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Simvastatin enhances bone morphogenetic protein receptor type II expression

Hong Hu, Arthur Sung, Guohua Zhao, Lingfang Shi, Daoming Qiu, Toshihiko Nishimura, Peter N. Kao *

Division of Pulmonary and Critical Care Medicine, Stanford University Medical Center, 300 Pasteur Drive, Stanford, CA 94305-5236, USA

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Abstract

Statins confer therapeutic benefits in systemic and pulmonary vascular diseases. Bone morphogenetic protein (BMP) receptors serve essential signaling functions in cardiovascular development and skeletal morphogenesis. Mutations in BMP receptor type II (BMPR2) are associated with human familial and idiopathic pulmonary arterial hypertension, and pathologic neointimal proliferation of vascular endothelial and smooth muscle cells within small pulmonary arteries. In severe experimental pulmonary hypertension, simvastatin reversed disease and conferred a 100% survival advantage. Here, modulation of BMPR2 gene expression by simvastatin is characterized in human embryonic kidney (HEK) 293T, pulmonary artery smooth muscle, and lung microvascular endothelial cells (HLMVECs). A 1.4 kb BMPR2 promoter containing Egr-1 binding sites confers reporter gene activation in 293T cells which is partially inhibited by simvastatin. Simvastatin enhances steady-state BMPR2 mRNA and protein expression in HLMVEC, through posttranscriptional mRNA stabilization. Simvastatin induction of BMPR2 expression may improve BMP–BMPR2 signaling thereby enhancing endothelial differentiation and function.

Keywords: Statins; Pulmonary hypertension; Transcription; Posttranscriptional RNA stabilization; Mevalonate

Statins, inhibitors of HMG-CoA reductase, confer substantial cardiovascular benefits that exceed their capacity to lower serum cholesterol. These beneficial effects include suppression of vascular inflammation and vascular smooth muscle cell proliferation [1–4], increases in endothelial nitric oxide production [5], and expression of antithrombotic modulators [6], and increases in circulating endothelial progenitor cells [7–9]. Multiple molecular mechanisms may underlie these effects including suppression of inflammatory transcription factors [10,11], modulation of endothelial differentiation and angiogenesis [12–14], and posttranscriptional stabilization of endothelial nitric oxide synthase (eNOS) RNA [15].

Insights into statins and vascular homeostasis may be gleaned from the effects of statins on skeletal development and bone formation. Statins promote bone formation through induction of bone morphogenetic protein (BMP)-2 and osteoblast differentiation [16–18]. The BMP ligand–receptor signaling pathways regulate skeletal morphogenesis as well as embryonic vascular development [19–22]. Type I and II transforming growth factor (TGF) and bone morphogenetic protein receptors (BMPRs) undergo ligand-mediated heterodimerization that activates serine-threonine phosphorylation of downstream SMADs [23,24].

A link between BMPR receptor type II (BMPR2) signaling and the human pulmonary vascular disease, idiopathic pulmonary arterial hypertension, was revealed when two independent groups identified mutations in BMPR2 as a genetic risk factor for the development of familial and sporadic primary pulmonary hypertension [25,26]. BMPR2 is

* Corresponding author. Fax: +1 650 725 5489.
E-mail address: peterkao@stanford.edu (P.N. Kao).

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expressed at high levels in normal pulmonary microvascular endothelial cells of humans [27] and mice [28], and signaling through BMPR2 is essential for embryonic vascular development and homeostasis [20,29]. Expression of BMPR2 protein was reduced in the lungs of all pulmonary hypertension patients examined, even in the absence of genetic mutations [27]. This observation suggests that deficient BMPR2 gene expression is associated with, and may contribute to, pulmonary vascular disease.

Recently, we demonstrated that simvastatin reversed established pulmonary hypertension and conferred a 100% survival advantage in pneumonectomized, monocrotaline-injected rats [4,10]. We proposed that the beneficial mechanisms of simvastatin in experimental pulmonary hypertension included suppression of vascular smooth muscle cell proliferation, induction of apoptosis in pathological neointimal smooth muscle cells, and enhancement of endothelial cell nitric oxide production. We translated these findings into an observational trial of simvastatin for treatment of human patients with pulmonary hypertension that demonstrated safety and suggested efficacy in terms of improved exercise tolerance, hemodynamics, and/or survival [30,31].

