The role of E-cadherin in mouse embryonic stem cell pluripotency

A thesis submitted to the University of Manchester for the degree of Doctor of Philosophy in the Faculty of Medical and Human Sciences

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School of Dentistry
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# Abbreviations

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<th>Description</th>
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<tbody>
<tr>
<td>AFP</td>
<td>α-fetoprotein</td>
</tr>
<tr>
<td>Alk</td>
<td>Activin receptor-like kinase</td>
</tr>
<tr>
<td>Aza</td>
<td>5-aza-2’-deoxycytidine</td>
</tr>
<tr>
<td>AJ</td>
<td>Adherens Junction</td>
</tr>
<tr>
<td>BD</td>
<td>Becton Dickinson</td>
</tr>
<tr>
<td>BMP</td>
<td>Bone morphogenetic protein</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CCC</td>
<td>Cytoplasmic cell adhesion complex</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
</tr>
<tr>
<td>cRNA</td>
<td>Complementary RNA</td>
</tr>
<tr>
<td>CpG</td>
<td>Cytosine-guanine</td>
</tr>
<tr>
<td>Ct</td>
<td>Cycle threshold</td>
</tr>
<tr>
<td>DAPI</td>
<td>4',6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DENT</td>
<td>Dysregulation of E-cadherin in neoplasm and tumourigenesis</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulphoxide</td>
</tr>
<tr>
<td>EB</td>
<td>Embryoid body</td>
</tr>
<tr>
<td>EC</td>
<td>Embryonal carcinoma</td>
</tr>
<tr>
<td>ECD</td>
<td>Extracellular cadherin domain</td>
</tr>
<tr>
<td>EGFR</td>
<td>Epidermal growth factor receptor</td>
</tr>
<tr>
<td>EMT</td>
<td>Epithelial-to-mesenchymal transition</td>
</tr>
<tr>
<td>Eno2</td>
<td>Enolase 2</td>
</tr>
<tr>
<td>ENPS</td>
<td>E-cadherin negative proliferating stem</td>
</tr>
<tr>
<td>EOMES</td>
<td>omesodermin</td>
</tr>
<tr>
<td>EphR</td>
<td>Ephrin receptor</td>
</tr>
<tr>
<td>EpiSC</td>
<td>Epiblast-derived stem cell</td>
</tr>
<tr>
<td>EPLIN</td>
<td>Epithelial protein lost in neoplasm</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular receptor kinase</td>
</tr>
<tr>
<td>ES</td>
<td>Embryonic stem</td>
</tr>
<tr>
<td>F-actin</td>
<td>Filamentous actin</td>
</tr>
<tr>
<td>FAB-SC</td>
<td>FGF, Activin- and BIO-derived stem cell</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>FC</td>
<td>Fold change</td>
</tr>
<tr>
<td>FGFR</td>
<td>Fibroblast growth factor receptor</td>
</tr>
<tr>
<td>FL</td>
<td>Full length</td>
</tr>
<tr>
<td>GCOS</td>
<td>GeneChip Operating Software</td>
</tr>
<tr>
<td>GO</td>
<td>Gene ontology</td>
</tr>
<tr>
<td>Grb2</td>
<td>Growth factor receptor-bound protein 2</td>
</tr>
<tr>
<td>GSK3β</td>
<td>Glycogen synthase kinase 3 β</td>
</tr>
<tr>
<td>HAV</td>
<td>Histidine-alanine-valine</td>
</tr>
<tr>
<td>hES</td>
<td>Human embryonic stem</td>
</tr>
<tr>
<td>hiPS</td>
<td>Human induced pluripotent stem</td>
</tr>
<tr>
<td>HNF4</td>
<td>Hepatic nuclear factor 4</td>
</tr>
<tr>
<td>ICM</td>
<td>Inner cell mass</td>
</tr>
<tr>
<td>IGFR</td>
<td>Insulin-like growth factor receptor</td>
</tr>
<tr>
<td>Id</td>
<td>Inhibitor of differentiation</td>
</tr>
<tr>
<td>iPS</td>
<td>Induced pluripotent stem</td>
</tr>
<tr>
<td>Jak</td>
<td>Janus-associated kinase</td>
</tr>
<tr>
<td>Jam2</td>
<td>Junctional adhesion molecule 2</td>
</tr>
<tr>
<td>Klf4</td>
<td>Kruppel-like factor 4</td>
</tr>
<tr>
<td>LIF</td>
<td>Leukaemia inhibitory factor</td>
</tr>
<tr>
<td>LIFR</td>
<td>LIF receptor</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MCS</td>
<td>Multiple cloning site</td>
</tr>
<tr>
<td>MEF</td>
<td>Mouse embryonic fibroblasts</td>
</tr>
<tr>
<td>MEF2c</td>
<td>Myocyte enhancing factor 2c</td>
</tr>
<tr>
<td>mES</td>
<td>Mouse embryonic stem</td>
</tr>
<tr>
<td>MET</td>
<td>Mesenchymal-to-epithelial transition</td>
</tr>
</tbody>
</table>
miRNA MicroRNA
MPECD Membrane proximal extracellular domain
mRNA Messenger RNA
Myh Myosin heavy chain
Myl Myosin light chain
Nef1 Neurofilament 1
P Phospho
p120ctn p120 catenin
p3 Passage 3
PBS Phosphate buffered saline
PcG Polycomb group
PCR Polymerase chain reaction
PE Phycoerythrin
PFA Paraformaldehyde
PI Propidium iodide
PI3K Phosphatidylinositol-3-kinase
PIPKIγ Type Iγ PI phosphate kinase
PRC Polycomb repressive complex
PS1 Presenillin 1
PTP Phosphotyrosine phosphatase
qPCR Quantitative PCR
RIPA Radioimmunoprecipitation assay
RNA Ribonucleic acid
RNAi RNA interference
RT Reverse transcription
RTK Receptor tyrosine kinase
Runx1 Runt-related transcription factor 1
SEM Standard error of the mean
SIP1 Smad-interacting protein 1
SR Serum replacement
SSEA-1 Stage-specific embryonic antigen-1

STAT3 Signal transducer and activator of transcription
T Brachyury
Tbx3 T-box factor 3
TFR Transferrin
TGFβ Transforming growth factor β
Trp2 Tryptophan 2
TTR Transthyretin
U Units
UTR Untranslated region
Wt Wild type
YAP Yes-associated protein
Abstract

To exploit pluripotent cells for regenerative medicine applications it will be necessary to understand the molecular mechanisms that govern pluripotency and lineage commitment within these cells. The mechanism by which LIF sustains ‘naïve’ pluripotency in mouse embryonic stem (mES) cell has recently been delineated; LIF signals to the core circuitry of pluripotency (Oct4, Sox2 and Nanog) via Jak/STAT3 and PI3K/Akt-mediated expression of Klf4 and Tbx3 respectively. E-cadherin has been shown to be required for LIF-dependent mES cell pluripotency since cell lines exhibiting low/no E-cadherin expression maintain pluripotency via Activin/Nodal. However, these cells maintain expression of the core circuitry of pluripotency, thus the role of E-cadherin in pluripotency remains elusive. To investigate this, we have characterised an E-cadherin negative proliferating stem (ENPS) cell line, generated by seeding wt mES cells at low density in the absence of LIF. These cells exhibit Activin/Nodal-dependent pluripotency marker expression but fail pluripotency tests such as EB differentiation and chimera generation and microarray analysis shows they lack naïve transcripts and express early lineage markers. We have also exploited two additional E-cadherin negative cell lines (Ecad⁻/ and EcadRNAi mES cells) to delineate the molecular mechanisms connecting E-cadherin to the core circuitry of pluripotency. These cells exhibit decreased expression of pluripotency markers Klf4 and Nanog, the latter a direct consequence of a lack of E-cadherin-mediated STAT3 activation. Interestingly, both ENPS and Ecad⁻/ mES cells can be ‘rescued’ to a naïve pluripotent state upon LIF stimulation. In ENPS cells, LIF supplementation induces restoration of E-cadherin expression, LIF-dependent pluripotency and EB and chimera generation abilities. In Ecad⁻/ mES cells, LIF supplementation restores LIF-dependent pluripotency via N-cadherin, thus demonstrating a novel role for N-cadherin in mES cell pluripotency. At high passage, ENPS cells (like some cancer cells) exhibit methylation of the E-cadherin promoter and PI3K-dependent increased survival compared to wt mES cells. Our findings provide a potential mechanism for the role of E-cadherin in induced pluripotent stem (iPS) cell generation, since STAT3 phosphorylation has recently been shown to be a limiting factor in this process. In addition, our data suggest E-cadherin can be manipulated to direct differentiation for regenerative medicine applications since ENPS cells exhibit a lineage bias towards neuroectoderm at the expense of endoderm specification.
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The Author

After achieving a 2.1 in Molecular Cell Biology from the University of York in 2008, during which I carried out a final year project in Dr Paul Genever’s lab entitled ‘Analysis of mesenchymal stem cell-endothelial cell interactions and their implications for tissue engineering,’ I pursued an MRes in Tissue Engineering for Regenerative Medicine at the University of Manchester. As part of my MRes I completed a 10 week research project in Dr Cathy Merry’s lab focusing on the role of 2-O-sulphated heparan sulphate in neural differentiation of mouse embryonic stem cells and a 25 week research project in Dr Chris Ward’s lab investigating the role of cell surface molecules in mouse embryonic stem cell pluripotency. After securing funding from the BBSRC to support my PhD (supervised by both Cathy and Chris) I continued this research, focusing specifically on the role of the cell adhesion molecule E-cadherin in mouse embryonic stem cell pluripotency.

Rationale for submitting a thesis in an alternative format

My rationale for submitting my thesis in an alternative format is primarily to facilitate manuscript submission. At the time of writing one manuscript has been published online in the journal Stem Cells and the other is being prepared for submission to Nature. This format is also preferred by the School of Dentistry in comparison to the standard format.

My thesis is structured in the following way: the general introduction and materials and methods sections are equivalent to those present in a standard PhD thesis. Chapter 3 constitutes the primary study of my PhD, investigating the role of E-cadherin in LIF-dependent pluripotency in mES cells, for which I generated all data. This manuscript has been accepted for publication in the journal Stem Cells (in press) and can be found in this format towards the end of this thesis. Chapter 4 outlines the characterisation of a novel E-cadherin negative proliferating stem cell line derived from mES cells. This study includes the initial characterisation of this cell line, carried out by Dr Francesca Soncin, Dr Chris Ward and Dr Maria Keramari and myself in addition to the cell counts, Annexin V/Dead cell apoptosis analysis.
and microarray analysis carried out by myself. At the time of writing, this manuscript, for which I am the first author, is in preparation for submission to Nature. Chapter 5 constitutes a general discussion of the results and their implications for the wider research field.

Acknowledgements

I would firstly like to thank my two co-supervisors, Chris and Cathy, for their support throughout my research. I would also like to thank all Ward and Merry lab members, past and present, particularly Sarah Ritson for her patience in teaching me lab techniques and Lisa Mohamet for her practical advice and help with my academic writing.

I would also like to thank the BBSRC and The School of Dentistry for funding this project. Last but not least, I’d like to thank my friends and family for their support and enthusiasm for my work throughout the past three years.
Chapter 1

General Introduction
Chapter 1 General Introduction

1.1 The biological roles of cadherins

Cell-cell contacts such as tight junctions, adherens junctions (AJs) and desmosomes are essential for tissue integrity and morphogenesis. Members of the cadherin superfamily, which can be subdivided into five main categories (type I classical cadherins, type II non-classical cadherins, desmosomal cadherins, protocadherins and other cadherin-related molecules), contribute to the formation of these complexes. For example, type I classical cadherins are key components of AJs and desmosomal cadherins such as desmoglein and desmocollin mediate desmosomal cell-cell contacts (Cavallaro and Christofori, 2004).

1.1.1 E-cadherin

Epithelial cadherin, or E-cadherin, is the prototype type I classical cadherin. Like all members of this group, E-cadherin is a single-span transmembrane glycoprotein and, as such, possesses both extracellular and intracellular domains. The extracellular domains of type I classical cadherins comprise five subunits, termed extracellular cadherin domains (ECDs). These ECDs are responsible for assembling the cis- and trans-homodimers that allow AJ generation. Although there is some controversy regarding the precise functions of distinct regions of E-cadherin in cell-cell adhesion, numerous studies have shown that the histidine-alanine-valine (HAV) domain, located at residues 79-81 of the most distal subunit, ECD1, is critical for cell-cell adhesion. For example, CHAVC, a peptide containing the HAV sequence, has been shown to abrogate cadherin-mediated cell-cell contact (Erez et al., 2004; Figure 1.1). A model for trans-homodimerisation whereby the tryptophan residue at position 2 (trp2) docks into a hydrophobic pocket created by the HAV domain on an adjacent cadherin molecule has been evidenced by mutations of trp2 and the alanine residue within the HAV domain, which abolish cadherin trans-homodimerisation (Pertz et al., 1999). However, the exact mechanism of AJ formation remains elusive, despite the linear zipper model being well-documented. Interestingly, the membrane proximal extracellular domain (ECD5) has also been
shown to be essential for adhesion since DECMA-1 binding to this domain has been shown to abolish cell-cell contact (Ozawa et al., 1990; Figure 1.1).

The intracellular domain of classical cadherins forms the cytoplasmic cell adhesion complex (CCC) through its interaction with various catenin molecules (including β-catenin and p120-catenin [p120<sup>ctn</sup>]). These interactions are required for cadherin-mediated adhesion since deletion of either the C-terminus or a membrane proximal sequence within the cytoplasmic region of classical cadherins has been shown to abrogate cell-cell contact due to uncoupling of β-catenin and p120<sup>ctn</sup> respectively (Kintner, 1992). In addition, mutation of α-catenin, which binds β-catenin within the CCC, has been shown to result in loss of cell-cell adhesion (Torres et al., 1997).

Eplithelial protein lost in neoplasm (EPLIN) is another key component of this complex. EPLIN has been shown to connect α-catenin to filamentous (F)-actin, thus anchoring the CCC to the cell cytoskeleton (Figure 1.2; Abe and Takeichi, 2008). Interestingly, in addition to its role in cell-cell adhesion, E-cadherin has been shown to play a key role in a diverse range of cellular processes. For example, microarray analysis of mouse (m) embryonic stem (ES) cells lacking the E-cadherin gene (Ecad<sup>−/−</sup> mES cells) has revealed additional roles for E-cadherin in metabolism, cell cycle control, apoptosis and multicellular organismal development (Soncin et al., 2011).
Figure 1.1. The 'linear zipper' model for cadherin-mediated cell-cell adhesion. A linear zipper model for classical cadherin-mediated cell-cell adhesion has been proposed whereby the HAV motif in EC1 creates a hydrophobic pocket for the Trp2 residue on an adjacent cadherin molecule to dock into. Binding to the HAV motif in ECD1 or ECD5, by CHAVC and DECMA-1 respectively, abrogates cadherin-mediated cell-cell adhesion. ECD: extracellular cadherin domain.
**Figure 1.2. E-cadherin and the CCC.** E-cadherin cell-cell contacts are anchored to F-actin via β-catenin, α-catenin and EPLIN. EPLIN: epithelial protein lost in neoplasm, p120<sup>ctn</sup>: p120 catenin, F-actin: filamentous actin.
1.1.1A E-cadherin expression in embryogenesis and tumorigenesis

Throughout embryogenesis, cadherins exhibit distinct spatio-temporal expression patterns, thus facilitating the tissue segregation that is essential for the development of an organism. The preference for homophilic binding exhibited by these molecules is key to their role in tissue segregation. One mechanism by which embryo morphogenesis is achieved is via epithelial-to-mesenchymal transition (EMT), which involves concomitant downregulation of E-cadherin and upregulation of N-cadherin, causing cells to lose polarity and acquire a more motile phenotype. EMT is initiated by Zinc-finger containing transcription factors such as Snail, Smad-interacting protein 1 (Sip1) and Slug and the basic helix-loop-helix transcription factor E12/E47 binding the E-cadherin promoter and repressing expression (Comjín et al., 2001; Cano et al., 2000; Hajra et al., 2002; Perez-Moreno et al., 2001; reviewed in Cavallaro and Christofori, 2004).

E-cadherin is the first cadherin to be expressed within the embryo, and its key role throughout embryogenesis is evidenced by the observation that 14% of genes regulated by E-cadherin in mES cells are involved in multicellular organismal development (Soncin et al., 2011). E-cadherin is initially expressed at the two cell stage and is maintained throughout compaction of the morula whereby it facilitates the segregation of the inner cell mass (ICM) from the surrounding trophectoderm epithelium within the blastocyst. Consequently, Ecad−/− embryos fail to complete this process, leading to arrested development prior to implantation (Larue et al., 1994). At later stages, E-cadherin is downregulated in an EMT event that allows cells to ingress the primitive streak during gastrulation, thus facilitating segregation of the three germ layers: endoderm, ectoderm and mesoderm (Hatta and Takeichi, 1986). EMT also occurs in the neural crest, allowing these cells to migrate away from the neural tube and give rise to neurons, glia and melanocytes along with the cartilage, bone and tissue of the cranium (Figure 1.3; Trainor et al., 2003).

EMTs also occur in the adult organism, where they are required for tissue regeneration and wound healing (Kalluri, 2009). However, aberrant EMT-like events
have also been implicated in metastasis of tumour cells, whereby these cells disseminate from the original tumour to a secondary site. This observation is of clinical importance since metastasis is the primary cause of death amongst cancer patients (Weigelt et al., 2005). Expression of E-cadherin in various cancer cell lines has therefore been studied extensively. For example, methylation of the E-cadherin promoter (in response to its transcriptional inactivation) has been observed in numerous cancer cells including gastric (Tamura et al., 2000), breast and prostate (Graff et al., 1995) and exposure to the DNA methyltransferase inhibitor 5-aza-2'-deoxycytidine (Aza) has been shown to reduce metastasis (Nam et al., 2004).

EMT is not an irreversible process, and mesenchymal-to-epithelial transitions (METs) also occur throughout embryogenesis and tumorigenesis. For example, during development MET-like events are involved in the terminal differentiation events involved in the formation of various organs (Davies, 1996; Li et al., 2011; Nakajima et al., 2000; Nakaya et al., 2004). Similarly, following the EMT-like event that allows dissemination of tumour cells to a secondary tumour site, cancer cells have been shown to revert via MET to an epithelial phenotype (reviewed in Yang and Weinberg, 2008).
Figure 1.3. Simplified diagram of mammalian embryogenesis. Following fertilisation, the embryo enters the cleavage stage whereupon it undergoes a number of cell divisions. The morula then undergoes compaction, where E-cadherin expression is crucial for the separation of the ICM and the trophectoderm. The naïve epiblast is then formed and the embryo implants into the wall of the uterus, whereupon the epiblast becomes primed for differentiation. E-cadherin is then downregulated in two key EMT events, gastrulation and neural crest migration. EMT: epithelial-to-mesenchymal transition, ICM: inner cell mass.
1.1.1B The role of E-cadherin in cell signalling

In addition to its physical role in tissue morphogenesis and tumourigenesis, E-cadherin has been shown to modulate the activity of various signalling cascades involved in these and other processes. Due to its sequestration of β-catenin, a key component in the canonical Wnt signalling pathway, many groups have postulated that E-cadherin expression may prevent transactivation of β-catenin target genes. There is some evidence for such a link between E-cadherin and Wnt activation (Orsulic et al., 1999), however, contradictory evidence suggests that β-catenin exists as two discrete functional pools, one of which binds E-cadherin and the other of which is involved in gene activation (Gottardi and Gumbiner, 2004). Importantly, data obtained by Soncin et al. (2011) in mES cells supports the latter hypothesis, since loss of E-cadherin in these cells did not induce transactivation of β-catenin target genes.

E-cadherin has also been shown to be involved in repressing ligand activation of various receptor tyrosine kinases (RTKs) such as the hepatocyte growth factor receptor c-Met (Davies et al., 2001), epidermal growth factor receptor (EGFR) (Hazan and Norton, 1998), insulin-like growth factor receptor (IGFR) (Morali et al., 2001) and ephrin receptor EphA2 (Zantek et al., 1999). Binding sites for several signalling molecules exist within the cytoplasmic region of E-cadherin. For example, type Iγ PI phosphate kinase (PIPKIγ), an enzyme responsible for generating the substrate for phosphatidylinositol-3-kinase (PI3K), has been shown to bind the cytoplasmic region of E-cadherin (Ling et al., 2007) along with the phosphotyrosine phosphatases (PTPs) µ (Brady-Kalnay et al., 1998) and PTP1B (Sheth et al., 2007) and presenillin 1 (PS1; Figure 1.4).

Microarray analysis of Ecad−/− mES cells has also revealed roles for E-cadherin in a wide range of signalling cascades including the Ras/Extracellular receptor kinase (ERK), FGF, BMP and PI3K/Akt pathways (Soncin 2011). However, the role of E-cadherin in many of these pathways differs between cell lines. For example, whereas the PI3K/Akt pathway has been shown to be activated by E-cadherin in...
keratinocytes (Calautti et al., 2005; Xie and Bikle, 2007) and Madin-darby canine kidney cells (Pece et al., 1999), it has been shown to be inhibited by E-cadherin in human ovarian cancer cells (Lau et al., 2011). Importantly, the PI3K/Akt pathway has been shown to be dysregulated in cancer more frequently than any other pathway (Hennessy et al., 2005) and thus regulation of this pathway by E-cadherin may provide one explanation for the role of E-cadherin in tumourigenesis. E-cadherin is a metastasis suppressor gene, as evidenced by the observation that forced E-cadherin expression reverses the metastatic phenotype of some cancer cell lines (Vleminckx et al., 1991). Importantly, the role of E-cadherin as a metastasis suppressor has led to the proposal of the dysregulation of E-cadherin in neoplasia and tumourigenesis (DENT) hypothesis whereby loss of E-cadherin alone (in the absence of EMT) is sufficient to induce tumourigenesis by altering the growth factor response of cells (Mohamet, Hawkins and Ward, 2011). In support of this theory, forced expression of E-cadherin in gut epithelium suppressed cellular proliferation and induced apoptosis (Hermiston et al., 1996). Mechanistically, E-cadherin has been shown to facilitate the formation of a β-catenin/caveolin-1 complex, preventing activation of the Survivin gene thus maintaining tissue homeostasis (Torres et al., 2007) and to prevent yes-associated protein (YAP)-mediated stimulation of proliferation (Kim et al., 2011).
Figure 1.4. Binding sites within the intracellular region of E-cadherin. The cytoplasmic region of E-cadherin has been shown to contain binding sites for p120\textsuperscript{ctn}, PS1, β-catenin, PIPK\textsubscript{Iγ} and PTP\textsubscript{μ}. Adapted from (van Roy and Berx, 2008). MPECD: membrane proximal extracellular domain, P120\textsuperscript{ctn}: p120 catenin, PS1: presenillin 1, PIPK\textsubscript{Iγ}: type Iγ PI phosphate kinase, PTP: phosphotyrosine phosphatase.
1.1.2 N-cadherin

Like E-cadherin, N-cadherin is a type I classical cadherin that mediates calcium-dependent cell-cell adhesion. As such, N-cadherin exhibits a high degree of structural homogeneity to E-cadherin, also possessing five ECDs with a HAV domain in ECD1 and a cytoplasmic region with binding sites for α-, β- and p120ctn (Straub et al., 2003).

