The microRNA-15 family inhibits the TGFβ-pathway in the heart

Anke J. Tijsen[1], Ingeborg van der Made[1], Maarten M. van den Hoogenhof[1], Wino J. Wijnen[1], Elza D. van Deel[2], Nina E. de Groot[1], Sergey Alekseev[1], Kees Fluiter[3], Blanche Schroen[4], Marie-José Goumans[5], Jolanda van der Velden[6], Dirk J. Duncker[2], Yigal M. Pinto[1], and Esther E. Creemers[1]*

[1]Department of Experimental Cardiology, Academic Medical Center, University of Amsterdam, Meibergdreef 15, 1105 AZ Amsterdam, The Netherlands; [2]Department of Cardiology, Thoraxcenter, Erasmus Medical Center, University Medical Center Rotterdam, Rotterdam, The Netherlands; [3]Department of Genome Analysis, Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands; [4]Department of Cardiology, Center for Heart Failure Research, Cardiovascular Research Institute Maastricht, Maastricht University, Maastricht, The Netherlands; [5]Department of Molecular and Cell Biology, Centre of Biomedical Genetics, Leiden University Medical Center, Leiden, The Netherlands; and [6]Laboratory for Physiology, Institute for Cardiovascular Research, VU University Medical Center, Amsterdam, The Netherlands

Aims	The overloaded heart remodels by cardiomyocyte hypertrophy and interstitial fibrosis, which contributes to the development of heart failure. Signalling via the TGFβ-pathway is crucial for this remodelling. Here we tested the hypothesis that microRNAs in the overloaded heart regulate this remodelling process via inhibition of the TGFβ-pathway.
Methods and results	We show that the miRNA-15 family, which we found to be up-regulated in the overloaded heart in multiple species, inhibits the TGFβ-pathway by targeting of TGFBR1 and several other genes within this pathway directly or indirectly, including p38, SMAD3, SMAD7, and endoglin. Inhibition of miR-15b by subcutaneous injections of LNA-based antimiRs in C57BL/6 mice subjected to transverse aorta constriction aggravated fibrosis and to a lesser extent also hypertrophy.
Conclusion	We identified the miR-15 family as a novel regulator of cardiac hypertrophy and fibrosis acting by inhibition of the TGFβ-pathway.
Keywords	Fibrosis • Hypertrophy • miRNA-15 family • TGFβ-pathway

1. Introduction

The heart responds to injury, caused by, e.g. myocardial infarction or sustained pressure overload through a number of structural alterations commonly referred to as cardiac remodelling. At the cellular level these alterations include cardiomyocyte hypertrophy, cardiomyocyte apoptosis, and changes in the expression of genes regulating energy metabolism, calcium handling, and genes normally expressed in the embryonic heart.[1] On the other hand, cardiac remodelling 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.[1] 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.[1]

Transforming growth factor β (TGFβ) is a cytokine that regulates both cardiomyocyte and fibroblast biology and its expression and signalling activity are increased during cardiac remodelling.[2,3] Both cell types express TGFβ1 and its two serine−threonine kinase receptors (TGFBR1 and 2).[4] In cardiac fibroblasts, binding of TGFβ to its receptors results in the activation of the canonical signalling 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.[5] A number of mouse models have established that activation of the canonical TGFβ-pathway in the heart promotes the development of fibrosis.[6,7] For example, heterozygous TGFβ1 deficient mice reveal attenuated fibrosis in the ageing heart,[7] while overexpression of TGFβ1 in transgenic mice results in increased interstitial fibrosis.[6] Also endoglin (eng), a TGFβ1 co-receptor required for signalling in the cardiac

* Corresponding author. Tel: +31 20 5668544; fax: +31 20 6976177, Email: e.e.creemers@amc.uva.nl

fibroblast was recently shown to be involved in the regulation of cardiac fibrosis, as transverse aorta constriction (TAC) in heterozygous endoglin deficient mice revealed attenuated cardiac fibrosis and preserved left-ventricular function.[8]

In cardiomyocytes, binding of TGFβ to its receptors leads to the 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.[9] Activation of the non-canonical TGFβ-pathway in myocytes during cardiac remodelling has been shown to regulate the development of myocyte hypertrophy.[6,10,11] Mice with cardiomyocyte-specific overexpression of constitutively active TAK1 showed an increased p38 phosphorylation, myocyte hypertrophy, and severe myocardial dysfunction.[10] Furthermore, Koitabashi *et al.*[11] 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 remodelling, 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 remodelling in rats and mice inhibited fibrosis, but did not affect cardiomyocyte hypertrophy.[11,12] Koitabashi *et al.*[11] 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 interstitial TGFβ signalling 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.[13,14] However, a phase III clinical trial investigating tranilast in restenosis revealed some adverse effects of this drug.[15] 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.[16,17] 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.[18]

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 miRNA expression 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,[19] and up-regulated in the diseased myocardium.[20] 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 fibrosis and to a lesser extent also cardiomyocyte hypertrophy, compared with the control antimiR group. In conclusion, we identified the miR-15 family as a regulator of cardiac hypertrophy and fibrosis acting by inhibition of the TGFβ-pathway.

2. Methods

2.1 Experimental animals

All animal studies were approved by the Institutional Animal Care and Use Committee of the University of Amsterdam (approval no. DCA102271) and in accordance with the guidelines of this institution and the Directive 2010/63/EU of the European Parliament.

Heart tissue of TGR(mRen2)27-rats (Ren2-rats) were used from a previous study.[21] These rats were anaesthetized with 50 mg/kg intraperitoneally injected pentobarbital to perform echocardiography. Thereafter, the heart was rapidly excised.

For the antimiR studies, we used 9-week-old C57BL/6JOlaHsd (Harlan) mice. In the first study, we injected them subcutaneously 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.[22] Therefore, mice were sedated with 4% isoflurane and intubated for mechanical ventilation with a gas mixture of O_2 and 2.5% isoflurane. Buprenorphine (0.05 mg/kg) was injected subcutaneously for surgical analgesia and repeated the first 2 days after TAC, when this deemed necessary based on the recovery of the mouse. After 4 weeks, mice were sedated with 4% isoflurane and anaesthesia is maintained with a gas mixture of O_2 and 2.5% isoflurane to perform echocardiography. Sedated mice were euthanized by 100% CO_2 ventilation. For further details on the animal studies see the Supplementary material online.

