Role of BMPR2 Alternative Splicing in Heritable Pulmonary Arterial Hypertension Penetrance

Joy Cogan, PhD; Eric Austin, MD; Lora Hedges, BS; Bethany Womack, BS; James West, PhD; James Loyd, MD; Rizwan Hamid, MD, PhD

Background—Bone morphogenic protein receptor 2 (BMPR2) gene mutations are the most common cause of heritable pulmonary arterial hypertension. However, only 20% of mutation carriers get clinical disease. Here, we explored the hypothesis that this reduced penetrance is due in part to an alteration in BMPR2 alternative splicing.

Methods and Results—Our data showed that BMPR2 has multiple alternative spliced variants. Two of these, isoform-A (full length) and isoform-B (missing exon 12), were expressed in all tissues analyzed. Analysis of cultured lymphocytes of 47 BMPR2 mutation–positive heritable pulmonary arterial hypertension patients and 35 BMPR2 mutation–positive unaffected carriers showed that patients had higher levels of isoform-B compared with isoform-A (B/A ratio) than carriers (P=0.002). Furthermore, compared with cells with a low B/A ratio, cells with a high B/A ratio had lower levels of unphosphorylated cofilin after BMP stimulation. Analysis of exon 12 sequences identified an exonic splice enhancer that binds serine arginine splicing factor 2 (SRSF2). Because SRSF2 promotes exon inclusion, reduced SRSF2 expression would mean that exon 12 would not be included in final BMPR2 mRNA (thus promoting increased isoform-B formation). Western blot analysis showed that SRSF2 expression was lower in cells from patients compared with cells from carriers and that siRNA-mediated knockdown of SRSF2 in pulmonary microvascular endothelial cells resulted in elevated levels of isoform-B compared with isoform-A, ie, an elevated B/A ratio.

Conclusions—Alterations in BMPR2 isoform ratios may provide an explanation of the reduced penetrance among BMPR2 mutation carriers. This ratio is controlled by an exonic splice enhancer in exon 12 and its associated splicing factor, SRSF2. (Circulation. 2012;126:1907-1916.)

Key Words: alternative splicing ■ bone morphogenetic protein receptors, type II ■ hypertension, pulmonary ■ penetrance ■ SRSF2 protein, human

Mutations in the BMPR2 gene have been shown to cause heritable pulmonary arterial hypertension (HPAH), an autosomal-dominant disorder with a variable age of onset.1 Although ≈82% of HPAH families have an identifiable mutation in the BMPR2 gene, only 20% of carriers ever develop disease.2 BMPR2 mutations can either produce stable transcripts or result in premature termination codons, resulting in the mutated transcript being rapidly degraded through the nonsense-mediated decay (NMD) pathway.3,4 NMD is an mRNA surveillance system that degrades transcripts containing premature termination codons to prevent translation of unnecessary or harmful transcripts.3 Thus, individuals with PAH and NMD+ BMPR2 mutations have disease caused by haploinsufficiency, whereas patients whose mutations are NMD− may have disease resulting from a dominant-negative mechanism. Reduced penetrance is seen in both groups of patients.

The molecular mechanisms that regulate the reduced penetrance remain poorly defined. Several studies have explored the hypothesis that reduced penetrance is due to the effect of modifying genes; however, no modifier genes have been identified that could explain reduced penetrance in the majority of patients.6–12 We recently reported that the expression of the nonmutated BMPR2 allele plays a role in HPAH penetrance.13 Patients had lower levels of expression of the normal BMPR2 allele than carriers. These data suggested for the first time that one of the important modifiers of BMPR2-related HPAH might in fact be the BMPR2 gene itself. We have delved further into which expression mechanisms of BMPR2 play a role in the reduced penetrance observed in HPAH, focusing particularly on alternative splicing.
Alternative splicing is a mechanism by which a single gene can generate multiple transcripts with likely different functions through internal deletion/skipping of exons in various combinations. Alternative splicing is a complex process that involves exonic and intronic acceptor and donor sites and intronic and exonic splice enhancers and silencers. A large number of proteins such as the serine-arginine–rich proteins and heterogeneous nuclear ribonucleoproteins (hnRNPs) bind to these enhancers and silencers and aid in splice-site recognition. The SR proteins bind to splicing enhancers and promote exon inclusion, whereas binding of hnRNPs acts as a splicing repressor promoting exon exclusion or skipping. At least 20 SR proteins have been identified, from which a smaller group of 7 are called “core” SR proteins: SRSF2 (SF2/ASF), SC35, SRp20, SRp75, SRp40, SRp55, and 9G8. The SR proteins bind to specific RNA sequences and assist in spliceosome recruitment through protein-protein interactions. This can have clinical consequences because alternative splicing has been shown to play a role in many human diseases.

