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MicroRNA-24 Regulates the Processing of Latent TGFβ1 During Cyclic Mechanical Stress in Human Trabecular Meshwork Cells Through Direct Targeting of FURIN

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Abstract

Cyclic mechanical stress (CMS) leads[Q1] to alterations of cellular functions in the trabecular meshwork (TM), including the up-regulation of transforming growth factor beta 1 (TGFβ1), that can potentially contribute to the pathogenesis of glaucoma. Although microRNAs (miRNAs) are known to play important roles in many biological functions, little is known about their potential involvement in the cellular responses elicited by mechanical stress. Here we analyzed changes in miRNA expression induced by CMS, and examined the possible role of miR-24 in the response of human TM cells to CMS. CMS induced the expression of miR-24 that led to the down regulation of the subtilisin-like proprotein convertase FURIN, which is known to play a major role in the processing of TGFβ1. FURIN was confirmed as a novel target of miR-24 by 3′ UTR luciferase assay and western blot. Overexpression of miR-24 resulted in a significant decrease in activated TGFβ1. This effect was mimicked by down regulation of FURIN by siRNA. Conversely, inhibition of miR-24 expression with a specific antagomir led to a small but significant increase in TGFβ1. Furthermore, the increase in active TGFβ1 induced by CMS in HTM cells was prevented by miR-24. Altogether, our results suggest that miRNAs might contribute to the regulation of responses to CMS in TM cells. Specifically, miR-24 might play an important role in modulating the induction of TGFβ1 mediated by CMS through direct targeting of FURIN.

Introduction

The trabecular meshwork (TM) and Schlemm's canal form the major route by which the aqueous humor exits the anterior chamber of the human eye and constitute the site of the abnormal increase in outflow resistance that leads to elevated intraocular pressure (IOP) in glaucoma (Fautsch and Johnson, 2006; Johnson, 2006; Overby et al., 2009; Tektas and Lutjen-Drecoll, 2009). There is abundant evidence that the TM is deformed when IOP increases and the anatomy is restored as the IOP decreases (Johnstone, 1979; Johnstone, 2004). Thus, it is likely that transient IOP oscillations such as those resulting from the ocular pulse lead to constant cycles of stretching and then relaxation of the TM cells. Previous studies have demonstrated that TM cells are sensitive to mechanical forces (Borras, 2003; WuDunn, 2009) and that cyclic mechanical stress (CMS) induces changes in cell morphology and gene expression that can potentially exert important effects on the physiology of the TM (Luna et al., 2009a, b; Ramos et al., 2009). Some of the responses elicited by CMS in TM cells, including the increased expression of MMP3 (Luna et al., 2009a) and release of PGE2 (Luna et al., 2009b), have been hypothesized to constitute homeostatic mechanisms aimed at increasing aqueous humor outflow in response to

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mechanical stress resulting from elevations in IOP (Luna et al., 2009a, b). In contrast, other responses mediated by CMS, such as increased expression of TGFβ1 (Liton et al., 2005), BMP2 (Luna et al., 2009a), CTGF (Chudgar et al., 2006), or increased cell contractility (Ramos et al., 2009) may contribute to pathological alterations of the TM and then secondarily further increase IOP. In spite of the physiologic relevance of the effects of CMS on the TM, the molecular mechanisms involved in the alterations of gene expression induced by CMS in TM cells are still poorly understood.

MicroRNAs (miRNAs) are known to play an important role in the regulation of many cellular functions by either repressing translation or inducing mRNA degradation of multiple specific gene targets (Ying et al., 2006; Bushati and Cohen, 2007). MiRNAs can participate in the regulation of gene expression changes induced by several types of stress (Marsit et al., 2006; Martin et al., 2010[Q2]), including hypoxic stress (Crosby et al., 2009), cold stress (Dresios et al., 2005), and oxidative stress (Cheng et al., 2009; Lin et al., 2009; Luna et al., 2009c). However, only a few studies have analyzed the potential involvement of miRNAs on the responses induced by mechanical forces in general (Qin et al. 2010a; Weber et al., 2010), and nothing is known about their role in the responses induced by CMS. Therefore, in the present study, we examined whether CMS could lead to changes in miRNA expression in HTM cells, and investigated the mechanisms by which some of these changes might influence the responses induced by CMS in HTM cells.

Material and Methods

Cell culture and treatments

Human trabecular meshwork (HTM) cell cultures were generated from cadaver eyes, with no history of eye disease, within 48 h post mortem as previously reported (Stamer et al., 1995). All procedures involving human tissue were conducted in accordance with the tenets of the Declaration of Helsinski. Cell cultures were maintained at 37ºC in 5% CO_2 in media (low glucose Dulbecco's Modified Eagle Medium with L-glutamine, 110 mg/ml sodium pyruvate, 10% fetal bovine serum, 100 μM non-essential amino-acids, 100 units/ml penicillin, 100 μg/ml streptomycin sulfate, and 0.25 μg/ml amphotericin B). All the reagents were obtained from Invitrogen Corporation (Carlsbad, CA). SB431542 (Tocris Bioscience, Ellisville, MO) a TGFβ1 inhibitor was added at 10 μM concentration in serum free media during 30 min after which cells were washed and TGFβ1 (Sigma, St Louis, MO) was added at a concentration of 1 ng/ml in serum free media, and cells were analyzed after 24 h.

