UNIVERSITY OF LIVERPOOL

The role of Heparan Sulphate in the Development of Mouse Embryoid Bodies

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Master in Philosophy

By
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Abbreviations

ASC Adult stem cell
BM Basement membrane
BMP Bone morphogenic protein
CEE Columnar epiblast epithelium
CRC Colorectal cancer
DAPI 4',6-diamidino-2-phenylindole
DMEM Dulbecco's modified eagles medium
EB Embryoid body
EEE Extraembryonic endoderm
ECM Extracellular matrix
EHS Engelbreth-Holm-Swarm
EMT Epithelial-to-mesenchymal transition
EXT1 Exostosin 1
EXT 2 Exostosin 2
FGF Fibroblast growth factor
GAG Glycosaminoglycan
GlcA Glucuronic acid
HS Heparan sulphate
HSPG Heparan sulphate proteglycans
ICM Inner cell mass
KLF Krüppel-like factor
mESC Mouse embryonic stem cell
LIF Leukemia Inhibitory factor
MEF Mouse embryonic fibroblasts
NEAA Non-essential amino acids
PBS Phosphate buffered saline
PE Parietal endoderm
PrE Primitive endoderm
qPCR Quantitative polymerase chain reaction
SD Standard deviation
STAT3 Signal transducer and activator of transcription 3
STO Mouse feeder layer
T Brachyury
TE Trophectoderm
WT Wildtype
VE Visceral endoderm

Abstract

Background:

The development of *in vitro* Embryoid Body (EB) formation facilitates the investigation of many aspects of cell differentiation occurring during early mammalian development. The function of various factors, such as fibroblast growth factors (FGFs) and their corresponding pathways within embryogenesis, has been widely studied. It is well documented that the polysaccharide Heparan Sulphate (HS) is essential for normal development within mouse embryonic stem cells (mESC); this is thought to be due its role as a co-factor in FGF signalling. Despite acting as a successful model for peri-implantation, HS-deficient EBs have yet to be fully characterised and it is undetermined if they develop in a similar way to EBs generated from wild-type (WT) cells.

Objectives:

The aim of this study was to investigate the role of HS in the development of mouse EBs at Day 4 derived from WT and EXT1-/- mESCs. The differentiation of Primitive Endoderm (PrE) and the subsequent synthesis of a basement membrane were primarily investigated using immunofluorescence and quantitative Polymerase Chain Reaction (qPCR). The expression of mesoderm, ectoderm and pluripotency markers were also analysed using these techniques.

Results:

EXT1-/- ESCs show less spontaneous differentiation as shown by increased Oct4 and nanog and lower levels of lineage markers. EXT1-/- EBs did not form BMs, with immunostaining for laminin-111 showing only weak intracellular staining in peripheral cells of day 4 EBs, and qPCR showing very low levels of Lama1 mRNA compared with WT EBs. EXT1-/- EBs failed to produce extraembryonic endoderm (EEE) at Day 4, based on morphological criteria and absence of immunostaining for megalin, and qPCR showed lower levels of the endodermal marker Pax6. EXT1-/- EBs expressed higher levels of Brachyury and Pax6 mRNA compared with the WT, as shown by qPCR. Results were not statistically significant.

Conclusions:

Analysis of immunofluoresence and qPCR indicated that an absence of HS results in decreased differentiation of endodermal lineages and as a consequence were unable to synthesis basement membranes. This could be due to the disruption of FGF signalling that regulates endodermal development. The ectodermal marker, Pax6, was also downregulated in EXT1-/- cells. Surprisingly, a lack of HS caused the up-regulation of the mesodermal marker, Brachyury. It is suggested that HS may play a role in the regulation in Krūppel-like factors (Klfs), in particular Klf5, which in turn are involved in the specification of mesoderm. Mesenchymal-like cells present within colorectal cancer have been shown to express nanog; this could explain the atypical up-regulation of the pluripotancy marker in the EXT1-/- EBs. Further directions would be the investigation of FGF signalling, gene expression of Klfs and characterising the phenotype of the mesodermal cells within EXT1-/- EBs.

1.Introduction

1.1 Early Mouse Development

1.1.1 Pre-implantation (from fertilisation to blastocyst formation)

Pre-implantation is the period of time that begins with fertilization and ends with the implantation of an embryo.
Embryo development commences when a spermatozoon fertilizes an oocyte in the ampulla of the uterine tube. Prior to fertilisation oocytes are suspended in 2nd meiotic division. On the completion of meiosis the cell becomes diploid and is known as a zygote. At approximately 30 hours post-fertilization the zygote undergoes cleavage, whereby rapid mitotic cell division forms progressively smaller blastomeres. The first cleavage produces two identical cells, which in turn, divide asynchronously to produce 4 cells, 8 cells, 16 cells, and so on. Over three days the zygote travels through the fallopian tube and completes three rounds of division. (Moore *et al.* 2013). In the mouse, compaction occurs around the eight-cell stage whereby the blastomeres are seen to maximize contact with each other via adherens junctions and tight junctions. E-cadherin, which is a Ca^2+-dependent cell-cell transmembrane protein, is a key component of the adherens junction, and expression levels increase around the time of compaction. Following compaction, individual blastomeres can no longer be distinguished from each other macroscopically (Ducibella *et al.,*1975; Hyafil *et al*. 1980; Vestweber et al. 1984). As the embryo enters the uterus it develops into a 16-32-cell ball and is referred to as the morula.

The next event is the formation of the blastocyst. At this point the first differentiation event in mammalian development occurs. This is the differentiation of trophectoderm. Trophectodem (TE) cells pump salt inside the morula, water follows through osmosis, and this is how the blastocoel cavity is formed. When TE cells differentiate Na ion channels localise to the apical

surface of the cells, and sodium potassium ATPase (sodium pump) localises to the basal surface, thus enabling Na to be pumped inside the morula.

Following formation of the blastocoel cavity, the inner cells are located to one pole of the blastocyst, and are referred to as the 'inner cell mass' (ICM). (Tarkowski et al. 1967)

The ICM comprises a population of pluripotent embryonic stem cells (ESCs) that give rise to three embryonic germ layers lines (endoderm ectoderm and mesoderm) that generate all the cell types of the embryo proper.
A number of transcription factors, including Oct-4 (Pou5fl), Sox2, Nanog and Sall4, play an important role in maintaining the pluripotency of ESCs (Jia-Chi *et al.* 2013). These factors are capable of inducing the expression of each other, and are essential for maintaining the self-renewing undifferentiated state of the ESCs in the ICM of the blastocyst (Fig.1) (Rodda *et al.* 2005).

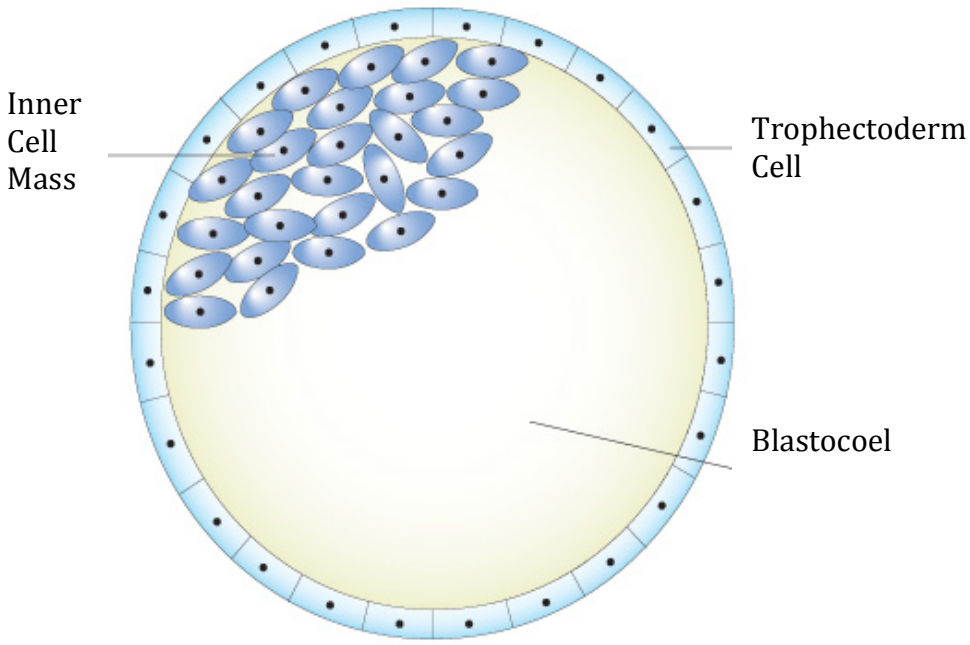

FIG 1. **Schematic representation of the blastocyst**. The trophectoderm cells pump fluid into the morula to form the blastocoel. The fluid-filled cavity surrounded by a ring of trophectoderm with the inner cell mass aggregated to one side.
Image taken from: E-learning Unit, SGUL. 2014. Blastocyst. [ONLINE] Available at:http://www.elu.sgul.ac.uk/rehash/guest/scorm/138/package/content/blastocyst.htm [Accessed 05 September 2014]

Following blastocyst formation, and shortly before implantation, the peripheral cells of the ICM that face the blastocoel cavity form the extraembryonic primitive endoderm (PrE), or hypoblast (Gilbert et al. 2000).

A function of the PrE is to secrete extracellular matrix (ECM) molecules in order to deposit a basement membrane (BM) between themselves and the remaining undifferentiated cells of the ICM. It does not give rise to any cells of the embryo proper. Shortly after implantation, the PrE differentiates into two other extraembryonic cell lineages, the parietal endoderm (PE), and visceral endoderm (VE). PE differentiates from PrE cells in contact with the trophectoderm. It migrates to cover the inner surface of the trophectoderm, forming the parietal yolk sac (Enders *et al.,* 1978) PE begins to produce large amounts of basement membrane (BM) components, including laminin and type IV collagen, which are incorporated into a thick BM called Reichert's membrane (Fig 2).

The PrE cells that directly overly the epiblast, differentiate to become visceral endoderm (VE) cells, which are columnar epithelial cells with extensive microvilli. (Yang *et al.* 2002).

Despite its name it has been previously understood that the visceral endoderm (VE) is strictly extraembryonic contributing to the yolk sac and does not contribute directly to organogenesis; however, recent studies suggest that VE may contribute to the gut lining of the embryo proper (Kwon *et al.* 2008;Tam et al. 1992).

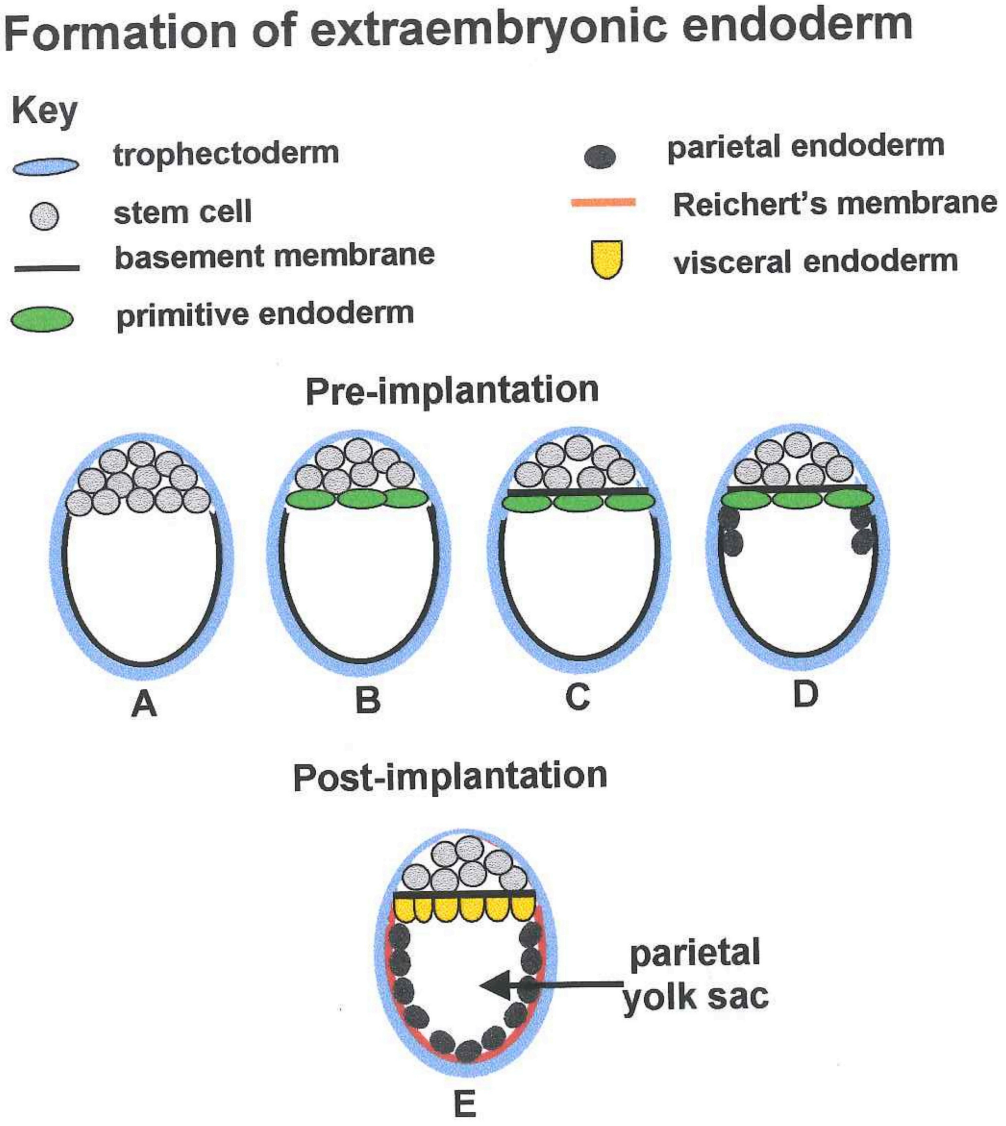

Fig 2. Schematic representation of EEE formation. (A) Upon blastocyst formation the ICM consists of equivalent stem cells. (B) Shortly afterwards, the stem cells on the surface of the ICM differentiate to become PrE. (C) The PrE deposits a BM between itself and the remaining undifferentiated stem cells. (D) Subsequently, the PrE cells that lie adjacent to the trophectodermal BM differentiate to become PE, migrate and deposit the Reichert's membrane. (E) Following implantation, the parietal yolk sac is formed. The PrE overlying the ICM that remain attached to their BM, differentiate to become VE. ***Taken from: Murray (2001) Early mouse development. Manuscripts. PhD. University of Liverpool.***

1.1.2 Mechanisms that regulate the differentiation of primitive endoderm

A number of transcription factors are active in the induction of PrE and can be used as markers.

Several of the Gata zinc finger transcription factors (Gata1-6) play a crucial role in the development and differentiation of a number of cell-types and are expressed in haematopoietic, endothelial, cardiac and gut-derived tissue (Koutsourakis *et al.* 1999). Gata6 has an important role in the differentiation of primitive, parietal and visceral extraembryonic lineages (Fujikura *et al.,* 2002) and Gata6 transformed mESCs demonstrate endoderm epithelial morphology (Li *et al.,* 2004;Ralston *et al.,* 2005). Gata6-null mESCs fail to develop and no recognizable extraembryonic endoderm (EEE) structure is present in Gata6-null embryos (Koutsourakis et al., 1999).

Gata6 acts downstream of the Fibroblast Growth Factors (FGF) signalling pathway (Morrisey *et al.,* 1998). FGF signaling is required for endoderm differentiation and disruption of the signaling via FGF4 results in abnormal gastrulation (Wilder *et al.,* 1997; Goldin et al. 2003). Disruption of FGF-receptor 2 (FGFR2) within mouse embryos leads to complete disintegration of the egg cylinder pre-empted by a lack of VE differentiation and termination of ICM development (Arman *et al.,* 1998). This establishes that Gata6 acts as an intermediary within the FGF signaling pathway and the loss of FGFR function inhibits endoderm specific transcription factors (Li *et al*., 2004); once Gata6 has been activated it works independently from FGF signaling.

It is important to note here Gata6 also regulates the expression of laminin genes needed for the deposition of basement membranes (BMs). Gata6 activates the synthesis of three polypeptide chains that make up Laminin-111; this is the main component of a BM. Similarly to endoderm differentiation, once Gata6 has been activated, FGF signaling is not required to induce laminin and Type IV collagen secretion (Li *et al*., 2004). PrE within mESCs has been shown to contain BM components within its cytoplasm. Following

differentiation into VE the BM components are extracellularly secreted at D3-4 to form a lattice structure (Li *et al*., 2004).

A second factor expressed in PE is Megalin or LRP2. Megalin is an endocytic receptor belonging to the LDL receptor family (Kerjaschki *et al* 1982). The majority of work concerning megalin is its role within the mammalian kidney (Christensen *et al*. 1998), however there is increasing evidence of its importance in embryonic development and hypotheses about its interactions with Sonic Hedgehog (Shh) (McCarthy *et al*. 2002). Megalin is expressed in the yolk sacs (Lundgren *et al*. 1997) and outer cells of pre-implantation mouse embryos (Fisher *et al*. 2001) and so is employed here as an early primitive endoderm marker.

1.1.3 Peri-implantation development (implantation to gastrulation)

Following implantation, epiblast cells polarise to form a pseudostratified epithelial layer called the primitive ectoderm. New cavity then forms at the centre of this epithelial layer, knows as the proamniotic cavity. It is at this stage that the embryo is called the egg cylinder.

With the development of the blastocyst and ICM, morphological asymmetry occurs and this is the beginning of the formation of axes within the mouse embryo. Axes are needed for the next stage of development to occur: Gastrulation (Selwood *et al*. 1992). This occurs at E6.5 and describes the period when the blastula develops into a trilaminar germ disc. A primitive streak is formed at the caudal end of the embryo through the migration of epiblast cells to the midline. The primitive node identifies the cephalic end of the embryo. The continual migration of epiblast cells towards the primitive streak causes invagination and the displacement of the visceral endoderm cells. This forms the definitive endoderm layer. (Tam *et al.* 1997)

The lateral spread of further epiblast cells create an intermediate layer known as the mesoderm These cells express Brachyury. The remaining cells form the ectoderm. In both mice and humans alike, Gastrulation consists of various morphogenetic processes that result in the formation of a gastrula with three germ layers that will give rise to all organs and tissue within the embryo.

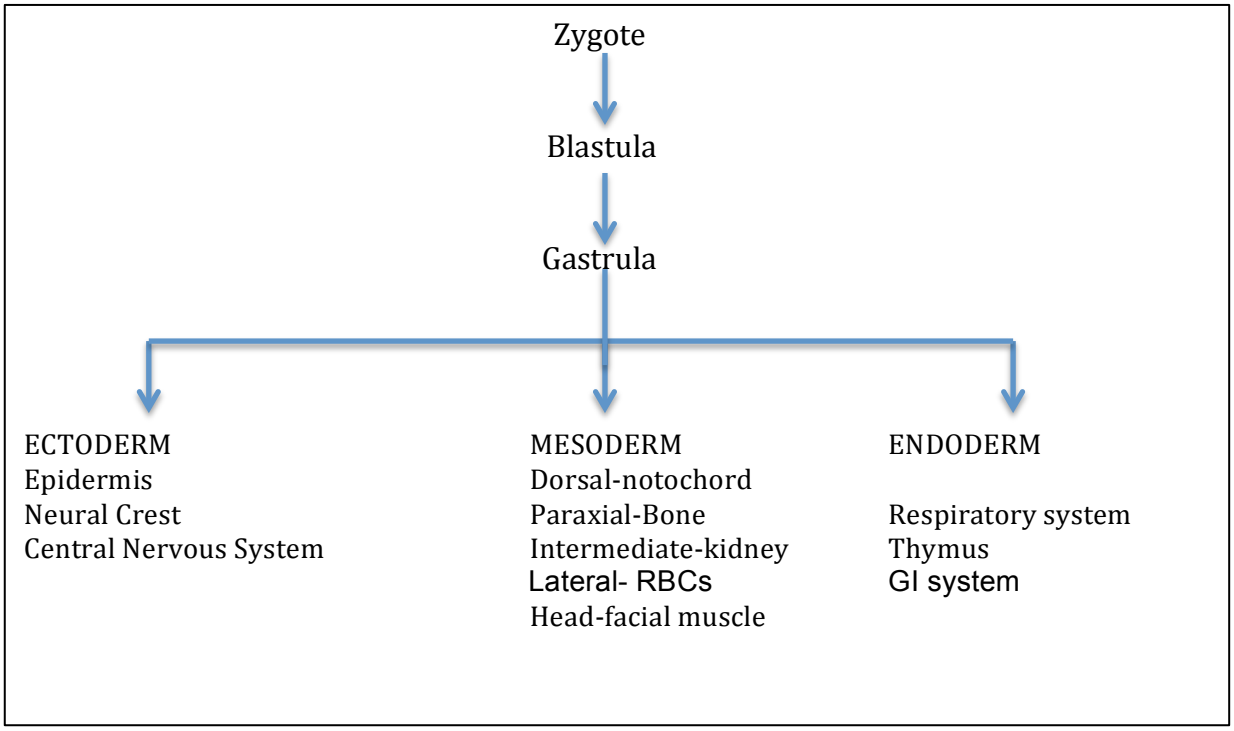

FIG 3. **Schematic flow diagram demonstrating the cell fate of the three primary germ layers**: Ectoderm, Mesoderm and Endoderm.

1.1.4 Mesoderm differentiation

Mesoderm differentiation is initially identified by the epithelial to mesenchymal transition (EMT) of epiblast cells (Lehembre *et al*., 2008). This occurs at the primitive streak; this is a transient structure formed along the posterior midline of the embryo (Tam and Behringer., 1997). Nascent mesoderm delaminates and migrates away from the primitive streak (Ciruna *et al*., 2001). The site at which the cells migrate through the medio-lateral axis results in the distinct population of cells.

The specification and patterning of mesodermal differentiation is regulated by FGF signaling. FGFs have potent mesoderm-inducing activity (Ciruna *et al*., 2001) and the disruption of FGFR results in an abnormal mesoderm cell layer (Slack *et al*., 1996; Isaacs *et al*., 1997). FGF8 has been identified as being expressed in early embryonic development (Crossley and Martin., 1995) and FGF8-/- embryos fail to form mesoderm as they are unable to migrate away from the Primitive Streak (Sun *et al*., 1999). Disruption of FGFR1 demonstrates the failure of the cells to undergo EMT and fail to traverse the Primitive Streak (Yamaguchi et al., 1994; Ciruna *et al*. 1997).

EMT is associated with the loss of E-Cadherin expression (Frame and Inman., 2008) and directly influences the differentiation and migration of mesoderm. E-Cadherin is needed to form adherens junctions that result in cell adhesion. During EMT, E-Cadherin is downregulated and the resulting nascent mesoderm does not express the cell-cell adhesion molecule (Damjanov *et al*., 1986). The zinc finger transcriptional repressor, Snail, regulates the repression of E-cadherin. Snail is commonly expressed within migratory cell populations involved in formation of the nascent mesoderm (Nieto *et al*., 1992) and overexpression induces EMT within mESCs (Batlle *et al*., 2000). The importance of Snail in embryonic development is demonstrated by the lethality of Snail-null embryos (Ciruna *et al*., 2001). Snail is regulated by FGF signaling.

A further important gene transiently transcribed in nascent and migrating mesoderm is Brachyury (T), which is expressed, in the intermediate and axial mesoderm from E7 making it a useful marker for mesoderm *in vitro*. Brachyury expression is dependent on FGF signaling via a regulatory loop (Kispert *et al*. 1994). In mice the absence of brachyury disrupts gastrulation and results in the death of the mouse shortly after. Caudal dysgenesis and absence of the posterior mesoderm indicates the mesodermal abnormalities that occur (Tam *et al*. 1992).

1.1.5 Definitive endoderm differentiation

Definitive endoderm goes on to form the mucosal lining of the embryonic gut and its associated organs such as liver and pancreas. (Bort *et al*., 2004; Molotkov *et al*., 2005; Tremblay and Zaret, 2005).

Wnt, FGF and BMP signaling pathways play a role in the differentiation of definitive endoderm. Studies have shown that the Activin/Nodal pathway is required for the formation of definitive endoderm. The result is dose-dependent whereby high levels of Nodal signalling will result in endoderm differentiation and lower signalling leads to mesoderm development (Kubo *et al*. 2004; D'Amour *et al*. 2005). Mouse embryos lacking Nodal, or ß-catenin fail to form a primitive streak (Conlon *et al*., 1994; Huelsken *et al*., 2000), suggesting that the canonical Wnt pathway and Nodal act in synergy to specify definitive endoderm. It has also been suggested that the inhibition of BMP4 pathways favours definitive endoderm differentiation (Li *et al*. 2011). Markers expressed in extraembryonic endoderm such as Gata6 are also expressed in definitive endoderm.

1.1.6 Ectoderm differentiation

Ectoderm differentiates to form the surface ectoderm (skin, hair etc.), neural crest (Peripheral Nervous System (PNS), melanocytes etc.) and neural tube (Central Nervous System (CNS)).

During gastrulation the ectoderm thickens to form the neural plate, acting as a platform for the development of the nervous system. This process occurs due to the inactivation of BMP4 inactivation of the growth factor BMP4, regulated by noggin, chordin and follistatin.

Transcription factors expressed in the ectoderm are Pax2 and Pax6. Overall, nine genes belong to the Pax family and play varying roles in embryogenesis, regulating cell-lineage specification, proliferation, migration, and survival of diverse cell and tissue types (Wang *et al*. 1998).

Paired box protein (Pax-6), which is a commonly used ectoderm marker, is a transcription factor present during embryonic development and a key regulatory gene in early neuroectoderm differentiation. Pax6-null mESCs cause failure of eye morphogenesis and severe abnormalities of brain development (Hogan *et al*. 1986, Pinson *et al*. 2006), whereas its over-expression guides cells towards a neuronal fate (Morrison and Scadden 2014).

1.1.7 Basement Membranes in early mammalian development

The BM is a form of specialized extracellular matrix that has various important roles within living organisms. BM is basolateral to all epithelia and endothelia in the body and can contribute to compartmentalization of tissue, regulate cell behavior, provide structural support and, of particular interest to this study, plays an important role in embryonic development. The diversity of the BM is down to its heterogeneous molecular composition. The composition of the BM was first investigated in the 1970s using the basement membrane-rich Engelbreth-Holm-Swarm (EHS) tumour (Timpl *et al*., 1979). As a result it was identified that BMs contain multiple substrates including type IV collagen, nidogen, sulfated proteoglycans, such as perlecan, and laminin (Martin and Timpl 1987; Leivo and Engvall 1988). These components comprise the 50 – 100nm BM structure. Type IV collagen and laminin form suprastructures which are bridged by sulphated proteoglycans and nidogens. (Aumailley and Smyth, 1998). This special structure is crucial for stability within the BM.

