Interleukin-6 Modulates the Expression of the Bone Morphogenetic Protein Receptor Type II Through a Novel STAT3–microRNA Cluster 17/92 Pathway

Matthias Brock, Michelle Trenkmann, Renate E. Gay, Beat A. Michel, Steffen Gay, Manuel Fischler, Silvia Ulrich, Rudolf Speich, Lars C. Huber

Abstract—Dysregulated expression of bone morphogenetic protein receptor type II (BMPR2) is a pathogenetic hallmark of pulmonary hypertension. Downregulation of BMPR2 protein but not mRNA has been observed in multiple animal models mimicking the disease, indicating a posttranscriptional mechanism of regulation. Because microRNAs (miRNAs) regulate gene expression mainly through inhibition of target gene translation, we hypothesized that miRNAs may play a role in the modulation of BMPR2. Performing a computational algorithm on the BMPR2 gene, several miRNAs encoded by the miRNA cluster 17/92 (miR-17/92) were retrieved as potential regulators. Ectopic overexpression of miR-17/92 resulted in a strong reduction of the BMPR2 protein, and a reporter gene system showed that BMPR2 is directly targeted by miR-17-5p and miR-20a. By stimulation experiments, we found that the miR-17/92 cluster is modulated by interleukin (IL)-6, a cytokine involved in the pathogenesis of pulmonary hypertension. Because IL-6 signaling is mainly mediated by STAT3 (signal transducer and activator of transcription 3), the expression of STAT3 was knocked down by small interfering RNA, which abolished the IL-6–mediated expression of miR-17/92. Consistent with these data, we found a highly conserved STAT3-binding site in the promoter region of the miR-17/92 gene (C13orf25). Promoter studies confirmed that IL-6 enhances transcription of C13orf25 through this distinct region. Finally, we showed that persistent activation of STAT3 leads to repressed protein expression of BMPR2. Taken together, we describe here a novel STAT3–miR-17/92—BMPR2 pathway, thus providing a mechanistic explanation for the loss of BMPR2 in the development of pulmonary hypertension. (Circ Res. 2009;104:1184-1191.)

Key Words: pulmonary hypertension ■ BMPR2 ■ miR-17/92 ■ interleukin-6 ■ STAT3

Pulmonary hypertension is a devastating condition defined by the sustained elevation of pulmonary vascular resistance that leads rapidly to right heart failure and death when left untreated. The pathogenesis of pulmonary hypertension is characterized by vascular remodeling and vasoconstriction. Many chemotactic and inflammatory factors have been associated with these vascular changes including interleukin (IL)-6 and transforming growth factor (TGF)β. In familial pulmonary arterial hypertension, germline mutations in the gene encoding the type II receptor of the bone morphogenetic protein (BMPR2) comprise a genetic hallmark of the disease. BMPR2 is a surface protein receptor that belongs to the transforming growth factor (TGF)β family. Its expression on endothelial and vascular smooth muscle cells mediates binding of bone morphogenetic proteins (BMPs) that have been identified as inhibitors of vascular smooth muscle cell proliferation while inducing cell death. Thus, it was suggested that the downregulation of BMPR2 might lead to significant alterations in these signaling cascades and, ultimately, to remodeling of the pulmonary vascular bed. Of interest, alterations in the surface expression of BMPR2 have also been described in nongenetic forms of pulmonary hypertension. In addition, loss of BMPR2 has been observed in several animal models mimicking the disease. The intracellular mechanisms leading to this downregulation however are yet to be elucidated. Studies focusing on the role of BMPR2 in the development of pulmonary arterial hypertension have observed reduced protein level of this surface receptor. Takahashi et al, for example, described the expression of BMPR2 in pulmonary arteries of rats under normal conditions and after exposure to hypoxic conditions. Consequently, hypoxia was found to reduce the expression of BMPR2 on the protein levels, whereas the levels of the corresponding mRNA were not affected adequately. Similarly, in the monocrotaline-induced rat model of pulmonary hypertension, the expression of the BMPR2 protein was rapidly reduced without initial effects on the mRNA levels.
These findings suggest a posttranscriptional mechanism, such as the involvement of microRNAs (miRNAs) that bind to their target mRNAs by Watson–Crick base pairing at distinct seed regions and, thus, alter mRNA stability or affect protein translation.

An accumulating body of evidence suggests that up to one-third of the human genome is regulated by miRNAs through posttranscriptional mechanisms. Consequently, miRNAs have been associated with various cellular processes including cell death, differentiation, and proliferation. Based on computational algorithms (ie, TargetScan) and the fact that surface protein receptors such as the TGFβ-receptor type II (TGFβR2) have already been shown to be regulated by miRNAs, we identified the miRNA cluster 17/92 as potential modulator of BMPR2 expression. In the present in vitro study, we used human pulmonary arterial endothelial cells (HPAECs), hepatocellular carcinoma (HepG2) cells, and human embryonic kidney (HEK)293 cells to address the following issues: (1) the role of microRNA (miR)-17/92 in the posttranscriptional regulation of BMPR2 expression; (2) the effects of inflammatory cytokines and growth factors on the expression of miR-17/92; and (3) the role of the transcription factor STAT3 (signal transducer and activator of transcription 3) as the master link between IL-6 and the modulation of BMPR2. Our data reveal for the first time a potential molecular mechanism explaining the downregulation of BMPR2 in the development of pulmonary arterial hypertension.

