Int J Clin Exp Pathol 2017;10(2):1703-1707
www.ijcep.com /ISSN:1936-2625/IJCEP0038549

Original Article

miR-192-5p, TGFβ2 and the expression of extracellular matrix moieties in of human trabecular meshwork cells

Ying Su, Feng Wang

Department of Ophthalmology, First Hospital of Harbin Medical University, Harbin 150001, China

Received August 18, 2016; Accepted October 10, 2016; Epub February 1, 2017; Published February 15, 2017

Abstract: Increased obstruction in trabecular meshwork (TM) especially juxtacanalicular tissue (JCT), is a major factor that contributes to primary open angle glaucoma (POAG). With POAG extracellular matrix (ECM) moieties such as fibronectin, collagen IV and elastin accumulate within the trabecular meshwork so the obstruction of the aqueous humor outflow pathway increased. MiR-192-5p is known to regulate the ECM deposits. In this study, we quantified the effects of miR-192-5p transfection on the expression of ECM components. MiR-192-5p mimic, inhibitor was used to transfect human trabecular meshwork cells (hTM) in *vitro*. Protein expression of fibronectin, collagen I, collagen IV and SPARC were measured using Western blot analysis. There was not a significant difference in the expression of fibronectin, laminin, collagen I, collagen IV and SPARC protein between the control plus transforming growth factor beta 2 (TGF-β2) group and the miR-192-5p mimic plus TGF-β2 group. After the transfection of hTM with miR-192 inhibitor, expression of fibronectin, laminin, collagen I, collagen IV and SPARC protein was depressed in the TM conditional medium transfected with *TGF-β2* group compared with the control group. Transfection with miR-192-5p inhibitor can decrease the expression of ECM components in cultured hTM. Our data suggest that miR-192-5p could serve as a new therapeutic for POAG in future studies.

Keywords: Glaucoma, trabecular meshwork, microRNA-192-5p, extracellular matrix

Introduction

Glaucoma ranks as third of the causes of blindnes [1, 2]. Elevated intraocular pressure plays an important role in the pathogenesis of glaucoma [3-5]. Increased resistance of the trabecular meshwork (TM), is a major factor that contributes to primary open angle glaucoma (POAG) [6-8]. Deposition of extracellular matrix (ECM) moieties such as fibronectin, collagen IV, elastin, within the TM, could potentially increase the obstruction of aqueous humor outflow through the TM [9-12].

Abnormal tissue repair is often associated with excessive ECM production that leads to fibrosis, including idiopathic pulmonary fibrosis [13] and liver fibrosis [14]. The epithelial-mesenchymal transition is confirmed as a biological process resulting in mesenchymal cell features, which can synthesize and organize ECM components [15]. Cytokine transforming growth factor, beta 2 (*TGF-β2*), is important for the regulation of ECM expression in POAG. miR-29, miR-192 and miR-200 significantly decrease the expression of ECM components in lung fibrosis, liver fibrosis, and kidney fibrosis [16-18]. miR-29 can also decrease the expression of moieties such as fibronectin, and collagen IV in TM cells [19-21]. However, the effect of miR-192 on the abnormal accumulation of ECM within in TM cells is unknown. In the present study, we tested whether transfection of miR-192-5p inhibitor decreased the expression of ECM in the TM.

Materials and methods

Trabecular meshwork cell culture and TGF-β2 treatment

HTM cell cultures were collected within 48 hours post mortem from six donors ranging in age from 25 to 56 years without eye disease. HTM cells were mixed after extraction from the donors TM tissues. All procedures involving human tissues obeyed the tenets of the Declaration of Helsinki. The ethics committee of Harbin Medical University, China, obtained

