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Adrenergic Repression of the Epigenetic Reader MeCP2 Facilitates Cardiac Adaptation in Chronic Heart Failure

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***Rationale*: In chronic heart failure, increased adrenergic activation contributes to structural remodeling and altered gene expression. Although adrenergic signaling alters histone modifications, it is unknown, whether it also affects other epigenetic processes, including DNA methylation and its recognition.**

***Objective*: The aim of this study was to identify the mechanism of regulation of the methyl-CpG–binding protein 2 (MeCP2) and its functional significance during cardiac pressure overload and unloading.**

***Methods and Results*: MeCP2 was identified as a reversibly repressed gene in mouse hearts after transverse aortic constriction and was normalized after removal of the constriction. Similarly, MeCP2 repression in human failing hearts resolved after unloading by a left ventricular assist device. The cluster miR-212/132 was upregulated after transverse aortic constriction or on activation of α_1- and β_1-adrenoceptors and miR-212/132 led to repression of MeCP2. Prevention of MeCP2 repression by a cardiomyocyte-specific, doxycycline-regulatable transgenic mouse model aggravated cardiac hypertrophy, fibrosis, and contractile dysfunction after transverse aortic constriction. Ablation of MeCP2 in cardiomyocytes facilitated recovery of failing hearts after reversible transverse aortic constriction. Genome-wide expression analysis, chromatin immunoprecipitation experiments, and DNA methylation analysis identified mitochondrial genes and their transcriptional regulators as MeCP2 target genes. Coincident with its repression, MeCP2 was removed from its target genes, whereas DNA methylation of MeCP2 target genes remained stable during pressure overload.**

***Conclusions*: These data connect adrenergic activation with a microRNA—MeCP2 epigenetic pathway that is important for cardiac adaptation during the development and recovery from heart failure. (*Circ Res*. 2015;117: 622-633. DOI: 10.1161/CIRCRESAHA.115.306721.)**

Key Words: DNA methylation ■ epigenomics ■ heart failure ■ MeCP2 protein ■ microRNAs ■ receptors, adrenergic

Cardiovascular diseases represent a leading cause of morbidity and mortality. Most drugs that are used to treat patients with chronic heart failure primarily target neuroendocrine mechanisms and they only secondarily affect structural remodeling of the failing heart.[1] Thus, new molecular targets and treatment strategies are urgently needed to improve the prognosis of chronic heart failure.[2]

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Nonstandard Abbreviations and Acronyms	
LVAD	left ventricular assist device
MeCP2	methyl-CpG–binding protein 2
rTAC	reversible transverse aortic constriction

Interestingly, even terminally failing hearts have the capacity to recover after mechanical unloading.[3] For patients with terminal heart failure, a left ventricular assist device (LVAD) may represent an important therapeutic option until a donor heart becomes available for transplantation. In some patients, mechanical unloading of the failing heart by an LVAD initiates a reverse remodeling process, which may ultimately lead to partial or even complete recovery of the function of the failing heart.[4,5] Several processes have been associated with reverse remodeling of the failing heart, including regression of cardiac hypertrophy and reduction of neuroendocrine activation.[6] However, the precise molecular mechanisms, which are responsible for functional improvement of failing hearts after mechanical unloading are unknown at present.

Several signaling mechanisms beyond cell surface receptors, which are activated by adrenergic signaling and other neuroendocrine compensatory systems, have been implied in the pathogenesis of chronic heart failure. These include a multitude of intracellular kinase signaling pathways and nuclear transcription factors.[7] In addition, several epigenetic processes, including microRNAs,[8] chromatin modifying enzymes, and proteins,[9–11] as well as DNA methylation[12–14] have been implicated in the development of heart failure.

Thus, we have searched for differentially expressed genes in human chronic heart failure and in experimental heart failure to identify epigenetic modulators, which are associated with the development and improvement of heart failure after mechanical unloading. We found methyl-CpG–binding protein 2 (MeCP2) to be repressed in chronic heart failure. MeCP2 is a member of the methylated DNA–binding domain family of proteins, which specifically recognize and bind to methylated DNA sequences.[15,16] MeCP2 may repress transcription, but it can also activate gene expression by recruiting coactivators.[17] This study demonstrates that MeCP2 repression by an adrenergic pathway involving miR-212/132 limits pathological hypertrophy, mitochondrial and contractile dysfunction, and facilitates functional recovery after mechanical unloading of failing hearts.

Methods

Animal Procedures

All animal procedures were approved by the responsible animal care committee (Regierungspräsidium Freiburg, Germany, approved protocol number G12/30) and they conformed to the Guide for the Care and Use of Laboratory Animals (8th edition, 2011).