Materials and Methods
An expanded Materials and Methods section is available in the online data supplement at http://circres.ahajournals.org.

Cell Culture
For cell culture, human embryonic kidney (HEK)293 cells, and human hepatocellular carcinoma (HepG2) cells were used. HPAECs were purchased from Cascade Biologics. All growth factors and stimulation agents (recombinant human IL-6, vascular endothelial growth factor [VEGF], platelet-derived growth factor [PDGF]) were purchased from R&D Systems.

Plasmid Construction
For overexpression of the miR-17/92 cluster, genomic DNA encoding miR-17/92 was amplified and cloned into pcDNA3.1/H9251 restriction sites as previously described. Cells were transfected using Lipofectamine 2000.

Real-Time RT-PCR Analysis
Total RNA was extracted using the RNeasy kit and quantification of specific RNA transcripts was performed by SYBR Green real-time PCRs using the ABI Prism 7700 Sequence Detection System.

Quantification of Mature miR-20a
Total RNA was extracted using the mirVana miRNA Isolation Kit. Mature miR-20a was detected by stem–loop reverse transcription, followed by SYBR Green real-time PCR and normalized to the expression of glyceraldehyde 3-phosphate dehydrogenase.

Western Blot Analysis
The following antibodies were used for Western blot: anti-human BMPR2, anti-human STAT3, anti-human phospho-STAT3, and anti-α-tubulin. Evaluation of the expression of specific proteins was performed by the Alpha Imager Software system via pixel quantification of the electronic image.

Reporter Gene Assay
A 1554-bp fragment of the 3′ untranslated region (3′UTR) of BMPR2 was amplified out of genomic DNA. The PCR product was XhoI digested and cloned into the XhoI restriction site of the pGL3 control vector. As negative control, the antisense construct was used according to Kuhn et al. HEK293 cells were transfected with the pGL3 control 3′UTR of BMPR2 “sense” or “antisense” construct. A vector encoding for the miR-17/92 cluster (pcDNA miR-17/92) and a vector for normalization (pRL-SV40) were added. For inhibition of endogenous miRNAs, a similar protocol was applied with the use of anti-miRs. Luciferase activity was measured using the Dual-Luciferase Reporter Assay System, and the values obtained were normalized to the activity of Renilla luciferase.

Nuclear Transfection
The endogenous expression of the human STAT3 gene was knocked down by validated small interfering (si)RNA for STAT3. Nuclear transfection of HPAECs was achieved by using the nucleofection kit HMVEC-L from Amaxa.

Statistics
For statistical analysis, GraphPad Prism Software was used.

Results
Computational Analysis Reveals BMPR2 As a Target of the miR-17/92 Cluster
Data from previous studies on the protein expression of BMPR2 in animal models of pulmonary hypertension suggested a posttranscriptional mechanism of regulation, possibly involving miRNAs. We thus performed a screening based on a computational algorithm (TargetScan, Whitehead Institute for Biomedical Research, www.targetscan.org) to identify distinct seed regions of miRNAs in the 3′UTR of the BMPR2 gene. To improve prediction rates the focus was put only on highly conserved seed regions. TargetScan retrieved several miRNAs for BMPR2, including miR-17-5p, miR-19a, miR-19b, miR-20a, and miR-92. Of interest, these miRNAs are encoded by one single miRNA cluster, miR-17/92 located at the chromosome 13q31 in the 3rd intron of the C13orf25 gene.

Overexpression of miR-17/92 Reduces the Expression of BMPR2
To verify the predicted miRNAs from the computational analysis on a functional basis, the entire cluster was cloned into a mammalian expression vector under the control of the SV40 promoter. Successful overexpression in HEK293 cells was confirmed by quantification of one representative mature miRNA derived from miR-17/92 (miR-20a) 72 hours after transfection. As shown in Figure 1A, the expression of miR-20a was significantly increased by 2.71-fold ($P=0.005$) as compared with the transfection with the empty vector (mock). The overexpression of the miR-17/92 cluster resulted in a significant decrease of BMPR2 on the protein level as analyzed by Western blot (0.66 ± 0.06 fold; $P=0.002$; Figure 1B). The time-dependent downregulation is shown in Figure 1 in the online data supplement. The corresponding levels of mRNA were also significantly reduced (0.89 ± 0.06 fold; $P=0.03$; Figure 1C). However, the changes observed on
miR-17/92 cluster (1.06±0.07 fold). These findings imply a direct interaction between the 3’UTR of BMPR2 and the miRNAs derived from the miR-17/92 cluster. To identify the impact of the distinct miRNAs, each endogenous miRNA from the cluster was inhibited by the use of antisense RNA molecules (anti-miRs). As shown in Figure 2B, these blocking experiments revealed a significantly higher relative luciferase activity for anti-miR-17-5p (1.31±0.21 fold; P=0.03) and anti-miR-20a (1.52±0.24 fold; P=0.008), indicating a pivotal role of these 2 miRNAs in the interaction with the BMPR2.

