HDACs Regulate miR-133a Expression in Pressure Overload–Induced Cardiac Fibrosis

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Background—MicroRNAs (miRNAs) and histone deacetylases (HDACs) serve a significant role in the pathogenesis of a variety of cardiovascular diseases. The transcriptional regulation of miRNAs is poorly understood in cardiac hypertrophy. We investigated whether the expression of miR-133a is epigenetically regulated by class I and IIb HDACs during hypertrophic remodeling.

Methods and Results—Transverse aortic constriction (TAC) was performed in CD1 mice to induce pressure overload hypertrophy. Mice were treated with class I and IIb HDAC inhibitor (HDACi) via drinking water for 2 and 4 weeks post TAC. miRNA expression was determined by real-time polymerase chain reaction. Echocardiography was performed at baseline and post TAC end points for structural and functional assessment. Chromatin immunoprecipitation was used to identify HDACs and transcription factors associated with miR-133a promoter. miR-133a expression was downregulated by 0.7- and 0.5-fold at 2 and 4 weeks post TAC, respectively, when compared with vehicle control (P<0.05). HDAC inhibition prevented this significant decrease 2 weeks post TAC and maintained miR-133a expression near vehicle control levels, which coincided with (1) a decrease in connective tissue growth factor expression, (2) a reduction in cardiac fibrosis and left atrium diameter (marker of end-diastolic pressure), suggesting an improvement in diastolic function. Chromatin immunoprecipitation analysis revealed that HDAC1 and HDAC2 are present on the miR-133a enhancer regions.

Conclusions—The results reveal that HDACs play a role in the regulation of pressure overload–induced miR-133a downregulation. This work is the first to provide insight into an epigenetic-miRNA regulatory pathway in pressure overload–induced cardiac fibrosis.

Key Words: microRNA ■ epigenomics ■ heart ventricles ■ histone deacetylase ■ hypertrophy

The left ventricle (LV) undergoes hypertrophic remodeling in response to chronic pressure overload (PO), a condition initiated by arterial hypertension or aortic stenosis that frequently leads to the development of chronic heart failure.1 Although the mechanisms by which PO leads to hypertrophic remodeling have not been completely defined, changes in microRNA (miRNA) expression makes an important contribution.2,3 In particular, miR-133a is abundantly expressed in the heart and plays a critical role in hypertrophy; miR-133a is downregulated in the LV and atria in multiple murine models of cardiac hypertrophy,4,5 in dilated atria from patients with mitral stenosis, and in ventricles from young patients having hypertrophic cardiomyopathy.6

In PO-induced remodeling, the myocardium is subjected to important modifications of the extracellular matrix resulting in myocardial fibrosis. Key proteins in this process are serum response factor (SRF), connective tissue growth factor (CTGF), and collagen type 1 (COL1a1), all of which are post-transcriptionally regulated by miR-133a.6,7 Thus, when miR-133a is downregulated during cardiac hypertrophy and LV dilation, these prohypertrophic and profibrotic factors are upregulated and contributed to the progression to heart failure.

Little is known about what regulates the PO-induced downregulation of miR-133a. Here, we examine whether epigenetic regulators may play a role in miR-133a expression in PO hypertrophy. Histone acetyltransferases and histone deacetylases (HDACs) are the enzymes that carry out the

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addition and the removal, respectively, of the acetyl moiety on lysine residues of histone proteins. HDAC inhibition reduced fibrosis in PO hypertrophy and blocked COL1a1 upregulation. In addition, HDACi treatment of rat neonatal cardiomyocytes upregulated miR-133a expression. Therefore, we hypothesize that HDACs regulate miR-133a expression during PO hypertrophy and that class I and IIb HDAC inhibition could attenuate pathological cardiac remodeling and improve cardiac function.

Methods

Transverse Aortic Constriction

The methods used to create transverse aortic constriction (TAC)–induced PO hypertrophy have been described previously. All CD1 mice were between 11 and 17 weeks of age at the time of TAC. Suberoylanilide hydroxamic acid (SAHA also known as vorinostat, cat no. S1047, Selleck, Houston, TX) was dissolved in 5 mol/L 2-hydroxypropyl-β-cyclodextrin, in the drinking water (75 mg/kg per day). Mice were studied 2 weeks (n=34, of which 16 received SAHA) or 4 weeks (n=29, of which 15 received SAHA) post TAC. Twenty-six mice did not undergo TAC and served as controls (of 26 mice, 10 received SAHA drug for 2 weeks). Vehicle water consisted of 5 mol/L 2-hydroxypropyl-β-cyclodextrin alone. All procedures performed were approved by the Institution Animal Care and Use Committee of the Medical University of South Carolina in accordance with National Institutes of Health guidelines.

