Cell fate specification and polarisation in mouse preimplantation epithelia

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Abstract

Understanding the establishment of polarity and the cell fate specification of epithelial cells is important for developmental biology, regenerative medicine and the study of cancer. In this thesis, models of pre-implantation epithelial development are used to investigate the relationship between these two processes.

The trophoblast is an extraembryonic epithelial tissue which contributes to the placenta. Addition of BMP4 to mouse and human embryonic stem (mES) cells grown in culture has been suggested to induce differentiation of cells to the trophoblast lineage. The use of this differentiation method was investigated as a possible model of trophoblast polarisation and cell fate specification. Unfortunately, with the protocol and reagents available this model did not appear to physiologically recapitulate trophoblast development and was not reliable.

The primitive endoderm is an epithelium which arises from the inner cell mass during mammalian pre-implantation development. It faces the blastocoel cavity and later gives rise to the extraembryonic parietal and visceral endoderm. When mES cells are grown in suspension they form aggregates of differentiating cells known as embryoid bodies. The outer-most cell layer of an embryoid body is an epithelial cell type comparable to the primitive endoderm. Embryoid bodies were used here to study the polarisation and cell fate specification of the primitive endoderm. The outer cells of these embryoid bodies were found to gradually acquire the hallmarks of polarised epithelial cells and express markers of primitive endoderm cell fate. The acquisition of epithelial polarity occurred prior to the maximal expression of cell fate markers.

Fgfr/Erk signalling is known to be required for specification of the primitive endoderm, but its role in polarisation of this tissue is less well understood. To investigate the function of this pathway in the primitive endoderm, embryoid bodies were cultured in the presence of a small molecule inhibitor of Mek. This inhibitor caused a loss of expression of markers of primitive endoderm cell fate and maintenance of the pluripotency marker Nanog. In addition, a mislocalisation of apico-basolateral markers and disruption of the epithelial barrier which normally blocks free diffusion across the epithelial cell layer occurred. Two inhibitors of the Fgf receptor elicited similar phenotypes, suggesting that Fgf receptor signalling promotes Erk-mediated polarisation. This data shows that the formation of a polarised primitive endoderm layer in embryoid bodies requires the Fgfr/Erk signalling pathway.

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"In everyone's life, at some time, our inner fire goes out. It is then burst into flame by an encounter with another human being. We should all be thankful for those people who rekindle the inner spirit." Albert Schweitzer

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List of Abbreviatons

3D	3-Dimensional
Afp	α-Fetoprotein
Amot	Angiomotin
Amotl-2	Angiomotin-like 2
Anova	Analysis of variance
aPkc	Atypical protein kinase c
APS	Ammonium Persulphate
BCA	Bicinchoninic acid
BMP	Bone Morphogenetic Protein
BSA	Bovine serum albumin
cDNA	Complementary Deoxyribonucleic acid
Dab-2	Disabled-2
DAPI	4',6-Diamidino-2-phenyindole, dilactate
Dlg	Discs-large
DMEM	Dulbecco's Modified Eagles Medium
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
E	Embryonic day
ECM	Extracellular matrix
EGF	Epidermal growth factor
EMT	Epithelial to mesenchymal transition
EPC	Ectoplacental cone
EpRas	Ha-Ras-transformed mammary epithelial cells
Erk	Extracellular signal-regulated kinase
ExE	Extraembryonic ectoderm
FACS	Fluorescence-activated cell sorting
FBS	Fetal Bovine Serum
Fgf	Fibroblast growth factor
Fgfr	Fibroblast growth factor
Gapdh	Glyceraldehyde 3 phosphate dehydrogenase
GMEM	Glasgow Minimum Essential Medium
Gsk-3	Glycogen synthase kinase-3
H&E	Haematoxylin & Eosin
hES	Human embryonic stem cell
Hnf4α	Hepatocyte nuclear factor 4 α
HRP	Horse radish peroxidase
IC-50	half maximal inhibitor concentration
ICM	Inner cell mass
Id	Inhibitor of Differentiation
IMDM	Iscove's Modified Dulbecco's Media
iPS	Induced pluripotent stem cells
Jam	Junctional adhesion molecules
LB	Luria Broth
Lgl	Lethal giant larvae
LIF	Leukemia Inhibitory factor
Lrp-2	Lipoprotein related protein (Megalin)
Magi	Membrane-associated guanylate kinase
Maguk	Membrane-associated guanylate kinase
Mapk	Mitogen-activated protein kinase

Mapkk	Mitogen-activated protein kinase kinase
MDCK	Madin-Darby Canine Kidney
Mefs	Mouse embryonic fibroblasts
mES	Mouse embryonic stem cell
mRNA	Messenger Ribonucleic acid
MTG	Monothioglycerol
N2B27	Defined mES cell media, 1:1 Neurobasal:DMEM F12 plus N2 and B2 supplements
NEAA	Non-essential amino acids
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
Pdgfrα	Platelet derived growth factor receptor α
Pdgfα	Platelet derived growth factor
PFA	Paraformaldehyde
PG	Plakoglobin
Pi3k	Phosphoinositide 3-kinase
Pkc	Protein kinase c
PKP	Plakophilins
PMSF	Phenylmethylsulphnoyl fluoride
ppErk	Diphosphorylated Extracellular signal-regulated kinase
PVDF	Polyvinylidene difluoride
q-PCR	Quantitative polymerase chain reaction
R63	E14tg2A mES cell line Clone R63
Rassf	Ras-association domain famil
RIPA	Radio-immunoprecipitation assay
RNA	Ribonucleic acid
SDS-PAGE	Sodium Dodecy Sulphate Poly-acrylamide gel electrophoresis
SEM	Standard error of the mean
TBS	Tris buffered saline
TBST	Tris buffered saline with 0.05% tween
TEM	Transmission electron microscopy
TEMED	Tetramethylethylenediamine
TER	Trans-epithelial resistance
Vegfr	Vascular endothelial growth factor receptor
Vol	Volume
Wt	Weight
Yap	Yes associated protein
Zo	Zona occludens
Stat	Signal Transducer and Activator of Transcription
Mek	Mitogen-activated extracellular signal-regulated kinase

Chapter 1

1 Introduction

1.1 Epithelia

Epithelial tissues line and separate different compartments of the body, they contain sheets of epithelial cells tightly packed together (St Johnston and Ahringer, 2010). The main function of an epithelium is to act as a selectively permeable barrier that regulates the transport of molecules between cellular compartments (St Johnston and Ahringer, 2010). To perform this function epithelial cells are apico-basolaterally polarised, meaning that they have a different lipid and protein composition in the apical and basolateral domains of the cell (Figure 1.1) (Müller, 2000; St Johnston and Ahringer, 2010). Epithelial cells contain specialised junctions between the cells to maintain the adherence of the cells together, and to regulate the movement of molecules across the epithelial sheet and between the epithelial cells (Figure 1.1).

Epithelia are divided into categories on the basis of the number of layers within the epithelium; simple epithelia have one layer, stratified are multilayered, and pseudostratified have only one layer but the nuclei are arranged so that it appears multilayered (Lee and Norden, 2013). Additionally, each of these types of epithelia can be classified on the basis of their shape. Squamous epithelia are wider than they are tall, cuboidal epithelia are equally wide as they are tall, and columnar epithelia are taller than they are wide.

Understanding the establishment and maintenance of epithelial polarity is important for two main reasons. Firstly, most cancers originate from epithelial tissues and a loss of polarity is a hallmark of cancer (Royer and Lu, 2011). Secondly, many tissues within an organism are epithelial, it is therefore important for developmental biology and regenerative medicine to understand the development of these tissues (Bryant and Mostov, 2008). This thesis will focus on the development of preimplantation epithelia in the mouse.

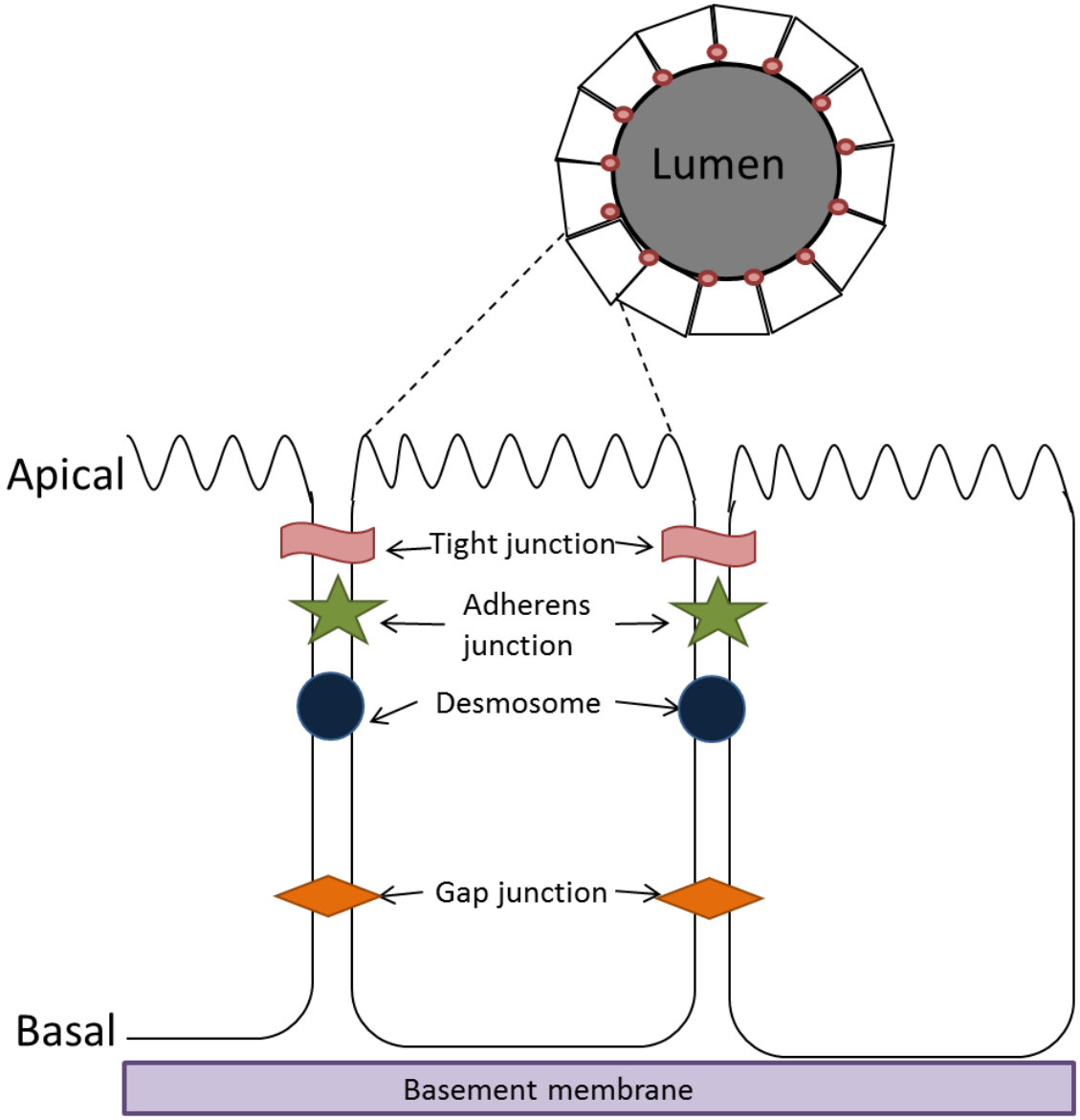


Figure 1.1: Polarised epithelial cells form an epithelial sheet which lines and separates compartments within an organism.
Epithelial cells have apico-basolateral polarity, they have different lipid and protein composition in these domains. Microvilli are present on the apical side, whilst the basement membrane forms on the basal side. They have four different types of junctions. Tight junctions are important in regulating transport of molecules across the epithelial sheet. Adherens junctions and desmosomes are critical for the adhesion of epithelial cells to each other, maintaining them in a sheet. Gap junctions allow the movement of ions and small molecules between adjacent cells. Epithelial cells can be layered in a stratified epithelia as shown here.

1.1.1 Polarity determinants

The polarisation of epithelial cells requires the function of complexes of polarity proteins (Figure 1.2). These proteins coordinate with each other to form and maintain a cell which is apico-basolaterally polarised and develops cell-cell junctions (Assémat et al., 2008).

Par complexes

The first polarity complex to be identified was the Par complex. Par polarity proteins were originally discovered in *C. elegans* for controlling the polarisation of P granules during cleavage, these mutations also result in defects in cleavage timing, patterning and localisation of the P granules (Kemphues et al., 1988). In Par-6 mutants; Par-1, Par-2, and Par-3 are mis-localised in early embryos suggesting that Par-6 is required to localise these other proteins (Watts et al., 1996). Later, Pkc-3, an atypical-Protein kinase c (aPkc) was shown to directly interact with Par-3 (Tabuse et al., 1998). These two proteins are co-dependent, and also depend on three other Par proteins (Tabuse et al., 1998). This established the idea that the Par proteins and aPkc form a complex together which is important for epithelial polarity (Tabuse et al., 1998). Homologues of the Par complex proteins exist in *Drosophila melanogaster,* similarly to *C. elegans* they are required for cellular polarity. The aPkc complex exhibits an apically restricted localisation in *Drosophila* epithelial cells (Benton and St Johnston, 2003). It has been shown that Par1 phosphorylates Bazooka (Drosophila Par3 homolog) thereby excluding the Bazooka-Par-6-aPkc complex from the lateral domain and restricting it to the apical domain (Benton and St Johnston, 2003).

In mammals there are two identified aPkc proteins. aPkcζ was first identified as a member of the Protein kinase c family. It was noted however that all other members of the Protein kinase c family have a tandem repeat of the characteristic cysteine-rich zinc-finger-like sequence in the regulatory domain, whilst Pkcζ has only one (Ono et al., 1989). This and other biochemical differences suggest it belongs to a separate related family, now known as aPkc (Ono et al., 1989). Both aPkcζ and aPkcλ/ι (λ in mouse, ι in human) are important for epithelial polarisation. aPkcλ has similar structural and biochemical properties to aPkcζ, and is therefore also part of the atypical family of Protein kinase c (Selbie et al., 1993). It was first shown in Madin darby canine kidney (MDCK) cells that aPkcλ co-localises at the tight-junction with Par-3 protein to which it can bind (Izumi et al., 1998). The calcium switch assay involves transferring cells to calcium free media which results in a loss of polarity. Upon transfer to media containing calcium the formation of epithelial junctions and polarisation can be observed. Expression of a dominant-negative point mutant in aPkcλ or aPkcζ in MDCK cells prevents Zona occludens-1 (Zo-1) and other tight junction components from localising at the tight junction and forming a tight junction after calcium switch, demonstrating that aPkc is required for tight junction formation (Suzuki et al., 2001).

Cdc42 is another protein which forms part of the Par polarity complex. In its active GTP-bound state, Cdc42 protein is linked to aPkc via Par-6 forming a complex with Par-3 (Joberty et al.,

2000). Overexpression of Par6 stimulates aPkcζ activity suggesting that the interaction of Par6 with aPkcζ regulates the activity of aPkcζ, this occurs in a Cdc42 dependent manner (Qiu et al., 2000). Overexpression of Par6 causes mis-localisation of Par3, Zo-1 and aPkcζ from the tight junction (Joberty et al., 2000). An activated mutant of Cdc42 also disrupts the co-localisation of Par3 and Zo-1, and tight junction formation suggesting that tight junction assembly depends on this complex. This therefore demonstrates that the Par3-Par6-Cdc42-aPkc complex is required for the establishment and maintenance of mammalian epithelial cell polarity.

Crumbs complex

One of the complexes present in apico-basolaterally polarised epithelial cells is the Crumbs complex. The Crumbs complex consists of three proteins which were all originally discovered in *Drosophila melanogaster.* Crumbs protein was the first protein of the complex to be identified (Tepass et al., 1990). It localises at the apical membrane of epithelial cells. Mutations in the Crumbs gene result in organisational defects in epithelia, suggesting it is required for the establishment and/or maintenance of cell polarity (Tepass et al., 1990). Overexpression of Crumbs causes an expansion of the apical plasma membrane of a cell (Wodarz et al., 1995). There are three mammalian Crumbs genes 1-3 which each have different expression patterns (Assémat et al., 2008). Crumbs 1 is expressed in the retina and brain, Crumbs2 has the same expression pattern as Crumbs 1 whilst also being expressed in the kidney. Crumbs 3 is expressed in skeletal muscles and all epithelial tissues. Mutations in Stardust, a different protein component of the Crumbs complex results in a similar phenotype in *Drosophila melanogaster* to that seen in Crumbs mutants suggesting that it is also required for apico-basolateral polarisation (Tepass and Knust, 1993). The mammalian homologue of Stardust is Pals1. The final component of the Crumbs complex is PatJ. The locus which encodes PatJ is Discs Lost; it encodes multiple proteins which when mutated cause mis-localisation of Crumbs, causing a loss of polarity (Bhat et al., 1999). dPatJ has since been shown to itself be required for polarisation of *Drosophila* epithelial cells (Nam and Choi, 2006).

Scribble complex

The last epithelial polarity complex described here is the scribble complex. It is composed of three components, Scribble, Lethal giant larvae (Lgl), and Discs-large (Dlg), and is required for the establishment of the apical domain. Mutations in the *Drosophila Melanogaster* Scribble protein cause mis-shaping of cells, a disruption of the wild-type monolayer organisation of epithelia, and a mislocalisation of apical proteins and adherens junctions to the basolateral domain (Bilder and Perrimon, 2000). Scribble localises in *Drosophila Melanogaster* at the

equivalent of the mammalian tight junction, known as the septate junction (Bilder and Perrimon, 2000). It is found in mammalian epithelial cells to localise at the adherens junction where its localisation is dependent upon E-cadherin, whilst the localisation of E-cadherin is dependent upon Scribble (Navarro et al., 2005). The scribble complex is also composed of Lgl and, Dlg which were both discovered in *Drosophila melanogaster* to be required for epithelial polarisation (Bilder et al., 2000). Dlg localises with Scribble at the septate junction, whilst Lgl mostly localises at the plasma membrane, but its expression is not polarised (Bilder et al., 2000). Genetic interaction of these three proteins; Scribble, Lgl, and Dlg suggest that they work in a common pathway (Bilder et al., 2000). In mammals Lgl and Dlg localise in epithelial cells to the lateral plasma membrane, Lgl also localises in the cytoplasm (Müller et al., 1995; Müsch et al., 2002).

Interactions between the polarity complexes

The polarity protein complexes interact with each other in order to maintain their localisation, activity, and the apico-basolateral polarisation of the cells (Figure 1.2). It has been shown in Drosophila, using multiple Lgl mutants, that Lgl can act as dose-dependent enhancer of Crumbs (Tanentzapf and Tepass, 2003). Lgl can also rescue a Crumbs or Stardust loss of function phenotype, suggesting that Lgl normally limits Crumbs. Dlg and Scribble mutants also have the same effect as Lgl in these experiments suggesting that the Scribble complex negatively regulates the function of the Crumbs complex. Additionally, *Drosophila melanogaster* embryos that lack Bazooka (Par-3 homolog) and Dlg have an identical phenotype to the Bazooka mutant embryos, suggesting that the scribble complex also inhibits the Par complex in Drosophila (Bilder et al., 2003). It has also been shown in Drosophila neuroblasts that aPkc can also inhibit the Scribble complex by phosphorylating Lgl (Betschinger et al., 2003). Phosphorylated Lgl can then no longer associate with the apical membrane or actin, restricting it to the basolateral membrane. Additionally, Lgl associates with the Par complex and inhibits the interaction of the complex with other partners suggesting a mutual antagonism between Lgl and the Par complex (Yamanaka et al., 2003). The interaction between Lgl and aPkc was confirmed in vivo in the vertebrate epithelial model *Xenopus laevis* (Chalmers et al., 2005). The overexpression of *Xenopus* Lgl2 in blastomeres has the same phenotype as an aPkc knockout, and Lgl can rescue the overapicalisation caused by overexpression of aPkc suggesting that Lgl and aPkc act antagonistically. The antagonistic effect of the Scribble complex on the Crumbs and Par complexes maintains the basolateral domain whilst the Crumbs, and Par complexes also antagonises Scribble activity, maintaining

the apical domain (Betschinger et al., 2003; Bilder et al., 2003; Chalmers et al., 2005; Yamanaka et al., 2003).

A computer model of the mutually antagonistic relationships between polarity complex proteins outlined above suggest that they are not sufficient to maintain polarity of the cells (Fletcher et al., 2012). A model however, including both mutual antagonism between apical and basal determinants and positive feedback of the apical determinants does generate and maintain polarity within a cell. Work in *Drosophila Melanogaster* has suggested that this positive feedback loop causes self-recruitment of apical determinants to the plasma membrane (Fletcher et al., 2012). Crumbs molecules recruit additional Crumbs molecules via the extracellular domain. Crumbs is phosphorylated by aPkc and Stardust and the aPkc-Par6-Cdc42 complex is recruited to the new Crumbs molecule. The phosphorylation of Crumbs by aPkc is required to stabilise it at the plasma membrane, preventing its endocytosis. The basal determinant Lgl acts to inhibit this positive feedback, therefore causing the endocytosis of Crumbs from the basolateral membrane.

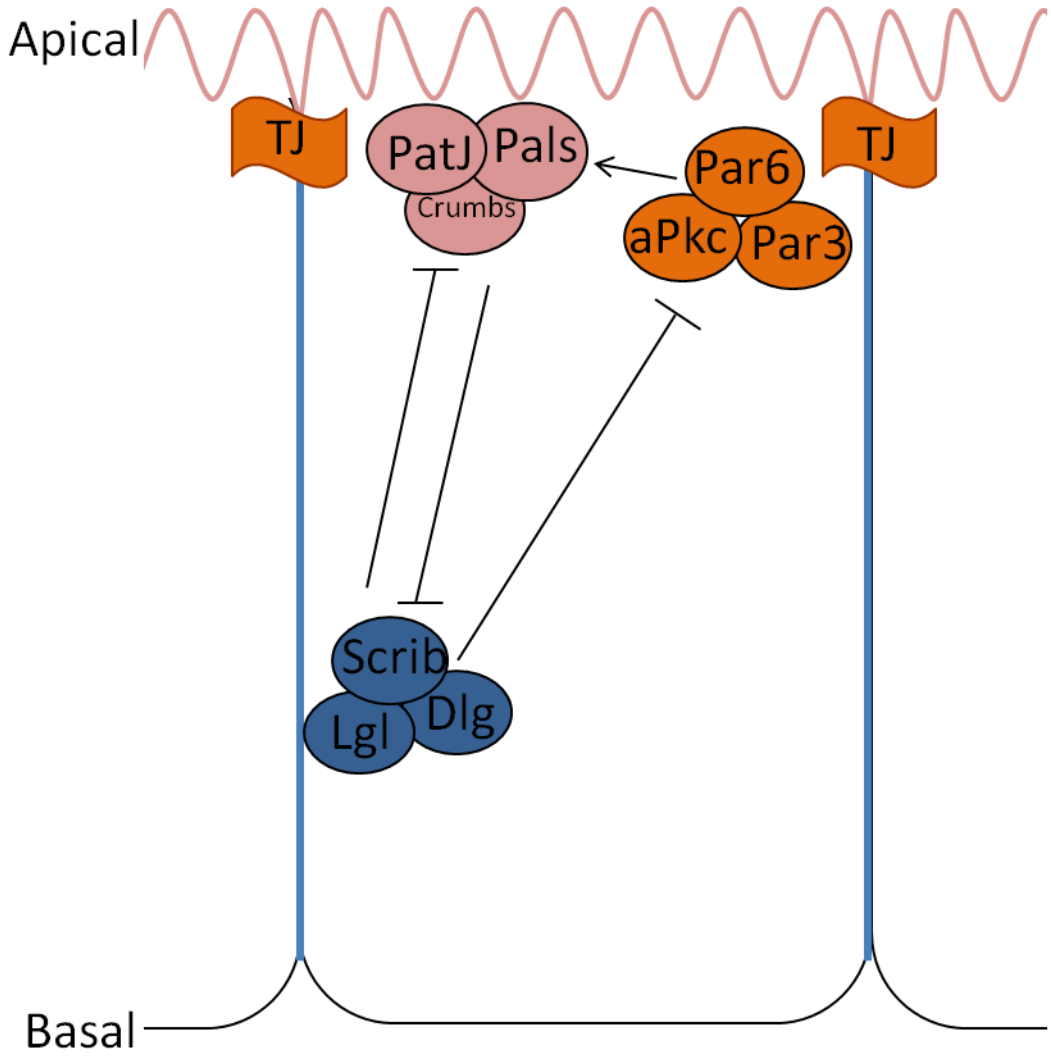


Figure 1.2: Protein components of the three epithelial polarity complexes
Epithelial polarity complexes are present within epithelial cells to establish and maintain the apico-basolateral polarisation of the cell. There are three main polarity complexes: Crumbs (Crumbs-PatJ-Pals) shown in pink, Par (Par6-Par3-aPkc) shown in orange, and Scribble (Scribble (Scribble-Dlg-Lgl) shown in blue. The Crumbs and Par complexes define the apical membrane domain, whilst the Scribble complex defines the basolateral membrane domain. The proteins within the complex interact with each other to ensure that each of the domains is maintained, and therefore the cell remains polarised (Modified from (Bryant and Mostov, 2008; Coradini et al., 2011).

1.1.2 Epithelial Junctions

In addition to maintaining polarity the polarity complexes help to maintain the four main types of junctions present in epithelial cells; tight junctions, adherens junctions, desmosomes and gap junctions. Each of these junctions is composed of different proteins and has a different role to contribute to the epithelial sheet (Figure 1.3).

Tight junctions

Tight junctions are the most apical junction found in epithelia (Balda and Matter, 1998; Matter and Balda, 2007; Shin et al., 2006; Steed et al., 2010). They function as a semi-permeable barrier to regulate movement of solutes between the two compartments which the epithelia separates (Anderson et al., 2004). It is predominantly their protein composition which makes them leakier allowing the transport of different solutes into the adjacent compartment (Anderson et al., 2004). Tight junctions were originally also thought to act as a fence, a phsycial blockade which prevents the diffusion of lipids and transmembrane proteins to maintain the polarity of the epithelial cell. Recent studies however, show that loss of two of the key components of the tight junction, Zo-1 and Zo-2 has no effect on the polarisation of the cells, suggesting that tight junctions may not have a role in maintaining the polarisation of the cell (Umeda et al., 2004).

Tight junctions are composed of transmembrane proteins which form the pore and act as the diffusion barrier. The transmembrane proteins bind to a cytoplasmic plaque of adaptor proteins which link the junction to the cytoskeleton as well as acting as a signalling platform (Figure 1.3) (Matter and Balda, 2007). There are three main families of transmembrane proteins present in the tight junction. The Jams (Junctional adhesion molecules) are members of an immunoglobulin subfamily, there are three Jams (A-C). At the tight junction Jams interact with Zo-1, Mupp1 and the polarity complex component Par-3 (Ebnet et al., 2004). The role of Jam-A binding to Par-3 suggests that it might be required for the initial polarisation of cells (Ebnet et al., 2004).

Claudins are another group of transmembrane proteins, they have four transmembrane domains (Furuse et al., 1998). At least 24 claudins have been identified (Shin et al., 2006). Claudins form the strands of the tight junction which are present around the lateral membrane of the cell. Expression of Claudin-2 in MDCK1 cells which do not usually express Claudin-2 decreases the transepithelial resistance (TER) of the cells to that of MDCK2 cells which do express Claudin-2 (Furuse et al., 2001). This suggests that it is the combination of Claudins which are present at the tight junction which regulates the paracellular permeability of the tight junction. Claudins each have different selectivity against charged solutes due to the presence of different charges in the extracellular loop which forms the pore, this regulates whether cations or anions are able to pass through the pore (Anderson et al., 2004). For example, in MDCK cells mutating negatively charged residues in the extracellular loop of Claudin-15 to positively charged residues reverses its selectivity from cations to anions

(Colegio et al., 2002). Similarly, substituting a negative charge for a positive charge in the extracellular loop of Claudin-4 increases paracellular Na^+ permeability.

The final group of transmembrane proteins within the tight junction is Occludin. Occludin also consists of four transmembrane domains, and contributes to the strands of the tight junction (Furuse et al., 1993). Overexpression of Occludin in MDCK cells results in an increase in TER suggesting that Occludin also functions in the regulation of paracellular transport in the tight junction (McCarthy et al., 1996). Mammalian Occludin proteins are highly homologous, having 90% amino acid sequence similarity with each other (Ando-Akatsuka et al., 1996). Occludin is phosphorylated by Ck2 at S408 which enables it to phosphorylate T404 and T400 (Raleigh et al., 2011). S408 phosphorylation is therefore the rate limiting step and potentially most important. When S408 is phosphorylated, more Occludin-Occludin interactions occur, and less Occludin is present at the tight junction, this releases Zo-1, and promotes cation flux through Claudin-1 and Claudin-2 pores. Conversely, when S408 is dephosphorylated, Occludin-Zo-1 interactions are stabilised, this prevents Claudin-1 and Claudin-2 but not Claudin-4 pore assembly or opening, causing paracellular cation flux to decrease.

The cytoplasmic plaque is essential for the transmembrane proteins to bind to, to attach the junction to the cytoskeleton, and to allow signalling. Zo proteins belong to the Membrane-associated guanylate kinase (Maguk) family of proteins, there are three Zo proteins (1-3) (Shin et al., 2006). Zo proteins interact with F-actin and actin-binding protein (Shin et al., 2006). Zo-1 is recruited to Cadherin-based adherens junctions prior to formation of tight junctions during polarisation of epithelial cells (Yonemura et al., 1995). Zo-$1^{-/-}$ epithelial cells are unable to form a tight junction initiated by Ca^{2+} switch, suggesting that Zo-1 is important in the formation of tight junctions (Umeda et al., 2004). The cytoplasmic plaque is also composed of membrane-associated guanylate kinase with inverted domain structure (Magi) proteins (eg. Magi-1) which are involved in signal transduction from the tight junction (Shin et al., 2006). Cingulin is also present at the cytoplasmic surface of epithelial tight junctions. Cingulin is important in linking the tight junction with the actin cytoskeleton as it interacts with many other tight junction components as well as actin (Shin et al., 2006).

Adherens junctions

Adherens junctions are localised slightly basal to the tight junctions in epithelia. Their main function is in regulating the adhesion of adjacent cells within the sheet and linking the

cytoskeletons of these cells to maintain the architecture of the epithelial sheet (Niessen and Gottardi, 2008; Rudini and Dejana, 2008).

The adherens junction is formed from two pairs of adhesive components (Figure 1.3). Cadherins are single-pass transmembrane proteins which are Ca^{2+}-dependent cell adhesion molecules (Ozawa et al., 1989). The cytoplasmic domain of Cadherins interacts with cytoplasmic proteins known as Catenins which link the adherens junction to the cytoskeleton (Ozawa et al., 1989). In the adherens junction, β-catenin or Plakoglobin (γ-catenin) binds directly to a specific recognition site within the Cadherin whilst also binding to α-catenin which binds to Actin (Ozawa et al., 1990; Rimm et al., 1995). Interestingly, β-catenin also has a very important role in signalling in the Wnt signalling cascade (Valenta et al., 2012). p120-catenin has important roles in regulating the Cadherin adhesions (Niessen and Gottardi, 2008).

The second pair of adhesive components is Nectin and Afadin. Nectins are Ca^{2+}-independent immunoglobulin-like cell-cell adhesion molecules (Irie et al., 2004; Takahashi et al., 1999). During polarisation it is Nectins which form initial 'spot-like' cell-cell contacts and recruit E-cadherin through Afadin and Catenins to induce the formation of the adherens junctions (Irie et al., 2004). Nectin interacts with Afadin, which links this complex to the actin cytoskeleton in adherens junctions (Takahashi et al., 1999).

Desmosomes

Like adherens junctions, Desmosomes are another type of junction which are important for the adherence of the cells to each other by tethering the intermediate filaments to the plasma membrane (Delva et al., 2009; Getsios et al., 2004). Desmosomes also consist of transmembrane proteins which regulate the adherence of cells (Figure 1.3). They attach to the cytoskeleton via the plaque proteins (Delva et al., 2009). The transmembrane Cadherin proteins Desmoglein and Desmocollin bind to outer dense plaque protein Plakoglobin and Plakophilin (Delva et al., 2009). Plakoglobin then binds to Desmoplakin linking the outer plaque to the inner plaque. The inner plaque is composed of the interaction between Desmoplakin and the Keratin filaments of the cytoskeleton (Delva et al., 2009).

Gap Junctions

Gap junctions are groups of intercellular channels creating a 2nm gap which permits the diffusion of ions and small molecules between cells (Goodenough and Paul, 2009). The gap-junction channels are made from hexamers of proteins known as Connexons (Figure 1.3).

Different connexins exist which can form homomeric or heteromeric hexamers (connexons) (Goodenough and Paul, 2009). This means that there are many different combinations of connexons which can be used to form a channel, these different combinations cause the channel to have different physiological properties, and will allow movement of different molecules. The gap junction channels are regulated by changing the conductance of single channels, or altering the number of channels present in the membrane.

Extracellular matrix (ECM)

Epithelia are attached to a basement membrane which is composed of extracellular matrix (ECM) proteins (Hynes, 2009). There are many extracellular matrix proteins such as type IV Collagens, Laminins, Fibronctin, Nidogen and Perlecan (Hynes, 2009). The basement membrane provides support to epithelial cell layers, as well as establishing the basal side of the cell. It is important in epithelia as well as other cells for cell signalling in differentiation, proliferation and survival (Hynes, 2009). Additionally, in other cell types the ECM is important as a substrate for migrating cells. Epithelial cells interact with the ECM through receptors such as Integrins, which link the cytoskeleton with the extracellular matrix (Barczyk et al., 2010). Through interaction with the basement membrane Integrins have a role in regulating tissue polarity (Akhtar and Streuli, 2013). In a 3 dimensional (3D) mammary-culture model, binding of β1-integrin to the basement membrane, and subsequent intracellular signalling through Integrin-linked kinase is required for the formation of the basolateral surface, but not the apical membrane (Akhtar and Streuli, 2013). β1-integrin orients the epithelial polarity through endocytosis of apical components from the cell basement membrane. This mechanism is required for the both establishment and maintenance of polarisation in these cells.

Epithelial junctions in mES cells and the primitive endoderm

There is currently limited understanding of whether mES cells have epithelial junctions, and what their role may be. E-cadherin cell-cell contacts exist in mES cells and are required to maintain pluripotency of the cells (Malaguti et al., 2013). β-catenin is also expressed in mES cells and is both nuclear and membrane-associated, it is the substrate of GSK-3 which regulates pluripotency (Kelly et al., 2011). The tight junction proteins Zo-1, Zo-2, Zo-3, Claudin-1, Occludin, and Cingulin are all expressed in mES cells (Xu et al., 2012). Zo-1 and Zo-2 are membrane associated in mES cells, Zo-1 regulates self-renewal and differentiation of mES cells. It is however unknown whether tight junctions are present in mES cells (Xu et al., 2012). Interestingly, Desmoplakin is not present in the cytoskeletal protein fraction of mES cells suggesting that mature desmosomes are not present (Eshkind et al., 2002). Desmoglein2

localises to small puncta in clusters on the cell membrane. Electron microscopy show many small cell-cell contact sites associated with small plaques which lack filament bundles, the identity of these junctions is unknown. Gap junctions are present in mES cells. Disruption of gap junction intercellular communication using pharmacological inhibitors, or siRNA of connexin43 causes a loss of pluripotency, and induces differentiation (Todorova et al., 2008). This suggests that gap junctions are important for mES cells to remain pluripotent and self-renew.

The presence of epithelial junctions in the primitive endoderm is less well characterised than in mES cells. E-cadherin and β-catenin are known to be expressed and localised in a lateral position in the primitive endoderm cells of an embryoid body (Moore et al., 2009; Wu et al., 2007), but to my knowledge the presence of functional adherens junction has not been examined *in vivo*. Similarly, localisation of the tight junction protein Zo-1 has been observed in primitive endoderm cells of an embyoid body, but to my knowledge further investigation of the role of this protein or other tight junctions proteins in the primitive endoderm has not been carried out (Wu et al., 2007). There is not, to my knowledge any published research about the existence of Desmosomes or Gap junctions in the primitive endoderm.

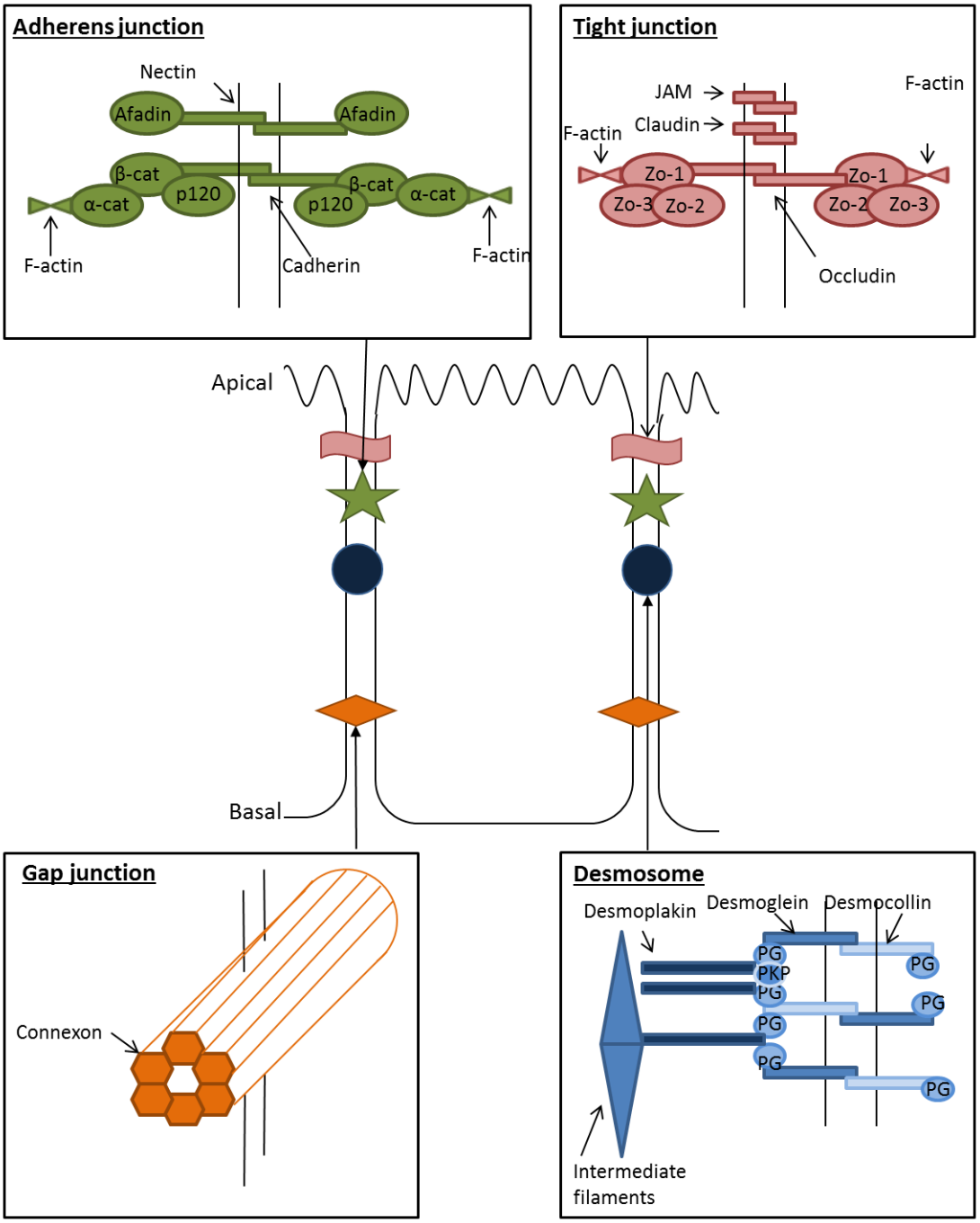


Figure 1.3: Structural components of epithelial cell junctions

Epithelia have four different types of junctions which each play an important role in the function of the epithelial sheet. The structural components of each junction are shown here. Tight junctions are the most apical junction, they regulate the movement of molecules across the epithelial sheet. They are composed of the transmembrane proteins Jam, Claudin, and Occludin, which all bind to cytoplasmic proteins Zo1-3 which interact with the Actin cytoskeleton. Adherens junctions localise basal to the tight junctions, and regulate the adherence of the cells to each other. There are two adhesion complexes 1) Nectin-Afadin, 2) Cadherin-Catenin, both Afadin and Catenins interact with the Actin cytoskeleton. Desmosomes also regulate the adhesion of the epithelial cells to each other. They are composed of the transmembrane proteins Desmogelin and Desmocollin, and the cytoplasmic proteins Plakoglobin (PG), Plakophilins (PKP), and Desmoplakin. Desmoplakin interacts with the intermediate filaments of the cytoskeleton. The most basal junction is the gap junction. This regulates movement of small molecules between adjacent epithelial cells. Gap junctions are composed of a hexamer of connexins known as a connexon which docks with other

connexons to form a channel across the plasma membranes. (Modified from (Delva et al., 2009; Goodenough and Paul, 2009; Martin-Belmonte and Perez-Moreno, 2012))

1.2 Mammalian pre-implantation development

Two epithelia form during pre-implantation development, the trophoblast and the primitive endoderm. Understanding their formation is important for our knowledge of the mechanisms of early development. Additionally, embryonic and extra-embryonic tissues provide a simple model which is relevant to the study of adult epithelial development.

Mammalian development begins with the fertilisation and subsequent fusion of the two gametes (Figure 1.4). Three cleavages follow which results in an embryo with double the total number of cells that existed before (2 to 4 to 8 cells), this produces the 8-cell stage embryo (Gilbert and Singer, 2000). At the 8-cell stage compaction occurs. Compaction involves the cells of the embryo maximising their contact with each other, this produces a tight ball of flattened cells (Gilbert and Singer, 2000). In the mouse, E-cadherin is required for the process of compaction (Shirayoshi et al., 1983). Until this stage in development all cells are totipotent, meaning that they can differentiate into any cell of the embryonic or extra-embryonic tissue (Tarkowski, 1959). Additionally, single-cell mRNA analysis has shown that until the late 8-cell stage there is no difference in expression patterns between cells (Guo et al., 2010).

During compaction cells develop intracellular apico-basolateral polarity, the outward facing membrane becomes the apical membrane and the inward facing becomes the basolateral (Cockburn and Rossant, 2010). For example, cell organelles localise in a polarised manner, actin localises apically, and some of the polarity determinants, for example Par3, Par6, aPkc, and Lgl develop their polarised localisation (Cockburn and Rossant, 2010).

After the compacted 8-cell stage, the embryo divides again to produce a morula which contains 16 cells (Cockburn and Rossant, 2010). At this stage there are some cells on the inside, and some cells on the outside. After another cleavage event, an internal cavity is created by a process known as cavitation. Cavitation involves an accumulation of Na^+ on the basolateral side of the outer cells driven by Na^+/K^+-ATPase (Watson and Kidder, 1988). The resulting osmotic gradient causes the outer cells to secrete fluid into the centre of the morula (Watson and Kidder, 1988). Tight junctions form to seal the cavity (Watson and Kidder, 1988). This creates a fluid filled cavity known as a blastocoel, and marks the formation of the blastocyst-stage embryo (Gilbert and Singer, 2000). It is at this stage that the first cell fate choice occurs, the formation of the trophoblast and the inner cell mass (ICM). The trophoblast is present around the outside of the blastocyst. The ICM, is formed from the remaining

totipotent cells, it is located to one side of the circle of trophoblast cells (Figure 1.4) (Cockburn and Rossant, 2010).

In the late blastocyst stage the cells of the ICM are segregated and form either the primitive endoderm, an extraembryonic endodermal epithelium, or cells of the epiblast which remain pluripotent (Saiz and Plusa, 2013; Schrode et al., 2013). The blastocyst embryo will then implant into the uterus of the mother and undergo gastrulation to continue its development to produce a fully grown embryo (Gilbert and Singer, 2000).

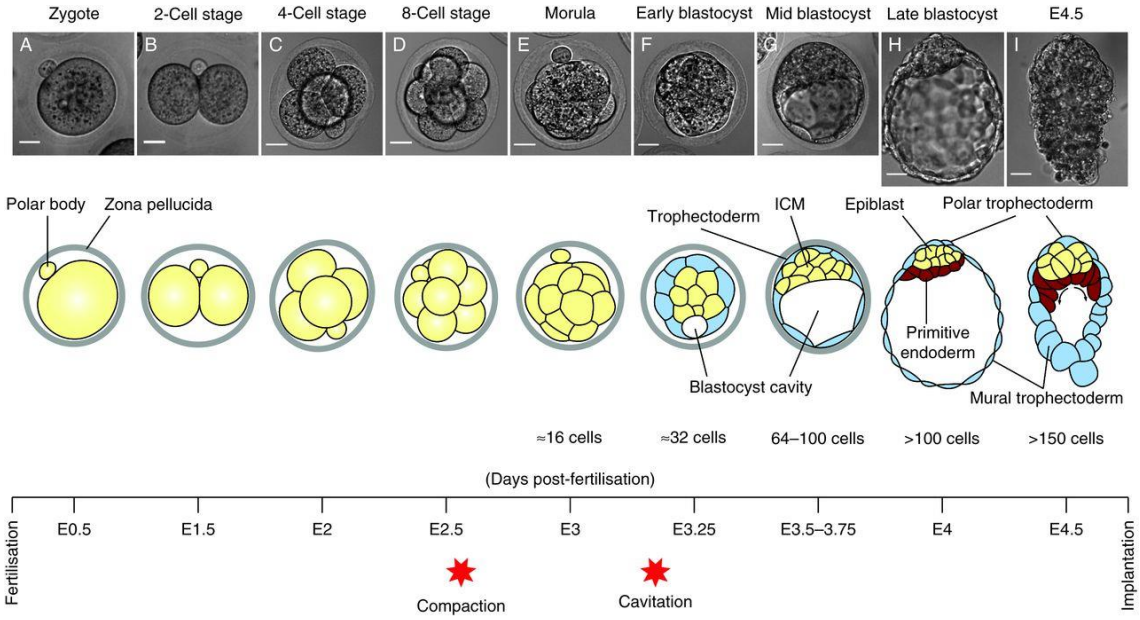


Figure 1.4: Mammalian pre-implantation development
Following the fusion of the egg and sperm a zygote is formed. Three rounds of cleavage then follow which produces the 8-cell stage embryo, until this stage there is no difference between the cells in the embryo. Subsequently, compaction occurs which forces the cells closer together. Following another round of cell division to form a 16-cell morula, cavitation takes place and the trophoblast and inner cell mass is specified. The second cell-fate decision which occurs is the formation of the epiblast and the primitive endoderm which is derived from the inner cell mass. The embryo then undergoes implantation and its development continues. (Adapted from (Saiz and Plusa, 2013))

1.2.1 Mouse embryonic stem cells

Properties of mES cells

Mouse embryonic stem (mES) cells are derived from the ICM of embryonic day (E) 3.5 embryos. They have two important properties. Firstly, they can self-renew, which means that they can produce at least one daughter cell which is identical to itself (Smith, 2001). Secondly, they are pluripotent, which means that they can produce all cell types found in the adult

organism, and will contribute to them in a chimera (Smith, 2001). Use of pluripotent cells to study development was first done with embryonal carcinoma cells. Embryonal carcinoma cells, the stem cells found in teratocarcinomas are able to contribute to all three germ layers (Martin and Evans, 1975).

mES cells are derived from the ICM of E3.5 embryos, they were first established in tissue culture by Evans MJ & Kaufmann MH and Martin G (Evans and Kaufman, 1981; Martin, 1981). Their isolation requires the flushing of E3.5 embryos from the uterus of the mother and subsequently plating them in culture on feeders for a number of days. Once the blastocyst has expanded the cells are disaggregated which produces a single cell suspension that is re-plated. These cells are then cultured and amplified to produce a mES cell line. mES cells are a more physiologically relevant way of studying mammalian development than embryonal carcinoma cells as they are derived from a cell type which exists during mouse embryonic development, and is not genetically transformed meaning that it is not oncogenic. Knowledge obtained from use of mES cells could allow therapeutic use of human embryonic stem (hES) cells in the future.

Pluripotency transcription factors

Since their derivation, our understanding of the molecular regulation of mES cell pluripotency has increased. There are three key transcription factors which are essential for the pluripotency of the ICM and mES cells, Oct3/4, Nanog and Sox2.

Oct4-deficient embryos develop to the blastocyst stage but their ICM is not pluripotent (Nichols et al., 1998). It is also impossible to derive or genetically produce Oct4 null mES cells suggesting that this transcription factor is required for the maintenance of mES cells (Nichols et al., 1998). Precise control of the expression of Oct3/4 is required by mES cells to maintain pluripotency (Niwa et al., 2000). If expression levels increase two-fold, cells differentiate into primitive endoderm and mesoderm, whilst a decrease in Oct3/4 causes differentiation of cells in to the trophoblast lineage.

Nanog deficient embryos fail to form an epiblast, and in this study Nanog-deficient mES cells were shown to lose pluripotency and differentiate into the extraembryonic endoderm (Mitsui et al., 2003). Overexpression of Nanog can maintain pluripotency of mES cells independently of Leukemia inhibitory factor (LIF)/Signal Transducer and Activator of Transcription-3 (Stat-3), and maintains Oct4 levels (Chambers et al., 2003; Mitsui et al., 2003). Expression of Nanog

fluctuates in mES cells, surprisingly mES cells were suggested by Chambers et al., (2007) to not be dependent on Nanog to maintain their pluripotency, in contradiction to the earlier study by Mitsui et al., (2003). Nanog null cells can contribute to all germ layers within chimaeras, showing that they are pluripotent, but they cannot form functional germ cells and are prone to differentiation (Chambers et al., 2007). Nanog is therefore required in the formation of the ICM, and for mES cells in culture it predisposes the cells to differentiation but it does not force them to commit to differentiation.

Inactivation of Sox2 in mouse embryos is homozygous lethal soon after implantation (Avilion et al., 2003). Analysis of the null embryos showed that Sox2 is required in the epiblast to prevent cell differentiation. Sox2 regulates expression of Oct3/4 through regulation of many transcription factors that maintain mES cells in a pluripotent state (Masui et al., 2007). Addition of Oct3/4 can therefore rescue Sox-2 null mES cells.

Signalling pathways required for pluripotency

In addition to transcription factors there are many signalling pathways which have been shown to be essential for pluripotency of mES cells. For example Fibroblast growth factor (Fgfr)/Extracellular signal-regulated kinase (Erk) signalling has been shown to have an important role in the control of pluripotency (Kunath et al., 2007; Stavridis et al., 2007). $Erk2^{-/-}$ mES cells do not differentiate when placed under protocols for mesoderm or neural differentiation, suggesting that Erk signalling is a stimulus for mES cells to exit self-renewal and differentiate (Kunath et al., 2007). Upstream of Erk signalling in the regulation of mES cell pluripotency is Fgf4, when LIF is withdrawn Fgf4-/- mES cells retain expression of Oct4, suggesting that the cells survive in an undifferentiated state, a decrease in ppErk1/2 levels is also observed (Kunath et al., 2007).

Stat3 signalling downstream of the gp130 receptor is also required for pluripotency of mES cells (Matsuda et al., 1999; Niwa et al., 1998). Induction of Stat3 in mES cells maintains mES cells in an undifferentiated state (Matsuda et al., 1999). Whilst expression of a Stat3 interfering mutant in mES cells cultured in the presence of LIF reduces self-renewal and promotes differentiation (Niwa et al., 1998). Stat3 regulates expression of the transcription factor Myc in mES cells, expression of Myc enables mES cells to self-renew and retain pluripotency in the absence of LIF (Cartwright et al., 2005).

Inhibition of Phosphoinositide 3-kinase (Pi3k) signalling also causes a reduction in the ability of LIF to maintain self-renewal (Paling et al., 2004). Expression of an activated form of Akt, a

downstream component of Pi3k signalling, maintains the pluripotency of mES cells in the absence of LIF (Watanabe et al., 2006). Inhibition of Pi3k signalling results in reduced expression of Nanog at both an RNA and protein level, suggesting this is one mechanism by which Pi3k signalling can regulate pluripotency of mES cells (Storm et al., 2007).

Additionally, Wnt signalling has been implicated in the control of mES cell pluripotency. Inhibition of Glycogen synthase kinase-3 (Gsk-3), a component of the Wnt signalling cascade maintains the pluripotency of mES cells, as shown by expression of Oct3/4 and Nanog (Sato et al., 2004). Upon differentiation of mES cells, Wnt signalling is endogenously downregulated. Gsk-3 double knockout mES cells are therefore unable to differentiate (Doble et al., 2007). One mechanism by which Gsk-3 mediates pluripotency is by increasing expression of protein and mRNA of Nanog and other pluripotency regulators (Sanchez-Ripoll et al., 2013).

Culture of mES cells

When they were first derived, mES cells were cultured with mitotically inactivated Mouse embryonic fibroblasts (MEFs), also known as feeders, and serum. These two components are both known to supply the mES cells with extrinsic factors to maintain their pluripotency and self-renewal (Evans and Kaufman, 1981; Martin, 1981). LIF is an essential factor produced by feeders which is required to maintain pluripotency of mES cells lines (Smith et al., 1988; Williams et al., 1988). If cultured with LIF mES cells can be cultured without feeders, whilst retaining their potential to form chimeric mice. LIF is not however sufficient to culture mES cells without serum. When mES cells are cultured with LIF and BMP without serum in a chemically defined system known as N2B27 their pluripotency and self-renewal are maintained (Ying et al., 2003a). LIF activates the transcription factor Stat3 (Matsuda et al., 1999; Niwa et al., 1998), whilst Bone morphogenetic protein (BMP) induces inhibitor of differentiation (Id) proteins (Ying et al., 2003a). Alternatively, mES cells can be cultured *in vitro* in the absence of serum or feeders in conditions known as 2i. 2i involves culture of mES cells in media containing a Gsk3β inhibitor and a Mitogen-activated extracellular signal-regulated kinase (Mek) inhibitor, culture of mES cells in this way maintains their pluripotency in a naïve state, making the culture more homogeneous (Ying et al., 2008). As cells can be cultured in 2i in the absence of LIF, this supports evidence that Erk and Wnt signalling are both essential for mES cell pluripotency.