1.1.2A The role of N-cadherin in embryogenesis

N-cadherin is so-called due to its ubiquitous expression throughout the nervous system, however, it has been shown to perform additional roles in heart formation (Piven et al., 2011), bone generation (Marie, 2002), chondrogenesis (Tuan, 2003) and skeletal muscle differentiation (George-Weinstein et al., 1997). Consequently, N-cadherin null mutant embryos arrest at day 10 of gestation, exhibiting malformation of the heart tube, somites and neural tube (Radice et al., 1997). The high degree of structural homogeneity between E- and N-cadherin allows N-cadherin to compensate for the role of E-cadherin in cell aggregation and chimera generation, under appropriate conditions, in mES cells. Interestingly, Ecadnull mES cells exhibiting forced expression of N-cadherin exhibit a tissue bias towards cartilage and neuroepithelium when injected subcutaneously into mice (Larue et al., 1996).

1.1.2B The role of N-cadherin in cell signalling

N-cadherin has also been shown to interact with various E-cadherin binding partners such as PTP1B (Lilien et al., 2002), PTPμ (Brady-Kalnay et al., 1998), PI3K (Li et al., 2001) and c-Met (Van Aken et al., 2003) (summarised in Table 1.1). However, the RTKs bound by the two cadherins differ significantly and may explain the differences in tissue specification they promote. For example, N-cadherin does not associate with IGF or EGFR but has been shown to stimulate various fibroblast growth factor receptors (FGFRs), even in the absence of FGF ligands (Williams et al., 2001). FGFs have been shown to be required for neural lineage specification (Stavridis et al., 2007) and the interaction between N-cadherin and FGFR has been
shown to be responsible for the acquisition of the motile phenotype that can lead to tumour cell metastasis (Cavallaro et al., 2001; Nieman et al., 1999) and for neurite outgrowth (Doherty and Walsh, 1996).

<table>
<thead>
<tr>
<th>Molecule</th>
<th>Interacts with E-cadherin?</th>
<th>Interacts with N-cadherin?</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-catenin</td>
<td>YES (Cavallaro et al., 2004)</td>
<td>YES (Straub et al., 2003)</td>
</tr>
<tr>
<td>α-catenin</td>
<td>YES (Cavallaro et al., 2004)</td>
<td>YES (Straub et al., 2003)</td>
</tr>
<tr>
<td>P120ctn</td>
<td>YES (Cavallaro et al., 2004)</td>
<td>YES (Straub et al., 2003)</td>
</tr>
<tr>
<td>PTP1B</td>
<td>YES (Sheth et al., 2007)</td>
<td>YES (Lilien et al., 2002)</td>
</tr>
<tr>
<td>PTPμ</td>
<td>YES (Brady kalnay et al., 1998)</td>
<td>YES (Brady kalnay et al., 1998)</td>
</tr>
<tr>
<td>c-Met</td>
<td>YES (Davies et al., 2001)</td>
<td>YES (van Aken et al., 2003)</td>
</tr>
<tr>
<td>PIPKΙγ</td>
<td>YES (Ling et al., 2007)</td>
<td>YES (Ling et al., 2007)</td>
</tr>
<tr>
<td>IGFR</td>
<td>YES (Morali et al., 2001)</td>
<td>NO (Cavallaro et al., 2004)</td>
</tr>
<tr>
<td>EGFR</td>
<td>YES (Hazan et al., 1998)</td>
<td>NO (Cavallaro et al., 2004)</td>
</tr>
<tr>
<td>c-Src</td>
<td>YES (Cavallaro et al., 2004)</td>
<td>YES (Cavallaro et al., 2004)</td>
</tr>
<tr>
<td>FGFR</td>
<td>NO (Cavallaro et al., 2004)</td>
<td>YES (Williams et al., 2001)</td>
</tr>
<tr>
<td>PI3K</td>
<td>YES (Pece et al., 1999)</td>
<td>YES (Li et al., 2001)</td>
</tr>
</tbody>
</table>

Table 1.1. Comparison of E-cadherin and N-cadherin binding partners.

1.2 Pluripotent stem cells

Since their original isolation from mouse (Evans and Kaufman, 1981; Martin, 1981) and human (Thomson et al., 1998) embryos, ES cells have generated great interest due to their ability to self-renew whilst retaining the potential to form every tissue within the adult organism (pluripotency). These abilities are already being harnessed to provide useful models of development and disease mechanisms (Park
et al., 2008) and for drug testing (Matsa et al., 2011) and may in the future be applied in cellular therapies (Ringe et al., 2002).

The isolation of ES cells was preceded by the in vitro propagation of embryonal carcinoma (EC) cells, a principle component of teratocarcinomas. The existence of similar cells in the early mouse embryo was suggested by implantation of these embryos into adult mice, which resulted in the generation of tumours (Stevens, 1970). The ability of ES cells to produce teratomas and to give rise to chimeric embryos and liveborn mice when injected into blastocysts are used to this day as pluripotency tests (Smith, 2001), along with the expression of pluripotency-associated genes.

1.2.1 The core circuitry of pluripotency

Pluripotency is supported in both mouse and human ES cells by the expression of a core circuitry of pluripotency-associated genes, Oct4, Sox2 and Nanog. Expression of the octamer-binding transcription factor Oct4 was initially shown to be restricted to the ICM of blastocysts by Nichols et al. (1998) and its expression in ES cells was later confirmed by Niwa et al. (2000). This group made the observation that precise levels of Oct4 expression are required for maintenance of pluripotency since overexpression of Oct4 led to endodermal and mesodermal differentiation and blockade of Oct4 induced trophoblast differentiation. Oct4 acts in combination with the HMG-binding factor Sox2 (Masui et al., 2007) and the two factors have been shown to form a heterodimeric complex which is then responsible for activating the expression of a multitude of pluripotency-associated genes (Botquin et al., 1998; Nishimoto et al., 1999; Yuan et al., 1995). Many Oct4/Sox2 target genes have also been shown to be shared with Nanog in both mouse (Loh et al., 2006) and human (Boyer et al., 2005) ES cells and both Sox2 (Masui et al., 2007) and Nanog (Niwa et al., 2009) have been shown to maintain Oct4 expression (Figure 1.5). Whilst Nanog null (Nanog⁻/⁻) mES cells exhibit a tendency to differentiate into primitive endoderm, these cells remain pluripotent, suggesting this factor is ultimately dispensable for mES cell pluripotency (Chambers et al., 2007). The expression of the
core circuitry of pluripotency can be maintained in mES cells either by supplementing the culture medium with leukaemia inhibitory factor (LIF) and bone morphogenetic protein 4 (BMP4) or by supplementing the culture medium with antagonists of differentiation-inducing pathways.

1.2.2 The role of LIF in mES cell pluripotency

Since the pluripotency of EC cells was shown to be supported by mitotically inactivated mouse embryonic fibroblasts (MEFs), mES cells were initially maintained on these feeder cells (Martin, 1981). LIF, an interleukin-6 family cytokine, was subsequently shown to be the primary cytokine produced by feeder cells (Rathjen et al., 1990) and its ability to replace the requirement of mES cells for MEFs was demonstrated by Smith and colleagues (1988). The LIF receptor (LIFR) consists of a gp130 subunit and a LIFR β subunit. When engaged, the LIFR triggers three parallel signal transduction networks, the Janus-associated kinase (Jak)/Signal transducer and activator of transcription (STAT3) pathway, the PI3K/Akt pathway and the Grb/Mitogen-activated protein kinase (MAPK) cascade. The Jak/STAT3 and PI3K/Akt pathways subsequently promote the expression of the core circuitry of pluripotency via the activation of Kruppel-like factor 4 (Klf4) and T-box factor 3 (Tbx3) respectively. Interestingly, forced expression of Klf4 or Tbx3 in mES cells was shown to support pluripotency in the absence of LIF, thus demonstrating the importance of these factors in mES cell pluripotency. LIF also exerts negative regulation of the core circuitry of pluripotency by promoting MAPK activation, thus preventing nuclear localisation of Tbx3 and therefore Nanog expression (Figure 1.5; Niwa et al., 2009). This is consistent with numerous studies that demonstrate that FGF stimulation of the MAPK pathway induces loss of mES cell pluripotency (Kunath et al., 2007; Stavridis et al., 2007) and that, therefore, culture of mES cells in medium supplemented with inhibitors of this pathway and the glycogen synthase kinase β inhibitor BIO (2i medium) can maintain mES cell pluripotency (Ying et al., 2008).

LIF can only prevent differentiation of mES cells in combination with bone morphogenetic proteins (BMPs), which can be provided by serum and act specifically to block neuroectodermal differentiation via activation of Inhibitor of
differentiation (Id) proteins (Figure 1.5) and blockade of extracellular receptor kinase (ERK) and p38 mitogen-activated protein kinase (MAPK) pathways, inhibitors of which have been shown to support mES cell self-renewal (Qi et al., 2004). mES cell pluripotency can also be maintained in serum-free medium supplemented with LIF and BMP alone (Ying, Nichols et al., 2003).
Figure 1.5. The maintenance of pluripotency in mES cells by LIF and BMP4. One signalling mechanism for the maintenance of the expression of Oct4, Sox2 and Nanog in mES cells is via LIF-dependent activation of the PI3K/Akt and JAK/STAT3 pathways in combination with activation of Id genes via BMP4/Smad signalling. BMP: bone morphogenetic protein, Grb2: growth factor receptor-bound protein 2, Id: inhibitor of differentiation, Jak: janus-associated kinase, Klf: kruppel-like factor, LIF: leukaemia inhibitory factor, LIFR: LIF receptor, MAPK: mitogen-activated protein kinase, PI3K: phosphatidylinositol-3-kinase, STAT: signal transducer and activator of transcription, Tbx: T-box factor, TGFβ: transforming growth factor β.
1.2.3 Epigenetic and microRNA regulation of pluripotency

In addition to the transcriptional control of pluripotency, recent work has highlighted the importance of epigenetic regulation in pluripotent cells (Azuara et al., 2006; Bernstein et al., 2006; Boyer et al., 2006b; Buszczak and Spradling, 2006). For example, chromatin, which comprises nucleosomes wrapped in DNA, has been shown to differ significantly between pluripotent cells and differentiated cells. Specifically, a high proportion of ES cell chromatin exists in the open conformation (euchromatin), which then becomes more condensed upon lineage specification, thus restricting access to transcriptional complexes (Figure 1.6; Boyer et al., 2006a; Efroni et al., 2008). The octamer of histone proteins that make up the nucleosome can also undergo post-translational modification of their lysine tails (Strahl and Allis, 2000) and these histone modification signatures have been shown to be conserved between mES and hES cells (Boyer et al., 2005). One example of histone modification enzymes is the polycomb group (PcG), which consists of polycomb repressive complex (PRC) 1 and PRC2. PRC2 has been shown to catalyse methylation of histone H3 on lysine 27 (a modification known as H3K27me2) which then recruits PRC1, resulting in transcriptional repression (Buszczak and Spradling, 2006). PcGs have been shown to be responsible for silencing genes associated with differentiation in ES cells, thus maintaining their pluripotent state (Azuara et al., 2006; Bernstein et al., 2006). For example, knock down of the Embryonic ectoderm development gene, a component of PRC2, has been shown to induce mES cell differentiation and neural derivatives of these cells were shown to exhibit a decrease in H3K27me2 and PcG binding (Boyer et al., 2005).

Interestingly, a high proportion of genes in ES cells are co-occupied by PRC2 and Oct4, Sox2 and Nanog complexes (Lee et al., 2006), suggesting these factors act concomitantly to maintain pluripotency. In support of this hypothesis, recruitment of PRC2 has been shown to be mediated by Oct4, Sox2 and Nanog in ES cells, whereby these factors bind to the promoter elements of genes involved in early cell fate decisions, blocking differentiation (Boyer et al., 2005; Boyer et al., 2006b). Oct4, Sox2 and Nanog have also been shown to interact with the nucleosome
remodelling and deacetylation complex NuRD (Liang et al., 2008). NuRD is responsible for mediating exit from pluripotency towards lineage specification as evidenced by the observation that knockdown of Mbd3 (a NuRD component) in mES cells induces LIF-independent self-renewal and a block in differentiation (Kaji et al., 2006).

DNA methylation is another epigenetic silencing mechanism whereby methyl groups are added to cytosine-guanine (CpG) islands within promoter DNA, resulting in inhibition of target gene expression, likely due to direct interference with transcription factor binding (Curradi et al., 2002). DNA methylation is essential for mammalian development, as evidenced by mutations of the three DNA methyltransferase (Dnmt) genes, Dnmt1, Dnmt3a and Dnmt3b, which have individually been shown to result in lethality in mouse models (Lei et al., 1996; Okano et al., 1999). Interestingly, the demethylating agent Aza has been shown to induce differentiation of mES cells to various somatic lineages, thus demonstrating the importance of DNA methylation in maintaining the pluripotent state (Taylor and Jones, 1979).

In addition to epigenetic regulation, there is accumulating evidence for the importance of microRNAs (miRNAs) in pluripotency and differentiation (Kashyap et al., 2009; Wilson et al., 2009). These small RNAs, of around 20-25 nucleotides in length, bind messenger RNA (mRNA), usually targeting it for degradation or repressing its translation. miRNAs therefore represent an additional post-translational gene-silencing mechanism (Kashyap et al., 2009). miRNAs have been implicated in ES cell fate acquisition. For example, miRNA-134 has been shown to mediate blockade of Nanog mRNA translation, thereby enhancing mES cell differentiation (Tay et al., 2008). The key role of miRNAs in pluripotency is evidenced by the observation that mES cells lacking Dicer, and therefore cannot generate miRNAs, exhibit differentiation defects both in vitro and in vivo (Kanellopoulou et al., 2005; Murchison et al., 2005).
Figure 1.6. Comparison of chromatin structure between pluripotent and differentiated cells. Whereas pluripotent cells possess a high proportion of chromatin in its open conformation, lineage committed cells possess more condensed chromatin, restricting transcription factor access.
1.2.4 Inducing pluripotency in somatic cells

Reprogramming of somatic cells to pluripotency was responsible for the seminal cloning of Dolly the sheep in 1997 and was achieved by somatic cell nuclear transfer (Wilmut et al., 1997). However, little was known about the reprogramming mechanism until Takahashi et al. (2006) identified four factors (Oct4, Sox2, Klf4 and c-Myc) from an initial screen of 24 candidate genes that were capable of generating induced pluripotent stem (iPS) cells from mouse fibroblasts. This pioneering research was closely followed by human iPS (hiPS) generation in 2007 by two separate groups (Takahashi et al., 2007; Yu et al., 2007), with the latter using Nanog and Lin28 reprogramming factors in the place of Klf4 and c-Myc.

These cells represent a significant advance in stem cell research as they address immunological and ethical issues associated with hES cells (Nakagawa and Yamanaka, 2010), can be patented in the European Union (Callaway, 2011) and present the unique opportunity to study disease mechanisms through the generation of iPS cells from patients suffering from various disorders. Conditions such as Parkinson’s disease (Byers et al., 2012), Huntingdon’s disease, Down’s syndrome, Duchenne muscular dystrophy (Park et al., 2008) and type I diabetes (Jeon et al., 2012) are amongst those being studied in this way. iPS cell technology may also aid in vitro toxicology testing (Webb, 2009), thus providing an alternative to animal-based models.

However, current challenges of iPS cell technology include the low efficiency of iPS cell generation along with various safety issues. For example, the use of viral vectors has been shown to cause random integration of oncogenes such as c-Myc (Okita et al., 2008). In recent years these challenges have been addressed in a variety of ways (reviewed in (Oh et al., 2012)), including narrowing down the factors used for reprogramming to Oct4 alone in neural stem cells (Kim et al., 2009b) and the use of plasmid DNA (Okita et al., 2008), miRNAs (Anokye-Danso et al., 2011; Liao et al., 2011), proteins (Kim et al., 2009a; Zhou et al., 2009), small molecules (Huangfu et al., 2008) and RNAs (Fusaki et al., 2009; Seki et al., 2010), the latter
potentially presenting the most promising breakthrough in terms of safety (Mostoslavsky, 2012). However, this method requires multiple transfections.

It is likely that epigenetic modifications, in addition to transgene manipulation, will be necessary to improve the efficiency of the reprogramming process. For example, iPS cells have been shown to retain ‘epigenetic memory’ of the phenotype from which they were originally derived (Ohi et al., 2011), likely due to incomplete promoter DNA methylation (Puri and Nagy, 2012), and this has been shown to restrict their differentiation potential (Muller et al., 2011). In support, small molecule inhibitors of histone deacetylases, histone demethylases and histone methyltransferases have been used to successfully enhance the reprogramming process (Huangfu et al., 2008). iPS cells and ES cells exhibit similarities in morphology, marker gene expression (Plath and Lowry, 2011), epigenetic patterning (Guenther et al., 2010) and some signature miRNAs (Wilson et al., 2009). However, some differences in gene expression (Chin et al., 2009) and miRNA expression have been observed (Wilson et al., 2009), along with differences in teratoma (Gutierrez-Aranda et al., 2010) and chimera (Zhao et al., 2009) formation abilities, thus raising the question of whether iPS cells are truly analogous to ES cells.

1.2.5 The role of E-cadherin in pluripotency

Interestingly, E-cadherin expression has been shown to be critical for miPS cell generation (Chen et al., 2011; Chen et al., 2010; Li et al., 2010b) and can replace Oct4 during the reprogramming process (Redmer et al., 2011), suggesting E-cadherin plays a key role in pluripotency acquisition. However, a mechanism for this is yet to be elucidated. The observation that E-cadherin downregulation occurs concomitantly with mES (Spencer et al., 2007) and hES (Eastham et al., 2007) cell differentiation provides further evidence for the importance of E-cadherin in pluripotency. The precise role of E-cadherin in pluripotency, however, remains a subject of debate. This role has been explored through the isolation of mouse epiblast-derived stem cells (EpiSCs; Brons et al., 2007; Tesar et al., 2007) and FGF,
Activin and BIO-derived stem cells (FAB-SCs), isolated in the presence of a LIF-blocking antibody (Chou et al., 2008), both of which exhibit low E-cadherin expression. For example, both cell lines fail to form chimeras and FAB-SCs cannot generate teratomas or give rise to EBs. Whilst EpiSCs can generate teratomas, EpiSC-derived EBs exhibit a tissue bias, predominantly giving rise to neuroectoderm (Tesar et al., 2007). However, these cells express comparable levels of pluripotency markers such as stage-specific embryonic antigen (SSEA-1), Oct4, Sox2 and Nanog to mES cells, suggesting cells cannot be described as differentiated simply due to a lack of E-cadherin, as proposed by Redmer et al. (2011).

It has recently been suggested that two pluripotent states exist: the naive and primed pluripotent states as described by Nichols and Smith (2009). These states correspond to the pre- and post-implantation epiblast respectively in vivo and mES cells and EpiSCs respectively in vitro. The primed pluripotent state may also be characterised by Activin/Nodal-dependent pluripotency since EpiSCs (Brons et al., 2007), FAB-SCs (Chou et al., 2008), hiPS cells (Vallier et al., 2009b), hES cells (Vallier et al., 2005) and the late epiblast in vivo (Mesnard et al., 2006) exhibit this growth factor dependence. However, the primed pluripotent state cannot be defined by Activin/Nodal dependence since miPS cells cultured in Activin/Nodal and FGF2 have also been shown to exhibit features of the naive pluripotent state (Di Stefano et al., 2010). Recent work from our lab has implicated the E-cadherin/β-catenin complex in the switch between LIF/BMP- and Activin/Nodal-dependent pluripotency since mES cells lacking either of these molecules exhibit Activin/Nodal-dependent pluripotency, as evidenced by the loss of pluripotency-associated markers in these cells upon exposure to SB431542, an inhibitor of the Activin receptor-like kinases (Alks) 4, 5 and 7 (Soncin et al, 2009). Reversible Activin/Nodal-mediated pluripotency was also induced in wild type (wt) D3 mES cells upon exposure to CHAVC or expression of a truncated form of E-cadherin lacking the β-catenin binding domain in Ecad−/− mES cells. However, Activin/Nodal-dependent pluripotency was not induced upon exposure of wtD3 mES cells to DECMA-1, indicating that specific regions of E-cadherin are required for LIF-dependent pluripotency and that it is not simply the loss of cell-cell contact that occurs
following treatment with CHAVC or DECMA that initiates the switch from LIF-dependent to Activin/Nodal-mediated pluripotency (Soncin et al., 2009). Microarray analysis has provided further evidence for the role of E-cadherin in LIF-dependent mES cell pluripotency. For example, the transcripts of various LIF signalling components such as Tbx3, Klf4 and Nanog have been shown to be downregulated in Ecad−/− mES cells compared to wtD3 mES cells and LIF supplementation was shown to exert minimal effects on the transcript profile of Ecad−/− mES cells (Soncin et al., 2011). Specifically, only two significant transcript alterations, Sp8 and STAT3, were observed between Ecad−/− mES cells cultured in the absence and presence of LIF. However, STAT3 is downstream of LIF signalling, suggesting these cells do have the potential to activate this pathway upon LIF stimulation.

