CHAPTER 4
THE MIRNA-15 FAMILY INHIBITS TGFβ SIGNALING IN THE HEART

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ABSTRACT
The overloaded heart remodels by cardiomyocyte hypertrophy and interstitial fibrosis, which contributes to the development of heart failure. Signaling via the TGFβ-pathway is crucial for this cardiac remodeling. Here we tested the hypothesis that microRNAs in the overloaded heart regulate this remodeling process via inhibition of the TGFβ-pathway. We show that the miRNA-15 family, which we found to be upregulated in the overloaded heart in multiple species, inhibits the TGFβ-pathway by directly targeting TGFBR1 and several other genes within this pathway, including p38, SMAD3, SMAD7 and endoglin. Inhibition of miR-15b by subcutaneous injections of LNA-based antimiRs in mice subjected to transverse aorta constriction aggravated cardiomyocyte hypertrophy and fibrosis. Finally, we show that when miR-15b is selectively overexpressed in cardiomyocytes in transgenic mice, it induces smaller hearts at baseline, but this selective overexpression in cardiomyocytes did not suffice to inhibit TAC-induced cardiac remodeling. In conclusion, we identified the miR-15 family as a regulator of cardiac hypertrophy and fibrosis acting by inhibition of the TGFβ-pathway.
INTRODUCTION

The heart responds to injury, caused by for example myocardial infarction or sustained pressure overload, through a number of structural alterations commonly referred to as cardiac remodeling. At the cellular level these alterations include cardiomyocyte hypertrophy, cardiomyocyte apoptosis and changes in expression of genes regulating energy metabolism, calcium handling and genes normally expressed in the embryonic heart. On the other hand cardiac remodeling is hallmarked by cardiac fibrosis, which is defined as the excessive accumulation of extracellular matrix (ECM) proteins in the interstitium and perivascular regions of the myocardium. Fibrosis increases stiffness of the heart and may eventually impair myocyte contractility, disrupt electrical coupling and cause tissue hypoxia, which together contribute to contractile dysfunction of the heart and the development of heart failure.

Transforming growth factor β (TGFβ) is a cytokine that regulates both cardiomyocyte and fibroblast biology and its expression and signaling activity are increased during cardiac remodeling. Both cell types express TGFβ1 and its two serine-threonine kinase receptors (TGFBR1 and 2). In cardiac fibroblasts binding of TGFβ to its receptors results in activation of the canonical signaling pathway, which comprises activation of SMAD2 and SMAD3 by phosphorylation. Subsequently, these phosphorylated SMADs form a complex with SMAD4 and together they translocate to the nucleus to act as a transcription factor, where they drive the expression of TGFβ-responsive genes. A number of mouse models have established that activation of the canonical TGFβ-pathway in the heart promotes the development of fibrosis. For example, heterozygous TGFβ1 deficient mice reveal attenuated fibrosis in the aging heart, while overexpression of TGFβ1 in transgenic mice results in increased interstitial fibrosis. Also endoglin (Eng), a TGFβ1 co-receptor required for signaling in the cardiac fibroblast was recently shown to be involved in the regulation of cardiac fibrosis, as transverse aorta constriction (TAC) in heterozygous Eng deficient mice revealed attenuated cardiac fibrosis and preserved left ventricular function.

In cardiomyocytes, binding of TGFβ to its receptors leads to activation of a non-canonical pathway involving the phosphorylation of TGFβ-activated-kinase 1 (TAK1) and p38 resulting in the activation of several transcription factors such as MEF2C, GATA4 and SRF. Activation of the non-canonical TGFβ-pathway in myocytes during cardiac remodeling has been shown to regulate the development of myocyte hypertrophy. Mice with cardiomyocyte-specific overexpression of constitutively active TAK1 showed an increased p38 phosphorylation, myocyte hypertrophy and severe myocardial dysfunction. Furthermore, Koitabashi et al. recently reported on the crucial role of TGFBR2 in cardiomyocyte hypertrophy after TAC, as they showed that in cardiomyocyte-specific TGFBR2 knockout mice, cardiac hypertrophy and fibrosis was inhibited and cardiac function improved.

Because of its critical involvement in cardiac remodeling, the inhibition of the TGFβ-pathway is a promising therapeutic target for heart failure. Inhibition of the TGFβ-pathway by neutralizing antibodies during pressure overload-induced cardiac remodeling in rats and mice
inhibited fibrosis, but did not affect cardiomyocyte hypertrophy. Koitabashi et al. further showed that these neutralizing antibodies were able to inhibit the canonical TGFβ-pathway in interstitial cells but not the non-canonical TGFβ-pathway in cardiomyocytes suggesting that only the interstitial TGFβ signaling is inhibited by these antibodies. Inhibition of the TGFβ-pathway by the non-specific drug tranilast, an inhibitor of TGFβ transcription, resulted in reduced levels of fibrosis in hypertensive rats independent of changes in blood pressure. However, a phase III clinical trial investigating tranilast in restenosis revealed some adverse effects of this drug. Together, these studies show that inhibition of the TGFβ-pathway might be used therapeutically to reduce fibrosis of the heart, but that novel ways to intervene in the TGFβ pathway are needed.

MicroRNAs (miRNAs) constitute a class of small RNA molecules that inhibit protein expression either through degradation of mRNA or interference with protein translation. Individual miRNAs regulate multiple mRNA targets and often these targets regulate one biological pathway, thereby potentiating the effect of one miRNA. MiRNAs have emerged as powerful regulators of almost every aspect of cardiac biology, including cardiomyocyte proliferation, hypertrophy and interstitial fibrosis. The ease to manipulate these miRNAs in vivo in numerous animal models and the recent success of the first human clinical trial for therapeutic suppression of hepatitis C virus replication using antimiRs triggered enthusiasm for miRNA-based therapeutics.

We set out this study to identify which miRNAs are able to regulate the TGFβ-pathway in the heart. Using target-prediction software and manipulation of miRNAs in cultured cells we identified the miR-15 family for its potential to inhibit the TGFβ-pathway. The miR-15 family consists of six highly conserved miRNAs (miR-15a/b, miR-16, miR-195, miR-497, miR-322), which are abundantly expressed in the heart, and upregulated in the diseased myocardium. We show that the miR-15 family inhibits multiple components of the TGFβ-pathway, including TGFBR1, SMAD3, SMAD7, p38 and endoglin. Inhibition of miR-15b in vivo, using LNA-based antimiRs in mice subjected to TAC, showed aggravated cardiomyocyte hypertrophy and fibrosis compared to the control antimir group. We generated a transgenic mouse model, in which we overexpressed miR-15b specifically in cardiomyocytes and these mice displayed smaller hearts with hypotrophic cardiomyocytes. Cardiac remodeling after TAC in terms of hypertrophy and fibrosis was not altered in these mice. In conclusion, we identified the miR-15 family as a regulator of cardiac hypertrophy and fibrosis acting by inhibition of the TGFβ-pathway. Our data also suggests that stimulation of the miR-15 family in cardiac fibroblasts may be of therapeutic interest to inhibit adverse cardiac remodeling in heart failure.
METHODS

Experimental animals
All animal studies were approved by the Institutional Animal Care and Use Committee of the University of Amsterdam and in accordance with the guidelines of this institution. Heart tissue of TGR(mRen2)27-rats (Ren2-rats, see supplemental methods) were used from a previous study. For the antimiR studies we used 9 week old C57BL/6JOlhHsd (Harlan) mice. In the first study we injected them intravenously with 5 mg/kg LNA based antimiRs (Ribotask) at two different timepoints (day 0 and day 5) and sacrificed them at day 7. In the second study we injected them subcutaneously with 5 mg/kg LNA based antimiRs at two different timepoints (day 0 and day 7). At day 3 or 4, mice were subjected to TAC surgery and after 4 weeks we performed echocardiography and sacrificed the mice.

To generate miR-15b transgenic mice, a mouse genomic fragment flanking miR-15b was subcloned in a cardiomyocyte-specific expression plasmid containing the α-MHC promoter and the human growth hormone polyA signal and injected in fertilized eggs. For further details on the mouse studies see the Supplemental methods.

Human samples
We included patients with end-stage hypertrophic cardiomyopathy, end-stage dilated cardiomyopathy and aorta stenosis. Left ventricular (LV) samples were obtained from hearts explanted during surgery or valve replacement surgery. As controls we used LV tissue of non-failing donors (not used for transplantation due to logistic reasons). The study protocol was approved by the local ethical committee and all patients gave informed consent.

Histological and molecular analysis
Methods of picrosirius red stainings, Hematoxyline & Azophloxine (H&A) stainings, microRNA in situ hybridizations, immunohistochemistry, luciferase assays, RNA and protein analysis are described in the Supplemental methods.

Cell culture and transfections
Neonatal rat ventricular myocytes and fibroblasts were isolated by enzymatic digestion of 1 to 2 day old rat hearts as described previously. These cells were transfected with 33 nmol/L mimic-miR15b or mimic-negative control #1 (Dharmacon) and 220 nmol/L antimiR-15b or antimiR-negative control (same antimiRs as used in the in vivo studies) using lipofectamine 2000 (Invitrogen) according to manufacturer's protocol. After 48 hours cells were harvested for RNA isolations.