1.3 Trophoblast

1.3.1 What is the trophoblast

The formation of the trophoblast epithelium and ICM is the first cell fate choice which occurs during embryonic development. This cell fate decision requires the formation of polarised outer cells, the trophoblast, and apolar inner cells, the ICM, from totipotent cells. The trophoblast, which is in direct contact with the ICM, is known as the polar trophoblast, this goes on to from the extraembronic ectoderm (ExE) which contains trophoblast stem cells, the ectoplacental cone (EPC) and secondary giant cells (Strumpf et al., 2005; Tanaka et al., 1998). The rest of the trophoblast, known as the mural trophoblast, stops proliferating and becomes primary giant cells (Tanaka et al., 1998). The placenta is critical during gestation for the development of the embryo and the health of the mother as it protects the embryo from immune rejection, supplies the correct amount of nutrients from the mother to the embryo, and safely removes all waste products (Harun et al., 2006).

1.3.2 Trophoblast specification: a transcription factor cascade

There are four main transcription factors known to be required for the specification of the trophoblast (Figure 1.5).

Tead4

mRNA of the transcription factor *Tead4* is reportedly expressed from the 2-cell embryo until the blastocyst stage, reaching its highest levels in the 8-cell embryos and morulae (Yagi et al., 2007). An alternative group did not detect its expression until the 4-cell stage but also observed it peaking at the 8-cell stage (Nishioka et al., 2008). Using immunostaining Tead4 protein was shown to be localised in the nuclei of both ICM and trophoblast cells in blastocyst stage embryos (Nishioka et al., 2008). Later at E6.5 *in situ* hybridisation suggests *Tead4* is primarily localised in the tissues derived from the trophoblast, such as the trophoblast stem cells, trophoblast cells of the EPC, the chorion and giant trophoblast cells (Yagi et al., 2007).

Inactivation of Tead4 results in a preimplantation lethal phenotype, producing embryos which lack a blastocoel cavity (Nishioka et al., 2008; Yagi et al., 2007). At the blastocyst stage Tead4$^{-/-}$ embryos do not express the trophoblast protein Cdx2, or mRNA of *Eomesodermin*, which is also required for specification of the trophoblast (Nishioka et al., 2008; Yagi et al., 2007). This suggests that Tead4 is upstream of Cdx2. All cells of the E3.5 Tead4-/- blastocyst express

pluripotency proteins Oct4 and Nanog (Nishioka et al., 2008; Yagi et al., 2007). If *Tead4* is disrupted after implantation, the embryo develops normally (Yagi et al., 2007). Constitutive activation of Tead4 in mES cells causes differentiation in to the trophoblast fate suggesting Tead4 is required and sufficient in this role (Nishioka et al., 2009). These experiments therefore suggest a role for Tead4 in the specification of the trophoblast during preimplantation development.

Cdx2

Caudal-type homeodomain protein Cdx2 is a transcription factor which is also critical for trophoblast specification. *Cdx2*, mRNA is first expressed from the 8-cell/early morula stage, at this early stage all cells are Oct4 and Cdx2 positive (Ralston and Rossant, 2008). At the blastocyst stage Cdx2 localises only in the nuclei of the cells of the trophoblast, and is downregulated in the cells of the ICM (Beck et al., 1995). It is subsequently expressed in the ExE, and a subset of the tissues derived from the trophoblast; chorion, ectoplacental canal, and allantoic bud (Beck et al., 1995). From E8.5, Cdx2 is expressed in embryonic tissues, predominantly in the posterior part of the gut, and in the tail bud (Beck et al., 1995).

Cdx2 homozygous null mutant mice die at the time of implantation (Chawengsaksophak et al., 1997). Mutants do not maintain a blastocoel, probably due to the mislocalisation of adherens and tight junction proteins at the late blastocyst stage (Strumpf et al., 2005). Expression of *Eomesodermin* is lower in Cdx2 null embryos, and markers of later trophoblast lineages are absent suggesting that the trophoblast is not specified in these mutants (Strumpf et al., 2005). The pluripotency markers Oct4 and Nanog are both expressed in all cells, both inner ICM and outer trophoblast cells of the Cdx2 null blastocysts, instead of being restricted to the ICM. This suggests that Cdx2 is required for the downregulation of Nanog and Oct4 in outer-cells (Strumpf et al., 2005). Activation of Cdx2 in mES cells can induce trophoblast differentiation (Niwa et al., 2005).

Interestingly, Cdx2 mutant mES cells contribute as frequently to the trophoblast in chimeric embryos as wild type cells, despite expressing high levels of Oct4 and Nanog (Ralston and Rossant, 2008). This shows that expression of Cdx2 is not required for trophoblast cells to form and therefore it is not required for the initial ICM, trophoblast cell fate allocation. Conversely, overexpression of Cdx2 by injection of mRNA in to cells of the embryo results in an increased contribution to the trophoblast, and a decreased contribution to the ICM through increasing the number of symmetric divisions (Jedrusik et al., 2008). Reducing Cdx2 levels in

cells of the embryo using RNAi has the inverse affect; a decreased contribution to the trophoblast, and an increased contribution to the ICM due to an increase in asymmetric divisions (Jedrusik et al., 2008). This study by Jedrusik et al., (2008) suggests that increased Cdx2 expression can influence trophoblast allocation, which is the opposite conclusion of the study by Ralston and Rossant (2008).

Recent work has shown the presence of maternal Cdx2 mRNA in embryos which could explain the difference in the experiments above, as any maternal Cdx2 would be present in the chimeras, whilst RNAi would remove both maternal and zygotic Cdx2 (Jedrusik et al., 2010; Wu et al., 2010). However, whether or not maternal Cdx2 does have a role in trophoblast development is controversial because two studies have reached directly opposing conclusions. One study showed that downregulation of both maternal and zygotic Cdx2 by dsRNA or siRNA causes developmental arrest, before blastocysts cavitation (Jedrusik et al., 2010). This is prior to the death observed in the zygotic null mutant suggesting that maternal Cdx2 has a role in trophoblast specification. Alternatively, a similar study depleted maternal and zygotic Cdx2 by siRNA and showed that embryos reached blastocysts stage, similarly to the zygotic only mutant (Wu et al., 2010). Authors therefore propose that maternal Cdx2 has no role in trophoblast specification, but zygotic Cdx2 is required for correct trophoblast development.

To try to clarify the role of maternal Cdx2, a conditional null allele strategy using the cre lox system was used, which avoids the use of injection and ensures a loss of maternal and zygotic Cdx2 from the beginning of development (Blij et al., 2012). A Cdx2 condiitonal allele was generated, cre-mediated recombination between the loxP sites caused deletion of the transcription start site and caused a nonsense frameshift. To generate germline null mice the Cdx2 conditional line was crossed with mice which expressed a female germline-specific Cre, Zp3-Cre. Germline null mice produced a similar number of offspring as control females when crossed with wild type males, suggesting maternal Cdx2 is not required for embryo development or female fertility (Blij et al., 2012). Production of maternal zygotic embryos from matings of germline null females with Cdx2 null males resulted in embryos which reached the blastocyst stage and then collapsed recapitulating the zygotic Cdx2 mutants(Blij et al., 2012). This study therefore suggests that there is no role for maternal Cdx2.

Gata3

Gata3 is a transcription factor required for trophoblast specification in parallel to Cdx2. It was first discovered for its role in transcriptional regulation of T-cell antigen receptor expression in

T-cell lymphocytes (Ko et al., 1991; Marine and Winoto, 1991; Oosterwegel et al., 1992). Gata3 is expressed in the embryonic central and peripheral nervous system, kidney, thymic rudiment and throughout T-lymphocyte differentiation (Ng et al., 1994; Oosterwegel et al., 1992). The high expression of Gata3 in differentiating trophoblast stem cells identified it as being potentially important in trophoblast specification (Ralston et al., 2010).

Overexpression of Gata3 in mES cells induces expression of trophoblast-specific genes. Overexpression of Cdx2 also induces expression of trophoblast-specific genes, some of which were the same as seen by Gata3, but many were induced by Cdx2 and not Gata3 and vice versa (Ralston et al., 2010). This suggests that Cdx2 and Gata3 have similar, but not completely overlapping roles in the trophoblast. Gata3 is still able to induce some trophoblast genes when overexpressed in a Cdx2 null mES cell line, but the expression of some trophoblast genes is missing (Ralston et al., 2010). This implies that the induction of expression of some trophoblast genes by Gata3 is Cdx2 dependent, but a subset are Cdx2-independent. Similarly to Cdx2, Gata3 expression is down-regulated in *Tead4-/-* embryos, but is expressed, suggesting that Tead4 is required for the maintenance, but not the initiation of Gata3 expression (Ralston et al., 2010). Gata3 expression is not affected in Cdx2 null embryos (Ralston et al., 2010). Gata3 is therefore thought to be act in parallel to Cdx2 under the regulation of Tead4, to induce expression of a similar but not identical set of trophoblast genes (Figure 1.5).

Eomesodermin

Downstream of both Gata3 and Cdx2 is the T-box transcription factor Eomesodermin. Eomesodermin is expressed in the mouse in the trophoblast lineage (Russ et al., 2000) and in the ExE of the egg cylinder before gastruation (Ciruna and Rossant, 1999). ExE is derived from the polar trophoblast. Eomesdoermin is subsequently present in the primitive streak, the posterior third of the epiblast and the nascent mesoderm. It is expressed in the distal and anterior visceral endoderm (Ciruna and Rossant, 1999). Later in development Eomesodermin is expressed in the developing brain (Kimura et al., 1999). It has also been shown to be expressed in trophoblast stem cells (Tanaka et al., 1998).

Eomesodermin null embryos are embryonic lethal, arresting soon after implantation (Russ et al., 2000). When cultured *in vitro* the trophoblast of the embryo spreads normally but does not form extensive outgrowths, whilst the blastocyst appears normal (Russ et al., 2000). The embryos cultured in vitro show normal expression of Cdx2, but markers of the differentiated trophoblast lineage trophoblast giant cells are absent. The correct localisation of Cdx2 and Oct4 in Eomesodermin$^{-/-}$ embryos shows that Eomesodermin null embryos die at a later time

point than the Cdx2 null embryos (Strumpf et al., 2005). This suggests that Eomesodermin is required at a later point in the development of the trophoblast than Cdx2 (Strumpf et al., 2005).

Elf5

Elf5 is an epigentically regulated transcription factor which is also essential for trophoblast specification. Elf5 is expressed in pre-implantation blastocysts, its expression is maintained throughout gastrulation in the ExE (Donnison et al., 2005). Elf5-deficient embryos cavitate normally, but are smaller than the wild-type control and lack any primitive streak formation (Donnison et al., 2005). $Elf5^{-/-}$ embryos do not form a chorion, it is thought that the absence of exchange with the mother through this tissue causes the midgestional death of these embryos (Donnison et al., 2005). Cdx2 is expressed in the polar trophoblast of $Elf5^{-/-}$ blastocysts, but by E5.5 neither Cdx2, Eomesodermin nor any other genes expressed in the trophoblast are expressed in the $Elf5^{-/-}$ embryos (Donnison et al., 2005). Additionally, trophoblast stem cells cannot be derived from the $Elf5^{-/-}$ embryos suggesting that Elf5 may be required for their maintenance (Donnison et al., 2005). Forced expression of Elf5 in wild-type mES cells causes an induction of Eomesoderm and Cdx2, proposing a role for Elf5 in reinforcing the trophoblast cell-lineage specification cascade (Ng et al., 2008). This may also explain why Cdx2 is present in $Elf5^{-/-}$ embryos, prior to Elf5 expression, but is not expressed later in the development of the trophoblast (Ng et al., 2008). Interestingly, Elf5 expression can also be regulated epigenetically, it is methylated when repressed in mES cells, and hypomethylated when expressed (Ng et al., 2008).

Pluripotency factors

Pluripotency factors which are usually expressed in the ICM also influence trophoblast cell fate specification (Figure 1.5). The ICM of $Oct4^{-/-}$ embryos is not pluripotent but instead expresses Troma1 suggesting that it becomes cells of the trophoblast lineage (Nichols et al., 1998). When trophoblast differentiation is induced by Cdx2 activation, Oct3/4 expression is repressed (Niwa et al., 2005). If mES cells have depleted Oct3/4 protein, Cdx2 does not suppress the Oct3/4 gene (Niwa et al., 2005). This suggests that Cdx2 represses Oct3/4 in cells differentiating to trophoblast in an Oct3/4 dependent manner. Additionally, Oct3/4 represses Cdx2, which suggests that there is an autoregulatory loop (Niwa et al., 2005). However, *In vivo* most $Oct4^{-/-}$ embryos do not express Cdx2 or Gata3 in the ICM, suggesting that Oct4 is not required for the initial repression of trophoblast genes (Ralston et al., 2010). The few $Oct4^{-/-}$ embryos which survive past implantation express Cdx2 and Gata3 in the epiblast as well as the

trophoblast suggesting that Oct4 is required for maintaining the repression of Cdx2 and Gata3 (Ralston et al., 2010).

Nanog and Cdx2 also reciprocally inhibit each other through binding to their promoters (Chen et al., 2009). Nanog overexpression suppresses upregulation of trophoblast markers in mES cells with conditional trophoblast differentiation induced by expression of an activated Ras allele upon addition of doxycyline. Nanog knockdown in this system upregulates expression of trophoblast markers (Chen et al., 2009). $Nanog^{-/-}$ embryos express low levels of Cdx2 in the ICM. Oct4-null embryos show a phenotype despite functional Nanog suggesting that the role of Oct4 is more important in the regulation of ICM/trophoblast cell fate specification than Nanog (Chen et al., 2009).

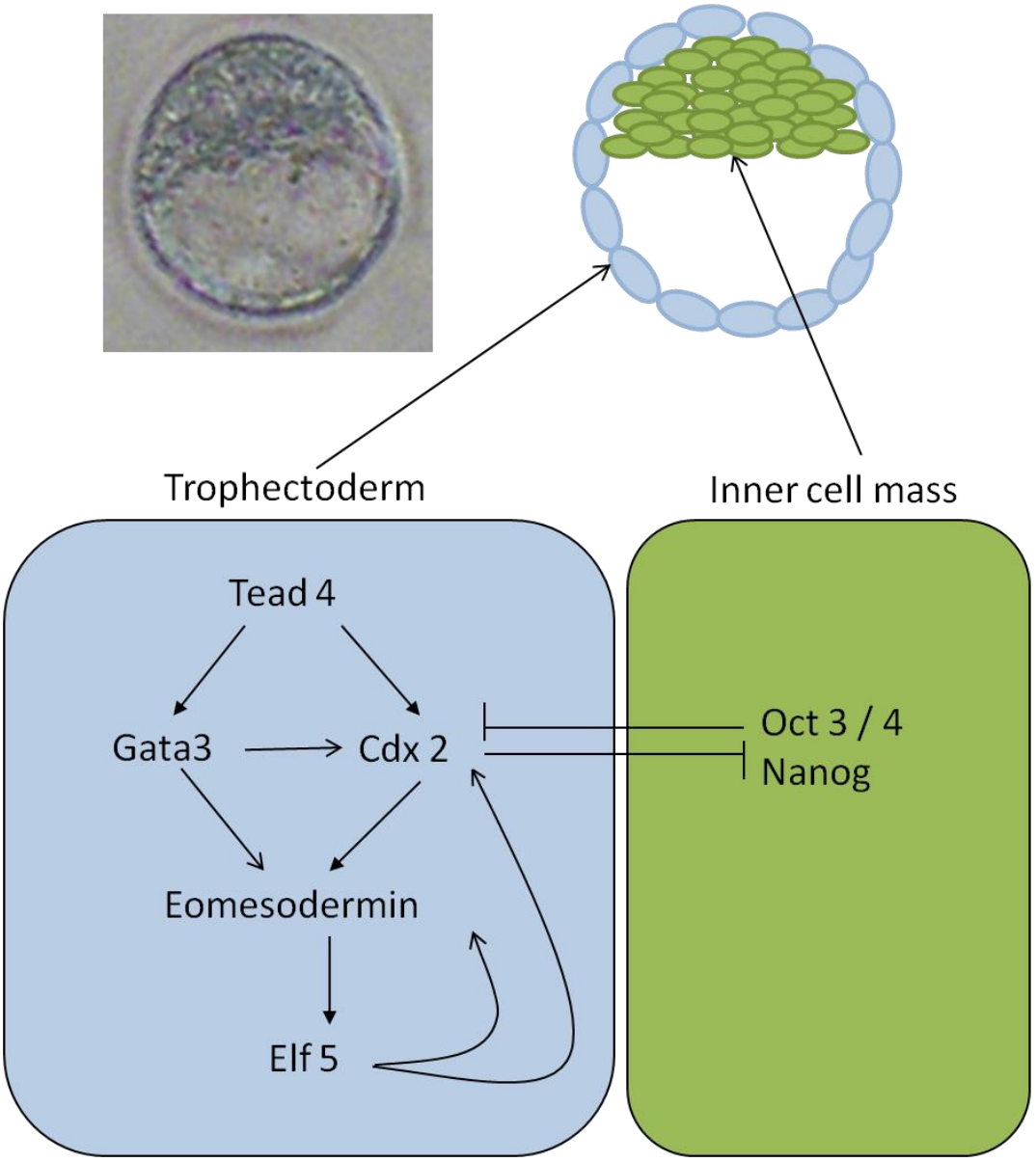


Figure 1.5: Specification of the trophoblast and the inner cell mass within a blastocyst embryo.

The trophoblast is the product of the first fate choice within the mammalian embryo, it is an epithelial sheet which forms the extraembryonic tissues critical for the development of the embryo. The inner cell mass contains pluripotent cells which form the embryo proper, it is from these cells which mouse embryonic stem cells can be derived. The inner cell mass contains two key transcription factors, Oct3/4 and Nanog which are essential to maintain its pluripotency. The trophoblast is specified by a cascade of transcription factors. At the top of the cascade is Tead4. Cdx2 inhibits the expression of Oct3/4 and Nanog in the trophoblast thereby ensuring the trophoblast becomes differentiated. Additionally, Oct3/4 and Nanog have been shown to inhibit Cdx2 in the inner cell mass, preventing its differentiation. (Adapted from (Sasaki, 2010))

1.3.3 Two models for trophoblast specification

There are two predominant models for how the morula, which is a ball of uniform cells segregates in to two cell populations, the trophoblast and the ICM, the positional model and the polarisation model Figure 1.6.

Positional model

The first model is the positional mode. At the morula stage some cells are inside with no contact to the outer surface of the embryo, around them are outside cells which are exposed to the outside environment (Tarkowski and Wróblewska, 1967). The positional model assumes that the inside and outside cells each experience different environmental conditions which directs the lineage choice they take (Tarkowski and Wróblewska, 1967). The inside cells will become the ICM, whilst the outside cells will differentiate into the trophoblast (Tarkowski and Wróblewska, 1967). Supporting evidence for this model first came from experiments when cells from a 4- or 8-cell embryos were placed on the outside of other 4- to 16-cell embryos. The transplanted cells tended to form the outer layer of the blastocyst, forming the trophoblast and yolk sac of the mouse (Hillman et al., 1972). However, recent work has suggested that it is possible to do this reaggregation with cells from the inner or outer of embryos until the 16-cell stage and they will contribute to either the trophoblast or ICM, suggesting it is not simply their position within the embryo which is important. (Suwińska et al., 2008).

Polarisation model

The second model of trophoblast development is that it is cell polarisation which regulates which cells form the trophoblast lineage and which form the ICM. Cell polarisation occurs during cell compaction, and is dependent upon cell-contact which also regulates the axis of polarisation (Ziomek and Johnson, 1980). To maintain this cell polarisation asymmetric cell division occurs in an 8-cell embryo producing one large polar cell, and a small apolar cell (Johnson and Ziomek, 1981). Some cells of the 8-cell embryo divide symmetrically generating two polar cells (Johnson and Ziomek, 1981). Polar cells are known to be present on the outside, and apolar cells are found in the inside of the embryo(Johnson and Ziomek, 1981). This understanding led to the birth of the polarisation model which suggests that the trophoblast, ICM lineage decision occurs through differential inheritance producing a polar and an apolar population of cells.

The outer trophoblast cells are morphologically distinct from the inner ICM cells, trophoblast cells are flattened, have polarised junctional complexes and polarised secondary lysosomes. The trophoblast polarises gradually, developing adherens junctions and an apical domain at the time of compaction in the 8-cell embryo, whilst tight junctions begin to form in the late morula (Fleming et al., 1989; Yamanaka et al., 2006). Additionally, the Par polarity complex protein Par3 localises to the apical surface of cells in the 8-cell stage embryo. At the 16-cell stage it extends to the lateral membrane, and by the blastocyst stage it is concentrated at apico-lateral surfaces and intercellular contacts (Plusa et al., 2005). A different Par polarity complex protein aPkc is diffusely localised in the cell, whilst being concentrated at the cell membrane as well as in the nucleus of 8-cell embryos. It subsequently become localises to the apico-lateral surface, and in the blastocyst is localised at the apical surface of trophoblast cells (Plusa et al., 2005). Downregulation of Par3 by RNAi or use of a dominant negative aPkc in a cell from the 4-cell stage embryo results in increased contribution of the injected cell to the ICM (Plusa et al., 2005). This occurs through a decrease in the number of symmetric divisions, and a change to the cell in an unknown manner which prevents it from remaining in the outer layer (Plusa et al., 2005). This therefore proves that manipulating the polarisation of the cells can affect their cell fate specification.

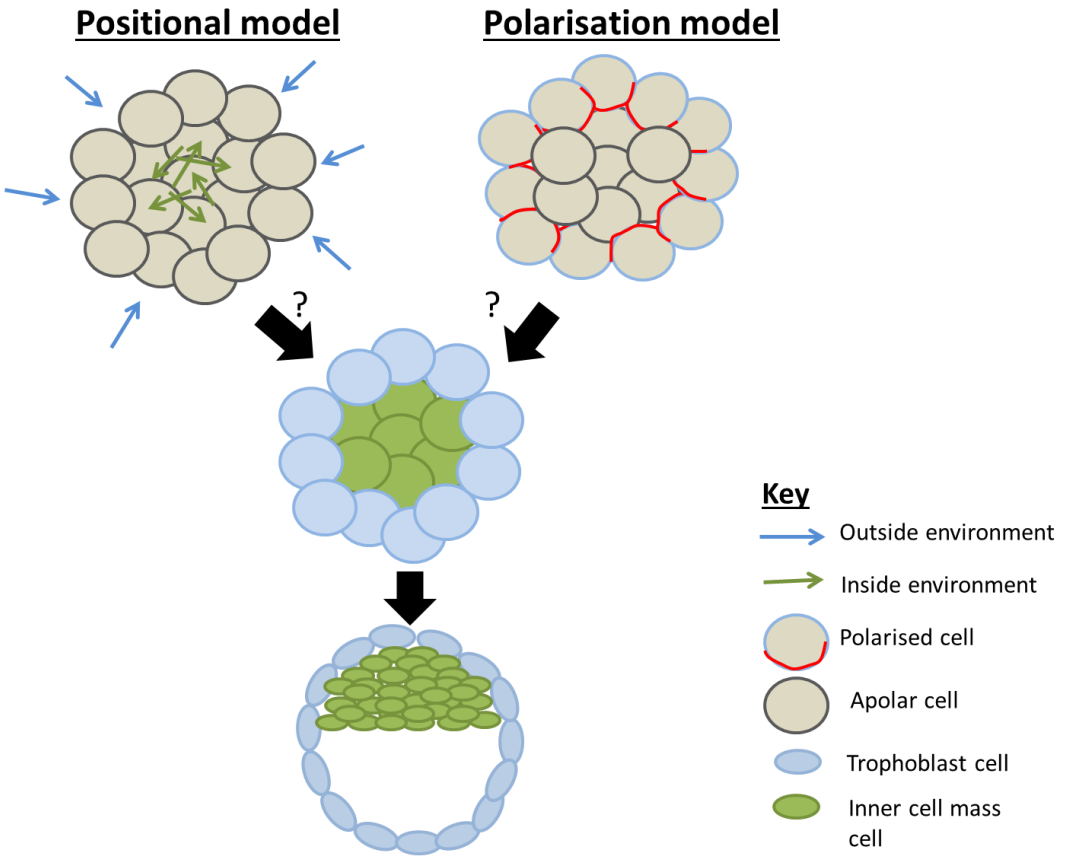


Figure 1.6: Two models for the development of the trophoblast and inner cell mass
The trophoblast and inner cell mass are formed from totipotent cells. There are two favoured models of their development. The positional model suggests that cells on the inside and outside experience different environments which results in their forming two different cell types. The inner cell mass are exposed to an inside environment, whilst the trophoblast is exposed to an outside environment. The polarisation model states that it is the polarity of the cell which dictates the fate of the cells. The polarised cells form the trophoblast, whilst the apolar cells become inner cell mass cells.

The Hippo pathway

The positional and polarisation models do not have to be exclusive. In recent years it has been shown that the Hippo signalling pathway is required for this cell fate decision, and current work suggests that it integrates both the positional and polarisation models (Hirate et al., 2012; Hirate et al., 2013). Hippo signalling involves the kinase Hippo which phosphorylates Mst 1 & 2 (Harvey and Hariharan, 2012). Mst1 & 2 and scaffold proteins Sav and Mob phosphorylate the kinase Lats1/2, which autoactivates and phosphorylates Yes associated protein (Yap), causing its inactivation and exit from the nucleus.

At the 8-cell stage all cells exhibit nuclear Yap, after this stage levels of nuclear Yap increase in outside cells, but is lower in inside cells where it was excluded from the nuclei (Hirate et al.,

2012; Nishioka et al., 2009). By the mid/late blastocyst stage nuclear Yap is exclusively expressed in outer cells (Nishioka et al., 2009). This restriction of nuclear Yap precedes the restriction of Cdx2 localisation to outer cells (Dietrich and Hiiragi, 2007; Niwa et al., 2005; Ralston and Rossant, 2008). Manipulation of the hippo pathway causes a change in the cell fate specification. For example injection of mRNA for Yap, into both cells of a 2-cell embryo causes more embryos to have high levels of Cdx2 in inside cells, suggesting it can effect Cdx2 expression (Nishioka et al., 2009).

The activation of the Hippo pathway has also been shown to be dependent on the polarisation of the outer cells. Disruption of the Par-aPkc complex *in vivo* results in an increase in phosphorylated-Yap in the outer cells as well as the inner cells (Hirate et al., 2013). Suggesting that phosphorylated Yap levels are dependent upon polarisation of the cells. This has been shown to occur through Angiomotin (Amot) and Angiomotin-like 2(Amotl2) which are adherens junction-associated proteins that are required for Hippo pathway activation and cell fate specification (Hirate et al., 2013). Outer polarised cells sequester Amot from basolateral adherens junctions to the apical domain through the Par-aPkc system suppressing Hippo signalling. Amot localises throughout the plasma membrane of inner cells because it binds to components of the adherens junction because there is no apical domain. This results in phosphorylation of Amot by Lats in the inner cells, leading to activation of the Hippo pathway (Hirate et al., 2013). Apical localisation of Amot therefore correlates with low Hippo signalling and nuclear localisation of Yap, whilst localisation of Amot throughout the plasma membrane correlates with active Hippo signalling and the localisation of Yap in the plasma membrane(Hirate et al., 2013). This mechanism therefore converts positional information through the polarisation state of the cell to affect Hippo signalling leading to fate specification of the trophoblast and ICM.

1.4 The Primitive endoderm

1.4.1 Physiological role of the primitive endoderm

After the trophoblast and ICM is specified, the next cell fate decision to take place during mammalian development occurs when cells of the ICM decide to follow either the primitive endoderm or epiblast cell fate (Cockburn and Rossant, 2010). The primitive endoderm contributes to the extra-embryonic endoderm whilst the epiblast forms the embryo proper (Gardner and Rossant, 1979). The primitive endoderm forms an epithelium which localises between the blastocoel cavity and the epiblast, these three components are surrounded by the trophoblast (Figure 1.4). Two subpopulations are formed from the primitive endoderm

(Gardner, 1982), the parietal and visceral endoderm give rise to the yolk sac, whilst the visceral endoderm also contributes to the gut endoderm (Kwon et al., 2008) and provides embryonic patterning signals (Saiz and Plusa, 2013).

1.4.2 Methods to study the development of the primitive endoderm

There are two main model systems used to study the development of the primitive endoderm, in vivo studies of the embryo and embryonic stem cell derived embryoid bodies (Figure 1.7).

In vivo studies

Mouse embryos are frequently used as a model system to probe the development of the primitive endoderm. Their use has many advantages as they allow good imaging, and the use of genetic reporter and knockout lines. Additionally, they can be manipulated with small molecules, and can be cultured *in vitro* to identify defects in pre-implantation lethal mice. Although performing experiments on embryos is the most physiologically relevant system, at this early stage in development it is however very technically challenging as the embryo is very small.

Embryoid bodies

Embryoid bodies are aggregates which form when embryonal carcinoma cells or mouse/human embryonic stem cells are cultured in suspension. They were first produced from embryonal carcinoma cells (Martin and Evans, 1975). The embryoid bodies formed were shown to comprise embryonal carcinoma cells on their inside covered by a layer of endodermal cells on the outside (Figure 1.7). Embryoid bodies can develop from simple embryoid bodies into cystic embryoid bodies which have a fluid-filled cavity. The inner cells of an embryoid body differentiate in to cell types of all three germ layers (Martin and Evans, 1975). Mouse embryonic stem cells also form embryoid bodies which can be either either simple of cystic, they have endoderm around their outside and cells of every germ layer on the inside (Martin, 1981).

Early studies suggested that there were similarities between the ordered differentiation of the embryoid body and the development of the early mouse embryo, highlighting their potential use as a model of embryonic development (Martin and Evans, 1975; Martin et al., 1977). Their development is reproducible and produces differentiated cell types such as visceral yolk sac, myocardial and hematopoetic cells (Doetschman et al., 1985). The invention of molecular techniques has allowed a more thorough comparison of the differentiation of embryoid bodies

with early embryonic differentiation. This comparison demonstrates that the pattern of gene expression observed in endodermal development *in vivo* is closely recapitulated by embryoid bodies, and that differentiation into cells of all three germ layers can occur (Abe et al., 1996; Leahy et al., 1999). These studies suggest that embryoid bodies are a good model of extraembryonic endoderm differentiation as well as differentiation of embryonic cells (Abe et al., 1996; Leahy et al., 1999).

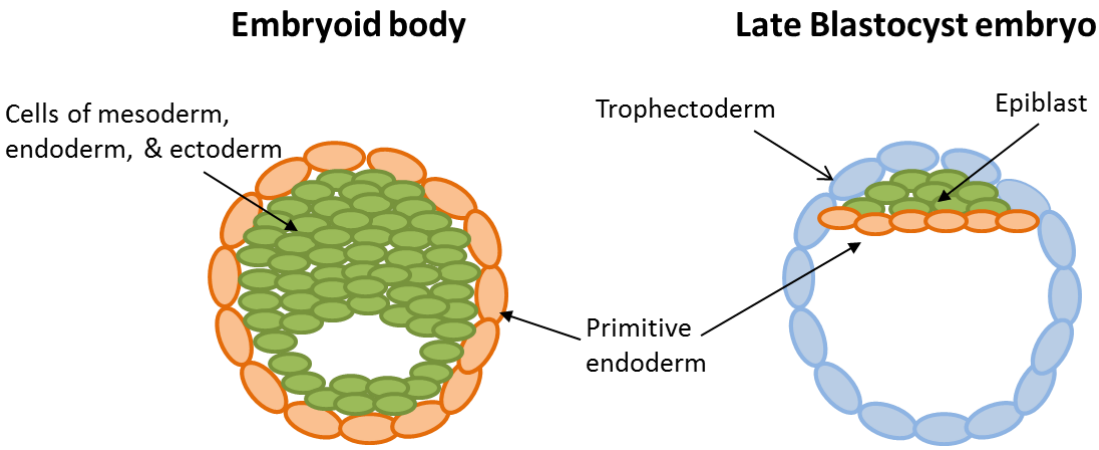


Figure 1.7 Morphology of an embryoid body in comparison to a mouse embryo
Embryoid bodies form when mES cells are cultured in suspension. Both embryoid bodies and mouse embryos are used as models of primitive endoderm development. The primitive endoderm is found in the outer layer of an embryoid body, whilst cells of the inside differentiate into cells of all three germ layers. Similarly, the epiblast of an embryo develops in to an embryo proper, and the primitive endoderm forms an epithelium between the epiblast and the blastocoel cavity.

1.4.3 Transcription factors important in the development of the primitive endoderm

There are four main transcription factors required for the early specification of the primitive endoderm (Gata6, Sox17, Gata4, and Sox7) (Figure 1.8).

Gata6 & Gata4

Expression pattern

Gata proteins are members of the zinc finger transcription factor family which bind a core GATA motif. As can be seen in Figure 1.9 Gata6 is expressed at the 32-cell stage in almost every cell of the inner cell mass, whilst expression of Gata4 is low (Guo et al., 2010). Gata4 and Gata6 are subsequently expressed in only a subset of cells of the inner cell mass at the 64-cell stage (Guo et al., 2010; Koutsourakis et al., 1999). Gata4 is rarely expressed in cells that are Nanog positive (Plusa et al., 2008), suggesting that Gata4 is a more specific marker of primitive endoderm cell fate choice than Gata6. Newly implanted blastocysts at E4.5 express both Gata4 and Gata6 in the primitive endoderm cells (Cai et al., 2008). At E4.75 the cells start to

move round extending beyond the ICM on to the blastocoel surface (Cai et al., 2008). By E5.0 a complete Gata4 and Gata6 positive visceral and parietal endoderm has formed which at first expresses both Gata4 and Gata6 (Cai et al., 2008). Mature parietal endoderm cells are Gata4 and Gata6 positive, whilst the matured visceral endoderm cells express only Gata4, not Gata6 (Cai et al., 2008).

During late embryonic and postnatal development Gata4 is expressed in the heart, intestinal epithelium, primitive endoderm and gonads (Arceci et al., 1993). Gata6 has a similar expression pattern to Gata4, it is expressed in the precardiac mesoderm, embryonic heart tube and the primitive gut (Morrisey et al., 1996). Gata6 is also expressed in arterial smooth muscle cells, the developing bronchi and the urogenital ridge and bladder (Morrisey et al., 1996).

Loss of Gata4

To investigate the function of Gata4, the phenotype of two Gata4 null mouse lines have been analysed. Gata4 null embryos are embryonic lethal dying prior to E10.5 (Kuo et al., 1997; Molkentin et al., 1997). One study found that the embryos die either at the egg cylinder stage (~E7.0), or past gastrulation (~E9.5) (Molkentin et al., 1997). Mutant embryos which die at the egg cylinder stage lack expression of visceral endoderm α-fetoprotein (Afp) mRNA, as detected by whole-mount *in situ* hybridisation, suggesting the visceral endoderm does not form properly (Molkentin et al., 1997). Those embryos which do develop passed the egg cylinder stage express Afp, and Hnf4α (Hepatocyte nuclear factor 4 α - a marker of the visceral endoderm) suggesting in some Gata4 null embryos there is a compensatory mechanism. These embryos subsequently die lacking a centralised heart tube, and foregut, and developed outside the yolk sac (Molkentin et al., 1997). Gata6 mRNA levels are upregulated two- to three-fold in these mutant embryos, as determined by semi-quantitative polymerase chain reaction (PCR), suggesting Gata6 may compensate for Gata4 allowing these embryos to develop further (Molkentin et al., 1997). Conversely, a different Gata4 null mouse line expresses high levels of Afp and Hnf4α mRNA in all embryos, as determined by in situ hybridisation, suggesting Gata4 is not required for formation of the visceral endoderm (Kuo et al., 1997). They suggest that the failure in closure of the yolk sac is due to the folding defect observed throughout the embryo instead of failure in formation of the visceral endoderm (Kuo et al., 1997).

Further information about the role Gata4 plays in the development of the primitive endoderm has come from *in vitro* studies using Gata4 null mES cells. These experiments give favour to

the conclusions reached by the study by Molkentin et al. (1997), rather than that of Kuo et al. (1997). Embryoid bodies formed from Gata4 deficient mES cells have no identifiable visceral endoderm by light and electron microscopy, and do not express markers of late extraembryonic endoderm development such as Afp or Hnf4α (Soudais et al., 1995). When these embryoid bodies are treated with retinoic acid, a stimulant of endoderm differentiation, visceral endoderm formation occurs, demonstrated by their morphology and expression of Afp. This is accompanied by induction of Gata6 expression suggesting a role for Gata6 in extra-embryonic endoderm development which is independent or downstreatm of Gata4 expression (Bielinska and Wilson, 1997).

Loss of Gata6

A Gata6 null mouse line has also been produced to investigate its role during primitive endoderm development. The mouse has been analysed by two groups producing two slightly different phenotypes. One of the investigations observed Gata6$^{-/-}$ mice dying between E6.5 and E7.5, showing a defect in endoderm differentiation as well as programmed cell death within the ExE (Morrisey et al., 1998). At E6.5 mutant embryos possessed an intact layer of extra-embryonic visceral endoderm and embryonic visceral endoderm, at E7.0 null embryos contained visceral and parietal endoderm, suggesting this is specified correctly in Gata6-deficient embryos (Morrisey et al., 1998). As determined by *in situ* hybridisation, Gata4 gene expression is largely reduced in the visceral and parietal endoderm of Gata6-deficient embryos, and Hnf4α mRNA could not be detected (Morrisey et al., 1998). This study therefore suggests that Gata6 is not required for the visceral and parietal endodermal tissues to form but is required for the maintenance of expression of extra-embryonic markers. The alternative study of the phenotype of Gata6 null embryos observed no recognisable endoderm structure at E5.5, and an absence of Gata4 positive parietal and visceral endoderm cells, as determined by immunostaining (Cai et al., 2008). These Gata6-null blastocysts exhibited no Gata4 or Gata6 positive cells, and the ICM did not have a covering of Gata4- and Gata6- positive primitive endoderm (Cai et al., 2008). No homozygous null E7.0 embryos were found in this investigation. Results from this study therefore suggest that Gata6 has a key role in the development of both the parietal and visceral endoderm or prior to specification of these lineages (Cai et al., 2008). Although the phenotype of these two mice is different, they both exhibit a failure in extraembryonic endoderm development, but disagree over for which stage it is important.

Further analysis of the role Gata6 plays in development has been carried out in embryoid bodies. Embryoid bodies produced from Gata6$^{-/-}$ ES cells do not express Afp, Hnf4α or Gata4, suggesting Gata6 is required for extraembryonic development (Morrisey et al., 1998). Formation of embryoid bodies from Gata6-null mES cells transfected with Gata4 causes expression of Disabled-2 (Dab2) protein which is an extra-embryonic endoderm marker (Cai et al., 2008). This suggests that in this circumstance Gata6 is not required for differentiation of ES cells in to visceral endoderm-like Dab2 positive cells, corroborating the observation that Gata6 is not expressed in the mature visceral endoderm (Cai et al., 2008). This demonstrates that Gata6 is required for specification of the primitive endoderm, and subsequently the parietal endoderm, but is not necessary for visceral endoderm development once the primitive endoderm is specified.

Increased expression of Gata6 or Gata4

To complement these loss of function studies, induced expression of either Gata6 or Gata4 in mES cells forces extraembryonic endoderm differentiation (Fujikura et al., 2002). Injection of dominant-negative Gata6 in to individual ICM cells causes ICM cells to preferentially contribute to the epiblast rather than the primitive endoderm suggesting that Gata6 maintains cells at the surface of the ICM (Meilhac et al., 2009). However, injection of Gata6 to cells of the deep ICM, did not change their localisation, therefore Gata6 is not sufficient to position cells of the ICM at the surface (Meilhac et al., 2009). Additionally, generating mosaic embryos with clones of cells with high levels of Gata6 had no affect on the progeny of the cell producing epiblast or primitive endoderm. But reducing it caused the cells to contribute more to the epiblast (Morris et al., 2010). Interestingly Gata6 and Gata4 are both induced when either Gata6 or Gata4 is overexpressed in mES cells, suggesting that they cross-regulate each other (Fujikura et al., 2002) (Figure 1.8).

Taking in to account all of the evidence from *in vivo* and *in vitro* studies, Gata6 and Gata4 function in early mouse embryonic differentiation is essential for extraembryonic endoderm development, and Gata6 is upstream of Gata4.

Sox 7 & 17

In addition to Gata6 and Gata4, the Sry-related HMG-box transcription factors Sox7 & 17 are required for specification of the primitive endoderm. Sox17 binds to, and activates a number of genes required for extraembryonic endoderm differentiation, such as Platelet derived growth factor receptor α (Pdgfrα), Fgfr2, basement membrane components, and reinforces

expression of Gata4 and Gata6 (Niakan et al., 2010). Sox17 has also be shown to inhibit transcription of pluripotency genes such as Sox2, Nanog and Oct4 (Niakan et al., 2010). Additionally, Sox17 may also have a role in migration of Gata4 and Gata6 to their correct localisation (Artus et al., 2011; Niakan et al., 2010). Gata4$^{-/-}$ embryoid bodies expressed Sox17, whilst Gata6$^{-/-}$ embryoid bodies lack Sox17 which suggests that initiation of Sox17 expression is upstream of Gata4 (Niakan et al., 2010).

In Sox17$^{-/-}$ embryos Sox7 expression is maintained, as shown by quantitative PCR (q-PCR) (Shimoda et al., 2007), and immunostaining (Artus et al., 2011). This suggests that Sox7 expression is not regulated by Sox17, and that Sox7 could compensate for loss of Sox17. Sox7 is expressed only in a subset of Pdgfrα-positive cells, at the 64-cell stage embryo, but only in cell of the primitive endoderm that are localised at the surface of the ICM. This suggests that Sox7 is a marker of cells which are committed to the primitive endoderm lineage, unlike Sox17, Gata6, and Gata4 (Artus et al., 2011). It also indicates that Sox7 is downstream of Gata6, Sox17 and Gata4. Silencing of Sox7 in embryonal carcinoma cell results in absence of Gata4 or Gata6 after differentiation with retinoic acid, and a loss of endodermal morphology (Futaki et al., 2004). This suggests that Sox7 has an important role in extraembryonic endoderm differentiation, and also that it is upstream of Gata4 or Gata6 which contradicts with the more recent findings of Artus et al. (2011).

Other transcription factors required for extra-embryonic endoderm development

There are many other important transcription factors required for mature extra-embryonic endoderm development.

Hnf-4α is expressed at E4.5 in the primitive endoderm, and is restricted to the visceral yolk sac from E5.5-8.5 (Duncan et al., 1994). During embryonic development, Hnf-4α is also present in the gut and nephrogenic tissue (Duncan et al., 1994). Hnf-4α null embryos die during development following retarded and abnormal gastrulation and cell death in the embryonic ectoderm (Chen et al., 1994). This phenotype can be rescued by addition of visceral endoderm from wild-type mice (Duncan et al., 1997). Embryoid bodies formed from Hnf4α null mES cells form primitive endoderm, confirmed by expression of Gata4, but they do not express proteins such as Afp which are known to be present in the visceral endoderm. This suggests that Hnf4α is essential for complete differentiation of the visceral endoderm but not the primitive endoderm (Duncan et al., 1997). Hnf4α can be regulated by Gata6, as in non-endodermal cells overexpression of Gata6 activates the Hnf4α promoter (Morrisey et al., 1998).

Afp was also used frequently in studies of visceral endoderm development before the identification of other markers. Using immunperoxidase and immunoprecipitation after radioactive labelling, it was identified that Afp is first detectable after E7 in the visceral endoderm and is not present in the parietal endoderm cells (Dziadek and Adamson, 1978).

Nanog

Similarly to the development of the trophoblast, Nanog has an important role in the specification of the primitive endoderm. When mES cells aggregate during embryoid body formation a downregulation in the pluripotency factor Nanog occurs in the primitive endoderm cells (Hamazaki et al., 2004). Induced overexpression of Nanog inhibits visceral endoderm differentiation in embryoid bodies (Hamazaki et al., 2004; Singh et al., 2007). Nanog can repress Gata6 expression directly by binding to the promoter region of the gene (Singh et al., 2007). It would therefore be expected that Nanog mutants have high expression of Gata4 and Gata6. Surprisingly, they do not express Gata4, but all cells of the ICM express Gata6 (Frankenberg et al., 2011; Messerschmidt and Kemler, 2010). Nanog mutant embryos can form primitive endoderm in mouse chimeras, the epiblast-derived tissues are formed by the wild-type cells, whilst the primitive endoderm derived cells are formed from the Nanog mutant cells (Messerschmidt and Kemler, 2010). These two studies therefore suggest that Nanog may have a non-cell autonomous role in primitive endoderm maintenance but not in its initial formation (Frankenberg et al., 2011; Messerschmidt and Kemler, 2010).

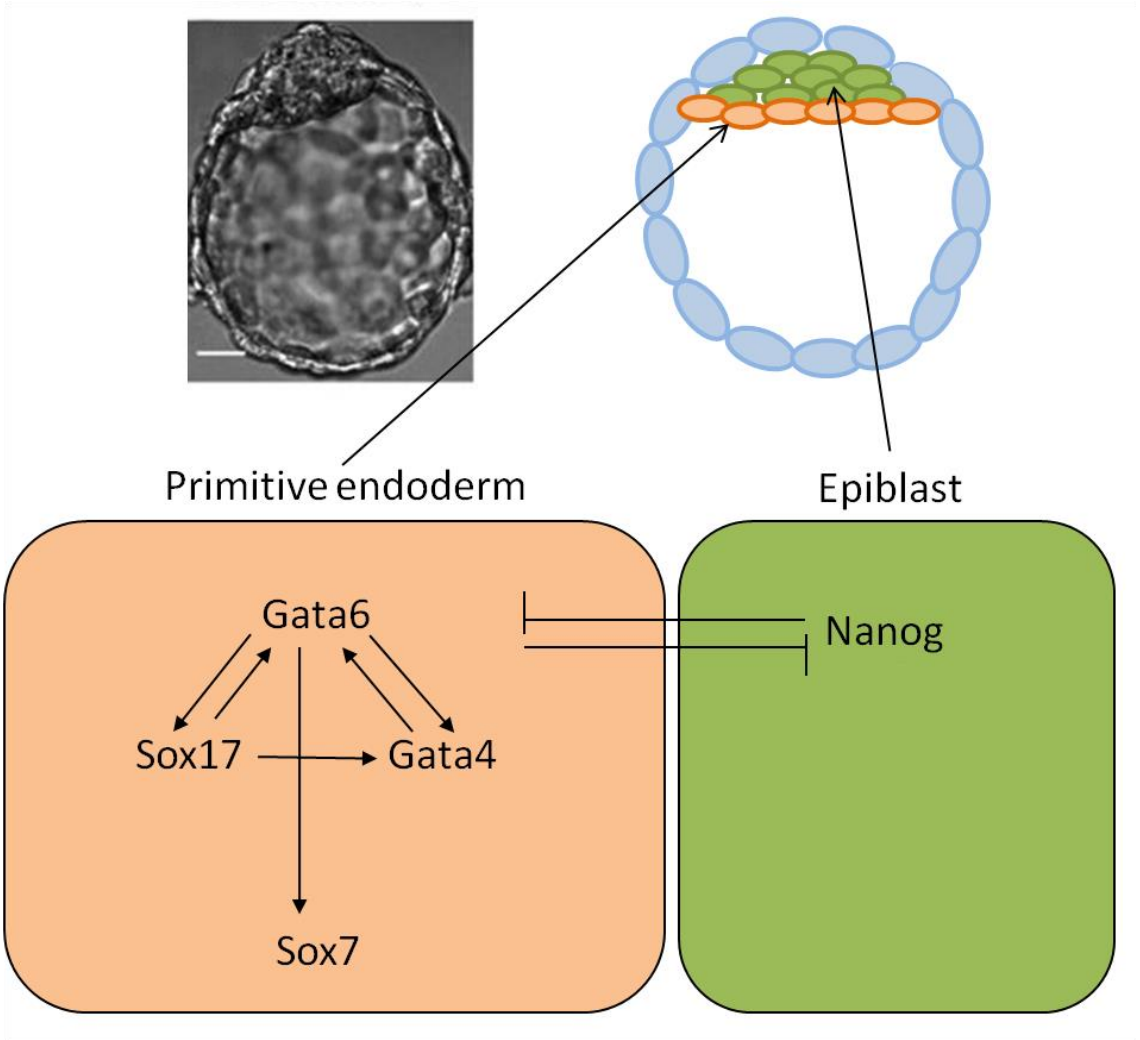


Figure 1.8: Transcription factors required for the specification of the ICM into the epiblast and the primitive endoderm.
The epiblast and the primitive endoderm are specified from the ICM in the second cell fate choice which occurs during embryonic development. The primitive endoderm forms the extraembryonic endoderm whilst the epiblast produces the embryo proper. The formation of the epiblast is predominantly dependent on the expression of the pluripotency transcription factors Nanog and Oct3/4. The fate specification of the primitive endoderm is regulated by four main transcription factors (Gata6, Sox17, Gata4 and Sox7). Gata6 is the first to be expressed, but is at first co-expressed in cells with Nanog. Gata4 and Sox17 are therefore the most specific early markers of the primitive endoderm. The pluripotency factors and Gata6 inhibit each other to prevent cells from changing fate once they are specified. Light microscopy image of the blastocyst is taken from (Saiz and Plusa, 2013).

1.4.4 Mechanisms of segregation of the primitive endoderm and epiblast

The mechanism used to segregate cells of the ICM into cells of the primitive endoderm and epiblast, and how these cells become positioned correctly in the embryo has been a central question in much of the research done in the field of primitive endoderm development.

Position-based model

Similarly to the trophoblast a very early model of primitive endoderm development was the position-based model. This hypothesis is based on observations that the primitive endoderm is present at the surface of the inner cell mass, and therefore it may be the location of these cells on the on the surface of the ICM, exposed to the blastocoel which causes them to become the primitive endoderm (Rossant, 1975). More recent studies have focussed on the asymmetric divisions from which the cells are derived. One study has shown that the asymmetric division from which the cells of the ICM are formed dictates whether it contributes to the epiblast or primitive endoderm (Morris et al., 2010). Conversely, an alternative study concluded using live cell tracing of mouse embryos from the eight-cell stage that there is no relationship between which asymmetric division the when the inner cells is generated and their cell fate commitment (Yamanaka et al., 2010). These studies therefore suggest that the position and origin of cells in the blasotcyst embryo may have a role in the primitive endoderm versus epiblast formation but that it is not very clear, or easy to detect.

Cell sorting

The alternative model of the mechanism of cell fate choice between epiblast and the primitive endoderm is that cells are specified to a cell fate and then sort into their compartments (Figure 1.9). Single-cell analysis of mRNA shows that many of the transcription factors that are restricted in the blastocyst are coexpressed in the 16-cell embryos (Guo et al., 2010). Until the 32-cell stage in mouse embryos the expression of Nanog and Gata6 protein overlaps in almost all cells (Plusa et al., 2008). At E3.5 Gata6 and Nanog are expressed in cells mainly in a mutually exclusive pattern with no bias to location within the ICM, thus creating a mosaic, salt-and-pepper expression pattern of these two transcription factors (Chazaud et al., 2006) (Figure 1.9). When traced, these cells give rise to cells of either the epiblast or primitive endoderm, but never both. This occurs even when single cells are removed and placed in different environments (Chazaud et al., 2006). The salt-and-pepper localisation of these transcription factors at the mRNA level has also been shown in single cell resolution (Guo et al., 2010). These results show that at this stage there is lineage segregation, but no positional restriction, suggesting that this second cell sorting model may be correct. Formation of the primitive endoderm as a segregated layer of Gata4-positive cells occurs in embryos between 80-100 cells (Plusa et al., 2008) (Figure 1.9).

Of the total number of primitive endoderm cells, 41% originate from the deeper ICM, suggesting that a lot of cell sorting is required in the development of the primitive endoderm

(Meilhac et al., 2009). Movement of cells in the blastocyst embryos was observed by Chazaud et al., 2006 (Chazaud et al., 2006). Time-lapse imaging has shown that all cells of the inner cell mass undergo movement, this movement can even involve a change in localisation to/from the ICM surface or deeper ICM (Meilhac et al., 2009). Cells expressing Pdgfrα (a primitive endoderm marker) exhibit a net movement towards the cavity, a primitive endoderm localisation (Meilhac et al., 2009). *In silico* results suggest that the best model is that position of the cell and cell sorting are involved in primitive endoderm development. This means that gene expression of a cell depends on its position, if these two components match the gene expression of the cell is less likely to change (Meilhac et al., 2009). This suggests that both positional and cell sorting mechanisms may play a role in the development of the primitive endoderm.

Potency of cells of the ICM in the blastocyst embryo

Subsequent studies have investigated the potency of the salt-and-pepper cells. Cells of the ICM in the early blastocyst embryo each have different potency (Grabarek et al., 2012). In the early ICM, a third of cells are bipotent whilst two thirds are restricted to either the primitive endoderm, or epiblast fate (Meilhac et al., 2009). This conclusion has been functionally reinforced by manipulating Fgf signalling which causes cells expressing either Gata6 or Nanog to change fate, demonstrating that the plasticity of cells remains until E4.0-E4.5 (Yamanaka et al., 2010). Therefore, until E4.0-E4.5 cells are progenitors but are not fully committed to their cell fate (Yamanaka et al., 2010). A more detailed study has shown that the loss of cell plasticity between the mid to late blastocyst stage, does not coincide with the appearance of mutually exclusive expression of Nanog and Gata6 (Grabarek et al., 2012). Instead, it is the exclusion of Oct4 which restricts the potency of cells of the ICM (Grabarek et al., 2012).