The Expression of miR-17/92 Correlates With The Activity of STAT3

A growing body of evidence suggests that the intracellular level of some miRNAs is regulated, at least in part, by the action of cytokines20 and growth factors.21 Because these factors have also been implicated in the pathogenesis of pulmonary hypertension,4 we stimulated HPAECs with IL-6, VEGF, and PDGF and quantified the expression of the preliminary transcript of miR-17/92 (C13orf25).

Figure 3A illustrates a significant upregulation of C13orf25 mRNA 30 minutes and 1 hour after the stimulation of HPAECs with IL-6 (1.98±0.19 fold; P=0.0018 and 1.87±0.33 fold; P=0.045 respectively) and VEGF (1.96±0.36 fold; P=0.045 and 1.58±0.03 fold; P=0.001 respectively) as compared with unstimulated control cells. 4 hours after stimulation, the mRNA levels reached baseline expression. The stimulation with PDGF did not affect the mRNA levels of C13orf25, probably because of the fact that HPAECs lack the receptor for PDGF (data not shown).

It was reported from previous experiments in endothelial cells, that IL-6 and VEGF share common signaling mechanisms including the STAT3 pathway.22,23 STAT3 is a major transcription factor, which is shuttled from the cytoplasm into the nucleus on activation by tyrosine phosphorylation of residue 705. When HPAECs were stimulated and analyzed by Western blotting, a strong phosphorylation of STAT3 could be observed for IL-6 after 30 minutes, and, to a lesser extent, after 1 hour. However, no phosphorylation could be detected after the addition of VEGF (Figure 3B). With respect to the data presented in Figure 3A, a correlation between activation of STAT3 and induction of the expression of C13orf25 can be postulated. According to this hypothesis, we performed siRNA experiments to knockdown the endogenous STAT3 for anti-miR-17-5p and anti-miR-20a. Data are shown as means±SD derived from 4 independent experiments.

Figure 2. Reporter gene studies on the interaction between 3’UTR of BMPR2 and the miR-17/92 cluster in HEK293 cells. A, Cotransfection of the pGL3 3’UTR of BMPR2 sense construct and the miR-17-92–overexpressing vector yielded a lower relative luciferase activity as compared with mock when transfected into HEK293 cells (0.76±0.12 fold; Figure 2A). The antisense construct was not affected by overexpression of the miR-17/92 cluster (1.06±0.07 fold). These findings imply a direct interaction between the 3’UTR of BMPR2 and the miRNAs derived from the miR-17/92 cluster. To identify the impact of the distinct miRNAs, each endogenous miRNA from the cluster was inhibited by the use of antisense RNA molecules (anti-miRs). As shown in Figure 2B, these blocking experiments revealed a significantly higher relative luciferase activity for anti-miR-17-5p (1.31±0.21 fold; P=0.03) and anti-miR-20a (1.52±0.24 fold; P=0.008), indicating a pivotal role of these 2 miRNAs in the interaction with the BMPR2.

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expression in HPAECs. Consequently, as shown in Figure 3C, the expression of STAT3 could be reduced by 50%. Stimulation experiments after established siRNA-mediated reduction of STAT3 are summarized in Figure 3D. Interestingly, the stimulatory effect of IL-6 on the expression of C13orf25 was almost completely abolished by the functional knockdown of STAT3 as compared with stimulated scrambled control cells (P = 0.0027). Consistent with the Western blot (Figure 3B), the difference observed between siRNA and scrambled transfected cells when stimulated with VEGF was statistically significant but considerably weaker than the difference on stimulation with IL-6 (P = 0.0428). To evaluate whether stimulation of HPAECs with IL-6 and VEGF might also affect the expression of mature miRNA derived from miR-17/92, expression levels of mature miR-20a were assessed and a significant upregulation was detected (1.44 ± 0.31 fold; P = 0.033). Data are shown as means ± SD derived from 4 independent experiments.

Identification of a Highly Conserved STAT3-Binding Site in the Promoter of C13orf25

Because the previous experiments revealed an essential role of STAT3 for the induction of C13orf25, as a next step, a screening for STAT3-binding sites in the promoter of C13orf25 was assessed (TFsearch, Computational Biology Research Center, Advanced Industrial Science and Technology [AIST], Japan; http://www.cbrc.jp/research/db/TFSEARCH.html) and revealed a binding site app. Upstream (100 bp) of the respective start codon. Alignment of this region with several mammalian species is shown in Figure 4A and underscores the evolutionary conservation of this binding site. To confirm this potential binding site, promoter activity studies were performed. We thus inserted the promoter of C13orf25 upstream to a luciferase reporter construct (pGL3 basic promoter wild-type [WT]). In addition, the sequence of the predicted binding site was altered by introduction of three point mutations and used as control (pGL3 basic promoter ΔSTAT3). Transfection was performed in an IL-6-responsive tumor cell line (HepG2). Stimulation of these cells with IL-6 yielded a higher relative luciferase activity of the promoter WT (1.62 ± 0.41 fold) as compared with the mutated promoter construct (ΔSTAT3, Figure 4E). Stimulation of HPAECs for 24 hours revealed significant upregulation of miR-20a after stimulation with IL-6 (P = 0.033). Data are shown as means ± SD derived from 4 independent experiments.

