

Pluripotent Stem Cell Model of Pulmonary Arterial Hypertension

PhD

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Abstract

Pulmonary arterial hypertension is a debilitating disease that results in obstruction of the pulmonary vessels resulting in right heart failure. Patients diagnosed with Group I heritable pulmonary arterial hypertension (HPAH) have a mean survival rate of 3 years. 70-80% of HPAH patients have a bone morphogenetic receptor 2 (*BMPR2*) mutation and, in particular, young women are predisposed to this fatal disease. Thus, *BMPR2* was identified to be one of the causes of PAH. However, this mutation is only 20% penetrant. Giving rise to several hypotheses such as haploinsufficiency, loss of function, gain of function, dominant negative and environmental insults leading to the progression of disease. Currently, these hypotheses have been investigated in animal models that do not fully recapitulate PAH in humans. In order to understand the function of *BMPR2* in human tissue development and disease we have utilised pluripotent stem cells as a human model system.

We developed a suite of human pluripotent stem cell (PSC) lines containing an allelic series of *BMPR2* mutations, including a patient-specific truncating mutation (2504delC). These PSC lines enabled the *in vitro* dissection of the role of *BMPR2* in the development and function of the major cell lineages of the blood vessel. Using these reagents, we identified a key role for *BMPR2* in the regulation of endothelial migration, cell fate decisions, angiogenesis and proliferation. Importantly, compensatory mechanisms, likely via alternate receptors such as ACVR2A/B, were identified that activated both SMAD-dependant and independent signalling when *BMPR2* was perturbed. Thus, *BMPR2* is not essential for the differentiation of PSCs to mesoderm,

cardiomyocytes, blood cells, smooth muscle cells (SMCs) and endothelial cells (ECs), *in vitro*. However, *BMPR2* mutant cardiomyocytes displayed an altered transcriptional response to BMP4 exposure. *BMPR2* mutant SMCs had cytoskeletal defects evident by distinct stress fibres. Phenotypic characterization of *BMPR2* mutant ECs revealed that a number of endothelial cellular functions relied upon *BMPR2*. Firstly, *BMPR2* mutants displayed defects in a tubule formation assay. Secondly, upon passaging *BMPR2* mutant ECs maintained a uniform morphology and expression of the EC surface markers, unlike wild type ECs. Furthermore, all *BMPR2* mutant genotypes displayed more rapid cell migration kinetics than wild type ECs. Metabolomic data and transcriptional data showed that *BMPR2* mutant ECs have dysregulated metabolism and related-gene expression. Taken together, *BMPR2* appears to have a protective function that maintains homeostasis of the vessel wall. These studies pave the way for future work to elucidate the mechanisms underlying *BMPR2* mediated PAH.

General Declaration

I declare that this thesis contains no material that has been accepted for any other degree or diploma at any university or equivalent institution. To the best of my knowledge and belief, this thesis contains no material previously published or written by any other person except where due referenced in the preface and text of this thesis.

This thesis consists of not more than 100,000 words. The ideas, development and writing of all chapters in this thesis were the principal responsibility of myself, the candidate, under the supervision and guidance of Dr David Elliott, Prof. Edouard Stanley and Dr Michael Cheung.

Overall, my contribution to the work included in this thesis is 100%.

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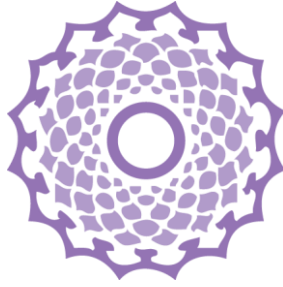
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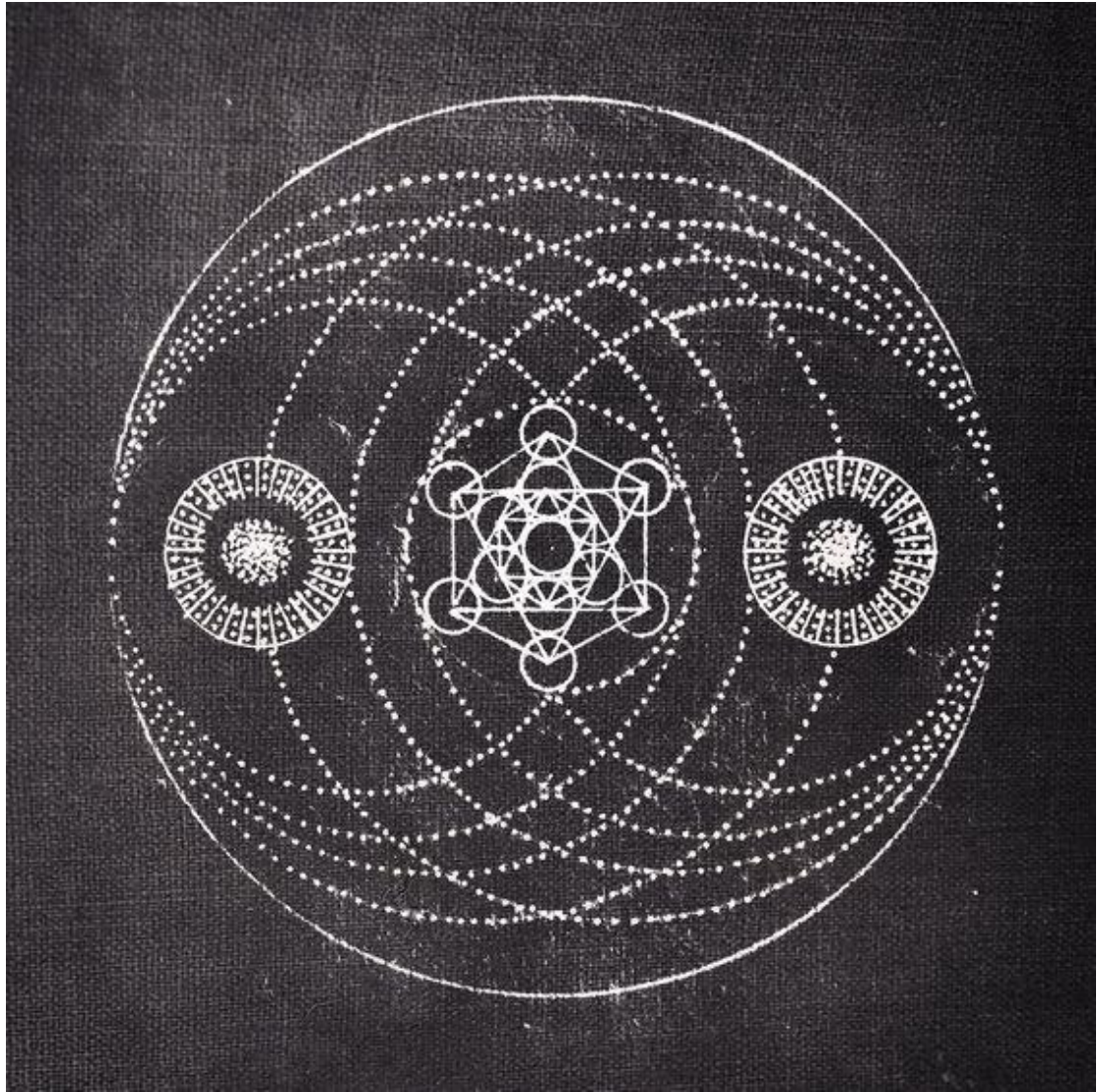
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The pursuit of understanding nature’s greatest mystery; existence, is what drives me. Ultimately, liberation from this question. “Science is the art of understanding”. From my perspective, the best sufferers are often the best at being happy, hence I will continue to embrace whatever comes and let go of whatever goes, to attain peace within.



“Through Nature, I learn to be free.”



List of Abbreviations

aa	Amino acid
ACVR2	Activin Receptor Type 2
Ac-LDL	Acetylated low density lipoprotein
AEL	Albumin essential lipids
APEL	Albumin polyvinylalcohol essential lipids
bFGF	Basic fibroblast growth factor
BMP	Bone morphogenic protein
BMPR2	Bone morphogenic receptor two
BF	Bright field
Bp	Base pair
BSA	Bovine serum albumin
Cas9	CRISPR associated protein 9
Cre	Cyclization recombination
CRISPR	Clustered regularly interspaced short palindromic repeats
CXCR4	Chemokine (C-X-C motif) receptor 4
D	Day
DAPI	4'-6' diamidino-2-phenylindole
DMH-1	Dorsomorphin homologue-1
DMSO	Dimethylbrose nucleic acid
E	Embryonic (day)
EB	Embryoid body
EC	Endothelial cell
E-CAD	E-cadherin
ECM	Extracellular matrix
EDTA	Ethylenediamine-tetra-acetic acid
EGF	Epidermal growth factor
eGFP	Enhanced green fluorescent protein
FACS	Fluorescence activated cell sorting
FBS	Fetal bovine serum
FGF	Fibroblast growth factor
G1-G6	Guide
hESC	Human embryonic stem cell
HET	Heterozygote
HUVEC	Human umbilical vein endothelial cell
IL-3	Interleukin 3
IL-6	Interleukin 6
KDR	Vascular endothelial growth factor receptor 2
KO	Knockout

μg	Microgram
μL	Microliter
μM	Micromolar
MEF	Mouse embryonic fibroblast
mM	Millimolar
mg	Milligram
MOPs	3-(N-morpholino)propanesulfonic acid
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
ng	Nanogram
NKX2-5	NK2 Homeobox 5
P1-P3	Probe
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PDGFR-α	Platelet-derived growth factor receptor alpha
PAH	Pulmonary arterial hypertension
PVDF	Polyvinylidene difluoride
PSC	Pluripotent stem cell
RNA	Ribonucleic acid
SCF	Stem cell factor
sgRNA	Single-guide RNA
SNP	Single nucleotide polymorphism
SMC	Smooth muscle cell
TGF-β	Transforming growth factor beta
VEGF	Vascular endothelial growth factor
vWF	Von willebrand factor
WNT	Wingless-related MMTV integration site

CHAPTER



Literature Review

Introduction

1.1 Clinical characteristics of Pulmonary Arterial Hypertension (PAH)

Pulmonary arterial hypertension (PAH) is a disease of the pulmonary vessels leading to right heart dysfunction and ultimately failure. The underlying causes of PAH range from genetic variation to environmental insults. It is estimated that almost 100 million people worldwide suffer from PAH (Simonneau et al. 2009). Patients diagnosed with PAH have a mean survival rate of 3 years (Archer, Weir, & Wilkins, 2010). This disease is more predominant in young women, with a 2.3:1 female to male ratio (Morrell 2010).

Initial PAH classifications, in 1973, designated only two categories, primary or secondary pulmonary hypertension. Primary PAH was defined by having identifiable causes or risk factors whereas secondary PAH was idiopathic. Subsequently, PAH nomenclature has evolved into several subgroups in order to accurately represent the clinical presentation of the disease. Pulmonary arterial hypertension (PAH) is now stratified into 5 categories. These 5 groups were categorized at the 4th World Symposium on Pulmonary Hypertension held by the World Health Organization (WHO) shown in **Table 1**.

For this review, the focus will be on Group I, heritable pulmonary artery hypertension (HPAH). Most heritable PAH are caused by a mutation in the *Bone Morphogenetic Protein Receptor, type II (BMPR2)* gene, which is a member of the transforming growth factor beta (TGF- β) receptor signalling

family. However, approximately 20% of individuals carrying a *BMPR2* mutation risk developing PAH (West et al. 2008a). The majority of disease associated *BMPR2* mutations are frame shift or nonsense mutations. Missense mutations of *BMPR2* are less common. In addition, improved sequencing technologies and genetic analysis has led to the identification of mutations in a range of other members of the TGF- β signalling cascade. These include *activin receptor like kinase type 1* (*ACVRL1* or *ALK1*), *endoglin* (*ENG*) and genes encoding intracellular components of the BMP pathway (e.g. *SMAD8*, *SMAD9*)(Soubrier et al. 2013). Given these findings we will discuss the molecular pathogenesis of PAH with a focus on BMP signalling.

Current Diagnostic classification of Pulmonary Hypertension:**Group I** Pulmonary Arterial Hypertension (PAH)

- 1.1 Idiopathic
- 1.2 Heritable (Formally known as familial pulmonary arterial hypertension)
- 1.3 Caused by Drugs & Toxins
- 1.4 Association with (APAH)
 - 1.4.1 Connective tissue disease
 - 1.4.2 Infection with HIV
 - 1.4.3 Portal hypertension
 - 1.4.4 Congenital heart disease
 - 1.4.5 Schistosomiasis
 - 1.4.6 Chronic haemolytic anaemia
- 1.5 Persistent pulmonary hypertension of the newborn

Group II

Pulmonary hypertension resulting from left heart disease mostly representing a large cohort of patients suffering from pulmonary venous hypertension.

Group III

All forms of pulmonary hypertension associated with alveolar hypoxia and/or disorders of the respiratory system.

Group IV

All embolic diseases including thrombosis leading to pulmonary hypertension.

Group V

Pulmonary hypertension that is associated with multiple disease affecting the pulmonary vessels.

Table 1. Classification of Pulmonary Hypertension. PAH is categorized into five main groups. Adapted from (Schermuly et al. 2011, Simonneau et al. 2004).

1.2 Clinical definition of PAH

There are few defining symptoms that reliably identify the onset of PAH. However, clinical presentation is often related either to right heart failure or association with other diseases. Persistent dyspnea (shortness of breath) on exertion is the most frequent symptom of PAH (David Montani and Sitbon 2013). Patients usually present with much higher levels of pulmonary arterial (PA) pressure with vague symptoms of increasing fatigue and dyspnea. Some

patients are diagnosed only after several fainting episodes, resulting from low cardiac output (David Montani and Sitbon 2013).

PAH is diagnosed by a persisting elevation of mean PA pressure above the average normal pressure of 25 mmHg at rest or 30 mmHg with exercise. It is also diagnosed by inserting a catheter with an inflated balloon wedging against the pulmonary artery. This pressure recorded with deflation of the balloon is known as pulmonary artery wedge pressure (PCWP). The normal range of PCWP is within 2-15mmH (Humbert and Trembath 2002).

Despite the low penetrance of *BMPR2* mutations, it accounts for 70-80% of HPAH and 11-40% of idiopathic cases with no family history (Machado et al. 2001). As all patients that have *BMPR2* mutations have heritable disease, hence, it was decided that the term “familial PAH” be abandoned in favor of the term “heritable PAH”. This also encompasses all idiopathic PAH with germline mutations and familial cases with or without identified mutations (Chaouat 2004).

1.3 Pathology of PAH

Pathologically, PAH is defined as a disease of the small pulmonary arteries, diagnosed by chronic obstruction or narrowing vasculature. This is caused by endothelial cell (EC) and smooth muscle cell (SMC) dysfunction. The consequence of this is a progressive effect of increased pulmonary vascular resistance (i.e. blood pressure), increased right ventricular output leading to right ventricular hypertrophy, and eventually heart failure (Humbert and Trembath 2002).

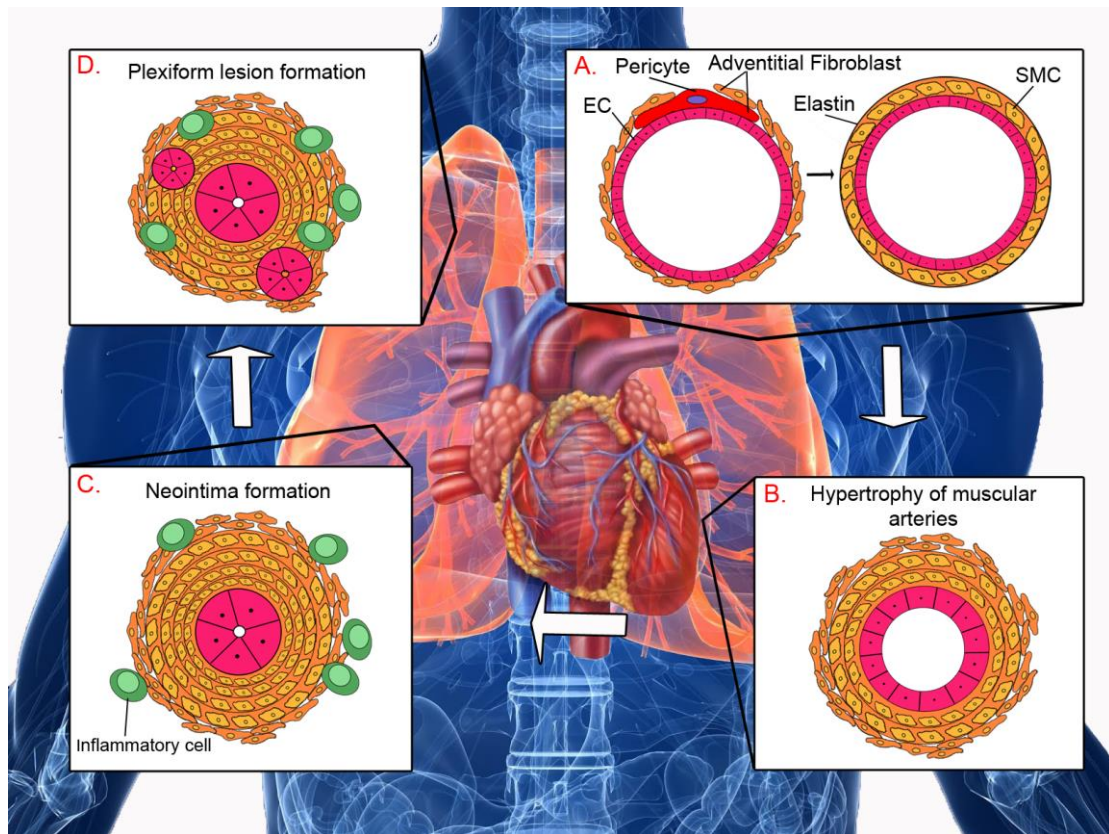


Figure 1. Schematic demonstrating the remodelling of pre-capillary arteries in a PAH patient. (A) The condition begins with abnormal distal muscle formation followed by **(B)** proliferation of fibroblast and SMC that causes hypertrophy of muscular capillaries. **(C)** This leads to formation of neointima and plexiform lesion, **(D)** resulting in severely narrowed blood vessels that increase pulmonary blood pressure.

The main factors that contribute to the increased pulmonary vascular resistance in PAH are vasoconstriction, vascular remodelling and thrombosis. However, obstruction and remodelling of the pulmonary artery by vascular proliferation is now considered the hallmark of PAH pathogenesis (Montani et al., 2005). The arterial wall is a dynamic three-dimensional structure that copes with fluctuations of blood pressure, flow, and shear stress taking place during development and in vascular function. In order to cope with these

factors, cellular and extracellular components are assembled in a specific manner with three distinct layers, the adventitia (outer), media (middle) and intima (inner). The outermost adventitia layer consists of fibroblasts, while the media layer is primarily composed of SMCs with one or two layers of elastic laminae. Finally, the innermost intima is composed of a single layer of ECs. Within each layer, there are extracellular matrix components such as collagen, elastin, fibronectin and proteoglycans, which are involved in architecture, tensile strength, elasticity, cell migration and proliferation. The process of pulmonary vascular remodelling involves all layers of the vessel wall and severe forms of PAH are characterized by thickening of all 3 layers of the pulmonary vessels because of hypertrophy/hyperplasia and increased deposition of extracellular matrix components (Humbert et al. 2003).

A common characteristic of PAH is the remodelling of the pulmonary artery and proliferation of smooth muscle cells in the small peripheral pulmonary arteries within the normally non-muscular respiratory acinus (**Fig. 1A**). The cellular process causing this remodelling is not fully understood. It has been shown in hypoxic models, that fibroblasts from the adventitia are the first to be activated during stimulus, resulting in their proliferation and migration into the medial and intima layers. These fibroblasts differentiate into smooth muscle-like cells that proliferate and produce matrix proteins that are deposited along the arterial walls (Misra et al. 2010). This repair mechanism, which normally occurs after injury to the vascular wall, can be altered in PAH patients leading to hypertrophy of the arterial walls (**Fig. 1B**).

Hypoxic pulmonary vasoconstriction occurs even at mild levels of alveolar hypoxia. At these levels of physiologic hypoxia, patients are asymptomatic with subsequent disease progression often undiagnosed (Bonnet et al. 2006). The severity of pulmonary hypertension increases when neointima occurs (**Fig. 1C**). Neointima describes the formation of a layer of myofibroblasts and extracellular matrix between the endothelium and the internal elastic lamina. Evidence suggests that upregulation of matrix metalloproteinases (MMP and MMP9) is involved in the migration of the adventitial fibroblasts (Marc Humbert et al., 2004a). Neovascularization, mainly of the adventitia, occurs concomitantly with thickening of the vascular walls although it can extend into the outer parts of the media (Davie et al., 2004).

Finally, the hallmark of severe PAH is the formation of plexiform lesions that contributes to vascular obstruction (**Fig. 1D**). Plexiform lesions consist of deposition of disorganized ECs, matrix proteins and fibroblasts and obstruction of the vascular lumen. These cells have been postulated to come from a specialized subpopulation of SMCs, proposed to be the daughters cells of a vascular stem cell pool (Passman et al. 2008), fibrocytes or transformed from ECs. The loss of distal PAs could be caused by apoptosis in ECs and/or pericytes. Interestingly, in patients with IPAH, 30% of plexiform lesions have a somatic frameshift mutation in the *transforming growth factor, beta receptor* (*TGF- β R2*) encoding a premature stop codon (Marc Humbert et al., 2004b). Further, 90% of plexiform lesions do not express *TGF- β R2* in contrast to normal ECs outside of the lesion. In addition, some PAH patients have somatic mutations in *TGF- β R2* that allow clonal expansion of ECs, which may contribute to plexiform formations and vascular remodelling (Yeager et al.

2001).

The inflammatory response plays a major role in PAH. Patients with severe cases of PAH associated with systemic inflammatory diseases such as systemic lupus erythematosus disease, respond to immunosuppression with improved hemodynamics and clinical outcomes (Bonnet et al. 2006). Approximately 30 to 40% of these patients have evidence of circulating auto-antibodies and elevated plasma concentrations of pro-inflammatory cytokines such as interleukin 1 (IL-1), interleukin-6 (IL-6), and chemokines such as fractalkine and MCP-1 (David Montani and Sitbon 2013). Inflammatory cells, such as lymphocytes B and T, macrophages, mastocytes and dendritic cells, are found in plexiform lesions of severe PAH (Balabanian et al. 2002). Chemokines, like RANTES and fractalkine are also overly expressed in the pulmonary vascular endothelium of PAH patients (Balabanian et al. 2002). Further, intravascular procoagulants (platelets) and fibronolytic (inhibits formation of blood clots/thrombosis) activities are altered in the pulmonary endothelium of PAH patients (Humbert et al. 2004). In addition to being a coagulant, platelets store and release pulmonary vasoconstriction factors such as thromboxane A₂, platelet derived growth factor (PDGF), serotonin (5-hydroxytryptamine (5-HT)), TGF- β , and vascular endothelial growth factor (VEGF). Thus, inflammatory cells and the cytokines they secrete are likely to play a role in the final stages of plexiform lesion formation.

Endothelial dysfunction in PAH patients is related to abnormal functioning of potassium channels and excessive vasoconstriction. Reduced expression of exogenous vasodilators such as nitric oxide (NO) and prostacyclin and

overexpression of vasoconstrictors like endothelin (ET)-1 alters vascular tone and promotes vascular remodelling (Humbert et al. 2004). PAH patients have been reported to have reduced expression of NO synthase in their lungs, which may permit proliferation of vascular SMCs (Giaid and Saleh 1995) and prostacyclin (Christman.B et al., 1992) which increases platelet aggregation.

1.4 Genetic causes of PAH

In 1997, Nichols *et al.* carried out a genome-wide microsatellite marker search using a set of polymorphic short-tandem repeat markers in 19 individuals with PAH from 6 families. A significant linkage to a marker on the long arm of chromosome 2 was identified. Subsequent genetic mapping defined the region containing the candidate causative gene to 2q31-33. Evaluation of 81 genes at this region led to identification of mutations in the *BMPR2* gene in all affected individuals (William C. Nichols 1997). Currently, there are more than 140 different *BMPR2* mutations associated with PAH cases. It is estimated that 11 to 40% of idiopathic PAH results from sporadic *BMPR2* mutations (Yu et al. 2005).

IPAH patients are reported to have significantly reduced wild type *BMPR2* expression in the pulmonary vasculature (Morrell 2006). These studies suggest that reduced expression of *BMPR2* may be important to the pathogenesis of PAH, even in the absence/presence of a genetic mutation. In addition, because the level of *BMPR2* expression in heritable cases was considerably lower than predicted from the state of haploinsufficiency, this suggests that some additional environmental or genetic factor may be

necessary to further reduce *BMPR2* expression below the threshold, which triggers profound vascular remodelling (Morrell 2006).

A rare group of patients carrying mutations in activin receptor-like kinase (*ALK1*), another member of the *TGF- β* receptor family develop PAH and hereditary hemorrhagic telangiectasia (HHT), known as Rendu-Olser disease. This autosomal dominant disease leads to abnormal formation of blood vessels in the skin, mucous linings, lungs, liver and brain (Farber 2006). Similar to *BMPR2* mutations, mutations in this type I receptor are believed to result in Smad-dependent growth. The resultant phenotype in Rendu-Olser patients suggests that the endothelium is in an activated proliferative state. BMP9 and BMP10 have been identified as ligands for a receptor complex comprising *BMPR2* and *ALK1*. Furthermore, BMP10 is expressed in the developing heart during embryogenesis (Chen et al. 2004) and BMP9 stimulates SMAD1/5/8 pathway in ECs. BMP9 and BMP10 are inhibitors of EC migration and proliferation (David et al. 2007, Morrell 2010). Thus, these molecules are important for regulating cellular homeostasis in the pulmonary vasculature.

In cases of germline mutations in *BMPR2*, a common aetiology associated with PAH suggests that BMPs have a pivotal role in maintaining normal pulmonary vascular physiology. Under normal conditions, BMP 2, 4, and 7 are known to suppress the growth of vascular SMCs. Therefore it is hypothesized that patients with *BMPR2* mutations might also be deficient in that process (Yu et al. 2008). Many reports state that *BMPR2* mutations lead to the loss of SMAD1/5/8 activity contributing to pulmonary remodelling and the onset of

PAH (Frump et al. 2013, Morrell 2010, Yu et al. 2008). Furthermore, reduced SMAD1 phosphorylation in cells of the media and intima layers of the pulmonary artery is observed in PAH patients (Aldred et al. 2006). These findings indicate that the down regulation of TGF- β /BMP signals via SMAD1/5/8 plays an important role in the pathogenesis of PAH (Shintani et al. 2009).

Two studies have shown that the mechanisms by which *BMPR2* mutants disrupt BMP/SMAD signalling are diverse and mutation specific (Nishihara et al. 2002). The missense mutations involving the substitution of cysteine residues within the ligand-binding or kinase domain of *BMPR2* leads to reduced trafficking of the mutant protein to the cell surface, a process that may interfere with BMP type I receptor trafficking (Morrell 2006). However, these mutations remain partially active because in both humans and in mouse models, individuals with these *BMPR2* mutations survive (Beppu et al. 2000), whereas mice that are homozygous null at the *Bmpr2* locus die in utero (Beppu et al., 2000). Mice carrying the disease related mutation do not spontaneously develop PAH but require further challenge (e.g. hypoxia and serotonin). In contrast, *Alk1*^{+/-} heterozygous deficient mice develop PAH demonstrating the importance of BMP signalling in PAH (Jerkic et al. 2011). This suggests that haploinsufficiency might be the underlying cause of heritable PAH in humans. Additionally, in a knockout mouse model targeted with a *BMPR2* hypomorphic construct that lacks half of the ligand-binding domain, *BMPR2* ^{$\Delta E2$} , homozygous mutants die mid-gestation with cardiovascular defects. These embryos display defects in the outflow tract of the heart, demonstrating the requirement for correct BMP signalling during

formation of the major pulmonary artery during embryogenesis (Delot, 2003).

BMPR2 belongs to a class of receptors in the *TGF- β* family and as many as 10–26% of patients with sporadic IPAH were found to carry a mutation in at least one member of the *TGF- β* receptor family (Machado et al. 2006). Du (Du et al. 2003) suggests that all forms of PAH have impaired vascular signalling network which comprises of *angiopoietin-1* (*ANGPT1*) and its endothelial-specific receptor, *TIE2*, *BMPR1A* and *BMPR2*. The IPAH cases have reduced *BMPR2* signalling as a result of the *Ang1/TIE2* mediated down regulation of *BMPR1A*. *BMPR1A* is a complementary type I receptor required for normal *BMPR2* signalling. Thus, this data links both genetic cases of PAH and the idiopathic cases to dysregulation of the BMP pathway. Furthermore, increased *ANGPT1* levels induces SMC hyperplasia and prevents endothelial apoptosis, both of which would lead to thickening of the PA wall (Rudge et al. 2003). Thus, *angiopoietin-1/TIE2* pathway plays a regulatory role in maintaining vasculature cellular homeostasis.

1.5 HPAH is prevalent in females

It is uncertain why heritable PAH as well as IPAH predominantly affects females, with a female to male ratio of 2.3:1 (Morell et al. 2010). This strongly suggests that hormonal influences predispose females to the condition (Aldred et al. 2006). The sex bias at presentation of the disease may implicate either a hormonal component or a role for an X-linked locus in disease predisposition. An alternative suggestion for the observed female predominance is that the genetic defects characteristic of the disease might

lead to male fetus loss (Pugh and Hemnes 2010). More females than males were born to subjects who were obligate carriers for the gene defect, suggesting selective disadvantage for male fetuses. Abnormal gender ratio of progeny was the first clinical finding to raise the possibility that the genes involved in PAH may play important roles during embryologic development (Sztrymf et al. 2007).

1.6 The Bone Morphogenetic Proteins (BMPS) and their receptors

Bone morphogenetic proteins (BMPs) are the largest group of cytokines within the TGF- β superfamily. BMPs were originally identified as molecules regulating growth and differentiation of bone and cartilage (Wozney et al., 1988). BMPs are now known to regulate development of a diverse number of cell lineages, including mesenchymal and epithelial cells. BMPs have essential roles in vertebrate embryogenesis including mesoderm induction, limb development, collagen synthesis, chondroblasts/osteoblasts maturation and hematopoiesis (Hogan 1996). Additionally, BMPs such as BMP2 and BMP4 have crucial roles in the induction of vertebrate cardiovascular development (Barron et al. 2000). Furthermore, BMP2 was found to inhibit vascular SMC proliferation in an *in-vivo* rat carotid artery balloon injury model (Nakaoka et al. 1997).

1.6.1 Type I and Type II receptors in the TGF- β family

BMPs interact with two classes of transmembrane receptors, called type I and type II receptors. There are seven type I receptors and three type II receptors for TGF- β cytokines. Type I receptors are denoted activin receptor like kinase1-7/ALK(1-7).

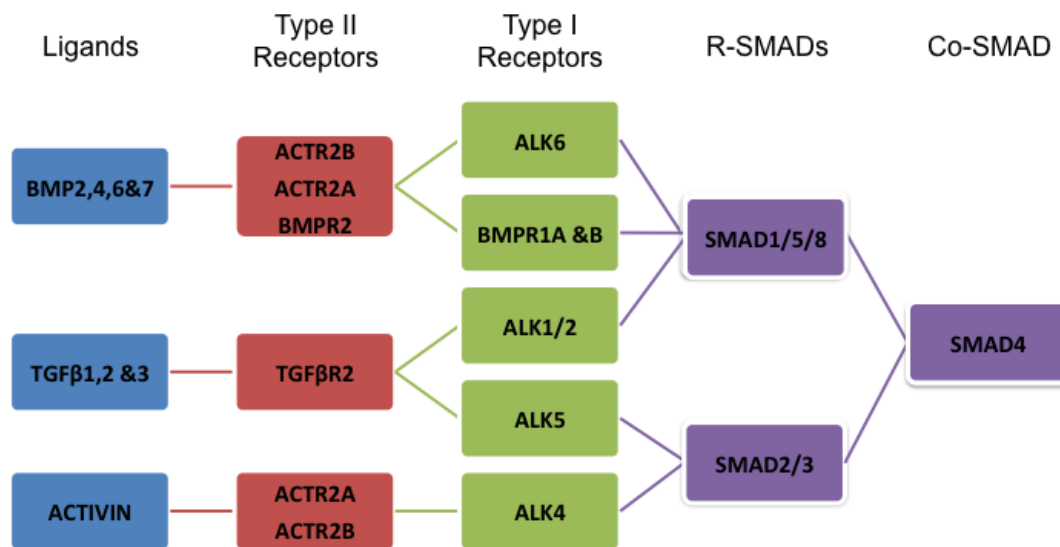


Figure 2. Schematic of relationship between ligands, type II and I receptors. ALKs are categorized into groups based on their structures and functions. ALK1 group consists of ALK1 and ALK2 while BMPR1 group consists of BMPR1A/ALK3 and BMPR1B/ALK6. The receptors of ALK1 group and BMPR1 group phosphorylates SMADs 1/5/8, whereas the TGF- β RI group phosphorylates SMADs 2/3. The type II receptors are BMPR2, activin receptor (ACTR2A) and (ACTR2B). BMPR2 is specific to BMPs, whereas ACTR2A and ACTR2B can bind to Activin, myostatin and BMPs. Type II receptors bind most BMP ligands and affect the binding preferences of BMPs to type I receptors (Miyazono et al. 2010).

Type II receptors have constitutively active cytoplasmic kinase domains however, are unable to activate signalling downstream without type I receptors. Type II receptors can form pre-formed complexes or be recruited to form complexes with type I receptors during ligand stimulation to further initiate cascading events (Caestecker and Meyrick 2001). Likewise, BMPs can bind to type I receptors in absence of type II receptors, but have greater binding affinity to receptor heterodimers and heterodimers are required for signal transduction initiation (Rozenzweig et al. 1995).

Furthermore, BMPs utilise other co-receptors in concert with the type I and II receptors. For example, glycosylphosphatidylinositol (GPI)-anchored proteins of the repulsive guidance molecule (RGM) family, *RGMa*, *b* (*DRAGON*) and *c* (*hemojuvelin*) are co-receptors for BMP2 and BMP4. These co-receptors enhance BMP signalling when co-expressed with type I and II receptors (Samad et al. 2005). Increased specificity of signalling results from the specific binding of BMPs to the RGM co-receptors, with BMP2 and 4 capable of binding but not BMP7 (Babitt et al., 2005).

Currently there are several ligands known to activate *BMPR2* intracellular signalling, namely BMP2, 3, 4, 6, 7, 9, 10, GDF5 (growth and differentiation factor) and GDF6. These ligands facilitate the assembly of type I/type II receptor heteromers to trigger phosphorylation and activation of type I kinases by constitutively active type II receptor kinases.

1.7 BMP Type II Receptor (*BMPR2*)

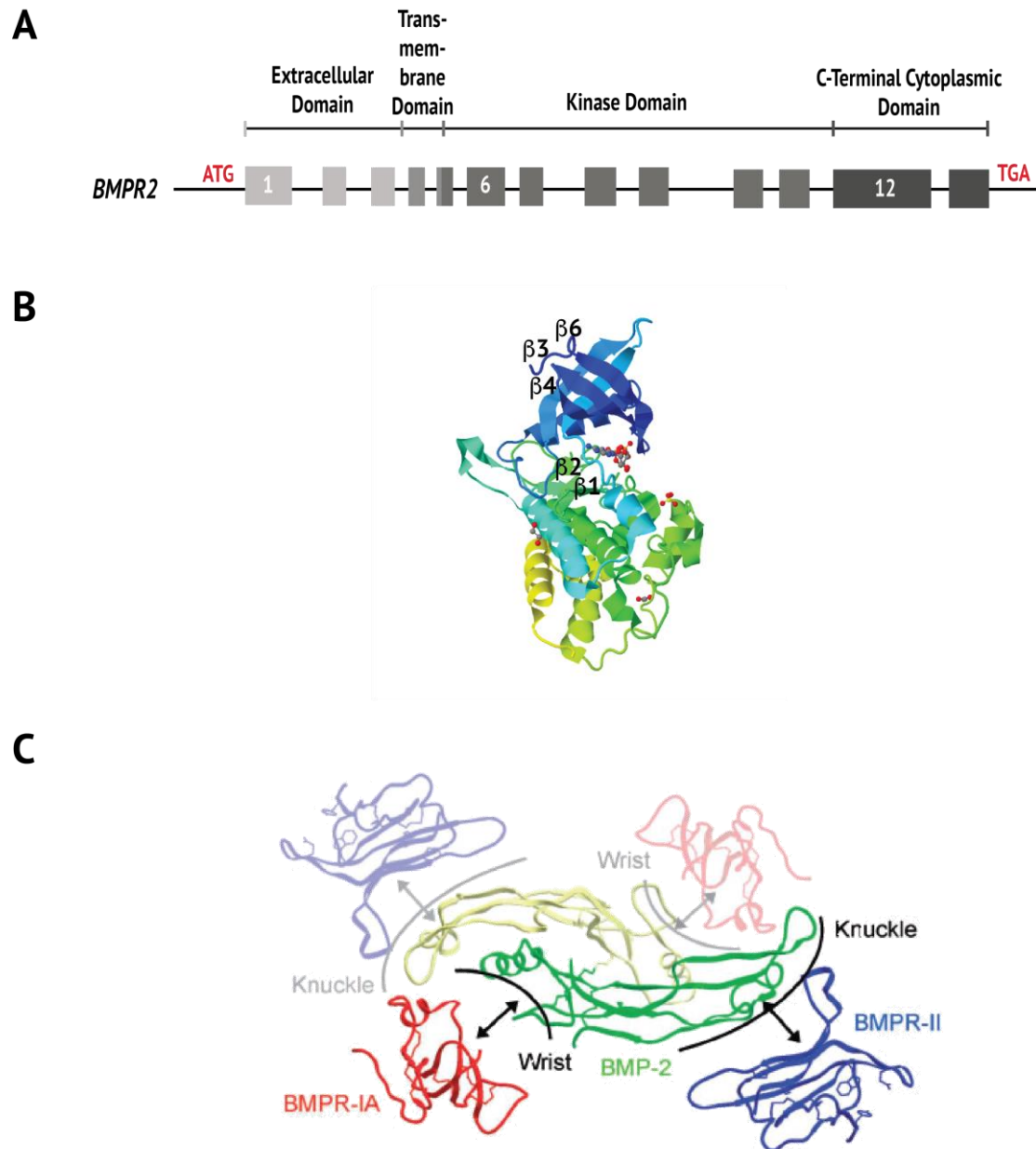


Figure 3. Schematic of *BMPR2* and its protein structure. **(A)** *BMPR2* consists of 13 exons that encode for the extracellular ligand-binding domain (exons 1-3), transmembrane membrane domain, (exons 4-5), kinase domain (exons 6-11) and the C-terminal tail domain (exons 12-13). **(B)** Crystal structure of *BMPR2* with a resolution of 2.35Å. Illustration from RCSB PDB: 3G2F (Chaikuad et al. 2009). **(C)** Top view of the ribbon diagram of a hypothetical interaction of BMP2 to *BMPR2* and *BMPR1A* in the cell membrane. Illustration from (Miyazono et al. 2010).

BMPR2 is distinguished from other type II receptors by its long carboxyl (C)-terminal sequence located after the intracellular kinase domain, which is encoded by exons 12 and 13 (**Fig. 3A**) (Morrell 2010). Both long and short forms of *BMPR2* have been isolated. The long form consisting of the C-terminal tail and the short form lacking the long C-terminal tail is expressed in most types of cells e.g. lungs, heart, kidney and brain. Currently, the exact function of the long *BMPR2* C-terminal tail is not known. It is proposed that the C-terminal domain interacts with cytosolic proteins that may modify signalling via alternate SMAD-independent pathways such as the Notch, MAPK, p38 kinase pathways and extracellular signal regulated kinases (Yu et al. 2005). For example, interactions between the *BMPR2* C-terminal region with the cytoskeletal regulators LIM kinase 1 (Foletta et al. 2003), TCTEX-1 (Machado et al. 2003), TRB3 (Chan et al. 2007) and TRPC (Yi Zhang and Xin Duan 2013) have been identified.

The short form splice variant of *BMPR2* is generated by alternative splicing of exon 12 and the subsequent exposure of a premature translation termination codon within exon 13. Thus, *BMPR2* short form lacks the long cytoplasmic domain. This *BMPR2* short form is still able to activate Smads *in vitro* (Kawabata et al., 1995). Alternative splicing is a mechanism that can generate multiple transcripts, with different functions, from a single gene by internal deletion or skipping of exons in various combinations. Alternatively, it can be a complex process involving exonic and intronic acceptor and donor sites and intronic and exonic splice enhancers and silencers (Keren, Lev-Maor, & Ast, 2010). As only 20% of carriers with a *BMPR2* mutation ever develop PAH, one factor that may play a role in the penetrance of PAH is the alternative

splicing of *BMPR2*. *BMPR2* mutations can produce stable transcripts or premature termination that result in truncated transcripts. These truncated transcripts are often degraded by nonsense mediated decay pathway (NMD). Therefore, the disease can result from either a dominant negative mechanism (stable RNA) or haploinsufficiency (NMD transcript). Furthermore, tissue-specific distant cis-regulatory elements typically control transcription factors and signaling molecules may also be at play e.g. Mouse *Bmp2*, *Bmp4*, *Bmp5* and *Gdf6* belonging to the BMP family have been well documented to be controlled by long-range regulatory sequences (Pregizer and Mortlock 2009). Nevertheless, both these groups have low penetrance of PAH (Cogan & Hamid, 2014). Moreover, down regulation of *BMPR2* even in the absence of mutations confirms that impairment of this signaling pathway is a common mechanism of PAH, regardless of genetic aetiology.

1.8 Ligand binding to receptor extracellular domains

The binding and formation of ligand-*BMPR1* and *BMPR2* depends on the structures of binary or ternary complexes of ligands and receptor extracellular domains. For example, BMP2 is a dimeric protein that has two receptor-binding motifs. The large epitope 1 (wrist epitope) in BMP2 is a high affinity-binding site for *BMPR1A* while the smaller epitope 2 (knuckle epitope) is a low affinity-binding site for *BMPR2* (**Fig. 3C**). The wrist epitope, comprising residues from both BMP2 subunits, contributes to binding to one *BMPR1A* molecule, while the knuckle epitope, constituted by residues of one subunit, binds to *BMPR2* (**Fig. 3C**). The extracellular domains of type II and type I receptors have several conserved cysteines, which are necessary for the

formation of three-dimensional structures with the ligand (Miyazono et al., 2010). Therefore, the formation of these three-dimensional structures has a significant role in activating the BMP signalling cascade.

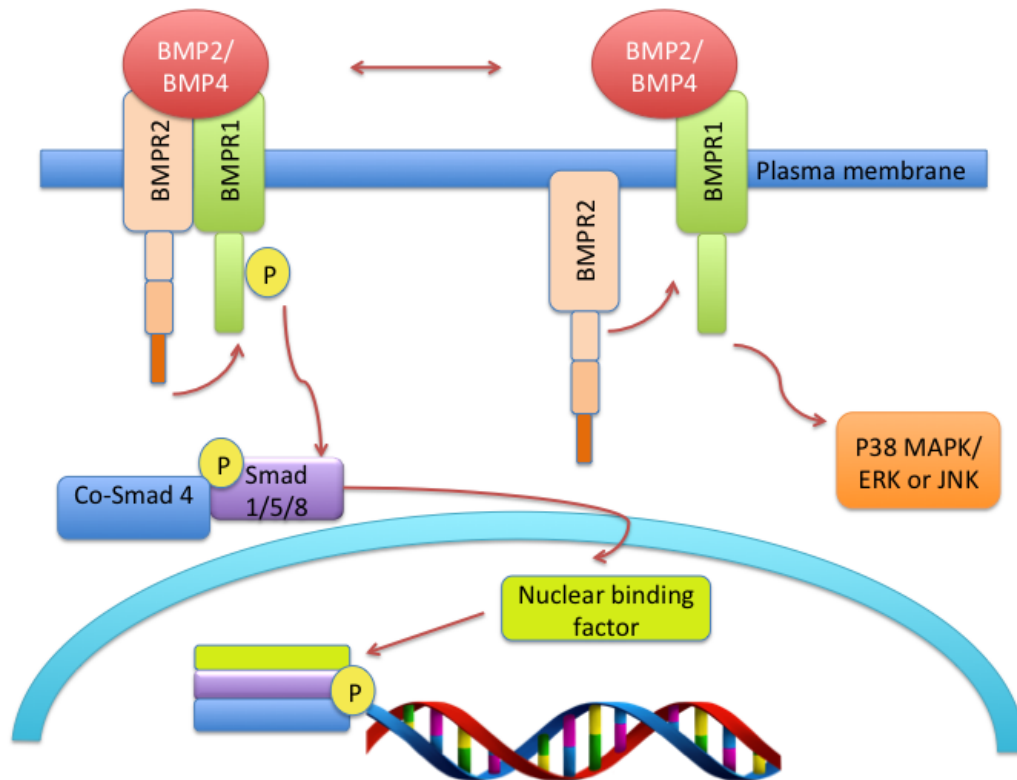


Figure 4. Illustration of BMP receptor complexes activating the canonical or non-canonical pathways. BMPs bind to pre-formed complexes to phosphorylate SMAD proteins and translocate into nucleus to form a binding complex with the nuclear binding factor to induce/suppress transcription of genes. BMPs can also recruit the formation of receptor complexes to activate non-canonical pathways such as p38MAPK and JNK.

Two signalling modes can occur during formation of ligand-type I and type II receptor hetero-oligomerization (**Fig. 4**). One mode forms a complex prior to ligand binding that activates signalling via canonical SMAD pathway. The

other mode is mediated by ligand binding to type I receptor and then recruitment of type II receptor activating signalling via non-canonical pathways such as p38 mitogen activated protein kinase (p38MAPK), protein kinase A (PKA) (Caestecker and Meyrick 2001) or c-Jun-NH2-terminal kinase (JNK) (Nohe et al., 2002). Engineered *BMPR2* mutants (*BMPR2TC3*) with a truncated cytoplasmic tail region were defective in binding to *BMPR1*, produced different BMP receptor oligomerization. *BMPR2TC3* that was 29 amino acids shorter than the naturally occurring *BMPR2* short isoform formed preformed complexes with *BMPR1A* but not with *BMPR1B*. Furthermore, deletion of the entire *BMPR2* kinase domain prevented complex formation with both *BMPR1* isoforms (Nohe et al., 2002). Importantly, this demonstrates that regions amino-terminal to the last 29 amino acids in the *BMPR2* cytoplasmic tail are essential for formation of pre-assembled complexes with *BMPR1B* in the absence of ligand, and the mechanism behind the disruption of BMP/SMAD signalling is mutation specific. This suggests that onset of PAH could be triggered by alternative non-canonical pathways. For example one of the functions of the *BMPR2* tail domain is to inhibit ALK2/ACVR1 mediated BMP7 signaling via the non-canonical BMP pathway (Leyton et al. 2013)

1.9 The BMP intracellular signalling cascade

After formation of the heteromeric complex, type II receptor kinases phosphorylate the serine-threonine GS box domain on the type 1 receptors that in turn phosphorylate cytoplasmic signalling proteins known as SMADs (**Fig. 3**). SMADs are transcriptional factors that are responsible for signal transduction in the TGF- β pathway. The activated *BMPR1* complex

phosphorylates SMAD 1/5/8 molecules. Upon phosphorylation, these BMP-specific RSMADs (Receptor regulated SMAD) form a complex with the co-SMAD (Common SMAD), Smad 4, and translocate into the nucleus to regulate transcription of specific genes. Additionally, there is an extra layer of BMP signalling regulation by endogenous inhibitors such as Noggin, Chordin, Follistatin and BAMBI (**Fig. 5**) (Humbert et al 2004).

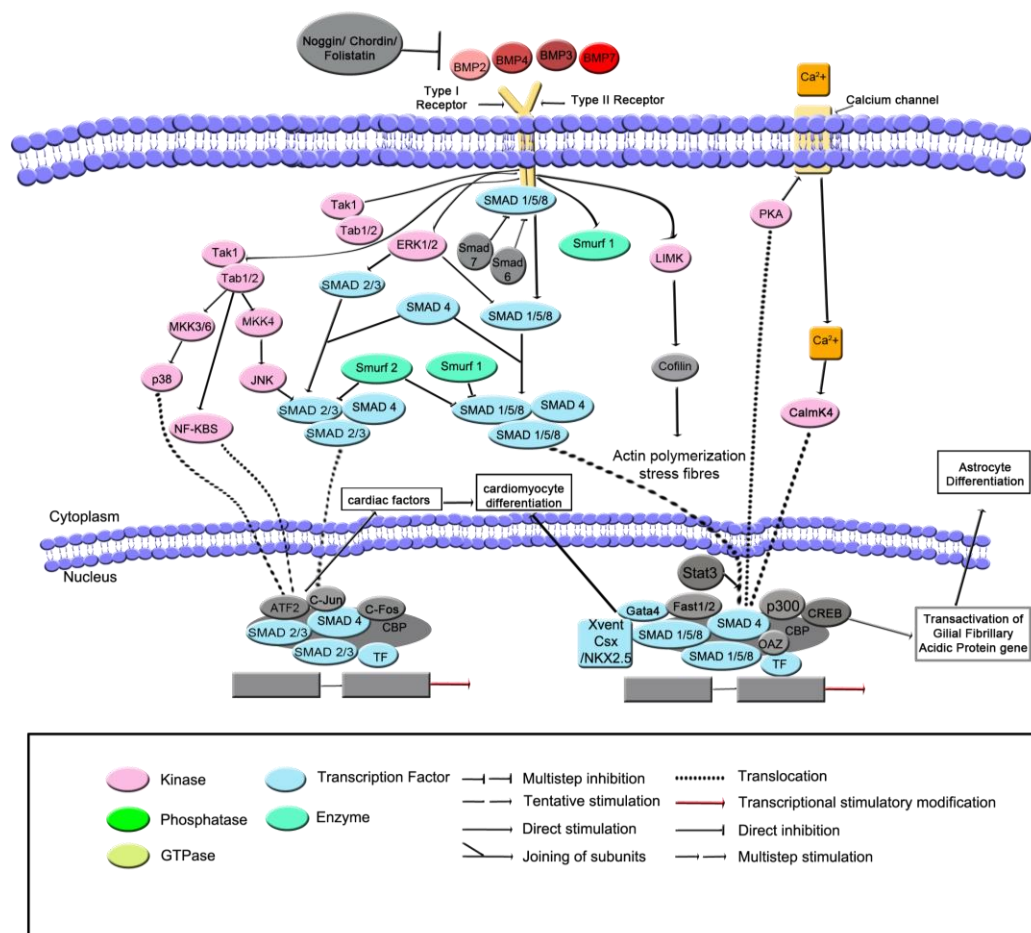


Figure 5. Diagram of BMP intracellular signalling pathway. Adapted from Cell Signalling Technology Inc.

1.9.1 SMAD transcriptional factors

In the nucleus, the SMAD N-terminal domain (MH1domain) binds with low affinity to the GCCG or CAGA motif in the promoter regions of many BMP-responsive genes (Shi et al. 1998). It was later identified that DNA binding co-factors can pair with SMAD2/3 or SMAD1/5/8. These DNA binding co-factors provide the specificity that enables SMADs to be recruited to promoters in response to BMPs in a cell type specific manner. For example, OAZ was identified as a DNA binding co-factor that specifically associates with Smads in presence of BMP2 and transcriptionally activates the homeobox regulator of *Xenopus* mesoderm and neural development, *Xvent2* (Hata et al. 2000). In addition, Smad complexes bind GC rich regions in certain promoters such as *Drosophila Tinman* (the homologue of human *NKX2-5*), which regulates the induction of visceral mesoderm, dorsal muscles, and the heart (Frasch 1995, Xu et al. 1998). Activation of *NKX2-5* by BMPs is mediated by SMAD1/4 and co-factor GATA4 and this is crucial during the early stages of cardiac development (Brown et al. 2004). Smad1-Smad4 and Smad3-Smad4 complexes bind to *Id1* promoter, which is a DNA-binding protein inhibitor that is mediated by BMPs (Massague et al. 2005). Interestingly, *Id1* expression in human embryonic stem cell (hESC) derived ECs is induced by TGF- β inhibition and is required for endothelial and vascular cell commitment (James et al. 2010). ISmads (inhibitory Smad), Smad6 and Smad7, represses BMP signalling competing with co-Smad4 for the binding to Smad1/5/8 (Nakashima et al. 2001). Other negative regulates of SMADs includes the SMAD ubiquitination regulatory factor (SMURF), which promotes SMAD destruction by the ubiquitin proteasome pathway.

SMADs interact with a wide range of tissue and cell specific transcription factors to regulate gene expression. For example, PASMC and ECs express a range of TGF- β family members such as BMPR2 and BMPR1B which bind to both TGF- β and BMP4 (Humbert and Trembath 2002). SMADs can also inhibit transcriptional activators to prevent gene expression. Smad3 inhibits myogenic differentiation when it binds to another myogenic inducer, MEF2C blocking its interaction with the co-activator GRIP-1 (Liu et al. 2004).

There is increasing evidence that alternate pathways such as p38MAPK pathway downstream of TAK1 (TGF-Beta Activated Kinase-1) can bypass the SMAD pathway and mediate BMPR2 signalling to modulate nuclear transcription and apoptosis (Rudarakanchana et al. 2002b). Studies of *Xenopus* development have led to the discovery of Smad cross-talk with other signalling networks such as the JNK/MAPK pathway. At mid-gastrulation *Xenopus* embryos lose the ability to generate mesoderm when induced with activin like signals due to the failure of Smad2 to translocate into the nucleus (Grainger and Gurdon 1989). However, a mutant Smad2 without MAPK sites was able to translocate into the nucleus when Activin was induced during gastrula stage and formed mesoderm suggesting a role for map kinases (Grimm and Gurdon 2002). The p38 and JNK pathways can modulate TGF- β gene responses depending on transcriptional factors controlled by MAP kinases (Nakashima et al. 2001).

Another pathway that is known to contribute to hypertrophic growth in cardiac tissue is the activation of CalmKIV (Saito et al. 2003). CalmKIV (Ca²⁺/calmodulin (Calm)-dependent Kinases) inhibits BMP signalling by

activating a CBP-binding substrate, thereby preventing CBP/p300 co-activating BMP-specific target genes. SMADs phosphorylate CBP/p300 to mediate activation of gene transcription. CBP/p300 is a histone acetyltransferase, which promotes transcription as acetylated histone results in an open chromatin structure (Bannister and Kouzarides 1996).

As described above, BMP signalling is a complex and finely regulated system. The signalling cascades that spread from *BMPR2* elicit a range of cellular responses. Given this complexity, *BMPR2* mutations that underlie PAH require careful molecular dissection to establish the mode-of-action. A number of animal models have been developed to study the pathogenesis of PAH.

1.10 Animal models

In this section, genetic animal models frequently used to study Group I PAH will be discussed. These include Fawn-Hooded rats, knockout and transgenic models. While the existing animal models inform on certain aspects of PAH, no animal model of pulmonary hypertension recapitulates the human condition (Schermlay et al. 2011). Furthermore, mice used in most studies have a mixed genetic background. Strain-specific genetic modifiers may modulate the propensity to develop PAH in mice carrying mutant *Bmpr2* alleles. This highlights the need for human systems to investigate PAH pathogenesis.

Animal Models	Species	Method	Duration /mortality	Intimal hyperplasia	Medial hypertrophy	Plexiform lesions	Vascular inflammation (HIF- α activity)	mPAP (mmHg)	RVH (Right Ventricular Hypertrophy)	Kv1.5
	Human PAH	Hereditary or spontaneous	~ 30% mortality at 3 years	+++	+++	+++	+	~30-60	Varies	↓
Fawn-hooded rats	Rats	Hereditary or can be exposed to hypoxia	1-2yrs	+	++	-	-	~30-50	Moderate	↓
Knockout Models										
BMPR2 heterozygous	Mice	Deletion of <i>BMPR2</i> kinase domain	-	-	+	-	+	~35-45	Mild	↓
Transgenic Models										
Dominant-negative BMPR2	Mice	Overexpression of mutant BMPR2 alleles	Stable up to 3 months	-	++	-	+	~35-45	Mild	↓
<i>BMPR2</i> knock down	Mice	shRNA knockdown of <i>BMPR2</i>	-	-	-	-	-	-	-	-
5-HTT overexpressing mouse	Mice	Transgenic mice exposed to hypoxia	2-4weeks/ Stable up to 3 months	-	+	-	-	~45-55	Mild	?

Table 2. Summary of genetic models used to study Group I PAH in humans.

1.10.1 Fawn Hooded Rats (FHR)

FHR/EurMcwiCrl is a specific strain of Fawn-Hooded Rats used as a model for PAH (Cowley et al. 2004). This strain of Fawn-Hooded Rats (FHR) develops PAH spontaneously through an increase in HIF-1 α because of platelet storage disorders that causes reduction in serotonin (5-hydroxytryptamine [5-HT]) uptake and storage and increases plasma serotonin levels (Aulakh et al. 1994). In humans, another cause of PAH is associated with an inherited platelet storage-pool disease whereby uptake and storage of 5-HT and biogenic amines by platelets is reduced (Hervé et al. 1989). The gene for 5-HT is localized to chromosome 17q11.1-q12 in humans (Gonzalez et al. 1998). PAH is heritable in FHR occurring in 68% of male and female offsprings (Kentera et al. 1988). FHR are allowed to age and monitored by echocardiography for the development of PAH. These rats are highly sensitive to hypoxia and at a higher altitude respond with marked vasoconstriction (Nagaoka et al. 2006). To advance PAH, FHR can be subjected to mild hypoxia that would normally not cause PAH in rodents (Cras et al. 2000).

FHR have increased ET-1 in the lungs (endothelin-1), decreased eNOS (endothelial nitric oxide synthase), platelet abnormalities and increased vasoconstrictor in response to serotonin, similar to the human PAH phenotype (Cras et al. 2000). However, another breed of rats known as Tester-Moriyama (TM), share a similar pigmentation and platelet disorder to FHR do not develop PAH when subjected to mild hypoxia. This suggests that serotonin pathway is not solely responsible for PAH in FHR (Nagaoka et al. 2001). The

pigmentation and platelet storage-pool deficiency in FHR is localized to chromosome 1 (Brown et al. 1996). This model recapitulates the proliferation of PASMC seen in human PAH, especially in response to epidermal growth factors. However, FHR do not develop vascular lesions commonly seen in severe stages of PAH in humans. It is believed that the decrease in mitochondrial expression of *SOD2* (superoxide dismutase 2) (Archer et al. 2010) in FHR results from the epigenetic silencing of the *SOD2* gene that leads to activation of HIF-1 α associated with fragmentation of mitochondria. Decreased *SOD2* expression represses mitochondrial redox signalling and increases activation of HIF-1 α (Archer et al. 2010). Mitochondrial fragmentation is a disorder in which two regulated processes, namely fission or fusion are increased or decreased, respectively. The increased fission of mitochondrial is caused by activation of dynamin-related protein 1 (DRP-1) (Marsboom et al. 2012). The mitochondrial fragmentation in FHR (and human PAH) relates in a large part to activation of dynamin-related protein (DRP-1) (Marsboom et al. 2012b). Inhibition of mitochondrial fission in PAH PASMC prevents mitotic division of mitochondria and arrests the cells in G2/M phase of the cell cycle. The resulting growth inhibition plus induction of apoptosis suggest this recently identified abnormality may contribute to PAH in the FHR model.