In humans and mice, BMPR2 has 13 exons and is alternatively spliced to produce 2 primary transcripts. Isoform-A is the full-length BMPR2 gene product and contains all 13 exons. Isoform-B is a much rarer transcript lacking exon 12. Whether this minor isoform is expressed in HPAH patients or has any role in the cellular BMPR-II function is not known. Studies have clearly shown that exon 12 is important for proper functioning of BMPR-II. Deletion of exon 12 is a common BMPR2 mutation found in HPAH patients, and previous studies have shown that it can disrupt BMPR-II function in a dominant-negative fashion. Furthermore, studies in mice have shown that overexpression of the exon 12–truncated Bmpr2 transcript results in pulmonary hypertension owing to complex vascular lesions consisting of the narrowing at branch points of resistance-level vessels and the dropout/occlusion of very small vessels. This experimental model of pulmonary hypertension is currently being studied.

In this study, we hypothesized that alternative splicing of BMPR2 plays a role in HPAH penetrance. To address this hypothesis, we performed BMPR2 isoform-A and isoform-B splicing in cells derived from patients and carriers. We found that patients expressed higher levels of isoform-B compared with isoform-A. Using expression analysis, sequencing, and target gene knockout, we then identified a splicing enhancer and its binding splicing factor, SRSF2, which appears to regulate the relative ratios of the BMPR2 isoforms in cells.

**Methods**

**PAH Patient Samples**

Cultured lymphocytes (CLs) from 47 BMPR2 mutation–positive HPAH patients and 35 BMPR2 mutation–positive unaffected carriers were used in this study (all the cell lines available in our cell repository at the time of study). Of the 47 patients, 27 had NMD+ mutations; in the carrier group, 20 had NMD+ mutations and 15 had NMD– mutations (the Table). Samples were collected with informed consent under a Vanderbilt University Institutional Review Board–approved protocol. Cells were established and grown and their BMPR2 mutation and NMD status was determined as previously described. The full range of these mutations has been previously described in detail.

**Determination of BMPR2 Isoform mRNA Levels**

cDNA was synthesized from 1 μg total cellular RNA isolated from the patient-derived CL cells or pooled human tissues (Human Universal Reference Total RNA; catalog No. 636538; Clontech, Mountain View, CA), including pulmonary microvascular endothelial cells (PMVECs), with the Superscript III cDNA Synthesis Kit (Life Technologies, Grand Island, NY). PMVECs were included in this analysis because they are an important cell in PAH pathogenesis. Taqman assays (4331182) were used for both isoform-A and isoform-B BMPR2 transcript detection by real-time polymerase chain reaction using Taqman Universal Master Mix and a 7500 Sequence Detection System (Applied Biosystems).基质体系和引物设计已详细描述（http://rulai.cshl.edu/cgi-bin/tools/ESE3/esefinder.cgi）。

**Western Blot Analysis**

Membranes were probed with primary antibody AF811 (R&D Systems Inc, Minneapolis, MN) for 1 hour and with secondary antibody 11-035-003 (Jackson Immunoresearch, West Grove, PA) for an additional hour. Detection was done with the Immobilon Western Blotting Substrate (Millipore, Billerica, MA). β-Actin was used as a loading control. For BMPR-II signaling analysis, we used phospho-cofilin antibody 3313 (Cell Signaling Technology) and cofilin antibody C8736 (Sigma).