Figure 3. Correlation between the expression of miR-17/92 and the activity of STAT3 in HPAECs. A, Quantification of the preliminary transcript of miR-17/92 (C13orf25). Stimulation of HPAECs with IL-6 and VEGF but not PDGF significantly increased the expression of C13orf25 mRNA. B, Phosphorylation of STAT3 at tyrosine residue 705 could be observed for IL-6 in a time-dependent manner, whereas no effect was seen for the stimulation with VEGF. C, siRNA-mediated knockdown reduced the expression of STAT3 by 50% on the protein level. D, The induction of C13orf25 mRNA by IL-6 was almost completely abolished by the functional knockdown of STAT3 as compared with stimulated scrambled control cells (P = 0.0027). The effect observed by stimulation with VEGF was less strong but still reached statistical significance (P = 0.0428). E, Stimulation of HPAECs for 24 hours revealed significant upregulation of miR-20a after stimulation with IL-6 (P = 0.033). Data are shown as means ± SD derived from 4 independent experiments.

Figure 4. Identification of a highly conserved STAT3-binding site in the promoter of C13orf25. A, A 33-bp region in the promoter of C13orf25 contains a predicted STAT3-binding site. Alignment of this region with several mammalian species highlights the evolutionary conservation of this binding site (adapted from Loffler et al30). B, Reporter gene assay studies. Stimulation of HepG2 cells with IL-6 resulted in a higher relative luciferase activity of the promoter WT (1.62 ± 0.41 fold) as compared with the mutated promoter construct (ΔSTAT3). Data are plotted as median including lower and upper whisker. Five independent experiments were performed.
Transfection of Persistent Activated STAT3 Downregulates the Expression Levels of BMPR2 in an In Vitro System

To investigate the question whether the activation of STAT3 might affect the protein levels of BMP2 through changes in the expression levels of miR-17/92, human STAT3, and a constitutively active form of human STAT3, STAT3-C, were cloned.24 To prevent experimental cross-reaction by overexpression of STAT3, HEK293 cells were used that express endogenous STAT3 in low amounts only. As positive readout, the mRNA expression of suppressor of cytokine signaling (SOCS3), a well-known target gene of STAT3,25 was quantified. Overexpression of the WT form of STAT3 resulted in increased levels of SOCS3 as compared with mock transfected cells (2.16±0.79 fold). This effect was even seen in HEK293 cells for miR-20a as for SOCS3, showing significant upgulation of mature miR-20a levels in STAT3 WT transfected cells (2.59±0.24 fold; Figure 5A), thus confirming the accuracy of this molecule. The same samples were further analyzed for the expression of miR-20a as a representative of mature miRNAs derived from miR-17/92 (Figure 5B). Consequently, a similar expression pattern was seen in HEK293 cells for miR-20a as for SOCS3, showing significantly increased miR-20a levels in STAT3 WT transfected cells (1.6±0.24 fold; P=0.016) and STAT3-C transfected cells (2.89±0.63 fold; P=0.03). These data emphasize that the STAT3 pathway modulates the expression of mature miRNAs encoded by the miR-17/92 cluster.

The data presented so far showed that (1) the BMP2 gene is regulated on a posttranscriptional level by miR-17-5p and miR-20a and (2) that the activation of STAT3 upregulates the preliminary transcript C13orf25 and the mature miR-20a. To link these findings, STAT3-C was overexpressed in HEK293 cells, and, subsequently, the BMP2 expression was analyzed after 96 hours (Figure 5C). Analysis of the mRNA levels of BMP2 revealed no significant changes (0.94±0.45-fold compared with mock) as shown in panel A. Conversely, the protein expression of BMP2 was found to be reduced by ≈30% after constitutive activation of STAT3 (Figure 5D).

Discussion

In the present study, we found that (1) the protein expression of BMP2 is modulated by the miR-17/92 cluster without affecting the BMP2 mRNA levels; (2) this regulatory effect is driven by 2 distinct miRNAs, ie, miR-17-5 and miR-20a, through conserved seed matches within the 3′UTR of BMP2; and (3) IL-6 regulates the expression of the miR-17/92 in HPAEC by signaling through STAT3. Moreover, we could show that (4) the promoter region of C13orf25 exhibits an evolutionary conserved STAT3-binding site and, finally, that (5) persistent activation of STAT3 leads to a strong upregulation of mature miR-20a, which, in turn, reduces the expression of BMP2 protein. Taken together, our findings offer a novel mechanistic explanation for the downregulation of BMP2, which has been repeatedly described as an important feature in the pathogenesis of pulmonary hypertension.

