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Co-dependence of BMPR2 and TGFβ in Elastic Fiber Assembly and its Perturbation in Pulmonary Arterial Hypertension

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Abstract

Objective—We determined in patients with pulmonary arterial (PA) hypertension (PAH) whether in addition to increased production of elastase by PA smooth muscle cells (SMC) previously reported, PA elastic fibers are susceptible to degradation owing to their abnormal assembly.

Approach and Results—Fibrillin-1 and elastin are the major components of elastic fibers, and fibrillin-1 binds bone morphogenetic proteins (BMPs) and the large latent complex of transforming growth factor-β1 (TGFβ1). Thus, we considered whether BMPs like TGFβ1 contribute to elastic fiber assembly and whether this process is perturbed in PAH particularly when the BMP receptor, BMPR2, is mutant. We also assessed whether in mice with *Bmpr2/1a* compound heterozygosity, elastic fibers are susceptible to degradation.

In PA SMC and adventitial fibroblasts (PAF), TGFβ1 increased elastin mRNA but the elevation in elastin protein, was dependent on BMPR2; TGFβ1 and BMP4, via BMPR2, increased extracellular accumulation of fibrillin-1. Both BMP4- and TGFβ1-stimulated elastic fiber assembly were impaired in idiopathic (I) PAH-PAF vs. control cells, particularly those with hereditary (H) PAH and a *BMPR2* mutation. This was related to profound reductions in elastin and fibrillin-1 mRNA. Elastin protein was increased in IPAH PAF by TGFβ1 but only minimally so in *BMPR2* mutant cells. Fibrillin-1 protein increased only modestly in IPAH or HPAH PAF stimulated with BMP4 or TGFβ1. In *Bmpr2/1a* heterozygote mice, reduced PA fibrillin-1 was

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associated with elastic fiber susceptibility to degradation and more severe pulmonary hypertension.

Conclusion—Disrupting BMPR2 impairs TGFβ1 and BMP4 mediated elastic fiber assembly and is of pathophysiologic significance in PAH.

Keywords

fibrillin-1; pulmonary hypertension; extracellular matrix; vascular biology; growth factors and cytokines

Introduction

Pulmonary arterial hypertension (PAH) is a disease characterized by a progressive loss and obliteration of distal pulmonary arteries (PAs) that result in elevation of PA pressure, increased pulmonary vascular resistance to flow, right-sided heart failure and high morbidity as well as mortality. Developing effective treatments for PAH will require an approach that reverses the fundamental mechanisms causing the pulmonary vascular pathology.

Mutations resulting in loss of function of bone morphogenetic protein receptor 2 (BMPR2) occur in >70% of patients with familial PAH (FPAH) and in 25% of those with idiopathic PAH (IPAH), collectively denoted as hereditary or HPAH[1, 2]. Reduced expression of *BMPR2* was shown in patients with IPAH without a mutation and even when PAH was related to other conditions (APAH)[3]. There is a low 20% penetrance of PAH in families carrying a *BMPR2* mutation that has been partially addressed by recent genetic studies, indicating that affected vs. non-affected family members have reduced expression of *BMPR2* from the normal allele[4] or polymorphisms causing heightened transforming growth factor beta (TGFβ)1 signaling[5]. Other studies documented a reduction in the BMPR2 ligand, bone morphogenetic protein (BMP) 4[6]. We have reported compensatory signaling pathways and gene expression in the unaffected *BMPR2* mutation carriers[7].

Elastic fibers provide elastic recoil to tissues such as the large arteries, lung, and skin. They consist of two major morphologically distinct components: elastin, a cross-linked polymer of tropoelastin, the monomeric secreted form of the protein[8], and fibrillins, primarily fibrillin-1, a large (350 kDa), cysteine-rich glycoprotein[9]. Fibrillin microfibrils are the pivotal structures involved in storage and regulation of growth factors of the TGFβ superfamily, including TGFβ1 and BMPs[10, 11]. While the role of TGFβ signaling in the formation of elastic fibers in arteries as well as in other tissues is well known[12], the contribution of BMPs to this process has not been studied.

Previous reports by our group have shown degradation of elastic fibers as a prominent feature of PAH, related to elevation in PA elastase activity identified as neutrophil elastase[13]. This enzyme is pivotal to vascular pathobiology since it releases mitogenic growth factors that are normally bound to intact elastic fibers[14] and other matrix components. Degradation products of elastin, elastin peptides, are highly pro-inflammatory[15], promoting the recruitment of activated inflammatory cells that produce cytokines and elastase that perpetuate adverse vascular remodeling. Mutations in the elastin gene (*ELN*) are associated

with vascular disorders causing stenosis of pulmonary and systemic arteries in Willliams syndrome[16], whereas mutations in the fibrillin-1 gene (*FBN1*) cause aneurysmal dilatation in Marfan syndrome[17].

Given the physical association of BMPs and TGFβ with fibrillin-1, we hypothesized that these growth factors could play a complementary role in the organization and stability of the elastic fiber. Our study uncovered a co-dependence between BMPR2 and TGFβ in the assembly of the elastic fiber. TGFβ-stimulated production of the elastin protein was BMPR2-dependent, as was BMP-and TGFβ–mediated production of fibrillin-1. Hence elastic fiber assembly was impaired in IPAH vascular cells, particularly in those cells from patients with HPAH and a *BMPR2* mutation. In transgenic mice with compound heterozygosity for *Bmpr2/1a*, that develop more severe pulmonary hypertension than wild type mice, we show production of a fibrillin-1 poor elastic fiber that is susceptible to elastase-mediated degradation. This is consistent with more advanced pathological features noted in HPAH patients with a *BMPR2* mutation[18] that have more rapid progression of disease[19].

Materials and Methods

Materials and Methods are available in the online-only Data Supplement.

Results

TGFβ1 increases elastin mRNA and protein; TGFβ1 and BMP4 increase fibrillin-1 protein

To investigate the relative roles of TGFβ1 and BMP4 signaling in the assembly of elastic fibers, we used human PA adventitial fibroblasts (PAF) and PA SMC at passages 3–6 cultured from donor control lungs from the Pulmonary Hypertension Breakthrough Initiative (see Materials and Methods). We found that TGFβ1 substantially increased *ELN* mRNA in PAF at 4h and 8h after stimulation (Fig. 1A) and elastin protein assessed at 48 h (Figure 1B). While BMP4 alone did not increase elastin mRNA or protein, it enhanced production of elastin protein when added to TGFβ1 (Figure 1B). Neither TGFβ1 nor BMP4 increased *FBN1* mRNA levels in PAF (Fig. 1C). However, BMP4 and TGFβ1 increased fibrillin-1 protein in PAF conditioned media (Fig. 1D). There was, however no increase when the two were administered together. BMP4 was chosen as the ligand that best produced fibrillin-1 following pilot studies that also assessed BMP2 and BMP7 (data not shown). We could not account for the BMP4-mediated increase in fibrillin-1 that accumulated in the conditioned media on the basis of an increase in fibronectin previously described[20] (Suppl. Fig. IA). We also investigated whether BMP4-mediated transcription of other elastin assembly proteins could promote accumulation of fibrillin-1, but saw no significant elevation in mRNA for emilin-1, lysyl oxidase, fibulin-5, and microfibrillar associated glycoprotein 1 or 2 (Suppl. Fig. IB). PAF were then stimulated with BMP4, TGFβ1 or both every other day for seven days to generate elastic fibers. We used both elastin (Figure 1E) and fibrillin-1 (Figure 1F) antibodies and applied quantitative morphometric techniques to images obtained by confocal microscopy to measure linear elastic immunoreactive fibers. Despite the fact that BMP4 did not increase elastin protein, we saw similar elastin immunoreactivity in the fibers when compared to TGFβ1. There was also an enhanced effect when the two were administered

together. As with the biochemical data, BMP4 and TGFβ each resulted in fibrillin immunoreactivity in the deposited fibers but combining the two agents did not result in an increase in fibrillin. This suggested that BMP4 made efficient use of constitutive elastin to assemble elastic fibers.

