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MicroRNA-27b Targets Gremlin 1 to Modulate Fibrotic Responses in Pulmonary Cells

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ABSTRACT

Fibrosis is a chronic disease characterized by an excessive deposition of scar tissue in the affected organs. A central mediator of this process is transforming growth factor-β (TGF-β), which stimulates the production of extracellular matrix proteins such as collagens. MicroRNAs (miRNAs) have been implicated in both fibrosis as well as in TGF-β signaling, but the extent of their regulation has not been fully defined. A functional screen was conducted using a library of miRNA inhibitors to identify miRNAs that affect TGF-β-induced type I collagen expression, a key event in the development of fibrosis. The inhibition of one miRNA in particular, miR-27b, caused a significant increase in type I collagen expression. We found that miR-27b directly targets Gremlin 1 by binding to its 3′-UTR, reducing its mRNA levels. TGF-β signaling decreased miR-27b expression and caused a corresponding increase in Gremlin 1 levels, suggesting that TGF-β regulates Gremlin 1 expression in part by modulating miR-27b expression. Reducing Gremlin 1 levels by either siRNA-mediated gene silencing or by using the miR-27b mimic inhibited the expression of several genes known to be involved in fibrosis, while increasing Gremlin 1 levels by the addition of either recombinant protein or the miR-27b inhibitor enhanced the expression of these genes. In summary, we have demonstrated that miR-27b targets Gremlin 1, and that this regulation likely represents an important control point in fibrotic pathways. J. Cell. Biochem. 115: 1539–1548, 2014.

KEY WORDS: MicroRNA; FIBROSIS; CELL BIOLOGY; CELL SIGNALING; GENE EXPRESSION; TRANSFORMING GROWTH FACTOR BETA (TGFbeta); GREMLIN 1

The formation of scar tissue is a normal part of tissue repair and wound healing in response to injury, but when left unchecked aberrant tissue repair and fibrosis can occur. Fibrosis is characterized by an excessive accumulation of extracellular matrix (ECM) proteins, which progressively remodels, destroys, and replaces the normal tissue architecture, ultimately resulting in organ failure. This process underlies the etiology of a number of life-threatening chronic diseases such as idiopathic pulmonary fibrosis (IPF), systemic sclerosis, and chronic kidney disease [Wynn, 2008]; however, the fundamental molecular drivers and control points that coordinate this aberrant tissue repair process are poorly understood.

Transforming growth factor-β (TGF-β) is a widely expressed cytokine that is considered to be a master regulator in tissue fibrosis, primarily signaling through serine/kinase receptors and Smad proteins. MicroRNAs (miRNAs) have been implicated in TGF-β signaling as well as in fibrosis, but the extent of their regulation has not been fully defined. The TGF-β signaling pathway regulates a wide variety of cell processes, and thus is subject to many levels of modulation by both positive and negative regulators. One protein of interest is Gremlin 1, a member of the CAN family of cysteine knot proteins that exists in both secreted and cell-associated forms [Topol et al., 1997, 2000; Pearce et al., 1999]. Although Gremlin 1 is well known as a bone morphogenetic protein (BMP) antagonist [Hsu et al., 1998; Pearce et al., 1999; Rider and Mulloy, 2010], it can also regulate cell functions in a BMP-independent manner [Chen et al., 2004; Stabile et al., 2007; Mitola et al., 2008, 2010; Kim et al., 2012a]. Of note, Gremlin 1 expression can be induced by TGF-β [McMahon et al., 2000; Koli et al., 2006], and has been shown to be highly expressed in fibrotic conditions [Dolan et al., 2005; Boers et al., 2006; Koli et al., 2006; Myllarniemi et al., 2008]. Recently, Rodrigues-Diez et al. [2012] demonstrated that Gremlin 1 is a profibrotic mediator downstream of TGF-β by showing that inhibition of endogenous Gremlin 1 prevented TGF-β-induced fibrotic phenotypes in renal tubulointerstitial cells. In addition, Farkas et al. [2011] found that Gremlin 1 overexpression resulted in epithelial activation and reversible fibrosis in rat lungs. An earlier study by Myllarniemi et al. has demonstrated that Gremlin 1

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expression is highly elevated in the lungs from mouse model of asbestos-induced pulmonary fibrosis, whereas BMP signaling was found to be significantly reduced. A similar upregulation of Gremlin 1 was also detected in lung biopsies of human idiopathic pulmonary fibrosis (IPF) patients [Myllarniemi et al., 2008]. Taken together, these results suggest that Gremlin 1 may be a key contributor in pathological tissue fibrosis.

miRNAs are known to be involved in regulating a diverse range of cellular and disease processes, including both fibrosis and TGF-β signaling [Inui et al., 2010; Jiang et al., 2010; Pandit et al., 2011; Vettori et al., 2012; Bowen et al., 2013]. These are small, evolutionarily conserved, noncoding RNA molecules that bind to complementary sequences on mRNAs, which are predominantly located within the 3′-UTR of their target genes [Bartel, 2004; Baek et al., 2008]. This serves to post-transcriptionally repress gene expression, primarily by reducing levels of the target mRNA, although miRNAs can also decrease expression by inducing deadenylation or repressing translation [Guo et al., 2010]. More than 1,800 miRNA sequences have now been identified in humans (http://www.mirbase.org, release 20 [Kozomara and Griffiths-Jones, 2011], and it is predicted that approximately 60% of genes are regulated by miRNAs [Friedman et al., 2009], therefore these molecules represent a significant method of gene regulation.

Here we sought to identify and characterize miRNAs that regulate cellular pro-fibrotic changes in response to TGF-β signaling. We used a miRNA inhibitor library to identify miRNAs that affect the induction of type I collagen, an abundant component of fibrotic lesions, and found that inhibition of miR-27b significantly increased collagen expression. We have discovered that miR-27b directly targets the 3′-UTR of Gremlin 1, and that inhibition of Gremlin 1 by miR-27b is repressed in the presence of TGF-β signaling. Importantly, de-coupling of this miR-27b/Gremlin-1 regulatory switch results in the induction of several genes known to be involved in tissue fibrosis, indicating that miR-27b and Gremlin 1 may play a key role in this disease.

