**MicroRNA-133 Modulates the β₁-Adrenergic Receptor Transduction Cascade**

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**Rationale:** The sympathetic nervous system plays a fundamental role in the regulation of myocardial function. During chronic pressure overload, overactivation of the sympathetic nervous system induces the release of catecholamines, which activate β-adrenergic receptors in cardiomyocytes and lead to increased heart rate and cardiac contractility. However, chronic stimulation of β-adrenergic receptors leads to impaired cardiac function, and β-blockers are widely used as therapeutic agents for the treatment of cardiac disease. MicroRNA-133 (miR-133) is highly expressed in the myocardium and is involved in controlling cardiac function through regulation of messenger RNA translation/stability.

**Objective:** To determine whether miR-133 affects β-adrenergic receptor signaling during progression to heart failure.

**Methods and Results:** Based on bioinformatic analysis, β₁-adrenergic receptor (β₁AR) and other components of the β₁AR signal transduction cascade, including adenylyl cyclase VI and the catalytic subunit of the cAMP-dependent protein kinase A, were predicted as direct targets of miR-133 and subsequently validated by experimental studies. Consistently, cAMP accumulation and activation of downstream targets were repressed by miR-133 overexpression in both neonatal and adult cardiomyocytes following selective β₁AR stimulation. Furthermore, gain-of-function and loss-of-function studies of miR-133 revealed its role in counteracting the deleterious apoptotic effects caused by chronic β₁AR stimulation. This was confirmed in vivo using a novel cardiac-specific TetON-miR-133 inducible transgenic mouse model. When subjected to transaortic constriction, TetON-miR-133 inducible transgenic mice maintained cardiac performance and showed attenuated apoptosis and reduced fibrosis compared with control mice.

**Conclusions:** miR-133 controls multiple components of the β₁AR transduction cascade and is cardioprotective during heart failure. *(Circ Res. 2014;115:273-283.)*

**Key Words:** adrenergic beta-1 receptor antagonists • cyclic AMP • heart failure • microRNAs • myocytes, cardiac

The sympathetic nervous system plays a pivotal role in the regulation of myocardial function. However, hyperactivation of this pathway can cause or accelerate cardiac pathology and is inversely correlated with survival.¹⁻⁴ These detrimental effects are caused by increased levels of circulating catecholamines, which act on cardiomyocytes mainly through chronic stimulation of β-adrenergic receptors (βARs). In the heart, different βAR subtypes are expressed with the β₁ and β₂ subtypes being the most abundant.⁵

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β₁-adrenergic receptor (β₁AR) belongs to a class of 7-transmembrane G protein–coupled receptors⁶ that, when stimulated, trigger the activation of G proteins,⁷ which, in turn, activate specific adenylyl cyclases (AC; ie, adenylyl cyclase V and adenylyl cyclase VI [AC₅₆] in the heart).⁸ This elicits a consequential accumulation of the second messenger cAMP, which activates the cAMP-dependent protein kinase A.
Nonstandard Abbreviations and Acronyms

<table>
<thead>
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<th>Abbreviation</th>
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<tr>
<td>AC</td>
<td>adenyl cyclase</td>
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<tr>
<td>AC&lt;sub&gt;c&lt;/sub&gt;</td>
<td>adenylate cyclase</td>
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<tr>
<td>β&lt;sub&gt;1&lt;/sub&gt;AR</td>
<td>β&lt;sub&gt;1&lt;/sub&gt;-adrenergic receptor</td>
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<tr>
<td>EPAC</td>
<td>exchange protein activated by cAMP</td>
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<td>miR-133</td>
<td>microRNA-133</td>
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<td>mRNA</td>
<td>messenger RNA</td>
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<td>nCM</td>
<td>neonatal cardiomyocyte</td>
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<td>PKA C-β</td>
<td>catalytic subunit β of cAMP-dependent protein kinase A</td>
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<td>PKA</td>
<td>protein kinase A</td>
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<td>TAC</td>
<td>transverse aortic constriction</td>
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<td>Tg133</td>
<td>TetON-miR-133 inducible transgenic mouse model</td>
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Results

miR-133 Modulates Components of the β<sub>1</sub>AR Signaling Cascade

In a bioinformatic search for potential targets of miR-133, the β<sub>1</sub>AR (adbr1) was among the predicted target (Online Table I). To determine whether miR-133 can directly target β<sub>1</sub>AR mRNA, its 3′ untranslated region was cloned into the psiCHECK-TM2 luciferase reporter vector and cotransfected with a miR-133 expressing plasmid into 293 cells. A 60% reduction in luciferase activity was found in the presence of miR-133, whereas mutation of the miR-133 target site completely abolished the repression (Figure 1A). Similar results were obtained using synthetic miR-133 mimics (data not shown). Further confirmation was obtained by immunoprecipitation with biotinylated miR-133 oligo on adult mouse heart homogenate following by polymerase chain reaction analysis for adbr1 mRNA on extracted total RNA (Figure 1D). Furthermore, in a loss-of-function experiment in neonatal cardiomyocytes (nCMs), repression of miR-133 by Ad-Decoy133<sup>19</sup> resulted in a ~60% upregulation of β<sub>1</sub>AR protein (Online Figure I). Taken together these results demonstrate that β<sub>1</sub>AR is a direct target of miR-133.