In PA SMC as in PAF we observed an increase in elastin mRNA (Suppl. Fig. IIA) and protein (Suppl. Fig. IIB) in response to TGFβ1, but no increase with BMP4. As in PAF there was no increase with either agonist in fibrillin-1 mRNA in PA SMC (Suppl. Fig. IIC). However, only BMP4 increased fibrillin-1 protein in PA SMC (Suppl. Fig. IID). In contrast to PAF, PA SMC did not assemble elastic fibers in culture as assessed by confocal microscopy. To determine whether PA SMC were secreting a factor that interfered with elastic fiber assembly, we cultured PA SMC in fibroblast conditioned media and PAF in PA SMC conditioned media (Suppl. Fig. III A and B). In keeping with our hypothesis PA SMC assembled elastic fibers in response to BMP4 or TGFβ1 when cultured in PAF conditioned media whereas PAF cultured in PA SMC conditioned media only produced scant elastic fibers in response to TGFβ1.

We then applied 2-dimensional gel electrophoresis as described in the Methods to identify a secreted factor that suppressed elastic fiber assembly in PA SMC conditioned media that was not present in PAF conditioned media (Suppl. Fig. IIIC). Of the proteins differentially produced the proteoglycan decorin (DCN) (Suppl. Fig. IIID) was known to suppress elastin assembly [21] and it was reduced in the fibroblast relative to PA SMC conditioned media at baseline and with BMP4 or TGFβ1 stimulation. We then reduced decorin in PA SMC by siRNA and showed production of dense elastic fibers, (Suppl. Fig. IIIE) and we also added decorin to PAF conditioned media and suppressed elastic fiber formation (Suppl. Fig. IIIF).

TGFβ1 production of elastin is BMPR2-dependent

To determine the role of BMPR2 in PAF production of elastin and fibrillin-1, we reduced BMPR2 by greater than 60% using siRNA (Fig. 2A). Interestingly we found that TGFβ1 production of elastin protein (Figure 2A) was greatly reduced as was BMP4 and TGFβ1 production of fibrillin-1 (Fig. 2B). Neither the BMPR2-dependent increase in elastin in response to TGFβ1, nor the BMP4-mediated increase in fibrillin-1 could be explained by changes in downstream effectors pSMAD1/5 or p-p38, or in microRNAs previously implicated downstream of BMPR2 such as miR21 or miR29[22, 23] (data not shown). Surprisingly, the TGFβ1 increase in elastin mRNA was preserved despite the loss of BMPR2 (Suppl. Fig. IV). This implied that BMPR2 may be necessary for TGFβ1 mediated translation of elastin mRNA or stability of the elastin protein. Loss of fibrillin-1 by siRNA did not interfere with elastin protein production in response to TGFβ1 (Figure 2A).

Non-targeting (control) siRNA did not affect the linear deposition of elastic fibers in response to BMP4 or TGFβ1 (Fig. 1C). As expected, the elastic fiber network produced following BMP4 stimulation was lost when BMPR2 was reduced by siRNA. While quantitatively the elastic fibers produced in response to TGFβ1 were not reduced with loss of BMPR2, they were more fragmented. This implies that the modest production of fibrillin and elastin in response to TGFβ1 was sufficient to allow for deposition of elastin fibers that

were structurally abnormal. However, experiments with fibrillin siRNA indicated that both TGFβ1 and BMP4 require fibrillin-1 to deposit and assemble elastic fibers.

Fibroblasts from PAH patients have impaired elastic fiber assembly

To investigate the consequence of BMPR2 dysfunction on elastic fiber formation in PAH, we compared PAF from unused donor lungs to PAF from lungs of patients with IPAH, and from those with a *BMPR2* mutation related to HPAH. There was a profound decrease in *ELN* mRNA at baseline with no or minimal response to BMP4 or TGFβ1 in IPAH patients including the group with a *BMPR2* mutation (Fig. 3A). TGFβ1-stimulated an increase in elastin protein in donor control and a more variable increase IPAH PAF, but there was a barely detectable increase in elastin protein in BMPR2 mutant cells (Fig. 3B). In PAF from patients with IPAH and in *BMPR2* mutant cells, there was a profound reduction in *FBN1* mRNA (Fig. 3C) that resulted in reduced fibrillin-1 protein in conditioned media in response to TGFβ1 or BMP4 (Fig. 3D). Thus in IPAH PAF, elastic fiber formation was greatly impaired in response to both BMP4 and TGFβ1, and in cells from the *BMPR2* mutant group, it was barely detectable (Fig. 3E). This is consistent with the loss of fibrillin-1 (Suppl. Fig. V) as well as elastin protein in the latter group.

To relate our findings in cultured PAF to the tissue, we determined whether elastin and fibrillin-1 are also reduced in PAs of PAH patients. Immunofluorescence revealed reduced fibrillin-1 in IPAH PAs, particularly in the internal elastic lamina, and a loss of both elastin and fibrillin-1 in PAs in lungs from patients with HPAH and a *BMPR2* mutation (Fig. 4).

The PA elastic laminae of *Bmpr2/1a* compound heterozygote mice are susceptible to degradation and have more severe pulmonary hypertension

We hypothesized that mice with impaired function of BMPR2 would have poorly assembled elastic fibers that were susceptible to degradation. To test this, mice with compound heterozygosity for *Bmpr2* and *1a (Bmpr2/1a)* were used. We pooled three groups of central PAs from four mice/pool and documented a marked decrease in fibrillin-1 protein without a comparable reduction in elastin in the compound heterozygotes compared to wild type (WT) mice (Fig. 5A). Aortae exhibited a similar abnormality (data not shown).

We then evaluated whether the fibrillin-1 poor elastic fibers in those PAs from *Bmpr2/1a* heterozygotes relative to WT mice, were susceptible to degradation. As in our previous studies[13] we perfused the vessels with either porcine pancreatic elastase or saline vehicle, and counted the number and size of fenestrations in the autofluorescent internal elastic lamina of the main PA by confocal microscopy. Both the area and number of fenestrations were increased twofold in the *Bmpr2/1a* heterozygote vs. WT elastic lamina following perfusion with elastase (Fig. 5B). We also determined whether a pathological insult resulting in pulmonary hypertension in association with an increase in lung elastase activity[24], would result in greater degradation of elastin in *Bmpr2/1* heterozygote vs. WT mice. Following administration of the VEGF receptor blocker Sugen 5416 by subcutaneous injection once a week during a three-week period of hypoxia (10% oxygen)[25], we observed more pronounced degradation of PA elastic fibers in the *Bmpr2/1* heterozygote vs. WT mice (Fig. 5B).