Furthermore, microarray analysis was used to compare the gene expression profiles of Ecad−/− mES cells and wtD3 mES cells with the previously described data for EpiSCs (Tesar et al., 2007) and FAB-SCs (Chou et al., 2008). Various pluripotency-associated transcripts, such as Rex1 and Nr0b1, were shown to be downregulated in all three cell lines compared to wt mES cells and EpiSC markers such as FGF5 were found to be upregulated in Ecad−/− mES cells. Together, these findings suggest that Ecad−/− mES cells may be representative of the primed pluripotent state. Whilst Ecad−/− mES cells were shown to be more similar to EpiSCs in their transcript profile than wtD3 mES cells, many differences in transcript expression were observed between all cell lines, thus confirming that Ecad−/− mES cells are a unique cell line (Soncin et al., 2009; 2011).

The similarity in growth factor dependence, gene expression profile, X chromosome activation status and morphology between hES cells and EpiSCs (Brons et al., 2007; summarised in Table S5.1) has led to the suggestion that hES cells are more representative of the primed than the naive pluripotent state (Rossant., 2008). As we cannot subject human pluripotent stem cells to rigorous tests of pluripotency such as chimera generation for ethical reasons, we do not know whether they are similar to mES cells or EpiSCs in this respect, however, acquisition of a naive pluripotent state in these cells may be necessary for the generation of all possible
cell types in regenerative medicine applications. Importantly, human pluripotent cells have been shown to be maintained in a more mES cell-like naive state by culture in mES cell culture conditions (Xu et al., 2010) or ectopic induction of reprogramming factors in the presence of LIF in either fibroblasts (Buecker et al., 2010) or hES cells (Hanna et al., 2010). In the latter study, inhibitors of glycogen synthase kinase 3 (GSK3) β and MAPK were also used, factors previously used in mES cell culture to activate the ‘ground state’ of naive pluripotency (Ying et al., 2008) and to enhance mES cell reprogramming (Guo et al., 2009). These ‘naive’ culture conditions induce both genetic and epigenetic reprogramming of iPS cells (Hanna et al., 2010). Interestingly, hES cells have been shown to be more reliant on E-cadherin signalling under these ‘2i’ culture conditions (Xu et al., 2010). FAB-SCs have also been shown to be reversed to the naive pluripotent state in which E-cadherin is upregulated concomitantly with pluripotency-associated markers and an increase in chimera and teratoma generation abilities is observed upon LIF stimulation (Chou et al., 2008). Similar results have been achieved in EpiSCs upon addition of LIF (Bao et al., 2009) or forced expression of Klf4 (Guo et al., 2009). However, no such reversal of Ecad−/− mES cells has been described and a mechanism for the role of E-cadherin in the transition between the primed and naive pluripotent states is therefore yet to be determined.
1.3 Aims

The primary objective of this project was to investigate the role of E-cadherin within the LIF signalling network in mES cells. It has previously been shown that E-cadherin expression promotes LIF-dependent mES cell pluripotency (Soncin et al., 2009) and since LIF has been shown to signal to the core circuitry of pluripotency (Niwa et al., 2009), this should aid our understanding of the general role of E-cadherin in the naive and primed pluripotent states and the transition between them.

Firstly, we compared the activation of the three branches of the LIF signalling cascade and the expression of various LIF signalling components between Ecad\(^{-}\) mES cells and their parental line, wtD3 mES cells. We then explored the hypothesis that E-cadherin regulates Nanog expression by comparing Nanog promoter activation between the two cell lines. Furthermore, we explored the region of E-cadherin involved in regulation of the LIF pathway in mES cells by transfecting Ecad\(^{-}\) mES cells with truncated fragments of E-cadherin and analysing LIF pathway activation. Finally, Ecad\(^{-}\) mES cells were stimulated with LIF to determine whether these cells are capable of LIF-dependent pluripotency, and the mechanisms involved in this process were then elucidated.

Secondly, we addressed the hypothesis that cell seeding density is a critical determinant of mES cell fate by plating wtD3 mES cells at low density in the absence of LIF, upon which we observed the generation of E-cadherin negative proliferating stem (ENPS) cells. These cells were also stimulated with LIF and, separately, transfected with E-cadherin to determine whether reversal of this process was possible. The mechanism by which E-cadherin was downregulated in these cells was examined, along with pluripotency marker expression, pluripotency-associated growth factor dependence, survival capacity and EB- and chimera-generation abilities. Affymetrix microarray analysis was performed to compare ENPS cells to wtD3 and Ecad\(^{-}\) mES cells, previously characterised in this way by Soncin et al. (2011), and to determine the pluripotent status of all three cell lines. Finally, these cells were analysed for a lineage bias in their differentiation capacity,
with the ultimate aim of manipulating cadherin expression to direct differentiation of pluripotent stem cells for regenerative medicine applications.
Chapter 2

General Materials and Methods
Chapter 2 General Materials and Methods

2.1 mES cell culture

The following cell lines were used in this project:

- Mouse ES-D3, strain 129S2/SvPas, ATCC number CRL-11632 (wtD3) mES cells,
- Mouse ES MESC20 (129/OLA mice) (MESC) Cells (donated by Dr R. Elder) transfected with E-cadherin RNA interference (RNAi) by Dr F. Soncin (EcadRNAi mES cells),
- Mouse ES MESC20 (129/OLA mice) (MESC) Cells (donated by Dr R. Elder) transfected with E-cadherin RNAi which was then inactivated using a TetR plasmid by Dr F. Soncin (EcadRNAiR mES cells),
- E-cadherin knockout D3 129Sv (Ecad\(^{-/}\)) mES cells (donated by Dr R. Kemmler),
- E-cadherin negative proliferating stem (ENPS) D3 mES cells (Ward lab),
- ENPS methylated (ENPS\(^{M}\)) D3 mES cells (Ward lab),
- ENPS reversed (ENPS\(^{R}\)) D3 mES cells (Ward lab).

Unless otherwise specified, mES cells were cultured on 0.1% (w/v) gelatin (Sigma)-coated plates in Knockout DMEM supplemented with 10% (v/v) fetal bovine serum (FBS) or serum replacement (SR), 50μm 2-mercaptoethanol (all Invitrogen), 2mM L-glutamine, 1% (v/v) non-essential amino acids (both PAA) and 1000U/ml LIF (Chemicon International; FBS-containing medium) at 37\(\)°C, 5% CO\(_2\). Cells were passaged every 2 days, when a maximum confluence of 70-80% was reached, using Trypsin/EDTA (PAA) to detach cells from the plates following two washes with Phosphate buffered saline solution (PBS, PAA). Where specified, cells were treated with appropriate inhibitors (Table 2.1)
<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Blocks</th>
<th>Concentration</th>
<th>Supplier</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>SB431542</td>
<td>Activin-like kinase receptors 4,5 and 7</td>
<td>10µM</td>
<td>Tocris</td>
<td>DMSO</td>
</tr>
<tr>
<td>LY294002</td>
<td>PI3K</td>
<td>10µM</td>
<td>Cell Signalling Technology</td>
<td>DMSO</td>
</tr>
<tr>
<td>E/N+</td>
<td>E- and N-cadherin</td>
<td>1mM</td>
<td>Bachem</td>
<td>E/N-</td>
</tr>
</tbody>
</table>

Table 2.1. Cells were treated with the appropriate concentrations of the inhibitors shown above.

2.1.1 Cell proliferation assays

w_tD3, ENPS and ENPS\textsuperscript{R} cell growth rates were compared over a 4 day period. 1 x 10\textsuperscript{5} cells were seeded in triplicate in a 6 well plate in FBS-containing medium. After the appropriate time period, cells were trypsinised and resuspended in 1ml PBS. 10µl of the cell suspension was then added to 10µl Trypan blue (Sigma) and counted using a Countess automated cell counter (Invitrogen). The mean values ± the standard error of the mean (SEM) for the triplicate samples were then plotted graphically. Significant results represent a p value of <0.05 in an unpaired two-tailed t-test. Fold change (FC) was calculated as cell number(ENPS)\textsubscript{days}/cell number(w_tD3 or ENPS\textsuperscript{R})\textsubscript{day} 4.

2.1.2 EB generation

To generate EBs, 1 x 10\textsuperscript{6} cells were cultured in suspension in bacterial grade dishes with 15ml of standard mES cell culture medium in the absence of LIF for 3 days. The culture medium was replenished daily.
2.1.3 Acclimatisation of cells to LIF/BMP medium and 2i medium

Cells were passaged (1:3 split) into increasing concentrations of LIF/BMP medium (ESGRO Complete Plus Clonal Grade Medium; Millipore), or 2i medium (iSTEM Embryonic Stem Cell Culture Medium; StemCells, Inc.) supplemented with 1000U/ml LIF, within FBS-containing medium every 2 days (Table 2.2). Trypsin was used until day 4, thereafter Cell dissociation buffer (Invitrogen) was used.

<table>
<thead>
<tr>
<th>Day</th>
<th>0</th>
<th>2</th>
<th>4</th>
<th>6</th>
<th>8</th>
<th>10</th>
<th>11</th>
<th>12</th>
</tr>
</thead>
<tbody>
<tr>
<td>% FBS medium</td>
<td>100</td>
<td>90</td>
<td>75</td>
<td>50</td>
<td>25</td>
<td>10</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>% LIF/BMP or 2i medium</td>
<td>0</td>
<td>10</td>
<td>25</td>
<td>50</td>
<td>75</td>
<td>90</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>

Table 2.2. Percentage of FBS-containing medium and LIF/BMP medium/2i medium at each day of mES cell acclimatisation to new media. All time points reflect cells being passaged except day 11 on which the medium was replenished.

2.1.4 Chimera generation

Cells were cultured as described in Section 2.1 before being trypsinised and reuspended in FBS-containing medium. 10-15 cells were then injected into 3.5 day old blastocysts and implanted into pseudo-pregnant BDF-1 female mice. Chimeric pups and germline transmission efficiency was determined by mating to a mouse genotype that enabled donor coat colour to be distinguished. Mice were housed according to Home Office guidelines and kept on a 12 hour light/dark cycle in which the dark period was from 7pm to 7am.

2.1.5 ENPS cell generation

ENPS cells were generated by plating wt mES cells at $5 \times 10^3$ cells/ml in FBS-containing medium in the absence of LIF. Cells were subsequently cultured under normal passaging regimen (i.e. 1 in 6 split every 2 days) for analysis. To generate
ENPS\textsuperscript{R} cells, LIF was added to ENPS cells prior to passage 3 (p3) and to generate ENPS\textsuperscript{M} cells, LIF was withdrawn from ENPS cells beyond p3.

2.2 Fluorescent flow cytometry

2.2.1 Staining protocol

Cells were harvested using Cell dissociation buffer, washed with 900µl PBS, centrifuged at 1000rpm for 2 minutes and resuspended in appropriate primary antibody (or isotype control antibody) at its optimal dilution (Table 2.3) in flow buffer (0.2% [w/v] bovine serum albumin [BSA], 0.1% [w/v] sodium azide in PBS) for 30 minutes on ice. Cells were then washed in PBS before being incubated in the appropriate phycoerythrin (PE)-conjugated secondary antibody (Santa Cruz) at its optimal dilution (1:200) in flow buffer for 30 minutes on ice in the dark. Finally, cells were washed and fixed in 500µl 1% (w/v) paraformaldehyde (PFA; Sigma) in PBS for analysis.

2.2.2 Sample analysis

Cells were analysed using a Becton Dickinson (BD) FACScalibur flow cytometer (BD bioscience) and Cell Quest Pro software. Initially, isotype control samples were used to gate the live cell population (R1; Figure 2.1) according to the side and forward scatter dot plot and to set up the laser parameters for non-specific staining. The antibody-probed samples were then analysed and merged with the isotype control results to produce the final plot. All data shown represents 1 x 10\textsuperscript{4} cells from the R1 population.
Figure 2.1. Analysis of fluorescent flow cytometry data. (A): Dot plot to show side scatter and forward scatter of the live cell population (R1). (B): Histogram to show the laser parameters for non-specific staining. (C): Histogram to show positive staining. (D): Histogram to show the merged isotype control peak and antibody peak. B-D represent the R1 population only.
2.2.3 Intracellular fluorescent flow cytometry

Cells were fixed in 1% (w/v) PFA for 10 minutes at room temperature before the cell membrane was permeabilised by incubating cells in ice-cold 70% (v/v) methanol at -20 °C overnight. Cells were then washed with 900 µl PBS and stained according to the above protocol using anti-Oct4, anti-Nanog and anti-Klf4 antibodies (Table 2.3).

<table>
<thead>
<tr>
<th>Target</th>
<th>Dilution</th>
<th>Ordering information</th>
</tr>
</thead>
<tbody>
<tr>
<td>E-cadherin (DECMA-1)</td>
<td>1:100</td>
<td>Sigma, U3254</td>
</tr>
<tr>
<td>SSEA-1</td>
<td>1:100</td>
<td>Santa Cruz, sc-21702</td>
</tr>
<tr>
<td>Oct4</td>
<td>1:100</td>
<td>Abcam, sc-5279</td>
</tr>
<tr>
<td>Klf4</td>
<td>1:100</td>
<td>R &amp; D systems, AF3158</td>
</tr>
<tr>
<td>Nanog</td>
<td>1:100</td>
<td>Abcam, ab80892</td>
</tr>
</tbody>
</table>

Table 2.3. Primary antibodies for fluorescent flow cytometry.

2.2.4.1 Annexin V/Propidium iodide assay

Cell viability was quantified using an Alexa fluor 488 Annexin V/Dead Cell Apoptosis Kit (Invitrogen) according to the manufacturer’s instructions. Whereas Annexin V binds phosphatidyl serine (present on the outer leaflet of apoptotic cells), Propidium iodide (PI) stains the nucleic acids in dead cells but is impermeant to live cells. Cells were harvested using Cell dissociation buffer before $1 \times 10^5$ cells were washed with PBS and resuspended in 100 µl 1X Annexin-binding buffer containing 5 µl Alexa fluor 488 Annexin V and 1 µl PI (100 µg/ml). Cells were then incubated at room temperature for 15 minutes in the dark before being prepared for analysis by the addition of 400 µl 1X Annexin-binding buffer and incubation on ice.
2.2.4.2 Annexin V/Propidium iodide assay analysis

Cells were stained and analysed by flow cytometry using 488nm excitation on the BD FACScalibur flow cytometer. Cell Quest Pro software was used to set up the laser parameters to generate 3 populations of cells according to Annexin V and PI staining; the Annexin V/PI⁻ live cell population (Figure 2.2a), the Annexin V⁺/PI⁺ dead cell population (Figure 2.2b) and the apoptotic Annexin V⁺/PI⁻ population (Figure 2.2c).

Figure 2.2. Annexin V/PI assay analysis. Cells were stained using an Alexa fluor 488 Annexin V/Dead Cell Apoptosis Kit then analysed by flow cytometry on a BD FACScalibur flow cytometer. (A): the Annexin V⁻/PI⁻ live cell population (B): the Annexin V⁺/PI⁺ dead cell population (C): the apoptotic Annexin V⁺/PI⁻ population.
2.3 Immunofluorescent cell imaging

Cells were fixed in 4% (w/v) PFA in PBS for 15 minutes at 4°C before being washed with PBS and incubated in the appropriate primary antibody at its optimal dilution (Table 2.4) in goat blocking serum (0.1% [w/v] BSA and 1% [v/v] goat serum in PBS) for 2 hours at room temperature. Cells were then washed with PBS and incubated in the appropriate Alexa fluor 488 conjugated secondary antibody in goat blocking serum (all 1:500, Invitrogen) at room temperature in the dark for 1 hour. Cells were then mounted using Vectorshield with 4’,6-diamidino-2-phenylindole (DAPI; Vector Laboratories) and imaged using a fluorescence microscope. The percentage of positive cells was quantified by taking the average count across three fields of view.

<table>
<thead>
<tr>
<th>Target</th>
<th>Dilution</th>
<th>Ordering information</th>
</tr>
</thead>
<tbody>
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<td>1:200</td>
<td>Abcam, ab19857</td>
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<tr>
<td>Sox2</td>
<td>1:500</td>
<td>Abcam, ab97959</td>
</tr>
<tr>
<td>Nanog</td>
<td>1:200</td>
<td>Abcam, ab80892</td>
</tr>
<tr>
<td>Klf4</td>
<td>1:200</td>
<td>R &amp; D systems, AF3158</td>
</tr>
<tr>
<td>N-cadherin</td>
<td>1:200</td>
<td>Santa Cruz, sc-7939</td>
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<tr>
<td>βIII-tubulin</td>
<td>1:1000</td>
<td>Abcam, ab41489</td>
</tr>
<tr>
<td>Nestin</td>
<td>1:250</td>
<td>Abcam, ab6142</td>
</tr>
<tr>
<td>Tyrosine hydroxylase</td>
<td>1:500</td>
<td>Millipore, AB152</td>
</tr>
</tbody>
</table>

Table 2.4. Primary antibodies for immunofluorescent cell imaging.
2.4 Nanog promoter Assay

2.4.1 Generation of Nanog promoter vectors

Primers were designed to amplify various fragments of the Nanog promoter region at the following positions: 4963 [5kb], 4547 [minus STAT3], 4432 [4.5kb], 3832 [4kb], 1087 [1kb] and 530 [0.5kb] bp upstream of the Nanog start site. The reverse primer was designed 162bp upstream of the start site within the untranslated region (Figure 2.3). Restriction enzyme sequences were tagged on to the 5’ end of each primer (forward primers: GGTACC [KpnI], reverse primer: CTCGAG [XhoI]). Promoter fragments were amplified by polymerase chain reactions (PCRs) containing 1µl genomic DNA template (extracted from wtD3 mES cells using a QIAamp DNA kit [Qiagen]), 8µl extensor reddymix (Abgene) and 1µl of the appropriate primer set (Table 2.5) according to the following programme: 94°C for 2 minutes, 35 cycles (94°C for 10 seconds, optimal annealing temperature for 1 minute, 68°C for 2 minutes) then 68°C for 10 minutes. The DNA was then precipitated, heated to 65°C for 5 minutes to remove annealed primers, run on a 1% (w/v) agarose gel and isolated using a QIAquick gel extraction kit (Qiagen). The insert and pGL3 Basic vector (Promega; Figure 2.4) were then digested using 2000U KpnI-HF and XhoI restriction enzymes (New England Biolabs) overnight at 37°C before being incubated with T4 DNA ligase (Roche). DH5α competent cells (Invitrogen) were transformed with the ligation reaction and ampicillin-resistant colonies picked and expanded before the plasmid was purified using a QIAPrep spin mini prep kit (Qiagen). Plasmids were then sequenced using ABI Prism 3100 Genetic Analyser (Applied Biosystems) to verify correct incorporation of the insert.
<table>
<thead>
<tr>
<th>Fragment</th>
<th>Forward primer (5’ to 3’)</th>
<th>Reverse primer (5’ to 3’)</th>
<th>Optimal annealing temperature (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5kb</td>
<td>ccctccctccctattcaaacc</td>
<td>ctctttctgtggaaggtcg</td>
<td>59.8</td>
</tr>
<tr>
<td>Minus STAT3</td>
<td>accaaaattacgtgccccctgagg</td>
<td>ctctttctgtggaaggtcg</td>
<td>59.8</td>
</tr>
<tr>
<td>4.5kb</td>
<td>gaggaacccctttgccctgtgtg</td>
<td>ctctttctgtggaaggtcg</td>
<td>59.8</td>
</tr>
<tr>
<td>4kb</td>
<td>aggtgccacatcttttggcttcg</td>
<td>ctctttctgtggaaggtcg</td>
<td>59.8</td>
</tr>
<tr>
<td>1kb</td>
<td>gccctttcccctctctgttcttg</td>
<td>ctctttctgtggaaggtcg</td>
<td>69.3</td>
</tr>
<tr>
<td>0.5kb</td>
<td>caatgtgaagagcaagcaaga</td>
<td>ctctttctgtggaaggtcg</td>
<td>69.3</td>
</tr>
</tbody>
</table>

Table 2.5. Primer sequences and annealing temperatures used to clone fragments of the Nanog promoter region into the pGL3 basic plasmid.
Figure 2.3. Primer sequences used for cloning the Nanog promoter region.

Sequence of approximately 5000bp of the Nanog promoter region and untranslated region (UTR; highlighted in yellow) depicting the primers used to amplify the (Top-Bottom): 5kb, Minus STAT3, 4.5kb, 4kb, 1kb and 0.5kb fragments of the Nanog promoter and the reverse primer (within the UTR). The STAT3 binding site is shown in green.
Figure 2.4. **pGL3 Basic vector map.** pGL3 Basic plasmid map to show the multiple cloning site (MCS), origin of replication (f1 ori), luciferase reporter gene (luc+), ampicillin resistance gene (Amp$^{R}$) and the SV40 poly A and synthetic poly A signals.
2.4.2 Amaxa transfection of mES cells

24 hours after passaging, cells were trypsinised and a cell count was performed as described in section 2.1.1. 2 x 10^5 cells were then pelleted by centrifugation (1000rpm for 1 minute) and resuspended in electroporation buffer containing 2µg FL-Ecad, Δβcat or Δβcat-Δp120\text{ctn} plasmids (previously described in (Soncin et al., 2009)) or the pGL3 Basic vector containing the Nanog promoter regions described above. Cells were then transferred to a cuvette from an ES Cell Electroporation Kit (Lonza), electroporated using an Amaxa Nucleofactor® system (Lonza; programme A30) and rapidly transferred to FBS-containing medium. Cells were analysed after 24 hours.

