Suppression of Type I Collagen Expression by miR-29b via PI3K, Akt, and Sp1 Pathway in Human Tenon’s Fibroblasts

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PURPOSE. To evaluate the expression profile of microRNAs (miRNAs) and their roles in human Tenon’s fibroblasts (HTFs), and to establish an miRNA-based gene-silencing method for antiﬁbroblast in vitro.

METHODS. The miRNA expression proﬁle was analyzed by microarray using quiescent and transforming growth factor beta 1 (TGFβ1)-stimulated primary HTFs, respectively. Candidate miRNAs were identiﬁed by quantitative RT-PCR. miRNAs potentially targeting ﬁbrosis-related genes were predicted using a published algorithm. Predicted ﬁbrosis-related genes regulated by candidate miRNAs were conﬁrmed by transfection of the miRNA into HTF culture (with or without TGFβ1 treatment), followed by quantitative RT-PCR and Western blot analysis.

RESULTS. In all, 38 miRNAs were identiﬁed to be upregulated and 31 downregulated, in TGFβ1-stimulated HTFs. Among those, the miR-29b, downregulated in TGFβ1-treated HTFs, targeted a cadre of mRNAs that encode proteins involved in ﬁbrosis, including PI3Kp85α, Sp1, and collagen type I alpha1 (Col1A1). Treatment of HTFs with TGFβ1 activated the PI3K/Akt/Sp1 pathway and, consequently, induced an increase in the expression of type I collagen. Overexpression of miR-29b inhibited the PI3K/Akt/Sp1 pathway and attenuated the expression of Col1A1.

CONCLUSIONS. miR-29b acted as a suppressor of type I collagen gene by repressing the PI3K/Akt/Sp1 pathway in HTFs. Overexpression of miR-29b protected subconjunctival tissues against collagen production and ﬁbrosis. These ﬁndings provided a novel rationale for the development of miRNA-based strategies for attenuating scar formation after glaucoma ﬁltering surgery. (Invest Ophthalmol Vis Sci. 2012;53:1670–1678) DOI:10.1167/iovs.11-8670

Conjunctival and scleral scarring remain as limiting factors in the success of ﬁltration surgery, and scarring in the sclerostomy site or bleb connective tissue are formed by excessive proliferation and aberrant extracellular matrix (ECM) production beneath the conjunctiva. The cytokine transforming growth factor beta (TGFβ) is a key mediator of wound healing, which drives the conversion of ﬁbroblasts to myoﬁbroblasts, which is primarily responsible for ﬁbrous tissue formation. The persistence of myoﬁbroblasts is associated with increased deposition of ECM, leading to tissue ﬁbrosis. Notably, collagen production by activated human Tenon’s ﬁbroblasts (HTFs) is regulated by TGFβ in an autocrine loop, which is accompanied by induction of TGFβ receptors. Thus, suppression of HTF activation and collagen expression is critical to establish therapeutic strategies for postsurgery scarring.

Recently, several reports described key roles of microRNAs (miRNAs) in organ ﬁbrosis, pointing to a new mode of regulation of ﬁbrotic processes. miRNAs are short, approximately 22-nucleotide RNAs that modulate gene expression by base pairing with the 3′-untranslated regions (UTRs) of miRNAs and inhibiting translation or promoting degradation of mRNA. miRNA is involved not only in the regulation of cell proliferation, apoptosis, and differentiation, but also in development and metabolism. In an effort to identify miRNAs with potential roles in bleb scarring, we evaluated the miRNA expression proﬁle using microarray, demonstrating that 38 miRNAs were upregulated and 31 downregulated, in activated HTFs, respectively. Among these miRNAs, we found miR-29b to be dramatically downregulated in the TGFβ1-activated HTFs.

