

The Function of E-cadherin in ES Cell Pluripotency

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1. Introduction

Stem cells have the potential to revolutionise medicine, providing treatment options for a wide range of diseases that currently lack a long term control strategy. In order for us to harness this potential, however, we need a thorough understanding of their self-renewal and differentiation capacities. Differentiation capacity, or 'potency', can be defined as the ability of a cell to give rise to every cell type within the developing embryo and its supporting tissues (totipotency), every tissue of the three germ layers: endoderm, ectoderm and mesoderm (pluripotency, Fig. 1.) or a restricted range of cell types (multipotency). The potency of a cell is dictated to some extent by the stage at which it is isolated from the embryo, which in turn affects its gene expression profile. For example, whereas embryonic stem (ES) cells are isolated from the inner cell mass (ICM) of the pre-implantation blastocyst, EpiS cells are derived from the later stage epiblast and exhibit significantly altered gene expression compared to mouse ES (mES) cells (Tesar *et al.*, 2007).

mES cells were first isolated from the ICM of the mouse blastocyst by two independent groups in 1981 (Evans and Kaufman, 1981; Martin, 1981) and have since been used as a model system in which to study mechanisms of development and disease. ES cells have also been isolated from other species including pig (Notarianni *et al.*, 1990), rabbit (Graves and Moreadith, 1993) and chicken (Pain *et al.*, 1996). In addition, pluripotent stem cells have been derived from cleavage-stage embryos, individual blastomeres (Chung *et al.*, 2006; Klimanskaya *et al.*, 2006; Wakayama *et al.*, 2007), parthenogenic embryos (Lin *et al.*, 2007; Mai *et al.*, 2007; Revazova *et al.*, 2007), trophoctoderm (Tanaka *et al.*, 1998) and extraembryonic endoderm (Kunath *et al.*, 2005). Pioneering work in 1998 by Thomson and colleagues (Thomson *et al.*) resulted in the derivation of human embryonic stem (hES) cells from human blastocysts. Whilst mES and hES cells differ greatly in their gene expression profile (Tesar *et al.*, 2007) and respond to distinct pluripotency-inducing signals (Daheron *et al.*, 2004; Vallier *et al.*, 2005), the core pluripotency regulatory network of Oct4, Sox2 and Nanog is conserved between the two species. Numerous studies have demonstrated the formation of an Oct4/Sox2 heterodimeric complex which is then responsible for activating the expression of a multitude of pluripotency-associated genes (Yuan *et al.*, 1995; Botquin *et al.*, 1998; Nishimoto *et al.*, 1999). Many of the Oct4/Sox2 gene targets have been shown to be shared with Nanog in both mES (Loh *et al.*, 2006) and hES cells (Boyer *et al.*, 2005). Insight into this core

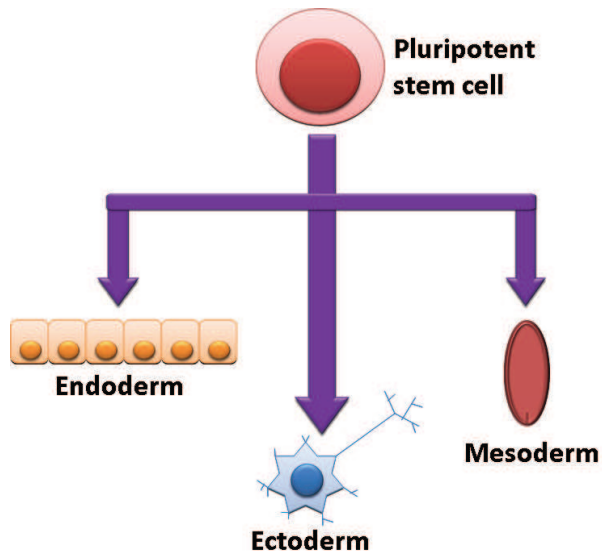


Fig. 1. Pluripotent stem cells are defined as possessing the ability to form all cells of the three primary germ layers (endoderm, ectoderm and mesoderm). Multipotent stem cells can form some of the cells of the germ layers and, therefore, exhibit more restricted potency compared to pluripotent cells.

regulatory network that governs the pluripotent state has recently led to the generation of induced pluripotent stem (iPS) cells from mouse (Takahashi and Yamanaka, 2006) and human somatic cells (Takahashi and Yamanaka, 2006; Yu *et al.*, 2007; Hanna *et al.*, 2008; Stadtfeld *et al.*, 2008). These cells, obtained by overexpression of a cocktail of pluripotency-associated genes (most commonly Oct4, Sox2, c-Myc and Klf4), have the potential to provide patient-specific cells for regenerative medicine strategies. The primary challenge of this research at present is the tumorigenicity of the transplanted cells, in one study 20% of mice injected with these cells developed tumors, likely to be due to the presence of the c-myc oncogene (Takahashi and Yamanaka, 2006). More recently, however, iPS cell generation from murine embryonic fibroblasts (MEFs) has been achieved without genetic alteration using recombinant proteins (Zhou *et al.*, 2009). These cells, termed piPS (protein-induced pluripotent stem) cells, demonstrated both self-renewal and pluripotent capacities both *in vitro* (i.e. embryoid body formation) and *in vivo* (chimera generation). iPS cells have also been generated from patients suffering from a range of disorders including diabetes mellitus and Parkinson's disease (Park *et al.*, 2008), with such cells providing a useful system with which to study the progression of specific disorders. Whereas iPS cells are more likely to be suitable for therapeutic applications (largely due to the ease of matching HLA types of donors to patients), ES cell research continues to provide valuable data about the pluripotent state to inform iPS cell research. This knowledge will be key to the translation of iPS cell technology from the laboratory to a clinical setting.