2.4.3 Dual luciferase reporter assay

Cells were transfected as described in Section 2.4.2 using 2µg of the appropriate plasmid (0.5kb, 1kb, 4kb, 4.5kb, 5kb, minus STAT3 or pGL3 as a control) along with 0.5µg Renilla plasmid. 24 hours after transfection, cells were lysed using a Dual Luciferase Reporter Assay System (Promega) according to the manufacturer’s instructions. Lysates were then analysed for luciferase and renilla activity using an Orion L Microplate Luminometer (Berthold Detection Systems). Luciferase activity was normalised using a vector containing the renilla gene and mean values (FC in promoter activity compared to the pGL3 plasmid control) were plotted graphically. All results reflect the mean ± SEM of 3 independent replicates and significant results represent a p value of <0.05 in an unpaired two-tailed t-test.

2.5 Western blot analysis

Cells were trypsinised, counted and resuspended in Radioimmunoprecipitation assay (RIPA) buffer (Sigma) containing protease inhibitor cocktail tablets (1 tablet per 10ml RIPA buffer; Roche) at a concentration of 2 x 10^7 cells/ml. Cells were then incubated on ice for 30 minutes before the lysate was centrifuged at 13000rpm for 5 minutes and cell debris was discarded. 20µl lysate was then incubated with 20µl laemmli buffer (Sigma) at 95°C for 5 minutes and loaded into a 10% (v/v) acrylamide gel (Table 2.6) to allow proteins to be separated by electrophoresis in a
Mini-PROTEAN 3 system (Bio-Rad) containing 500ml running buffer (25mM Tris Base, 0.1% [w/v] SDS [both Sigma] and 190mM glycine [Fisher Scientific]) at 120V for 90 minutes. 5µl of Amersham Full-Range Rainbow Molecular Weight Marker (GE Healthcare) was loaded alongside protein samples to allow determination of molecular weight (kDa) and therefore confirm specific antibody binding. Proteins were then electrotransferred onto Hybond-ECL nitrocellulose membrane (Amersham Biosciences) by being sandwiched between 12 layers of filter paper, previously conditioned in blotting buffer (25mM Tris Base, 190mM glycine and 20% [v/v] methanol) along with the membrane, within a Trans blot SD20 blotter (Jencons). Non-specific binding to the membrane was blocked in blocking buffer (5% [w/v] dry milk in TBS [10mM TRIS, 15mM NaCl in PBS]) at room temperature on a rocking platform for 30 minutes. The membrane was then incubated in the appropriate primary antibody (Table 2.7) at its optimal dilution in blocking buffer at 4°C overnight with the exception of the pERK1/2 antibody which was blocked in 5% (w/v) bovine serum albumin (Sigma) in TBS. The membrane was then washed 3 times in TBST (TBS + 10% Tween) before being incubated in the appropriate HRP-conjugated secondary antibody (Dako, 1:2000) in blocking buffer for 1 hour at room temperature. The membrane was then washed again in TBST and developed using enhanced chemiluminescence (Amersham Biosciences). The membrane was exposed to an autoradiographic film (Hyperfilm ECL; Amersham Biosciences) for the appropriate time period in an X-ray cassette before the film was manually developed in a dark room using Kodak GBX developer and fixer solutions (Sigma). For phospho-westerns, the media was replenished for two 30 minute periods prior to sample collection and samples were obtained using cell scrapers (Greiner Bio-one) rather than trypsinisation. Results shown are representative of 3 repeats.
<table>
<thead>
<tr>
<th>10% separating gel</th>
<th>Stacking gel</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.3ml 30% acrylamide/bisacrylamide</td>
<td>670µl 30% acrylamide/bisacrylamide</td>
<td>Bio-Rad</td>
</tr>
<tr>
<td>2.5ml 1.5M Tris HCl pH8.8/0.4% (w/v) SDS</td>
<td>1ml 0.5M Tris HCl pH6.8/0.4% (w/v) SDS</td>
<td>Sigma</td>
</tr>
<tr>
<td>4.2ml dH₂O</td>
<td>2.3ml dH₂O</td>
<td>N/A</td>
</tr>
<tr>
<td>50µl 10% (v/v) APS</td>
<td>30µl 10% (v/v) APS</td>
<td>Sigma</td>
</tr>
<tr>
<td>10µl TEMED</td>
<td>5µl TEMED</td>
<td>Sigma</td>
</tr>
</tbody>
</table>

Table 2.6. Components of 10% (v/v) acrylamide gels.

<table>
<thead>
<tr>
<th>Target</th>
<th>Dilution</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>Akt</td>
<td>1:200</td>
<td>Cell Signalling Technology</td>
</tr>
<tr>
<td>Phospho-Akt (pAkt; Ser473)</td>
<td>1:200</td>
<td>Cell Signalling Technology</td>
</tr>
<tr>
<td>Phospho-STAT3 (pSTAT3; Tyr705)</td>
<td>1:200</td>
<td>Cell Signalling Technology</td>
</tr>
<tr>
<td>STAT3</td>
<td>1:200</td>
<td>Santa Cruz</td>
</tr>
<tr>
<td>ERK1/2</td>
<td>1:200</td>
<td>Cell Signalling Technology</td>
</tr>
<tr>
<td>Phospho-ERK1/2 (pERK1/2; Thr202/Try204)</td>
<td>1:200</td>
<td>Cell Signalling Technology</td>
</tr>
<tr>
<td>Nanog</td>
<td>1:200</td>
<td>Abcam</td>
</tr>
<tr>
<td>Klf4</td>
<td>1:500</td>
<td>R &amp; D systems</td>
</tr>
<tr>
<td>α-tubulin</td>
<td>1:2000</td>
<td>Sigma</td>
</tr>
</tbody>
</table>

Table 2.7. Primary antibodies for western blot analysis.
2.6.1 RNA extraction, DNase treatment and RNA purification

Nucleic acids were obtained from cells using 500µl Tri reagent (Sigma) and either extracted immediately or stored at -80°C for future use. 50µl chloroform (Sigma) was then added and samples were vortexed for 15 seconds, incubated on ice for 5 minutes and centrifuged at 10000rpm for 15 minutes. The upper aqueous layer was then removed and the nucleic acids from this fraction precipitated using 200µl isopropanol for 15 minutes on ice followed by an additional 15 minute centrifugation at 10000rpm. The pellet was then washed twice in 800µl 70% (v/v) ethanol and resuspended in 40 µl nuclease-free water (Ambion). A DNase digestion was then performed for 2 hours at 37°C using 2.5 units (u) RQ1 DNase, 5µl 10x RQ1 DNase buffer, 40u RNAsin (all Promega) and 1.5µl nuclease-free water to remove any contaminating DNA. The reaction was then terminated using 10µl Stop solution (Promega) and the RNA was purified by phenol/chloroform extraction using 50µl phenol and 30µl chloroform, then precipitated using 10µl 3M NaOAc (pH 4.8), 150µl 95% (v/v) ethanol and 1µl glycogen (Roche) before being washed with 50µl 80% (v/v) ethanol and resuspended in 20µl nuclease free water. The RNA quality was then assessed by determining the 260/280 ratio using a Nanodrop ND-1000 spectrophotometer (Thermo Scientific, 260/280: 2.0 for pure RNA) and an additional DNase digestion performed if this value was lower than 1.9.

2.6.2 Generation of cDNA by reverse transcription

cDNA was synthesised by reverse transcription (RT) from 1µg RNA using a high capacity RNA-to-cDNA Kit (Applied Biosystems) according to the manufacturers’ instructions. Samples were centrifuged at 13000rpm for 5 minutes, aliquoted and stored at -20°C prior to analysis.

2.6.3 RT-PCR and agarose gel electrophoresis

Each RT-PCR reaction consisted of 1µl cDNA, 1µl 50pmol/µl forward and reverse primers (designed using the Primer 3 website http://frodo.wi.mit.edu/primer3/; Table 2.8) and 8µl Reddymix PCR Master Mix (Abgene). Unless specified in Table 2.8, products were amplified in a 2720 Thermal Cycler (Applied Biosystems) using the following programme: 94°C for 5 minutes, 25 cycles (94°C for 30 seconds, 60°C
for 40 seconds, 75°C for 45 seconds), 72°C for 7 minutes. Samples were then visualised on a 2% (w/v) agarose gel containing 8% (v/v) Safeview Nucleic Acid Stain (NBS Biologicals) using a UVI Pro Platinum Transilluminator (UVITec). 3 biological repeats were performed for each RT-PCR reaction and representative results are shown. –RT reactions showed no bands after 25 cycles.
<table>
<thead>
<tr>
<th>RT-PCR Primer product</th>
<th>Forward primer (5’ to 3’)</th>
<th>Reverse primer (5’ to 3’)</th>
<th>Size [bp]</th>
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<td>60</td>
</tr>
<tr>
<td>Sox2</td>
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<td>Hepatic nuclear factor 4 (HNF4)</td>
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<td>Transthyretin (Ttr)</td>
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<td>Myocyte enhancer factor 2c (Mef2c)</td>
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<td>Methylated E-cadherin promoter</td>
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<td>Unmethylated E-cadherin promoter</td>
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Table 2.8. Primer sequences used in qPCR and RT-PCR.
2.7 Quantitative real-time PCR

SYBR Green I emits fluorescence when bound to double stranded DNA and, as such, can be used to quantify levels of gene expression (relative to a gene of reference) within a cDNA sample from a particular cell line. Primers for use in quantitative real-time PCR (qPCR; Table 2.8) were designed using the Universal Probe Library software supplied by Roche Applied Science (www.roche-applied-science.com) and sequences verified using the Basic Local Alignment Sequence Tool on the National Centre for Biotechnology Information website (http://www.ncbi.nlm.nih.gov/). Triplicate samples containing 12.5µl SYBR Green PCR Master Mix (AmpliTaq Gold Polymerase, SYBR Green I dye, dNTPs and the passive internal reference ROX dye in reaction buffer; Applied Biosystems), 7.35µl RNase free H₂O, 0.075µl each of appropriate forward and reverse primers (100pmol/µl) and 5µl cDNA (2ng/µl, prepared as described in Section 2.6.2) were loaded into 96-well MicroAmp Optical Reaction plates (Applied Biosystems). Products were then amplified in a ABI 7500 Real Time PCR System (Applied Biosystems) using the following programme: 10 minute initial denaturation at 95°C, followed by 40 cycles (95°C for 30 seconds, 60°C for 30 seconds, 75°C for 35 seconds) then a final extension step (72°C for 10 minutes). Dissociation of the SYBR Green I dye was then performed under the following conditions: 95°C for 15 seconds, 60°C for 1 minute, 95°C for 15 seconds. qPCR results were analysed using the ABI 7500 system SDS software to give cycle threshold (Ct) values (the cycle number at which fluorescence due to amplification of the PCR product exceeds the background fluorescence threshold) and Ct values accepted or rejected according to the quality of the melting curve obtained (a single peak indicates a single gene product; Figure 2.5). Ct values for each gene were normalised to 18S transcripts to give ΔCt values (ΔCt = target gene Ct – 18s Ct) then ΔΔCt values (ΔCt_{sample} – ΔCt_{wtD3}) were obtained. Mean $2^{-\Delta\Delta Ct}$ values, as a measure of relative transcript expression, were then plotted on a graph with error bars reflecting the SEM for each set of values. The reaction was repeated to include 3 biological replicates. –RT reactions showed no amplification after 40 cycles.
Figure 2.5. Examples of SYBR Green dissociation peaks. (A): A single peak represents amplification of a single gene product. (B): Multiple peaks represent amplification of contaminating gene products.

2.8 Microarray analysis

Microarray analysis was performed on wtD3 mES cells, ENPS<sup>R</sup> cells and 2 ENPS<sup>M</sup> cell lines (ENPS1 and ENPS2) cultured in FBS-containing medium. RNA was extracted as described in section 2.6.1. Samples were analysed by the Microarray Facility of Life Sciences (The University of Manchester, UK) according to the following protocol.

2.8.1 cDNA preparation for the array

Samples were prepared according to the Affymetrix GeneChip Technical manual. Unless specified, all reagents were provided by Affymetrix UK. Firstly, RNA quality was determined using the RNA 6000 Nano assay and run on an Agilent 2100 Bioanalyser (Agilent Technologies) as described in the manufacturer’s guide. cDNA was then synthesised from 10ng RNA using a two-cycle synthesis kit and the nucleic acids purified using the GeneChip Sample Clean up Module Kit according to the manufacturer’s instructions. The GeneChip IVT Labelling Kit was used to amplify biotin-labelled cRNA which was then cleaned with the GeneChip Sample Clean up Module Kit. cRNA was then quantified using a Nanodrop ND-1000 spectrophotometer before 15µg cRNA was fragmented at 94°C for 30mins in 5x
fragmentation buffer. Hybridisation cocktails were prepared according to the manual and loaded onto a mouse genome Mouse430_2 chip for 16 hours at 45°C on a rotating platform (60rpm). GeneChips were then loaded onto a Fluidics station, washed and labelled with streptavidin-conjugated R-PE (Invitrogen) using the EukGe W S2v5 protocol and controlled with the Affymetrix GeneChip Operating Software v1.4 (GCOS). The GeneChips were then analysed on the GeneChip Scanner 3000 (Agilent Technologies) using the GCOS software.

2.8.2 Analysis of results

Quality control was performed with dChip (V2005) (Li and Hung Wong, 2001) using the default settings. The GCRMA package in Bioconductor was then used to perform background correction, quantile normalisation and gene expression analysis (Han et al., 2004) and Partek Genomics Solution software v6.4 (Partek Inc.) was used to perform principle component analysis (Smyth, 2004). Differential expression analyses between all 4 cell lines were performed with Limma using the lmFit and eBayes functions (Smyth, 2004). Gene lists of differentially expressed genes were controlled for false discovery rate errors using the q-value method (Storey and Tibshirani, 2003). A q-value <0.05 and FC ≥ 2.5 were used as parameters for statistically significant alterations and the DAVID website (http://david.abcc.ncifcrf.gov/) used to group transcripts according to Gene Ontology (GO) terms.
Chapter 3

E-cadherin and, in its absence, N-cadherin promotes Nanog expression in mES cells via STAT3 phosphorylation

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Author contributions: K.H.: Conception and design, collection and assembly of data, data analysis and interpretation, manuscript writing, final approval of manuscript; L.M.: manuscript writing, provision of study material, final approval of manuscript; S.R.: Conception and design, manuscript writing; C.M.W.: Conception and design, financial support, provision of study material, collection and assembly of data, data analysis and interpretation, manuscript writing, final approval of manuscript; C.L.R.M.: Conception and design, manuscript writing.
3.1 Abstract

We have recently shown that loss of E-cadherin in mouse embryonic stem (mES) cells results in significant alterations to both the transcriptome and hierarchy of pluripotency-associated signalling pathways. Here we show that E-cadherin promotes Klf4 and Nanog transcript and protein expression in mES cells via STAT3 phosphorylation and that β-catenin, and its binding region in E-cadherin, is required for this function. To further investigate the role of E-cadherin in LIF-dependent pluripotency, E-cadherin null (Ecad<sup>−/−</sup>) mES cells were cultured in LIF/BMP supplemented medium. Under these conditions, Ecad<sup>−/−</sup> mES cells exhibited partial restoration of cell-cell contact and STAT3 phosphorylation and upregulated Klf4, Nanog and N-cadherin transcripts and protein. Abrogation of N-cadherin using an inhibitory peptide caused loss of pSTAT3, Klf4 and Nanog in these cells, demonstrating that N-cadherin supports LIF-dependent pluripotency in this context. We therefore identify a novel molecular mechanism linking E- and N-cadherin to the core circuitry of pluripotency in mES cells. This mechanism may explain the recently documented role of E-cadherin in efficient induced pluripotent stem (iPS) cell reprogramming.

3.2 Introduction

Understanding the molecular mechanisms that govern pluripotency in ES cells is key to the derivation of novel stem cell types (e.g. iPS cells), the use of these cells as models of disease (Park et al., 2008) and the application of such cells in clinical therapies (Ringe et al., 2002). Pluripotency can be maintained in vitro in mES cells by leukaemia inhibitory factor (LIF) and bone morphogenetic proteins (Ying et al., 2003; BMPs), whereas human (h) ES cells and hiPS cells require Activin/Nodal and fibroblast growth factor 2 (FGF2) to maintain this state (Vallier et al., 2005; Vallier et al., 2009b). Despite the variation in exogenous cues supporting pluripotency between the two species, the core circuitry of pluripotency, Oct4, Sox2 and Nanog, is conserved. Recent examination of the relationship of LIF to this core circuitry in mES cells has revealed two parallel-functioning pathways (Jak/STAT3 and PI3K/Akt)
that maintain Oct4, Sox2 and Nanog expression via Kruppel-like factor 4 (Klf4) and T-box factor 3 (Tbx3). LIF has also been shown to stimulate the MAPK/ERK pathway in these cells (Niwa et al., 2009). Critically, artificial activation of STAT3 has been shown to maintain self-renewal of mES cells in the absence of LIF, demonstrating the importance of this protein in mES cell pluripotency (Matsuda et al., 1999).

In recent years, E-cadherin has emerged as a key factor in the maintenance of pluripotency. For example, E-cadherin has been implicated in the regulation of signalling pathway hierarchy in mouse stem cells (Chou et al., 2008; Soncin et al., 2009), enhanced iPS cell generation (Chen et al., 2011; Chen et al., 2010; Li et al., 2010b; Redmer et al., 2011) and in promoting pluripotent hES cell expansion when utilised as a substratum (Nagaoka et al., 2010). Moreover, we have recently shown that E-cadherin null (Ecad−/−) mES cells exhibit alterations in over 2000 gene transcripts when compared to wild type (wt) mES cells and that these changes are associated with a wide range of cellular processes (Soncin et al., 2011). E-cadherin is a well-characterised member of the classical cadherin family. Structurally, it is a single-pass transmembrane glycoprotein with a HAV motif within its extracellular region and a β-catenin binding domain within its cytoplasmic region, the latter facilitating interaction with the actin cytoskeleton via α-catenin (Cavallaro and Christofori, 2004). Ecad−/− embryos fail to form a trophectodermal epithelium or to undergo compaction due to loss of cell-cell contact (Larue et al., 1994), thus demonstrating the importance of this adhesion molecule for embryogenesis. mES cells derived from Ecad−/− embryos or β-catenin null (βcat−/−) embryos exhibit a single-celled mesenchymal phenotype (Soncin et al., 2009) and maintain pluripotency marker expression independently of LIF, instead using the Activin/Nodal pathway. As such, Ecad−/− and βcat−/− exhibit more similarity to hES cells, hiPS cells, FAB-SCs (Chou et al., 2008) and EpiSCs (Brons et al., 2007; Tesar et al., 2007) than wt mES cells.

N-cadherin is also a member of the classical cadherin family and, as such, exhibits a high degree of structural homogeneity to E-cadherin. N-cadherin possesses a HAV motif within its extracellular region and has been shown to form a complex with β-
catenin (Wahl et al., 2003). However, unlike E-cadherin, N-cadherin is not expressed in ES cells, instead it is rapidly upregulated in both hES and mES cells upon induction of differentiation in a process akin to an epithelial-to-mesenchymal transition (EMT) event (Eastham et al., 2007; Spencer et al., 2007). Forced expression of N-cadherin in Ecad<sup>−/−</sup> mES cells has been shown to rescue cell-cell contact and increase the frequency of chimera generation (Larue et al., 1996). To date, however, there is no evidence that N-cadherin can compensate for the role of E-cadherin in maintaining LIF-dependent mES cell pluripotency.

In this study, we have investigated the function of E-cadherin in LIF-dependent pluripotency. We show that E-cadherin positively regulates Klf4 and Nanog transcript and protein expression via STAT3 phosphorylation in mES cells and that its β-catenin binding domain is critical for this function. Furthermore, culture of Ecad<sup>−/−</sup> mES cells in LIF/BMP-supplemented medium in the absence of Activin/Nodal leads to restoration of cell-cell contact, STAT3 phosphorylation and enhancement of Klf4 and Nanog transcript and protein expression. This is likely to be via an N-cadherin-dependent mechanism in Ecad<sup>−/−</sup> mES cells and an E-cadherin-dependent mechanism in wt mES cells since abrogation of these proteins in the respective cells when cultured in LIF/BMP medium results in loss of pSTAT3 and decreased Klf4 and Nanog expression. Therefore, either E-cadherin or N-cadherin can facilitate LIF-dependent pluripotency in mES cells and in their absence no other factor acts to fulfil this function.

3.3 Materials and Methods

**Culture of mouse embryonic stem cells**

All cell lines used in this study have been described previously (Soncin et al., 2009). Unless otherwise specified, mES cells were cultured on 0.1% gelatin (Sigma)-coated plates in Knockout DMEM supplemented with 10% fetal bovine serum (FBS), 50μM 2-mercaptoethanol (all Invitrogen), 2mM L-glutamine, 1% non-essential amino acids (both PAA) and 1000U/ml LIF (Chemicon International) at 37°C, 5% CO₂. Cells were passaged every 2 days, when a maximum confluence of 70-80% was reached. Cells
were acclimatised to culture in ESGRO Complete Plus Clonal Grade Medium (LIF/BMP medium; Millipore), or 2i medium (iSTEM Embryonic Stem Cell Culture Medium, Stem Cells Inc.) supplemented with 1000U/ml LIF, by passage in increasing concentrations of these media within FBS-containing medium (Table 2.2). At days 0-4 cells were detached from plates using Trypsin/EDTA (PAA), thereafter cell dissociation buffer (Gibco) was used. Appropriate cells were treated with a 1mM concentration of an E- and N-cadherin peptide inhibitor (Devemy and Blaschuk, 2009) (E/N+) or a control peptide (Devemy and Blaschuk, 2008) (E/N-), a 10μM concentration of the Alk inhibitor, SB431542 (Tocris) or Jak inhibitor I (Calbiochem) for 8 days. Controls were treated with DMSO.

**Amaxa Transfection of mES cells**

24 hours after passaging, 2x10^6 cells were transfected as previously described with FL-Ecad, Δβcat, Δβcat-Δp120ctn or empty control plasmids or Nanog promoter regions within the pGL3 basic plasmid.