MATERIALS AND METHODS

CELL CULTURE

A549 human alveolar epithelial cells (American Type Culture Collection; ATCC, CCL-185) and primary lung fibroblast cells (ATCC CCL-210) were grown in F-12K medium (American Type Culture Collection) supplemented with 10% fetal bovine serum (FBS; HyClone). MRC-5 lung fibroblasts (ATCC, CCL-171) were grown in Eagle's minimum essential medium (ATCC) supplemented with 10% FBS (HyClone). For stimulation with TGF-β, A549 cells were transferred to serum-free medium for 24 h and then treated with either a vehicle control containing 0.4 mM HCl and 0.1% BSA or with 2 ng/ml TGF-β (R & D Systems) for the times shown in the text. Recombinant Gremlin 1 (R & D Systems) and BMP-2 (Pfizer, Inc.) were added at the concentrations and for the times indicated within the text.

miRNA AND siRNA TRANSFECTIONS

A miRNA hairpin inhibitor library containing 879 miRNA inhibitors was purchased from Thermo Scientific Dharmacon. A549 cells were transfected with one of the following: one of the 879 miRNA inhibitors, a nonspecific miRNA control inhibitor, a nonspecific miRNA control mimic, miR-27b inhibitor, miR-27b mimic (all miRNAs from Thermo Scientific Dharmacon), a nonspecific control siRNA, or Grem1 siRNA (Life Technologies). Transfection mixtures contained 0.25 μl of Lipofectamine 2000 (Life Technologies), 25 μl of Opti-MEM (Life Technologies) and either a miRNA inhibitor (50 nM), miRNA mimic (25 nM), or an siRNA (25 nM). The transfection mixture was incubated at room temperature for 10 min, then added to a 96-well plate. Cells (1×10^4) in medium containing 10% FBS were seeded in each well and incubated at 37°C. After 24 h, cells were serum-starved for an additional 24 h, after which they were treated with either vehicle control or with TGF-β for 48 h. Cells were then harvested and the RNA extracted and analyzed by quantitative RT-PCR (qPCR).

QUANTITATIVE RT-PCR

RNA extracts were prepared from cells using either an RNeasy kit (Qiagen) or a TaqMan gene expression Cells-to-Ct kit (Life Technologies) according to the manufacturer's protocol. The mRNA expression levels were measured using gene-specific TaqMan gene expression assays (Life Technologies) and then normalized to β-glucuronidase (GUSB). MiRNA extracts were prepared using a miRNeasy kit (Qiagen) and a TaqMan miRNA Reverse Transcription Kit (Life Technologies) in accordance with the manufacturer's instructions. MiRNA expression levels were quantified using the TaqMan miRNA assays, normalized to RNU48. Relative expression was calculated using the $\Delta\Delta C_T$ method.

IMMUNOBLOT ANALYSIS

Whole-cell extracts were prepared by lysing cells with radioimmunoprecipitation assay lysis buffer (50 mM Tris [pH 8.0], 150 mM NaCl, 1% NP-40, 0.5% deoxycholic acid, 0.1% SDS) containing protease inhibitors (Roche Applied Science). Proteins were separated by electrophoresis in 10% Bis–Tris gels (Life Technologies), transferred to a nitrocellulose membrane, and incubated with anti-Gremlin 1 (Abgent) and anti-β-actin (Cell Signaling Technology). Immunoblots were visualized using a goat anti-rabbit horseradish peroxidase-conjugated secondary antibody (Bio-Rad) and chemiluminescence (PerkinElmer).

3′-UTR LUCIFERASE ASSAY

Luciferase reporter constructs containing the Gremlin 1 3′-UTR with either an intact or mutated miR-27b binding site were generated by OriGene. The constructs were co-transfected into A549 cells along with either a miRNA inhibitor or mimic nontargeting control, miR-27b inhibitor, or miR-27b mimic using Lipofectamine LTX (Life Technologies) according to the manufacturer's instructions. pRL-TK (Promega) was used to normalize transfection efficiency. Cells were serum-starved for 24 h, and then treated with either vehicle control or TGF-β for 24 h. Firefly and *Renilla* luciferase activities were measured with the Dual-Glo Luciferase Assay System (Promega).

CELL PROLIFERATION ASSAY

Cell proliferation was evaluated by the colorimetric bromodeoxyuridine (BrdU) incorporation assay. Briefly, human fetal lung

fibroblast cell line MRC-5 and primary human lung fibroblasts were grown in serum-free conditions for 24 h, after which they were either left untreated as a control, or treated with either recombinant Gremlin 1, BMP2, or a combination of the two at the concentrations indicated within the text. After 48 h of treatment, proliferation was measured with the BrdU Cell Proliferation ELISA kit (Roche Applied Science) according to the manufacturer's instructions.

STATISTICAL ANALYSES

Data are presented as the means ± standard error of the mean. Statistical comparisons were performed using an unpaired two-tailed Student's *t*-test. Values of $P < 0.05$ were considered to be significant.

RESULTS

FUNCTIONAL SCREEN IDENTIFIED miR-27b AS A REGULATOR OF TYPE I COLLAGEN

A number of miRNAs have been implicated in the regulation of the fibrotic response, however given the extent of miRNA regulation there are likely to be many more that play a role in this process. Therefore, our first objective was to use functional profiling to determine which miRNAs regulate the expression of a key profibrotic gene in a relevant cell-based in vitro assay. We chose to focus on the TGF-β-induced profibrotic gene signature in A549 lung epithelial cells, since these cells are known to produce a robust fibrotic response to TGF-β treatment [Kasai et al., 2005; Kim et al., 2012b]. Although several pro-fibrotic genes were regulated by stimulation with TGF-β (Suppl. Fig. 1), we observed the greatest response in type I collagen levels, a gene known to be downstream of TGF-β signaling [Ignotz et al., 1987]. We found that A549 cells exhibited a time-dependent increase in type I collagen expression in response to TGF-β treatment, achieving a 50-fold increase in expression after 48 h as compared to the vehicle control treated cells (Fig. 1A). Since increased deposition of type I collagen is known to be a major feature of tissue fibrosis, we chose to follow the expression of this gene in the subsequent miRNA screen as an indicator of a profibrotic phenotype.

