

Transforming Growth Factor- β 1-induced Human Subconjunctival Fibrosis is Mediated by MicroRNA 143/145 Expression

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PURPOSE. To investigate the roles and pathways of microRNAs 143 and 145 in transforming growth factor (TGF)- β 1-induced human subconjunctival fibrosis.

METHODS. Human tenon's capsule fibroblasts (HTFs) were obtained from a healthy eye. After treating cultured HTFs with TGF- β 1, the expression of microRNAs 143 and 145 was evaluated using polymerase chain reaction. To identify the pathways of TGF- β 1-induced microRNA 143/145 expression, HTFs were treated with specific inhibitors of p38MAPK, PI3K/Akt, JNK, ERK, and with siRNAs for SMAD2 and SMAD4. Mutagenesis studies were performed to evaluate the role of the CarG box and SMAD-binding element (SBE). To investigate the role of microRNA 143/145 in TGF- β 1-induced myofibroblast transdifferentiation, microRNA 143/145 mimics and microRNA 143/145 inhibitors were applied to the HTFs.

RESULTS. Array analysis revealed that TGF- β 1 induced the expression of microRNA 143/145 in a dose- and time-dependent manner. When inhibitors and siRNAs for p38MAPK, PI3K/Akt, ERK, and JNK were applied, the TGF- β 1-induced expression of microRNA 143/145 was inhibited; however, SMAD2 and SMAD4 inhibition did not affect the TGF- β 1-induced expression of these microRNAs. In the mutagenesis studies, both the CarG box and SBE were associated with TGF- β 1-induced expression of microRNA 143/145. Mimics of microRNA 143/145 induced increased myofibroblast formation, whereas their inhibitors had the opposite effect.

CONCLUSIONS. TGF- β 1-induced human subconjunctival fibrosis was mediated by the expression of microRNA 143/145, mainly via SMAD-independent pathways. Inhibition of TGF- β 1-induced microRNA 143/145 expression in HTFs might represent a novel strategy to prevent subconjunctival fibrosis.

Keywords: glaucoma, fibrosis, TGF-beta, microRNA, conjunctiva

Given that a high intraocular pressure (IOP) is the most important factor for the development and progression of glaucoma,¹⁻³ current treatment strategies focus on controlling the IOP by medications or surgical procedures. Conventional glaucoma surgeries, such as trabeculectomy or implantation of glaucoma drainage devices are based on the concept that the aqueous humor produced by the ciliary body is drained into the subconjunctival space by an artificial drainage route, thereby reducing the IOP. The main cause of glaucoma surgery failure is excessive subconjunctival fibrosis and closure of the aqueous humor drainage route caused by proliferation of human Tenon's capsule fibroblasts (HTFs) and subsequent increase in the production of the extracellular matrix (ECM) and collagen contraction.⁴⁻⁶

Transforming growth factor (TGF)- β is a key mediator of scar formation during wound healing. It drives the proliferation of fibroblasts, the conversion of fibroblasts into myofibroblasts, and increases the deposition of the ECM.^{7,8} Thus, TGF- β and its signaling pathways are considered important therapeutic

targets to prevent excessive scarring. The TGF- β signaling pathways in fibrosis are classified into SMAD (Sma/'mothers against decapentaplegic' [Mad])-dependent pathways and SMAD-independent pathways, including mitogen-activated protein kinase (MAPK), phosphoinositide 3-kinase (PI3K)/Akt, Abelson nonreceptor tyrosine kinase (c-Abl), and Rho GTPase pathways.^{7,8} Among the members of the MAPK family, p38MAPK, c-Jun-N-terminal kinase (JNK), and extracellular signal-regulated kinase (ERK) are activated by TGF- β .^{7,8}

MicroRNAs (miRNAs) are small noncoding RNAs that bind to target mRNAs to regulate gene expression.⁹⁻¹¹ MiRNAs are powerful modulators in cell proliferation and differentiation,⁹⁻¹¹ and are aberrantly expressed in various disorders, such as cancer, cardiovascular diseases, neurologic disorders, and infectious diseases.⁹⁻¹¹ Thus, miRNAs are considered as diagnostic markers, prognostic markers, and therapeutic targets in various pathologic conditions.⁹⁻¹¹ Currently, little is known about the role of miRNAs in subconjunctival fibrosis.¹²⁻¹⁶ We hypothesized that if miRNAs play a role in the TGF- β 1-induced



subconjunctival fibrosis, modulation of this pathway would help to prevent excessive subconjunctival fibrosis after glaucoma surgery. Therefore, the present study aimed to investigate the roles and pathways of miRNAs in TGF- β 1-induced human subconjunctival fibrosis.

MATERIALS AND METHODS

Cell Cultures and Chemicals

This research followed the tenets of the Declaration of Helsinki and received approval from the institutional review board. HTFs were obtained from the subconjunctival Tenon's capsule.¹⁷⁻¹⁹ In brief, Tenon's capsule was excised during the strabismus surgery in a healthy eye without the presence of ocular disorders with the exception of strabismus. The HTFs were cultured at 37°C in a 5% humidified CO₂ environment in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS), 50 μ g/mL penicillin, and 50 μ g/mL streptomycin. The cells used for these studies had been passaged between three and six times. For TGF- β 1 treatment, growing cells were seeded in plates and cultured until 90% confluence. Following serum starvation overnight, the cells were treated with TGF- β 1 (10 ng/mL) for 24 or 48 hours. For inhibitor studies, cells were pretreated with the indicated signaling inhibitors 30 minutes before TGF- β 1 treatment. SB203580 (specific inhibitor of p38MAPK), LY294002 (inhibitor of PI3K), and U0126 (inhibitor of ERK1/2) were from Cell Signaling Technology (Danvers, MA, USA). SP600125 (inhibitor of JNK) was from Calbiochem (Darmstadt, Germany) and SH5 (inhibitor of Akt) was from Abcam (Cambridge, UK). Except when used in the dose-dependency studies, each inhibitor was used at a concentration of 10 μ M.

