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Original Article

TGF-β_2 stimulates Tenon's capsule fibroblast proliferation in patients with glaucoma via suppression of miR-29b expression regulated by Nrf2

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Abstract: Purpose: To investigate the role of transforming growth factor-β_2 (TGF-β_2) in Tenon's capsule fibroblasts proliferation from glaucoma patients and the effect of nuclear factor (erythroid-derived 2)-like 2 (Nrf2) and miR-29b mRNA in this process. Methods: Tenon's capsule fibroblasts obtained from patients who had undergone selective glaucoma surgery (GTFs) were cultured and stimulated with 5 ng/mL TGF-β_2 for 1, 3, 5, and 7 days. MTS assay was performed to detect the cell viability. Expression of Nrf2 and miR-29b was analyzed with western blot, RT-PCR and Chromatin immunoprecipitation assay (ChIP) in human fibroblast SX1412-B exposed to TGF-β_2. Results: MTS assay showed that TGF-β_2 was more stimulatory on GTFs proliferation than controls. At the same time, TGF-β_2 exerted an intenser effect of decreasing the Nrf2 protein and miR-29b mRNA levels in GTFs, and the level of miR-29b was effectively regulated by Ad-Nrf2. In addition, ChIP assay suggested that TGF-β_2 down-regulated miR-29b expression through repressing the binding of Nrf2 to the promoter of miR-29b. Finally, we found that overexpression Nrf2 in GTFs reduced the proliferation effect on GTFs induced by TGF-β_2, while miR-29b inhibitor reversed this effect. Conclusion: This study suggests that TGF-β_2 has a time-effect relationship with Tenon's capsule fibroblasts proliferation from glaucoma patients, and it stimulates Tenon's capsule fibroblast proliferation via suppression of miR-29b expression regulated by Nrf2.

Keywords: Transforming growth factor-β_2, miR-29b, glaucoma, Tenon's capsule fibroblast, proliferation, Nrf2

Introduction

Glaucoma is a primary cause of irreversible blindness all over the world, characterized by irreversible apoptosis of retinal ganglion cells (RGCs) and cupping of the optic disk [1, 2]. To date, the most effective treatment available for glaucoma appears to be glaucoma filtering surgery; however, excessive scarring of the filtering bleb is the most important cause of surgical failure [3]. Some activities of human Tenon's capsule fibroblasts are implicated in the complex wound healing processes, containing proliferation, migration, synthesis of extracellular matrix components (ECM), and collagen contraction. Moreover, Tenon's capsule fibroblasts may be activated by cytokines and growth factors and believed to be the key cells involved in the sub-conjunctival wound healing response. The cytokine transforming growth factor-β (TGF-β) has been recognized as a pivotal mediator in conjunctival scarring, wound healing and extracellular matrix (ECM) synthesis [4, 5]. Among all three identified TGF-β isoforms, TGF-β_2 is the cardinal isoform involved in ocular scarring diseases such as conjunctival scarring and fibrosis [6, 7]. Animal models of conjunctival scarring and other human Tenon's capsule fibroblasts related diseases have confirmed that TGF-β can stimulate the activities of human Tenon's capsule fibroblasts. However, the physiological and pathological contexts involved in Tenon's capsule fibroblast proliferation in patients with glaucoma remains to be determined.

It has been proposed that several common mechanisms were involved in triggering RGC death in glaucoma, including compromised blood flow at the optic nerve, glutamate excitotoxicity and oxidative stress [8-10]. Among these primary mechanisms, oxidative stress is

thought to be an important mechanism of cell death in open-angle glaucoma (POAG) [11]. However, the precise nature of glaucoma damage caused by oxidative stress remains unclear. Nuclear factor (erythroid-derived 2)-like 2 (Nrf2) is a key transcription factor of antioxidant and Phase II detoxification genes, activated by oxidative stress and the presence of electrophiles [12]. Emerging recognition suggests that Nrf2 may protect cells from oxidative stress via the over-production of antioxidants and detoxifiation proteins [13, 14]. Despite the Nrf2 signaling pathway has been well researched, less is known about the possibility of microRNAs (miRNAs) transcript regulation by Nrf2.