Previous studies identiﬁed that miR-29 targeted a cadre of miRNAs, which encoded profibrotic proteins in various cells showing great potential in liver, heart, kidneys, and other body organs in the treatment of ﬁbrotic diseases. Class I phosphoinositide 3-kinase (PI3K) is composed of a p110 catalytic subunit and a regulatory p85α subunit. Akt is a 60kDa PH (pleckstrin homology) Ser/Thr kinase, which is one of the major intermediary molecules down the PI3K signaling pathway, and its activation by TGFβ transmits the signal to downstream physiologic processes. Functionally, Akt acts downstream of PI3K and upstream of Sp1. Activated PI3K generates several phosphoinositides, leading to Akt activation by phosphorylation at Thr308 and Ser473 by phosphoinositide-dependent kinase-1. Activation of the PI3K/Akt/Sp1 pathway facilitated the collagen synthesis in the ﬁbroblast. Thereby TGFβ promoted their invasiveness by inducing Col1A1 expression via the sequential actions of PI3K, Akt, and Sp1.

As a usually irreversible disease, postsurgery scarring has treatment challenges similar to those of organ ﬁbrosis. Detection of the same miRNA expression means that common antifibrotic strategies might exist. Successful miRNA therapy, which has been used for other organ ﬁbrosis, might be possible for the treatment of postoperative scar formation.

Together with previous studies on the functional role of miR-29 in ﬁbrosis of multiple organs and its involvement in the PI3K/Akt/Sp1 pathway, we speculated that miR-29b is related...
with proliferation of HTFs and fibrosis resistance. We performed a systematic analysis of the effects of miR-29b on biological properties of HTFs and investigated its action mechanisms based on miRNA expression profile and interpretation of bioinformatics.

Until now, the function of miR-29b in HTFs has remained largely unknown. These findings may add to our current knowledge about the characteristics of HTFs, and provide a novel therapeutic strategy against the activation and progression of bleb scarring after glaucoma filtering surgery.

**Materials and Methods**

**Cell Culture and Treatment**

In compliance with the provisions of the Declaration of Helsinki, human subconjunctival fibroblasts were obtained from excised Tenon’s capsule specimens (thus HTFs) during strabismus surgery. Unless otherwise stated, cell cultures were maintained in Dulbecco’s modified Eagle’s medium (DMEM; Invitrogen, Grand Island, NY) containing 10% fetal bovine serum (FBS), 1% L-glutamine (2 mM), 50 μg/mL penicillin, and 50 μg/mL streptomycin at 37°C in 5% CO2 atmosphere. All experiments used HTFs from passage 3 to 6 cultures. Cultures were allowed to reach 80% to 85% confluence and were treated with 10 ng/mL TGFβ1 (PeproTech, London, UK) in serum-free medium for 48 hours. All experiments were repeated at least three times.

**miRNA Microarray**

Total RNAs were extracted from cultured primary HTF cells and were analyzed using an miRNA microarray (KangChen Bio-Tech, Inc., Shanghai, China). Procedures were performed as described by the manufacturer (http://www.KangChen.com.cn). Briefly, miRNAs were separated from 30 to 50 μg total RNAs using a commercial miRNA isolation kit (Ambion Inc., Austin, TX). Purified miRNAs were labeled with a commercial kit (miRCURY Hy3/Hy5 Labeling Kit; Exiqon, Vedbæk, Denmark). The labeled samples (Hy3) and labeled reference pool (Hy5) RNA samples were then mixed pairwise and hybridized (to the miRCURY LNA Array, version 14.0; Exiqon). The hybridization was performed in accordance with the manufacturer’s array manual (miRCURY LNA Array Manual; Exiqon). After hybridization, the slides were washed (Wash Buffer Kit; Exiqon), dried, and scanned on an array scanner (Axon GenePix 4000B; Molecular Devices, Sunnyvale, CA). A commercial kit was used (GenePix pro V6.0; Molecular Devices) to read the raw intensity of the image. The results were subjected to unsupervised hierarchical clustering (Cluster 3.0) and TreeView analysis (Stanford University, Stanford, CA).

**Quantitative RT-PCR for Evaluating the Expression of miR-29b**

Primary HTFs from the control and the TGFβ1-treated groups were used for total RNA preparation using a commercial kit (Ambion miRNA Isolation Kit; Ambion Inc.; each group contained HTFs from three patients. Real-time quantification of miR expression was performed using probes (TaqMan Probes; Applied Biosystems, Foster City, CA) specific for miR-29b using a commercial kit (TaqMan microRNA assays kit; Applied Biosystems) according to the manufacturer’s protocol. Data were normalized to the expression of U6 small nuclear ribonucleic acid (snRNA).

