Function of Bmpr1a in ES cell differentiation and cell competition.

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ABSTRACT

Bone morphogenetic protein (BMP) 4 signalling via BMPR1A is required for the maintenance of the epiblast in the early embryo, and for self-renewal of pluripotent mouse embryonic stem (ES) cells by inhibiting neural differentiation. In this study, the self-renewal and differentiation abilities of ES cells lacking BMPR1A were investigated. *Bmpr1a*-null ES cells did not respond to BMP4 but retained a degree of SMAD1/5/8 activation and *Id1* expression. This activation was likely due to BMP7 signalling via ACVR1. The observation that *Bmpr1a$^{-/-}$* ES cells showed no self-renewal or pluripotency defects suggested that signalling by BMPs of the 60a subgroup (such as BMP7) can also maintain pluripotency. When *Bmpr1a$^{-/-}$* ES cells were differentiated, although they did form derivatives of the three germ layers, they displayed a higher propensity to undergo neurectodermal specification than control cells, likely due to their lower levels of BMP signalling.

Cell Competition is the process by which viable cells are eliminated in the presence of metabolically more active or fitter cells. In *Drosophila* this process depends on dMyc levels and on limiting amounts of the survival factor *Decapentaplegic* (homologous to the mammalian BMPs). When *Bmpr1a$^{-/-}$* ES cells were co-cultured with wild-type cells, they gradually disappeared from the culture and were therefore out-competed. This cell competition was enhanced by limiting the amounts of survival and growth factors and could be rescued by restoring BMP4 signalling in *Bmpr1a$^{-/-}$* cells. In co-culture, *Bmpr1a$^{-/-}$* ES cells showed no significant changes in apoptosis but had a decreased cell cycle rate and increased levels of differentiation. Concomitantly, higher c-MYC levels were observed in wild-type cells due to increased protein stability. The out-competition of Bmpr1a$^{-/-}$ cells was dependent on differentiation as it could be prevented by inhibiting this process. These results suggest that during development cell competition may be an important mechanism controlling cell fate and survival.

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ABBREVIATIONS

2i	dual inhibition (PD0325901 + CHIR99021)
3i	triple inhibition (SU5402+PD184352+CHIR99021)
AP	alkaline phosphatase
A-P	Anterior - Posterior
APC	allophycocyanin
AVE	anterior visceral endoderm
BMP	bone morphogenetic protein
bp	base pair
BrdU	5-bromo-2-deoxyuridine
BSA	bovine serum albumin
CAG	CMV early enhancer/chicken β actin
CMV	cytomegalovirus
CCCP	carbonyl cyanide m-chlorophenylhydrazone
cDNA	complementary DNA
CM	conditioned medium
DAPI	4,6-diamidino-2-phenylindole
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dpc	days post coitum
DTT	dihiothreitol
EC	embryonic carcinoma
EDTA	ethylene-dinitrilo tetraacetic acid
EG	embryonic germ
EGFP	enhanced green fluorescent protein
EMT	epithelial to mesenchymal transition
EpiSC	post-implantation epiblast-derived stem cells
ERK	extracellular regulated kinase
ES	embryonic stem
ExE	extraembryonic ectoderm
FACS	fluorescence-activated cell sorting
FC	flow cytometry
FCS	foetal calf serum
FGF	fibroblast growth factor
GFP	green fluorescent protein
GSC	germline stem cell
GSK	glycogen synthase kinase 3
h	hour
hES	human ES
ICM	inner cell mass
iPS cell	induced pluripotent stem cell
JAK	Janus-associated tyrosine kinases

JNK	c-Jun amino-terminal kinase
Kb	kilobase (pair)
kDa	kiloDalton
LB	lysogeny broth
LIF	leukemia inhibitory factor
M	molar
MAPK	mitogen-activated protein kinase
MEK	mitogen activated protein kinase/ERK kinase
min	minute
mRNA	messenger RNA
NP-40	nonidet P-40
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PE	primitive endoderm
PFA	paraformaldehyde
PGC	primordial germ cells
PI	propidium iodide
PS	primitive streak
qRT-PCR	quantitative reverse-transcription PCR
res	restored function
RNA	ribonucleic acid
rpm	rotations per minute
RT	room temperature
RT-PCR	reverse-transcription PCR
s.d.	standard deviation
SDS	sodium dodecyl sulphate
shRNA	short harpin RNA
SNARF	seminaphthorhodafluor
STAT	latent signal transducer activator of transcription factor
TE	trophectoderm
TF	transcription factor
TGF	transforming growth factor
Tris	(hydroxymethyl)methylamine
VE	visceral endoderm
WT	wild-type

1. INTRODUCTION

1.1. Early Mammalian development

1.1.1. Preimplantation embryo development

During embryonic development, specific programmes of gene expression are progressively established leading to cell commitment into specific lineages and axis patterning (Arnold and Robertson, 2009). The expansion of early progenitor cells in the embryo, while gradually becoming more specialised and restricted in their developmental potential, is regulated by key signalling pathways and by developmentally regulated transcription factors.

Mammalian embryonic development starts in the oviduct with fertilisation, when the haploid sperm and oocyte fuse generating a 1-cell embryo, the fertilized egg. During the first two days of development following fertilization, a series of mitotic cell divisions leads to the formation of an 8-cell embryo, a cluster of undifferentiated cells. These 8 cells (or blastomeres) subsequently undergo compactation and a further round of division, forming the 16-cell compacted morula (Johnson and McConnell, 2004). The late compacted morula then experiences the first lineage segregation, as the polarized outer layer of cells differentiates into the trophectoderm, giving rise to the blastocyst at 3.5 days post coitum (dpc). The early mouse blastocyst comprises a cavity (the blastocoel) and two distinct cell populations (Figure 1.1A): the trophectoderm (TE), the precursor of all the trophoblast lineages that form the fetal part of the placenta, and the inner cell mass (ICM), which will give rise to all the embryonic tissues and to the lineages of the yolk sac. The ICM is positioned to one side of the TE, designated polar TE, whilst the mural TE surrounds the blastocoel. The asymmetric location of the ICM defines the Embryonic-Abembryonic (Em-Ab) axis.
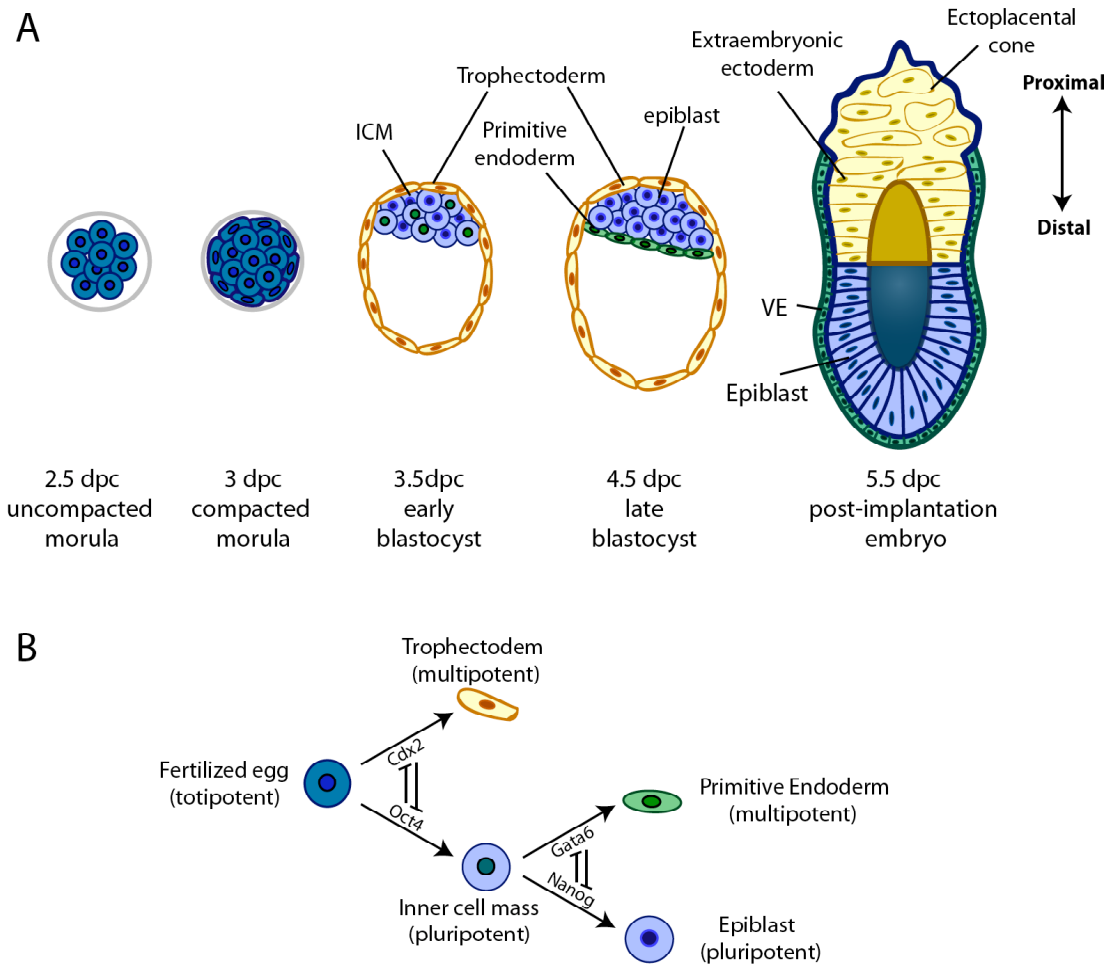
Figure 1.1 Schematic representation of the early stages of mouse development, when the first cell fat decisions are made.

A) *Representation of the events from fertilisation to implantation*. During the two days after fertilisation the mouse embryo undergoes cell divisions to form the 8-cell morula, which then suffers compactation, giving rise to the compacted morula. At 3.5 dpc the conceptus becomes a multilayered blastocyst. The outermost layer is the trophectoderm (TE; shown in yellow), which later gives rise to extraembryonic tissues such as the ectoplacental cone and eventually forms the placenta; The inner cell mass (ICM; shown in light blue) is a mass of pluripotent cells, which go on to form every tissue of the animal itself. By 4.0 dpc a subpopulation of cells within the ICM move to the inner surface and form the primitive endoderm (PE; shown in green). At 4.5 dpc the embryo implants into the uterine wall and undergoes morphogenetic changes to form the egg cylinder. The ICM has become the embryonic ectoderm, which is referred to as the epiblast (also shown in light blue). **B)** *Genes responsible for lineage segregation*. Flow diagram showing the genes responsible for converting a totipotent precursor cell into the three lineages of the late blastocyst. Note the reciprocal inhibitory interactions that stabilize and reinforce lineage commitment.

This first cell fate decision is mediated by cell polarization and the mutually exclusive expression of *Oct4* (*Pou5f1*) and *Cdx2* (Jedrusik et al., 2008; Niwa et al., 2005; Ralston and Rossant, 2008; Strumpf et al., 2005). These transcription factors are initially co-expressed in all cells of the compacted morula. Subsequently, *Cdx2* and *Oct4* expression become restricted to the TE and ICM, respectively, by a mechanism that might depend on asymmetrical cell divisions of the morula (Jedrusik et al., 2008) and reciprocal repression of transcription (Niwa et al., 2005). OCT4 maintains pluripotency in the ICM (Nichols et al., 1998) whilst CDX2 expression is essential for the integrity and expansion of the TE lineage (Strumpf et al., 2005). Additionally, within the ICM of the early blastocyst, a mosaic and random "salt and pepper" expression of the transcriptions factors Nanog and GATA6 can be observed (Chazaud et al., 2006). Chazaud and coworkers have suggested that *Nanog* and *Gata-6* expressing cells are already committed for the subsequent lineage segregation, which takes place just before implantation. *Gata-6*-positive cells are thus sorted to the distal surface of the ICM, where they form the hypoblast or primitive endoderm (PE), and *Nanog*-positive cells exclusively give rise to the pluripotent epiblast (Figure 1.1B). Lineage tracing and chimera studies in the mouse have shown that these lineages are restricted in fate by the time of implantation. The epiblast (or embryonic ectoderm) will give rise to all tissues of the embryo proper whereas derivatives of the TE and the PE give rise to the extraembryonic structures that support the intra-uterine development of the embryo and act as signalling sources to pattern the underlying embryonic tissues (reviewed in Rossant, 2004).

As depicted here, with each cell division during embryonic development, cells increase their degree of differentiation and, inevitably, become more and more restricted in their developmental potential (reviewed in Arnold and Robertson, 2009). The fertilised egg and cells of the 2- and the 4-cell embryo are totipotent as they can

give rise to every cell type of the organism (including extra-embryonic lineages). By the blastocyst stage, the cells of the epiblast are still able to give rise to all somatic lineages but have lost the ability to generate cells of the extra-embryonic tissues (placenta and extra-embryonic membranes). These cells are thus called pluripotent and can differentiate into cells of the three somatic germ layers (ectoderm, mesoderm, endoderm) as well as primordial germ cells. The cells from the TE and PE are multipotent since they can differentiate exclusively into trophoblast, and visceral and parietal endoderm lineages, respectively (Bielinska et al., 1999; Rossant, 2001).

This view has recently been questioned by Silva, Nichols and Smith (Nichols and Smith, 2009; Silva and Smith, 2008), who argue that the egg and blastomeres have a restricted developmental potential, being able to directly generate only two cell types, the trophoblast and the ICM. Subsequent development thus requires the acquisition of further potency by the ICM. Therefore, the authors suggest that the epiblast of the late blastocyst does not represent a restriction in potency from the egg; conversely, it constitutes a "ground state", a completely unrestricted population with the potency and epigenetic flexibility to generate all the embryonic lineages during development.

Epigenetic regulation, including regulation at the level of DNA, histones and nuclear organization, is also an essential part in the establishment and, more importantly, maintenance of a given cellular identity throughout development (reviewed in Kiefer, 2007; and Ng and Gurdon, 2008). However, this level of regulation will not be explored here.

At 4.5 dpc the blastocyst hatches from the protective outer zona pellucida and implants into the uterine wall. At this stage the conceptus is referred to as the egg cylinder.

1.1.2. Implantation and axis formation

Implantation and formation of the egg-cylinder embryo

During implantation, between 4.5 and 5.0 dpc, the embryo attaches to the endometrial surface and the trophectoderm invades the uterine epithelium, anchoring to the uterine connective tissue.

For successful implantation to occur both the uterus and the blastocyst must be receptive, that is, have achieved implantation competency. There is a "window" of implantation, which depends on locally produced signalling molecules, including cytokines, growth factors, homeobox transcription factors, lipid mediators and morphogens (Wang and Dey, 2006). These signals, together with ovarian hormones, serve as autocrine, paracrine and juxtacrine factors to specify uterine receptivity.

At the peri-implantation stage the mouse embryo undergoes a series of morphogenetic changes to form the "egg cylinder" (Figure 1.1A). The polar TE expands in response to fibroblast growth factor (FGF) 4 secreted by the epiblast (Tanaka et al., 1998), and forms the extraembryonic ectoderm (ExE) and the ectoplacental cone, the progenitors of the placenta. This pushes the epiblast towards the distal pole of the conceptus into the blastocoel cavity. At this stage the PE differentiates to become parietal endoderm, which migrates from the surface of the ICM and forms a protective membrane that envelops the entire egg cylinder, and visceral endoderm (VE). The VE constitutes a continuous cell monolayer that overlies both the ExE and the epiblast, and will eventually form part of the yolk sac (Arnold and Robertson, 2009).

A further morphogenetic process that takes place around the time of implantation is the formation of the pro-amniotic cavity, which forms in the centre of the epiblast converting it into a cup-shaped columnar epithelium. This process, named cavitation, is the result of a combination of apoptotic signals from the visceral endoderm (Coucouvanis and Martin, 1995) and selective cell survival mediated by adhesion to the basement membrane between the VE and epiblast (Murray and Edgar, 2000). Coucouvanis and Martin have also shown that Bone Morphogenetic Protein (BMP) signalling is capable of promoting, and is required for, differentiation of the VE and cavitation (Coucouvanis and Martin, 1999). The surviving epiblast cells that line the cavity become polarized, as the contact with the basement membrane defines an apical and basal surface, and form a pseudostratified columnar epithelium.

Maintenance of pluripotency of the epiblast at the egg-cylinder stage also requires the TGF-β-related molecules Nodal and BMPs, as in their absence the epiblast prematurely differentiates into neurectoderm (Camus et al., 2006; Di-Gregorio et al., 2007; Mesnard et al., 2006).

Establishing the anterior–posterior (A-P) axis.

Just after implantation, reciprocal signalling between the epiblast, ExE and VE, by secreted growth factors of the TGF-β (Nodal and BMPs), Wnt and FGF families, leads to regionalised gene-expression patterns in these tissues. This sets up the embryonic axis, marking the start of embryonic patterning, and is required for subsequent developmental steps such as cell lineage allocation and tissue differentiation.

Around 5.25 dpc, Nodal signalling via SMAD2 leads to the specification of the distal visceral endoderm (DVE) at the distal tip of the embryo (Brennan et al., 2001). The DVE will act as a specialised signalling centre that orquestrates anterior-posterior (A-

P) axis development. Inhibitory signals from the ExE prevent DVE induction in the proximal VE, restricting it to the distal tip of the conceptus (Richardson et al., 2006; Rodriguez et al., 2005). At 5.5 dpc the DVE moves towards the prospective anterior side of the embryo forming the anterior VE (AVE), and signalling by this tissue establishes embryonic A-P polarity (Beddington and Robertson, 1998; Thomas and Beddington, 1996).

Nodal signalling also has an important role in driving DVE migration, as reduction in the level of Nodal transcription prevents it (Lowe et al., 2001). The exact mechanism that directs the coordinated movement of the DVE remains unknown but both differential proliferation and active migration have been suggested to play a role (reviewed in Srinivas, 2006). In particular, *Dkk1* expression at the prospective anterior may act as an attractive signal for directing the active migration of DVE cells (Kimura-Yoshida et al., 2005).

The AVE cells express extracellular Nodal and Wnt-signalling antagonists including cerberus-like-1 (*Cer1*), left–right determination factor 1 (*Lefty1*) and Dickkopf homologue 1 (*Dkk1*), attenuating Nodal and Wnt signalling and thus maintaining the anterior character of the adjacent epiblast (Srinivas, 2006).

Positioning of the AVE and the consequent A-P molecular asymmetry precedes any morphological sign of embryonic pattern.

1.1.3. Gastrulation and germ layer formation

During gastrulation the three embryonic germ layers, ectoderm, mesoderm and definitive endoderm (DE), are generated and these will constitute the progenitor lineages of all the embryonic tissues. At later stages of development, ectoderm derivatives will form neural tissues, neural crest and skin. Mesoderm cells form many cell types of internal organs like muscle, bone, cartilage, connective tissues,

vasculature and blood. The outer layer of definitive endoderm gives rise to the gut tube and associated organs, such as the lungs, liver and pancreas.

At around 6.0 dpc, epiblast cells begin to converge towards the proximal posterior pole of the embryo to form the primitive streak, which marks the onset of gastrulation (Beddington and Robertson, 1999; Tam and Behringer, 1997). At the primitive streak cells undergo an epithelial to mesenchymal transition (EMT), ingress in between the epiblast and the overlying VE, and subsequently emerge as mesoderm or incorporate the definitive endoderm (reviewed in Tam and Behringer, 1997).

High levels of Nodal, BMP, and Wnt signalling at the proximal posterior epiblast are required for primitive streak (PS) induction. Auto-regulatory reciprocal interactions between the epiblast and the ExE maintain high levels of these morphogens at the appropriate location (reviewed in Tam and Loebel, 2007). Mutants in which the activation of these pathways has been compromised fail to form mesoderm and do not gastrulate (Conlon et al., 1994; Liu et al., 1999; Mishina et al., 1995; Tam and Loebel, 2007). Conversely, embryos that lack expression of Wnt and TGF-β antagonists exhibit the formation of multiple or enlarged primitive streaks (reviewed in Tam and Loebel, 2007).

The PS, initially induced at the proximal posterior pole of the epiblast, progressively elongates and extends to the distal tip of the embryo. Molecular analyses and lineage mapping studies have shown that different regions of the primitive streak differ in gene expression patterns, signalling environments and developmental potential. The allocation of gastrulating cells to specific lineages is thus temporally and spatially controlled (Lawson, 1999; Lawson et al., 1991) by differences in signalling strength of BMP4, Nodal, Wnt3 or Wnt3a, and FGF8 (reviewed in Tam and Loebel, 2007).

The first mobilized epiblast cells ingress trough the posterior part of the PS giving rise to the extraembryonic mesoderm. As gastrulation proceeds, cells migrate

through more anterior parts of the PS and generate cranial, cardiac and paraxial mesoderm, and subsequently axial mesendoderm. Definitive endoderm develops from epiblast cells that transit the most anterior region of the PS (reviewed in Tam and Loebel, 2007). In the anterior end of the primitive streak, another specialised structure can be distinguished, the node, which is an important signalling centre for A-P and left–right patterning. In contrast to mesoderm and definitive endoderm, the ectoderm lineage derives from the anterior region of the epiblast that does not enter the PS .

At the molecular level, genes such as Brachyury (*T*), are expressed throughout the PS, whereas others are found preferentially in posterior (*Cdx2*, *HoxB1*), intermediate (*Mixl1*, *Lhx1*) or anterior regions (*Foxa2* and *Gsc*) (reviewed in Arnold and Robertson, 2009; and Tam and Loebel, 2007).

Segregation of primordial germ cells (PGCs) from the somatic lineages at gastrulation requires signals from the extraembryonic ectoderm and visceral endoderm (reviewed in Hayashi et al., 2007). Around 5.5 dpc, a group of cells at the proximal epiblast start expressing Fragilis in response to BMP4 signals from the ExE. Subsequently, some of these cells acquire Blimp1 expression, becoming restricted PGC precursors. After gastrulation, the PGC precursors locate to the posterior proximal region, where they undergo specification to form the population of Stella-positive PGCs. PGCs undergo extensive epigenetic reprogramming to maintain pluripotency throughout the life cycle.

1.2.   Pluripotent stem cell lines

Pluripotency, the capacity to generate all cell types, is a cellular property transiently found *in vivo* in the ICM of the mammalian blastocyst (reviewed in Rossant, 2007)

and conserved until 7.5 dpc only in some regions of the epiblast (Beddington, 1982; Beddington, 1983; Diwan and Stevens, 1976; Lawson et al., 1991) and later in PGCs (Donovan, 1998). Pluripotency can also be found *ex vivo* in stem cell lines, such as embryonic stem (ES) cells, derived and maintained under defined growth conditions that promote proliferation while preventing differentiation (Chambers and Smith, 2004; Niwa, 2007; Silva and Smith, 2008)

Embryonal carcinoma cells were the first pluripotent cells to be propagated *in vitro* (reviewed in Solter, 2006). Since then, pluripotent stem cell lines have been derived from different species, distinct tissues of the embryo, at different stages of development an even from reprogrammed adult somatic tissues (reviewed in Rossant, 2008).

1.2.1. Embryonal carcinoma (EC) cells

EC cells are derived from teratocarcinomas, malignant germ cell tumours that comprise both undifferentiated cells and differentiated derivatives of all three germ layers. When fragments of teratocarcinomas were put in culture, a variety of cell types was obtained, among them some that could be clonally expanded and that had the potential to form new teratomas once transplanted into host animals (reviewed in Solter, 2006). The use of a layer of feeder cells, usually fibroblasts, allowed the successful expansion of these pluripotent cells with little differentiation, and hence the establishment of EC cell lines. EC cells can be clonally derived from single cells, are capable of both self-renewal and multilineage differentiation, being even competent to contribute to various somatic cell types upon injection into mouse blastocysts (Papaioannou et al., 1978). Despite an abnormal karyotype, EC cells have similar developmental properties, and express markers in common with the pluripotent cells of the early embryo (Andrews et al., 2005). The isolation of EC lines

made possible the definition of conditions that allowed self-renewal and also some models of differentiation later used in ES cells.

1.2.2. Embryonic stem (ES) cells

ES cells were derived for the first time in 1981 (Evans and Kaufman, 1981; Martin, 1981) from the ICM of mouse blastocysts, using the same culture conditions as the ones previously used for the isolation of embryonal carcinoma (EC) cells (reviewed in Solter, 2006). However, only more than 15 years later were the first human ES (hES) cell lines isolated (Thomson et al., 1998).

Two essential properties characterise ES cells: self-renewal and pluripotency. Given their ability to self-renew, under appropriate conditions, ES cells can be maintained indefinitely in culture in an undifferentiated state without losing their pluripotency. Both mouse and human ES cells can differentiate *in vitro* into a variety of cell types, and after ectopic transplantation give rise to teratomas containing derivatives of the three germ layers (reviewed in Yu and Thomson, 2008). When mouse ES cells are reintroduced into developing blastocysts, they are readily incorporated into the ICM and re-enter embryonic development generating chimeric animals with contribution to all embryonic tissues, including germ cells (Bradley et al., 1984).

The self-renewal and multi-lineage differentiation abilities make stem cells uniquely convenient for regenerative medicine, tissue repair and gene therapy applications (reviewed in Murry and Keller, 2008), reasons for which stem cells have become the focus of many current studies. Additionally, their potential for *in vitro* differentiation makes them a useful model for the study of embryonic development at the cellular and molecular level (reviewed in Nishikawa et al., 2007). Finally, ES cells can be genetically modified, providing a means of interfering with genetic functions and creating lines that bear specific markers. The ability for germline transmission allows

these modifications to be passed to the mouse progeny creating transgenic mouse lines (reviewed in Raymond and Soriano, 2006).

Genetic control of Pluripotency

Cell fate during development is decided by transcription factors that act as molecular switches to activate or repress specific gene expression programmes. The activity of transcription factors, however, is mainly controlled by stimuli from the extracellular environment. Lineage specification is thus determined by both extrinsic and intrinsic factors.

Three main transcription factors have been suggested to form a core network that controls the maintenance of pluripotency in mouse ES cells (Figure 1.2) (reviewed in Boiani and Scholer, 2005; Boyer et al., 2006; and Chambers and Smith, 2004). These are the POU-family transcription factor Oct4 (Nichols et al., 1998; Niwa et al., 2000), the homeodomain DNA-binding protein Nanog (Chambers et al., 2003; Mitsui et al., 2003) and the SOX-family transcription factor Sox2 (Avilion et al., 2003). These three transcription factors are all highly expressed in the inner cell mass and epiblast of the mouse embryo and in undifferentiated ES cells (reviewed in Niwa, 2007).

Null mutations of each of these genes results in early embryonic lethality due to the inability to maintain pluripotent cells (Avilion et al., 2003; Mitsui et al., 2003; Nichols et al., 1998). A key role of the Oct4, Sox2 and Nanog transcription factors seems to be to counteract differentiation by continuously suppressing functional expression and activity of lineage specification factors, thereby promoting self-renewal and maintaining pluripotency (Niwa, 2007; Silva and Smith, 2008; Smith, 2005). Co-regulatory and auto-regulatory mechanisms appear to link the three factors in a self-

reinforcing circuit (reviewed in Niwa, 2007). This network of transcriptional regulators is also conserved in human (h)ES cells.

Oct4 expression in the mouse is restricted to early embryos and germ cells (Scholer et al. 1989). Homozygous deletion of this gene results in an embryo consisting exclusively of trophectoderm tissues due to a failure in the formation of the ICM (Nichols et al., 1998). *In vitro*, Oct4 expression is a hallmark of both mouse and human ES cells and is lost upon differentiation; overexpression of this protein causes differentiation into endoderm and mesoderm, whereas its repression leads to differentiation into trophoblast (Niwa et al., 2000). This clearly suggests that the precise regulation of the levels of Oct4, within a narrow range, is required to sustain self-renewal.

Nanog expression, as for *Oct4*, decreases rapidly as ES cells differentiate (Chambers et al., 2003; Mitsui et al., 2003). Mouse ES cells lacking Nanog are still able to proliferate undifferentiated but show a greater tendency for spontaneous differentiation towards extra-embryonic endoderm and cannot give rise to mature germ cells (Chambers et al., 2003; Mitsui et al., 2003). Conversely, over-expression of Nanog sustains mouse ES cells in the absence of exogenous cytokines (LIF and BMPs) that would otherwise be required for self-renewal (Chambers et al., 2003; Ying et al., 2003a).

Sox2, a member of the SOX (SRY-related high mobility group (HMG)-box) family of transcription factors, also plays a key role in the self-renewal and pluripotency of ES cells. Unlike Oct4 and Nanog, Sox2 expression is not restricted to pluripotent cells as it is also detected in early neural lineages (Ellis et al., 2004; Li et al., 1998) in addition to the ICM, epiblast, and germ cells (Avilion et al., 2003). *Sox2*-null embryos die around implantation due to a failure in epiblast development (Avilion et al., 2003). Also, similarly to what is observed for Oct4, Sox2 deletion in ES cells results in differentiation to trophectoderm (Masui et al., 2007). Indeed, Sox2 and Oct4 are

transcriptional partners that regulate the expression of several pluripotency-associated genes, including Fgf4, Lefty1 and Nanog. This is achieved by the binding of both transcription factors to their respective motifs in Oct-Sox enhancers, which are highly active in undifferentiated ES cells but not in differentiated cells (Chew et al., 2005; Tomioka et al., 2002; Yuan et al., 1995). Oct4 and Sox2 are regulated by positive self-reinforcing loops, as the Oct-Sox enhancers are also important in promoting the expression of Oct3/4 and Sox2 themselves (Chew et al., 2005; Okumura-Nakanishi et al., 2005; Richardson et al., 2006; Tomioka et al., 2002). However, a recent report has argued that Sox2 is dispensable at the Oct-Sox enhancers and that, instead, it promotes pluripotency by regulating transcription factors that maintain the appropriate levels of Oct4 expression (Masui et al., 2007).

The DNA-binding sites for Oct4, Sox2, and Nanog have been extensively studied using a genome-wide approach (Boyer et al., 2005; Loh et al., 2006). These studies have shown that, although the mouse and hES-cell models are different from each other and show very specific targets, the three transcription factors share many target genes in both of them, including a large number of developmentally important transcription factors. Additionally, these studies have confirmed that Oct4, Sox2, and Nanog positively influence their own transcription.
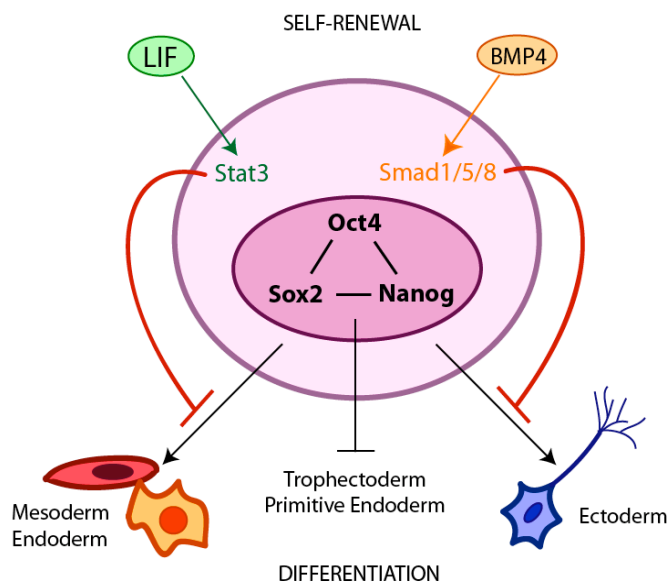
Figure 1.2 Intrinsic and extrinsic control of ES-cell self renewal.

LIF and BMP act together to block differentiation. LIF activates STAT3 and blocks non-neural differentiation. BMP blocks neural differentiation by induction of Id's. The intrinsic transcriptional regulators Nanog, Oct4 and Sox2 act to maintain an undifferentiated phenotype (adapted from Chambers and Smith, 2004).

An extended transcriptional network

Lately it has become evident that the network that controls pluripotency is more complex and involves other players (Kim et al., 2008). For example, EC cells express all three transcription factors at considerable levels, yet they do not have the same potential as pluripotent ES cells (Chambers and Smith, 2004). This strongly suggests that other transcription factors and regulators are required to either establish or retain pluripotency. Another piece of evidence is the reprogramming of somatic cells mouse embryonic fibroblasts to ES-like pluripotent stem cells by the induced expression of defined factors outside the traditional core such as Klf4 and c-Myc, in addition to Oct4 and Sox2 (Takahashi and Yamanaka, 2006). In fact, new regulators of pluripotency (such as Esrrb, Dax1, Sall4, Rex1, Stat3, among others) and their interaction networks, have recently been identified by shRNA knock-down screens (Ivanova et al., 2006), proteomics (Wang et al., 2006) and analysis of transcription

factor binding sites and gene expression data (Zhou et al., 2007). The analysis of target promoters of candidate transcription factors, including reprogramming factors, has shown that promoters bound by a high number of these regulators are generally active in the pluripotent state and become repressed upon differentiation (Kim et al., 2008). The extent and complexity of the pluripotency transcriptional network will continue to expand as our understanding of ES cells improves and new tools for their study become available (Orkin et al., 2008).

Extrinsic regulators of mouse ES cell self-renewal

The culture conditions in which ES cells have been maintained over the years since their derivation have become progressively more defined. This has been both a cause and a consequence of a better understanding of the pathways that control ES cell self-renewal and differentiation.

Mouse ES cells generally require extrinsic factors in the culture medium to maintain the undifferentiated state. The first mouse ES cell lines were derived using fibroblast feeder layers and serum (Evans and Kaufman, 1981; Martin, 1981). The feeder cells, besides providing a matrix for cell attachment, secrete factors that suppress spontaneous differentiation of ES cells *in vitro*. Medium conditioned by the culture of specific cell types was found to be able to sustain ES cells in the absence of feeders, and the factor in the conditioned medium responsible for this effect was identified as the cytokine leukemia inhibitory factor (LIF) (Smith et al., 1988; Williams et al., 1988). LIF and related cytokines act via LIFR/gp130 receptors, activating Janus-associated tyrosine kinases (JAK)/latent signal transducer activator of transcription factor (STAT)3 (Yoshida et al., 1994), and Shp2/ERK mitogen-activated protein kinase (MAPK) cascades (Takahashi-Tezuka et al., 1998). The self-renewal effect of LIF is mediated via STAT3 and its activation alone is sufficient for maintenance of mouse ES cells in the presence of serum (Matsuda et al., 1999; Niwa et al., 1998). ERK

activation, however, predisposes cells to differentiation (Kunath et al., 2007) and its suppression promotes ES cell self-renewal (Burdon et al., 1999). Thus the proliferative effect of LIF on mouse ES cells requires a finely tuned balance between positive and negative effectors. In addition to repressing differentiation, LIF also promotes growth and viability of ES cells (Duval et al., 2000). Myc activation has been reported to be a critical effector of LIF-induced self-renewal (Cartwright et al., 2005). Myc is highly expressed in undifferentiated mouse ES cells and its levels promptly go down after LIF withdrawal. Cartwright and coworkers have shown that Myc is a STAT3 transcriptional target in ES cells, that it blocks differentiation, and that sustained MYC activity maintains ES-cell self renewal in the absence of LIF. Also, the downregulation of MYC protein levels during differentiation is dependent on threonine 58 phosphorylation and consequent GSK3-mediated degradation (Cartwright et al., 2005).

In serum-free medium, LIF/STAT3 alone is insufficient to prevent mouse ES cell differentiation as some neural differentiation still occurs. However, in combination with BMPs, ES cell self-renewal is sustained (Figure 1.2) (Ying et al., 2003a). BMPs induce the expression of ID (inhibitor of differentiation) proteins through activation of the SMAD pathway, thereby repressing differentiation towards neural fates. Ying and coworkers have shown that the overexpression of Ids could indeed promote mouse ES cell proliferation in the presence of LIF alone without the need for either BMPs or serum. Qi and coworkers, however, replaced exogenous BMP4 in the culture medium by ERK and p38 inhibitors as a means to maintain ES self-renewal, arguing that BMPs act through inhibition of MAPK pathways independently of SMADs (Qi et al., 2004).

The ground state

As described above, the activation of specific transcriptional regulators, in particular STAT3 and SMADs, via stimulation by extrinsic factors is generally considered necessary for ES cell maintenance. However, a recent report by Ying and coworkers has questioned this traditional view showing that extrinsic stimuli are dispensable for self-renewal as long as auto-inductive differentiation signals are repressed (Ying et al., 2008). FGF4 is produced in an autocrine fashion by undifferentiated ES cells. FGF4, among other extrinsic factors, activates the ERK signalling cascade, instructing ES cells to exit self-renewal and enter a primed state, where they become responsive to inductive cues for lineage specification (Kunath et al., 2007; Stavridis et al., 2007). Blockade of this pathway using chemical inhibitors enhances the growth of undifferentiated ES cells (Burdon et al., 1999; Sato et al., 2004), and in combination with suppression of glycogen synthase kinase-3 (GSK3) is sufficient to sustain ES cell self-renewal (Ying et al., 2008). The use of selective small-molecule inhibitors to suppress FGF receptor tyrosine kinases and ERK signalling (by SU5402 and PD184352, respectively) in serum-free medium, significantly reduces spontaneous differentiation of ES cells, except for occasional neural differentiation, but it is insufficient to maintain ES-cell propagation due to impaired growth and survival. GSK3 inhibition, on the other hand, suppresses neural specification and enhances growth capacity, promoting non-neural differentiation. However, the combination of the three inhibitors (3i) results in a highly efficient expansion of undifferentiated colonies, even at a low cell density (Ying et al., 2008). The 3i combination of inhibitors allowed the derivation of ES cells from refractory strains and when these were injected into morulae resulted in high-contribution chimeras and germline transmission. A more potent MEK inhibitor, PD0325901, is sufficient to sustain ES cells in combination with the GSK3 inhibitor (2i), confirming the central role of ERK, downstream of FGF, in promoting ES-cell escape from self-renewal.

The authors have also shown that the contribution of GSK3 inhibition, beyond limiting differentiation, is mainly to maintain metabolic activity, biosynthetic capacity and overall viability, in this way possibly increasing the threshold for commitment (Ying et al., 2008). Additionally, the 2i condition, on feeders or with addition of LIF, was successfully used for the derivation of rat ES cells (Buehr et al., 2008; Li et al., 2008), a previously refractory species, and enhanced the isolation of induced pluripotent stem cell (iPSC) lines with full pluripotent capacity (Guo et al., 2009; Silva et al., 2008). These observations led the authors to suggest that inhibition of autoinductive differentiation is sufficient to maintain ES cells in a fully unrestricted and intrinsically self-maintaining basal state, the "ground state" of pluripotency (Nichols and Smith, 2009; Silva and Smith, 2008).

Differentiation of mouse ES cells

The ability of ES cells to differentiate into derivatives of the three primary germ layers establishes their potential, both for clinical applications and as models for the *in vitro* study of developmental processes. However, for their use in these areas it is essential to be able to control ES cell differentiation and to direct their development along specific pathways.

Several protocols have been developed for the generation of a broad spectrum of cell types from ES cells (reviewed in Murry and Keller, 2008). These can be grouped into three basic approaches: 1) the formation of three-dimensional aggregates known as embryoid bodies (EBs), 2) the culture of ES cells as monolayers on extracellular matrix proteins, and 3) the culture of ES cells on supportive stromal layers. Efforts have been made towards the use of progressively more defined conditions, such as the use of serum-free media with specific inducers to direct differentiation. Also, ES-cell differentiation has recently been approached from a developmental biology perspective, allowing the recapitulation of key events that regulate early lineage

commitment in the embryo. This results in the efficient and reproducible generation of highly enriched differentiated cell populations (Murry and Keller, 2008).

When grown as aggregates in suspension, in the absence of LIF, ES cells form embryoid Bodies (EBs). In these structures, differentiation proceeds into the three germ layers, in a manner reminiscent of pre- and peri-implantation development. EB differentiation recapitulates embryonic events such as the formation of an external endoderm layer, differentiation of a columnar epithelium, formation of a central cavity and mesoderm specification (Coucouvanis and Martin, 1995; Martin et al., 1977). However, this is a poorly controlled system in which serum factors play undefined roles, and gives rise to very heterogeneous cell populations.