As an abundant class of noncoding small (~22 nucleotides) RNAs, miRNAs are under the transcriptional control of transcription factors and function as modulating gene expression at the post-transcriptional level [15, 16]. Specifically, miR-29b has been demonstrated to regulate multiple genes coding for ECM proteins, including multiple collagens, fibrillins, and elastin. Recent studies have suggested that miR-29 family served as an important mediator in the development of tissue fibrosis [17, 18]. Furthermore, conformational studies revealed that only miR-29b exhibited a significant change in expression in response to Nrf2 activation in the lymphoblast cell line [19]. MiR-29b is also a positive regulator of osteoblast differentiation and controls the expression of collagens in differentiated osteoblasts [17].

To gain more insight into the potential role of Nrf2 regulation miR-29b in TGF-β_2 function, we investigated the effects of TGF-β_2 on Tenon's capsule fibroblast proliferation from patients with a diagnosis of glaucoma (GTFs) or patients who had undergone cataract surgery (HTFs), analyzed the changes in miR-29b expression mediated by Nrf2, and evaluated whether alterations in miR-29b expression might alter the effects induced by TGF-β_2 in Tenon's capsule fibroblast proliferation.

Materials and methods

Primary tissue procurement and culture

The research procedures followed the rules of the Declaration of Helsinki. Informed consent was received from the patients. Biopsies of human Tenon's capsules were obtained from patients who had undergone glaucoma surgery (GTFs) and patients (n = 10; without any topical eye treatment; HTFs) who had undergone trauma or cataract surgery. Patients' age ranged from 20 to 58 years with a mean age of 34.21 years. To establish explanted cultures, the biopsies were dissected into 3×3 mm sections and placed in 35 mm^2 culture dishes (Falcon, Becton-Dickinson) in Dulbecco's modified Eagle's medium (DMEM; Gibco) containing 10% (v/v) fetal calf serum (FBS). Migrated the cells from the explanted tissue and they were then incubated at 37°C in a humidified 95% air/5% CO_2 atmosphere. Cells between the third and sixth passages were used in this study. Cultures were allowed to reach about 80% confluence. After 24 h of serum starvation in serum-free DMEM, the cells were incubated in serum-free DMEM with or without human recombinant TGF-β_2 (R&D Systems) for 1, 3, 5, and 7 days. A final concentration of TGF-β_2 was 5.0 ng/ml.

MTS assay

GTFs and HTFs were plated in 96-well plates (Falcon) at a density of 4.0×10^4 cells/well and stimulated with 5 ng/mL of TGF-β_2. MTS assay was performed by adding 20 mL of Cell-Titer 96 Aqueous One Solution Reagent (Promega Corporation) per well with 100 mL serum-free DMEM, and then incubated at 37°C in humidified 95% air/5% CO_2 for 4 h. The optical density of the fluid in each well was read at 490 nm in spectrophotometer (Titertek Multiscan, Flow Lab). Wells containing basic medium served as controls.

Transfection

The design and construction of Ad-Nrf2 and Ad-GFP were completed by Shanghai Genechem Co., Ltd, China. Nrf2 levels of cells were over-expressed or knocked down by transfected with Ad-Nrf2 or Ad-GFP, and miR-29b of cells were over-expressed or knocked down by transfected with miR-29b mimic or miR-29b inhibitor. Cells were transfected with the final concentration of 100 nM miR-27a mimic or miR-27a inhibitor or negative control miRNA using Lipofectamine 2000 reagent (Invitrogen, USA) according to the manufacturer's instructions. miR-29b, miR-29b inhibitor and negative control miRNA were produced by Ribobio Co., Ltd. (RIBOBIO, China).

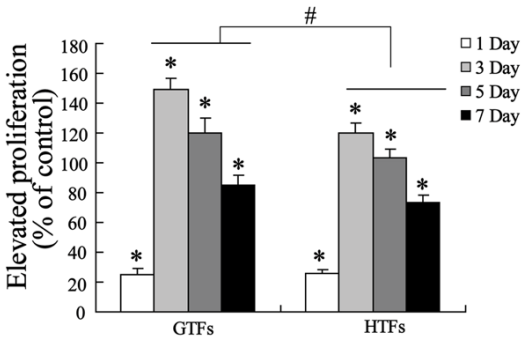

Figure 1. GTF (A) and HTF (B) proliferation was examined using MTS assay. Results were mean ± SD of 3 independent experiments. The value on day 0 served as the control of relative elevated proliferation rate, *VS day 0, #GTFs VS HTFs, P<0.05.

