The Hypoxia-Inducible MicroRNA Cluster
miR-199a~214 Targets Myocardial PPARδ
and Impairs Mitochondrial Fatty Acid Oxidation

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SUMMARY

Peroxisome proliferator-activated receptor δ (PPARδ) is a critical regulator of energy metabolism in the heart. Here, we propose a mechanism that integrates two deleterious characteristics of heart failure, hypoxia and a metabolic shift toward glycolysis, involving the microRNA cluster miR-199a~214 and PPARδ. We demonstrate that under hemodynamic stress, cardiac hypoxia activates DNM3os, a noncoding transcript that harbors the microRNA cluster miR-199a~214, which shares PPARδ as common target. To address the significance of miR-199a~214 induction and concomitant PPARδ repression, we performed antagonim-based silencing of both microRNAs and subjected mice to biomechanical stress to induce heart failure. Remarkably, antagonim-treated animals displayed improved cardiac function and restored mitochondrial fatty acid oxidation. Taken together, our data suggest a mechanism whereby miR-199a~214 actively represses cardiac PPARδ expression, facilitating a metabolic shift from predominant reliance on fatty acid utilization in the healthy myocardium toward increased reliance on glucose metabolism at the onset of heart failure.

INTRODUCTION

Despite the remarkable metabolic flexibility of the heart regarding nutritional status and cardiac demand, several studies demonstrated abnormalities in cardiac lipid homeostasis and energy production as a consistent feature of heart failure (HF) (Barger and Kelly, 2000; Razeghi et al., 2001). Due to limited oxygen and fatty acid availability, the fetal heart mainly relies on anaerobic glucose utilization. In contrast, rising cardiac work and the abundance of fatty acids in the postnatal heart bring about increased reliance on mitochondrial fatty acid oxidation (FAO). Hemodynamically stressed hearts exhibit a return to the fetal metabolic pattern that is hallmarkmed by impaired mitochondrial FAO and a shift to further reliance on glucose metabolism (Rajabi et al., 2007; Razeghi et al., 2001). Due to the strict aerobic nature of the heart and the inability to generate sufficient energy under anaerobic conditions, reliance on glycolysis has a major impact on available ATP levels (Di Lisa et al., 2007). As such, hypoxia is considered to be a characteristic of the failing heart (Giordano, 2005; Sabbah et al., 2000; Tanaka et al., 1994).

Regulation of the metabolic profile by activity of the peroxisome proliferator-activated receptor δ (PPARδ) has been shown to play a role in the metabolic switch from FAO to glycolysis (Berkart et al., 2007). Heart muscle-restricted deletion of PPARδ resulted in progressive lipid accumulation, cardiac hypertrophy, and congestive heart failure (Cheng et al., 2004). Conversely, selective overexpression of PPARδ in the mouse heart provoked an increase in myocardial glucose utilization with no myocardial
lipid accumulation due to preserved FAO and resistance to cardiac disease induced by ischemia reperfusion injury (Burkart et al., 2007). These studies clearly illustrate the importance of PPARδ signaling in energy homeostasis and pathogenesis of heart failure. Despite this, whether and how PPARδ is regulated remains unclear.

Small noncoding RNAs (~18–24 nucleotides), such as microRNAs (miRNAs, miRs), have been shown to be a fascinating mechanism, coordinating complex programs of gene expression (da Costa Martins et al., 2008; Thum et al., 2007; van Rooij et al., 2007). By regulating the stability and translation of messenger RNAs by base-pairing with the 3′ UTRs, miRNAs have been shown to impact gene expression in cardiovascular disease (da Costa Martins et al., 2008; Thum et al., 2007). In a comparative study among animal models of pathological cardiac remodeling, miR-199a and miR-214 showed increased expression in these animal models as well as in samples of failing human hearts (van Rooij et al., 2006). In addition, expression of these miRNAs induced a hypertrophic response in cardiomyocytes, suggesting that miR-199a and miR-214 are involved in the processes leading to cardiac disease (van Rooij et al., 2006). Despite their apparent role in cardiac remodeling, the mechanisms driving the regulation of miR-199a and miR-214 in the failing heart and how these miRNAs provoke deleterious cardiac remodeling remain undefined.

We report here that the miRNA cluster miR-199a–214, embedded in chromosome 1 in a large noncoding RNA, Dnm3os, actively represses PPARδ. Furthermore, myocardial hypoxia provokes Dnm3os activation and concomitant miR-199a–214 expression. Increased expression of miR-199a and miR-214 promotes decreased cardiac PPARδ expression, mitochondrial disarray and I band widening, and decreased mitochondrial fatty acid oxidative capacity. Conversely, antago-
mir-based silencing of miR-199a–214 in mice subjected to pressure overload derepressed cardiac PPARδ levels, normalized mitochondrial fatty acid oxidation, and improved cardiac structure and function. Taken together, our data suggest a mechanism whereby myocardial hypoxia, a hallmark of heart failure, induces expression of members of the miRNA cluster miR-199a–214, which actively downregulate cardiac PPARδ expression, provoking a switch toward a mitochondrial glycolytic metabolic profile that contributes to characteristics of heart failure.