Stimulation of β<sub>1</sub>AR with catecholamines regulates a wide range of biological processes through the activation of intermediate signaling components, such as the second messenger cAMP and subsequently PKA. Thus, to determine whether additional regulators of the β<sub>1</sub>AR transduction cascade are potentially targeted by miR-133, we performed bioinformatic analysis, luciferase assays, target pull down, and miR-133 potentiating experiments in nCMs. Our results revealed that both AC<sub>c</sub> (adcy6), one of the main AC isoforms expressed in cardiomyocytes,<sup>21</sup> and the PKA C-β (prkacb) are targeted by miR-133 (Online Table I; Figure 1B and 1D; Online Figures I and IIA). In addition, EPAC, an additional component of the β<sub>1</sub>AR signaling cascade, was also shown to be a predicted target of miR-133 (Online Table I) and validated by luciferase and immunoprecipitation assays (Online Figure IIB and IIC).

Thus, miR-133 can directly target multiple signal transduction molecules of the β<sub>1</sub>AR/cAMP/PKA pathway, consistent with recent evidence that a single microRNA can target several components of the same pathway.<sup>24,25</sup>

miR-133 Modulates Activation of β<sub>1</sub>AR Signaling in Cardiomyocytes

Based on the results described above, we sought to determine the effect of miR-133 on the activation of β<sub>1</sub>AR-dependent signaling using cAMP imaging in living cultured cardiomyocytes. To this aim, control and Ad-miR-133-infected cardiomyocytes were transiently transfected with the fluorescent cAMP sensor EPAC1-cAMPS and challenged by β<sub>1</sub>AR-selective stimulation through administration of norepinephrine (10 nmol/L) in the presence of the β<sub>1</sub>AR-selective antagonist ICI-118,551 (100 nmol/L). As expected, this resulted in a rapid increase in cAMP levels in control cells, whereas the response was significantly reduced in miR-133 overexpressing cardiomyocytes (peak ΔR/R<sub>c</sub> [%]: Ad-miR-133=12.96±0.05; control=34.12±0.04; P<0.01; Figure 2A and 2B). In support of these findings, miR-133 overexpression resulted in a 60% reduction in β<sub>1</sub>AR protein expression (Online Figure 1C).

Methods

A detailed description of methods is provided in the Online Data Supplement.
of the specificity of miR-133 in targeting β1AR-dependent signaling, stimulation of cardiomyocytes with the β1AR-selective agonist clenbuterol (10 µmol/L) caused a comparable increase in cAMP in miR-133 overexpressing and control cardiomyocytes (Figure 2C). In addition, no effects of miR-133 on mRNA levels of β2AR were found (Online Figure IIIB).

Because we found ACVI to be negatively regulated by miR-133 (Figure 1B and 1D; Online Figures I and IIA), we next sought to determine whether miR-133 overexpression may have an effect on the ability of cardiomyocytes to synthesize cAMP in response to direct activation of ACs. We thus performed an in-cell AC activity assay by measuring the synthesis rate and maximal [cAMP] elicited by a submaximal concentration of the direct AC activator forskolin (5 µmol/L). Notably, miR-133 overexpression reduced both the maximal amount and the rate of cAMP accumulation (steady-state cAMP ΔR/Rₐ [%]: AdmiR-133=22.0±2.9; control=35.0±6.8; P<0.01; accumulation rate [dR/min]: AdmiR-133=0.56±0.01; control=0.1±0.02, P<0.01; Figure 3A and 3B).

Based on our identification of PKA C-β as a target of miR-133 (Figure 1C and 1D and Online Figure I) as well as the reduced cAMP response (Figure 2A and 2B) and PKA-dependent phosphorylation of phospholamban in miR-133-overexpressing compared with control nCMs (Online Figure IIIA), we next hypothesized that PKA activity is reduced in miR-133-overexpressing nCMs. To test this, we transfected nCMs
β receptor (10 nmol/L) and measured in single cells on μ, Quantification of the average cAMP change (100 mol/L) was elicited with forskolin (25 mol/L) and isobutylmethylxantine cAMPS (representative traces). Maximal cAMP accumulation cardiomyocytes transfected with the cAMP sensor Epac1-(ICI; 100 nmol/L) in control (blue) and Ad-miR-133 (red) neonatal of infection=25. CLB indicates clenbuterol; Ctl, control; FRSK, IBMX, isobutylmethylxantine; and NE, norepinephrine. of correlation of dynamic changes in FRET with the fluorescent PKA activity reporter AKAR3 that al-

Figure 2. MicroRNA-133 (miR-133) modulates β1-adrenergic receptor (β1AR) signaling in vitro. A. Effect of norepinephrine (10 nmol/L) on cAMP levels on β1AR blockage with ICI-118,551 (ICI; 100 nmol/L) in control (blue) and Ad-miR-133 (red) neonatal cardiomyocytes transfected with the cAMP sensor Epac1-cAMPS (representative trace). Maximal cAMP accumulation was elicited with forskolin (25 μmol/L) and isobutylmethylxantine (100 μmol/L) B. Quantification of the average cAMP change measured in single cells on β1AR stimulation with norepinephrine (10 μmol/L) and β1AR blockage with ICI (100 nmol/L). Values are expressed as % change in the 480 nm/545 nm emission ratio of Epac1-cAMP following norepinephrine stimulation and normalized to the maximal response obtained with forskolin plus isobutylmethylxantine. cAMP accumulation was highly (≈60%) reduced in miR-133 overexpressing cardiomyocytes (Ad-miR-133, n=9; controls, n=10). C. Quantification of the average cAMP change measured in single cells on selective β1AR stimulation with clenbuterol (10 μmol/L), showing no significant changes in β1AR response (Ad-miR-133, n=8; control, n=10). ***P<0.001. No significant differences were observed in Ad-empty infected cells compared with control cells. Multiplicity of infection=25. CLB indicates clenbuterol; Ctl, control; FRSK, forskolin; IBMX, isobutylmethylxantine; and NE, norepinephrine.

with the fluorescent PKA activity reporter AKAR3 that allows for correlation of dynamic changes in FRET with the kinetics of PKA phosphorylation. For this purpose, we treated AKAR3-expressing nCMs with a cell-permeable cAMP analog (10 μmol/L) to activate PKA directly. As expected, the rate of PKA phosphorylation of AKAR3 was significantly lower in miR-133-overexpressing nCMs compared with control cells after the treatment with the cAMP analog (phosphorylation rate [dR/min] Ad-miR-133=1.7±0.4; control=3.6±0.4; P<0.01; Figure 3C).