Cyclic mechanical stress (CMS)

HTM cell cultures (passages 3–5) were plated on type I collagen-coated flexible silicone bottom plates (Flexcell, Hillsborough, NC). For miRNA analysis cells were grown to confluence, and for transfections with miRNAs, cells were plated at 50–70% confluency, transfected 24 h later and subjected to CMS 72 h after transfection. Medium was switched to serum-free DMEM 2 h before CMS and cells were stretched for 3 or 16 h (20% stretching, 1 cycle per second), using the computer-controlled, vacuum-operated FX-3000 Flexercell Strain Unit (Flexcell, Hillsborough, NC). Frequency of 1 cycle per second was selected to mimic cardiac frequency. Control cells were cultured under the same conditions but no mechanical force was applied.

Transfections

HTM cells were transfected with hsa-miR-24 mimic or control mimic (scramble) (20–40 pmolar) (Dharmacon, Chicago, IL) using an endothelial nucleofactor Kit (Lonza, Basel, Switzerland) or lipofectamine 2000 (Invitrogen), following manufacturer's instructions. Co-transfections of 293A cells with luciferase 3′UTR constructs (0.3 μg), miR-24 mimic or

control mimic (20 pmolar) were accomplished using Effectene (Qiagen, Valencia, CA) following manufacturer's instructions.

RNA isolation, Quantitative PCR (Q-PCR), and miRNA PCR arrays

Total RNA was isolated using a RNeasy kit (Qiagen Inc.) or Trizol (Invitrogen) according to the manufacturer's instructions. RNA yields were measured using RiboGreen fluorescent dye (Invitrogen). First strand cDNA was synthesized from total RNA (1 μg) by reverse transcription using oligodT and Superscript II reverse transcriptase (Invitrogen) according to manufacturer's instructions. Q-PCR reactions were performed in 20 μl mixture containing 1 μl of the cDNA preparation, 1X iQ SYBR Green Supermix (Biorad, Hercules, CA), using the following PCR parameters: 95ºC for 5 min followed by 50 cycles of 95ºC for 15 sec, 65ºC for 15 sec, and 72ºC for 15 sec. β-Actin was used as an internal standard of mRNA expression. The absence of non-specific products was confirmed by both the analysis of the melt curves and by electrophoresis in 3% Super acryl-Agarose gels. The primers used for Q-PCR amplification are shown in Table 1. MicroRNAs were extracted from total RNA using RT^2 qPCR-Grade miRNA isolation kit (SABiosciences, Frederick, MD). For PCR Arrays, miRNAs cDNA (100 ng) were amplified using RT^2 miRNA First Strand Kit and RT^2 miRNA PCR Array (MAH-001A) following manufacturer's instructions (SABiosciences, Frederick, MD). For miR-24 amplification we used TaqMan microRNA reverse transcription kit, specific primers for hsa-miR-24 and U6B, as a standard, and 25 ng of enriched small microRNAs. Q-PCR products were amplified using TaqMan® Universal PCR Master Mix, following manufacturer's instructions (Applied Biosystems, Foster City, CA). The fluorescence threshold value (Ct) was calculated using the iCycle system software. The results were expressed as mean value ± SD in three independent experiments.

Gene microarray analysis

Gene array analysis was conducted in three independent sets of transfections with either miR-24 mimic or mimic control of the same HTM cell line. Total RNA was extracted 3 days post-transfection using RNeasy kit (Qiagen), amplified (1 round amplification) using One cycle target labeling and control reagents (Affymetrix, Santa Clara, CA) and hybridized to Human Genome U133A2 Arrays (Affymetrix) at the Duke University Microarray facility. Raw data were normalized using quantile normalization and analyzed using GeneSpring 10 (Silicon Genetics). ANOVA test was performed (*P*-values ≤ 0.05) for genes differentially expressed using the Benjamin and Hochberg False Discovery Rate correction test. The list of genes was compared to three databases that predict targets for microRNAs: Microcosm (http://www.ebi.ac.uk/enright-srv/microcosm/htdocs/targets/v5/), TargetScan (http://www.targetscan.org), and PicTar-Vert (http://pictar.mdc-berlin.de/). To study the potential biological significance of the changes observed in the arrays, we performed network analysis of differentially expressed genes using Metacore pathway analysis (GeneGo, St. Joseph, MI).

Interaction between miR-24 and *FURIN* 3′-untranslated region (3′-UTR)

The entire 3′UTR from *FURIN* was amplified using the following primers *FURIN* 3′-F-ggCTCGAGgcaagaggggtggagactgc and *FURIN* 3′-R-gggGCGGCCGCctgtgcaccaacccagcatc, respectively, which carried XhoI and NotI restriction sites in the forward or the reverse position. PCR amplifications from 3′UTR and the complementary sequences were confirmed by sequencing and cloned into XhoI and NotI sites downstream of Renilla luciferase in the psiCheck2 vector (Promega, Madison, WI). For analysis of luciferase activity, 293A cells were seeded in 12 well plates, 24 h before transfection, transfected with psicheck 3′UTR or the complementary sequence from *FURIN* (0.3 μg), and miRNAs for miR-24 mimic or control mimic (20 pmolar). Luciferase was

measured using the Dual Luciferase Kit (Promega, Madison, WI) following manufacturer's instructions and read in a TD-20/20 luminometer (Turner Designs, Sunnyvale, CA).