HDAC Activity Assay

HDAC activity was measured with the homogenous fluorescence release HDAC deacetylase assay as previously described. AMC (7-amino-4-methyl coumarin) fluorescence was measured using Fluoroskan Ascent from Labsystems at excitation 355/emission 460, and background signals from predeveloped blanks were subtracted. The data were standardized using control, and the absolute deacetylation and background signals from predeveloped blanks were subtracted. Fluoroskan Ascent from Labsystems at excitation 355/emission 460, and background signals from predeveloped blanks were subtracted. Fluoroskan Ascent from Labsystems at excitation 355/emission 460, and background signals from predeveloped blanks were subtracted.

Immunoblotting

LV myocardial samples were homogenized in lysis buffer, supernatant and pellet fractions for each sample run on SDS-PAGE, and Western blotting was performed using CTGF antibody (1:2500, Abcam, cat no. ab6992), acetylated histone H3 (Cell Signaling no. 9702), and α-tubulin (Sigma, T9026). Band intensity was quantified using ImageJ 1.47i software (National Institutes of Health, http://imagej.nih.gov/ij/).

RNA Isolation

Left ventricular myocardial tissue (30–50 mg) was placed in RNAlater and then homogenized in Qiazol Lysis Reagent, and total RNA (including miRNA and small RNA molecules) was purified with the mirNeasy Mini Kit (cat no. 217004, Qiagen, Valencia, CA). Total and small RNA concentration was determined with Agilent RNA 6000 Nano Kit on the Agilent Bioanalyzer 2100 (Agilent, Santa Clara, CA).

miRNA Quantification

Using the Taqman microRNA Reverse Transcription Kit and Taqman microRNA Assays (Life Technologies), isolated RNA (10 ng) was reverse transcribed, and expression level of mature miR-133a (assay ID: 002246, miR-133a-1 and miR-133a-2 combined) was determined using a CFX96 Real Time System/C1000 thermal cycler (Biorad, Hercules, CA). The relative expression of miR-133a was determined and normalized to the small nuclear RNA RNU48 (NC_000010.11, assay ID: 001093) using the comparative cycle threshold (CT) method. Relative expression intensity values were calculated as 2−∆∆CT, in which ACT are CT values normalized to the referent control RNUSB.

Chromatin Immunoprecipitation

Chromatin immunoprecipitation (ChIP) assays were performed as previously described with some modifications. Fifty milligrams of heart tissue from each group were minced in cold PBS with phenylmethylsulfonyl fluoride, homogenized, and crosslinked with formaldehyde (10 minutes). Crosslinking activity was quenched with the addition of glycine for 5 minutes then suspended in SDS lysis buffer and incubated on ice for 30 minutes. The cell lysates were sonicated for 6 cycles (10 s on and 10 s off), and the cell debris was spun down. The supernatants were diluted with ChiP dilution buffer on a scale of 1:4 and used immediately for immunoprecipitation. Samples were precleared with 30 μL of ChiP grade protein A/G Plus Agarose beads (Thermo Scientific, 26161) for 30 minutes at 4°C. Ten microliters from each IgG sample were saved at 4°C for input controls. Antibody of interest was added according to the experimental design at a concentration of 5 μg per sample (Santa Cruz antibodies: HDAC1 [cat no. sc7872], HDAC2 [cat no. sc7899X], HDAC3 [cat no. sc11417], and SRF [cat no. 13029]). The proximal promoter for enhancer regions, miR-133a1 and miR-133a2, was polymerase chain reaction amplified from the immunoprecipitated and nonimmunoprecipitated (input control) chromatin using sense and antisense primers (Table in the Data Supplement). Primers that amplified regions distal to the proximal promoter were also used as negative polymerase chain reaction controls.