RNA isolation and quantitative real-time PCR

Quantitative real-time reverse transcription-PCR (RT-PCR) was performed to examine the expression of miR-29b mRNA. In brief, GTFs/HTFs exposed to TGF-β_2 and human fibroblast SX1412-B were plated in 60 mm^2 dishes (Falcon) at a density of 2×10^4 cells/dish and stimulated as earlier. Total RNA was extracted from fibroblasts using the Trizol RNA extraction reagent (Invitrogen Corp.) according to the manufacturer's specifications. Purified RNA was reverse-transcribed using a SYBR Prime ScriptTM RT-PCR Kit (TaKaRa Corp.). Real-time quantification of miR-29b mRNA was performed on an ABI PRISM 7000 Sequence Detection System using SYBR Green I as the reporter dye (TaKaRa Corp.). The comparative Ct method was employed, and the relative quantity of miR-29b mRNA was expressed as fold change = $2^{-\Delta\Delta Ct}$.

Western blot analysis

Whole cellular proteins were isolated from primary cultured GTFs and HTFs. Briefly, lysis buffer containing 1.6% Triton X-100, 5 M urea, 0.1 mM leupeptin, and 1.5 mM phenylmethylsulfonyl fluoride was used to obtain total cell lysates. After centrifugation at 12,000 g for 30 min, the supernatant was removed and stored at -80°C. Protein concentrations were measured using a BCA assay kit according to the manufacturer's instructions (Shenergy Biocolor BioScience and Technology Company). Equal amounts of protein (15 mg) were subjected to 8% sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to PVDF membranes, which were blocked in 5% skim milk/PBS + 0.05% Tween-20. The membranes were probed with primary antibodies against Nrf2 (1:1,000 dilution; Biovision) and β-actin (1:1,000 dilution; Cell Signaling technology) following the ECL-Western blot protocol (Amersham Life Sciences), and the secondary antibodies were HRP-conjugated goat anti-rabbit (1:1,000 dilution; Cell Signaling Technology). The enhanced chemiluminescence (ECL) detection method was used, and blots were exposed to film for 30 s. Signals were quantified using the Image Quant Image Analysis Software (Amersham Biosciences).

Cell cultures

The human fibroblast SX1412-B (ATCC, USA) was cultured in low glucose Dulbecco's Modified Eagle Medium with 10% fetal bovine serum, 100 μM nonessential amino acids, 100 U/ml penicillin, 100 μg/ml streptomycin sulfate, and 0.25 μg/ml amphotericin B maintained at 37°C in 5% CO_2. All the reagents were obtained from Invitrogen Corporation (Carlsbad, CA).

Chromatin immunoprecipitation assay

The chromatin immunoprecipitation assay (ChIP) assay was performed as described previously. Briefly, soluble chromatin from human fibroblast SX1412-B exposed to TGF-β_2 was incubated with anti-Nrf2 antibody (sc-722) and normal rabbit immunoglobulin G (IgG). Immunoprecipitated DNAs with protein A/G-agarose were purified and dissolved in 20 L of distilled H_2O. Each 2 L of DNA was used for PCR analysis. The PCR products were separated by electrophoresis on 2% agarose gels and stained with ethidium bromide.

Statistical analysis

All data were expressed as mean ± standard deviation (SD). Data from Western blots, ChIP assay, and real-time PCR studies were analyzed using a one-way analysis of variance (ANOVA) followed by *t* test. Statistical analysis was performed in SPSS 16.0 (SPSS, Chicago, IL). *P*-values less than 0.05 ($P<0.05$) was considered significant.