**Identification of Exonic Splice Enhancer**

BMPR2 sequences were scanned for exonic splice enhancers with ESE Finder 3.0 as previously described (http://rulai.cshl.edu/cgi-bin/tools/ESE3/esefinder.cgi).

**SRSF2 Knockdown in PMVECs**

We used Silencer Select Pre-Designed siRNA (Life Technologies) to knock down SRSF2 (catalog No. s12730). PMVECs (Lonza, Basel, Switzerland) were transfected with 5 nmol/L siRNA (final concentration) with Lipofectamine RNAiMAX transfection reagent (Life Technologies) as instructed by the manufacturer. The transfected cells were incubated for 24 hours, followed by RNA and protein extraction for real-time polymerase chain reaction and Western blot (WB) analyses.
Statistics
Categorical variables were summarized by frequencies, with dichotomous variables assessed for normality of distribution and subsequently compared by use of the $\chi^2$ test. We compared continuous variables using Student t tests for normally distributed variables and Mann-Whitney U tests for nonnormally distributed variables. To adjust for potential bias related to sex and age, multivariate linear regression was used to assess the relationship of $\text{BMPR2}$ isoform ratios to sex and age. An $\alpha$ level of 0.05 was chosen, and values of $P<0.05$ were considered statistically significant. All $P$ values were 2 sided. Analyses were performed with Prism 5 for Mac OS X software package (GraphPad Software Inc, La Jolla, CA) and SPSS (version 19; IBM SPSS Statistics, Armonk, NY).

Results
$\text{BMPR2}$ Is Alternatively Spliced
To determine the full extent of human $\text{BMPR2}$ (chromosome 2, at 2q33-q34, from 203240945–203432480; National Center for Biotechnology 37, August 2010) alternative splicing, we first analyzed 537 GenBank accessions using alignment tools in the National Center for Biotechnology databases. Only expressed sequence tags matching the genomic sequence with >99% accuracy over their entire length were further evaluated. These analyses showed that the $\text{BMPR2}$ gene contains 19 distinct introns and 6 alternatively spliced potential variants (Figure 1A). Expression and sequence analyses of mRNA isolated from a variety of normal and HPAH patient tissues showed that most tissues expressed isoform-A (full length) and isoform-B (exon 12 is spliced out), although isoform-A expression was significantly higher than isoform-B expression (Figure 1B). Interestingly, lung tissues had one of the highest levels of expression of both isoforms (Figure 1A and 1B).

Affected $\text{BMPR2}$ Mutation Carriers Have Higher Amounts of Isoform-B mRNA Relative to Isoform-A
In light of the relative importance of exon 12 in BMPR-II function, we hypothesized that isoform-B (lacking exon12) cellular expression levels are important for HPAH pathogenesis. To test our hypothesis, we measured the relative abundance of isoform-A and -B mRNA in CLs derived from HPAH patients (47 cell lines) or carriers (35 cell lines) by isoform-specific real-time polymerase chain reaction and, in a smaller subset, by WB analysis. This number reflected all the cell lines available in our repository at that time. Characteristics of the affected mutation carrier and unaffected mutation carrier samples analyzed are shown in the Table. Forty-seven CL cell lines carried NMD$^+$ $\text{BMPR2}$ mutations and 35 carried NMD$^-$ $\text{BMPR2}$ mutations. The affected $\text{BMPR2}$...
mutation carrier group contained more female individuals who were younger at the time of sample acquisition compared with the unaffected BMPR2 mutation carrier group, as expected. RNA and protein expression data were analyzed in an aggregate fashion and showed that patients had significantly higher expression of isoform-B relative to isoform-A (B/A ratio) than unaffected mutation carriers \((P=0.002;\) Figure 2). This association held even when samples were analyzed on the basis of the type of mutation, NMD\(^+\) \((P=0.02)\) versus NMD\(^-\) \((P=0.03;\) Figure I in the online-only Data Supplement).

