

Investigating the Mechanism of Cell Competition in mESCs

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Abstract

Cell competition is a type of cell-cell interaction first described in *Drosophila* by Morato and Ripoll (1976), whereupon the co-existence of two cell populations with different metabolic properties or growth rates results in the growth of the stronger population at the expense of the weaker one. BMP2 and 4 are the *Drosophila* homologues of Decapentaplegic which has been shown to have roles in cell competition. Based on data from studies on *Drosophila* models of cell competition, the lab generated mESCs null for *Bmpr1a* and investigated their behaviour when co-cultured with wild-type mESCs as a mammalian model of cell competition.

This report further investigates changes in proliferation and apoptosis among competing cells and begins to investigate the signalling pathways which drive loser cells to initiate apoptosis. We report that apoptosis is caspase-dependent and possibly mediated by ERK and Wnt signalling as modulating these pathways changes the dynamics of competing cells. The report utilises a transwell growth system to demonstrate that cell competition is mediated by unknown signalling factors, an observation which has also been described in models of competing cells in *Drosophila*. The report shows that differentiation of mESCs is an important inducing factor for cell competition as naïve mESCs do not compete. Lastly this study shows that cell competition is a general mechanism of maintaining stem cell quality as other mutations which incur a growth or metabolic disadvantage as mESCs with defects in autophagy or are tetraploid are also eliminated by cell competition when co-cultured with wild-type cells.

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Abbreviations

2i	media containing PD0325901 and CHIRON99201
4n	tetraploid
A-P	anterior-posterior
APC	allophycocyanin
ATG	autophagy
AVE	anterior visceral endoderm
BMP	bone morphogenic proteins
BMPR	bone morphogenic protein receptor
BSA	bovine serum albumin
CRD	cysteine-rich domain
DAPI	4,6-diamidino-2-phenylindole
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
DVE	distal visceral endoderm
E	embryonic day
EDTA	ethylene-dinitrilo tetaacetic acid
EGF	epidermal growth factors
EGFP	enhanced green fluorescent protein
EMT	epithelial-to-mesenchymal transition
EpiSC	epiblast stem cell
ERK	extracellular regulated kinase
ExE	extraembryonic endoderm
FACs	flow assisted cell sorting/flow cytometry
FCS	foetal calf serum
FGF	fibroblast growth factor
FGFR	fibroblast growth factor receptor
FRS2 α	FGFR substrate 2 α
GDF	growth differentiation factor
GDP	guanosine diphosphate
GFP	green fluorescent protein

GMEM	Glasgow's modified eagle medium
GS box	domain containing a large number of glycine and serine residues
GSK	glycogen synthase kinase
GTP	guanosine triphosphate
h	hour
HSPCs	haematopoietic stem and progenitor cells
HSPGs	heparin sulphate proteoglycans
ICM	inner cell mass
Id	inhibitor of differentiation
iPS	induced pluripotent stem cell
JAK	Janus-associated tyrosine kinases
JNK	c-Jun amino-terminal kinase
LDL	low density lipoprotein
LIF	leukaemia inhibitory factor
LN	natural logarithm
L-R	left-right
M	molar
MAPK	mitogen activated protein kinase
MEK	mitogen activated protein kinase/ERK kinase
mESC	murine embryonic stem cell
min	minute
NEAA	non-essential amino acids
NP-40	nonidet P-40
PBS	phospho-buffered saline
PCP	planar cell polarity pathway
P-D	proximal-distal
PE	parietal endoderm
PGC	primordial germ cell
PI	propidium iodide
PI3K	phosphatidylinositol-3 kinase
PLC	phospholipase C

PrE	primitive endoderm
PS	primitive streak
PS media	media containing PD184352 and SU5402
PVDF	polyvinylidene fluoride
Rp	ribosomal protein
RT	room temperature
s.e.m	standard error of the mean
SDS	sodium dodecyl sulphate
Sos	Son of Sevenless
STAT	signal activator and transducer
t	time
TBS	tris buffered saline
TBST	tris buffered saline/Tween-20
TE	trophectoderm
TF	transcription factor
TGF- β	transforming growth factor- β
Tris	(hydroxymethyl)methylamine
TRITC	tetramethylrhodamine-5-(and-6)-isothiocyanate
TUNEL	terminal deoxynucleotidyl transferase dUTP nick end labelling
VE	visceral endoderm
WT	wild-type

Chapter 1 - Introduction

1.1 Early Mammalian Development

1.1.1 The Preimplantation Embryo

Murine development starts in the female oviduct, where the haploid sperm and oocyte fuse to form a fertilised egg. This totipotent cell gives rise to the whole embryo through a series of cell divisions and gradual restrictions in cell fate.

1.1.2 First Cell Fate Decision – Inner Cell Mass or Trophectoderm?

By E2.5 the fertilized oocyte has undergone multiple cell divisions and is comprised of eight blastomeres. At this stage the embryo undergoes compaction, which increases cell-cell contact and adhesion between blastomeres and generates apical-basal polarity in each cell (Pratt et al., 1982). After compaction the cells undergo two more rounds of asymmetric cell division and generate cells positioned on the inside of the blastocyst and cells positioned on the outside.

Although it is not known how this is initiated, all cells of the compacted morula can express caudal-type homeobox protein 2 (*Cdx2*) and Pou5f1 transcription factor *Oct3/4*; however expression of these genes starts to become segregated by E3.0 (Jedrusik et al., 2008; Niwa et al., 2005). Specification of inside (Inner Cell Mass - ICM) and outside (Trophectoderm -TE) cell fate is mediated by the Hippo signalling pathway, which integrates cell polarity and cell adhesion cues into cell fate. The inside-outside polarity and differences in cell adhesion result in changes in localisation of members of the Hippo signalling pathway.

In outside cells, which have established apical-basal polarity Hippo signalling is repressed by localisation of *Angiomotin* (*Amot*) to the apical membrane, bound to actin and inactive. This allows for the nuclear translocation of the transcriptional co-activator Yes-associated protein (Yap1). Yap1 binds to its partner TEA domain family transcription factor 4 (Tead4) and activates expression of TE -specific genes such as *Cdx2* (Nishioka et al., 2009; Yagi et al., 2007). Inside cells of the compacted morula are apolar and form adherens junctions with

other neighbouring cells. Members of the Hippo signalling pathway, such as *Nf2* and *Lats1/2* kinase are localised there in a functional complex with *Amot*. *Amot* binds to *Nf2* via its N-terminus, and *Lats1/2* kinase phosphorylates *Amot* at S167 and stabilises the complex. Yap is phosphorylated and remains cytoplasmic, and does not translocate to the nucleus as in outside cells, and these cells become ICM (Cockburn et al., 2013; Hirate et al., 2013; Manzanares and Rodriguez, 2013)

Expression of the *Oct3/4* is mutually exclusive of *Cdx2* and its expression becomes gradually restricted to inside cells fated to become inner cell mass (ICM). By E3.5 the early blastocyst is comprised of two distinct cell types, the ICM, which expresses *Oct3/4* and the overlying TE, which expresses *Cdx2* (Jedrusik et al., 2008; Niwa et al., 2005; Strumpf et al., 2005). *Oct4* is essential for maintaining pluripotency within the ICM (Nichols et al., 1998); whereas *Tead4* and *Cdx2* are required for maintenance and expansion of the TE (Strumpf et al., 2005). The cavity of the blastocyst forms, primarily as secretion of intracellular vacuoles which when externalized coalesce forming the blastocoel. Due to the constraints of the surrounding zona pellucida, this creates a compressed elongated shape with the ICM at one end, generating the embryonic-abembryonic axis (Rossant and Tam, 2009). Morphological changes in the embryo and key associated lineage-specific genes are illustrated in Figure 1.1. (Niwa, 2007).

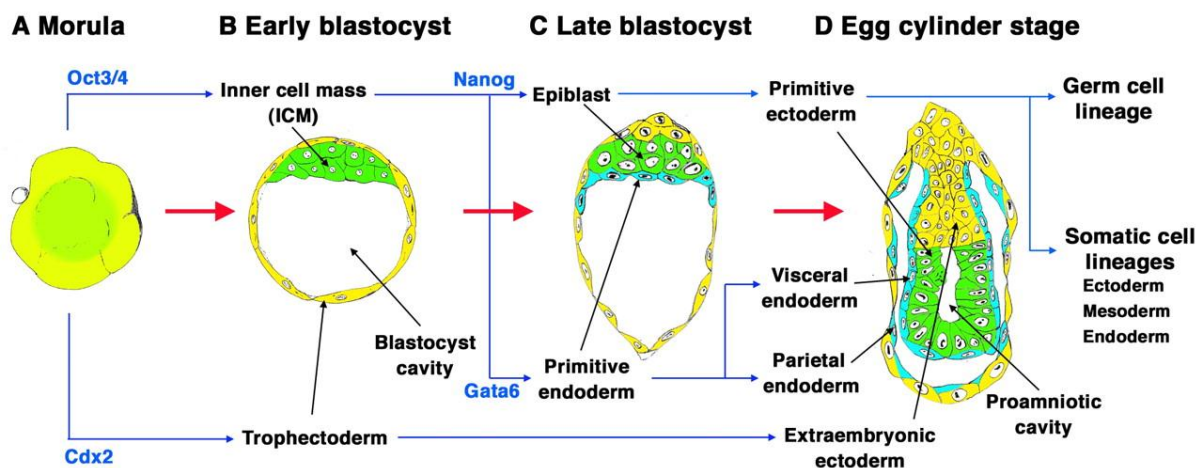


Figure 1.1 Early murine development (Niwa, 2007)

Schematic showing the different stages of murine early development. As development progresses, the embryo undergoes morphological changes and cells within the embryo differentiate. The different cell types and major signalling proteins expressed in each cell type are shown.

1.1.3 Second cell fate – PrE or Epiblast?

At E3.5 the ICM is not a homogenous population, throughout cells express either Nanog or GATA6 in a random “salt and pepper” pattern, suggesting that their fate is already determined as either primitive endoderm (PrE) or epiblast (also known as embryonic ectoderm) (Chazaud et al., 2006). GATA6-expressing cells are sorted, in a *Disabled-2* (*Dab2*) dependent manner, to the distal surface of the ICM and fated to become PrE (Yang et al., 2002); whilst Nanog and Oct4 expressing cells remain within the ICM and become epiblast. There is recent evidence that Oct4 expressing cells retain less plasticity than GATA6-positive PrE fated cells, and are thus less able to respond to signals inducing and maintaining PrE fate, hence their ability to remain undifferentiated and within the ICM (Grabarek et al., 2012; Kang et al., 2013). This view has been challenged by Frum and co-workers who show that Fibroblast Growth Factor (FGF) 4 signalling from neighbouring ICM fated cells induce *Oct4* mediated signalling in neighbouring cells to induce PrE specific gene expression (Frum et al., 2013; Livigni and Brickman, 2013). By E4.5 the ICM is segregated into these two different populations with PrE cells overlying the pluripotent epiblast.

1.1.4 Implantation and Formation of the Egg-cylinder

At E4.5 the blastocyst hatches from the zona pellucida and implants into the uterine wall. For implantation to be successful, both the peri-implantation embryo and uterine wall need to be ‘competent’ to allow attachment and invasion of the embryo into the uterine wall. Locally produced signals from the embryo alongside ovarian hormones specify uterine receptivity (Wang and Dey, 2006). Blastocyst activation (competence) is also determined by multiple factors including changes in cell cycle; signalling and energy metabolic pathways. Xie and co-workers (2008) have implicated the Wnt signalling pathway in blastocyst implantation, as mutants lacking maternal and zygotic β -catenin fail to implant and remain dormant (Xie et al., 2008).

Concurrent with implantation the embryo undergoes morphological changes, transforming from a blastocyst to form the ‘egg-cylinder’ stage embryo, as illustrated in Figure 1.1 D (Niwa, 2007). The TE proliferates to form the ectoplacental cone at the proximal tip of the

egg cylinder with the extraembryonic ectoderm (ExE) underlying it, two structures which eventually form the embryo supporting tissues such as the placenta. The PrE differentiates into two further structures; the parietal endoderm (PE) and the visceral endoderm (VE). The PE migrates from the surface of the ICM and contacts the maternal tissue directly. In contrast the VE overlies the epiblast and the ExE (Arnold and Robertson, 2009).

Proliferation of the ExE forces the epiblast to move towards the distal part of the embryo. At this point the epiblast is a solid mass of cells surrounded by the PE. During a process known as cavitation this ball of cells forms a pseudostratified columnar epithelium surrounding a central pro-amniotic cavity. Bone Morphogenetic Protein (BMP) signals from within the epiblast are required for VE differentiation but also act as pro-apoptotic signals for inner cells of the epiblast. The remaining epiblast cells survive due to pro-survival signals mediated by their adherence to the basal membrane between them and the overlying VE. The epiblast becomes a polarized layer of cells surrounding a cavity, with apical surfaces facing towards the cavity and a basal surface adhered to the underlying basal membrane (Coucouvanis and Martin, 1995, 1999; Rossant and Tam, 2009).

1.1.5 Formation of the Anterior-Posterior (A-P) axis

Interplay between the signalling pathways of the transforming growth factor- β (TGF β); Wnt and FGF families set up the embryonic axis and pattern the embryo. These pathways generate regionalised areas of gene expression that are required for subsequent development of cell lineages and tissue differentiation (Tam and Loebel, 2007; Tam et al., 2006).

Around E5.0 Nodal preproteins are produced in the ExE tissues and cleaved by convertases Fucin and Pace4 (*Spc1/Spc4*) to pattern the adjacent epiblast (Beck et al., 2002; Brennan et al., 2001). Nodal signals within the epiblast set up a feedback loop to maintain BMP signals in the ExE. BMP signalling from the ExE back to the epiblast induces *Wnt* signals and maintains Nodal signalling (Brennan et al., 2001; Lu et al., 2001; Quail et al., 2013; Shen, 2007; Vincent et al., 2003). In the overlying VE Smad2 is phosphorylated by Nodal signals but these are restricted to the most distal part of the VE by inhibitory Smad1 signals caused by BMP signalling in the overlying ExE (Rodriguez et al., 2005; Yamamoto et al., 2009).

This results in the formation of the DVE a signalling centre where pSmad2; *Foxa2*; *Lhx1* and *Eomes* act in concert to induce expression of Nodal signalling inhibitors *Lefty1/2* and *Cerberus-like (Cerl)* and *Wnt* inhibitor *Dkkopf* (Belo et al., 2000; Kimura-Yoshida et al., 2005; Meno et al., 2001; Nowotschin et al., 2013; Yamamoto et al., 2004). These inhibitory signals from the newly formed DVE restrict Nodal and Wnt expression to the then, proximal epiblast resulting in the formation of the proximal-distal axis.

By E5.5 the DVE is visible as a thickened part of the VE with columnar morphology of cells with heterogeneous expression markers including *Hex*; *Hesx1*; homeobox protein *Lhx1* (*Lim1*) and *Cerberus-like 1 (Cerl)* (Belo et al., 1997; Shawlot et al., 1999; Srinivas, 2006; Srinivas et al., 2004; Thomas and Beddington, 1996; Thomas et al., 1998). Around this time the DVE lose their columnar morphology, become cuboidal and actively migrate to the anterior side of the embryo forming the AVE (Srinivas et al., 2004). Migration of the DVE to the anterior side of the embryo to form the AVE is facilitated by a variety of mechanisms, including Nodal-driven differential proliferation of the epiblast; (Stuckey et al., 2011b; Yamamoto et al., 2004) and formation of multi-cellular rosettes within the VE to help the DVE migrate as a coherent orderly group of cells (Takaoka and Hamada, 2012). In addition to changes in proliferation and cell movement, Wnt and Nodal signals and their antagonists *Lefty*; *Cerl* and *Dkk1*, act as repulsive and attractant cues respectively to guide DVE migration (Kimura-Yoshida et al., 2005; Srinivas, 2006; Stern and Downs, 2012; Yamamoto et al., 2004).

By E6.0 DVE cells have migrated to extra-embryonic/embryonic border, which inhibits further movement and redirects movement of the DVE laterally where they lose expression of DVE markers (Trichas et al., 2011; Trichas et al., 2012). However, AVE is still being formed at the distal part of the VE and previously formed AVE starts to migrate towards the proximal side of the embryo until the AVE fully occupies the anterior side of the embryo by E6.5 (Srinivas et al., 2004; Takaoka and Hamada, 2012; Takaoka et al., 2011; Tam and Loebel, 2007; Trichas et al., 2011; Trichas et al., 2012). Like the DVE, AVE signals maintain the anterior character of the adjacent epiblast and consequently generate an A-P molecular asymmetry of Nodal and Wnt signalling throughout the epiblast prior to gastrulation (Srinivas, 2006).

1.1.6 Gastrulation and Formation of the Germ Layers

The onset of gastrulation is marked by a movement of epiblast cells converging on the proximal posterior pole of the embryo to form the primitive streak (PS) at around E6.0 (Beddington and Robertson, 1999; Tam and Behringer, 1997; Tam et al., 2006). High levels of Nodal; Wnt and BMP signalling at the proximal posterior epiblast are required for PS induction, and these levels are maintained by auto-regulatory and reciprocal signals between the epiblast and ExE (Tam and Loebel, 2007). At the primitive streak, cells undergo an epithelial-to-mesenchymal transition (EMT) and ingress into the posterior epiblast at the junction with the ExE. Over time more epiblast cells become incorporated into the primitive streak as it migrates and lengthens up to the distal tip of the embryo. Once incorporated primitive streak cells subsequently emerge as mesoderm or incorporate the definitive endoderm (Tam and Behringer, 1997).

Different regions of the primitive streak differ in gene expression; microenvironment and thus developmental potential, allocating gastrulating cells to specific lineages (Lawson et al., 1991; Tzouanacou et al., 2009). Epiblast cells which are the first to ingress into the PS give rise to extraembryonic mesoderm. As the PS elongates and migrates towards the distal tip of the embryo, cells ingress through more anterior parts of the PS and thus give rise to progressively more anterior cell fates. Mesoderm fates arise from epiblast cells which ingress through the more posterior parts of the PS, whilst definitive endoderm develops from epiblast cells that transit the most anterior region of the PS. At the most anterior part of the PS a specialised signalling structure, the node is distinguished and acts as an important signalling centre for A-P and left-right (L-R) patterning. Lastly, ectoderm arises from anterior epiblast which is thought not to enter the PS (Tam and Loebel, 2007). A small subset of cells is set aside during development as primordial germ cells (PGCs).

1.2 Pluripotent Stem Cell Lines

1.2.1 Embryonic Stem Cells

During development two populations of cells can be isolated from different developmental time points in the embryo and grown indefinitely in culture. The first of these are murine embryonic stem cells (mESCs) which can be derived from the ICM of mouse blastocysts. These were derived for the first time in 1981 and characterised by two essential properties – their ability to self-renew under appropriate conditions; and their ability to differentiate into derivatives of the three germ layers (Evans and Kaufman, 1981; Martin, 1981). Isolation of an equivalent population from human embryos only took place 17 years later (Thomson et al., 1998).

Under appropriate conditions, mESCs can be maintained indefinitely in culture without losing pluripotency and can be directed to fates from all three germ layers via appropriate stimuli allowing development to be studied at the cellular and molecular level (Nishikawa et al., 2007; Yu and Thomson, 2008). Once differentiation protocols have been developed to a degree of sufficient purity, ideally mESCs could be used for regenerative medicine. In addition to directed differentiation *in vitro*, mESCs can be reincorporated into the ICM of developing mouse blastocysts to generate chimaeras with contribution to all embryonic tissues, including germ cells and therefore progeny (Bradley et al., 1984). This allows *in vivo* studies of transgenic mice with targeted inherited mutations and markers (Raymond and Soriano, 2006).

1.2.2 Extrinsic Regulators of mESC Self-Renewal

Cell fate during development is determined by repression or activation of transcription factors in response to extrinsic cues produced by surrounding cells and microenvironment. In culture conditions, extrinsic cues are added to the culture media and produced by surrounding cells as well as the coating of the cell culture plates. As a consequence the culture conditions for maintaining mESCs have been progressively defined since their derivation allowing better understanding of how pluripotency and self-renewal are controlled. The first mESCs were cultured on layers of “feeder cells” – mitotically inactive fibroblasts and serum. Feeder cell layers serve the dual purpose of providing an adhesion surface as well as producing a signal that inhibits mESC differentiation (Smith and Hooper, 1987). Subsequent analysis identified Leukaemia Inhibitory Factor (LIF) – a member of the IL6 family of cytokines, as required for

self-renewal. LIF binds the transmembrane receptor gp130 (Heinrich et al., 2003). The LIF/gp130 complex activates downstream tyrosine phosphorylation, and eventual nuclear translocation of the Janus Kinase signal transducer and activator of transcription3, (Jak/STAT) (Matsuda et al., 1999; Niwa et al., 1998). LIF/STAT3 signalling activates downstream pluripotency targets, including a known pluripotency inductive factor cMyc (Cartwright et al., 2005).

In serum-free medium LIF/STAT3 alone is not sufficient to prevent mESC differentiation. In LIF alone mESCs differentiate to a neural fate (Ying et al., 2003). In addition to LIF without serum, BMPs are required to maintain mESC self-renewal. BMPs induce the expression of inhibitor of differentiation (*Id*) proteins via the SMAD signalling pathway and suppress neural differentiation. Upon LIF withdrawal, *Id*-expressing mESCs differentiate to non-neural fates suggesting that *Id* genes block lineage-specific transcription factors and therefore mESC self-renewal in response to LIF/STAT (Ying et al., 2003). Qi and co-workers have made an alternative argument that exogenous BMP signals act to inhibit p38 and ERK signalling to maintain mESC self-renewal independent of SMAD signalling and can be replaced with MAPK inhibitors (Qi et al., 2004).

1.2.3 Intrinsic Regulation of mESC Self-Renewal

In response to extrinsic cues, cell fate during development is determined by transcription factors which activate or repress specific gene expression programmes. In addition, changes in their organisation orchestrate germ layer fate selection (Thomson et al., 2011). Three main transcription factors; *Oct4*; the homeodomain DNA-binding protein *Nanog* and SOX-family transcription factor *Sox2* are expressed highly in the ICM and epiblast of the mouse embryo and in undifferentiated mESCs (Avilion et al., 2003; Chambers et al., 2003; Loh et al., 2006; Nichols et al., 1998; Niwa, 2007).

Oct4 expression in the mouse is restricted to blastomeres, pluripotent early embryo cells, the ICM and epiblast and the germ cell lineage. *In vitro* cultured pluripotent cell lines – embryonic carcinoma; ES and embryonic germ cells express *Oct4* (Chambers and Smith, 2004). Within the embryo levels of *Oct4* are tightly regulated as specific levels of expression

are required to maintain pluripotency. Repression or deletion of Oct4 within the ICM or its *in vitro* counterpart leads to a lack of ICM specification and consequently differentiation to trophoctoderm tissue whereas a less than two-fold increase causes differentiation to PrE and mesoderm (Nichols et al., 1998; Niwa, 2007). *Oct4* was shown to act specifically to induce PrE fate triggered by FGF4 produced by neighbouring epiblast cells (Frum et al., 2013).

Nanog was identified as part of a screen for critical factors that can maintain mESC pluripotency independently of LIF/STAT3 signalling. Nanog expression in pluripotent cell lines and the early mouse ICM and epiblast is similar to Oct4 (Chambers et al., 2003; Mitsui et al., 2003). Nanog null mutants can form ICM but fail to develop epiblast. Nanog^{-/-} mESCs can be derived but do not give rise to germ cells and tend to differentiation into extra embryonic endoderm (Chambers et al., 2003; Mitsui et al., 2003). Conversely overexpression of Nanog was capable of maintaining mESC self-renewal independently of LIF/STAT3 (Chambers et al., 2003; Mitsui et al., 2003; Pan and Thomson, 2007).

Unlike Oct4 and Nanog, Sox2 expression is not restricted to pluripotent lineages. Sox2 is first expressed in the morula and blastocyst before being restricted to the ICM and epiblast (Avilion et al., 2003). At post-implantation stages expression of Sox2 becomes restricted to presumptive anterior neurectoderm and is used throughout development; in the adult and during differentiation protocols as a marker for multipotent neural stem cells (Ellis et al., 2004). As with *Oct4*, *Sox2* null embryos have abnormal ICM and fail to specify epiblast (Avilion et al., 2003). In the absence of *Sox2* mESCs differentiate into trophoctoderm cells (Masui et al., 2007).

Several genes associated with pluripotency, including Oct4; Sox2 and Nanog, contain enhancer binding sites for Oct4 and Sox2, and it was previously thought that as the two were transcription factor partners, both bind to these enhancers and regulate their expression (Chew et al., 2005; Kuroda et al., 2005; Nishimoto et al., 1999; Tomioka et al., 2002; Yuan et al., 1995). However analysis of *Sox2* null mESCs shows that regulation of Oct4-Sox2 enhancers can occur in the absence of Sox2. Masui and co-workers conclude that the essential role of Sox2 is to stabilize mESCs in a pluripotent state by maintaining a specific level of Oct3/4 expression (Masui et al., 2007).

1.2.4 The Ground State of Embryonic Stem Cells

In order for ES cell therapy to be used in clinical treatments, they must be able to be maintained in conditions free of animal products. Conventional mESC culture requires Foetal Calf Serum (FCS) which contains secreted BMPs and exogenous LIF to be added to cultures to maintain mESCs in an undifferentiated state. Culture requirements such as FCS needed to be replaced with synthetic counterparts. Furthermore analysis of culture conditions used to maintain mESCs in culture have given valuable insight into the cues that induce differentiation. These studies raised the prospect that mESCs could intrinsically maintain an undifferentiated state if protected from external differentiation-inducing cues - a “ground state” of embryonic self-renewal (Ying et al., 2008). Oct4 induces paracrine FGF4 secretion by undifferentiated mESCs and activates ERK signalling. FGF induced ERK signalling in combination with other signalling factors primes mESCs to exit self-renewal and become responsive to lineage induction cues (Kunath et al., 2007; Stravidis et al., 2007). Inhibition of ERK signalling maintains mESC self-renewal and pluripotency (Burdon et al., 1999). Ying and co-workers suppressed ERK signalling at the FGF receptor and ERK kinase levels (using SU5402 and PD184352 (PS media) respectively) and showed that this could maintain undifferentiated mESCs in the absence of FCS with occasional neuronal differentiation. Further refinement of serum free culture conditions replaced PS media with the more potent MEK1/2 inhibitor PD0325901 and added a GSK3 inhibitor to suppress the remaining neural differentiation and promote mESC viability. The resultant media combination was termed 2i and was sufficient to maintain and propagate mESCs at low clonal density (Ying et al., 2008).

1.2.5 Epiblast Stem Cells

A second population of pluripotent stem cells can be derived from the epiblast of the late blastocyst of E5.5 to 6.5 post-implantation mouse embryos. EpiSCs express transcription factors known to regulate pluripotency (namely Oct4; Sox2 and Nanog), maintain their genomic integrity and differentiate into derivatives of the three germ layers as well as primordial germ cells (Brons et al., 2007; Tesar et al., 2007). However, ES and EpiSCs depend upon different signal transduction pathways to maintain their pluripotent state. Using

culture conditions designed for maintenance of human ESCs, EpiSCs can be maintained in chemically defined medium containing Activin A and FGF2 (Brons et al., 2007; Tesar et al., 2007). On a molecular level EpiSCs express different marker genes to mESCs, rather than Rex1 or Gbx2, EpiSCs express markers found in the *in vivo* epiblast – including FGF5 and Nodal (Brons et al., 2007). Lastly, analysis of their capacity for integration into pre-implantation-stage mouse embryos (both the morula and blastocyst stage) suggests that EpiSCs may either be unable to develop into the early cell lineages, such as the ICM or that the pre-implantation embryo is not a compatible environment for EpiSCs (Brons et al., 2007; Tesar et al., 2007). However, EpiSCs readily form chimaeras when introduced into postimplantation epiblast, if introduced prior to E8.5, a stage where epiblast pluripotency is lost (Huang et al., 2012). Differentiation of EpiSCs is also different to mESCs. Inhibition of Activin/Nodal signalling results in cells adopting neurectoderm lineage (Tesar et al., 2007). Addition of BMPs, extrinsic signals in mESC maintenance, results in specification of EpiSCs into primordial germ cells (Bao et al., 2009; Chenoweth and Tesar, 2010).

1.3 TGF β Signalling

The TGF β super-family signalling pathway is divided into two subfamilies. The first is comprised of BMP (Bone Morphogenetic Protein)/GDF (Growth and Differentiation Factor)/MIS (Muellerian Inhibiting Substance) subfamily and the second is the TGF- β /Activin/Nodal subfamily. Subfamilies are defined by sequence similarity and activation of specific downstream signalling pathways. The TGF-beta signalling superfamily is illustrated in Figure 1.2 below (Shi and Massague et al., 2003).

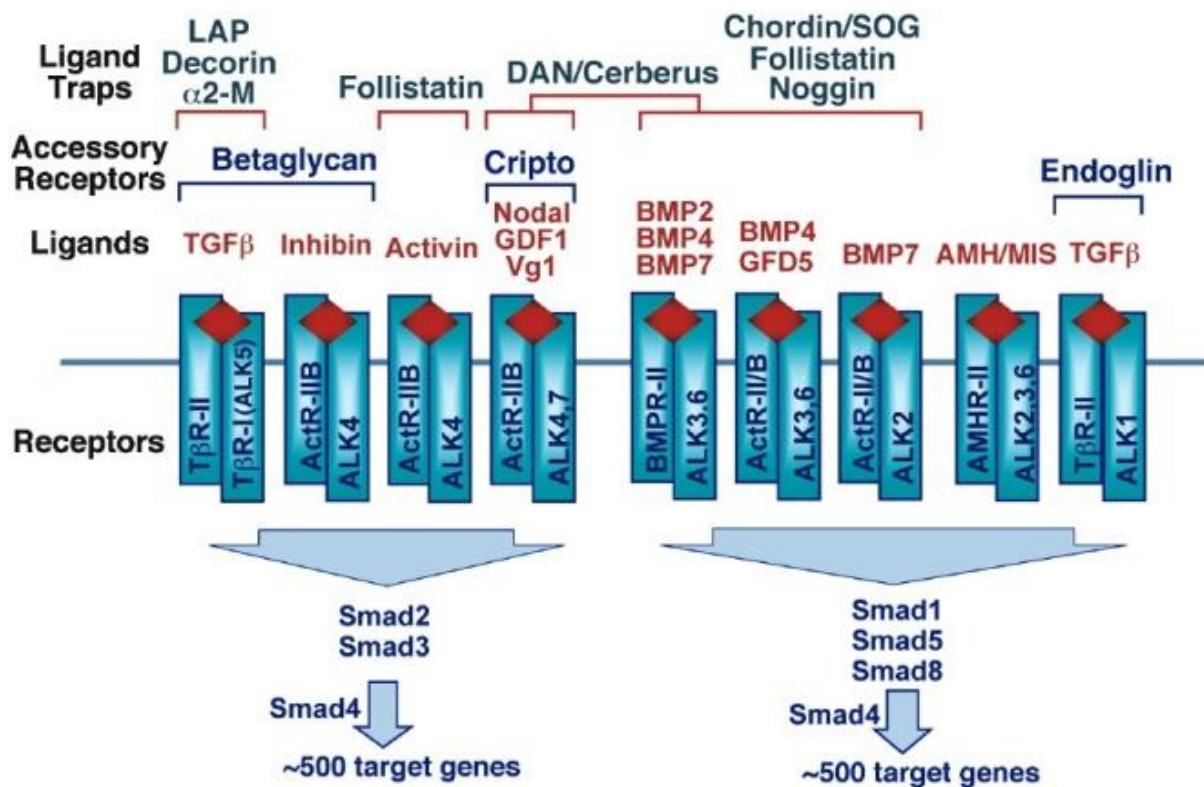


Figure 1.2 The TGF-β Superfamily of Signalling (Shi and Massague., 2003).

Figure showing the TGF-beta superfamily of signalling. Ligands (red) bind to receptor heterodimers comprised of Type I/Type II receptors. Once bound, receptors phosphorylate specific downstream SMADs, which form a complex with Co-SMAD-4 and translocate to the nucleus to affect target gene transcription. Figure taken directly from Shi and Massague, 2003.

TGF-β signalling ligands form a dimer which bind to a receptor complex composed of two Type I and two Type II receptors to activate downstream signalling (Heldin et al., 1997; reviewed by Shi and Massague, 2003). Due to the range of members of the TGF-β superfamily, various nomenclature have been used for the different ligands and receptors involved. Alternative nomenclature used for TGF-β Type I receptors are summarized in the table below:

Acvr1	ALK1
Actr1	ALK2

Bmpr1a	ALK3
Actr1b	ALK4
TβR1	ALK5
Bmpr1b	ALK6
Acvr1c	ALK7

Table 1. Alternative nomenclature used in TGFβ Signalling.

Table summarising alternative nomenclature used in TGFβ signalling (Shi and Massague, 2003; Tsuchida et al., 2004).

BMP ligands have high affinity for Type I receptors; upon binding these facilitate binding to type II receptors. In contrast, members of the TGF-β/Activin/Nodal subfamily ligands have higher binding affinity to Type II receptors. Ligand binding to the Type II receptor allows recruitment and subsequent binding of the Type I receptor (Massague et al., 1998). The specific Type I/Type II receptors bound by each ligand are illustrated in Figure 1.3.1. Interaction between Type I and Type II receptors upon ligand binding results in the phosphorylation of the GS-box region of the Type I receptor by the constitutively active Type II receptor. Once activated these phosphorylate intracellular signalling proteins called SMADs. TGF-β; Activin/Nodal and Inhibin signal downstream receptor phosphorylation of (R-) SMADs 2/3, whilst BMP/MIS/GDF related ligands signals are mediated via R-SMADs 1/5/8. All SMADs form complexes with Co-SMAD 4 prior to nuclear translocation to affect transcription. Inhibitor SMADs 6 and 7 act as negative regulators of these signalling pathways, either through directly binding to activated Type I receptors and preventing their phosphorylation (reviewed by ten Dijke et al., 2000; Yan et al., 2009) or through competitive binding for receptor Smads with Co-Smad 4 (Hata et al., 1998).

1.3.1 BMP Signalling

BMPs were originally identified in bone and comprise a large subgroup within the transforming growth factor-β (TGFβ) family. There have been around 20 BMP ligands identified (Bragdon et al., 2011); which can be further subdivided into functional subgroups based on sequence similarity and downstream activity. One subgroup is comprised of BMP2;

BMP4 and the *Drosophila* Decapentaplegic (Dpp) gene which have known roles in development and bind mainly to the type 1 BMP receptor (BMPR) 1a and 1b. Other subgroups include the OP-1 group, which is comprised of BMP5/6/7/8a and the *Drosophila gbb-60A*; and then the growth differentiation factor-5 (GDF) group made up of GDF5/6 and 7. Due to the variety of BMPRs and their binding affinities; posttranslational modifications and expression patterns, BMPs have diverse biological functions throughout development; adult tissue homeostasis and disease (Miyazono et al., 2010; Shi and Massague, 2003).

BMP receptors (BMPRs) are located at the cell surface and are serine/threonine kinase receptors. They are composed of a short extracellular domain, a transmembrane domain and the intracellular serine/threonine kinase domain. These fall into two subgroups, type I and type II. Five BMP type I receptors have been identified: Acvr1; Actr1; Bmpr1a; Actr1b and Bmpr1b (alternative nomenclature is summarised in Table 1). In addition to these there are three type II BMP receptors: Bmpr2; ActrIIa and ActrIIb. Further complexity arises with BMP ligand binding to these receptors, as different receptors bind with different affinities to different ligands and other Type I/Type II receptors (Bragdon et al., 2011; Heldin et al., 1997).

BMP ligand binding to BMPRs activates the specifically via phosphorylation of R-SMADs 1/5/8 which bind to the co-receptor (Co-) SMAD4 and translocate to the nucleus to activate downstream transcription factors. In addition to signalling through SMADs, BMP signalling interacts with many other signalling pathways such as Nodal; Wnt; Hedgehog and the MAPK cascades (Guo and Wang, 2009).

1.3.1.1 BMP signalling in early mouse development and mESCs

In mESCs, BMP signals act in conjunction with LIF to maintain self-renewal and pluripotency by upregulating expression of *Id* genes (Ying et al., 2003). BMP4 has also been shown to specifically maintain mESC self-renewal by inhibiting MAPK signalling (Qi et al., 2004).

BMPs play a variety of roles throughout mouse development. BMP4 is essential for gastrulation and embryos lacking both alleles die between E6.5 and 9.5 many arresting at the egg cylinder stage (Winnier et al., 1995). Homozygous mutations in the closely related BMP2 ligand are also embryonic lethal, although embryos develop further and are able to undergo gastrulation, mutants die due to defects in heart formation and proamniotic canal closure (Zhang and Bradley, 1996). Closer analysis of *Bmp2*^{-/-} embryos reveal heart and foregut tissues are specified properly but display perturbed morphogenesis that can be rescued by restoring BMP2 signalling in the epiblast (Madabhushi and Lacy, 2011).

Due to the redundancy of BMP ligands, mutants for the BMPRs display more severe phenotypes. BMPR2 is the type II receptor that specifically binds BMP ligands. During early development BMPR2 is expressed in the one, two-cell and blastocyst stage embryos and throughout the embryonic and extraembryonic tissues of E6.0 to E7.5 embryos (Beppu et al., 2000; Roelen et al., 1997). *BMPR2*^{-/-} mice arrest at the egg cylinder stage, show growth abnormality by E6.5 and are reabsorbed by E9.5. Analyses of these mutants show that they fail to pattern the epiblast, do not gastrulate or form mesoderm, although display no defects in VE differentiation (Beppu et al., 2000). Embryos null for either of the other Type II receptors *ActRIIa* or *ActRIIb* develop to term, although both receptors may rescue the phenotype of the other (Chang et al., 2001; Song et al., 1999).

Analysis of the Type I BMP receptors expression patterns shows that they are temporally regulated which may suggest specificity of BMP ligand binding during development. *BMPR1b* is not expressed earlier than E9.5 so is unlikely to have roles in early embryonic development (Dewulf et al., 1995). *Acvr1* is expressed in extraembryonic tissues such as the VE and later also in extra-embryonic mesoderm and ectoderm as well as the mesoderm. Mutants lacking *Acvr1* show defects in gastrulation, with abnormal VE and mesoderm formation as well as defects in formation of the L-R axis, indicating roles for BMP signalling in patterning extraembryonic tissues as well as the epiblast (Gu et al., 1999; Kishigami et al., 2004).

Bmpr1a is first detected in the blastocyst, but remains ubiquitously expressed throughout both extra- and embryonic tissues (Mishina et al., 1995; Roelen et al., 1997). Similar to

BMPR2^{-/-} mice, BMPR1a homozygous mutants are smaller by E7.0, and are reabsorbed by E9.5 with defects in mesoderm formation (Mishina et al., 1995). Later it was demonstrated that Bmpr1a signalling functions during gastrulation to guide AVE migration through regulation of *Dkk1* expression, an attractive cue that guides the AVE to the anterior side of the embryo (Miura et al., 2010). Analysis of a conditional Bmpr1a^{-/-} mutant, which lacks expression in the epiblast reveal that Bmpr1a^{-/-} mESCs are not restricted to any cell fate in chimaeric embryos, but play a role in regulating signalling sources that promote anterior cell fates (Davis et al., 2004). Further analysis of Bmpr1a^{-/-} mutants showed that BMP signalling via the BMPR1a receptor is required to promote epiblast proliferation prior to gastrulation, by suppressing premature neural differentiation (Di-Gregorio et al., 2007).

Surprisingly the Smads which transduce BMP signals also have specific functions during mouse development. Smad1 is specifically required for formation of extra-embryonic tissues, and Smad1^{-/-} mutants die due to lack of formation of chorioallantoic fusion (Lechleider et al., 2001). Smad5^{-/-} mutants are also embryonic lethal, and die during mid-gestation with heart gut and amnion defects, and defects in patterning anterior structures (Chang et al., 1999). Like BMP2 mutants, mesoderm derivatives are formed but incorrectly patterned leading to defects in their formation. Unlike Smads1 and 5, Smad8 homozygous mutants are viable and fertile. In addition to specific roles, Smads show cooperative signalling, where heterozygous mutants for single Smad alleles can partially rescue defects in others (Arnold et al., 2006).

1.3.2 Nodal Signalling

Nodal signalling is part of the TGF- β /Activin/Nodal subfamily of ligands and part of the TGF- β superfamily of signalling proteins. Like BMPs, Nodal signals are transduced via heterodimers of serine/threonine kinases at the cell surface. Nodal precursor ligands are secreted from cells and processed by proprotein convertases such as Pace4 and Furin into functional ligands in a complex at the cell surface (Beck et al., 2002; Blanchet et al., 2008; Mesnard et al., 2006). Once at the cell membrane Nodal signals are transduced via the type 1 receptor Actr1b or the Nodal-specific Acvr1c in a complex with Type II receptor ActRIIB (Reissmann et al., 2001; Shen, 2007; Shi and Massague, 2003). Once activated the

serine/threonine kinase heterodimers phosphorylates SMAD2/3, which bind to receptor SMAD4 and translocate to the nucleus (Kishigami and Mishina, 2005). At the nucleus the SMAD complex interacts with winged-helix transcription factor FoxH1 and the Mixer subclass of homeodomain proteins to activate target genes (Shen, 2007).

Transduction of Nodal signals also requires the EGF-CFC family of co-receptors/ligands. In the mammalian genome two of these receptors are present: Cripto and Cryptic (Schier and Shen, 2000; Shen et al., 1997; Yan et al., 2002). *Cripto* acts via recruiting Furin and PACE4, two proteases which cleave Nodal precursor ligands (Blanchet et al., 2008). Also at the receptor level extracellular inhibitors *Lefty1/2* act as competitive inhibitors of Nodal by binding to its serine/threonine kinase receptors and preventing ligand binding (Meno et al., 1999; Sakuma et al., 2002; Shen, 2007).

