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MicroRNA-15b is decreased during cardiac fibrosis and inhibits cardiac fibroblast activation through targeting of the small GTPase intermediates Growth Factor Receptor-Bound 2 (Grb2) and Son-of-Sevenless homologue (SOS) 1 and SOS2

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ABSTRACT

Background
In the heart, chronic injury results in the accumulation of extracellular matrix (ECM). The accumulation of fibrotic tissue might culminate in the progressive decline in cardiac function and, ultimately, heart failure. A pivotal process in cardiac fibrogenesis is fibroblast activation, entailing fibroblast proliferation, myofibroblast differentiation and excessive ECM production. Although canonical Transforming Growth Factor β (TGF-β) signaling is well-established as an inducer of cardiac fibroblasts activation, non-canonical TGF-β signaling via small GTPases has emerged as a second regulatory mechanism of fibroblast activation. We found that the expression of microRNA (miR)-15b is decreased in fibrotic hearts and identified the small GTPase intermediates Growth Factor Receptor-Bound 2 (Grb2) and Son-of-Sevenless homologue (SOS) 1 and SOS2 as putative targets of miR-15b. Therefore, we hypothesize that miR-15b might inhibit cardiac fibroblast activation by the inhibition of non-canonical TGF-β signaling.

Methods
Cardiac fibrosis was induced in mice by transverse aortic constriction and miR-15b expression was analyzed in the hearts of mice with and without aortic constriction. Binding of miR-15b to the 3'-UTR region of its putative gene targets was investigated using dual luciferase reporter assays. In vitro, cardiac fibroblasts transfected with miR-15b mimics or scrambled control sequences were stimulated with TGF-β1 and the gene expression of small GTPase intermediates (Grb2, SOS1 and SOS2), ECM components (COL1A1 and COL3A1) and myofibroblast markers (ACTA2 and CNN1) were analyzed. At the functional level, gel contraction assays were performed and fibroblast proliferation was assessed. Finally, the activity of the small GTPase Ras was measured in TGF-β-stimulated cardiac fibroblasts that were transfected with miR-15b mimics or scrambled control sequences.

Results
Aortic constriction induced left ventricular fibrosis in mice. In the fibrotic mouse hearts, miR-15b expression was analyzed in the hearts of mice with and without aortic constriction. Binding of miR-15b to the 3'-UTR region of its putative gene targets was investigated using dual luciferase reporter assays. In vitro, cardiac fibroblasts transfected with miR-15b mimics or scrambled control sequences were stimulated with TGF-β1 and the gene expression of small GTPase intermediates (Grb2, SOS1 and SOS2), ECM components (COL1A1 and COL3A1) and myofibroblast markers (ACTA2 and CNN1) were analyzed. At the functional level, gel contraction assays were performed and fibroblast proliferation was assessed. Finally, the activity of the small GTPase Ras was measured in TGF-β-stimulated cardiac fibroblasts that were transfected with miR-15b mimics or scrambled control sequences.

Conclusion
During cardiac fibrosis, miR-15b expression is decreased, which culminates in the activation of cardiac fibroblasts. Maintaining the expression of miR-15b, via the exogenous delivery of miR-15b mimics precludes cardiac fibroblast activation by TGF-β1, which might be due to the decreased expression of the small GTPase intermediates Grb2, SOS1 and SOS2. Therefore, the decrease of miR-15b expression during cardiac fibrosis might contribute to the progression of heart failure and could thus be used as a therapeutic target.

INTRODUCTION

Chronic fibroproliferative disease, leading to organ failure, is the cause of nearly 45% of deaths in the developed world1. A hallmark feature of fibrosis in any organ is fibroblast activation, i.e. fibroblast proliferation, myofibroblast differentiation and ECM deposition1-4. Chronic injury leads to a damage repair response by the resident tissue cells4. However, if this repair response becomes dysregulated and fibroblast activation persists, it might culminate in organ fibrosis.

In the healthy heart, ECM production and degradation are in homeostasis. In response to chronic injury, ECM accumulates in the cardiac tissue, which results in stiffening of the heart and reduced cardiac contractility. Furthermore, cardiac ECM accumulation can impair the electrophysiological coupling of cardiomyocytes 4 and might result in compensatory cardiac hypertrophy. Combined, these processes might result in the progressive loss of cardiac function and finally heart failure5. Transforming Growth Factor (TGF) B1 and B2, play a pivotal role in the development of heart failure through the induction of cardiac fibroblast activation6-14.

TGF-β induces a pro-fibrotic response through the activation of its canonical and non-canonical signal transduction pathways. The binding of TGF-β to its canonical receptor complex composed of TGF-β receptor (TGFBR) 1 and TGFBR27, results in the activation of Smad2/3 complexes, which is extensively studied in the context of cardiac fibrosis8. Yet, non-canonical TGF-β signaling also contributes to the development and progression of cardiac fibrosis7-10. Recently, non-canonical TGF-β signal transduction via TGFBR1/2 and the associated small GTPases has emerged as regulatory mechanism of fibroblast activation and fibrosis11-13. Small GTPases are cell membrane-bound G-proteins, which alternate between an active GTP and an inactive GDP state14. When TGF-β binds to its receptor complex, the small GTPase intermediates Growth Factor Receptor-Bound 2 (Grb2) and Son-of-Sevenless homologue (SOS) 1 and SOS2 are recruited to the receptor complex, which activate the downstream GTPases 8,11. Ultimately, the TGF-β-induced activation of small GTPases might culminate in fibroblast activation12,13, and the inhibition the small GTPase Ras can limit fibrogenesis14,15.