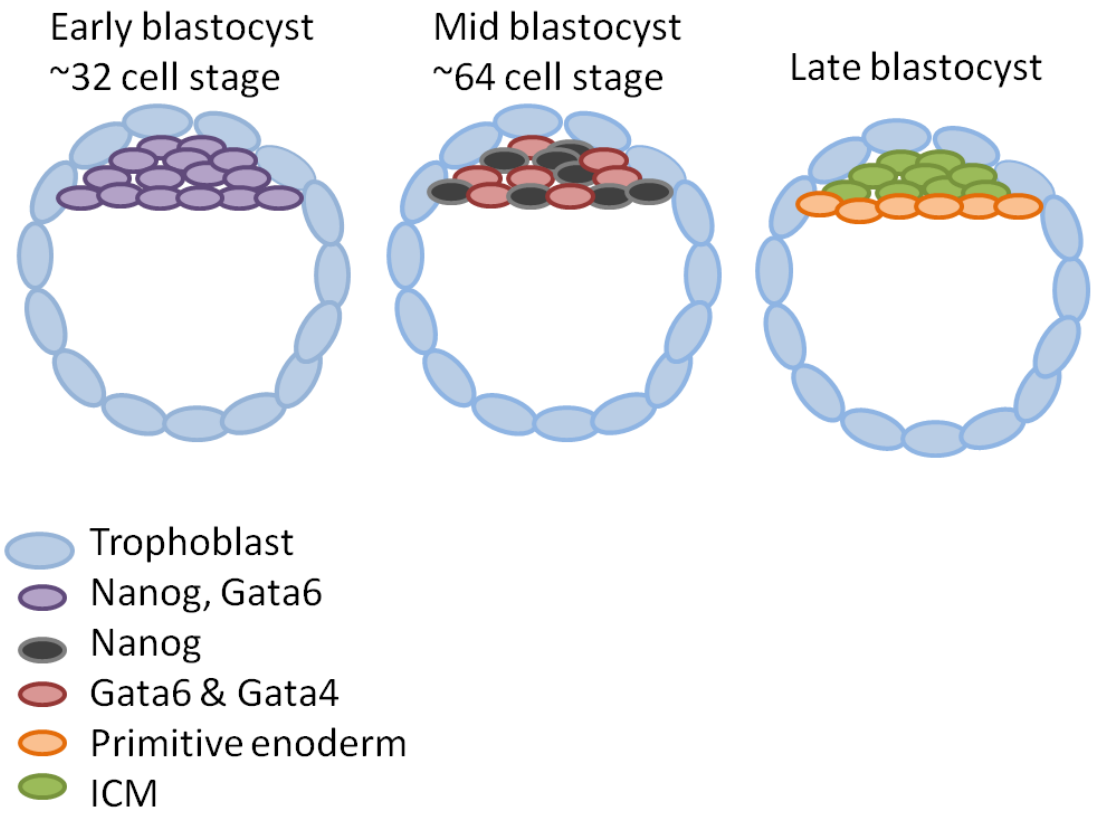


Figure 1.9 The pattern of expression of transcription factors during embryonic development provides a model of how the primitive endoderm and epiblast are specified
The expression pattern of transcription factors required for epiblast and primitive endoderm specification has been determined using immunostaining of mouse embryos and single-cell mRNA anlaysis (Chazaud et al., 2006; Guo et al., 2010; Plusa et al., 2008). In the early blastocyst stage embryo Nanog and Gata6 are expressed in all nuclei of the ICM. By the mid-blastocyst stage some cells express Gata6 and Gata4 whilst others express only Nanog. These cells are organised in a 'salt-and-pepper' pattern, meaning that both types are interdispersed throughout the ICM. The cells then sort, so that by the late-blastocyst stage the primitive endoderm Gata6, Gata4 positive cells line the blastocoel, whilst the Nanog positive epiblast cells localise between the primitive endoderm and the trophoblast on one side of the embryo. (Modified from (Saiz and Plusa, 2013).

1.4.5 Role for Fgfr/Erk signalling in primitive endoderm development

Fgfr/Erk signalling has an important role in the specification of the primitive endoderm lineage.

Fgfr/Erk signalling cascades

Fibroblast growth factors (Fgfs) are secreted glycoproteins which signal through Fgf receptors to regulate many developmental pathways (Turner and Grose, 2010). There are 18 known ligands of Fgf signalling, and 4 receptors (Fgfr1-4) (Turner and Grose, 2010). Fgf receptors are tyrosine kinase receptors which form dimers upon ligand binding, causing the activation of an

intracellular kinase. This leads to intermolecular transphosphorylation of the tyrosine kinase domains and intracellular tail of the Fgf receptor (Turner and Grose, 2010). The phosphorylated tyrosine residues function as docking sites for adaptors to initiate intracellular signalling. Erk, PI3k, Plcγ, p38 Mapk and Jun N-terminal kinase, Stat and protein S6 kinase 2 pathways are all signalling pathways downstream of Fgf receptor activation (Turner and Grose, 2010).

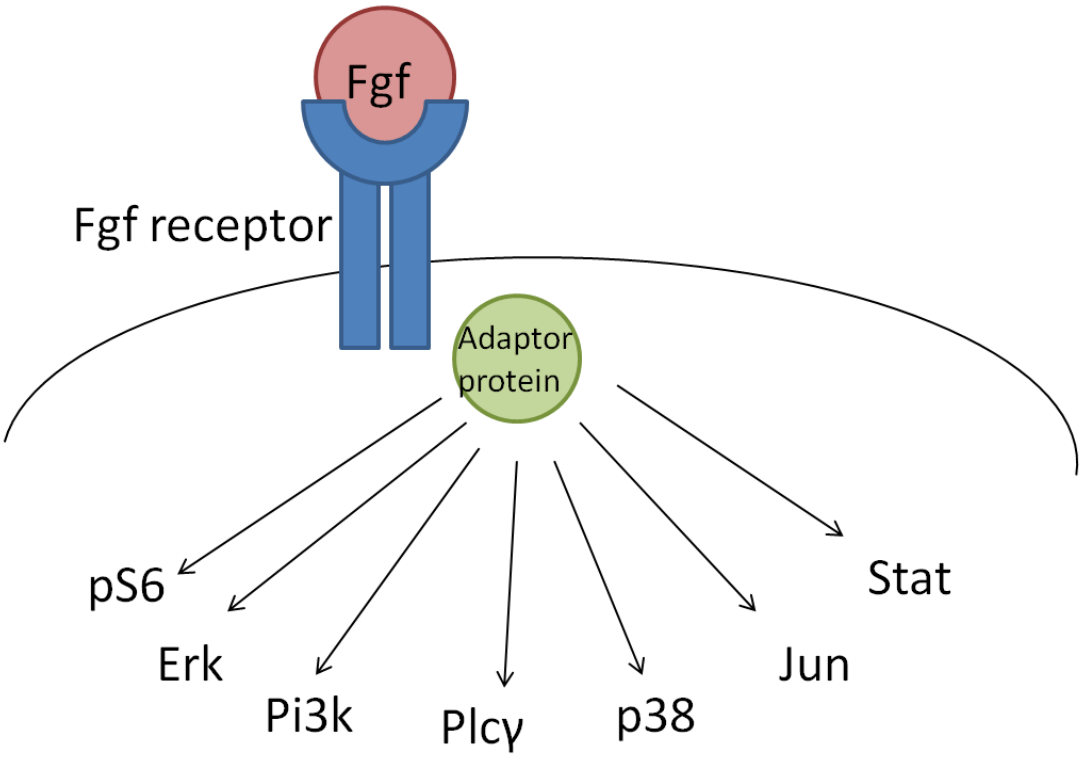


Figure 1.10 Fibroblast growth factor (Fgf) signalling cascade
The receptor tyrosine kinase Fgf receptor is activated by the binding of Fgf. This causes trans-phosphorylation of the tyrosine kinase domains of the receptor which allows the binding of adaptor proteins to the intracellular region of the Fgf receptor and a signalling cascade. There are many signalling cascades downstream of the Fgf receptor such as: pS6, Erk, Pi3K, Plcγ, p38, Jun and Stat.

Mammalian Mitogen-activated protein kinase (Mapk) signalling consists of 4 Mapk signalling pathways (Erk1/2, c-jun amino-terminal kinases, p38 kinases and Erk5) (Roberts and Der, 2007). These cascades consists of at least three levels of proteins, Mapk kinase kinase (Mapkk), a Mapk kinase, and a Mapk, each of which is activated by phosphorylation (Roberts and Der, 2007; Roskoski, 2012). For this study it is the Erk signalling cascade which is most relevant (Figure 1.11). The Erk pathway is activated by growth factor-stimulated cell surface receptors, Raf serine/threonine kinases are the Mapkk, this phosphorylates and activates the dual-specificity protein kinase Mek1/2 (Mapkk) (Roberts and Der, 2007; Roskoski, 2012). Mek1/2 phosphorylates and activates Erk1/2 Mapk which is a serine-threonine kinase that

phosphorylates and regulates the activity of many proteins (Roberts and Der, 2007; Roskoski, 2012).

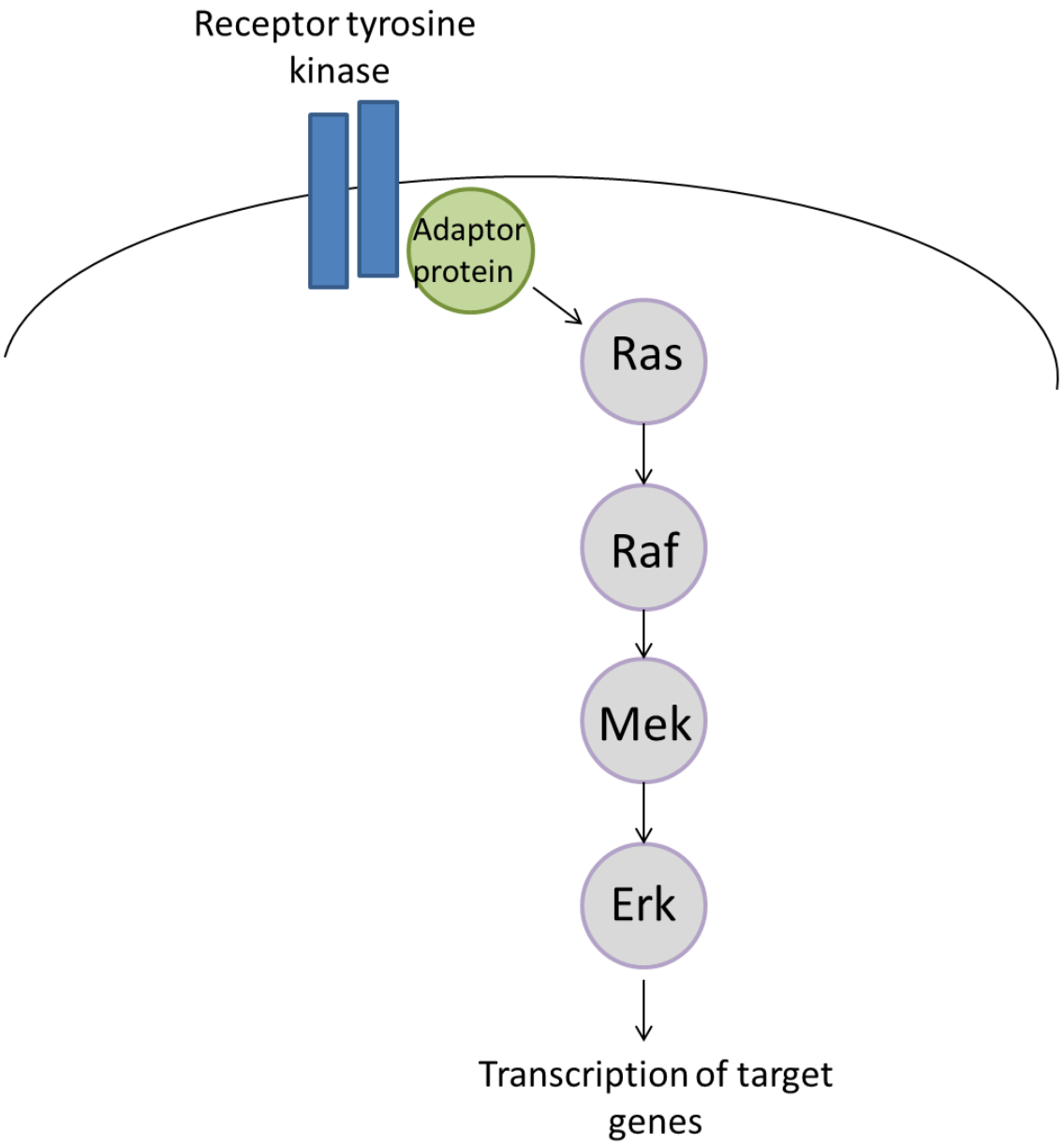


Figure 1.11 The Erk signalling cascade
The Erk signalling cascade is one of the pathways of Mitogen-activated protein kinase (Mapk) signalling. As shown here Erk signalling begins with receptor tyrosine kinase activation, this causes activation via Ras of Raf, and subsequently Mek1/2, and Erk1/2. Activated Erk1/2 can then phosphorylate and regulate the activity of many proteins, leading to the transcription of genes.

Evidence for a role of Fgfr signalling in primitive endoderm specification

Fgf4 is thought to be required for development of the primitive endoderm. Fgf4 is expressed in embryos from the 1-cell stage. In the blastocyst embryo it localises to the ICM but is not detected in the trophoblast (Rappolee et al., 1994). Treatment of embryos with Fgf4 and

heparin causes all cells of the ICM to become Gata6 positive (Rappolee et al., 1994; Yamanaka et al., 2010), and can rescue primitive endoderm development, demonstrated by Sox17 and Gata4 expression in Nanog mutants (Frankenberg et al., 2011). Fewer $Fgf4^{-/-}$ mES cells undergo differentiation upon addition of retinoic acid, and many die suggesting a deficiency either in proliferation and/or survival of the differentiated cells, addition of recombinant Fgf4 rescues this phenotype (Wilder et al., 1997). Fgf4 null embryos are embryonic lethal after implantation, (Feldman et al., 1995), they lack expression of Hnf4α in the primitive endoderm, suggesting a key role for Fgf4 in primitive endoderm development (Goldin and Papaioannou, 2003). More detailed analysis of $Fgf4^{-/-}$ embryos shows that Gata6 and Nanog colocalise until the 32-cell stage as is seen in the wild-type embryo, but by the 64-cell stage only a small number of weakly Gata6 positive cells can be detected, whilst Gata4 was never detected (Kang et al., 2013). This mutant cannot be rescued by addition of exogenous Fgf at a uniform concentration suggesting *in vivo* it is regional differences in Fgf concentration which produce the salt-and-pepper pattern. This suggests that Fgf4 signalling is not required for initial expression of Gata6 but is required after the 32-cell stage in its maintenance and/or formation of the salt and pepper pattern. It either directly or as a consequence of loss of Gata6 regulates Gata4 expression and primitive endoderm specification.

Fgf4 activates the Fgf receptor during primitive endoderm development. Both Fgfr1 (previously known as Bek), and Fgfr2 (previously known as Kgfr together with Flg) are expressed in the ICM of blastocyst embryos (Orr-Urtreger et al., 1991). As Fgf-4 is also expressed in the cells of the ICM, this suggests that Fgfr signalling after activation by Fgf4 may have a role in the development of the inner cell mass (Niswander and Martin, 1992). $Fgfr1^{-/-}$ mice are embryonic lethal prior to or during gastrulation, and display a ruffled extraembryonic endoderm (Deng et al., 1994; Yamaguchi et al., 1994). Fgfr2-/- mice also die a few hours after implantation with a collapsed yolk cavity, there is no distinguishable primitive endoderm in the blastocyst embryo *in vivo* or when the embryos are cultured *in vitro* (Arman et al., 1998). Embryoid bodies made from mES cells with a dominant negative Fgfr2 (dnFgfr2) do not form Hnf4α or Afp expressing visceral endoderm on the outside of the embryoid body, and do not cavitate (Chen et al., 2000). Embryoid bodies made with $Fgfr1^{-/-}$ mES cells also do not express Afp (Esner et al., 2002). Fgfr2 activation in mES cells downregulates Nanog gene transcription through activation of the Mek pathway, forcing their differentiation to the primitive endoderm cell fate (Santostefano et al., 2012).

Evidence for a key role for Fgf4 and Fgfr2 in the correct development of the inner cell mass is reinforced by single-cell expression data showing that the strongest inverse correlation within the cells of the ICM is between Fgfr2 and Fgf4 (Guo et al., 2010). This is caused by a decrease in Fgfr2, and an increase of Fgf4 in a subpopulation of 32-cell ICM cells. The other cells of the ICM have a high level of Fgfr2 and a low level of Fgf4 as is seen in all cells at the 16-cell stage. In conclusion this evidence suggests that Fgf4 signals via Fgfr1 and/or Fgfr2 in an essential role during primitive endoderm development.

Erk signalling is required for primitive endoderm specification

The adaptor protein Grb2, which is required for receptor tyrosine kinase signalling has been shown to be required for primitive endoderm development (Cheng et al., 1998). A more detailed analysis of the $Grb2^{-/-}$ mouse phenotype demonstrated that Gata6 expression is lost and all ICM cells are Nanog positive (Chazaud et al., 2006). Accordingly, when induced to undergo endoderm differentiation, $Grb2^{-/-}$ mES cells which are transfected with Gata6 or Gata4 are able to form primitive endoderm in embryoid bodies, suggesting that Grb2 signalling acts upstream of Gata6 and Gata4 (Wang et al., 2011). Interestingly, forced expression of H-Ras in Grb2-deficient mES cells promotes endoderm differentiation in embryoid bodies, suggesting Grb2 functions through activation of Ras in this context (Cheng et al., 1998).

One of the downstream signalling pathways of Ras is the Erk signalling cascade. There is strong evidence to suggest that Erk signalling is required for primitive endoderm development. Embryoid bodies formed from mES cells transfected with a constitutively active Mek mutant have repressed Nanog expression, and primitive endoderm differentiation occurs (Hamazaki et al., 2006). This suggests that the Erk signalling pathway mediates Nanog repression, which is known to be required for differentiation of ICM cells into primitive endoderm and is known to be affected by Fgfr signalling. Additionally, embryoid bodies formed from dominant negative Ras mutant mES cells have reduced expression of Gata6, Gata4 and Afp (Yoshida-Koide et al., 2004). Of three Ras mutants which selectively activated either the RalGef/Ral, Pi3k/Akt or Raf/Mek/Erk, only the Ras mutant which could activate the Raf/Mek/Erk signalling cascade could induce expression of the extraembryonic genes (Yoshida-Koide et al., 2004). This suggests that Ras signalling effects extraembryonic endoderm differentiation through the Erk signalling cascade. This was confirmed by an experiment when embryoid bodies were cultured with 10µM of the Mek inhibitor U0126 and a significant decrease in expression of extraembryonic endoderm markers was observed (Yoshida-Koide et al., 2004).

This therefore led to the hypothesis that the mosaic expression of epiblast and primitive endoderm markers is dependent upon an Fgfr/Grb2-Ras-Mek/Erk signalling cascade. Subsequent work culturing mouse embryos with both Fgfr and Mek inhibitors has supported this hypothesis. Treatment of embryos with inhibitors of the Fgf receptor and Mek (100nM PD173074 & 0.5uM PD0325901 Yamanaka et al. (2010), or 0.8µM PD184352 and 2µM SU5402 or 1µM PD0325901 and 100nM PD173074 Nichols et al. (2009)) causes ICM cells to express Nanog whilst repressing primitive endoderm markers Gata6 & 4 (Nichols et al., 2009; Yamanaka et al., 2010). No effect was seen on the total number of cells of the ICM, therefore the phenotype is caused by a cell fate specification reassignment rather than loss of the primitive endoderm lineage cells (Nichols et al., 2009). When embryos are treated with both inhibitors from the 8-cell stage to the late blastocyst they show the cell fate defect, however, if the embryos are treated with both inhibitors from the 8-16 cell stage only until the early blastocyst stage and then replaced in control media they have no cell fate phenotype (Yamanaka et al., 2010). Additionally, if embryos are treated from the early blastocyst stage they show an upregulation of Nanog and downregulation of Gata6 (Yamanaka et al., 2010). This suggests that the Fgfr/Erk signalling cascade is essential for regulating the primitive endoderm and epiblast cell fate after the early blastocyst stage.

1.4.6 Other signalling pathways involved in development of the primitive endoderm

In addition to a requirement for Fgfr/Erk signalling in the development of the primitive endoderm there is evidence for the activity of other signalling pathways.

Platelet derived growth factor (Pdgf)

Platelet derived growth factor (Pdgf) exists as a homodimer or heterodimer and binds to the Pdgfr (Wang and Song, 1996). Pdgfr is a receptor tyrosine kinase which also exists as either a homodimer or a heterodimer of two subunits, α and β. Pdgfr is known to stimulate many signalling events following activation, three of them are Ras-Mapk, Pi3k and Plcγ signalling (Andrae et al., 2008). Expression of Pdgfrα in F9 embryonal carcinoma cells in the developing parietal endoderm of embryoid bodies is regulated by binding of Gata4 to an enhancer within the Pdfgrα promoter, suggesting that this signalling process may be involved in primitive endoderm development. Gata6 is also required for the activation of Pdgfrα expression, whilst more recent evidence suggests that Gata4 and Gata6 are required for its maintenance (Artus et al., 2010). Pdgfrα has been identified and used as an early marker of the primitive endoderm lineage (Plusa et al., 2008).

Pdgf signalling has a functional role in primitive endoderm development (Artus et al., 2010). Inhibition of Pdgf results in XEN cells (Extraembryonic endoderm stem cells) exiting the cell cycle, resulting in reduced proliferation, suggesting that Pdgf signalling is required as a mitogen for XEN cells via Mek and Pkc signalling (Artus et al., 2010). It is unsurprising therefore that Pdgf is required for the expansion/maintenance of the primitive endoderm *in vivo.* Embryos lacking Pdgf signalling also have increased caspase-dependent apoptosis, suggesting that it is required for the survival of primitive endoderm cells in the ICM (Artus et al., 2013). Pdgf signalling does not however affect cell sorting or lineage specification within the ICM.

Pi3k signalling

Phosphatidyl inositol 3-kinase (Pi3k) signalling was suggested to be the downstream signalling pathway from the Fgf receptor (Chen et al., 2000). This was suggested because embryoid bodies made from mES cells with a dominant negative form of the Fgf receptor did not have altered ppERK levels in comparison to wild-type, whilst Akt was activated 4-10 fold more than wild-type embryoid body. Additionally, inhibition of Pi3k signalling using 10mM LY294002 almost completely inhibited embryoid body differentiation but this is a very high concentration of LY294002, and therefore could be due to an off target or toxic effect of the compound (See 5.3.6). Synthesis and localisation of Laminin-1 and Collagen IV for the basement membrane of the primitive endoderm in embryoid bodies requires Akt, a downstream component of Pi3k signalling following activation of the Fgf receptor (Li et al., 2001b). Inhibition of Pi3k signalling has been shown to have no effect on the downregulation of Nanog which occurs in the outer layer of cells during embryoid body formation (Hamazaki et al., 2006). This suggests that Pi3k does not regulate Nanog during primitive endoderm cell fate specification. In conclusion, evidence suggests there is a role for Pi3k signalling in primitive endoderm development but further work is required to full understand it.

1.4.7 Polarisation in the development of the primitive endoderm

As outlined above, there has been a lot of work investigating the mechanisms regulating primitive endoderm cell fate specification. As the primitive endoderm is an epithelium, the cells are apico-basolaterally polarised. Some work has been done to investigate the role this polarisation may have in the development of the primitive endoderm.

E-cadherin

E-cadherin is polarised in cells of the primitive endoderm (Moore et al., 2009). Mutation in a sequence essential for Ca^{2+} binding of E-cadherin, is embryonic lethal after the morula stage

and embryos are not capable of forming normal blastocysts (Riethmacher et al., 1995). Surprisingly, E-cad$^{-/-}$ mES cells form embryoid bodies, and undergo normal primitive endoderm differentiation, but cavitation does not occur (Rula et al., 2007). In chimeric embryoid bodies, E-cadherin null embryonal carcinoma cells predominantly migrate to the outer layer, whilst wild-type cells form the inner cells, therefore sorting according to their adhesion (Moore et al., 2009). However, when wild-type cells are differentiated (with retinoic acid) and chimeric embryoid bodies are formed with E-cadherin null embryonal carcinoma cells, the E-cadherin positive primitive endoderm like cells form the outer layer, and the E-cadherin null embryonic carcinoma cells form the inner cells (Moore et al., 2009). This suggests that in this experiment it is the differentiation state of the embryonal carcinoma cells which controls the sorting rather than their adhesion. This proposes that the adhesive state of cells can cause their sorting but that *in vivo* it may be the fact that the primitive endoderm progenitor cells have started to differentiate which controls their positioning rather than a difference in adhesion of the cells.

Cdc42 and aPkc

Cdc42 is a protein present in the Par polarity complex, embryoid bodies formed from Cdc42$^{-/-}$ mES cells have Afp positive endodermal cells but they do not form a pseduostratified columnar epithelium, and the embryoid body does not form a cavity (Wu et al., 2007). A normal basement membrane is however present, alongside polarised α6 integrin distribution (Wu et al., 2007). The tight junction protein Zo-1 is expressed at reduced levels and electron microscopy demonstrates that between most cells the tight junctions are absent, additionally aPkc is not polarised (Wu et al., 2007). This suggests that Cdc42 is required for the polarisation of the extraembryonic endoderm but not basement membrane formation or its cell fate specification. A reduction in phosphorylated aPkc suggests that in the Cdc42 mutant embryoid bodies it is the lack of active aPkc which induces the phenotype observed (Wu et al., 2007).

Study of the role of aPkc in embryos also shows an important role for aPkc in primitive endoderm development although the phenotype is not the same as that observed in Cdc42 null embryoid bodies. aPkc localises to cytoplasm, nuclei and cell contacts of cells within the early blastocyst (Saiz et al., 2013). In mid and late blastocyst embryos aPkc levels are heterogeneous within the ICM, being expressed highly in cells which are positive for Gata4. Embryoid bodies made from mES cells with a dominant negative aPkcζ show a loss of polarisation demonstrated by incomplete tight junctions, and adherens junction belts (Wu et al., 2007). Inhibition of aPkc in embryos causes primitive endoderm cells to fail to become anchored at the blastocoel cavity, suggesting a requirement for aPkc in the sorting of the

primitive endoderm (Saiz et al., 2013). No effect is observed on early primitive endoderm markers (eg. Pdgfrα), but later there is an appearance of cells which lack both Nanog and Gata4 which is never observed in normal embryos (Saiz et al., 2013). A disruption in the apical domain of the primitive endoderm is also observed , shown by disruption of Lrp2 (megalin), and Dab2 (Saiz et al., 2013). This suggests that in addition to being essential for sorting, aPkc is also required for maturation of the primitive endoderm cell fate.

Lrp2 (megalin)

Lrp2 is a low-density lipoprotein receptor-related protein (Lrp), which is also known as megalin. Its localisation becomes polarised during primitive endoderm development. It is expressed at very low levels in the 8-cell morula stage but is upregulated in E3.5 and E4.5 embryos (Gerbe et al., 2008). In E3.5 embryos Lrp2 is homogeneously distributed in the cytoplasm of Nanog negative cells of the ICM contributing to the salt-and-pepper pattern. Expression of Lrp2 gradually increases and polarises once the cells reach the ICM surface, in E4.5 embryos it localises to the apical side of primitive endoderm cells (Gerbe et al., 2008). As was observed with expression of aPkc, localisation of Lrp2 suggests that prior to cell sorting the cells prepare for epithelialisation but proteins do not have a polarised localisation until they reach the ICM surface where they form an epithelium.

Dab2

Dab2 is a cargo-selective adaptor protein which mediates clathrin-coated vesicle endocytosis. Like Lrp2 it is expressed from E4.5 in the apical of visceral and parietal endoderm cells (Gerbe et al., 2008; Morrisey et al., 2000; Yang et al., 2002). $Dab2^{-/-}$ mice have disrupted development following implantation at E5.5, and the embryos die at E6.5 (Yang et al., 2002). The $Dab2^{-/-}$ mice have Gata4 positive cells but they are disorganised, the mice die due to a defect in visceral endoderm organisation as well as a failure in cell proliferation (Yang et al., 2002). The role for Dab2 in endoderm sorting was confirmed in chimeric embryoid bodies formed from Dab2-suppressed and wild-type F9 embryonal carcinoma cells. The Dab2-suppressed cells localise to the inside of the sphere whilst the wild-type cells localised to the outer layer (Rula et al., 2007). Some of the cells of the ICM of the $Dab2^{-/-}$ mice express Gata4, but they also have Laminin-positive cells, suggesting that the endoderm differentiation does occur but the positioning and epithelial organisation of the cells does not occur (Yang et al., 2007). Both E-cadherin and Lrp2 lose their polarised localisation in $Dab2^{-/-}$ mice, and endodcytic vesicles containing E-cadherin can also be seen, this suggests that Dab2 is required for the localisation and polarisation of extraembryonic endoderm cells (Yang et al., 2007). Interestingly Dab2 is directly activated by Gata6, but not Gata4 (Morrisey et al., 2000). These studies therefore

suggest a requirement for polarisation of the visceral endoderm in its development, which regulates extraembryonic endodermal cell positioning.

Basement membrane

Laminin is a component of the basement membrane, its role in primitive endoderm development has been well studied. In embryoid bodies made from F9 embryonic carcinoma cells addition of exogenous Laminin prevents accumulation of a basement membrane, disrupts the organisation of the epithelium, and prevents expression of Afp (Grover et al., 1983). Treatment with an anti-Laminin antibody also causes a failure in the organisation of the epithelium suggesting that levels of Laminin within the basement membrane are important to organise the epithelium of the primitive endoderm (Grover et al., 1983). Embryoid bodies made with Laminin $\gamma1^{-/-}$ mES cells do not have a basement membrane, and do not cavitate (Murray and Edgar, 2000). These embryoid bodies do however have visceral and parietal endoderm cells, but do not form an organised epithelium on the outside of the embryoid body (Murray and Edgar, 2001). This suggests that Laminin and/or the basement membrane is essential for cavitation and extraembryonic endoderm organisation in embryoid bodies but not cell differentiation. This is the opposite conclusion to that obtained from analysis of $Dab2^{-/-}$, suggesting a role for polarised proteins other than Laminin in the differentiation of the primitive endoderm. The phenotype observed in the *in vitro* studies outlined above is reinforced by study of Laminin $\gamma1^{-/-}$ mouse embryos. These embryos die just after E5.5, they have primitive endoderm cells but they remain in the ICM (Smyth et al., 1999). The expression of Laminin-1 is controlled by Gata6 induced by Fgf (Li et al., 2004). These studies all suggest that Laminin may be required for organisation of the endodermal epithelium and cavitation. Collagen-IV is also a component of the basement membrane. In E3.75 embryos it is expressed in cells within the ICM in a salt-and-pepper manner suggesting that prior to cell sorting the cells express basement membrane components but their localisation is not polarised (Gerbe et al., 2008).

β1-integrins are cell-surface receptors which connect the cell to the basement membrane. β1-integrin homozygous null embryos die just after implantation (Fässler and Meyer, 1995). Analysis of β1-integrin null nice show a retarded growth of the ICM and although Laminin positive cells are present they form clumps instead of a monolayer (Stephens et al., 1995). More detailed investigation of the role of β1-integrin in endoderm differentiation has been done in embryoid bodies made from β1-integrin mutant mES cells. No continuous layer of endoderm forms on the periphery of the embryoid body, instead clusters of cells are observed (Liu et al., 2009). No difference in expression levels of Gata4 or Afp are detected, but a large

reduction in expression of basement membrane components and Dab2 is seen (Liu et al., 2009). This agrees with the conclusions formed from studies on Laminin, and suggests that the basement membrane is essential for cell sorting but not for cell fate specification.

In conclusion, proteins which are apico-basolaterally polarised in the primitive endoderm seem to have an important role in the organisation of the primitive endoderm epithelium. However, it has not been investigated in depth what relationship this has to other mechanisms, such as cell fate specification, known to be required for primitive endoderm development.

1.5 Aims

The goal of this thesis is to investigate the relationship between polarisation and cell fate specification in preimplantation epithelia. Specifically there are three aims:

1. Establish if BMP4 induced trophoblast differentiation in mES cells can be used as a model of polarisation and cell fate specification.

2. Establish if the development of the primitive endoderm in embryoid bodies can be used as model of preimplantation epithelial polarisation and cell fate specification.

3. Use the most appropriate model to investigate the role of cell signalling in polarisation and cell fate specification of preimplantation epithelia.

Chapter 2

2 Materials and Methods

2.1 mES cell culture

2.1.1 Materials

Table 2.1: Materials required for routine mES cell culture, culture in N2B27 media, and methylcellulose and hanging drop embryoid body formation.

Product	Supplier	Catalogue Number
Media		
Dulbecco's Modified Eagles Medium(DMEM)	Invitrogen	41965039
Knockout DMEM	Invitrogen	10829018
Glasgow Minimum Essential Medium (GMEM)	Invitrogen	21710025
DMEM/F12	Invitrogen	21331046
Neurobasal media	Invitrogen	21103049
Iscove's Modified Dulbecco's Media (IMDM)	Invitrogen	21980032
ES-Cult	Stem Cell Technologies	M3120
Serum		
Fetal Bovine Serum (FBS, ES Cell Screened HyClone	ThermoScientific	SH30070.03E
FBS (used for embryoid body formation)	Invitrogen	16000-044
Knockout Serum replacement	Invitrogen	10828-028
Media supplements		
2-Mercaptoethanol	Biorad	161-0710
Glutamine	Invitrogen	25030-024
100x Non-essential amino acids	Invitrogen	11140-035
Pyruvate	Invitrogen	11360-039
N2 supplement (100x)	Invitrogen	17502048
B27 supplement (50x)	Invitrogen	17504044
Monothioglycerol (MTG)	Sigma	M6145
Bovine serum albumin (BSA) Fraction V	Invitrogen	V15260
Holo-Transferrin	Sigma	T0665
L-Ascorbic Acid	Sigma	A4544
Insulin	Sigma	I2767
Cell culture plasticware		
92mm x17mm tissue culture dish	Nunc	150350
58mm x 15mm tissue culture dish	Nunc	150288
6-well dishes	Nunc	140685
PET membrane Transwell insert	Corning	3470
30mm Petri Dish Triple Vent	Sterilin	121V
6-well ultra low-adherent plates	Corning	3471
Cytokines		
BMP4	Stemgent	03-0007
BMP4	R&D diagnostics	314-BP-010
ESGRO (LIF)	Chemicon	ESG1106
Other		

Porcine Gelatin	Sigma	G1890
Plasma Fibronectin	Invitrogen	33010018
Urea	Sigma	U5378
Matrigel	Becton Dickinson	354277
0.05% Trypsin-EDTA	Invitrogen	25300-062
10x Phosphate buffered saline (PBS)	Invitrogen	14200-067
Sterile water	Baxter	UKF7114
Dimethyl dulfoxide (DMSO)	Sigma	D2650
PD-0325901	Axon MedChem	Axon 1408
AZD-4547	Santa Cruz	Sc-364421
PD-173074	Selleckchem	S1264
1m	School of Chemistry and Astbury Centre for Structural Molecular Biology, University of Leeds	Gift

Cell Culture Media

Table 2.2: Media recipes required for routine mES cell culture, culture in N2B27 media, and methylcellulose and hanging drop embryoid body formation.

Cell culture media	Media constituents
Knock-out DMEM media	Knockout DMEM 15% (vol/vol) Knockout serum replacement 2mM L-glutamine 0.1mM 2-mercaptoepthanol 0.1mM Non-essential amino acids
mES cell Freezing Media	Media A GMEM 0.1mM Non-essential amino acids 2mM L-glutamine 0.1mM 2-mercaptoethanol 1mM Pyruvate Media B Media A 20% DMSO
N2B27 Media	1:1 mixture of Media A and Media B Media A DMEM/F12 N2 supplement 50µg/ml BSA fraction V 2mM Glutamine Monothiolglycerol Media B Neurobasal media B27 supplement
Methylcellulose Embryoid Body Media	40% ES-cult 15% Fetal Bovine Serum 200µg/ml Holo-transferrin 50µM 2-Mercaptoethanol 50µg/ml L-Ascorbic acid 10µg/ml Insulin 3.5% IMDM
Hanging Drop Embryoid Body Media	DMEM 20% FBS 50µM 2-mercaptoethanol

2.1.2 Routine maintienance of mES cells

The E14tg2A mES cell line (Clone R63) was a kind gift of Dr Owen Witte, UCLA, California (Era and Witte, 2000). mESCs were routinely cultured on dishes coated in 0.1% (wt/vol) porcine gelatin in Knockout DMEM media supplemented 10^3 units/ml murine LIIF. Occasionally, cells were cultured with 1i, addition of 2µM 1m (GSK3β inhibitor (Bone et al., 2009)), to maintain

the pluripotency of the cells. For routine cell passage cells were trypsined in 0.05% trypsin-EDTA for five minutes. Trypsin was subsequently neutralised with media, cells were then centrifuged at 1000rpm for 5 minutes. Cells were counted using a Neubauer Haemocytometer, the re-suspended cells were plated at a density of 500,000 cells per 10 cm dish. Cell passaging was usually performed every other day. Alternatively, when cultured for 3 days cells were plated at a density of 200,000 cells per 10cm dish or equivalent.

2.1.3 mES cell freezing and thawing

In order to freeze cells, they were trypsinised as outlined above and then resuspended in ice cold media A at a density of $1x10^6$ cells/ml. Ice cold media B was then added in a drop-wise fashion to produce a cell density of $1x10^6$ cells/ml. 1ml of the cell suspension was frozen in 1ml vials overnight in the -80^{o}C freezer, and subsequently frozen in liquid nitrogen for long-term storage.

Thawing of mES cells was done as quickly as possible. 9mls of Knockout DMEM media was added to the 1ml of frozen cells and centrifuged at 1000rpm for 5 minutes. Each vial containing $1x10^6$ cells was subsequently resuspended in 10mls media and plated on a 92mmx17mm cell culture dish.

2.1.4 Culture of cells in N2B27 media

mES cells were trypsinised as previously described, but resuspended in N2B27 media following centrifugation. They were then plated onto 0.1% gelatin-coated (unless otherwise stated) 0.4µm pore size, 6.5mm diameter membrane PET membrane Transwell inserts, or 6-well plates in N2B27 media at the relevant density (usually 12,000 cells/filter). Alternatively 1% gelatin, 0.05mg/ml bovine plasma Fibronectin with addition of 0.08M Urea or Matrigel were used to coat the Transwell inserts. The media was then supplemented with 10ng/ml BMP4, 10ng/ml BMP4 and LIF or N2B27 media alone as described in results section. Cells were cultured for 4-5 days as stated. Light microscopy images were taken using the AMG Evos microscope.

2.1.5 Formation of embryoid bodies

For methylcellulose embryoid body formation, cells were tryspinised in 0.05% Trypsin-EDTA and resuspended at a density of $1x10^5$/ml in IMDM. The cell suspension was added to the methylcellulose embryoid body media, vortexed, and allowed to stand for 5-10 minutes.

Subsequently, 2ml media was added to each 30mm dish. Two 30mm dishes containing media and one containing water with no lid were placed in a 10mm dish and incubated for the desired duration.

For hanging drop embryoid body formation, cells were tryspinised in 0.05% Trypsin-EDTA and resuspended at a density of 40,000 cells/ml in hanging drop embryoid body media. Inhibitors were added if needed. Small-molecule inhibitors were added to the cell suspension at concentrations indicated in results chapters; 0.5µM-4µM PD-0325901, 1µM-8µM AZD-4547, 100nM PD-173074. Control embryoid bodies were formed from media containing the same DMSO percentage as used with the inhibitors. Drops of the cell suspension (25µl) containing 1000 cells each were pipetted on to the inside of the lid of a 10cm tissue culture dish. 10mls of PBS was placed in the bottom of the plate and the lid carefully placed back on. If cultured for longer than 5 days, on day 5 embryoid bodies were washed using PBS, put in fresh media containing inhibitors, if required, and plated on a ultra-low adherent dish. Images of unstained embryoid bodies were taken on a Leica MZFLIII microscope.

2.2 Immunocytochemistry

2.2.1 Materials

Table 2.3: Materials required for immunocytochemistry of cells and embryoid bodies as well as biotinylation of embryoid bodies.

Product	Supplier	Catalogue Number
Paraformaldehyde (PFA)	Sigma	P6148
Methanol	Sigma	65550
Tween	Sigma	P1379
Tween	Sigma	P7949
Triton	Sigma	T8787
BSA	Sigma	A4503
BSA	Sigma	A2153
PBS tablets	Oxoid	BR0014G
Mowiol	Calbiochem	475904
Glycerol	Sigma	G6279
Trizma	Sigma	T3253
Glass slides	SLS	MIC 3804
Coverslips No1 (11mm Dia)	SLS	MIC3302
Glass pipettes	VWR	612-1701
Fetal Bovine Serum	Lonza	14-801FH
EZ-Link Sulfo-NHS-LC-Biotin	Thermo Scientific	21335
Streptavidin, DyLight 488 Conjugated	Thermo Scientific	21832

Table 2.4: Primary antibodies used for Immunofluorescence and Western Blotting
Supplier and catalogue number of each antibody used. Dilution of each antibody used is shown. Abbreviations: IF = Immunofluorescence, WB = western blotting.

Antibody	Supplier	Catalogue Number	Species	Dilution
α-Tubulin	Sigma	T9026	Mouse	WB - 1:5000
β-catenin	Cell Signalling Technology	9562	Rabbit	IF – 1:100 WB - 1:5000
Cdx2	Biogenex	MU392A-UC	Mouse	IF 1:100
Cleaved Caspase-3	Cell Signalling Technology	9661	Rabbit	IF: 1:200
E-cadherin	Invitrogen	33-4000	Mouse	IF – 1:25 WB 1:1000
Eomesodermin	Abcam	Ab23345	Rabbit	IF – 1:100
Fibronectin	Sigma	F3648	Rabbit	IF – 1:250 WB – 1:1000
Glyceraldehyde 3-phosphate dehydrogenase (Gapdh)	Ambion	AM4300	Mouse	WB - 1:40,000
Gata4	Santa Cruz	Sc-9053	Rabbit	IF - 1:100 WB – 1:1000
Gata6	R&D systems	AF1700	Goat	IF – 1:100 WB – 1:1000
Hnf4α	Santa Cruz	Sc-8987	Rabbit	IF – 1:100
Laminin	Abcam	Ab11575-20	Rabbit	IF – 1:100
Nanog	eBioscience	14-5761	Rat	IF1:200
Occludin	Invitrogen	33-1500	Mouse	IF - 1:25
aPkc-ζ/λ	Santa Cruz Biotechnology	Sc-216	Rabbit	IF - 1:100 WB – 1:1000
Diphosphorylated Erk (ppErk)	Sigma	M9692	Mouse	WB 1:5000
Total Erk	Cell Signalling Technology	4695S	Rabbit	WB 1:5000
Zo-1	Invitrogen	33-9100	Mouse	IF – 1:25
Zo-1	Invitrogen	40-2200	Rabbit	WB 1:500

Table 2.5: Seconday antibodies used for Immunofluorescence

Product	Supplier	Catalogue Code	Dilution
Secondary antibodies			
Goat α-mouse IgG AlexFluor 488	Molecular Probes	A11001	1:500
Goat α-mouse IgG DyLight 546	Newmarket Biosciences	GtxMu-003-E2550NHSX	1:200
Rhodamine Red-C-AffiniPure Donkey α-mouse IgG	Jackson ImmunoResearch	711-295-150	1:500
Goat α-mouse IgG AlexFluor 488	Invitrogen	A11029	1:500
Goat α-rabbit IgG AlexFluor 488	Molecular Probes	A11008	1:500
Goat α-rabbit IgG AlexFluor 488	Invitrogen	A11070	1:500
Goat α-rabbit IgG AlexFluor 568	Molecular Probes	A11011	1:500
Rhodamine Red-C-AffiniPure Donkey α-Rabbit IgG	Jackson ImmunoResearch	711-295-152	1:500
Donkey α-goat IgG DyLight 633	Newmarket Biosciences	DkxGt-003-D633NHSX	1:200
Fluorescein Rabbit α-goat IgG	Vector laboratories	FI-5000	1:500
Goat α-rat IgG AlexFluor 488	Molecular probes	A11006	1:200
DAPI, dilactate	Sigma	D9564	1:1000

2.2.2 Buffers

Table 2.6: Recipes for buffers required for immunocytochemistry

Buffer	Buffer constituents
Mowiol	2.5g Mowiol 6g glycerol 6ml water 12ml 0.2M Tris (pH8.5)

2.2.3 Immunofluorescence staining of cells grown in N2B27 media

Cells were grown on Transwell inserts as outlined above for 4-5 days and were subsequently washed with PBS and fixed with 4% PFA for 1 – 1.5 hours. Cells were then washed in PBS and permeabilised in ice cold methanol for five minutes at -20˚C. They were subsequently washed three times in PBS and blocked in PBS containing 10% FBS (vol/vol) for 30 minutes. Cells were washed three times in 2% FBS in PBS (vol/vol). Primary antibodies (Table 2.4) were diluted in 2% FBS in PBS and incubated on cells for at least two hours at room temperature. Cells were then washed in 2% FBS in PBS five times for five minutes per wash, and were incubated with secondary antibodies (Table 2.5) diluted in 2% FBS in PBS for at least one hour at room

temperature. Cells were lastly washed five times for five minutes in PBS with 2% FBS. The inserts were then cut out and mounted in Mowiol with a coverslip. Staining was visualised using a Zeiss LSM 510 Meta confocal microscope.

Quantification of immunofluorescence was done by analysis of 10 fields of view across each insert. Areas of cells in a monolayer away from the colony were randomly chosen using the DAPI channel to avoid bias. Images within one experiment were taken using only the same settings on the confocal microscope and were processed using Photoshop CS2 (Adobe) with the same settings. The total number of cells was counted using the DAPI channel, and a percentage of Cdx2 positive cells calculated. Statistical significance was determined using a one-way analysis of variance (ANOVA) repeated measures with a Tukey's post-hoc test through GraphPad Prism5.

2.2.4 Immunofluorescence staining of embryoid bodies

At the appropriate time point, embryoid bodies were fixed with 4% (wt/vol) PFA in PBS for 1 hour and permeabilised in 0.25% triton (vol/vol) in PBS for 20 minutes. Embryoid bodies were washed 5 times in 0.1% tween (vol/vol) in PBS. Non-specific binding was blocked by 3% BSA (wt/vol) + 0.1% tween in PBS for 30 minutes. Primary (Table 2.4) and secondary (Table 2.5) antibodies were diluted in 3% BSA + 0.1% tween (vol/vol) in PBS. Embryoid bodies were incubated with primary antibodies for two hours at the concentration stated in Table 2.4, and with secondary antibodies for one hour. DAPI (1:1000) was added to the secondary antibody. Embryoid bodies were washed five times with 0.1% tween in PBS following all antibody incubations.

Stained embryoid bodies were mounted in Mowiol within the middle of two 13mm diameter stationery self-adhesive reinforcement rings stacked on top of each other, a coverslip was placed over the top. Immunostained embryoid bodies were examined on a Zeiss LSM510 Meta or a Leica SP5 laser-scanning confocal microscope. Image processing was done using Photoshop CS2 (Adobe) and Image J (NIH). Quantification was done by analysis of a field of view of at least three embryoid bodies per condition per experiment for at least three independent experiments. This gave at least 181 cells per experiment. Areas were randomly chosen using only the DAPI channel to avoid bias. Counts were done of surface layer cells only using the cell counter plugin in Image J (NIH). For statistical analysis a 1-way ANOVA with a Dunnetts post-hoc test, or a paired t-test were performed using GraphPad Prism 5.

2.2.5 Biotinylation

The biotinylation method was adapted from previous protocols (Chalmers et al., 2006; Minsuk and Keller, 1997). Embryoid bodies were washed in PBS, and incubated with 10mM EZ-Link Sulfo-NHS-LC-Biotin diluted in DMEM for 30 minutes at room temperature. Two washes in DMEM were performed followed by a wash in PBS. Embryoid bodies were then fixed in 4% PFA in PBS for one hour, permeabilised in 0.25% triton (vol/vol) in PBS, for 20 minutes and blocked for 30 minutes in 3% BSA (wt/vol) in 0.1% tween (vol/vol) in PBS. 1:100 DyLight 488 conjugated Streptavidin diluted in DMEM was added to embryoid bodies for two hours, they were subsequently washed five times with 0.1% tween in PBS. Embryoid bodies were mounted and imaged as stated in the immunofluorescence protocol of embryoid bodies.

2.3 Histology

2.3.1 Materials

Table 2.7: Materials required for gelatin-embedding histology, and haematoxylin and eosin (H&E) staining of embryoid bodies

Product	Supplier	Catalogue Number
Gelatin	Sigma	G2500
Sucrose	Fisher	S/8560/53
OCT	Raymond Lamb	Lamb/OCT
Ethanol	Sigma	32221
Hydrocholoric acid	Sigma	258148
Erlichs Haematoxylin	Raymond Lamb	Lamb/190d
Eosin	Raymond Lamb	Lamb/100d
Histoclear	Raymond Lamb	HS-200
DPX mountant for histology	Sigma	06522
Gelatin-coated microscope slides	Fisher	MNJ-800-010F

2.3.2 Gelatin Embedding and Cryosectioning

For histological analysis, embryoid bodies were fixed in 4% (wt/vol) PFA for 1 hour at room temperature and washed twice in PBS. Gelatin embedding was then carried out as previously described (Stern and Holland). Embryoid bodies were put sequentially into 5% sucrose/PBS and then 20% sucrose/PBS at 4˚C until they sank. Embryoid bodies were placed in pre-warmed 7.5% gelatin in 15% sucrose/PBS at 38˚C to infiltrate until they sank. Lastly, embryoid bodies were transferred from 7.5% gelatin in 15% sucrose/PBS to a plastic mold, they were then left to set at room temperature. Embryoid body-gelatin molds were sometimes stored in the fridge (4˚C) covered with cling film, or in the -80^{o}C freezer. Alternatively, embryoid body-gelatin blocks were removed from the molds by freezing them on dry ice and were used the same day for cryosectioning. Embryoid body-gelatin blocks were mounted onto cryostat chucks with OCT and sectioned into 5µm slices at -22^{o}C using a Leica CM1850 cryostat.

2.3.3 Haematoxylin and Eosin (H&E) staining

For H&E staining slides were removed from the -80^{o}C freezer and left at room temperature for 10 min. To remove gelatin, the slides were washed for 45 min in PBS prewarmed to 38^{o}C and were then washed twice for 5 min in PBS at room temperature. Slides containing gelatin cryosections were incubated sequentially in: 100% ethanol 2 minutes, 95% ethanol 1 minutes, 90% ethanol 1 minutes, 70% ethanol 1 minute, 50% ethanol 1 minute, H_2O 2 minutes, Erlichs haematoxylin 2 minutes, Running tap water 3 minutes, 1% HCl in 70% ethanol 30 seconds, 1%

NH_3 in 70% ethanol 1 minute, 70% ethanol 1 minute, 0.5% eosin in H_2O 5 minutes, 95% ethanol 15 seconds, 100% ethanol 2 minutes, Histoclear mount 1 2 minutes, Histoclear mount 2 for 2 minutes. Slides were mounted with the non-aqueous mounting medium for microscopy DPX and covered with cover slips. Finally, they were left to dry overnight at room temperature.

2.4 Molecular Biology

2.4.1 Materials

Table 2.8: Materials required for RNA extraction and qPCR.

Product	Supplier	Catalogue Number
RNA extraction		
TRIzol	Invitrogen	15596-018
Chloroform	Fisher	C/4960/17
Isopropanol	Fisher	P/7500/17
Ethanol	Fisher	E/0650DF/17
Sterile water	Baxter	UKF7114
Agarose	Invitrogen	15510-027
Reverse Transcription		
DNase1	Ambion	AM2235
Rnasin Plus RNase inhibitor	Promega	8170G
Oligo$(dT)_{15}$ Primer	Promega	C1101
Omniscript RT kit	Qiagen	205111
PCR		
HotStarTaq DNA polymerase	Qiagen	203203
1.25mM dNTPs	Invitrogen	10297-018
qPCR reagents		
LightCycler Fast Start DNA master SYBR Green 1	Roche	12 239 264 001
Capillaries	Roche	04 929 292 001
Cloning reagents		
pcDNA3.1/V5-His TOPO TA expression kit	Invitrogen	K961020
Bacto-Tryptone	Sigma Aldrich	T9410
Bacto-Yeast	BD	212750
NaCl	Sigma Aldrich	S9888
Ampicillin sodium salt	Sigma Aldrich	A9518
Miniprep kit	Sigma Aldrich	PLN70

Table 2.9: Primers used for q-PCR and their appropriate annealing temperatures.

Gene	Forward Primer (5' – 3')	Reverse Primer (5' – 3')	Annealing temperature
β-actin	TAGGCACCAGGGTGTGATGG	CATGGCTGGGGTGTTGAAGG	60˚C
Pou5 (Oct3/4)	GGCGTTCTCTTTGGAAAGGTG	CTCGAACCACATCCTTCTCT	58˚C
Nanog	CTCTTCAAGGCAGCCCTGAT	CCATTGCTAGTCTTCAACCAC	60˚C
Cdx2	GTGCGAGTGGATGCGGAAGC	TGAGGCTGGGAAGGTTGTGG	67˚C
Elf5	TTCGCTCGCAAGGTTACTCC	GCTCCCTGTCTTCCCATTCC	66˚C
Eomesodermin	GCAGGGCAATAAGATGTACG	GAACTGTGTCTCTGAGAAGG	62˚C
Gata3	GGGTTCGGATGTAAGTCGAG	CCACAGTGGGGTAGAGGTTG	60˚C
Brachyury	CATGTACTCTTCTTGCTGG	GGTCTCGGGAAAGCAGTGGC	58˚C

Table 2.10: Primers used for Topo cloning

T7 (5' – 3')	BGH reverse (5' – 3')	Annealing temperature
TAATACGACTCACTATAGGG	TAGAAGGCACAGTCGAGG	55˚C

2.4.2 Buffers

Table 2.11: Recipes for reagents required for molecular biology techniques

Buffer	Buffer constituents
Luria Broth (LB)	10g Bacto-tryptone 5g Bacto-yeast extract 10g NaCl 1L H_2O pH7.0

2.4.3 RNA extraction and Reverse-transcription

RNA extraction was carried out using TRIzol. Choloroform was then added to all samples and centrifuged at 13,000 rpm for 15 minutes at 4˚C. To the upper phase an equal volume of isopropanol was added and left for 10 minutes followed by centrifugation for 10 minutes at 13,000 rpm for 10 minutes. The pellet was washed in cold 75% ethanol, and this was spun at 13,000 rpm for five minutes. The ethanol was removed and the pellet allowed to air dry. The pellet was then resuspended in 20ul water, if necessary samples were heated to 65˚C for 5 minutes to aid solubilisation. The RNA concentration was determined using a BioTek Synergy HT platereader and the Gen5 (v1.05) software. The purity of RNA was visually inspected by running 1µg of each RNA sample by electrophoresis on a 1.2% agarose gel.