1.3.2.1 Nodal Signalling in early mouse development and mESCs

Nodal signalling has multiple evolutionally conserved roles during embryogenesis. *In vitro* studies of Nodal signalling in embryonic stem cells revealed that Activin/Nodal signalling is not required for self-renewal or pluripotency of mESCs but may promote BMP signalling by repressing Smad7. This stimulates BMP signalling to promote self-renewal (Galvin et al., 2010). However, Activin/Nodal signalling is required at later stages of development to maintain self-renewal and pluripotency of later derived EpiSCs and induction of mesoderm and endoderm cell fates (Brons et al., 2007; Fei et al., 2010; Mesnard et al., 2006; Takenaga et al., 2007).

Mutants lacking *Nodal* or its intracellular signal transducer *Smad2* fail to form mesoderm or a primitive streak and are embryonic lethal around E8.5 (Conlon et al., 1994; Nomura and Li, 1998; Zhou et al., 1993). Surprisingly mice mutants lacking the Nodal specific type I receptor *Acvr1c* are viable and fertile, showing no defects during development whilst *Actr1b*^{-/-} phenocopy *Nodal* and *Smad2* knockout mice suggesting *Acvr1c* mutations can be rescued by *Acvr1c* (Gu et al., 1998; Jornvall et al., 2004). Unlike *Smad2*, *Smad3* knockout mice are fertile and viable with no overt defects during embryogenesis, suggesting Activin/Nodal signals during development are mediated by *Smad2* (Zhu et al., 1998). Embryonic lethality

in *Nodal* signalling pathway mutants are due to a failure to pattern the epiblast in conjunction with BMP and Wnt signals. These signals specify the DVE and thus establish the proximal-distal axis, and its later migration to form the AVE and specify the anterior-posterior axis in conjunction with p38 signalling (Brennan et al., 2001; Chu and Shen, 2010; Clements et al., 2011; Lu et al., 2001; Varlet et al., 1997).

1.4 FGF Signalling

The fibroblast growth factor family is a diverse evolutionally conserved family of secreted proteins which are required to activate multiple signalling pathways. They are present in *Drosophila melanogaster* and *C.elegans* but are most diverse in vertebrates. In mouse there have been 22 ligands identified – *FGF1-18*; *FGF20-23*. FGF ligands are secreted into the extracellular environment and create a binding complex with FGF tyrosine kinase receptors and heparin sulphate proteoglycans (HSPGs) to activate a variety of downstream signalling cascades. The FGF ligands all share a similar conserved internal core of 28 amino acid residues and proteins range in molecular weight from 17 to 34kDa in vertebrates (Ornitz et al., 2000). Most FGF ligands have a secretory sequence at the N terminus, a heparin binding site and a distinct FGFR binding site.

FGF receptors (FGFRs) are intracellular tyrosine kinases (Thisse and Thisse, 2006). These are encoded by four *FGFR* genes to produce a variety of splice variants. FGFRs diversify the ability of FGFs to activate different signalling pathways through modifications to their extracellular ligand binding domains, ability to bind to FGFs and heparin sulphate proteoglycans (HSPGs) and binding to other FGFRs. Once a complex has been established between FGFs; FGRs and HSPGs it results in phosphorylation of tyrosines in the intracellular domain of the FGFR leading to activation of downstream signalling pathways.

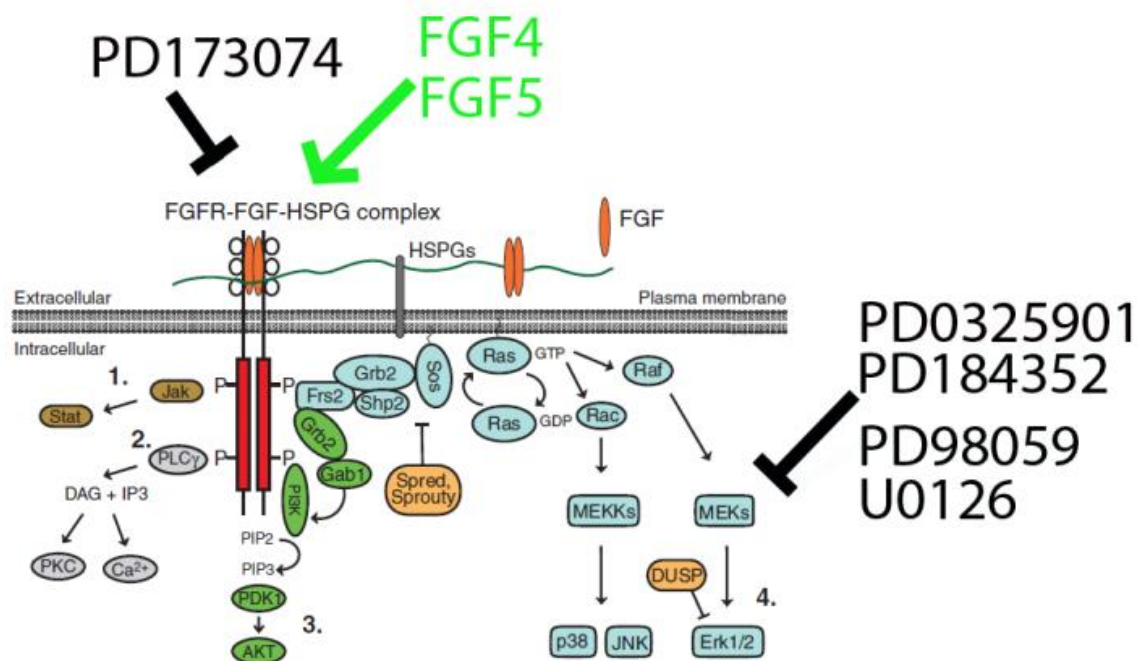


Figure 1.3 Overview of the FGF-FGFR signalling pathway (Lanner and Rossant, 2010)

FGF ligands bind to FGFRs in a complex with heparin sulphate proteoglycans. This results in activation of the FGFR receptor complex and activation of downstream signalling events. The FGFR complex can activate a number of downstream signalling targets: 1. the JAK/STAT signalling pathway; 2. The PLCγ/Ca²⁺ sensing pathway; 3. The PI3K/AKT signalling pathway, and lastly 4. The Ras/MAPK signalling pathway which leads to activation of effector MAPKs - p38; JNK and ERK1/2 (figure taken directly from Rossant and Lanner, 2010). Reagents used during experiments to affect this signalling pathway in mESCs are indicated on the figure.

FGF-ligand complexes usually signal via the Ras/MAP kinase pathway. Other signalling pathways activated via FGFRs include the PCP pathway; PLCγ/Ca²⁺; JAK/STAT and PI3K/AKT pathways. In addition to ERK, the Ras/MAPK pathway also activates the JNK and p38 kinases illustrated in Figure 1.3 (Lanner and Rossant, 2010; Villegas et al., 2010). In activation of the ERK1/2 MAPKs, once the FGF ligand is bound the activated FGFR interacts with FGFR substrate 2α (FRS2α), allowing Grb2/Son of Sevenless (Sos) binding. Sos catalyzes the exchange of GDP for GTP on Ras and Ras activation of downstream MAPKs leading to ERK1/2. These enter the nucleus and phosphorylate target transcription factors.

FGF signalling is tightly regulated at multiple levels to determine the timing duration and spread of the signal. A growing number of proteins have been identified that regulate the activity of FGF signalling pathways both at the extracellular and intracellular level. The first identified inhibitors of FGF signalling were the 4 secreted extracellular *Sprouty* and the 2 related *Spred* proteins (Hacohen et al., 1998; Wakioka et al., 2001; Yu et al., 2011). Other intracellular modulators of FGF signalling are the dual specificity phosphatases (DUSPs) (Alonso et al., 2004; Li et al., 2007) and *Sef* (Il7rd – Mouse Genome Informatics), an intracellular regulator that targets the FGFR1 and 2 receptors during development (Tsang et al., 2002).

1.4.1 FGF Signalling in early mouse development and mESCs

In vitro Kunath and co-workers showed that ERK signalling in the ICM acts as a cue priming mESCs to differentiate (Kunath et al., 2007). This was confirmed by Ying and colleagues who demonstrated that inhibition of ERK signalling by small chemical inhibitors, in conjunction with GSK3 β inhibition could maintain mESC self-renewal and pluripotency indefinitely (Ying et al., 2008). FGF/ERK signalling promotes competency of mESCs to differentiate towards EpiSCs, and neural fate (Stravidis et al., 2007).

In vivo FGF signalling is required progressively throughout development. The earliest requirements have been shown in maintenance of trophoblast cells (Georgiades and Rossant, 2006). FGF4 acting via ERK signalling has been shown to act as an auto-inductive cue for ICM differentiation and lineage commitment to PrE fate (Frum et al., 2013; Kunath et al., 2007). Analysis of mutants null for members of the FGF signalling pathway during early development confirms this requirement. Mice mutant for FGF4/FGFR2 or Grb2 fail to specify PrE (Arman et al., 1998; Chazaud et al., 2006; Feldman et al., 1995). Further work has also shown that the requirement for FGF signalling for specification of PrE fate may not be due to ERK signalling alone. Plusa and colleagues have also seen a requirement for PI3K signalling in specification of PrE cell fate, an alternative target of FGF-FGFR signalling (Plusa et al., 2008). Due to the requirement of FGF signalling in specifying PrE, FGF mutant mESCs differentiated *in vitro* as EBs also fail to develop VE. As a consequence of this, these mutants also fail to cavitate (Chang et al., 2001; Esner et al., 2002). The PI3K signalling

pathway may also have a role in cavitation as some FGF mutant defects can be rescued via forced induction of PI3K signalling (Li et al., 2001). FGF signalling is also required during gastrulation, as loss of function mutations in FGFR1 result in embryonic lethality around E7.5 to E9.5 due to defects in paraxial mesoderm formation and movement of the primitive streak (reviewed by Dorey et al., 2010; Deng et al., 1994; Yamaguchi et al., 1994).

1.5 Wnt Signalling

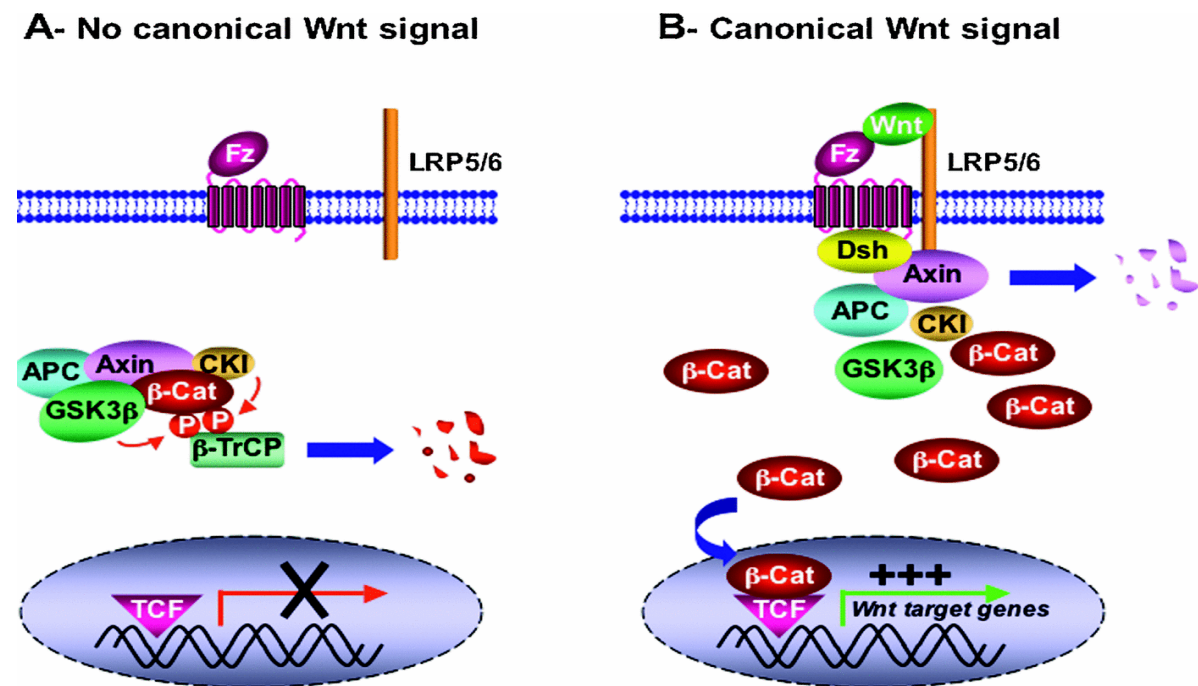


Figure 1.4 Overview of the Wnt signalling pathway (Pinto and Clevers, 2005)

(A) In the absence of Wnt signalling, the Wnt degradation complex phosphorylates β -catenin which leads to its degradation in the cytoplasm. (B) Wnt ligand binding to its receptors Fz and LRP5/6 causes translocation of Wnt degradation complex proteins to the membrane. This allows β -catenin to accumulate in the cytoplasm and translocate to the nucleus, and transcription of Wnt target genes by binding to transcription factors of the TCF/LEF family.

The Wnt signalling pathway is an evolutionally conserved signalling pathway with diverse roles in cell polarity; differentiation and embryogenesis (Logan and Nusse, 2004). There are two main signalling pathways which utilise Wnt signalling components, the canonical pathway, which involves Wnt signalling via Frizzled receptors (Fz) and β -catenin and the non-canonical pathway signals independently of β -catenin but has equally diverse roles in

cell behaviour and development (Strutt, 2003; Veeman et al., 2003). This work will focus on the canonical Wnt signalling pathway as described in Figure 1.4 (Pinto and Clevers, 2005).

Wnt ligands are defined by amino acid sequence and 19 members are expressed in the mouse genome. All Wnt proteins include a secretion signal sequence, several charged amino acid residues and an abundance of potential glycosylation sites (Mikels and Nusse, 2006). Upon synthesis Wnt proteins undergo extensive posttranslational modifications, including palmitoylation; glycosylation and lipid modifications (Sengupta et al., 2010; Tanaka et al., 2000; Willert et al., 2003). One particular protein which plays an essential role in Wnt posttranslational modification is *porcupine* (*porc*) which was first discovered in Wg processing in *Drosophila* (Kadowaki et al., 1996; Tanaka et al., 2000). Mutants lacking *porc* activity produce Wnt signals normally but fail to be secreted from producing cells significantly reducing Wnt signalling activity (Biechele et al., 2011; Zhai et al., 2004). Once modified, Wnt proteins are transported to the apical cell surface and secreted. Secreted Wnt ligands bind to a Fz/low density lipoprotein (LDL) receptor-related protein (LRP5/6) complex on the extracellular surface of neighbouring cells (Bhanot et al., 1996). Fzds are a family of seven transmembrane spanning receptors, with a cysteine-rich domain (CRD) in the ligand binding extracellular N-terminus. It is the CRD domain which mediates Wnt ligand binding at the extracellular surface (Wang et al., 1996). The C-terminus of the Fz receptor activates the canonical Wnt signalling pathway via a conserved Lys-Thr-X-X-X-Trp sequence which relocates to the membrane and phosphorylates *Dishevelled* (*Dvl*) (Chen et al., 2003; Huang and Klein, 2004; Umbhauer et al., 2000). LRP5/6 acts as a co-receptor in Wnt signalling with Fz and is also essential for Wnt signalling by recruiting Axin, a negative regulator of Wnt signalling, to the membrane when in a complex with Fz and a Wnt ligand (Mao et al., 2001; Tolwinski et al., 2003; Wehrli et al., 2000).

In the absence of a Wnt ligand the intracellular Wnt signalling co-activator non membrane bound β -catenin is sequestered in the cytosol and phosphorylated by a protein complex comprised of Dvl; glycogen synthase kinase-3 β (GSK3 β); Axin; Adenomatous Polyposis Coli (APC); Caesin Kinase1 (CK1) and protein phosphatase 2A (PP2a) (Kimelman and Xu, 2006) leading to β -catenin degradation. Upon Wnt ligand binding, Dvl and Axin are relocalised to the membrane and the β -catenin degradation complex is destabilised. β -catenin

accumulates within the cytosol and translocates to the nucleus where it acts as transcriptional coactivator (Itoh et al., 1998; Liu et al., 2002; Mao et al., 2001; Rubinfeld et al., 1996; Tolwinski and Weischaus, 2004). The main signalling targets of nuclear β -catenin are the TCF/LEF family of transcription factors. In vertebrates the TCF/Lef family of transcription factors has four paralogues TCF1,3,4 and Lef1 in addition to splice variants (Archbold et al., 2012; Hoppler and Kavanagh, 2006; Valenta et al., 2012).

Extracellular antagonists of the Wnt signalling pathway prevent ligand-receptor interactions by two different mechanisms. Extracellular signalling molecules can bind to Wnt receptors and prevent Wnt ligand binding, examples of these molecules include Frzb (also known as sFRP3) and its four homologues (sFRP1/2-4/5) form the sFRP (secreted Frizzled Receptor Protein) family; Wnt inhibitory factor 1 (Wif1) (Hsieh et al., 1999) and Cerberus (Katoh and Katoh, 2006; Kawano and Kypta, 2003). The second family of Wnt receptor antagonists bind and cause internalisation of Wnt receptor LRP5/6, including Wise (Lintern et al., 2009) and the Dkkopf family of proteins (Dkk1-4) (Mao et al., 2002).

Intracellular modulation of Wnt signalling can be achieved through modulating movement of Wnt signalling components within the cell (Fu et al., 2009), availability of receptors at the cell surface (Yamamoto et al., 2005) and availability of “free” cytosolic pool of β -catenin (Orsulic et al., 1999) among other mechanisms.

1.5.1 Wnt Signalling in early mouse development and mESCs

Wnt proteins have important roles in embryonic development as well as in tissue homeostasis and stem cell maintenance after birth (Clevers, 2006; Logan and Nusse, 2004). In addition Wnt signalling acts as a morphogen so signalling intensity and duration affect different cell fates. Wnt knockout mice models have been developed to study the role of specific ligands during mouse development, and phenotypes range from embryonic lethality to no morphological changes (van Amerongen and Berns, 2006). Wnt expression in the embryo is first detected in the mouse blastocyst with Wnt9a expressed within the TE (Kemp et al., 2005; Pfister et al., 2007). Canonical Wnt signalling has roles in specifying the primitive streak. Mutants in components of Wnt signalling; Wnt3; β -catenin and *Porc* mice fail to

form the primitive streak and eventually undergo apoptosis and are reabsorbed (Biechele et al., 2011; Huelsken and Birchmeier, 2001; Liu et al., 1999). Conversely mutants lacking negative regulators of the Wnt signalling pathway *Axin1*; *Tcf3*; *Dkk1* develop ectopic axial structures and posteriorization of the embryo and lose neural structures (Kim et al., 2000; Merrill et al., 2004; Mukhopadhyay et al., 2001; Zeng et al., 1997). This suggests that Wnt signalling is not required for formation of anterior, head structures and is required to specify posterior cell fates.

Wnt signalling is required in mESCs to maintain cell viability in the absence of other extrinsic factors (Ying et al., 2008). However the role of transcriptional Wnt signalling is controversial. In the absence of Wnt signalling, mESCs differentiate more quickly to an EpiSC fate (ten Berge et al., 2011). However further analysis of β -catenin in self-renewal and pluripotency reveals that β -catenin maintains self-renewal and pluripotency through two separate mechanisms, relieve of Tcf3 mediated repression of pluripotency required genes (Wray et al., 2011) and modulation of membrane bound pluripotency factor Oct4 (Faunes et al., 2013).

1.6 Cell Competition

Cell competition is a phenomenon where the coexistence of two cell populations with different metabolic properties or growth rates results in growth of the stronger population (termed “winners”) at the expense of the weaker one (“losers”) (Baker and Li, 2008; de Beco et al., 2012; Diaz and Moreno, 2005). Later models of cell competition also included the concept of “supercompetition”, where cells that obtain a growth advantage, through randomly acquired mutations, are able to eliminate their surrounding wild-type neighbours. A model of cell competition and ‘supercompetition’ is illustrated in Figure 1.5 (Moreno, 2009). This process of recognition and elimination of vulnerable, mispatterned or abnormal cells during tissue growth has demonstrated important roles in tissue homeostasis; organ size control; stem cell maintenance and cancer.

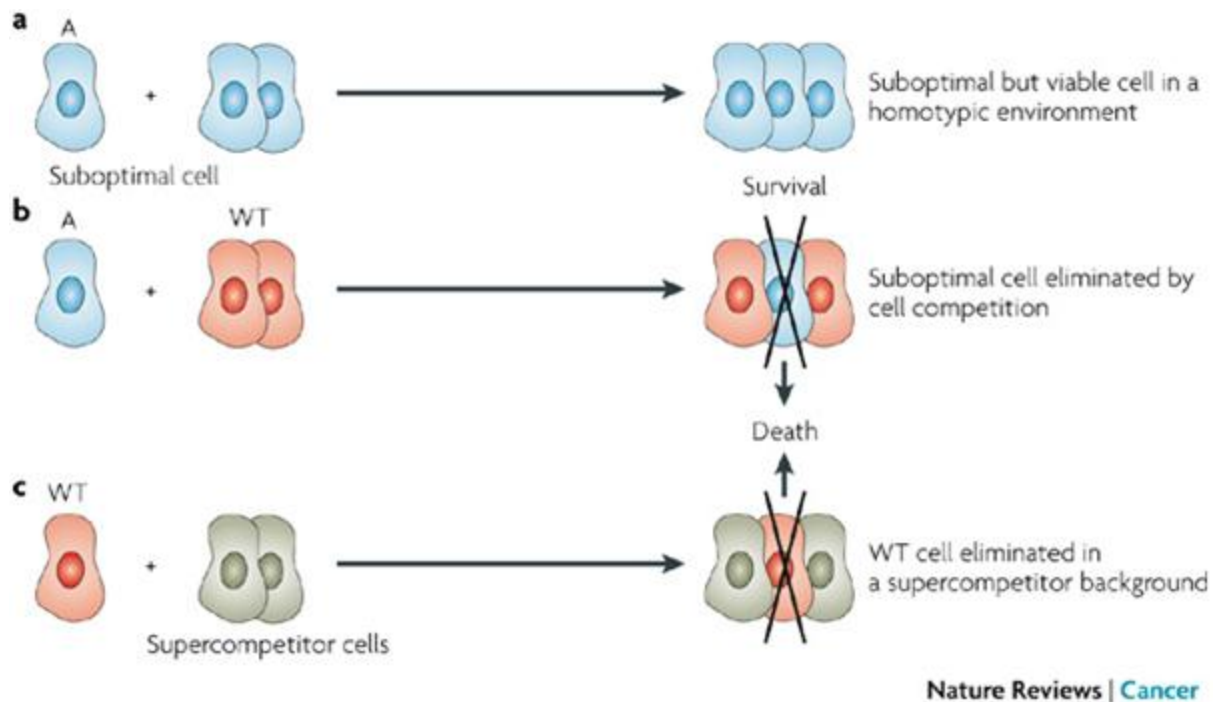


Figure 1.5 Cell Competition (Moreno et al., 2009)

Suboptimal cells grown in a monotypic environment survive. When suboptimal cells are introduced against a wild-type background, they are eliminated by cell competition. When a wild-type cell is introduced against a back-ground of cells with a growth advantage (supercompetitors) wild-type cells are eliminated by cell competition

Cell competition was first described in *Drosophila melanogaster* in 1975 in flies heterozygous for *Minute* mutations, which affect ribosomal protein-encoding (Rp) genes. Whilst flies homozygous for these mutations are lethal, heterozygous *Minute* mutants (M/+) flies are slow-growing but viable (Lambertsson, 1998). In their paper Morata and Ripoll induced M/+ clones against a wild-type background in the developing epithelia of the wing and discovered these cells were eliminated during development despite their viability in homozygous M/+ flies. The loss of an otherwise viable population of cells in a heterozygous population was termed cell competition (Morata and Ripoll, 1975).

Since being first described further hallmarks of cell competition have been identified; first that although mutant cells are lost by apoptosis or delamination from competing populations, winner cells over-proliferate to maintain organ size (de la Cova et al., 2004). Furthermore, cell competition is determined by compartment boundaries, with competition occurring only in the posterior portion of the wing disc and not the anterior compartment (Simpson and

Morata, 1981). A number of factors have also since been identified as sufficient to induce cell competition in various models.

1.6.1 Cell Competition induced by differences in *Dpp* signal transduction

M/+ mutants transduce lower amounts of *decapentaplegic* (*dpp*) compared to their surrounding wild-type neighbours due to their slower proliferation rate (Moreno et al., 2002). In response to lower levels of *dpp* M/+ cells upregulate expression of the transcriptional repressor *brinker* and its co-activator dNAB followed by upregulation of the JNK pathway and apoptosis (Moreno et al., 2002; Ziv et al., 2009). Conversely, constitutive activation of the *dpp* pathway in M/+ cells promoted their survival (Burke and Basler, 1996). Therefore, *Dpp* signalling may be a general mechanism of monitoring cell fitness to identify abnormal cells.

1.6.2 Cell Competition induced by relative differences in Myc expression

Myc is a transcription factor involved in regulating the expression of many genes across a range of cellular processes affecting cell growth; apoptosis and ribosome biogenesis. Hypomorphic alleles of dMyc are not embryonic lethal but result in reduced body size, cell size and viability (Johnston et al., 1999). Similar to *Minute* mutants, clones of cells expressing hypomorphic dMyc alleles are eliminated from the developing *drosophila* wing disc during development (Johnston et al., 1999). Like M/+ induced competition, dMyc hypomorphic cells are protected by compartment boundaries (de la Cova et al., 2004). Moreno and Basler (2004) introduced dMyc overexpressing clones with 4x and 2x endogenous dMyc levels. Analysis of their behaviour against a wild-type background showed that these clones were able to overtake large areas of the wing disc similar to regular cell competition (Moreno and Basler, 2004). These experiments identify dMyc overexpressing cells as ‘supercompetitors’ and highlight the importance of relative expression levels in competing cultures.

1.6.3 Cell competition induced by differences in Wingless signalling

Vincent et al., (2011) investigated the role of *wingless* (*Wg* – a *Drosophila* homologue of Wnt signalling) in cell competition (Vincent et al., 2011). Similar to *Dpp*; signalling via the Wnt pathway is a major regulator of development in *Drosophila* and functions as a survival factor in the developing wing disc. *Wg* insensitive cells, deficient in either of the *Wg* receptors *Frizzled* (*Fz/Fz2*) or the co-receptor *arrow*, are viable in a homogenous environment, however when introduced against a wild-type background *Wg* signalling mutants are eliminated by apoptosis dependent cell competition. Conversely over-expressing mutants, for example those lacking components of the β -catenin destruction complex APC (adenomatous polyposis coli) or Axin are able to eliminate their wild-type neighbours. Further analysis of *Wg*-induced cell competition revealed that it is *Myc*; and *Minute* independent. This suggests that cell competition is a general mechanism that can be triggered by multiple signalling pathways leading to apoptosis of loser cells.

1.6.4 Fitness modulators in Cell Competition

Salvador-Warts-Hippo pathway

Identifying changes in *Dpp*; *Wg* and *dMyc* as cell competition triggers garnered interest into identifying other signalling pathways sufficient to induce cell competition. Primarily Tyler et al., (2007) performed an unbiased genetic screen for mutations sufficient to rescue haplosufficient *RpL36* mutant clones against a wild-type background in *Drosophila* (Tyler et al., 2007). This screen identified members of the *Salvador-Warts-Hippo* pathway as sufficient to both rescue mutant clones, and transform these cells into ‘supercompetitors’ capable of eliminating their wild-type neighbours. This pathway plays a major regulatory role in controlling cell-cycle progression; growth and apoptosis as well as defining the fate of pre-implantation mESCs in the mouse embryo (Nishioka et al., 2009; Pan, 2010).

Cell Polarity pathways

Cell polarity refers to the specialized localisation of specific proteins at the cell membrane, such as the apical or basal surfaces. This sequestering of specific proteins are required for cells to interact with each other and their microenvironment. Mutations in central regulators of apico-basal polarity *Lethal giant larvae* (*Lgl*) and *Disc large* (*Dlg*); *Scribble* and *Crumbs*,

as well as *Lgl* interaction partner *Mahjong* have all been related to cell competition (Hafezi et al., 2012; Menendez et al., 2010; Norman et al., 2011; Tamori et al., 2010). These models of cell competition differ from the original Dpp; Wg and Myc models as homozygous mutants for these genes are non-viable. Homozygous mutant flies for these genes form neoplastic tumours during development as cells overproliferate and do not regulate organ size. Whilst these mutant cells are viable and able to proliferate rapidly, tumour formation eventually leads to death of the fly larvae. These cell polarity genes are of interest as models of cell competition as in the presence of wild-type cells, *lgl* and *scrib* knockout mutant clones do not form tumours and are instead eliminated by apoptosis (Menendez et al., 2010). When mutants for cell polarity genes interact with wild-type neighbours, these cells undergo apoptosis and are extruded from the tissue. Models of cell polarity mutants in cell competition have been observed both within the *Drosophila* wing disc and in mammalian Madin-Darby Canine Kidney (MCDK) cells (Hafezi et al., 2012; Menendez et al., 2010; Norman et al., 2011).

1.6.5 Recognising mutant cells during Competition

Secreted Factors

Cell competition has been shown to be partly mediated by secreted factors. S2 *Drosophila* cells grown in culture media conditioned by heterogeneous cell populations with different dMyc expression levels can induce competition in naïve homogenous cultures. This suggests that during competition *Drosophila* epithelial S2 cells secrete an unknown “killing signal” which is selectively transduced by the weaker population (Senoo-Matsuda and Johnston, 2007). Conversely mutant cells can also secrete a “survival protein” which allows them temporary protection against cell death. Portela et al., 2010 identified *dSPARC*, a secreted glycoprotein that acts as a protective barrier against competition-induced apoptosis (Portela et al., 2010). Ectopic expression of *dSPARC* is sufficient to prevent *Minute* and dMyc competition induced apoptosis. *dSPARC* secretion may therefore be a general response to apoptotic signals to allow cells time to recover after damage.

The Flower code

Changes in cell surface protein expression have also been demonstrated to be involved in cell competition. Rhiner and co-workers describe *Flower* (*Fwe*) an ubiquitously expressed extracellular surface protein, posttranslationally modified into different isoforms in response to cell metabolic rate (Rhiner et al., 2010). Cells expressing the same isoform of *Fwe* do not compete. In *Minute* mediated cell competition, mutant cells express one of two truncated forms of *Fwe* termed *Fwe*^{loseA} or *Fwe*^{loseB} whilst winner cells express an isoform termed *Fwe*^{ubi}. Ectopic expression of either truncated isoform results in loss of otherwise wild-type cells whereas expression of the ubiquitous isoform is sufficient to rescue a mutant cell from being eliminated (Rhiner et al., 2010).

The Flower code is particularly interesting as it provides a “read-out” for cell viability. Of interest is whether this read-out using alternative isoforms of *Fwe* could be applicable to mammalian systems, such as cell competition in the epiblast or in other systems. Further evidence of the role of *Fwe* in competitive interactions between cells was described within the developing *Drosophila* retina, where loser isoforms of *Fwe* are expressed at the surface of stunted photoreceptors prior to induced apoptosis (Merino et al., 2013; Pichaud et al., 2013). This suggests that the role of *Fwe* in identifying cells as suboptimal is conserved throughout different tissue types.

To determine whether *Fwe* is also conserved in other species, *Fwe* was assessed in mouse models. In mice, *Fwe* has a single predicted homologue termed *mFwe* and is spliced into four separate isoforms. Expression of *mFwe* was also analysed in adult tissues and found to have the highest expression in the eyes and brain, similar to *Drosophila* *Fwe*. Interestingly, when different isoforms of *Fwe* were expressed within these tissues, apoptosis was not triggered as would have been expected in cell competition. However when transfected into *Drosophila* models of cell competition, expression of *mFwe* isoforms 1 or 3 is sufficient to trigger apoptosis in the expressing cell (Petrova et al., 2012). This discrepancy may be related to cell competition being active within different developmental boundaries or times between *Drosophila* and mice. This data suggests that *Fwe* and its role in cell competition is evolutionarily conserved between species, and highlights changes in *Fwe* isoforms as a potential bio-marker for early changes in cancer progression.

1.6.6 Cell Competition – apoptosis and engulfment

Cell competition is limited mostly by compartment boundaries, and is modulated partially by secreted factors and cellular distances. Once identified as “losers” these cells are removed from competing environments. TUNEL or activated caspase-3 staining co-localises with “loser” cells confirming that “loser” cells are eliminated by apoptosis. However, the signalling pathways preceding apoptosis vary dependent upon the model of cell competition (de la Cova et al., 2004; Moreno and Basler, 2004; Moreno et al., 2002).

In addition to apoptosis, engulfment may play a role in removing loser cells. Li and Baker (2007) observed that winner cells actively eliminate loser cells during competition (Li and Baker, 2007). As they examined the boundaries of wild-type and M/+ cells in *Minute* mediated cell competition they observed wild-type cells in the process of engulfing mutant cell debris. These studies showed that the ability to engulf loser cells is required for cell competition and sufficient to induce competition where no growth difference is present. However, the role of engulfment during cell competition is controversial as a later paper was unable to replicate these results, and concluded that “winner” cells were still able to induce apoptosis in “loser” cells in M/+ mediated competition, despite lacking genes required for engulfment such as *drpr* and *wasp*. Instead, they observed that apoptotic “loser” cells were extruded from the basal epithelium and the resultant cellular debris was engulfed by circulating haemocytes (Lolo et al., 2012).

1.6.7 Mammalian models of cell competition

Cell competition has also been described in various mammalian systems. Some evidence suggests that cell competition may also occur in the mouse for example; *Belly spot and tail* (*Bst*) heterozygote cells are defective in ribosomal protein L24 and behave similarly to *Drosophila Minutes*. When injected with R26-*lacZ* mESCs, *Bst*/+ blastocysts retain a greater contribution of R26-*lacZ* cells at E12.5 when compared to similarly injected wild-type blastocysts, suggesting defects in *Bst*/+ cells’ ability to contribute to chimaeras (Oliver et al., 2004). Competitive-like interactions have also been described between donor wild-type and diseased host liver hepatocytes during liver regeneration in rats (Oertel et al., 2006).

Tamori et al., (2010) were the first authors to describe cell competition in a mammalian epithelial model using Martin Canine-Darby Kidney (MCDK) epithelial cells deficient in a scaffolding protein *Mah-jong* (Tamori et al., 2010). *Mah-jong* deficient MCDK cells (*Mahj*^{-/-}) grow normally and show no defects. However when they are grown in apposition with wild-type MCDK cells *Mahj*^{-/-} cells upregulate JNK signalling and undergo elimination by apoptosis. This model adheres most strenuously to cell competition hallmarks described in *Drosophila* as mutant cells are viable in homogenous culture and are eliminated by apoptosis in heterogeneous populations.

Also in 2010, two papers were published describing p53-mediated hematopoietic stem cell and progenitor cells as a model of cell competition (Bondar and Medzhitov, 2010; Green, 2010; Marusyk et al., 2010). Bondar and Medzhitov, (2010) irradiated haematopoietic stem and progenitor cells and introduced them with wild-type progenitors into lethally irradiated recipients. Whilst irradiated HSPCs could repopulate bone marrow as a homogeneously treated population, in the presence of non-irradiated progenitors treated HSPCs contributed a significantly lower proportion to later HSPC population than non-irradiated HSPCs. In this model outcompeted irradiated cells undergo p53 mediated senescence and do not proliferate. Loss or reduction in p53 in irradiated HSPCs eliminated the competitive advantage of wild-type HSPCs as irradiated HSPCs no longer induced senescence in response to the presence of wild-type neighbours.

Most recently, cell competition in mouse models of development have been described. The first paper, from Claveria et al., (2013) describes a super-competition model using a genetic mosaic model to induce relative expressions of *Myc* in the mouse epiblast. In this model, alleles with differential *Myc* expression were expressed under EYFP or EGFP in the epiblast, and the ratio of fluorophore expression analysed (Claveria et al., 2013). Using this model, the authors show that cell competition occurs in the epiblast, results in and that cell competition is dependent upon apoptosis in loser (relatively lower *Myc* expressing) cells. This paper is of particular interest as it describes cell competition *in vivo* as a normally occurring phenomenon during development to eliminate lower *Myc*-expressing cells in the epiblast.

The second paper, by Sancho et al., (2013) also describes cell competition during mouse development. In this work a series of mutant cell lines (*Bmpr1a*^{-/-}; *Atg5*^{-/-} and 4n) mESCs as well as *Myc* over-expressing mESCs are competed in co-culture against wild-type mESCs. The authors go on to show that during differentiation in culture over a four-day period, wild-type mESCs eliminate neighbouring mutant cell lines and are out-competed by *Myc* over-expressing mESCs (Sancho et al., 2013; and this work). The authors go on to show that this elimination of neighbouring cells in co-culture is apoptosis dependent and mediated by secreted factors. Additionally, the authors go on to show that prior to elimination of the loser population, *Myc* is upregulated in the winner population, suggesting that relative expression of *Myc* is the driving force behind cell competition. These results are also demonstrated *in vivo* using the mouse epiblast at E5.5 to E7.5 similar to work shown by Claveria et al., (2013) (Claveria et al., 2013; Sancho et al., 2013; and this work).

1.6.8 Roles for cell competition

Quality control

Cell competition has been proposed to have three roles, the first as a quality control mechanism. During development, cell populations, such as the *Drosophila* imaginal disc or eye, proliferate and differentiate rapidly in response to various environmental changes. Cell competition would ensure that suboptimal, mispatterned or abnormal cells undergo apoptosis during development and not contribute to the adult tissue (Moreno and Basler, 2004; Moreno et al., 2002; Tyler et al., 2007).

Cancer

Cell competition may also be involved during cancer development, as a tumour promoter mechanism. “Field cancerization” is a term used to describe a possible early event in cancer where mutant cells proliferate rapidly without inducing morphological changes due to compensatory loss of surrounding wild-type cells, as described in cell competition (Moreno, 2009).

Conversely, cell competition could also act as a tumour suppressing mechanism depending on context. Cells which acquire a mutation in a tumour suppressor gene against a wild-type

background would be eliminated by apoptosis. Examples of both models of cell competition have been described earlier in addition to the known oncogene *Myc* being identified as sufficient to induce competitive behaviour (de la Cova et al., 2004; Menendez et al., 2010; Moreno and Basler, 2004; Tamori et al., 2010).

Regeneration

The third proposed role for cell competition is in regeneration. In response to tissue damage, tissues able to regenerate proliferate rapidly, differentiate to replace lost tissue and return to quiescence when appropriate organ size is restored. However if cells from a healthy donor are transplanted near the damaged area, “suboptimal” host cells undergo apoptosis and donor cells proliferate to replace the damaged organ. During this process a large area of host tissue is overtaken in the process, yet organ size is still maintained and the replaced tissue is functional (Bondar and Medzhitov, 2010; Oertel et al., 2006). This has clinical applications as non-functional tissues and organs could be damaged and then replaced with functional donor tissue at the expense of host cells.

1.7 Autophagy

Autophagy is a process by which cytoplasmic components, including organelles are degraded by the lysosome. Macroautophagy (hereafter referred to as “autophagy”) is of particular interest as it is through this method that cytosolic contents are delivered to the lysosome by specialised compartments called autophagosomes. Autophagy has physiological and morphological roles in the cell. It acts as an adaptive response to starvation or stress to breakdown excess proteins back into their amino acid components and provide energy when needed. Due to this autophagy is closely coupled to the nutrient-sensing metabolism pathway, regulated by mammalian target of rapamycin (mTOR) (Lum *et al.*, 2005, Yang and Klionsky, 2010).

Autophagy also acts as a quality control mechanism for intracellular proteins and organelles, where long-lived or excess cytosolic components are trafficked into the autophagosome pathway and degraded. This allows the cell to ‘refresh’ its cytosolic contents and to remove damaged or misfolded organelles and proteins which would otherwise remain. Lastly,

autophagy has roles during development, the earliest of which acts to convert the fertilised oocyte to an unpatterned totipotent cell. Many components of the autophagy pathway are required at different points of embryogenesis emphasising the importance of autophagy in development (reviewed by Mizushima and Levine, 2010).

Autophagy is regulated by a central 'master' regulator called TOR, a serine-threonine kinase. TOR is highly conserved and as illustrated in Figure 1.6 incorporates signals from both growth factors and nutrient signals and the energy status of the cell (Yang and Klionsky, 2010). Although TOR serves as the central component in two complexes in mammals; mTORC1 and mTORC2, Whilst both complexes are involved in autophagy, only mTORC 1 is directly involved in the regulation of autophagy. mTORC1 comprises of mTOR; raptor (regulatory associated protein of mTOR) which regulates autophagy inhibition on addition of rapamycin (Oshiro et al., 2004) ; GβL (also known as mLST8) and PRAS40 (proline-rich Akt substrate of 40kDa (reviewed by Ravikumar et al., 2010).

In response to low levels of nutrients or high amounts of abnormally-folded proteins in the cell the autophagy pathway degrades these components back into its constituent parts in order to be recycled. This occurs via a series of sequential steps involving components of the autophagy pathway which mark components for degradation. These components are then encompassed into vesicles called autophagosomes. These eventually fuse with lysosomes within the cell containing enzymes which degrade their contents allowing them to be reused to form further proteins or provide energy for the cell.