MicroRNAs (miRs) are small (20-25 nucleotides), non-coding RNAs that regulate gene expression at the post-transcriptional level through their interaction with their target mRNA16. MiRs interact with their target mRNAs through imperfect base pairing to the 3’ untranslated region (3’UTR) of its target mRNA. Upon the formation of a miR-mRNA duplex, translational repression of the mRNA occurs by either the degradation of the mRNA or by blocking of translation initiation or elongation11. In cardiac physiology, miRs are one of the regulatory mechanisms that orchestrate

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proliferation, differentiation and angiogenesis, whereas in cardiac pathology, dysregulated miR expression might induce or facilitate the pathogenesis.

We found that miR-15b expression is decreased during cardiac fibrosis and identified miR-15b as a putative modulator of non-canonical TGF-β signaling via the small GTPases. Using bioinformatics tools (i.e., TargetScan and mirANDA algorithm), we uncovered that miR-15b might target the small GTPases Grb2, SOS1 and SOS2. We hypothesized that miR-15b might inhibit cardiac fibroblast activation by targeting these small GTPase intermediates. Subsequently, we investigated the role of miR-15b on non-canonical TGF-β signaling during cardiac fibroblast activation.

**MATERIALS AND METHODS**

**Transverse aortic constriction cardiac fibrosis mouse model**

Male C57Bl/6j mice, aged 10-12 weeks, were purchased (Harlan, Horst, The Netherlands). Animals were randomly assigned to the experimental groups. Aortic banding was performed to induce cardiac fibrosis in 3 groups as described previously. In brief, under anesthesia, the ascending aorta was visualized through an incision in the second intercostal space. A suture was placed around the aorta and a blunt 27G needle was placed onto the aorta. The suture was tightened around the aorta and needle to constrict the aorta after which the needle was removed to allow a standardized opening of the aorta. In sham animals, the same operative procedure was performed, except for placement of the constricting suture. Two weeks after the aortic banding, animals were sacrificed by exsanguination under general anesthesia. Hearts were collected for histochemistry and gene expression analyses. The local Committee for Animal Experimentation (University of Groningen, University Medical Centre Groningen) approved all experimental procedures.

**Cardiac fibroblast culture, microRNA transfection and stimulation**

Experiments were conducted with mouse or human cardiac fibroblasts (CF). Mouse CF were isolated from adult mouse hearts. Hearts were minced into small tissue pieces and subjected to 0.1% collagenase (Roche Diagnostics, Mannheim, Germany) digestion. Digested tissue was filtered through a 70µm cell strainer to obtain a single cell suspension. Cells were pelleted by centrifugation and plated at a density of 5,000 cells/cm². Cells were selected by puromycin (6µg/ml; Invitrogen, Carlsbad, CA).

Human CF were purchased (Promocell, Heidelberg, Germany) and culture expanded in DMEM with 10% FBS, 2mM L-glutamine, 1% penicillin/streptomycin, 10ng/mL recombinant human fibroblast growth factor 2 (FGF-2; Peprotech, Rocky Hill, NJ) and 1x Insulin-Transferrin-Selenium (ITS; Gibco, Grand Island, NY). Approximately a week before transfection and stimulation, CF were switched to culture medium without FGF-2 and ITS.

CF were transfected with pre-miR-15b (cat# PM10904, Ambion Life Technologies, Carlsbad, CA) or scrambled miR control sequences (cat# AM171110, Ambion Life Technologies, Carlsbad, CA) with the siRNA Reagent System (Santa Cruz Biotechnology, Heidelberg, Germany) in accordance with the manufacturer’s instructions. For lentiviral expression of miR-15b or scrambled control sequences, pGreenPuro-miR-15b and pGreenPuro-SCR were generated by unidirectional cloning of oligonucleotides (Biolegio, Leiden, The Netherlands) encoding the pre-miR-15b or a scrambled sequence into the BamH1/EcoR1 sites of the pGreenPuro vector (Systembio). Vectors were enumerated in competent Top10 E.coli (Invitrogen, Carlsbad, CA) and isolated using the Qiagen miniprep kit. Correct insertion of the oligonucleotides was validated by sequencing (Basesearch, Leiden, The Netherlands). HEK293T cells were transfected with pGP-miR-15b or pGP-SCR, pVSVG (envelope plasmid) and pCMV-R8.91 (gag-pol 2nd generation packaging plasmid) using the Endofectin-Lenti (Gene Copoeia, Rockville, MD) transfection reagent. 24-72 hours post-transfection, viral supernatants were collected in DMEM/F-12, centrifuged at 500g for 10 min and filtered through a 0.45µm PES filter to remove any remaining cell debris. Viral supernatants were supplemented with polybrene (6µg/ml; Sigma, St. Louis, MO) and applied on 30% confluent CF for three consecutive rounds of 24h exposure. Transduced CF were passaged once and transduced cells were selected by puromycin (6µg/ml; Invitrogen, Carlsbad, CA).