Results

Effect of TGF-β_2 on fibroblasts proliferation

To detect the effect of TGF-β_2 on fibroblasts proliferation, cell viability of GTFs/HTFs were

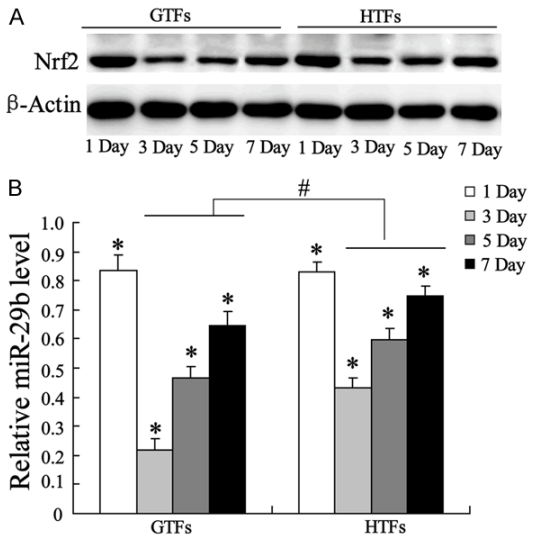

Figure 2. Nrf2 protein expression (A) and miR-29b mRNA (B) in the activated GTFs and HTFs after exposure to TGF-β_2 were analyzed by western blot method and real-time PCR, respectively. Results were mean ± SD of 3 independent experiments. The value on day 0 was as the control, *VS day 0, #GTFs VS HTFs, $P<0.05$.

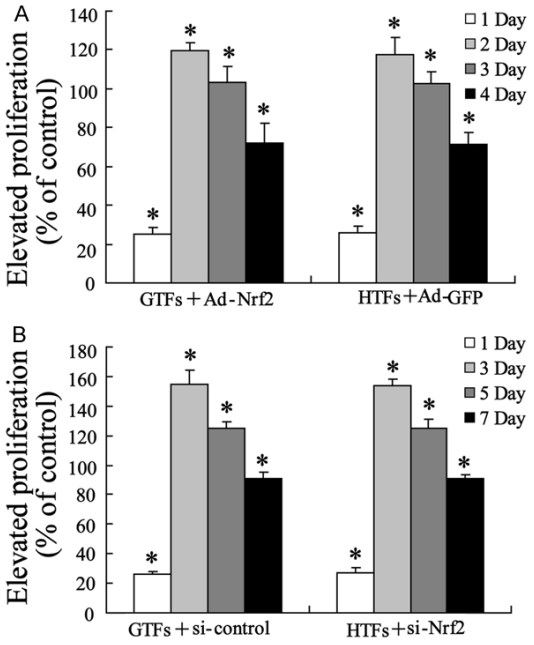

Figure 3. Effect of overexpression Nrf2 in GTFs (A) and knockdown Nrf2 expression in HTFs (B) on the proliferation of fibroblasts. Results were mean ± SD of 3 independent experiments. The value on day 0 was as the control of relative elevated proliferation rate, *VS day 0, $P<0.05$.

analyzed with MTS assay. As demonstrated in **Figure 1**, at the concentration of 5.0 ng/mL, TGF-β_2 significantly stimulated GTFs/HTFs proliferation compared with their own control after days 1, 3, 5, and 7 ($P<0.05$). In addition, the cell proliferation of GTFs/HTFs exposed to TGF-β_2 presented in a time-dependent manner. Comparing with HTFs proliferation, TGF-β_2 appeared to be more stimulatory on GTFs proliferation, and the peak proliferation rate was 148% on day 3 after treatment in GTFs.

Expression of Nrf2 and miR-29b after exposure to TGF-β_2 in fibroblasts

It has been shown that TGF-β_2 treatment significantly reduced miR-29b expression in trabecular meshwork cells, and Nrf2 can regulate miR-29b expression [20, 21]. We therefore examined whether TGF-β_2 can decrease nuclear Nrf2 expression and its target gene miR-29b in GTFs. Then western blot and real-time PCR were performed. The results summarized in **Figure 2**. As we could see, Nrf2 protein (**Figure 2A**) and miR-29b mRNA expression (**Figure 2B**) were showed time-dependent down-regulation in GTFs/HTFs. Opposite to the corresponding rates of cell proliferation, the Nrf2 protein and miR-29b mRNA levels reached their minima at 5 ng/mL TGF-β_2 after 3 days of culture in GTFs/HTFs and then enhanced. Obviously, TGF-β_2 exerted an intenser effect of decreasing the Nrf2 protein and miR-29b mRNA levels on GTFs than that on HTFs.