Protein extraction and Western blotting

For protein extraction, cells were harvested 72 h after transfection, washed in PBS and lysated in 1X cold RIPA buffer, and protein concentration was determined using Micro BCA Protein Assay Kit (Pierce, Rockford, IL). Equal loading was run in 8% SDS–PAGE and transferred to PVDF membranes. Membranes were incubated overnight at 4ºC, with antibodies against FURIN or tubulin (Santa Cruz Biotechnology, Santa Cruz, CA). Blots were developed using a chemiluminescence detection system (ECL-Plus from Amersham, Buckinghamshire, UK).

TGFβ1 and TGFβ2 measurements

TGFβ1 and TGFβ2 were measured 72 h after transfection using Quantikine Human TGFβ1 and TGFβ2 (R&D systems, Minneapolis, MN) following manufacturer's instructions. These are "sandwich" enzyme linked immunoassays that measure activated TGFβs. For CMS, supernatants were collected immediately after stretching (3 or 16 h). For other TGFβ measurements, supernatant was collected after 48 h, all media was serum free.

Results

Cyclic mechanical stress induced changes in miRNAs

Three HTM cell lines were seeded by triplicates in collagen bioflex plates and subjected to CMS for 3 h at 20% stretching, 1 cycle per second. Non-mechanical force was applied to the controls. MicroRNAs were extracted from stressed and control cells and analyzed using miRNA PCR Arrays (Supplemental Material). Seven miRNAs were consistently up-regulated in all cell lines (miR-16, miR-27a, miR-27b, miR-7, let-7f, miR-26a, and miR-24) and another nine were significantly up-regulated in two cell lines (Fig. 1).

Changes in gene expression induced by miR-24

Since miR-24 is believed to be involved in the regulation of TGFβ1 signaling, which has been associated with multiple pathologic conditions associated with mechanical stress, we investigated the differences in gene expression induced by miR-24. For this purpose, one HTM cell line was transfected by triplicate with miR-24 or control mimic and the expression profile analyzed using Affymetrix U133A2 chips. Ninety-four genes were significantly ($P \leq 0.05$) up- or down-regulated by miR-24 more than 1.5-fold. Twenty-one of these genes were predicted as putative targets of miR-24 by computational analysis (Table 2 shows genes up- or down- regulated by more than 1.8-fold). To validate Affymetrix microarray data, changes in expression of 12 genes were analyzed by Q-PCR in three independent HTM cell lines (Fig. 2A). To identify pathways, the genes significantly up- or down-regulated by more than 1.5-fold were analyzed using Metacore pathway analysis. The four canonical pathways more significantly affected by miR-24 were Immune Response IL-17 signaling pathway (*P*-value 5.5×10^{-4}), Cytokine production by Th17 cells in cystic fibrosis (8.5×10^{-3}), Immune response IL-1 signaling pathway (7.3×10^{-3}), and Immune response PGE2 signaling pathway (7×10^{-3}) (Fig. 2B).

Targeting of *FURIN* by miR-24

FURIN was among the genes significantly down-regulated by miR-24 and computational predictions indicated that miR-24 shares complementarity with the 3′UTR of *FURIN*. We confirmed that the 3′UTR of *FURIN* interacts with miR-24 using the psiCheck2 luciferase assay system. MiR-24 reduced the luciferase activity in cells co-transfected with FURIN

3′UTR compared to control (scrambled). This effect was prevented when the 3′UTR complementary sequence of *FURIN* was used. *FURIN* down-regulation was confirmed at protein level after transfection with miR-24 or scrambled (Fig. 3A,B).

Regulation of TGFβ1 by miR-24

FURIN is known to play an important role in TGFβ1 processing. To evaluate whether miR-24 could affect the expression of active TGFβ1 we transfected two HTM cell lines with miR-24 or scramble control and measured the activated TGFβ1 and TGFβ2 by ELISA. TGFβ1 was significantly down-regulated by miR-24 by 83 and 54%, and miR-24 antagomir increased activated TGFβ1 by 21 and 22% (Fig. 4A,B). TGFβ2 was not significantly affected by miR-24 in the same experiments (data not showed).

RNA interference against FURIN down-regulated TGFβ1 expression

To evaluate whether specific down-regulation of *FURIN* was enough to inhibit the expression of active TGFβ1 in HTM cells, we transfected two cell lines with small interference RNA against *FURIN* or scramble RNA and measured the activated TGFβ1 by ELISA. si*FURIN* down-regulated significantly (88 and 69%) the amount of activated TGFβ1 compared to the control (Fig. 4C). The knockdown of *FURIN* was confirmed by Q-PCR (Fig. 4D).

MiR-24 prevented the up-regulation of active TGFβ1 induced by CMS

Two HTM cell lines transfected with scramble or miR-24 were subjected to CMS, during 16 h and the amount of activated TGFβ1 was measured by ELISA. TGFβ1 increased significantly in cells subjected to CMS compared to non-stressed cells by 64 and 47%. Cells transfected with miR-24 and subjected to CMS increased TGFβ1 by 35 and 5%, respectively (Fig. 5).