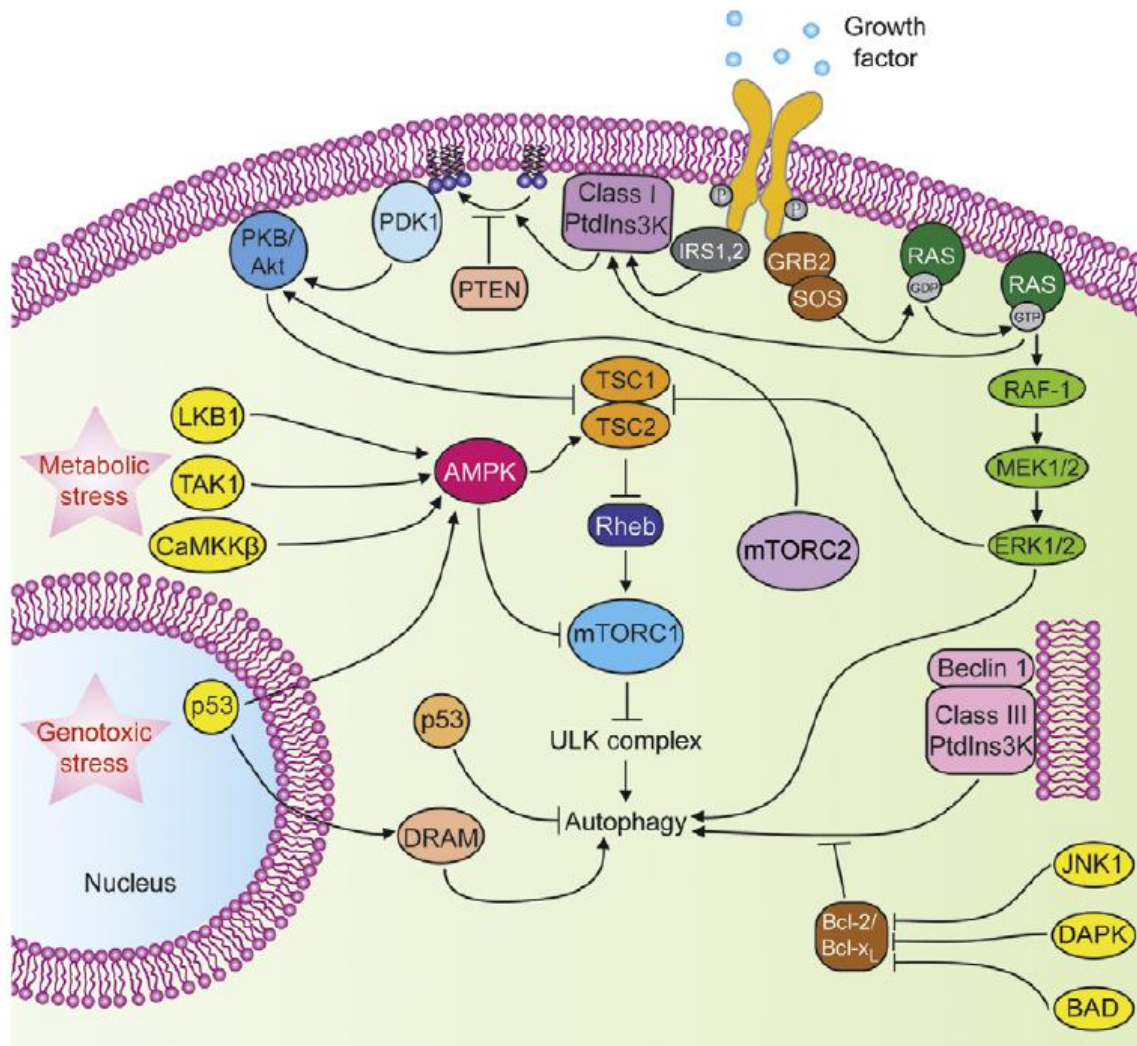


Figure 1.6 Overview of the signalling pathways regulating autophagy (Yang and Klionsky, 2010).

Figure showing an overview of the signalling pathways which affect autophagy. Growth factors; cell stress and nutrients all feed into autophagy pathway which is involved in regulating cell growth and energy status (Yang and Klionsky, 2010).

Both *ATG5* and *Beclin1* are components of the autophagy pathway in mammalian systems. *ATG5* forms a conjugate complex with *ATG12*, which is required for formation of the autophagosome isolation membrane where cytosolic components are degraded (Mizushima *et al.*, 2001). *Beclin1* acts in a variety of roles, some autophagy-independent and regulates autophagy through interaction with class I PI3K kinases. The class I PI3K kinase complex made up partly of ATG14 and Beclin-1 mediate localisation of autophagy components to the preautophagosome site (PAS) (reviewed by Cao and Klionsky, 2007). It is worth mentioning a third component of the autophagy machinery LC3 which is expressed at the surface of the

autophagolysosome where unwanted components are degraded. In response to increased levels of autophagy, LC3 is cleaved from its original state LC3-I to a smaller fragment LC3-II and this conversion serves as a marker for levels of autophagy (reviewed by Tanida *et al.*, 2004).

Qu *et al.*, 2006 used an LC3-GFP reporter line as a marker for autophagy, and found this process to be clearly occurring within wild-type mESCs. They then used two separate cell lines, *atg5* and *beclin1* null ESCs to analyse an additional role for autophagy during cavitation in development. Embryoid bodies (EBs) comprised of either *atg5* or *beclin-1* ESCs fail to cavitate. This failure to cavitate differs from S2 EBs which do not respond to BMP mediated cell death signals from the visceral endoderm (VE) (Coucouvanis and Martin, 1999). Qu, *et al.*, (2006) observe apoptosis, suggesting these cells are responsive to BMP signalling, but cell corpses are not cleared and remain within the EB. These experiments indicated that autophagy is required for cell engulfment. ES cells are highly proliferative and long-lived so it is highly plausible that autophagy is required as a cell homeostasis mechanism not only to remove mis-folded proteins and supply energy within the cell but also to remove dead cells from the environment.

Autophagy has a debated role within cell competition. Engulfment of neighbouring loser cells during cell competition within the *Drosophila* wing disc was observed by Li and Baker (2007). They went on to hypothesise that winner cells “eat” their way through the neighbouring loser population to perpetrate cell competition (Li and Baker, 2007). However, this view was later challenged by Lolo and colleagues who could not recreate these experiments and hypothesised that loser cells were actually extruded from the basement membrane and engulfed by circulating hemocytes (Lolo *et al.*, 2012). Whilst the exact method of removal of loser cells from a competing environment is still under debate, models of cell competition using mESCs and *in vivo* mouse studies have also observed winner cells engulfing their losing neighbours during competition (this work, Sancho *et al.*, 2013; Claveria *et al.*, 2013). Whether this is an active part of cell competition or a mechanism to remove loser cell debris is unknown and requires further investigation.

1.8 Apoptosis in mESCs

Apoptosis occurs throughout the life of an organism. It can be triggered by a variety of causes, such as a reduction in growth factor binding, withdrawal of non-apoptotic factors or excessive cell stress. All cell types undergo apoptosis, although the stimuli and sensitivity of response depends upon cell type. In response to such stimuli, a variety of changes occur within the cell. Morphologically, cells, including mESCs, undergoing apoptosis show certain features, such as blebbing; cell swelling; vacuole formation and disruption of the cell membrane (Elmore, 2007; Lawen, 2003). The cell eventually disintegrates and in some cases, including cell competition, the debris is engulfed into neighbouring surviving cells (Claveria et al., 2013; Li and Baker, 2007; Sancho et al., 2013). These morphological changes are accompanied by changes at the cell surface, due to disruption of the cell membrane. Markers staining for inner cell membrane residues, in particular AnnexinV which binds to phosphatidylserine at the cell surface can be used to assess levels of apoptosis.

In response to stimuli apoptosis signals via extrinsic and/or intrinsic initiator pathways. The extrinsic pathways regulating apoptosis involves the binding of extracellular ligands to “death receptors” at the cell surface causing activation of downstream caspases (Elmore et al., 2007). Interestingly mESCs do not express any members of the extrinsic apoptotic pathway and therefore initiate apoptosis only through intrinsic signalling pathways (Khromov et al., 2012; Nishitai et al., 2004). The intrinsic pathways that trigger apoptosis are also diverse. As mentioned earlier, these can be triggered by a variety of stimuli. All of these stimuli cause changes in the inner mitochondrial membrane and cause release of pro-apoptotic factors contained therein (Saelens et al., 2004). These factors, such as *Cytochrome-c* and DIABLO, form complexes with other proteins such as Apaf-1 and subsequently recruit and activate the intrinsic initiator procaspase-9 to its cleaved active form, caspase-9 (Elmore et al., 2007).

Both the extrinsic and intrinsic apoptosis pathways culminate in activation of executor caspases, caspase3,6-7. Expression of activated, (cleaved) caspase-3 can also be used as a late non-reversible marker to assess apoptosis. Activation of these executor caspases leads to disassembly of the cell structure, DNA fragmentation and death of the cell, where the remaining debris is engulfed by neighbouring cells (Lawen et al., 2003)

1.9 Aims of this Study

A mammalian model of cell competition was developed using mESCs null for *Bmpr1a* (Sancho et al., 2013). These cells do not express the type I BMP receptor and transduce a lower level of BMP signal. When grown in co-culture with wild type mESCs in conditions where growth factors are limiting, *Bmpr1a* null cells are rapidly out-competed. This model has been used in this study to further elucidate mammalian cell competition.

This study focuses on different aspects of cell competition. First the report describes how BMPRIa null cells are removed from competing co-cultures with wild-type mESCs by analysing proliferation; apoptosis and upstream signalling cascades known to be activated in response to stress. This study goes on to address is if the elimination of *Bmpr1a*^{-/-} mESCs depends on cell-cell contact or is mediated by secreted factors, using a transwell assay system. This same model is also used to test whether cell competition is a conserved mechanism by investigating how wild-type mutant mESCs interact. Lastly this study investigates cell competition at the molecular level by studying the signalling pathways which affect the elimination of *Bmpr1a*^{-/-} cells. For this the ERK and WNT signalling pathways are analysed.

This work will help demonstrate that cell competition is a conserved mechanism involved in embryonic stem cell fitness and begin to investigate how embryonic stem cells interact with each other to determine fitness levels.

Chapter 2 – Materials and Methods

2.1 Tissue Culture Methods

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

2.1.1 mESC Lines

E14Tg2a.IV mESCs were a gift from Austin Smith (Nichols et al., 1990). *Bmpr1a*^{-/-} FR124 cell line were generated from Oct4-GFP 129_SvEv transgenic mice a gift by Yuji Mishina (Qi et al., 2004). *Atg5*-all^{-/-fltx} mESCs were generated from an R1 ES parent cell line (129 ES background) gifted by Mizushima's lab (Mizushima et al., 2001). *Bmpr1a*^{-/-} mESC line C1 were derived from *Bmpr1a*^{-/-} mice maintained on a 129SvCC background and established from blastocyst outgrowths maintained by Margarida Sancho (Mishina et al., 1995; Sancho et al., 2013). 4n (tetraploid) mESC cell line were generated by Margarida Sancho from E14Tg2a.IV mESCs during GFP electroporation experiments and were determined to have 80 chromosomes by karyotyping (Sancho et al., 2013). E14-gfp8; C1-gfp6 and 4n-GFP mESC lines were stably transfected with eGFP under the control of the CAG promoter by Margarida Sancho (Sancho et al., 2013).

2.1.2 mESC Maintenance

mESCs were maintained as undifferentiated cells on 0.1% gelatine-coated flasks (Nunc, Thermo Fisher). Cells were grown in Glasgow's Modified Eagle Medium (GMEM) supplemented with 10% (v/v) foetal calf serum (FCS), 1X non-essential amino acids (NEAA), 1x Sodium Pyruvate; 2mM L-glutamine, 0.1mM β -mercaptoethanol and 1:500 LIF (produced in house by Sara Pozzi and Kim Smith). Cell washes were done with phosphate-buffered saline (PBS). mESCs were routinely dissociated with trypsin-EDTA and passaged 1:8 every 2-3 days. For future use cells were frozen in media containing 10% tissue-culture grade dimethyl sulfoxide (DMSO, Sigma) and 90% FCS, and stored in liquid nitrogen. All cells were cultured at 37°C in 5% CO₂.

2.1.3 Cell Competition Assays

Confluent mESCs were dissociated with Trypsin-EDTA and counted using a *Neubaur improved* cell counting chamber. 4.2×10^4 cells/cm² were plated onto 0.1% gelatin-coated 6 well plates (Nunc, Thermo Fisher). Cells were plated as each genotype separately or in co-culture as a mixture of control and mutant mESCs at either 50:50 or 40:60 ratios depending on cell genotype. After plating cells were maintained in ES media without LIF and changed daily thereafter for the duration of the experiment.

E14-GFP8 and FR124 *Bmpr1a*^{-/-} mESCs were used in cell competitions to determine if GFP loss was responsible for the loss of GFP⁺ mESCs during cell competition (Figure 3.2B). E14-gfp8 cells were used in competition with *ATG5*^{-/-} mESC competitions. Tetraploid mESCs generated from GFP electroporated E14Tg2a.IV mESCs were used as the mutant cell line for tetraploid experiments (Sancho et al., 2013). Wild-type E14-gfp8 and FR124 *Bmpr1a*^{-/-} cells were also used to confirm results for pancaspase inhibition experiments (Figure 3.4H). For all other experiments E14tg2a.IV mESCs were used as wild-type controls. Unless stated otherwise C1-gfp6 *Bmpr1a*^{-/-} mESCs derived mice maintained on a 129SvCC background and established from blastocyst outgrowths maintained by Margarida Sancho were used as the mutant mESCs in all other experiments (Sancho et al., 2013).

For transwell cell competition assays 4.2×10^4 cells/cm² control or mutant cells were plated onto wells of a 6 well plate coated with 0.1% gelatin. In a separate 6 well plate transwells (Millipore) were also coated with 0.1% gelatin and 4.2×10^4 cells/cm² control or mutant cells were plated onto them. Cells were left for 4-5 hours before cells in transwell were placed overlying those plated directly onto the plate.

For growth curve analysis cells were plated for separate and co-cultures and then counted daily. The cell count graphs represent the total cell number of each genotype counted. For cells grown in co-culture, the total number of cells was counted and the relative proportion of each genotype present was determined by flow cytometry (FACs). This proportion was then multiplied by the total cell number in co-culture to determine the cell number of each genotype present. As control and mutant cells represent a proportion of the total cell number in co-cultures, these numbers are often lower than those grown in separate cultures.

The bar graphs represent the ratio of control to mutant cells in separate and co-culture. This was determined by FACs analysis of each culture. For co-cultures the population was analysed as a whole population. For analysis of separate cultures, each population was trypsinised and mixed in equal volumes before analysis by FACs.

2.1.4 Growth Rate Analysis

Growth rates were calculated for each cell type in each condition daily. The growth rate (cell doublings per day) for each cell type was calculated as equals natural logarithm, (LN) (final number/initial number) divided by Ln (2). Unless stated otherwise a minimum of three independent experiments were performed and the average $\pm$ s.e.m plotted.

2.1.5 Growth Factor/Inhibitor Treatments

Cell competition assays were set up as described in 2.1.3. All growth factors/inhibitors are listed in Table 1 below, in order and concentration used. 1 μ g/ml heparin was used as a vehicle control for FG4 and FGF5 experiments (Sigma). Unless stated otherwise DMSO was used as a vehicle control. Inhibitors were added either 4-5 hours after plating, with fresh media, or 2 days after plating. Unless stated otherwise, a minimum of three independent experiments were performed and average $\pm$ s.e.m plotted.

Reagent	Concentration	Target	Company
Z-VAD-FMK	100 μ M	Caspases	R&D Systems
SP600125	5 μ M	JNK	Sigma
SB203580	20 μ M	p38	Merck
Rapamycin	10ng/ml	mTOR	Cayman Chemicals
LY294002	25 μ M	PI3K	Cayman Chemicals
Wortmannin	1 μ M	PI3K	Sigma
SB431542	10 μ M	ALK5/4/7	Cayman Chemicals
PD0325901	1 μ M	MEK1/2	Cayman Chemicals

Reagent	Concentration	Target	Company
PD184352	2 μ M	MEK1/2	Santa Cruz
PD98059	12.5 μ M	MEK1/2	Cell Signalling
U0126	10 μ M	MEK1/2	Cell Signalling
PD173074	100ng/ml	FGFR1/3	Cayman Chemicals
FGF4	5ng/ml	FGFR1c/2c	R&D Systems
FGF5	10ng/ml	FGFR1c/2c	R&D Systems
IWR-1	2 μ M	Axin1/2	Calbiochem
IWP-2	2 μ M	Porcupine	Calbiochem
CHIRON99201	3 μ M	GSK3 β	Sigma

Table 2. Reagents used during Cell Competition Assays

Reagents used to modify cell behaviour during cell competition listed in order of use. Reagents are described at the concentration used to affect cell competition dynamics and the company supplier.

2.2 Flow Cytometry (FACs) Analysis

2.2.1 GFP and PI analysis of live cells

All FACs analysis was performed on a BD LSR2 cytometer with FlowJo software. For GFP expression analysis in separate and co-cultures, mESCs were washed with PBS, trypsinised, pelleted and resuspended in FACs buffer (3% FCS in PBS). For exclusion of dead cells, cells were stained with propidium iodide (PI). 3 μ l of 1mg/ml PI solution (Sigma) was added per 500 μ l of cell suspension prior to FACs analysis. PI⁺ cells were excluded prior to analysis of GFP expression ratios. Fluorophore expression channels GFP <525/50 B-A> channel PI <610/20 B-A> channel.

2.2.2 AnnexinV/PI staining for early/late apoptotic cells

AnnexinV is used as an early marker of apoptosis by staining exposed phosphatidylserine caused by disruption of cell membranes. PI is used as a late marker of apoptosis by binding to exposed nucleic acids. Cells in separate or co-culture were harvested and pelleted. Cell pellets were resuspended in 100 μ l AnnexinV binding buffer (0.1% BSA in 10mM HEOES,

140mM NaCl, 2.5mM CaCl₂, pH7.4) with 3µl of APC-conjugated AnnexinV (Molecular Probes). Cell pellets were incubated at RT in the dark for 15 min. 5µl of 1mg/ml PI was then added to the cell suspension and cells incubated at RT in the dark for a further 5min. After this incubation period, 400µl of annexin-binding buffer was added to each sample and immediately analysed. AnnexinV-APC fluorescence was detected in the APC channel < APC-A>, in correlation with PI and GFP.

2.3 Live Cell Imaging

Cells were plated as described during cell competition assays. Cells were grown for 2 days before 6ml of media was added to each well. Cells were then transferred to a humidified chamber set at 37°C and 5% CO₂ connected to an Ultraview microscope and Hamamatsu software. Cells were imaged every 5 minutes using both brightfield phase contrast and GFP channels. Data was correlated and analysed using Velocity 6.1 software to generate short time-lapse movies.

2.4 Protein Analysis

2.4.1 Western Blot

4x10⁵ cells were plated onto gelatin coated 6-well plates and grown for 2 days in GMEM+Serum. Inhibitors were then added at increasing concentrations per well for 24 hours. PD0325901 (Cayman Chemicals) added at 1;2;5;10 and 20µM; PD184352 (Santa Cruz) added at 2;5;10;20 or 50µM; PD98059 (Cell Signalling) added at 12.5;25;37.5;50 and 75µM; U0126 (Cell Signalling) added at 10 and 20µM; PD173074 added at 100;200;500;1000 and 2000ng/ml.

Cells were washed with ice-cold PBS before being lysed on ice with RIPA buffer (150mM NaCl; 50mM Tris pH8; 0.5% sodium deoxycholate, 0.1% SDS, 1% NP-40) containing Complete Mini Protease Inhibitors (Roche) and Phosphatase Inhibitors Set II (Calbiochem). Protein extracts were quantified using the Bradford method (reagent from Biorad). After denaturation (10min at 95°C) 5-40µg of protein were loaded and separated in a 12% pre-

made acrylamide gel (XT-Criterion, Biorad) in 1x XT-MOPS Buffer (Biorad). Proteins were then transferred onto a PVDF membrane (Amersham) using vertical wet transfer for 1 hour at 100V at 4°C. Membranes were blocked in 5% milk in TBS/0.1% Tween (TBST) containing protease inhibitors at RT for 1 hour before incubation with primary antibody in 5% BSA/TBST overnight at 4°C. Primary antibodies used were mouse anti phospho-ERK (1:10,000 Sigma) and rabbit anti α -tubulin (1:5000 Cell Signalling).

The following day membranes were washed in TBST and incubated in peroxidase-conjugated secondary antibody (anti-mouse 1:5000 or anti-rabbit 1:2000, from Santa Cruz) in 5% milk/TBST at RT for 1h. Membranes were washed again in TBST and rinsed in PBS and chemiluminescence assayed using ECL-Plus Western blotting detection system (Amersham Biosciences). Blots were visualised on a Hyperfilm ECL X-ray film (Amersham Biosciences).

2.4.2 Immunofluorescence

For immunofluorescences analysis cells were grown on glass coverslips coated with 10ng/ml human plasma fibronectin (Millipore).

Coverslips were washed in PBS and fixed in 4% paraformaldehyde (PFA) in PBS for 10min. Fixed cells were washed again with PBS and permeabilized with 0.4% Triton-X100 in PBS for 5min, before washing with PBS. Samples were then incubated in blocking solution (10% BSA, 0.1% Triton-X100 in PBS) for 30min. This was followed by incubation in primary antibody: rabbit anti-cleaved caspase3 (1:100 Cell Signalling); rabbit anti-GFP (1:400 Molecular Probes) was diluted in blocking solution o/n at 4°C in a humid chamber. Coverslips were then washed with PBS and incubated with secondary antibodies coupled to appropriate fluorophores (1:300 Molecular probes) diluted in blocking solution for 45min at RT in the dark.

Cells were washed 2x more in PBS. For some experiments cells were then incubated in Phalloidin-TRITC (1:1000/diluted in PBS) for 40min at RT in the dark. Finally cells were washed twice in PBS before being mounted in Vectashield with DAPI (Vector). Samples

were visualised using an SP5 Leica laser-scanning confocal microscope. Images were processed using Leica confocal software and Adobe Photoshop CS4.

Chapter 3 - Characterisation of Cell Competition between wild-type and *Bmpr1a*^{-/-} mESCs

3.1 Introduction

Cell competition is a type of cell-cell interaction where the co-existence of two cell populations with differing growth or metabolic properties results in the loss of the weaker ‘loser’ population and a compensatory increase in proliferation of the stronger ‘winner’ population (Levayer and Moreno, 2013; Wagstaff et al., 2013). In addition to other factors, differences in signal transduction of BMP homologue in *Drosophila Dpp* have been shown to induce cell competition in the epithelial wing disc of developing flies (Burke and Basler, 1996; Moreno and Basler, 2004; Moreno et al., 2002). However, the mechanisms underlying cell competition are not universal with different models triggered and resolved in a context-dependent manner.

Cell competition has been shown to have multiple roles, in tissue homeostasis, repair, organ size and stem cell maintenance and is a conserved mechanism that is present in mammalian models in addition to *Drosophila* (de la Cova et al., 2004; Jin et al., 2008; Oertel et al., 2006; Rhiner et al., 2009; Tamori et al., 2010). The hypothesis that cell competition acts as a protective mechanism against “unfit” cells is intriguing in the context of stem cells which are required to proliferate and self-renew over long time periods, yet still remain competent to differentiate into specialised cell types. In the mouse embryo, two pluripotent stem cell populations are generated, the earlier derived ICM, from which mESCs are obtained from and the later forming and more specialized epiblast, from which EpiSCs are derived from. Both populations are highly proliferative, and subject to random mutations that could immediately affect their viability or that may damage the organism only at later stages of development.

The majority of competitive interactions are resolved by apoptosis of the weaker population of cells and compensatory proliferation of the stronger ‘winner’ cell population (Abrams, 2002; Morata and Ripoll, 1975). However, the mechanism to induce apoptosis differs between systems such as induction of JNK-dependent apoptosis in the *Drosophila* imaginal

disc (Menendez et al., 2010; Moreno and Basler, 2004; Moreno et al., 2002; Portela et al., 2010; Tamori et al., 2010) or induction of pro-apoptotic genes such as *hid* in the developing *Drosophila* wing disc (de la Cova et al., 2004). Other models have been shown to be non-apoptosis dependent such as the mouse haematopoietic system where loser cells undergo p53-induced senescence (Bondar and Medzhitov, 2010) or the *Drosophila* germ stem cell niche where cells are displaced and differentiate (Jin et al., 2008; Rhiner et al., 2009).

Primarily, this chapter introduces the model used to investigate cell competition in mammalian pluripotent stem cells and for this uses mESC cultures. The purpose of this chapter is to determine how competing wildtype and *Bmpr1a*^{-/-} mESCs interact. This chapter also investigates the growth rate of competing and monotypic cultures and how competition is resolved by investigating some of the cellular pathways involved in cell stress and apoptosis.

3.2 Results

3.2.1 *Bmpr1a*^{-/-} mESCs are progressively lost upon coculture with wild-type mESCs

Cell competition is a biological phenomenon where suboptimal cells are eliminated by apoptosis when confronted with a faster growing or fitter population (Levayer et al., 2013).

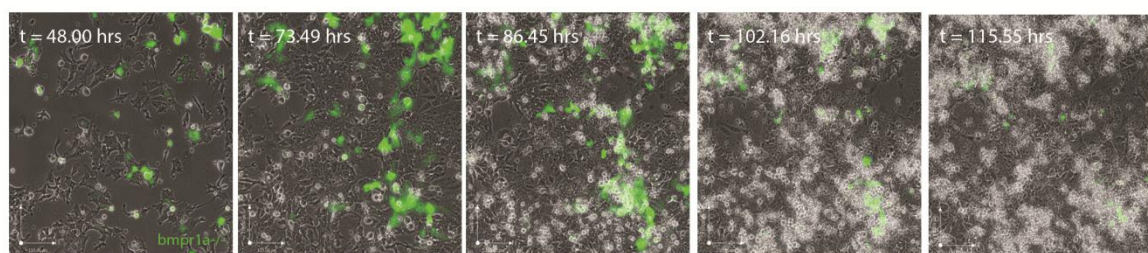


Figure 3.1 *Bmpr1a*^{-/-} mESCs are out-competed when co-cultured with wild-type mESCs

Time-lapse imaging of co-cultured wild-type and *Bmpr1a*^{-/-}-GFP ESCs. Wild-type and *Bmpr1a*^{-/-}-GFP ESCs were mixed together at a 40:60 ratio and the two cell types were co-cultured in GMEM + Serum media. Represented are overlapping fluorescence (GFP) and brightfield images of the co-cultures. t= time in hours (hrs.).

In

Drosophila, differences in being able to transduce Dpp have been shown to induce cell competition between populations within the same wing disc (Burke and Basler, 1996; Moreno and Basler, 2004; Moreno et al., 2002). The mammalian homologues of Dpp are BMP2/4, which signal via the *Bmpr1a* receptor in the early mouse embryo (Di-Gregorio et al., 2007; Mishina et al., 1995). Previous work in the lab and by other groups isolated and established *Bmpr1a*^{-/-} mESCs as cell lines that could be maintained in conventional culture conditions (GMEM supplemented with LIF and FCS) (Qi et al., 2004; Sancho et al., 2013).

Further analysis show that *Bmpr1a*^{-/-} mESCs display lower levels of BMP signalling than wild-type cells and do not respond to BMP2/4. However although these cells exhibit premature neural differentiation in the epiblast, mosaic knockout mice have shown *Bmpr1a*^{-/-} cells present in tissues derived from all the germ layers, in a similar manner to wild-type controls (Davis et al., 2004; Di-Gregorio et al., 2007; Sancho et al., 2013). Using these mutant cells, a co-culture system was set up by the lab to examine the behaviour of *Bmpr1a*^{-/-} mutant cells when coexisting with wild-type cells. In this system *Bmpr1a*^{-/-} mESCs were co-cultured with wild-type E14 mESCs in GMEM plus serum and the relative proportion of each cell type in the culture analysed (Sancho et al., 2013). In order to visualise this process a time-lapse experiment was set up of *Bmpr1a*^{-/-} mESCs labelled with GFP in co-culture with wild-type E14 mESCs. Figure 3.1 shows the overlap of brightfield and fluorescent images at various time-points illustrating the gradual loss of GFP⁺ mESCs from co-culture. As the experiment progresses, areas of previous high GFP expression are replaced with large numbers of rounded pro-apoptotic mESCs. Whilst this experiment allows us to visualise cell competition in real-time as active cells rather than fixed images it does not allow quantification of the loss of *Bmpr1a*^{-/-} mESCs from co-culture. This experiment also does not explain how *Bmpr1a*^{-/-} mESCs are lost from co-culture, although judging by the rounded cell morphology it is suggestive that *Bmpr1a*^{-/-} mESCs are subject to apoptosis.

3.2.2 Loss of *Bmpr1a*^{-/-} mESCs from co-culture with wild-type mESCs is not due to GFP expression

To allow for a quantitative representation of cell competition the ratio of wild-type to

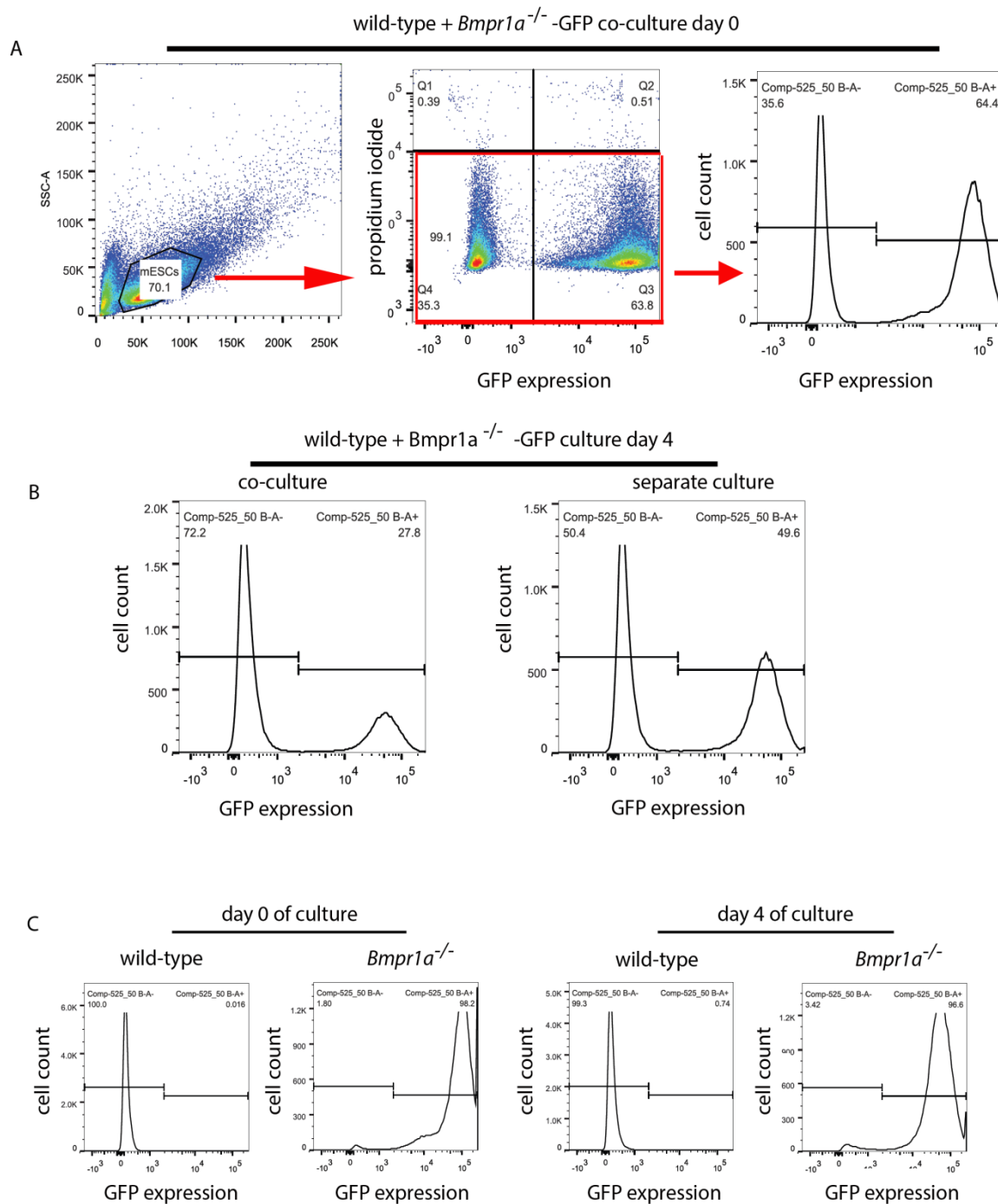


Figure 3.2 Flow cytometry data of wild-type and *Bmpr1a*^{-/-} mESCs during cell competition

(A) Wild-type and *Bmpr1a*^{-/-}-GFP mESCs at day 0 of cell competition, cells plated as co-culture, flow cytometry data of total population, gated for mESCs, then gated again to exclude (dead) propidium iodide positively stained cells (red box) before analysis as a histogram. (B) Flow cytometry data showing proportion of wild-type and *Bmpr1a*^{-/-}-GFP mESCs after 4 days of culture in GMEM+Serum. Data highlights the different contribution of each cell type in separate and co-culture. (C) Wild-type and *Bmpr1a*^{-/-}-GFP mESCs at day 0 and day 4 of cell competition to demonstrate how many cells have a high enough GFP expression to be easily distinguished from unlabelled cells in culture.

Bmpr1a^{-/-} - GFP mESCs was analysed by flow cytometry (FACs) daily and normalised to the original ratio of cells seeded at day 0 (40:60 unless otherwise stated to correct for the slight proliferation defect shown in *Bmpr1a*^{-/-} mESCs in culture) (shown as flow cytometry data in Figure 3.2). By day 4 of co-culture, *Bmpr1a*^{-/-} mESCs contribute a significantly lower proportion of cells in co-culture compared to separate culture, suggesting they have been outcompeted (at day 4 co-culture ratio of wild-type mESCs to *Bmpr1a*^{-/-}-GFP mESCs = 5.48±1.08 compared to separate culture ratio 1.68±0.27 n=3 p≤0.01). This data is shown in graphical form in Figure 3.3A. To exclude a possible effect of GFP expression affecting competition wild-type E14 mESCs expressing GFP under the CAG promoter (E14-GFP) and FR124-lacZ *Bmpr1a*^{-/-} mESCs were also analysed in the same manner. After 4 days of co-culture, the ratio of wild-type/*Bmpr1a*^{-/-} mESCs was significantly higher in co-cultures compared to separate cultures, as shown in Figure 3.3B (ratio wild-type/ *Bmpr1a*^{-/-} co-culture 5.26±0.4 compared to separate culture ratio 2.14±0.54 average ±s.e.m n=3 p≤0.01 students t-test). Analysis of the contribution of competing cultures results in a significant proportion of

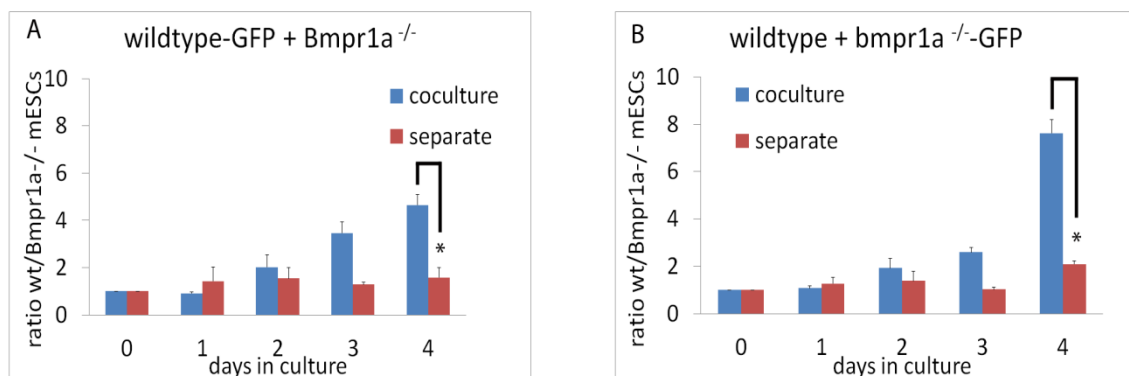


Figure 3.3 Cell Competition between wild-type and *Bmpr1a*^{-/-} mESCs is not affect by GFP expression

Wildtype and *Bmpr1a*^{-/-} mESCs were grown in GMEM+Serum in separate and co-culture for four days and the ratio of GFP positive to negative mESCs in each condition was analysed by flow cytometry. Graphical representation of FACs data of (A) wild-type E14 and C1-gfp6 *Bmpr1a*^{-/-} mESCs and (B) wild-type E14-GFP and FR124-lacZ *Bmpr1a*^{-/-} mESCs in separate and co-cultures. All numbers are normalised to the starting population ratio (40:60 wild-type to *Bmpr1a*^{-/-}) n=3 average±s.e.m p ≤0.01

co-cultures consisting of wild-type mESCs compared to monotypic cultures.

3.2.3 Wild-type cells proliferate significantly faster upon co-culture with *Bmpr1a*^{-/-} mESCs

One of the hallmarks of cell competition is the over-proliferation of the stronger ‘winner’ population in competitive cultures, to maintain organ size and compensate for the loss of the loser population (de la Cova et al., 2004). In order to address whether changes in cell growth rate occur during competition between mESCs, wild-type and *Bmpr1a*^{-/-} mESCs were plated and the total number of cells in cultures were counted daily and shown for separate and co-culture, Figure 3.4 A. In separate cultures, both wild-type and *Bmpr1a*^{-/-} mESCs show similar growth curves. Observation of wild-type and *Bmpr1a*^{-/-} mESC co-cultures shows that *Bmpr1a*^{-/-} mESCs appear to undergo growth arrest around day 2-3 of co-culture and are actively lost from co-culture by day 4. To investigate further, the growth rate of wild-type and *Bmpr1a*^{-/-} mESCs was investigated by analysing population doubling times in separate and co-cultures, using the following equation $\text{Ln}(\text{final cell number}/\text{initial cell number})/\text{Ln}(2)$.

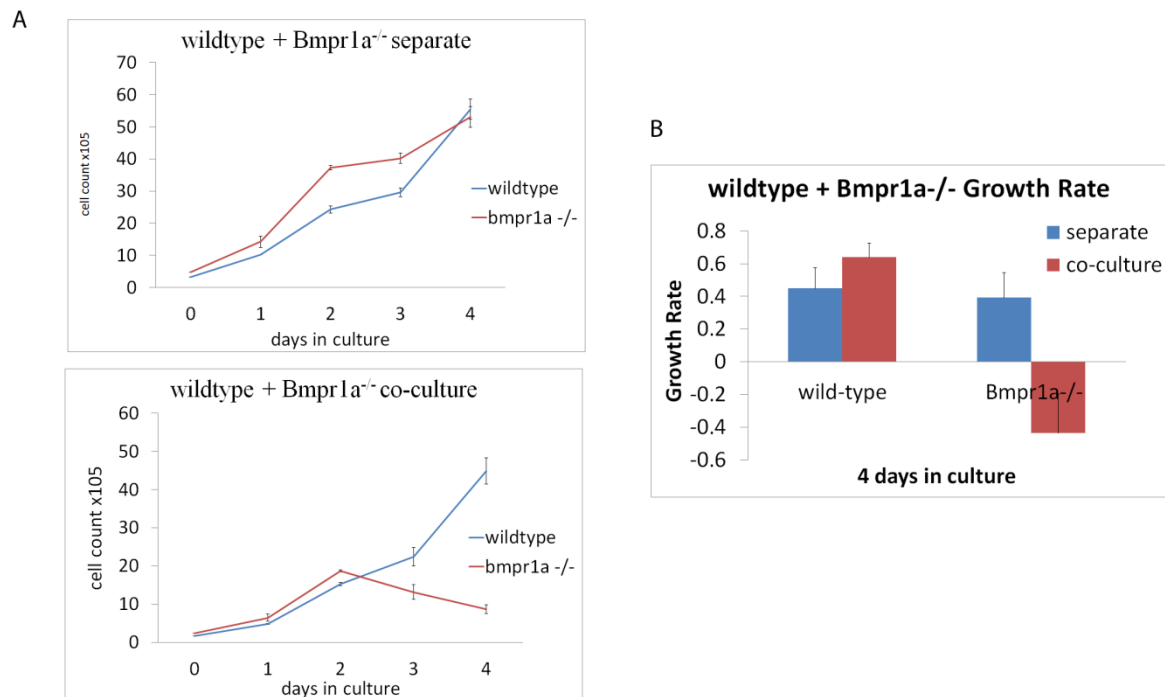


Figure 3.4 Wild-type cells over-proliferate when co-cultured with *Bmpr1a*^{-/-} mESCs

(A) Total number of cells in separate (top) and co-culture conditions (bottom) grown in GMEM+Serum. Number of cells in co-culture populations is derived from the total number of cells multiplied by the percentage of GFP⁺ cells in that population as analysed by FACs on a daily basis. Data shown n=3, average±s.e.m. **(B)** Differences in growth rate between wild-type and *Bmpr1a*^{-/-} ESCs – growth rate is calculated as $\text{Ln}(\text{final cell number}/\text{initial cell number})/\text{Ln}(2)$ n=3 average±s.e.m *p≤0.05

By day 4 of co-culture, there is a significant increase in proliferation of wild-type cells in co-culture when compared to separate culture conditions with a corresponding decrease in the growth rate of *Bmpr1a*^{-/-} mESCs, shown in Figure 3.4B (wild-type growth rate separate 0.40±0.1 co-culture 0.62±0.07 n=3 *Bmpr1a*^{-/-} growth rate separate 0.35±0.12 co-culture - 0.37±0.18 average±SEM p≤0.05). Therefore, in response to cell competition wild-type mESCs overproliferate compared to mono-typic cultures. This is similar to cell behaviour seen in *Drosophila* models of cell competition (de la Cova et al., 2004).

3.2.4 *Bmpr1a*^{-/-} mESCs are lost from competing cultures by apoptosis

A second hallmark of cell competition is that loser cells in a population are eliminated by apoptosis (Abrams, 2002; de la Cova et al., 2004; Moreno et al., 2002). To investigate if higher levels of apoptosis were visible in co-cultures compared to separate monocultures of wild-type and *Bmpr1a*^{-/-} mESCs, two methods of analysing apoptosis were used. Primarily separate and co-cultures of mESCs were fixed and stained at day 3 of culture for cleaved-caspase3, a late apoptotic marker, Figure 3.5A-C. Unfortunately it is difficult to qualitatively state that any condition has a higher level of cleaved caspase3 staining. In addition, caspase3 cleavage and activation is a late stage in apoptosis, by this stage many pro-apoptotic mESCs may have detached and lost before analysis.