In order to develop an appropriate control for the FHR model of PAH, the FHR-BN1 consomic rat was developed. This line was generated by the introgression of chromosome 1 from the hypoxia resistant Brown Norway rats into an isogenic FHR background (Cowley et al. 2004). These rats demonstrate that the key genes responsible for PAH in FHR animals are

found on chromosome 1. Thus, the PAH-initiating gene in FHR is likely present on chromosome 1 (Bonnet et al. 2006). However, in humans the majority of HPAH cases are linked to mutations in *BMPR2* which is located on chromosome 2. Therefore, FHR may not be a true representation of PAH in humans. Furthermore, the main disadvantage of using this model is the slow development of PH in FHRs, although exposing FHRs to mild hypoxia can accelerate the process. Also, FHRs develop simplification of alveolar, raising the issue that FHR is not a pure model of PAH (Cras et al. 2000).

1.10.2 Mouse models of *BMPR2* mediated PAH

Transgenic mice expressing the disease related *BMPR2* mutation model have been developed to investigate PAH. However, many attempts have been made in mice, to recapitulate the haploinsufficiency in PAH patients with *BMPR2* mutations to no avail (Beppu et al., 2000). Mice that are heterozygous for the *Bmpr2* null allele showed only modest increase of PAH pressure and pulmonary vascular resistance (Beppu 2010). It appears that mice have a lower threshold of *Bmpr2* signalling required for the maintenance of vascular function compared to humans. However, homozygous knockout of *Bmpr2* in mice is embryonic lethal, and this suggests that PAH patients harboring heterozygous *BMPR2* mutations have sufficient levels of *BMPR2* to develop past gastrulation (Hong et al. 2008).

1.10.3 Dominant negative transgenic mice

Another method used in mice to study *BMPR2* associated PAH is via overexpression of human mutant *BMPR2* isoforms in an inducible system.

Overexpression of a kinase-dead, dominant-negative BMPR2 mutant protein lacking the entire intracellular domain in smooth muscle lead to mild PAH and modest medial thickness of PA (West et al. 2004). Subsequently, a second series of mice expressing smooth muscle-specific doxycycline-inducible *BMPR2* mutation with an arginine to stop codon at amino acid 899 (SM22-rtTA x TetO₇-*BMPR2*^{R899X}) was developed (West et al. 2008a). Although, not a known pathogenic mutation, mice overexpressing *BMPR2*^{R899X} developed PH and severe pulmonary vascular pruning. Overexpression of this dominant-negative *BMPR2* variant is associated with down regulation of voltage gated potassium channel Kv1.5, and results in PA vasoconstriction. Nifedipine, a selective L-type calcium channel blocker, reduces PH in these mice. This suggests that activation of L-type calcium channels, caused by reduced expression of Kv1.5 channels and membrane depolarization, mediates PH in these mice (Katharine A. Young 2006).

1.10.4 Conditional knockout mice

To circumvent the embryonic lethality of *Bmpr2* null mice, a conditional Cre-lox knockout mice strain has been developed (Beppu et al. (2005)). loxP sequences were introduced flanking exons 4 and 5, allowing conditional deletion of the transmembrane domain in cells expressing the Cre recombinase. A Cre transgene selectively expressed in endothelial cells was used in these conditional knockout mice to generate PH (Hong et al. 2008). It was found that PAH developed in some of the mice when *Bmpr2* was deleted in mature animals. These mice had media hypertrophy of the small Pas and right ventricular hypertrophy (RVH) (Yu et al. 2005).

1.10.5 shRNA *BMPR2* knockdown mice

Silencing *BMPR2* expression by RNA interference does not cause PAH, although knockdown of *BMPR2* is approximately 90% (Beppu et al. 2005). It causes other symptoms such as gastrointestinal hyperplasia, incomplete mural cell coverage of vessel walls and mucosal hemorrhage (Beppu et al. 2005). BMP receptor appears to regulate angiogenesis by maintaining the endothelial migratory signalling system. Therefore when this process is disrupted, there is malformation of the vessel walls. This result supports the notion that down regulation of *BMPR2* alone may not be sufficient to induce PAH.

1.10.6 5-HTT overexpression in mice

Serotonin (5-HT) is known to induce proliferation of PASMC and migration of fibroblasts causing vasoconstriction. Expression of serotonin transporter (5-HTT) is increased in Group 1 PAH patients (Eddahibi et al. 2001). Transgenic overexpression of 5-HTT in SMC-specific manner using the SM22 promoter results in muscularized distal PAs and increased proliferation of PASMCs (MacLean et al. 2004). Interestingly, when monocrotaline (a toxic alkaloid known to cause PAH in humans), which alone is insufficient to cause PH in mice, is given to 5-HTT overexpressing mice results to an increase in right ventricular systolic pressure (RVSP) (25-35mmHg). However, PAH symptoms and histological changes are modest in this model. There has been difficulties in recapitulating PAH in animal models, thus there is a need to develop human based models of this disease.

1.11 Human pluripotent stem cells (hPSCs) for disease modelling

Pluripotent stem cells (PSCs) have the ability to differentiate into any of the three germ layers, endoderm, mesoderm and ectoderm (Evans and Kaufman 1981, Martin 1981, Thomson et al. 1998). The two types of PSCs are embryonic stem cells (ESCs), derived from the inner cell mass of the embryo and genetically reprogrammed induced pluripotent stem cells (iPSCs).

HESCs were first isolated from human blastocysts by Thomson and colleagues in 1998. HESCs derived from inner cell mass of human blastocysts have the ability to differentiate into 3 germ layers. iPSCs were first generated in 2006 from mouse cells by Shinya Yamanaka's team at Kyoto University, Japan, and by James Thomson's team at the University of Wisconsin-Madison (Yoshida and Yamanaka 2010). Mouse fibroblasts were transduced with 4 transcriptional factors (c-myc, Oct3/4, sox2 and klf4) using retrovirus transduction. Reprogrammed cells were then selected by the expression of a Bgeo cassette (a fusion of the Bgalactosidase and neomycin resistance genes) driven by the mouse *Fbx15* promoter. These *Fbx15* derived iPSCs gave rise to teratomas with histological evidence of cells differentiating into 3 germ layers when subcutaneously injected into nude mice (Takahashi and Yamanaka 2006). Subsequently, in 2007 human iPSCs were derived from human fibroblasts (Takahashi et al. 2007).

The ability to reprogram somatic cells from any individual, with their unique genotype, into iPSCs allows many diseases to be studied *in vitro*. The production of iPSCs does not require embryos, unlike the production of ESCs.

Therefore, iPSCs are easier to acquire and circumvent ethical concerns expressed by some groups regarding the derivation of hESCs. Phenotypically, iPSCs resemble ESCs in many aspects, such as expression of similar pluripotent markers and immortality in culture (Yoshida & Yamanaka, 2010). Importantly, differentiation protocols developed for ESCs can be applied to iPSCs. Thus, iPSCs derived from patients bearing genetic mutations can be differentiated into the affected cell types. Previously, cardiovascular defects, such as PAH, were difficult to study because they were difficult to model in the laboratory. Further, as discussed previously, although animal models proved useful in elucidating several mechanisms in PAH, most rodent models do not fully recapitulate the phenotype of the disease in humans. To date a large number of monogenic cardiovascular diseases utilising hiPSC derived from patients with pathogenic mutations have been modelled, such as hypertrophic cardiomyopathy (heterozygous missense mutation in *MYH7*) (Lan et al. 2013), long QT syndrome (heterozygous missense mutation in *KCNH2*, *SCN5A*) (Matsa et al. 2013; Ma et al. 2013; Bellin et al. 2013; Terrenoire et al. 2012) and supravalvular aortic stenosis syndrome (mutation in elastin (*ELN*) gene causing vascular smooth muscle dysfunction) (Ge et al. 2012). However, PAH has yet to be modelled *in vitro* using iPSC. Therefore, following on from iPSC models of other cardiac defects, we hypothesize that some aspects of the molecular pathology of PAH will be able to be studied using iPSCs from PAH patients.

1.12 Genome editing

In 2007, Mario Capecchi, Martin Evans, and Oliver Smithies won the Nobel Prize in Physiology or Medicine for developing specific gene modification techniques in mouse ESCs, this technology enabled the creation of “knockout” mice (Mak 2007). This powerful technology is applicable to iPSCs enabling the further dissection of the genetic causes of human disease. Editing PSC genome allows the production of lines carrying disease causing genetic mutations. Furthermore, isogenic control cell lines can be generated from patient specific iPSCs by repairing the disease associated genetic lesion. These modified lines act as important controls to support the characterization of disease phenotypes. Genome editing can also be used for insertion of “safety” genes, marker genes for specific cell types (via fluorescent proteins behind specific promoters) and inducible genes to switch on and off factors within the differentiating or proliferating cells (Svendsen 2013). These methods provide useful tools to researchers interested in studying various gene abnormalities involved in disease pathogenesis as well as elucidating novel pathways.

1.12.1 Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) technology

With platforms such as ZFNs and TALENs, genome editing has become a powerful tool and has opened doors to the discovery of other genome editing systems (Gaj et al. 2013). Recently, a new approach using clustered regularly interspaced short palindromic repeats (CRISPR) based on a bacterial

CRISPR-associated protein 9 nuclease from *Streptococcus pyogenes* (Cas9) has been developed. This system is unique and adaptable because it relies on RNA as the moiety for targeting the nuclease to the desired DNA sequence. In contrast to ZFN and TALEN systems that use protein-DNA interactions for targeting, RNA-guided nucleases (RGNs, such as CRISPR) use base-pairing rules between engineered RNA and the target DNA site (Sander and Joung 2014).

CRISPR systems were first discovered in bacteria as an immune mechanism to protect themselves from foreign invasion of nucleic acids such as plasmids or viruses. Type II CRISPR systems incorporate sequences from invading DNA between CRISPR repeat sequences encoded as arrays within the bacterial host genome. Transcripts from the CRISPR repeat arrays are processed into CRISPR RNAs invading DNA, known as “protospacer” sequence, and part of the CRISPR repeat. Each crRNA hybridizes with a second RNA, known as the transactivating CRISPR RNA (tracrRNA), and these two RNA form a complex with the Cas9 nuclease. The protospacer-encoded portion of the crRNA directs Cas9 to cleave complementary target-DNA sequences when they are adjacent to the protospacer adjacent motifs (PAM). The target sequence must contain the PAM site or the protospacer sequence will not incorporate into the CRISPR locus.

This type II CRISPR system from the *S.pyogenes* has been adapted to introduce sequence specific double stranded breaks (DSBs) and genome editing into eukaryotes. The Cas9 nuclease and guide RNA (gRNA), consists of the crRNA and fixed tracrRNA. Approximately 20 nucleotides at the 5' end

of the gRNA with the pam site motif 5'-NGG, directs Cas9 to a specific target DNA site using standard RNA-DNA complementary base pairing rules. Cas9 nuclease induces DSBs at the target site and insertions or deletion of specific nucleotides can be introduced via non-homologous end joining (NHEJ) or HDR (homology directed repair) pathways. NHEJ can introduce deletion or insertion mutations of variable lengths causing frame shifts or deletion of the targeted site, in contrast, precise modification or insertion from single stranded or double stranded DNA donor template can repair or introduce a point mutation. Using these techniques, we hope to utilise these strategies to correct and create point mutations in *BMPR2* in order to further investigate the disease causing mechanisms behind PAH.

Summary

PAH is a fatal disease characterized by chronic obstruction of the pulmonary arteries that affects certain carriers of the *BMPR2* mutation. Although, *BMPR2* mutations underlie PAH, it is not fully penetrant, indicating the genetic complexity of the disease. *BMPR2* is involved in the BMP signalling cascade that is important for cardiovascular development and maintenance. Thus, it has been suggested that affected patients are predisposed to the disease due to alterations in this pathway.

Animal models are frequently used to study the pathogenesis of PAH. However, these models do not fully recapitulate human PAH. A more appropriate model would be to use human PSCs carrying pathogenic mutations in *BMPR2*, allowing the disease to be modelled *in vitro*. In this

context, this thesis aims to develop a suite of cellular reagents to facilitate the cellular and molecular dissection of *BMPR2* function, which will underpin future studies focused on arresting PAH disease progress by identifying signalling networks amenable to pharmaceutical intervention.

Aim of this study

BMPR2 is a serine/threonine receptor kinase belonging to the transforming growth factor- β (TGF- β) family that is a receptor for the BMP class of cytokines. BMPs play an important role in embryonic development and maintaining tissue homeostasis. Further, perturbation of the BMP signalling pathway causes a range of diseases including vascular disorders such as PAH. Importantly, *BMPR2* mutations are found in the majority of PAH cases. Therefore, we sought to examine the function of human *BMPR2* in vascular development and function.

The major aim of this thesis was to elucidate the downstream consequences of perturbed *BMPR2* signalling in cardiovascular cell lineages. To address this aim, I produced multiple genetically modified PSC lines to generate an allelic series of *BMPR2* mutations. These cellular reagents were used to investigate the requirement for *BMPR2* in the formation and function of mesodermal cells, cardiomyocytes, smooth muscle cells and endothelial cells. Strikingly, perturbed *BMPR2* did not affect the differentiation of these cardiovascular cell types, *in vitro*. However, cellular phenotyping demonstrated that *BMPR2* regulates endothelial cell morphology and migration. Dysregulation of endothelial cell functions mediated by *BMPR2*

may impair endothelial cell activity in the pulmonary blood vessels contributing to both disease onset and progression in PAH.

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CHAPTER



Materials and Methods

Introduction

We utilised human induced pluripotent stem cell lines (hiPSCs) and human embryonic stem cells (ESCs) as *in vitro* models for investigating *BMP2* mutations associated with PAH. This chapter herein describes the general protocols and techniques used to facilitate this project.

The first section will describe general routine techniques used, followed by the generation of reagents using CRISPR. The third section will describe the experimental methods used to conduct our studies.

A brief explanation of what CRISPR technology is; CRISPR, an acronym for Clustered Regularly Interspaced Palindromic Repeats, are a class of bacterial endonucleases that have been adapted for facilitating the precise editing of a variety of different genomes. Discovered in *Streptococcus pyogenes*, as a defense mechanism against bacteriophage invasion, the type II CRISPR/Cas-9 system uses an RNA-guide (RGEN), which guides the Cas9 to cleave target DNA sequences.

Transcripts from the CRISPR repeat arrays are processed into CRISPR RNAs (crRNAs), each harboring a variable sequence transcribed from the invading DNA, known as the “protospacer” sequence, and part of the CRISPR repeat (**Fig. 1**). Each crRNA hybridizes with a second RNA, known as the transactivating CRISPR RNA (tracrRNA), and these two RNAs complex with the Cas9 nuclease (Sander and Joung 2014).

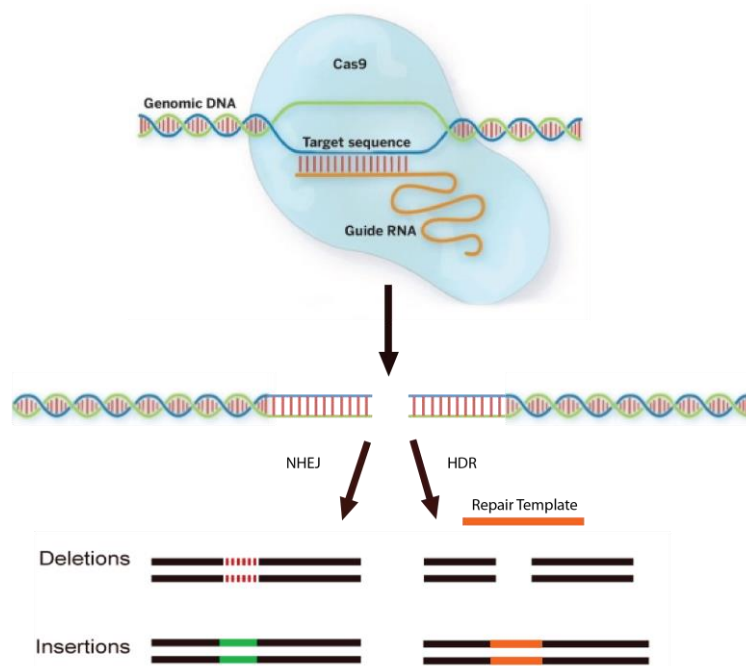


Figure 1. Schematic of CRISPR mechanism. Cas9 finds homology to sequence and makes double stranded nicks in target sequence. Left arrow denoting non-homologous end-joining pathway (NHEJ) creates mutations such as deletion or insertions (indels) of single to several nucleotides into the locus of the targeted region. While the right arrow shows that the nicks are combined by the presence of a repair template through homology directed repair (HDR).

2.1 Routine methods

2.1.1 Culturing of hPSCs

A patient derived induced pluripotent stem cell hiPSC *BMPR2*^{2504delC/W}, harbouring a 2504delC mutation in *BMPR2* and a normal control derived hiPSC, iPSC^W were used in this study. Four genetically modified cell lines (refer to **section 2.2**) were made from HES3 derived *NKX2-5*^{eGFP/W} as the backbone. These four HES3 cell lines will be referred accordingly as i) *BMPR2*^{DelTail/W} carrying a heterozygous mutation in *BMPR2* tail domain similar

to patient hiPSC *BMPR2*^{2504delC/W}, ii) *HES3 BMPR2*^{Null} lacking ATG in exon 1 of *BMPR2*, iii) *HES3 BMPR2*^{DelKinase} carrying an in-frame deletion in the kinase domain of *BMPR2* and iv) *HES3 BMPR2*^{DelTail} a C-terminal tail domain null in *BMPR2*. All cultures were incubated at 37°C in a humidified atmosphere with 5% carbon dioxide. HESCs were routinely enzymatically passaged twice a week onto either 25cm², 75cm² or 150cm² flasks at ratios ranging from 1:2 to 1:10, dependent on the application. Unless otherwise stated, all culture media and supplements were purchased from Life Technologies. These cell lines were routinely maintained in a medium containing DMEM/F12, 20% (v/v) knockout serum replacement, 10mM non-essential amino acids, 2mM L-glutamine, penicillin/streptomycin, 50mM 2β-mercaptoethanol, and 10ng ml⁻¹ bFGF (R&D) on flasks seeded with a specific density of irradiated mouse embryonic fibroblasts (MEFs) at seeding density as previously described (Costa et al. 2007, Elliott et al. 2011).

2.1.2 Polymerase Chain Reaction (PCR)

PCR was carried out in 25µl or 50µl volumes using either GoTaq Green Master Mix (Promega Cat # M7122) or Platinum Taq DNA Polymerase High Fidelity (Invitrogen Cat # 11304011), enzyme kit. The amount of DNA template used per reaction varied according to the quality and source of the DNA (e.g. cloned, plasmid or genomic). Amplifications were performed on a Mastercycler EP Gradient S (Eppendorf), PTC-200 Peltier Thermal Cycler (MJ Research) or 2720 Thermal Cycler (Applied Biosystems), with cycling parameters appropriate to primer pair melting temperatures (T_m), DNA template size and enzyme utilised. Where high purity DNA was required, the

MinElute or the QiaQuick PCR Purification Kit (Qiagen Cat # 28104) was used to purify DNA fragments from reducing presence of primers, nucleotides, polymerases and salts.

2.1.3 Quantitative Polymerase Chain Reaction (qPCR)

High Pure Isolation RNA kit by Roche (Roche Cat # 11828665001) or ISOLATE II RNA Mini Kit (Bioline, Cat # BIO52072) was used to extract total RNA from cells according to manufacturer's instructions. cDNA synthesis of the minimum to 2.5µg of RNA were reverse transcribed in a 20 µL final reaction volume using SuperScript® VILO™ kit (Invitrogen Cat # 11754050). Gene expression levels were determined by real time quantitative PCR using TaqMan gene expression assays with TaqMan Universal PCR Master Mix (Applied Biosystems, Cat # 4304437) and were quantified on the ABI 7300 Real-Time PCR detection system (Applied Biosystems). The Taqman assays utilised are listed in the Supplementary Table 5. QPCR was performed under standard cycling conditions (1 cycle of optimization at 50°C for 2min; 1 cycle of initial denaturation at 95°C for 10min; 40 cycles of denaturation at 95°C for 15s and annealing/extension at 60°C for 1min). Relative changes in gene expression were calculated using the $\Delta\Delta C_t$ -method. Gene expressions levels were normalized to GAPDH for the analysis (Livak and Schmittgen 2001).

2.2 Correction of *BMPR2*^{2504delC/W} patient and generation of an allelic series of *BMPR2* mutated PSC cell lines

2.2.1 Design of guide RNA for CRISPR targeting

Using the genomic sequence of *BMPR2*, guides were designed next to pam sites adjacent to the locus of c.2504delC mutation in order for Cas9 to cleave the targeted site for homology directed repair (HDR). A 70 base oligonucleotide repair template was designed to replace the missing cytosine in the mutant allele of *BMPR2*^{2504delC/W}. In addition, the repair template had synonymous substitutions of Valine and Serine incorporated for screening purposes. One screening primer was designed to have homology to the synonymous mutations that were present in the repair template. These guides were blasted for any off targets using an online program developed by MIT (<http://crispr.mit.edu/>). Based on this program, RNA guides with the lowest off target score were selected for all of the *BMPR2* knockout experiments. Additional overhangs were incorporated into the CRISPR guide oligonucleotides (**Fig. 2**) before submitting for synthesis by Sigma-Aldrich.

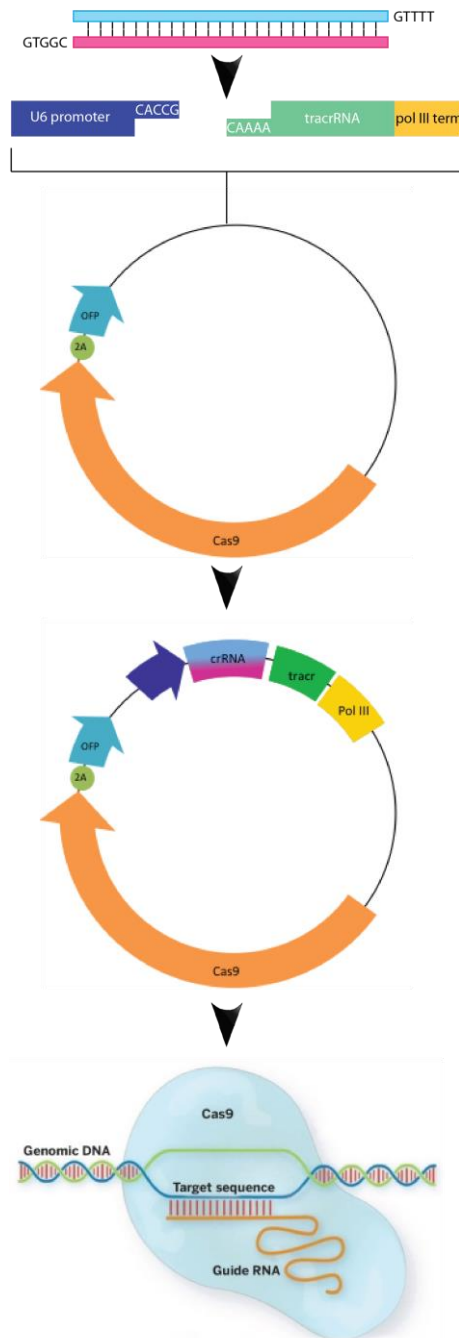


Figure 2. Flow diagram of the process of recombining the homology arms to Cas9 vector. Design of guide RNA should be next to a pam site (NGG) and have overhangs GTGGC on the 5' end and GTTTT on the 3' end. The top and bottom oligonucleotides are annealed before ligating to the cloning site of the linearized PX458 Cas9 vector.

2.2.2 Annealing of oligonucleotide

Forward strand oligonucleotide (200µm TE Buffer)	5µl
Reverse strand oligonucleotide (200µm TE Buffer)	5µl
10x Oligonucleotide Annealing Buffer (100mM Tris-HCL, pH8.0, 10mM EDTA pH8.0, 1M NaCl)	2µl
DNase/RNase - Free water	8µl
Total volume	20µl

To anneal the forward strand and reverse strand oligonucleotides, the following reagents (shown in the table above) were combined in an eppendorf tube and incubated at 95°C for 4 mins on a heat block. After heating, tubes were removed and allowed to cool slowly to 25°C on the bench top (5-10mins). Once cooled, tubes were gently mixed and centrifuged briefly ~ 5 secs. Annealed oligonucleotides were serially diluted to 3 concentrations, 1 in 10, 1 in 100 (500nm), and 1 in 1000.

2.2.3 Restriction endonuclease linearization of pSpCas9 (BB)-2A-GFP (PX458)

The CRISPR/Cas9 vector pSpCas9 (BB)-2A-GFP (PX458) (Addgene Cat # 48138) was utilised in the following experiments. Restriction endonuclease digests were performed in 20µl or 50µl volumes, using 200ng-5µg purified DNA template, and restriction enzyme bbsI (New England Biolabs Cat # R0539L) 5000 units/ml with compatible buffer recommended by the manufacturer. 1mg/ml Bovine Serum Albumin (New England Biolabs) was included when required. Reactions were incubated at 37°C and allowed to

proceed for 3hrs, before being assessed on an agarose gel. Subsequently, purification of linearized vector was carried out by gel extraction.

2.2.4 Gel and PCR purification of linearized vector

Following gel electrophoresis, DNA fragments were exposed under long wavelength ultraviolet light and fragments of interests were excised with a scalpel blade, weighed and transferred into eppendorf tubes for gel extraction purposes. Extraction and purification of DNA was performed using the QIAQuick Gel Extraction Kit (Qiagen Cat # 28704), in accordance with the manufacturer's protocol. For the purpose of purifying PCR products, PCR reactions are carried out as per PCR protocol using the same kit.

2.2.5 Transformation of competent *E.coli* cells

Transformation of *Escherichia coli* (*E.coli*) cells was carried out by a chemical based method. For heat-shock transformation, 2µl ligation or recombination mixture was added to 50µl chemically competent DH10B *E.coli* cells, followed by incubation on ice for 30min before heat shocked at 42°C for 30s and then placing back onto the ice for 2min. Following transformation, 950µl of pre-warmed S.O.C. medium (NEB Cat # C2987H) was added to the transformed cells and then incubated on a horizontal shaker (200rpm) at 37°C for 1hr. Cells were spread onto pre-warmed LB-Agar plates containing 50µg/ml ampicillin and left overnight at 37°C. In cloning steps that incorporated β-galactosidase blue/white colony screening, Isopropyl β-D-1-thiogalactopyranoside (IPTG) and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) were spread onto the LB-agar plates prior to

application of the transformation cultures. The following day, antibiotic-resistant colonies were picked and cultured overnight at 37° C in 2mls of LB-Broth containing the same concentration of selective agent. Purification of plasmid DNA from the bacterial cultures is as described above in **section 2.2.4**. Extracted plasmid DNA from clones were examined either by restriction enzyme digestion or DNA sequencing.

2.2.6 Ligation of annealed oligonucleotides to vector

5X Ligation Buffer	4µl
Linearized PX458 vector	2µl
ds Annealed oligonucleotide (5nM)	2µl
DNase/RNase - free water	11µl
T4 DNA Ligase	1µl
Total volume	20µl

To ligate annealed oligonucleotides to vector, reagents shown (in table above) were mixed together in eppendorf tubes. Reactions were incubated at room temperature (25-27°C) for 10mins – overnight. The next day, the ligation mixtures were transformed into competent cells as per manufacturer's protocol (**section 2.2.5**). The transformation reactions were then plated onto LB agar plates containing appropriate antibiotic and incubated overnight at 37°C. Antibiotic resistant colonies were picked and cultured for minipreps. Extraction of plasmid DNA from these colonies was carried out using the Qiaprep mini kit (Qiagen Cat # 27104) and submitted for sequencing. After sequence verification, clones with the correct sequence and orientation were

cultured for Maxiprep (Qiagen Cat # 12165) and plasmid DNA was extracted according to the protocol as described in the kit.

2.2.7 Transfection of PX458 into cell lines

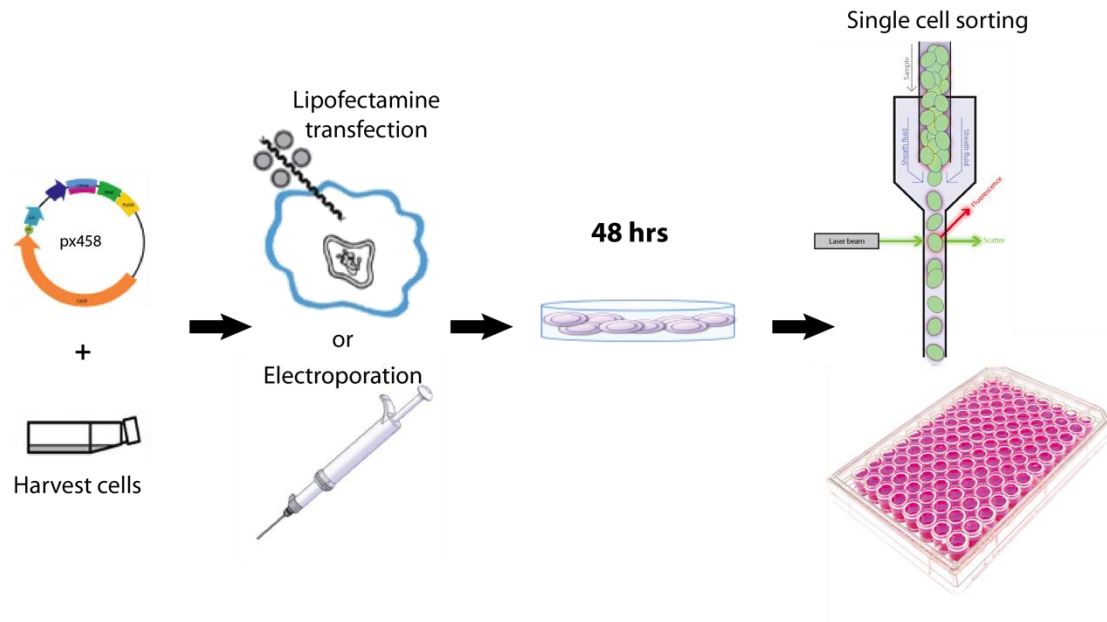


Figure 3. Flow diagram outlines the workflow for gene targeting using two different methods of transfection. 1×10^7 hPSCs were transfected with $5 \mu\text{g}$ of targeting vector by lipofectamine or electroporation methods. Transfected cells that were electroporated were plated back down onto culture dishes. Both techniques used to transfect cells required recovery for 48hrs before harvesting for single cell sorting of GFP^+ cells into a 96 well plate by flow cytometry.

Transfection of cells with vector DNA was carried out either by chemical or electroporation method. Lipid based transfection was performed using Lipofectamine™ RNAiMAX (Invitrogen Catalog # 13778-075 - 0.75ml) and Lipofectamine™ 2000 (Invitrogen Catalog # 11668-019 - 1.5ml). The day prior transfection, 70-80% confluent cells were harvested with TrypLE™ and plated onto 6 well dishes with a seeding density of approximately 1×10^6 per well. On

the day of transfection, the following reagents were prepared according to manufacturers recommendations. Below shows the table that lists the dilutions of reagents required.

Opti-MEM® Medium (Invitrogen)	} Mix A: Add together	150µl x 4
Lipofectamine™ 2000		6µl, 9µl, 12µl, 15µl
Opti-MEM® Medium	} Mix B: Add together	700µl
Vector DNA		14µg
Dispense	Mix A & B: 150 µl+ 150 µl	250µl per well

Four dilutions of Lipofectamine™ 2000 were tested to determine optimum transfection efficiency.

Opti-MEM® Medium (Invitrogen)	} Mix A: Add together	150µl
Lipofectamine™ RNAiMAX		9µl
SiRNA/Oligo (10 µm)	} Mix B: Add together	0.6µg in 150µl
Opti-MEM® Medium		150µl
Dispense	Mix A & B: 150 µl+ 150 µl	250µl per well

Both reactions were incubated for 5 mins before adding to wells in a 6 well dish. One well was left as the control containing only Lipofectamine™ 2000 + Lipofectamine™ RNAiMAX. Following 48hrs incubation, cells in a humidified 37°C/5% CO₂ incubator were harvested and single cell sorted for GFP⁺ into 96 wells containing MEFs seeded at an appropriate density.

Preparation of starting materials for electroporation

1. Flask of cells grown up to required numbers (70- 80% confluency)
2. Penstrep Free growth media (Hes Media / iPSC Media)
3. Plate down feeders onto required culture dishes (approximately 4 x 6cm dishes)
4. Purified and concentrated plasmid DNA

A day prior electroporation, cells were passaged such that on the day of the experiment, cells were approximately 70-80% confluent. The range of cell numbers for each tip were as follows: $5 \times 10^4 - 2 \times 10^5$ cells per each 10 μ L Neon® Tip and $5 \times 10^5 - 2 \times 10^6$ cells per each 100 μ L Neon® Tip. 6-well plates seeded with a total of 1.2×10^6 of MEFs were also prepared.

On the day of the electroporation, pre-plated MEFs on 6 well plates containing MEF media were then replaced with 0.5 ml of hES medium containing serum and supplements without antibiotics for pre-warming in a humidified 37°C/5% CO₂ incubator. Cells for electroporation were rinsed using warm PBS (without Ca²⁺ and Mg²⁺) and harvested using TrpLE™ for 4 mins at 37°C to obtain a single cell suspension. After incubation, TrypLE™ was neutralised using PBS, followed by centrifugation at 1500 RPM for 3 mins for cell collection. After decanting supernatant, the cells were resuspended in PBS and the total cell number was determined using a haemocytometer. For a 100 μ L NEON tip, 1×10^6 cells are required. The required number of cells was determined and resuspended accordingly (~0.75 ml for 10 μ L Neon® Tip or 7.5 ml for 100 μ L Neon® Tip). 1×10^6 cells were transferred to a 1.5ml microcentrifuge tube and centrifuged at 100–400 $\times$ g for 5 minutes at room temperature. After pelleting the cells, PBS was aspirated and the cell pellet was re-suspended in

re-suspension Buffer R at a final density of 1.0×10^6 cells/100 μ L, after which DNA was added. The cells were gently pipetted to obtain a single cell suspension and maintained on ice, as cells in this buffer at room temperature have reduced cell viability and transfection efficiency after 30 minutes. Before carrying out the electroporation, Neon® Tube was filled with electrolytic Buffer and inserted into the Neon® Pipette Station. The Neon® device program for the electroporation was chosen according to the cell type used. The cells and DNA mixture was then drawn up using the Neon® Pipette with the 100 μ L Neon® Tip. This contraption was inserted into the Neon® Tube for electroporation to occur. After electroporation, the cells were transferred drop wise into pre-prepared 6 well dishes with warmed media (containing no antibiotics) and left in humidified 37°C/5% CO₂ incubator for two days of recovery before single cell sorting of GFP⁺ cells into 96 well plates for colony formation.

2.2.8 Picking and screening of colonies

After 2 weeks, sizable colonies were picked and transferred onto prepared 48 wells with MEFs. One third of the colony was passaged on while two thirds were boiled in TE buffer for extraction of DNA. PCR was carried out with the screening primers and DNA from clones. The desired clones were selected after analysis of the gel electrophoresis results.

2.2.9 TOPO Cloning of clones

TOPO Cloning method was utilized to validate the sequences of the clones. TOPO vector was introduced into One Shot TOP10 competent cells

(Invitrogen) as shown in the table below and per manufacturer's instructions. X-Gal screening identified the correct recombinants and the white colonies were picked, purified by miniprep and sent for further analysis by sequencing.

Fresh PCR product	0.5 - 4 μ l
Salt Solution	1 μ l
TOPO vector	1 μ l
DNase/RNase - free water	Make up to 6 μ l
Total volume	6 μ l

2.2.10 Sequencing of plasmids and PCR products

Automated sequencing analysis was performed at AGRF facility at WEHI Melbourne, Australia. Samples that were submitted for sequence analysis comprised of 600-1200ng plasmid DNA template or 10-100ng PCR product, ~3.2pmol primer and dH₂O to a final volume of 12 μ l.

2.2.11 Single Nucleotide Polymorphism (SNP) analysis of clones

Clones were grown in T25 flasks to 80% confluency and harvested as a cell pellet. Samples were submitted to Victorian Clinical Genetic Services (VCGS, MCRI) for single nucleotide polymorphism (SNP) analysis. All cell lines used for functional experiments retained normal karyotype.

2.2.12 Summarised flow cytometric analysis of GFP⁺ cells expressing CRISPR Cas9 vector and PCR screening of clones derived from flow cytometry sort.

Taken together, flow cytometric analysis revealed that *BMPR2^{DelKinase}* had the highest GFP⁺ population at 50% followed by *BMPR2^{DelTail}* at 38% and *BMPR2^{Null}* at 31% transfection efficiency (**Fig. 5A**). Remarkably, electroporation method resulted in a significantly higher transfection efficiency of Cas9 complexes into the cells compared to the liposome-mediated method used previously. The variability in transfection efficiency during electroporation can be attributed to a number of reasons such as age of cells, confluency of cells and quality/quantity of Cas9 expression vectors used (**Fig. 5B**). sgRNA G1 and G2 targeting exon 1 of *BMPR2* in *NKX2-5^{eGFP/W}* resulted in 1 clone B (#55) that had a biallelic deletion between G1 and G2. In contrast, sgRNA G3 and G4 targeting exon 6 resulted in 79 clones with biallelic deletions between the two guides while sgRNA G5 and G6 targeting exon 12 resulted in 50 clones with biallelic deletions between the two guides (**Fig. 5B**). Initially we hoped that genotyping of the clones could be simplified by creating extensive excisions that were detectable by PCR screening. It is clear from our screening results that not only small and large indels can be undetectable by PCR screening, but even indels that occur beyond the range of the sequencing primers can be overlooked. Therefore it has become imperative to have several validation methods in place when screening for desired clones.

Since the same CRISPR/Cas9 sgRNA utilised in the correction experiment was used for this experiment, we compared the transfection efficiencies between the two DNA repair mechanism, HDR and NHEJ. Cells that used NHEJ to repair the DNA nicks thus incorporating the transient expression of GFP had a 4 fold increase of 8% in transfection efficiency compared to cells that that used HDR in *BMPR2*^{2504delC} correction which resulted in only 2 % GFP⁺ cells, signifying that HDR recombination occurred at a lower frequency as opposed to the preferred NHEJ recombination (**Fig. 4**). However, this transfection efficiency may vary across cell types and it could be that iPSCs are more susceptible to death during manipulation than hESC.

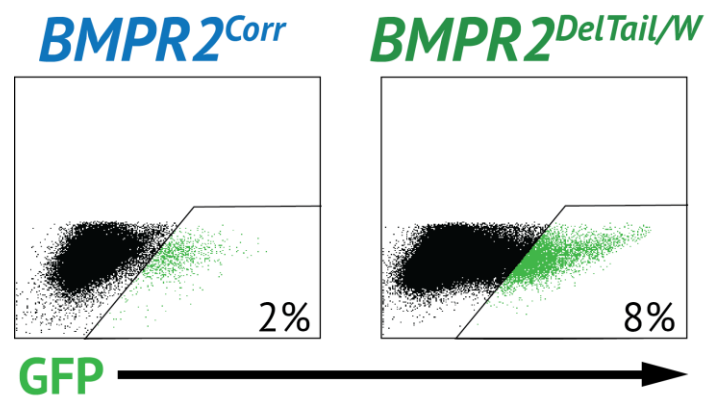


Figure 4. Transfection efficiencies of cells using HDR and NHEJ DNA repair pathway. Flow cytometry graphs showing the percentage of GFP⁺ cells 48hrs after lipofectamine transfection of PSCS with a plasmid encoding Cas9, GFP and expressing the same CRISPR guide designed to target c-terminal domain of *BMPR2*. *BMPR2^{Corr}* had a 2% GFP⁺ population whilst *BMPR2^{DelTail/W}* had 4-fold increase of GFP⁺ at 8%. HDR is known to occur at a lower frequency compared to NHEJ pathway; however, the cell type could also affect transfection efficiency.

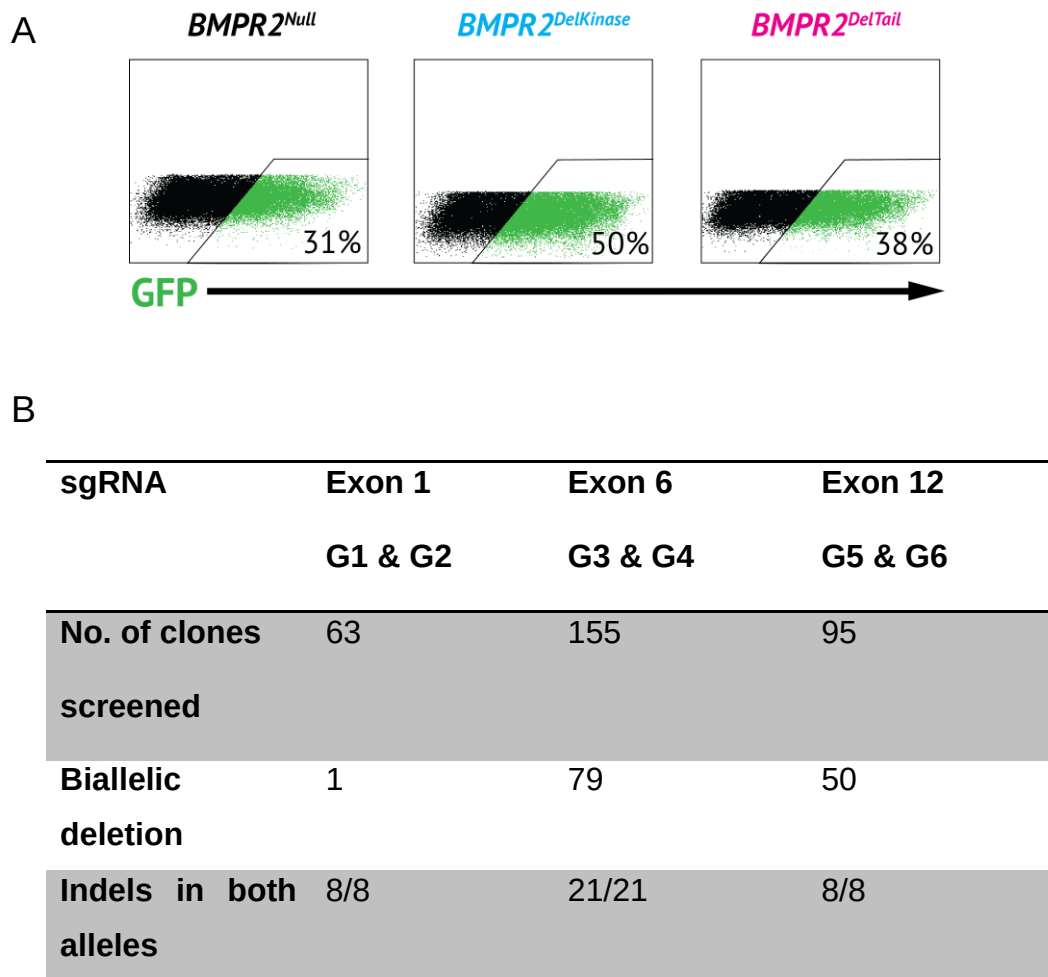


Figure 5. (A) Flow cytometric analysis of total GFP⁺ cells, 48hrs after electroporating PSCs with a plasmid encoding Cas9, GFP and sgRNAs designed to target the various domains of BMPR2. *BMPR2^{DelKinase}* had the highest GFP⁺ population at 50% followed by *BMPR2^{DelTail}* at 38% and *BMPR2^{Null}* at 31% transfection efficiency. The age of cells, confluency of cells and quality/quantity of Cas9 expression vectors used during electroporation can result in variability of transfection efficiency. By using the electroporation method, transfection efficiency has improved vastly compared to liposome-mediated method previously used. (B) Table depicts the number of clones screened from each targeting event, by each pair of sgRNA Cas 9 binding to the different domains of BMPR2. Followed by the number of clones that had the desired homologous deletion and the number of clones that had the desired deletion in one allele but small indels detected in the other allele.

2.3 Differentiation methods

2.3.1 Mesoderm differentiation of human pluripotent stem cells (hPSCs) using the spin embryoid body method

HESC culture and differentiation were performed as previously described (Elliott et al. 2011). Briefly, hESC lines were harvested at day 0 and resuspended in either APEL medium ($1\mu\text{g ml}^{-1}$ Insulin) (Ng et al. 2008) or StemDiffAPEL (Stem Cell Technologies, Cat# 05210) at 3.5×10^4 cells per 96 well containing 5-40ng/ml of BMP4 (R&D Systems, Cat# 314-BP), 5-40 ng/ml ACTIVIN A (R&D Systems, Cat# 338-AC) for BMP4 and ACTIVIN A dosage studies. 25 μl of APEL media containing 2x the desired concentration of growth factors were dispensed uniformly throughout the 96 well round-bottom non-adherent plate. Subsequently, cells were uniformly distributed at 25 μl per well containing the differentiation medium. To promote embryoid body (EB) formation, plates were centrifuged at 480g for 3 minutes before incubation at 37°C, 5% CO₂. At day 3, EBs were collected and enzymatically dissociated with TrypLE™ for approximately 5 mins at 37°C, rinsed with PBS and pelleted in 15ml falcon tubes. Dissociated EBs were then filtered with cell strainer and labelled with surface antibodies PDGFR- α and EpCam (Bd Biosciences) for FACS analysis. (For FACS methods see **section 2.3.8**)

2.3.2 Blood assays

Blood assays were carried out as previously described in Ng et al. (2008).

At day 0 of differentiation, growth factors as shown in the table below were added into 75mls of APEL media. Cells were harvested as described before

and quantified to give the total cell number for the required seeding density of 50×10^4 cells per plate. 80 μ l suspended cells in differentiation media was dispensed uniformly across 5 x 96 well round-bottom plates. Plates were spun in the centrifuge for 3 mins to aggregate cells into EBs and incubated in humidified 37°C/5% CO₂ incubator.

Concentrations of growth factors used at Day 0 of differentiation

Growth Factor	Stock	Final	25mls
BMP4	100ng/ μ l	20ng/ml	5 μ l
VEGF	100ng/ μ l	30ng/ml	7.5 μ l
SCF	100ng/ μ l	40ng/ml	10 μ l
Act A	100ng/ μ l	20ng/ml	5 μ l

Day 2- BL-CFC Assay - 1 plate of EBs from each cell line was harvested in TrypLE™ for 7mins at 37°C. EBs were dissociated with a Gilson pipette and centrifuged to remove TrypLE™. Dissociated EBs were resuspended in 500 μ L of APEL medium for quantification. Each well required 3000 cells and for each cell line, 3 x 24 wells was setup. The required number of cells was then added into MC-STAPEL (diluted to 1% using 2 x STAPEL supplements + STAPEL medium - PFHMII + day 3 blood growth factors). 2ml aliquots of MC with cells in 3 x yellow-capped tubes were mixed and 500 μ L dispensed into each 24 wells.

Day 3 growth factors added to 40mls of MC-STAPEL

Growth Factors	Stock ng/ml	Final ng/ml	40mls
SCF	100	100	40µL
VEGF	100	50	20µL
IL-3	100	50	20µL
IL-6	100	50	20µL
TPO	100	50	20µL
BMP4	100	20	8µL
FGF2	100	10	4µL
EPREX	2u/µL	2u/µL	40µL

Day 4- Stage II medium change - Half a plate of EBs from each cell line was harvested for FACS analysis on Day 4 with the following cell surface markers (**Supplementary 4**). 60µL of media from each well of the remaining 6 plates from each cell line were removed and replaced with 100µL of Stage II medium. For 6 plates, a total of 95mls of APEL media was required.

Growth Factors	Stock ng/ml	Final ng/ml	38mls
VEGF	100	50	19µL
SCF	100	50	19µL
IGF	100	25	9.5µL
FGF2	100	10	4.5µL

Day 6- Plate down of EBs onto Geltrex coated 6 well dishes. 2 x 6 wells were required for each cell line. 30 EBs were plated down into each well with Stage II medium and left to attach overnight. The next day Stage II medium was replaced with Stage III medium [3mls per well]. NOTE: For 4-wells at 2mls per well = 8mls IMDM + 40µL Pen/Strep + 40µL GFR-matrigel.

Day 7- Stage III medium change. Stage II medium was aspirated from wells and replaced with 3mls of Stage III medium supplemented with Pen/Strep to combat bacterial contamination.

Growth Factors	Stock ng /ml	Final ng/ml	12mls
SCF	100	50	6 μ L
VEGF	100	50	6 μ L
TPO	100	25	3 μ L
FLT3	100	25	3 μ L
IL3	100	50	6 μ L
FGF2	100	10	1.2 μ L
EPREX	2 u/ μ L	3 u/ μ L	40 μ L

DAY 7- FACS Analyses. One and a half plates of each cell line was harvested and dissociated using TrypLE™ for 18 minutes at 37°C. Cells were separated into required number of tubes and stained with CD31-APC, CD34-PECy7, CXCR4-BV, CD43-APC, CD73-APC, GLY-APC, KDR-APC, EpCAM-BV and CD45-BV (**Supplementary 4**) for FACS analysis.

DAY 12- FACS Analyses. Remaining 6-wells of each cell line were dissociated using TrypLE™ for 22 minutes at 37°C. Dissociated cells were separate into tubes and stained with CD31-APC, CD34-PECy7, CXCR4-BV, CD43-APC, CD73-APC, GLY-APC and CD45-BV (**Supplementary 4**) for FACS analysis.

2.3.3 Monolayer definitive endoderm differentiation of *BMPR2*^{2504delC/W} and iPSC^W

Definitive endoderm monolayer differentiation assay was carried out with *BMPR2*^{2504delC/W} and iPSC^W cell lines. Cells were harvested and seeded onto

Geltrex coated 12 well plates at 1×10^6 per well and induced with ACTIVIN A (100ng/ml), PI-103(50nM) inhibitor of phosphatidylinositol 3-kinase (PI3K)/Akt and target of rapamycin complex 1 (mTORC1) and the Wnt pathway activator CHIR99021 (2 μ M) for 24hrs. After 24hrs, media was replaced with BMP antagonist DMH-1 (1 μ M) and ACTIVIN A (100ng/ml) in differentiation media AEL(Ng et al. 2008). At day 3, endoderm cells were harvested and labelled with surface antibodies conjugated CXCR4-PECy7 and EpCam-APC and analysed by flow cytometry. Cells were also extracted for total RNA and quantified by qPCR with Taqman Probes (**Supplementary 5**).

2.3.4 Monolayer cardiac differentiation

For monolayer cardiac differentiation, the direct differentiation protocol was adapted from Lian et al. (2012). Single-cell suspension of PSCs were obtained by dissociating 70-80% confluent cell lines with TrypLE™ (Cat# 12605028). Viable cells (0.8×10^6 cells/well) were re-suspended in RPMI+B27 medium without insulin supplemented with 2mM L-glutamine, penicillin/streptomycin (cardiac differentiation media) containing 12 μ M CHIR99021 (Tocris, Cat# 4423), 80 ng ml⁻¹ ACTIVIN A, 50 μ g ml⁻¹ Ascorbic Acid (Sigma, Cat# A92902) and seeded onto a 12-well plate (Corning) coated with diluted (1:100) Geltrex™ (Life Technologies, Cat# A1413202). After 24hrs (day 1 of differentiation), medium was aspirated and replaced with fresh differentiation medium supplemented with 5 μ M IWR-1 (Sigma Aldrich, Cat# 0161) and 50 μ g ml⁻¹ Ascorbic Acid. On day 3 of differentiation, the IWR-1+Ascorbic acid differentiation medium was removed and replenished with

RPMI+B27 medium every two days. Cells were then maintained in RPMI+B27 medium until harvested for analysis.

2.3.5 Endothelial differentiation

Endothelial differentiation protocol was adapted from the monolayer cardiomyocyte differentiation protocol. Enzymatically dissociated cells were plated down as previously described in the cardiac differentiation protocol and the first 24-48hrs, cells were cultured in similar medium containing 80ng/ml ACTIVIN A and 12nm CHIR99021 in RPMI+B27 media. After 24/48hrs later, culture media was refreshed with RPMI+B27 and IWR-1 with additional two cytokines, 30ng/ml VEGF (PeproTech, Cat# 100-20) and 50ng/ml SCF (PeproTech, Cat# 300-07). Cultures were replenished every 2 days with the second media until day 6. At day 6, cells were harvested and labelled with cellular surface markers CD31 and CD34 (**Supplementary 4**) as described in the FACS staining protocol for flow cytometry sorting. The positive CD31⁺CD34⁺ population of endothelial progenitors were collected in FACS tubes, centrifuged at 1500 RPM for 3 mins, decanted before resuspending with HUVEC media, EGM-2MV Bulletkit (Lonza CC-3202) and plated onto 6 well dishes pre-coated with fibronectin (1ug/ml) at a cell seeding density of approximately 0.5×10^6 per well.

2.3.6 Smooth muscle cell differentiation

Day 0-2 Phase I – Smooth muscle cell differentiation protocol was optimized following Lian et al. (2014). Besides generating smooth muscle cells, this protocol can be used to generate endothelial cells by using a different

specification media after the initial stages. Cells were harvested as previously described and seeded onto 6 well dish pre-coated with Geltrex® at a cell seeding density of 0.5×10^6 cells per cm^2 . Cells were supplemented with LaSR basal medium, which consists of Advanced™ DMEM/F12, 2.5mM (ThermoFisher Scientific Cat# 12634028 GlutaMAX, and 60mg/ml ascorbic acid (Sigma Aldrich, Cat# A8960) and 6-10mM CHIR99021 for 2 days.

Day 3 Phase II – Media was removed and cultures were replenished with LaSR basal medium for a further of 3-4 days

Day 5 Phase III – Harvested cells were labelled with surface marker CD34 for cell sorting according to previously described methods. CD34⁺ cells were collected in a tube and plated down onto collagen IV coated 6 well dishes supplemented with medium 231 (ThermoFisher Scientific Cat# M231500) + smooth muscle differentiation supplement (SMDS) (ThermoFisher Scientific Cat# S0085) for further differentiation of CD34⁺ progenitors to smooth muscle cells. After 4 days in Phase III differentiation media, cells were passaged and replaced with smooth muscle cell maintenance media for growth consisting of medium 231 + smooth muscle growth supplement (SMGS) (ThermoFisher Scientific Cat# S00725).

2.3.7 Fluorescence-activated cell sorting of differentiating hPSCs

For cardiac and endothelial enrichment, FACS was performed on hPSC lines based on eGFP⁺ expression for cardiac cells and CRISPR experiments, while endothelial cells were labelled with CD31 and CD34 surface markers and sorted for CD31⁺CD34⁺ using BD INFLUX cell sorter (Becton Dickinson).

Cells were sorted on days 5, 6, 7, 10 or 14 depending on the type of differentiation and were dissociated and filtered through a 40µM cell strainer (Falcon, Cat# Corning, Inc. 352340). Single cell suspensions were re-suspended in FACS wash (Phosphate-Buffered Saline Buffer + 2% (v/v) foetal calf serum) containing propidium iodide (PI). Live cells were gated on the basis of side scatter, forward scatter and propidium iodide exclusion. Flow cytometric gates were set using IgG isotype controls. Flow cytometric analysis of acquired or sorted populations was performed using Flowlogic Software (Inivai Technologies).

2.3.8 Flow cytometric analysis

For flow cytometric analysis of EBs and sorting of live cells, differentiated hESCs were dissociated with TrypLE™ to form a single cell suspension. Briefly, samples were incubated with primary antibodies for 30min and washed using FACS wash buffer solution (i.e. 2% FBS in PBS). For control stains, cells were labelled with non-specific antibodies (IgG). All antibodies were diluted and incubated in FACS wash/FACS block buffer (2% FBS, 1% BSA, 2mM EDTA). For samples incubated with unconjugated primary antibodies e.g. PDGFR-α, a three-step stain was carried out. Following incubation with unconjugated primary antibodies, samples were rinsed with FACS wash. Samples were then incubated with a secondary fluorophore conjugated goat anti-mouse IgG antibody. Subsequently, samples were incubated with a second primary antibody e.g. EpCam conjugated with PE-Cy7. Lastly, labelled cells are re-suspended in FACS wash + PI for analysis. For isotype controls, cells labelled with unconjugated mouse IgG isotype

controls were labelled with PE secondary antibodies. Sample detection was performed using FACS Fortessa (BD Pharmingen™) and FACS data analysed using the software, Flow Logic™.

2.3.9 Intracellular flow cytometry

Intracellular flow cytometry was performed as previously described (Mossman et al. 2005). Briefly, hESCs were dissociated with TrypLE™ to a single cell suspension then fixed and permeabilised using BD Cytofix/Cytoperm (BD Biosciences) for 20min at 4°C. Cells were washed twice using 1X BD Perm/Wash (BD Biosciences), which was used as both the wash buffer and antibody diluent. Cells were incubated in an optimal concentration of the primary antibody or equivalent amount of isotype control for 30min at 4°C in the dark. Labelled cells were washed twice in 1X BD Perm/Wash, before being stained with an appropriate fluorochrome-conjugated secondary antibody using the same conditions as above. Following two washes in 1X BD Perm/Wash, cells were re-suspended in FACS wash prior to flow cytometric analysis. Isotype control cells were labelled with purified mouse IgG2b (BD Biosciences #556351), while unconjugated primary antibodies were detected with APC-conjugated goat anti-mouse IgG (BD Biosciences #550826).

2.3.10 Immunofluorescence and microscopy

Cells were seeded onto either plastic coverslips (Thermo Fisher Scientific Nunc™ Thermanox™ Coverslips) or chamber slides. Media was removed from cells and rinsed with PBS before fixing and permeabilizing in 4% paraformaldehyde with 0.1% triton X-100 for 10-15mins. Cells were then

rinsed prior to blocking in 5% Bovine serum albumin (BSA)/goat serum diluted in TBS/PBS buffer. After blocking, cells were incubated with primary antibodies overnight at 4°C. This was followed by incubation of either Rabbit or Mouse Alexa Fluor 568/647 conjugated secondary antibodies (Molecular Probes). Detection of immunofluorescence and image capture was carried out by confocal microscope (LSM 780 Zeiss).