RESULTS

The miR-199a:214 Cluster Is Increased in Human and Mouse Heart Failure

Recent cardiac microRNA profiling studies from our group and others have shown that the expression of both miR-199a and miR-214 is increased in various models of heart failure (da Costa Martins et al., 2010; Leptidis et al., 2013; van Rooij et al., 2006). Human miR-199a–214 is encoded by a large noncoding RNA, DNM3os, which is transcribed in the opposite strand of the DNM3 gene (Figure 1A). Both mature miRNAs, hsa-miR-199a-2-5p and hsa-miR-214-3p, are evolutionary conserved among several species (Figure 1A). Northern blot analysis confirmed that both miR-199a and miR-214 are upregulated in hearts from mice subjected to pressure overload by transverse aortic constriction (TAC) (Figures 1B and 1C). Quantitative RT-PCR also showed an increase in DNM3os expression in TAC-operated mice, verifying the clustered expression of both miRs in failing mouse hearts (Figure 1D). An increased expression of DNM3os, along with the encoded miRNA cluster miR-199a–214, was also evident in biopsies of cardiac tissue from heart failure patients (Figure 1E).

Mechanistically, miR-199a and miR-214 are predicted to target multiple genes according to several public data sets. Interestingly, we identified PPARδ as a predicted common target of both microRNAs. Indeed, investigation of the 3′ UTR of PPARδ revealed an evolutionarily conserved seed region for miR-214 in both human and mouse PPARδ and a less-conserved seed region for miR-199a (Figure 1F). Next, we examined whether PPARδ levels are affected in biopsies of cardiac tissue from heart failure patients. Western blot analysis in biopsies of cardiac tissue from heart failure patients showed lower protein levels of PPARδ compared with human control heart tissue (Figures 1G and 1H).

To further confirm the functionality of the seed regions of miR-199a and miR-214, we fused the 3′ UTR of PPARδ to a luciferase...
reporter gene, generating miRNA expression reporter constructs (Figure 1I). Coexpression of synthetic miR-199a and/or miR-214 decreased PPARδ 3′ UTR reporter activity (Figure 1J), while mutating the seed regions for miR-214 in the PPARδ 3′ UTR reporter construct abrogated the inhibitory effect of miR-214 and miR-199a. Mutating the seed region for miR-199a only had an inhibitory effect on miR-199a transfection (Figure 1J; Figure S1A available online), indicating a dominant effect of miR-214 on PPARδ repression and establishing the causative link between posttranslational control by the miRNA cluster miR-199a–214 on PPARδ. To further verify whether increased miR-199a–214 levels are directly responsible for the decrease of PPARδ protein levels in the failing hearts, we overexpressed synthetic precursors for miR-199a and/or miR-214 in a dose-dependent manner and observed efficient downregulation of endogenous PPARδ expression (Figure 1K). Finally, we overexpressed a form of PPARδ lacking a 3′ UTR and demonstrated that this form of PPARδ was insensitive to precursor transfection of miR-199a and miR-214 (Figures 1L and 1M). Taken together, these data demonstrate that miR-199a and miR-214, as well as their host gene Dnm3os, are increased in human and mouse heart failure and directly target PPARδ.

Hypoxia Drives the Expression of miR-199a–214 through Hif1α/Twist1

Myocardial hypoxia has been associated with a variety of clinical conditions, including ischemic heart disease (IHD), systemic hypertension, and pathological cardiac hypertrophy, where the hypoxia-inducible transcription factor 1α (Hif1α) drives hypoxia-inducible gene expression (Lei et al., 2008; Rey and Semenza, 2010). One Hif1α downstream mechanism employs the activation of the transcription factor Twist1 by binding directly to the hypoxia response element (HRE) in the Twist1 proximal promoter (Yang et al., 2008). Interestingly, DNM3os has been identified as a target gene for the helix-loop-helix transcription factor Twist1 by binding directly to the hypoxia-inducible transcription factor 1α (Loebel et al., 2005). To verify the functionality of this element, a luciferase reporter harboring the proximal DNM3os promoter region was generated and tested for Hif1α or Twist1 sensitivity (Figure 2A). The DNM3os-luc reporter demonstrated activation upon cotransfection with Hif1α or Twist1, while the enhancer box (E box)-mutated DNM3os-luc reporter construct showed no activation under the same conditions (Figure 2B).

Dnm3os homozygous-deficient mice died within 1 month of birth accompanied by skeletal abnormalities, defects in dorsal neural arches and spinous processes of the vertebrae, osteopenia, and reduced expression of its host genes miR-199a and miR-214 (Watanabe et al., 2008). We reasoned that Dnm3os-deficient hearts may display derepression of PPARδ due to the reduced expression of miR-199a–214. In line, western blotting demonstrated increased PPARδ expression in Dnm3os-deficient hearts compared to hearts from wild-type (WT) littermates (Figures 2C and 2D). The premature death of juvenile Dnm3os-deficient mice precluded further functional studies.

The PPARδ 3′ UTR reporter also showed strong inhibition of luciferase activity under hypoxia, with peak activity on day 3 (Figure 2E), confirming both the hypoxia-sensitive and microRNA-mediated posttranslational regulation of PPARδ. Quantitative RT-PCR confirmed a significant increase of endogenous miR-199a, miR-214, and Dnm3os transcript abundance in cardiomyocytes upon exposure to hypoxia (2% O2) (Figure 2F), indicating hypoxia as a primary stimulus for the expression of mature miR-199a and miR-214 via transcriptional regulation of DNM3os. Locked nucleotide acid (LNA) knockdown probes for miR-199a and/or miR-214 exhibited a strong and specific repression of the target miRs as quantified by PCR (Figures S1B and S1C). LNA-based knockdown of miR-199a and miR-214 inhibited the hypoxia-mediated PPARδ downregulation (Figure 2G). Interestingly, knockdown of miR-214 abrogated the hypoxia-induced downregulation of PPARδ more effectively than when miR-199a was targeted (Figure 2G). These results indicate a more pronounced role for miR-214 than miR-199a in hypoxia-mediated knockdown of PPARδ. Ventricular deletion of Hif1α in mice was previously used to implicate a Hif1α-PPARγ axis in hypertrophy-induced PPARγ activation, metabolic reprogramming, and contractile dysfunction (Krishnan et al., 2009). Accordingly, we tested whether cardiac-specific Hif1α deletion would also abrogate PPARδ expression under baseline conditions or after pressure overload. Western blotting demonstrated that PPARδ expression was reduced after pressure overload, and this reduction was efficiently prevented by cardiac-specific Hif1α deletion (Figures 2H and 2I). In conclusion, the data demonstrate that hypoxia serves as an upstream stimulus for Dnm3os and miR-199a–214 expression in the heart in a hypoxia- and/or Hif1α-dependent manner.