Altogether, these results demonstrate that miR-133 is directly involved in the modulation of the β1AR signaling pathway by acting at multiple levels downstream of β1AR (β1AR–ACV–PKA).

miR-133 Modulates Activation of β1AR Signaling In Vivo

To determine whether miR-133-dependent modulation of the β1AR signaling pathway is taking place also in vivo, we generated an inducible cardiac-specific TetON-miR-133 inducible transgenic mouse model (Tg133) in which the pri-miR-133a-2 sequence (chromosome 2) was inserted downstream of a TetON activation system.26 When fed with doxycycline-supplemented diet, Tg133 mice showed a 3.15- and 1.65-fold overexpression of pri-miR-133a-2 and mature miR-133, respectively, while no effect was observed on the expression of endogenous pri-miR-133a-1 (chromosome 18; Figure 4A). Consistent with the overexpression of miR-133, WHSC2, a previously validated target of miR-133,19 was downregulated in Tg133 versus control mice (Online Figure IVB). Furthermore, an inverse relationship between miR-133 and β1AR expression was confirmed in a receptor-density assay (Figure 4B). In agreement with results from a previous study,21 induced Tg133 mice did not show any major phenotypic abnormalities compared with control mice as evaluated by echocardiographic analysis (data not shown).

However, in agreement with our in vitro data on neonatal cells (see above), freshly isolated adult cardiomyocytes from Tg133 mice hearts showed blunted cAMP accumulation after the treatment with 100 nmol/L of the β1AR-selective agonist xamoterol (81.9±2 pmol/mg [cAMP/protein] versus 52.7±6 pmol/mg in control and Tg133 cardiomyocytes, respectively; Figure 4C). Consistently, acute induction of miR-133 in Tg133 mice resulted in reduced left ventricular hemodynamic performance in response to dobutamine stimulation (Figure 4D). We next asked whether in a condition of chronic activation of β1AR signaling detrimental to cardiac performance,27,28 that is, during left ventricular pressure overload induced by transverse aortic constriction (TAC), induction of miR-133 expression would be sufficient to prevent alterations in cardiac function. In support of this hypothesis, Tg133 mice subjected to TAC showed preserved cardiac performance and attenuated cardiac remodeling as demonstrated by echocardiography and histological analyses (Figures 5A and 6C; Online Table III; Online Figure IVC). In agreement with our in vitro data, a reduction in mRNA and protein levels as well as density of β1AR was observed in Tg133 mice compared with control mice 1 week after TAC (Figure 5B; Online Figure VIB), which was accompanied by decreased cAMP/PKA-dependent phosphorylation of phospholamban (Online Figures IVB and VIB). At the immuno-histochemical level, no difference at basal level was found (data not shown), whereas a strong reduction in apoptosis...
and fibrosis was observed in Tg133 mice 3 and 12 weeks after TAC (Figure 6C; Online Figure VB).

To evaluate how the observed cardioprotection in Tg133 mice relates to that mediated by pharmacological blockade of β₁ signaling, C57J/B6 mice were subjected to TAC, whereafter half received treatment with a clinically relevant dose of metoprolol (350 mg/kg/d). Echocardiographic analysis revealed a reduction in the TAC-induced decrease in fractional shortening in metoprolol-treated mice (Online Figure VA) to an extent that is comparable to or lower than that observed in Tg133 mice. Furthermore, metoprolol administration prevented the expected downregulation of miR-133 1 week post TAC and maintained miR-133 at levels comparable to those of the sham group (Figure 5C). On the contrary, no significant differences in miR-133 levels between metoprolol-treated and untreated mice were found 3 weeks after TAC.

Altogether, these data show that miR-133 modulates the β₁AR transduction axis both in vitro and in vivo and suggest that the partial protective effect of β-blockers might be due to the preservation of miR-133 expression levels during the first phase of pressure overload, which is the time frame when miR-133 expression is decreased.

miR-133 Protects Cardiomyocytes From β₁AR-Induced Apoptosis In Vitro and In Vivo

Because prolonged stimulation of cardiomyocytes with β₁AR has previously been shown to be associated with activation of apoptotic cell death, we hypothesized that miR-133 overexpression affects nCM susceptibility to apoptosis in response to chronic βAR activation. To determine this, we stimulated Ad-miR-133-infected and control nCMs with different doses of norepinephrine in the presence of the β₂AR antagonist ICI-118,551. Immunostaining for the apoptotic marker caspase-3 after 72 hours of treatment revealed a significant reduction in the fraction of caspase-3-positive nCMs in norepinephrine-treated miR-133 overexpressing nCMs compared with control cells (Figure 6A; Online Figure VIIA), indicating that miR-133 overexpression protects nCMs from β₁AR-induced apoptosis. Conversely, miR-133 downregulation, as obtained by infection with Ad-Decoy133, resulted in a significant increase in nCM apoptosis (Figure 6B), accompanied by a considerable
decrease in cell density in response to prolonged \( \beta_1 \)-AR activation (Online Figure VIIIB). In agreement with our in vitro data, the fraction of apoptotic myocardial cells was significantly lower in Tg133 mice compared with control mice both 1 and 3 weeks after TAC (Figure 6C; Online Figure VB). This was accompanied by reduced fibrosis and resulted in cardioprotection in Tg133 mice (Figure 6C). In line with this, Tg133 mice at 1 week after TAC showed reduced levels of the \( \beta_1 \)- but not \( \beta_2 \)-adrenergic receptor kinase (G protein–coupled receptor kinases 2 and 3, respectively; Figure 6D; Online Figure IVD), which has previously been shown to exert a prodeath function.13 Likewise, also the mRNA levels of identified miR-133 targets were downregulated in Tg133 mice after TAC (Figure 5B; Online Figure VIA).