**Transient Transfection of miRNA and Treated Groups**

Chemically modified RNA oligonucleotides comprising a sequence complementary to the mature miR-29b (miR-29b inhibitor, anti-miR-29b) were used to inhibit miR-29b activity. The miR-29b mimic is a double-stranded construct consisting of a guide and passenger strands. An oligonucleotide containing a four-base mismatch (mm miR-29b, nontargeting scramble RNA) was used as a negative control. The miR-29b inhibitor, mimic, and their negative controls were all obtained from a commercial supplier (GenePharma, Shanghai, China). The artificially designed oligonucleotides, with either mimicking sequences or antisense sequences and nontargeting scramble RNAs, were transfected into primary HTFs isolated from the HTF tissue, using a com-

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**Figure 1.** Bar chart representation of miRNAs that displayed twofold or more change in expression in activated HTFs after 48 hours of TGFβ1 stimulation as determined by microarray analysis. Values were expressed as fold change compared with quiescent primary HTFs.
mmercial reagent (Lipofectamine 2000; Invitrogen, Carlsbad, CA), at a final concentration of 50 nM, respectively. Briefly, the cells were plated in antibiotic-free medium supplemented with 10% FBS at a density of 1–2 × 10^5 cells/mL 24 hours before the transfection. miRNA and a commercial reagent (Lipofectamine 2000; Invitrogen) were mixed at a ratio of 25 (pmol):1 (µL) in a commercial medium (Opti-MEM I reduced medium; Invitrogen) and incubated for 20 minutes at room temperature. The media complexes (miRNA-Lipofectamine 2000; Invitrogen) were then added to fibroblast-culture serum-free medium. After 6 hours, the culture medium was changed, and TGFβ1 was added at a concentration of 10 ng/mL. For the pharmacologic inhibition group, cells were treated with 10 µM of a kinase inhibitor (LY294002; Cell Signaling Technologies, Danvers, MA) for 1 hour before the TGFβ1 stimulation.

Subset I (without TGFβ1 Treatment). (1) untreated group (control); (2) negative control for miR-29b mimic (mimic NC); (3) miR-29b mimic (mimic); (4) negative control for inhibitor (inhibitor NC); (5) miR-29b inhibitor (inhibitor).

Subset II (with TGFβ1 Treatment). (1) TGFβ1 (10 ng/mL); (2) TGFβ1 + mimic NC; (3) TGFβ1 + mimic; (4) TGFβ1 + inhibitor NC; (5) TGFβ1 + inhibitor; (6) TGFβ1 + LY294002 (10 µM).

**RNA Isolation and Quantitative RT-PCR**

Total RNA from cultured cells was isolated using a commercial kit (mirVana miRNA Isolation Kit; Ambion, Inc.) according to the manufacturer’s instructions. Concentrations of the extracted RNAs were measured using a spectrophotometer. cDNAs were synthesized using 10 ng of total RNA and used for real-time PCR. The forward and reverse primers used were: alpha 1 (I) collagen (Col1A1): Forward 5'-GGT GTG CGA TGA CG-3', Reverse 5'-GCA TTT GGT CGG TGG GTG-3'; Sp1: Forward 5'-GCA CCT ATT CCC TCA TCC TTT C-3', Reverse 5'-TTG GCG TTA CTG TTC TGG-3'; PI3Kp85α: Forward 5'-TTG AGA ATG AAA TGC-3', Reverse 5'-ATT CAG CCA TTC ATT CCA CC-3'; and GAPDH: Forward 5'-GAA GGT CGG AGT CAA CGG ATT T-3', Reverse 5'-CCT GGA AGA TGG TGA TGG GAT T-3'. Real-Time PCR was performed using a commercial fluorescent probe (SYBR Green PCR Master Mix; Applied Biosystems) according to the manufacturer’s instructions. All experiments were done in triplicate. The ΔΔCt method was applied to calculate the relative differences between control and treated groups, and results were expressed as fold changes in gene expression.