2.3.11 Live cell imaging, fluorescence and bright field microscopy

Bright field and immunofluorescence images were acquired either by the Axiovert 200 Inverted fluorescent microscope, equipped with a Zeiss AxioCam or the confocal microscope Zeiss LSM 780. All images were processed with AxioVision software (Carl Zeiss Microscope). Post-analyses of images were processed in Image J.

2.4 Western blotting

2.4.1 Transfection of 293T cells with protein expression SMAD1

Controls for phosphorylation studies were generated by transiently transfecting HEK293T cells with a SMAD1 protein expression vector. HEK293T cells were passaged onto 3 x 3.5 cm dishes to 70-80% confluency, the day prior transfection. Transfection of 3 x 3.5 cm dishes required 363µl of Opti-MEM® (ThermoFisher Scientific Cat# 31985062) containing 0.02µg/ml vector plasmid and 50µl of FuGENE®6 transfection reagent (Promega Cat# E2691) was mixed in a tube and vortexed. For easy detection of transfection efficiency, 5µg of eGFP plasmid at a ratio of 1:10 FuGENE®6 was added in

the reaction. The mixture was incubated for 5-10mins (no longer than 40mins). 125µl of the mix was added drop wise while swirling to each well containing cells. Two ratios 3:1 and 6:1 of FuGENE®6:protein expression vector was tested and 6:1 gave the best transfection efficiency based on the observed eGFP⁺ expression. Two days after transfection, HEK293T cells were treated with/without BMP (100ng/ml) for 30mins before harvesting for protein (refer to **section 2.4.2**).

2.4.2 Harvesting protein lysates from cells

For BMP treatment, confluent cells on 10cm dishes were incubated with 100ng/ml BMP4 in serum starving medium for a desired period of time. After incubation, cells were rinsed twice with cold PBS before addition of 200µl – 1ml of either RIPA or NP40 buffer supplemented with PhosSTOP (Roche Cat# 04906845001) and cOmplete™, EDTA-free Protease Inhibitor Cocktail (Roche Cat# 04693132001). After a short incubation in the buffer, cells were scraped to collect the protein lysates. Protein lysates collected in eppendorf tubes were sonicated to release protein from the cells. After sonication, protein lysates were centrifuged for 20mins at 4°C and at top speed. The supernatants were collected and transferred into fresh eppendorf tubes to be stored at -20°C or at -80°C for long-term storage.

2.4.3 Protein quantification

The DC™ protein assay (BioRad Cat# 5000111) was utilised for the quantification of protein lysates. Working reagent A was prepared by adding 20µl of reagent S to 1ml of reagent A (This working reagent A' is stable for 1

week). Standard curve of 5 serial dilutions of a protein standard (BioRad Cat# 5000007) from 0.2mg/ml to about 1.5mg/ml protein was prepared. 5µl of the standards and samples were transferred into a clean, dry 96 well micro-titer plate. Following that, 25µl of working reagent A and 200µl of reagent B were added into each well containing standards and samples. After 15mins, absorbances were read at 750 nm within the hour. The plates were then mixed for 30secs and read by a micro plate detector (Tecan Infinite 200 PRO multimode reader) that had a mixing function. The absorbances were read at a wavelength of 750nm. Protein quantifications were processed in Image J.

2.4.4 Immunoprecipitation of protein lysates

Immunoprecipitations were carried out using Dynabeads® Protein G Kit (ThermoFisher Scientific Cat# 10007) according to manufacturer's description. 50µl of Protein G beads were transferred into each tube and buffer was removed by placing tubes on the magnet holder provided. The beads would adhere to the magnet allowing easy removal of the supernatant. Antibodies were diluted to a concentration of 1-10µg in 200µl of Ab Binding and Washing Buffer for each sample. Beads with antibodies (Dynabeads-Ab) were incubated for 10mins at room temperature with rotation. Subsequently, beads were placed on the magnet for removal of antibodies and washed twice with 200µl of Ab Binding and Washing Buffer. Protein samples containing antigen (Ag) (100-1000µl) were then added into tubes with Dynabeads-Ab complexes and incubated for 10mins at room temperature with rotation. After incubation, protein samples were removed again by placing into the magnet holder and the beads were washed twice with 200µl of Washing Buffer. After

removal of the wash buffer, Dynabeads-Ab-Ag mixture was transferred into fresh eppendorf tubes. 20µl of elution buffer was added to each Dynabeads-Ab-Ag complex in each tube and incubated for 2mins at room temperature. The tubes were placed into the magnet holder in order to bind the Dynabeads, the eluted Ab-Ag complex was collected and transferred into fresh eppendorf tubes. The eluted Ab-Ag complex was then boiled in 10µl of NuPage® LDS sample buffer and DTT reducing agent for 10mins at 70°C prior to gel loading.

2.4.5 Preparing protein for running on SDS page gel

Protein lysate ~ 20-100 µg per well	Xµl
Nupage LDS loading buffer	2.5µl
DTT (0.5mM)	1µl
Make up to 10 µl H ₂ O	Xµl
Total volume	20µl

For each protein sample, reagents were mixed in an eppendorf tube with a pipette and heated at 70°C for 10mins before running on SDS page gel.

2.4.6 Running SDS page electrophoresis

Packaging of the Novex pre-cast gel (Invitrogen) was removed and gel was rinsed with H₂O. The comb was removed from the gel before insertion into the electrophoresis tank. Protein ladder and pre-boiled protein lysates containing loading dye were loaded into the wells of the gel and ran with appropriate

voltages (e.g 0.5 Amp for overnight runs or 150-200v for 1-2hrs) until the loading dye reached the bottom of the gel.

2.5 Cell proliferation MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assays

The MTT assay was initially performed using Vybrant® MTT Cell Proliferation Assay Kit (ThermoFisher Scientific Cat# V13154) which is a colorimetric assay for assessing cell metabolic and proliferation activity. The NAD(P)H-dependent cellular oxidoreductase enzymes may, under defined conditions, reflect the number of viable cells present. These enzymes are capable of reducing the tetrazolium dye MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide to its insoluble formazan, which turns it to a purple colour that can be read by a spectrophotometer and quantified using a standard curve. Standards had a known number of cells seeded into 96 well plates and were carried out in triplicates. Standards allowed for the quantification of cell numbers in the samples. Subsequent MTT assays were performed using MTT (Sigma Aldrich Cat# 298-93-1) powder. MTT was dissolved in H₂O to a concentration of 0.5mg/ml. Existing media in 96 well plates with cells were removed and replaced with 50µl of serum/phenol/ascorbic acid free media (phenol free DMEM-F12/GlutaMax/Glucose/ITS-X), followed by 5µl of 0.5mg/ml MTT and incubated in a humidified incubator at 37°C for 4 hrs. Post 4 hrs, 50 µl of 1mg of sodium dodecyl sulphate, SDS (Sigma Aldrich Cat# 151-21-3) dissolved in 1ml of 10mM HCL-H₂O was added to each well and incubated for a further 4

hrs at 37°C before absorbances were read by a detector (Tecan Infinite 200 PRO multimode reader) at a wavelength of 570nm.

2.6 Scratch assays

Scratch assays were conducted using Passage 1 endothelial cells post day 6 of sorting. 0.5×10^5 cells in 50 μ L of EGM-2V media were seeded in a concentrated drop in the middle of each 12 well and carefully transferred to a humidified 37°C/5% CO₂ incubator for 2hrs. After 2hrs, the plate was viewed under the microscope to ensure that the cells have adhered to the plastic. APEL media prepared earlier with VEGF (50ng/ml) was then added to each well gently as not to perturb the cells. The next day, scratches were made with a pipette tip down the middle of each concentrated area of cells and replenished with APEL media containing relevant growth factors for the study. After which, the plates were immediately transferred to a pre-warmed incubator chamber attached to the stage of the confocal microscope for live cell imaging. Live-cell imaging was setup by indicating positions for each scratch and the time course was set for the confocal to capture images at 30min intervals for a period of 18hrs. Captured images were then quantified manually by measuring area closure according to time. All experiments were carried out in triplicates.

2.7 Angiogenesis assays

Angiogenesis assays were carried out according to Arnaoutova and Kleinman (2010) published protocol. Briefly, confluent endothelial cells were passaged the day prior carrying out the assay. Required amount of Geltrex® was

thawed overnight on ice at 4°C or thawed on ice if in smaller aliquots before use. 50-100µl of thawed Geltrex® was added gently into 96 well imaging plates with no air bubbles and left to set in the incubator at 37°C for at least 20mins. Meanwhile, endothelial cells were harvested and re-suspended at 1.5×10^6 per ml of suspension media, EGM-2-Basal with/without growth factors such as VEGF/BMP4/bFGF or in complete growth factor EGM-2V. 100µl containing 1.5×10^4 of cells in suspension was added gently on top of each Geltrex® coated well. Plates were returned to incubator and time was noted for time course purposes. N.B: Wells at the edges surrounding the 96 well imaging plates were filled with water to prevent the samples from drying out in the incubator. Additionally, the confocal microscope is not able to image the wells closest to the edges. Images were taken periodically at 1, 5, 8, 24hrs with Zeiss Bright Field Microscope.

2.8 Statistical analysis

Statistical analyses for all experimental outcomes were performed with Microsoft Excel version 14.7.2 and GraphPad Prism v7. Results are presented as mean $\pm$ S.E.M. and were compared using a 2-tailed Student t-test or one or two-way ANOVA (significance was assigned for $P < 0.05$). Unless otherwise stated, all experiments were done in triplicates ($n=3$).

Supplementary Figures

Supplementary Table 1. List of sgRNA sequences used in study.

Gene	sgRNA Sequence 5'- 3'	Repair Oligo 5'- 3'
<i>BMPR2</i> Exon 10	TATGCAGGTTCTCGTGTCT	GAAACCATCCCACCTTTTGAG GATATGCAGGTTCTCGTAAG CAGGGAAAAACAGAGACCCA AGTTCCCAGA
	sgRNA G1 Sequence	sgRNA G2 Sequence
<i>BMPR2</i> Exon 1	CTGTATTGTGATACGGGC	TACCATGGACCATCCTGC
	sgRNA G3 Sequence	sgRNA G4 Sequence
<i>BMPR2</i> Exon 6	GATTGGCCGAGGTCGATA	ACTATAAAGCGGGCAATGTT
	sgRNA G5 Sequence	sgRNA G6 Sequence
<i>BMPR2</i> Exon 10	CTCTGGTACGGATTCCCCTG	TATGCAGGTTCTCGTGTCT

Supplementary Table 2. List of primers used during PCR screen for CRISPR targeted clones.

Gene	Forward primer 5'- 3'	Reverse primer 5'- 3'
<i>BMPR2</i> Exon 10	ACCAGATGGCTTTTCAGAC AG	CAGGCTATTTTCTTTCCAGG C
<i>BMPR2</i> Exon 1	TAAGGAATCCTGCCTTCCC G	GGGGGACGCATGGCGAA
<i>BMPR2</i> Exon 6	GATTGGCCGAGGTCGATA	ACTATAAAGCGGGCAATGTT
<i>BMPR2</i> Exon 10	GATTTCCAAATGTGCCTGA AGG	AAACAAAATGGAAGTAAACT GAC

Supplementary Table 3. List of primers designed between the two sgRNAs used during PCR re-screen for validation of CRISPR targeted clones.

	Forward primer 5'- 3'	Reverse primer 5'- 3'
<i>BMP2</i> Exon 1	GATCAGTCCACGGGAGAG AAG	CAGCGAGGAAGTCATCCCTG
<i>BMP2</i> Exon 6	TCCTTGGATGAGCGTCCAG T	TAAAGCGGGCAATGTTGTCA
<i>BMP2</i> Exon 10	CAGAGGTTGGAAACCATCC CA	AGTGGGATGGTTTCCAACCT

Supplementary Table 4. List of antibodies used in this study.

Antibody	Dilution Factor	Supplier & catalog #
Mouse Anti-Human α – Actinin	1:200	Sigma, A7811
Anti-human CD31 APC	1:100	BioLegend, 303115
Mouse Anti-human CD31	1:200	DAKO, M0823
Anti-human CD34 PE/Cy7	1:50	BioLegend, 343516
Anti-human CD43	1:100	BioLegend, 343205
Anti-human CD44 APC	1:100	BioLegend, 103012
Anti-human CD45 BV	1:100	BioLegend, 103134
Anti-human CD73	1:100	BioLegend, 344008
Anti-human CXCR4/CD184	1:100	BioLegend, 306518
Anti-human EpCam/CD326 Cy7	1:50	BioLegend, 324222
Anti-human Glycophorin A	1:200	BioLegend, 306608
Anti-human KDR / CD309 APC	1:100	BioLegend, 359909
Anti-human Ki-67 PE	1:100	BioLegend, 350504
Rabbit Anti-human NKX2-5	1:200	AbCAM, 35842
Anti-human SIRPA PE	1:100	BioLegend, 323806
Anti-human SSEA 647	1:100	BioLegend, 125607
Anti-human PDGFR α /CD140a	1:100	BD Pharmingen, 556001

Anti-human PECAM-1/CD31 APC	1:100	BioLegend, 303102
Anti-human TIE2/CD202b	1:100	BD Pharmingen, 557039
Mouse Anti-Human Troponin I	1:200	Abcam, ab47003
Mouse Anti-Human Troponin T	1:200	Abcam, ab8295
Rabbit Anti-Human Troponin T	1:200	Abcam, ab45932
Anti-human vWF	1:200	DAKO, M0616
Anti-human VCAM1 APC	1:100	BioLegend, 105718
WESTERN BLOT Antibodies		
Mouse GAPDH	1:5000	Abcam, ab9484
Rabbit Phospho-Smad1/5/9	1:1000	Cell Signalling, 13820
Smad 1	1:1000	Cell Signalling, 9743
Rabbit Phospho-Smad 2/3	1:1000	Cell Signalling, 8828
Mouse Phospho-p38 MAPK	1:2000	Cell Signalling, 9216
Goat Anti-human BMPR-II	0.1µg/mL	R&D systems, AF811

Supplementary Table 5. List of Taqman assays used in gene expression studies.

Taqman Probe	Thermo Fisher Scientific catalog #
ACTA2	Hs00426835_g1
ACVR2A	Hs00155658_m1
AKT	Hs00178289_m1
BMP2	Hs00154192_m1
BMP4	Hs00370078_m1
BMPR2	Hs00176148_m1
BMPR2	Hs01574530_m1
BMPR2	Hs01556137_m1
EFNA1	Hs00358886_m1
EFNAB2	Hs01031833_m1

GATA4	Hs00171403_m1
GAPDH	Hs99999905_m1
HEY1	Hs01114113_m1
HEY2	Hs00232622_m1
ID1	Hs0367657_s1
KDR	Hs00176676_m1
MYL2	Hs00166405_m1
MYH6	Hs01101425_m1
NOTCH1	Hs00413187_m1
NPPA	Hs00383231_m1
NKX2-5	Hs00231763_m1
TBX5	Hs01052563_m1
TBX20	Hs00396596_m1
SMAD1	Hs01077084_m1
SMAD7	Hs00998193_m1
vWF	Hs00169795_m1

CHAPTER



Generation of an Allelic Series of *BMPR2* Mutant Cell Lines

Introduction

BMPR2 is important for pulmonary arterial homeostasis and mutations in *BMPR2* are commonly found (>70 %) in patients with heritable pulmonary arterial hypertension (PAH) (Machado et al. 2009). In this chapter, we describe the generation of tools to investigate the molecular mechanisms underlying the cellular phenotype of PAH caused by mutations in *BMPR2*.

The genetic background of individual pluripotent stem cell (PSC) lines is a potential source of variability that may affect *in vitro* disease modelling. There are two potential solutions to this problem: correcting the disease causing mutation in a patient derived iPSC line or introducing the disease causing mutation into a wild type PSC line. Either way, for the “disease in a dish” approach to be successful, it is essential to set up an experimental system in which the disease-causing genetic lesion is the sole variable. However, until relatively recently, difficulties in performing precise genetic modifications in PSC has hindered the creation of isogenic cell lines (Soldner et al. 2011). Genome editing using CRISPR/Cas9 based methods has facilitated the rapid and precise genetic engineering required for the efficient production of isogenic pairs of PSC lines (Mali et al. 2013). We used this technology to generate a panel of isogenic cell lines from PSCs that contained a series of *BMPR2* mutations pertinent to PAH.

Results

3.1 Correction of iPSC *BMPR2*^{2504delC/W}

We utilised an iPSC cell line made from skin fibroblasts of a female PAH patient carrying a single cytosine deletion in exon 10 (c.2504delCFs60), which encodes the C-terminal end of the kinase domain. This single nucleotide deletion results in a frameshift leading to a pre-mature stop codon, truncating the cytoplasmic tail domain of the protein (**Fig. 3.1**).

As a prelude to studying the effect of this mutation, we created an isogenic control cell line by correcting the mutation using CRISPR mediated homologous directed recombination. We designed a 20 base single stranded guide RNA to complement the sequences adjacent to the PAM site (NGG) closest to c.2504delC mutation. The repair template used was a 70 base oligonucleotide that re-introduced the deleted cytosine and included a series of synonymous mutations. These silent mutations altered the codon sequence used for Valine (GTG→GTA) and Serine (TCT→AGC) at position 453 and 454 aa in the *BMPR2* gene (**Fig. 3.2 A**) and ensure that the repaired allele is no longer susceptible to Cas9 cleavage. Furthermore, these changes in the corrected *BMPR2* allele were used to enable PCR based screening for clones that had incorporated the repair template (**Fig. 3.2 B**).

The parental *BMPR2*^{2504delC/W} cell line was co-transfected with the repair oligonucleotide and the CRISPR expression plasmid carry the sgRNA (PX458, see **Chapter 2, 2.2**). To derive single clones, transfected cells,

identified on the basis of GFP⁺ expression, were isolated by flow cytometric sorting and deposited into each well of a 96 well plate. PCR screening of 99 clones identified three clones with the rescue template incorporated into the *BMPR2* locus (**Fig. 3.2 B**). Sequencing analysis of isolated PCR products from these clones demonstrated that only a single clone had the desired genotype, i.e. *BMPR2*^{Corr/W} (**Fig. 3.2 C**). Furthermore, *BMRP2* expression levels were restored in the *BMPR2*^{Corr/W} line compared to the parental *BMPR2*^{2504delC/W} line (**Fig. 3.2 D**). However, karyotyping by single nucleotide polymorphism (SNP) analysis revealed that the *BMPR2*^{Corr/W} PSC line contained multiple chromosomal abnormalities. Therefore, this *BMPR2*^{Corr/W} cell line could not be used as an appropriate control for the original *BMPR2*^{2504delC/W} iPSC line (**Appendix Fig. 1**). We found that *BMPR2*^{2504delC/W} is an unstable iPSC line because it has the propensity to acquire chromosomal abnormalities even after rescue efforts by single cell sorting to isolate a clone with normal karyotype, thus precluding its use for further experiments.

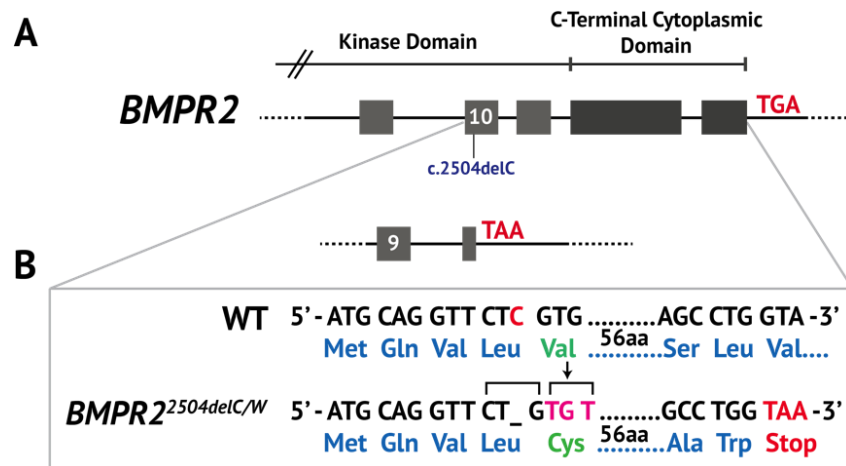


Figure 3.1. Schematic showing the point mutation *c.2504delC* in *BMPR2* that a PAH patient is carrying. (A) This patient carries a heterozygote point mutation *c.2504delC* in exon 10 that encodes the C-terminal of the kinase domain in *BMPR2*. Skin fibroblast from this patient was reprogrammed into a PSC line; *BMPR2*^{c.2504delC/W} **(B)** *BMPR2*^{c.2504delC/W} has a single nucleotide Cytosine deletion that results in a frameshift mutation changing Valine to Cysteine, resulting in a pre-mature stop codon 60 aa later. The pre-mature stop codon results in a *BMPR2* protein that lacks the C-terminal tail domain. The mRNA transcript produced by this mutation is believed to be eliminated by nonsense-mediated decay (NMD).

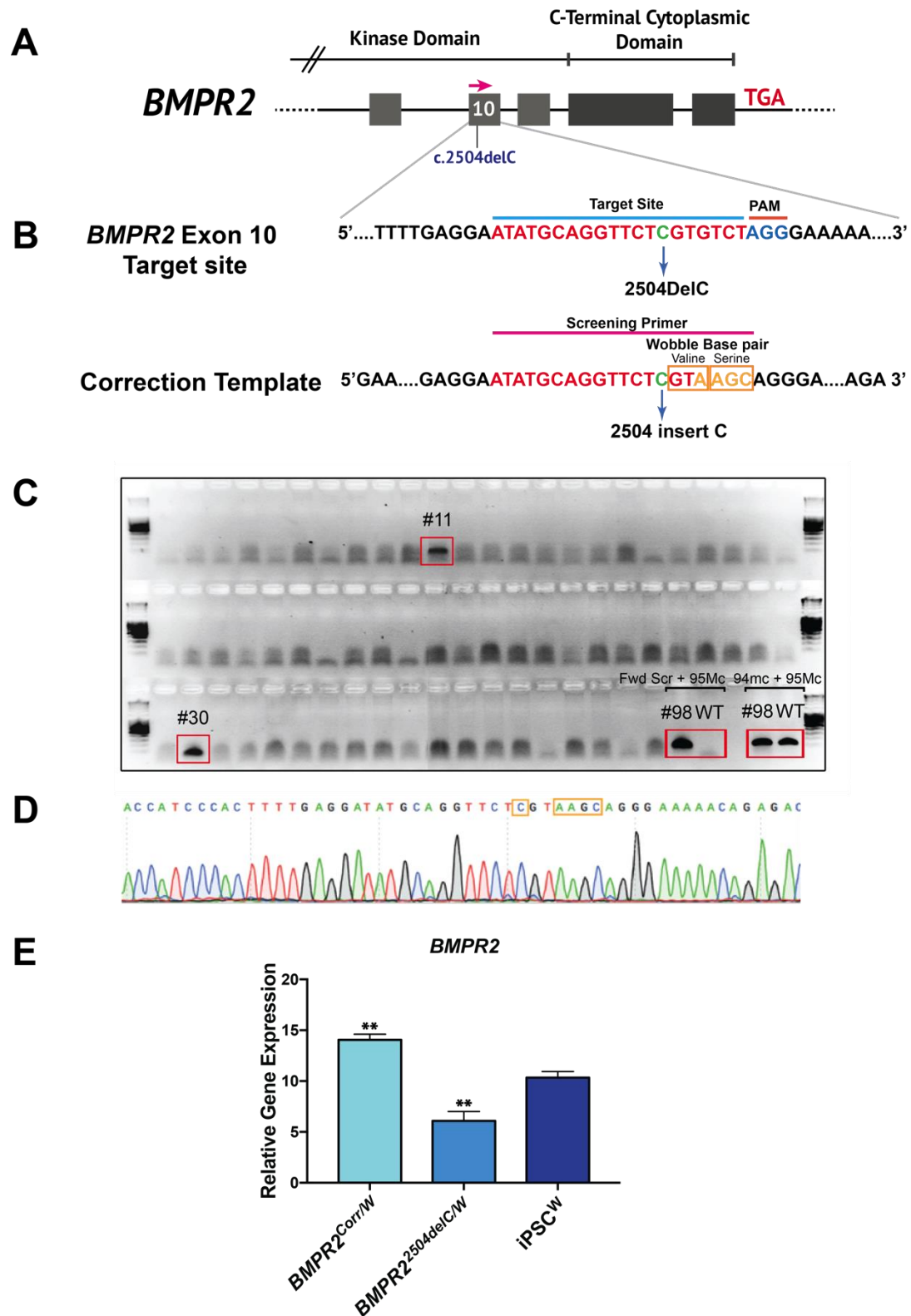


Figure 3.2. Correction of a pathogenic BMPR2 mutation. (A) Schematic showing the point mutation c.2504delC in BMPR2 for repair. Pink arrow indicates the location of the forward screening primer. **(B)** Genomic loci of target site for repair in BMPR2^{2504delC/W}. Design of 70 base oligonucleotide repair template containing synonymous mutations (highlighted in orange) and PCR screening primer to identify successful incorporation of repair template in the BMPR2^{2504delC/W} allele. **(C)** Gel electrophoresis of PCR screening detected only 3 clones out of the 99 clones that had incorporated the repair template. However, only one clone had the correct insertion. **(D)** Chromatogram of sequencing result from DNA of clone #98 showing correct insertion of the repair oligonucleotide correcting 2504delC in the BMPR2 locus. **(E)** Gene expression profile of BMPR2 from RNA extraction of corrected clone #98 BMPR2^{Corr/W}, BMPR2^{2504delC/W} and iPSC^W. BMPR2 expression seemed to be restored in BMPR2^{Corr/W} (**p<0.0001) in comparison to parental line BMPR2^{2504delC/W} (**p<0.003). Statistical analyses were carried out using Tukey's multiple comparisons test. Data show mean expression of BMPR2 levels (n=3; ±S.E.M.).

3.2 Novel frameshift mutations at the *BMPR2* locus in hESCs

To generate a series of novel mutations analogous to the pathogenic *BMPR2*^{2504delC/W}, we took advantage of the propensity of PSC to use the non-homologous end-joining (NHEJ) pathway to repair CRISPR/Cas9 cleavage. When used for DNA repair NHEJ commonly causes insertions or deletions (called indels) which occur at a higher frequency than HDR (Paix et al. 2015). Therefore, this approach circumvents the low efficiency of HDR observed when repairing the *BMPR2*^{2504delC/W} mutation (**Section 3.1**) and is predicted to result in similar truncating mutations. Furthermore, we chose to introduce these mutations into a PSC line carrying sequences encoding the enhanced Green Fluorescent Protein (eGFP) at the *NKX2-5* locus (Elliott et al. 2011). *NKX2-5* is a homeobox protein and an early marker of cardiogenesis (Schwartz and Olson 1999). The *NKX2-5*^{eGFP/W} reporter line is useful for isolating cardiovascular cell lineages (**Fig. 3.4 A**) (Elliott et al. 2011, Skelton et al. 2014). By generating mutations in *BMPR2* in the *NKX2-5*^{eGFP/W} reporter line, we can utilize the reporter to enrich for cardiomyocytes allowing us to study the effects of *BMPR2* deficiency on cardiomyocytes.

After lipofectamine transfection of the Cas9 expression vector/CRISPR guide plasmid, single transfected cells (as judged by GFP⁺ expression from the Cas9 expression vector) were distributed into each well of a 96 well plate using single cell flow cytometry sorting. We generated *BMPR2*^{DelTail/W} by introducing small deletions through NHEJ after cleaving *BMPR2* at c.2504 with the same CRISPR/Cas9 sgRNA used in the correction experiment. A total of 100 colonies from the single cell flow cytometry sorting were screened

for indels by PCR. PCR fragments spanning the genetically modified region of 10 potential heterozygous clones (**Fig. 3.3**) were TOPO cloned into *E.coli* competent cells and plasmid DNA was sent for sequencing. This analysis indicated that 2 of the 10 clones were heterozygote for a deletion in the C-terminal tail domain of *BMPR2* gene (**Fig. 3.4 C**). In each case, these heterozygote clones had a deletion of several nucleotides at a point within the gene that corresponded to the predicted CRISPR/Cas9 cut site (**Fig. 3.4 C**). These deletions generated a premature stop codon in exon 10, which encodes the C-terminus of the *BMPR2* kinase domain (**Fig. 3.4 D**). Thus, these mutations, designated *BMPR2^{DelTail}*, are similar to the patient iPSC *BMPR2^{2504delC}* mutation with both encoding a truncated protein lacking the tail domain. Gene expression analysis showed that *BMPR2* mRNA levels in two individual *BMPR2^{DelTail/W}* clones were similar to those seen in our patient iPSCs, *BMPR2^{2504delC/W}* ($p < 0.01$, $n = 3$) (**Fig. 3.5**) and were significantly reduced relative to the isogenic wild type controls (*BMPR2^{DelTail/W}* A $p < 0.04$, $n = 3$ and *BMPR2^{DelTail/W}* B $p < 0.01$, $n = 3$), *NKX2-5^{eGFP/W}* and wild type iPSC^W.

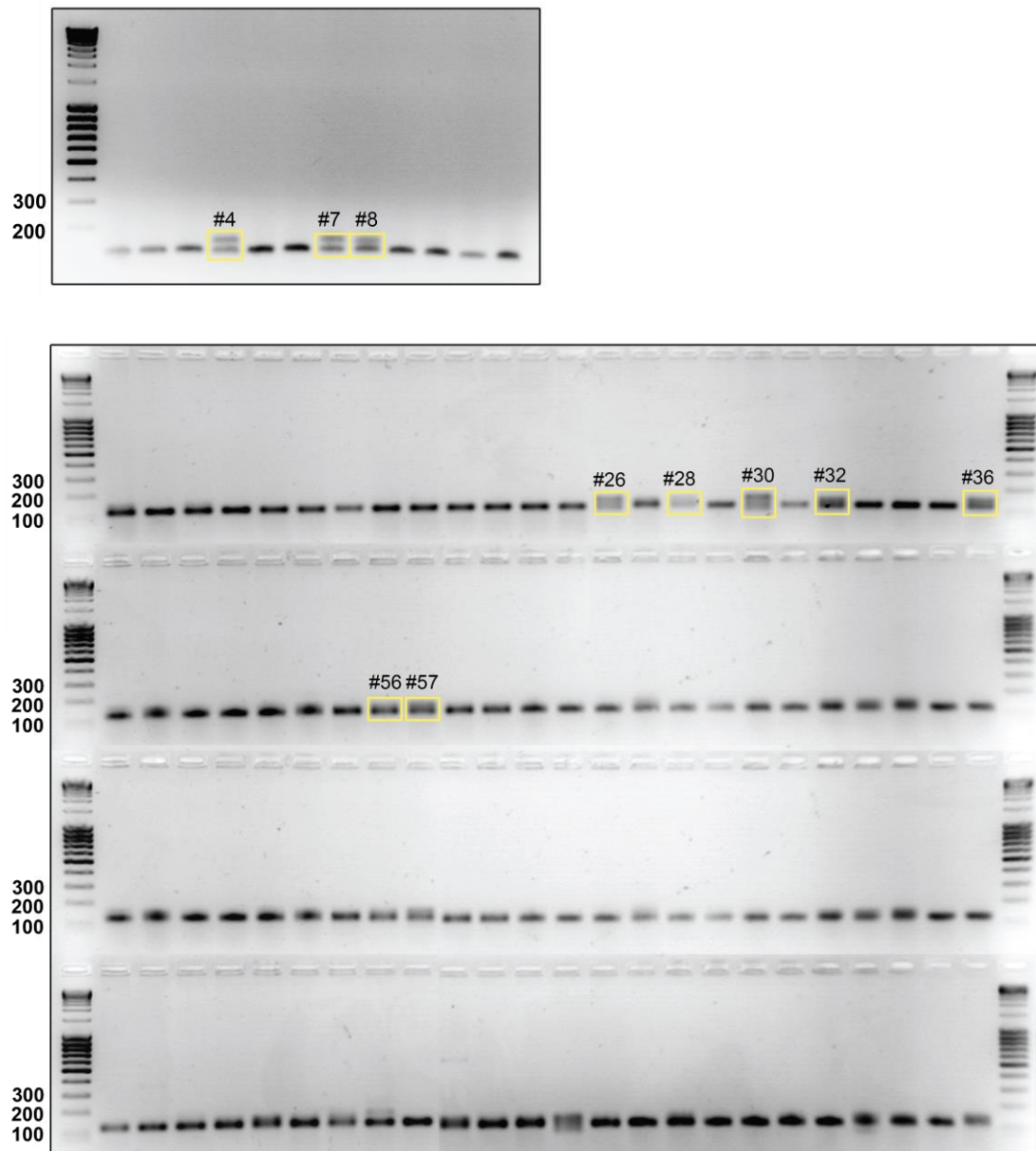


Figure 3.3 Gel electrophoresis of PCR screening for CRISPR/Cas9-sgRNA targeted clones. 10 clones were shortlisted as heterozygotes and PCR fragments were TOPO cloned into plasmids and extraction of plasmid DNA for further analysis by sequencing to select clones with the desired deletions. After sequence analysis, 2 clones, #4 (Clone A) and #36 (Clone B) were selected.

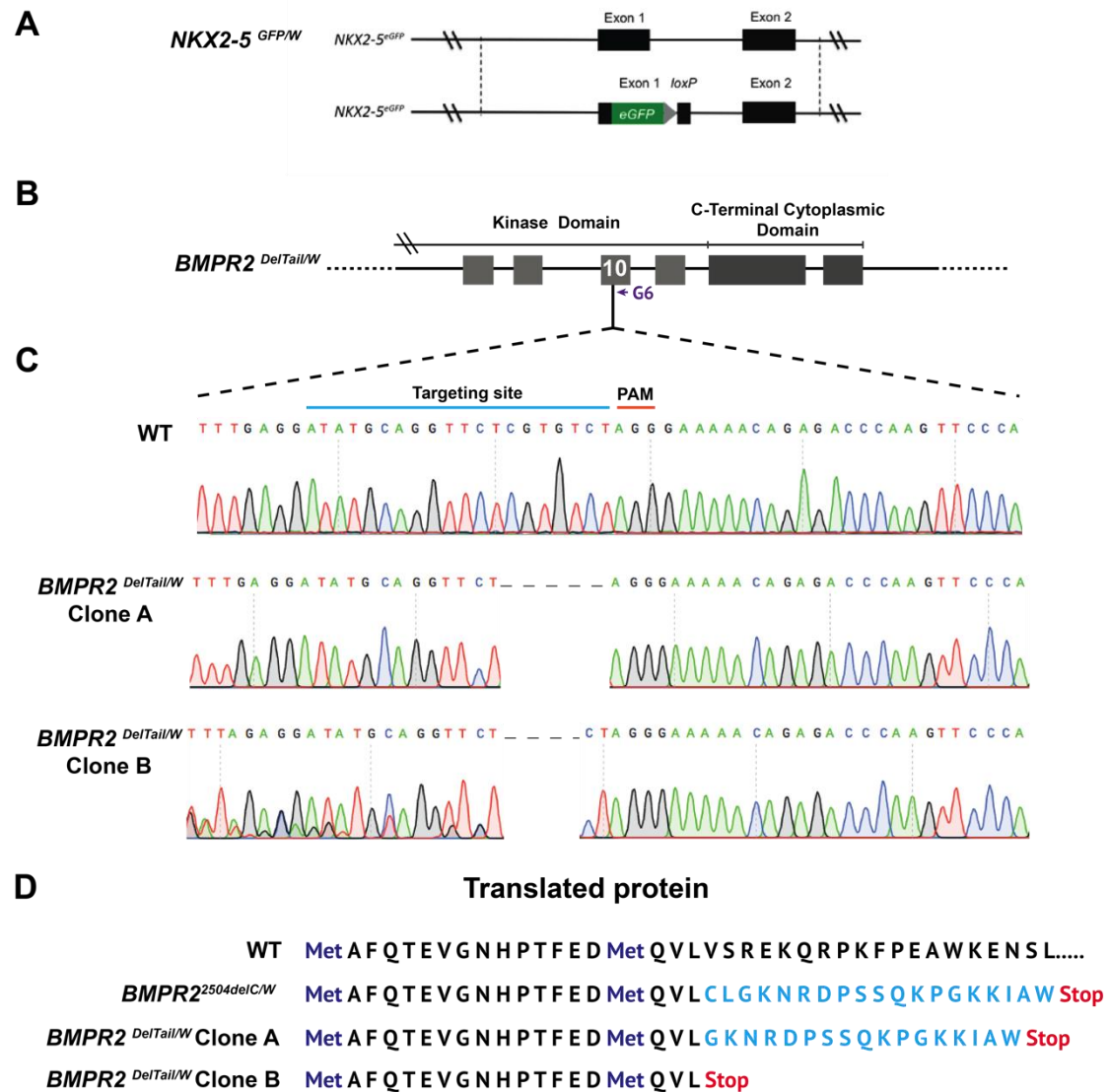


Figure 3.4 Generation of $BMPR2^{DelTail/W}$ in $HES3\ NKX2-5^{eGFP/W}$ (A) Schematic showing exon 1 of $NKX2-5^{eGFP/W}$ hESC with eGFP reporter tag. **(B)** The exon 10 encoding the C-terminal of kinase domain of $BMPR2$ was targeted in the $HES3\ NKX2-5^{GFP/W}$ hESC line. G6 denotes the location of the sgRNA used to target the gene **(C)** Sequence of wild type, clone A and clone B $BMPR2^{DelTail/W}$ show several base pair deletions within the target site. **(D)** Snap shot of amino acid (aa) sequence translations in $BMPR2$ of wild type, patient PSC line $BMPR2^{2504delC}$ and $BMPR2^{DelTail/W}$ clones at the mutation sites. $BMPR2^{DelTail/W}$ clones harbouring these deletions similarly cause a premature stop codon in $BMPR2$ akin to patient iPSC $BMPR2^{2504delC/W}$.

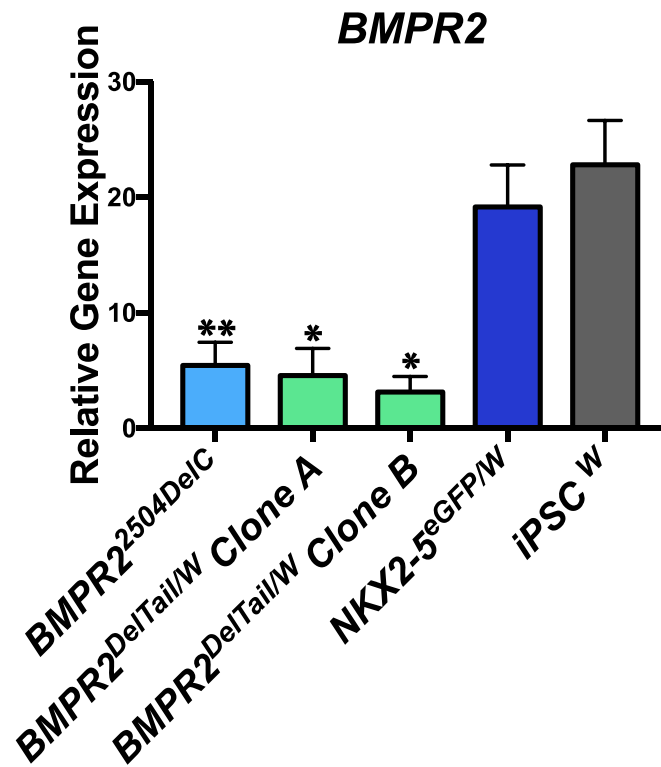


Figure 3.5 Gene expression analysis of BMPR2 from RNA extraction of CRISPR targeted clones A and B. BMPR2^{delTail/W}A (* $p < 0.04$) and BMPR2^{delTail/W}B (* $p < 0.01$) show significant reductions in BMPR2 expressions compared to parental line NKX2-5^{eGFP/W} and similar expression levels to patient BMPR2^{2504delC/W} (** $p < 0.01$ vs iPSC^W). In contrast, NKX2-5^{eGFP/W} and iPSC^W have significant elevated levels of BMPR2. Statistical analyses were conducted using Tukey's multiple comparisons test. Data show mean expression of BMPR2 levels ($n=3$; $\pm$ S.E.M.).

3.3 Generation of an allelic series of homozygote *BMPR2* mutants

In vivo studies of heterozygous *BMPR2* germline mutations demonstrated that *BMPR2*^{+/-} mice are viable and reproduce normally (Beppu 2010). These mice also exhibit mild PAH. In humans PAH, heterozygous mutation of the *BMPR2* tail domain is approximately 30% penetrant, with most carriers being asymptomatic (Frump et al. 2013). We reasoned that a similar level of phenotypic penetrance in an *in vitro* PSC model might be difficult to detect, particularly as the appropriate cell type (i.e. endothelial or smooth muscle cells) to study had yet to be determined. To place ourselves in a position in which the phenotype might be more robust, we derived an allelic series of *BMPR2* homozygote mutants that targeted different domains within *BMPR2*. In some cases, the phenotype of the corresponding mutation in the mouse was profound, potentially providing a reference point for some of the mutations predicated to have a less severe effect. For example, studies have shown that mice homozygous for a deletion of *BMPR2* kinase domain do not develop past gastrulation, whereas heterozygous mice carrying the same mutation appear to be normal (Beppu et al. 2000).

To generate the *BMPR2* allelic series, we designed 3 pairs of CRISPR guides (sgRNA) that targeted the extracellular domain, the kinase domain and C-terminal cytoplasmic tail domain of *BMPR2*. As demonstrated in previous CRISPR/Cas9 based genome editing experiments, single sgRNAs most frequently produce insertions or deletions (indels) of one to several nucleotides. Therefore, to ensure homozygous deletion and complete disruption of the gene, and to facilitate the identification of PSC clones with

the desired modifications, we chose to partially delete an exon from each different domain in the *BMPR2* locus. This strategy was also put in place so it was possible to directly screen for PSC clones carrying a homozygous deletion without the need to use DNA sequencing as part of the screening process. **Fig. 3.6 A** is a schematic of the *BMPR2* locus showing the regions targeted to generate the allelic series of *BMPR2* mutant cell lines. To generate *BMPR2*^{Null}, a complete *BMPR2* null line, a pair of sgRNAs, G1 and G2 (**Fig. 3.6 A G1 & G2**) were designed to remove a DNA fragment of 164 base pairs encompassing part of 5'UTR and start codon (ATG) in exon 1 of *BMPR2* that encodes part of the extracellular ligand-binding domain. A second pair of sgRNAs, G3 and G4 (**Fig. 3.6 A G3 & G4**) were designed to remove 146 base pairs within exon 6, which encodes part of the *BMPR2* kinase domain. The third pair of sgRNAs, G5 and G6 (**Fig. 3.6 A G5 & G6**) were designed to remove 89 base pairs from the splice acceptor site of exon 10, generating homozygote versions of deletions similar to those present in our patient iPSC line *BMPR2*^{2504delC/W}. In addition, each pair of sgRNAs was designed to produce a deletion that was not a multiple of 3 base pairs, creating a predictable frameshift mutation that would most likely result in a non-functional polypeptide. **Fig. 3.6 B** shows examples of sequencing results representing two clones of each *BMPR2* knockout class confirmed to have successful targeted sites utilising CRISPR techniques.

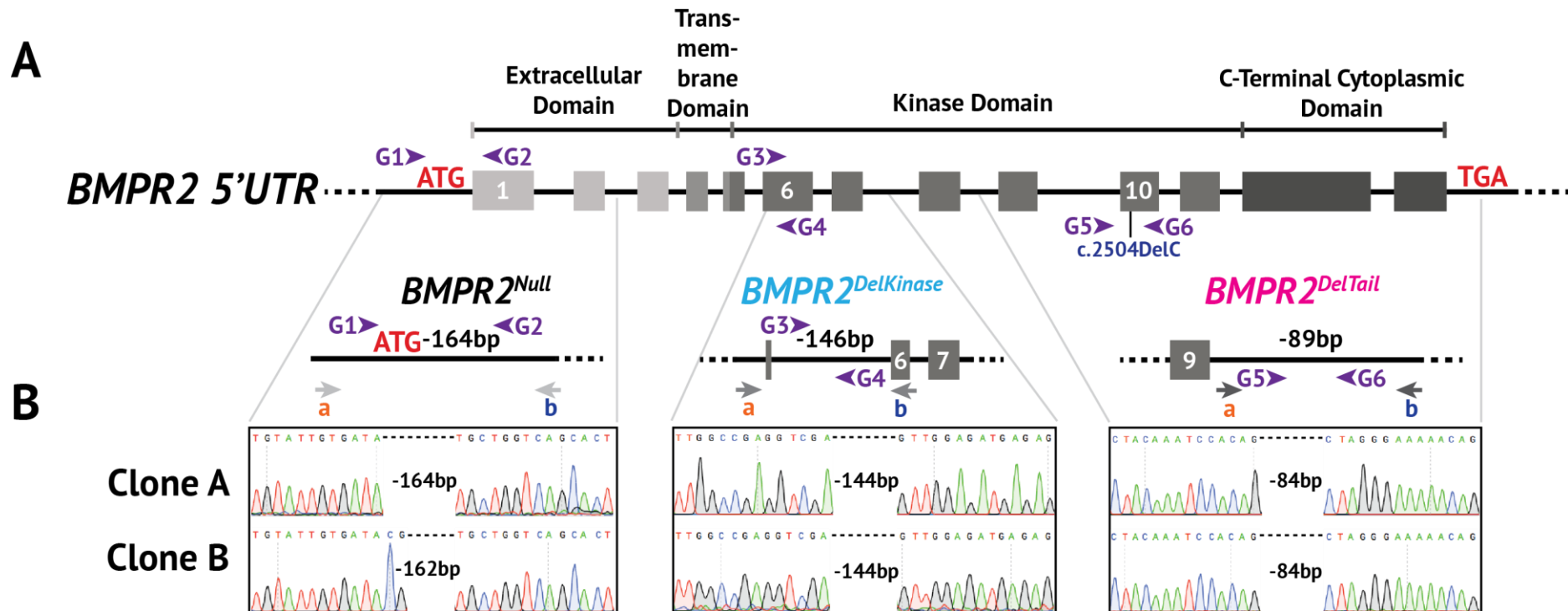


Figure 3.6 Schematic of BMPR2 Gene containing 13 exons and 12 introns. (A) Exons 1-3 encode the extracellular domain, exons 4-5 encode of the transmembrane domain, exons 5-11 encode of the kinase domain and exons 12-13 encode the C-terminal cytoplasmic tail domain. c.2504DelC highlighted in blue indicates the point mutation in our patient derived iPSC line. The missing cytosine causes a frameshift mutation and is predicted to induce a pre-mature stop codon thus truncating the tail domain of BMPR2 protein (A. Left to right) Dual sgRNA guides were designed to create a complete knockout of BMPR2 (G1&G2), BMPR2 kinase domain mutant (G3 & G4) and a BMPR2 tail domain mutant (G5 & G6) in HES3 NKX2-5^{eGFP/W} hESC line. (B) Below each region are the predicted transcript after the removal of the sequences by the sgRNAs. Arrows denoted by a and b represent the forward and reverse screening primers used during the PCR screen of targeted clones. Followed by sequencing results from two clones, A and B with confirmed deletions after successful targeting with the Cas9 sgRNAs.

3.4 The workflow and screening processes for the generation of an allelic series of homozygote *BMPR2* mutants

The overall workflow for the generation of each mutation type is shown in **Fig. 3.7**. Although our experimental design was set up to enable direct identification of clones containing a homozygous deletion, this prediction relied on both CRISPR guides working efficiently. In reality, this outcome was rare, and so, ultimately, PCR fragments representing the targeted regions required sequencing. Using conditions described in **Chapter 2, 2.2**, we electroporated cells with each pair of sgRNA/Cas9-T2A-GFP expression vectors into the cardiac reporter line *NKX2-5^{eGFP/W}*. After 48 hours, GFP⁺ cells that were transfected with CRISPR expression plasmids were single cell sorted into wells of a 96 well plate. Following approximately 2 weeks, resultant colonies were then screened via PCR using primers that corresponded to sequences on either side of the DNA interval predicted to be deleted by each sgRNA/CRISPR pair. Clones that had the desired excisions were shortlisted to the next stage of validation, which involved sequencing of each *BMPR2* allele. PCR products from selected clones were cloned into TOPO plasmids and subsequently 4 recombinant plasmids representing each deletion type were sequenced. As a final validation step, PSC clones representing each deletion type were re-screened using a PCR strategy that utilised primers designed to anneal to sequences within the deleted interval in combination with each of the external screening primers. This step ensured that the PSC clones were genotypically homozygous for the *BMPR2* deletion, as a PCR

product would amplify only from cultures containing the wild type *BMPR2* allele.

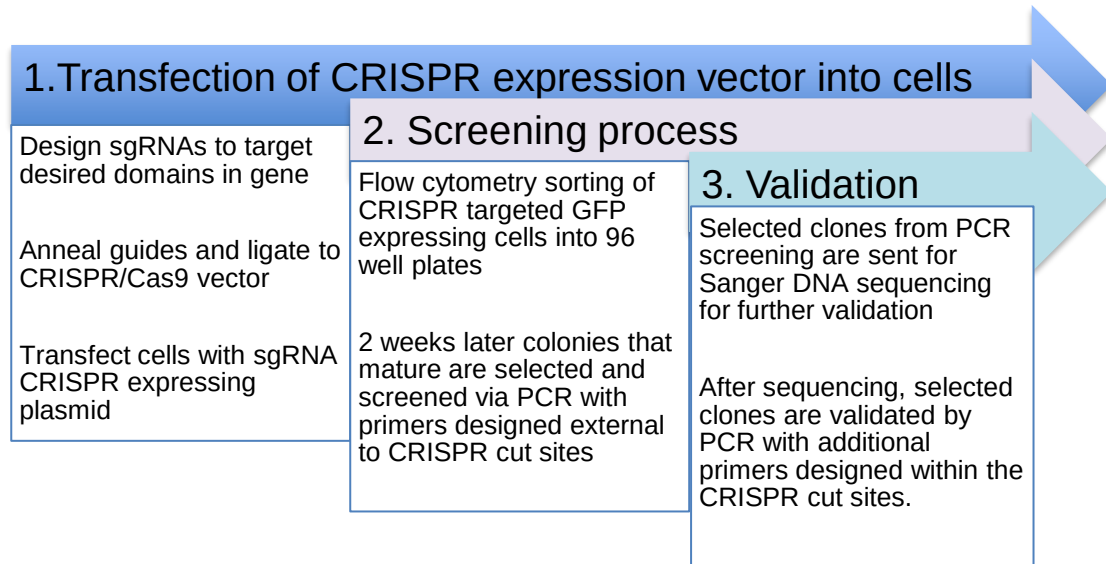


Figure 3.7 Flow chart describing overall workflow using CRISPR/Cas9 system to modify a cell line. Brief overview of the workflow: 1) Transfection of CRISPR expression plasmid into cells. 2) Screen for colonies. 3) Validate desired clones.

3.5 Generation of hESC *BMPR2*^{Null} carrying deletion of *BMPR2* exon 1

Following single cell sorting of cells transfected with sgRNA CRISPR vector G1 and G2, targeting exon 1, 63 clones were screened by PCR with primers located outside G1 and G2 binding sites denoted in **Fig. 3.8 A** as (a & b). Only 5 clones were identified by PCR as biallelically targeted (red boxes, **Fig. 3.8 B**), their PCR products were purified and then TOPO cloned into plasmids for further analysis by DNA Sanger sequencing. Sequencing analysis showed that of these 5 clones, only 1 clone, B (#55), had 162 nucleotides removed in both alleles. The other 4 clones (red boxes, **Fig. 3.8 B**) had heterozygous deletions and large insertions in their other allele that were undetected by our sequencing primers. Further isolation of the larger PCR fragment for sequencing had to be carried out to determine if the insertions lead to disruption of the open reading frame. We selected a second clone denoted A (#41) out of the 4, for experiments after determining that the sequences of the larger PCR fragment did indeed disrupt the ORF (**Fig. 3.11 C**). Sequencing analysis revealed that clone A, (#41) had the targeted deletion of 164 nucleotides in one allele and a monoallelic insertion of 481 nucleotides 52 bases after the ATG start codon that leads to a frameshift mutation (**Fig. 3.11 C**). From our PCR screen, we observed clones to have potential heterozygote deletions and 8 of these clones (yellow boxes, **Fig. 3.8 B**) were shortlisted. Although the aim was to acquire homozygote *BMPR2* mutants, we reasoned that having heterozygote deletions for each set of homozygote *BMPR2* mutants would provide additional resources to investigate *BMPR2* function. However, these 8 clones were rejected, after sequencing results revealed random indels in their putative wild type alleles.

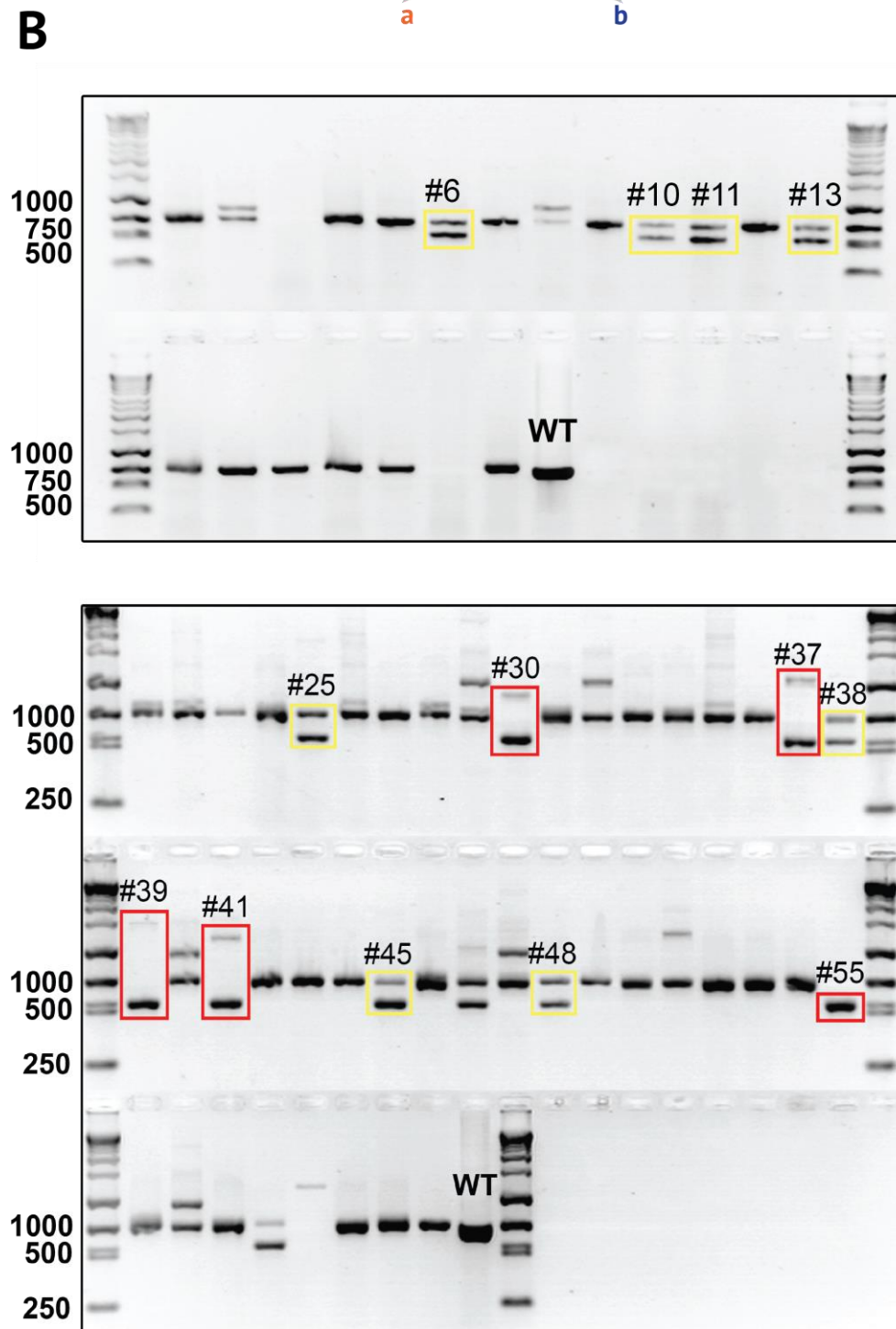
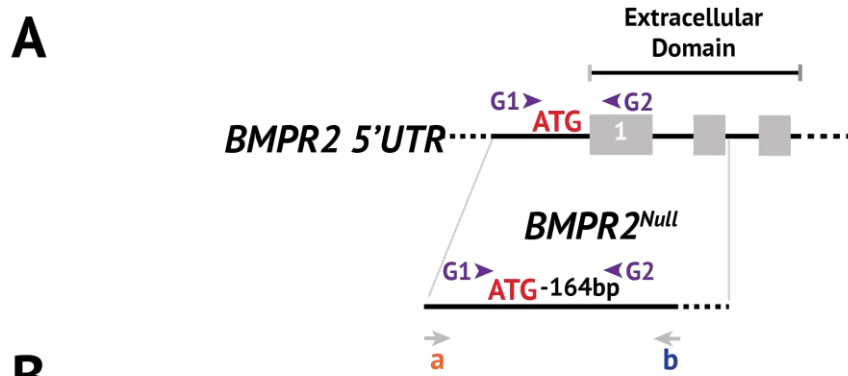


Figure 3.8 Gel electrophoresis of PCR screen to identify *BMPR2*^{Null} clones that were targeted with dual CRISPR/Cas9 sgRNAs in exon 1 of *BMPR2*. (A) Schematic of *BMPR2* showing the targeting sites of sgRNAs G1 & G2 in exon 1 and the predicted outcome of the protein transcript after excision of sequences between the two sgRNAs. Arrows denoted by **a** & **b** show the location of the primers used in the PCR screen. (B) Image of an agarose gel showing the results of the PCR screen of 63 clones derived from exon 1 *BMPR2* Cas9 sgRNA targeting. Only 1 clone, B (#55) of the 5 sequenced (red boxes) had biallelic deletions between G1 and G2. The other 4 clones had large insertions in one of their alleles and the desired deletion in their other allele. Further analyses of these insertions had to be carried out by sequencing the extracted larger PCR fragment because it was not detected by initial sequencing. Yellow boxes highlight 8 putative heterozygotes that had monoallelic deletions. However, results from sequencing showed that these “heterozygotes” had small indels in their putative wild type allele.