Taken together, these data demonstrate that by targeting multiple components of the \( \beta_1 \)-AR-signaling cascade, miR-133 protects cardiomyocytes from \( \beta_1 \)-AR-induced apoptosis.

**Discussion**

In this study, we provide the first evidence that miR-133 controls the \( \beta_1 \)-AR system at multiple levels along its signaling axis in cardiomyocytes. In particular, we show that miR-133, by targeting the \( \beta_1 \)-receptor itself and its downstream effectors AC\( \gamma_\) and PKA C-\( \beta \), is a key modulator of the \( \beta_1 \)-AR-mediated accumulation of the second messenger cAMP (Figure 7). This result is in line with a growing body of evidence demonstrating that microRNAs play a role in modulating specific receptor pathways in cardiomyocytes.24–25 For example miR-1, which together with miR-133 is one of the most highly expressed microRNAs in the heart and is transcribed from the same bicistronic unit as miR-133, was found by us and others to regulate the insulin-like growth factor 1/insulin-like growth factor 1 receptor signal transduction pathway,25,34 a key regulator of cardiac muscle trophism and function.

**Figure 4. MicroRNA-133 (miR-133) affects \( \beta \)-adrenergic stimulation in vivo.** A, messenger RNA expression levels of mature and precursor miR-133 in TetON-miR-133 inducible transgenic mice (Tg133) compared with control mice 2 days after doxycycline administration (n=11). B, Cardiac \( \beta_1 \)-adrenergic receptor (\( \beta_1 \)-AR) density, as evaluated in a radioligand binding assay, was significantly reduced in Tg133 mice compared with control mice after doxycycline administration (n=11). C, Effect of selective \( \beta_1 \)-AR stimulation with xamoterol on cAMP accumulation in adult cardiomyocytes isolated from Tg133 and control mice. No cAMP increase was observed in Tg133 cardiomyocytes (n=4). D, Evaluation of max dP/dt during dobutamine stress test in Tg133 mice and control mice after doxycycline administration (control, n=8; Tg133, n=9). Bars indicate SEM. *P<0.05; **P<0.01; ***P<0.005; ****P<0.001. Ctl indicates control; and Dox, doxycycline.
Recent studies have demonstrated a role of βAR signaling in controlling microRNA expression. For instance, a group of 18 microRNAs was reported to be differentially expressed in the heart in response to βAR agonist or antagonist treatment. Similarly, Kim et al recently showed altered expression levels of a number of microRNAs in response to treatment with the β2-arrestin 1-biased βAR agonist carvedilol. In another study, β-blockers were found to induce serum responsive factor–mediated inhibition of miR-1 expression, which appeared to be correlated with PKA-dependent phosphorylation of serum responsive factor in response to βAR activation. These data correlate with a study on patients with chronic heart failure, where altered microRNA expression was found to be associated with reduced catecholamine sensitivity. Finally, the microRNA let-7f was found to target βAR directly and to be negatively regulated by β2-agonist stimulation. However, a direct link between microRNAs and the β1AR cascade has not previously been demonstrated.

Our data show that miR-133 specifically interferes with the βAR signaling pathway and excludes any potential effect of the microRNA on β2AR signaling. In fact, no effect on the cAMP accumulation rate was observed in miR-133-overexpressing cardiomyocytes treated with βAR-selective agonist. The miR-133 targeting of ACV, which has been found to be selectively coupled to β1AR, further supports its pathway specificity for the β1 axis. On the contrary, adenylate cyclase V, which is not a miR-133-predicted target, is predominantly associated with activation of β2AR signaling. Thus, miR-133 overexpression in cardiomyocytes should change the balance between β1- and β2-dependent effects toward the β2AR-dependent signaling pathway, resulting in cardioprotection and counteraction of deleterious effects due to activation of the β1AR. Apoptosis is among the detrimental processes that are triggered by β1AR activation, consistent with the reported antiapoptotic role of miR-133. In agreement with this, both our in vitro and in vivo data (Figure 6) show that miR-133 protects cardiomyocytes against β1AR-induced apoptosis. Furthermore, mRNA levels of grk2, which is upregulated in heart failure exerting a prodeath function, were downregulated in Tg133 mice 1 week after TAC (Figure 6D). On the contrary, no differences were found in mRNA levels of grk3, which is mainly associated with endothelin receptors.
In this study, we also demonstrated that miR-133 targets EPAC (Online Figure IIB and IIC), which is involved in the modulation of cAMP levels through a negative feedback loop between the cAMP-specific phosphodiesterase 4D3 and PKA.42 Thus, the decreased cAMP levels in response to miR-133 overexpression may be not only due to downregulation of the miR-133 targets β1AR, ACVI, and PKA C-β but also due to enhanced phosphodiesterase 4D3 activity consequent to EPAC downregulation. Furthermore, EPAC has been shown to be associated with Ca2+/calmodulin kinase II and β-arrestin at the β1AR,43–45 suggesting that by targeting EPAC, miR-133 affects the Ca2+/calmodulin kinase II–dependent signaling in the failing heart. Future studies are needed to shed more light on this matter.