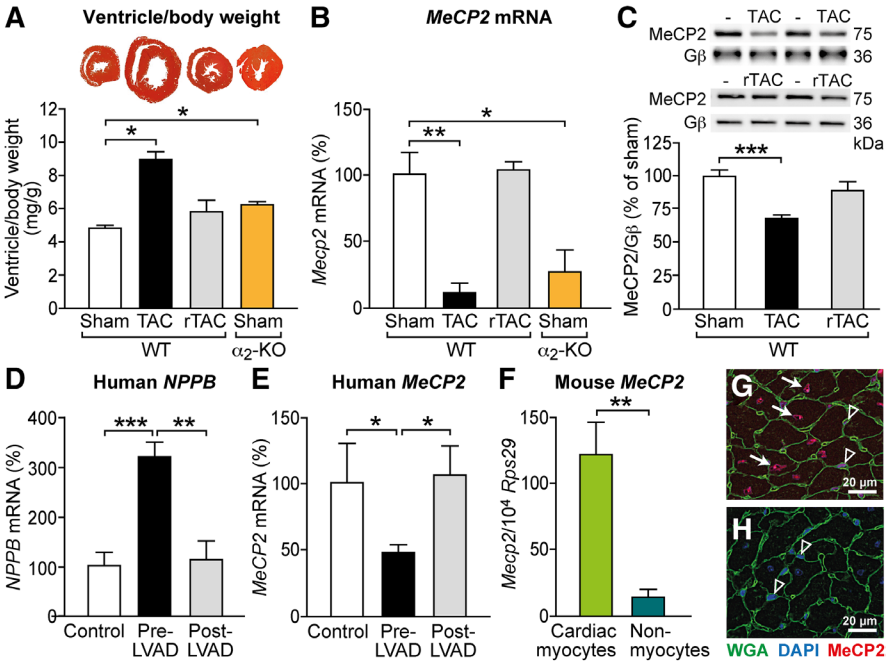

Figure 1. Expression of methyl-CpG–binding protein 2 (MeCP2) in mouse and human heart failure. A–C, Expression of MeCP2 in male mouse hearts after left ventricular pressure overload as induced by transverse aortic constriction (TAC) for 4 weeks followed by removal of the aortic stenosis (rTAC) for 4 weeks. α_2-Knockout (KO) mice with targeted ablation of α_2-adrenoceptor expression (genotype *Adra2a*$^{-/-}$*2b*$^{-/-}$*2c*$^{-/-}$; n=6–9 per group). **A**, Ventricle weight/body weight ratios. Expression of *MeCP2* mRNA (**B**; n=6–9) and protein (**C**; n=5–8). **D** and **E**, Brain natriuretic peptide (*NPPB*) and *MeCP2* mRNA levels in biopsies from nonfailing or failing human hearts before and after left ventricular assist device (LVAD) implantation (n=5–7 per group). **F**, *MeCP2* mRNA levels in adult mouse ventricular cardiomyocytes when compared with nonmyocytes (n=5, 8-week-old male C57BL/6 N mice). **G** and **H**, Immunohistochemical staining showed localization of MeCP2 (red) primarily in cardiomyocyte nuclei (arrows) but not in nonmyocyte nuclei (triangles). Staining with anti-MeCP2 (**G**) or control IgG (**H**), wheat germ agglutinin (WGA) to identify cell membranes, 4′,6-diamidino-2-phenylindole (DAPI) for nuclei staining in 8-week-old male C57BL/6 N mice. Mean±SEM; **P*<0.05; ***P*<0.01; ****P*<0.001. WT indicates wild-type.

Mouse Models

Constitutive expression of MeCP2 in cardiomyocytes was achieved by generating transgenic mice expressing MeCP2 under control of the α-myosin heavy chain gene promoter. For cardiomyocyte-specific and doxycycline-regulatable expression of MeCP2, a binary transgenic line was generated, termed MeCP2-TG. Cardiomyocyte-specific ablation of MeCP2 was achieved by crossing a floxed *MeCP2* allele (strain B6.129P2-MeCP2^{tm1Bird}/J, Jackson Laboratory)[18] with MLC2a-Cre mice.[19] *MiR-212/132* transgenic mice contained a 486-bp genomic region of the *miR-212/132* locus under control of the α-myosin heavy chain gene promoter.[20]

Human Cardiac Biopsies

LV biopsies were obtained during surgery for LVAD implantation or explantation during heart transplantation. Control biopsies were obtained during aortic surgery. Studies were approved by the Ethics Committee of the University of Freiburg with informed consent of the patients (protocol number 10006/11).

Isolation of Cardiac Myocytes

Cardiomyocytes were isolated from ventricles of neonatal rats (postnatal days P0-P3) by trypsin incubation. Adult mouse cardiomyocytes were obtained by Langendorff perfusion with 0.25 mg/mL Liberase enzyme solution (DH Research Grade, Roche).

Transverse Aortic Constriction and Reversible Transverse Aortic Constriction

Mice (aged 12 weeks) were anesthetized with isoflurane 2% vol/vol in oxygen. The transverse aorta was constricted using a 27G cannula as place holder. For ventricular unloading, the constriction was surgically removed at the indicated times after transverse aortic constriction (TAC).