We determined whether the susceptibility of elastic fibers to degradation could be related to more severe pulmonary hypertension in the *Bmpr2/1a* vs. control mice. A decrease in pulmonary artery acceleration time in *Bmpr2/1a* heterozygotes when compared to WT controls (Fig. 5C) was associated with a small but significant increase in right ventricular systolic pressure (Fig. 5D) and with more severe right ventricular hypertrophy (Fig. 5E) following Sugen and hypoxia.

Immunofluorescence of elastin and fibrillin-1 in intrapulmonary arteries assessed at the level of the terminal respiratory unit showed fragmentation of elastic fibers in association with pulmonary hypertension, that was more severe in the *Bmpr2/1a* heterozygotes vs. WT mice (Fig. 5F, G). This was associated with a greater loss of alveolar wall and duct arteries relative to alveoli (Fig. 5H). Muscularization of distal vessels was similar (Suppl. Figure VI).

Discussion

While previous studies have focused on the antagonistic effects of BMPs and TGFβ in PAH[5], our work indicates that, as in development[26], both growth factors that co-occupy the same extracellular matrix protein, fibrillin-1[11, 27], take part in assembling elastic fibers. In this paper we show a previously unappreciated dependence of both TGFβ1 and BMP4 on BMPR2 in the assembly of the two main components of the elastic fiber, elastin and fibrillin-1 (Fig. 6). Neither the dependence on BMPR2 of TGFβ1-mediated production of elastin and fibrillin, nor the regulation of fibrillin-1 accumulation by BMPs had been previously established. In IPAH and HPAH *BMPR2* mutant PAF, the profound reduction in *ELN* and *FBN1* mRNA is related to the impaired production of elastic fibers. The reduction in PA fibrillin-1 in *Bmpr2/1a* heterozygote mice is associated with susceptibility of the elastic fibers to degradation, with more fragmented elastin, a greater loss of distal arteries and more severe pulmonary hypertension in response to VEGF receptor blockade and chronic hypoxia.

Elastic laminae are an integral component of the PA and are fragmented in PAs of PAH patients in association with occlusive changes. Breakdown of elastic laminae is observed with neointimal formation that occurs with age[28] and with vascular inflammation[29], and yet little attention has been paid to whether the underlying composition of the elastic fibers might increase susceptibility to degradation. It is not surprising that TGFβ1 stimulated an increase in elastin mRNA and protein in both PA SMC and PAF, based upon previous studies[30]. It had also been shown that TGFβ stimulates production of fibrillin-1[31] although this was not studied in vascular cells.

Previous models of elastic fiber formation in culture used epithelial cells, dermal fibroblasts or rat smooth muscle cells[32–35]. For the present study, we developed a cell culture system that is robust in assessing factors that contribute to assembly of the elastic fibers in the vessel wall. Our studies revealed the importance of fibrillin-1 production for the formation of stable elastic fibers in culture. It is known that TGFβ1 stimulates elastin synthesis by a mechanism involving stabilization of the mRNA[36]. Loss of BMPR2 function likely impairs the stability of the elastin protein as the TGFβ1 mediated increase in elastin mRNA is preserved. The

reduction in elastin protein may be related to metabolic changes associated with loss of BMPR2[37] that could perturb post-translational modifications necessary for elastin protein stability. This and the mechanism that explains the profound reduction in *ELN* and *FBN1* mRNA in PAH cells will be of interest to pursue in future studies. Despite the failure of TGFβ1 to increase *ELN* mRNA in IPAH PAF, elastin protein was still increased. In *BMPR2* mutant PAF, TGFβ could not increase elastin protein. Impaired stimulation of fibrillin-1 protein by both BMP4 and TGFβ1 may be largely related to the low mRNA levels in IPAH and *BMPR2* mutant HPAH PAF. This is reflected in fibrillin-poor PAs in the tissue sections of IPAH patients. The reduction in elastin production in response to TGFβ1 that was prominent in PAH cells with a BMPR2 mutation is reflected in the more profound loss of elastin in elastic fibers in the PAs from these patients.

We found that the elastic fibers in the PAs of *Bmpr2/1a* compound heterozygote compared to WT mice are more susceptible to degradation by exogenous administration of elastase. It is the endogenous lung elastase associated with exposure to Sugen 5416 and chronic hypoxia[24] that resulted in elastic fiber degradation in the PAs of both genotypes. However, the enhanced susceptibility to degradation observed in the *Bmpr2/1a* compound heterozygote compared to WT mice can be ascribed to the decrease in fibrillin-1. It was unexpected to find no reduction in elastin protein in the PAs of the *Bmpr2/1a* compound heterozygote compared to WT mice despite the fact that TGFβ1 is dependent on BMPR2 to produce elastic fibers. Perhaps reproducing this dependency in the mice requires greater deficiency of BMPR2 than haploinsufficiency, as was achieved with *BMPR2* siRNA, or as is observed in the PAH subgroup with a *BMPR2* mutation.

Despite the loss of fibrillin-1 and susceptibility of the elastic fiber to degradation, we did not see the aneurysmal dilatation observed when there is a mutation in *FBN1*, as in Marfan syndrome[38]. Recently SMC apoptosis has been described in transgenic mice with a mutation in *FBN1*[39] and loss of BMPR2 signaling by reduced *BMPR2* can lead to resistance to apoptosis in SMC[40]. *Bmpr2* heterozygous mice have no[41] or little[42] resting elevation of pulmonary arterial pressure under normal conditions or even with hypoxia alone[41]. We reasoned that compound heterozygosity might bring out a more consistent increase in hypoxia-induced pulmonary hypertension, but it was necessary to use an additional stimulus, i.e., VEGF receptor blockade. It is interesting that the pathological features associated with the more severe pulmonary hypertension related to a greater loss of distal arteries rather than to increased muscularization, but we anticipate that this feature, along with a greater increase in RVSP and RVH, may progress over time.

While heightened activity of TGFβ has been previously related to reduced activity of BMPR2 in pulmonary hypertension[43, 44] our study shows a co-dependence between BMPR2 and TGFβ1 in assembling the key components of the elastic fiber. This reinforces the need for strategies that evaluate therapies for their ability to restore a balance between TGFβ and BMPR2 signaling.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- In pulmonary arterial smooth muscle cells and fibroblasts, TGFβ1 increases elastin mRNA and protein and fibrillin protein in a BMPR2 dependent manner and BMP4 also requires BMPR2 to increase extracellular fibrillin-1.
- Elastic fiber assembly is impaired in pulmonary arterial fibroblasts from patients with IPAH, particularly when there is HPAH and *BMPR2* mutation.
- Greater degradation of elastic fibers is apparent in IPAH patient pulmonary arteries particularly in those with HPAH and a BMP2 mutation.
- Mice with compound heterozygosity for *Bmpr2/1a* have fibrillin-1 poor elastic fibers that are susceptible to degradation by elastase or following exposure to VEGF receptor blockade and hypoxia.