Sample Preparation

Total RNA was harvested from HTFs in the absence or presence of TGF- β 1 for 48 hours using the TRI Reagent (Molecular Research Center, Cincinnati, OH, USA) according to the manufacturer's instructions. After validating the effect of TGF- β 1 on the induction of HTF gene expression using quantitative RT-PCR, RNA samples were applied to a miRNA array.²⁰ The total RNA pellet was dissolved in Nuclease-free water, and its quality and quantity was assessed using an bioanalyzer system (Agilent 2100; Agilent Technologies, Santa Clara, CA, USA). Gene expression was analyzed using a miRNA array (GeneChip Affymetrix miRNA3.0, miRBase Version 17; Affymetrix, Santa Clara, CA, USA), which comprised approximately 1,733 representative human mature miRNAs.

Microarray

DNA labeled at its 3' end with biotin was prepared according to the standard Affymetrix protocol from 1000 ng of total RNA (Expression Analysis Technical Manual, 2001, Affymetrix). The biotin-labeled DNA was hybridized for 16 to 18 hours at 48°C on the miRNA array (Affymetrix). The GeneChips were washed and stained on a fluidics station (Affymetrix Fluidics Station 450; Affymetrix). GeneChips were scanned using a commercial scanner (Affymetrix GeneChip Scanner 3000 7G; Affymetrix). The data were analyzed using robust multiarray average (RMA) and detection above background (DABG) analysis using the Affymetrix default analysis settings and global scaling as the normalization method. The normalized and log transformed intensity values were then analyzed using commercial software (Expression Console software v1.3; Affymetrix). Fold-change filters required that the miRNAs be present in at least a 2-fold and a 0.5-fold increase of the up- and downregulated miRNAs,

respectively. Analysis of the variation in miRNA expression was conducted for the predicted targets. Hierarchical clustering was used to cluster the data that showed similar behavior across experiments using commercial software (GeneSpring GX 12.6; Agilent Technologies). The clustering algorithm was Euclidean distance with average linkage.

Secondary miRNA Analysis

We analyzed the results of primary screening of miRNAs and selected secondary targeting miRNAs. The cut-off values for the secondary targeting miRNAs were 1) base log value of the signal of more than 7.0 and 2) log ratio between TGF- β 1 treated fibroblast and control is more than 1.0. Finally, two miRNAs, miRNA 143 and miRNA 145, were selected. Both miRNAs were upregulated in the primary screening test (Fig. 1). Total RNA was extracted from cultured HTFs, and cDNA synthesis was carried out using a first strand cDNA synthesis kit (GE Healthcare, IL, USA). Semiquantitative PCR and SYBR green-based real-time PCR (MyIQ, Thermo Fisher Scientific, Waltham, MA, USA) were used to determine the miRNA expression levels.

RNA Extraction and RT-PCR

Total RNA was extracted from cultured HTFs using TRI REAGENT and cDNA synthesis was carried out using a first strand synthesis system (SuperScript III First strand synthesis System Invitrogen, Carlsbad, CA, USA) and using a Mir-X miRNA first strand cDNA synthesis kit (Clontech, Mountain View, CA, USA). Semiquantitative PCR was performed using a SYBR green-based real-time PCR kit (Stratagene, Agilent Technologies). Control housekeeping genes were *Gapdh* (for mRNA) and U6 (for miRNA). The primers used for the different target mRNAs and miRNAs are listed in supplemental Table 1.

Western Blotting

Cells were rinsed with phosphate-buffered saline (PBS) twice, and protein was extracted in cold lysis buffer containing 1% protease inhibitor mixture (Sigma-Aldrich Corp., St. Louis, MO, USA).²⁰ The protein concentration was determined using a detergent-compatible protein assay (Bio-Rad, Hercules, CA, USA). Equal amounts of protein were resolved by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE), transferred onto nitrocellulose membranes, blocked with 5% nonfat milk for 1 hour, and then incubated with the indicated primary antibodies overnight at 4°C. After a 1-hour incubation with the appropriate secondary antibody, the specific signals were revealed using an enhanced chemiluminescence reagent (Pierce, Thermo Fisher Scientific).²⁰ The primary antibodies recognizing serum response factor (SRF) were purchased from Santa Cruz Biotechnology (Dallas, TX, USA).