Common to the different protocols, the first step in the differentiation pathway is generally the development of a population resembling the epiblast of the mouse embryo. When induced with Wnt, activin, BMP or serum, ES cells generate a Brachyury-positive, primitive streak-like population (reviewed in Murry and Keller, 2008). Subsequent manipulation of the BMP, Wnt and Nodal pathways in ES-cell cultures modulates differentiation into representatives of specific germ layers and cell types (Figure 1.3) in a way that resembles the developmental programme of the embryo (reviewed in Murry and Keller, 2008). Therefore, the period of exposure and sequence of addition of particular factors can considerably alter the differentiation pathway induced. Also resembling what happens in the embryo, in the absence of PS-inducing signals, ES cells differentiate into the neurectoderm lineage (Tropepe et al., 2001; Ying et al., 2003b).
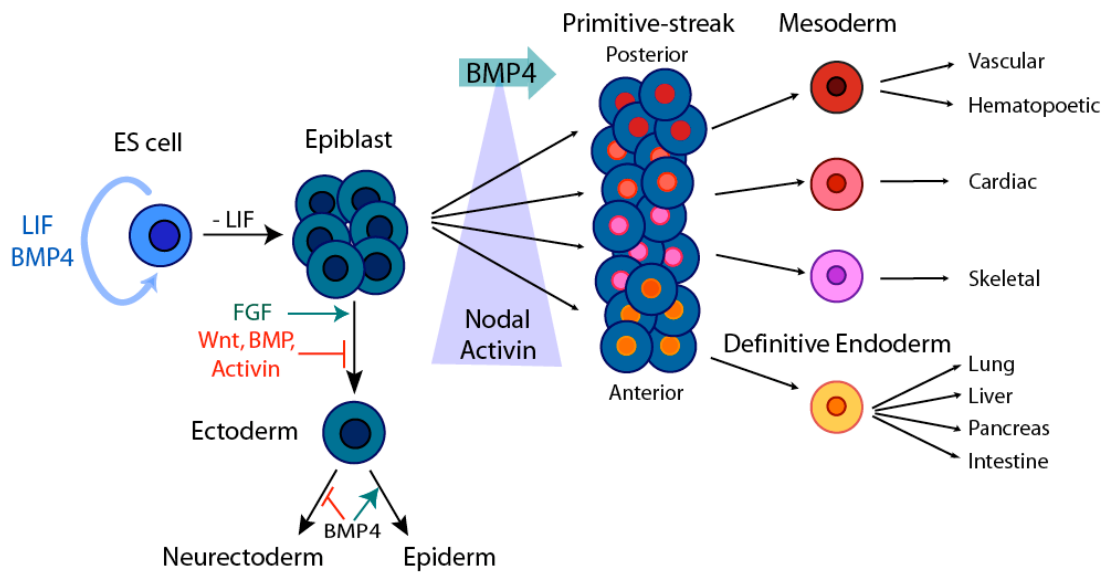
Figure 1.3 Schematic representation of ES-cell differentiation into primitive ectoderm and the primary germ layers and the main signalling pathways involved in these choices.

A hypothetical primitive streak is shown consisting of both posterior and anterior populations from which the different cell types are established. BMP4 is shown to function to induce posterior mesoderm and skin. A gradient of activin/nodal signaling is indicated, with low concentrations inducing more posterior populations and high concentrations inducing endoderm, indicative of the anterior primitive streak. FGF is shown to play a role in neural induction, whereas Wnt, BMP, and activin are inhibitors of the early stages of this pathway.

Human ES cells

More than 15 years after the isolation of mouse ES cells, the first human ES (hES) cell lines were derived (Thomson et al., 1998). hES cells are different from mouse ES cells in several aspects (reviewed in Yu and Thomson, 2008). Self-renewal is maintained by the activation of different signalling pathways, some important transcription factors and specific markers are differentially expressed, and the developmental potential seems to partially differ. Nevertheless, ES cells of both species share the two properties that define them: pluripotency and self-renewal.

Similarly to the mouse, the core transcription factors OCT4, SOX2 and NANOG also maintain self-renewal in hES cells. OCT4 expression is lost when hES cells differentiate and its repression leads to commitment to the trophoblast lineage (Matin et al., 2004). NANOG overexpression enables feeder-independent growth of hES cells and improves cloning efficiency (Darr et al., 2006) and its repression also predisposes to differentiation to extraembryonic lineages (Hyslop et al., 2005)

However, even though at the molecular level mouse and hES cells seem to be controlled by the same transcription factors, the signalling pathways that maintain them differ significantly (reviewed in Rao, 2004; Yu and Thomson, 2008). In contrast to the mouse, LIF/STAT3 activation is not able to maintain hES self-renewal (Daheron et al., 2004; Thomson et al., 1998). Instead, FGF and TGF-β/Activin/Nodal signalling are of central importance to the self-renewal of hES cells (Vallier et al., 2005) and suppression of BMP activity improves hES cell maintenance (Xu et al., 2005). The events downstream of these pathways responsible for sustaining self-renewal are still not well understood. In the presence of BMP4, hES cells undergo differentiation to trophoblast cells (Xu et al., 2002). This fate is not generally obtained in mouse ES-cell cultures, unless in genetically modified cells, suggesting human and mouse ES cells also have different developmental potential.

Whether the differences between mouse and hES cells are due to species variability, or whether they correspond to different stages of embryonic development, is still unclear.

1.2.3. Epiblast Stem cells

Recently, pluripotent stem cell lines have been established from epiblasts isolated from 5.5 to 6.5 dpc post-implantation mouse embryos (Brons et al., 2007; Tesar et al., 2007). These epiblast stem cells (EpiSCs) are capable of self-renewing *in vitro*

and are pluripotent. They express the core transcription factors known to regulate pluripotency (Oct4, Sox2 and Nanog), maintain their genomic integrity, and are capable of differentiating into various cell types of the three germ layers, both *in vitro* and in teratomas. However, these cells differ from mouse ES cells in many essential aspects and, interestingly, share key features with hES cells.

The gene expression profile of EpiSCs is different from that of mouse ES cells and consistent with its post-implantation epiblast origin. This suggests that EpiSCs and mouse ES cells represent two distinct pluripotent states, comparable to the post-implantation epiblast and the pre-implantation ICM, respectively. Also, EpiSCs do not incorporate into chimeric embryos after blastocyst injection or morula aggregation suggesting that they are incompatible with the pre-implantation embryo environment (Brons et al., 2007; Tesar et al., 2007).

Similar to hES cells, the derivation and self-renewal of EpiSCs requires FGF and Activin/Nodal signalling, whereas in LIF and serum/BMP4 they promptly differentiate (Brons et al., 2007; Tesar et al., 2007). Additionally, EpiSCs and hES cells have common mechanisms of epigenetic regulation of transcription, which are distinct from mouse ES cells (Tesar et al., 2007).

When mouse ES cells are transferred to EpiSCs culture conditions they continue to proliferate and, after passaging, give rise to relatively homogeneous and EpiSCs-like cultures (Guo et al., 2009). After this differentiation step, the cells obtained have stable alterations in gene expression (equivalent to the post-implantation epiblast), growth factor dependence and epigenetic status similar to EpiScs. Indeed, this represents a stable state that cannot be reversed unless by the induced expression of *Klf4* in 2i+LIF culture conditions (Guo et al., 2009).

1.2.4. Embryonic germ (EG) cells

Embryonic germ (EG) cells can be derived from PGCs between 8.5 and 11.5 dpc, using a combination of stem cell factor (SCF), LIF, and FGF in the presence of a feeder layer (Matsui et al., 1992; Resnick et al., 1992). EG cells are morphologically identical to mouse ES cells and express typical ES cell markers such as SSEA-1 and Oct4. Also, they differentiate *in vitro* into a variety of cell types, and upon blastocyst injection, can contribute extensively to chimeric mice including to germ cells (Labosky et al., 1994; Matsui et al., 1992). However, EG cells retain epigenetic features of the original PGCs which are distinct from those of ES cells (Labosky et al., 1994).

1.2.5. Induced pluripotent stem (iPS) cells

Nuclear reprogramming defines a switch in nuclear gene expression from a differentiated cell to an embryonic-like state or to another cell type (Gurdon and Melton, 2008). This can be achieved by somatic cell nuclear transfer, cell fusion, induction of pluripotency by ectopic gene expression, or direct reprogramming. Reprogramming has been an area of great interest due to its potential application for cell replacement without the hazard of immune rejection or the ethical problems of using human embryos.

A great advance in this field was made with the discovery of a method for inducing direct reprogramming to pluripotent stem cells by simply introducing a small number of "reprogramming factors" into differentiated cells (Takahashi and Yamanaka, 2006). Takahashi and Yamanaka showed that the viral transfection of four transcription factors, Oct3/4, Sox2, c-Myc, and Klf4, into mouse fibroblasts under ES-cell culture conditions, is sufficient to direct the appearance of ES-like cells, designated induced pluripotent stem (or iPS) cells. These cells exhibited morphology and growth properties similar to ES cells, expressed ES cell marker genes, generated teratomas

containing tissues from all three germ layers, and following blastocyst injection, iPS cells contributed to mouse embryonic development (but did not give rise to adult chimeras) (Takahashi and Yamanaka, 2006). Additional selection for Nanog expression generates germline-competent iPS cells with increased ES-cell-like gene expression and DNA methylation patterns (Okita et al., 2007).

These four factors were selected from a screen that started with 24 candidate genes (Takahashi and Yamanaka, 2006). Among these, the requirement for Oct4 and Sox2 is not surprising given the well established key role of these transcription factors in self-renewal. Klf4 and c-Myc, however, were more unexpected. c-Myc has several downstream targets that enhance proliferation and transformation, thus probably having a central role in promoting cell growth. Additionally, c-Myc is regulated by STAT3 and has a important functions in self-renewal and maintenance of pluripotency in mouse ES cells (Cartwright et al., 2005). However, given the known oncogenic role of c-Myc, and the observation that mice obtained by injection of iPS reprogrammed with this factor frequently develop tumours (Okita et al., 2007), retroviral introduction of c-Myc should be avoided for clinical application. Klf4 is a Kruppel-like transcription factor that has been associated with both tumour suppression and oncogenesis (Evans and Liu, 2008). Also, Klf4 is highly expressed in undifferentiated mouse ES cells and has a positive effect in self-renewal. It represses p53 directly and in this way it might contribute to activation of Nanog and other ES cell-specific genes (Evans and Liu, 2008; Lewitzky and Yamanaka, 2007).

Since the original derivation of iPS cells, many iPS lines have been established from various differentiated cell types, including adult human cells (Yamanaka, 2008). In these studies, several combinations of reprogramming factors were explored and different ways of delivering them were used. Some of the initial problems of iPS generation are thus now starting to be overcome: new methods are being developed in which c-Myc induced expression is not required (Nakagawa et al., 2008; Wernig et

al., 2008), the reprogramming factors can be introduced without the need for retroviral insertion (Kaji et al., 2009; Okita et al., 2008), and reprogramming efficiency is increased (reviewed in Amabile and Meissner, 2009). Therefore, the use of iPS cells may eventually provide a suitable source of different cell types for patient-specific cell replacement therapy in humans and of disease-specific cell lines to test potential therapeutic agents (Yamanaka, 2008).

1.3. BMP signalling

Bone Morphogenetic proteins (BMPs) are secreted growth factors members of the transforming growth factor-β (TGF-β) superfamily. They have been implicated in a variety of functions, including many cell fate decisions during developmental processes (Chen et al., 2004b), and have a critical role in maintaining ES cell self-renewal (Qi et al., 2004; Ying et al., 2003a).

1.3.1. BMP/TGF-β signalling pathway

BMPs are members of the TGF-β family of molecules and are subdivided into three main groups based on their structure and function (Miyazono et al., 2005): BMP2, BMP4 and the *Drosophila* Decapentaplegic (Dpp) gene product form the BMP2/4 subgroup; BMP5, BMP6, BMP7, BMP8, and the *Drosophila gbb-60A* form the OP-1 group; the growth differentiation factor-5 (GDF-5), GDF-6 and GDF-7 belong to the GDF-5 group. The biological functions of BMPs are diverse since they have distinct spatiotemporal expression profiles and bind to different receptors with different affinities (Miyazono et al., 2005; Shi and Massague, 2003).

Members of the TGF-β superfamily bind to a receptor complex composed of two distinct receptor types known as type I and type II receptors (Heldin et al., 1997; Shi

and Massague, 2003) (Figure 1.4). Both receptor types are required for signal transduction and contain serine/threonine kinase domains in their intracellular portions. The type II receptor kinases are constitutively active; upon ligand binding, hetero-tetrameric complexes composed of two molecules of each receptor type are formed (Shi and Massague, 2003) and type II receptor kinases transphosphorylate the GS (Gly-Ser) domain of type I receptors. Active type I receptors phosphorylate receptor-regulated SMADs (R-SMADs), which in turn associate with common-partner SMADs (Co-SMAD) (Heldin et al., 1997) (Figure 1.4). The R-SMAD/Co-SMAD complexes then translocate into the nucleus and regulate the expression of target genes in cooperation with transcription factors, co-activators and co-repressors. Type 1 BMP receptors are therefore critical for determining the specificity of downstream SMAD signalling.
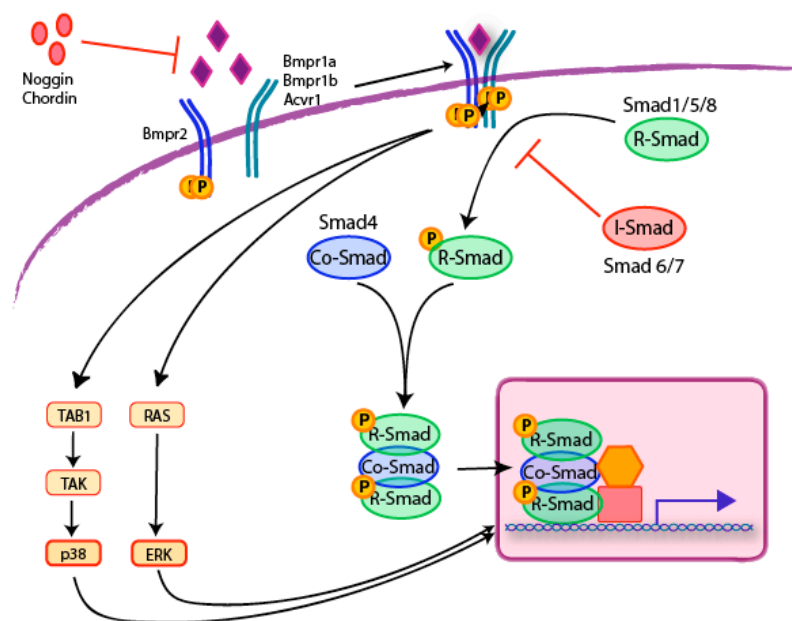
Figure 1.4 The Bone Morphogenetic Protein (BMP) signalling pathway.

BMPs bind to receptor complexes composed of type I and II receptors, which upon activation lead to Smad phosphorylation and ultimately to modulation of the expression of target genes. Alternative transducers of BMPs include p38 and possibly other MAPK pathways.

BMPs interact with three distinct type II receptors: BMP type II receptor (BMPR-II), activin type II receptor (ACVR-II) and activin type IIB receptor (ACVR-IIB).

Regarding type I receptors, three receptors have been shown to bind BMPs: ACVR1, BMPR1A and BMPR1B (also known as ALK2, ALK3 and ALK6) (Miyazono et al., 2005; Shi and Massague, 2003). These receptors have different and dynamic expression patterns during early mouse development (Roelen et al., 1997). *Acvr1* is expressed from the one-cell to the blastocyst stage, *Bmpr1b* transcripts are detected from the one-cell zygote to the uncompacted morula, and *Bmpr1a* is present in blastocysts (Roelen et al., 1997) and is the only type I BMP receptor expressed in the epiblast of post-implantation embryos (Mishina et al., 1995). This suggests that different receptor complexes can be formed at different developmental stages. *Bmpr2*, *Bmpr1a* and *Bmpr1b* mRNAs were also detected in undifferentiated and differentiated embryonal carcinoma and embryonic stem cells (Roelen et al., 1997).

BMP type I receptors specifically phosphorylate the R-SMADs 1, 5 and 8, whereas SMADs 2 and 3 are activated by activin and TGF-β type-I receptors. SMAD 4 is the only Co-SMAD in mammals, and is shared by both BMP and TGF-β/activin signalling pathways (Heldin et al., 1997; Miyazono et al., 2000; ten Dijke et al., 2000). SMAD 6 and 7 are inhibitory SMADs (I-SMADs), which act in opposition to signal transducing R- and Co-SMADs, forming stable associations with activated type I receptors and thus preventing the phosphorylation of R-SMADs (ten Dijke et al., 2000) (Figure 1.4).

BMP2 and 4 preferentially bind to BMPR1A and 1B type I receptors, whereas proteins of the OP-1 group bind to ACVR1 and BMPR1B. Those of the GDF5 group bind to BMPR1B, but not efficiently to other receptors (Miyazono et al., 2005).

ID (inhibitors of differentiation) proteins are one of the most crucial targets of BMPs in many cell types (Hollnagel et al., 1999; Lopez-Rovira et al., 2002; Ogata et al., 1993) and may be responsible for their biological activities (Kowanetz et al., 2004; Nakashima et al., 2001; Norton et al., 1998; Yokota and Mori, 2002). They constitute

a family of helix-loop-helix transcription factors that regulate a variety of cellular responses including cell growth, differentiation, tumorigenesis and neoplastic transformation (Norton, 2000), generally acting as positive regulators of cell proliferation and negative regulators of differentiation (Miyazono and Miyazawa, 2002; Norton et al., 1998).

BMP signaling is precisely regulated at various levels, and an important part of this regulation occurs extracellulary, mainly by BMP antagonists (Figure 1.4). Numerous extracellular BMP antagonists have been described; on the basis of protein sequence alignment these can be categorized into subgroups including noggin, the chordin family, twisted gastrulation, and the Dan family (Yanagita, 2009). BMP antagonists bind with high affinity to BMPs and have been shown to prevent the interaction of BMP proteins with their particular receptors. However, recently published studies have also described agonist activities of formerly recognised BMP antagonists, depending on the cellular context, developmental stage and concentration of the binding protein (Moreno-Miralles et al., 2009).

Even though SMADs are the main transducers of BMP signalling, there is evidence that MAP kinases, particularly p38, act as an alternative pathway downstream of BMPs. The activation of the MAPK pathway by BMP2/4 is mediated by the activation of TGF-β-activated kinase (TAK1) and subsequent phosphorylation of p38 stress-activated protein kinase (Iwasaki et al., 1999; Kimura et al., 2000; Nohe et al., 2004; Shibuya et al., 1998; Yamaguchi et al., 1995). BMP2 has been shown to induce apoptosis through the TAK1-p38 pathway and, interestingly, SMAD 6 and 7 have been shown to have an inhibitory effect on the same pathway (Kimura et al., 2000).

Additionally, BMP signaling establishes extensive communication with other signalling pathways. Highly complex and context-dependent cross-talk has already been reported between TGF-β/BMP and a variety of other pathways, including

MAPK, phosphatidylinositol-3 kinase/Akt, Wnt, Hedgehog and Notch (Guo and Wang, 2009).

1.3.2. BMP signalling in mouse development and ES cells

The BMP family of proteins has pleiotropic roles during development and after birth in many different organisms. BMP ligands, receptors, and signal transducers are fairly conserved among species, with identified homologues in *C. elegans, Drosophila*, and vertebrates.

Besides potent bone inducing properties, BMPs have diverse roles during vertebrate embryogenesis, as already described. Many of the functions of BMPs in mammalian development have been revealed by loss-of-function analysis of BMP ligands and their receptors in the mouse (Chen et al., 2004a; Zhao, 2003). Analysis of null mutations for BMP2 and BMP4 in mice have revealed important roles for these proteins in primordial germ cell (PGC) induction, localization and survival (Fujiwara et al., 2001; Lawson et al., 1999), mesoderm formation and patterning (Winnier et al., 1995), extra-embryonic mesoderm and cardiac development (Zhang and Bradley, 1996), primitive streak formation and left-right patterning (Fujiwara et al., 2002).

Mutations in either type I or type II BMP receptors display more severe phenotypes, suggesting functional redundancy between BMP proteins. *Bmpr2* and *Bmpr1a*-null embryos show a defect in epiblast proliferation and the initiation of gastrulation, having no mesoderm formation and ectopic neural differentiation (Beppu et al., 2000; Di-Gregorio et al., 2007; Mishina et al., 1995), while in *Acvr1a* deficient embryos there is a later defect in gastrulation progression (Gu et al., 1999). SMAD4 deficient embryos also show a similar phenotype having growth retardation, failure to gastrulate and to express mesodermal markers and showing abnormal endodermal development (Sirard et al., 1998).

BMPs were first reported to be involved in the maintenance of stem cells in *Drosophila* where the BMP2/4 homologue Decapentaplegic (Dpp) is specifically required to maintain female germline stem cells and promote their proliferation (Xie and Spradling, 1998).

In mouse ES cells, BMPs have been shown to antagonise neural differentiation and promote differentiation into non-neural fates (Johansson and Wiles, 1995; Tropepe et al., 2001; Ying et al., 2003a; Ying et al., 2003b). However, in combination with LIF, BMP proteins sustain self-renewal and pluripotency (see chapter 1.2.2). This role for BMPs is mediated by activation of the ID proteins via the SMAD pathway (Ying et al., 2003a). The blockage of lineage-specific transcription factors by ID proteins enables the response to LIF/STAT3, an essential step for self-renewal maintenance (Niwa et al., 1998; Smith et al., 1988). However, overexpression of BMP, even in the presence of LIF, leads to differentiation into non-neural fates (Johansson and Wiles, 1995; Ying et al., 2003a), indicating that a balance of SMAD and STAT signalling determines the choice between self-renewal and differentiation (Ying et al., 2003a).

BMP4 has also been shown to be required for ES cell self-renewal by inhibiting the mitogen activated kinase (MAPK) pathways ERK and p38, as inhibitors of ERK and p38 MAPKs mimic the effect of BMP4 on ES cells (Qi et al., 2004). Importantly, by using a p38 inhibitor, Qi and coworkers could derive ES cells from *Bmpr1a*$^{-/-}$ blastocysts, something that had not been possible in the absence of the inhibitors (Qi et al., 2004; Zhao, 2003).

1.4. Cell Competition

Cell competition is a type of cell-cell interaction in which the coexistence of two cell populations with different metabolic properties or growth rates, results in growth of the stronger population at the expense of the weaker one (Diaz and Moreno, 2005; Johnston, 2009; Tyler et al., 2007). This process of recognition and elimination of vulnerable, mispatterned or abnormal cells during tissue growth plays an important role in tissue homeostasis, organ size control, and stem cell maintenance.

1.4.1. Cell Competition in the Drosophila wing

Out-competition of Minute mutants

Cell competition was first described in Minute mosaics in the epithelium of the *Drosophila* wing. Minute (M/+) mutants are deficient in a ribosomal protein (Rp)–encoding gene (Lambertsson, 1998), making them slow-growing but viable. Morata and Ripoll induced clones of wild-type (+/+) cells in the epithelium of the developing wing of M/+ flies, and found that, surprisingly, the +/+ cells could overtake a big part of the wing (Morata and Ripoll, 1975). The finding that sibling cells compete for contribution to the adult tissues led the authors to define cell competition as a struggle between slow-growing M/+ cells (termed "losers") and faster-growing wild-type cells ("winners"). Subsequent work by Morata and Simpson showed that cell competition is the result of local interactions between slow and faster-growing cells, with an intensity proportional to the differences in growth, and that the size and pattern of the adult wing is maintained during this process (Simpson, 1979; Simpson and Morata, 1981). It must be emphasized that the Minute cells are only non-viable when growing next to cells with higher metabolic activity, and, therefore, cell competition relies upon interactions between the two cell types.

Moreno and coworkers further investigated the mechanism for the elimination of M/+ cells in the wing mosaics, showing that the cells with reduced ribosomal activity are eliminated by apoptosis (Moreno et al., 2002). Elimination of the M/+ cells is accompanied by compensatory proliferation of wild-type cells, replacing apoptotic cells during cell competition, thereby maintaining the total number of cells in the developing tissue, and the normal size and shape of the resulting organ.

These experiments demonstrated that the potential for proliferation and survival of a cell, and ultimaely its contribution to fully developed tissues, is determined by the interactions with its neighbours.

Competition in response to Myc levels

More recently, cells with different levels of the growth regulator Myc were also shown to experience cell competition (de la Cova et al., 2004; Moreno and Basler, 2004). Cells with reduced expression of dMyc (Moreno and Basler, 2004), when in contact with metabolically more active cells, are eliminated from the *Drosophila* wing, despite being viable when among cells of the same genotype. Conversely, dMyc over-expression induces super-competition. Cells overexpressing dMyc over-proliferate and expand at the expense of WT surrounding cells, which are eliminated by apoptosis, maintaining total cell numbers (de la Cova et al., 2004; Moreno and Basler, 2004).

Monitoring cell fitness

Interestingly, promoting cellular growth by other mechanisms, such as the overexpression of the phosophoinositide 3-kinase Dp110 or of cyclin D/Cdk4, do not cause supercompetition (de la Cova et al., 2004). Further studies are needed to

clarify which growth pathways induce cell competition, and what distinguishes these from the ones that do not.

In both models of cell competition (M/+ and Myc-dependent), ribosomal biogenesis is impaired, either directly by Minute mutations, or via the action of Myc-target genes. Ribosomal activity may thus be compared between cell types, by a yet unknown mechanism, as an indicator of the relative metabolic state or cell fitness (Johnston, 2009).

Involvement of Dpp/BMP signalling

The cells eliminated by competition display a deficit in growth factor signalling (Moreno and Basler, 2004; Moreno et al., 2002), suggesting that competition for extracellular ligands present in limited concentrations might be the mechanism that supports cell competition. Metabolically less active cells, with a lower ability to translate limiting amounts of survival and growth proteins, would not obtain enough survival factors and consequently would die. Therefore, the capacity to translate limiting growth factors would determine the competitive behaviour of neighbouring cells (Diaz and Moreno, 2005).

Decapentaplegic (Dpp), a BMP homologue, is a key growth and survival factor during development of the *Drosophila* wing (Burke and Basler, 1996; Martin-Castellanos and Edgar, 2002). Cells with a disadvantage in competing for, or in transducing, Dpp show increased expression of the transcription repressor *brinker* and activation of the c-Jun amino-terminal kinase (JNK) pathway, which in turn triggers apoptosis in these cells (Moreno et al., 2002). Constitutive activation of the Dpp pathway enhances the survival of M/+ clones (Moreno et al., 2002) and of lower dMyc-expressing cells (Moreno and Basler, 2004), thus reducing cell competition. Furthermore, in a genetic screen for mutations that induce survival of M/+ cells during cell competition, most of

the genes identified enhance Dpp signalling activity (Tyler et al., 2007). However, mutations in which important Dpp transducers are absent were also able to rescue M/+ clones and no known JNK pathway mutations were identified in this screen (Tyler et al., 2007). Additionally, in other studies no differences in Dpp signalling were observed in competing cells (de la Cova et al., 2004; Li and Baker, 2007) and in the absence of JNK signalling, cell competition still occurred (de la Cova et al., 2004). Therefore, Dpp signalling does seem to play an important role during cell competition, but other mechanisms and signalling pathways are also likely to be involved.

Mechanism of cell competition

Triggering of apoptosis is a consistent mechanism for the elimination of out-competed cells in the epithelium of *Drosophila* wing in both models described (de la Cova et al., 2004; Diaz and Moreno, 2005; Moreno et al., 2002). Recently, it was shown that cell death is indeed essential for cell competition to occur, that it takes place at the boundary between the two cell populations, and that apoptotic cells are engulfed by the neighbouring "winner" cells (Li and Baker, 2007). Surprisingly, more than a passive response to the presence of dying cells, engulfment is required for cell competition to occur. Several engulfment genes are required specifically in wild-type cells for the killing and elimination of M/+ cells, and in the absence of these genes, competition is prevented. Conversely, ectopic activation of engulfment effectors could promote death and engulfment of cells that do not differ in growth rate (Li and Baker, 2007). Thus engulfment genes seem to act downstream of growth differences to eliminate cells with reduced metabolic activity. However, not all out-competed cells are engulfed by winners, since it has been described that many delaminate and are extruded from the epithelium, possibly for phagocytosis by hemocytes (Moreno and Basler, 2004).

A more detailed study of the mechanisms involved in cell competition has been facilitated by the development of an *in vitro* model of cell competition with *Drosophila* cell lines expressing different levels of dMyc (Senoo-Matsuda and Johnston, 2007). In this model, co-culture of cells with different levels of dMyc expression induces apoptosis specifically in lower-expressing dMyc cells and over-proliferation of cells with higher dMyc expression. These effects do not require physical contact but instead are mediated by soluble factors, as they can be induced by medium conditioned by competing cells. This suggests that cells recognize their competitive status using a mutual sensing mechanism involving production of diffusible factors. For the production of these factors the medium has to be conditioned by both cell types, an observation that once again emphasizes the fact that cell competition is instigated by the relative, not absolute, "fitness" of each cell population. The presence of both cell types therefore allows the comparison of metabolic levels and recognition of the winner or loser status.

In summary, cell competition in *Drosophila* appears to proceed through a series of discrete steps: local sensing and recognition of cellular differences, production of diffusible factors, signalling that activates stress pathways and apoptotic suicide of loser cells, activation of an engulfment program, and growth stimulation of winner cells (reviewed in Johnston, 2009).

1.4.2. Niche Occupancy: Stem Cell competition

A different type of competition at the cellular level takes place in the *Drosophila* gonad, where both somatic stem cells and germline stem cells (GSCs) compete for occupancy of the particular niche or microenvironment (Jin et al., 2008; Nystul and

Spradling, 2007). During niche competition, stem cell fitness is also compared and "winners" and "losers" are recognized. However, unlike cell competition in somatic epithelia, the "weaker" cells are not killed but instead displaced from the stem cell niche (reviewed in Johnston, 2009).

The GSC niche in the *Drosophila* developing ovary contains many different cell types in relatively close proximity, including stem cells, their accompanying support cells (the cap cells), and the differentiating stem cell-progeny. GSCs associate directly with cap cells via adherent junctions and are anchored firmly by E-cadherin–dependent interactions (reviewed in Kirilly and Xie, 2007). The tight GSC–cap cell association ensures that the GSC receives proximity-dependent signals, such as Dpp, from the niche. High Dpp activity is required to repress expression of the differentiation-promoting genes *bam* and *bgcn* in the GSC. GSCs divide asymmetrically, allowing one daughter to remain in the niche while the other moves away from the niche and differentiates. Niche residency is essential for a functional GSC and for continued production of new daughter cells. Cells lacking the Dpp-repressed differentiation genes (*bam* and *bgcn*) cannot differentiate and out-compete wild-type GSCs for niche residency, probably due to increased E-cadherin expression (Jin et al., 2008).

Myc expression has also been shown to induce cell competition in the GSC niche: cells with relatively higher expression of dMyc out-compete wild-type stem cells without affecting total stem cell numbers (Rhiner et al., 2009). Also, a naturally occurring cell competition border formed by high dMyc-expressing stem cells and low dMyc-expressing progeny has been described. This may facilitate the concentration of the niche self-renewal factor Dpp in metabolically active, high dMyc-expressing stem cells (Rhiner et al., 2009).

Niche space for somatic follicle stem cells (FSCs), the progenitors of the follicular epithelium that surrounds the germline cysts, is also subject to competition (Nystul and Spradling, 2007).

Niche stem cell competition and epithelial cell competition are thus fundamentally different. In both types of cell competition there is a struggle for supremacy. However, competition for niche occupancy is adhesion-based and leads to differentiation of out-competed cells, whereas competition between disc epithelial cells involves a direct cell-cell comparison of metabolic status determining the death of weaker cells. Nevertheless, in both cases, cell competition is a subtle but important mechanism that ensures optimal organ function and promotes homeostasis.

1.4.3. Cell competition in homeostasis and disease

Competition in mammalian cells

Some evidence suggests that cell competition may also occur in the mouse. *Belly spot and tail* (*Bst*) heterozygous mice (defective for the ribosomal protein L24) behave in a similar way to the Minute mutants, and *Bst*$^{+/-}$ cells show a competitive disadvantage in chimera colonisation (Oliver et al., 2004). Also, competitive-like interactions have been described between highly proliferative transplanted wild-type cells and diseased host hepatocytes during liver regeneration in rats (Oertel et al., 2006)

Cell competition in development and disease

Competition between cells is an efficient mechanism of quality selection. It regulates the balance between proliferation, differentiation and apoptosis in expanding or dynamic tissues, hence controlling cell number and optimizing tissue fitness and organ function. Cell competition may therefore be involved in the homeostatic processes that regulate organ size and quality control during development (Adachi-

Yamada and O'Connor, 2002), as well as in tissue repair and regeneration (Oertel et al., 2006).

However, cell competition may not only be involved in maintaining homeostasis. The expansion of one cell population at the expense of another is a hallmark of cancer. Indeed, both stem cell competition and cell competition are emerging models for tumorigenesis (reviewed in Baker and Li, 2008; and Rhiner and Moreno, 2009). The acquisition of mutations that confer a growth advantage in a subset of cells in a tissue or organ, along with the killing of their neighbours, is a recognized part of tumour progression. Likewise, mutations that increase adhesiveness or "stemness" of winner stem cells could out-compete wild-type cells and lead to tumour formation.

During *Drosophila* embryonic development, naturally occurring discontinuities in the reception of morphogens also lead to apoptosis of cells at the discontinuity boundary by activating the JNK pathway (Adachi-Yamada and O'Connor, 2002; Manjon et al., 2007). This is most likely related to the process of cell competition, and may represent an evolutionarily important mechanism that helps preventing abnormal tissue specification and growth during development.

1.5. Aims of this study

BMP4 signalling via BMPR1A has been shown to be required for the maintenance of the epiblast population in the early embryo, and for self-renewal of pluripotent mouse embryonic stem (ES) cells, by inhibiting differentiation into neural fates.

The first aim of this study was to further explore the role of BMP signalling via BMPR1A in mouse ES cell self-renewal and differentiation. For this purpose, *Bmpr1a-null* ES cells were initially derived and characterised in terms of signalling activation and gene expression patterns. Next, the self-renewal ability of these cells was analysed, as well as their potential to differentiate into different lineages in

embryoid bodies and by monolayer differentiation. A possible compensation mechanism that may be sustaining ES cells in the absence of BMPR1A was also investigated.

A second aim of this study was to analyse the role of BMP signalling in a related but distinct context: cell competition in ES cells. BMP/Dpp signalling has an important role in cell competition in *Drosophila*, both in the somatic epithelium and in the GSC stem cell niche, contributing to the selection of "fitter" cells during development.

Making use of the *Bmpr1a-null* ES cells, in which BMP activation is lower than in wild-type cells, a co-culture system was developed for the study of cell competition. In order to determine the mechanism by which *Bmpr1a-null* cells were being out-competed, the proliferation, apoptosis and differentiation of the two cell types were analysed in co-cultures. The involvement of c-MYC in these competitive interactions, as well as the role of extracellular BMP concentrations and of secreted soluble factors, were also investigated.

This study will therefore help to clarify the function of BMP4 in ES cells and extend its scope beyond the traditional view, investigating new roles for this pathway during mammalian development.

2. MATERIALS AND METHODS

2.1. Tissue Culture Methods

All tissue culture reagents were from Gibco (Invitrogen) unless stated otherwise.

E14Tg2a.IV ES cells were a gift from Austin Smith; Bmpr1a mutant FR88 and FR124 cell lines (Qi et al., 2004) were kindly provided by Yuji Mishina.

2.1.1. ES cell maintenance

ES cells were maintained in an undifferentiated state on 0.1% gelatine-coated flasks (Nunc, Thermo Fisher) in Dulbelcco's Modified Eagle Medium (DMEM) supplemented with 15% (v/v) foetal calf serum (FCS; Sigma), 1X Dulbelcco's non-essential amino acids (DNAA), 2 mM L-glutamine, 0.1 mM β-mercaptoethanol and 1500 U/ml ESGRO$^{®}$ leukemia inhibitory factor (LIF; Chemicon-Millipore). ES cells were routinely dissociated with trypsin-EDTA and frozen for future use in media containing 10% tissue-culture grade dimethyl sulfoxide (DMSO, Sigma), 90% FCS. All cells were cultured at 37ºC in an atmosphere with 5% CO_2.

2.1.2. Blastocyst outgrowths and ES cell derivation

Bmpr1a$^{+/-}$ (Mishina et al., 1995) mice were maintained on a 129SvCC genetic background. For the derivation of ES cells, 3.5 dpc blastocysts were harvested from Bmpr1a$^{+/-}$ intercrosses and cultured on gelatine-coated 4-well plates (Corning Life Sciences) in ES cell medium containing 15% FCS and LIF. After 6-7 days in culture, the ICM of the explants was picked, dissected out from the trophoblast cells, trypsinized and mechanically dissociated, and replated into gelatinised 4-well plates.

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Three to four days later, ES-like colonies were picked, trypsinised and each transferred to a new 4-well plate. Subsequently grown ES colonies were expanded and treated as established ES cell lines. ES cell lines were subsequently genotyped and karyotyped.

For blastocyst outgrowth assays, blastocysts obtained from $Bmpr1a^{+/-}$ intercrosses were cultured on gelatine-coated 48 well plates in serum-free medium with BMP4 and LIF (ESGRO Clonal Grade, Chemicon) or supplemented with 200ng/µl BMP7 (R&D Systems) for 10 days and then stained for alkaline phosphatase activity. Outgrowth assays were carried out by Aida di Gregorio.

2.1.3. Electroporation and establishment of transgenic ES cell lines

Prior to electroporation, ES cells were plated into a gelatine-coated 75 cm^2 tissue culture flask (Nunc, Thermo Fisher) and grown until confluent. Cells were trypsinised and counted using a haemocytometer. Approximately 3 x 10^7 cells were used for electroporation in 1 ml DMEM without any additives. 30 µg of linear DNA was added to the cell suspension in a 0.4cm electroporation cuvette (BioRad) and cells were electroprated using a BioRad Genepulser electroporation unit. Cells were exposed to two pulses; the first was at 240 volts, and the second at 230 volts, with capacitance set to 500 µF. After second pulse the cuvette was tapped on the bench and placed on ice for 10 minutes. The cells were then plated on gelatinised 10 cm culture plates (Nunc, Thermo Fisher) in normal ES-cell growth medium (DMEM with 15% FCS and LIF). One to two days after the electroporation, media containing puromycin (Sigma) at a final concentration of 1.5 µg/ml was added to the plates to select for colonies positive for transgene expression.

After 8 to 10 days of puromycin selection, 20 to 50 colonies were picked into gelatinised, flat bottom 96 well plates. These clones were subsequently expanded in normal ES cell culture conditions (DMEM with 15% FCS and LIF), screened for expression of the transgene, karyotyped and frozen.

2.1.4. Karyotyping ES cells

ES cells were plated into a gelatine-coated 25 cm^2 tissue culture flask (Nunc, Thermo Fisher) and grown until 50-75% confluent. When cells were ready for karyotyping, the medium was changed and 2 hrs later Demecolcine (Sigma) was added directly to the medium to a final concentration of 20 ng/ml. After 1 hr, cells were trypsinized and washed once in PBS. The pellet was resuspended in 5 ml hypotonic solution (0.56% w/v KCl) until a single cell suspension was obtained. Cells were kept in hypotonic solution at RT for exactly 6 mins, then spun and the supernatant removed, leaving a drop to resuspend the pellet in. 5 ml of ice-cold fixative (Methanol: Acetic acid, 3:1 made up fresh) was added dropwise, whilst flicking the tube. After 5 mins at RT, cells were spun down, and the fixing procedure was repeated a further 3 times. After the last fixation step, cells were resuspended in a final volume of 1 ml fixative. Metaphase spreads were obtained by dropping a drop of cell suspension from a height of around 40 cm onto acid-washed slides (treated overnight with 5% acetic acid in ethanol). After drying thoroughly, the slides were stained in 10% Giemsa's (BDH Lab Supplies) in PBS for 20 mins. Slides were then washed gently in tap water twice, with a final wash in dH2O. Slides were left to dry overnight and coverslips were mounted using DPX medium (BDH Lab Supplies). Slides were observed on a normal dissection microscope. Murine nuclei possess a full complement of 40 chromosomes. The chromosomes of 10 to 20 nuclei were counted and a 10% tolerance for variability in chromosome number was accepted.