Collagen Content by Light Microscopy

Collagen content of the LV myocardium was determined by picrosirius red–stained images captured with polarized light was performed. Five fields chosen at random from each mouse were scanned with SigmaScan software. Fields with large blood vessels were excluded from the analysis. The areas examined were distributed throughout the myocardium from the subendocardium to the subepicardium and excluded the epicardial surface. Collagen volume fraction was calculated as the area stained by picrosirius red divided by the total area of interest.
Myocardial Fibroblasts Cultures

Primary cultures of myocardial fibroblasts were established from LV myocardial biopsies (2x2 mm) from patients (n=5) with end-stage nonischemic DCM presenting for heart transplantation and from normal individuals (n=4) using a previously described outgrowth techniques. The protocols used in this study were reviewed and approved by the Medical University of South Carolina Review Board for Human Research (HR no. 8076, approval date 12-6-2013) before the initiation of this study. Previous studies have shown that adult myocardial fibroblasts retain pathological phenotype acquired in vivo through early passages in cell cultures. Cells were treated with HDACi SAHA at 10 μmol/L concentration for 24 hours with a refresher 1 hour before collection.

HDAC2 was silenced in the DCM cells using human HDAC2-siRNA (Life Technology, CA; cat no. S6494 and control negative no. 1) along with lipofectamine RNAiMAX transfection reagent (cat no. 13778-150) and OptiMEM (cat no. 11058-021). Once confluent, DCM cells were transfected with HDAC2-siRNA and scramble-siRNA (5 μmol/L) for 3 hours. The media were then replaced with complete fibroblast growth media (PromoCell, cat no. C23020) for 2 days. HDACi SAHA (10 μmol/L) was added to the media 24 hours and 1 hour before lifting cells. Cells were lifted with TripLE, pelleted, and collected in sample buffer.

Statistical Analysis

GraphPad Prism 6 (GraphPad Software, Inc.) was used for statistical analysis. We performed Kolmogorov–Smirnov analysis to test for normality. In addition, when applicable, we used 1-way ANOVA (with Tukey post-test), 2-way ANOVA (with Bonferroni post-test), nonparametric Kruskal–Wallis test (with Dunn multiple comparison post-test) for comparison of groups with ≤5 data points and unpaired t tests when necessary to determine statistical significance. P values ≤0.05 were considered significant. Values for all measurements were expressed as the mean± SEM.

Results

Class I and Class II HDAC Activities During PO Hypertrophy

The HDAC activity was assessed in homogenized tissue from the LV of outbred CD1 male mice 72 hours, 1 week, and 2 weeks post TAC. When compared with sham mice, class I HDAC activity increased by 97% and class II HDAC activity increased by 79% at 1 week post TAC (Figure 1A and 1B). Both class I and II HDAC activities remained elevated at 2 weeks post TAC. At 72 hours post TAC, class I and II HDAC activities remained unchanged.

Effect of Class I and IIB HDAC Inhibition on PO-Induced Downregulation of miR-133a

The expression of miR-133a was not significantly changed 1 week post TAC, and SAHA treatment had no effect on miR-133a levels. miR-133a expression is significantly reduced 2 and 4 weeks post TAC (Figure 2A). SAHA prevented the...
PO-induced downregulation of miR-133a at 2 weeks post TAC. The effect of HDAC inhibition on miR-133a expression is still evident at 4 weeks post TAC but is not as profound as seen at 2 weeks. These findings suggest that class I and IIb HDACs regulate changes in miR-133a expression during PO-induced cardiac remodeling and fibrosis. miR-133a expression in control mice on vehicle and SAHA for 2 weeks was not significantly different, suggesting that class I and IIb HDAC inhibition does not affect endogenous miR-133a expression levels in the normal myocardium. Increased acetylation level of histone H3 and α-tubulin, which are known substrates for class I and IIb HDACs, demonstrates that SAHA treatment effectively inhibited HDAC activity (Figure 2B).

Effect of HDAC Inhibition on miR-133a’s Fibrotic Targets and Cardiac Fibrosis

Many profibrotic genes are silenced by miR-133a, including CTGF and COL1a1. Previous studies have shown that the pathological downregulation of miR-133a expression during PO cardiac remodeling allows CTGF and COL1a1 levels to increase, which contributes to collagen synthesis and fibrosis. To test whether the restoration of miR-133a expression caused by HDAC inhibition affects the expression of its profibrotic targets and the development of cardiac fibrosis, we measured CTGF protein levels and examined the extent of cardiac fibrosis with collagen volume fraction in the hearts of TAC mice after 1, 2, and 4 weeks of PO cardiac remodeling. CTGF protein abundance was not significantly changed from control mice after 1 week of PO, and HDAC inhibition had no significant effect on CTGF protein levels (Figure 3A). CTGF protein abundance was increased by 75% after 2 weeks of PO and 61% after 4 weeks of PO (Figure 3C). HDAC inhibition prevented the PO-dependent increase in CTGF protein levels compared with vehicle at 2 weeks post TAC. Although not significant, CTGF levels were still decreased compared with vehicle at 4 weeks post TAC. Importantly, CTGF protein abundance in the HDACi-treated mice after 2 and 4 weeks of PO was not significantly different from control+vehicle, suggesting that class I and IIb HDAC inhibition prevents the PO-dependent increase in CTGF abundance after TAC.