Small Interfering RNA transfections

Small interfering RNAs (siRNAs) targeting *SRF* (439240) and *SMAD4* (4390824) were purchased from Ambion (Thermo Fisher Scientific). The ON-TARGET plusSMART pool siRNAs targeting human *SMAD2* (NM_005901) and *MYOCD* (myocardin; NM_153604) were from Dharmacon siRNA Technologies (Lafayette, CO, USA). A scrambled siRNA duplex was used as a negative control. A transfection reagent (Lipofectamine 2000; Invitrogen) was used to deliver the siRNAs into cells according to the manufacturer's instructions. Following overnight siRNA transfection, cells were refed with fresh growth medium for 24 hours before the indicated treatments.²⁰ RNA or protein was extracted at 48 to 72 hours after transfection; qPCR and

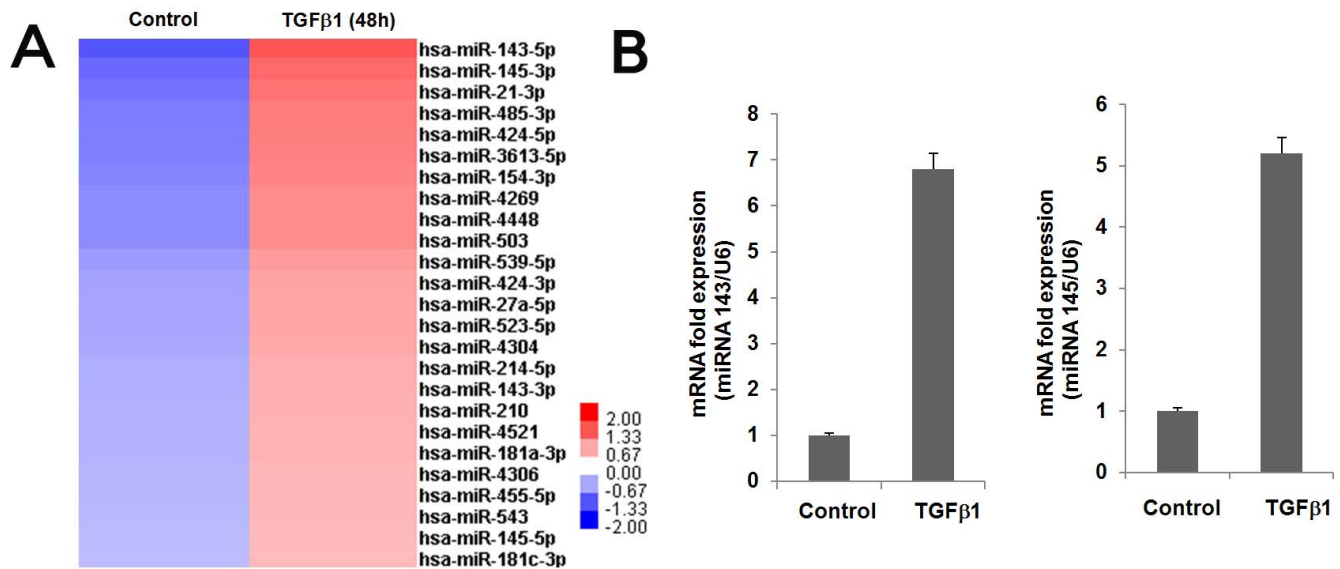


FIGURE 1. Microarray results of TGF- β 1-induced miRNA expression in HTF. (A) Array analysis revealed expression of various TGF- β 1-induced miRNAs in HTF. (B) Polymerase chain reaction analysis revealed that miRNAs 143 and 145 showed the strongest expression in HTF.

Western blotting were used to determine the knockdown efficiency.²⁰

Cloning and Mutagenesis of the miRNA 143 and 145 Enhancer

The enhancer element from the promoter of the gene encoding human miRNAs 143 and 145, encompassing a conserved CarG box (CC[A/T]₆GG) and a putative SMAD binding element (SBE), was PCR-amplified from genomic DNA-derived from human coronary artery smooth muscle cells using a high-fidelity polymerase (Roche Applied Science, Basel, Switzerland). The primers used were human-miRNA 143/145 enhancer, forward 5'-GATACAGATCTAGAGGGAAAGGGCTTGTTAGGAT-3' and reverse 5'-GATACAGATCTAAAGGTGATGTTGAACCAGGTGGC-3'; human-miRNA 143/145 enhancer CarG Mut, forward 5'-CAGAGCAGCCTGGGTCTATAAGGAAGGAGCTCAGG-3' and reverse 5'-CCTGAGCTCCTTCCCTTATAGACCCAGGCTGCTCTG-3'; human-miRNA 143/145 enhancer SBE Mut, forward 5'-CTGGCGAGAAAACCTACATAGCCACTGTT-3' and reverse 5'-AACAGTGGCTATGTAGGTTTTCTCGCCAG-3'. The enhancer was then cloned into the BglII site of the pGL3 luciferase reporter containing a minimal thymidine kinase promoter (Promega Corp., Madison, WI, USA). Point mutations of the CarG and SBE sites were made using the mutagenesis kit (QuikChange; Stratagene, La Jolla, CA, USA).²⁰

Transfection and Luciferase Assays

Lipofectamine 2000 was used to transfect the reporters into HTFs. Cells were seeded in 24-well plates and grown until 90% confluence. Transfections were performed according to the manufacturer's instructions. Six hours after transfection, cells were refed with 10% FBS for 24 hours, and serum starvation lasted for 24 hours, following which, HTF cells were treated with TGF- β 1 (10 ng/mL) for 24 hours. To confirm the absence of contamination, or any unexpected effects of TGF- β 1 on cell proliferation, a *Renilla* reporter gene (Promega Corp.) was included as an internal control. Cell lysates were prepared for the luciferase assay according to the manufacturer's instructions (Promega Corp.). All transfections were performed in

quadruplicate and repeated in at least three independent experiments.²⁰ Data were analyzed using graphing software (GraphPad Prism Software version 4.0; GraphPad Software, La Jolla, CA, USA) and expressed as the normalized fold-increase over controls \pm the standard deviation (SD).²⁰