**Western Blot Analysis**

Cells were rinsed with ice-cold PBS, and total cell proteins were extracted using RIPA lysis buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM EDTA, 1% Na3VO4, 0.5 µg/ml leupeptin, and 1 mM phenylmethanesulfonyl fluoride [PMSF]). After centrifugation for 10 minutes at 14,000g, the supernatant was collected and used for Western blot analysis. Proteins (5 µg/lane) were subjected to SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes (Hybond; GE Healthcare, Piscataway, NJ). Membranes were blocked with TBST (20 mM Tris, 137 mM NaCl [pH 7.4], and 0.02% Tween 20) containing 5% nonfat dry milk, then incubated with various primary antibodies diluted in TBST.
for 24 hours at 4°C, and washed three times with TBST. Detection of primary antibodies was achieved by incubating membranes with horseradish peroxidase–conjugated anti-mouse or anti-rabbit antibody diluted 1:1000 in TBST for 1 hour at room temperature, followed by three washes with TBST. Immunoreactive proteins were visualized using chemiluminescence detection reagents (ECL; GE Healthcare) on autoradiograph films. Anti-Col1A1 antibody was purchased commercially (Santa Cruz Biotechnology, Santa Cruz, CA). Monoclonal antibodies to PI3Kp85, phospho-PI3Kp85, Sp1, Akt, and phospho-Akt-Ser473 were all obtained commercially (Cell Signaling Technologies). β-Actin (antibody purchased from Sigma-Aldrich, St. Louis, MO) was used as the loading control in all cases.

**MTT Assay**

The capacity for cellular proliferation was measured with a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Twenty-four hours after RNA transfection, cells were seeded into 96-well culture plates for 24, 48, and 72 hours. The cells were then incubated with 20 μL MTT (5 mg/mL) for 4 hours at 37°C and 150 μL DMSO was added to solubilize the crystals for 20 minutes at room temperature. The optical density was determined with a spectrophotometer (Multiskan MK3; Thermo Fisher Scientific, Waltham, MA) at a wavelength of 570 nm.

**Statistical Analysis**

Statistical evaluation was performed using Student’s t-test to compare the differences between treated groups and control groups. The data were expressed as mean ± SEM. All experiments were performed in triplicate. P < 0.05 was considered to be statistically significant.

**RESULTS**

**Expression of miR-29b Was Downregulated in TGFβ1-Stimulated HTFs**

In an effort to identify miRNAs involved in scarring after filtration surgery, we induced HTFs by TGFβ1 administration and compared the expression profile of miRNAs in TGFβ1-stimulated HTFs with the nontreated primary HTFs control group from the same passages (Fig. 1). Among 9360 individual miRNAs represented on the microarrays, the expression of 69 miRNAs was significantly altered in TGFβ1-stimulated HTFs; 38 miRNAs were upregulated ≥twofold and 31 miRNAs were downregulated ≥twofold.

miR microarray analyses revealed that the level of miR-29b in the TGFβ1-stimulated HTFs showed a significant decrease of 2.32-fold compared with its untreated counterparts. It was validated by quantitative real-time RT-PCR. Figure 2 shows the normalized (to U6 snRNA) level of miR-29b in the HTFs of control and TGFβ1-stimulated groups. A significant (P < 0.01) decrease in the level of miR-29b was observed in the myofibroblasts compared with the control.

**Downregulation of PI3Kp85, Sp1, and Col1A1 mRNAs with miR-29b Mimic**

Prior studies have identified that miR-29b directly targeted the 3’-UTR of PI3Kp85, Sp1, and Col1A1 mRNAs in different cells8–10; we therefore tested whether overexpression of miR-29b reduces the expression of these genes in this specific cell type. HTFs were transfected with either the negative control or miR-29b mimic (GenePharma, Shanghai, China) using commercial reagents (Lipofectamine 2000 and Plus Reagent; Invitrogen). The transfection efficiency was optimized according to the fluorescent intensity of reporter dye (FAM) (Fig. 3). The level of miR-29b expression in fibroblast cultures increased strikingly 6 hours posttransfection (Fig. 3B). Then, the media were replaced with fresh media and incubated in the presence of 50 nM miR-29b mimic and a scramble RNA control using a commercial reagent (Lipofectamine 2000; Invitrogen). After 6 hours, the medium was replaced with DMEM containing 10% FBS with or without 10 ng/mL TGFβ1, and the culture was maintained for another 48 hours before being used for RNA preparation. Overexpression of miR-29b significantly downregulated the mRNA level of PI3Kp85 (A), Sp1 (B), and Col1A1 (C) in HTF cells with or without TGFβ1 treatment. *P < 0.05, **P < 0.01.
or the absence of TGFβ1 (10 ng/mL) for 48 hours to study the effects of miR-29b overexpression on PI3Kp85α, Sp1, and Col1A1 mRNA levels. Our results showed that transient transfection of miR-29b mimic significantly inhibited the expression of PI3Kp85α, Sp1, and Col1A1 miRNAs (Fig. 4) in both control and in TGFβ1-stimulated HTFs. Our observations of the role of miR-29b on the regulation of collagen type I and similar mechanisms agreed with former reports that have been reported in other cells.11,12,19,20 It was implied that miR-29b was a potent miRNA regulating collagen production in HTFs.