**Colony forming assays**

Cells were trypsinised and serial dilutions were plated in a 96 well plate in the appropriate media. Wells containing a single cell were identified and when 70-80% confluence was reached, cells from these wells were passaged into 24-, 12- and 6-well plates for analysis.

**Fluorescent flow cytometry**

Cells were harvested using cell dissociation buffer, centrifuged and washed with PBS before being resuspended in 100μl DECMA-1 (1:100 dilution; Sigma) in FACS buffer (0.2% bovine serum albumin, 0.1% sodium azide in PBS) for 30 minutes on ice. Cells were then washed with PBS, resuspended in PE-conjugated secondary antibody (1:100 dilution; Santa Cruz) in FACS buffer and incubated for 30 minutes on ice. Cells were then washed and fixed in 1% w/v paraformaldehyde (PFA) in PBS. Cell fluorescence was then quantified using a Becton Dickinson FACS Calibur. Viable cells were gated using forward and side scatter and all data represents cells from this population. For intracellular flow cytometry, cells were fixed in 1% PFA for 10
minutes at room temperature before the membrane was permeabilised by incubating cells in ice-cold 70% methanol at -20°C overnight. Cells were then washed with PBS and stained according to the above protocol using an Oct4, Nanog (Abcam) or Klf4 antibody (R&D systems, all 1:100).

Immunofluorescent cell imaging
Cells were fixed in 4% w/v PFA in PBS for 15 minutes before being prepared as previously described (Barrow et al., 2006) using the following primary antibodies: N-cadherin, (1:200 dilution; Santa Cruz) Klf4, Nanog and Oct4 (all 1:200; Abcam) followed by the appropriate Alexa-Fluor 488-conjugated secondary antibody (1:500; Invitrogen). Images were visualised using a fluorescence microscope and processed using Adobe Photoshop version 6.0.

RT-PCR
RNA was harvested using TRI reagent (Sigma), treated with DNase (Promega) and phenol/chloroform purified. cDNA was synthesised as previously described (Ward et al., 2003). Products were amplified for 25 cycles at the optimal primer annealing temperature (Table 2.8).

Quantitative real-time PCR
Triplicate samples containing appropriate forward and reverse primers (100pmol/μl; Table 2.8) were analysed as previously described with 18S ribosomal RNA as the endogenous control. All results show the standard error of the mean (SEM) of 3 independent replicates.

Western blot analysis
Cells were trypsinised and lysed using RIPA buffer (Sigma) containing protease inhibitor cocktail tablets (Roche Applied Science). The lysate from 4x10^5 cells was incubated on ice for 30 minutes and boiled in reducing conditions. Proteins were then separated using SDS-PAGE and electrotransferred onto Hybond-ECL nitrocellulose membrane (Amersham Biosciences). The membrane was incubated in blocking buffer (5% dry milk in TBS [10mM TRIS, 15mM NaCl in PBS]) with the
exception of the phospho-ERK1/2 (pERK1/2; Thr202/Tyr204) antibody, which was blocked in 5% BSA in TBS at room temperature for 30 minutes. The membrane was then incubated in primary antibody (α-tubulin [Sigma; 1:2000], KLF4 [R&D systems], STAT3 [Santa Cruz], phospho-STAT3 [pSTAT3; Tyr705], phospho-Akt [pAkt; Ser473], Akt, pERK1/2, ERK1/2 [all Cell Signalling Technology], N-cadherin and Nanog [Abcam], all 1:200) in blocking buffer at 4°C overnight. The membrane was washed 3 times with TBST (TBS + 10% Tween) before being incubated in the appropriate HRP-conjugated secondary antibody (Dako; all 1:2000) in blocking buffer for 1 hour at room temperature. The membrane was then washed as previously described and developed using enhanced chemiluminescence (Amersham Biosciences). For phospho-westerns, the media was replenished for two 30 minute periods prior to sample collection.

**Nanog promoter analysis**

Regions upstream of the Nanog transcriptional start site (530, 1087, 3832, 4432, 4547 [minus STAT3] and 4963bp) were amplified by PCR and ligated into the pGL3 basic plasmid (Promega). Vectors were amplified in DH5α competent cells (Invitrogen) and sequenced prior to use. Vectors were transfected into mES cells using an Amaxa Nucleofactor II system (Amaxa Biosystems; programme A30). 24 hours post-transfection, cells were lysed using a Dual Luciferase Reporter Assay System (Promega) according to the manufacturer’s instructions. Lysates were analysed for luciferase activity using an Orion L Microplate Luminometer (Berthold Detection Systems). Luciferase activity was normalised using a vector containing the Renilla gene and mean values were plotted graphically. All results reflect the SEM of 3 independent replicates.

3.4 Results

**Klf4 and Nanog are positively regulated by E-cadherin**

The current understanding of Nanog regulation via LIF signalling in mES cells is shown in Figure 3.1a. To explore the role of E-cadherin in this transduction network, Ecad<sup>−/−</sup> mES cells and the parental cell line wtD3 mES cells were analysed
along with MESC20 mES cells exhibiting RNA interference of E-cadherin (EcadRNAi mES cells) and a clone of these cells in which E-cadherin inhibition has been reversed (EcadRNAiR mES cells). Ecad<sup>−/−</sup> and EcadRNAi mES cells lack cell surface E-cadherin as evidenced by flow cytometry analysis (Figure 3.1b). RT-PCR analysis of these cells cultured in FBS-containing medium supplemented with LIF (FBS-containing medium) confirmed the expression of Oct4, Nanog and Klf4 transcripts (Figure 3.1c) and Klf4 and Nanog proteins (Figure 3.1d) in wtD3 and EcadRNAiR mES cells. However, Ecad<sup>−/−</sup> and EcadRNAi mES cells exhibited decreased Nanog and Klf4 transcript (Figure 3.1c) and protein (Figure 3.1d) levels in comparison to controls. Following the observation of decreased Nanog protein levels in Ecad<sup>−/−</sup> and EcadRNAi mES cells in comparison to the control cell lines, a possible role for E-cadherin in the regulation of Nanog transcription was explored in further detail. In agreement with the RT-PCR data, qPCR analysis of Nanog transcript expression levels indicated decreased transcript expression in Ecad<sup>−/−</sup> and EcadRNAi mES cells in comparison to control cell lines (Figure 3.1e).
**Figure 3.1.** Ecad<sup>+/−</sup> and EcadRNAi mES cells lack expression of LIF signalling components. (A): A simplified depiction of the current understanding of the LIF signalling network in mES cells and its connection to Nanog (Adapted from Niwa et al., 2009). (B): Flow cytometry analysis of E-cadherin expression in Ecad<sup>+/−</sup>, EcadRNAi (both pink), wtD3 and EcadRNAiR mES cells (both green) cultured in FBS-containing medium. The purple peak represents an isotype control. (C): RT-PCR analysis of Oct4, Nanog, Klf4 and β-tubulin housekeeping transcripts in Ecad<sup>+/−</sup>, EcadRNAi mES cells and control cells cultured in FBS-containing medium. (D): Western blot analysis of Klf4 and Nanog and α-tubulin housekeeping protein expression in Ecad<sup>+/−</sup> and EcadRNAi mES cells and control cells cultured in FBS-containing medium. (E): qPCR analysis of Nanog transcript expression in Ecad<sup>+/−</sup>, wtD3, EcadRNAi and EcadRNAiR mES cells. Expression levels were normalised to 18S rRNA transcripts and wtD3 mES
cell transcripts were given a value of 1. Error bars reflect the standard error of the mean (SEM) for 3 biological replicates.

**Ecad<sup>−/−</sup> mES cells lack pSTAT3 activation of the Nanog promoter**

To further investigate the regulation of Nanog by E-cadherin, Ecad<sup>−/−</sup> and wtD3 mES cells were transfected with a luciferase reporter gene transcriptionally regulated by various truncated fragments of the Nanog promoter region. Results indicated low levels of transcriptional activation of Nanog in response to transfection with each of the vectors in Ecad<sup>−/−</sup> mES cells whereas wtD3 mES cells exhibited a significant increase in luciferase activity when transfected with the 5kb vector in comparison to when transfected with the 4.5kb vector (Figure 3.2a; p<0.05). It has previously been shown that Nanog transcript expression is upregulated in mES cells by STAT3 and Brachyury (T) binding to regions 4956 and 4912bp upstream of the Nanog promoter respectively (Suzuki et al., 2006). Deletion of the STAT3 and T binding elements within the Nanog promoter vector resulted in significantly decreased promoter activity in wtD3 mES cells whereas Ecad<sup>−/−</sup> mES cells exhibited similar activity compared to the 5kb vector (Figure 3.2a; minus STAT3; p<0.05). RT-PCR analysis of T transcripts demonstrated that T is not expressed in wtD3 mES cells cultured in FBS-containing medium (Figure 3.2b) and therefore cannot be responsible for the differences in luciferase activity observed between Ecad<sup>−/−</sup> and wtD3 mES cells. However, as observed previously (Soncin et al., 2009), T was upregulated in wtD3 mES cells upon withdrawal of LIF for 8 days (Figure 3.2b).

These findings suggest a requirement for STAT3 activation of the Nanog promoter in wtD3 mES cells, indicating that the low activity of the Nanog promoter observed in Ecad<sup>−/−</sup> mES cells may be due to a lack of STAT3 transcriptional enhancement in these cells. To test this hypothesis, the levels of pSTAT3 in wtD3 and Ecad<sup>−/−</sup> mES cells were assessed by western blot analysis. Whilst total STAT3 demonstrated comparable levels between Ecad<sup>−/−</sup> and wtD3 mES cells (Figure 3.2c; STAT3), Ecad<sup>−/−</sup> mES cells lacked pSTAT3 (Figure 3.2c; pSTAT3). Together, these results suggest that the decreased Nanog expression observed in Ecad<sup>−/−</sup> mES cells reflects a lack of pSTAT3 activation of the Nanog promoter in these cells.
To explore the role of E-cadherin in the activation of other branches of the LIF pathway we also assessed Akt and ERK1/2 phosphorylation by western blotting. This technique demonstrated that whereas pERK1/2 is absent in Ecad<sup>−/−</sup> mES cells (Figure 3.2d), pAkt is present in Ecad<sup>−/−</sup> mES cells but absent in wtD3 mES cells (Figure 3.2e). However, since these pathways are activated by a wide range of alternative pathways (whereas STAT3 is activated by LIF alone) we did not pursue this analysis further.
Figure 3.2. Ecad\textsuperscript{+/−} mES cells lack STAT3 activation of the Nanog promoter. (A): mES cells grown in FBS-containing medium were transfected with a luciferase reporter vector containing truncated fragments of the Nanog promoter region ([0.5kb [530bp], 1kb [1087bp], 4kb [3832bp], 4.5kb [4432bp], minus STAT3 [4547bp] and 5kb [4963bp]]). Cells were then analysed for luciferase activity 24 hours after transfection. Results were normalised using the renilla gene and the FC over the activity of the empty pGL3 control plasmid was plotted graphically. Error bars reflect the SEM of 3 independent experiments. *p<0.05; unpaired t test. (B): RT-PCR analysis of Brachyury (T) in wtD3 mES cells in FBS-containing media with LIF and after 8 days of LIF withdrawal. (C): Western blot analysis of STAT3 activation in wtD3 and Ecad\textsuperscript{−/−} mES cells cultured in FBS-containing medium. (D): Western blot
analysis of ERK1/2 and (E): Akt activation in wtD3 and Ecad⁻/⁻ mES cells cultured in FBS-containing medium. FC: fold change.
The β-catenin binding region of E-cadherin is required for STAT3 phosphorylation

To determine the region of E-cadherin required for STAT3 activation in mES cells, Ecad<sup>+/−</sup> mES cells were transiently transfected with cDNA encoding full length E-cadherin (FL-Ecad), E-cadherin lacking the β-catenin binding domain (Δβcat) and E-cadherin lacking the entire cytoplasmic domain<sup>28</sup> (Δβcat-Δp120<sup>ctn</sup>). Whereas all three expression vectors conferred an increase in cell surface E-cadherin protein on Ecad<sup>+/−</sup> mES cells, as evidenced by flow cytometry analysis, only full length E-cadherin restored cell-cell contact in these cells (Figure 3.3a). In addition, western blot analysis revealed that only full length E-cadherin was able to restore STAT3 phosphorylation in Ecad<sup>+/−</sup> mES cells and that βcat<sup>+/−</sup> mES cells also lack pSTAT3 (Figure 3.3b). Together, these results indicate that the β-catenin binding domain of E-cadherin is crucial for its role in STAT3 activation.
**Figure 3.3.** The β-catenin binding region of E-cadherin is required for STAT3 activation in mES cells. (A): Phase contrast images of Ecad<sup>−/−</sup> mES cells cultured in FBS-containing medium and transfected with full length E-cadherin (Ecad-FL), E-cadherin lacking the β-catenin binding domain (Δβcat) and E-cadherin lacking the entire cytoplasmic domain (Δβcat-Δp120<sub>ctn</sub>; scale bars: 100µm). Cells were analysed by flow cytometry for cell surface E-cadherin expression (green peaks). Purple peaks represent isotype controls. (B): Ecad<sup>−/−</sup> mES cells transfected with truncated fragments of E-cadherin were analysed for pSTAT3 using western blotting. βcat<sup>−/−</sup> mES cells cultured in FBS-containing medium were also assessed for pSTAT3 using western blotting.
**Ecad\(^{-/}\) mES cells can maintain pluripotency via LIF/BMP**

We have previously stated that Ecad\(^{-/}\) mES cells maintain viability upon culture in LIF/BMP medium (Soncin et al., 2009). Ecad\(^{-/}\) mES cells cultured under these conditions exhibited partial restoration of cell-cell contact and Oct4 expression was maintained in >95% of the wtD3 and Ecad\(^{-/}\) mES cell populations, including when exposed to the Activin/Nodal receptor inhibitor SB431542. Conversely, Ecad\(^{-/}\) mES cells cultured in FBS-containing medium lost Oct4 expression under these conditions (Figure 3.4a), as previously shown (Soncin et al., 2009). Furthermore, Oct4 expression was lost in both wtD3 and Ecad\(^{-/}\) mES cells when cultured in LIF/BMP medium supplemented with a Jak inhibitor (Figure 3.4b), confirming the requirement for Jak/STAT3 signalling to maintain pluripotency under these conditions. In addition, Ecad\(^{-/}\) mES cells exhibited partial restoration of Klf4 and Nanog transcripts (Figure 3.4c, e) and protein (Figure 3.4d) when cultured in LIF/BMP medium in comparison to when grown in FBS-containing medium. Immunofluorescent and flow cytometry analysis of Klf4 and Nanog expression in Ecad\(^{-/}\) mES cells confirmed these results and demonstrated that transfection of Ecad\(^{-/}\) mES cells with FL-Ecad increased Klf4 and Nanog protein expression (Figure S3.1a-b). Western blot analysis also demonstrated partial restoration of STAT3 phosphorylation in Ecad\(^{-/}\) mES cells cultured in LIF/BMP (Figure 3.4f). Together, these results demonstrate that Ecad\(^{-/}\) mES cells possess functional LIF/BMP signalling pathways that can be activated to support pluripotency marker expression in the absence of Activin/Nodal signalling.
Figure 3.4. Ecad$^{-/-}$ mES cells maintain pluripotency marker expression and upregulate LIF signalling components when cultured in LIF/BMP medium. (A): Phase contrast images, flow cytometry analysis and immunofluorescent analysis of Oct4 (green) protein expression (scale bars: 100µm) in wtD3 and Ecad$^{-/-}$ mES cells cultured either in FBS-medium or LIF/BMP medium and either untreated, treated with the Alk inhibitor SB431542 or treated with its control, DMSO. Nuclei are stained with DAPI (blue). (B): Phase contrast images (Scale bars: 100µm) and immunofluorescent analysis of Oct4 expression (green; scale bars: 50µm) in wtD3 and Ecad$^{-/-}$ mES cells cultured in LIF/BMP medium supplemented with a Jak inhibitor or its control, DMSO. (C): RT-PCR analysis of Oct4, Nanog and Klf4 transcripts in wtD3 and Ecad$^{-/-}$ mES cells grown in LIF/BMP medium. (D): qPCR analysis of Nanog and Klf4 transcript expression in mES cells cultured in FBS-containing medium or LIF/BMP medium. Error bars reflect the SEM for 3 replicates. Expression levels are related to wtD3 mES cell transcripts, which were assigned a value of 1. (E): Western blot analysis of Klf4 and Nanog protein expression in wtD3 and Ecad$^{-/-}$ mES cells grown in FBS-containing medium or LIF/BMP medium. (F): Western blot analysis of STAT3 phosphorylation in mES cells cultured in FBS-containing medium or LIF/BMP medium.
N-cadherin promotes Ecad<sup>−/−</sup> mES cell pluripotency marker expression in LIF/BMP medium

We hypothesised that the restoration of cell-cell contact observed in Ecad<sup>−/−</sup> mES cells cultured in LIF/BMP medium was due to de novo expression of N-cadherin, since N-cadherin has previously been shown to rescue cell-cell adhesion in Ecad<sup>−/−</sup> mES cells (Larue et al., 1996). N-cadherin transcripts were not detected in wtD3 or Ecad<sup>−/−</sup> mES cells cultured in FBS-containing medium, however, Ecad<sup>−/−</sup> mES cells cultured in LIF/BMP medium exhibited expression of N-cadherin transcripts (Figure 3.5a) and protein (Figure 3.5b). Furthermore, N-cadherin protein expression was localised at cell-cell contacts (Figure 3.5c) and treatment of the cells with an E-and N-cadherin peptide inhibitor (E/N+) abrogated cell-cell contact in both wtD3 and Ecad<sup>−/−</sup> mES cells (Figure 3.5d). These results suggest that N-cadherin maintains cell-cell contact between Ecad<sup>−/−</sup> mES cells when cultured in LIF/BMP medium. qPCR analysis of Nanog transcript expression levels in cells grown in LIF/BMP medium demonstrated decreased expression in both wtD3 and Ecad<sup>−/−</sup> mES cells when treated with the E/N+ peptide compared to untreated cells or cells exposed to the control peptide (E/N−; Figure 3.5e). Treatment of wtD3 and Ecad<sup>−/−</sup> mES cells cultured in LIF/BMP medium with the E/N+ peptide had no effect on Oct4 expression as determined by flow cytometry (Figure 3.5f), however, exposure to this peptide resulted in the loss of Klf4 transcripts whereas cells treated with the E/N- peptide and untreated cells maintained Klf4 transcript and protein expression (Figure 3.5g). Furthermore, STAT3 phosphorylation was abolished in both wtD3 and Ecad<sup>−/−</sup> mES cells following treatment with the E/N+ peptide for 48 hours (Figure 3.5h). Together, these results indicate that N-cadherin can compensate for E-cadherin-dependent STAT3 phosphorylation in mES cells. Interestingly, similar results were obtained in Ecad<sup>−/−</sup> mES cells cultured in 2i medium (Figure S3.3).
Figure 3.5. N-cadherin promotes the expression of LIF signalling components in Ecad\textsuperscript{-/-} mES cells cultured in LIF/BMP medium. (A): RT-PCR analysis of N-cadherin transcripts in mES cells grown in FBS-containing medium or LIF/BMP medium. (B): Western blot analysis and (C): Immunofluorescent analysis of N-cadherin protein expression (green) in wtD3 and Ecad\textsuperscript{-/-} mES cells cultured in LIF/BMP medium. DAPI (blue) was used to stain the nuclei. Scale bars: 25µm or 10µm (inset). (D): Morphology of wtD3 and Ecad\textsuperscript{-/-} mES cells cultured in LIF/BMP medium and exposed to an E- and N-cadherin peptide inhibitor (E/N+) or control peptide (E/N-). Scale bars: 100µm. (E): qPCR analysis of Nanog transcript expression in mES cells grown in LIF/BMP medium with E/N+, E/N- or untreated (LIF/BMP). Error bars reflect the SEM of 3 independent experiments. (F): Flow cytometry analysis of Oct4 expression in wtD3 and Ecad\textsuperscript{-/-} mES cells grown in LIF/BMP medium and E/N+ (blue), E/N- (green) or untreated (pink). (G): RT-PCR analysis of Klf4 transcripts in mES cells cultured in LIF/BMP medium and E/N+, E/N- or untreated (LIF/BMP). (H): Western blot analysis of pSTAT3 and Klf4 expression in wtD3 and Ecad\textsuperscript{-/-} mES cells cultured for 6 days in LIF/BMP medium with E/N+, E/N- or no treatment (LIF/BMP).
Figure 3.6. The role of E- and N-cadherin in LIF signalling and mES cell pluripotency. (A): According to the data presented here, the β-catenin binding domain of E-cadherin is crucial for STAT3 phosphorylation and subsequent expression of Klf4 and Nanog in mES cells, thus facilitating LIF-dependent pluripotency in mES cells. (B): E-cadherin or, in its absence, N-cadherin expression is required for STAT3 phosphorylation, elevated Klf4 and Nanog expression, LIF/BMP-mediated pluripotency and expression of an ES cell-like phenotype. In the absence of E- and N-cadherin, mES cells adopt a more epiblast-like phenotype, exhibiting a lack of STAT3 activation, decreased Klf4 and Nanog expression and Activin/Nodal-mediated pluripotency.
3.5.1 Discussion

In this study we have dissected a novel signalling pathway that connects E-cadherin and N-cadherin to the core circuitry of pluripotency in mES cells. Firstly, we show that E-cadherin is required for STAT3 phosphorylation and subsequent enhancement of Klf4 and Nanog transcript and protein expression. Interestingly, blockade of E-cadherin causes direct inhibition of STAT3 phosphorylation since treatment of wtD3 mES cells with the E/N+ peptide for 3 hours resulted in loss of STAT3 phosphorylation (Figure S3.1c). Importantly, the observation of decreased pSTAT3, Klf4 and Nanog in Ecad−/− mES cells is not due to phenotypic drift during prolonged culture or isolation of an aberrant clone since we have repeated these results in EcadRNAi mES cells and clones from single-cell low passage Ecad−/− mES cells (Figure S3.2). Furthermore, we demonstrate that β-catenin and its binding domain within the cytoplasmic region of E-cadherin is required for STAT3 activation (Figure 3.6a). These results support our recent finding that LIF supplementation exerted little effect on the transcriptome of Ecad−/− mES cells (Soncin et al., 2011), presumably as a result of the lack of STAT3 phosphorylation in these cells. In the absence of E-cadherin we show that N-cadherin can compensate for the role of E-cadherin in STAT3 phosphorylation and positive regulation of Klf4 and Nanog. To our knowledge, this is the first demonstration that N-cadherin can function in mES cells to maintain pluripotency marker expression.

