Role of miRNAs in pathologic fibrosis in the glaucomatous optic nerve head

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INTRODUCTION

Glaucoma is the leading cause of irreversible vision loss and is associated with fibrotic changes to the optic nerve head (ONH). Sensitivity to intraocular pressure (IOP) is a prominent risk factor for the development and progression of glaucoma. The IOP pressure transduced to the optic nerve head (ONH), an area where unmyelinated axons pass, causes progressive loss of RGC axons. The lamina cribrosa (LC) region of the ONH is the initial site of injury in glaucoma. The LC is posteriorly displaced, axons bundles are disorganized and astrocytes are reactivated. This results in a disruption of the extracellular matrix (ECM) balance in the ONH. For example, increased expression of collagens type (COL) I, III, IV, V and VI, and fibronectin (FN) are observed in glaucomatous ONH. Our previous study showed the expression of TGFβ2 is elevated in the ONH of glaucoma eyes compared to normal eyes. Recently, miRNAs have added additional complexity as posttranscriptional epigenetic regulators of gene expression. miRNAs are small non-coding RNA molecules that silence gene expression. Altered expression of growth factors can result in differential expression of miRNAs and increased synthesis of ECM proteins. The purpose of this study was to determine: (a) differences in the expression of profibrotic and anti-fibrotic miRNAs in ONH astrocytes (ONA) or LC cells treated with or without TGFβ2 and (b) whether candidate miRNAs regulate the synthesis of ECM proteins in ONHA and LC cells.

DESIGN & METHODS

Primary human ONH cell culture: Well-characterized primary human ONH astrocytes (ONHA) were characterized as glial fibrillary acidic protein (GFAP) positive and alpha-smooth muscle actin (αSMA) negative. In contrast, LC cells were characterized as GFAP negative and αSMA positive.

miRNA PCR arrays:
ONHA and LC cells were treated with 5ng/ml TGFβ2 or with control for 24hrs. A SYBR green-based real-time PCR miRNA array examined 84 fibrotic miRNAs (MZ117; Qiagen, Valencia CA) and expression analyzed on a web based data analysis tool (https://www.qiagen.com/us/shop/genes-and-pathways/data-analysis-center-overview-page/).

Quantitative RT PCR:
Initial denaturation at 95℃ for 30 seconds followed by 40 cycles of 95℃ for 10 seconds; 60 ℃ for 30 seconds followed by melting curve step. PCR was performed on a real-time thermal cycler (model CFX96;Bio-Rad Laboratories). The expression of miRNAs were normalized to control using the ΔΔ cycle thresholds (Ct) method.

Transfection studies:
ONHA and LC cells were transfected with candidate miRNAs mimics or inhibitors at 10nM or with all stars negative control (10nM) to confirm computational target site predictions (TargetScan database available online; http://www.targetscan.org).

Immunocytochemistry staining:
Primary ONH cells were transfected with or without TGFβ2 (5ng/ml), fixed and stained for FN and COL type I and IV.

Immunofluorescent staining:
Immunofluorescent staining was performed in normal and glaucomatous human ONH tissue for FN and COLIV. Formalin fixed, paraffin embedded tissues were sectioned and stained with antibodies for FN and COLIV. Negative control consisted of PBS-superblock without primary antibody (not shown).

RESULTS

Figure 1. Scatter plots of differentially expressed human mature miRNAs treated with TGFβ2 in A) ONHA and B) LC cells.
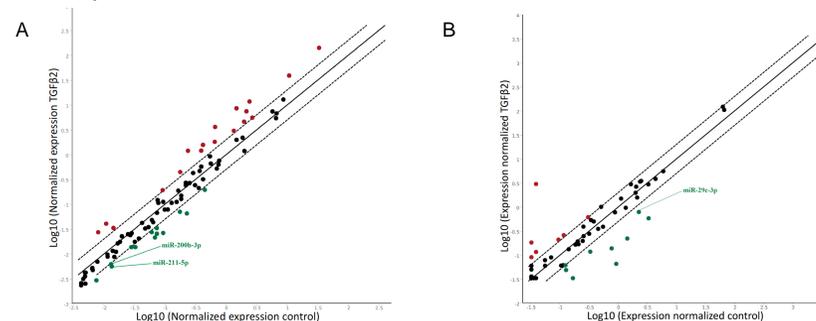

Scatter plots were generated by plotting the normalized log expression data obtained from cells treated with TGFβ2 against control cells. Diagonal lines represent the 2-fold threshold for differentially expressed miRNAs. Red points indicate miRNAs upregulated and green points are miRNAs downregulated by at least 2-fold.