3.6 Generation of hESC line *BMPR2^{DelKinase}* carrying an in-frame deletion within exon 6 of *BMPR2*

A total of 155 clones representing cells transfected with sgRNAs G3 and G4 targeting exon 6 *BMPR2* were isolated and PCR screened with primers (denoted in **Fig. 3.9 A** as **a** and **b**) that bound to regions outside of G3 and G4 (**Fig. 3.9 A**). 6 clones with potential heterozygote deletions (yellow boxes, **Fig. 3.9 B**) and 13 clones with homozygote deletions (red boxes, **Fig. 3.9 B**) were selected from the PCR screen for further analysis by sequencing. All 13 clones with putative homologous deletions were validated by sequencing and confirmed to have biallelic deletions of the region between the two sgRNAs. In this case, the 144 bp deletion does not disrupt the reading frame (ie. remains in frame) and, thus, encodes a *BMPR2* lacking only the kinase domain. All 6 heterozygote clones with putative monoallelic deletions were found to have random indels in their putative wild type alleles after sequencing analysis. Based on the PCR band pattern of the 13 clones confirmed to have biallelic deletions, we could genotype a total of 79 clones with biallelic deletions from the PCR screen. Subsequently, all 21 putative heterozygote clones genotyped by PCR were sequenced and were found to contain random indels in their putative wild type allele. Therefore, no heterozygotes were acquired from this experiment.

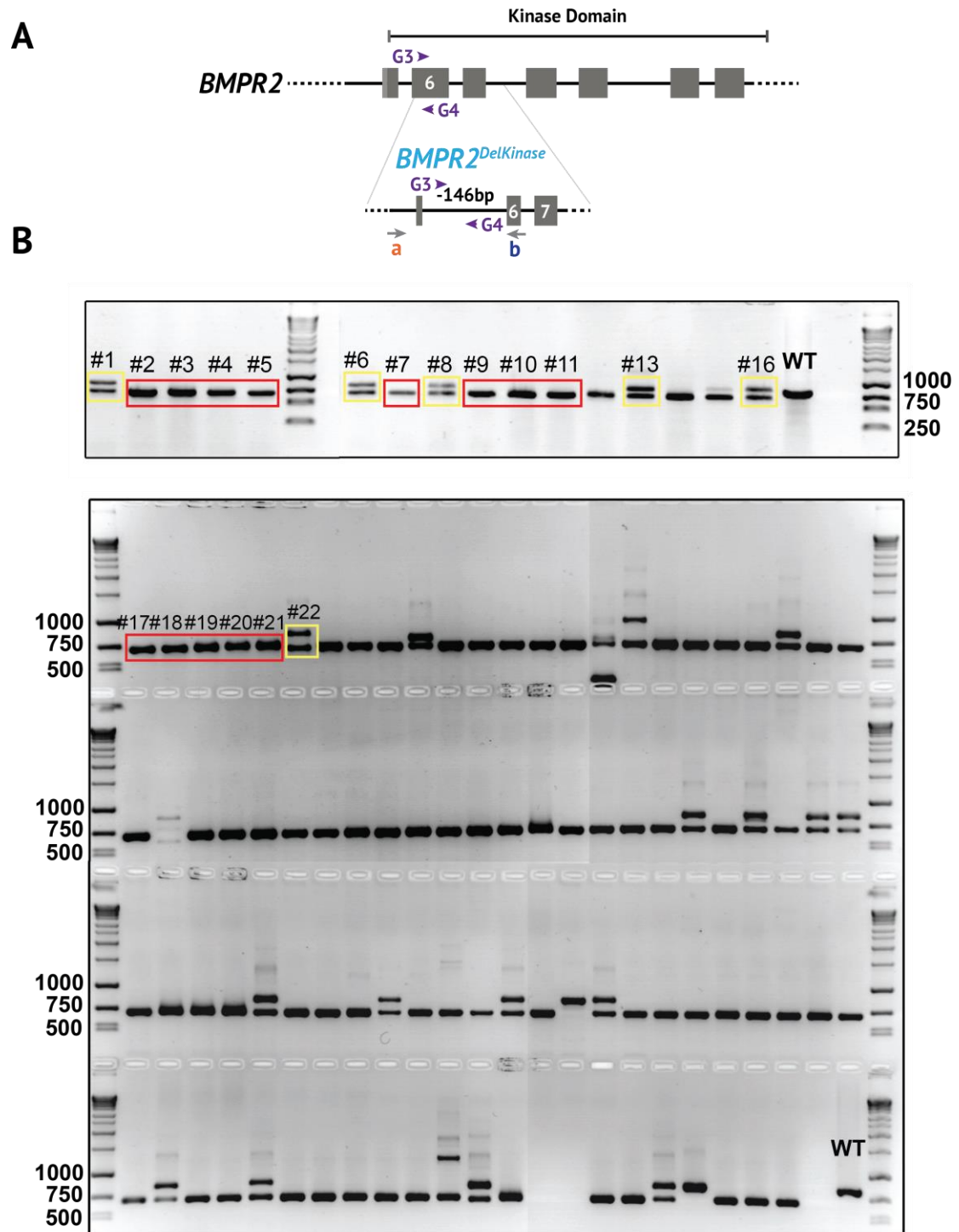
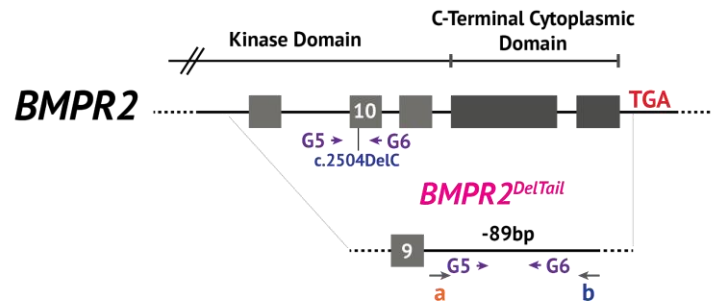


Figure 3.9 Gel electrophoresis of PCR screen to identify *BMPR2*^{DelKinase} clones that were targeted with dual CRISPR/Cas9 sgRNAs in exon 6 of *BMPR2*. (A) Schematic of *BMPR2* showing the targeting sites of sgRNAs G3 & G4 in exon 6 and the predicted outcome of the protein transcript after excision of sequences between the two sgRNAs. Arrows denoted by **a** & **b** show the location of the primers used in the PCR screen. (B) Agarose gel image show the results of a PCR screen of 155 clones derived from exon 6 *BMPR2* Cas9 sgRNA G3 and G4 targeting. Of these 155 clones, 79 clones were genotyped as homozygotes containing biallelic deletions inferred from band patterns of the PCR screening. 10 of these putative homozygotes (highlighted with red boxes) were selected for analysis by sequencing. Sequencing results validated that these 10 clones have homozygous deletions between sgRNAs G3 and G4. 6 heterozygote clones (yellow boxes) presumed to have monoallelic deletions were submitted for sequence analysis and results indicated small indels in their putative wild type alleles. Subsequently 21 of these putative “heterozygotes” were sequenced and random indels were detected in all of them.

3.7 Generation of hESC line *BMPR2^{DelTail}* carrying deletion of *BMPR2* exon 10

A total of 95 clones representing cells transfected with exon 10 sgRNAs G5 and G6 truncating the C- terminal kinase domain of *BMPR2* were isolated after single cell sorting and screened via PCR (**Fig. 3.10 A**). 10 clones were identified by PCR to have putative biallelic deletions (red boxes, **Fig. 3.10 B**) and 8 clones presumed to have monoallelic deletions (yellow boxes, **Fig. 3.10 B**) were selected for further analysis by sequencing. Once again, all 8 clones with putative monoallelic deletions contained random indels in their putative wild type allele. In contrast, PCR fragments representing the 10 homozygote clones genotyped with putative biallelic deletions lacked sequences corresponding to the region between the two sgRNAs G5 and G6. Based on the PCR band pattern, a total of 50 colonies were genotyped to contain putative biallelic deletions from the PCR screen (**Fig. 3.10 B**). Subsequently, two homozygote *BMPR2* mutant clones were selected for further functional experiments based on their sequence analysis: clone A (#91) had a deletion of 84 nucleotides in both alleles while clone B (#94) had an 84bp deletion in allele 1 and an 83bp deletion in allele 2.

A



B

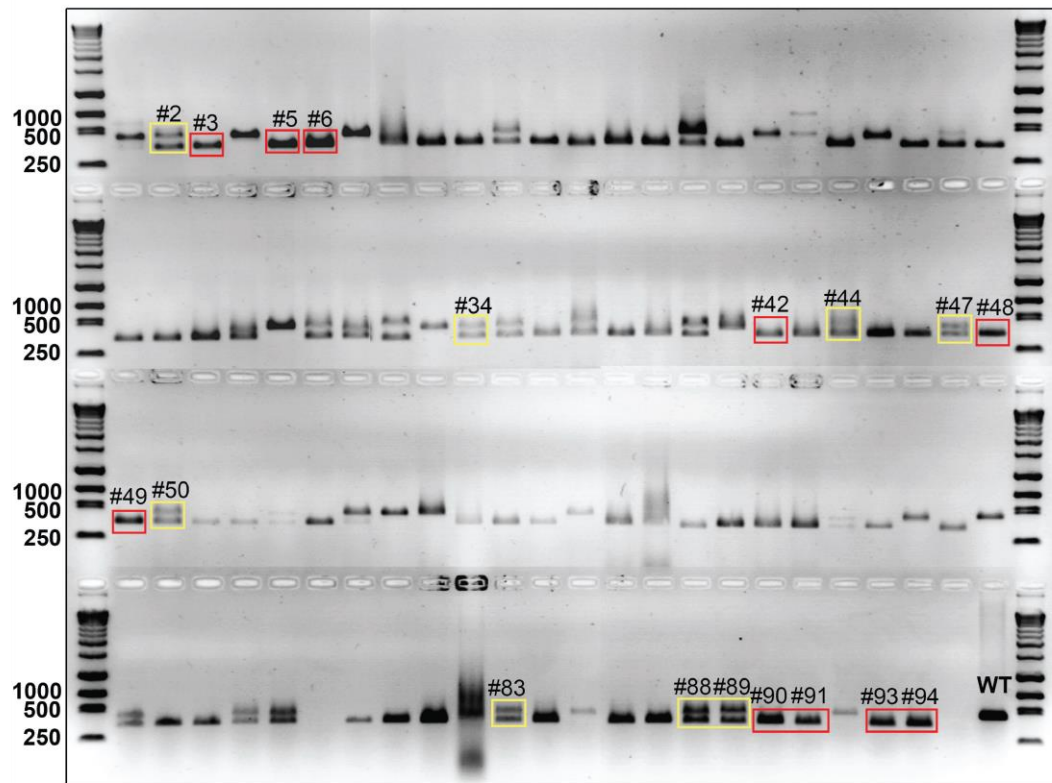


Figure 3.10 Gel electrophoresis of PCR screen to identify *BMPR2*^{DelTail} clones that were targeted with dual CRISPR/Cas9 sgRNAs in exon 10 of *BMPR2*. (A) Schematic of *BMPR2* showing the targeting sites of sgRNAs G5 & G6 in exon 10 and the predicted outcome of the protein transcript after excision of sequences between the two sgRNAs. Arrows denoted by **a** & **b** show the location of the primers used in the PCR screen. c.2504delC highlighted in blue indicates the point mutation in our patient derived iPSC line, *BMPR2*^{2504delC/W}. (B) Image of an agarose gel showing the results of a PCR screen of 95 clones derived from exon 10 *BMPR2* CRISPR/Cas9 sgRNA G5 and G6 targeting. Of these 95 clones screened, 50 clones were genotyped to have biallelic deletions. Sequence analysis of the 10 putative homozygote clones genotyped to have biallelic deletions (highlighted in red boxes) and the 8 putative heterozygote clones genotyped to have monoallelic deletions (highlighted in yellow boxes), confirmed that all 10 homozygote clones had biallelic deletions between G5 and G6 whereas the 8 clones genotyped to have putative monoallelic deletions were found to have random indels present in their putative wild type alleles.

3.8 PCR validation of homozygote *BMPR2* mutants

For those clones representing mutants that were predicted to have homozygous deletions, we re-screened two clones from each targeting experiment using primers that bound to sequences corresponding to the interval between the two CRISPR/Cas9 sgRNAs binding sites, referred to as internal primers. Each of these internal primers denoted (**d→** & **c←**) were paired with each external screening primer, (**a→** & **b←**) that lay outside the Cas9 sgRNA bindings sites (**Fig. 3.11 A**). In this figure, tracks containing PCR products generated with a reverse external primer and a forward internal primer are indicated by (**d→+b←**) whilst those corresponding to products generated with forward external primers and reverse internal primers are indicated by (**a→+c←**) (**Fig. 3.11 B**). If the sequences between the two sgRNAs were indeed deleted then this experiment should not yield a PCR product. All selected clones except for clone A (#41) yielded no amplicons in this re-screening experiment (**Fig. 3.11 B**). As mentioned previously (**section 3.5**), a fainter larger PCR fragment was amplified from DNA representing, clone A, (#41) *BMPR2^{Null}*, using the original screening primers (**a→+b←**) (highlighted with blue box, **Fig. 3.11 B**). Anomalous PCR products were also observed when DNA representing this clone was amplified with separate sets of internal primers and external primers (highlighted with orange boxes, **Fig. 3.11 *BMPR2^{Null}* gel**). To clarify the structure of DNA represented by this anomalous PCR product, the fragment was subjected to sequence analysis. This analysis revealed that the larger PCR fragment was due to an insertion of 481 bases (**Allele 1, Fig. 3.11 C**). Given that this large insertion is not a multiple of 3 and is located 3' of the ATG start codon, we predicted that this

insertion would produce a frameshift resulting in a non-functional polypeptide. In addition, earlier sequencing results had confirmed that the other allele (**Allele 2, Fig. 3.11 C**) of clone A (#41) had a 164 nucleotide deletion which removed the ATG (start codon), thus rendering this clone functionally null for *BMPR2*.

Finally, PSC clones containing the mutations characterised above were sent for karyotype analysis using the SNP array profiling. These results verified that all of the clones had a normal molecular karyotype. Next, we assessed these clones for the expression of pluripotency markers using flow cytometry (**Fig. 3.12**). Profiles from each clone showed robust expression of stem cell surface markers, SSEA4, EpCAM, TRA181 and CD9 (**Fig. 3.12**). After validation, we proceeded to use these clones to investigate the role of *BMPR2* in cardiovascular cell lineages, which are described in Chapters 4 and 5.

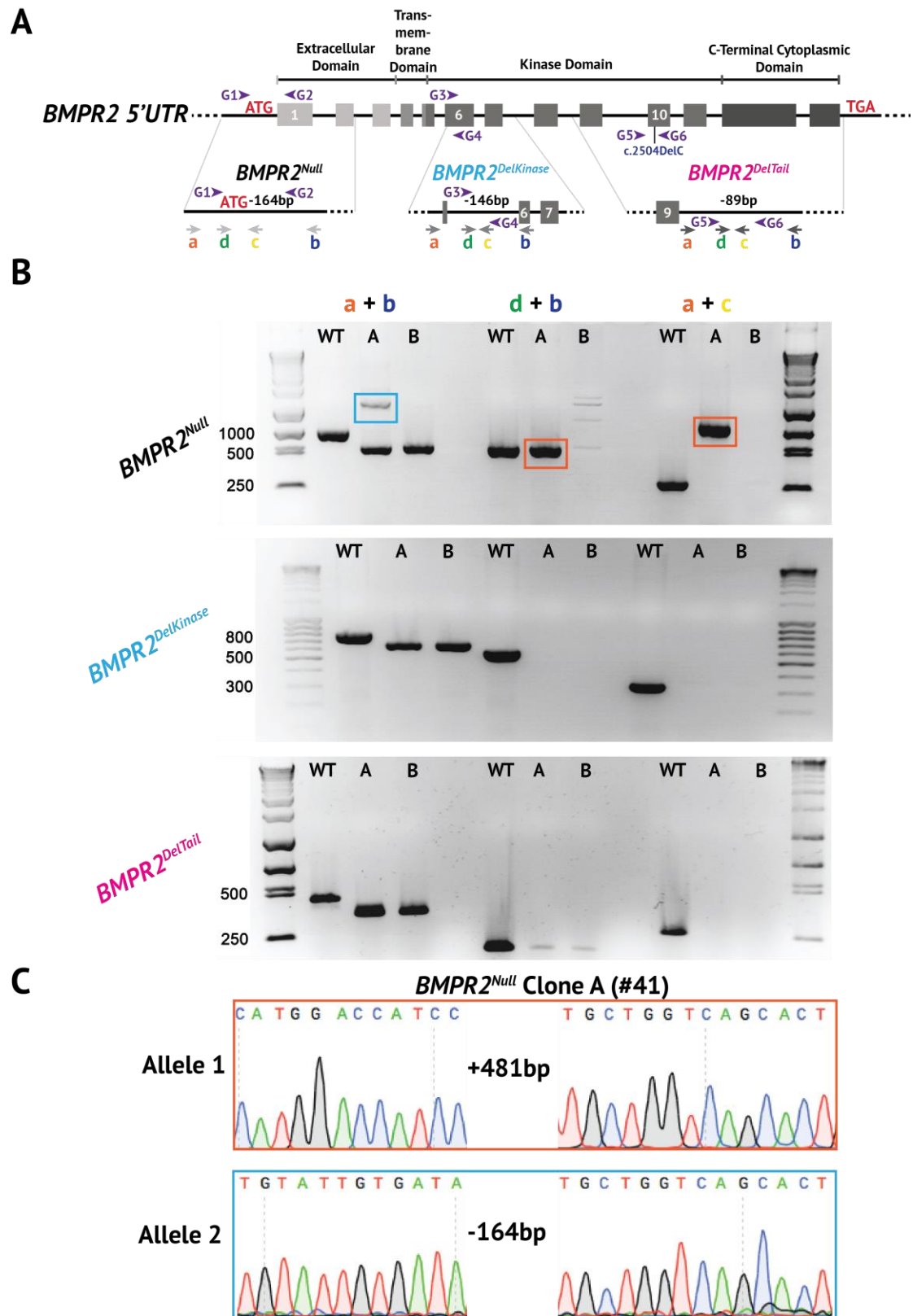


Figure 3.11 Further validation by PCR of selected BMPR2 mutant PSC clones carrying desired biallelic deletions in each domain of BMPR2. (A) Schematic of BMPR2 Gene containing 13 exons and 12 introns showing the sites targeted by the CRISPR guides. To generate, PSC line BMPR2^{Null}, **G1** & **G2** are sgRNAs designed to remove a portion of the 5'UTR and ATG of exon 1 resulting in a complete BMPR2 null. **G3** & **G4** are sgRNAs designed to remove a portion within exon 6 of BMPR2, resulting in PSC line BMPR2^{DelKinase} with an in-frame deletion in the kinase domain. **G5** & **G6** are sgRNAs designed to remove an intron-exon region in exon 10 resulting in a PSC line BMPR2^{DelTail} with a truncated BMPR2 transcript missing the C-terminal tail domain. To further verify that BMPR2 mutant clones contained the desired biallelic deletions, PCR was carried out with each external screening primer denoted by arrows labelled **a**➔ & **b**↠ paired with internal primers designed to bind within the CRISPR binding sites denoted by arrows labelled **c**↠ & **d**➔. (B) Images of agarose gels showing PCR products generated from PCR re-screening of selected BMPR2 clones to confirm deleted regions. In this experiment, any amplification of PCR product would mean regions were not completely excised. (**a**➔ + **b**↠) primers located external of CRISPR cleavage sites are the original primers used in the screening of targeted clones. The next primer set (**d**➔ + **b**↠) indicate forward internal primers are paired with reverse external primers while in the third primer pair (**a**➔ + **c**↠) indicates forward external primers are paired with reverse internal primers. The **top agarose gel BMPR2^{Null}** shows PCR bands of products amplified using screening primers (**a**➔ + **b**↠) spanning across deleted regions in exon 1 of BMPR2. Wild type (WT parental line control); lanes A & B are BMPR2^{Null} clones. Denoted by a **Blue box**, a fainter larger PCR fragment is observed from the DNA of clone A (#41). The amplification of a PCR product with primers (**d**➔ + **b**↠) and the larger PCR fragment with primers (**a**➔ + **c**↠) of clone A of BMPR2^{Null} was further analysed by sequencing (highlighted with orange boxes). The **second agarose gel BMPR2^{DelKinase}** shows the results of PCR analysis with primers designed across deleted regions in exon 6 kinase domain of BMPR2. Wild type (WT parental line control); lanes A & B are BMPR2^{DelKinase} clones. Primer sets (**d**➔ + **b**↠) and (**a**➔ + **c**↠) yielded no PCR amplicons, hence confirming

complete deletion of sequences between **G3** & **G4**. The **bottom agarose gel** **BMPR2^{DelTail}** shows the results of PCR analysis with primers designed across deleted regions in exon 10 of BMPR2. Wild type (WT parental line control); lanes A & B are BMPR2^{DelTail} clones. Primer sets (**d** → + **b** ←) and (**a** → + **c** ←) yielded no PCR amplicons, hence confirming complete deletion of sequences between **G5** & **G6**. **(C)** Sequencing results of the anomalous PCR fragment from BMPR2^{Null} clone A (#41) (blue box in BMPR2^{Null} agarose gel highlights the anomalous insertion). The amplified fragment from primer pair (**a** → + **c** ←) highlighted with an orange box was extracted and sent for sequencing. Results from sequencing of the fragment indicated that BMPR2^{Null} clone A (#41) had an insertion of 481 nucleotides in Allele 1 that is after the ATG start codon and the desired deletion of 164 nucleotides in Allele 2. This insertion causes a frameshift thus it is predicted to disrupt the ORF.

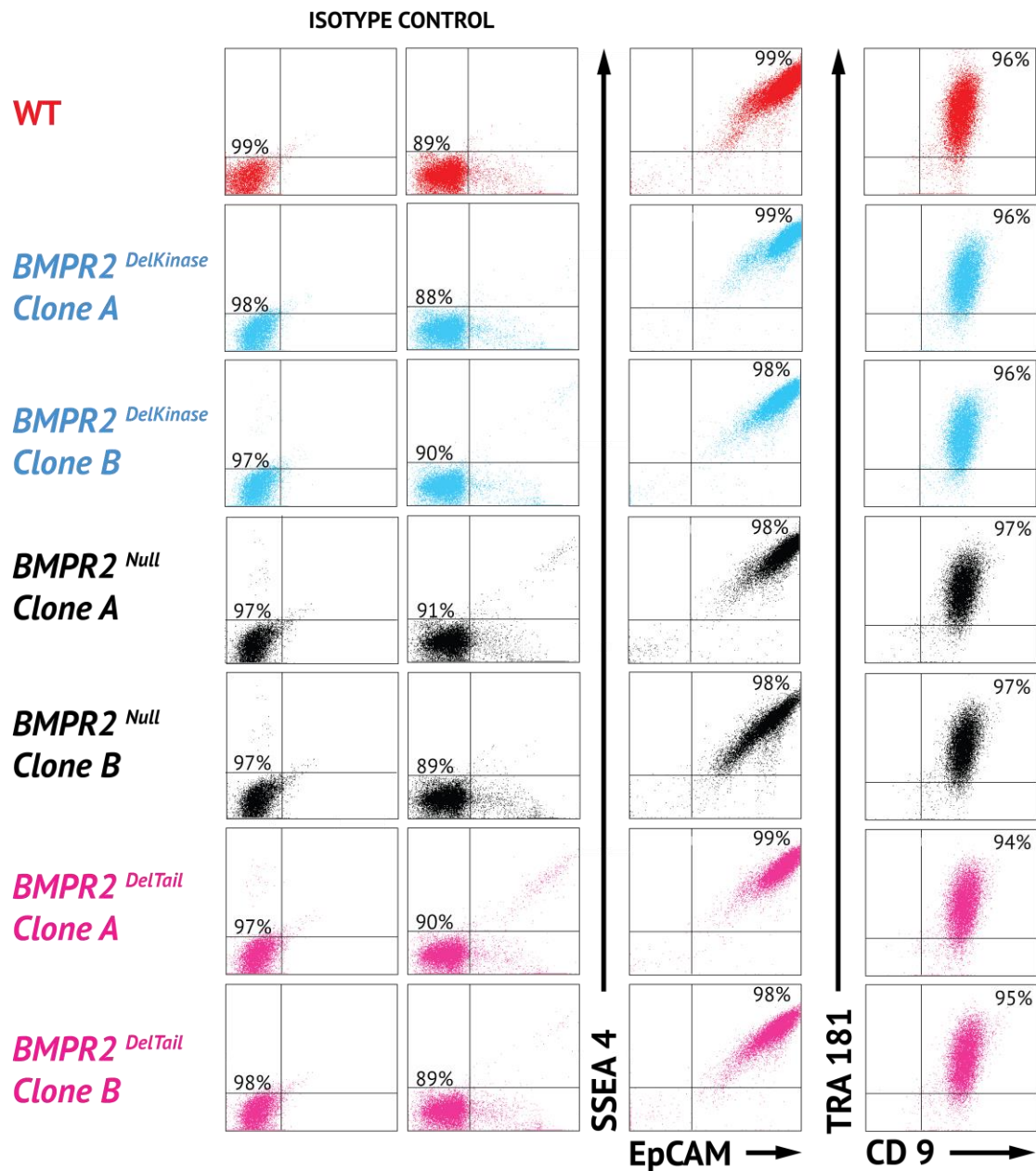


Figure 3.12 *BMPR2* mutant PSCs express pluripotent markers. First two panels show the isotype controls. Flow cytometric analysis shows that all *BMPR2* mutant hESC lines express the pluripotency surface markers SSEA 4, EpCAM, TRA181 and CD9. Numbers represent percentage of total cells in each quadrant.

3.9 Functional analysis of PSCs containing *BMPR2* mutations

We used Taqman qPCR probes to examine the expression of *BMPR2* in *BMPR2* mutant PSCs, isogenic control *NKX2-5^{eGFP/W}*, patient *BMPR2^{2504delC/W}* and iPSC^W cell lines. The first probe, P1, detects transcripts containing exons 1 and 2 (**Fig. 3.13, P1**). This analysis showed that *BMPR2^{Null}* ($p < 0.001$, $n = 3$) PSCs, in which sequences surrounding exon 1 had been deleted, had negligible expression of *BMPR2* (**Fig. 3.13, P1**). Similarly, experiments using this probe indicated that *BMPR2^{DelTail}* ($p < 0.02$, $n = 3$) PSCs had significantly lower *BMPR2* expression compared to their isogenic counterpart, *NKX2-5^{eGFP/W}* hESCs (**Fig. 3.13, P1**). In experiments using probe P2 (**Fig. 3.13, P2**), which detects transcripts containing exons 9 and 10, we found the expression of *BMPR2* in *BMPR2^{Null}* ($p < 0.01$, $n = 3$) PSCs was significantly reduced relative to that observed in parental PSCs and that *BMPR2* expression in *BMPR2^{DelTail}* ($p < 0.001$, $n = 3$) PSCs was negligible. Analysis of *BMPR2^{DelKinase}* PSCs using the P2 probe set indicated that *BMPR2* expression was only slightly reduced relative to that seen in the parental *NKX2-5^{eGFP/W}* line. The third probe (**Fig. 3.13, P3**), detected sequences between exons 11 and 12 (**Fig. 3.13, P3**). Results obtained using this probe suggested that *BMPR2* expressions were significantly lower in *BMPR2^{Null}* and *BMPR2^{DelTail}* PSCs in comparison to the parental line *NKX2-5^{eGFP/W}*. Therefore, irrespective of which probe was used, *BMPR2* expression levels for *BMPR2^{Null}* ($p < 0.02$, $n = 3$) and *BMPR2^{DelTail}* ($p < 0.03$, $n = 3$) were consistently lower than the parental *NKX2-5^{eGFP/W}* line. Conversely, *BMPR2^{DelKinase}* PSCs appeared to have normal expression of *BMPR2* compared to isogenic control. This could be due to transcripts being expressed, as mutation was an in-frame deletion and hence nonsense-

mediated decay (NMD) an mRNA surveillance mechanism detecting errors that results in pre-mature stop codons was not activated.

A



B

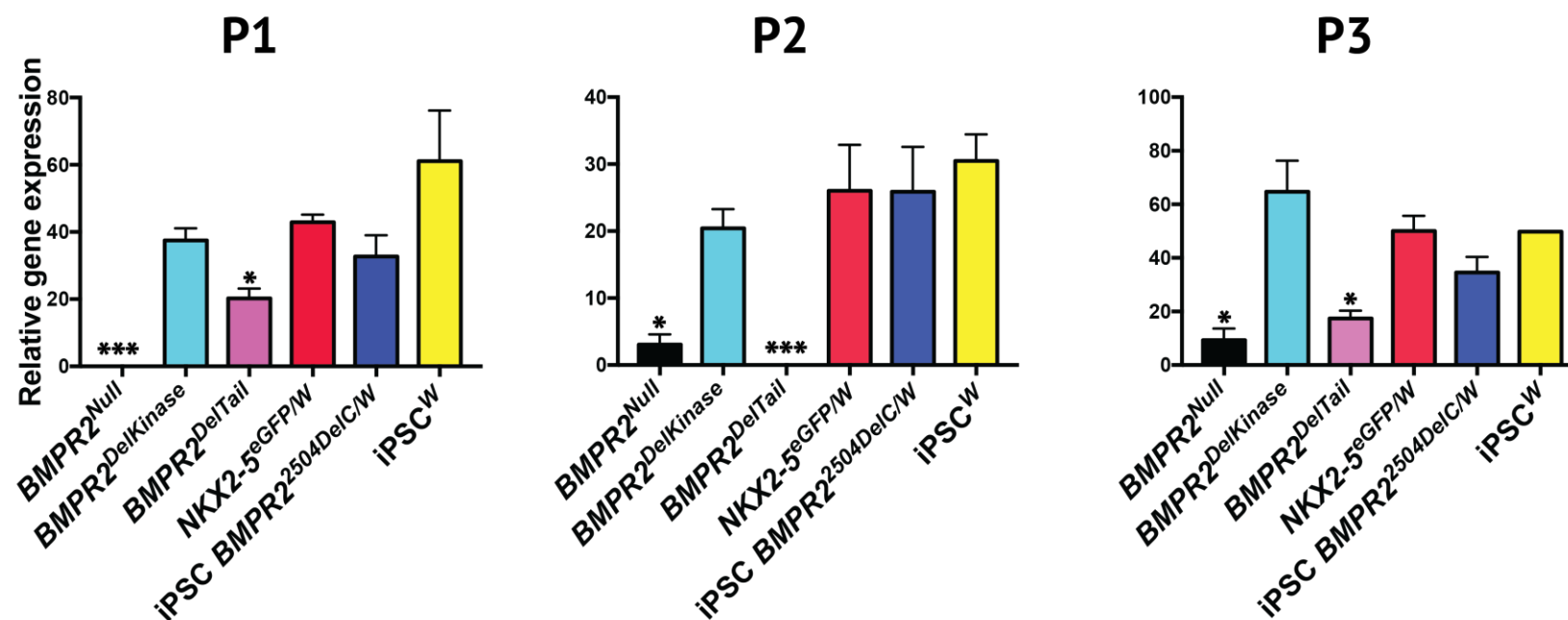


Figure 3.13 BMPR2 expression in BMPR2 mutant PSC lines were quantified by qPCR (A) Schematic map of the BMPR2 locus showing the location of Taqman Probes spanning different exons. These probes were used to quantify the BMPR2 expression levels in BMPR2 mutant clones by qPCR **(From left to right labelled as P1 – P3).** **(B)** Relative gene expression of BMPR2 in mutant and control cell lines was determined by qPCR and normalised to GAPDH. The 3 probes showed consistent reduction of BMPR2 transcripts in BMPR2 mutants compared to the isogenic control, with the exception of the BMPR2^{DelKinase} PSC line. P1 histogram shows negligible expression of BMPR2 transcripts in BMPR2^{Null} (**p<0.001) while reduced transcripts was observed in BMPR2^{DelTail} (*p<0.02). P2 histogram shows reduction of BMPR2 transcripts in BMPR2^{Null} (*p<0.01) and negligible expression in BMPR2^{DelTail} (***p<0.001). P3 histogram shows reduction of BMPR2 transcripts in BMPR2^{Null} (*p<0.02) and BMPR2^{DelTail} (*p<0.03). (n=3; ±S.E.M.). Statistical analyses were conducted using Dunnett's and Tukey's multiple comparisons test.

Discussion

We generated a series of cell lines using CRISPR/Cas9 genome editing to introduce mutations into the *BMP2* gene, including changes analogous to those observed in PAH patients. We also used homology directed repair (HDR) to correct a mutation present in an iPSC line derived from a patient with PAH, thus generating an isogenic control for this line. However, the use of these patient derived lines was precluded due to the rapid accumulation of chromosomal abnormalities in both the patient and corrected cell lines. In our experiments, HDR was a very low frequency event. The general efficiency of the HDR pathway is reported to be < 12% (Paix et al. 2015). In our case, we had a rate of 3%, of the 3 clones targeted with the repair template; only 1 clone had integrated the 70 base pair oligonucleotide repair template into the mutated allele, the other two clones had partial integration of the repair template in both of their alleles. Typically, the non-homologous end-joining pathway (NHEJ), which frequently produces indels, is utilised more often than HDR. Some studies have shown that chemical inhibition of NHEJ forcing the cells to use HDR, improves the efficiency of HDR-mediated genome editing (Chu et al. 2015, Maruyama et al. 2015). Even so, HDR remains a low-efficiency process and chemical manipulations may be toxic to the cells. It is also important to note, that HDR only occurs during S and G2 phases, where DNA replication is completed and sister chromatids serve as repair templates (Howden et al. 2015). On the other hand, NHEJ can occur at any point of the cell cycle (Heyer et al. 2010). Therefore, in this context, controlling the Cas9 activity during specific cell cycle stages can minimize undesirable indels

(Howden et al. 2015). This is achieved by fusing a Cas9 nuclease to a peptide derived from the human Geminin protein (SpCas9-Gem) to facilitate its degradation during the G1 phase of the cell cycle where NHEJ predominates, thus limiting introduction of indels at the target locus.

Table 3.1 This table shows a summary of the generated cell lines containing the various *BMPR2* mutations, their predicted protein and outcome.

Cell line	Mutation	Protein prediction	Consequence
<i>iPSC BMPR2</i> ^{2504delC/W}	Heterozygote deletion of cytosine at position c.2504 of <i>BMPR2</i>	Premature stop codon truncating protein	Missing tail domain in mutant allele
<i>hESC BMPR2</i> ^{DelTail/W}	Heterozygote <i>BMPR2</i> mutants of Tail domain	Premature stop codon truncating protein	Missing tail domain in mutant allele
<i>hESC BMPR2</i> ^{DelTail}	Homozygote deletion of 84 bases in exon 10 encoding the C-terminal of the kinase domain	Disrupted acceptor splice sequence causing truncation of protein	Truncation of the tail domain
<i>hESC BMPR2</i> ^{Null}	Homozygote deletion of 162 bases containing ATG in exon 1	Disrupted ORF of signal peptide	Disrupted protein transcript
<i>hESC BMPR2</i> ^{DelKinase}	Homozygote deletion of 144 bases within exon 6 that encodes the kinase domain	In-frame mutation of the catalytic domain of serine threonine Kinases	Shorter kinase domain missing an ATP-active binding site, no NMD

To date, there are over 370 mutations identified in the *BMPR2* gene of HPAH patients (Machado et al. 2015) the majority result in frameshift or non-sense mutations. However, only 30% of these carriers with *BMPR2* mutation will

develop clinical HPAH (Frump et al. 2016). Mutations of *BMPR2* are found throughout every exon except exon 13. One hypothesis states that the nature of *BMPR2* mutation could affect the penetrance/severity of the disease (Frump et al. 2016). In addition, Frump et al. (2016) reported that patients with *BMPR2* mutations who express mutant *BMPR2* protein that escape non-sense mediated messenger RNA decay (NMD-mutations) actually develop more severe disease than HPAH patients with NMD+ mutations who do not express *BMPR2* mutant proteins. Furthermore, mutations that introduce a premature termination codon can also lead to splicing anomalies including exon skipping or alteration of exonic and intronic splicing enhancer (ESE and ISE) and suppressors (ESSs and ISSs) (Nasim et al. 2008). Conversely, according to Girerd et al. (2015), the severity of the disease can be related to the mutation position and there is a genotype-phenotype link in *BMPR2* mutation carriers. The prematurely terminated protein products such as the mutants *BMPR2^{DelTail/W}* and *BMPR2^{2504delC/W}* may act as dominant negatives. For example, Leyton et al. (2013) reported an enhanced BMP7 signalling transduced via tail domain negative mutant *Bmpr2^{ΔtD}* in mice while *Bmpr2^{ΔtD/ΔtD}* mice died in utero at E7.5-E8.5. Furthermore, siRNA knockdown of cells from *Bmpr2^{ΔtD/ΔtD}* mice to selectively deplete BMP receptors showed that the tail domain of *Bmpr2* inhibits *Acvr1*-mediated BMP7 signalling (Leyton et al. 2013). Interestingly, a shorter isoform of *BMPR2* without the cytoplasmic tail has preserved BMP signalling (Cogan et al. 2012). Recently, younger PAH patients have been reported to carry mutations in the 5'UTR that have decreased transcription of *BMPR2* (Wang et al. 2016). The expression levels and plasma membrane levels of *BMPR2* has been shown to be

determined by two processes, translational regulation of the protein and endocytosis/degradation which has a mild modulatory effect (A. R. Amsalem et al. 2015). Both of these mechanisms enhance the expression of *BMPR2* short form relative to the long form at the cell surface resulting in activation of the SMAD1/5/8 pathway increasing their intensities. However, the key regulator in balancing activation of the canonical versus the non-canonical signals by BMPs could be controlled by alternative splicing of *BMPR2*. Thus, the allelic series of *BMPR2* mutants generated here permit further *in vitro* experiments to investigate *BMPR2* function.

Limitations of CRISPR technique in this study

The advantage of using CRISPR/Cas9 gene editing to create genetic knockouts is its simplicity. However, some limitations concerning this technology involve the design of the guides being restricted by the PAM sites, cleavage efficiencies and inefficient screening method. Of recent, other variants of Cas9 have been developed to bind to wider range of PAM sites that are derivatives of NGG, addressing the issue of restriction by NGG PAM sites (Murovec et al. 2017).

Two separate sgRNA targeting sequences, each having 100% homology to the target DNA, may not have equivalent cleavage efficiencies (Hua Fu et al. 2014). This was observed in our experiments while using the dual sgRNA approach to remove a genomic fragment, due to variability in cleavage efficiency of the flanking sgRNAs, excision was not always achieved. Cleavage efficiency may increase or decrease depending upon the specific nucleotides within the selected target sequence (Wu et al. 2014). For example, sgRNA targeting sequences containing a G nucleotide at position 20

(1 bp upstream of the PAM) may be more efficacious than sgRNAs containing a C nucleotide at the same position in spite of being a perfect match for the target sequence (Wu et al. 2014). Therefore, close examination of predicted on-target and off-target activity of each potential sgRNA targeting sequence is necessary to design the best sgRNA for each experiment. Lastly, the imprecise nature of NHEJ requires several validation methods as “check points” when screening for desired clones. These limitations notwithstanding, CRISPR mediated genome editing has facilitated the construction of a series of isogenic PSCs mutated at the *BMPR2* locus. In the subsequent chapters, these lines are used to dissect *BMPR2* function in a range of mesodermal cell lineages.

CHAPTER



Mesoderm Formation and Cardiogenesis

Introduction

BMP signalling plays important roles in the specification and functioning of a number of cell lineages including nascent mesodermal cells, cardiomyocytes, smooth muscle cells (SMCs) and endothelial cells (ECs) (Zhang and Li 2005). Thus, disruption of *BMPR2* may impact these cell types and, in turn, the function of the cardiovascular system may be compromised. In this chapter we will examine *BMPR2* function during *in vitro* differentiation of cardiac mesoderm and cardiomyocytes. To set the scene for this analysis, the text below briefly outlines the developmental relationship between these lineages and what is known about the role of *BMPR2* in their genesis and function.

BMP signalling in mesoderm formation

Mesoderm formation occurs during the process of gastrulation, a morphogenetic event that converts the 2 layered embryo into a 3-layered structure comprising the three germ layers, ectoderm, mesoderm and endoderm (**Fig. 4.1**). These germ layers subsequently give rise to all of the organs and structures present in the body (**Fig. 4.2**). The ectodermal cells form the nervous system and epidermal tissues whilst the endoderm produces the gut lining and derivatives of the gut, including organs such as the liver, pancreas and lung (**Fig. 4.2**). Mesodermal cells give rise to a host of tissues and cell types that include those forming the cardiovascular system, blood, skeletal muscle, bone and kidneys (**Fig. 4.2**).

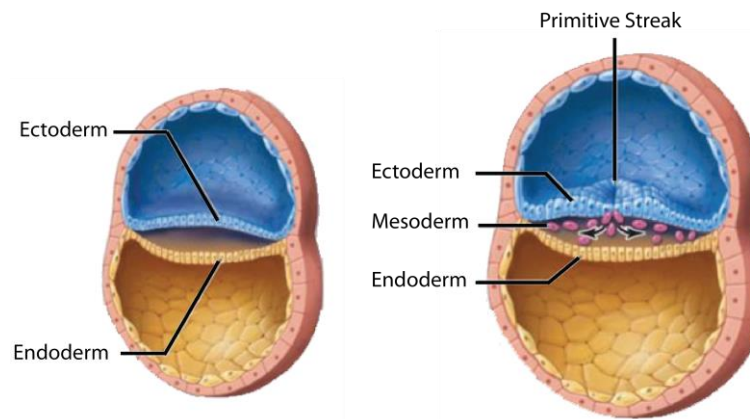


Figure 4.1. Schematic diagram showing the development of the blastocyst during gastrulation. The primitive streak arises from the epiblast and ingresses by moving between the ectoderm and endoderm to form the mesoderm layer. Adapted from (The McGraw-Hill Companies, 2000).

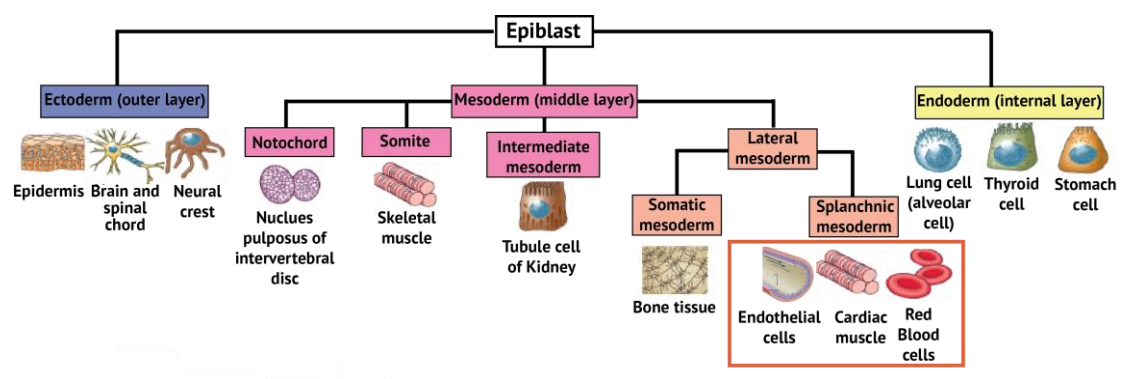


Figure 4.2 Cell lineage tree of the three germ layer derivatives. During gastrulation, the primitive streak arises from the epiblast and the formation of the 3 germ layers begins. These germ layers subsequently go on to form the various organs of the body. Highlighted in the red box are the cell types that arise from the mesoderm population that are most affected by the loss of BMPR2. Illustration adapted from Stem Cells: Scientific progress and future research directions (2001).

Gene targeting studies in the mouse have shown that BMP signals are required for gastrulation. For example, *Bmpr1A* mutants fail to form mesoderm and consequently fail to generate the typical three-layered embryo (Mishina et al. 1995). Similarly, *Bmp4* knockout mouse embryos die between E6.5 and E9.5, due to defective mesoderm formation (Winnier et al. 1995). Embryos lacking *ActR1A* arrest at the early gastrulation stage and display disrupted mesoderm formation (Gu et al. 1999, Mishina et al. 1999). Conversely, embryos deficient in either *ActR2A* or *ActR2B* develop to term with no gross defects in mesoderm formation (Matzuk et al. 1995, Oh and Li 1997), whereas double knockout embryos arrest development at the pre-gastrulation egg cylinder stage (Song et al. 1999). Taken together these studies demonstrate that correct BMP signalling is essential for early mesoderm development.

In the context of our studies, in *Bmpr2* null mouse embryos gastrulation failed as no mesoderm was formed and the embryos arrested at the egg cylinder stage (Beppu et al. 2000). *Bmpr2* mutant embryos displayed severe growth retardation and there was no morphologically discernable primitive streak. Further characterization of these embryos by whole-mount in situ hybridization revealed mesoderm markers such as *Brachyury (T)* and *HNF-3 β* were not expressed. In contrast, control embryos expressed these genes within the primitive streak. These results confirmed that *Bmpr2* null mice embryos did not form mesoderm (Beppu et al. 2000).

***BMPR2* and Cardiogenesis**

In vivo studies in mammals indicate that ablation of components of the BMP signalling network can result in defective heart development leading to embryonic lethality (Beppu et al. 2009). In the mouse, receptors that bind and transduce BMP signals, *Acvr2A*, *Acvr2B* and *Bmpr2*, are expressed during cardiomyocyte formation and in cardiomyocytes once they have formed (Beppu et al. 2000, Feijen et al. 1994). To evaluate the impact that *BMPR2* deficiency had on cardiogenesis, conditional knockout mice were generated using a *Mox2*-Cre transgene to overcome early embryonic lethality. The *Mox2*-Cre mouse line expresses the Cre recombinase from the *Mox2* locus. This locus drives expression of Cre throughout the epiblast following implantation of the embryo (Tallquist and Soriano 2000). *BMPR2^{fllox/-}; Mox2-Cre* mice exhibited structural cardiac defects including double-outlet right ventricle, ventricular septal defect (VSD), atrioventricular (AV) cushion defects, and thickened valve leaflets. In another study, mice homozygous for a *BMPR2* hypomorphic allele, which has residual BMP signalling activity, die at mid-gestation and exhibit skeletal and cardiovascular abnormalities including persistent truncus arteriosus (PTA), interrupted aortic arch, and absent semilunar valve formation (Delot 2003).

In light of the above, we expect that mutations disrupting *BMPR2* may impact the formation or function of iPSC-derived cardiomyocytes. Furthermore, BMP signalling is required for mesoderm formation *in vivo*. Considering the severe mesodermal phenotype in *BMPR2* null mice embryos, we sought to examine mesoderm induction during *in vitro* differentiation of cell lines with impaired

BMPR2 signalling. Our allelic series of *BMPR2* mutants (Chapter 3) were introduced into a HES3 *NKX2-5^{eGFP/W}* hESC reporter line, which permits investigation of the differentiation and function of cardiomyocytes with altered *BMPR2*. Firstly, in contrast to the murine *in vivo* situation, mesoderm differentiation in all human *BMPR2* mutant PSC lines was unaffected. We also demonstrate that cardiac mesoderm formation and cardiomyocyte differentiation is not perturbed in the *BMPR2* mutants. However, gene expression profiling suggests that cardiomyogenesis may be compromised when *BMPR2* signalling is disrupted. This is discussed in the context of prevalence of right ventricular heart failure in PAH patients carrying *BMPR2* mutations.

Results

4.1 BMP4 mediated mesoderm induction is not impaired in *BMPR2* heterozygotes

Given the importance of BMP signalling during gastrulation, we sought to examine the response of *BMPR2*^{2504delC/W} iPSC line to BMP4 exposure during mesoderm differentiation *in vitro*. We developed a BMP4 dose response assay to investigate mesoderm formation. Spin embryoid bodies (EBs) made from PSCs were treated with a range of BMP4 concentrations (0 to 40 ng/ml of BMP4) in a media free from serum and animal constituents denoted APEL (Ng et al. 2008). To quantify production of mesodermal and endodermal cell types, flow cytometry was used to assay the expression of PDGFR- α (mesoderm marker) and EpCAM (epithelial/endoderm marker) at day 3 of the differentiation. This timepoint corresponds to the primitive streak stage of embryogenesis, at the time the three germ layers begins to form.

Results of day 3 BMP dose response assays indicated that *BMPR2*^{2504delC/W} was capable of differentiating to mesoderm after induction with BMP4 (**Fig. 4.3 A**). Furthermore, results indicate that *BMPR2*^{2504delC/W} did not have an altered response to BMP4 (**Fig. 4.3 A**). We repeated this experiment with a hESC line (*BMPR2*^{DelTail/W}) carrying a similar mutation to our patient's cell line (i.e. *BMPR2*^{2504delC/W}) in which the *BMPR2* tail domain was truncated due to a pre-mature stop codon. The BMP4 response was consistent in both *BMPR2*^{DelTail/W} and the isogenic wild type control, *NKX2-5*^{eGFP/W} (**Fig. 4.3 B**). In addition, the BMP4 dose response of both *BMPR2*^{2504delC/W} and

BMPR2^{DelTail/W} during mesoderm differentiation was similar (**Fig. 4.3 A & B**). Furthermore, BMP4 activation of canonical BMP signalling was demonstrated via immunostaining of phosphorylated SMAD1/5/8 localised to the nuclei of patient PSC *BMPR2^{2504delC/W}* and wild type iPSC^W (**Fig. 4.4 A, B & E**). The levels of SMAD1/5/8 expression, quantified by measuring the immunofluorescence intensity, was consistent in both wild type and *BMPR2^{2504delC/W}* PSCs, showing increased expression during BMP4 treatment (**Fig. 4.4 E**). Conversely, increase in total SMAD 1 expression was observed only in wild type and not in *BMPR2^{2504delC/W}* PSCs during BMP4 treatment (**Fig. 4.4 C, D & F**). Our results show that, when present as a heterozygote mutation, 2504delC in *BMPR2* does not prevent signalling through the canonical BMP pathway.

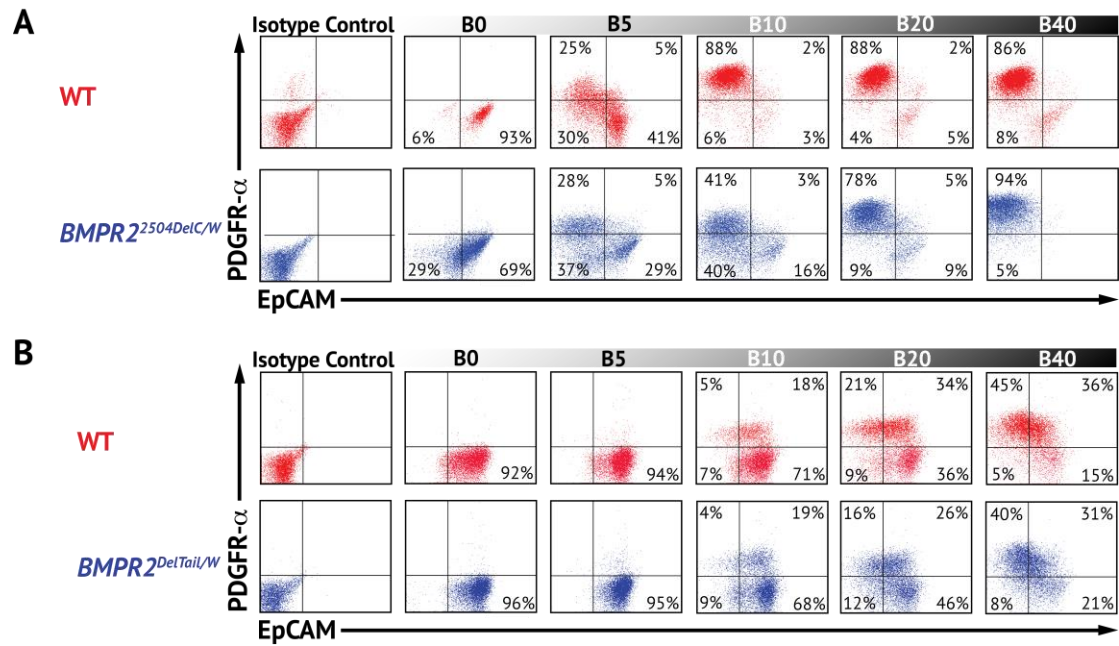


Figure 4.3 Mesoderm inductions of BMPR2 mutants are not impaired, in vivo. Flow cytometric analysis of PDGFR- α and EpCAM expression from day 3 of **(A)** wild type hiPSC and patient derived *BMPR2*^{2504delC/W} iPSCs **(B)** Differentiating parental wild type hESC *NKX2-5*^{eGFP/W} and hESC *BMPR2*^{DelTail/W} were subjected to increasing doses of BMP4 (indicated as Bn, where n=[BMP] in ng/ml). Isotype controls are shown in the left most plots. From this analysis, *BMPR2*^{2504delC/W} and *BMPR2*^{DelTail/W} PSCs containing a heterozygote mutation in the tail domain of *BMPR2* differentiate as efficiently as respective wild type controls. Numbers represent percentage of total cells in each quadrant.

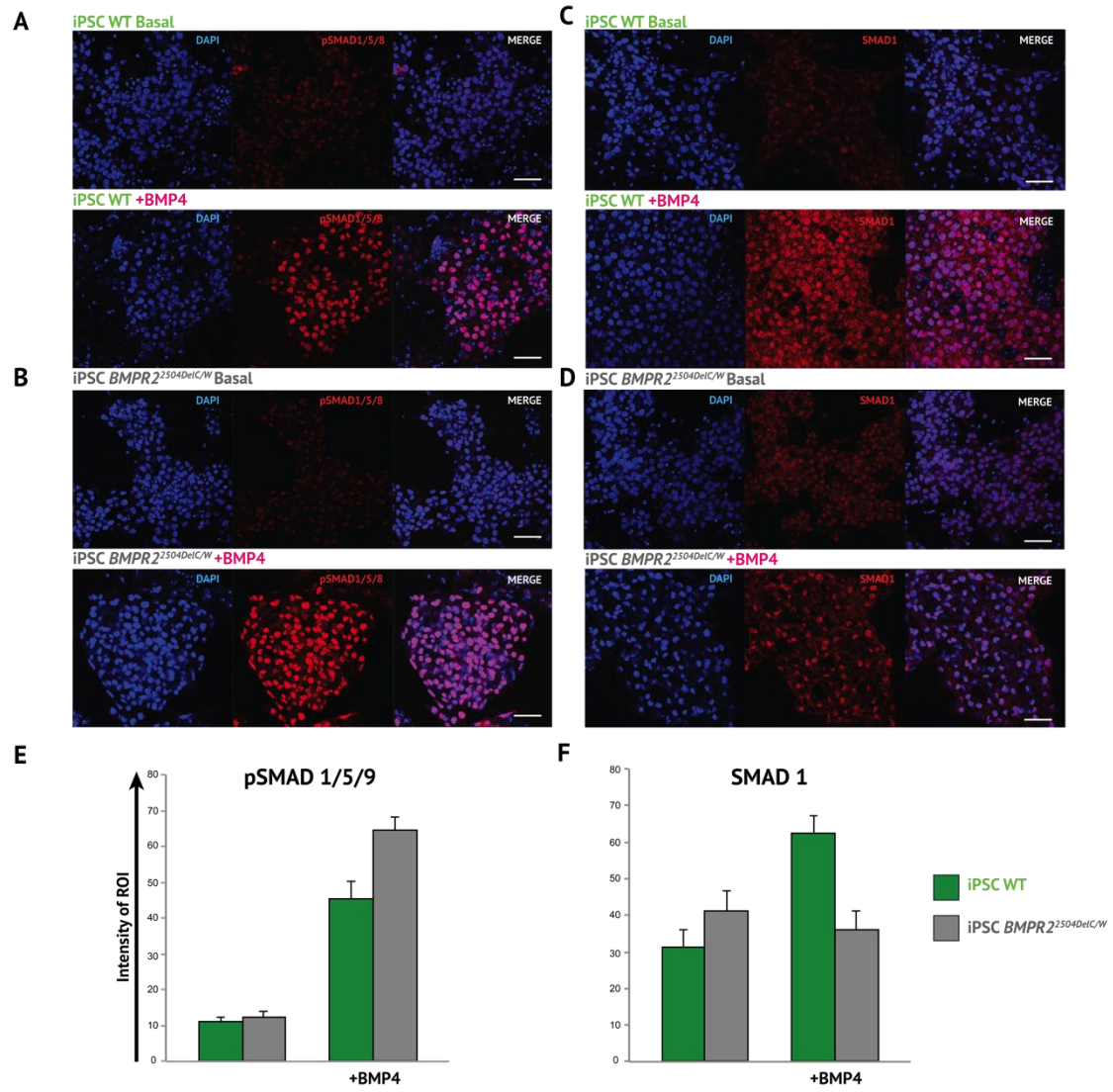
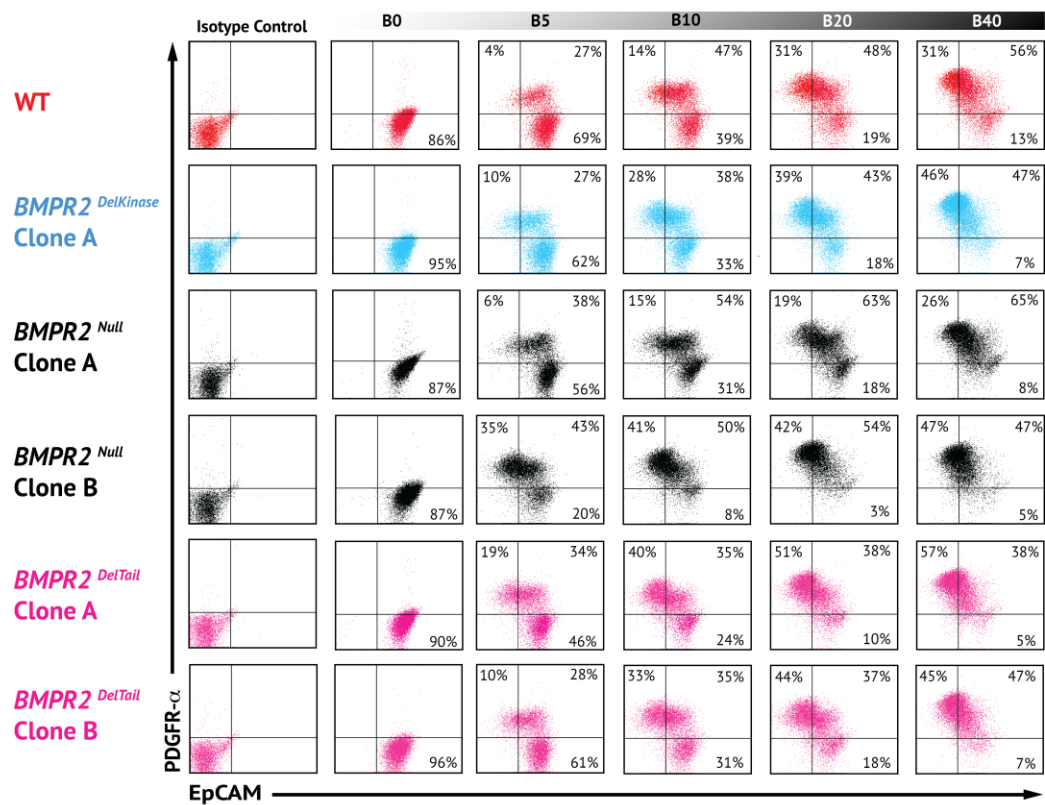


Figure 4.4 BMP4 treatment induces pSMAD1/5/8 nuclear translocation in both *BMPR2*^{2504delC/W} and *iPSC*^W. PSCs were treated with 100ng/ml of BMP4 for 30mins. Detection of phosphorylated SMAD1/5/8 complex and SMAD1 protein was carried out with anti-pSMAD1/5/8 and SMAD1 antibodies. **(A)** Top panel shows untreated *iPSC*^W cultures and bottom panel shows BMP4 treated *iPSC*^W cultures labelled with anti-pSMAD1/5/8 antibody. **(B)** Top panel shows untreated *BMPR2*^{2504delC/W} cultures and bottom panel shows BMP4 treated *BMPR2*^{2504delC/W} cultures labelled with anti-pSMAD1/5/8 antibody. Note nuclear accumulation of pSMAD1/5/8 complex after BMP4 treatment for both cell lines. **(C)** Top panel shows *iPSC*^W without BMP4 treatment, bottom panel shows BMP4 treated cultures labelled with anti-SMAD 1 antibody **(D)** Top panel shows *BMPR2*^{2504delC/W} without BMP4 treatment, bottom panel shows BMP4 treated cultures labelled with anti-SMAD 1 antibody. Nuclei were stained with DAPI. Single confocal sections are shown (scale bars = 60µM). **(E)** Quantification of pSMAD1/5/8 expression via measurement of immunofluorescence intensity demonstrates that *BMPR2*^{2504delC/W} phospho-SMAD1/5/8 levels are comparable to *iPSC*^W. **(F)** Graph representing immunofluorescence intensity of ROI showing *BMPR2*^{2504delC/W} and *iPSC*^W expressing SMAD1 levels. *iPSC*^W had an increase in SMAD1 levels. However, *BMPR2*^{2504delC/W} did not up regulate SMAD 1 even after BMP4 treatment. ROI = region of interest, regions were quantified using consistent areas for both cell lines.