2.2 Human samples

We included patients with end-stage hypertrophic cardiomyopathy (HCM), 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 conformed with the Declaration of Helsinki and approved by the local Ethical Committee. All patients gave informed consent.

2.3 Histological and molecular analysis

Methods of picrosirius red stainings, Hematoxyline & Azophloxine (H&A) stainings, microRNA *in situ* hybridizations, luciferase assays, and RNA analysis are described in the Supplementary material online, Methods.

2.4 Cell culture and transfections

Neonatal rat ventricular myocytes and fibroblasts were isolated by enzymatic digestion of 1–2-day-old rat hearts as described previously.[23] 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 h 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 phRL-renilla luciferase (Promega) using genejammer (Agilent Technologies). After 24 h cells were lysed for luciferase assays. HepG2 cells were co-transfected with 200 ng CAGA-luciferase reporter and 5 ng phRL-renilla luciferase using genejammer according to the manufacturer's protocol. The next day, cells were stimulated with six different concentrations of recombinant TGFβ1 (Tebu-Bio) ranging from 0 to 2 ng/mL. After 24 h of stimulation cells were lysed for luciferase assays.

2.5 Statistical analysis

Data are shown as mean $\pm$ SEM and sample sizes are mentioned in the figure legends per individual experiments. Two-way ANOVA was performed to compare values in multiple group experiments with two main treatments. In case of a significant interaction between the two main factors, an independent samples *t*-test (or one-way ANOVA with Tukey's *post-hoc* in case of more than two groups) was reported. For the CAGA-experiments, linear regression analysis was performed to compare the slopes of luciferase activation by TGFβ with and without miR-15 manipulation.

3. Results

3.1 The miR-15 family is predicted to target multiple genes in the TGFβ-pathway

Since the TGFβ-pathway is critically involved in cardiac remodelling, we searched for miRNAs that are abundantly expressed in the heart and are capable of regulating the TGFβ-pathway. To identify which miRNAs may regulate the TGFβ-pathway, we undertook a bioinformatics 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 (rodent and human miR-15a/b, miR-16, miR-195, miR-497, and the rodent miR-322 with its human homologue miR-424), which are all abundantly expressed in the heart, both in cardiomyocytes and fibroblasts.[19] 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. 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 1*, see Supplementary material online, *Figure S1*).

3.2 The miR-15 family is up-regulated in cardiac hypertrophy and heart failure

In an independent study, we identified miR-15b as a miRNA up-regulated in the left ventricle of Ren2-rats, a model of hypertension-induced hypertrophy and heart failure (see Supplementary material online, *Figure S2A*). We also found miR-15b and its family member miR-16 significantly up-regulated in human end-stage HCM compared with healthy donor hearts (*Figure 1B*), while the other family members showed a trend towards up-regulation. To determine whether the miR-15 family is also up-regulated in other forms of human hypertrophy and heart failure, we conducted real-time PCR of all miR-15 family members on left ventricular (LV) samples of patients with end-stage dilated cardiomyopathy and aorta stenosis and compared it with the expression in healthy donor hearts. As shown in *Figure 1C*, miR-15b, miR-16, and miR-195 are also significantly up-regulated in pressure overloaded hearts due to aortic stenosis. In a second rodent model, we subjected mice to 4 weeks of TAC, to induce pressure overload hypertrophy and measured all miR-15 family members by real-time PCR. Also in these hearts, we observed an increased expression of the miR-15 family (*Figure 1D*, note that the rodent miR-322 is the homologue of human miR-424). The up-regulation of miR-15b in failing

Ren2-rat hearts and its significant up-regulation in human HCM and aorta stenosis patients was the rationale to further focus our studies on this particular miRNA.

To identify the cell type(s) responsible for miR-15b expression in the heart, we performed *in situ* hybridization on paraffin sections of adult wild-type 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. In cardiomyocytes, we detected a nuclear and cytoplasmic staining, of which the nuclear staining most probably represents staining of miRNA precursors and the cytoplasmic staining of the mature miRNA. Real-time PCR of the miR-15 family on equal amounts of RNA isolated from cultured neonatal rat cardiomyocytes and fibroblasts confirmed that this miRNA family is expressed in both cell types, and that except for miR-322, which is higher expressed in fibroblasts, the level of expression is comparable (*Figure 1F*).

3.3 The miR-15 family 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.[24] 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*, see Supplementary material online, *Figure S3A*). Since the expression of miR-15b in HepG2 cells is relatively low compared with other miR-15 family members (see Supplementary material online, *Figure S3B*), we reasoned that knockdown of miR-15b in these cells would not be sufficient to increase activation of the CAGA reporter. Therefore, we decided to inhibit the complete miR-15 family by using tiny LNA oligonucleotides directed against the common seed sequence. This resulted in increased luciferase activity of the CAGA-reporter upon TGFβ stimulation (*Figure 2B*, see Supplementary material online, *Figure S3C*). Together, these luciferase assays provide the first *in vitro* evidence that the miR-15 family suppresses the canonical TGFβ-pathway.