AnnexinV staining is also used to identify pro- apoptotic cells before caspase activation by identifying cells whose phospholipid cell membrane is disrupted. The percentage of AnnexinV^{+ve}/PI^{-ve} mESCs in separate and co-cultures of wild-type and *Bmpr1a*^{-/-} mESCs were analysed by flow cytometry but no significant differences in the percentage of pro-apoptotic cells in each population were observed (Figure 3.5 D).

Previous reports in the literature have investigated whether inhibiting apoptosis in competing cultures is sufficient to rescue loser cells (Abrams, 2002; de la Cova et al., 2004; Moreno et al., 2002). To test if this was the case for competing mESC cultures these were treated from the second day of co-culture with a DMSO control or 100µM pan-caspase inhibitor Z-VAD-FMK and stained with anti-GFP; Phalloidin and DAPI to visualise individual cells (Figure 3.5E, F). Inhibition of caspase activity was confined to the latter half of the experiment to minimise non-apoptotic cell death in response to caspase inhibition and DMSO uptake. This

analysis revealed that competing cultures treated with DMSO contained large amounts of *Bmpr1a*^{-/-}-GFP^{+ve} cellular debris (highlighted by white arrows) suggesting that mutant cells were dying in these cultures. In contrast to this pan-caspase inhibitor treated cultures showed low amounts of cellular debris and a higher proportion of GFP^{+ve} mESCs in comparison. To quantify if inhibition of apoptosis was sufficient to prevent competition between wild-type and *Bmpr1a*^{-/-} mESCs, competing and separate cultures of wild-type and *Bmpr1a*^{-/-} mESCs were treated with 100μM of pan-caspase inhibitor Z-VAD FMK from the second day of co-

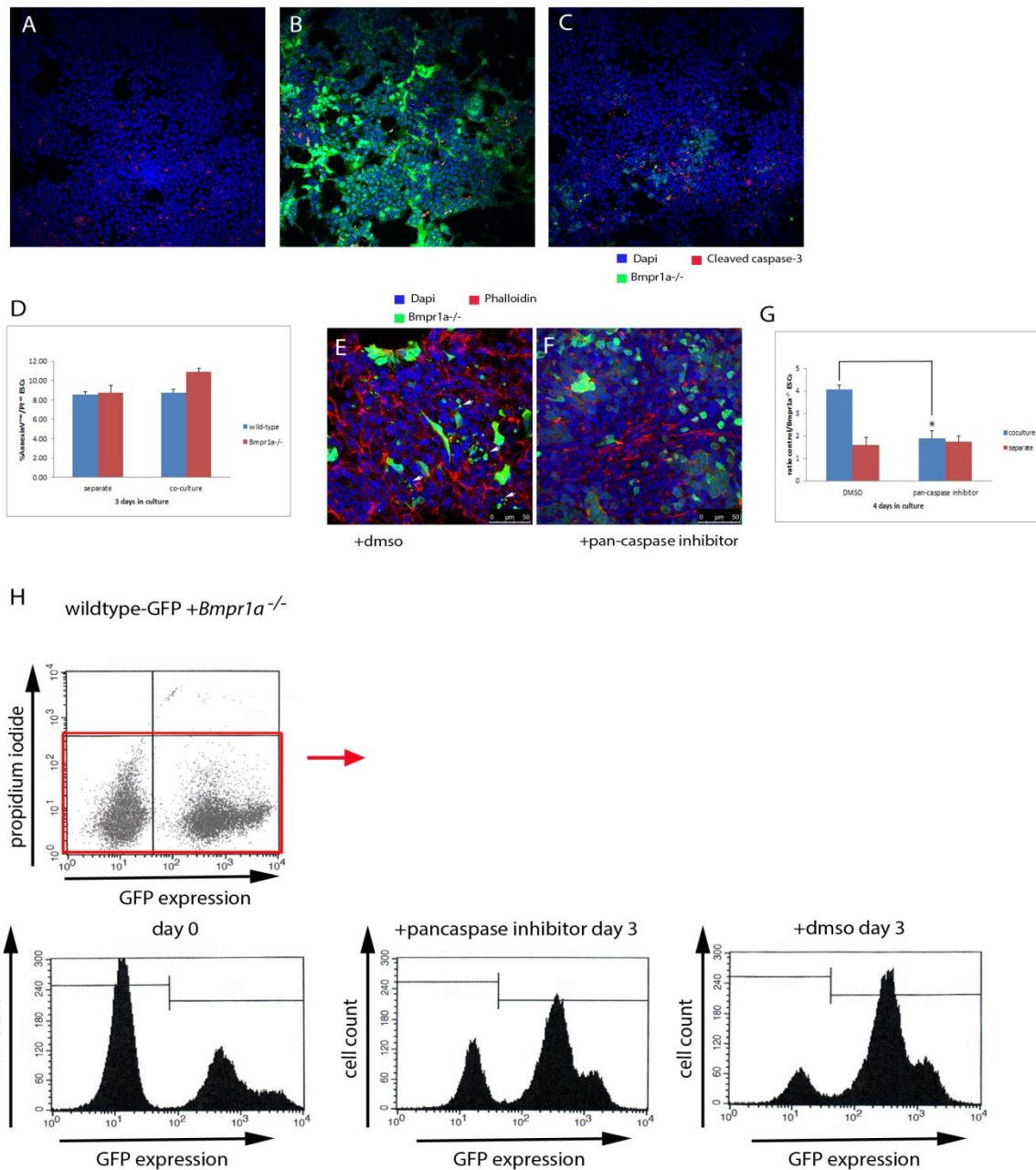


Figure 3.5 Cell competition between wild-type and *Bmpr1a*^{-/-} mESCs is apoptosis dependent

(A) wild-type (B) *Bmpr1a*^{-/-} and (C) co-cultures of mESCs fixed and stained for cleaved-caspase3 at day 3 of culture. Cell nuclei are visualised with Dapi. (D) Graphical representation of the percentage of AnnexinV⁺/PI⁻ mESCs in separate and co-cultures of wild-type and *Bmpr1a*^{-/-} mESCs (average±s.e.m n=3). (E) Comparison of fixed competing cultures when treated with DMSO (control) or (F) 100uM Z-VAD-FMK at day 3 of culture. Cell nuclei were stained with DAPI, *Bmpr1a*^{-/-} mESCs were labelled with GFP and cell actin stained with Phalloidin-TRITC. White arrows highlight *Bmpr1a*^{-/-}-GFP cellular debris in cultures treated with DMSO that is absent in pan-caspase inhibitor treated cultures. (G) Ratio of wild-type/*Bmpr1a*^{-/-} mESCs in separate and co-culture analysed by FACS after 3 days of co-culture shows a significant rescue of cell competition between wild-type and *Bmpr1a*^{-/-} mESCs by addition of 100uM Z-VAD-FMK pan-caspase inhibitor from day 2 of culture (average±s.e.m n=5 p≤0.01). (H) Flow cytometry data showing wild-type-GFP transfected cells co-cultured with *Bmpr1a*^{-/-} ESCs treated with pancaspase inhibitor or DMSO control at day 0 and 3 days after co-culture.

culture and the ratio of wild-type to *Bmpr1a*^{-/-} mESCs was calculated and normalised to the first day of treatment. Inhibition of caspase activity was sufficient to rescue *Bmpr1a*^{-/-} mESC elimination during competition, shown in Figure 3.5G (Z-VAD-FMK co-culture 1.98±0.17 separate 1.74±0.35; DMSO control co-culture 4.08±0.31 separate 1.59±0.48 data shown as average±SEM n=5 p≤0.01). Analysis of competing cultures reveals GFP⁺ *Bmpr1a*^{-/-} mESC debris and a significant reduction in the proportion of *Bmpr1a*^{-/-} mESCs in competing cultures, both of which are rescued in the presence of the pan-caspase inhibitor Z-VAD-FMK suggesting *Bmpr1a*^{-/-} mESCs are eliminated by apoptosis upon co-culture with wild-type mESCs.

This data is also represented directly as analysed by flow cytometry in Figure 3.5H. In this experiment, wildtype-GFP mESCs and *Bmpr1a*^{-/-} mESCs are plated either separately or in co-culture at a 40:60 wild-type to *Bmpr1a*^{-/-} mESCs and treated with either pan-caspase inhibitor or DMSO for 2 days and analysed on the third (3) day of co-culture, similar to data represented graphically in Figure 3.5G. As also seen with wildtype and *Bmpr1a*^{-/-}-GFP mESC cell competition experiments, cell competition was significantly reduced upon addition of pancaspase inhibitor ZVAD-FMK compared to DMSO controls.

3.2.5 Inhibition of JNK or p38 signalling is insufficient to rescue *Bmpr1a*^{-/-} mESCs from cell competition

Caspase activation is one of the last steps to occur during the apoptotic process. Upstream signalling cascades respond to cellular stresses and if unresolved, lead to apoptosis. It is of interest to identify which signalling pathways mediate competitive stress upstream of caspase activation and apoptosis, as this would help elucidate how cells sense changes in 'fitness' and trigger competition. It is likely that a variety of triggers exist to mediate competition, which signals via diverse pathways before converging on caspase activation and apoptosis, as seen in other cell competition models (Levayer and Moreno, 2013; Wagstaff et al., 2013).

In previous studies of competing cultures, JNK signalling and activation of downstream pro-apoptosis signals were found to be a common response to cell stress (Weston and Davis, 2007), and is described in both *Drosophila* and mammalian models of cell competition (de la Cova et al., 2004; Moreno and Basler, 2004; Tamori et al., 2010). To investigate if JNK signalling also affects competition between *Bmpr1a*^{-/-} and wild-type mESCs, 5µM of JNK inhibitor SP600125 (Bennett et al., 2001) was added from the initial day of culture and replaced daily. As a separate target of interest the p38 pathway was also investigated. Like JNK signalling, activation of p38 is also induced in response to cellular stress (Ono and Han, 2000). Therefore competing cultures were treated from the initial day of competition with 20µM of p38 inhibitor SB203580 and replaced daily with fresh media (Clements et al., 2011; Cuenda et al., 1995). DMSO was added to control cultures as a vehicle control in both experiments. Growth curves of separate and co-cultured wild-type and *Bmpr1a*^{-/-} mESCs were counted daily and plotted, shown in Figure 3.6.

Wild-type mESCs treated with 5µM SP600125 resulted in a profound reduction in cell number of wild-type cells, although it is unclear whether this is due to higher levels of apoptosis or growth arrest. This effect is not seen in co-cultures or in monocultures of *Bmpr1a*^{-/-} mESCs (Figure 3.6). Due to this change in growth upon JNK inhibition specifically in wild-type mESCs it is difficult to analyse the involvement of JNK signalling in cell competition. The ratio of wild-type to *Bmpr1a*^{-/-} mESCs in separate compared to co-cultures is significantly higher in co-culture due to the very low number of wild-type mESCs upon separate culture, suggesting that cell competition still occurs although not to the same extent (JNK inhibitor treated co-culture 1.98±0.03 separate 0.63±0.11 p≤0.05, DMSO control co-culture 3.08±0.10 separate 1.09±0.07 data shown as average±sem n=2 representative experiment shown). Reports in the literature show that mESCs which lack JNK isoforms 1 and 2 proliferate significantly faster than wild-type mESCs, so it is possible that the growth defect observed may be due to the cytotoxicity of the inhibitor SP600125 (Davies et al., 2000; Xu and Davis, 2010). Other JNK inhibitors are described in the literature which could be investigated and target individual JNK isoforms (Bogoyevitch et al., 2010). However, it is interesting to note that *Bmpr1a*^{-/-} mESCs display no growth rate phenotype in response to compound JNK signalling inhibition.

Wild-type and *Bmpr1a*^{-/-} mESCs grown with p38 inhibitor were cultured as separate and co-cultures of both genotypes. Growth curves were similar in separate cultures of wild-type and *Bmpr1a*^{-/-} mESCs. The growth curves of wild-type mESCs in co-culture was similar to that of separate cultures, but *Bmpr1a*^{-/-} mESCs undergo growth arrest and are progressively lost from co-culture, shown in Figure 3.6D-E. Inhibition of p38 signalling has no significant effect on wild-type or *Bmpr1a*^{-/-} mESC cell growth or cell competition as shown in Figure 3.6(D-F) suggesting that it is not involved in cell competition (p38 inhibitor co-culture 6.97±0.77 separate 2.95±0.5 compared to DMSO co-culture 4.57±1.25 separate 2.33±0.40 data shown as average±sem n=3, Figure 3.6F). This data indicates that JNK or p38 signalling pathways are not required during competition between wild-type and *Bmpr1a*^{-/-} mESCs though this may be due to redundancy between the different signalling pathways mediating competition (de la Cova et al., 2004; Tamori et al., 2010).

3.3 Discussion

The model described throughout the majority of this work investigates competitive interactions between wild-type and *Bmpr1a*^{-/-} mESCs. This mutation was investigated due to previous observations in *Drosophila* cell competition models in the epithelial wing disc of developing flies (Burke and Basler, 1996; Moreno and Basler, 2004; Moreno et al., 2002). In these experiments, differences in signal transduction of *Dpp* (*Decapentaplegic*) the *Drosophila* homologue of BMP 2 and 4 is sufficient to induce these cells to be outcompeted by neighbouring wild-type cells. *Bmpr1a*^{-/-} is the mammalian receptor for BMP2 and 4 and *Bmpr1a*^{-/-} mESCs have been shown to have reduced pSMAD 1/5/8 compared to wild-type cells in response to BMP 2 or 4 (Sancho et al., 2013). Morphologically, *Bmpr1a*^{-/-} mESCs are similar to wild-type cells and show similar abilities to self-renew and growth rates both as mESCs and during the four days of differentiation, when grown separately (Sancho et al., 2013 and this work). These features, similar to that seen in *Drosophila* models of cell competition, suggest that the interaction of *Bmpr1a*^{-/-} mESCs upon co-culture with wild-type cells is a good mammalian model for studying cell competition.

Analysis of differentiation of wild-type and *Bmpr1a*^{-/-} mESCs upon separate and co-culture at day 0 and day 4 do not show significant differences in gene expression for specific germ

layers (Sancho et al., 2013 supplemental data). Additionally, *in vivo* whilst *Bmpr1a*^{-/-} embryos the epiblast undergoes premature neural differentiation (Di-Gregorio et al., 2007) this defect is not seen in mosaic *Bmpr1a*^{-/-} embryos, where mutant cells can contribute to all three germ layers (Davis et al., 2004; Di-Gregorio et al., 2007). These data do not suggest that a deficiency in acquiring particular cell fates cause *Bmpr1a*^{-/-} mESCs to undergo apoptosis upon co-culture with wild-type counterparts.

To determine whether the loss of *Bmpr1a*^{-/-} mESCs from co-culture with wild-type cells was the sole cause determining their elimination, *Bmpr1a*^{-/-} mESCs were re-transfected with *Bmpr1a* (termed *Bmpr1a*GOF mESCs). *Bmpr1a*GOF mESCs were cultured separately or in co-culture with either *Bmpr1a*^{-/-} mESCs or wild-type mESCs. After 4 days of co-culture the ratio of each cell type per culture condition was analysed by FACs. Interestingly *Bmpr1a*GOF mESCs were able to out-compete *Bmpr1a*^{-/-} mESCs upon co-culture but showed no competitive behaviour when co-cultured with wild-type mESCs (Sancho et al., 2013). This data shows that loss of *Bmpr1a*^{-/-} in mESCs triggers cell competition and results in the elimination of this mutant population when confronted with wild-type mESCs or mESCs where *Bmpr1a* function is restored.

This chapter has shown that by inhibiting caspase activity the elimination of mutant cells is rescued and therefore that in mESCs cell competition is resolved by apoptosis of *Bmpr1a*^{-/-} cells. It is therefore likely that the attempts to analyse cell death in competitive cultures by AnnexinV staining (an early apoptotic marker of cell phospholipid membrane disorganisation) and activated (cleaved) caspase 3 analysis described in this chapter were both inconclusive due to loss of GFP expression in mutant cells and detachment of apoptotic cells from culture as shown in Figure 3.1. In addition cleaved caspase3 staining was difficult to quantify, as positively stained debris could not always be identified as belonging to one or more cells. The choice to prevent caspase activation was aimed at minimising loss of GFP expression and delamination of apoptotic mESCs to ensure an accurate analysis of the proportion of wildtype and *Bmpr1a*^{-/-} mESCs.

The addition of pancaspase inhibitor ZVAD-FMK was sufficient to significantly rescue the elimination of *Bmpr1a*^{-/-} mESCs from cell competition upon co-culture with wild-type

mESCs, regardless of GFP expression (similar results observed with both wild-type-GFP +*Bmpr1a*^{-/-} mESCs and wild-type +*Bmpr1a*^{-/-}-GFP mESCs). Interestingly the data indicated that despite the inhibition of caspase activation a non-significant proportion of *Bmpr1a*^{-/-} mESCs were still being lost upon co-culture with wild-type mESCs. This data suggests that *Bmpr1a*^{-/-} mESCs are also eliminated by other methods in addition to caspase activation, such as delamination, engulfment or necrosis (non-caspase dependent cell death). A second possible explanation could be due to the increase in growth rate of wild-type mESCs upon co-culture with *Bmpr1a*^{-/-} mESCs. Although, this increase in growth rate is not sufficient to result in a significantly higher proportion of wild-type cells without also triggering apoptosis in neighbouring *Bmpr1a*^{-/-} mESCs

In response to cell competition analysis of growth rate changes in cells upon competition show that wild-type mESCs exhibit higher growth rates upon co-culture compared to separate cultures. This has also been observed in previous models of cell competition as a mechanism to ensure reproducible organ size and to maintain total cell number (de la Cova et al., 2004; Norman et al., 2011). During development the epiblast is a rapidly proliferating mass of cells and is required to increase in cell number by upto 5-fold between E5.5 to E6.5dpc in the mouse embryo and commence gastrulation (Snow, 1977; Stuckey et al., 2011a). This time-frame prior to gastrulation and posterior patterning in the epiblast around E6.5 coincides with the developmental state of competing mESCs *in vivo* (Claveria et al., 2013; Sancho et al., 2013). This suggests that cell competition promotes higher proliferation in winner cells upon competition and maintains rapid proliferation in the epiblast so loss of mutant cells in the epiblast doesn't result in developmental delay or differences in patterning the epiblast.

Whilst it has been shown that ultimately *Bmpr1a*^{-/-} mESCs are lost from competing cultures by apoptosis, the upstream signalling pathways that induce cell death were not identified. In some *Drosophila* models of cell competition, a reduction in levels of *Dpp* signal transduction has been shown to result in upregulation of JNK signalling leading to apoptosis (Moreno et al., 2002). Furthermore extensive reports in the literature highlight multiple roles in JNK signalling in mediating cell stress; apoptosis and during mammalian development, including during cavitation, making it a logical target of investigation (Barr and Bogoyevitch, 2001; Maekawa et al., 2005; Sabapathy et al., 1999; Xu et al., 2010).

However, it has been difficult to analyse the role of JNK signalling in cell competition between mESCs due to the effects of JNK inhibitor SP600125 on wild-type mESCs. In mESCs only the JNK 1 and 2 isoforms are present as the third isoform JNK3 is restricted to the brain and neural tissue (Davis, 2000). Furthermore mESCs can be derived from JNK1; 2 and compound JNK1/2 knockout mice which exhibit no self-renewal or proliferation defects (Xu et al., 2010). These data indicate that the cytotoxicity observed in wild-type mESCs is due to the inhibitor used and not the lack of JNK signalling, although this does not address why this cytotoxicity is not present in *Bmpr1a*^{-/-} mESC mono-cultures. Another possible explanation for the cytotoxicity of wild-type but not *Bmpr1a*^{-/-} mESCs could be due to the changes in differentiation ability. *JNK1/2*^{-/-} mESCs are defective in mesoderm differentiation, a trait shared by *Bmpr1a*^{-/-} mESCs (Xu et al., 2010). It could be that this inability to differentiate into mesodermal lineages triggers apoptosis in wild-type mESCs upon JNK inhibition, but *Bmpr1a*^{-/-} mESCs may have adapted in culture to adopt other fates, thus escaping apoptosis. Other methods to inhibit JNK signalling, including non-anthropyrazole inhibitors could be used, or target the downstream JNK signalling substrate c-Jun to perturb JNK signalling with fewer cytotoxic side effects (Bogoyevitch et al., 2010).

p38 signalling has also been linked to apoptosis in response to cell stress and cell competition (Kyriakis et al., 1997; Norman et al., 2011; Ono and Han, 2000). In addition the p38 MAPK pathway is involved in embryonic development and inhibition of the p38 MAPK pathway allows maintenance of embryonic stem cells and derivation of embryonic stem cells from recalcitrant cell lines (Natale et al., 2004; Qi et al., 2004; Zohn et al., 2006). Whilst the *Bmpr1a*^{-/-} mESCs used in this work can be maintained in the absence of p38 inhibition, it was hypothesised that this pathway may trigger apoptosis in these cells in response to competitive stress. However, treatment of cultures with a p38 inhibitor has no effect on the dynamics of cell competition, suggesting that imbalances in p38 signalling between wild-type and *Bmpr1a*^{-/-} mESCs are not the driving force for competition.

This chapter has begun to investigate the signalling pathways that may be involved in cell competition, however further investigation is needed. Within the embryo there are a variety of interacting signalling pathways acting to pattern the epiblast and surrounding tissues, such

as Nodal; Wnt; FGF (Tam and Loebel, 2007; Tam et al., 2006). An inability to transduce such signals or differential responses to such signals in a single mutated cell surrounded by wild-type cells could trigger cell competition, rendering such signalling pathways targets of future investigation.

Chapter 4 – Investigating the Signalling factors involved mediating cell competition between wild-type and *Bmpr1a*^{-/-} mESCs

4.1 Introduction

One of the fundamental questions invoked during studies of cell competition is how do cells sense their relative fitness levels, particularly what marks a cell as a “loser” or a “winner” during competition, and what if any response can be used to mark the change in cell identity prior to apoptosis. Two possible mechanisms have been suggested by which cells interact, cell-cell contact-mediated communication and uptake of unknown signalling factors.

Senoo-Matsuda and Johnston first demonstrated the existence of secreted factors mediating cell behaviour in cultures of *Drosophila* epithelial S2 cells with differing levels of dMyc. In their experiments, naïve cultures of control or *dMyc* overexpressing cells were treated with media from control and *dMyc* overexpressing cells. In response wild-type cells underwent apoptosis whilst *dMyc* overexpressing cells overproliferated (Senoo-Matsuda and Johnston, 2007). These experiments suggest the presence of unknown secreted factors that mediate cell competition. This mechanism has been postulated to be a part of cell competition in mammalian systems, although it is thought to be a short ranged mechanism (Claveria et al., 2013).

Rhiner et al., also investigated how cells sense each other's' relative fitness and described the existence of the *Flower* (*Fwe*) code that could also explain how cells respond differently to the same stimulus. Using the *Drosophila* wing imaginal disc model, Rhiner and coworkers showed that winner and loser cells display different isoforms of *Flower*, an ubiquitous protein expressed at the cell membrane. In this model winner cells displayed a full-length isoform of the protein termed *fwe*^{ubi} whilst loser cells expressed a truncated form of the protein *fwe*^{lose}-*A/fwe*^{lose-B}. Forced expression of either loser isoforms of *fwe* in non-competing populations is sufficient to induce apoptosis in those cells. However, interpretation of the *fwe* code required direct cell-cell contact and therefore is likely to involve a different mechanism to that described in *Drosophila* epithelial cells (Rhiner et al., 2010).

During early development, the vertebrate body plan is laid down. Through inductive interactions that are mediated by intracellular signalling factors, such as Wnt, FGF and TFG β family members. These cues serve to pattern the embryo and direct cell fate. Differences in these signalling pathways may serve as a “read-out” of cell viability and thus may be involved during cell competition (Tam et al., 2006).

In this chapter we first investigate whether secreted factors are required for cell competition to occur using a transwell assay that prevents cell-cell contact. This chapter then goes on to investigate the role of some of the signalling pathways involved during embryonic development. In particular LIF; Nodal; Wnt and FGF signalling pathways were investigated due to their roles in patterning the early embryo. Through a series of inhibition assays, the roles of various signalling pathways were perturbed during the co-culture of wild-type and *Bmpr1a*^{-/-} mESCs and their effects on cell competition dynamics investigated.

4.2 Results

4.2.1 *Bmpr1a*^{-/-} mESCs are lost from co-culture with wild-type mESCs despite no cell-cell contact

In order to investigate whether cell-cell contact was required for cell competition between mESCs we utilised a transwell system. In this system, shown in Figure 4.1A, cells are grown on the base of the well; whilst another group of cells are grown on the transwell filter lying 1.2mm above. In separate cultures, cells are grown with cells of the same genotype grown in the overlying transwell, and in co-culture, cells of another genotype are used. Cells were grown in this way for 4 days and the number of cells in the base of the 6 well plate were counted daily, and shown in Figure 4.1B. The genotype of cells grown in the base of the well are listed first and the genotype of those grown in the transwell filter is shown in brackets.

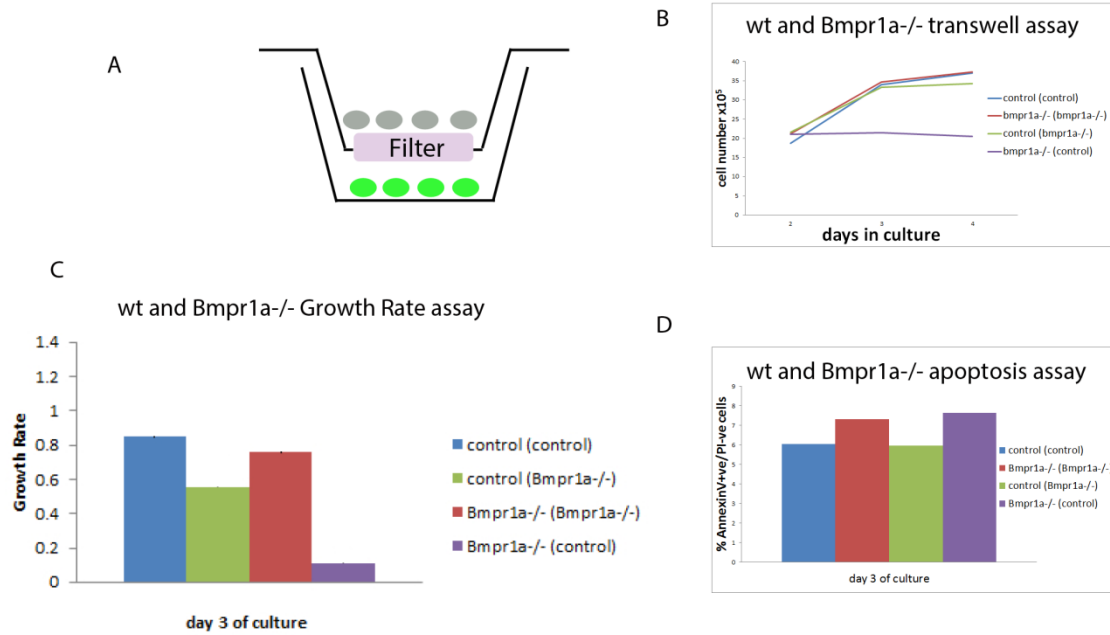


Figure 4.1 Cell-cell contact is not required for out-competition of *Bmpr1a*^{-/-} mESCs upon co-culture with wild-type mESCs

(A) Model of the transwell system. Cells of one genotype are grown at the base of a 6 well plate (green circles) whilst those of another genotype (grey circles) are grown atop a filter situated 1.2mm above the base of the well. (B) Growth curves showing cell number per day. Cells are grown as shown in (A) with the genotype grown on the filter shown in brackets. (Data shown as average cell number. At 4 days of culture $n=3$ $p \leq 0.01$; for previous days' growth $n=2$) (C) Changes in growth rate in cultures between days 2 and 3 of culture. (D) Percentage of AnnexinV⁺/PI⁻ cells grown for 3 days in culture. In both experiments, cells grown in the base of the well are listed first. The genotypes of the cells grown in transwells are listed in brackets. (data shown as average cell number as $n=2$).

Growth rate is calculated as the natural log (final number/initial number)/Ln (2) to account for exponential growth or mESCs, in each case data is shown as average $\pm$ s.e.m. Analysis of growth rates between days 2 and 3 of culture show a marked reduction in the growth rate of *Bmpr1a*^{-/-} mESCs when grown with overlaying wild-type cells *Bmpr1a*^{-/-} (control) 0.11 ± 0.08 compared to separate *Bmpr1a*^{-/-} mESC culture; *Bmpr1a*^{-/-} (*Bmpr1a*^{-/-}) 0.76 ± 0.20 (data shown as average cell number $n=2$). This reduction is similar to what is observed when analysing the growth curves of wild-type/*Bmpr1a*^{-/-} mESCs co-cultured in the same well (Appendix Table 1). Interestingly, there is also a reduction in the growth rate between wild-type cells grown with overlaying *Bmpr1a*^{-/-} mESCs (co-culture) compared to wild-type cells with cells of the same genotype in the transwell above (separate). The growth rate of cells grown in

transwell are shown in Figure 4.1C (control (control) 0.85 ± 0.33 ; control (*Bmpr1a*^{-/-}) 0.56 ± 0.18 ; *Bmpr1a*^{-/-} (*Bmpr1a*^{-/-}) 0.76 ± 0.20 ; *Bmpr1a*^{-/-} (control) 0.11 ± 0.08 ; data shown as average $\pm$ s.e.m n=2). As the growth rate has only been calculated for two experiments, further work is needed to confirm if any of these changes in growth rate are significant. This experiment suggests that secreted factors are involved in inducing apoptosis and growth arrest of competing mESCs.

Following the observation that no cell-cell contact is required for the elimination of *Bmpr1a*^{-/-} mESC when in co-culture with wild-type mESCs, the question of how *Bmpr1a*^{-/-} mESCs are lost upon co-culture with wild-type mESCs arose. Primarily this was thought to be due to secreted factors from wild-type cells triggering apoptosis in neighbouring *Bmpr1a*^{-/-} mESCs. To investigate this, the number of apoptotic cells in various culture conditions was analysed. To quantify levels of apoptosis occurring in each growth condition, cells were grown for 3 days in transwell culture. Cells grown in the base of the well were stained and analysed by FACs for AnnexinV⁺/PI⁻ to determine the percentage of pro-apoptotic cells in each population. There are no significant changes in the percentage of pro-apoptotic cells in each condition, data shown in Figure 4.1D (%AnnexinV⁺/PI⁻ mESCs at day 3 co-culture with the same genotype: wild-type 6.03 ± 1.29 *Bmpr1a*^{-/-} 7.30 ± 2.10 ; co- culture with cells of different genotype wild-type 5.97 ± 0.87 *Bmpr1a*^{-/-} 7.63 ± 2.22 data shown as average $\pm$ s.e.m n=3 p $\leq$ 0.05). Given that caspase inhibition blocks the elimination of *Bmpr1a*^{-/-} mESCs when in co-culture with control cells (chapter 3), this observation suggests in the transwell assays *Bmpr1a*^{-/-} mESCs could be extruded from the mESC colonies very early on during the apoptotic process. Alternatively the growth arrest observed in these assays could be due to secreted factors mediating a cell cycle block in loser cells and cell-cell contact being necessary for their elimination. Further work is required to investigate these aspects.

4.2.2 Activin/Nodal Signalling is not involved in mediating cell-competition between wild-type and *Bmpr1a*^{-/-} mESCs.

In mouse development Nodal signalling is required for maintenance of EpiSCs and for the formation of mesoderm and endoderm (Chenoweth and Tesar, 2010; Fei et al., 2010; Mesnard et al., 2006; Takenaga et al., 2007). The involvement of Activin/Nodal signalling

during cell competition was investigated by using SB431542, a chemical inhibitor against the serine/threonine kinase receptors involved in transducing these signals – ALK5; the activin type I receptor ALK4 and the nodal specific type I receptor ALK7 (Clements et al., 2011; Inman et al., 2002; Laping et al., 2002).

First, Activin/Nodal signalling was inhibited from the onset of cell competition. Co-cultures of *Bmpr1a*^{-/-} and wild-type mESCs were plated as a 40:60 ratio as *Bmpr1a*^{-/-} mESCs have a slight growth defect in culture. All cultures had media replaced daily supplemented with either 10µM SB431542 or an equal volume of DMSO. Analysis of the growth curves of separate cultures of wild-type and *Bmpr1a*^{-/-} mESCs in inhibitor treated cultures shows that addition of 10µM SB431542 daily to cultures was not cytotoxic to mESCs compared to DMSO controls. In separate cultures both wild-type and *Bmpr1a*^{-/-} mESCs show similar growth curves. In co-cultures of *Bmpr1a*^{-/-} and wild-type mESCs in DMSO, *Bmpr1a*^{-/-} mESCs undergo growth arrest after two days and are progressively lost from culture. In contrast co-cultures treated daily with 10µM SB431542 show similar growth curves to separate cultures. These data are shown in Figure 4.2A-B. After four days of culture the ratio of wild-type to *Bmpr1a*^{-/-} mESCs in separate and co-cultures of 10µM SB431542 or DMSO treated were analysed by FACs to determine the ratio of wild-type cells to *Bmpr1a*^{-/-} mESCs in separate and co-cultures. The ratio of wild-type to *Bmpr1a*^{-/-} mESCs after 4 days of culture in 10µM SB431542, shown in Figure 4.2C, shows significant rescue of *Bmpr1a*^{-/-} mESCs compared to DMSO treated cells (ratio of wild-type/*Bmpr1a*^{-/-} mESCs in 10µM SB431542 treated co-culture 1.52±0.32 separate 1.20±0.15 DMSO co-culture 10.66±3.17 separate 2.01±0.24 n=3 p≤0.05). This suggests that addition of SB431542 to competing cultures is sufficient to rescue cell competition if added from day 0 of culture.

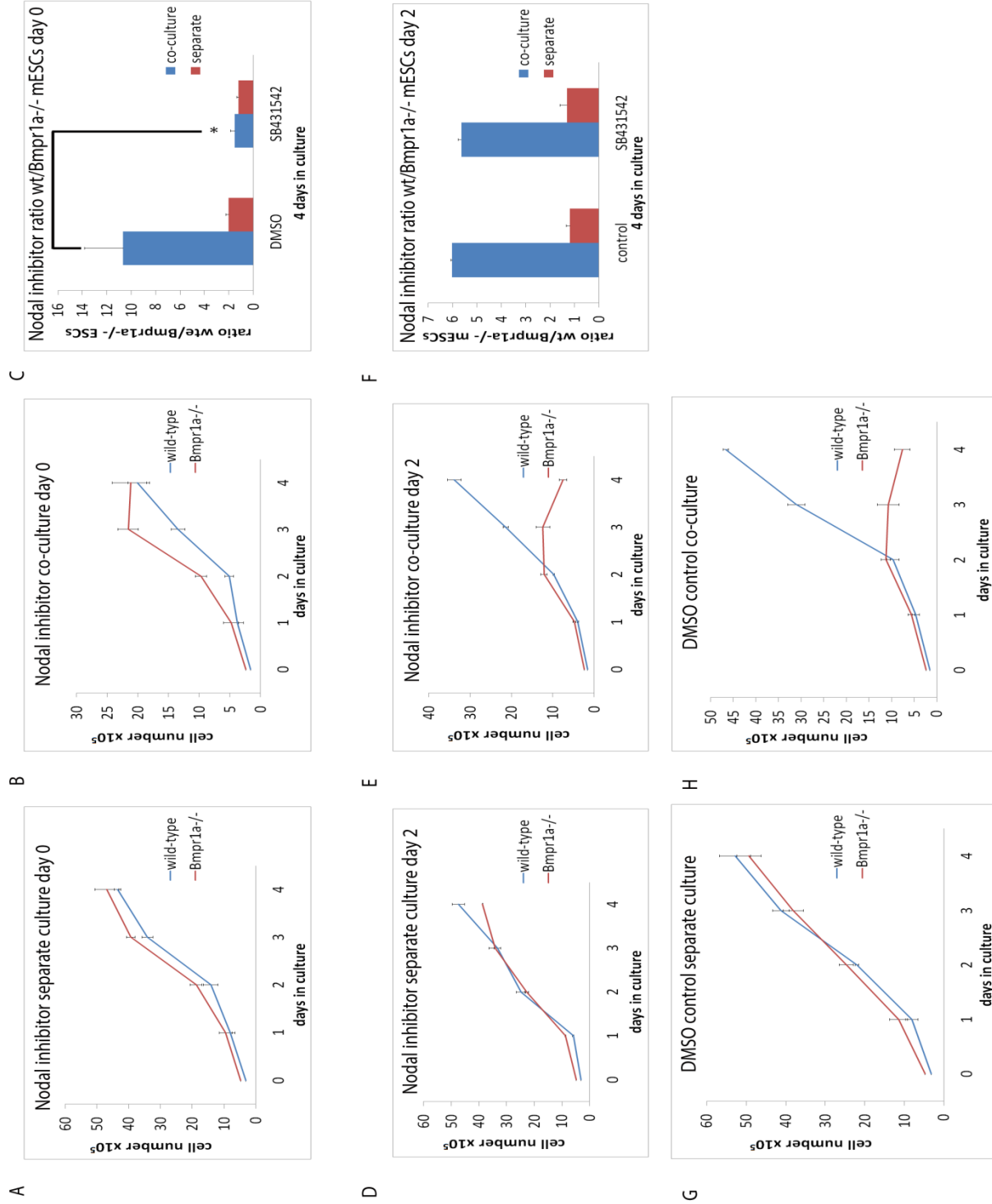
Figure 4.2 Activin/Nodal is not involved in mediating cell

competition between wild-type and *Bmpr1a*^{-/-} mESCs

(A-C) Wild-type and *Bmpr1a*^{-/-} ESCs were plated as (A) separate and (B) co-cultures and treated daily with 10uM Activin/Nodal inhibitor SB431542. (C) The ratio of control/*Bmpr1a*^{-/-} ESCs analysed at

day 4 of co-culture and compared with DMSO treated cultures (D-F) Wild-type and *Bmpr1a*^{-/-} ESCs were plated as (D) separate and (E) co-cultures and treated from day 2 of co-culture with 10uM SB431542. (F) The ratio of control/*Bmpr1a*^{-/-}

ESCs analysed at day 4 of co-culture and compared with DMSO treated cultures normalised back to ratio of wild-type/*Bmpr1a*^{-/-} ESCs at day 2 of culture. (G-H) DMSO controls of separate (G) and co-cultures (H) data shown as average±s.e.m n=3 p≤0.05

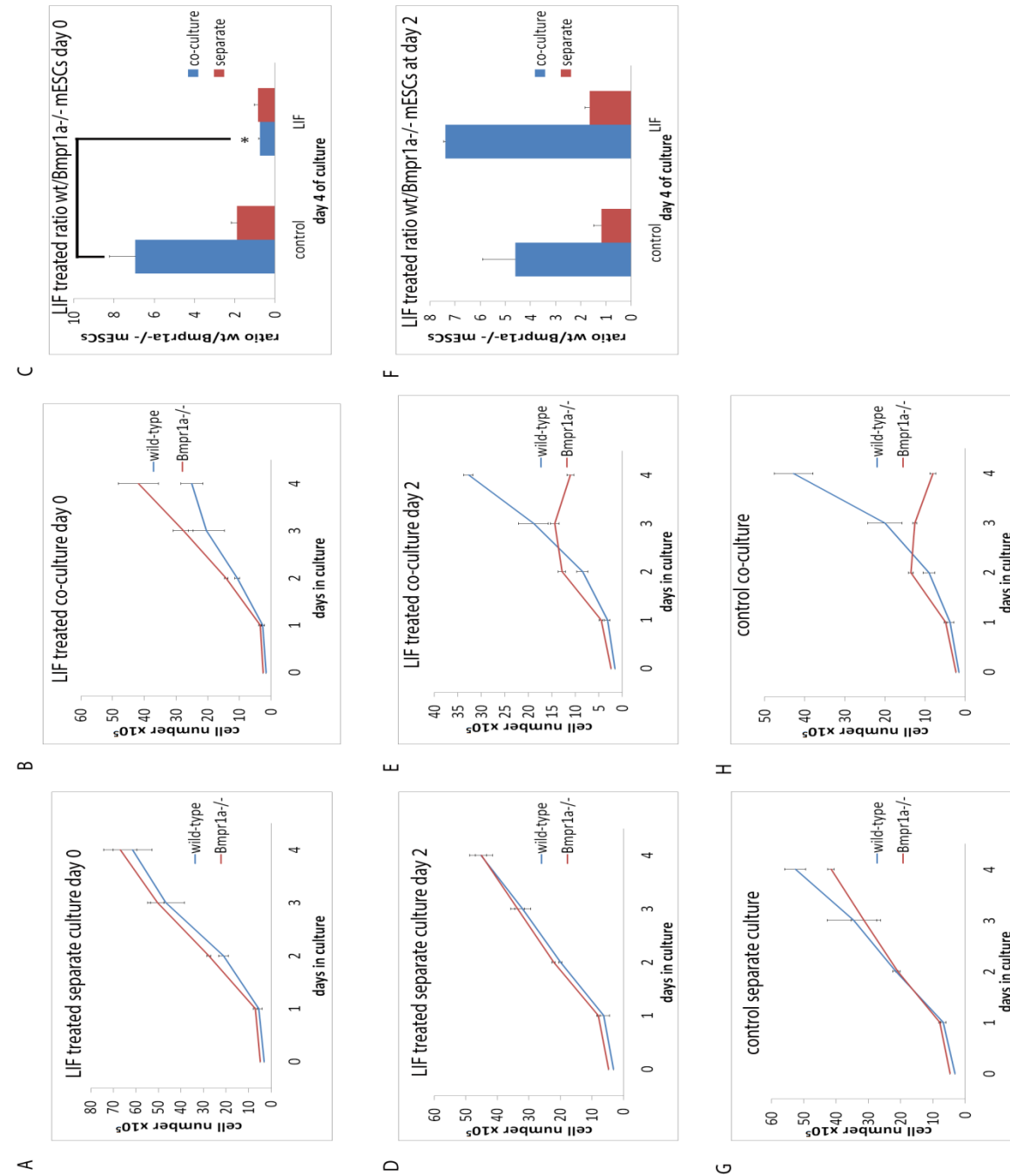


Within this model of cell competition wild-type and *Bmpr1a*^{-/-} cells are plated as mESCs and then grown in GMEM+Serum, which allows undirected differentiation of mESCs. Cell competition is generally induced around the second day of culture in our model. By day 2 of culture, mESCs have begun to differentiate (Sancho et al., 2013). Nodal has been implicated in maintaining pluripotency in mESCs, by preventing inhibition by Smad7 of BMP-mediated signalling (Galvin et al., 2010). To determine if Activin/Nodal signalling is sufficient to prevent cell competition once mESCs have begun to differentiate cultures were treated from day 2 with 10µM SB431542. Analysis of growth curves of separate wild-type and *Bmpr1a*^{-/-} mESCs showed no differences in the growth of either cell population. In contrast analysis of the growth curves of wild-type and *Bmpr1a*^{-/-} mESC co-cultures (Figure 4.2C-D) showed that *Bmpr1a*^{-/-} mESCs underwent growth arrest from day 2 of co-culture despite inhibition of Nodal/Activin signalling. The ratio of wild-type to *Bmpr1a*^{-/-} mESCs in treated cultures compared to DMSO controls were also investigated by FACs analysis. These ratios were normalised to the ratio at day 2. Analysis of ratios of wild-type to *Bmpr1a*^{-/-} mESCs in culture when Activin/Nodal signalling was inhibited from day 2 of culture was not significantly different from DMSO controls, (ratio of wild-type to *Bmpr1a*^{-/-} mESCs treated with 10µM SB431542 from day 2 co-culture 5.64±0.12 separate 1.31±0.27 compared to DMSO control co-culture 6.02±0.06 separate 1.18±0.16 average±s.e.m n=3 p≤0.05 shown in Figure 4.2F). This experiment shows that inhibition of Activin/Nodal signalling is insufficient to rescue *Bmpr1a*^{-/-} mESCs from cell competition once cells have initiated differentiation.