TGFβ1 up-regulated miR-24 expression

In order to know if a feedback exists between miR-24 and TGFβ1, we treated two HTM cell lines with 1 ng per ml of TGFβ1 or TGFβ1 plus a TGFβ1 inhibitor (SB431542, 10 μM) and analyzed the expression of miR24 by Q-PCR. TGFβ1 increased significantly the expression of miR-24 and SB431542 abolished this increase (Fig. 6).

Discussion

Cellular responses to CMS are believed to play an important role in the physiology of the outflow pathway. Our results showed that CMS induced the expression of several miRNAs in HTM cells and that one of them, miR-24, can contribute to the regulation of the levels of TGFβ1 induced by CMS in HTM cells.

To our knowledge the only two previous studies addressing the effects of mechanical stress on miRNA expression in mammalian cells have been conducted in vascular endothelial cells using shear stress as a model (Qin et al., 2010a; Weber et al., 2010). Although the cell types and mode of mechanical stress reported in these publications were very different from those used in our study, an interesting common effect of shear stress in vascular endothelial cells and CMS in HTM cells was the up-regulation of members of the miR-23a/24-2/27a and miR-23b/24-1/27b paralog clusters. Therefore, up-regulation of miRNAs from these two clusters might constitute a common response to different forms of mechanical stress in several cell types.

The MicroRNA-23b/24-1/27b cluster has been demonstrated to regulate transforming growth factor-beta (TGFβ)/bone morphogenetic protein signaling and liver stem cell

differentiation by different mechanisms including the direct targeting of Smads (Rogler et al., 2009). Specifically, miR-24, which is expressed from both miR-23a/24-2/27a and miR-23b/24-1/27b clusters, has been shown to antagonize with TGFβ signaling through post-transcriptional regulation of Tribbles-like protein-3 (Trb3) (Chan et al., 2009Q4). Trb3 mediates degradation of the SMAD specific E3 ubiquitin protein ligase 1, Smurf1 (Chan et al., 2007), which is involved in degradation of Smads and facilitates the antagonistic action of Smad7 by targeting Smad7 at the plasma membrane (Suzuki et al., 2002). Another example of the antagonistic activity of miR-24 with the TGFβ superfamily has been reported in hematopoietic progenitor cells where miR-24 targets the activin type I receptor ALK4 (ACVR1B) that interferes with activin-induced Smad 2 phosphorylation thus delaying activin-induced maturation of hematopoietic progenitor cells in cell cultures (Wang et al., 2008).

Induction of TGFβ is a common response to mechanical stress in several cell types (Yasuda et al., 1996; Li et al., 1998; Skutek et al., 2001; Sakata et al., 2004; Mohamed and Boriek, 2010) including TM (Liton et al., 2005). Such induction of TGFβ contributes to the pathogenesis of multiple conditions such as cystic fibrosis, liver disease, lung fibrosis, tubulointerstitial fibrosis in the kidney, myocardial fibrosis, glomerular sclerosis, diabetic nephropathy, and asthma (Yasuda et al., 1996; Hirakata et al., 1997; Ihn, 2002a, b; Kelly et al., 2003; Lee et al., 2006; Wolf and Ziyadeh, 2007; Kassiri et al., 2009; Mohamed and Boriek, 2010; Rohatgi and Flores, 2010). TGFβ1 is believed to play an important role in both the normal physiology of the TM and the pathogenesis of this tissue in glaucoma (Zhao et al., 2004; Tan et al., 2006; Acott and Kelley, 2008; Fatma et al., 2009; Fuchshofer and Tamm, 2009; Agarwal and Agarwal, 2010). For example, over-expression of TGFβ1 in rat eyes changed the morphology of the anterior segment and increased IOP, and elevation of TGFβ1 has been associated with pseudoexfoliative and neovascular glaucomas (Yu et al., 2007; Robertson et al., 2010; Schlotzer-Schrehardt et al., 2001; Zenkel et al., 2010).

Given the potential involvement of miR-24 in the response to mechanical stress in different cell types and the relevance of TGFβ in the pathogenic responses induced by mechanical stress, we analyzed the influence of this miRNA on gene expression in HTM cells. Our results showed that miR-24 induced significant changes in expression of several genes involved in immune response and cytokine production, including the previously reported target ACVR1B (ALK4, fold −1.94). Our results also showed a significant decrease in expression of the Subtilisin-like proprotein convertase FURIN (fold −1.84), which was confirmed as a novel bona fide target of this microRNA. Targeting of FURIN by miR-24 can be particularly relevant to the understanding of the antagonistic effects of this microRNA with the TGFβ pathway since FURIN is known to play a major role in the processing of TGFβ1 (Dubois et al., 2001; Kusakabe et al., 2008).