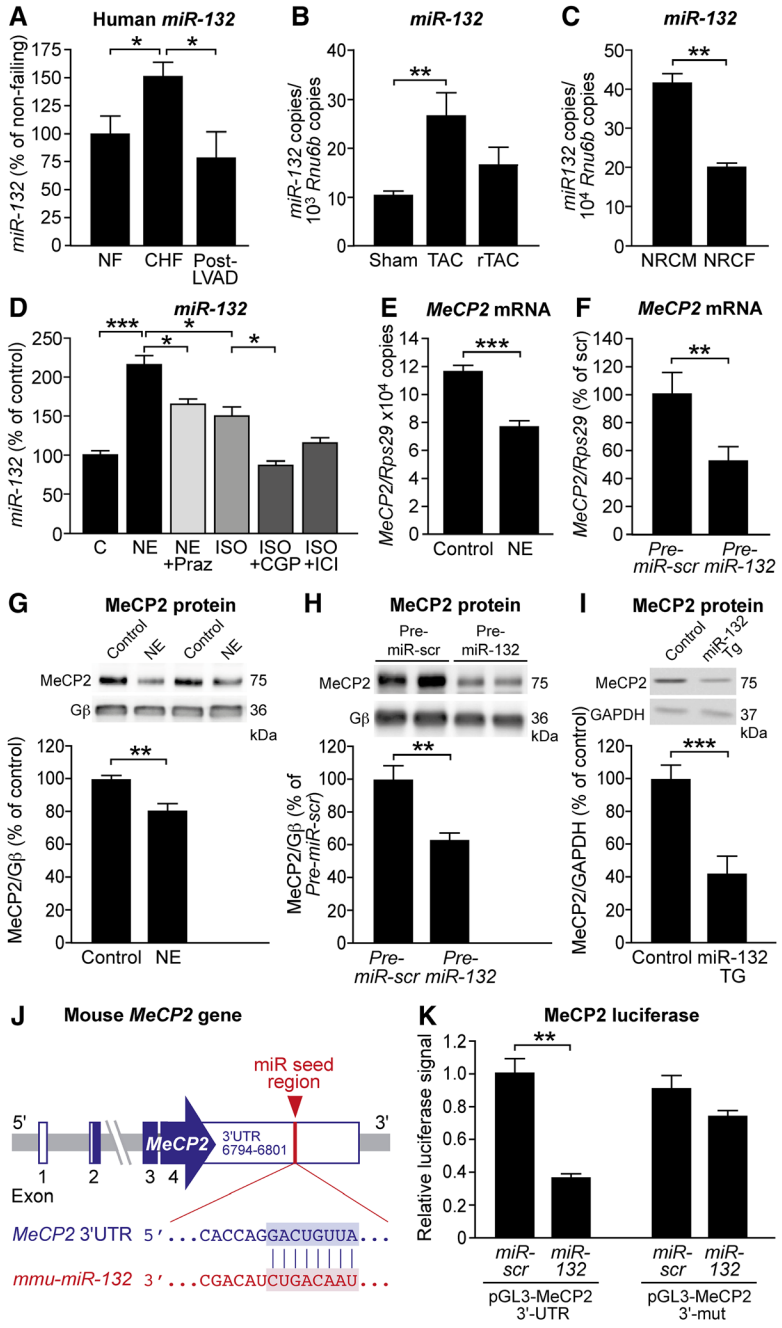

Figure 2. *MiR-132* is upregulated by adrenergic activation and targets *MeCP2* for repression. **A** and **B**, Upregulation of *miR-132* in biopsies from human failing hearts (n=5–7). **B**, *MiR-132* was increased in hypertrophic mouse hearts after transverse aortic constriction (TAC) but not after reversible TAC (rTAC; n=5 per group, C57BL6/N). **C**, *MiR-132* was found at higher levels in neonatal rat cardiomyocytes (NRCMs) when compared with neonatal rat nonmyocytes (NRCFs, n=5 per group from 2 independent isolations). **D**, *MiR-132* expression in neonatal rat cardiomyocytes after stimulation with norepinephrine (40 μmol/L, 72 hours) or isoproterenol (10 μmol/L, 72 hours) and inhibition of α_1-adrenoceptors (prazosin, 1 μmol/L), β_1-adrenoceptors (CGP20712A, 500 nmol/L), or β_2-adrenoceptors (ICI118551, 100 nmol/L; n=3–4 experiments). **E**, Stimulation of neonatal rat cardiomyocytes with norepinephrine (40 μmol/L, 72 hours) decreased *MeCP2* mRNA (n=6 per group from 2 independent isolations). **F**, Transfection of neonatal rat cardiac myocytes withr pre–*miR-132* but not with scrambled miR (pre–*miR-scr*) reduced *MeCP2* levels (n=6 per group). **G–I**, Methyl-CpG–binding protein 2 (MeCP2) protein levels in cardiac myocytes (n=4–6 per group) and mouse hearts from wild-type and *miR-132* overexpressing transgenic hearts (n=8–10 per group; **I**). **J** and **K**, The mouse *MeCP2* gene displaying the 3′ part with the seed region for *miR-132*. Luciferase signal (**K**) of vectors containing the wild-type or mutated 3′ *MeCP2* region (n=6–8 per group). Mean±SEM; **P*<0.05, ***P*<0.01, ****P*<0.001. CHF indicates chronic heart failure pre-LVAD; NF, nonfailing hearts; post-LVAD, after ventricular unloading by a left ventricular assist device; and TG mice, transgenic mice.

Respiratory Measurements
Mitochondria were isolated from whole left ventricular mouse tissue.[21] Ventricular myofibrils were prepared as described.[22]

Luciferase Assay
The *MeCP2* 3′-untranslated region region was amplified by polymerase chain reaction and cloned into the pGL3-control vector (Promega). For the luciferase assay, neonatal rat cardiac myocytes were electroporated with pGL3-MeCP2 plasmids and pre-miRs were transfected using the Amaxa Rat Cardiomyocyte Nucleofector kit.