2.1.5. Alkaline Phosphatase staining

Staining was performed using the Alkaline Phosphatase kit from Sigma. Cells were fixed in Citrate-acetone-formaldehyde fixative solution for 30 seconds and subsequently rinsed in deionised water for 45 seconds. Cells were then incubated with alkaline-dye mixture at room temperature for 15 minutes, protected from the light, rinsed for 2 minutes in deionised water, left to dry, and finally visualised and images acquired using a Leica MZ FLIII dissection microscope with a Leica Image Manager 50 software package.

2.1.6. β-Galactosidase staining

Cells were fixed for 10 minutes at 4°C in fixing solution (0.2% Gluteraldehyde, 2% Formaldehyde in PBT), and after that washed in rinse solution, pH 7.3 (0.1M Phosphate Buffer, 0.1% Sodium Deoxycholate, 0.2% Igepal, 5 mM EGTA, 2 mM $MgCl_2$). Cells were then incubated in stain solution (5 mM Potassium Ferrocyanide, 5 mM Potassium Ferricyanide and 1 mg/ml X-gal in rinse solution) overnight at 37°C in a humid chamber. The day after cells were washed in PBS and visualised and images acquired using a Leica MZ FLIII dissection microscope with a Leica Image Manager 50 software package.

2.1.7. Crystal violet staining

For analysis of cell growth, ES cells were plated in triplicate in 24-well plates at a density of 1×10^4 cells per well. A plate was fixed with glutaraldehyde (Sigma-Aldrich; 0.5% in PBS) 6 hours after plating the cells and then every day for 4 days, and stained with a solution of 0.2% crystal violet (in dH_2O) for 30 minutes at room

temperature (RT). The plates were then gently but thoroughly washed in dH_2O and left to dry. Relative cell number was determined by dissolving the crystal violet stain in 1M acetic acid and measuring its absorbance at 595 nm.

2.1.8. ES cell differentiation

For ES cell differentiation by embryoid body (EB) formation, $2x10^6$ cells were plated per in a 10cm non-gelatinised bacterial petri dish in complete DMEM medium (with FCS and all additives) in the absence of LIF. Medium was changed every two days during the course of differentiation.

For adherent monolayer differentiation in Basal medium, cells were plated on gelatine-coated dishes (Nunc, Thermo Fisher) at low ($1x10^6$ cells per well of a 6-well plate) or medium ($4x10^6$ cells per 6-well) confluency in complete DMEM medium (with FCS and all additives) in the absence of LIF. 6 hours after plating, the cells were washed and serum-free ESGRO Complete™ Basal Medium (Millipore) was added to the wells. Medium was changed every day during the desired period of differentiation.

Monolayer differentiation in the presence of BMPs was performed as previously described with the exception that 10 ng/ml BMP4 (R&D Systems) ou 25 ng/ml BMP7 (R&D Systems) were added to the Basal culture medium.

For monolayer neural differentiation (Ying et al., 2003b), cells were plated at relatively high confluency the day before starting differentiation so that they were around 70-80% confluent and in exponential growth phase at the time of starting the differentiation protocol. ES cells were then trypsinised, washed twice in serum-free medium and counted using a haemocytometer. $1x10^5$ cells were plated per well of

gelatine-coated 6-well plates (Nunc, Thermo Fisher) in N2B27 medium (Stem Cell Sciences). After this, medium was changed every 2 days for the desired period of differentiation.

2.2. Plasmid construction and bacterial transformation

Enzymes and buffers were all from New England Biolabs. For plasmid constriction, 2 µg of insert and 2 µg of vector were digested with appropriate restriction enzymes following manufacturer's instruction. Digestion products were run on 1% agarose gels with the 100 bp or 1 Kb ladders and bands of correct size were purified using QIAquick Gel Extraction Kit (Qiagen) according to manufacturer's instructions. When for blunt-blunt end ligations, the purified DNA was treated for 30 min with 1 µl Antartic Phosphatase at 37°C and then the phosphatase inactivated by 15 min at 65°C. 100 ng of vector and a three-molar excess of insert were ligated with 1 µl (400 U) of T4 DNA ligase overnight at 16°C in a total volume of 10 µl. 2 µl of the ligation mixture were then transformed into XL10-Gold ultracompetent cells (Stratagene) according to the manufacture's protocol. Briefly, 90 µl of XL10 cells were mixed with 4 µl β-mercaptoethanol and 2 µl of the ligation mixture and incubated on ice for 30 min. For heat-shock transformation the cells were incubated at 42°C for 30 seconds and then on ice for 2 min. Pre-heated SOB broth was then added to the cells and these were incubated at 37°C for 1 hour with shaking at 250rpm. The transformation mixture was then plated into Lysogeny Broth (LB) agar supplemented with 50 µg/µl of ampicillin or other appropriate antibiotic and incubated overnight at 37°C. The day after, individual colonies were picked into LB broth containing 50 µg/µl of ampicillin or other appropriate antibiotic and incubated overnight at 37°C with agitation. DNA was extracted from exponentially growing cultures with the Miniprep or Maxipreps kits (Qiagen) according to manufacturer's instructions and final DNA diluted in double

distilled water. The plasmids obtained after each cloning step were subjected to restriction digests to confirm the insertion and sequenced at the MRC Clinical Sciences Centre sequencing facility.

pPyCAGIP-EGFP construct
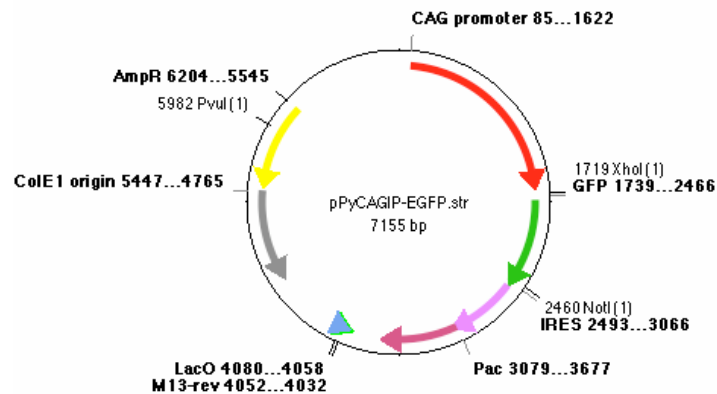
Figure 2.1 Map of the pPyCAGIP-EGFP expression vector.

To generate the PyCAGIP-GFP vector, the EGFP coding sequence was excised from the EGFP-N2 plasmid (Clontech) and inserted into the pPyGAGIP (a gift from Ian Chambers) (Chambers et al., 2003) downstream of the CAG promoter using the XhoI and NotI sites. The PyCAGIP-GFP vector was then grown, linearised by PvuI digestion, purified by phenol-chloroform extraction (Sigma) and electroporated into ES cells.

pPyCAGIP-Bmpr1a-2A-EGFP construct
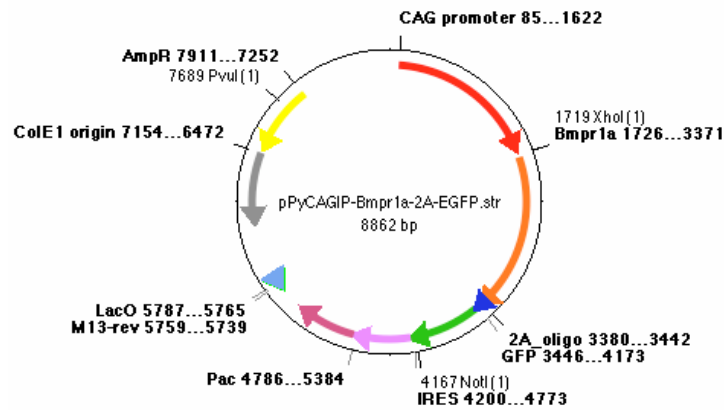
Figure 2.2 Map of the pPyCAGIP-Bmpr1a-2A-EGFP expression vector.

The Bmpr1a coding sequence (excluding the stop codon) was amplified from the Bmpr1a cDNA using the PCR primers Fw *5'*-<u>CTCGAG</u>CAGCAGGACV AGTCATTCAA-*3'* and Rv *5'*-<u>GTCGAC</u>AATCTTTACATCCTGGGATTC-*3'* which introduced flanking restriction sites for XhoI and SalI (underlined). The PCR mix was as follows: 100 ng of cDNA, 0.2 mM of each primer (Sigma-Genosys), 0.2 mM of dNTPs (Roche Diagnostics), 1 µl Formamide (Fluka), 1.25 µl Dimethyl Sulphoxide (DMSO, Sigma), 10 units Pfu Ultra and 5µl 10x Pfu PCR buffer (Promega) in a total volume of 50 µl. PCR conditions included an initial denaturation step at 92 ºC for 3 minutes, followed by 15 amplification cycles of 15 seconds at 92 ºC, 35 seconds at 58ºC and 2 min at 68 ºC. This was followed by a further 15 cycles of 15 seconds at 92 ºC, 35 seconds at 58 ºC and 2 min 25 sec at 68 ºC. Finally there was a final elongation step of 15 minutes at 68 ºC. The resulting PCR products were size-fractionated by electrophoresis in a 1% agarose gel containing ethidium bromide, visualised under ultraviolet light and the DNA band removed from the gel and purified using Qiaquick gel purification kit (Qiagen). The PCR product was then blunt cloned into pCR-Blunt II-TOPO using the Zero Blunt TOPO PCR Cloning Kit (Invitrogen).

The following synthetic oligos with the sequence for the viral 2A, XhoI and NcoI

protruding ends (underlined) and 3' phosphorylated ends were ordered from Sigma-Aldrich: *s 5'*-<u>TCGAG</u>GGCAGTGGAGAGGGCAGAGGAAGTCTGCTAACATGCGG TGACGTCGAGGAGAATCCTGGCCCAT<u>C</u>-P-*3'; as 5'*-<u>CATGGA</u>TGGGCCAGGATT CTCCTCGACGTCACCGCATGTTAGCAGACTTCCTCTGCCCTCTCCACTGCCCTC <u>GAG</u>-P-*3'*. These synthetic oligos were resuspended in TE buffer (10 mM Tris pH7.6, 1 mM EDTA), and 5 µg of each oligo diluted in annealing buffer (100 mM Tris pH7.4, 150 mM NaCl, 10mM EDTA) to a final volume of 100 µl, incubated at 95°C for 10 min and allowed to cool slowly to room temperature for annealing. The annealed oligos were then ligated to XhoI/NcoI pUC21 plasmids where the EGFP had already been subcloned at the XhoI and NotI sites. The resulting plasmids were sequenced (MRC Clinical Sciences Centre sequencing facility) and plasmids with the correct sequence selected for subsequent cloning steps.

The 2A-EGFP fragment was excised and cloned into the pPyGAGIP plasmid (a gift from Ian Chambers) using XhoI and NotI. Finally, the Bmpr1a coding sequence was inserted into the pPyGAGIP-2A-EGFP at the XhoI site using XhoI and SalI sites. The automatic destruction of the SalI/XhoI site allowed determining the direction of the insertion. The PyCAGIP-Bmpr1a-2A-EGFP vector was then grown, linearised by PvuI digestion, purified by phenol-chloroform extraction (Sigma) and electroporated into ES cells.

2.3.   Genomic PCR analysis

2.3.1. Cell lysis for genotyping

Pellets of approximately 1×10^5 cells were digested in 30 to 50 µl of lysis buffer (50 mM Tris-HCl, pH 8.5, 1 mM EDTA, 0.5% Tween-20) overnight at 55°C in the presence of 6 µg/ml Proteinase K. Proteinase K was inactivated by incubation of

lysates at 95 °C for 10 mins. Samples were vortexed, spun down and stored at 4°C.

2 µl of lysate were used per 50 µl PCR reaction.

2.3.2. *Genotyping and sexing ES-cell lines by PCR*

Bmpr1a^{-/-} (Mishina et al., 1995) ES cells were genotyped by PCR using the following primers: F0 (*5'-AGACTGCCTTGGGAAAAGCGC-3'*), and F5 (*5'-GGACTATGGA CACACA ATGGC-3'*) to amplify the wild-type allele (280 bp) and F3 (*5'-CTCTGAATTTCTA GTCCACATCTGC-3'*) and F5 to amplify the mutant allele (190 bp), by standard PCR.

ES cells were sexed by PCR using the primers Ube1xa (*5'-TGGTCTGGACCC AAACGCTGTCCACA-3'*) and Ube1xb (*5'-GGCAGCAGCCATCACATAATCCAG ATG-3'*) that give bands of distinct sizes for the UBE1X and UBE1Y genes on the X and Y chromosome, respectively (Chuma and Nakatsuji, 2001).

The standard PCR reaction mix was made up in a 50 µl total volume as follows: 2 µl DNA lysate, 1 unit of Amplitaq, 10 µmol of each primer (Sigma), 5 µl 10x buffer and 0.2 mM of each dNTP (all Roche Diagnostics). PCR was performed on a Peltier thermal cycler (PTC-100) using the following program: initial denaturation step at 95°C for 5 mins, then 40 amplification cycles of 30 secs at 94 °C, 30 secs annealing (at 60°C for the *Bmpr1a^{-/-}* genotyping PCR and 66 °C for the Ube1 sexing PCR), 45 secs at 72 °C, followed by a final elongation step of 5 mins at 72 °C. The resulting PCR products were size-fractionated by electrophoresis in 1% agarose gels containing ethidium bromide (Sigma) and visualised under UV light.

2.4. RNA and gene expression analysis

2.4.1. RNA extraction and cDNA synthesis

RNA extraction was performed using TRIzol reagent (Invitrogen) and residual genomic DNA was eliminated using the *in column* DNase digestion set (Qiagen) followed by the RNeasy (Qiagen) RNA Cleanup protocol.

1.5µg of total RNA was then reverse transcribed using SuperScript III Reverse Transcriptase system (Invitrogen, Life Technologies). 1 µl of nonamer primers (Sigma), 1.5 µg of total RNA and RNase free water were mixed to a final volume of 10 µl. After a 5 minute incubation at 65°C the samples were put on ice and a mix containing 2 µl of 10 mM dNTP mix (Roche), 2 µl of 0.1 M DTT, 4 µl of 5X first strand buffer, 1 µl of Rnase inhibitor (Roche) and 1 µl of 200 U/µl Superscript III was added. A reaction mixture without the enzyme was also set up as a negative control (designated "-RT"). The mixture was incubated at 25°C for 5 min, 42°C for 1 h 30 min and at 70°C for 15 min. The cDNAs were diluted 1:10 in dH_2O and kept at -20°C until PCR analysis.

2.4.2. Quantitative RT-PCR analysis

Quantitative PCR (qRT-PCR) analysis was carried out using an OpticonII™ DNA engine (MJ Research Inc.) and Opticon Monitor software (MJ Research Inc.). PCR reactions included Sybr-Green PCR Mastermix (Qiagen), 300nM primers and 2µl of diluted cDNA template in a 30µl reaction volume. PCR conditions were as follow: 95°C for 15 min, then 40 cycles of 94°C for 15 sec, 60°C for 30 sec, 72°C for 30 sec, followed by plate-read. Each measurement was performed in triplicate and data normalised according to the expression of selected housekeeping genes (Ohl et al., 2005). RNAs from mouse embryo head, spleen, liver and trophoblast stem (TS) cells

were used as positive controls. Negative controls included reactions without reverse transcriptase (-RT). Sequences of the specific primers used for qRT-PCR amplification are indicated in Table A1 (in Appendixes section).

2.4.3. Microarray analysis

RNA samples were obtained as previously described (section 2.4.1). Sample labelling, hybridization to the mouse Gene 1.0 ST Array system (Affymetrix), and data acquisition were performed by UCL Genomics at the Institute of Child Health. Normalisation and statistical analysis of the resulting array data was performed by Marion Leleu using GeneSpring software.

2.5. Methods for protein analysis

2.5.1. Western Blot

$5x10^5$ cells were plated on gelatin-coated 6-well plates in ES medium, allowed to grow for 24 hours, and then changed to serum-free medium (ESGRO Basal medium, Chemicon) for overnight starvation prior to stimulation with 10, 50, or 500 ng/µl BMP4; 27 ng/ml or 250 ng/ml BMP7; 25 ng/ml FGF4; 25 ng/ml Activin; 50 or 250 ng/µl Noggin; 50 or 250 ng/µl BMPR1A-FC (all from R&D Systems), or 3000units/ml LIF (Chemicon).

Cells were lysed in RIPA buffer (150 mM NaCl, 50 mM Tris pH8, 0.5% sodium deoxycholate, 0.1% SDS, 1% NP-40) containing Complete Mini Protease Inhibitors (Roche). Protein extracts were then quantified usind the Bradford Method (reagent from BioRad). After denaturation (10 min at 95°C), 5 µg of protein were loaded and separated in a 10% acrylamide/Tris-HCl gel in glycine/Tris-HCl buffer. Proteins were then transferred onto a polyscreen PVDF membrane (Amersham) using vertical wet

transfer. Membranes were blocked in 0.1uM sodium orthovanadate / 5% milk in TBS / 0.1% Tween (TBST) for 1 hour before incubation with primary antibody in 5% BSA/TBST (or 5% milk/TBST for c-MYC), overnight at 4ºC. The following antibodies and concentrations were used: rabbit anti phospho-SMAD1/5/8 (1:1000), rabbit anti phospho-SMAD2 (1:1000), rabbit anti phospho-p38 (1:1000), rabbit anti phospho-ERK1/2 (1:2000), rabbit anti phospho-STAT3 (1:1000), rabbit anti α-tubulin (1:1500) (all from Cell Signalling), rabbit anti-cMYC (N-262 clone, 1:200), mouse anti-PCNA (1:5000, Santa Cruz).

On the following day, membranes were washed in TBST and subsequently incubated in peroxidase-conjugated secondary antibody (anti-rabbit, 1:2000, from Santa Cruz or anti-mouse 1:5000, from Sigma) in 5% milk/TBST for 1 hour at room temperature. Finally, membranes were washed in TBST, and chemiluminescence assayed using ECL-Plus Western blotting detection system (Amersham Biosciences) and visualised on a Hyperfilm ECL X-ray film (Amersham Biosciences).

2.5.2. *Immunofluorescence*

For immunofluorescence analysis cells were grown on gelatine-coated glass coverslips. For BrdU incorporation cells in exponential growth phase were exposed to 20 µM BrdU for appropriate periods of time (5, 45 or 120) minutes before fixation.

Coverslips were removed from culture plates, washed in PBS and fixed in 2% paraformaldehyde (PFA) in PBS for 20 min. Fixed samples were washed in PBS and, for intracellular staining, permeabilised with 0.4% Triton X-100 for 5 min. Samples were then incubated in blocking solution [2.5% bovine serum albumin (BSA), 0.05% Tween20, 10% Normal Goat Serum (Vector) in PBS] for 30 min followed by incubation in primary antibody: rabbit anti-cleaved caspase3 antibody

(1:100, Cell Signalling) was diluted in blocking solution; rat anti-BrdU antibody anti-BrdU (Abcam, Cat no. ab6326) was incubated in DNase and DNase buffer (Ambion) diluted in blocking solution. Cells were incubated in primary antibodies for 2 h at room temperature in a humid chamber. Coverslips were subsequently washed in washing buffer (0.2% BSA, 0.05% Tween20 in PBS; 3x5 min) and incubated with secondary antibodies coupled with appropriated fluorophores (Molecular Probes) diluted in blocking solution for 45 min. Finally, cells were washed twice in wash buffer (5 min), once in PBS (3 min) and mounted in Vectashield (Vector) with DAPI (0.1 µg/ml). Samples were visualised using a SP1 Leica laser-scanning confocal microscope. Images were processed using Leica Confocal software and Adobe Photoshop CS2.

2.6. Flow Cytometry (FC) analysis

All flow cytometry (FC) analyses were performed in a FACScalibur (BD Biosciences) with CellQuest software.

2.6.1. GFP and PI analysis of live cells

For the analysis of GFP expression in single and mixed cell cultures, cells were trypsinised, washed and resuspended in FACS buffer (3% FCS in PBS). For propidium iodide (PI) exclusion, 5 µl of 1mg/ml PI solution (Sigma) were added per ml of cell suspension. EGFP expression was assessed using the FL1 detection channel and propidium iodide staining was detected in the FL2 detection channel.

2.6.2. Methods for cell cycle analysis by FC

PI cell cycle profiles

For the analysis of cell cycle profiles by Flow Cytometry, cells were trypsinised and washed twice in cold PBS without Ca^{2+} and Mg^{2+}. Approximately $1x10^6$ cells were pelleted and fixed in 10 ml of ice-cold 70% ethanol (VWR) added dropwise. Cells were washed twice and the resuspended in 1 ml of PI staining buffer (50 μg/ml PI, 0.05% NP-40, 1 mg/ml RNaseA in PBS without Ca^{2+} and Mg^{2+}). Cells were then incubated in PI staining buffer for 30 min in the dark and immediately analysed. PI fluorescence was assessed using the FL2 detection channel.

SNARF-1 analysis

For loading of the carboxyl seminaphthorhodafluor (SNARF-1) dye, SNARF-1 solution (Molecular Probes) was added to the cell suspension at a final concentration of 12.5 μM and incubated for 20 minutes at room temperature in the dark. Cells were then washed twice in 5%FCS/PBS, resuspended in ES cell medium (containing FCS and LIF), and plated in gelatine-coated dishes. SNARF1 is cell permeable in the acetomethyl ester form and diffuses passively into the cells where after deacetylation it is captured by cellular esterases. Once bound intracellularly this dye is symmetrically diluted in the daughter cells after each cell division. Cells were collected at regular time intervals (0. 12, 24, and 48 hours after loading) and resuspended in FACS buffer for analysis. SNARF-1 fluorescence was detected in the FL3 channel and was combined with GFP detection in the FL1 channel.

Analysis of BrdU incorporation

For analysis of BrdU incorporation, cells in exponential growth phase were exposed to 20 μM BrdU during 5, 45 or 120 minutes before fixation.

After incubation with BrdU, cells were trypsinised, washed and fixed in 4% paraformaldehyde (PFA) for 20 min at room temperature. Next, cells were washed in PBS and permeabilised in 0.5% Triton X-100, 5%BSA in PBS for 30 min at room temperature. Samples were then treated with DNase at a final concentration of 300 μg/ml in DNase buffer and PBS for 1 hour at 37°C and subsequently washed and incubated with APC-conjugated anti-BrdU antibody (BD Pharmingen) diluted 1:50 in 1% BSA, 0.05% Tween20 in PBS for 30 min at room temperature in the dark. After this period the cells were washed and resuspended in PBS with 3%FCS for flow cytometry analysis. BrdU staining was detected in the FL4 channel and was combined with GFP detection in the FL1 channel.

2.6.3. Methods for apoptosis analysis by FC

Annexin V staining

Annexin V staining was used to assess the exposure of phosphatidylserine caused by loss of phospholipid asymmetry in the plasma membrane during the initial stages of apoptosis. Cells were harvested and approximately $2x10^5$ cells were pelleted. These were ressuspended in 100 μl of annexin-binding buffer (0.1% BSA in 10mM HEPES, 140mM NaCl, 2.5mM $CaCl_2$, pH7.4) with 5 μl of APC-conjugated annexinV (Molecular Probes) and incubated at RT in the dark for 15 min. After this period, 10 μl of 1 mg/ml PI was added and cells were incubated in the dark for further 5 min. After the incubation period, 400 μl of annexin-binding buffer were added to each sample and these were immediately analysed. AnnexinV-APC fluorescence was detected in the FL4 channel, PI fluorescence was detected in FL2, and GFP detected in the FL1 channel.

Analysis of mitochondrial membrane potential using DiIC1(5)

The use of the the cationic lipophilic dye DiIC1(5) allowed assessing changes in mitochondrial membrane potential, a feature that is lost during apoptosis. When loaded to the cells, this dye accumulates primarily in mitochondria with active membrane potential and is released when this is lost during apoptosis, leading to a decrease in the fluorescence intensity of the dye (Galluzzi et al., 2007b). For the loading of DiIC1(5) (Molecular Probes) into the cells, ES cells were trypsinised and $1x10^6$ cells were ressuspended in 1 ml of pre-warmed medium. DiIC1(5) was added to each sample to a final concentration of 50nM and these were incubated at 37ºC in 5%CO2 atmosphere for 20 minutes. The cells were then washed in pre-warmed PBS, ressuspended in 100 µl of FACS buffer (3%FCS, PBS) to which 5 µl of 1mg/ml PI were added and incubated for further 10 min at 37ºC (the temperature is important to maintain membrane potential). After the incubation period, 400 µl of FACS buffer were added and the samples were immediately analysed. DiIC1(5) fluorescence was detected in the FL4 channel, PI fluorescence was detected in FL2, and GFP detected in the FL1 channel.

To control for the efficiency of the method, one sample was treated with carbonyl cyanide m-chlorophenylhydrazone (CCCP), a compound that disrupts mitochondrial membrane potential. For this sample the procedure was the same as previously described except for CCCP being added to a final concentration of 50µM simultaneously with DiIC1(5).

2.6.4. SSEA-1 staining

Analysis of the SSEA1 surface antigen was performed as follows: cells were trypsinised,washed twice in FACS buffer (PBS, 3% FCS), and $1x10^6$ cells were resuspended in APC-conjugated-SSEA1 antibody (R&D Systems; diluted 1:10 in

FACS buffer) and incubated 30 min at room temperature. The cells were then washed twice and resuspended in 500 µl of FACS buffer for analysis. APC fluorescence was detected in the FL4 channel and was combined with GFP detection in the FL1 channel.

2.6.5. *Fluorescence-activated cell sorting (FACS)*

For the separation of cells in mixed cultures of cells, based on GFP expression, the cells were trypsinised, washed and resuspended in FACS buffer (3%FCS in PBS). PI was added to the cell suspension just before sorting to allow for dead cell exclusion at a final concentration of 5 µg/ml. The cells were then FACS sorted (GFP-positive and GFP-negative populations isolated) using a FACSAria cell sorter (BD Biosciences) at the MRC Clinical Sciences Centre Flow Cytometry facility. The samples were kept on ice or refrigerated during the whole procedure. After sorting, the cells were pelleted and used for RNA or protein analysis.

3. DERIVATION AND CHARACTERIZATION OF *Bmpr1a*^{-/-} ES CELLS

3.1. Introduction

BMP signalling plays important roles during mammalian development, namely determining embryonic patterning and cell fate specification (Kishigami and Mishina, 2005; Kitisin et al., 2007). In mouse ES cells, BMPs block neural differentiation, thereby maintaining self-renewal and pluripotency in combination with LIF (Ying et al., 2003a).

Activation of SMAD1/5/8 is the main pathway downstream of BMP stimulation (Shi and Massague, 2003). However, MAPK signalling, in particular p38 and ERK, can also be activated in response to BMPs (Nohe et al., 2004). Both SMAD activation, and the consequent expression of ID proteins (Ying et al., 2003a), and MAPK inhibition (Qi et al., 2004) have been suggested to be important in the self-renewal activity of BMPs.

BMPR1A is a type I BMP receptor that is specifically bound by BMP4 and BMP2, and with lower affinity by BMP6 and BMP7. Upon ligand binding, BMPR1A forms a heteromeric complex with BMPR2, whose constitutively active kinase phosphorylates and activates BMPR1A, thus initiating intracellular signalling by SMAD1/5/8 or MAPK (Nohe et al., 2004). Both BMPR1A and the other type I BMP receptors, BMPR1B and ACVR1, seem to activate the same downstream signalling pathways, even though different functions during embryonic development have been described (Kishigami and Mishina, 2005; Nohe et al., 2004).

Bmpr1a is expressed in the mouse embryo from the blastocyst stage (Roelen et al., 1997), and is ubiquitously expressed in the epiblast and extraembryonic region at gastrulation (Mishina et al., 1995), whereas the two other type I BMP receptors are only expressed at different stages and in a more restricted manner (Dewulf et al., 1995; Roelen et al., 1994). Even though *Bmpr1a* is the main type I BMP receptor expressed in the blastocyst (Roelen et al., 1997), *Bmpr1a-null* embryos do not show any pre-implantation defects, suggesting that it is not required at this stage, or that other mechanisms can compensate for its absence. In the pre-gastrulation egg-cylinder embryo, BMP signalling via BMPR1A is required to maintain epiblast pluripotency by inhibiting premature neural differentiation (Di-Gregorio et al., 2007). *Bmpr1a-null* embryos also show a defect in epiblast proliferation and in the initiation of gastrulation, having no mesoderm formation (Beppu et al., 2000; Mishina et al., 1995). This phenotype is more severe than that of *Bmp2* or *Bmp4* knockouts, indicating that functional redundancy exits between BMP proteins (Mishina, 2003).

In mouse ES cells, BMPR1A is highly expressed and apparently required for their derivation, given the difficulty in isolating ES cells devoid of this receptor (Qi et al., 2004). However, inhibition of p38 MAPK by SB203580 allowed the derivation of *Bmpr1a-null* ES cells, on feeder cells and using medium supplemented with LIF and serum (Qi et al., 2004), suggesting that the p38 pathway acts downstream of BMPs in maintaining self-renewal.

In order to better understand the role of BMP signalling via BMPR1A in mouse ES cells, ES cells were derived from *Bmpr1a-null* blastocysts and their signalling activation, self-renewal ability, and gene expression profile were analysed. A possible mechanism that may be compensating for the lack of BMPR1A in these cells was also investigated.

3.2. Results

3.2.1. ES cells can be isolated from Bmpr1a$^{-/-}$ embryos

To investigate the importance of BMP signalling via BMPR1A in maintaining self-renewal and pluripotency, the ability to isolate ES cells from *Bmpr1a*$^{-/-}$ embryos was tested. Blastocysts obtained from crosses of *Bmpr1a* heterozygous (+/-) mice (Mishina et al., 1995) were cultured on gelatine-coated dishes in ES cell medium (containing LIF and FCS) to generate outgrowths. Control and *Bmpr1a*$^{-/-}$ outgrowths had a similar morphology and were positive for alkaline phosphatase (AP) activity (a marker of undifferentiated cells) (Figure 3.1A). The outgrowths were then dissociated and replated to establish ES cell lines. Two ES cell lines were obtained and genotyped using primers specific for the *Bmpr1a-null* mutation (Mishina et al., 1995) showing that the 3.5 cells are wild-type (WT) and the C1 are null for *Bmpr1a* (Figure 3.1C). C1-*Bmpr1a*$^{-/-}$ cells could be passaged at least 30 times whilst still retaining the ability to form AP-positive colonies (Figure 3.1A) with characteristic ES-cell morphology (Figure 3.1B), and maintained a normal karyotype (Figure 3.1D). PCR analysis of the *Ube1* gene, which gives different sized products for the X and Y linked copies (primers provided by TB Nesterova), revealed that both cell lines derived are XX (Figure 3.1E).
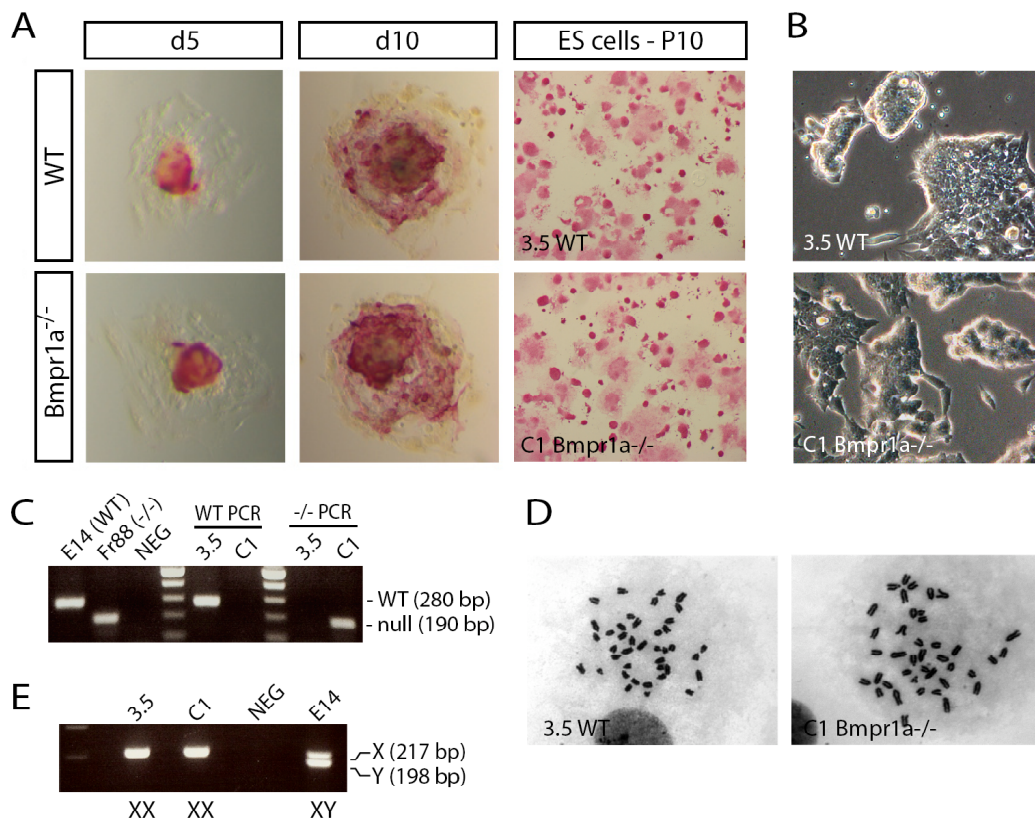
Figure 3.1 ES cell derivation from *Bmpr1a⁻/⁻* mouse embryos.

(A) Blastocysts obtained from *Bmpr1a⁺/⁻* intercrosses were cultured on gelatine-coated dishes in ES cell medium containing FCS and LIF. The outgrowths were subsequently disaggregated and replated in order to establish ES cell lines. Staining for alkaline phosphatase activity shows that *Bmpr1a⁻/⁻* outgrowths after 5 (d5) and 10 (d10) days in culture, and the established C1-*Bmpr1a⁻/⁻* ES cell line have levels of pluripotency similar to wild-type (WT) controls. **(B)** Bright field images of 3.5 WT and C1-*Bmpr1a⁻/⁻* ES cells. **(C)** DNA extracted from the newly derived 3.5 and C1 ES cell lines was analysed by PCR using primers specific for the *Bmpr1a* WT (WT PCR, 280bp PCR product) and mutant (-/-PCR, 190bp product) alleles (Mishina et al., 1995). DNA samples obtained from the previously established E14 and Fr88 (Qi et al., 2004) cell lines were used as positive controls for the WT and mutant alleles, respectively. PCR genotyping showed that 3.5 cells are WT and C1 cells are *Bmpr1a-null* (-/-). **(D)** Metaphase spreads were performed to confirm that the derived cell lines had normal chromosomal numbers. **(E)** PCR analysis of the *Ube1* gene using primers that generate different sized products for the X (217bp) and Y (198bp) linked copies. The XY E14 cell line was used as positive control. Both the newly derived 3.5 and C1 cell lines were XX.

3.2.2. BMP4 cannot signal in Bmpr1a$^{-/-}$ ES cells

BMP signalling cooperates with LIF/STAT3 in the maintenance of ES cell pluripotency and self-renewal (Ying et al., 2003a). Activation of SMAD1/5/8 is the main pathway downstream of BMPs (Shi and Massague, 2003), but p38 and ERK signalling have also been reported to be activated in response to BMP stimulation (Nohe et al., 2004; Ying et al., 2003a).

The activation of intracellular signalling pathways in wild-type (WT) and *Bmpr1a*$^{-/-}$ cells in response to BMP4 and LIF was analysed. Cells were serum starved overnight and then stimulated for 1 hour with 50 or 500 ng/ml of BMP4, LIF (3000 Units/ml), LIF and BMP4 (50 ng/ml), or ES cell medium containing LIF and FCS. The activation of SMAD1/5/8, p38 and ERK was then investigated by immunoblotting.

As shown in Figure 3.2, BMP4 stimulates SMAD1/5/8 phosphorylation in WT ES cells, but not in C1-*Bmpr1a* mutant cells. Even at increased BMP4 concentration (500 ng/ml), which could lead to binding to lower affinity receptors, no phosphorylation of SMAD1/5/8 was observed in *Bmpr1a*-null cells. At this concentration, activation of SMAD1/5/8 in WT ES cells was not increased compared to stimulation with 50 ng/ml BMP4, suggesting saturation of the receptors. No difference in SMAD activation was seen when cells were stimulated with BMP4 in combination with LIF, indicating that the effect of BMP4 in SMAD signalling is independent of that cytokine.

The ability of BMP4 to stimulate p38 and ERK signalling, as shown by Ying et al. (2003), or to antagonise these MAPK pathways in ES cells as suggested by Qi and coworkers (Qi et al., 2004), was next investigated. Neither increased phosphorylation of p38 and ERK1/2 in *Bmpr1a*$^{-/-}$ cells, nor diminished signalling in WT cells were observed upon BMP4 stimulation (Figure 3.2). These observations suggest that BMP4 signalling is not required to block the activity of these pathways as proposed

by Qi et al. (2004). On the contrary, BMP4 stimulation led to a slight increase in p38 phosphorylation in WT but not in *Bmpr1a*^{-/-} cells indicating that activated BMPR1A can also signal via p38 in ES cells. These results show that self-renewal in *Bmpr1a*^{-/-} ES cells cannot be maintained by BMP4, as BMP4 does not stimulate any of the known BMP signal transducers in these cells.
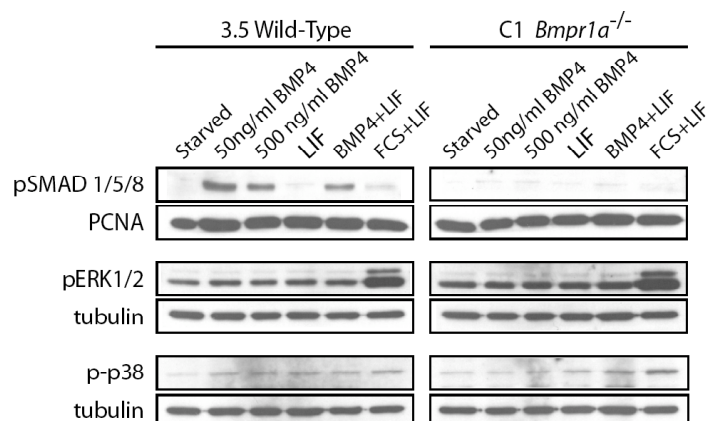
Figure 3.2 Bmpr1a^{-/-} cells do not respond to BMP4 via the Smad1/5/8, p38 or ERK pathways.

C1-*Bmpr1a*^{-/-} and control 3.5 WT cells were starved overnight and subsequently treated for 1 hour with 50 ng/ml BMP4, 500 ng/ml BMP4, LIF (1500U/ml), BMP4 (50 ng/ml) plus LIF, 15% FCS plus LIF or left untreated. Protein lysates were obtained, separated by electrophoresis and analysed by western blotting using antibodies against phosphorylated (p-)SMAD1/5/8, p-p38 and p-p44/42 (ERK1/2). PCNA and α-tubulin were used as loading controls. BMP4 stimulated phosphorylation of SMAD1/5/8 in 3.5-WT but not in C1-*Bmpr1a*^{-/-} ES cells. No significant activation of p38 or ERK MAP kinases by BMP4 was observed in either cell line.