COS7 and HepG2 cells were cultured under standard conditions. Cells were transfected at day 1 with 33 nmol/L mimic-miR15b, mimic-negative control #1, 100 nmol/L tiny-miR15 or tiny-miR negative control (Ribotask) using lipofectamine 2000. At day 2, COS7 cells were co-transfected with 10 ng pmIR-report vector (Ambion) containing the 3'UTR of TGFBR1, TGFBR2,
SMAD3, SMAD4, SMAD7, p38 or endoglin and with 5 ng pRL-renilla luciferase (Promega) using genejammer (Agilent Technologies). After 24 hours cells were lysed for luciferase assays. HepG2 cells were co-transfected with 200 ng CAGA-luciferase reporter and 5 ng pRL-renilla luciferase using genejammer according to the manufacturer’s protocol. The next day, cells were stimulated with 6 different concentrations of recombinant TGFβ1 (Tebu-Bio) ranging from 0-2 ng/ml. After 24 hours of stimulation cells were lysed for luciferase assays.

Statistical analysis
Data are shown as mean +/- SEM and sample sizes are mentioned in the Figure legends per individual experiment. Student’s t-test was performed to compare the differences between group means. For the CAGA-experiments linear regression analysis was performed to compare the slopes of luciferase activation by TGFβ. p-values<0.05 were considered significant

RESULTS
The miR-15 family is predicted to target multiple genes in the TGFβ-pathway
Since the TGFβ-pathway is critically involved in cardiac remodeling, we searched for miRNAs that are abundantly expressed in the heart and are capable of regulating the TGFβ-pathway. To identify which miRNA may regulate the TGFβ-pathway we undertook a bioinformatical approach using miRanda and Targetscan software. Our attention was drawn to a set of miRNAs belonging to the same family, the miR-15 family. The miR-15 family consists of six highly conserved miRNAs (miR-15a/b, miR-16, miR-195, miR-497, miR-322), which are all abundantly expressed in the heart, both in cardiomyocytes and fibroblasts.13 These family-members contain the same ‘seed’ sequence (Figure 1A), which is the critical part of a miRNA for target recognition. Therefore they are expected to show an overlap in mRNA targets and thus to regulate the same cellular processes.

Interestingly, many genes of the canonical TGFβ-pathway (TGFBR1, TGFBR2, TGFBR3, endoglin, SMAD2, SMAD3, SMAD4, SMAD7), but also several genes in the non-canonical TGFβ-pathway (TGFBR1, TGFBR2, TRAF6, TAK1, p38) are predicted targets of the miR-15 family. Moreover, most of these predicted targets contain more than one putative miR-15 family binding site in their 3’UTR and many binding sites are evolutionarily conserved to rat and human (Table I, Supplemental figure 1).

The miR-15 family is upregulated in cardiac hypertrophy and heart failure
In an independent study, we identified miR-15b as a miRNA upregulated in the left ventricle (LV) of Ren2-rats, a model of hypertension-induced hypertrophy and heart failure (Figure 1B). In a second rodent model, we subjected mice to 4 weeks of TAC, which induces pressure overload hypertrophy and measured all miR-15 family members by real-time PCR. Also in these hearts we observed an increased expression of miR-15b and its family members (Figure 1C). To determine whether the miR-15 family is also upregulated in human hypertrophy and heart failure, we
Figure 1. MiR-15 expression in the heart. A) miR-15b is part of the miR-15 family that includes 6 miRNAs, which all share a common seed sequence. Differences in nucleotides compared to the miR-15b sequence are depicted in red. B) miR-15b expression in hypertrophied and failing Ren2-rat hearts, as detected by microarrays (n=2). C) The complete miR-15 family is upregulated after 4 weeks of TAC in mice (n=5). D) In human hearts, upregulation of the miR-15 family is also observed in end-stage hypertrophic cardiomyopathy (n=8), end-stage dilated cardiomyopathy (n=5), and aorta stenosis patients (n=10) compared to healthy control hearts (n=5). E) In situ hybridization shows expression of miR-15b in cardiomyocytes (open arrows) and interstitial cells (black arrows). Scale bar=50 μm. F) Real-time PCR shows comparable miR-15b levels in cultured cardiomyocytes and fibroblasts (n=3). MiRNA levels in real-time PCR were normalized to U6. *p-value<0.05 compared to the control group.
conducted real-time PCR of all miR-15 family members on LV samples of patients with end-stage hypertrophic cardiomyopathy, end-stage dilated cardiomyopathy and aorta stenosis and compared it to the expression in healthy donor hearts. As shown in Figure 1D, all miR-15 family members are significantly upregulated in human hypertrophy and heart failure.

To identify the cell type(s) responsible for miR-15b expression in the heart, we performed in situ hybridization on paraﬃn sections of adult wildtype mice using LNA-based probes against miR-15b and a negative control sequence. As shown in Figure 1E we detected miR-15b in both cardiomyocytes and interstitial cells, which largely consist of ﬁbroblasts, whereas the negative control sequence showed almost no signal. Real-time PCR of miR-15b on equal amounts of RNA isolated from cultured neonatal rat cardiomyocytes and ﬁbroblasts conﬁrmed that this miRNA is expressed in both cell types, and that the level of expression is comparable (Figure 1F).

MiR-15 directly targets multiple genes in the TGFβ-pathway

To explore whether the miR-15 family is able to regulate the canonical TGFβ-pathway we used a CAGA-luciferase reporter, which is speciﬁcally activated by phosphorylated SMAD2 and SMAD3. Overexpression of the CAGA reporter along with miR-15b in HepG2 cells led to a decrease in luciferase activity upon TGFβ stimulation, which indicates that miR-15b inhibits the canonical TGFβ-pathway (Figure 2A, Supplemental ﬁgure 2A). In support of this model, inhibition of the miR-15 family using tiny LNA oligonucleotides directed against the common seed sequence of the family, resulted in increased luciferase activity of the CAGA-reporter upon TGFβ stimulation (Figure 2B, Supplemental ﬁgure 2B). Together, these luciferase assays provide the first in vitro evidence that miR-15 family members inhibit the canonical TGFβ-pathway.

Table I: Predicted miR-15 family targets in the TGFβ-pathway.

<table>
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<tr>
<th>Gene</th>
<th>Canonical / non-canonical pathway</th>
<th>Predicted binding sites in Mouse</th>
<th>Conserved binding sites</th>
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<tr>
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Predicted miR-15 family targets in the TGFβ-pathway with the number of predicted binding sites in the mouse genome and their conservation to rat and human.
Figure 2. The miR-15 family inhibits the TGFβ-pathway. A) Overexpression of miR-15b in HepG2 cells transfected with a CAGA-luciferase reporter results in decreased luciferase activity compared with the negative control (NC) mimic, which indicates that the canonical TGFβ-pathway is inhibited. B) Inhibition of the miR-15 family in HepG2 cells results in an increased activation of the CAGA-luciferase reporter by TGFβ. N=6, p<0.001 for the difference in slope of luciferase activation between the NC and miR-15 transfected cells.

Evidence that the canonical TGFβ-pathway is suppressed by the miR-15 family.

To investigate whether the miR-15 family is able to regulate the expression of the predicted targets of the TGFβ-pathway in a cardiac cell system, we transfected neonatal rat cardiomyocytes and fibroblasts with mimics and antimiRs to overexpress or inhibit miR-15b levels and measured mRNA levels by real-time PCR. For successful overexpression or knockdown of miR-15b in these cells we refer to Supplemental figure 2C and D. In cardiomyocytes (Figure 3A), overexpression of miR-15b resulted in decreased mRNA levels of p38, SMAD2, SMAD3, SMAD7 and endoglin and a non-significant down-regulation of TGFBR1 and TGFBR2. No effect was seen on TGFBR3 and SMAD4 expression. Conversely, inhibition of miR-15b in cardiomyocytes resulted in a significant upregulation of p38, TGFBR1, TGFBR2, TGFBR3, SMAD2, SMAD3, SMAD7, and endoglin, while SMAD4 levels were not changed (Figure 3A). Together this indicates that in cardiomyocytes, the transcript levels of p38, SMAD2, SMAD3, SMAD7 and endoglin are tightly regulated by miR-15b. Moreover, the observation that TGFBR1 and TGFBR2 mRNA is significantly upregulated after inhibition of miR-15b suggests that the expression of these genes is also regulated by miR-15b. SMAD4 was not regulated by overexpression neither by inhibition of miR-15b. We also performed these experiments in cultured neonatal cardiac fibroblasts and observed similar trends, however the response of these genes to miR-15b upregulation or knockdown was less pronounced than in cardiomyocytes (Figure 3B). A possible explanation for the more subtle responses to miR-15b manipulation probably relates to the tendency of these cells to undergo a phenotypic switch to myofibroblasts in cell culture and to the high basal expression and activity of the TGFβ pathway in these differentiated cells. Overall, many of the predicted target genes...
showed a trend towards downregulation after miR-15b overexpression in cardiac fibroblasts and an upregulation after inhibition of miR-15b.