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 (see Supplementary material online, *Figure S3D* and *S3E*) and measured mRNA levels by real-time PCR. As can be seen from see Supplementary material online, *Figure S2B*, all miR-15 family members are expressed in neonatal hearts, supporting the use of neonatal cells for knockdown experiments. In neonatal cardiomyocytes (*Figure 3A*), overexpression of miR-15b resulted in decreased mRNA levels of p38, SMAD2, SMAD3, and endoglin. No significant effects were seen on TGFBR1, TGFBR2, TGFBR3, SMAD4, and SMAD7. Conversely, transfection of cardiomyocytes with antimiR-15b resulted in a significant up-regulation of p38, TGFBR1, TGFBR2, TGFBR3, SMAD3, and SMAD7, while SMAD2, SMAD4, and endoglin levels were not changed (*Figure 3A*). Together this indicates that in cardiomyocytes, the transcript levels of p38, SMAD3, and SMAD7 are tightly regulated by the miR-15 family. Moreover, the observation that TGFBR1, TGFBR2, and SMAD7 mRNA is significantly up-regulated after inhibition of miR-15b and SMAD2 and endoglin mRNA is significantly down-regulated after overexpression of miR-15b suggests that the expression of these genes is also regulated
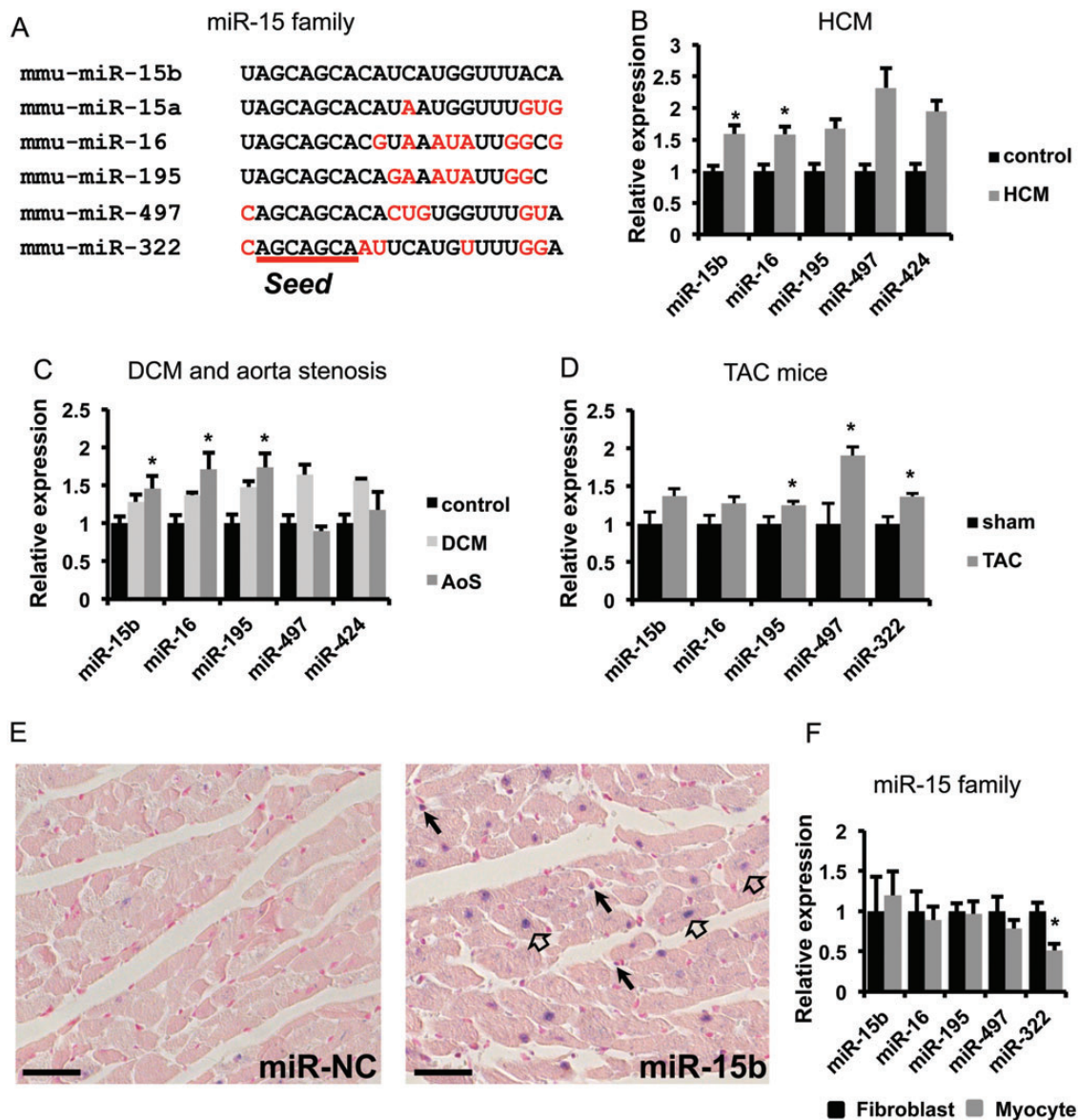
Figure 1 MiR-15 family expression in the heart. (*A*) miR-15b is part of the miR-15 family that includes six miRNAs, which all share a common seed sequence. Differences in nucleotides compared with the miR-15b sequence are depicted in red. (*B*) The miR-15 family is up-regulated in human end-stage HCM (*n* = 8) compared with healthy control hearts (*n* = 5). (*C*) In human hearts, up-regulation of the miR-15 family is also observed in end-stage dilated cardiomyopathy (*n* = 5), and aorta stenosis patients (*n* = 10) compared with healthy control hearts (*n* = 5). (*D*) The complete miR-15 family is up-regulated after 4 weeks of TAC in mice (*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 expression levels of the miR-15 family in cultured cardiomyocytes and fibroblasts, except for miR-322 which is higher expressed in fibroblasts (*n* = 3). MiRNA levels in real-time PCR were normalized to U6. *P-value < 0.05 compared with the control group.

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 up-regulation or knockdown was less pronounced compared with the 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.[25] Overall,

many of the predicted target genes showed a trend towards down-regulation after miR-15b overexpression in cardiac fibroblasts and an up-regulation after inhibition of miR-15b.

Because miRNA effects can be more pronounced on the protein than on the mRNA level, we investigated whether miR-15b overexpression would be able to regulate TGFβ receptors expression at the protein level. Therefore, we performed western blotting using lysates of cardiomyocytes in which miR-15b was overexpressed with miR-15b mimics. In these experiments, we detected a reduced protein level of TGFBR1 and TGFBR3 after overexpression of miR-15b but not of TGFBR2 (*Figure 3C*).

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 (see Supplementary material online, *Figure S3F*). Knockdown of the miR-15 family in COS7 cells resulted in an up-regulation of the luciferase activity of the reporters containing the 3′UTR of TGFBR1, SMAD3, SMAD7, p38, and endoglin (*Figure 3D*), suggesting that the miR-15 family is able to repress the expression of these genes by interaction with 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 target SMAD2 or TGFBR3.

To 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 subcutaneously with LNA-based antimiR-15b or antimiR-NC twice and sacrificed the mice 1 week after the first injection (*Figure 4A*). Real-time PCR revealed the complete loss of miR-15b and an inhibition of the other miR-15 family members in the myocardium of these mice (*Figure 4B*). The loss of the miR-15 family members in these mice resulted in a significant up-regulation of TGFBR1 and SMAD3 mRNA, a trend towards up-regulation of p38, TGFBR2, TGFBR3, SMAD4, SMAD7, and endoglin mRNA, and no effect on SMAD2 mRNA levels (*Figure 4C*).