4.2.3 LIF mediated signalling is not involved in cell competition between wild-type and *Bmpr1a*^{-/-} mESCs.

LIF signalling is essential for the maintenance of mESC pluripotency via activation of the Jak-Stat3 signalling pathway resulting in *Tbx3*-mediated activation of *Sox2* and *Klf4*-mediated activation of *Oct3/4* (Niwa et al., 2009). Previous work in *Drosophila* showed that differences in STAT signalling, a downstream target of LIF in mESCs, is sufficient to trigger competition in the wing disc of developing larvae (Rodrigues et al., 2012). Recently published work by Sancho et al., and Claveria et al., have highlighted the importance of *cMyc*

Figure 4.3 LIF signalling is not involved in mediating cell competition between wild-type and *Bmpr1a*^{-/-} mESCs
(A-C) Wild-type and *Bmpr1a*^{-/-} ESCs were plated as **(A)** separate and **(B)** co-cultures and treated daily with LIF. **(C)** The ratio of control/*Bmpr1a*^{-/-} ESCs analysed at day 4 of co-culture and compared with control untreated cultures. **(D-F)** Wild-type and *Bmpr1a*^{-/-} ESCs were plated as **(D)** separate and **(E)** co-cultures and treated from day 2 of co-culture with LIF. **(F)** The ratio of control/*Bmpr1a*^{-/-} ESCs analysed at day 4 of co-culture and compared with DMSO treated cultures normalised back to ratio of wild-type/*Bmpr1a*^{-/-} ESCs at day 2 of culture. **(G-H)** DMSO controls of separate **(G)** and co-cultures **(H)** data shown as average±s.e.m n=3 p≤0.05.



in mediating cell competition in mESCs and *c-Myc* is a known target of LIF (Cartwright et al., 2005; Claveria et al., 2013; Sancho et al., 2013).

The role of LIF mediated signalling in cell competition was investigated. Separate and co-cultures of wild-type and *Bmpr1a*^{-/-} mESCs were plated, as previously described (chapter 3). LIF was added with fresh media daily, to all cultures of *Bmpr1a*^{-/-} and wild-type mESCs from the onset of the experiment. Total cell numbers of separate and co-cultures of wild-type and *Bmpr1a*^{-/-} mESCs were counted daily and compared with control cultures where LIF was not added. In separate cultures for both LIF supplemented and control treated conditions, both wild-type and *Bmpr1a*^{-/-} mESCs show similar growth curves. In co-cultures of wild-type and *Bmpr1a*^{-/-} mESCs treated with LIF from day 0 of competition, no growth arrest or loss of *Bmpr1a*^{-/-} mESCs is observed (Figure 4.3A-B).

When the ratio of wild-type to *Bmpr1a*^{-/-} mESCs in separate and co-cultures are quantified by FACS analysis, after 4 days of culture it could be observed that there is a significant rescue of *Bmpr1a*^{-/-} mESCs in LIF treated cultures compared to untreated controls, shown in Figure 4.3C (LIF treated co-culture 0.76±0.07 separate 0.86±0.17 untreated co-culture 6.94±1.29 separate 1.88±0.029 data shown as average±s.e.m n=3 p≤0.05). This suggests that LIF rescues *Bmpr1a*^{-/-} mESCs from out-competition by wild-type mESCs.

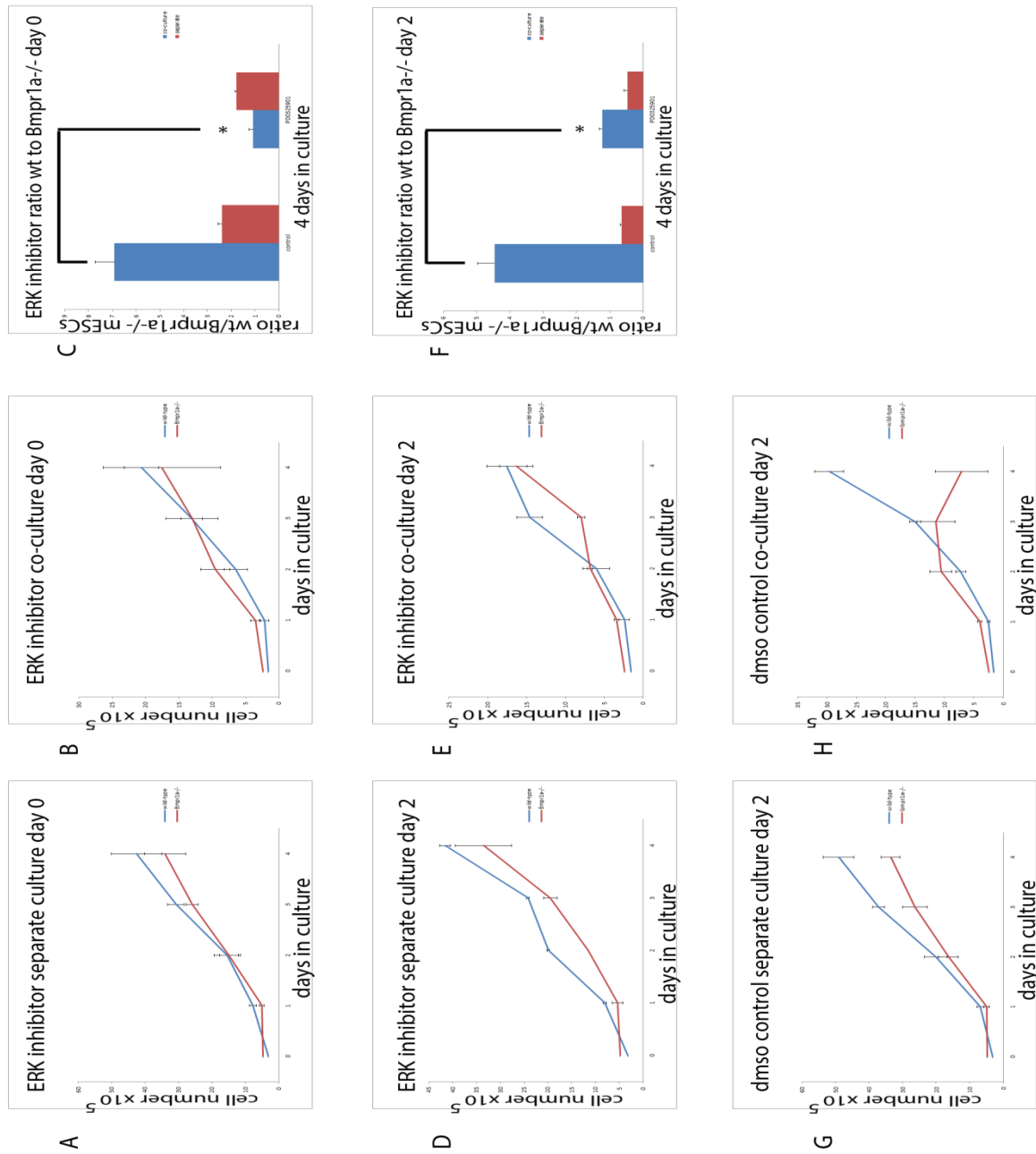
mESCs cultured in GMEM supplemented with Serum and LIF can be maintained indefinitely as a pluripotent undifferentiated population in culture. To investigate if addition of LIF is sufficient to rescue *Bmpr1a*^{-/-} mESCs from competition once cells have started to differentiate, LIF was added to separate and co-cultures of wild-type and *Bmpr1a*^{-/-} mESCs from day 2 of culture. As shown in Figure 4.3D-E the growth curves of separate wild-type and *Bmpr1a*^{-/-} mESCs are similar. In co-cultures of wild-type and *Bmpr1a*^{-/-} mESCs where LIF is only added from the second day of culture, *Bmpr1a*^{-/-} mESCs behave similarly to untreated cultures, therefore undergo growth arrest at day 2 and are progressively lost from culture thereafter. When the proportion of each genotype to the total cell population is quantified by FACS analysis and the ratio of wild-type to *Bmpr1a*^{-/-} mESCs plotted normalised to day 2, it could be observed that cultures treated with LIF from day 2 show no

significant change compared to untreated controls, Figure 4.3F (co-culture 4.61 ± 0.41 separate 1.18 ± 0.05 compared to untreated control co-culture 7.38 ± 1.01 separate 1.65 ± 0.15 average $\pm$ s.e.m $n=3$ $p \leq 0.05$). This indicates that once mESCs have initiated differentiation LIF mediated signalling is unable to prevent cell competition between wild-type and *Bmpr1a*^{-/-} mESCs, suggesting this pathway is not involved in cell competition.

4.2.4 Inhibition of FGF/ERK signalling prevents cell-competition between wild-type and *Bmpr1a*^{-/-} mESCs

Similar to *Nodal*; FGF/ERK signalling is essential throughout mouse development. Null mutations in FGF signalling members are embryonic lethal as early as E4.0 whereupon *Fgf4* mutants die due to defects in PrE and TE formation (Feldman et al., 1995; Goldin and Papaioannou, 2003; Yamanaka and Ralson, 2010). Furthermore, FGF mediated activation of ERK1/2 acts as an auto-inductive cue for mESCs to initiate differentiation (Kunath et al., 2007). To investigate the role of FGF/ERK signalling in cell competition, separate and co-cultures of wild-type and *Bmpr1a*^{-/-} mESCs were plated in a 40:60 ratio. 1 μ M of MEK1/2 inhibitor PD0325901 or an equivalent volume of DMSO was added to cultures with fresh media daily. Shown in Figure 4.4 are the growth curves of separate and competing cultures of wild-type and *Bmpr1a*^{-/-} mESCs treated with 1 μ M PD0325901 or DMSO added from day 0. In separate cultures wild-type and *Bmpr1a*^{-/-} mESCs show similar growth curves, and this is also reflected by the analysis of growth rates, in Appendix Table.1 (growth rate separate wild-type 1.17 ± 0.10 ; *Bmpr1a*^{-/-} 1.48 ± 0.25 average $\pm$ s.e.m $n=3$ $p=0.62$). In competing cultures of wild-type and *Bmpr1a*^{-/-} mESCs treated with DMSO, *Bmpr1a*^{-/-} mESCs growth arrest by day 2 and are progressively lost from culture between days 3 and 4. This growth arrest and removal of *Bmpr1a*^{-/-} mESCs from co-culture with wild-type cells is not observed in cultures treated with 1 μ M PD0325901. This observation is confirmed when the ratios of wild-type and *Bmpr1a*^{-/-} mESCs in separate and co-culture conditions are quantified by FACs analysis and normalised to the day 0. The ratio of wild-type/*Bmpr1a*^{-/-} mESCs from day 0 in 1 μ M PD0325901 treated co-culture was similar to the ratio of PD0325901 treated separate cultures and significantly lower than that of DMSO treated co-cultures (ratio of wild-type/*Bmpr1a*^{-/-} mESCs from day 0 in 1 μ M PD0325901 1.08 ± 0.17 separate 1.78 ± 0.06 compared to DMSO control co-cultures 6.92 ± 0.80 separate 2.40 ± 0.16 data shown as average $\pm$ s.e.m $n=3$ $p \leq 0.05$).

Figure 4.4 Inhibition of FGF/ERK signalling is sufficient to rescue cell competition after the onset of differentiation between wild-type and *Bmpr1a*^{-/-} mESCs
(A-C) Wild-type and *Bmpr1a*^{-/-} ESCs were plated as **(A)** separate and **(B)** co-cultures and treated daily with 1uM PD0325901. **(C)** The ratio of control/*Bmpr1a*^{-/-} ESCs analysed at day 4 of co-culture and compared with control untreated cultures. **(D-F)** Wild-type and *Bmpr1a*^{-/-} ESCs were plated as **(D)** separate and **(E)** co-cultures and treated from day 2 of co-culture with 1uM PD0325901. **(F)** The ratio of control/*Bmpr1a*^{-/-} ESCs analysed at day 4 of co-culture and compared with DMSO treated cultures normalised back to ratio of wild-type/*Bmpr1a*^{-/-} ESCs at day 2 of culture. **(G-H)** DMSO controls of separate **(G)** and co-cultures **(H)** data shown as average±s.e.m n=3 p≤0.05.



Given that *Lif* and inhibiting Nodal signalling only blocked cell competition of pluripotent mESCs, it is possible that similar may occur with FGF/ERK signalling. To investigate this competing and separate cultures of wild-type and *Bmpr1a*^{-/-} mESCs were grown in GMEM+Serum for 2 days, therefore allowing cells to differentiate. After 2 days of culture the media was supplemented with 1µM PD0325901 or DMSO. Under these conditions wild-type and *Bmpr1a*^{-/-} mESCs in separate cultures show similar growth curves and growth rates (between day 2 and 3) upon addition of 1µM PD0325901 compared to DMSO controls, shown in Figure 4.4 and Appendix Table 1. In contrast to this *Bmpr1a*^{-/-} mESCs in co-culture did not undergo growth arrest when treated with *Bmpr1a*^{-/-} mESCs, as occurred with DMSO controls. Analysis of the ratio of wild-type to *Bmpr1a*^{-/-} mESCs treated with 1µM PD0325901 normalised to day 2 co-culture confirms that this inhibitor rescues the elimination of *Bmpr1a*^{-/-} mESCs (ratio of wild-type to *Bmpr1a*^{-/-} mESCs, 1µM PD0325901 from day 2 co-culture 1.23±0.08 separate 0.85±0.10 compared to DMSO control co-culture 4.47±0.49 separate 1.92±0.03 average±s.e.m n=3 p≤0.05; Figure 4.4F). This suggests that even if added after the onset of differentiation, inhibition of FGF/ERK signalling is sufficient to rescue *Bmpr1a*^{-/-} mESCs from competition.

As described, PD0325901 prevented the elimination of *Bmpr1a*^{-/-} mESCs by wild-type mESCs even when added after the onset of differentiation (day 2 of cell competition) and western blot analysis shows that 1µM PD0325901 is sufficient to completely inhibit ERK1/2 phosphorylation.

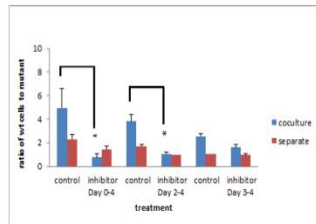
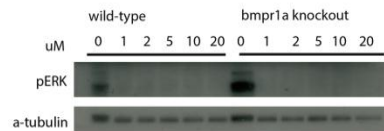
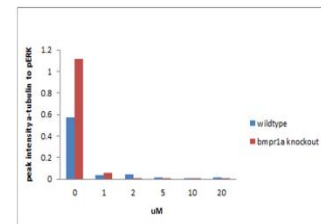
Figure 4.5 Complete suppression of ERK1/2 phosphorylation is required to prevent cell competition between wild-type and *Bmpr1a*^{-/-} mESCs
ERK1/2 MAPK Inhibitors

Effect on Cell Competition

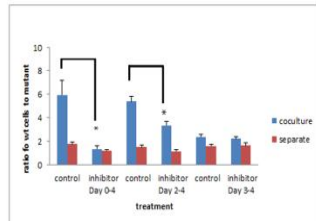
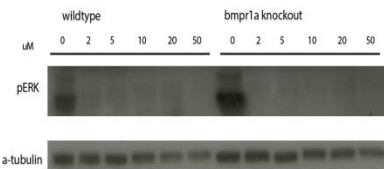
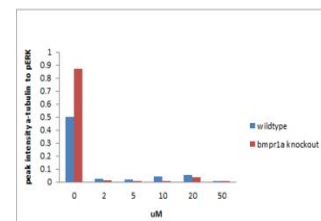
Western Blot

Quantification of Western Blot

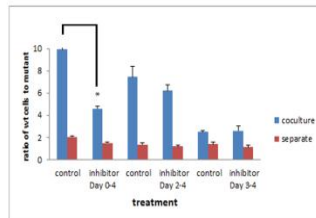
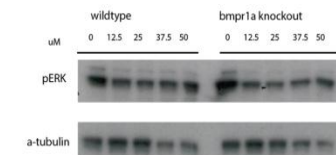
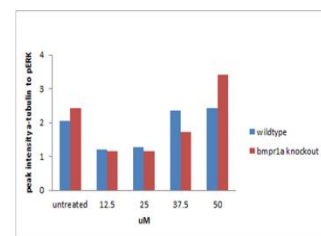
A PD0325901



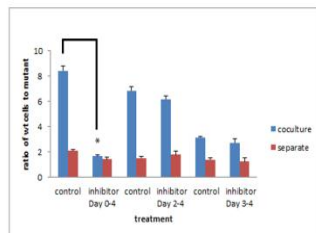
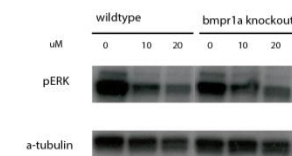
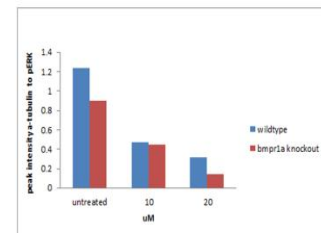
B PD184352



C PD98059



D U0126



E PD173074 - FGFR inhibitor

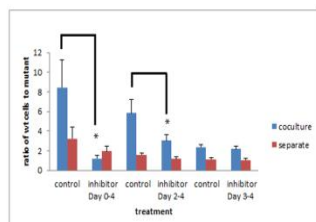
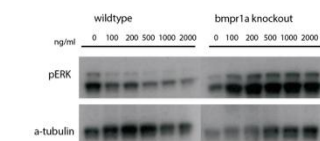
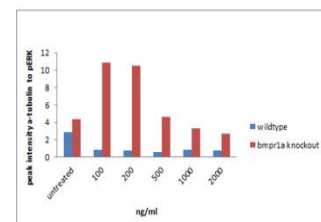


Figure 4.5 Complete suppression of ERK1/2 phosphorylation is required to prevent cell competition between wild-type and *Bmpr1a*^{-/-} mESCs

Wild-type and *Bmpr1a*^{-/-} ESCs were cultured separately or in co-cultures and the ratio of wild-type/*Bmpr1a*^{-/-} mESCs after four days of culture was normalised back to the first day of inhibitor treatment and plotted compared to equivalent DMSO controls. As an indication of inhibitor potency the levels of phosphorylated ERK1/2 were determined by western blot and quantified with α -tubulin used as an indicator of protein content. Effect of (A) 100ng/ml of FGFR inhibitor PD173074 (B) 10uM MEK1/2 inhibitor U0126 (C) 12.5uM PD98059 (D) 1uM PD0325901 (E) 2uM PD184352 on the ratio of wild-type/*Bmpr1a*^{-/-} ESCs during cell competition when added at day 0; day2 and day3 of culture; and ERK1/2 phosphorylation. (Data shown as average $\pm$ s.e.m n=3 p $\leq$ 0.05)

To further test if FGF/ERK signalling was sufficient to rescue cell competition between wild-type and *Bmpr1a*^{-/-} mESCs, a second inhibitor, PD184352, was investigated. In Figure 4.5B Western blot analysis of PD184352 showed that 2 μ M PD184352 is sufficient to block pERK1/2 as previously described (Kunath et al., 2007). Comparison of the ratio of wild-type to *Bmpr1a*^{-/-} mESCs in cultures supplemented daily with 2 μ M PD184352 from day 0 of competition found it to be significantly lower than DMSO control co-cultures and similar to separate cultures (ratio of wild-type to *Bmpr1a*^{-/-} mESCs 2 μ M PD184352 day 0 co-culture 1.33 $\pm$ 0.30 separate 1.22 $\pm$ 0.08 DMSO control 5.95 $\pm$ 1.21 separate 1.76 $\pm$ 0.19 data shown as average $\pm$ s.e.m n=3 p $\leq$ 0.05). When 2 μ M PD184352 was added from day 2 of culture, after mESCs had begun to differentiate, the ratio of wild-type to *Bmpr1a*^{-/-} mESCs was also significantly lower than DMSO control co-cultures (day 2 treated co-culture 3.35 $\pm$ 0.31 separate 1.14 $\pm$ 0.12 DMSO control 5.39 $\pm$ 0.41 separate 1.51 $\pm$ 0.19 (average $\pm$ s.e.m n=3 p $\leq$ 0.05).

PD98059 has a similar mode of action to PD184352, acting by preventing ERK1/2 activation, but is not as effective at suppressing FGF/ERK signalling (Davies et al., 2000). 12.5 μ M PD98059 was added to separate and co-cultures of wild-type and *Bmpr1a*^{-/-} mESCs from day 0 of cell competition and replaced daily with fresh media. After 4 days of culture the ratio of wild-type to *Bmpr1a*^{-/-} mESCs in cultures treated with 12.5 μ M PD98059 or DMSO was quantified by FACs and normalised. As shown in Figure 4.5C although treatment of wild-type and *Bmpr1a*^{-/-} mESC co-cultures with 12.5 μ M PD98059 did not completely rescue the elimination of *Bmpr1a*^{-/-} mESCs it leads to a significantly lower ratio of wild-type to *Bmpr1a*^{-/-} mESCs compared to DMSO treated co-cultures (ratio wild-type/*Bmpr1a*^{-/-} mESCs 12.5 μ M

PD98059 day 0 co-culture 4.61 ± 0.22 separate 1.52 ± 0.09 DMSO control co-culture 9.91 ± 0.59 separate 2.07 ± 0.06 (average $\pm$ s.e.m $n=3$ $p \leq 0.05$). $12.5 \mu\text{M}$ PD98059 was also added to separate and co-cultures of wild-type and *Bmpr1a*^{-/-} mESCs after 2 days of culture in GMEM+SeruM. However under these conditions no significant differences could be observed in the ratio of wild-type to *Bmpr1a*^{-/-} mESCs in co-culture compared to DMSO (ratio wild-type/*Bmpr1a*^{-/-} mESCs treated co-culture 6.22 ± 0.51 separate 1.20 ± 0.11 DMSO control co-culture 7.46 ± 0.96 separate 1.39 ± 0.16). Analysis of the efficiency of PD98059 to block ERK1/2 revealed that $12.5 \mu\text{M}$ only led to a partial blockage of ERK1/2 phosphorylation (fold change wild-type 0.6X reduction compared to control; *Bmpr1a*^{-/-} 0.5X reduction, Figure 4.5C). This suggests that partial suppression of ERK signalling is not sufficient to rescue *Bmpr1a*^{-/-} from cell competition in co-culture with wild-type mESCs.

Next U0126, a chemical inhibitor of MEK1/2 signalling but with fewer non-specific effects than PD98059 (Davies et al., 2000) was tested. The effect of U0126 on ERK1/2 phosphorylation was analysed by western blot (Figure 4.5D) and it was observed that $10 \mu\text{M}$ only caused 0.5 fold reduction in ERK1/2 phosphorylation. To analyse the effects of this level of pERK1/2 inhibition on mESCs separate and co-cultures of wild-type and *Bmpr1a*^{-/-} mESCs were plated (starting ratio 40:60 wildtype:*Bmpr1a*^{-/-} mESCs). $10 \mu\text{M}$ U0126 was added daily from the onset of cell competition and the ratio of wild-type to *Bmpr1a*^{-/-} mESCs analysed after 4 days of culture in each condition. As observed with PD98059, partial suppression of FGF/ERK signalling from the onset of culture was sufficient to partially rescue *Bmpr1a*^{-/-} mESCs from elimination compared to DMSO controls (ratio wild-type/*Bmpr1a*^{-/-} mESCs $10 \mu\text{M}$ U0126 day 0 co-culture 1.65 ± 0.13 separate 1.41 ± 0.20 DMSO control co-culture 8.39 ± 0.38 separate 2.12 ± 0.04). The effect of PD98059 on the co-culture of wild-type and *Bmpr1a*^{-/-} mESCs once they had started differentiate was also analysed. When co-cultures treated 2 days after culture with $10 \mu\text{M}$ U0126 were compared to DMSO controls, no significant difference in ratio of wild-type/*Bmpr1a*^{-/-} mESCs was observed (day 2 treated co-culture 6.15 ± 0.29 separate 1.78 ± 0.29 DMSO control co-culture 6.82 ± 0.34 separate 1.51 ± 0.14 all data shown as average $\pm$ s.e.m $n=3$ $p \leq 0.05$). These data suggest that complete suppression of FGF/ERK signalling is required to prevent cell competition between wild-type and *Bmpr1a*^{-/-} mESCs after the onset of differentiation.

PD173074 acts as a small chemical inhibitor of FGFR1 by preventing its autophosphorylation (Bansal et al., 2003). PD173074 therefore acts by suppressing the response to FGF ligand binding to its receptor rather than acting on ERK1/2 phosphorylation. To determine the concentration of PD173074 required to suppress ERK1/2 phosphorylation wild-type and *Bmpr1a*^{-/-} mESCs were treated with increasing concentrations of inhibitor. The level of pERK1/2 at each concentration in both cell lines was analysed by western blot, Figure 4.5E. Surprisingly PD173074 affected wild-type and *Bmpr1a*^{-/-} mESCs differently. In wild-type cells addition of increasing concentrations of PD173074 resulted in an increased reduction in PD173074. In contrast, when treated with lower concentrations of PD173074 *Bmpr1a*^{-/-} mESCs show higher levels of pERK1/2.

Next the effect of PD173074 on competition was analysed. Adding 100ng/ml PD173074 to separate and co-cultures of wild-type and *Bmpr1a*^{-/-} mESCs allowed to analyse the effect of suppression of FGF/ERK signalling in wild-type mESCs, whilst upregulating it in *Bmpr1a*^{-/-} mESCs. Separate and co-cultures of wild-type and *Bmpr1a*^{-/-} mESCs were plated and treated from day 0 with 100ng/ml PD173074, replaced daily with fresh media. After 4 days of culture the ratio of wild-type and *Bmpr1a*^{-/-} mESCs in co-culture was significantly lower compared to DMSO controls (Figure 4.5E 100ng/ml PD173074 day 0 co-culture 1.37±0.32 separate 2.15±0.49 DMSO control 10.05±2.19 separate 2.69±0.51). As with previous experiments the effects of adding the inhibitor from day 2 was analysed by studying the ratio of wild-type to *Bmpr1a*^{-/-} mESCs in separate and co-culture. The ratio of wild-type to *Bmpr1a*^{-/-} mESCs co-cultured from 2 day with PD173074 was compared to similarly treated DMSO controls, and found to be significantly lower in treated cultures (day 2 treated co-culture 3.37±0.58 separate 1.17±0.23 DMSO control co-culture 6.83±0.96 separate 1.63±0.24 data shown as average±s.e.m n=3 p≤0.05). This data suggests that a change in FGF/ERK signalling in ‘winner’ cells mediates cell competition in wild-type and *Bmpr1a*^{-/-} mESCs.

The effect of each inhibitor was determined by their ability to significantly alter the ratio of wild-type to *Bmpr1a*^{-/-} mESCs after 4 days of culture. As with both Activin/Nodal and LIF experiments, the effect of FGF/ERK inhibition on cell competition was investigated at two different time points, from the onset of competition (cells are mESCs - inhibitor from day 0) or after 2 or 3 days of culture to allow mESCs to initiate differentiation (day 2/ day 3

inhibitor). In each case, the ratio of wild-type to *Bmpr1a*^{-/-} mESCs were normalised to the population ratio at the onset of inhibitor treatment. Once normalised, ratios of wild-type to *Bmpr1a*^{-/-} mESCs were compared by students' t-test to DMSO controls cultured at the same time to determine whether changes were significant. To establish the efficiency of each inhibitor western blot for ERK1/2 phosphorylation was used as an indicator of their ability to prevent ERK1/2 phosphorylation. In all cases, addition of inhibitor from day 3 of culture was never sufficient to rescue cell competition (shown in Figure 4.5 ratios shown in Appendix Table 2). It is likely that by that point the process is too far along to be efficiently reversed.

4.2.5 Addition of FGF ligands to competing cultures does not promote cell competition between wild-type and *Bmpr1a*^{-/-} mESCs

In early murine development, FGF4 is paracrine factor produced by cells of the ICM and acts as an autoinductive cue to promote differentiation and specific PrE cell fate (Kunath et al., 2007; Frum et al., 2013). As mESCs differentiate and become EpiSCs, FGF4 is downregulated in favour of FGF5, which is often used as a marker of EpiSCs (Hebert et al., 1994; reviewed by Rossant and Lanner, 2010). Both FGF4 and FGF5 have different affinities for the FGFRs. These differences in receptor binding affinity are likely to reflect specificity activation of ligand targets.

Inhibition of FGF/ERK signalling, as analysed by western blotting for pERK, was able to block cell competition, at both the mESC state and after the onset of differentiation. To investigate if promoting FGF signalling was sufficient to promote cell competition, FGF4 or FGF5 was added to separate and co-cultures of wild-type and *Bmpr1a*^{-/-} mESCs. FGF4 or FGF5 was added to cultures daily at 5ng/ml and 10ng/ml daily in the presence of 1ug/ml heparin (Kunath et al., 2007). 1ug/ml heparin was used as a volume control for these experiments.

Figure 4.6 Addition of FGF4 or

FGF5 does not affect cell

competition between wild-type and *Bmpr1a*^{-/-} mESCs

(A-C) Wild-type and *Bmpr1a*^{-/-} ESCs were plated as **(A)** separate and **(B)** co-cultures and treated daily with 5ng/ml FGF4 in 1ug/ml heparin

(C) The ratio of control/*Bmpr1a*^{-/-} ESCs analysed at day 4 of co-culture

and compared with 1ug/ml heparin - only treated cultures **(D-F)** Wild-

type and *Bmpr1a*^{-/-} ESCs were plated as **(D)** separate and **(E)** co-cultures and treated daily with 10ng/ml FGF5

in 1ug/ml heparin. **(F)** The ratio of control/*Bmpr1a*^{-/-} ESCs analysed at

day 4 of co-culture and compared

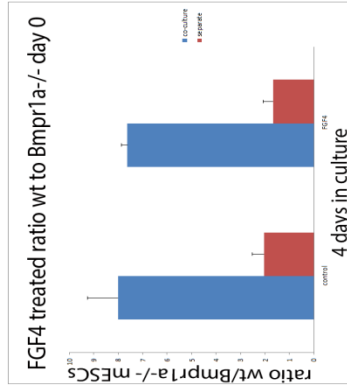
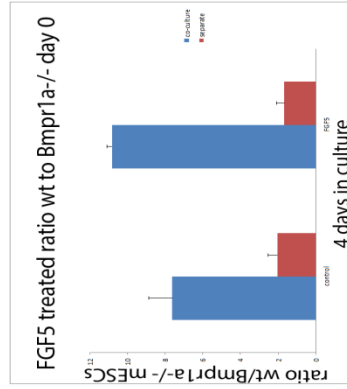
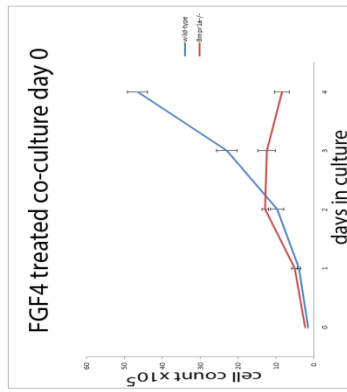
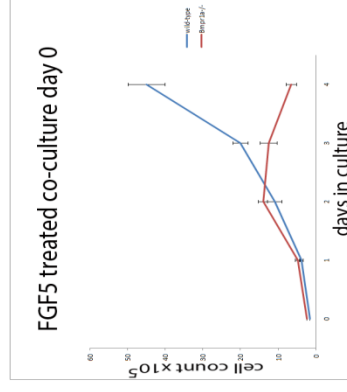
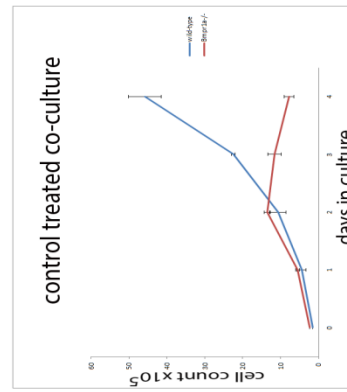
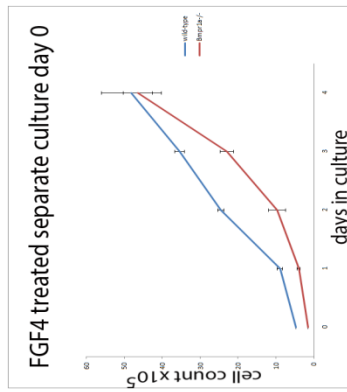
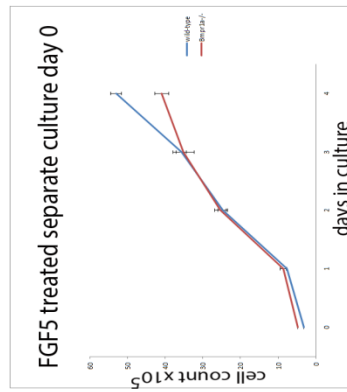
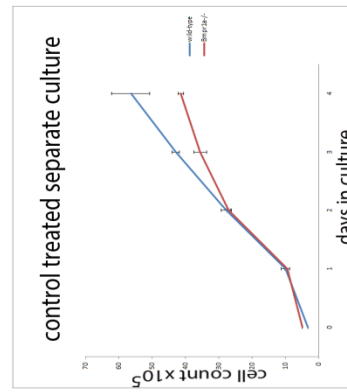
1ug/ml heparin-only cultures after 4

days of co-culture **(G-H)** 1ug/ml

heparin controls of separate **(G)** and

co-cultures **(H)**. (data shown as

average±s.e.m n=3 p ≤0.05).



In separate cultures treated with 5ng/ml FGF4 and 1ug/ml heparin wild-type and *Bmpr1a*^{-/-} show similar growth curves (Figure 4.6). However, in co-culture with wild-type cells, *Bmpr1a*^{-/-} mESCs growth arrest after 2 days and are progressively lost from culture. After 4 days of culture, separate and co-cultures of wild-type and *Bmpr1a*^{-/-} mESCs were analysed by FACs. This was to quantify if the loss of *Bmpr1a*^{-/-} mESCs from co-culture with wild-type mESCs was more pronounced in the presence of excess FGF, shown in Figure 4.6. When the ratio of wild-type and *Bmpr1a*^{-/-} mESCs in co-cultures grown with additional FGF4 was compared to heparin treated controls, no significant difference was observed (ratio wild-type/*Bmpr1a*^{-/-} mESCs 5ng/ml FGF4 co-culture 7.62±0.25 separate 1.67±0.40; 1ug/ml heparin only 8.01±1.25 separate 2.04±0.48; average±s.e.m n=3 p≤0.05).

Similar to FGF4- treated cultures, wild-type and *Bmpr1a*^{-/-} mESCs grown separately supplemented with 10ng/ml FGF5 also show similar growth rates. When cells of both genotypes are grown in co-culture, *Bmpr1a*^{-/-} mESCs growth arrest and are progressively lost from culture, similar to FGF4-treated and 1ug/ml heparin control cultures. After 4 days of culture, the ratio of wild-type and *Bmpr1a*^{-/-} mESCs in separate and co-cultures were quantified by FACs analysis (shown in Figure 4.6F). However, when these ratios were compared to 1ug/ml heparin control cultures no significant difference in these ratios was observed (ratio wild-type/*Bmpr1a*^{-/-} mESCs 10ng/ml FGF5 co-culture 10.82±2.63 separate 2.01±0.23; 1ug/ml heparin co-culture 7.62±0.81 separate 2.15±0.46 data shown as average±s.e.m n=3 p≤0.05). These data show that addition of excess FGF4 or FGF5 does not promote cell competition in cultures of wild-type and *Bmpr1a*^{-/-} mESCs. All FGF/ERK signalling modulation experiments are summarized in Table 3.

Reagent	Concentration	Effect on Cell competition at day 0	Effect on Cell competition at day 2
PD0325901 MEK 1/2 inhibitor	1 μ M	Rescue	Rescue
PD184352 MEK 1/2 inhibitor	2 μ M	Rescue	Rescue
PD98059 MEK 1/2 inhibitor	12.5 μ M	Rescue	No Rescue
U0126 MEK1/2 inhibitor	10 μ M	Rescue	No Rescue
PD173074 FGFR inhibitor	1000ng/ml	Rescue	Rescue
FGF4 FGFR1c/2c ligand	5ng/ml	No change	N/A
FGF5 FGFR1c/2c ligand	10ng/ml	No change	N/A

Table 3. Summary of Results of FGF/ERK modulation experiments on Cell Competition between wild-type and *Bmpr1a*^{-/-} mESCs.

The table above summarises the results seen when the FGF/ERK signalling pathway was perturbed using different small molecule signalling molecules to affect activation of the FGF/ERK signalling pathway.

4.2.6 Inhibition of Wnt signalling is sufficient to significantly promote cell competition dynamics between wild-type and *Bmpr1a*^{-/-} mESCs.

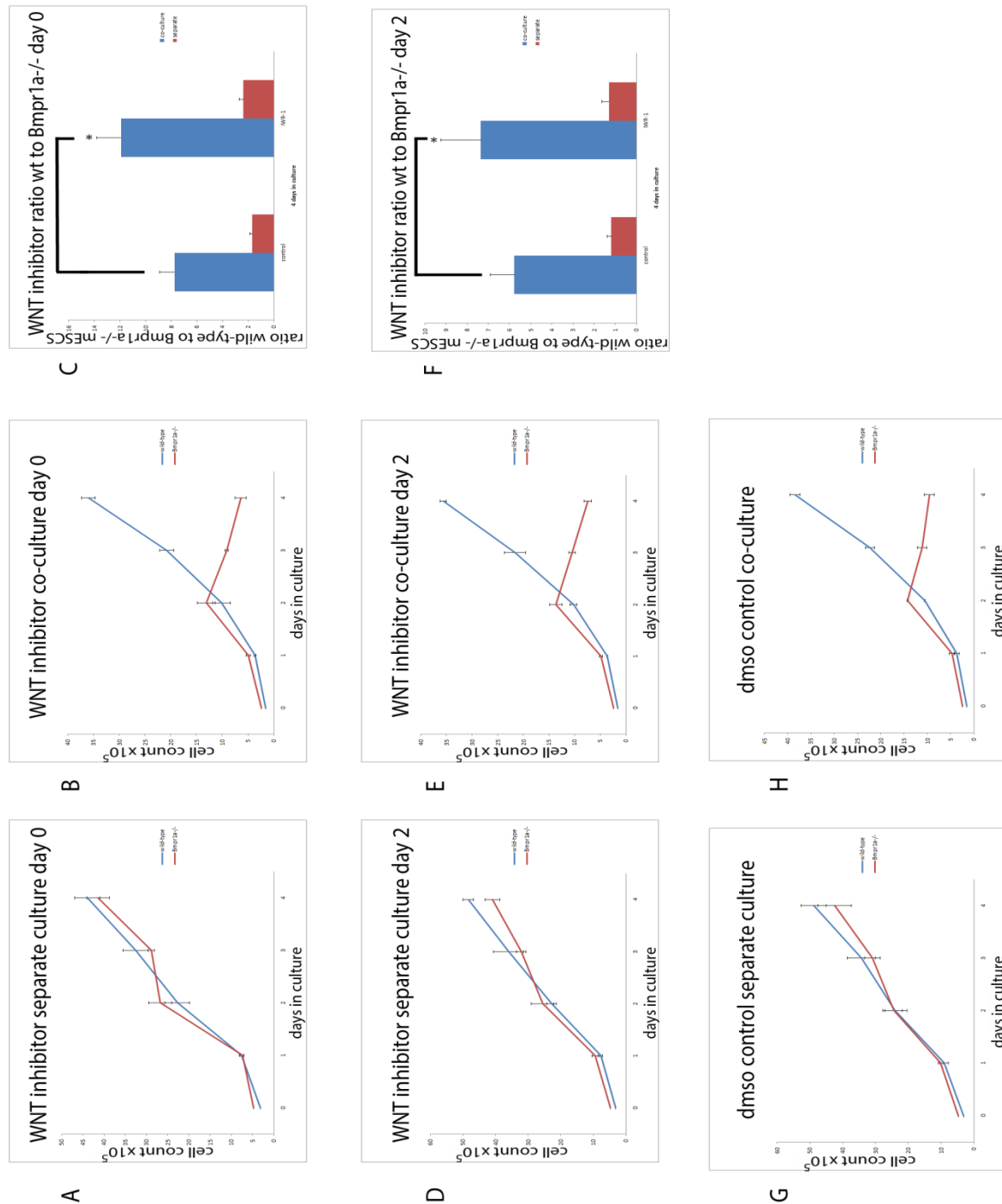
Wnt signalling is required throughout development in addition to Activin/Nodal; BMP and FGF signalling pathways. In mESCs, Wnt signalling is required to maintain mESC self-renewal and pluripotency, in part by alleviating Tcf3-mediated repression of core pluripotency transcription factors (Niwa, 2011; ten Berge et al., 2011). However in EpiSCs,

Wnt signalling is required for both differentiation and pluripotency. Nuclear transduction of β -catenin in EpiSCs promotes differentiation, whilst retention of high levels of β -catenin in the cytoplasm maintains pluripotency (Kim et al., 2013).