4.2 *BMPR2* is not required for human mesoderm specification, *in vitro*

We examined the role of *BMPR2* signalling during *in vitro* mesoderm formation using the allelic series of *BMPR2* modified lines described in Chapter 3. This series included PSC lines containing a deletion of sequences in exon 1, generating a complete *BMPR2* null (*BMPR2^{Null}*), an in-frame deletion within exon 6 encoding the kinase domain (*BMPR2^{DelKinase}*) and a splice acceptor site deletion in exon 10 encoding C-terminal sequences of the kinase domain, resulting in a tail domain null (*BMPR2^{DelTail}*). Spin EBs formed from wild type and *BMPR2* mutants were differentiated for 3 days in medium supplemented with incremental doses of BMP4. Flow cytometric analysis of day 3 EBs revealed that wild type and *BMPR2* mutant EBs efficiently formed mesoderm, as indicated by up regulation of PDGFR- α and down regulation of EpCAM (**Fig. 4.5 A**). Furthermore, heat map analysis showed consistent PDGFR- α expression between the *BMPR2* mutants and wild type differentiating PSCs in response to BMP4 (**Fig. 4.5 B**). Furthermore, we subjected *BMPR2^{2504delC/W}* to a definitive endoderm differentiation protocol that included the use of exogenous BMP4 and found no differences in endoderm differentiation compared to wild type PSCs (**Appendices Fig. 2 & 3**). Taken together, our results indicate that *BMPR2* is not required for human mesoderm formation *in vitro*. Moreover, evidence that loss of *BMPR2* can be compensated by other receptors such as *ACTR2* has been demonstrated. This is explored further in the following section.

A



B

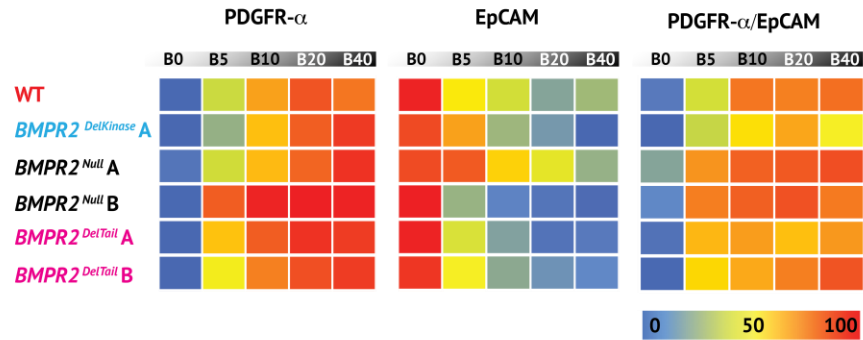


Figure 4.5 BMPR2 is not required for human mesoderm formation, in vitro. Flow cytometric analysis showing EpCAM and PDGFR- α expression from day 3 induction of mesoderm in EBs **(A)** Parental line hESC NKX2-5^{eGFP/W} and homozygote BMPR2 mutants were subjected to increasing doses of BMP4 (indicated as Bn, where n=[BMP] in ng/ml). Isotype controls are shown in the first plots. Results demonstrate that the allelic series of BMPR2 mutants have similar differentiation patterns to the wild type. **(B)** Heatmap analysis shows the averages of percentage positive cells expressing PDGFR- α and EpCAM at day 3 of the differentiation. As illustrated in the heatmap, the mesoderm differential pattern of BMPR2 mutants and wild type show consistency in response to BMP4 (n=3).

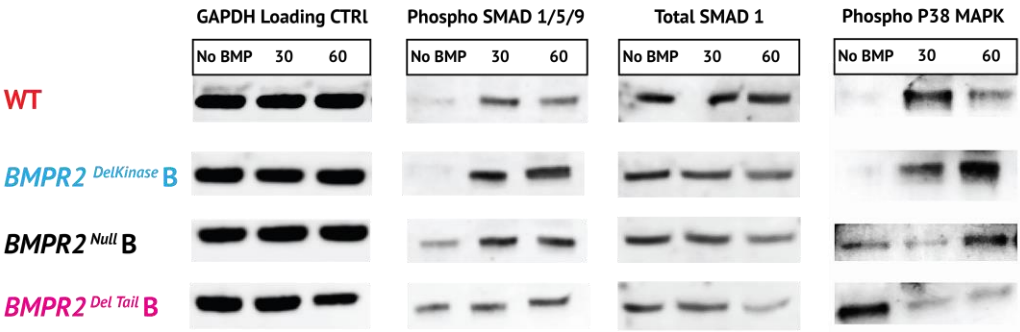
4.3 *BMPR2* modulates BMP signalling in PSCs

Canonical BMP signalling requires the formation of a heterotetramer of two transmembrane serine/threonine kinases, a type 2 receptor (*BMPR2*) and a type 1 receptor (*BMPR1A* or *BMPR1B*) (Gilboa et al. 2000). However, studies by Yu et al. 2008 have shown that *ACTR2* (Activin receptor type 2) can bind BMPs and recruit *BMPR1* to activate the BMP signalling pathway (Yu et al. 2008). Indeed, BMP ligands signal through the three type 2 receptors, *BMPR2*, *ACTR2A* and *ACTR2B* in combination with any of the three type I receptors *ACVR1*, *BMPR1A*, *BMPR1B* (respectively, ALK2, ALK3 and ALK6). This promiscuity in the use of different BMPRs and ACTRs is not reciprocal: *ACTR2* can bind ACTIVINs and BMPs but *BMPR2* binds only BMPs (Mueller and Nickel 2012).

In view of the above, we hypothesized that BMP signalling might still occur in cells lacking a functional *BMPR2*. In order to assess this theory, we examined proteins involved in the downstream signalling of these receptors in cells representing the allelic series of *BMPR2* mutations. PSCs were serum starved for 24hrs and treated with 100 ng/mL of BMP4 for 30 and 60mins. Protein lysates from each treatment group were prepared and the expression of phosphorylated SMADs analysed using an immunoprecipitation/Western blot assay. A GAPDH loading control demonstrated that equivalent amounts of protein extract from each genotype and condition were used in these experiments (**Fig. 4.6 A**). Phosphorylated SMAD1/5/9 was observed in all PSC lines that were treated with BMP4 (**Fig. 4.6 A**). This observation indicates that BMP4 activated the canonical BMP signalling pathway in

BMPR2 mutant cells, possibly via receptors *ACTR2A* and *ACTR2B*. Furthermore, phosphorylated SMAD1/5/9 was observed in untreated *BMPR2^{Null}* and *BMPR2^{DelTail}* PSCs (**Fig. 4.6 B**). In contrast, the parental wild type and the *BMPR2^{DelKinase}* mutant lacking the kinase domain had no phosphorylated SMAD1/5/9 prior to BMP4 addition (**Fig. 4.6 B**). Moreover, in samples treated for 60mins, the amount of phosphorylated SMAD1/5/9 was higher in all of the *BMPR2* mutants in comparison to wild type. *BMPR2* wild type PSCs had lower levels of phospho-SMAD1/5/9 after 60mins incubation with BMP4 compared to 30mins (**Fig. 4.6 B**). These results suggest that disruption of *BMPR2* impaired regulation of the BMP/SMAD signalling system. In addition to these post-translational effects, the total levels of SMAD1, a transducer of canonical BMP signalling, were noticeably reduced after 60mins of BMP4 treatment in all mutant PSCs (**Fig. 4.6 B**). Paralleling the results observed with phospho-SMAD1/5/9, the level of phosphorylated p38MAPK, a component of the non-canonical BMP signalling pathway, was elevated in untreated *BMPR2^{Null}* and *BMPR2^{DelTail}* PSCs compared to wild type PSCs and *BMPR2^{DelKinase}* (**Fig. 4.6 B**). Furthermore, in wild type *BMPR2^{DelKinase}* and *BMPR2^{Null}*, the kinetics of p38MAPK phosphorylation mirrored the changes observed in SMAD1/5/9 phosphorylation during BMP4 exposure. However, in *BMPR2^{DelTail}* PSCs p38MAPK phosphorylation was reduced after the addition of BMP4 (**Fig. 4.6**). This finding is consistent with the known role of the *BMPR2* tail domain in mediating the non-canonical pathway (Iwasaki et al. 1999). Taken together, these results suggest that disruption of *BMPR2* causes signalling dysfunction in both the canonical and non-canonical BMP signalling pathways.

A



B

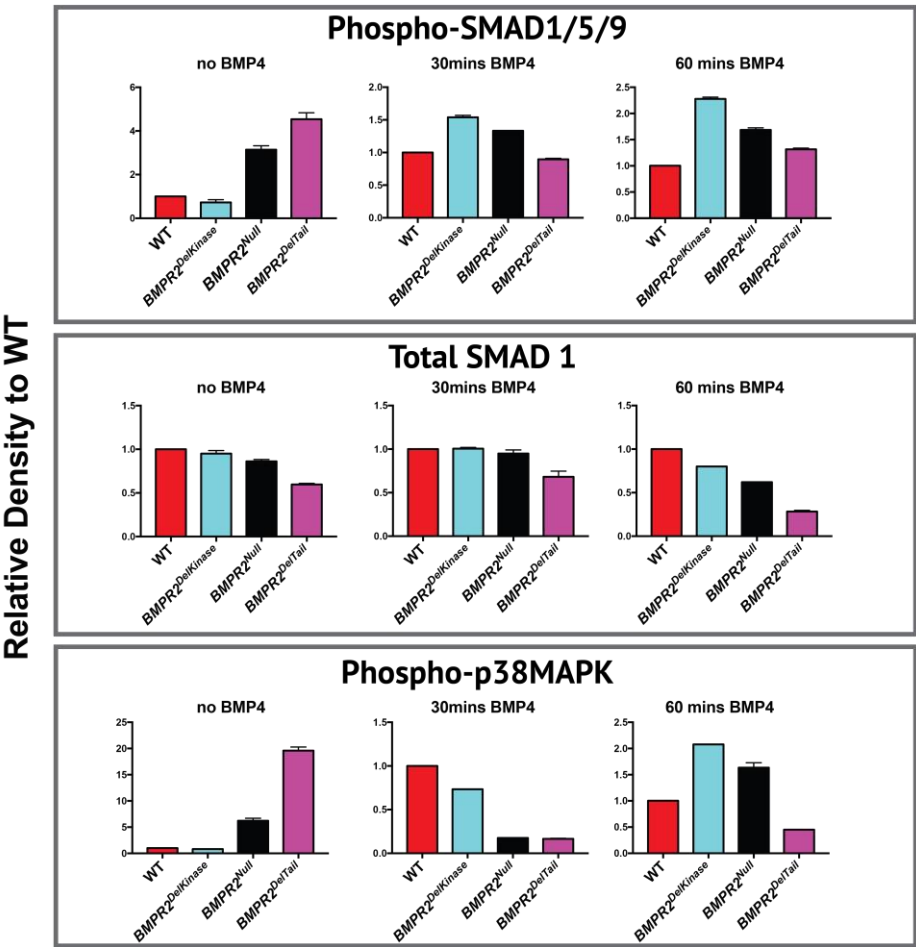


Figure 4.6 Disruption of both canonical and non-canonical BMP signalling in BMPR2 mutant PSCs. Western blot analyses of phospho-SMAD1/5/9, total SMAD1 and phospho-p38MAPK in PSCs treated with BMP4. **(A)** Protein lysates were extracted from isogenic control HES NKX2-5^{eGFP/W} and BMPR2 mutants that were untreated and induced with 100ng/mL of BMP4 for 30mins and 60mins. From left to right, GAPDH loading control, phospho-SMAD1/5/9, total SMAD1 and phospho-p38MAPK. Samples represent untreated cultures (No BMP) and cultures treated with BMP4 for 30 and 60mins. **(B)** Histogram represents densities of protein samples measured relative to wild type protein blots. Relative to wild type, BMPR2 mutant phospho-SMAD1/5/9 and phospho-p38MAPK signals are dysregulated.

4.4 *BMPR2* is not essential for cardiomyocyte differentiation

Pulmonary arterial hypertension often leads to right heart failure, potentially due to narrowing of the arterial capillaries, which subsequently places additional stress on the heart. We considered whether a lack of *BMPR2* could also contribute to cardiac dysfunction and whether mutations in *BMPR2* might directly affect cardiomyocyte development. To test this theory, we examined the differentiation of wild type and *BMPR2* mutant PSCs towards cardiomyocytes using a highly efficient monolayer cardiac differentiation method (Lian et al. 2012, Skelton et al. 2016). The cardiac differentiation time course is illustrated in **Fig. 4.7**.

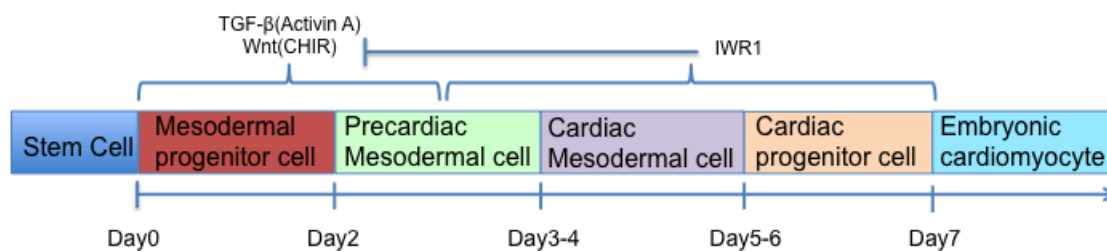


Figure 4.7 Schematic representation of the time course of the PSC monolayer cardiac differentiation protocol. Days when cytokines and small molecules are added and removed are indicated. For this protocol, CHIR and ACTIVIN A are added in base media RPMI 1640 + B-27® at day 0 and removed 24hrs later followed by refreshment with RPMI 1640 + B-27® and IWR1 subsequently every 48hrs until day 7. After day 7, media is replenished with RPMI 1640 + B-27®.

All cell lines differentiated into cardiomyocytes, as evidenced by the ability of cell monolayers to undergo spontaneous contraction. To further characterise these cultures, day 14 cardiomyocytes were immunostained with definitive cardiac markers such as *NKX2-5*, an early cardiac transcription factor, and sarcomeric α -ACTININ (**Fig 4.8**). This analysis showed that, as anticipated, *NKX2-5* immunostaining was associated with eGFP⁺ cells. In addition, wild type and *BMPR2* mutant cardiomyocytes expressed sarcomeric α -ACTININ (**Fig 4.8**), cardiac TROPONIN I and TROPONIN T (**Fig. 4.9**), suggesting that cardiomyogenesis was unaffected by the loss of *BMPR2* or perturbation of *BMPR2* signalling.

Flow cytometric analysis confirmed that *BMPR2* mutant PSCs efficiently differentiate into cardiomyocytes, identified by expression of eGFP⁺ and the cell surface markers, SIRPA⁺ and VCAM1⁺ (**Fig.4.10**) (Skelton et al. 2014). A summary of this analysis demonstrates that the efficiency of differentiation is relatively consistent across all cell lines tested (**Fig 4.11 C & D**). While line-to-line variation was observed, *BMPR2* mutants showed statistically significant decrease in the proportion of eGFP⁺ cells to wild type parental line: *BMPR2^{DelKinase}* $p < 0.001$, *BMPR2^{NullA}* $p < 0.01$, *BMPR2^{NullB}* $p < 0.0002$, *BMPR2^{DelTailA}* $p < 0.0002$ and *BMPR2^{DelTailB}* $p < 0.02$, $n = 4$ (**Fig. 4.11 A**). Furthermore, endothelial progenitors expressing CD34⁺ were found at higher proportions in *BMPR2* mutant cultures in comparison to wild type cardiomyocyte cultures ($p < 0.05$, $n = 4$) (**Fig. 4.11 B**). Endothelial progenitor cell production and function is further analysed in Chapter 5.

To further determine the impact of altered *BMPR2* signalling in *BMPR2*

mutant cardiomyocytes, we performed gene expression profiling. RNA was extracted from eGFP⁺ cardiomyocytes isolated by flow cytometry at day 10 of differentiation (i.e. early cardiomyocytes) and analysed by qPCR. Wild type and *BMPR2* mutant cardiac gene expression profiles revealed no differences in the expression levels of the cardiac markers *NKX2-5*, *TBX5*, *GATA4*, *NPPA* and *TBX20* (**Fig. 4.12**). However, *BMPR2* expression levels in *BMPR2* mutant cells were reduced and no *BMPR2* transcripts were found in the *BMPR2*^{Nulls} (**Fig. 4.12**).

Preliminary studies conducted with patient cell line *BMPR2*^{2504delC/W}, demonstrated that this cell line efficiently differentiated into cardiomyocytes as shown in flow cytometric detection of a SIRPA⁺VCAM1⁺ population and immunostaining of cells expressing NKX2-5, α -ACTININ, TROPONIN I and TROPONIN T (**Fig. 4.13 A & D**). In contrast, qPCR cardiac gene profiling of sorted SIRPA⁺VCAM1⁺ *BMPR2*^{2504delC/W} cardiomyocytes displayed a dramatic reduction in level of *NKX2-5* but increased transcription of the cardiac myofilament markers, *MYL2* (myosin light chain 2) and *MYH6* (myosin heavy chain α) compared to iPSC^W (**Fig. 4.13 C**). *NKX2-5* is a known target of SMADs (Brown et al. 2004) and the lower transcript level observed may be the result of reduced *BMPR2* signalling due to nonsense mediated decay (NMD) of the *BMPR2*^{2504delC} transcript (see **Chapter 3, Fig. 3.13**). Nevertheless, as shown by the efficient production of contractile monolayers that express cardiac markers, including NKX2-5, (**Fig. 4.13**) the *BMPR2*^{2504delC} allele does not grossly impair cardiogenesis.

To investigate whether *BMPR2* mediates cardiomyocyte response to BMP4, we added exogenous BMP4 to differentiating cultures and measured gene expression by qPCR. Relative to *BMPR2* mutants, wild type cardiomyocytes have significantly higher transcript levels of the cardiac transcription factors *NKX2-5* ($p < 0.05$, $n = 3$), *GATA4* ($p < 0.0001$, $n = 3$) and the BMP pathway components *SMAD1* ($p < 0.05$, $n = 3$) and *ID1* ($p < 0.05$, $n = 3$). In addition, transcripts encoding the transmembrane proteins *NOTCH1* ($p < 0.005$, $n = 3$) and *EFNA1* ($p < 0.01$, $n = 3$) (**Fig. 4.14**) were also at higher levels in wild type cardiomyocytes after BMP4 treatment. These data suggest that *BMPR2* is an important mediator of BMP4 signalling in cardiomyocytes. This impaired transcriptional response to exogenous BMP4 in *BMPR2* mutants suggests that *BMPR2* mutations may compromise cardiomyocyte development or function pre-disposing PAH patient carrying a *BMPR2* mutation to a higher risk of cardiac failure.

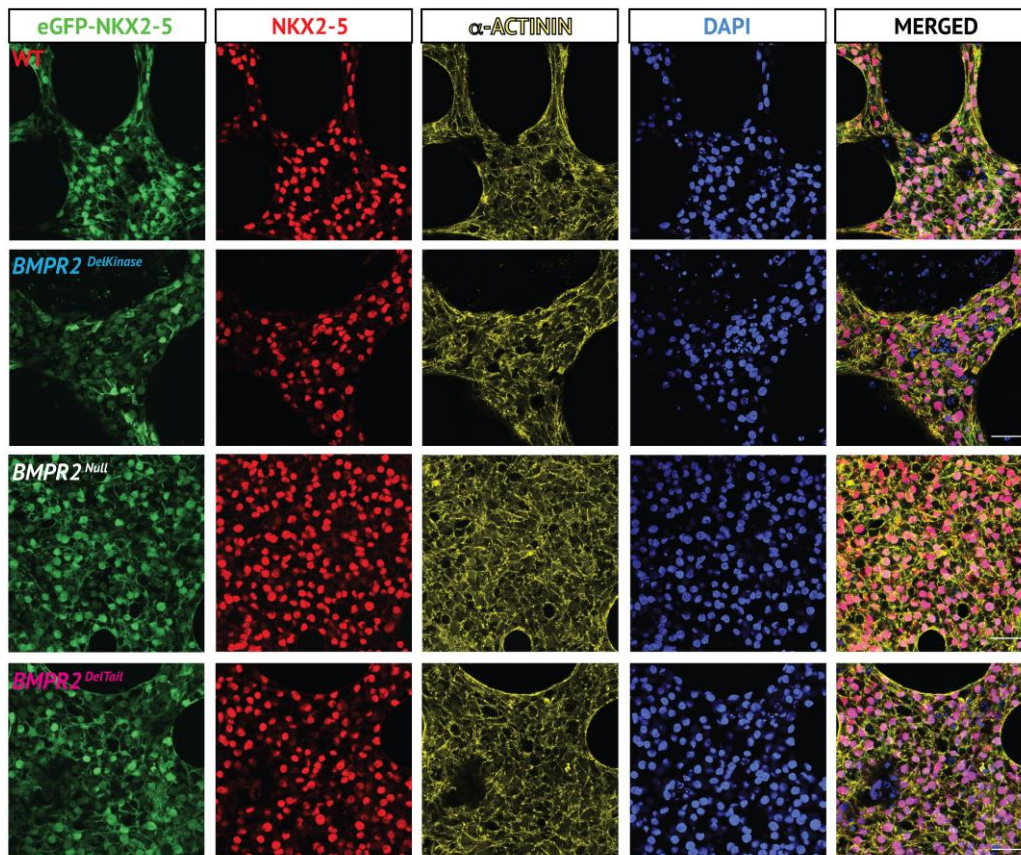


Figure 4.8 *BMPR2* mutant cardiomyocytes express the cardiac transcription factor *NKX2-5* and the myofilament protein α -ACTININ. Confocal sections of day 14 wild type and *BMPR2* mutant cardiomyocytes. Cells were labelled with antibodies directed against *NKX2-5* and α -ACTININ (as indicated). All genotypes differentiated into cardiomyocytes and expressed *NKX2-5*, α -ACTININ, which marks the sarcomeric structures, and eGFP, which reports transcription of *NKX2-5*. Nuclei were counterstained with DAPI. Scale bar = 50 μ m.

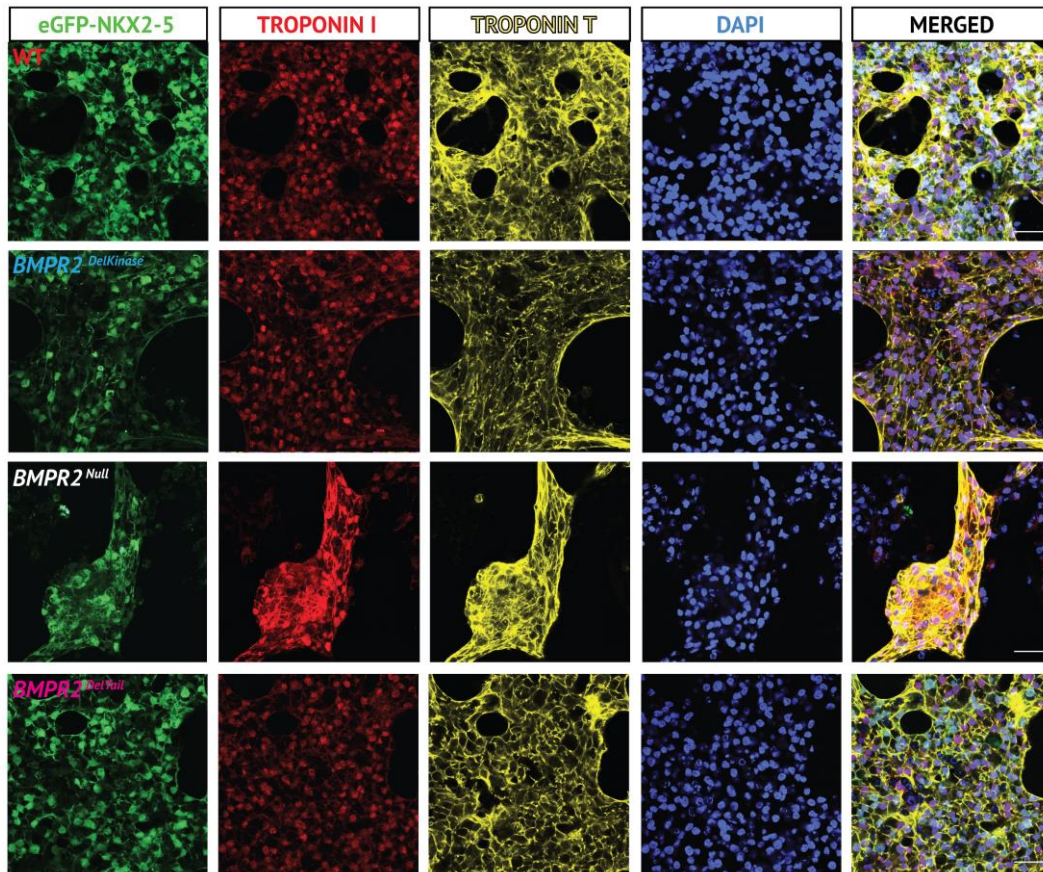


Figure 4.9 *BMPR2* mutant cardiomyocytes express the cardiac myofilament proteins *TROPONIN-I* and *TROPONIN-T*. Confocal sections of day 14 wild type and *BMPR2* mutant cardiomyocytes. Cells were labelled with antibodies directed against *TROPONIN-I* and *TROPONIN-T* (as indicated). Wild type and *BMPR2* mutant PSCs differentiate into cardiomyocytes and express *TROPONIN I* and *TROPONIN T*. These proteins expressed in cardiac and skeletal muscle are both important for contractile function. eGFP positive cells also indicate that *NKX2-5* is expressed. Nuclei were counterstained with DAPI. Scale bar = 50 μ m.

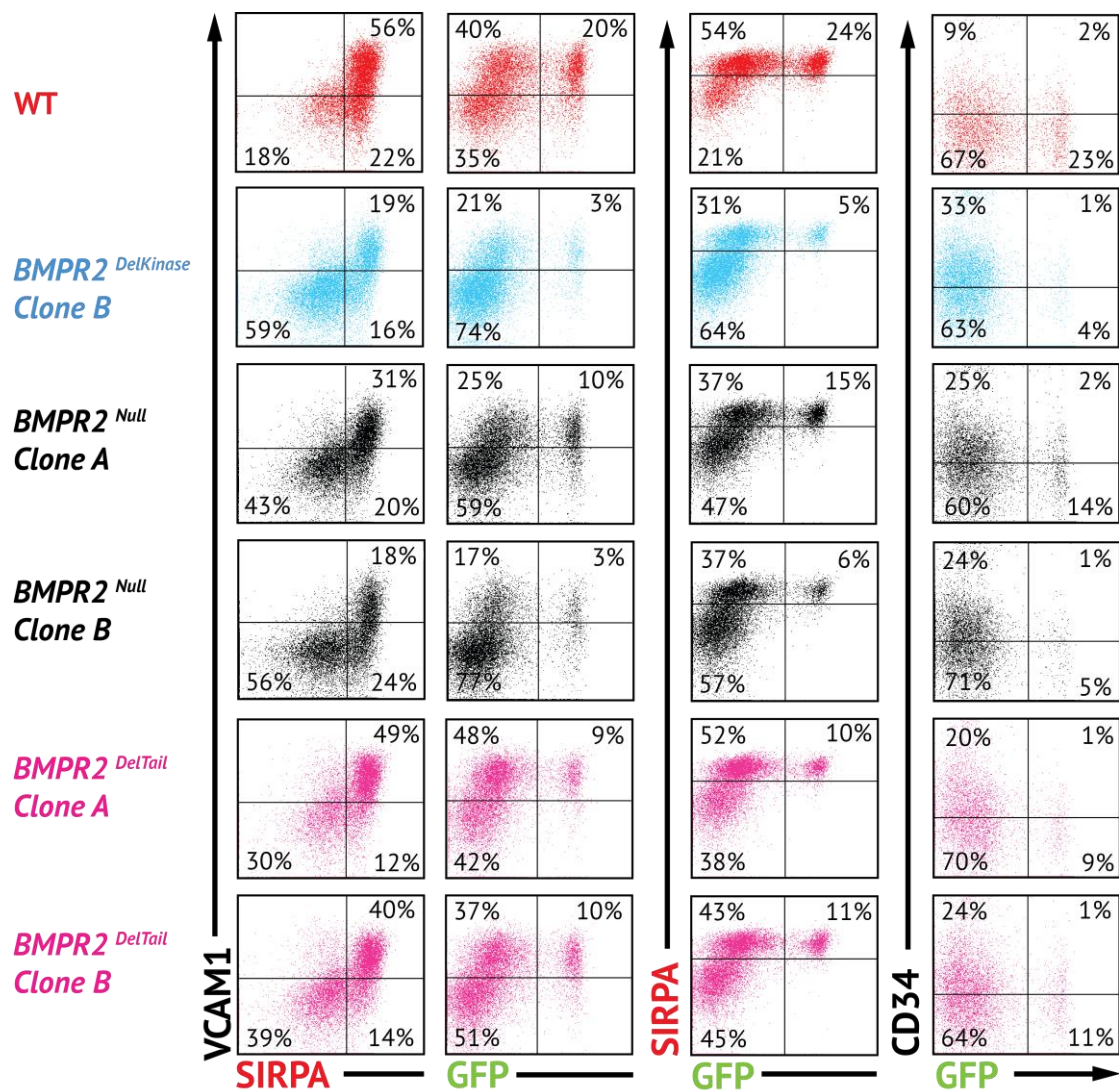


Figure 4.10 *BMPR2* mutant iPSC efficiently generate VCAM1/SIRPA positive cardiomyocytes and CD34 positive endothelial cells. Flow cytometric analysis of unsorted day 14 cardiomyocytes. Cardiomyocytes express eGFP⁺, VCAM1⁺ and SIRPA⁺. CD34⁺ marks the endothelial progenitor population in the cultures. All *BMPR2* mutant PSC genotypes differentiated into SIRPA⁺VCAM1⁺ cardiomyocytes that are positive for GFP⁺ (NKX2-5⁺). The numbers represent percentage of total cells in each quadrant.

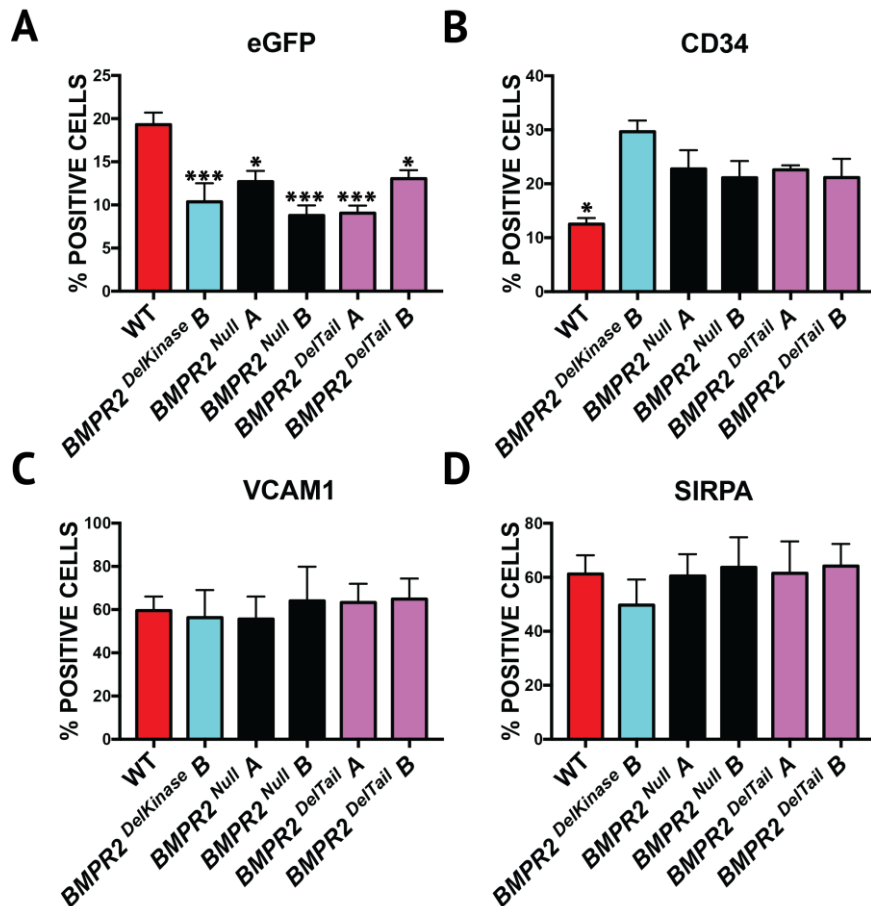


Figure 4.11 BMPR2 mutants have reduced NKX2-5⁺ cardiomyocytes and increased CD34⁺ endothelial cells in a cardiac differentiation protocol. Histograms summarising the results of flow cytometric analysis comparing the percentage positive day 14 wild type and BMPR2 mutant cultures expressing (A) eGFP⁺-NKX2-5 (B) VCAM1⁺ (C) SIRPA⁺ and (D) CD34⁺. This analysis revealed that the frequency of VCAM1⁺ and SIRPA⁺ cells present in cultures representing wild type and BMPR2 mutant cardiomyocytes are consistent. In contrast, BMPR2 mutant cardiomyocyte cultures have significantly lower percentages of cells expressing eGFP⁺ (Dunnett's test, adjusted p-values: BMPR2^{DelKinase} *** $p < 0.001$, BMPR2^{Null}A * $p < 0.01$, BMPR2^{Null}B *** $p < 0.0002$, BMPR2^{DelTail}A, *** $p < 0.0002$ and BMPR2^{DelTail}B * $p < 0.02$), while the percentage of CD34⁺ expressing cells was significantly lower in wild type cardiomyocyte cultures suggesting that lack of BMPR2 favoured differentiation of CD34⁺ progenitor cells (* $p < 0.01$, using Dunnett's multiple comparison test) ($n=4$; $\pm$ S.E.M.).

qPCR Profile of D10 Sorted Cardiomyocytes

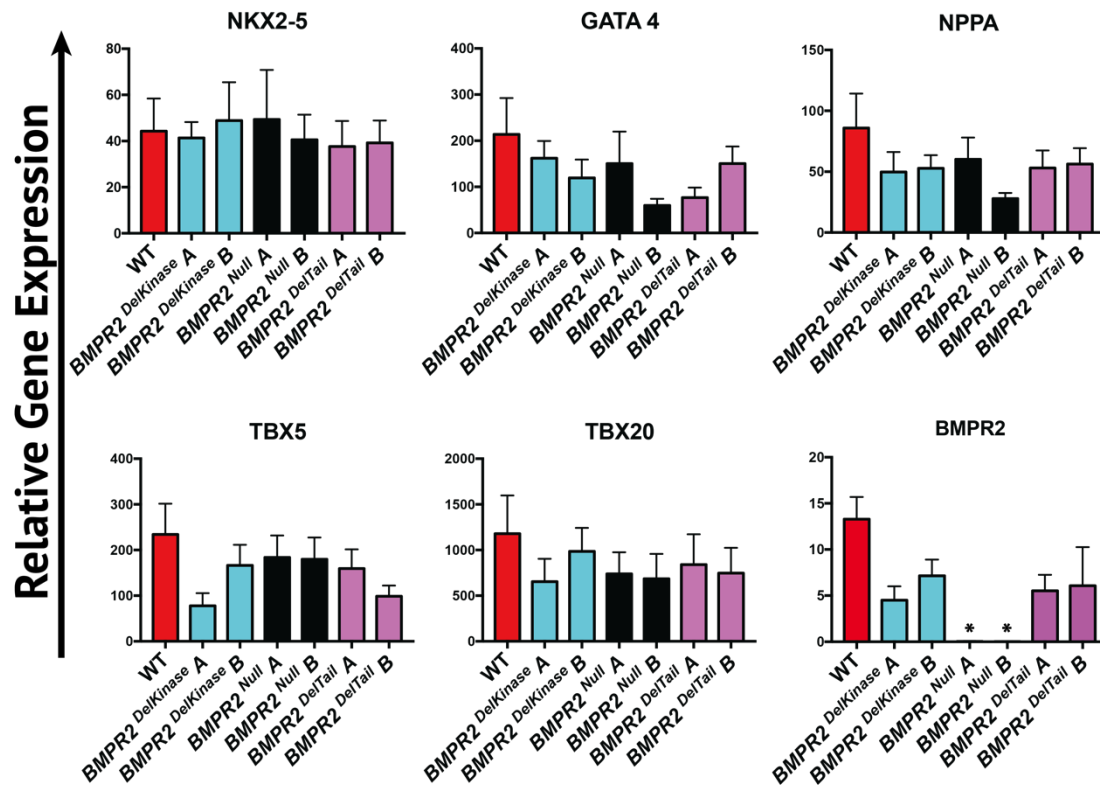


Figure 4.12. BMPR2 mutant cardiomyocytes express cardiac markers. QPCR cardiac gene expression profile of day 10 sorted GFP⁺ cardiomyocytes. Expressions of NKX2-5 are shown to be consistent throughout all cell lines as cells were sorted based on eGFP⁺ expression. Expression of cardiac genes in BMPR2 mutant cardiomyocytes showed no differences to wild type cardiomyocytes. While BMPR2 expressions are negligible in BMPR2^{Null} cardiomyocytes (Dunnett's test: BMPR2^{Null} A **p*<0.03, BMPR2^{Null} B **p*<0.01) and are slightly down regulated in BMPR2^{DelKinase} and BMPR2^{DelTail} (*n*=4; ±S.E.M.).

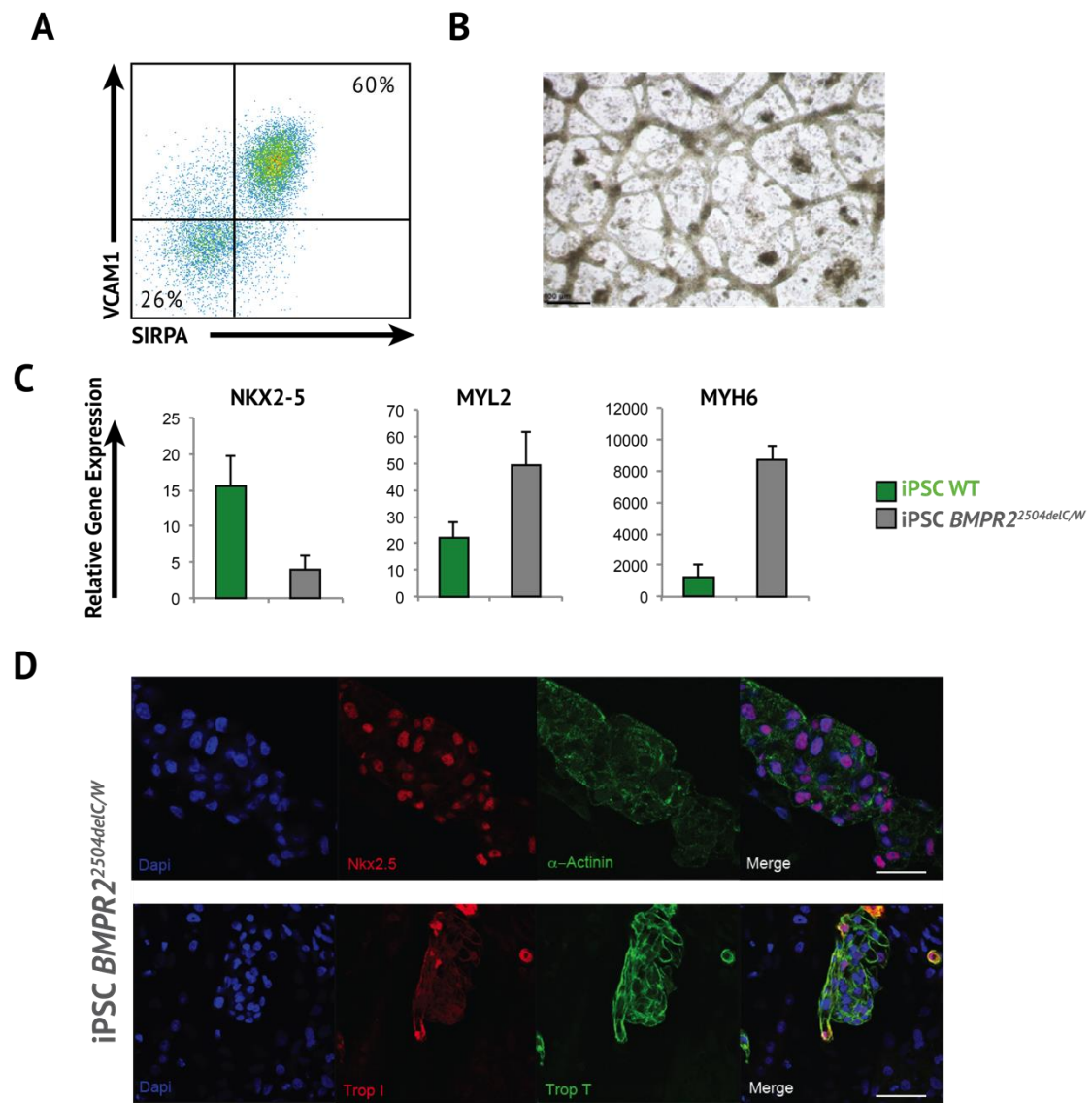


Figure 4.13 Characterization of patient PSC line *BMPR2*^{2504delC/W} differentiated into cardiomyocytes. (A) Representative FACS analysis of *BMPR2*^{2504delC/W} cardiomyocytes stained with SIRPA and VCAM1 antibodies. Double positive cells (top right quadrant) are enriched for cardiomyocytes (B) Still frame of a video taken from contracting *BMPR2*^{2504delC/W} cardiomyocytes. Scale bar = 100µM. (C) Relative gene expression of cardiac markers was quantified by real-time qPCR and normalized against GAPDH. *BMPR2*^{2504delC/W} and iPSC^W cardiomyocytes show expression of cardiac markers NKX2-5, MYL2 and MYH6. (Error bars display ±S.E.M., n=4). (D) Single confocal sections showing expression of NKX2-5, α-ACTININ (Top panel) and TROPONIN I (TROP I), TROPONIN T (TROP T) (Bottom panel) in cardiomyocytes derived from *BMPR2*^{2504delC/W} PSCs. Myofilament proteins show expected striated pattern while the transcription factor NKX2-5 displays the expected nuclear localization. Nuclei were counterstained with DAPI. Scale bars = 50µm.

qPCR Profile of D10 Sorted BMP4 Treated Cardiomyocytes

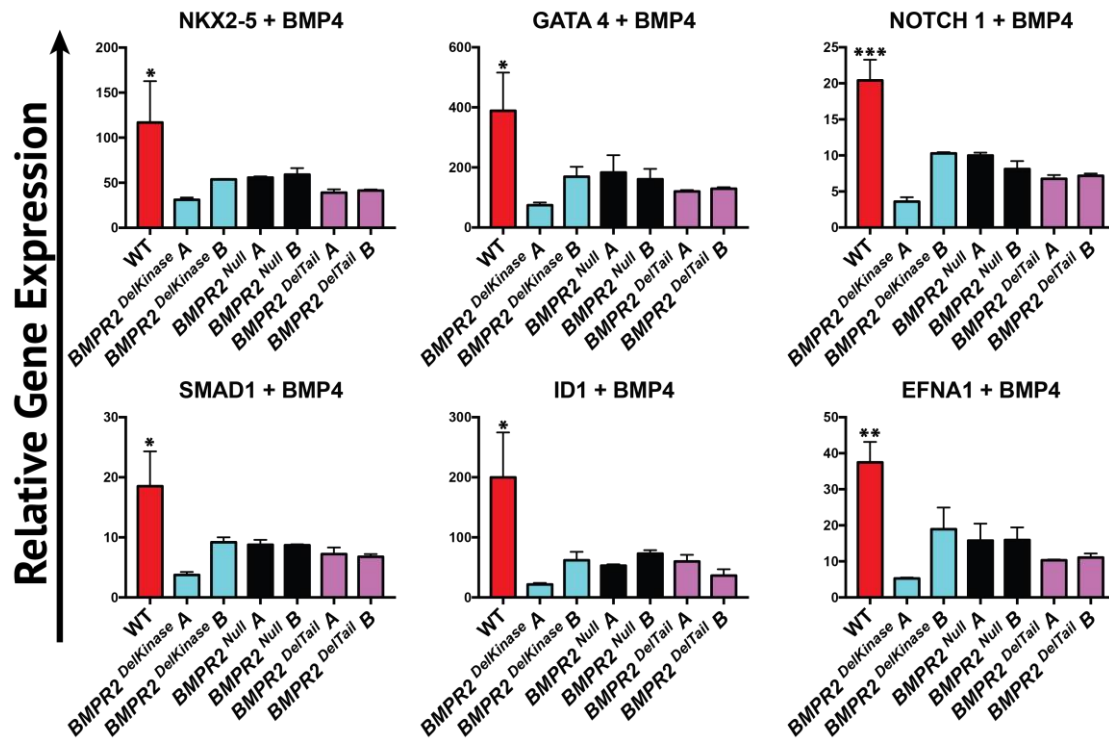


Figure 4.14 Expression profiling of cardiomyocyte response to BMP4 exposure. QPCR profile of day 10 cardiomyocytes treated with 100ng/mL BMP4 isolated by flow cytometry sorting based on eGFP⁺ expression. QPCR profile of cardiac markers NKX2-5 (* $p < 0.05$) and GATA4 (* $p < 0.0001$) were up regulated in wild type cardiomyocytes in presence of BMP4. In addition, wild type cardiomyocytes had up regulated expressions of NOTCH 1 (*** $p < 0.005$), SMAD1 (* $p < 0.05$), ID1 (* $p < 0.05$) and EFNA1 (** $p < 0.01$), while transcriptions of these genes were not up regulated in BMPR2 mutant cardiomyocytes ($n=3$; $\pm$ S.E.M.). Statistical analyses were conducted using Dunnett's multiple comparisons test.

Discussion

In this chapter, we investigated the functional consequences of *BMPR2* disruption for mesoderm and cardiac development. Contrary to prior studies by Beppu et al. (2000) in the mouse reporting that *Bmpr2* knockout precluded mesoderm formation, our data demonstrated that *BMPR2* deficiency did not perturb mesoderm formation in the allelic series of *BMPR2* mutant PSCs (**Fig. 4.5**). Furthermore, heterozygote *BMPR2* cell lines, patient PSC line *BMPR2*^{2504delC/W} and HES3 *BMPR2*^{DelTail/W}, likewise differentiate into mesodermal cells and retain the ability to transduce canonical BMP signalling (**Fig. 4.3 & Fig. 4.4**). Our data are consistent with several reports suggesting that the C-terminal tail domain of *BMPR2* is not essential in activating the canonical BMP signalling (Chan et al. 2007, Machado et al. 2003, Nishihara et al. 2002, West et al. 2008b).

There are a number of possible explanations as to why *BMPR2* seems to be dispensable for mesoderm formation during human PSC differentiation *in vitro*. The most straightforward explanation is that mice and humans use fundamentally different processes to regulate this event. However, because this pathway is conserved across all other species so far examined (Wong et al. 2006), this possibility seems unlikely. A more plausible explanation is that, *in vivo*, the correct positioning of BMP producing and responding cells is required for gastrulation to proceed. During embryonic development in mice, cells of the inner cell mass (which resemble ESCs) express high levels of BMP ligands whilst the trophectoderm (the extra-embryonic tissue) expresses *Bmpr2*. Deficiency of *Bmpr2* during this pre-implantation stage precludes the

correct development of extra-embryonic lineages, primitive endoderm and trophoctoderm (Graham et al. 2014). Therefore, it is possible that the subsequent failure of gastrulation in *Bmpr2* null embryos may reflect the downstream consequences of perturbed development of extra-embryonic tissues, which later in development become a major source of BMP4 (Graham et al. 2014). However, even if this explanation holds true, the fact that human PSCs lacking *BMPR2* efficiently form mesoderm implies that other receptors (eg. *ACVR2A* and *ACVR2B*) must be able to compensate for loss of *BMPR2* in an *in vitro* setting. In this regard, PSCs also express *BMPR1*, *BMPR2*, *ACVR2A* and *ACVR2B* (Bhattacharya et al. 2004, Pera et al. 2004), all of which have been shown to bind and transduce BMP signals.

BMP4 induced *BMPR2* mutant PSCs were able to transduce signals through the canonical BMP pathway, possibly via compensatory up regulation of the receptors *ACVR2A* and *ACVR2B* binding to BMPs and forming heterodimers with *BMPR1*, as previously reported (Lowery et al. 2015, Yu et al. 2005). However, it may be that only *ACVR2A* and not *ACVR2B* can compensate for the absence of *BMPR2*, as is the case in PSMCs (Leyton et al. 2013). Furthermore, *BMPR2* mutant proteins truncated within the tail domain have been demonstrated to retain ability to transduce signals via the canonical BMP pathway (i.e. SMAD signalling) (Rudarakanchana et al. 2002a). Nevertheless, our immunoprecipitation results demonstrated impaired SMAD phosphorylation status in all of the *BMPR2* mutants. This finding is consistent with previous literature suggesting that *BMPR2* deficiency causes a dysregulation in SMAD signals (Nishihara et al. 2002, Rudarakanchana et al.

2002b, Yu et al. 2005). Levels of phosphorylated SMAD1/5/8 was detected in *BMPR2^{Null}* and *BMPR2^{DelTail}* PSCs even in the absence of exogenous BMPs, which was not observed in wild type and *BMPR2^{DelKinase}* suggesting that *BMPR2* may be required to negatively regulate endogenous SMAD phosphorylation (**Fig. 4.6 A**). Furthermore, SMAD proteins continuously shuttle between the cytoplasm and the nucleus even when unstimulated (Watanabe et al. 2000). Moreover, there is compelling evidence that the bulk of SMADs accumulated in the nucleus in response to signal is not degraded there. Evidence suggests that such shuttling provides an elegant sensing mechanism ensuring that the concentration of active SMADs in the nucleus reflects the actual signal strength at any given time. For example, SMAD2 relocates to the cytoplasm upon inactivation of the signal when unphosphorylated, suggesting that nuclear export is accompanied by dephosphorylation (Inman et al. 2002). In addition, we establish that *BMPR2* deficiency impairs the non-canonical BMP (SMAD independent) signalling pathway. This was observed by an increase of phosphorylation levels of p38MAPK expression detected in untreated *BMPR2^{Null}* and at significant levels in *BMPR2^{DelTail}* PSCs (**Fig. 4.6 B**). Moreover, there was an overall increased and delayed response of phospho-p38MAPK expression in BMP4 induced *BMPR2^{DelKinase}* and *BMPR2^{Null}* after an hour (**Fig. 4.6 B**). Rudarakanchana et al. (2002a) reported that overexpression of mutant *BMPR2* receptors, including versions lacking a cytoplasmic tail, in NMuMG (mammary gland cells) led to activation of p38MAPK in the absence of exogenous ligand, whereas overexpression of the wild type receptor required the presence of BMP4 to activate p38MAPK. In addition to the MAPK

pathway, BMP signalling has been found to affect PI3K/Akt, P/kc and Rho-GTPases pathways (Wang et al. 2014). A second possibility is that the elevated SMAD1/5/9 and p38MAPK phosphorylation levels might originate from either ACTIVIN or TGF- β signalling pathways. If this was the case, then this might imply that one of *BMPR2*'s multifactorial functions was to suppress signalling emanating from other TGF- β signalling systems. Such a function would be consistent with the well recognised cross regulation of signalling pathways representing different members of the TGF- β family.

Investigating altered *BMPR2* signalling in cardiogenesis

Another novel aspect of this study demonstrated that *BMPR2* mutant PSCs have the capacity to differentiate into cardiomyocytes in an *in vitro* setting. *BMPR2* mutant cardiomyocytes were characterized by immunostaining of cardiac markers such as *NKX2-5*, *α -ACTININ*, *TROPONIN I*, *TROPONIN T*, flow cytometry expression of eGFP⁺ (*NKX2-5*), SIRPA⁺, VCAM1⁺ and qPCR gene expression analysis of cardiac markers.

Unsorted *BMPR2* mutant and wild type cardiomyocyte cultures contained consistent populations of SIRPA⁺VCAM1⁺ expressing cells (**Fig. 4.11 C & D**), *BMPR2* mutant eGFP⁺ populations were significantly lower than in wild type cultures (**Fig. 4.11 A**). In contrast, CD34⁺ population in *BMPR2* mutant cardiomyocyte cultures were significantly increased suggesting that the disruption to *BMPR2* may enhance endothelial progenitors *in vitro* (**Fig. 4.11 B**).

Subsequently, we examined the gene expression profiles of *BMPR2* mutant cardiomyocytes and found no statistical differences in the expression levels of

cardiac markers *NKX2-5*, *TBX5*, *GATA4*, *NPPA* and *TBX20* (**Fig. 4.12**). In contrast, BMP4 treated wild type cardiomyocytes showed increased gene transcription of cardiac markers *NKX2-5*, *GATA4* and BMP signalling pathway components *SMAD1* and *ID1* (**Fig. 4.14**). While lower levels of these gene transcripts were observed in BMP4 treated *BMPR2* mutant cardiomyocytes. Furthermore, *NOTCH1* and *EFNA1*, genes that are important for the structural maintenance of the myocardium, were not up regulated in *BMPR2* mutant cardiomyocytes after exposure to BMP4 (**Fig. 4.14**).

BMP signalling occurs through binding to heteromeric serine/ threonine kinase type 1 or 2 BMP receptors, activating phosphorylation of the type 1 by the type 2 receptor. The type 1 receptor (*BMPR1A* or *BMPR1B*, of which only *BMPR1A* is expressed in the heart) binds and phosphorylates the pathway-specific SMAD1/5/8. SMAD transcription factors are known to be involved in embryonic and cardiac development. *NKX2-5* a well-known cardiac transcription factor was found to have an upstream gene enhancer composed of highly repeated SMAD and GATA binding sites (Brown et al. 2004). BMP signalling directly activates *NKX2-5* through SMAD1/4 transcription factors, and co-depends with GATA factors. Impaired *BMPR2* signalling would impact downstream components in the BMP/SMAD pathways.

Collectively, these data suggests that disruption of *BMPR2* signalling affects the canonical (i.e. SMAD dependents) and non-canonical (i.e. SMAD independent) regulation of the BMP pathway, contributing to the pathogenesis of PAH. In addition, certain *BMPR2* mutations e.g. *BMPR2^{DelKinase}*, can act in a dominant negative fashion thereby affecting other BMP receptors. However, our results show that mesoderm and cardiomyocyte differentiation are not

affected by the disruption of *BMPR2* *in vitro*. In contrast, *BMPR2* mutant and wild type cardiomyocytes elicit different responses in the presence of exogenous BMP4. This suggests that disrupted *BMPR2* in cardiomyocytes may increase their susceptibility to environmental insults that can constitute to the progression of right ventricular heart failure in PAH patients carrying *BMPR2* mutations.

CHAPTER



Vasculogenesis and Angiogenesis

Introduction

Vasculogenesis and angiogenesis

The pathological phenotype in PAH patients is characterized by narrowing and thickening of the small pulmonary blood vessels. Affected patients exhibit vascular remodelling of all layers of the vessel. This includes thickening of the intima layer (lined by endothelial cells), hypertrophy of the smooth muscle cells in the medial layer, adventitial fibrosis and occluded vessels by *in situ* thrombosis (Yuan and Rubin 2005). Furthermore, *BMPR2* expression levels in endothelial cells (ECs) and smooth muscle cells (SMCs) of PAH patients, both carriers and non-carriers of *BMPR2* mutations, were significantly reduced (Atkinson et al. 2002, Du et al. 2003). In this chapter we will examine the effects of disrupted *BMPR2* function during *in vitro* differentiation and development of mesoderm derivatives such as blood cells and vascular cell types, SMCs and ECs.

