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Molecular Medicine

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

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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

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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.[13] 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 antagomirs (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.[14–16]

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 (eg, 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 are 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 extracorporal 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 *mir*Vana miRNA isolation kit (Ambion) according to the protocol of the manufacturers. Total and miRNA-specific cDNA was generated with iScript cDNA synthesis kit (Bio-Rad), and *mir*Vana 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$\pm$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 $\leq$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).

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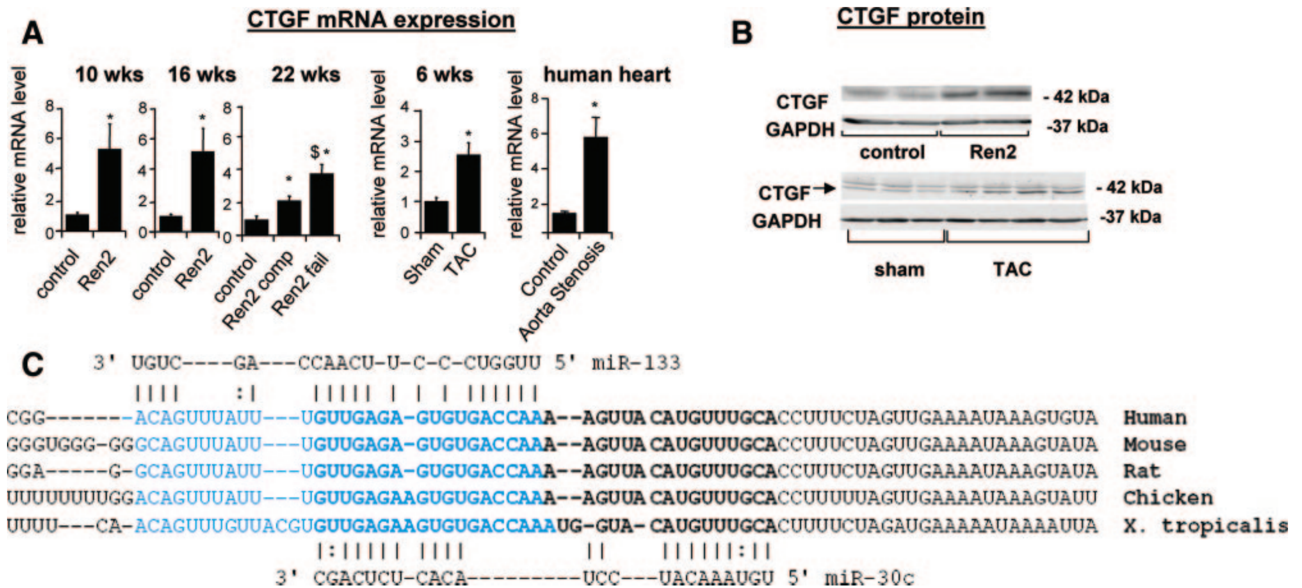

Figure 1. CTGF expression is elevated in pathological LVH. A, CTGF mRNA is elevated in hearts of 10-week-old Ren-2 rats (n=9), 16-week-old Ren-2 rats (n=7), and failing Ren-2 rats (n=6) and, to a lesser extent, in compensated Ren-2 rats (n=5), as analyzed by real-time PCR. CTGF mRNA is also elevated after TAC (n=5) compared to sham-operated mice (n=10) and in cardiac biopsies of patients with LVH (aorta stenosis, n=9) compared to "healthy" human myocardium (CABG, n=4). CTGF expression was normalized for expression of the housekeeping gene PGK-1 and presented as mean±SEM. *$P<0.05$ compared to control or sham heart, \$$P<0.05$ compared to compensated Ren-2 hearts. B, Western blot for CTGF and GAPDH in hearts of 16-week-old Ren-2 rats (n=4/group) and TAC mice (n=4/group). C, Sequence alignment between miR-133 (blue) and miR-30c (bold) and the 3′-UTR of CTGF in several species.