To first investigate whether perturbing Wnt signalling would affect cell competition dynamics, two recently developed Wnt signalling inhibitors IWR-1 and IWP2 were utilised (Chen et al., 2009). IWR-1 is a tankyrase inhibitor, which prevents the degradation of Axin1/2, members of the β -catenin proteasome. Treatment of cells with IWR-1 significantly reduces β -catenin mediated Wnt signalling (Huang et al., 2009). Separate and co-cultures of *Bmpr1a*^{-/-} mESCs were plated, (with a starting ratio of 40:60 *Bmpr1a*^{-/-} mESCs in co-culture) and 2 μ M IWR-1 was added daily. As Wnt signalling has been shown to affect mESCs and mEpiSCs differently the effect of perturbing Wnt signalling was investigated both in mESCs and after cells had begun to differentiate.

Figure 4.7 Inhibition of Wnt signalling using small molecule inhibitor IWR-1 rescues cell competition between wild-type and *Bmpr1a*^{-/-} mESCs

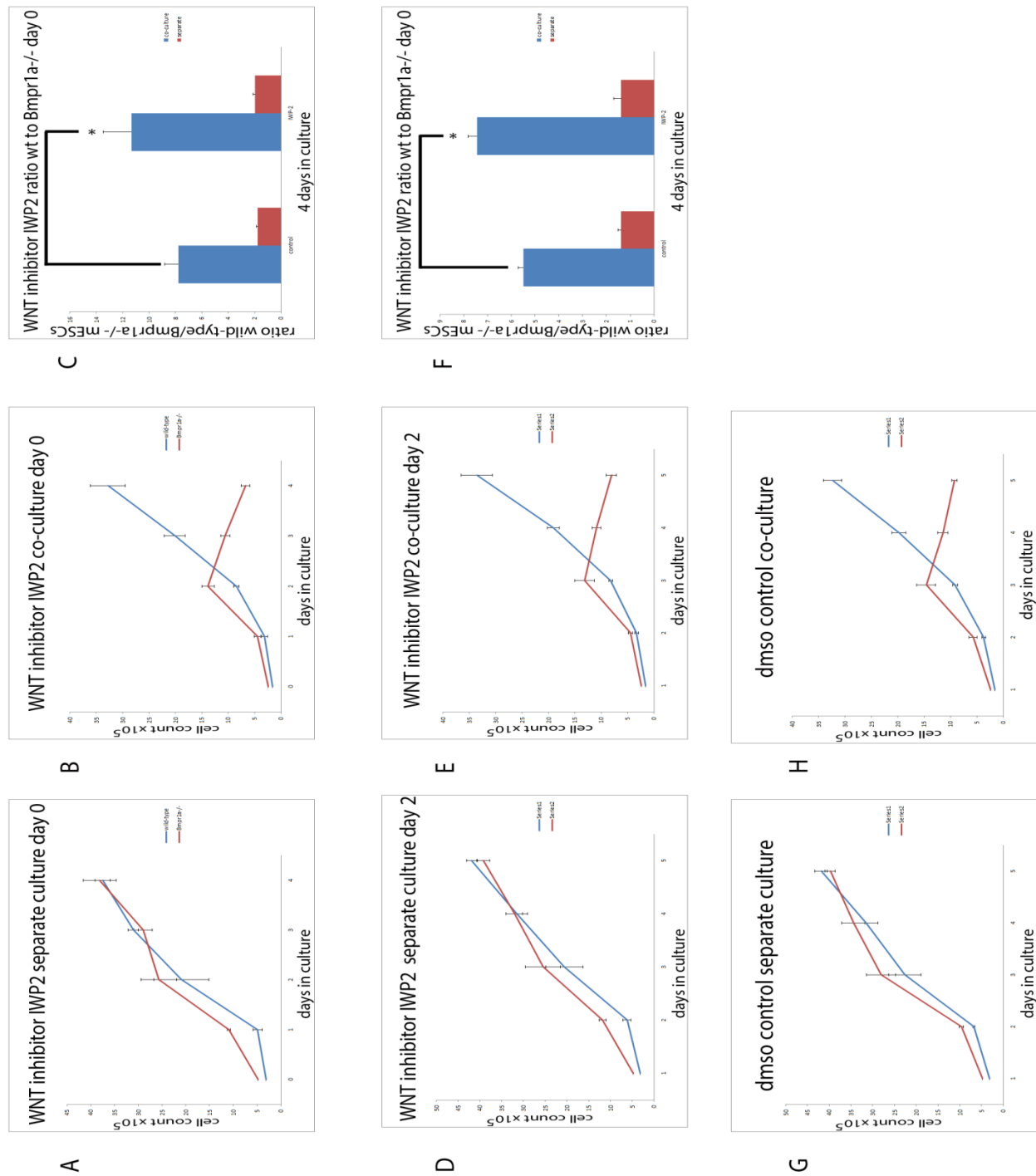
(A-C) Wild-type and *Bmpr1a*^{-/-} ESCs were plated as (A) separate and (B) co-cultures and treated daily with 2μM tankyrase inhibitor IWR-1. (C) The ratio of control/*Bmpr1a*^{-/-} ESCs analysed at day 4 of co-culture and compared with DMSO treated cultures (D-F) Wild-type and *Bmpr1a*^{-/-} ESCs were plated as (D) separate and (E) co-cultures and treated from day 2 of co-culture with 2μM IWR-1. (F) The ratio of control/*Bmpr1a*^{-/-} ESCs analysed at day 4 of co-culture and compared with DMSO treated cultures normalised to the ratio of wild-type/*Bmpr1a*^{-/-} ESCs at day 2 of culture (G-H) DMSO controls of separate (G) and co-cultures (H), (data shown as average±s.e.m.n=3 p ≤0.05).



Addition of 2 μ M IWR in separate cultures of wild-type and *Bmpr1a*^{-/-} mESCs did not affect cell growth; either by comparison of growth curves or analysis of growth rates (Appendix, Figure 1). However, in wild-type and *Bmpr1a*^{-/-} mESCs in co-cultures shows that after two days of culture, *Bmpr1a*^{-/-} mESCs are progressively lost from culture, reflected by a significant reduction in their growth rate compared to separate cultures. After 4 days of culture analysis of the ratio of wild-type/*Bmpr1a*^{-/-} mESCs in separate and co-culture compared to DMSO controls revealed is a significantly higher proportion of wild-type mESCs in co-cultures treated with 2 μ M IWR-1 compared to DMSO treated cultures, Figure 4.7 (ratio wild-type/*Bmpr1a*^{-/-} mESCs IWR-1 co-culture 11.91 $\pm$ 1.89 separate 2.37 $\pm$ 0.33 DMSO control co-culture 7.75 $\pm$ 1.13 separate 1.68 $\pm$ 0.20).

To investigate if inhibiting Wnt signalling promotes cell competition after cells have begun to differentiate separate and co-cultures of wild-type and *Bmpr1a*^{-/-} mESCs were cultured for 2 days in GMEM+Serum. After 2 days of culture 2 μ M IWR-1 was added daily to these cultures with DMSO treated cultures used as a vehicle control. Analysis of the growth curves and growth rates of wild-type and *Bmpr1a*^{-/-} mESCs shows that inhibition of Wnt signalling after cells have started to differentiate does not affect cell growth in separate culture conditions (growth rate wild-type treated 0.64 $\pm$ 0.10; DMSO 0.44 $\pm$ 0.04 p=0.91 *Bmpr1a*^{-/-} treated 0.35 $\pm$ 0.11; DMSO 0.39 $\pm$ 0.12 p=0.74 average $\pm$ s.e.m n=3 shown in Appendix Table 1). In contrast when the growth curves of wild-type and *Bmpr1a*^{-/-} mESCs in co-culture were analysed, it could be observed that *Bmpr1a*^{-/-} mESCs are also progressively lost from culture. Analysis of growth rates shows that *Bmpr1a*^{-/-} mESCs are not lost faster in co-cultures treated with IWR-1 compared to DMSO controls, Figure 4.7C (growth rate *Bmpr1a*^{-/-} co-culture treated -0.37 $\pm$ 0.08; DMSO -0.33 $\pm$ 0.15 average $\pm$ s.e.m n=3 p=0.74). However when the ratio of wild-type to *Bmpr1a*^{-/-} mESCs in treated co-cultures are compared to DMSO controls, there is a significant increase in the proportion of wild-type mESCs, Figure 4.7F (day 2 treated 7.35 $\pm$ 0.10 separate 1.29 $\pm$ 0.08 DMSO co-culture 5.77 $\pm$ 0.45 separate 1.18 $\pm$ 0.10; data shown as average $\pm$ s.e.m n=3 p $\leq$ 0.05). This suggests that *Bmpr1a*^{-/-} mESCs are being lost more quickly in IWR-1 treated cultures compared to DMSO controls, but that this is due to differences other than cell growth.

Figure 4.8 Inhibition of Wnt signalling using a second small molecule Wnt pathway inhibitor (IWP2) also inhibits cell competition between wild-type and *Bmpr1a*^{-/-} mESCs



To confirm these results a second Wnt signalling inhibitor, IWP-2 which targets *Porcupine* (*Porc*), was used. *Porc* encodes a protein which modifies Wnt ligands post-transcriptionally by adding a palmitoyl group to ligands (Kadowaki et al., 1996; Tanaka et al., 2000). This modification renders Wnt ligands soluble and thus able to travel through the extracellular space. In contrast to IWR-1 inhibition, treatment of cells with IWP-2 would also help to investigate if changes in Wnt signalling are due to β -catenin mediated signalling or non-canonical effects of Wnt binding to receptor ligands.

Wild-type and *Bmpr1a*^{-/-} mESCs were plated in separate and co-cultures at a starting ratio of 40:60 and 2 μ M IWP-2 was added from day 0, data shown in Figure 4.8. Comparison of the growth curves shows similar growth between wild-type and *Bmpr1a*^{-/-} mESCs when grown separately, reflected by growth rates (wild-type 0.66 $\pm$ 0.26; *Bmpr1a*^{-/-} 0.20 $\pm$ 0.17 average $\pm$ s.e.m n=3 p=0.08 shown in Appendix Table 1). In co-cultures wild-type mESCs *Bmpr1a*^{-/-} mESCs growth arrest and are progressively removed from culture, as shown by a significant reduction in the growth rate of *Bmpr1a*^{-/-} mESCs after 3 days in co-culture (growth rate between day 2 and day 3 separate 0.20 $\pm$ 0.17 co-culture -0.38 $\pm$ 0.17 average $\pm$ s.e.m n=3 p $\leq$ 0.05 shown in Appendix 1). After 4 days of culture supplemented with 2 μ M IWP-2 the ratio of wild-type to *Bmpr1a*^{-/-} mESCs in treated cultures were compared to DMSO controls. Data shown in Figure 4.8 shows that in co-cultures of wild-type and *Bmpr1a*^{-/-} mESCs there is a significantly higher proportion of wild-type mESCs when Wnt signalling is inhibited compared to controls (ratio of wild-type/*Bmpr1a*^{-/-} mESCs normalised back to the first day of treatment, day 0 2 μ M IWP-2 co-culture 11.34 $\pm$ 2.13 separate 1.95 $\pm$ 0.17 DMSO control co-culture 7.78 $\pm$ 1.03 separate 1.76 $\pm$ 0.11).

A second experiment was performed to investigate if IWP-2 inhibition of Wnt signalling was also sufficient to affect cell competition dynamics after the onset of differentiation. Separate and co-cultures of wild-type and *Bmpr1a*^{-/-} mESCs were plated and allowed to differentiate in GMEM+Serum for 2 days. After 2 days culture medium was supplemented with 2 μ M IWP-2 or an equal volume of DMSO. Analysis of the growth curves of cells grown in separate culture show no significant differences (growth rate between day 2 and day 3 wild-type 0.66 $\pm$ 0.14; *Bmpr1a*^{-/-} 0.36 $\pm$ 0.11 average $\pm$ s.e.m n=3 p=0.24, shown in Appendix Table 1). However, as with IWR-1 treated and DMSO control co-cultures, after 2 days of co-culture

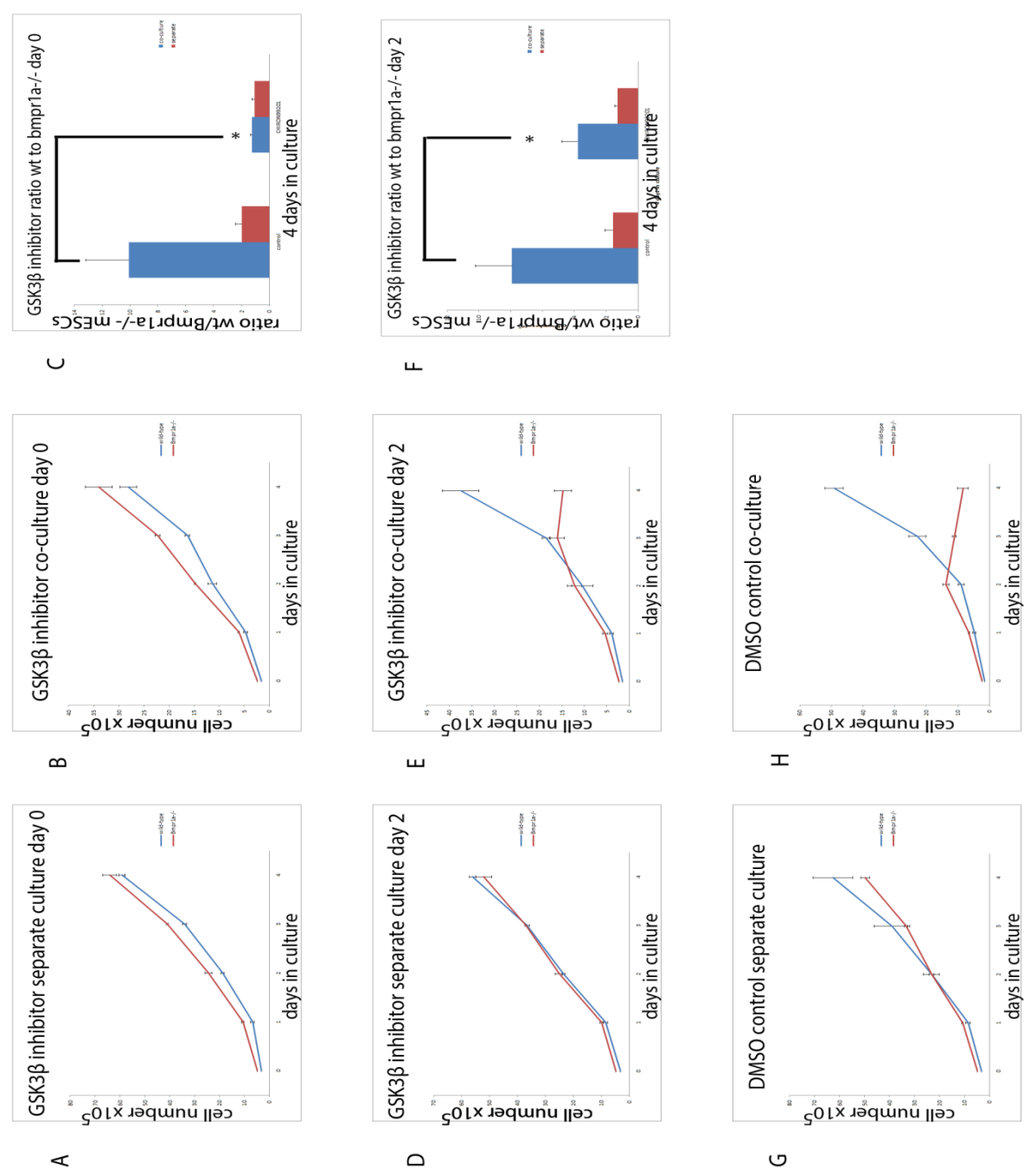
inhibition of Wnt signalling still resulted in growth arrest and removal from culture of *Bmpr1a*^{-/-} mESCs. Analysis of growth rates demonstrates that this removal of *Bmpr1a*^{-/-} mESCs compared to DMSO co-cultures is not due to changes in cell growth (growth ratio between day 2 and day 3 of co-culture 2 μ M IWP-2 -0.25 $\pm$ 0.24 DMSO -0.20 $\pm$ 0.22 average $\pm$ s.e.m n=3 p=0.67). To compare if the change in proportion of *Bmpr1a*^{-/-} mESCs in culture was significantly different to DMSO controls, the ratio of wild-type to *Bmpr1a*^{-/-} mESCs in separate and co-cultures was quantified by FACs analysis. There is a significant reduction in the ratio of wild-type/*Bmpr1a*^{-/-} mESCs in IWP-2 treated co-cultures compared to DMSO controls, once normalised to the ratio of wild-type/*Bmpr1a*^{-/-} mESCs after 2 days of culture data shown in Figure 4.8 (ratio wild-type/*Bmpr1a*^{-/-} mESCs IWP-2 day 2 treated co-culture 7.45 $\pm$ 0.36 separate 1.38 $\pm$ 0.31 DMSO control 5.50 $\pm$ 0.21 separate 1.38 $\pm$ 0.13 average $\pm$ s.e.m n=3 p $\leq$ 0.05).

These data show that suppression of *Wnt* mediated signalling promotes cell competition dynamics and therefore has the opposite effect to that observed with suppressing FGF/ERK signalling. Furthermore these data show that these changes are likely to be affected by canonical β -catenin mediated changes in Wnt signal transduction.

4.2.7 Promoting Wnt signalling is sufficient to rescue *Bmpr1a*^{-/-} mESCs upon competition with wild-type mESCs.

The converse experiment, investigating the effect of promoting Wnt signalling was carried out using the GSK3 β inhibitor CHIRON99201. Like Axin1/2 GSK3 β is also a member of the β -catenin proteasome. In addition with other proteins GSK3 β binds to β -catenin and phosphorylates it. Phosphorylation of β -catenin targets it to the lysosome and results in its degradation. Inhibition of GSK3 β results in disassociation of the β -catenin phosphorylation complex. In response to Wnt signals, GSK3 β is inhibited and β -catenin accumulates in the cytoplasm and translocates to the nucleus to target transcription.

Figure 4.9 Promoting Wnt signalling through inhibition of GSK3 β , rescues cell competition between wild-type and *Bmpr1a*^{-/-} mESCs
(A-C) Wild-type and *Bmpr1a*^{-/-} ESCs were plated as **(A)** separate and **(B)** co-cultures and treated daily with 3uM GSK3 β inhibitor CHIRON99201. **(C)** The ratio of control/*Bmpr1a*^{-/-} ESCs analysed at day 4 of co-culture and compared with DMSO treated cultures. **(D-F)** Wild-type and *Bmpr1a*^{-/-} ESCs were plated as **(D)** separate and **(E)** co-cultures and treated from day 2 of co-culture with 3uM CHIRON99201. **(F)** The ratio of control/*Bmpr1a*^{-/-} ESCs analysed at day 4 of co-culture and compared with DMSO treated cultures normalised to the ratio of wild-type/*Bmpr1a*^{-/-} ESCs at day 2 of culture **(G-H)** DMSO controls of separate **(G)** and co-cultures **(H)**. (data shown as average $\pm$ s.e.m n=3 p $\leq$ 0.05).



Separate and co-cultures of wild-type and *Bmpr1a*^{-/-} mESCs were plated and 3μM CHIRON99201, a GSK3β inhibitor, was added from the day 0, data shown in Figure 4.9. In contrast to DMSO controls where *Bmpr1a*^{-/-} mESCs are lost in co-culture, wild-type and *Bmpr1a*^{-/-} mESCs s cultured separately or together in the presence of CHIRON displayed similar growth curves. Despite this wild-type mESCs show significantly higher growth rates in co-culture compared to separate cultures between days 2 and 3 of culture co-culture 0.86±0.01 separate 0.53±0.05 average±s.e.m n=3 p≤0.05). This increase in growth rate is not accompanied by a significant reduction in growth rate of *Bmpr1a*^{-/-} mESCs (growth rates co-culture 0.64±0.04 separate 0.60±0.10 average±s.e.m n=3 p=0.85). To compare if this rescue of *Bmpr1a*^{-/-} mESCs is significant the ratio of wild-type and *Bmpr1a*^{-/-} mESCs in separate and co-culture were analysed after 4 days by FACs, Figure 4.9C. These data was normalised to the starting ratio of wild-type:*Bmpr1a*^{-/-} mESCs (40:60) and the ratio in co-culture was found to be significantly lower compared to DMSO controls, data (ratio of wild- type/*Bmpr1a*^{-/-} mESCs 3μM CHIRON99201 day 0 co-culture 1.24±0.11 separate 1.07±0.17 DMSO control co-culture 10.08±3.08 separate 1.97±0.47).

Wnt signalling is involved in maintaining mESC self-renewal and pluripotency and therefore the question if promoting Wnt signalling sufficient to rescue cell competition after the onset of differentiation was next asked. To investigate this separate and co-cultures of wild-type and *Bmpr1a*^{-/-} mESCs were plated and left to differentiate in GMEM+Serum for 2 days. After 2 days 3μM CHIRON99201 was added daily to cultures with fresh media. Growth curves of separate cultures show no changes compared to DMSO controls, shown in Figure 4.9 and Appendix 1 (growth rate at day 3 wild-type CHIRON99201: 0.67±0.14; DMSO 0.71±0.09 p=0.58; *Bmpr1a*^{-/-} CHIRON99201 0.55±0.07; DMSO 0.47±0.13 p=24). Similarly, the growth curve of wild-type mESCs in co-culture with *Bmpr1a*^{-/-} mESCs show no differences compared to DMSO controls or separate cultures (growth rate wild-type co-culture treatment 0.88±0.23 compared to DMSO 1.24±0.11 p=0.13). However, when compared to the growth rate of wild-type mESCs in separate cultures treated with CHIRON99201, cells in co-culture grow significantly faster (separate wild-type 0.66±0.14 average±s.e.m n=3 p≤0.05). In contrast to this, while in DMSO controls *Bmpr1a*^{-/-} mESCs growth arrest after two days of co-culture with wild-type mESCs and are progressively lost from culture after this, in co-cultures supplemented with 3μM CHIRON99201 this growth

arrest only commences at day 3. After 4 days of culture FACs analysis was used to quantify the ratio of wild-type to *Bmpr1a*^{-/-} mESCs in separate and co-cultures. These data were normalised to the ratio of wild-type/*Bmpr1a*^{-/-} mESCs when the inhibitors were added (day2). In co-cultures of wild-type and *Bmpr1a*^{-/-} mESCs supplemented for 2 days with 3μM CHIRON99201, there is a significantly higher proportion of wild-type cells in co-culture compared to separate cultures. However, when this difference was compared to DMSO control co-cultures, it was significantly lower, suggesting a partial rescue of *Bmpr1a*^{-/-} mESCs occurred (Figure 4.9F; ratio wild-type/*Bmpr1a*^{-/-} mESCs day 2 treated co-culture 3.79±0.99 separate 1.30±0.25 DMSO control co-culture 7.92±2.28 separate 1.59±0.17 (data shown as average±s.e.m n=3 p<0.05). Promoting Wnt signalling after differentiation is sufficient to rescue *Bmpr1a*^{-/-} mESCs from cell competition with wild-type mESCs but this rescue is partial.

4.3 Discussion

What roles do cell-cell contact and signalling factors have in cell competition? Senoo-Matsuda and Johnston first demonstrated the role of secreted factors in cell competition in cultures of *Drosophila* epithelial cells. Using transwell culture experiments, they showed that wild-type cells grown with dMyc overexpressing cells were eliminated by apoptosis even if these populations were not in contact with each other (Senoo-Matsuda and Johnston, 2007).

A second series of experiments performed by Rhiner and co-workers seem to contradict this finding (Rhiner et al., 2010). Using microarray analysis of competing *dMyc* over-expressing cells against a wild-type background, Rhiner and colleagues identified a number of genes differentially expressed in separate and competing populations. One of these genes encoded the cell-surface receptor *Flower* (*Fwe*). *Fwe* is a transmembrane synaptic vesicle Ca²⁺ channel involved in endocytosis (Yao et al., 2009). Analysis of *Fwe* isoform expressions showed that in the presence of winner cells, loser cells changed expression of full length *Fwe* receptor to a truncated form. In large clones of *Fwe*^{loseA/loseB} cells, apoptosis was significantly higher at the clone border with wild-type cells. Similarly, *Fwe*^{loseA/loseB}-GFP *Drosophila* epithelial cells survived in co-cultures with wild-type cells when plated at low confluencies where no cell contact occurred (Rhiner et al., 2010)

In this thesis the requirement for cell-cell contact during cell competition was investigated. Using transwell co-cultures of mESCs with different genotypes (wild-type vs. *Bmpr1a*^{-/-}/Atg5^{-/-} or 4n) it was observed that cell competition occurred without cell-cell contact. One possible explanation why cell competition has been shown to have different requirements for cell contact in different contexts is that both cell contact and secreted factors may be involved in cell competition. For example it is possible that in the absence of cell-cell contact secreted factors alone may mediate cell competition, but when cells are grown in contact with each other there may be an additive effect of long and short range signals.

Interestingly, analysis of growth rates of wild-type mESCs grown in transwell co-culture compared to physical co-culture was changed. As shown in chapter 3 the presence of *Bmpr1a*^{-/-} mESCs significantly increases the growth rate of wild-type mESCs in co-culture, where cells can communicate via cell-cell boundaries. Similar observations have also been made in *Drosophila* models of cell competition (de la Cova et al., 2004). However, in transwells with overlying *Bmpr1a*^{-/-} mESCs, the growth rate of wild-type cells are lower compared to wild-type cells with cells of the same genotype overlying them. It could be that cell-cell contact with loser cells drives the proliferation of winner cells during cell competition, whilst secreted factors trigger their elimination.

Though there are no significant changes in apoptosis using transwell co-culture, this does not preclude that *Bmpr1a*^{-/-} mESCs are being eliminated from co-culture with wild-type mESCs by other mechanisms (Degterev and Yuan, 2008; Vincent et al., 2013). An alternative possibility is that analysis of AnnexinV levels does not accurately reflect differences in apoptosis. The analysis done in this chapter was only carried out on the cells remaining attached to the base of the tissue culture well. As was observed in Figure 3.1, many mESCs detach from culture prior to analysis. Thus, this data may not truly reflect the actual percentages of apoptotic and dying cells in competing cell cultures, as a number of cells may have already been lost by extrusion, or engulfed by their neighbours. Inhibition of caspase activation was sufficient to rescue *Bmpr1a*^{-/-} mESCs from cell competition upon co-culture with wild-type cells (chapter 3); it may be interesting to see whether this rescue occurs in transwell co-culture as well.

Given that secreted factors were found to mediate cell competition in mESCs, this chapter also investigated which signalling pathways affect cell competition dynamics between wild-type and *Bmpr1a*^{-/-} mESCs. In the literature, Nodal signalling has been shown to help maintain mESCs in a pluripotent state by mediating BMP signalling (Galvin et al., 2010). Activin/Nodal signalling is also required for EpiSC pluripotency (Brons et al., 2007; Tesar et al., 2007). Interestingly, inhibition of Activin/Nodal signalling in co-cultures was sufficient to prevent cell competition while cells remain in the undifferentiated naïve ES state, but not once cells had been allowed to begin differentiating.

LIF/STAT mediated signalling is also a known requirement for mESC pluripotency in conventional ES media (GMEM supplemented with LIF and Serum). LIF acts by activating STAT3 signalling, a known target of which is *c-Myc*. Culture of cells in ES media completely rescues *Bmpr1a*^{-/-} mESCs from competition with wild-type cells. However, LIF is unable to rescue loser cells from competition once cells have begun differentiating. This could be due to down-regulation of the LIF/gp130 receptor rendering cells less susceptible to signalling (Sancho et al., 2013).

The role of the FGF/ERK signalling pathway in cell competition in wild-type and *Bmpr1a*^{-/-} mESCs was also investigated. This chapter shows that inhibiting ERK signalling at different levels had different effects. Complete suppression of ERK rescued cell competition after cells had started differentiating, but partial suppression did not. Suppression of FGF/ERK signalling has been shown to promote mESC self-renewal and pluripotency as part of the 2i culture system (Ying et al., 2008). In this system suppressing pERK also suppresses Myc (Ying et al., 2008). Further evidence that ERK signalling acts upstream of Myc stabilisation was presented by Sears and colleagues (2002). In their work they describe a mechanism where ERK acts as a positive modulator of Myc stability via phosphorylation of the S62 residue (Sears et al., 2002).

Therefore changes in Myc stability may explain why FGF/ERK inhibition could rescue cells from competition. This would also provide an explanation for the cell competition rescue in PD173074 treated cultures where ERK phosphorylation was suppressed only in wild-type

cells. Inhibition of pERK in wild-type cells would also suppress cMyc upregulation in those cells, thereby preventing a difference between cMyc expression between wild-type and mutant cells (Ying et al., 2008; Sancho et al., 2013). Without the transient upregulation of cMyc in winner cells, cell competition does not occur (Sancho et al., 2013).

However, experiments where additional FGF4 or FGF5 were added to cultures did not affect cell competition. This could be due to the FGF concentrations used for these experiments being insufficient to significantly upregulate ERK signalling in wild-type cells.

Alternatively, given that FGF signalling pathway is comprised of 22 known ligands (Villegas et al., 2010). It could be that different ligands or a combination of ligands are required to promote cell competition.

Finally the role of Wnt signalling in cell competition was also investigated. Both inhibition and stimulation of Wnt signalling were able to change cell competition dynamics. When Wnt signalling is inhibited, cell competition dynamics are more pronounced. In cultures where GSK3 β is repressed, cell competition is also inhibited. In mESCs and mEpiSCs, Wnt signalling has been shown to mediate self-renewal and pluripotency via localisation of β -catenin (reviewed by Niwa et al., 2011; Kim et al., 2013). Wnt signalling has been shown to act by inhibiting GSK3 β activity (Metcalf and Bienz, 2011; Wu and Pan, 2010). GSK3 β phosphorylates the T58 residue on cMyc, a modification that targets it for degradation. Therefore, like the ERK signalling pathway, modulation of the Wnt signalling pathway, and therefore GSK3 β activity could affect Myc stability. cMyc could either be more unstable in loser cells or, via repression of GSK3 β activity, more stable in winner cells.

Both FGF and Wnt signalling may converge on *c-Myc* to drive cell competition. *Myc* signalling has been reported to trigger cell competition in *Drosophila* (de la Cova et al., 2004; Moreno and Basler, 2004). In co-cultures of wild-type mESCs with cells of mutant genotype (*Bmpr1a*^{-/-}; *Atg5*^{-/-} and 4n), cMyc is transiently upregulated in wild-type cells during competition (day 3-4 of co-culture) (Sancho et al., 2013). Recently another group has also shown that relative differences in Myc expression can drive cell competition *in vivo* during early mouse development (Claveria et al., 2013).

One question that arises from these experiments is why are mESCs resistant to competition? One explanation is that this pluripotent state also has high levels of *cMyc*, rendering cells less susceptible to relative changes in expression. In addition questions still remain as to the precise role of the ERK and Wnt signalling pathways in cell competition. As both the FGF/ERK and WNT signalling pathways also affect differentiation of mESCs it could be that affecting the process of differentiation could affect cell competition. As changes in *c-Myc* expression is the common trigger of cell competition, it is likely that these factors help regulate the relative expression of *c-Myc* between winner and loser cells (Sancho et al., 2013). FGF signalling is known to maintain mESC pluripotency, and thus, maintaining the relatively high *c-Myc* expression of that state (Kunath et al., 2007). This could therefore make winner cells less able to sense the relative differences in *Myc* expression that would indicate a loser cell and trigger competition. The converse would apply to competitions where WNT signalling was modified as WNTs are required to drive mESC differentiation

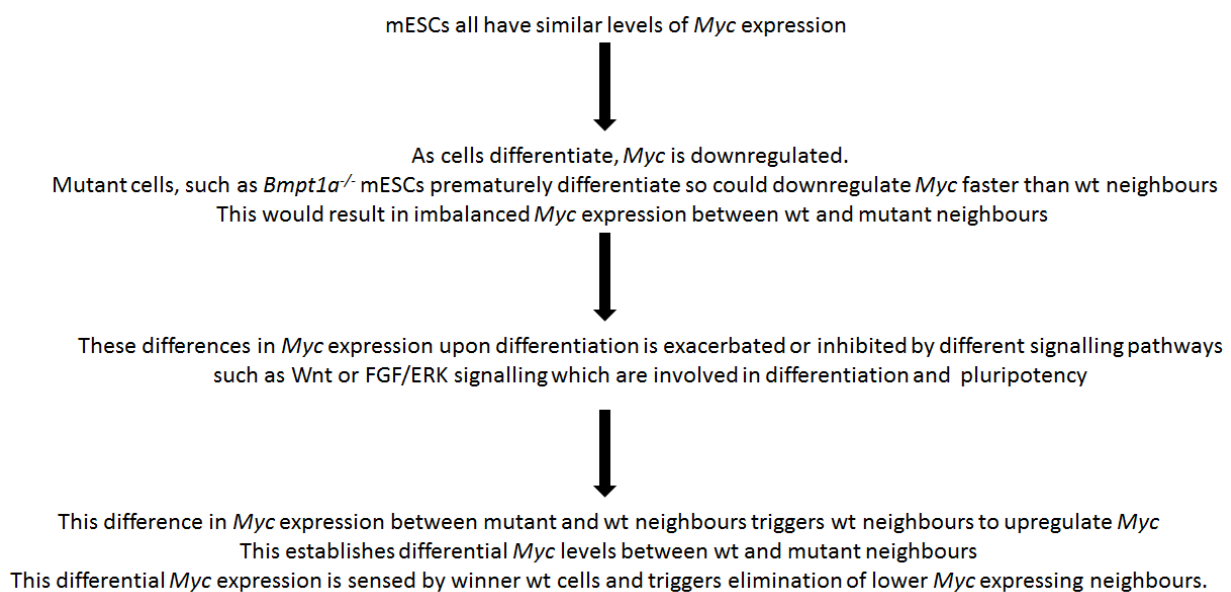


Figure 4.10 Model for how differentiation and signalling factors may affect cell competition

mESCs have homogenously high levels of *c-Myc* expression. As cells begin to differentiate this level of high *c-Myc* expression is lost. This rate may be dependent upon differentiation, which is affected by signalling factors such as members of the FGF or WNT pathways. This results in the first step during cell competition where mutual sensing of *c-Myc* expression allows cells to identify neighbours cells with lower *c-Myc* indicating them as “unfit” or mispatterned. In response to mutual sensing this establishes differential *c-Myc* expression between “fit” and “unfit” cells. Cells continue to mutually sense the *c-Myc* expression levels of neighbouring cells leading to activation of apoptosis and elimination of “unfit” cells with relatively lower *c-Myc* expression (Sancho et al., 2013).

(Altasi et al., 2013). However this explanation still leaves some questions as it is unknown if there are differences in signal transduction between winner and loser cells and whether this would directly result in upregulation of *c-Myc* in winner cells? It would also be interesting to investigate how changes in *c-Myc* regulation induce apoptosis in loser cells and survival of winner cells. Lastly it would be of interest to investigate if other pathways independent of *c-Myc* induction affect cell competition. This hypothesis is summarised in the chart below, Figure 4.10.

To begin to answer these questions, first it needs to be proved that modulation of the ERK or Wnt signalling pathways affect *c-Myc* upregulation in winner cells. Once this is established, the next line of questioning would be how is this change accomplished? Does ERK and Wnt signalling act as described in the literature by phosphorylating specific residues on *c-Myc* that affect its stability (Cartwright et al., 2005; Sears et al., 2002)? To investigate this analysis of signalling activity in each cell type could be investigated at the onset of differentiation.

An additional question would be whether differences in Wnt and FGF signalling in winner or loser cells alone are enough to prevent cell competition? This would require the generation of mutant cell lines also deficient in FGF or Wnt signal transduction or investigating whether cells with impaired FGF/Wnt signalling could outcompete other mutant genotypes.

Chapter 5 – Investigating the Role of Autophagy during Cell Competition

5.1 Introduction

Autophagy is a process by which cytoplasmic components, including organelles are degraded by the lysosome. Macroautophagy (hereafter referred to as “autophagy”) is of particular interest as it is through this method that cytosolic contents are delivered to the lysosome by specialised compartments called autophagosomes. Phagocytosis of neighbouring cell debris (also known as “engulfment”) once internalized, is degraded by the autophagy pathway.

Autophagy is regulated by the serine/threonine kinase mammalian target of rapamycin (mTOR) (Lum et al., 2005). *ATG5* and *Beclin1* are also components of the autophagy pathway in mammalian systems. *ATG5* forms a conjugate complex with *ATG12*, which is required for formation of the autophagosome isolation membrane where cytosolic components are degraded (Mizushima et al., 2001). *Beclin1* acts in a variety of roles, some autophagy-independent and regulates autophagy through interaction with class I PI3K kinases. The class I PI3K kinase complex made up partly of ATG14 and Beclin-1 mediate localisation of autophagy components to the preautophagosome site (PAS) (Cao and Klionsky, 2007).

Autophagy has physiological and morphological roles in the cell. It acts as an adaptive response to starvation or stress to breakdown excess proteins back into their amino acid components and provide energy when needed. Due to this autophagy is closely coupled to the nutrient-sensing metabolism pathway. In addition, autophagy acts as a quality control mechanism for intracellular proteins and organelles, where long-lived or excess cytosolic components are trafficked into the autophagosome pathway and degraded. This allows the cell to ‘refresh’ its cytosolic contents and to remove damaged or misfolded organelles and proteins which would otherwise remain.

Autophagy is active throughout embryonic development, in particular its role in clearing apoptotic cellular debris. In *Atg5* and *Beclin1* null mutant EBs cavitation does not occur due

to the persistence of apoptotic corpses in the fluid-filled centre of the EB, suggesting the autophagy pathway may be involved in clearance of cellular debris (Coucovanis and Martin, 1999; Mizushima and Levine, 2010; Qu et al., 2007; Tsukamoto et al., 2008). Considering this role in apoptotic corpse clearing and the controversial hypothesis that during cell competition winner cells “eat” their way through competing populations, the role of autophagy and its regulatory pathways were investigated in this chapter (Li and Baker, 2007; Lolo et al., 2012).

5.2 Results

5.2.1 Cell debris in competing cultures is internalised by neighbouring wild-type mESCs

Observation of competing wild-type and *Bmpr1a*^{-/-} co-cultures show large amounts of GFP⁺ cellular debris (see Figure 3.3). Previous work in *Drosophila* illustrated that cell competition may progress through competing populations by wild-type neighbours “eating” their way through surrounding “loser” cells (Li and Baker, 2007). To investigate whether a similar process was occurring during competition between mESCs, monotypic and co-cultures of

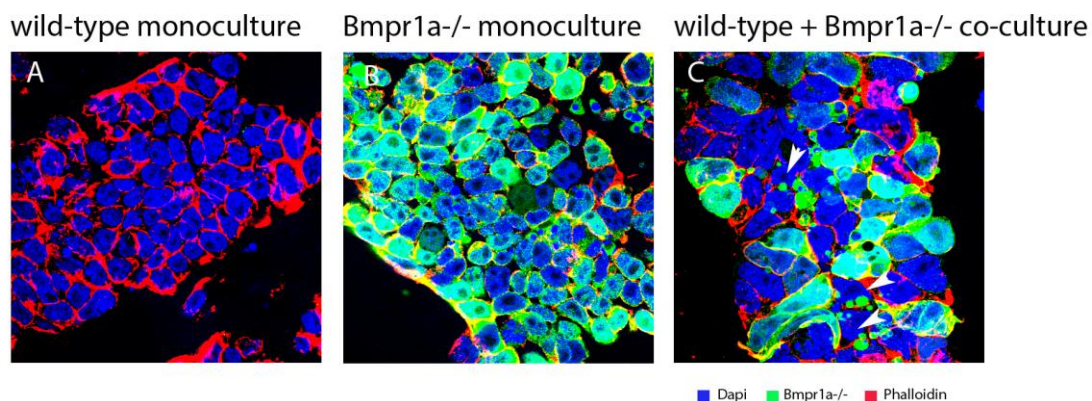


Figure 5.1 Internalised cell debris is present in wild-type cells of competing cultures, but absent in monotypic cultures of wild-type and *Bmpr1a*^{-/-} ESCs in the same conditions.

(A) Monoculture of wild-type, (B) *Bmpr1a*^{-/-} and (C) co-cultures of both cell lines were grown for 3 days in GMEM+Serum, 1 micron sections of colonies were examined by confocal microscopy. Cell nuclei were stained with DAPI, *Bmpr1a*^{-/-} ESCs were labelled with GFP and cell actin stained with Phalloidin-TRITC. White arrows highlight *Bmpr1a*^{-/-}-GFP cellular debris.

wild-type and *Bmpr1a*^{-/-} mESCs were cultured for 3 days, fixed and stained. DAPI was used to stain for cell nuclei, and Phalloidin-TRITC used to visualise cell actin and therefore cell boundaries. To distinguish between each cell type *Bmpr1a*^{-/-}-GFP mESCs were identified by staining for GFP. Confocal microscopy was used to examine 1micron sections through colonies. Monotypic cultures of wild-type (A) and *Bmpr1a*^{-/-} (B) mESCs showed very little cellular debris. However in Figure 5.1C when sections of co-cultures of wild-type and *Bmpr1a*^{-/-} mESCs were examined we identified GFP⁺ debris from *Bmpr1a*^{-/-} mESCs within and at the cell membrane boundaries of neighbouring wild-type mESCs (white arrows).