Laminin is one of the most abundant proteins present in the BM and is essential for its formation. Laminin-111 is the first major laminin expressed during the peri-implantation period (Shim *et al*., 1996; Smyth *et al*., 1999) and is essential for basement membrane formation and early embryogenesis (Urbano *et al*., 2009).. It is described as a heterotrimeric glycoprotein because it is composed of three separate subunits that resemble a three-pronged fork (Colognato and Yurchenco, 2000). Three short arms are formed by a different chain and one long arm is composed of the three assembled coiled chains (Hunter *et al,* 1989). The three shorter arms allow binding to other laminin molecules, while the long arm acts as an anchor binding tissue cells to the membrane. The structure is composed of three polypeptide chains (alpha, beta, and gamma). There are five α chains (LAMA1-5), four β chains (LAMB1-4), and three ϒ chains (LAMC1-3) currently identified which exist in at least 12 heterotrimeric combinations (laminin isoforms 1 to 12) with different tissue distributions and functions (Siler *et al.,* 2000). The α subunits are thought to initiate cell-surface adhesion via receptors and also plays a role within the self-assembly of the BM. The β and ϒ chains play a structural role within the ECM structure and although are secreted first, rely on the presence of Lama1 to form the essential trimer. β 1 and ϒ 1 are detected at the 2-cell stage of the embryo, whilst alpha 1 is detected at the 8-16 cell stage (Cooper and MacQueen 1983; Dziadek and Timpl 1985). As a result the Lama1 chain appears to be the rate-limiting step in BM assembly (Miner and Yurchenco *et al*., 2004) (Fig.4).

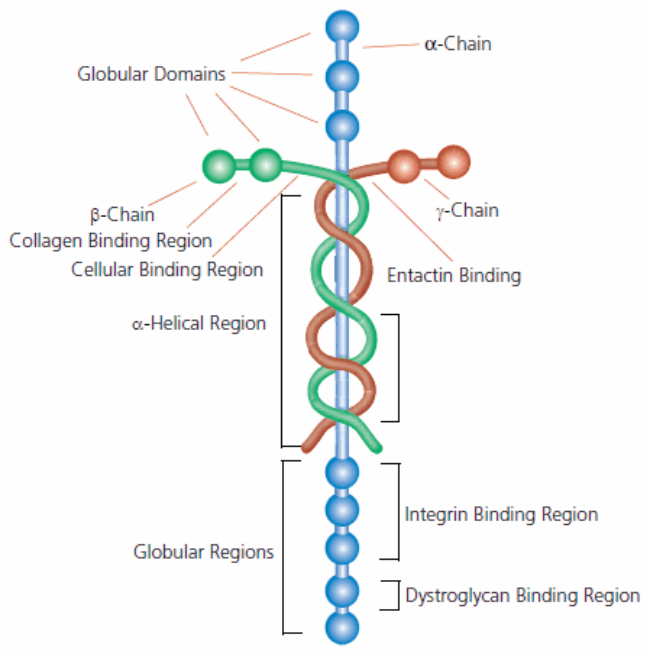

FIG 4. Laminin. Schematic diagram outlining the heterotrimeric structure of laminin identifying the α, β and γ chains. Laminins are composed of a central ~400 kDa alpha chain with a varying number of globular regions and two ~200 kDa chains (beta and gamma) with helical a-helical and globular regions.

Image taken from: Sigma-Aldrich (2014) Laminin. Available at: *http://www.sigmaaldrich.com/life-science/biochemicals/biochemical-products.html?TablePage=21735645. Accessed: 01/09/14.*

Basement membranes start to be deposited in the early embryo shortly after the trophectoderm differentiates. The first BM is deposited by the trophectoderm cells and is required for the migration of the PE cells to form the parietal yolk sac. The second BM is deposited by PrE cells. It is deposited at the basal surface of the PrE, forming a barrier between themselves and the epiblast. Later the PE cells deposit the Reichert's membrane, a thickened BM, between itself and the trophectoderm. (Enders *et al.,* 1978).

The use of mutant murine embryos and knockout gene mESCs have demonstrated the importance of laminin to the BM structure and hence highlighted the role of the BM in embryo development. Without laminin-111 normal embryogenesis will not occur due to failure of BM formation and leads to lethality of the embryo (Smyth *et al.*1999). Laminin is also required for epiblast polarization, cavitation of the EB and for protection of adherent cells from apoptosis (Murray and Edgar., 2000). Disruption of Lama1 gene results in failure of extraembryonic membrane formation and this formation of the RM (Miner *et al*., 2004). Lama1 has also been indicated in the differentiation of endoderm within mESC and essential for VE differentiation. (Akerlund *et al*., 2009; Higuchi *et al*. 2010). β1-null mESC show a similar picture but this is thought to be because its own expression governs that of Lama1 (Aumailley *et al*., 2000). mESC embryos deficient in the laminin γ1 chain are unable to form a BM and result in failure of primitive ectoderm epithelialization and the death of the homozygous mutant embryos at E5.5, through failed organization of the parietal yolk sac (Smyth *et al*., 1999).

1.2 Embryoid bodies

1.2.1 Stem cells

Stem cells are unspecified cells defined by their ability to self-renew and their capacity to differentiate into a specialised cell or cells. This differential potential varies depending on cell type and allows the subdivision of stem cells. Totipotent stem cells can give rise to all intra and extraembryonic tissues; pluripotent cells are limited to forming the three germ cell layers; multipotent stem cells differentiate into a subset of cell lineages; unipotent stem cells only have the capacity to differentiate into one mature lineage. Stem cells tend to be categorised into 2 categories: adult (also referred to as somatic) stem cells, and pluripotent stem cells, which include embryonic stem cells (ESCs), embryonic germ cells and induced pluripotent stem cells.

Adult stem cells (ASCs) are undifferentiated cells found within differentiated cells of a tissue or an organ. They often have a multi- or unipotent potential and differentiate into cell colonies specific to the tissue or organ. ASCs generate 'progenitor' cells, which like stem cells, can generate different types of specialised cells, but have a more limited capacity to self-renew. Adult stem/progenitor cells maintain the integrity of the tissue and are responsible for repair after injury by functioning in a microenvironment referred to as the "stem cell niche" (Morrison and Scadden *et al.* 2014).
Numerous studies have reported the presence of ASCs within a variety of tissues (Kim *et al.,* 2005; Le *grand et al.,* 2007) and interest remains about their therapeutic value. Initial work during the 1950s identified haematopoietic and mesenchymal stem cells that give rise to mesodermal hemangioblast precursor cells and are commonly used successfully in the treatment of patients with multiple myeloma or leukaemia.

1.2.2 Embryonic stem cells

Embryonic stem cells (ESCs) are derived from the inner cell mass of the pre-implanted blastocyst (Martin and Kaufman *et al*., 1981) and hold both the capacity to self-renew indefinitely and to differentiate into all three primary embryonic germ layers: ectoderm, mesoderm and endoderm. ESCs can be cultured successfully *in vitro* and due to their ability for unlimited expansion, have potential applications in not only regenerative medicine but also in the investigation of early mammalian development and lineage specification.

The first mouse embryonic stem cells were isolated in 1981 (Evans and Kaufman, 1981) from the inner cell mass of day 3.5 - 4 blastocysts. The multipotency of murine embryonic stem cells is evident from previous work undertaken (Itskovitz-Eldor *et al.,* 2000). mESC have been successfully injected into the blastocyst cavity and implanted in pseudo- pregnant mice with the result of the expression of all cell types in the chimeric progeny mice (Capecchi *et al.,* 1989; Rossant *et al.,* 1989). mESC have also been shown to be capable of inducing teratomas comprising of endodermal, ectodermal and mesodermal cell lines when injected subcutaneously into syngeneic mice (Wobus *et al.,* 1984). Finally, the *in vitro* aggregation of mESC results in formation of embryoid bodies (EBs) that demonstrate differentiation similar to that of a peri-implantation embryo.

mESCs can be maintained *in vitro* in tailored culture conditions and expanded without loss of their capacity to differentiate to all cell lineages. Typically, mESCs are cultured in a serum-based medium as a monolayer on a mouse embryonic fibroblast (MEF) feeder layer; these secrete precursors of components of the extracellular matrix (ECM) and growth factors, increase plating efficiency and maintain pluripotency. Serum-free systems have also been developed using matrigel or laminin supplemented with growth factors such as FGF (Wang *et al.,* 2005) The discovery of leukemia Inhibitory Factor (LIF) allowed the culture of cells on gelatin-coated tissue culture dishes

without a MEF feeder layer, as it served to maintain the cells in an undifferentiated state (Chambers and Smith., 2004). Despite the presence of LIF in culture, mESCs that are located on the periphery of a colony will often undergo spontaneous differentiation (Singh *et al.,* 2007;Tanaka *et al.,* 2008). The ease in which mESCs can be genetically manipulated has allowed the *in vivo* analysis of genes via transgenic, chimeric and knockout mice.

The isolation of human embryonic stem cells hESCs cells happened at a much later date in 1998 and were derived from inner cell mass of blastocyst stage embryos that developed in culture within 5 days of fertilization of the oocyte (Thomson *et al.,*1998). There are currently sixty-four different derivations of hESCs listed in the NIH registry for research use (http://escr.nih.gov). Protocol for the culture of hESCs is similar to that of mESCs with the use of a MEF feeder layer but unlike the murine cells, hESCs do not require LIF for maintenance of pluripotency (Reubinoff *et al.,* 2000; Thomson *et al.,* 1998). Nevertheless similarities are apparent between the two ESCs concerning the expression of the pluripotency markers Oct4 and Nanog (Ginis *et al.* 2003).

1.3 Regulation of mESC pluripotency

The ability of ESCs to self-renew and remain pluripotent is governed by a number of cell signaling pathways, such as the LIF-STAT3 (Signal transducer and activator of transcription 3) pathway, and key transcription factors such as Oct4 and Nanog.

1.3.1 LIF/STAT3 pathway

Leukemia inhibitory factor (LIF) is an interleukin 6 class cytokine that in conjunction with the downstream effector STAT3 maintains, pluripotency of mESCs. LIF signaling is initiated through dimerization of LIF-R and gp130. The next step is the phosphorylation of these cytokine receptors on to tyrosine receptors via the Jak family non-receptor tyrosine kinases (Carter-Su et al.,1998) followed by the recruitment of STAT3. Studies have shown STAT3 to be a vital part of the pathway and its inactivation within LIF-maintained

mESCs results in spontaneous differentiation (Niwa *et al.,* 1998). The LIF/STAT3 pathway also promotes pluripotency through the selective enhancement of Klf4 (Krüppel- like transcription factor 4) expression. mESCs overexpressing Klf4 have increased resistance to differentiation (Niwa *et al.,* 2009) and have the ability to reprogram somatic cells to a pluripotent state (Takahashi and Yamanaka, 2006).

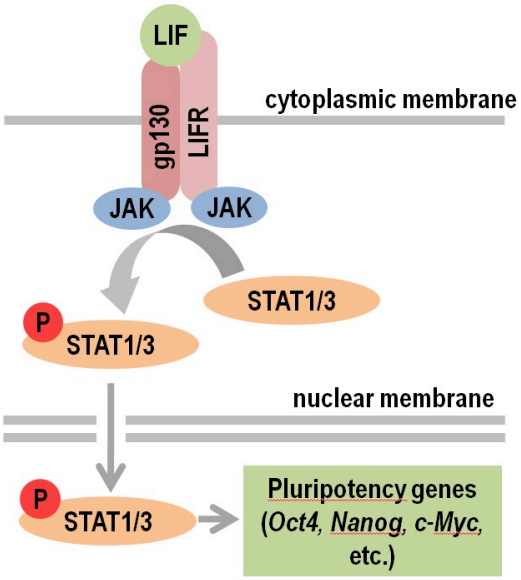

FIG 5. Schematic flow diagrams detailing LIF/STAT pathway.

Taken from: ***Arabadjiev et al. (2012)*** *Of mice and men – differential mechanisms of maintaining the undifferentiated state in mESC and hESC. Biodiscovery 3: 1.*

1.3.2 Oct-4

Octamer-4 (Oct4) is a 352 amino acid protein encoded by the gene *POU5F1*, located on chromosome 6 of the human genome and a key transcription factor for the formation of self-renewing pluripotent stem cells (Nichols *et al*. 1998) . This transcription factor is expressed in the ICM, epiblast and primordial germ cells and its expression level dictates ESC fate (Niwa *et al.* 2000). Oct4-deficient mice are unable to develop beyond the pre-implantation stage with under-expression resulting in trophectoderm differentiation (Nichols *et al*., 1998). In contrast, high-expression of Oct-4 promotes endoderm and

mesoderm differentiation, showing the importance of oct4 in the initial cell fate decisions during mammalian development (Thomson *et al*., 2011).

1.3.3 Nanog

The Homeobox protein Nanog is expressed in pluripotent cells of mouse pre-implantation embryos, ESCs, and EGCs (Embryonic Germ Cells). It was previously thought that the biochemical pathway, LIF/gp130/Stat3, singularly maintained the self-renewal of mESCs (Burdon *et al*. 2002); however, Nanog can maintain pluripotency of ESCs in the absence of LIF (Mitsui *et al*. 2003). Nanog-deficient mESCs lose pluripotency and differentiate to EEE (Mitsui *et al*., 2003).

1.4 Embryoid body development

If ESCs are cultured in suspension rather than adherent culture, they clump together to form aggregates called embryoid bodies (EBs). EBs are so-called because in the absence of LIF, their development mimics that of the early embryo (Robertson *et al*., 1987).

After the initial development of cell aggregates the EBs begin to differentiate into PrE at day 2 (Murray and Edgar ESCs within the centre of the EB and the surrounding PrE (Robertson., 1987). The BM formed within the EBs appears thicker than the BM underlying the PrE *in vivo* and can sometimes resemble Reichert's membrane (Salamat *et al*., 1995) This is thought to be due to the fact that in EBs, PE cells (derived from PrE) are unable to migrate onto the trophectodermal BM, because this cell type does not form in EBs, and so instead, a Reichert's-like membrane can be observed separating the outer extra-embryonic endoderm from the undifferentiated ESCs within the centre of the EB (Roberston *et al*., 1987). PrE can be distinguished from PE cells because the former are squamous epithelial cells, where as the latter are unpolarised migratory cells that sometimes form multi-layers on the surface of the EB. In addition to giving rise to PE cells, as in the embryo, PrE also gives rise to VE cells, which are tall columnar epithelial cells with large apical vacuoles (Takito et al., 2004).

Following deposition of the PrE BM, the inner cells in contact with this BM polarize to form a pseudostratified ectoderm epithelium. Once formed, cells positioned at the apical surface of this epithelium undergo apoptosis, and a proamniotic-like cavity is formed. The ectodermal epithelium that forms in the EBs can generate derivatives of the three embryonic germ layers, but differentiation from this stage onwards is chaotic. For instance, various specialized cells are observed, such as beating cardiomyocytes and blood islands, but they are not organized into recognizable structures (Robertson *et al*., 1987).

Although there are differences between mouse embryo and EB development, there are many similarities, which make the EB an excellent model for

investigating differentiation events occurring during peri-implantation development.

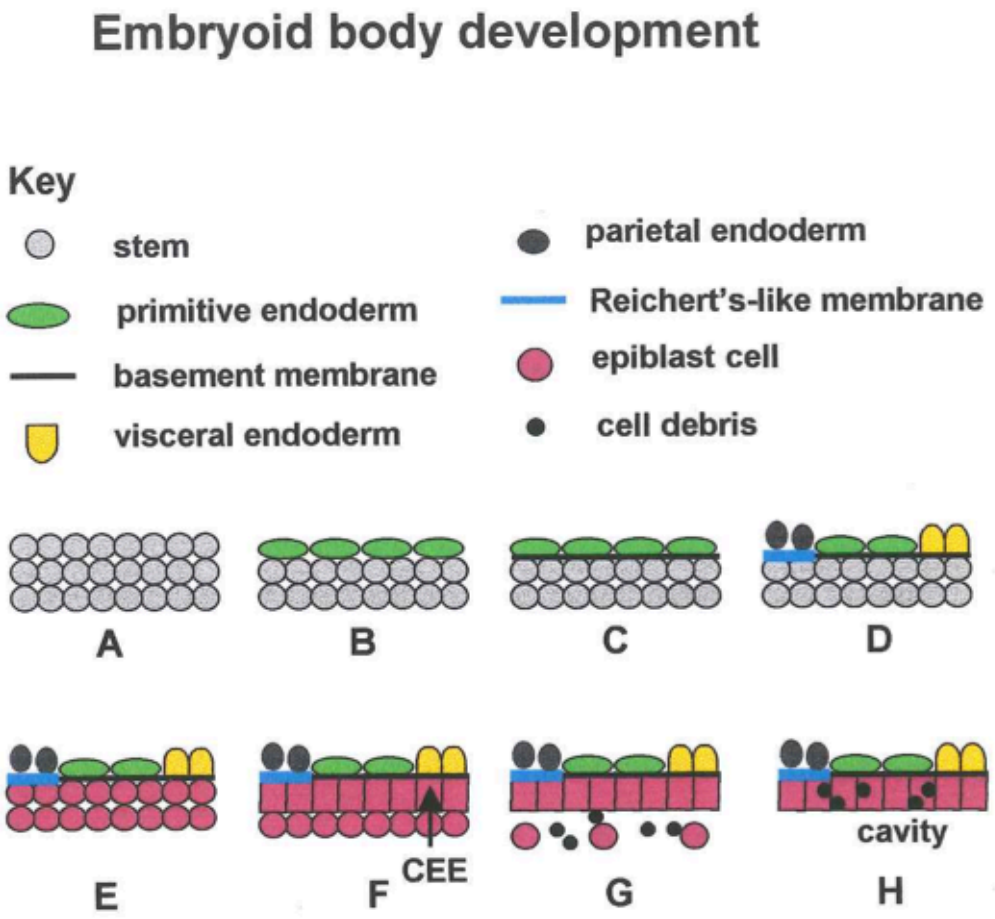

Fig 6 Schematic representation of EB development. (A) The first stage involves the clumping together of undifferentiated ESC to form a cellular aggregate. (B) The cells on the surface of the aggregate then differentiate to become PrE. (C) The PrE cells deposit a BM between themselves and the undifferentiated core cells of the EB. (D) Some of the PrE cells differentiate to become visceral and parietal endoderm cells. The PE cells deposit a Reichert's-like membrane. (E) The ESC within the core of the EB differentiate to become epiblast cells. (F) The epiblast cells in contact with the BM polarise to form columnar epiblast epithelium (CEE). (G) Epiblast cells positioned on the apical surface of the CEE detach and undergo programmed cell death. (H) The cell debris is phagocytosed by the cells of CEE to reveal the cavity. ***Taken from: Murray (2001) Early mouse development. PhD. University of Liverpool.***

1.5 Heparan sulphate

Proteoglycans are a diverse group of molecules found in both the ECM and on the cell surface. They interact with chemokines, growth factors, and morphogens, and research has indicated their importance for modulating signaling pathways such as FGF BMP. They consist of a core protein to which one or more complex carbohydrates or Glycosaminoglycan (GAG) chains can be attached at specific sites (Mizumoto *et al*., 2005). These GAG chains are classified into different groups according to the structure of the polysaccharide, and include molecules such as dermatan sulfate (DS), keratan sulfate (KS), different isomeric forms of chondroitin sulfate (CS), heparin, and heparan sulfate (HS) (Esko *et al*., 2009).

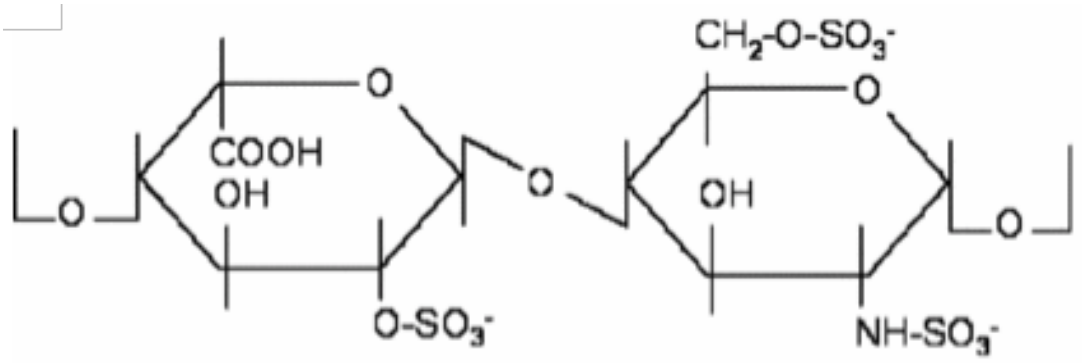

Fig.7 Schematic structure of the most common disaccharide repeat of heparan sulphate

Heparan sulphate is a polysaccharide chain that consists of repeating disaccharide units of hexuronic acid (HexA) and glucosamine residues and has a structure of – HexAα/β1-GlcNα 1, 4 - . HexA can be a C-5 epimer β D glucaronic acid (GlcN) or α L iduronic acid (idoA) and glucosamine residues may be N-acetylated, N-sulphated or rarely N-substituted. O-Sulphation of the HS molecule is variable but most commonly occurs on the C-2 of IdoA and GLcA and c-6 and c-3 of GlcN (Mizumoto *et al*., 2005). It is found on most cell surfaces and within the extracellular matrix.

Heparin is structurally similar, but approximately 100% of the GlcN is N-sulphated compared to 50% within HS (Gallagher and Walker., 1985). The distribution of heparin also differs as it is restricted to mast cells and also found on neural precursors (Kusche-Gullberg *et al*., 1998).

Heparan sulphate proteoglycans (HSPGs) interact with a variety of ligands including growth factors. The relative positioning of the carboxyl and sulphate groups give the HSPGs variation that allow interaction with a large variety of ligands (Sarrazin *et al*., 2011). The HSPG molecule consists of a protein core that is attached to numerous HS chains and they are categorized relative to their distribution (Lander *et al*., 1998).

1.5.1 Biosynthesis of Heparan sulphate

Heparan sulphate is one of the most complex structures belonging to the GAG family. The biosynthesis of HS chains produces a vast array of HS saccharides resulting in a diverse expression of function. Limited studies have been conducted on the biosynthesis of HS in comparison to heparin and as a result most of the knowledge on the formation of N-sulphated glycosaminoglycans is based on these. Although it is probable the mechanisms controlling the final composition of the HS have differences, the proposed basic steps for HS biosynthesis are outlined below.

Biosynthesis begins with the formation of the protein core, the structure of which is very poorly understood. A tetrasaccharide linkage region is then assembled on the protein and is known as the GAG-protein link region

(GlcAβ1–3Galβ1–3Galβ1–4Xylβ1-O-Ser) (Esko and Selleck., 2002). Within the endoplasmic reticulum and cis-Golgi, a xylose monosaccharide covalently attaches to a serine residue within the protein core, via xylotransferase. This is followed by the addition of two galactose residues to the Xyl residue and is completed by the addition of GlcA, each reaction being catalyzed by galactosyltransferases II and I and by glucuronosytransferase I respectively (Mizumoto *et al*., 2005).

The next step is the assembly of the non-sulphated polysaccharide. The uridiine diphospate (UDP)-sugars, UDP-N- acetylglucosamine (GlcNAc) and glucuronic acid (GlcA) sequentially transfer to a primed protein acceptor. This mechanism occurs within the Golgi apparatus and results in a repeating disaccharide chain (Mizumoto *et al*., 2005). This mechanism is mediated by related enzymes whose genes are members of the exostoses (EXT) gene family of tumour suppressors (Zak *et al*., 2002). The final step is the modification of the uniform chain to produce a variety of HS saccharides.

1.5.2 Role of HS in ESC differentiation and embryo development

The role of Heparan Sulphate in mESC pluripotency and cell lineage commitment of ESCs has been studied using cells that are either HS-deficient or have undersulphated HS (Kraushaar *et al*. 2013).

EXT1-/-	EXT1 null mESC (Kraushaar *et al*., 2012)
EXT1cn/cn	mESCs with the ablation of a conditional EXT1 allele *in vitro* (Kraushaar *et al*., 2012)
Ndst1/2-/-	N-deacetylase/N-sulfotransferase (heparan glucosaminyl) 1 / 2 null mESC (Holmborn *et al*., 2004)
siRNA-EXT1	mESCs stably transinfected to knockdown gene expression of EXT1 (Sasaki *et al*., 2008)
EXT1-KD	mESCs transiently transinfected to knockdown gene expression of EXT1(Sasaki *et al*., 2008)

Table 1. Undersulphated or HS-deficient ESCs commonly employed in studies.

The importance of HS in early embryonic development has been clearly highlighted in a number of studies that show the lethality of HS-deficient embryos (Lin *et al*., 2000; Stickens *et al*., 2005).

It is theorised that it is the remodelling of the HS structure and so the affinity of HS to specific cell signalling molecules that controls the cell lineage commitment of mESCs (Kraushaar et el. 2013). In mESC, HS chains are usually under-sulphated and are thought to act to maintain pluripotency. As differentiation occurs up-regulation of sulfotransferase mRNAs appears to increase sulphation of HS chains and regulate cell-lineage decisions (Johnson *et al*., 2007).

The role of HS in mesoderm differentiation is not completely clear. Kraushaar *et al* concluded that EXT1 cn/cn mESCs could not express brachyury and were incapable of producing mesoderm (Kraushaar *et al*., 2012). The addition of HS in this study appeared to rescue mesoderm differentiation. This work conflicts with Holley *et al* who showed HS-deficient EBs could express brachyury, indicating that nascent mesoderm had differentiated, but there appeared to be defects in mesoderm maturation (Holley *et al*., 2011).

Neuronal differentiation also seems to be directly affected by a lack of HS *in vitro* owing to a lack of FGF signalling needed for neurogenesis (Coumoul *et al.,* 2003) Mutant ESCs have failed to differentiate into neural progenitors and also failed to express nestin, a protein normally expressed in neural cells (Forsberg *et al.* 2012).

1.5.3 Role of HS in regulating signalling pathways

1.5.3.1 Heparan sulphate and FGF signaling

Fibroblast growth factors (FGFs) are a family of 22 small polypeptide Fibroblast Growth Factor Ligands of varying homology and function that are indicated in numerous biological processes.