CTGF Harbors Phylogenetically Conserved Binding Sites for miR-133 and miR-30c

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 reported[14,23–25] 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 cardiac myocytes 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

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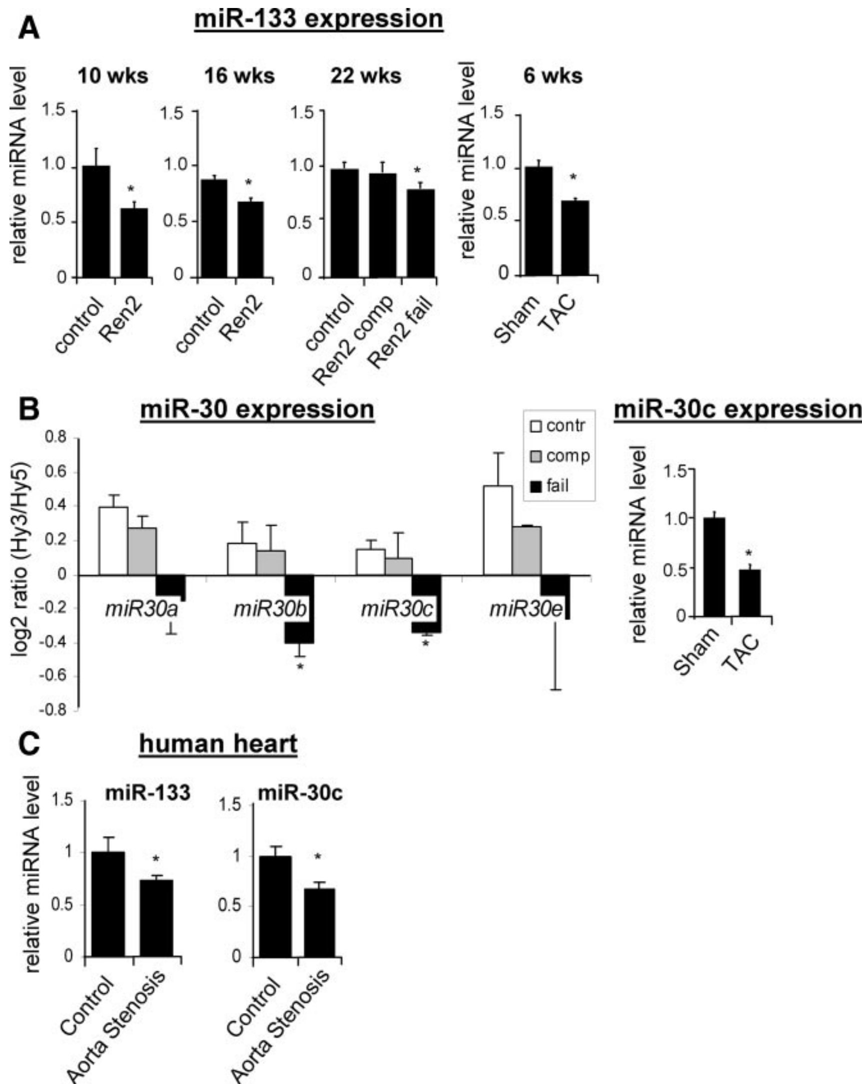

Figure 2. Loss of mature miR-133 and miR-30 in cardiac hypertrophy and failure. A, Mature miR-133 is downregulated in the hearts of 10-week-old Ren-2 rats (n=9), in 16-week-old Ren-2 rats (n=6), in failing Ren2 hearts (n=6), and in hypertrophic mouse hearts (TAC surgery, n=5), as detected by real-time PCR. B, Loss of expression of multiple members of the miR-30 family in failing rat hearts (detected by miRNA arrays, Exiqon) and in hypertrophic mouse hearts (TAC surgery, n=5). C, Real-time PCR analysis on cardiac biopsies of patients with LVH (aorta stenosis, n=9) compared to healthy human myocardium (CABG, n=4) for mature miR-133 and mature miR-30c. miRNA expression was normalized for expression of PGK1 and presented as mean±SEM. *$P<0.05$ compared to control or sham heart.