Figure 2. Over-expression of miR-200b-3p decreases TGFβ2 induced FN and COLIV expression in ONHA.
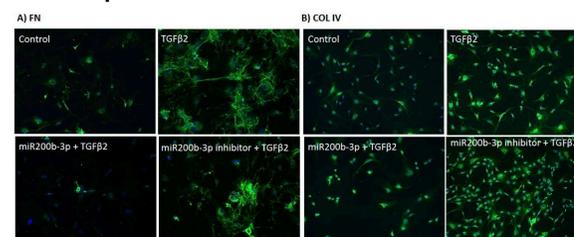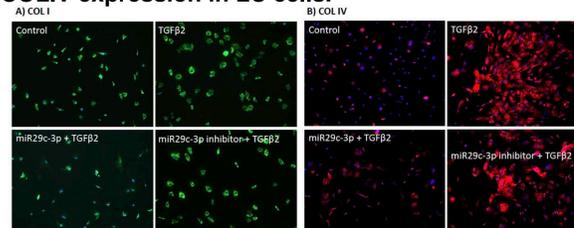

ONHA transfected with miR-200b mimic decreased TGFβ2 induced A) FN and COLIV expression. This effect was reversed using an inhibitor of miR-200b. Non-targeting control and inhibitor control did not effect expression of FN or COLIV (data not shown).

Figure 3. Over-expression of miR-211-5p decreases TGFβ2 induced FN and COLIV expression in ONHA.
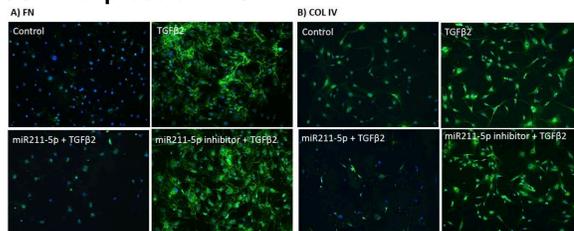

ONHA transfected with miR-211 mimic decreased TGFβ2 induced A) FN and COLIV expression. This effect was reversed using an inhibitor of miR-211. Non-targeting control and inhibitor control did not effect expression of FN or COLIV (data not shown).

Figure 4. Over-expression of miR-29c-3p decreases TGFβ2 induced COLI and COLIV expression in LC cells.

LC cells transfected with miR-29c mimic decreased TGFβ2 induced A) COLI and B) COLIV expression. This effect was reversed using an inhibitor of miR-29c. Non-targeting control and inhibitor control did not effect expression of COLI or COLIV (data not shown).

Figure 5. FN and COLIV expression in human ONH tissue.
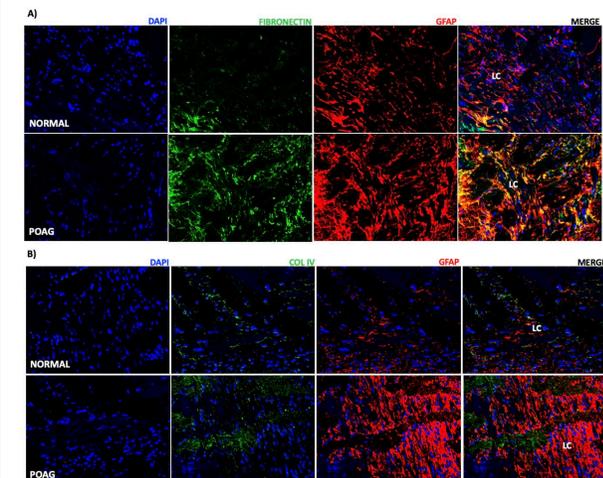
TGFβ2 downregulation of anti-fibrotic miRNAs in ONH cells is associated with an increased expression of A) FN and B) COLIV in human POAG ONH tissue sections compared to aged matched normal controls.

CONCLUSIONS

- TGFβ2 treatment resulted in both upregulated and downregulated miRNAs in ONHA and LC cells.
- Using anti-fibrotic miRNAs identified in our miRNA PCR arrays, we were able to alter the expression of fibrotic ECM proteins in ONH cells.
  - Transfection of miR-200b-3p and miR-211-5p mimics decreased TGFβ2-induced FN and COL IV expression in ONHA, while inhibitors of these miRNA enhanced TGFβ2-induced FN and COL IV expression.
  - Transfection of miR-29c-3p mimic decreased TGFβ2-induced COL I and IV expression in LC cells while inhibiting endogenous miR-29c-3p reversed this effect.
- Decreased expression of anti-fibrotic miRNAs correlated to an increased expression of FN and COL IV in human POAG ONH tissue compared to aged matched normal eyes.
- TGFβ2 downregulates anti-fibrotic miRNAs to create a profibrotic environment that may lead to pathogenic ONH remodelling.

NEXT STEPS

In future studies, we plan to examine anti-fibrotic miRNAs in a glaucoma in-vivo mouse model to determine if over-expressing anti-fibrotic miRNAs prevent ONH damage.

ACKNOWLEDGEMENTS