MiRNA overexpression and Knockdown Studies

Growing HTFs were transfected with 30 nM of precursor miRNA mimics or antisense miRNA inhibitors (also known as anti-miRNAs) using siPORT NeoFX (Applied Biosystems, Foster City, CA, USA), following the manufacturer's instructions.²⁰ RNA or protein was harvested 72 hours after transfection. Expression levels of miRNAs and α -smooth muscle actin (SMA), a biomarker of myofibroblast formation, were quantified using semiquantitative PCR (Stratagene, Agilent Technologies).

RESULTS

TGF- β 1-Induced the Expression of miRNAs 143 and 145 in HTFs

Analysis of the miRNA array revealed that TGF- β 1 induced the expression of various miRNAs in HTFs. Among them, miRNA 143 and 145 showed the strongest expression levels (Fig. 1). Time- and dose-dependent upregulation of miRNAs 143 and 145 by TGF- β 1 treatment was validated using PCR. These results suggested that miRNAs 143 and 145 play a role in TGF- β 1-induced subconjunctival fibrosis.

SRF and MYOCD Contribute to TGF- β 1-Induced Expression of miRNAs 143 and 145

It has been reported that the promoter region of miRNA 143 and 145 contains a conserved CarG box that is responsive to both SRF and its coactivator MYOCD.^{20,21} To determine the induction mechanism of miRNAs 143 and 145, SRF and MYOCD expression was assessed. After treatment with TGF- β 1, both SRF and MYOCD expression increased in a time-dependent manner. Next, siRNAs were used to knock down the expression of SRF and MYOCD, which induced a decrease

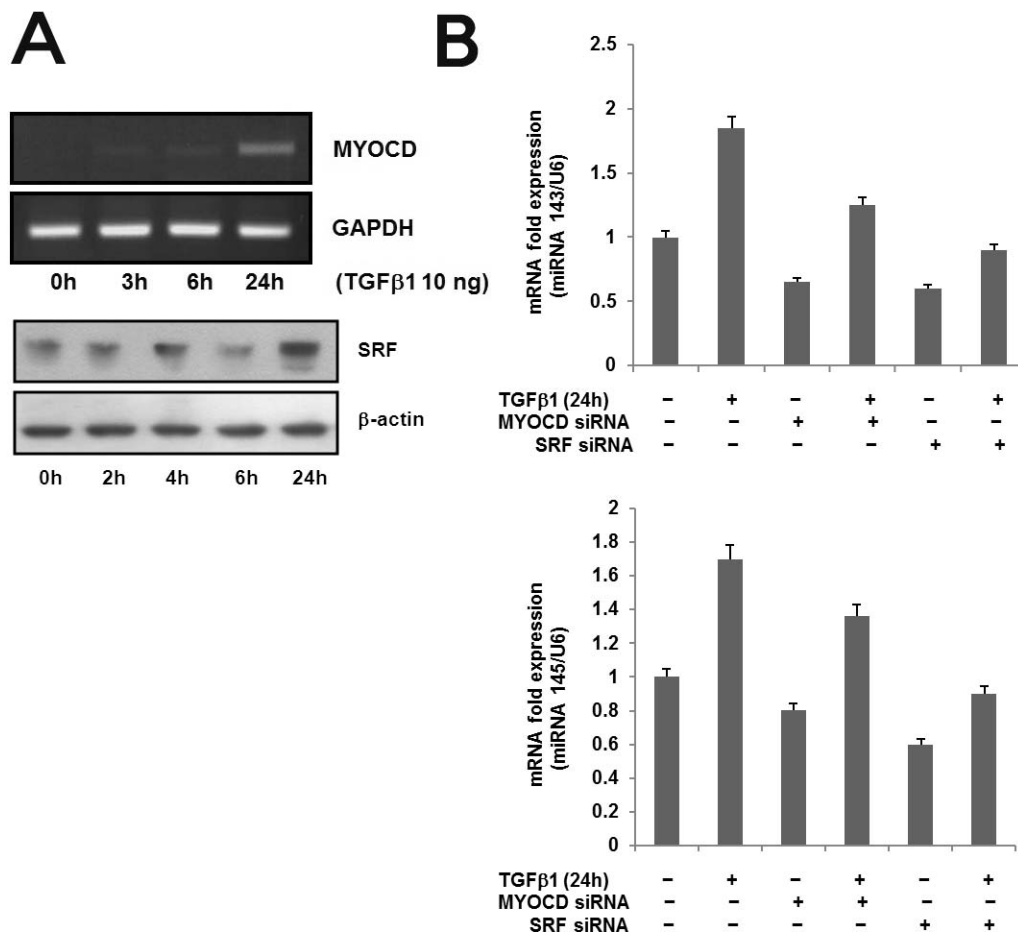


FIGURE 2. TGF- β 1-induced MYOCD and SRF expression in HTF. (A) Polymerase chain reaction and Western blot revealed TGF- β 1-induced MYOCD and SRF expression in HTF in a time-dependent manner. (B) When siRNAs to MYOCD and SRF were treated, TGF- β 1-induced miRNA 143/145 expression was decreased.

in the TGF- β 1-induced expression of miRNAs 143 and 145 (mean \pm SD fold expression of 0.90 ± 0.04 to 1.36 ± 0.03 with siRNAs compared to 1.70 ± 0.04 to 1.85 ± 0.04 without siRNAs, Fig. 2). These results suggested that SRF and MYOCD contribute to the TGF- β 1-induced expression of miRNAs 143 and 145.

