miR-133 and miR-30 Regulate Connective Tissue Growth Factor
Implications for a Role of MicroRNAs in Myocardial Matrix Remodeling

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Abstract—The myocardium of the failing heart undergoes a number of structural alterations, most notably hypertrophy of cardiac myocytes and an increase in extracellular matrix proteins, often seen as primary fibrosis. Connective tissue growth factor (CTGF) is a key molecule in the process of fibrosis and therefore seems an attractive therapeutic target. Regulation of CTGF expression at the promoter level has been studied extensively, but it is unknown how CTGF transcripts are regulated at the posttranscriptional level. Here we provide several lines of evidence to show that CTGF is importantly regulated by 2 major cardiac microRNAs (miRNAs), miR-133 and miR-30. First, the expression of both miRNAs was inversely related to the amount of CTGF in 2 rodent models of heart disease and in human pathological left ventricular hypertrophy. Second, in cultured cardiomyocytes and fibroblasts, knockdown of these miRNAs increased CTGF levels. Third, overexpression of miR-133 or miR-30c decreased CTGF levels, which was accompanied by decreased production of collagens. Fourth, we show that CTGF is a direct target of these miRNAs, because they directly interact with the 3’ untranslated region of CTGF. Taken together, our results indicate that miR-133 and miR-30 importantly limit the production of CTGF. We also provide evidence that the decrease of these 2 miRNAs in pathological left ventricular hypertrophy allows CTGF levels to increase, which contributes to collagen synthesis. In conclusion, our results show that both miR-133 and miR-30 directly downregulate CTGF, a key profibrotic protein, and thereby establish an important role for these miRNAs in the control of structural changes in the extracellular matrix of the myocardium. (Circ Res. 2009;104:170-178.)

Key Words: fibrosis ■ miRNAs ■ heart failure ■ CTGF

A well-organized extracellular matrix (ECM) is necessary to maintain strength and functional integrity of cardiac tissue and is involved in communication between the different cells in the heart.1 In response to numerous pathological stimuli, such as hypertension and pressure loading, ECM proteins accumulate excessively in the heart. This process alters mechanical stiffness and electric properties, which adversely affects the function of the heart.2

Connective tissue growth factor (CTGF) is a secreted protein that has been identified as a powerful inducer of ECM synthesis.3–5 The importance of CTGF is recognized in many different forms of pathology and has been described in different organs, including the heart, to be an important mediator of tissue fibrosis.3,6–9 CTGF has thus emerged as a new target for the therapeutic intervention in fibrotic diseases. CTGF expression is induced by transforming growth factor (TGF)β and other prohypertrophic stimuli such as endothelin.3 In the healthy heart, CTGF is predominantly expressed in fibroblasts; however, in the process of cardiac remodeling, CTGF is also secreted by cardiac myocytes.3,6,10 It has been traditionally assumed that growth factors such as TGFβ and endothelin regulate CTGF expression exclusively in fibroblasts. However, it is now recognized that cardiac myocytes also produce CTGF in response to the same stimuli, thereby providing the signal to surround themselves with increased ECM.6

Regulation of CTGF by microRNAs (miRNAs) has not yet been described. miRNAs have emerged as a new class of posttranscriptional regulators of gene function, and growing evidence indicates that miRNAs play key roles in growth, development, and stress responses of the heart.11–13 miRNAs are ~22 nucleotides in length and inhibit translation by base
pairing with the 3' untranslated region (3' UTR) of specific messenger RNA transcripts. The estimated number of miRNA genes is as high as 1000 in the human genome, and together they are estimated to regulate as many as 30% of messenger RNA transcripts. Specific evidence that miRNAs are required for adequate form and function of the heart was reported recently from 3 independent studies using knockout mice and antagonirs (anti-miRNAs). These particular studies showed that 3 muscle-specific miRNAs (miR-1, -133, and -208) regulate protein levels by repressing translation of genes involved in cardiac contractility, hypertrophy, and electric conductance.