3.4 *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 remodelling, we injected the LNA-based antimiRs subcutaneously in mice and subjected them to TAC or sham surgery (*Figure 4D*). Mice were sacrificed after 4 weeks of TAC and the loss of miR-15b in the heart was confirmed by northern blot (*Figure 4E*). 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 up-regulation of the complete miR-15 family after TAC (*Figure 4F*). The level of down-regulation 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 (with concomitant down-regulation of some of the miR-15 family members) in TAC-induced cardiac remodelling resulted in a stronger increase in heart weight corrected for tibia length compared with the antimiR negative control (antimiR-NC) injected mice (*Figure 5A*). Next, we investigated whether the fibrotic response, a process mainly governed by cardiac fibroblasts was affected. Therefore, we determined the area of collagen fibres by means of picrosirius red staining in cardiac sections. Strikingly, we found that upon loss of miR-15b, fibrosis was more strongly induced after 4 weeks of TAC

Table 1 Predicted miR-15 family targets in the TGFβ-pathway

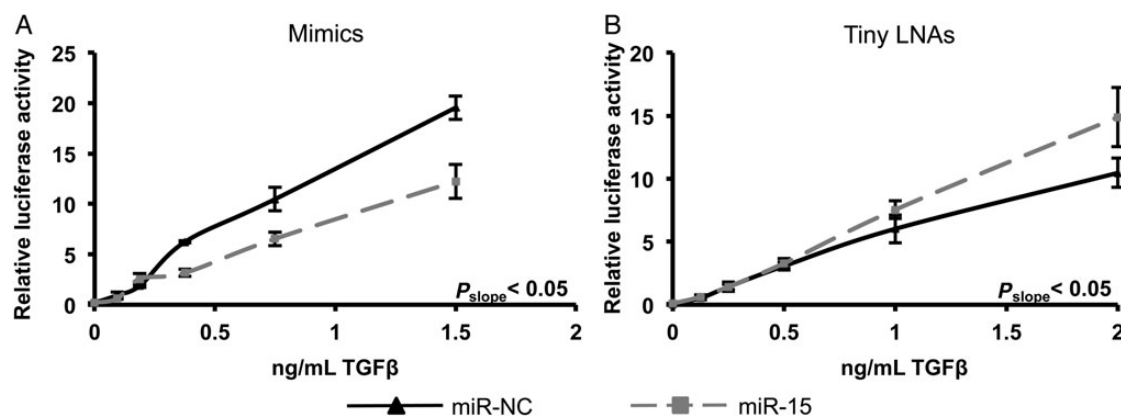| Gene	Canonical/non-canonical pathway	Predicted binding sites in mouse	Conserved binding sites to rat and human
TGFBR1	Both	6	5
TGFBR2	Both	2	0
TGFBR3	Canonical	3	1
Endoglin	Canonical	1	0
SMAD2	Canonical	2	1
SMAD3	Canonical	5	3
SMAD4	Canonical	2	0
SMAD7	Canonical	1	1
TRAF6	Non-canonical	3	0
TAK1	Non-canonical	1	1
p38	Non-canonical	2	1

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. $n = 6$, $P < 0.05$ for the difference in slope of luciferase activation between the NC and miR-15 transfected cells.
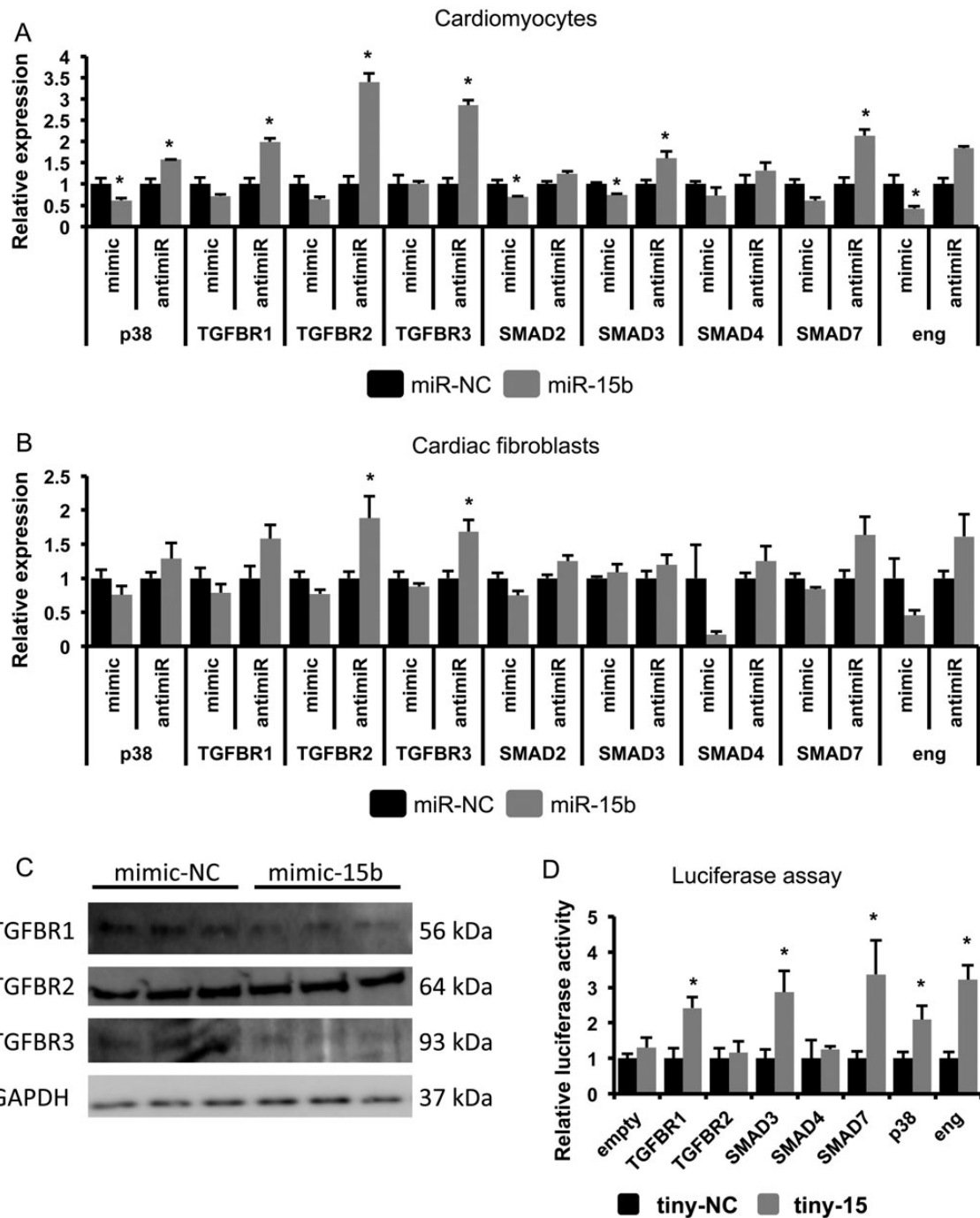
Figure 3 The miR-15 family targets numerous genes in the TGFβ-pathway. (*A*) mRNA levels detected by real-time PCR of predicted miR-15 target genes in cardiomyocytes and (*B*) fibroblasts with overexpression (mimic) and knockdown (antimiR) of miR-15b levels. mRNA levels are normalized to Hypoxanthine Phosphoribosyltransferase 1 (HPRT) levels. (*C*) Protein levels of TGFBR1 and 3, but not of TGFBR2, are down-regulated after overexpression of miR15b in cardiomyocytes. GAPDH is shown as loading control. (*D*) Luciferase assays using reporters with the respective 3′UTR subcloned behind the luciferase gene suggest 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 with negative control (NC) transfected cells.