Vasculogenesis is the process of de novo blood vessel formation, while angiogenesis is the sprouting (sprouting angiogenesis) and/or splitting (non-sprouting angiogenesis) of new blood vessels from existing ones (Risau 1997). There are two main cell types that make up blood vessels, ECs and SMCs (**Fig. 5.1**). Both cell types are thought to arise from a common precursor, the hemangioblast, that eventually gives rise to blood cells and angioblasts, the latter generating the vascular cells such as ECs and SMCs (Shalaby et al. 1997). One important and well-understood role of transforming growth factor beta (TGF- β) signalling in angiogenesis is that of promoting vessel muscularization (Ten Dijke and Arthur 2007). The TGF- β superfamily is

evolutionary conserved and includes pleiotropic ligands including the ACTIVINS and bone morphogenetic proteins (BMPs). Vascular SMCs (arteries and veins) or pericytes (capillaries) cover nascent vessels and are required for both vessel stability and function (**Fig. 5.1**). Muscularization is achieved when ECs promote SMC or pericyte differentiation of neighbouring mesenchymal cells via paracrine TGF- β 1 based signalling. In addition, cell-cell communication through gap-junctions between ECs and adjacent mesenchymal cells also regulate SMC differentiation (Hirschi et al. 2003).

In the context of this study, BMP signalling is one of the key paracrine pathways regulating blood vessel development, maintenance and function (Deckers et al. 2002, Lowery and de Caestecker 2010, Valdimarsdottir et al. 2002). Both BMP2 and BMP4 have been shown to stimulate EC migration and tube formation activity, consistent with their ability to promote neo-angiogenesis (Smadja et al. 2008). Furthermore, BMP4 signals downstream of Indian Hedgehog and is essential for promoting EC differentiation from hESCs (Little and Mullins 2009). BMP2 was found to enhance pulmonary arterial endothelial cell (PAEC) survival and proliferation *in vitro* (Teichert-Kuliszewska et al. 2006). Moreover, BMP2 induces the expression of endothelial nitric oxide synthase (eNOS), which is important for proper vascular function and vasodilation. The loss of BMP-induced eNOS expression has been reported to cause pulmonary arterial EC dysfunction that leads to increase in pulmonary vasoconstriction (Anderson et al. 2010, Frank et al. 2008). Thus, BMP signalling plays multiple roles in vessel formation and homeostasis.

During vessel formation, mesodermal progenitor cells migrate in response to endoderm-derived signals that include fibroblast growth factor (FGF) and BMP4 to sites of vascularization. Initially, this takes place in the extra-embryonic visceral mesenchyme of the yolk sac (Coultas et al. 2005). Subsequently, these two key signalling components drive the differentiation of mesodermal angioblast precursor cells towards the endothelial and hematopoietic cell fate (Marcelo et al. 2013). ECs express BMPs, and by this mechanism suppress vascular SMC proliferation and increase expression of smooth muscle differentiation markers (Glienke et al. 2000, Willette et al. 1999). Furthermore, BMP2/4 and BMP5/7 deficient mice showed abnormal development in heart and vasculature (Goumans and Mummery 2000).

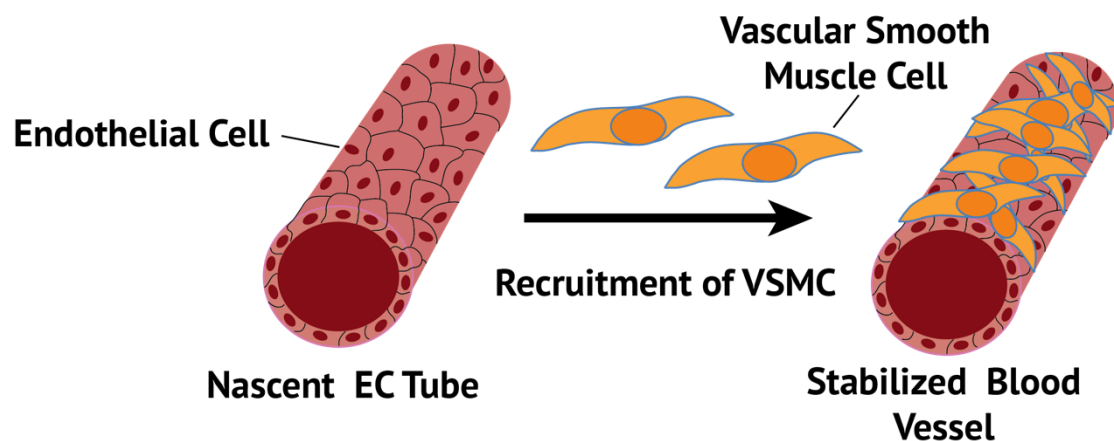


Figure 5.1 *Endothelial cells form the nascent tube. ECs send out signals to recruit VSMCs. After the recruitment of VSMCs, the blood vessel becomes stabilized.*

The role of BMP signalling in the pathogenesis of PAH is complex. This is due to the combinatorial expression of BMP ligands, receptors and antagonists in the ECs and SMCs that form the blood vessel (Glienke et al. 2000, Yu et al.

2008). As a result, the consequences of perturbation of *BMPR2* may be buffered by alternate components of the BMP signalling network. Furthermore, there are spatial restrictions on expression of components of the BMP network. For example, microvascular ECs and human umbilical vein EC (HUVEC) undergo apoptosis during exposure to BMP4, while ECs localized in the small arteries are highly resistant to BMP4. This phenomenon is directly regulated by inhibitory Smads (I-Smads) (Kiyono and Shibuya 2006). This highly specialized deployment, coupled with the inherent redundancy of the BMP network may serve to lower the penetrance of *BMPR2* mutations leading to PAH.

In this study, we used established SMC and hematopoietic differentiation protocols and developed an EC differentiation protocol to enable the characterisation of *BMPR2* mutant vascular progenitors. We found that impaired *BMPR2* signalling alters the cytoskeletal structures in *BMPR2* mutant SMCs and changes the cellular phenotype of ECs. Additionally, hematopoietic potential of *BMPR2* deficient mesodermal progenitors was reduced. Observations from our functional studies of ECs, including scratch-migration and MTT proliferation assays, lead us to conclude that *BMPR2* plays a role in balancing proliferation-differentiation and in cellular migration. These findings are discussed in the context of PAH pathogenesis.

Results

5.1 Analysis of hematopoietic capacity of CD34⁺/CD31⁺ progenitors isolated in optimised differentiation conditions

Endothelial cells arise from a bi-potential precursor of the blood and vascular systems known as the hemangioblast (Choi et al. 1998, Sabin 1920). BMP signalling is crucial for the specification of hemangioblasts and the subsequent development to hematopoietic lineages (Sadlon et al. 2004). Therefore, we sought to determine if *BMPR2* is required for the differentiation of hematopoietic cells. Using a well-established, embryoid body (EB) based differentiation protocol (Davis et al. 2008, Ng et al. 2016, Pick et al. 2007) in animal produce free defined media (APEL), we examined hematopoiesis in wild type and *BMPR2*^{Null} PSCs.

In this experiment, we subjected wild type and *BMPR2*^{Null} PSCs to a hematopoietic and blast-colony differentiation protocol that included FGF2 and BMP4, denoted control group. In parallel, exogenous FGF2 or BMP4 was omitted in control arms of the experiment. No difference was observed between the genotypes in the production of the CD43 and Glycophorin (data not shown) positive erythroid cells (**Fig. 5.2 A**). However, flow cytometric analysis of hematopoietic differentiation showed that perturbed *BMPR2* impaired lineage specification during primitive (i.e. extra-embryonic like) hematopoiesis. The proportion of *BMPR2*^{Null} myeloid/hematopoietic progenitors as defined by co-expression of CD34 and CD43 was reduced compared to wild type ($p < 0.04$, $n = 3$) (**Fig. 5.2 B**). In the -FGF2/+BMP4 group,

wild type and *BMPR2^{Null}* display a similar proportion of CD34⁺CD43⁺ cells (**Fig. 5.2 A**). Similarly, removal of BMP4 resulted in the absence of the CD34⁺CD43⁺ population in both wild type and *BMPR2^{Null}* cultures. These results suggest that deficiency in *BMPR2* reduces the specification of primitive myeloid/haematopoietic progenitors but not the erythroid population. Nevertheless, BMP4 is required for hematopoiesis as cultures lacking BMP4 failed to generate any hematopoietic lineage. These results are concordant with our own laboratory findings as well as other literature documenting primitive hematopoiesis suppression when BMP signalling has been inhibited or is absent in mouse and human ES cell cultures (Adelman et al. 2002, Lengerke et al. 2009).

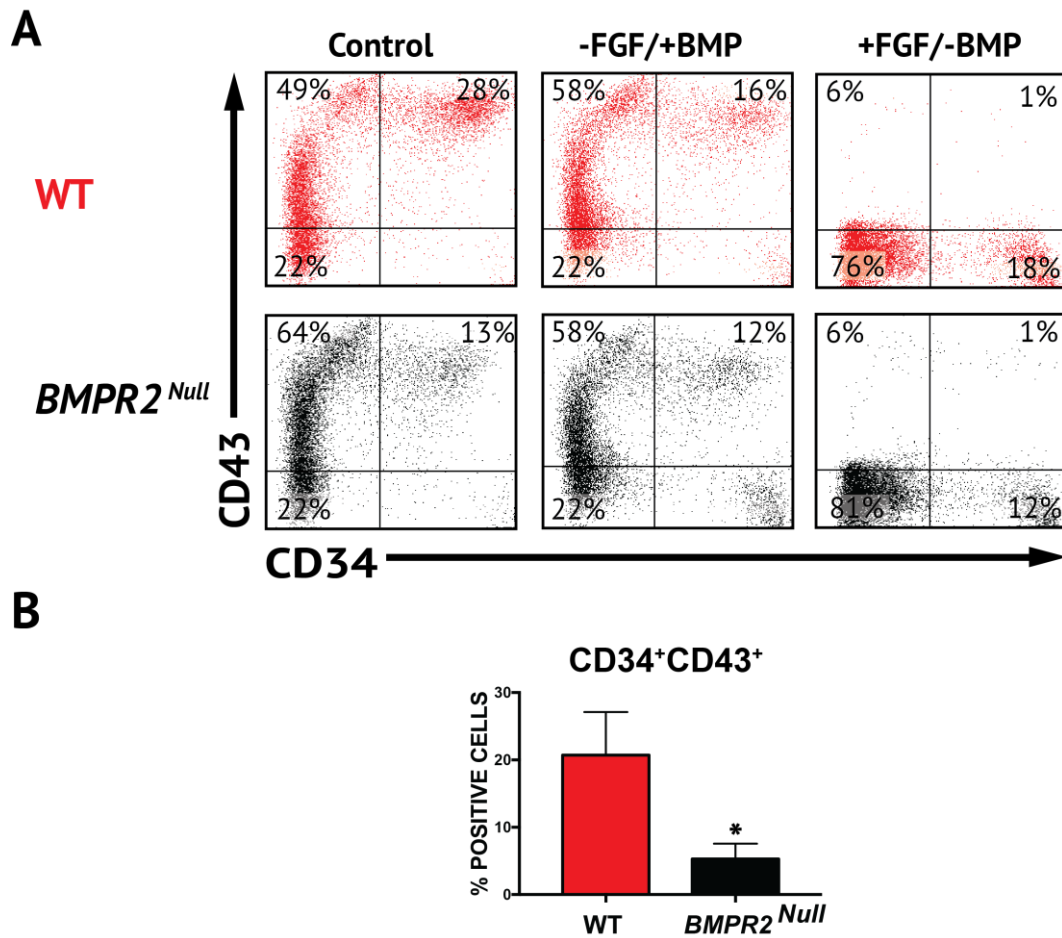


Figure 5.2 Analysis of the hematopoiesis capacity of BMPR2^{Null} versus wild type. (A) Flow cytometric analysis of day 12 primitive hematopoietic differentiation of wild type and BMPR2^{Null} PSCs subjected to the standard protocol, without FGF2 (-FGF2/+BMP4) and without BMP4 (+FGF2/-BMP4). Wild type and BMPR2^{Null} in control group show a population of cells that express CD43⁺ hematopoietic marker demonstrating that hematopoietic differentiation was not affected by perturbed BMPR2. While the CD34⁺CD43⁺ population was reduced in both -FGF2 groups, this reduction is also observed in the BMPR2^{Null} control group. However, no CD43⁺ population was observed in both -BMP4 groups. (B) Histogram depicts CD34⁺CD43⁺ population showing a reduction in BMPR2^{Null} versus wild type in the control group (**p*<0.04 using paired Student *t*-test). The numbers denote percentage of total cells in each quadrant (*n*=3; ±S.E.M.).

5.2 Characterization of *BMPR2* mutant smooth muscle cells (SMCs)

Here we investigate the effects of *BMPR2* deficiency in SMCs. To accomplish this, we optimised a published SMC differentiation protocol for our PSC cell lines (Lian et al. 2014). Briefly, vascular progenitors that express surface markers CD31⁺ and CD34⁺ were isolated by FACS and subsequently, further differentiated into SMCs. CHIR99021 concentration was optimised to improve production of the CD31⁺ CD34⁺ population (**Fig. 5.3 A**). CD31⁺CD34⁺ vascular progenitors, isolated at day 5, were cultured in vascular smooth muscle differentiation medium on collagen IV-coated plates to promote SMC differentiation. At day 10 of differentiation, these cells expressed the SMC surface markers CD44⁺ and PDGFR- α ⁺, and no longer expressed the endothelial markers CD31⁺ CD34⁺ (**Fig. 5.3 B**).

In vitro *BMPR2* mutant SMCs after 3 passages showed altered morphology in comparison to wild type SMCs (**Fig. 5.4 A**). These passage 3 *BMPR2* mutant SMCs exhibited smooth muscle actin positive stress fibre cytoskeletal defects that were noticeably different to wild type SMCs (**Fig. 5.4 A**). Furthermore, we observed that after 3 passages, both clones of *BMPR2*^{DelTail} SMCs were sparse in culture, suggesting that either the survival or proliferation capacity of these cells was compromised (**Fig. 5.4 B**). In contrast, *BMPR2*^{DelKinase} and *BMPR2*^{Null} SMCs displayed cytoskeletal differences to wild type SMCs but no apparent reduction in survival or proliferation (**Fig. 5.4 A, B**). Taken together these data demonstrate that disruption of *BMPR2* affects cytoskeletal organization in SMCs.

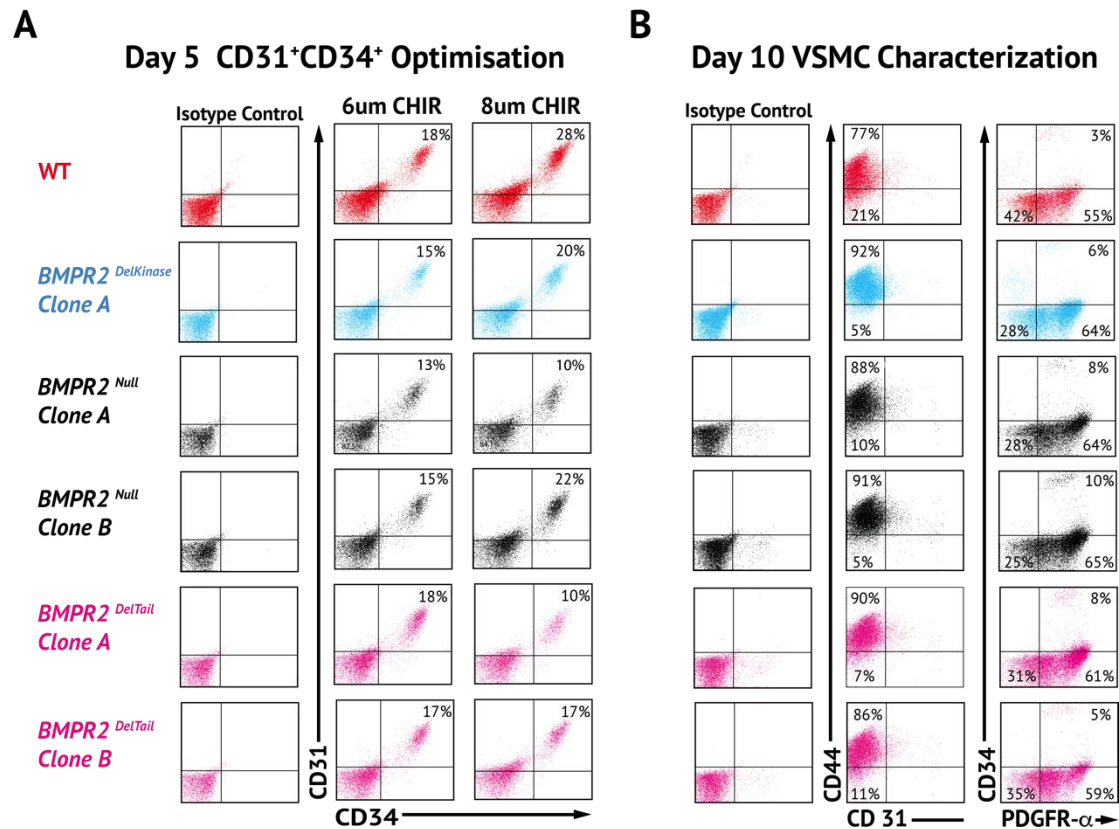


Figure 5.3 Flow cytometry profile of vascular progenitors expressing CD31⁺CD34⁺ (A) The population of CD31⁺CD34⁺ vascular progenitors were isolated at day 5 of differentiation by flow cytometry sorting. The optimum CHIR99021 concentration was determined via flow cytometric analysis. The CHIR99021 concentration at 8μm yielded the greatest CD31⁺CD34⁺ population and this condition was used for subsequent differentiations. **(B)** Day 10 SMCs were characterized with SMC markers CD44⁺ and PDGFR-α⁺. At day 10, post isolation of CD31⁺CD34⁺ by flow cytometry sorting, SMCs cultured in SMC differentiation media lose their CD31⁺CD34⁺ expression and show positive expression of SMC markers PDGFR-α⁺ and CD44⁺.

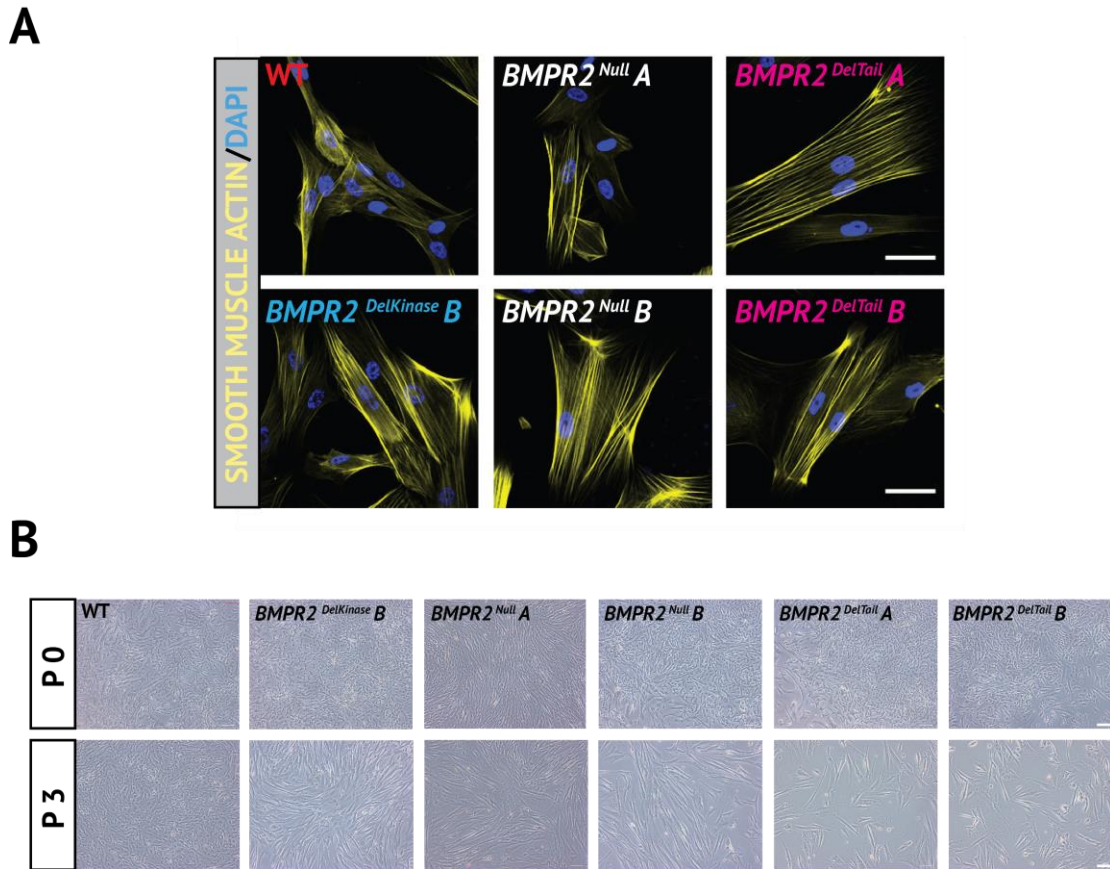


Figure 5.4 Microscopic images of SMCs differentiated from BMPR2 mutant and wild type PSCs. (A) Single confocal images show SMCs immunostained with smooth muscle actin (SMA) antibody. SMA staining reveals distinct differences in stress fibre organizations between BMPR2 mutant and wild type SMCs. Nuclei were counterstained with DAPI. **(B)** Bright field images on the top panel show wild type and BMPR2 mutant SMCs at passage 0 post flow cytometry sorting. Bottom panel images show SMCs after 3 passages, cultured in SMC growth media. Passage 3 BMPR2 mutant SMCs were observed to be morphologically distinct to wild type SMCs. Furthermore, both clones of *BMPR2^{DelTail}* SMCs appear sparse in culture, showing a difference in growth/death capacity compared to the other BMPR2 mutant genotypes and wild type. Notably, cytoskeletal defects were observed in all BMPR2 mutant SMCs genotypes. Scale bars = 50um.

5.3 Characterization of *BMPR2* mutant endothelial cells (ECs)

The intima, the layer of ECs lining the blood vessels, is abnormally thickened in PAH patients, in part due to EC dysfunction (Teichert-Kuliszewska et al. 2006, Valdimarsdottir et al. 2002). ECs from PAH patients carrying a mutation in *BMPR2* have increased susceptibility to apoptosis, which impairs proper vessel wall function. Subsequently, as PAH progresses, alterations to the intima provide an environment in which after initial EC apoptosis, proliferative ECs take over (Taraseviciene-Stewart et al. 2001). As a result, vessel wall thickness is increased leading to arterial constriction (Taraseviciene-Stewart et al. 2001).

To investigate *BMPR2* deficiency in ECs, we subjected our *BMPR2* mutant PSCs to an EC differentiation protocol adapted from the cardiac differentiation protocol. This protocol efficiently generates mesoderm progenitors (**Fig. 5.5 & 5.6 A**) and we sought to manipulate culture conditions to favour EC specification (**Fig. 5.6 A**). In particular, the cytokines vascular endothelial growth factor (VEGF) and stem cell factor (SCF) have been shown to promote the specification and expansion of ECs (Costa et al. 2013, Nourse et al. 2009). Therefore, to drive mesoderm progenitors to an endothelial progenitor stage, VEGF and SCF were added at day 2 to our standard cardiomyocyte monolayer differentiation protocol (**Chapter 4, Fig. 4.7 & Fig. 5.6 A**). In this protocol production of endothelial progenitors marked by CD31⁺CD34⁺ peaked at day 6 of the differentiation (**Fig. 5.5 & Fig. 5.6 B**). Under the same differentiation conditions, *BMPR2* mutants consistently generated a significantly larger CD31⁺CD34⁺ population than wild type PSCs ($p < 0.05$,

n=20, for all *BMPR2* mutant genotypes) (**Fig. 5.6 C**). This occurrence of increased CD34⁺ expressing cells was also observed in *BMPR2* mutant cardiomyocyte differentiations (**Chapter 4, Fig. 4.11**), suggesting that *BMPR2* modulates, in some manner, the specification of CD34⁺ endothelial progenitor cells.

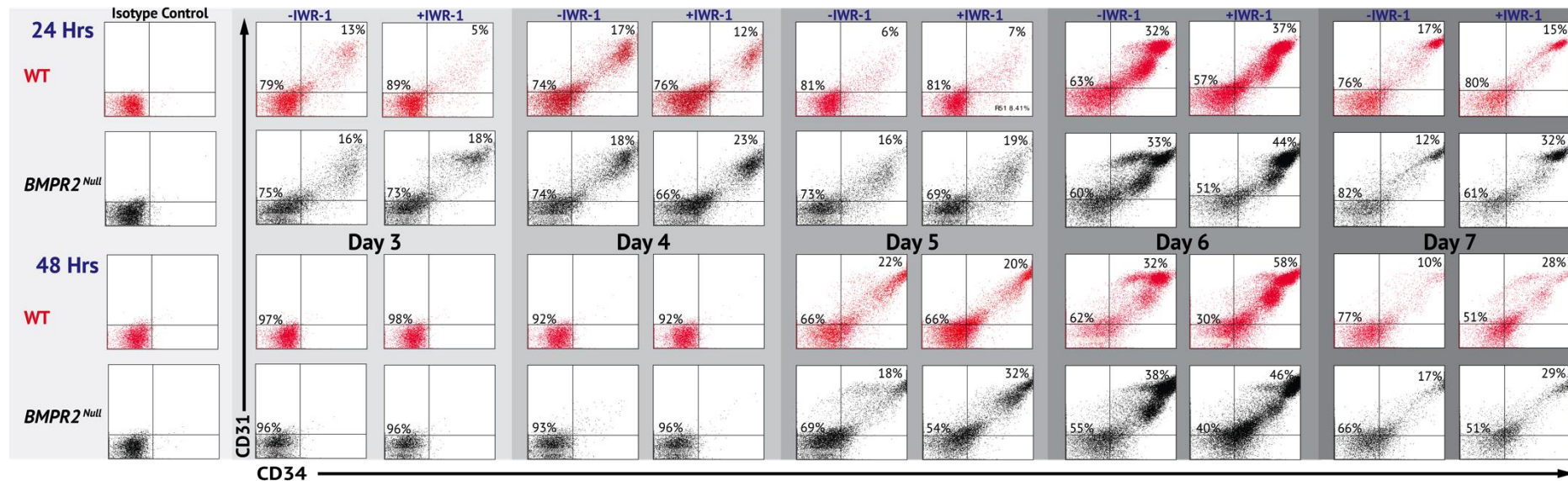


Figure 5.5. Flow cytometric analysis of EC differentiation to optimise differentiation conditions. The CD31⁺CD34⁺ population in EC differentiations peaked at day 6. There were no significant differences between addition of the factors after 24hrs or 48hrs. However, we chose to include IWR-1 as it improved the production of CD31⁺CD34⁺. After day 7 there is a mark decrease in CD31⁺CD34⁺ expression in cells.

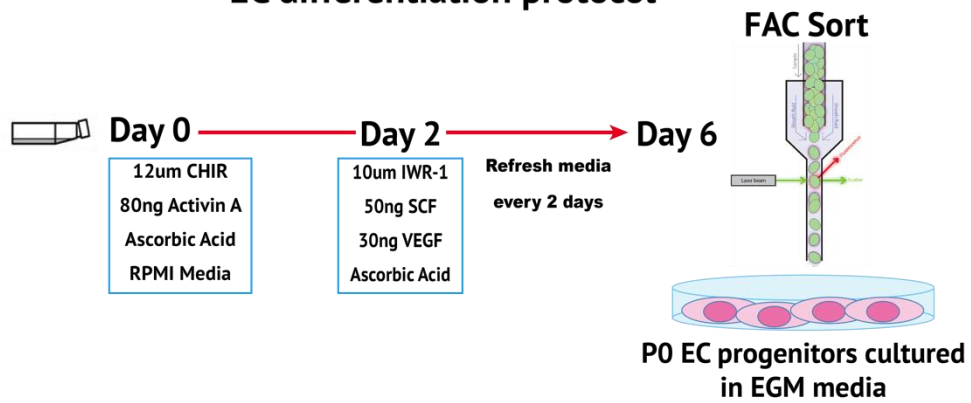
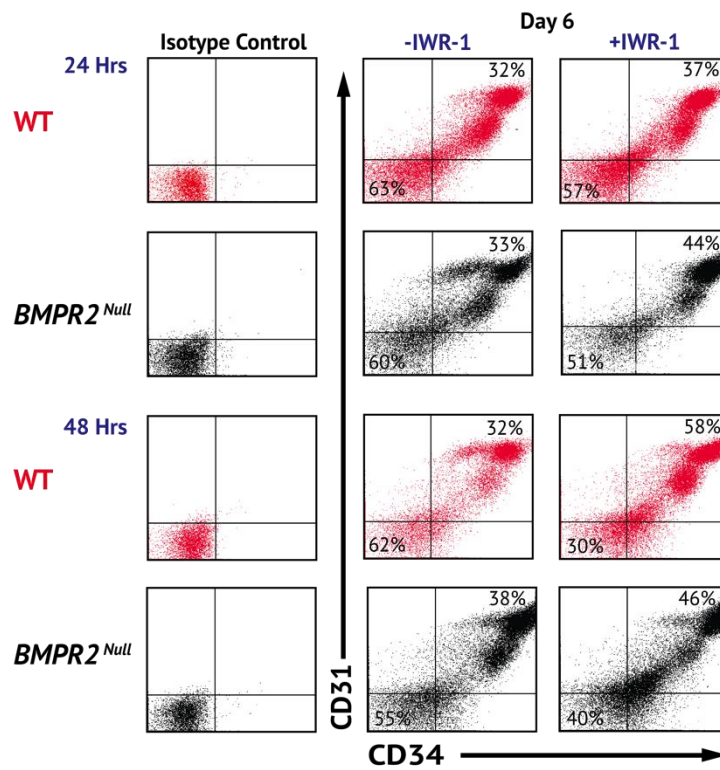
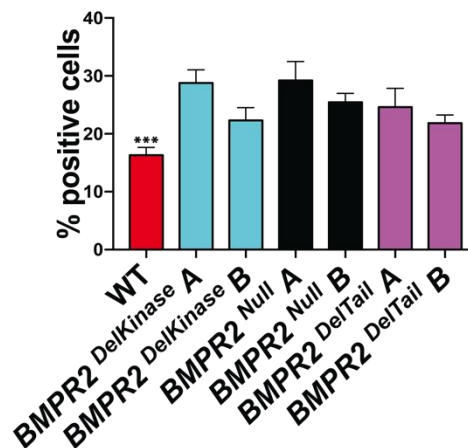
A**EC differentiation protocol****B****Optimisation of CD31⁺CD34⁺ population****C****Day 6 FAC Sorted CD31⁺CD34⁺ population**

Figure 5.6 Differentiation conditions were optimised to yield the highest CD31⁺CD34⁺ population in order to obtain greater quantities of ECs. (A) Schematic illustrating EC differentiation protocol adapted from our cardiomyocyte protocol to generate ECs. On day 6, flow cytometry sorting isolates CD31⁺CD34⁺ population that marks for the EC progenitors. **(B)** Flow cytometric analysis show that EC differentiation at day 6 and addition of IWR-1 generated the greatest quantity of endothelial progenitors marked by CD31⁺CD34⁺ expression. Cytokines SCF and VEGF are added following +/- IWR-1 in RPMI-B27 based media after 24hr and 48 hrs. The condition that generated the largest population of CD31⁺CD34⁺ was applied to all subsequent differentiations. After day 6 of differentiation, CD31⁺CD34⁺ population reduces drastically (**Fig. 5.5**). **(C)** Histogram depicts the percentages of day 6 CD31⁺CD34⁺ expressing cells in wild type and BMPR2 mutant cultures. Wild type differentiations have significantly lower CD31⁺CD34⁺ population compared to BMPR2 mutant cultures (WT *** $p < 0.0002$ using one-way ANOVA, BMPR2^{DelKinaseA} $p < 0.001$, BMPR2^{DelKinaseB} $p < 0.03$, BMPR2^{NullA} $p < 0.0002$, BMPR2^{NullB} $p < 0.002$, BMPR2^{DelTailA} $p < 0.02$, BMPR2^{DelTailB} $p < 0.05$ using Dunnett's multiple comparison test) ($n=20$; $\pm$ S.E.M.).

5.4 Characterization of *BMPR2* mutant ECs

ECs show a degree of plasticity and are capable of responding to environmental cues. For example, ECs have been shown to sprout from transplant into host tissue connecting the transplanted tissue to the host circulatory system (Noden 1989, Poole and Coffin 1989). To study ECs, we isolated day 6 CD31⁺CD34⁺ endothelial progenitors by FACS and cultured these cells in HUVEC media (Lonza, EGM™-2 BulletKit™) to further differentiate progenitors into ECs (**Fig. 5.6 A**). Immunohistochemistry of ECs show wild type and *BMPR2* mutant ECs express the EC surface markers CD31 and von willebrand factor (vWF) (**Fig. 5.7**). Furthermore, the ECs produced by our differentiation protocol are functional as demonstrated by the uptake of acetylated low-density lipoprotein (Ac-LDL) and the capacity to form tubes *in vitro* (**Fig. 5.7**). To examine vasculogenesis in *BMPR2* mutant ECs, passage 1 ECs were seeded onto a layer of Geltrex® matrix and left for 24 hours (Arnaoutova and Kleinman 2010). After 24 hours, formation of tubules was quantified. The average tubule length and number of rings formed were consistent in wild type and *BMPR2* mutant EC lines (**Fig. 5.8**). However, the number of junctions (i.e. new sprouts/splits from existing tubules) formed ($p < 0.009$, $n = 3$) and the number of tubules ($p < 0.02$, $n = 3$) were significantly higher in *BMPR2*^{Null} (**Fig. 5.8**). In contrast, *BMPR2*^{DelTail} had a significantly reduced number of tubules ($p < 0.002$, $n = 3$) (**Fig. 5.8**). Phenotypically, *BMPR2*^{DelTail} ECs remain as a monolayer rather than self-organising into tubules (right panel **Fig. 5.7**). These data demonstrate that we have generated ECs that display classical endothelial characteristics (**Fig. 5.7**) and that *BMPR2* signalling has important roles in tubule sprouting *in vitro*.

For all genotypes, ECs could only be enzymatically passaged 3 times, equivalent to 18 days post-sort culture. During the course of enzymatic passaging of EC lines, an observable difference between wild type and *BMPR2* mutant EC cultures emerged. At passage 3 wild type cultures appeared morphologically distinct and consisted of an apparent heterogeneous cell population (**Fig. 5.9**). To analyse this difference, ECs at passage 2 and 3 were assayed by flow cytometry (**Fig. 5.10 A & B**). Flow cytometric analysis of passage 2 wild type and *BMPR2* mutant ECs were positive for EC markers CD31⁺CD34⁺, KDR⁺TIE2⁺, CD44⁺ and negative for PDGFR- α ⁻ (**Fig. 5.10 A**). However, passage 3 ECs analysed by flow cytometry revealed that wild type ECs had progressively less uniform CD31⁺CD34⁺ and KDR⁺TIE2⁺ profiles, while consistent expression of these markers were retained in *BMPR2* mutant ECs (**Fig. 5.10 B**). There was an emergent population expressing the known SMC/mesenchymal stromal cell markers PDGFR- α and CD44 in wild type ECs but not in the *BMPR2* mutant EC cultures (**Fig. 5.10 B**).

The identification of a PDGFR- α ⁺CD44⁺ population in cultured wild type ECs led us to question if *BMPR2* signalling was required for the trans-differentiation of CD34⁺ endothelial precursors towards SMCs (**Fig. 5.10 C**). In order to examine the requirement of BMP signalling for the emergence of the PDGFR- α ⁺CD44⁺ population, isolated wild type and *BMPR2* mutant CD31⁺CD34⁺ endothelial progenitors were placed into three groups: i) ECs cultured in normal EGM-2 media; ii) ECs cultured in EGM-2 supplemented

with BMP4 and ii) ECs cultured in EGM-2 supplemented with DMH-1 (**Fig. 5.11 A**). DMH-1 (dorsomorphin homologue-1) is a specific small molecule inhibitor of BMPR1 that inhibits the cascade of SMAD1/5/8 signalling in the canonical BMP pathway. EGM-2 media was required for this experiment as APEL media (supplemented with 30ng VEGF) was unable to support any other cell types besides ECs (data not shown). Flow cytometric analysis of the untreated wild type ECs showed an emergent population of PDGFR- α^+ that was not observed in the *BMPR2* mutant EC cultures (**Fig. 5.11 B**). Furthermore, the addition of BMP4 seemed to drive wild type ECs towards the PDGFR- α^+ cell fate (**Fig. 5.11 C**). The addition of DMH-1 (inhibitor of BMPR1/SMAD signalling pathway) demonstrated that inhibition of the BMP signalling pathway results in a cellular profile resembling the cultured *BMPR2* mutant ECs, i.e. uniform CD31 $^+$ CD34 $^+$ and KDR $^+$ TIE2 $^+$ and no appearance of the PDGFR α^+ CD44 $^+$ population (**Fig. 5.11 D**). These findings are consistent with the morphological changes observed (**Fig. 5.9**) during the culture of wild type and *BMPR2* mutant ECs. The proliferation capacity of wild type and *BMPR2* mutant ECs are examined later in this Chapter (**Section 5.7**).

We next sought to analyse the gene expression of *BMPR2* mutant and wild type ECs. Isolated CD31 $^+$ CD34 $^+$ endothelial progenitors were cultured for 4 days and then extracted for RNA to carry out qPCR analysis (**Fig. 5.12**). We demonstrate that wild type and *BMPR2* mutant ECs express similar levels of specific endothelial markers, KDR (vascular endothelial growth factor receptor 2) and vWF (von-Willebrand factor) (**Fig. 5.12**). Gene expression analysis of passage 1 *BMPR2* mutant and wild type ECs revealed no substantial

differences for gene transcripts of *NOTCH1*, *SMAD1*, *BMP2*, *BMP4*, and *HEY1* (downstream target of NOTCH) and *ACTA2* (expressed by SMCs). Conversely, there was a significant decrease in expression of the gene encoding the transmembrane ligand *EPHRIN-B2* in *BMPR2* mutants compared to wild type ECs (*BMPR2^{DelKinase}* $p < 0.007$, *BMPR2^{Null}* $p < 0.01$ & *BMPR2^{DelTail}* $p < 0.006$, $n=5$). This reduction in *EPHRIN-B2* expression suggests that perturbed *BMPR2* in ECs may alter cellular communication as mediated by the *EPH-B2/EPHRIN-B2* interaction. As in PSCs and cardiomyocytes, *BMPR2^{Null}* ECs have negligible transcription of *BMPR2* ($p < 0.0001$, $n=5$) and moderate reduction in *BMPR2^{DelKinase}* and *BMPR2^{DelTail}* mutants. In terms of the BMP signalling network, *BMPR2^{DelKinase}* ($p < 0.03$, $n=5$) and *BMPR2^{Null}* ($p < 0.04$, $n=5$) had significantly increased *ACVR2A* transcripts, while *BMPR2^{DelTail}* had an insignificant increase. We hypothesize that *ACVR2A* may compensate for the *BMPR2* mutations affecting the BMP canonical pathway (**Fig. 5.12**).

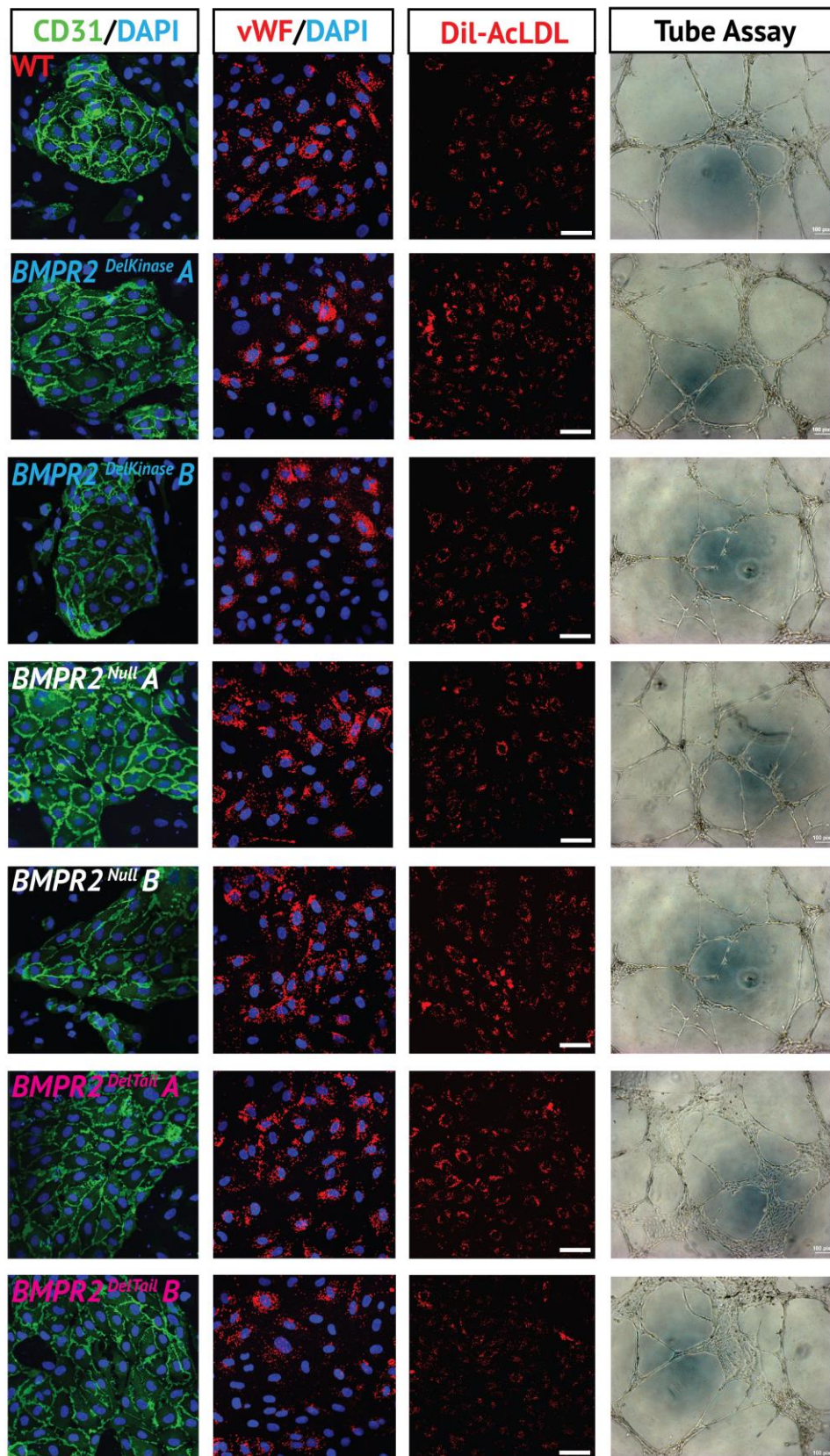


Figure 5.7 Analysis of ECs isolated from wild type and BMPR2 mutant iPSCs. Confocal images of wild type and BMPR2 mutant ECs (top to bottom) expressing CD31, vWF, up-take of Dil-AcLDL and brightfield images of tube formation assay. From first to second columns, ECs are positive for CD31 and vWF. Nuclei were counterstained with Dapi. ECs uptake Dil-AcLDL (third column) and form micro-tubes (scale bars = 100 μ m) when cultured on Geltrex® (forth column). Scale bars = 50 μ m.

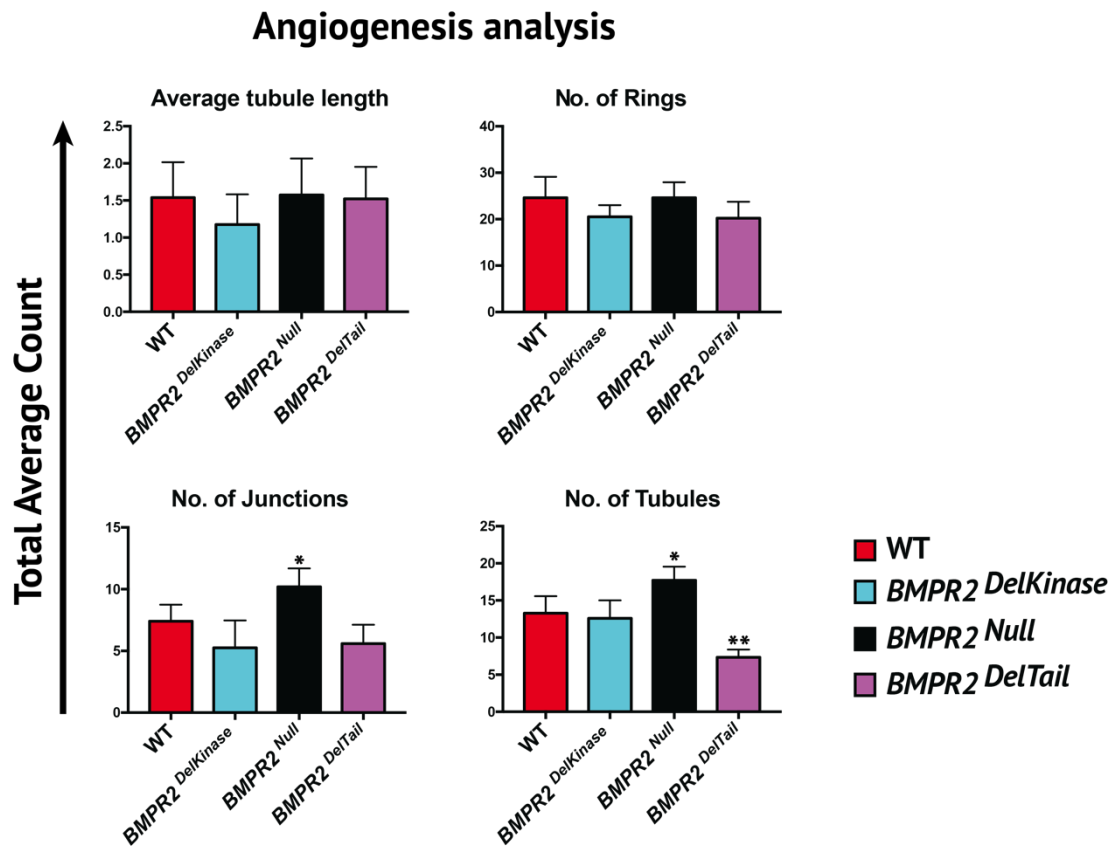


Figure 5.8 Tubule formation is impaired when BMPR2 signalling is disrupted. Histograms depict angiogenesis analyses of two clones combined from each BMPR2 mutant genotype and wild type (i.e. quantification of tube assays presented in the fourth column of **Fig. 5.7**). A uniform number of ECs were seeded onto Geltrex® matrix and scored after 24 hours. The average tubule length and number of rings were consistent in all cell lines. The number of junctions formed and the number of tubules was significantly higher in BMPR2^{Null} (* $p < 0.009$ & * $p < 0.02$ respectively, relative to wild type). However, significantly reduced number of formed tubules was observed in BMPR2^{DelTail} (** $p < 0.002$ relative to wild type) ($n = 3$; $\pm$ S.E.M.). Statistical analysis was conducted using Student's paired *t*-test.

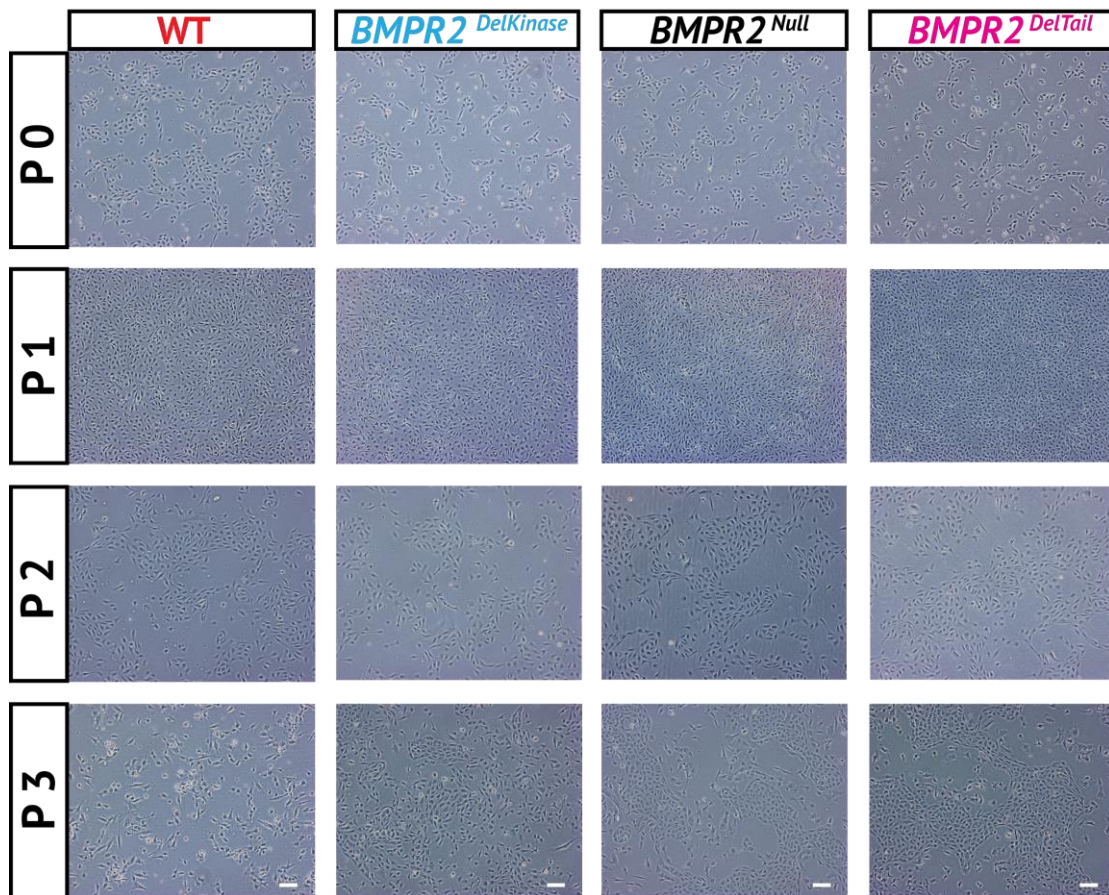
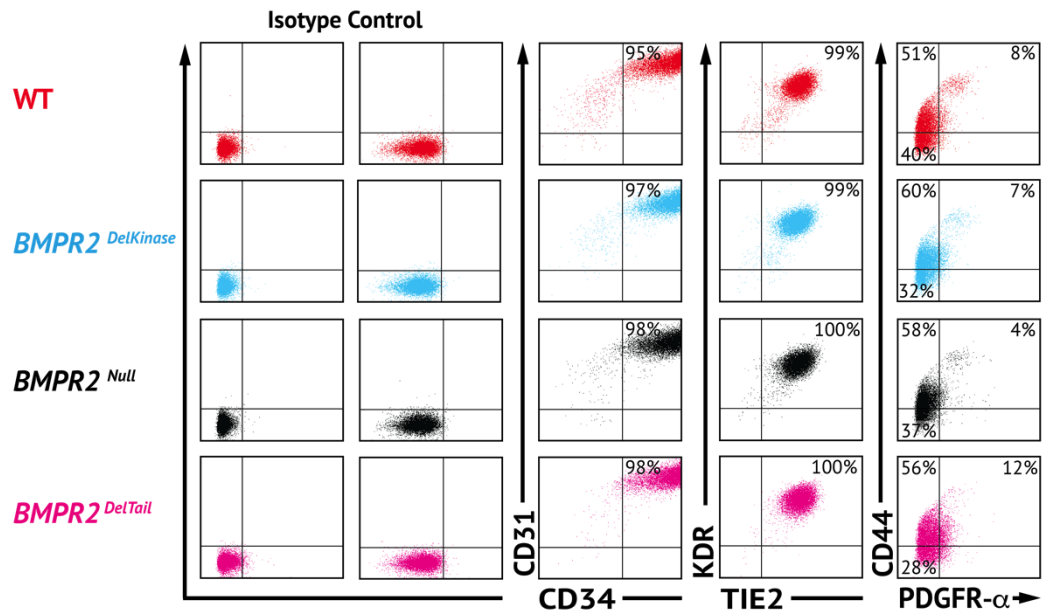


Figure 5.9 Cell morphology changes during culture of wild type ECs. Bright field images of wild type and BMPR2 mutant ECs from passage 0 (post sort) to passage 3 in vitro. ECs enzymatically passaged 3 times equates to 18 days in culture from day 0 of differentiation to isolation of CD31⁺CD34⁺ endothelial progenitors. Passage 3 wild type cells are morphologically distinct to BMPR2 mutant ECs. Passage 3 Wild type ECs also have a prevalence of a different subtype of cells in culture. Scale bars = 100 μ m.

A

Post-sort ECs express EC markers after 2 passages



B

Post-sort ECs after 3 passages

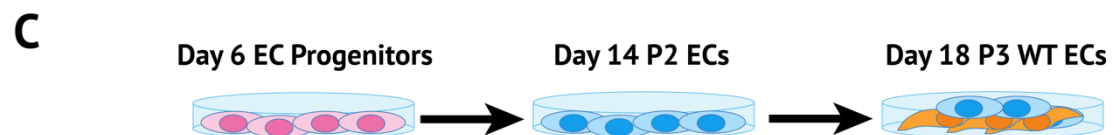
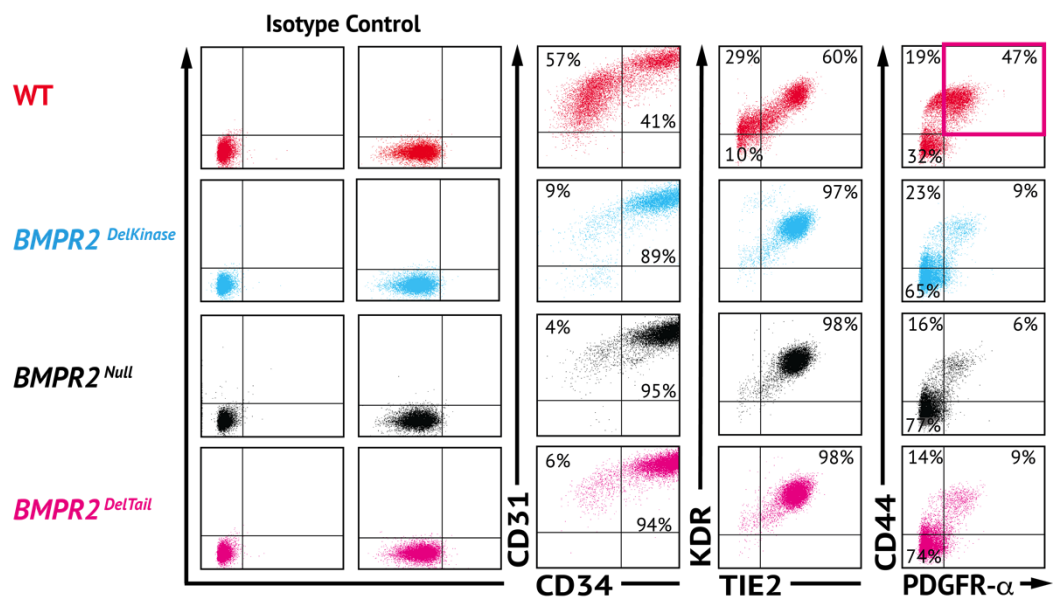


Figure 5.10 BMPR2 mutant ECs develop a different cell surface marker profile during culture. (A) FACS profile analysis of passage 2 wild type and BMPR2 mutant ECs. Left panel shows isotype controls (performed separately). Followed by CD31 and CD34, KDR and TIE2 and CD44 and PDGFR- α FACS plots. Wild type and BMPR2 mutant EC lines retain similar EC phenotype after 2 enzymatic passages in culture post day 6 flow cytometry isolation of CD31⁺CD34⁺ population. **(B)** FACS profile analysis of passage 3 wild type and BMPR2 mutant ECs. Wild type ECs diverge from BMPR2 mutant EC phenotype after 3 passages in culture indicated by loss of CD31⁺CD34⁺, KDR⁺TIE2⁺ and gain in CD44⁺PDGFR- α ⁺ expression, whereas BMPR2 mutants retain their EC phenotype. **(C)** Schematic shows the heterogeneity of cell types observed in wild type cultures after approximately 18 days, equivalent to three rounds of passaging.