TGFβs are secreted as pro-proteins and cleaved to mature TGFβs and their latency associated peptides (LAPs) by the convertase family of endoproteases. After cleavage TGFβs and LAP remain non-covalently attached as latent complexes and secreted to the pericellular space associated with the ECM (Koli et al., 2001; Annes et al., 2003; ten Dijke and Arthur, 2007) (Dubois et al., 2001; Kusakabe et al., 2008; Hynes, 2009). Consistent with the role of FURIN on the processing of TGFβ1, miR-24 was able to decrease the levels of active TGFβ1 in HTM cell cultures, and this effect was mimicked by the inhibition of FURIN expression with a specific siRNA. The observed lack of effects of miR-24 on the presence of active TGFβ2 was also consistent with the known insensitivity of latent TGFβ2 to FURIN that results from the differences between the tertiary structure of the LAP regions of TGFβ1 and TGFβ2 (Kusakabe et al., 2008).

Our results also indicated that induction of miR-24 might contribute to regulate the amount of TGFβ1 activated by CMS in HTM cells since miR-24 mimic decreased and miR-24 antagomir increased the levels of active TGFβ1 produced by HTM. Furthermore, the observation that miR-24 was up-regulated by TGFβ1 in HTM cells suggests the presence of a negative feedback loop between TGFβ1 and miR-24 in which miR-24 might be up-regulated as a homeostatic response to increased levels of active TGFβ1. Thus, the up-regulation of miR-24 during CMS could potentially serve to prevent excessive activation of TGFβ1 and limit some of the pathogenic effects of this cytokine in the outflow pathway. The up-regulation of miR-24 by TGFβ1 observed in HTM cells may be cell-type or context dependent, since TGFβ1 has been reported to both, repress miR-24 transcription during skeletal muscle differentiation (Konishi et al., 1991), and induce the expression of the miR-23a/27a/24 cluster in Huh-7 cells (Huang et al., 2008).

In addition to its role in regulating the TGFβ superfamily, miR-24 is believed to act as a tumor suppressor by regulating cell cycle progression, apoptosis, and DNA damage responses through several validated targets (Lal et al., 2009a, b; Mishra et al., 2009; Qin et al., 2010b; Takagi et al., 2010; Zaidi et al., 2009). The observed targeting of *FURIN* by miR-24 could also be particularly relevant to the tumor suppressor effects mediated by miR-24. FURIN is known to play an important role in the acquisition of malignant phenotype and metastatic potential in tumor cells by processing growth factors (Basak et al., 2010), by enable tumors to evade the antiangiogenic effects of sema3B (Varshavsky et al., 2008), and also inhibition of FURIN has been shown to significantly inhibit invasion and migration of human cancer cells (Bassi et al., 2001; Zhou et al., 2009).

In conclusion, CMS induced significant alterations in the expression of several microRNAs that could contribute to the regulation of some of the responses to mechanical stress in HTM cells. Specifically, up-regulation of miR-24 and the subsequent down-regulation of its target *FURIN* may serve as a homeostatic mechanism to limit the amount of TGFβ1 activated by CMS and to prevent some potentially pathogenic effects of this cytokine in the outflow pathway.

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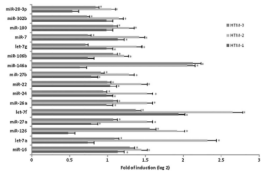

Fig. 1.

Changes in miRNA expression[Q3] induced by CMS in HTM cells. Three primary HTM cell lines were subjected to CMS (20% stretching, 1 cycle per second) for 3 h. Changes in microRNA expression were analyzed using miRNA PCR Arrays (MAH-001A). Control cells were incubated under the same conditions but no mechanical force was applied. The figure represents the logarithm of fold change in expression induced by CMS. Bars represent standard deviation from three different experiments; one asterisks means $P \leq 0.05$.

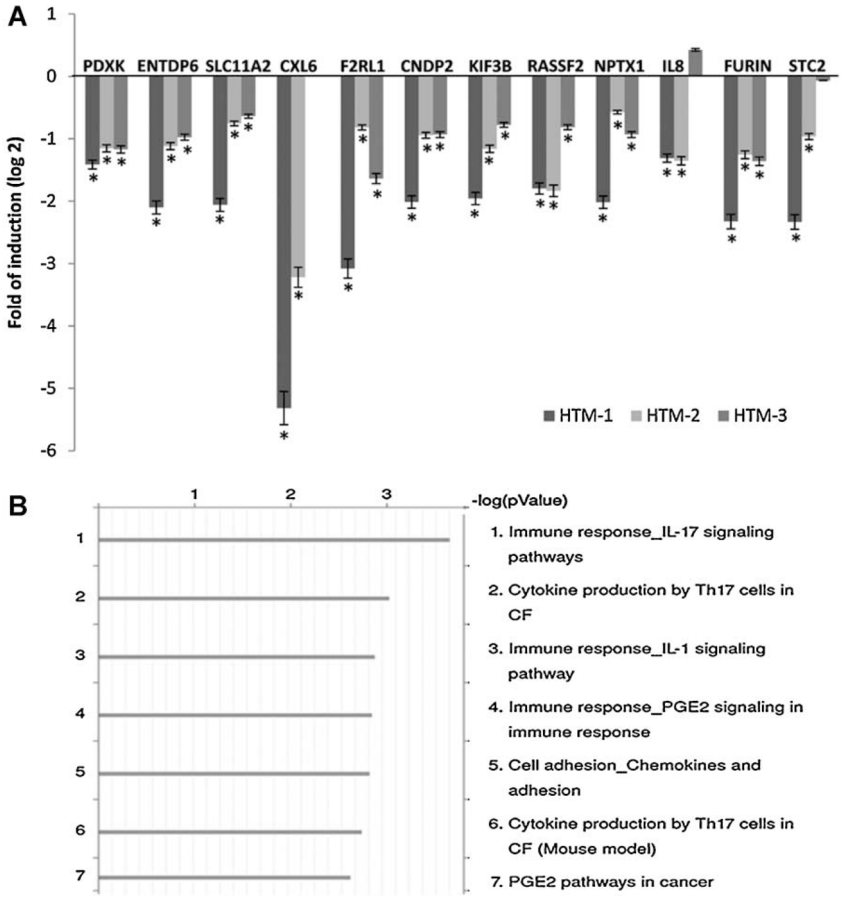

Fig. 2.

