated with 5-azacytidine, cadmium chloride, sodium arsenite and valproic acid at an (A) IC\textsubscript{10} and (B) a 3-log fold lower than IC\textsubscript{10} concentrations under normoxia (20\% O\textsubscript{2}) and hypoxia (0.5\% O\textsubscript{2}). A two-way ANOVA statistical analysis was performed to demonstrate if the compound treatment, the oxygen tension or the interaction (if any) of the two factors (compound treatment and oxygen tension) affect the expression of pluripotency markers OCT4, NANOG, SOX2, epigenetically-defined biomarkers GLIS2, HMGA1, PFDN5, and epigenetic markers JMJD2C, TET1, TET2 and TET3. P values are presented for each factor and the interaction (if any) of the two factors for each marker examined. Both compound concentrations and the oxygen tension, and the interaction between the two factors found to have a significant effect on the expression of all markers.

RH1 cells exposed to an IC\textsubscript{10} concentration of 5-azacytidine under normoxia showed a significant reduction of the pluripotency markers OCT4, NANOG, epigenetically-defined biomarkers GLIS2, HMGA1, PFDN5 and TET1 while JMJD2C and TET2 were notably induced (Figure 6.4). Cadmium chloride-treated cells showed results similar to the 5-azacytidine-treated cultures along with a significant decrease of TET3. Furthermore, a mixed response was
observed upon treatment with sodium arsenite under normoxia as there was a significant decrease of OCT4, GLIS2, HMGA1, PFDN5 and TET3 whereas NANOG and TET2 were considerably increased. Treatment with valproic acid led to the significant augmentation of the pluripotency markers OCT4, NANOG, SOX2 and the dioxygenases JMJD2C, TET1, TET2 and TET3.

RH1 cells showed an increased expression of the core pluripotency markers, JMJD2C and TET2, while the biomarkers GLIS2, HMGA1 and PFDN5 remained reduced under hypoxic conditions at an IC$_{10}$ concentration of 5-azacytidine (Figure 6.4). At the same culture conditions, cadmium chloride-treated cells demonstrated a mixed response as NANOG, HMGA1, PFDN5, TET1 and TET3 were significantly downregulated, JMJD2C was notably upregulated and the rest of markers were expressed at baseline transcript levels. In addition, a mixed response was observed in the case of sodium arsenite-treated RH1 cells under hypoxia as OCT4, NANOG, SOX2, JMJD2C, TET1 and TET2 were significantly upregulated while the biomarkers GLIS2, HMGA1 and PFDN5 were considerably downregulated. Lastly, exposure of cells to valproic acid resulted in the significant upregulation of markers except GLIS2 and HMGA1.

When RH1 cells were exposed to the lower concentration of 5-azacytidine the effects were similar to the ones observed at an IC$_{10}$ concentration under
normoxia, only at a lower level (Figure 6.4). Cadmium chloride-treated cells followed a similar trend of marker expression when exposed at the lower concentration as at the IC_{10} concentration, except TET2 and TET3, whose expression was close to baseline levels. In the case of sodium arsenite-treated cells, the lower concentration effect on the marker expression was less pronounced compared to IC_{10}. Treatment with valproic acid showed a similar marker expression trend as the IC_{10} with the exception of TET2 and TET3 downregulation.

Also, the expression of OCT4, TET1 and TET3 was significantly decreased compared to the IC_{10} in RH1 cells under hypoxia treated with the lower concentration of 5-azacytidine (Figure 6.4). Cadmium chloride-treated cells, contrary to the IC_{10} concentration, exhibited significantly reduced expression of OCT4 and increased expression of NANOG and SOX2. The biomarkers GLIS2, HMGA1, PFDN5 and JMJD2C showed a similar tendency, while TET1 and TET3 were notably increased contrary to the decreased expression at the IC_{10} exposure. Moreover, treatment with the lower concentration of sodium arsenite produced a less pronounced effect under hypoxia compared to that of the IC_{10}. The pluripotency markers, NANOG and SOX2, were expressed close to baseline levels compared to the significant upregulation observed at the higher concentration. The biomarkers GLIS2, HMGA1 and PFDN5 were affected differently as they were upregulated compared to the significant downregulation
at IC_{10} exposure. Also, the TETs were affected in a different way with the lower concentration downregulating TET1 and TET3 expression. Finally, exposure of cells to valproic acid demonstrated a less pronounced and more constant effects on marker expression as not all markers were upregulated as observed for the IC_{10} concentration.

\[ (A, \text{IC}_{10} \text{ concentration}) \]
Valproic Acid

(B, 3-log fold lower concentration)

5-Azacytidine
Figure 6.4. Subcytotoxic effects of 5-azacytidine, cadmium chloride, sodium arsenite and valproic acid on pluripotent and epigenetic genes in RH1 human ES cells. RH1 cells were treated with 5-azacytidine, cadmium chloride, sodium arsenite and valproic acid at an (A) IC$_{10}$ and (B) a 3-log fold lower than IC$_{10}$ concentrations under normoxia (20% O$_2$) and hypoxia (0.5% O$_2$) for seven days. RNA was isolated at day 7 and qRT-PCR was performed to assess the expression of pluripotency markers OCT4, NANO, SOX2, biomarkers GLIS2, HMGA1, PFDN5 and epigenetic markers JMJD2C, TET1, TET2 and TET3. The samples were normalised against β-actin. The untreated controls have a value of 1.0. Asterisks indicate levels of statistical significance (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001) of compound-treated cultures with respect to untreated cultures under normoxia and hypoxia, respectively, as calculated by Student's t-test.

Subsequently, as in the case of H9 cells, a two-way ANOVA test was performed to test if the expression of the genes OCT4, NANO, SOX2, GLIS2, HMGA1, PFDN5, JMJD2C, TET1/2/3 is affected by the two experimental factors. As
illustrated in Table 6.3, at an IC$_{10}$ concentration, the effects of the compound treatment and oxygen tension as well as the interaction between the two are extremely significant (****p<0.0001) for all genes similar to H9 cells. At the 3-log fold lower concentration, the effects vary from not significant (ns) to extremely significant (****p<0.0001), with the majority being extremely significant.

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<th>Compound Treatment</th>
<th>Interaction</th>
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Table 6.3. Two-way ANOVA statistical analysis of the effects of compound treatment and oxygen tension on pluripotency, biomarker and TET genes in RH1 human ES cells. RH1 cells were treated with 5-azacytidine, cadmium chloride, sodium arsenite and valproic acid at an (A) IC\textsubscript{10} and (B) a 3-log fold lower than IC\textsubscript{10} concentrations under normoxia (20% O\textsubscript{2}) and hypoxia (0.5% O\textsubscript{2}). A two-way ANOVA statistical analysis was performed to demonstrate if the compound treatment, the oxygen tension or the interaction (if any) of these two experimental factors affect the expression of pluripotency markers OCT4, NANOG, SOX2, epigenetically-defined biomarkers GLIS2, HMGA1, PFDN5, and dioxygenases JMJD2C, TET1, TET2 and TET3. A summary of p values are presented for each factor and the interaction (if any) of these two factors for each marker examined in RH1 human ES cells. At an IC\textsubscript{10} concentration the effects of the compound treatment or oxygen tension and the interaction between the two were determined as significant for all genes. At the 3-log fold lower concentration, the effects in all three cases varied from not significant to extremely significant with the majority been extremely significant.
Immunocytochemistry was performed for the pluripotent markers OCT4 and NANOG in both human ES cell lines to establish if there were corresponding changes in protein levels in human ES cells treated with all four test compounds. Figure 6.5 shows that the untreated control H9 cells maintained OCT4 and NANOG expression under both oxygen tensions. However, cells treated with the compounds under normoxia at an IC$_{10}$ concentration showed decreased levels of OCT4 and NANOG, except in the case of valproic acid where the protein levels remained unchanged. On the contrary, under hypoxia, the expression of OCT4 and NANOG in the 5-azacytidine and sodium arsenite-treated human ES cells remained unaffected similar to valproic acid while in the case of cadmium chloride-treated cells there is an apparent decrease in protein levels when compared to the untreated control. Cells treated with the lower concentration of compounds showed a similar but less pronounced response under normoxia with the IC$_{10}$ concentration and with the exception of cadmium chloride under hypoxic conditions where the OCT4 and NANOG protein levels did not decrease.
(A1, IC\textsubscript{10} concentration, Normoxia)
(A2, IC_{10} concentration, Hypoxia)
(B1, 3-log fold lower concentration, Normoxia)

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Figure 6.5. Subcytotoxic effects of 5-azacytidine, cadmium chloride, sodium arsenite and valproic acid on H9 human ES cell pluripotency. H9 cells were treated with each compound at an IC$_{10}$ (A1, A2) and a 3-log fold lower (B1, B2) concentrations under normoxic (20% O$_2$) (A1, B1) and hypoxic (0.5% O$_2$) (A2, B2) culture conditions for seven days. Samples were fixed with 4% PFA/PBS at the end of the culture period. Representative images of immunohistochemical analysis for the pluripotency markers OCT4 (green) and NANOG (red) are illustrated. DAPI (blue) was used as a nuclear staining. These images were captured using Zeiss Observer fluorescence microscope.
and prepared using the Zeiss AxioVision 4.8.2 software. The scale bar represents 100 μm.

As observed in H9 cells, at an IC_{10} concentration under normoxic culture conditions RH1 compound-treated cells showed decreased expression of the pluripotency markers OCT4 and NANOG, while under a hypoxic atmosphere only cadmium chloride-treated cells showed reduction at the protein level (Figure 6.6). RH1 cells exposed to the lower concentration of compounds retained the OCT4 and NANOG protein levels under both normoxic and hypoxic conditions.
(A1, IC$_{10}$ concentration, Normoxia)
(A2, IC_{10} concentration, Hypoxia)
(B1, 3-log fold lower concentration, Normoxia)
Figure 6.6. Subcytotoxic effects of 5-azacytidine, cadmium chloride, sodium arsenite and valproic acid on RH1 human ES cell pluripotency. RH1 cells were treated with each compound at an IC$_{10}$ (A1, A2) and a 3-log fold lower (B1, B2) concentration under normoxic (20% O$_2$) (A1, B1) and hypoxic (0.5% O$_2$) (A2, B2) culture conditions for seven days. Samples were fixed with 4% PFA/PBS at the end of the culture period. Representative images of immunohistochemical analysis for the pluripotency markers OCT4 (green) and NANOG (red) are illustrated. DAPI (blue) was used as a nuclear staining. These images were captured using Zeiss Observer.
fluorescence microscope and prepared using the Zeiss AxioVision 4.8.2 software. The scale bar represents 100 μm.

As previously stated, the epigenetic marker 5-hmC plays an important role in ES cell pluripotency and the TET family (TET1-3) of dioxygenases is responsible for the conversion of 5-mC to 5-hmC. Hence, to further explore the subcytotoxic effects of the four compounds under normoxia and hypoxia on the epigenetic status of human ES cells, the expression of the epigenetic marks 5-mC and 5-hmC was evaluated by immunocytochemistry. Also, the effects of the compound treatments and simultaneous exposure to different oxygen atmospheres on 5-hmC was quantified using an ELISA.

Immunocytochemical assessment of 5-mC and 5-hmC at a nuclear level of the untreated controls under normoxia and hypoxia in H9 cells demonstrated apparent detection of both (Figure 6.7). There were detectable levels of 5-hmC in all cases under normoxic and hypoxic conditions with cadmium chloride showing the lowest levels of expression. Interestingly, under normoxic culture conditions the expression of 5-mC was nearly undetectable in cells treated with 5-azacytidine, cadmium chloride and sodium arsenite but not with valproic acid whereas, under hypoxic conditions only the cadmium chloride-treated cells had significantly lost 5-mC expression. The observations were similar for both compound concentrations tested.
(A1, IC<sub>10</sub> concentration, Normoxia)

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(A2, IC_{10} concentration; Hypoxia)

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(B1, 3-log fold lower concentration, Normoxia)
(B2, 3-log fold lower concentration, Hypoxia)

**Figure 6.7.** Subcytotoxic effects of 5-azacytidine, cadmium chloride, sodium arsenite and valproic acid on the expression of 5-mC and 5-hmC in H9 human ES cells. H9 cells were treated with each of the test compounds at (A1, A2) an IC_{10} and (B1, B2) a 3-log fold lower concentration under normoxic (20% O_2) (A1, B1) and hypoxic (0.5% O_2) (A2, B2) culture conditions for seven days. Samples were fixed with 4% PFA/PBS at day 7. Images of immunohistochemical analysis for 5-methylcytosine (5-mC, green) and 5-hydroxymethylcytosine (5-hmC, red) are illustrated. DAPI (blue) represents nuclear staining. The immunostaining was recorded using a Zeiss Observer
fluorescence microscope. All the images were prepared using the Zeiss AxioVision 4.8.2 software. The scale bar represents 100 µm.