The cell surface receptor BMP2 is essential for the modulation of differentiation, proliferation and the fibrous matrix production of both endothelial and smooth muscle cells.26,7 Changes in the expression of BMP2 thus might promote vascular remodeling as observed in the arterial vessels of patients with pulmonary hypertension. This hypothesis is supported by the fact that the loss of BMP2 resulting from germline mutations is a hallmark of genetic forms of pulmonary hypertension and, moreover, that reduced BMP2 levels in the pulmonary arteries have been described in several animal models of pulmonary hypertension.8,10 The regulation of BMP2, however, is poorly understood to date, but results from previous studies suggest a posttranscriptional mechanism of regulation.8,10 In this field, miRNAs have emerged as novel molecular players. To our knowledge, this study comprises the first data on the modulation of BMP2 by miRNAs.

We approached the experiments by performing a computational screening that revealed multiple miRNAs as potential regulators of BMP2. The highest prediction rates, however, were yielded for the miRNAs derived from the miR-17/92 cluster located in the third intron of the C13orf25 gene. It was
previously shown that this polycistronic region encodes for 6 mature miRNAs (ie, the miR-17/92 cluster: miR-17-5p, miR-18a, miR-19a, miR-19b, miR-20a, and miR-92).18 To investigate the role of these miRNAs in the regulation of BMPR2, we used a mammalian pcDNA expression vector encoding for miR-17/92.Transient overexpression of this cluster led to a reduction of BMPR2 protein levels, whereas the mRNA levels remained unchanged. These results confirmed the hypothesis of a posttranscriptional role of miR-17/92 in the regulation of BMPR2. To prove whether these miRNAs directly interact with the 3’UTR of BMPR2, we further constructed a reporter gene system containing a luciferase gene and the predicted seed matches for miR-17/92. This assay showed repressed luciferase activity following overexpression of miR-17/92, thus verifying a direct binding of these miRNAs. Conversely, elevated levels of luciferase activity were found after blocking the endogenous miRNAs by using individually designed anti-miRs. In particular, we could show that the 3’UTR of BMPR2 is a direct target of 2 distinct miRNAs, ie, miR-17-5p and miR-20a, derived from the miR-17/92 cluster. Consistent with these data, TGFβR2, another receptor from the identical protein family, is also targeted by the miR-17/92 cluster.13 TGFβR2 is a major mediator of tissue fibrosis and has been associated with the pathogenesis of pulmonary hypertension.27 Moreover, it has been found that the TGFβR2 protein is almost absent in plexiform lesions that characterize the aberrant endothelial proliferation in idiopathic pulmonary arterial hypertension.28 Whether the miRNAs derived from the cluster 17/92 regulate TGFβR2 and BMPR2 alike and, thus, might provide a final common pathway in the remodeling of pulmonary arterial vessels must be addressed by further studies.

The gene C13orf25 was first described as target for chromosomal amplification in malignant lymphoma.29 This gene attracted primary attention after several studies suggested oncogenic activities probably attributable to targeting tumor suppressor genes, such as Bim and PTEN.30 Moreover, Suarez et al showed that the miR-17/92 cluster is pivotally involved in the angiogenic sprouting of human endothelial cells.31 Recent observations demonstrated that the expression pattern of miRNAs derived from miR-17/92 is regulated by a number of known transcription factors. O’Donnell et al, for example, described the modulation of miR-17/92 by c-Myc.14 and the data on this regulatory network have recently been extended by Woods et al showing a direct interaction of E2F3 with the promoter region of C13orf25.32

In our experiments, we provide evidence that the promoter of C13orf25 also bears a functional binding site for STAT3, which controls the transcription of several genes involved in the inflammatory response.33 By applying promoter studies, we confirmed a STAT3-responsive region located ~100 bp upstream of the start codon of C13orf25. The functional importance of these findings is highlighted by the fact that this region was found to be phylogenetically conserved among mammalian species. In all species investigated, the distance of the STAT3-binding site to the respective sequence encoding for miR-17/92 revealed to span between 3200 and 4500 bp. Interestingly, a similar distance was described recently for the other known STAT3-regulated miRNA (ie, miR-21),20 indicating a common evolutionary assembly of miRNA genes regulated by this transcription factor.