To further investigate whether the predicted targets are direct miR-15 family targets, we subcloned the 3'UTRs of these genes behind a luciferase reporter. Since the complete miR-15 family is expressed at high levels in COS7 cells, we chose to perform luciferase assays in this cell type after inhibition of the whole miR-15 family using tiny LNA transfections (Supplemental figure 2E). Knockdown of the miR-15 family in COS7 cells resulted in an upregulation of the luciferase activity of the reporters containing the 3'UTR of TGFBR1, SMAD3, SMAD7, p38 and endoglin (Figure 3C), indicating that the miR-15 family is able to regulate these genes via direct binding to their 3'UTRs. The luciferase activity of the reporters containing the 3'UTR of TGFBR2 and SMAD4 was not influenced by inhibition of the miR-15 family, indicating that these predicted targets are not direct miR-15 family target genes. For technical reasons we were unable to subclone the 3'UTR of SMAD2 and TGFBR3 behind the luciferase reporter and we could therefore not test whether the miR-15 family was able to directly target SMAD2 or TGFBR3.

To further investigate whether the miR-15 family is able to regulate these identified targets in vivo, we inhibited miR-15b levels in mice. We injected mice intravenously with LNA-based antimiR-15b or PBS as vehicle control twice and sacrificed the mice one week after the first injection (Figure 4A). Real-time PCR revealed the complete loss of miR-15b in the myocardium of these mice (Figure 4B). The loss of miR-15b in these mice resulted in a 1.8-fold upregulation of TGFBR1 mRNA, a trend towards upregulation of SMAD3 mRNA, and no effect on mRNA levels of p38, TGFBR2, TGFBR3, SMAD2, SMAD4, SMAD7, and endoglin (Figure 4C). These data clearly illustrate that miR-15b controls the mRNA levels of TGFBR1 in the heart.

In vivo inhibition of miR-15b during sustained pressure overload of the heart results in increased hypertrophy and fibrosis

To study the effect of global loss of miR-15b on cardiac remodeling, we injected the LNA-based antimiRs subcutaneously in mice and subjected them to TAC or sham surgery (Figure 5A). Mice were sacrificed after 4 weeks of TAC and the loss of miR-15b in the heart was confirmed by Northern blot (Figure 5B). Real-time PCR revealed that the other family members were, although to a lesser extent, also significantly inhibited in the antimiR-15b injected mice and that the antimiR-15b injections were able to prevent the upregulation of the complete miR-15 family after TAC (Figure 5C). The level of downregulation of the other miR-15 family members was in accordance to the extent of sequence homology to mature miR-15b (Figure 1A).

Loss of miR-15b in TAC-induced cardiac remodeling affected processes in both cardiomyocytes and fibroblasts. There was a stronger increase in heart weight corrected for tibia length in response to TAC in the antimiR-15b injected mice compared to the antimiR negative control (antimiR-NC) injected mice (Figure 6A). Next, we investigated whether the fibrotic response, a process mainly governed by cardiac fibroblasts was affected. Therefore we determined the area of collagen fibers by means of picrosirius red stainings in cardiac sections.
Figure 3. The miR-15 family directly targets numerous genes in the TGFβ-pathway. A) mRNA levels detected by real-time PCR of predicted miR-15 target genes in cardiomyocytes and B) fibroblasts with overexpression (mimic) and knockdown (antimiR) of miR-15b levels. mRNA levels are normalized to HPRT levels. C) Luciferase assays using reporters with the respective 3’UTR subcloned behind the luciferase gene reveal direct targeting of TGFBR1, SMAD3, SMAD7, p38 and endoglin after knockdown of the miR-15 family using tiny LNAs. N=3 for real-time PCR and luciferase assays. *p<0.05 compared to negative control (NC) transfected cells.
Figure 4. Inhibition of miR-15b in mice results in upregulation of target genes. A) Design of the antimiR study. B) Real-time PCR shows complete loss of miR-15b in antimiR injected mice. MiRNA levels are normalized to U6. C) Real-time PCR of predicted miR-15 family target genes shows upregulation of TGFBR1 and a trend towards upregulation of SMAD3 in the myocardium after inhibition of miR-15b. mRNA levels are normalized to HPRT levels. N=4 for PBS injected mice and n=5 for antimiR-15b injected mice. *p<0.05 compared to PBS control.

Strikingly, we found that upon loss of miR-15b, fibrosis was more strongly induced after 4 weeks of TAC (Figure 6B and C). Excessive fibrosis in antimiR-15b treated mice was detected when we quantified images of the whole LV, but also when zooming in into specific areas of the LV, such as the LV free wall and the papillary muscles. Fibrosis was however not significantly altered in the septum of the heart (Supplemental figure 3A).

We also determined whether cardiomyocyte size was affected by the loss of miR-15b by measuring cell size area in Hematoxyline & Azophloxine (H&A) stained sections of the antimiR injected mice. As shown by the representative images in Figure 6D and the quantifications in Figure 6E this revealed an increased hypertrophic response to TAC in the antimiR-15b injected mice compared to the antimiR-NC injected mice. This increase in cardiac hypertrophy was accompanied by a trend towards a stronger upregulation of the hypertrophic marker genes ANF and βMHC, but these differences did not reach statistical significance (Supplemental figure 3B). Although loss of miR-15b during TAC-induced cardiac remodeling resulted in enhanced
hypertrophic and fibrotic responses, this did not affect cardiac function at 4 weeks after TAC as shown by a similar decrease in fractional shortening between antimiR-15b and antimiR-NC injected mice (Supplemental figure 3C).

We next tested whether the TGFβ-pathway was increasingly activated in the LV of these antimiR injected mice by measuring downstream ECM-related targets of the canonical TGFβ-pathway such as collagens, CTGF, TIMP1 and periostrin (postn). By means of real-time PCR we show that TIMP1 and postn are increasingly expressed after TAC in the antimiR-15b compared...
Figure 6. Inhibition of miR-15b aggravates cardiac remodeling after TAC. A) Inhibition of miR-15b results in a stronger increase in heart weight corrected for tibia length after TAC. B) Representative pictures of picrosirius red sections to determine fibrosis, which is increased after inhibition of miR-15b as quantified in C). D) Representative pictures of H&E sections, which were used to measure cell sizes, which are quantified in E) and show a stronger increase in cardiomyocyte hypertrophy after inhibition of miR-15b. E) Some downstream target genes of the TGFβ-pathway are significantly increased after TAC surgery in the antimiR-15b injected animals (TIMP, POSTN), while other targets show a trend towards increased expression (COL1a1, CTGF). N=5 antimiR-NC sham, n=8 antimiR-NC TAC, n=6 antimiR-15b sham, n=9 antimiR-15b TAC. Scale bar = 50 μm, *p<0.05 compared to sham and #p<0.05 compared to antimiR-NC.
to the antimiR-NC injected mice (Figure 6F). Col1a1 and CTGF showed a trend towards enhanced upregulation after TAC in the antimiR-15b mice. Together, these results indicate that the canonical TGFβ-pathway is more strongly activated upon pressure overload in the antimiR-15b compared to the antimiR-NC injected mice.

**Cardiomyocyte-specific overexpression of miR-15b results in smaller hearts, but does not protect against fibrosis and hypertrophy**

Multiple cell types of the heart, including myocytes, fibroblasts and endothelial cells express the miR-15 family, as well as many of the components of the TGFβ-pathway. To be able to further pinpoint the role of the miR-15 family in the cardiomyocyte we generated transgenic mouse lines that overexpress the miR-15b precursor driven by the cardiomyocyte-specific αMHC-promoter. This resulted in a 1500-fold upregulation of pri-miR-15b (Supplemental figure 4A) and a 3- and 4-fold induction of mature miR-15b in line A and C respectively, as shown by real-time PCR (Figure 7A) and confirmed by Northern blot analysis (Figure 7B).

The effects of miR-15b overexpression were immediately apparent on visual inspection; the hearts of adult miR-15b transgenic mice were substantially smaller than the hearts of their littermate controls (Figure 7C). This is shown by a reduction in heart weight corrected for tibia length in both mouse lines at age 8-12 weeks, 17-24 weeks and 8-12 months (Figure 7D and Supplemental figure 4B). Echocardiography revealed that overexpression of miR-15b in transgenic mice resulted in a decreased internal LV diameter in diastole in the 8-12 months old mice of line A (Supplemental figure 4C). The reduction in heart size did not affect cardiac function, as no differences in fractional shortening between miR-15b transgenic mice and their littermate controls were detected by echocardiography (Supplemental figure 4D).

