Contributions of *BMPR2* mutations and extrinsic factors to cellular phenotypes of pulmonary arterial hypertension revealed by iPSC modeling

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To the Editor,

Reduced bone morphogenetic protein receptor 2 (BMPR2) signaling is central to the pathobiology of pulmonary arterial hypertension (PAH). However, the reduced penetrance of *BMPR2* mutations in families suggests that other factors are required to establish disease (1). To date, it has proved difficult to elucidate these factors due to a lack of appropriate models. Sa *et al.* (2017) developed an iPSC-EC model of PAH that recapitulated some of the previously described phenotypes of patient-derived PAECs, as well as appropriate responsiveness to Elafin and FK506 (2). This demonstrated a potential utility of iPSCs in modeling PAECs in PAH. However, other phenotypes such as inner mitochondrial membrane (IMM) hyperpolarization, could not be recapitulated. Therefore, there is a need to better understand the contribution of *BMPR2* mutations to PAH-associated phenotypes and the requirement for other factors in this process. Two advantages of iPSCs in disease modeling are their amenability to genome editing and their differentiation into specific cell types under serum-free, chemically-defined conditions. This allows the assessment of the impact of a *BMPR2* mutation without the confounding effects of genetic differences between cell lines, and to determine the impact of controlled exposure to extrinsic factors that may influence the acquisition of a diseased state. In addition, no iPSC-smooth muscle cell (SMC) model of PAH has yet been described. We have addressed these issues.

Methods

Using CRISPR-Cas9-mediated homologous recombination in a wild-type iPSC line, two isogenic sub-lines carrying either (i) a known causal *BMPR2* mutation (W9X) (referred to as C2 $W9X^{+/-}$), or (ii) a deletion of exon 1 (C2 ΔExon1) were generated. Serum-free, chemically-defined iPSC differentiation protocols were used to generate iPSC-SMCs and iPSC-ECs. This was achieved by differentiating iPSCs into iPSC-SMCs via either a lateral plate mesoderm (LM), paraxial mesoderm (PM) or neural ectoderm (NE) lineage followed by 12 days in TGF-β1 and PDGF-BB +/- BMP4 (Figure 1A) (3), and into ECs via FGF-2-, BMP4- and LY294002-induced mesoderm followed by FGF-2 and VEGF +/- BMP4. iPSC-SMCs were compared to adult distal and proximal PASMCs by microarray analysis. Cells were used post-differentiation and in chemically-defined conditions, and exposed to additional factors such as serum, BMP4 and TNFα. Key PAH-associated cellular phenotypes, including altered apoptosis (via caspase cleavage and annexin/PI staining), proliferation (via DNA content and cell counts) and IMM polarization (via TMRE staining), which are cellular changes common to both SMCs and ECs (4), were assessed.

Results and Discussion

The *BMPR2* mutations introduced into C2-iPSCs resulted in *BMPR2* haploinsufficiency in an otherwise isogenic background compared to the wild-type parent iPSC line. This approach removed the effects that different genetic modifiers (5) may have on the penetrance of cellular phenotypes.

Derivation of iPSC-SMCs and -ECs that perfectly represent adult pulmonary artery smooth muscle cells (PASMCs) and PAECs is yet to be achieved. Therefore, our goal was to generate iPSC-derived SMCs (iPSC-SMCs) that recapitulated some of the important functional responses of adult-derived distal PASMCs, as well as iPSC-ECs with enhanced

expression of arterial markers, that could be used as surrogates for adult pulmonary vascular cells. The lineage-specific differentiation protocols used generated iPSC-SMCs expressing SMA, CALPONIN and MYH11 (Figure 1A and data not shown) that had a contractile phenotype (data not shown), as well as iPSC-ECs which were enriched for arterial-specific EC markers including *ACVRL1*, *CLDN5*, *EFNB2*, *NOTCH1*, *JAG1* and *JAG2 and* able to form vascular networks (data not shown) (6). Principle component analysis of microarray gene expression data for 171 SMC-associated genes showed that LM-SMCs were more similar to distal PASMCs compared to PM- and NE-derived SMCs (Figure 1B). In addition, LM-SMCs were not growth suppressed by BMP4 (50 ng/ml) and were less apoptotic when treated with BMP4, similar to the responses previously described in distal PASMCs from donors (Figures 1C and 1D) (7).