Gene Expression Analysis
Total RNA was isolated from cardiac tissue using the RNeasy fibrous tissue kit (Qiagen, Hilden, Germany). For quantitative polymerase chain reaction, 1 µg of total RNA was transcribed (Qiagen, Reverse Transcription Kit). Gene expression was analyzed with Illumina Mouse WG-6 v2.0 Expression BeadChips.

Isolation of Cardiomyocyte Nuclei
For purification of cardiomyocyte nuclei, magnetic-assisted sorting with pericentriolar material 1 (PCM1) antibody was used.[14,23]

Chromatin Immunoprecipitation Followed by Next Generation Sequencing
Chromatin was fixed for 2 minutes in 1% paraformaldehyde. Nuclei were sheared in lysis buffer (50 mmol/L Tris-HCl; pH, 8.0, 10 mmol/L EDTA; 1% SDS) for 30 minutes in a Bioruptor (Diagenode, 30 s on/off) to obtain 100 to 400 bp DNA fragments and precipitated with anti-MeCP2 antibody and protein A Dynabeads (Life technologies). Libraries were sequenced on a HiSeq 2000 (50 bp, Illumina). MeCP2 chromatin immunoprecipitation followed by next generation sequencing reads were mapped to the mouse genome (mm9 assembly). DNA CpG-methylation density was calculated from published cardiomyocyte-specific methylomes.[14]

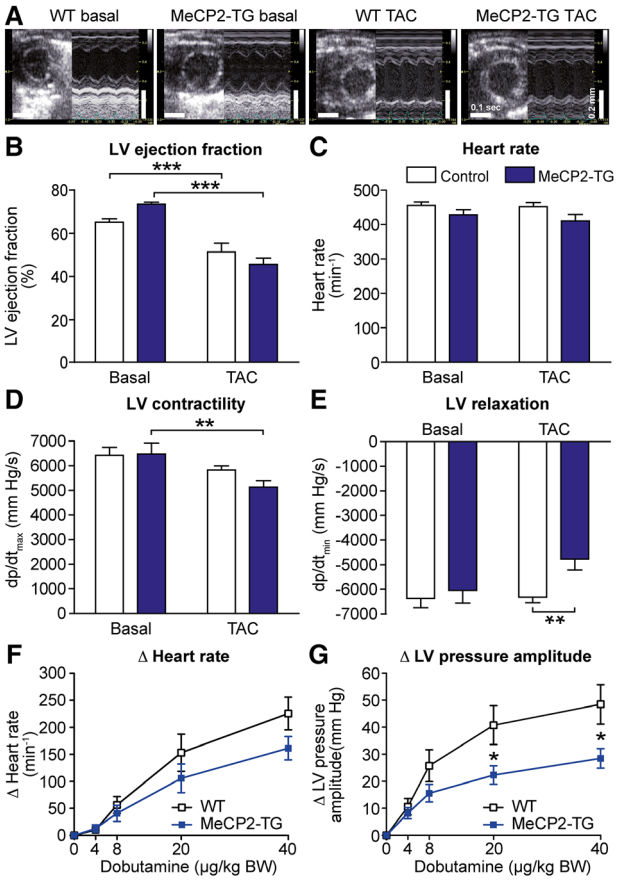

Figure 3. Cardiac function in methyl-CpG–binding protein 2-transgenic (MeCP2-TG) mice. A–E, Echocardiography (**A–C**; n=4–14 per group) and left ventricular catheterization (**D–E**; n=5–9 per group) of mouse hearts before and after 4 weeks of cardiac pressure overload (transverse aortic constriction [TAC]). **F** and **G**, Heart rate and left ventricle (LV) pressure response to intravenous cumulative infusion of dobutamine (n=8–10 per group, 10- to 12-week-old male MeCP2-TG and littermate control mice). Mean±SEM; **P*<0.05, ***P*<0.01, ****P*<0.001. WT indicates wild-type.

Results

MeCP2 Is Repressed in Mouse and Human Heart Failure
A mouse model with left ventricular pressure overload because of TAC was used to identify differentially expressed genes after TAC, which returned to baseline on ventricular unloading (Figure 1A). By gene array expression screening, we found that MeCP2 mRNA and protein were significantly downregulated in response to TAC (Figure 1B and 1C) and were normalized after removal of the aortic stenosis (reversible TAC [rTAC]; Figure 1B and 1C). *MeCP2* repression was also observed in a mouse model with increased catecholamine release because of genetic ablation of all 3 α_2-adrenoceptor subtypes (genotype *Adra2a*$^{-/-}$*2b*$^{-/-}$*2c*$^{-/-}$; Figure 1A and 1B).[24,25] These findings were validated in biopsies from human failing hearts before and after cardiac unloading because of implantation of an LVAD (Figure 1D and 1E). *MeCP2* mRNA was significantly repressed in failing versus nonfailing human hearts and expression was normalized after unloading by an LVAD (Figure 1E). Overall, 30 genes were identified which were regulated in the same manner as MeCP2 in failing versus unloaded human hearts, but not all these genes were expressed in cardiomyocytes. Importantly, MeCP2 was found to be primarily expressed in cardiomyocytes versus nonmyocytes (Figure 1F–1H).