Here we characterized simvastatin modulation of BMPR2 gene expression in vitro, at the levels of transcription, posttranscriptional mRNA stabilization, and protein expression. Simvastatin enhances BMPR2 expression in human lung microvascular endothelial cells (HLMVEC). We propose that simvastatin induction of BMP ligand [16,17] and BMPR2 receptor expression may modulate endothelial differentiation [14] and promote angiogenesis [32].

Materials and methods

Cell culture and chemicals. Human embryonic kidney (HEK) 293T cells (ATCC) were cultured in a Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (GIBCO). Human pulmonary artery smooth muscle cells (HPASMC) and human lung microvascular endothelial cells (HLMVEC) were purchased from Clonetics and cultured in the recommended specific media, SmBm and EBM-2 (Clonetics). Simvastatin was a gift from Merck (Merck-Rahway, NJ, USA) and was converted to the active form by alkaline hydrolysis, as described [33]. Mevalonic acid, actinomycin D, and general chemicals were purchased from Sigma.

Generation of BMPR2 promoter-luciferase reporter genes. The 5′ flanking sequence of the human BMPR2 gene, located at Chromosome 2q33, was identified from GenBank (NT_005403.15|Hs2_5560:53449001–53451614). PCR primers were designed to amplify 1.4 kb of the proximal promoter upstream of the predicted transcription start site and including the 5′-UTR. The 5′ primer sequence was 5′-GTACGGACAGA CGCGCGCACGCAGCGGGACG and the 3′ sequence was 5′-AGGGT CAGAACTAGTTCCCGACTGC. Genomic PCR was performed in a 50 μl reaction mixture containing template 1 μg human genomic DNA, 100 ng each primer, dNTPs, and 0.5 μl LA Taq (TaKara, Shuzo, Japan) in LA PCR buffer II. After an initial denaturation step at 95 °C for 10 min, a two-step reaction was performed at 94 °C for 20 s and 68 °C for 2 min, repeated for 35 cycles, followed by a final extension at 72 °C for 10 min. The PCR product was purified by using a gel extraction kit (Qiagen) and cloned in the Topo TA Cloning pCR2.1 vector (Invitrogen). Positive clones were confirmed by DNA sequencing (ABI Prism, PAN facility, Stanford). Nested deletions of the BMPR2 promoter were generated by PCR and cloned directionally upstream of the firefly luciferase reporter gene of pGL3 vector (Promega).

BMPR2 promoter analysis in transiently transfected HEK293T cells. HEK293T cells and HPASMCs were seeded at a density of 7×10^5 cells per well (35 mm) in six-well culture plates and incubated overnight in 2 ml DMEM/FBS of SmBm media at 37 °C in a CO_2 incubator. Transient transfections using 3 μg BMPR2-firefly luciferase and 1 μg of elongation factor 1-α *Renilla* luciferase reporters were performed in quadruplicate using 7 μl Lipofectamine reagent (Invitrogen) in a total volume of 1 ml. Following a 5 h incubation, the transfection solution was replaced with fresh media and the incubation continued for 24 h. Cells were washed once with phosphate buffered saline and then harvested directly in 500 μl of Dual Luciferase lysis buffer (Promega). Whole cell extracts were analyzed for firefly and *Renilla* luciferase activities (Dual Luciferase Reporter Assay System, Promega) using a luminometer (Analytical Luminescence Laboratory Monolight 2010). The normalized BMPR2 promoter activity is calculated as the ratio of firefly to *Renilla* luciferase activities.

RNA expression analysis. Total RNA from HEK293T cells, HPASMC, and HLMVEC was isolated using Trizol (Invitrogen), fractionated and analyzed by Northern hybridization as described [34]. For analysis of steady-state RNA levels, cells were incubated with simvastatin and/or mevalonate for 24 h. For analysis of RNA stability, cells were exposed to 5 μg/ml actinomycin D in the absence or presence of simvastatin for 8 h.

BMPR2 protein analysis. HLMVECs were cultured for 24 h in the absence or presence of simvastatin and mevalonate, then whole cell extracts was prepared. Thirty micrograms of proteins were fractionated by SDS–PAGE (4–12% gradient gels) and transferred electrophoretically to nitrocellulose. Detection was achieved using primary anti-BMPR2 mouse monoclonal antibody (Becton–Dickinson Transduction (BD) 612992, 1:200 dilution) and secondary detection with goat anti-mouse IgG coupled to horseradish peroxidase (1:3000 dilution). Detection was performed using enhanced chemiluminescence (Amersham). Detection of actin as a loading control was performed using primary antibody (BD, at 1:1000 dilution).