There remains some controversy within the literature regarding the requirement for E-cadherin in maintaining mES cell pluripotency. For example, Redmer et al. (2011) suggest that abrogation of E-cadherin expression in wt mES cells leads to their differentiation. This is inconsistent with our findings; wt mES cells can be cultured in the presence of the E-cadherin inhibiting antibody DECMA-1 whilst retaining pluripotency marker expression and the ability to form cells representative of the three primary germ layers (Mohamet et al., 2010). Therefore, whilst loss of E-cadherin leads to decreased Nanog expression levels, which could be interpreted as differentiation, the ability to reverse this phenotype demonstrates that differentiation has not occurred. Fundamentally, the ability of N-
cadherin to maintain elevated levels of Nanog protein expression in Ecad<sup>−/−</sup> mES cells demonstrates that loss of E-cadherin alone is insufficient to induce differentiation in mES cells, at least under the conditions described in this study. Instead, we suggest that loss of E-cadherin in mES cells leads to a reversible pluripotent epiblast-like phenotype. This is supported by recent evidence that EpiSCs can be reprogrammed to a mES cell-like naive pluripotent state (Yang et al., 2010), suggesting that LIF stimulation, under appropriate conditions, reprograms Ecad<sup>−/−</sup> mES cells to naïve pluripotency, as observed in FAB-SCs (Chou et al., 2008).

The ability of N-cadherin to promote STAT3 phosphorylation, and subsequent Klf4 and Nanog expression in the absence of E-cadherin in mES cells was unexpected and provides a novel function for this protein in the maintenance of mES cell pluripotency. E-cadherin expression is shown here to be dominant over N-cadherin expression in wt mES cells since the latter is not upregulated in these cells upon LIF stimulation. Therefore, it appears that N-cadherin only functions as a regulator of STAT3 phosphorylation in the absence of E-cadherin. It is likely that this function of N-cadherin is limited to in vitro culture of mES cells since E-cadherin does not exhibit redundancy during embryogenesis (Larue et al., 1994), despite the ability of N-cadherin to rescue the chimera-forming abilities of Ecad<sup>−/−</sup> mES cells to some extent (Larue et al., 1996). Our data suggests that only E- and N-cadherin can promote STAT3 phosphorylation in mES cells. Whilst it is possible that VE-cadherin could also function in a similar manner, since this protein also contains a β-catenin binding domain, treatment of wt mES cells with an E- and N-cadherin inhibitory peptide was sufficient to abrogate STAT3 phosphorylation.

There is a clear hierarchy in mES cell pluripotent pathway regulation in both the presence and absence of E-cadherin expression (Figure 3.6b). Firstly, the dominant pluripotency pathway/cadherin regulator in wt mES cells is LIF/E-cadherin. In the absence of E-cadherin, mES cells can bypass the LIF pathway, instead using Activin/Nodal to maintain pluripotency marker expression, albeit with decreased Klf4 and Nanog expression. Alternatively, when stimulated with LIF in the absence of Activin/Nodal, mES cells can maintain STAT3 phosphorylation and LIF-dependent
pluripotency via N-cadherin. Our data suggest that the Activin/Nodal pathway exhibits priority over the LIF/BMP pathway in the absence of E-cadherin since Ecad<sup>−/−</sup> mES cells maintain pluripotency marker expression via the former when cultured in FBS-containing medium supplemented with LIF. Ecad<sup>−/−</sup> mES cells also maintain pluripotency marker expression when cultured in GSK3 and MAPK inhibitors (2i medium; Figure S3.3), as previously shown (Ying et al., 2008), suggesting these cells also possess a ‘ground state’ of self-renewal. Interestingly, these cells exhibit N-cadherin-dependent STAT3 phosphorylation and upregulation of Klf4 and Nanog in a similar manner to when cultured in LIF/BMP medium.

During ES cell differentiation, the cells undergo an EMT-like event which is associated with an E- to N-cadherin switch and increased cell motility (Eastham et al., 2007; Spencer et al., 2007). However, the upregulation of N-cadherin that occurs in Ecad<sup>−/−</sup> mES cells cultured in LIF/BMP medium is not a true EMT event as it leads to increased Nanog expression and restored cell-cell contact, which is in contrast to the function of N-cadherin in EMT. To our knowledge, this is the first description of the ability of N-cadherin to promote pluripotency marker expression, albeit in a mutant (Ecad<sup>−/−</sup> mES) cell line.

### 3.5.2 Conclusion

In summary, we demonstrate that E-cadherin is required for STAT3 phosphorylation in mES cells, resulting in positive regulation of Klf4 and Nanog transcripts and protein and, ultimately, facilitating LIF-dependent maintenance of pluripotency. Interestingly, this may explain the requirement for E-cadherin in iPS cell reprogramming since STAT3 phosphorylation has recently been shown to be a limiting factor within this process (Yang et al., 2010). In addition, we show for the first time that N-cadherin can compensate for this function of E-cadherin in mES cells cultured in LIF/BMP medium, thus providing a novel function for this adhesion protein in mES cell pluripotency. Redmer et al. (2011) have recently demonstrated that expression of E-cadherin can replace Oct4 in the derivation of mouse iPS cells and it may be interesting to determine whether N-cadherin can also exhibit such an
effect. The findings presented here contribute to our understanding of the role of cadherins in potentiating pluripotency networks and may aid our understanding of iPS cell derivation.
Figure S3.1. E-cadherin and N-cadherin promote STAT3 phosphorylation and Klf4 and Nanog expression in mES cells. (A): Immunofluorescent and flow cytometry analysis of Klf4 and Nanog protein expression in Ecad<sup>−/−</sup> mES cells cultured in FBS-containing medium or LIF/BMP medium. (B): Immunofluorescent and flow cytometry analysis of Klf4 and Nanog protein expression in Ecad<sup>−/−</sup> mES cells transfected with FL-Ecad or the control plasmid, pCMV. (C): Western blot analysis for pSTAT3 in wtD3 mES cells treated with E/N<sup>+</sup> or the control peptide, E/N for 3 hours in FBS-containing medium. All scale bars: 100μm.
Figure S3.2. **Confirmation of results from single cells.** **(A):** RT-PCR analysis of N-cadherin and Klf4 transcripts in wtD3 and Ecad\(^{-}\) mES cells cultured in FBS-containing medium and LIF/BMP medium. **(B):** qPCR analysis of Klf4 and Nanog transcripts in wtD3 and Ecad\(^{-}\) mES cells in wtD3 and Ecad\(^{-}\) mES cells cultured in FBS-containing medium and LIF/BMP medium. **(C):** Flow cytometry and immunofluorescent analysis of Oct4 expression (green) wtD3 and Ecad\(^{-}\) mES cells cultured in FBS-containing medium and LIF/BMP medium and treated with the Alk inhibitor SB431542. Nuclei were stained with DAPI (blue). Scale bars: 100µm. **(D):** Western blot analysis of pSTAT3, STAT3, **(E):** Nanog and α-tubulin protein expression in wtD3 and Ecad\(^{-}\) mES cells cultured in FBS-containing medium or LIF/BMP medium.
Figure S3.3. Ecad<sup>−/−</sup> mES cells exhibit N-cadherin-mediated STAT3 phosphorylation and upregulation of Klf4 and Nanog when cultured in 2i medium. (A): Phase contrast images of wtD3 and Ecad<sup>−/−</sup> mES cells grown in 2i medium. (B): Immunostaining for Oct4, Nanog, Klf4 and N-cadherin in Ecad<sup>−/−</sup> mES cells grown in 2i medium. (C): Phase contrast images of Ecad<sup>−/−</sup> mES cells grown in 2i medium and exposed to the E/N+ peptide inhibitor or its control, E/N-. (D): Western blot analysis of STAT3 phosphorylation in wtD3 and Ecad<sup>−/−</sup> mES cells grown in FBS-containing medium and 2i medium with the E- and N-cadherin inhibitory peptide E/N+, the control peptide E/N- or untreated (2i). (E): RT-PCR analysis of Klf4, Nanog and N-cadherin in Ecad<sup>−/−</sup> mES cells grown in 2i medium and exposed to the E/N+ peptide, the control peptide, E/N-, or untreated (2i). (F): qPCR analysis of Klf4 and Nanog transcript expression in Ecad<sup>−/−</sup> mES cells grown in 2i medium and exposed to the E/N+ peptide, the control peptide, E/N-, or untreated (2i). All scale bars: 100µm.
Chapter 4

Novel cell lines isolated from mES cells exhibiting \textit{de novo} inhibition of E-cadherin

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4.1 Abstract

We have previously demonstrated that an epithelial-to-mesenchymal transition (EMT)-like event, whereby E-cadherin is downregulated concomitantly with N-cadherin upregulation, characterises differentiation of both mouse (m) (Spencer et al., 2007) and human (h) (Eastham et al., 2007) embryonic stem (ES) cells. However, we have also shown that loss of E-cadherin in mES cells can induce a reversible Activin/Nodal-dependent pluripotent phenotype (Soncin et al., 2009) whereby LIF withdrawal does not induce differentiation and cells express pluripotency-associated markers but lack the ability to generate embryoid bodies (EBs; Mohamet et al., 2010). In this study we explore this paradox. Here we show that seeding mES cells at low density in the absence of LIF induces a transiently reversible E-cadherin*/Oct4*/Nanog*/Sox2* cell phenotype, whereas higher seeding densities induce mES cell differentiation. E-cadherin*/Oct4*/Nanog*/Sox2* cells, termed E-cadherin negative proliferating stem (ENPS) cells, exhibit increased survival in comparison to wild type (wt) mES cells, maintain pluripotency marker expression via Activin/Nodal and fail to form EBs or chimeras. Addition of LIF to early passage (<p3) ENPS cells leads to re-expression of E-cadherin, reduced cell survival and restoration of EB and chimera generation capacities (ENPSR cells). However, culture of ENPS cells without LIF for >p3 results in de novo methylation of the E-cadherin promoter which cannot be reversed by LIF supplementation (ENPSM cells). Furthermore, differentiation of ENPSM cells in the absence of exogenous factors results in a decreased proportion of endoderm and an increased proportion of neuroectoderm cells within the population compared to wt mES cells. ENPS cells therefore provide novel insight into pluripotency and differentiation and the epigenetic regulation of E-cadherin. Furthermore, our findings suggest that cadherin modulation can be utilised to direct differentiation of pluripotent cells for use in cell-based therapies.

4.2 Introduction

Pluripotent stem cells, such as ES cells, are derived from the inner cell mass (ICM) of pre-implantation blastocysts and can be grown in culture indefinitely under appropriate
conditions (Chou et al., 2008; Ward et al., 2002; Ying et al., 2003), whilst retaining the ability to differentiate into cells representative of all embryonic and adult tissues (Smith, 2001). These cells therefore show promise for exploitation as model systems of development/disease and in regenerative medicine. Maintenance of pluripotency in mES cells is assessed by EB differentiation and teratoma and chimera generation abilities along with the expression of core intrinsic factors such as Nanog (Chambers et al., 2003), Sox2 (Masui et al., 2007) and Oct4 (Niwa et al., 2000). Expression of these genes is regulated by LIF/BMP in wt mES cells (Niwa et al., 2009) and Activin/Nodal in mouse epiblast-derived EpiSCs (Brons et al., 2007; Tesar et al., 2007), FAB-SCs (Chou et al., 2008), E-cadherin null (Ecad^-/-) mES cells (Soncin et al., 2009) and human pluripotent stem cells (Vallier et al., 2005; Vallier et al., 2009a). E-cadherin, the prototype classical cadherin, is essential for embryogenesis since Ecad^-/- embryos fail to develop beyond the blastocyst stage (Larue et al., 1994), reflecting loss of epithelial integrity in both the trophectoderm and the ICM (Kan et al., 2007; Larue et al., 1994). Coordinated epithelial-to-mesenchymal transitions (EMTs), whereby downregulation of E-cadherin occurs concomitantly with upregulation of N-cadherin, are also responsible for tissue segregation within the embryo. For example, cells of the epiblast undergo EMT to allow ingression within the primitive streak throughout gastrulation (Kalluri and Weinberg, 2009) and the neuroepithelium of the dorsal neural tube undergoes EMT to allow neural crest generation (Ahlstrom and Erickson, 2009). The loss of epithelial integrity that occurs during EMT has also been implicated in tumourigenesis whereby downregulation of E-cadherin, either by methylation of the promoter (Graff et al., 1995; Nam et al., 2004; Tamura et al., 2000) or degradation of E-cadherin protein (Craig and Brady-Kalnay, 2011), allows the acquisition of a more motile phenotype and thus tumour metastasis. The mesenchymal phenotype acquired upon E-cadherin loss has also been shown to confer resistance to apoptosis on some cancer cell lines (Polyak and Weinberg, 2009).

Whilst cells that maintain pluripotency via Activin/Nodal have been isolated from pre- and post-implantation tissues of mouse embryos, to date, such cells have not been derived from ES cells in the absence of exogenous growth factors (Rathjen et al., 1999). These studies have evidenced a role for E-cadherin in pluripotency whereby cells with
low E-cadherin expression such as EpiSCs and FAB-SCs fail to form EBs or incorporate into the germline of mice. In addition, E-cadherin has been shown to be required for reprogramming of somatic cells to pluripotency (Chen et al., 2011; Chen et al., 2010). However, the role of E-cadherin in pluripotency remains incompletely defined. We have previously demonstrated that an EMT-like event occurs during both mES and hES cell differentiation (Eastham et al., 2007; Spencer et al., 2007) and that seeding ES cells at low density results in rapid loss of cell surface E-cadherin (Spencer et al., 2007). In this study we show that seeding mES cells at low density in the absence of LIF can induce a transiently reversible E-cadherin+/Oct4+/Nanog+/Sox2+ cell population with increased propensities for survival and neuroectodermal differentiation whereas higher seeding densities lead to mES cell differentiation.

4.3 Materials and Methods

Culture of mES cells

Unless otherwise specified, mES cells were cultured on 0.1% gelatin (Sigma)-coated plates in Knockout DMEM supplemented with 10% fetal bovine serum (FBS), 50μm 2-mercaptoethanol (all Invitrogen), 2mM L-glutamine, 1% non-essential amino acids (both PAA) and 1000U/ml LIF (Chemicon International) at 37°C, 5% CO2. Cells were passaged every 2 days, when a maximum confluence of 70-80% was reached, using Trypsin/EDTA (PAA) to detach cells from the plates following two washes with Phosphate Buffered Saline solution (PBS, PAA). Where specified, cells were treated with 10μM of the Activin receptor-like kinase (Alk) inhibitor SB431542 (Tocris) or the PI3K inhibitor LY294002 (Cell Signalling Technology) or dimethylsulfoxide (DMSO) as a control. EBs (Mohamet et al., 2010) and chimeras (Ward et al., 2002) were generated as previously described.

Amaxa transfection

2 x 10⁶ cells were transfected with full-length E-cadherin (pEcad) or a control plasmid (pCMV) as described previously (Soncin et al., 2009).

Fluorescent flow cytometry analysis

Cells were harvested using Cell dissociation buffer before being washed with 900μl PBS
and resuspended in appropriate primary antibody (or isotype control) at its optimal dilution (1:100) in flow buffer (0.2% BSA w/v, 0.1% sodium azide w/v in PBS) for 30 minutes on ice. Primary antibodies were as follows: anti-E-cadherin (Decma-1, Sigma) and anti-Stage-Specific Embryonic Antigen (SSEA-1; Santa Cruz). Cells were then washed in PBS before being incubated in the appropriate PE-conjugated secondary antibody (Santa Cruz) at its optimal dilution (1:200) in flow buffer for 30 minutes on ice in the dark. Finally, cells were washed and fixed in 500µl 1% w/v paraformaldehyde (PFA) in PBS for analysis using a FACSCalibur (BD Biosciences). Viable cells were gated using forward scatter and side scatter and all data shown represents quantification of the fluorescence emitted by this population.

**Annexin V/Propidium iodide (PI) assay**
Cell viability was quantified using an Alexa Fluor 488 annexin V/Dead Cell Apoptosis Kit according to the manufacturer’s instructions. Briefly, cells were harvested using Cell dissociation buffer before 1 x 10^5 cells were washed with PBS and resuspended in 100µl 1X annexin-binding buffer containing 5µl Alexa Fluor 488 annexin V and 1µl PI (100µg/ml). Cells were then incubated at room temperature for 15 minutes in the dark before being prepared for analysis by the addition of 400µl 1X annexin-binding buffer and incubation on ice. Cell fluorescence was quantified using a Becton Dickinson FACS Calibur (the fluorescence emission at 530nm and 575nm was measured using 488nm excitation).

**Immunofluorescent cell analysis**
Cells were fixed in situ using 4% (w/v) PFA (Sigma) in PBS for 15 minutes at 4°C before being washed with PBS and incubated in the appropriate primary antibody at its optimal dilution in goat blocking serum (0.1% BSA and 1% goat serum in PBS) for 2 hours at room temperature. Primary antibodies were as follows: anti-Oct4 (1:200), anti-Sox2 (1:500), anti-Nanog (1:200), anti-Nestin (1:250), anti-beta-III-tubulin (βIIIIT; 1:1000; All Abcam), and anti-Tyrosine hydroxylase (TH; 1:500; Millipore). Cells were then washed with PBS and incubated in the appropriate Alexa-fluor 488 conjugated secondary antibody in goat blocking serum (all 1:500, Invitrogen) at room temperature in the dark. Cells were then mounted using Vectorshield with 4’,6-diamidino-2-phenylindole (DAPI;
Vector Laboratories) and imaged using a fluorescence microscope.

**RT-PCR**

Total RNA was isolated from cells using the RNeasy kit (Qiagen) according to the manufacturer’s instructions. 1µg of RNA was then reverse transcribed to cDNA as previously described (Ward et al., 2003). Primer sequences can be found in Table 2.8.

**Quantitative PCR**

Triplicate samples containing appropriate forward and reverse primers (100pmol/µl; Table 2.8) were analysed as previously described (Soncin et al., 2011) with 18s rRNA as an endogenous control. All results represent the standard error of the mean (SEM) of 3 independent replicates.

**Western blot**

4 x 10⁵ cells were lysed, boiled in reducing conditions, separated and electrotransferred onto nitrocellulose membrane as previously described (Ward et al., 2003). The membrane was probed using the appropriate primary antibody (anti-pAkt [Ser473] and anti-Akt, both Cell Signalling Technology, 1:200 dilution) followed by the appropriate secondary antibody (1:2000; Roche).

**Microarray analysis**

RNA was extracted from the cells by phenol/chloroform extraction and microarray analysis was performed as described previously (Soncin et al., 2011).

**4.4 Results**

**Low density seeding of wt mES cells in the absence of LIF leads to the generation of an E-cadherin*/Oct4*/Nanog*/Sox2* cell population**

wtD3 mES cells seeded at 5 x 10³ cells/ml or 2 x 10⁴ cells/ml in the absence of LIF in gelatin-coated plates were assessed for colony morphology. Whereas wtD3 mES cells seeded at the lower density exhibited a relatively homogenous and dispersed population after 2 days (Figure 4.1a(i); d2), cells seeded at 2 x 10⁴ cells/ml grew mostly
as individual colonies (Figure 4.1a(ii); d2). Subsequent passaging of the cells (1 in 6 split) resulted in low density seeded cells remaining as a relatively homogenous and dispersed population after 8 days (Figure 4.1a(i); d8) whereas at higher seeding density cells exhibited a characteristic differentiated morphology (Figure 4.1a(ii); d8). Analysis of E-cadherin expression in the cells shown in Figure 4.1a(i) demonstrated loss of cell surface expression and transcripts (Figure 4.1b(i); -LIF) compared to wtD3 mES cells (Figure 4.1b(i);+LIF). These cells were termed E-cadherin negative proliferating stem (ENPS) cells. ENPS cells maintained protein expression of the pluripotency markers stage-specific embryonic antigen (SSEA1; Figure 4.1b(ii)), Nanog, Oct4 and Sox2 (Figure 4.1c).

It has previously been shown that EB formation is impaired in FAB-SCs, cells that exhibit low E-cadherin expression (Chou et al., 2008), and Ecad<sup>−/−</sup> mES cells (Mohamet et al., 2010). Therefore the EB formation capacity of ENPS cells was tested. Whereas wtD3 mES cells formed characteristic EBs in suspension, ENPS cells remained as loosely associated cell aggregates (Figure 4.1d). Interestingly, Activin/Nodal signalling was shown to be required for ENPS generation since no ENPS cells were formed upon exposure of wtD3 mES cells to the Activin receptor-like kinase inhibitor SB431542 under ENPS generation conditions. Instead, mES cells adopted a differentiated morphology (Figure 4.1e(i)) and lost SSEA-1 expression more rapidly (Figure 4.1e(ii)) than controls (+DMSO) under these conditions, even when plated at higher densities (data not shown).

ENPS cells have since been isolated from several independent mES cell lines, although successful derivation rates of such cells are varied (Table 4.1).