MiR-212/132 Is Upregulated by Adrenergic Activation and Targets *MeCP2*
As increased circulating norepinephrine levels in α_2-knockout mice (Figure 1B) led to downregulation of *MeCP2* in vivo, we searched for microRNAs that are induced by adrenergic activation and which repress *MeCP2*. We identified the cluster *miR-212/132* as a candidate for *MeCP2* repression (Figure 2). *MiR-132* expression was elevated in human and mouse failing hearts and expression was normalized in response to mechanical unloading (Figure 2A and 2B). *MiR-132* was significantly higher expressed in cardiomyocytes when compared with cardiac fibroblasts (Figure 2C). In vitro experiments in isolated cardiomyocytes showed that adrenoceptor stimulation with norepinephrine led to induction of *miR-132*, which was paralleled by repression of *MeCP2* mRNA (Figure 2D and 2E). The effect of norepinephrine was partially blocked by the α_1-adrenoceptor antagonist prazosin (Figure 2D). The β-adrenoceptor agonist isoproterenol also led to *miR-132* induction and this effect was completely prevented by the β_1-specific antagonist CGP20712A (Figure 2D). Thus, adrenergic induction of *miR-132* involved both α_1- and β_1-adrenoceptors. Consistent with this finding, *miR-132* was significantly induced by activation of Gq or Gs proteins by *Pasteurella multocida* toxin[26] or by Cholera toxin,[27] respectively (Online Figure IA). In vivo, adrenergic stimulation by

isoproterenol/phenylephrine for 7 days also induced *miR-212/132* (Online Figure IIA and IIB). Incubation of cardiomyocytes with pre–*miR-132* but not with scrambled pre-miR reduced *MeCP2* mRNA levels (Figure 2F). These results were reproduced on the protein level, with norepinephrine and *miR-132* repressing MeCP2 in cardiomyocytes (Figure 2G and 2H) and in transgenic mice with cardiac myocyte-specific expression of *miR-132* (Figure 2I).[20] Activity of luciferase fused to the wild-type *MeCP2* gene 3′-untranslated region containing the miR seed region but not luciferase fused to mutated 3′-untranslated region was significantly repressed by pre–*miR-132* (Figure 2J and 2K), suggesting a direct effect of *miR-132* on *MeCP2* expression in vitro and in vivo. Similar results were obtained for *miR-212* (Online Figure IB–IF). Because *miR-212/132* were previously shown to repress FOXO3 in response to adrenergic or angiotensin II stimulation,[20] expression of *miR-212/132*, MeCP2, and FOXO3 were validated in vivo. Although isoproterenol/phenylephrine repressed MeCP2 but not FOXO3 (Online Figure IIC and IID), angiotensin II was able to significantly decrease both FOXO3 and MeCP2 levels in the heart (Online Figure III).

Mouse Models With Cardiomyocyte-Specific Expression or Ablation of MeCP2

To search for a potential causal role of MeCP2 in the development of cardiac hypertrophy and failure, 3 mouse models with expression or ablation of MeCP2 in cardiomyocytes were generated (Online Figures IV and V). Overexpression of MeCP2