High Relative B/A Ratio Adversely Affects Signaling From BMPR-II Receptor

Because patients’ cells had a higher relative B/A ratio than carriers’ cells, we next determined whether the higher B/A ratio had any effect on BMPR-II function on a cellular level. BMPR2 exon 12 encodes an intracytoplasmic domain, which is important for BMPR-II interactions and regulation of actin through LIM kinase 1. A normally functioning cytoplasmic domain inhibits the ability of LIM kinase to phosphorylate the actin-binding/modulating protein cofilin, resulting in decreased phosphorylated cofilin (p-cofilin) and increased unphosphorylated cofilin in cells.\(^{37}\) On the other hand, a malfunctioning or absent cytoplasmic domain diminishes the inhibitory effects of BMPR-II on LIMK, resulting in increased p-cofilin and decreased cofilin.\(^{37,38}\)

We proceeded to determine whether there were differences in cofilin phosphorylation between CL cell lines with high B/A ratios and cell lines with low B/A ratios. We stimulated exponentially growing CLs with high and low B/A ratios with 10 ng BMP for 1 hour and 20 ng BMP for 2 hours. Our data show that cell lines with high relative B/A ratios had significantly lower cofilin/p-cofilin ratios than cell lines with low B/A isoform ratios \((2.6\pm0.17\) versus \(3.4\pm0.15;\) \(P=0.01;\) \(n=4)\) as measured by WB densitometry; in other words, cells with high B/A ratios had lower cofilin and higher p-cofilin, whereas cells with low B/A ratios had higher cofilin and lower p-cofilin, suggesting that there was defective/reduced functioning of BMPR-II receptor in these cell lines (Figure 3A).

We then determined whether the cofilin signaling in cell lines with high and low B/A ratios was different in response to higher or sustained stimulation of the BMPR-II receptor. We thus exposed high and low B/A ratio cell lines to higher doses of BMP (20 ng) for 2 hours and then compared the levels of p-cofilin and cofilin. These data showed that although longer exposure of the low B/A ratio cell lines to a higher dose of BMP significantly increased the cofilin/p-cofilin ratio as measured by WB densitometry, similar treatment of the high B/A ratio cell lines did not \((10.6\pm0.8 [n=8] \text{ versus } 2.7\pm0.17 [n=6];\) \(P=0.0001;\) Figure 3B). This suggested that cell lines with higher relative B/A ratios have significantly decreased capacity to respond to higher/longer BMP signaling compared with lower B/A ratio cell lines (Figure 3B). Thus, data presented in Figure 3A and 3B conclusively show that the relative increase in BMPR2 isoform-B levels affects the cofilin signaling function of the BMPR-II receptor.

**Figure 2.** BMPR2 isoform ratios in cultured lymphocytes (CLs) derived from carriers (light gray; \(n=35\)) and affected individuals (dark gray; \(n=47\)). A, Relative isoform-B versus isoform-A (B/A) mRNA ratio as determined by isoform-specific real-time polymerase chain reaction (PCR). Real-time PCR analysis was done 3 times with each sample run in triplicate. Results from 1 representative experiment are shown as box-and-whiskers plots with whiskers indicating Tukey whiskers and extreme data points indicated by filled circles. B and C, Western blot validation of the B/A ratios. Total cellular protein extracts from CL cell lines from 9 affected individuals with the highest B/A ratios and 7 carriers with the lowest B/A ratios were analyzed. Isoform mRNA expression data were analyzed in a nonparametric (Mann-Whitney \(U\) test) manner; Western blot densitometry data were analyzed with the Student \(t\) test. An \(\alpha\) level of 0.05 was chosen, and values of \(P<0.05\) were considered statistically significant. All \(P\) values were 2 tailed.