All RNA samples were treated with DNase 1 and Rnasin Plus RNase inhibitor for 20 minutes at 37^{o}C before complementary Deoxyribonucleic acid (cDNA) synthesis. 1µg of RNA was reverse-transcribed using Oligo(dT)$_{15}$ primers, 5mM (0.5mM final concentration) dNTPs and Omniscript at 40˚C for one hour, followed by 70˚C for 15 minutes.

2.4.4 PCR and q-PCR

All primers used in q-PCR were first optimised using PCR of 1ul (1/25th) of the calibrator reverse-transcription product (R63 in N2B27 with BMP4 and R63 cells in Knockout DMEM with LIF). The PCR reaction had a total volume of 25µl, with the addition of the following components 2.5µl 10X PCR buffer, 1.25mM (0.2mM final concentration) dNTPs, 25pmol (0.4pmol final concentration) forward and reverse primer (Table 2.9), 0.2µl HotStarTaq DNA polymerase. The PCR program run on a Techne TC-512 involved an initial denaturation for 5 minutes at 95˚C, each cycle consisted of: 30 seconds denaturation at 94˚C followed by annealing at the appropriate temperature, followed by 1 minute elongation at 72˚C. The final elongation step was at 72˚C for 5 minutes. Products were then separated using a 2% agarose gel and visualised. Primer details are given in Table 2.9.

q-PCR reactions were set up with a total volume of 10µl consisting of: 2µl of SYBR green mastermix, 25mM $MgCl_2$ (4mM final concentration), 25pmol of each primer (4pmols final concentration) and 2µl of template. Capillaries were centrifuged at 4000 rpm for 30 seconds, subsequently samples were run in the Roche LightCycler1.5. The cycle run was: 10 minute pre-incubation at 95˚C, the amplification consisted of 10 seconds at 95˚C followed by 5 seconds at the primer specific annealing temperature followed by 16 seconds at 72˚C, this amplification was repeated 40 times. Melt-curve analyses were performed to ensure that only one product was formed, PCR efficiencies were determined through production of a standard curve using different dilutions of template control cDNA. The efficiency was used to determine the relative quantification values for calibrator-normalised target gene expression, normalised to β-actin using LightCycler software (v4.0). These values were normalised setting the BMP4+LIF d3 average as 1. Each cDNA sample was analysed in duplicate in the same run, and each experiment was repeated three times. Statistical significance was determined using a one-way repeated measures ANOVA with a Tukey's post-hoc test using GraphPad Prism 5.

2.4.5 Cloning

Due to the low levels of Elf5 expression in the calibrator sample, cloning was used to generate DNA to produce a standard curve for q-PCR. PCR was carried out on the calibrator sample using Elf5 primers in table 1, with 66˚C annealing temperature. The PCR product was cloned in to the pcDNA3.1/V5-His-Topo plasmid vector as outlined in the Topo TA cloning kit instructions. Colonies were picked and grown in LB with 100ng/ml ampicilin. PCR was used to confirm presence of Elf5-fragment (primers in Table 2.10). A miniprep was performed according to instructions in the kit to obtain *Elf5* DNA to dilute to produce a standard curve for qPCR.

2.5 SDS-PAGE

2.5.1 Materials

Table 2.12: Reagents required for (Sodium dodecyl sulphate polyacrylamide gel electrophoresis) SDS-PAGE sample extraction and quantification, blotting and protein resolution.

Product	Supplier	Catalogue Number
Western blot - Sample extraction and preparation		
NaCl	Sigma	S9888
NaCl	Fisher	S/3160/53
Trizma	Sigma	T6066
Nonidet P40	VWR	560092-L
NP-40	Sigma	18896
Sodium Deoxycolate	Fisons Laboratory Equipment	S3500/45
Sodium Deoxycolate	Sigma	30970
Sodium Dodcyl Sulphate	Sigma	L3771
Benzonase	Sigma	E1014
PBS	Invitrogen	14200
Glycerol	Sigma	G5150
Bromophenol blue	Fisons	13/P620/44
NuPAGE LDS Sample Buffer (4x)	Life Technologies	NP0008
NuPAGE Sample Reducing Agent (10x)	Life Technologies	NP0009
Protease inhibitor cocktail		
Sodium Vanadate	Sigma	S-6508
Sodium Molybdate	BDH	10254
Sodium Fluoride	Sigma	S6521
Phenylmethylsulphnoyl fluoride (PMSF)	Sigma	P7626
Pepstatin A	Sigma	P5318
Aprotinin	Roche	236624
Leupeptin	Sigma	L8511
Soyabean trpsin inhibitor	Roche	109886
Phosphatase Inhibitor Cocktail 2	Sigma	P5726
Phosphatase Inhibitor Cocktail 3	Sigma	P0044
cOmplete,Mini, EDTA-free (Protease inhibitor cocktail)	Roche	04 693 159 001
Bicinchoninic acid (BCA) protein quantification assay		
Albumin standard	Thermo Scientific	23209
Pierce BCA Protein Assay Reagent A	Thermo Scientific	23227
BCA Protein Assay Reagent B	Thermo Scientific	1859078
Western blot – protein resolution and immunoblotting		
Acrylamide/bis acrylamide (37:5:1)	Bio-rad	161-0158
Ammonium Persulfate (APS)	Sigma	A3678
Tetramethylethylenediamine (TEMED)	Sigma	T9281
Glycine	Sigma	G8898
Tween	Sigma	P1379
NuPAGE MOPS SDS Running Buffer	Life Technologies	NP0001
NuPAGE Antioxidant	Life Technologies	NP0005

NuPAGE Novex 4-12% Bis-Tris Gels, 1.0mm, 10 well	Life Technologies	NP0321
NuPAGE Novex 4-12% Bis-Tris Gels, 1.0mm, 12 well	Life Technologies	NP0322
EZ-Run Prestained Rec Protein Ladder	Fisher	BP03603-500
Bio-rad broad range molecular weight	Bio-rad	161-0317
Full-Range Rainbow Molecular Weight Markers	GE Healthcare	RPN800E
Methanol	Sigma	34860
Glycine	Sigma	G7126
Trizma base	Sigma	G7126
3mm Chromatography paper	Whatmann	3030917
Western blotting filter paper	Thermo Scientific	88600F
Hybond ECL Nitrocellulose membrane	GE Healthcare	RPN303D
Hybond-P Polyvinylidene difluoride (PVDF) membrane	GE Healthcare	RPN303F
Ponceau S solution	Sigma	P7170
BSA	Sigma	A3059
Ovalbumin	Sigma	A5378
Sodium azide	BDH	10396
Amersham Hyperfilm ECL	GE Healthcare	28-9068-37
ECL Prime Western Blotting Detection kit	GE Healthcare	RPN2232
ECL 2 Western Blotting Substrate	Thermo Scientific	80196
Secondary antibodies		
Polyclonal goat anti-mouse Immunoglobulin/Horse radish peroxidise (HRP)	DAKO	P0447
Polyclonal goat anti-rabbit Immunoglobulin/HRP	DAKO	P0449
Polyclonal goat anti-goat Immunoglobulin/HRP	DAKO	P0448
Anti-Mouse IgG, HRP-linked species-specific whole antibody (from sheep)	GE	NA931
Anti-Rabbit IgG, HRP-linked species-specific whole antibody (from donkey)	GE	NA934

2.5.2 Buffers

Table 2.13: Recipes or buffers required for SDS-PAGE.

Buffer	Buffer constituents
Radio-immunoprecipitation assay (RIPA) buffer	150mM NaCl 50mM Tris HCl pH8 1% NP-40 0.5% Na Deoxycolate 0.1% SDS 25units/ml Benzonase nuclease (exclude if sonicate samples) H_2O
Protease/Phosphatase inhibitor home-made cocktail	1mM sodium Vanadate 1mM Sodium Molybdate 10mM Sodium Gluoride 10µg/ml PMSF 0.7µg/ml Pepstatin A 10µg/ml Aprotinin 10µg/ml Leupeptin 10µg/ml Soyabean trypsin inhibitor
5x Sample Buffer	5% SDS 50% Glycerol 200mM Tris HCl pH6.8 dH_2O Bromophenol Blue 5% 2-mercaptoethanol
Running Buffer	25mM Trizma 192mM Glycine 0.1%(wt/vol) SDS H_2O
Transfer Buffer	39mM Glycine 48mM Tris base 0.00375% SDS 20% (vol/vol) Methanol H_2O
Tris Buffered Saline (TBS)	20mM Tris-HCl pH7.5 150mM NaCl
TBS tween (TBST)	1x TBS 0.05% Tween
Nitrocellulose Stripping Buffer	0.2M Tris pH6.7 2% SDS 0.1M 2-mercaptoethanol H_2O

Running gel

Table 2.14: Volume of each component required to make 3x1mm SDS-PAGE running gel. Percentage running gel used for each protein is stated in text.

	6.5% Running gel	10% Running gel
Acrylamide	3.25mls	5mls
MilliQ H_2O	6.1mls	4.35mls
1M Tris pH8.8	5.6mls	5.6mls
10% SDS	0.25mls	0.25mls
10% APS	50 µl	50 µl
TEMED	20µl	20µl

Stacking gel

Table 2.15: Volume of each component required to make 4x1mm SDS-PAGE stacking gel.

	5% stacking gel
Acrylamide	1.67
MilliQ H_2O	6.0
1M Tris pH6.8	1.25
10% SDS	0.15
10% APS	50 µl
TEMED	20µl

2.5.3 Cell lysates

Lysates were prepared by washing embryoid bodies twice in PBS. Ice-cold RIPA buffer was added supplemented with either Phosphatase inhibitor cocktails 2 and 3 and c*O*mplete Mini, EDTA-free protease inhibitor cocktail or the protease/phosphatase inhibitor home-made cocktail. If Benzonase was not added to the RIPA buffer samples were sonicated with a Soniprep150. Samples were kept at -20^{o}C until required. Protein concentrations were determined using the BCA assay (Thermo Scientific) according to the manufacturer's directions.

2.5.4 Immunoblotting

Cell lysates were separated by SDS-PAGE using 6.5% (Fibronectin only) or 10% (all other proteins) polyacrylamide gels using the Bio-Rad system or gradient Novex Bis-Tris Gels using an XCell SureLock Mini Cell (Invitrogen). Immunoblotting was performed using a semi-dry blotter (Amersham Biosciences, Multiphor II) or the Bio-Rad wet-transfer system in transfer buffer for one hour. When using nitrocellulose and blotting for Fibronectin transfer was carried out for two hours. Nitrocellulose or PVDF membranes were blocked for one hour in 5% milk or 5% BSA for one hour and then incubated with primary antibodies overnight at 4^{o}C, appropriate secondary antibody was added for one hour. Immunoblotting was carried out using primary antibodies at the concentrations stated in Table 2.4. Anti-rabbit, anti-mouse, and anti-goat

secondary antibodies conjugated to HRP were used at 1:10,000 (DAKO), or 1:5000 (GE). After both primary and secondary antibody incubations five 10 minute washes in TBST were performed.

Blots were developed using ECL prime (GE Healthcare) or ECL2 (Thermo Scientific) according to manufacturer's directions. Detection was carried out using an ImageQuant RT ECL system or Amersham Hyperfilm ECL with quantification using ImageJ (NIH). As required nitrocellulose blots were stripped for 60 minutes at 55^{o}C in stripping buffer PVDF blots were stripped for 10 minutes at room temperature using 0.5M NaOH. Blots were subsequently washed in TBST and then blocked using either 5% BSA or 5% Milk. Each blot was normalised to a loading control, either Gapdh or α-Tubulin as stated on graph. For statistical analysis a 1-way ANOVA with a Dunnetts post-hoc test was performed using GraphPad Prism 5.

Chapter 3

3 BMP4 as a stimulus of mES cell differentiation to trophoblast

3.1 Introduction

3.1.1 Trophoblast

The trophoblast is formed following the first cell fate decision which occurs during mammalian embryonic development (Further details in 1.3.1). Totipotent cells form either the ICM, which produces the embryo and other extraembryonic tissues, or the trophoblast (Cockburn and Rossant, 2010). The trophoblast is an extra-embryonic epithelial tissue which contributes differentiated cell types to form the placenta.

A lot is known about the fate specification of the trophoblast, it requires the transcription factors Tead4 (Yagi et al., 2007), Cdx2 (Strumpf et al., 2005), Gata3 (Ralston et al., 2010), Eomesodermin (Russ et al., 2000) and Elf5 (Ng et al., 2008). A mutual antagonism between these factors and pluripotency transcription factors is also essential (Further details in 1.3.2). Increasingly, the polarisation of the trophoblast is thought to be essential for its development (Further details in 1.3.3). Most recently adherens junctions present in the polar trophoblast, but absent from the apolar cells of the ICM have been shown to regulate Hippo signalling which is required for this cell fate decision (Hirate et al., 2013).

3.1.2 Evidence for a role of BMP4 in driving embryonic stem cell differentiation to form trophoblast cells

For this project the use of BMP4 to drive embryonic stem differentiation to the trophoblast may make a good model to study pre-implantation epithelia, there are however some controversies within this field. Research to date in this area is presented below.

hES cells

Addition of BMP4 to hES cells induces expression of trophoblast genes, and secretion of placental hormones, these changes are blocked by addition of Noggin, a BMP antagonist (Xu et al., 2002). The differentiation of hES cells to trophoblast cells by BMP4 causes inhibition of Activin/Nodal signalling, therefore addition of Activin A as well as BMP4 can also prevent trophoblast differentiation (Wu et al., 2008). Short-term exposure of hES cells to BMP4 induces mesodermal differentiation (Zhang et al., 2008). If BMP signalling is subsequently inhibited in these cells they can differentiate into mature mesoderm lineages such as cardiac and hematopoietic cells.

Recently, the BMP4-induced phenotype in hES cells has been re-examined (Bernardo et al., 2011). BMP4 induced cells express the trophoblast marker CDX2, but in these cells high levels of BRACHYURY were also expressed which suggested they were a mesodermal cell type. The expression of these two proteins was essential for expression of additional mesodermal genes as well as other trophoblast genes. The expression of CDX2 in these cells was also dependent upon expression of BRACHYURY. Although BMP4 induced expression of CDX2 and other trophoblast markers in these cells, ELF5 was highly methylated and was expressed only at low levels, where as in human trophoblast lineages it is hypomethylated. This suggests that in hES cells BMP4 induces a mesodermal cell type which expresses CDX2 and some other transcription factors required for trophoblast differentiation, but does not induce true trophoblast cells. However, the results published in the paper by Bernardo et al., (2011) were directly contradicted by Amita et al., (2013) who showed a much higher percentage of hES cells became KERATIN 7 positive after culture with BMP4, suggesting they were trophoblast. These cells also expressed HLA and ELF5 which was not observed by Bernardo et al., (2011), and only expressed very low levels of BRACHYURY (Amita et al., 2013). Amita et al., (2013) do however suggest that the substratum upon which the cells were grown and the media in which they were grown during differentiation in the Bernardo et al (2011) paper reduced the efficiency and speed of the differentiation when examining some aspects of the phenotype.

Further investigation in to this area has shown that inhibition of FGF is required for BMP4 to induce differentiation to trophoblast as it maintains NANOG through the ERK signalling pathway (Sudheer et al., 2012; Yu et al., 2011). Instead culture with BMP4 and FGF produces mesoderm, endothelial, and trophoblast progenitors, in distinct populations (Drukker et al., 2012). Additionally, trophoblast cells have been produced from human induced pluripotent stem (iPS) cells reprogrammed from human fibroblasts, using BMP4 (Chen et al., 2013). These cells express Cdx2, Eomesodermin, Gata3 and other trophoblast associated genes. If successful, this technique of trophoblast formation from human iPS cells could be very useful to investigate the causes and mechanisms of placental diseases using human iPS cells derived from patients.

mES cells

A possible role for BMP4 in the differentiation of mES cells to the trophoblast has also been suggested, but less well studied. When mES cells are cultured in serum on Collagen IV expression of mesodermal genes is induced, as well as trophoblast markers (Schenke-Layland et al., 2007). These cells do however express trophoblast-restricted genes which demonstrates

the presence of different trophoblast subtypes such as trophoblast stem cells, spongiotrophoblast cells and labyrinthe trophoblast cells. No trophoblast markers were observed when using Collagen I, Laminin or Fibronectin as substrates for culture. The differentiation on Collagen IV could not be recapitulated with cells that had been maintained on 0.1% gelatin instead of feeders, suggesting the previous culture conditions affect the potential of mES cells to differentiated in to the trophoblast lineage.

When cultured in defined conditions in a monolayer, addition of BMP4 causes upregulation of many trophoblast marker genes such as *Cdx2*, and *Gata3* as detected by q-PCR (Hayashi et al., 2010). However, some genes such as *Eomesodermin* are not upregulated. Addition of Noggin, a BMP antagonist decreases the expression of trophoblast transcription factors. This BMP induced affect is through Smad proteins and the differentiation is promoted by Fibronectin and Laminin extracellular matrix, but not type 1 Collagen or PDL. These results contradict those of Schenke-Layland et al. (2007) as they disagree over which extracellular matrices will support differentiation to cells which express trophoblast markers. This may be because the experiment by Schenke-Layland et al. (2007) was performed in cells that had been maintained on feeders, rather than on gelatin, and also were carried out in the presence of serum rather than in a chemically defined system.

A different study has shown that culture of mES cells in serum without LIF on 0.2% gelatin-coated dishes causes an upregulation of trophoblast markers in comparison to the same treatment of mES cells which are cultured on MEFs (Peng et al., 2011). This study does not however compare to any other extracellular matrices. Interestingly, noggin seems to up-regulate this differentiation suggesting that BMP negatively influences this differentiation step rather than being used to induce it.

3.1.3 N2B27 media

N2B27 media is a serum-free culture medium which is used to accurately control cell culture conditions (Ying et al., 2003a). It requires the addition of N2 and B27 supplements to DMEM/F12 and Neurobasal media. This media provides a chemically defined environment which provides an ideal situation for studying the effects of extrinsic factors on differentiation of mES cells (Smith, 2001; Ying et al., 2003a).

When cultured in N2B27 media alone mES cells form a neuroectodermal cell type (Ying et al., 2003b). Pluripotency of mES cells is maintained when mES cells are cultured in N2B27 media

with addition of LIF and BMP4 (Ying et al., 2003a). Addition of only BMP4 to the N2B27 media prevents neural differentiation, instead large, non-neural flattened cells appear (Ying et al., 2003b). These cells look cobblestoned as epithelial cells do suggesting that they may have formed an epithelial cell type (Ying et al., 2003a). Recent work has shown that in these cell culture conditions BMP4 maintains expression of E-cadherin, further suggesting that an epithelial cell type may be formed (Malaguti et al., 2013). These cobblestoned cells could therefore be a good model to study embryonic epithelial development.

Previous work in the Chalmers lab produced preliminary results that suggested that addition of BMP4 to mES cells cultured on 0.1% gelatin in N2B27 media caused differentiation of cells to a trophoblast-like cell type. A downregulation of pluripotency proteins was observed by immunostaining and PCR. The cells also expressed RNA for many markers of trophoblast cells types, and expressed Cdx2 protein as shown by immunoctochemistry.

3.1.4 Aims

Building on previous work in the Chalmers lab, the main goal of this chapter is to determine whether differentiation of mES cells induced by BMP4 in N2B27 media would be a useful model of pre-implantation embryonic epithelial development. This will require two interrelated aims to be fulfilled:

- Characterise the identity of cells grown in mES cells with addition of BMP4. Establish whether or not they are polarised, and whether they faithfully recapitulate the development of the trophoblast lineage.
- Determine whether growth of mES cells in N2B27 media supplemented with BMP4 would be a useful model of trophoblast development which could be manipulated and analysed with a combination of techniques.

3.2 Results

3.2.1 Cells cultured in N2B27 media supplemented with BMP4 exhibit a cobblestone morphology

The first step in characterising the response of mES cells to growth in N2B27 media supplemented with LIF and BMP4, or BMP4 alone was to see if we observed similar morphological changes to previous reports. R63 mouse embryonic stem cells were grown in N2B27 supplemented with both BMP4 and LIF, or BMP4 alone (Figure 3.1). Cells cultured in N2B27 supplemented with BMP4 and LIF formed colonies of cells which resembled those observed when mES cells were routinely passaged in Knockout DMEM + LIF (Figure 3.1A). When N2B27 media was supplemented with BMP4 alone the cells formed monolayered regions of cells which were flat and cobblestone-like with some colonies (areas with multiple layers of cells) still present (Figure 3.1B).

3.2.2 Zo-1 localises in a polarised position when mES cells are cultured in N2B27 media supplemented with BMP4

Having observed an epithelial-like morphology when mouse embryonic stem cells were cultured in N2B27 supplemented with BMP4, the localisation of the tight junction marker Zo-1 was investigated using immunocytochemistry to determine if the cells formed were epithelial (Figure 3.2). Zo-1 formed a puncta at the border of the apical and basolateral regions of the membrane between the nuclei in monolayer regions of the culture as well as in cells on the top layer of the colony. Some cells deeper within the colony also expressed Zo-1 but its exact localisation within the cells was not clear due to the large nuclei and their proximity to each other. This stereotypical localisation of Zo-1 suggests that when mES cells were grown in N2B27 supplemented with BMP4 those in the monolayer and on the top layer of the colony had tight junctions and were therefore likely to be apico-basolaterally polarised.

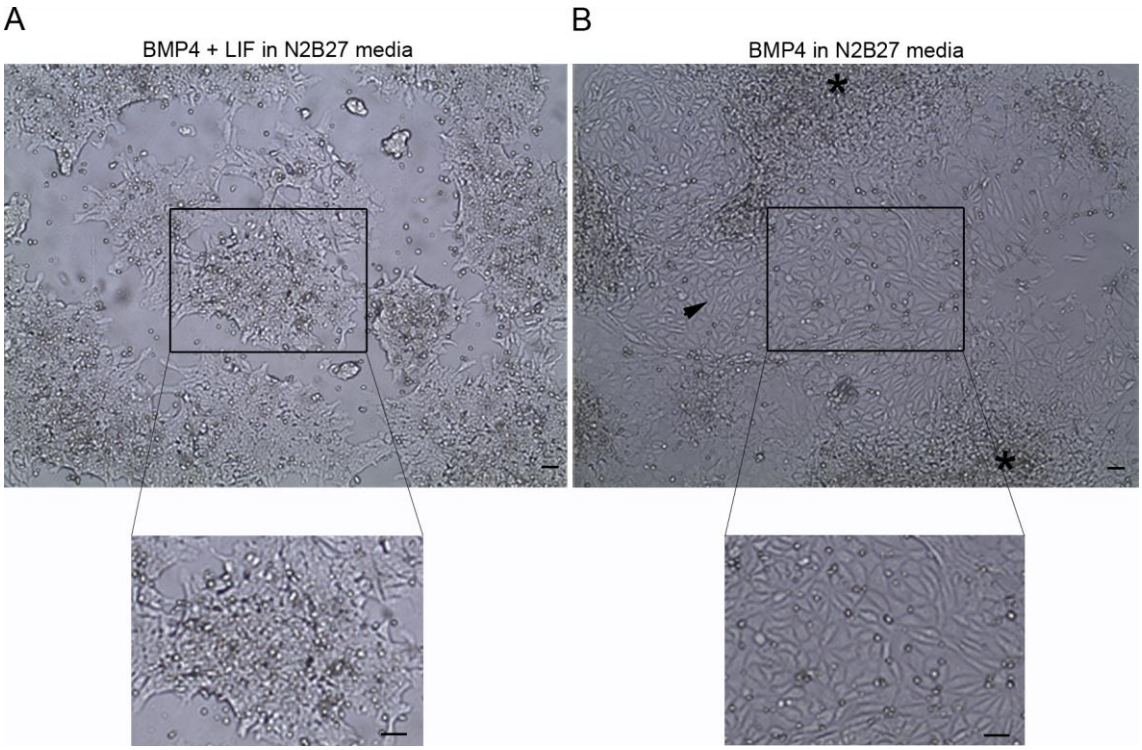


Figure 3.1: mES cells cultured in N2B27 media supplemented with BMP4 had a cobblestone morphology

Light microscopy images of mouse embryonic stem cells after 4 days of culture in N2B27 with addition of different supplements. (A) Mouse embryonic stem cells cultured in N2B27 media supplemented with BMP4 and LIF formed colonies of cells. (B) Mouse embryonic stem cells cultured in N2B27 media supplemented with BMP4 formed a sheet around the colonies these cells had a cobblestone-like morphology (shown by arrowheads). Colonies are highlighted with a *. Scale bars represent 10µm.

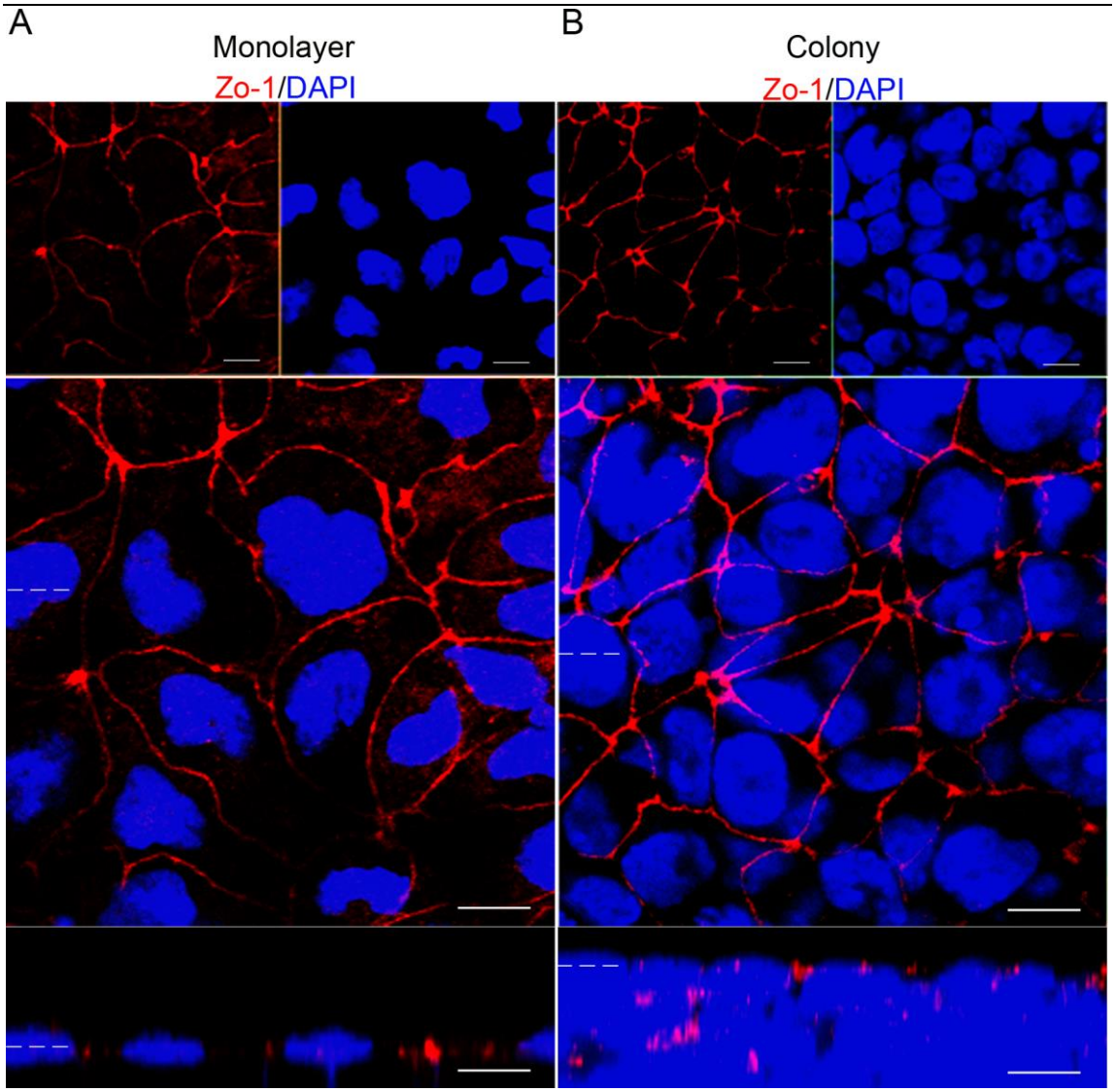


Figure 3.2: Zo-1 localised to junctions of cells cultured in N2B27 media supplemented with BMP4 in both the monolayer and the top layer of cells in a colony

Mouse embryonic stem cells were cultured in N2B27 media supplemented with BMP4 for 5 days. Localisation of the tight-junction marker Zo-1 was determined by immunostaining. Zo-1 localised (A) In cells present in a monolayer at an apical puncta between cells in a location typical of tight-junction. (B) In cells found in the top layer of a colony at an apical puncta, but is also found in cells deeper within the colony. Representative images from three independent experiments are shown. Dotted lines represent position that the relevant orthogonal or aerial images were taken. Scale bars represent 10µm.

3.2.3 Culture of cells with BMP4 causes induction of expression of a subset of markers of the trophoblast lineage.

Having observed that mES cells cultured in N2B27 and BMP4 formed an apparently epithelial cell type with tight junctions, q-PCR was used to identify which cell type was produced. RNA was extracted on days 3, 4, and 5. Expression of *Cdx2*, a transcription factor required for trophoblast differentiation was higher in BMP4 treated cells than those cultured in N2B27 supplemented with BMP4 and LIF or in media alone (Figure 3.3A). There was a two-fold increase in *Cdx2* expression on day 4 compared with day 3 in cells cultured in BMP4, but subsequently the expression returned to the same levels as the cells cultured with BMP4 and LIF. Similarly, the expression of *Eomesodermin*, another transcription factor required for trophoblast differentiation, was higher in cells cultured in N2B27 supplemented with BMP4 than those with BMP4 and LIF, or those without supplements (Figure 3.3B). The expression of *Eomesodermin* was high on day 3, and decreased gradually over time. *Gata3* expression was also higher in cells cultured in N2B27 media supplemented with BMP4 than in the other conditions (Figure 3.3C). The levels were low on day 3, but increased on day 4, and remained high on day 5. Elf5 is epigenetically regulated and is critical for trophoblast differentiation (Ng et al., 2008). There was no induction of *Elf5* (Figure 3.3D). This suggests that *Cdx2*, *Eomesodermin* and *Gata3* transcription factors were upregulated upon addition of BMP4. However, *Elf5* was not induced suggesting upregulation of only a subset of trophoblast genes occurred. This argues BMP4 may only cause a partial induction of trophoblast cell fate.

The mesodermal transcription factor *Brachyury* has been shown to be expressed in hESCs, mEpiSCs, and mES cells when they are cultured with BMP4 in chemically defined media (Bernardo et al., 2011; Malaguti et al., 2013). qPCR shows that *Brachyury* was not expressed in cells treated here with BMP4 and LIF, or in media alone (Figure 3.3E). Conversely, on day 3 *Brachyury* was expressed in cells treated with BMP4, its expression level in these cells was almost 7 fold greater than those cultured in BMP4 and LIF. On day 5 the levels of expression decreased by a half, and on day 5 it decreased to only 1.75 fold higher than cells treated with BMP4 and LIF for 5 days. This suggests that cells in this culture system expressed the mesodermal transcription factor *Brachyury* as well as a subset of transcription factors required for trophoblast differentiation.

Having observed an induction of some but not all trophoblast markers expression of transcription factors known to be required for pluripotency were quantified using q-PCR (Figure 3.4). The expression levels of *Pou5* in cells cultured in all conditions fluctuated

between days, there was no clear trend when comparing either across the time points or between conditions on any of the individual days (Figure 3.4A). Expression of *Nanog* was the same on days 3 and 4 in both cells cultured with BMP4 and LIF and BMP4 only (Figure 3.4B). *Nanog* levels were lower in cells cultured in N2B27. On day 5 there was a statistically significant decrease in expression of *Nanog* in cells cultured with BMP4 only in comparison to BMP4 + LIF, whilst *Nanog* levels increased in cells cultured with BMP4 and LIF. Overall, there is no clear trend in expression of the pluripotency genes *Pou5* between conditions or over the time course, whilst there is a decrease in *Nanog* in BMP4 treated cells on day 5 in comparison to cells cultured with both BMP4 and LIF. This suggests that only on day 5 is there a reduction in pluripotency when cells are grown with BMP4 in N2B27 media.

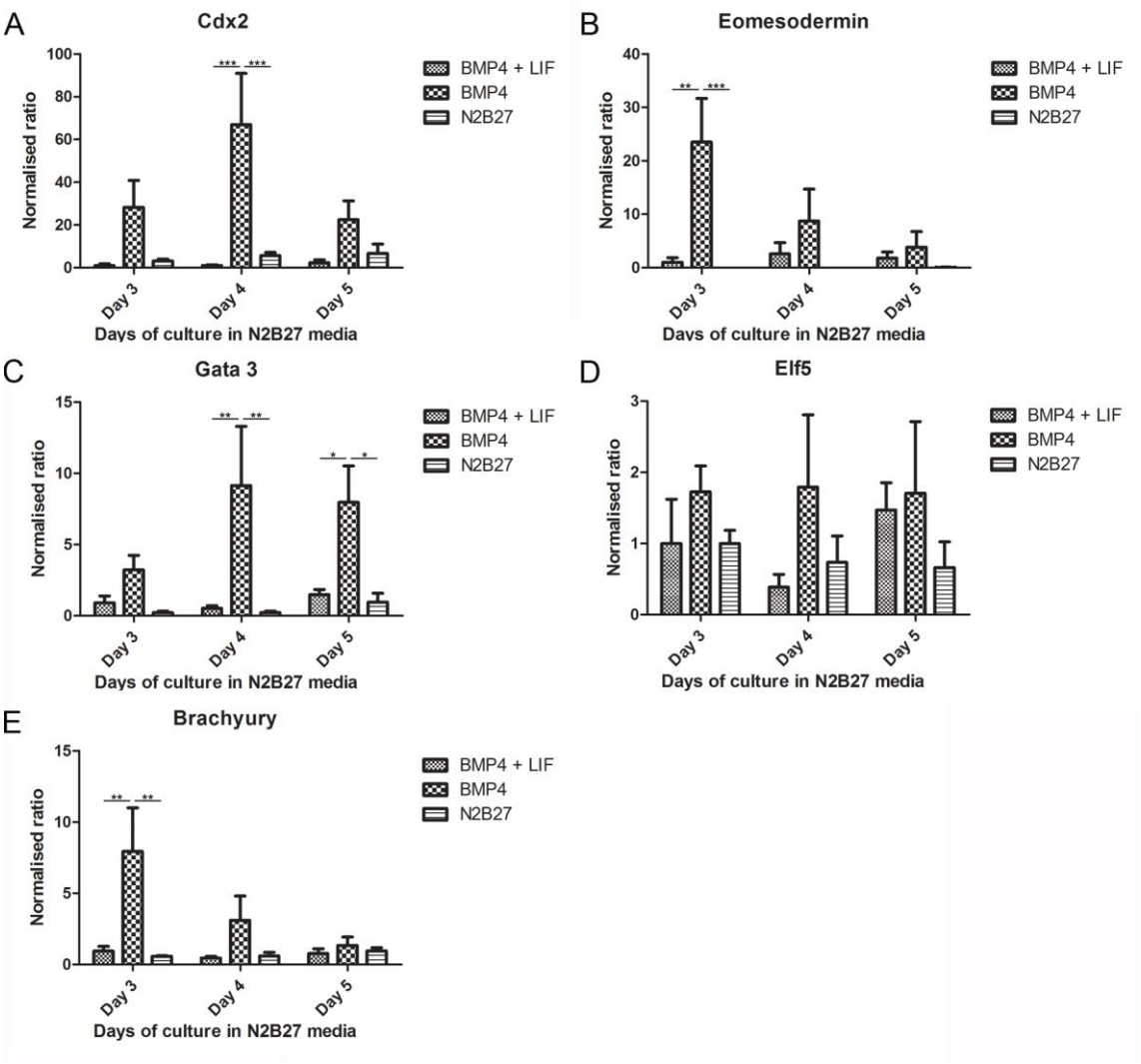


Figure 3.3 Cells cultured in N2B27 supplemented with BMP4 expressed a subset of trophoblast transcription factors and also express mesoderm transcription factor Brachyury
q-PCR analysis for the trophoblast markers (A) *Cdx2*, (B) *Eomesodermin,* (C) *Gata3* and (D) *Elf5* and mesoderm marker (E) *Brachyury* on samples cultured for 3, 4 or 5 days in N2B27 media supplemented with BMP4 plus LIF, N2B27 supplemented with BMP4 or in N2B27 media without supplement. *Cdx2*, *Eomesodermin*, *Gata3*, and *Brachyury* were highly expressed upon addition of BMP4, whilst *Elf5* was not expressed. Expression levels were normalised to *β-actin* and for each target gene to the BMP4 with LIF d3 sample. Data is from three independent experiments, error bars present Standard error of the mean (SEM). Statistical significance was determined with a Two-way ANOVA with a Bonferroni post-hoc test (* P=0.1-0.5, ** p=0.001-0.01, *** p<0.001).

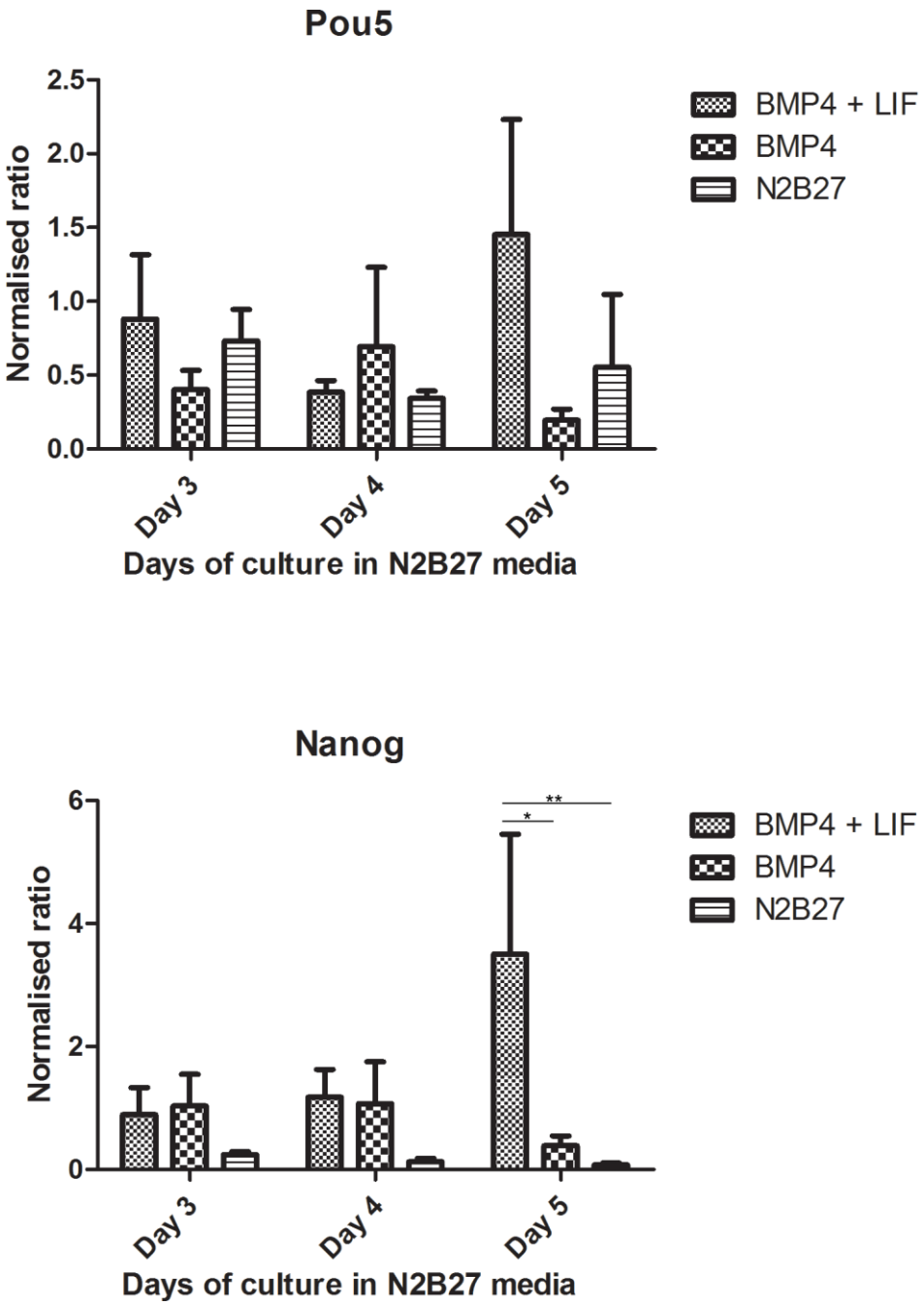


Figure 3.4 Expression of pluripotency markers Pou5 and Nanog when mouse embryonic stem cells were cultured in N2B27 supplemented with BMP4 and LIF, BMP4, or media only.
q-PCR analysis of expression of pluripotency markers (A) *Pou5* and (B) *Nanog* was carried out on samples cultured for 3, 4 or 5 days in N2B27 media supplemented with BMP4 plus LIF, N2B27 supplemented with BMP4 or in N2B27 media without supplement. There is no clear trend in expression of either *Pou5* or *Nanog* in any of the culture conditions or time points. Expression was normalised to *β-actin* and for each target gene to the BMP4 with LIF d3 sample. Data is from three independent experiments, error bars present SEM. Statistical significance was determined with a Two-way ANOVA with a Bonferroni post-hoc test (* P=0.1-0.5, ** p=0.001-0.01, *** p<0.001).

3.2.4 Cdx2 and Eomesodermin localise to the nuclei of cells but are not co-expressed

As RNA expression of a subset of trophoblast transcription factors was observed immunostaining was carried out to observe the localisation of the protein in the cells. Surprisingly, neither Cdx2 nor Eomesdoermin could be detected in cells of the monolayer (Figure 3.5). In the colonies, Cdx2 localised in the nuclei of the cells in the top layer, and also in some cells deeper in the colony. Eomesodermin also localised in the nuclei of cells but in a different subset of cells to Cdx2. This suggests that although RNA was present for both of these proteins they may not be expressed in the same cells but instead in different cell populations.

3.2.5 The phenotype observed in cells grown in BMP4 was very variable

Previous work in the Chalmers lab had observed localisation of Cdx2 in a large proportion of nuclei of cells in the monolayer as well as the upper cells in the colony. Different culture conditions were tested to identify the cause of the reduced number of cells in the monolayer expressing Cdx2 (Table 3.1). Optimisation of the cell culture density, cell line, BMP4 supplier, media used to neutralise the trypsin, culture volume and culture vessel did not change the observed expression pattern of Cdx2 assessed by immunostaining. The last thing to be optimised was the substrate used to coat the transwell filter on which the embryonic stem cells grow (Figure 3.6). Interestingly, the number of Cdx2 positive cells observed in the monolayer was dependent on the substrate upon which they grew. On Matrigel, Fibronectin and 1% Gelatin less than 10% of cells were Cdx2 positive. When using 0.1% gelatin almost 50% of cells in the monolayer had Cdx2 positive nuclei, suggesting that cells growing on this substrate favour differentiation in to a Cdx2 positive cell type.

All previous experiments outlined above had also been carried out on a 0.1% gelatin coated cell culture vessel. When doing this experiment a sudden increase in the number of Cdx2 positive nuclei in the monolayers was observed (Figure 3.6, Figure 3.7). The percentage of cells in the monolayer which were positive for Cdx2 in this experiment is much more similar to the response seen previously in the Chalmers lab. Cdx2 was still also expressed in the nuclei of the upper cells of the colony (Figure 3.7) as seen previously in this thesis (Figure 3.5) and previously by the Chalmers lab.

Unfortunately, the observed frequency of Cdx2 positive nuclei did not remain consistently at 50%, in the majority of subsequent experiments this result could not be repeated. Cdx2 positive nuclei were only found in the upper layer of the colony. The fluctuations in expression

of Cdx2 in the cells of the monolayer grown in N2B27 media supplemented with BMP4 suggest that this system did not give consistent, reliable results with the reagents available.

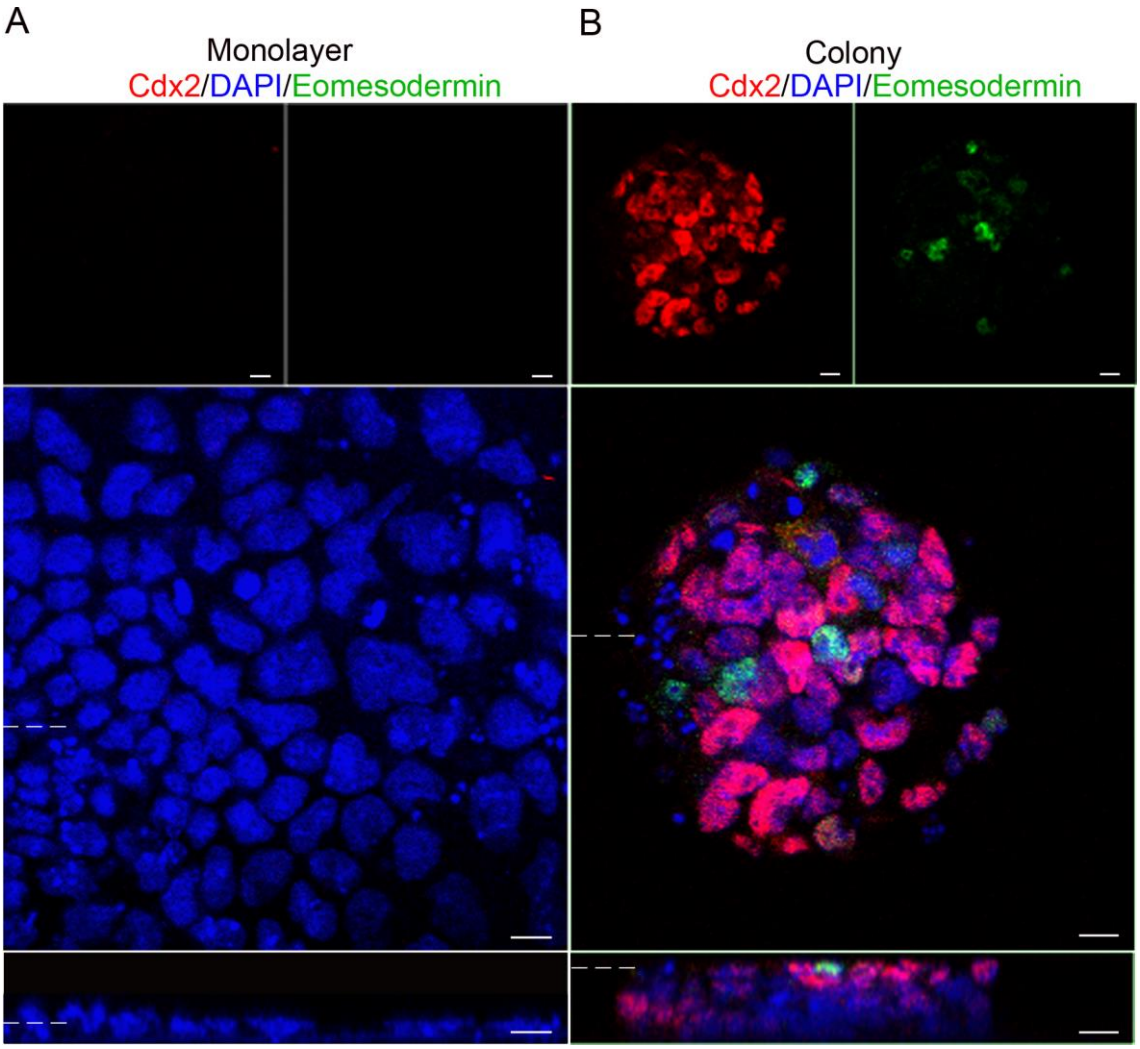


Figure 3.5: Mouse embryonic stem cells found in colonies after culture in N2B27 supplemented with BMP4 expressed either Cdx2 or Eomesodermin, but not in the same cells, cells in the monolayer did not express either.

Mouse embryonic stem cells were culture in N2B27 suplemented with BMP4 for 5 days. Localisation of the trophoblast proteins Cdx2 and Eomesodermin were determined using immunostaining. (A) Cells which were flat and in a monolayer did not express either Cdx2 or Eomesodermin. (B) Cells on the outer-layer of the colony expressed either Cdx2 or Eomesodermin but not in the same cells. Representative images from two independent experiments are shown. Dotted lines represent position that the relevant orthogonal or aerial images were taken. Scale bars represent 10µm.

Table 3.1 Modification of culture conditions did not increase the number of Cdx2 positive cells present in the monolayer after culture in N2B27 media supplemented with BMP4.
mES cells were cultured in transwell inserts in N2B27 media with addition of BMP4. Culture conditions (Variable) were changed in order to optimise conditions. Differences in the cell phenotype was analysed using cell morphology and immunostaining for a trophoblast lineage marker (Cdx2). None of these changes in culture condition increased the number of cells in the monolayer which expressed the transcription factor Cdx2.

Variable	Conditions tested	Outcome
Cell culture density	R63: 12,000(n=2) 18,000 (n=4), 25,000 (n=1)	At higher cell densities: too many cells with few monolayer regions and therefore imaging and analysis very difficult. At lower cell densities: too few cells, cells died, insufficient cells alive for analysis. The threshold at which these two extremes were reached seemed to change over time. No Cdx2 expression in monolayer region.
Cell line	Sox1GFP(n=3) R63 (n=6)	No Cdx2 expression in monolayer region.
BMP4 supplier	R&D (n=3) Stemgent (n=6)	No Cdx2 expression in monolayer region.
Media to neutralise trypsin	N2B27 media (n=2) Knockout media (n=7)	No Cdx2 expression in monolayer region.
Culture volume	1ml (n=6) 600µl (n=3)	No Cdx2 expression in monolayer region.
Culture vessel	Coverslip (n=1) Transwell insert (n=9)	No Cdx2 expression in monolayer region.

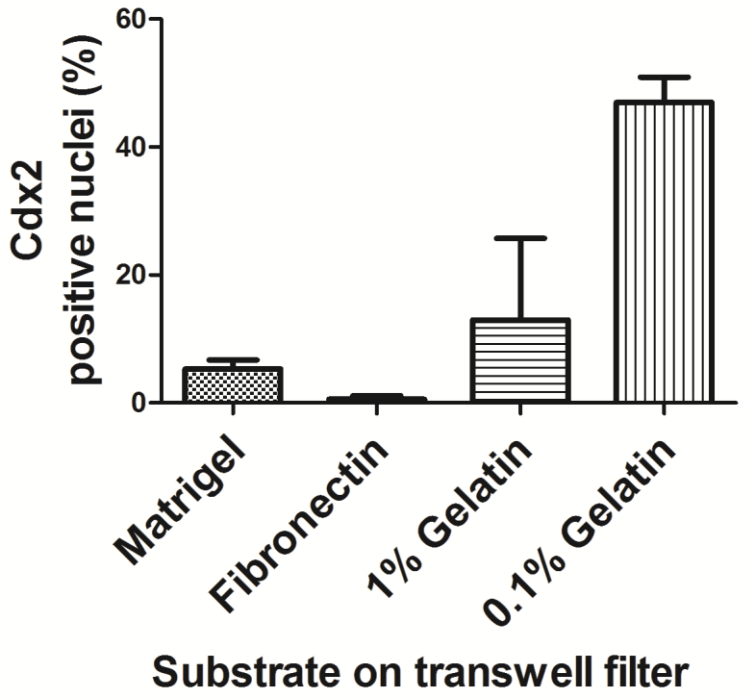


Figure 3.6 Mouse embryonic stem cells grown on 0.1% gelatin in N2B27 media supplemented with BMP4 had more Cdx2 positive cells that on other substrates.
Mouse embryonic stem cells were cultured in N2B27 media supplemented with BMP4. Cells were immunostained for Cdx2 and the number of positive cells present in the monolayer was counted. Most Cdx2 positive nuclei were observed when cells were cultured on 0.1% gelatin coated transwell filters. Data is from at least two independent experiments, error bars present SEM. Statistical significance was determined with a one-way ANOVA with a Tukey's post-hoc test (* P=0.1-0.5, ** p=0.001-0.01, *** p<0.001).