Under these serum-free, chemically-defined conditions, *BMPR2* heterozygosity alone was sufficient to cause reduced apoptosis and increased proliferation in iPSC-SMCs (Figures 2A and 2B). However, *BMPR2* heterozygosity in iPSC-ECs required additional exposure to serum to manifest increased proliferation and apoptosis (Figures 2C-2F). These findings were confirmed by performing cell counts to assess proliferation, and annexin-V-FITC propidium iodide staining to measure apoptosis (data not shown). Taken together, these results demonstrate a clear difference in the contribution of *BMPR2* heterozygosity to establishing disease phenotypes in SMCs and ECs, and therefore highlights an important difference between these cell types. By contrast, neither cell type displayed hyperpolarized IMMs as they emerged from the serum-free iPSC differentiation protocols (Figures 2G and 2I). Only after serum +/- TNFα exposure for 1 week for iPSC-ECs (Figure 2J), and serum + TNFα exposure for 1 week or serum-only for 2 weeks for iPSC-SMCs (Figures 2H, 2K and 2L), did these cells acquire IMM hyperpolarization. IMM hyperpolarization is a key factor in pulmonary vascular remodeling, but how a hyperpolarized state is established in the context

of *BMPR2* heterozygosity was not known. Recently, BMP9 was shown to reverse PAH in rodent models, mainly via its action on PAECs (8). In iPSC-ECs, IMM hyperpolarization could be prevented by exposure to BMP9 (1 ng/ml) (Figure 2J), potentially exposing one of the possible modes of action of BMP9 in reversing PAH. Remarkably, *BMPR2*$^{+/-}$ iPSC-SMCs demonstrated prolonged hyperpolarization despite withdrawal of TNFα (Figure 2L). This suggests that transient exposure to a disease-triggering agent may be sufficient to drive the progression of disease in a *BMPR2* mutation carrier.

The significance of these findings is that this iPSC system can be used to address the controversial question of whether genetic reduction of BMPR2 alone is necessary and/or sufficient for establishing the major cellular phenotypes associated with PAH. This would be extremely difficult to address in patient-derived primary cells. The use of specialized differentiation protocols with minimal interference from extrinsic factors allowed the effect of *BMPR2* heterozygosity in SMCs and ECs to be shown definitively. Extrinsic factors were then added in a highly controlled manner to show their impact on establishing PAH-associated cellular phenotypes. In essence, it was possible to transition cells from a pre-diseased to a diseased state, opening the way to discovering new druggable pathways to prevent or reverse PAH. Although the generation of pulmonary SMCs and ECs from iPSCs is yet to be achieved, the differentiation protocols used in this study produced cells that recapitulated key phenotypes found in diseased adult PASMCs and PAECs. Thus, these protocols will have broad impact for those modeling pulmonary vascular diseases, and also for those using pulmonary organoids and pulmonary artery-on-chip technologies to study epithelial-endothelial cell interactions in the alveoli and for drug screening. Finally, this study defines an iPSC-derived SMC model of PAH. Only EC and mesenchymal iPSC models of PAH have been described previously, and the mesenchymal model did not recapitulate the pro-proliferative phenotype of SMCs from patients with PAH (9).

Figure 1. Generation of PASMC-like iPSC-SMCs.