In recent years, evidence for the function of E-cadherin in regulating self-renewal signalling pathways, cell surface localisation of receptors and survival of ES and iPS cells has emerged.

In this chapter, we discuss the function of E-cadherin in regulating pluripotent signalling pathways in ES cells, the importance of E-cadherin expression in hES cells and a mesenchymal-epithelial transition event that appears to dictate efficient derivation of iPSC cells. In addition, we discuss epithelial-mesenchymal transition during human and mouse ES cell differentiation and highlight similarities between this process and tumour cell metastasis.

2. Embryonic stem cell self-renewal

2.1 mES cell self-renewal

Mouse ES cells were originally isolated and maintained in an undifferentiated state by co-culture with mitotically inactivated MEFs. More recently, however, advances have been made to more clearly define the specific combinations of signals that activate key pluripotency pathways. The interleukin-6 family cytokine leukaemia inhibitory factor (LIF) has been shown to be crucial for mES cell self-renewal *in vitro*. Upon LIF engagement, gp130 forms a heterodimeric complex with the LIF receptor β subunit allowing its activation and the subsequent stimulation of three parallel signalling cascades; phosphatidylinositol-3-OH kinase (PI3K)/Akt, Janus kinase (Jak)/signal transducer and activator of transcription 3 (STAT3) and Grb2/mitogen activated protein kinase (MAPK). PI3K/Akt and Jak/STAT3 pathways converge to activate the core pluripotency mediators Sox2 and Nanog whilst the Grb/MAPK pathway inhibits Tbx3-mediated stimulation of Nanog and Sox2 (Niwa *et al.*, 2009) (Fig. 2).

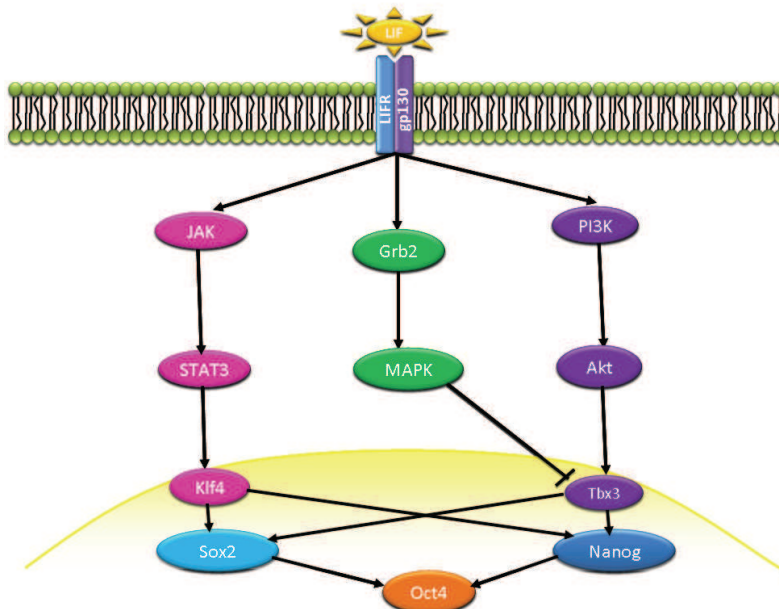


Fig. 2. The LIFR/gp130 signalling pathway stimulates expression of Sox2 and Nanog in mouse ES cells via various intermediate components. Adapted from Niwa *et al.*, 2009 (Niwa *et al.*, 2009).

Whilst LIF has been shown to prevent mesodermal and endodermal differentiation, the transforming growth factor β (Tgf β) family member bone morphogenic protein 4 (Bmp4) is also required to block neuroectoderm lineage specification within mES cell cultures. Bmp4 has been shown to perform this function by activating transcription of the inhibitor of differentiation (Id) genes via Smads1/5/8 (Ying *et al.*, 2003). Together, LIF and BMP4 are the major components of a media formulation known as Clonal Grade Medium (Millipore, Watford, UK) which can maintain mES cell pluripotency in the absence of animal serum. mES cells can also be maintained in an undifferentiated state in the absence of LIF and Bmp4 in medium supplemented with antagonists of MAPK and glycogen synthase 3 (Ying *et al.*, 2008). Additional pathways that have been linked to self-renewal include the PI3K/Akt signalling cascade, likely due to its key role in LIF signal propagation, and the canonical Wnt pathway. Canonical Wnt pathway activation has been shown to support self-renewal in both mES and hES cells (Sato *et al.*, 2004) and, more recently, to derive FAB stem cells, so-called due to the factors used in their isolation (Fibroblast growth factor 2 (Fgf2), Activin and BIO, the latter a Wnt pathway activator) (Chou *et al.*, 2008).