To identify miRNAs that affect the induction of type I collagen, we performed a functional screen using a miRNA hairpin inhibitor library containing 879 miRNA inhibitors. A549 cells were transfected with either a nonspecific miRNA control inhibitor or a miRNA

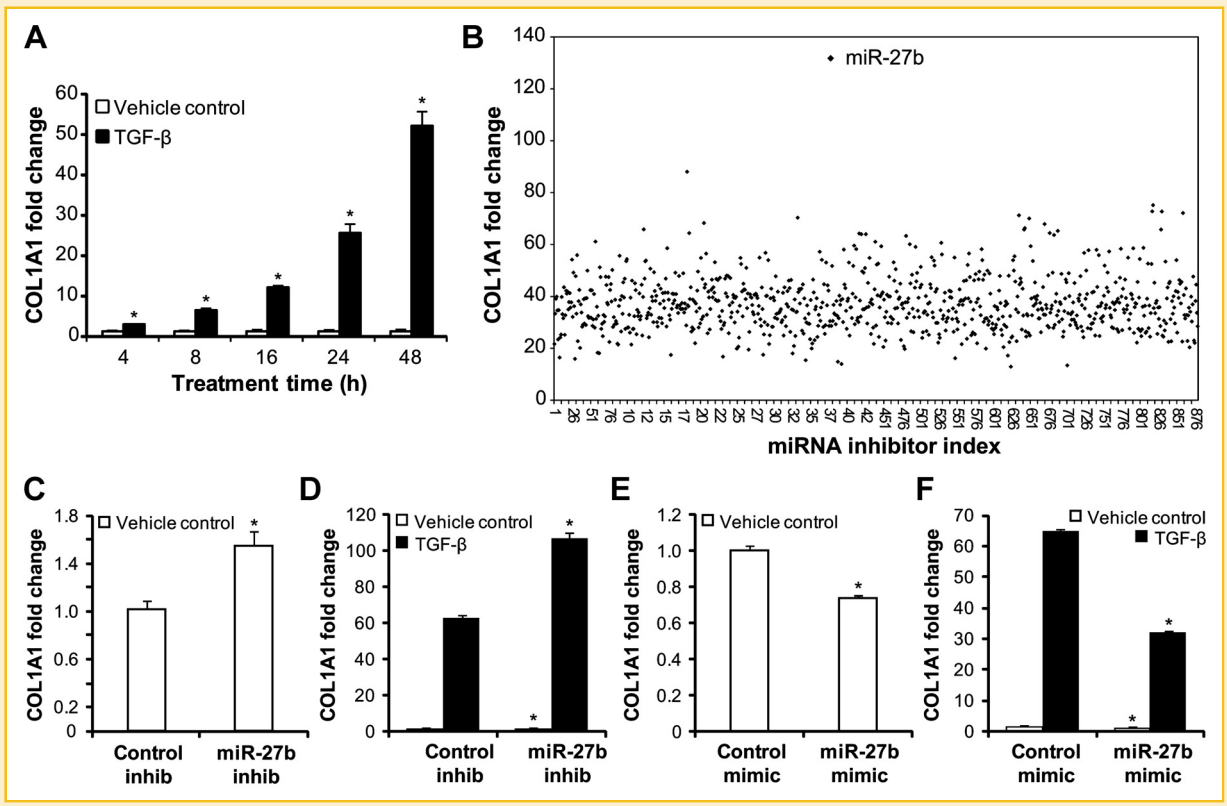

Fig. 1. TGF-β-induced type I collagen expression is regulated by miR-27b. A: A549 cells were serum starved for 24 h and then treated with either a vehicle control or TGF-β for the indicated times. qRT-PCR was used to quantify the levels of type I collagen mRNA, normalized to GUSB. Data are presented as the fold induction of type I collagen relative to the vehicle control. Values are the means ± SEM of three independent replicates. B: A549 cells were transfected with miRNAs from a library of 879 miRNA inhibitors. Following TGF-β treatment for 48 h, the induction of type I collagen mRNA was measured using qRT-PCR. The data points represent the TGF-β-induced collagen expression relative to the vehicle control for each individual miRNA in the library. Values are the means of duplicate experiments. Type I collagen mRNA expression was measured as above in A549 cells transfected with either control miRNA inhibitor or miR-27b inhibitor, and then treated with either a vehicle control (C) or TGF-β (D) for 48 h. n = 3. Type I collagen mRNA expression was measured in A549 cells transfected with either control miRNA mimic or miR-27b mimic and then treated with either vehicle control (E) or TGF-β (F) for 48 h. n = 3. *$P < 0.05$ for all panels.

inhibitor from the library, and then treated with either a vehicle control or TGF-β for 48 h (Fig. 1B). The screen identified several miRNA inhibitors that increased TGF-β-induced type I collagen expression by >1.5-fold (miR-27b, miR-139-5p, miR-768-3p, miR-767-5p, and miR-876-5p), however the miR-27b inhibitor had the greatest effect on gene expression. Inhibition of miR-27b resulted in an ~130-fold increase in type I collagen expression following TGF-β stimulation, versus an average ~50-fold increase in identically stimulated cells that were transfected with the miRNA control inhibitor (Fig. 1B). These results suggested that miR-27b may play an important role in the regulation of collagen I gene expression. Consequently, we sought to characterize this regulatory pathway in more detail.

To confirm the results from the screen, we repeated the experiment using an individually purchased miR-27b inhibitor. Compared to the control inhibitor, the miR-27b inhibitor caused a ~1.5-fold increase in type I collagen gene expression in unstimulated A549 cells (Fig. 1C). TGF-β treatment caused an ~60-fold induction in collagen in cells transfected with the control inhibitor, and inhibition of miR-27b further increased the induction of type I collagen gene expression to 110-fold (Fig. 1D). To further establish that miR-27b can modulate type I collagen expression, we measured the effects of the miR-27b mimic, which has a sequence identical to that of the endogenous miRNA. As expected, transfection of the miR-27b mimic decreased basal levels of type I collagen gene expression in unstimulated cells as compared to cells transfected with the control miRNA mimic (Fig. 1E). Interestingly the miR-27b mimic caused a greater reduction (~50%) in TGF-β-induced type I collagen gene expression (Fig. 1F). These results suggest that miR-27b is an important regulator of type I collagen gene expression, particularly in settings that support tissue fibrosis.