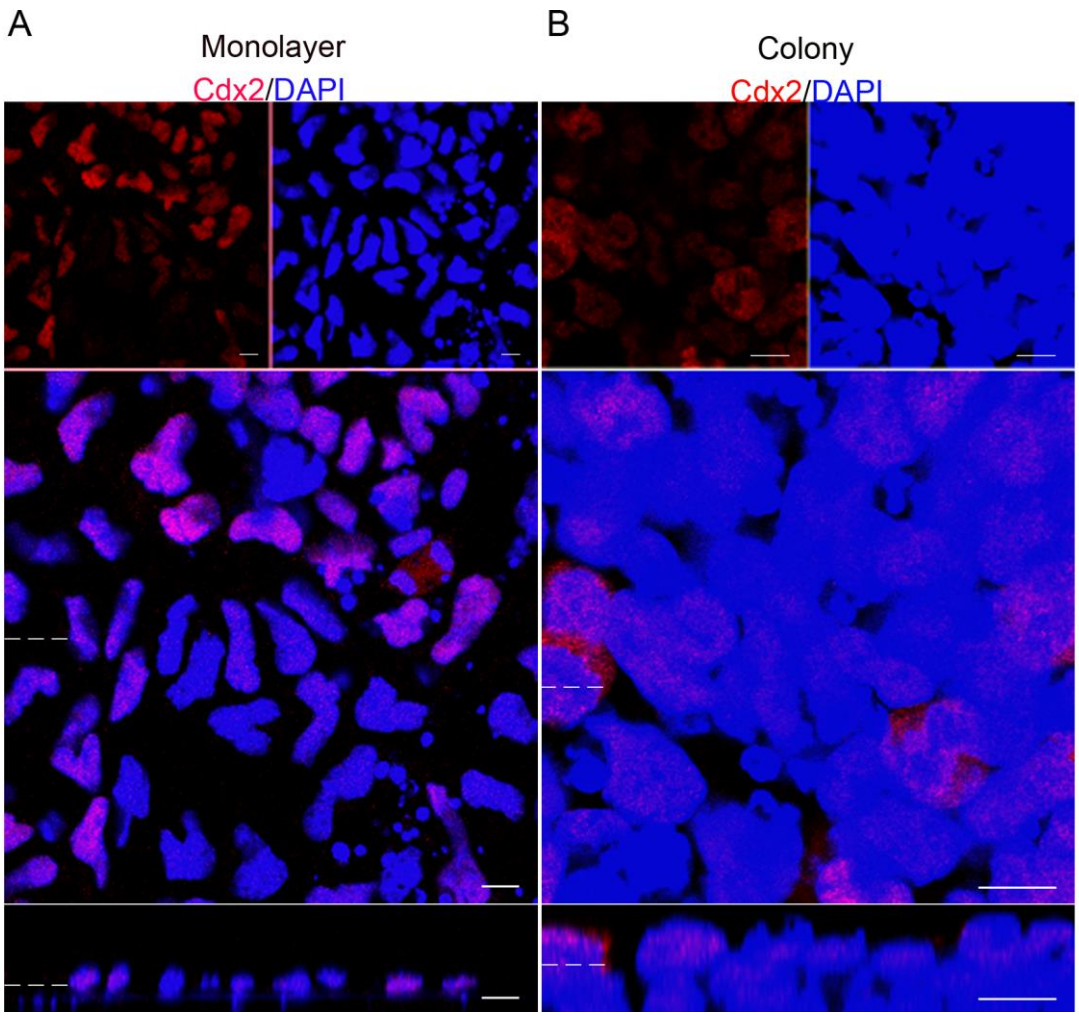


Figure 3.7 Cdx2 localised to the nuclei of cells present in both the monolayer and the top cell layer of a colony after culture in N2B27 media supplemented with BMP4.
mES cells were cultured in N2B27 media supplemented with BMP4 for 5 days. Localisation of Cdx2 was assessed by immunostaining. (A) Many cells which are flat and in a monolayer express Cdx2 in the nuclei. (B) Cells in the outer-layer of the colony express Cdx2 in nuclei, but not underlying cells. Representative images from two independent experiments are shown. Dotted lines represent position that the relevant orthogonal or aerial images were taken. Scale bars represent 10µm.

3.3 Discussion

3.3.1 Summary

Data presented here shows that growth of mES cells in chemically defined media supplemented with BMP4 caused differentiation of mES cells. The cells which were produced expressed a subset of transcription factors required for trophoblast development, such as *Cdx2*, *Eomesodermin* and *Gata3* but did not express *Elf5*. Localisation of Eomesodermin and Cdx2 protein was observed in different populations of cells, and were not co-expressed. Interestingly these cells also expressed the mesodermal marker *Brachyury,* which is not thought to be expressed in trophoblast cells. The lack of *Elf5* and activation of *Brachyury* suggests that this may not be a physiologically relevant model of trophoblast development. Additionally, the model did not produce a consistent phenotype. Overall, these results suggest that addition of BMP4 to mES cells in a chemically defined system with the reagents available would not be a useful model to investigate trophoblast development.

3.3.2 Culture of mES cells in BMP4 produces a polarised cell type

Culture of mES cells in BMP4 resulted in formation of cells in a monolayer, and also cells in a multi-layered area which is referred to here as a colony. To our knowledge other than the localisation of Cadherins (Malaguti et al., 2013; Schenke-Layland et al., 2007), the localisation of proteins usually apico-basolaterally localised in an epithelium has not been previously carried out on mES cells following culture with BMP4. Treatment of BMP4 maintains E-cadherin expression in cells (Malaguti et al., 2013), P-cadherin (Cdh-3) has also been previously shown to localise at the membrane of mES cells cultured with BMP4 (Hayashi et al., 2010; Schenke-Layland et al., 2007). Zo-1 localised between the apical and lateral regions of each cells in the monolayer, as well cells in the outer-layer of the colony and some cells deeper within the colony. This suggests that these cells may have had tight junctions, and may therefore have been apico-basolaterally polarised. To confirm the presence of tight junctions it would be necessary to use electon microscopy to visualise them and to observe the localisation of other proteins present in the tight-junction such as Claudins, Occludin, and Jams. To determine whether the cells are apico-basolaterally polarised it would be necessary to determine the localisation of proteins present within the three polarity complexes, Par, Crumbs, and Scribble. It would also be useful to determine the localisation of proteins found in other junctions, such as the adherens junction.

3.3.3 An unidentified cell-type is formed from addition of BMP4

Having observed that the addition of BMP4 caused localisation of Zo-1 in mES cells in the position expected for an apico-basolateral polarised cell, the identity of these cells was identified. To enhance this analysis it would have been advantageous to have mouse trophoblast tissue, or trophoblast stem cells as a control in both the q-PCR and the immunostaining experiments. This would have allowed a direct comparison of expression levels observed upon addition of BMP4, and those seen *in vivo.* Cells were cultured in BMP4 and LIF which maintains mES cell pluripotency and therefore acts as an undifferentiated control. There was no effect on the expression of the Oct4 gene *Pou5,* but a decrease in *Nanog* levels in cells cultured with BMP4 in comparison to the BMP4 + LIF control on day 5 which suggests that there may have been a reduction in pluripotency at this time point. Additionally, the upregulation of *Cdx2*, *Eomesodermin*, and *Gata3* in comparison to the mES cells maintained in BMP4 and LIF suggested that these cells expressed a subset of trophoblast genes. Other groups have also previously observed BMP4-induced upregulation of genes expressed in the trophoblast (Hayashi et al., 2010; Peng et al., 2011; Schenke-Layland et al., 2007). Surprisingly, expression of *Elf5*, an epigenetically regulated transcription factor was not observed. Some papers have shown the expression of *Elf5* following culture of hES cells with BMP4 (Amita et al., 2013; Li et al., 2013), but Bernardo et al (2011) observed only very low levels of its expression.

Immunostaining of a colony region formed shows that expression of Cdx2 and Eomesodermin protein was in two different cell populations and that these transcription factors were not expressed in the same cells. To our knowledge the co-localisation of these proteins or other transcription factors required for trophoblast differentiation has not been shown after culture of mES or hES cells with BMP4. The lack of *Elf5* expression, and presence of Cdx2 and Eomesodermin protein in two separate populations suggests that addition of BMP4 has not induced cells of a true trophoblast lineage. Differentiation to the trophoblast lineage in hES cells has been shown to be dependent upon inhibition of Fgf by maintaining Nanog (Sudheer et al., 2012; Yu et al., 2011). This suggests perhaps addition of an Fgf receptor inhibitor would enhance the differentiation of mES cells to trophoblast, this approach should be taken with caution though as there are known differences between regulation of differentiation and pluripotency in human and mouse ES cells.

In addition to expression of transcription factors expressed in the trophoblast, expression of the mesodermal gene *Brachyury* was also observed in BMP4-treated mES cells. Brachyury is expressed in the same cells as Cdx2 and other trophoblast markers when hES cells are cultured

with BMP4 (Bernardo et al., 2011). In contrast, in mES cells high levels of BMP4 have been shown to prime cells for mesoderm, resulting in Brachyury positive cells but which do not express other markers of mesodermal differentiation (Malaguti et al., 2013). In order to confirm if cells express both Cdx2 and Brachyury or are primed for mesoderm it would first be necessary to carry out immunostaining or fluorescence-activated cell sorting (FACS) analysis to determine whether Cdx2 and Brachyury are expressed in the same cells or are present in different cell populations. Additionally, it would be useful to determine if any other mesodermal proteins are expressed in these cells. If the cells are Brachyury positive, Cdx2 positive, and express mesodermal markers they would seem to fit the description of hES cells treated with BMP4 (Bernardo et al., 2011). Alternatively, if the Brachyury positive cells do not express mesodermal markers and are E-cadherin positive then they are more likely to be the mesoderm-primed cells which are induced by BMP4 in mES cells (Malaguti et al., 2013). Additionally, if Brachyury and Cdx2 are not expressed in separate cells it would suggest that they are two distinct populations, a mesodermal (primed or committed) and a trophoblast progenitor as suggested in hES cells following BMP4 induction (Drukker et al., 2012).

3.3.4 mES cell differentiation by BMP4 is dependent on extracellular matrix?

The differentiation of mES cells to the trophoblast cell fate has previously been shown to be dependent on the extracellular matrix. In this study I showed that no Cdx2 positive cells were present when cells were cultured on Fibronectin and very few Cdx2 positive cells were observed when the cells were grown on Matrigel or 1% Gelatin. The absence of Cdx2 positive cells when cultured on Fibronectin contradicts the findings of Hayashi *et al.* (2010). This may be because they used a different serum-free system. A previous study has also shown that the presence of mesodermal genes is dependent upon the extracellular matrix, when grown on Collagen IV they were present, but when cells were grown on Collagen I, Laminin or Fibronectin they were not expressed (Schenke-Layland et al., 2007). This is also contradicted by my finding as *Brachyury* is expressed here when cells are cultured on 0.1% gelatin. This may be because Schenke-Layland *et al.* (2007) did not use a serum-free system, and therefore these results are not directly comparable. Overall this study and others suggest that the extracellular matrix upon which mES cells are grown when treated with BMP4 is important for their differentiation, but there is not yet a clear picture of how and why.

3.3.5 Growth of mES cells in BMP4 as a model of pre-implantation epithelial development

The aim of this thesis is to investigate the mechanisms responsible for the polarisation and cell fate specification of pre-implantation epithelia. The data obtained to characterise the cell type

produced from addition of BMP4 to mES cells suggested that it did not produce a homogeneous population and did not seem to recapitulate trophoblast development as *Elf5* was not expressed, and the mesodermal marker *Brachyury* was expressed. This suggests that this may not be a very physiologically relevant model with which to study development of the murine trophoblast. A comparison of the differentiation of hES cells following addition of BMP5, 10, and 13 showed that BMP10 caused differentiation faster, and led to a small upregulation of mesodermal genes (Lichtner et al., 2013). Future studies with mES cells could look at the effects of other BMPs on this differentiation step which might allow optimisation of the trophoblast differentiation.

An additional downfall of this system is the variability observed between experiments. Although sometimes the cells grew and produced cells which seemed to express trophoblast markers, frequently this did not happen. Optimisation of many of the different assay conditions and reagents did not produce cells with expression of Cdx2. However, on occasion after many consecutive experiments failing to produce any Cdx2 positive nuclei, almost 50% of cells in the monolayer were found to be positive for Cdx2. It is therefore perhaps the state of the cells prior to differentiation which is important, and therefore the constituents of the media used in routine cell culture, or the extra-cellular matrix upon which the cells are routinely cultured that could be important. This unreliability in addition to the concerns in how physiologically relevant this model is suggests that it would be difficult to investigate the development of the trophoblast using this model system with the current protocol.

3.3.6 Conclusion

Growth of mES cells in N2B27, a chemically defined system caused cells to differentiate in to a cell type which expressed some transcription factors required for trophoblast development (*Eomesodermin*, *Cdx2* and *Gata3*), but not *Elf5*. Additionally, *Brachyury* a protein required for mesoderm differentiation was expressed. This suggests that these cells do not represent a physiologically relevant model of trophoblast differentiation. Lastly, this model seemed to be inconsistent and unreliable. For these two reasons in its current form, it would not make a good model of trophoblast development and therefore an alternative model of pre-implantation development should be investigated.

Chapter 4

4 Embryoid bodies as a model of primitive endoderm specification and epithelial polarisation

4.1 Introduction

The culture of mES cells in N2B27 media supplemented with BMP4 did not produce a reproducible physiologically relevant phenotype. Unfortunately for this project the protocol used here would therefore not make a useful model of mammalian pre-implantation epithelial development. An alternative model of differentiation of mES cells to a pre-implantation epithelial cell type is explored in this chapter. Embryoid bodies are used as a model of primitive endoderm development.

4.1.1 Primitive endoderm

The primitive endoderm forms an epithelium which localises between the blastocoel cavity and the epiblast, these three components are surrounded by the trophoblast. Two subpopulations are formed from the primitive endoderm, the parietal and visceral endoderm give rise to the yolk sac, whilst the visceral endoderm also contributes to the gut endoderm and provides embryonic patterning signals (Saiz and Plusa, 2013). The primitive endoderm is formed from a key cell fate decision in early mammalian development when cells in the ICM decide to follow either the primitive endoderm or epiblast cell fate (Cockburn and Rossant, 2010). Cells of the epiblast give rise to the embryo proper. Recently, there has been a rapid increase in our understanding of how the primitive endoderm versus epiblast cell fate decision is regulated (Further detail in 1.4). This has come from studies in mouse embryos, as well as work using embryoid bodies. Cells initially express both epiblast (eg. Nanog) and primitive endoderm markers (eg. Gata6 and Gata4), the expression then resolves into a salt-and-pepper pattern within the ICM, where cells express either epiblast or primitive endoderm markers (Chazaud et al., 2006; Guo et al., 2010; Plusa et al., 2008). The cells of the primitive endoderm then migrate to their final position facing the blastocoel cavity where they form an epithelial sheet. It is thought that Fgfr/Erk signalling regulates the mosaic expression of the primitive endoderm markers in the ICM (Lanner and Rossant, 2010).

4.1.2 Polarisation of the primitive endoderm

In contrast to our growing understanding of cell fate specification less is known about the mechanisms which regulate polarisation of primitive endoderm cells (Further detail in 1.4.7). A number of studies have shown a polarised localisation of proteins within the epithelial cells of the primitive endoderm. For example, E-cadherin is localised at the basolateral membrane of primitive endoderm cells in an embryoid body (Moore et al., 2009). aPkc, Dab2 and Lrp2 localise to the apical membrane of embryonic primitive endoderm cells (Gerbe et al., 2008; Wu

et al., 2007), and a constituent of the basement membrane, Laminin has been reported to be required for proper epithelial organisation (Murray and Edgar, 2000). These studies therefore show that the primitive endoderm is polarised, but the role that epithelial polarisation has in primitive endoderm specification has not been extensively studied.

4.1.3 Embryoid bodies

To investigate the development of the primitive endoderm this chapter will investigate the use of embryoid bodies. When mES cells are cultured in suspension they form aggregates known as embryoid bodies (Martin, 1981; Martin and Evans, 1975). Embryoid bodies undergo differentiation to cells types of all three germ layers; ectoderm, endoderm and mesoderm (Martin and Evans, 1975). The cell layer on the outside of the embryoid body represents the primitive endoderm (Martin, 1981), embryoid bodies can therefore be used to study primitive endoderm development. For example, some of the earliest papers to identify a role for Gata4 and Gata6 in the development of the primitive endoderm used embryoid bodies formed from $Gata4^{-/-}$ (Soudais et al., 1995) or $Gata6^{-/-}$ (Morrisey et al., 1998) mES cells. Additionally, two of the first papers implicating the Fgf receptor in primitive endoderm development made embryoid bodies with a dominant negative Fgfr2 (Chen et al., 2000) or $Fgfr1^{-/-}$ mES cells (Esner et al., 2002) and demonstrated that there was no visceral endoderm development.

As well as being used as a model of primitive endoderm cell fate specification, the development of the outer layer of the embryoid body has been previously used as a model of epithelial polarisation. A very important paper in the understanding of epithelial tight junctions used $Occludin^{-/-}$ mES cells to show that even in the absence of Occludin the cell layer on the outside of the embryoid body still form polarised epithelial cells with tight junctions in which Zo-1 is correctly localised (Saitou et al., 1998). This therefore provided evidence for the first time that there are additional transmembrane proteins, later identified as Claudins and Jams, which are present within the tight junction and can provide a barrier in the absence of Occludin. Additionally, embryoid bodies formed using $Afadin^{-/-}$ (Komura et al., 2008), and $Cingluin^{-/-}$ (Guillemot et al., 2004) mES have further elucidated the role of these proteins in epithelial cell junction formation.

Embryoid bodies can be formed in three main ways (Kurosawa, 2007). The simplest way is culturing mES cells in suspension in a bacterial culture dish in the absence of factors required for pluripotency (LIF, or feeders). mES cells do not adhere to these plates and instead stick to each other forming aggregates. The number of cells which form each aggregate can vary, this

therefore produces embryoid bodies which are highly heterogenous in size, shape, and differentiation stage. Alternatively, embryoid bodies can be formed in hanging drop culture. A drop of cell suspension is placed on an inverted lid so that each drop contains a known number of cells. The cells are forced to the bottom of the drop by gravity and form an aggregate. This method results in embryoid bodies which are more homogeneous as they are all formed from the same number of cells at the same time, although some variation remains. The final method which is frequently used for embryoid body formation is methylcellulose, where a single-cell suspension is seeded into semisolid methylcellulose media. The cells remain as single cells and each develops into an aggregate creating clonal embryoid bodies from single cells. There are a range of methods to make embryoid bodies with different advantages and disadvantages, the tehcnique can be chosen in order to match the needs of the experiment.

4.1.4 Aims

The goal of this chapter is to determine whether the formation of the primitive endoderm of an embryoid body would be a good model with which to investigate primitive endoderm specification and polarisation. This will hopefully also provide a clear description of the normal cell fate specification and cell polarisation of the primitive endoderm in embryoid bodies.

This aim can be split into three parts:

1. Establish optimum culture conditions of embryoid bodies to study primitive endoderm development.
2. Characterise the temporal pattern of cell fate specification of the primitive endoderm in an embryoid body
3. Observe the localisation of markers apico-basolaterally polarised during the development of the primitive endoderm in an embryoid body.

4.2 Results

4.2.1 Embryoid bodies formed in methylcellulose gradually develop into a polarised primitive endoderm

In order to use embryoid bodies as a model of epithelial specification and polarisation characterisation of their development was carried out. The first culture system used to make embryoid bodies was the semi-solid material methylcellulose. This method was chosen as it should produce homogeneous, clonal embryoid bodies as is regularly used in the haematopoietic field (Bone and Welham, 2007). Embryoid bodies were formed from individual mES cells using the semi-solid material methylcellulose and brightfield images taken (Figure 4.1). The embryoid bodies appeared round after 7 days of culture, by day 14 the embryoid bodies themselves were still a round shape, but a lot of cellular material was present around them (Figure 4.1). After 21 days of culture the embryoid bodies were more circular, but some were also smaller in size, and the cellular material surrounding them remained (Figure 4.1). This surrounding material suggested that they were not healthy.

To assess whether embryoid bodies would be a good model of primitive endoderm specification the localisation of Gata4, a transcription factor required for primitive endoderm development, was assessed using whole-mount immunostaining (Figure 4.2). On day 7 there were very occassionaly weakly positive nuclei for Gata4, by day 14 many of the nuclei were positive for Gata4. This suggests that the outer layer of cells of the embryoid body formed in methylcellulose were gradually became specified to the primitive endoderm fate.

Having established that the cells of the outer-layer of the embryoid body formed primitive endoderm cells by day 14, the localisation of proteins required for a polarised epithelium was determined using whole-mount immunostaining. This would help to determine if embryoid bodies would be a good model of epithelial polarisation as well as primitive endoderm specification. aPkcζ/γ localised in the nuclei of the outer layer of cells on day 7 as well as in the apical and lateral membranes (Figure 4.3A). By day 14 aPkcζ/γ was restricted to the apical surface (Figure 4.3A). On both days 7 and 14 the tight junction protein Zo-1 predominantly localised at an apical puncta between the apical and basal domains of the outer cells of the embryoid body. From the aerial view Zo-1 formed a defined line around the periphery of each of the outer cells (Figure 4.3B). On day 14, an additional tight junction protein Occludin localised in a similar position to Zo-1 at an apical puncta between the apical and basal domains of the outer cells (Figure 4.4). β-catenin, and E-cadherin also both localised at the lateral side

of the cells in the outer-layer (Figure 4.4). Suggesting that by day 14 the cells in the outer layer were apcio-basolaterally polarised.

The localisation of aPkcζ/γ and Zo-1 demonstrates that the outer cell layer was partly polarised on day 7. By day 14 aPkcλ/γ, Zo-1, Occludin, β-catenin and E-cadherin were localised in a localisation that suggested that the outer cell layer was at this time point apico-basolaterally polarised. This highlights that this could be a good model to capture the process of epithelial polarisation, and maintenance of polarisation in the primitive endoderm.

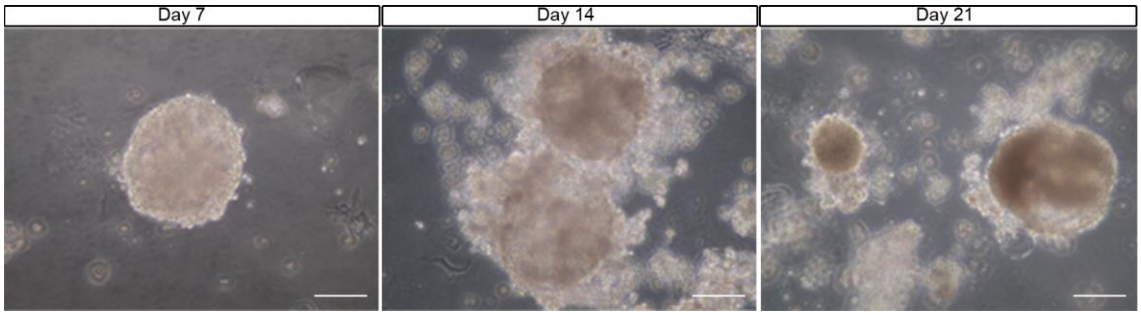


Figure 4.1: Embryoid bodies grown in methylcellulose formed round aggregates of cells but an increasing amount of cell debris was present.

Embryoid bodies were produced from R63 mES cells in methylcellulose. Development of the embryoid body was monitored over 21 days. Light microscopy images show the gradual increase in size and roundedness of the embryoid bodies. There was also an increase in the amount of debris present around the embryoid bodies. Representative images from at least three independent experiments are shown. Scale bars 100µm.

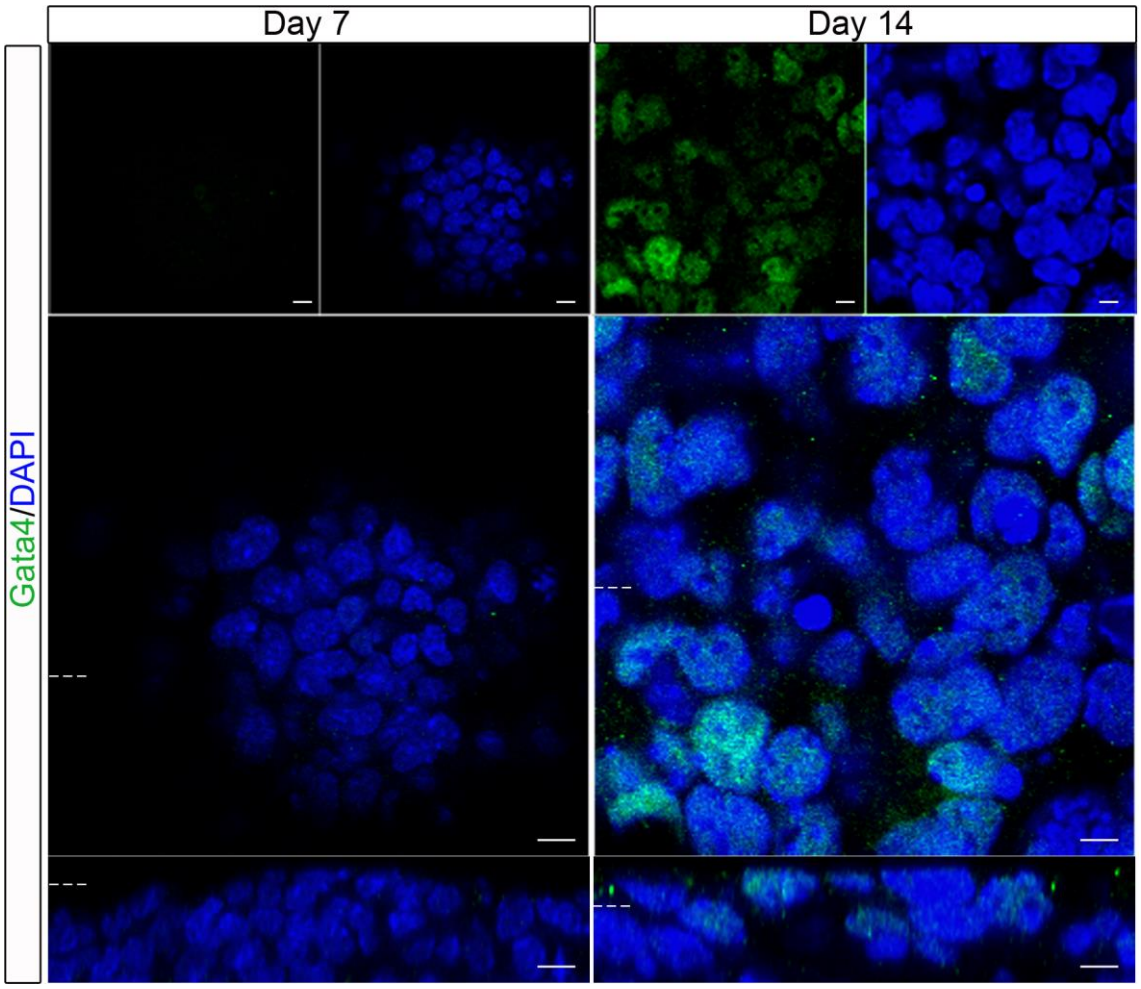


Figure 4.2: Gata4 expression increased over time in embryoid bodies cultured in methylcellulose

Embryoid bodies were produced from R63 mES cells in methylcellulose. Development of the embryoid body was monitored over 14 days. Whole-mount immunostaining shows localisation of Gata4. Very few nuclei expressed Gata4 on day 7, whilst many nuclei were positive by day 14. Representative images from two independent experiments are shown. Dotted lines represent position that the relevant orthogonal or aerial images were taken. Scale bars 10µm.

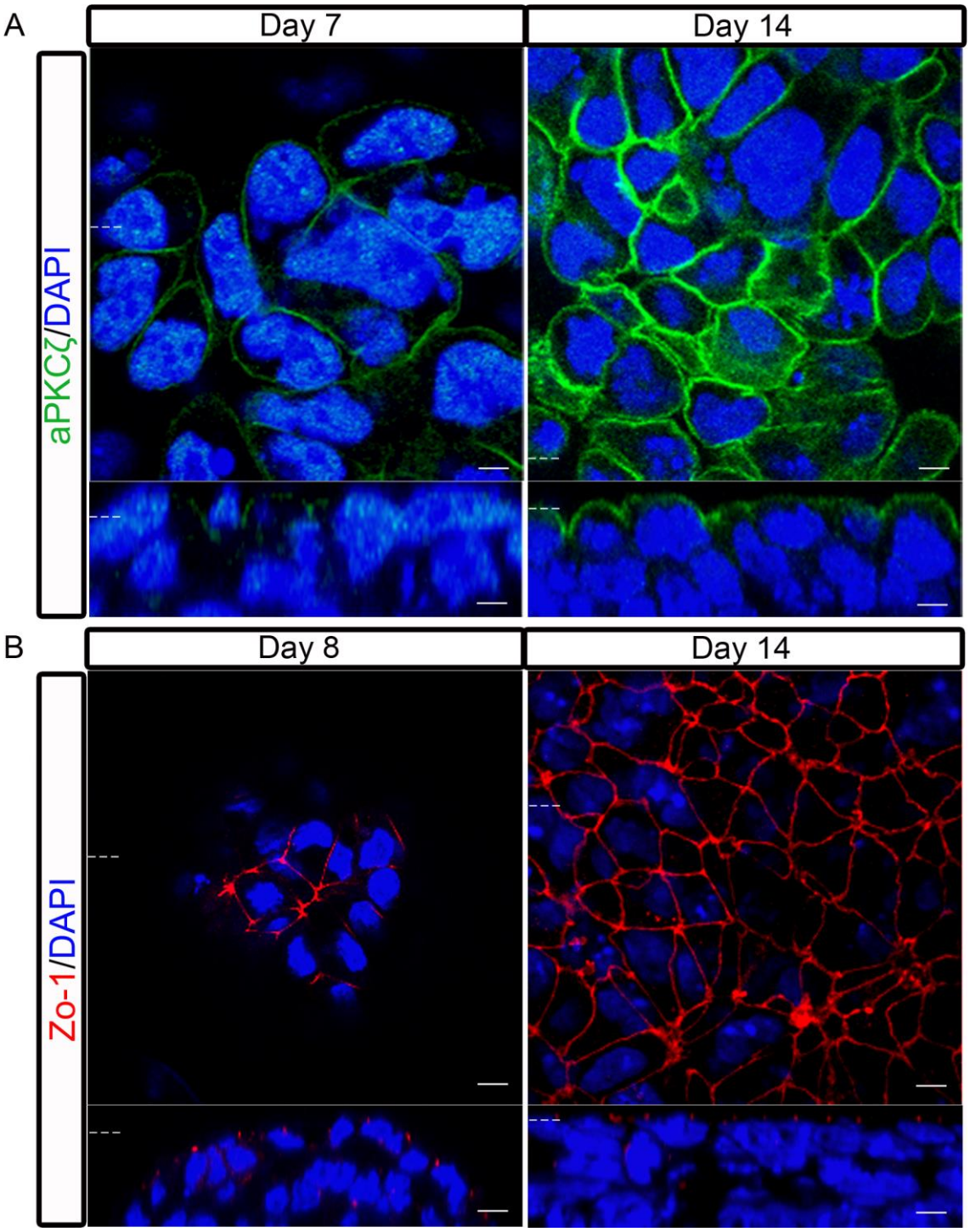


Figure 4.3: Embryoid bodies grown in methylcellulose gradually developed apico-basolateral polarity

Embryoid bodies were produced from R63 mES cells in methylcellulose. Development of the embryoid body was monitored over 14 days. Localisation of proteins which show apico-basolateral polarity in epithelia was examined in the primitive endoderm layer of embryoid bodies using whole-mount immunostaining. (A) aPkcζ/γ showed predominantly a nuclear localisation on day 7, but was apically localised on day 14. (B) The tight junction protein Zo-1 showed a polarised localisation on both day 7 and 14. Representative images from two independent experiments are shown. Dotted lines represent position that the relevant orthogonal or aerial images were taken. Scale bars 10µm.

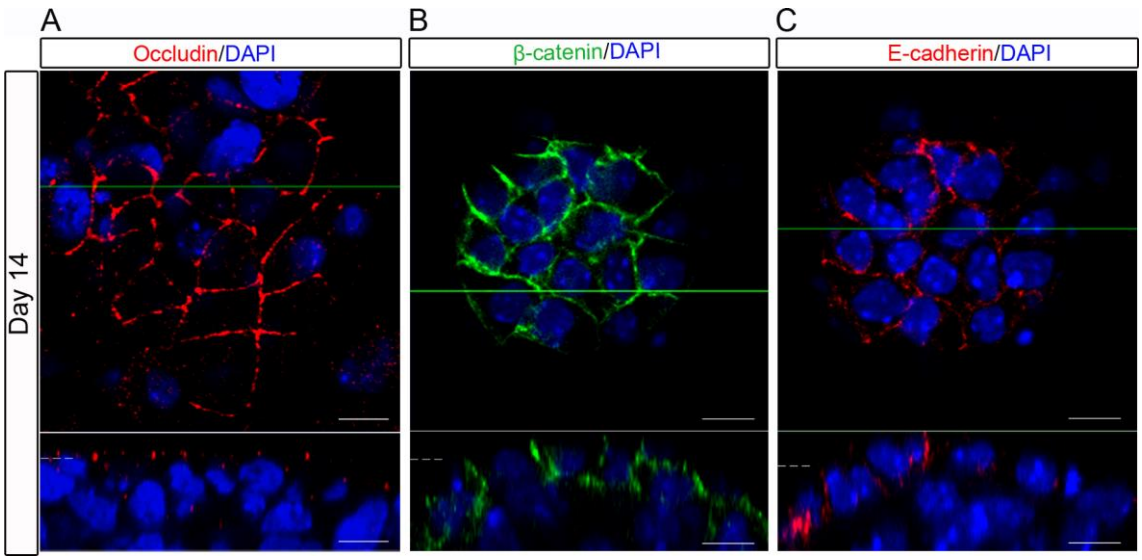


Figure 4.4: Embryoid bodies grown in methylcellulose were apico-basolaterally polarised by day 14.

Embryoid bodies were produced from R63 mES cells in methylcellulose. Development of the embryoid body was monitored after 14 days in culture. Localisation of proteins which show apico-basolateral polarity in epithelia was examined in the primitive endoderm layer of embryoid bodies using whole-mount immunostaining. (A) Occludin was present at a puncta at the border of the apical and lateral membrane of cells in the outer layer of the embryoid body. (B) β-catenin and (C) E-cadherin both localised at the lateral membrane of cells on the outer layer of the embryoid body. Representative images from one experiment is shown. Dotted lines represent position the aerial position was take. Green line represents position the orthogonal image was taken. Scale bars 10µm.

4.2.2 Embryoid bodies cultured in hanging drops have a defined histological epithelial cell layer

Information obtained from the embryoid bodies grown in methylcellulose suggested that this would be a good model system to study both epithelial polarisation and the fate specification of the primitive endoderm. However, when they were grown in the methylcellulose a lot of debris was present and the embryoid bodies looked increasingly less healthy, and even shrink. Additionally, when culturing the embryoid bodies in this way they required culturing for at least 14 days, and perhaps even 21 days to observe expression of genes expressed in the primitive endoderm later than Gata4 such as Hnf4α. Culture of embryoid bodies in an alternative system, a hanging drop was therefore attempted. This technique forms an embryoid body from 1000 cells which are within a drop on an inverted lid.

The morphology of the hanging drop embryoid body was assessed using brightfield microscopy and histological analysis. Hanging drop embryoid bodies on day 3 were round and had a fairly homogeneous morphology (Figure 4.5). From the sections it could be seen that the outer layer of cells was distinct from the inner cells (Figure 4.5 shown by arrow). The inner cells had large nuclei, little cytoplasm and were homogeneous suggesting that they had not differentiated (Figure 4.5).

Over time the embryoid bodies became larger, and the outer layer became more defined as a continuous squamous epithelium (Figure 4.5 Arrow). On day 5 small holes appeared within the embryoid bodies, these holes appeared larger at later time points suggesting cavitation (Figure 4.5 shown by *). Additionally, nuclei of the inner cells became smaller (Figure 4.4 shown by >) and there were also anuclear areas suggesting cell differentiation or death (Figure 4.5 shown by #). Over time, the embryoid bodies showed increased heterogeneity in gross morphology, and at day 7 and 10 balloon-like cysts were present (Figure 4.5), which resemble those observed previously (Martin et al., 1977).

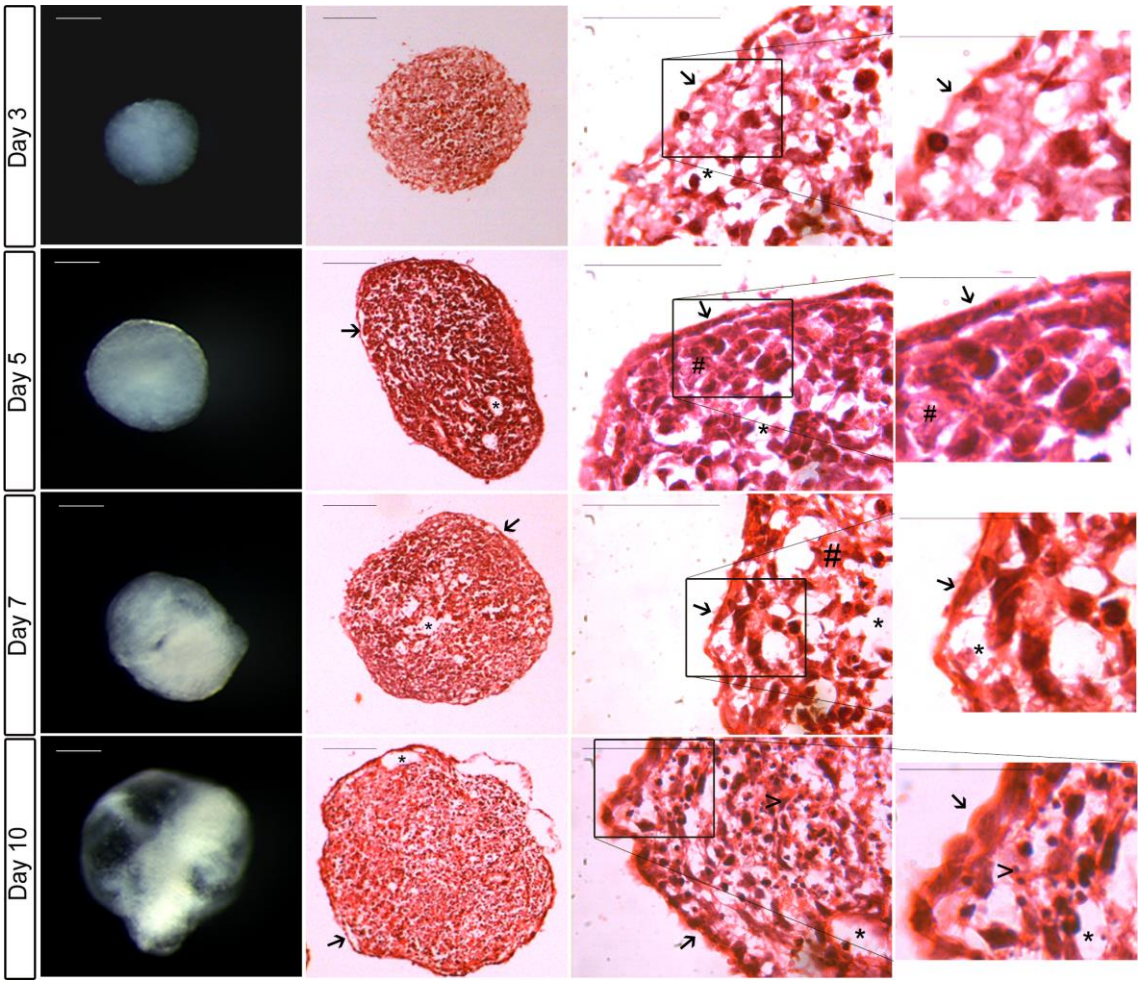


Figure 4.5: Hanging drop embryoid bodies became larger, more heterogeneous and cavitated during development

Embryoid bodies were formed from R63 mES cells using the hanging drop method. (First column) Light microscopy shows the gradual increase in size of the embryoid bodies as well as increased heterogeneity, loss of circularity and formation of cystic cavities at later timepoints. (Second, Third and fourth columns) Haematoxylin and eosin staining of cryosections shows a more defined epithelial outer layer (arrow) over time, as well as the appearance of small holes (*) anuclear areas (#) and small nuclei (>) at later time points. Representative images from at least independent experiments are shown. Scale bars: First column 200µm, Second column 100µM, Third column 50µm, Fourth column 25µm.

4.2.3 Embryoid bodies express primitive endoderm cell fate specification markers by day 7

Having observed the histological development of the hanging drop embryoid bodies, whole-mount immunostaining was used to characterise the temporal development of their primitive endoderm. A small percentage of nuclei were positive for the primitive endoderm fate markers Gata6, Gata4 or Hnf4α on day 3 (Figure 4.6A). As the embryoid bodies grew, the number of nuclei which expressed these markers increased, reaching a peak on day 7 (Figure 4.6A). Additionally, expression of the pluripotency protein Nanog decreased (Figure 4.6B). It was expressed in approximately 35% of nuclei on day 3, this fell to 7% on day 5 and no cells expressed Nanog by day 7.

Expression of Gata6 and Gata4 protein was also assessed using western blotting (Figure 4.7). Gata6 expression slightly decreased on day 3 in comparison to day 0, but on day 5 increased to almost double the levels present on day 0 (Figure 4.7A). There was subsequently a decrease in expression levels, on day 10 Gata6 expression was the same as that observed on day 0 (Figure 4.7A). The pattern of expression of Gata4 is very similar to that of Gata6, on days 0 and 3 the expression levels were the same, but on day 5 the expression increased approximately two-fold (Figure 4.7B). The expression levels of Gata4 then decreased, and on day 10 the protein levels were slightly reduced in comparison to day 0 (Figure 4.7B). However, the only statistically significant change was the increase in expression of Gata6 on day 5 in comparison do day 0. Therefore there is expression of these proteins in undifferentiated mES cells, perhaps due to spontaneous differentiation, but expression increases during embryoid body formation.

Increasing expression of the primitive endoderm fate markers (Gata6, Gata4 and Hnf4α), and loss of the pluripotency marker Nanog suggests that the outer layer of the embryoid body was undergoing a developmental program similar to that observed in the embryonic primitive endoderm. Strong Gata4 expression was observed in hanging drop embryoid bodies by immunostaining on day 7, whilst in methylcellulose embryoid bodies Gata4 was not strongly expressed until day 14. Additionally the hanging drop embryoid bodies remained healthy throughout the period of time their development was investigated. This suggests that culture of embryoid bodies in hanging drops rather than methylcellulose may provide a good model of primitive endoderm specification.

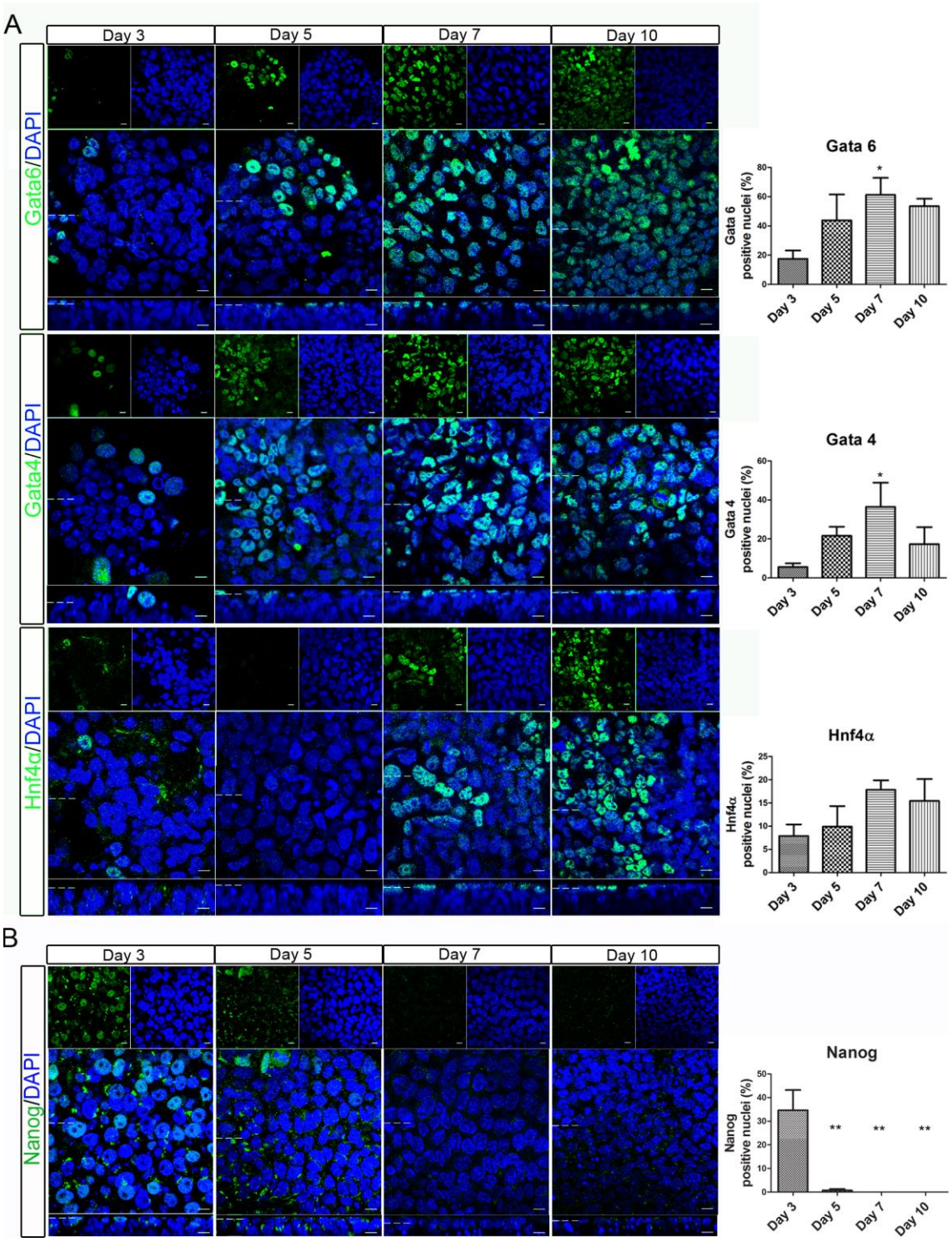


Figure 4.6: The number of nuclei expressing primitive endoderm cell fate markers gradually increased in hanging drop embryoid bodies.

Embryoid bodies were produced from R63 mES cells using the hanging drop method. Development of the embryoid body was monitored over ten days. Whole-mount immunostaining shows nuclear localisation of Gata6, Gata4, and Hnf4α on days 3, 5, 7, and 10 of embryoid body development. The percentage of positive nuclei for each protein is shown graphically. The number of positive nuclei increased, reaching a maximum on day 7. Data is from at least 3 independent experiments, error bars are SEM. Statistical analysis is a one-way Anova with a Dunnett's post-hoc test (* P=0.1-0.5, ** p=0.001-0.01, *** p<0.001). Dotted lines represent position that the relevant orthogonal or aerial images were taken. Scale bars 10µm.

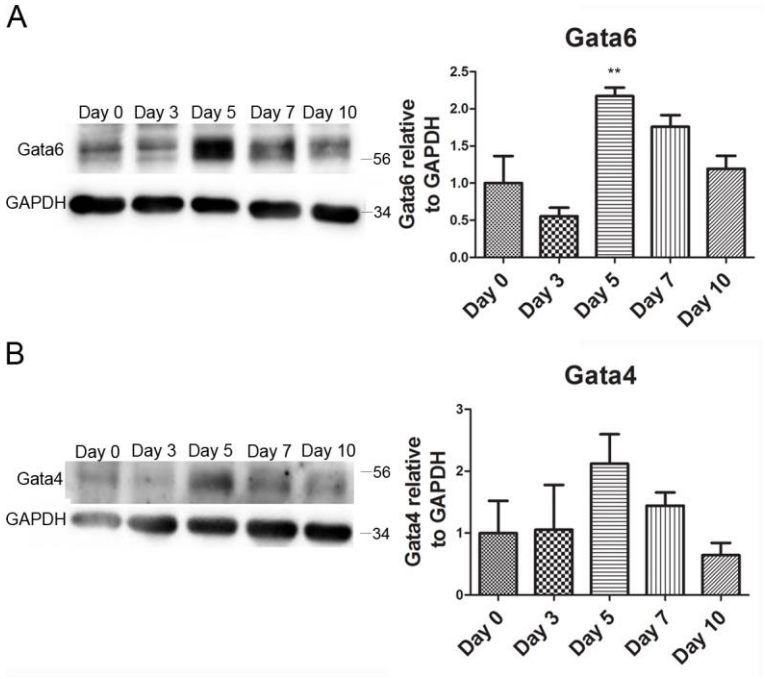


Figure 4.7: Expression of Gata6 and Gata4 in hanging drop embryoid bodies peaked on day 5 and subsequently decreased

Embryoid bodies were produced from R63 mES cells using the hanging drop method. Development of the embryoid body was monitored over ten days. Western blotting shows that expression of (A) Gata6 and (B) Gata4 increased to a peak on day 5, and then gradually decreased until day 10. A representative blot and quantification from 3 independent experiments is shown. Error bars represent SEM. Statistical analysis is a one-way Anova with a Dunnett's post-hoc test (* P=0.1-0.5, ** p=0.001-0.01, *** p<0.001).

4.2.4 The trophoblast marker Cdx2 is expressed in the outer-layer of the embryoid body

Having observed expression of transcription factors expressed in the primitive endoderm in the outer layer of the embryoid body I wanted to determine if trophoblast cells were also forming. To do this the expression of Cdx2, a transcription factor expressed during trophoblast development was examined.

Perhaps surprisingly it was observed by whole-mount immunostaining that Cdx2 expressing cells were present in the outer layer of the embryoid body. Cdx2 was expressed at low levels in the outer layer of cells on day 3, and then in an increasing number of nuclei, reaching a peak on day 7 (Figure 4.8). By day 10 the number of positive nuclei decreased. The percentage of Cdx2 positive nuclei in the outer layer was lower than the number of Gata6 positive nuclei suggesting that the predominant cell type in the outer layer was the primitive endoderm, not the trophoblast.

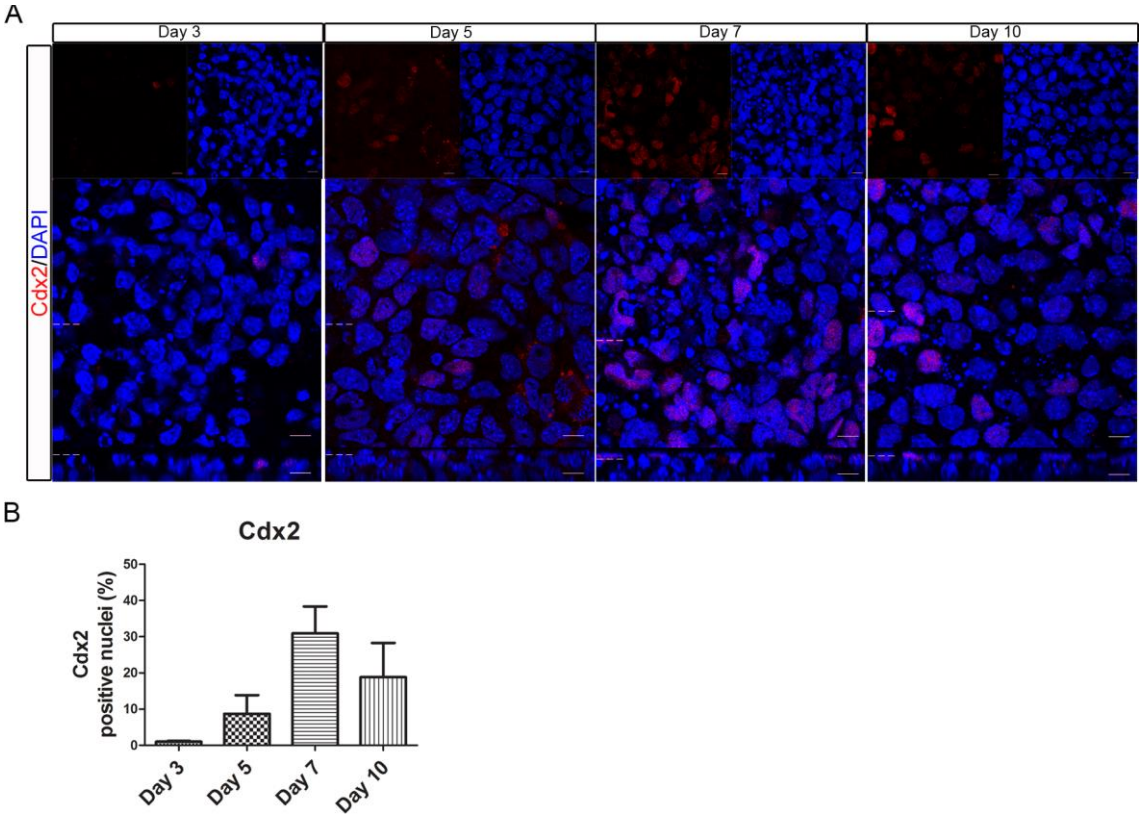


Figure 4.8: The trophoblast cell fate marker Cdx2 was gradually expressed in an increasing number of nuclei of the outer-cell layer.
Embryoid bodies were produced from R63 mES cells using the hanging drop method. Development of the embryoid body was monitored over ten days. (A) Whole-mount immunostaining showed a nuclear localisation of Cdx2 on days 3, 5, 7 and 10 of embryoid body development. (B) The percentage of Cdx2 positive nuclei in the outer cell layer are shown graphically. The number of positive nuclei increased, reaching a maximum on day 7. Data is from 3 independent experiments (Day 3, 7, and 10), and 2 independent experiments (Day 5). Error bars are standard error of the mean (SEM). Statistical analysis is a one-way Anova with a Dunnett's post-hoc test (* P=0.1-0.5, ** p=0.001-0.01, *** p<0.001). Dotted lines represent position that the relevant orthogonal or aerial images were taken. Scale bars 10µm.

4.2.5 The N-terminal Rassf protein Rassf8 is expressed in the nuclei of cells in the outer layer of the embryoid body

Previous work in the Chalmers lab has investigated the role of Ras-association domain family (Rassf) proteins (Recino et al., 2010; Sherwood et al., 2008). The defining feature of this family is the presence of the RA (Ras association) Ras binding domain (Sherwood et al., 2010). Little is known about Rassf8, but a role for dRassf8 in the regulation of adherens junction in *Drosophila* has been identified (Langton et al., 2009). dRassf8 interacts with dASPP protein, this complex regulates the ability of dCsk to phosphorylate Src42A. Src42A itself promotes adherens junction remodelling through E-cahderin and Armadillo. Furthermore, Rassf8 has been shown to colocalise with adherens junction components in non-small cell lung cancer cell lines (Lock et al., 2010).

As there is evidence for a role for Rassf8 in adherens junctions within epithelia whole-mount immunostaining was carried out on the embryoid bodies to determine whether Rassf8 was expressed in the outer layer, and where it was localised (Figure 4.9). Surprisingly, whole-mount immunostaining showed a nuclear localisation of Rassf8 in the outer-layer of cells in the embryoid body suggesting it wasn't associated with the adherens junction in this epithelium (Figure 4.9A). Quantification of the number of nuclei expressing Rassf8 showed that over time the number of positive nuclei increased, reaching almost 50% on day 10 (Figure 4.9B). Some regions of the embryoid body had a high number of positive nuclei, whilst others had very few positive nuclei. This suggests that Rassf8 is expressed in nuclei of cells in the outer-layer of the embryoid body and may therefore be involved in primitive endoderm development. The Rassf8 antibody was used in western blotting to characterise the antibody and confirm the changes in protein expression. Many non-specific bands of a similar molecular weight to Rassf8 were present with the antibody preventing any anlsysi of protein expression levels, and raising the question of the specificity of the antibody. This antibody has however been used in the study of Rassf8 in non-small cell lung cancer cell lines, after Rassf8 RNAi the western blot band disappeared which suggests that this antibody is specific (Lock et al., 2010). Use of an alternative antibody which binds to a different epitope did not produce any positive results in immunostaining, and also produced many non-specific bands in western blot.