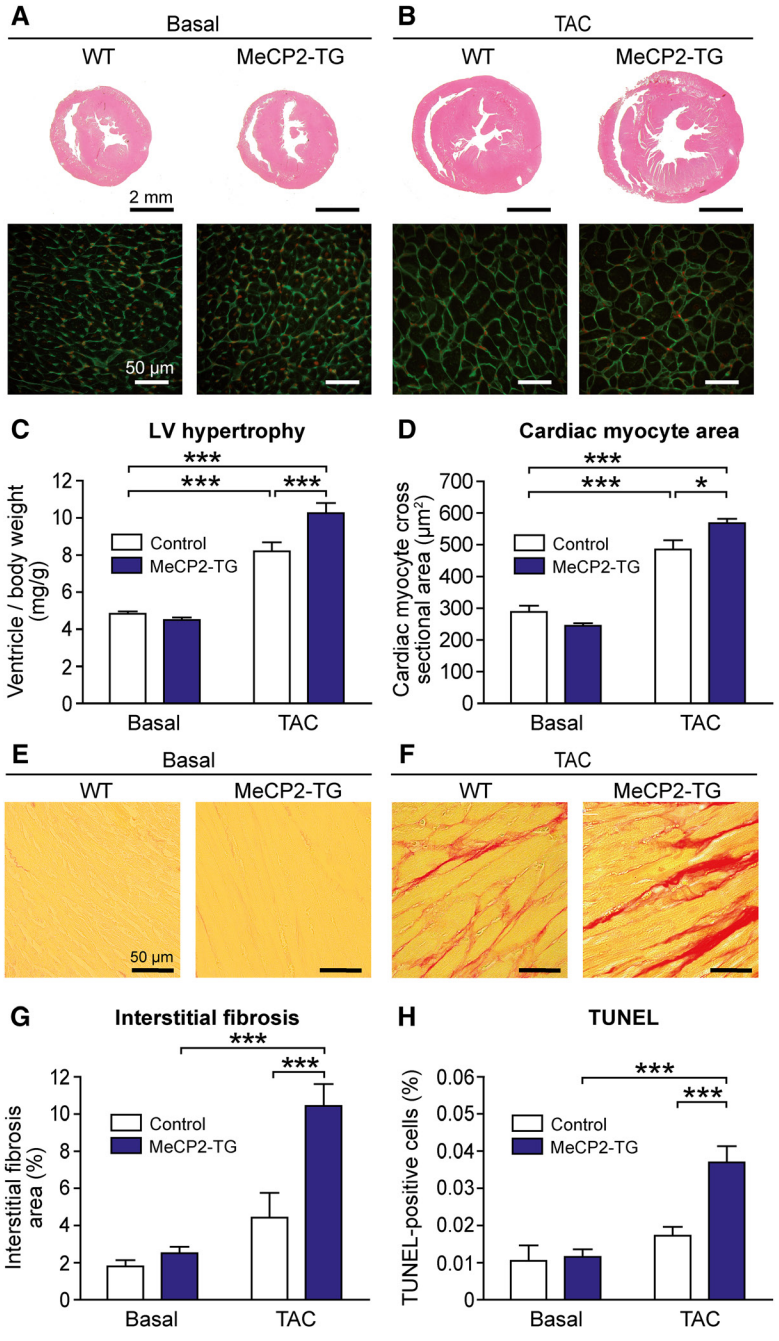

Figure 4. Cardiac hypertrophy and fibrosis in methyl-CpG–binding protein 2-transgenic (MeCP2-TG) mice. A and **B**, Hematoxylin-eosin (**top**) and wheat germ agglutinin stained (lower panels) cardiac sections of sham (control) and transverse aortic constriction (TAC)–operated mice. Ventricular weight/body weight ratios (n=4–15 per group; **C**) and cardiac myocyte cross-sectional areas (n=4–9 per group; **D**). **E**–**G**, Interstitial cardiac fibrosis identified by Sirius red staining and morphometric analysis of fibrotic areas (**G**; n=4–9 per group). **H**, TUNEL (TdT-mediated dUTP-biotin nick end labeling) staining to detect apoptotic cells (n=5–10 per group). Mean±SEM; **P*<0.05; ***P*<0.01; ****P*<0.001. LV indicates left ventricle.

under control of the α-myosin heavy chain gene promoter led to postnatal lethality because of severe cardiomyopathy with cardiomyocyte hypertrophy, atrial enlargement, and massive interstitial cardiac fibrosis (Online Figure IVA–IVC). To facilitate spatial and temporal control of cardiac MeCP2 expression, MeCP2 was controlled by a cardiomyocyte-specific, tetracycline-regulatable expression system (MeCP2-TG; Online Figure VA–VC). Transgene induction completely prevented downregulation of *MeCP2* after pressure overload (Online Figure VB).

Cardiomyocyte-specific ablation of MeCP2 was achieved by crossing a floxed *MeCP2* allele with MLC2a-Cre mice (MeCP2-knockout; Online Figure VD–VF).[19] *MeCP2* gene deletion reduced cardiac tissue *MeCP2* mRNA to levels, which were similar to those observed in wild-type mice after TAC (Online Figure VE versus Figure 1B). Cardiomyocyte-specific targeting of the *MeCP2* gene also reduced MeCP2 protein levels, further supporting that cardiomyocytes are the major source of MeCP2 expression in the heart (Online Figure VF).

To test whether prevention of MeCP2 repression in the doxycycline-controlled MeCP2-TG model was protective or maladaptive in heart failure, mice were subjected to TAC. Cardiac function was assessed by echocardiography and by direct LV catheterization (Figure 3A–3G). LV ejection fraction was similar in MeCP2-TG and control mice at baseline and after TAC (Figure 3A and 3B). LV contractility was significantly reduced in MeCP2-TG mice after pressure overload and LV relaxation was impaired in MeCP2-TG versus control mice after TAC (Figure 3D and 3E). Cardiac contractile reserve during dobutamine stimulation showed significantly smaller increases in LV pressure amplitude in transgenic mice when compared with wild-type mice without significant differences in heart rate between genotypes (Figure 3F and 3G).

Ventricular weight and cardiomyocyte cross sections were not altered in MeCP2-TG or MeCP2-knockout mice at baseline (Figure 4A–4D; Online Figure VI). However, prevention of MeCP2 downregulation in MeCP2-TG mice significantly aggravated hypertrophic, fibrotic, and apoptotic responses to TAC (Figure 4A–4H) demonstrating that prevention of MeCP2 repression facilitated maladaptive cardiac remodeling. In contrast, cardiomyocyte-specific loss of MeCP2 did not affect cardiac morphology or LV function (Online Figure VI).