In normal cells, the expression and phosphorylation of STAT3 is finely balanced by negative feedback loops including the activation of SOCS proteins.25 These feedback mechanisms, however, might be bypassed through persistent upstream signaling or through knock down of inhibitory proteins, leading to constitutively activated STAT3. Such phenomena have been found in several human tumors.34 Of interest, a constitutive activation of STAT3 has also been described in human arterial endothelial cells derived from patients with pulmonary hypertension.35 In general, the initiation of aberrant signaling by overexpression of persistently activated STAT3 displays an interesting experimental approach to identify novel miRNAs regulated directly or indirectly through inflammatory responses. The results obtained by such strategies might help to understand the reasons for altered miRNA expression profiles in these conditions. Along this line, we constructed such an expression vector for STAT3,24 and subsequent transfection of this vector promptly resulted in increased levels of mature miR-20a as compared with mock or STAT3 WT transfected cells. Because this experimental setup reduced the expression of BMPR2 protein but not the respective mRNA, we suggest that this effect is driven, at least in part, by the upregulation of miRNAs derived from the cluster miR-17/92.

STAT3 was first described in the downstream signaling of IL-6 modulating acute phase gene expression.36 Intriguingly, patients with pulmonary hypertension were found to have higher serum levels of IL-6 as compared with healthy controls,3 and the ectopic administration of IL-6 has been observed to induce a mild elevation of the pulmonary arterial pressure in mice.37,38 Moreover, the important role of IL-6 in the pathogenesis of pulmonary hypertension has been underpinned by a recent study in transgenic mice overexpressing IL-6. When compared with their WT counterparts, these animals developed increased ventricular systolic pressures, right ventricular hypertrophy, and pulmonary vasculopathic changes indicative for pulmonary hypertension.5 In the present study, we thus investigated the influence of IL-6 on the expression of C13orf25 in HPACs and found that IL-6 induced the gene expression of miR-17/92 in a STAT3-dependent manner.

Our data offer a direct link between the action of IL-6 and the expression of the miR-17/92 cluster. Because STAT3 has been shown to activate also the transcription of the c-myc gene,39 our findings highlight the role of IL-6 in the regulatory pathway that controls the expression of miR-17/92 and thus complements this network by a novel piece (Figure 6).

Finally, our data allow us to conclude that increased IL-6 signaling leads to the downregulation of BMPR2, based on a phylogenetically conserved STAT3–miR-17/92 pathway. It could be speculated that STAT3 plays an important role in the development of pulmonary hypertension, in particular because it has been shown that a persistently activated STAT3 promotes cell survival of HPACs derived from patients with pulmonary arterial hypertension. Because we could further show that STAT3 regulates the BMPR2 expression through transcriptional activation of miR-17/92, one might postulate...
this cluster as a highly specific target for the causative treatment of pulmonary hypertension. Because inhibitors of miRNAs are not presently available in a clinical setting, the inhibition of STAT3 activation by anticytokine therapies directed against IL-6 might provide a feasible alternative to restore functional levels of BMPR2.

Taken together, we provide here, to our knowledge, for the first time a mechanistic explanation for the loss of BMPR2 in pulmonary hypertension, shedding novel light on the pathogenesis of this disease and related conditions.

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**Disclosures**

None.

**References**

26. Loffler D, Brocke-Heidrich K, Pfeifer G, Stosits C, Hackermuller J, Kretzschmar DL, Kjaergaard J, Gramatzki M, Blumert C, Cvijic H, Ullmann AK, Stadler PF, Horn F. Interleukin-6 dependent survival of IIFK/H9252R2. Here, we provide evidence that the related BMPR2 is modulated by miR-17-92 alike.

Figure 6. Mechanism of regulation of BMPR2. The transcription of miR-17/92 is regulated by c-Myc and E2F3.14,32 In the present study, we demonstrate that the promoter region of miR-17/92 also bears a functional binding site for STAT3. The latent transcription factor STAT3 is the major mediator of IL-6 signaling. With respect to the fact that STAT3 induces the expression of c-Myc,26 IL-6 emerges as a new modulator of miR-17/92. It is known that miRNAs derived from this cluster target the surface receptor TGFBR2.13 Here, we provide evidence that the related BMPR2 is modulated by miR-17/92 alike.


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**Supplement Material**

**Materials and Methods**

*Cell culture*

Human embryonic kidney (HEK)293 cells and human hepatocellular carcinoma (HepG2) cells were grown in Dulbecco’s minimum essential medium (Gibco-Invitrogen, Basel, Switzerland) supplemented with 10% fetal calf serum (FCS), 2mM L-glutamine, 50U/ml penicillin/streptomycin, 0.2% Fungizone, and 10mM HEPES (all reagents provided by Gibco-Invitrogen) at 37°C in a humidified atmosphere of 5% CO₂. Human pulmonary artery endothelial cells (HPAEC) were purchased from Cascade Biologics (Invitrogen, Basel, Switzerland) and were cultured in Medium 200 (Invitrogen) according to the manufacturer’s instructions.

After an incubation period of 24h in starvation medium (0.5% FCS), recombinant human IL-6, VEGF or PDGF were added at a final concentration of 20ng/ml each. All growth factors and stimulation agents were purchased from R&D Systems (Abingdon, United Kingdom).