Here we hypothesize that miRNAs also regulate genes involved in fibrosis of the heart. We undertook a bioinformatics approach to identify miRNAs that target CTGF and describe 2 miRNAs, miR-133 and miR-30, both consistently downregulated in several models of pathological hypertrophy and heart failure as regulators of CTGF expression. This provides a novel mechanism in heart failure, where upstream signals that promote tissue fibrosis (e.g., TGFβ) are accompanied by a loss of inhibitory action by relevant miRNAs, so that production of CTGF protein is amplified, thereby further contributing to cardiac fibrosis.

**Materials and Methods**

**Animal Models**

The homozygous Ren2 rat is a model of hypertension-induced heart failure. Here the mouse renin-2 gene has been introduced into the rat genome, causing activation of the renin–angiotensin system and resulting in cardiac hypertrophy by the age of 8 weeks and heart failure before the age of 18 weeks.17,18 Cardiac hypertrophy develops invariably. Some of these rats rapidly progress to heart failure, whereas other similarly hypertensive littermates remain compensated.19 Heart function of 10-week-old Ren-2 and Sprague–Dawley rats was monitored by serial echocardiography at 10, 12, 15, 16, 18, 19, and 21 weeks of age, and animals were euthanized at 15 to 18 weeks on clinical signs of heart failure (HF-prone rats) or at 21 weeks when clinical signs of failure had not appeared (compensated rats). Another group of Sprague–Dawley rats were monitored and euthanized at 10 and 16 weeks of age, and their hearts were used as controls. Male and female C57Bl6 mice were subjected to transverse aortic binding or sham surgery as has been previously described.20 The number of animals used per experiment is described in the figure legends. All animal experiments were approved by the Animal Care and Ethics Committee of the University of Maastricht.

**Patients**

Nine patients with isolated aortic stenosis undergoing valve replacement surgery were included in the study. Four patients undergoing coronary artery bypass grafting (CABG) were included as nonhypertrophic controls.21 CABG patients had normal ejection fraction, no unstable angina, and no history of myocardial infarction or left ventricular (LV) hypertrophy (LVH).21 During open-heart surgery, but before extracorporeal circulation, 2 to 3 transmural needle biopsies were taken from the anterior LV, and snap-frozen in liquid nitrogen for RNA isolation. The institutional ethics committee of the University Hospital Maastricht approved the study, and all patients gave informed consent.

**Cell Culture**

Rat ventricular myocytes (RCMs) and fibroblasts (RCFs) were isolated by enzymatic digestion of 1- to 2-day-old neonatal rats as described previously.22 Transductions and transfections of these cells are described in the expanded Materials and Methods section, available in the online data supplement at http://circres.ahajournals.org.

**Real-Time PCR**

To detect miRNAs from cells or tissues, total RNA was isolated using the mirVana miRNA isolation kit (Ambion) according to the protocol of the manufacturer. Total and miRNA-specific cDNA was generated with iScript cDNA synthesis kit (Bio-Rad), and mirVana quantitative RT-PCR primer sets for miR-133 and miR-30c (30033 and 30144, Ambion).

**Western Blotting**

Protein was isolated after grinding frozen heart tissue with radioimmunoprecipitation assay buffer. Cells were lysed with standard sample buffer, sheared through a 23-gauge needle and boiled for 5 minutes. Primary antibodies for the detection of CTGF and GAPDH were, respectively, ab6992 (Abcam) and 6C5 (RDI).

**miRNA Reporter Assays**

Primers and strategies to clone miR-overexpression plasmids and CTGF 3'-UTR reporter plasmids are described in the online data supplement.

**In Situ Hybridization**

LNA hybridization probes complementary to human mature miR-133b (38033–05) and a scrambled probe (99001–05) with 3'-digoxigenin conjugate were purchased from Exiqon (Vedbaek, Denmark). miRNA in situ hybridization was performed as described at http://www.exiqon.com/insitu.

**Statistical Analysis**

Data are shown as means±SEM, and sample sizes are mentioned in the figure legends per individual experiment. Student’s t test was performed to compare the difference between means. Probability values of ≤0.05 were considered statistically significant.