Ablation of MeCP2 Expression Facilitates Recovery From Pressure Overload

The effect of MeCP2 on recovery from cardiac pressure overload was tested by removing the aortic stenosis after 4 weeks of TAC (Figure 5). Rapidly after removal of the aortic constriction (rTAC), the aortic arch was remodeled as visualized by magnetic resonance imaging (Figure 5A) and by echocardiography (Figure 5B and 5C). Within 14 days of rTAC, LV fractional shortening (Figure 5D) recovered to >60% of the control values before TAC. Doxycycline-regulated expression of MeCP2 impeded functional recovery, whereas ablation of MeCP2 significantly facilitated cardiac recovery (Figure 5E). Regression of ventricular and cardiomyocyte

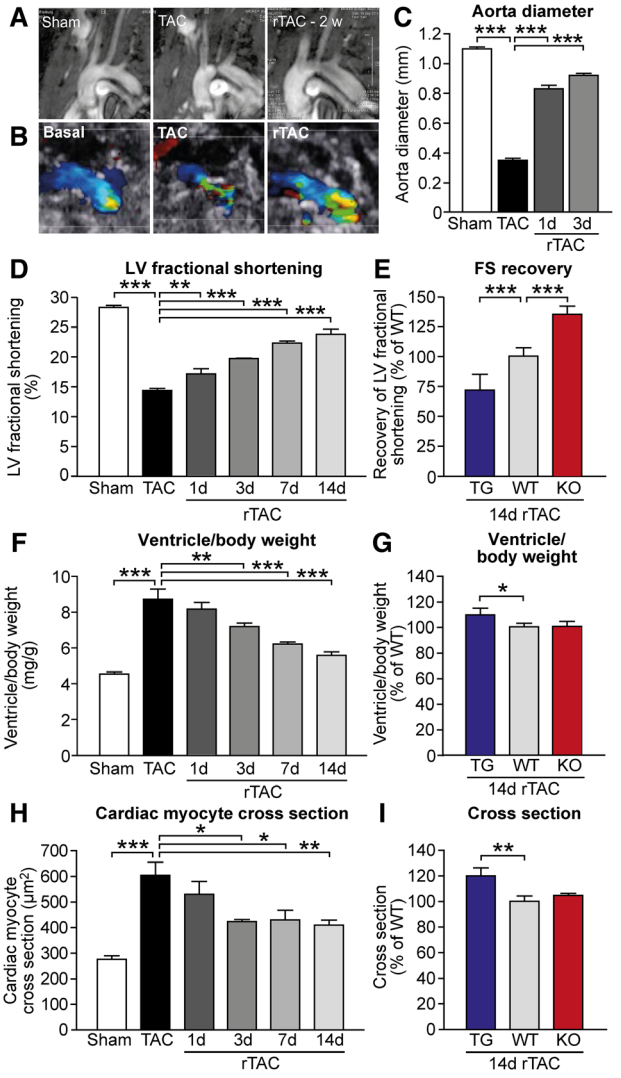

Figure 5. Cardiac remodeling after reversible left ventricular pressure overload (reversible transverse aortic constriction [rTAC]) in mice with cardiomyocyte-specific expression (methyl-CpG–binding protein 2-transgenic [MeCP2-TG] mice) or ablation of MeCP2-knockout [KO]. Left ventricular pressure overload was induced by TAC for 4 weeks followed by removal of the stenosis (rTAC) for 1 to 14 days. MRI (**A**) and color Doppler echocardiography (**B**) of the aortic arch in sham-operated wild-type (WT) mice (left panels), 3 weeks after TAC (middle panels) and 2 weeks after removal of the aortic stenosis (rTAC, **right**). **C**, Aortic arch diameter at the site of the TAC stenosis as determined by echocardiography (n=5 per group). **D** and **E**, Left ventricular (LV) fractional shortening at baseline, after 4 weeks of TAC and after rTAC for indicated times (**D**, n=5 and **E**, n=7–12). **F** and **G**, Ventricle weight/body weight ratios after TAC and rTAC in WT, MeCP2-TG, and MeCP2-KO mice (**F**, n=5 and **G**, n=7–12). **H** and **I**, Cardiac myocyte cross-sectional areas as determined by wheat germ agglutinin staining of cardiac sections (**H**, n=5 and **I**, n=7–12). Data from 8- to 12-week-old male C57BL/6 N mice (**A**–**D**, **F**, and **H**) or MeCP2-TG and MeCP2-KO and littermate controls (**E**, **G**, and **I**). Mean±SEM; **P*<0.05; ***P*<0.01; ****P*<0.001.

hypertrophy started immediately after rTAC (Figure 5F). Within 14 days after rTAC, ventricular weight and cardiac myocyte cross-sectional areas rapidly approached pre-TAC values (Figure 5F and 5H). Recovery of cardiac hypertrophy as induced by mechanical unloading (rTAC) was significantly

attenuated in MeCP2-TG but not in MeCP2-deficient mice (Figure 5G and 5I).

Differential Gene Expression in MeCP2-TG Hearts

To identify the molecular function of MeCP2 in the heart, gene expression profiling was performed in all genotypes at baseline, after TAC and rTAC (Figure 6A and 6B, Online Figure VIIA and VIIB; Online Table III). Genes that were differentially expressed in MeCP2-TG versus wild-type mice after TAC were analyzed in detail (Figure 6A). The vast majority of MeCP2-TG regulated genes after TAC were not affected by pressure overload versus sham conditions in wild-type mice (Figure 6A, third column). When these genes were filtered according to their expression level in cardiomyocytes,[14] 60% of genes which were upregulated in MeCP2-TG hearts after TAC were not expressed in cardiomyocytes (<1 fragments per kilobase of exon per million fragments mapped [FPKM][14]). This group of genes showed a significant enrichment of genes involved in inflammatory processes and response to wounding (Figure 6B, upper group), suggesting that they were upregulated in noncardiomyocyte cells. In contrast, 94% of genes (181 of 192) that were downregulated in MeCP2-TG hearts after TAC were significantly expressed in cardiomyocytes (>1 FPKM[14]). Gene ontology analysis of this group revealed a significant enrichment of genes involved in metabolic processes (Figure 6B, lower group). When genes that were repressed by MeCP2 were mapped onto mitochondrial pathways, fatty acid and ketone metabolism as well as mitochondrial transcription factors and their coregulators appeared to be most affected by MeCP2 (Online Figure VIIC–VIIE).