After transfection and prior to stimulation, cells were synchronized for 24h in DMEM with 0.5% FBS, 2mM L-glutamine and 1%penicillin/streptomycin. For experiments, CF were stimulated with 5ng/mL TGF-β1 (R&D Biosystems, Abingdon, UK) and/or 10μM of the small GTPase inhibitor farnesyl thiosalicylic acid (FTS; Cayman Chemical, Ann Arbor, MI) in DMEM/F-12 supplemented with polybrene (6µg/ml; Sigma, St. Louis, MO) and 100ng/mL Bone morphogenetic protein (BMP7; R&D Biosystems, Abingdon, UK) or 10% FBS, 2mM L-glutamine and 1% penicillin/streptomycin. DMSO was used as vehicle control. Cells were cultured under experimental conditions for 24 hours (proliferation assessment by Ki67 immunofluorescence) or 72h (other assays).

**Histology**

Mouse hearts were formalin fixed and paraffin embedded. Four µm transverse sections were deparaaffinized and stained with Masson’s Trichrome to enumerate in Masson’s Trichrome negative nuclei were counted and enumerated in 5 random fields (10x visual field). Labeled nuclei were counted and enumerated in Masson’s Trichrome-stained sections. Between each staining solution, slides were extensively washed with demi-water. Finally, slides were incubated with 1% glacial Acetic Acid, were mounted in toluene solution and visualized by light microscopy.
Gene and microRNA transcript analysis
Total RNA was isolated using Trizol Reagent (Invitrogen, Carlsbad, CA) in accordance with the manufacturer’s instructions. RNA quantity was assessed spectrophotometrically (Nanodrop, Wilmington, DE) and RNA integrity was assessed by gel electrophoresis. For gene expression analysis, 500ng total RNA was reverse transcribed into cDNA using the First Strand cDNA synthesis kit and random hexamer primers (Applied Biosystems, Carlsbad, CA) according to the manufacturer’s instructions. Quantitative polymerase chain reaction (qPCR) was performed using 10ng cDNA, human or mouse gene specific primers (all from Biolegio, Leiden, the Netherlands; Primer sequences, see Table 1) and Fast Start SYBR green (Roche, Almere, the Netherlands).

For miR expression analysis, RNA was reverse transcribed using the TaqMan microRNA reverse transcription kit (Applied Biosystems, Carlsbad, CA) and specific miR-15b and RNU-6B stem loop primers. For qPCR analysis, 20ng cDNA and specific primers for miR-15b and RNU-6B and a common antisense primer (all from Biolegio, Leiden, the Netherlands; Primer, see Table 2) and Fast Start SYBR green were used. For mRNA and miR expression analyses, reactions were run on a Via7 real time PCR machine (Applied Biosystems, Carlsbad, CA) and analyzed using the Via7 software. All reactions were performed in duplicate. Gene expression was calculated using the ΔΔCt method. GAPDH (for mRNA) and RNU-6B (for miRs) were used as reference genes. Data are presented as fold change compared to control.

3′UTR reporter assays
3′UTR reporter assays were performed as described previously. In brief, 3′UTR fragments were isolated from a cDNA pool derived from various human tissues using specific primers (Biolegio, Leiden, The Netherlands). Primer sequences are found in Table 3. Primers were extended with restriction sequences and amplification was performed. The amplicon size was validated by agarose gel electrophoresis. The purified amplicons were cloned into a dual luciferase reporter vector, psiCHECK-2 (Promega, Madison, WI). COS7 cells were transfected with the UTR reporter plasmids and 50nM pre-miR-15b or scrambled control (Ambion Life Technologies, Carlsbad, CA). After 48h, luciferase activity was assayed using the Dual-Glo Luciferase Assay kit (Promega, Madison, WI) to assess binding of miR-15b to the reporter plasmids.

Immunoblot analyses
Protein levels of Grb2 and SOS1 were determined by use of immunoblot analyses. The immunoblot procedures were conducted as previously described. The primary antibodies rabbit anti-Grb2 (1:1,000; cat# sc-255, Santa Cruz Biotehnologies, Santa Cruz, CA), rabbit anti-SOS1 (1:2,000; cat# D377T, Cell Signaling Technology, Beverly, MA), rabbit anti-β-actin (1:1,000; cat# 4967L, Cell Signaling Technology, Beverly, MA) and rabbit anti-GAPDH (1:1,000; cat# ab9485, Abcam, Cambridge, UK) were used. The secondary antibody goat anti-rabbit IRdye680 (1:10,000; cat# 925-68021, Li-Cor Biosciences, Bad Homburg, Germany) was used. Proteins were visualized using an Odyssey infrared imaging system (Li-Cor Biosciences, Bad Homburg, Germany). Densitometry analysis was performed using the TotalLab software (Nonlinear Dynamics, Newcastle-upon-Tyne, UK).