GREMLIN 1 IS A PREDICTED TARGET OF miR-27b

We next sought to identify the predicted gene targets of miR-27b. MiRNAs regulate expression by binding to complementary sequences in their target mRNA, which are typically located within the 3′-UTR. Efficient binding is thought to require a perfect match between the miRNA and the mRNA located at positions 2–8 of the miRNA, known as the seed region [Lewis et al., 2003]. Computational analyses were performed with TargetScan [Friedman et al., 2009] and PicTar [Krek et al., 2005] to predict the potential mRNA targets of miR-27b. Because such short sequences can occur frequently by chance within the genome, it is difficult to identify true physiological targets. We therefore limited our predictions to sites that were conserved in mammals across multiple species, since it is more likely that evolutionarily conserved sequences are functionally relevant. One predicted target of interest was Gremlin 1, a protein that is known to be induced in fibrosis, particularly in the lung and kidney [Dolan et al., 2005; Boers et al., 2006; Koli et al., 2006; Myllarniemi et al., 2008]. There was a perfect match between the seed sequence of miR-27b and a region in the 3′-UTR of Gremlin 1 (Fig. 2A), and furthermore, this binding site is identical among mammals (Fig. 2B). This suggested that Gremlin 1 may be regulated by miR-27b, thus we proceeded to experimentally verify this prediction.

miR-27b AND GREMLIN 1 EXPRESSION LEVELS ARE INVERSELY REGULATED BY TGF-β SIGNALING

We first determined how TGF-β signaling might regulate the expression of miR-27b and Gremlin 1 in A549 lung epithelial cells. Previous studies have shown that Gremlin 1 is induced by TGF-β in other cell types [McMahon et al., 2000; Koli et al., 2006]. As expected, we found that TGF-β treatment caused a dramatic increase in Gremlin 1 gene expression, which reached a maximum induction of 30-fold after 48 h (Fig. 3A). In a parallel experiment, A549 cells were similarly stimulated with TGF-β, but this time a miRNA-specific RNA preparation protocol was used in order to measure miR-27b gene expression. In contrast to the Gremlin 1 expression patterns, TGF-β stimulation reduced miR-27b levels (Fig. 3B). Therefore TGF-β signaling inversely regulates miR-27b and Gremlin 1 expression levels.

miR-27b DIRECTLY TARGETS THE GREMLIN 1 3′-UTR

To determine whether or not miR-27b regulates Gremlin 1 gene expression, A549 cells were first transfected with the miR-27b inhibitor. Our results show that inhibition of miR-27b increased the basal mRNA levels of Gremlin 1 by almost threefold (Fig. 4A). Stimulation with TGF-β caused a strong induction of Gremlin 1 gene expression in these cells, and inhibition of miR-27b resulted in an additional 25% increase in the TGF-β-induced Gremlin 1 gene expression as compared to identically treated cells that were

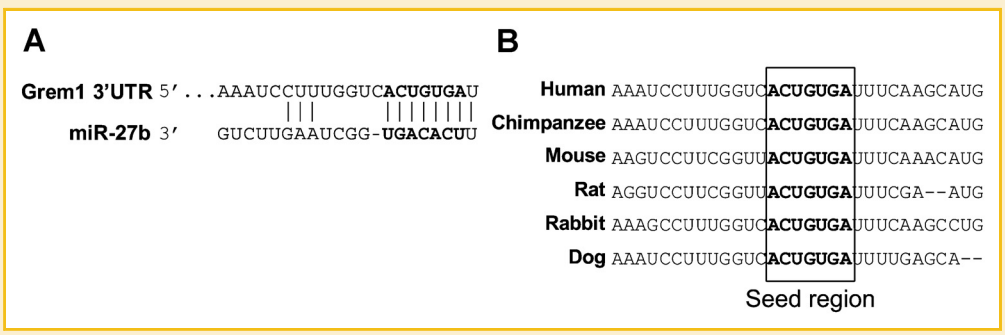

Fig. 2. miR-27b is predicted to target Gremlin 1. A: Computational analyses using TargetScan and Pictar identified Gremlin 1 as a predicted target of miR-27b. The Gremlin 1 3′-UTR has an exact match to the critical seed sequence of miR-27b at positions 2–7. B: Conservation among mammals of the miR-27b binding site sequence located within the Gremlin 1 3′-UTR, highlighted in gray.

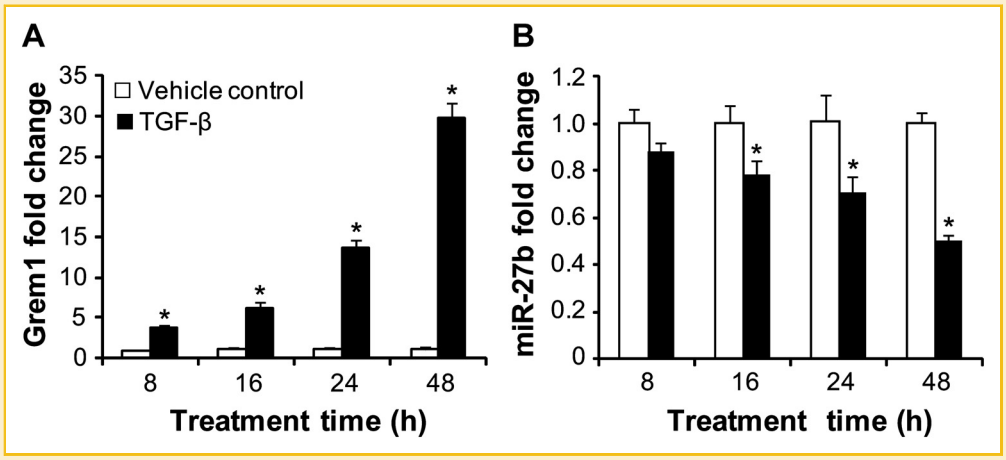

Fig. 3. miR-27b and Gremlin 1 are inversely regulated by TGF-β signaling. A549 cells were serum starved for 24 h and then treated with either a vehicle control or TGF-β for the indicated times. qRT-PCR was used to quantify both (A) Gremlin 1 mRNA expression and (B) miR-27b expression. Data are presented as the fold induction relative to the vehicle control. Values are the means ± SEM of three independent replicates. *$P < 0.05$.

transfected with the control inhibitor (Fig. 4B). In addition, the miR-27b inhibitor also increased levels of Gremlin 1 protein (Fig. 4C). When the miR-27b mimic was transfected into the cells, it did not have a significant inhibitory effect on Gremlin 1 expression in the unstimulated cells (Fig. 4D). This was not entirely unexpected; however, as in A549 cells we detected high levels of endogenous miR-27b and low levels of endogenous Gremlin 1. We theorize that the system was saturated, and that adding additional miR-27b was not able to have any additional effects on basal Gremlin 1 levels. When A549 cells were treated with TGF-β, endogenous miRNA-27b levels became much lower (Fig. 3B). Transfection of exogenous miR-27b mimic into TGF-β-treated cells now caused a marked decrease in both Gremlin 1 mRNA levels (~50%) (Fig. 4E) and Gremlin 1 protein level (Fig. 4F).

