Altered MicroRNA Processing in Heritable Pulmonary Arterial Hypertension

An Important Role for Smad-8

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Rationale: Heritable pulmonary arterial hypertension (HPAH) is primarily caused by mutations of the bone morphogenetic protein (BMP) type-II receptor (*BMPR2*). Recent identification of mutations in the downstream mediator Smad-8 (gene, *SMAD9*) was surprising, because loss of Smad-8 function in canonical BMP signaling is largely compensated by Smad-1 and -5. We therefore hypothesized that noncanonical pathways may play an important role in PAH.

Objectives: To determine whether HPAH mutations disrupt noncanonical Smad-mediated microRNA (miR) processing.

Methods: Expression of *miR-21*, *miR-27a*, and *miR-100* was studied in pulmonary artery endothelial (PAEC) and pulmonary artery smooth muscle cells (PASM) from explant lungs of patients with PAH.

Measurements and Main Results: *SMAD9* mutation completely abrogated miR induction, whereas canonical signaling was only reduced by one-third. *miR-21* levels actually decreased, suggesting that residual canonical signaling uses up or degrades existing *miR-21*. *BMPR2* mutations also led to loss of miR induction in two of three cases. HPAH cells proliferated faster than other PAH or controls. *miR-21* and *miR-27a* each showed antiproliferative effects in PAEC and PASM, and PAEC growth rate after BMP treatment correlated strongly with *miR-21* fold-change. Overexpression of *SMAD9* corrected miR processing and reversed the hyperproliferative phenotype.

Conclusions: HPAH-associated mutations engender a primary defect in noncanonical miR processing, whereas canonical BMP signaling is partially maintained. Smad-8 is essential for this miR pathway and its loss was not complemented by Smad-1 and -5; this may represent the first nonredundant role for Smad-8. Induction of *miR-21* and *miR-27a* may be a critical component of BMP-induced growth suppression, loss of which likely contributes to vascular cell proliferation in HPAH.

Keywords: bone morphogenetic protein signaling; mutation; endothelium; smooth muscle

Pulmonary arterial hypertension (PAH) is a sustained elevation of pulmonary artery pressure resulting from vascular remodeling

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

SMAD9 mutations have been associated with heritable pulmonary arterial hypertension (PAH) but they do not significantly alter canonical bone morphogenetic protein (BMP) signaling, so the mechanism whereby they contribute to the pathogenesis of PAH is unclear.

What This Study Adds to the Field

We show that *SMAD9* is essential for BMP-mediated noncanonical microRNA processing. Heterozygous *SMAD9* or *BMPR2* mutations had a greater impact on microRNA processing than canonical BMP signaling. Gene replacement corrected dysregulated microRNA maturation and restored BMP-mediated growth suppression. These results define a new nonredundant role for Smad-8 and emphasize the importance of noncanonical BMP pathways in the pathogenesis of PAH.

and vessel narrowing that progressively obliterates the precapillary pulmonary arteries. It may be idiopathic (IPAH) or associated with an underlying condition, such as collagen vascular disease or congenital heart defect (APAH). After a recent reclassification, individuals with an identifiable genetic mutation or known family history are grouped together as heritable PAH (HPAH) (1). The primary genetic predisposition is a heterozygous mutation of the bone morphogenetic protein (BMP) receptor type-II (*BMPR-II*; gene symbol, *BMPR2*) (2–5). Recently, mutations affecting Smad-8 (gene symbol, *SMAD9*), a downstream mediator of BMP signaling, were also reported (6). Smad-8 is closely related to two other mediators, Smad-1 and -5, which are collectively known as the receptor Smads (R-Smads). In canonical BMP signaling, R-Smads are phosphorylated by the activated receptor complex, whereupon they associate with Smad-4 and translocate to the nucleus. Given this functional overlap between the R-Smads in canonical signaling, it was somewhat surprising that heterozygous Smad-8 mutations could cause PAH. We therefore sought to identify nonredundant roles for Smad-8 in the pulmonary vasculature, with an emphasis on noncanonical pathways.