**Results**

**CTGF Expression Is Increased in Pathological LVH**

Real-time PCR and Western blotting show that CTGF levels are substantially increased in 2 established rodent models of cardiac hypertrophy and in patients with LVH (Figure 1). In the first model, the Ren2 rat model of hypertension-induced LVH, rats develop severe hypertension at 8 weeks of age. With similar levels of LVH, 50% of the littermates either quickly progress to heart failure, whereas the other 50% stay compensated for prolonged periods of time. By using sequential tissue sampling in these ren2 rats, we show that CTGF mRNA and protein is already upregulated in the early phases of hypertrophy (Figure 1A and 1B). In a second model, we subjected mice to thoracic aortic banding (transverse aortic constriction [TAC]), which induces hypertrophy by increased afterload of the heart and is accompanied by significant fibrosis (data not shown). Also in these hearts, we observed an increased expression of CTGF (Figure 1A and 1B). To determine whether CTGF expression is also increased in human heart disease, we conducted real-time PCR on cardiac biopsies of patients with aortic valve stenosis (ventricular hypertrophy) and CABG patients (nonhypertrophic). We found that in the hypertrophic human hearts, CTGF expression was induced at least 5-fold compared to the control ventricles (Figure 1A).
To explore whether miRNAs could regulate CTGF, we undertook a bioinformatic approach using miRanda software (www.microrna.org) and found 10 miRNAs that could potentially target CTGF mRNA. The 2 most notable miRNAs were miR-133 and miR-30c, because those miRNAs were significantly downregulated in miRNA arrays performed on hypertrophic and failing hearts of the hypertensive Ren2 rat model (data not shown). Alignment of the 3'-UTR of CTGF among a wide range of species (human, rat, mouse, chicken, and Xenopus tropicalis) revealed that the predicted binding sites for miR-133 and miR-30c are highly conserved during evolution, suggesting the potential importance of these binding sites in CTGF (Figure 1C).

miR-133 and miR-30c Are Downregulated in Pathological LVH

In Figure 2A, we extend the previously reported14,23–25 loss of mature miR-133 in hypertrophic hearts, in 2 rodent models of LVH and heart failure. Already very early in the course of pathological LVH, in 10-week-old Ren2 rats, we observed a significant downregulation in the expression of miR-133. At later stages, during the transition to heart failure, mature miR-133 levels continued to be repressed compared to the Sprague–Dawley controls. Also in TAC hearts, we observed a loss of miR-133 when compared to the sham operated controls (Figure 2A, right).

Several miR-30 family members are abundantly expressed in the cardiomyocytes (Figure I in the online data supplement). Strikingly, we observed a robust downregulation of multiple members of the miR-30 family in the LV of failing Ren-2 hearts (Figure 2B and supplemental Figure II). miR-30c was also significantly downregulated in the diseased LV of the banded mice (Figure 2B) and in cardiac biopsies from LVH patients (Figure 2C). Together, these results of reduced miR-133 and miR-30c expression in several forms of pressure loading–induced LVH raise the intriguing possibility that loss of miR-133 and miR-30 actually cause accumulation of CTGF protein and thereby contribute to tissue fibrosis in the diseased heart.

miR-133 Is Expressed Mainly in Cardiomyocytes

To examine the cell types responsible for expression of miR-133 and miR-30c in the heart, we performed real-time PCR on equal amounts of RNA isolated from cultured rat cardiomyocytes and cultured rat cardiac fibroblasts and normalized the expression for GAPDH levels. As shown in Figure 3A, we detected 15-fold higher miR-133 levels in myocytes than fibroblasts, indicating that miR-133 expression is highly specific for cardiac myocytes. Mature miR30c levels were 2-fold higher in cardiac fibroblasts compared to cardiac myocytes (Figure 3A). Next, we performed in situ hybridizations using LNA probes for mature miR-133 in frozen sections of normal rat hearts. As shown in Figure 3C and 3D mature miR-133 was detected at high levels in the rat heart, whereas a scrambled probe serving as a negative control showed no signal (Figure 3B). High-powered magnifications revealed that mature miR-133 is concentrated in cytoplasmic foci in the proximity of the nucleus (Figure 3D). The presence of miR-133 in those so called P-bodies, the cellular compartment where miRNA and miRNA-repressed mRNAs are located in mammalian cells,26 indicates that miR-133 is actively involved in posttranscriptional regulation.
in cardiomyocytes. Notably, no miR-133 signal was detected in vascular structures, which further indicates that cardiomyocytes exclusively express this miRNA in the heart.