(*A*) Wild-type and *BMPR2*$^{+/-}$ iPSCs differentiated into iPSC-SMCs from lateral plate mesoderm (LM), paraxial mesoderm (PM) and neuroectoderm (NE) express smooth muscle actin (SMA) and calponin (green), (DAPI: blue) (scale bar 20 μm). (***B***) Gene expression patterns of all samples (human pulmonary artery smooth muscle cells (PASMCs), paraxial mesoderm (PM-SMCs), neuroectoderm (NE-SMCs), and lateral plate mesoderm (LM-SMC) smooth muscle cells) were analyzed using Illumina HumanHT-12 v4 Expression BeadChip microarrays. Gene expression patterns of samples were sorted based on similarity by hierarchical clustering. For this analysis, 171 SMC-specific genes were selected based on the wikipathway database (WikiPathway WP2064 revision 47071). The 2D principal component analysis for differential gene expression are plotted in (***B)***. Legend squares: Red (PASMCs), blue (LM-SMCs), dark green (NE-SMCs) and pink (PM-SMCs). (***C***) LM-SMCs are not growth suppressed by BMP4 (50 ng/ml), unlike PM- and NE-SMCs. (***D***) The apoptotic response in wild-type LM-SMCs is reduced in the presence of exogenous BMP4 (50 ng/ml), as previously described for distal PASMCs. Data in (***C*)** and (***D*)** presented as mean ± s.e.m. of 3 biological replicates (* $P < 0.05$, *** $P < 0.001$, one-way ANOVA (**C**) and Student's *t* test (**D**)).