MicroRNAs (miRs) are evolutionarily conserved small non-coding RNA molecules that negatively regulate gene expression. The primary miR transcript is processed into a 60–80 nucleotide precursor (pre-miR) molecule by the p68-Drosha microprocessor complex. This pre-miR is then exported from the nucleus and converted to the mature 18–24 nucleotide miR by DICER. Recently, it was shown that BMP signaling directly controls processing of a subset of miRs through a noncanonical role of

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TABLE 1. CHARACTERISTICS OF PATIENT CELLS STUDIED

Subject	WHO Class	Age	Sex	Germline Mutation	Somatic Mutation (15)
HPAH-1	1.2	50	F	<i>BMPR2</i> deletion exons 4–5	None
HPAH-2	1.2	41	M	<i>BMPR2</i> deletion exons 1–8	Monosomy 13, 20% of PAEC
HPAH-3	1.2	26	F	<i>SMAD9</i> R294X	None
HPAH-4	1.2	33	F	<i>BMPR2</i> R321X	None
IPAH	1.1	49	M	None	None
APAH	1.4	45	F	None	Monosomy X, 46% of PAEC

Definition of abbreviations: APAH = associated pulmonary arterial hypertension; HPAH = heritable pulmonary arterial hypertension; IPAH = idiopathic pulmonary arterial hypertension; PAEC = pulmonary artery endothelial cells; WHO = World Health Organization.

R-Smads (7). BMP stimulation promotes Smad-1 and -5 to recruit the primary miR transcript into a p68-Drosha complex. Levels of pre- and mature-miR increase two- to threefold, whereas the primary miR transcript does not change, confirming this is a post-transcriptional mechanism of regulation. This noncanonical pathway is independent of Smad-4. miRs regulated by the R-Smads contain the consensus binding sequence CAGAC and include *miR-21*, *miR-100*, *miR-199a-5p*, and *miR-27a* (7, 8).

Although this pathway was first defined in pulmonary artery smooth muscle cells (PASMC) (7), several important questions pertinent to PAH remain unanswered. Pulmonary artery endothelial cells (PAEC) have not been studied, the role of Smad-8 was not examined, and previous studies were only performed with short-interfering RNA (siRNA) knockdown of Smad-1 and -5, so the effects of naturally occurring PAH mutations are unknown. Here we test whether Smad-8 is important in miR processing and analyze PAEC and PASMC from patients with PAH to determine whether HPAH mutations result in dysregulated miR biogenesis. Some of the results of this study have been previously reported in the form of an abstract (9, 10).

METHODS

Study Population

PAEC and PASMC from explant lungs of patients with PAH were isolated as previously described (11–13). Patients comprised four HPAH, one IPAH, and one APAH (Table 1). Control cells were isolated from failed donor lungs or purchased commercially (Lonza, Allendale, NJ). Cells were maintained in EGM-2 (PAEC) or SmGM-2 (PASMC) medium (Lonza) and used at passages four to nine. Analysis for mutations in *BMPR2*, *SMAD9*, and somatic chromosome abnormalities was performed as described (5, 14, 15). Three *BMPR2* mutations and one *SMAD9* were identified (Table 1). Studies were approved by the Cleveland Clinic Institutional Review Board and all subjects gave written informed consent.