The signalling ability of *Bmpr1a*^{-/-} ES cells was further investigated by looking at the activation of SMAD1/5/8, SMAD2, p38, and STAT3 in response to FCS+LIF, BMP4, LIF, FGF4 and Activin after 1 and 24 hours (Figure 3.3A). As expected, in FCS plus LIF all the pathways analysed were activated, even though this activation was stronger for the shorter pulse. For both stimulus durations, SMAD1/5/8 and p38 activation were lower in C1-*Bmpr1a*^{-/-} compared to 3.5-WT cells in FCS+LIF. As

before, BMP4 induced strong phosphorylation of SMAD1/5/8 in WT cells, but not in *Bmpr1a⁻/⁻* cells, and had no significant effects on the other pathways. Apart from SMAD1/5/8 activation in response to BMP4 and to FCS+LIF, no major signalling differences were observed between the two cell types.
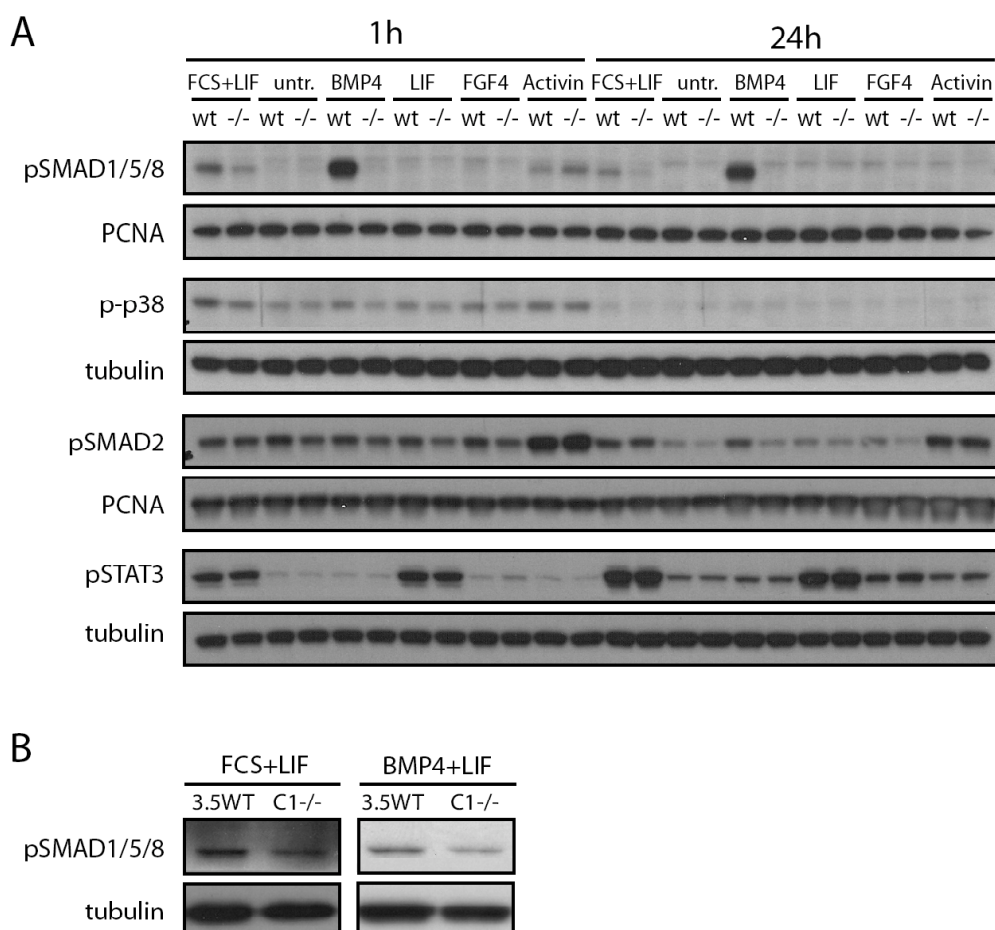
Figure 3.3 Bmpr1a$^{-/-}$ cells do not respond to BMP4 nor show altered response to other stimulus but have SMAD1/5/8 activation after long-term culture in ES-cell maintenance conditions.

(A) Western blot analysis of protein lysates obtained from 3.5-WT (WT) and C1-*Bmpr1a$^{-/-}$* (-/-) cells after overnight starvation followed by 1 or 24 hour treatments with FCS plus LIF (1500U/ml), 10 ng/ml BMP4, 1500U/ml LIF, 25 ng/ml FGF4, 25 ng/ml Activin, or no stimulus (untreated). Specific antibodies were used to detect activation of the SMAD1/5/8, p38, SMAD2, and STAT3 pathways; PCNA and α-tubulin were used as loading controls. C1-*Bmpr1a$^{-/-}$* ES cells showed similar responses to the different stimuli compared to the control WT cells, except for the BMP4 treatment to which the mutant cells did not respond. **(B)** Analysis of SMAD1/5/8 activation by western blotting after 4 days culture in medium containing FCS plus LIF or BMP4 plus LIF. C1-*Bmpr1a$^{-/-}$* ES cells show lower levels but still clear SMAD1/5/8 phosphorylation in both these conditions.

SMAD1/5/8 phosphorylation was also analysed in ES cells maintained for 4 days either in medium with FCS and LIF, or in serum-free medium with BMP4 and LIF. In both these conditions $Bmpr1a^{-/-}$ cells showed a lower, but evident, level of SMAD1/5/8 activation (Figure 3.3B). This observation was particularly surprising for the serum-free condition given that BMP4 cannot stimulate SMAD1/5/8 in the absence of BMPR1A. This indicates that either long-term stimulation with BMP4 can activate low affinity receptors in these cells, or that in BMP4+LIF medium SMAD1/5/8 phosphorylation is indirectly induced, possibly via autocrine stimulation. The residual SMAD1/5/8 activation is probably required and sufficient to maintain the self-renewal of $Bmpr1a^{-/-}$ ES cells.

3.2.3. Bmpr1a$^{-/-}$ ES cells do not have self-renewal defects.

In order to investigate the self-renewal of C1-$Bmpr1a^{-/-}$ ES cells, these and control 3.5-WT cells were plated at a low density on gelatinised plates, grown for 6 days in different culture conditions and finally stained for alkaline phosphatase activity (Figure 3.4A). To overcome the low plating efficiencies of some conditions, the cells were all plated in medium containing FCS and 6 hours later washed in PBS and the media replaced. As shown in Figure 3.4B, quantification of AP staining revealed that the self-renewal ability of C1 $Bmpr1a^{-/-}$ ES cells is not significantly different from that of WT ES cells in any of the conditions tested. The similar levels of AP staining in ES-cell medium (containing FCS and LIF) had initially led us to hypothesise that compensatory factors in the serum could be maintaining self-renewal in $Bmpr1a^{-/-}$ cells. Given that C1-$Bmpr1a^{-/-}$ cells do not respond to BMP4 (Figures 3.2 and 3.3), these cells should not be able to self-renew in serum-free medium with BMP4 (10 ng/ml) and LIF, conditions that are generally sufficient to maintain ES cells (Ying et al., 2003a). However, even in these serum-free conditions (BMP4+LIF), $Bmpr1a^{-/-}$

cells formed an only slightly lower number of colonies, with equivalent levels of alkaline phosphatase activity compared to WT cells (41% high and 53% medium stained in WT cells compared to 35% high and 60% medium stained colonies in null cells). The highest degree of differentiation for both WT and *Bmpr1a*^{-/-} cells was observed in serum-free Basal medium. Conversely, the use of 2i conditions (with MEK and GSK3 inhibitors) maintained highly pluripotent colonies, as shown by the levels of AP staining, which are in fact considerably stronger than those obtained for the usual ES culture conditions (FCS+LIF).
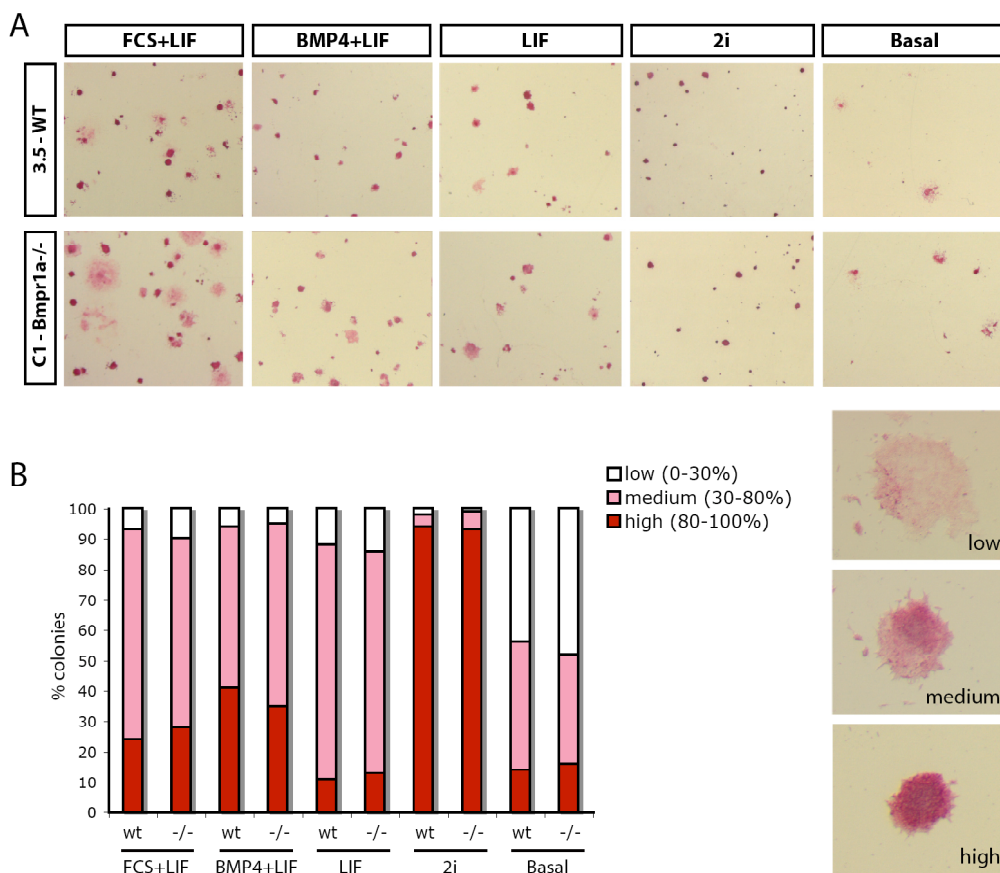
Figure 3.4 Bmpr1a^{-/-} ES cells do not show defects when grown at clonal density.

(A) 3.5-WT and C1-*Bmpr1a*^{-/-} cells were plated at low density (1000 cells per 9.6 cm[2] well of a 6-well plate) in serum-containing medium, and 6 hours later replaced by Basal medium alone or with LIF (1500U/ml), FCS plus LIF, BMP4 (10 ng/ml) plus LIF, or 2i (PD0325901 + CHIR99021). Alkaline phosphatase activity was assessed 6 days after culture in these conditions. **(B)** ES-cell colonies were classified as having high, medium or low alkaline phosphatase activity according to the amount of staining as well as the staining intensity (representative images are shown on the right). For the quantification of alkaline phosphatase

activity 100 arbitrary colonies were assessed. No major differences were observed between the two cell types.

The proliferation and viability of wild-type and *Bmpr1a*^{-/-} ES cells were examined by looking at cell cycle profiles and crystal violet growth curves. As shown in Figure 3.5A, the propidium iodide cell cycle profiles of C1-*Bmpr1a*^{-/-} cells growing in normal ES-cell maintenance conditions (FCS+LIF) were very similar to those of control ES cells. Quantification of the percentages of cells in S and G2/M phases showed that these were comparable for both cell types (3.5-WT: 39.4%±1.9 cell in S phase, 32.2%±2.1 cells in G2/M; C1-*Bmpr1a*^{-/-}: 39.1%±2.6 cells in S, 31.2%±1.6 cells in G2/M phase; Figure 3.5A). The growth of the two cell types in different culture conditions was next analysed by staining the cells with crystal violet, a dye that binds to DNA, allowing visualisation of cell numbers (Figure 3.5B). Quantification of crystal violet staining of WT and C1-*Bmpr1a*^{-/-} ES cells during the first four days after plating at relatively low cell densities showed that the two cell types have similar growth curves both in serum-containing and in serum-free conditions (Figure 3.5C). However, both cell types experienced a moderate decrease in proliferation rate in serum–free medium compared to cells grown in FCS-containing medium (Figure 3.5C).

In summary, even though C1-*Bmpr1a*^{-/-} cells did not respond to BMP4 (Figures 3.2 and 3.3), they could be maintained both in FCS plus LIF and in BMP4 plus LIF conditions, without major differences in morphology (Figure 3.1), alkaline phosphatase activity (Figure 3.4) or proliferation rate (Figure 3.5C), compared to WT cells. Therefore, these results show that C1-*Bmpr1a*^{-/-} ES cells do not have proliferation and self-renewal defects, neither in serum-containing nor in serum-free conditions.
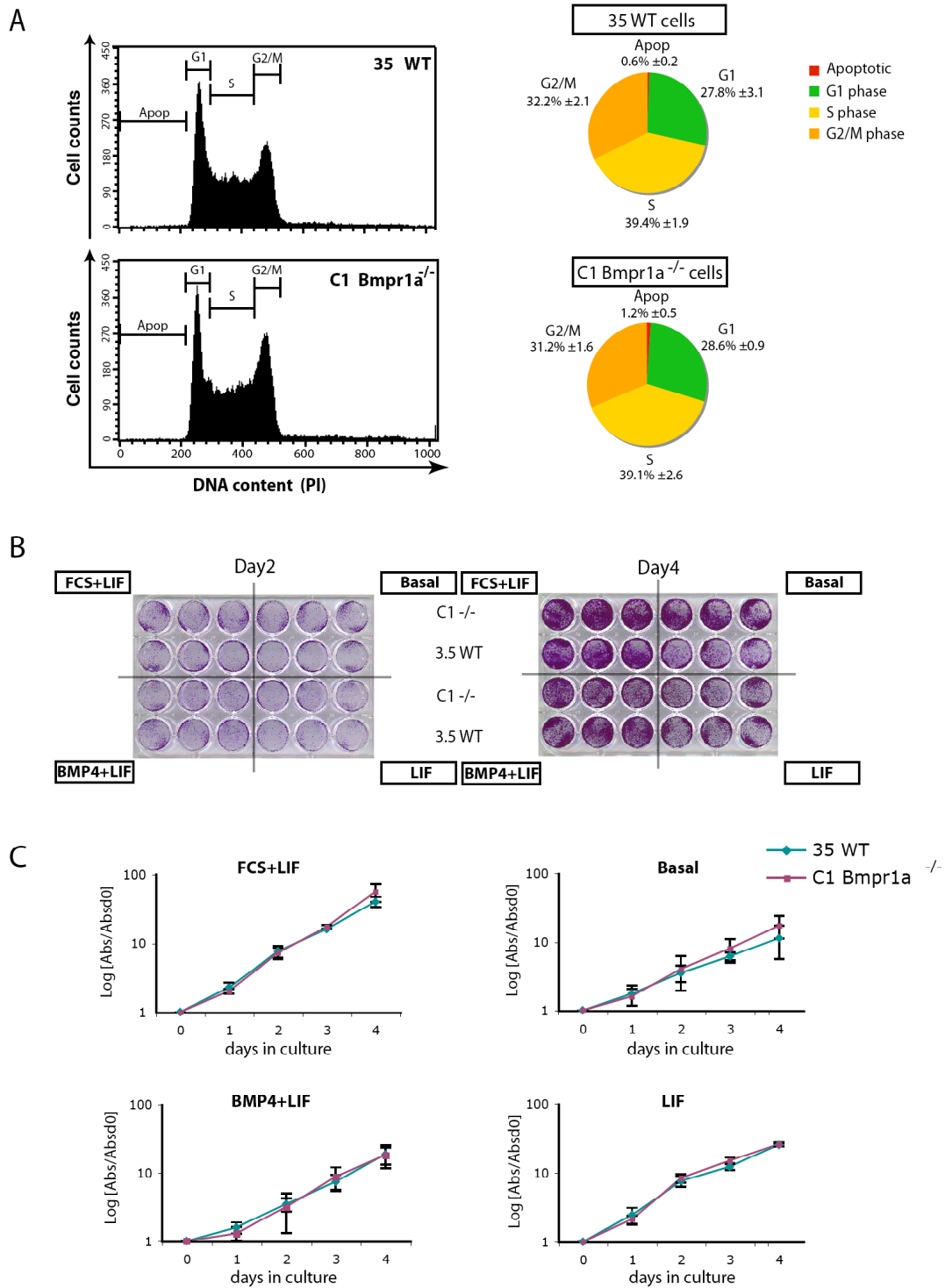
Figure 3.5. *Bmpr1a^-/-* and WT ES cells have similar growth patterns.

(A) The cell cycle profiles of 3.5-WT and C1-*Bmpr1a^-/-* cells were assessed by flow cytometry after propidium iodide staining. The pie charts represent the percentage of cells in each cell cycle phase (average of 3 independent assays +/- standard deviation) in each cell type. **(B)** 3.5-WT and C1-*Bmpr1a^-/-* ES cells were grown in basal medium only or with LIF, BMP4+LIF or FCS+LIF for 4 days. During this period a plate was fixed every day and stained for crystal violet to assess cell numbers. Representative plates of the staining observed at days 2 and 4

are shown. **(C)** Spectrophotometric quantification of the crystal violet staining shows that 3.5-WT and C1-$Bmpr1a^{-/-}$ ES cells have a similar growth rates in all the conditions tested. Data was normalised to the initial number of cells plated (absorbance at 595nm obtained at the beginning of the experiment, d0); data shown as mean +/- standard deviation (s.d.) of 3 independent experiments (n=3).

3.2.4. *$Bmpr1a^{-/-}$ and control ES cells show similar gene expression patterns*

To characterise the pluripotency of C1-$Bmpr1a^{-/-}$ cells in ES-cell maintenance conditions, the expression of the pluripotency markers *Oct4* and *Nanog*, as well as that of lineage specific markers such as *Flk1* and *Nkx2.5* for mesoderm, *Msx1* and *Sox1* for neuroectoderm, *Gata 4* and *6* for endoderm and *Eomes* and *Cdx2* for trophoblast, was analysed by quantitative RT-PCR. As shown in Figure 3.6A, no significant differences were observed between WT and mutant cells in the levels of expression of any of the genes analysed. These results indicate that $Bmpr1a^{-/-}$ ES cells do not have pluripotency defects.

The gene expression pattern of $Bmpr1a^{-/-}$ ES cells was further characterized by looking at global gene expression profiles by Microarrays, and comparing them to those of WT cells. In this experiment, the independently derived ES cell lines Fr88 and Fr124 $Bmpr1a^{-/-}$ cells (Qi et al., 2004), and E14 WT cells were also included. Clustering analysis showed that the gene expression profiles of mutant cells do not greatly differ from those of WT cells (Figure 3.6B). Also, the different WT and null ES cells do not cluster together, indicating that there is no specific change in the gene expression profiles between mutant and control cells.
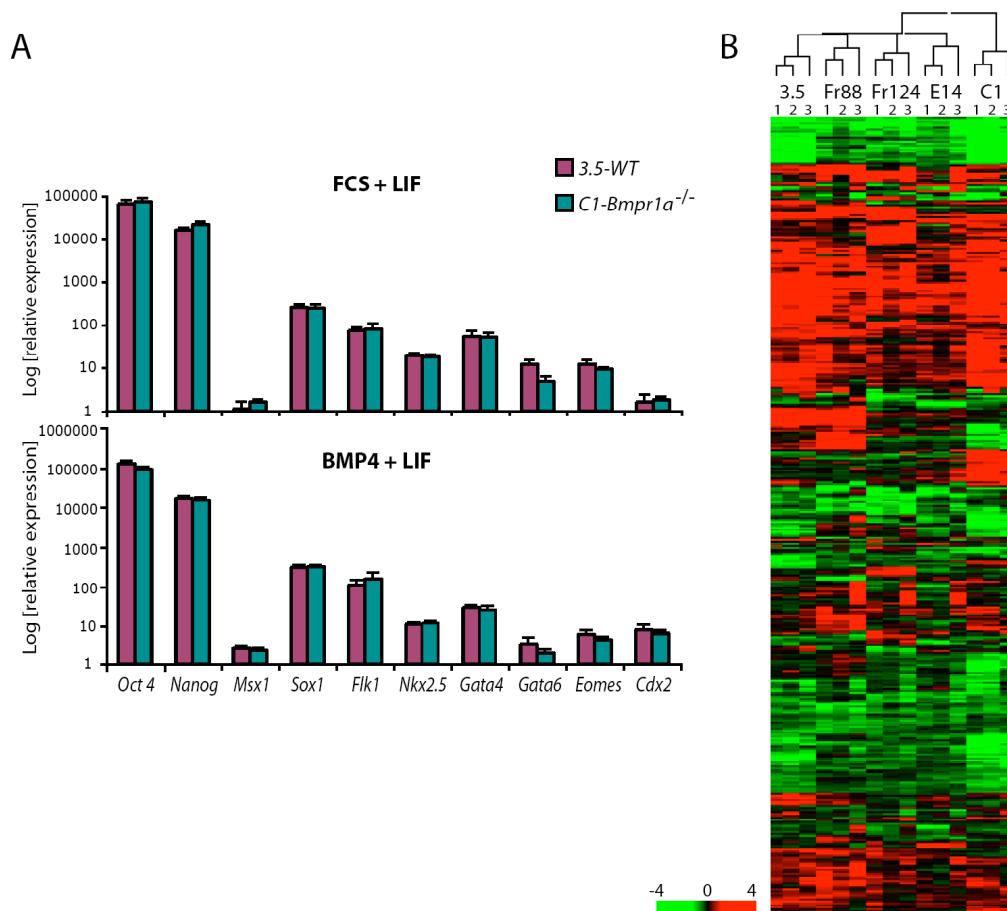
Figure 3.6 Gene expression analysis in *Bmpr1a$^{-/-}$* and WT ES cells

(A) Quantitative RT-PCR analysis of the expression of markers of pluripotency (*Oct4, Nanog*), neuroectoderm (*Sox1, Msx1*), mesoderm (*Flk1, Nkx2.5*), endoderm (*Gata4, Gata6*) and trophectoderm (*Eomes, Cdx2*) in 3.5-WT and C1-*Bmpr1a$^{-/-}$* ES cells grown in FCS+LIF or BMP4+LIF for 3 days. No significant differences in gene expression were observed between the two cell types. Expression values were normalised to *Hmbs and βActin* expression; data shown as mean +/- s.d. (n=3). **(B)** The genome-wide gene expression pattern of 2 independent WT ES cell lines (E14 and 3.5) and 3 *Bmpr1a$^{-/-}$* ES cell lines (C1, Fr88 and Fr124) maintened in LIF and FCS was analysed using the Affimetrix Gene 1.0 Arrays. Hierarchical clustering analysis and heat map representation of the gene expression patterns show that WT and *Bmpr1a$^{-/-}$* ES cell lines do not cluster together.

3.2.5. Bmpr1a$^{-/-}$ *ES cells respond to BMP7 stimulation*

The previous results have suggested that a low activation of the SMAD1/5/8 pathway is sufficient to sustain ES-cell self-renewal. *Bmpr1a*$^{-/-}$ ES cells were able to maintain this low activation even in serum-free conditions (only with BMP4 and LIF), indicating that this can be achieved via an indirect, BMPR1A-independent, mechanism. Therefore, one possibility is that ES cells secrete other BMPs that stimulate SMAD1/5/8 phosphorylation in an autocrine manner. Alternatively, the low levels of SMAD1/5/8 phosphorylation observed could be insufficient to induce the expression of Id genes and so other pathways have to compensate for this.

To address this issue, the expression levels of the BMP target genes *Id1*, *2* and *3* were analysed by quantitative RT-PCR. Consistent with the lower activation of the SMAD1/5/8 pathway in C1-*Bmpr1a*$^{-/-}$ cells, expression of *Id* genes was also reduced in comparison to control cells (Figure 3.7A). However, *Id* expression was still detectable both in serum containing and in serum-free medium with LIF and BMP4. In particular, Id1 expression in BMP4 plus LIF, even though it was considerably upregulated in WT cells, in *Bmpr1a*$^{-/-}$ cells showed levels comparable to those of both cell types in FCS and LIF. This confirms that there is still activation of the BMP pathway in the absence of BMPR1A and that it is sufficient to counteract ES-cell differentiation by inducing the expression of *Id* genes.

The activation of SMAD1/5/8 and expression of *Id1* in *Bmpr1a*$^{-/-}$ ES cells could be explained by autocrine stimulation by BMPs other than BMP4, something that has previously been reported to occur in ES cells (Monteiro et al., 2004). To investigate the mechanism by which *Bmpr1a*$^{-/-}$ cells maintain this activation in the absence of BMP4 signalling, expression of the different type I BMP receptors and of the type II receptor *Bmpr2* were analysed. As seen in Figure 3.7A, no increase was observed in the expression of either receptor in *Bmpr1a*$^{-/-}$ cells, both in FCS plus LIF and in serum-free conditions with LIF and BMP4. Importantly, even though *Bmpr1b*

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expression is hardly detectable in both cell types, *Acvr1* is expressed at considerable levels. The expression of BMP ligands was also examined and, in addition to *Bmp4*, *Bmp7* was also found to be significantly expressed in ES cells. This expression of *Bmp7* was almost 3-fold higher in C1-*Bmpr1a*$^{-/-}$ cells than in 3.5-WT cells in serum-free conditions containing LIF and BMP4 (Figure 3.7A).

BMP7 is a member of the OP-1 subgroup, which binds to, and preferentially activates, the type I BMP receptor ACVR1 (Podos and Ferguson, 1999; ten Dijke et al., 1994). Given that both *Bmp7* and its high affinity receptor are expressed in *Bmpr1a*$^{-/-}$ ES cells (Figure 3.7A), autocrine signalling could be one mechanism by which SMAD1/5/8 activation is stimulated in these cells. The observation that, in contrast to BMP4, BMP7 was capable of activating the phosphorylation of SMAD1/5/8 in *Bmpr1a*$^{-/-}$ ES cells (Figure 3.7B) strengthened this hypothesis.

To further test the hypothesis that BMP7 was capable of maintaining self-renewal in the absence of BMP4 signalling, blastocyst outgrowth assays were carried out by Aida di Gregorio, in our lab. Initially, *Bmpr1a*$^{-/-}$ blastocysts were cultured in the presence of BMP4 and LIF. In these conditions *Bmpr1a*$^{-/-}$ blastocysts showed severely impaired proliferation and variable levels of alkaline phosphatase activity compared to WT blatocysts (Figure 3.7C). This indicates that BMP4 is unable to sustain self-renewal of the inner cell mass in these embryos. The capacity of BMP7 to rescue the defects of *Bmpr1a*$^{-/-}$ blastocyst outgrowths was subsequently analysed. When *Bmpr1a*$^{-/-}$ blastocysts were cultured in the presence of BMP7 and LIF they formed outgrowths that were equivalent both in size and alkaline phosphatase activity to control outgrowths (Figure 3.7C).
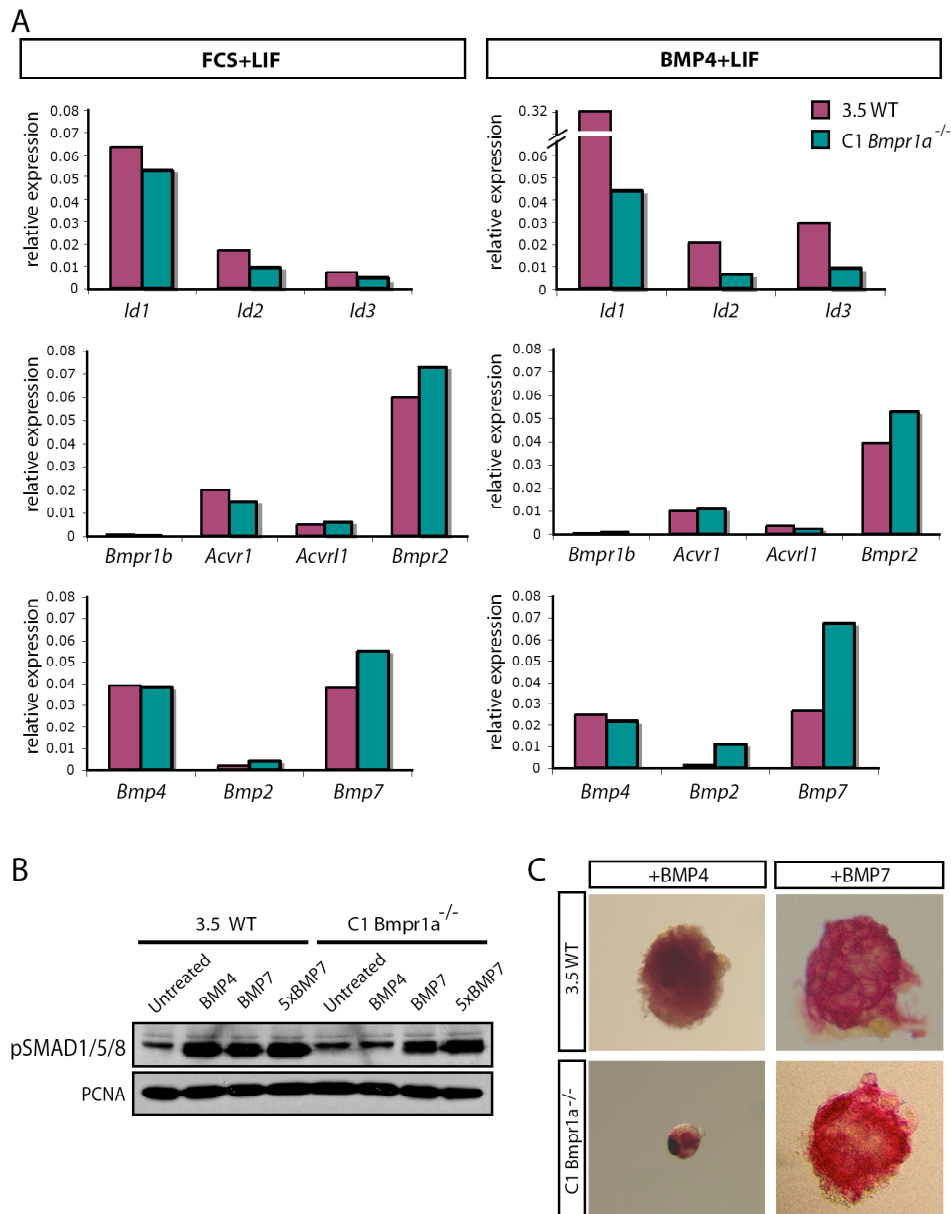
Figure 3.7 Bmpr1a$^{-/-}$ cells upregulate Bmp7 expression, can respond to BMP7 signalling and in this way maintain their self-renewal.

(A) Quantitative RT-PCR analysis of gene expression of BMP targets (*Id1, Id2, Id3*), BMP receptors (*Bmpr1b, Acvr1, Acvrl1, Bmpr2*) and BMP ligands (*Bmp4, Bmp2, Bmp7*) in 3.5-WT and C1-*Bmpr1a$^{-/-}$* ES cells in medium containing LIF and FCS or LIF and BMP4. Mutant cells express BMP target genes and in serum-free medium have increased expression of *Bmp7*. Data was normalised to *Hmbs and PGK1* expression. **(B)** Western blot analysis showing that BMP7 stimulation induces SMAD1/5/8 phosphorylation both in 3.5-WT and C1-*Bmpr1a$^{-/-}$* ES cells. PCNA was used as loading control. **(C)** *Bmpr1a$^{-/-}$* embryos showed self-renewal defects when cultured in serum-free conditions with BMP4, as shown by decreased proliferation and alkaline phosphatase staining. The self-renewal defects were rescued when BMP7 was added to the culture conditions (published in Di-Gregorio et al., 2007).

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These results indicate that signalling by BMPs of the OP-1 subgroup via ACVR1 may be maintaining self-renewal in the absence of BMP4/BMPR1a signalling, both in ES cells and the early embryo.

3.3. Discussion

BMPs are necessary, in combination with LIF, to sustain self-renewal of pluripotent ES cells (Qi et al., 2004; Ying et al., 2003a; Ying et al., 2003b). *Bmpr1a* is the type I BMP receptor most highly expressed in undifferentiated ES cells (Qi et al., 2004; Ying et al., 2003a), in the pluripotent ICM and in the early epiblast of the mouse embryo (Mishina et al., 1995; Roelen et al., 1997). Recently, our group has shown that signalling via BMPR1A is required during mouse development to maintain the pluripotency of the epiblast and prevent precocious neural differentiation of this tissue (Di-Gregorio et al., 2007)

Whether the ability to maintain ES cell self-renewal is restricted to specific BMP ligands and receptors, or is a general property of this family of proteins is still an unanswered question. Furthermore, the mechanisms by which BMPs exert their effects on ES cells and the downstream signalling pathways involved, is also controversial. *Bmpr1a*^{-/-} ES cells had previously only been successfully isolated using a p38 inhibitor (Qi et al., 2004). However, in this study, *Bmpr1a*^{-/-} ES cells were derived in the absence of inhibitors, in *feeder*-free conditions. This can be explained by the fact that all the previous efforts to isolate ES cells from *Bmpr1a*^{-/-} blastocysts had been carried out on a fibroblast *feeder* layer (of which the main function is the production of LIF), with the addition of LIF to the medium (Qi et al., 2004), thus using a double source this cytokine. This may have inhibited the generation of *Bmpr1a*^{-/-} ES cells since there has to be a finely tuned balance between the levels of BMP/SMAD and LIF/Stat3 signalling to maintain self-renewal and pluripotency in ES

cells (Ying et al., 2003a). A more detailed study of the interactions between these two pathways would be needed to understand how this balance determines the maintenance of undifferentiated ES cells. Alternatively a difference in the composition of the serum (mainly in concentration of BMPs) could account for these differences.

Three main mechanisms have been proposed to explain how BMPs sustain self-renewal in combination with LIF. Ying and coworkers have shown that this can be achieved through an increase in Id expression due to SMAD activation (Ying et al., 2003a). Another report suggested that self-renewal is dependent on BMP-mediated inhibition of the p38 and ERK differentiation promoting pathways (Qi et al., 2004). Finally, more recently, a negative feedback mechanism has been reported in which the combined action of BMPs and LIF lead to the up-regulation of *Nanog*, thus preventing BMP-induced mesoderm specification and ES-cell differentiation in general (Suzuki et al., 2006).

In the present study, BMP4 stimulation of ES cells led to an increase of SMAD1/5/8 phosphorylation and a slight activation of p38, but no significant changes in the activation of the ERK pathway were observed. Furthermore, C1-*Bmpr1a*^{-/-} cells did not respond to BMP4 stimulation by any of the known BMP transducers in ES cells. However, these cells could self-renew and retained pluripotency, as indicated by alkaline phosphatase activity, cell cycle profiles and gene expression patterns. This suggests that BMP4 signalling via BMPR1A is not essential for ES cell maintenance, and that its absence is compensated by other mechanisms. To further confirm that *Bmpr1a*^{-/-} ES cells do not show any pluripotency defects, the ability of these cells to give rise to all the different cell lineages should be analysed using *in vitro* differentiation assays (see next chapter) and, ultimately by generation of chimeras.

Although the levels of BMP signalling are decreased in *Bmpr1a*^{-/-} cells, these cells still retain SMAD1/5/8 phosphorylation and expression of the target gene *Id1*, both in

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the presence of FCS and, more surprisingly, in serum-free conditions. This low activation of the SMAD1/5/8 pathway is probably sufficient for these cells to propagate in serum-free media.

Overexpression of the inhibitory *Smad6* or *Smad7* has shown that inhibition of SMAD1/5/8 activation makes BMP4 and serum ineffective in suppressing ES-cell neural differentiation (Gambaro et al., 2006; Ying et al., 2003a). Also, inhibition of p38 using a specific chemical inhibitor had no effect on the ability of BMP4 to support self-renewal in combination with LIF, nor did it inhibit ES-cell differentiation in LIF only (Ying et al., 2003a). These reports, together with the results of the present study, confirm that SMAD1/5/8 activation is the main pathway involved in the maintenance of self-renewal downstream of BMPs.

The lack of self-renewal defects observed in *Bmpr1a$^{-/-}$* cells and in blastocyst outgrowths cultured in serum suggests that secreted factors are compensating for the deficiency in transducing BMP2/4 by these cells. The activation of SMAD1/5/8 observed in *Bmpr1a$^{-/-}$* ES cells cultured in serum free condition, indicates that these factors could be other BMPs produced in an autocrine fashion. It is shown here that Bmp7, a BMP of the OP-1 subgroup, is up-regulated in *Bmpr1a$^{-/-}$* cells, and that it can activate its specific type I receptor Acvr1 inducing SMAD1/5/8 phosphorylation in these cells. Furthermore, addition of BMP7 rescues the self-renewal defects of *Bmpr1a$^{-/-}$* blastocysts grown in serum-free medium. Expression of BMP7 by mouse ES cells, as well as their ability to respond to autocrine loops, have also been previously reported (Monteiro et al., 2004). Therefore, autocrine BMP7 signalling via ACVR1 is the likely mechanism by which SMAD1/5/8 activation is stimulated in *Bmpr1a$^{-/-}$* cells, allowing their maintenance independently of an exogenous source of BMPs. This indicates that, in the absence of BMP4/BMPR1A signalling, members of the OP-1 subgroup, like BMP7, can maintain self-renewal and pluripotency in ES cells.

In the mouse embryo it has been shown that BMP2/4 signalling via BMPR1a is the main source of BMP signalling that inhibits neural fate prior to gastrulation (Di-Gregorio et al., 2007). However, as gastrulation commences, the sources of BMP2/4 in the AVE and extra-embryonic ectoderm start to be displaced from being in contact with the embryo, and BMPs of the 60A subgroup, such as BMP7, start to be expressed (Solloway and Robertson, 1999). Therefore, signalling via ACVR1 at this stage is likely to cooperate with BMP2/4 to reinforce the inhibition of neural induction.

4. DEVELOPMENTAL POTENTIAL OF *Bmpr1a*$^{-/-}$ ES CELLS

4.1. Introduction

During gastrulation, the pluripotent epiblast gives rise to the three primary germ layers, ectoderm, mesoderm and definitive endoderm (Beddington and Robertson, 1999; Tam and Behringer, 1997), the progenitor lineages of all the embryonic tissues. According to the spatio-temporal position of primitive streak (PS) ingression, epiblast cells are exposed to different levels of BMP, Nodal, FGF and Wnt signalling that will determine the patterning of the emerging mesoderm and endoderm cells (reviewed in Tam and Loebel, 2007). Neural and surface ectoderm cells are formed in the anterior region of the epiblast, which does not enter the PS, in response to the low levels of BMPs signalling present in this region.

Pluripotent stem cell lines can differentiate *in vitro* and give rise to derivatives of the three germ layers. Two types of pluripotent stem cells have been derived from the epiblast of early embryos: ES cells, isolated from the epiblast of the blastocyst-stage embryo (Evans and Kaufman, 1981; Martin, 1981), and epiblast stem cells (EpiSCs), obtained from the post-implantation epithelial epiblast (Brons et al., 2007; Tesar et al., 2007). Whereas ES cell self-renewal is sustained by LIF and BMP4 (or serum), EpiSCs require FGF and Activin/Nodal signalling for their maintenance. Although EpiSCs express the core pluripotency transcription factors *Oct4*, *Sox2* and *Nanog*, their global gene expression pattern is different from that of mouse ES cells, and related to their post-implantation epiblast origin (Brons et al., 2007; Tesar et al., 2007). Gene expression and epigenetic regulation analysis have revealed that ES and EpiSCs represent two distinct pluripotent states, comparable to the pre-implantation ICM and the post-implantation epiblast, respectively. ES cells can stably

differentiate into an EpiSC-like state when put in appropriate culture conditions (Guo et al., 2009).

Both ES cells and EpiSCs, in accordance with the developmental potential of their *in vivo* counterparts, are capable of differentiating into various cell types of the three germ layers. This can be accomplished *in vitro*, either via the formation of three-dimensional aggregates known as embryoid bodies (EBs), or by culturing ES cells on extracellular matrix proteins or on supportive stromal layers (reviewed in Murry and Keller, 2008).