Validation of Affymetrix microarray data and analysis of canonical pathways affected by miR-24. Panel A represents the logarithm of the fold change on gene expression of HTM cells transfected with miR-24 mimic compared to cells transfected with scramble control in three primary cell lines. The expression of 12 genes significantly down-regulated on the array (Gene symbol: *PDXK*, *ENTDP6*, *SLC11A2*, *CXXL6*, *F2RL1*, *CNDP2*, *KIF3B*, *RASF2*, *NPTX1*, *IL8*, *FURIN*, and *STC2*) were analyzed by Q-PCR. Bars represent standard deviation from three different experiments; one asteriskmeans $P \leq 0.05$. Panel B represents the seven canonical pathways most significantly affected by miR-24 mimic compared to controls and was generated using Metacore pathway analysis and the genes significantly up- or down-regulated by more than 1.5-fold ($P \leq 0.05$) on the Affymetrix U133A2 arrays (CF = cystic fibrosis).

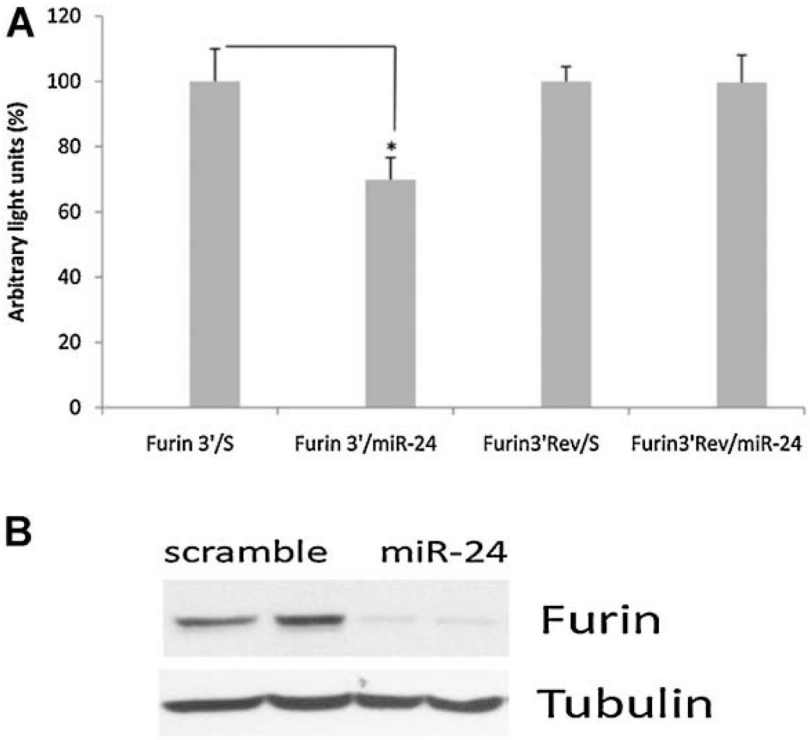

Fig. 3.

FURIN is a novel target of miR-24. Panel A represents the luciferase activity (%) in 293 cells co-transfected with psicheckvectors, containing the 3′UTR or complementary sequence (R) from FURIN, and miR-24, compared to cells co-transfected with the same vectors and scramble control. Bars represent standard deviation from three different experiments; two asterisks means $P \leq 0.01$. In panel B HTM cells were transfected with miR-24 mimic or scramble control and the expression of FURIN and tubulin were assayed by Western blot 3 days after transfection.

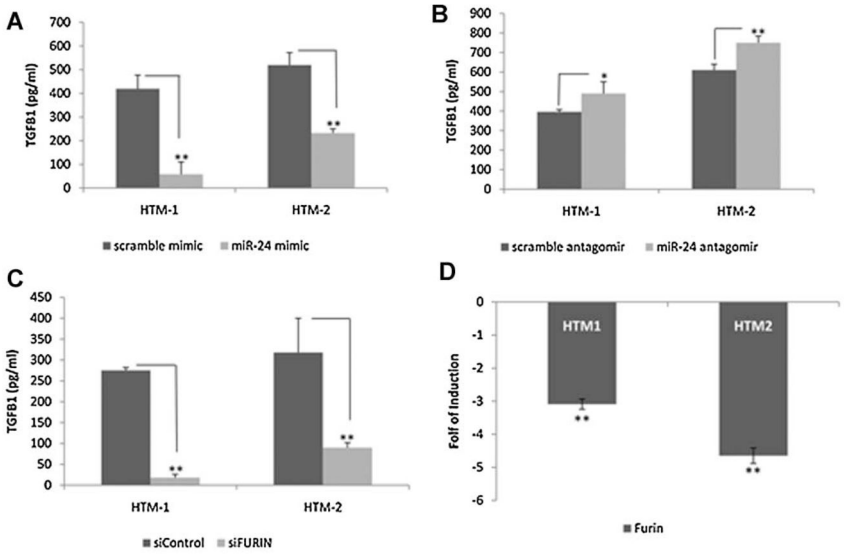

Fig. 4.