Cell Culture and Treatments

PAEC were plated at 8.8×10^4 cells per well and PASMC at 4.2×10^4 cells per well in 12-well plates. siRNA knockdown was initiated 24 hours after seeding. Transfections were performed using DharmaFECT-1 with gene-specific siRNA pools (Dharmacon, Lafayette, CO) at a final concentration of 100 nM. siGENOME nontargeting siRNA Pool-2 (Dharmacon) was used as a negative control. After a further 24 hours, cells were treated with 3-nM BMP4 (PASMC) or BMP9 (PAEC). This choice of ligands reflects known tissue-specific responsiveness; BMP9 is the only ligand that strongly induces BMP signaling in PAEC, whereas PASMC respond weakly to BMP9 and strongly to BMP2 and BMP4 (16). Each experimental condition was performed in triplicate in independent wells. Total RNA was extracted using miRNeasy mini kit (Qiagen, Valencia, CA), reverse transcribed, and analyzed by real-time quantitative polymerase chain reaction. The effects of overexpression or inhibition of miRs on cell proliferation was studied using 2,3-Bis(2-methoxy-4-nitro-5-sulphophenyl)-2H-tetrazolium-5-carbox-anilide assay and

live cell counts. Changes in endogenous *miR-21* levels with BMP stimulation were followed by transfecting PAEC with γ - ^{32}P -labeled *miR-21*. Correction of the *SMAD9* mutation in PAEC was performed by introducing an expression construct using nucleofection. Detailed methods for these experiments are provided in the online supplement.

Statistical Analysis

Statistical significance of pairwise comparisons was determined by two-tailed *t* test, or with Mann-Whitney rank-sum test if the data were not normally distributed. Multiple-group data were tested using one-way analysis of variance (ANOVA) followed by pairwise multiple comparison procedures (Holm-Sidak method). All tests were performed using the SigmaStat v3.5 statistical package (Systat Software Inc., Chicago, IL).

Western Blot and Coimmunoprecipitation Assays

To validate siRNA knockdown at the protein level, 50 μg of protein was resolved on a 10% sodium dodecyl sulfate polyacrylamide gel under denaturing conditions, semidry transferred to polyvinylidene fluoride membrane (Biorad, Hercules, CA) and immunoblotted with anti-BMPR2 (#B19720–050, 1:200; BD Biosciences, San Jose, CA,) or anti-SMAD4 (#9515, 1:500; Cell Signaling, Danvers, MA). An antiactin antibody (#sc-1615, 1:2000; Santa Cruz Biotechnology, Santa Cruz, CA) was used as a loading control. RNA-immunoprecipitation (IP) and protein co-IP experiments were performed as detailed in the online supplement.

RESULTS

BMPR2 is Essential for Smad-mediated miR Processing

Before studying the role of *SMAD9*, we first tested whether miR processing was likely to be significantly altered in HPAH by determining the effect of loss of BMPR-II function. siRNA knockdown of *BMPR2* or *SMAD4* in PAEC and PASMC each achieved at least an 80% reduction in mRNA and protein levels compared with a control nontargeting siRNA pool (see Figure E1 in the online supplement). BMP stimulation of control cells induced a twofold to three fold increase in pre- and mature-*miR-21* levels, which was completely abrogated by *BMPR2* knockdown (Figure 1). *miR-27a* and *miR-100* show a similar pattern (see Figure E2). In contrast, siRNA knockdown of *SMAD4* had no effect on these miRs, whereas induction of *ID1* and *miR-181b*, downstream targets known to be regulated by canonical BMP signaling (7, 17), was blocked by *SMAD4* knockdown (Figure 1; see Figure E2). These results demonstrate that BMPR-II is essential for Smad-mediated miR processing and confirm that siRNA knockdown of *SMAD4* effectively distinguishes between the canonical and noncanonical pathways.

Loss of SMAD9 Profoundly Affects miR Processing but Not Canonical BMP Signaling

We then sought to determine whether Smad-8 promotes miR processing in the same manner as Smad-1 and -5 and if so,

Figure 1. Expression of *miR-21* and its precursors in pulmonary artery endothelial cells (PAEC) (A) and pulmonary artery smooth muscle cells (PASC) (B) after short-interfering RNA knockdown (KD) of *BMPR2* or *SMAD4*. Bone morphogenetic protein (BMP) stimulation leads to an increase in pre- and mature-*miR-21* in control and *SMAD4*-KD but not *BMPR2*-KD cells; ** $P < 0.001$. Basal level of mature *miR-21* is significantly lower in *BMPR2*-KD PAEC than control cells; § $P < 0.001$. miR = microRNA.