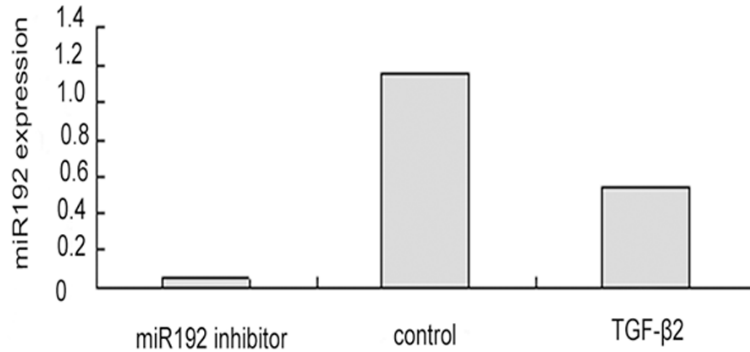

Figure 1. Real-time PCR analysis showed the presence of the miR-192 family in the TM. TGF-β2 enhanced the expression of miR-192. There was a statistically significant reduction of miR-192 expression by TGF-β2 compared with the control group ($P < 0.01$), whereas there is significant decrease of miR-192 levels after transfection of TM with miR-192 inhibitor.

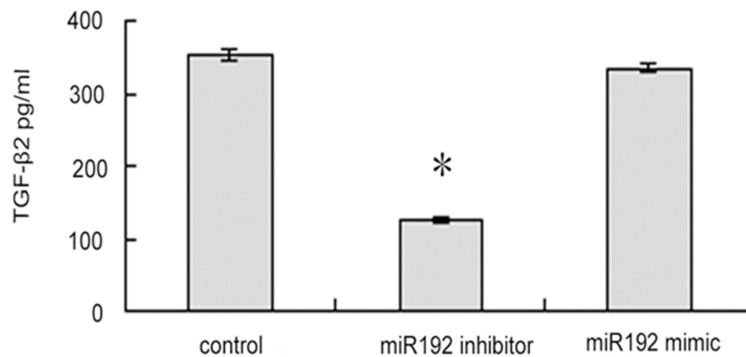

Figure 2. Effect of miR-192 inhibitor on TGF-β2 expression in hTM. $*P < 0.01$ compared with other groups. The levels of TGF-β2 protein in hTM transfected with miR-192 inhibitor were significantly reduced ($P < 0.01$, **Figure 3**). There was no significant difference in TGF-β2 expression between the group treated with miR-192 mimic and the control group ($P > 0.05$, **Figure 3**).

written approved from all of the donors to participate in the study and the study.

The lens, cornea, retina, iris and ciliary body were extracted first. Then, TM cells between Decement's membrane and the scleral spur were dissected and placed in a culture dish. The cell culture medium was a Dulbecco's Modified Eagle's Medium (low glucose) supplemented with 10% fetal bovine serum, L-glutamine (0.292 mg/ml), penicillin (100 units/ml), streptomycin (0.1 mg/ml), and amphotericin B (4 mg/ml) (HyClone Labs, Logan, UT, USA). rTM cells between passages 5 and 8 were selected for our experiment. In the TGF-β2 (Sigma Aldrich, St. Louis, MO, USA) treatment group, cells were treated with 2 ng/mL TGF-β2 for 24 hours [21].

MiR-192-5p transfection

HTM cells were transfected with miR-192-5p mimic, inhibitor and their negative control RNA (Qiagen, Valencia, CA, USA) using Liptofectamine 2000 reagent (Invitrogen™, Life Technologies, Grand Island, NY, USA). Then, cells were incubated at 37°C in a CO2 incubator for 24-48 h. They were then assayed as described below [19].

RNA isolation and quantitative-PCR

We used a TRIzol RNA isolation system to prepare total RNA. The first strand of cDNA in miScript RT buffer was incubated at 37°C for 60 min, and 95°C for 5 min. A 7300 Sequence Detection system was used to perform real-time quantification. The results were analyzed as mean value ± SE [19].