Untreated RH1 cells controls maintained under normoxia and hypoxia showed detectable expression of 5-mC and 5-hmC (Figure 6.8). Similar to H9 cells, exposure to each of the four compounds under these conditions resulted in evident levels of 5-hmC detected at an IC$_{10}$ concentration with cadmium chloride showing the lowest levels of expression. At the same concentration, the expression of 5-mC under normoxia was significantly decreased with the exception being the valproic acid while under hypoxia only cadmium chloride-treated cells showed significant reduction. Further, RH1 cells treated at the lower concentration of compounds under normoxia led to a considerable reduction in 5-hmC levels only in the case of cadmium chloride, while under hypoxia all treated cells showed 5-hmC expression. As far as the expression of 5-mC is concerned, under normoxia, only cells treated with valproic acid expressed 5-mC whereas under hypoxia only cadmium chloride decreased 5-mC expression.
(A1, IC_{10} concentration, Normoxia)

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(A2, IC₁₀ concentration, Hypoxia)
(B1, 3-log fold lower concentration, Normoxia)
Figure 6.8. Subcytotoxic effects of 5-azacytidine, cadmium chloride, sodium arsenite and valproic acid on the expression of 5-mC and 5-hmC in RH1 human ES cells. RH1 cells were treated with each of the test compounds at (A1, A2) an IC$_{10}$ and (B1, B2) a 3-log fold lower concentration under normoxic (20% O$_2$) (A1, B1) and hypoxic (0.5% O$_2$) (A2, B2) culture conditions for seven days. Samples were fixed with 4% PFA/PBS at day 7. Images of immunohistochemical analysis for 5-methylcytosine (5-mC, green) and 5-hydroxymethylcytosine (5-hmC, red) are illustrated. DAPI (blue) represents nuclear staining. The immunostaining was recorded using a Zeiss Observer.
fluorescence microscope. All the images were prepared using the Zeiss AxioVision 4.8.2 software. The scale bar represents 100 μm.

Since immunocytochemical assessment of 5-hmC is only qualitative and at times difficult to distinguish differences in expression, it was important to determine 5-hmC status quantitatively in treated human ES cells. Therefore, a DNA ELISA assay for quantifying 5-hmC was used according to manufacturer's protocol as described in previous chapters (Section 2.7 and 3.2.4). Triplicate biological samples for all compound treatments under both oxygen conditions were assessed for 5-hmC expression. Each biological sample was represented by a technical triplicate.

In H9 cells the untreated control cultures maintained under hypoxia had significantly higher levels of 5-hmC than the normoxic conditions when exposed to both tested concentrations (Figure 6.9). In the case of the IC_{10} concentration, cells treated with 5-azacytidine, cadmium chloride and sodium arsenite showed considerably lower 5-hmC expression contrary to the significant increase observed in the valproic acid-treated cells (Figure 6.9 A). Under hypoxic conditions, all compound treatments resulted in a significant increase of 5-hmC. Furthermore, the levels of 5-hmC were significantly higher under the hypoxic culture conditions compared to the normoxia for the untreated control and all the compound treatments.
In the case of H9 cells treated at the lower concentration of compounds, under normoxia the levels of 5-hmC were lower but only 5-azacytidine and cadmium chloride-treated cells reached significance (Figure 6.9 B). Under hypoxia, cadmium chloride and sodium arsenite-treated cultures had significantly lower levels of 5-hmC whereas 5-azacytine and valproic acid-treated cells retained 5-hmC expression close to that of the hypoxic untreated control. Also, comparing the hypoxic to the normoxic environment in terms of 5-hmC expression, it is the hypoxic cultures of the untreated control and the valproic acid which demonstrated significantly higher levels.
Figure 6.9. Subcytotoxic effects of 5-azacytidine, cadmium chloride, sodium arsenite and valproic acid on the epigenetic marker 5-hmC in H9 human ES cells. H9 cells were treated with 5-azacytidine, cadmium chloride, sodium arsenite and valproic acid at an (A) IC_{10} and (B) a 3-log fold lower than IC_{10} concentration under normoxic (20% O_2) and hypoxic (0.5% O_2) atmospheric conditions for seven days. DNA was isolated at day 7 and triplicate biological samples were quantified for 5-hmC using the Quest 5-hmC™ DNA ELISA kit. Each biological replicate was represented by a technical triplicate. Data are shown as mean values ± S.D. and the asterisks signify statistical difference (*p<0.05, **p<0.01, ***p<0.001) of compound-treated cultures with respect to untreated cultures under normoxia and hypoxia, respectively, as calculated by one-way ANOVA with Dunnett’s post-hoc test. Student’s t-test was performed for the
comparison between hypoxia and normoxia for each culture group (indicated with bracket).

RH1 cells treated with IC$_{10}$ concentration of 5-azacytidine, cadmium chloride and sodium arsenite under normoxia showed a significant reduction of 5-hmC expression, while valproic acid treatment had no effect. Hypoxia had no significant effect on 5-hmC as the expression levels were close to those of the hypoxic untreated control with the exception of cadmium chloride-treated cells where 5-hmC was significantly decreased. In addition, RH1 cells treated at the lower concentration of compounds under normoxia led to the significant reduction of 5-hmC with 5-azacytidine and cadmium chloride treatment whereas sodium arsenite and valproic acid had no significant effects. As far as the effect of compounds under hypoxia is concerned, a significant reduction in 5-hmC levels was observed only in cadmium chloride and sodium arsenite treated RH1 cells.
(A, IC_{10} concentration)
Figure 6.10. Subcytotoxic effects of 5-azacytidine, cadmium chloride, sodium arsenite and valproic acid on the epigenetic marker 5-hmC in RH1 human ES cells. RH1 cells were treated with 5-azacytidine, cadmium chloride, sodium arsenite and valproic acid at an (A) IC₁₀ and (B) a 3-log fold lower than IC₁₀ concentration under normoxia (20% O₂) and hypoxia (0.5% O₂) for seven days. DNA was isolated at day 7 and triplicate biological samples were assessed for global levels of 5-hmC using the Quest 5-hmC™ DNA ELISA kit. Each biological replicate was represented by a technical triplicate. Data are shown as mean values ± S.D. and the asterisks signify statistical difference (**p<0.01, ***p<0.001) of compound-treated cultures with respect to untreated cultures under normoxia and hypoxia, respectively; as calculated by one-way ANOVA with Dunnett’s post-hoc test. Student’s t-test was performed for the comparison between hypoxia and normoxia for each culture group (indicated with bracket).
Finally, a two-way ANOVA test was performed to test whether the expression of 5-hmC is affected by the compound treatments, the oxygen tension and the interaction between these two factors. The results are presented in Table 6.4. The data revealed that for both test concentrations and both human ES cell lines, the effects of the compound treatment or oxygen tension and the interaction between the two are extremely significant.

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Table 6.4. Two-way ANOVA statistical analysis of the effects of compound treatment and oxygen tension on 5-hmC content in human ES cells. H9 and RH1 human ES cells were treated with 5-azacytidine, cadmium chloride, sodium arsenite and valproic acid at an IC_{10} and a 3-log fold lower than IC_{10} concentration under normoxic (20% O₂) and hypoxic (0.5% O₂) conditions. A two-way ANOVA statistical analysis was performed to show if the compound treatment, the oxygen tension or the interaction (if any) of the two experimental factors affect the expression of 5-hmC. P values are presented for each case (compound treatment, oxygen tension and their interaction) examined in H9 and RH1 cells. The two-way ANOVA analysis shown that for both compound concentrations and both human ES cell lines the effects of the
compound treatment or oxygen tension and the interaction between the two were extremely significant.

6.2.1.3 Treatment of human ES cells with subcytotoxic concentrations of 5-azacytidine, cadmium chloride and sodium arsenite but not valproic acid lead to cellular differentiation under normoxic conditions

The loss of an undifferentiated human ES cell phenotype, reduction in pluripotency markers and induced differentiation under normoxia following exposure to 5-azacytidine, cadmium chloride and sodium arsenite for both H9 and RH1 cells did not clearly indicate into which lineages they may have differentiated. For this reason, to establish whether 5-azacytidine, cadmium chloride and sodium arsenite led to a specific lineage, qRT-PCR analysis for lineage-associated markers was performed. Quadruplicate biological samples for each compound were assessed for CDX2 (trophoblast), Brachyury (mesoderm), AFP (endoderm) and PAX6 (ectoderm) expression.

Treatment of H9 cells with 5-azacytidine showed a significant increase of the endodermal marker AFP, while the expression of CDX2, Brachyury and PAX6
were decreased at both concentrations (Figure 6.11). In the case of cadmium chloride-treated cells, Brachyury was significantly elevated at both concentrations tested. Further, sodium arsenite-treated cells showed a significant increase in Brachyury transcript levels at an IC_{10} concentration but not in the lower concentration. As expected, lineage-associate marker expression in the valproic acid-treated cells was close to baseline transcript levels confirming the maintenance of an undifferentiated human ES cell phenotype observed at the morphological, mRNA and protein expression level.

(A, IC_{10} concentration)
Figure 6.11. Embryonic lineage marker expression in H9 human ES cells following compound treatment under normoxic culture conditions. Quadruplicate cultures of H9 cells were treated with 5-azacytidine, cadmium chloride, sodium arsenite and valproic acid at an (A) IC_{10} and (B) a 3-log fold lower than IC_{10} concentration for seven days. RNA was isolated at day 7 and qRT-PCR was performed to assess the expression of lineage markers CDX2 (trophoblast), Brachyury (mesoderm), AFP (endoderm) and PAX6 (ectoderm). Each biological sample was represented by triplicate technical replicates. Observed changes are relative to β-actin expression and normalised to the untreated control which has a value of 1.0. Asterisks indicate levels of statistical significance (*p<0.05, **p<0.01, ***p<0.001) as calculated by Student’s t-test.

Similarly to H9 cells, 5-azacytidine-treated RH1 cells demonstrated a significant increase of the endodermal (AFP) marker at both concentrations (Figure 6.12). Contrary to cadmium chloride-treated H9 cells, RH1 showed a notable induction
of CDX2 which was observed at both concentrations. In addition, sodium arsenite-treated RH1 cells, and in agreement with the H9 cells, showed an augmented expression of the mesodermal marker, Brachyury. Lastly, treatment of RH1 cells with valproic acid led to the significant reduction of CDX2 and Brachyury at an IC_{10} concentration while the expression of all lineage markers remained close to baseline transcript levels when cells were exposed to the lower concentration.