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Strain</th>
<th>Attempts</th>
<th>ENPS lines derive</th>
<th>% efficiency</th>
</tr>
</thead>
<tbody>
<tr>
<td>D3</td>
<td>129/Sv+c/+p</td>
<td>8</td>
<td>2</td>
<td>25</td>
</tr>
<tr>
<td>MESC20</td>
<td>129/OLA</td>
<td>10</td>
<td>2</td>
<td>20</td>
</tr>
<tr>
<td>E14TG2a</td>
<td>129/OLA</td>
<td>10</td>
<td>0</td>
<td>0*</td>
</tr>
</tbody>
</table>

*Table 4.1. Efficiency of ENPS cell generation from mES cell lines.* One clone was isolated that exhibited partial inhibition of E-cadherin.
Cumulative cell counts demonstrated almost a 1.5-fold increase in ENPS cell numbers compared to wtD3 mES cells over a period of 4 days (Figure 4.1f(i)). To determine whether this observation was due to altered proliferation rates or increased cell survival an Annexin V/Propidium iodide (PI) assay was performed, revealing a significant decrease in Annexin V⁺/PI⁺ (non-viable) ENPS cells and a significant increase in Annexin V⁻/PI⁻ (viable) ENPS cells compared to wtD3 mES cells (Figure 4.1f(ii); *p<0.05), suggesting that the increased ENPS cell number observed is due to increased survival of this cell line. To determine whether ENPS cells exhibited Activin/Nodal-dependent pluripotency, as previously observed in Ecad⁻/⁻ mES cells (Soncin et al., 2009), these cells were exposed to SB431542. Whereas wtD3 mES cells maintained an undifferentiated morphology (Figure 4.1g(i)) and expression of pluripotency-associated transcripts (Figure 4.1g(ii)) under these conditions, ENPS cells exhibited differentiated morphology (Figure 4.1g(i)) and loss of Oct4 and Nanog transcripts (Figure 4.1g(ii)), thus confirming that ENPS cells exhibit Activin/Nodal-dependent pluripotency.
Figure 4.1. Low density \((5 \times 10^3 \text{ cells/ml})\) seeding of mES cells in the absence of LIF results in the generation of an Activin/Nodal-dependent E-cadherin⁻/Oct4⁻/Sox2⁺/Nanog⁺ cell population with increased survival capacity. (A): WtD3 mES cells were seeded at \(5 \times 10^3 \text{ cells/ml}\) or \(2 \times 10^4 \text{ cells/ml}\) in the absence of LIF for 8 days. Whereas the former cells lose cell-cell contact the latter exhibited differentiated morphology. (B): Flow cytometry analysis of (i) E-cadherin and (ii) SSEA-1 expression in wtD3 mES cells seeded at \(5 \times 10^3 \text{ cells/ml}\) in the absence of LIF for 8 days (-LIF) or wtD3 mES cells (+LIF). (C): Immunofluorescent staining of ENPS cells for pluripotency marker expression (green). The nuclei were stained with DAPI (blue). (D): Phase contrast images of EB formation in wtD3 and ENPS cells. (E): (i) Phase contrast images to show the morphology of wtD3 mES cells seeded at \(5 \times 10^3 \text{ cells/ml}\) in the presence/absence of LIF, the Alk inhibitor SB431542 and its control, DMSO. (ii) Flow cytometry analysis of SSEA-1 expression in wtD3 mES cells seeded at \(5 \times 10^3 \text{ cells/ml}\) in the presence/absence of LIF, the Alk inhibitor SB431542 and its control, DMSO. (F): (i) Cumulative cell counts of wtD3 and ENPS cells over a 4 day period. *p<0.05. (ii) Annexin V/Dead cell analysis of wtD3 and ENPS cells. * p<0.05. (G): Phase contrast images of wtD3 and ENPS cells exposed to SB431542 or its control, DMSO. (ii) RT-PCR analysis of pluripotency marker transcript expression in wtD3 and ENPS cells when exposed to SB431542 or its control, DMSO. DMSO: Dimethyl sulfoxide, ENPS: E-cadherin Negative Proliferating Stem, EB: Embryoid body. All scale bars: 100µm.
The ENPS cell phenotype can be reversed by forced expression of E-cadherin or LIF stimulation at low passage

To determine whether Activin/Nodal-dependent pluripotency exhibited by ENPS cells could be reversed by re-expression of E-cadherin these cells were transfected with an E-cadherin expression plasmid (pEcad). Upon exposure to SB431542, ENPS cells transfected with pEcad exhibited an undifferentiated morphology in the majority of the cell population (Figure 4.2a(i)) and maintenance of pluripotency-associated transcripts (Figure 4.2a(ii)) whereas control cells, transfected with an empty plasmid (pCMV), exhibited differentiated morphology (Figure 4.2a(i)) and loss of pluripotency-associated transcripts (Figure 4.2a(ii)) under these conditions. In addition, to determine whether loss of E-cadherin in ENPS cells could be reversed by LIF supplementation, as demonstrated by Chou et al. in FAB-SCs (2008), we cultured low passage (<p3) ENPS cells in the presence of LIF. Within 3 days of LIF stimulation, ENPS cells exhibited increased cell surface E-cadherin expression (Figure 4.2b(i)), restoration of cell-cell contact (Figure 4.2b(ii)) and were capable of forming EBs in suspension culture (Figure 4.2b(iii)). These cells, termed ENPS cells, also exhibited restoration of LIF-dependent pluripotency, since they maintained an undifferentiated morphology (Figure 4.2c(i)) and pluripotency marker expression (Figure 4.2c(ii)) upon exposure to SB431542. Subsequent removal of LIF from ENPS cells did not induce an ENPS cell phenotype but led to their differentiation (data not shown).

To determine whether ENPS cells are pluripotent, the chimera-generation ability of these cells was assessed. This analysis demonstrated low chimerism and the absence of germline transmission in ENPS cells whereas ENPS cells exhibited similar chimeric and germline transmission rates to wtD3 mES cells (Table 4.2). In addition, we observed that prolonged culture of ENPS cells (>p3) in the absence of LIF resulted in the inability of LIF supplementation to restore E-cadherin expression (Figure 4.2d(i)) or cell-cell contact (Figure 4.2d(ii)). We therefore explored the possibility that the irreversible loss of E-cadherin resulted from methylation of the E-cadherin promoter in these cells, as previously described in cancer cell lines (Graff et al., 1995; Nam et al., 2004; Tamura et al., 2000). PCR analysis of CpG spanning regions of the E-cadherin promoter using methylation-specific and -non-specific primers suggested that these cells exhibited...
methylation of the E-cadherin promoter (Figure 4.2e(i)). In addition, when treated with the DNA methyltransferase inhibitor 5-Aza-2’-deoxycytidine (Aza), these cells exhibited restoration of E-cadherin transcript expression (Figure 4.2e(ii)). Together, these results confirm that prolonged culture of ENPS cells in the absence of LIF leads to de novo methylation of CpG islands within the E-cadherin promoter. These cells were termed ENPS\textsuperscript{M} cells.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>% Chimeras</th>
<th>% germ line transmission</th>
</tr>
</thead>
<tbody>
<tr>
<td>wtD3</td>
<td>48</td>
<td>16.7</td>
</tr>
<tr>
<td>ENPS\textsuperscript{M}</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>ENPS\textsuperscript{R}</td>
<td>44</td>
<td>13.6</td>
</tr>
</tbody>
</table>

Table 4.2. Chimera and germline efficiency of wtD3, ENPS\textsuperscript{M} and ENPS\textsuperscript{R} cell lines.

ENPS: E-cadherin negative proliferating stem.
Figure 4.2. The ENPS cell phenotype can be reversed by forced expression of E-cadherin or LIF stimulation at low passage. (A): (i) Phase contrast images of ENPS cells transfected with an E-cadherin expression plasmid (pEcad) or control plasmid (pCMV) and exposed to SB431542 or its control, DMSO. (ii) RT-PCR analysis of pluripotency marker expression in ENPS cells transfected with pEcad or pCMV and exposed to SB431542. (B): (i) Flow cytometry analysis of E-cadherin expression in untreated (green), following 3 days of LIF stimulation (blue) and following 7 days of LIF stimulation (pink). (ii) Phase contrast images of monolayer and (iii) suspension cultured ENPS cells and ENPS cells to which LIF has been added (ENPSR cells). (C): (i) Phase contrast images of ENPSR cells exposed to SB431542 or its control, DMSO. (ii) RT-PCR analysis of pluripotency marker expression in ENPSR cells exposed to SB431542 or its control, DMSO. (D): (i) Flow cytometry analysis of E-cadherin in ENPS cells (pink), wtD3 mES cells (green) and high passage (>p3) ENPS cells stimulated with LIF. (ii) Phase contrast image of high passage (>p3) ENPS cells stimulated with LIF. (E): (i) RT-PCR analysis of methylation specific (M) and unmethylation specific (U) transcripts in wtD3 and ENPSM cells. (ii) RT-PCR analysis of E-cadherin transcripts in wtD3 and ENPSM cells treated with the methylation inhibitor AZA or its control, DMSO. All scale bars: 100µm. AZA: 5-Aza-2’-deoxycytidine, ENPS: E-cadherin negative proliferating stem.
ENPS cells exhibit PI3K/Akt-mediated increased survival capacity in comparison to ENPS\textsuperscript{R} cells

Similar to that observed in wtD3 mES cells, ENPS\textsuperscript{R} cells exhibited almost 1.5-fold decreased cell numbers (Figure 4.3a) and decreased survival (Figure 4.3b) in comparison to ENPS cells. To explore a potential mechanism for the increased survival of ENPS cells compared to ENPS\textsuperscript{R} cells, levels of activation of the PI3K/Akt pathway were determined by western blot. This revealed an increase in Akt phosphorylation in ENPS cells in comparison to ENPS\textsuperscript{R} cells (Figure 4.3c). Cell counts were also performed on ENPS cells in the presence of the PI3K inhibitor LY294002, revealing approximately a 2-fold decrease in cell numbers upon exposure to this inhibitor in comparison to control cells (DMSO; Figure 4.3d(i)). An Annexin V/PI assay performed under these conditions also indicated a significant increase in Annexin V\textsuperscript{+}/PI\textsuperscript{+} (non-viable) cells and a significant decrease in Annexin V\textsuperscript{-}/PI\textsuperscript{-} (viable) cells upon exposure to LY294002 in comparison to control cells (DMSO; Figure 4.3d(ii); *p<0.05). Together, these results suggest that the PI3K/Akt pathway plays a role in the increased survival of ENPS cells compared to ENPS\textsuperscript{R} and wtD3 mES cells.
Figure 4.3. ENPS cells exhibit PI3K/Akt-mediated increased survival capacity in comparison to ENPS\textsuperscript{R} cells. (A): Cumulative cell counts of ENPS and ENPS\textsuperscript{R} cells over a 4 day period. *p<0.05. (B): Annexin V/Dead cell analysis of ENPS and ENPS\textsuperscript{R} cells. *p<0.05. (C): Western blot analysis of phosphorylated Akt (pAkt) and total Akt in ENPS and ENPS\textsuperscript{R} cells exposed to the PI3K inhibitor LY294002 or its control, DMSO. (D): (i) Cumulative cell counts of ENPS cells exposed to LY294002 or its control, DMSO, over a 4 day period. *p<0.05. (ii) Annexin V/Dead cell analysis of ENPS cells exposed to LY294002 or its control, DMSO. *p<0.05. DMSO: dimethylsulphoxide, ENPS: E-cadherin negative proliferating stem.
ENPS\textsuperscript{M} cells exhibit a neuroectodermal bias in their differentiation capacity

Microarray analysis of wtD3 mES cells, two independent ENPS\textsuperscript{M} cell lines (ENPS1 and ENPS2; derived from wtD3 mES cells) and ENPS\textsuperscript{R} cells (derived from the ENPS1 cell line) demonstrated significant transcriptional variations between wtD3/ENPS\textsuperscript{R} and ENPS1/2 cells but not within these populations (Figure 4.4a; more detailed analysis in Supplementary data). Gene ontology (GO) analysis revealed upregulation of transcripts associated with neurogenesis in ENPS\textsuperscript{M} cells compared to wtD3 mES cells (Table 4.3). To determine whether ENPS\textsuperscript{M} cells exhibit this germ layer bias \textit{in vitro} we cultured wtD3 and ENPS\textsuperscript{M} cells in adherent culture in 3% serum/7% serum replacement (SR) for 14 days. This resulted in the loss or decrease in expression of the pluripotency-associated transcripts Oct4, Nanog and Rex1 in ENPS\textsuperscript{M} cells whilst wtD3 mES cells maintained expression of these transcripts (Figure 4.4b(i)). Interestingly, ENPS\textsuperscript{M} cells exhibited decreased expression of endoderm-specific transcripts (Figure 4.4b(ii)) and elevated expression of neuroectoderm markers (Figure 4.4b(iii)) compared to wtD3 mES cells whereas similar levels of mesoderm-specific transcripts were expressed by wtD3 and ENPS\textsuperscript{M} cells (Figure 4.4b(iv)). In addition, differentiated ENPS\textsuperscript{M} cells expressed βIIIT, Nestin and TH proteins whereas wtD3 mES cells lacked TH immunoreactivity and exhibited a lower proportion of cells expressing the other neural markers (Figure 4.4c). Together, these results demonstrate that differentiation of ENPS\textsuperscript{M} cells in the absence of exogenous factors results in enrichment of neuroectoderm cells within the population.
Figure 4.4. ENPS\textsuperscript{M} cells exhibit a propensity for neural differentiation in vitro. (A): Dendrogram demonstrating clustering analysis of RNA microarray data for wtD3 mES cells (purple), ENPS\textsuperscript{R} cells (green) and two independently derived ENPS cell lines, ENPS1 (red) and ENPS2 (blue). (B): RT-PCR analysis of markers associated with (i) pluripotency (ii) endoderm (iii) ectoderm and (iv) mesoderm in ENPS\textsuperscript{M} and wtD3 mES cells following 14 days of culture in 3%FBS/7%SR. (C): Immunofluorescent staining of neuroectodermal markers in ENPS\textsuperscript{M} and wtD3 mES cells following 14 days of culture in 3%FBS/7%SR. Scale bars: 100µm. AFP: α-fetoprotein, β\textsuperscript{IIIT}: β\textsuperscript{IIIT}ubulin, Eno2: Enolase 2, ENPS: E-cadherin negative proliferating stem, Hnf4: Hepatic nuclear factor 4, Mef2c: Myocyte enhancing factor 2c, Myh: Myosin heavy chain, Myl: Myosin light chain, Nef1: Neurofilament 1, Runx1: Runt-related transcription factor 1, TH: Tyrosine hydroxylase, TFR: Transferrin, TTR: Transthyretin.
<table>
<thead>
<tr>
<th>Gene</th>
<th>ENPS1 (fold increase compared to wtD3)</th>
<th>ENPS2 (fold increase compared to wtD3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cd24a</td>
<td>NS</td>
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</tr>
<tr>
<td>Irquois related homeobox 3</td>
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<td>4.0</td>
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<tr>
<td>Acyl-CoA synthetase long-chain family member 6</td>
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<td>3.2</td>
</tr>
<tr>
<td>Adhesion molecule with Ig-like domain 1</td>
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<td>NS</td>
</tr>
<tr>
<td>Amyloid beta precursor protein-binding, family B, member 1</td>
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<td>3.2</td>
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<tr>
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<td>FGF8</td>
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</tr>
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</tr>
<tr>
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</tr>
<tr>
<td>Tissue inhibitor of metalloproteinase 2</td>
<td>3.0</td>
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</tr>
</tbody>
</table>

Table 4.3. Comparison of neurogenesis-associated transcript expression between wtD3 and ENPS<sup>M</sup> cells.
4.5.1 Discussion

In this study we describe the first isolation of an E-cadherin/Oct4+/Sox2+/Nanog+ cell line (ENPSM cells) from mES cells. Our data suggests that the generation of ENPSM cells represents an EMT-like event since Snail and Sip1 transcripts were detected during ENPSM cell derivation (Figure S4.1), thus suggesting that these proteins are responsible for the reversible repression of E-cadherin in these cells and that LIF negatively regulates these factors in mES cells. Transformation of mES cells into ENPSM cells is likely to be an intrinsic cellular process since exposure of wt mES cells to ENPSM cell-conditioned medium did not induce an ENPSM cell-like phenotype (data not shown).

Interestingly, a high proportion of transcripts associated with LIF withdrawal in mES cells are shared with ENPSM cells (Table S4.1), suggesting that ENPSM cells may be representative of the ‘reversibly committed’ state described by Trouillas et al. (2009). This observation also provides further evidence for a LIF/E-cadherin positive feedback loop, as previously observed in FAB-SCs (Chou et al., 2008), whereby withdrawal of LIF induces methylation of the E-cadherin promoter and loss of E-cadherin induces LIF-independent pluripotency. The observation that ENPSM cells fail to form EBs or chimeras and exhibit Activin/Nodal-dependent pluripotency marker expression is consistent with previous data obtained from EpiSC (Brons et al., 2007; Tesar et al., 2007) and FAB-SC (Chou et al., 2008) cell lines and may indicate that ENPSM cells lack naïve pluripotency. However, EpiSCs, FAB-SCs, Ecad−/− mES cells (Soncin et al., 2009) and ENPSM cells maintain wt expression levels of pluripotency markers such as SSEA-1, Oct4 and Sox2, thus questioning the idea that loss of E-cadherin leads to differentiation, as proposed by Redmer et al. (2011). Despite expressing high levels of various pluripotency-associated markers, ENPSM cells exhibit decreased Nanog expression compared to wtD3 mES cells (data not shown) and lose expression of Oct4, Nanog and Rex1 more rapidly than wtD3 mES cells upon in vitro differentiation, suggesting that these cells are primed for differentiation, as described by Nichols et al. (Nichols and Smith, 2009). In support, the microarray data obtained for ENPSM cells indicates that transcripts associated with positive regulation of cell differentiation are upregulated in ENPSM cells compared to wtD3 mES cells (Figure S4.2). Together, these results corroborate our previously
published data showing that an EMT-like event occurs during ES cell differentiation (Eastham et al., 2007; Spencer et al., 2007). Our findings also provide evidence for a transiently reversible Activin/Nodal-dependent stage in mES cell differentiation (Figure S4.3).

Microarray analysis suggested an upregulation of transcripts associated with neurogenesis in ENPS\textsuperscript{M} cells compared to wtD3 mES cells; this data was confirmed by the demonstration of a neuroectodermal bias in the differentiation of ENPS\textsuperscript{M} cells compared to wtD3 mES cells. In support of this finding, it has previously been shown that blockade of EMT reduces the efficiency of neural differentiation in mES cells (Li et al., 2011), thus suggesting that the N-cadherin expression that occurs concomitantly with downregulation of E-cadherin in EMT is potentially responsible for the increased efficiency of neuroectodermal differentiation observed in ENPS\textsuperscript{M} cells compared to wtD3 mES cells. Interestingly, N-cadherin has been shown to be upregulated in EpiSCs (Bao et al., 2009) and ENPS1 cells exhibit a 2-fold increase in N-cadherin transcripts in comparison to wtD3 mES cells (data not shown) and this difference may be enhanced upon induction of differentiation. The EMT event that accompanies neural differentiation \textit{in vitro} is likely to reflect the formation of the neural crest \textit{in vivo} (Ahlstrom and Erickson, 2009).

Importantly, the acquisition of the ‘primed’ pluripotent state in ENPS cells is reversible. Both E-cadherin re-expression and LIF stimulation at low passage were shown to reverse the phenotype to a wt-like ‘naive’ pluripotent state in which cell-cell contact, E-cadherin expression and EB and chimera generation abilities were restored (ENPS\textsuperscript{R} cells). Reversal of the primed pluripotent state has also been observed in EpiSCs upon LIF stimulation (Bao et al., 2009) and by overexpression of Klf4 (Guo et al., 2009) and in FAB-SCs upon LIF stimulation or transfection with E-cadherin (Chou et al., 2008). This mesenchymal-to-epithelial transition event also accompanies induced pluripotent stem cell generation (Vallier et al., 2009a). However, at higher passage, ENPS cells exhibit methylation-induced silencing of the E-cadherin promoter and can no longer be ‘rescued’ by LIF supplementation (ENPS\textsuperscript{M} cells) despite the ability of full length E-cadherin to reverse this phenotype. Together, these results support our previous description of loss of E-
cadherin as a *reversible* step towards commitment to differentiation (Hawkins et al., 2012).