*Plasmid construction*

For overexpression of the miR-17/92 cluster, genomic DNA encoding miR-17/92 was amplified and cloned into pcDNA3.1 + (Invitrogen) between the HindIII and EcoRI restriction sites as previously described. The following primers were used: sense 5’- AAA CTT AAG CTT CTA AAT GGA CCT CAT ATC TTT GAG - 3’; anti-sense 5’- CTG CAG AAT TCG AAA ACA AGA CAA GAT GTA TTT ACA C - 3’. The full-length sequence of human STAT3 was amplified out of cDNA derived from HepG2 cells and cloned into pcDNA3.1 + (Invitrogen) between the HindIII and XhoI restriction sites. The following primers were used: sense 5’- AAA CTT AAG CTT GCC GCC ATG GCC CAA TGG AAT CAG - 3’; anti-sense 5’- CTA GAC TCG AGT CAC ATG GGG GAG GTA GC - 3’. For
construction of a constitutively active form of human STAT3, called STAT3-C, the amino acid residues A661 and N663 were mutated to cysteine, respectively. The following primers were used: sense 5’ - CAT GGA TTG TAC CTG CAT CCT GGT GTC TCC ACT G - 3’; anti-sense 5’ - CCA GGA TGC AGG TAC AAT CCA TGA TCT TAT AGC - 3’. The correct sequence of each insert was confirmed by sequencing.

For transfection, HEK293 cells were seeded on 6- or 12-well plates at a density of 5 x 10^5 cells per ml in an antibiotic-free medium. The next day, cells were transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions.

**Real time RT-PCR analysis**

Total RNA was extracted using the RNeasy kit (Qiagen, Basel, Switzerland) according to the manufacturer's instructions. Quantification of specific RNA transcripts was performed by SYBR Green real-time PCRs, using the ABI Prism 7700 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). SYBR Green real-time PCR was performed for human bone morphogenetic protein receptor type II (BMPR2, sense 5’ - AGC CCA ACA GTC AAT CCA ATG - 3’; anti-sense 5’ - GGT TGC GTT CAT TCT GCA TAG - 3’), human suppressor of cytokine signaling 3 (SOCS3, sense 5’ - CTG TAC CTG GGT GGA TGG AG - 3’; anti-sense 5’ - TGA AAG ATG TCC CGT CTC CT - 3’), human signal transducer and activator of transcription (STAT3, sense 5’ - TTC ACT TGG GTG GAG AAG GAC A - 3’; anti-sense 5’ - CGG ACT GGA TCT GGG TCT TAC C - 3’), and human preliminary transcript of the miR-17/92 cluster (C13orf25, sense 5’ - TTG CTA AGT GGA AGC CAG AAG - 3’; anti-sense 5’ - CAT CCA CGT GGC AAA ACA T - 3’). To confirm specific amplification by the SYBR Green PCR, a dissociation curve analysis was performed for each primer pair, and both non-RT negative controls and water controls were used for these analyses. The amounts of loaded RNA were normalized by using a predeveloped 18S ribosomal RNA control kit (Applied Biosystems). Differential gene expression was calculated
with the threshold cycle ($C_t$), and relative quantification was calculated with the comparative
$C_t$ method.

**Quantification of mature microRNA-20a**

Total RNA was extracted using the mirVana miRNA Isolation Kit (Applied Biosystems). Mature microRNA-20a was detected by stem-loop reverse transcription followed by SYBR Green real-time PCR, using the ABI Prism 7700 Sequence Detection System (Applied Biosystems). Obtained signals were normalized to the expression of glyceraldehyde 3-phosphate dehydrogenase (GAPDH, sense 5’ - GGG AAG CTT GTC ATC AAT GGA - 3’; anti-sense 5’ - TCT CGC TCC TGG AAG ATG GT - 3’).

**Western blot analysis**

For protein extraction confluent cells were lysed using RIPA buffer (150mM NaCl, 50mM Tris-HCl, 250μM EDTA, 5mM NaF, 1% Triton X-100, 1% Deoxycholic acid). Whole-cell lysates (40 μg) were separated by 10% SDS-PAGE and the proteins were transferred to nitrocellulose or polyvinylidene fluoride membrane, respectively. The following antibodies were used for Western blot: anti - human BMPR2 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), anti - human STAT3 (R&D Systems), anti - human Phospho-STAT3 (Tyr705, Cell Signaling Technology Inc., Beverly, MA, USA) and anti - α-Tubulin (Sigma, St. Louis, MO, USA). Evaluation of the expression of specific proteins was performed by the Alpha Imager Software system (Alpha Innotech, San Leandro, CA, USA) via pixel quantification of the electronic image.