**BMPR2 Exon 12 Contains a Splice Enhancer That Binds Splicing Factor SRSF2**

Exon inclusion and exclusion are determined by exonic and intronic splicing enhancers and silencers and the various splicing factors that bind to these. We analyzed the 1280-bp sequence of BMPR2 exon 12 using matrixes for the prediction of sequences required for binding of splicing factors (http://rulai.cshl.edu/cgi-bin/tools/ESE3/esefinder).\(^{35,36}\) This analysis revealed a large number of possible enhancer sequences that met the matrix threshold (Figure 4A). To identify which, if any, of these putative enhancer sequences are involved in exon 12 inclusion versus exclusion, we
queried our large HPAH BMPR2 mutation database for naturally occurring mutations in exon 12 that resulted in the exclusion (deletion) of exon 12 from the BMPR2 transcript. Our query identified a 2695C>T change in exon 12 of the full-length BMPR2 transcript (NM_001204) that, when present, resulted in skipping/loss of exon 12.26 Further analysis of the sequence around this change using the splice enhancer prediction matrix revealed that this sequence is predicted to bind splicing factor SRSF2 (Figure 4B) and that the C>T change alters the most important nucleotide of the consensus enhancer sequence, resulting in complete disruption of the SRSF2 binding site (Figure 4C and 4D). These data suggest that exon 12 contains at least 1 splicing enhancer that binds splicing factor SRSF2 and can regulate BMPR2 exon 12 inclusion/exclusion.

**SRSF2 Regulates BMPR2 Exon 12 Inclusion**

Because naturally occurring mutations in the SRSF2 binding sequence lead to exon skipping, we wondered whether SRSF2 plays a direct role in BMPR2 alternative splicing and thus cellular levels of isoform-A and -B. Generally, it is well known that SRSF2 promotes exon inclusion. Thus, we first determined whether there is any difference in cellular SRSF2 levels between the high (patients) and low (carriers) B/A ratio cell lines. Six cell lines with a high B/A ratio and 5 cell lines with a low B/A ratio were analyzed by WB analysis. We also analyzed levels of 2 other splicing factors, hnRNPA1 and SRPK, with binding sites in exon 12. This analysis showed that CL cell lines with a high B/A ratio had low levels of SRSF2 compared with cell lines with a low B/A ratio, as measured by WB densitometry, and that this difference was significant (2.72x10^6±3.36x10^5 [n=5] versus 4.72x10^6±3.6x10^5 [n=6]; P=0.003; Figure 5A).

There were no statistical differences in levels of SRPK (4.52x10^5±7.25x10^4 [n=5] versus 5.59x10^5±5.56x10^4 [n=6]; P=0.3; Figure 5B) or hnRNPA1 (3.18x10^5±4.81x10^4 [n=5] versus 2.06x10^5±3.07x10^4 [n=6]; P=0.08; Figure 5C) between cells with high and low B/A ratios as measured by WB densitometry, although hnRNPA1 levels were suggestive of an association. These data indicate that SRSF2 might be a more important regulator of BMPR2 isoform ratios than SRPK and hnRNPA1 in the cell lines analyzed. They are also consistent with the splicing patterns because (as discussed above) SRSF2 promotes exon inclusion, so we would expect that cell lines with low levels of isoform-B would have higher amounts of SRSF2 and vice versa. To more directly identify a role for SRSF2 in BMPR2 alternative splicing, we used siRNA to knock down SRSF2 (Figure 5D) in PMVECs. We then tested the relative quantities of BMPR2 isoform-A and -B in these cells. Although control siRNA did not alter the B/A ratio, SRSF2 knockdown resulted in a higher B/A ratio (Figure 5E). These data are consistent with the notion that SRSF2 regulates BMPR2 isoform levels.

**Discussion**

One of the most perplexing features of BMPR2-associated HPAH is the observed reduced penetrance. Nearly 80% of mutation carriers never manifest disease, but they can produce offspring who do.13 Here, we present data that suggest that alternative splicing of BMPR2 contributes to this reduced penetrance.