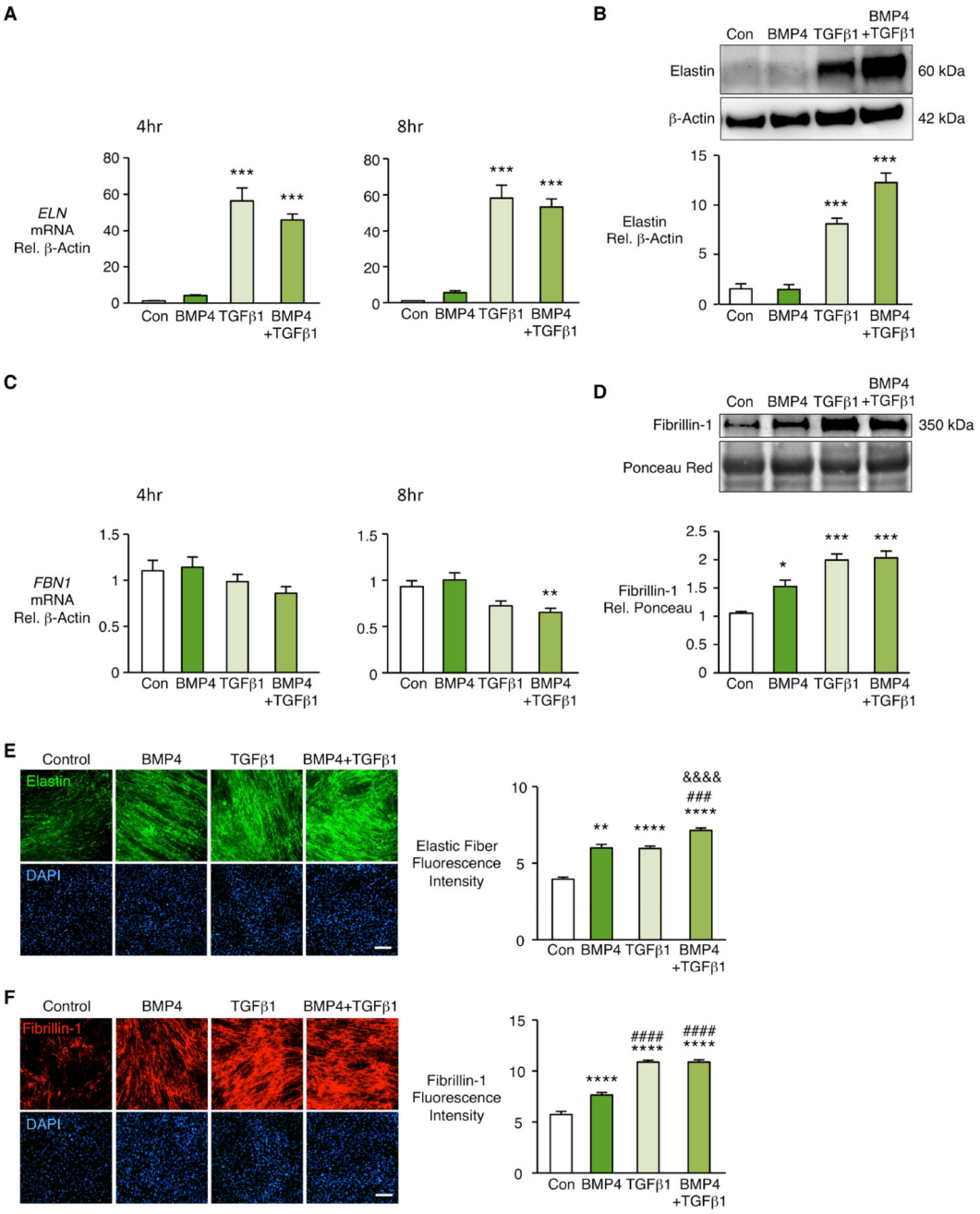

Figure 1. TGFβ1 increases elastin mRNA and protein and TGFβ1 and BMP4 increase extracellular fibrillin-1 in human pulmonary artery fibroblasts

Human pulmonary artery fibroblasts (PAF) isolated from unused donor lungs and used between passages 3–6 were stimulated with BMP4 (10ng/ml), TGFβ1 (2ng/ml), BMP4+TGFβ1, or vehicle (Con). **(A, C)** Fold change in mRNA of *ELN* and *FBN1* was measured four and eight hours after stimulation. **(B, D)** Representative immunoblots above and densitometry below for elastin in cell lysates and fibrillin-1 in conditioned media 48h after stimulation. Beta-actin and Ponceau staining were used as loading controls for cell lysates and conditioned media respectively. Fibrillin-1 is normalized to Con. Bars represent

Mean±SEM of n=3–5 independent experiments, *p<0.05, **p<0.01, ***p<0.001 vs. Con, by one-way ANOVA and post-hoc Bonferroni test.

(E, F) PAF of donor controls were seeded in glass chamber slides and grown for four days to confluence. Cells were starved overnight, then stimulated every other day for seven days with vehicle (Con), BMP4, TGFβ1 or BMP4+TGFβ1. Elastic fibers were visualized by indirect immunofluorescence of elastin **(E)** and fibrillin-1 **(F)**. Right, fluorescence intensities quantified by Image J software and normalized to cell number assessed by nuclei DAPI staining. Scale bar=100μm. Bars represent Mean±SEM of n=4 independent experiments, **p<0.01, ****p<0.0001 vs. Con; $^{\#\#\#}$p<0.001, $^{\#\#\#\#}$p<0.0001 vs. BMP4; $^{\&\&\&\&}$p<0.0001 vs. TGFβ1, by two-way ANOVA and post-hoc Bonferroni test.