The first step in the ES-cell differentiation pathway is generally the formation of a cell population resembling the epiblast of the mouse embryo. When stimulated with Wnt, activin, BMP or serum, ES cells generate a Brachyury-positive, primitive streak-like population (reviewed in Murry and Keller, 2008). Subsequent manipulation of the BMP, Wnt and Nodal pathways in ES-cell cultures modulates differentiation into specific germ layers and cell types in a way that resembles what happens in the embryo (reviewed in Murry and Keller, 2008).

In vivo, when the PS-inducing pathways (Wnt, Nodal and BMP) are not activated, epiblast cells differentiate into the neuroectoderm lineage. ES cells in comparable conditions follow an identical fate. Several neural differentiation protocols have been developed, some involving an initial step of EB formation or co-culture with different cell types, leading to the production of neural precursors with varying efficiencies (Kawasaki et al., 2000; Keller, 2005; Li et al., 1998; Ying et al., 2003b). Importantly, when ES cells are cultured at low density in serum-free medium in an adherent monoculture, the appearance of a high number of *Nestin* (Tropepe et al., 2001) or *Sox1*-positive cells (Ying et al., 2003b) can be observed, indicating neuroectodermal specification. LIF reduces the efficiency of neural differentiation (Ying et al., 2003b), which is in accordance with its role in maintaining undifferentiated ES cells (Chambers and Smith, 2004; Ying et al., 2003a; Ying et al., 2003b), even though it

mainly inhibits the development of non-neural cell types (Tropepe et al., 2001; Ying et al., 2003a) and also maintains cell survival in minimal conditions (Duval et al., 2000; Tropepe et al., 2001; Ying et al., 2008). Furthermore, monolayer neural differentiation is dependent on autocrine FGF signalling and inhibited by BMPs, which induce alternative fates (Tropepe et al., 2001; Ying et al., 2003b). Wnt and activin/Nodal activation also suppress neural differentiation by inducing alternative fates (reviewed in Murry and Keller, 2008). This is consistent with the observation that these pathways are not active in the region of ectoderm induction in the early embryo. Notch signalling is also a key player in establishment of neural progenitor cells (Lowell et al., 2006), mainly promoting cell survival and expansion of the progenitors by blocking their differentiation (Androutsellis-Theotokis et al., 2006). ES-cell derived neural precursors can afterwards be directed to particular neuronal subtypes using factor combinations known to regulate these steps during development (Murry and Keller, 2008).

In addition to the neural lineages, ectodermal precursors also give rise to epidermal cells. The mammalian epidermis is derived from surface ectoderm through a process of cell fate selection and lineage progression that results in the stratified squamous epithelium (Turksen and Troy, 1998). Epidermal epithelial cells can be identified by the expression of keratin intermediate filaments, the composition of which is specific for particular types of epithelial differentiation and development. Keratin 8 (*Krt8*) and *Krt18* are expressed earliest during embryonic development and characterize simple epithelium, the epidermal progenitor marker *Krt19* is expressed in embryonic, simple, and stratified epithelia, and *Krt5* and *Krt14* are expressed in mature keratinocytes. In the epidermis, keratinocytes that undergo terminal differentiation to epidermal cells of the cornified layer start expressing *Krt1*, *Krt10* and proteins of the cornified envelope, such as involucrin (Turksen and Troy, 1998).

ES cells can differentiate to epidermis in embryoid bodies (EBs). Simple epithelial markers (*Krt 8*, *18*, and *19*) can be detected by 12-15 days of EB culture, and expression of *Krt10*, *Krt14* and involucrin from 21 days, indicating that keratinocyte differentiation has taken place (Bagutti et al., 1996). Coraux et al. (2003) developed a protocol in which ES cells were cultured on secreted matrix proteins in the absence of LIF, resulting in the formation of mouse skin *in vitro*, with both epidermal and dermal layers. Addition of BMP4 from the fourth day of culture totally prevented neural commitment while promoting commitment to *Krt8/18*-positive epidermal progenitors, which, in the presence of serum, further differentiated to *Krt5/14*-positive keratinocytes (Coraux et al., 2003). Subsequent studies showed that, besides inducing epidermal differentiation, BMP4 further determines the choice between ectodermal fates by inducing the SMAD-dependent apoptosis of Sox1-positive neural precursors (Gambaro et al., 2006).

BMP4 has thus been suggested to be the main factor regulating the commitment of ESC-derived ectodermal precursors to the alternative ectodermal fates, inducing epidermal differentiation while inhibiting neural specification (Aberdam et al., 2007a; Kawasaki et al., 2000; Munoz-Sanjuan and Brivanlou, 2002).

In this chapter, the pluripotency of $Bmpr1a^{-/-}$ ES cells was tested *in vitro*. With this aim, their ability to differentiate into cells of the three germ layers was analysed using both embryoid bodies and monolayer differentiation protocols.

4.2. Results

4.2.1. Embryoid Bodies of Bmpr1a$^{-/-}$ *cells express markers of the three primary germ layers.*

To test the general ability of C1-*Bmpr1a*$^{-/-}$ ES cells to differentiate into derivatives of the three primary germ layers, the expression of lineage-specific genes was analysed during embryoid body formation. Embryoid bodies are structures that form when ES cells are grown in suspension as aggregates, in the absence of LIF, in which differentiation proceeds into the three germ layers in a manner reminiscent of peri-implantation development (Keller, 1995). EB differentiation recapitulates embryonic events such as the formation of an external endoderm layer, differentiation of a columnar epithelium, formation of a central cavity and mesoderm specification (Coucouvanis and Martin, 1995; Martin et al., 1977). The signals that direct differentiation during EB formation are largely unknown given the undefined composition of the serum in the culture medium, in addition to the factors produced by the cells themselves, which have an increased importance due to their close proximity.

C1-*Bmpr1a*$^{-/-}$ and control 3.5-WT ES cells were thus grown in suspension in the absence of LIF for 12 days, RNA was extracted at days 0 (ES), 4, 8 and 12 of differentiation, and the expression of genes specific of pluripotent ES cells (*Oct4* and *Nanog*), neuroectoderm (*Sox1* and *Pax6*), mesoderm (*Flk1* and *T*), endoderm (*Gata4* and *Gata6*) and trophectoderm (*Cdx2* and *Eomes*) was analysed at these time-points (Figure 4.1).

In ES-cell conditions (FCS+LIF) the expression level of the pluripotency genes *Oct4* and *Nanog* was somewhat higher in C1-*Bmpr1a*$^{-/-}$ compared to control ES cells, even though no increased expression in any of the differentiation markers was observed.
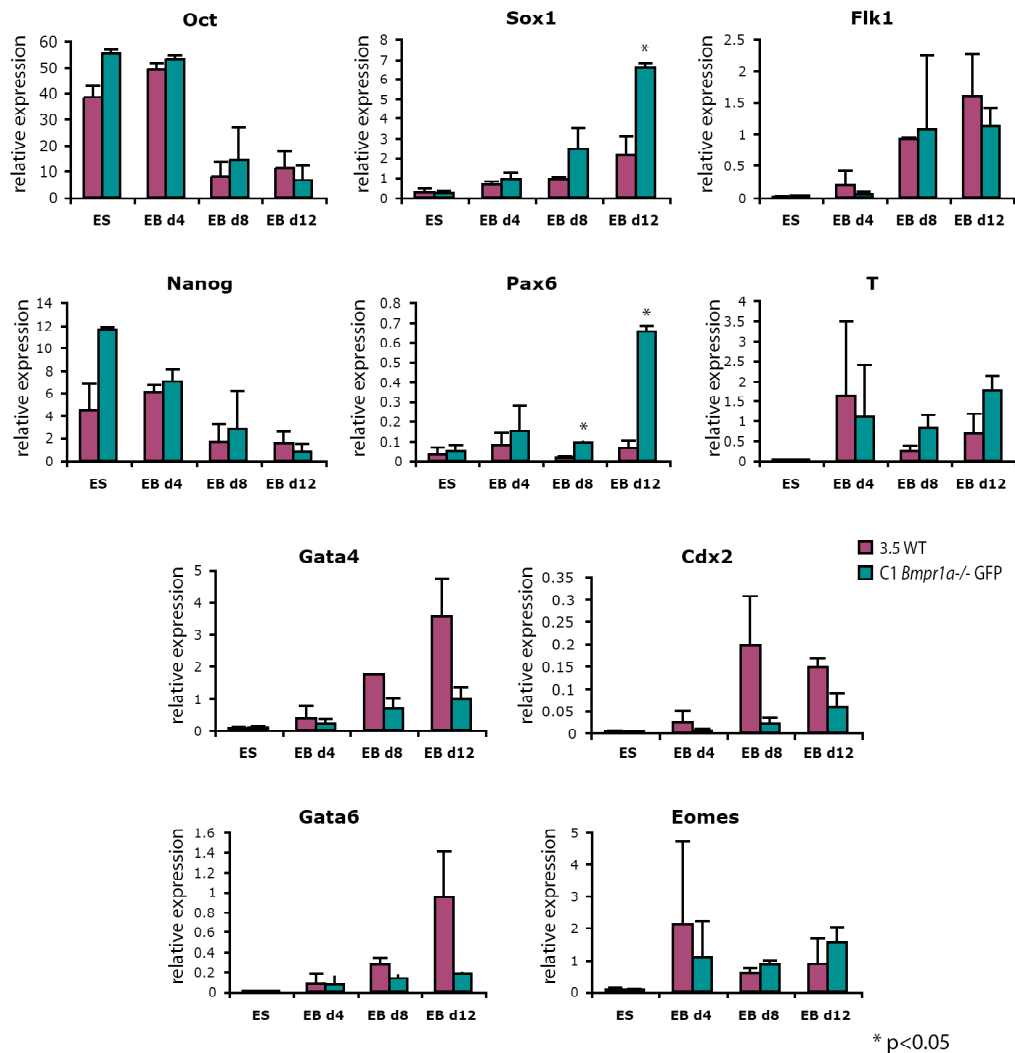
Figure 4.1 *Bmpr1a^{-/-}* ES cells can differentiate into derivatives of the three germ layers but have propensity for neural commitment during embryoid body formation.

C1-*Bmpr1a^{-/-}* and control 3.5-WT ES cells were grown in suspension in the absence of LIF for 12 days and gene expression was analysed by qRT-PCR at days 0 (ES), 4, 8 and 12 of embryoid body (EB) formation. The expression of genes specific of pluripotent ES cells (*Oct4* and *Nanog*), neuroectoderm (*Sox1* and *Pax6*), mesoderm (*Flk1* and *T*), endoderm (*Gata4* and *Gata6*) and trophectoderm (*Cdx2* and *Eomes*) was analysed by qRT-PCR. During differentiation there is an increase in expression of markers of all the different lineages in both cell types but in *Bmpr1a^{-/-}* ES cells the upregulation of neural genes is significantly higher that in WT cells (*, $p < 0.05$, student's t-test). All expression values were normalised to *Hmbs and βActin* expression; data shown as mean +/- s.d. of 3 independent experiments.

As seen in Figure 4.1, during the first four days of EB formation there was no significant increase in expression of lineage specific genes, even though a tendency for increased *T* and *Eomes* expression was observed in both cell types. This suggests that by this stage a PS-like population of cells was starting to appear.

By day 8 of differentiation a considerable downregulation of the pluripotency transcription factors *Oct4* and *Nanog* was observed both in 3.5-WT and C1-*Bmpr1a$^{-/-}$* cells. Simultaneously, increased expression of the neuroectoderm marker *Sox1*, the mesodermal *Flk1*, and the endoderm transcription factors *Gata 4* and *Gata 6* were detected.

From day 8, but most significantly at day 12 of EB formation, a significantly higher expression of *Sox1* (3-fold, $p<0.05$) and *Pax6* (10-fold, $p<0.05$), was detected in C1-*Bmpr1a$^{-/-}$* cells compared to 3.5-WT controls. This suggests a higher commitment of *Bmpr1a-null* cells to the neuroectodermal lineage. At this stage of differentiation, lower expression of *Gata 4* and *Gata 6* were also observed in C1-*Bmpr1a$^{-/-}$* cells, suggesting a concomitant lower propensity for the endodermal fate. This differentiation can be either to definitive endoderm or to visceral endoderm, as BMP signalling has also been involved in commitment to this lineage (Coucouvanis and Martin, 1999). No significant differences were observed in mesodermal commitment between the two cell types.

Since ES cells do not generally give rise to extraembryonic lineages, the increase in expression of *Cdx2* and *Eomes* shown in Figure 4.1 probably does not indicate specification to the trophectoderm lineage. Alternatively, as *Cdx2* is also involved in gut endoderm development (Gao et al., 2009), and *Eomes* is important for mesoderm formation and specification of the definitive endoderm lineage (Arnold et al., 2008), expression of these genes is most likely related to specification to mesoderm and definitive endoderm.

4.2.2. Bmpr1a^{-/-} cells show a tendency for ectodermal specification in basal medium

In another approach to test the developmental potential of *Bmpr1a*^{-/-} ES cells, these and control E14-WT cells were grown in adherent culture, at medium confluency, in serum-free Basal (ESGRO) medium. In this study, besides the C1-*Bmpr1a*^{-/-} cells, the two independently derived ES cell lines Fr88 and Fr124-*Bmpr1a*^{-/-} cells (Qi et al., 2004) were also included.

Unlike what happened in the previous system, in this differentiation model no unknown exogenous factors from the serum were present. However, the relatively high confluency at which the cells were cultured allowed their response to endogenous secreted factors. The gene expression patterns of the E14-WT and the C1, Fr88 and Fr124-*Bmpr1a*^{-/-} cells grown for 3 days in these conditions were analysed by microarrays.

The differentiation pathway chosen by the different cell lines using this system was investigated by looking at a set of candidate genes representative of pluripotent ES cells, EpiSCs, neuroectoderm, epiderm, mesoderm and endoderm cells (Figure 4.2).

During the 3 days of differentiation, the expression of pluripotency genes was significantly downregulated, at approximately the same extent both in WT and *Bmpr1a*^{-/-} cells. This shows that in the absence of LIF or any other exogenous factors, *Bmpr1a*^{-/-} ES cells, similarly to WT cells, do not have the ability to self-renew and therefore start differentiating.

To determine whether ES cells in these conditions differentiate into an EpiSC-like state, the expression of genes characteristic of these stem cells was examined. The epiblast genes *Fgf5* and *Otx2* were indeed expressed at low levels in the ES cell condition and considerably upregulated after 3 days in basal medium, both in *Bmpr1-null* and control cells. However, other markers of EpiSCs such as *Nodal*, *Lefty 1* and

Lefty 2 decreased in expression upon differentiation. It is known that mouse ES cells express high levels of several TGFβ signalling factors, including Nodal, and exhibit strong autocrine SMAD2/3 signalling (Mavrakis et al., 2007). This activation of the Nodal pathway has been suggested to be required for the propagation of mouse ES cells (Ogawa et al., 2007). EpiSCs, which represent a developmentally more differentiated stage, require exogenous Nodal/Activin signals (Brons et al., 2007; Tesar et al., 2007), which were not present in the culture conditions in this assay. In the embryo, high Nodal signalling is also found in the early epiblast and becomes restricted to only a few cells is the posterior epiblast before gastrulation (Conlon et al., 1994). The decreased expression of *Nodal*, *Lefty 1* and *Lefty 2,* with similar levels in *Bmpr1a*$^{-/-}$ and control cells, therefore suggests that both cell types have differentiated into a more anterior-like epiblast population.

Differentiation into the neuroectodermal fate was next investigated. Even though the culture conditions used do not particularly induce neural differentiation, increased expression of specific neural genes such as *Six3* and *Hesx1* was observed in some cell lines. Interestingly, *Nestin* expression was considerably increased in basal medium in all the mutant cell lines, but not in WT cells, suggesting an increased tendency of mutant cells to undergo neural specification.

The expression of cytokeratins gives an indication of the degree of specification to the epidermal fate. After 3 days of LIF and FCS withdrawal, an upregulation of the early epidermal markers keratins 8, 18 and 19 was evident in *Bmpr1a-null* cells but not in E14-WT cells. No differences were observed in expression of the keratinocyte marker *Krt14* (which is present only later in epidermal development).
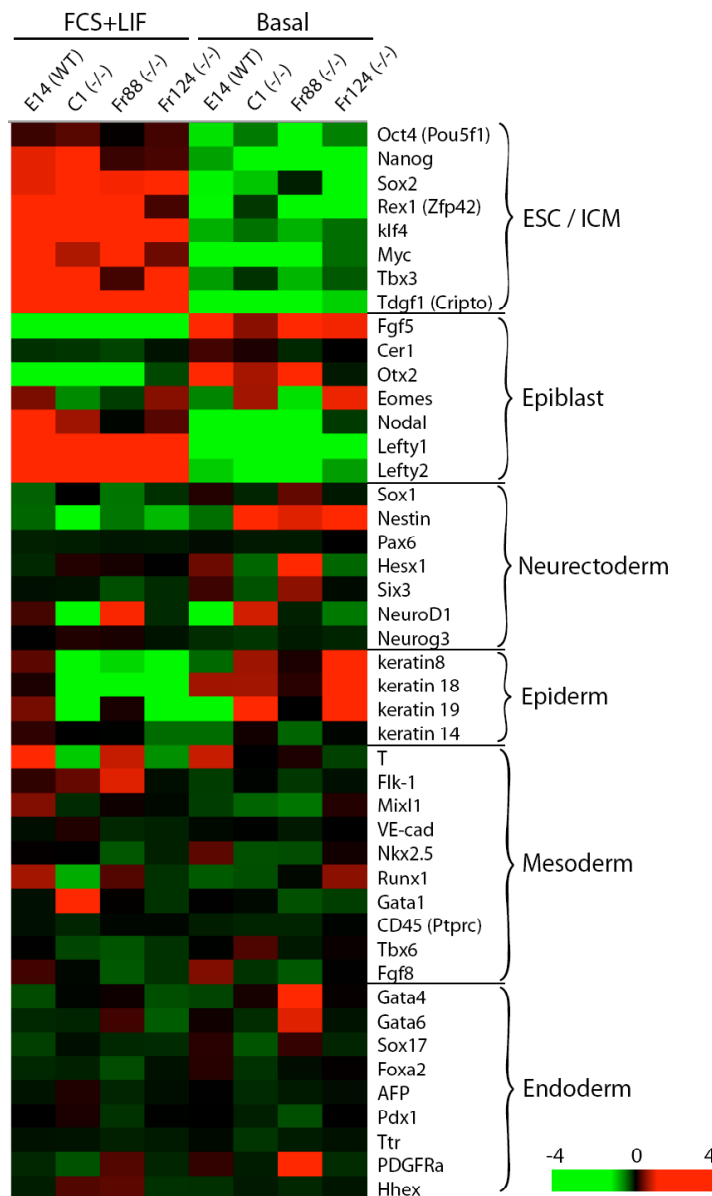
Figure 4.2 *Bmpr1a*$^{-/-}$ **ES cells show increased ectodermal commitment.**

Microarray gene expression heat map representing the relative expression of lineage specific markers in Fr88, Fr124 and C1 *Bmpr1a*$^{-/-}$ cells and control E14-WT ES cells, in ES maintenance conditions (with FCS+LIF), and after 3 days differentiation in basal medium. For each gene (rows) the heat map colours the average gene expression of 3 independent experiments for each sample (columns) in units of standard deviation from the mean across all samples. Increased expression is coloured in shades of red and decreased expression in shades of green according to the scale shown at the bottom of the map.

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Together, these results show that culture of ES cells at medium confluency in serum-free basal medium is permissive of ectodermal specification and that *Bmpr1a-null* cells have a higher tendency to differentiate into this fate compared to WT cells.

Expression of genes related to specification to mesodermal and endodermal fates was also analysed but no significant changes were observed in these culture conditions.

4.2.3. Increased neural specification of Bmpr1a$^{-/-}$ cells during monolayer differentiation

Given the apparent propensity for neural differentiation of *Bmpr1a-null* ES cells observed in EB formation and serum free conditions, this issue was further studied using the monolayer neural differentiation protocol (Ying et al., 2003b). The adherent monoculture of ES cells at low density in serum-free medium minimizes the presence of inductive signals and favours neural specification (Tropepe et al., 2001; Ying et al., 2003b).

As seen in Figure 4.3A, after four days of monolayer differentiation both C1-*Bmpr1a*$^{-/-}$ and control 3.5-WT ES cells exhibited the appearance of colonies with a rosette conformation typical of neuroepithelial cells (Ying et al., 2003b).

Expression analysis revealed a rapid decrease in *Nanog* expression from the first day of LIF and serum withdrawal, whereas *Oct4* expression was maintained for longer (Figure 4.3A). The decrease in expression levels of these pluripotency factors was similar for C1-*Bmpr1a*$^{-/-}$ and 3.5-WT cells.

The expression of the neural progenitor markers *Sox1* and *Pax6* gradually increased during the course of differentiation and by the fourth day they were significantly overexpressed in *Bmpr1a-null* compared to WT cells. Once again, C1-*Bmpr1a*$^{-/-}$ cells

underwent neural commitment more efficiently in differentiation conditions that favour this fate.
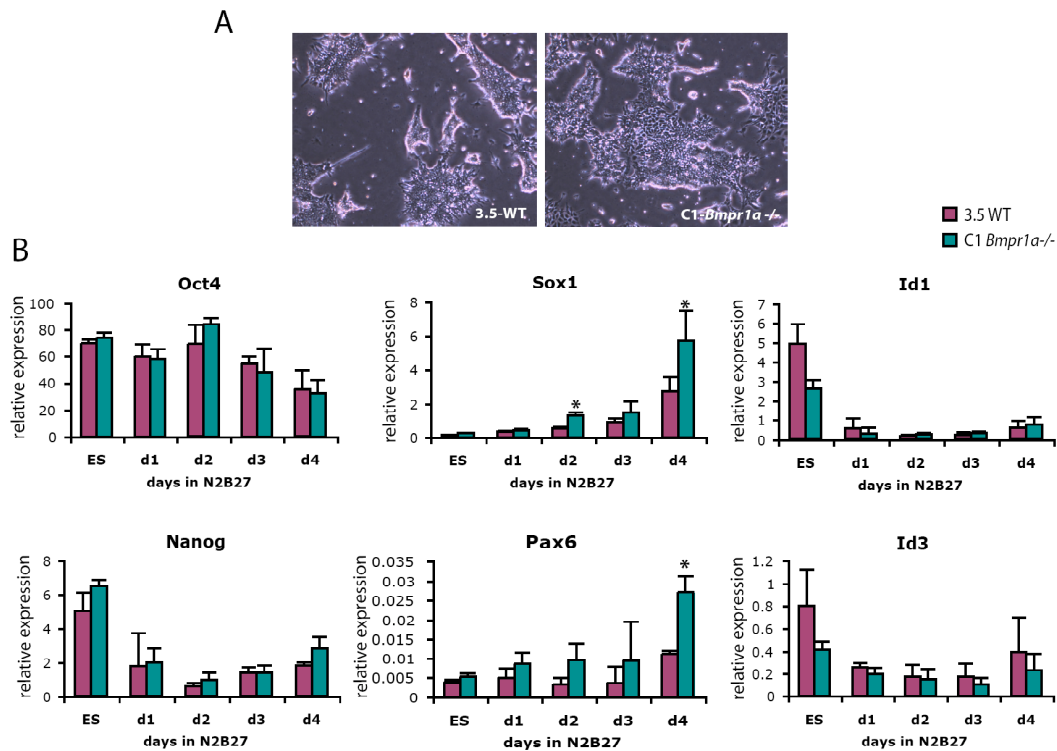
Figure 4.3 *Bmpr1a*$^{-/-}$ ES cells have increased propensity for neural differentiation.

(A) Bright field images of 3.5-WT and C1-*Bmpr1a*$^{-/-}$ ES cells after 4 days of monolayer neural differentiation in N2B27 (Ying et al., 2003b). **(B)** qRT-PCR expression analysis of the pluripotency markers *Oct4* and *Nanog*, neural markers *Sox1* and *Pax6*, and the BMP target genes *Id1* and *Id3*, during the first four days of monolayer neural differentiation. At the fourth day of differentiation C1-*Bmpr1a*$^{-/-}$ cells show upregulation of neural markers compared to control 3.5-WT cells. Expression values were normalised to *Hmbs* and *Hprt1* expression; data shown as mean +/- s.d. (n=3); *, $p < 0.05$, student's t-test.

BMPs inhibit differentiation, particularly neural commitment, by inducing the expression of Id proteins which then act as inhibitors of basic helix-loop-helix (bHLH) neurogenic transcription factors (Norton et al., 1998; Ruzinova and Benezra, 2003; Ying et al., 2003a). As shown in Figure 4.3B, *Id1* and *Id3* expression was relatively higher in 3.5-WT than in C1-*Bmpr1a*$^{-/-}$ ES cells maintained in LIF+FCS, although the expression levels in the mutant cells seem to be sufficient to maintain them in an

undifferentiated state. After LIF and serum withdrawal the levels of expression of *Id1* and *Id3* declined and similarly low expression levels were maintained through the course of differentiation for 3.5-WT and C1-*Bmpr1a*$^{-/-}$ cells. Therefore, the increased neural specification observed in *Bmpr1a*$^{-/-}$ cells by day 4 of differentiation cannot be explained by a lower expression of Id genes at this stage.

Activation of SMAD1/5/8, the main signalling pathway downstream of BMPs and the one that leads to Id induction (Ying et al., 2003a), was also investigated during neural differentiation. In ES-cell maintenance conditions (with serum and LIF), SMAD1/5/8 phosphorylation was higher in WT than in *Bmpr1a*$^{-/-}$ cells. Figure 4.4 shows that after 1 day in serum-free differentiation conditions the levels of SMAD1/5/8 activation have dropped for both cell types but remain relatively higher in WT cells. However, from day 2 of differentiation SMAD1/5/8 phosphorylation was practically undetected both in 3.5-WT and C1-*Bmpr1a*$^{-/-}$ cells. This observation suggests that, in agreement with what was seen with the expression of Id genes, it is the initially lower activation of BMP signalling in mutant cells that facilitates their increased neural specification.

Low activity of SMAD2/3, the other branch of TGFβ signalling, is also thought to play a role in neural induction. Nodal mutant embryos show premature neuronal differentiation of the epiblast (Camus et al., 2006) and in human ES cells inhibition of Nodal signalling promotes neuronal specification, indicating a role for this pathway in controlling early neural development of pluripotent cells (Smith et al., 2008).

For these reasons the levels of pSMAD2/3 were analysed during neural differentiation. Activation of the SMAD2/3 pathway is high in ES cells (Figure 4.4) and as differentiation was initiated the levels of activation dropped significantly. However, from the second day of differentiation, pSMAD2/3 levels increased again and remained relatively high during the next 2 days of the monolayer neural differentiation. Interestingly, SMAD2/3 phosphorylation was lower in C1-*Bmpr1a*$^{-/-}$

compared to WT controls throughout the differentiation protocol, which might also explain the increased neural differentiation observed in these cells.
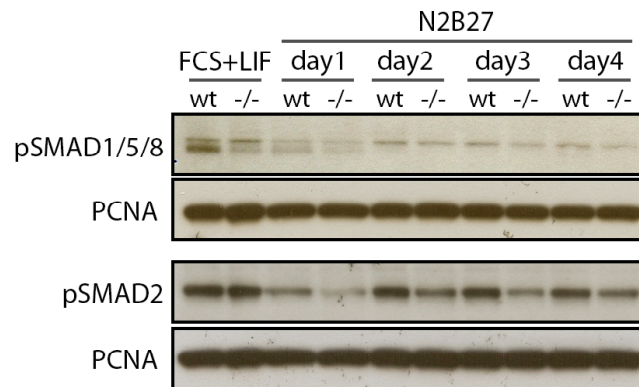
Figure 4.4 Analysis of SMAD1/5/8 and SMAD2 activation during neural differentiation.

Western blot analysis of SMAD1/5/8 and SMAD2 phosphorylation in 3.5-WT and C1-*Bmpr1a*^{-/-} cells in medium containing FCS plus LIF and during the first four days of the monolayer neural differentiation protocol. SMAD1/5/8 activation is higher in 3.5-WT than in C1-*Bmpr1a*^{-/-} cells at the onset of differentiation, but signalling in both cell types decreases to similar levels during the first couple of days after LIF and serum withdrawal. SMAD2 has a more complex activation pattern during neural differentiation. PCNA was used as loading control.

4.2.4. *Epidermal differentiation in response to BMPs*

Finally, differentiation in response to BMPs was studied, again using a monolayer serum-free differentiation system. C1-*Bmpr1a*^{-/-} and control 3.5-WT ES cells were cultured at relatively low confluency in basal medium to which 10 μg/ml BMP4, 25 μg/ml BMP7 or BMP4+LIF were added for 4 days.

In BMP+LIF most cells were maintained in an undifferentiated state as can be seen by the compact colony morphology in Figure 4.5 and the high levels of expression of the pluripotency factors in Figure 4.6. However, both the cell morphology and the expression of some lineage-specific genes (namely *Sox1* and *Nestin*) indicated a somewhat higher amount of differentiation in C1-*Bmpr1a*^{-/-} cells.

In basal medium some rosettes typical of neural progenitor cells were observed both in WT and in *Bmpr1a⁻/⁻* cells (Figure 4.5). Gene expression analysis revealed that in this condition the expression levels of pluripotency factors, primarily *Nanog*, were considerably reduced, and the neural precursor markers *Sox1* and *Nestin* were moderately upregulated (Figure 4.6). This suggested that even though this condition does not particularly favour the neural fate, some degree of neural specification was occurring in the cultures, at a similar extent in both cell types.
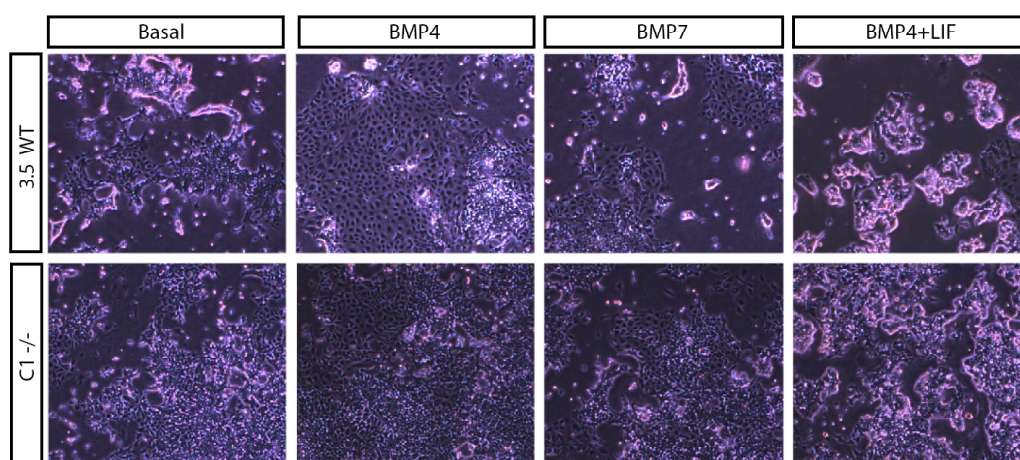
Figure 4.5 Differentiation in the presence of BMP4 and BMP7.

Bright field images of 3.5-WT and C1-*Bmpr1a⁻/⁻* ES cells after 4 days of adherent culture in serum-free basal medium only, or with 10 ng/ml BMP4, 25 ng/ml BMP7 or BMP4 and LIF. BMPs induce the appearance of flattened differentiated cells in both cell types. This type of differentiation is more evident in 3.5-WT cells in the presence of BMP4.

When BMP4 was added at 10 μg/ml to basal media, WT ES cells adopted an epidermal-like morphology and formed big epithelial sheets as shown in Figure 4.5. Compared to the basal condition, WT cells showed decreased expression of *Nestin*, faintly higher levels of expression of *Gata4* and *Flk1*, and a considerable overexpression of the epidermal progenitor markers *Krt18* and *Krt14* (Figure 4.6). This indicates that BMP4 counteracted specification to the neural fate while promoting alternative fates, particularly epidermal commitment. C1-*Bmpr1a⁻/⁻* cells, however, did not show the same kind of response. In these cells, both *Sox1* and

Nestin were still highly expressed in the presence of BMP4 and the expression of keratins was lower than in WT cells. Surprisingly though, the C1-*Bmpr1a*$^{-/-}$ cells did show a degree of response to BMP4 stimulation since morphological differences could be seen in these cells compared to culture in the absence of BMP4 and some expression of *Krt14* and *Krt18* was detected. This may indicate that the prolonged exposure to BMP4 may facilitate binding to other low-affinity type I BMP receptors, eliciting a much weaker response.

Similar to the effect of BMP4, exposure of WT ES cells to BMP7 induced the generation of cells with an epithelial morphology (Figure 4.5) and an upregulation in endodermal, mesodermal and epidermal genes (Figure 4.6). Unlike what happened with BMP4 treatment, *Bmpr1a*$^{-/-}$ cells were able to respond to BMP7 and adopted a similar fate to the WT controls.
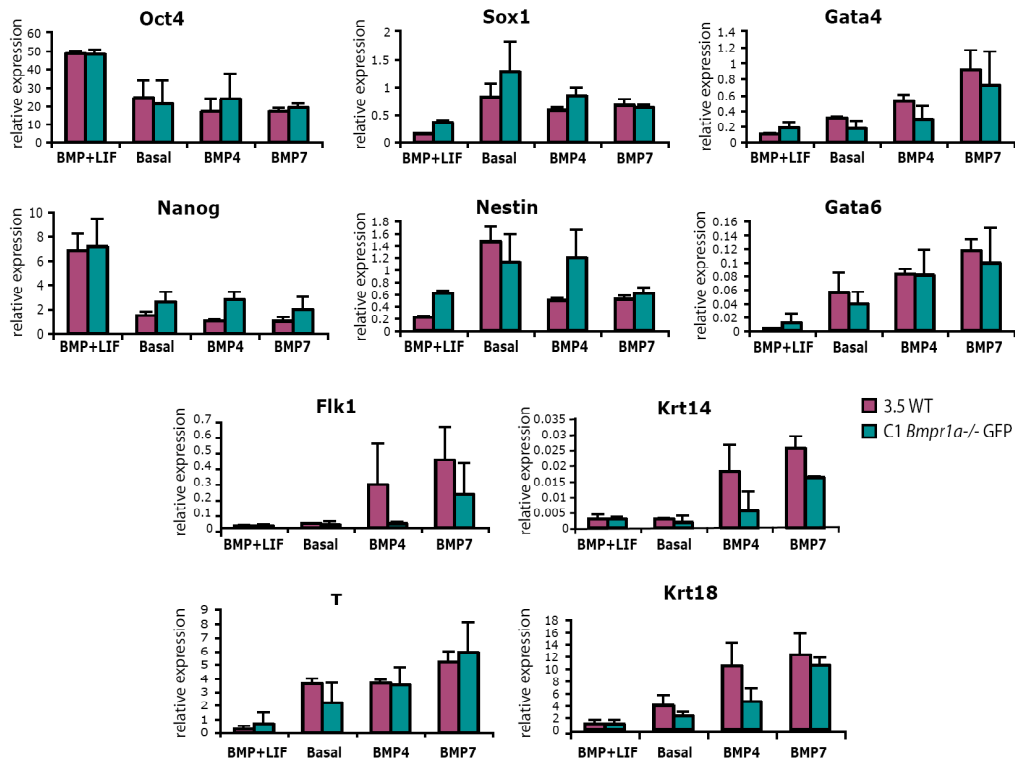
Figure 4.6 Gene expression analysis during ES-cell differentiation induced by BMP4 and BMP7.

qRT-PCR expression analysis of the pluripotency markers *Oct4* and *Nanog*, neural markers *Sox1* and *Nestin*, endoderm markers *Gata4* and *Gata6*, mesodermal markers *Flk1* and *T* and the epidermal markers *Krt14* and *Krt18* at the fourth day of differentiation in adherent culture in serum-free basal medium only or with BMP4+LIF, BMP4 or BMP7. The expression analysis shows that addition of BMP4 to the medium represses neural differentiation and promotes non-neural differentiation. C1-*Bmpr1a*$^{-/-}$ cells upregulate epidermal markers in the same way as control 3.5-WT cells in the presence of BMP7 but not in the presence of BMP4. Expression values were normalised to *Hmbs and βActin* expression; data shown as mean +/- s.d. (n=2).

4.3. Discussion

BMPs have an important role in regulating the fate of different cell types, both during mouse embryonic development (Di-Gregorio et al., 2007; Kishigami and Mishina, 2005; Kitisin et al., 2007; ten Dijke et al., 2003) and in *in vitro* ES cell differentiation (reviewed in Gadue et al., 2005; Murry and Keller, 2008).

In the epiblast of the early embryo, signalling via BMPR1A is required to maintain pluripotency and prevent premature neural differentiation (Di-Gregorio et al., 2007). At a slightly later stage, *Bmpr1a-null* embryos show a defect in the initiation of gastrulation, not being able to form mesoderm or endoderm (Beppu et al., 2000; Mishina et al., 1995). BMP4 has also been suggested to be the main factor regulating the cell fate choice of ectodermal precursors, preventing neural specification while inducing epidermal differentiation (Aberdam et al., 2007a; Kawasaki et al., 2000; Munoz-Sanjuan and Brivanlou, 2002)

Bmpr1a$^{-/-}$ ES cells do not respond to BMP4 but are able of self-renewing and can therefore be maintained in culture in a pluripotent state (see Chapter 3). In this chapter, the developmental potential of *Bmpr1a*$^{-/-}$ ES cells was analysed by studying their ability to differentiate into different lineages *in vitro*.

C1-*Bmpr1a*$^{-/-}$ cells were able to give rise to derivatives of the three germ layers in embryoid bodies. However, higher levels of expression of the neural progenitor markers *Sox1* and *Pax6* suggested a greater propensity for the *Bmpr1a-null* cells to commit to the neuroectodermal lineage. When cultured in serum free basal medium, ectodermal specification was favoured in *Bmpr1a-null* cells, as indicated by the increased expression of *Nestin* and of the early epidermal markers keratins 8, 18 and 19. *Bmpr1a*$^{-/-}$ cells have a lower activation of the BMP signalling pathway, therefore the observed tendency of these cells to undergo neural specification agrees with the requirement for low BMP signalling during neural induction in the embryo (Arnold and

Robertson, 2009; Hemmati-Brivanlou and Melton, 1997; Kishigami and Mishina, 2005). On the other hand, it was shown here that C1-*Bmpr1a$^{-/-}$* cells also express higher levels of *Bmp7* and can respond to BMP7 stimulation (see previous chapter). In case BMP7 has an epidermal-inducing activity similar to the one described for BMP4 (Aberdam et al., 2007b; Coraux et al., 2003), response to this auto-stimulatory signal could be responsible for the observed epidermal specification. The upregulation of both neural and epidermal markers in basal medium may therefore indicate that both these ectodermal fates were being favoured in these culture conditions, probably depending on specific local microenvironments. Alternatively, *Bmpr1a$^{-/-}$* cells could be differentiating into bipotent *Sox1*-positive ectodermal precursors, capable of generating both neural and epidermal cells depending on external signals, an intermediate stage previously speculated to exist by Aberdam and coworkers (Aberdam et al., 2007a). It would be interesting to further explore this issue by analysing protein expression of markers of both lineages and, in case of co-expression, to investigate the developmental potential of these cells.

The monolayer neural differentiation protocol allowed a more detailed study of the ability of C1-*Bmpr1a$^{-/-}$* cells to undergo neural commitment. In agreement with what was observed in embryoid bodies and in basal conditions, C1-*Bmpr1a$^{-/-}$* cells in monolayer differentiation showed a higher propensity to undergo neural commitment, as indicated by the higher expression of the neural progenitor markers *Sox1* and *Pax6* at the fourth day of the differentiation protocol. Although the expression of Id genes at this stage of differentiation was equally low in both cell types, in undifferentiated WT cells *Id1* and *Id3* expression was higher. Id proteins are induced in response to BMP signalling and act as repressors of basic helix-loop-helix (bHLH) neurogenic transcription factors (Norton et al., 1998; Ruzinova and Benezra, 2003; Ying et al., 2003a). Decreased levels of Id proteins in *Bmpr1a-null* cells at the initial stages of neural commitment could thus lead to a faster or more efficient initiation of

the differentiation programme. Activation of SMAD1/5/8, which leads to induction of Id proteins, was also higher in WT cells at the beginning of differentiation but not after the first couple of days of the differentiation protocol. This observation once again suggests that the initial stages of neural differentiation could determine the efficiency of the process.