Ras activation assays
Ras activation was assessed using a Ras Activation ELISA assay kit (Millipore, Temecula, CA) in accordance with the manufacturer’s instructions. Cells were lysed in the supplied lysis buffer with a protease inhibitor (Sigma Aldrich, St. Louis, MO). The samples were sonicated and the protein concentrations were measured using the Quick Start Bradford protein assay (BioRad Laboratories, Hercules, CA). 100μg protein per experimental condition was used. 5 minutes after addition of the chemiluminescent substrates, the signal was recorded for 1 second on a Luminoskan ASCENT imaging system (Li-Cor Biosciences, Bad Homburg, Germany). Densitometry analysis was performed using the TotalLab software (Nonlinear Dynamics, Newcastle-upon-Tyne, UK).

Table 1 | Primer sequences for mRNA qPCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward sequence</th>
<th>Reverse sequence</th>
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<tr>
<td>ACT2</td>
<td>CTGTCCAGCCATTCAT</td>
<td>TCAAGTCGAGTTTGT</td>
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<td>CNI1</td>
<td>CCAACCATACAGGTGAG</td>
<td>TCACTGTTTTCCTGG</td>
</tr>
<tr>
<td>COL1A1</td>
<td>GGGATCCTGGACCTTAA</td>
<td>GGAACACCTGTCCTCA</td>
</tr>
<tr>
<td>COL3A1</td>
<td>CTGGACCCCCAGGTTCCTT</td>
<td>CATCTGATCTCCTCTCA</td>
</tr>
<tr>
<td>Grb2</td>
<td>CCAAGAATCATAGAATAATGACACCA</td>
<td>CCGTGTTTTTGAAGATCATTT</td>
</tr>
<tr>
<td>SOS1</td>
<td>GAGTCAAGGTTTCTTTCACCA</td>
<td>GACTCTTAGGCTTTTTG</td>
</tr>
<tr>
<td>SOS2</td>
<td>GGTTCGAGCCTTTTGAAG</td>
<td>GATAAGGGCTTCAAGATACGA</td>
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Table 2 | Primer sequences for miR cDNA synthesis and qPCR

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<th>Stemloop</th>
<th>Forward sequence</th>
<th>Reverse sequence</th>
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<td>miR-15b</td>
<td>GGTGTCACGCTCGGAGGTTCGCACTGTGCAGGAGTTTGGCAGAGAAACC</td>
<td>CCGGTCAGCGGCCGTCGG</td>
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<td>RNU6B</td>
<td>GTTCGTAGGAGGTCGGTACGAGTGTGCAGGAGTTTGGCAGAGAAACC</td>
<td>CCGGTCAGCGGCCGTCGG</td>
<td>TCGGGTACGCAGCAGCATCAT</td>
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Table 3 | Primer sequences 3′UTR reporter assays / 3′UTR fragments

<table>
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<th>miRNA</th>
<th>Stemloop</th>
<th>Forward sequence</th>
<th>Reverse sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Grb2</td>
<td>GAGGAGAAGACCTATTTTAAAG</td>
<td>GAAGAATCCGAGTTTATTTTATTAC</td>
<td></td>
</tr>
<tr>
<td>SOS1</td>
<td>GTTCCTCTGACTGGAGATCATTT</td>
<td>GTGGGCTAGAAAGCTT</td>
<td></td>
</tr>
<tr>
<td>SOS2</td>
<td>CTTTACATATGATCATTT</td>
<td>CTTTAAATTTAAGACTGGGGTG</td>
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</tbody>
</table>
Chapter 8

MicroRNA-15b inhibits cardiac fibroblast activation through targeting of Grb2 and SOS1 and 2

MIR-15b mimics target the small GTPase intermediates Grb2, SOS1 and SOS2

Online bioinformatics tools (TARGETScan and the miRANDA algorithm) imply that the downstream mediators of non-canonical TGF-β signaling, i.e., the small GTPase intermediates Grb2, SOS1 and SOS2 are putative targets of miR-15b (Fig. 2A). The co-transfection of Grb2, SOS1 and SOS2 reporter constructs with miR-15b mimics into COS7 cells reduced the reporter activity compared to the scrambled controls (Fig. 2B), indicating a direct repression of miR-15b on the 3'-UTRs of these genes. Thus, we established that Grb2, SOS1 and SOS2 are genuine miR-15b targets.

Gel contraction assay

For gel contraction assays, CF transfected with pre-miR15b or a scrambled control sequence were treated with TGF-β1 and/or FTS or vehicle controls for 3 days. Afterwards, CF were detached and the cell number was determined using a hemocytometer (Beckman Coulter, Palo Alto, CA). CF were resuspended in DMEM with 0.5% FBS and mixed with high concentration rat tail collagen (Corning, New York, NY) and the pH of this mixture was adjusted using NaOH (Sigma-Aldrich, St. Louis, MO), HEPES (Gibco, Grand Island, NY) and 10xPBS according to the manufacturer’s instructions. 100μl of this cell-collagen mixture was pipetted into the wells of a 96 wells plate. The gels were made in triplicate for each experimental condition. The final gels consisted of 3x10^5 cells/mL and 2.4mg/mL collagen. The collagens gels were allowed to polymerize for one hour after which the gels were circumferentially detached with a 25G needle. Appropriate media with TGF-β1 and/or FTS or vehicle controls were added and the gels were allowed to contract. 72h after detachment, the collagens gels were scanned with a flatbed scanner. The gel surface area was determined using ImageJ (NIH, Bethesda, ML).