2.2 hES cell self-renewal

Unlike mES cells, hES cell pluripotency is not reliant upon the LIF signalling network (Daheron *et al.*, 2004). Instead, hES cell self-renewal has been shown to be supported by the Tgf β family ligands Activin and Nodal, in combination with Fgf2 (Vallier *et al.*, 2005) (Fig. 3.). When bound to their Activin-like kinase (Alk) receptors, Activin, Nodal and Tgf β initiate a signalling cascade involving the phosphorylation of Smads2/3. These transcription factors

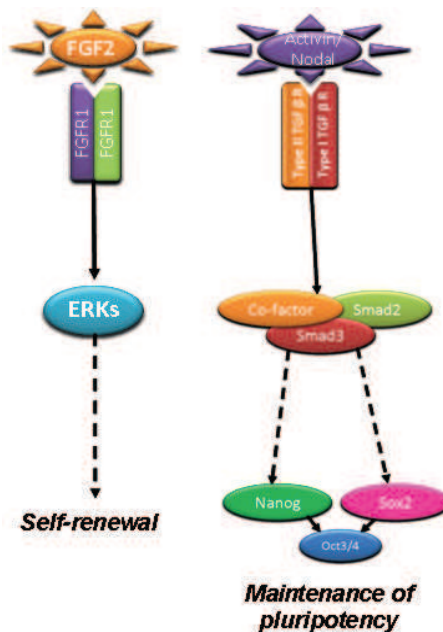


Fig. 3. Pathways associated with pluripotency and self-renewal in hES, mouse E-cadherin/-ES cells and EpiSCs.

then form a complex with Smad4 allowing them to translocate to the nucleus and activate target genes (Massague and Chen, 2000), including Nanog (Vallier *et al.*, 2009). Interestingly, mouse EpiS cells have been shown to require the same molecular milieu as hES cells and human iPS cells to maintain pluripotency (Brons *et al.*, 2007) and their global gene expression pattern also more closely resembles the hES cell transcriptome than that of mES cells (Tesar *et al.*, 2007).

3. E-cadherin

Both mES and hES cells grow as tight colonies and their integrity is maintained by cell-cell contacts, such as adherens junctions (AJ), of which a major component is E-cadherin (Fig. 4). E-cadherin is a well-characterised member of the calcium-dependent cadherin superfamily. E-cadherin is a glycoprotein and is categorised as a type 1 classical cadherin due to its possession of a histidine-alanine-valine (HAV) sequence in the extracellular domain. The role of cell-cell adhesion remains fundamental throughout embryogenesis and E-cadherin plays a critical role in the sorting of mixed cell populations to allow tissue segregation (Cavallaro and Christofori, 2004). This key developmental role is evidenced by studies in E-cadherin null (-/-) mutant embryos, which fail to form a trophectodermal epithelium or compact and form a blastocyst cavity due to the loss of cell-cell contact (Larue *et al.*, 1994). The role of E-cadherin in cell-cell adhesion is facilitated by its five extracellular (EC) domains and a cytoplasmic region, the latter of which allows stabilisation of the molecule at the cell membrane via its interaction with p120^{cas}, β -catenin and α -E-catenin

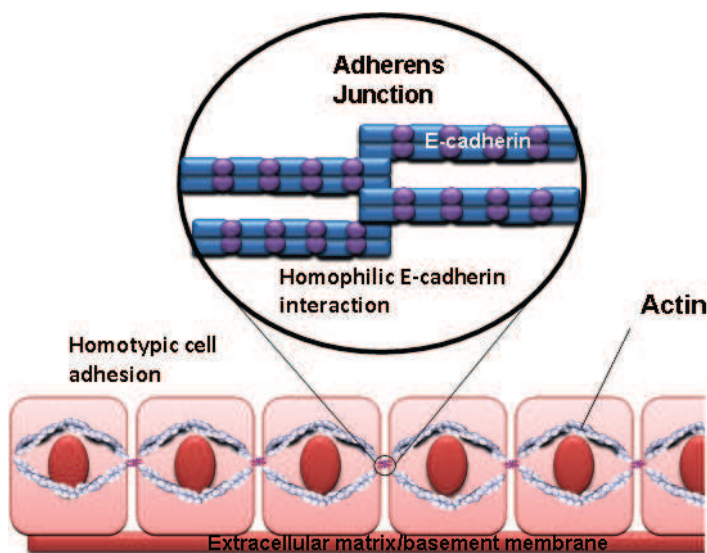


Fig. 4. Diagrammatic representation of adherens junctions formed by E-cadherin interactions. E-cadherin cis-homodimers interact with identical homodimers on neighbouring cells (homophilic interaction) with cell-cell adhesion most often associated with identical cells (homotypic adhesion). For simplicity, only E-cadherin is represented in the AJ.