Conditional Targeted Deletion of PPARδ Causes Severe Cardiac Dysfunction

To investigate whether maintenance of PPARδ expression is required for normal myocardial homeostasis and to bypass the early embryonic lethality of PPARδ null mice (Barak et al., 2002), we provoked deletion of a floxed PPARδ (PPARδF/F) allele using a tamoxifen-inducible Cre recombinase under the control of the cardiac-specific α-myosin heavy-chain promoter (αMHC). Specificity of PPARδ gene deletion was shown by real-time RT-PCR for all three endogenous PPAR isoforms, demonstrating that our genetic intervention did not affect PPARα or PPARγ transcripts, but resulted in a strong and selective downregulation of PPARδ transcripts (Figure 2A). We forced PPARδ gene deletion at the age of 8 weeks and noted that within 5 days after tamoxifen delivery, αMHC-MCM-PPARδF/F mice displayed signs of inactivity and a weak condition compared to all control groups. Indeed, up to 25% of tamoxifen-treated αMHC-MCM-PPARδF/F mice died within 1 week after initiation of the treatment, and this mortality rate increased to 75% during the following 2 weeks (data not shown). Upon autopsy, tamoxifen-treated αMHC-MCM-PPARδF/F displayed severely enlarged hearts (Figure S2B).

Cardiac tissue from these mice revealed intricate features of clinical heart failure, including hypertrophied myofibers, myocyte disarray, and interstitial fibrosis in hearts (Figure S2B), which was reflected by doubling of heart weight at the whole-organ level (Figure S2C). These data demonstrate that conditional cardiac-specific deletion of PPARδ in the adult heart causes rapid cardiac remodeling, which results in multiple clinical signs of end-stage heart failure and reduced survivability. Cardiac geometry and function was assessed noninvasively by echocardiography at 2 weeks after tamoxifen treatment (Figure S2D; Table S1). At this time point, tamoxifen-treated αMHC-MCM-PPARδF/F
animals demonstrated a significant decline in cardiac contractility, as evidenced by a 50% decrease in fractional shortening (Figure S2E), and severe left ventricular dilation (Figure S2F). These data indicate that conditional cardiac-specific deletion of PPARδ provoked progressive functional and geometrical deterioration consistent with a heart failure phenotype, including potent reactivation of stress-induced embryonic genes such as Acta1, Nppb, Myh7, and Nppa (Figure S2G). Moreover, we noted a substantial decrease in transcript abundance for Ctd6 and hadha, without changes in Slc2a1 transcript abundance (Figure S2H), indicating a reduction in fatty acid transport capacity and oxidation. Conclusively, deletion of PPARδ in the adult heart induces rapid and spontaneous cardiac dysfunction, induction of fetal hypertrophic marker genes, and selective downregulation of genes involved in fatty acid metabolism.

**miR-214 Silencing Improves Cardiac Contractility and Derepresses PPARδ**

Antagonirs are RNA-like oligonucleotides that are reverse compliment to mature microRNAs and harbor various modifications for ribonuclease (RNase) protection and pharmacologic properties such as enhanced tissue and cellular uptake. Antagonirs efficiently silence microRNAs in most tissues in vivo (Krützfeldt et al., 2005). To intervene in the targeted downregulation of PPARδ by miR-199a–214 in cardiac disease conditions, we subjected mice to transverse aortic constriction pressure overload...
Figure 3. Silencing miR-214 or miR-199a Differentially Improves Cardiac Dysfunction
(A) Design of antagomir treatment study.
(B) Quantitative RT-PCR analysis of miR-199a, miR-214, miR-199b, miR-107, miR-133a, miR-25, and miR-181a expression in mice hearts after vehicle or antagomir-199a treatment.
(C) Quantitative RT-PCR analysis of miR-199a, miR-214, miR-199b, miR-107, miR-133a, miR-25, and miR-181a expression in mice hearts after vehicle or antagomir-214 treatment.
for 6 weeks and treated them with antagonists for either miR-199a or miR-214 for 3 consecutive days at 3-week intervals (Figure 3A). To verify silencing efficiency of antagoni-199a and antagoni-214, quantitative RT-PCR was performed (Figures 3B and 3C). The specificity of the antagonists used was verified by measuring the expression of other microRNAs (Figures 3B and 3C). Sham-operated mice treated with vehicle, antagoni-199a, antagoni-214 showed no signs of histopathology or alterations in heart size (Figures S3A–S3C). Vehicle-treated mice showed substantial cardiac enlargement, displayed hypertrophied myofibers, myocyte disarray, and interstitial fibrosis upon hemodynamic stress (Figure 3D), and displayed significantly increased heart weights (Figure 3E). No significant reduction in heart weight was observed in antagoni-199a or antagoni-214-treated mice that underwent TAC surgery (Figures 3D and 3E). In contrast, pressure overload induced a pronounced decrease in systolic and diastolic contractility observed in vehicle-treated mice subjected to TAC surgery was significantly improved by either antagoni-199a or antagoni-214 treatment (Figures 3H–3J; Table 1). Furthermore, antagoni-214 treatment was able to fully derepress PPARα levels following hemodynamic stress (Figures 3K and 3L). To analyze the effect of antagoni-214 treatment on metabolism, we performed RT-PCRs for both fatty acid and glucose marker genes. Cardiac pressure overload did not alter the expression of genes implicated in glucose metabolism (Figure 3M). In contrast, pressure overload induced a pronounced reduction in key genes involved in fatty acid metabolism, including acyl-coenzyme A dehydrogenase, medium-chain (Acadm), CD36 antigen (Cd36), and diacylglycerol O-acyltransferase 2 (Dgat2), which were fully restored upon antagoni-214 treatment (Figure 3N). Antagoni-214 treatment did not influence the reactivation of stress-induced fetal genes, including Nppa, Nppb, Acta1, and Myh7 (Figure S3D). Taken together, these data demonstrate that single miR-199a or miR-214