**miR-133 and miR-30c Regulate CTGF mRNA and Protein Levels**

We then performed a series of functional studies to determine the role of miR-133 and miR-30 in the regulation of CTGF. First, we transfected cultured cardiac myocytes with RNA oligonucleotides, complementary to mature miR-133b (miR-133 inhibitor) or a scrambled sequence that served as control (Figure 4A). Successful knockdown of miRNAs in these cells was confirmed and shown in supplemental Figure 3. This experiment demonstrates that specific knockdown of miR-133b robustly induces CTGF mRNA and protein levels. Next, we tested whether miR-133 overexpression is sufficient to repress CTGF levels. For this purpose, we overexpressed miR-133a (by using double-stranded RNA oligos comprising the mature miR-133a sequence) or miR-133b using a lentiviral approach in cardiac myocytes and fibroblasts. Real-time PCR showed >1000-fold increase of pre–miR-133b (data not shown), whereas mature miR-133b was only induced 1.5- to 2.0-fold in myocytes (supplemental Figure III, A) and 14-fold in fibroblasts (supplemental Figure III, B). The observed difference between pre- and mature miRNA levels in cardiac myocytes suggests that processing of pre–miR-133b into mature miR-133b has a low efficiency in these cells. Nevertheless, the 1.5- to 2.0-fold increase of mature miR-133b was sufficient to blunt endogenous CTGF mRNA and protein levels substantially in cultured cardiac myocytes (Figure 4B) and fibroblasts (Figure 4C). Overexpression of miR-133a using mimic oligos reached much higher miR-133 levels, and, as a consequence, this resulted in a more robust downregulation of CTGF mRNA (Figure 4B) and fibroblasts (Figure 4C). The miR-133b–induced downregulation of CTGF, in turn, resulted in a significant decrease in profibrotic signaling, as indicated by reduced production of collagen type I and III mRNA in cardiac fibroblasts (Figure 4C).

Also miR-30c appeared a potent regulator of CTGF expression: silencing of miR-30c in cultured cardiac myocytes using miRNA inhibitors induced CTGF mRNA and protein levels 2- to 3-fold (Figure 5A and supplemental Figure III, D). Also in fibroblasts, CTGF protein levels largely depended on endogenous miR-30, as demonstrated by knockdown of
miR-30c in these cells (Figure 5C). Increased CTGF protein levels in cardiac fibroblasts attributable to a reduction of endogenous miR-30c levels resulted in profibrotic signaling, as evidenced by increased expression of collagen type I and fibronectin (Figure 5C). To test whether overexpression of miR-30c is sufficient to blunt CTGF expression, we transfected cardiomyocytes (supplemental Figure III, D) and fibroblasts (supplemental Figure IV) with double-stranded RNA oligos comprising the mature miR-30c sequence (miR-30 mimic) or a nontargeting control miRNA. In conclusion, miR-30c mimic was sufficient to repress CTGF mRNA and protein in both cardiac myocytes and fibroblasts (Figure 5B and supplemental Figure IV, respectively).

Combined knockdown of miR-133 and miR-30 did not result in a synergistic or additive increase in CTGF expression (data not shown). We attribute this to the fact that the predicted miRNA binding sites in the 3′-UTR of CTGF for miR-133 and miR-30 display considerable overlap (Figure 1C) and therefore may spatially hinder each other from binding the 3′-UTR simultaneously. Moreover, occupancy of CTGF mRNA by both miRNAs simultaneously is of limited physiological relevance because the miR-133 is expressed exclusively in cardiac myocytes, whereas miR-30c is expressed predominantly in fibroblasts.

CTGF Is a Direct Target of miR-133 and miR-30

The above suggests that CTGF is regulated by miR-133 and miR-30c in pathological LVH. However, the CTGF regulation by those miRNAs could be indirect. To test whether the putative miR-133 target sequence in CTGF 3′-UTR (Figure 1C) directly regulates protein levels of CTGF, we inserted the full-length 3′-UTR of the CTGF transcript into a luciferase expression plasmid (pMir-report), which we then transfected into COS1 cells. Cotransfection of this luciferase reporter with miR-133, but not a control miRNA, resulted in a significant decrease in luciferase activity. Deletion of the miRNA binding site, only 28 nucleotides within the 1023-nt UTR, abrogated the repressive effect of miR-133 on luciferase activity (Figure 6A), indicating that the expression of CTGF is extremely sensitive to the miR-binding sequence within the 3′-UTR. miR-133 also dose-dependently inhibited the synthesis of Flag-tagged MEF2C expression cassette linked to the CTGF 3′-UTR binding sequence, but not the mutant CTGF 3′-UTR, in which the miR-133 target site was deleted (Figure 6B).