Figure 1

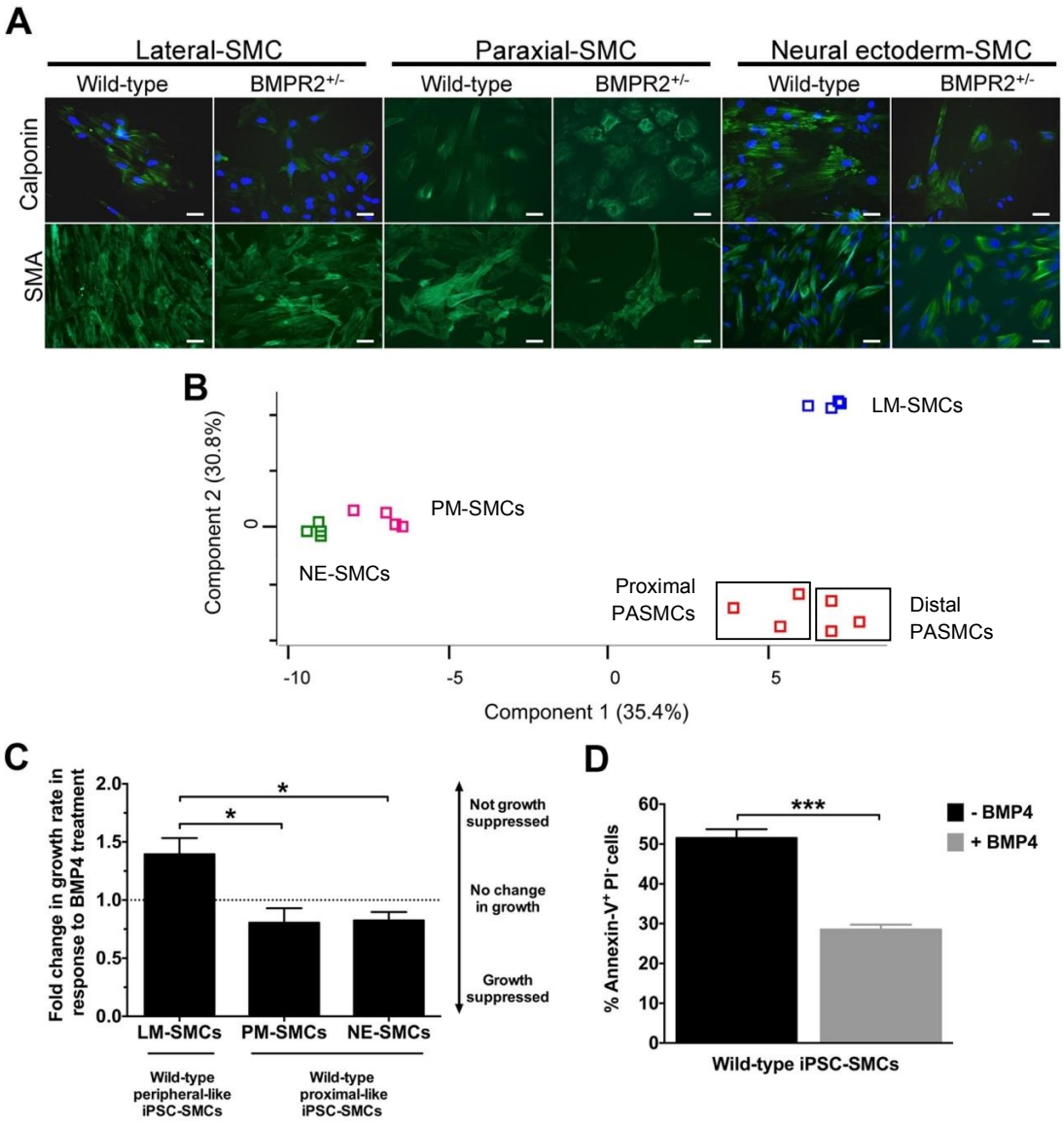
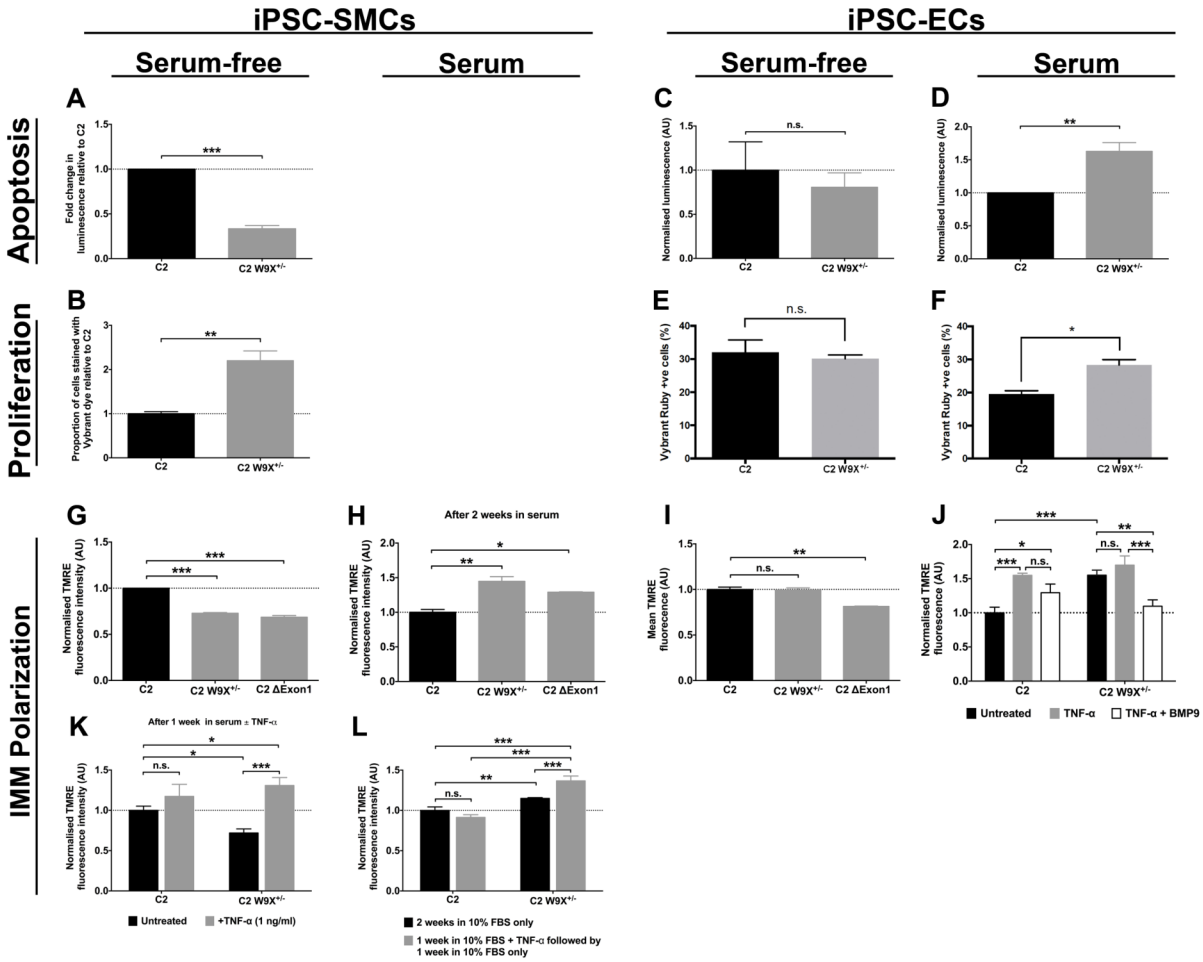