Previous studies on transgenic mice constitutively overexpressing miR-133 have shown major discrepancies, most likely owing to the different approaches used. In fact, whereas miR-133 overexpression driven by an early embryonic promoter (β-myosin heavy chain) caused a lethal phenotype with detrimental cardiac defects in survivors to adulthood,20 a normal basal cardiac phenotype was obtained when transgenic miR-133 was stably overexpressed at a later postdevelopmental phase under the control of the α-myosin heavy chain promoter.21 In agreement with the latter study, our echocardiographic analysis on induced adult Tg133 mice showed no abnormalities under basal resting conditions compared with control mice, although a reduction in fractional shortening was observed with extended time of microRNA induction (Online Figure IVC). On the other hand, whereas the α-myosin heavy chain-transgenic mouse model showed impaired cardiac performance in response to ventricular pressure overload, our inducible miR-133 transgenic mice exhibited...
preserved fractional shortening and attenuated cardiac remodeling (Figures 5 and 6). These apparently contrasting results might be due to the different modalities of miR-133 expression and possibly to compensatory mechanisms that might be activated when miR-133 is constitutively overexpressed.

Conclusions
In this study, we demonstrate with several complementary approaches that miR-133 targets distinct components of the β₁AR pathway and provide evidence suggesting that restoration of miR-133 levels in patients might be used as a potential novel therapeutic strategy for the treatment of cardiovascular diseases. This is in line with our observation that downregulation of miR-133 levels in condition of pressure overload can be counteracted by administration of a clinically relevant β-blocker. Current therapeutic strategies are mainly designed to achieve inhibition of endogenous levels of specific microRNAs in vivo through the use of microRNA-antisense oligos. For example, downregulation of various microRNAs have been shown to have beneficial effect on cardiac fibrosis and remodeling, and neangiogenesis. As of yet, overexpression of microRNAs in vivo has only been achieved through the use of viral vectors, which as opposed to antisense technology, do not allow for transient microRNA modulation. Thus, the development of improved strategies for efficient and specific expression of microRNAs in the heart is needed before microRNA replacement can be envisioned as a therapeutic strategy.

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Disclosures

None.

References


### Novelty and Significance

**What Is Known?**
- MicroRNA-133 (miR-133) expression is inversely related to cardiac hypertrophy and human cardiac disease.
- β-blockers are used for the treatment of cardiovascular diseases.
- β1-adrenergic receptor (β1AR) signaling exerts proapoptotic effects, whereas miR-133 plays an antiapoptotic role.

**What New Information Does This Article Contribute?**
- miR-133 directly targets the β1AR transduction cascade at the level of β1AR (adbr1), adenylate cyclase VI (Adcy6), and catalytic subunit β of cAMP-dependent protein kinase A (PrkacB).
- miR-133 overexpression protects cardiomyocytes against β1AR-dependent apoptosis.
- β-blockers prevent miR-133 downregulation in response to ventricular pressure overload after transverse aortic constriction, and transgenic mice with inducible and cardiac-specific miR-133 show cardioprotection after transverse aortic constriction.

In conditions of pathological stress, activation of the β1AR signaling is a causative factor in the chain of events leading to the decline in cardiac function. In line with this, therapeutic use of β-blockers has been shown to be beneficial in heart failure. Based on a combination of in vitro and in vivo approaches, we demonstrate that the β1AR transduction cascade is directly regulated by miR-133, which is downregulated in response of ventricular pressure overload. In particular, we found that (1) miR-133 overexpression reduces cAMP accumulation in β1AR-agonist-treated neonatal and adult cardiomyocytes and (2) mice with inducible and cardiac-specific overexpression of miR-133 show cardioprotection during transverse aortic constriction by preventing, at least in part, the activation of pathological effects (eg, apoptosis) due on β1AR activation. Thus, we demonstrate for the first time a direct correlation between β1-adrenergic signaling and miR-133 and provide proof of concept that restoration of miR-133 levels in conditions of heart failure might be used as a potential novel therapeutic strategy.
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Supplemental Material

MiR-133 modulates the β1Adrenergic Receptor transduction cascade

Castaldi, MiR-133 targets β1AR pathway

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Target prediction
Prediction of miR-133 targets was performed using publicly available algorithms: TargetScan (http://www.targetscan.org/), miRanda (http://www.microrna.org/), PicTar (http://pictar.bio.nyu.edu/), PITA (http://genie.weizmann.ac.il/pubs/mir07/mir07_prediction.html), and miRDIP (http://ophid.utoronto.ca/mirDIP/). 3'UTRs of predicted targets were uploaded on the PITA database (http://genie.weizmann.ac.il/pubs/mir07/mir07_prediction.html) and scanned for miR-133 binding sites (Online Table I).

Luciferase assay
A 3' UTR segment of the adrb1, adcy6, prkacb, and epac mRNAs were subcloned by standard procedures into the psiCHECK-TM2 vector (Promega) immediately downstream of the stop codon of the Renilla luciferase gene. The miR-133 mature sequence was cloned into the pcDNA6.2-GW/EmGFP-miR vector according to the manufacturer’s protocol (Invitrogen). 2.5x10^5 293 cells were transfected with psiCHECK-3'UTR and pcDNA6.2-GW/EmGFP-miR-133 plasmids. 48 h post transfection, cells were lysed and luciferase activity was measured as described by the manufacturer (Promega). To confirm specific targeting of the 3’UTR of target genes by miR-133, site-specific mutagenesis at the predicted sites for each target was performed using the QuikChange Site-Directed Mutagenesis Kit as described by the manufacturer (Stratagene). Mutated constructs were then tested in luciferase assays and rescue of chemiluminescent signal in the presence of miR-133 identified real targeting sites.