From this study it is not clear whether this different susceptibility of C1-*Bmpr1a*$^{-/-}$ cells to neural differentiation is a direct consequence of the differences in phosphorylated SMAD1/5/8 and in Id expression at the onset of differentiation. Clarification of this matter would require a more detailed study of the RNA and protein levels of Ids during the initial period of differentiation and an analysis of how these relate to the expression of bHLH neurogenic transcription factors. However, the observation that the most significant differences in expression of neural markers are only detected 4 days after the onset of differentiation suggests that an Id-independent mechanism is responsible for these differences. One hypothesis is that the lower levels of BMP signalling in *Bmpr1a*$^{-/-}$ cells in undifferentiated conditions determine a more permissive epigenetic state of the promoters of genes involved in neural specification. To verify this hypothesis, an investigation of the epigenetic status of these promoters should be performed, as well as an analysis of the expression of epigenetic regulators suggested to be involved in lineage specification, such as the transcriptional repressor REST and the Polycomb Group proteins (Ballas and Mandel, 2005; Rajasekhar and Begemann, 2007).

Another question that remains is whether C1-*Bmpr1a*$^{-/-}$ cells undergo faster or more efficient neural differentiation in relation to WT cells. The observation that the levels of pluripotency markers do not decrease faster in mutant cells during the first days of differentiation suggests a higher efficiency of the process but does not definitively answer the question. To resolve this issue, a careful study of the timing of onset of early markers of differentiation would have to be made, as well as a quantification of

the amount of neural progenitors and neurons obtained at different stages of differentiation (including later stages than the ones analysed here).

The epidermal-inducing activity of BMP4 during mouse ES cell-differentiation is well documented (Aberdam et al., 2007b; Coraux et al., 2003). A similar role for BMP7 in the mouse has not yet been reported but, in this study, the increased expression of the early epidermal markers *Krt8*, *Krt18* and *Krt19* in C1-*Bmpr1a*$^{-/-}$ cells in basal medium had already suggested that auto-stimulation of these cells by other BMPs could also be driving epidermal differentiation. This aspect was thus further investigated by culturing ES cells at low density in serum-free medium in the presence of BMP4 or BMP7. In these conditions, ES cells undergo commitment preferentially to the epidermal lineage, as observed by the upregulation of the epidermal progenitor markers *Krt18* and *Krt14* in WT cells, both in response to BMP4 and to BMP7. *Bmpr1a*$^{-/-}$ cells are not responsive to BMP4 but do respond to BMP7 (see previous chapter). In agreement with this, C1-*Bmpr1a*$^{-/-}$ cells showed deficient epidermal differentiation in response to BMP4 but followed a similar fate to WT cells when treated with BMP7, indicating that they have an equivalent potential for epidermal commitment when in adequate conditions.

Confirmation of full pluripotency of *Bmpr1a*$^{-/-}$ ES cells can only be provided *in vivo* by analysing their contribution to embryonic development following blastocyst injection. Even though the cells derived in this study have not been submitted to this final test, Fr124-*Bmpr1a*$^{-/-}$ ES cells, derived in the presence of p38 inhibitor, could give rise to derivatives of the three germ layers in chimeras (Di-Gregorio et al., 2007).

In summary, these results show that although *Bmpr1a*$^{-/-}$ cells cannot respond to BMP4, they are pluripotent and have the ability to differentiate into derivatives of the three primary germ layers. However, the lower level of BMP activation in these cells predisposes them for ectodermal commitment.

5. CELL COMPETITION IN ES CELLS

5.1. Introduction

Cell competition is the process by which cells are eliminated by the presence of metabolically more active or faster proliferating cells (Diaz and Moreno, 2005; Johnston, 2009; Tyler et al., 2007).

This type of cell interaction is responsible for the elimination of Minute (M/+) mutant and lower *dMyc* expressing cells from the epithelium of the *Drosophila* wing in mosaic organisms (de la Cova et al., 2004; Johnston et al., 1999; Morata and Ripoll, 1975; Moreno and Basler, 2004; Simpson, 1979).

Transduction of the BMP homologue Dpp has been suggested to be one of the main factors determining the competitive potential of cells in the *Drosophila* wing epithelium (Moreno and Basler, 2004; Moreno et al., 2002). Besides acting as a morphogen important for patterning and cell fate specification in wing imaginal discs, Dpp is a key growth and survival factor during development of the *Drosophila* wing (Burke and Basler, 1996; Martin-Castellanos and Edgar, 2002). Clones in which loss of function mutations are induced in proteins required for transduction of Dpp/BMP signalling show impaired cell growth and proliferation and thus are eliminated from the wing epithelium during development (Burke and Basler, 1996). Conversely ectopic expression of *Dpp* results in over-proliferation of surrounding cells, indicating that it has a direct role in promoting cell proliferation in the wing imaginal discs. Cells in which protein synthesis and biometabolic function are compromised, such as the M/+ and low-expressing *Myc* mutant cells, are likely to have a reduced capacity to internalize and transduce survival and growth factors such as Dpp. This

disadvantage in competing for limiting amounts of extracellular survival factors may therefore cause lower levels of Dpp/BMP signaling which lead to increased expression of the transcription repressor *brinker* and activation of the c-Jun amino-terminal kinase (JNK) pathway, triggering apoptosis and out-competition (Moreno et al., 2002). Further evidence for the importance of Dpp/BMP signalling in cell competition comes from two observations. In the first place, constitutive activation of the Dpp pathway enhances the survival of M/+ clones (Moreno et al., 2002) and of lower *dMyc*-expressing cells (Moreno and Basler, 2004), thus reducing cell competition. Second, most genes identified in a genetic screen for mutations that induce survival of M/+ cells during cell competition enhance Dpp activity (Tyler et al., 2007). However, competition for the Dpp survival signal probably does not represent a universal mechanism by which weaker cells are eliminated. In some studies no differences in Dpp/BMP signalling were observed in competing cells (de la Cova et al., 2004; Li and Baker, 2007) and in the absence of JNK signalling, cell competition still occurred (de la Cova et al., 2004).

In the ovarian stem cell niche Dpp/BMP signalling sustains self-renewal of germline stem cells (GSCs) by directly repressing the expression of the bag-of-marbles (bam) differentiation factor (Chen and McKearin, 2003; Song et al., 2004). GSCs are firmly anchored to the niche cap cells via adherent junctions, thereby ensuring exposure to high concentrations of the Dpp self-renewal signal secreted by these (reviewed in Kirilly and Xie, 2007). Cells further away from the niche have lower levels of Dpp activation and thus fail to repress bam transcription and differentiate (Chen and McKearin, 2003; Kirilly and Xie, 2007; Rhiner et al., 2009; Song et al., 2004). GSCs expressing different levels of *dMyc* have also been reported to experience competitive interactions. Differences in *dMyc* expression lead to differential metabolic activity, protein synthesis and endocytosis, and therefore to different efficiencies in the uptake and transduction of Dpp. Ultimately, these differences determine the

niche exclusion and differentiation of cells with lower activation of the Dpp pathway. In normal situations, *dMyc* is highly expressed in GSCs and considerably downregulated in their progeny (Maines et al., 2004). This has been suggested to create a naturally occurring cell competition border which facilitates the concentration of the niche self-renewal factor Dpp in metabolically active high *dMyc* GSCs (Rhiner et al., 2009). Differentiation-defective *bam* or *bgcn* mutant GSCs also out-compete normal cells displacing them from the niche independently of dMyc and Dpp/BMP signalling (Jin et al., 2008). Therefore, unlike cell competition in the wing epithelium, where cell-cell comparison of metabolic status determines apoptosis of weaker cells, competition for niche occupancy is adhesion-based and leads to differentiation of out-competed cells (reviewed in Johnston, 2009).

In mammals, cell competition has only been suggested to occur in chimeras of *Belly spot and tail* (*Bst*) mutant cells, that carry a mutation in the Rpl24 ribosomal protein (Oliver et al., 2004) and during liver regeneration in rats (Oertel et al., 2006). However, the mechanism of competition in these cases has not been appreciably explored.

In this study, in order to investigate whether cell competition occurs between ES cells, and particularly to determine the role of BMP signalling in this process, a co-culture system was developed using *Bmpr1a*$^{-/-}$ ES cells. Cells with lower levels of BMP signalling were found to be out-competed, particularly in conditions of limiting amounts of growth and survival factors.

5.2. Results

5.2.1. Bmpr1a$^{-/-}$ cells are out-competed in co-cultures with WT cells

Given the involvement of Dpp/BMP signalling in cell competition in the *Drosophila* wing and ovarian stem cell niche cells (Burke and Basler, 1996; Moreno and Basler, 2004; Moreno et al., 2002; Rhiner et al., 2009), and their central role in mouse ES-cell maintenance (Qi et al., 2004; Ying et al., 2003a), we have hypothesized that this pathway may also cause cell competition in ES cells.

As a first approach to test if competitive interactions can be observed among ES cells, Fr124 (*Bmpr1a*$^{-/-}$-LacZ) cells were mixed in equal numbers with E14 (WT) cells and co-cultured in ES cell maintenance conditions, being passaged every three days. Mutant cells could be identified in the mixture since they contain the *lacZ* gene, thus expressing the beta-galactosidase reporter. When cultured together with E14-WT cells, Fr124-*Bmpr1a*$^{-/-}$ cells were progressively eliminated, being practically absent from the co-culture after 5 to 6 passages (Figure 5.1A).

To better follow and analyse this process, GFP-expressing C1-*Bmpr1a*$^{-/-}$ cells were generated by electroporating an expression vector in which EGFP expression is driven by the ubiquitous CAG promoter (CMV Enhancer/chicken β-Actin Promoter). Similarly to what was observed for the Fr124- *Bmpr1a*$^{-/-}$-lacZ marked cells, C1-*Bmpr1a*$^{-/-}$-GFP cells were progressively out-competed from co-cultures with 3.5-WT cells (Figure 5.1B).
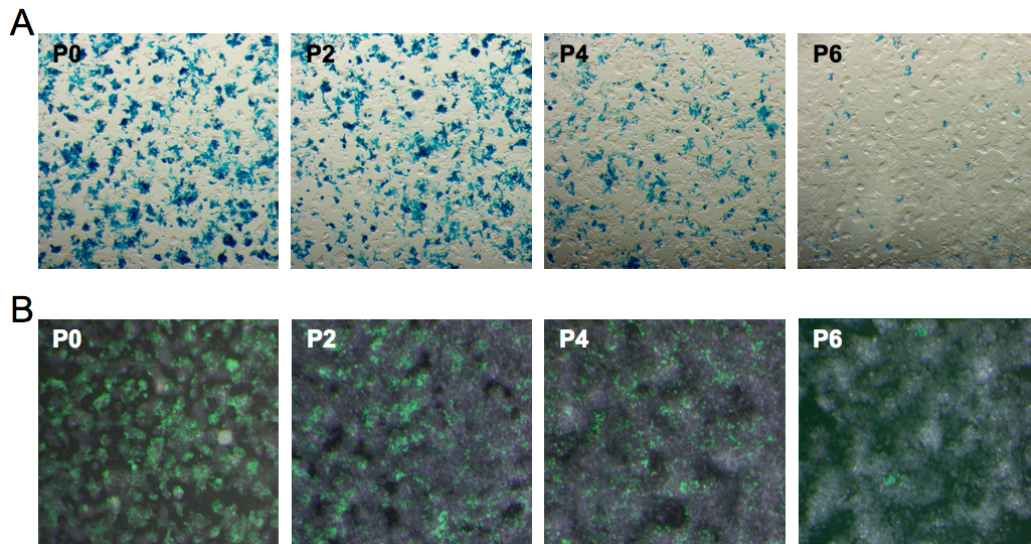
Figure 5.1 *Bmpr1a*^{-/-} ES cells are out-competed when co-cultured with WT cells.

(A) Equal numbers of E14-WT and Fr124-*Bmpr1a*^{-/-}-*LacZ* ES cells were mixed together and the two cell types were co-cultured in ES cell maintenance conditions containing LIF and FCS and passaged every 3 days. Represented are bright field images of *β*-galactosidase staining of the co-cultures 2 days after plating (P0) and after 2, 4 and 6 passages (P2, P4, P6) showing that Fr124-*Bmpr1a*^{-/-}-*LacZ* ES cells are gradually out-competed from co-cultures. **(B)** Co-culture of 3.5-WT and C1-*Bmpr1a*^{-/-}-GFP ES cells, in a similar way to the experiment described in A. Represented are overlapping fluorescence (GFP) and dark field images of the co-cultures.

Expression of GFP by one of the cell types in the co-cultures allowed their analysis by Flow Cytometry (FC). FC analysis showed that cells lost GFP as they died, and so dead cells had to be excluded from subsequent studies based on propidium iodide (PI) staining. PI binds to double stranded DNA by intercalating between base pairs, but is excluded from cells with intact plasma membranes, therefore allowing the distinction between live and dead cells.

To identify competitive interactions between the two cell types, the behaviour of the cells in co-cultures was always compared to that of the cells cultured separately. For this analysis, cells from separate cultures were dissociated and similar volumes of both cell suspensions were mixed before FC analysis.

As shown in Figures 5.2A and B, quantification of the percentage of GFP-positive and -negative cells in the co-cultures showed that approximately 10% of C1-*Bmpr1a$^{-/-}$*-GFP cells were lost every three days, at the time of passaging. This was significantly different to the behaviour of the cells when they were cultured separately. To exclude the possibilities that GFP expression was causing a competitive disadvantage to the cells or that the cells were losing GFP expression during co-culture, the reverse experiment was also performed, in which 3.5-WT-GFP cells were mixed with non-marked C1-*Bmpr1a$^{-/-}$* cells. When this was done, C1-*Bmpr1a$^{-/-}$* cells were equally eliminated from the culture (Figure 5.2C) confirming the results previously obtained. Finally, to determine whether *Bmpr1a$^{-/-}$* cells were being lost due to adhesion defects that could impair their replating efficiency at the time of passaging, the percentages of each cell type in co-cultures were counted every day during 5 passages. This analysis showed that cells were progressively lost from co-cultures and not specifically at the time they were split (Figure 5.2D).
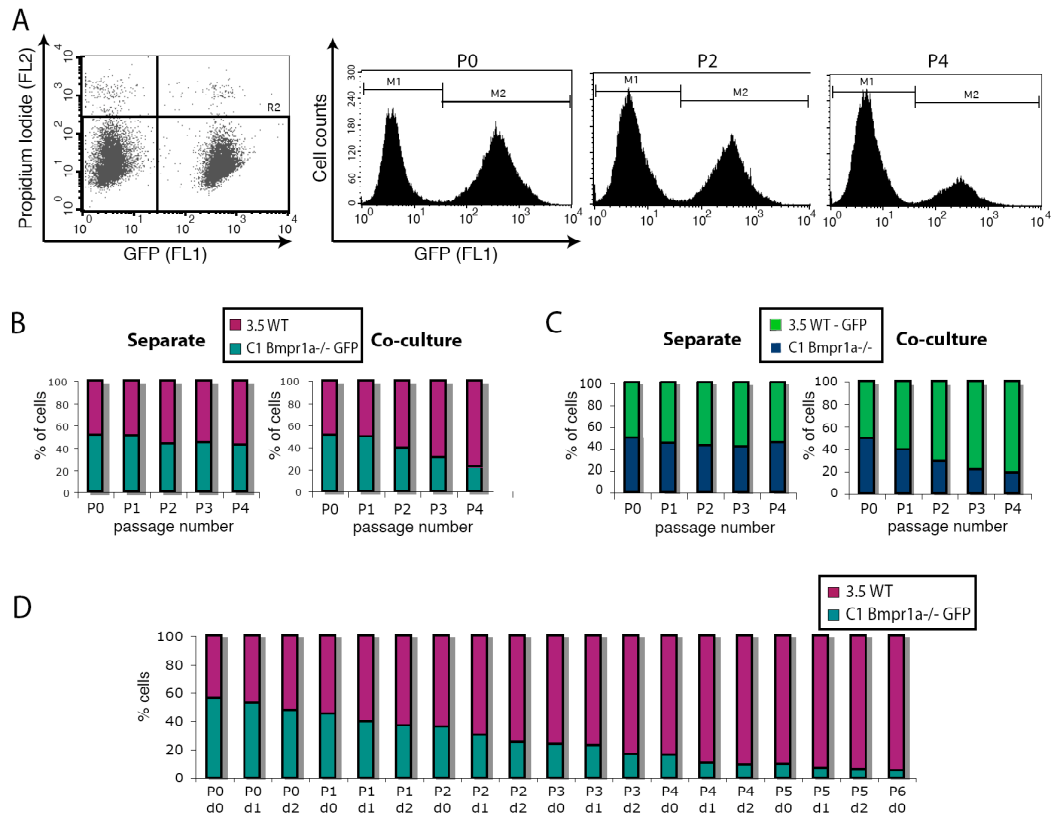
Figure 5.2 *Bmpr1a^-/-* ES cells are out-competed when co-cultured with WT cells.

(A) Flow cytometry (FC) analysis of GFP expression in co-cultures of 3.5-WT and C1-*Bmpr1a^-/-*-GFP ES cells allowed the quantification of the percentages of each cell type present at the time of each passage. The live-cell population was gated excluding propidium iodide (PI)-positive cells from the analysis. Represented are the histograms obtained by FC analysis of GFP fluorescence at the time of plating (P0), and at the second (P2) and fourth (P4) passages. **(B)** Graphical representation of FC analysis of the percentages of GFP-positive (C1-*Bmpr1a^-/-*-GFP cells) and -negative (3.5-WT cells) cells present in co-cultures and separate cultures at the beginning of the experiment (P0) and at the time of each of the first four passages (P1-4). **(C)** Graphical representation of FC analysis of the percentages of GFP-positive (3.5-WT-GFP cells) and -negative (C1-*Bmpr1a^-/-* cells) cells present in co-cultures and separate cultures at the beginning of the experiment (P0) and at the time of each of the first four passages (P1-4). **(D)** Percentage of GFP-positive and -negative cells present in co-cultures of C1-*Bmpr1a^-/-*-GFP cells and 3.5-WT cells as assessed by FC analysis every day over the period of 6 passages. All data shown is from representative experiments.

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5.2.2. Cell Competition is enhanced in conditions of limiting amounts of growth and survival factors

Co-cultures of WT and *Bmpr1a*-null cells in ES-cell maintenance medium (containing FCS and LIF, in addition to other supplements commonly required for cell growth), showed that the latter cells were out-competed. However, this process was slow, requiring 5 to 6 passages (along more than two weeks) for the elimination of mutant cells. Therefore, to investigate which factors could enhance cell competition, different culture conditions were tested.

With this aim, co-cultures were firstly performed at different confluencies. Figure 5.3A shows that when this was done, even though no major differences were observed, out-competition was slightly more efficient at high confluency (Figure 5.3A), suggesting that growth factors becoming limiting enhances this process.

Cells were then cultured in serum-free conditions, with limiting amounts of growth and survival factors (Figure 5.3B). The ratio between the percentages of the two cell types represents the growth advantage of WT over *Bmpr1a*$^{-/-}$-GFP cells. Comparison of this parameter between cells grown separately and in co-cultures allows visualization of the extent of competitive interactions as opposed to the effect that each growth condition has on the cells on their own.

Starting with equal number of WT and *Bmpr1a*$^{-/-}$-GFP cells, after 4 days in normal ESC maintenance conditions (FCS+LIF), the ratio between the two cell types was maintained when the cells were cultured separately, and only slightly changed in co-cultures (Figure 5.3B), which is in accordance with previous results. In serum-free conditions, however, 3.5-WT cells showed a growth advantage over C1-*Bmpr1a*$^{-/-}$-GFP cells in monotypic cultures. The decreased viability of *Bmpr1a*$^{-/-}$-GFP cells was observed both in Basal medium only, and when BMP4, LIF or both these factors were added to the cultures. Figure 5.3B shows that in Basal medium, the WT/*Bmpr1a*$^{-/-}$ ratio was significantly higher in co-cultures than in separate cultures,

indicating a high degree of competition. Interestingly, addition of LIF to the cultures caused inhibition of the competitive interactions. Cell competition was thus maximal in Basal medium and, therefore, this was the condition chosen for further studies.
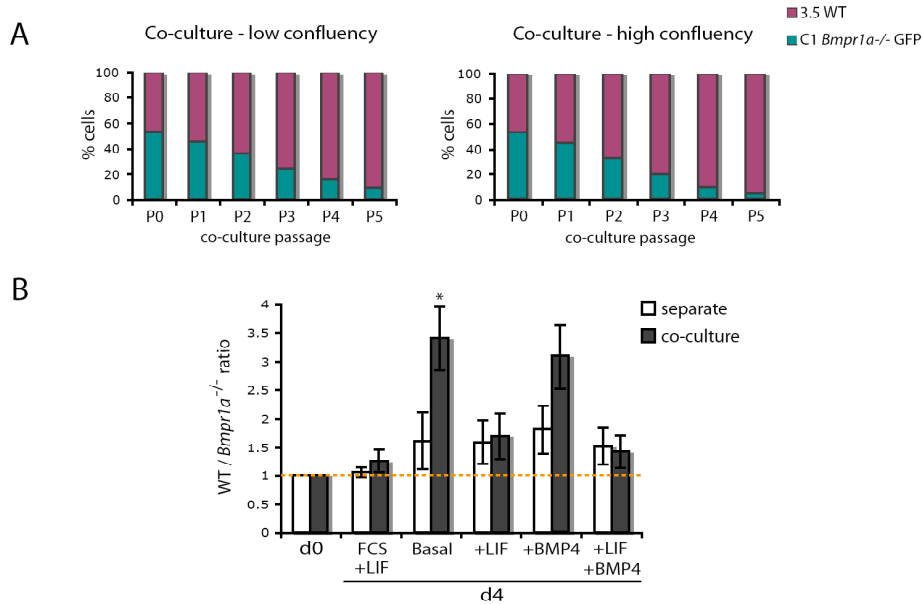
Figure 5.3 Out-competition of *Bmpr1a*$^{-/-}$ ES cells is enhanced in conditions of limiting survival and growth factors.

(A) Graphical representation of flow cytometry (FC) analysis of the percentages of GFP-positive (C1-*Bmpr1a*$^{-/-}$-GFP cells) and -negative (3.5-WT cells) populations of cells present in co-cultures at the beginning of the experiment (P0) and at the time of the first five passages (P1-5). Co-cultures were carried out at low (left; approximately 1x10^5 cells plated per well of a 6-well plate) and high (right, approximately 8x10^5 cells plated per well of a 6-well plate) confluencies in medium containing FCS+LIF. (B) Co-cultures and separate cultures of 3.5-WT and C1-*Bmpr1a*$^{-/-}$-GFP ES cells were performed in medium containing FCS plus LIF or in basal medium in the absence (Basal) or presence of LIF, BMP4 or BMP4 plus LIF. The percentages of GFP-positive and -negative cells were assessed by FC at the beginning of the experiment (d0) and after 4 days in these culture conditions (d4). Represented are the ratios of the percentages of WT to Bmpr1a-/- cells (i.e. GFP-negative 3.5-WT to GFP-positive C1-*Bmpr1a*$^{-/-}$ cells). This parameter indicates the growth advantage that WT cells have over *Bmpr1a*$^{-/-}$-GFP cells. Comparison of the ratios obtained for separate cultures and co-cultures allows determining if competitive interactions exist between the two cell types studied. Orange dashed line represents the WT/-/- ratio at the beginning of the experiment (ratio=1), which would be maintained if neither cell line had a growth advantage over the other one. Maximal competitive interactions were obtained in serum-free basal medium. Data shown as mean +/- s.d. (n=3)

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As shown in Figure 5.4A, FC analysis of the percentages of GFP cells in co-cultures confirmed that out-competition of $Bmpr1a^{-/-}$ cells was considerably accelerated in Basal medium. After 4 days in co-culture only 10 to 20% of $Bmpr1a^{-/-}$-GFP cells were present, allowing the study of cell competition without the need for passaging the cells. Combining cell counting and GFP quantification by FC it was possible to follow the growth pattern of the 2 cell types in co-cultures (Figure 5.4B). Even though a growth disadvantage of C1-$Bmpr1a^{-/-}$-GFP cells in Basal medium was evident in separate cultures, when in co-culture this disadvantage was greatly increased. Importantly, between the third and fourth day of co-culture, the number of C1-$Bmpr1a^{-/-}$-GFP cells actually decreased whereas 3.5-WT cells continued proliferating. To determine whether this competitive behaviour was specific to the 3.5-WT and C1-$Bmpr1a^{-/-}$ cells lines, the same experiment was performed using independently derived cells, and this time with GFP being expressed by the WT cells. The growth curves obtained when control E14-GFP cells were co-cultured with Fr124-$Bmpr1a^{-/-}$ cells again showed out-competition of the ones with a deficiency in BMP signalling, which was in fact even more pronounced than with the C1-$Bmpr1a^{-/-}$ cells (Figure 5.4C).
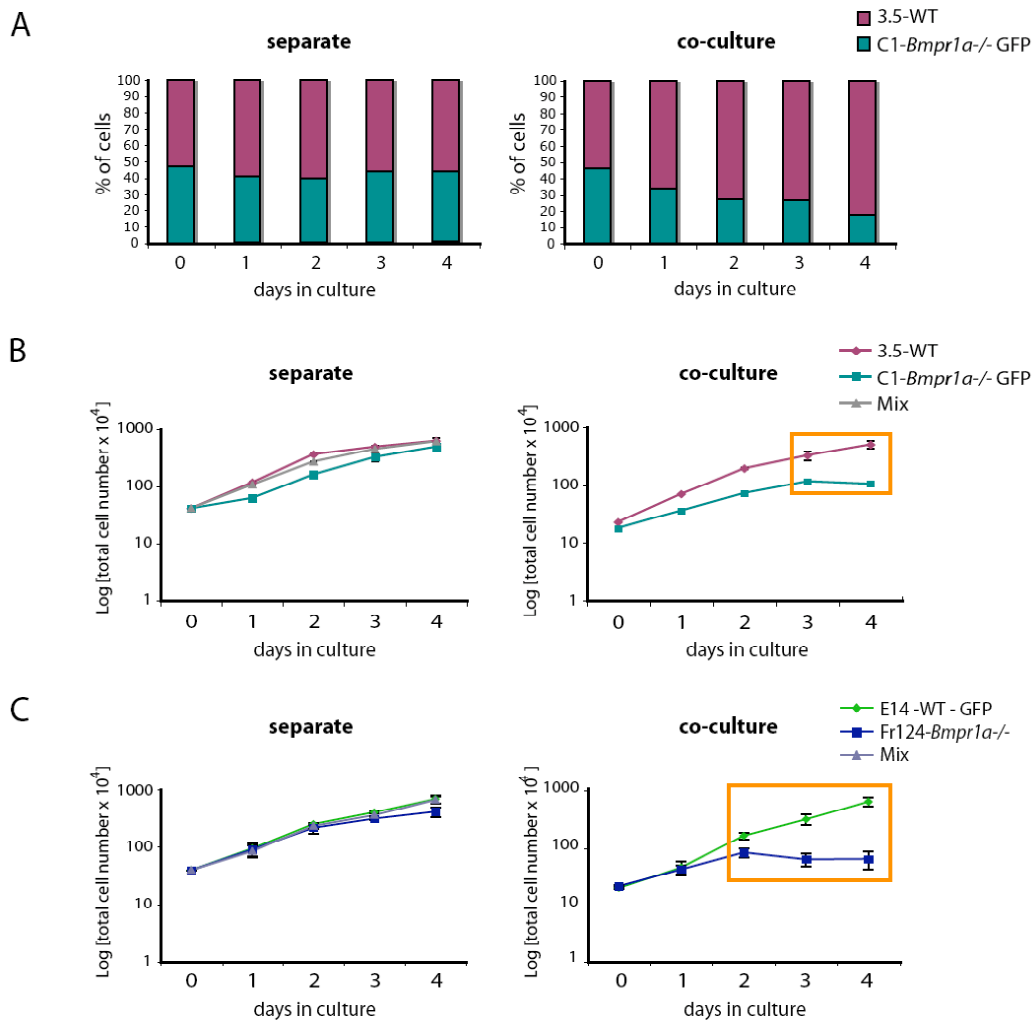
Figure 5.4 Out-competition of *Bmpr1a*$^{-/-}$ ES cells in serum-free medium.

(A) The percentages of GFP-positive (C1-*Bmpr1a*$^{-/-}$-GFP cells) and -negative (3.5-WT cells) cell populations in separate cultures (left) and co-cultures (right) were analysed by flow cytometry (FC) at the beginning of the experiment (d0) and at each day during the following 4 days of culture in Basal medium (d1-4). One representative experiment is shown. **(B)** Growth curves of 3.5-WT and C1-*Bmpr1a*$^{-/-}$-GFP ES cells in separate cultures (left) and co-cultures (right) in basal medium were determined by combining data obtained by cell counting and FC analysis. Orange box highlights the period of co-culture when the cell behaviour significantly differs from the one of separate cultures, indicating competitive interactions. Data shown as mean +/- s.d. (n=3). **(C)** Growth curves of E14-WT-GFP and Fr124-*Bmpr1a*$^{-/-}$ ES cells in separate cultures (left) and co-cultures (right) in basal medium. Orange box highlights the period of co-culture when competitive interactions occur. Data shown as mean +/- s.d. (n=3)

However, among the different ES-cell clones used in co-culture experiments, one of the controls in which BMP signalling was normal, but that showed a considerable

growth disadvantage, and abnormal colony morphology, was out-competed by *Bmpr1a*$^{-/-}$ cells (Figure 5.5). Given the growth defects of this cell line, this experiment does not contradict the previous data, but shows that other deficiencies besides faulty BMP transduction can cause cell competition.
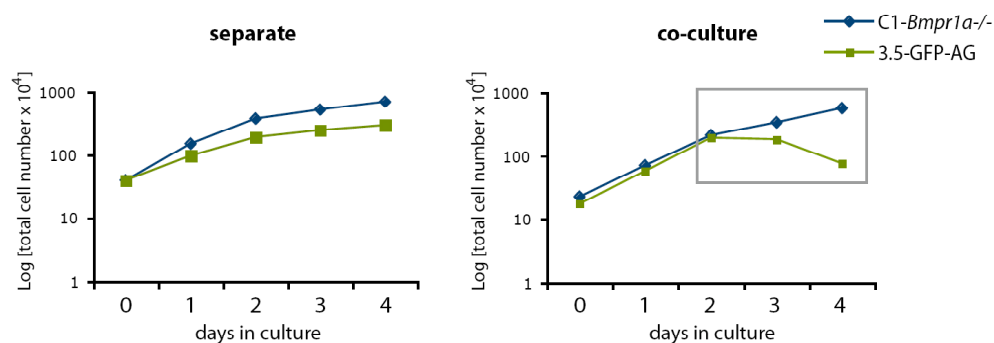
Figure 5.5 BMP transduction defects are not the only trigger of ES cell competition.

Growth curves of separate cultures (left) and co-cultures (right) of 3.5-GFP-AG and C1-*Bmpr1a*$^{-/-}$ ES cells in basal medium show that *Bmpr1a*$^{-/-}$ ES cells can out-compete cells with higher BMP transduction ability but which have impaired growth. Grey box highlights the period of co-culture when competitive interactions occur (n=1)

5.2.3. Out-competition of Bmpr1a$^{-/-}$ cells is inhibited when BMP4 signalling is restored.

Analysis of co-cultures has shown that *Bmpr1a*$^{-/-}$ cells are out-competed by WT cells, particularly in conditions of limiting amounts of growth factors. To confirm that the cause for out-competition of *Bmpr1a*$^{-/-}$ cells in this system is the deficiency in BMP signal transduction, *Bmpr1a* expression was restored in mutant cells. This was achieved by creating an expression vector containing the coding sequences for *Bmpr1a*, EGFP and puromycin N-acetyl-transferase (Pac, which confers puromycin resistance) all driven by the ubiquitous CAG promoter (Figure 5.6A). In this vector, the sequence for the viral 2A peptide was used as a linker region between the *Bmpr1a* and EGFP cDNAs. Therefore, the two sequences should be co-translated

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but, given the highly inefficient peptide bond formation between glycine and proline residues within the 2A peptide, are easily cleaved and generate 2 unfused, yet stoichiometric, proteins (Ryan and Drew, 1994; Trichas et al., 2008). This was followed by an internal ribosome entry site (IRES) and then the Pac coding sequence.

After electroporation of the *Bmpr1a*-GFP expression vector into C1-*Bmpr1a*$^{-/-}$ cells, colonies were selected for puromycin resistance and screened for GFP expression. However, for some unknown reason, puromycin-resistant colonies did not show GFP expression. In order to determine whether *Bmpr1a* was being expressed and, more importantly, if it was functional, cells were stimulated with BMP4 and tested for SMAD 1/5/8 phosphorylation. Figure 5.6B shows that the parental C1-*Bmpr1a*$^{-/-}$ cells did not respond to BMP4 stimulation, as previously shown, but cells carrying the CAG-*Bmpr1a*-GFP transgene showed activation of SMAD1/5/8, indicating that they express a functional BMPR1A receptor. These cells were thus nam

ed C1-*Bmpr1a*^{res} (res, restored function).

When co-culturing C1-*Bmpr1a*^{res} cells with control 3.5-WT-GFP-expressing cells, no cell competition was observed (Figure 5.6C), unlike what happened with the parental C1-*Bmpr1a*$^{-/-}$ cells (compare with Figure 5.4B). Conversely, in co-cultures of C1-*Bmpr1a*^{res} cells and C1-*Bmpr1a*$^{-/-}$ GFP^{+} cells, C1-*Bmpr1a*^{res} cells out-competed the parental ones, (Figure 5.6D). These results show that expression of BMPR1A rescued the competitive disadvantage of the C1-*Bmpr1a*$^{-/-}$ cell line, indicating that this disadvantage is due to a deficiency in transducing BMP signalling.
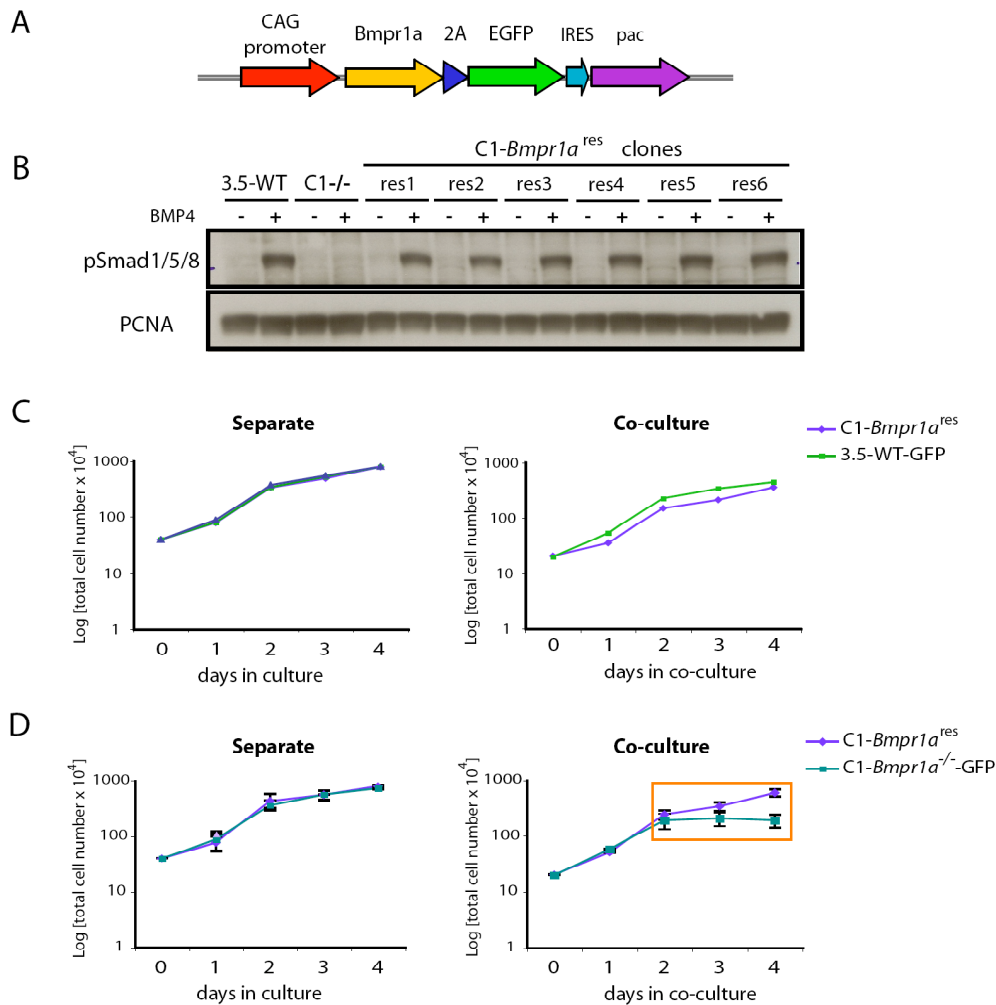
Figure 5.6 Out-competition of *Bmpr1a^-/-^* ES cells is rescued by restoring *Bmpr1a* expression in these cells

(A) Schematic representation of the vector constructed to express *Bmpr1a* in ES cells (CAG-Bmpr1a-GFP transgene). The coding sequences for *Bmpr1a* and for *EGFP* (linked by the viral 2A sequence) were directionally cloned into the pPyCAGIP episomal expression vector, creating an expression cassette where *Bmpr1a, EGFP* and the puromycin resistance gene (*pac*) were all driven by the ubiquitous CAG promoter. The CAG-Bmpr1a-GFP transgene was electroporated into C1-*Bmpr1a^-/-^* ES cells and colonies were selected for puromycin resistence and screened for GFP expression. No GFP expression was detected. (B) Western blot analysis showed that BMP4 stimulation induces SMAD1/5/8 phosphorylation in C1-*Bmpr1a^-/-^* ES cell clones carrying the CAG-Bmpr1a-GFP transgene (C1-*Bmpr1a*^res1-6^ cells), even though it does not activate this pathway in the parental cells, indicating that the transgene leads to expression of functional Bmpr1a. (C) Growth curves of 3.5-WT-GFP and C1-*Bmpr1a*^res^ ES cells in separate cultures (left) and co-cultures (right) in basal medium. (D) Growth curves of C1-*Bmpr1a^-/-^*-GFP and C1-*Bmpr1a*^res^ ES cells in separate cultures (left) and co-cultures (right) in basal medium. Orange box highlights the period of co-culture when competitive interactions occur. Data shown as mean +/- s.d. (n=2)

These experiments demonstrated that co-culture of *Bmpr1a*$^{-/-}$ with control WT ES cells in Basal medium, a condition in which growth and survival factors are scarce, provide a good *in vitro* system to study the role of BMP signalling in cell competition in ES cells.

5.3. Discussion

The process of cell competition, initially described and mainly studied in *Drosophila*, represents an important mechanism for quality selection during development and maintenance of tissue homeostasis (Adachi-Yamada and O'Connor, 2004; Baker and Li, 2008; Johnston, 2009; Leevers and McNeill, 2005). Cell competition studies in the epithelium of the *Drosophila* wing have shown that the levels of protein synthesis and biometabolic function determine the competitive potential of cells in a heterogeneous environment (de la Cova et al., 2004; Johnston et al., 1999; Morata and Ripoll, 1975; Moreno and Basler, 2004; Simpson, 1979), probably due to differential abilities to internalize and transduce growth and survival signals (Moreno and Basler, 2004; Moreno et al., 2002). In the *Drosophila* ovary, competition for occupancy of the stem cell niche has an important role controlling the balance between differentiation and self-renewal of GSCs (Diaz and Moreno, 2005; Jin et al., 2008; Johnston, 2009; Rhiner et al., 2009).