HSPGs are essential for FGF signaling *in vivo* (Lin *et al*., 1999) and a lack heparan sulphate results in a failure to trigger further cascade pathways downstream. This is because they operate using a co-receptor system consisting of tyrosine kinase receptors (FGFR1-5) and the appropriate FGFto form a 2:2:2 FGF:FGFR:heparan dimer (Fig.8).

Studies have concentrated on the role of HS in the development of specific cell types, including neuronal cells where a lack of HS results in a failure of neural differentiation (Johnson *et al*., 2007). Specification of primitive endoderm relies upon the Grb2/Ras/ERK pathway that acts downstream of the FGF signalling, and this differentiation event could thus be dependent upon the present of HS.

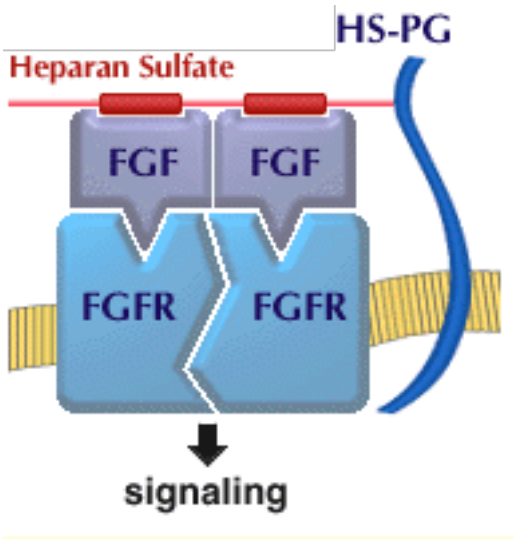

FIG 8. Schematic detailing the role of HS in FGF-FGFR binding.
Image taken from: GlycoWorld. (1997) Proteoglycan- A01. Available at: *http://www.glycoforum.gr.jp/science/word/proteoglycan/PGA01E.html. Accessed on: 01/09/14.*

1.5.3.2 Heparan sulphate and BMP signaling

The bone morphogenic proteins (BMPs) are a subclass of more than 20 members of the TGF-beta family (transforming growth factor). Its signaling inhibits the expression of Nanog and activates the expression of differentiation genes via Smad/1/5/8. A lack of HS leads to the degradation of BMP4, revealing that HS stabilizes BMP4 to promote BMP signaling. BMP is involved in the differentiation of mesoderm (Winnier et al., 2005). Interestingly it has been suggested that BMPs are also required for maintenance of mESCs pluripotency in a study examining self-renewal in serum-free conditions (Galvin *et al*. 2010). However this role is thought to be in the inhibition of neuroectoderm differentiation (Wilson et al., 1995).

1.5.4 Role of Exostosin Glycosyltransferase-1 (Ext1) in development

The Exostosin Glycosyltransferase-1 (*EXT1)* gene codes for the protein exostosin-1 and is located on the long (q) arm of chromosome 8 at position 24.11.(base pair 117,799,362 to base pair 118,111,818). Exostosin-1 is located in the Golgi apparatus where it functions to modify enzymes and proteins and specifically binds to EXT-2 to form a polymerase that modifies Heparan Sulphate (McCormick *et al*. , 1998). The absence of EXT1 or EXT2 leads to lack of HS synthesis within the cells. Gene-silencing strategies have been used to investigate the roles of EXT1 and EXT2 heparan sulfate chain elongation indicating a decreased amount of the proteins leads to the production of shorter HS chains (Busse *et al*. 2007). Clinically, EXT1 and EXT2 have been linked to the autosomal dominant hereditary disorder Hereditary multiple exostoses (HME) as a result of the absence of HS affecting the signaling response in exostosis chondrocytes (Hecht *et al*. 2002; Hecht *et al*. 2005). Inheritance is autosomal dominant with almost complete penetrance resulting in benign osteochondromas; theses are often painless but have cosmetic implications on the patient (Peterson, 1989).

Lin *et al*. demonstrate that Ext1-/- mouse embryos display defects in extraembryonic tissues an have an early lethality (Lin *et al*., 2000). However, a problem with this study is that it is very difficult to analyse the embryos at this stage of development due to their small size. For instance, it is unclear from the results if there are really any problems with EEE, nor is it possible to say whether the problems with gastrulation are simply due to delayed development. Within the same study EBs were cultured and cell lineage examined, although in limited detail. For instance, images of EBs are only shown at day 10, at which stage, EB development has becomes very chaotic and no longer becomes an appropriate model for early embryonic development.

1.6 Aims

1. Identify differences in pluripotency and spontaneous differentiation between WT and EXT1-/- mESC
2. Develop EBs from EXT1-/- mESC that are truly HS-deficient
3. Evaluate the PrE differentiation and BM synthesis within EXT1-/- EBs
4. Identify the requirement for HS in mesoderm and ectoderm lineages

2. Materials and methods

2.1 Materials

2.1.1 Cell lines

The STO (SIM (Sandos inbred mouse) 6-thioguanine resistant, ouabain resistant) mouse embryonic fibroblast cell line was a gift from Dr Neil Smyth, University of Southampton, UK. The wild-type E14 and Ext1-/- E14 ESC lines were a gift from Dr Cathy Merry, University of Manchester, UK.

2.1.2 STO cell and EB culture medium

STO medium consisted of high glucose DMEM (Sigma) supplemented with 10 % FBS (Gibco), 2mM L-glutamine (Gibco) and 1% Non-essential amino acids (NEAA) (Gibco).

2.1.3 mESC culture medium

ESC medium consisted of high glucose DMEM (Sigma) supplemented with 15% Foetal Bovine Serum (FBS) (ESC grade from Sigma) 2mM L-glutamine (Gibco), 1% Non-essential Amino Acids (NEAA) (Gibco), 50μM 2-mercaptoethanol (Gibco)and 1000 U / mL leukaemia inhibitory factor (LIF)(Millipore).

2.2 Cell culture

2.2.1 Routine STO culture

STO cells were cultured in STO medium in 10 cm tissue culture dishes (Corning) that had been coated with 0.1 % gelatin. When 80-90% confluent, STOs were cultured as follows: STO medium was aspirated and cells were washed once with 5ml PBS (w/o Ca^{2+} and Mg^{2+}). The cells were then trypsinised with the addition of 4.5ml of PBS and 0.5ml 10x trypsin/EDTA for 3-5 min, and subsequently neutralized with the addition of 5ml STO medium. The cell suspension was transferred to a 15ml conical tube and centrifuged at 1000 rpm for 2.5 minutes. Supernatant was removed and cells re-suspended in 6ml STO medium. 1ml was transferred to each of 6 10cm gelatinized

tissue culture dishes containing 9ml each of STO medium. STO cells were were sub- cultured every fourth day at a ratio of 1:6 and incubated at 37°C and 5 % CO_2.

2.2.2 Preparation of STO feeder cells

The STO medium was reduced to 5ml and 20μg/ml of Mitomycin C (Sigma) was added for 2 hours at 37°C and 5 % CO_2 in a humidified incubator. After the 2h incubation period, the medium was aspirated and the cells washed X3 with PBS. The cells were then trypsinised and centrifuged as described above. The pellet was resuspend in 6ml STO medium and 0.5ml transferred to each of twelve 3.5cm gelatinized tissue culture dishes containing 1ml STO medium. STO feeder cells were allowed to settle overnight and used within 7-10 days. As Mitomycin-C is an alkylating agent that mitotically arrests cells extra care was taken when handling.

2.2.3 Routine mESC culture on STO feeders

E14 WT and E14 EXT1-/- mESCs were cultured separately on 3.5 cm tissue culture dishes (Nunc) coated with 0.1 % gelatine in mESC medium. mESCs were cultured as follows: medium was removed from and cells were washed once with PBS. Cells were trypsinised and centrifuged as described for STO cells. The cell pellet was suspended in 3ml mESC medium and 0.5ml transferred to each of 6 3.5cm STO feeder dishes. Typically, mESCs were sub-cultured every fourth day at a ratio of 1:6, with medium being changed every 2 days, and incubated at 37°C and 5 % CO_2.

2.2.4 Routine mESC culture on gelatin

Prior to EB formation both WT and EXT1-/- were sub-cultured twice on gelatin coated dishes w/o STO feeders. Trypsinisation was the same as described above. Following resuspension in mESC medium, STO cells were depleted as follows: mESCs were transferred to a gelatinized 6cm dish. After 20 minutes the cell suspension was aspirated from the 6cm dish and transferred to a second 6cm gelatinized dish. This process was repeated a second time and

the cells incubated at 37°C and 5% CO_2. According to experience and number of cells present under microscope cells were split 1:1, 1:2 or 1:3.

2.2.5 Freezing cells

Medium was removed from the culture dish and cells were washed once with PBS, followed by trypsinisation with 1 X trypsin/EDTA for 3-5 minutes and were neutralised with equal volumes of STO medium. After centrifugation at 1000rpm for 2.5 minutes, medium was aspirated and cells re-suspended in 0.5ml cell culture freezing medium (Gibco) and transferred to a cryovial. The cryovial was placed in a freezing chamber containing isopropanol overnight at - 80°C and transferred to liquid nitrogen for long-term storage.

2.2.6 Thawing of cells

The cryovial containing cells was removed from liquid nitrogen and thawed rapidly in a water bath at 37°C. 500μl of STO medium was added to the cryovial to resuspend the cells and transferred to 9ml of STO medium within a 15ml conical tube. This was centrifuged at 1000rpm for 2.5 minutes and the medium removed and replaced with appropriate medium. The cells were finally transferred to the appropriate culture dish and incubated at 37°C 5 % CO_2.

2.2.7 Embryoid Body Formation

Medium was removed from 3.5 cm dish of E14 mESCs and cells washed once with PBS. 1 mL 1xtrypsin/EDTA was added for 3-5 min, and neutralised with 1 mL mESC medium, before centrifugation at 1000rpm for 2.5 min. The medium was removed and cells were re-suspended in 1ml of EB medium. Cells were counted using haemocytometer (cells/mL) and mESCs were plated at a density of 75 x 10^3 cells/ml onto 10 cm bacteriological dishes (Sarstedt) containing 9ml EB medium. The medium was changed every other day by swirling the dish in a circular motion in order to collect the EBs in the centre of the dish. Old medium was then gently aspirated from the edge of the dish. Alternatively, if cell debris present, EBs were transferred to 15ml conical tube and left to settle at the bottom. Old medium was aspirated and replaced with

fresh medium and transferred to new bacteriological dish.

2.2.8 Fixation of mESCs

In order to allow immunofluorescence analysis of the mESC they were cultured in 8 well tissue culture chamber slides. A confluent dish was trypsinised and centrifuged and the pellet resuspended in 3.2 ml mESC medium. 400μl of the cell suspension was added to each of the 0.1% gelatinized chambers.

The medium was removed from the chamber well slides containing mESC and 4% paraformaldehyde (PFA)* was added. After 5min incubation at room temperature, the PFA was aspirated and the cells washed X3 in PBS. Cells were stored at 4°C in PBS for up to 1 week before immunostaining.
* For 1L of 4% PFA, 40g of paraformaldehyde powder was added to 1L of 1x PBS.

2.2.9 Fixation of EBs

EBs were transferred to a conical tube and allowed to settle at the bottom or were centrifuged at 800rpm for 1 minute. The medium was aspirated and ~4mls 4% PFA was added. Following 10-20 min incubation (depending on EB age) EBs were washed X3 in PBS and stored at 4°C in PBS for up to 1 week before gelatin embedding.

2.2.10 Gelatin embedding of EBs

PBS was aspirated from the fixed EBs and ~10mls of 15% sucrose (in PBS) added. These were left to soak overnight at 4°C. The next day the sucrose solution was aspirated and ~4mls 7.5% gelatin solution (molten) added (made up in 15% sucrose in PBS). These were incubated at 37°C in a water bath for 30-60mins. Simultaneously, a few millimeters of the molten gelatin were poured into weighing boats and allowed to set. After the elapsed time most of the gelatin solution from the EBs was removed leaving ~100-500μl in the conical tube. The EBs were carefully pipetted in a droplet of gelatin onto the set gelatin in the weighing boat.

After setting the EBs were cut out of the weighing boat and mounted on cork disks with cryomountant (OCT). The gelatin embedded EBs were then covered in cryomountant and placed on dry ice until they were frozen. They were then stored at -20°C in aluminium foil.

2.2.11 Sectioning of EBs

Frozen sections of EBs were prepared using a cryostat set at – 20°C and 10µm thickness. Once cut, sections were transferred to microscope slides (Thermo-scientific, Superfrost). These were stored at -20°C before being used for immunostaining.

2.2.12 Immunostaining of EBs

A hydrophobic pen was used encircle the EBs on the slides before being placed in a coplin jar filled with PBS in water bath at 37°C for approximately 20 minutes. After all the gelatine had been removed the slides were air-dried and 10% Bovine Serum (BS) in PBS (Sigma) was added as a blocking solution at room temperature for 60 minutes. This prevents nonspecific binding of applied antibodies. After incubation, the blocking solution was removed and the primary antibody solution was added, and samples were incubated at 4°C overnight in a humidified chamber. Primary antibodies are indicated in Table 2. The primary antibody solution consisted of 1% BS made up using 1xPBS and the primary antibody of interest at the appropriate concentration e.g. 1 in 200. After incubation, samples were washed three times in PBS. Secondary antibody solution was then added and incubated at room temperature in the dark for 2 hours in a humidified chamber. Secondary antibodies are indicated in Table 3. After incubation with secondary antibody solution, samples were washed three times in PBS. Finally, the nuclear stain 4',6-diamidino-2-phenylindole (DAPI) was applied at a concentration of 1:100,000 and samples were incubated at room temperature in the dark for 5 minutes. Slides were washed three times in PBS and a coverslip added using fluorescent mountant (Dako). To make the mounting more permanent and prevent spoiling the edges of the slides were sealed with nail varnish.

2.2.13 Immunostaining of mESCs

A solution of 10 % FBS (Gibco), 0.1 % Triton X-100 in PBS was added to the slides and incubated with blocking solution at room temperature for 60 minutes. After incubation, the blocking solution was removed and the primary antibody solution was added, cells were incubated at 4°C for 2 hours. The procedure then followed the protocol for EB immunostaining.

All solutions were centrifuged for 5 min at 13,000 rpm prior to use and staining was visualized using an inverted microscope (Leica microsystems, 291185).

The primary antibody solutions consisted of 1% Bovine serum and the appropriate concentration of primary antibody in 1x PBS. 0.1% Triton was added to permeabilise the mESC.

Target Molecule	Animal Raised in and Clonality	Concentration	Manufacturer	Reference /ID Number
Oct 4	mouse monoclonal IgG_{2b}	1:500	Santa-Cruz	sc-5279
Nanog	Rabbit polyclonal	1:500	Abcam	ab80892
Laminin	Rabbit polyclonal	1:500	Sigma-Aldrich	L9393
Megalin (LRP2)	Mouse Monoclonal IgG_1	1:500	Acris	DM3613P
Gata6	Rabbit polyclonal IgG	1:200	Santa-Cruz	sc-9055
Brachyury	Goat Polyclonal IgG	1:200	Santa- Cruz	sc-17745
Heparan Sulphate (10E4 epitope)	Mouse monoclonal IgM	1:500	Amsbio	370255-s

Table 2. Primary antibodies

	Conjugated Fluorphor	Reference/ID
Anti-mouse IgG2b	**594 (red)**	**Alexa-Fluor** A21145
Goat anti Mouse IgG1	594 (Red)	AlexaFluor A21125
Chick anti Rabbit IgG	488 (Green)	Life technologies A21441
Goat anti-mouse IgM	488 (Green)	AlexaFluor A21042

Table 3. Secondary antibodies

2.2.14 Quantitative Polymerase Chain Reaction (qPCR)

mESC and EBs were transferred to a microfuge tube and 500 µl Trizol (Invitrogen) added. Samples were stored at 4ºC for up to 1 week prior to RNA extraction.

2.2.15 RNA extraction

To begin the extraction 100µl chloroform (Sigma Aldrich) was added to the sample in ½ ml Trizol. The sample was then shaken vigorously for 15 seconds to mix. The samples were then centrifuged for 15 minutes at 13000rpm at 4°C. After the centrifugation, there were 3 separate phases visible: lower phase; interphase and upper aqueous phase. The lower phase contains protein and DNA and the upper phase contains the RNA. The topmost clear layer containing the RNA was transferred to a new microfuge tube containing 1 µl of 1µg/µl solution of glycogen and 500µl isopropanol (Sigma Aldrich) added. To increase the yield of RNA the samples were stored overnight at -20ºC. The following day, the samples were centrifuged for 10 minutes at 13000rpm, after which, an RNA pellet was visible at the bottom of the tube. The isopropanol was discarded and the pellet was then washed in 1ml of 75% ethanol (Sigma Aldrich) (nuclease free water was used for

dilution). The sample was then centrifuged for 5 minutes at 7,500rpm. Following RNA extraction a nanodrop spectrophotometer was used for quantification. Here 1µl of sample of nuclease free water was initially placed on the instrument and measured as a 'blank' to create a baseline measurement followed by each RNA sample.

2.2.16 DNAase treatment

The ethanol was discarded, pellet air-dried briefly, and dissolved in 12µl nuclease-free water. 8µl of the RNA solution was transferred into a 0.2ml microfuge tube. 1µl DNase buffer (Promega) was added followed by 1µl DNAse enzyme (Promega). The sample was then incubated at 37ºC for 30 minutes. After incubation, 1µl 'Stop' buffer (Promega) was added to cease the reaction and incubated for a further 15 minutes at 60ºC.

2.2.17 Complementary DNA (cDNA) synthesis

8µl of DNase-treated RNA were transferred into 0.2ml microfuge tube and the following reactants added:

- **1µl of a 100ng/µl stock solution of random hexamers (Qiagen)**
- **1µl Deoxyribonucleotide Triphosphate (dNTP) mix (10mM stock) (Invitrogen)**
- **4µl nuclease-free water (Fisher Bioreagents) to give a final volume of 14µl**

This was then incubated at 65^{o}C for 5 min and placed immediately on ice. The following reagents were then added to the 0.2ml microfuge tube, giving a final reaction volume of 20µl:

- **1µl 5x buffer (Invitrogen)**
- **1µl 0.1M Dithiothreitol (DTT)(Invitrogen)**
- **1µl Superscript III (Invitrogen)**

The sample was incubated at 25°C for 5 minutes and then at 50°C for 60 minutes. Finally' to denature the Superscript III the sample was incubated at 70°C for 15 min. After the incubation was complete the cDNA was stored in a freezer at -20°C

2.2.18 Quantitative PCR

Reactants were assembled in a 200µl tube with a final reaction volume of 20µl with the following constituents per reaction:

- **10µl 2X SYBR Taq master mix (Sigma Aldrich),**
- **7µl Nuclease free water,**
- **1µl Template e.g. cDNA or Nuclease Free Water for 'no template' controls**
- **1µl Forward Primer (6.25pmol/µl)**
- **1µl Reverse Primer (6.25pmol/µl)**

The samples were loaded into the PCR machine Rotorgene 3000 (Corbett) and the reaction started using the following parameters: Hold 95°C 6 minutes. Cycling 95°C 6 Seconds, 58°C 20 seconds, 72°C 30 seconds. For this study 40 cycles were used.

In each PCR series 3 technical replicates were prepared for housekeeping/reference primers and 2 technical replicates prepared for primers recognising genes of interest. Three biological replicates of the mESC and Day4 EBs of WT and EXT1-/- origin were analysed.

The housekeeping gene used to normalise gene expression was glyceraldehyde-3-phosphate dehydrogenase (GAPDH). GAPDH is a gene that encodes a member of the glyceraldehyde-3-phosphate dehydrogenase protein family.

After each cycling step had finished, melt analysis was performed to confirm that the specific product had been amplified and that there were no primer dimers present. No template control samples were used to confirm the absence of any contamination.

2.2.19 Calculation of Relative gene expression

Values were normalised to GAPDH levels and the delta-delta Ct method was used to calculate relative change in expression between the WT and EXT1-/- samples. Delta-delta Ct requires the Cycles to Threshold (Ct) for a gene of interest and is compared to the (Ct) value for the housekeeping gene to determine relative expression. A constant 'Threshold' was set at the point in which the reaction became exponential; this was 0.4398 and was used for each experimental condition. As there were three biological replicates these were averaged and the standard deviation and standard error calculated.

Relative change in expression compared to normal conditions =

$2^{-\Delta\Delta Ct}$ or $2^{(\text{Average Ct of WT primer} - \text{Average Ct of EXT1-/-})}$

After determining the relative quantity of each gene the relative expression was determined by comparison to the relative quantity of the housekeeping gene, GAPDH.

Relative Gene Expression (ΔΔCt) =

$$\frac{\text{(Relative Quantity of Gene of Interest)}}{\text{(relative quantity of Housekeeping Gene)}}$$

The Students t-test was used to test the significance of the results.

Name	Primer (Sense)	Primer (Antisense)	Product length
GAPDH [1]	F: TGAAGCAGGCATCTGAGGG	R: CGAAGGTGGAAGAGTGGGAG	102bp
Bry[1]	F: CATCGGAACAGCTCTCCAACCTAT	R: GTGGGCTGGCGTTATGACTCA	136bp
Nanog[2]	F: AAGCAGAAGATGCGGACTGT	R: GTGCTGAGCCCTTCTGAATC	232bp
Oct4[3]	F: TGGAGACTTTGCAGCCTGAG	R: CTTCAGCAGCTTGGCAAACTG	188bp
Pax6[4]	GAGAAGAGAAGAGAAACTGAGGAACCAGA	ATGGGTTGGCAAAGCACTGTACG	201bp
EXT1 [1]	F: GGAGTTGCCATTCTCCGA	R: TAAGCCTCCCACAAGAACTG	153bp
Megalin [2]	F: GGCCACCAGTTCACTTGCT	R: TGAGATGCATCGTCCAGAC	171bp
Lama1[3]	F: CCGACAACCTCCTCTTCTACC	R: TCTCCACTGCGAGAAAGTCA	60bp
Gata6[5]	GACTCCTACTTCCTCTTCTTCTAATTCAGA	ACCTGAATACTTGAGGTCACTGTTCTC	151bp

Table 4. Primers used for qPCR analysis.

2.2.20 Gel Electrophoresis

Gel electrophoresis was performed using the products of a PCR reaction to confirm the absence of *Ext1* expression in Ext1-/- ESCs and that the PCR products of the analysed genes were of the expected size.

2.2.20.1 Gel Preparation

A 2 % agarose gel was prepared by adding 3g Agarose (Bioline) to 150ml of 1X Tris-Acetate EDTA (TAE) buffer and heated in an 800W microwave for 1 minute to produce a gel solution. After cooling, 3μl of a 10mg/ml stock of ethidium bromide** (Sigma Aldrich) was added to the solution. The gel was then poured into an electrophoresis cassette, a comb added and left to set for approximately 30 minutes. The cassette was then placed within the Electrophoresis Chamber (SCIE-PLAS) and submerged in 1X TAE running buffer . 5μl of 5X loading buffer was added to each 10μl PCR sample to give total volume of 25μl and each well loaded up with the appropriate PCR product. A Hyperladder IV (100bp) (Abcam) was pipetted into one of the wells to act as the reference. The electrophoresis ran at 75mV for 20 minutes.
The gel was examined under ultraviolet light and images taken using Gene Flash.

3. Morphological analysis of WT and EXT1-/- mESC and EBs

3.1 Introduction

3.1.1 Morphology of Mouse Embryonic Stem Cells

3.1.1.1mESC

Mouse embryonic stem cells (mESCs) are derived from the inner cell mass of the pre-implantation blastocyst (Evans and Kaufman, 1981) and are characterized by their pluripotency capacity, self-renewal and limitless proliferation (Niwa *et al*., 2007; Pera and Tam, 2010).

mESCc can be maintained *in vitro* by culturing them in 20% foetal bovine serum (FBS) on a layer of feeder cells, which are typically mitomycin C-treated mouse embryonic fibroblasts. mESCs can also be maintained successfully in the absence of feeders with the addition of the cytokine, leukaemia inhibitory factor (LIF), which leads to the activation and nuclear translocation of signal transducer and activation of transcription 3 (STAT3) (Burdon *et al.,* 2002). These accepted protocols allow the expansion of the pluripotent cells for extended periods of time without the loss of their capacity to contribute to all cell lineages (Wiles *et al*., 1993).

Successful culture of the mESC line is represented by the cell colonies demonstrating a characteristic undifferentiated morphology. Colonies are amorphous and cells demonstrate a large nuclear:cytoplasmic ratio when examined under a microscope. Nucleoli are prominent and due to the cells adhering closely to each other within compact colonies, it can be difficult to identify individual cells (StemCell technologies).

Although the above culture conditions can be used to expand mESCs, it is usually the case that 100% of the population will not consist entirely of undifferentiated cells, as there is typically some spontaneous differentiation occurring, which can be detected by observing the colony morphology using phase contrast microscopy. Differentiated cells are usually observed at the colony periphery and are readily identified by their tendency to be less

adherent to the undifferentiated mESCs within the colony (Hayashi *et al*., 2008; Toyooka *et al*., 2008). A small degree of spontaneous differentiation is a good indicator that the cells are still pluripotent. However, if there is excessive differentiation, the undifferentiated mESCs can become outnumbered by their differentiated progeny. Gross differentiation is recognised by the flattening of the cells and the disappearance of typical compact colonies.

3.1.1.2 Ext1-/- mESCs

EXT1-/- mESCs have a genetic mutation in the EXT1 gene; this gene functions to elongate the HS chain via the production of two co-polymerase enzymes that mediate the alternating addition of N-acetyl-glucosamine (GlcNAc) residues followed by D-glucuronic acid (GlcA). The result is that EXT1-/- mESCs are HS-deficient (McCormick *et al*., 1998) as they can only generate defective endogenous HS (Lin *et al*., 2000). HS regulates various biological reactions by interacting with extracellular signalling molecules such as the fibroblast growth factor (FGF) family. It has been reported that HS is needed for maintenance of self-renewal of mESC and that HS-deficient mESC proliferate slowly and demonstrate spontaneous differentiation of extraembryonic endoderm lineage (Sasaki *et al*., 2008).

In contrast there is evidence that HS is needed for differentiation of mESCs (Kraushaar *et al*., 2013) and EXT1-/- deficient cells can be successfully maintained in 2D- culture and display the characteristic morphology of pluripotent cells.