Table 1. Morphometric and Echocardiographic Characteristics of Wild-Type Mice Treated with Vehicle, Antagomir-199a, or Antagomir-214 and Subjected to Sham or Transverse Aortic Constriction Surgery

<table>
<thead>
<tr>
<th></th>
<th>Sham Vehicle</th>
<th>Antagomir-199a</th>
<th>Antagomir-214</th>
<th>TAC Vehicle</th>
<th>Antagomir-199a</th>
<th>Antagomir-214</th>
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<tbody>
<tr>
<td>n</td>
<td>8</td>
<td>8</td>
<td>8</td>
<td>8</td>
<td>9</td>
<td>9</td>
</tr>
<tr>
<td>BW (g)</td>
<td>29 ± 1</td>
<td>27 ± 1</td>
<td>26 ± 1</td>
<td>26 ± 1</td>
<td>29 ± 1</td>
<td>28 ± 1</td>
</tr>
<tr>
<td>LV mass (mg)</td>
<td>98 ± 4</td>
<td>102 ± 8</td>
<td>102 ± 8</td>
<td>130 ± 6*</td>
<td>155 ± 12*</td>
<td>139 ± 10*</td>
</tr>
<tr>
<td>LV mass/BW (mg/g)</td>
<td>3.50 ± 0.18</td>
<td>3.80 ± 0.30</td>
<td>3.96 ± 0.58</td>
<td>4.95 ± 0.20*</td>
<td>5.49 ± 0.48*</td>
<td>5.07 ± 0.33*</td>
</tr>
<tr>
<td>IVSd (mm)</td>
<td>0.86 ± 0.04</td>
<td>0.90 ± 0.04</td>
<td>0.89 ± 0.06</td>
<td>0.99 ± 0.07*</td>
<td>1.07 ± 0.05*</td>
<td>0.99 ± 0.04*</td>
</tr>
<tr>
<td>IVSs (mm)</td>
<td>1.31 ± 0.11</td>
<td>1.30 ± 0.08</td>
<td>1.25 ± 0.06</td>
<td>1.32 ± 0.08</td>
<td>1.46 ± 0.08*</td>
<td>1.35 ± 0.06</td>
</tr>
<tr>
<td>LVIDd (mm)</td>
<td>3.86 ± 0.11</td>
<td>3.69 ± 0.13</td>
<td>3.81 ± 0.08</td>
<td>4.19 ± 0.05*</td>
<td>4.07 ± 0.14*</td>
<td>4.24 ± 0.13*</td>
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<tr>
<td>LVIDs (mm)</td>
<td>2.36 ± 0.08</td>
<td>2.41 ± 0.15</td>
<td>2.62 ± 0.11</td>
<td>3.30 ± 0.08*</td>
<td>3.07 ± 0.17*</td>
<td>3.01 ± 0.15*</td>
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<tr>
<td>LVPWd (mm)</td>
<td>0.91 ± 0.07</td>
<td>1.00 ± 0.09</td>
<td>0.93 ± 0.09</td>
<td>0.94 ± 0.07</td>
<td>1.17 ± 0.11*</td>
<td>1.00 ± 0.06</td>
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<tr>
<td>LVPWs (mm)</td>
<td>1.33 ± 0.06</td>
<td>1.37 ± 0.09</td>
<td>1.34 ± 0.09</td>
<td>1.18 ± 0.08</td>
<td>1.41 ± 0.10*</td>
<td>1.30 ± 0.05*</td>
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<tr>
<td>EF (%)</td>
<td>71 ± 1</td>
<td>75 ± 3</td>
<td>72 ± 2</td>
<td>51 ± 5*</td>
<td>56 ± 4*</td>
<td>64 ± 2**</td>
</tr>
<tr>
<td>FS (%)</td>
<td>39 ± 4</td>
<td>38 ± 3</td>
<td>35 ± 2</td>
<td>21 ± 1*</td>
<td>25 ± 3*</td>
<td>29 ± 1*</td>
</tr>
<tr>
<td>E/A (mm/s)</td>
<td>1.45 ± 0.08</td>
<td>1.57 ± 0.12</td>
<td>1.58 ± 0.13</td>
<td>2.04 ± 0.13*</td>
<td>1.58 ± 0.14*</td>
<td>1.72 ± 0.13*</td>
</tr>
</tbody>
</table>