Figure 3. Impact of histone deacetylase (HDAC) inhibition onto miR-133a’s targets and fibrosis. A, Quantification of connective tissue growth factor (CTGF) protein abundance (normalized to β-actin) 1, 2, and 4 wk post transverse aortic constriction (TAC) surgery demonstrating the effect of HDAC inhibition on pressure overloaded hearts (#P≤0.05 vs 2wkTAC+vehicle determined by Kruskal–Wallis \[P=0.0194\] with Dunn multiple comparisons post-test). B, Collagen volume fraction (CVF) was quantified from picrosirius red (PSR)–stained left ventricular (LV) myocardium under polarized light microscopy. In the vehicle group (white bars), collagen volume fraction was significantly increased 2 and 4 wk post TAC (*P≤0.05 vs control+vehicle, determined by 2-way ANOVA with Tukey multiple comparisons test). CVF was not significantly increased with HDAC inhibitor (HDACi) treatment at 1wkTAC and 2wkTAC when compared to control + HDACi. CVF did significantly increase over control + HDACi in the 4wkTAC group (&P≤0.05 vs control+HDACi). HDAC inhibition with suberoylanilide hydroxamic acid significantly reduced collagen deposition at 2 and 4 wk post TAC (#P≤0.05 vs 2wkTAC+vehicle and 4wkTAC+vehicle determined by 2-way ANOVA with Bonferroni multiple comparisons post-test). C, Images of PSR-stained LV myocardium under polarized light: larger collagen fibers are bright yellow or orange/red, and the thinner ones are green.
Myocardial interstitial collagen content was quantified using collagen volume fraction from picrosirius red–stained tissue sections. Collagen volume fraction was not changed at 1 week but was significantly increased after both 2 and 4 weeks of PO when compared with control (Figure 3B and 3C). This represents an increase in interstitial insoluble fibrillar collagen content. Inhibition of HDAC activity significantly reduced collagen deposition after 2 and 4 weeks of PO when compared with vehicle alone.

**Effect of HDAC Inhibition on LV Structure and Function**

To determine whether the structure and the function of the myocardium were affected by HDAC inhibition, we examined several structural and functional parameters (Figure 4). The LA diameter (Figure 4A), LVW/body weight (Figure 4B), LVW/tibia length (Figure 4C), and passive stiffness of the LV myocardium (Figure 4F) were significantly increased 2 and 4 weeks post TAC in vehicle-treated mice when compared with baseline. HDAC inhibition significantly decreased the LA diameter (Figure 4A) and normalized the stiffness of myocardium 2 and 4 weeks post TAC when compared with baseline. However, HDAC inhibition had no effect on cardiac hypertrophy (LVW/body weight and LVW/tibia length) and systolic function of the heart (LV ejection fraction in Figure 4D and LV end-diastolic volume in Figure 4E).

**HDAC1 and HDAC2 Are Associated With miR-133a Enhancer Regions**

miR-133a belongs to the miR-133a/133b family and is associated with the miR-1 family; miR-1–2/133a-1 and miR-1–1/133a-2 originate from bicistronic transcripts on chromosomes 18 and 2, respectively. Enhancer regions have been identified regulating miR-133a-1 and miR-133a-2 expression. On chromosome 2, the intronic region between miR-1–1 and miR-133a-2 stem loops has an intragenic enhancer region regulated by SRF via CArG-binding domains, myocardin via E-box domains, and myocyte enhancer factor-2 (MEF2) via the MEF2 box. On chromosome 18, the intronic region between miR-133a-1 and miR-1–2 stem loops also has an intragenic enhancer region regulated by MEF2 and myocardin. Although miR-133a-1 and miR-1–2 and miR-133a-2 and miR-1–1 each share a common enhancer region, miR-133a can be differentially regulated from miR-1. Because there is increased evidence for the role of class I HDACs in cardiac pathologies, we performed ChIP assay for HDAC1, 2, and 3 on heart tissues from control and 2-week TAC mice. Figure 5B shows that HDAC1 and the transcription factor SRF are present on both enhancer regions in all experimental conditions and that HDAC3 is not associated with either enhancer region under any of the conditions. HDAC2 is not present on either enhancer region in the untreated controls but is recruited to the miR-133a-2 enhancer region in the 2-week PO ventricles. Acetylation of histones is positively correlated with increased transcription. Therefore, if miR-133a transcriptional regulation is mediated primarily by histone acetylation, we would predict the enhancer to have less acetylation in PO, where miR-133a expression drops. But unexpectedly, histone H3 acetylation is greater in the 2-week post-TAC tissue when compared with control (Figure I in the Data Supplement).