Effects of changes in expression of miR-24 and FURIN on the production of active TGFβ1 in HTM cells. Activated TGFβ1 (pg/ml) was measured by ELISA on cell culture supernatant from two HTM cell lines transfected with miR-24 mimic, scramble control, si*FURIN* or siControl. Panel A represents TGFβ1 expression on cells transfected with miR-24 mimic or scramble control. Panel B represents a similar experiment in cell lines transfected with miR-24 antagomir or scramble antagomir. Panel C represents cell lines transfected with RNA interference against *FURIN* (si*FURIN*)or RNA interference control (siControl). Panel D shows the down-regulation of *FURIN* mRNA in the same experiments, measured by Q-PCR. Bars represent standard deviation from three different experiments. One, two, and three asterisks means $P \leq 0.05$, 0.01, and 0.005.

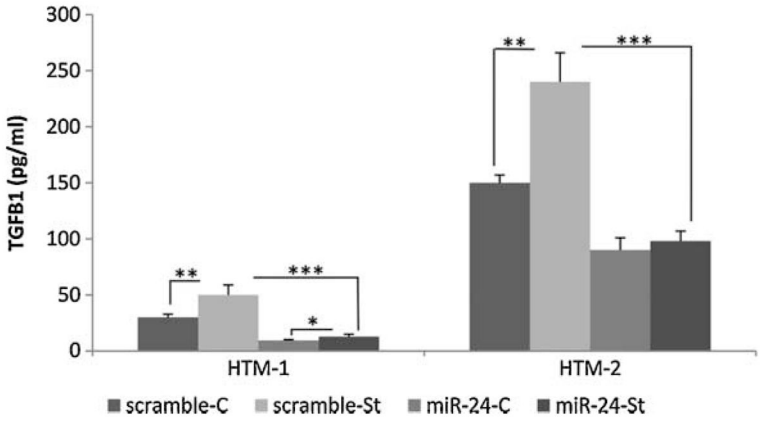

Fig. 5.

Increase in active TGFβ1 induced by CMS was prevented by miR-24. Figure represents the amount of TGFβ1 (pg/ml) in two HTM cell lines transfected with miR-24 or scramble control and subjected to CMS for 16 h (St) or no mechanical force was applied in the control (C). Bars represent standard deviation from three different experiments. One, two, and three asterisks means $P \leq 0.05$, 0.01, and 0.001.

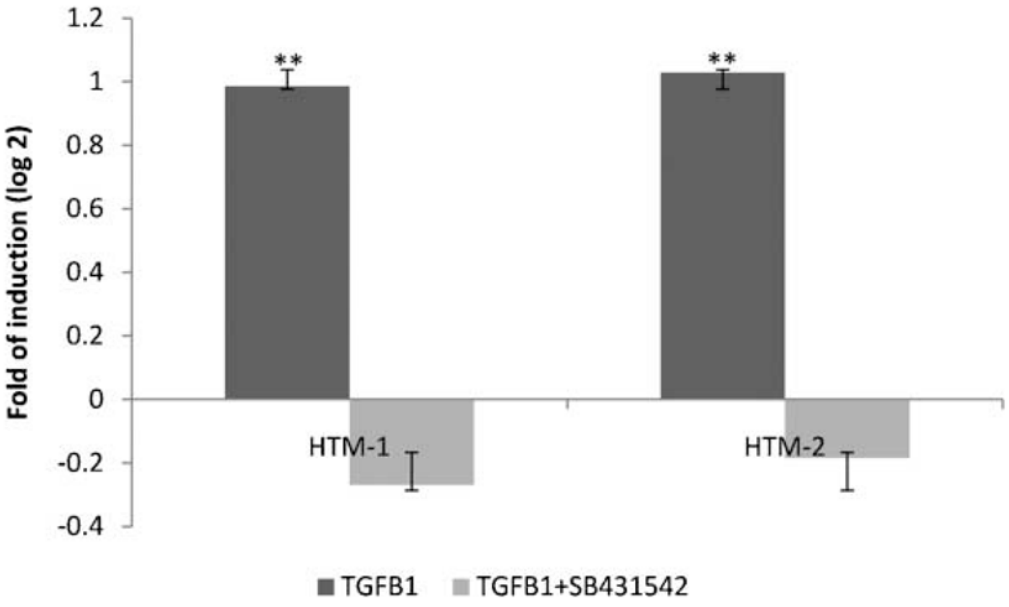

Fig. 6.

TGFβ1 increased expression of miR-24. Figure represents the logarithm of the fold change of miR-24 after 24 h of treatment with TGFβ1 (1 ng/ml) or TGFβ1 plus SB431542 (10 μM), a TGFβ1 inhibitor. Bars represent standard deviation from three different experiments in two HTM cell lines; two asterisks means $P \leq 0.01$.