Statistical analysis. Significance of the differences between the experimental conditions was determined by Student's t test. The data presented are means ± standard deviation. Statistical significance was indicated by a value of $p < 0.05$.

Results

Functional analysis of transcriptional regulation by human BMPR2 proximal promoter regulatory sequences

We obtained the 5′ flanking sequence of the human BMPR2 gene through GenBank (NT_005403.15|Hs2_5560: 53449001–53451614) and used the MAT Inspector program (Genomatix, Germany) to analyze potential regulatory sequences. The inferred proximal BMPR2 promoter reveals the presence of TATA and CCATT boxes, and potential binding sites for regulatory transcription factors that include Egr-1, Myf5, Sp1, Smad 3, 4, and HMGI(Y) (Fig. 1A).

We performed genomic PCR amplification and cloned a 1.4 kb (−1252/+191 bp) proximal BMPR2 promoter sequence upstream of a firefly luciferase reporter gene. We transfected this BMPR2 promoter-firefly luciferase reporter, together with a normalizing elongation factor 1-α *Renilla* luciferase normalizing vector into HEK293T cells, and demonstrated transcriptional activity (Fig. 1B). Our analyses showed that the presence of increasing BMPR2 5′ flanking sequence was associated with greater

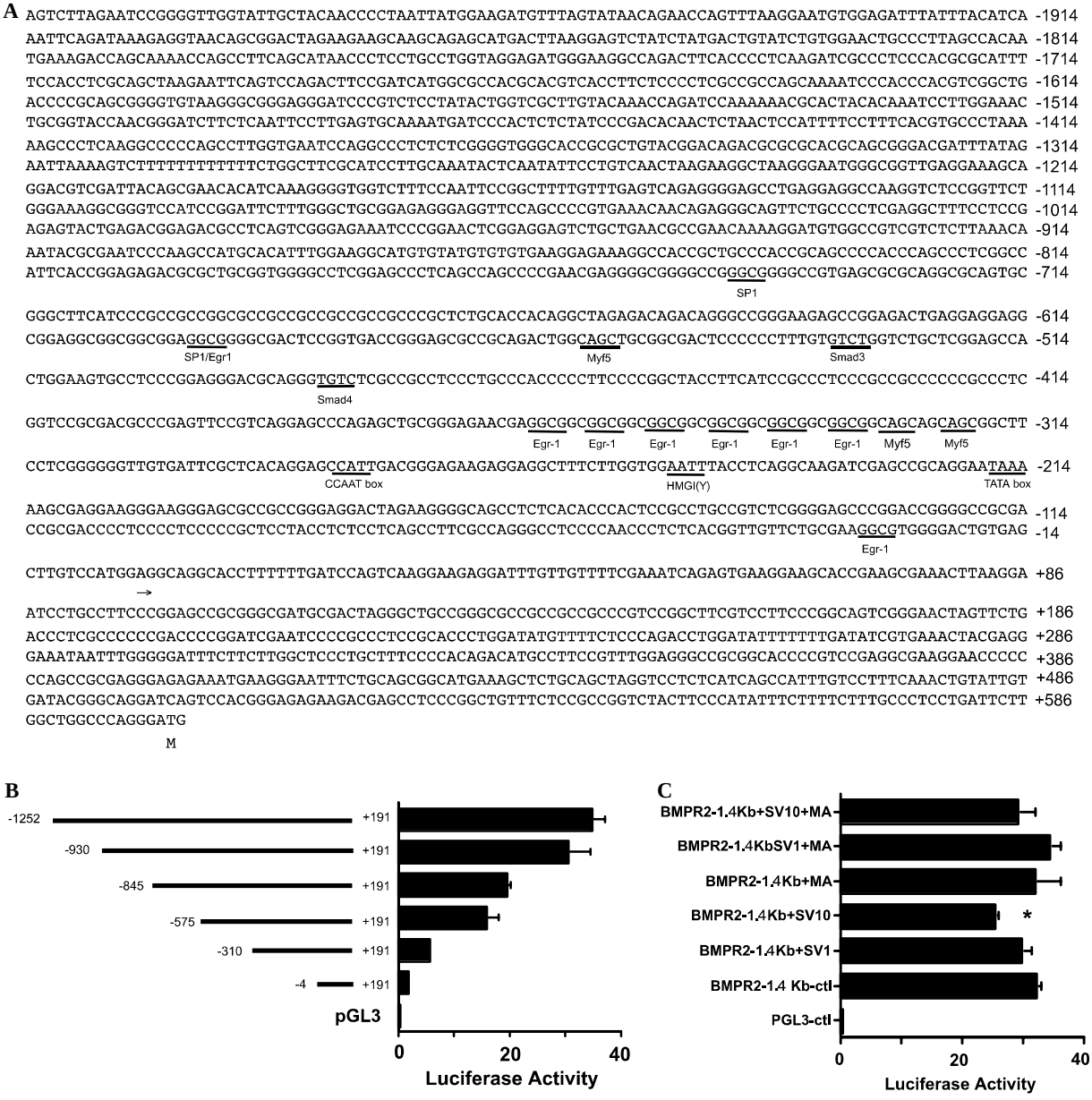

Fig. 1. Analysis of the proximal promoter of the human 5′ bone morphogenetic protein receptor type II. (A) 5′ flanking sequence and predicted transcription factor binding sites using MAT Inspector (Genomatix, Muchen, Germany). (B) Functional analysis of transcriptional regulatory sequences using the indicated BMPR2 promoter-firefly luciferase reporter genes. Transient transfections in HEK293T cells were each normalized by cotransfection of elongation factor 1-α *Renilla* luciferase. (C) Effects of simvastatin (1 or 10 μM) in the absence or presence of mevalonate (100 μM) on the activity of 1.4 kb BMPR2-luciferase are shown. For all experiments, the data are presented as means ± SD ($n = 4$; $^*p < 0.05$). SV1, simvastatin 1 μM; SV10, simvastatin 10 μM; MA, mevalonate; pGL3-ctl, pGL3 empty vector as control.