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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 (supplemental Figure III, C). 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

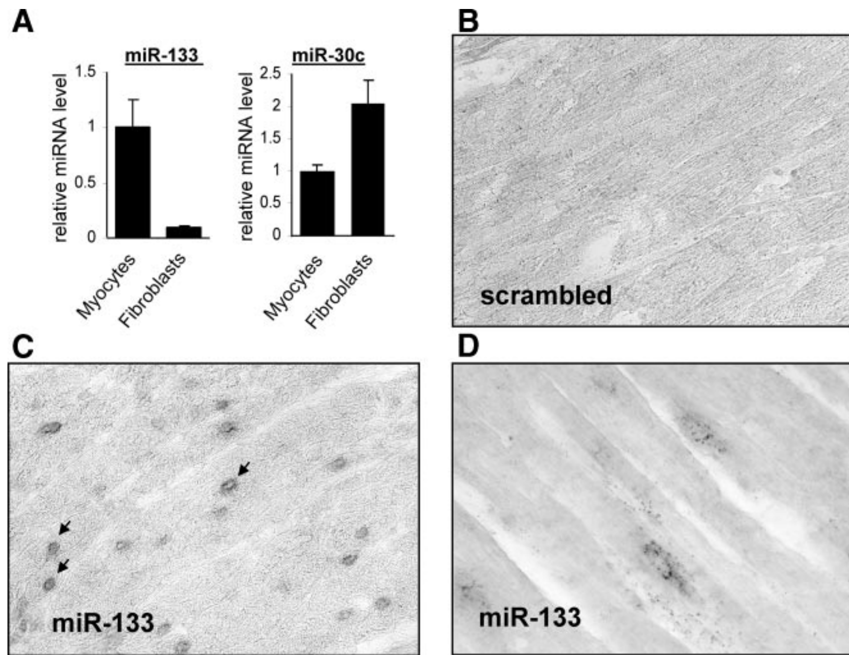

Figure 3. miR-133 is expressed in cardiac myocytes and miR-30c mainly in fibroblasts. A, Real-time PCR detection of mature miR-133 and miR-30c in cultured neonatal rat cardiac myocytes and fibroblasts corrected for GAPDH expression. B through D, In situ hybridization on frozen sections of healthy rat heart with 3′-digoxigenin–labeled LNA probe for a scrambled (B) and miRNA-133 probe (C) at ×100 magnification. D, High-powered magnification (×400) shows mature miR-133 in cytoplasmic foci in the proximity of the nucleus (P-bodies).

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.

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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.[2] 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 Spinale[27]). 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