This experiment demonstrates that wild-type neighbours in competing cultures may internalise part of the cellular debris produced by post-apoptotic *Bmpr1a*^{-/-} mESCs. However, there are a variety of caveats this experiment does not address such as the mechanisms used to internalise debris from neighbouring cells, how this debris is degraded and whether this is an active or passive process during cell competition. Further experiments are required to co-localize debris with markers for cellular compartments, and to investigate how mESCs internalize neighbouring cellular debris.

5.2.2 Inhibition of mTOR significantly increases cell competition

The breakdown of large intracellular organelles or internalized cellular debris usually occurs via the autophagy pathway (Florey and Overholtzer, 2012). One of the major regulators of autophagy is the serine/threonine kinase mTOR, the central component of mTORC1, a complex that regulates autophagy (Yang and Klionsky, 2010). Rapamycin inhibits autophagy by causing disassociation of the mTORC1 complex and thus relieving inhibition of autophagy (Ballou and Lin, 2008; Oshiro et al., 2004; Pattingre et al., 2008). To investigate whether the mTOR signalling pathway is involved in cell competition 10ng/ml rapamycin was added to cultures and replaced with fresh media daily. The growth curves of wild-type and *Bmpr1a*^{-/-} mESCs in separate and co-cultures were counted and plotted daily, (Figure 5.2A). In separate cultures, wild-type and *Bmpr1a*^{-/-} mESCs have similar growth curves.

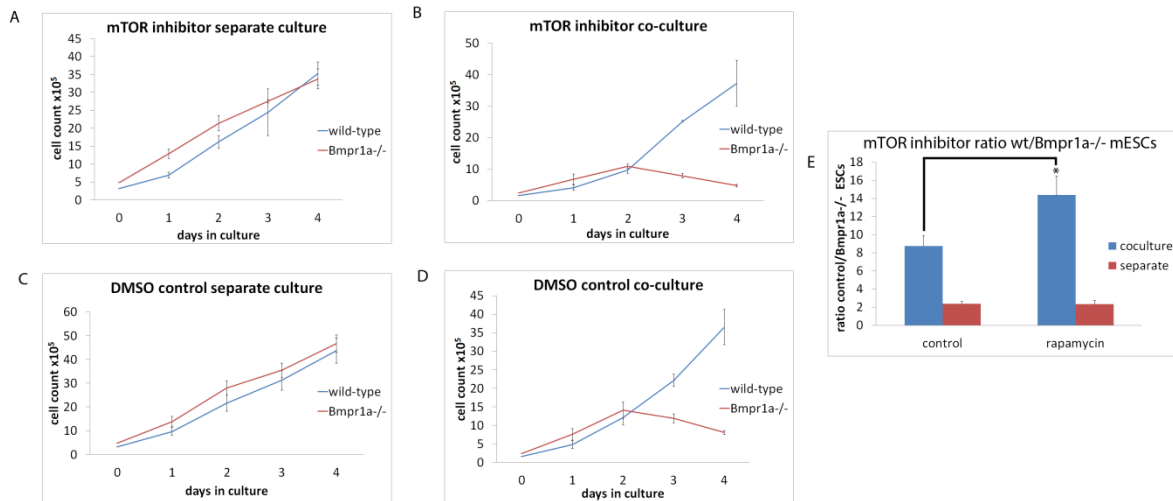


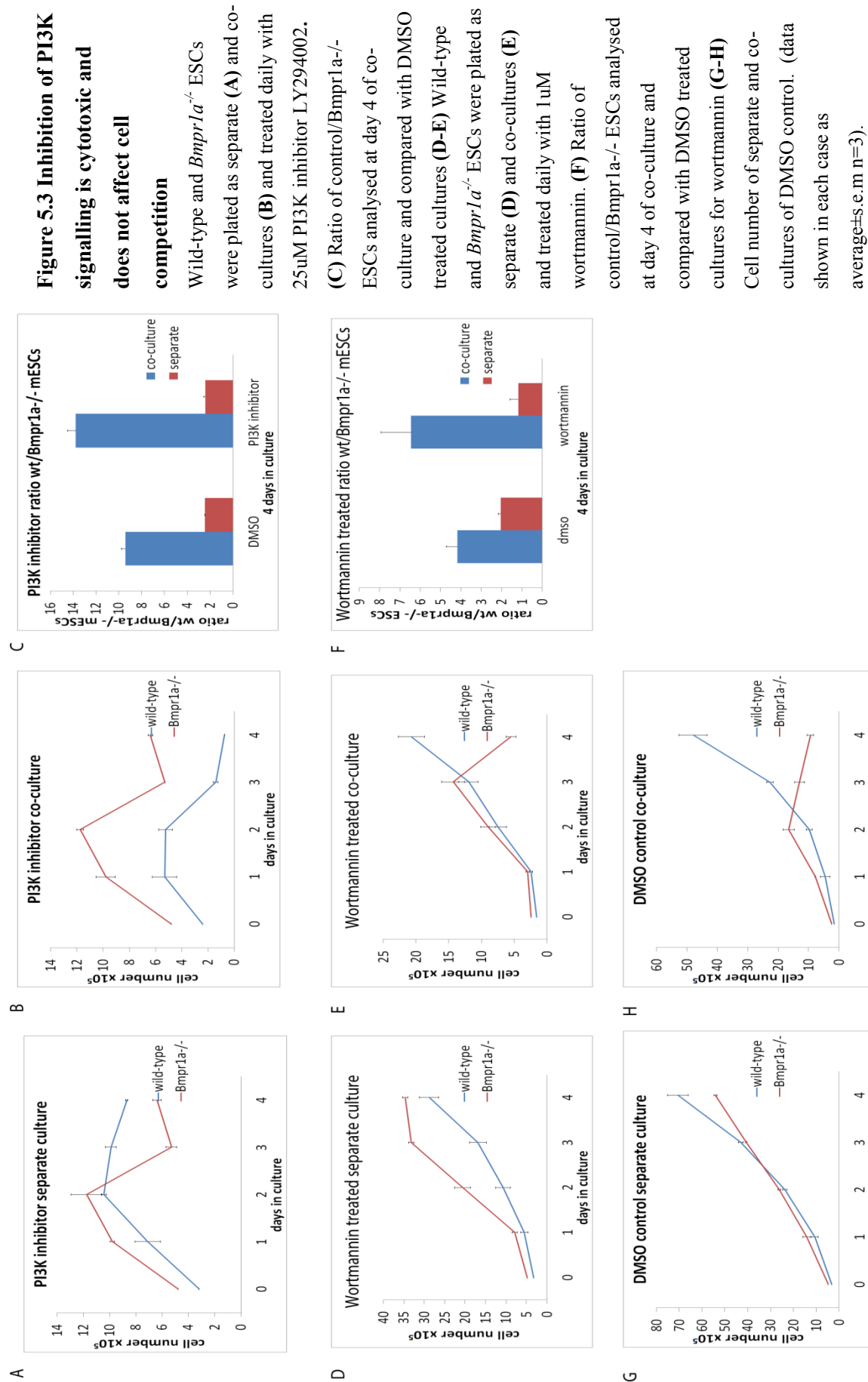
Figure 5.2 Inhibition of mTOR signalling significantly increases cell competition between wild-type and *Bmpr1a*^{-/-} mESCs

Growth curves of separate (A) and co-culture (B) wild-type and *Bmpr1a*^{-/-} ESCs grown with the addition of 10ng/ml rapamycin to culture media compared to DMSO control separate (C) and co-cultures (D). (E) Ratio of wild-type to *Bmpr1a*^{-/-} ESCs cultured after 4 days in the presence of rapamycin or DMSO and normalised to the starting ratio of cells. In the presence of rapamycin, there is a significantly higher ratio of wild-type to *Bmpr1a*^{-/-} ESCs (average±s.e.m n=3 p<0.01).

In co-cultures with wild-type cells, *Bmpr1a*^{-/-} mESCs growth arrest after 2 days and are rapidly eliminated from co-cultures (Figure 5.2B). In addition, comparison of the growth curves of rapamycin treated cultures compared to DMSO controls indicated a greater elimination of mutant cells. After 4 days of culture the ratio of wild-type to *Bmpr1a*^{-/-} mESCs in co-cultures was analysed by FACs. In rapamycin-treated cultures the proportion of wild-type cells in co-culture was significantly higher than in DMSO controls shown in Figure 5.2E (rapamycin treated co-culture 14.36±2.09 separate 2.33±0.44 DMSO co-culture 8.74±1.15 separate 2.37±0.26 n=3 average±s.e.m p<0.01). This suggests that the mTOR signalling pathway inhibition is sufficient to enhance cell competition, although whether it does this by inducing increased levels of autophagy or some other mechanism involved in nutrient or energy sensing is unclear.

5.2.3 Inhibition of PI3K signalling to inhibit autophagy does not affect cell competition between wild-type and *Bmpr1a*^{-/-} mESCs

Given the previous results, it was hypothesised that inhibition of autophagy, could prevent cell competition (Li and Baker, 2007). PI3K signalling has been shown to regulate autophagy complexes (Blommaert et al., 1997; Yang and Klionsky, 2010). To investigate whether inhibiting PI3K affects cell competition, 25 μ M of PI3K inhibitor LY294002 was added to cultures daily (Storm et al., 2007). Growth curves of separate and co-cultures treated with 25 μ M LY294002 show that LY294002 is may be highly cytotoxic to mESCs as large numbers of cells are lost from cultures compared to untreated DMSO control growth curves (Figure 5.3 A-B, G-H). Despite high numbers of cells being lost from culture, the ratio of wild-type to *Bmpr1a*^{-/-} mESCs was analysed at day 4 of competition, but no rescue of *Bmpr1a*^{-/-} mESCs was observed (Figure 5.3C), (ratio wild-type/*Bmpr1a*^{-/-} mESCs after 4 days of culture LY294002 treated co-culture 13.29 $\pm$ 0.71 separate 2.43 $\pm$ 0.06; DMSO control 9.44 $\pm$ 0.34 separate 2.46 $\pm$ 0.15 average $\pm$ s.e.m n=3).



Due to the high levels of cell death observed in response to LY294002 addition to culture media, a second PI3K inhibitor, wortmannin was used (Powis et al., 1994). This was to determine if the lack of rescue *Bmpr1a*^{-/-} mESCs was dependent on PI3K signalling inhibition or other non-specific effects of LY294002. Cell numbers in separate and co-cultures treated daily with 1µM wortmannin were counted and plotted as growth curves (Figure 5.3D-E). In separate cultures wild-type and *Bmpr1a*^{-/-} mESCs show similar growth curves, compared to DMSO controls (Figure 5.3). However, in co-cultures, *Bmpr1a*^{-/-} mESCs growth arrest after 2 days and are gradually lost from culture. After 4 days of co-culture the ratio of wild-type to *Bmpr1a*^{-/-} mESCs in wortmannin treated cultures was analysed by FACs and were compared by students' t-test against DMSO controls. No significant differences in either separate or co-cultures was observed, shown in Figure 5.3F (wortmannin treated co-culture 5.49±1.46 separate 1.18±0.41 DMSO co-culture 4.18±0.53 separate 2.04±0.10 with similar ratios if just cell numbers are investigated and normalised back to d0 of culture co-culture 5.67±1.07 separate 1.25±0.08 DMSO co-culture 3.71±0.48 separate 1.81±0.15 data shown as average±s.e.m n=3 p≤0.05). The ratio of separate cells in DMSO cultures seems quite high despite similar growth curves, as cell numbers are normalised to d0, so an increase in wild-type cell numbers compared to *Bmpr1a*^{-/-} mESCs is exaggerated due to the lower starting proportions (40:60 wild-type/*Bmpr1a*^{-/-} mESCs at d0). However, despite higher wild-type to *Bmpr1a*^{-/-} mESC ratios in treated cultures, PI3K signalling inhibition does not rescue of *Bmpr1a*^{-/-} mESCs from competing cultures as there is no significant difference between ratio of DMSO and wortmannin treated cultures at day 4 of competition.

5.2.4 Atg5^{-/-} mESCs are out-competed upon co-culture with wild-type mESCs.

AuTophagy (ATG) proteins are major components of the autophagy pathway (Itakura and Mizushima, 2010). *Atg5* is required for elongation of the autophagosome membrane in conjunction with its partner *Atg12* (Mizushima et al., 2001). *Atg5*^{-/-} mESCs were kindly gifted by the Mizushima lab and have been extensively characterised (Mizushima et al., 2001). *Atg5*^{-/-} mESCs can be maintained indefinitely in culture and transgenic mice with random deletion of *Atg5* under the control of *Cre*-reporter develop into neonates, suggesting that they can contribute to all germ layers (Mizushima and Levine, 2010; Mizushima et al., 2001; Takamura et al., 2011)

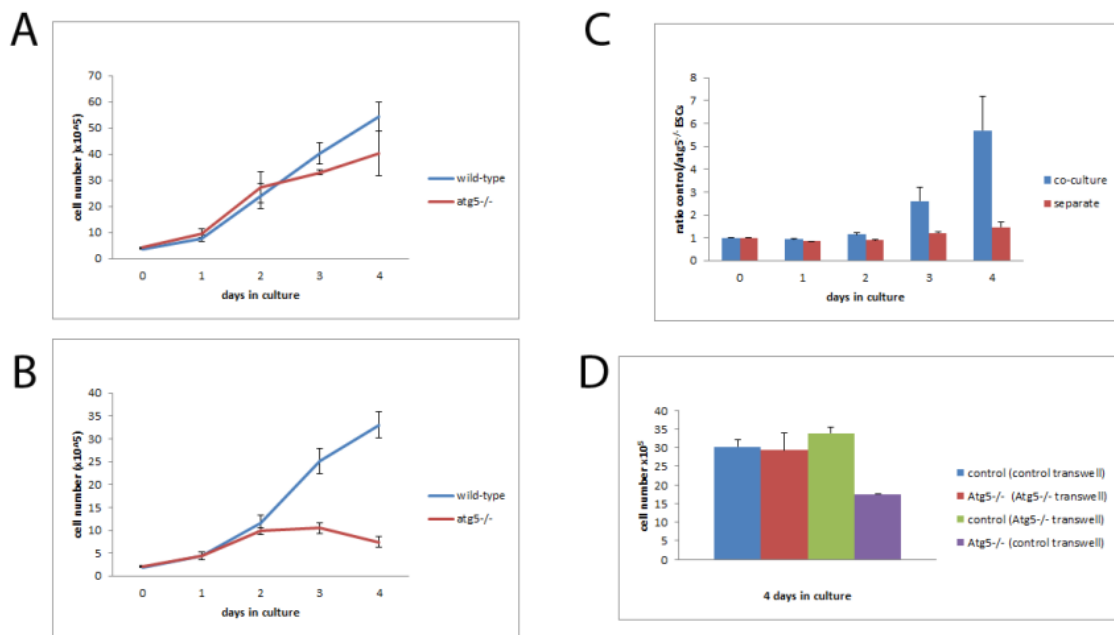


Figure 5.4 *Atg5*^{-/-} ESCs are lost from co-culture with wild-type ESCs

(A) Separate and (B) co-cultures of wild-type and *Atg5*^{-/-} ESCs are grown in GMEM+Serum for 4 days and cell numbers counted daily. (C) Ratio of wild-type to *Atg5*^{-/-} ESCs in separate and co-cultures analysed daily by FACs (average±s.e.m n=3 p≤0.01). (D) Total cell number after 4 days of transwell co-culture. Cells were grown as shown in Figure 4.1A with the genotype grown on the filter shown in brackets (n=2 average±s.e.m).

As an alternative method of investigating autophagy in cell competition, autophagy-deficient *Atg5*^{-/-} mESCs were competed against wild-type cells, shown in Figure 5.4. In order to identify each cell type, wild-type mESCs were expressing GFP. *Atg5*^{-/-} and wild-type mESCs were cultured separately and in co-culture with cell numbers and the ratio of wild-type to *Atg5*^{-/-} mESCs analysed daily (Figure 5.4).

Wild-type and *Atg5*^{-/-} mESCs grown in monoculture show similar growth curves. However, in co-cultures with wild-type mESCs, *Atg5*^{-/-} mESCs growth arrest and are progressively lost from culture (Figure 5.4B). To quantify the proportion of each cell type, separate and co-cultures were analysed by FACs and the ratio of wild-type to *Atg5*^{-/-} mESCs normalised to the starting ratio at day 0 of culture (50:50 wild-type/*Atg5*^{-/-}) and plotted daily, shown in Figure 5.4C. By day 4 of culture, the ratio of wild-type to *Atg5*^{-/-} mESCs was significantly higher in co-culture compared to separate cultures, indicating that autophagy-deficient *Atg5*^{-/-} mESCs

were out-competed by wild-type mESCs (ratio of wild-type to *Atg5*^{-/-} mESCs co-culture 5.70±1.49 separate 1.48±0.22 data shown as average±s.e.m n=3 p≤0.05). This is similar to what has been observed with *Bmpr1a*^{-/-} mESCs upon co-culture with wild-type cells. It may be of interest to compete wild-type mESCs with other autophagy-deficient cell lines should also be used to investigate if this is a general response.

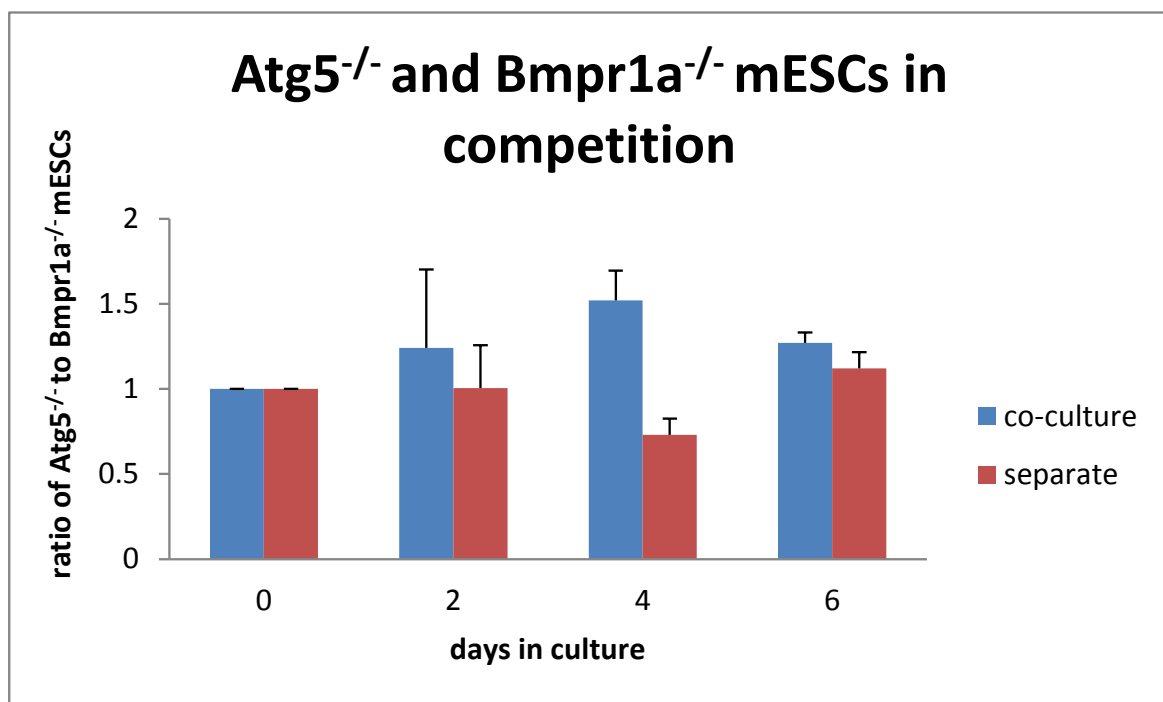


Figure 5.5 *Atg5*^{-/-} and *Bmpr1a*^{-/-} mESCs do not undergo cell competition in co-culture

Atg5^{-/-} and *Bmpr1a*^{-/-} mESCs were cultured separately and in co-culture for up to 6 days in GMEM+Serum. Every 2 days the ratio of *Atg5*^{-/-} to *Bmpr1a*^{-/-} mESCs in separate and co-cultures were analysed by FACs (averages±sem n=3).

Both *Atg5*^{-/-} and *Bmpr1a*^{-/-} mESCs are out-competed when co-cultured with wild-type cells. To investigate whether *Atg5*^{-/-} or *Bmpr1a*^{-/-} mESCs would be able to recognise each other as defective and therefore compete, both cell lines were cultured separately or in co-culture for up to 6 days in GMEM+Serum and the ratio of *Atg5*^{-/-} to *Bmpr1a*^{-/-} mESCs analysed every 2 days by FACs, shown in Figure 5.5. Interestingly, no significant difference in the ratio of each cell type was observed even after 6 days of co-culture (ratio of *Atg5*^{-/-} to *Bmpr1a*^{-/-}

mESCs after 6 days of co-culture 1.27 ± 0.06 compared to separate culture 1.12 ± 0.10 average $\pm$ sem n=3).

Previous experiments, shown in Figure 4.1, illustrated that secreted factors are involved in mediating cell competition between wild-type and *Bmpr1a*^{-/-} mESCs.

To investigate whether cell competition occurs between mESCs regardless of mutant phenotype and whether the requirement of secreted factors in mediating this process is general rather than genotype specific, wild-type and *Atg5*^{-/-} mESCs and wild-type and tetraploid (4n) mESCs were investigated using the same cell competition model as wild-type

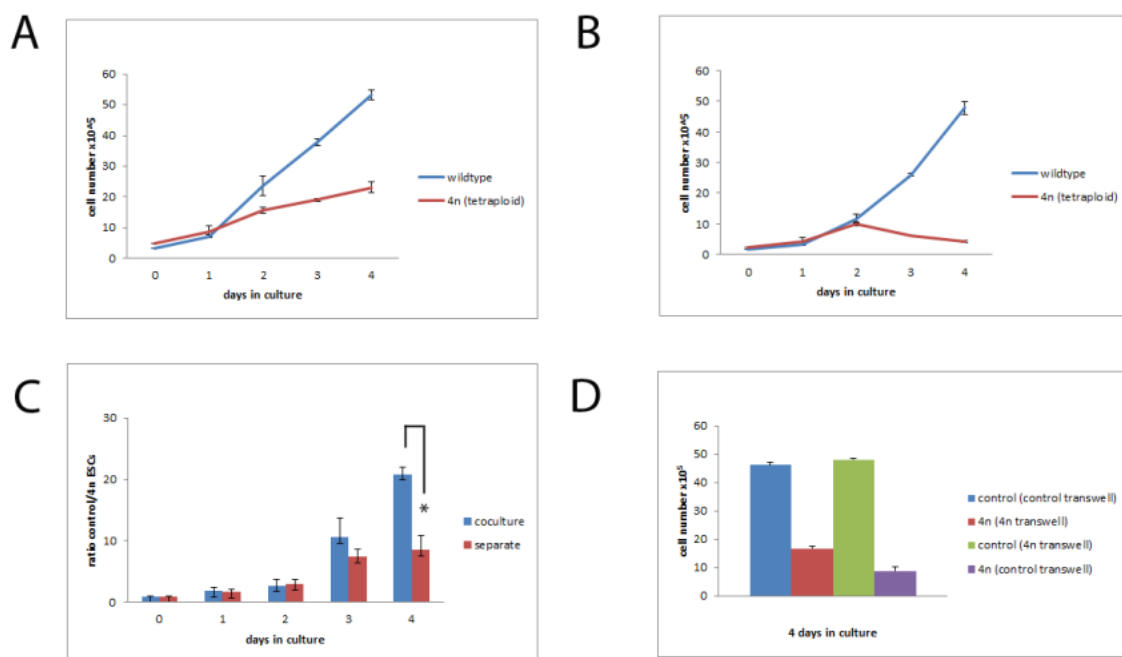


Figure 5.6 Tetraploid (4n) ESCs are lost from co-culture with wild-type ESCs

(A) Separate and (B) co-cultures of wild-type and 4n ESCs are grown in GMEM+Serum for 4 days and cell numbers counted daily. (C) Ratio of wild-type to 4n ESCs in separate and co-cultures analysed daily by FACs (average $\pm$ s.e.m n=3 $p \leq 0.01$). (D) Total cell number after 4 days of transwell co-culture. Cells were grown as shown in Figure 4.1A with the genotype grown on the filter shown in brackets (n=2 average $\pm$ s.e.m).

and *Bmpr1a*^{-/-} mESCs.

Wild-type and *Atg5*^{-/-} mESCs were grown in culture with cells of the same or different genotype grown in an overlying transwell to investigate the role of secreted factors during

competition. Cell numbers were counted and plotted at day 4 shown in Figure 5.4D, with the genotype of cells in overlying transwells shown in brackets; (control (control) 30.20 ± 1.27 ; *Atg5^{-/-}* (*Atg5^{-/-}*) 29.25 ± 4.77 ; control (*Atg5^{-/-}*) 33.90 ± 0.21 ; *Atg5^{-/-}* (control) 17.45 ± 1.52 data shown as average $\pm$ s.e.m n=2). Despite the lack of cell-cell contact, *Atg5^{-/-}* mESCs display a growth defect upon co-culture with wild-type mESCs suggesting wild-type mESCs release secreted-factor(s) that affect *Atg5^{-/-}* mESC growth.

Tetraploid (4n) mESCs were co-cultured with wild-type cells to observe whether they are also eliminated by cell competition. In separate cultures, wild-type cells have significantly higher cell numbers; however growth rate analysis shows no significant differences (Appendix Table 1). Upon co-culture with wild-type mESCs, 4n mESCs growth arrest after two days and are lost from co-culture, Figure 5.6C. This behaviour is similar to that observed with *Bmpr1a^{-/-}* and *Atg5^{-/-}* mESCs, shown in Figure 5.4. This is reflected in a significantly higher ratio of wild-type/4n mESCs in co-cultures compared to separate cultures after 4 days of culture when populations were analysed by FACs (at day 4 of culture ratio of wild-type to 4n mESCs co-culture 20.87 ± 1.13 separate 8.48 ± 2.31 average $\pm$ s.e.m n=3 $p \leq 0.05$).

Wild-type mESCs have been shown in this work to be able to eliminate both *Bmpr1a^{-/-}* and *Atg5^{-/-}* mESCs in the absence of cell-cell contact. To investigate if this was also the case during competition between 4n and wild-type cells, cells of both genotypes were with transwells of the same or different genotype overlying them, as described in Figure 4.1. After 4 days of co-culture in transwells, cells grown in the underlying wells were counted. Compared to co-culture of cells with the same genotype in the overlying transwell, the number of 4n mESCs co-cultured with wild-type mESCs in the overlying transwell was lower, shown in Fig.5.6D although further experiments are required for statistical analysis (cell number in transwell co-culture control (control) 46.25 ± 0.88 ; 4n (4n) 16.80 ± 0.71 ; control (4n) 48.00 ± 0.42 ; 4n (control) 8.85 ± 1.31 (data shown as average $\pm$ s.e.m ratios n=3 transwell culture n=2). This data suggests that cell competition and the role of secreted factor(s) is a general mechanism between wild-type mESCs and mESCs with mutant genotypes.

5.3 Discussion

In this chapter, the role of autophagy during cell competition was investigated. The observation that winner or circulating cells “eat” their surrounding loser neighbours has been observed in both *Drosophila* and mouse models of cell competition (Claveria et al., 2013; Li and Baker, 2007; Lolo et al., 2012; Sancho et al., 2013). Our data confirms this as *Bmpr1a*^{-/-} GFP^{+ve} debris was observed within neighbouring wild-type mESCs. This chapter aimed to address the role of autophagy and its regulatory pathways during cell competition between mESCs.

Whilst mutant cell debris was observed within neighbouring wild-type mESCs during cell competition (Figure 5,1), this may not significantly alter the proportion of wild-type and mutant cells analysed by flow cytometry in separate and co-cultures. Primarily, instances whereupon endocytosed cell debris was found within neighbouring cells was very rare, and may contribute a non-significant proportion of cells if analysed by FACs as a mutant cell rather than a wild-type cell. One possible experiment to exclude the possibility of analysing wild-type cells with endocytosed mutant cell debris as mutant cells would be to label wild-type and mutant cell types with separate fluorophores. In this instance, the amount of endocytosed material would in most cases be much less fluorescent than the fluorophores expressed by the cell and would more likely result in more specific sorting by FACs.

Autophagy is regulated by the mTOR signalling pathway, and acts to inhibit autophagy to basal levels. Rapamycin causes dissociation of the mTOR signalling complex and relieves autophagy from this inhibition (Ballou and Lin, 2008; Oshiro et al., 2004). In contrast, the nucleation and assembly of the phagophore membrane where unwanted cellular components are degraded requires formation of a class III PI3K complex and PI3K inhibitors such as wortmannin are used to inhibit autophagy (Blommaart et al., 1997; Klionsky and Deretic, 2010; Liang et al., 1999; Sun et al., 2008). Whilst addition of rapamycin to competing cultures was sufficient to significantly increase cell competition dynamics, it was more difficult to interpret data from inhibiting PI3K signalling. One of the inhibitors used, LY294002, was highly cytotoxic suggesting that non-specific effects could affect the interpretation of the data from these experiments, and no inhibition or significant reduction in cell competition was observed with a second inhibitor, wortmannin.

Two points raised in this chapter are of interest, primarily the differences in cell behaviour on LY294002 treatment, compared to wortmannin treatment. Reports in the literature have described LY294002 non-specific effects, including affecting kinases in metabolism; DNA binding and transcription and ATPases, and is therefore likely to affect cell proliferation and survival (Gharbi et al., 2007). This data suggests that LY294002 affects cell survival in mESCs in a non-PI3K dependent manner, as no changes in cell growth were observed compared to DMSO controls when wortmannin, a second PI3K inhibitor was used.

Secondly are the non-opposing effects of rapamycin induced mTOR inhibition compared to PI3K inhibition with wortmannin. Ideally as they both act on opposing sides of the autophagy pathway, they should have opposite effects on cell competition. However, the data shown in this chapter demonstrates this is not the case. It is likely that other signalling pathways regulating autophagy, such as the AMPK and p53 signalling pathways are sufficient to mediate autophagy independent of PI3K, whereas rapamycin, which acts directly on mTOR, may be less susceptible to regulation by other kinases (Ravikumar et al., 2010; Yang and Klionsky, 2010).

An alternative method to modifying the signalling pathways involved in mediating cell competition was to observe the behaviour of wild-type mESCs upon confrontation with mESCs with defective autophagy. Upon co-culture with wild-type mESCs, *Atg5^{-/-}* mESCs were lost from culture, reminiscent of what is observed upon *Bmpr1a^{-/-}*/wild-type mESC co-culture. This data suggests that defects in autophagy are sufficient to induce cell competition. Although this experiment only partially addresses the hypothesis that autophagy is required for cell competition, as autophagy is required for multiple roles in the cell including energy homeostasis and accumulation of old or misfolded proteins (Yang and Klionsky, 2010). However, this experiment does begin to address the idea of cell competition as a general phenomenon between optimal and suboptimal cells. It begins to investigate the idea that differences in energy homeostasis may trigger cell competition. As autophagy also has roles in energy homeostasis, it would be interesting to investigate whether differences in the energy-sensing machinery within cells is involved in cell competition. This could be done through looking at phosphorylation of the energy-sensing kinase AMPK.

Interestingly, *Atg5*^{-/-} mESCs were unable to recognise and out-compete *Bmpr1a*^{-/-} mESCs in co-culture, suggesting a role for autophagy during cell competition. This does suggest that components of the autophagy or energy-sensing pathways are involved in recognition of mutant cell lines by wild-type or winner cells during competition. However the particular molecular mechanisms involved would require further study.

Additionally, these experiments do not address whether winner cells in competing cultures “eat” their way through the losing population. Components of the autophagy machinery are known to be involved in phagocytosis of apoptotic corpse clearance, and this engulfment of neighbouring mutant neighbours has been observed during competition (Claveria et al., 2013; Qu et al., 2007). It would be helpful to determine whether autophagy has an active or passive role during cell competition. However, it may be that this process of eliminating loser neighbours during cell competition is only part of how mutant cells are removed. It is likely that in addition to engulfment, loser cells are removed by apoptosis and extrusion from competing environments (Vincent et al., 2013).

Analysis of autophagy deficient mice by development mosaic mutation models revealed that such mice developed multiple tumours in the liver, derived specifically from *Atg5*^{-/-} hepatocytes, despite the presence of wild-type neighbours (Takamura et al., 2011). This is despite the fact that *Atg5*^{-/-} cells contribute to all cell types. There is also evidence in the literature to suggest that components of the autophagy pathway also function as tumour suppressors (Yue et al., 2003). This suggests that competitive interactions between wild-type and *Atg5*^{-/-} mESCs or their equivalent which could arise during development would target and remove these cells before they could contribute to the adult. This would coincide with reports in the literature of other pro-cancerous mutations in such as *Scribble*; *Dlg* and *Lgl* mutants that are also regulated by cell competition (Chen et al., 2012; Hafezi et al., 2012; Menendez et al., 2010; Norman et al., 2011; Tamori et al., 2010).

To continue research into the role of autophagy during cell competition it is worth mentioning two further components of the autophagy machinery. LC3 is expressed at the surface of the autophagolysosome (fusion of the autophagosome with lysosome) where unwanted components are degraded. In response to increased levels of autophagy, LC3 is

cleaved from its original state LC3-I to a smaller fragment LC3-II and this conversion serves as a marker for levels of autophagy (Klionsky et al., 2008; Mizushima, 2004; Tanida et al., 2004). Secondly p62 is a protein which recognises toxic cellular waste and is continuously cleared by basal levels of autophagy. A lack of autophagy leads to accumulation of the p62 protein therefore analysis p62 degradation is also used as a marker to examine levels of autophagy. (Lamark et al., 2009; Rusten and Stenmark, 2010). Western blotting for changes in these markers during cell competition would be useful to discover if the autophagy pathways are differently regulated between winner and loser cells during competition.

Chapter 6 – General Discussion

Cell competition has been described using a variety of model systems. Despite the diversity of competition triggers, many of these models share similar hallmarks whereupon outcompeted cells are ultimately eliminated by apoptosis or extruded and later engulfed, and remaining ‘winner’ cells over-proliferate in a compensatory manner (de Beco et al., 2012; Levayer and Moreno, 2013; Tamori and Deng, 2011; Vincent et al., 2013). In contrast to these models, other reports of cell competition, such as occupancy of the *Drosophila* stem cell niche by GSCs or of the mouse bone marrow by HSPCs do not exhibit these hallmarks. Instead, GSCs in the *Drosophila* stem cell niche become displaced and differentiate whilst HSPCs become senescent when outcompeted (Bondar and Medzhitov, 2010; Rhiner and Moreno, 2009).

In the embryo, the epiblast differentiates to generate all the somatic cells of the adult and this process starts with formation of the three germ layers occurs during gastrulation. Prior to gastrulation the epiblast rapidly proliferates to increase in cell number (Snow, 1977; Stuckey et al., 2011a). During such periods of rapid proliferation the likelihood of mutated or mispatterned cells arising is quite high, and whilst these mutations may not be lethal, they could lead to defects in adult tissues or the organism. At the onset of gastrulation, apoptosis in the embryo is also increased (Spruce et al., 2010). This increase in apoptosis correlates with increased proliferation making it likely that cell competition is a mechanism to recognise and remove mispatterned or mutated cells from the embryo before they can be incorporated into adult tissues.

This work investigated cell competition between wild-type mESCs when confronted with other mESCs of mutant genotypes, (shown in this work: *Bmpr1a*^{-/-}; *Atg5*^{-/-} and 4n mESCs). During the first step of differentiation (commitment from naive to primed mESCs) mutant cells in this model are eliminated by caspase-dependent apoptosis, rather than differentiating or entering senescence. Elimination of mutant cells is accompanied by an increase in growth rate in winner cells (Sancho et al., 2013, and this work).

One hypothesis is that cell competition could be linked to changes in signal transduction, as secreted factors have been shown to mediate cell competition between mESCs. A variety of signalling pathways mediate their effects by binding of extracellular secreted ligands to cell surface receptors, including the FGF and Wnt signalling pathways. Defects in *Wg* have been shown to trigger cell competition in *Drosophila* (Vincent et al., 2011). It is shown here that manipulation of both Wnt and FGF signalling in vitro are sufficient to determine the outcome of cell competition between mESCs, and after cells have begun differentiating. These signalling pathways have also been shown to rescue defects caused by loss of *Bmpr1a*^{-/-} in the embryo. In *Bmpr1a*^{null/flox}Sox2Cre embryos, *Bmpr1a* is lost specifically throughout the epiblast. These mice fail to specify the AVE due to aberrant migration of the DVE, and show a marked reduction in expression of Wnt throughout the embryo. Addition of Wnt ligands to cultured mutant embryos restores expression of Wnt signalling inhibitors, *Dkk1* and *Cer1*, at the anterior side of the embryo, directing AVE migration (Miura et al., 2010). A second model to study the function of BMP signalling in the epiblast was generated using the *Mox2Cre* line leading to *Bmpr1a* being lost in a mosaic pattern in the epiblast. These studies found that *Bmpr1a*^{null/flox}*Mox2Cre* embryos displayed normal AVE migration and initiated gastrulation. However commitment of cell fate through the primitive streak is perturbed in these embryos resulting in mispatterning of paraxial mesoderm. Interestingly the authors reported that inhibition of FGF signalling restores normal timing of paraxial mesoderm recruitment in *Bmpr1a*^{null/flox}*Mox2Cre* embryos (Miura et al., 2006). These observations show that defects in BMP signalling can be rescued by changes in FGF and Wnt signalling.

Could differences in signal transduction between winner and loser cells cause temporary stability of *c-Myc* in winner cells, and at the same time, cause mutant cells to begin to be eliminated from co-cultures (Sancho et al., 2013)? Relative differences in *c-Myc* expression have been shown to induce cell competition in the absence of other mutations and may be a conserved trigger in mediating competition within *Drosophila* (de la Cova et al., 2004; Moreno and Basler, 2004); and mouse models (Claveria et al., 2013; Sancho et al., 2013).

Evidence in the literature indicates *c-Myc* relative expression between cells is what marks winner cells from loser cell (Claveria et al., 2013; Sancho et al., 2013). As both FGF and Wnt signalling pathways were able to affect the outcome of cell competition between

mESCs, the question arises whether these signalling pathways affect *c-Myc* stability. The onset of cell competition in mESCs coincides with the differentiation of the epiblast (Claveria et al., 2013; Sancho et al., 2013). Interestingly, both FGF/ERK and WNT signalling are involved in differentiation, and could affect cell competition due to their effects on changing *c-Myc* expression upon differentiation. For example, inhibition of GSK3 β results in increased *c-Myc* stability (Cartwright et al., 2005), this would highlight relative changes in *c-Myc* expression between winner and loser cells, resulting in the observed increase in cell competition. In contrast, MEK inhibition results in decreased *c-Myc* stability (Ying et al., 2008). In this instance, the relative differences in *c-Myc* expression between winner and loser cells would be lost, allowing survival of mutant (in this work *Bmpr1a*^{-/-}) mESCs.

In addition to signalling pathways, the autophagy pathway was also investigated in this thesis. The clearance of apoptotic cells by wild-type neighbours was shown during cell competition when Li and Baker observed a wild-type cell in the process of engulfing a neighbouring mutant cell (Li and Baker, 2007). In addition, defects in engulfment have also been shown to render cells susceptible to being outcompeted when grown beside wild-type neighbours (Li and Baker, 2007). In competing mESC cultures, clearance of apoptotic corpses/neighbouring cells by engulfment has been observed, both in this work and by others (Claveria et al., 2013; Sancho et al., 2013). We investigated the role of autophagy as genes involved in autophagy have been shown to have a role in clearance of apoptotic corpses during early development. *Atg5* and *Beclin1* (*Atg6*) are both components of autophagy and embryoid bodies null for these genes cavitate, in that cells at the centre of the embryoid body undergo apoptosis. However, the fluid-filled space at the centre of these mutant embryoid bodies is not formed due to persistence of apoptotic corpses (Qu et al., 2007). This suggests that components of the autophagy pathway may also be involved in engulfment. We show in this work that *Atg5*^{-/-} mESCs cells defective in autophagy but which may also be involved in engulfment of apoptotic corpses are removed from co-cultures by cell competition (Sancho et al., 2013). It is unclear why a possible defect in engulfment results in elimination by cell competition, and this inability probably masks other mutations which trigger cell competition.

Another hypothesis could be that autophagy is also involved in cell competition via its roles in energy homeostasis. Autophagy is induced in response to signalling pathways in the cell that regulate nutrient sensing and cell metabolism, such as AMP-activated protein kinase (AMPK) or via growth factor binding downstream of the PKB/Akt pathway (Gwinn et al., 2008; Yang and Klionsky, 2010). The lower growth rate of mutant cells when co-cultured with wild-type cells could be caused by a lower ability for nutrient uptake, due to the presence of faster-growing neighbours. One hypothesis would be that loser cells increase autophagy in order to degrade unneeded organelles or proteins, thus allowing recycling of the resulting amino acids for the synthesis of proteins essential for cell survival. Autophagy deficient *Atg5*^{-/-} mESCs also display defects linked to low levels of ATP (Qu et al., 2007). In addition to low levels of ATP, *Atg5*^{-/-} mESCs would be unable to degrade intracellular components for protein synthesis and therefore unable to cope with long periods of starvation, resulting in their elimination from competing cultures.