SMAD-Independent Pathways Play a Key Role in TGF- β 1-Induced Expression of miRNAs 143 and 145

Previous studies reported that SMAD-independent pathways, including p38MAPK, JNK, ERK, and PI3K/Akt pathways, transduce TGF- β 1 signals.^{7,8} To investigate the role of these pathways in the TGF- β 1-induced expression of miRNAs 143 and 145 in HTFs, the effects of SB203580 (inhibitor of p38MAPK), SP600125 (inhibitor of JNK), U0126 (inhibitor of ERK1/2), LY294002 (inhibitor of PI3K), and SH5 (inhibitor of Akt) on the expression of miRNAs 143 and 145 were analyzed. Based on the inhibitor study, all these inhibitors blocked the TGF- β 1-induced expression of miRNAs 143 and 145 (mean \pm SD fold expression of 0.70 ± 0.04 to 6.20 ± 0.05 with inhibitors compared to 1.60 ± 0.04 to 9.00 ± 0.05 without inhibitors, Fig. 3). Additionally, SB203580 and SH5 also blocked the expression SRF and MYOCD. These findings suggested that SMAD-independent pathways, including the p38MAPK, JNK, ERK, and PI3K/Akt pathways, play a role in the TGF- β 1-induced expression of miRNAs 143 and 145 in HTFs.

TGF- β 1-Induced Expression of miRNAs 143 and 145 Is SMAD Pathway-Independent

In addition to SMAD-independent pathways, TGF- β 1-induced myofibroblast transdifferentiation is also mediated by SMAD-dependent pathways.^{7,8} Thus, the effect of SMAD2 and SMAD4 on the TGF- β 1-induced expression of miRNA 143 and 145 in HTFs was assessed. When siRNAs targeting SMAD2 and SMAD4 were applied, the TGF- β 1-induced expression of miRNAs 143 and 145 was not significantly affected (mean \pm SD fold expression of 1.18 ± 0.04 to 4.28 ± 0.05 with siRNAs compared to 1.90 ± 0.04 to 3.22 ± 0.05 without siRNAs, Fig. 3). This finding suggested that the SMAD-dependent pathways are not the principal pathways mediating TGF- β 1-induced expression of miRNAs 143 and 145 in HTFs.

TGF- β 1 Targets an Upstream Enhance of miRNAs 143 and 145

To evaluate the role of the CarG box and SBE in the TGF- β 1-induced expression of miRNAs 143 and 145, each site was mutated either separately or simultaneously. Mutation of either the CarG box or SBE induced an attenuated response to TGF- β 1 and the CarG/SBE double mutation showed greater attenuation of TGF- β 1 stimulation (Fig. 4). This result suggested that TGF- β 1 activates the expression of miRNAs 143 and 145 via the upstream enhancer region containing the CarG box and SBE.