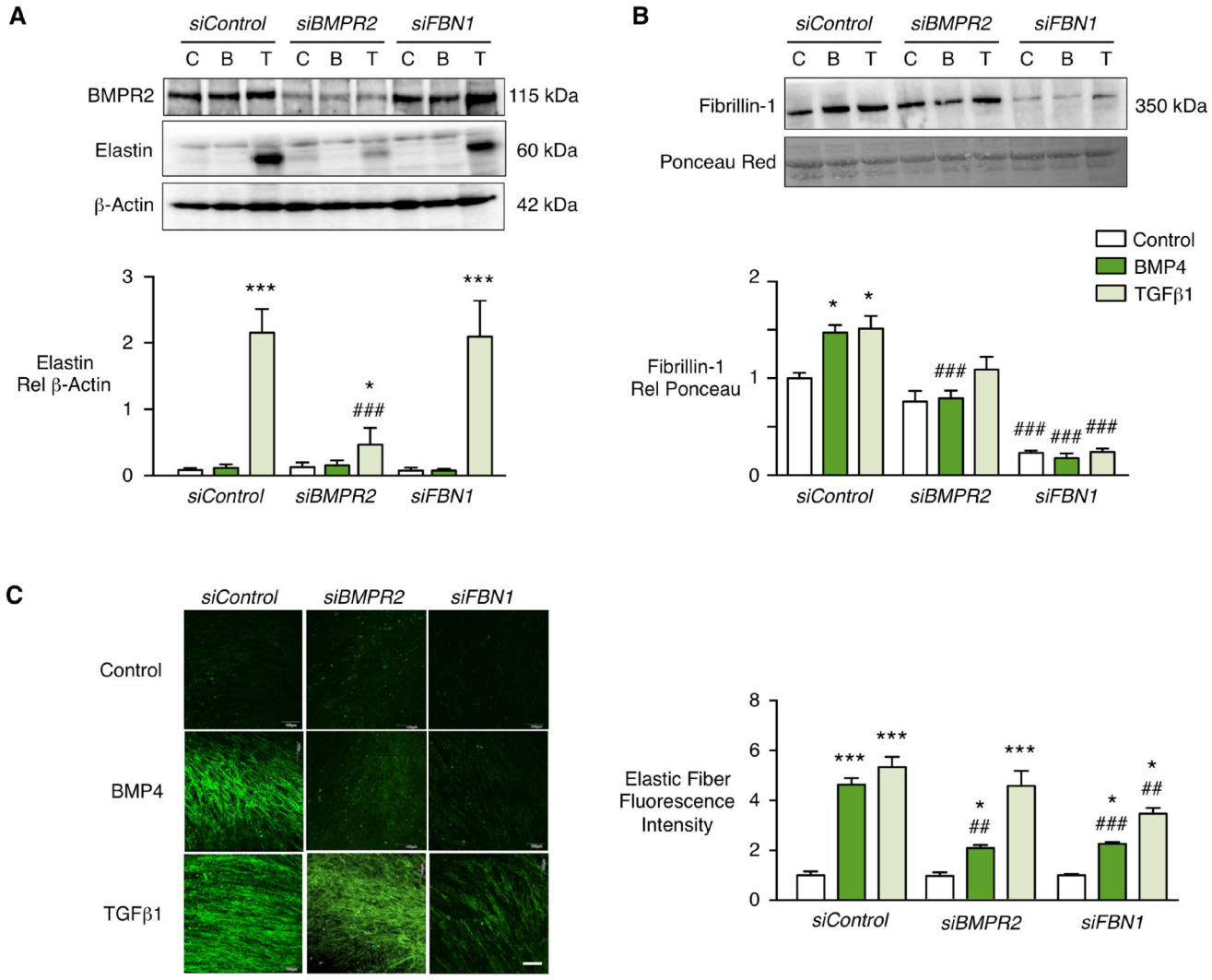

Figure 2. BMPR2 and fibrillin-1 are required for PAF elastic fiber formation

PAF of donor controls were transfected with siRNA oligonucleotides targeting *BMPR2* or *FBN1*, or with non-targeting siRNA (*siControl*). Starting 24h after transfection, the cells were stimulated for 48h with BMP4 (10ng/mL), TGFβ1 (2ng/mL) or vehicle (Control). **(A, B)** Representative immunoblot above and densitometry below for elastin and BMPR2 in cell lysates **(A)** and fibrillin-1 in conditioned media **(B)** of PAF donor controls. β-actin and Ponceau were used as loading controls for cell lysates and conditioned media respectively. **(C)** To assess elastic fibers cells were stimulated every other day for seven days and then visualized by indirect immunofluorescence of elastin and quantified by Image J software. Note that reduction of BMPR2 and fibrillin-1 inhibited BMP4-induced elastic fiber formation, and reduced fibrillin-1 also inhibited TGFβ1-induced fiber formation. The elastic fibers induced by TGFβ1 appear more fragmented in cells treated with BMPR2 siRNA. Scale bar=100μm. Bars represent Mean±SEM of n=3 independent experiments, *p<0.05, ***p<0.001 vs. unstimulated Control; ##p<0.01, ###p<0.001 vs. non-targeting siRNA (*siControl*) by two-way ANOVA and post-hoc Bonferroni test.

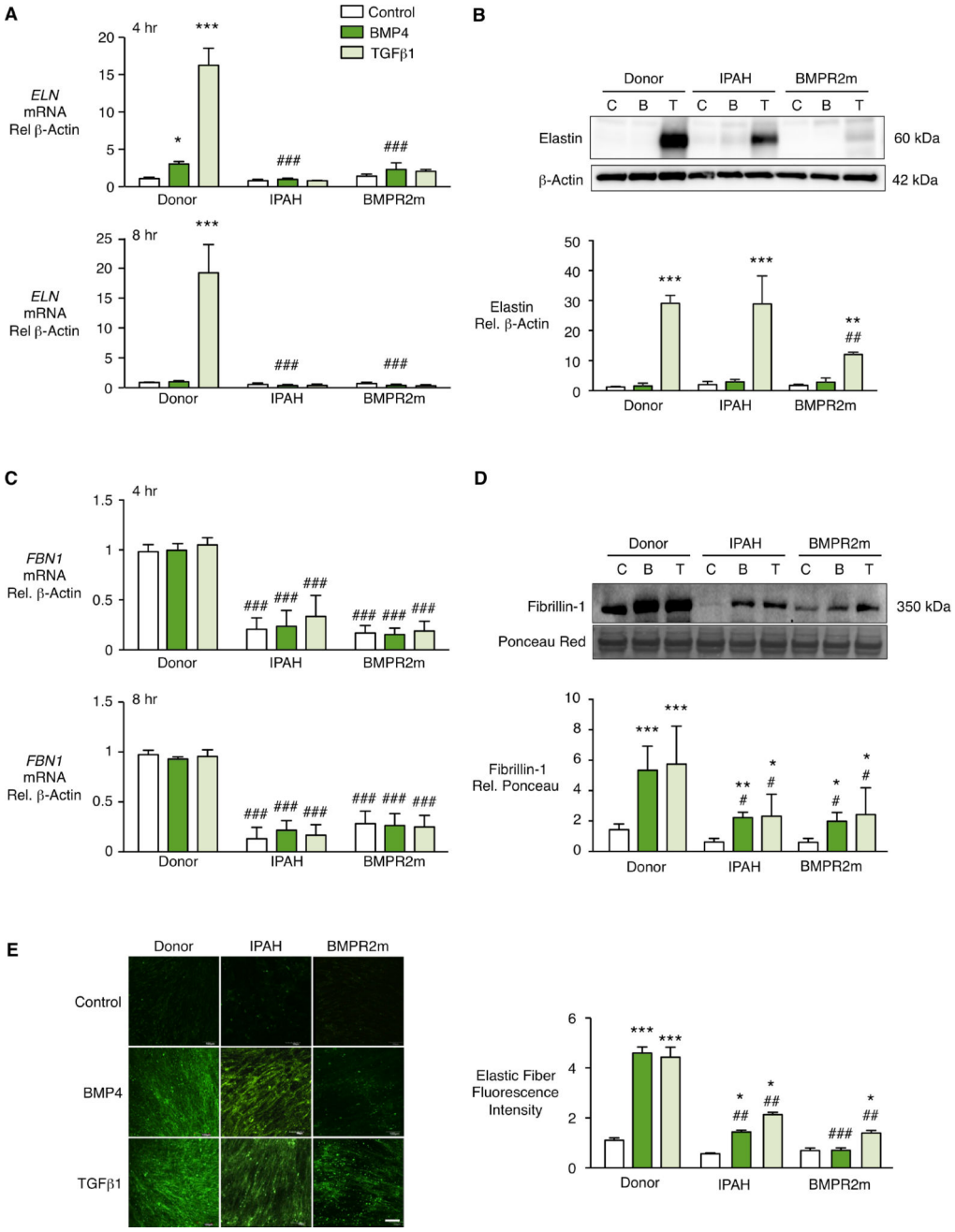

Figure 3. Reduced PAF elastic fibers from IPAH and HPAH with *BMPR2* mutation (BMPR2m) related to elastin and fibrillin-1