Both in the *Drosophila* wing and in the ovarian stem cell niche, cells that uptake and transduce Dpp/BMP signalling more efficiently have a competitive advantage over cells with lower activation of this pathway (Burke and Basler, 1996; Moreno and Basler, 2004; Moreno et al., 2002; Rhiner et al., 2009)

In mouse ES cells, BMP signalling plays a crucial role sustaining self-renewal and pluripotency in combination with LIF (Qi et al., 2004; Ying et al., 2003a). *Bmpr1a*-null

ES cells have reduced levels of activation of the BMP pathway but do not show self-renewal or pluripotency defects (see previous chapters).

In this study, *Bmpr1a*-null ES cells were cultured together with control WT cells in order to investigate whether competitive interactions occur between ES cells with differential activation of the BMP pathway. The generation of a GFP-labelled *Bmpr1a*$^{-/-}$ ES cell line allowed the study of cell behaviour in co-cultures with other cell types without having to disturb the system. Furthermore, it facilitates multiparametric analysis by FC and sorting of the cells.

Mixed cultures of *Bmpr1a*$^{-/-}$ and WT cells in medium containing FCS and LIF, conditions generally used for ES-cell maintenance, revealed that the cells with lower activation of the BMP signalling pathway are gradually out-competed, even though in these conditions this is a slow process. However, limiting the availability of growth and survival factors by culturing the cells in Basal serum-free medium increased the competitive disadvantage of *Bmpr1a*$^{-/-}$ cells, thereby greatly enhancing ES-cell competition.

Definitive proof that BMP signalling, and not other factors intrinsic to the ES-cell lines used in the co-culture experiments, determines the competitive potential of ES cells was provided by restoring *Bmpr1a* expression in mutant *Bmpr1a*$^{-/-}$ ES cells. Expression of functional BMPR1A re-established the ability to respond to BMP activation and inhibited the out-competition of *Bmpr1a*$^{-/-}$ ES cells.

However, differences in BMP signalling do not always determine the ES-cell competition outcome. *Bmpr1a*$^{-/-}$ ES were exhibited a "winner" behaviour when co-cultured with a cell line with severely impaired growth. Given the growth defects of this cell line, this observation does not contradict the previous data. Nevertheless, it shows that other deficiencies besides faulty BMP transduction can be responsible for a competitive disadvantage of ES cells when in the presence of "fitter" cells.

Taken together, these results demonstrate that the potential for proliferation and survival of an ES cell is determined by interactions with its neighbours. In particular, they show that, similarly to what occurs during the cell competition in the Drosophila wing epithelium and stem cell niche, defective BMP signalling confers a competitive disadvantage to ES cells when in the presence of WT cells. Moreover, the co-culture of $Bmpr1a^{-/-}$ ES cells with control WT cells in Basal serum-free conditions was established as a suitable *in vitro* system to study competitive interactions between ES cells with differing abilities to respond to BMP signalling.

Cell competition has been suggested to ensure normal development, organ size and homeostasis in *Drosophila* (Adachi-Yamada and O'Connor, 2004; Diaz and Moreno, 2005; Johnston, 2009). The results of the present study, together with the described out-competition of the ribosomal-defective $Bst^{+/-}$ cells during chimera colonisation (Oliver et al., 2004), suggest that cell competition may also represent an important mechanism that ensures tissue fitness and homeostasis during mammalian development. In addition, cell competition has been reported during organ regeneration (Oertel et al., 2006) and is likely to be involved in tumour formation (reviewed in Baker and Li, 2008; and Rhiner and Moreno, 2009). Therefore, understanding the mechanism and regulation of cell competition is an important issue in disease and developmental biology.

6. INVESTIGATING THE MECHANISM OF OUT-COMPETITION OF *BMPR1A$^{-/-}$* ES CELLS

6.1. Introduction

Given the likely involvement of cell competition in ensuring homeostatic balance and optimal function during development and tissue regeneration (Adachi-Yamada and O'Connor, 2004; Baker and Li, 2008; Diaz and Moreno, 2005; Oertel et al., 2006; Rhiner and Moreno, 2009), it is important to understand the processes by which cells compare their relative "fitness" and eliminate weaker ones during competitive interactions.

In the epithelium of the *Drosophila* wing, cells with a lower protein synthesis and metabolic activity such as M/+ and lower dMyc-expressing cells are killed by surrounding more competitive cells. In turn, these over-proliferate thus maintaining normal tissue size and morphology (de la Cova et al., 2004; Johnston et al., 1999; Morata and Ripoll, 1975; Moreno and Basler, 2004; Moreno et al., 2002). During liver regeneration in the rat, increased apoptosis of diseased cells and overproliferation of WT transplanted fetal liver cells has also been observed (Oertel et al., 2006).

Distinct mechanisms have been suggested to be responsible for triggering apoptosis of the out-competed cells in the *Drosophila* wing. Moreno and coworkers have suggested that the lower protein synthesis activity of these cells impairs their capacity to internalise and transduce survival factors, in particular Dpp, thus activating JNK-dependent apoptosis (Moreno and Basler, 2004; Moreno et al., 2002). They find that stimulation of endocytic uptake, constitutive activation of the Dpp pathway, inhibition of JNK activation, and blocking of apoptosis, all prevent cell competition (Moreno and Basler, 2004; Moreno et al., 2002). In this view, competition

is driven by the differential ability for internalization of limiting concentrations of the Dpp survival factor, leading to apoptosis of less competitive cells due to the transduction of insufficient amounts of survival signal (Diaz and Moreno, 2005).

Studies by Johnston and coworkers indicate a different mechanism, independent of Dpp transduction, for the out-competition of lower dMyc expressing cells. These authors suggest that cell competition is executed via induction of the proapoptotic gene *hid*, and that cells do not compete for a limiting ligand but rather communicate via a short-range signal that allows them to sense and respond to local differences in dMyc levels (de la Cova et al., 2004). This communication does not require physical interaction between the two cell types, and the observation that it can be achieved in single cultures by medium conditioned by competing co-cultures indicates that it is mediated by soluble factors (Senoo-Matsuda and Johnston, 2007). The production of these active soluble factors requires the presence of both cell types during the process so that relative levels of dMyc expression can be compared and a "winner"/"loser" status established (Senoo-Matsuda and Johnston, 2007).

A different type of cell competition takes place in the *Drosophila* ovary, where stem cells struggle for niche occupancy, resulting in the displacement and differentiation of weaker cells (Jin et al., 2008; Nystul and Spradling, 2007; Rhiner et al., 2009). Maintenance of GSCs (germline stem cells) requires the Dpp differentiation-repressing signal from the supporting cap cells in the stem cell niche (Kirilly and Xie, 2007). Both GSCs lacking the Dpp-repressed differentiation genes *bam* and *bgcn* and GSCs with relatively higher expression of dMyc have an advantage in competing for niche residency (Jin et al., 2008; Rhiner et al., 2009). The process by which GSCs compare their relative fitness for niche occupancy is also still unclear but the relative ability to adhere to cap cells (Jin et al., 2008) and competition for the Dpp signal (Rhiner et al., 2009) have both been suggested to be involved.

In summary, two essentially different types of competitive interactions have been described, in which determinants, effectors and sensing mechanisms specific to each type result in distinct outcomes. Cell competition in the wing epithelium leads to apoptotic death, whereas stem cell competition for niche occupancy determines the displacement and differentiation of less competitive cells. How cells sense the relative competitive potential is still unclear.

In our system, *Bmpr1a$^{-/-}$* mouse ES cells are out-competed when co-cultured with WT cells. This chapter aims to investigate how *Bmpr1a$^{-/-}$* cells are being out-competed. With this purpose, several methods were used to detect variations in apoptosis, cell proliferation, and differentiation in WT and *Bmpr1a$^{-/-}$* cells in co-cultures, both in medium containing FCS and LIF, and in basal medium. The potential involvement of c-Myc in this process, as well as the mechanism used to sense differences in BMP signalling between the two cell types, were also investigated.

6.2. Results

6.2.1. No significant differences in apoptosis or cell proliferation were observed in co-cultures of Bmpr1a$^{-/-}$ and WT cells in medium containing FCS and LIF.

The mechanism responsible for the out-competition of C1-*Bmpr1a$^{-/-}$* cells in co-cultures with WT cells was first investigated in ES-cell maintenance conditions, containing LIF and FCS. In these conditions, C1-*Bmpr1a$^{-/-}$* cells were eliminated from co-cultures after 5 to 6 passages, corresponding to 15 to 20 days (see previous chapter). To determine whether out-competed cells were undergoing apoptosis, immunostaining for activated (cleaved) caspase3 was initially performed in co-

cultures at different passages (Figure 6.1A). Quantification of apoptotic cells based on immunostainings was difficult due to the compact structure of the ES cell colonies and diffuse GFP expression. Preliminary analysis of this experiment revealed that there was a higher proportion of GFP-negative cells expressing activated caspase-3. However, it was likely that C1-*Bmpr1a*$^{-/-}$GFP cells lost GFP expression as they died and, therefore, apoptosis analysis using this assay could be misleading. Apoptosis analysis had thus to be limited to early apoptotic cells in live cultures, where dead cells (necrotic and late apoptotic cells) could be identified based on propidium iodide (PI) staining. Flow cytometry (FC) analysis of allophycocyanin (APC)-conjugated annexin V in combination with PI exclusion of dead cells was then used to assess apoptosis activation in GFP-positive (C1-*Bmpr1a*$^{-/-}$) and -negative (3.5-WT) cells in co-cultures along 4 passages. As shown in Figure 6.1B no differences in annexin V staining were observed between cells grown separately and in co-culture, or between the two cell types within the co-cultures, for any of the time-points analysed. Apoptosis was further investigated using DiIC1(5), a cationic lipophilic dye which accumulates primarily in mitochondria with active membrane potential, a feature that is lost during apoptosis leading to a decrease in the fluorescence intensity of the dye (Galluzzi et al., 2007b). The efficiency of this method in mouse ES cells was first tested treating the cells with carbonyl cyanide m-chlorophenylhydrazone (CCCP), a compound that disrupts mitochondrial membrane potential. As expected, a decrease in DiIC1(5) fluorescence was observed after exposure to CCCP. However, no differences in the DiIC1(5) intensity were detected between the two cell types in co-cultures (Figure 6.1C).
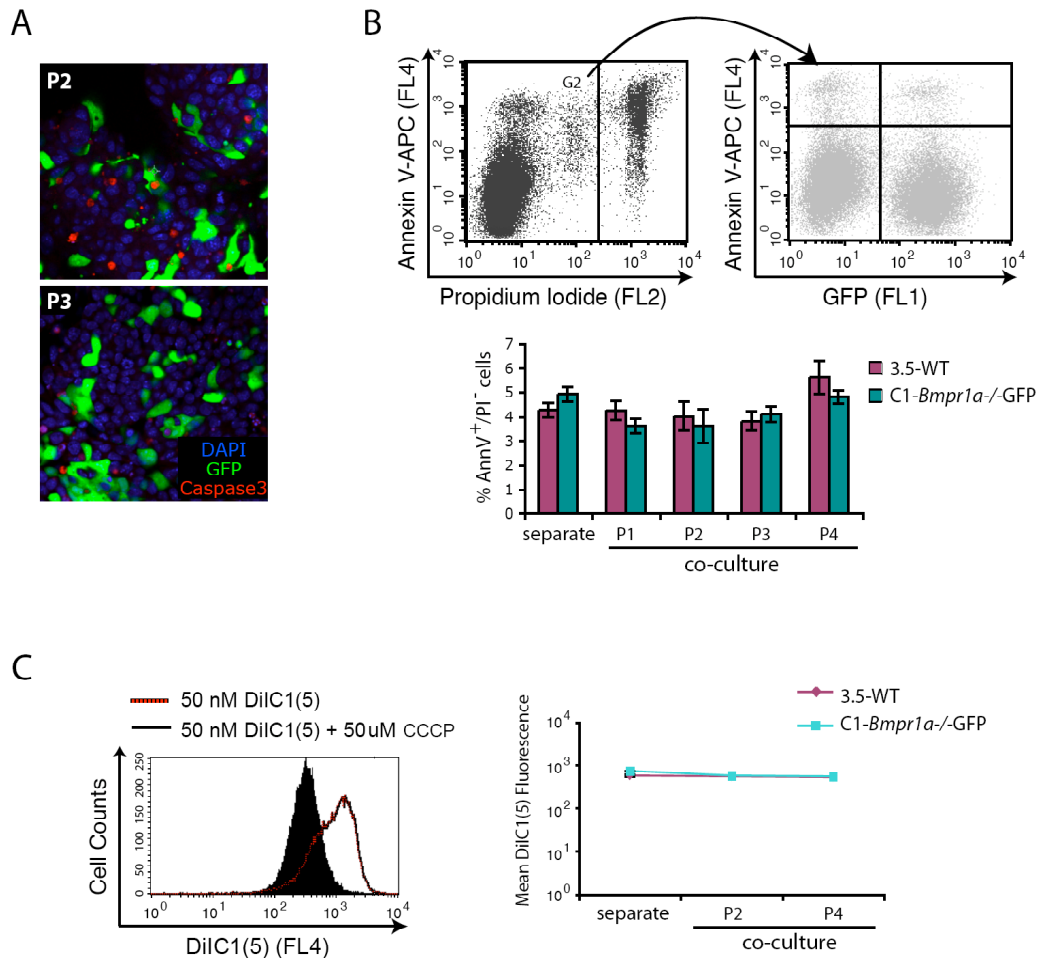
Figure 6.1 Apoptosis analysis of co-cultures of *Bmpr1a*$^{-/-}$ and WT ES cells in medium containing LIF and FCS.

(A) Confocal images showing projections of multiple optical z-sections after immunostaining for cleaved caspase 3 (red) in co-cultures of 3.5-WT and C1-*Bmpr1a*$^{-/-}$-GFP ES cells at passage 2 (P2) and 3 (P3). **(B)** Flow cytometry (FC) analysis of annexinV-APC and PI staining in co-cultures of 3.5-WT and C1-*Bmpr1a*$^{-/-}$-GFP ES cells. PI-positive (dead) cells were excluded from the analysis. The percentage of annexinV-positive/PI-negative cells within the GFP-positive and the GFP-negative populations was assessed in separate cultures and at passages 1 to 4 (P1-4) of co-cultures. Data shown as mean +/- s.d. (n=3) **(C)** Changes in mitochondrial membrane potential were assessed by FC analysis of fluorescence intensity of the lipophilic dye DilC1(5) (far red emission). CCCP exposure was used as a positive control for induced apoptosis (left). No differences were observed in DilC1(5) mean fluorescence intensities between the GFP-positive and -negative populations in co-cultures in medium containing FCS+LIF.

Changes in cell proliferation during do-culture in ES cell medium were subsequently

investigated. Bromodeoxyuridine (BrdU), a synthetic thymidine analogue, gets incorporated into a cell's DNA when the cell is dividing (during the S-phase of the cell cycle) and can thus be used as a measure of cell proliferation. Co-cultures were incubated for 5 minutes in 20μM BrdU, immunostained and analysed by confocal microscopy. This analysis suggested that BrdU was incorporated mainly by GFP-negative cells (Figure 6.2A) but quantification of this difference was difficult. To facilitate and make quantification more accurate, BrdU incorporation was subsequently analysed by FC. As shown in Figure 6.2B, FC analysis after different periods of exposure to BrdU demonstrated that a lower percentage of C1-*Bmpr1a*$^{-/-}$-GFP cells in co-cultures incorporated BrdU, indicating a lower number of proliferating cells, both compared to 3.5-WT cells in the same co-cultures, and to C1-*Bmpr1a*$^{-/-}$-GFP in separate cultures. However, this difference was small and not statistically significant ($p > 0.05$, student's t test).

Cell proliferation in co-cultures was also assessed using the SNARF-1 dilution method (Magg and Albert, 2007). The carboxyl seminaphthorhodafluor (SNARF-1) dye is cell permeable in the acetomethyl ester form and diffuses passively into the cells where after deacetylation it is captured by cellular esterases. Once bound intracellularly this dye is symmetrically diluted in the daughter cells after each cell division and its dilution can therefore be used as a proliferation indicator. Unlike more commonly used proliferation dyes, such as CFSE, the far red emission of SNARF-1 allows its use in combination with GFP. SNARF-1 was loaded into co-cultures at passage 3 and the fluorescence intensity was analysed by FC up to 72 hours after loading. After 72h the fluorescence intensity was almost at the background level and so no further analysis was possible. This analysis revealed that the two cell types in co-culture had very similar proliferation rates, and only a very small difference in fluorescence intensity indicated that 3.5-WT cells proliferate slightly faster than C1-*Bmpr1a*$^{-/-}$-GFP cells in co-cultures (Figure 6.2C).
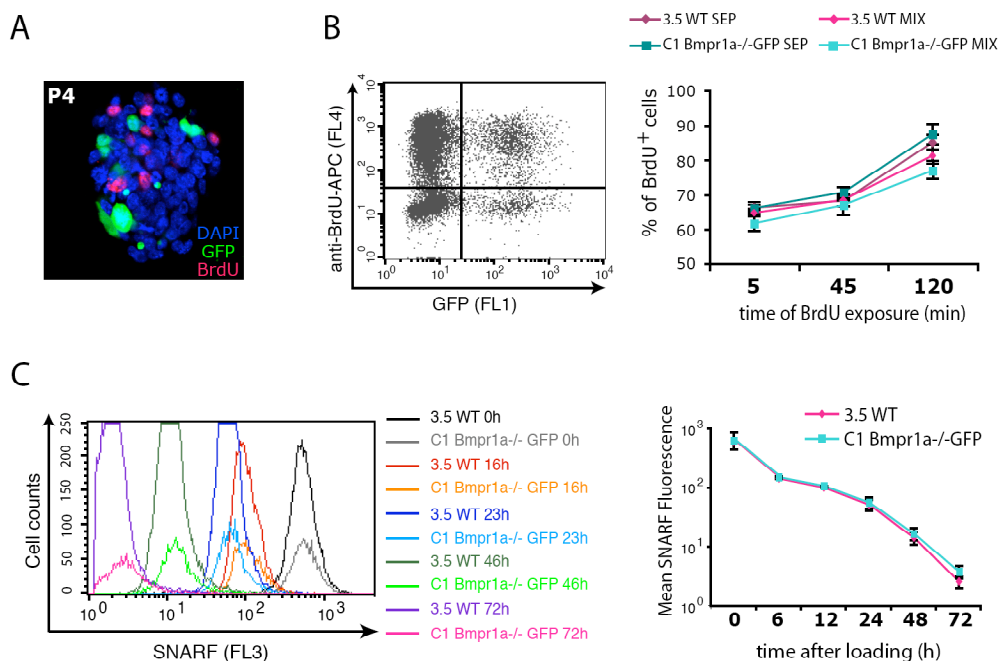
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Figure 6.2 Proliferation analysis of co-cultures of *Bmpr1a⁻ᐟ⁻* and WT ES cells in medium containing LIF and FCS.

(A) Confocal image showing the projection of multiple optical z-sections after immunostaining for BrdU incorporation (red) in co-cultures of 3.5-WT and C1-*Bmpr1a⁻ᐟ⁻*-GFP ES cells at passage (P)4. (B) Flow cytometry (FC) analysis of immunostaining for BrdU incorporation after 5, 45 and 120 minutes of BrdU exposure. No significant differences were observed in BrdU incorporation between separate cultures and co-cultures (passage3) of 3.5-WT and C1-*Bmpr1a⁻ᐟ⁻*-GFP ES cells in medium containing FCS+LIF. Data shown as mean +/- s.d. (n=3) (C) Histograms of SNARF fluorescence analysis by FC (left) and graphical representation of mean fluorescence intensities (right), just after loading of the dye (0h), and at different time points after loading. Data shown as mean +/- s.d. (n=2)

The cell cycle profiles of sorted cells after co-culture was also analysed but no differences were observed (Figure A.2, in appendixes section)

Overall, these results show that the co-culture of C1-*Bmpr1a⁻ᐟ⁻*-GFP cells with 3.5-WT cells in ES-cell maintenance conditions (containing LIF and FCS) does not induce changes in apoptosis but does seem to cause slightly reduced cell proliferation of C1-*Bmpr1a⁻ᐟ⁻* cells.

6.2.2. Reduced proliferation and increased differentiation of Bmpr1a^{-/-} cells in serum-free medium lead to their out-competition from co-cultures with WT cells.

Since the co-culture of *Bmpr1a*^{-/-} and WT cells in basal (serum-free) medium considerably enhanced cell competition, the role of apoptosis and cell proliferation in the out-competition of *Bmpr1a*^{-/-} cells was subsequently analysed in these conditions.

As can be seen in Figure 6.3A, after 4 days in co-culture, the levels of annexin V staining of C1-*Bmpr1a*^{-/-}-GFP cells were very similar to the ones of this cell type in separate cultures. Regarding 3.5-WT cells, a small decrease in apoptosis was observed in co-cultures, but this was not statistically significant ($p > 0.05$, student's t-test).

Apoptotic cell death was also investigated in competing co-cultures of E14-WT-GFP and Fr124-*Bmpr1a*^{-/-} ES cells. In this case, the number of early apoptotic annexin V-positive/PI-negative Fr124-*Bmpr1a*^{-/-} cells was moderately increased in co-cultures in comparison to separate cultures ($p > 0.05$, student's t test) and did not change in E14-WT-GFP cells (Figure 6.3B).

These results suggest that apoptosis is not a major factor leading to the out-competition of *Bmpr1a*^{-/-} cells in basal medium.
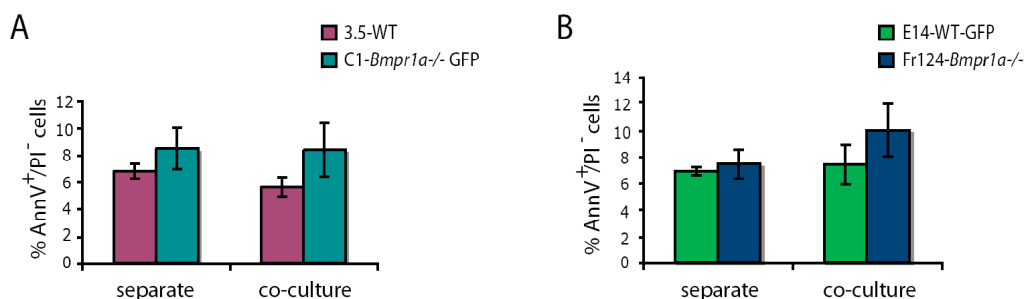
Figure 6.3 Apoptosis analysis of co-cultures of *Bmpr1a*^{-/-} and WT ES cells in basal medium.

Figure 6.3 Apoptosis analysis of co-cultures of Bmpr1a-/- and WT ES cells in basal medium.

Graphical representation of the percentage of annexinV-positive/PI-negative cells obtained by flow cytometry analysis for the GFP-positive and GFP-negative cell populations in separate cultures and co-cultures of (A) 3.5-WT and C1-*Bmpr1a*$^{-/-}$-GFP ES cells, and (B) E14-WT-GFP and Fr124-*Bmpr1a*$^{-/-}$ ES cells, in serum-free basal medium. Data shown as mean +/- s.d. (n=3)

Changes in cell proliferation in serum-free co-cultures were next investigated. As shown in Figure 6.4A and 6.4B the number of BrdU-positive *Bmpr1a*$^{-/-}$ cells in co-cultures was lower than in separate cultures and than in co-cultured WT cells. This was true both for co-cultures of C1-*Bmpr1a*$^{-/-}$-GFP with 3.5-WT cells and of Fr124-*Bmpr1a*$^{-/-}$ with E14-WT-GFP cells. These differences were not statistically significant (p>0.05, student's t test), which may be due to the variability in competition efficiency between assays, as well as the low number of biological replicates (n=3). However, the tendency for a lower number of *Bmpr1a*$^{-/-}$ cells in S-phase in co-cultures was constant and reproducible.

These results indicate that the co-culture of *Bmpr1a*$^{-/-}$ with WT ES cells induces a decrease in proliferation of the cells with lower BMP signalling activation, hence contributing to their out-competition.
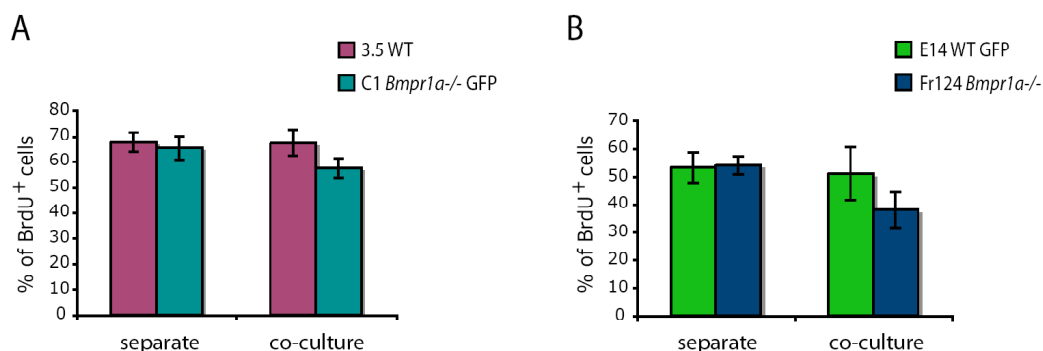
Figure 6.4 *Bmpr1a*$^{-/-}$ ES cells show decreased proliferation when co-cultured with WT cells in basal medium.

Percentage of cells positive for BrdU incoporation after a 2 hour BrdU exposure in separate cultures and co-cultures of **(A)** 3.5-WT and C1-*Bmpr1a*$^{-/-}$-GFP ES cells, and **(B)** E14-WT-GFP and Fr124-*Bmpr1a*$^{-/-}$ ES cells, in serum-free basal medium. Data shown as mean+/-s.d. (n=3)

Given the close association between cell-cycle regulation and the self-renewal/differentiation choice in ES cells (Burdon et al., 2002; Orford and Scadden, 2008; Singh and Dalton, 2009), and the role of BMP4 in ES-cell self-renewal (Qi et al., 2004; Ying et al., 2003a) we hypothesised that $Bmpr1a^{-/-}$ cells could be differentiating in result of the competitive interactions with WT cells.

For the study of ES cell differentiation, cells in co-cultures were stained for the pluripotency marker SSEA-1 and analysed by FC. Figure 6.5A shows that a significantly higher percentage of SSEA-1 negative (differentiated) C1-$Bmpr1a^{-/-}$cells cells were found in co-cultures compared to separate cultures (nearly 2-fold increase, p<0.05, student's t-test). The role of differentiation in the out-competition of $Bmpr1a^{-/-}$ cells was further confirmed by performing co-cultures in conditions in which differentiation is inhibited. LIF promotes self-renewal by activating the STAT3 transcription factor (Matsuda et al., 1999; Niwa et al., 1998; Smith et al., 1988; Williams et al., 1988), and is capable of maintaining relatively undifferentiated cultures, even though it only completely inhibits differentiation in combination with serum or BMPs (Ying et al., 2003a). Differentiation is also efficiently blocked, and self-renewal maintained, in the presence of chemical inhibitors of ERK (PD0325901) and GSK3 (CHIR99021) signalling (2i) (Ying et al., 2008). As shown in Figure 6.5B cell competition was completely inhibited both by LIF and 2i conditions. Surprisingly, even in the presence of each inhibitor separately (PD or CHIR) competition was blocked to a similar extent to the 2i. The role of differentiation in the out-competition of $Bmpr1a^{-/-}$ ES cells was also tested in co-cultures of the $Bmpr1a$-null Fr124 and E14-WT cells. Even though in this case a consistent increase in SSEA1-negative $Bmpr1a^{-/-}$ cells in co-cultures was not observed (Figure 6.5C), LIF and the ERK and GSK3 inhibitors were able to block cell competition, in a similar way to the co-cultures of C1-$Bmpr1a^{-/-}$ and 3.5-WT cells (Figure 6.5D).
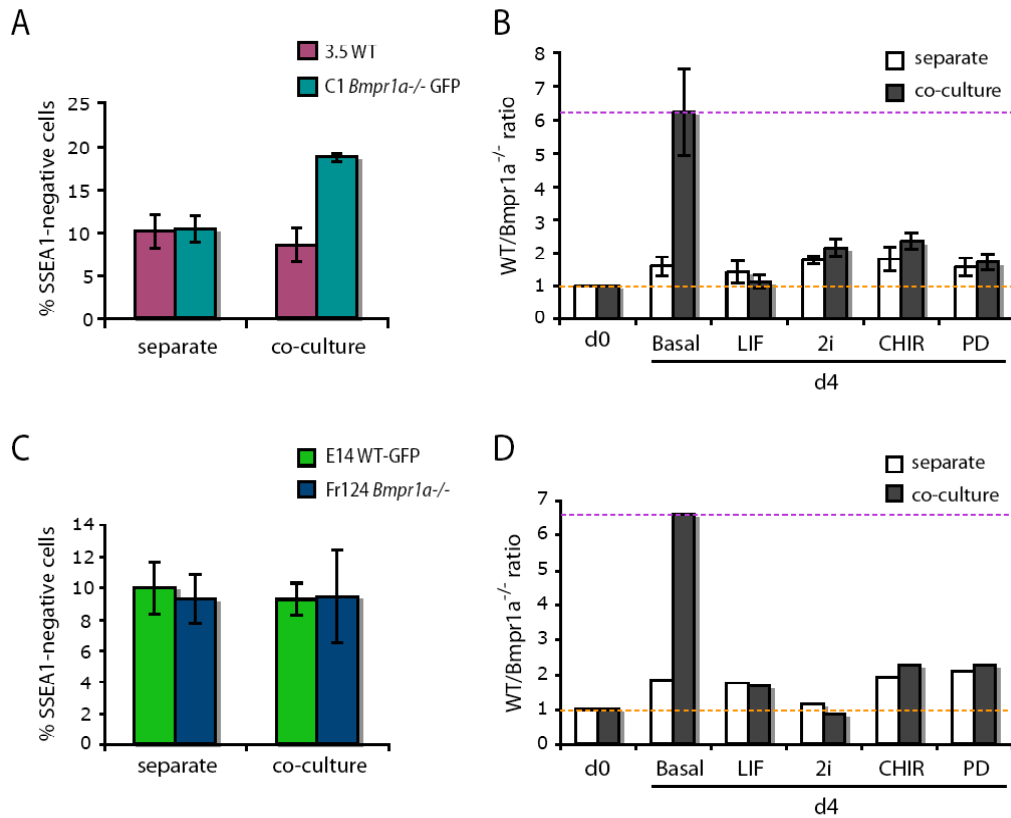
Figure 6.5 Inhibition of differentiation prevents the out-competition of *Bmpr1a$^{-/-}$* ES cells from co-cultures with WT cells.

(A) Expression of the mouse ES-cell marker SSEA1 was assessed by flow cytometry (FC) after immunostaining of live cells. The increased number of SSEA1-negative cells in co-cultured C1-*Bmpr1a$^{-/-}$*-GFP ES cells is indicative of increased differentiation of these cells. Data shown as mean +/- s.d. (n=3) **(B)** Representation of the WT/Bmpr1a$^{-/-}$ ratios (3.5-WTcells/C1-*Bmpr1a$^{-/-}$*-GFP cells) obtained when cells were grown in separate cultures and in co-cultures for 4 days (d4) in different growth conditions. The orange dashed line represents the WT/Bmpr1a$^{-/-}$ ratio at the beginning of the experiment (d0, ratio=1 would be maintained if neither of the cell lines had a growth advantage over the other one) and the purple dashed line represents the WT/Bmpr1a$^{-/-}$ ratio in serum-free basal medium, where significant competitive interactions are observed. Inhibition of differentiation by the addition of LIF, 2i (MEK inhibitor PD0325901 plus GSK3 inhibitor CHIR99021), or each inhibitor separately (PD or CHIR) to basal medium also prevented competitive interactions between 3.5-WT and C1-*Bmpr1a$^{-/-}$*-GFP ES cells. **(C)** Percentage of SSEA1-negative cells in co-cultures of E14-WT-GFP and Fr124-*Bmpr1a$^{-/-}$* ES cells. **(D)** Representation of WT/Bmpr1a$^{-/-}$ ratios obtained for separate cultures and co-cultures of WT-GFP and Fr124-*Bmpr1a$^{-/-}$* ES cells after 4 days (d4) in basal medium and in conditions where differentiation is inhibited (n=1).

To find out whether *Bmpr1a*^{-/-} cells are differentiating into any particular fate, the expression of lineage specific markers was analysed. As shown in Figure 6.6, only *Krt18* and *Krt14* show some degree of up-regulation in C1-*Bmpr1a*^{-/-} cells in co-cultures, indicating differentiation into early epidermis (Turksen and Troy, 1998). Concomitantly, *Bmpr1a*^{-/-} ES cells down-regulate the neural precursor marker Nestin in co-culture.
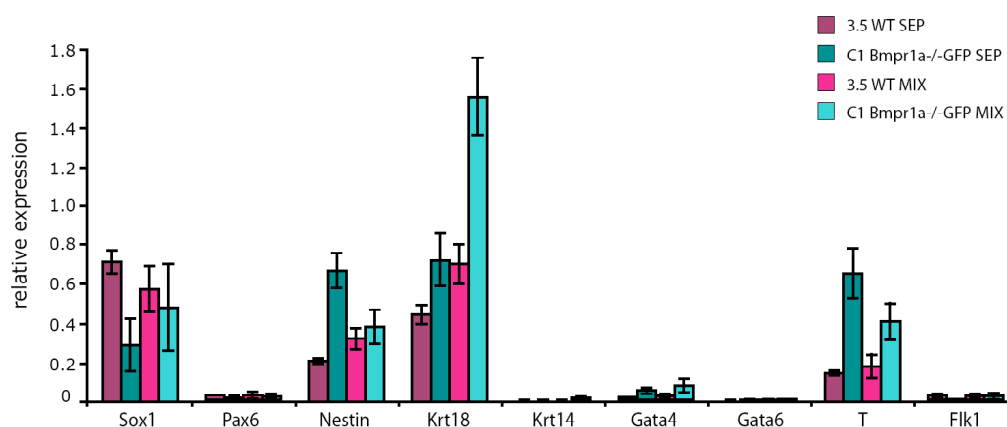
Figure 6.6 Out-competed *Bmpr1a*^{-/-} ES cells show upregulation of epidermal and downregulation of neural specific genes.

qRT-PCR gene expression analysis of neural (*Sox1, Pax6* and *Nestin*), epidermal (*Krt18* and *Krt14*), endodermal (*Gata4* and *Gata6*) and mesodermal (*Flk1* and *T*) lineage specific markers. Gene expression was analysed in 3.5-WT and C1-*Bmpr1a*^{-/-}-GFP ES cells cultured separately (sep) and in FACS sorted co-cultures (mix) at the fourth day of serum-free culture. Expression values were normalised to *Hmbs* and *Hprt1* expression; data shown as mean +/- s.d. (n=2).

6.2.3. "Winner" cells in competing co-cultures have increased levels of c-MYC.

In *Drosophila*, *dMyc* expression levels determine cell competitive interactions between cells, both in the wing epithelium and in the stem cell ovary (de la Cova et al., 2004; Moreno and Basler, 2004; Rhiner et al., 2009). The MYC family of transcription factors is implicated in a variety of biological processes (reviewed in

Eilers and Eisenman, 2008; Meyer and Penn, 2008), among them cell cycle regulation (Obaya et al., 1999; Oster et al., 2002) and control of ES-cell self-renewal (Cartwright et al., 2005; Singh and Dalton, 2009). Therefore, we asked whether c-MYC has a role in the out-competition of *Bmpr1a*$^{-/-}$ ES cells.

We first analysed the *c-Myc* mRNA levels in 3.5-WT and C1-*Bmpr1a*$^{-/-}$-GFP cells in ES-cell maintenance conditions (with LIF and FCS), in FACS sorted co-cultures after 3 and 4 days in basal medium, and in control separate cultures at the same stages. As shown in Figure 6.7A, C1-*Bmpr1a*$^{-/-}$ ES cells express higher levels *c-Myc* than matched WT cells in FSC+LIF. After LIF withdrawal, the levels of *c-Myc* expression drop in both cell types, but a relatively higher expression is maintained in C1-*Bmpr1a*$^{-/-}$ cells in separate cultures. However, when the two cell types are mixed, *c-Myc* expression is similar in the two cell types.

The levels of c-MYC protein at these same stages were next analysed by western blot. To control for the potential protein degradation during the FACS sorting required to separate mutant and WT cells in the co-cultures, protein lysates from separate cultures were obtained both before and after submitting the cells to FACS.

Regarding the samples that did not go through FACS sorting, it can be seen in Figure 6.7B that c-MYC expression was significantly lower in cells grown in serum free basal medium than in the maintenance conditions containing serum and LIF. After 3 days in basal medium, c-MYC levels were higher in C1-*Bmpr1a*$^{-/-}$ ES cells than in control WT cells, which is in agreement with the mRNA expression levels, but this difference was lost by the fourth day in culture. Comparing these with the expression levels of matched samples that were submitted to FACS sorting, it is clear that a substantial amount of protein degradation takes place during the duration of the sorting procedure (Figure 6.7B). We can thus infer that the co-culture samples underwent similar levels of degradation. This makes even more surprising the observation that, at the third day of co-culture in basal medium, c-MYC could be

detected at considerably higher levels in co-cultured 3.5-WT cells than in co-cultured C1-*Bmpr1a*$^{-/-}$ cells medium (Figure 6.7B and Figure A.3 in Appendixes section). No difference was observed when the cells were grown separately or at the fourth day of co-culture in basal medium (Figure 6.7B).
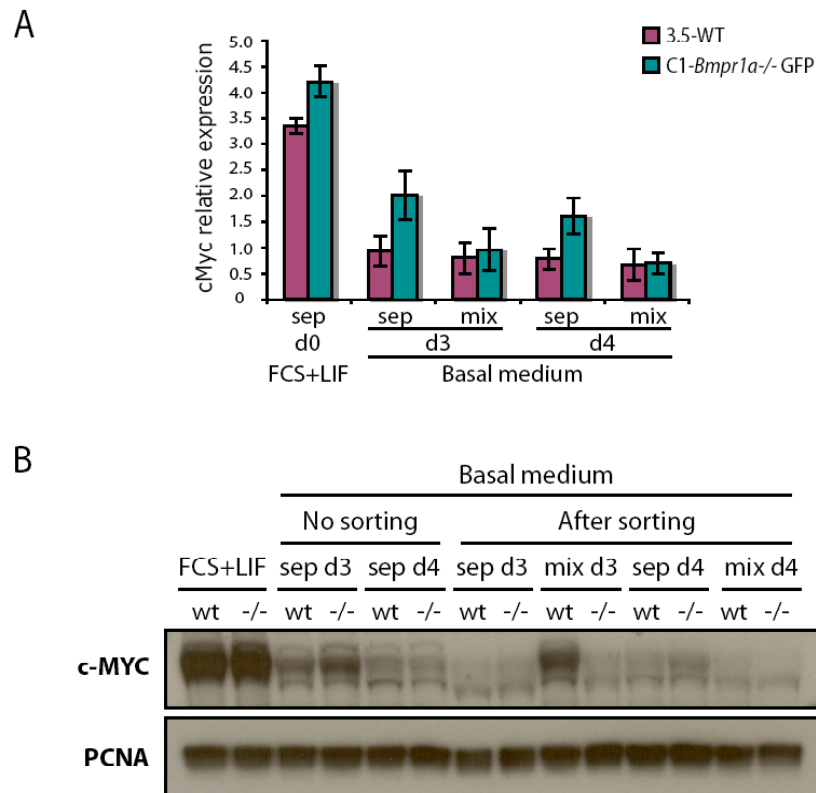
Figure 6.7 WT ES cells have increased c-MYC levels when co-cultured with *Bmpr1a*$^{-/-}$ ES cells.