Based on the results of our antimiR study, where miR-15b was shown to inhibit hypertrophy of individual cardiomyocytes, we investigated whether a difference in cell size might underlie the smaller hearts of the miR-15b transgenic mice. Therefore, we quantified cell sizes of cardiomyocytes in H&E stained sections of adult miR-15b transgenic hearts and this revealed that transgenic mice indeed have smaller cardiomyocytes compared to the wildtype controls (Figure 7E and F). A recent study has implicated the miR-15 family in the proliferation of cardiomyocytes at neonatal age, by regulating the cell cycle gene checkpoint kinase 1 (Chek1). Based on this study, we tested whether proliferation may also have been affected in the miR-15b transgenic heart at neonatal day 3 (p3). We performed immunohistochemistry of phospho-histoneH3 and antigen Ki-67, commonly used markers of proliferation, but found no differences between miR-15b transgenic p3 hearts compared to their controls (Supplemental figure 4E).

Interestingly, we did observe a downregulation of Chek1 in the transgenic hearts (Figure 7G).

To investigate whether increased miR-15b levels in the cardiomyocytes would be sufficient to protect against pathological hypertrophy, we performed TAC surgery in 8 week old miR-15b transgenic mice. Interestingly, we did not detect any differences in cardiac hypertrophy after 3 weeks of TAC, as evidenced by comparable increases in heart weight and expression...
Figure 7. Cardiomyocyte-specific miR-15b transgenic mice have smaller hearts. A) Overexpression of mature miR-15b is confirmed by real-time PCR and B) by Northern blot, which show a 4- and 3-fold overexpression in two independent mouse lines. Real-time PCR is normalized to U6 and comprised of n=5 for all groups. C) Representative hearts of wildtype and transgenic mice. Scale bar = 1 mm D) Heart weight corrected for tibia length is decreased in miR-15b transgenic mice of line A. E) Representative H&E stained sections of wildtype and transgenic mice at 8-12 weeks of age. Scale bar = 50 μm. F) Cell size quantification in H&E stained sections. G) At neonatal day 3 heart weights corrected for body weights are similar between wildtype and transgenic mice, while mRNA levels of Chek1 (involved in proliferation) and p38 (involved in myocyte growth) are decreased in transgenic mice. H) Protein levels of TGFBR1 are downregulated in adult transgenic mice as shown by Western blot. N=6 wildtype and transgenic at 8-12 weeks and 17-24 weeks, n=4 wildtype and 10 transgene at 8-12 months. *p<0.05 compared to wildtype mice.
of hypertrophic marker genes in miR-15b transgenic and wildtype mice (Supplemental figure 5A and B). Furthermore, cardiomyocyte cell sizes were not different between TAC operated transgenic and wildtype mice, indicating that cardiomyocyte growth was not inhibited in the transgenic animals (Supplemental figure 5C). Also the fibrotic response was not affected in the miR-15b transgenic mice, as fibrosis and the increase in expression of ECM related genes was comparable between transgenics and wildtypes (Supplemental figure 5D-F). Finally, echocardiography revealed no difference in cardiac function between miR-15b transgenic and wildtype mice, as shown by a comparable decrease in fractional shortening upon TAC surgery (Supplemental figure 5G). These results indicate that cardiomyocyte-specific overexpression of miR-15b is not sufficient to inhibit cardiac remodeling after pressure overload.

Despite a lack of effect on pathological remodeling in miR-15b transgenic mice at 3 weeks after TAC, we could observe repression of some miR-15b target genes in the non-canonical TGFβ-pathway in the transgenic mice. We found mRNA levels of p38 reduced in hearts of transgenic mice at postnatal day 3 (Figure 7G) and protein levels of TGFBR1 significantly reduced in adult miR-15b transgenic mice (Figure 7H).

DISCUSSION
The studies described here have identified the miR-15 family for its potential to inhibit the TGFβ-pathway, a critical pathway in the regulation of cardiac fibrosis and hypertrophy. The specific components of the canonical and non-canonical TGFβ-pathway that are targeted by the miR-15 family are depicted in Figure 8. Inhibition of the miR-15 family in vivo, using LNA-based antimiRs against miR-15b in mice subjected to TAC, showed aggravated cardiomyocyte hypertrophy and fibrosis compared to the antimiR-NC group. This is in line with previous studies, where increased TGFβ signaling in both fibroblasts and myocytes also resulted in cardiac hypertrophy and interstitial fibrosis. By generating a transgenic mouse line, overexpressing miR-15b under the αMHC promoter, we further investigated the role of miR-15b specifically in cardiomyocytes. These mice displayed smaller hearts, comprised of hypotrophic cardiomyocytes, as could be expected with reduced TGFβ signaling in these cells. Interestingly, cardiac remodeling after TAC in terms of hypertrophy and fibrosis was not altered in these transgenic mice, indicating that overexpression of miR-15b in the cardiomyocyte is not sufficient to affect pathological remodeling of the heart.

Thus, cardiac remodeling was modulated after inhibition of miR-15b by antimiR injections, but not after overexpression of miR-15b in transgenic mice. Several explanations might underlie this difference. First, in the two mouse models we manipulated miR-15b levels in different cell types. In the antimiR injected animals we inhibited miR-15b in all cell types of the heart, whereas in miR-15b transgenic mice, miR-15b was specifically overexpressed in cardiomyocytes. Since miR-15b levels were not upregulated in fibroblasts in the transgenic mice, it is not surprising that we did not observe an inhibition on ECM deposition in this model. Second, there is a difference in the regulation of the family members of miR-15 in the two
CHAPTER 4

models. In the antimiR injected animals, we completely lost miR-15b expression in the heart and additionally, prevented the upregulation normally seen in sustained pressure overload of the other miR-15 family members. This resulted in a substantial downregulation of the complete miR-15 family, which likely had additive effects on the regulation of miR-15 targets. In the miR-15b transgenic mice we observed a 3-4 fold overexpression of only miR-15b and not of the other family members, an increase that may not have been high enough to control the TGFβ-pathway during pathological remodeling. In conclusion, the net effect on manipulating miR-15 targets was probably higher in the antimiR study, when compared to the overexpression model.

Third, differences between the two models might have been induced by differences in timing of miR-15b regulation. In the antimiR injected animals, injections and thus inhibition of miR-15b was performed 2-3 days before TAC surgery, while in the transgenic animals the overexpression was induced immediately after birth, which might have triggered compensation mechanisms in these transgenic mice.

Our data show that multiple miR-15 family members are upregulated in rat and human hypertrophy and heart failure. These family members comprise the same 'seed' for mRNA recognition and are expected to show an overlap in targets. We show that the miR-15 family targets both activators (TGFBR1, SMAD3, endoglin) and repressors (SMAD7) of TGFβ signaling; however the net effect seems to be 'repression of SMAD signaling' as shown by the luciferase

Figure 8. Putative model for the regulation of the TGFβ-pathway by the miR-15 family. The canonical TGFβ-pathway is mainly active in cardiac fibroblasts and the non-canonical pathway in cardiomyocytes. MiR-15 directly represses the expression of several genes in the canonical (TGFBR1, endoglin, SMAD3, SMAD7) and non-canonical TGFβ-pathway (TGFBR1, p38), miR-15 depicted in red. The expression of SMAD2 was also regulated by miR-15, but as we were unable to test whether it is a direct target we depicted the miR-15 for this target in purple.
assays using the CAGA-reporter and by the excessive cardiac fibrosis observed in the antimiR-15b treated mice. The regulation of TGFβ signaling at multiple levels by one miRNA family indicates that this miRNA family can act as a potent repressor to limit excessive expression of ECM genes. The increase in miR-15 family members in the remodeling heart suggests that it is part of a feedback mechanism to limit TGFβ activity.

Although we identified multiple components of the TGFβ-pathway to be regulated and directly targeted by the miR-15 family, only two of these targets, TGFBR1 and p38 appeared to be modulated in vivo. The 3’UTR of TGFBR1 contains 5 conserved miR-15 binding sites, which was the largest number of all predicted miR-15 targets in the TGFβ-pathway (Table I). We found TGFBR1 levels regulated in cultured cardiomyocytes and fibroblasts after inhibition and overexpression of miR-15b. In vivo the TGFBR1 was significantly upregulated on the mRNA level after inhibition of miR-15b in mice injected with antimiR-15b and downregulated at the protein level in adult miR-15b transgenic mouse hearts. Interestingly, our results in the miR-15b transgenes, showing no effect on cardiac hypertrophy in response to TAC are in line with the identification of TGFBR1 as one of the most important miR-15 targets. In this regard, Koitabashi et al. showed that the cardiomyocyte-specific TGFBR1 knockout mice subjected to TAC also did not show reduced hypertrophy, albeit these mice did display modestly reduced fibrosis.11 The differences in TAC-induced fibrosis in the miR-15b transgenic mice and cardiomyocyte-specific TGFBR1 knockout mice might be explained by the level of downregulation of TGFBR1, which is smaller in our cardiomyocyte specific miR-15b transgenic mice compared to the TGFBR1 knockout mice.