Western blot analysis

Conditioned medium was collected after transfection in TM cells in a serum-free medium containing 0.5 mg/mL BSA (HyClone Labs, Logan, UT, USA). Absorbance spectroscopy was used to measure the protein concentration at 278 nm. Membranes were incubated with primary antibodies against fibronectin, laminin, collagen I, collagen IV and SPARC (secreted protein acidic and rich in cysteine) (Santa Cruz Biotechnology, Dallas, Texas, USA) overnight at 4°C, followed by incubation with secondary antibodies. The membrane was then assayed using an enhanced chemiluminescent kit (ECL, Thermo Scientific, Pittsburgh PA, USA) and scanned with ChemiDoc™ Doc XRS+ system (Bio-Rad, Hercules, CA, USA). Values were expressed as fold change relative to the control and normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Santa Cruz Biotechnology, Dallas, Texas, USA) [21].

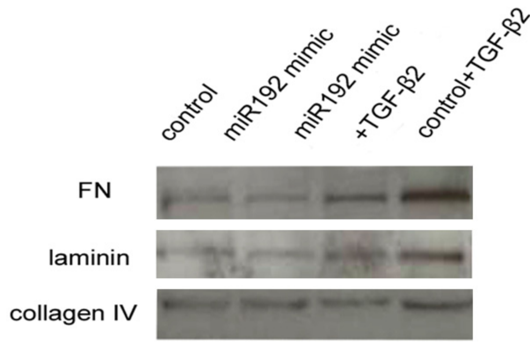

Figure 3. Transfection with miR-192 mimic on ECM expression in hTM. *$P < 0.01$ compared with other groups. There was a significant increase in the expression of fibronectin, laminin, collagen I, collagen IV and SPARC protein in the control plus TGF-β2 group compared with the miR-192 mimic plus TGF-β2 group ($P < 0.01$). There was no significant difference between the control group and the miR-192 mimic treated group or the miR-192 + TGF-β2 treated group ($P > 0.5$).

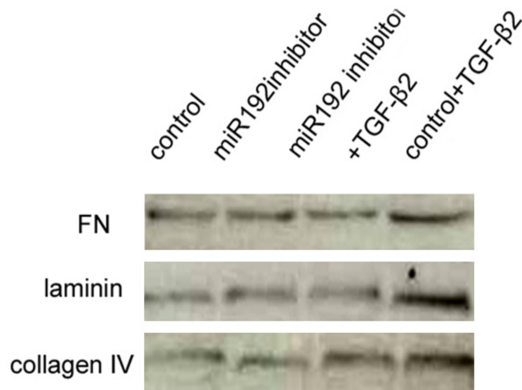

Figure 4. Downregulation expression of fibronectin, collagen I, collagen IV, SPARC and laminin protein by transfection with miR-192 inhibitor. After the transfection of miR-192 inhibitor, expression of fibronectin, collagen I, collagen IV, SPARC and laminin protein was depressed in the TM conditional medium compared with *the TGF-β2* group plus control group ($P < 0.01$).

Statistical analysis

All computations were carried out using the software of SPSS version 18.0 for Windows. All data are presented as the mean ± SD and have been evaluated for normality of distribution. Difference between mean values was tested for statistical significance with student t test, with a P value < 0.05 indicative of statistical significance.

Results

Expression of miR-192 in hTM

Our result showed that transfection did not change the cellular morphology. Real-time PCR analysis revealed the presence of the miR-192 in the TM (**Figure 1**). To determine whether the miR-192 family functions as a regulator of TGF-β2-mediated ECM synthesis, miR-192 expression was measured after 24 hours of incubation of TM cells with TGF-β2. Our result showed that a statistically significant induction of miRNA-192 levels by TGF-β2 compared with the control group, whereas real-time PCR analysis showed that there was a significant decrease of miR-192 levels after transfection with a miR-192 inhibitor (**Figure 1**).