Elastic fiber formation by PAF from donor controls, IPAH and HPAH with a *BMPR2* mutation (*BMPR2m*) analyzed as described in Figure 2. Fold change in *ELN* and *FBN1* mRNA **(A, C)** measured four and eight hours following stimulation by BMP4 (10ng/ml), TGFβ1 (2ng/ml), or vehicle Control. Representative immunoblot and densitometry for elastin in the cell lysates and fibrillin-1 in the conditioned media **(B, D)** measured 48 hours after stimulation. **(E)** Elastic fibers were visualized by indirect immunofluorescence of elastin and quantified by Image J software. Scale bar=100μm. β–Actin and Ponceau were

used as loading controls. Bars represent Mean±SEM of n=3 different cell lines (donor controls) per condition, *p<0.05, **p<0.001, ***p<0.001 vs. unstimulated (Control); #p<0.05, ##p<0.01, ###p<0.001 vs. Donor, by two-way ANOVA and post-hoc Bonferroni test.

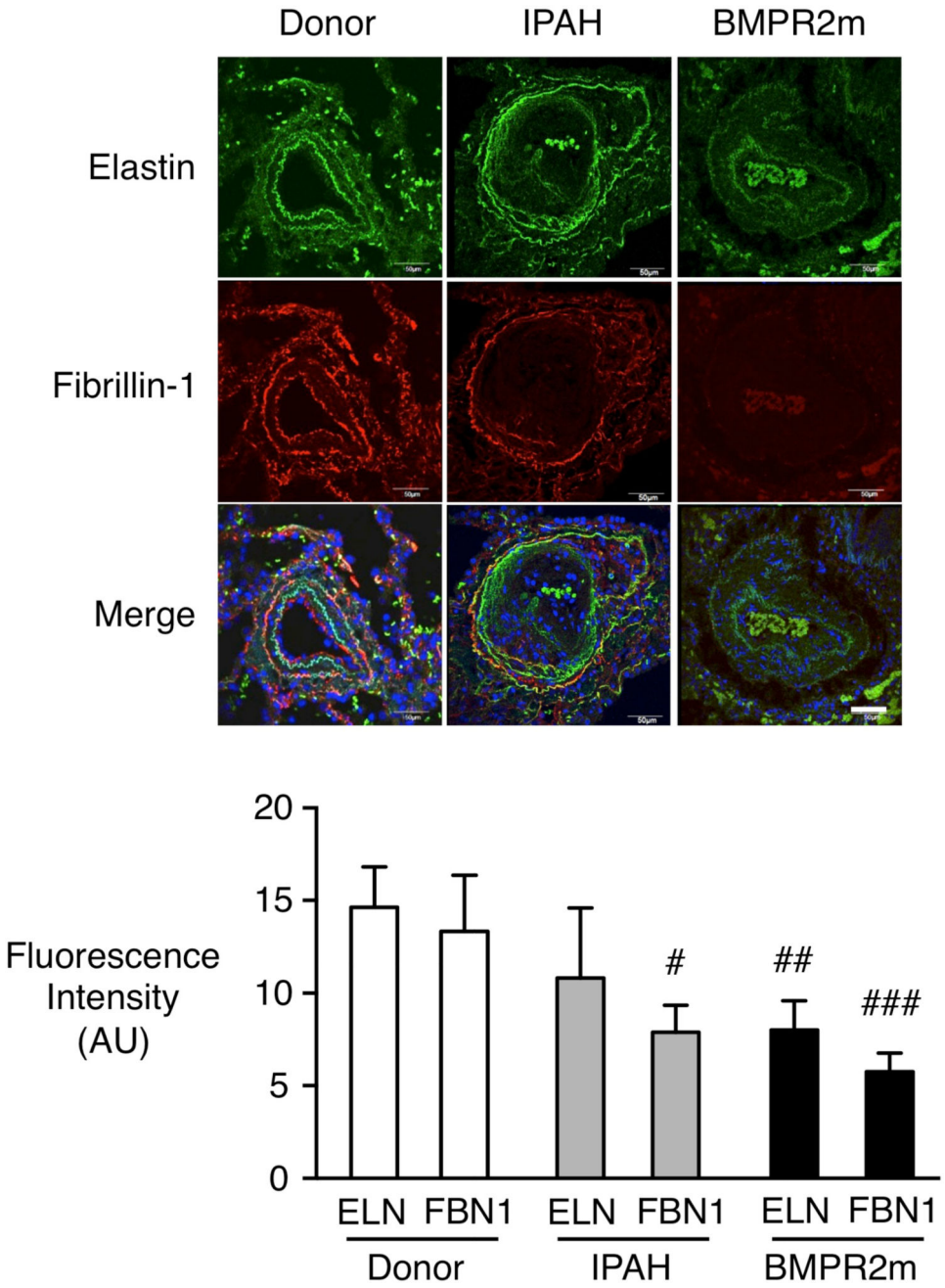

Figure 4. Reduced PA elastin and fibrillin-1 in IPAH and HPAH pulmonary arteries with a BMPR2 mutation vs. donor control

Representative PAs in lung tissue sections from donor control, IPAH and HPAH with a *BMPR2* mutation (*BMPR2m*) were immunostained for elastin (green) and fibrillin-1 (red) and quantified below. Note that in PAs at the level of the terminal bronchiolus from IPAH lungs the elastic laminae are fragmented associated with a reduction in fibrillin-1, and in the *BMPR2m* there is substantial loss of elastic laminae associated with a decrease in both fibrillin-1 and elastin. Fluorescence intensity of elastin fibers was quantified by ImageJ. Bars represent Mean±SEM for donor control (n=5), IPAH (n=4) or BMPR2m

(n=4). #p<0.05, ##p<0.01, ###p<0.001 vs. Donor by 2-way ANOVA and post-hoc Bonferroni test. Scale bar=50μm.