Besides providing the first evidence that miR-15 regulates multiple components of the TGFβ-pathway in the heart, our experiments also confirm the findings of a previous study by Porrello et al., in which cardiomyocyte-specific overexpression of another miR-15 family member was shown to result in smaller hearts.26 In that study, miR-195 overexpression driven by the βMHC promoter resulted in smaller hearts due to a reduction in cardiomyocyte proliferation, as evidenced by a decrease in phospho-HistoneH3 stainings in one day old miR-195 transgenic mice. Using gene profiling and argonaute-2 immunoprecipitation approaches, miR-195 was shown to regulate the expression of a number of cell cycle genes, including Chek1, which was subsequently identified as a direct target of miR-195.26 In the present study we show that overexpression of miR-15b also results in smaller hearts and we confirm the inhibition of the target gene Chek1 in three day old miR-15b transgenic mice. Besides this reported inhibition of cardiomyocyte proliferation by the miR-15 family, we also detected differences in cell size of cardiomyocytes in the adult miR-15b transgenes. This indicates that the smaller hearts in our miR-15b transgenic mice partly resulted from reduced growth of individual cardiomyocytes.

Support for this concept is provided by the observed downregulation of the direct targets TGFBR1 and p38 in the hearts of miR-15b transgenic mice. An important difference between the miR-15b transgenic mice in the present study and the miR-195 transgenic mice described by the Olson group is the development of heart failure. The miR-195 transgenic mice, both
under control of the αMHC- and βMHC-promoter developed heart failure, while the miR-15b transgenic mice (αMHC) did not show any signs of heart failure until at least one year of age. An explanation for this difference may relate to the absolute levels of mature miRNAs of the miR-15 family, which were much higher in the miR-195 transgenic animals. In this regard, miR-195 has a higher endogenous expression in the heart and in both transgenic models this miRNA was overexpressed by more than 20-fold, while the endogenous expression of miR-15b is lower and our mice overexpressed this miRNA only 4-fold.

Two recent studies revealed that inhibition of the miR-15 family resulted in decreased infarct sizes after MI. Porrello et al. injected neonatal mice with antimiRs to inhibit miR-15 and subjected these mice to MI at 3 weeks of age. This resulted in increased regeneration of the infarcted myocardium due to proliferation of cardiomyocytes. Hullinger et al. injected adult mice with tiny LNAs at the moment of reperfusion in an ischemia-reperfusion model. Inhibition of miR-15 in this model protected hypoxic cardiomyocytes against apoptosis and this decreased the infarct size. As a consequence, these studies have suggested to inhibit miR-15 therapeutically after MI to reduce infarct size. Although inhibition of miRNAs as a therapeutic target seems feasible for some miRNAs, for example inhibition of miR-122 in hepatitis C patients appeared safe and effective in the first clinical trial to antimiR-based therapeutics, this approach might be more difficult for the miR-15 family after MI. This because miR-15 is ubiquitously expressed in the human body and therefore significant side effects might be expected. In this regard, the genetic loss of the miR-15/16 locus appears to be involved in the development of leukemia. Despite the exciting recent findings that inhibition of miR-15 immediately after MI protects against cardiomyocyte apoptosis, increases proliferation of surviving cardiomyocytes and improves cardiac function, our data indicate that on the long term adverse cardiac remodeling may occur due to unrestrained activation of the TGFβ-pathway.

In conclusion, we identified the miR-15 family as a regulator of cardiac hypertrophy and fibrosis by targeting multiple components of the TGFβ-pathway. Our data suggests that stimulation of the miR-15 family in cardiac fibroblasts may be of therapeutic interest to limit adverse remodeling in heart failure. Overexpression of miR-15 family members using fibroblast-specific promoters may be sufficient to limit cardiac fibrosis, and therefore these studies are awaited with great interest.
REFERENCES


MIRNA-15 INHIBITS TGFB SIGNALING

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22. van Deel ED, de Boer M, Kuster DW, Boontje NM, Holemans P, Sipido KR, van der Velden J, Duncker DJ. Exercise training does not improve cardiac function in compensated or decompensated left ventricular hypertrophy induced by aortic stenosis. *J Mol Cell Cardiol* 2011;50:1017-25.


SUPPLEMENTAL METHODS

Experimental animals

The Ren2-rat study was approved by the Institutional Animal Care and Use Committee of the Maastricht University. All other animal studies were approved by the Institutional Animal Care and Use Committee of the University of Amsterdam and in accordance with the guidelines of this institution.

TGR(ΔRen2)27-rats

In the Ren2-rats, the mouse renin-2 gene is introduced into the rat genome, which causes activation of the renin-angiotensin system. In homozygous rats this results in hypertension-induced cardiac hypertrophy at 8 weeks of age and heart failure before 18 weeks of age.1 The cardiac hypertrophy develops invariably, while some of these rats rapidly progress to heart failure and other similarly hypertensive littersmates remain compensated. In this study heart function of Ren2-rats was monitored by serial echocardiography at 10, 12, 15, 16, 18, 19, and 21 weeks of age. Rats were euthanized at 15 to 18 weeks on signs of heart failure (failing rats) or at 21 weeks when no signs of heart failure appeared (compensated rats). The hearts of Sprague-Dawley rats were used as controls. Total RNA was isolated from left ventricular (LV) tissue of 2 Sprague-Dawley control rats, 2 compensated Ren2-rats and 2 failing Ren2-rats using the mirVana miRNA isolation kit (Ambion) according to manufacturer’s protocol. 5 μg of total RNA was used for miRCURY™ LNA Arrays version 8.1 (Exiqon).

AntimiR-injected mice

In the first study C57BL/6J(OlaHsd (Harlan) mice at 9 weeks of age were injected intravenously with 5 mg/kg antimiRs (Ribotask) or PBS as vehicle control at two different timepoints (day 0 and day 5). AntimiRs consisted of locked nucleic acid (LNA) bases and 2’-O-methyl RNA bases in a ratio 1:2, where all nucleotides were phosphorothioated. The antimiRs were designed to perfectly complement the complete mature miR-15b sequence. Mice were sacrificed at day 7.

In the second study C57BL/6J(OlaHsd (Harlan) mice at 9 weeks of age were injected subcutaneously with 5 mg/kg antimiR-15b or a negative control antimiR (Ribotask) at two different timepoints (day 0 and day 7). The negative control sequence (Supplemental Table 1) is reported before.3 At day 3 or 4, mice were subjected to TAC surgery as described below. Mice were subjected to echocardiography and sacrificed 4 weeks after TAC surgery.

Supplemental Table 1. Sequences of miRNA-inhibitors

<table>
<thead>
<tr>
<th>AntimiR</th>
<th>Sequence (LNA, m= 2’O methyl RNA)</th>
</tr>
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<tbody>
<tr>
<td>antimiR-15</td>
<td>5’- IT mG mU IA mA mA IC mC mA IT mG mA IT mG mU mC mU IG mC mL mU IA -3’</td>
</tr>
<tr>
<td>antimiR-NC</td>
<td>5’- IA mC mA IC mA IC mA IC mC mC mC mU IG mU mC IA mC mA IT mU mC mC IA -3’</td>
</tr>
<tr>
<td>Tiny-miR15</td>
<td>5’- IG IT IT IC IT IC IC IT -3’</td>
</tr>
<tr>
<td>Tiny-miRNC</td>
<td>5’- IA IT IT IT IT IA IA IC -3’</td>
</tr>
</tbody>
</table>

Transgenic mice

To generate transgenic mice the mouse genomic fragment flanking pri-miR-15b was subcloned into the αMHC clone 26 vector, which contains the cardiomyocyte-specific αMHC promoter and the human growth hormone polyA signal.2 The pri-miR-15b sequence was amplified from mouse genomic DNA using the primers in Supplemental Table 2. The vector was linearized using Sall and pronuclear
injected with standard procedures in a FVB/NHsd strain. For genotyping we used a primer in the αMHC promoter (5’-GAAATGCAGTGTTAGGAAAGT-3’) in combination with the reverse primer used for cloning of the pri-miR-15b sequence. The mice were maintained on a mixed genetic background and all mice used in this study were from an F2 generation of the FVB strain backcrossed with C57BL/6J/OlaHsd mice. In all studies transgenic mice were compared to their wildtype littermates.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Vector</th>
<th>Primer sequence (restriction sites underlined)</th>
</tr>
</thead>
</table>
| Pri-miR-15b | αMHC clone 26 | Fw 5’-ACTGCTCAGAGTGTTAGGAAAGT-3’
|          |               | Rv 5’-ACTGCTCAGAGTGTTAGGAAAGT-3’
| 3’UTR TGFBR1 | pmiR-REPORT™ | Fw 5’-ACTGACGCGTTCTCTCTGTTGCTGAGTGTGTC-3’
|          |               | Rv 5’-ACTGACGCGTTCTCTCTGTTGCTGAGTGTGTC-3’
| 3’UTR TGFBR2 | pmiR-REPORT™ | Fw 5’-ACTGGTTTAAACTCCACTGCCACTTGCAC-3’
|          |               | Rv 5’-ACTGGTTTAAACTCCACTGCCACTTGCAC-3’
| 3’UTR SMAD3 | pmiR-REPORT™ | Fw 5’-ACTGACTAGTTCAAAGAACACCCAGTCCTCACTCAAC-3’
|          |               | Rv 5’-ACTGACTAGTTCAAAGAACACCCAGTCCTCACTCAAC-3’
| 3’UTR SMAD4 | pmiR-REPORT™ | Fw 5’-ACTGACTAGTTCAAAGAACACCCAGTCCTCACTCAAC-3’
|          |               | Rv 5’-ACTGACTAGTTCAAAGAACACCCAGTCCTCACTCAAC-3’
| 3’UTR SMAD7 | pmiR-REPORT™ | Fw 5’-ACTGACTAGTTCAAAGAACACCCAGTCCTCACTCAAC-3’
|          |               | Rv 5’-ACTGACTAGTTCAAAGAACACCCAGTCCTCACTCAAC-3’
| 3’UTR p38  | pmiR-REPORT™ | Fw 5’-ACTGACTAGTTCAAAGAACACCCAGTCCTCACTCAAC-3’
|          |               | Rv 5’-ACTGACTAGTTCAAAGAACACCCAGTCCTCACTCAAC-3’
| 3’UTR eng  | pmiR-REPORT™ | Fw 5’-ACTGACTAGTTCAAAGAACACCCAGTCCTCACTCAAC-3’
|          |               | Rv 5’-ACTGACTAGTTCAAAGAACACCCAGTCCTCACTCAAC-3’