whether *SMAD9* mutations would dysregulate this process. RNA-IP experiments confirmed that all three miRs bind to Smad-8 in a BMP-dependent manner (Figure 2) and protein co-IP confirmed association of Smad-8 with p68 (see Figure E3). Because previous studies only examined dual-knockdown of *SMAD1* and *SMAD5* in PASC (7), we also studied the effects of knocking down these genes individually in both PAEC and PASC. siRNA pools targeting individual *SMADs* were confirmed to be gene-specific (see Figure E4). Functional overlap between the R-Smads engenders some redundancy in the canonical BMP signaling pathway. Consistent with this, siRNA knockdown of individual R-Smads led to attenuation but not complete inhibition of canonical BMP signaling (Figure 3, *IDI*). Notably, *SMAD9* knockdown had the least impact. In marked contrast, *miR-21* processing was completely blocked by knockdown of any individual R-Smad, including *SMAD9*, in both cell types (Figure 3). Similar results were obtained for *miR-100*, *-27a*, and *-199a-5p* (see Figure E5). These results demonstrate that Smad-8 is essential for the noncanonical BMP-mediated miR processing pathway. Furthermore, unlike the canonical signaling pathway, loss of any individual R-Smad is not compensated by the remaining two, leading to a dominant-negative effect on miR biogenesis.

miR-21 Levels Decrease with Canonical BMP Signaling

Knockdown of individual R-Smads not only blocked miR induction but the levels of pre- and mature-*miR-21* actually decreased by up to 70% compared with controls cells (Figure 3). This effect was seen in PAEC and PASC and was BMP-dependent. A

timecourse in *SMAD1* knockdown PAEC showed that pre- and mature-*miR-21* levels gradually declined for 16 hours post-BMP stimulation, then plateaued at around 40% of pretreatment levels until 20 hours and subsequently began to rise again, although they had still not returned to pretreatment levels at the 48-hour time point (Figure 4). Primary *miR-21* levels remained relatively constant.

These results suggest that blocking noncanonical *miR-21* induction unmasks a decrease in existing *miR-21* driven by canonical BMP-signaling. To test this hypothesis, we first performed siRNA knockdown of p68 to block noncanonical miR processing independently of the Smads. BMP stimulation of p68-knockdown PAEC led to a threefold reduction in pre-*miR-21* and a fivefold decrease in the mature miR, whereas inhibition of the canonical and noncanonical pathways with *SMAD1/4* double knockdown gave no overall change (see Figure E6). We then transfected control and siRNA-treated PAEC with radiolabeled mature *miR-21*. In this way we could isolate what happens to the *miR-21* already present at the time of stimulation, because any additional *miR-21* newly induced by BMP9 would not be labeled. BMP stimulation of control PAEC led to a 50% decrease in radiolabeled *miR-21* after 24 hours, whereas no change was seen in *SMAD4*-knockdown cells (Figure 5). This further supports our hypothesis that canonical signaling by Smad-4 leads to a decrease in *miR-21* levels. Cells with siRNA knockdown of *SMAD1* or *SMAD9* showed decreases comparable with control cells (Figure 5), consistent with our previous evidence that they retain some canonical BMP signaling (Figure 3). Treatment with α -amanitin, puromycin, or cyclohexamide blocked the decrease, suggesting that *de novo* RNA transcription and protein

Figure 2. RNA-immunoprecipitation (IP) of Flag-tagged Smad-8 in pulmonary artery endothelial cells. (A) Quantitative polymerase chain reaction demonstrates little or no primary microRNA (miR) bound to Smad-8 in vehicle-treated cells and significant induction of binding with bone morphogenetic protein (BMP)-9 treatment. Pre-*miR-16* is not regulated by BMPs (see Figure E15) and showed no detectable binding to Smad-8. Control IPs with nonspecific IgG were negative for all miRs. No reverse transcriptase (RT) denotes a negative control with no RT, confirming there is no DNA contamination. (B) Western blot analysis with anti-Flag antibody confirms specificity of the pulldown and equal efficiency in vehicle and BMP9-treated samples.