Our data show that cells from patients are more likely to have higher levels of isoform-B relative to levels of isoform-A (B/A ratio) compared with cells from carriers. Thus, if the wild-type BMPR2 allele in a BMPR2 mutation carrier is spliced in such a manner that there is more isoform-B relative to isoform-A, then that mutation carrier is more likely to develop PAH.

We further show that cells with high B/A isoform ratios have higher p-cofilin and lower cofilin (a reduced capacity to unphosphorylate cofilin) under both short-term and sustained BMP stimulation. Thus, increased levels of isoform-B relative to isoform-A reduce the ability of the
cells to inhibit cofilin phosphorylation in response to BMP. This increase in B/A ratio is due in part to an exonic splice enhancer that binds an SR splicing factor, SRSF2. Carriers had higher cellular levels of SRSF2 than patients, and SRSF2 knockdown in PMVECs resulted in increased B/A isoform ratios, implicating SRSF2 directly in BMPR2 alternative splicing in a lung cell line thought to be important in HPAH pathogenesis. Thus, the data presented here add HPAH to the list of pulmonary diseases in which alternative splicing plays a role.

Our data showing that cellular isoform-B levels may have a role in BMPR-II function and HPAH penetrance are not surprising given that isoform-B lacks exon 12 of the BMPR2 gene. At 1280 bp, exon 12 is the largest exon of the gene and forms part of the intracytoplasmic tail domain that is important for interactions of BMPR-II with β-actin. BMPR2 mutations that result in skipping of exon 12 cause HPAH through what is thought to be a dominant-negative effect because overexpression of exon 12–truncated Bmpr2 in mice produces pulmonary hypertension. One of the more surprising observations presented here was that CL cell lines with increased B/A isoform ratios have significantly decreased capacity to respond to overstimulation of the BMPR-II receptor compared with cell lines with low B/A ratios. Thus, in the setting in which 1 BMPR2 allele is abnormal because of a mutation, additional stress on BMPR2-II signaling in the form of increased expression of a naturally occurring dominant-negative isoform (isoform-B)
likely further impairs receptor function and is thus more likely to contribute to disease.

We have previously shown that expression of the wild-type BMPR2 allele also plays a role in disease penetrance in that patients are more likely to have lower levels of wild-type BMPR2 expression than unaffected mutation carriers.13 This, together with the data presented here, suggests that the BMPR2 gene itself may be the most important modifier of HPAH penetrance. Because of the limitations of our earlier study, we are unable at this time to determine the relative contributions of wild-type BMPR2 expression and BMPR2 alternative splicing to the penetrance of HPAH. It is likely, however, that there is some overlap, at a molecular level, between these mechanisms.