Effects of Nrf2 expression on proliferation of fibroblasts

To determine the effect of Nrf2 expression on GTFs/HTFs proliferation, GTFs were transfected Ad-Nrf2 and its control Ad-GFP. As shown in **Figure 3A**, similar to the above results, TGF-β_2 still significantly stimulated GTFs/HTFs proliferation. Notablely, there was no difference in proliferation of GTFs and HTFs after exposure to TGF-β_2. Additionally, we detected the effect of knockdown Nrf2 expression through transfection si-Nrf2 into HTFs and si-control into GTFs. We could see that elevation proliferation in HTFs after exposure to TGF-β_2 was also the same as in GTFs (**Figure 3B**).

Nrf2 regulated the expression of miR-29b in human fibroblast SX1412-B

To investigate the effect of Nrf2 on miR-29b expression in human fibroblast SX1412-B,

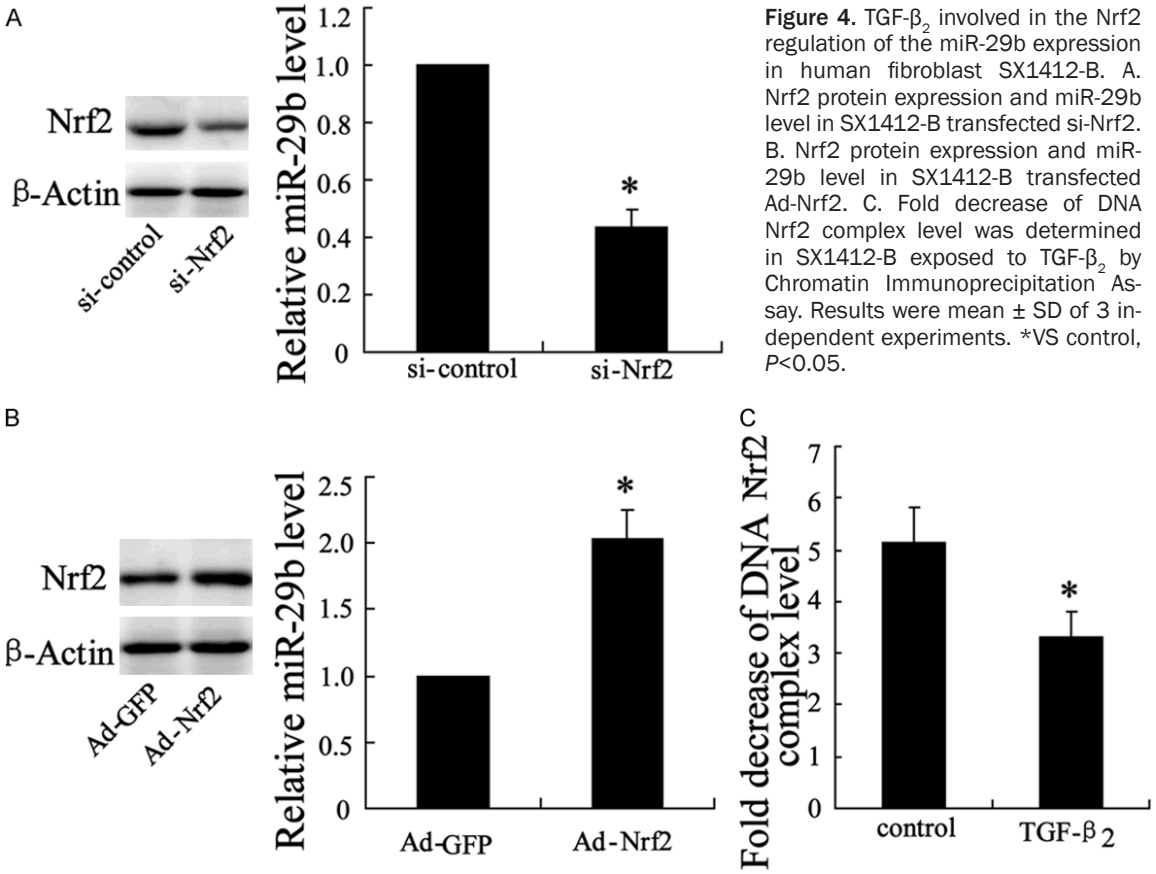

Figure 4. TGF-β_2 involved in the Nrf2 regulation of the miR-29b expression in human fibroblast SX1412-B. A. Nrf2 protein expression and miR-29b level in SX1412-B transfected si-Nrf2. B. Nrf2 protein expression and miR-29b level in SX1412-B transfected Ad-Nrf2. C. Fold decrease of DNA Nrf2 complex level was determined in SX1412-B exposed to TGF-β_2 by Chromatin Immunoprecipitation Assay. Results were mean ± SD of 3 independent experiments. *VS control, $P<0.05$.