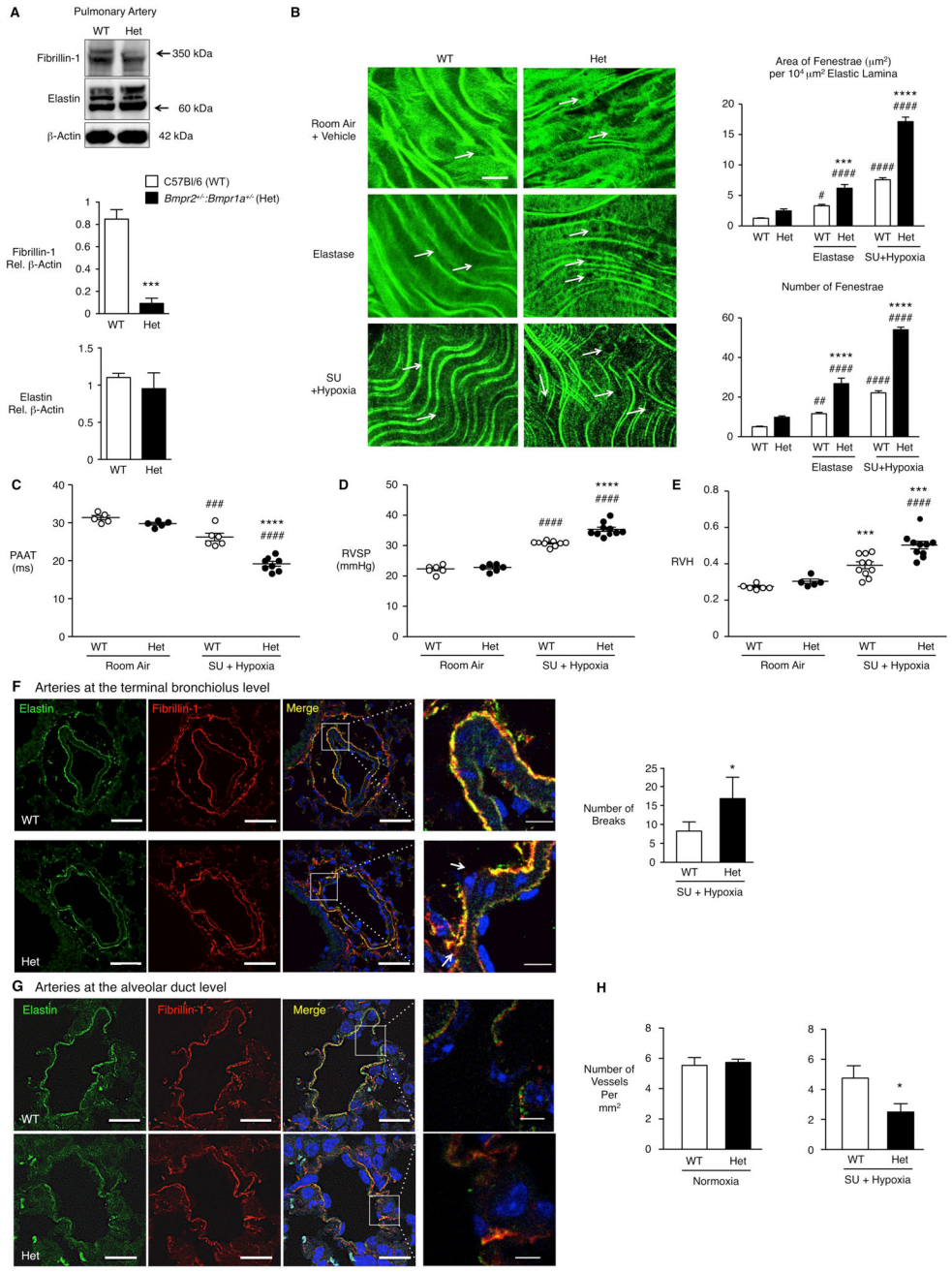

Figure 5. Reduced fibrillin-1 and heightened susceptibility to elastic fiber degradation in *Bmpr2/1a compound heterozygote* mice

(A) Representative immunoblot and densitometric analysis of elastin and fibrillin-1 of three sets of four pooled main PAs from *Bmpr2/1a* (Het) and wild type (WT) mice. Proteins were assessed on a reducing gel and the bands designated by the arrows were quantified. n=3 pools of Het or WT. **(B)** Representative confocal images of internal elastic lamina of central PAs from Het and WT mice. Central PAs were incubated with vehicle (PBS, top row) or porcine pancreatic elastase (5ng/mL, middle). On the bottom are PAs from mice exposed to Sugen 5416 (SU) and hypoxia as described in **(C)** below. Note the increase in size and number of fenestrations in the Het vs. WT PAs at baseline, following elastase treatment, or

after Sugen and hypoxia (arrows). (SU+ hypoxia). On the right, quantification of number and area of fenestrations per total area of elastin assessed in six separate fields per condition (n=5/group). Scale bar=30μm.

(C–H) Het and WT mice were exposed to room air (normoxia), or 10% O_2 (hypoxia) for three weeks following subcutaneous injection of VEGF receptor blocker Sugen 5416. **(C)** Pulmonary artery acceleration time (PAAT) measured as described in "Methods". n=5–6 (WT) or 5–8 (Het) mice. **(D, E)** Development of PAH assessed by the right ventricular systolic pressure (RVSP, **D**) and right ventricular hypertrophy (RVH; **E**) given by the Fulton index (weight of RV/left ventricle and septum, RV/LV+S). Bars indicate Mean±SEM of n=5–6 mice for normoxia and n=9–10 mice for hypoxia. **(F)** Representative images of PAs at the level of the terminal bronchiolus stained for elastin and fibrillin-1. Note the increased number of breaks in the elastic lamina of Het vs. WT. Scale bars=50μm, 10μm in the higher magnification panels, right column. n=3. **(G)** Representative images of arteries at the alveolar duct level stained for elastin and fibrillin-1 from the het and WT mice exposed to hypoxia and Sugen. Scale bars=20μm, and 5μm in the higher magnification panels, right column. **(H)** Quantification of number of vessels per mm^2 in normoxia and hypoxia. n=3. Bars represent Mean±SEM. In **A, F** and **H**, *p<0.05, **p<0.01, ***p<0.001 vs. WT by t-test; In **B, C, D, E**, ***p<0.001 and ****p<0.0001, Het vs. WT; $^{\#}$p<0.05, $^{\#\#}$p<0.01, $^{\#\#\#}$p<0.001 and $^{\#\#\#\#}$p<0.0001, SU+Hypoxia vs. Normoxia, same genotype, by two-way ANOVA and post-hoc Bonferroni test. In **A**, 3 sets of pooled PAs (n=4) from female (F) mice were used. For confocal measurements of elastin fenestrations, 3F and 2 males (M) in each genotype in each condition. PAAT, in RA, 3F, 2M of each genotype, and in Su+hypoxia 6M WT and 8M het. RVSP: in RA 3F and 3M of each genotype and in SU+/Hypoxia 9M WT and 10M hets. For RVH, 3M and 3F RA and 10M of each genotype in SU+Hypoxia.