The above results demonstrate that miR-27b can regulate Gremlin 1 expression, however they do not prove that there is a direct interaction between miR-27b and the mRNA of Gremlin 1. To further substantiate that miR-27b specifically and directly represses Gremlin 1 expression, luciferase reporter assays were performed using a construct that contained the Gremlin 1 3′-UTR sequence inserted downstream of the luciferase gene (Fig. 5A). The reporter vector was then co-transfected into A549 cells with the miR-27b inhibitor, the miR-27b mimic, or their controls. Introduction of the miR-27b inhibitor significantly increased luciferase activity, indicating that suppression of the endogenous miR-27b prevented the luciferase mRNA from being degraded (Fig. 5B). Since we had previously found that the miR-27b mimic had a strong effect on Gremlin 1 gene expression in A549 cells treated with TGF-β but not in the untreated cells (Fig. 4D,E), presumably due to high endogenous miRNA27b levels in unstimulated A549 cells, we evaluated the effect of the miR-27b mimic on the luciferase reporter in the presence of TGF-β to suppress endogenous miR-27b expression. The mimic significantly decreased luciferase activity (Fig. 5C), further demonstrating that miR-27b targets the Gremlin 1 3′-UTR to cause mRNA degradation. We then mutated the seed region in the putative miR-27b binding site within the Gremlin 1 3′-UTR to confirm that miR-27b was binding to this sequence (Fig. 5A). Indeed, the regulatory effects on the luciferase reporter observed for the miR-27b inhibitor or mimic were essentially abrogated when we replaced the wild-type Gremlin 1 3′-UTR with the mutated version (Fig. 5B,C), indicating that the miR-27b binding site had been abolished. Taken together, our results confirm that miR-27b targets Gremlin 1, resulting in degradation of Gremlin 1 mRNA.

MODULATING GREMLIN 1 LEVELS REGULATES THE EXPRESSION OF GENES INVOLVED IN FIBROSIS

Our next step was to determine if miR-27b and/or Gremlin 1 mediate the expression of genes that are known to be implicated in fibrosis. In addition to type I collagen, we selected connective tissue growth factor (CTGF), plasminogen activator inhibitor-1 (PAI-1), and E-cadherin (CDH1). These genes have been shown to be regulated by TGF-β, and are often used as markers of fibrosis. We therefore measured the expression changes of these genes in response to miR-27b and Gremlin 1 perturbation.

A549 cells were first transfected with either the control inhibitor or miR-27b inhibitor and then treated with either vehicle or with TGF-β for 48 h. We found that inhibition of miR-27b significantly increased CTGF levels in both the untreated as well as the TGF-β treated cells, and also increased the induction of PAI-1 in response to TGF-β (Fig. 6A). In addition, the miR-27b inhibitor significantly decreased E-cadherin expression in TGF-β treated cells (Fig. 6A). Since inhibition of miR-27b increased Gremlin 1, we theorized that increasing Gremlin 1 levels by the addition of recombinant protein should have the same effects on gene expression as the miR-27b inhibitor. Indeed, levels of type I collagen, CTGF and PAI-1 increased in cells treated with recombinant Gremlin 1, and expression of E-cadherin decreased (Fig. 6B). As expected, the miR-27b mimic had the opposite effects as the inhibitor, significantly decreasing CTGF and PAI-1 expression and increasing E-cadherin expression (Fig. 6C), particularly in the cells treated with TGF-β. To confirm

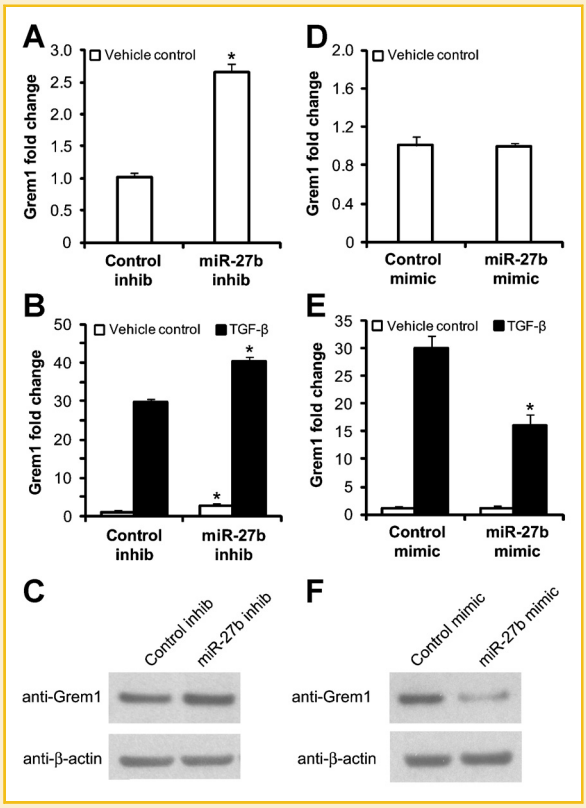

Fig. 4. miR-27b regulates Gremlin 1 expression. A: Basal Gremlin 1 mRNA expression in A549 cells transfected with either control miRNA inhibitor or the miR-27b inhibitor. Data are shown as the fold increase relative to the control inhibitor, and represent the mean of three experiments ± SEM. B: A549 cells were transfected with either control miRNA inhibitor or miR-27b inhibitor. Cells were treated with TGF-β for 48 h, after which Gremlin 1 mRNA induction was measured by qPCR. Data are presented as the fold increase of Gremlin 1 relative to the cells transfected with the control inhibitor and treated with vehicle control. n = 3. C: Immunoblots of whole cell lysates from A549 cells transfected with either control or miR-27b inhibitor, then treated with TGF-β for 48 h. β-actin was used as a loading control. D: Gremlin 1 mRNA levels in A549 cells transfected with either control miRNA mimic or the miR-27b mimic. Data are shown as the fold increase relative to the control mimic. n = 3. E: A549 cells were transfected with either control miRNA mimic or the miR-27b mimic. Cells were treated with TGF-β for 48 h, after which Gremlin 1 mRNA induction was measured by qPCR. Data are presented as the fold increase relative to the vehicle control-treated cells transfected with the control mimic. $n = 3$. F: Immunoblots of whole cell lysates from A549 cells transfected with either control mimic or miR-27b mimic, then treated with TGF-β for 48 h. *$P < 0.05$.

that regulation of these genes occurred via Gremlin 1, we measured the changes in expression of these genes in response to siRNA-mediated knockdown of Gremlin 1, which effectively reduced Gremlin 1 protein levels (Suppl. Fig. 2). Similar to the miR-27b mimic, Gremlin 1 siRNA markedly repressed expression of type I collagen, CTGF, and PAI-1, and increased E-cadherin (Fig. 6D), suggesting that Gremlin 1 is involved in the regulation of these genes. These data demonstrate that augmenting Gremlin 1 expression by either inhibiting miR-27b or by adding recombinant protein increased the expression of profibrotic genes, such as type I collagen, and reduced expression of genes that may inhibit fibrosis, such as E-cadherin. Conversely, reducing Gremlin 1 levels by either siRNA or by the miR-27b mimic had the reverse effects. Taken together, our results indicate that miR-27b modulates the expression of genes that are known to be involved in fibrosis, and that it does this in part by regulating Gremlin 1.