(A) qRT-PCR analysis of *cMyc* gene expression in separate cultures (sep) and FACS sorted co-cultures (mix) of 3.5-WT and C1-*Bmpr1a*$^{-/-}$-GFP ES cells grown in ES-cell maintenance conditions containing FCS+LIF, and at the third (d3) and fourth (d4) days of culture in serum free conditions (Basal medium). Expression values were normalised to *Hmbs* and *Hprt1* expression; data shown as mean +/- s.d. (n=2). (B) Protein lysates were obtained from 3.5-WT and C1-*Bmpr1a*$^{-/-}$-GFP ES cells in the following conditions: separately cultured in FCS+LIF, separately cultured (sep) in serum free (basal) medium, before and after FACS sorting, at the third (d3) and fourth (d4) day of separate culture (sep), and after co-culture (mix) in serum free (basal) medium, after FACS sorting, at the third (d3) and fourth (d4) day of culture. The expression of c-MYC protein in these lysates was analysed by western blot. PCNA was used as loading control.

The higher levels of c-MYC observed in WT cells at the third day of co-culture could be due to a considerably increased expression of this protein. This is an unlikely hypothesis given the RNA expression, but it cannot be excluded as it could be regulated at the translational level or differences in transcription could have been present at earlier stages. Alternatively, the observed differences could be due to an increased stability of c-MYC in the WT cells when co-cultured with $Bmpr1a^{-/-}$ ES cells.

6.2.4. Investigating how ES cells sense differences in BMP signalling during competition.

Finally, the mechanism by which ES cells in competing co-cultures compare their relative levels of BMP signalling and determine the differentiation of $Bmpr1a^{-/-}$ cells was investigated.

To determine whether $Bmpr1a^{-/-}$ and WT cells were competing for limiting amounts of BMPs in the culture medium, co-cultures were carried out in the presence of BMP ligands and of BMP antagonists.

Adding BMP4 and BMP7 to the culture conditions has intrinsically different effects in terms of SMAD1//5/8 activation in WT and $Bmpr1a^{-/-}$ cells. BMP4 increases the disparity in BMP activation between the two cell types, whereas BMP7 induces activation of the pathway in both of them (see Figure 3.7B in chapter 3). Surprisingly, none of these ligands significantly altered the competition outcome in co-cultures (Figure 6.8A).

To investigate the effect of further limiting BMP availability in culture, the competition assays were performed in the presence of the BMP antagonist Noggin and of the fusion protein BMPR1A-FC, a soluble dominant negative form of BMPR1A. The efficiency of different concentrations of Noggin and of BMPR1A-FC was initially

tested (Figure A.4, in appendixes section). Both 50 and 250 ng/ml of Noggin were able to block Smad1/5/8 activation in response to 10 ng/ml BMP4, whereas inhibition by BMPR1A-FC was only effective at the higher concentration. The addition of Noggin and BMPR1A-FC at concentrations that efficiently blocked Smad1/5/8 phosphorylation led to a small and but not significant increase in competitive interactions (Figure 6.8B).
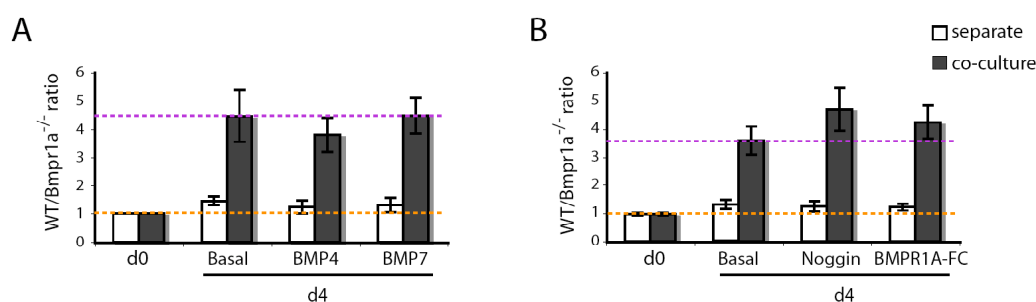
Figure 6.8 Cell competition between WT and *Bmpr1a*^{-/-} ES cells in the presence of BMP ligands and inhibitors.

(A) Representation of the WT/Bmpr1a^{-/-} ratios (3.5-WTcells/C1-*Bmpr1a*^{-/-}-GFP cells) in separate cultures and in co-cultures of the two cell types after 4 days (d4) in the absence (Basal) or presence of BMP4 or BMP7. **(B)** WT/Bmpr1a^{-/-} ratios in separate cultures and in co-cultures, after 4 days growth in the presence or absence of Noggin and the BMPR1A-FC chimera. The orange dashed line represents the WT/Bmpr1a^{-/-} ratio at the beginning of the experiment (d0, ratio=1) and the purple dashed line represents the WT/Bmpr1a^{-/-} ratio in serum-free basal medium, where significant competitive interactions are observed. All data is represented as mean +/- s.d. (n=2).

Together, these results indicate that WT and *Bmpr1a*^{-/-} cells in mixed cultures do not compete for limiting amounts of BMPs. However competitive interactions may be enhanced when BMP availability is additionally restricted by inhibition of autocrine or paracrine signals.

If $Bmpr1a^{-/-}$ and WT ES cells do not compete for limited BMP availability, another mechanism must ensure that they communicate their relative degree of BMP signal transduction. In the epithelium of the *Drosophila* wing, this process has been reported to be mediated by soluble factors produced by both cell types, although these cells do not necessarily have to be in physical contact (Senoo-Matsuda and Johnston, 2007).

We thus tested whether a similar mechanism may be allowing the cells to perceive and respond to differences in BMP transduction in neighbouring ES cells. With this aim, 3.5-WT and C1-$Bmpr1a^{-/-}$-GFP cells were grown in basal media conditioned by each cell type separately, or by 3.5-WT and C1-$Bmpr1a^{-/-}$-GFP co-cultures. The cell growth after 4 days culture in these conditions was assessed in terms of the number of viable cells present in the culture. Preliminary results (Figure 6.9) showed that cells grown in conditioned medium (CM) have a significantly reduced proliferation or viability, likely due to nutrient depletion of the medium while it was being conditioned. These experiments also indicated that medium conditioned by 3.5-WT cells as well as medium conditioned by competing co-cultures gives a small growth disadvantage to C1-$Bmpr1a^{-/-}$-GFP cells, which is not observed when these cells are grown in medium conditioned by C1-$Bmpr1a^{-/-}$-GFP cells. However, these differences could be explained by differential nutrient depletion in the medium. Nutrient depletion is highly dependent on the cell density, which may not be exactly the same in the different cultures along the duration of the experiment due to small differences in cell proliferation (see Figure 5.4B). For this reason, different assays must be designed to test the role of soluble factors in cell competition, such as the culture of the two cell types in chambers that prevent their physical contact but allow sharing the same culture medium, and hence the exchange of soluble factors.
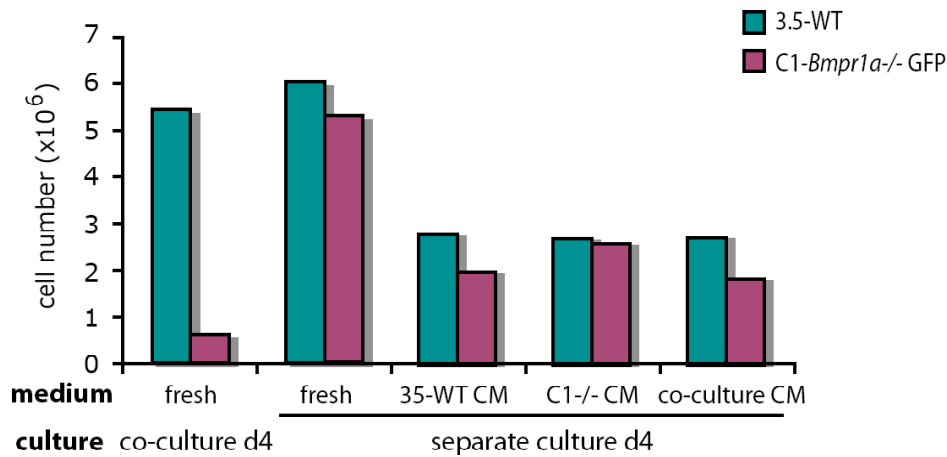
Figure 6.9 Analysis of role of secreted factors in cell competition.

3.5-WT and C1-*Bmpr1a$^{-/-}$*-GFP ES cells were cultured in fresh basal medium, or in medium previously conditioned (CM) for 24 hours by each cell type grown separately or by co-cultures of both cell types. The number of viable cells in each growth condition was counted at the fourth day of culture.

6.3. Discussion

In the previous chapter, *Bmpr1a$^{-/-}$* and WT ES cells were shown to establish competitive interactions. When co-cultured, these two cell types exhibited a growth behaviour different to the one shown in separate cultures, leading to the out-competition of the cells with lower BMP activation. This process was observed to be more prominent in basal medium, a condition in which survival and growth factors are very limiting. But how do cells perceive the differences in signalling activation and determine their response to those differences?

Fundamentally, two processes can be responsible for the out-competition of *Bmpr1a$^{-/-}$* in this system: increased cell death, or changes in cell cycle and proliferation. Both of these possibilities were investigated in co-cultures of *Bmpr1a$^{-/-}$* and WT ES cells. This study was initially carried out in cultures containing LIF and serum, where no significant differences in apoptosis or cell proliferation were identified by any of the

different methods used to study these cellular processes. This is probably due to the slowness of the process in these culture conditions as it takes 5 to 6 passages (around 15 to 20 days), for the *Bmpr1a*$^{-/-}$ cells to be completely eliminated from the co-cultures. In serum-free basal medium cell competition was more efficient but no increased apoptosis of *Bmpr1a*$^{-/-}$ cells was observed. However, this observation does not rule out the contribution of cell death to the competition process. A factor to take into consideration is that the study of cell death in these ES cell cultures is complicated by two technical problems: first, dead cells generally detach from the ES-cell colony and start floating and, second, GFP expression is lost as cells die. Although annexin V staining identifies one of the early features of the apoptotic process, the loss of phospholipid asymmetry in the plasma membrane, it cannot be excluded that ES cells die very soon after this. Also, apoptosis is the most common and best studied form of programmed cell death but not the only one (Degterev and Yuan, 2008; Edinger and Thompson, 2004; Galluzzi et al., 2007a) and so features of other types of cell death should also be analysed, such as the activation of specific pathways and effectors.

Regarding the study of cell cycle changes during cell competition, a reduced number of proliferating (BrdU-positive) *Bmpr1a*$^{-/-}$ cells was observed in competing co-cultures when compared to cells in separate cultures. Staining for the pluripotency marker SSEA-1 revealed that the decrease in *Bmpr1a*$^{-/-}$ proliferating cells was accompanied by their increased differentiation. The upregulation of *Krt18* observed in *Bmpr1a*$^{-/-}$ cells in mixed cultures suggests differentiation into simple epithelium (early epidermis), what was initially startling given the described role of BMPs in promoting epidermal differentiation (see chapter 4). However, a more careful analysis of the published data showed that the BMP4 epidermal-inducing activity has been reported to be restricted to a short window between the 4th and 8th day of differentiation (Coraux et al., 2003). Also, our previous results show that culture in BMP4 from the

onset of differentiation is permissive of the epidermal fate (see Figure 4.6, chapter 4) but we do not know whether this choice would be favoured in the absence of BMP signalling during initiation of the differentiation process.

An alternative hypothesis is that cell competition mimics what has been suggested to occur in *Xenopus* and chick embryos at the border between cells with high and low BMP signalling, where a form of cell communication has been proposed to be required for the induction of different fates (Linker et al., 2009). In a similar way, *Bmpr1a*$^{-/-}$ ES cells would normally undergo neural differentiation as they have very low levels of BMP signalling. However, if they are adjacent to cells that are responding to higher concentrations of BMPs, they are instructed not to initiate neural differentiation but rather to initiate epidermal differentiation. This model would suggest that cell differentiation is not only regulated by the overall levels of signalling, but also by the relative levels of signalling perceived by neighbouring cells.

The crucial role of differentiation in the out-competition of *Bmpr1a*$^{-/-}$ cells was confirmed by the observation that in the presence of LIF and in 2i, conditions that inhibit ES-cell differentiation, cell competition is prevented. Surprisingly, the single activity of each of the components of the 2i (the MEK inhibitor PD0325901 and the GSK3 inhibitor CHIR99021) also prevented the out-competition of *Bmpr1a*$^{-/-}$ cells. Inhibition of ERK signalling in serum free medium in the absence of LIF suppresses differentiation even though cell viability is compromised (Ying et al., 2008). Given the short duration of our assay (4 days), ERK inhibition did not significantly impair cell viability. However, its differentiation-suppressing effect did block cell competition. Regarding GSK3 inhibition, it has been suggested to be an important effector mechanism of two different self-renewal signals, possibly converging in a common target. GSK3 activity antagonises Wnt signalling, so its inhibition would increase Wnt signalling activation, which has been described to maintain pluripotency of mouse and human ES cells (Sato et al., 2004). Furthermore, GSK3 inhibition has also been

reported to act downstream of LIF/STAT3 as a mechanism to maintain MYC stability and thereby sustain ES-cell self-renewal (Cartwright et al., 2005).

Several lines of evidence point to a likely involvement of MYC in the out-competition of *Bmpr1a*$^{-/-}$ cells: 1) dMyc is involved in cell competition both in the *Drosophila* wing epithelium (de la Cova et al., 2004; Johnston et al., 1999; Moreno and Basler, 2004) and in the ovarian stem cell niche (Rhiner et al., 2009); 2) the cell cycle changes in out-competed *Bmpr1a*$^{-/-}$ cells are likely to be regulated by MYC (Obaya et al., 1999; Oster et al., 2002; Singh and Dalton, 2009); 3) the increased differentiation of *Bmpr1a*$^{-/-}$ cells could be caused by low MYC levels (Cartwright et al., 2005; Leon et al., 2009; Singh and Dalton, 2009); and 4) the suppression of cell competition by GSK3 inhibition could be due to a stabilisation of MYC in *Bmpr1a*$^{-/-}$ ES cells (Cartwright et al., 2005).

qRT-PCR analysis showed that *c-Myc* mRNA levels were not higher in WT cells than in *Bmpr1a*$^{-/-}$ ES cells in conditions where cell competition could be observed. Indeed, *c-Myc* was found to be more highly expressed in *Bmpr1a*$^{-/-}$ cells than in WT cells, both in ES-cell maintenance conditions containing FCS and LIF, and up to after 4 days culture in serum-free basal medium.

Besides transcriptional regulation and RNA turnover, post-translational mechanisms also play a very important role in regulating MYC expression, specifically at the level of protein degradation/stability (Meyer and Penn, 2008). In particular, phosphorylation of Thr58 by GSK3 directs MYC ubiquitination and proteasomal degradation. Analysis of c-MYC protein levels revealed that, indeed, increased levels are present in WT cells in competing co-cultures. This is probably due to increased protein stability, even though confirmation of this requires the analysis of GSK3 activity and Thr58 phosphorylation in both cell types in mixed cultures. It is thus logical to reason that increased c-MYC stability is an important effector, downstream of BMP signalling, in determining the out-competition of *Bmpr1a*$^{-/-}$ ES cells. However,

the importance of c-MYC stabilisation during cell competition should be further confirmed, for example by analysing the competitive behaviour of cells expressing a stabilised form of c-MYC (with the T58A mutation (Cartwright et al., 2005)).

An important question in the study of cell competition is how cells are able to perceive their competitive potential in relation to their neighbours. The approaches taken here to start investigating this matter have not been able to provide a clear answer. They have shown that cells in co-cultures do not compete for limiting amounts of BMPs, but also that competitive interactions are maximized when autocrine and paracrine BMP signals are also inhibited. This indicates that comparison of BMP signalling activity is important for cell competition, but other factors must also be mediating this process. The communication mechanism by which ES cells compare their competitive levels and instruct appropriate responses remain elusive.

Overall, these findings establish a system in which competitive interactions between ES cells lead to the elimination of the cells with lower BMP signaling. Comparison of BMP transduction abilities, via a still unidentified mechanism, leads to a stabilisation of c-MYC in more competitive cells, and to reduced cell proliferation, increased differentiation and, therefore, out-competition, of cells with a lower ability to transduce BMP signaling.

Stem cell interactions such as the ones described here may significantly contribute to the balance between self-renewal and differentiation, and ensure the elimination of abnormal or less adapted cells. Therefore, it may be an important mechanism regulating normal development and tissue homeostasis.

GENERAL DISCUSSION

BMPR1A is the type I BMP receptor most highly expressed in the pluripotent ICM and early epiblast of the mouse embryo (Mishina et al., 1995; Roelen et al., 1997), and in undifferentiated ES cells (Qi et al., 2004; Ying et al., 2003a). Signalling via BMPR1A is required in the mouse embryo to maintain pluripotency and prevent precocious neural differentiation of the epiblast (Di-Gregorio et al., 2007) and for mesoderm and endoderm formation (Beppu et al., 2000; Mishina et al., 1995). In mouse ES cells, BMP4 signalling inhibits neural differentiation, maintaining self-renewal and pluripotency in combination with LIF (Ying et al., 2003a), and previous efforts to derive *Bmpr1a-null* ES cells required inhibition of the p38 MAPK pathway (Qi et al., 2004).

The first aim of this thesis was to analyse in more depth the role of BMPR1A in mouse ES-cell self-renewal and differentiation, as well as the downstream effectors involved in regulating these processes. In this study *Bmpr1a$^{-/-}$* ES cells were derived in the absence of MAPK inhibitors. It is likely that this was possible due to the right balance between BMP/SMAD and LIF/STAT3 activation being achieved in the ES cell derivation conditions used. These cells could be maintained in culture for long periods in an undifferentiated state and although they did not activate any of the known intracellular responses to BMP4, they were able to self-renew and showed normal proliferation and gene expression patterns. The work presented indicates that *Bmpr1a$^{-/-}$* ES-cell self-renewal is likely to be sustained by the presence of residual SMAD1/5/8 phosphorylation which is sufficient to maintain the expression of the target gene *Id1*. The low SMAD1/5/8 activation was maintained even in serum-free medium containing only LIF and BMP4. Evidence presented in this study indicates that this SMAD1/5/8 activation is probably due to autocrine stimulation of ACVR1 by BMPs of the OP-1 subgroup such as BMP7. Together, these results indicate that

SMAD1/5/8 is the main pathway involved in sustaining self-renewal downstream of BMPs. Additionally, they show that the ability to maintain ES-cell self-renewal and pluripotency is not restricted to BMP4/BMPR1A as signalling by members of the OP-1 subgroup, like BMP7, elicits similar effects.

Analysis of the differentiation of $Bmpr1a^{-/-}$ cells in different conditions showed that these cells are able to give rise to derivatives of the 3 gem layers, and therefore that they do not have pluripotency defects. However, they seem to have a predisposition for neural specification, which is in accordance with the described requirement for low BMP signalling during neural induction in the embryo (Arnold and Robertson, 2009; Hemmati-Brivanlou and Melton, 1997; Kishigami and Mishina, 2005) and the *in vitro* function of BMPs in blocking ES-cell neural differentiation (Ying et al., 2003a; Ying et al., 2003b). The mechanism by which the lower BMP signalling levels in undifferentiated cells determine this tendency for increased neural differentiation is not clear in the present study. One possibility is that this could be a direct consequence of the differences in pSMAD1/5/8 and Id expression at the onset of differentiation, which could accelerate the differentiation process. However, the similar levels of expression of pluripotency and epiblast markers between mutant and control cells suggests that additional factors determine this increased neural differentiation. A possible alternative explanation is that, due to the lower BMP signalling, a permissive epigenetic state is established at the promoters of neurogenic genes, therefore allowing an accelerated neurogenesis once differentiation along the neural path is initiated. Clarification of this issue would contribute to the understanding of neural specification in the gastrulating mouse embryo where the BMP levels are kept low in certain epiblast populations due to the local expression of BMP antagonists (Arnold and Robertson, 2009; Kishigami and Mishina, 2005).

A further observation made in this study was that in serum-free conditions at relatively high confluencies, differentiation of $Bmpr1a^{-/-}$ cells to an ectodermal fate

was favoured, with upregulation of markers of both neural and epidermal lineages. This observation raises the interesting possibility of the existence of a bipotent ectodermal precursor, previously postulated by Aberdam et al. (2007a). Further differentiation of this precursor to neural or epidermal lineages would be directed by external signals, in particular BMPs, or even possibly by mechanisms of cell-cell communication.

In the second part of this study, the existence of competitive interactions between ES cells with differential levels of BMP signalling activation was investigated. Cell competition was first described to occur during the growth of the *Drosophila* wing epithelium where cells with higher ribosomal or biometabolic activity eliminate weaker cells in mosaic organisms (Diaz and Moreno, 2005; Johnston, 2009). Dpp is an important growth and survival factor during development of the *Drosophila* wing (Burke and Basler, 1996; Martin-Castellanos and Edgar, 2002) and the efficiency in Dpp uptake is one of the factors that determines the competitive potential of cells in this system (Moreno and Basler, 2004; Moreno et al., 2002). In the Drosophila ovary Dpp sustains self-renewal of GSCs by repressing the expression of differentiation factors (Chen and McKearin, 2003; Song et al., 2004) and it has also been involved in stem cell competition for niche residency of cells with different levels of dMyc expression (Rhiner et al., 2009).

In this study, the co-culture of *Bmpr1a*$^{-/-}$ and WT ES cells led to changes in the behaviour of these cells, which resulted in the out-competition of those with lower levels of BMP activation. This process was inhibited when BMP signalling activity was re-established in *Bmpr1a*$^{-/-}$ cells, confirming that defective BMP transduction confers a competitive disadvantage to ES cells. However, it was also shown here that the ability for BMP signalling transduction is not the only factor that can induce competition in ES cells.

The competitive interactions between WT and $Bmpr1a^{-/-}$ cells were considerably increased in serum-free basal medium. In co-cultures in these conditions, a reduced cell proliferation and concomitant increased differentiation were observed in $Bmpr1a^{-/-}$ cells. No evidence for the involvement of cell death in the out-competition of $Bmpr1a^{-/-}$ cells was found, although this matter requires further analysis.

One important aspect to consider in these experiments is that the conditions that promote cell competition, besides being of very limiting amounts of survival and growth factors, are also conditions that induce ES-cell differentiation. When differentiation was prevented by the presence of LIF or in 2i medium, the out-competition of $Bmpr1a^{-/-}$ cells was also prevented. Therefore, an intriguing hypothesis is that the undifferentiated ES-cell state is protective of cell competition, and only when the differentiation programme is initiated the cells become more vulnerable or responsive to competitive interactions. Differentiation is certainly involved in the out-competition of $Bmpr1a^{-/-}$ cells, but is it a trigger or a consequence of the competitive interactions?

A striking way in which WT cells responded to the presence of $Bmpr1a^{-/-}$ cells was by increasing the levels of c-MYC protein, probably by increasing its stability. Increased Myc levels have been described to be involved in cell competition in both systems where this process has been studied in *Drosophila* (de la Cova et al., 2004; Johnston et al., 1999; Moreno and Basler, 2004; Rhiner et al., 2009). The observed c-MYC stabilisation could be achieved via the inhibition of GSK3 activity, which has been reported to direct MYC proteosomal degradation, but the mechanisms involved in the cell-cell comparison that leads to these changes are not known. The meaning of this increased stability is also unclear. High levels of c-MYC have been described to sustain self-renewal in the absence of LIF (Cartwright et al., 2005), and ectopic Myc expression promotes the stem cell state during reprogramming (Knoepfler, 2008; Takahashi and Yamanaka, 2006) and supports a "metastable" pluripotent state (Hanna et al., 2009). Therefore, one possibility would be that c-MYC stabilization

could be sustaining the undifferentiated state of WT cells, while *Bmpr1a*^{-/-} cells differentiated, as expected in response to LIF withdrawal. However, SEEA-1 staining shows that the pluripotency of WT cells is only slightly increased in co-cultures. c-MYC could also be regulating cell-cycle progression in WT cells (Singh and Dalton, 2009) but again, changes in the number of proliferating cells were only observed in *Bmpr1a*^{-/-} cells and not WT cells. Therefore, c-MYC stabilisation in WT cells seems to be part of a mechanism that will instruct changes in *Bmpr1a*^{-/-} cells rather than directly changing the behaviour of WT cells. The specific role of c-MYC in this process, or the other players with which it interacts, should be subject of future studies.

The specific changes in the behaviour of *Bmpr1a*^{-/-} cells that lead to their out-competition are also elusive. The most likely hypothesis is that WT cells are able to induce increased differentiation or instruct cell fate specification of *Bmpr1a*^{-/-} cells in co-culture. As previously described, the culture of *Bmpr1a*^{-/-} cells in basal medium at medium confluency, the same conditions in which the co-culture assays were performed, induces ectodermal differentiation. In these conditions, increased expression of both neural an epidermal markers was observed in *Bmpr1a*^{-/-} cells, although it is not known yet whether this was in the same cell (a bipotent ectodermal precursor) or in different cells within the culture. In any case, our findings suggest that co-culture of *Bmpr1a*^{-/-} ES cells with WT cells prevents them from undergoing neural differentiation and drives them towards an epidermal cell fate. The increased differentiation may then lead to these cells being diluted out from co-cultures, or their inability to respond to BMP4 may hinder progression of the epidermal differentiation programme causing cell-cycle arrest or cell death. In line with this, it would also be interesting perform the co-culture experiments in conditions that favour neural differentiation, using the monolayer differentiation protocol.
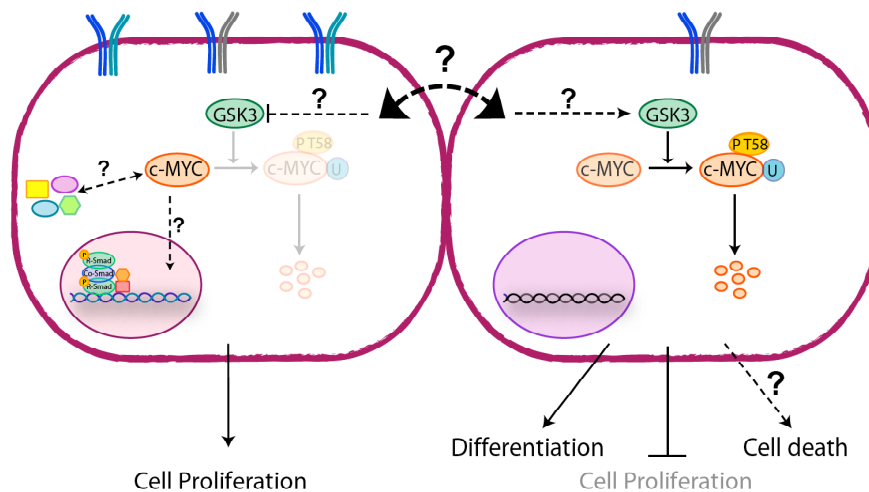
Figure 6.10 A model for cell competition between WT and *Bmpr1a*^-/- ES cells.

Competitive interactions between WT (left) and *Bmpr1a*^-/- (right) ES cells lead to the elimination of the cells with lower BMP signaling. Comparison of BMP transduction abilities, via a still unidentified mechanism (?), leads to the stabilisation of c-MYC in more competitive cells. Stabilised c-MYC in WT cells, either by interacting with other effectors or via regulation of gene transcription, maintains proliferation in these cells and instructs differentiation and reduced cell proliferation in *Bmpr1a*^-/-cells, eventually leading to their out-competition. The role of cell death in this process is still unclear (?).

As summarised in Figure 7.1, in this study a system was established where competitive interactions between ES cells lead to the elimination of the cells with lower BMP signalling. An unidentified mechanism allows comparison of the relative BMP transduction abilities between the two cell types, leading to a stabilisation of c-MYC in more competitive cells. c-MYC increased levels are probably part of a cascade of events that instruct reduced cell proliferation and increased differentiation or alternative cell fate choices in cells with a lower ability to transduce BMP signalling, eventually leading to their out-competition. Therefore, many intriguing questions remain. How do cells perceive the relative signalling abilities of neighbouring cells? Besides BMP transduction ability, which other factors determine competitive interactions between ES cells? Which effectors are involved, together with c-MYC, in responding to the relative competitive differences? How do cells

instruct the "winner" or "loser" behaviour to each other? These are just some of the many questions that this study raised for future investigations.

The existence of cell competition *in vivo* is another important issue to resolve. As a first approach to an *in vivo* system, the behaviour of co-cultures of cells with differential BMP signalling during embryoid body differentiation should be investigated (as embryoid bodies recapitulate many of the events during early embryonic development). For further *in vivo* analysis, mutant clones should be specifically induced in wild-type mouse embryos (and vice-versa) by mitotic recombination (Liu et al., 2002) and the proliferative and apoptotic behaviour of this clones investigated.

Overall, the system described here shows that the potential for proliferation and survival, and possibly the cell fate choices, of a differentiating cell are determined by interactions with its neighbours. These interactions may be fundamental in regulating the balance between self-renewal and differentiation, and selecting fitter cells during mammalian development and tissue homeostasis. For example, competitive interactions may be involved in many processes during development such as cavitation of the peri-implantation embryo, where cell death takes place in response to BMP signalling levels (Coucouvanis and Martin, 1995; Coucouvanis and Martin, 1999) or in the maintenance of adult stem cell niches such as in the haematopoietic system where c-MYC controls the balance between stem cell self-renewal and differentiation (Wilson et al., 2004). Furthermore, expansion of one cell population at the expense of another is a characteristic of tumour progression (reviewed in Baker and Li, 2008; and Rhiner and Moreno, 2009). Therefore, understanding the determinants, mechanisms and regulation of cell competition is a fundamental biological question.

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APPENDIXES

Table 1 Primers for the amplification of transcripts by quantitative RT-PCR.

| Gene	Forward sequence	Reverse sequence
Gapdh	TGCACCACCAACTGCTTAGC	GGCATGGACTGTGGTCATGAG
Hprt1	TGACACTGGTAAAACAATGCA	GGTCCTTTTCACCAGCAAGCT
Hmbs	ACTGGTGGAGTCTGGAGTCTAGATGGC	GCCAGGCTGATGCCCAGGTT
βActin	CTAAGGCCAACCGTGAAAAG	ACCAGAGGCATACAGGGACA
Pgk1	GCTGTTCCAAGCATCAAATTCT	CCCTTCCCTTCTTCCTCTACAT
Oct4	CGTGGAGACTTTGCAGCCTG	GCTTGGCAAACTGTTCTAGCTCCT
Nanog	CTTACAAGGGTCTGCTACTGAGATGC	TGCTTCCTGGCAAGGACCTT
Sox1	GCGAGATGATCAGCATGTACC	TAGTGCTGTGGCAGCGAGT
Msx1	GCCTCTCGGCCATTTCTCAG	CGGTTGGTCTTGTGCTTGCG
Pax6	ACTTCAGTACCAGGGCAACC	TCATCCGAGTCTTCTCCGTTA
Nestin	CTGCAGGCCACTGAAAAGTT	TCTGACTCTGTAGACCCTGCTTC
Krt14	CCTCTGGCTCTCAGTCATCC	GAGACCACCTTGCCATCG
Krt18	GACGCTGAGACCACACTCAC	CTCCATCTGTGCCTTGTATCG
Flk-1	AGAACATTTGTCCGAGTTCACA	CGGACTTGACTGCCCACT
Nkx2.5	CCCCAAGTGCTCTCCTGCTTTCCC	GCCATCCGTCTCGGCTTTGTCCA
Bry(T)	CGACCACAAAGATGTAATGGAG	CCAGCACCAGGAACAAGC
Gata4	GAGGCTCAGCCGCAGTTGCAG	CGGCTAAAGAAGCCTAGTCCTTGCTT
Gata6	GACTCCTACTTCCTCTTCTTCTAATTCAGA	ACCTGAATACTTGAGGTCACTGTTCTC
Cdx2	CACCATCAGGAGGAAAAGTGA	CTGCGGTTCTGAAACCAAAT
Eomes	TTCACCTTCTCAGAGACACAGTTCAT	GAGTTAACCTGTCATTTTCTGAAGCC
Bmp4	GAGGAGTTTCCATCACGAAGA	GCTCTGCCGAGGAGATCA
Bmp2	CGGACTGCGGTCTCCTAA	GGGGAAGCAGCAACACTAGA
Bmp7	CGAGACCTTCCAGATCACAGT	CAGCAAGAAGAGGTCCGACT
Bmpr1a	CTCATTTCCATGGCTGTCTG	CGACCCCTGCTTGAGATACT
Bmpr1b	CCCGGCCATAAGTGAAGA	GGGTGGGGGCTGTACTCT
Acvr1	ATTGAAGGGCTCATCACCAC	AAGACCGGAGCCACTTCC
Acvrl1	ACACCCACCATCCCTAACC	ACCAGCACTCTCTCATCATCTG
Bmpr2	GAGCCCTCCCTTGACCTG	GTATCGACCCCGTCCAATC
Id1	GCGAGATCAGTGCCTTGG	CTCCTGAAGGGCTGGAGTC
Id2	GACAGAACCAGGCGTCCA	AGCTCAGAAGGGAATTCAGATG
Id3	CAAGAGGAGCTTTTGCCACT	GAGAGAGGGTCCCAGAGTCC
c-Myc	CCTAGTGCTGCATGAGGAGAC	CCTCATCTTCTTGCTCTTCTTCA
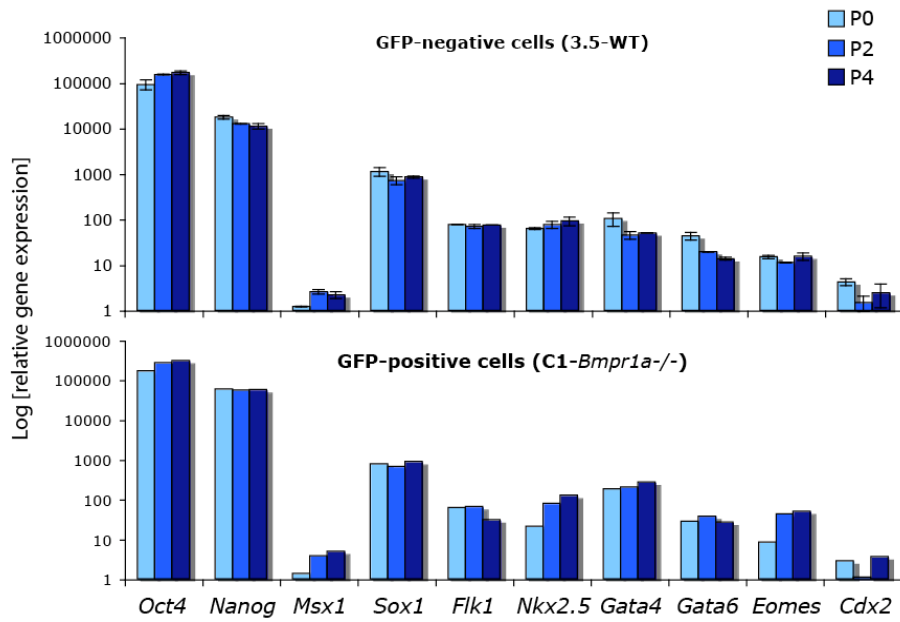
Figure A.1 Gene Expression in 3.5-WT and C1-Bmpr1a-/- co-cultures.

Gene expression analysis by qRT-PCR of lineage specific markers in 3.5-WT and C1-Bmpr1a-/- GFP cells sorted before (P0) and after co-culture for 2 (P2) or 4 (P4) passages
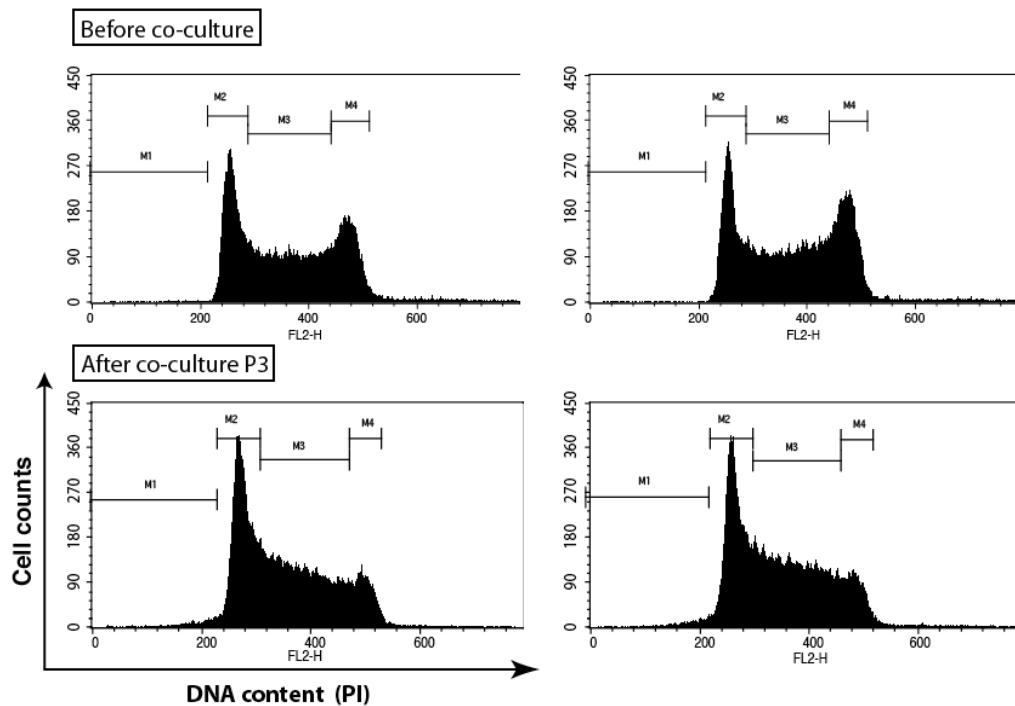

Figure A.2 Cell cycle profiles of cells in co-cultures.

Cell cycle profiles of 3.5-WT and C1-Bmpr1a-/- GFP cells sorted before (top pannel) and after 3 passages in co-culture (bottom panel)
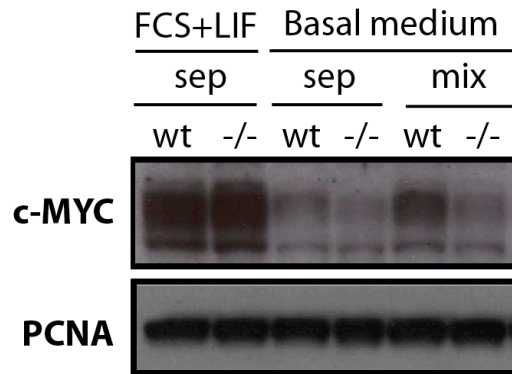
Figure A.3 c-MYC protein expression in 3.5-WT and C1-Bmpr1a-/- co-cultures.

Western Blot analysis of c-MYC expression in 3.5-WT and C1-*Bmpr1a$^{-/-}$*-GFP ES cells separately cultured in FCS+LIF, and after FACS sorting of cells separately cultured (sep) or co-cultured (mix) for 3 days in serum free (Basal) medium. PCNA was used as loading control.
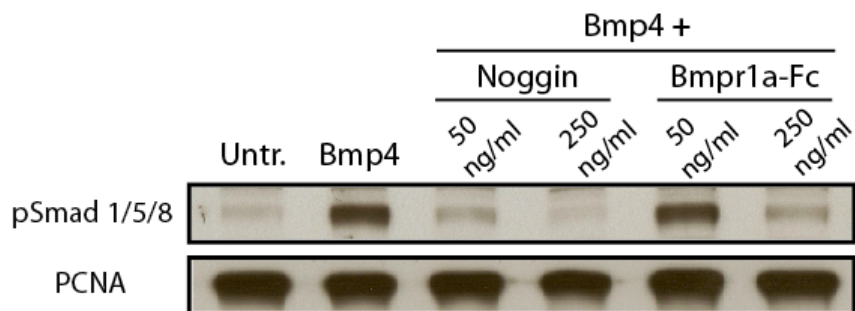
Figure A.4 Effect of Noggin and Bmpr1a-Fc in SMAD1/5/8 activation.

Western Blot showing the effects of different concentrations of Noggin and Bmpr1a-Fc in the inhibition of BMP signalling in ES cells.