**Reporter gene assay**

A 1554 bp fragment of the 3’untranslated region (UTR) of BMPR2 was amplified out of genomic DNA using the following primer: sense 5’ - TAA TTC TAG AGC ATC ATT TAA
ACA TGC AGA - 3'; anti-sense 5' - CGA CTC TAG ACA TCA GTT TGC AAA TTA ATA G - 3'. The PCR product was XbaI digested and cloned into the XbaI restriction site of the pGL3 control vector (Promega AG, Dübendorf, Switzerland). The correct sequence and orientation of the insert was confirmed by sequencing. As negative control, the anti-sense construct was used according to Kuhn et al.4

For transfection, HEK293 cells were plated in 12-well plates at a density of $5 \times 10^5$ cells per ml in an antibiotic-free medium. The next day, cells were transfected with the pGL3 control 3'UTR of BMPR2 “sense” or “anti-sense” construct (150ng/well, respectively) using Lipofectamine 2000. Moreover, a vector encoding for the miR-17/92 cluster (pcDNA miR-17/92, 770ng/well) and a vector for normalisation (pRL-SV40, Promega; 80ng/well) were added. For inhibition of endogenous miRNAs, a similar protocol was applied with the use of anti-miRs (anti-miR-17-5p, anti-miR-19a, anti-miR-20a, anti-miR-92, all from Applied Biosystem; 100nM/well) instead of the miR-17/92 encoding vector. After 24h, the cells were lysed, and luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega). The values obtained were normalized to the activity of Renilla luciferase (pRL-SV40).

A 1351 bp fragment of the promoter of C13orf25 was amplified from human genomic DNA as previously described5 using the following primer: sense 5' - GGG CTC GAG ATC TTT TCA GAT TTG GCC TTT TAT TTT - 3'; anti-sense 5' - AAT GCC AAG CTT AGG AGA GCT TCG CGG AGG AG - 3'. The PCR product was digested with BgIII and HindIII and cloned into the pGL3 basic vector (Promega). A mutation was introduced into the STAT3 binding site using the following primers: sense 5’ - TAT GT C CT T GAG AAT TCC GGA ATT TCC TG - 3'; anti-sense 5’ - TCT CAA GG A CAT AAT TGT TAA AAG TGA GG - 3' (alterations underlined). The correct sequence of each insert was confirmed by sequencing.

For transfection, HepG2 cells were plated in 12-well plates at a density of $5 \times 10^5$ cells per ml in an antibiotic-free medium. The next day, cells were co-transfected either with the pGL3
basic promoter of C13orf25 wildtype (pGL3 basic promoter WT) or with the pGL3 basic promoter of C13orf25 ΔSTAT3 (pGL3 basic promoter ΔSTAT3, each 140ng/well) and pRL-SV40 (60ng/well) using FuGENE6 (Roche Diagnostics AG, Rotkreuz, Switzerland). After 6h, the cells were serum starved and stimulated with IL-6 (20ng/ml) for a time period of 4h. Luciferase activity was measured as mentioned above.

**Nuclear transfection**

To knock down the endogenous expression of the human STAT3 gene validated siRNA for STAT3 was purchased from Qiagen (Hs_STAT3_7 HP Validated siRNA). Nuclear transfection of HPAEC was achieved by using the nucleofection kit HMVEC-L from Amaxa (Amaxa GmbH, Cologne, Germany), according to the manufacturer’s protocol. Briefly, cells were resuspended in nucleofection solution at a density of $5 \times 10^5$ cells per 100$\mu$l. For each transfection, 100$\mu$l of cell suspension were mixed with siRNA or scrambled negative control to obtain a final concentration of 100nM RNA. The suspension was pipetted into a cuvette and pulsed in a Nucleofector device using program S-05. Subsequently, cells were diluted with prewarmed medium and transferred into a 6-well plate. Following an incubation period of 24h, cells were serum starved and stimulated with the respective stimulation agent.

**Statistics**

For statistical analysis, GraphPad Prism Software (GraphPad Software, San Diego, CA, USA) was used. To compare samples, the paired or unpaired t-test was applied and a p-value < 0.05 was considered to be statistically significant. All data are shown as mean ± SD.
References


Online Figure I

Overexpression of miR-17/92 in HEK293 cells results in time-dependent down-regulation of BMPR2.

HEK293 cells were transfected with the pcDNA miR-17/92 vector and protein was collected after 24h, 48h, 72h and 96h. Maximum effect of transient overexpression of the miR-17/92 cluster was observed by Western blot analysis after 72h resulting in a decrease of app. 30% of BMPR2 protein.

Online Figure II

Reporter gene studies on the interaction between 3’UTR of BMPR2 and the miR-17/92 cluster in HEK293 cells.

Reporter system with a part of the 3’UTR region of BMPR2 (35 – 1589 bp) containing the predicted seed matches for miR-17-5p, miR-19a, miR-19b, miR-20a and miR-92.
Online Figure I

IB: BMPR2

IB: α-Tubulin

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Online Figure II

5’ Luciferase 3’ UTR of BMPR2 mRNA 3’

203 bp – TTTGCAC – 209 bp (seed match for miR-19a/b)
206 bp – GCACTTT – 212 bp (seed match for miR-17-5p & miR-20a)
508 bp – GTGCAATA – 515 bp (seed match for miR-92)
642 bp – TTTGCACA – 649 bp (seed match for miR-19a/b)
1523 bp – GCACTTT – 1529 bp (seed match for miR-17-5p & miR-20a)