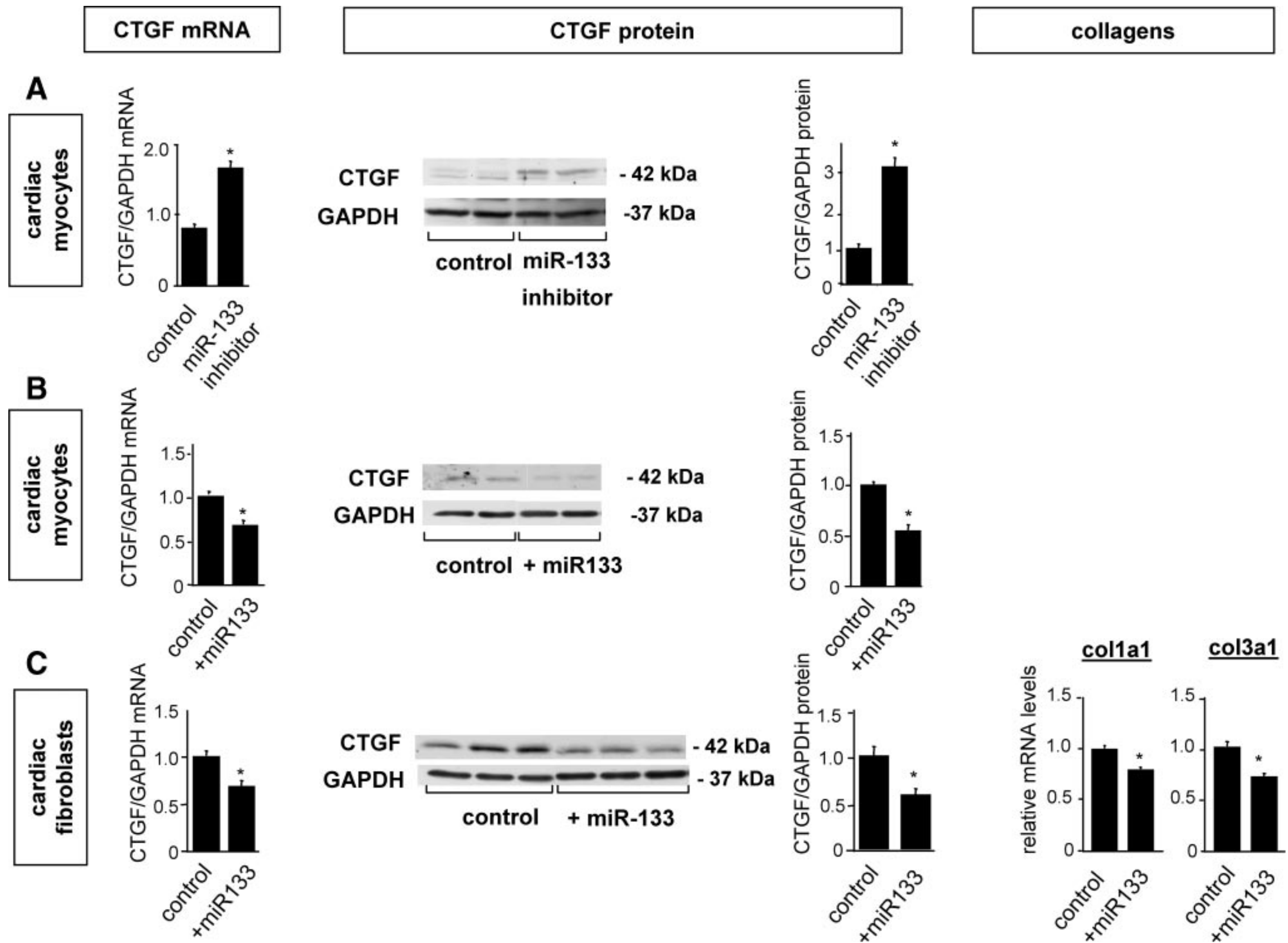

Figure 4. miR-133 regulates CTGF mRNA and protein levels. Knockdown (A) and overexpression (B and C) experiments of miR-133 in cultured myocytes and fibroblasts. For degree of miR-133 knockdown and overexpression, see supplemental Figure I (A). A, Knockdown of miR-133, by transfection of miR-133 inhibitor 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.

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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