RNA immunoprecipitation
A synthetic biotinylated miR-133 oligo (IBA BioTAGnology) was used to immunoprecipitate the miR:RNA-target binding complexes from heart homogenate as previously described.1 mRNA targets were then amplified by PCR (primers are listed in Online Table II).

RNA isolation and quantification
Total RNA was extracted using TRIZol Reagent (Life Technologies). Reverse transcription of RNA for miR-133, U6, and Rnu5g expression analysis was performed using the mirCURY LNA™ Universal RT microRNA PCR Polyadenylation and cDNA synthesis kit (Exiqon). Quantitative polymerase chain reaction (qPCR) was performed with microRNA LNA™ PCR primers (Exiqon) using the Fast SYBR Green Master Mix (Life Technologies). Reverse transcription of RNA for pri-mir-133a-1, pri-mir-133a-2, as well as adrb1, adrb2, adcy6, prkacb, grk2, and grk3 expression analyses was performed using the High Capacity cDNA Reverse Transcription Kit (Life Technologies), following by qPCR with custom designed
oligos (shown in Online Table II) using the Fast SYBR Green Master Mix (Life Technologies). Relative expression analyses were performed using the \( \Delta \Delta C(t) \) method.

**Neonatal cardiomyocyte isolation and culture**

Hearts from 1-2 days old Sprague Dawley rats were minced in ADS (5 mM glucose, 106 mM NaCl, 5.3 mM KCl, 20 mM Hepes, 0.8 mM Na$_2$HPO$_4$, and 0.4 mM MgSO$_4$, pH 7.4) and enzymatically dissociated with collagenase A (0.4 mg/ml) (Roche) and pancreatin (1.2 mg/ml) (Sigma). Cells were cultured for 24 hours in medium containing 67% DMEM, 17.5% M199, 10% HS, 5% NCS, L-glutamine, and antibiotics (all from Invitrogen), plated on laminin-coated dishes (10 µg/cm$^2$) (BD) at a density of 6x10$^5$ cells/cm$^2$, and maintained in a humidified atmosphere (5% CO$_2$) at 37°C. At the second day, cardiomyocytes (CMs) were cultured in a medium with low serum concentration.$^2$

**FRET imaging of cultured cardiomyocytes**

24 hours after seeding, neonatal CMs (nCMs) were transfected with the appropriate FRET-based sensor, GaMPs (for cAMP accumulation measurements, courtesy of Dr. Martin Lohse) or the fluorescent PKA activity reporter AKAR3 (for PKA activity, kind gift of Dr. Jin Zhang) using transfectin (Bio-Rad) as recommended by the manufacturer. After five hours, the medium was changed and approximately 5x10$^5$ nCMs were infected with either Ad-Empty or Ad-miR133 at different multiplicity of infection (MOI) as stated in the Results section and maintained in complete culture medium until the start of the imaging experiments. Cells were transferred to Hepes-buffered Ringer-modified saline (in mmol/L, 125 NaCl, 5 KCl, 1 Na$_2$PO$_4$, 1 MgSO$_4$, 5.5 glucose, 1.8 CaCl$_2$, 20 Hepes, pH 7.4) kept at room temperature, and imaged on an inverted Olympus IX50 microscope coupled to a CCD camera (Sensicam QE, PCO) and a custom built beam-splitter optical device (F. Mammano, Venetian Institute of Molecular Medicine). Images were acquired using custom developed software and processed using ImageJ (National Institutes of Health). FRET changes were determined by measuring the background-subtracted 480 nm/535 nm fluorescence emission upon excitation at 430 nm. FRET values were expressed as $AR/R_b$, where $R_b$ is the ratio at $t = 0$ s and $AR = R - R_b$. Norepinephrine (NE), forskolin (FRSK), clenbuterol (CLB), ICI-118,551 (ICI), and Isobutylmethylxantine (IBMX) (all from Sigma) were delivered to the cells using separate perfusion lanes. For the AC activity assay, the rate of cAMP increase was estimated by calculating the slope of CFP/YFP emission ratio change (dR/dt) in GaMP transfected cells between 100 and 150 sec after FRSK addition. For the PKA activity assay, AKAR transfected cells were incubated with 10 mmol/L of the cell permeable cAMP analogue 8-(4-Chlorophenylthio)-N6-phenyladenosine-3',5'-cyclic monophosphate (8-CPT-6-Phe-cAMP, Biolog, DE), and the 535 nm/480 nm emission ratio was compared in Ad-miR133 vs. control cells. No differences were observed in Ad-Empty infected cells compared to control (Ctl) cells (data not shown).

**cAMP assay in freshly isolated adult cardiomyocytes**

CMs were isolated from 2-month-old wild-type and Tg133 male mice using standard enzymatic techniques, whereafter calcium was gradually reintroduced.$^2$ cAMP accumulation in response to selective $\beta_1$AR stimulation was evaluated in freshly isolated cell through incubation with increasing concentrations (0.1, 1, and 10 µmol/L) of xamoterol (Tocris) for 15 minutes at 37°C, after which cAMP levels were measured using the Cyclic AMP EIA Kit (Cayman) following the manufacturer’s instructions.