Statistical analyses

The data are derived from at least three independent experiments, unless stated otherwise. For statistical analyses, GraphPad Prism version 5 (GraphPad Software Inc., La Jolla, CA) was used. Data are presented as mean±SEM, unless stated otherwise. Data were analyzed using a one-way analysis of variance (ANOVA) with a Bonferroni post-hoc test, unless stated otherwise. P-values <0.05 were considered statistically significant.

RESULTS

MIR-15b expression is decreased in cardiac fibrosis and in TGF-β1-activated cardiac fibroblasts

Aortic banding in mice caused a distortion of the cardiac architecture and the deposition of high amounts of ECM between the muscle fibers, compared to the compact myocardial tissue in the sham control hearts (Fig. 1A). On average, aortic banding for 14 days increased the ECM amount in the left ventricle from ~3 to ~8% (Fig. 1B). In non-fibrotic hearts, miR-15b expression could be readily detected, whereas in the hearts of banded mice, miR-15b expression was decreased (Fig. 1C).

We questioned if the decrease in miR-15b expression levels would also occur in TGF-β1-activated cardiac fibroblasts. CF readily expressed miR-15b, however, in response to TGF-β1 stimulation, CF decreased the expression of miR-15b (Fig. 1D).

ab7817, Abcam, Cambridge, UK) primary antibodies. The secondary antibodies, donkey anti-rabbit AlexaFluor 594 and donkey anti-mouse AlexaFluor 594 (both 1:500; both from Molecular Probes, Carlsbad, CA) were used for signal development. Images were acquired using a Zeiss Axio Observer. Z1 microscope (Carl Zeiss, Mainz, Germany) in fluorescence mode. The images were analyzed using the TissueFAXS Immunofluorescence Cell Analysis system (TissueGnostics USA Inc., Tarzana, CA).

Gel contraction assay

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We questioned if the decrease in miR-15b expression levels would also occur in TGF-β1-activated cardiac fibroblasts. CF readily expressed miR-15b, however, in response to TGF-β1 stimulation, CF decreased the expression of miR-15b (Fig. 1D).

Figure 1 | MicroRNA-15b levels are decreased in fibrotic mouse hearts and in TGF-β1-stimulated mouse cardiac fibroblasts. (A) Masson's trichrome staining of a healthy (sham-operated) mouse heart and a mouse heart after transverse aortic constriction (TAC). (B) Quantification of the fibrotic area in hearts of normal (sham operated) mice and of mice that underwent TAC. MiR-15b levels in (C) normal hearts and TAC hearts and in (D) unstimulated and TGF-β1-activated mouse cardiac fibroblasts. **P<0.01 (Mann-Whitney Test)
In TGF-β1-stimulated CF, the expression of Grb2, SOS1 and SOS2 increased compared to the non-stimulated CF (Fig. 3A-C). Next, we questioned if miR-15b might preclude the increase in expression of Grb2, SOS1 and SOS2 in TGF-β1-stimulated CF. CF transfected with miR-15b mimics showed reduced expression of Grb2, SOS1 and SOS2 compared to the unstimulated control cells and to the TGF-β1-stimulated CF (Fig 3A-C). Based on preliminary experiments (n=1), TGF-β1 stimulation seems to decrease the Grb2 protein level (Fig. 3D,E), whereas the SOS1 protein level seems to be slightly increased (Fig. 3F,G) compared to unstimulated control cells. In TGF-β1-stimulated miR-15b transfected cells, the Grb2 and SOS protein levels decreased (Fig 3D-G) compared to TGF-β1-stimulated cells. In combination, these data suggest that miR-15b regulates the expression of the small GTPase intermediates Grb2, SOS1 and SOS2 in TGF-β-stimulated CF.

Concurrently, after aortic banding, the Grb2 expression level tended to be increased (Fig. 3H) and SOS1 and SOS2 expression levels are higher in the banded hearts compared to the non-banded hearts (Fig. 3I,J).

Figure 2 | MiR-15b directly targets small GTPase intermediates Grb2, SOS1 and SOS2. (A) In silico analysis identified Grb2, SOS1 and SOS2 as putative miR-15b targets, with a single binding site in the 3’UTR region of these genes. (B) Luciferase reporter assays in miR-15b transfected COS7 cells with Grb2, SOS1 and SOS2 reporter constructs. Data are mean±SEM. **P<0.01 (one-way ANOVA and Bonferroni post-hoc test)
miR-15b decreases the expression of ECM and cytoskeleton components and contractility

Since miR-15b levels are decreased during cardiac fibroblast activation, we questioned if maintaining miR-15b expression could inhibit fibroblast activation, i.e. fibroblast proliferation, myofibroblast differentiation and ECM deposition. Therefore, we firstly investigated the proliferation in TGF-β1-stimulated CF (Fig. 4A). The addition of TGF-β1 to CF did not increase the cell proliferation as compared to unstimulated CF (Fig. 4B). Transfection of CF with miR-15b mimics or a pharmacological inhibitor of small GTPases (FTS), did not alter the proliferation as compared to unstimulated cells (Fig. 4B). However, in TGF-β1-stimulated CF, miR-15b transfection decreased CF proliferation in all three independent experiments, albeit these results did not reach statistical difference due to the large interexperimental variability (Fig. 4B).