(*Figure 5B* 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. In these regions, we found that antimiR-15b treatment increased collagen deposition after TAC ∼3.5-fold compared with antimiR-NC injected mice. Interestingly, this antimiR-15b mediated

increase in fibrosis was not seen in the septum of the heart (see Supplementary material online, *Figure S4A*).

We also determined whether cardiomyocyte size was affected after antimiR-15b treatment, by measuring cell size area in H&A-stained sections of the antimiR injected mice. As shown by the representative images in *Figure 5D* and the quantifications in *Figure 5E*, this revealed a
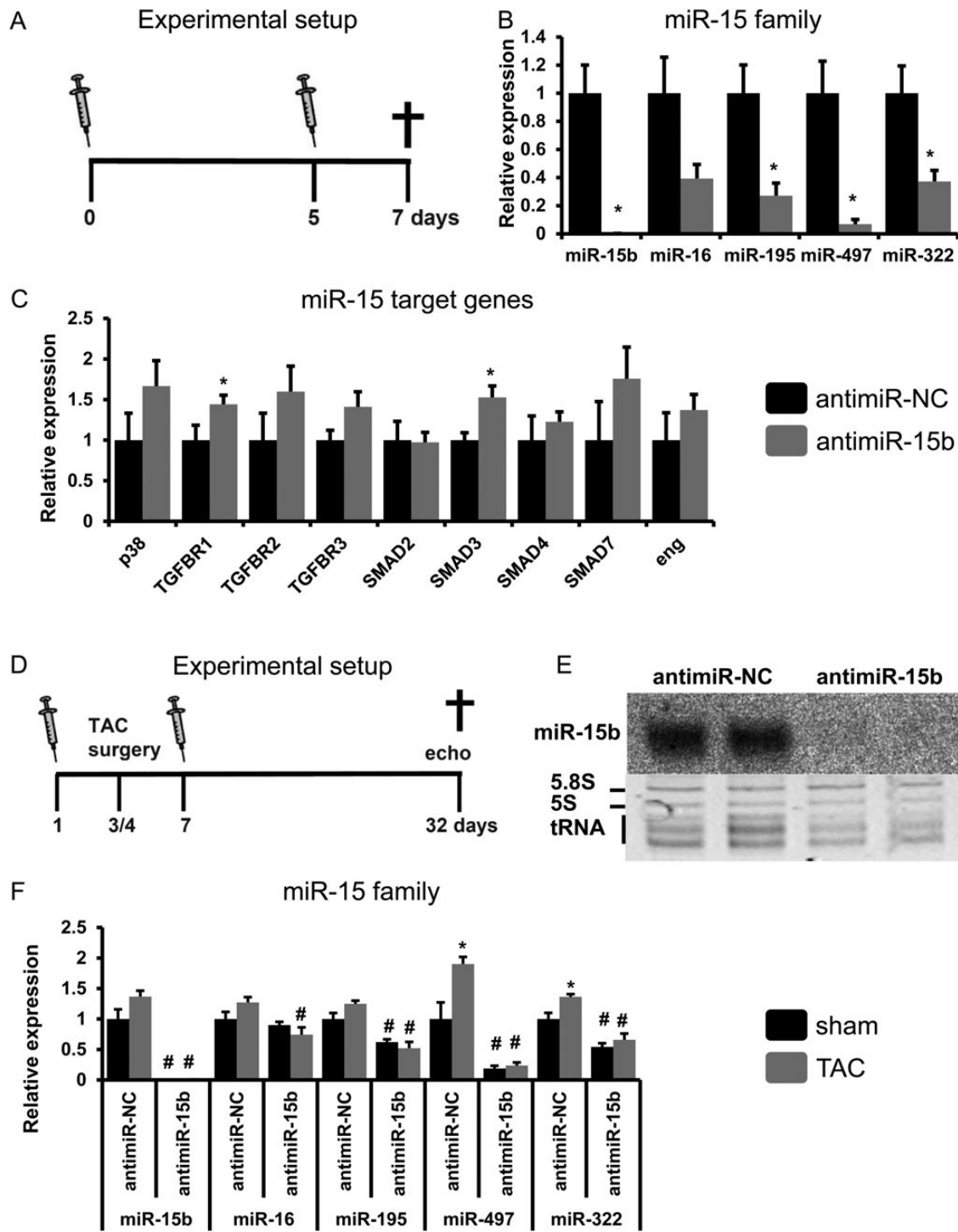
Figure 4 Inhibition of miR-15b in mice results in the up-regulation of target genes. (A) Design of the first antimiR study. (B) Real-time PCR shows complete loss of miR-15b and a strong inhibition of the other family members in antimiR-15b injected mice. (C) Real-time PCR of predicted miR-15 family target genes shows up-regulation of TGFBR1 and SMAD3 in the myocardium after injections of antimiR-15b. (D) Design of the second antimiR study. (E) Northern blot shows complete loss of miR-15b in antimiR injected mice. (F) Real-time PCR shows the inhibition of miR-15 family members by antimiR-injections. MiRNA levels are normalized to U6 and mRNA levels to HPRT levels. $n = 5$ for antimiR-NC and antimiR-15b injected mice. $*P < 0.05$ compared with antimiR-NC control (A/B/C). $n = 5$ antimiR-NC sham, $n = 8$ antimiR-NC TAC, $n = 6$ antimiR-15b sham, $n = 9$ antimiR-15b TAC. $*P < 0.05$ compared with sham and $^\#P < 0.05$ compared with antimiR-NC (D/E/F).