**Western blot analysis**

Cells or tissues were lysed in RIPA buffer containing (in mmol/L) 10 Tris HCl pH7.2, 150 NaCl, 5 EDTA, 1% Triton-X, 0.1% SDS, complete protease inhibitor cocktail (Roche), 100 µmol/L Sodium Orthovanadate, and 10 mmol/L P-nitrophenylphosphate. 30 µg of extracted proteins were loaded on 4-12% acrylamide gels and Western blot analysis was performed using the following antibodies: $\beta_1$AR (SantaCruz, sc-568), WHSC2 (Abcam, Ad1481), PLN
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(Novus Biological, NEB300-582), PLN P-16 (Abcam, Ab15000), ACV1 (Sigma SAB2100054), PRKACB (Sigma SAB1302543), α-tubulin (Abcam 18251), and GAPDH (Cell Signaling, 2128); HRP-secondary antibodies were from Cell Signaling. ECL (Thermo Fisher Scientific) was used for protein detection using a Chemidoc MP Imaging System (Bio-Rad) and densitometry analysis was performed using Image Lab software 5.0 (Bio-Rad).

Immunofluorescence analysis
Cultured CMs were fixed with 4% paraformaldehyde (PFA) for 30 min at 4°C, permeabilized with 1X PBS supplemented with 1% BSA and 0.1% Triton X-100 (all from Sigma) and stained with antibodies against cleaved caspase-3 antibody (Cell Signaling Technology, 9662) or cardiac troponin I3 diluted in 1X PBS supplemented with 1% BSA for 2 hours at 37°C. Cy3-conjugated secondary antibodies from the Jackson ImmunoResearch Laboratories were used and nuclei were counterstained with DAPI (Sigma). Cells were analyzed using a Leica TCS SP5 confocal microscope.

β1AR Receptor Density
Myocardial sarcolemmal membranes were prepared by homogenizing whole hearts in ice-cold buffer containing 50 mM HEPES (pH 7.3), 150 mM KCl, and 5 mM EDTA. Total βAR density was determined by incubating 25 µg of the above homogenate with a saturating concentration of 125I-cyanopindolol (125I-CYP) (Perkin Elmer) in 500 µl of binding buffer. Nonspecific binding was determined in the presence of 20 µmol/L alprenolol (Perkin Elmer). Binding assays were conducted at 37°C for 60 min and terminated by rapid vacuum filtration over glass fiber filters, which were subsequently washed and counted in a gamma counter. Specific binding was reported as fmol of receptor per mg of membrane protein.

Apoptosis assay
Non-infected, Ad-Empty-, and Ad-miR133 infected nCMs were subjected to chronic treatment (72 hours) with 0.1, 1, and 10 (µmol/L) NE either in the absence or in presence of 0.1, 1, and 10 (µmol/L) of the β2AR antagonist ICI, and compared to non-treated cells. The same experimental protocol was followed in CMs infected with Ad-Decoy2 and relative non-infected and Ad-Empty infected controls. At the end of the pharmacological treatment, nCMs were fixed and co-stained with antibodies against cleaved caspase-3 and cardiac troponin I as described above. Neonatal CMs were analyzed using a confocal microscope and cell density as well as the number of caspase-3 positive CMs/total nCMs were estimated. Three coverslips for each experimental group were analyzed and a total of three different cell cultures were tested.

Histochemical analysis
Histochemical analyses were performed on 5 µm thick paraffin sections of heart specimens. H&E- and Azan Mallory-stainings were performed as previously described.5 For immunofluorescence staining, slides were heated in 10 mM sodium citrate (pH 6.0) for antigen retrieval. After rinsing in dH2O, sections were incubated with antibodies against smooth muscle actin (Sigma, A2547) and desmin (Abcam, AB8592), and subsequently Cy2 and Cy3-conjugated secondary antibodies (The Jackson ImmunoResearch Laboratories) before counterstaining with Toto-3 (Life Technologies). Apoptosis was determined using the In Situ Cell Death Detection AP Kit (Boehringer Mannheim). Specimens pretreated with DNase I served as positive control. Fluorescence acquisition was performed using a Leica TCS SP5 confocal microscope.
Animals
All mouse procedures on mice were performed according to institutional guidelines in compliance with national (4D.L. N.116, G.U., suppl. 40, 18-2-1992) and international law and policies (EEC Council-Directive 86/609, OJ L 358,1,12-12-1987; NIH Guide for the Care and Use of Laboratory Animals, US National Research Council-1996 and new directive 2010/63/EU). The protocol was approved by the Italian Ministry of Health. Special attention was paid to animal welfare and to minimize the number of animals used and their suffering. A targeting construct for inducible miR-133 expression was generated by cloning a DNA fragment containing the Tet operon, the minimal CMV promoter, the miR-133a-2 sequence, and a polyA termination sequence. Positive founder mice (F0) were crossed with mice expressing the reverse tetracycline-responsive transcriptional activator rtTA under the control of the cardiac-specific α-Myosin Heavy Chain (α-MHC), kindly provided by Dr. Wolfgang Dillmann (University of California San Diego, CA). Double transgenic mice (Tg) were backcrossed for six generations into the C57Bl/6J strain background. All experiments were performed on 12-week-old male mice with at least 8 animals per group. Induction of cardiac-specific expression of miR-133 in Tg mice was obtained by administration of doxycycline (Dox, 500 mg/Kg) to their food pellet as described in the text. Ctl receive the same Dox treatment as Tg133. For hemodynamic studies, cardiac catheterization was performed using a 1.4 French (0.46 mm) conductance catheter (Millar Instruments Inc.) inserted retrograde through the right carotid artery into the left ventricle, where pressure was recorded. A polyethylene-50 catheter was placed into the right external jugular vein for dobutamine infusion. True zero was obtained in saline at the end of each experiment and any offset was corrected. After bilateral vagotomy, basal pressures were again measured, and β-adrenergic responsiveness was assessed with graded doses of dobutamine, as described. In β-blocker experiments, 2-month-old C57Bl/6J male mice were subjected to transverse aortic constriction (TAC), and metoprolol (350mg/kg/day, Sigma) was given in the drinking water as previously reported. For each group, sham operated animals were used as controls. B-mode echocardiography and TAC were performed using standard techniques.