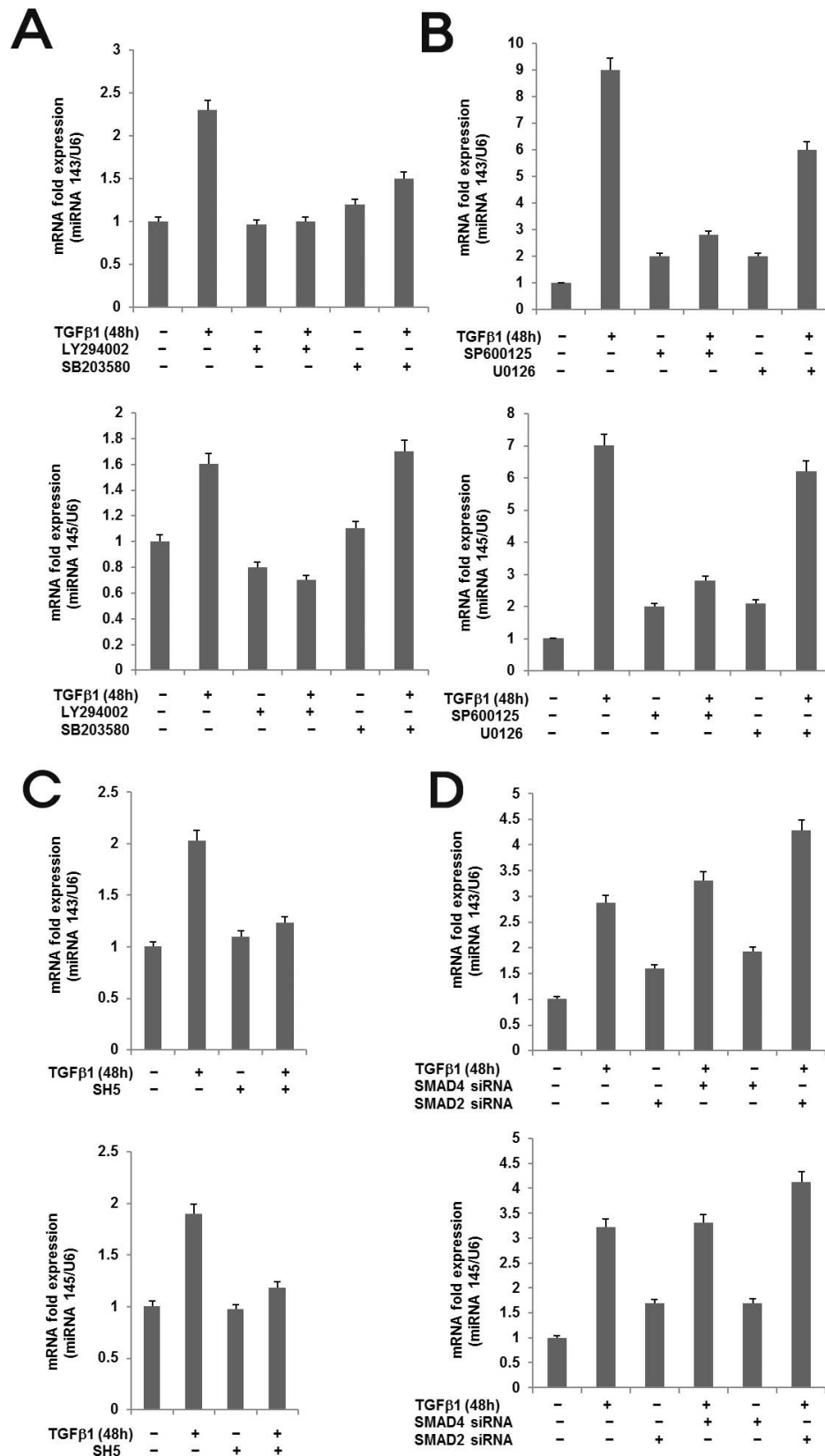


FIGURE 3. Role of p38MAPK, JNK, ERK1/2, PI3K, Akt, SMAD2, and SMAD4 in TGF-β1-induced miRNA expression in human Tenon's capsule fibroblast. (A-C) Inhibitors of p38MAPK (SB203580), JNK (SP600125), ERK1/2 (U0126), PI3K (LY294002), and Akt (SH5) revealed decreased expression of TGF-β1-induced miRNA 143/145. (D) However, treatment of siRNAs to SMAD2 and SMAD4 did not affect TGF-β1-induced miRNA 143/145 expression.

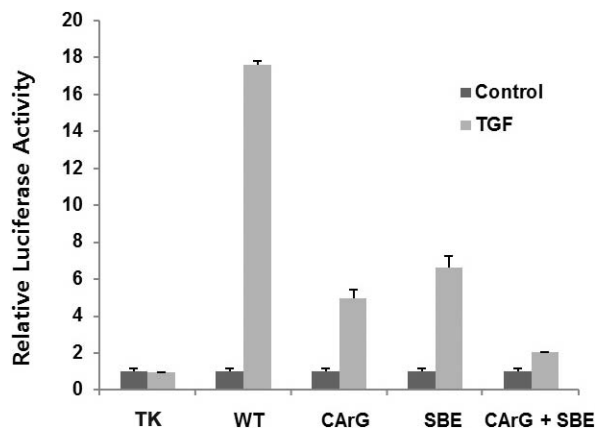


FIGURE 4. Luciferase assays of CARg and SMAD binding element (SBE) mutations. Mutation of CARg and SBE revealed attenuated response to transforming growth factor (TGF)- β 1-induced miRNA 143/145 expression compared to the vector (TK) and wild type (WT). CARg and SBE double mutation showed greater attenuation.

MiRNA 143 and 145 Contribute to TGF- β 1-Induced Myofibroblast Transdifferentiation

To investigate the role of miRNAs 143 and 145 in TGF- β 1-induced myofibroblast transdifferentiation, miRNA mimics and miRNA inhibitors were applied to HTFs. After transfection,

mimics of miRNAs 143 and 145 induced increased expression of α -SMA (mean \pm SD fold expression of 8.32 ± 0.05 for miRNA 143 mimic and 45.00 ± 0.52 for miRNA 145 mimic), and the inhibitors of miRNAs 143 and 145 had the opposite effect (mean \pm SD fold expression of 0.44 ± 0.02 for miRNA 143 inhibitor and 0.44 ± 0.02 for miRNA 145 inhibitor, Fig. 5). These results implied that activation or inhibition of miRNAs 143 and 145 would affect TGF- β 1-induced myofibroblast transdifferentiation.

DISCUSSION

In the present study, we observed that TGF- β 1 induced the expression of miRNAs 143 and 145 in HTFs, mainly via SMAD-independent pathways. Mimics of miRNAs 143 and 145 induced increased myofibroblast formation, whereas inhibitors of miRNAs 143 and 145 led to decreased myofibroblast formation. These findings suggest that TGF- β 1-induced expression of miRNAs 143 and 145 plays an important role in subconjunctival fibrosis. To the best of our knowledge, this is the first study to report the roles and pathways associated with miRNA 143 and 145 expression in human subconjunctival fibrosis.