(A, IC_{10} concentration)
Figure 6.12. Embryonic lineage marker expression in RH1 human ES cells following compound treatment under normoxic culture conditions. Quadruplicate cultures of RH1 cells were treated with 5-azacytidine, cadmium chloride, sodium arsenite and valproic acid at an (A) IC$_{10}$ and (B) a 3-log fold lower than IC$_{10}$ concentration for seven days. RNA was isolated at day 7 and qRT-PCR was performed to assess the expression of lineage markers CDX2 (trophoblast), Brachyury (mesoderm), AFP (endoderm) and PAX6 (ectoderm). Each biological sample was represented by triplicate technical replicates. Observed changes are relative to β-actin expression and normalised to the untreated control which has a value of 1.0. Asterisks indicate levels of statistical significance (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001) as calculated by Student's t-test.
6.2.1.4 Subcytotoxic effects of 5-azacytidine, cadmium chloride, sodium arsenite and valproic acid on the mRNA expression of antioxidant defence genes in human ES cells

Toxic environmental compounds such as cadmium chloride and sodium arsenite are known to induce oxidative stress and interfere with the cellular antioxidant defence system (Ercal, et al., 2001). Cadmium chloride has a dual effect on antioxidant capacity by inducing oxidative stress via the inhibition of antioxidants and by activating antioxidants as a consequence of an unbalanced redox state (Cuypers, et al., 2010). Treatment with cadmium chloride has been shown to induce and reduce SOD expression and decrease GPXs (Cuypers, et al., 2010). Furthermore, sodium arsenite has been reported to increase or decrease antioxidant defence genes in various model systems (Ercal, et al., 2001). For this reason, the expression of the major antioxidant defence genes GPX1, GPX4, SOD1 and SOD2 was measured by qRT-PCR. Quadruplicate biological samples were assessed for H9 and RH1 cells exposed to both concentrations under normoxia and hypoxia.

Figure 6.13 shows the expression levels of the four antioxidant genes in H9 compound-treated cells. At an IC$_{10}$ concentration under normoxia, treatment with 5-azacytidine, cadmium chloride and sodium arsenite caused a significant decrease in the expression of antioxidants, while valproic acid leads to a modest
decrease of GPX4, SOD1, SOD2 and a significant increase of GPX1. Under hypoxia, 5-azacytidine and cadmium chloride treatment of H9 cells resulted in the significant downregulation of antioxidant genes, whereas sodium arsenite and valproic acid resulted in a significant decrease of GPX4, and GPX1 and SOD1, respectively. When H9 cells were exposed to the lower concentration of compounds under normoxia (Figure 6.13), 5-azacytidine and cadmium chloride-treated cells showed a significant attenuated antioxidant expression, except for GPX4 and SOD2 in 5-azacytidine-treated cells. Moreover, sodium arsenite treatment resulted in the significant downregulation of antioxidants while valproic acid showed only a considerable increase in GPX1. Under hypoxia, 5-azacytidine and cadmium chloride treatment of H9 cells resulted in the significant downregulation of antioxidant genes. Sodium arsenite-treated cells showed a significant decrease in GPX1 and GPX4 genes, whereas valproic acid resulted in a modest increase of GPX4.
(A, IC_{10} concentration)
Sodium Arsenite

Valproic Acid

Log_{10} mRNA Fold Difference

GPX1  GPX4  SOD1  SOD2

NORMOXIA
HYPOXIA
(B, 3-log fold lower concentration)

5-Azacytidine

Cadmium Chloride

Log_{10} mRNA Fold Difference
Figure 6.13. Subcytotoxic effects of 5-azacytidine, cadmium chloride, sodium arsenite and valproic acid on the mRNA expression of antioxidant defence genes in H9 human ES cells. Quadruplicate cultures of H9 cells were treated with 5-azacytidine, cadmium chloride, sodium arsenite and valproic acid at an (A) IC_{10} and (B) a 3-log fold lower than IC_{10} concentration for seven days under normoxia and hypoxia. Total RNA was isolated at day 7 and qRT-PCR was performed to assess the expression of antioxidant genes GPX1, GPX4, SOD1 and SOD2. Each biological sample was represented by triplicate technical replicates. Observed changes are relative to β-actin expression and normalised to the untreated control. Asterisks indicate levels of statistical significance (*p<0.05, **p<0.01, ****p<0.0001) of compound-treated cultures with respect to untreated cultures under normoxia and hypoxia, respectively; as calculated by Student’s t-test.

In RH1 cells, at an IC_{10} concentration and under normoxic conditions all antioxidants were significantly downregulated, while under hypoxia only GPX1 and SOD1 were notably reduced (Figure 6.14). Treatment with cadmium chloride led to a significant decrease of antioxidants under both oxygen tensions except SOD2 under hypoxia. Furthermore, sodium arsenite-treated cells showed a considerable attenuation of all antioxidants with the exception of GPX4 under hypoxia. Significant downregulation of all antioxidants under both normoxia and hypoxia was observed in valproic acid-treated cells.

At the lower concentration, 5-azacytidine and cadmium chloride resulted in a significant reduction of antioxidant expression under normoxia and hypoxia in RH1 cells (Figure 6.14). Sodium arsenite treatment led to the significant
decrease of GPX1 and SOD1 under normoxia and all antioxidants under hypoxia. Lastly, exposure to valproic acid showed decreased antioxidant expression under normoxic conditions, a modest increase of GPX1 and SOD1, as well as reduced SOD2 and GPX4 at baseline levels.

(A, IC10 concentration)
(B, 3-log fold lower concentration)
Figure 6.14. Subcytotoxic effects of 5-azacytidine, cadmium chloride, sodium arsenite and valproic acid on the mRNA expression of antioxidant defence genes in RH1 human ES cells. Quadruplicate cultures of RH1 cells were treated with 5-azacytidine, cadmium chloride, sodium arsenite and valproic acid at an (A) IC₁₀ and (B) a 3-log fold lower than IC₁₀ concentration for seven days under normoxia and hypoxia. Total RNA was isolated at day 7 and qRT-PCR was performed to assess the expression of antioxidant genes GPX1, GPX4, SOD1 and SOD2. Each biological sample was represented by triplicate technical replicates. Observed changes are relative to β-actin expression and normalised to the untreated control which has a value of 1.0. Asterisks indicate levels of statistical significance (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001) of compound-treated cultures with respect to untreated cultures under normoxia and hypoxia, respectively; as calculated by Student’s t-test.
6.2.2. Hypoxia generates reactive oxygen species (ROS) in H9 and RH1 human ES cells

It has been reported that hypoxia increases the generation of ROS (Chandel, et al., 1998; reviewed in López-Lázaro, 2006; Hamanaka and Chandel, 2009; reviewed by Zepeda, et al., 2013). To examine whether H9 and RH1 cells generate ROS under hypoxic culture conditions, cells were exposed to hypoxia for seven days and compared against the control cultures maintained at normoxia. The production of ROS was measured by the sensitive fluorescence dye, dihydrodichlorofluorescein diacetate (H$_2$DCF-DA), using a microplate reader. Once the H$_2$DCF-DA is added to cells in culture, the H$_2$DCF is oxidised to DCF and results in green fluorescence. The brightness of the fluorescence is considered to reflect the extent to which ROS are present (Karlsson, et al., 2010).

Quadruplicate biological samples of H9 and RH1 cells were assessed for DCF fluorescence. An increase in fluorescence revealed that hypoxia elicited a significant (**p<0.0001) elevation of ROS in both human ES cell lines over the seven day culture period (Figure 6.15). The level of autofluorescence was measured in control untreated H9 and RH1 cells exposed to hypoxia and
normoxia without the H$_2$DCF-DA dye (described as untreated ctrl_unstained) in every experiment.

**Figure 6.15. Generation of ROS in human ES cells maintained under hypoxia.** H9 and RH1 cells were exposed to normoxic and hypoxic atmospheres for seven days. At day 7, ROS levels were determined using the fluorescent dye H$_2$DCF-DA. The DCF fluorescence was measured and error bars represent the mean ± S.D. of quadruplicate biological replicates. Hypoxia demonstrates significant elevation of ROS compared to the normoxic control. Bar graph represents five independent experiments. Data are shown as mean values ± S.D. and the asterisks indicate levels of statistical significance (****p<0.0001) as calculated by Student’s t-test. Abbreviations: ROS, reactive oxygen species; H$_2$DCF-DA: 2’,7’-dichlorodihydrofluorescein diacetate; DCF: 2’,7’-dichlorofluorescein; hESCs, human embryonic stem cells.
6.2.3 Generation of ROS following treatment with 5-azacytidine, cadmium chloride, sodium arsenite and valproic acid under normoxia and hypoxia in human ES cells

Apart from hypoxia, the test compounds 5-azacytidine, cadmium chloride, sodium arsenite and valproic acid have been demonstrated to cause an increase in the production of ROS (Arany, et al., 2011; Tian, et al., 2013). Hence, to determine whether ROS are generated upon treatment with 5-azacytidine, cadmium chloride, sodium arsenite or valproic acid, both human ES cell lines were treated at both concentrations and maintained under normoxia or hypoxia for seven days. The production of ROS was measured by the fluorescent dye H$_2$DCF-DA at two different time points (days 1 and 7). As aforementioned, when the H$_2$DCF-DA is added to cells in culture the H$_2$DCF is oxidised to DCF and the result is green fluorescence. The DCF fluorescence was determined for compound-treated human ES cells exposed to normoxic and hypoxic atmospheres. Buthionine sulfoximine (BSO) was used as a positive control as it has been shown to generate ROS in human ES cell cultures (Ji, et al., 2010).

As mentioned previously, the level of autofluorescence was measured in control untreated H9 and RH1 cells exposed under hypoxic and normoxic atmospheres without the H$_2$DCF-DA dye in all experiments. Also, any autofluorescence that
might have resulted from the test compounds themselves (each compound dissolved in water - in the absence of human ES cells) was determined and found to be not significant.

As can be seen in Figure 6.16, H9 cells demonstrated ROS generation within 24 hours with both concentrations of compounds. The untreated controls under hypoxia showed a significant increase over normoxia at both concentrations and time points. Cells exposed to an IC_{10} concentration under normoxia showed elevated ROS only in the case of valproic acid on day 1. Under hypoxia at day 1 only cadmium chloride and sodium arsenite increased ROS significantly, however, all compounds caused a considerably higher production of ROS by day 7. In addition, exposure of H9 cells to the lower concentration appeared to increase ROS levels under normoxia in all compound treatments except 5-azacytidine at day 1 but not at day 7. As in the case of the IC_{10} concentration under hypoxia, cells showed significant generation of ROS in all cases, the exception being 5-azacytidine at day 7. Moreover, hypoxic cultures demonstrated significantly higher ROS levels when compared to the normoxic conditions except for IC_{10} concentration valproic acid at day 1.
(A, IC$_{10}$ concentration)
(B, 3-log fold lower concentration)
Figure 6.16. Generation of ROS following treatment with 5-azacytidine, cadmium chloride, sodium arsenite and valproic acid under normoxia and hypoxia in H9 human ES cells. H9 cells were treated with each compound at an (A) IC_{10} and (B) a 3-log fold lower than IC_{10} concentration for seven days under normoxia and hypoxia. ROS levels were determined at days 1 and 7 using H_{2}DCF-DA. The DCF fluorescence was measured and error bars represent the mean ± SD of quadruplicate biological replicates. BSO was used as a positive control. Bar graphs represent six and seven independent experiments for day 1 and 7, respectively. The asterisks signify statistical difference (*p<0.05, **p<0.01, ***p<0.001) of compound-treated cultures with respect to untreated cultures under normoxia and hypoxia, respectively; as calculated by one-way
ANOVA with Dunnett’s post-hoc test. The statistical significance of the hypoxic cultures compared to the normoxic has been calculated by Student’s t-test (indicated with bracket). Abbreviations: ROS, reactive oxygen species; H$_2$DCF-DA: 2’,7’-dichlorodihydrofluorescein diacetate; DCF: 2’,7’-dichlorofluorescein; BSO: L-Buthionine sulfoximine.