slightly increased hypertrophic response to TAC in the antimiR-15b injected mice compared with the antimiR-NC injected mice. This increase in cardiac hypertrophy was accompanied by a trend towards a stronger up-regulation of the hypertrophic marker genes ANF and βMHC, but these differences did not reach statistical significance (see Supplementary material online, *Figure S4B*). Although loss of miR-15b and its family members during TAC-induced cardiac remodelling resulted in enhanced hypertrophic and fibrotic responses, this did not affect cardiac systolic function at 4 weeks after TAC as shown by a similar decrease in fractional shortening (see Supplementary material online, *Figure S4C*). Echocardiography also revealed increased anterior wall thickness (indicative of more hypertrophy) after TAC and antimiR-15b treatment, but these increases did not reach statistical significance (see Supplementary material online, *Figure S4D* and *S4E*). There was no difference in left-ventricular internal diameter in diastole between antimiR-15b and antimiR-NC injected mice (see Supplementary material online, *Figure S4F*).

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 periostin (postn). By means of real-time PCR, we show that the ECM-related target gene postn is increasingly expressed after TAC in the antimiR-15b compared with the antimiR-NC injected mice. Col1a1, CTGF, and TIMP1 showed a trend towards enhanced up-regulation after TAC in the antimiR-15b mice (*Figure 5F*). Interestingly, TGFβ1 ligand is also up-regulated in the antimiR-15b treated sham mice compared with the antimiR-NC treated sham mice (see Supplementary material online, *Figure S4G*).

4. 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 6*. Inhibition of the miR-15 family *in vivo*, using LNA-based antimiRs against miR-15b in mice subjected to TAC, showed aggravated fibrosis and to a lesser extent also cardiomyocyte hypertrophy, compared with the antimiR-NC group. This is in line with previous studies, where increased TGFβ signalling in the heart also resulted in cardiac hypertrophy and interstitial fibrosis.[6]

Our data show that multiple miR-15 family members are up-regulated in rodent 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. In our antimiR-15b treated animals, we not only completely lost miR-15b expression in the heart, but also to a lesser extent the other family members, and additionally, we prevented the up-regulation normally seen in sustained pressure overload. This resulted in a substantial down-regulation of the complete miR-15 family, which likely had additive effects on the regulation of miR-15 family targets and therefore the observed phenotype is most probably due to this down-regulation of the multiple members of the miR-15 family. We show that the miR-15 family targets both activators (TGFBR1, SMAD3, endoglin) and repressors (SMAD7) of TGFβ signalling, however, the net effect seems to be 'repression of SMAD signalling' as shown by the luciferase assays using the CAGA-reporter and by the excessive cardiac fibrosis observed in the antimiR-15b treated mice. The regulation of TGFβ signalling 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 remodelling 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 targeted by the miR-15 family, only TGFBR1 and SMAD3 appeared to be significantly modulated *in vivo*. The 3′UTR of TGFBR1 and SMAD3 contain, respectively, 5 and 3 conserved miR-15 family binding sites, which were the largest numbers of all predicted miR-15 family targets in the TGFβ-pathway (*Table 1*). We found TGFBR1 mRNA and protein and SMAD3 mRNA levels regulated in cultured cardiomyocytes and fibroblasts after inhibition and overexpression of miR-15b. *In vivo*, TGFBR1 and SMAD3 were significantly up-regulated on the mRNA level in mice injected with antimiR-15b.

Our studies suggest that several genes of the TGFβ-pathway are direct targets of the miR-15 family. We have shown that the expression of multiple TGFβ-pathway genes are tightly regulated both by miR-15 knockdown and overexpression in cultured cardiac cells. Moreover, we have shown by luciferase assays that the 3′UTR of these TGFβ-pathway genes are sensitized to members of the miR-15 family. Nevertheless, to conclusively assess a direct action of the miR-15 family on these target genes, site-specific mutagenesis at the level of the binding site in the 3′UTR of the luciferase constructs would be required. This was, however, not feasible due to the high number of predicted binding sites in these 3′UTRs (as shown in see Supplementary material online, *Figure S1*) and therefore still leaves the possibility for an indirect role of this miRNA family on the TGFβ-pathway.

Fibrosis and hypertrophy are both regulated by TGFβ signalling, however, the precise interplay between the downstream components of the pathway is different for these biological processes, also because they are largely regulated in different cell types (see *Figure 6*). Divakaran *et al.*[26] recently provided evidence that somatic SMAD3 knockout mice have increased cardiac hypertrophy, but are partly protected from the development of fibrosis. This shows that SMAD3 is required for fibrosis, but not for the development of hypertrophy in the mouse heart. This is in line with our antimiR-15b study, where we observe increased SMAD3 expression and a major effect on fibrosis and to a lesser extent hypertrophy. Increased TGFBR1 in antimiR-15b treated mice likely contributed to the increased fibrotic response after TAC as well. In this regard, Koitabashi *et al.*[11] showed that the *cardiomyocyte-specific* TGFBR1 knockout mice subjected to TAC displayed reduced fibrosis in their hearts, while the hypertrophic response was unaffected. The hypertrophic phenotype after TAC that we observed after knockdown of the miR-15 family is probably caused by altered expression of other miR-15 family targets such as p38 and TGFBR2.[11]

Cross-talk between cardiomyocytes and fibroblasts may also have contributed to the dual effects that we observed *in vivo* after miR-15 inhibition. It is known that cardiomyocytes and fibroblasts communicate through release of paracrine factors, direct cell−cell interactions and cell interactions with the ECM.[27] It is therefore possible that fibrosis affected metabolism of cardiomyocytes or that hypertrophy in cardiomyocytes had indirect effects on the fibrotic response of the fibroblast. It is impossible to pinpoint which target in which cell type is exactly responsible for the hypertrophic or the fibrotic phenotype after antimiR-15b treatment *in vivo*, since indirect effects or cross-talk between cells may have added to the phenotype.