3.1.2 Morphology of Embryoid Bodies

3.1.2.1 Wildtype EBs

The culture of mESCs in suspension will result in the formation of aggregates or embryoid bodies (Martin *et al*., 1977). EBs undergo differentiation into the three cell lineages and represent a model of peri-implantation development of the mammalian embryo. EB development demonstrates compaction at day 2, and the appearance of squamous epithelial cells at the periphery of the EBs represents the differentiation of primitive endoderm (PrE) (Murray and Edgar 2000). At Day 4 the PrE has differentiated to form visceral endoderm consisting of tall columnar epithelial cells and parietal endoderm that form multilayers on the periphery of the EB. Parietal endodermal cells deposit a thick BM that is representative of the Reichert's membrane (Smith and Strickland, 1981).

3.1.2.2 EXT1-/ EBs

mESCs that are HS-deficient are also capable of forming EBs, but they have not been fully characterised, and as yet, it is not clear if they develop in a similar way to EBs generated from wild-type cells. (Lin *et al*. 2000; Stickens *et al*., 2005)

The aim of this chapter is to determine if there are any obvious morphological differences between EXT1-/- and WT ESCs and their derived EBs.

3.2 Results

3.2.1 Morphological analysis of EXT1-/- mESCs using phase contrastmicroscopy

To determine if there were any obvious morphological differences between WT and EXT1-/- ESCs, the two cell types were cultured on feeder cells generated from mouse embryonic fibroblasts (STO line), and following 3 days in culture, were analysed using phase contrast microscopy.

It was found that there were no noticeable differences between the two cells types. In both WT and EXT1-/- mESC cultures, the cells shows a typical morphology, with high nuclear:cytoplasmic ratio, prominent nucleoli, and formed compact colonies (Fig. 9).
Although the proliferation rate was not formally tested, for both cell lines, it was necessary to subculture every 2 to 3 days, which suggested a similar rate of expansion

These results suggested that HS is not required to maintain mESCs. However, it is possible that the STO feeder cells could provide a source of HS for the EXT1-/- ESCs. To test this, the WT and EXT1-/- cells were cultured for two passages on gelatin-coated dishes to remove the STO feeder cells and their morphology was then analysed using phase contrast microscopy. The results showed EXT1-/- mESC demonstrated typical morphology of ESCs and no differences were identified between the two cell-lines colonies despite even in the absence of exogenous HS provided by STO feeder cells (Fig 9).

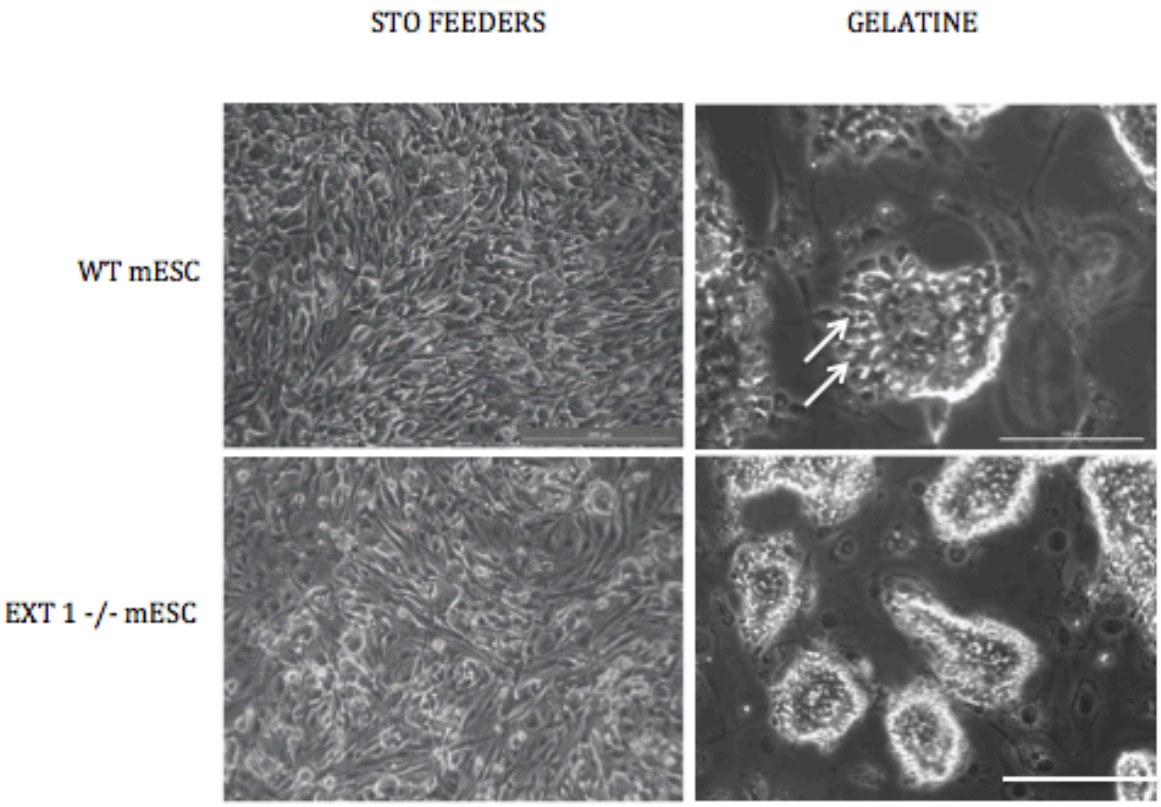

Fig.9 Wildtype mESC and EXT1-/- mESC were cultured on STO feeder cells and gelatine coated dishes for three days in 15% FBS medium and the morphology assessed. Note the defined colonies and high nuclear-to-cytoplasm ratio and prominent nucleoli (arrows) of the mESC cultured on gelatine.
Scale 200µm.

3.2.2 Morphological analysis of EBs derived from EXT1-/- mESCs using phase contrast microscopy

To test if lack of HS affected EB development, WT and EXT1-/- ESCs were cultured for 2 passages on gelatin-coated dishes to remove the STO feeder cells, and were then plated in non-adherent dishes to promote aggregation and EB formation. After 4 days, the EBs were fixed, and frozen sections prepared for analysis. Specifically, the size of the EBs was compared between the two groups by measuring their diameter, and the EBs were assessed to see if there was any evidence of extra-embryonic endoderm (i.e., primitive, visceral or parietal) formation at the EB periphery. These endodermal types can be usually be identified by their morphology and location. For instance, primitive endoderm are squamous epithelial cells that form a monolayer at the surface; visceral endoderm are tall columnar epithelial cells that form a monolayer at the surface; parietal endoderm are migratory cells that can form multilayers at the surface. Furthermore, the basement membrane underlying the endoderm can usually be identified as it appears as a dark line under phase contrast microscopy.

3.2.3 EB size

The size of the EBs derived from WT mESC were relatively consistent and had an average diameter of 113 μm ranging from 90- 120 μm.
In comparison the EXT1-/- EBs demonstratad a heterogenous size with the formation of frequently smaller or markedly larger EBs to that of the WT EBs. The average diameter of the eXT1-/- EBs was 89.6μm with a range of (25-160 μm) (Fig. 10). There were three biological replicates used with a total of 16 EBs in both populations.

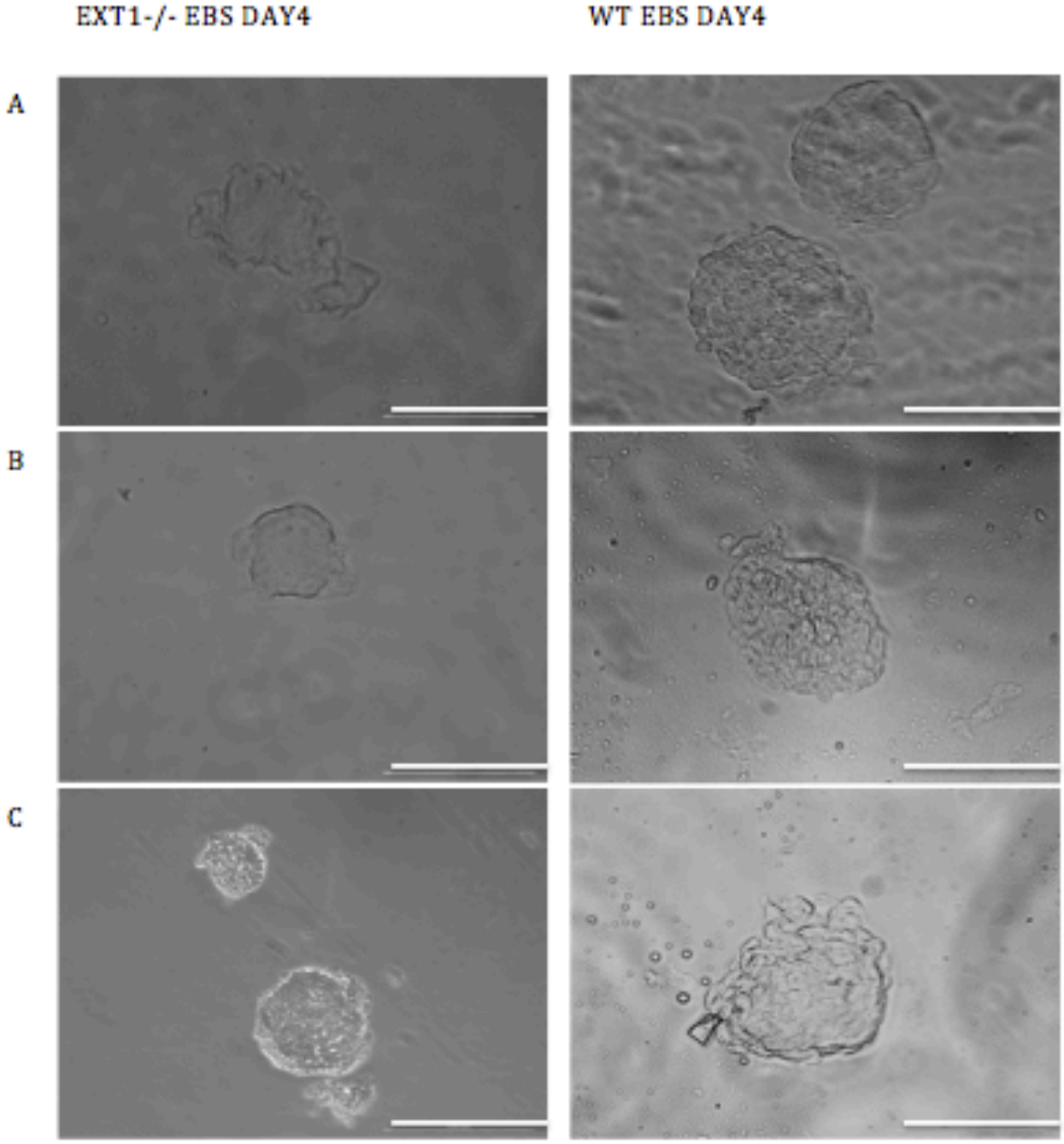

Fig 10. **Morphological analysis of day 4 WT and EXT1-/- EBs.** Phase contrast microscopy of EB frozen sections show that WT EBs have a more consistent size (100-120μm) compare with that of the EXT1-/- EBs (25-120 μm). All EBs presented throughout this study were analysed for size and reulst discussed in section 3.2.3. Scale bar, 100μm.

3.2.4 EB differentiation

WT populations demonstrated differentiation that was expected by EBs. Basement Membranes were assumed to be present within the EBs demonstrated by the dark line separating the inner cells of the EB from the cells on the periphery. These were continuous, indicating successful BM synthesis and required confirmation by staining for Laminin-111. The cells on the outside of the apparent BM were typical of extra-embryonic endoderm (EEE) that is typically seen at Day4 in WT EBs (Fig.11) There was no evidence of a squamous epithelial monolayer suggestive of Primitive Endoderm. However, this is not unexpected, as it does not normally appear until Day 6.

The EXT1-/- EBs exhibited a less spherical, asymmetrical morphology compared to the WT EBs. Within this population there was no clear appearance of a BM. These EBs also lacked peripheral cells representative of EEE.

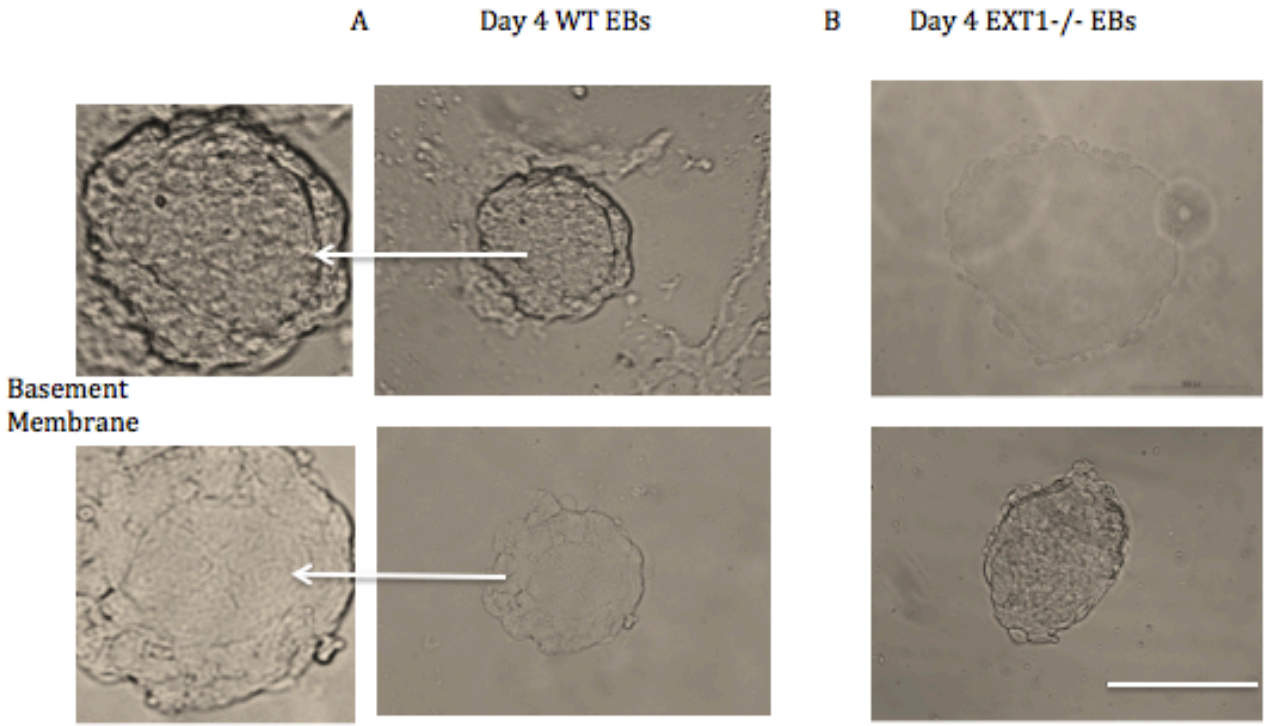

Fig. 11. Morphological analysis of day 4 WT and EXT1-/- EBs. Phase contrast microscopy of EB frozen sections show that WT embryoid bodies have an outer layer of endoderm with an underlying basement membrane (arrow) (A) whereas EXT1-/- EBs appear to lack an endoderm layer and basement membrane (B). EBs are representative of EBs from 2 different biological replicates. Scale bar, 100 μm.

3.3 Discussion

Prior to EB formation, WT and EXT1-/- mESC had demonstrated typical morphological characteristics of undifferentiated cells with no differences in appearance (Fig 9) Analysis identified differences in the number of differentiating EBs between the two cell-lines and suggested that endoderm differentiation is defective in the EXT1-/- EBs. There was no endodermal like morphology present on the periphery of the WT EBs and no obvious signs of a BM(Fig.11).

This suggests that HS is necessary for endoderm differentiation, possibly because it is required for FGF signaling, which is known to be required for primitive endoderm differentiation (Arman *et al*., 1998). However, to confirm these results, it is necessary to investigate the expression pattern of endoderm markers. This will be undertaken in the chapter 5.

4. Immunofluorescence investigations of WT and EXT1-/- mESC and EBs

4.1 Introduction

Pluripotent mESCs express a range of characteristic markers. Most commonly, Oct4 and Nanog are indicative of the cells capacity for self-renewal and prevent the onset of differentiation. (Medvedev, Shevchenko *et al*., 2008; Wang *et al*., 2008).

However, the expression of Nanog is heterogenous within mESC colonies (Singh *et al*., 2007). Expansion of the cells will lead to the formation of multi-layered colonies and it is within these that often other transcription factors, such as Brachyury are expressed within mesodermal cells (Tanaka. 2008).

Successful EB development relies on the mESC line to be pluripotent in order for differentiation to represent that of the peri-implantation embryo.

4.1.1Expression of key lineage markers in mouse embryos and mESC-derived EBs

EBs undergo differentiation similar to that of an embryo. Screening for key markers expressed by the different cell types in the early embryo can confirm the identity of the cells that form within the EB.

The first differentiation event during normal EB development is the differentiation of primitive endoderm cells (PrE) on the periphery of the EB which typically occurs at day 2 (Murray *et al*., 2001). It is not completely clear whether PrE differentiation is triggered because cells find themselves on the surface of the EB, or whether they differentiate inside the EB and then migrate to the periphery.

True pluripotency of mESCs *in vitro* is established through the expression of Oct4 and Nanog (Medvedev, Shevchenko *et al*. 2008; Wang *et al*. 2008). The formation of EBs results in differentiation, with the presence of PrE on the periphery by Day 4. *Nanog* downregulation is essential for ES-cell

differentiation into PrE upon aggregation (Hamazaki *et al*., 2004) and similarly, *Oct4* expression is downregulated in trophectodermal cells but maintained in the inner cell mas of the embryo (Kehler *et al*., 2004; Boiani *et al*., 2002). Work utilising EBs has demonstated that the expression of Oct 4 and Nanog decreases over time. Localisation of Oct4 expression becomes limited to a few inner cells in EBs. Nanog expression and distribution displayed a similar trend to that of Oct4 (Williams, 2013).

The glycoprotein, megalin, or LRP2, is a marker of PrE. Megalin is expressed in the trophectoderm of pre-implantation embryos (Fisher *et al*., 2006), and following blastocyst formation, is expressed in the PrE, where it is located in endosomes (Drake *et al*., 2004). Following implantation, megalin is present on the apical surface of the visceral endoderm (VE). Megalin expression follows a similar expression pattern in EBs (Moore et al., 2009).

4.1.2 Laminin-111

Laminin-111 is a heterotrimeric extracellular matrix protein composed of α1, β1, and γ1 chains that is crucial for early basement membrane assembly (Miner and Yurchenco,, 2004). Basement membranes themselves play an important role in embryogenesis and are needed for normal cell differentiation to occur. (Miner and Yurchenco, 2004) In mESC that have dysfunctional BMs the PrE fails to epithelialize (Murray *et al*., 2001), while mesodermal differentiation appears to be accelerated (Fujiwara *et al*., 2007). Morphological studies in the previous chapter suggested that the EXT1-/- EBs failed to deposit a BM. This will be confirmed here by performing immunostaining for laminin.

4.1.3 Culture conditions used for expanding mESCs can influence how EBs develop

Previous unpublished work from the Liverpool Stem Cell Group has shown that if mESCs are cultured for several passages in the absence of feeders, they are unable to generate normal EBs. Specifically, PrE differentiation and BM deposition did not occur in EBs derived from mESCs cultured for prolonged periods in the absence of feeder layers. It is therefore important that mESCs are cultured in the presence of feeders in order to generate EBs that mimic embryo development. However, a potential problem with this approach in the current study is that it is possible that HS from the feeder cells could be transferred to the EXT1-/- mESCs. Therefore, to check this, in this chapter, immunostaining for HS using the antibody 10E4 was performed to investigate if HS is present on EXT1-/- mESCs cultured in the presence of feeders, and whether culturing the EXT1-/- mESCs in the absence of feeders for 1 or 2 passages was able to remove the HS.

4.2 Objectives

In the previous chapter, morphological analysis of EXT1-/- EBs using phase contrast microscopy suggested that in contrast to WT EBs, extra-embryonic endoderm cells had not differentiated at the periphery of the EXT1-/- EBs, nor had a BM been deposited between the PrE and inner cells of the EB. The objectives of this chapter were as follows:

1. Perform immunostaining for HS in mESCs and EBs to confirm that levels are depleted in EXT1-null samples, and to investigate the localisation of HS in the WT samples.
2. Perform immunostaining for megalin and laminin-111 on mESCs to investigate if these markers are expressed prior to EB formation.
3. Confirm whether PrE differentiation and BM deposition occurs in EXT1-/- EBs by performing immunostaining for the PrE marker, megalin, and the BM protein, laminin-111.

4.3 Results

4.3.1 Heparan Sulphate expression in EXT1-/- mESC

The mESCs within this study were grown on a feeder layer for expansion. It was reasoned that the STO cells could produce HS and possibly transfer the polysaccharide onto the HS-deficient mESC during co-culture. For this reason, immunostaining for HS was performed on mESCs cultured on feeders, and following 2 passages on gelatin-coated dishes in the absence of feeders.

The results showed that in the presence of STO feeder cells, HS can be detected on colonies of EXT1-/- ESCs, but following 2 passage on gelatin-coated dishes in the absence of feeder cells, HS could no longer be detected. In contrast, HS could be detected on colonies of WT ESCs cultured for 2 passages in the absence of feeder cells (Fig.12). These results confirmed that HS is depleted in EXT1-/- mESCs, but that when co-cultured with feeder cells, HS appears to be transferred from the feeder cells to the mESCs, Therefore, for EB formation, ESCs were cultured without feeder cells for two passages prior to making EBs to ensure that STO-derived HS would not be able to affect EB development.

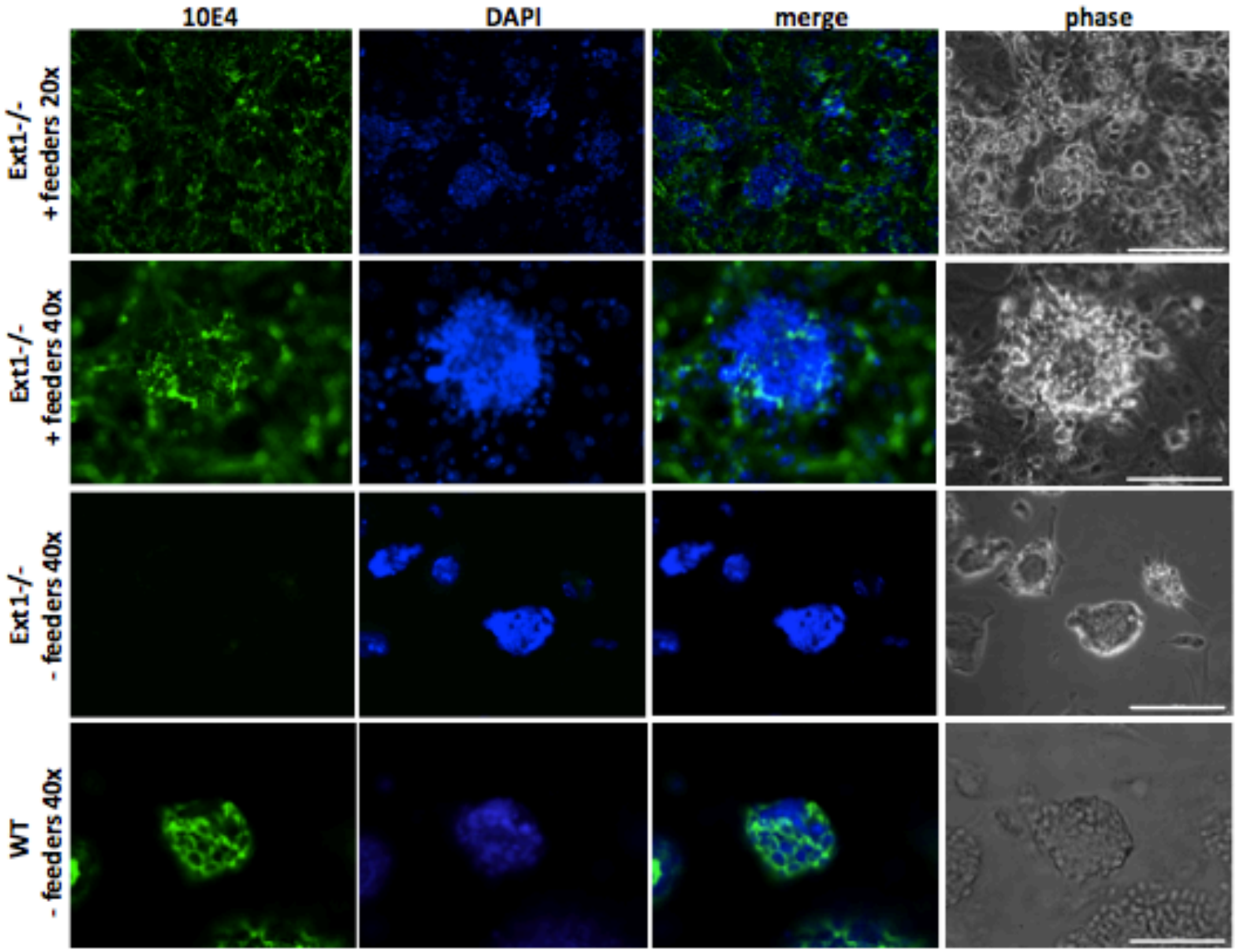

Fig. 12 Immunofluorescence staining for heparan sulphate in WT and Ext1-/- ESCs. ESCs were immunostained with the10E4 antibody that recognises N-sulphated glucosamine residues present in heparan sulphate. Note that when Ext1-/- ESCs are cultured on STO feeder cells, positive staining is detected on cells within the Ext1-/- colonies (top two panels). In the absence of STO feeder cells, positive staining is absent from Ext1-/- cells but is detected on WT ESCs (bottom two panels). The images are representative of ESC colonies from a single experiment. Scale bar in top panel is 200μm, and in bottom three panels, is 100μm.

4.3.2 Immunofluorescence staining for megalin and laminin-111 i WT and Ext1-/- ESCs.

Due to a previous report that suggested PrE cells spontaneously differentiate and then migrate to the surface of EBs (Moore *et al*., 2009), the expression of the megalin and laminin-111 was checked using immunofluorescence in the WT and EXT1-/- populations prior to EB formation.

The results showed that no megalin was detected in EXT1-/- mESCs, indicating a complete absence of spontaneous differentiation in these cultures. In the WT samples, cells expressing high levels of megalin were detected, and in most cases, appeared to be at the periphery of mESC colonies, though this could not be confirmed given that confocal microscopy was not performed (Fig. 13).