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**Figure 4. Structural and functional assessment. A, D, and E.** The left atrial (LA) diameter, left ventricular ejection fraction (LVEF), and LV end-diastolic volume (EDV) were obtained based on echocardiograph data. **B and C.** Body weight (BW), LV weight (LVW), and tibia length (TL) were measured 2 and 4 wk post transverse aortic constriction (TAC). **F.** Passive stiffness of the LV myocardium. *P<0.05 vs baseline. #P<0.05 vs vehicle within same group. HDACi indicates histone deacetylase inhibitor.
Effect of HDAC Inhibition on miR-133a Expression in Cardiac Fibroblasts From Patients With DCM

Given the profound effect of HDAC inhibition on fibrotic gene expression in treated murine hearts and the antifibrotic profile of miR-133a, we investigated the effect of HDAC inhibition on miR-133a expression in cardiac fibroblasts isolated from DCM and normal patients. Consistent with our murine data, miR-133a is significantly lower in DCM fibroblasts when compared with normal fibroblasts (Figure 6A). Importantly, HDAC inhibition significantly stimulated miR-133a expression in DCM fibroblasts (Figure 6C) but had no effect on miR-133a expression in the normal fibroblasts (Figure 6B).

Figure 5. miR-133a enhancer regions and regulation. A. Two bicistronic gene clusters each encoding miR-1–2/miR133a-1 and miR-1-1/miR133a-2 located on chromosome 18 and 2, respectively. cis-regulatory elements that direct muscle-specific expression of each locus are indicated by black boxes, and the transcription factors that act through these elements are shown with black arrows. The distance between the 2 bicistronic miRNAs is shown by green arrows, and the regions targeted by the chromatin immunoprecipitation (ChIP) primers with pink arrows. Asterisk denotes newly identified serum response factor (SRF)-binding element. HDAC2 is present on miR-133a enhancer regions in association with the transcription factor SRF.

B. Murine ventricular tissue samples were analyzed by ChIP to determine whether endogenous class I HDACs (HDAC1, HDAC2, and HDAC3), as well as SRF, are associated with miR-133a enhancer regions (antibodies for HDACs and SRF from Santa Cruz). A negative control, using rabbit IgG as the precipitating antibody, was run to demonstrate the specificity of the ChIP assay. Input DNA from the samples before immunoprecipitation is also included as a positive control and loading control. HDACi indicates histone deacetylase inhibitor; MEF2, myocyte enhancer factor-2; MyoD, myocardin; TAC, transverse aortic constriction; and veh, vehicle.

Figure 6. Levels of miR-133a expression in cardiac fibroblasts from 5 patients with dilated cardiomyopathy (DCM) and 4 normal individuals. Cells were treated with histone deacetylase inhibitor (HDACi) suberoylanilide hydroxamic acid (SAHA) at 10 μmol/L for 24 h. Data analyzed by unpaired t test. A, Comparison between miR-133a expression level in normal and DCM cell lines (*P≤0.05 vs normal). B, Fold change in miR-133a expression between normal cells treated with HDACi SAHA and untreated (*P≤0.05 vs normal). C, Fold change in miR-133a expression between DCM cells treated with HDACi SAHA and untreated (*P≤0.05 vs DCM). CT indicates cycle threshold.
Effect of HDAC2 Knockdown on miR-133a Expression in DCM Fibroblasts

Because HDAC2 is recruited to the miR-133a-2 enhancer region in the 2-week PO ventricles, we examined the effect of HDAC2 silencing using HDAC2-siRNA in the DCM cells. HDAC2-siRNA knocked down HDAC2 expression in the DCM cells compared with control siRNA (Figure 7A and 7B). HDAC2 knockdown did not result in the increase of miR-133a expression similar to that observed with HDAC inhibition (control+HDACi). But interestingly, HDAC2 knockdown prevented SAHA-stimulated miR-133a expression, indicating that although HDAC2 may not be required for miR-133a downregulation in DCM cells, its activity plays a role in the partial restoration of expression seen with HDACi treatment.