Figure 3. Short-interfering RNA knockdown (KD) of any individual R-Smad (*SMAD1*, *SMAD5*, or *SMAD9*) leads to a paradoxical decrease in *miR-21* levels after bone morphogenetic protein (BMP) stimulation; $^{\dagger}P \leq 0.002$. Similar effects are seen in pulmonary artery endothelial cells (PAEC) (A) and pulmonary artery smooth muscle cells (PASM) (B). In contrast, expression of *ID1*, a downstream target of canonical BMP signaling, is still induced in both cell types; $^{**}P < 0.001$. Basal level of mature *miR-21* is significantly lower in *Smad*-KD PAEC than control cells; $^{\S}P \leq 0.006$. miR = microRNA.

synthesis is required. This effect may be specific to *miR-21*, because there was no decrease in *miR-27a* (Figure 5).

miR Processing Is Defective in Patients with PAH and Germline Mutations

Most PAH-associated *BMPR2* mutations lead to haploinsufficiency and approximately 50% of normal gene activity (4). In contrast, siRNA knockdown reduces mRNA levels by 80–90% (see Figure E1). Thus, it was important to determine the relevance of our results in siRNA-treated cells to the *in vivo* human disease process. We analyzed miR processing in cells isolated from explant lungs of four patients with HPAH with heterozygous germline mutations of *BMPR2* or *SMAD9* (Table 1). Three of these cases (hereafter referred to as Group-A) showed no significant induction of *miR-21*, *miR-27a*, or *miR-100* in PAEC (Figure 6). Indeed, consistent with our siRNA data, *miR-21* levels in these cases actually decreased when stimulated with BMP9. The fourth case (HPAH-4) showed significant induction of all three miRs, as did PAEC from two patients without mutations (Figure 6). In contrast, *ID1* expression was significantly up-regulated in Group-A cases, although the fold-change was smaller than in controls and other patients (Figure 6). Likewise, *miR-181b* was significantly induced in two of the three Group-A patients (see Figure E7). It was also notable that basal levels of *miR-21* were significantly lower in all PAH cases, with the largest reduction in Group-A cells (see Figure E8). Similar results were obtained in PAH PASM, with the interesting exception that induction of *miR-199a-5p* was completely absent in R321X (see Figure E9).

miR-21 and *miR-27a* Are Antiproliferative in PAEC and PASM

To investigate the physiologic effects of altered miR regulation in the pulmonary artery, we performed a growth curve analysis of

control PAEC transfected with either pre-*miR-21* or a *miR-21* inhibitor molecule and counted live cells at each time point. Overexpression of pre-*miR-21* significantly reduced cell growth and was partially dose dependent (see Figure E10). Conversely, inhibition of endogenous *miR-21* with an anti-miR significantly accelerated cell growth, again with some evidence of a dose response. One-way ANOVA of cell counts at 72 hours was

Figure 4. Timecourse of *miR-21* expression in control (open symbols) and *SMAD1*-knockdown (KD) (solid symbols) pulmonary artery endothelial cells after bone morphogenetic protein-9 treatment at time zero. Data are normalized to untreated cells at time zero. Primary *miR-21* levels in both control and *SMAD1*-KD cells were essentially constant around 1; control cells are omitted for clarity. miR = microRNA.