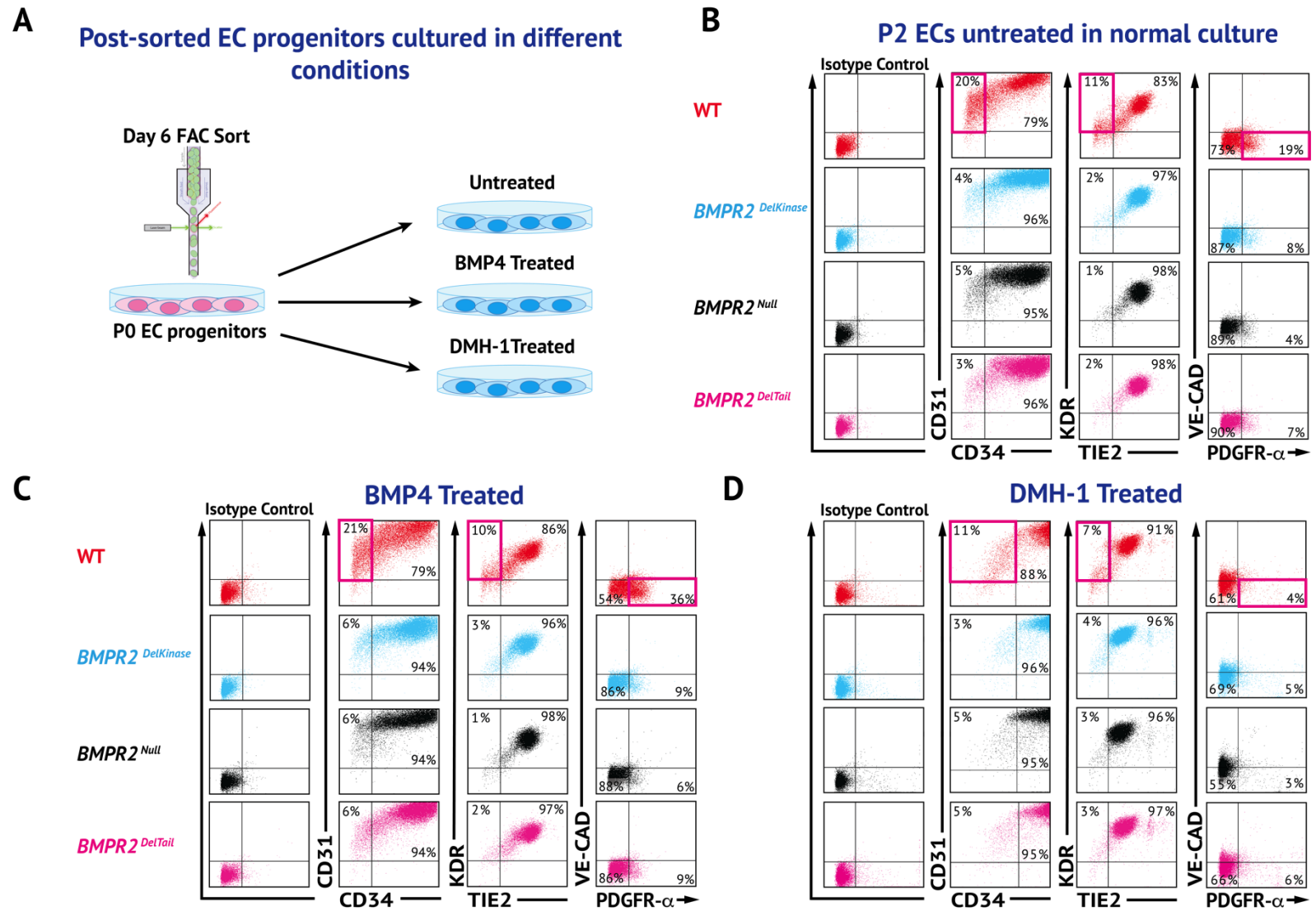


Figure 5.11 Modulation of BMP signalling alters cell marker profile of cultured ECs. (A) Day 6 CD31⁺CD34⁺ BMPR2 mutant and wild type cells were isolated and distributed into 3 groups each. First group was cultured in normal EGM-2 media, while the second group was supplemented with 50ng of BMP4 and the third group was supplemented with 2 μ m DMH-1. **(B)** FACS analysis of passage 2 wild type and BMPR2 mutant ECs cultured in EGM-2 media. Left panel shows isotype controls. Followed by CD31 and CD34⁺, KDR and TIE2 and VE-CADHERIN and PDGFR- α . At passage 2, in wild type ECs a population of cells lost CD31, CD34, KDR, TIE2 expression and a PDGFR- α ⁺ population emerged (highlighted in pink). **(C)** FACS profile analysis of passage 2 wild type and BMPR2 mutant ECs cultured in EGM-2 media supplemented with 50ng of BMP4. Left panel shows isotype controls. Followed by CD31 and CD34, KDR and TIE2, and VE-CADHERIN and PDGFR- α . Wild type ECs show a reduction of CD31⁺CD34⁺, KDR⁺TIE2⁺ and an increased population of PDGFR- α ⁺ compared to the BMPR2 mutant ECs, the untreated and the DMH-1 treated groups (highlighted in pink). **(D)** FACS profile analysis of passage 2 wild type and BMPR2 mutant ECs cultured in EGM-2 media supplemented with 2 μ m DMH-1. Left panel shows isotype controls. Followed by CD31 and CD34, KDR and TIE2, and VE-CADHERIN and PDGFR- α . Wild type ECs retain similar EC phenotype to BMPR2 mutant ECs, with uniform expression levels of CD31⁺CD34⁺, KDR⁺TIE2⁺ and negligible expression of PDGFR- α ⁻ (highlighted in pink). The numbers represent percentage of total cells in each quadrant.

P1 EC qPCR Profile

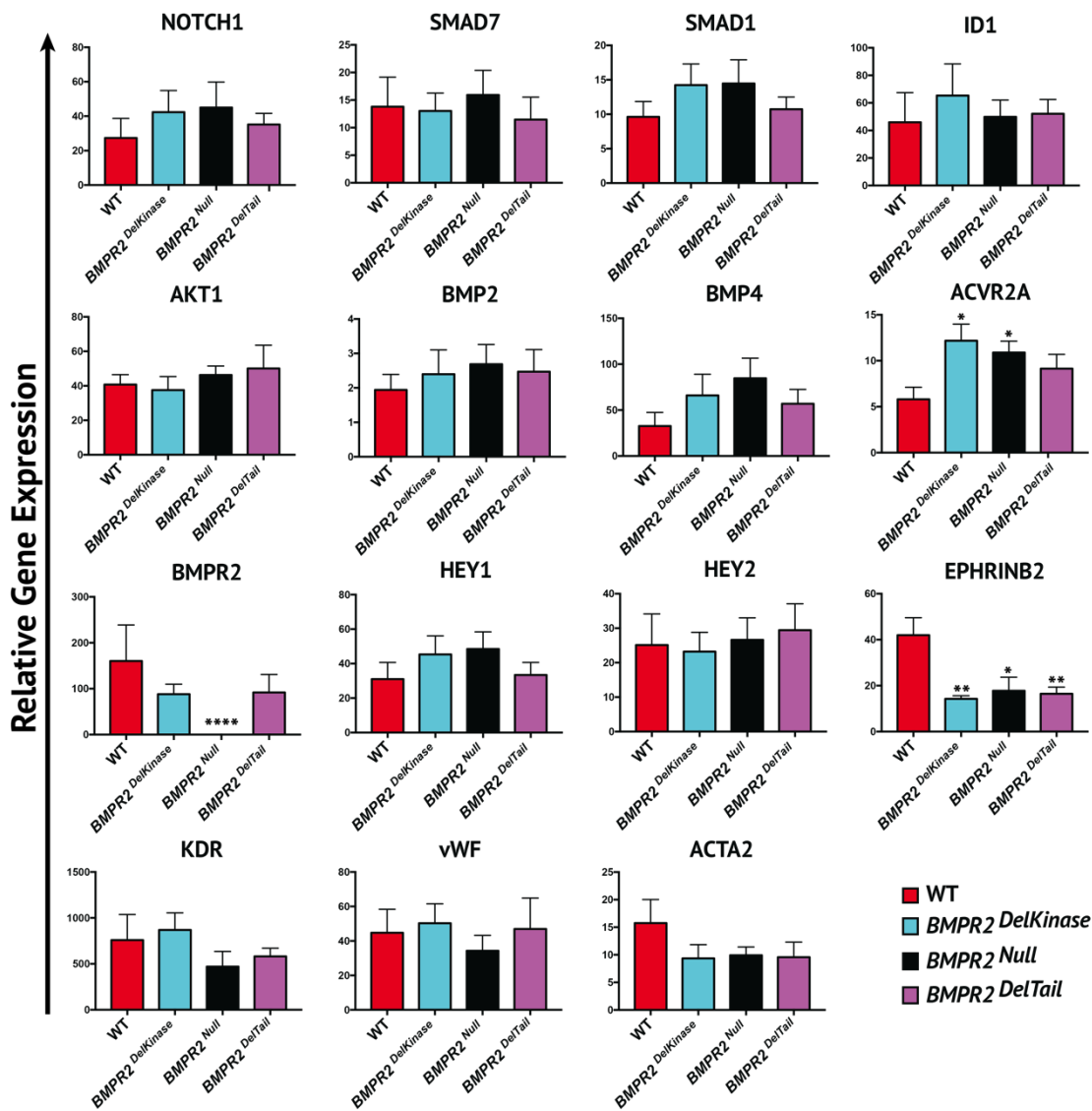


Figure 5.12 BMPR2 is required for EPHRIN-B2 expression and ACVR2A is up regulated in BMPR2 mutants. Gene expression quantified from Clones A and B of each mutant line was combined for this analysis. Wild type and BMPR2 mutant ECs show consistent expression of EC markers KDR and vWF. No statistical differences were observed in gene transcript levels of NOTCH1, SMAD7, SMAD1, ID1, AKT1, BMP2, BMP4, HEY1, HEY2 and ACTA2. In contrast, there was significant increase of ACVR2A transcripts in *BMPR2^{DelKinase}* and *BMPR2^{Null}* (**p*<0.03 & **p*<0.04, respectively using unpaired t-test) and significant decrease in EPHRIN-B2 gene transcript levels in all BMPR2 mutant genotypes (*BMPR2^{DelKinase}* ***p*<0.007, *BMPR2^{Null}* **p*<0.01 & *BMPR2^{DelTail}* ***p*<0.006, Tukey's multiple comparison test). Furthermore, negligible transcripts of BMPR2 in *BMPR2^{Null}* (*****p*<0.0001, unpaired t-test) and modest decrease in *BMPR2^{DelKinase}* and *BMPR2^{DelTail}* were observed (*n*=5; ±S.E.M.).

5.5 Functional studies of *BMPR2* mutant ECs (scratch-migration assay)

The scratch-migration assay is a simple functional assay that is often used for *in vitro* cell migration studies (Liang et al. 2007). This assay was established to mimic cell migration during wound healing. To assess the behaviour of the *BMPR2* mutant ECs, we performed a scratch-migration assay. Briefly, passage 1 ECs were seeded in 24 well dishes, allowed to settle for 2hrs before the media was changed to serum free media, (APEL plus 30ng of VEGF) for EC maintenance and at 24 hour the EC monolayer was scratched. Scratches were made with a pipette tip down the middle of each EC culture. Live cell imaging was programmed to image the series of wells at 30 min intervals overnight (**Fig. 5.13 A**). Preliminary scratch experiments revealed that in control media (APEL + 30ng VEGF), *BMPR2^{Null}* ECs (highlighted in pink) covered scratches completely after 24hrs while wild type cells did not (**Fig. 5.13 B**). Subsequently, we were able to demonstrate that inhibiting BMPR1/SMAD signalling with DMH-1 resulted in wild type ECs covering scratches completely. Thus, inhibition of canonical BMP signalling in wild type ECs leads to a *BMPR2^{Null}* like migration response over the scratched area (highlighted in pink, **Fig. 5.13 C**). Importantly, wild type ECs in control media did not cover the scratch completely after 24hrs. Further, this result was recapitulated utilising automated confocal microscopy at 30min intervals. Wild type ECs in control media repeatedly failed to close scratches after 210mins, whereas *BMPR2* mutant ECs covered the scratched area completely (**Fig. 5.14**). The percentage of the area versus time that the ECs took to cover the scratches was quantified (**Fig. 5.15 A**). This analysis demonstrated that wild type ECs have an average of <50% scratch coverage compared to *BMPR2*

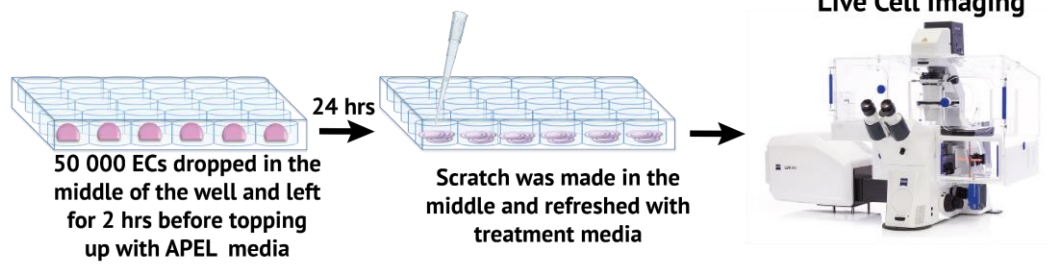
mutant ECs that covered an average of 70-80% of the scratch area after 8hrs (**Fig. 5.15 A**). Furthermore, *BMPR2^{DelKinase}* ($p < 0.02$, $n = 5$) and *BMPR2^{DelTail}* ($p < 0.05$, $n = 5$) have significantly increased scratch coverage rates over wild type ECs (**Fig. 5.15 A**).

In order to validate our genetic findings implicating *BMPR2* as a determinant of cellular migration rate we utilised the BMP antagonist DMH-1 to ascertain if canonical BMP signalling inhibited the rate of cell migration. Wild type ECs show increased scratch coverage rates similarly to *BMPR2* mutant ECs after DMH-1 treatment (**Fig. 5.15 A**). In contrast, addition of NOGGIN, an antagonist that inhibits BMP ligand binding to all BMP receptors, decreased the migration rates in all of the cell lines (**Fig. 5.15 A**). Furthermore, NOGGIN treated ECs were disorientated and tended to migrate in a disorderly manner, however, this requires further quantification (**Fig. 5.15 A**). Subsequently, we wanted to test if addition of TGF- β 1 ligand that binds to the TGF- β 1/2 receptors to transduce SMAD 2/3 pathway had any effect on *BMPR2* mutant ECs. Results show significant increase in scratch coverage in TGF- β 1 treated wild type ECs compared to *BMPR2^{Null}* ECs ($p < 0.03$, $n = 5$) but there were no statistical differences between *BMPR2^{DelKinase}* and *BMPR2^{DelTail}* EC lines. Overall, treatment with TGF- β 1+ PI3K inhibitor (i.e. inhibitor of AKT/mTOR pathway), PI3K inhibitor and SB431542 (TGF- β 1 inhibitor) did not cause any significant changes between wild type and *BMPR2* mutant ECs.

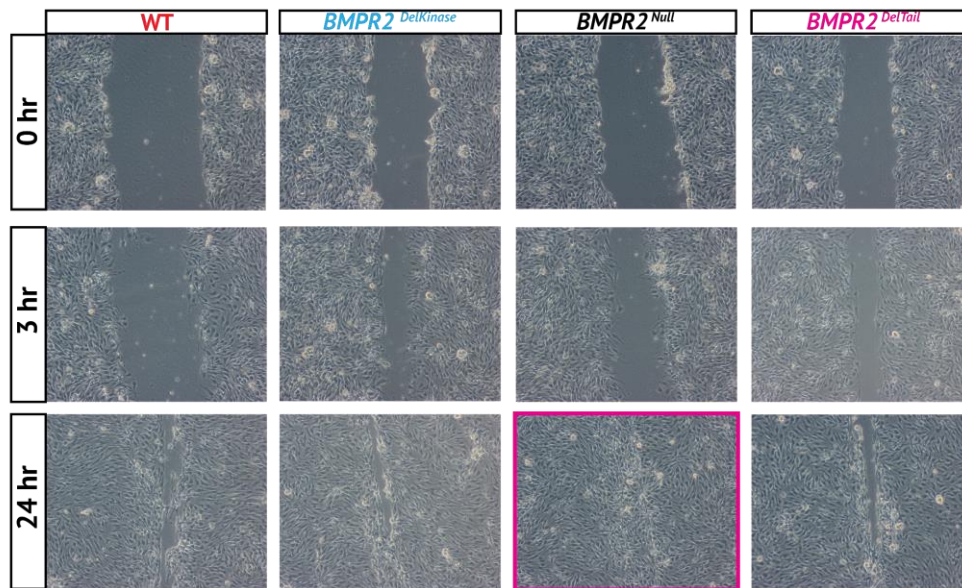
To assess the EC migration rates, the slopes of the percentage area covered versus time graphs were compared in histograms (**Fig. 5.15 B**). In control media, wild type ECs displayed a slower migration rate than *BMPR2* mutant

ECs (**Fig. 5.15 B**). The addition of DMH-1 to wild type cells, however, increased this migration rate to that of *BMPR2* mutant ECs. In contrast, addition of NOGGIN decreased the migration rates in all cell lines ($p < 0.02$ NOGGIN vs. DMH-1, $p < 0.04$ NOGGIN vs. BMP4) (**Fig. 5.15 B**). Induction with TGF- β 1, TGF β 1+ PI3K inhibitor, PI3K inhibitor and SB431542 (TGF- β 1 inhibitor) did not alter the relative migration rates between wild type and *BMPR2* mutant ECs (**Fig. 5.15 B**). Of note, migration of ECs in order to cover the scratches occurred over a short period of time i.e. < 210 mins, hence proliferation of ECs were ruled out. Furthermore, detailed image analyses of the scratch assays determined that cells were migrating and not proliferating.

A



B



C

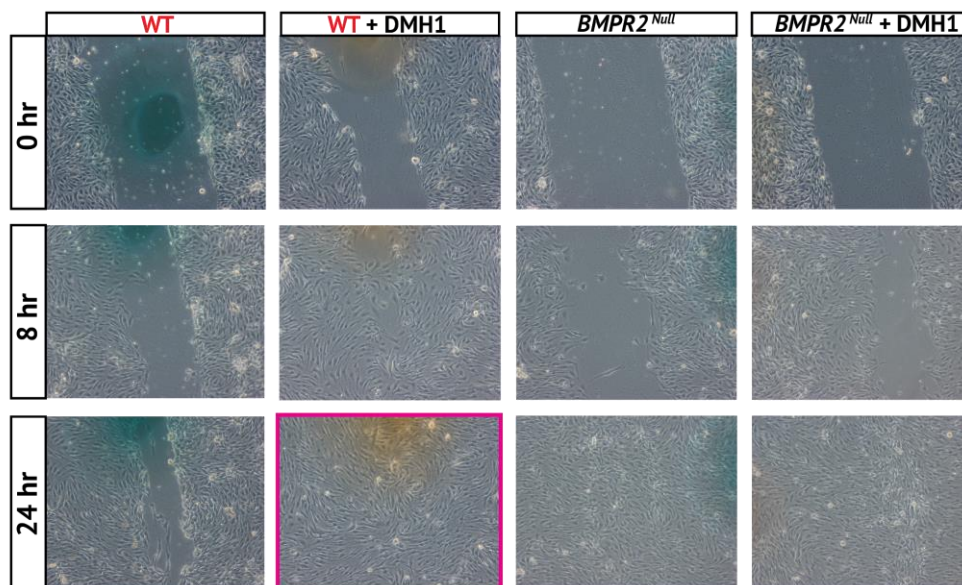


Figure 5.13 Scratch-migration assays of wild type and BMPR2 mutant ECs over 24hr period in control media and treatment with DMH-1. (A) Work flow of live cell imaging for EC scratch assays begins with seeding 50000 ECs in 50 μ l of media in the middle of each well and incubating for 2hrs. Once cells settle, wells are topped up with APEL media supplemented with 30ng of VEGF for 24hr serum starvation. The next day, scratches are made with a pipette tip down the middle of the concentrated cell drop and refreshed with treatment media before proceeding to live cell imaging overnight. **(B)** Bright field images showing scratch assays of wild type and BMPR2 mutant ECs in control media (APEL + 30ng VEGF) taken at 0, 3 and 24hrs post scratches. These preliminary results from scratch assays revealed that BMPR2^{Null} (highlighted in pink) covered scratches completely after 24hrs while wild type cells did not. **(C)** Bright field images showing scratch assays of wild type and BMPR2^{Null} treated with DMH-1 in control media taken at 0, 8 and 24hrs post scratch. Addition of DMH-1 to wild type cells invariantly covered scratches, displaying a similar behaviour to BMPR2^{Null} (highlighted in pink).

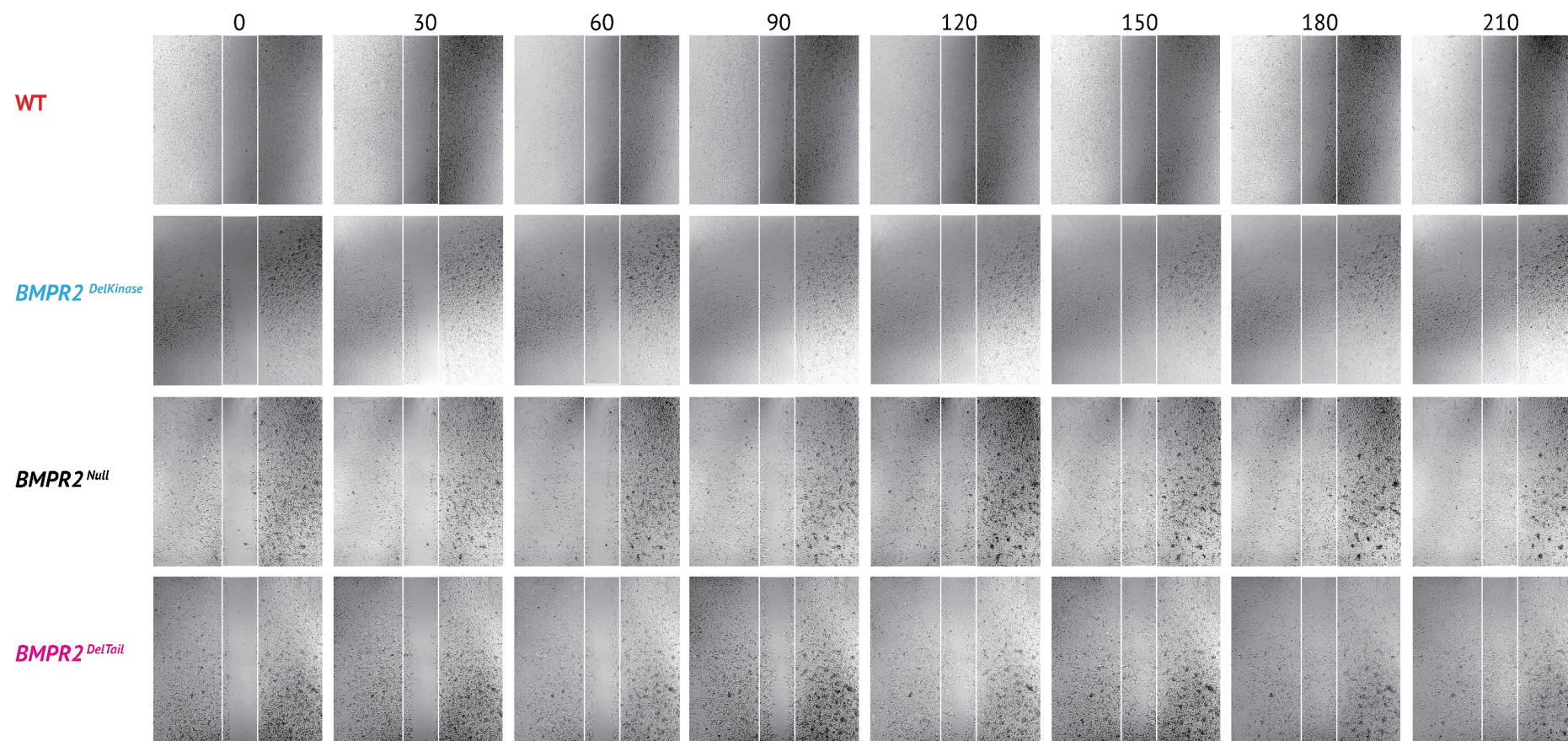


Figure 5.14 Time-lapse images of wild type and BMPR2 mutant ECs scratch assays. (A) Bright field images of passage 1 ECs scratch assays captured periodically at every 30min interval by confocal microscopy. Wild type ECs in control medium after 210mins did not cover the scratch completely. In contrast, all genotypes of BMPR2 mutant EC scratches were completely covered at 210mins.

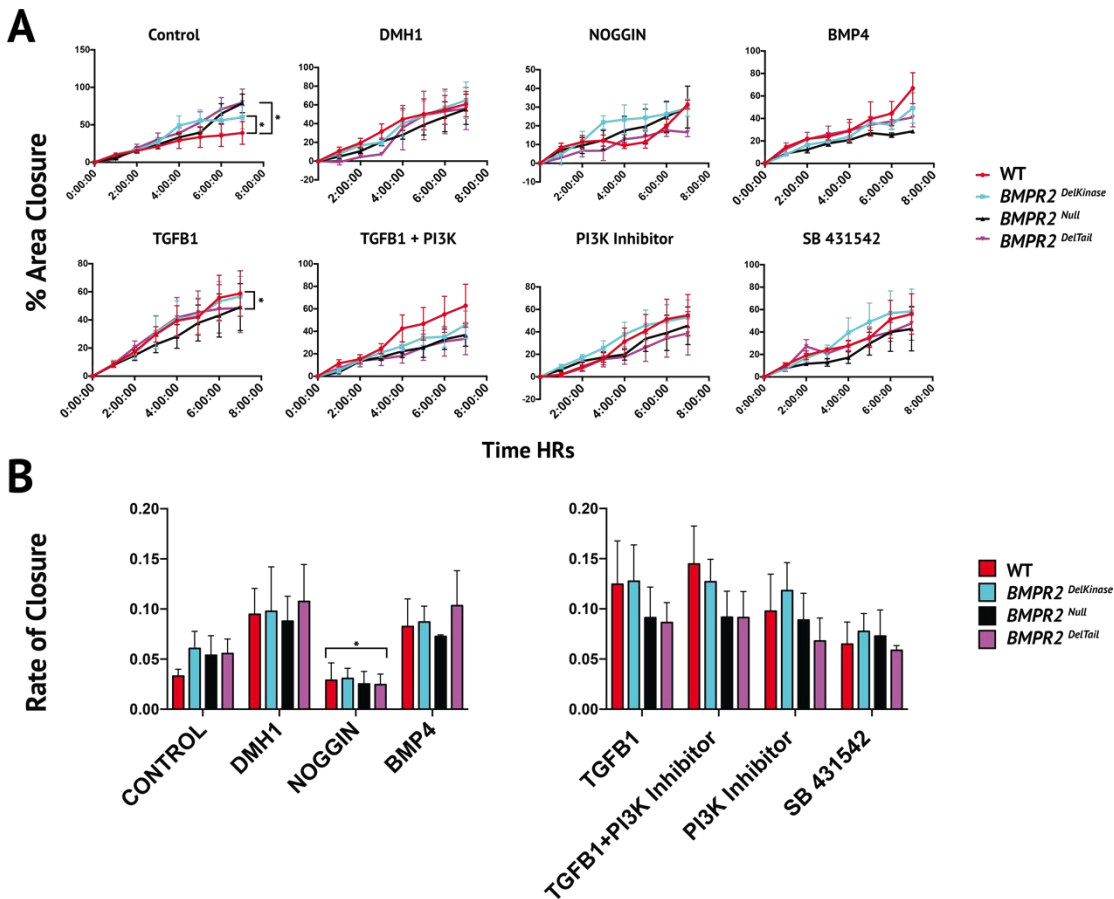


Figure 5.15 Migration rates of wild type and BMPR2 mutant ECs in control media and treated with DMH-1, NOGGIN, BMP4, TGF- β 1, PI3k inhibitor and SB431542. (A) Graphs show percentage of area covered by ECs induced in media with various factors versus time. In control media, wild type ECs has a coverage area average of <50% compared to BMPR2 mutant ECs that has a coverage area of <80%. BMPR2^{DelKinase} (* p <0.02) and BMPR2^{DelTail} (* p <0.05) have significant increases in coverage over wild type ECs. Whilst, DMH-1 treated wild type and BMPR2 mutant ECs migrate and cover scratches at an equal rate. In contrast, NOGGIN treated wild type and BMPR2 mutant ECs become seemingly disorientated and decrease in their coverage rates. In TGF- β 1 treated cultures, there was a significant increase in coverage rate of wild type ECs (* p <0.03) relative to BMPR2^{Null} ECs. Overall no statistical differences were observed in coverage rates of BMP4, TGF- β 1+PI3K inhibitor and SB431542 induced wild type and BMPR2 mutant ECs. **(B)** Histogram diagram depicts the migratory rates at which the ECs took to cover scratches. As shown in control media, wild type cells migrate at a slower rate compared to BMPR2 mutants. The addition of DMH-1 to wild type cells however, increased this rate comparable to BMPR2 mutants. In contrast, addition of NOGGIN decreased the migration rates in all cell lines (* p <0.02 NOGGIN vs DMH-1, * p <0.04 NOGGIN vs BMP4, Tukey's multiple comparison test). Although addition of TGF- β 1 increased rates of scratch closure in wild type compared to BMPR2^{Null} ECs (p <0.03, Dunett's T test), overall migration rates were similar across BMPR2^{DelKinase} and BMPR2^{DelTail} EC lines. Induction with TGF- β 1+ PI3K inhibitor, PI3K inhibitor and SB431542 (TGF- β 1 inhibitor) did not seem to present any significant changes (n =5, $\pm$ S.E.M.).

5.6 Proliferation assay of ECs (MTT assay)

The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay is commonly used to determine growth rates. MTT is a yellow tetrazolium salt that is taken up by cells and cleaved by a mitochondrial dehydrogenase enzyme to produce purple/blue formazan crystals, which are accumulated in living cells. These crystals are dissolved by the addition of DMSO to solubilize the formazan product. This colorimetric assay can then be quantified using an ELISA plate reader (Staton et al. 2009).

Here, the MTT assay is employed for the assessment of the proliferative capacities of wild type and *BMPR2* mutant ECs. Notably, for the following experiments, ECs from the same isolated batch were used. Cell viability of *BMPR2* mutant and wild type ECs were determined by serially diluting known numbers of ECs in a 96 well plate in APEL media, 24hrs later, ECs were incubated with MTT for 8 hours and absorbances assayed to determine cell proliferation. Cell viability is determined by the absorbances at different cell seeding densities. The viability chart shows consistency in wild type and *BMPR2* mutant EC numbers 24hrs post cell seeding, demonstrating that there were no differences in cell viability between wild type and *BMPR2* mutant ECs (**Fig. 5.16 A**). To determine proliferation rates, wild type and *BMPR2* mutant ECs were serially diluted with control media (APEL + 30ng VEGF) in 96 well plates and assayed with MTT reagents 72hrs later. *BMPR2^{DelKinase}* ($p < 0.01$, $n=5$), *BMPR2^{Null}* ($p < 0.02$, $n=5$) and *BMPR2^{DelTail}* ($p < 0.04$, $n=5$) showed a significant increase in proliferation rates compared to wild type ECs (**Fig. 5.16 B**). Subsequently, we examined *BMPR2* mutant ECs response to BMP4,

BMP6, BMP9, ACTIVIN A and bFGF in a range of concentrations. In addition, ECs were treated with DMH-1 (BMPRI/SMADs Inhibitor) and SB431542 (TGF- β 1 inhibitor). A uniform number of wild type and *BMPR2* mutant ECs were seeded into 96 well plates and treated with the range of small molecules and growth factors for 3 days and then assayed using MTT reagents. Heat map analysis of the MTT assays show increased proliferation of *BMPR2* mutant ECs cultured with BMP4, BMP6 and BMP9, and this was particularly pronounced in *BMPR2^{DelTail}* ECs compared to the other *BMPR2* mutant genotypes (**Fig. 5.16 B**). Conversely, when ECs were induced with bFGF alone and bFGF+VEGF, proliferation increased in wild type ECs but to a lesser extent in the *BMPR2* mutant ECs (**Fig. 5.16 B**). When treated with inhibitors DMH-1 and SB431542, no differences in proliferation were observed between the genotypes (**Fig. 5.16 B**). Taken together these data support the notion that *BMPR2* may function to regulate proliferation of ECs.

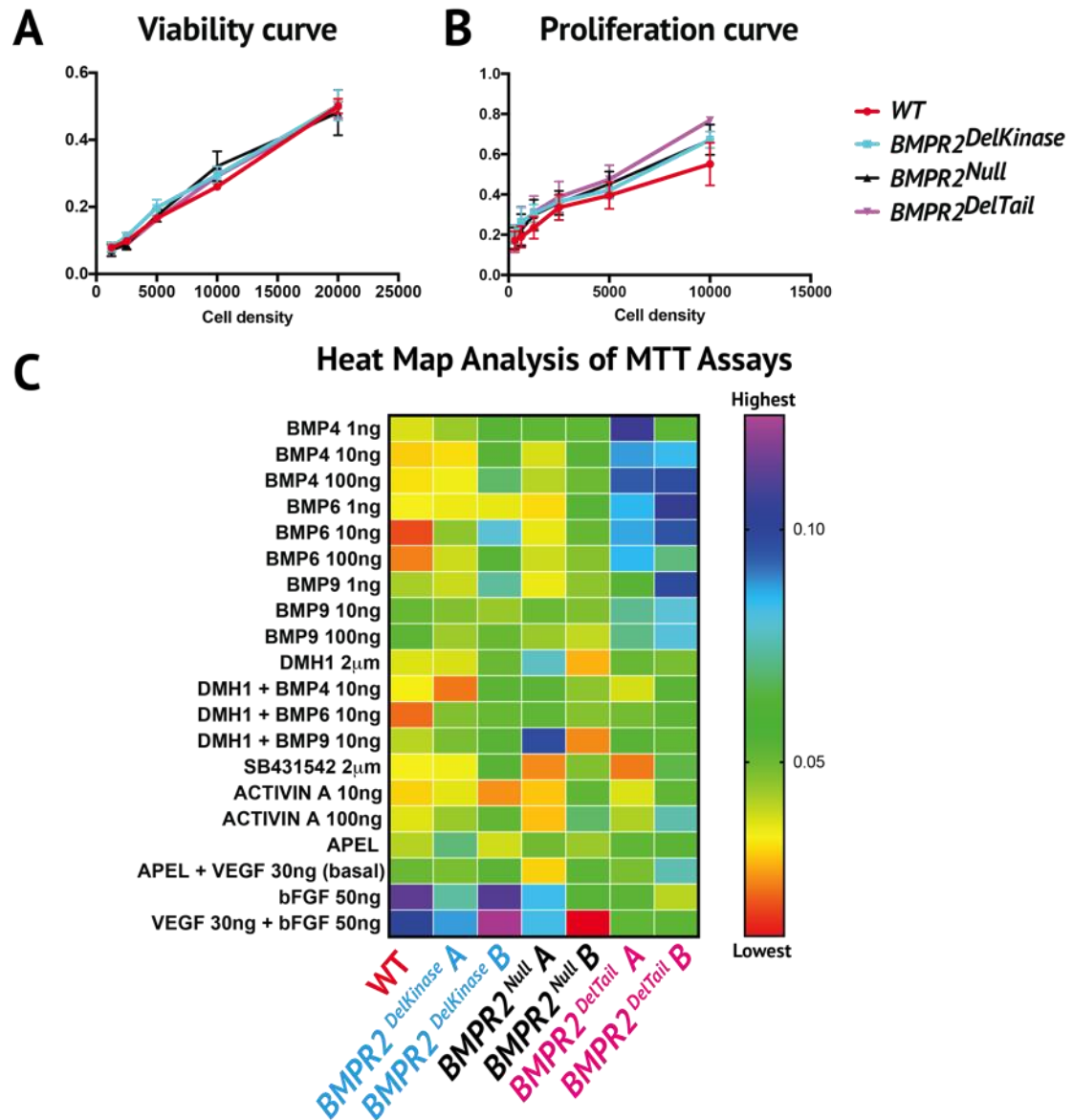


Figure 5.16 BMPR2 mutant ECs have altered proliferation kinetics. (A) Graph shows the viability curve of BMPR2 mutants and wild type ECs. BMPR2 mutant ECs and wild type were seeded into 96 well plates that were serially diluted with APEL media. After 24hrs, plates were assayed with MTT reagents and absorbances were measured using a plate reader. Cell viability is determined by the absorbances plotted against the cell density. Viability curves demonstrate the consistency of wild type and mutant ECs 24hrs post cell seeding. **(B)** Proliferation curve of BMPR2 mutants and wild type ECs were carried out by seeding 10,000 BMPR2 mutant and wild type ECs into 96 well plates and serially diluting. After 3 days in normal media, plates were subjected to MTT assays to determine proliferation curve. *BMPR2^{DelKinase}* (* $p < 0.01$), *BMPR2^{Null}* (* $p < 0.02$) and *BMPR2^{DelTail}* (* $p < 0.04$) have significant increase in proliferation rates compared to wild type ECs. Statistics were carried out using paired t-test. **(C)** Heat map analysis of the average absorbances obtained from MTT assays of BMPR2 mutants and wild type ECs treated with various growth factors. Subsequently, 10,000 ECs were seeded into 96 wells and treated with various growth factors, BMPs 4, 6 & 9, ACTIVIN A and bFGF in different concentrations. In addition, ECs were induced with inhibitor of BMPR1/SMADs, DMH-1 and TGF- β 1 inhibitor, SB431542 for 3 days before being treated with MTT and assayed. Heat map analysis of MTT assays shows increased proliferation in mutant ECs when induced with BMPs 4,6, and 9. This was more pronounced in *BMPR2^{DelTail}* ECs compared to the other BMPR2 mutant genotypes. Conversely, treatment with bFGF increased proliferation in wild type ECs but to a lesser extent in BMPR2 mutant ECs. Overall, BMPR2 mutant ECs treated with BMPs and ACTIVIN A had increased proliferation rates compared to wild type. However, BMPR2 mutant ECs treated with inhibitors DMH-1 and SB431542 showed consistency in proliferation rates to wild type ECs ($p < 0.0001$) ($n=5$; $\pm$ S.E.M.). Statistical analysis were conducted using Student paired t-test and two-way ANOVA.

5.7 Metabolomic profile of wild type and *BMPR2* mutant ECs

The phenotypic changes in motility and proliferation of *BMPR2* mutant ECs may be due to altered energetics. Both cellular motility and proliferation are ATP-dependant. To determine if *BMPR2* mutant ECs have impaired metabolism, we profiled the metabolites in wild type and *BMPR2* mutant EC cultures.

The metabolomics study of wild type and *BMPR2* mutant ECs was performed using liquid chromatogram-mass spectrometry (LC-MS). While the metabolome for all mutant *BMPR2* lines was examined preliminary analysis of showed that among the different *BMPR2* mutant genotypes, *BMPR2^{DelKinase}* (i.e. in-frame deletion resulting in disruption to the kinase domain) was the most distinct from wild type ECs. The metabolome profile of *BMPR2^{DelKinase}* day 4 post-sort ECs had 22 significantly altered metabolites compared to wild type (**Table 1**). The largest changes in *BMPR2^{DelKinase}* ECs are reduced adenosine, D-glucose-6-phosphate, malic acid and increased in NADH, CMP and aspartic acid. Adenosine is formed from cAMP (cyclic adenosine monophosphate) derived from hydrolysis of intracellular ATP and ADP (Adenosine diphosphate) and is particularly important in regulating coronary blood flow (Newby 1984). TCA cycle components important for carbohydrate, fat and protein metabolism, for example malic acid, pyruvic, citric acid and maleic acid (**Table 1**) were reduced in *BMPR2^{DelKinase}*. Metabolites that were also reduced in *BMPR2^{DelKinase}* such as acetoacetate and 2-Ketoglutaric acid indicate impaired ketogenesis, a biochemical process that breaks down fatty acids during low levels of blood glucose (**Table 1**). Furthermore, components of the glycolysis cycle for example, CMP, serine and D-glucose-

6-phosphate were altered. Taken together, metabolome data reveals that *BMPR2* mutation affecting the kinase domain results in changes in fatty acid oxidation, glycolysis and glucose oxidation. These significant changes affect mitochondrial oxidative phosphorylation, which is the core mechanism of cellular respiration.

Table 1. Metabolites that are significantly altered in targeted cell matrix (in order of greatest fold change). Pink shows metabolites that are decreased while blue shows metabolites that are increased in *BMPR2^{DelKinase}* relative to wild type ECs. Statistical analyses were conducted using Student's t-test.

No.	Metabolite	p-value	BH Adjusted p-value	Fold change	Putative/ Absolute*
1	Adenosine	0.0025	0.018667	-1.62	Absolute
2	D-Glucose-6-phosphate	0.00079	0.010539	-1.49	Absolute
3	Malic Acid	0.00002	0.001293	-1.26	Absolute
4	2-Ketoglutaric acid	0.00067	0.010539	-1.22	Absolute
5	Maleic acid	0.00003	0.001293	-1.20	Absolute
6	Pyruvic acid	0.0047	0.023013	-1.03	Absolute
7	Isocitric acid	0.0035	0.01929	-0.92	Absolute
8	Lactic acid	0.0056	0.027538	-0.90	Absolute
9	Citric acid	0.007	0.027538	-0.86	Absolute
10	UDP-Galactose	0.002	0.018667	-0.77	Absolute
11	UDP-Glucose	0.0023	0.018667	-0.77	Absolute
12	Glycero-3-phosphoethanolamine	0.0047	0.023013	-0.71	Putative
13	Acetoacetate	0.00047	0.009838	-0.47	Absolute
14	2-Hydroxyglutaric acid	0.0027	0.018691	-0.27	Absolute
15	Asparagine	0.0047	0.025813	0.21	Absolute
16	Glycylglycine	0.0079	0.029884	0.21	Absolute
17	Glutamic Acid	0.0022	0.018667	0.41	Absolute
18	Alanine	0.0035	0.01929	0.43	Absolute
19	Serine	0.00039	0.009838	0.49	Absolute
20	Aspartic acid	0.00089	0.010539	0.67	Absolute
21	CMP	0.0069	0.027538	0.71	Absolute
22	NADH	0.0033	0.01929	0.76	Absolute

5.8 Expression profiling of day 4 wild type and *BMPR2* mutant ECs

Transcriptomic analysis was performed on RNA isolated from ECs that had been cultured for 4 days in standard endothelial media (EGM2) after enrichment by FACS isolation (refer to **Fig. 5.6**). To identify differentially expressed transcripts, pairwise comparison between wild type and each mutant genotype was performed and transcripts with a minimum twofold change ($p < 0.05$; $n = 3$) were identified. Genes with the highest differential expression patterns are listed in Appendices Table 1 to Table 3. This analysis demonstrated that *BMPR2* transcript levels in our *BMPR2* mutant ECs were down regulated, consistent with our previous findings (refer to **Fig. 5.12**). We hypothesized that one of the key mitochondrial biogenesis genes, peroxisome proliferator-activated receptor- γ (*PPAR γ*), would be dysregulated. *BMPR2* is known to regulate *PPAR γ* -mediated gene transcription in ECs and SMCs (Guignabert et al. 2009, Hansmann et al. 2008). Furthermore, in ECs *BMPR2* activates *PPAR γ* and induces expression of genes that regulate endothelial cell survival and angiogenesis (Ryan et al. 2013). Prominent among these genes is *APELIN receptor* (*APLNR*), which encodes a G-protein coupled receptor that functions in endothelial cell homeostasis. Consistent with these previous findings, both *PPAR γ* and *APLNR* were up regulated in all *BMPR2* mutant genotypes.

We found a total of 38 down regulated genes and 257 up regulated genes that were common to all three *BMPR2* mutant genotypes relative to wild type (**Fig. 5.17 A & B**). Expression of key transcripts for a number of cellular processes consistent with the *BMPR2* mutant phenotypes are disrupted in *BMPR2* mutants. For example, genes encoding proteins involved in microtubule

binding (*JAKMIP2*), cellular adhesion and motility (*ITGA4*), cell fate and developmental (*SRY-BOX*, *HOXA2*, *5HHIP*), vessel development and homeostasis (*ANGPT1*, *COL8A1*), serotonin (5HT) type receptor mediated signalling pathway (*HTR1B*, *HTR2C*), metabolic processes (*ABCA13*, *CHDH*, *CYP4A22*), and ion channel activity (*TRPC4*, *DLK1*, *KCNH8*) had differential expression profiles (**Fig. 5.17 A & B**).

Following on from this, we wanted to correlate the transcriptional and metabolomics data. Therefore, we submitted the list of genes that were differentially regulated with a minimum of twofold change in *BMPR2^{DelKinase}* relative to wild type for gene ontology (GO) analysis (**Fig. 5.18 A & B**). GO analysis showed a number of metabolic processes were dysregulated in *BMPR2^{DelKinase}*, these included DNA metabolic process, RNA metabolic process, primary metabolic process and nitrogen compound metabolic process. These data demonstrate that the metabolic differences between *BMPR2^{DelKinase}* and wild type ECs are underpinned by altered gene regulation. Furthermore, the differentially expressed gene lists were enriched for genes involved in chromatin organization ($p < 8.92E-42$), DNA replication ($p < 3.24E-06$), cellular adhesion ($p < 3.3E-13$), cell communication ($p < 2.1E-05$) and translational ($p < 7.8E-0$) (**Fig. 5.18 A & B**). Thus, deletion of the *BMPR2* kinase domain results in significant alterations to the EC gene expression profile, which mediates the changes in cellular morphology and behaviour we have observed.

Major GO terms for example, chromatin organization and assembly, cell cycle and epigenetic modifiers were listed in GO analysis of genes that were up-

regulated in the wild type ECs compared to *BMPR2^{DelKinase}* ECs. These GO terms suggest that the emergence of PDGFR- α^+ cells observed in wild type EC cultures may be the consequence of wild type ECs trans-differentiating into SMCs. This cell fate change requires extensive chromatin remodelling to facilitate the phenotypic switch. Furthermore, PDGFR- α^+ cells may be more proliferative, hence may underlie the increased expression of cell cycle genes in wild type ECs.

By contrast, *BMPR2^{DelKinase}* ECs are phenotypically efficient wound healers. This is supported by the enrichment of GO terms for increased metabolic processes, cell adhesion, cell-cell adhesion, cell-communication and cell-matrix adhesion. These GO terms are indicative of an EC population with capacity to rapidly respond to signalling cues. Further, these data suggest that *BMPR2* kinase domain is a key mediator of cellular energetics and extracellular matrix interaction.

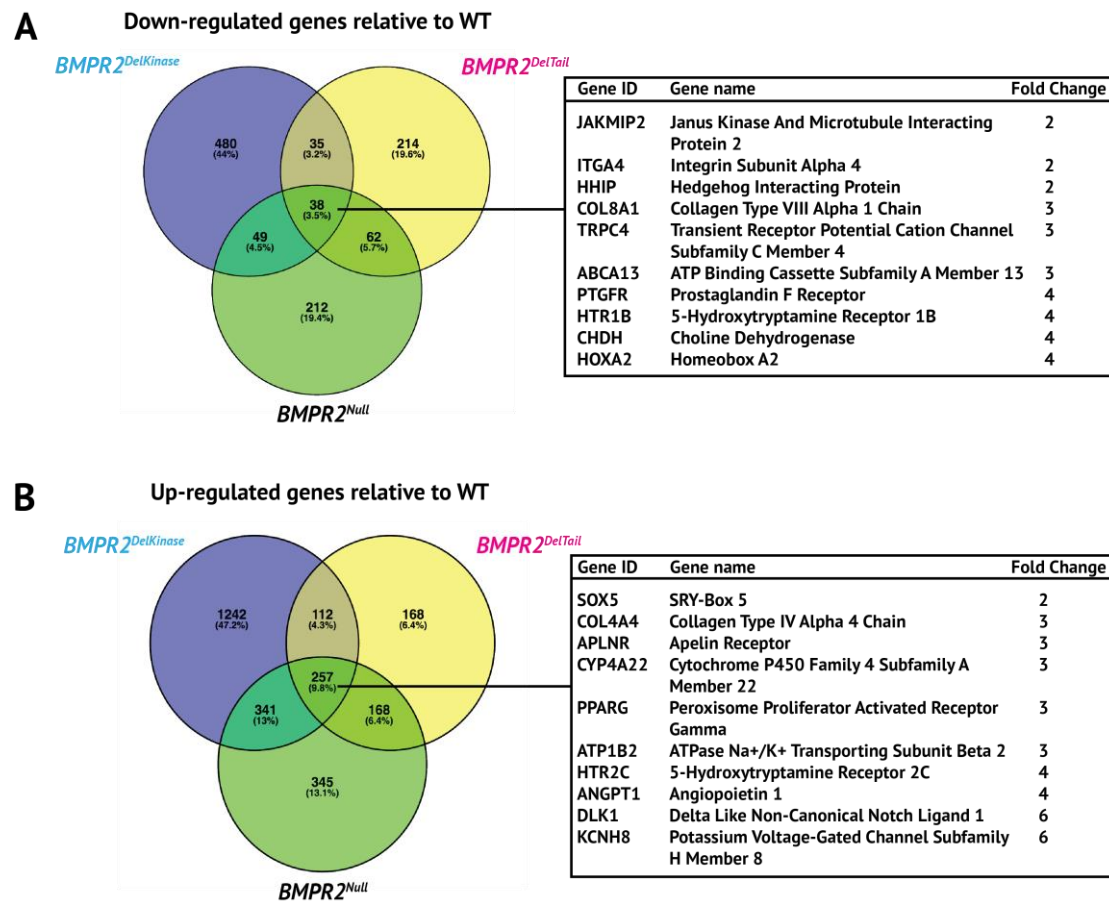
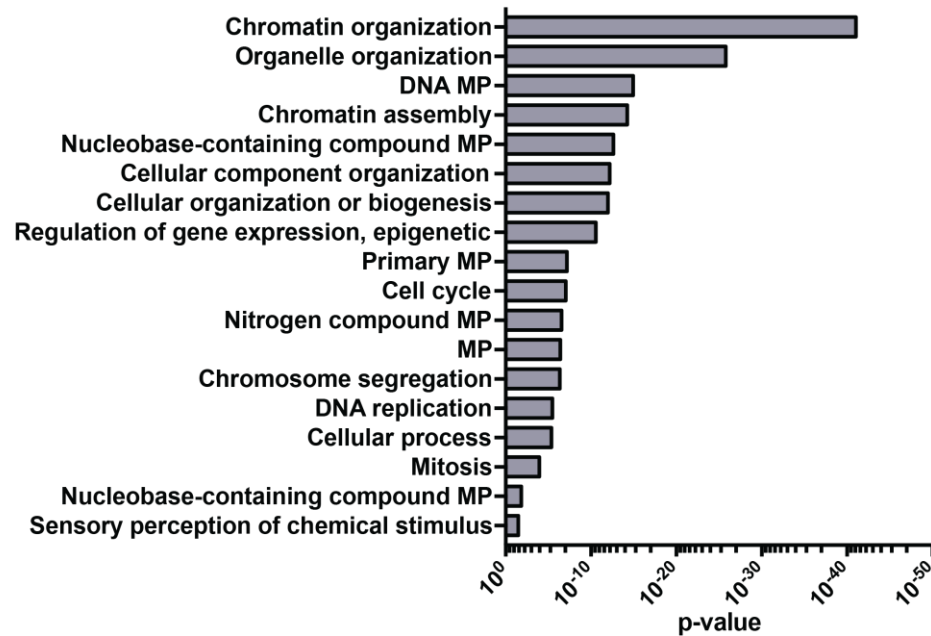


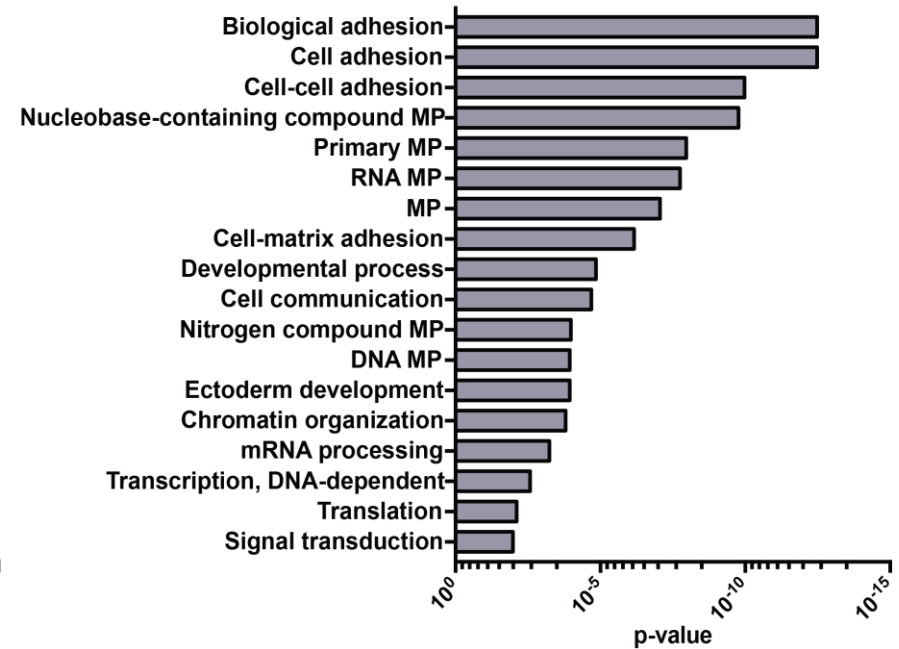
Figure 5.17 Venn diagrams showing the number of dysregulated genes that are shared among the three *BMPR2* mutant EC genotypes relative to wild type ECs. **(A)** The three *BMPR2* mutant genotypes commonly share 38 genes that are significantly down regulated relative to wild type. Examples of 10 of these down-regulated genes are shown on the right with fold change relative to wild type. **(B)** There are 257 up-regulated genes commonly shared among the three genotypes of *BMPR2* mutant ECs relative to wild type ECs. Examples of 10 of these up-regulated genes are shown on the right with fold change relative to wild type.

A

**Down-regulated genes in *BMPR2*^{DelKinase}
relative to WT**

**B**

**Up-regulated genes in *BMPR2*^{DelKinase}
relative to WT**



MP - Metabolic Process

Figure 5.18 Gene ontology (GO) analysis of *BMPR2^{DelKinase}* ECs relative to wild type ECs (A) Bar graphs represent p-values of the GO terms for the down-regulated genes in *BMPR2^{DelKinase}* ECs relative to wild type ECs. Genes that were up regulated in wild type ECs are involved in chromatin remodelling, epigenetic modifiers and cell cycle processes. These GO terms may be indicative of the phenotypic shift that we observe in the wild type ECs and not in the *BMPR2^{DelKinase}* ECs. **(B)** Bar graphs represent p-values of the GO terms for the up-regulated genes in *BMPR2^{DelKinase}* ECs relative to wild type ECs. GO analysis show up regulated genes in *BMPR2^{DelKinase}* are involved in cell-adhesion, cell-matrix adhesion, cell communication and metabolic processes. These GO terms reflect our metabolomics findings and in vitro data suggesting that perturbation to the kinase domain of *BMPR2* disrupts these key processes.

Discussion

The commonly held hypothesis for the aetiology of hereditary PAH is enhanced cell proliferation and apoptosis of vascular cell types due to *BMPR2* mutations leading to PA remodelling. Alternatively, dysregulation of BMP dependent signals may lead to EC and SMC dysfunction. In addition, high expression of *BMPR2* is predominantly localized in lung ECs suggesting that haploinsufficiency in *BMPR2* mutations causes PAH progression (Jeffery et al. 2005). Our aim was to investigate the functional consequences of altered *BMPR2* during mesoderm differentiation and its derivatives, blood cells, SMCs and ECs. These studies were facilitated by a series of PSC lines harbouring a range of *BMPR2* mutations described in Chapter 3. Our findings demonstrate that the differentiation efficiency of *BMPR2* mutant PSCs to blood, SMCs and ECs was not affected. Firstly, we examined the hematopoietic potential of CD34⁺ precursors in our *BMPR2* mutant lines before focusing on the cell types most affected by impaired *BMPR2* during progression of PAH such as SMCs and ECs.

The emergence of hematopoietic and EC cell types from the extra-embryonic tissue in the yolk sac develops from a common bi-potential progenitor known as the hemangioblast (Choi et al. 1998, Sabin 1920). This observation that blood cells develop in close proximity to endothelial cells in the yolk sac is reinforced by a number of studies demonstrating that progenitors of both lineages share the expression of a number of genes including CD34, Flt-3 (FMS-like tyrosine kinase 3) ligand, VEGFR-1 (vascular endothelial growth

factor receptor-1) and C-kit (Garcia-Porrero et al. 1998, Manaia et al. 2000). Since BMP signalling is an important regulator of hematopoiesis, we reasoned that this process might be affected by *BMPR2* disruption. Therefore, using an EB differentiation model, we assessed the hematopoietic capacity of CD34⁺ precursors in our *BMPR2* mutant lines. From our results, we demonstrated that *BMPR2* deficient progenitors gave rise to erythrocytes (CD43⁺) under appropriate culture conditions but were compromised in a certain sub population of blood cells namely the CD34⁺CD43⁺ myeloid/progenitor cells. Several studies have shown the importance of BMP4 in hematopoiesis and perturbation to the BMP signalling pathway highly results in impaired hematopoiesis development (Adelman et al. 2002, Lengerke et al. 2009). Our data also demonstrate that without exogenous BMP4 erythrocytes are not formed during blood differentiation of wild type and *BMPR2*^{Null} cells *in vitro*. Taken together, these data suggest that *BMPR2* deficiency reduces the specification of myeloid/haematopoietic progenitors but not the erythroid population. Although disruption to *BMPR2* did not affect primitive hematopoietic differentiation, it is plausible that perhaps there could be downstream effects in definitive hematopoiesis.

We demonstrated that wild type and *BMPR2* mutant PSCs differentiated into SMCs consistently. However, *in vitro* cultures of *BMPR2* mutant SMCs displayed cytoskeletal defects emphasized by smooth muscle actin stress fibres after 3 enzymatic passages. This was evident in *BMPR2*^{De/Tail} SMCs appearing to have reduced proliferation/viability compared to the other *BMPR2* mutant genotypes, although further quantification by proliferation

studies is required. *BMPR2* is known to interact with the cytoskeleton and human genetic studies of PAH patients have confirmed alterations in cytoskeletal pathways such as the Ras/Rho GTPase (Johnson et al. 2012). The *BMPR2* cytoplasmic tail domain is known to directly bind and regulate several cytoskeletal dynamic components such as LIMK1 (Foletta et al. 2003), c-Src (Wong et al. 2005) and Tctex-1 (Machado et al. 2003). For example, the interaction between LIMK1 and *BMPR2* tail domain during BMP4 stimulation increases phosphorylation of cofilin, which in turn changes the organization of the actin cytoskeleton and sub-cellular localisation of LIMK1 (Foletta et al. 2003). Furthermore, *BMPR2* has been shown to regulate cytoskeletal functions including adhesion and migration (Burton et al. 2010, Gamell et al. 2008).

ECs are major regulators of vascular function and homeostasis, thus EC dysfunction is recognised as the underlying cause of PAH (Budhiraja et al. 2004). ECs also play major roles in angiogenesis and vasculogenesis (Risau 1997). To examine the angiogenic capacity of our *BMPR2* mutant ECs, wild type and *BMPR2* mutant ECs were subjected to angiogenesis assays. Tubules formed from *BMPR2* mutant ECs were structurally distinct from wild type tubules. In particular, *BMPR2^{DelTail}* had thin tubules and cells that persisted as a monolayer. Hence, *BMPR2^{DelTail}* had the lowest number of junctions and tubules formed. In contrast, *BMPR2^{Null}* had the highest number of junctions and tubules. These findings are in line with reports of gene expression profiling experiments in mice and cells cultured from mice with *Bmpr2* mutation specific to the cytoplasmic tail domain showing changes in

metabolic, cytoskeletal, adhesion, and microtubule-related genes (Johnson et al. 2012, Majka et al. 2011, West et al. 2008b). Furthermore, these data suggest that the location of the mutation in *BMPR2*, or if the mutant transcript undergoes nonsense mediated decay, may contribute to disease progression via different mechanisms.

We found that *BMPR2* deficiency contributes to altered expression and signalling during development of vascular progenitors. Our results show that inhibiting *SMAD* signalling with DMH-1 (inhibitor of *BMPR1/SMAD* signals) led to wild type ECs behaving similarly to *BMPR2* mutant ECs, retaining consistent CD31⁺CD34⁺, KDR⁺TIE2⁺ phenotype for several passages. Conversely, addition of BMP4 prompted wild type EC progenitors to adopt a PDGFR- α ⁺CD44⁺ cell phenotype. From our gene expression analysis, we observed modest increase in *BMPR2* mutant ECs of the transcripts for *NOTCH1*, *HEY1*, *SMAD1*, *BMP2* and *BMP4*. In addition, *BMPR2*^{DelKinase} and *BMPR2*^{Null} ECs had a significant increase in *ACVR2A* transcript levels, suggesting that loss of *BMPR2* may be compensated via *ACVR2A*. It has been shown that overexpression of NOTCH1 and its (HESR1) HEY 1 target blocks angiogenic sprouting (Gridley 2007) and knockout of either gene results in cardiovascular defects, with problems in vascular remodelling, arteriovenous specification, septation, and cushion formation (Fischer et al. 2004). However, none of these markers were significantly altered in our system.