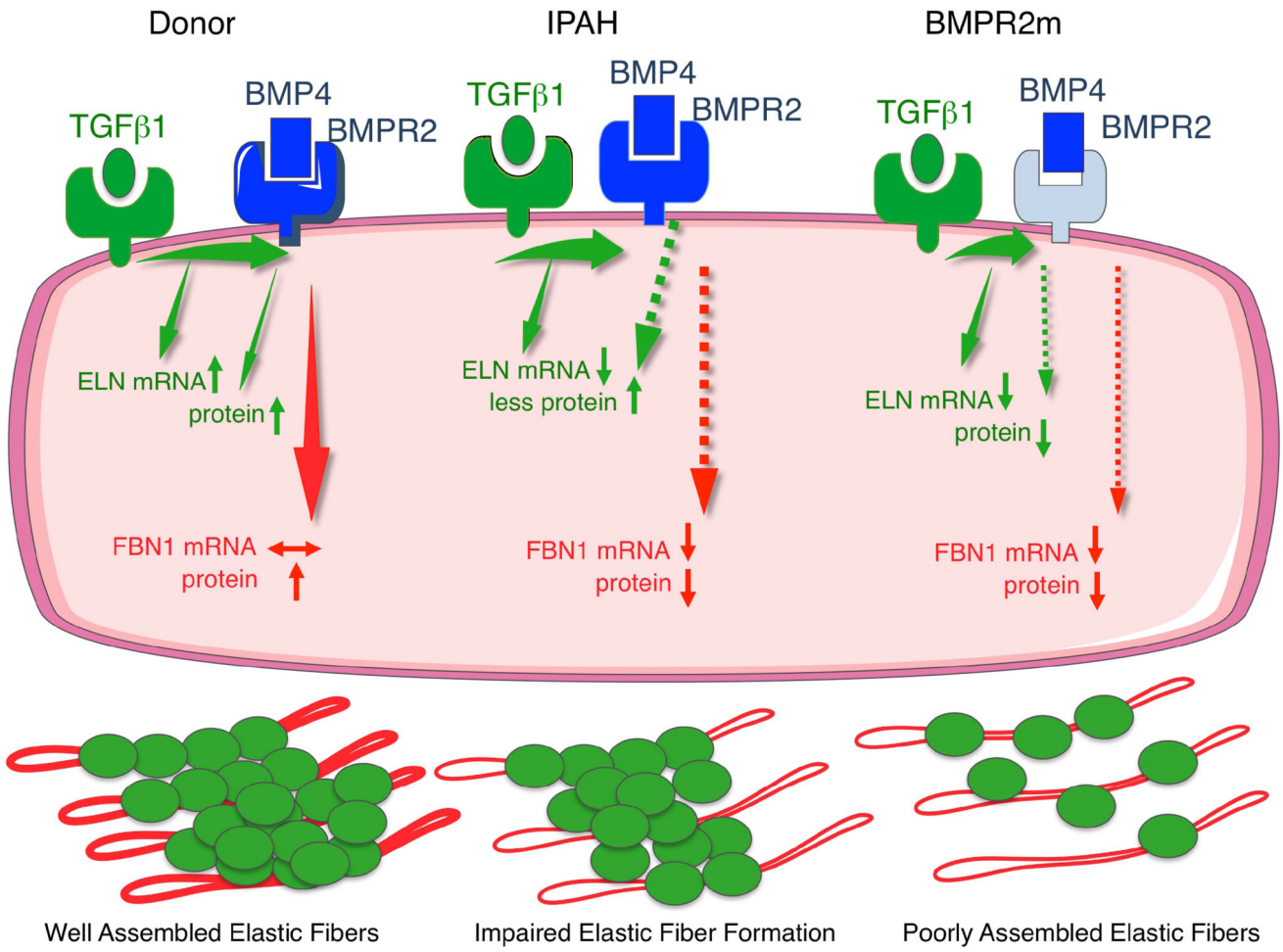

Figure 6.

Model of elastic fiber formation in control, IPAH and HPAH with a BMPR2 mutation. While TGFβ1 stimulates elastin mRNA, production of elastin and fibrillin-1 proteins are both largely BMPR2 dependent. TGFβ1 and BMP4 via BMPR2 increase fibrillin-1. In IPAH the elastic fiber formation is impaired due to reduced fibrillin-1 mRNA and protein and elastin mRNA, although some elastin protein is produced in response to TGFβ1. When there is a mutation in BMPR2, fibrillin-1 and elastin protein are both markedly decreased in response to both TGFβ1 and BMP4, leading to poorly assembled elastic fibers.

Table 1

Characteristics of Patients and Controls

A. Controls (unused donor lungs)

Study ID	Age-Gender	Cause of Death	Study
Don-1	60-F	Head Trauma Intracranial: Hemorrhage/Stroke; Death from Natural Causes	IF; WB; EF
Don-2	26-M	Gunshot wound to the head	qPCR; IF; WB; EF
Don-3	47-M	Head Trauma; Bicycle vs. Car Accident	qPCR; IF; WB; EF
Don-4	46-F	Cerebrovascular/Stroke; Intracranial Hemorrhage	qPCR; IF; WB
Don-5	25-M	Intracranial Hemorrhage	IF; WB
Don-6	56-F	Cerebrovascular Accident	IF; WB
Don-7	36-F	Subarachnoid hemorrhage	qPCR; WB
Don-8	1-M	Anoxia/Drowning	qPCR; WB

B. IPAH Patients

ID	Age-Gender	Known Mutation	PAP (s/d/m)	PVR (WU)	6MW (m)	PAH Medications	Study
IPAH-1	40-F	None	62/24/36	N/A	233	treprostinil ambrisentansildenafil	qPCR; IF; WB; EF
IPAH-2	40-M	None	118/49/64	73	420	sildenafil ambrisentantreprostinil	qPCR; IF; WB; EF
IPAH-3	51-M	None	41/19/30	6.09	378	sildenafil epoprostenol	qPCR; IF; WB; EF
IPAH-4	56-F	None	83/39/57	11.41	137	sildenafil ambrisentantreprostinil	IF; WB
IPAH-5	25-M	None	65/15/36	N/A	511	sildenafil epoprostenol treprostinil	IF; WB
IPAH-6	49-F	None	100/50/75	16.76	326	ambrisentan sildenafil epoprostenol	IF; WB

C. HPAH Patients with *BMPR2* Mutation

ID	Age-Gender	Known Mutation	PAP (s/d/m)	PVR (WU)	6MW (m)	PAH Medications	Study
BMPR2m-1	27-F	*BMPR2* c.76+5G>GA, probable mutation, not in dbSNP; may disrupt splicing SMAD9 – No	110/49/69	12.11	421	sildenafil, treprostinil bosentan iloprost	qPCR; IF; WB; EF
BMPR2m-2	33-F	*BMPR2:* c.961C>T; p.R321X, Nonsense, exon 7, c. 961C>CT (nucleotide change), p.321R>R/X (amino acid change) SMAD9 – No	87/29/48	9.74	288	bosentan treprostinil sildenafil epoprostenol	qPCR; IF; WB; EF
BMPR2m-3	33-F	*BMPR2:* c.1471C>T; p.R491W, missense, exon 11, c. 1471C>CT (nucleotide change), p.491R>R/W (amino acid change) SMAD9-No	75/33/48	15.57	326	epoprostenilbosentan sildenafil treprostinil	qPCR;IF; WB; EF

C. HPAH Patients with *BMPR2* Mutation

ID	Age-Gender	Known Mutation	PAP (s/d/m)	PVR (WU)	6MW (m)	PAH Medications	Study
BMPR2m-4	37-M	*BMPR2* c.1471C>T; p.R491W, missense, exon 11	119/51/77	14.22	308	sildenafil, sitaxsentan, ambrisentanepoprostenol Imatinib (investigational medication) treprostinil	IF; WB; EF

IPAH, idiopathic pulmonary arterial hypertension

HPAH, patients with familial pulmonary arterial hypertension classified separately because they were mutant for *BMPR2* (*Bmpr2m*)

Hemodynamic data from catheterization was obtained from studies performed closest to transplantation. PAH medications are listed according to total drug exposure during treatment period of patient, not necessarily in combination.

PAP, pulmonary artery pressure (mmHg), s: systolic, d: diastolic, m: mean

PVR, pulmonary vascular resistance (dynes/sec•cm^{-5}) (Baseline Fick PVR)

6MW, distance (m) walked in 6 minutes

NA, data not available

IF, Immunofluorescence

WB, protein expression assayed by western immunoblot

qPCR, gene expression assayed by qPCR

EF, fiber formation in culture

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