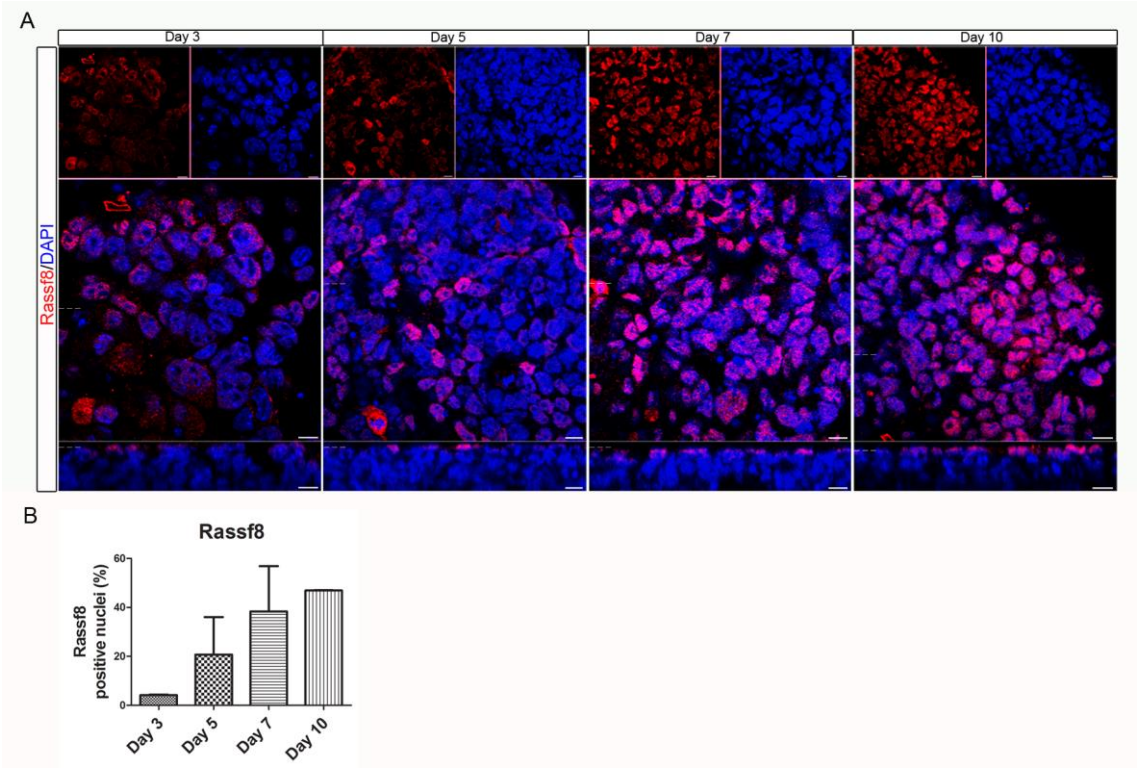


Figure 4.9: The N-terminal Rassf protein Rassf8 was expressed in the nuclei of cells in the outer-layer of embryoid bodies

Embryoid bodies were produced from R63 mES cells using the hanging drop method. Development of the embryoid body was monitored over ten days. (A) Whole-mount immunostaining showing nuclear localisation of Rassf8 on days 3, 5, 7 and 10 of embryoid body development. (B) The percentage of Rassf8 positive nuclei in the outer cell layer are shown graphically. The number of positive nuclei increased, reaching a maximum on day 10. Data is from 2 independent experiments. Error bars are SEM. Dotted lines represent position that the relevant orthogonal or aerial images were taken. Scale bars 10µm.

4.2.6 Primitive endoderm of the embryoid body develops apico-basolateral polarity by day 5

The previous work in this chapter suggested that the hanging drop embryoid bodies appear to be primarily following a developmental pathway which recapitulates primitive endoderm development. As polarity has been shown to change as the primitive endoderm forms (Saiz et al., 2013), whole-mount immunostaining was used to observe the localisation of commonly used markers of epithelial polarisation in embryoid bodies. After 3 days of culture, the embryoid bodies showed expression of aPkcζ/λ in the cytoplasm of the outer layer of cells (Figure 4.10A). By day 5, aPkcζ/λ localisation was mostly apically restricted across the whole of the outer surface of cells of the embryoid body, this localisation remained unchanged on day 7 and 10. Zo-1 was localised to the border between the apical and lateral side of the epithelial cells, forming rings which outlined the cells by day 3 (Figure 4.10B). By day 5 the apical puncta were uniformly present in cells of the outer layer. Additionally the localisation of the tight junction protein Occludin was observed (Figure 4.10C). The quality of the immunostaining was not optimal but from the images collected it would seem that on day 3 the protein was localised in the cytoplasm of the outer layer of cells. By day 5 the protein was localised mostly at the membrane of the outer cell layers at a puncta between the apical and basal compartments, there was also protein localised in the cytoplasm. On days 7 and 10 the staining observed at the membrane was stronger than on day 5, but Occludin was still localised in the cytoplasm and some was apical of the outer cell layer.

β-catenin, an adherens junction protein was predominantly present at the lateral side of cells of the outermost cell layer from day 3, but in some areas was also apically localised (Figure 4.11A). By day 5, β-catenin was consistently localised to the lateral side of the outer-most cell layer. The localisation of the adherens junction protein E-cadherin was also observed (Figure 4.11B). The immunostaining for this protein was not very clear though so it was difficult to precisely define its localisation. On day 3 the protein seemed to be predominantly localised in the lateral membrane of the cells, but was also present basal of the outer-layer of cells. By day 5 and 7 the protein was localised mostly in the lateral membrane of the cells, although it remained in the cytoplasm, or apical membrane of some cells. On day 10, the E-cadherin protein was localised mostly to the lateral membrane of the outer-layer of cells.

Fibronectin is a basement membrane protein, on day 3 it was localised to patches directly below the outermost cell layer, and deeper into the embryoid body (Figure 4.12A). This localisation gradually became more specific, by day 5 the staining was more restricted to the

basal side of the outermost cell layer, whilst there were still patches of apical Fibronectin. By day 7 the Fibronectin was restricted to below the basal-side of the outermost cells and no staining was observed in cells below the outer cell layer. Additionally, the localisation of the basement membrane protein Laminin was investigated (Figure 4.12B). The protein was localised around all membranes of the outer layer of cells on day 3. On days 5 and 7 it became increasingly more restricted to the apical membrane. By day 10 the Laminin was exclusively restricted to the apical of the outer layer of the cells. The absence of Laminin from the basement membrane, and its apical localisation is surprising (See discussion).

The gradual localisation of the apico-basolaterally polarised proteins (aPkcζ/γ, Zo-1, Occludin, β-catenin, E-cadherin and Fibronectin) shows that the epithelium surrounding the embryoid body began to polarise on day 3 and had clear polarisation by day 5. This suggests that the outer layer of cells of the embryoid bodies grown in hanging drops would be a good model of epithelial polarisation as well as primitive endoderm cell fate specification.

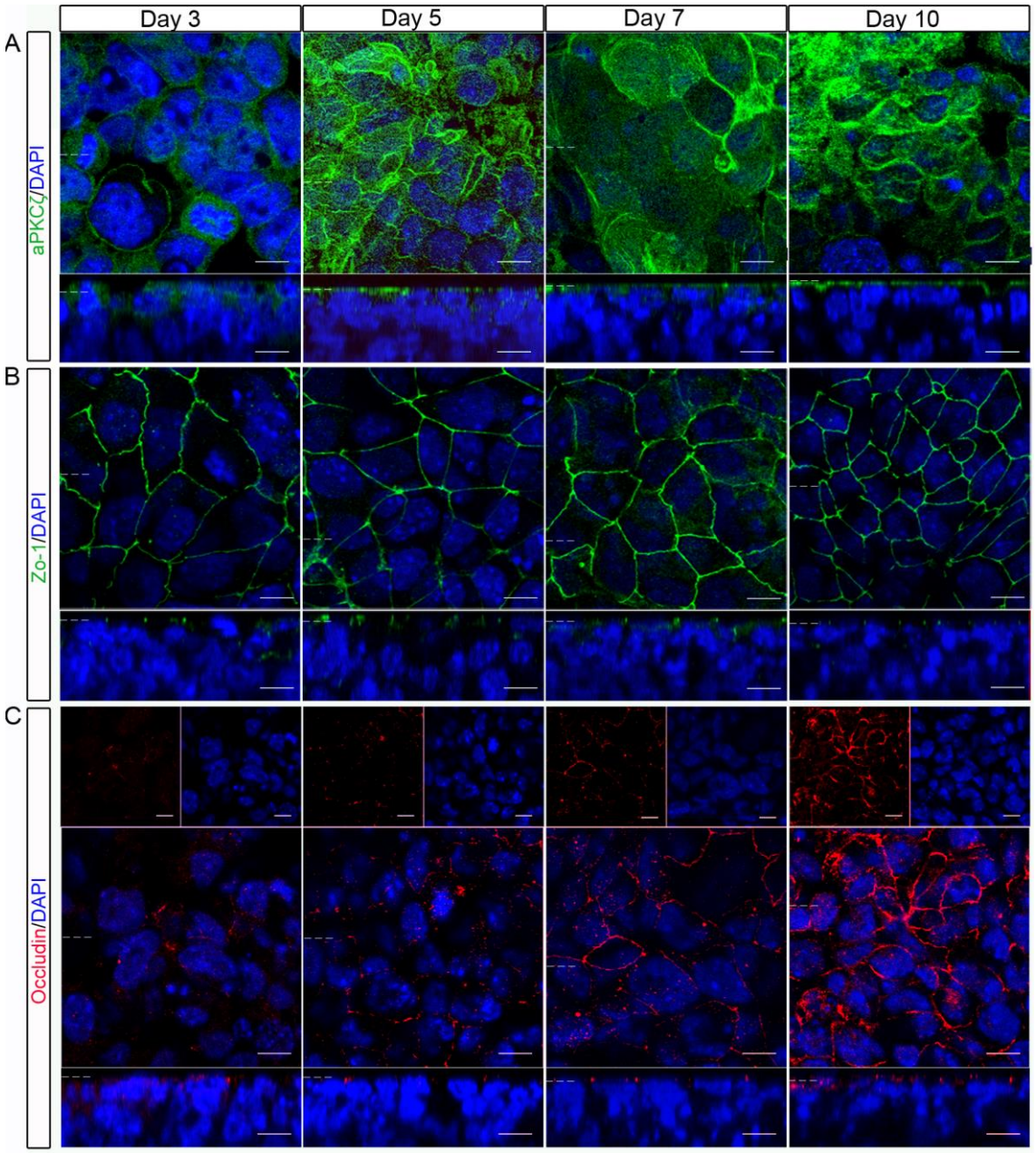


Figure 4.10: The outer-layer of embryoid bodies gradually developed polarised localisation of apical polarity and tight junction components

Localisation of proteins which usually show apical and junctional polarity in epithelia were examined in the primitive endoderm layer of embryoid bodies using whole-mount immunostaining. (A) The apical polarity complex protein aPkcζ/λ shows cytoplasmic localisation on day 3, but was apically localised from day 5. Tight-junction protein (B) Zo-1 showed a polarised localisation from day 3 onwards. (C) Occludin showed a polarised localisation from day 5. Representative images from at least three independent experiments are shown. Dotted lines represent position that the relevant orthogonal or aerial images were taken. Scale bars 10μm.

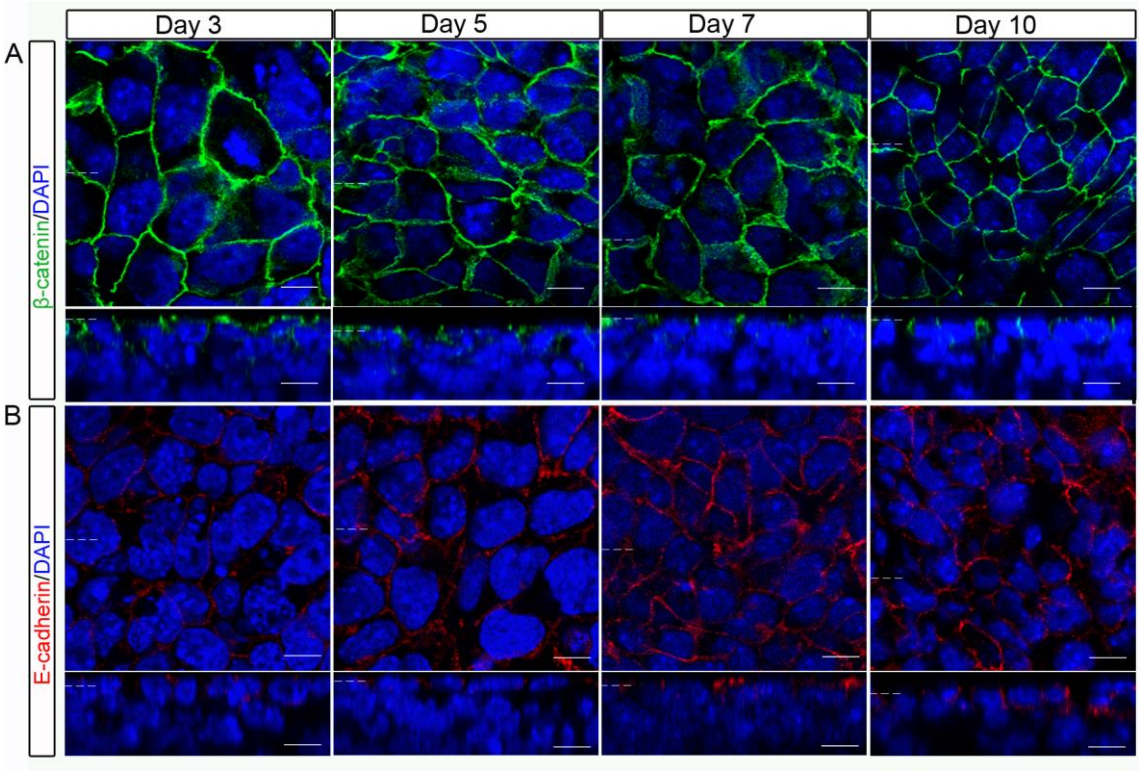


Figure 4.11: The outer-layer of embryoid bodies gradually developed a polarised localisation of adherens junction proteins

Localisation of adherens junction proteins which are usually localised at the lateral membrane in epithelia were examined in the primitive endoderm layer of embryoid bodies using whole-mount immunostaining. (A) β-catenin showed apical and basolateral localisation at day 3, but by day 5 became more restricted to the lateral sides of cells. (B) E-cadherin was localised in disorganised puncta on day 3, by day 5 a predominantly lateral localisation was observed and maintained throughout development. Representative images from at least three independent experiments are shown. Dotted lines represent position that the relevant orthogonal or aerial images were taken. Scale bars 10µm.

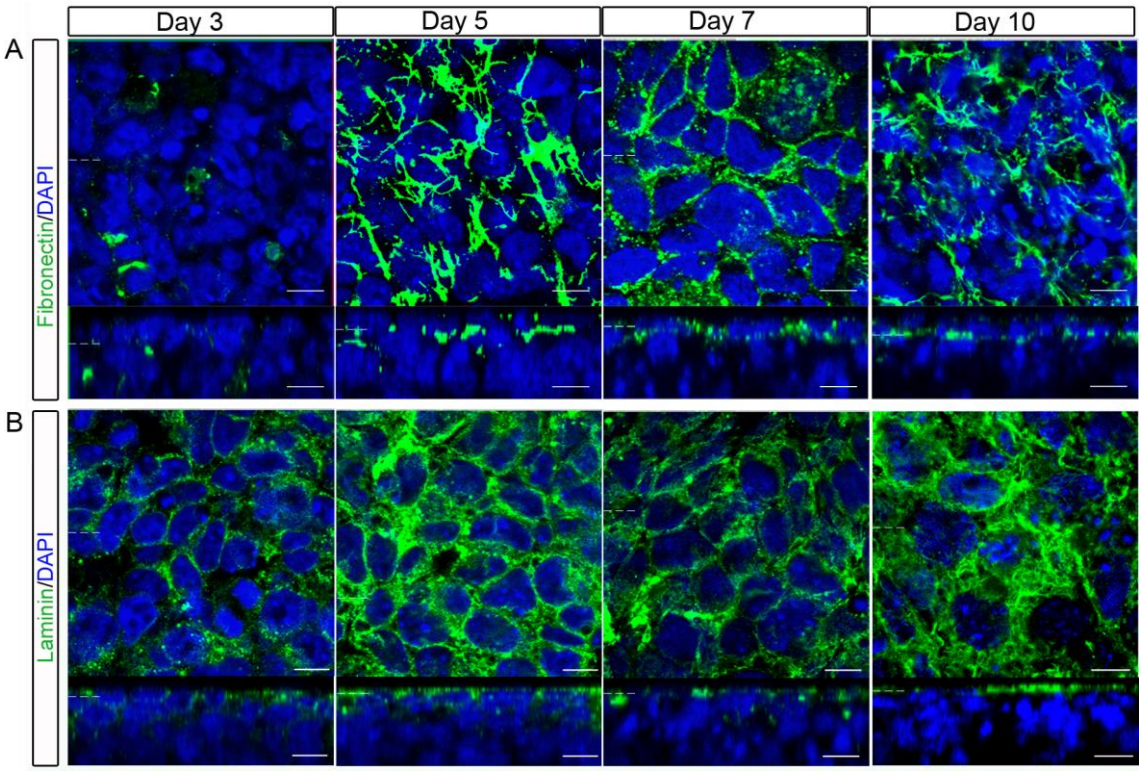


Figure 4.12 The outer-layer of embryoid bodies gradually developed an organised Fibronectin basement membrane, but not Laminin

Localisation of basement membrane proteins were examined in the primitive endoderm layer of embryoid bodies using whole-mount immunostaining. (A) The basement membrane protein Fibronectin formed aggregates on day 3, but from day 5 to day 10 showed gradually increasing staining at the basal side of the outer layer of cells. The epithelia remained polarised at 10 days. (B) The basement membrane protein Laminin was lateral and apically localised on days 3 and 5. After 7 and 10 days of culture the protein was predominantly localised apical to the outer cell layer. Representative images from at least three independent experiments are shown. Dotted lines represent position that the relevant orthogonal or aerial images were taken. Scale bars 10µm.

4.2.7 Epithelial polarity proteins are not synthesised de novo

Having observed the gradual polarisation of the primitive endoderm, western blotting was carried out to quantify their expression levels to allow comparison between changes in localisation with changes in expression levels. aPkcζ was expressed in pluripotent mES cells, the levels then changed little over the time course examined (Figure 4.13A). The adherens junction protein E-cadherin was expressed on day 0, the expression levels then gradually decreased over time, by day 10 E-cadherin protein expression was a third of that observed on day 0 (Figure 4.13B). The adherens junction protein β-catenin also showed a decrease in expression on day 3 which was not statistically significant, the expression did not subsequently change further, on day 10 the decrease was just over a half compared to day 0 (Figure 4.13C). Expression of Fibronectin, the basement membrane protein increased gradually over the 10 days, it was almost nine times higher on day 10 than on day 0. These western blots suggest that the aPkcζ/γ, E-cadherin, and β-catenin proteins may have been relocalised rather than synthesised *de novo*, whilst Fibronectin was synthesised *de novo*.

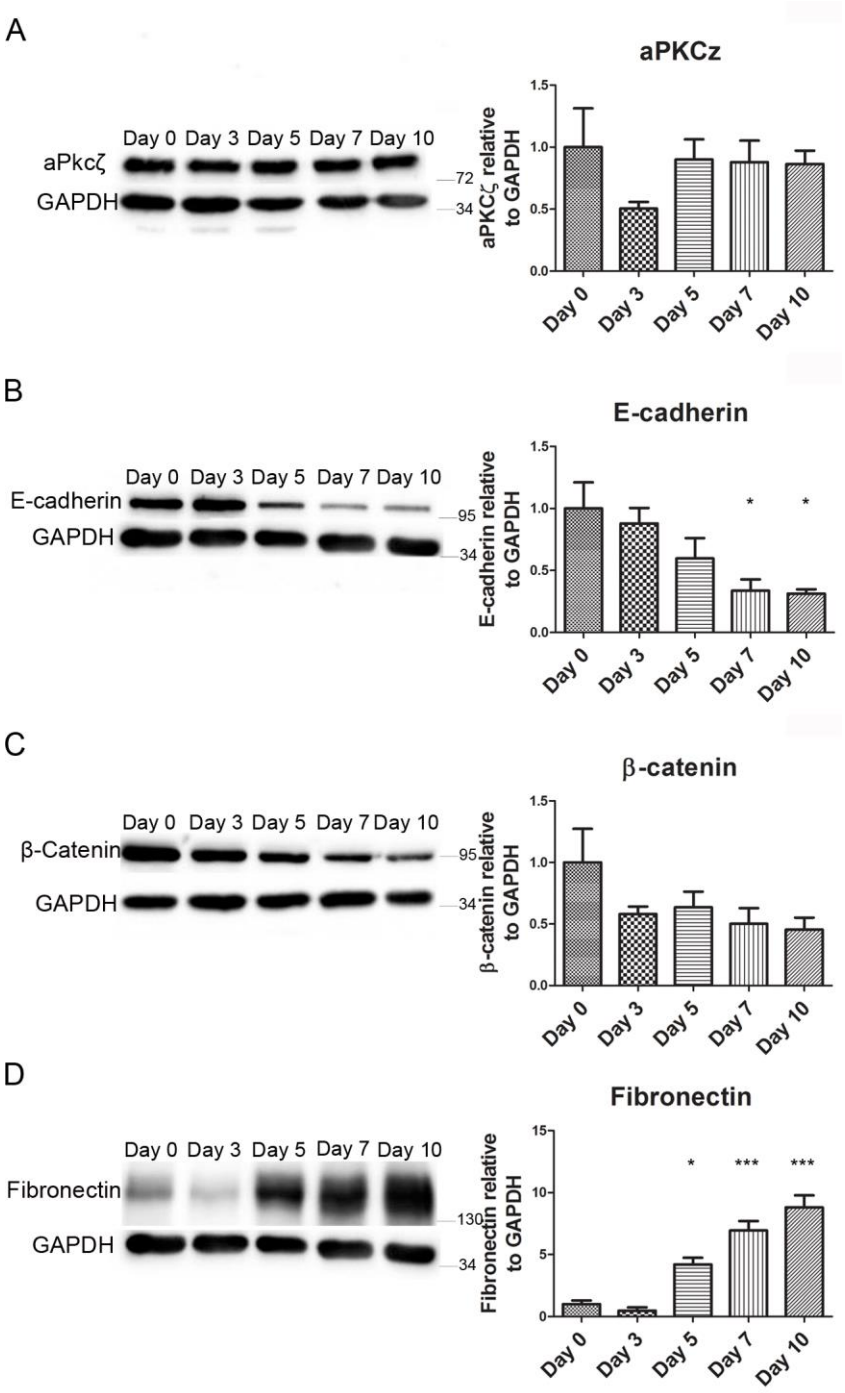


Figure 4.13: The apico-basolateral polarised proteins aPkcζ/λ, E-cadherin and β-catenin were not synthesised de novo during primitive endoderm development but Fibronectin was

Expression levels of apico-basolateral polarised proteins were determined across embryoid body development using western blotting. (A) Polarity complex protein aPkcζ/γ expression did not change markedly over time. Expression of adherens junction protein (B) E-cadherin decreased throughout the development of the embryoid body whilst (C) β-catenin decreased on day 3 in comparison to day 0 but did not then change. (D) Fibronectin, a basement membrane protein, expression increased over development. A representative blot and quantification from 3 independent experiments is shown. Error bars represent SEM. Statistical analysis is a one-way Anova with a Dunnett's post-hoc test (* P=0.1-0.5, ** p=0.001-0.01, *** p<0.001).

4.3 Discussion

4.3.1 Summary

Results presented in this chapter demonstrate that cells in the outer layer of embryoid bodies grown in methylcellulose did not express primitive endoderm cell fate specification markers until day 14. After this period of time the embryoid bodies appeared unhealthy, and a lot of debris was present in the cell culture media. Conversely, hanging drop embryoid bodies gradually polarised and expressed primitive endoderm cell fate specification markers over 10 days. This suggested that hanging drop embryoid bodies could be a good model of the development of the primitive endoderm epithelium. The cells of the outer layer expressed Zo-1 in an apico-basolateral localised position on day 3 in all cells of the embryoid body, whilst Nanog was still expressed. Additionally, all proteins examined except Occludin showed a polarised localisation on day 5 indictating that the cells are polarised. Maximal nuclear localisation of Gata6, Gata4, and Hnf4α occurred on day 7 suggesting primitive endoderm cell fate specification did not occur until day 7. This leads us to propose that polarisation of the outer cell layer of an embryoid body may commence prior to cell fate specification.

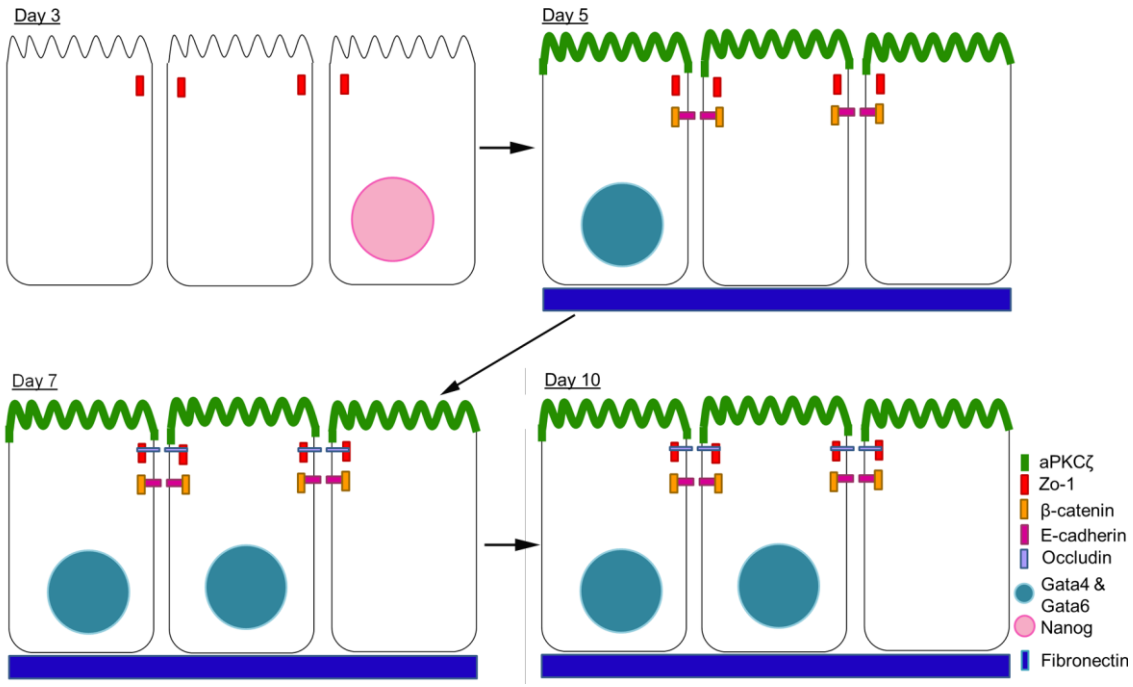


Figure 4.14: Epithelial polarity proteins began to polarise prior to maximal localisation of Gata4 and Gata6 in the outer layer of an embryoid body
Whole-mount immunostaining was carried out on hanging drop embryoid bodies after 3, 5, 7 and 10 days of culture to determine the localisation of epithelial polarity proteins and primitive enedoderm cell fate markers. A model was generated showing the order in which epithelial polarity proteins and primitive endoderm cell fate specification markers are localised.

4.3.2 Growth of embryoid bodies in methylcellulose with this protocol is not a useful model for study of primitive endoderm development

The data suggests that the embryoid bodies grown in methylceullose were not suitable as a model of primitive endoderm epithelial polarisation or specification for this project. Embryoid bodies produced in methylcellulose have previously been used to study development, particularly in the area of haematopoiesis (Bone and Welham, 2007; Wiles and Keller, 1991). Embryoid bodies formed in methylcellulose looked morphologically unhealthy when cultured for more than 7 days, they shrank in size, and began to lose their roundness which they had acquired during their development. This probably occurred because the embryoid bodies were cultured in the methylcellulose for the whole culture period, up to 3 weeks, without added nutrients, or removal of any waste products. Experiments were carried out to add serum or place the embryoid bodies in suspension culture after they had formed but this resulted in the embryoid bodies clumping together and therefore losing their homogeneity and clonal identity. If the embryoid bodies grown in methylcellulose had been used as a model system their health status could have influenced the results causing false conclusions to be drawn. Additionally, having to culture the embryoid bodies for as long as 14 days would not practically have been ideal. The formation of embryoid bodies in methylcellulose could be useful for future experiments if clonal identity was important, or to look at the earliest stages of polarisation by looking at additional time points between day 0 and day 14. These experiments did however demonstrate that use of embryoid bodies would be a powerful system with which to investigate both epithelial polarisation and primitive endoderm cell fate specification but an alternative culture method was required.

4.3.3 Hanging drop embryoid bodies are cavitated, cystic and form a continuous squamous epithelium

In comparison to the embryoid bodies formed in methylcellulose, those formed in hanging drops quickly formed large round balls of cells. This is likely to be because each embryoid body was formed in a drop containing 1000 cells, whilst the methylcellulose embryoid bodies were formed from a single cell. The histology showed the beginning of the appearance of cavities in the hanging drop embryoid bodies, and within the embryoid body cells appeared to be dying. There were also cysts present which resembled those previously observed (Martin et al., 1977).

The histological analysis of the embryoid bodies showed the appearance of an increasingly defined simple squamous epithelium. *In vivo* visceral endoderm overlying the distal pole of

the egg cylinder is squamous, whilst the proximal cells, overlying the extraembryonic ectoderm are cuboidal (Gardner, 1983). This therefore suggests that histologically the visceral endoderm which formed in these embryoid bodies is more similar to distal visceral endoderm than the proximal visceral endoderm. To further analyse the morphology of these cells Transmission electron microscopy (TEM) could be performed. The suggestion that this represents distal visceral endoderm is supported by analysis of the localisation of extraembryonic endoderm markers in embryoid bodies which concluded that the cells on the outer layer of an embryoid are most similar to distal visceral endoderm cells (Artus et al., 2010).

4.3.4 Hanging drop embryoid bodies recapitulate some aspects of development of the primitive endoderm

Having observed the formation of a clearly defined epithelium the identity of these cells was determined using whole-mount immunostaining for transcription factors essential for primitive endoderm cell fate specification. The expression of Gata6, Gata4, and Hnf4α in the nuclei of cells, and the loss of expression of nuclear Nanog in the outer layer suggests that it formed the primitive endoderm. We therefore propose that this would be a good model to study the gradual fate specification of this tissue. To confirm this in the future it could be useful to analyse the expression of other primitive endoderm markers such as Sox17, and Afp.

Embryoid bodies do not however recapitulate all aspects of primitive endoderm development. During mouse development the primitive endoderm is specified from the ICM during which time the embryo consists of only 64-100 cells. At this stage a trophoblast epithelium is present, within which the ICM and the blastocoel cavity resides (Saiz and Plusa, 2013). Conversely, the embryoid bodies were formed from drops of 1000 cells which formed a ball of cells from which the primitive endoderm is specified. An additional downfall is that the techniques used here do not allow analysis of the initial expression of Gata6 and Gata4, or the cell sorting observed during primitive endoderm formation in the embryo as this occurs deep within the embryoid body. The sorting has been analysed previously by others in embryoid bodies by doing cell mixing experiments to produce embryoid bodies formed from different genetically engineered, or wild type cells (Rula et al., 2007). They then analysed the sorting of cells using immunohistochemistry, and immunofluorescence staining of sections. Experiments performed in this thesis were carried out using whole-mount immunostaining and confocal microscopy which does not allow imaging deep in to the embryoid body. Whilst optimising imaging techniques immunofluoresecence staining of sections was attempted but proved difficult to produce reproducible results, or analyse the location of the cells within the embryoid body. Optimisation of this technique would however allow the system described

here to be used to investigate apects of the cell sorting during primitive endoderm development.

Western blotting analysis of the expression of the primitive endoderm transcription factors showed that the peak in the expression of Gata6 and Gata4 occurred on day 5 of their development. This was surprising as the peak in the number of positive nuclei was on day 7. There are two reasons why this may be. Firstly, the expression of the transcription factors in each individual nucleus may be high in day 5 embryoid bodies, after this time point more nuclei were positive but each nucleus may have expressed lower levels of the protein. An alternative reason for this discrepancy is that this analysis was taken from protein of the whole embryoid body, not just the outer-layer of cells. These transcription factors are known to be expressed during the development of other tissues (Arceci et al., 1993; Duncan et al., 1997; Morrisey et al., 1996). The western blot may therefore represent the temporal change in an alternative differentiation step of cells within the embryoid body rather than that of the primitive endoderm. To improve this analysis, and differentiate between these explanations it would be useful to sort the cells of the embryoid body using a FACS, and then perform western blotting. This would provide information on the protein expression levels within exclusively the primitive endoderm cells.

4.3.5 Cdx2 is expressed in the nuclei of cells in the outer-layer of an embryoid body

In addition to expression of primitive endoderm markers, expression of Cdx2 was also observed. Cdx2 is a transcription factor which is critical for trophoblast specification, and also has a role in the development of the gut. Its expression in embryoid bodies has not to my knowledge been previously described. The number of cells in the outer-layer which expressed it increased during embryoid body development and peaked on day 7, this is similar to the pattern observed with the primitive endoderm markers. Cdx2 is not restricted to the nuclei of cells of the outer-layer, but is also expressed in the deeper layers of the embryoid body which can be imaged with the confocal. This suggests that this Cdx2 positive population was not restricted to the outer-layer which the primitive endoderm markers were.

There are many experiments which could be performed to further define this population of cells. To identify if the primitive endoderm and Cdx2 expressing populations are the same or separate it would be interesting to observe whether the Cdx2 positive nuclei co-express any primitive endoderm markers. It would be expected that they do not as I have found no evidence in the literature for a physiological cell population which expresses Cdx2 and Gata6

and/or Gata4. It would also be interesting to determine whether these cells are following a trophoblast cell fate or an alternative lineage by observing whether other transcription factors required for the development of the trophoblast are also expressed in these Cdx2 positive cells. In hES cells and mouse epiblast stem cells a population of cells which co-express Brachyury and Cdx2 can be formed following addition of BMP (Bernardo et al., 2011). It may be that formation of embryoid bodies is also causing the production of this cell population from mES cells. Determining whether the cells co-express Brachyury may therefore help to identify what the identity of the Cdx2 positive cell population is.

4.3.6 Rassf8 is expressed in the nuclei of cells in the outer layer of the embryoid body

The localisation of the N-terminal Rassf protein Rassf8 to the nuclei of cells in the outer layer of the embryoid body was unexpected. Rassf8 has previously been suggested to be localised at the adherens junction (Langton et al., 2009; Lock et al., 2010). A nuclear localisation of Rassf8 has previously been observed in epithelial tumours but the function of nuclear Rassf8 has not been investigated to date (Falvella et al., 2006).

The increasing number of nuclei which express Rassf8 during the development of the embryoid body demonstrates that as the primitive endoderm develops the number of cells expressing the protein increase. This suggests that this protein may be required for primitive endoderm cell fate specification and/or polarisation. It would be useful to confirm the localisation of Rassf8 using immunoblotting for Rassf8 with lysates from different subcellular fractions. To investigate the role of Rassf8 in the primitive endoderm, derivation of $Rassf8^{-/-}$ mES cells was attempted but there was not sufficient time to characterise the cells. A full characterisation of the cells would confirm that they do not express Rassf8, and analyse their capacity to self-renew and their pluripotentcy. Having characterised the cells they could be used to make embryoid bodies, and analysis of the primitive endoderm cell fate specification and polarisation of the outer layer could be carried out.

4.3.7 Apico-basolateral polarisation can be interrogated using embryoid bodies

The polarised localisation of a number of proteins has been reported before in the primitive endoderm (Gerbe et al., 2008; Moore et al., 2009; Murray and Edgar, 2001; Rula et al., 2007; Yang et al., 2007). aPkc has been previously shown to localise at the apical membrane of the primitive endoderm of the mature E4.5 primitive endoderm (Saiz et al., 2013), and in embryoid bodies (Wu et al., 2007) as observed here too. Zo-1 was previously observed in the lateral membrane but in a less defined point than observed here (Wu et al., 2007). E-cadherin has

been previously been shown to localise basolaterally in the primitive endoderm of embryoid bodies which is consistent with my findings (Moore et al., 2009). This suggests that my description of the polarised localisation of these proteins is consistent with previous reports.

The localisation of Laminin apical to the outer-cell layer in the embryoid body is of great surprise. It has previously been shown to be localised in the basement membrane of embryoid bodies formed from embryonal carcimona cells (Grover et al., 1983) and has been shown to be required for the accumulation of a basement membrane, cavitation, and formation of an organised epithelium in embryoid bodies (Grover et al., 1983; Murray and Edgar, 2000, 2001). The antibody used to determine the localisation of Laminin here has been used by others in mouse tissue (Buniello et al., 2013), which suggests that it does recognise the correct protein. To confirm this localisation an alternative antibody could be tested in this model.

The localisation of multiple epithelial polarity proteins during primitive endoderm development has not previously been examined. The results presented here show that the primitive endoderm of an embryoid body gradually polarises in an order which is similar to that observed in other epithelia (Reviewed by (Martin-Belmonte and Perez-Moreno, 2012)). Zo-1, E-cadherin, and β-catenin localise at junctions at the initial stage of cell polarisation, followed by localisation of aPkc at the apical membrane, Occludin at the tight junction, and deposition of Fibronectin to form a basement membrane (Figure 4.14). This highlights the potential utility of this model system in the study of primitive endoderm polarisation. To extend this analysis it would be useful to look at other proteins which are apico-basolaterally localised in epithelia, such as additional tight junction, adherens junction, and polarity complex proteins. Additionally it would be useful to carry out the biotin assay used in Chapter 5 to analyse the epithelial barrier formation of the outer cell layer throughout the development of the embryoid body.

4.3.8 Epithelial polarity proteins are not synthesised de novo

Analysis of the expression of the apico-basolateral polarity proteins demonstrated that all of the polarity proteins were expressed in mES cells prior to embryoid body formation and that with the exception of Fibronectin their levels did not increase throughout the development of the embryoid body. This suggests that all the proteins examined, except Fibronectin had to be relocalised rather than synthesised *de novo.* Conversely the expression of Fibronectin increased markedly over the time which leads me to propose that this protein was synthesised during the development of the embryoid body.

Expression of β-catenin and E-cadherin decreased, there are two possible reasons for this. Firstly, mES cells have been shown to express these two proteins, whilst the deep differentiated cells within the embryoid body may not express these proteins. Formation of the embryoid body from mES, and the subsequent differentiation of these cells which expressed these proteins would therefore result in a decrease in the total protein levels present despite levels being maintained in the outer cells. Secondly, the embryoid body grew over time, which assuming this means there would be more cells, the primitive endoderm would therefore account for a smaller proportion of the total protein present and cause a decrease in the total protein of interest within an embryoid body.

4.3.9 Conclusion

A gradual polarisation and fate specification of the primitive endoderm in hanging drop embryoid bodies can be clearly observed. It therefore seems that the primitive endoderm of an embryoid body has a number of characteristics which will make it a valuable model for future experiments. It is a 3D model composed of a variety of non-transformed cell types, which gradually polarise and express cell fate markers. There are also a growing number of knockout and transgenic ES cell lines which could be studied in this system (Dolgin, 2011; Skarnes et al., 2011). In particular, embryoid bodies seem well suited for studying the relationship between polarisation and fate specification in a developing epithelium.

Chapter 5

5 Formation of a polarised primitive endoderm layer in embryoid bodies requires Fgfr/Erk signalling

5.1 Introduction

5.1.1 Regulation of cell fate specification and polarisation in the primitive endoderm

In chapter 4 the temporal order of cell fate specification and polarisation of the outer layer of cells was characterised in embryoid bodies cultured using the hanging drop culture method. These studies suggested that the outer-layer of the embryoid body gradually polarised, and that maximal expression of primitive endoderm cell fate specification markers occurred after the onset of polarisation. This suggested that embryoid bodies would make a good model to study the regulation of these processes which is the aim of this chapter.

Fgf4 activation of Fgfr signalling via the Raf/Mek/Erk signalling (Erk signalling) pathway has an important role in promoting primitive endoderm cell fate specification (Further detail in 1.4.5). In contrast to our growing understanding of cell fate specification, less is known about the mechanisms which regulate the polarisation of primitive endoderm cells. It is not yet known if the Erk signalling pathway is required for polarisation of the primitive endoderm. In this chapter I aim to inhibit signalling pathways and investigate their role in the polarisation of primitive endoderm cells.

5.1.2 Inhibitors of the Fgf receptor and Erk signalling

In order to investigate the requirement for Fgfr/Erk signalling in primitive endoderm cell fate specification and polarisation, small molecule inhibitors were used. Fgfr and Erk signalling is outlined in section 1.4.5.

To inhibit Fgf receptors in this study AZD-4547 was mainly used. AZD-4547 is an ATP-competitive tyrosine kinase inhibitor of Fgf receptors 1,2, and 3 (Chell et al., 2013; Gavine et al., 2012). AZD-4547 has an *in vitro* IC-50 (half maximal inhibitor concentration) of 0.2nM, 2.5nM and 1.8nM, and a cellular IC-50 of 12nM, 2nM and 40nM for Fgfr 1,2 and 3 respectively (Gavine et al., 2012). Its selectivity was assessed against many kinases, the next nearest kinase it inhibits is recombinant Vascular endothelial growth factor receptor 2 (Vegfr2) against which it has an IC-50 of 24nM showing that it is at least 9-fold more selective for Fgfr1,2 and 3 than any other kinases tested (Gavine et al., 2012). It inhibits Fgfr phosphorylation and therefore associated downstream signalling through Frs2, Plcγ, and Mapk (Gavine et al., 2012). In some experiments the Fgf receptor inhibitior PD-173074 was used (Figure 5.15). PD-173074 was

developed as an inhibitor of both Vegfr and the Fgf receptor 1 (Mohammadi et al., 1998). It has an *in vitro* IC-50 of 21.5nM against Fgfr1, and a cellular IC-50 of 1-5nM. The cellular IC-50 of PD-173074 for Vegfr2 is 100-200nM. This provides high selectivity as it inhibits the next nearest kinase Pdgfr, with an *in vitro* IC-50 of 17.6μM, and c-Src with an IC-50 of 19.8 μM.

To disrupt the Erk signalling pathway a Mek inhibitor, PD-0325901, was used. PD-0325901 is a non-ATP-competitive inhibitor of Mek developed by Pfizer (Sebolt-Leopold and Herrera, 2004). It is highly selective, demonstrated by its inability to inhibit many serine/threonine and tyrosine kinases (Sebolt-Leopold and Herrera, 2004). Its selectivity is thought to occur because it binds to a binding pocket in a region which is not homologous to other kinases (Sebolt-Leopold and Herrera, 2004). The PD-0325901 is also highly potent with an *in vitro* IC_{50} of 1nM against activated Mek1 and 2 (Sebolt-Leopold and Herrera, 2004), and 0.33nM IC_{50} in colon 26 cells (Barrett et al., 2008; Thompson and Lyons, 2005). *In vivo* PD-0325901 can causes a dose-dependent decrease in ppErk in liver and lung (Brown et al., 2007) and has shown anticancer activity in 6 of 7 human tumour xenografts models (Sebolt-Leopold and Herrera, 2004; Thompson and Lyons, 2005).

5.1.3 Pi3k signalling and its inhibition

Phosphoinositide 3-kinase (Pi3k) signalling begins with the activation of Pi3ks by activators (Vanhaesebroeck et al., 2010). There are 14 Pi3ks which are separated in to 4 classes (I – IV) (Gharbi et al., 2007). Pi3ks subsequently phosphorylate one of three different phosphatidylinositold lipids; PtdIns, PtdIns-4-phosphate, and PtdIns-4,5,bisphosphate (Vanhaesebroeck et al., 2010). The phosphorylated form of these three lipids is bound by many different effector proteins, regulating the localisation and function of the proteins. Lipid phosphatases degrade or interconvert 3-phosphoinoisitides thereby negatively regulating Pi3k signalling. Pi3k signalling is involved in many processes such as migration, survival and cell-cycle progression.

In this chapter LY-294002 a small molecule inhibitor of Pi3k is used. LY-294002 has an IC-50 against Pi3k of 1.4μM (Vlahos et al., 1994). LY-294002 inhibits all Pi3k classes as well as other off-target proteins such as mammalian target of rapamycin (mTor), Casein kinase 2 (Ck2) and Gsk3β (Gharbi et al., 2007). LY-294002 is used here as it is a good first inhibitor of Pi3k signalling to use before using isoform specific inhibitor.

5.1.4 Assays for epithelial barrier function

One of the main functions of epithelia is to provide a barrier to prevent movement of molecules across the epithelial cell layer (St Johnston and Ahringer, 2010). A loss of epithelial cell polarisation would be expected to cause a disruption in the barrier function. There are many ways to assess epithelial barrier function and its properties. One method to study the function of an epithelial barrier is by addition of a membrane-imperbeable biotin, which covalently links to amino groups on extracellular proteins in the membrane of an epithelium. The biotin can be visualised using steptavidin. In an intact barrier the biotin will only bind to proteins on the apical membrane, and not the lateral or basal membranes (Chalmers et al., 2006; Minsuk and Keller, 1997), an alternative approach is to determine the TER which is a measure of ion conductance (Buschmann et al., 2013). Ion-selectivity of the epithelial barrier can be determined by measuring PNa^{+}/PCl^{-}, whilst the size selectivity of the ionic barrier can be determined by measuring paracellular flux of other ions, with the same charge but of a different size. The paracellular flux of macromolecules, demonstrating the leakiness of the tight junction barrier can be determined by addition of FITC-dextran of different known molecular weights to determine how much dextran of each size can cross the barrier.

5.1.5 Aims

Having thoroughly characterised the cell fate specification and polarisation of the primitive endoderm in embryoid bodies in Chapter 4, in this Chapter I investigate the mechanisms which promote the polarisation of the primitive endoderm. This goal can be broken down in to two aims:

1. Investigate whether the Fgfr/Erk signalling pathway is required for polarisation of this tissue using a small molecule inhibitors of the Fgf receptor and the Erk signalling pathway.
2. Assess what effect inhibiting Pi3k signalling has on polarisation and cell fate specification of the primitive endoderm layer of the embryoid body.

5.2 Results

5.2.1 Inhibition of Mek with PD-0325901 results in a loss of Gata4 and Gata6 expression in embryoid bodies

To investigate the role of Mek signalling in cell polarisation PD-0325901, a potent Mek inhibitor (Brown et al., 2007; Sebolt-Leopold and Herrera, 2004) was added to the cell suspension (day 0) and analysis of the phenotype of the embryoid bodies was carried out after 7 days of development (Figure 5.1A). Diphosphorylated Erk1 and 2 (ppErk1/2) was markedly decreased (Figure 5.1B) demonstrating that, when added to the media PD-0325901 successfully inhibited Mek signalling.

There was a slight reduction in size of the embryoid bodies at the highest dose tested (4µM) in comparison to embryoid bodies treated with vehicle, but this was not statistically significant (Figure 5.1C). A statistically significant increase in the circularity of the embryoid body (Figure 5.1D) in comparison to control was observed suggesting that Erk signalling regulates the shape which the embryoid body forms.

To establish if the addition of PD-0325901 caused an increase in apoptosis, whole-mount immunostaining for cleaved Caspase-3 was performed (Figure 5.1E). No significant change in the number of nuclei positive for cleaved Caspase-3 was observed in the outer layer of the embryoid bodies showing that inhibiting Mek did not cause an increase in apoptosis that might have affected the development of these cells.

Disruption of Mek-signalling causes a loss of Gata6 and Gata4 expression, and a maintenance in Nanog expression preventing the cell fate specification of the primitive endoderm both *in vivo* and *in vitro* (Chazaud et al., 2006; Cheng et al., 1998). The expression levels of Gata6, Gata4 and Nanog were analysed to assess the effect Mek inhibition has in this system. Western blotting of embryoid bodies showed a dose-dependent decrease in Gata6 and Gata4 protein expression levels, resulting in very little protein remaining at the highest concentration of PD-0325901 (4µM) (Figure 5.2A & B). This result was confirmed using whole-mount immunostaining of Gata6 (Figure 5.2C). In contrast, the percentage of Nanog positive nuclei increased from 0% in control to an average of 76% upon addition of 4µM PD-0325901 (Figure 5.2D). This confirms that upon inhibition of Mek the cells of the outer-layer of the embryoid body failed to become primitive endoderm cells, but instead remained pluripotent.