Statistical analysis
All data are expressed as mean ± s.e.m. Comparison between experimental groups was performed using the unpaired Student’s t-test or ANOVA tests using Prism 6.0 software (GraphPad Software, Inc.). A value of at least P < 0.05 was considered statistically significant.
Online Figures

Online Figure I. MiR-133 loss-of-function upregulates protein levels of β1AR, Adcy6, and Prkacb. Western Blot and densitometric analysis for β1AR, ACv1, and Prkacb on total protein lysates from nCMs infected or not with Ad-Decoy133 (MOI = 50; n = 3). Tubulin was used as loading control. *, P<0.05.
**Online Figure II.** **MiR-133 targets Adcy6 and Epac**

**A.** Luciferase reporter activity for all Adcy6 predicted sites (n=3).

**B.** Conserved binding site for miR-133 in the 3’UTR of epac (up) and corresponding luciferase reporter activity (bottom) (n=3). For sequence alignments, filled and empty dots (●) represent conserved and non-conserved wobble pairs, respectively; continuous and dashed lines represent conserved or non-conserved residues, respectively.

**C.** Representative PCR analysis of epac mRNA co-immunoprecipitated with biotinylated miR-133 oligo. Input = pre-immunoprecipitated RNA (positive control); miR-133 = biotinylated miR-133; neg; Control = no oligo (n= 3). *, P<0.05; **, P < 0.01; ***, P < 0.001.
Online Figure III. β₁ARs, but not β₂ARs are targeted by miR-133. A, Western blot and densitometric analyses for β₁AR and PLN (total and phosphorylated form at the PKA consensus site, Ser16) on cell lysate from NE-treated miR-133 and control (non-infected and AdEmpty infected) nCMs (n = 3). GAPDH was used as loading control. B, Adrb2 mRNA levels in NE-treated nCMs infected with Ad-miR133 compared to Ctl (n = 3). *, P<0.05.
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Online Figure IV. Tg133 mice A, Cell size measurements of single adult CMs isolated from Tg133 and Ctl mice 1 week after Dox administration (n = 600, 3 animals per group). B, Western blot analysis for expression of WHSC2 (top), β1AR, and PLN (total and phosphorylated form at the PKA consensus site, Ser16) (bottom) on left ventricular extracts from Tg133 and Ctl mice after 2 days of Dox administration (n = 3). GAPDH was used as loading control. C, Fractional shortening (%) in Tg133 and Ctl sham mice fed with normal or Dox-supplemented diet (n = 6). *, P<0.05**, P < 0.01; ***, P < 0.001. D, qRT-PCR on left ventricular extracts from Tg133 and Ctl mice after 2 days of Dox administration (n = 5).
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**Online Figure V.** Metoprolol administration to TAC mice. **A,** Fractional shortening (%) in C57J/B6 mice administered or not with metoprolol at baseline as well as 1 and 3 weeks after TAC (n = 10). **B,** Quantification of apoptotic cells in heart sections from Tg133 and Ctl mice 1, 3, and 12 weeks after TAC. *, P<0.05; ***, P<0.005.
**Online Figure VI. Tg133 mice subjected to TAC.**

**A,** qRT-PCR analysis for miR-133 target mRNAs in Tg133 compared to Ctl mice 1 week after TAC (n = 3).

**B,** Western blot and densitometric analysis for β1AR and PLN (phosphorylated/total) on left ventricular lysates from Tg133 and Ctl mice 1 week after TAC. *, P<0.05; **, P<0.001.
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**Online Figure VII.** MiR-133 determines the apoptotic response following βAR-chronic stimulation. **A,** Percentage of caspase-3 positive nCMs following NE-stimulation in the presence of the α-blocker prazosin (1 µmol/L). **B,** Cell density evaluated in cultures of Ctl and Ad-Decoy133 infected nCMs both at baseline and following 72 hours of treatment with NE in the presence of ICI (n=150 cells per group). *P < 0.05; *** P < 0.005.
**Online Table I. Bioinformatic target prediction for miR-133.** Validated targets are highlighted. dGduplex considers the binding free energy of the miRNA-target duplex, where the miRNA and the target pair according to the pairing forces imposed by the seed; dGopen describes the free energy lost by unpairing the target-site nucleotides; ddG represents the total miR-target interaction score that equals the difference between dGduplex and dGopen.

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### Online Table II. PCR and qPCR primers.

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#### qPCR

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### Online table III. Echocardiographic parameters of Tg133 and Clt mice at different time points post TAC.

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IVSd, diastolic septal wall thickness; LVIDd, left ventricular end-diastolic inner diameter; LVPWd, left ventricular posterior wall thickness in diastole; IVSs, systolic intraventricular septal wall thickness; LVIDs, left ventricular end-systolic inner diameter; LVPWs, left ventricular posterior wall thickness in systole; FS, left ventricular fractional shortening; HR, heart rate; TAC, transverse aortic constriction. *P < 0.05.
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References