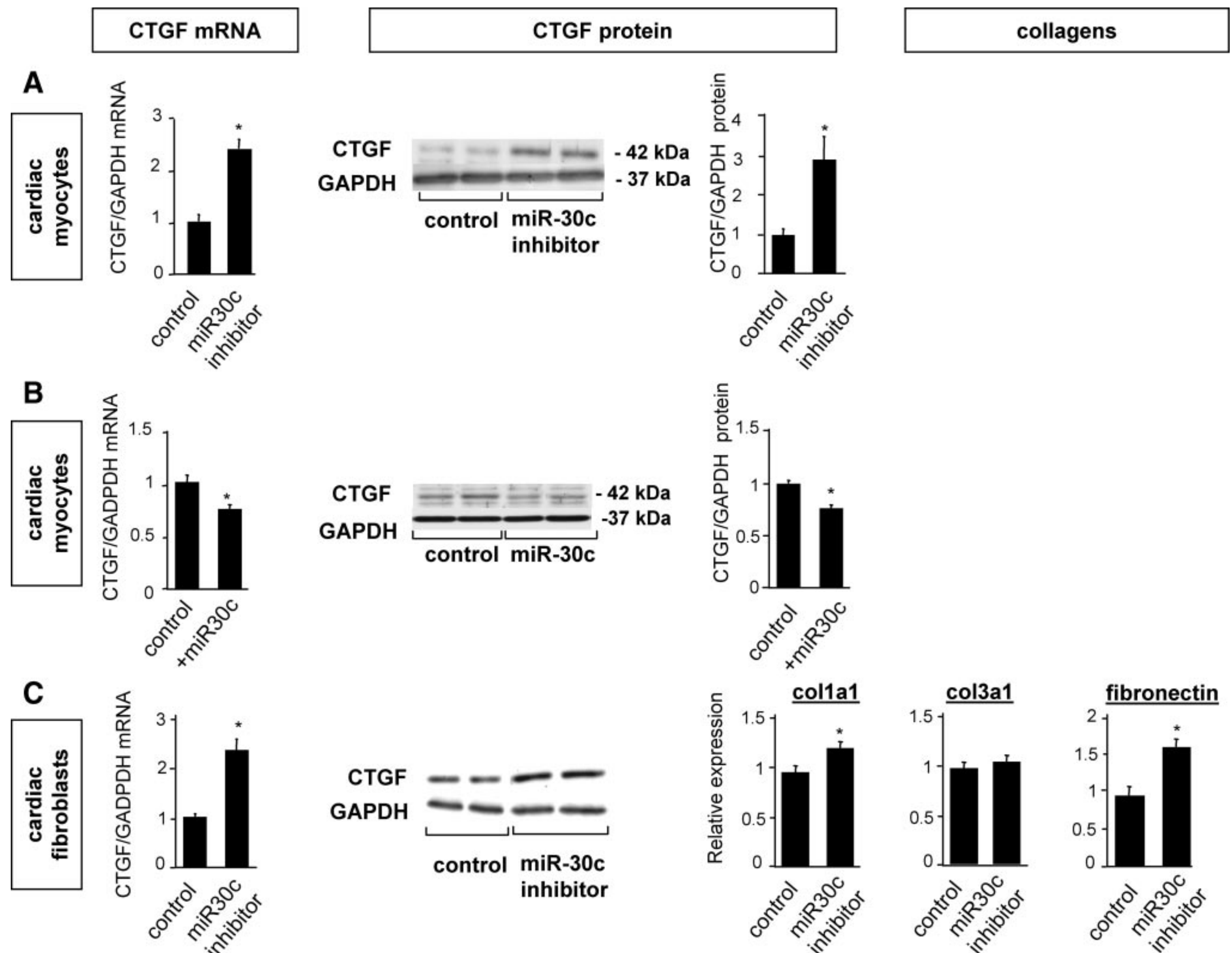

Figure 5. miR-30c regulates CTGF mRNA and protein levels. Knockdown (A) and overexpression (B and C) experiments of miR-30c in cultured myocytes and fibroblasts. Degree of miR-30c knockdown and overexpression is shown in supplemental Figure I. A, miR-30c inhibitor enhances CTGF mRNA and protein levels in cultured cardiac myocytes, as analyzed by real-time PCR and Western blot, respectively. Quantification of protein bands is shown at right. B, Overexpression of miR30c results in reduced CTGF mRNA and protein in cultured cardiac myocytes. C, Knockdown of endogenous miR-30c in fibroblasts increases CTGF levels and results in enhanced collagen type I and fibronectin expression. *$P<0.05$ 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.[29] 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.[30] 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