miR-29b Inhibits TGFβ1-Induced Akt Phosphorylation without Affecting the Total Akt Level in HTFs

Our finding of the level of miR-29b being downregulated in TGFβ1-stimulated HTFs prompted us to study its role in the effects of TGFβ1 on HTFs. TGFβ1 has been well known to activate the PI3K and Akt, a Ser/Thr kinase, pathway by binding to its receptor. We decided to study whether the level of miR-29b would affect this PI3K/Akt pathway by transfecting the HTF cells with either 50 nM of the miR-29b mimic or negative control, which had a scramble sequence. LY294002, a well established PI3K inhibitor, was used as a positive control and in TGFβ1-treated HTFs. Our observations of the role of miR-29b on the regulation of collagen type I and similar mechanisms agreed with former reports that have been reported in other cells.11,12,19,20 It was implied that miR-29b was a potent miRNA regulating collagen production in HTFs.

miR-29b Regulates the Expression of PI3K

The finding of miR-29b inhibiting only TGFβ1-stimulated Akt phosphorylation without altering total Akt levels prompted us to further look for direct targets of miR-29b in intermediates upstream of Akt. Using TargetScan (http://www.targetscan.org) for target identification and analysis, we found that the p85α regulatory subunit of PI3K harbors a binding site for miR-29b at positions 331 to 337 of its 3′-UTR (Fig. 613). PI3Ks constitute a family of enzymes involved in a variety of cellular functions, such as cell growth, proliferation, differentiation, motility, survival, and intracellular trafficking. Class I PI3Ks are heterodimeric molecules composed of a regulatory (p110) and a catalytic subunit (p85). There are five variants of the p85 regulatory subunit, designated p85α, p55α, p50α, p85β, or p55γ. p110 is stabilized only when it binds to p85.

miR-29b Suppresses TGFβ1-Mediated Activation of Col1A1 Gene Expression

Results until now demonstrated that overexpression of miR-29b in HTFs significantly decreased the expression of p85α subunit at both the mRNA and the protein levels (Figs. 4A and 7). This implied that the p85α was targeted by endogenous miR-29b. The counteracting effect of miR-29b on TGFβ1-stimulated Akt phosphorylation might therefore begin with direct inhibition of PI3Kp85α by miR-29b, which in turn leads to reduced phosphorylation of the downstream Akt, although the total Akt level is unaltered.

miR-29b overexpression significantly inhibited the expression of Col1A1 in HTFs. Transient transfection of miR-29b mimic significantly inhibited type I collagen miRNA and protein expression (Figs. 4A and 7). This implied that the p85a regulatory subunit of PI3K harbors a binding site for miR-29b at positions 331 to 337 of its 3′-UTR. Because PI3K and Akt act upstream of Sp1,22 and the transcription activity of the human Col1A1 could be positively regulated by Sp1,25 modulation of the level or of the transcriptional activity of Sp1 might play an important role in the regulation of collagen gene expression. In addition, according to the predicted binding site of miR-29b on the 3′-UTR of the p85α regulatory subunit of PI3K, the seed region of miR-29b is complementary to the 3′-UTR.
to TargetScan, our laboratory and others have recently revealed that Sp1 and Col1A1 have one or two predicted target sites in their 3′-UTRs for miR-29b, respectively (Fig. 8). So we further investigated the cellular effects of miR-29b and TGFβ1 on Sp1. Our results indicated that in the presence of miR-29b, both Col1A1 and Sp1 miRNA and protein levels decreased, as determined by real-time RT-PCR and Western blotting analysis (Figs. 4B, 4C and Fig. 9). These effects were blocked at the presence of anti-miR-29b (Fig. 9), suggesting that the expressions of Col1A1 and Sp1 were regulated by endogenous miR-29b. TGFβ1 stimulation activated Sp1, which in turn led to upregulated expression of Col1A1. Thus, the miR-29b-mediated suppression of type I collagen expression in HTFs can be twofold: (1) directly inhibit the expression of Col1A1 mRNA at its 3′-UTR and (2) negative regulation of its transcriptional activator, Sp1. Such an effect of miR-29b on TGFβ1-stimulated Col1A1 expression pointed toward a novel angle of regulation of the PI3K/Akt and Col1A1 pathways in HTFs.