Figure 5. Splicing factor levels in high and low B/A ratio cell lines. A, SRSF2 levels as detected by Western blot analysis and shown as densitometry units in low (light gray; n=5) and high (dark gray; n=6) B/A ratio cell lines. B, SRPK1 levels as detected by Western blot analysis and shown as densitometry units in low (light gray; n=5) and high (dark gray; n=6) B/A ratio cell lines. C, heterogeneous nuclear ribonucleoprotein 1 (hnRNP1) levels as detected by Western blot analysis and shown as densitometry units in low (light gray; n=5) and high (dark gray; n=6) B/A ratio cell lines. D, Real-time polymerase chain reaction (PCR; graph) and Western blot (inset) confirmation of siRNA-mediated SRSF2 knockdown in PMVECs. E, Determination of B/A ratio by real-time PCR in pulmonary microvascular endothelial cells after siRNA-mediated knockdown of SRSF2. An α level of 0.05 was chosen, and values of P<0.05 were considered statistically significant. All P values were 2 tailed.
In this study, we identified 1 splice enhancer and 1 splicing factor, SRSF2, that regulate BMPR2 alternative splicing. SPRK1 and hnRNP A1 levels were not different between the high and low B/A isoform ratio cell lines. This finding may suggest that these splicing factors are not involved in BMPR2 alternative splicing or that our WB analyses were not adequately powered to detect a difference in the expression of these splicing factors. As shown in Figure 5A, exon 12 contains a large number of possible enhancer and silencer sequences, thus increasing the likelihood that additional enhancers and their associated factors play a role in BMPR2 alternative splicing. Our hnRNP A1 expression data hint at this possibility. hnRNP A1 is an exon exclusion factor, and its increased function and levels can lead to the exclusion of an exon. So, one would predict that hnRNP A1 would be higher in cells lines with high B/A ratios and low in cells lines with low B/A ratios. Our data are suggestive of this (Figure 5C). Thus, the molecular mechanisms that lead to a high B/A ratio in patients might include both low SRSF2 and high hnRNP A1. It is likely that alterations of SRSF2 levels also affect alternative splicing of many other genes in addition to BMPR2, so the eventual effect on penetrance may be due to a global effect on alternative splicing. Elucidation of the global alternative splicing eventual effect on penetrance may be due to a global effect on splicing of many other genes in addition to BMPR2, s ot h e SP RK1 and hnRNP A1 levels were not different between the high and low B/A isoform ratio cell lines. This may suggest that these splicing factors are not involved in BMPR2 alternative splicing or that our WB analyses were not adequately powered to detect a difference in the expression of these splicing factors. As shown in Figure 5A, exon 12 contains a large number of possible enhancer and silencer sequences, thus increasing the likelihood that additional enhancers and their associated factors play a role in BMPR2 alternative splicing. Our hnRNP A1 expression data hint at this possibility. hnRNP A1 is an exon exclusion factor, and its increased function and levels can lead to the exclusion of an exon. So, one would predict that hnRNP A1 would be higher in cells lines with high B/A ratios and low in cells lines with low B/A ratios. Our data are suggestive of this (Figure 5C). Thus, the molecular mechanisms that lead to a high B/A ratio in patients might include both low SRSF2 and high hnRNP A1. It is likely that alterations of SRSF2 levels also affect alternative splicing of many other genes in addition to BMPR2, so the eventual effect on penetrance may be due to a global effect on alternative splicing. Elucidation of the global alternative splicing pattern is important and may provide further insights into the pathways and cellular processes involved. In addition to cellular levels, processes like phosphorylation, poly(ADP-ribosylation), sumoylation, and/or arginine methylation can also affect splicing factor function; however, whether any of these is at play here is not known and requires significant additional work.

At this time, we do not know why SRSF2 expression is higher in some mutation carriers. It is known that SRSF2 can autoregulate its expression by activating the NMD pathway; however, expression levels of SRSF2 have not previously been associated with any disease. Similarly, the role of any polymorphisms in SRSF2 expression or function remains undefined. Interestingly, mutations in SRSF2 were recently shown to be associated with worse outcomes in myelodysplastic syndromes. SRSF2 can also autoregulate its function by posttranslational modifications. Thus, the higher expression of SRSF2 in some mutation carriers may be secondary to one of the reasons discussed above or simply baseline expression differences resulting from cis or trans regulatory effects on the SRSF2 gene.

Obviously, in a patient, there might be other mechanisms at play. Several studies, including our own, have shown that environment, including exposure to drugs and chemicals, can alter splicing factor levels. Thus, dynamic changes to alternative splicing, in response to medication, drugs, and environmental signals, could lead to added stress on BMPR-II signaling and push a mutation carrier toward disease. This may be particularly important when HPAH patients are under treatment in which the pharmacological milieu may in fact act to dynamically alter alternative splicing, leading to worsening of disease. However, because our analyses were done on CL cells, the differences in SRSF2 expression reported here are more likely to be due to genetic/inherited factors. Nevertheless, our studies suggest that further investigations are needed to determine the effects of environmental signals (eg, hypoxia and oxygen) and the common pharmacological therapies typically used in HPAH on BMPR2 and global alternative splicing. We also do not know whether other splicing isoforms (identified through our expressed sequence tag analysis; Figure 1) might also play a role in HPAH pathogenesis. Certainly, we did not detect any evidence of their expression in patient-derived CLs or normal PMVECs. However, we do not know whether the same is the case in patient-derived PMVECs as well. Those studies will require additional resources such as induced pluripotent stem cell–derived PMVECs from HPAH patients, which currently are not available.