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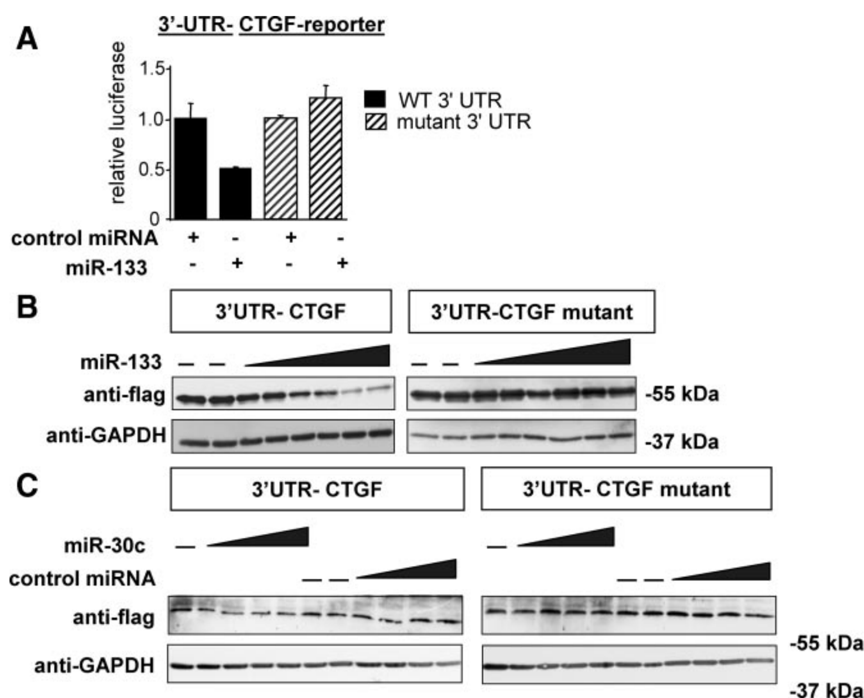

Figure 6. CTGF is a direct target of miR-133 and miR-30c. A, Cos cells were transfected with CTGF–3′-UTR luciferase constructs, together with expression plasmids for miR-133 or a scrambled miRNA (control miRNA). miR-133 overexpression decreased luciferase activity of the wild-type but not the mutant 3′-UTR where the miR-133 binding site was deleted. B, Dose-dependent inhibition of Flag-CTGF–3′-UTR by miR-133, as demonstrated by Western blot with Flag antibody. C, Western blot on Cos cells transfected with Flag-CTGF–3′-UTR and increasing concentrations of miR-30c or a control (scrambled) miRNA.

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[14] because in vivo administration of antagomir-133 was responsible for the induction of spontaneous hypertrophy.[14] Unfortunately, these authors did not examine tissue fibrosis in these hearts, so it is unknown whether these antagomir-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.[14] It was reported recently that miR-133 also directly regulates a key splicing factor (nPTB) during muscle development and maturation.[29] nPTB and its homolog regulate many exons that are spliced in a muscle-specific manner, such as α-actinin, cardiac troponin T, and α-tropomyosin.[29] Furthermore, miR-133 has also been described to repress HERG K^+ channel expression in cardiac cells.[31] In diabetic hearts, ERG protein depression contributes to repolarization slowing, QT prolongation, and associated arrhythmias.[31]

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.[31] 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.[32] 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.[33] This miRNA controls fibrosis by directly targeting a panel of ECM mRNAs, including collagens, fibrillins, and elastins,[32] and, like miR-133 and miR-30c, is downregulated in hypertrophied myocardium.[24] In conclusion, these data strongly suggest that dysregulation of specific miRNAs in the heart may contribute to ECM-dependent pathophysiology of the heart.

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Disclosures

None.

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