activation of the reporter gene. An incremental 2.5-fold increase in transcriptional activity occurred with inclusion of sequences from −310 to −575 bp that include the Egr-1 and Myf5 binding sites (Fig. 1B). We observed similar results in transfected HPASMCs (data not shown). HML-VECs were not amenable to transient transfections for BMPR2 promoter analysis.

The effects of simvastatin were characterized on the activity of the 1.4 kb BMPR2 promoter-luciferase reporter gene (Fig. 1C). We observed a moderate (∼20%) inhibition of BMPR2-luciferase activity in the presence of 10 μM simvastatin (Fig. 1C). Coincubation with mevalonate showed a trend toward reversal of the simvastatin effect (Fig. 1C).

Simvastatin induces BMPR2 steady-state mRNA expression

The steady-state expression of BMPR2 mRNA and protein is determined by the rates of transcription, posttranscriptional stabilization, translation, and degradation. We characterized steady-state BMPR2 mRNA expression, and its modulation by simvastatin and mevalonate, using Northern analyses in HEK293T cells (Fig. 2A) and

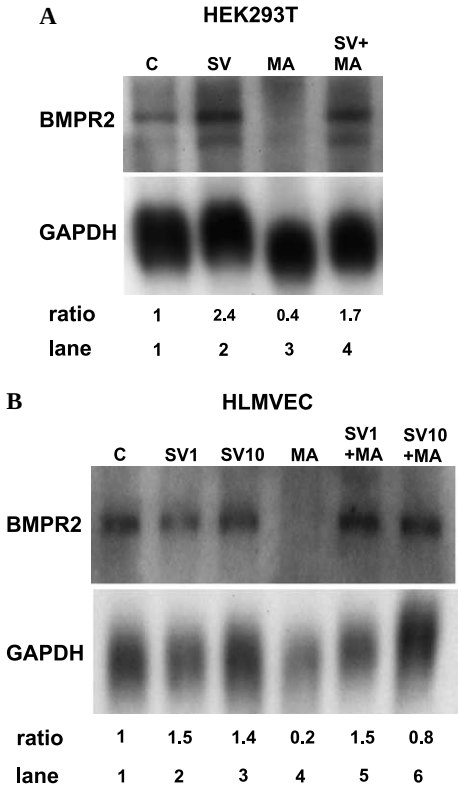

Fig. 2. Northern analysis of BMPR2 mRNA steady-state expression and induction by simvastatin. (A) HEK293T cells were treated with simvastatin (100 μM) in the absence or presence of mevalonate (100 μM) for 24 h before total RNA was isolated. (B) Human lung microvascular endothelial cells (HLMVECs) were treated with simvastatin (1 or 10 μM) in the absence or presence of mevalonate (100 μM) for 24 h before total RNA was isolated. Hybridization was performed for GAPDH as a loading control. The expression BMPR2 mRNA normalized by that of GAPDH is presented beneath each lane. SV1, simvastatin 1 μM; SV10, simvastatin 10 μM; MA, mevalonate.