Regarding laminin immunostaining, it was found that in EXT1-/- cultures, positive staining was only detected in the few remaining STO cells, and virtually no staining was detected in the EXT1-/- mESCs. On the other hand, many cells within the WT mESC colonies expressed laminin, but there was no evidence of BM deposition (Fig. 14).

These results indicate a complete absence of spontaneous differentiation in the EXT1-/- ESCs but suggest that some spontaneous differentiation is occurring in the WT cultures.

4.3.3 Immunostaining for heparan sulphate in EXT1-/- and WT EBs

To investigate HS expression, day 4 EBs were fixed and frozen sections prepared and immunostained with 10E4.

No positive staining for HS was observed in the EXT1-/- EBs (Fig.15). In contrast, HS was detected in the WT EBs, and it appeared that inner EB cells had higher levels of HS than the peripheral cells (Fig.16).

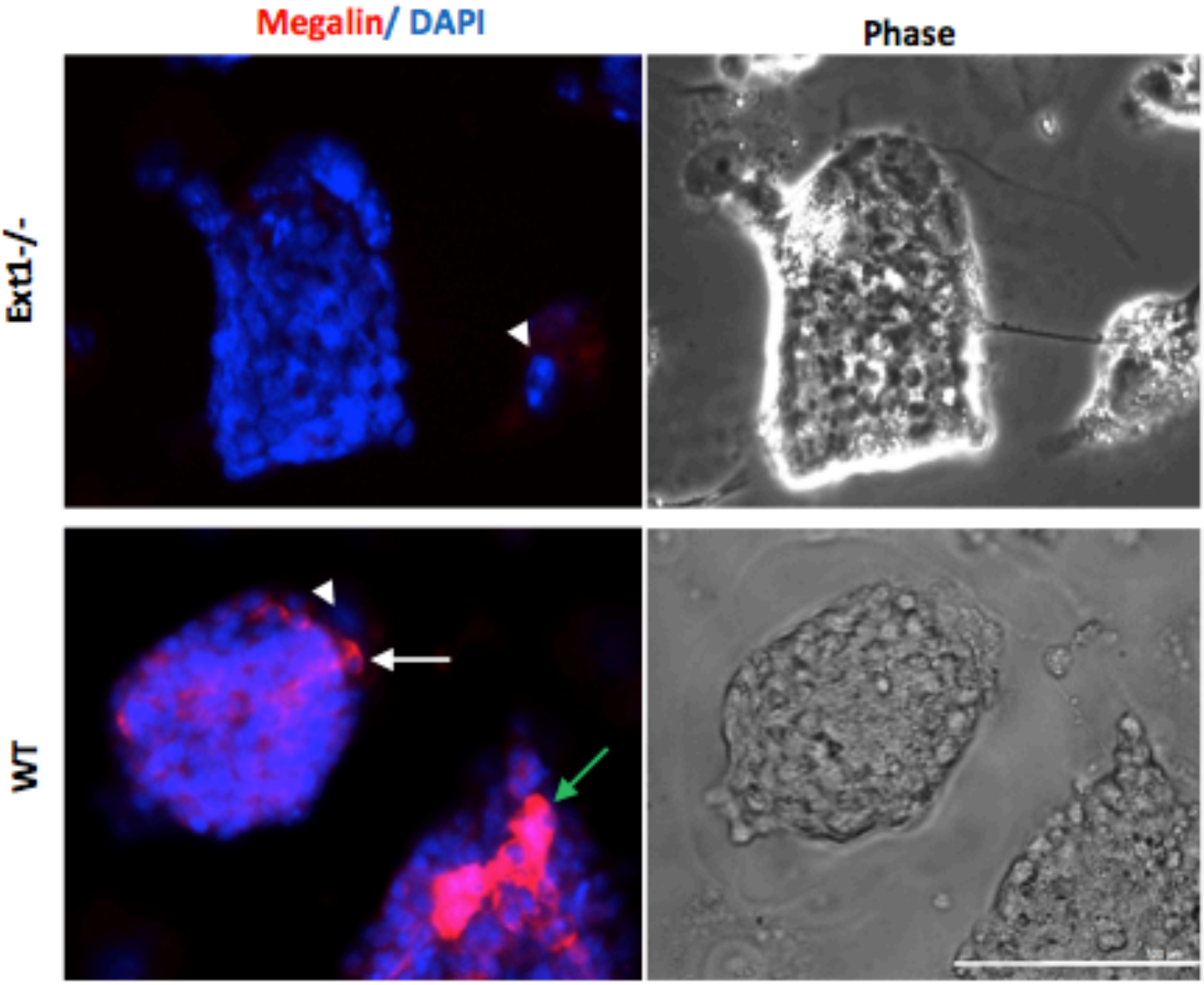

Fig. 13. Immunofluorescence staining for megalin in WT and Ext1-/- ESCs. ESCs were immunostained with an antibody that recognises megalin. Positive staining is absent from Ext1-/- ESCs but is detected in WT ESCs. Some cells at the periphery of the WT colonies (white arrow) or that appear to be on the surface of multi-layered colonies (green arrow) have more intense staining. Staining is absent from remnant feeder cells (arrowhead). The images are representative of ESC colonies from a single experiment. Scale bar is 100µm.

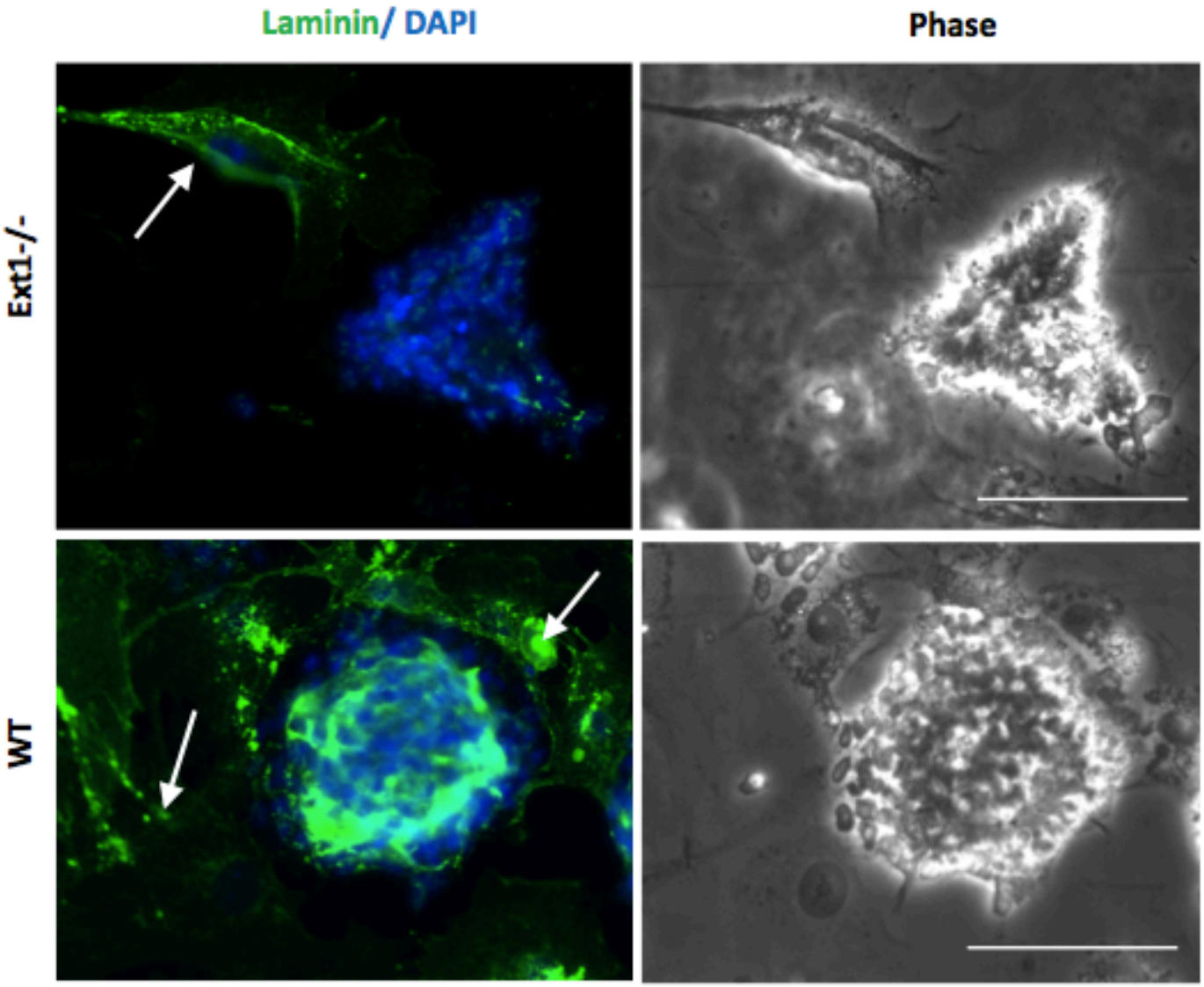

Fig. 14 Immunofluorescence staining for laminin-111 in WT and Ext1-/- ESCs. ESCs were immunostained with an antibody that recognises all three chains of laminin-111. Positive staining is absent from Ext1-/- cells but is detected in WT ESCs, where it appears to be present within the cells. There is no evidence of basement membranes in the WT ESC colonies. Positive staining is also detected within remnant STO feeder cells (arrows). The images are representative of ESC colonies from a single experiment. Scale bar is 100µm.

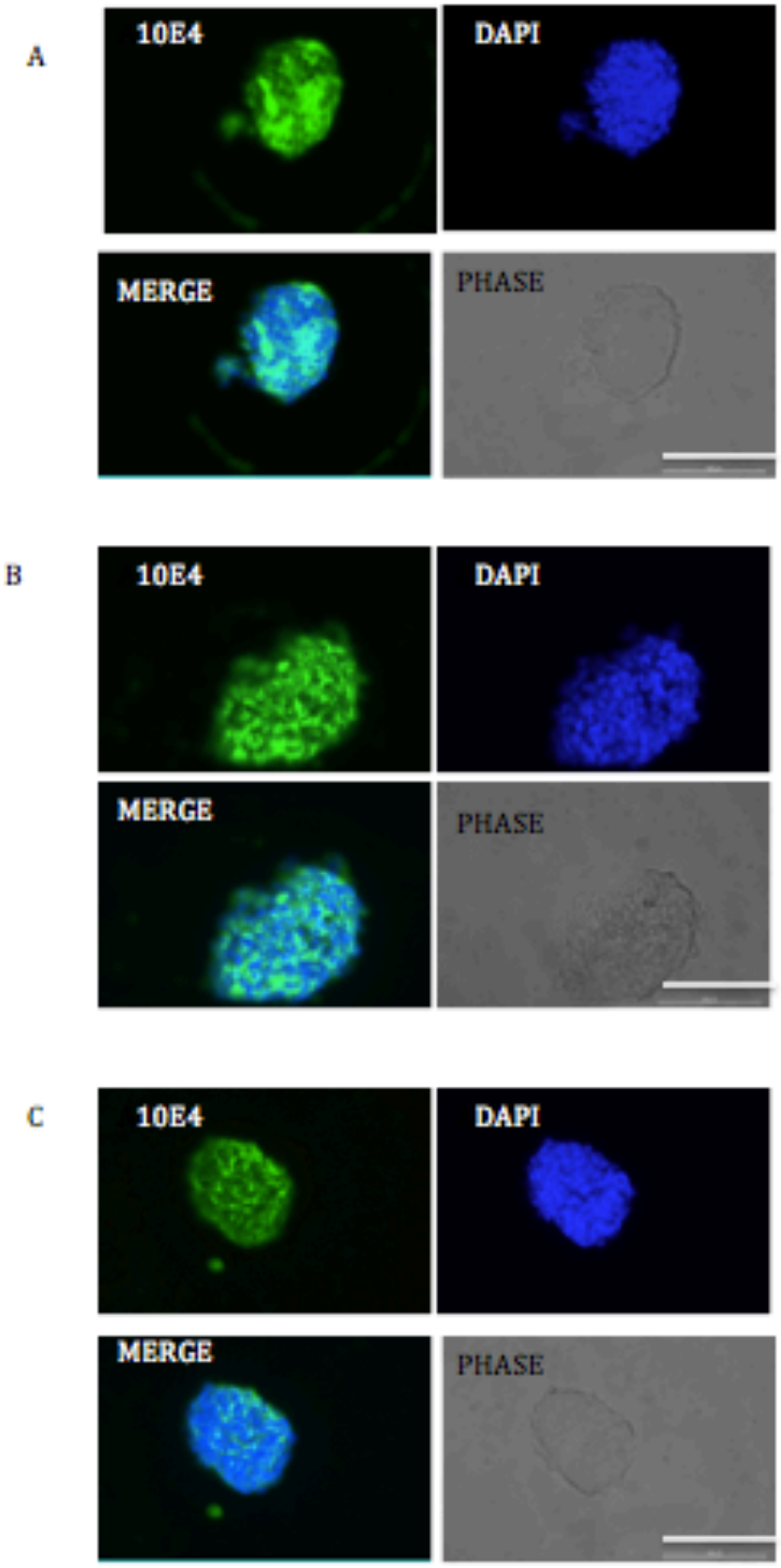

Fig. 15 Immunofluorescence staining for 10E4 in frozen sections of WT day 4 EBs. The images show representative EBs from 3 biological replicates. Note the lack of positive staining of10E4 in the WT EBs. A is sample/batch 1; B is sample 2; C is sample 3. Scale bar, 100 µm.

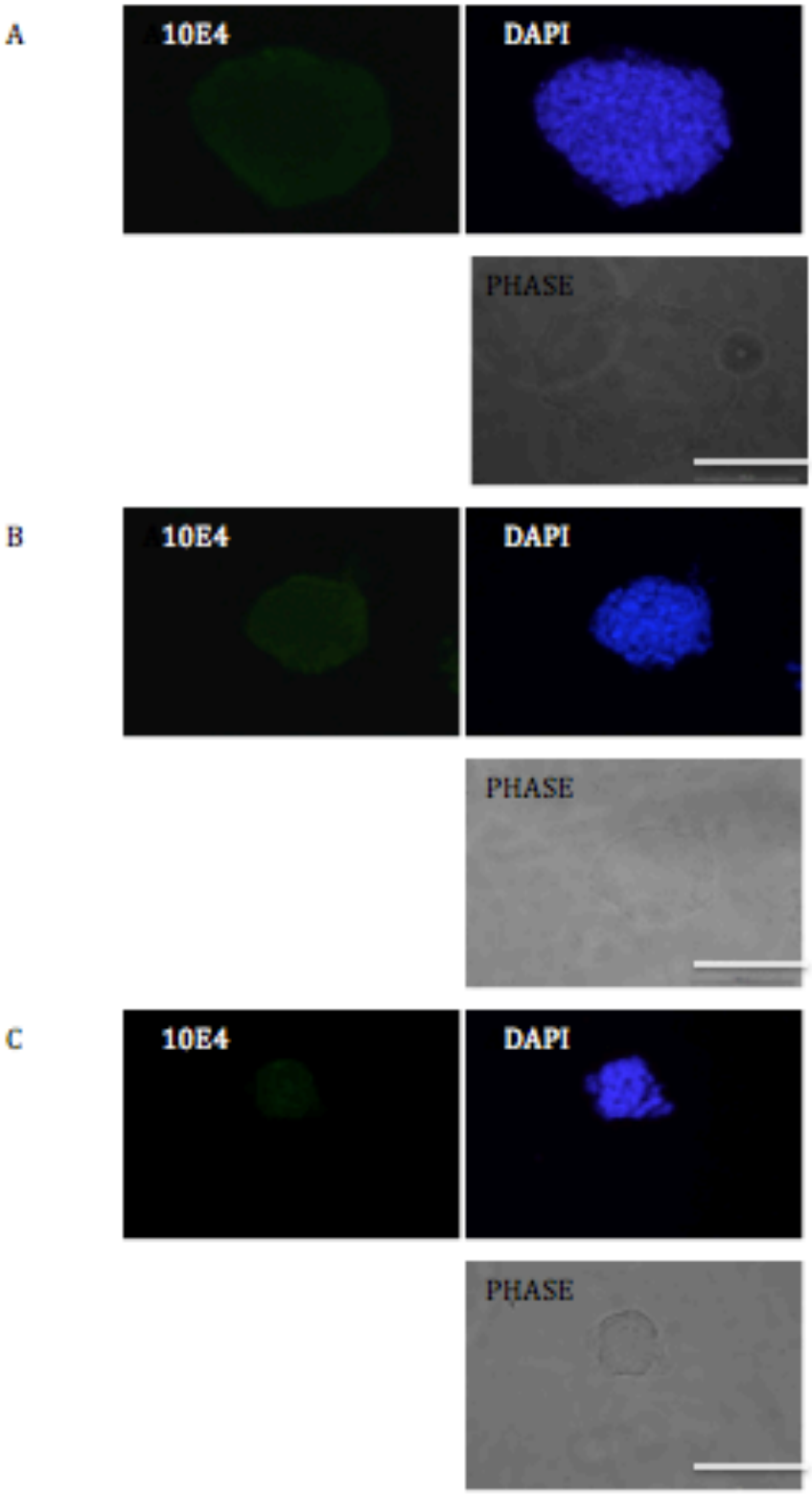

Fig. 16 Immunofluorescence staining for 10E4 in frozen sections of EXT1-/- day 4 EBs. The images show representative EBs from 3 biological replicates. Note the lack of 10E4 in the EXT1-/- EBs. A is sample/batch 1; B is sample 2; C is sample 3. Scale bar, A B 100 µm C 200 µm.

Megalin and nanog dual immunostaining was performed to investigate if PrE had formed in the day 4 EXT1 -/- EBs. In WT EBs, it was found that the peripheral cells expressed megalin, whereas the inner cells did not (Fig. 17) Some of the peripheral cells appeared to be VE as they were tall columnar cells on the EB periphery. In these cells, megalin appeared to be mainly localized to the apical surface. In PrE cells in the WT EBs, megalin staining appeared to be cytoplasmic. This is consistent with the expression profile in the embryos, where expression in the PrE cells at the surface of the inner cell mass was reported to be within endosomes, whereas in VE cells, it was localized to the apical surface (Maurer *et al*, 2005) (Fig. 17.1). In contrast to WT EBs, no megalin staining was detected in EXT1-/- EBs, consistent with the fact that no PrE had differentiated in these EBs (Fig. 18).
In the WT EBs, it was found that nanog was expressed in the nuclei of a few inner cells which is expected as the inner cells would be expected to start differentiating by day 4. Surprisingly, almost no nanog+ cells were detected in the EXT1-/- EBs, suggesting that despite the fact that PrE had failed to differentiate in the EBs, the ESCs within the EBs were nevertheless differentiating.

4.3.5 Basement Membrane deposition within the WT and EXT1-/- EBs

Dual immunostaining for laminin and Oct4 showed that in WT EBs, a BM was present between the outer extra- embryonic endoderm cells and inner cells, and that Oct4 was only detected in the nuclei of inner cells, reflecting the staining pattern observed with nanog (Fig. 19). The EXT1-/- EBs showed little evidence of any laminin expression and BMs were completely absent. However, some peripheral cells appeared to have increased levels of laminin. Similarly to the staining pattern observed with nanog antibodies, almost no cells within the EXT1-/- EBs expressed Oct4 (Fig.20).

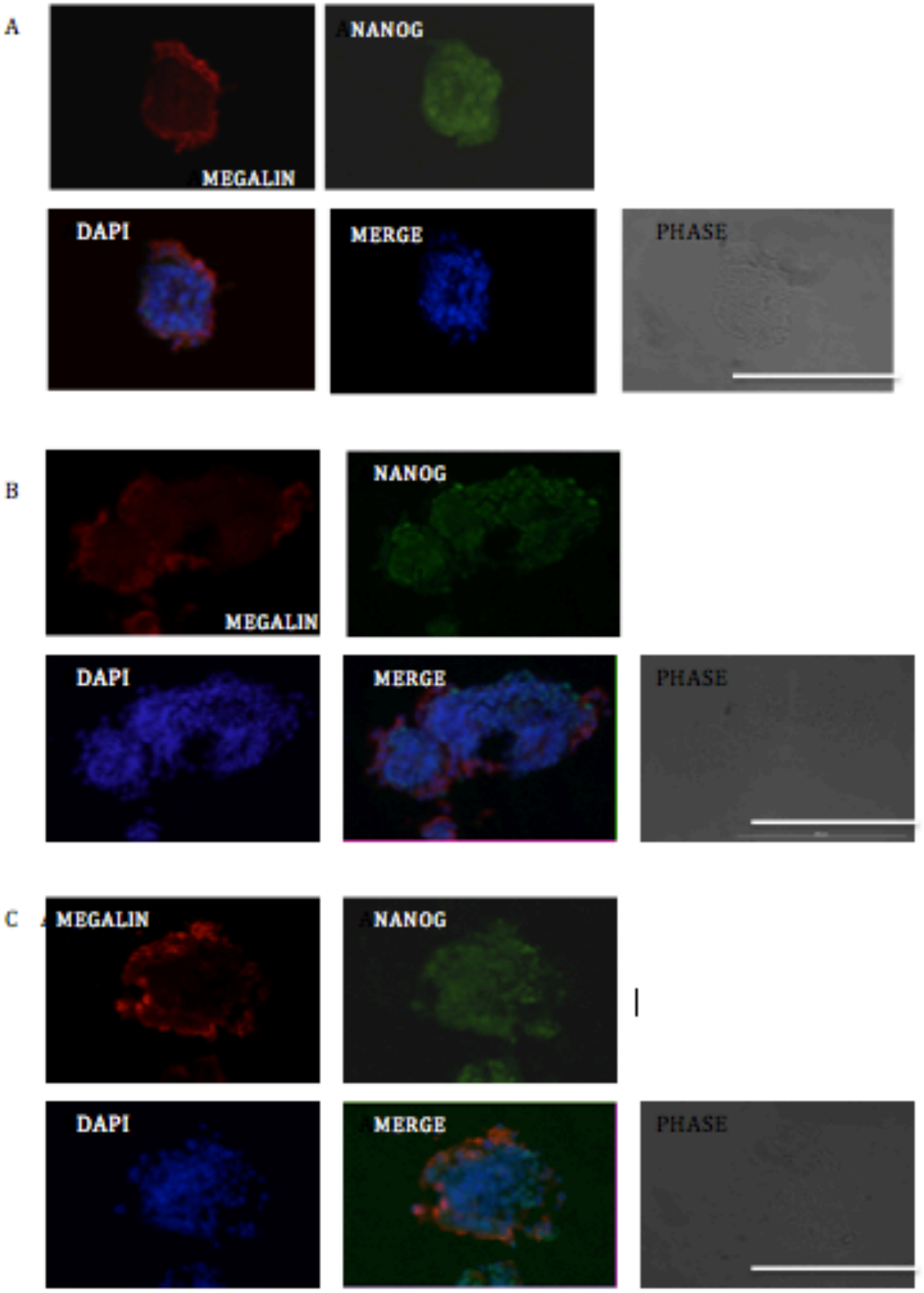

Fig. 17 Immunofluorescence staining for megalin and nanog in frozen sections of WT day 4 EBs. The images show representative EBs from 3 biological replicates. Note the presence of megalin+ cells positioned on the periphery of the EBs. Nanog is only detected in cell within the core of the EB. A is sample/batch 1; B is sample 2; C is sample 3. Scale bar, 200 µm.

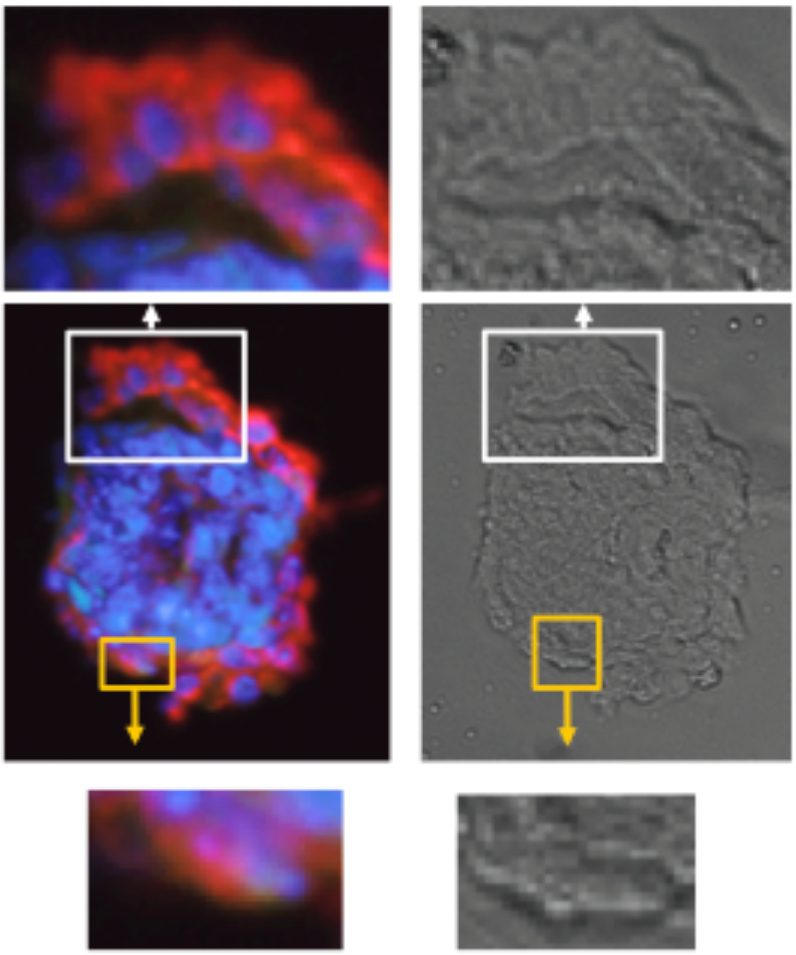

Fig. 17.1 Enlargement of the area within the white box shows a group of visceral endoderm (VE) cells on the EB periphery. These cells can be identified as VE because they are tall columnar epithelial cells that form a monolayer on the EB periphery. In these cells, megalin is localised to the apical surface. Enlargement of the area within the yellow box shows a PrE cell on the EB periphery. This cell can be identified as PrE because it is a squamous cell in a monolayer at the EB periphery. In this cell, megalin is not localised to the apical surface as it is in the VE cells, but instead, appears to be cytoplasmic.