Transverse aorta constriction surgery
To induce LV overload we surgically performed transverse aorta constriction (TAC) in 8 week old male antimiR-injected and transgenic mice. Mice were sedated with 4% isoflurane and intubated for mechanical ventilation. Mice were ventilated with a gas mixture of O2 and 2.5% isoflurane to maintain anesthesia and placed on a heating pad to maintain body temperature at 37°C. Buprenorphine (0.05 mg/kg) was injected subcutaneously for postsurgical analgesia. Thoracotomy was performed above the first rib just lateral of the sternum. The aortic arch was constricted (6-0 silk suture) together with a 27G (Ø 0.42 mm) needle between the truncus brachiocephalicus and the arteria carotis communis sinistra. Immediately after constriction the needle was removed to restore blood flow. The sham surgery was performed identically, but without the aortic ligation. The administration of buprenorphine (0.05 mg/kg s.c.) was repeated the first 2 days after TAC, when this deemed necessary based on the recovery of the mouse. Mice were sacrificed after 3 and 4 weeks of TAC in the transgenic and antimiR-injected mice experiments respectively.

Echocardiography
We measured LV function and dimensions by transthoracic two-dimensional echocardiography using a Vevo 770 Ultrasound (Visual Sonics) equipped with a 30-MHz linear array transducer. Mice were sedated on 4% isoflurane and anesthesia was maintained by a mixture of O2 and 2.5% isoflurane.
M-mode tracings in parasternal short axis view at the height of the papillary muscle were used to measure LV internal diameter at end-systole and end-diastole. Fractional shortening was calculated from these internal diameters using the following formula: ((LV end-diastolic diameter - LV end-systolic diameter) / LV end-diastolic diameter) x 100%

**Tissue collection**

Immediately after euthanization of the mice, hearts were injected with 1 mol/L KCl to induce relaxation of cardiac myocytes, fluid was removed from the lumen and hearts were weighed. The upper half of the heart was fixed in 4% paraformaldehyde for histological analysis. The lower half was divided in 2 parts, cutted through the septum and LV free wall, which were snap-frozen in liquid nitrogen for RNA and protein analysis respectively.

**Human samples**

We included patients with end-stage hypertrophic cardiomyopathy, end-stage dilated cardiomyopathy and aorta stenosis. Left ventricular (LV) samples were obtained from hearts explanted during surgery or valve replacement surgery. As controls we used LV tissue of non-failing donors (not used for transplantation due to logistic reasons), which had no history of cardiac abnormalities, normal ECG and normal ventricular function on echocardiography within 24 hours of heart transplantation. The study protocol was approved by the local ethical committees and all patients gave informed consent.

**Histological analysis**

Mouse hearts were fixed overnight in 4% paraformaldehyde, transferred to 70% ethanol until embedding, and embedded in paraffin using standard techniques.

Sections of 5 μm thickness were stained with hematoxylin and azophloxin by dewaxing, hydration, 10 min incubation with hematoxylin, 10 min rinsing with running tap water, 2 min rinsing with bidistilled water, 3 min incubation with azophloxin, 1 min differentiation in bidistilled water, dehydration, and mounting with entellan (Merck). Pictures of these sections were taken with a light microscope (Axiophot) at 20x magnification, with 5 pictures in the LV free wall, 5 in the septum and 5 in the papillary muscles. To determine cell size we measured the cross-sectional area of ~10 individual transversely cut cardiomyocytes per picture (~150 per heart) using the image processing software Scion Image.

To determine the collagen content in mouse hearts, we stained sections of 5 μm thickness with picrosirius red. Therefore sections were dewaxed, hydrated, incubated for 60 min in picrosirius red solution, 2 min differentiated under continuously moving in 0.01 N HCl dehydrated, and mounted in entellan (Merck). Pictures of these sections were taken with a light microscope (Axiophot) at 20x magnification, 10 pictures in the LV free wall, 10 in the septum, and 5 in the papillary muscles. Perivascular fibrosis was manually omitted from the pictures. The Sirius red positive areas in each picture were automatically calculated as a percentage of the total tissue area using an in house made quantification macro in ImagePro 6.2.

For in situ hybridizations we used sections of 7 μm thickness. These sections were dewaxed, hydrated and 5 min equilibrated in phosphate buffered saline (PBS). Sections were 15 min incubated with 1-Methylimidazole buffer (1% v/v 1-Methylimidazol pH 8.0, 0.3 mol/L NaCl), 30 minutes incubated in EDC (0.16 mol/L 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide in 1-Methylimidazole buffer), washed in PBS, proteinase-K treated for 5 min at 37ºC, washed with PBS, 15 min incubated in 1-Methylimidazole buffer, 30 minutes incubated in EDC, washed in PBS, 10 min fixed in 4% parafix,
washed in PBS, 20 min incubated in 3% H₂O₂ in PBS, and washed in PBS. Then sections were prehybridized for 30 min in hybridization mix (10 mmol/L Hepes pH 7.5, 600 mmol/L NaCl, 50% v/v Formamide, 1 mmol/L EDTA, 0.1% w/v Ficoll 400, 0.1% polyvinylpyrolidone, 0.1% BSA, 500 µg/ml Haring sperm DNA) at 60°C, 1.5 hr hybridized in 5 min pre-boiled hybridization mix with 500 nmol/L LNA probes (Riboblock, Supplemental table 3) at 60°C and then washed at 60°C in 2x SSC (0.3 mol/L NaCl, 30 mmol/L Trisodium Citrate), 0.5x SSC and 0.2x SSC respectively. Detection of the bound probes was performed by 30 min blocking with 2% w/v blocking powder (Roche) in PBS-T, 45 min incubation with anti-FITC-POD (Roche) 1:4 in blocking solution, washing with PBS-T, 5 min Tyramide Signal amplification (TSA, PerkinElmer), washing in PBS-T, 45 min incubation with anti-FITC-AP 1:200 in Blocking solution, washing in PBS-T, washing in NTM solution (100 mmol/L Tris pH9.5, 100 mmol/L NaCl, 50 mM MgCl₂), incubation in NBT/BCIP (Roche) 1:50 in NTM solution, washing in bidistilled water, 15 min incubation in nuclear red solution, washing in distilled water followed by dehydration of the slides and mounting with entellan. Pictures were taken at a 20X magnification with a light microscope (Axiophot).