SX1412-B were transfected with si-Nrf2 or Ad-Nrf2 to down-regulate or over-express the expression of Nrf2. As shown in **Figure 4**, the level of miR-29b was effectively regulated by si-Nrf2 or Ad-Nrf2. Down-regulated Nrf2 decreased the quantity of miR-29b (**Figure 4A**), and the level of miR-29b was also up-regulated by over-expression Nrf2 (**Figure 4B**). To validate the role of TGF-β_2 in Nrf2 regulation miR-29b, Chromatin Immunoprecipitation Assay was performed in human fibroblast SX1412-B exposed to TGF-β_2. **Figure 4C** revealed that fold decrease of DNA Nrf2 complex level was significantly lower in SX1412-B exposed to TGF-β_2 comparing to the control. This result suggested that TGF-β_2 repressed the binding of Nrf2 to the promoter of miR-29b.

MiR-29b reversed the effects of TGF-β_2 on proliferation of fibroblasts via Nrf2 pathway

As indicated previously, TGF-β_2 involved in the Nrf2 regulation of the miR-29b expression through inhibiting the binding of Nrf2 to the promoter of miR-29b. We further examined the role of miR-29b in the effects of TGF-β_2 on proliferation of fibroblasts via Nrf2 pathway. As shown in **Figure 5**, overexpression Nrf2 in GTFs reduced the proliferation effect on GTFs induced by TGF-β_2, while miR-29b inhibitor reversed this effect. Correspondingly, down-regulated Nrf2 in HTFs increased the proliferation effect on HTFs induced by TGF-β_2, and this effect could be reversed by miR-29b mimic.

Discussion

The activation of Tenon's capsule fibroblasts performed an essential role in conjunctival scarring and acted as the central regulatory element of the scarring reaction. As a transforming growth factor, a large body of evidence showed that TGF-β_2 had a stimulatory effect on fibroblast activity, such as fibroblast-mediated collagen contraction, fibroblast proliferation, and fibroblast migration [22-25]. This stimulation has been reported to occur in a biphasic, concentration-dependent manner, with different peak activities associated with different fibroblast functions. In addition to these stud-

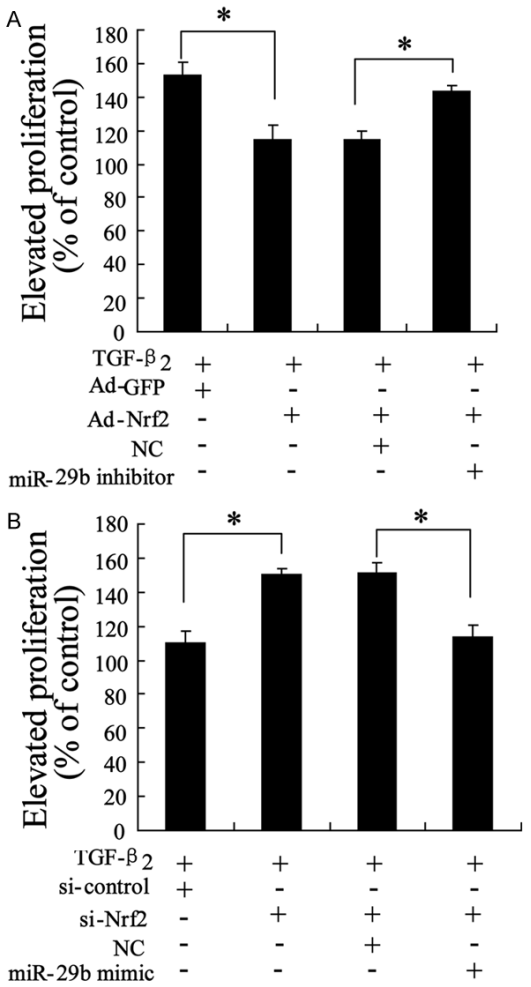

Figure 5. MiR-29b reversed the effects of TGF-β_2 on proliferation of fibroblasts via Nrf2 pathway. A. Elevated proliferation of GTFs. B. Elevated proliferation of HTFs; Results were mean ± SD of 3 independent experiments. *VS control, $P<0.05$.

ies, the present work comparatively investigated the effect of TGF-β_2 on Tenon's capsule fibroblast proliferation in patients with glaucoma and the related pathway involved in this process.