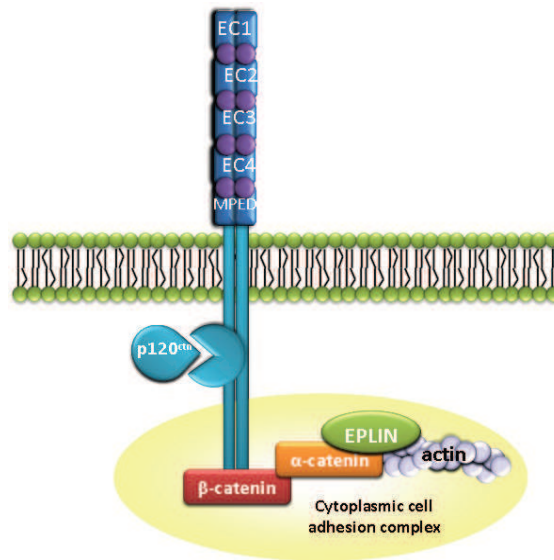


Fig. 5. Diagrammatic representation of E-cadherin cis-homodimers and the cytoplasmic cell adhesion complex. EC - extracellular domain; MPED - membrane proximal extracellular domain.

(possibly via Epithelial Protein Lost In Neoplasm). Together these molecules make up the Cytoplasmic Cell adhesion Complex (CCC) which anchors E-cadherin to the actin cytoskeleton (Fig. 5.). There is some controversy surrounding the roles of specific regions of E-cadherin in cell-cell adhesion, however, there is evidence for a critical role of the HAV domain, located on residues 79-81 of EC1, in cell-cell contact. The HAV domain is thought to form a hydrophobic pocket into which a Tryptophan residue (Trp2) from an adjacent E-cadherin molecule docks (Cavallaro and Christofori, 2004). Mutations of Trp2 and the Alanine residue of the HAV domain, W2A and A80I respectively, were shown to abolish trans- but not cis-homodimerisation of E-cadherin molecules, thus demonstrating the key roles of these amino acids in the formation of E-cadherin-mediated cell-cell contact (Pertz *et al.*, 1999). Additional roles for EC4 and the membrane proximal extracellular domain (MPED) of E-cadherin in homophilic adhesion have been evidenced by the E-cadherin targeting antibody DECMA-1, which abolishes cell-cell contact by interacting with these regions (Ozawa *et al.*, 1990).

3.1 Function of E-cadherin in ES cells

3.1.1 E-cadherin regulates localisation of cell surface molecules

Loss of E-cadherin in ES cells imparts significant alterations within the cellular architecture and can result in mis-localisation of cell surface proteins. For example, we have demonstrated that loss of E-cadherin in mouse and hES cells results in translocation of the promigratory molecule 5T4 from the cytoplasm to the plasma membrane and that this is associated with altered actin cytoskeleton arrangement and induction of cell polarisation (Eastham *et al.*, 2007; Spencer *et al.*, 2007). In addition, it has been reported that E-cadherin mediated cell-cell contact regulates expression of Eph receptors and Ephrins (Orsulic and

Kemler, 2000). Therefore, E-cadherin appears to play a critical role in regulating cellular architecture and, thus, localisation of plasma membrane proteins. This is supported by our unpublished observations which suggest that E-cadherin also functions to maintain plasma membrane localisation of a range of proteoglycans in ES cells (Soncin et al, unpublished data). As a result, E-cadherin should not be seen as simply a cell adhesion protein but an important molecule for maintaining the integrity of epithelium and ES cell colonies. Surprisingly, little has been reported on the function of altered E-cadherin expression in cell surface protein localisation. Since loss of E-cadherin is a defining event in tumour cell metastasis it is possible that cell surface proteins regulated by E-cadherin expression could provide novel targets for tumour therapies.

3.1.2 E-cadherin expression regulates signalling pathways in pluripotent cells

We have recently shown that E-cadherin^{-/-} mouse ES cells do not respond to LIF when cultured in medium supplemented with foetal bovine serum (FBS). Although E-cadherin^{-/-} ES cells exhibit an undifferentiated phenotype in medium supplemented with FBS/LIF, the cells maintain pluripotency and self-renewal by utilising Activin, Nodal and Fgf2 present within ES-screened FBS (Soncin *et al.*, 2009) (Fig. 3.). E-cadherin^{-/-} ES cells can be cultured in an undifferentiated state in serum-free medium supplemented with Activin A, Nodal and Fgf2 and exposure of the cells to the Activin-like kinase receptors (Alks)-4, -5 and -7 inhibitor (SB431542) under these conditions induces their differentiation (Soncin *et al.*, 2009). Mutant E-cadherin protein analysis in our lab demonstrated that the β -catenin binding domain of E-cadherin is critical for LIF/Bmp-mediated pluripotency in ES cells. Although E-cadherin^{-/-} ES cells utilise Activin/Nodal signalling as a default pluripotent pathway in serum-supplemented medium they are also able to maintain pluripotency via LIF/BMP signals in the absence of Activin/Nodal (Soncin *et al.*, 2009). Therefore, E-cadherin^{-/-} ES cells possess a functional "ground state" pluripotent signalling network (Ying *et al.*, 2008) as well as the ability to circumvent this pathway by utilising Activin and Nodal. Our results suggested that mES cells exhibit a hierarchy of pluripotency signalling pathways and this was confirmed by our observation that reversible Activin/Nodal-dependent pluripotency could be induced in wild type (wt)ES cells by their treatment with an E-cadherin homodimerisation-inhibiting peptide (CHAVC). Interestingly, β -catenin null (-/-) ES cells are also able to maintain an undifferentiated state in the absence of LIF and, similar to that observed in E-cadherin^{-/-} ES cells, this is achieved via Activin, Nodal and Fgf2 signalling. Therefore, the CCC appears to play a major function in regulating LIF-dependent pluripotency and self-renewal of mES cells.