**miR-29b Inhibits HTF Proliferation In Vitro**

As Figure 10 shows, TGFβ1 (10 ng/mL) treatment significantly increased the proliferation of HTFs at all time points. To provide evidence that miR-29b was indeed involved in the growth of HTFs, HTFs were transiently transfected with miR-29b mimic, miR-29b inhibitor, and their respective negative controls. According to the results of the MTT assay, we found that the HTFs, which transfected with miR-29b mimic, had a significant growth inhibition at different degrees (Fig. 10). There was a significant decrease in the cell proliferation of HTFs compared with control at 72 hours. However, proliferation of the cells transfected with miR-29b inhibitor was increased compared with that of the cells transfected with anti-miR-29b negative control and blank control. Our studies suggested that upregulation of miR-29b might inhibit the proliferation of HTFs in vitro.

**DISCUSSION**

The present study revealed several novel findings in the cellular events of activated HTFs. First, we found an altered expression profile of miRNAs induced by TGFβ1 in HTFs using miRNA array analysis. Second, we showed that miR-29b suppressed type I collagen gene expression and cellular proliferation by modulation of the PI3K/Akt/Sp1 signaling pathway. After glaucoma filtration surgery, miR-29b was significantly downregulated in the TGFβ1-activated primary HTFs, which contributed to ECM-dependent pathophysiology. Finally, we used an in vitro model to show that elevating intracellular miR-29b expression by exogenous miR-29b delivery may provide an effective way of attenuating collagen deposit and, thus, to some extent, influence the formation of scarring. For the first time, our in vitro studies showed a novel rationale for the development of miRNA-based strategies for antiscarring of subconjunctival tissue after glaucoma filtering surgery.

**miRNAs have been identified as critical regulators of fibrotic processes, and have shown great potential in the treatment of fibrosis in multiple body organs.** HTFs, the major fibrotic cells in the Tenon’s tissue, acted as the inducer of bleb scarring after their transdifferentiation into myofibroblast-like cells. However, there is still very limited understanding on miRNA’s regulation in HTFs. Therefore, the dissection of miRNAs as vital molecules responsible for the fine-tuning of varied pathways that culminated into scarring opened up a novel area in glaucoma surgery research.