We next investigated if miR-15b mimics or FTS could inhibit the TGF-β1-induced expression of ECM genes. TGF-β1 stimulation of CF increased the gene expression of COL1A1 and COL3A1 (Fig. 5). Addition of FTS did not alter COL1A1 (Fig. 4A) or COL3A1 (Fig. 4B) gene expression, compared to unstimulated control CF, however, in FTS prevented induction of COL1A1 and COL3A1 gene expression by TGF-β1. In CF transfected with miR-15b mimics, the induction of COL1A1 (Fig. 5A) and COL3A1 expression (Fig. 5B) following TGF-β1 stimulation was precluded.

Finally, we assessed if miR-15b mimics or FTS could also inhibit myofibroblast differentiation and examined the expression of ACTA2 and CNN1 and CF contraction at the functional level. TGF-β1 stimulation of CF resulted in the increased gene expression of ACTA2 and CNN1 (Fig. 6A,B), which was abolished by the addition of FTS or the transfection of miR-15b mimics (Fig. 6A,B). On protein level, the percentage of α-SMA positive cells decreased in TGF-β1-activated CF compared to unstimulated CF (Fig. 6C,D). However, in TGF-β1-activated, miR-15b transfected CF, α-SMA levels seemed to decrease even further compared to TGF-β1-stimulated scrambled control CF (Fig. 6C,D). We questioned if the reduced expression of ACTA2 and CNN1 in miR-15b-expressing fibroblasts would culminate in a decreased contractile capacity (Fig. 7). CF have a baseline contractile capacity (Fig. 7B), which is elevated by the addition of TGF-β1 (Fig. 7B). In miR-15b-expressing CF, this enlargement by TGF-β1 stimulation was largely abrogated (Fig. 7B) and the contractile capacity remained at baseline levels.

TGF-β1-induced activation of cardiac fibroblasts is dependent on small GTPase activation

As the transfection of miR-15b mimics in CF decreased the expression of the small GTPase intermediates Grb2 and SOS2 and precluded fibroblast activation, we questioned if non-canonical TGF-β signaling via the small GTPases result in fibroblast activation. We inhibited small GTPase activation using the inhibitor FTS (Fig. 5 and 6). When small GTPase signaling was inhibited during TGF-β1-induced CF activation, the increase in the expression of COL1A1 and COL3A1 (Fig. 5A,B) and ACTA2 and CNN1 (Fig. 6A,B) was precluded as compared to TGF-β1-stimulated CF. These data suggest that non-canonical TGF-β signaling via the small GTPases is required for fibroblast activation.

Figure 4 | Proliferation of cardiac fibroblasts is not altered by TGF-β1 stimulation, however miR-15b transfection tended to decrease proliferation. (A) Representative pictures of immunofluorescent staining for proliferation marker Ki67 in mouse cardiac fibroblasts (B) Percentage of Ki67+ cells (one-way ANOVA and Bonferroni post-hoc test). Scale bar represents 100µm.
MicroRNA-15b inhibits cardiac fibroblast activation through targeting of Grb2 and SOS1 and 2

Figure 5 | miR-15b transfection inhibits TGF-β1-induced ECM production in mouse cardiac fibroblasts. Gene expression levels of (A) COL1A1 and (B) COL3A1. Data are mean±SEM. **P<0.01, ***P<0.001, ****P<0.0001 (one-way ANOVA and Bonferroni post-hoc test).

Because miR-15b reduces the expression of the small GTPase mediators Grb2, SOS1 and SOS2, we questioned if this would ultimately also reduce the activation of the small GTPase Ras. TGF-β1 stimulation or FTS inhibition did not change Ras activation, compared to unstimulated CF (Fig. 8). Also, miR-15b transfection decreased Ras activation as compared to unstimulated scrambled control cells (Fig. 8), however, this difference was not statistically significant due to the large interexperimental variability. In TGF-β1-stimulated cells miR-15b transfection did not alter Ras activation compared to the TGF-β1-stimulated scrambled control cells (Fig. 8).

BMP-7 treatment restores miR-15b expression levels, reduces Grb2, SOS1 and SOS2 expression and inhibits fibroblast activation

BMP7 is an antagonist of TGF-β1 signaling\(^2\). Therefore, we questioned if BMP7 could increase miR-15b expression levels to antagonize fibroblast activation. BMP7 alone did not significantly alter the miR-15b expression (Figure 9A) in unstimulated CF, but in TGF-β1-stimulated CF, the miR-15b expression levels were maintained at baseline level by the addition of BMP7 (Fig 9A). Also, BMP7 treatment inhibited the increase of SOS1 and SOS2 expression, compared to TGF-β1-stimulated CF (Fig 9B,C). BMP7 stimulation did not alter the expression of ACTA2 and CNN1 in presence or absence of TGF-β1 (p>0.05, data not shown). The COL1A1 and COL3A1 gene expression was increased by TGF-β1 stimulation (Fig 9D,E). The increase in COL1A1 expression by TGF-β1 stimulation could be partially inhibited by BMP7 addition compared to TGF-β1-stimulated CF (Fig 9D), whereas the TGF-β1-induced increase in COL3A1 expression was inhibited almost completely compared to TGF-β1-stimulated CF (Fig. 9E).