We have performed global gene array analysis of E-cadherin^{-/-} mES cells and detected >2000 transcript alterations compared to the wild-type parental cell line (Soncin et al, unpublished). Surprisingly, the altered transcripts detected in the array of E-cadherin^{-/-} ES cells were not only confined to cell adhesion and motility but also genes associated with a range of biological functions, such as primary metabolic processes, catabolism, apoptosis and differentiation (Soncin et al, unpublished data). E-cadherin^{-/-} ES cells also exhibited a transcriptional phenotype more similar to epiblast-derived cells, suggesting that loss of E-cadherin expression may determine the ICM-to-epiblast transition. Therefore, E-cadherin does not function solely as an adhesion molecule in mES cells but also to regulate transcription associated with a diverse range of cell functions, maintain appropriate growth factor responsiveness of the cells and retain plasma membrane localisation of a range of molecules.

3.1.3 Culture of mES cells in shake flask suspension culture using an E-cadherin neutralising antibody

Adherent methods for the culture of ES cells are cumbersome, result in significant batch-to-batch variation and are costly and labour-intensive. Given the requirement in regenerative medicine to reproducibly derive sufficient numbers of cells of a consistent quality in a cost-effective manner we have been investigating the use of bioreactors for the suspension culture of ES cells. The advantage of such an approach over existing adherent methods is the ability to design a scaleable, non-intensive and relatively homogeneous high cell volume density microenvironment that can be monitored *in silico*. E-cadherin has been demonstrated to be the cause of aggregation of ES cells in suspension culture (Fok and Zandstra, 2005) and several groups have suggested that abrogation of E-cadherin in ES cells results in low cell viability (Fok and Zandstra, 2005) and could adversely affect the pluripotent status of the cells when cultured in bioreactors (Dang *et al.*, 2004). However, our data in E-cadherin^{-/-} ES cells demonstrated that these cells could be cultured in static suspension culture and maintain pluripotent marker expression over 30d (Mohamet *et al.*, 2010). Furthermore, we have recently demonstrated that wild type mES cells can be cultured as a near single cell suspension over prolonged periods in scalable shake flasks in the absence of additional media supplements (Mohamet *et al.*, 2010). Using the E-cadherin neutralising antibody DECMA-1 we have shown that wtES cells exhibit doubling times of $15.6\text{h}\pm 4.7$ and mean-fold increase in viable cell numbers over 48h of 16 ± 0.9 . Under these conditions, wtES cells could be cultured for 15d whilst maintaining expression of pluripotency markers and high cell viability. In addition, the cells exhibited a normal karyotype and, subsequently, were able to differentiate to cells representative of the three primary germ layers (Mohamet *et al.*, 2010). Culture of ES cells in shake flasks provides a useful cost-effective method which significantly decreases the requirement for technical input and plastic consumables associated with current adherent methods.

3.1.4 E-cadherin expression enhances hES cell colony formation and self-renewal

Several studies have identified the Rho-associated kinase (ROCK) inhibitor Y-27632 as a potent factor for increasing the survival of dissociated hES cells (Watanabe *et al.*, 2007). Recently, it has emerged that Y-27632 is likely to function by stabilising E-cadherin protein at the plasma membrane, allowing re-aggregation of the dissociated cells (Li *et al.*, 2009). Stabilisation of E-cadherin at the cell surface of disaggregated hES cells was associated with expression of the apoptotic inhibitory gene Bcl-XL and inhibition of the pro-apoptotic gene Caspase-3 (Li *et al.*, 2009). Forced expression of E-cadherin in dissociated hES cells increased clonogenicity up to 20-fold and cells lacking E-cadherin were shown to undergo cell death or differentiation within 48h. Similarly, Xu and colleagues (Xu *et al.*, 2009) identified a small molecule, Thiazovivin (Tzv), which was observed to promote cell aggregation in suspension by inhibiting endocytosis of E-cadherin. The authors identified ROCK as a direct target of Tzv and concluded that inhibition of the ROCK pathway reflected the increased survival of hES cells treated with Tzv. It has also been shown that functional interactions between small GTPase Rap1 and E-cadherin is responsible for regulating self-renewal of hES cells (Li *et al.*, 2009). Colony formation and self-renewal of hES cells was found to be suppressed by inhibition of Rap1 as a consequence of altered endocytic recycling of E-cadherin. We have observed that culture of hES cells with the E-cadherin neutralising antibody SHE78.7 results in decreased proliferation of the cells (Eastham *et al.*, 2007), perhaps reflecting degradation of Rap1 in these cells (Li *et al.*, 2009). Therefore, stabilisation of E-cadherin at the plasma membrane appears to be critical for the survival of disaggregated hES cells.