The use of CLs is a relative weakness of this study. However, the use of patient CLs is an unavoidable limitation owing to the unavailability of patient PMVECs in sufficient numbers. Furthermore, because splicing could be affected by the overall disease milieu of a patient, primary cells from patients may not be an ideal model to study baseline genetic variation in splicing patterns even if they were available. Thus, a relative advantage of CLs is that they are removed from the immediate environment and consequently are not affected by any potential drug or paracrine disease effects. Additionally, CLs can be obtained easily not only from HPAH patients but also from asymptomatic mutation carriers and control subjects. A second concern about the use of CLs is whether the process of establishing these cell lines can in itself lead to changes in gene expression. However, we feel that this is unlikely to be the case for 2 reasons. First, even if the derivation influenced gene expression and/or splicing, we expect that it would influence affected and unaffected CLs equally. Second, we have previously shown that the derivation of CL cell lines did not affect BMPR2 gene expression. Thus, it is likely that the differences observed here reflect underlying genetic differences. Therefore, even though CLs have several relative weaknesses when used as a discovery tool, they can help formulate hypotheses that can then be tested in a cell line of interest. This study provides a good example of this concept.

Conclusions
We have shown that alternative splicing of BMPR2 has a role in HPAH penetrance. If a mutation carrier splices his or her BMPR2 gene in a manner that results in more isoform-B transcript relative to isoform-A, then that mutation carrier is more likely to develop PAH. In light of what is known about how drugs and environmental factors can affect splicing, our findings have implications for HPAH pathogenesis, treatment, and follow-up.

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None.
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One of the most perplexing features of heritable pulmonary arterial hypertension is its reduced penetrance. Nearly 80% of mutation carriers have no clinical symptoms, but they can produce offspring who are affected by heritable pulmonary arterial hypertension. Thus, disease development cannot be predicted in a mutation carrier, creating anxiety in the patient and uncertainty about treatment in the physician. The data presented in this article point to a novel explanation for the reduced penetrance seen in heritable pulmonary arterial hypertension. Our data show that BMPR2 alternative splicing plays a role in this reduced penetrance. BMPR2 mutation carriers were more likely to have pulmonary arterial hypertension if they had higher levels of an alternatively spliced BMPR2 transcript, isoform-B, relative to the full-length BMPR2 transcript. Thus, our data suggest that although a BMPR2 mutation creates baseline susceptibility, an important determinant of disease penetrance appears to be the higher relative expression of the alternative spliced BMPR2 isoform-B. These data emphasize the importance of BMPR2 alternative splicing in heritable pulmonary arterial hypertension and raise an intriguing question: Can a predictive model of disease based on expression levels of the BMPR2 alternative splicing be developed? This predictive model will have obvious clinical utility in predicting which mutation carriers may eventually develop disease and thus will require appropriate follow-up. Because splicing can be manipulated in vivo by drugs, our findings suggest that manipulation of BMPR2 alternative splicing should be explored as a potential new intervention in heritable pulmonary arterial hypertension.
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Supplementary Figure 1

**A**

Comparison of Relative (B/A) Isoform mRNA Ratios between Carriers (n=20) and Affected (n=27) individuals. The P value is 0.02.

**B**

Comparison of Relative (B/A) Isoform mRNA Ratios between Carriers (n=15) and Affected (n=20) individuals. The P value is 0.03.
Figure Legend for supplementary figure 1.

Supplementary Figure 1. BMPR2 isoform ratios as determined by real-time PCR in CLs from carriers/unaffected (light gray) and affected/patients (dark gray) containing NMD+ (A) and NMD- (B) mutations. Data are shown as box and whiskers plots with whiskers indicating Tukey whiskers and extreme data points indicated by filled circles. An α-level of 0.05 was chosen, and P-values < 0.05 were considered statistically significant.