IC$_{10}$ concentration significantly increased ROS levels in RH1 cells following treatment with cadmium chloride, sodium arsenite and valproic acid under normoxia on day 1 while under hypoxia only sodium arsenite caused a considerable increase (Figure 6.17). By day 7, none of the compounds induced ROS generation under normoxic conditions contrary to the hypoxic where all compounds showed significant elevations. Further, only cadmium chloride and sodium arsenite significantly increased ROS at the lower concentrations under normoxia at day 1, while under hypoxia all compounds, except 5-azacytidine, showed augmented ROS levels. At day 7, under normoxia none of the compound treatments produced ROS contrary to hypoxia where all compounds showed a significant increase in ROS. Similar to H9 cells, cultures under a hypoxic atmosphere showed a significantly higher ROS generation in comparison to normoxia for all conditions, with the exception of valproic acid at an IC$_{10}$ concentration on day 1.
(A, IC$_{10}$ concentration)
(B, 3-log fold lower concentration)
Figure 6.17. Generation of ROS following treatment with 5-azacytidine, cadmium chloride, sodium arsenite and valproic acid under normoxia and hypoxia in RH1 human ES cells. RH1 cells were treated with each compound at an (A) IC_{10} and (B) a 3-log fold lower than IC_{10} concentration for seven days under normoxia and hypoxia. ROS levels were determined on days 1 and 7 using H$_2$DCF-DA. The DCF fluorescence was measured and error bars represent the mean values ± SD of quadruplicate biological replicates. BSO was used as a positive control. Bar graphs represent six and seven independent experiments for day 1 and 7, respectively. The asterisks signify statistical difference (*p<0.05, **p<0.01, ***p<0.001) of compound-treated cultures with respect to untreated cultures under normoxia and hypoxia, respectively; as calculated by one-way ANOVA with Dunnett’s post-hoc test. The statistical significance of the hypoxic cultures compared to the normoxic has been calculated by Student’s t-test.
(indicated with bracket). Abbreviations: ROS, reactive oxygen species; H$_2$DCF-DA: 2',7'-dichlorodihydrofluorescein diacetate; DCF: 2',7'-dichlorofluorescein.

As before, a two-way ANOVA test was performed to test if the generation of ROS is affected by the compound treatment, the oxygen tension and the interaction between the two experimental factors. It was shown that for both test concentrations and cell lines the effects of the compound treatment or oxygen tension and the interaction between the two are considered extremely significant except in the case of RH1 cells exposed to the lower concentration which showed that the interaction between the two factors was not significant on day 1.

6.2.4. Compound treatment of 5-azacytidine, cadmium chloride, sodium arsenite and valproic acid under hypoxia induces HO-1 expression in human ES cells

The role of oxidative stress in environmental compounds such as arsenite and cadmium chloride has been investigated in toxicity studies by measuring the levels of heme oxygenase-1 (HO-1), a known redox-regulated protein (Teng, et al., 2013). HO-1 is also documented to act as a stress response gene (Lee, et al., 2005; Ruiz-Ramos, et al., 2009). Studies have demonstrated that treatment
of human cells with either sodium arsenite or cadmium chloride, apart from leading to the generation of ROS, induces the expression of HO-1 (Masuya, et al., 1998; Lee, et al., 2005; Ruiz-Ramos, et al., 2009; Lii, et al., 2011). Additionally, HO-1 is induced in response to hypoxia (Masuya, et al., 1998). Hence, the effects of the compounds on HO-1 mRNA expression levels was assessed as it has been identified as an oxidative stress responsive gene, and all test compounds generate ROS, especially under hypoxic atmospheric conditions. Quadruplicate biological samples of H9 and RH1 cells treated with each of the four test compounds under normoxia and hypoxia were assessed for HO-1 expression by qRT-PCR.

Figure 6.18 shows the expression levels of HO-1 in treated H9 cells maintained under normoxic and hypoxic atmospheres exposed to both tested concentrations. None of the compounds induced HO-1 expression under normoxia contrary to the significant induction under hypoxia compared to the untreated controls for both concentrations. Also, treated cultures under hypoxia were found to have significantly elevated levels of HO-1 compared to the normoxia.
(A, IC$_{10}$ concentration)
Figure 6.18. Compound treatment of 5-azacytidine, cadmium chloride, sodium arsenite and valproic acid under hypoxia induces HO-1 mRNA expression in H9 human ES cells. H9 cells were treated with each compound at an (A) IC$_{10}$ and (B) a 3-log fold lower than IC$_{10}$ concentration for seven days. Total RNA was isolated on day 7 and qRT-PCR was employed to assess the transcript levels of the redox-regulated HO-1. Observed changes are relative to β-actin expression and normalised to the untreated cultures. The expression levels in control untreated cells are set as 1.0. Asterisks indicate levels of statistical significance (**p<0.01, ***p<0.001) of compound-treated cultures with respect to untreated cultures under normoxia and hypoxia, respectively; as calculated by one-way ANOVA with Dunnett’s post-hoc test. Student’s t-test was performed for the comparison between hypoxia and normoxia for each compound treatment (indicated with bracket. Abbreviation: HO-1, Heme Oxygenase-1.)
Further, RH1 treated cells under normoxic conditions did not show any significant expression of HO-1 contrary to those maintained under hypoxia when exposed to the IC$_{10}$ concentration in comparison to the untreated controls. The same effects were observed when cells were exposed to the lower concentrations, except in the case of valproic acid. Hypoxic maintenance of compound-treated cells resulted in a significant induction compared to the normoxic atmosphere (Figure 6.19).

**A, IC$_{10}$ concentration**
Figure 6.19. Compound treatment of 5-azacytidine, cadmium chloride, sodium arsenite and valproic acid under hypoxia induces HO-1 mRNA expression in RH1 human ES cells. RH1 cells were treated with each compound at an (A) IC$_{10}$ and (B) a 3-log fold lower than IC$_{10}$ concentration for seven days. RNA was isolated on day 7 and qRT-PCR was employed to assess the transcript levels of HO-1. Data shown are normalised against β-actin and the expression levels in control untreated cells are set as 1.0. Asterisks indicate levels of statistical significance (*p<0.05, **p<0.01, ***p<0.001) of compound-treated cultures with respect to untreated cultures under normoxia and hypoxia, respectively; as calculated by one-way ANOVA with Dunnett’s post-hoc test. Student’s t-test was performed for the comparison between hypoxia and normoxia for each compound treatment (indicated with bracket). Abbreviation: HO-1, Heme Oxygenase-1.
In order to determine if the compound treatment, the oxygen tension or the interaction (if any) of these two factors affected the expression of HO-1, a two-way ANOVA test was performed for H9 and RH1 cells. Table 6.5 presents the summary of \( p \) values for both tested concentrations and human ES cell lines. As it can be seen, in both cell lines and concentrations all three cases showed significance.

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Table 6.5. Two-way ANOVA statistical analysis of the effects of compound treatment and oxygen tension on HO-1 expression in human ES cells. H9 and RH1 human ES cells were treated with 5-azacytidine, cadmium chloride, sodium arsenite and valproic acid at an IC\textsubscript{10} and a 3-log fold lower than IC\textsubscript{10} concentration under normoxic and hypoxic conditions. A two-way ANOVA statistical analysis was performed to demonstrate if the compound treatment, the oxygen tension or the interaction (if any) of these two factors affect the expression of HO-1. \( p \) values are presented for each case (compound treatment, oxygen tension and their interaction) for H9 and RH1 cells.
The analysis revealed that in both cell lines and tested compound concentrations all three cases showed statistical significance. Abbreviation: HO-1, Heme Oxygenase-1.

6.2.5. 5-Azacytidine, cadmium chloride, sodium arsenite and valproic acid cause depolarisation of the mitochondrial membrane in human ES cells

Studies have shown that arsenite causes ROS-related depolarisation of the mitochondrial membrane potential (Liu, et al., 2005; Ruiz-Ramos, et al., 2009). Observations that the test compounds in this study generated ROS led to ascertain the status of the mitochondrial membrane potential in H9 and RH1 human ES cells. The mitochondrial fluorescence dye, rhodamine 123, was used to assess any possible changes in human ES cell mitochondrial membrane potential with treatments under normoxia and hypoxia. Loss of rhodamine 123 from the mitochondria results in attenuated fluorescence intensity indicating depolarisation of the mitochondrial membrane. The fluorescent intensity for each quadruplicate biological sample for each concentration, compound treatment and oxygen tension was assessed for rhodamine 123 using a microplate reader.
In H9 cells, untreated controls showed no difference in rhodamine 123 fluorescence indicating that hypoxic exposure itself did not result in an alteration of the mitochondrial membrane potential (Figure 6.20). Under both normoxic and hypoxic atmospheres and at both subcytotoxic concentrations, all compounds led to a significant (**p<0.001) loss of rhodamine 123 fluorescence, indicating depolarisation of the membrane potential. Also, hypoxic and compound-treated cells showed significant reduction in rhodamine 123 when compared to the normoxic and compound-treated cells, with the exception of sodium arsenite and valproic acid at the lower concentration.

\[A, \text{IC}_{10} \text{ concentration}\]
Figure 6.20. Subcytotoxic effects of compound treatment on the MMP of H9 human ES cells under normoxia and hypoxia. H9 cells were treated with 5-azacytidine, cadmium chloride, sodium arsenite and valproic acid at an (A) IC_{10} and (B) a 3-log fold lower than IC_{10} concentration for seven days under normoxia and hypoxia. On day 7, the MMP was measured using Rhodamine 123 and the fluorescence intensity was determined. Error bars represent the mean values ± S.D. of quadruplicate biological replicates. Bar graphs represent three independent experiments. The asterisks signify statistical difference (***p<0.001) of compound-treated cultures with respect to untreated cultures under normoxia and hypoxia, respectively; as calculated by one-way ANOVA with Dunnett’s post-hoc test. Student’s t-test was performed for the comparison between hypoxia and normoxia for each compound treatment (indicated with bracket). Abbreviation: MMP, Mitochondrial Membrane Potential.
In RH1 cell cultures, the rhodamine 123 fluorescence was of similar intensity in the normoxic and hypoxic untreated controls (Figure 6.21). On the contrary, when cells were exposed to an IC$_{10}$ concentration under both oxygen tensions, all compound treatments led to a significant (***p<0.001) reduction when compared to the normoxic and hypoxic untreated controls. Also, all four groups of compound-treated cultures under hypoxia showed significantly reduced fluorescence, indicating significant depolarisation of mitochondrial membrane potential. Similar to the IC$_{10}$ concentration, exposure to the lower concentration resulted in a significant (***p<0.001) reduction of rhodamine 123 fluorescence under normoxia and hypoxia for all compound treatments. When comparing the compound-treated cultures under hypoxia to normoxia, only 5-azacytidine resulted in a significant decrease.
(A, IC$_{10}$ concentration)

(B, 3-log fold lower concentration)
Figure 6.21. Subcytotoxic effects of compound treatment on the MMP of RH1 human ES cells under normoxia and hypoxia. RH1 cells were treated with 5-azacytidine, cadmium chloride, sodium arsenite and valproic acid at an (A) IC_{10} and (B) a 3-log fold lower than IC_{10} concentration for seven days under normoxia and hypoxia. On day 7, the MMP was measured using Rhodamine 123 and the fluorescence intensity was determined. Error bars represent the mean values ± S.D. of quadruplicate biological replicates. Bar graphs represent three independent experiments. The asterisks signify statistical difference (**p<0.001) of compound-treated cultures with respect to untreated cultures under normoxia and hypoxia, respectively; as calculated by one-way ANOVA with Dunnett’s post-hoc test. Student’s t-test was performed for the comparison between hypoxia and normoxia for each compound treatment (indicated with bracket).