Several studies have utilised plates coated with E-cadherin-Fc protein to demonstrate increased survival of hES cells (Nagaoka *et al.*, 2010 ; Xu *et al.*, 2009). Culture of hES or iPS cells on E-cadherin-Fc coated plates exhibited a normal karyotype and maintained pluripotent marker expression over 60 days (Nagaoka *et al.*, 2010). These cells were subsequently induced to form cells representative of the three primary germ layers by their culture as embryoid bodies or following formation of teratomas. The plating efficiency of disaggregated hES cells was found to be decreased by proteolytic degradation of cell surface E-cadherin which could be prevented by the use of non-proteolytic dissociation buffer. As a result, E-cadherin-Fc coated plates may represent a useful method to provide a defined substratum for the growth of human ES and iPS cells.

3.1.5 E-cadherin expression enhances iPS cell derivation

Derivation of iPS cells represents a useful alternative to the use of human embryos for isolation of pluripotent cells as well as allowing the investigation of genetic mutations during cell lineage formation. Recently it has been suggested that nuclear reprogramming of mouse fibroblasts to iPS cells requires a mesenchymal-epithelial event (MET) (Li *et al.*, 2010.). When MET was blocked during iPS cell-induction the derivation of such cells was found to be significantly reduced. In addition, low chimaera forming ability of iPS cells was associated with low levels of E-cadherin protein expression. However, recent evidence has suggested that low chimaera-forming ability of cells is associated with inefficient incorporation of the cells into the ICM, rather than a true lack of pluripotency of the cells (Li *et al.*, 2010; Chou *et al.*, 2008).

Improved iPS cell derivation has been demonstrated by Chen and colleagues (Chen *et al.*, 2010) who isolated two small molecules that enhanced E-cadherin expression. To confirm the function of E-cadherin in the iPS-derivation procedure they forced expression of E-cadherin in MEFs and showed that this increased iPS-derivation rates 4-fold. Inhibition of E-cadherin expression using RNAi or an inhibitory peptide during iPS cell derivation lead to decreased iPS cell isolation. Interestingly, in contrast to signalling pathway alterations described by Soncin *et al.* (Soncin *et al.*, 2009), the β -catenin binding domain of E-cadherin was not required for efficient iPS cell derivation and instead was dependent on the extracellular domain of the protein. We have previously demonstrated that the β -catenin domain of E-cadherin is required to restore cell-cell contact in E-cadherin-/- ES cells (Soncin *et al.*, 2009), suggesting that the function of E-cadherin in iPS cell derivation reflects a requirement for cell-extracellular matrix (ECM) interaction rather than cell-cell adhesion. Therefore, E-cadherin appears to function via two discreet mechanisms; firstly, as a regulator of pluripotent signalling pathways via the CCC and, secondly, as an enhancer of ES cell-ECM interactions to aid cell survival.

4. Loss of E-cadherin during ES cell differentiation

4.1 Epithelial-mesenchymal transition

Epithelial-mesenchymal transition (EMT) events involve the conversion of a cell from an epithelial to a more motile mesenchymal phenotype. This process is associated with a switch from E-cadherin expression to a less adhesive cadherin, such as N-cadherin. Coordinated EMT events are important during embryogenesis, for example, to allow the ingress of epiblast cells within the primitive streak during gastrulation. However, aberrant EMT-like events have also been implicated in tumorigenesis whereby a cell is transformed to a more

metastatic phenotype, leading to tumor cell invasion of the surrounding tissues. Therefore, E-cadherin is considered to be a metastasis suppressor gene (Vleminckx *et al.*, 1991). The role of E-cadherin down-regulation during tumorigenesis is likely to be two-fold; loss of E-cadherin-mediated cell-cell contact initiates morphological changes as well as affecting the signal transduction status of the cell (Mohamet *et al.*, 2011). EMT has been shown to be stimulated by various signalling cascades and loss of E-cadherin has been shown to affect signalling via a variety of receptor tyrosine kinases, such as, the epidermal growth factor receptor and the hepatocyte growth factor receptor c-Met (reviewed in Cavallaro and Christofori, 2004). The upregulation of N-cadherin that occurs during EMT events can also affect signalling pathways such as those mediated by Fgf receptor 1 and may contribute to the increased cell survival and invasiveness that is characteristic of cells that have undergone an EMT event (Suyama *et al.*, 2002; Cavallaro and Christofori, 2004).

4.1.1 EMT during ES cell differentiation

As well as their potential use in regenerative therapies ES cells are an excellent model system for elucidating mechanisms involved in development and disease. We have demonstrated that an EMT event occurs during mouse (Spencer *et al.*, 2007) and human (Eastham *et al.*, 2007) ES cell differentiation and that this exhibits striking similarity to processes associated with ingression of epiblast cells within the primitive streak and tumour cell metastasis. The ES cell EMT event is associated with an E- to N-cadherin switch (Fig. 6.), upregulation of Snail, Slug and Sip1 (E-cadherin transcript repressors) and increased gelatinase activity and cellular motility (Fig. 7.). Whilst cell density can affect the extent to which the E- to N-cadherin switch occurs in adherent ES cell culture, EMT is often observed during embryoid body culture (Ward, unpublished).