Previous studies investigated the expression of several miRNAs in HTFs. For instance, Li et al.¹² evaluated the expression of miRNAs in TGF- β 1-stimulated HTFs and found that miRNA-29b suppressed type I collagen expression via the PI3K/Akt/Sp1 pathway. In a subsequent study, they reported

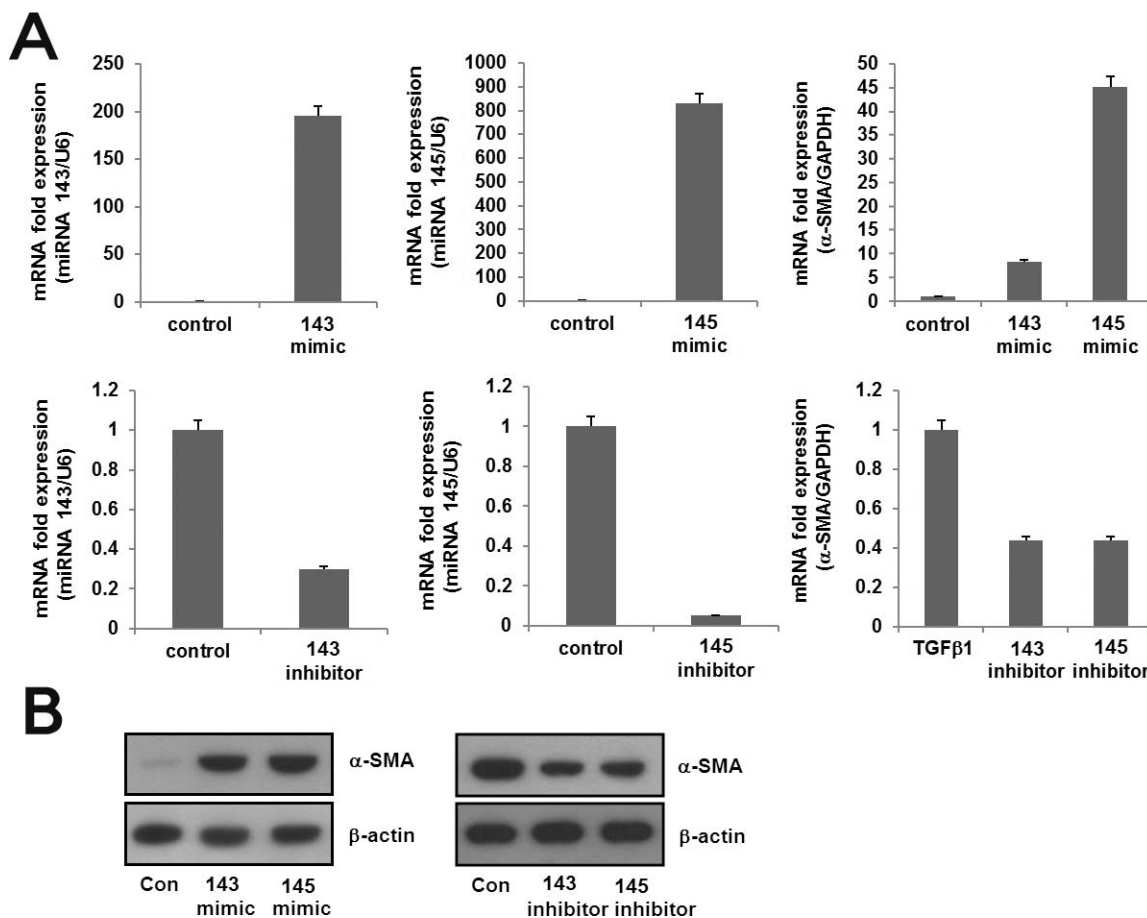


FIGURE 5. Role of miRNA 143/145 in TGF- β 1-induced myofibroblast transdifferentiation. (A) Polymerase chain reaction analysis showed increased or decreased expression of miRNA 143/145 after transfection of miRNA 143/145 mimics and inhibitors, respectively. (B) miRNA 143/145 mimics and inhibitors induced increased and decreased expression of α -smooth muscle actin (SMA), respectively.

that subconjunctival injection of lentivirus-mediated miRNA-29b inhibited the proliferation of fibroblasts and reduced collagen deposition in rabbit eyes after trabeculectomy.¹³ Ran et al.¹⁴ also reported that TGF- β 2 stimulates HTF proliferation in patients with glaucoma via suppression of miRNA-29b expression. Tong et al.¹⁵ investigated HTF proliferation stimulated by TGF- β 1 and reported that miRNA-200b stimulated the proliferation of HTFs by targeting p27/kip1 (also known as cyclin dependent kinase inhibitor 1B) and RND3 (Rho family GTPase 3). Xu et al.¹⁶ reported that the miRNA-216b/Beclin 1 axis regulates autophagy and apoptosis in HTFs upon hydroxytamoxifen exposure. These findings implied that various miRNAs might contribute to the regulation of HTF proliferation and differentiation.

MiRNAs 143 and 145 were reported to regulate the contractile phenotype of vascular smooth muscle cells.²⁰⁻²² For instance, miRNAs 143 and 145 regulate the proliferation and differentiation of vascular smooth muscle cells and act as signaling molecules for the communication between vascular endothelial cells and smooth muscle cells.²² Thus, miRNAs 143 and 145 are considered as key mediators in various cardiovascular diseases, including ischemic heart disease, hypertension, and atherosclerosis.²² The results of our array analysis revealed expression levels of various TGF- β 1-induced miRNAs in HTFs. Among them, miRNAs 143 and 145 showed the strongest expression. These findings suggested that miRNAs 143 and 145 would be key factors and therapeutic targets in subconjunctival fibrosis, in addition to cardiovascular diseases.