**4.5.2 Conclusion**

We describe the isolation of a novel cell line, ENPS$^M$ cells. Whilst it is unclear as to whether ENPS$^M$ cells are a physiologically relevant cell type, it is possible that they are representative of epiblast cells undergoing EMT to allow ingress of the primitive streak (Kalluri, 2009). ENPS$^M$ cells may provide novel insight into epigenetic regulation of E-cadherin in cancer. For example, methylation of the E-cadherin promoter is observed in many carcinomas (Graff et al., 1995; Nam et al., 2004; Tamura et al., 2000) and ENPS$^M$ cells may therefore provide an *in vitro* model within which to study this process in detail. The increased survival observed in ENPS$^M$ cells in comparison to wtD3 mES cells has also been observed in the MCF7 breast cancer cell line upon inhibition of E-cadherin (Miazga, personal communication), thus supporting this idea and suggesting that the PI3K/Akt survival pathway could be targeted therapeutically. Furthermore, the observation of a differentiation bias towards neuroectoderm, at the expense of endoderm, in ENPS$^M$ cells may aid the directed differentiation of pluripotent cells for regenerative medicine applications.
**Figure S4.1.** Expression of EMT-inducing factor gene expression in ENPS cells and *wtD3* mES cells. ENPS: E-cadherin negative proliferating stem, Mmp: Matrix metalloproteinase, Sip1: Smad-interacting protein 1.
<table>
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<tr>
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<th>mES-LIF</th>
<th>ENPS1</th>
<th>ENPS2</th>
</tr>
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<tbody>
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<td>▼(1.3)</td>
<td>▼(1.2)</td>
</tr>
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<tr>
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<td>▲(13.0)</td>
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</tr>
<tr>
<td>Dnmt3a</td>
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Table S4.1. Comparison of wt mES cell transcript expression following 24 hours of LIF withdrawal and ENPSM cell transcript expression (Adapted from Trouillas et al., 2009).
Figure S4.2. Microarray analysis of ENPS<sup>M</sup> cells. (A): GO analysis of the functions of genes that are upregulated in ENPS<sup>M</sup> cells. (B): GO analysis of the functions of genes that are downregulated in ENPS<sup>M</sup> cells. ENPS: E-cadherin negative proliferating stem. GO: gene ontology.
mES cell differentiation

**NAIVE PLURIPOTENCY**

- LIF-dependent

- mES/ENPS<sup>R</sup> cell

- E-cadherin

**PRIMED PLURIPOTENCY**

- Activin/Nodal-dependent

- ENPS<sup>M</sup> cell

- Terminal differentiated lineages

High density

Low density

- -LIF

- +LIF

- E-cadherin
Figure S4.3. A model of the stages of mES cell differentiation. Upon LIF withdrawal, mES cells progress towards differentiation through an intermediate E-cadherin negative proliferating stem cell (ENPS) cell stage, similar to the primed pluripotent state, whereby E-cadherin is downregulated and mES cells use Activin/Nodal to maintain pluripotency. Following further LIF withdrawal, mES cells either commit fully to differentiation or adopt a stable phenotype in which the E-cadherin promoter is methylated (ENPS\textsuperscript{M} cells). The latter cell fate decision is dependent on cell seeding density, with low cell seeding densities giving rise to ENPS\textsuperscript{M} cells and high cell seeding densities promoting cell differentiation.
4.6 Supplementary data

Microarray analysis

In total, 2472 genes were differentially expressed in ENPS1 cells and 2135 in ENPS2 cells compared to wtD3 mES cells (according to our threshold criteria: FC±2.5; q≤0.05). Analysis of genes expressed in wtD3, ENPS1 and ENPS2 cells revealed that 12547 genes were expressed in all cell lines and 1436 genes were expressed exclusively in wtD3 (Figure S4.4a). Of the genes identified as being upregulated in ENPSM cells, 1612 were shared, 1065 were expressed only in ENPS1 and 1418 were expressed only in ENPS2 (Figure S4.4b). Of the genes identified as being downregulated in ENPSM cells, 1658 were shared, 1466 were expressed only in ENPS1 and 435 were expressed only in ENPS2 cells (Figure S4.4c). We also observed a high degree of similarity in the 25 most downregulated genes shared between ENPS1 and ENPS2 cells, with 17 of the 25 most downregulated genes being conserved between the two cell lines, whereas the 25 most upregulated genes showed more variation (Table S4.2). RT-PCR analysis confirmed downregulation of Rex1/Zfp42, Fbxo15, Klf4, Tbx3, Dax1/Nr0b1, Daz1 and Junctional adhesion molecule 2 (Jam2) transcripts in ENPS2 cells (Figure S4.4d) and quantitative real-time PCR confirmed downregulation of Nanog and LIFR and upregulation of Eomesodermin (EOMES) in this cell line compared to wtD3 mES cells (Figure S4.4e). GO terms associated with the shared upregulated genes included cell differentiation, cell motility, membrane organisation and intracellular signalling cascade (Figure S4.2a). Together with our observation of increased survival in ENPS cells (Figure 4.3), the upregulation of transcripts associated with cell motility in ENPSM cells (Figure S4.2a) may have implications for tumorigenesis, supporting the previously demonstrated association of loss of E-cadherin and cancer progression (Vleminkx et al., 1991). GO terms associated with the shared downregulated genes were quite distinct from those associated with the upregulated genes (Figure S4.2a-b), with terms including regulation of gene expression and chromatin organisation. Interestingly, a high proportion of genes in both the downregulated and upregulated categories were associated with multicellular organismal development (16% and 13% respectively).
Figure S4.4. Confirmation of microarray data by RT-PCR and qPCR. (A): Analysis of gene expression in wtD3 mES cells and two ENPS$^M$ cell lines, ENPS1 and ENPS2 showing the number of genes conserved between the cell lines. (B): Analysis of genes that are upregulated in ENPS1 and ENPS2 cells compared to wtD3 mES cells showing the number of upregulated genes conserved between the two cell lines. (C): Analysis of genes that are downregulated in ENPS1 and ENPS2 cells compared to wtD3 mES cells showing the number of upregulated genes conserved between the two cell lines. (D): RT-PCR analysis of transcripts that are downregulated in ENPS$^M$ cells compared to wtD3 mES cells. (E): qPCR analysis of Nanog, LIFR and EOMES to show the relative expression of these transcripts in ENPS$^M$ cells compared to wtD3 mES cells. Expression levels were normalised to 18S rRNA transcripts and wtD3 mES cell transcripts were given a value of 1. Error bars reflect the standard error of the mean (SEM) for 3 biological replicates. LIFR: leukaemia inhibitory factor receptor, EOMES: Eomesodermin, ENPS: E-cadherin negative proliferating stem, Jam2: Junctional adhesion molecule 2.
Table S4.2. Comparison of the top 25 most upregulated (green) and downregulated (red) transcripts between ENPS1 and ENPS2 cells. Gene transcripts conserved between the cell lines are shown in bold.
Chapter 5
General Discussion
Chapter 5 General Discussion

In this study we have analysed the role of E-cadherin in mES cell pluripotency, thus providing novel insight into the previously documented role of E-cadherin in LIF-dependent self-renewal (Soncin et al., 2009) and, ultimately, its role in the transition between the naïve and primed pluripotent states. Firstly, we describe a novel mechanism connecting E-cadherin to the core circuitry of pluripotency in mES cells. Specifically, we show that E-cadherin promotes Klf4 and Nanog expression in mES cells, the latter via direct induction of STAT3 phosphorylation. This mechanism may explain the recently documented requirement for E-cadherin in miPS cell reprogramming (Chen et al., 2011; Chen et al., 2010; Li et al., 2010b; Redmer et al., 2011), since STAT3 phosphorylation has been shown to be a limiting factor in this process (Yang et al., 2010). In addition, this finding may explain the low levels of STAT3 activation and Klf4 and Nanog expression observed in EpiSCs (Yang et al., 2010) since these cells also express low levels of E-cadherin (Tesar et al., 2007). The lack of activation of the LIF pathway in EpiSCs and Ecad<sup>−/−</sup> mES cells corresponds to the LIF-independent pluripotency exhibited by these cells (Brons et al., 2007; Soncin et al., 2009) and the low level expression of Socs3 observed in both EpiSCs (Yang et al., 2010) and Ecad<sup>−/−</sup> mES cells (Soncin et al., 2011), since Socs3 is a direct target of LIF signalling. Interestingly, Tbx3 downregulation has also been observed in Ecad<sup>−/−</sup> mES cells (Figure S5.1) and EpiSCs (Yang et al., 2010) in comparison to wt mES cells, despite the increase in Akt phosphorylation in Ecad<sup>−/−</sup> mES cells demonstrated in this study. This suggests that Tbx3, in addition to Klf4 and Nanog, may be under the control of STAT3. This is contrary to the model presented by Niwa et al. (2009).

We provide the first demonstration of a role for N-cadherin in mES cell pluripotency since culture of Ecad<sup>−/−</sup> mES cells in LIF/BMP medium induced N-cadherin expression, STAT3 activation and Klf4 and Nanog expression. LIF pathway activation in these cells was shown to be due to N-cadherin since blockade of N-cadherin using an inhibitory peptide induced loss of these factors. Interestingly, recent data generated in our laboratory indicates that N-cadherin can also compensate for the role of E-cadherin in LIF-dependent pluripotency in FBS-containing medium (Wai
Lau, personal communication). Whereas exposure of wtD3 mES cells to either DECMA-1 or the E/N+ peptide induced loss of cell-cell contact, only the latter induced loss of STAT3 phosphorylation in these cells, suggesting that ECD1 but not ECD5 of E- and N-cadherin is involved in LIF-dependent pluripotency (DECMA-1 data not shown). Since E- and N-cadherin both directly enhance STAT3 phosphorylation, further comparison of the binding partners between the two cadherins (such as those shown in Table 1.1) may allow a mechanism for their role in STAT3 phosphorylation to be identified. For example, in this study we show that the β-catenin binding site, conserved between E- and N-cadherin, is required for STAT3 phosphorylation. However, we are unable to distinguish between the roles of β-catenin and PIPKιγ in this process since both molecules bind within this region of E- and N-cadherin (Figure 1.4; Ling et al., 2007). Mutation of the PIPKιγ binding site of the cytoplasmic region of E-cadherin, preventing PIPKιγ but not β-catenin from binding E-cadherin, has been documented (Suriano et al., 2003) and may be exploited to address this question. Preliminary data obtained from Ecad−/− mES cells expressing this mutated form of E-cadherin indicated a potential role for PIPKιγ in Tbx3 and Nanog expression (Figure S5.2) but these findings remain inconclusive due to time restrictions. Further characterisation of these cells, along with additional targeted mutations of E- and N-cadherin and immunoprecipitation of E- and N-cadherin and β-catenin should be performed to allow a mechanism for the interaction between these molecules and STAT3 to be elucidated.

The isolation of a novel E-cadherin+/Oct4+/Nanog+/Sox2+ ENPSM cell line has also advanced our understanding of the role of E-cadherin in mES cell pluripotency. These cells exhibit a high degree of similarity to Ecad−/− mES cells and EpiSCs. For example, all cell lines display Activin/Nodal-dependent pluripotency (Brons et al., 2007; Larue et al., 1994; Soncin et al., 2009), decreased activation of the LIF pathway (including decreased LIFR transcript expression (Soncin et al., 2011; Yang et al., 2010)), defects in chimera generation (Brons et al., 2007; Larue et al., 1994), and concomitant downregulation of naïve pluripotency markers such as FGF4, Rex1 and Nr0b1 and upregulation of primed pluripotency markers such as FGF5 and EOMES compared to wtD3 mES cells (Rossant, 2008; Soncin et al., 2011; Tesar et al.,
We also show that ENPS\textsuperscript{M} cells lose pluripotency marker expression more readily than wtD3 mES cells upon differentiation-inducing conditions, however, their pluripotency was confirmed by the observation that ENPS\textsuperscript{M} cells express markers from all three germ layers upon differentiation, albeit with a reduced capacity for endoderm differentiation. Despite similarities, there are various differences in gene expression between ENPS\textsuperscript{M} cells and EpiSCs (Figure S5.3), as previously shown between Ecad\textsuperscript{-/} mES cells and EpiSCs (Soncin et al., 2009). In addition, EpiSCs have been shown to be competent in EB formation (Brons et al., 2007; Tesar et al., 2007), whereas Ecad\textsuperscript{-/} mES cells (Mohamet et al., 2010) and ENPS\textsuperscript{M} cells are not. Furthermore, STAT3 protein expression has been shown to be decreased in EpiSCs (Yang et al., 2010) compared to wt mES cells but we show here that STAT3 expression is similar between Ecad\textsuperscript{-/} and wtD3 mES cells. We also demonstrate lack of Brachyury/T transcript expression in Ecad\textsuperscript{-/} mES cells, as previously shown (Soncin et al., 2009), whereas EpiSCs have been shown to express Brachyury/T (Yang et al., 2010). Perhaps most importantly, however, we have shown that Ecad\textsuperscript{-/} mES cells are competent in both colony forming assays and for maintenance of pluripotency in 2i medium supplemented with LIF, whereas EpiSCs exhibit differentiation and death when cultured in this medium (Guo et al., 2009; Yang et al., 2010) and fail clonal expansion tests (Hanna et al., 2010). Together, these findings suggest that E-cadherin (or N-cadherin) is required for the transition from the primed pluripotent state to the naïve pluripotent state since Ecad\textsuperscript{-/} and ENPS\textsuperscript{M} cells, like EpiSCs, exhibit many features of the primed pluripotent state. Importantly, both Ecad\textsuperscript{-/} and ENPS cell lines can be converted to a naïve-like pluripotent state upon LIF stimulation or forced expression of E-cadherin. However, our data also suggest that multiple subcategories exist within the primed pluripotent state that vary in their transcriptional profile, as suggested by Colman and Dreesen (2009). In support, Chou et al (2008) state that whilst FAB-SCs exhibit much similarity to EpiSCs they are phenotypically distinct cell lines, as evidenced by microarray and miRNA comparisons.

Our findings corroborate previously obtained data indicating that STAT3 directly binds the Nanog promoter and enhances Nanog expression in mES cells (Suzuki et
al., 2006). We identify a region within the Nanog promoter containing a STAT3 binding motif that decreases Nanog expression upon its deletion. Whilst Nanog has been shown to inhibit differentiation of mES cells it is dispensable for the maintenance of pluripotency in these cells (Chambers et al., 2007; Mitsui et al., 2003) and recent findings suggest that it is required for the transition from the primed to the naïve pluripotent state. For example, Nanog has been shown to be required during the final stages of iPS cell reprogramming in vitro and specification of the naïve pluripotent epiblast in vivo (Theunissen and Silva, 2011). This may be explained by its role in X chromosome activation, a property exhibited by naïve but not primed pluripotent cells (Navarro et al., 2008). It is therefore likely that the role of E-cadherin in the acquisition of naïve pluripotency is via its upregulation of Nanog via STAT3 phosphorylation.

The conversion of Ecad<sup>−/−</sup> mES cells and ENPS cells to naïve wt-like mES cells, either by forced expression of E-cadherin or LIF stimulation, may constitute a reprogramming event similar to that observed in FAB-SCs (Chou et al., 2008) and EpiSCs (Yang et al., 2010) upon LIF pathway stimulation or ectopic expression of either Klf4 or Nanog (Silva et al., 2009). Interestingly, culture in 2i medium is often used to enhance reprogramming of EpiSCs (Guo et al., 2009; Silva et al., 2008b; Yang et al., 2010) and we observed a similar reprogramming event when Ecad<sup>−/−</sup> mES cells were cultured in this medium. Nanog expression has been shown to be critical to survival in 2i medium (Silva et al., 2009) so it is likely that N-cadherin-mediated upregulation of Nanog via STAT3 phosphorylation allows Ecad<sup>−/−</sup> mES cells to thrive in this context, since N-cadherin was observed at the surface of these cells. ENPS cell reprogramming at low passage to ENPS<sup>®</sup> cells resulted in restoration of chimera and EB generation abilities along with LIF-dependent pluripotency within these cells, however, LIF supplementation alone is insufficient for reprogramming at high passage due to methylation of the E-cadherin promoter. This is similar to that observed in EpiSCs, which have been shown to require forced gene expression for reprogramming (Guo et al., 2009; Silva et al., 2008b; Yang et al., 2010), suggesting that the E-cadherin promoter may also be methylated in EpiSCs. In
support, Guo et al. (2009) report that reprogramming of EpiSCs by culture in 2i medium did not occur beyond p4 unless Klf4 was overexpressed in these cells.

Interestingly, the de novo methylation of the E-cadherin promoter exhibited by ENPS\textsuperscript{M} cells has also been observed in various cancer cell lines (Graff et al., 1995; Tamura et al., 2000) and is associated with increased tumour invasiveness (Nam et al., 2004) and patient mortality (Graziano et al., 2004). ENPS\textsuperscript{M} cells may therefore present a useful model system within which to study tumourigenesis. For example, these cells exhibited an increased survival capacity compared to wtD3 mES cells which has also been observed in the MCF7 breast cancer cell line (Miazga, personal communication). This increased survival was abolished in ENPS\textsuperscript{M} cells upon PI3K/Akt pathway inhibition. Interestingly, both ENPS\textsuperscript{M} and Ecad\textsuperscript{-/-} mES cells exhibit increased Akt phosphorylation compared to wtD3 mES cells. The precise regulation of the PI3K/Akt pathway in these cells should therefore be examined as this may aid the identification of potential therapeutic targets related to this pathway (reviewed in (Hennessy et al., 2005)). The Wnt pathway has also been associated with increased cell survival (Masckauchán et al., 2005), however, this is unlikely to be involved in ENPS\textsuperscript{M} cells since loss of E-cadherin has been shown to have no effect on the levels of β-catenin-mediated target gene transactivation in mES cells (Soncin et al., 2011).

It has been proposed that primed pluripotent cells represent a useful model within which to explore mechanisms involved in directed differentiation since they may be more likely to exhibit lineage bias (Nichols and Smith, 2009). Insight into these mechanisms will be crucial to the transition of human pluripotent cells from the laboratory to the clinic. To this end, we compared the differentiation capacity of ENPS\textsuperscript{M} cells to that of wtD3 mES cells. Interestingly, our data demonstrates that loss of E-cadherin in mES cells does confer a bias towards neuroectodermal and away from endodermal lineage specification on the cells. These findings corroborate previous data which demonstrates that an E-cadherin-based substratum can promote neural differentiation in mES and miPS cells (Haque et al., 2012), that blockade of EMT prevents neural differentiation in mES cells (Li et al., 2011) and
that EpiSCs predominantly give rise to neuroectoderm upon in vitro EB-mediated differentiation (Tesar et al., 2007). Together, these results demonstrate that E-cadherin performs a pivotal role in cell fate acquisition in addition to pluripotency within mES cells.

FGF5 was shown to be upregulated in Ecad−/− mES cells and both ENPSM cell lines in comparison to wtD3 mES cells. Since FGF5 has been shown to be required for neural specification (Stavridis et al., 2007), this provides a potential mechanism by which loss of E-cadherin may confer a neural lineage bias on the cells. In addition, N-cadherin expression has previously been shown to promote differentiation of Ecad−/− mES cells to neuroepithelium in vivo (Larue et al., 1996), suggesting a similar mechanism may be operating in vitro in ENPS cells to promote neuroectodermal specification. As described above, N-cadherin has been shown to activate various FGFR subtypes and a discrete period of FGF-mediated ERK1/2 activation has been shown to be required for neural specification of mES cells in vitro (Stavridis et al., 2007), thus providing a potential mechanism for N-cadherin-mediated enhancement of neuroectoderm differentiation observed in ENPS cells. Paradoxically, we observed decreased levels of pERK1/2 in Ecad−/− mES cells compared to wtD3 mES cells but this discrepancy is likely due to the narrow time window in which ERK1/2 phosphorylation is required for neural specification. To test the hypothesis that upregulation of N-cadherin in ENPSM cells confers a neural lineage bias on the cells, wtD3 mES cells should be exposed to the E/N+ peptide (which targets both E- and N-cadherin) and lineage bias assessed.

Whilst the primed pluripotent state is useful for determining the mechanisms involved in early cell fate decisions, hiPS cells in the naïve pluripotent state represent the ideal platform for differentiation into a wide range of lineages for regenerative medicine applications. Various groups have demonstrated the acquisition of a more naïve pluripotent phenotype within hES and hiPS cells grown in mES cell culture conditions (Hanna et al., 2010; Silva et al., 2008a; Xu et al., 2010). Under these conditions hiPS cell reprogramming is achieved more rapidly (Silva et al., 2008a). This may be due to the upregulation of E-cadherin observed in
hES cells under these conditions (Xu et al., 2010), since E-cadherin has been shown to be critical for hES cell self-renewal (Li et al., 2010a), along with miPS cell reprogramming (Chen et al., 2011; Chen et al., 2010; Li et al., 2010b; Redmer et al., 2011) and potentially hiPS cell reprogramming, since human epithelial cells have been shown to be reprogrammed more efficiently than fibroblasts (Aasen et al., 2008) and EMT inhibitors such as SB431542 have been shown to decrease reprogramming efficiency (Lin et al., 2009). It would therefore be interesting to determine whether the same mechanism, whereby E-cadherin mediates direct activation of Nanog via STAT3 phosphorylation, connects E-cadherin to the core circuitry of pluripotency in human pluripotent cells as in mES cells. Determining the molecular mechanisms associated with the role of E-cadherin (and other cadherins) in hES and hiPS cell pluripotency and early cell fate decisions should aid in the eventual translation of such cells to a clinical setting.
Appendix 1: Supplementary data

Figure S5.1. Tbx3 is downregulated in Ecad<sup>−/−</sup> mES cells in comparison to wtD3 mES cells when cultured in FBS-containing medium.
Figure S5.2. Mutation of the PIPKIγ binding domain within the cytoplasmic region of E-cadherin does not restore Tbx3 and Nanog expression.
Figure S5.3. Conserved genes expressed by EpiSCs, ENPS$^M$ cells and Ecad$^{-/-}$ mES cells.
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Table S5.1 Comparison of the features of various pluripotent cell lines.
Appendix 2: Company addresses

Abcam plc.
332 Cambridge Science Park
Cambridge CB4 0WN

Abgene
(see Thermo Scientific)

Affymetrix UK Ltd
Voyager, Mercury Park
Wycombe Lane
Wooburn Green
High Wycombe
HP10 0HH

Agilent Technologies Ltd
South Queensferry
West Lothian
EH30 9TG

Ambion
(see Life Technologies)

Amersham Biosciences
(see GE Healthcare)

Applied Biosystems
(see Life Technology)

Bachem
Hauptstrasse 144
4416 Bubendorf
Switzerland

Berthold Detection Systems
Bleichstraße 56-68
75173 Pforzheim
Germany

BD Biosciences
2350 Qume Drive
San Jose, California
USA, 95131

Calbiochem
(see Millipore)

Cell Signalling Technology
(see New England Biolabs)

Chemicon International
(see Millipore)

Dako UK Ltd.
Cambridge House
St Thomas Place
Ely CB7 4EX
Cambridgeshire

GE Healthcare
The Grove Centre
White Lion Road
Amersham HP7 9LL
Bucks

Gibco
(see Life Technologies)

Invitrogen Ltd.
(see Life Technologies)

Life Technologies
3175 Staley Road
Grand Island, NY 14072
USA

Lonza Group Ltd
Muenchensteinerstrasse 38
CH-4002 Basel
Switzerland

NBS Biologicals
14 Tower Square
Huntingdon
Cambridgeshire
PE29 7DT
New England Biolabs
240 County Road
Ipswich, MA

PAA Laboratories Ltd.
Termare Close
Houndstone Business Park
Yeovil BA22 8YG
Somerset

Partek Inc
12747 Olive Blvd
Suite 205
St Louis
Missouri
63141
USA

Promega UK Ltd.
Delta House
Southampton Science Park
Southampton SO16 7NS

Qiagen UK Ltd.
QIAGEN House
Fleming Way
Crawley
West Sussex, RH10 9NQ

Roche Applied Science
Millipore UK Ltd.
Unit 3&5, The Courtyards
Hatters Lane
Watford WD18 8YH

Thermo Scientific
Abgene House, Blenheim Road
Epsom, KT19 9AP

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