Discussion

In this study, we examined the epigenetic regulation of miR-133a in the heart during PO-induced cardiac remodeling and fibrosis. We demonstrated that (1) class I and II HDAC activities are increased during PO remodeling. (2) Treatment with the class I and IIb HDACi SAHA significantly attenuated the PO-induced decrease in miR-133a expression (after 2 and 4 weeks of PO). (3) HDAC inhibition significantly diminished PO-induced upregulation of CTGF protein abundance and collagen deposition, suggesting that the effect of HDAC inhibition on miR-133a expression is reflected on its downstream fibrotic targets. (4) Importantly, the effect of HDAC inhibition on miR-133a expression is also correlated with a reduction in LA diameter and passive stiffness in TAC mice, suggesting improved diastolic function. (5) HDAC1 and HDAC2 are associated with miR-133a-1 and miR-133a-2 enhancer regions. (6) Consistent with what we see in the murine model, HDAC inhibition increased the depressed expression of miR-133a found in human fibroblast isolated from DCM patients. These data support the hypothesis that acetylation regulates the expression of miR-133a during PO cardiac fibrosis, and it is the first report of the direct association of HDAC1 and HDAC2 with miR-133a promoter by ChiP.

Importance and Crosstalk of miRNAs and HDACs

miRNAs have been identified as central players in the pathogenesis of various cardiovascular diseases.2 miRNAs can regulate the expression of many target genes; therefore, the change in expression in 1 miRNA can affect the expression of entire gene networks contributing to complex pathological phenotypes. Recent reports have demonstrated that the expression pattern of many miRNAs shifts at the onset of cardiac diseases. Based on murine hypertrophic models, van Rooij et al36 identified 28 differentially expressed miRNAs and many of them were also pathologically regulated in human failing hearts. Of special interest, miR-133a is downregulated in the LV and atria in multiple murine models of PO-induced cardiac remodeling,4,5 is highly expressed in cardiac and skeletal muscle, and is one of the most highly expressed miRNA in cardiomyocytes (although it is also detectable in cardiac fibroblasts). Its expression is decreased when compared with normal in dilated atria from patients with mitral stenosis and in myectomies of ventricles from young patients undergoing curative surgery for hypertrophic cardiomyopathy.

Although the biogenesis of miRNAs is well understood, little is known about the regulation of miRNA expression. Interestingly, the miRNA expression profile from heart failure patients is highly similar to the profile of fetally expressed miRNAs,37 in agreement with the reactivation of the fetal gene program during PO-induced cardiac remodeling.38 Interestingly, HDAC inhibition suppresses the reactivation of
several fetal genes in murine models of cardiac hypertrophy. Increasing evidence shows that many miRNAs are regulated by epigenetic mechanisms. The majority of these are regulated by DNA methylation but histone modification can induce or suppress miRNA expression. Aberrant histone acetylation has been shown to affect miRNA expression in a variety of cancers, including colorectal cancer. HDAC inhibition alters the expression of miR-15a, miR-16, and miR-29 samples. The expression of miR-15a/16 is epigenetically silenced in chronic lymphocytic leukemia via the recruitment of HDAC3 to the promoter by MYC and nuclear factor-κB recruitment of HDAC4 silences miR-23 expression in human leukemic Jurkat cells. Expression of antiangiogenic miR-17–92 cluster in endothelial cells can be modulated by HDAC9. Not surprisingly, this works both ways. HDACs and histone acetyltransferases are among the targets of miRNAs. HDAC4 is targeted by miR-1-1 in human hepatocellular carcinoma cells, and HDAC1 is regulated by miR-449a in prostate cancer cells.