To investigate the pathways associated with the expression of miRNAs 143 and 145 in HTFs, chemical inhibitors and siRNAs were applied, and mutagenesis studies were performed. The binding of SRF and its coactivator MYOCD to the CARG box activates gene transcription and this phenomenon is considered a master switch for the expression of contractile and cytoskeletal genes.²³ In the present study, TGF- β 1 induced SRF and MYOCD expression, and knockdown of SRF and MYOCD using siRNAs reduced the expression levels of miRNAs 143 and 145. In the mutagenesis study, mutation of the CARG box induced decreased expression of miRNAs 143 and 145. Previous studies also reported that SRF and MYOCD regulated the expression of miRNAs 143 and 145 in gastrointestinal and vascular smooth muscle cells.²⁰⁻²⁴ These results confirmed the role of SRF and MYOCD binding to the CARG box in the expression of miRNAs 143 and 145 in various types of cells.

When MAPK pathways, including p38MAPK, JNK, and ERK pathways, were inhibited, the expression levels of miRNAs 143 and 145 in HTFs decreased. Long and Miano²⁰ also reported that the p38MAPK-SRF-MYOCD axis is essential for the TGF- β 1-induced expression of miRNAs 143 and 145 in human coronary artery smooth muscle cells. In addition, inhibition of the PI3K/Akt pathway reduced the expression levels of miRNAs 143 and 145 in HTFs. The PI3K/Akt pathway has been reported to play a diverse role in fibroblasts.^{12,13,17-19} For instance, in our previous studies, TGF- β 2 induced human subconjunctival fibrosis by activation of the PI3K/Akt pathway¹⁷ and lithium chloride inhibited TGF- β 1-induced myofibroblast transdifferentiation via the PI3K/Akt pathway in HTFs.¹⁹ Li et al.¹² and Yu et al.¹³ also reported that miRNA-29b suppressed type I collagen expression via the PI3K/Akt pathway. These results implied the essential roles of SMAD-independent pathways in the TGF- β 1-induced expression of miRNAs 143 and 145 in HTFs.

Long and Miano²⁰ reported that in human coronary artery smooth cells, both SMAD-dependent and SMAD-independent (p38MAPK) pathways were involved in TGF- β 1-induced expression of miRNAs 143 and 145. In the human prostate, binding of SMAD2 and SMAD3 to SBE induced the expression

of miRNAs 143 and 145.²⁵ Additionally, miRNA 143 expression in human gastric fibroblasts is SMAD dependent.²⁶ In contrast, the results of the present study revealed that inhibition of SMAD2 and SMAD4 using siRNAs did not inhibit the expression of miRNAs 143 and 145 in HTFs, which suggested that SMAD-dependent pathways may not play a key role in the TGF- β 1-induced expression of miRNAs 143 and 145. This discrepancy may be explained by different signal pathways that are active among different types of cells.

In the present study, transfection of inhibitors of miRNAs 143 and 145 induced inhibition of myofibroblast formation in HTFs. Therefore, theoretically, inhibition of TGF- β 1-induced expression of miRNAs 143 and 145 by blocking MAPK (p38MAPK, JNK, ERK) or PI3K/Akt pathways, mutagenesis of miRNA promoters (CARG box, SBE), or direct administration of inhibitors of miRNAs 143 and 145 might provide a novel miRNA-based treatment strategy to prevent excessive subconjunctival fibrosis. A recent study revealed that the application of a p38MAPK inhibitor to HTFs inhibited their proliferation *in vitro*.²⁷ Furthermore, injection of a p38MAPK inhibitor into subconjunctival tissue of rabbit eyes after glaucoma surgery significantly improved the surgical outcomes.²⁷ These results implied that inhibition of p38MAPK might be a promising strategy to reduce fibrosis. Therefore, further studies regarding the effect of inhibiting the expression of miRNAs 143 and 145 expression on subconjunctival fibrosis *in vivo* are required.

Currently, various agents, including mitomycin-C (MMC), 5-fluorouracil (5-FU), or anti-VEGF therapy have been used during or after glaucoma surgery to prevent excessive subconjunctival fibrosis.^{5,6,28,29} MMC and 5-FU are strong antifibrotic agents that reduce proliferation of HTFs. However, because of their nonspecific mechanism of action and induction of widespread cell death and apoptosis, MMC and 5-FU application can lead to complications, such as corneal and conjunctival toxicity, hypotony maculopathy, wound leakage, and infection.^{5,6} Recently, anti-VEGF therapy was introduced to reduce subconjunctival fibrosis after glaucoma surgery.^{28,29} However, to date, its effect remains controversial. A possible advantage of a miRNA-based treatment strategy over MMC, 5-FU, or anti-VEGF application is that inhibition of miRNAs may specifically affect the targeted cells, whereas the application of MMC, 5-FU, or anti-VEGF affects various cell types. Therefore, miRNA-based treatment may induce fewer complications compared with MMC, 5-FU, or anti-VEGF. Further studies comparing the anti-fibrotic effect of inhibiting miRNAs 143 and 145 inhibition with the effects of MMC, 5-FU, or anti-VEGF are required.

In conclusion, TGF- β 1-induced human subconjunctival fibrosis is mediated by the expression of miRNAs 143 and 145, mainly via SMAD-independent pathways. Inhibition of TGF- β 1-induced expression of miRNAs 143 and 145 in HTFs might represent a novel strategy to prevent subconjunctival fibrosis.

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