A significant reduction in EPHRIN-B2 expression in *BMPR2* mutant ECs was observed. Therefore, EPH-B2/EPHRIN-B2 signalling, which is important in regulating cell migration and proliferation, is impaired in *BMPR2* mutant ECs. Furthermore, this finding correlates with the increased motility of *BMPR2* mutant ECs compared to wild type ECs in control media. Others have reported that down regulation of Eph-Ephrins, jagged-2 and Sema3c may underlie defects seen in PAH patients (Kim et al. 2012, Rhodes et al. 2015). Swift and Weinstein (2009) state that phosphatidylinositol 3-kinase/Akt (PI3K) activation downstream of KDR (VEGFR2) suppresses the MEK/ERK pathway, thereby reducing expression of the arterial marker EPHRIN-B2 and promoting a venous phenotype. It is plausible that *BMPR2* deficiency up-regulates PI3K/AKT pathway suppressing EPHRIN-B2 altering specification of arterial ECs. Animal studies have shown that ephrin-B2 is required for remodelling of arteries and veins (Wang et al. 1998). Furthermore, *BMPR2* mutant ECs treated with exogenous BMP4, BMP6, BMP9 and ACTIVIN A had an overall increase in proliferation over wild type ECs. This increase was especially heightened in *BMPR2^{DelTail}* ECs. BMP4, BMP6 and BMP9 are known to activate the canonical and non-canonical BMP pathway regulating apoptosis, proliferation, migration and tube formation (Orlova et al. 2011, Valdimarsdottir et al. 2002, Wang et al. 2014). ACTIVIN A binds to ACVR2A or ACVR2B to form a complex with type 1 receptors (ACTR1A & ACTR1B) leading to SMAD2/3 activation (Walton et al. 2012). However, there are reports stating that ACTIVIN A can bind to *BMPR2* (Attisano et al. 1993, Rejon et al. 2013). Of note, these observations suggest that impaired *BMPR2* may lead to

activation of alternate pathways such as p38MAPK signalling contributing to PAH progression.

The metabolome data presented shows that deletion of the *BMPR2* kinase domain results in alteration of fatty acid oxidation, glycolysis and glucose oxidation. The most significant change in *BMPR2^{DelKinase}* ECs is a reduction in metabolites such as adenosine and TCA cycle components malic acid, pyruvic, citric acid and maleic acid. *BMPR2^{DelKinase}* ECs had a significant reduction in fatty acid oxidation components and changes in the glycolysis pathway (**Table 1**). Alterations in glucose oxidation alongside mitochondrial oxidation phosphorylation has been demonstrated in the pulmonary artery endothelium of PAH patients (Sutendra et al. 2010, Xu et al. 2007). Furthermore, PAH associated with chronic hypoxia has been linked to an imbalance between glycolysis, glucose oxidation and fatty acid oxidation (Fijalkowska et al. 2010). Therefore, the *in vitro* studies presented here may offer insights to the disruption of the metabolic program of ECs carrying *BMPR2* mutations. However, further experiments are required to fully define the impact of disrupting *BMPR2* signalling on EC metabolism.

In order to define the downstream transcriptional networks regulated by *BMPR2* we performed RNA-seq on cultured ECs from all four *BMPR2* genotypes. While this data has only undergone preliminary analysis it has revealed that a number of key genes such as *PPAR γ* (transcriptional regulator), *JAKMIP2* (microtubule binding), *ITGA4* (cellular adhesion), *SRY-BOX5*, *HOXA2*, *5HHIP* (cell fate and developmental genes), *ABCA13*, *CHDH*,

CYP4A22 (metabolic components), *COL4A4*, *COL4A2* (extracellular matrix components), *ANGPT1*, *COL8A1* (vessel development and homeostasis), *HTR1B*, *HTR2C* (serotonin (5HT) type receptor mediated signalling pathway), *PTGFR*, *APLNR* (G-protein coupled receptor activity) and *TRPC4*, *DLK1*, *KCNH8* (ion channel activity) are commonly dysregulated in *BMPR2* mutants. In the context of the cellular and metabolomics findings, *PPAR γ* a known transcriptional regulator (Kliwer et al. 1994, Poulsen et al. 2012) of mitochondrial biogenesis is up regulated consistent with the altered metabolome in *BMPR2* mutant ECs. Therefore, we expect that further dissection of this transcriptional data set will identify transcription networks that may be perturbed in PAH.

We observed up regulation of cytoskeletal and cell motility markers that are consistent with the distinct tubule morphology and enhanced motility of the *BMPR2* mutant ECs. Furthermore, up regulation of chromatin re-modellers and cell cycle regulation may correlate to the appearance of PDGFR- α positive population in wild type cells that is lacking in *BMPR2* mutant EC cultures. In addition, the outcome of GO analysis of differentially regulated genes in *BMPR2^{DelKinase}* ECs show an enrichment for genes associated with cellular energetics, which correlates to the metabolomics findings.

Taken together, our data set suggests that the normal role of *BMPR2* in the adult pulmonary vasculature is akin to a gatekeeper mediating responses that are necessary for optimal cellular function in an environment that requires rapid adaptation to physical stresses. Suppression of *BMPR2* signalling

results in defective cell-cell interaction and cytoskeletal dynamics that would impair vascular homeostasis and angiogenesis or angiogenic repair. Furthermore, we observed alterations to proliferation/apoptosis and differentiation decisions in *BMPR2* mutants that may contribute to disease pathogenesis. Results from our allelic series of *BMPR2* mutations show that there are different mechanisms at play such as loss of function or gain of function, this may depend on the location of the mutation in the gene. Finally, *BMPR2* is required for regulated cellular metabolism and this may link to the phenotypes observed in culture. Therefore, in the disease setting, it is likely that ECs and SMCs heterozygous for *BMPR2* mutations within the vessel wall will have a maladaptive response to environmental stressors that is mediated by the molecular and cellular pathways described in this chapter.

CHAPTER



General Discussion

Introduction

This thesis describes the generation of genetically modified PSC lines that were employed to investigate the role of *BMPR2* in development and to define the role of *BMPR2* in the major cell lineages of the blood vessel. Furthermore, this work demonstrates that PSC lines harbouring a series of *BMPR2* mutations are capable of differentiating into a number of mesodermal cell lineages. In summary: Chapter 1 reviews the literature detailing the clinical characteristics of PAH, the BMP ligands and their receptors, animal models of PAH and finally using PSCs for modelling PAH. Chapter 2 outlines the methodology used in the work presented here from gene editing to functional experiments. Chapter 3 focuses on the generation of the allelic series of *BMPR2* mutants used in this study. We utilised CRISPR/Cas 9 to repair and modify the *BMPR2* locus in PSCs to acquire cellular reagents for functional studies. Chapter 4 illustrates the mesoderm and cardiac differentiation protocols employed in order for us to examine the cell types affected by *BMPR2* deficiency. From our studies, we show that *BMPR2* deficiency does not affect mesoderm and cardiac differentiation. However, disrupted *BMPR2* may contribute to a suboptimal function in cardiomyocytes when exposed to exogenous BMP4, which is consistent with an overall higher risk of cardiac failure in PAH patients that are *BMPR2* mutation carriers. In Chapter 5, we examined cell types that were most affected by the loss of *BMPR2*, such as blood lineages, SMCs and ECs. We also demonstrate that PSCs differentiated to these cell types *in vitro* despite having impaired *BMPR2* signalling. In addition, mutations in different domains of *BMPR2* led to

different cellular phenotypes *in vitro*. The complexity of the molecular and cellular pathways regulated by BMPR2 may, in part, explain the aetiology of PAH. Collectively, this work has set the foundation towards a deeper understanding of *BMPR2*-related PAH pathogenesis, which may subsequently contribute to gene and drug therapy.

Summary of key findings

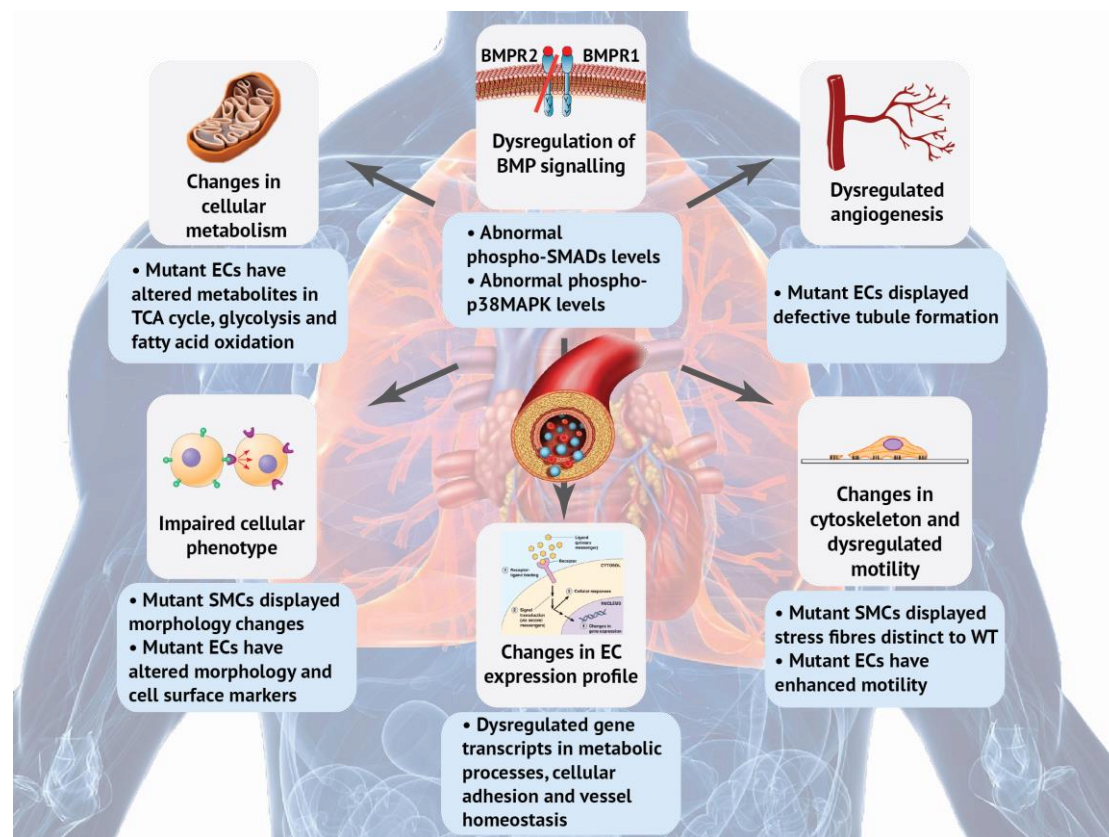


Figure 6.1 Schematic shows an overview of EC dysfunction as a result of *BMPR2* mutation. We found that *BMPR2* mutant ECs showed changes in metabolism, cellular phenotype, motility, cellular expression and tubule formation (angiogenesis). While *BMPR2* mutant SMCs showed changes in cellular morphology and cytoskeleton in comparison to wild type derivatives. See Chapter 7, Appendix, Table 4 for summary of results.

6.1 Generation of an allelic series of *BMPR2* mutant cell lines

Chapter 3 details the generation of the reagents required for investigating *BMPR2* function. The use of PSCs and the timely discovery of CRISPR/Cas9 to mediate genome editing have enabled us to efficiently modify the *BMPR2* locus. We have a complete *BMPR2* null and two PSC lines that express mutant *BMPR2* proteins lacking C-terminal tail domain and disrupted kinase domain. However, wild type and mutant *BMPR2* protein was undetected either by western blotting or immunofluorescence. To circumvent this problem an epitope tag can be engineered into the *BMPR2* coding sequence to enable detection of *BMPR2* protein, since there are no commercial anti-*BMPR2* antibodies that can reliably detect the *BMPR2* protein.

6.2 Despite abnormal pSMAD1/5/8 and p38MAPK phosphorylation levels, *BMPR2* mutants efficiently differentiate into mesoderm

In Chapter 4, we investigated impaired function of *BMPR2* during mesodermal and cardiac differentiation. We found that BMP4 induced mesoderm differentiation was not affected in *BMPR2* mutant human PSCs. In contrast, murine knockout studies of *Bmpr2* report a lack of mesoderm formation (Beppu et al. 2000). This discrepancy in the requirement for *Bmpr2* signalling for mesodermal development between mice and humans may reflect interspecies differences. Alternatively, the *in vitro* differentiation of hPSC may be permissive for mesodermal formation as it lacks the precise spatial temporal constraints of the embryo (Graham et al. 2014).

We investigated the BMP signalling cascade in both wild type and *BMPR2* mutant PSCs. Western blot profiling demonstrated that *BMPR2* mutant PSCs induced with BMP4, activated the canonical BMP signalling pathway suggesting compensatory signalling via receptor *ACVR2A/B*. An alternate signalling mechanism used by *BMPR2* is p38MAPK, a downstream effector protein of the non-canonical BMP signalling pathway. Western blot profiling in PSCs showed that phosphorylated-p38MAPK was detected in the absence of BMP4 in cultures lacking the *BMPR2* tail domain. This suggests that the tail domain is required to modulate p38MAPK phosphorylation. These results demonstrate that impaired *BMPR2* function causes an abnormal signalling cascade when mutants were treated with exogenous BMPs. Nevertheless, all *BMPR2* mutant lines efficiently generated mesoderm *in vitro*.

6.3 *BMPR2* disruption does not affect cardiomyocyte differentiation

Another novel aspect of this study showed that *BMPR2* disruption did not affect cardiomyocyte differentiation. Gene expression signatures were not significantly different between wild type and *BMPR2* mutant cardiomyocytes. Conversely, treatment with exogenous BMP4 revealed a different response in gene expression between wild type and *BMPR2* mutant cardiomyocytes. For example, gene transcripts for *NKX2-5*, *GATA4*, *NOTCH1*, *SMAD1*, *ID1* and *EFNA1* were upregulated in wild type but not in *BMPR2* mutant cardiomyocytes. This result suggests that *BMPR2* mutations may directly impact the function of cardiomyocytes in PAH patients. Moreover, Van Der Bruggen et al. (2016) reported that PAH patients carrying mutations in the *BMPR2* gene presented with more severely compromised right ventricle

function despite having similar mean pulmonary arterial pressures and pulmonary vascular resistance to non-carriers. In addition, the differences were not a result of cardiac adaptation but due to increased hypertrophy, fibrosis and cardiac metabolism in PAH patients who carried *BMPR2* mutations (Van Der Bruggen et al. 2016).

6.4 *BMPR2* mutant SMCs have altered cytoskeletal dynamics

The C-terminal cytoplasmic tail domain of *BMPR2* has been reported to interact with cytoskeletal organizational proteins such as LIMK (Foletta et al. 2003), TCTEX (Machado et al. 2003), and SRC (Wong et al. 2005). In line with these reports, our results show that *BMPR2* mutant SMCs have cytoskeletal structures that are morphologically distinct to wild type SMCs. Moreover, *BMPR2^{DelTail}*, i.e. a truncated *BMPR2* protein lacking the tail domain, SMCs had altered proliferation-apoptosis rate to wild type SMCs *in vitro*. Johnson et al. (2012) reported that in Rosa26-Bmpr2^{R899X} mice, an established PAH model, the cytoskeletal architecture of pulmonary microvascular ECs was abnormal. In addition, whole lung gene expression studies from Rosa26-Bmpr2^{R899X} mice showed that genes in the Rho GTPase pathway (Rac1 activation) were affected. Following on from this, *BMPR2* mutations in different domains stably transfected into pulmonary microvascular ECs revealed that cytoskeletal function was compromised in all *BMPR2* mutant EC genotypes (Johnson et al. 2012). Protein expression profiling of PASMCs from PAH patients revealed increased expression of eIF2/mTOR/p70S6K, RhoA/actin cytoskeleton/integrin and protein ubiquitination, these pathways are closely related and have critical functions

in smooth muscle proliferation, apoptosis, contraction and cellular stress during the pathophysiology of PAH (Yao et al. 2015). Cytoskeletal organization anomalies of *BMPR2* mutant SMCs may be one of the contributing factors leading to the onset of PAH pathogenesis. However, further investigation by proteomic profiling of our *BMPR2* mutant SMCs is required to understand how perturbed *BMPR2* affects these signalling networks.

6.5 *BMPR2* mutant have increased CD34⁺ progenitors in cardiac and EC differentiation conditions

Cardiomyocyte and EC differentiation resulted in significantly greater populations of CD34⁺ expressing cells in all *BMPR2* mutant genotypes. It has been reported that there are greater numbers of CD34⁺CD133⁺ endothelial progenitor cells derived from colony forming units of endothelial-like cells (CFU-EC) among IPAH ECs compared to control ECs (Asosingh et al. 2008). The mechanisms driving EC proliferation in PAH might be similar to those used during embryonic blood vessel development (vasculogenesis/angiogenesis). Furthermore, EC proliferation occurs from stem cell niches within the compromised pulmonary blood vessel wall, thus activating multiple progenitors that may trans-differentiate into ECs, leading to the generation of plexiform lesions frequently found in severe PAH patients (Xu and Erzurum 2010). Therefore, increased production of CD34⁺ EC progenitors observed in all *BMPR2* mutant cell lines suggest that *BMPR2* mutations predispose individuals to PAH due to over specification of proliferative CD34⁺ EC progenitors within the blood vessel wall. Recent

studies have begun to dissect the signal transduction pathways and transcription factors that regulate the specification of ECs into different subtypes including, arterial, venous and lymphatic ECs (Alex Le et al. 2010). Understanding the EC differentiation and endothelial progenitors (EPC), particularly how they are regulated in health and disease may contribute to our understanding of PAH progression.

6.6 *BMPR2* deficiency does not affect EC differentiation but affects tubule formation

We found that EC differentiation was not affected by perturbation of *BMPR2* and demonstrated that *BMPR2* mutant ECs were positive for EC markers such as CD31 and von Willebrand factor (vWF). Furthermore, *BMPR2* mutant ECs were able to uptake acetylated LDL and formed tubules in appropriate culture conditions, both of which are functional properties of ECs. However, tubules formed by *BMPR2* mutant ECs were observed to be morphologically distinct from wild type EC tubules. Additionally, there were morphological differences among the tubules formed by the different genotypes of *BMPR2* mutant ECs. In particular, *BMPR2^{DelTail}* ECs formed fewer tubules and these were thinner than the wildtype. In contrast, *BMPR2^{Null}* ECs had a propensity to form more tubules compared to wild type ECs. These findings suggest that perturbed *BMPR2* signalling affects the regulation of processes required for vasculogenesis/tubulogenesis. Furthermore, these results are concordant with reports that pulmonary arterial ECs derived from PAH patients form defective tubules and that *BMPR2* mutations specific to the cytoplasmic tail domain affect metabolic, cytoskeletal, adhesion and microtubule-related genes

(Johnson et al. 2012, Majka et al. 2011, Masri et al. 2007, West et al. 2008b).

In addition, these findings are consistent with the notion that *BMPR2* plays an important role in regulating cytoskeletal dynamics.

6.7 Gene expression profiling of *BMPR2* mutant ECs

Gene expression profiling of wild type and *BMPR2* mutant ECs showed consistent expression of EC markers *KDR* and *vWF* and no statistical differences in gene transcript levels of *NOTCH1*, *SMAD7*, *SMAD1*, *ID1*, *AKT1*, *BMP2*, *BMP4*, *HEY1*, *HEY2* and *ACTA2*. In contrast, there was significant increase of *ACVR2A* transcripts in *BMPR2^{DelKinase}* and *BMPR2^{Null}* ECs and significant decrease in *EPHRIN-B2* gene transcript levels in all *BMPR2* mutant EC genotypes. Increased transcription of *ACVR2A* in *BMPR2* null and kinase deficient mutant ECs, which inhibit the canonical BMP pathway, may provide functional compensation for disrupted *BMPR2* signalling via increased *ACVR2A*. Besides binding to ACTIVINs, *ACVR2A* is also known to bind to BMPs to form heterodimers with *BMPR1* (Lavery et al. 2008). Furthermore, *BMPR2* mutant ECs displayed decreased *EPHRIN-B2* transcripts. *EPHRIN-B2* is a transmembrane protein that is highly expressed in human arterial ECs. *EPHRIN-B2* interacts with Eph receptors to regulate angiogenesis and modulates the motility and cellular morphology of ECs, primarily by stimulating downstream effectors that regulate actin cytoskeletal organization through the Rho small GTPases (Bochenek et al. 2010). This finding is further supported by scratch-migration assays demonstrating that *BMPR2* mutant ECs have an enhanced migration capacity. Future experiments will focus on determining the precise role of *EPHRIN-B2* signalling in EC function.

6.8 *BMPR2* regulates cellular migration in ECs

Our findings implicate *BMPR2* in the regulation of EC migration. Therefore, to ascertain if the BMP/SMAD canonical signalling regulates the process of migration, we utilised the BMP antagonist DMH-1 to inhibit BMP canonical signalling. Wild type ECs treated with DMH-1 had increased migration rates, consistent with that of the *BMPR2* mutants in scratch-migration assays. In contrast, treatment with NOGGIN, which binds BMPs preventing interaction with receptors thereby abolishing all BMP signalling, decreased migration rates in wild type and *BMPR2* mutant ECs. ECs derived from PAH patients have enhanced migration *in vitro* and in *BMPR2* knockdown experiments in human pulmonary arterial ECs results in increased motility and pSMAD3 activation via *ACTR2A* (de Jesus Perez et al. 2009, Masri et al. 2007). Moreover, directed migration is necessary for ECs to converge and form vessels (Adams and Alitalo 2007). In addition, previous studies show that BMP2 induces EC migration and vascular tube formation (Deckers et al. 2002, Langenfeld and Langenfeld 2004). Therefore, these findings demonstrate that impaired *BMPR2* affects cellular migration that in turn impacts upon vasculogenesis/tubulogenesis. In addition, there is increased basal level of apoptosis of normal PAECs in PAH patients (Teichert-Kuliszewska et al. 2006). This stimulates an injury response, whereby the precursor cells migrate to the site of injury in order to replace the initial increase of apoptotic ECs. This response appears to be overly active in patients with *BMPR2* mutations. Furthermore, our results demonstrate that *BMPR2* mutant ECs migrated more rapidly than wild type and hence more

cells may arrive at the injury site than necessary, this suggests one mechanistic contribution to the thickened vessel walls in these patients.

6.9 *BMPR2* deficiency maintain an EC expression profile

Subsequently, we found that *in vitro* cultures of wild type ECs appear morphologically distinct to *BMPR2* mutant ECs after several enzymatic passages. Flow cytometry expression profiling of earlier passages of wild type and *BMPR2* mutant ECs express markers such as CD31, CD34, KDR, TIE2 and not PDGFR- α . Flow cytometric analyses of later passages of *BMPR2* mutant ECs show that these cells maintain the characteristic EC expression profile. Wild type ECs however, began to show changes in cell surface marker expression, in particular a population of PDGFR- α positive cells emerged and lower cell numbers maintained expression of CD31, CD34, KDR and TIE2. Following on from this, we showed that inhibiting the BMP/SMAD canonical pathway in wild type ECs resulted in the maintenance of the EC marker positive population observed in the *BMPR2* mutant ECs.

6.10 *BMPR2* mutant ECs have increased metabolism/proliferation capacity when exposed to exogenous BMPs

Wild type and *BMPR2* mutant ECs demonstrated similar viability after enzymatic passaging and cell seeding. However, *BMPR2*^{DelTail} ECs had a significant increase in proliferation rates compared to wild type ECs. Heat map analysis of the MTT assays demonstrated that *BMPR2* mutant ECs cultured with BMP4, BMP6 and BMP9, had higher proliferative rates than wild type ECs. This was particularly pronounced in *BMPR2*^{DelTail} ECs compared to

the other *BMPR2* mutant genotypes. Conversely, when the proliferative cytokines bFGF and VEGF were added to cultures, proliferation increased in wild type ECs but to a lesser extent in the *BMPR2* mutant ECs. This occurrence is explained by the tight cross talk between FGF and BMP signalling pathways. Antagonism of FGF signalling by BMP ligands has been found to be necessary for specifying cardiomyocyte cell fate in the early embryo and in embryonic stem cells (Tirosh-Finkel et al. 2010). Hence, BMP and FGF signalling pathways act via inter- and intra-regulatory loops in multiple tissues in order to coordinate the balance between proliferation and differentiation of mesodermal progenitors. No differences in proliferation rates were observed between the *BMPR2* mutant genotypes and wild type ECs, when treated with inhibitors DMH-1 and SB431542 (TGF- β 1 inhibitor). Pulmonary arterial ECs of PAH patients have been shown to have increased proliferation, migration and alterations to tube formation compared to cells derived from healthy control lungs (Masri et al. 2007). Taken together, the data presented indicate that *BMPR2* is important in regulation of proliferation-apoptosis, angiogenesis, cell-cell communication and cellular motility in ECs.

6.11 *BMPR2* plays a key role in EC metabolism

Our findings thus far have shown that ECs display enhanced motility, dysregulated tubule formation and increased proliferation capacity, all of which requires increased amount of ATP and in turn impacts upon mitochondria function. We postulate that *BMPR2* may play a role in the regulation of metabolism. The metabolome profile showed metabolic differences between wild type and *BMPR2^{DelKinase}* ECs. There was a reduction

in metabolites such as adenosine, a derivative of cAMP (cyclic adenosine monophosphate) derived from hydrolysis of intracellular ATP-ADP and TCA cycle components, for example malic acid, pyruvic, citric acid and maleic acid (**Table 1**). Acetoacetate and 2-Ketoglutaric acid reductions in *BMPR2^{DelKinase}* ECs also indicate changes in fatty acid oxidation. Furthermore, alterations in glycolysis cycle components for example, CMP, serine and D-glucose-6-phosphate were observed in *BMPR2^{DelKinase}* ECs. Collectively, metabolome analysis reveals that *BMPR2* mutation affecting the kinase domain results in changes in fatty acid oxidation, glycolysis and glucose oxidation. These significant changes affect mitochondrial oxidative phosphorylation. Further, altered mitochondrial metabolism associated with glycolysis has been described in PAH (Archer et al. 2008). *BMPR2* mutants have cytoskeletal defects in SMCs and possibly, altered cytoskeletal dynamics in ECs. Interactions between mitochondria and the cytoskeleton are essential for normal mitochondrial morphology, motility and distribution (Boldogh and Pon 2006). In addition, *BMPR2* siRNA knockdown in human pulmonary arterial ECs and ECs from PAH patients consistently show that during normoxia (10-21% oxygen tension) enhanced mitochondria biogenesis is driven by increased p53, PGC1 α , NRF2 and TFAM (Diebold et al. 2015). Furthermore, PAH ECs with impaired *BMPR2* have increased mitochondrial membrane potential, ATP production and glycolysis leading to mitochondrial fission. In contrast, loss of *BMPR2* during re-oxygenation after hypoxia impairs p53, PGC1 α , TFAM, and decreased mitochondrial membrane potential and ATP and mtDNA deletion (Diebold et al. 2015). Together, these findings further

support the notion that *BMPR2* mutation resulting in PAH is linked to mitochondrial dysfunction.

6.12 Identifying *BMPR2*-dependent transcription networks

Examining the gene expression signatures of wild type and *BMPR2* mutant ECs provides an opportunity to identify the transcriptional networks that drive pathogenesis in *BMPR2* related PAH. For example, *BMPR2* regulates *PPAR γ* , a key transcription factor for mitochondrial biogenesis, via multiple signalling pathways. In our stem cell model, it is clear that *PPAR γ* expression is reliant on normal *BMPR2* signalling. *PPAR γ* is highly expressed in normal ECs and reduced in PAH patients (Ameshima et al. 2003). In ECs, *BMPR2* activates *PPAR γ* and induces expression of genes that regulate EC survival and angiogenesis such as *APLNR* (Guignabert et al. 2009). However, in some cases lung tissue from PAH patients showed decrease expression of *PPAR γ* and its downstream target *APOE*, which may reflect the advanced disease state of these biopsies (Ameshima et al. 2003, Geraci et al. 2001). In addition, *Ppar γ* ^{-/-} mice displayed defects in vasculature structure as well as a lack of balance between pro- and anti-angiogenic factors (Nadra et al. 2010). The importance of efficient mitochondrial function to angiogenesis underscores the requirement for correct *BMPR2* signalling for pulmonary arterial formation and homeostasis.

GO assessment of the differentially regulated genes in *BMPR2* mutant ECs listed a number of key terms that were dysregulated, such as metabolic processes, cytoskeletal organization, cellular adhesion, locomotion and cell-

cell communication. These processes are indicative of the phenotypic characteristics observed in the *BMPR2* mutant ECs for example impaired tubule formation and enhanced motility. Processes such as chromatin remodelling and cell cycle regulation were impaired in *BMPR2* mutant ECs and in part may explain the morphological differences observed in wild type ECs. In addition, dysregulation of cellular energetics in *BMPR2* mutant ECs are reflective of the findings in the metabolomics study. Collectively, preliminary analysis of the metabolomics and transcriptomic data has highlighted several key pathways that may provide insights into the mechanism of perturbed *BMPR2* signalling in ECs. Nevertheless, it is important to note that the *BMPR2* mutants examined in this study are homozygous deletions, not the pathogenic point mutations found in PAH patients. Therefore, further experiments will be required to elucidate the precise pathogenic mechanisms associated with *BMPR2* mutations found in patients.

6.13 Conclusion and future developments

BMPR2 is expressed throughout the developing embryo and is required for the maintenance of the adult vasculature. It is highly expressed on the vascular endothelium of the pulmonary arteries in lung tissue (Morrell 2006), thus suggesting that *BMPR2* deficiency incapacitates the normal adaptive response of the endothelium in the vessels of the lungs. Levels of *BMPR2* transcripts and proteins are found to be reduced in the lungs of both heritable and idiopathic PAH patients, regardless of the presence of *BMPR2* mutations (Nasim et al. 2012). We hypothesize that PAH is not caused by *BMPR2*

mutation alone but is triggered by either external stressors and stimuli or unfavourable genetic interactions. For example, RNA sequencing experiments, found that COL4A1, COL4A2, and EFNA1 were reduced in PAH patients (Rhodes et al. 2015). Further, reduction by siRNA of these genes inhibited pulmonary endothelial adhesion, migration, and tube formation (Rhodes et al. 2015). Collagen IV is a trigger for tubule cells to localize BMP guidance cues and BMP pathway activation results in a subset of tubule cells that drive to outgrowth through the body cavity (Bunt et al. 2010). Loss of collagen IV, or abrogation of BMP signalling results in tubule misrouting and defective organ shape and positioning (Bunt et al. 2010). Much like the causality dilemma, we are uncertain if disease occurs because the original capillary architecture is somewhat defective or whether this damage is secondary to the cause.

An emerging hypothesis is that the root cause of PAH is due to mitochondrial defects in *BMPR2* carriers (Diebold et al. 2015, Fessel et al. 2012). In addition, to our own observations, several groups have provided compelling evidence for a shift in glucose metabolism from oxidative phosphorylation to glycolysis known as “the Warburg effect” (i.e. identified in abnormal proliferating cells such as cancer cells) in experimental and human PAH. Furthermore, upregulation of glutamine metabolism, changes in fatty acid metabolism and disruption of other major metabolic pathways is observed in PAH patients (Fessel et al. 2012, Tudor et al. 2012). The linkage between *BMPR2* and mitochondrial function is possibly related to *BMPR2* interaction with F-actin related pathways and cytoskeletal trafficking. These are the central pathways regulating mitochondrial fission and fusion and defects in

mitochondrial fission and fusion have been implicated in pulmonary hypertension and oxidant stress (Lane et al. 2011). EC mitochondria are mainly responsible for increase reactive oxygen species (ROS) and enhanced mitochondrial fission and/or attenuated fusion may lead to mitochondrial fragmentation thus disrupting endothelial physiological function (Pangare and Makino 2012). Abnormal mitochondrial biogenesis and disturbance of mitochondrial autophagy increase the accumulation of damaged mitochondria, such as irreversibly depolarized or leaky mitochondria that promote apoptosis. Further, *BMPR2* signalling may act on mitochondrial homeostasis via the downstream target peroxisome proliferator-activated receptor γ (*PPAR γ*) and other downstream effectors (Galetto et al. 2001). *PPAR γ* is best known for its role in glucose metabolism and adipogenesis, but is a potential target for a causative role in PAH as it is highly expressed in the lungs and has been shown to have vasoprotective effects in systemic vascular disease (Duan et al. 2008). *PPAR γ* regulates multiple pathways known to be important in PAH including endothelin-1, MCP-1, and eNOS (Farber and Loscalzo 2004, Itoh et al. 2006). Finally, *PPAR γ* can block the pERK signal, and it induces expression of genes that prevent cell division, namely, the cell cycle inhibitors p27 and p21 (Hansmann et al. 2008).

In summary, our data have identified a number of key *BMPR2*-dependant pathways that are important for both EC and SMC function and homeostasis. We have generated a collection of cellular tools that have permitted a detailed examination of the role of *BMPR2*. Currently, we are conducting ongoing analysis using 'omics as a tool to identify key signalling networks. This work

may identify targets that are amenable to pharmaceutical intervention to impede PAH disease progression. The cellular phenotyping of *BMPR2* mutant lines presented here has provided a number of insights into the molecular basis of PAH pathogenesis and serves as a framework for the future examination of patient-specific PAH models.

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CHAPTER



Appendices

Appendices

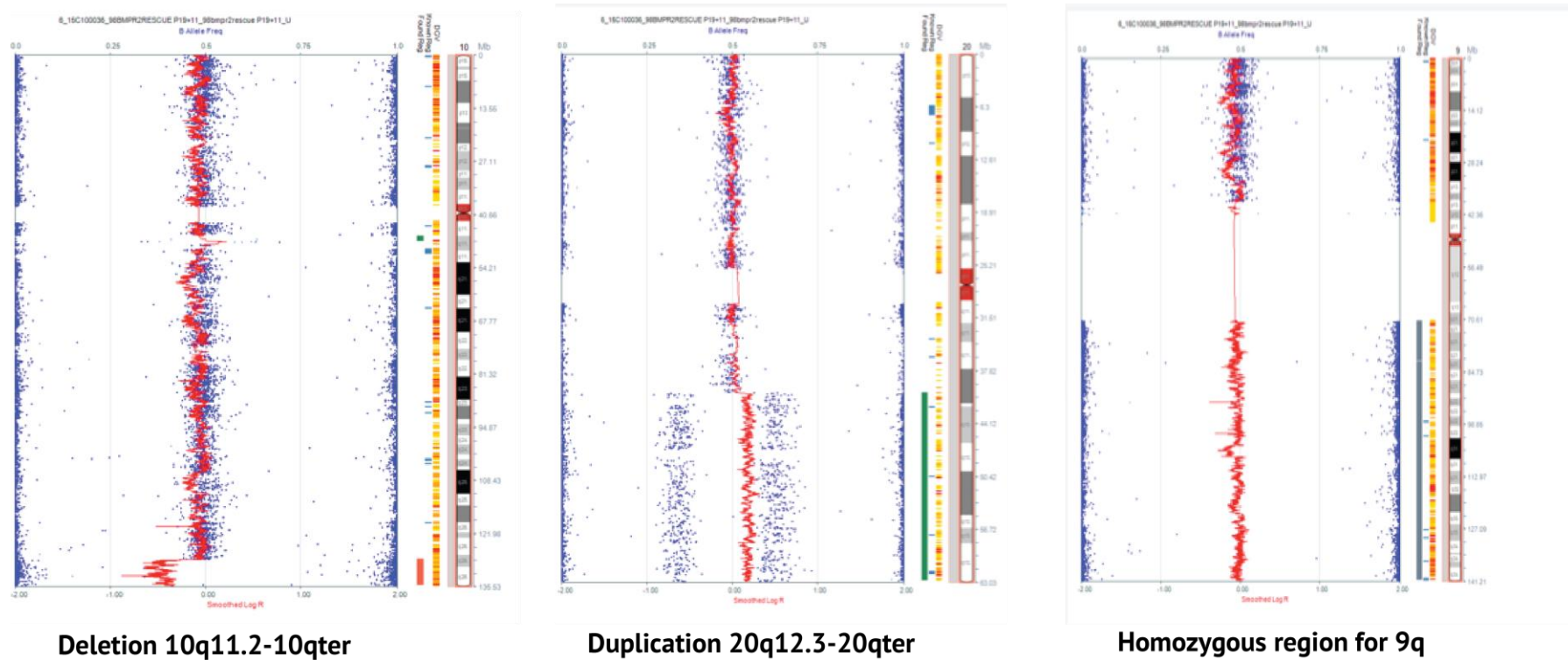


Figure 1. Results from SNP analysis of *Bmpr2*^{Corr} show abnormal karyotype of PSCs.

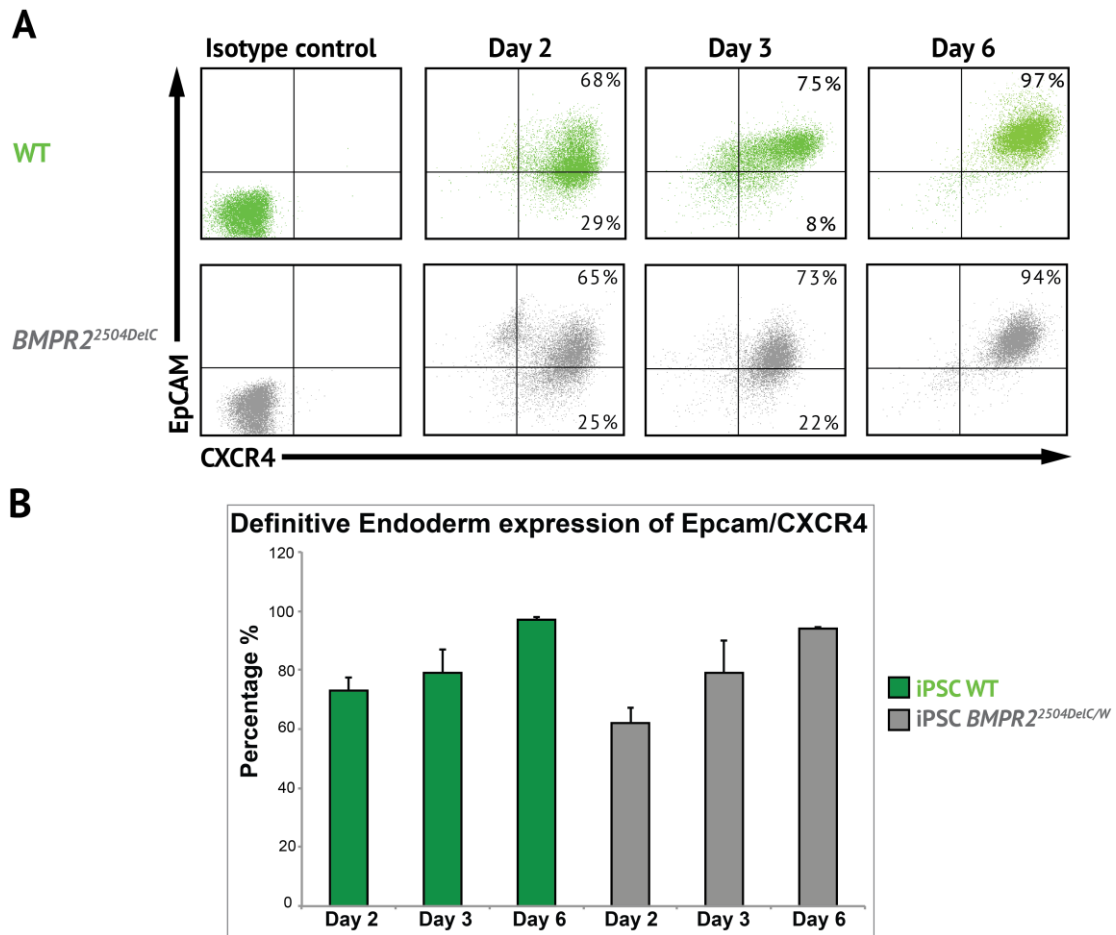


Figure 2. *BMPR2*^{2504delC/W} iPSC display normal endodermal differentiation. (A) FACS plots showing EpCAM and CXCR4 expression from days 2, 3 and 6 of differentiating *BMPR2*^{2504delC/W} and *iPSC*^W demonstrating high efficiency of endoderm differentiation. Isotype controls are shown in the first plots. (B) Graph shows mean percentage positive cells co-expressing EpCAM and CXCR4 during days 2, 3 and 6. Results indicate that *BMPR2*^{2504delC/W} has similar differentiation patterns compared to *iPSC*^W. Error bars display $n=3$; $\pm$ S.E.M.

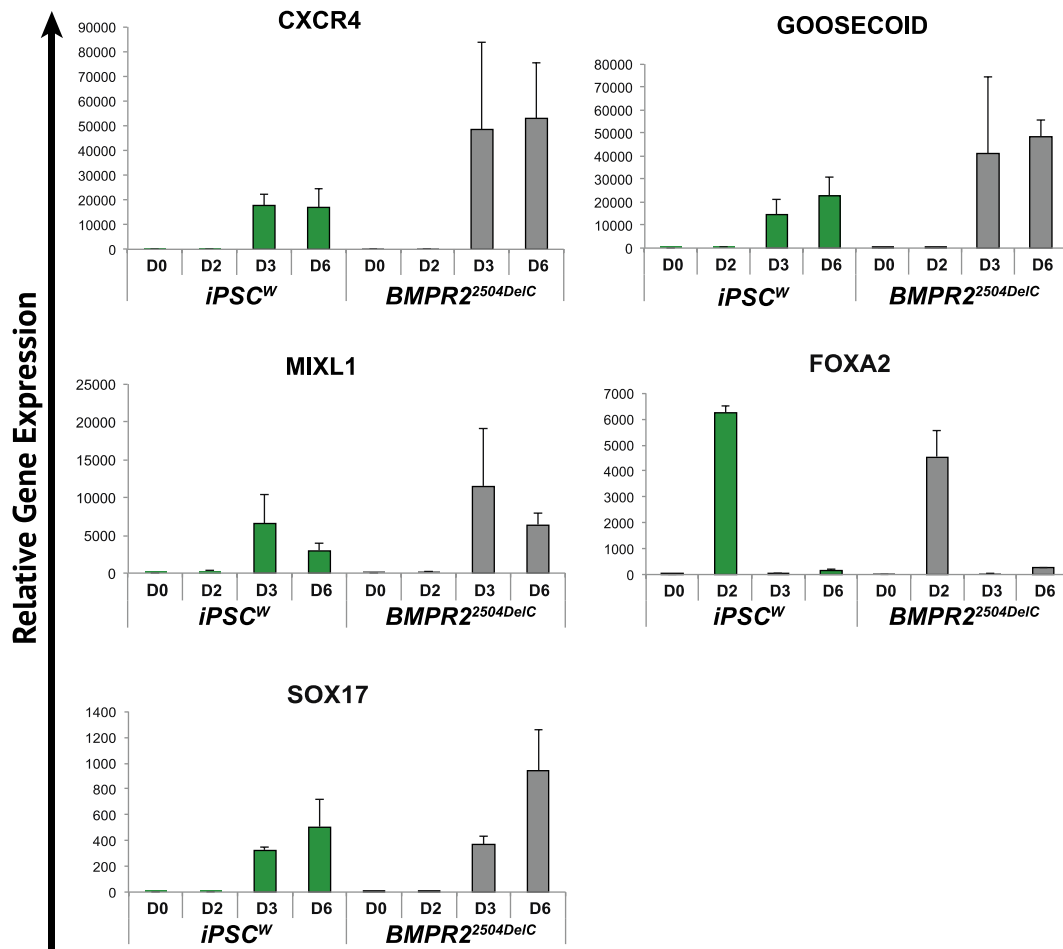


Figure 3. Gene expression profiles of endoderm markers during differentiation of *BMPR2*^{2504delC/W} and *iPSC*^W. Data show mean expression of genes at day 0, 2, 3 and 6 of differentiation. Green bars represent *iPSC*^W, grey bars represent *BMPR2*^{2504delC/W} and error bars display $n=3$; $\pm$ S.E.M.

ENTREZID	SYMBOL	GENE NAME	logFC	AveExpr	t	P.Value	adj.P.Val	B
1277	COL1A1	collagen type I alpha 1 chain	-3.62	5.76	-5.77	1.07E-05	6.15E-02	3.36
780854	SNORD3D	small nucleolar RNA, C/D box 3D	-3.56	2.68	-2.04	5.43E-02	2.50E-01	-3.90
1129	CHRM2	cholinergic receptor muscarinic 2	-3.48	1.12	-4.19	4.31E-04	1.28E-01	-1.22
100874205	ATP11A-AS1	ATP11A antisense RNA 1	-3.37	0.48	-3.09	5.63E-03	1.90E-01	-2.66
84627	ZNF469	zinc finger protein 469	-3.31	-1.05	-4.30	3.30E-04	1.25E-01	-0.92
50832	TAS2R4	taste 2 receptor member 4	-3.27	0.16	-3.72	1.30E-03	1.64E-01	-2.00
285758	LINC01268	long intergenic non-protein coding RNA 1268	-3.27	-0.62	-2.66	1.49E-02	2.01E-01	-3.26
5308	PITX2	paired like homeodomain 2	-3.23	1.16	-3.34	3.20E-03	1.87E-01	-2.12
970	CD70	CD70 molecule	-3.22	-1.22	-3.35	3.08E-03	1.85E-01	-2.60
5166	PDK4	pyruvate dehydrogenase kinase 4	-3.15	2.75	-3.56	1.90E-03	1.66E-01	-1.30
101927969	LOC101927969	uncharacterized LOC101927969	-3.13	-0.88	-2.74	1.24E-02	2.01E-01	-2.97
57828	CATSPERG	cation channel sperm associated auxiliary subunit gamma	-3.04	0.67	-2.84	1.00E-02	1.98E-01	-3.03
55511	SAGE1	sarcoma antigen 1	-3.00	-1.15	-3.74	1.23E-03	1.64E-01	-2.02
202151	RANBP3L	RAN binding protein 3 like	-2.95	-0.03	-3.04	6.31E-03	1.90E-01	-2.93
3691	ITGB4	integrin subunit beta 4	-2.93	1.27	-3.31	3.36E-03	1.89E-01	-2.51
6252	RTN1	reticulon 1	2.40	-0.46	2.43	2.43E-02	2.12E-01	-3.49
11264	PXMP4	peroxisomal membrane protein 4	2.55	-1.44	3.56	1.90E-03	1.66E-01	-2.50
27164	SALL3	spalt like transcription factor 3	2.57	0.47	2.43	2.46E-02	2.12E-01	-3.51
85417	CCNB3	cyclin B3	2.67	-1.21	2.88	9.04E-03	1.97E-01	-2.97
101927229	LOC101927229	uncharacterized LOC101927229	2.71	-1.46	3.74	1.25E-03	1.64E-01	-1.98
2674	GFRA1	GDNF family receptor alpha 1	2.74	-1.16	3.67	1.45E-03	1.66E-01	-1.82
100128054	PLCE1-AS1	PLCE1 antisense RNA 1	2.74	-1.46	3.62	1.65E-03	1.66E-01	-1.87
100216001	LINC00704	long intergenic non-protein coding RNA 704	2.75	-0.79	2.59	1.73E-02	2.06E-01	-3.06
4103	MAGEA4	MAGE family member A4	2.77	-1.14	3.85	9.64E-04	1.64E-01	-1.81
255738	PCSK9	proprotein convertase subtilisin/kexin type 9	2.87	0.16	3.21	4.29E-03	1.90E-01	-2.62
51266	CLEC1B	C-type lectin domain family 1 member B	2.89	-0.69	1.35	1.90E-01	4.21E-01	-4.58
2353	FOS	Fos proto-oncogene, AP-1 transcription factor subunit	2.91	2.99	1.68	1.08E-01	3.28E-01	-4.40
50487	PLA2G3	phospholipase A2 group III	2.92	-0.45	2.60	1.70E-02	2.05E-01	-3.13
10117	ENAM	enamelin	3.08	-1.12	3.49	2.21E-03	1.74E-01	-2.07
9173	IL1RL1	interleukin 1 receptor like 1	3.70	1.26	3.45	2.47E-03	1.77E-01	-1.57

Table 1. Transcriptome results of the top 30 genes that are differentially regulated in *BMPR2^{DelKinase}* ECs relative to wild type ECs. Highlighted in pink are 15 down-regulated genes and in blue are 15 up-regulated genes in *BMPR2^{DelKinase}*. Included in the table are LogFC showing the fold change relative to wild type, p-values and adjusted p-values.

ENTREZID	SYMBOL	GENE NAME	logFC	AveExpr	t	P.Value	adj.P.Val	B
3046	HBE1	hemoglobin subunit epsilon 1	-4.73	1.80	-1.63	1.17E-01	7.96E-01	-4.40
5023	P2RX1	purinergic receptor P2X 1	-4.43	-0.23	-2.63	1.58E-02	5.72E-01	-3.26
8832	CD84	CD84 molecule	-4.28	-1.62	-4.27	3.58E-04	1.87E-01	-1.42
50486	G0S2	G0/G1 switch 2	-4.22	-0.41	-4.47	2.22E-04	1.58E-01	-1.21
5742	PTGS1	prostaglandin-endoperoxide synthase 1	-4.13	2.97	-2.86	9.52E-03	5.03E-01	-2.73
5341	PLEK	pleckstrin	-4.00	-0.44	-2.35	2.90E-02	6.20E-01	-3.45
3048	HBG2	hemoglobin subunit gamma 2	-3.94	0.47	-1.90	7.21E-02	7.29E-01	-4.02
255631	COL24A1	collagen type XXIV alpha 1 chain	-3.84	-0.78	-2.52	2.00E-02	5.79E-01	-3.34
54	ACP5	acid phosphatase 5, tartrate resistant	-3.65	3.66	-5.12	4.77E-05	6.57E-02	2.13
638	BIK	BCL2 interacting killer	-3.63	2.15	-2.58	1.75E-02	5.75E-01	-3.12
5308	PITX2	paired like homeodomain 2	-3.58	1.16	-3.77	1.16E-03	3.01E-01	-1.37
151	ADRA2B	adrenoceptor alpha 2B	-3.54	1.67	-3.58	1.82E-03	3.15E-01	-1.82
3764	KCNJ8	potassium voltage-gated channel subfamily J member 8	-3.49	-1.27	-3.51	2.13E-03	3.15E-01	-2.34
6693	SPN	sialophorin	-3.35	1.51	-3.25	3.90E-03	3.92E-01	-2.45
170392	OIT3	oncoprotein induced transcript 3	-3.35	2.36	-2.20	3.92E-02	6.64E-01	-3.61
9037	SEMA5A	semaphorin 5A	2.43	5.07	3.36	3.01E-03	3.70E-01	-1.79
387914	SHISA2	shisa family member 2	2.48	1.58	2.10	4.86E-02	7.01E-01	-3.78
84935	MEDAG	mesenteric estrogen dependent adipogenesis	2.51	-1.34	3.22	4.17E-03	3.92E-01	-2.50
11249	NXPH2	neurexophilin 2	2.58	0.50	3.46	2.42E-03	3.34E-01	-1.64
8590	OR6A2	olfactory receptor family 6 subfamily A member 2	2.58	-0.58	3.28	3.65E-03	3.92E-01	-2.62
3855	KRT7	keratin 7	2.73	0.30	3.01	6.82E-03	4.49E-01	-2.29
105370045	LOC105370045	filamin-interacting protein FAM101A	2.84	-1.37	3.86	9.35E-04	2.73E-01	-1.85
7306	TYRP1	tyrosinase related protein 1	2.94	-1.22	3.51	2.16E-03	3.15E-01	-1.88
3024	HIST1H1A	histone cluster 1 H1 family member a	3.02	0.99	2.06	5.25E-02	7.04E-01	-3.83
51142	CHCHD2	coiled-coil-helix-coiled-coil-helix domain containing 2	3.06	4.37	9.81	3.34E-09	5.75E-05	10.13
1734	DIO2	deiodinase, iodothyronine type II	3.24	-0.64	3.43	2.57E-03	3.47E-01	-2.25
6529	SLC6A1	solute carrier family 6 member 1	3.33	2.08	2.43	2.44E-02	5.98E-01	-3.28
6406	SEMG1	semenogelin I	3.50	-0.52	3.43	2.58E-03	3.47E-01	-1.69
4103	MAGEA4	MAGE family member A4	4.34	-1.14	5.57	1.70E-05	5.28E-02	-0.08
100128054	PLCE1-AS1	PLCE1 antisense RNA 1	4.44	-1.46	4.97	6.87E-05	8.02E-02	-0.56

Table 2. Transcriptome results of the top 30 genes that are differentially regulated in *BMPR2^{Null}* ECs relative to wild type ECs. Highlighted in pink are 15 down-regulated genes and in blue are 15 up-regulated genes in *BMPR2^{Null}* ECs. Included in the table are LogFC showing the fold change relative to wild type, p-values and adjusted p-values.

ENTREZID	SYMBOL	GENE NAME	logFC	AveExpr	t	P.Value	adj.P.Val	B
1277	COL1A1	collagen type I alpha 1 chain	-3.62	5.76	-5.77	1.07E-05	6.15E-02	3.36
780854	SNORD3D	small nucleolar RNA, C/D box 3D	-3.56	2.68	-2.04	5.43E-02	2.50E-01	-3.90
1129	CHRM2	cholinergic receptor muscarinic 2	-3.48	1.12	-4.19	4.31E-04	1.28E-01	-1.22
100874205	ATP11A-AS1	ATP11A antisense RNA 1	-3.37	0.48	-3.09	5.63E-03	1.90E-01	-2.66
84627	ZNF469	zinc finger protein 469	-3.31	-1.05	-4.30	3.30E-04	1.25E-01	-0.92
50832	TAS2R4	taste 2 receptor member 4	-3.27	0.16	-3.72	1.30E-03	1.64E-01	-2.00
285758	LINC01268	long intergenic non-protein coding RNA 1268	-3.27	-0.62	-2.66	1.49E-02	2.01E-01	-3.26
5308	PITX2	paired like homeodomain 2	-3.23	1.16	-3.34	3.20E-03	1.87E-01	-2.12
970	CD70	CD70 molecule	-3.22	-1.22	-3.35	3.08E-03	1.85E-01	-2.60
5166	PDK4	pyruvate dehydrogenase kinase 4	-3.15	2.75	-3.56	1.90E-03	1.66E-01	-1.30
101927969	LOC101927969	uncharacterized LOC101927969	-3.13	-0.88	-2.74	1.24E-02	2.01E-01	-2.97
57828	CATSPERG	cation channel sperm associated auxiliary subunit gamma	-3.04	0.67	-2.84	1.00E-02	1.98E-01	-3.03
55511	SAGE1	sarcoma antigen 1	-3.00	-1.15	-3.74	1.23E-03	1.64E-01	-2.02
202151	RANBP3L	RAN binding protein 3 like	-2.95	-0.03	-3.04	6.31E-03	1.90E-01	-2.93
6252	RTN1	reticulon 1	2.40	-0.46	2.43	2.43E-02	2.12E-01	-3.49
11264	PXMP4	peroxisomal membrane protein 4	2.55	-1.44	3.56	1.90E-03	1.66E-01	-2.50
27164	SALL3	spalt like transcription factor 3	2.57	0.47	2.43	2.46E-02	2.12E-01	-3.51
85417	CCNB3	cyclin B3	2.67	-1.21	2.88	9.04E-03	1.97E-01	-2.97
101927229	LOC101927229	uncharacterized LOC101927229	2.71	-1.46	3.74	1.25E-03	1.64E-01	-1.98
2674	GFRA1	GDNF family receptor alpha 1	2.74	-1.16	3.67	1.45E-03	1.66E-01	-1.82
100128054	PLCE1-AS1	PLCE1 antisense RNA 1	2.74	-1.46	3.62	1.65E-03	1.66E-01	-1.87
100216001	LINC00704	long intergenic non-protein coding RNA 704	2.75	-0.79	2.59	1.73E-02	2.06E-01	-3.06
4103	MAGEA4	MAGE family member A4	2.77	-1.14	3.85	9.64E-04	1.64E-01	-1.81
255738	PCSK9	proprotein convertase subtilisin/kexin type 9	2.87	0.16	3.21	4.29E-03	1.90E-01	-2.62
51266	CLEC1B	C-type lectin domain family 1 member B	2.89	-0.69	1.35	1.90E-01	4.21E-01	-4.58
2353	FOS	Fos proto-oncogene, AP-1 transcription factor subunit	2.91	2.99	1.68	1.08E-01	3.28E-01	-4.40
50487	PLA2G3	phospholipase A2 group III	2.92	-0.45	2.60	1.70E-02	2.05E-01	-3.13
10117	ENAM	enamelin	3.08	-1.12	3.49	2.21E-03	1.74E-01	-2.07
9173	IL1RL1	interleukin 1 receptor like 1	3.70	1.26	3.45	2.47E-03	1.77E-01	-1.57

Table 3. Transcriptome results of the top 30 genes that are differentially regulated in *BMPR2^{DelTail}* ECs relative to wild type ECs. Highlighted in pink are 15 down-regulated genes and in blue are 15 up-regulated genes in *BMPR2^{DelTail}* ECs. Included in the table are LogFC showing the fold change relative to wild type, P-values and adjusted p-values.

Cell line	Mesoderm Formation	Phosphorylation p38 MAPK 1hr	Phosphorylation SMADs 1hr (Density)	Total SMAD 1hr	Cardiac Differentiation	Blood Cells	Smooth muscle cells	Endothelial cells	PDGF- α Positive cells	Proliferation rate (cell number vs. time)	Angiogenesis	Scratch assay rate (area vs time)
Wild type	✓	1=normal levels	1	1	✓	✓	✓	✓	✗	Ref.	Nicely formed structures	Ref.
<i>BMPR2^{Null}</i>	✓	1.6	1.6	0.6	✓	✓	✓	✓	✓	* $p<0.02$	Thinly formed tubes	Insignificant but increased
<i>BMPR2^{DelKinase}</i>	✓	2	2	0.8	✓	✓	✓	✓	✓	* $p<0.01$	Thinly formed tubes	$p<0.02$
<i>BMPR2^{DelTail}</i>	✓	0.5	1.4	0.3	✓	✓	✓	✓	✓	* $p<0.04$	Thin tubes and some unformed monolayer of ECs	$p<0.05$

Table 4. Summary results of *BMPR2* mutants relative to wild type. Overall, *BMPR2* mutants have dysfunctional canonical and non-canonical signalling, defective tubes formed, increased proliferation and motility rates.