TABLE 1

Primers used for Quantitative PCR

Gene symbol	Forward 5′-3′	Reverse 5′-3′
CNDP2	GCACAGCCACAAGAAAGACA	ATGGAGAACTTGCCAACCAC
F2RL1	GATGTGGTCCAAACCCTCTG	TTTTGCCACTTAGAATAGCATTTG
KIF3B	CCAGGGAGCTGAAACTCAAG	TCTGGACGAATCATCATGGA
RASF2	GGTCTTCCTGCACTTGAAGC	CACATAGGTGGCTGCTCAGA
CXCL6	TTGAAGAGTGTGGGGGAAAG	CCACAGCCTTTTCGGTAAGA
SLC11A2	ATGTCCTTCCTGGACTGTGG	CACACTGGCTCTGATGGCTA
NTPX1	TGTACGCCTTCACTGTCTGC	TGATGACAAAAGGCAGCTTG
PDXK	GTCTCCGTGTTTGTCCCTGT	TTTTCACAAAGCACGACTGG
ENTDP6	TTGCCTCTTCCTTGGGTATG	ACGTCACTCAAGCAGCACAG
STC2	TCTGCACCTCGGCCATCCAG	TCAGAATACTCAGACTGTTC
IL8	AGGACAAGAGCCAGGAAGAA	ACTGCACCTTCACACAGAGC
FURIN	ACAACTATGGGACGCTGACC	TGGACACAGCTCTTCTGGTG
ACTB	CCTCGCCTTTGCCGATCCG	GCCGGAGCCGTTGTCGACG

TABLE 2

Genes up- or down-regulated by 1.8-fold in human trabecular meshwork cells after over expression of miR-24 mimic

Gene symbol	Fold change	Regulation	*P*-value	Database	Gene title
PTHLH	3.0565975	Down	3.97E-04	M,T,P	Parathyroid hormone-like hormone
CNDP2	2.4332333	Down	4.35E-05		CNDP dipeptidase 2
IL8	2.2841344	Down	1.86E-04		Interleukin 8
F2RL1	2.2676232	Down	4.22E-05	T	Coagulation factor II (thrombin) receptor-like 1
IL8	2.2576025	Down	4.99E-04	P	Interleukin 8
KIF3B	2.2168906	Down	2.55E-05		Kinesin family member 3B
GALNT12	2.1888528	Down	0.0029251	M	UDP-N-acetyl-alpha-D-galactosamine
RASSF2	2.15163	Down	2.69E-06		Ras associationdomain family member 2
CXCL6	2.13901	Down	0.0017257		Chemokine (C-X-C motif) ligand 6
SLC11A2	2.0708907	Down	1.35E-04		Solute carrier family 11, member 2
C14orf2	2.0584471	Down	2.15E-05		Chromosome 14 open reading frame 2
ANXA10	2.0433328	Down	3.64E-04		Annexin A10
NPTX1	2.027866	Down	0.0187071		Neuronal pentraxin I
PDXK	2.0209773	Down	1.01E-05	T,P	Pyridoxal kinase
ENTPD6	2.0142882	Down	1.31E-04		Ectonucleoside triphosphate diphosphohydrolase 6
KCNK3	1.9923251	Down	1.82E-04	T,P	Potassium channel, subfamily K, member 3
STC2	1.9881191	Down	1.43E-05	T	Stanniocalcin 2
HHLA3	1.9669507	Down	0.0013616		HERV-H LTR-associating 3
CXCL1	1.9485825	Down	2.07E-04		Chemokine (C-X-C motif) ligand 1
ACVR1B	1.9390614	Down	2.84E-04	M,T	Activin A receptor, type IB
MED16	1.9286143	Down	6.86E-04		Mediator complex subunit 16
STC2	1.9134991	Down	5.14E-05		Stanniocalcin 2
SYT1	1.9131047	Up	0.0040418		Synaptotagmin I
MICB	1.8921673	Down	6.76E-05		MHC class I polypeptide-related sequence B
AVL9	1.8742172	Down	2.70E-06		AVL9 homolog (S. cerevisiase)
PTGS2	1.8690909	Down	6.22E-05		Prostaglandin-endoperoxide synthase 2
GRINA	1.8647411	Down	0.0017328		Glutamate receptor, ionotropic
BTN3A2/BTN3A3	1.857413	Down	1.95E-04		Butyrophilin, subfamily 3, member A2//A3
FURIN	1.8437475	Down	9.03E-04		Furin (paired basic amino acid cleaving enzyme)

Gene symbol	Fold change	Regulation	*P*-value	Database	Gene title
STC1	1.8405004	Down	2.87E-05		Stanniocalcin 1
RAP2C	1.839736	Down	4.87E-04		RAP2C, member of RAS oncogene family
ADAM12	1.8122648	Down	6.18E-04		ADAM metallopeptidase domain 12
GREM1	1.8109983	Down	5.69E-04		Gremlin 1, cysteine knot superfamily, homolog
GREM1	1.8081648	Down	1.07E-04	M	Gremlin 1, cysteine knot superfamily, homolog

M, T, P means Microcosm, TargetScan, and PicTar-Vert miRNA target prediction databases.