Supplemental Table 3. Probe sequences for in situ hybridization

<table>
<thead>
<tr>
<th>Probe</th>
<th>Sequence (l=LNA, m= 2’O methyl RNA, FAm=FAM label)</th>
</tr>
</thead>
<tbody>
<tr>
<td>miR-15</td>
<td>5’- FAm lG mU mA lA mA mC lC mA lU IG mA mU lG IC mU mG lC -3’</td>
</tr>
<tr>
<td>miR-NC</td>
<td>5’- FAm lG mU mC lG lU mC lA mU mC lG mA lC IT mU lJ IG mU mA -3’</td>
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</table>

For immunofluorescence we used 5 µm thick sections, which were dewaxed, hydrated, and equilibrated in PBS. This was followed by antigen retrieval by 6 min of pressure cooking in antigen unmasking solution (Vector Laboratories, diluted 1:100 in bidest), 10 min incubation on ice, and washing in PBS. Sections were 2.5 hr incubated on RT with the first antibodies 1:200 in 0.1% triton in PBS (K67: rabbit polyclonal, monosan, psx1028; p-histoneH3: rabbit polyclonal, cell signaling, 97015; cTNI goat polyclonal, Hytest, 4T212), washed in PBS, and incubated first 1 hr at 37°C with the second antibody donkey-anti-goat Alexa-488 and after washing with PBS 1 hr at 37°C with the second antibody goat-anti-rabbit Alexa-568. Sections were washed again with PBS and 30 min stained at RT with DAPI 1:1000 in PBS. After washing with PBS, sections were mounted in Glycerol/PBS. Images were acquired with a fluorescence microscope (DM6000) at 10x magnification and the percentage of Ki67 or pHistoneH3 positive cardiomyocyte nuclei was calculated using an in house macro in Matlab. 1

Cell culture and transfections
Cardiac myocytes and fibroblasts
Neonatal rat cardiac myocytes and fibroblast were isolated from 1-2 day old Lewis rats, which were sacrificed by decapitation. Hearts were carefully taken out, atria removed and LV cut into small pieces. Ventricles were enzymatic digested using 0.05% collagenase I (Invitrogen) and 0.5% pancreatin (Sigma) in Hanks’ balanced salt solution (Sigma). Cells were pre-plated twice for 1 hr in DMEM 11966 (Invitrogen) supplemented with 10% horse serum, 5% newborn calf serum, 0.16% glucose and antibiotics (plating medium) to separate cardiac myocytes and fibroblasts. After pre-plating cardiac fibroblasts were cultured on DMEM 41965 (Invitrogen) supplemented with 10% non-heat inactivated fetal calf serum and antibiotics, while cardiac myocytes were plated at a density of 1*10⁴ cells per well in 6-wellplates coated with 1% gelatin (Fluka) in plating medium. After 2 days, plating medium was
changed into experimental medium (DMEM 11966/M199 4:1, 0.16% glucose, 2% BSA, antibiotics) overnight and cells were transfected at day 3. Cardiac fibroblasts of passage 2 were plated at a density of $1.5 \times 10^6$ per plate in 6-well plates for transfections.

Both cell types were transfected with 33 nmol/L mimic-miR15b or mimic-negative control #1 (Dharmacon) and in separate experiments with 220 nmol/L antimiR-15b or antimiR-NC (Ribotask) on plain medium using lipofectamine 2000 (Invitrogen) according to manufacturer's protocol and incubated for 6 hours. After 6 hours medium was changed into experimental medium for cardiac myocytes and standard culture medium described above for cardiac fibroblasts. After 48 hours cells were washed with PBS and RNA was isolated using TRIzol.

COS-7 cells
COS-7 cells were cultured in DMEM 41966 (Invitrogen) supplemented with 10% heat-inactivated fetal calf serum and antibiotics. Cells were plated in 24 wells-plates at a density of $1 \times 10^6$ cells per plate for transfections. At day 1 these cells were transfected with 100 nmol/L tiny-miR15 or tiny-NC on plain medium using lipofectamine 2000 according to manufacturer's protocol. Tiny LNAs are fully phosphorothioated LNA oligonucleotides directed against the seed sequence of the miR-15 family and the sequence is depicted in Supplemental table 1. After 6 hours, transfection medium was changed into standard culture medium. At day 2 cells were co-transfected with 10 ng pmir-report empty (Ambion) or this vector with the 3'UTR of TGFBR1, TGFBR2, SMAD3, SMAD4, SMAD7, p38 or endoglin and with 5 ng phRL-renilla luciferase (Promega) using genejammer (Agilent technologies) according to manufacturer's protocol on standard culture medium. After 24 hours cells were washed with PBS and lysed for luciferase assays.

HepG2 cells
HepG2 cells were cultured in DMEM/F12 medium (Invitrogen) supplemented with 10% non heat-inactivated fetal calf serum and antibiotics. Cells were plated in 24 wells-plates at a density of $2 \times 10^6$ cells per plate for transfections. At day 1 these cells were transfected with 33 nmol/L mimic-miR15b, mimic-negative control #1, 100 nmol/L tiny-miR15 or tiny-NC on plain medium using lipofectamine 2000 according to manufacturer's protocol. After 6 hours, transfection medium was changed into serum-free standard culture medium. At day 2 cells were co-transfected with 200 ng CAGA-luciferase reporter and 5 ng phRL-renilla luciferase on serum-free standard culture medium using genejammer according to manufacturer's protocol. At day 3 cells were stimulated in serum-free standard culture medium with 6 different concentrations of recombinant TGFβ1 (Tebu-Bio) ranging from 0-2 ng/ml. After 24 hours of stimulation cells were washed with PBS and lysed for luciferase assays.

Luciferase assays
Cell were washed in PBS and lysed in 1x lysis buffer (Renilla reporter assay system Promega) diluted in milli-Q by 15 min shaking at RT and 1 freeze-thaw cycle. 20 μl lysate was used to measure luciferase and Renilla luciferase activity in white 96-wellsplates (cellstar). We used the Glomax multi detection system to add the substrate and measure the emitted light with 3 sec delay after addition of the substrate and 1 sec of integration. We used 100 ul of luciferin substrate (Biafiltr) and 50 ul of Renilla substrate (Promega). Renilla luciferase activity was used to normalize luciferase activity per well for cell densities and transfection efficiencies.
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RNA analysis

Except for the Ren2-rats, where RNA was isolated using the mirVana miRNA isolation kit according to manufacturer’s protocol, all RNA was isolated using TRIzol (Invitrogen) according to manufacturer’s protocol.

Northern blot

For northern blot we used a starfire probe (Integrated DNA Technologies) with a sequence perfectly complementary to the mature miR-15b sequence. This probe was labeled using the StarFire Kit (Integrated DNA Technologies) and purified on an Illustra ProbeQuant G50 Micro Column (GE Healthcare) both according to manufacturer’s protocol. 3 µg total RNA was loaded and separated on a 16% acrylamide gel. To confirm equal loading gels were stained with ethidium bromide. Afterwards RNA is transferred to a Hybond N+ membrane, UV crosslinked (0.2 J/cm²), and baked 2 hr at 80°C. Labeled probes were hybridized at 39°C in hybridization buffer (7% SDS, 200 mM Na₂HPO₄, pH7.2) overnight. The next day membranes were washed at 42°C with 2x SSPE buffer (0.36 mol/L NaCl, 200 mmol/L NaH₂PO₄, 20 mmol/L EDTA, 0.1% DEPC, 0.1% SDS, pH 7.4) and exposed to Phospho-image film overnight.

cDNA preparation and Q-PCR using the mirVana system

This system was used to measure miRNA levels in the transgenic mice. MiRNA-specific cDNA was generated of 400 ng of total RNA using the Iscript cDNA synthesis kit (Bio-Rad) and the mirVana quantitative RT-PCR primer sets (Ambion) according to manufacturer’s protocol. This cDNA was amplified using the mirVana quantitative RT-PCR primer sets (Ambion) and LightCycler 480 SYBR green master I (Roche) in a LightCycler 480 system II using the following program: 95°C for 3 minutes and 40 cycli of 95°C for 15 seconds and 60°C for 45 seconds. All samples in the qPCRs were measured in duplo.

cDNA preparation and Q-PCR using the Taqman system

This system was used to determine miRNA levels in RNA isolated from anti-miR injected mice and from cells transfected with tiny LNAs. MiRNA-specific cDNA was generated of 400 ng of total RNA using the Taqman miRNA reverse transcription kit (Applied Biosystems) and the RT-primer of the Taqman miRNA assay (Applied Biosystems) according to manufacturer’s protocol. cDNA was amplified using the Taqman miRNA assay (Applied Biosystems) and LightCycler 480 probes master (Roche) in a lightcycler 480 system II using the following program: 95°C for 10 minutes and 40 cycli of 95°C for 15 seconds and 60°C for 1 minute.

cDNA preparation and Q-PCR using the miScript system

This system was used to measure mRNA levels in all experiments and miRNA levels in human samples and cells transfected with antimiRs and mimics. Total cDNA was generated of 400 ng of total RNA using the miScript reverse transcription kit (Qiagen) according to the manufacturer’s protocol. Real-time PCR was performed using the LightCycler 480 High Resolution Melting Master (Roche). The reaction was performed according to manufacturer’s protocol in a mixture with 2.5 mmol/L MgCl₂ and with 8 times diluted cDNA. The cDNA was amplified in a LightCycler 480 system II using the following program: 95°C for 10 minutes and 40 cycli of 95°C for 45 seconds, annealing temperature for 45 seconds, and 72°C for 45 seconds, where the annealing temperature was 55°C for miRNA amplification and 60°C for mRNA amplification. Primer sequences are included in Supplemental table 4.
<table>
<thead>
<tr>
<th>Gene</th>
<th>Species</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>miRNA general reverse</td>
<td>all</td>
<td>5'-GAATCGAGCACCAGTTACGC-3'</td>
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<tr>
<td>miR-15b</td>
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<td>miR-16</td>
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Protein analysis
Protein was extracted from LV tissue in RIPA buffer (50 mmol/L Tris-HCl pH 8.0, 150 mmol/L NaCl, 1% Igepal CA-630, 0.5% Sodium deoxycholate, 0.1% sodium dodecyl sulfate) with addition of protease inhibitor cocktail (Roche) and 1 mmol/L Sodium Orthovanadate. Protein concentrations were measured using the BCA protein assay kit (Pierce) according to manufacturer’s protocol.