Based on the observed up-regulation of TGFBR1 and SMAD3 mRNA (*Figure 4B*) and the increased expression of TGFβ1 ligand mRNA (see Supplementary material online, *Figure S4G*) after antimiR-15b treatment one could expect to find an increase in cardiac fibrosis in the sham mice
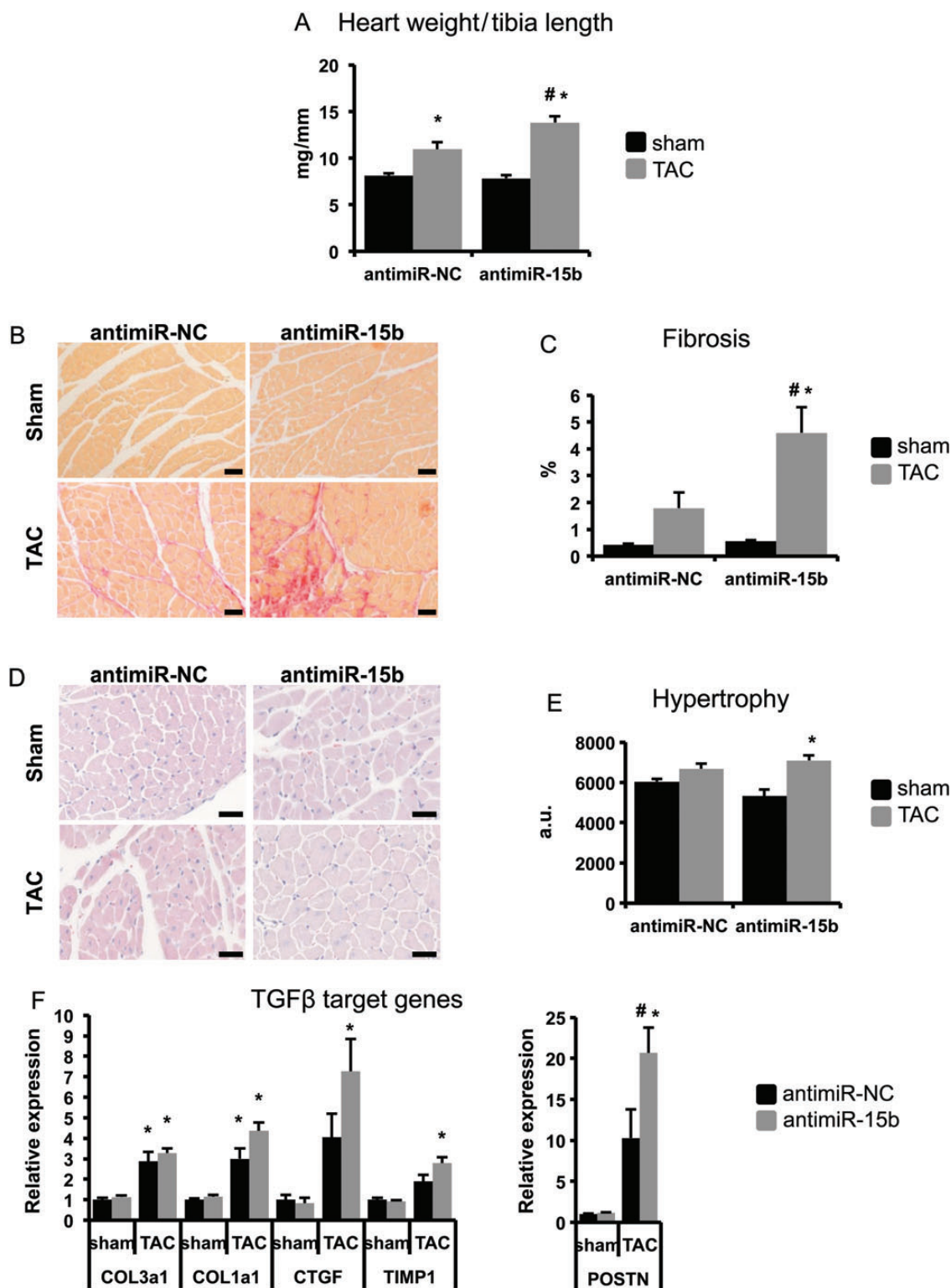
Figure 5 Inhibition of miR-15b aggravates cardiac remodelling after TAC. (*A*) Inhibition of miR-15b results in a stronger increase in heart weight corrected for tibia length after TAC. (*B*) Representative pictures of picrosirius red sections to determine fibrosis, which is increased after inhibition of miR-15b as quantified in (*C*). (*D*) Representative pictures of H&A 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. Arbitrary units (a.u.) denote the number of pixels per myocyte in the cross-sectional plane. (*F*) Some downstream target genes of the TGFβ-pathway are significantly increased after TAC surgery in the antimiR-15b injected animals (POSTN), while other targets show a trend towards increased expression (COL1a1, CTGF, TIMP1). $n = 5$ 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 with sham and $^{\#}P < 0.05$ compared with antimiR-NC.