Figure 5. Autoradiograph and quantitation of exogenous ³²P-labeled *miR-21* levels 24 hours after transfection, normalized to 5S ribosomal RNA to control for the amount of total RNA loaded. Densitometric ratios were then normalized to the untreated control in each experiment. Bone morphogenetic protein (BMP) stimulation leads to a decrease in *miR-21* levels, which is blocked by *SMAD4* short-interfering RNA knockdown (KD) but not *SMAD1* or *SMAD9*, suggesting it is mediated by the canonical pathway. There is no decrease in cells treated with inhibitors of transcription (α-amanitin) or translation (puromycin or cyclohexamide). *miR-27a* levels are unchanged with BMP treatment. miR = microRNA.

statistically significant ($P < 0.001$), with highly significant pairwise differences between pre-miR or anti-miR versus control. Cotransfection of equimolar amounts of pre- and anti-miR was not significantly different from untransfected cells, demonstrating this is a specific effect of *miR-21* that can be competitively reversed. Negative control pre- and anti-miR constructs also did not differ from control cells (data not shown).

We then analyzed the growth parameters of patient PAEC and PASC using 2,3-Bis(2-methoxy-4-nitro-5-sulfonyl)-2H-tetrazolium-5-carbox-anilide assays. Cells without mutations proliferated at the same rate as controls, whereas the three Group-A cases with abnormal miR regulation proliferated significantly faster (see Figure E11A). Cells from case HPAH-4 (*BMP2*-R321X) were intermediate between these two groups. Because R321X is predicted to cause nonsense-mediated RNA decay, leading to haploinsufficiency, we hypothesized that perhaps the intermediate phenotype reflected higher expression of the wild-type allele. Quantitative polymerase chain reaction analysis confirmed that *BMP2* expression was 75% of control, despite complete nonsense-mediated RNA decay of the mutant allele (see Figure E12).

BMP stimulation led to a decrease in PAEC and PASC proliferation, as previously reported (18). However, under these conditions Group-A cells still grew faster than unstimulated control cells (see Figure E11B). Indeed, the proliferation rate was highly correlated with the BMP-induced *miR-21* fold-change

($R^2 = 0.99$; $P < 0.001$) (Figure 7). Overexpression of *miR-21* induced further growth suppression and restored a proliferation rate comparable with unstimulated control cells (see Figure E12). This was not caused by increased apoptosis (data not shown). *miR-27a* showed a similar effect but with less suppression of control and nonmutant PAH cells (see Figure E12). Overexpression of *miR-100* was slightly proliferative but the magnitude of the effect was very small (data not shown).

Overexpression of *SMAD9* in Mutant PAEC Corrects miR Processing, Proliferation Rate, and BMP Responsiveness

Although overexpression of *miR-21* or *miR-27a* could normalize proliferation rates of Group-A PAEC, it does not restore BMP-responsiveness and the cells still proliferate faster than BMP-treated controls. We therefore sought to restore *SMAD9* levels and determine whether this would correct both the miR processing defect and cell proliferation. Overexpression of wild-type *SMAD9* in control PAEC had no major effects; canonical signaling and miR processing were unchanged or slightly increased and cell proliferation over a 72-hour period post-transfection was not significantly different than the mock-transfected or green fluorescent protein-transfected cells (Figure 8). Introduction of this construct into the *SMAD9*-mutant PAEC not only normalized their baseline proliferation rate to that of control cells but also completely restored the additional

Figure 6. Fold-change of microRNAs (miR) and *ID1* in patient pulmonary artery endothelial cells (PAEC) when treated with bone morphogenetic protein (BMP)-9. Three of four patients with germline mutations show a significant decrease in *miR-21* relative to basal levels ($^S P < 0.01$) and nonsignificant changes in *miR-27a* and *miR-100*, whereas *ID1* is significantly increased ($^{**} P < 0.001$) albeit with a smaller fold-change relative to control cells. The remaining patients showed significant induction of all miRs and *ID1*, with fold-changes that did not differ from control cells. APAH = associated pulmonary arterial hypertension; IPAH = idiopathic pulmonary arterial hypertension.

growth suppressive effects of BMP9 stimulation (Figure 8). MiR fold-change was also normalized (Figure 8), but interestingly, the basal level of *miR-21* remained significantly lower than control cells (see Figure E14).