For western blots protein was diluted in a sample buffer (17.5% glycerol, 6% SDS, 250 mmol/L TRIS-HCl pH 6.7, 10% β-mercapto-ethanol) and denatured 10 min at 95°C. 30 µg of protein was loaded and separated on a 10% acrylamide gel and transferred to a methanol activated polyvinylidene
fluoride (PVDF) membrane (Millipore). This membrane was blocked 1 hr in 3.5% protifar in TBST and incubated in the first antibodies overnight at 4°C (TGFBR1: rabbit polyclonal, 1:500, sancta cruz, sc-398; GAPDH: mouse monoclonal, 1:10,000, Fitzgerald, G109a) The next day membranes were washed in TBST and at least 2 hr incubated in the second antibody at RT (1:5000, HRP-linked). Bands were detected using the ECL prime western blotting detection reagent (Amersham) and images acquired using the ImageQuant LAS4000 (GE Healthcare).

Plasmid constructions
The luciferase reporter plasmids for TGFBR1, TGFBR2, SMAD3, SMAD4, SMAD7, p38, and endoglin were constructed by PCR-amplification of the 3'UTRs from mouse genomic DNA with the primers in Supplemental table 2. These fragments were cloned into the pMIR-REPORT™ luciferase vector (Ambion) downstream of the luciferase coding region. All generated constructs were sequence verified.

The CAGA-luc reporter was a kind gift of prof. Goumans (Leiden university medical center). This plasmid contains 12 times the CAGACA sequence in front of the luciferase reporter (pGL3-basic vector, Promega).

Statistical analysis
Data are shown as mean +/- SEM and sample sizes are mentioned in the Figure legends per individual experiment. Student’s t-test was performed to compare the differences between means. For the CAGA-experiments linear regression analysis was performed to compare the slopes of luciferase activation by TGFβ. Probability values <0.05 were considered statistically significant.
CHAPTER 4

A

TGFBR1 3'-ACAUUUG---GCUGAC---GGACCU-5' miR-15
Mouse 5' - CCAUCGGAGG---GCCGUGUGGACCUGU-3'
Rat 5' - CUAAGCGG---GCCGUGUGGACCUGU-3'
Human 5' - UUAGUGUGUGGACCUGU-3'

3'-ACAUUUG---GCCGUGUGGACCUGU-5' miR-15

TGFBR1 3'-ACAUUUG---GCUGAC---GGACCU-5' miR-15
Mouse 5' - GUUGGCGG---GCCGUGUGGACCUGU-3'
Rat 5' - GUUGGCGG---GCCGUGUGGACCUGU-3'
Human 5' - AUUGGCGG---GCCGUGUGGACCUGU-3'

TGFBR1 3'-ACAUUUG---GCUGAC---GGACCU-5' miR-15
Mouse 5' - GCCGUGUGGACCUGU-3'
Rat 5' - GCCGUGUGGACCUGU-3'
Human 5' - GCCGUGUGGACCUGU-3'

TGFBR1 3'-ACAUUUG---GCUGAC---GGACCU-5' miR-15
Mouse 5' - GCCGUGUGGACCUGU-3'
Rat 5' - GCCGUGUGGACCUGU-3'
Human 5' - GCCGUGUGGACCUGU-3'

TGFBR2 3'-ACAUUUG---GCUGAC---GGACCU-5' miR-15
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Rat 5' - CUGUUGGACCUGU-3'
Human 5' - CUGUUGGACCUGU-3'

TGFBR2 3'-ACAUUUG---GCUGAC---GGACCU-5' miR-15
Mouse 5' - ACCGUUGGACCUGU-3'
Rat 5' - ACCGUUGGACCUGU-3'
Human 5' - ACCGUUGGACCUGU-3'
C

**TGFB3**

3'-ACAUUGUGUACUCACGACGAA-5' miR-15

Mouse 5'- UUCUUGUACUGUGACGCGCUU-3'

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Human 5'- GUCUCGUGUGACGCGACGCUU-3'

**TGFB3**

3'-ACAUUGUGUACUCACGACGAA-5' miR-15

Mouse 5'- CCUCUCUUGUGACGCGACGCUU-3'

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**TGFB3**

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**SMAD2**

3'-ACAUUGUGUACUCACGACGAA-5' miR-15

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Human 5'- GUCUCGUGUGACGCGACGCUU-3'

**SMAD2**

3'-ACAUUGUGUACUCACGACGAA-5' miR-15

Mouse 5'- CUGUGUGUGACGCGACGCUU-3'

Rat 5'- CUGUGUGUGACGCGACGCUU-3'

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**SMAD2**

3'-ACAUUGUGUACUCACGACGAA-5' miR-15

Mouse 5'- GUCUCGUGUGACGCGACGCUU-3'

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**SMAD4**

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**SMAD4**

3'-ACAUUGUGUACUCACGACGAA-5' miR-15

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Supplemental figure 1. Predicted miR-15 family target sites in the genes of the TGFβ-pathway. Schematic representation of the predicted miR-15 family target sites and their evolutionary conservation in the 3’UTR of A) TGFBR1, B) TGFBR2, C) TGFBR3, D) SMAD2, E) SMAD4, F) SMAD3, G) SMAD7, H) endoglin, and I) p38. Underlined, nucleotides belonging to the target site. Grey, nucleotides not conserved compared to mouse.
Supplemental figure 2. Confirmation of overexpression and knockdown after mimic and inhibitor transfections. A) Mimic miR-15b transfection in HepG2 cells results in overexpression of miR-15b. B) Tiny LNA transfection in HepG2 cells results in inhibition of miR-15b, miR-16 and miR-195. MiR-497 was not inhibited, but this miRNA has a very low expression in HepG2 cells. C) Mimic transfections in cardiac myocytes and fibroblasts results in a strong overexpression of miR-15b, while D) antimiR transfections resulted in a strong inhibition of miR-15b in these cells. E) Tiny LNA transfections in COS7-cells inhibited the expression levels of miR-15 family members, and also in these cells miR-497 was relatively low expressed. N=3 for all experiments, *p<0.05 compared to negative control transfected cells.
Supplemental figure 3. Cardiac remodeling is aggravated after inhibition of miR-15b. A) The fibrotic response to TAC is increased after inhibition of miR-15b in the LV free wall and in the papillary muscle, but not in the septum. B) There is a trend towards a stronger increase in the hypertrophic markers ANF and βMHC after miR-15b inhibition in TAC. C) Although TAC induced cardiac remodeling is aggravated after inhibition of miR-15b, the decline in cardiac function as measured by fractional shortening is similar in antimiR-15b and antimiR-NC injected mice. N=5 antimiR-NC sham, n=8 antimiR-NC TAC, n=6 antimiR-15b sham, n=9 antimiR-15b TAC. *p<0.05 compared to sham, **p<0.05 compared to antimiR-NC.
Supplemental figure 4. MiR-15b transgenic mice have smaller hearts. A) Real-time PCR shows a 1500-fold upregulation of pri-miR-15b in miR-15b transgenic mice. Normalized to HPR, n=2. B) Heart weight corrected for tibia length is decreased in miR-15b transgenic mice of line C. C) Echocardiography in line A reveals a decline in LV internal diameter in 8-12 month old miR-15b transgenic mice and D) no differences in fractional shortening between wildtype and transgenic mice. E) No difference in Ki67 or phospho-HistoneH3 (pH3) positive cardiomyocytes was detected between wildtype and transgenic mice of line A at neonatal day 3. N=6 wildtype, 6 transgene line A; 6 wildtype, 5 transgene line C at 8-12 weeks, n=4 wildtype, 6 transgene line C at 17-24 weeks, n=4 wildtype, 10 transgene line A; 9 wildtype, 5 transgene line C at 8-12 months, n=6 wildtype, 10 transgene at neonatal day 3. *p<0.05 compared to wildtype mice.
Supplemental figure 5. Cardiomyocyte specific miR-15b transgenic mice display similar pressure-overload induced cardiac remodeling as their wildtype littermates. A) Wildtype and transgenic mice have a similar increase in heart weight corrected for tibia length, B) in the hypertrophic markers ANF and BNP, and in C) cardiomyocyte hypertrophy measured in H&E stained sections. D) Representative images of picrosirius red stained sections show no difference in fibrotic response to TAC in transgenic and wildtype mice, which is confirmed by quantification E) and by real-time PCR of fibrotic genes F). G) Wildtype and transgenic mice also display a similar decrease in fractional shortening 3 weeks after TAC surgery. N=6 wildtype sham, n=10 wildtype TAC, n=6 transgenic sham, n=7 transgenic TAC. *p<0.05 compared to sham, +p<0.05 compared to wildtype mice.
REFERENCES