Lastly, to demonstrate if the compound treatment, the oxygen atmosphere or the interaction of these two experimental factors affected the mitochondrial membrane potential, a two-way ANOVA statistical test was performed for both human ES cell lines. Table 6.6 presents the p value summary for both cell lines and concentrations. As shown, in both cell lines and concentrations all cases demonstrated extreme significance except from the interaction between the two experimental factors in RH1 cells exposed to the lower compound concentration.
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Table 6.6. A two-way ANOVA statistical analysis of the effects of compound treatment and oxygen tension on the MMP in human ES cells. H9 and RH1 cells were treated with 5-azacytidine, cadmium chloride, sodium arsenite and valproic acid at an IC<sub>10</sub> and a 3-log fold lower than IC<sub>10</sub> concentration under normoxic and hypoxic conditions. A two-way ANOVA statistical analysis was carried out to demonstrate if the compound treatment, the oxygen tension or the interaction (if any) of the two factors affect the mitochondrial membrane potential. A summary of the p values are presented for each case (compound treatment, oxygen tension and their interaction) for H9 and RH1 cells. The statistical analysis revealed that in both cell lines and tested compound concentrations all three cases showed extreme levels of significance except from the interaction between the two experimental factors in RH1 cells exposed to the lower compound concentration. Abbreviations: ns, non significant; MMP, Mitochondrial Membrane Potential.
6.2.5.1 N-acetyl-cysteine (NAC) improves mitochondrial membrane potential in human ES cells following treatment with 5-azacytidine, cadmium chloride, sodium arsenite and valproic acid

As shown earlier, the ability of H9 and RH1 cells to maintain mitochondrial membrane potential is reduced upon treatment with each of the four test compounds under both normoxic and hypoxic culture conditions. This reduction was more pronounced in compound-treated cells maintained under hypoxia where ROS generation is significantly higher than the control cultures. Since, NAC is known as a redox modulator (Murphy, et al., 2011) and has been shown to protect against mitochondrial membrane drop (Liu, et al., 2005; Berniakovich, et al., 2012), the compound-treated human ES cell cultures under both oxygen atmospheres were treated with NAC to examine whether it improves the mitochondrial membrane potential of the cells.

Figure 6.22 presents the effect of NAC supplementation in H9 compound-treated cultures. Under normoxia and hypoxia, the mitochondrial membrane potential is still significantly lower (***(p<0.001) than the untreated controls at both subcytotoxic concentrations. Nevertheless, the mitochondrial membrane potential has recovered under the hypoxic compound-treated cultures at significantly higher levels compared to the normoxic compound-treated cultures,
except of valproic acid under normoxia at an IC$_{10}$ concentration and normoxia and hypoxia at the lower concentration. This observation suggests that the loss of mitochondrial membrane potential is ROS-dependent.

(A, IC$_{10}$ concentration)
(B, 3-log fold lower concentration)
Figure 6.22. NAC improves MMP following compound treatment in H9 human ES cells. H9 cells were treated with 5-azacytidine, cadmium chloride, sodium arsenite and valproic acid at an (A) IC\textsubscript{10} and (B) a 3-log fold lower than IC\textsubscript{10} concentration supplemented with the redox modulator NAC under normoxia and hypoxia for seven days. On day 7, the MMP was measured using Rhodamine 123 and the fluorescence intensity was recorded. Error bars represent the mean values ± S.D. of quadruplicate biological replicates. Bar graphs represent three independent experiments. The asterisks signify statistical difference (***p<0.001) of compound-treated cultures with respect to untreated cultures under normoxia and hypoxia, respectively; as calculated by one-way ANOVA with Dunnett’s post-hoc test. Student’s t-test was performed for the comparison between hypoxia and normoxia for each compound treatment (indicated with bracket). Abbreviation: NAC, N-acetyl-cysteine; MMP, Mitochondrial Membrane Potential.
Similar observations were made in RH1 compound-treated cultures as treatment with NAC demonstrated that under both oxygen atmospheres and concentrations, the mitochondrial membrane potential is considerably attenuated (**p<0.001) compared to the untreated controls (Figure 6.23). In parallel to H9 cells, the recovery of the mitochondrial membrane potential in the hypoxic compound-treated cultures compared to the normoxic was significant, with the exception being valproic acid under normoxia and hypoxia at the lower concentration.

(A, IC_{10} concentration)
(B, 3-log fold lower concentration)
Figure 6.23. NAC improves MMP following compound treatment in RH1 human ES cells. RH1 cells were treated with 5-azacytidine, cadmium chloride, sodium arsenite and valproic acid at an (A) IC$_{10}$ and (B) a 3-log fold lower than IC$_{10}$ concentration supplemented with the redox modulator NAC under normoxia and hypoxia for seven days. On day 7, the MMP was determined using Rhodamine 123 and the fluorescence intensity was measured. Error bars represent the mean values ± S.D. of quadruplicate biological replicates. Bar graphs represent three independent experiments. The asterisks signify statistical difference (**p<0.001) of compound-treated cultures with respect to untreated cultures under normoxia and hypoxia, respectively; as calculated by one-way ANOVA with Dunnett’s post-hoc test. Student’s t-test was performed for the comparison between hypoxia and normoxia for each compound treatment (indicated with bracket). Abbreviation: MMP, Mitochondrial Membrane Potential; NAC, N-acetyl-cysteine.
6.2.6. Hypoxia-inducible factor-1α (HIF-1α) expression is ROS-dependent

The test compounds used in this study are known to induce or inhibit the expression of HIF-1α, a regulator of the hypoxic response in mammalian cells. 5-azacytidine and sodium arsenite (Duyndam, et al., 2001; Arany, et al., 2011; Tian, et al., 2013) have been shown to induce HIF-1α expression while cadmium chloride and valproic acid have an inhibitory effect (Chun, et al., 2000; Kim, et al., 2007; Vengellur, et al., 2011; Lee and Kim, 2012). Furthermore, the expression of HIF-1α is enhanced by ROS (Chandel, et al., 1998), which all four test compounds are known to generate, especially under hypoxic conditions in this study. Hence, the expression levels of HIF-1α were determined by qRT-PCR for all compounds under normoxic and hypoxic culture conditions for both concentrations and human ES cell lines. Desferrioxamine (DFO) and cobalt chloride (CoCl₂) were used as positive reference compounds to ensure the stabilisation of HIF-1α under normoxic conditions (Groenman, et al., 2007; Lee, et al., 2013).

As the treatment end in this study is seven days and it is known that the expression of HIF-1α in human ES cells is lost after ~ 48 hrs under hypoxic conditions (Cameron, et al., 2008; Forristal, et al., 2010), it was essential to
investigate the expression of HIF-1α for the H9 and RH1 untreated cells under normoxia and hypoxia at different time points over the 7-day culture period. Quadruplicate biological samples were collected at days 1, 3 and 7 of culture and hypoxic H9 and RH1 human ES cells were compared directly to the normoxic. It was found that there is expression of HIF-1α at an mRNA level (Figure 6.24) under hypoxia but not of significance which compared with previous studies (Forsyth, et al., 2008; Westfall, et al., 2008; Forristal, et al., 2010). However, the expression of HIF-1α was significantly reduced by day 3 and eventually lost by day 7 of culture under hypoxia in both cell lines.

Figure 6.24. HIF-1α mRNA expression in H9 and RH1 human ES cells under hypoxia. H9 and RH1 cells were maintained under normoxia (20% O₂) and hypoxia (0.5% O₂) for seven days. Total RNA was isolated on days 1, 3 and 7 and qRT-PCR was employed to assess the transcript levels of HIF-1α. Data shown are normalised
against β-actin. The expression level in normoxic cells is set as 1.0. Asterisks indicate levels of statistical significance (*p<0.05, **p<0.001, ***p<0.0001) as calculated by Student’s t-test. × denotes that no target transcript was detectable.

Further, as shown in Figure 6.25, the positive reference compounds DFO and CoCl₂ successfully induced HIF-1α under normoxia. All four compounds under normoxic atmosphere at both subcytotoxic concentrations significantly downregulated the expression of HIF-1α, except at the lower concentration in RH1 valproic acid-treated cells where it is downregulated but not significantly. Under hypoxic culture conditions, HIF-1α is significantly upregulated in 5-azacytidine and sodium arsenite-treated H9 and RH1 cells at both concentrations while cadmium chloride and valproic acid significantly decreased the HIF-1α mRNA transcript levels. These observations corroborate with published work in other model systems that 5-azacytidine and sodium arsenite induce HIF-1α whereas cadmium chloride and valproic acid reduce it. Also, the effects of all compounds on HIF-1α expression under hypoxia were statistically significant when compared to the normoxic human ES cell cultures.
(A1, H9 hESCs, IC_{10} concentration)
(A2, H9 hESCs, 3-log fold lower concentration)
(B1, RH1 hESCs, IC$_{10}$ concentration)
Figure 6.25. The expression of HIF-1α in compound-treated human ES cells under normoxia and hypoxia. H9 (A1, A2) and RH1 (B1, B2) cells were treated with 5-azacytidine, cadmium chloride, sodium arsenite and valproic acid (1: IC$_{10}$ and 2: 3-log fold lower concentration) for seven days. RNA was isolated on day 7 and qRT-PCR was employed to assess mRNA levels of HIF-1α. DFO (100 μM) and CoCl$_2$ (100 μM) were used as positive reference compounds for the induction of HIF-1α under normoxia. Data normalised against β-actin and the expression level in control untreated cells is set as 1.0. The asterisks signify statistical difference (*p<0.05, **p<0.01, ***p<0.001) of compound-treated cultures with respect to untreated cultures under normoxia and hypoxia, respectively; as calculated by one-way ANOVA with Dunnett’s post-hoc test. The designated P values represent the statistical significance of hypoxic cultures against the normoxic for each test compound (indicated with bracket) as calculated by
Next, in order to examine whether ROS are responsible for the induction of HIF-1α in the compound-treated human ES cells, the effect of the antioxidant NAC, known to reduce ROS was examined under hypoxia at an IC_{10} concentration when ROS is shown to be predominantly elevated. Hence, H9 and RH1 cells were treated with all test compounds under hypoxic conditions for seven days in the presence and absence of NAC. Figure 6.26 shows that treatment with NAC significantly downregulates HIF-1α in H9 and RH1 cells in the case of 5-azacytidine and sodium arsenite contrary to its upregulation in the absence of NAC, indicating that HIF-1α expression is mediated by elevated levels of ROS. At the same time, HIF-1α was further significantly reduced with cadmium chloride and valproic acid treatment in both cell lines.
Figure 6.26. Compound-treated human ES cell cultures supplemented with the antioxidant NAC decreased HIF-1α expression under hypoxia. (A) H9 and (B) RH1 cells were treated with 5-azacytidine, cadmium chloride, sodium arsenite and valproic acid at an IC_{10} concentration under hypoxia for seven days. RNA was isolated on day 7 and qRT-PCR was employed to assess the transcript levels of HIF-1α. Data normalised against β-actin. The expression of HIF-1α is significantly reduced upon all compound treatments under hypoxia in both cell lines. Asterisks indicate levels of statistical significance (*p<0.05, **p<0.01, ****p<0.0001) of the hypoxic cultures supplemented with the antioxidant NAC against the hypoxic in the absence of NAC for each test compound as calculated by Student’s t-test. × denotes that no target transcript was detectable. Abbreviations: HIF-1α, Hypoxia Inducible Factor-1α; NAC, N-acetyl-cysteine; hESCs, human embryonic stem cells.

6.3 Discussion

The aim of this chapter was to establish a human ES cell-based toxicology test system with which to evaluate the subcytotoxic effects of compounds known to disrupt the epigenome and affect development. The results presented in previous chapters of this thesis identified epigenetically-defined biomarkers of pluripotent human ES cell identity which were then applied to the toxicology test in order to evaluate their sensitivity. Furthermore, the study was performed under normoxic and hypoxic conditions as hypoxic maintenance of ES cells resembles their original environment since early development occurs in low oxygen tensions, and some of the selected epigenetic biomarkers, such as the
dioxygenases TET1 and JMJD2C, require oxygen for their enzymatic activity. The potential mechanisms by which subcytotoxic exposure to the test compounds induced differentiation and how hypoxia exerted a protective effect against differentiation, were then investigated.

Exposure of human ES cells to the selected test compounds, 5-azacytidine, cadmium chloride, sodium arsenite and valproic acid, at two different subcytotoxic concentrations under normoxic conditions, induced differentiation, with the exception of valproic acid. On the contrary, exposure to hypoxic conditions, protected human ES cells against the differentiation-inducing effects of subcytotoxic exposure to 5-azacytidine and sodium arsenite but not that of cadmium chloride. Next, qRT-PCR analysis was performed to assess the effects of subcytotoxic exposure on undifferentiated human ES cell renewal and epigenetic biomarker expression and evaluate whether these effects were conserved in hypoxia which several studies using human ES cells have shown to be important for the maintenance of an undifferentiated cell state (Ezashi, et al., 2005; Forsyth, et al., 2006; Forristal, et al., 2010; Lim, et al., 2011; Närvä, et al., 2013). The data revealed that in normoxia, all compound treatments at subcytotoxic concentrations, except valproic acid, downregulated the majority of pluripotency and epigenetic biomarker expression whereas a hypoxic environment favoured the upregulation of these markers with the exception of cadmium chloride. This is consistent with the observed morphology. The two-
way ANOVA test showed that both the compounds and the oxygen tension individually had statistically significant effects on gene expression. In addition, the interaction between these two factors was also significant.