Effect of miR-192-5p inhibitor on TGF-β2 expression in hTM

The levels of TGF-β2 protein in hTM transfected with miR-192-5p inhibitor were significantly reduced ($P < 0.01$, **Figure 2**). There was no significant difference in TGF-β2 expression between the group treated with miR-192-5p mimic and the control group ($P > 0.05$, **Figure 2**).

Transfection with miR-192-5p mimic on ECM expression in hTM

There was a significant increase in the expression of fibronectin, laminin, collagen I, collagen IV and SPARC protein in the control plus TGF-β2 group compared with the miR-192 mimic plus TGF-β2 group ($P < 0.01$, **Figure 3**). There was no significant difference between the control group and the miR-192 mimic treated group or the miR-192-5p + TGF-β2 treated group ($P > 0.5$).

Expression of fibronectin, laminin, collagen I, collagen IV and SPARC after transfection with miR-192-5p inhibitor

To investigate the effect of the miR-192-5p on ECM expression in the TM, we inhibited its expression and tested its effect on ECM synthesis in the TM. After the transfection of TM with miR-192-5p inhibitor, expression of fibronectin, laminin, collagen I, collagen IV and SPARC protein levels were lower in the TM conditional medium compared with the TGF-β2 and control group ($P < 0.01$, **Figure 4**).

Discussion

Several miRNAs, including miR-23, -29, -192, -194, and -215, are highly expressed in the trabecular meshwork [22-26] which are associated with fibrosis of the kidney, lung and liver [27, 28]. Overexpression of miR-29a can down regulate the expression of ECM in the TM [29, 30]. *In vitro*, miR-192 mediates *TGF-β*-induced collagen expression [31]. It has been confirmed that miR-192 expression was suppressed by *TGF-β1* in human tubular epithelial cells (TEC) and fibrogenesis can be increased when there isn't miR-192 in diabetic nephropathy [32-34].

Our study bridges the gap of knowledge regarding the incomplete and imprecise knowledge of the role of miR-192-5p in the etiology of POAG.

Based on our data, miR-192-5p was a mediator of TGF-β which can induce deposition of ECM in the hTM as evidenced by the findings that overexpression of miR-192-5p mimics enhanced TGF-β2-induced ECM expression, whereas inhibition of miR-192-5p by overexpressing miR-192 inhibitor blocked fibronectin, laminin, collagen I, collagen IV, SPARC expression in response to TGF-β2. Above all, our results suggest that miR-192-5p is upregulated by TGF-β2 and leads to ECM accumulation in the hTM.

Conclusion

We found that miR-192-5p can down regulate the expression of ECM in TM conditional medium. These data are significant because miR-192-5p may be a target in developing novel therapeutics for the treatment of POAG. In future studies we plan to test if transfection of hTM with miR-192 can lower IOP in *vivo* by decreasing ECM expression.

Acknowledgements

This work was supported by the grant of natural science, the science foundation of China (81100659, 81470633), the grant of returned overseas scholars of Chinese ministry of education, the grant of returned overseas Chinese scholars of Heilongjiang province of China (LC2013C33/H1204), Scientific foundation of education ministry of China (200923071200-03), and the Postdoctral foundation of China (20080430137, 200902418).

Disclosure of conflict of interest

None.

Address correspondence to: Feng Wang, Department of Ophthalmology, First Hospital, Harbin Medical University, Harbin 150001, China. Tel: 86-13936673269; Fax: 86-13936673269; E-mail: wangfd@126.com