Data are expressed as means ± SEM. TAC, transverse aortic constriction; sham, sham-operated control group; BW, body weight; LV, left ventricular; IVSd, interventricular septal thickness at end diastole; IVSs, interventricular septal thickness at end systole; LVIDd, left ventricular internal dimension at end diastole; LVIDs, left ventricular internal dimension at end systole; LVPWd, left ventricular posterior wall thickness at end diastole; LVPWs, left ventricular posterior wall thickness at end systole; EF, ejection fraction; FS, fractional shortening; E/A, Doppler E/A ratio. *p < 0.05 versus sham group treated with control antagonist; †p < 0.05 versus experimental group.
Figure 4. Antagomir-Mediated Silencing of Both mir-199a and miR-214 Attenuates Cardiac Remodeling

(A) Design of antagomir treatment study.
(B) Pressure gradients across the transverse aorta measured noninvasively to validate the TAC procedure.
(C) Representative images of H&E (top panels), Sirius red-stained (middle panels), or WGA-labeled (lower panels) histological sections of mice hearts after 6 weeks of sham or TAC surgery, treated with vehicle or antagomirs.
(D) Gravimetric analysis of corrected heart weights of mice after 6 weeks of sham or TAC surgery, treated with vehicle or antagomirs.
(E) Representative M-mode images in mice hearts after 6 weeks of sham or TAC surgery, treated with vehicle or antagomirs.
(F and G) Quantification by echocardiography of LV internal diameter at systole (LVIDs) (F) and fractional shortening (%FS) (G) of mice hearts after 6 weeks of sham or TAC surgery, treated with vehicle or antagomirs.
(H) Western blot analysis of endogenous PPARδ and GAPDH levels in mice hearts after 6 weeks of sham or TAC surgery, treated with vehicle or antagomirs.

(legend continued on next page)
antagomir-mediated silencing mildly improved cardiac remodeling and differentially restored cardiac contractility, PPARδ expression, and key genes involved in fatty energy metabolism.

**miR-199a–214 Silencing Attenuates Cardiac Remodeling and Dysfunction**

Next, we intervened in miR-199a–214 induction in cardiac disease conditions by subjecting mice to transverse aortic constriction pressure overload for 6 weeks and treating them with antagomirs for both miR-199a and miR-214 for 3 consecutive days at 3-week intervals (Figure 4A). To ensure equal pressure gradients among the experimental groups, noninvasive pressure gradients across the transverse aorta were measured (Figure 4B). Vehicle-treated mice showed substantial cardiac enlargement upon biomechanical stress (Figure 4C), whereas antagomir-199a–214-treated mice displayed significantly reduced heart weights (Figure 4D). Sham-operated mice treated with vehicle or antagomir-199a–214 showed no signs of histopathology (Figure 4C). In contrast, cardiac tissue of vehicle-treated mice subjected to pressure overload displayed hypertrophied myofibers, myocyte disarray, and interstitial fibrosis, while mice treated with antagomir-199a–214 displayed normal myocyte arrangement and significantly reduced hypertrophy and fibrosis (Figure 4C; Figure S4A). Analysis of cardiac function by M-mode echocardiography at 6 weeks showed an increase in LVID and a proportional decrease in systolic contractility (FS) in mice subjected to pressure overload and treated with vehicle (Figures 4E–4G; Figure S4A). Analysis of cardiac function by M-mode echocardiography at 6 weeks showed an increase in LVID and a proportional decrease in systolic contractility (FS) in mice subjected to pressure overload and treated with vehicle (Figures 4E–4G; Figure S4A). Analysis of cardiac function by M-mode echocardiography at 6 weeks showed an increase in LVID and a proportional decrease in systolic contractility (FS) in mice subjected to pressure overload and treated with vehicle (Figures 4E–4G; Table 2).

Importantly, treatment with antagomir-199a–214 derepressed PPARδ levels even in sham-operated animals and restored PPARδ expression following hemodynamic stress (Figures 4H and 4I), but did not influence PPARδ expression (Figure S4B), further underscoring a specific role for PPARδ. To analyze the effect of antagomir treatment on metabolism in these hearts, we performed RT-PCRs for both fatty acid and glucose marker genes. Neither cardiac pressure overload nor antagomir-199a–214 treatment altered the expression of genes implicated in glucose metabolism, including those encoding solute carrier family 2 facilitated glucose transporter member 1 and member 4 (Slc2a1, Slc2a4) and hexokinase 1 (Hk1), in mouse hearts exposed to pressure overload (Figure 4J). In contrast, cardiac pressure overload induced pronounced changes in key genes involved in fatty acid metabolism, including acyl-coenzyme A dehydrogenase, long chain (Acadl) and Acadm, indicating restoration of fatty acid metabolism (Figure 4K).