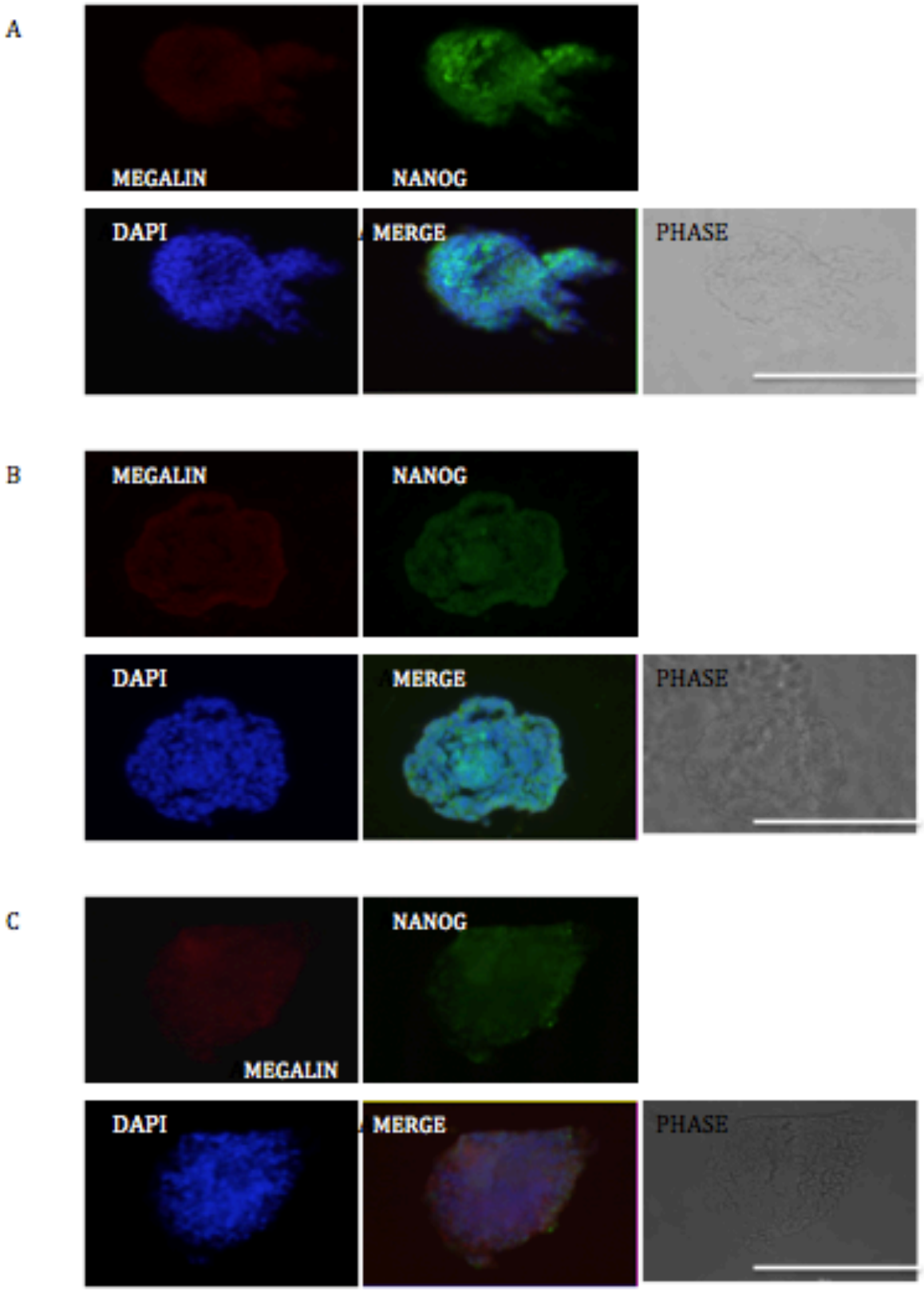

Fig. 18 Immunofluorescence staining for megalin and nanog in frozen sections of EXT1-/- day 4 EBs. The images show representative EBs from 3 biological replicates. There is no evidence of megalin+ cells on the periphery of the EBs and most EBs do not contain any nanog+ cells. A is sample/batch 1; B is sample 2; C is sample 3. Scale bar, 200 μm.

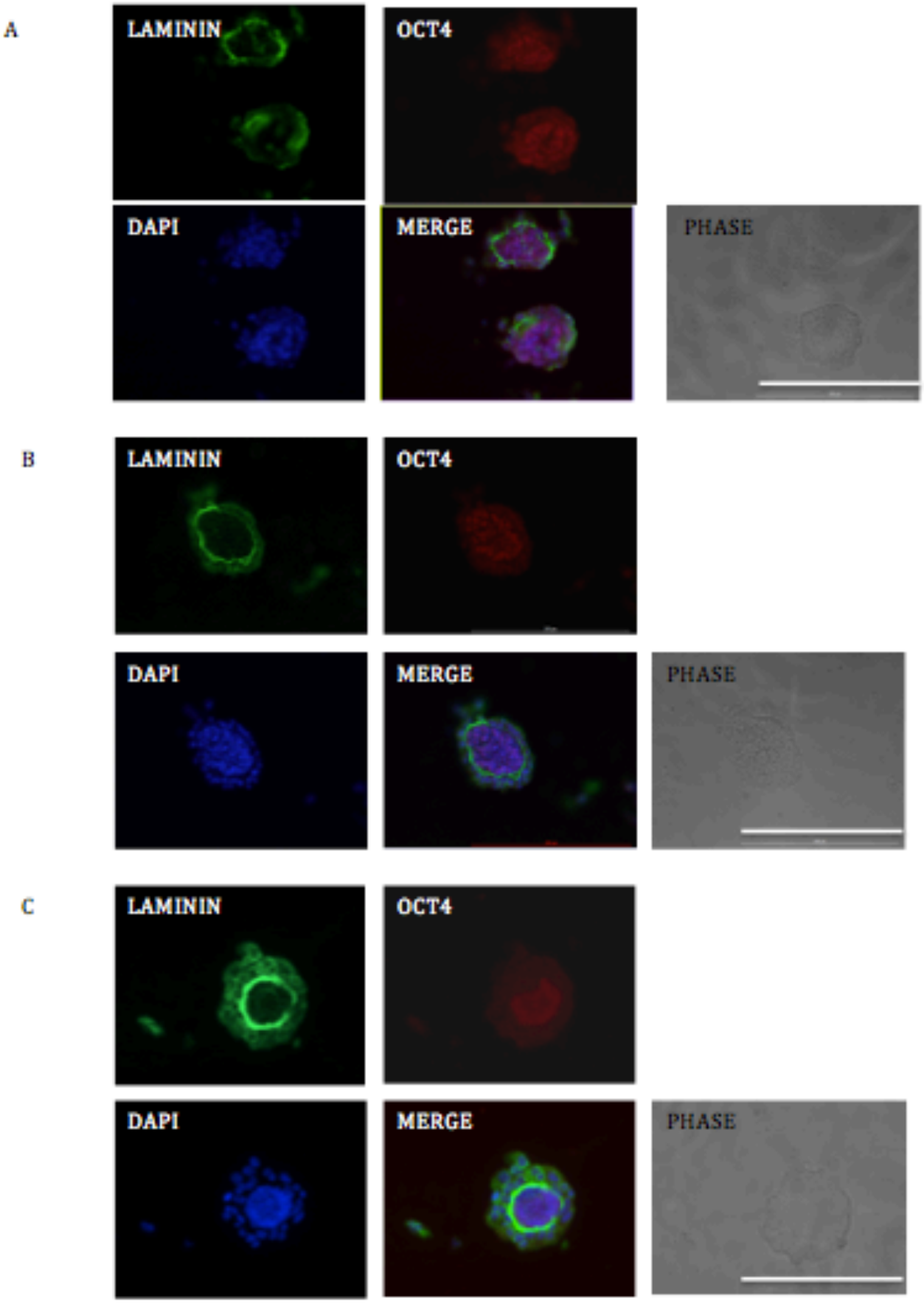

Fig. 19 Immunofluorescence staining for laminin and Oct4 in frozen sections of WT day 4 EBs. The images show representative EBs from 3 biological replicates. Note the laminin+ cell sin the basement membrane of the EBs and the oct4+ cells within the middle of the EBs. A is sample/batch 1; B is sample 2; C is sample 3. Scale bar, 200μm.

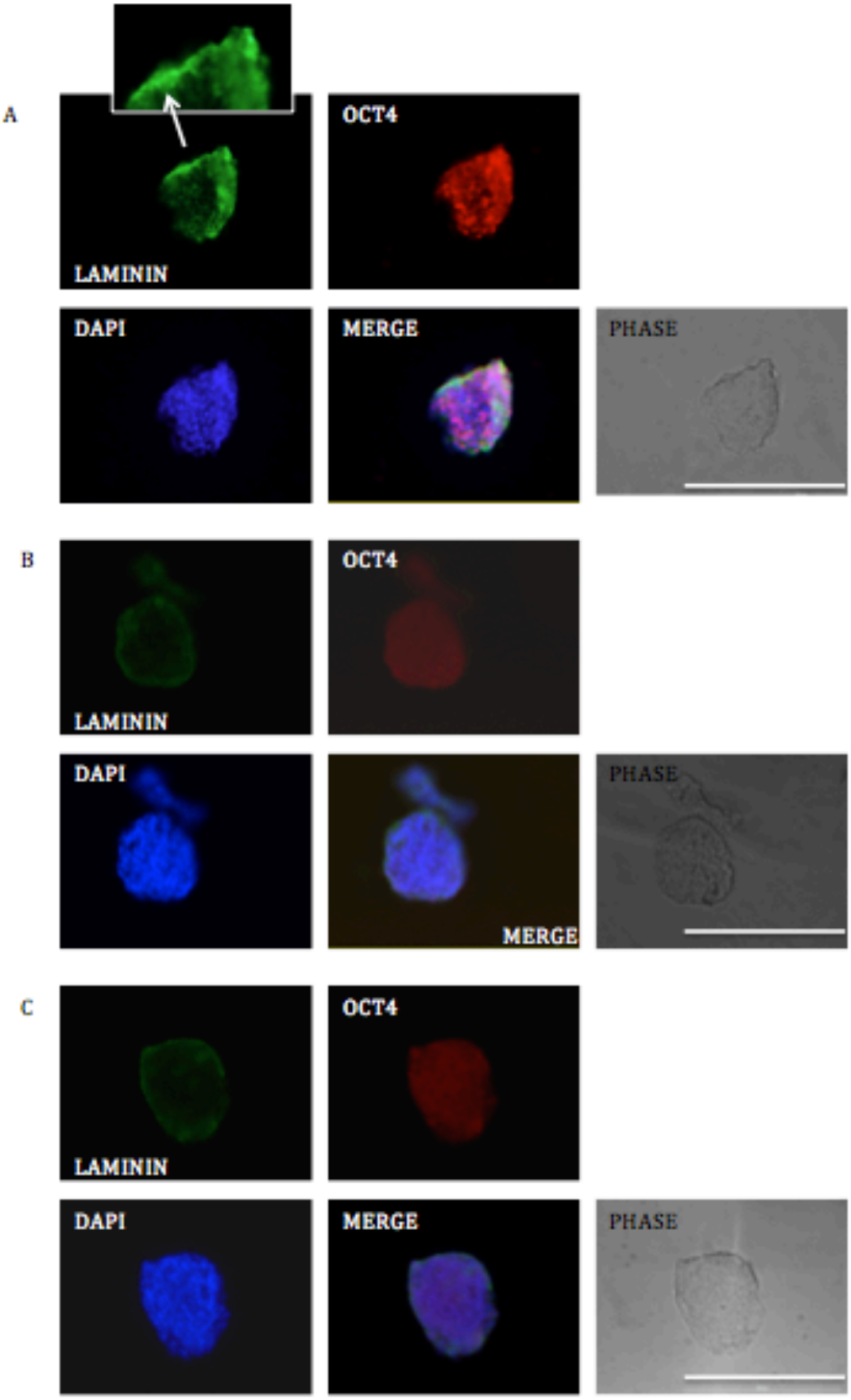

Fig. 20 Immunofluorescence staining for laminin and oct4 in frozen sections of EXT1-/- day 4 EBs. The images show representative EBs from 3 biological replicates. Sample A shows evidence of Laminin+ cells on its periphery and also shows oct4+ cells throughout. There is no evidence of Laminin+ cells in of EBs and they do not contain any oct4+ cells. A is sample/batch 1; B is sample 2; C is sample 3. Scale bar, 200 μm.

4.4 Discussion

In contrast to the results obtained here, a previous paper had suggested that EXT1-/- EBs could produce endoderm despite their lack of HS (Lin *et al*. 2000). However, in this earlier report, it appeared that EBs had been generated directly from EXT1-/- ESCs cultured on feeder cells, so in this case, HS derived from the feeder cells might have been sufficient to facilitate the FGF signaling that is known to be required for PrE differentiation (Arman *et al*., 1998). To test if this were the case, it would be interesting to generate EBs directly from EXT1-/- ESCs cultured on feeder cells, and following 2 passages on gelatin, see if PrE was able to differentiate in the former conditions but not the latter. Immunostaining prior to EB development also demonstrated an absence of Megalin staining indicating that EXT1-/- mESC were unable to differentiate into endodermal cells, whereas a number of cells at the periphery of the WT colonies or cells that appear to be on the surface of multi-layered colonies presented spontaneous differentiation.

Given the lack of PrE in the EXT1-/- EBs, it was not surprising that BMs were also lacking, because it is known that the BM is synthesized by the PrE cells (Murray *et al*., 2001). However, despite the lack of BM deposition, it was found that peripheral cells of EXT1-/- EBs appears to display intracellular laminin staining. It is known that during the early stages of development, the beta1 and gamma1 chains of laminin are expressed prior to the alpha1 chain being expressed. Expression of the alpha 1 chain is the rate-limiting step for BM synthesis, and before it is turned on, the beta and gamma chains accumulate within the cells (Miner and Yurchenco *et al*., 2004). It is known that expression of the alpha1 chain of laminin is dependent on FGF signaling (Lonai, 2005), which is in turn dependent on HS (Yayon *et al*., 1991). It is therefore possible that in the EXT1-/- EBs, cells at the periphery might have begun to differentiate to the PrE lineage and upregulated the beta1 and gamma1 laminin chains, but due to the lack of HS, the alpha 1 chain could not be expressed, preventing normal PrE differentiation.

Due to the lack of PrE differentiation in the EXT1-/- EBs, it was surprising that most of the inner cells no longer expressed the pluripotency markers, Oct4

and nanog, because differentiation of inner cells is thought to depend on signals from the extra-embryonic endoderm (Murray *et al*., 2001). This raised the question of whether the inner cells were differentiating to mesoderm or ectoderm, or were simply losing their pluripotency. This question will be addressed in the next chapter by using qPCR to look at the expression levels of various lineage markers in the EXT1-/- EBs.

5. Quantitative Polymerase Chain Reactions of WT and EXT1-/- mESC and EBs

5.1 Introduction

In the previous chapter it was found that PrE failed to differentiate in EXT1-/- EBs and there was no evidence of BM deposition. However, the cells within the EXT1-/- EBs down-regulated Oct4 and nanog, raising the possibility that they were differentiating to other lineages. In this chapter, Real-time Quantitative Polymerase Chain Reaction (qPCR) will be employed to quantify the expression levels of different lineage markers in the EXT1-/- ESCs and EBs, relative to levels in WT ESCs and EBs.

5.1.1 Expression of the pluripotency markers, Oct4 and nanog

In the previous chapter, it appeared that compared with WT EBs, fewer cells in the EXT1-/- EBs were positive for the pluripotency markers, Oct4 and nanog. Therefore, qPCR analysis will be performed here to investigate if these markers are expressed at significantly lower levels in the EXT1-/- samples.

5.1.2 Expression of the extra-embryonic endoderm markers, megalin, Gata6 and Lama1

Most of the markers of EEE are also expressed by definitive endoderm and it can therefore be difficult to distinguish these two lineages. However, given that the EBs under investigation in this study are only at day 4, a stage before primitive ectoderm has formed (the cell type that gives rise to mesoderm, definitive ectoderm and definitive endoderm), then it is likely that extra-embryonic endoderm will be the predominant lineage.

Expression levels of megalin, Gata6 and Lama1 will be investigated here. Megalin is first expressed in PrE and is also expressed in VE (for further information on this marker see section 4.1.1 of previous chapter).

Gata6 is a member of the Gata factors which regulates PrE (Capo-Chichi *et al*. 2005); they also play a role in the regulation of key regulators of both extraembryonic and definitive endoderm differentiation. Gata6 is a key

regulator of endoderm differentiation that is often expressed within cells. Gata6-null embryos are unable to regulate PrE differentiation and therefore lack the cell lineage.

Laminin-111 is the first major laminin expressed during the peri-implantation period (Shim *et al*., 1996; Smyth *et al*., 1999) and is essential for basement membrane formation and early embryogenesis (Huang *et al*., 2003; Urbano *et al*., 2009). It is classically described as a cross-shaped molecule comprising α1,β1 and Y1 chains and its structure is detailed earlier in 1.1.5. The α subunits initiate cell-surface adhesion via receptors and also plays a role within the self-assembly of the BM. The Laminin alpha chain appears to be the rate-limiting step in BM assembly, because in the absence of this chain, the beta and gamma chains accumulate in the cell and are not secreted (Miner and Yurchenco, 2004).

5.1.3 Expression of the mesodermal marker, brachyury

Onset of expression of Brachyur*y* (T) marks the specification of intermediate and axial mesoderm at the time of gastrulation (Wilkinson *et al*.,1990; Kispert and Herrmann 1994) and normal differentiation has been identified in WT EBs. mESC lacking the EXT1 gene have been shown to be incapable of producing mesoderm (Kraushaar *et al*., 2012). Conflicting work has identified the presence nascent mesoderm within EXT-null EBs (Holley *et al*., 2011).

5.1.4 Expression of the ectodermal marker, Pax6

Evidence of neuroectodermal development can be identified by the presence of the Pax transcription factors, in particular Pax6. Neuronal differentiation seems to be directly affected by a lack of HS *in vitro* owing to a lack of FGF signalling needed for neurogenesis (Coumoul *et al*., 2003, Partanen *et al*., 2007). As a result, Pax6 was used to investigate any indication of neuroectodermal differentiation within the EB populations.

5.2 Objectives

In the previous chapter, immunofluorescence analysis suggested that there was little evidence of spontaneous differentiation in EXT1-/- ESCs. Analysis of EBs confirmed that EEE cells had not differentiated at the periphery of the EXT1-/- EBs, but that inner cells appeared to have differentiated as shown by a lack of Oct4+ and nanog+ cells. The objectives of this chapter were as follows:

1. Confirm that EXT1-/- mRNA is not expressed in the EXT1-/- ESCs and EBs using qualitative RT-PCR.
2. Using qPCR, determine the expression levels of the pluripotency and lineage markers indicated above in EXT1-/- ESCs relative to WT ESCs in order to confirm whether there is less spontaneous differentiation in EXT1-/- ESCs
3. Using qPCR, determine the expression levels of the pluripotency markers, Oct4 and nanog in EXT1-/- day 4 EBs relative to WT EBs in order to confirm whether more differentiation has taken place in the EXT1-/- EBs.
4. Using qPCR, determine the levels of the lineage markers indicated above in EXT1-/- day 4 EBs relative to WT EBs in order to confirm that mRNA levels of PrE markers are lower in the EXT1-/- EBs, and to investigate if there is an up-regulation in the mesodermal and/or ectodermal markers.

5.3 Results

5.3.1.1 Confirmation of absence of *Ext1* expression in Ext1-/- ESCs using RT-PCR.

The conclusions of this study rely on the depletion of HS in the EXT1-/- mESC. Immunofluorescence showed that after passaging on gelatine there was no HS expressed on the EXT1-/- mESC. In order to confirm this RT-PCR was carried out on WT mESC and EXT1-/- mESCs. Using a real-time PCR instrument, it was found that no amplification could be detected when the EXT1-/- ESCs were used as template. PCR products were analysed using 2% ethidium bromide gel electrophoresis to confirm absence of *EXT1* within the EXT1-/- ESCs. No Ext1 band (153bp) was detected in the Ext1-/- sample whereas a clear band can be seen for the WT sample. RT-PCR for Gapdh (102bp) shows an equal amount of template from the wild-type and Ext1-/- samples was used (Fig.21). This result confirms the phenotype of the EXT1-/- ESCs.

5.3.1.2 Confirmation that the PCR products of the analysed genes are of the expected size.

In order to allow accurate analysis of the relative gene expression within the samples it was important to validate the PCR primers and confirm that the products of the analysed gene were of the expected size. cDNA for WT D4 EBs was used as the positive control. Using a real-time PCR instrument, it was found during the amplification stage of the reaction, all primer pairs produced exponential curves, and melting point analysis performed at the end of the cycling stage showed that all primer pairs produced a single melting peak with no evidence of primer dimers. Gel electrophoresis showed that the bands obtained with each primer pair were of the expected size (Fig. 22).

Fig 21. Confirmation of absence of *Ext1* expression in Ext1-/- ESCs using RT-PCR. PCR products were analysed using 2% ethidium bromide gel electrophoresis. No Ext1 band (153bp) was detected in the Ext1-/- sample. RT-PCR for Gapdh (102bp) shows an equal amount of template from the wild type and Ext1-/- samples was used.

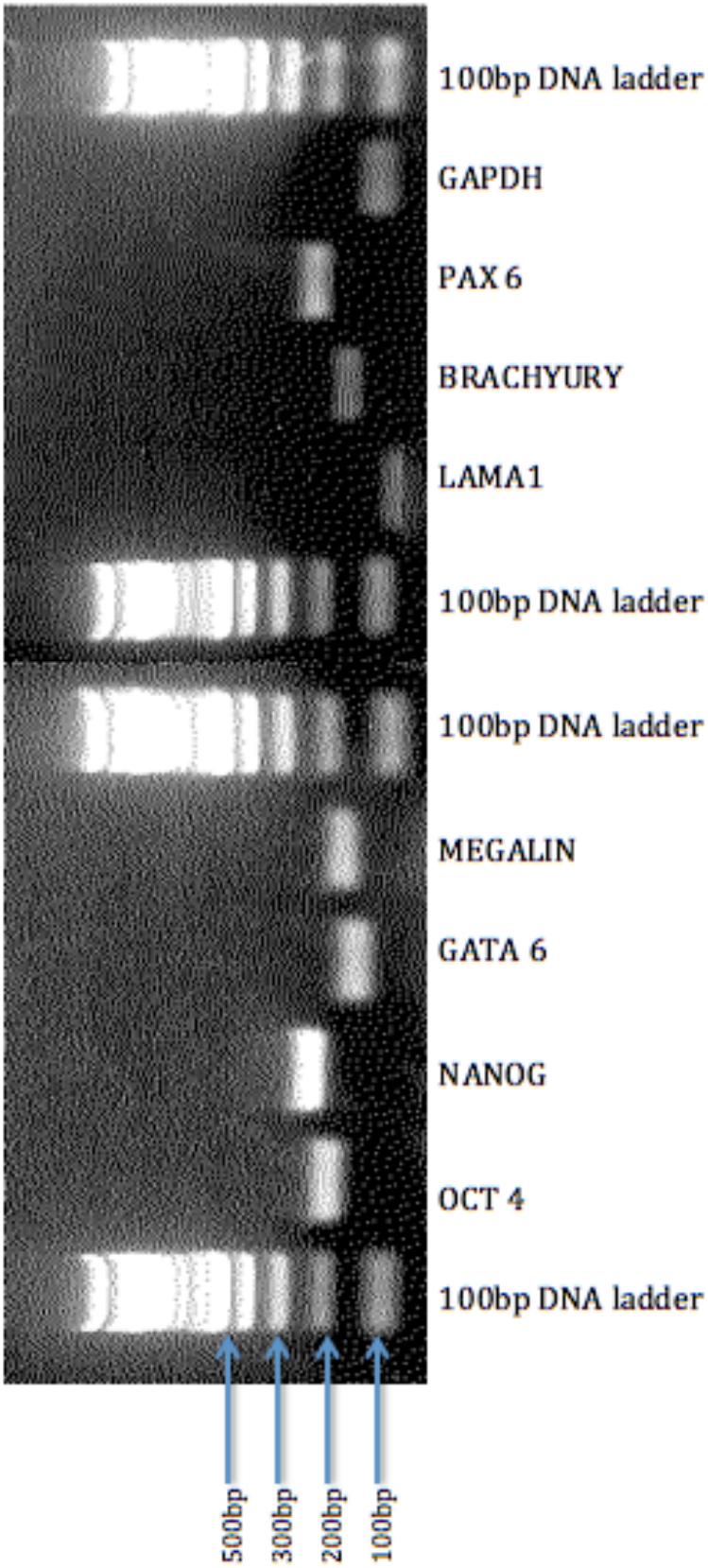

Fig 22. Confirmation that the PCR products of the analysed genes are of the expected size. PCR products were analysed using 2% ethidium bromide gel electrophoresis. All products were of the expected size; i.e., Oct4, 188 bp; Nanog, 232 bp, Gata6, 151bp, Megalin, 171bp; Lama1, 60bp; Brachyury, 136bp; Pax6, 201bp; Gapdh, 102bp.

5.3.2 Relative gene expression in EXT1-/- mESC and EBs

RT qPCR was carried out on cDNA extracted from 3 biological replicates of the WT and EXT1-/- mESCs samples each consisting of cDNA from mESC and D4 EBs. The housekeeping gene used was glyceraldehyde 3-phosphate dehydrogenase (GAPDH); this has been validated for its use for ESC derived gene transcription studies (Murphy and Polak. 2002). Two technical replicates were carried on each gene of interest in each reaction series.

The delta-delta method for calculating the change in gene expression in the embryoid bodies was used, whereby WT GAPDH acted as the reference. The average of three biological replicates was taken and plotted.

5.3.3 mESC

5.3.3.1 Relative expression of pluripotency markers in mESC

Oct4 and Nanog primers were used to quantify the pluripotency gene expression in the WT and EXT1-/- mESC.

Expression levels of *Oct4* and *Nanog* were higher in the Ext1-/- mESC compared to the WT ESCs, but the difference was not statistically significant ($p>0.05$, Student t-test; n=3) (Fig. 23). Oct4 demonstrated an average 27 fold increase in expression within the EXT1-/- meanwhile Nanog showed a 321 fold increase. A large standard error exists for these results with gene expression showing great variation within the sample. However it can be said that pluripotency markers were consistently higher within the EXT1-/- mESC, which would be consistent with the fact that there appeared to be less spontaneous differentiation in the EXT1-/- ESCs.