primary human lung microvascular endothelial cells (Fig. 2B). The major BMPR2 mRNA present in both cell types was consistent with the known size of 11 kb (Fig. 2). Simvastatin (1, 10, and 100 μM) induced steady-state BMPR2 mRNA. Treatment with mevalonate (100 μM) specifically decreased BMPR2 mRNA expression in HEK293T cells (Fig. 2A, lane 3) and HLMVECs (Fig. 2B, lane 4). Interestingly, simvastatin reversed the inhibitory effects of mevalonate and restored BMPR2 mRNA expression in HEK293T cells (Fig. 2A, lane 4 vs. 3) and HLMVECs (Fig. 2B, lanes 5, 6 vs. 4).

Simvastatin induces posttranscriptional stabilization of BMPR2 mRNA

Although our BMPR2 promoter studies showed that simvastatin inhibited reporter gene transcription (Fig. 1C), our Northern analyses showed that simvastatin increased endogenous steady-state BMPR2 mRNA levels (Fig. 2). These findings suggested to us that simvastatin modulates BMPR2 expression through posttranscriptional mRNA stabilization, as was previously demonstrated for

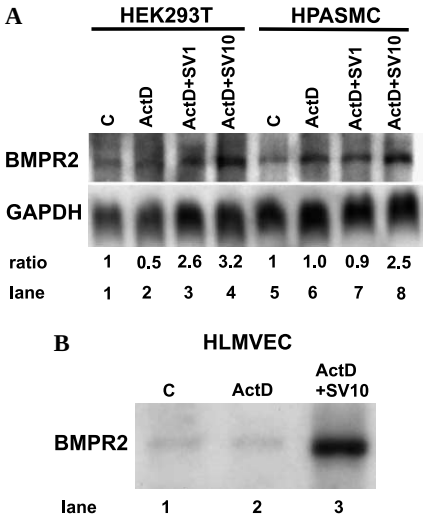

Fig. 3. Posttranscriptional stabilization of BMPR2 mRNA by simvastatin. HEK293T, HPASMC (A) and HLMVEC (B) were cultured for 8 h in the presence of simvastatin (1 or 10 μM) and actinomycin D (5 μg/ml) to block transcription, and total RNA was isolated and analyzed by Northern hybridization. C, control; ActD, actinomycin D; SV1, simvastatin 1 μM; SV10, simvastatin 10 μM.

eNOS [5]. To test this hypothesis, we characterized the effects of simvastatin on BMPR2 mRNA levels in the presence of actinomycin D to block transcription (Fig. 3).

We used actinomycin D treatment to block transcription and then measured BMPR2 mRNA levels in HEK293T, HPASMC, and HLMVECs (Fig. 3). Our results reveal at least twofold induction of BMPR2 mRNA by simvastatin (10 μM) in actinomycin D-treated HEK293T, HPASMC, and HLMVECs (Fig. 3A, lanes 3, 4 vs. 1, 2 and lane 8 vs. 5, 6, and Fig. 3B, lane 3 vs. 1, 2).

Simvastatin induces BMPR2 protein expression

We used Western immunoblotting to characterize the effects of simvastatin on BMPR2 protein expression in HLMVECs. Of three different BMPR2 antibodies evaluated, only one (recommended by H. Beppu, personal communication) showed specific recognition of a single species of the correct molecular weight. Simvastatin at 1 and 10 μM induced BMPR2 protein expression approximately twofold (Fig. 4, lanes 2, 3 vs. 1). Simvastatin induction of BMPR2 protein expression was decreased by coincubation with mevalonate (Fig. 4, lane 6 vs. 3).

Taken together, these studies demonstrate that simvastatin enhances BMPR2 expression in HLMVEC through mechanisms that include posttranscriptional stabilization of BMPR2 mRNA.