**Table 2. Echocardiographic Analysis of Mice Following Sham Operation or Transverse Aortic Constriction with or without Double Antagomir-199a and Antagomir-214 Treatment**

<table>
<thead>
<tr>
<th></th>
<th>Sham Vehicle</th>
<th>Antagomir-199a–214</th>
<th>TAC Vehicle</th>
<th>Antagomir-199a–214</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>7</td>
<td>6</td>
<td>7</td>
<td>6</td>
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<tr>
<td>IVSd (mm)</td>
<td>0.98 ± 0.10</td>
<td>0.83 ± 0.14</td>
<td>1.29 ± 0.07</td>
<td>1.23 ± 0.14</td>
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<td>IVSs (mm)</td>
<td>1.79 ± 0.05</td>
<td>1.68 ± 0.07</td>
<td>1.74 ± 0.06</td>
<td>1.98 ± 0.09</td>
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<td>LVIDs (mm)</td>
<td>2.55 ± 0.22</td>
<td>2.10 ± 0.13</td>
<td>3.75 ± 0.17</td>
<td>2.52 ± 0.26</td>
</tr>
<tr>
<td>LVIDd (mm)</td>
<td>4.35 ± 0.12</td>
<td>4.12 ± 0.22</td>
<td>4.78 ± 0.14</td>
<td>4.38 ± 0.18</td>
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<td>LVPWd (mm)</td>
<td>1.80 ± 0.08</td>
<td>2.09 ± 0.08</td>
<td>1.49 ± 0.10</td>
<td>1.97 ± 0.16</td>
</tr>
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<td>LVPWd (mm)</td>
<td>0.91 ± 0.07</td>
<td>1.27 ± 0.14</td>
<td>1.11 ± 0.07</td>
<td>1.23 ± 0.08</td>
</tr>
<tr>
<td>FS (%)</td>
<td>47 ± 1</td>
<td>45 ± 1</td>
<td>25 ± 1</td>
<td>38 ± 1*</td>
</tr>
</tbody>
</table>

Data are expressed as means ± SEM. LV, left ventricular; IVSd, interventricular septal thickness at end diastole; IVSs, interventricular septal thickness at end systole; LVIDd, left ventricular internal dimension at end diastole; LVIDs, left ventricular internal dimension at end systole; LVPWd, left ventricular posterior wall thickness at end diastole; LVPWs, left ventricular posterior wall thickness at end systole; FS, fractional shortening.*p < 0.05 versus sham group treated with vehicle; #p < 0.05 versus experimental group.

(i) Quantification of GAPDH-corrected protein levels of PPARδ.

(ii) Real-time PCR analysis of transcript abundance for glucose metabolism marker genes (J) and fatty acid metabolism marker genes (K) in mouse hearts after 6 weeks of sham or TAC surgery, treated with vehicle or antagomirs. *p < 0.05 versus corresponding control group; #p < 0.05 versus corresponding experimental group (error bars are SEM). See also Figure S4.
substrate-dependent changes in the mitochondrial oxidative capacity of the heart upon pressure overload and treatment with antagomir-199a/C24 (Figures 5C and 5D). Thus, when isolated cardiac mitochondria were fueled with the carbohydrate-derived substrate pyruvate, ADP-stimulated, oligomycin-insensitive as well as FCCP-induced respiration was similar across all groups (Figure 5C). On the other hand, pressure overload considerably reduced mitochondrial fatty acid oxidative capacity, as evidenced by a significant reduction in both the ADP-stimulated (~25%) and the maximally uncoupled respiration (~20%) upon the fatty acid substrate palmitoyl-CoA (Figure 5D). The leak rate (state 4 respiration) remained unaffected by pressure overload. Interestingly, the reduced fatty acid oxidative capacity upon pressure overload was completely restored by antagomir-mediated silencing of miR-199a and miR-214 derepresses PPARδ expression and normalizes cardiac energy homeostasis. *p < 0.05 versus corresponding control group; #p < 0.05 versus corresponding experimental group (error bars are SEM). See also Figure S5 and Table S2.
antagomir-based silencing of the miRNA cluster miR-199a~214, indicating that both ADP-stimulated and the maximally un-coupled respiration rate upon palmitoyl-CoA + carnitine was similar to the respective respiration rates observed in control animals. Single microRNAs likely simultaneously influence expression of up to 100 target genes (Hu et al., 2012). Given the strong restoration of mitochondrial fatty acid oxidative capacity and cardiac contractility of the hemodynamically challenged heart achieved by antagomir-199a~214, we interrogated (using an unbiased proteomics screen) whether additional proteins were influenced either directly by silencing miR-199a~214 or indirectly by reactivation of PPARδ (Table S2). Accordingly, we detected an additional 20 differentially expressed proteins (five of which were mitochondrial) in pressure-overloaded hearts upon miR-199a~214 silencing, many of which harbored potential miR-199a (Figure S5B) or miR-214 seed regions and/or contained PPAR response elements (PPREs) in their promoter region (Figure S5E), suggesting that miR-199a~214 silencing pro- vokes specific alterations on the cardiac mitochondrial prote- ome in addition to PPARδ derepression. Altogether, our data suggest a mechanism whereby myocardial hypoxia, a character- istic of heart failure, induces expression of members of the miRNA cluster miR-199a~214 that actively downregulate several mitochondrial and cardiac targets including PPARδ, provoking a switch toward a glycolytic metabolic profile that contributes to heart failure (Figure S5F).

DISCUSSION

Although the regulation of metabolism is modulated by a variety of factors, the molecular mechanisms that drive the metabolic shift in the failing heart are still incompletely understood. The hemodynamically challenged myocardium exhibits a return to the fetal metabolic pattern that is hallmarked by impaired mitochon- drial fatty acid oxidation and a shift to further reliance on glucose metabolism (Rajabi et al., 2007; Razeghi et al., 2001). Here, we propose a mechanism that integrates two characteristics of heart failure, hypoxia and a metabolic shift toward glycolysis, involving a microRNA cluster and a variety of target genes including PPARδ. We demonstrate that under hemodynamic stress, regional hypoxia in the heart activates Dnm3os, a non-coding RNA transcript that harbors the miRNA cluster miR-199a~214, in a HIF-dependent and/or HIF-independent manner. As one mechanistic explanation, the miRNA-mediated repres- sion of PPARδ activity participates in a specific defect in mitochon- drial respiration using fatty acids as substrate, resulting in a metabolic shift of the heart from predominant reliance on fatty acid utilization in the healthy myocardium toward increased reli- ance on glucose metabolism in the failing heart.