Interestingly the signalling pathways identified in this thesis as affecting cell competition are also involved in the regulation of autophagy. FGF signalling has been shown to directly stimulate autophagy (Yang and Klionsky, 2010). In contrast, Wnt signalling has been shown to suppress the autophagy pathway via repression of p62 (Petherick et al., 2013). A possible mechanism for the comparison of cellular fitness could be through changes in autophagy downstream of FGF and Wnt signals. An alternative hypothesis is that changes in activation of metabolic sensing kinases (such as AMPK) caused by differences energy homeostasis and nutrient signalling between neighbouring cells triggers cell competition. For example, when treated with rapamycin, cell competition was significantly enhanced. This could be due to differences in nutrient uptake between winner and loser cells being exacerbated as intracellular resources are degraded faster when autophagy is stimulated.

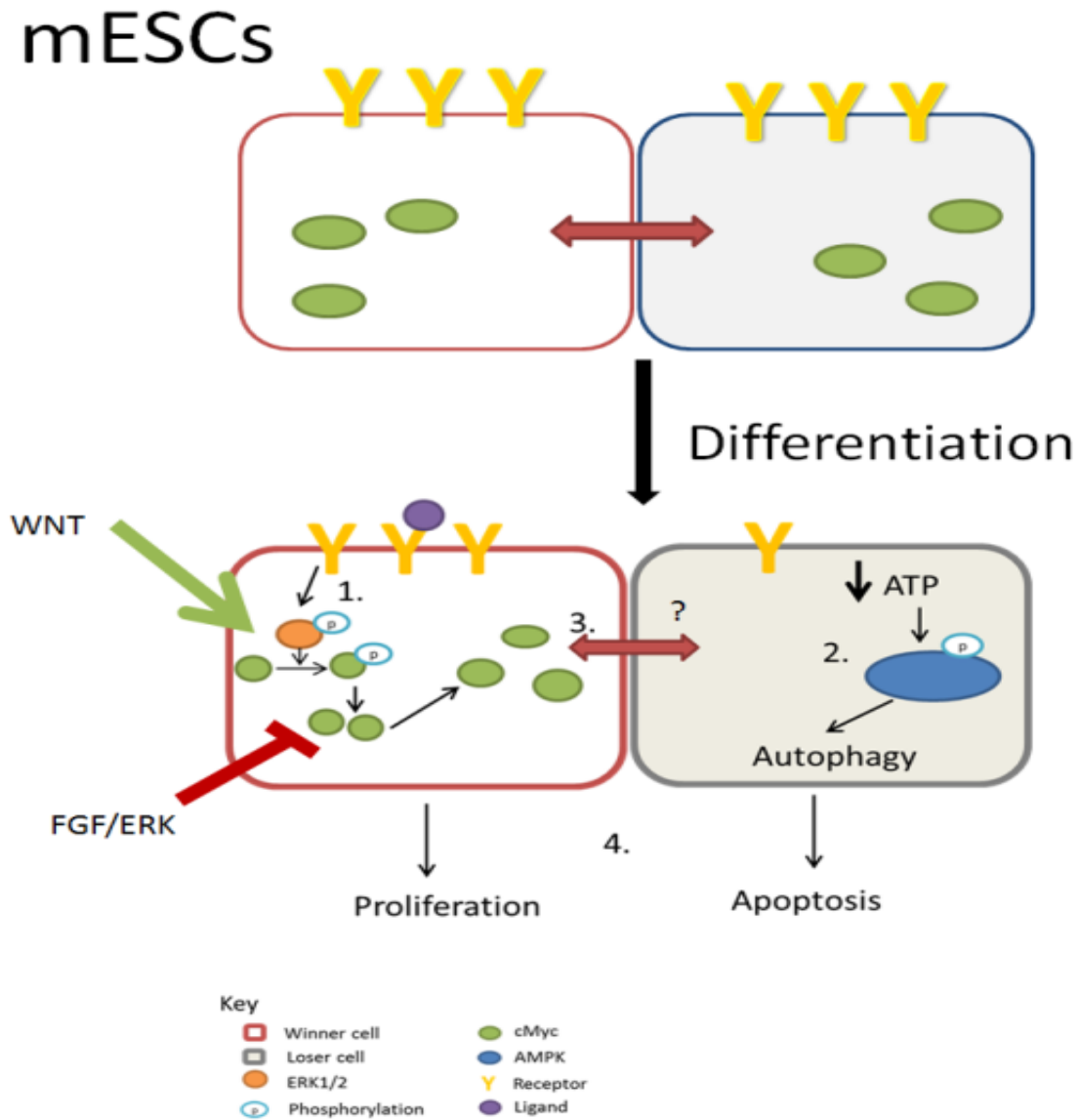


Figure 6.1 Possible model for cell competition between mESCs

Hypothetical model for how ESCs compete. As mESCs homogenously high levels of *c-Myc* are present in all cells and no imbalances are seen. Upon differentiation, levels of *c-Myc* expression are reduced leading to imbalances in *c-Myc* between competing cells. These imbalances are mediated by differentiation factors such as WNT or FGF/ERK which stabilise or lower *c-Myc* expression. 1. Binding of nutrients and growth factor ligands at the cell surface of winner cells leads to increases in *c-Myc* stability. 2, Loser cells are unable to uptake ligands or nutrients due to the presence of faster growing neighbouring cells, therefore intracellular levels of ATP drop, leading to AMPK phosphorylation and upregulation of autophagy. 3. Cells sense relative differences in *c-Myc* and nutrient sensing, through an unknown mechanism (?). 4. Differences in *c-Myc* stability and nutrient uptake trigger proliferation in winner cells and apoptosis in loser cells.

Cell competition is likely to be a multi-step process and a possible model is illustrated in Figure 6.1. In competing cultures, wild-type cells would be more efficient at internalizing

higher levels of nutrients and specific ligands whereas loser cells would not. Furthermore, due to the presence of winner cells internalising survival and growth factors faster, loser cells would also be subjected to a higher energy demand. As described in the literature during mouse development, relative *c-Myc* expression between cells drives cell competition (Claveria et al., 2013; Sancho et al., 2013; . It is therefore possible to hypothesise that internalisation of growth and signalling factors; changes in energy homeostasis or protein ribosynthesis result either directly or indirectly into changes in *c-Myc* expression within the cell (Claveria et al., 2013; Sancho et al., 2013).

It is also possible that these changes could co-ordinate with rates of cell differentiation, to avoid mispatterning of cells, as the competitive nature of cells occurs during mouse development, from E3.5 to E10.5 in mouse (Claveria et al., 2013). In this environment cells could be continuously monitoring each other for fitness, and one mechanism is through relative *c-Myc* expression. Cells with prolonged relatively lower *c-Myc* would be classified as “unfit” or mispatterned by their neighbours. Cells would then use this mutual sensing mechanism to class relative levels of *c-Myc* expression allowing winner cells to identify loser cells, triggering a still unknown mechanism to remove loser cells. This would ensure that the epiblast is a homogenously fit population from which to derive all adult cell populations (Claveria et al., 2013). It is also still not clear whether winner cells remove loser cells by inducing apoptosis, promoting their extrusion or engulfment or, more likely, a combination of these methods (Vincent et al., 2013).

Once competitive interactions have been triggered, winner cells identify and remove loser cells from their environment. In a number of models of cell competition loser cells upregulate cell stress pathways prior to apoptosis, such as JNK signalling and this leads to activation of proapoptotic genes (Menendez et al., 2010; Moreno and Basler, 2004; Moreno et al., 2002; Portela et al., 2010; Tamori et al., 2010). However, in this work and others, disruption of JNK signalling was unable to prevent competition (de la Cova et al., 2004; Rodrigues et al., 2012 and this work). Therefore the mechanisms that winner cells induce apoptosis in loser cells could be context dependent. Considering how efficient cell competition is in removing large numbers of mutant cells in cultures, it is also possible that multiple signalling pathways lead to differential apoptosis and proliferation during

competition. There are a variety of pathways to investigate during this process. As suggested earlier, removal of loser cells from competing cultures could occur via a combination of three different mechanisms; apoptosis; extrusion and engulfment (Vincent et al., 2013). We have shown that caspase inhibition is sufficient to rescue *Bmpr1a*^{-/-} mESCs from competition in co-culture with wild-type cells (Sancho et al., 2013). Inhibition of RHO kinase has been suggested to prevent live cell extrusion (Gu et al., 2011) and it could be interesting to investigate whether this could rescue the elimination of mutant cells during competition. Also, wild-type cells increase their growth rate in separate compared to co-cultures, even though it is still not clear how this is regulated. It could be that transient upregulation of *c-Myc* in winner cells prior to elimination of mutant cells also promotes proliferation (Sancho et al., 2013).

In conclusion, cell competition illustrates that cell survival is ultimately dependent upon its microenvironment and neighbours within the organism. It acts as a selection mechanism from very early stages during mammalian development for the fittest cells that will contribute to the adult. Models of cell competition in adult tissues, such as stem cell niches, show that this mechanism remains active throughout the lifespan of the organism. It is possible that competitive interactions also occur when tissues start proliferating such as in cancer and tissue regeneration following injury, as well as during development (Oertel et al., 2006; Stuckey et al., 2011a). In the case of cancer, it is possible that cell competition selects for mutated faster growing cells, particularly if the mutation results in upregulation of *c-Myc* in cancerous cells. In these cases, an understanding of cell competition may help provide therapies to prevent mutant cells from eliminating un-mutated neighbours and prevent metastasis. Therefore, the study of cell competition, its triggers and mechanism are instrumental in understanding cell survival in a variety of contexts.

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Appendix

A.1 Growth Rates=Ln (final number of cells/initial number of cells) /

LN (2)

Appendix Table 1 – Master List of Growth Rates

Growth Rate Control and Bmpr1a^{-/-} (GFP) in GMEM+Serum

	Control		
	Separate	Co-culture	t-test
D0-1	0.94±0.33	1.05±0.32	0.86
D1-2	1.75±0.25	1.67±0.25	0.61
D2-3	1.00±0.15	1.27±0.16	0.05
D3-4	0.45±0.13	0.64±0.09	0.02
	Bmpr1a ^{-/-}		
	Separate	Co-culture	t-test
D0-1	0.43±0.25	0.75±0.2	0.07
D1-2	1.67±0.18	1.73±0.22	0.71
D2-3	0.86±0.04	0.09±0.18	0.02
D3-4	0.39±0.15	-0.44±0.24	0.07

Growth Rate Control (GFP) and Atg5^{-/-} in GMEM+Serum

Control			
	Separate	Co-culture	t-test
D0-1	1.01±0.18	1.17±0.17	0.50
D1-2	1.59±0.12	1.42±0.08	0.47
D2-3	0.79±0.14	1.12±0.04	0.06
D3-4	0.43±0.04	0.40±0.06	0.53

Atg5^{-/-}			
	Separate	Co-culture	t-test
D0-1	1.16±0.22	1.04±0.023	0.48
D1-2	1.48±0.1	1.19±0.12	0.33
D2-3	0.34±0.25	0.07±0.2	0.15
D3-4	0.21±0.23	0.51±0.18	0.00

Growth Rate Control and 4n (tetraploid) in GMEM+Serum

Control			
	Separate	Co-culture	t-test
D0-1	1.16±0.08	1.08±0.14	0.67
D1-2	1.69±0.10	1.76±0.24	0.78
D2-3	0.71±0.12	1.18±0.14	0.02
D3-4	0.49±0.08	0.87±0.07	0.01

4n (tetraploid)			
	Separate	Co-culture	t-test
D0-1	0.79±0.24	0.72±0.30	0.56
D1-2	0.91±0.17	1.32±0.25	0.22
D2-3	0.28±0.05	0.66±0.10	0.01
D3-4	0.27±0.12	-0.58±0.07	0.02

Growth Rate Control and Bmpr1a^{-/-} mESCs in transwell

Control			
	Separate	Co-culture	t-test
D2-3	0.85±0.33	0.66±0.08	
D3-4	0.41±0.16	0.23±0.03	

Bmpr1a^{-/-}			
	Separate	Co-culture	t-test
D2-3	0.76±0.20	0.11±0.08	

D3-4 0.21±0.16 0.06±0.18

Growth Rate Control +Bmpr1a/- in 10ng/ml rapamycin

Day 0

	Control - 10ng/ml rapamycin			Control DMSO		
	separate	co-culture	t-test	separate	co-culture	t-test
D0-1	1.08±0.13	1.31±0.18	0.38	1.55±0.19	1.50±0.23	0.73
D1-2	1.24±0.14	1.28±0.13	0.94	1.17±0.34	1.38±0.35	0.16
D2-3	0.50±0.33	1.39±0.12	0.25	0.54±0.29	0.90±0.14	0.33
D3-4	0.63±0.21	0.50±0.22	0.85	0.49±0.24	0.70±0.06	0.54

	Bmpr1a/- - 10ng/ml rapamycin			Bmpr1a/- DMSO		
	separate	co-culture	t-test	separate	co-culture	t-test
D0-1	1.41±0.11	1.43±0.23	0.94	1.50±0.16	1.59±0.21	0.37
D1-2	0.73±0.04	0.74±0.17	0.96	1.03±0.21	0.91±0.30	0.46
D2-3	0.38±0.09	-0.47±0.12	0.09	0.35±0.04	-0.21±0.11	0.03
D3-4	0.28±0.10	-0.74±0.16	0.02	0.40±0.04	-0.55±0.07	0.00

Growth Rate Control +Bmpr1a/- in 1μM wortmannin

Day 0

	Control - 1μM wortmannin			Control DMSO		
	separate	co-culture	t-test	separate	co-culture	t-test
D0-1	0.61±0.13	0.43±0.12	0.33	0.69±0.06	0.57±0.12	0.41
D1-2	0.77±0.12	1.45±0.17	0.04	1.63±0.01	1.68±0.03	0.47
D2-3	0.52±0.10	0.71±0.06	0.09	0.26±0.07	0.95±0.22	0.09
D3-4	0.66±0.10	0.97±0.19	0.78	1.14±0.08	0.94±0.13	0.11

	Bmpr1a/- - 1μM wortmannin			Bmpr1a/- DMSO		
	separate	co-culture	t-test	separate	co-culture	t-test
D0-1	0.69±0.12	0.21±0.09	0.03	0.75±0.08	0.52±0.05	0.34
D1-2	1.36±0.04	1.47±0.13	0.50	1.70±0.11	1.81±0.06	0.61
D2-3	0.82±0.12	0.73±0.04	0.41	0.41±0.14	0.36±0.14	0.86
D3-4	0.06±0.03	-0.83±0.17	0.05	0.23±0.06	-0.44±0.23	0.20

Growth Rate Control +Bmpr1a/- in 10μM SB431542

Day 0

	Control - 10μM SB431542			Control DMSO		
	separate	co-culture	t-test	separate	co-culture	t-test
D0-1	1.29±0.18	1.15±0.26	0.39	1.29±0.18	1.50±0.16	0.14
D1-2	0.82±0.02	0.49±0.17	0.29	1.49±0.19	1.08±0.03	0.19

D2-3	1.30±0.14	1.43±0.06	0.35	0.90±0.02	1.70±0.14	0.02
D3-4	0.35±0.03	0.58±0.02	0.02	0.36±0.27	0.59±0.05	0.27

Bmpr1a^{-/-} - 10μM SB431542

	separate	co-culture	t-test
D0-1	0.92±0.20	0.90±0.26	0.89
D1-2	1.03±0.11	1.10±0.17	0.71
D2-3	1.08±0.10	1.15±0.17	0.55
D3-4	0.25±0.11	-0.05±0.07	0.24

Bmpr1a^{-/-} DMSO

	separate	co-culture	t-test
D0-1	1.20±0.19	1.20±0.13	1.00
D1-2	1.15±0.15	1.02±0.05	0.47
D2-3	0.63±0.11	-0.14±0.33	0.14
D3-4	0.37±0.06	-0.51±0.06	0.00

Day 2

Control - 10μM SB431542

	separate	co-culture	t-test
D0-1	0.88±0.04	1.30±0.04	0.02
D1-2	2.07±0.10	1.31±0.03	0.10
D2-3	0.43±0.09	1.13±0.04	0.22
D3-4	0.51±0.07	0.66±0.07	0.70

Control DMSO

	separate	co-culture	t-test
D0-1	0.88±0.04	1.30±0.04	0.02
D1-2	2.07±0.10	1.31±0.03	0.10
D2-3	0.67±0.08	1.35±0.08	0.28
D3-4	0.53±0.02	0.90±0.15	0.38

Bmpr1a^{-/-} - 10μM SB431542

	separate	co-culture	t-test
D0-1	0.87±0.02	0.99±0.06	0.58
D1-2	1.37±0.01	1.34±0.01	0.14
D2-3	0.58±0.08	0.02±0.06	0.05
D3-4	0.18±0.06	-0.71±0.01	0.07

Bmpr1a^{-/-} DMSO

	separate	co-culture	t-test
D0-1	0.87±0.02	0.99±0.06	0.58
D1-2	1.37±0.01	1.34±0.01	0.14
D2-3	0.78±0.02	0.01±0.08	0.16
D3-4	0.22±0.07	-0.32±0.16	0.21

Growth Rate Control +Bmpr1a^{-/-} in LIF

Day 0

Control - LIF

	separate	co-culture	t-test
D0-1	0.70±0.25	0.61±0.21	0.79
D1-2	2.01±0.15	2.12±0.18	0.69
D2-3	1.12±0.08	0.84±0.19	0.24
D3-4	0.41±0.11	0.36±0.24	0.84

Control DMSO

	separate	co-culture	t-test
D0-1	1.07±0.13	1.17±0.26	0.64
D1-2	1.69±0.14	1.30±0.10	0.06
D2-3	0.60±0.22	1.11±0.10	0.21
D3-4	0.68±0.18	1.15±0.13	0.06

Bmpr1a^{-/-} - LIF

	separate	co-culture	t-test
D0-1	0.52±0.14	0.48±0.13	0.91
D1-2	2.01±0.17	2.09±0.13	0.85
D2-3	0.86±0.09	0.95±0.14	0.65
D3-4	0.40±0.08	0.58±0.21	0.43

Bmpr1a^{-/-} DMSO

	separate	co-culture	t-test
D0-1	0.73±0.04	1.02±0.08	0.11
D1-2	1.39±0.08	1.47±0.11	0.65
D2-3	0.57±0.12	-0.11±0.08	0.02
D3-4	0.43±0.13	-0.65±0.05	0.05

Day 2						
Control - LIF				Control DMSO		
	separate	co-culture	t-test	separate	co-culture	t-test
D0-1	0.80±0.36	0.96±0.19	0.61	0.80±0.36	0.96±0.19	0.61
D1-2	1.84±0.33	1.43±0.07	0.43	1.84±0.33	1.42±0.07	0.42
D2-3	0.67±0.07	1.13±0.07	0.03	0.71±0.11	1.17±0.06	0.02
D3-4	0.51±0.06	0.84±0.17	0.23	0.44±0.01	0.92±0.11	0.08

Bmpr1a^{-/-} - LIF				Bmpr1a^{-/-} DMSO		
	separate	co-culture	t-test	separate	co-culture	t-test
D0-1	0.72±0.07	0.89±0.10	0.40	0.72±0.07	0.89±0.10	0.40
D1-2	1.48±0.05	1.53±0.06	0.78	1.48±0.05	1.52±0.06	0.82
D2-3	0.59±0.08	0.16±0.02	0.04	0.51±0.10	0.06±0.07	0.02
D3-4	0.42±0.15	-0.37±0.09	0.04	0.43±0.07	-0.81±0.11	0.03

Growth Rate Control +Bmpr1a^{-/-} in 1μM PD0325901

Day 0						
Control - 1μM PD0325901				Control DMSO		
	separate	co-culture	t-test	separate	co-culture	t-test
D0-1	1.00±0.17	0.29±.12	0.21	0.72±0.14	0.68±0.09	0.92
D1-2	1.02±0.18	1.38±0.21	0.58	1.53±0.12	1.33±0.13	0.61
D2-3	1.17±0.10	1.48±0.25	0.62	1.19±0.14	1.10±0.05	0.32
D3-4	0.63±0.14	0.44±0.11	0.61	0.53±0.08	1.10±0.06	0.08

Bmpr1a^{-/-} - 1μM PD0325901				Bmpr1a^{-/-} DMSO		
	separate	co-culture	t-test	separate	co-culture	t-test
D0-1	0.19±0.13	0.48±0.12	0.28	0.10±0.13	0.80±0.11	0.03
D1-2	1.20±0.08	1.24±0.08	0.88	1.45±0.17	1.28±0.08	0.57
D2-3	1.02±0.14	1.06±0.09	0.93	0.63±0.25	0.22±0.08	0.28
D3-4	0.65±0.014	0.72±0.08	0.70	0.71±0.14	-0.31±0.16	0.01

Day 2						
Control - 1μM PD0325901				Control DMSO		
	separate	co-culture	t-test	separate	co-culture	t-test
D0-1	1.34±0.04	0.59±0.08	0.21	1.25±0.04	0.59±0.08	0.21
D1-2	1.30±0.03	1.33±0.02	0.85	1.30±0.03	1.33±0.02	0.85
D2-3	0.28±0.00	1.27±0.03	0.03	0.92±0.15	1.29±0.11	0.61
D3-4	0.78±0.04	0.26±0.07	0.26	0.32±0.12	1.05±0.10	0.34

Bmpr1a^{-/-} - 1μM PD0325901				Bmpr1a^{-/-} DMSO		
	separate	co-culture	t-test	separate	co-culture	t-test

D0-1	0.12±0.19	0.51±0.08	0.33	0.12±0.19	0.51±0.08	0.33
D1-2	1.15±0.19	1.00±0.04	0.72	1.15±0.19	1.00±0.04	0.72
D2-3	0.75±0.06	0.22±0.01	0.17	0.90±0.01	0.48±0.08	0.26
D3-4	0.75±0.10	1.02±0.17	0.33	0.49±0.06	-0.29±0.03	0.15

Growth Rate Control +Bmpr1a-/- in 2μM PD184352

Day 0

	Control - 2μM PD184352			Control DMSO		
	separate	co-culture	t-test	separate	co-culture	t-test
D0-1	0.74±0.09	1.09±0.10	0.08	0.90±0.12	1.01±0.07	0.45
D1-2	1.89±0.19	1.13±0.14	0.16	1.73±0.11	1.36±0.10	0.28
D2-3	0.75±0.10	0.99±0.05	0.37	0.99±0.05	1.33±0.03	0.11
D3-4	0.45±0.12	0.58±0.12	0.30	0.55±0.17	0.98±0.18	0.02

	Bmpr1a-/- - 2μM PD184352			Bmpr1a-/- DMSO		
	separate	co-culture	t-test	separate	co-culture	t-test
D0-1	0.71±0.13	1.10±0.16	0.02	0.75±0.14	1.00±0.16	0.33
D1-2	1.58±0.09	1.30±0.13	0.12	1.50±0.15	1.42±0.04	0.78
D2-3	0.75±0.06	0.62±0.07	0.21	0.61±0.06	0.19±.07	0.14
D3-4	0.33±0.07	0.53±0.14	0.55	0.51±0.12	-0.35±0.08	0.02

Day 2

	Control - 2μM PD184352			Control DMSO		
	separate	co-culture	t-test	separate	co-culture	t-test
D0-1	0.99±0.15	0.85±0.02	0.53	0.99±0.15	0.85±0.02	0.53
D1-2	1.57±0.20	1.31±0.05	0.49	1.57±0.20	1.31±0.05	0.49
D2-3	0.67±0.13	1.42±0.07	0.02	0.87±0.07	1.41±0.04	0.01
D3-4	0.53±0.09	0.66±0.11	0.39	0.58±0.09	1.08±0.15	0.05

	Bmpr1a-/- - 2μM PD184352			Bmpr1a-/- DMSO		
	separate	co-culture	t-test	separate	co-culture	t-test
D0-1	0.74±0.24	0.86±0.11	0.57	0.74±0.24	0.86±0.11	0.57
D1-2	1.40±0.19	1.40±0.03	0.99	1.40±0.19	1.40±0.03	0.99
D2-3	0.59±0.08	0.24±0.13	0.04	0.77±0.05	0.36±0.09	0.02
D3-4	0.48±0.05	0.13±0.08	0.08	0.46±0.02	-0.30±0.02	0.00

Growth Rate Control +Bmpr1a-/- in 12.5μM PD98059

Day 0

	Control - 12.5μM PD98059			Control DMSO		
	separate	co-culture	t-test	separate	co-culture	t-test

D0-1	1.00±0.24	1.08±.16	0.59	1.26±0.19	1.35±.17	0.57
D1-2	1.49±0.14	1.27±0.15	0.01	1.45±0.12	1.17±0.12	0.05
D2-3	1.08±0.01	1.31±0.07	0.12	0.98±0.08	1.50±0.03	0.08
D3-4	0.36±0.11	0.92±0.06	0.03	0.57±0.05	0.87±0.06	0.00

Bmpr1a^{-/-} - 12.5μM PD98059				Bmpr1a^{-/-} DMSO		
	separate	co-culture	t-test	separate	co-culture	t-test
D0-1	0.87±0.22	0.93±0.22	0.72	0.97±0.26	1.17±0.22	0.26
D1-2	1.24±0.19	1.35±0.12	0.67	1.36±0.18	1.16±.09	0.26
D2-3	0.81±0.07	0.13±0.05	0.04	0.41±0.05	-0.45±0.02	0.00
D3-4	0.43±0.02	0.05±0.09	0.10	0.62±0.09	-0.22±0.11	0.02

Day 2

Control - 12.5μM PD98059				Control DMSO		
	separate	co-culture	t-test	separate	co-culture	t-test
D0-1	1.39±0.19	1.34±0.19	0.67	1.39±0.19	1.34±0.19	0.67
D1-2	1.20±0.17	1.14±0.16	0.81	1.20±0.17	1.14±0.16	0.81
D2-3	0.92±0.09	1.50±0.00	0.04	1.02±0.10	1.56±0.01	0.05
D3-4	0.39±0.03	0.70±0.07	0.12	0.60±0.10	0.78±0.04	0.22

Bmpr1a^{-/-} - 12.5μM PD98059				Bmpr1a^{-/-} DMSO		
	separate	co-culture	t-test	separate	co-culture	t-test
D0-1	0.94±0.25	1.10±0.23	0.24	0.94±0.25	1.10±0.23	0.24
D1-2	1.32±0.16	1.16±0.13	0.35	1.32±0.16	1.16±0.13	0.35
D2-3	0.76±0.03	-0.11±0.03	0.01	0.63±0.07	-0.15±0.03	0.02
D3-4	0.29±0.06	-0.31±0.12	0.03	0.52±0.07	-0.38±0.15	0.03

Growth Rate Control +Bmpr1a^{-/-} in 10μM U0126

Day 0

Control - 10μM U0126				Control DMSO		
	separate	co-culture	t-test	separate	co-culture	t-test
D0-1	1.36±0.02	1.58±0.03	0.01	1.20±0.17	1.72±.06	0.51
D1-2	1.06±0.02	0.82±0.03	0.02	1.18±.17	0.96±0.02	0.48
D2-3	0.80±0.02	1.03±0.02	0.04	1.05±0.06	1.25±.05	0.21
D3-4	0.43±0.12	0.57±0.15	0.31	0.39±0.03	1.05±.04	0.02

Bmpr1a^{-/-} - 10μM U0126				Bmpr1a^{-/-} DMSO		
	separate	co-culture	t-test	separate	co-culture	t-test
D0-1	0.80±0.14	1.28±0.07	0.04	0.98±0.16	1.28±.05	0.21
D1-2	1.17±0.15	0.88±0.06	0.31	1.31±0.09	1.14±0.01	0.37
D2-3	0.78±0.05	0.69±0.09	0.36	0.45±0.11	-0.35±0.03	0.02

D3-4	0.22±0.05	0.11±0.14	0.62	0.38±.04	-0.49±0.03	0.00
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Day 2

Control - 10µM U0126

	separate	co-culture	t-test
D0-1	1.50±0.06	1.55±0.08	0.82
D1-2	1.58±0.21	1.09±0.05	0.31
D2-3	0.62±0.16	1.27±0.10	0.03
D3-4	-0.07±0.05	0.47±0.13	0.09

Control DMSO

	separate	co-culture	t-test
D0-1	1.50±0.06	1.55±0.08	0.82
D1-2	1.58±0.21	1.09±0.05	0.31
D2-3	0.61±0.26	1.26±0.01	0.21
D3-4	0.30±0.17	0.71±0.04	0.15

Bmpr1a-/- - 10µM U0126

	separate	co-culture	t-test
D0-1	1.00±0.15	1.31±0.04	0.17
D1-2	1.38±0.17	1.10±0.08	0.29
D2-3	0.44±0.10	-0.07±0.04	0.12
D3-4	0.01±0.07	-0.99±0.17	0.07

Bmpr1a-/- DMSO

	separate	co-culture	t-test
D0-1	1.00±0.15	1.31±0.04	0.17
D1-2	1.38±0.17	1.10±0.08	0.29
D2-3	0.45±0.10	-0.13±0.06	0.04
D3-4	0.26±0.09	-0.65±0.03	0.03

Growth Rate Control +Bmpr1a-/- in 100ng/ml PD173074

Day 0

Control - 100ng/ml PD173074

	separate	co-culture	t-test
D0-1	0.90±0.12	0.96±0.13	0.90
D1-2	1.54±0.12	0.97±0.20	0.10
D2-3	0.75±0.15	1.02±0.06	0.25
D3-4	0.44±0.18	0.48±0.12	0.58

Control DMSO

	separate	co-culture	t-test
D0-1	1.22±0.08	1.19±.08	0.69
D1-2	1.53±0.06	1.35±0.15	0.41
D2-3	0.99±.13	1.18±0.16	0.76
D3-4	0.57±0.11	0.90±0.08	0.08

Bmpr1a-/- - 100ng/ml PD173074

	separate	co-culture	t-test
D0-1	0.44±0.15	0.74±0.14	0.01
D1-2	1.05±0.11	1.00±0.25	0.29
D2-3	0.80±0.07	0.92±0.03	0.36
D3-4	0.58±.10	0.45±0.18	0.88

Bmpr1a-/- DMSO

	separate	co-culture	t-test
D0-1	0.51±0.04	0.92±0.13	0.02
D1-2	1.42±0.17	1.13±0.23	0.21
D2-3	0.48±.02	-0.08±0.12	0.03
D3-4	0.59±.06	-0.53±.30	0.15

Day 2

Control - 100ng/ml PD173074

	separate	co-culture	t-test
D0-1	1.19±0.08	0.65±0.21	0.17
D1-2	1.47±0.08	1.82±0.14	0.12
D2-3	0.73±0.01	1.00±0.07	0.11
D3-4	0.10±0.07	0.34±0.20	0.30

Control DMSO

	separate	co-culture	t-test
D0-1	1.19±.08	0.66±0.21	0.18
D1-2	1.47±.08	1.81±0.14	0.12
D2-3	0.78±0.01	1.01±0.10	0.19
D3-4	0.61±0.02	1.25±.15	0.08

Bmpr1a-/- - 100ng/ml PD173074				Bmpr1a-/- DMSO		
	separate	co-culture	t-test	separate	co-culture	t-test
D0-1	0.27±0.07	0.27±0.17	0.99	0.27±0.07	0.26±0.17	0.96
D1-2	1.74±0.05	1.83±0.12	0.45	1.74±0.05	1.84±0.11	0.42
D2-3	0.09±0.04	-0.53±0.02	0.01	0.38±0.04	0.03±0.05	0.02
D3-4	0.42±0.05	0.15±0.14	0.18	0.64±0.06	-0.51±0.10	0.03

Control - 5ng/ml FGF4				Control 1ug/ml Heparin		
	separate	co-culture	t-test	separate	co-culture	t-test
D0-1	1.22±0.10	1.29±0.13	0.71	1.61±0.13	1.40±0.25	0.82
D1-2	1.62±0.10	1.26±0.06	0.19	1.50±0.09	1.28±0.15	0.44
D2-3	0.62±0.06	1.27±0.07	0.02	0.63±0.08	1.13±0.18	0.06
D3-4	0.71±0.11	1.03±0.13	0.09	0.38±0.13	1.01±0.08	0.00

Bmpr1a-/- - 100ng/ml PD173074				Bmpr1a-/- 1ug/ml Heparin		
	separate	co-culture	t-test	separate	co-culture	t-test
D0-1	0.90±0.04	1.06±0.16	0.61	0.97±0.07	1.19±0.08	0.68
D1-2	1.44±0.07	1.36±0.09	0.21	1.52±0.07	1.31±0.11	0.95
D2-3	0.54±0.14	-0.09±0.13	0.04	0.39±0.06	-0.26±0.18	0.03
D3-4	0.44±0.06	-0.60±0.12	0.04	0.22±0.07	-0.58±0.06	0.00

Growth Rate Control +Bmpr1a-/- in 10ng/ml FGF5

Day 0

Control - 10ng/ml FGF5				Control 1ug/ml Heparin		
	separate	co-culture	t-test	separate	co-culture	t-test
D0-1	1.27±0.02	1.22±0.14	0.52	1.61±0.13	1.40±0.25	0.82
D1-2	1.67±0.05	1.5±0.08	0.54	1.50±0.09	1.28±0.15	0.44
D2-3	0.53±0.03	0.91±0.07	0.08	0.63±0.08	1.13±0.18	0.06
D3-4	0.57±0.02	1.16±0.02	0.02	0.38±0.13	1.01±0.08	0.00

Bmpr1a-/- - 10ng/ml FGF5				Bmpr1a-/- 1ug/ml Heparin		
	separate	co-culture	t-test	separate	co-culture	t-test
D0-1	0.83±0.10	0.97±0.16	0.43	0.97±0.07	1.19±0.08	0.68
D1-2	1.56±0.08	1.57±0.07	0.49	1.52±0.07	1.31±0.11	0.95
D2-3	0.47±0.12	-0.21±0.12	0.19	0.39±0.06	-0.26±0.18	0.03
D3-4	0.23±0.05	-0.95±0.04	0.02	0.22±0.07	-0.58±0.06	0.00

Growth Rate Control +Bmpr1a-/- in 2μM IWR-1

Day 0

	Control - 2μM IWR-1			Control DMSO		
	separate	co-culture	t-test	separate	co-culture	t-test
D0-1	1.23±0.07	1.17±0.06	0.13	1.48±0.15	1.17±.12	0.36
D1-2	1.57±0.10	1.46±.15	0.31	1.40±0.19	1.55±0.13	0.76
D2-3	0.53±0.05	1.07±0.10	0.04	0.52±.05	1.06±0.11	0.14
D3-4	0.44±0.03	0.80±0.10	0.08	0.52±0.05	0.78±0.14	0.17

	Bmpr1a/- - 2μM IWR-1			Bmpr1a/- DMSO		
	separate	co-culture	t-test	separate	co-culture	t-test
D0-1	0.62±0.06	1.04±0.08	0.17	1.07±0.07	0.97±.11	0.72
D1-2	1.85±0.11	1.38±0.18	0.10	1.26±0.19	1.60±.12	0.50
D2-3	0.12±0.10	-0.50±0.16	0.03	0.35±0.10	-0.37±0.09	0.04
D3-4	0.52±.05	-0.54±0.19	0.05	0.45±0.04	-0.22±0.08	0.03

Day 2

	Control - 2μM IWR-1			Control DMSO		
	separate	co-culture	t-test	separate	co-culture	t-test
D0-1	1.25±0.06	1.20±0.04	0.54	1.25±0.06	1.20±.04	0.54
D1-2	1.58±.02	1.47±0.07	0.49	1.58±0.02	1.47±0.07	0.49
D2-3	0.64±0.10	1.08±0.04	0.10	0.44±0.04	1.06±0.05	0.03
D3-4	0.45±0.12	0.73±0.11	0.01	0.49±.05	0.61±0.06	0.01

	Bmpr1a/- - 2μM IWR-1			Bmpr1a/- DMSO		
	separate	co-culture	t-test	separate	co-culture	t-test
D0-1	0.95±0.11	1.01±.06	0.63	0.95±0.11	1.01±.06	0.63
D1-2	1.43±0.25	1.48±0.13	0.79	1.43±0.25	1.48±.13	0.79
D2-3	0.35±0.11	-0.37±0.08	0.01	0.39±0.12	-0.33±0.15	0.00
D3-4	0.35±0.03	-0.51±0.13	0.06	0.31±0.04	-0.32±0.07	0.05

Growth Rate Control +Bmpr1a/- in 2μM IWP-2

Day 0

	Control - 2μM IWP-2			Control DMSO		
	separate	co-culture	t-test	separate	co-culture	t-test
D0-1	0.56±0.23	0.90±0.20	0.05	1.09±0.04	1.20±0.11	0.49
D1-2	2.05±0.26	1.50±0.26	0.35	1.70±0.18	1.31±.16	0.04
D2-3	0.66±0.26	1.24±.17	0.39	0.51±0.17	1.11±0.08	0.09
D3-4	0.27±0.07	0.71±0.21	0.15	0.42±0.12	0.71±.11	0.09

	Bmpr1a/- - 2μM IWP-2			Bmpr1a/- DMSO		
	separate	co-culture	t-test	separate	co-culture	t-test
D0-1	1.19±0.03	0.88±.15	0.23	1.00±0.05	1.22±0.15	0.45

D1-2	1.20±0.12	1.63±0.17	0.37	1.53±0.14	1.36±.25	0.48
D2-3	0.20±0.17	-0.38±0.17	0.04	0.30±0.07	-0.34±0.21	0.12
D3-4	0.39±.11	-0.66±0.20	0.07	0.22±0.05	-0.30±0.12	0.17

Day 2

Control - 2μM IWP-2				Control DMSO		
	separate	co-culture	t-test	separate	co-culture	t-test
D0-1	0.92±0.16	1.03±0.11	0.69	0.92±0.16	1.03±0.11	0.69
D1-2	1.71±0.21	1.33±0.09	0.46	1.71±.21	1.33±0.09	0.46
D2-3	0.66±0.15	1.21±0.03	0.14	0.62±.12	1.33±0.12	0.15
D3-4	0.42±0.09	0.81±0.15	0.08	0.40±.10	0.61±0.21	0.31

Bmpr1a/- - 2μM IWP-2				Bmpr1a/- DMSO		
	separate	co-culture	t-test	separate	co-culture	t-test
D0-1	1.29±.07	0.88±0.10	0.05	1.29±.07	0.88±0.10	0.05
D1-2	1.08±0.14	1.54±.08	0.10	1.08±0.14	1.54±.08	0.10
D2-3	0.36±0.11	-0.25±0.21	0.09	0.36±0.16	-0.20±0.22	0.08
D3-4	0.29±0.04	-0.44±0.19	0.15	0.39±0.11	-0.27±.19	0.08

Growth Rate Control +Bmpr1a/- in 3μM CHIRON99201

Day 0

Control - 3μM CHIRON99201				Control DMSO		
	separate	co-culture	t-test	separate	co-culture	t-test
D0-1	1.06±0.11	1.55±0.09	0.02	1.40±0.58	1.56±0.10	0.58
D1-2	1.49±0.09	1.27±0.07	0.04	1.43±0.04	0.92±0.13	0.10
D2-3	0.86±0.01	0.53±0.05	0.03	0.73±0.15	1.33±0.11	0.02
D3-4	0.79±0.01	0.78±0.08	0.91	0.71±0.10	1.13±0.08	0.05

Bmpr1a/- - 3μM CHIRON99201				Bmpr1a/- DMSO		
	separate	co-culture	t-test	separate	co-culture	t-test
D0-1	1.15±0.04	1.34±0.02	0.16	1.16±0.12	1.43±0.04	0.32
D1-2	1.19±0.08	1.28±0.03	0.45	1.11±0.05	1.08±0.10	0.92
D2-3	0.76±0.07	0.59±0.01	0.29	0.52±0.07	-0.30±0.04	0.00
D3-4	0.64±.04	0.60±0.10	0.85	0.57±0.14	-0.48±0.22	0.07

Day 2

Control - 3μM CHIRON99201				Control DMSO		
	separate	co-culture	t-test	separate	co-culture	t-test
D0-1	1.37±0.17	1.29±0.13	0.72	1.37±0.17	1.29±0.13	0.72
D1-2	1.46±0.07	1.35±0.22	0.30	1.46±0.07	1.19±0.15	0.30
D2-3	0.67±0.14	0.88±0.23	0.03	0.71±0.09	1.24±0.11	0.05

D3-4	0.62±0.14	1.01±0.14	0.06	0.70±.12	1.14±0.09	0.11
Bmpr1a^{-/-} - 3μM CHIRON99201				Bmpr1a^{-/-} DMSO		
	separate	co-culture	t-test	separate	co-culture	t-test
D0-1	1.04±0.15	1.15±0.11	0.93	1.04±0.15	1.15±0.11	0.93
D1-2	1.35±0.17	1.16±0.22	0.30	1.35±.17	1.36±0.13	0.30
D2-3	0.55±0.07	0.41±0.24	0.01	0.47±0.13	-0.11±0.11	0.02
D3-4	0.50±0.10	-0.14±0.07	0.00	0.53±0.08	-0.39±0.15	0.02

A.2 Ratio wild-type/*Bmpr1a*^{-/-} mESCs normalised to day 3 of treatment

Appendix Table 2 - Ratio of wild-type/*Bmpr1a*^{-/-} mESCs after 3 days of culture All data normalised to ratio after 3 days of culture.

All data shown as average $\pm$ s.e.m; unless otherwise stated n=3

	1μM PD0325901	DMSO	t-test
co-culture	1.15	2.95	
separate	0.47	0.65	n=1
	2μM PD184352	DMSO	
co-culture	2.21 $\pm$ 0.19	2.36 $\pm$ 0.21	0.14
separate	1.62 $\pm$ 0.22	1.60 $\pm$ 0.11	
	12.5μM PD98059	DMSO	
co-culture	2.61 $\pm$ 0.43	2.51 $\pm$ 0.13	0.79
separate	1.12 $\pm$ 0.23	1.41 $\pm$ 0.17	
	10μM U0126	DMSO	
co-culture	2.71 $\pm$ 0.31	3.14 $\pm$ 0.09	0.26
separate	1.25 $\pm$ 0.30	1.40 $\pm$ 0.12	
	100ng/ml PD173074	DMSO	
co-culture	1.90 $\pm$ 0.34	2.10 $\pm$ 0.20	n=2
separate	0.84 $\pm$ 0.16	1.05 $\pm$ 0.24	