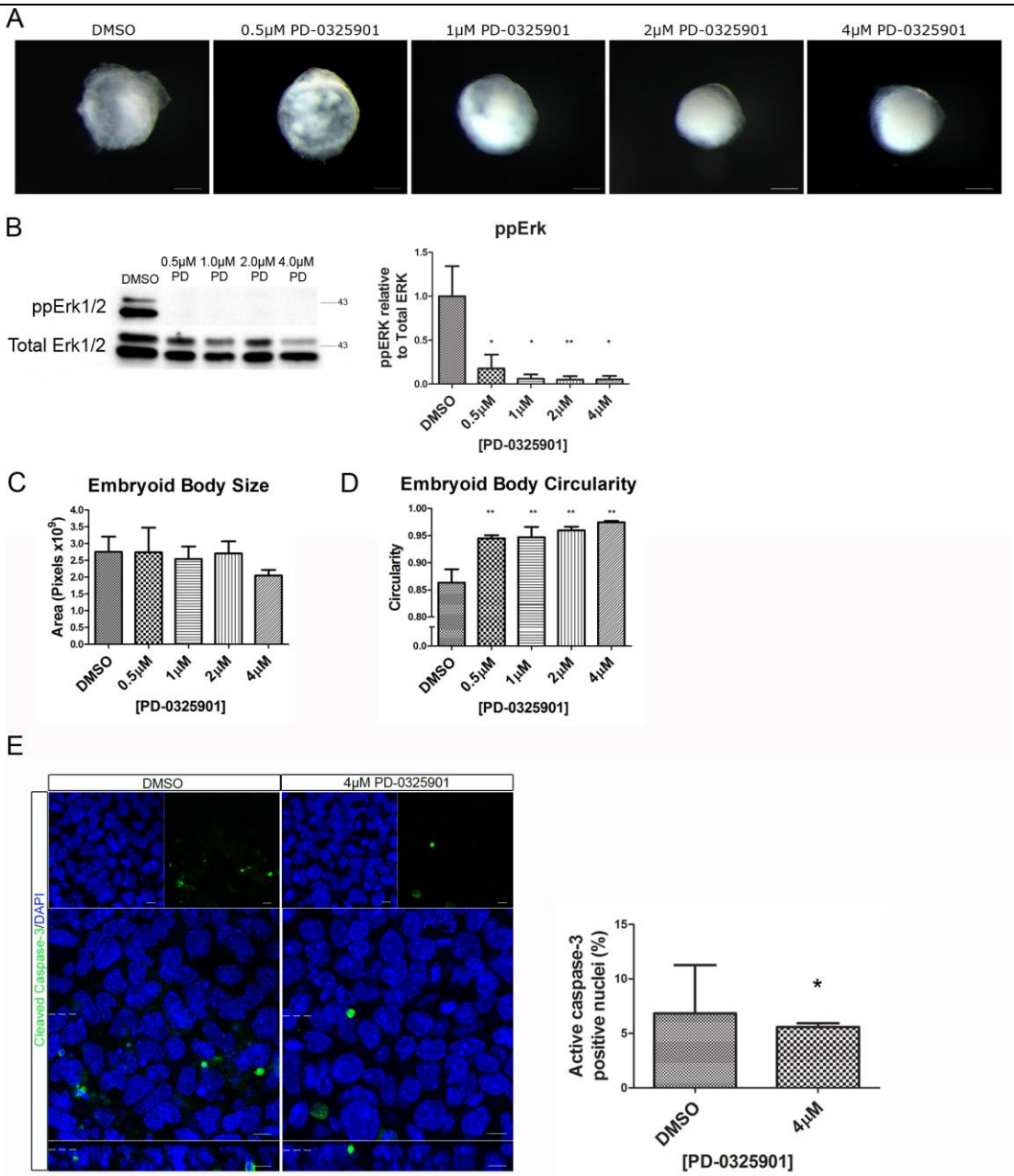


Figure 5.1: Addition of PD-0325901 inhibited Erk phosphorylation and resulted in more circular embryoid bodies

Embryoid bodies were grown in different concentrations of PD-0325901 or 0.04% DMSO for 7 days. (A) Light microscopy images show morphology of the embryoid bodies following inhibitor treatment. The embryoid bodies appeared slightly smaller and rounder. Scale bars 200µm. (B) Western blotting demonstrates that PD-0325901 reduced levels of diphosphorylated Erk1/2. A representative blot and quantification from 3 independent experiments is shown. (C) Quantification of the size of the embryoid bodies suggests that there was a slight reduction in size, but this is not statistically significant. (D) Measurement of the circularity of the embryoid bodies shows that inhibition of Mek caused a statistical increase in circularity. (E) Whole-mount immunostaining of cleaved Caspase-3. A slight reduction in the number of cleaved Caspase-3 nuclei is observed upon treatment with 4µM PD-0325901 in comparison to 0.04% DMSO suggesting that less apoptosis occurs in the outer-layer of these embryoid bodies. A representative image from 3 independent experiments is shown. Data is from 3 independent experiments, error bars are SEM. Statistical analysis is (B-D) a one-way Anova with a Dunnett's post-hoc test, (E) a paired t-test. (* P=0.1-0.5, ** p=0.001-0.01, *** p<0.001)

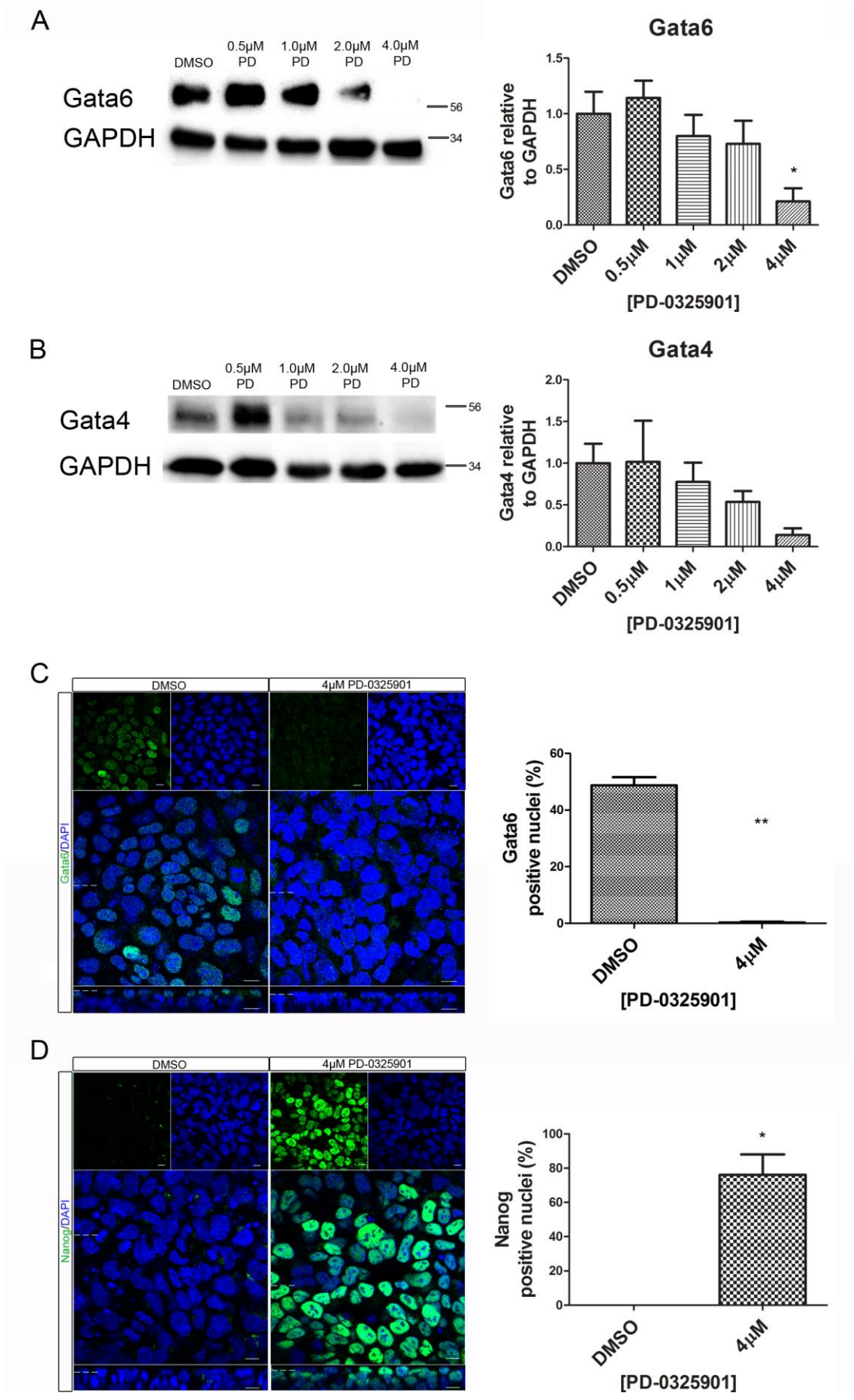

A
DMSO
0.5μM PD
1.0μM PD
2.0μM PD
4.0μM PD
Gata6
GAPDH
56
34
Gata6
Gata6 relative to GAPDH
1.5
1.0
0.5
0.0
*
DMSO
0.5μM
1μM
2μM
4μM
[PD-0325901]
B
DMSO
0.5μM PD
1.0μM PD
2.0μM PD
4.0μM PD
Gata4
GAPDH
56
34
Gata4
Gata4 relative to GAPDH
2.0
1.5
1.0
0.5
0.0
DMSO
0.5μM
1μM
2μM
4μM
[PD-0325901]
C
DMSO
4μM PD-0325901
Gata6/DAPI
Gata6 positive nuclei (%)
60
40
20
0
**
DMSO
4μM
[PD-0325901]
D
DMSO
4μM PD-0325901
Nanog/DAPI
Nanog positive nuclei (%)
100
80
60
40
20
0
*
DMSO
4μM
[PD-0325901]

Figure 5.2: Reduced expression of primitive endoderm markers Gata4 and Gata6 and increased expression of Nanog was observed in embryoid bodies upon inhibition of Mek.
Embryoid bodies were grown in different concentrations of PD-0325901 or 0.04% DMSO for 7 days. Expression levels of (A) Gata6, and (B) Gata4 were analysed using western blotting. A representative blot and quantification from 3 independent experiments is shown for each marker. A dose dependent decrease in expression of both proteins was observed. Statistical analysis is a one-way Anova with a Dunnett's post-hoc test. Whole-mount immunostaining of (C) Gata6 and (D) Nanog after treatment of embryoid bodies with 4μM PD-0325901. A reduction in the percentage of nuclei expressing Gata6 was observed whilst there was an increase in the percentage of nuclei expressing Nanog. A representative image from 3 independent experiments is shown. Scale bars 10μm. Dotted lines represent position that the relevant orthogonal or aerial images were taken. Statistical test is a paired T-test. Data is from 3 independent experiments, error bars represent SEM. (* P=0.1-0.5, ** p=0.001-0.01, *** p<0.001).

5.2.2 Mek inhibition disrupts epithelial polarity

Having observed a disruption in the specification of the primitive endoderm cell fate following inhibition of Mek signalling (Figure 5.2), embryoid bodies were cultured in PD-0325901 and the localisation of polarity and junctional proteins examined (Figure 5.3 & Figure 5.4). The addition of 2 µM PD-0325901 appeared to cause maximal inhibition of ppErk1/2, but 4 µM caused a bigger reduction in Gata4/6 staining. The expression of these proteins in the primitive endoderm is Mek dependent (Yoshida-Koide et al., 2004) arguing that in this system there may be a low level of Mek signalling which is inhibited by addition of 4µM PD-0325901 in place of 2µM PD-0325901. For this reason 4µM PD-0325901 was chosen for subsequent experiments.

aPkcζ/λ was apically localised in the control embryoid bodies, but when treated with the Mek inhibitor aPkcζ/λ was also present in the cytoplasm and in cell layers below the outer layer (Figure 5.3A). After treatment with the Mek inhibitor for seven days, the tight-junction protein Zo-1 remained localised at the apical side of the outer layer cells but did not form a belt-like structure around the cells (Figure 5.3B). Occasional labelled junctions remained, but the majority of protein was present in isolated puncta. This suggested that inhibition of Mek disrupted the formation of the tight junctions and the localisation of aPkcζ/λ.

The localisation of the adherens junction proteins E-cadherin and β-catenin was also disrupted by treatment with the Mek inhibitor. E-cadherin staining was not very strong, but it would seem that, unlike in the DMSO control, when the embryoid bodies were cultured with the Mek inhibitor E-cadherin was present apically as well as laterally, and in layers below that of the outer layer (Figure 5.4A). β-catenin localised apically as well as laterally in the outer cell layer of embryoid bodies treated with PD-0325901 (Figure 5.4B) and was observed in the 2nd layer of cells (the layer below the outer-layer). This localisation was not observed in the controls (Figure 5.4A&B). Lastly, a disruption in the localisation of the basement membrane protein Fibronectin was observed (Figure 5.4C). Upon addition of PD-0325901, Fibronectin formed small aggregates of protein instead of a fibrous network as observed in the control embryoid bodies. In addition, the Fibronectin protein in embryoid bodies treated with PD-0325901 was not basally restricted, but was present in all cell layers observed (Figure 5.4C). The mislocalisation of the polarity complex protein aPkcζ/λ, as well as tight junction, adherens junction and basement membrane proteins shows that the apico-basolateral polarity of the outer layer of embryoid bodies was disrupted following Mek inhibition.

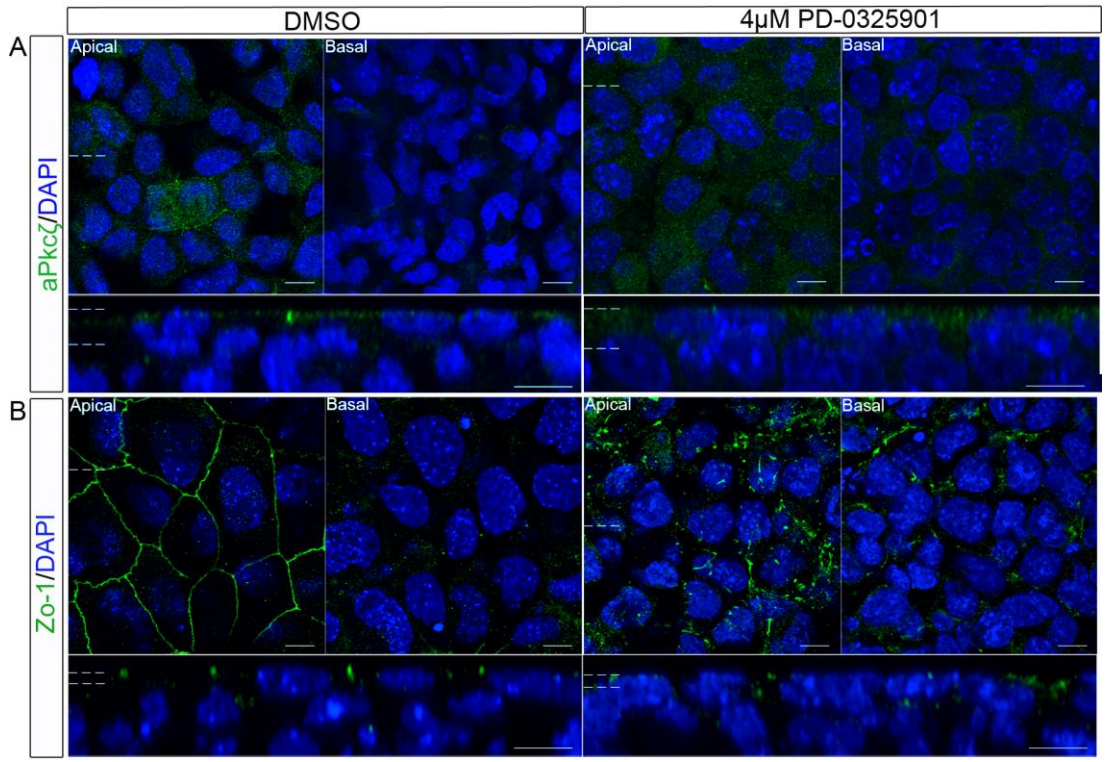


Figure 5.3: Inhibition of Mek disrupted the normal localisation of polarity complex protein aPkcζ/λ and the tight junction protein Zo-1 in the outer layer of embryoid bodies.
Embryoid bodies were treated with 4μM PD-0325901, a Mek inhibitor, or 0.04% DMSO for 7 days. Localisation of proteins which normally polarise in the primitive endoderm epithelium were assessed using whole-mount immunostaining. (A) aPkcζ/λ a member of a polarity complex, and (B) Zo-1 a tight junction protein were both shown to have an altered localisation when grown with a Mek inhibitor, suggesting a disruption in the apico-basolateral polarity of these cells. Representative images from three independent experiments are shown. Scale bars 10μm. Dotted lines represent position that the relevant orthogonal or aerial images were taken.

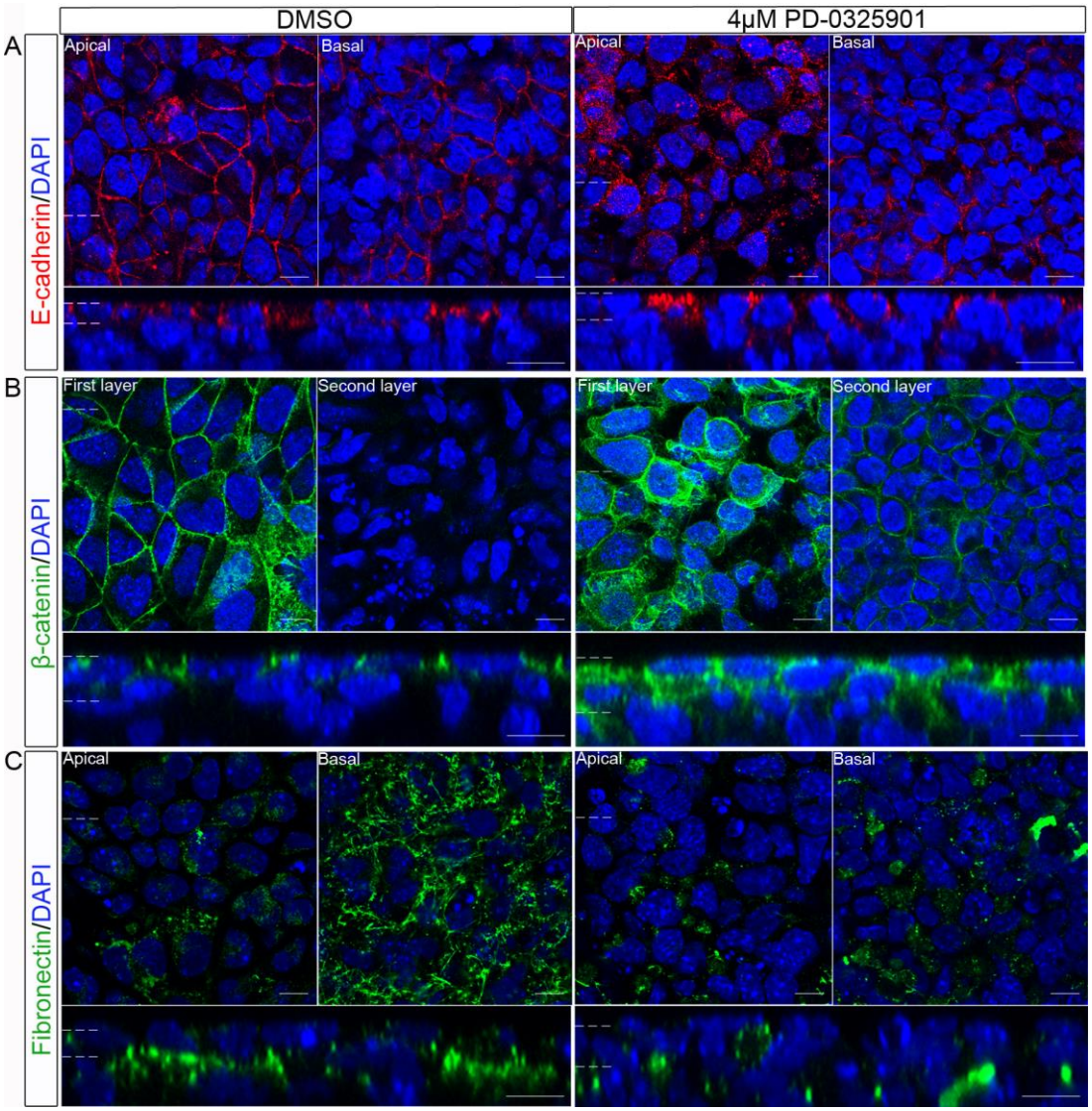


Figure 5.4: Inhibition of Mek disrupted the normal localisation of adherens junction and basement membrane proteins in the outer layer of embryoid bodies.
Embryoid bodies were treated with 4μM PD-0325901, a Mek inhibitor, or 0.04% DMSO for 7 days. Localisation of proteins which normally polarise in the primitive endoderm epithelium were assessed using whole-mount immunostaining. (A) E-cadherin, (B) β-catenin both proteins in the adherens junction and (C) the basement membrane protein Fibronectin, were all shown to have an altered localisation when grown with a Mek inhibitor, suggesting a disruption in the apico-basolateral polarity of these cells. Representative images from three independent experiments are shown. Scale bars 10μm. Dotted lines represent position that the relevant orthogonal or aerial images were taken.

5.2.3 Inhibition of Mek has no effect on the expression of junctional and polarity proteins

Having observed a mislocalisation of the polarity and junctional proteins upon Mek inhibition, the expression levels of these proteins was assessed using western blotting (Figure 5.5). No statistically significant effect was observed in expression of aPkcζ/λ or β-catenin (Figure 5.5A &C). There was also no statistically significant effect on expression of E-cadherin, however a slight increase in expression was seen at increasing doses of the inhibitor (Figure 5.5B). Additionally a non-statistically significant increase in expression of Fibronectin was observed when embryoid bodies were treated with 1μM and 2μM PD-0325901, but not 4μM PD-0325901 (Figure 5.5D). These results suggest that there may be some regulation of the expression of these proteins at a whole embryoid body level, but predominantly the effect caused by inhibition of Mek is a change in the localisation of these proteins.

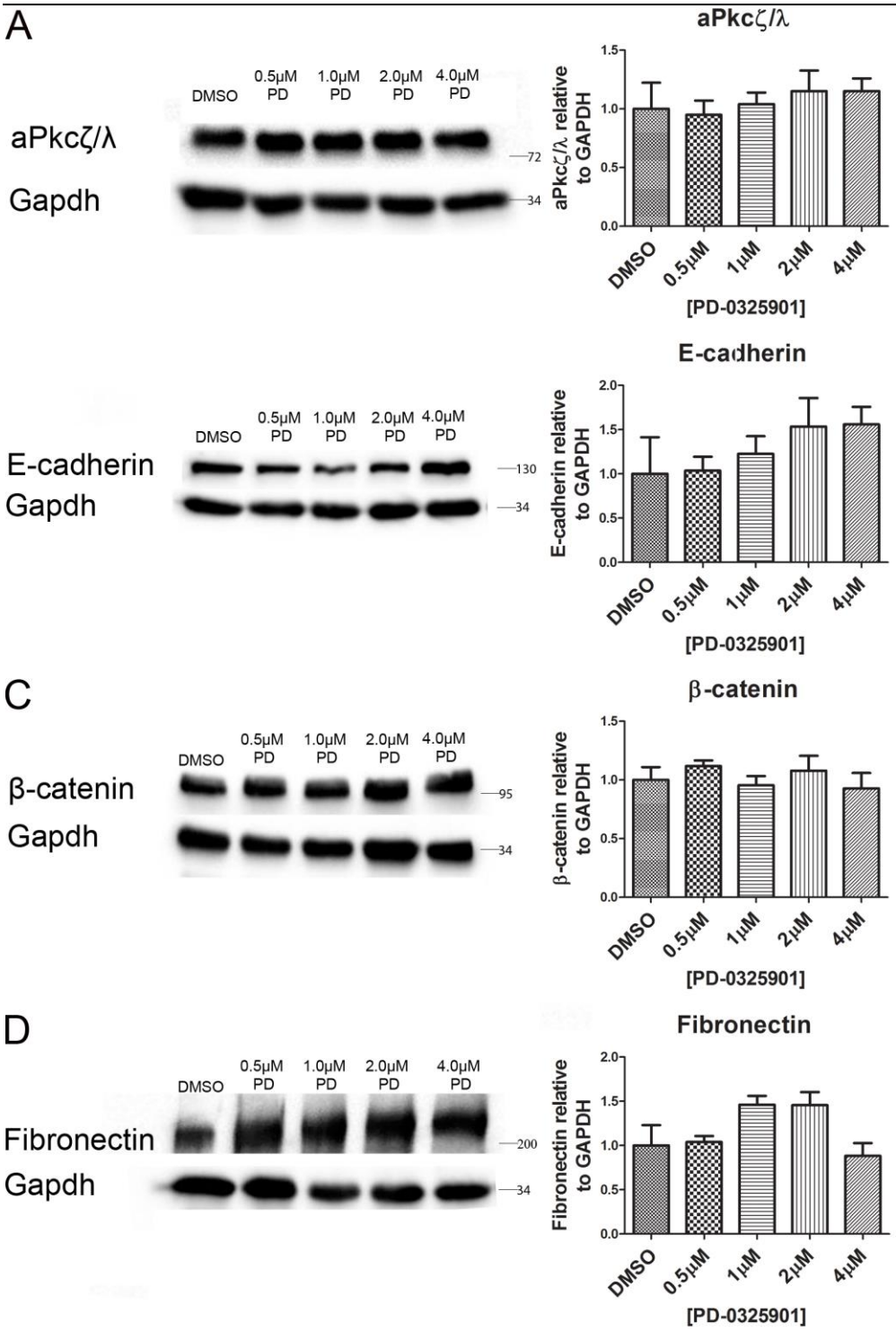


Figure 5.5: Mek inhibition had no effect on the expression of junction and polarity proteins. Embryoid bodies were grown in different concentrations of PD-0325901 or 0.04% DMSO for 7 days. Expression levels of (A) polarity complex protein aPkcζ/λ, (B) E-cadherin, (C)β-catenin adherens junction proteins, and (D) basement membrane protein Fibronectin were analysed using western blotting. No effect was seen on expression of aPkcζ/λ, or β-catenin, whilst a small dose dependent increase in expression of both E-cadherin and Fibronectin was observed. A representative blot and quantification from 3 independent experiments is shown for each marker, error bars represent SEM. Statistical analysis is a one-way Anova with a Dunnett's post-hoc test (* P=0.1-0.5, ** p=0.001-0.01, *** p<0.001).

5.2.4 Loss of Gata6 and Gata4 expression upon inhibition of Fgf receptor signalling

As disruption of the Erk signalling cascade resulted in a disruption in the apico-basolateral polarity of the outer-layer of the embryoid body, I wished to identify the receptor responsible for Erk activation in this system. A leading candidate for this was the Fgf receptor, as its inhibition has previously been shown to decrease expression of Gata6 and Gata4, disrupting cell fate specification in the primitive endoderm (Arman et al., 1998; Feldman et al., 1995). A newly developed potent and selective inhibitor of the Fgf receptor, AZD-4547, was used to treat embryoid bodies (Figure 5.6A). This inhibitor was chosen as it is more selective than previously available compounds such as PD-173074 (Gavine et al., 2012; Mohammadi et al., 1998). Addition of this inhibitor caused a reduction in ppErk1 and 2 (Figure 5.6B). Some ppERK expression remained, perhaps due to other signalling pathways acting upstream of Mek, such as GPCRs, Integrins or other Receptor tyrosine kinases. Addition of AZD-4547 produced embryoid bodies which were smaller than controls even at the lowest dose tested (1uM) (Figure 5.6A&C). There was also a dose dependent increase in the circularity of the embryoid bodies, resulting in embryoid bodies which were significantly more circular than controls when cultured with 4µM and 8µM PD-0325901 (Figure 5.6A&D). To determine if addition of AZD-4547 caused an effect on apoptosis whole-mount immunostaining for cleaved Caspase-3 was carried out. There was a small, but not statistically significant, increase in the number of nuclei in the outer-layer of the embryoid body which were positive for cleaved Caspase-3 (Figure 6E). The majority of cells (>85%) remained Caspase negative, arguing that any changes in development of the primitive endoderm are not due to cell death (Figure 5.6).

A dose-dependent decrease in expression levels of both Gata4 and Gata6 was observed by western blotting when embryoid bodies were grown in increasing concentrations of the Fgf receptor inhibitor (Figure 5.7A&B). Additionally, whole-mount immunostaining confirmed a reduction in Gata6 positive nuclei when 4µM AZD-4547 was used (Figure 5.7C). Nanog expression increased from 0% in control to an average of 82% upon inhibition of the Fgf receptor (Figure 7D). This suggests that Fgf receptor signalling is activating Erk to drive an increase in size, heterogeneity of shape, loss of Nanog, and expression of primitive endoderm fate markers in embryoid bodies.

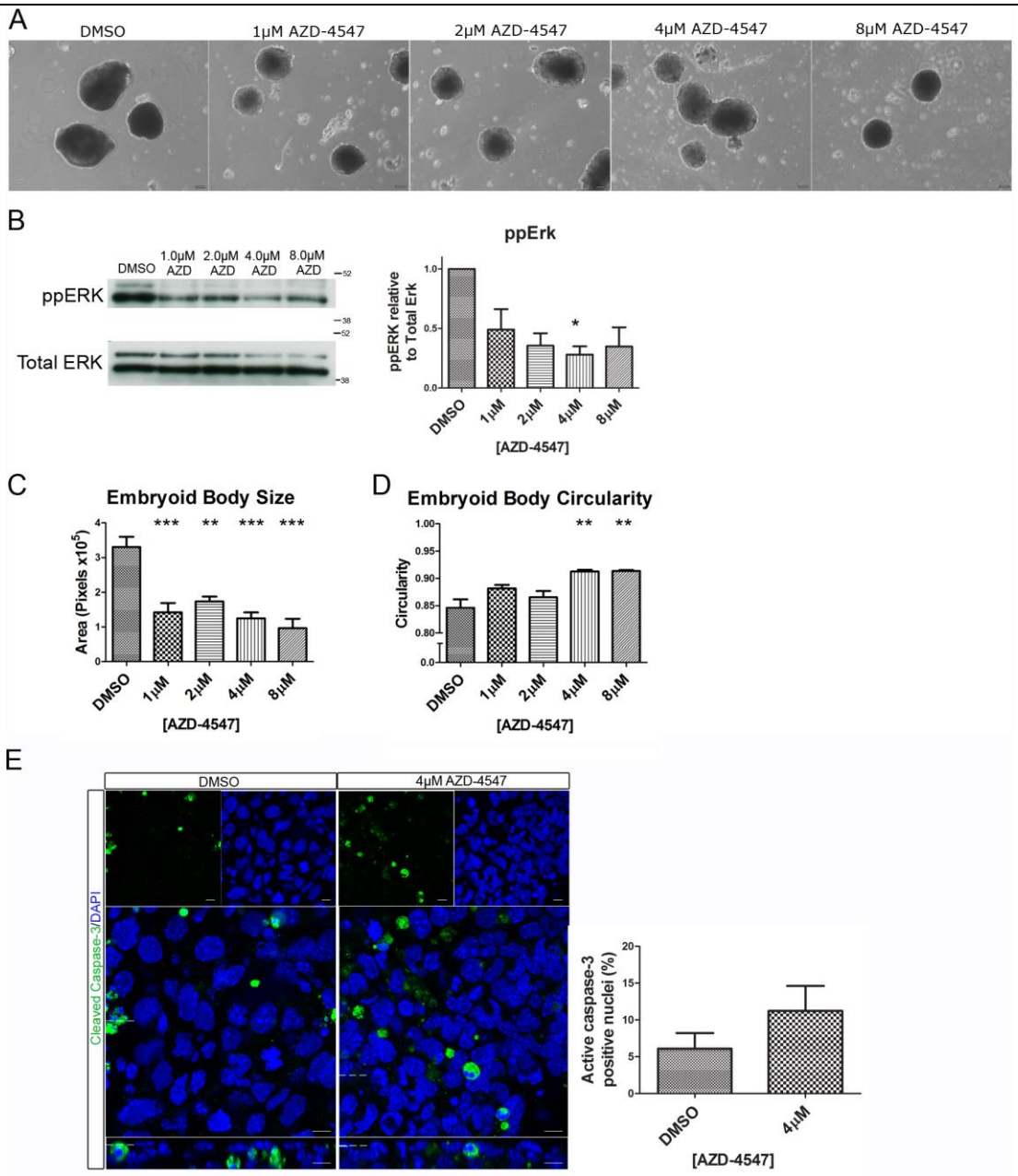
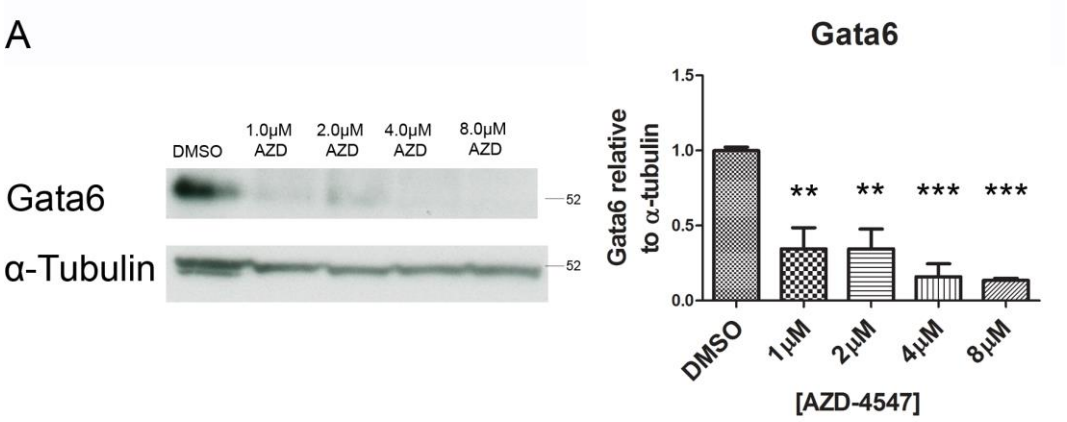
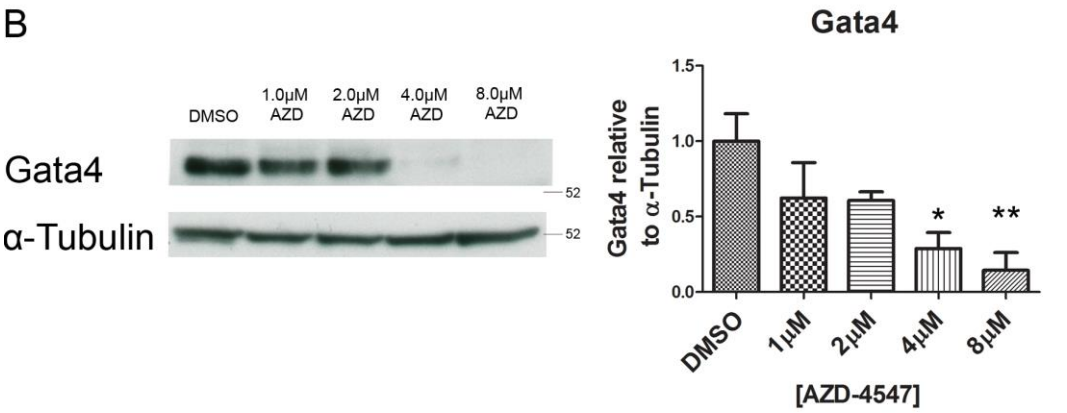
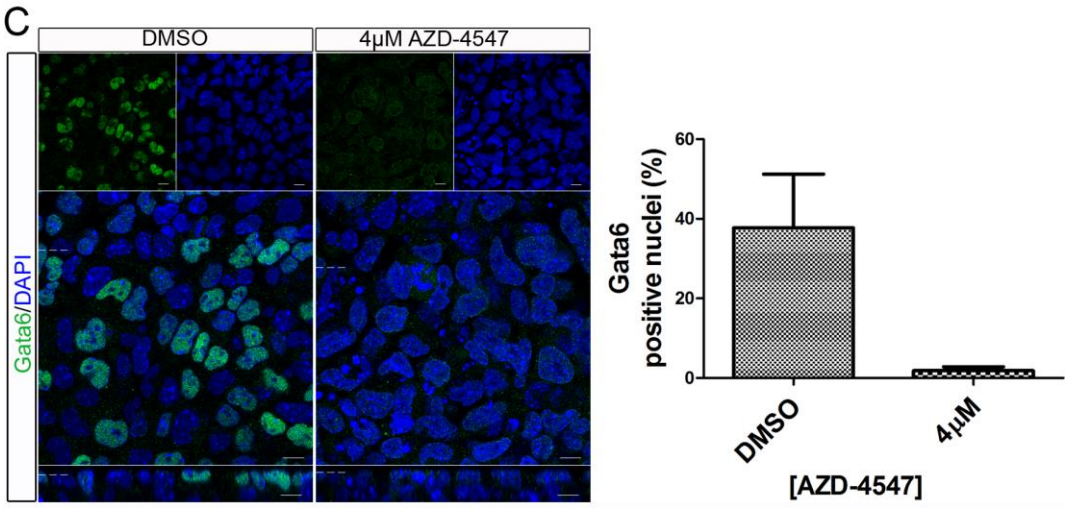


Figure 5.6: Addition of AZD-4547 inhibited Erk phosphorylation and resulted in smaller and more circular embryoid bodies.

Embryoid bodies were grown in different concentrations of AZD-4547 for 7 days. (A) Light microscopy images show a change in morphology of the embryoid bodies. Scale bars 100μm. (B) Western blotting demonstrates that AZD-4547 reduced levels of diphosphorylated Erk1/2. A representative blot and quantification from 3 independent experiments is shown for each marker. (C) Inhibition of the Fgf receptor caused a significant reduction in size of the embryoid bodies. (D) Inhibition of the Fgf receptor caused a statistically significant increase in circularity. (E) Whole-mount immunostaining of cleaved Caspase-3 in embryoid bodies treated with 4μM AZD-4547 or 0.04% DMSO. A small non-statistically significant increase in the number of cleaved Caspase-3 nuclei is observed upon treatment with AZD-4547 suggesting that more apoptosis may occurs in the outer-layer of these embryoid bodies. A representative image from 3 independent experiments is shown. Data is from 3 independent experiments, error bars represent SEM. Statistical analysis is (B-D) a one-way Anova with a Dunnett's post-hoc test, (E) a paired t-test (* P=0.1-0.5, ** p=0.001-0.01, *** p<0.001).


A
Gata6
DMSO
1.0μM AZD
2.0μM AZD
4.0μM AZD
8.0μM AZD
Gata6
α-Tubulin
52
52
Gata6 relative to α-tubulin
1.5
1.0
0.5
0.0
**
**
***
***
DMSO
1μM
2μM
4μM
8μM
[AZD-4547]


B
Gata4
DMSO
1.0μM AZD
2.0μM AZD
4.0μM AZD
8.0μM AZD
Gata4
α-Tubulin
52
52
Gata4 relative to α-Tubulin
1.5
1.0
0.5
0.0
*
**
DMSO
1μM
2μM
4μM
8μM
[AZD-4547]


C
DMSO
4μM AZD-4547
Gata6/DAPI
Gata6 positive nuclei (%)
60
40
20
0
DMSO
4μM
[AZD-4547]

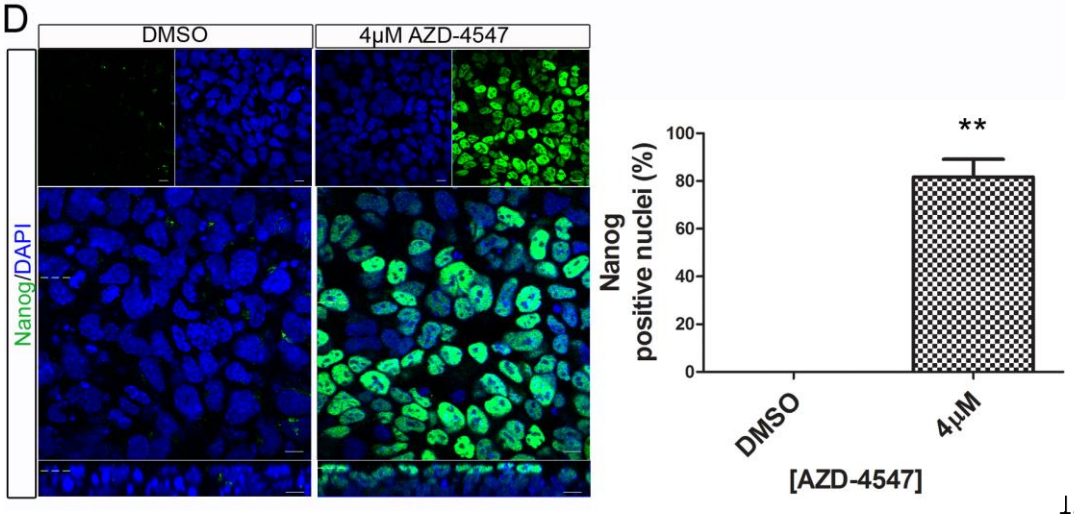

D
DMSO
4μM AZD-4547
Nanog/DAPI
Nanog positive nuclei (%)
100
80
60
40
20
0
**
DMSO
4μM
[AZD-4547]

Figure 5.7: Reduced expression of the primitive endoderm markers Gata4 and Gata6, and increased expression of Nanog was observed in embryoid bodies following inhibitor of the Fgf receptor.

Embryoid bodies were grown in different concentrations of AZD-4547 or0.08% DMSO for 7 days. Expression levels of (A) Gata6, and (B) Gata4 were analysed using western blotting. A representative blot and quantification from 3 independent experiments is shown for each marker. A dose dependent decrease in expression of both proteins was observed. Statistical analysis is a one-way Anova with a Dunnett's post-hoc test. Whole-mount immunostaining of (C) Gata6 and (D) Nanog after treatment of embryoid bodies with 4µM AZD-4547 or 0.04% DMSO, A reduction in the percentage of nuclei expressing Gata6 was observed. The percentage of nuclei expressing Nanog increased. Scale bars 10µm. Dotted lines represent position that the relevant orthogonal or aerial images were taken. Statistical analysis is a paired t-test. Data is from 3 independent experiments, error bars represent SEM. (* P=0.1-0.5, ** p=0.001-0.01, *** p<0.001)

5.2.5 Loss of apico-basolateral polarity upon Fgf receptor inhibition

Having observed a loss in expression of primitive endoderm markers, I examined apico-basolateral polarity components following inhibition of the Fgf receptor. This analysis was performed using 4μM AZD-4547 as a clear inhibition of ppERK, loss of Gata6 and Gata4 expression and, morphological changes were observed at this concentration. Following inhibition of the Fgf receptor, aPkcζ/λ localised throughout the cytoplasm of the outer-layer and was found in the layers below (Figure 5.8A). Zo-1 localised to apical puncta, but did not form a belt around the periphery of the cells, instead only patches or short lines of Zo-1 were detected (Figure 5.8B). A disruption in adherens junction proteins was also observed. E-cadherin localised apically and laterally of the outer-cell layer as well as in cells below the outer layer (Figure 5.9A). β-catenin was observed apically in some cells and was also found in the cells below the outer layer (Figure 5.9B). Localisation of Fibronectin was also investigated. The protein was spread throughout the outer layer, as well as in layers below and was present in patches, rather than forming the fibrous network observed in controls (Figure 5.9C). The mislocalisation of these proteins was similar to that seen when embryoid bodies were treated with the Mek inhibitor, suggesting that Mek dependent polarisation is driven, at least in part, by activation of the Fgf receptor.

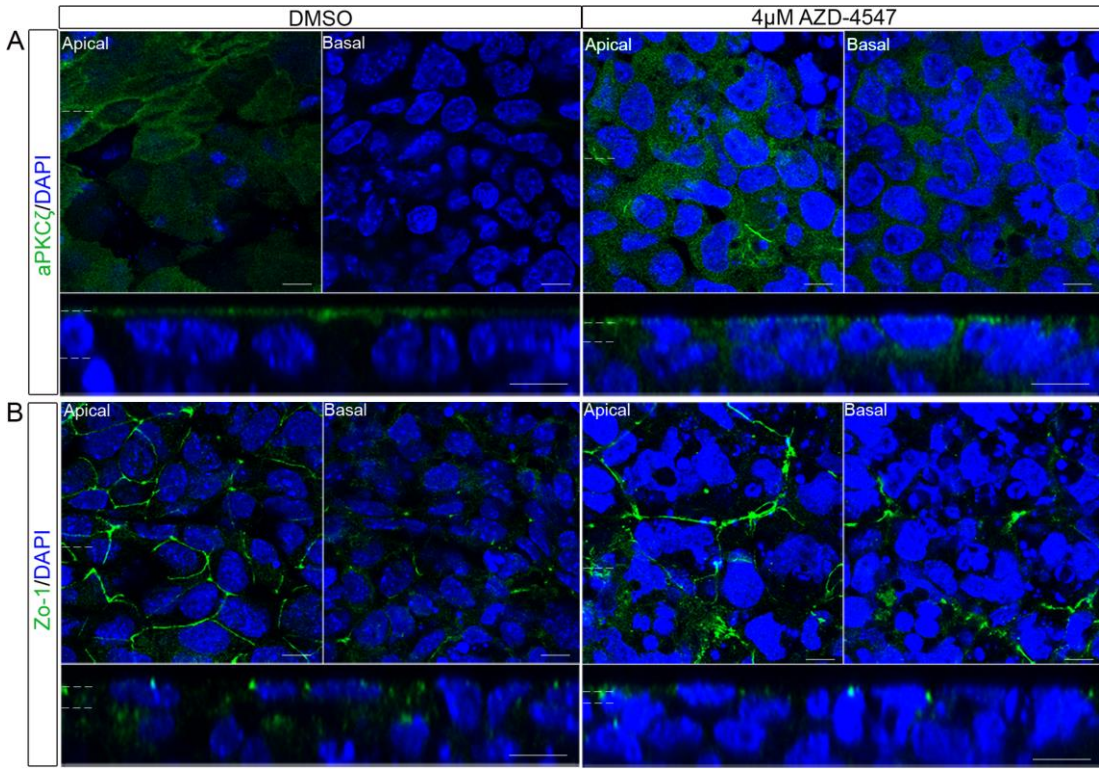


Figure 5.8: Inhibition of the Fgf receptor disrupted the normal localisation of polarity complex protein aPkcζ/λ and tight junction protein Zo-1 in the outer layer of embryoid bodies

Embryoid bodies were treated with the Fgf receptor inhibitor 4μM AZD-4547, or 0.04% DMSO for 7 days. Whole-mount immunostaining demonstrated the localisation of proteins normally polarised in the primitive endoderm. (A) aPkcζ/λ a member of a polarity complex, and (B) Zo-1 a tight junction protein, were shown to lose their apico-basolateral polarised localisation when grown with an Fgf receptor inhibitor suggesting a disruption in the apico-basolateral polarity of these cells. Representative images from three independent experiments are shown. Scale bars 10μm. Dotted lines represent position that the relevant orthogonal or aerial images were taken.

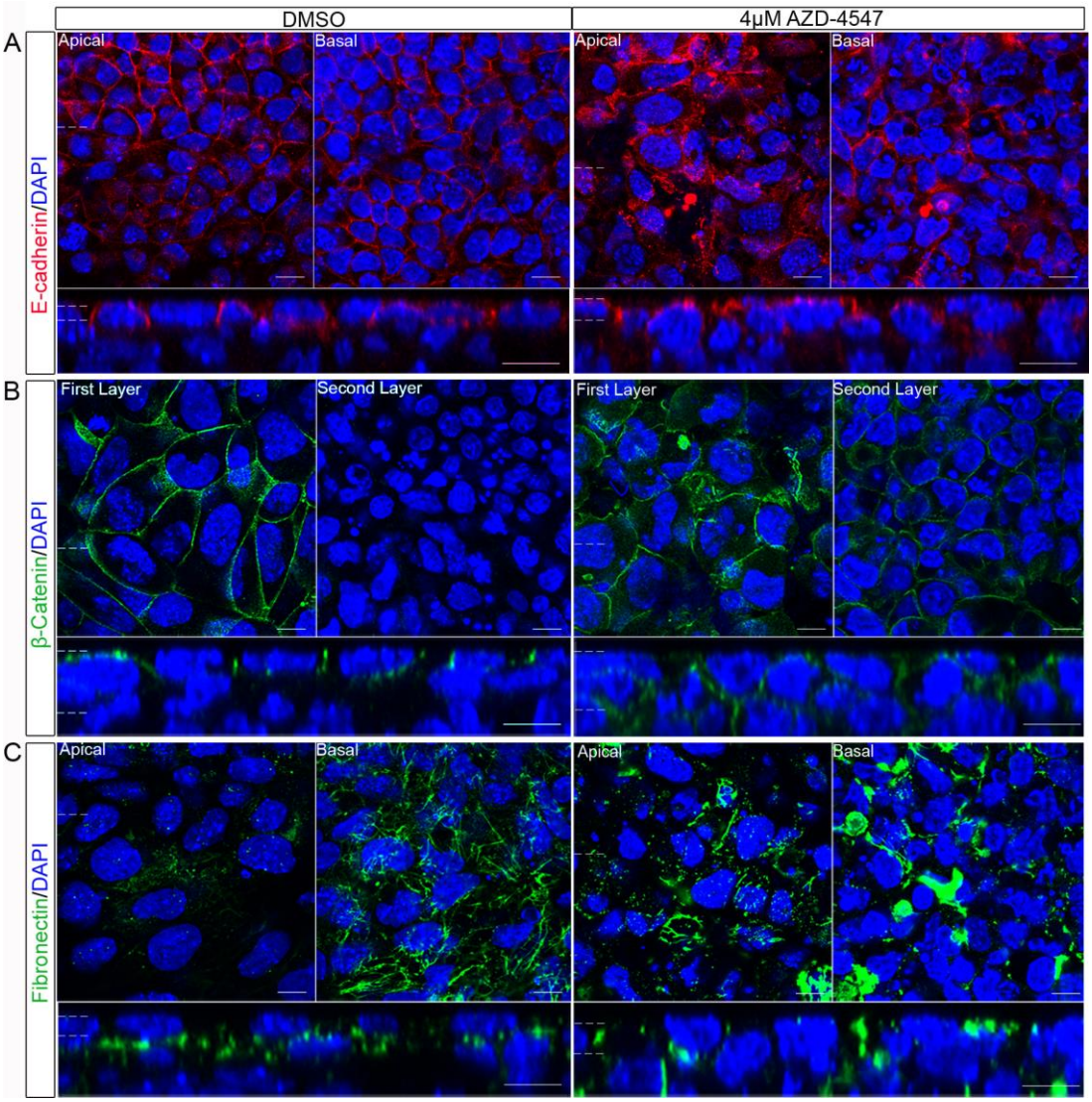


Figure 5.9: Inhibition of the Fgf receptor disrupted the normal localisation of adherens junction and basement membrane proteins in the outer layer of embryoid bodies

Embryoid bodies were treated with the Fgf receptor inhibitor 4μM AZD-4547, or 0.04% DMSO for 7 days. Whole-mount immunostaining demonstrated the localisation of proteins normally polarised in the primitive endoderm. (A) E-cadherin, (B) β-catenin both proteins in the adherens junction and (C) the basement membrane protein Fibronectin, were shown to lose their apico-basolateral polarised localisation when embryoid bodies were grown with an Fgf receptor inhibitor suggesting a disruption in the apico-basolateral polarity of these cells. Representative images from three independent experiments are shown. Scale bars 10μm. Dotted lines represent position that the relevant orthogonal or aerial images were taken.

5.2.6 The effect of Fgfr inhibition on the expression of junctional and polarity proteins

The expression of junctional and polarity proteins was quantified using western blotting because a mislocalisation of polarity and junctional proteins had been observed upon inhibition of the Fgf receptor (Figure 5.10). There was a non-statistically significant increase in expression of the polarity complex protein aPkcζ/λ at increasing doses of the Fgf receptor inhibitor (Figure 5.10A). There are two splice variants of Zo-1, the longer isoform has an 80 amino acid domain known as the α domain, and therefore known as Zo-$1^{\alpha+}$, whilst the less common form lacks the α-domain and is therefore known as Zo-$1^{\alpha-}$ (Willott et al., 1992). Zo-$1^{\alpha+}$ is not very highly expressed in the vehicle control embryoid bodies, but there is a large, although not statistically significant increase in expression of Zo-$1^{\alpha+}$, following inhibition of the Fgf receptor suggesting it caused a change in the splicing of this gene (Figure 5.10B). Expression of Zo-$1^{\alpha-}$ shows a non-statistically significant decrease in expression (Figure 5.10B). A statistically significant ~3-fold increase in E-cadherin expression was observed over the vehicle control at all concentrations of AZD-4547 (Figure 5.10C). A small non-statistically significant decrease in expression of the adherens junction protein β-catenin (Figure 5.10D) was also observed. No effect was seen on the expression of Fibronectin protein in the presence of AZD-4547 (Figure 5.10E). These western blots suggest that regulation by the Fgf receptor of apico-basolaterally polarised proteins varies depending upon the protein, and in most cases small trends are seen which are not statistically significant. The statistically significant increase in E-cadherin observed is particularly interesting as a small decrease in expression of β-catenin was observed suggesting that the effect of Fgf receptor inhibition on expression of these two adherens junction proteins is different.

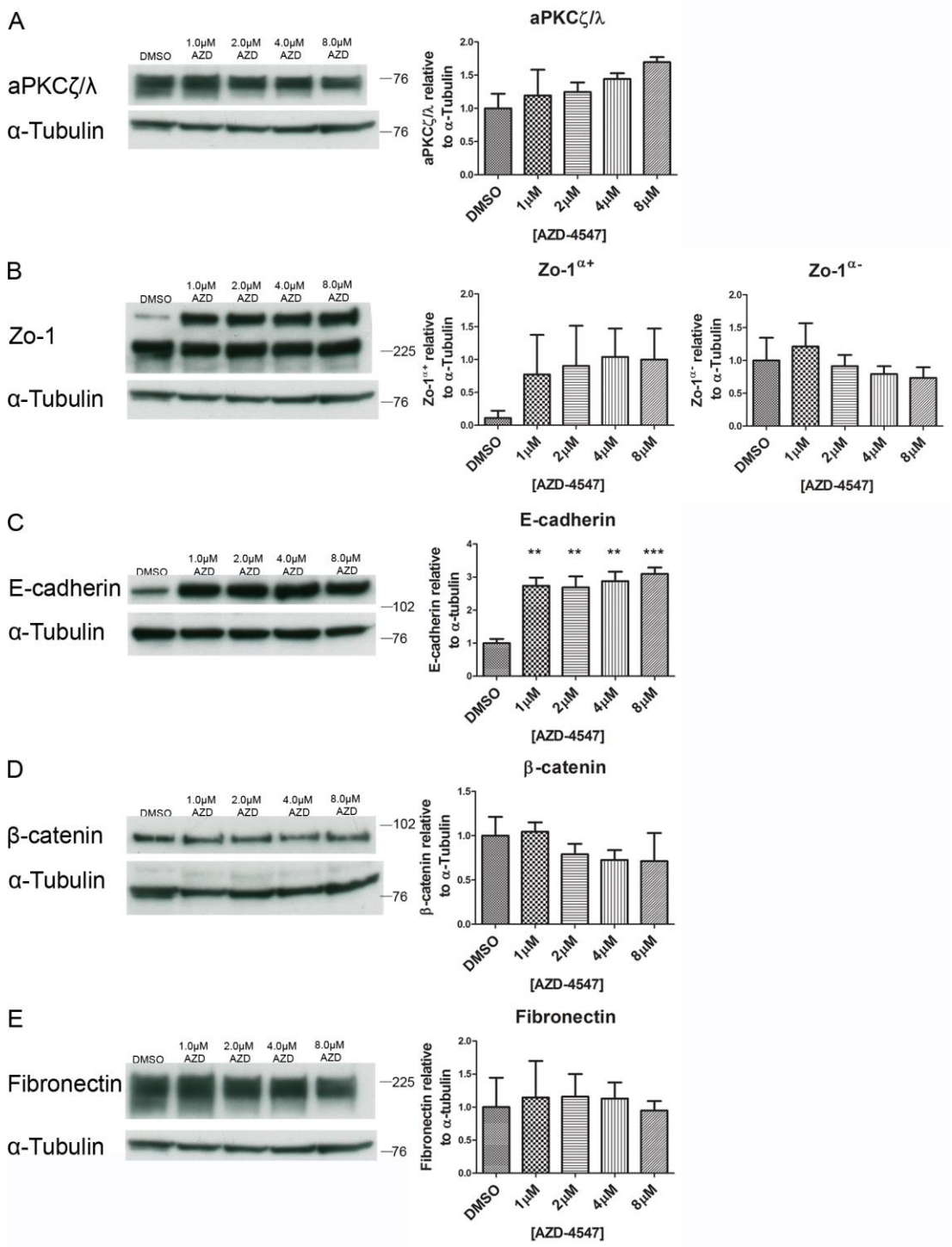


Figure 5.10: The Effect of Fgf receptor inhibition on the expression of junction and polarity proteins

Embryoid bodies were grown in different concentrations of AZD-4547 or 0.08% DMSO for 7 days. Expression levels of (A) polarity complex protein aPkcζ/λ, (B) tight junction protein Zo-1, adherens junction proteins (C) E-cadherin, (D)β-catenin, and (D) basement membrane protein Fibronectin were analysed using western blotting. A representative blot and quantification from 3 independent experiments is shown for each marker. No effect was seen on Fibronectin expression, whilst a small increase in aPkcζ and Zo-$1^{\alpha+}$, and a small decrease in Zo-$1^{\alpha-}$ and β-catenin was observed. Fgf receptor inhibition caused a large, statistically significant increase in expression of E-cadherin. Data is from 3 independent experiments, error bars represent SEM. Statistical analysis is a one-way Anova with a Dunnett's post-hoc test (* p=0.1-0.5, ** p=0.001-0.01, *** p<0.001).

5.2.7 Inhibition of the Fgf receptor with PD-173074 also causes a disruption in polarisation of the outer-layer of cells

Treatment of embryoid bodies with the recently developed Fgfr inhibitor AZD-4547 caused a mis-localisation of apico-basolaterally polarised proteins. To confirm the specificity of this effect and allow comparison with previous literature, the polarisation of embryoid bodies was observed using the structurally distinct and more commonly used inhibitor PD-173074. This is an effective inhibitor of Fgfr1, but also inhibits Vegfr2 (Mohammadi et al., 1998). The polarisation of the primitive endoderm was observed following treatment with 0.1µM PD-173074 which is the concentration commonly used for studying primitive endoderm *in vivo* (Nichols et al., 2009; Yamanaka et al., 2010). In these *in vivo* studies it is often used in combination with a Mek inhibitor (Nichols et al., 2009; Yamanaka et al., 2010). Therefore, embryoid bodies were also treated with 0.1µM PD-173074 and 1µM PD-0325901, the combination and concentration of inhibitors previously used to assess primitive endoderm development (Nichols et al., 2009; Yamanaka et al., 2010).

The tight-junction protein Zo-1 localised to junctions in control embryoid bodies, but this localisation was disrupted following treatment with either 0.1µM PD-173074 or the combination of 0.1µM PD-173074 and 1µM PD-0325901 (Figure 5.11). The localisation of the adherens junction protein β-catenin was also disrupted following treatment with PD-173074 and when treated with the combined inhibitors (Figure 5.11). The use of two structurally distinct inhibitors of the Fgf receptor, which gave very similar results, argues that the phenotypes described above are caused by specifically inhibiting Fgfr signalling.