DISCUSSION

Alterations in BMP signaling clearly play an important role in the pathogenesis of PAH; all known HPAH mutations affect genes in the pathway (2–5, 19–21) and there is evidence for down-regulation of BMPR-II in the lungs of other types of human PAH (22) and hypoxia and monocrotaline animal models (23–25). It was therefore surprising when a *SMAD9* mutation was identified (6), because loss of Smad-8 function is largely compensated by the overlapping functions of Smad-1 and -5 (26). Thus, the *SMAD9* knockout mouse is viable, whereas *SMAD1* and *SMAD5* knockouts are both embryonic lethal (27–29). The *SMAD9* knockout does show evidence of spontaneous pulmonary vascular remodeling, supporting a role in PAH (30). We therefore hypothesized that the functions of BMP signaling most critical to PAH may be those where Smad-8 function is nonredundant. Furthermore, mutations in *SMAD4*, the co-Smad that mediates canonical BMP signaling, predispose to juvenile polyposis and colorectal cancer (31) but are not known to cause PAH, suggesting that noncanonical pathways may be especially important in the pathogenesis of PAH. The recent identification of a miR processing pathway mediated by Smad-1 and -5 and independent of Smad-4 (7) was an attractive candidate fulfilling these criteria, but the importance of Smad-8 has not been investigated. Here we tested whether Smad-8 is required for miR processing and analyzed the effects of naturally occurring heterozygous *BMPR2* and *SMAD9* mutations in explant cells from the lungs of patients with PAH.

Our data clearly show that Smad-8 is essential for miR processing, with complete loss of induction in *SMAD9* siRNA knockdown cells. Remarkably, the same effect was seen in a patient with only a heterozygous mutation and this defect could be corrected by overexpressing wild-type *SMAD9*. Canonical BMP signaling remained relatively intact, with less than a 50% reduction in fold-change of downstream target genes. Because Smad-1 and -5 had previously only been investigated in a combined siRNA knockdown, we also tested specific knockdown of the genes individually and found that Smad-1, -5, and -8 are required for miR induction. Given this apparently dominant-negative effect of losing a single R-Smad, we hypothesized that the miR processing pathway requires formation of a complex comprising all three R-Smads to bind the p68/Drosha complex. Canonical BMP signaling is a precedent for this, where two phosphorylated R-Smads form a heterotrimeric

complex with Smad-4. However, our efforts to prove this through coimmunoprecipitation of endogenous Smad-p68 complexes have been hampered by the lack of a specific Smad-8 antibody. These data also suggest that interaction of the R-Smads with Smad-4 may be favored kinetically over p68-Drosha, and so noncanonical miR processing is competitively disadvantaged by *SMAD9* mutation. Further studies are required to fully characterize the nature of the complex molecular interactions involved in these two pathways.

Another remarkable finding was that dysregulation of miR processing, either by siRNA knockdown or germline mutation, was associated with a decrease in *miR-21* levels on ligand stimulation. This led us to hypothesize that induction of *miR-21* by the noncanonical pathway is balanced by a decrease mediated by canonical BMP signaling. In normal PAEC the induction outweighs the decrease, whereas mutations that block miR processing but leave canonical signaling partially intact lead to an overall reduction in *miR-21* levels. Transfection of radiolabeled *miR-21* into control and various siRNA-treated PAEC supported this hypothesis. It is presently unclear whether the decrease represents active degradation of *miR-21* or if it is used up by a target mRNA induced by canonical BMP signaling. Physiologically, however, it may be critically important, because *miR-21* was clearly antiproliferative in PAEC and SMC and their growth rate after BMP stimulation correlated strongly with the change in their *miR-21* levels.

Baseline proliferation rates of cells from all four HPAH cases were significantly higher than controls or mutation-negative patients. Notably, the *SMAD9* mutation case clustered tightly with the two *BMPR2* mutations that dysregulated miR

Figure 7. The growth rate of patient and control pulmonary artery endothelial cells (PAEC) stimulated with bone morphogenetic protein (BMP)-9 strongly correlates with the change in their *miR-21* levels. miR = microRNA.