The exact reason for the EMT event during ES cell differentiation remains unclear, although it is likely to reflect early events associated with embryogenesis. Furthermore, EMT during ES cell differentiation appears to be a regulated event, unlike the process associated with tumour cell metastasis. We have shown that loss of E-cadherin during ES cell EMT is associated with lack of pluripotent transcripts in E-cadherin-negative cells (Spencer *et al.*, 2007). As a result, E-cadherin expression can be used as a useful non-invasive tool to assess the pluripotent status of ES cells (Spencer *et al.*, 2011). Since EMT/metastasis is difficult to study *in vivo*, ES cells may provide a useful model system for the study of this process (Eastham *et al.*, 2007; Spencer *et al.*, 2007).

Our analysis of the EMT event during mES cell differentiation has enabled an understanding of the hierarchy of this process. For example, whilst induced loss of E-cadherin expression in ES cells resulted in increased cellular motility it did not result in an EMT-like event, with transcripts for Snail, Slug, Sip1 and MMP2/9 remaining unchanged (Spencer *et al.*, 2007). This suggests that loss of E-cadherin is not responsible for induction of EMT in ES cells. We also assessed EMT during differentiation of N-cadherin-/- mES cells and showed that these cells exhibited a characteristic EMT event similar to that of the parental cell line. Therefore, localisation of N-cadherin at the plasma membrane, and possible subsequent activation of signalling cascades during this process, are unlikely to be critical for the onset of EMT in mES cells, although N-cadherin-/- ES cells did exhibit significantly decreased motility compared to wtES cells. Therefore, whilst loss of E-cadherin and gain of N-cadherin are associated with an EMT event during ES cell differentiation these proteins do not appear to initiate nor control this process.

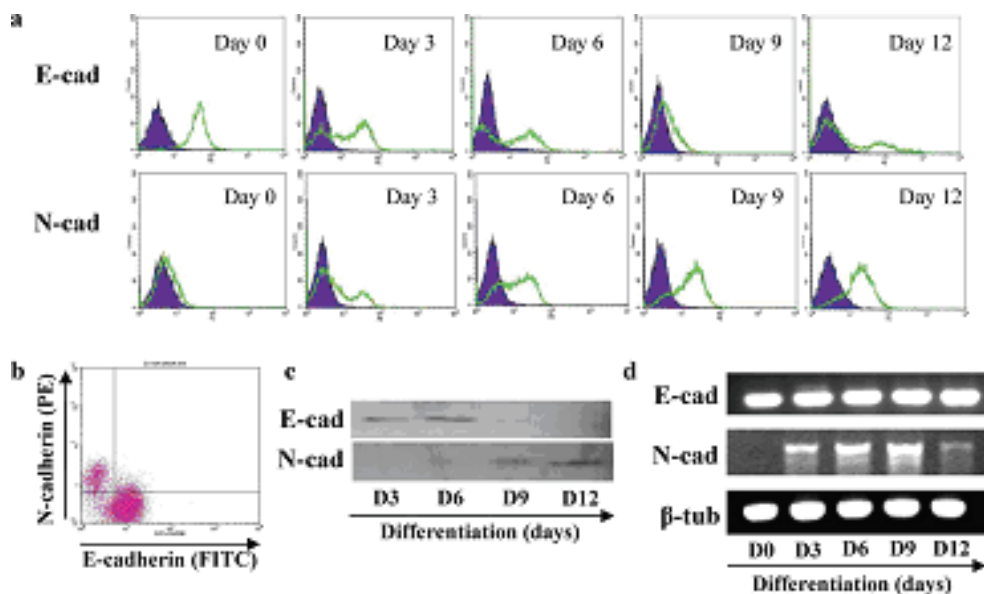


Fig. 6. An EMT event occurs during mES cell differentiation. (a) Mouse ES cell spontaneous differentiation is associated with loss of cell surface E-cadherin (E-cad) protein and gain of cell surface N-cadherin (N-cad) protein. (b) Fluorescent flow cytometry dual staining for E- and N-cadherin on ES cells differentiated for 3d. (c) Western blot analysis of total cellular E- (E-cad) or N-cadherin (N-cad) proteins in ES cells differentiated for 3, 6, 9 and 12 d. (d) RT-PCR analysis of E- (E-cad) and N-cadherin (N-cad) and β -tubulin (β -tub; control) transcript expression during ES cell differentiation. Images in these figures have been previously published in full or in part by ASCB (<http://www.molbiolcell.org/cgi/content/full/18/8/2838#F1>)