References

[1] Quigley HA, Broman AT. The number of people with glaucoma worldwide in 2010 and 2020. Br J Ophthalmol 2006; 90: 262-267.
[2] Lutjen-Drecoll E. Morphological changes in glaucomatous eyes and the role of TGFbeta2 for the pathogenesis of the disease. Exp Eye Res 2005; 81: 1-4.
[3] Zhu Y, Zhang L, Sasaki Y, Milbrandt J, Gidday JM. Protection of Mouse Retinal Ganglion Cell Axons and Soma from Glaucomatous and Ischemic Injury by Cytoplasmic Overexpression of Nmnat1. Invest Ophthalmol Vis Sci 2013; 54: 25-36.
[4] Chung EJ, Lee HK, Jung SA, Lee SJ, Chee HY, Sohn YH. Transduction of PTEN proteins using the tat domain modulates TGF-β1-mediated signaling pathways and transdifferentiation in subconjunctival fibroblasts. Invest Ophthalmol Vis Sci 2012; 53: 379-86.
[5] Takai Y, Tanito M, Ohira A. Multiplex cytokine analysis of aqueous humor in eyes with primary open-angle glaucoma, exfoliation glaucoma, and cataract. Invest Ophthalmol Vis Sci 2012; 53: 241-7.
[6] Zenkel M, Krysta A, Pasutto F, Juenemann A, Kruse FE, Schlötzer-Schrehardt U. Regulation of lysyl oxidase-like 1 (LOXL1) and elastin-related genes by pathogenic factors associated with pseudoexfoliation syndrome. Invest Ophthalmol Vis Sci 2011; 52: 8488-95.
[7] Tektas OY, Lutjen-Drecoll E. Structural changes of the trabecular meshwork in different kinds of glaucoma. Exp Eye Res 2009; 88: 769-775.
[8] Roberts AB, Heine UI, Flanders KC, Sporn MB. Transforming growth factor-beta. Major role in regulation of extracellular matrix. Ann N Y Acad Sci 1990; 580: 225-232.
[9] Bollinger KE, Crabb JS, Yuan X, Putliwala T, Clark AF, Crabb JW. Quantitative proteomics: TGFβ2 signaling in trabecular meshwork cells. Invest Ophthalmol Vis Sci 2011; 52: 8287-94.
[10] Han H, Wecker T, Grehn F, Schlunck G. Elasticity-dependent modulation of TGF-β responses in human trabecular meshwork cells. Invest Ophthalmol Vis Sci 2011; 52: 2889-96.
[11] Yu AL, Birke K, Moriniere J, Welge-Lüssen U. TGF-{beta}2 induces senescence-associated