This study confirmed that TGF-β_2 stimulated both GTFs and HTFs efficiently in a time-dependent manner, and the proliferation effect of TGF-β_2 on GTFs was more significant. In addition, we found that the peak proliferation rate appeared at 3 days post-exposure to TGF-β_2, which was also validated in previous investigations [26, 27]. Increasing evidence showed that the miR-29 family was the best characterized miRNA associated with TGF-β-mediated fibrosis [18, 28, 29]. Coralia Luna et al suggested that TGF-β_2 significantly and consistently decreased the expression of miR-29b in trabecular meshwork cells [30]. As a member of the Cap 'n' Collar basic leucine zipper transcription factor family, Nrf2 was responsible for miRNA transcription. Kurinna et al. demonstrated that Nrf2 activated expression of miR-29b in keratinocytes [31]. Additionally, chromatin immunoprecipitation followed by sequencing (ChIP-Seq) revealed that Nrf2 binded to promoters of genes encoding miRNAs, although the functional consequences remain largely unknown. To date, the main risk factor for primary open-angle glaucoma was elevated intraocular pressure (IOP). Oxidative stress was reported to trigger degeneration in the human trabecular meshwork and its endothelial cell components, subsequently leading to an increase in IOP and glaucoma. Moreover, activity of Nrf2 was strongly enhanced in response to electrophilic and oxidative stress, which results in stabilization and nuclear accumulation of Nrf2.

In order to make clear the potential mechanism of the stimulation effect of TGF-β_2, we investigated the levels of Nrf2 protein and miR-29b mRNA expression. Our results showed that Nrf2 protein and miR-29b mRNA expression were detected time-dependent down-regulation in GTFs/HTFs post-exposed to TGF-β_2, reached their minima after 3 days and then enhanced. Moreover, TGF-β_2 exerted a stronger effect on GTFs than that on HTFs. Taken together, this study indicated that Nrf2 and miR-29b were involved in the stimulation effect of TGF-β_2 on GTFs/HTFs.

To further confirm the role of Nrf2 in GTFs proliferation, Nrf2 levels were regulated via transfecting Ad-Nrf2 into GTFs or si-Nrf2 into HTFs to overexpress or knock-down Nrf2. As a result, there was no difference in proliferation of GTFs and HTFs after exposure to TGF-β_2 in this two cases. Combined the above findings, it was suggested that overexpression Nrf2 may attenuate the proliferation of GTFs induced by TGF-β_2. Next, we examined the relationship of Nrf2 and miR-29b in human fibroblast SX1412-B. The level of miR-29b mRNA was effectively and positively regulated by Nrf2. Chromatin Immunoprecipitation Assay supported that TGF-β_2 can repress the binding of Nrf2 to the promoter of miR-29b. Based on this findings, we further examined the role of miR-29b in the effects of TGF-β_2 on proliferation of fibroblasts

via Nrf2 pathway. Finally, we observed that overexpression Nrf2 in GTFs reduced TGF-β_2-induced proliferation effect on GTFs, and miR-29b inhibitor might reversed this effect. Together, these data implied that TGF-β_2 stimulates Tenon's capsule fibroblast proliferation in patients with glaucoma and it exerted the function via suppression miR-29b expression regulated by Nrf2.

In conclusion, our results suggested that TGF-β_2 played an important role in proliferation of Tenon's capsule fibroblasts from glaucoma patients. Regulation of miR-29b expression by Nrf2 was implicated in TGF-β_2 promoting proliferation of Tenon's capsule fibroblasts from glaucoma patients. This finding may offer a new insight for more precise and rational design of conjunctive drug and/or gene delivery nanosystems. As for glaucoma patients, miR-29b could play an important role in modulating the pathogenic effects of TGF-β_2 on the outflow pathway in glaucoma.

Disclosure of conflict of interest

None.

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