**Control of Scarring by miR-29b via the PI3K/Akt/Sp1 Signaling Pathway**

In the present study, we investigated the intracellular TGFβ1 signal transduction involved in the expression of collagen in...
primary subconjunctival fibroblasts and our results indicated that miR-29b suppressed the expression of collagen by attenuating the action of TGFβ1-induced signal cascade. Because collagen gene expression was regulated by miR-192 through its interaction with the transcriptional repressor E-box, we further examined Sp1, which was a transcriptional regulator of interaction with the transcriptional repressor E-box, we further examined Sp1, which was a transcriptional regulator of interaction with the transcriptional repressor E-box. We found that activated Akt was involved in the modulation of TGFβ1-induced expression of Col1A1. The suppression effect of miR-29b on the phosphorylation of Akt was a consequence of miR-29b’s negative regulation of the p85α regulatory subunit of PI3K by possible targeting to its 3’-UTR. In our study, we confirmed the inhibitory effect of miR-29b on PI3Kp85α expression in HTF cells by showing that overexpression of miR-29b significantly decreased the levels of the p85α subunit of PI3K. As a consequence, the elevated level of miR-29b led to decreased expression of Col1A1. These together with the fact that miR-29 levels were decreased in the myofibroblasts (Fig. 2) suggested that the decreased levels of miR-29b might be responsible for the increased levels of p-PI3Kp85α, P-Akt, and Col1A1, as observed in vitro by mechanisms described earlier. In other words, through directly targeting PI3K/ Akt pathway, we found that miR-29b did not affect the total Akt level, although TGFβ1-stimulated phosphorylation of Akt was significantly inhibited. In addition, a PI3K-specific inhibitor (LY294002) was shown (1) to inhibit TGFβ1-induced Akt phosphorylation and (2) to reduce the TGFβ-induced expression of Col1A1. These results indicated that activated Akt was involved in the modulation of TGFβ1-induced expression of Col1A1. The suppression effect of miR-29b on the phosphorylation of Akt was a consequence of miR-29b’s negative regulation of the p85α regulatory subunit of PI3K by possible targeting to its 3’-UTR. In our study, we confirmed the inhibitory effect of miR-29b on PI3Kp85α expression in HTF cells by showing that overexpression of miR-29b significantly decreased the levels of the p85α subunit of PI3K. As a consequence, the elevated level of miR-29b led to decreased expression of Col1A1. These together with the fact that miR-29 levels were decreased in the myofibroblasts (Fig. 2) suggested that the decreased levels of miR-29b might be responsible for the increased levels of p-PI3Kp85α, P-Akt, and Col1A1, as observed in vitro by mechanisms described earlier. In other words, through directly targeting 3’-UTRs of PI3Kp85α, Sp1, and Col1A1, miR-29b regulated collagen gene expression by modulating phosphorylation of the PI3K/Akt/Sp1 signal pathway in HTFs (Fig. 1A)13.

Although our results suggested a key role for miR-29b in the control of bleb scarring, it was important to note that there was not a direct one-to-one stoichiometric relationship between the levels of miR-29b and collagen expression. For example, overexpression of miR-29b mimic was accompanied by a striking decrease in collagen expression at both mRNA and protein levels, whereas miR-29b antisense inhibitor only slightly induced collagen expression. We speculated that a target gene may be bound with multitypes of miRNAs, and the effect of knocking down one single type of miRNA is not significant. Moreover, additional regulatory steps might be involved in the actions of miR-29b and in the control of scarring. Overall, the inhibition of miR-29b expression by TGFβ may lead to aberrant augmentation of collagen deposition in HTFs and played a role in reduction of the success rate in glaucoma filtration surgery (Fig. 1B)13.

**Therapeutic Opportunities**

Our results confirmed that overexpression of miR-29b in HTFs can effectively cause targeted gene silencing, providing new insights into the involvement of miRNAs in the formation of bleb scarring. Together with previous studies on the functional role of miR-29 in the cardiac, liver, or lung fibrosis, we speculated that the miR-29 family may be a key factor in the formation of tissue scarring of multiple body organs. In this

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**Figure 9.** Effects of miR-29b on the expression of Sp1 and Col1A1 in HTFs in the presence (+) or the absence (−) of TGFβ1. HTFs were transfected with 50 nM of miR-29b mimic, anti-miR-29b, or scramble RNA using a commercial reagent (Li-pofectamine 2000; Invitrogen) for 6 hours. Cells were then treated with or without TGFβ1 (10 ng/mL) for 48 hours before being used for Western blot analysis.

**Figure 10.** miR-29b inhibits HTFs proliferation in vitro. The primary HTFs were incubated with TGFβ1 (10 ng/mL) and transiently transfected with miR-29b mimics, miR-29b inhibitor, and their respective negative control, after which cell proliferation was measured using the MTT assay. Data are the mean ± SEM of results from three independent experiments. Con, control; mimic NC: mimic negative control; inhibitor NC: inhibitor negative control.
regard, elevating miR-29b expression by miRNA delivery represents an intriguing therapeutic approach in systematic fibrotic diseases.

In conclusion, to our knowledge, this was the first report toward unraveling the role of miR-29b in the fine-tuning of a fibrotic pathway involved in bleb scarring, providing a novel rationale for the development of miRNA-based strategies for the attenuation of scar formation after glaucoma filtering surgery.

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