GREMLIN 1 INDUCES FIBROBLAST PROLIFERATION IN A BMP-DEPENDENT MANNER

One of the key features of pulmonary fibrosis is uncontrolled proliferation of fibroblasts, preceded by a recurrent, long-lasting injury to the alveolar epithelium. Because Gremlin 1 levels are highly elevated in fibrotic lungs [Myllarniemi et al., 2008], we explored the effect of Gremlin 1 on lung fibroblast cell proliferation, and examined the interplay between Gremlin 1 and BMP-2 in the regulation of cell proliferation (Fig. 7). While treatment of both the MRC-5 fibroblast cells and primary human lung fibroblasts with BMP-2 inhibited cell proliferation in a dose-dependent manner, we found that co-treatment with recombinant Gremlin 1 reversed BMP-mediated repression of cell proliferation. Conversely, treatment of cells with Gremlin 1 alone resulted in a significant increase in cell proliferation, and addition of BMP-2 blocked this effect. These results suggest that Gremlin 1 may contribute to fibrosis by regulating fibroblast cell proliferation, likely by antagonizing BMP signaling.

DISCUSSION

miRNAs control a diverse range of biological processes such as cell proliferation, differentiation, and cell death, as well as many others. As a result of this wide breadth of regulation, aberrant expression of miRNAs can lead to the development of multiple pathologies. Chronic tissue fibrosis is one such disorder in which miRNAs have been implicated; however, the extent of this regulation remains largely unknown. In this study, we sought to identify miRNAs with a potential role in fibrosis, and to characterize the regulatory mechanisms that lead to the gene expression changes associated with fibrotic processes.

To determine which miRNAs might be involved in fibrosis, we chose to use a miRNA inhibitor library to inhibit individual miRNAs in order to assess their effects on the expression of type I collagen, one of the key ECM component in fibrotic lesions. These inhibitors are chemically enhanced oligonucleotides designed to specifically suppress the activity of endogenous miRNAs, allowing for the effects of the individual miRNAs to be determined. This approach, which measures result of suppressing the activity of an endogenous miRNA, is likely to be more functionally relevant than using miRNA mimics, in which nonphysiological levels of miRNAs are introduced into the cells. Of the 879 inhibitors that were screened, we identified miR-27b as a potent regulator of several genes known to be involved in fibrosis, including type I collagen. We further demonstrated that miR-27b can directly target the profibrotic gene Gremlin 1 by specifically binding to its 3′-UTR. Interestingly, TGF-β treatment downregulated miR-27b, similar to what has been seen in cardiomyocytes [Wang et al., 2012], and at the same time upregulated Gremlin 1 expression. The inverse correlation between

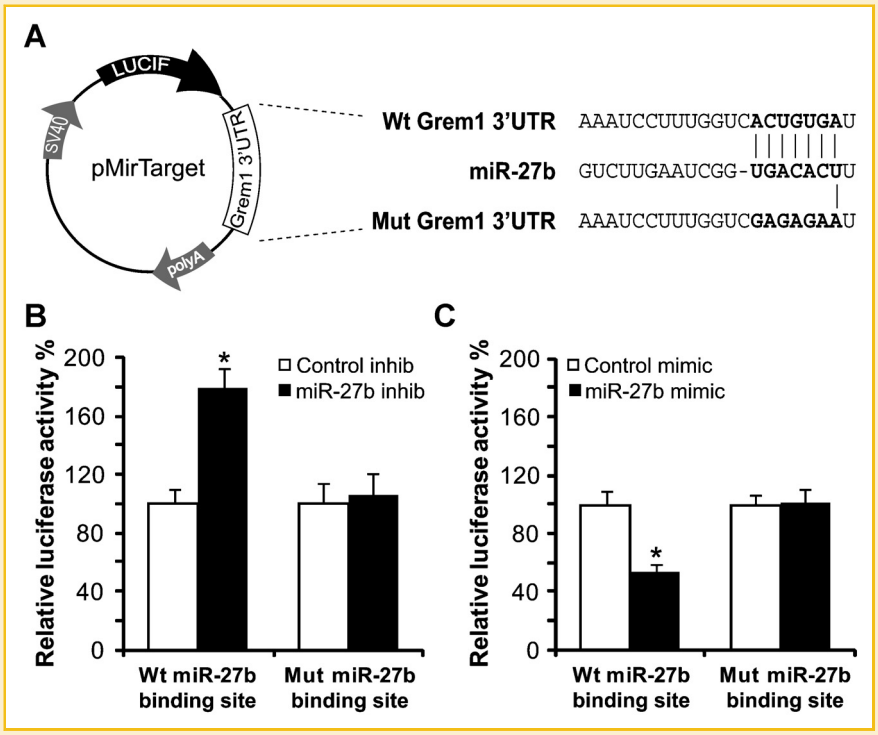

Fig. 5. miR-27b directly binds to the Gremlin 1 3′-UTR. A: Schematic of the pMirTarget reporter construct. The sequences of the wild-type (wt) Gremlin 1 3′-UTR, miR-27b, and the mutated (mut) Gremlin 1 3′-UTR are shown to the right, with bars indicating a sequence match. B: Luciferase reporter activity in A549 cells co-transfected with either control inhibitor or the miR-27b inhibitor and a vector containing either the wild-type Gremlin 1 3′-UTR or the mutated Gremlin 1 3′-UTR. Data are expressed as luciferase values relative to the control and are the ratio of firefly to *Renilla* luciferase activity. n = 6. C: Luciferase reporter activities in A549 cells co-transfected with either the control mimic or the miR-27b mimic along with a vector containing either the wild-type Gremlin 1 3′-UTR or the mutated Gremlin 1 3′-UTR. Data are expressed as luciferase values relative to the control and are the ratio of firefly to *Renilla* luciferase activity. n = 6. *$P < 0.05$ for all panels.

the gene expression patterns of miR-27b and Gremlin 1 in response to TGF-β stimulation suggests that TGF-β-mediated induction of Gremlin 1 expression is partially achieved by reducing the levels of miR-27b. Additional mechanisms likely contribute to the induction of Gremlin 1 gene expression by TGF-β however, since Gremlin 1 induction was detected prior to a substantial decrease in miR-27b levels.