To test whether the putative miR-30 target sequence in CTGF 3′-UTR could mediate translational repression, we performed the same type of Western blot–based reporter assay as described above. Cotransfection of the expression vectors containing miR-30c and Flag-MEF2C–3′-UTR–CTGF showed less Flag-tagged protein compared to the scrambled miRNA, but not when the miR-30c binding site is deleted from the construct (Figure 6C). Overall, these results show that miR-133 and miR-30 can directly influence CTGF protein levels through specific binding to its 3′-UTR.

Discussion

In response to numerous pathological stimuli, such as hypertension and pressure loading, ECM proteins accumulate excessively in the heart. This process alters mechanical and electric properties, which adversely affects the function of the heart. The ECM of the healthy heart is subjected to a balanced turnover: on the one hand, new ECM components are synthesized by cardiac fibroblasts; and on the other hand, ECM components are continuously degraded by the action of a large family of matrix metalloproteinases, which are produced by multiple cell types in the heart (reviewed by Spinale27). The accumulation of ECM proteins in hearts of patients with LVH and heart failure has been attributed to an imbalance in the ratio of matrix metalloproteinases to their inhibitors (TIMPs) in favor of reduced proteolytic activity, as...
well as an increased production of ECM components by cardiac fibroblasts.\(^{27,28}\)

CTGF, a profibrotic growth factor, is considered a key molecule in the control of ECM synthesis and may serve as a diagnostic marker and therapeutic target for cardiac fibrosis and heart failure.\(^3,8\) Regulation of CTGF expression at the promoter level has been studied extensively. One of the most potent inducers of CTGF is TGFβ, which regulates expression through a signaling cascade requiring Smads, protein kinase C/ras/MEK/extracellular signal-regulated kinase, and an Ets-1 binding element in the CTGF promoter.\(^3\) Little is known about how CTGF transcripts are regulated at the posttranscriptional level, and regulation of CTGF expression by miRNAs has not yet been described.

In the present study, we show that miR-133 and miR-30c are powerful negative regulators of CTGF expression in the heart. miR-133 and miR-30 families are among the most highly expressed miRNAs in cardiac myocytes (supplemental Figure I), and their levels decrease substantially in the course of pathological hypertrophy, leading to overt heart failure.

We observed a loss of these miRNAs in multiple models (Ren2 rat heart, mice subjected to TAC) and in human LVH. Importantly, downregulation of miR-133 and miR-30c occurred already very early in LVH, before any sign of loss of function, as shown in the 10-week-old Ren-2 rats. Early loss of these miRNAs suggests that this downregulation does not represent a disease consequence caused by, for instance, cell death, inflammation, or fibrosis, but rather represents an evolutionarily conserved mechanism that contributes to adverse cardiac remodeling and tissue fibrosis. Downregulation of these particular miRNAs in response to cardiac stress is in line with previously published miRNA array results on RNA isolated from hearts of mice subjected to TAC and constitutive calcineurin A signaling and that of the failing human heart.\(^{23–25}\)

Here, we provide several lines of evidence showing that CTGF is regulated by miR-133 and miR-30c in pathological LVH. First, the expression of miR-133 and miR-30c was inversely related to the amount of CTGF mRNA and protein in 2 rodent models of heart disease and in human pathological