Interestingly, the expression of oxygenases (two of which belong to the epigenetically-defined biomarkers) appears to be higher under hypoxia (especially at an IC$_{10}$ concentration) with the exception of cadmium chloride. As mentioned previously, members of the TET and Jumonji-domain containing histone demethylases belong to the human superfamily of Fe(II) and 2-OG-depedent oxygenases which require molecular oxygen for their enzymatic activity. Since they utilise oxygen as a co-substrate in the hydroxylation and demethylation reactions it could be expected that their activity would be compromised under low oxygen tensions (Perez-Perri, et al., 2011). Accumulating evidence from various studies has demonstrated that even under hypoxia, most of the members of the Jumonji-domain containing histone demethylases are transcriptionally active (Beyer, et al., 2008; Pollard, et al., 2008; Wellmann, et al., 2008; Xia, et al., 2009; Yang, et al., 2009; Perez-Perri, et al., 2011). It has therefore been proposed that the induction of Jumonji-domain containing histone demethylases under low oxygen conditions is a compensatory mechanism in response to compromised oxygen availability (Xia, et al., 2009; Perez-Perri, et al., 2011). The study of Xia, et al. (2009), using different breast cancer cell lines exposed to 0.5% O$_2$ to investigate and identify
direct targets of HIF-1 transactivation reported that there was a marked enrichment for the 2-OG dioxygenase family. The authors further demonstrated that dioxygenases are HIF-1 targets and that JMJD2C is among those Jumonji-domain containing histone demethylases that are direct HIF-1 targets. Based on all these findings and our observations it could be suggested that the TET members are also HIF-1 targets acting as compensatory mechanisms in response to hypoxia (0.5% O$_2$). Future studies of similar work as in Xia, et al. (2009) to investigate whether TET1-3 are HIF-1 targets in human ES cells are required to test this hypothesis. Identification of HIF-1 binding sites by ChIP using human ES cells and siRNA knockdown of HIF-1$\alpha$ and HIF-2$\alpha$ in human ES cells under hypoxic conditions would provide evidence to support or reject this hypothesis.

Immunostaining and ELISA assays were performed to assess the effects of the test compounds on the epigenetic markers, 5-mC and 5-hmC. The results demonstrate that exposure of human ES cells to 5-azacytidine, cadmium chloride and sodium arsenite under normoxic conditions significantly attenuated the expression of 5-mC, while under hypoxia only the cadmium chloride-treated cells lost 5-mC expression. Taking into account that 5-azacytidine, cadmium chloride and sodium arsenite are known to inhibit DNMT activity (Takiguchi, et al., 2003; Tsuji-Takayama, et al., 2004; Reichard, et al., 2007; Braiteh, et al., 2008; Ren, et al., 2011), the expression of DNMT1, DNMT3A and DNMT3B
should also be confirmed with qRT-PCR. Moreover, the test compounds could cause a reduction or inhibition of DNMT activity via depletion of SAM, the methyl donor needed for methylation of DNA and histones by DNMTs (Cyr and Domann, 2011; Ren, et al., 2011). Upon exposure to oxidative stress, the need for GSH can surpass the cellular ability to produce it resulting in the decline of the methionine pool and subsequent reduction in SAM availability (Cyr and Domann, 2011). Therefore, it would worthwhile to examine the GSH production and SAM availability upon compound and/or different oxygen tension exposure of human ES cells.

Given that the test compounds used in this study are known to induce changes in the cellular antioxidant defence system and generate ROS, along with the capacity of hypoxia to trigger ROS generation, it was important to investigate the impact of treatments on the antioxidants GPX1, GPX4, SOD1 and SOD2. The qRT-PCR analysis revealed a decreased mRNA expression of the antioxidants GPX1, GPX4, SOD1 and SOD2 indicating oxidative stress. This result was further confirmed by the detection of ROS. The production of ROS was measured using the sensitive fluorescence dye H$_2$DCF-DA. As aforementioned, the brightness of the fluorescence is considered to reflect the extent to which ROS are present (Batandier, et al., 2002; Karlsson, et al., 2010). This work confirmed that hypoxia causes a significant increase in the production of ROS compared to normoxic controls. Although the generation of ROS under hypoxia
is a controversial issue, others have demonstrated (mitochondrial) ROS generation under hypoxic culture conditions (Chandel, et al., 1998; Brunelle, et al., 2005; Guzy, et al., 2005; Mansfield, et al., 2005; reviewed by López-Lázaro, 2006, Hamanaka and Chandel, 2009, Zepeda, et al., 2013).

Furthermore, the expression of HO-1 was assessed as it has been identified as a biomarker responsive to oxidative stress (Lee, et al., 2005; Ruiz-Ramos, et al., 2009) and is also induced in response to hypoxia (Masuya, et al., 1998). It has been reported that sodium arsenite and cadmium induce HO-1 expression (Lee, et al., 2005; Ruiz-Ramos, et al., 2009; Li, et al., 2011; Teng, et al., 2013). HO-1 was found to be significant upregulated by all four compounds under hypoxia indicating that the combination of low oxygen tension and compound exposure creates a more oxidative stressed environment, which concurs with the observation that ROS levels are more elevated under the same exposure combination. The two-way ANOVA statistical test revealed that both environmental factors contribute significantly to the observed effects on HO-1 expression and that the interaction between the two is also statistically significant. Additionally, the effect of the same experimental conditions on the mitochondrial membrane potential was determined and found to be significantly lower than the untreated control groups in all compound treatments and oxygen tensions with the 2-way ANOVA, indicating that the interaction of the two was statistically significant. The fact that supplementation of the treated cultures with
the redox-modulator NAC, known to prevent loss of mitochondrial membrane potential (Liu, et al., 2005), improves it shows that ROS are responsible for the observed loss in these cultures. It has to be noted that although NAC improved the mitochondrial membrane potential, it was still lower than the untreated normoxic and hypoxic controls. Given the increased ROS production, the possibility of DNA oxidative damage, especially since compounds such as arsenic which produce ROS have been shown to promote oxidative damage in mammalian cells, must be considered (Kessel, et al., 2002). Future work to check for biomarkers of oxidative damage, such as the formation of 8-hydroxyguanosine, is required.

Augmented levels of ROS during hypoxia have been documented to stabilise the transcription factor HIF-1α (Chandel, et al., 1998). In the present study, the expression of HIF-1α was monitored over seven days under normoxia and hypoxia (in the absence of compounds) and it was found to be expressed 24 hrs following hypoxic exposed which significantly decreased by day 3 and was depleted by day 7. This finding is consistent with previous studies where HIF-1α was shown to be at its highest level at 24 hrs in human ES cells exposed to hypoxia and reduced after seven days (Cameron, et al., 2008; Forristal, et al., 2010; Närväs, et al., 2013). HIF-1α was not expressed in normoxia but under hypoxia it was stabilised in the 5-azacytidine and sodium arsenite-treated human ES cells. The fact that HIF-1α is not expressed at day 7 in hypoxia,
indicates that the significant upregulation of HIF-1α is due to further exposure of the cells to 5-azacytidine and sodium arsenite. In contrast, human ES cells treated with cadmium chloride and valproic acid, known to inhibit HIF-1α (Chun, et al., 2000; Kim, et al., 2007; Vengellur, et al., 2011; Lee and Kim, 2012), did not display HIF-1α expression indicating that ROS is indeed the cause of HIF-1α induction. Since other studies have identified mitochondrial ROS production as a requirement for HIF-1α under hypoxia, it would be valuable to identify the source of ROS generation in this system. In order to do so, mitochondrial electron transport inhibitors, such as rotenone and myxothiazol, could be employed in future studies. The use of these inhibitors has been shown to prevent ROS production and inhibit the hypoxic induction of HIF-1α (reviewed in Hamanaka and Chandel, 2009, Zepeda, et al., 2013).

In order to further examine if HIF-1α expression was indeed due to the presence of ROS, compound-treated human ES cells were cultured in the presence of the antioxidant NAC which is known to reduce ROS. Antioxidant supplementation of the cultures resulted in the significant reduction of HIF-1α indicating that ROS are responsible for hypoxic HIF-1α induction. The observation that antioxidant treatment reduces the hypoxic HIF-1α response suggesting that ROS production is responsible for the propagation of the hypoxic signal has been presented in the study of Chandel, et al. (1998) where mitochondrial ROS were
shown to be required for the hypoxic activation of HIF-1α (Chandel, et al., 1998; Hamanaka and Chandel, 2009).

To date, a myriad of studies using various cellular models of involving hypoxia have supported their observations based on the dogma that HIF hydroxylases (PHDs and FIH) downregulate and activate HIF-α under normoxic conditions while under suppressing their activity in hypoxia due to the need for molecular oxygen for hydroxylation, thereby augmenting HIF-1α levels (Loenarz, et al., 2008; Zepeda, et al., 2013). A recent study (Närvä, et al., 2013), describing the hypoxic response of three different human ES cell lines (one of which is H9 used in the current study) has demonstrated for the first time that all HIF prolyl hydroxylase (PHD1-3) are expressed in human ES cells until day 7 of culture. The authors also reported that PHD2 and PHD3 were further induced under 24 hour hypoxic exposure in all human ES cells tested. It was the intention of this study to examine the expression of PHD1-3 as they belong to the same human 2-OG and Fe(II)-dependent superfamily of oxygenases as the TET1-3 and JMJD2C but unfortunately due to time limitations this has not been performed. However, following the observations of Närvä, et al. (2013), it is now necessary to test the response of PHDs at 0.5% O2 tension in order to examine if the response would be similar to that of the study at 4% O2. Furthermore, the study of Song, et al. (2011) examining the genome-wide distribution of 5-hmC in mouse cerebellum at different stages of development determined 5,425 genes
that were 5-hmC-enriched and identified pathways that were linked with neurodegenerative disorders, angiogenesis and hypoxia. Interestingly, all these pathways have been associated with oxidative stress (Song, et al., 2011). Hence, based on the observations from this study that there was 5-hmC enrichment in genes associated with hypoxia, the fact that the TETs require dioxygen to catalyse the oxidation of 5-mC to 5-hmC and the observations from the current study that TETs are significantly upregulated under hypoxia, one could speculate (as the authors of the Song, et al. study) that the oxidation of 5-mC to 5-hmC by TETs may also represent an additional oxygen-sensing pathway in mammalian cells (Song, et al., 2011).

In summary, a human ES cell-based toxicology test system has been developed which can be used to assess the subcytotoxic effects of compounds known to disrupt the epigenome and affect development. The system was used to assess their impact on the maintenance of an undifferentiated human ES cell state as reflected by alterations in pluripotency markers, epigenetically-defined biomarkers and changes in global 5-hmC levels as well as the expression of genes responsible for this epigenetic modification (TET1-3). The epigenetically-defined biomarkers of pluripotent human ES cell identity (GLIS2, HMGA1, PFDN5, JMJD2C and TET1) could serve as biomarkers for subcytotoxicity screenings of compounds at an epigenetic level as their expression has been shown to be altered upon compound exposure along with monitoring the
expression of 5-hmC. Furthermore, this study established that hypoxia protected compound-treated human ES cells against differentiation possibly via an oxidative mechanism that leads to the upregulation of TET and JMJD2C dioxygenases, known to play an essential role in the maintenance of a human ES cell undifferentiated state.
Chapter 7

General Discussion
Throughout their life, humans are exposed to a myriad of xenobiotics such as environmental pollutants, pharmaceutical agents and chemical compounds involving health risks which can result in abnormality of the offspring, when the exposure takes place during embryonic stages of development or tissue and organ damages, and even cancer development later in adulthood (Wobus and Löser, 2011). There is a battery of systems available assessing toxicity mainly based on transformed cell lines, primary cells, animal models or animal derived cells which involve high cost, ethical issues and most importantly do not sufficiently mirror the human responses. Hence, the evident need to develop more robust predictive toxicology models as close as possible to human physiology brought pluripotent stem cells into focus as a potential developmental system on predicting toxicities (Laustriat, et al., 2010; Wobus and Löser, 2011).