Studies in animal models of fibrosis have not reached a consensus on the importance of miR-27b in disease pathology. For example, Xie et al. [2011] demonstrated a downregulation of miR-27b in the lungs of mice that were administered with bleomycin, a widely used animal model of lung fibrosis [Moeller et al., 2008; Moore and Hogaboam, 2008], whereas other investigators report no changes in miR-27b expression following bleomycin treatment [Liu et al., 2010; Cushing et al., 2011]. Nevertheless, an analysis of these studies reveals little overlap in the sets of differentially expressed miRNAs, which may reflect differences in their experimental designs. Furthermore, it is well known that animal models of bleomycin-induced pulmonary fibrosis do not accurately reproduce the characteristics of human IPF [Borzone et al., 2001], therefore the results from human studies are likely to be more relevant. Oak et al. [2011] measured the miRNA expression profiles in lung biopsies obtained from normal lungs as well as those from patients with IPF that exhibited either a clinically slow or rapid progression of disease. They found that a number of miRNAs were differentially expressed in the fibrotic lungs, and moreover, that miRNA expression was related to the rate of disease progression. Interestingly, miR-27b was downregulated more than threefold in patients with a progressively fast disease progression as compared to the normal lung, but was not significantly decreased in patients with a slow progression. These results suggest that miRNA expression patterns are related to the severity of fibrosis, and furthermore, that decreased miR-27b levels may be an indicator of patients at risk for rapid disease progression.

A similar story is emerging around the role of miR-27b in liver fibrosis, although results are somewhat contradictory between different investigators. In an effort to predict liver transplant patients at risk for later developing fibrosis, Gehrau et al. [2013] determined the expression patterns of miRNAs in biopsies collected from patients 3 years after receiving a liver transplant. They found that miR-27b was one of six miRNAs that was downregulated in patients who developed fibrosis post-transplant compared to those patients who did not progress to disease, suggesting that loss of miR-27b might play a role in the development of liver fibrosis. In contrast, an in vitro study suggested that miR-27b might be profibrotic in activated hepatic stellate cells, since reducing miR-27b levels caused a decrease in the proliferation of these cells [Ji et al., 2009]. These are only two studies, however, so there is a need for additional work to fully define the functions of miR-27b in liver fibrosis.