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**Figure 4.** miR-133 regulates CTGF mRNA and protein levels. Knockdown (A) and overexpression (B and C) experiments of miR-133 in cultured myocytes and fibroblasts. For degree of miR-133 knockdown and overexpression, see supplemental Figure I (A). A, Knockdown of miR-133, by transfection of miR-133 inhibitor in cultured cardiac myocytes, enhances CTGF mRNA and protein levels, as measured by real-time PCR and Western blot, respectively. Quantification of protein bands reveals a 3-fold increase of CTGF protein after knockdown of miR-133 (right). Lentiviral overexpression of miR-133 represses CTGF mRNA and protein levels in cardiac myocytes (B) and fibroblasts (C) to similar extents. Loss of CTGF protein affects fibrotic gene expression, as evidenced by a concomitant decrease in procollagen type 1 and type 3 expression in the miR-133–treated fibroblasts (C, right). \(^*\)P<0.05 compared to control treated cells.
LVH. Second, in both neonatal rat cardiomyocytes and fibroblasts, miR-133 or miR-30c knockdown increased CTGF levels by more than 100% at the mRNA level and 300% at the protein level. Third, overexpression of miR-133 or miR-30c resulted in a significant downregulation of CTGF, which was accompanied with a decrease in the production of collagens. Fourth, we show that miR-133 and miR-30 can directly influence protein levels through specific binding to the 3'UTR of CTGF. Taken together, our results indicate that in the healthy heart, miR-133 and miR-30 importantly limit the amount of CTGF produced, not only by repressing translation but also by degrading its mRNA. Our in vitro studies also provide evidence that loss of these miRNAs in the course of LVH allows for more intense profibrotic signaling, at least in part mediated by the increase in CTGF. Strikingly, 34 of the 42 mammalian collagen genes are also predicted targets of miR-133, and many of these collagens have large numbers of miRNA binding sites in their UTRs. This suggests a major role for miR-133 in preventing collagen synthesis in cardiomyocytes and this underlines an important role for myocytes in determining the quality of their surrounding ECM.

The 2 main cell types in the heart, myocytes and fibroblasts are both well-established sources of CTGF production and secretion. Our results show that fine-tuning of CTGF protein levels is regulated by miRNAs in a cell type–specific manner. Whereas cardiac myocytes express miR-133 and miR-30c to regulate CTGF protein levels, fibroblasts mainly seem to possess miR-30 to inhibit CTGF expression. The existence of cell type–specific miRNAs regulating CTGF levels emphasizes the importance of tight regulation of CTGF protein levels in the heart.

Kubota et al recently reported that the 3'UTR of CTGF contains an 80-base minimal cis-acting element that is capable of repressing gene expression. Alignment of miR-133 and miR-30 binding sites with this repressive element and CTGF mRNA revealed that the miR-binding sites did not reside within these 80 bases but were situated approximately
800 bases downstream of the element. The repressive element may possess signal sequences for mRNA export from the nucleus or bind factors involved in mRNA stability, such as other miRNAs. The existence of other functional regulatory RNA elements in the 3’-UTR of CTGF further underscores the fundamental need for precise regulation of CTGF protein production in tissue.

Earlier reports showed that miR-133 controls cardiac hypertrophy because in vivo administration of antagomir-133 was responsible for the induction of spontaneous hypertrophy. Unfortunately, these authors did not examine tissue fibrosis in these hearts, so it is unknown whether these antagonir-treated mice displayed increased cardiac fibrosis. RhoA (GTP-GDP exchange protein), Cdc42 (signal transduction kinase implicated in hypertrophy), and WHSC2 (nuclear factor involved in cardiogenesis) were identified as bona fide targets of miR-133 in the heart. It was reported recently that miR-133 also directly regulates a key splicing factor (nPTB) during muscle development and maturation. Furthermore, miR-133 has also been described to repress HERG K+ channel expression in cardiac cells. In diabetic hearts, ERG protein depression contributes to repolarization slowing, QT prolongation, and associated arrhythmias.

The picture emerges that 1 single miRNA can importantly influence a number of mechanisms that all are crucial to the adverse remodeling of the pressure loaded myocardium. The broad effects of miR-133 in targeting vital mechanisms in the remodeling myocardium imply that overexpression or normalization of miR-133 levels in vivo might have powerful beneficial effects, not only to prevent pathological cardiac growth but also, as revealed by this study, possibly to limit excessive fibrosis. However, the finding that increasing miR-133 expression results in lowered ERG protein and may induce cardiac conduction abnormalities poses significant hurdles to the possible therapeutic manipulation of miR-133. The miR-30 family is among the most highly expressed miRNAs in the heart, and, to our knowledge, we are the first to report a target and possible function of this miRNA. Perhaps strategies to enhance expression of miR-30 to prevent or regress cardiac fibrosis will be more promising for therapy than targeting miR-133.