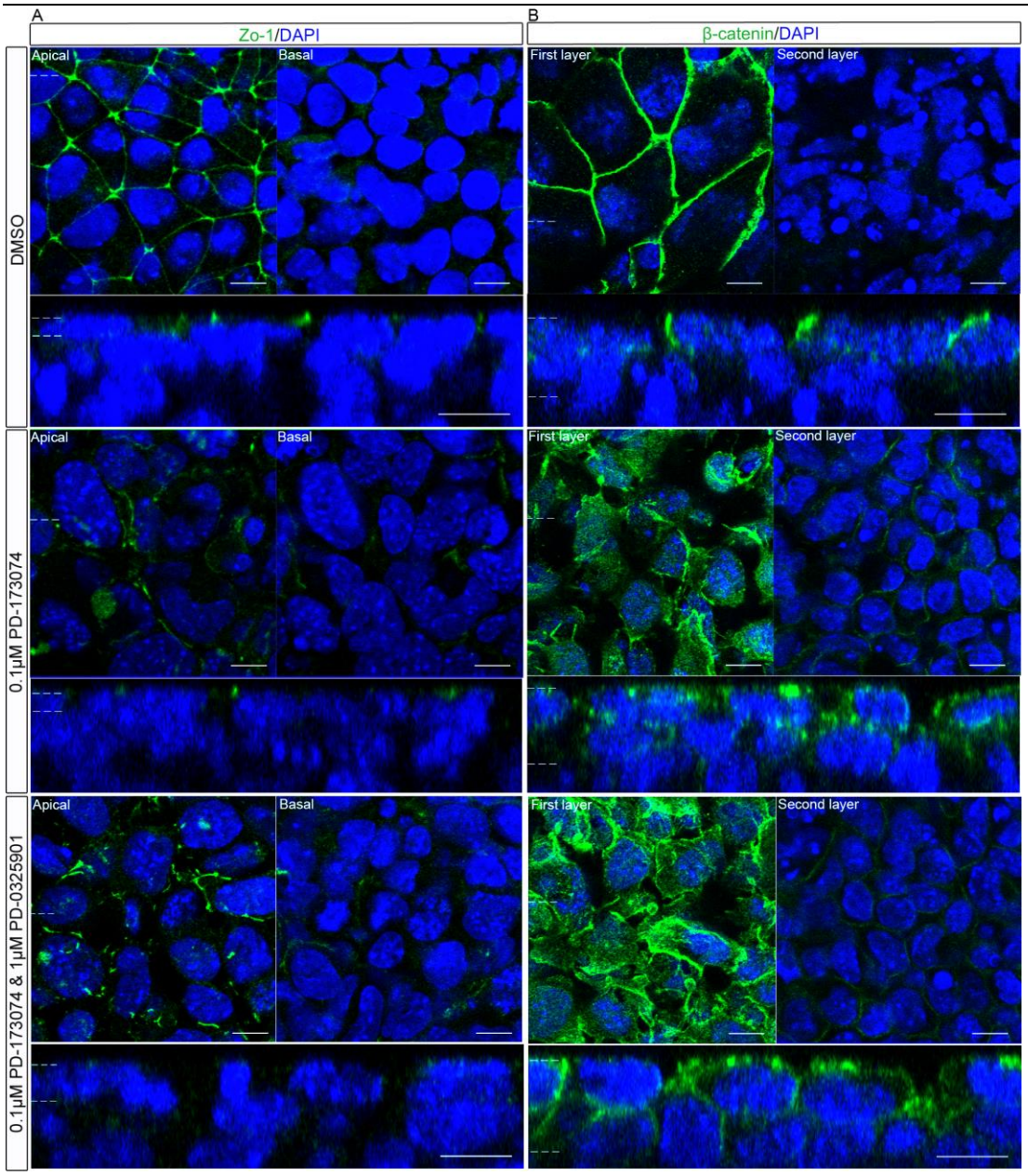


Figure 5.11 Inhibition of the Fgf receptor with PD-173074 or the Fgf receptor and Mek disrupted the normal localisation of polarity and junction proteins in the outer layer of embryoid bodies

Embryoid bodies were treated with 0.1μM of the Fgf receptor inhibitor PD-173074, 0.1μM PD-173074 and 1 μMPD-0325901 or 0.02% DMSO for 7 days. Whole-mount immunostaining demonstrated the localisation of proteins normally polarised in the primitive endoderm. (A) Zo-1 a tight junction protein and (B) β-catenin a protein in the adherens junction were shown to lose their normal polarised localisation when embryoid bodies were incubated with 0.1μM PD-173074 or 0.1μM PD-173074 and 1μM PD-0325901. This suggests a disruption in the apico-basolateral polarisation of these cells. A representative image from 3 independent experiments is shown. Scale bars 10μm. Dotted lines represent position that the relevant orthogonal or aerial images were taken.

5.2.8 Pi3k signalling regulates expression of Gata4 but not localisation of Zo-1

Only a small decrease in ppErk levels was observed when the Fgf receptor was inhibited, yet there was almost a full depletion of Gata6/4. This suggested that there may be an additional signalling pathway downstream of the Fgf receptor which regulates the development of the primitive endoderm (Figure 5.12). To test if Phosphatidylinositol 3-kinase (Pi3k) signalling is required for the specification and polarisation of the primitive endoderm the broad-spectrum inhibitor of Pi3k LY-294002 (Gharbi et al., 2007; Vlahos et al., 1994) was used (Figure 5.13A).

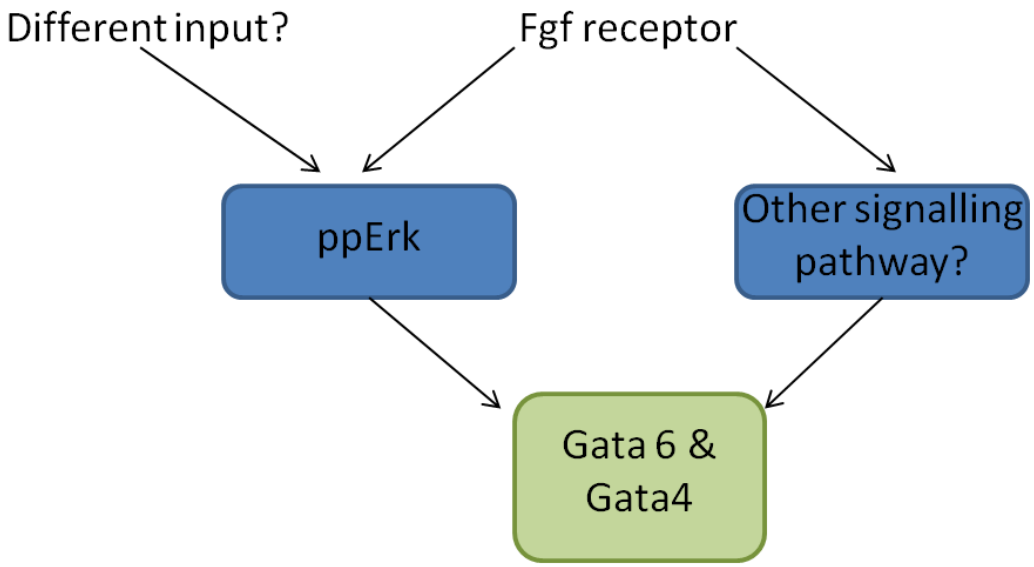
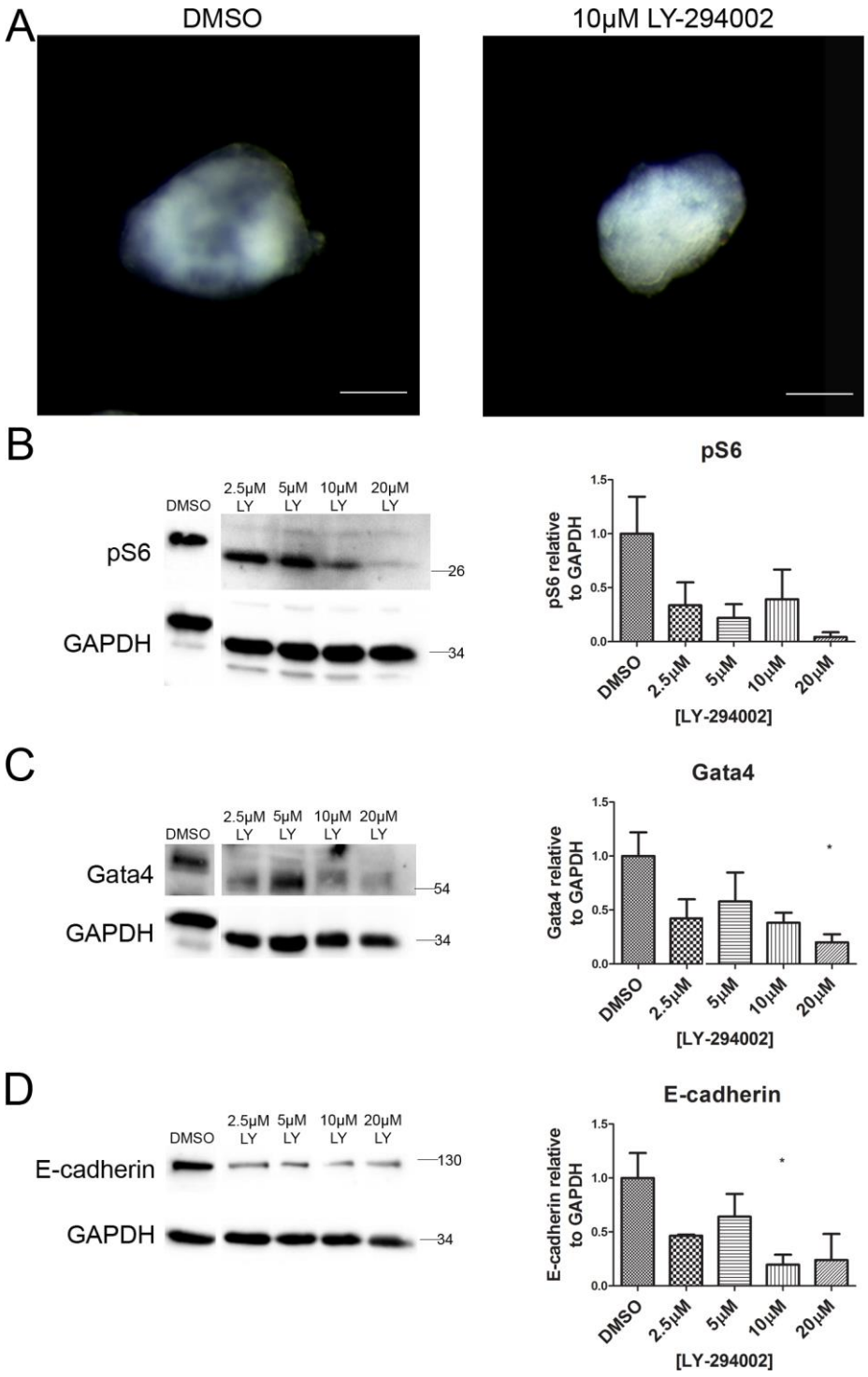


Figure 5.12: Signalling pathways responsible for cell fate specification in the primitive endoderm.
Fgf receptor and Erk signalling regulate the cell fate specification, demonstrated by reduced expression of Gata6 and Gata4 upon inhibition of these signalling cascades. An incomplete decrease in ppErk levels upon Fgf receptor inhibition suggests two things. Firstly, that an additional upstream signalling pathway to the Fgf receptor is also responsible for ppERK expression in the embryoid body. Secondly, as a complete loss of Gata6 and Gata4 was observed following inhibition of the Fgf receptor despite the incomplete loss of ppErk there may be an alternative signalling pathway to Erk downstream of the Fgf receptor regulating primitive endoderm cell fate specification.

Western blotting was used to determine at what concentration LY-294002 successfully inhibited the Pi3K signalling pathway (Figure 5.13B). Culture of embryoid bodies in LY-294002 caused a decrease in pS6 levels, although it was not statistically significant. There was little effect on the morphology of the embryoid bodies when cultured for 7 days in 10µM LY-294002 (Figure 5.13A). No obvious change in size, or shape of the embryoid body in comparison to the vehicle control was observed. Interestingly, inhibition of Pi3k caused a decrease in expression of the primitive endoderm cell fate marker Gata4, (Figure 5.13C), suggesting that Pi3K signalling may regulate primitive endoderm cell fate specification. Inhibition of Pi3k with LY-

294002 also caused a decrease in expression of the adherens junction protein E-cadherin suggesting that expression of this protein which is required for epithelial polarisation is regulated by Pi3k signalling, and may therefore affect epithelial polarisation.

To investigate the effects of Pi3k inhibition on the localisation of polarisation and cell fate specification markers in the outer layer of cells of the embryoid body, whole-mount immunostaining was carried out (Figure 5.14). Surprisingly, in contrast to the data from the western-blotting there was still expression of Gata4 in the nucleus of cells in the outer-cell layer after treatment with 10µM LY-294002 (Figure 5.14A). This suggests that these cells have maintained their primitive endoderm cell fate. Additionally, the localisation of tight-junction protein Zo-1 appeared unaffected by inhibition of Pi3K, suggesting that these outer cells still have tight junctions, and may still be polarised (Figure 5.14B). This suggests that there may not be any affect on the fate specification or polarisation of the outer-cell layer upon inhibition of Pi3k. Instead it may be other signalling pathways which are responsible for these processes downstream of Fgfr in addition to Erk signalling. Further work is required to confirm these findings and further investigate the role of other signalling pathways.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20μM LY
pS6
GAPDH
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34
pS6
pS6 relative to GAPDH
[LY-294002]
C
Gata4
GAPDH
54
34
Gata4
Gata4 relative to GAPDH
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D
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GAPDH
130
34
E-cadherin
E-cadherin relative to GAPDH
[LY-294002]

Figure 5.13: Addition of LY-294002 inhibited phosphorylation of S6-ribosomal protein, and expression of Gata4 and E-cadherin, but had no effect on the morphology of the embryoid bodies.

Embryoid bodies were grown in different concentrations of LY-294002 for 7 days. (A) Light microscopy images show no change in the morphology of the embryoid bodies. Scale bars 100μm. Western blotting was used to demonstrate the effect that LY-294002 had on expression of (B) phosphorylated S6-ribosomal protein (C) primitive endoderm protein Gata4 (D) adherens junction protein E-cadherin. Inhibition of Pi3k caused a reduction in levels of phosphorylated S6-ribosomal protein, as well as the primitive endoderm marker Gata4, and adherens junction protein E-cadherin. A representative blot and quantification from 3 independent experiments is shown for each marker. Data is from 3 independent experiments, error bars represent SEM. Statistical analysis is a one-way Anova with a Dunnett's post-hoc test (* P=0.1-0.5, ** p=0.001-0.01, *** p<0.001).

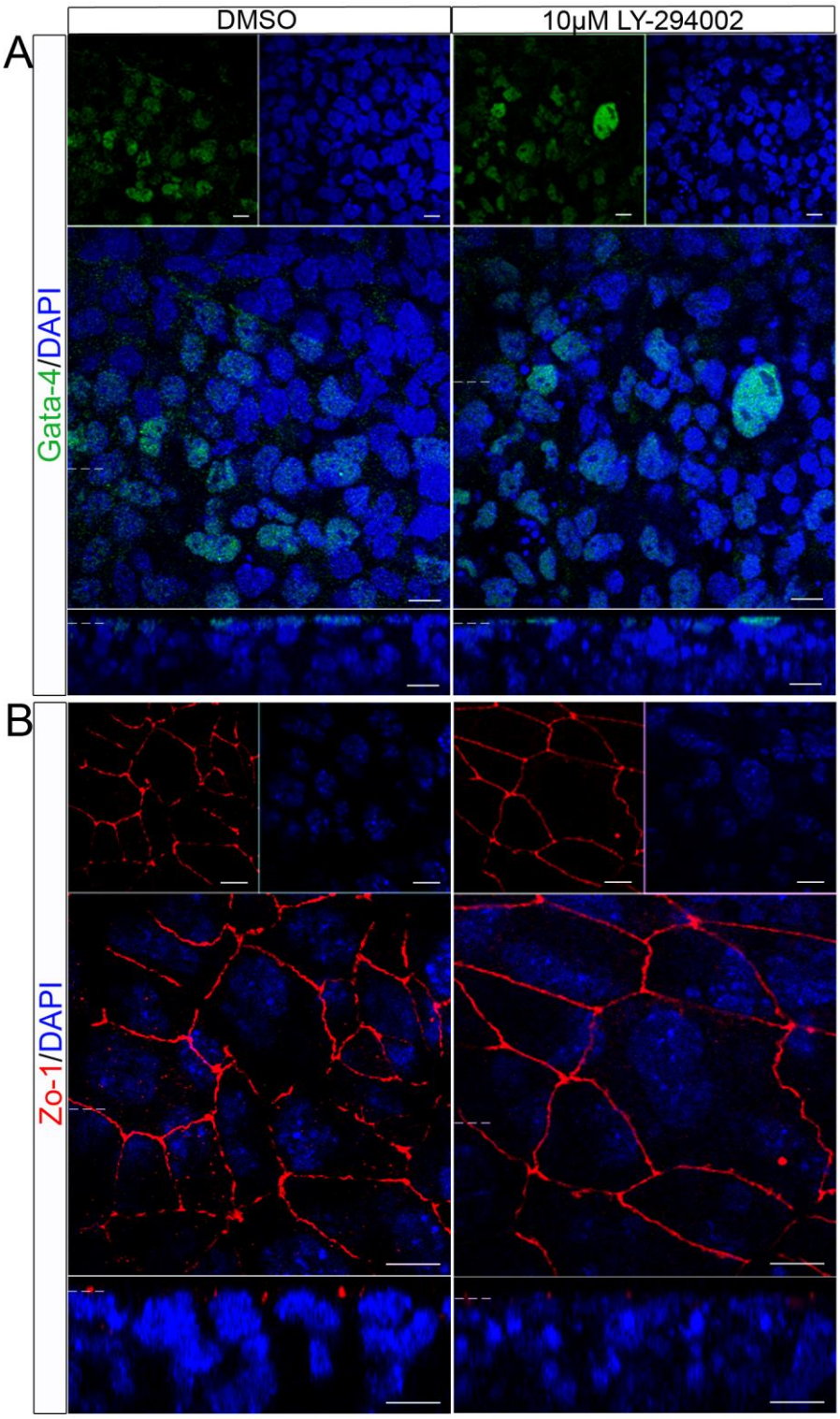

Figure 5.14: Localisation of primitive endoderm marker Gata4 and tight junction marker Zo-1 in the outer-layer of embryoid bodies is unaffected by inhibition of Pi3k

Embryoid bodies were treated with the Pi3k inhibitor 10μM LY-294002, or DMSO for 7 days. Whole-mount immunostaining demonstrated the localisation of (A) primitive endoderm marker Gata4, and (B) tight-junction protein Zo-1. Neither show any change in localisation when grown with a Pi3k inhibitor suggesting that the development of these cells is unaffected. Representative images from two independent experiments are shown. Scale bars 10μm. Dotted lines represent position that the relevant orthogonal or aerial images were taken.

5.2.9 Inhibition of Mek or the Fgf receptor signalling results in a loss of epithelial barrier function

The maintenance of a diffusion barrier is a common function of epithelial layers (St Johnston and Ahringer, 2010) . Therefore investigated the final experiment of this thesis investigated if, in addition to the mislocalisation of apico-basolateral polarity proteins described above, the cells of the embryoid bodies had defects in their barrier function. The diffusion of a membrane-impermeable biotin, which covalently links to amino groups on extracellular proteins, was used as a test for epithelial barrier function. The bound biotin can be readily visualised using fluorescently labelled streptavidin (Figure 5.15). In control embryoid bodies (Figure 5.15A&B), binding of biotin was restricted to the outer, apical layer of the embryoid bodies. When embryoid bodies were treated with the Mek inhibitor (PD-0325901) or Fgf receptor inhibitor (AZD-4547), the biotin was observed throughout the lateral side of the outer-layer cells, and in deeper cell layers (Figure 5.15 A&B). This demonstrated that the barrier function of the embryoid body epithelium was disrupted following inhibition of signalling by Mek or the Fgf receptor.

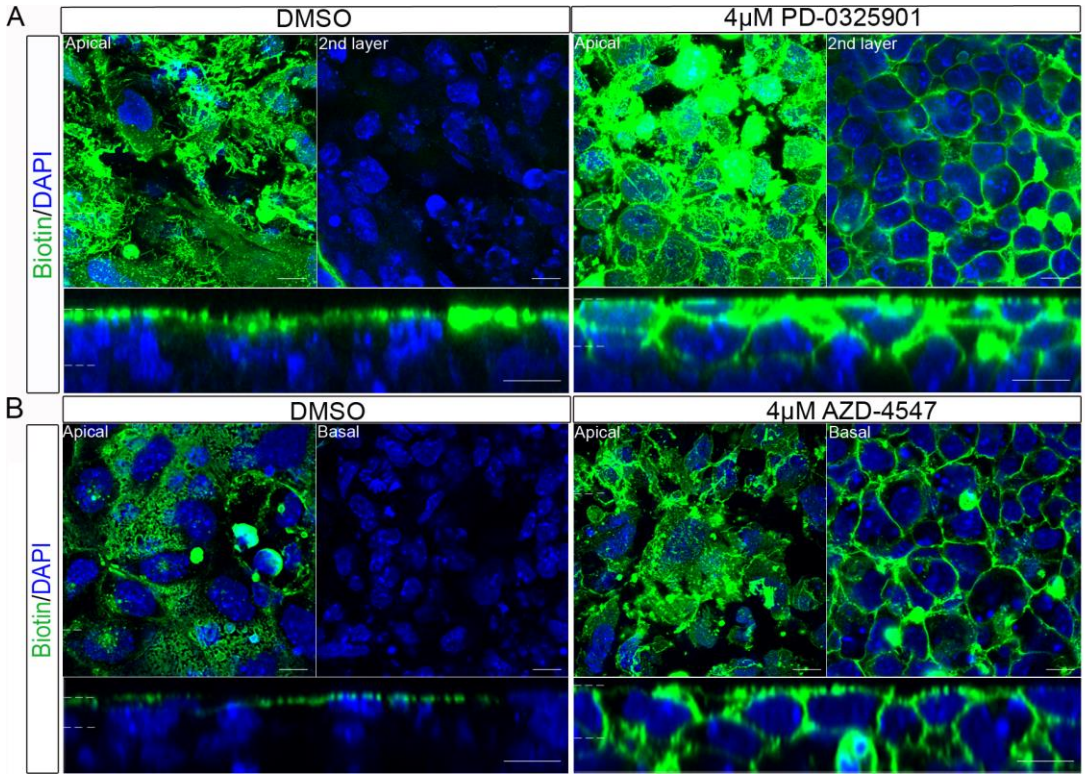

Figure 5.15: Inhibition of the Fgf receptor or Mek caused a loss in barrier function of the cells in the outer layer of the embryoid body.
Embryoid bodies were treated with (A) 0.04% DMSO, or 4μM PD-0325901(Mek inhibitor), (B) 0.04% DMSO or 4μM AZD-4547 (Fgf receptor inhibitor) for 7 days. A membrane impermeable biotin which covalently links to amino groups was added to the embryoid bodies and subsequently visualised using Alexa-fluor-488-conjugated Streptavidin. The biotin was largely restricted to the apical surface of the embryoid body in DMSO controls. Following treatment with either a Mek or an Fgf receptor inhibitor, biotin also bound to basolateral membrane proteins of the outer cell layer and the membranes of cells under this layer. This suggests that the normal epithelial barrier has been disrupted. Representative images from three independent experiments are shown. Scale bars 10μm. Dotted lines represent position that the relevant orthogonal or aerial images were taken.

5.3 Discussion

5.3.1 Summary

In this chapter, I investigated the role of different signalling cascades in the polarisation of the primitive endoderm using embryoid bodies as a model system. My data demonstrates that inhibition of either Mek or the Fgf receptor caused a loss of primitive endoderm markers, maintenance of Nanog and mislocalisation of all the polarity proteins examined; polarity complex protein aPkcζ/λ, tight junction protein Zo-1, adherens junction proteins β-catenin and E-cadherin, and basement membrane protein Fibronectin. Associated with the disruption in polarity and junctional protein localisation was a loss of the barrier function of the epithelium. This argues that Fgf receptor signalling activates Erk 1 and 2 which then promotes cell fate specification, polarisation and establishment of a functional epithelial barrier. Preliminary data also suggests that Pi3k signalling may not have a role in the cell fate specification or polarisation of the primitive endoderm.

5.3.2 Embryoid bodies as a model of primitive endoderm development

Embryoid bodies have previously been used as a model of primitive endoderm development (Further detail in 1.4). Work presented here provides further evidence that the requirement for Fgfr/Erk signalling in embryos is recapitulated in embryoid bodies. This suggests that embryoid bodies are a relevant model for primitive endoderm development as well as epithelial polarisation as discussed in Chapter 4.

5.3.3 Fgfr/Erk signalling is required for the formation of cystic embryoid bodies

Inhibition of Fgfr or Erk signalling clearly causes a change in the morphology of the embryoid bodies (Figure 5.1 and Figure 5.6). The increase in circularity of the embryoid bodies upon inhibition of Fgfr and Erk is due to a loss of cystic cavities, which results in the embryoid bodies appearing more white, and less hollow when viewed using a microscope. Gata4$^{-/-}$ embryoid bodies are also smaller than control and do not have cysts (Soudais et al., 1995). As a similar change in morphology is observed here after inhibition of Fgfr or Mek, it may be the inhibition of primitive endoderm cell fate which is the cause of this change in morphology. Additionally, no cavitation is observed when the basement membrane is disrupted when embryoid bodies are formed with Laminin-1$^{-/-}$ mES cells suggesting that it could also be the loss of polarisation of the embryoid bodies which prevents cavitation, resulting in formation of a rounder embryoid body. To further investigate this change in morphology it would be useful to

perform H&E staining on histological sections of the embryoid bodies to observe any morphological changes within the embryoid body such as an absence of cavitation.

The change in size and morphology of embryoid bodies is different in magnitude depending on whether the Fgfr or Erk inhibitor is used. Inhibition of Mek only caused a reduction in the size of the embryoid body at the highest concentration, whilst the circularity of the embryoid body was affected at all concentrations of the inhibitor. The Fgfr inhibitor caused a similar increase in circularity as the Mek inhibitor, but there was a significant reduction in the size of the embryoid body at all concentrations of the Fgfr inhibitor. There are multiple signalling pathways downstream of the Fgf receptor (Turner and Grose, 2010), it may be that one of these additional pathways may also regulate embryoid body size, perhaps through regulation of apoptosis, cell proliferation or cell size, whilst Erk signalling regulates the morphology of the embryoid body more than the size.

5.3.4 Fgfr/Erk signalling is required for cell fate specification and polarisation of the outer-layer of the embryoid body

The Fgfr/Erk signalling pathway is known to be required for cell fate specification of the primitive endoderm (Lanner and Rossant, 2010). My data confirms that inhibition of either the Fgf receptor or Mek signalling causes a loss of primitive endoderm cell fate markers and maintenance of the pluripotency protein Nanog. Interestingly, inhibition of the Fgf receptor caused a stronger reduction of primitive endoderm fate markers, despite inducing less inhibition of Erk than the Mek inhibitor. This suggests that another receptor may be activating Mek (Figure 5.12). This observation also raises the possibility that the Fgf receptor might be activating other signalling pathways, such as Pi3k-Akt, Stat, Plcγ, p38 Mapk, and Jnk (Turner and Grose, 2010) to promote expression of Gata4 and Gata6. If this hypothesis was true addition of Fgf to embryoid bodies treated with the Mek inhibitor would cause a upregulation of Gata6 and Gata4 through an Erk signalling independent pathway. To test which other signalling cascades were involved the effect of Fgf receptor inhibition on phosphorylation of proteins in different signalling cascades could be assessed by western blotting. Subseuqently, the effect of inhibition of these other signalling cascades on cell fate specification and polarisation of the outer-layer of cells of the embryoid body could be determined.

My data also show that Fgfr/Erk signalling is required for the formation of a polarised primitive endoderm layer with an efficient barrier function. This hypothesis is supported by several other findings. Firstly, aPkc does not polarise in the primitive endoderm of embryos grown in

2i medium, which includes a Mek and a Gsk3β inhibitor (Saiz et al., 2013). Secondly, expression of a truncated form of Fgfr2 in mES cells has been reported to disrupt basement membrane formation (Li et al., 2001a), while Grb2 knockout embryoid bodies show an absence of Laminin basal to the outer cell layer (Cheng et al., 1998). Combining my data with previous studies leads us to propose that the Fgfr/MAPK signalling pathway promotes polarisation of the primitive endoderm as well as its fate. There are at least three possibilities to explain this dual role (Figure: 5.16); 1) Fgfr/Erk signalling regulates primitive endoderm cell fate specification which then promotes polarisation. 2) Fgfr/Erk signalling initiates the polarisation of the primitive endoderm which then drives fate specification. 3) Mapk independently regulates both the polarisation and fate determination of the primitive endoderm.

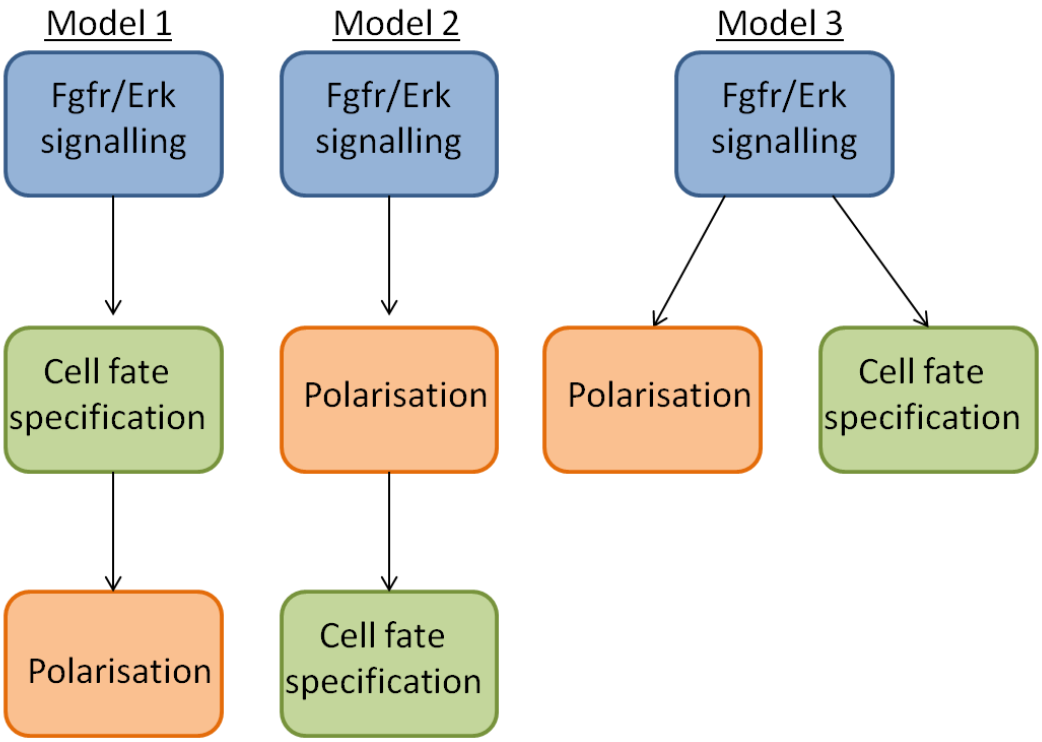

Figure: 5.16: Three possible explanations for the dual role of Fgfr/Erk signalling in primitive endoderm development.
Results presented in this chapter show that Fgfr/Erk signalling is required for the formation of a polarised primitive endoderm layer. This suggests that the process of cell polarisation and cell fate specification may be dependent upon each other (Models 1 & 2), or that these two processes are independent but both regulated by Fgfr/Erk signalling (Model 3).

In embryoid bodies, the cells start to polarise prior to the maximal nuclear localisation of Gata6, Gata4, and Hnf4α (Figure 4.6, Figure 4.10, Figure 4.11, Figure 4.12). This suggests that in embryoid bodies the onset of polarisation occurs before cell fate specification and argues against model one where cell fate regulators promote polarisation. There is also evidence that disrupting polarisation of the primitive endoderm does not affect fate determination, arguing

against model two. Embryoid bodies formed with mES cells which do not express Laminin γ1, a key component of the basement membrane, have primitive endoderm cells in the outer-layer although they are not properly organised (Murray and Edgar, 2000, 2001). Additionally, E-cadherin $^{-/-}$ ES cells showed normal primitive endoderm development, but the embryoid bodies did not form a cavity, consistent with defects in epithelial barrier formation (Rula et al., 2007). In Dab2 null mouse embryos cells of the primitive endoderm lose their apico-basolateral polarity. In these embryos Gata4 expression was observed, but positive cells were positioned throughout the epiblast, suggesting a failure in positioning but not fate regulation (Rula et al., 2007; Yang et al., 2007).

The studies described above argue against the idea of polarity regulating cell fate (model 2). However, there is also evidence to support the model that loss of proteins required for epithelial polarisation can disrupt primitive endoderm fate specification. In Integrin β1 -/- embryoid bodies, α-fetoprotein (a marker of visceral endoderm) is only expressed in clusters of endoderm cells, Dab2 protein was not observed by immunofluorescence, and Gata4 became cytoplasmic (Liu et al., 2009). This suggests that the absence of integrins disrupts the development of the primitive endoderm. The mutant embryoid bodies had no ppErk1/2, and had reduced p38 and Akt activation suggesting that integrins control endoderm differentiation via the Raf/Erk and Akt signalling pathways (Liu et al., 2009). Live-imaging of embryos treated with an aPkc inhibitor revealed that the primitive endoderm cells migrate but fail to remain on the ICM surface when they reach the cavity. This resulted in primitive endoderm cells being present throughout the epiblast rather than forming a distinct layer on the edge of the blastocoel (Saiz et al., 2013). No effect was seen on early markers of the primitive endoderm fate (Pdgfrα, and Gata6), but some cells were negative for both Nanog and Gata4, suggesting a defect in primitive endoderm maturation (Saiz et al., 2013). Co-transfection experiments also showed that the Dab2 promoter can be transactivated by forced expression of Gata6, in NIH-3T3 cells (Morrisey et al., 2000) suggesting the polarity regulator Dab2 may be a downstream target of the cell fate regulator Gata6. In summary, current evidence appears to support the idea of a hybrid model where early regulation of primitive endoderm fate acts upstream of polarisation, but that aPkc and possibly other regulators of polarity, are required for the maturation of the primitive endoderm fate. This is clearly a complex process, and the mechanistic elucidation of Fgfr/Erk signalling's dual role in cell fate determination and cell polarisation, and how these two processes are interlinked and interact will be a key goal for future work (Further detail in 6.3).

5.3.5 Fgfr/Erk regulation of protein expression differs depending on protein

The effect of Fgfr or Erk inhibition on the localisation of polarity proteins is very similar. However, the effect on expression of polarity proteins appears to be dependent upon whether it is the Fgf receptor or Erk which is being inhibited (Table 5.1)

Table 5.1: Summary of effect of inhibition of Mek or the Fgf receptor on expression of proteins usually apico-basolaterally localised in the primitive endoderm.

	Mek inhibition (4μM PD-0325901)	**Fgfr inhibition (4μM AZD-4547)**
aPkcζ/λ	Possible small increase	Possible small increase
Zo-1	Unknown	Possible Increase in Zo-1α+, no change in Zo-1α-
β-catenin	No change	Possible small decrease
E-cadherin	Possible increase	Statistically significant increase
Fibronectin	Possible small increase	No change

A different effect is seen following Fgf receptor or Erk inhibition on the expression of every protein observed except E-cadherin. There are three possible reasons for this; 1) the effects observed are mostly very small, not statistically significant and therefore may not be biologically relevant but instead are just noise; 2) these two inhibitors aren't effecting exactly the same intercellular signalling pathways and therefore are effecting protein expression in different ways in the primitive endoderm; 3) the protein levels assessed are across the whole embryoid body, whilst the investigation of the localisation of these proteins was restricted only to the first few layers of the embryoid body. These two signalling pathways may have different effects on the inner layers of the embryoid body not visible during the confocal analysis of the whole-mount immunostaining, whilst having the same effect on protein expression in the outer-layers of cells.

A significant increase in E-cadherin is observed upon inhibition of the Fgf receptor whilst a small increase is detected when Erk is inhibited. Interestingly, no effect is seen on β-catenin levels upon Erk signalling, whilst inhibition of the Fgf receptor causes a small decrease. Both β-catenin and E-cadherin are components of the adherens junction but different effects are observed on their protein expression levels. This suggests that expression of the proteins present in this junction is differently regulated event though a similar effect on their localisation is observed with the two inhibitors. This may occur because β-catenin is known to have a role in signalling and its expression levels may change depending on the differentiation of the other cells within the embryoid body.

An increase in expression of the Zo-$1^{\alpha+}$ splice variant was observed upon inhibition of the Fgf receptor. Late expression of Zo-$1^{\alpha+}$ and its intracellular localisation has been suggested to be a rate-limiting step in the synthesis of the tight junction (Sheth et al., 1997). *In vivo* Zo-$1^{\alpha-}$ tight junction formation occurs from the 8-cell stage after compaction, whilst the Zo-$1^{\alpha+}$ tight junction assembly occurs during the 32-stage prior to blastocoel accumulation, and is localised in perinuclear sites before incorporation into the tight junction (Sheth et al., 1997). As a disruption in the localisation of Zo-1 was observed upon inhibition of the Fgf receptor it could be that the Zo-$1^{\alpha+}$ is expressed because the cell is undergoing tight junction assembly and is not polarised.

5.3.6 Pi 3-kinase may not regulate primitive endoderm specification or Zo-1 localisation

Inhibition of the Fgf receptor resulted in only a small decrease in ppErk levels, suggesting that there may be additional signalling pathways downstream of the Fgf receptor which regulates the development of the primitive endoderm (Figure 5.12). Data presented here suggests that although Pi3k signalling regulates the expression levels of Gata4, it is not required for localisation of Gata4 to the nuclei of primitive endoderm cells. This is in contrast to results published by others which suggest that Fgfr regulates primitive endoderm specification through the Plcγ1 and Pi3k-Akt pathway rather than through Erk signalling (Chen et al., 2000). The difference in results may be because in my study 10**μ**M LY-294002 was added only on day 0 and day 5, whilst Chen et al. (2000) added 10**m**M LY-294002 in fresh media every day. At this concentration, ~7000 times its enzymatic IC-50 (1.4μM) (Vlahos et al., 1994), it is possible that this compound is causing toxic affects, as well as many off-target effects. In the presence of LY294002, Nanog repression is enhanced which suggests that the Pi3k-Akt pathway is not responsible for Nanog repression (Hamazaki et al., 2006; Hamazaki et al., 2004). As Nanog repression is a critical event in primitive endoderm specification this suggests that Pi3k signalling does not regulate this aspect of primitive endoderm development. Investigating the effect of PI3K inhibition on localisation and expression of additional proteins required for primitive endoderm specification would be essential to make a clear conclusion as to the role of this signalling cascade in primitive endoderm development.

Pi3k-Akt signalling regulates transcription and localisation of Laminin β1 and collagen in embryoid bodies (Li et al., 2001b). No change in the localisation of the tight junction protein Zo-1 was observed in this study upon inhibition of Pi3k signalling, suggesting it does not regulate its localisation. Interestingly, there was a decrease in expression of E-cadherin, but there was not sufficient time to investigate its localisation. To clarify the effect of Pi3k

signalling on epithelial polarisation in this system it would be beneficial to investigate the localisation of other proteins which have been shown to be apico-basolaterally localised under normal conditions.

5.3.7 Additional signalling pathways upstream of Erk signalling

As only a small decrease in ppErk levels was observed when the Fgf receptor was inhibited there must be other inputs than the Fgf receptor upstream of Erk (Figure 5.12). These could be other receptor tyrosine kinases eg. Egfr (Roskoski, 2012), or alternatively G-protein coupled receptors (Sugden and Clerk, 1997), Integrins (Giancotti and Ruoslahti, 1999), or Calcium (Agell et al., 2002).

5.3.8 Conclusion

Here, with my extensively characterised embryoid body model of primitive endoderm specification and polarisation I investigated the mechanisms which promote polarisation of the primitive endoderm. My results demonstrate that the Fgfr/Erk pathway is required for the formation of a polarised primitive endoderm cell layer with in embryoid bodies. Treatment with small molecule inhibitors of Mek or the Fgf receptor caused a mislocalisation of polarity complex, tight junction, adherens junction and basement membrane proteins which normally show apico-basolateral polarisation in the primitive endoderm cells. Additionally, disruption of the epithelial barrier, which normally blocks free diffusion across the epithelium of the primitive endoderm, was observed. These results show that Fgf receptor driven Erk signalling is required for the formation of an endoderm with apico-basolateral polarity and epithelial barrier function in embryoid bodies.

Chapter 6

6 Final Discussion and Future work

6.1 Summary

The aim of this thesis was to establish a model to investigate the relationship between polarisation and cell fate specification in preimplantation epithelia. When mES cells were cultured with BMP4, cells expressed transcription factors required for trophoblast development but did not express *Elf5*, the trophoblast markers Cdx2 and Eomesodermin did not colocalise, and there was expression of the mesodermal transcription factor *Brachyury*. BMP4 did not therefore induce differentiation of mES cells to cells with characteristics which are known to be typical of trophoblast cells. Additionally, there was a lot of experimental variation. It was therefore decided that BMP4 induced trophoblast differentiation would not make a good a model of the development of preimplantation epithelia for this thesis.

Subsequent work focused on the use of embryoid bodies as a model of primitive endoderm development. The outer-layer of an embryoid body forms the primitive endoderm. Using hanging drop embryoid bodies the gradual fate specification and polarisation of the primitive endoderm was observed. It was discovered that the peak in number of positive nuclei for primitive endoderm cell fate markers such as Gata6 and Gata4, and the complete loss of pluripotency marker Nanog occurred after some proteins showed apico-basolateral polarisation.

Having established the use of hanging drop embryoid bodies as a model of primitive endoderm development the regulation of the primitive endoderm polarisation was investigated. It has previously been shown that the Fgfr/Erk signalling cascade is required for primitive endoderm cell fate specification. Results presented here showed that this signalling cascade was required for the formation of a polarised primitive endoderm. Inhibition of either the Fgf receptor, Erk signalling, or both these components resulted in a mis-localisation of epithelial polarity and junction proteins and a disruption of barrier function. This suggests that the Fgfr/Erk signalling cascade is required for the formation of a polarised primitive endoderm and that the processes of cell polarisation and cell fate specification may be inter-related and potentially dependent upon each other during primitive endoderm development.

6.2 Embryoid bodies as a model of preimplantation epithelial development

As outlined above the development of the primitive endoderm in embryoid bodies was chosen to be used in this thesis as a model of primitive endoderm development. There are many

advantages to using this model. It is a reliable 3D model composed of a variety of non-transformed cell types, which gradually polarise and express cell fate markers. In order to probe their development it is possible to manipulate the embryoid bodies using small-molecule inhibitors as shown in this thesis, as well using the many transgenic mES cell lines which are available (Dolgin, 2011; Skarnes et al., 2011). This model is also amenable to many techniques for analysis, such as whole-mount immunostaining, western blotting and PCR. The use of these techniques for manipulation and analysis of development allows thorough investigation of epithelial polarisation, and primitive endoderm fate cell fate specification individually. They also provide a good model to study the regulation of these events, and the relationship between them, some experiments which could be carried out using this system to examine this question in the future are proposed in 6.3.

There are however limitations to the use of embryoid bodies. For example, the protein analysis performed here was done using whole embryoid body lysates and therefore analysis was potentially confused by the expression of proteins in the non-primitive endoderm cells within the embryoid body. A potential solution to this would be to sort the primitive endoderm cells using FACS to allow expression analysis of only this cell population instead of the whole-embryoid body. Additionally, when investigating the development of the primitive endoderm in mouse embryos, because they are smaller it is possible to confocal through the embryo which facilitates analysis of the sorting of these cells. A possible solution to this would be to develop a protocol of sectioning the embryoid bodies and performing immunhistochemistry to examine the inner cells of the embryoid body. A final limitation is that use of embryoid bodies is a good model to investigate mechanisms but it is not an *in vivo* model, and is therefore unlikely to fully recapitulate all aspects of tissue development. It is therefore essential to confirm major findings from embryoid bodies *in vivo.*

6.3 The relationship between polarisation and cell fate specification in the primitive endoderm

The role that different polarity proteins have in the development of the primitive endoderm has been previously investigated (Further detail in 1.4.7). There has however been very limited work which has studied the relationship between primitive endoderm cell fate specification and polarisation. Work presented here in chapter 5 identified a role for the Fgfr/Erk signalling pathway in the development of a polarised primitive endoderm. Additionally, results outlined in chapter 4 suggested a gradual polarisation of the primitive endoderm which is established prior to maximal expression of cell fate specification proteins.

These two results suggest that there may be an inter-dependent relationship between primitive endoderm polarisation and fate specification, alternatively these two events may be regulated by the same mechanism independently, (Figure: 5.16) (Further discussion in 5.3.4).

There are many possible future experiments which could be performed to begin to investigate the relationship between primitive endoderm polarisation, cell fate specification and the Fgfr/Erk signalling cascade. One such experiment is to further investigate the temporal relationship between these two events by trying to isolate the two events. For example, in all experiments described in Chapter 5 the small-molecule inhibitors were added on day 0, and also when the media was replenished on day 5, all analysis was subsequently done on day 7. It would be interesting to analyse the phenotype observed at other time points, such as on day 3 when some proteins have already polarised, but few cells express primitive endoderm markers. Additionally, adding the inhibitors at different time points during the development of the embryoid bodies may provide more information. Addition of the inhibitor on day 3 would allow the outer-layer to begin to polarise but full fate specification would not be expected to occur prior to treatment. Alternatively the inhibitor could be added from day 0 until day 3, during which time it is known that the outer cell layer polarises but complete cell fate specification does not occur. The removal of the inhibitor on day 3 would then allow the embryoid bodies to potentially recover polarity and/or cell fate and may provide us with further information as to the relationship between these two processes.

A different approach to investigate the relationship between these two components and Fgfr/Erk signalling would be to try to rescue the phenotype we have observed after addition of the Fgfr or Mek inhibitors. This could be either by inducing cellular polarisation or cell fate specification in the embryoid body. For example, a basement membrane component could be added to the outside of the embryoid body treated with the small molecular inhibitor to try and induce a polarity in the cells, it would be interesting to see if these cells subsequently acquire primitive endoderm fate markers, or just develop a polarity. If they do not acquire primitive endoderm fate it would suggest that primitive endoderm cell fate specification is not directly downstream of polarisation. Addition of exogenous laminin to embryoid bodies has previously been shown to prevent accumulation of a basement membrane, and expression of the endoderm marker Afp, but this was carried out on normal developed embryoid bodies (Grover et al., 1983). This suggests that addition of a basement membrane component may not induce polarity in the primitive endoderm cells as hoped. One approach to rescuing cell fate specification would be to induce *Gata6* or *Gata4* expression after embryoid body

formation and treatment with small molecules. This could perhaps be done using an inducible cell line, so that addition of a chemical such as doxycylcine would induce expression of the gene and therefore potentially stimulate primitive endoderm fate specification in these cells. If inducing primitive endoderm fate specification rescued both the cell polarisation and the cell fate specification it would suggest that cell polarisation can be regulated by cell fate specifcation and cell fate specification is regulated by Fgfr/Erk signalling. Alternatively if inducing epithelial polarisation in the cells rescues both the cell polarisation and cell fate specification it suggests cell polarisation can regulate cell fate specification and cell polarisation is directly regulated by Fgfr/Erk signalling. If both rescuing cell fate and cell polarity individually rescue the full phenotype it would suggest a complicated inter-dependent relationship between these two events which would require investigation with further experiments. Lastly, if each approach induced only the cell polarisation or cell fate specification expected it would suggest that the Fgfr/Erk signalling cascade regulates these two events independently.

An alternative approach to investigating the relationship between the cell fate specification and cell polarisation of the primitive endoderm in embryoid bodies would be to disrupt these processes directly and analyse the phenotype. For example, Gata6 and Gata4 knockout mES cells already exist, their phenotype in embryoid bodies has been analysed with respect to primitive endoderm fate specification (Bielinska and Wilson, 1997; Morrisey et al., 1998; Soudais et al., 1995), but the polarisation of the outer-layer of cells was not investigated. Additionally, cell polarisation could be disrupted using small molecule inhibitors such as an aPkc inhibitor. aPkc inhibitors have been used before *in vivo* but without a comprehensive study of the localisation of multiple polarity and junction proteins (Saiz et al., 2013) . E-cadherin blocking antibodies are available and have been used previously, and would potentially disrupt cell polarisation (Malaguti et al., 2013). Alternatively, there are many mES cell lines which are available that could be used to disrupt epithelial polarisation for example mES cell lines are available which are null for polarity and junction components, for example Zo-1$^{-/-}$(Xu et al., 2012) Afadin$^{-/-}$ (Komura et al., 2008), and Cingulin$^{-/-}$ (Guillemot et al., 2004). It would be important with all these approaches aimed at disrupting polarisation to ensure that apico-basolateral polarisation is disrupted, as loss of some components which are polarised does not cause mis-polarisation of the whole cell, for example Occludin$^{-/-}$ embryoid bodies (Saitou et al., 1998). Alternatively, a small scale chemical screen for polarity regulators could be performed on embryoid bodies, using known inhibitors of key pathways. This could identify novel signalling pathways which regulate primitive endoderm polarisation, and/or cell fate

specification which would increase our knowledge about both of these events and might futher elucidate the relationship between these two events.

6.4 The role for Fgfr/Erk signalling in epithelial polarisation

The focus of this PhD was has been the development of embryonic epithelia, but a key question is whether this is relevant to adult epithelia. Work presented here suggests that Fgfr/Erk signalling is required for the polarisation of the cells of the primitive endoderm. Conversely, there is a lot of evidence that the Erk signalling pathway can cause the opposite effect in other cell types, therefore resulting in epithelial-mesenchymal transition (EMT). In an EMT-induced cell line HOC313 cells (a head an neck squamous cell carcinoma cell line), inhibition of Fgf with a small molecule inhibitor causes reduced expression of Snail1 and 2 (EMT genes) , and induces E-cadherin expression (Nguyen et al., 2013). Suggesting Fgfr/Erk signalling is required for epithelial to mesenchymal transition.

Ha-Ras-transformed mammary epithelial cells (EpRas) cells are a different polarised cell line used to investigate EMT. When EpRas cells are cultured in collagen gels with an Erk signalling inhibitor more than 95% of TGFβ-induced EMT structures fail to form (Janda et al., 2002). This suggests that Erk signalling is required for TGFβ-induced EMT. Ras mutants which are known to selectively signal through either the Erk or Pi3k pathway were used to probe the requirements for Erk or Pi3k signalling in this system. The mutants with overactive Erk signalling had a spindle-shaped morphology, were positive for the EMT marker vimentin, and lost the epithelial marker E-cadherin (Janda et al., 2002). Conversely, the cells with increased Pi3k signalling showed redistribution of E-cadherin, but did not upregulate vimentin (Janda et al., 2002). These two experiments therefore demonstrated that TGFβ signals through Erk inducing EMT in cells, which involves a loss of polarisation, therefore having the opposite effect to that which it has in the primitive endoderm.

However, the role that Fgfr/Erk signalling has in epithelial polarisation is very complex and context dependent. For example in Caco-2 cells the differentiation state of the cells or the stimulus changes the effect Erk signalling has on tight junctions (Aggarwal et al., 2011). Epidermal growth factor (EGF), a stimulus of Erk signalling, makes H_2O_2 induced barrier disruption worse in undifferentiated cells, whilst when EGF was added to differentiated cells the disruption of the barrier was reduced (Aggarwal et al., 2011). This suggests that Erk signalling has two opposing effects on tight junction stability depending upon the

differentiation state of the cells. When Caco-2 cells are undifferentiated stimulation of Erk signalling causes disassembly of the tight junction, conversely, in differentiated cells Erk signalling results in assembly of the tight junction, as is observed in the work presented here.

The studies presented above (Aggarwal et al., 2011; Janda et al., 2002; Nguyen et al., 2013) suggest that the role of the Fgfr/Erk signalling pathway in epithelial polarity is context dependent. Therefore, the requirement we have identified for the Fgfr/Erk signalling pathway in the establishment of a polarised epithelium in embryoid bodies may be observed in other systems. In depth studies are required to determine the effect of Fgfr and Erk inhibition on the polarisation of other embryonic epithelia as well as to see if they have a role in the maintenance of polarisation in adult epithelia.

To help these comparisons to other systems it would be interesting to investigate the mechanism by which Fgfr/Erk signalling regulates polarisation in the primitive endoderm of embryoid bodies. As Erk signalling is known to phosphorylate many transcription factors, protein kinases and phosphatases and other proteins directly (Roskoski, 2012) a candidate approach would be a good way to start investigating this. The first step in epithelial polarisation involves the adhesion of the basal surface of the cell by integrin receptors to the basement membrane (Martin-Belmonte and Perez-Moreno, 2012). Initial adhesions between cells are formed from Nectin-Afadin associating with Par3, followed by recruitment of other junction proteins to produce a primordial adhesion. It could be one of the proteins which is involved in the establishment of epithelial polarisation that is the target directly, or indirectly of the Fgfr/Erk signalling. Alternatively, it could be the maintenance of the polarisation which is regulated by Fgfr/Erk and therefore it could be any protein or proteins which are involved in epithelial polarisation that is a direct or indirect target of this signalling cascade.

6.5 Final conclusion

In summary, work presented in this thesis highlights embryoid bodies as a good model for studying the regulation of primitive endoderm polarisation and the relationship between epithelial cell fate specification and polarisation. Results demonstrated that in the outer-layer of embryoid bodies the onset of polarisation occurred prior to maximal expression of primitive endoderm fate markers. The work also showed that the Fgfr/Erk signalling pathway is required for polarisation and barrier formation in the primitive endoderm of embryoid bodies. This poses the question of whether there is a relationship between primitive endoderm

polarisation and cell fate specification which future studies could investigate. These studies are likely to be relevant to the development of other embryonic and adult epithelia.

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