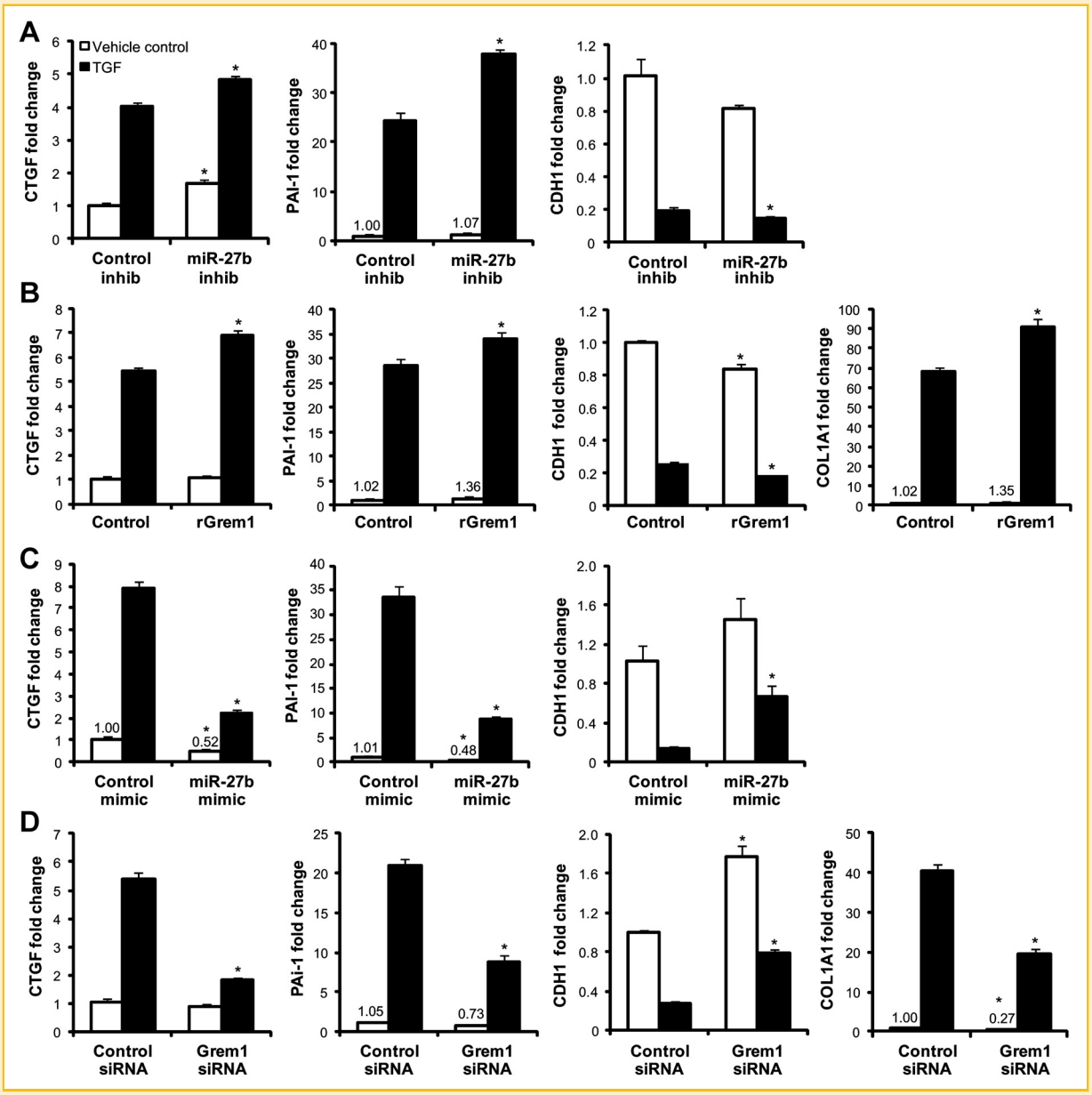

Fig. 6. Modulation of Gremlin 1 levels with either siRNA or miR-27b regulates expression of fibrotic genes. A: A549 lung epithelial cells were transfected with control miRNA inhibitor or the miR-27b inhibitor and either treated with a vehicle control or with TGF-β for 48 h. qRT-PCR was used to measure the mRNA levels of the shown here. Data are presented as the fold increase relative to the control inhibitor treated with vehicle control. B: Cells were treated with either vehicle control or TGF-β in the absence ("Control") or presence or recombinant Gremlin 1 ("rGrem1"). Data are presented as the fold increase relative to the cells subjected to vehicle control only. C: A549 cells were transfected with control miRNA mimic or the miR-27b mimic and either treated with a vehicle control or with TGF-β for 48 h. Data are shown as the fold increase relative to the control mimic treated with vehicle control. D: Cells were transfected with either a nonspecific control siRNA or Gremlin siRNA, and then treated with vehicle control or TGF-β for 48 h. Data represent the fold induction relative to the cells transfected with nonspecific siRNA and treated with vehicle control. For all panels, values are the means ± SEM of three independent replicates and $^*P < 0.05$.

The mechanism by which Gremlin 1 exerts its effects on cell functions remains poorly defined. In IPF, it is believed that repetitive cycles of epithelial cell injury and activation provoke fibrotic changes in fibroblasts, including uncontrolled proliferation of these cells. Our data using in vitro cell culture systems suggest that one potential mechanism by which Gremlin 1, which is highly increased in fibrotic lungs, may contribute to fibrosis pathology is by regulating fibroblast cell proliferation in a BMP-dependent manner. Although Gremlin 1 is best known for regulating cell functions by antagonizing BMPs, a number of studies have shown that Gremlin 1 can also exert its effects in a BMP-independent manner. We have found that the BMP pathway does not seem to play a role in reversing

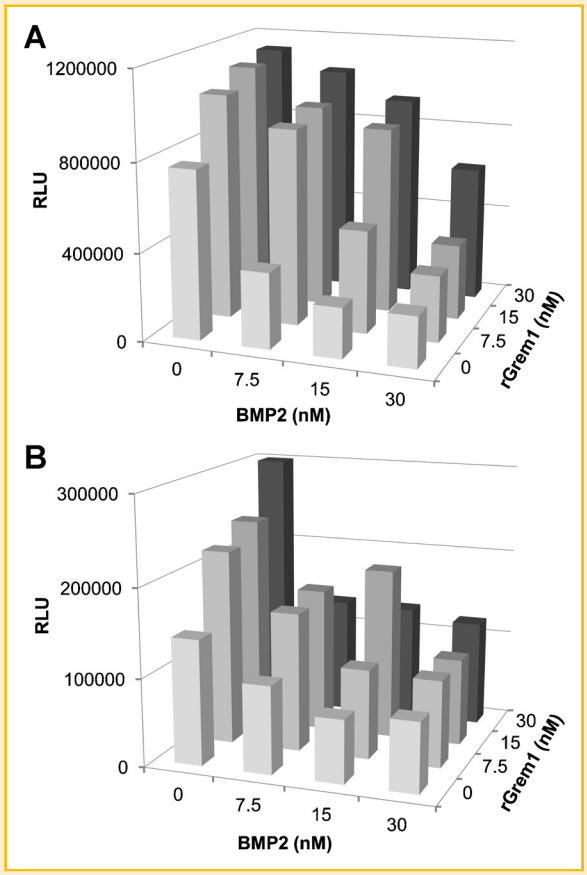

Fig. 7. Gremlin 1 induces proliferation of fibroblast cells. The effect of Gremlin 1 and BMP-2 on the proliferation of both MRC-5 lung fibroblast cells (A) and primary lung fibroblasts (B) was measured using the BrdU proliferation assay. Cells were grown in serum-free medium for 24 h, after which they were treated for 48 h with recombinant Gremlin 1 (rGrem1; 7.5, 15, or 30 nM) in the presence or absence of BMP-2 (7.5, 15, or 30 nM). Data are presented as the mean relative light units (RLU) of three independent values.

TGF-β-induced profibrotic gene expression in A549 cells, as treatment of TGF-β-stimulated A549 cells with recombinant BMP-2, -4, or -7 did not attenuate TGF-β-mediated cellular responses (Suppl. Fig. 3). Likewise, Murray et al. determined that BMP-7 did not inhibit the expression of genes induced by TGF-β stimulation in either A549 cells or fibroblasts [Murray et al., 2008]. Several mechanisms of BMP-independent Gremlin 1 regulation have been proposed. Gremlin 1 was shown to bind to vascular endothelial growth factor receptor-2 (VEGFR2) in endothelial cells, thus activating the VEGFR2 pathway to modulate angiogenesis in a BMP-independent manner [Mitola et al., 2010]. Gremlin 1 is able to bind to the surface of endothelial cells via heparin-sulfate proteoglycans [Stabile et al., 2007], which can also act as co-receptors to modulate the interaction between Gremlin 1 and VEGFR2 [Chiodelli et al., 2011]. In addition, Gremlin 1 was found to regulate the transcription factor NF-κB by upregulating the expression of angiopoientin-1, also by a mechanism independent of BMP inhibition [Mitola et al., 2008], and Chen et al. [2004] showed that Gremlin binds to both Slit1 and Slit2 proteins. More relevant to our study is the finding that Gremlin 1 can directly bind to the cell surface of A549 cells [Kim et al., 2012a]. Interestingly, this binding was unaffected by the addition of BMPs and was not mediated by the VEGFR2 receptor, which was not expressed in these cells. Thus, we believe that Gremlin 1 may exerts its pro-fibrotic effects by antagonizing BMP's anti-proliferative activity in fibroblast cells, but in epithelial cells such as the A549 cells it does so using a BMP-independent mechanism, although the protein binding and signaling events that drive this response have yet to be uncovered.

In conclusion, our study further establishes the importance of miRNAs in mediating the changes in gene expression that occur during fibrosis. Specifically, we have shown for the first time that miR-27b regulates Gremlin 1 expression by directly binding to its 3′-UTR, causing a decrease in Gremlin levels. We further show that miR-27b and Gremlin 1 are inversely regulated by TGF-β signaling, suggesting that the reduction of miR-27b levels in response to TGF-β stimulation partially mediates the increase in Gremlin 1 expression. Importantly, we have demonstrated that the expression of key profibrotic genes is regulated by both miR-27b and Gremlin 1, indicating that they play a significant role in the fibrotic response.

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