Figure 8. (A) Overexpression of *SMAD9* normalizes the bone morphogenetic protein (BMP)-induced fold-change of all three microRNAs (miRs) in pulmonary artery endothelial cells (PAEC) with the *SMAD9*-R294X mutation; * $P < 0.05$ in analysis of variance *post hoc* pairwise test relative to fold-change in mock-treated control cells; ns = not significant. (B) Baseline proliferation rate and growth suppression in response to BMP9 are also completely corrected (*post hoc* P values vs. control at 72 h: R294X + GFP, $P < 0.001$; R294X + SMAD9, $P = 0.138$; R294X + SMAD9 + BMP9 vs. control + BMP9, $P = 0.39$). Mock-treated cells and control-GFP are omitted for clarity. GFP = green fluorescent protein.

processing, despite their differential effects on canonical signaling. The fourth case was intriguing, with an intermediate phenotype correlating with elevated wild-type *BMPR2* expression. Up-regulation of the wild-type allele has been proposed as a mechanism for reduced penetrance in HPAH (32), but in this case it was not protective. One of the limitations of working with explant cells is they derive from late-stage disease, have accumulated many changes, and may be influenced by previous drug treatments. Yet, overexpression of *SMAD9* in mutant PAEC normalized their baseline proliferation rate, corrected miR processing, and restored BMP-mediated growth suppression to levels comparable with mock-transfected control cells. This suggests that despite the many other changes in these cells from late-stage disease lungs, their hyperproliferation is almost completely accounted for by the primary mutation and can be corrected by gene replacement.

Cells from patients with PAH also had lower basal levels of *miR-21* than controls, consistent with recent data on whole-lung sections (33). Interestingly, this was not corrected by overexpression of *SMAD9*, even though the BMP-induced fold-change was normalized, suggesting that the mechanism may be independent of the BMP pathway. Further support for a pathogenic role for *miR-21* comes from the rat monocrotaline model of pulmonary hypertension where it is also down-regulated (33). Indeed, given that monocrotaline leads to reduced *BMPR2* expression and Smad signaling (24, 25, 34), the molecular basis for *miR-21* down-regulation in this model may be analogous to our data in human HPAH. Together, these data raise the possibility that miR replacement might have therapeutic potential in PAH. However, considerable caution is needed because in many other tissues, *miR-21* is widely considered to be oncogenic, with evidence that

overexpression leads to increased proliferation and decreased apoptosis (35, 36). In the lung, overexpression of *miR-21* in myofibroblasts has been implicated in the pathogenesis of idiopathic pulmonary fibrosis (37). Thus, any therapeutic strategies require very specific targeting to the pulmonary vasculature to avoid potentially serious adverse consequences. Additionally, our data show that multiple miRs are dysregulated in HPAH, further complicating miR-based therapeutic approaches. In contrast, we show that overexpressing wild-type *SMAD9* in mutant cells effectively restored normal processing for multiple different miRs. Overexpression in control cells did not significantly alter BMP signaling or cell proliferation, suggesting that “overcorrection” is not detrimental. This suggests that approaches to correct HPAH mutations by increasing the availability of functional protein and correcting receptor stoichiometry (38, 39) might achieve better physiologic results by simultaneously correcting multiple different canonical and noncanonical functions of BMP signaling with fewer adverse consequences.

In conclusion, our data show that heterozygous mutations of *BMPR2* or *SMAD9* are sufficient to abrogate noncanonical BMP-mediated miR processing, in contrast with canonical BMP signaling, where the response is only attenuated. This difference is particularly marked for *SMAD9* mutations, defining possibly the first nonredundant role for Smad-8. Cell proliferation was strongly correlated with *miR-21* levels, suggesting that aberrant miR processing may play an important role in the pathogenesis of PAH.

Author Disclosures are available with the text of this article at www.atsjournals.org.

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