Figure 6 | Mesenchymal marker expression is decreased in miR-15b transfected, TGF-β1-stimulated cardiac fibroblasts. Gene expression of (A) ACTA2 or αSMA and (B) CNN1 or Calponin in mouse cardiac fibroblasts. (D) Representative pictures of immunofluorescent stainings for αSMA and (C) quantification of the percentage of αSMA positive human cardiac fibroblasts (n=1). Data are mean±SEM. **P<0.01, ***P<0.001, ****P<0.0001 (one-way ANOVA and Bonferroni post-hoc test).
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A.

Control  miR-15b  TGF-β1  miR15b + TGF-β1

Figure 7 | MiR-15b decreases contractility of human cardiac fibroblasts. (A) Photographs of representative collagen gels and (B) quantification of the percentage of gel contraction by human cardiac fibroblasts. *P<0.05, **P<0.01. (one-way ANOVA with Fisher’s LSD test). Scale bar represents 200µm.

DISCUSSION

Here, we show that miR-15b levels are decreased during cardiac fibrosis and during TGF-β1-induced cardiac fibroblast activation. We demonstrate that Grb2, SOS1 and SOS2 are targets of miR-15b. During TGF-β1-induced cardiac fibroblast activation, the expression of the small GTPase intermediates (Grb2, SOS1 and SOS2), ECM components (COL1A1 and COL3A1) and the myofibroblast markers (ACTA2 and CCN1) is readily induced at the gene level. Transfection of cardiac fibroblasts with miR-15b mimics decreases the expression of the small GTPase intermediates of Grb2, SOS1 and SOS2 in TGF-β1-stimulated CF, putatively leading to the observed inhibition of TGF-β1-induced cardiac fibroblast activation (summarized in Fig. 10). Particularly, we demonstrate that miR-15b is a novel regulator of cardiac fibroblast activation and might thus represent a new possibility for therapeutic intervention.

Unexpectedly, in our experiment we did not find an induction of cardiac fibroblast proliferation upon TGF-β1 stimulation. TGF-β is known to induce growth arrest or apoptosis in endothelial, epithelial and hematopoietic cells. In fibroblasts, the effect of TGF-β seems to be context dependent, since it has been reported that TGF-β does not lead to proliferation directly, but induces the expression of pro-mitotic factors such as FGF-2 or CTGF and/or is dependent on the presence of mitogenic stimuli in the culture media, such as PDGF or EGF. Under hypoxic conditions, the inhibition of TGF-β signaling by the overexpression of TGFBR3 even leads to a decrease in apoptosis, suggesting that TGF-β1 can be pro-apoptotic under certain conditions. Thus, the traditional view that TGF-β1 induces fibroblast proliferation might be a biased view.

We found that miR-15b mimics seemed to decrease Ras activation in unstimulated CF, as compared to scrambled control CF. However, in TGF-β1-stimulated miR-15b mimics failed to decrease Ras activation compared to TGF-β1-stimulated scrambled control CF, suggesting compensatory activation of Ras by alternative upstream mediators in presence of TGF-β. However, the miR-15b mimics did decrease the Grb2, SOS1 and SOS2 and concurrently inhibited cardiac fibroblast activation. Thus, our data suggest that the inhibition of cardiac fibroblast activation by miR-15b only partially relies on Ras inactivation. This finding might be explained by the interaction of Grb2, SOS1 and SOS2 with small GTPases other than Ras. For example, Grb2 can interact with RAB13, SOS1 with RAF and Rac, and SOS2 with Rho and Rac, thus creating alternative signaling routes. Activation of the small GTPases RAF, Rac and Rho might contribute to fibroblast activation. Ultimately, the activation of the small GTPases leads to the induction of Mitogen-activated protein
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kinase kinase/Extracellular signal-regulated kinase (MEK/ERK), phosphoinositide 3-kinase/Akt (PI3K/Akt) and p38/Mitogen activated protein kinase (p38/MAPK) signaling (Fig. 10), which are all implicated in fibroblast activation. Moreover, the activation of p38/MAPK signaling by Grb2 has been reported during fibrogenesis. For example, Grb$^{+/-}$ mice are protected against transverse aortic constriction induced cardiac fibrosis. It has been suggested that Grb2 can activate p38 directly, without the intermediation of small GTPases (Fig. 10). Thus, the observed inhibition of fibroblast activation by miR-15b might be mediated by a decrease in Grb2, SOS1 and SOS2, leading to less activation of small GTPases such as RAF, Rac and Rho and/or by a decrease in Grb2-induced p38 activation. Thus, other small GTPases such as RAF, Rac and Rho should be investigated to further clarify how miR-15b can inhibit fibroblast activation.

Interestingly, gain-of-function mutations of SOS1 and SOS2 can cause Noonan syndrome 4 and 9, respectively. Noonan syndrome is characterized by craniofacial anomalies, but patients affected by these syndromes also have a high risk (20-30%) of developing the fibroproliferative heart disease hypertrophic cardiomyopathy. Additionally, gain-of-function mutations of SOS1 can lead to hereditary gingival fibromatosis, a fibroproliferative disease of the oral mucosa.