Nowadays, there is significant progress occurring in developing in vitro models for predictive toxicology using pluripotent stem cells. Human embryonic stem cells and their derivatives constitute potential models for investigating the effects of environmental toxicants and drug candidates on human health (Cezar, 2007; Pouton and Haynes, 2007; Laustriat, et al., 2010). At their undifferentiated state, ES cells can serve as a model for developmental toxicity while the derivative cell types such as hepatocytes, cardiomyocytes, keratinocytes and neurons are being used as models for hepatotoxicity, cardiotoxicity, skin toxicity and neurotoxicity, respectively (Laustriat, et al., 2010).
The overall aim of this thesis was to develop a human ES cell-based toxicology test system with which to evaluate the subcytotoxic effects of compounds on epigenetic determinants of undifferentiated human embryonic stem cell phenotype. Initially, in our group, Pells, et al. (unpublished) identified a group of ~200 hESC expressed genes whose associated CpG islands possess a unique and conserved human ESC-specific methylation patterns as compared with differentiated tissues (male and female blood, spleen, muscle, brain). Gene ontology analysis of these gene groups showed that expressed genes with associated CGIs that were unmethylated or methylated specifically in human ES cells were enriched by transcriptional activators and repressors, respectively. Subsequently, candidates were selected from the transcriptional activator (GLIS2 and HMGA1) and repressor (PFDN5) data sets in order to investigate a potential functional role in pluripotent human embryonic stem cell identity. Small interfering RNA targeting human ES cells of these three candidates along with the dioxygenases JMJD2C and TET1 mediating the hydroxymethylation of histones and DNA, respectively, as well as the other two members of the TET family TET2 and TET3 was performed. I have demonstrated that the individual knockdown of GLIS2, HMGA1, PFDN5, JMJD2C, TET1, TET2, TET3 and triple-knockdown of TET1-3 in two human ES cell lines (H9 and RH1) resulted in the loss of the undifferentiated human ES cell phenotype and pluripotency. Further, knockdown of these genes led to the induction of differentiation, loss of genome
hydroxymethylation and gene-dependent perturbation of germinal lineage associated with gene expression.

The same human ES cell lines were used and established a culture test system in order to evaluate the effects of subcytotoxic (IC_{10} and 3-log fold lower concentrations) exposures to compounds known to disrupt the epigenome and development in vivo. In this study, I have investigated a set of four test compounds (5-azacytidine, cadmium chloride, sodium arsenite and valproic acid) which have been selected based on their effect on germinal lineage determination and epigenetic mechanisms affecting gene expression and cellular phenotype. Both human ES cell lines were exposed to each compound and maintained under normoxic (20% O_2) and hypoxic (0.5% O_2) atmospheric culture conditions. The reasons why I chose to perform the study not only under normoxic but under hypoxic culture conditions as well are due to the fact that hypoxic maintenance of ES cells resembles the in vivo embryonic development, human ES cells have been shown to be favoured by hypoxia as low oxygen tensions improve morphology, diminish spontaneous differentiation, increase proliferation and enhance expression of pluripotency markers (Ezashi et al., 2005; Forsyth et al., 2006; Westfall, et al., 2008; Forristal, et al., 2010; Rajala, et al., 2011), and two of our selected epigenetic biomarkers (JMJD2C and TET1) require oxygen for their enzymatic function.
I have discovered that hypoxia significantly induces the generation of reactive oxygen species which is further augmented by the exposure of human ES cells to each of the four test compounds which are known to elevate ROS (Barchowsky, 1999; Na, et al., 2003; Tong, et al., 2005; Defoort, et al., 2006; Zhang, et al., 2010; Arany, et al., 2011; Sciandrello, et al., 2011; Tung and Winn, 2011; Pourahmad, et al., 2012; Tian, et al., 2013). This effect has been correlated with elevated expression of the dioxygenases, increased expression of pluripotency-associated markers and 5-hydroxymethylcytosine. Elevated reactive oxygen species in human ES cell cultures were accompanied by reduced expression of cellular antioxidant enzymes, augmented expression of heme oxygenase-1 (HO-1), a responsive gene to oxidative stress (Masuya, et al., 1998; Lee, et al., 2005; Ruiz-Ramos, et al., 2009; Li, et al., 2011; Teng, et al., 2013) and loss of mitochondrial membrane potential (MMP). Further, I have shown that 5-azacytidine, cadmium chloride and sodium arsenite but not valproic acid induced differentiation under an atmospheric environment which was correlated with loss of genome hydroxymethylation and induction of ROS. This effect was revealed to be blocked under hypoxic conditions, except in the case of cadmium chloride, along with augmented reactive oxygen species production, higher levels of pluripotency-associated markers and 5-hmC. In addition, an interesting finding was the elevation of HIF-1α in the cases of 5-azacytidine and sodium arsenite-treated human ES cells cultures under hypoxic culture conditions contrary to cadmium chloride and valproic acid-treated
cultures. The compounds 5-azacytidine and sodium arsenite have been known to induce HIF-1α (Duyndam, et al., 2001; Arany, et al., 2011; Tian, et al., 2013) while cadmium chloride and valproic acid inhibit HIF-1α expression (Chun, et al., 2000; Kim, et al., 2007; Vengellur, et al., 2011; Lee and Kim, 2012). Also, the expression of HIF-1α has been shown to be enhanced by ROS (Chandel, et al., 1998) which all four compounds have been shown to generate in this study; especially under hypoxic conditions. Hence, I then investigated whether ROS are the responsible source for the induction of HIF-1α in compound-treated human ES cells. To do so, all compound-treated human ES cell cultures were supplemented with the antioxidant N-acetyl-cysteine (a precursor of glutathione) which has been shown to reduce ROS at an IC_{10} concentration and upon hypoxic exposure due to the fact that those were the culture conditions where ROS was predominantly induced. N-acetyl-cysteine successfully reduced the expression of HIF-1α in all compound treatments opposite to its upregulation in the absence of the antioxidant demonstrating that the HIF-1α expression is mediated by elevated ROS levels in human ES cells.

Taken all together and given the evidence that ROS and dioxygenases are elevated under hypoxia; compounds such as 5-azacytidine and sodium arsenite induce the expression of HIF-1α, the antioxidant N-acetyl-cysteine attenuates HIF-1α and dioxygenases JMJD2C and TETs (Appendix XIV), I propose a
model showing the potential interactions between hypoxia, compounds which generate reactive oxygen species and elevation of HIF-1α and dioxygenases (Figure 7.1). As aforementioned, all the test compounds with the exception of valproic acid induce differentiation under normoxia. It has been confirmed by another study that ROS are responsible for inducing differentiation of human ES cells under normoxic conditions (Ji, et al., 2010). This study has demonstrated that treatment of human ES cells with buthionine sulfoximine, which is an inhibitor of intracellular glutathione and enriches ROS levels, generated ROS as detected using DCF-DA staining. On the contrary, upon hypoxic exposure, all our test compounds except cadmium chloride blocked differentiation. A possible sequence of events may include hypoxia-induced elevation of ROS which activates HIF-1α (López-Lázaro, 2006; Zepeda, et al., 2013), which in turn activates the expression of JMJD2C and TETs. Oxidation of histones and DNA by these enzymes may serve the twin benefit of reducing ROS, thereby protecting cells from DNA damage induced by ROS, and catalysing a more open configuration of chromatin and DNA methylation, both preserving a pluripotent cell phenotype by preventing silencing of core pluripotency transcription factors.
Figure 7.1 Model showing the potential relationships between hypoxia, compounds generating ROS, HIF-1α and dioxygenases JMJD2C and TETs.

It has been confirmed by other studies that Jumonji-domain containing demethylases are direct targets of the HIF transcription factor (Beyer, et al., 2008; Pollard, et al., 2008; Wellmann, et al., 2008; Krieg, et al., 2010; Melvin and Rocha, 2012). JMJD2C was included in this group of demethylases where under hypoxia and upon siRNA targeted against HIF-1α, JMJD2C expression
was significantly reduced (Pollard, et al., 2008). These findings evidently suggest the involvement of Jumonji-domain containing demethylases and dioxygenases in the response to hypoxia. Also, the fact that the antioxidant N-acetyl-cysteine decreases the expression of HIF-1α and subsequently the expression of dioxygenases JMJD2C and TETs (Appendix XIV) in my study reinforces the notion that JMJD2C is induced in a HIF-1α-dependent manner. This can be further explored by employing siRNA against HIF-1α under 0.5% hypoxia in the compound-test culture system to investigate whether JMJD2C and TETs will be downregulated. Also, this can be tested via exogenous overexpression of HIF-1α under normoxic conditions in order to examine if HIF-1α overexpression will increase the expression of JMJD2C and TETs (which otherwise are downregulated upon compound-treatment of 5-azacytidine, cadmium, chloride and sodium arsenite) and exert protection against differentiation.

Furthermore, treatment with cadmium chloride which creates oxidative stress in human ES cells but inhibits HIF-1α was shown to downregulate dioxygenases and maintain differentiation under hypoxic culture conditions, especially in H9 human ES cells, underlining the significant role of HIF-1α in our test culture system. Similar to cadmium chloride, valproic acid treatment led to an oxidative stressed culture environment and inhibit HIF-1α but showed to have maintained
the human ES cell undifferentiated phenotype both under normoxic and hypoxic conditions. Valproic acid is a histone deacetylase inhibitor (specific to class I HDACs) (Lee and Kim, 2012) and therefore would have the effect of inducing histone acetylation, and therefore leading to a more open configuration of chromatin preserving a pluripotent cell phenotype by preventing silencing of core pluripotent transcription factors. The fact that valproic acid inhibits HIF-1α but maintains pluripotency under hypoxia has been observed in mESC cultures as well (Lee and Kim, 2012). HIF-1α can be modulated by oxidative stress in various ways. There are cases where hypoxia generates reactive oxygen species and oxidative stress and elicits a cellular protective effect. Hypoxic preconditioning is shown to be protective in neurons as it involves activation of HIF-1 and downstream induction of HIF-1 target genes (Ma, et al., 2010). Specifically, the study of Liu, et al., (2005) has clearly demonstrated induction of HIF-1α via ROS under hypoxia. Induced HIF-1α and its downstream target erythropoietin were found to induce a neuroprotective effect in wild-type mouse neurons while this effect was lost in neurons from SOD1 transgenic mice, indicating that oxidative stress occurring during hypoxia is involved in neuroprotection (Liu, et al., 2005; Ma, 2010).