While this work was being completed, Liu et al reported an essential role for miR-133a in the control of cardiac gene expression and function. They showed that combined deletion of both miR-133a-1 and miR-133a-2 in mice causes lethal ventricular–septal defects in half of the embryos, whereas double mutant mice that survive to adulthood succumb to dilated cardiomyopathy and heart failure. Whether the severe fibrosis that was observed in these failing hearts is regulated by CTGF is currently unknown but will be interesting for future studies. Another interesting study from the same laboratory recently described another miRNA, miR-29, to act as a regulator of cardiac fibrosis. This miRNA controls fibrosis by directly targeting a panel of ECM mRNAs, including collagens, fibrillins, and elastins, and, like miR-133 and miR-30c, is downregulated in hypertrophied myocardium. In conclusion, these data strongly suggest that dysregulation of specific miRNAs in the heart may contribute to ECM-dependent pathophysiology of the heart.

Sources of Funding
This work was supported by Netherlands Heart Foundation grants NHS2007-B77 and NHS2003-T302. Y.M.P. is an established investigator of the Netherlands Heart Foundation.

Disclosures
None.
References


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Circ Res. 2009;104:170-178; originally published online December 18, 2008;
doi: 10.1161/CIRCRESAHA.108.182535

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
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Data Supplement (unedited) at:
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miRNA Expression profiling in cultured neonatal rat cardiomyocytes
MiRNA arrays (Exiqon) were performed on RNA isolated from cultured rat cardiomyocytes. miRNAs are ranked from left to right on the basis of their expression level (spot intensities). Arrow heads indicate expression levels of miR-133 and miR-30 family members.
**Loss of mature miR-30c in ren2 rats**
Real-time PCR for mature miR-30c in ren2 rats at several ages, demonstrating the most robust loss of miR-30c in the early stages of hyperrophy. MiRNA expression was normalized for expression of PGK1 and presented as mean ± sem. *: p<0.05 compared to control or sham heart. n<5 per group.
Levels of knock-down and overexpression of miRNAs in primary cells.

A) Real-time PCR for mature miR-133b in cardiac myocytes transfected with miR-133b inhibitor (left panel) or after lentiviral overexpression of miR-133b (right panel). B) Robust overexpression of miR-133b in isolated rat cardiac fibroblasts after transduction with a lentivirus containing pri-miR-133b. C) Overexpression of miR-133a (mimic, left panel) in cultured cardiomyocytes resulted in robust down-regulation of CTGF mRNA (right panel), as determined by real-time PCR. D) Real-time PCR for mature miR-30c in cardiac myocytes transfected with miRNA inhibitor (left panel) or mimic (right panel) for miR-30c. Mature miRNA expression is normalized for GAPDH expression in the same samples. Data presented as mean ± SEM. * p < 0.05 compared to control.
Overexpression of miR-30c in cultured cardiac fibroblasts represses CTGF mRNA and protein
Real-time PCR for miR-30c in isolated fibroblasts transfected with a control or miR-30c mimic reveals a robust overexpression of miR30c (left panel). This results in decreased mRNA (real-time PCR) and protein levels (Western blot). As a control we used a scrambled miRNA mimic duplex.
Transduction and transfection of cultured RCMs and RCFs
Lentivirus for overexpression of miRNAs of interest were generated using pcDH1 expression vectors (pcDH1 Lentivectors, System Biosciences). For lentiviral miRNA overexpression, 2.5 x 10^5 RCMs per well were plated on gelatinized six-well plates and cultured overnight in DMEM/M199 (4:1) media (supplemented with 10% horse serum, 5% newborn calf serum, glucose, gentamycin and AraC) and 1.5 x 10^5 RCFs per well were plated in six well plates and cultured overnight in DMEM (supplemented with 10% foetal bovine serum and gentamycin). The next day, the RCMs and RCFs were placed on low serum media and transduced with miR133b, miR-30c or the control-miRNA virus, facilitated by sequa-brene (Sigma Aldrich). After puromycin selection (3µg/ml), infection efficiencies were above 80%. After 5 days of culturing, cells were harvested for RNA or protein isolation. For miRNA inhibitor or mimic transfection, 1 x 10^6 RCMs, and 1.5 x 10^6 RCFs per well were plated on six-well plates and cultured overnight (see above). Then the cells were placed on low serum for 24 hr. The next day they were placed on serum-free media without antibiotics for 4 hr. Cells were then transfected with 100 nM miRiDian rno-miR-133b miRNA inhibitor (I-320198-00), miRiDian rno-miR-30c miRNA inhibitor (I-320075-03), miRiDian miRNA inhibitor Negative Control #2 (IN-002000-01), miRiDian rno-miR-30c miRNA mimic (C-320075-03) and miRiDian miRNA mimic negative control #2 (CN-002000-01) (all obtained from Dharmacon) using Lipofectamin 2000 (11668, Invitrogen) according to manufacturers protocol. After 48 hr of culturing, cells were isolated for RNA or protein isolation.