We show here that BMP7 treatment of cardiac fibroblasts can maintain the expression of miR-15b and partially abrogates inhibits TGF-β1-induced fibroblast activation. Previously, BMP7 has been shown to reduce cardiac and kidney fibrosis by the induction of Smad1/5/8 phosphorylation, which antagonizes the action of TGF-β1-induced Smad 2/3 phosphorylation. Here, we demonstrate that BMP7 might limit fibroblast activation due to the decreased expression of the small GTPase intermediates Grb2, SOS1 and SOS2. We did not dissect the mechanism of BMP7-induction of miR-15b maintenance. A better understanding of how miR-15b levels are regulated by BMP7, could elucidate new regulatory mechanisms of non-canonical TGF-β signaling, which might be used to interfere in fibroblast activation and fibrogenesis.

In our experiments, we focused on the miR-15b mediated inhibition of non-canonical TGF-β signaling via the small GTPase intermediates Grb2 and SOS1 and SOS2 in cardiac fibroblast activation. In accordance with our findings, a decrease in miR-15b levels has been found in hepatic  

Figure 9 | BMP7 maintains miR-15b expression in TGF-β1-stimulated cardiac fibroblasts. Gene expression levels of (A) miR15b, (B) SOS1, (C) SOS2, (D) COL1A1 and (E) COL3A1 in mouse cardiac fibroblasts. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001. (one-way ANOVA and Bonferroni post-hoc test)

These pathologies underline the importance of SOS1 and SOS2 signaling in fibrogenesis, yet their molecular mechanisms need further elucidation.

We show here that BMP7 treatment of cardiac fibroblasts can maintain the expression of miR-15b and partially abrogates inhibits TGF-β1-induced fibroblast activation. Previously, BMP7 has been shown to reduce cardiac and kidney fibrosis by the induction of Smad1/5/8 phosphorylation, which antagonizes the action of TGF-β1-induced Smad 2/3 phosphorylation. Here, we demonstrate that BMP7 might limit fibroblast activation due to the decreased expression of the small GTPase intermediates Grb2, SOS1 and SOS2. We did not dissect the mechanism of BMP7-induced miR-15b maintenance. A better understanding of how miR-15b levels are regulated by BMP7, could elucidate new regulatory mechanisms of non-canonical TGF-β signaling, which might be used to interfere in fibroblast activation and fibrogenesis.

In our experiments, we focused on the miR-15b mediated inhibition of non-canonical TGF-β signaling via the small GTPase intermediates Grb2 and SOS1 and SOS2 in cardiac fibroblast activation. In accordance with our findings, a decrease in miR-15b levels has been found in hepatic
fibroblast activation, which culminated in a decrease in apoptosis, as Bcl2 is a target of miR-15b. Also, in vascular smooth muscle cell activation, a decrease in miR-15b expression levels coincides with a decrease in the expression of Yes-associated Protein (YAP). Moreover, the inhibition of miR-15b after transverse aortic constriction in mice leads to an increase in cardiac fibrosis: in this particular study miR-15b was shown to target TGFBR1 and TGFBR2 in cardiac fibroblasts. Based on these findings and our results, miR-15b might inhibit TGF-β-induced activation of cardiac fibroblasts, since the TGFBR1/2 complex initiates activation of both canonical and non-canonical TGF-β signaling. Thus, this would explain our observation that miR-15b precludes TGF-β1-induced cardiac fibroblast activation, even though we did not observe differences in activation of the small GTPase Ras. Concluding, upon the inhibition of miR-15b, cardiac fibroblasts acquire the ability to be activated by TGF-β via its canonical and non-canonical signal transduction pathways. Hence, in cardiac pathologies where fibrotic tissue accumulation is present (e.g. pressure overload induced cardiac hypertrophy), the expression of miR-15b in fibroblasts could inhibit fibroblast activation and might prevent the loss of cardiac function and the development of heart failure in the long run.

In other cardiac injury models (i.e. in Ren2 hypertrophic cardiomyopathy and after coronary artery ligation), miR-15b levels were increased during disease. In most of these studies, miR-15b is studied in the cellular context of cardiomyocytes. MiR-15b inhibition in cardiomyocytes leads to a higher number of viable cardiomyocytes and a decreased infarct size whereas miR-15b overexpression leads to increased cardiomyocyte apoptosis. Thus, the function of miR-15b is cell type and context dependent. Hence, in pathology where the cardiomyocyte apoptosis is found (e.g. myocardial infarction), low miR-15b expression levels may be favorable, whereas in pathologies where fibroblast activation leads to ECM accumulation (e.g. cardiac fibrosis) high miR-15b levels might be protective.

In conclusion, we have shown that miR-15b is decreased in fibrotic hearts and during TGF-β-induced fibroblast activation. Transfection of miR-15b mimics in TGF-β-stimulated cardiac fibroblasts results in a decrease in the expression of the small GTPase intermediates Grb2, SOS1 and SOS2 and precludes fibroblast activation. The decrease of miR-15b in cardiac fibrosis might contribute to cardiac fibrogenesis and the progression of cardiac failure and restoration of miR-15b expression levels might thus be used as a novel antifibrogenic therapy.

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REFERENCES


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