Although the interplay between epigenetic regulators and miRNAs is beginning to be worked out in cancer, little is known about this complex interaction in the heart. One recent article examining the antagonisms between IP3R1 and miR-133a demonstrated that the treatment of rat neonatal cardiomyocytes with the pan-HDACi trichostatin A results in the upregulation of miR-133a. Intrigued by this observation and by studies showing that both restoring miR-133a levels and treatment with HDAC inhibitors suppress pathological cardiac remodeling and myocardial fibrosis, we examined the regulation of miR-133a by HDACs. Here, we found that miR-133a is significantly downregulated 2 and 4 weeks post TAC, in agreement with previous studies, and made the novel discovery that HDAC inhibition prevented the PO-induced downregulation of miR-133a expression. Interestingly, SAHA treatment in animals that did not receive TAC did not result in any increase in the basal level of miR-133a. Drawnel et al found that trichostatin A treatment induced miR-133a expression by 80%. This discrepancy may be because of the difference of an in vitro response in rat neonatal ventricular myocytes versus our study in an in vivo mouse heart. Our results show that HDAC inhibition had an impact only on the pathological expression of miR-133a in the TAC mice and restored basal levels. Importantly, the restoration of miR-133a levels prevented the increase in CTGF protein content and collagen volume fraction. It is noteworthy that the increase in HDAC activity seen in this study at 1 week post TAC did not result in the downregulation of miR-133a yet inhibition of HDAC activity at 2 and 4 weeks post TAC results in restoration of miR-133a to control levels. These data suggest that the other factors are also required. Therefore, HDAC activity is required but not sufficient for repression of miR-133a expression. Identification of additional corepressor(s) and determining the role of class I HDACs in the regulation of miR-133a need further evaluation. Previous studies have demonstrated that class I and IIb HDACs can mediate the expression of many other genes, which contribute to cardiac pathologies. Therefore, SAHA treatment would be expected to affect the expression of many other genes in addition to miR-133a that contribute in one way or another to PO cardiac remodeling and fibrosis. Identifying these additional targets, which contribute to fibrosis, awaits further evaluation.

**HDAC1 and HDAC2 Are Present on miR-133a Enhancer Regions**

HDAC1 and HDAC2 with the miR-133a-1 and miR-133a-2 enhancer regions. Although HDAC1’s association with the miR-133a enhancer regions is similar in the control and pressure overloaded heart, HDAC2 is recruited to the miR-133a-2 enhancer region in the pressure overloaded ventricle. Overexpression of HDAC2 induces hypertrophy. HDAC2 null mice generated by cardiac-conditional knockout remain susceptible to isoproterenol and PO-induced cardiac hypertrophy, whereas HDAC2 null mice generated by lacZ insertion resist hypertrophic remodeling induced by PO stress. Kee et al found that induction of HSP70 in response to exogenous hypertrophic stimuli is a key regulatory mechanism to activate HDAC2 in the early phase of cardiac hypertrophy and concluded that HDAC2 is required for hypertrophic responses in the heart.

To better understand the role of HDAC2 in miR-133a regulation in an in vitro setting, we used siRNA knockdown in the human DCM cells. HDAC2 knockdown did not result in miR-133a upregulation. These data were not completely unexpected. HDAC1 and HDAC2 are recruited by transcription factors either as homodimers or heterodimers or as part of multifactor repressor complexes. Although HDAC1 and HDAC2 have some distinctive deacetylase targets, the common theme of many HDAC1/HDAC2 knockdown and knock-out studies is redundancy and compensation. In addition, HDAC knockdown may exert a completely different effect than inhibiting its catalytic activity. This may be the case for miR-133a regulation, HDAC1 may be able to compensate for the loss of HDAC2, but in this case inhibition of HDAC activity did not result in the stimulation of miR-133a expression.

We had expected to find a class I HDAC present after TAC, and indeed there is more HDAC2 detected on miR-133a-2 enhancer with TAC than in control hearts. But we anticipated that treatment with SAHA would reduce the presence of HDACs on the enhancer chromatin. Our finding that HDAC inhibition enhanced the stabilization of HDAC2 with the miR-133a enhancer chromatin in control and TAC mice was unexpected. Importantly, even though more HDAC2 is present on the miR-133a enhancer, SAHA treatment inhibits the deacetylase activity. This is not the first observation showing that in addition to inhibiting enzymatic activity, HDAC inhibitors can also stabilize HDAC repressor complexes. Nebbioso et al demonstrated that treatment with the HDACi MC1568 stabilizes the interaction of MEF2 with the HDAC4-NCOR-HDAC3 repressor complex. Both their findings and ours suggest that HDAC inhibitors can affect molecular mechanisms beyond inhibiting catalytic activity. Because SAHA treatment with TAC prevents miR-133a downregulation, we assume that stabilization of the corepressor complex does not hinder the recruitment of coactivators, such as histone acetyltransferases, to the miR-133a enhancer.