A
Lateral-SMC
Paraxial-SMC
Neural ectoderm-SMC
Wild-type
BMPR2+/-
Wild-type
BMPR2+/-
Wild-type
BMPR2+/-
Calponin
SMA
B
LM-SMCs
PM-SMCs
NE-SMCs
Proximal PASMCs
Distal PASMCs
Component 2 (30.8%)
Component 1 (35.4%)
0
-10
-5
0
5
C
Fold change in growth rate in response to BMP4 treatment
2.0
1.5
1.0
0.5
0.0
*
*
Not growth suppressed
No change in growth
Growth suppressed
LM-SMCs
PM-SMCs
NE-SMCs
Wild-type peripheral-like iPSC-SMCs
Wild-type proximal-like iPSC-SMCs
D
% Annexin-V+ PI- cells
60
50
40
30
20
10
0
***
- BMP4
+ BMP4
Wild-type iPSC-SMCs

Figure 2. Effect of *BMPR2* heterozygosity on proliferation, apoptosis and inner mitochondrial membrane polarization of iPSC-SMCs and iPSC-ECs. C2 W9X$^{+/-}$ iPSC-SMCs are significantly less apoptotic (***A***) and more proliferative (***B***) compared to wild-type C2 iPSC-SMCs under serum-free conditions, assessed using the Caspase-3/7 Glo assay (***A***) and by measuring double-stranded DNA content by Vybrant DyeCycle Ruby staining (***B***). By contrast, there was no significant difference in apoptosis (***C***) and proliferation (***E***) between C2 and C2 W9X$^{+/-}$ iPSC-ECs under serum-free conditions. C2 W9X$^{+/-}$ iPSC-ECs became significantly more apoptotic (***D***) and proliferative (***F***) relative to isogenic wild-type C2 iPSC-ECs after exposure to 10% FBS for 1 week. (***G***) Serum-free *BMPR2*$^{+/-}$ iPSC-SMCs display a hypopolarized inner mitochondrial membrane (IMM) compared to isogenic wild-type cells, assessed using TMRE staining and flow cytometry. *BMPR2*$^{+/-}$ iPSC-SMCs became hyperpolarized compared to isogenic wild-type cells after two weeks of exposure to serum (***H***) or after one week of exposure to serum and TNFα (1 ng/ml) (***K***). (***L***) After one week of exposure to serum + TNFα, TNFα was removed and all cells cultured for one further week in serum only to see if the polarization state would recover. The polarization state of *BMPR2*$^{+/-}$ iPSC-SMCs did not normalize and was significantly higher than exposure to serum only for 2 weeks, and was also significantly higher than in isogenic wild-types treated the same way. (***I***) *BMPR2*$^{+/-}$ iPSC-ECs do not have a hyperpolarized IMM relative to the isogenic wild-type under serum-free conditions, but become significantly hyperpolarized after exposure to serum for one week (***J***). Wild-type iPSC-ECs showed significantly higher TMRE fluorescence after TNFα treatment (1 ng/ml), suggesting that TNFα increases inner mitochondrial membrane hyperpolarization. Co-treatment of C2 W9X$^{+/-}$ iPSC-ECs with TNFα (1 ng/ml) and BMP9 (1 ng/ml) for one week resulted in significantly reduced TMRE fluorescence and hence IMM polarization compared to the effect of TNFα alone. Data presented as mean ± s.e.m of the results from 3 independent differentiations (***A-F***) and 3 technical replicates per differentiation

(* P < 0.05, ** P < 0.01, *** P < 0.001, Student's *t* test (***A-F***), one-way ANOVA (***G-I***) or two-way ANOVA (***J-L***)).

Figure 2



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