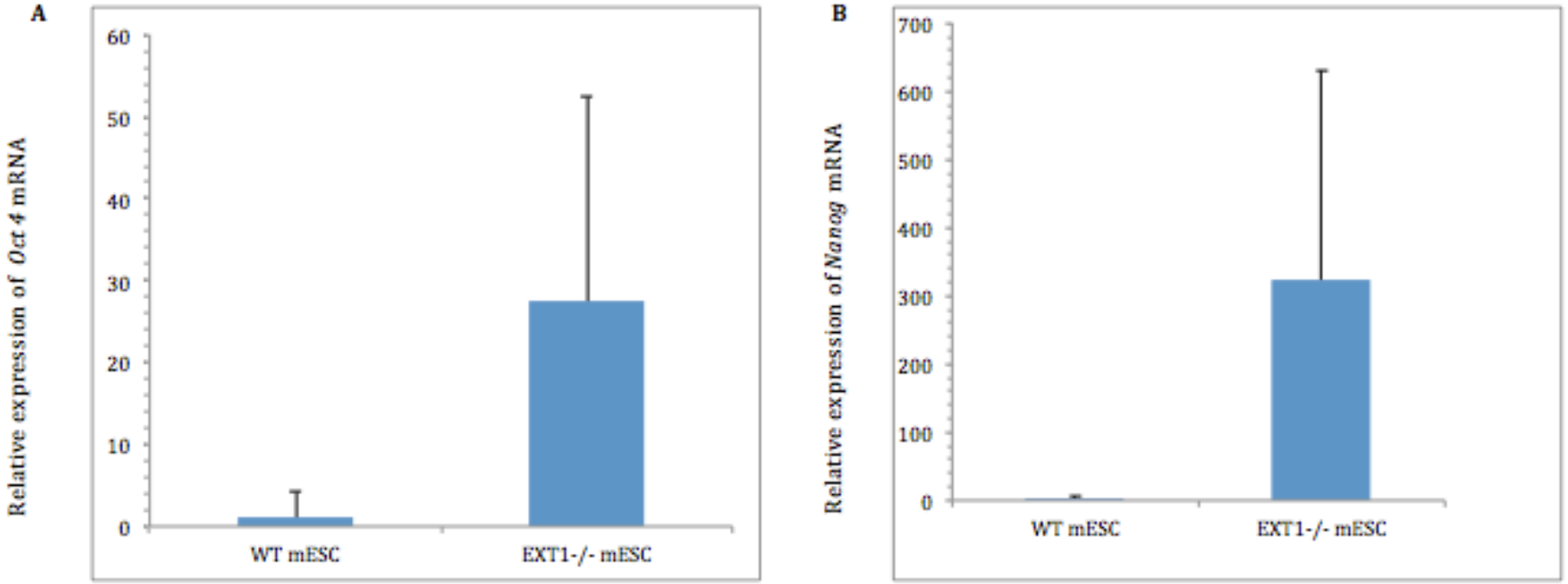

Fig.23. The relative expression of pluripotency markers in WT mESC and EXT1-/- mESC. Expression of pluripotency markers, *Oct4* and *Nanog*, in WT and Ext1-/- mESC. Real-time quantitative RT-PCR was performed on WT and Ext1-/- cells cultured for 2 passages in the absence of STO feeder cells. The reference gene was *Gapdh*. Expression levels of *Oct4* (A) and *Nanog* (B) were higher in the Ext1-/- mESC compared to the WT sample, but the difference was not statistically significant ($p>0.05$, Student t-test; $n=3$). Error bars represent standard error of the mean.

5.3.3.2 Relative expression of endoderm markers in mESC

qPCR showed higher expression levels of megalin and Lama1 in the WT ESCs compare with EXT1-/- ESCs, which is consistent with the immunofluorescence data obtained in chapter 2, and shows that in the WT ESCs, the extent of spontaneous differentiation to EEE is higher than in EXT1-/- ESCs (Fig.24). However, the difference was not statistically significant, which is likely due to the high degree of variation between samples ($p>0.05$, Student t-test; n=3). Expression levels of Gata6 were also investigated because it is known that this is an early marker of PrE differentiation, and that Gata6 expression is required for PrE differentiation (Cai *et al*., 2008). Similarly to the other PrE markers, Gata6 mRNA levels were higher in WT ESCs, but again, the increase was not significantly higher than in EXT-/- ESCs (Fig.25).

5.3.3.3 Relative expression of neuroectoderm and mesoderm markers in mESC

A similar picture was seen for the neuroectoderm marker Pax6 indicating that expression in EXT1-/- mESC is relatively decreased compared with WT mESC. Once again this was not statistically significant (Fig.26).
There was not sufficient data to analyse the expression of Brachyury and so quantify the mesoderm differentiation within the mESC. These missing data are due to the samples not reaching the threshold value in the RT-qPCR which in itself is a finding as it implies that there was very little RNA present in the samples at the time of fixation, which is expected as at this point in time the cells should be undifferentiated.

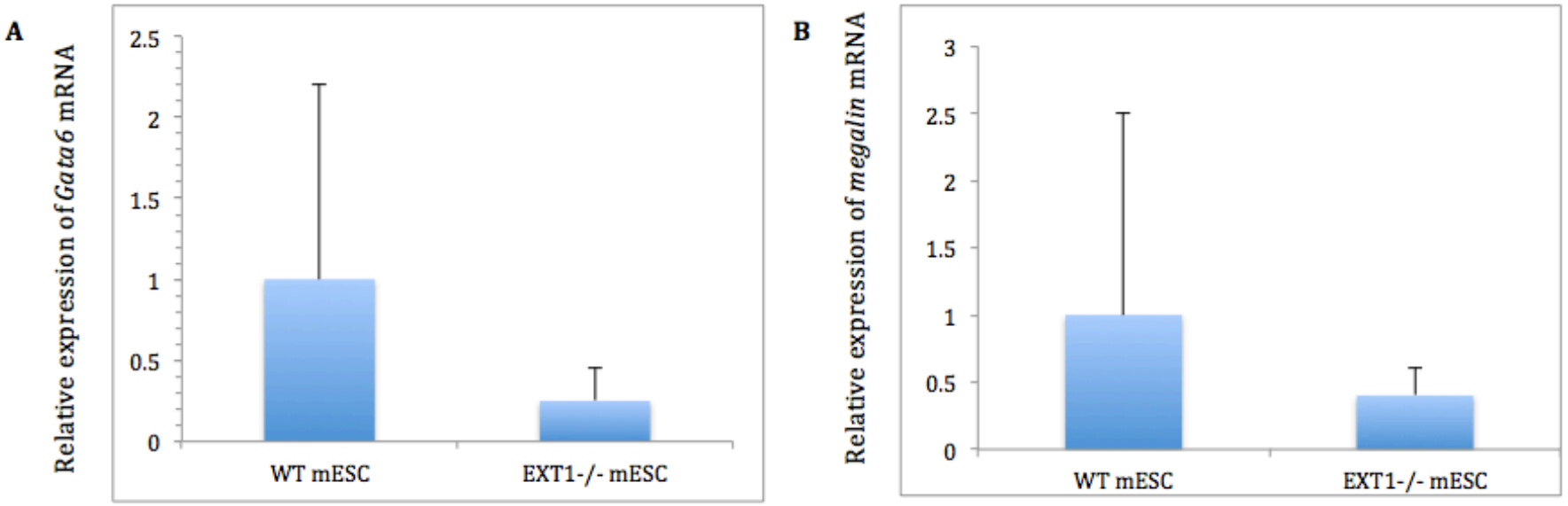

Fig. 24. Expression of primitive endoderm lineage markers, *Gata6* and *megalin*, in WT and Ext1-/- mESC. Real-time quantitative RT-PCR was performed on WT and Ext1-/- cells cultured for 2 passages in the absence of STO feeder cells. The reference gene was *Gapdh*. Expression levels of *Gata6* (A) and *megalin* (B) were lower in the Ext1-/- mESC compared to the WT sample, but the difference was not statistically significant ($p>0.05$, Student t-test; $n=3$). Error bars represent standard error of the mean.

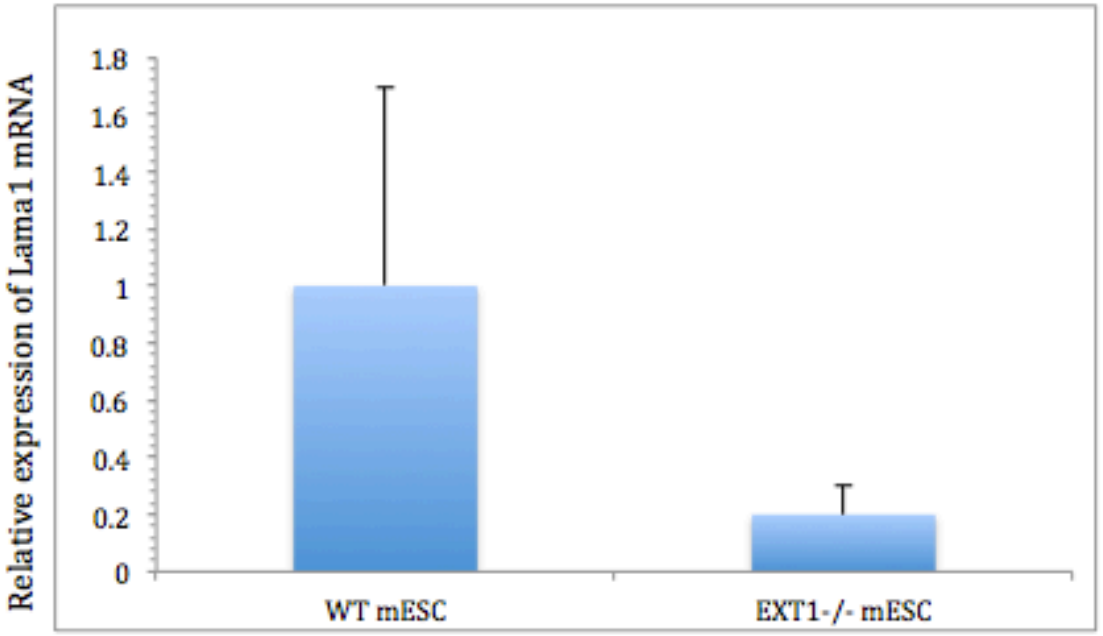

Fig. 25. Expression of *Lama1* in WT and EXT1-/- mESC. Real-time quantitative RT-PCR was performed on WT and Ext1-/- cells cultured for 2 passages in the absence of STO feeder cells. The reference gene was *Gapdh*. Expression levels were lower in the Ext1-/- mESC compared to the WT sample, but the difference was not statistically significant ($p>0.05$, Student t-test; $n=3$). Error bars represent standard error of the mean.

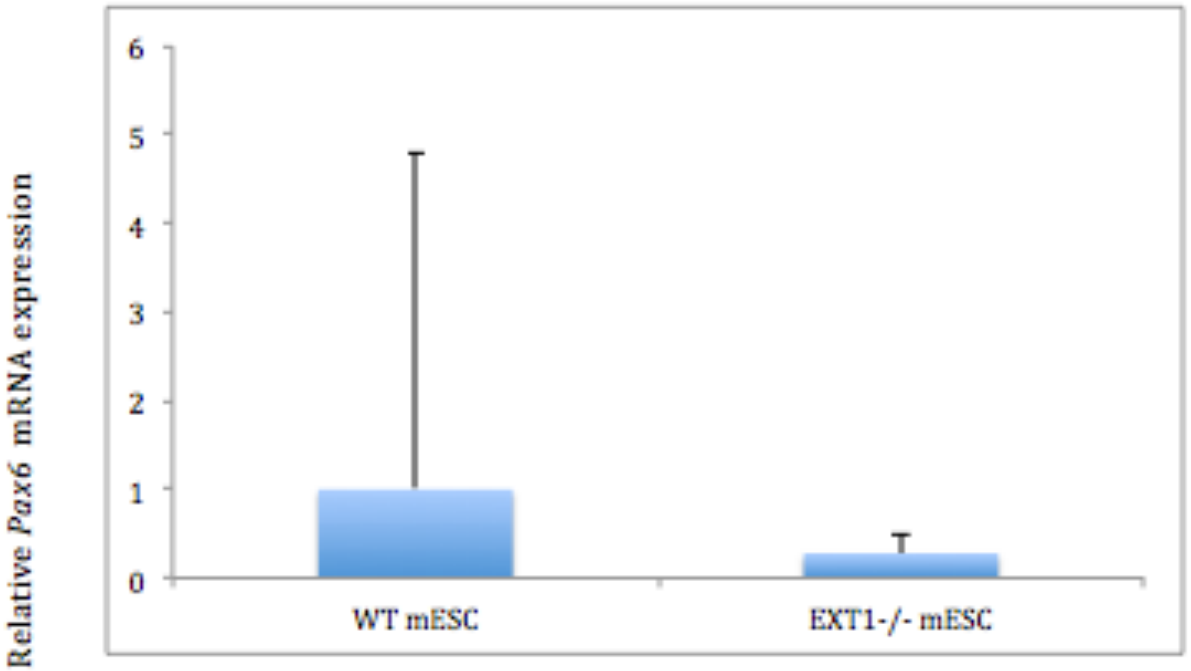

Fig. 26. The relative expression of neuroectoderm lineage markers in WT mESC and EXT1-/- mESC. Real-time quantitative RT-PCR was performed on WT and Ext1-/- cells cultured for 2 passages in the absence of STO feeder cells. The reference gene was *Gapdh*. Expression levels of *Pax6* were lower in the Ext1-/- mESC compared to the WT sample, but the difference was not statistically significant ($p>0.05$, Student t-test; $n=3$). Error bars represent standard error of the mean.

5.3.4 Relative expression in D4 EBs

5.3.4.1 Relative expression of pluripotency markers in EBs

Expression of Oct4 in EXT1-/- EBs was relatively decreased compared with WT EBs. This result is consistent with the findings from the immunofluorescence that indicated very little Oct4 was present in the EXT1-/- EBs by day 4. The results were however not statistically significant. Nanog on the other hand was consistently expressed at a higher level within the EXT1-/- EBs (Fig.27). This was surprising because immunofluorescence did not show any Nanog positive staining for the EBs as D4. This result will be discussed later.

5.3.4.2 Relative expression of endoderm markers in EBs

The expression levels of the extra-embryonic endoderm markers was increased in the WT EBs as was expected. This is seen in Fig. 28 indicating the increased expression levels of *Gata6* and *megalin*. Although consistently higher, the difference was not statistically significant ($p>0.05$, Student t-test; n=3). The increased expression of Megalin supports the immunofluorescence findings from the previous experiment.

LamA1 expression levels were lower in the Ext1-/- EBs compared with the WT sample (Fig. 29). This is consistent with the morphological analysis of the frozen EB sections and also the immunofluorescence carried out using Laminin-111 antibody that showed a lack of BM within the mutant population.

5.3.4.3 Relative expression of neuroectoderm and mesoderm in EBs

The expression levels of Pax 6 were higher in the Day4 WT EBs suggesting the formation of neuroectoderm within the embryoid bodies (Fig.30).

An interesting result concerned that of the expression of Brachyury. RT-PCR indicated that mesoderm differentiation was occurring in the EXT1-/- EBs and expression levels were higher in the Ext1-/- EBs compared to the WT EBs (Fig.31). Once again this was not statistically significant ($p>0.05$, Student t-test; n=3), but due to its consistent increased expression suggests that despite the lack of HS mesoderm differentiation can occur.

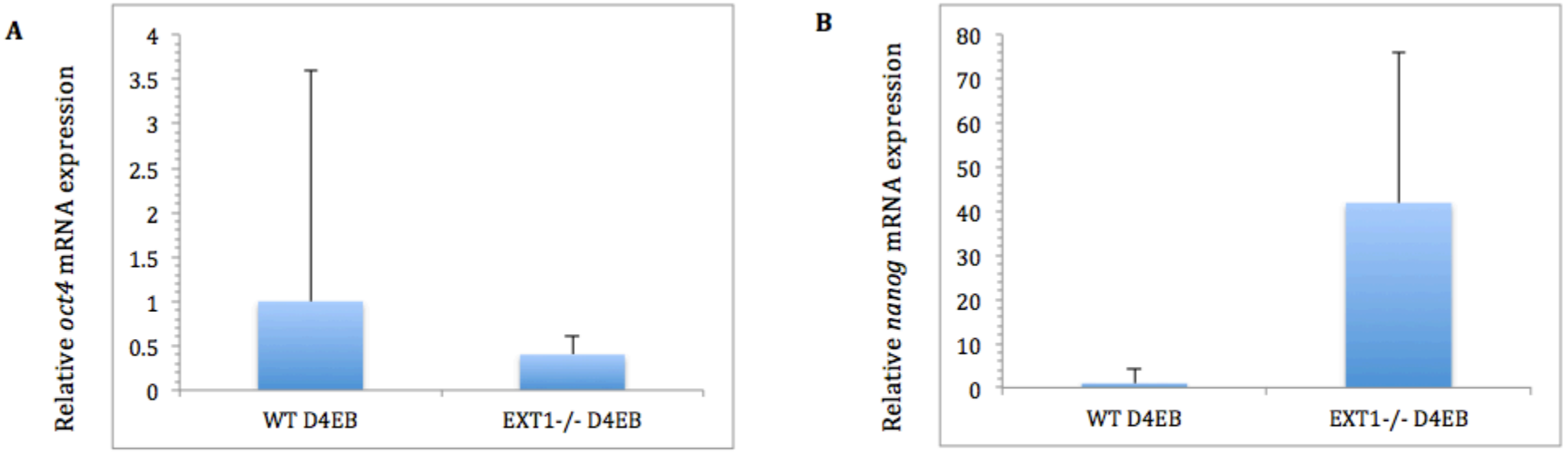

FIG.27. Expression of pluripotency markers, *Oct4* and *nanog*, in WT and Ext1-/- Day 4 EBs. Real-time quantitative RT-PCR was performed on WT and Ext1-/- cells cultured for 2 passages in the absence of STO feeder cells. The reference gene was *Gapdh*. Expression levels of *Oct4* (A) and *nanog* (B) were higher in the Ext1-/- mESC compared to the WT sample, but the difference was not statistically sigificant ($p>0.05$, Student t-test; $n=3$). Error bars represent standard error of the mean.

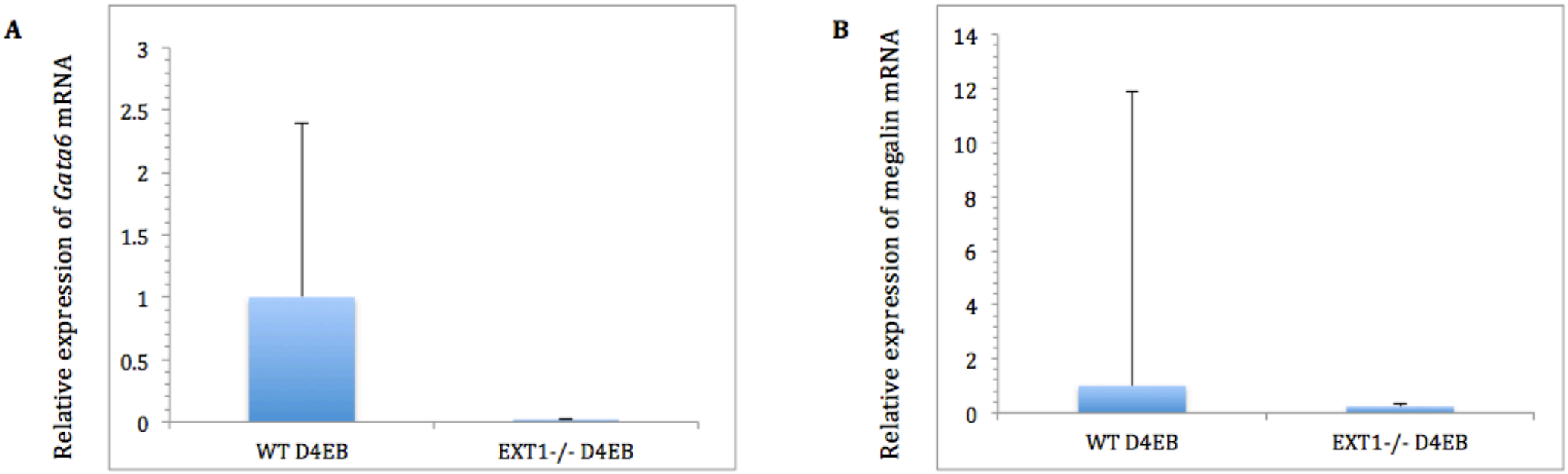

Fig. 28. Expression of primitive endoderm lineage markers, *Gata6* and *megalin*, in WT and Ext1-/ day 4 EBs. Real-time quantitative RT-PCR was performed on WT and Ext1-/- day 4 EBs. The reference gene was *Gapdh*. Expression levels of *Gata6* (A) and *megalin* (B) were lower in the Ext1-/- EBs compared to the WT sample, but the difference was not statistically significant ($p>0.05$, Student t-test; n=3). Error bars represent standard error of the mean.

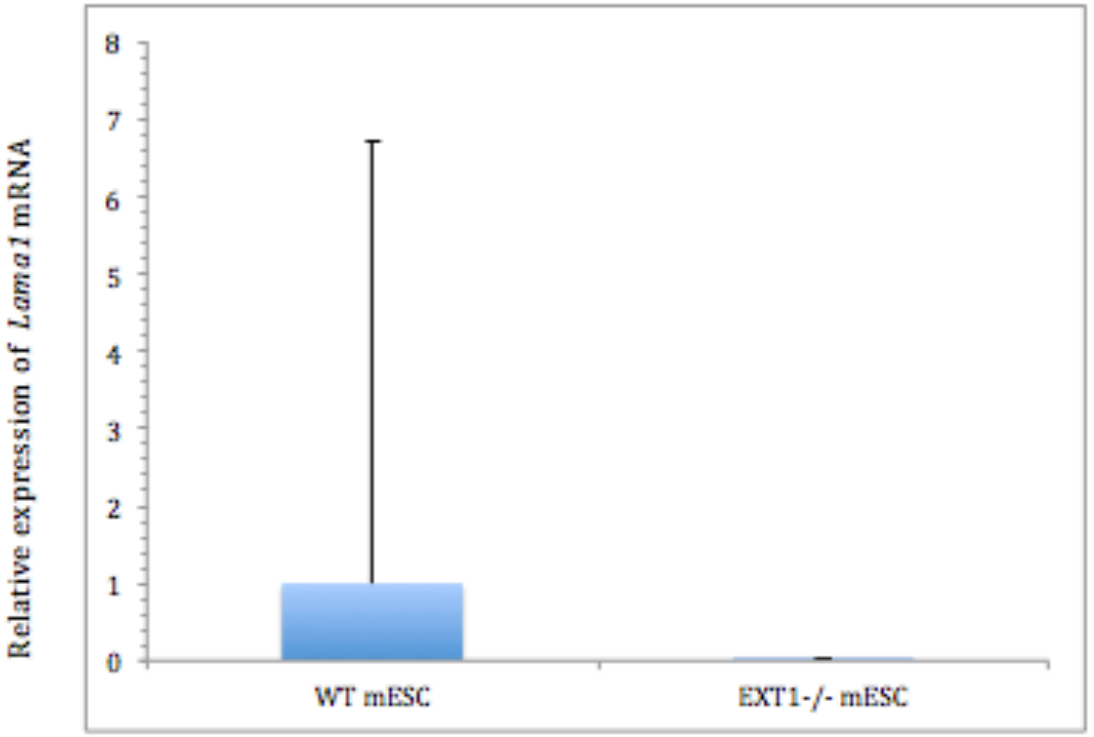

Fig. 29 .Expression of *Lama1* in WT and EXT1-/- day 4 EBs. Real-time quantitative RT-PCR was performed on WT and Ext1-/- day 4 EBs. The reference gene was *Gapdh*. Expression levels were lower in the Ext1-/- EBs compared to the WT EBs, but the difference was not statistically significant ($p>0.05$, Student t-test; $n=3$). Error bars represent standard error of the mean.

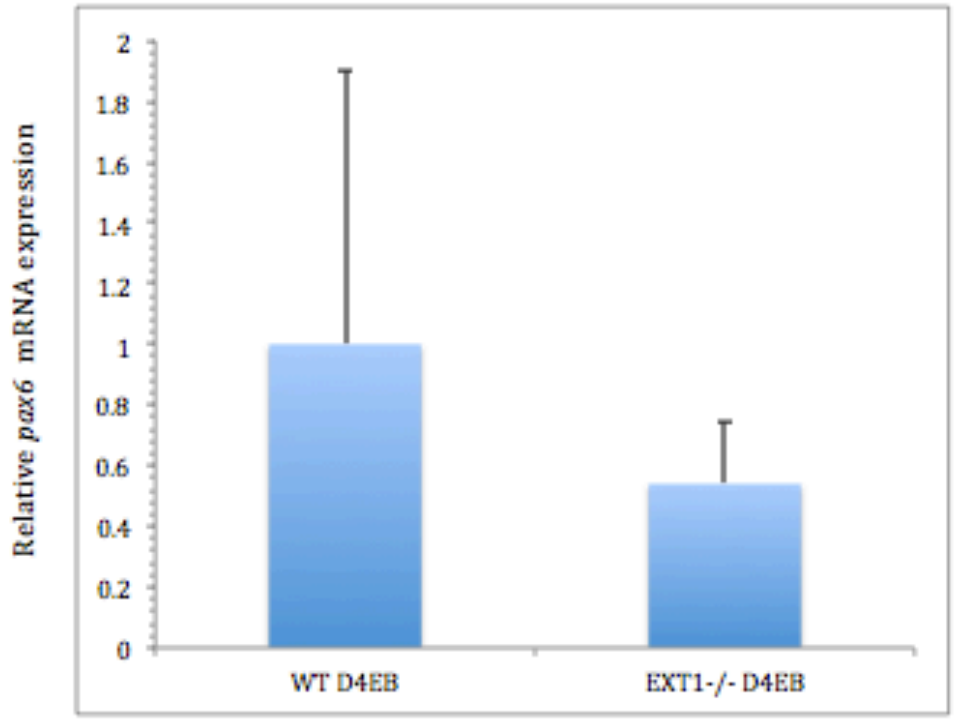

Fig. 30. Expression of neuroectoderm lineage marker, *Pax6*, in WT and EXT1-/- day 4 EBs. Real-time quantitative RT-PCR was performed on WT and Ext1-/- day 4 EBs. The reference gene was *Gapdh*. Expression levels were lower in the Ext1-/- EBs compared to the WT EBs, but the difference was not statistically significant ($p>0.05$, Student t-test; $n=3$). Error bars represent standard error of the mean.