Recently, Gan and colleagues demonstrated that muscle-spe- cific overexpression of PPARδ increased glucose oxidation in mitochondria through the reprogramming of glucose utilization pathways via interaction with the exercise-inducible AMP-acti- vated protein kinase (AMPK) and myocyte enhancer factor 2A (MEF2A) (Gan et al., 2011). This effect may be selective for skele- tal muscle, given that in our study we did not observe enhanced glucose utilization after reactivation of PPARδ in the hemody- namically stressed heart, but further lend evidence toward a role for PPARδ in the regulation of mitochondrial fueling.

Targeted expression of an activated form of PPARδ in skeletal muscle induced a muscle fiber type switch, hereby conferring resistance to obesity and improved metabolic profiles, in part through increased mitochondrial function (Wang et al., 2004). Furthermore, conditional transgenic mice expressing a constitu- tively active form of PPARδ in cardiomyocytes displayed enhanced mitochondrial capacity at baseline and under condi- tions of pressure overload (Liu et al., 2011). In contrast, Wang et al. (2010) demonstrated that inducible gene targeting of PPARδ in the adult heart resulted in decreased mitochondrial function, concomitant with a hypertrophic response and decreased cardiac function. These data confirm our findings that restoring PPARδ levels in the heart maintains oxidative ca- pacity of mitochondria and protects against heart failure. Conversely, our proteomics analysis of the miR-199a~214-silenced myocardium has given us a first unbiased indication of additional factors further involved in the phenotypic changes we observed. As expected, a large number of them are mito- chondria specific (Coq9, Dlat, Uqcrfs1, Naduf2, Hsp1, and Nnt); however, their precise regulation by this pathway remains to be experimentally validated. As a first attempt to elucidate their potential dependency on a miR-199a~214/PPARδ circuitry, we identified a number of potential PPREs as well as a number of potential binding sites for miR-199a and/or miR-214. These data suggest possible first or second degree interactions with our proposed mechanism and open up future studies to elucidate their function in cardiac energy metabolism.

As a key determinant of progression to heart failure, hypoxia is postulated to be a driving force for adverse cardiac remodeling. Pressure overload and ischemia often occur together clinically, as in patients with hypertension and coronary disease. As miR-214 was recently demonstrated to act as a regulator of cardio- myocyte Ca2+ homeostasis and survival during acute cardiac ischemia-reperfusion injury (Aurora et al., 2012), the possible use of future miR-214 therapeutics may be of more benefit in chronic cardiac (hypertrophic) remodeling processes rather than acute cardiac ischemic conditions. Indeed, pathologically hypertrophied hearts show significant decreased capillary den- sity and associated hypoxia (Friedls et al., 2004). In a mouse model with cardiomyocyte-restricted deletion of the von Hippel-Lindau protein (VHL), a component of the E3 ubiquitin ligase that inhibits Hifα, chronic activation of the Hifα hypoxia response pathway is observed (Lei et al., 2008), resulting in pro- gressive cardiac degeneration with lipid accumulation and decreased mitochondrial number leading to severe heart failure. Next to its role in modulating the fate of mesenchymal cell pop- ulation during development, Twist1 has been also implicated in a wide range of neoplasias, including gastric, liver, and breast cancers (Kareth and Tuveson, 2004; Mironchik et al., 2005; Niu et al., 2007). Analysis of Twist1 expression showed a ubiquitous expression in various mouse tissues with diverse expression patterns, with relatively high expression in the cardiac ventricle (Lu et al., 2011), which is in line with the proposed regulatory role for Dnm3os expression. Dnm3os-deficient mice display de- fects in skeletal formation and body growth during embryonic development (Watanabe et al., 2008). Taken together, our data show that miR-199a and miR-214 play a pivotal role in PPARδ-mediated regulation of cardiac mitochon- drial substrate fluxes with impact on cardiac structure and

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function, resulting in a metabolic shift of the heart from predomi-
nant reliance on fatty acid utilization in the healthy myocardium
toward increased reliance on glucose metabolism in the failing
heart. Our observations indicate that human heart failure arises
from derangements in gene regulatory circuits, where molecular
understanding of these circuits will aid in predicting sites of ther-
apneic intervention.

EXPERIMENTAL PROCEDURES

Human Heart Samples
Tissue was taken from the left ventricular free wall of patients with end-stage
heart failure secondary to ischemic heart disease or from patients undergoing
heart transplantation because of terminal heart failure. Control tissue was
taken from the left ventricular free wall of refused donor hearts.

Mouse Models
Mouse models included Dnm3os-1/2 mice (Watanabe et al., 2008), Hif1α−/− mice
(Ryan et al., 1998) interbred with myosin light chain 2v (MLC2v)-Cre
mice (Chen et al., 2018) (Krishnan et al., 2009), and PPARδ−/− mice (Jackson
Laboratories) interbred with mice harboring a tamoxifen-regulated form of
Cre recombinase (MerCreMer) under control of the murine Myh6 promoter
(Solah et al., 2001). All protocols were performed according to institutional
guidelines and approved by local Animal Care and Use Committees.