In order to strengthen the capacity and further develop our human ES cell-based toxicology model it would be desirable to perform siRNA targeting on more genes which possess a human ES cell unique and conserved pattern of CpG
island methylation from the gene sets already identified by Pells, et al. (unpublished data) in order to study their functional role in human ES cell pluripotency and effect on the epigenome. Genes that would affect the human ES cell undifferentiated state as the ones described in this study can subsequently be tested in our compound test culture system in order to identify additional biomarkers of a pluripotent stem cell phenotype whose epigenetic profile has been altered by test compounds and one could use to predict in vivo developmental toxicity. Additionally, the compound-treated human ES cells under normoxia were differentiated towards specific germinal lineages. 5-azacytidine induced both human ES cell lines towards endoderm (AFP), sodium arsenite towards mesoderm (Brachyury) and cadmium chloride towards mesoderm (Brachyury) for H9 cells and trophoblast (CDX2) for RH1 cells. It is absolutely necessary to screen more lineage-associate markers to substantiate germinal lineage specification. Confirmation of the methylation and hydroxymethylation status of these genes can serve as an early indication to predict whether a test compound can have a biased effect on germinal lineage specification. Further, future development can include the use of induced pluripotent stem cells to establish a more advanced pluripotent stem cell based model by enabling assessment of variations in toxicological response in relation to donor representative genetic polymorphism/mutations.
Further, the elevated ROS production, elevation of the responsive gene to oxidative stress HO-1, decline of cellular antioxidants and loss of mitochondrial membrane potential during hypoxia and upon compound treatment indicated that an oxidant stress is generated at low oxygen tensions in human ES cells and requires investigation into determining which cellular compartments in human ES cells is responsible for the oxidant signal seen during hypoxia. To do so, future work could utilise the technology of SERS nanosensors developed by Dr Campbell's group (University of Edinburgh) via the use of gold nanoparticles to measure SERS spectra. Modification of these particles with molecules whose SERS spectrum alters depending on oxidation state, the measurement of reduced and oxidized species and eventually the intracellular redox potential is feasible; especially since the nanoparticles can be targeted and retained in the cellular compartment of interest (Auchinvoile, et al., 2011).

In this study, a low oxygen, triple-gas incubator in which oxygen was displaced by nitrogen to achieve 0.5% O$_2$ atmosphere was utilized. The fact that human ES cells are unaffected by the very short exposure to atmospheric air while feeding the cultures is reflected in the observations of the unaffected undifferentiated human ES cell morphology and the fact that when cultures were exposed to atmospheric conditions the cells were differentiating. Also, I have further minimized the possibility of changes in oxygen tension by placing the culture media in the low oxygen incubator for several hours prior to feeding.
However, for future long-term hypoxia (e.g. one month or longer) experiments where more tissue culture passaging and feeding procedures will be required, full hypoxic workstation would be the most suitable equipment.

A myriad of studies nowadays have reported various epigenetic mechanisms that effectively regulate gene expression in cells. Among various stimuli responsible for epigenetic modifications is the oxidative stress (reviewed in Cyr and Domann, 2011). Oxidative stress can perturb several epigenetic enzymes in a direct and indirect manner by affecting the citric acid cycle (CAC) or causing impairment in SAM production (SAM being the methylating agent involved in DNA methylation) (Shahrzad, et al., 2007). Also, due to oxidative damage, modified bases in DNA can be formed and it has been suggested that in some cells the formation of 5-hmC could be the result of oxidative stress (Dahl, et al., 2011, Münzel, et al., 2011; Hitchler and Domann, 2012). Redox interactions with epigenetic processes can also assist or act as signals to drive normal development (reviewed in Cyr and Domann, 2011). This thesis has demonstrated the interactions forming between oxidative stress, hypoxic response and involvement of epigenetic enzymes such as the Jumonji-domain containing demethylases and the TET hydroxylases in human embryonic stem cells and provided further evidence into the notion that epigenetic events can be regulated by toxicological stimuli.
The human ES cell-based toxicology model established in this thesis offers the advantage of reducing significantly the need of primary cell lines and animal use for developmental toxicity by identifying genes whose silencing via epigenetic mechanisms in undifferentiated human ES cells could have the potential to act as early warning indicators for the effects of a toxicant or a test compound; and one could predict whether these can have a biased effect on embryonic lineage specification. One could also predict which exposure would be harmful for human health and provide aid in the diagnosis of various disorders (Arita and Costa, 2009; Baccarelli and Bollati, 2009).


models of Parkinson’s disease. Biochimica et Biophysica Acta (BBA) – Molecular Basis of Disease 1362 (1), 77-86.


and differentiation of human multipotent mesenchymal stromal cells. BMC Cell Biology 11: 11.


cytochrome c impairs cellular oxygen sensing and hypoxic HIF-alpha activation. Cell Metabolism 1 (6), 393-399.


### Appendix I

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</tr>
<tr>
<td>NANOG</td>
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List of primary and secondary antibodies used for immunocytochemistry with details about dilutions.
## Appendix II

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Cycling protocol used for qRT-PCR.
### Appendix III

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**List of primer sequences used for qRT-PCR.** *Primer sequences used in Paul de Sousa’s laboratory. §Primer sequences adapted from the literature. †Primer...*
sequences adapted from Prof A Sachinidis laboratory (i.e. for study comparison). §Piccoli, et al., (2007); Danet, et al., (2003).
### Appendix IV

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**List of sequences used for the small interference RNA experiments** (these sequences had been designed by previous members of the de Sousa laboratory).
siRNA-mediated knockdown controls YAP1 and OCT4 in H9 and RH1 human ES cells. H9 and RH1 cells were transfected with siRNA (twice, 24 hrs apart) directed against YAP1 (control), OCT4 (positive control) and IDS-null (negative control). RNA was harvested following siRNA treatment and transcript levels of YAP1 and OCT4 were assayed by qRT-PCR. The expression level of each gene was normalised to the GAPDH and expressed compared to the negative control IDS-null, which has a value of 1.0. Data sets from chapters 3, 4 and 5 of this thesis have been combined to demonstrate the successful knockdown of the genes YAP1 and OCT4. Asterisks indicating levels of statistical significance (****p<0.0001) as calculated by Student's t-test.
### Appendix VI

**(A) Genes with Me-CGI in Human ES cells**

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### (B) Genes with UnMe-CGI in Human ES cells

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**Human ES cell-specific gene associated methylation.** Genes whose associated CGI is (A) methylated in human ES cells and unmethylated in somatic tissues or (B) unmethylated in human ES cell and methylated in somatic tissues.
Appendix VII

Standard curve constructed by using a Control DNA Set and utilised to quantify the percentage of 5-hmC in DNA samples isolated from H9 human ES cells treated with siRNA against IDS-null, YAP1, OCT4, GLIS2, HMGA1 and PFDN5. The standard curve was constructed by plotting the absorbance (measured at 405 nm) values of the control DNA samples A-E included in the Quest 5-hmC™ DNA ELISA Kit (see Table 2.1). Data are plotted as absorbance (Y-axis) versus the percentage of 5-hmC (X-axis). Samples A and E are the negative and positive controls, respectively. The percentage of 5-hmC in any given DNA sample presented in Figure 3.17 was calculated using the linear equation:

\[ y = 0.822x + 0.0577 \]

with a coefficient of determination \( R^2 = 0.9866 \).
regression equation $y = a + bx$; in this case is $\% \text{ 5-hmC} = \frac{\text{absorbance} - y\text{-intercept}}{\text{Slope}} \Rightarrow \% \text{ 5-hmC} = \frac{\text{absorbance} - 0.0577}{0.822}$. 
Appendix VIII

Standard curve constructed by using a Control DNA Set and utilised to quantify the percentage of 5-hmC in DNA samples isolated from RH1 human ES cells treated with siRNA against IDS-null, YAP1, OCT4, GLIS2, HMGA1 and PFDN5. The standard curve was constructed by plotting the absorbance (measured at 405 nm) values of the control DNA samples A-E included in the Quest 5-hmC™ DNA ELISA Kit (see Table 2.1). Data are plotted as absorbance (Y-axis) versus the percentage of 5-hmC (X-axis). Samples A and E are the negative and positive controls, respectively. The percentage of 5-hmC in any given DNA sample presented in Figure 3.20 was calculated using

\[
y = 0.8235x + 0.0568 \\
R^2 = 0.9882
\]
the linear regression equation $y = a + bx$; in this case is $% 5$-hmC = (absorbance – y-intercept) / Slope $\Rightarrow$ $% 5$-hmC = (absorbance – 0.0568) / 0.8235).
Standard curve constructed by using a Control DNA Set and utilised to quantify the percentage of 5-hmC in DNA samples isolated from H9 human ES cells treated with siRNA against IDS-null, YAP1, OCT4, TET1, TET2, TET3 and TET1-3. The standard curve was constructed by plotting the absorbance (measured at 405 nm) values of the control DNA samples A-E included in the Quest 5-hmC™ DNA ELISA Kit (see Table 2.1). Data are plotted as absorbance (Y-axis) versus the percentage of 5-hmC (X-axis). Samples A and E are the negative and positive controls, respectively. The percentage of 5-hmC in any given DNA sample presented in Figure 4.18 was calculated using
the linear regression equation $y = a + bx$; in this case is $\% \ 5$-hmC = (absorbance – y-intercept) / Slope $\Rightarrow \% \ 5$-hmC = (absorbance – 0.0563) / 0.8243.
Appendix X

Standard curve constructed by using a Control DNA Set and utilised to quantify the percentage of 5-hmC in DNA samples isolated from RH1 human ES cells treated with siRNA against IDS-null, YAP1, OCT4, TET1, TET2, TET3 and TET1-3. The standard curve was constructed by plotting the absorbance (measured at 405 nm) values of the control DNA samples A-E included in the Quest 5-hmC™ DNA ELISA Kit (see Table 2.1). Data are plotted as absorbance (Y-axis) versus the percentage of 5-hmC (X-axis). Samples A and E are the negative and positive controls, respectively. The percentage of 5-hmC in any given DNA sample presented in Figure 4.19 was calculated using the linear regression equation \( y = a + bx \); in this case is \( \% \) 5-hmC = (absorbance – y-intercept) / Slope \( \Rightarrow \% \) 5-hmC = (absorbance – 0.057) / 0.83).
Appendix XI

Standard curve constructed by using a Control DNA Set and utilised to quantify the percentage of 5-hmC in DNA samples isolated from H9 human ES cells treated with siRNA against IDS-null, YAP1, OCT4 and JMJD2C. The standard curve was constructed by plotting the absorbance (measured at 405 nm) values of the control DNA samples A-E included in the Quest 5-hmC™ DNA ELISA Kit (see Table 2.1). Data are plotted as absorbance (Y-axis) versus the percentage of 5-hmC (X-axis). Samples A and E are the negative and positive controls, respectively. The percentage of 5-hmC in any given DNA sample presented in Figure 5.12 was calculated using the linear regression equation \( y = a + bx \); in this instance is \( \% \text{ 5-hmC} = \frac{\text{absorbance} - y \text{-intercept}}{\text{Slope}} \Rightarrow \% \text{ 5-hmC} = \frac{\text{absorbance} - 0.0566}{0.8282} \).
Standard curve constructed by using a Control DNA Set and utilised to quantify the percentage of 5-hmC in DNA samples isolated from RH1 human ES cells treated with siRNA against IDS-null, YAP1, OCT4 and JMJD2C. The standard curve was constructed by plotting the absorbance (measured at 405 nm) values of the control DNA samples A-E included in the Quest 5-hmC™ DNA ELISA Kit (see Table 2.1). Data are plotted as absorbance (Y-axis) versus the percentage of 5-hmC (X-axis). Samples A and E are the negative and positive controls, respectively. The percentage of 5-hmC in any given DNA sample presented in Figure 5.14 was calculated using the linear regression equation $y = a + bx$; in this instance is $\%~5\text{-hmC} = (\text{absorbance} - y\text{-intercept}) / \text{Slope} \Rightarrow \%~5\text{-hmC} = (\text{absorbance} - 0.0564) / 0.8248$. 
Range finder compound (valproic acid) cytotoxicity curve for effect on viability of human ES cells (H9 and RH1) following seven days of culture. Cell-Titer Blue Viability Assay was performed. IC$_{10}$ values at which exposure to valproic acid resulted at 10% reduction of cell viability compared with untreated control. The green-coloured point indicates the IC$_{10}$ concentration. Table indicates the tested concentrations.

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Sodium arsenite-treated human ES cells supplemented with the antioxidant NAC decreased the expression of dioxygenases under hypoxia. H9 and RH1 cells were treated with sodium arsenite at an IC$_{10}$ concentration for seven days. RNA was isolated at day 7 and qRT-PCR was performed to assess the transcript levels of dioxygenases JMJD2C, TET1, TET2
and TET3. Data normalised against β-actin. The asterisks indicate levels of statistical significance (***p<0.001, ****p<0.0001) of the hypoxic cultures supplemented with the antioxidant NAC against the hypoxic in the absence of NAC as calculated by Student’s t-test.