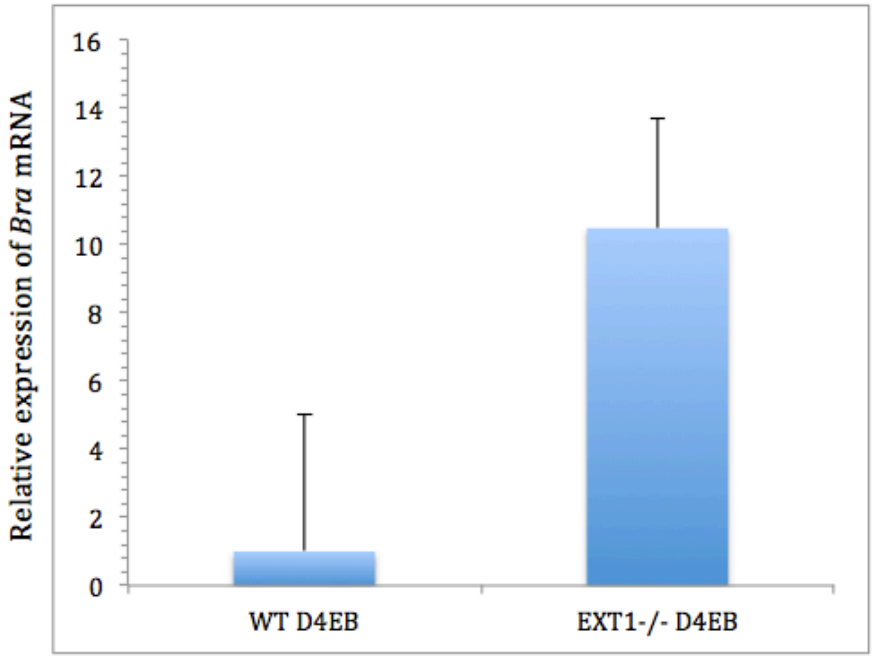

Fig. 31. Expression of mesodermal lineage marker, *Brachyury*, in WT and EXT1-/- day 4 EBs. Real-time quantitative RT-PCR was performed on WT and Ext1-/- day 4 EBs. The reference gene was *Gapdh*. Expression levels were higher in the Ext1-/- EBs compared to the WT EBs, but the difference was not statistically significant ($p>0.05$, Student t-test; $n=3$). Error bars represent standard error of the mean.

5.4 Discussion

Although there was a clear trend in the results for Oct4 and nanog to be upregulated in EXT1-/- ESCs and all lineage markers to be downregulated, the results were not significant. This is likely due to the fact there was a great deal of variation between the biological replicates. It is important to note that when comparing Ext1-/- and WT results within each single replicate, it was almost always the case that the results followed the above trend, but surprisingly, there were large variations between the replicates, with the 2nd replicate displaying most variation. For instance, regarding Oct4, the difference in Ct values between Oct4 and Gapdh for the 1st biological replicate was 4.8 (WT) and 2.7 (Ext1-/-); for the 2nd biological replicate was 12.9 (WT) and 6.7 (Ext1-/-); and for the 3rd biological replicate was 3.5 (WT) and 3.4 (Ext1-/-). It is possible that these differences might have been due to variability in the quality of the STO feeders layers used between replicates. Interestingly, on analysing the raw CT data, it was found that as well as having lower levels of Oct4 and nanog, the ESCs from the 2nd biological replicate also had lower expression levels of all lineage markers except for Pax6, which was upregulated. For instance, the difference in Ct values between Pax6 and Gapdh for the 1st replicate was 9.3 (WT) and 11.2 (Ext1-/-); for the 2nd replicate was 4.6 (WT) and 8.2 (Ext1-/-); and for the 3rd replicate was 10.5 (WT) and 11.5 (Ext1-/-). This suggests that there was a higher degree of spontaneous differentiation to the ectoderm lineage in the 2nd biological replicate.

A similar situation was seen with the EB data, with the same trend in marker expression being observed when comparing WT and Ext1-/- results within a single replicate, but there being quite a bit of variability between replicates. For instance, for the interesting result relating to the upregulation of brachyury in the Ext1-/- EBs, the difference in Ct values between Brachyury and Gapdh for the 1st replicate was 9.7 (WT) and 5.7 (Ext1-/-); for the 2nd replicate was 10.7 (WT) and 8.3 (Ext1-/-); and for the 3rd replicate was 15.5 (WT) and 12.2 (Ext1-/-).

Although the results were not significant, there was tendency for the PrE markers to be expressed at lower levels in the Ext1-/- EBs, which was consistent with the immunofluorescence data obtained in chapter 4. As discussed in chapter 4, it is likely that this is due to insufficient FGF signalling caused by a lack of HS in the Ext1-/- cells (Kraushaar *et al*., 2012). Limited work has been conducted on HS-deficient EBs. Lin *et al*. utilized EXT1-/- EBs and concluded that a lack of HS leads to the impairment of EEE at days 3 and 5 culture and remained low after day 7. The endodermal marker Transferrin was detected in both the wild-type and homozygous mutant EBs at day 5, however remained at a low level at later stages in EXT1-/- clones (Lin *et al*., 2000). It is important to note however that the results from this study are not very robust due to the quality of the EBs cultured. It can be noted that of the EBs pictured within the paper, the labeling of Visceral Endoderm (VE) on the WT EBs is not accurate and suggests that they had not developed the expected phenotype of an Embryoid Body. EBs were also analysed up to Day 12, at which point development becomes very chaotic and is not representative of early embryonic development. EBs developed form the EXT1-/- mESC also demonstrate a thick BM characteristic of Reichert's membrane. As previously discussed it seems that HS is essential for the deposition of a BM owing to the presence of endodermal cells and so secretion of Lama1. The presence of a Reichert-like structure within Lin *et al*s. EBs suggests that the model is not truly HS-deficient. Work within our laboratory has indicated that plating density ($75x10^3$ cells/ml) and serum batch is crucial for getting the EBs to develop like the early embryo and the morphology of the WT EBs within this study represented normal development

An interesting result was the increased expression levels of brachyury in the Ext1-/- EBs compared with the WT EBs, which suggested that the Ext1-/- cells within the EBs had a tendency to differentiate towards the mesoderm lineage. This result is surprising because it is known that in the mouse embryo, FGF signalling is required for gastrulation (Schulte-Merker *et al*., 1995; Ciruna *et al*., 2001) (differentiation of nascent mesoderm) as well as for PrE differentiation, so it might have been expected that if a deficiency in HS prevented the FGF signalling required for PrE differentiation, it might also

have prevented the FGF signalling required for mesoderm differentiation. Kraushaar *et al*. reported that Brachyury (T) failed to be up-regulated within EXT1-/- mESCs, failing to form mesoderm cells. The addition of heparin fully restored T expression and demonstrated the need for HS for mESCs to successfully differentiate into a mesoderm lineage. However, the methods carried out by Kraushaar *et al*. are not suitable for examining differentiation of ESCs that normally occur within embryonic development. The mutant mESCs are examined as a 2D monolayer and do not represent the 3D nature of the embryo. Within our results it is not clear if the brachyury-expressing 'mesoderm' cells within Ext1-/- EBs are equivalent to the mesoderm cells that differentiate in WT EBs, and a thorough characterisation of their phenotype would be required. Nevertheless, the results obtained in this chapter suggest that in contrast to WT EBs, which at day4, show extensive extra-embryonic endoderm differentiation and little mesoderm differentiation, Ext1-/- EBs show no evidence of EEE differentiation, but a high degree of mesoderm differentiation. A recent paper has shown that the transcription factors, Klf4 and Klf5 inhibit the differentiation of extra-embryonic endoderm and mesoderm, respectively, and that down-regulation of Klf5 enhances mesoderm differentiation. It would therefore be interesting to investigate the expression levels of these two molecules in the WT and Ext1-/- ESCs and EBs (Aksoy *et al*., 2014).

Another surprising result was the up-regulation of nanog mRNA in the Ext1-/- EBs, despite there being no nanog+ cells detected using immunostaining (see previous chapter). A recent paper has reported that in colorectal cancer cells, and other cell lines, brachyury induces nanog expression, and this is associated with epithelial to mesenchymal transition (Sarkar *et al*., 2012). It is therefore possible that in the early stages of EB development, nanog is switched off in the Ext1-/- EBs, but following the induction of brachyury expression, is turned on again in 'mesodermal' cells. This could be tested by performing qPCR analysis on day 2 EBs, and undertaking dual immunostaining with antibodies to detect brachyury and nanog. The expression of nanog by mesodermal cells is not observed in the developing embryo, and instead, nanog is down-regulated in the primitive ectoderm prior

to gastrulation. Therefore, if it transpired that mesodermal cells within Ext1-/- EBs did express nanog, then it would suggest that these mesodermal cells might have an abnormal phenotype.

The final investigation carried out was to determine the role of Heparan Sulphate in ectodermal differentiation. As with the other germ cell lineages, it was hypothesized that the FGF signaling regulating ectodermal differentiation would be disrupted due to the absence of HS (Ying *et al*., 2003). Previous work indicates that FGFR1 is expressed throughout WT mESCs (Walshe and Mason 2000; Blak *et al*. 2005; Trokovic *et al*. 2005) and the specific inactivation of FGF8 leads to cell death within the early neuroectodermal cells (McMahon *et al*. 1992; Chi *et al*. 2003). Our results presented a decreased expression of Pax6 within the Ext1-/- EBs in comparison to the WT population. This demonstrates that HS is vital for the differentiation of ectoderm within mESCs and is an essential component of normal gastrulation.

6. General Discussion

Analysis of cellular interactions during the early stages of development can prove challenging due to the inaccessibility of peri-implantation embryos and the difficulty of isolating cells *in vivo*. The development of *in vitro* EB formation has now facilitated the investigation of many aspects of cell differentiation occurring during early mammalian embryogenesis.

Heparan sulphate (HS) is a complex sulphated polysaccharide that occurs as a proteoglycan (HSPG). It is expressed at the cell surface on all animal cells and acts as an essential co-receptor for many proteins such as growth factors that regulate growth and differentiation. The importance of HS in early embryonic development has been clearly highlighted in a number of studies that demonstrate the lethality of HS-deficient embryos (Lin et al., 2000; Stickens et al., 2005). The targeted disruption of the EXT1 gene results in HS-deficient mESC and have been utilised to study the effects of HS on differentiation in multiple studies. mESCs that are HS-deficient are capable of forming EBs but these EBs have yet to be fully characterised; it is still undetermined if they develop in a similar way to EBs generated from WT cells (Lin et al. 2000). The aim of this study was to investigate the role of HS in the development of mouse EBs by comparing the development of EBs derived from WT and EXT1-/- mESCs.

Results within this study suggest:

- EXT1-/- ESCs show less spontaneous differentiation as shown by increased Oct4 and nanog and lower levels of lineage markers

- Inner cells within Ext1-/- EBs differentiate more quickly than those within WT EBs, as shown by immunofluorescence for Oct4 and nanog

- HS is needed for BM synthesis
    - EXT1-/- EBs did not form BMs, with immunostaining for laminin-111 showing only weak intracellular staining in peripheral cells of day 4 EBs, and qPCR showing very low levels of Lama1 mRNA compared with WT EBs.

- A lack of HS results in decreased differentiation of Endodermal and Ectodermal cell lineages
    - EXT1-/- EBs failed to produce EEE at Day 4, based on morphological criteria and absence of immunostaining for megalin, and qPCR showed lower levels of endodermal markers
    - EXT1-/- EBs expressed lower levels of neuroectodermal marker Pax6, as shown by qPCR.

- A lack of HS causes the up-regulation of mesodermal markers
    - EXT1-/- EBs expressed higher levels of Brachyury mRNA at compared to the WT, as shown by qPCR.

Previous work undertaken by Lin et al. had attempted to characterise EXT1-/- EBs as discussed throughout this work (Lin et al. 2000). However within that paper EBs had been cultured directly from mESCs cultured on feeder cells. Work undertaken in this study demonstrated that exogenous HS is readily transferred to the HS-null cells hence masking their true phenotype. We noted that passage of mESCs on gelatine prior to EB formation successfully

depleted STO feeder cells and resulted in the development of EBs that were HS-deficient.

This study supports the idea that HS is required for the differentiation of EEE in mESC-derived EBs. WT EBs displayed normal EB development with the appearance of EEE on the periphery of the aggregates at Day4. In contrast morphological analysis of the EXT1-/- population suggested a complete lack of EEE. To confirm these results immunostaining for Megalin was carried out. Positive staining for megalin confirmed the presence of EEE within the WT EBs with no expression highlighted within the mutant EBs. Final qPCR experiments indicated that Gata6, Megalin and Lama1 were all decreased within the HS-null mESC and the derived EBs. From these separate experiments it is clear that endodermal differentiation was negatively affected by the lack of HS present in the cells.

The role of EEE in the synthesis of BMs in the early mouse embryo has been highlighted in many existing studies and discussed throughout this thesis. Our results indicated that there was no evidence of BMs in the Ext1-/- EBs, which probably resulted from the lack of EEE differentiation. Immunostaining for Laminin-111 showed some increased staining in the peripheral cells of Ext1-/- EBs, but qPCR showed lower expression levels of Lama1 mRNA. Lama1 is the rate-limiting step in the production of Laminin-111 (Miner and Yurchenco et al., 2004), the main component of the BM and is required for the assembly and secretion of the laminin trimer. The reduced levels of Lama1 in the Ext1-/- EBs, is the likely explanation for the intracellular staining observed in the peripheral cells of Ext1-/- EBs.

A surprising result was that EXT1-/- EBs had a tendency to differentiate towards the mesoderm lineage. Although not statistically significant, an absence of HS resulted in an increased expression of Brachyury. This was unexpected because it has been reported that Ext1-/- embryos fail to gastrulate normally (Lin et al., 2000), and it is known that HS is required for FGF signalling, and that mesoderm differentiation in the embryo appears to require FGFs.

A final result indicated that neuroectoderm differentiation also relies on the presence of HS. Although only qPCR was carried out to determine ectodermal differentiation, a decreased expression of Pax6 was observed within the Ext1-/- EBs. This is not surprising as previous FACS analysis has revealed a significant increase in HS as cells commit to a neural lineage (Johnson et al., 2007) and the EXT1 gene is expressed throughout the neural tube at E9.5 within mice (Siekmann *et al.,2004*). Work on the embryonic mouse brain indicates that the disruption of EXT1 causes numerous patterning defects and suggests a pivotal role of HS in midline axon guidance (Inatani et al., 2003). The phenotype of these embryos suggests that *Fgf8* signalling is probably the most critically disrupted pathway that leads to these abnormalities.

It is well documented that Gata6 acts downstream of the Fibroblast Growth Factors (FGF) signalling pathway in order to regulate endoderm differentiation (Li et al., 2004; Ralston et al., 2005). The disruption of FGF signalling and its effects upon Gata6 expression have been shown to have a detrimental effect upon normal embryonic development. A lack of Gata6 can lead to abnormal gastrulation (Goldin and Papaioannou., 2003) and the complete disintegration of the egg cylinder due to a lack of VE differentiation (Arman et al., 1998). Successful FGF signaling in vivo relies on the presence of HSPGs as they function via a co-receptor system (Lin et al., 1999). Considering current papers investigating the relationship between HS and Endoderm it can be suggested from our results that an absence of endoderm differentiation is due to the disruption of FGF signaling.

Similarly to the differentiation of endoderm and the synthesis of BMs, the specification and patterning of mesodermal differentiation is also regulated by FGF signaling. The epithelial to mesenchymal transition (EMT) is directly influenced by the expression of Snail which down-regulates E-cadherin and works downstream of FGFs. Due to these factors it was hypothesized that mesodermal differentiation would also be decreased within the EXT1-/- EBs, but instead, an upregulation was observed. Our findings could be associated with Krüppel like factors (Klfs) which are zinc-finger containing transcription factors associated with the development and differentiation of the embryo

(Pearson et al.,2008). Recent work by Aksoy et al. identified the potential role of Krüppel like factors 4 (Klf4) and 5 (Klf5) in the development of endoderm and mesoderm (Aksoy et al., 2014). Klf4 and Klf5 have a similar distribution within embryonic tissue and have an antagonistic relationship that controls gene expression. The expression of both these factors is regulated by the LIF/STAT3 pathway. Original work noted their importance in the maintenance of pluripotency whereby there is high expression of Klfs within mESC that decreases after differentiation (Bourillot et al., 2010). Klf4 is also one of the original four factors shown to reprogram somatic cells to a pluripotent state (Takahashi and Yamanaka, 2006). The overexpression of these factors increases the self-renewal of mESC thus delaying the differentiation of the cells that is typically induced by the culture of EBs (Ema et al., 2008). Conversely the functional inactivation of Klfs induces spontaneous differentiation (Bourillot et al., 2010). More recently Aksoy et al. noted the specific potential roles of Klf4 and Kl5 expression in cell fate specification. The knockdown of Klf4 via RNA interference increased the expression of endodermal markers such as Gata6 within mESC and specifically enhances development of visceral and definitive endoderm. Of interest to this study is that the knockdown of Klf5 resulted in an increase in the expression of mesodermal markers, including Brachyury (T), and enhanced differentiation towards mesodermal lineage (Aksoy et al., 2014). This suggests that Klf5 interferes with the commitment of mesendoderm cells into mesoderm lineage. From our work it can be suggested that HS may play a potential role in the regulation of Klf4 and Klf5 gene expression. The presence of HS may enable the expression of Klf5, and combined with other regulatory mechanisms, result in the normal mesodermal differentiation found within WT EBs. The lack of HS within the EXT1-/- null population may lead to reduced levels of Klf5, which in turn, could lead to an increase in mesdodermal development. As Klf4 and Klf5 act antagonistically, if Klf5 levels were reduced in Ext1-/- EBs, then it is possible that Klf4 would be increased, hence the decreased level of endodermal differentiation within the cells. However, Aksoy et al. noted that collectively their results indicated that the knockdown of both Klf4 and Klf5 expression results in an enhancement of mesodermal differentiation; the effect of Klf5 knockdown being much more pronounced. Therefore, the

absence of HS may interfere with the expression of Klfs as a whole.

Recent work identified the expression of Brachyury in mesenchymal-like cells present in colorectal cancer (CRC) (Sarkar et al., 2012). Brachyury + cell lines expressed the Nanog2 pseudogene which is functionally identical and indistinguishable from Nanog1and it was suggested that Brachyury may be involved in the regulation of Nanog. The fact that Brachyury mRNA was upregulated but there was an absence of Nanog + cells in the Ext1-/- was not surprising. mRNA is often expressed before the presence of the protein. It is probable that if samples had been collected at Day 5 immunofluorescence would have been positive. As suggested in chapter 5 it is therefore possible that in the early stages of EB development, nanog is switched off in the Ext1-/- EBs, but following the induction of brachyury expression, is turned on again in 'mesodermal' cells. In the developing embryo this relationship is not observed and Nanog is consistently downregulated as the embryo develops. If the mesodermal cells within EXT1-/- EBs were shown to express Nanog then it is possible that they will have an abnormal phenotype.

Although our results indicate the importance of HS in differentiation, they were not statistically significant. The biological replicates demonstrated great variance in regard to gene expression. This may be due to the quality of the STO cell feeder layer that the mESC were initially cultured on. A low-density feeder layer can result in increased spontaneous differentiation within the mESC and result in the development of EBs with varying characteristics. In future, it would be recommended to include 6 biological replicates and to ensure STO feeder cell quality was consistent between replicates by using a similar passage number of STOs and ensuring they were discarded by 1 week following mitomycin-C treatment.

The scope of this study was limited due to time constraints and focused on the differentiation events of EBs occurring by Day 4. Collection and analysis of EBs at Day 2 and at a later time point in development i.e. Day 6, would allow analysis of differentiation events over time. This would demonstrate if development is delayed within the HS-deficient EBs.

A further limitation is that this project only employed the E14 mESC line (Hooper, Hardy et al. 1987) for investigation. A variety of mESC lines are available for culture and each is genetically distinct, and it is thus possible that the behaviour observed within this study are specific to this line.

6.1 Future Directions

The results from this project raised further questions that represent potential future directions and follow-up experiments.

EXT1-/- mESCs were shown to develop abnormal EBs owing to the absence of HS. Heparin is a close structural relative or subset of the HS family, and unlike HS, which is found synthesised by all mammalian cells, heparin is restricted to mast cells, where its primary function is to store histamine and proteases (Montgomery *et al.,* 1992). Heparin is well-studied and is frequently used as a proxy molecule for HS (Rabenstein 2002;, Presto *et al.* 2008). To confirm if the lack of PrE is due to an absence of HS, exogenous Heparin or HS could be added to the EXT1-/- EBs. This has been done previously by the Merry group in Manchester (adding HS to rescue neuronal differentiation in 2D culture). From this it would be possible to discover if HS needs to be present continuously, or only at the start of EB development to trigger PrE differentiation, and if addition of HS reduces the extent of mesoderm differentiation in the Ext1-/- EBs. It would be important to investigate whether it is the disruption of FGF signalling that leads to abnormal development. Experimental methods such as western blotting could be used to assess activation of FGF signalling and ELISA could detect activation of FGF through specific receptor dependence.

After our results indicated the upregulation of the mesoderm marker, brachyury within the EXT1-/- EBs, it would be important to carry out characterisation of mesodermal cells and observe any differences in the phenotype of mesodermal cells of the HS-null and those of WT EBs. Characterisation would involve expression profiling via microarray for mRNA and also proteomics for protein. Differentiation potential could be checked by investigating their ability to generate mesodermal derivatives such as kidney

cells, as has previously been done by the Liverpool Stem Cell group (Rak-Raszewska et al., 2012).

Considering the results presented by Aksoy et al. it would be interesting to investigate the role of Klf4 and Klf5 within EXT1-/- EBs. In order to substantiate these hypotheses it would be necessary to quantify the expression of Klf4 and Klf5 within HS-deficient mESC and identify the effect this has on their mesodermal lineage capacity.

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Appendix

set 1

ext1-/-

cal.Oct4	ext. ES.Oct		Delta Ct	base 2	R
14.88	17.3		-2.42	0.1868562	0.2698071
cal.gapdh	ext. ES.gapdh				
12.65	13.18		-0.53	0.6925547	
cal.nanog	ext nan				
17.7	17.75		-0.05	0.9659363	1.3947437
cal.bra	ext bra				
22.36	18.86		3.5	11.313708	16.336194
cal.gata	ext gata				
18.56	24		-5.44	0.0230355	0.0332616
cal.pax6	ext pax6				
22.01	23.65		-1.64	0.3208565	0.463294
cal.mega	ext mega				
15.66	18.85		-3.19	0.1095757	0.1582196
cal.lam	ext. lam		-5.75	0.0185814	0.0268302
16.53	22.28				

set 2

ext1-/-

cal. Gap	ext Gap	Delta ct	base 2	R
12.71	16.78	-4.07	0.0595399	
cal.nan	ext nan			
20.19	17.48	2.71	6.5432165	109.89638
cal.oct4	ext oct			
15.33	20.19	-4.86	0.0344345	0.5783441
cal.Pax6	ext pax			
22.55	28.31	-5.76	0.018453	0.3099269
cal.bra	ext bra			
23.45	25.11	-1.66	0.3164391	5.3147433
cal. Gata6	ext gata			
18.05	32.17	-14.12	5.616E-05	0.0009433
cal. Meg	ext. meg			
16.39	25.7	-9.31	0.0015755	0.0264608
cal.lam	ext lam			
15.97	25.33	-9.36	0.0015218	0.0255594

set 3

ext1-/-

cal.gap	gap	Delta Ct	base 2	R
11.95	13.63	-1.68	0.312083	
cal.nan	ext nan			
20.32	18.15	2.17	4.500234	14.42001
cal oct	oct			
18.91	23.19	-4.28	0.051474	0.164938
cal bra	bra			
27.4	25.79	1.61	3.052518	9.781122
cal pax6	pax6			
21.77	23.67	-1.9	0.267943	0.858565
cal mega	megalin			
25.52	28.18	-2.66	0.15822	0.50698
cal lama	lama			
24.94	27.53	-2.59	0.166086	0.532185
cal gata	gata			
20.83	28.47	-7.64	0.0050134	0.0160643

gap	average	sd
oct	0.3377	0.2149
nan	41.90371	59.24244
bra	11.31862	6.90228
gata6	0.01676	0.01617
pax6	0.54393	0.28307
mega	0.23055	0.24829
lama	0.19486	0.29213

Relative change data for D4 Ebs (three biological replicates)

SET 1	EXT1-/-				SET 2	EXT1-/-				SET 3	EXT1-/-			
cal.gap	ext es gap													
13.74	13.86	-0.12	0.9201877		12.62	15.35	-2.73	0.150726		9.88	16.78	-6.9	0.0083732	
cal.0ct4	ext oct 4													
18.49	16.57	1.92	3.7842306	4.1124553	25.54	22	3.54	11.63178	77.171701	13.42	20.19	-6.77	0.0091628	1.0942937
cal.nanog	ext.nanog													
18.68	16.71	1.97	3.9176812	4.2574807	29.54	22.39	7.15	142.02489	942.27215	14.86	17.48	-2.62	0.1626677	19.427118
cal.bra	ext bra													
25.83	26.69	-0.86	0.5509526	0.5987394	26.85	not detected			0	22.74	not detected			
cal. Gata6	ext gata6													
22.23	26.11	-3.88	0.0679209	0.073812	26	29.75	-3.75	0.0743254	0.6438423	19.6	32.17	-12.57	0.0001645	0.0196408
cal. Pax6	ext pax6													
23.08	25.07	-1.99	0.2517389	0.2735734	17.21	23.63	-6.42	0.0116785	0.0774817	20.34	28.31	-7.97	0.0039883	0.476319
cal. Mega	ext mega													
21.12	22.84	-1.72	0.3035487	0.329877	25.49	28.73	-3.24	0.1058432	0.7022224	16.36	25.7	-9.34	0.001543	0.1842837
cal.lama	ext lama													
20.59	24.49	-3.9	0.0669858	0.0727958	22.14	26	-3.86	0.0688691	0.4569157	15.93	25.33	-9.4	0.0014802	0.1767767

		WT	R	SD
gap		12.08		
oct		19.15	27.459483	43.07848
nanog		21.026667	321.98558	537.23747
bra		25.14	0.2993697	
gata6		22.61	0.2457651	0.34581
pax6		20.21	0.2757914	0.19943
megalin		20.99	0.405461	0.26711
laminin		19.553333	0.2354961	0.19868

Relative change data for mESC (three biological replicates)