Aortic Banding and Transthoracic Echocardiography
Transverse aortic constriction or sham surgery was performed in 2- to 3-
month-old Bl6CBAF1 mice by subjecting the aorta to a defined 21G con-
traction between the first and second truncus of the aortic arch as described
previously (Bouraja et al., 2008; Rockman et al., 1991). Noninvasive, Doppler
echocardiographic analysis was performed as described previously in detail
(da Costa Martins et al., 2010).

Antagomir Administration
Antagomirs, 20–23 nt long RNA oligos complementary to miR-199a or miR-
214, were purchased from Fidelity Systems or Integrated DNA Technologies
and synthesized essentially as previously described (Kruetzfeldt et al., 2005),
except that cholesterol was linked through a hydroxypropynol linkage. All anta-
gomirs were 2′-Ome modified, contained a 3′ cholesterol-TEG (15 atom
triethylene glycol), 2′ phosphate (PT) bonds at the very first 5′ end,
and PT bonds between the last 3′ bases. All antagomirs were HPLC purified
and desalted before use.

Northern Blot Analysis
Northern blot analysis was performed as described previously (da Costa
Martins et al., 2010) using 3′-digoxigenin-labeled locked nucleic acid oligonucleo-
tides for hsa-miR-199a, hsa-miR-214, mmu-miR-199a, mmu-miR-214, or U6
small nuclear RNA (Rnu6-2) and detected with an antibody to 3′-digoxigenin
(Roche).

MicroRNA and mRNA Real-Time PCR
Primer sequences are described in Table S3. Real-time PCR was performed
using miScript SYBR Green PCR Kit (QIAGEN) on a Bio-Rad iCycler.

Primary Cardiomyocytes, Transient Transfections, and Luciferase
Reporter Assays
Cardiomyocyte cultures were isolated by enzymatic dissociation of 1- to 2-
day-old neonatal rat hearts, as described previously (De Windt et al., 2000),
and transfected with miR-199a, miR-214, or scrambled-miR precursor mole-
cules (Ambion) using oligofectamine (Invitrogen). Human embryonic kidney
(HEK) 293 cells were transfected with pmiR reporter plasmids harboring the
3′ UTR of human PPARδ using FuGENE 6 (Roche) reagent, followed by trans-
fection with miR-199a, miR-214, or scrambled miR precursor molecules using
oligofectamine. For cotransfection assays, pGL3-Dnm3os promoter con-
structs were cotransfected with constructs expressing a stabilized form of
HIF1α and/or Twist1, pRL-TK, containing the thymidine kinase promoter driving
Renilla luciferase, was included to correct for transfection efficiency.

Western Blot Analysis
Immunoprecipitation, SDS PAGE electrophoresis, and blotting were per-
formed as described previously (De Windt et al., 2000). Primary antibodies
that were used for western blotting include polyclonal anti-PPAR-delta
(Abcam), anti-PPAR-alpha (Abcam), monoclonal anti-GAPDH (Chemicon),
and monoclonal total OXPHOS cocktail (MitoSciences), followed by corre-
spanding horseradish peroxidase (HRP)-conjugated secondary antibodies
and enhanced chemiluminescence (ECL) detection.

Histological Analysis and Immunofluorescence Microscopy
Hearts were arrested in diastole, paraffin embedded, sectioned at 4 μm, and
stained with fluorescein isothiocyanate (FITC)-labeled wheat germ agglutinin
(WGA), hematoxylin and eosin (H&E), Sirius red, or biotinylated Griffonia sim-
plicifolia lectin I (GS-I) to visualize cardiac vascularization.

Isolation of Cardiac Mitochondria and Mitochondrial Respirometry
Cardiac tissue was processed for mitochondrial isolation by mechanical Potter
homogenization, repeated centrifugation, and gentle resuspension. Mitochon-
drial respiratory rates were analyzed using an oxygraph (OROBOROS Instru-
ments) (Hoeks et al., 2010).

Proteomics Analysis
Cardiac tissue was diced, PBS washed with proteinase inhibitors (Sigma-
Aldrich), and incubated with 0.1% SDS, and the SDS solution was stored
frozen. Next, samples were incubated with 4 M guanidine hydrochloride with
proteinase inhibitors, proteins were precipitated by centrifugation, and the pel-
lets were kept at –20 °C. The gel bands were subjected to in-gel digestion with
trypsin, and tryptic peptides were separated on a Nanoflow LC System (Dio-
nex UltiMate 3000), eluted with a 40 min gradient, and the column (Dionex Pep-
Map C18) was coupled to a nanospray source (PicoView). Spectra were
collected from an ion trap mass analyzer (LTQ Orbitrap XL). Tandem mass
spectrometry (MS/MS) was performed on the top six ions in each MS scan us-
ing the data-dependent acquisition mode with dynamic exclusion enabled. MS
spectra were separately analyzed in MaxQuant Software. To construct a MS/
MS peak list file, up to the top eight peaks per 100 Da window were extracted
and submitted to search against a concatenated forward and reverse version
of the UniProtKB/Swiss-Prot mouse database. A principal component analysis
was performed using the normalized high/low ratios of all proteins from all
samples. Significant differences were identified using the Bioconductor limma
package and Bayesian statistics to moderate variance across proteins and
calculate a p value.

Statistical Analysis
The results are presented as mean ± SEM. All statistical analyses were per-
formed with Prism software (GraphPad), consisting of an ANOVA test followed
by Tukey’s post hoc test when group differences were detected at the 5% sig-
nificance level or Student’s t test when comparing two experimental groups.
Differences were considered significant when p < 0.05.

SUPPLEMENTAL INFORMATION
Supplemental Information includes Supplemental Experimental Procedures,
five figures, and three tables and can be found with this article online at
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