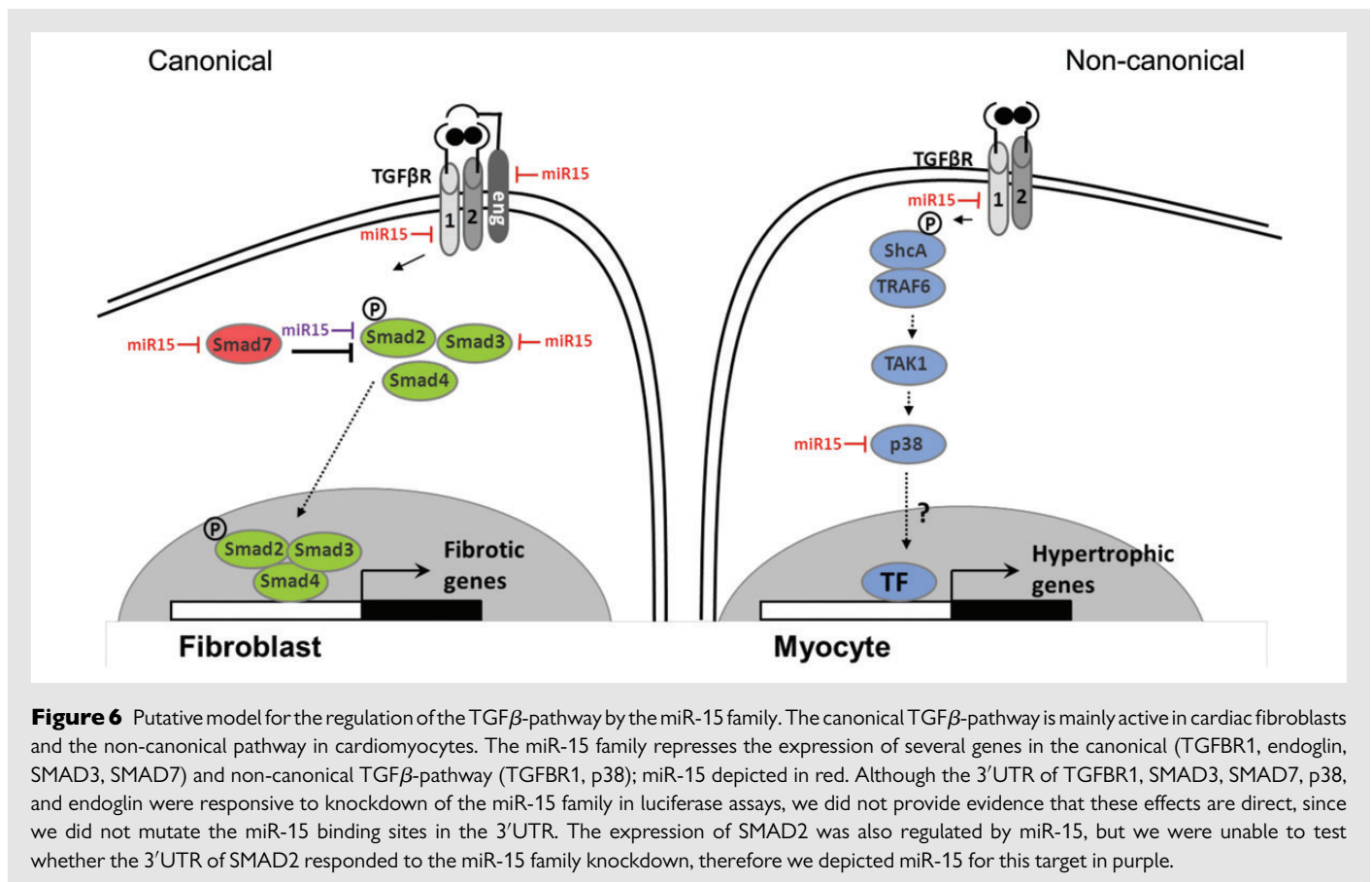**Figure 6** Putative model for the regulation of the TGFβ-pathway by the miR-15 family. The canonical TGFβ-pathway is mainly active in cardiac fibroblasts and the non-canonical pathway in cardiomyocytes. The miR-15 family represses the expression of several genes in the canonical (TGFBR1, endoglin, SMAD3, SMAD7) and non-canonical TGFβ-pathway (TGFBR1, p38); miR-15 depicted in red. Although the 3′UTR of TGFBR1, SMAD3, SMAD7, p38, and endoglin were responsive to knockdown of the miR-15 family in luciferase assays, we did not provide evidence that these effects are direct, since we did not mutate the miR-15 binding sites in the 3′UTR. The expression of SMAD2 was also regulated by miR-15, but we were unable to test whether the 3′UTR of SMAD2 responded to the miR-15 family knockdown, therefore we depicted miR-15 for this target in purple.

that were treated 4 weeks with antimiR-15b. However, we did not find more fibrosis in the 4 weeks-treated sham hearts. We believe this relates to the low activity of the TGFβ pathway in the healthy heart. Apparently, under healthy conditions, a 1.4-fold increase in TGFBR1 mRNA and a 1.5-fold increase in SMAD3 mRNA expression are not sufficient to cause fibrosis. It is interesting that we also found an increase in TGFβ1 mRNA, but at this stage we do not know whether this also translated into a concomitant increase in bioactive TGFβ1 ligand at the protein level. In the pressure-overloaded heart on the other hand, the TGFβ pathway is activated and more easily manipulated, as can be seen by the aggravated fibrotic response after antimiR-15b treatment. It is quite common that during normal tissue homeostasis, the function of a particular miRNA is redundant, while under pathological conditions such as cardiac stress induced by pressure overload its function becomes significant.[17]

Cardiac fibrosis increases stiffness of the heart, which will primarily impact on relaxation of the ventricle and it therefore may lead to diastolic dysfunction. Eventually, cardiac fibrosis may 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.[1] Contractility was measured by fractional shortening in echocardiography, but after 4 weeks of antimiR-15b treatment, we did not observe cardiac dysfunction. However, as contractility of the heart is only affected later after the development of fibrosis, it might be that our time-point of sacrificing the mice after the TAC operation (4 weeks) was too early to see a difference in fractional shortening. Since cardiac fibrosis at first mainly affects diastolic function, it would be very interesting to know whether diastolic function would be affected

after antimiR-15b treatment. In this regard, we consider it a limitation of our study that diastolic function could not be measured directly (e.g. by mitral flow velocity measurements and tissue Doppler assessment of LV relaxation).

Previous studies have revealed the importance of the miR-15 family in cardiomyocytes. In a study by Porrello et al.,[28] cardiomyocyte-specific overexpression of the miR-15 family member, miR-195, driven by the βMHC promoter resulted in smaller hearts due to a reduction in cardiomyocyte proliferation in the embryonic heart. MiR-195 was shown to regulate the expression of a number of cell cycle genes, including Chek1, which was identified as a direct target of miR-195.[28] These effects of miR-195 suggested that inhibition of the miR-15 family after myocardial infarction (MI) might increase proliferation of cardiomyocytes in the borderzone of the infarcted region and thus decrease infarct sizes. To study this, Porrello et al.[29] injected neonatal mice with antimiRs to inhibit miR-15 and subjected these mice to MI at 3 weeks of age. This indeed resulted in increased regeneration of the infarcted myocardium due to proliferation of cardiomyocytes. Hullinger et al.[19] injected adult mice with tiny LNAs at the moment of reperfusion in an ischaemia–reperfusion model. Inhibition of the miR-15 family in this model protected hypoxic cardiomyocytes against apoptosis and this decreased the infarct size. As a consequence, these studies have suggested that the miR-15 family can be used therapeutically to reduce infarct size after MI. 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,[18] this approach might be more difficult for the miR-15 family after MI. The reason for this is the fact that the miR-15

family is ubiquitously expressed in the 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 leukaemia.[30] Despite the exciting recent findings that inhibition of the miR-15 family immediately after MI protects against cardiomyocyte apoptosis, increases proliferation of surviving cardiomyocytes, and improves cardiac function,[19,29] our data indicate that on the long-term adverse cardiac remodelling may occur due to unrestrained activation of the TGFβ-pathway.

Supplementary material

Supplementary material is available at *Cardiovascular Research* online.

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