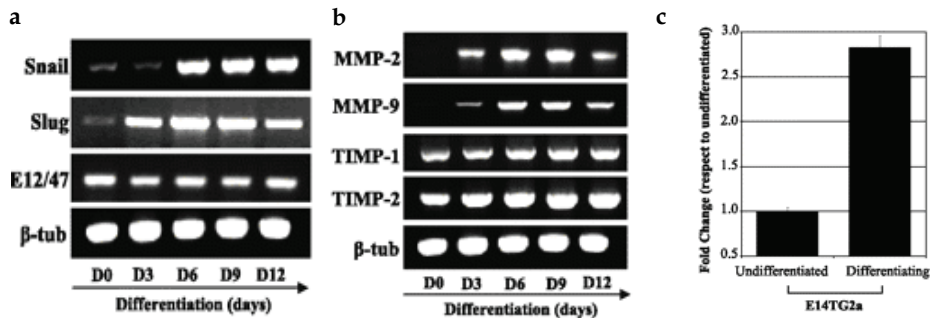


Fig. 7. Mouse ES cell differentiation is associated with expression of the E-cadherin repressors Snail and Slug, matrix metalloproteinase activity, and increased motility. (a) Transcript expression of Snail, Slug, E12/E47, and β -tubulin (β -tub; control) in undifferentiated ES cells (day 0) and in cells differentiated for 3, 6, 9, and 12 d. (b) Transcript expression of MMP-2 and -9, tissue inhibitor of metalloproteinase (TIMP)-1 and -2 and β -tubulin (β -tub; control) in undifferentiated and differentiating ES cells. (c) Cellular motility of undifferentiated and differentiating (3 d in the absence of LIF) wild-type ES cells was assessed using Costar Transwell 5- μ m pore size plates. Data represents the fold change in motility compared with undifferentiated cells. Images in these figures have been previously published in full or in part by ASCB (<http://www.molbiolcell.org/cgi/content/full/18/8/2838#F1>)

5. Conclusion

E-cadherin is emerging as a key regulator of human and mouse ES cell pluripotency and self-renewal and is challenging long-held views of signalling pathways in these cells. For example, we have demonstrated that mES cells possess at least two functional pluripotent pathways that are dependent upon E-cadherin protein expression levels (Soncin *et al.*, 2009). Whilst the exact mechanisms controlling the switch between LIF/BMP and Activin/Nodal pathways in mES cells are not fully understood it is clear that E-cadherin functions to maintain the hierarchy of these independent pathways. We have also observed that abrogation of E-cadherin in hES cells can alter dependence of the cells to FGF2 signalling (Ward, unpublished), suggesting that E-cadherin also functions to regulate pluripotency pathways in human cells.

Current evidence suggests that E-cadherin plays several roles in ES cells. Firstly, E-cadherin functions to maintain cell-cell contact and this is likely to effect localisation of plasma membrane proteins. Our demonstration that E-cadherin inhibits cell surface localisation of the pro-migratory factor 5T4 (Eastham *et al.*, 2007; Spencer *et al.*, 2007) suggests that E-cadherin expression exerts a physical effect on the localisation of plasma membrane proteins. Unpublished data in our lab has revealed that loss of E-cadherin in mES cells does not affect cell surface expression of gp130 or the LIFR (Hawkins, unpublished), suggesting that alterations in signalling pathways in E-cadherin^{-/-} mES cells are not due to changes in the localisation of pluripotency-associated receptors. The exact reason for the switch from LIF/BMP to Activin/Nodal dependent pluripotency in mES cells lacking E-cadherin is not clear. Indeed, we have found that inhibition of E-cadherin-mediated cell-cell contact using

the neutralising antibody DECMA-1 does not stimulate Activin/Nodal-dependent pluripotency in mES cells. Therefore, the exact region(s) of E-cadherin which regulate LIF/BMP-dependent pluripotency in mES cells remain unknown. The third function of E-cadherin appears to be in regulating the expression of several thousand transcripts in mES cells. Whilst many of these mRNA changes are likely to reflect indirect transcriptional regulation by E-cadherin, it serves to highlight the importance of this protein in maintaining cellular homeostasis. The fourth function of E-cadherin in ES cells appears to be specific to human pluripotent cells: Enhancement of cell survival in dissociated cell populations. mES cells do not exhibit significant cell death when dissociated into single cells, indeed, this is desirable for maintenance of an undifferentiated population, and E-cadherin^{-/-} ES cells exhibit almost 2-fold increased proliferation compared to wt mES cells (Soncin *et al.*, 2009). The effect of loss of E-cadherin in disaggregated hES cells appears to reflect endocytic cycling of the protein leading to decreased stability at the cell surface. However, hES cells are able to proliferate, albeit more slowly, upon inhibition of E-cadherin and maintain expression of pluripotent markers (Eastham *et al.*, 2007). The key difference between loss of E-cadherin expression in hES and mES cells, therefore, appears to be cellular disaggregation in the absence of a substratum. For example, whilst hES cells maintain viability in adherent culture following treatment with the E-cadherin neutralising antibody SHE78.7, the majority of the population die within 24h when cultured in suspension (Mohamet *et al.*, 2010). In summary, E-cadherin exhibits a range of functions in ES cells which result in stabilisation of epithelial integrity and associated signalling pathways. As a result, E-cadherin should no longer be classed as a mere cell adhesion protein but as a fundamental regulator of ES cell homeostasis and identity.

6. References

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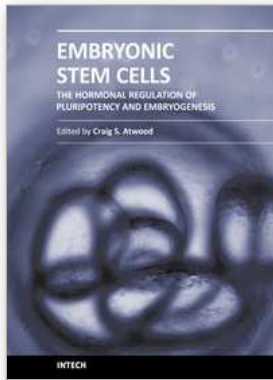
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