Discussion

This is the first study to characterize the regulation of BMPR2 gene expression, and its modulation by simvastatin, in HLMVECs. We used complementary methods to

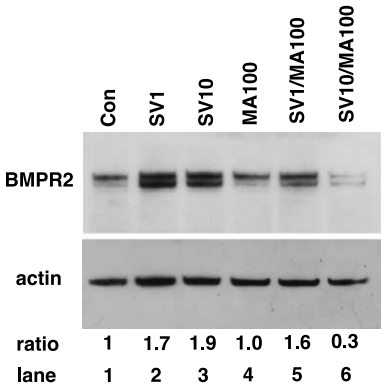

Fig. 4. Simvastatin induces BMPR2 protein expression in HLMVEC. Cells were cultured in the presence of simvastatin (1 or 10 μM) in the absence or presence of mevalonate (100 μM) for 16 h and then extracted cellular proteins were fractionated by SDS–PAGE and BMPR2 was detected by immunoblotting. The expression of actin is shown as a loading control. Con, control; SV1, simvastatin 1 μM; SV10, simvastatin 10 μM; MA100, mevalonate 100 μM.

study BMPR2 transcriptional regulation, RNA expression and stability, and protein expression.

We identified 5′ flanking sequences upstream of the human BMPR2 putative transcription start site that conferred activation on a linked luciferase reporter gene. Our BMPR2 promoter analysis demonstrated a 2.5-fold increase in basal transcription with the addition of sequences between −310 and −575 bp. This domain contains six tandem repeats of binding sites for Egr-1, a zinc-finger transcription factor that is highly expressed in the brain, heart, and lungs. Egr-1 stimulated transcription of human TGF-β receptor type II through binding to proximal promoter sequences [35]. The proximal BMPR2 promoter region also contains multiple binding sites for Myf-5, a basic helix–loop–helix transcription factor involved in myogenic differentiation [36], and binding sites for SMAD3 and SMAD4, potential downstream mediators of BMPR signaling. The actual contributions of these transcription factors to BMRP2 gene transcription remain to be determined. Simvastatin treatment moderately inhibited BMPR2-luciferase activity.

In contrast to simvastatin's inhibition of the BMPR2 transcription reporter activity, simvastatin increased steady-state endogenous BMPR2 mRNA expression. We next demonstrated that simvastatin enhanced BMPR2 mRNA expression while transcription was blocked by actinomycin D. These results established that simvastatin increased posttranscriptional stability of BMPR2 mRNA. The exact RNA sequences that regulate BMPR2 mRNA stabilization by simvastatin remain to be determined. The human BMPR2 gene contains 7 kb of 3′-untranslated region (UTR) sequence, with 11 occurrences of the sequence UAUUUA, known to contribute to posttranscriptional regulation of mRNA turnover. Posttranscriptional stabilization of eNOS mRNA by cerivastatin in tumor necrosis factor α-treated bovine aortic endothelial cells involved suppression of (destabilizing) cytosolic 3′-UTR binding proteins [37]. Further complexity in the posttranscriptional regulation of eNOS mRNA arises due to the selective tissue expression of an overlapping natural antisense transcript [38]. Posttranscriptional regulatory mechanisms that involve antisense and double-stranded RNAs hold the potential to modulate the tissue-specific expression of diverse genes, including BMPR2 [39]. We suggest that the reduced pulmonary endothelial expression of BMPR2 observed in the majority of patients with pulmonary hypertension [27] might arise from posttranscriptional downregulation of BMPR2 RNA.

This study reveals regulation of BMPR2 gene expression by simvastatin that is likely relevant to pulmonary vascular homeostasis and to novel therapeutic approaches to pulmonary hypertension. ten Dijke and co-workers [32] demonstrated that BMP signaling activates endothelial phosphorylation of Smad 1/5/8 and expression of Id1, thereby promoting endothelial migration and tube formation in support of angiogenesis. Fluvastatin treatment of human dermal microvascular endothelial cells similarly induced Id1 expression [14]. These two studies reveal a convergence of BMP–BMPR2 and statin signaling in the induction of endothelial Id1 and suggest that these pathways may be synergistic.

Simvastatin enhances BMPR2 gene expression in human lung microvascular endothelial cells in vitro. We propose that statin treatment may synergize with BMP–BMPR2 signaling in endothelial cells to promote angiogenesis and nitric oxide production. The observed remarkable therapeutic benefits of simvastatin in experimental pulmonary hypertension [40] may involve enhancement (or restoration) of BMP–BMPR2 expression and signaling leading to improved endothelial function and regression of vascular disease.

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