changes in human trabecular meshwork cells. Invest Ophthalmol Vis Sci 2010; 51: 5718-23.
[12] Robertson JV, Golesic E, Gauldie J, West-Mays JA. Ocular gene transfer of active TGF-beta induces changes in anterior segment morphology and elevated IOP in rats. Invest Ophthalmol Vis Sci 2010; 51: 308-18.
[13] Wang F, Qi LX, Su Y, Yan QH, Teng Y. Inhibition of Cell Proliferation of Tenon's Capsule Fibroblast by S-Phase Kinase-Interacting Protein 2 Targeting SiRNA through Increasing p27 Protein Level. Invest Ophthalmol Vis Sci 2010; 51: 1475-1482.
[14] Su Y, Wang F, Yan Q, Teng Y, Cui H. Inhibition of proliferation of rabbit lens epithelial cells by S-phase kinase-interacting protein 2 targeting small interfering RNA. Mol Vis 2010; 16: 907-915.
[15] Laping NJ, Grygielko E, Mathur A. Inhibition of transforming growth factor (TGF)-beta1-induced extracellular matrix with a novel inhibitor of the TGF-beta type I receptor kinase activity: SB-431542. Mol Pharmacol 2002; 62: 58-64.
[16] Sawyer JS, Anderson BD, Beight DW. Synthesis and activity of new aryl- and heteroaryl-substituted pyrazole inhibitors of the transforming growth factor-beta type I receptor kinase domain. J Med Chem 2003; 46: 3953-3956.
[17] Tripathi RC, Chan WF, Li J, Tripathi BJ. Trabecular cells express the TGF-beta 2 gene and secrete the cytokine. Exp Eye Res 1994; 58: 523-528.
[18] Luna C, Li G, Qiu J, Epstein DL, Gonzalez P. Cross-talk between miR-29 and Transforming Growth Factor-Betas in Trabecular Meshwork Cells. Invest Ophthalmol Vis Sci 2011; 52: 3567-3572.
[19] Villarreal G Jr, Oh DJ, Kang MH, Rhee DJ. Coordinated regulation of extracellular matrix synthesis by the MicroRNA-29 family in the trabecular meshwork. Invest Ophthalmol Vis Sci 2011; 52: 3391-3397.
[20] Su Y, Yang CY, Li Z, Xu F, Zhang L, Wang F, Zhao S. Smad7 siRNA inhibit expression of extracellular matrix in trabecular meshwork cells treated with TGF-β2. Molecular Vision 2012; 18: 1881-1884.
[21] Omori S, Kitagawa H, Koike J. Activated extracellular signalregulated kinase correlates with cyst formation and transforming growth factor-beta expression in fetal obstructive uropathy. Kidney Int 2008; 73: 1031-1037.
[22] Dobaczewski M, Gonzalez-Quesada C, Frangogiannis NG. The extracellular matrix as a modulator of the inflammatory and reparative response following myocardial infarction. J Mol Cell Cardiol 2010; 48: 504-511.
[23] Gill S, Wight TN, Frevert CW. Proteoglycans: key regulators of pulmonary inflammation and the innate immune response to lung infection. Anat Rec (Hoboken) 2010; 293: 968-981.
[24] Evanko SP, Potter-Perigo S, Johnson PY, Wight TN. Organization of hyaluronan and versican in the extracellular matrix of human fibroblasts treated with the viral mimetic poly I: C. J Histochem Cytochem 2009; 57: 1041-1060.
[25] Winter J, Jung S, Keller S, Gregory RI, Diederichs S. Many roads to maturity: microRNA biogenesis pathways and their regulation. Nat Cell Biol 2009; 11: 228-234.
[26] Kim VN, Han J, Siomi MC. Biogenesis of small RNAs in animals. Nat Rev Mol Cell Biol 2009; 10: 126-139.
[27] Lewis BP, Shih IH, Jones-Rhoades MW, Bartel DP, Burge CB. Prediction of mammalian microRNA targets. Cell 2003; 115: 787-798.
[28] Doench JG, Sharp PA. Specificity of microRNA target selection in translational repression. Genes Dev 2004; 18: 504-511.
[29] Didiano D, Hobert O. Perfect seed pairing is not a generally reliable predictor for miRNA-target interactions. Nat Struct Mol Biol 2006; 13: 849-851.
[30] Deshpande SD, Putta S, Wang M, Lai JY, Bitzer M, Nelson RG, Lanting LL, Kato M, Natarajan R. Transforming growth factor- β -induced cross talk between p53 and a microRNA in the pathogenesis of diabetic nephropathy. Diabetes 2013; 62: 3151-62.
[31] Saetrom P, Heale BS, Snove O Jr, Aagaard L, Alluin J, Rossi JJ. Distance constraints between microRNA target sites dictate efficacy and cooperativity. Nucleic Acids Res 2007; 35: 2333-2342.
[32] Long D, Lee R, Williams P, Chan CY, Ambros V, Ding Y. Potent effect of target structure on microRNA function. Nat Struct Mol Biol 2007; 14: 287-294.
[33] Vasudevan S, Steitz JA. AU-rich-element-mediated upregulation of translation by FXR1 and Argonaute 2. Cell 2007; 128: 1105-1118.
[34] Barzegar-Fallah A, Alimoradi H, Razmi A, Dehpour AR, Asgari M, Shafiei M. Inhibition of calcineurin/NFAT pathway plays an essential role in renoprotective effect of tropisetron in early stage of diabetic nephropathy. Euro J Pharmacy 2015; 767: 152-9.