Acetylation of lysine residues in the N-termini of histones correlates with increased transcription, and heterochromatic regions are generally hypoacetylated. Our data are contrary...
to simple histone deacetylation as a major factor accounting for the repression of miR-133a expression in PO hypertrophy. It is possible that HDAC2 regulates miR-133a expression by the deacetylation of or interaction with transcription factors, coactivators, or corepressors on the miR-133a enhancer. SRF and MEF2 have been shown to play a role in cardiac expression of miR-133a-1 and miR-133a-2.8,9,11 We identified the presence of an additional SRF-binding (CArG) element between miR-1 and miR-133a-1 (Figure S; Figure II in the Data Supplement). Both SRF and MEF2 have been shown to undergo acetylation that may affect their activity or interaction with positive or negative cofactors.56,57 Hsp70-Hsp90 Organizing Protein is one of SRF’s cofactors that inhibits SRF transcriptional activity by recruiting a corepressor complex that includes HDAC2.58 Clearly much work remains to identify the exact mechanism by which HDACs regulate miR-133a expression in PO cardiac remodeling and fibrosis.

In conclusion, this is the first study focused on the complex interaction between miRNAs and epigenetic regulators in heart disease. Certainly, there is a need to better understand the mechanisms of epigenetic regulation of miRNAs. The potential of HDAC inhibitors and other epigenetic drugs to induce or repress the deregulated miRNAs may provide innovative therapies to reset the epigenome in heart disease.

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None.

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**CLINICAL PERSPECTIVE**

The hearts of patients with hypertension or aortic stenosis are subject to pressure overload stress that leads to concentric left ventricular hypertrophic remodeling. Many of the patients with hypertrophic remodeling will progress to heart failure with preserved ejection fraction. Heart failure with preserved ejection fraction is considered predominantly a syndrome, which pathophysiologically involves diastolic abnormalities (relaxation and/or stiffness) with poor prognosis and the existing treatments remain unsuccessful. MicroRNAs play key roles in hearts responding to hypertrophic stress. miR-133a in particular is critical in negatively regulating genes, which contribute to fibrosis and directly affect diastolic heart function. miR-133a is downregulated in cardiac hypertrophy. This study demonstrates that miR-133a expression in pressure overload hypertrophy is regulated by histone deacetylases, which are key factors in the epigenetic machinery. Histone deacetylase inhibition using vorinostat (FDA approved for cutaneous T cell lymphoma) results in the preservation of miR-133a expression levels in murine pressure overloaded ventricles. The effect of histone deacetylase inhibition on miR-133a expression is correlated with decreased fibrosis and an improvement in diastolic function in the murine model. Regulation of microRNAs by inducing epigenetic changes may lead to innovative therapeutic strategies in the treatment of hypertrophic heart diseases.
HDACs Regulate miR-133a Expression in Pressure Overload–Induced Cardiac Fibrosis

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**SUPPLEMENTAL MATERIAL**

**Supplemental Table:** ChIP primers designed for miR-133a enhancer regions.

<table>
<thead>
<tr>
<th>Region</th>
<th>Forward primer (5’ to 3’)</th>
<th>Reverse primer (5’ to 3’)</th>
<th>Size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>miR-133a1</td>
<td>TGAAGATAGCACTTGCTGGT</td>
<td>GGCACTGCTGACACTGCTA</td>
<td>226</td>
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<tr>
<td>miR-133a2</td>
<td>GGGTCTCTTTCCCTGAGGTC</td>
<td>ATGACAGGTTCCAGGACAGC</td>
<td>187</td>
</tr>
</tbody>
</table>

**Supplemental Figure 1:** Analysis of miR-133a2 enhancer region by ChIP to determine association with HDAC2, SRF, acetylated-histone H3 and total histone H3. Murine ventricular tissue samples were analyzed by chromatin immunoprecipitation (ChIP) to determine whether endogenous HDAC2 (sc7899X), SRF (sc13029), histone H3 (Millipore 06-755) and acetyl-histone H3 (AceH3, Millipore 06-599) are associated with miR-133a2 enhancer region. A negative control, using rabbit IgG as the precipitating antibody, was run to demonstrate the specificity of the ChIP assay. Input DNA from the samples before immunoprecipitation (IP) is also included as a positive control and loading control. Abbreviations: “veh” for vehicle, and “HDACi” for HDAC inhibitor.
**Supplemental Figure 2:** Enhancer region miR-133a1. SRF binds to a newly identified CArG element on miR-133a1 enhancer region (red box). Previously identified MEF2 and Ebox elements are shown in green and yellow respectively.