Luciferase assays and western blot based reporter assay
For luciferase assays, we transiently transfected (GeneJammer, Stratagene) β-galactosidase control plasmid (50 ng), wild-type or mutant luciferase-CTGF-3'UTR reporter (25 ng) and pCDH1-miR-133b or pCDH1-miR-30c overexpressing construct (25 ng) into 40-50% confluent COS1 cells, grown in a 24-well plate. At 48 hr post transfection, cells were lysed and assayed for luciferase activity. β-galactosidase was assayed to normalize luciferase results for cell densities and transfection efficiency. For the western blot-based miRNA reporter assay, we transfected wild-type or mutant flag Mef2C-CTGF-3'UTR (25 ng), miR-133 or miR-30c overexpression construct (50, 100 and 200 ng), and empty pCDH1 as filler into COS1 cells as described above. At 48 hr post transfection, cells were harvested for Western blot using flag antibody (Sigma).
**Plasmid construction**

*pCDH1-miR133b*: rno-miR-133b precursor DNA was PCR-amplified from rat genomic DNA with the primers:

5'-ACTGGAATTCTGAGCTGTTAGCAGGTAATG-3' and
5'-ACTGGCGGCCGCTATGATCTCTTGTGACCTGGG-3'.

This fragment (375 bp) was cloned into the pCDH1-MCS1-EF1-Puro vector (System Biosciences) under the control of a CMV promoter.

*pCDH1-miR30c*: rno-miR-30c-1 precursor DNA was PCR-amplified from rat genomic DNA with the primers:

5'-AGCTGAATTCTTTTACTCAGCCCATGTGGTCG-3' and
5'-AGCTGCGGCCGCGCATTCTGTCCGATCTGGGTGTTGGTG-3'.

This fragment (263 bp) was cloned into the pCDH1-MCS1-EF1-Puro vector (System Biosciences) under the control of a CMV promoter.

*pCDH1-control-miRNA*: this vector is based on the pcDNATM6.2-GW/miR-neg control plasmid (Invitrogen) which contains an insert that can form a hairpin structure that is processed into mature miRNA but is predicted not to target any known vertebrate gene. Luciferase reporter plasmid was constructed by PCR-amplifying the 3’UTR from rat CTGF with the primers: 5’-GAGTAAGGGACACGAACT-3’ and 5’-GAAAGGTGCAAACATGTAAC-3’. This 1023 bp fragment was cloned into the pMIR-REPORTTM Luciferase vector (Ambion) downstream of the luciferase coding region, creating luciferase-CTGF 3’UTR. The complete miR-133 binding site (indicated in blue in Fig. 1C) was deleted by PCR-based mutagenesis creating the luciferase CTGF-3’UTR mutant. Mef2C-flag reporter plasmids were constructed by cloning the Mef2C open reading frame (flag-tagged) behind the CMV-promoter of the pcDNA3.1 vector (Invitrogen). Next, we subcloned the rat CTGF 3’UTR and rat CTGF 3’UTR mutant downstream of the Mef2C open reading frame, creating Mef2c-flag CTGF-3’UTR and Mef2C-flag-CTGF-3’UTR mutant reporter constructs. All generated constructs were sequence verified.