Mitochondrial Structure and Function in MeCP2 Transgenic Mice

Downregulation of metabolic genes suggested that MeCP2 might affect mitochondrial function. Electron microscopy of

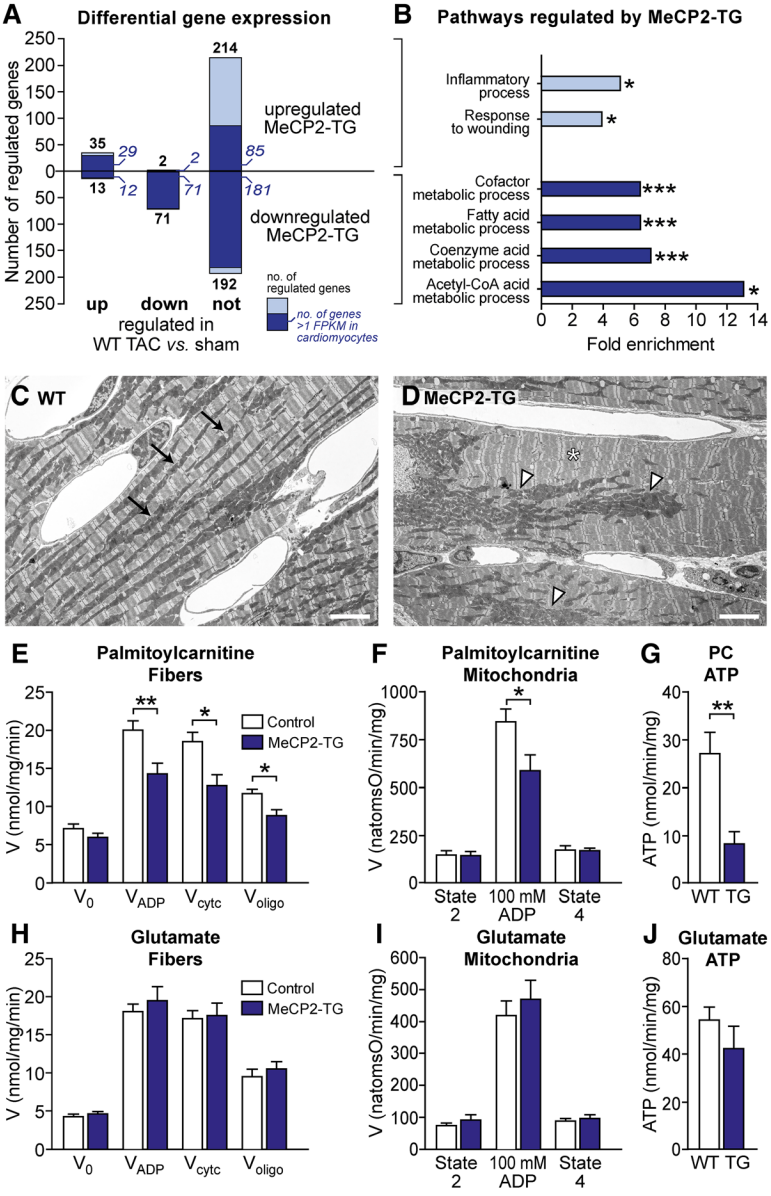

Figure 6. Differential gene expression and mitochondrial morphology and function in methyl-CpG–binding protein 2-transgenic (MeCP2-TG) mice. Differential gene expression (**A**) and associated gene ontology pathways (**B**) in MeCP2-TG vs wild-type (WT) hearts after transverse aortic constriction (TAC). Genes that were upregulated or downregulated (>1.5-fold; *P*<0.05) in MeCP2-TG hearts were grouped according to their regulation in WT TAC vs sham hearts (columns 1–3) and were classified as not expressed in cardiomyocytes (light blue, <1 fragments per kilobase of exon per million fragments mapped [FPKM][14]) or as expressed (dark blue, >1 FPKM[14]). Electron microscopy images of left ventricular sections from 10-week-old male wild-type (**C**) and MeCP2-TG hearts (**D**) revealed interfibrillar distribution of mitochondria in WT hearts (**C**, arrows) and perinuclear clustering (**D**, arrowheads) and loss of interfibrillar mitochondria (**D**, asterisk) in MeCP2-TG hearts (n=4 mice per group). **E**, **F**, **H**, **I**, Respiratory function of cardiac fibers (**E** and **H**, n=5–7 per group) or isolated cardiac mitochondria (**F** and **I**, n=5–10 per group) in the presence of palmitoylcarnitine and malate (**E** and **F**) or glutamate and malate (**H** and **I**) as substrates. Cardiac ATP tissue content in hearts incubated in the presence of palmitoylcarnitine/malate (**G**) or glutamate/malate (**J**, n=5–6 per group). Mean±SEM; **P*<0.05; ***P*<0.01.

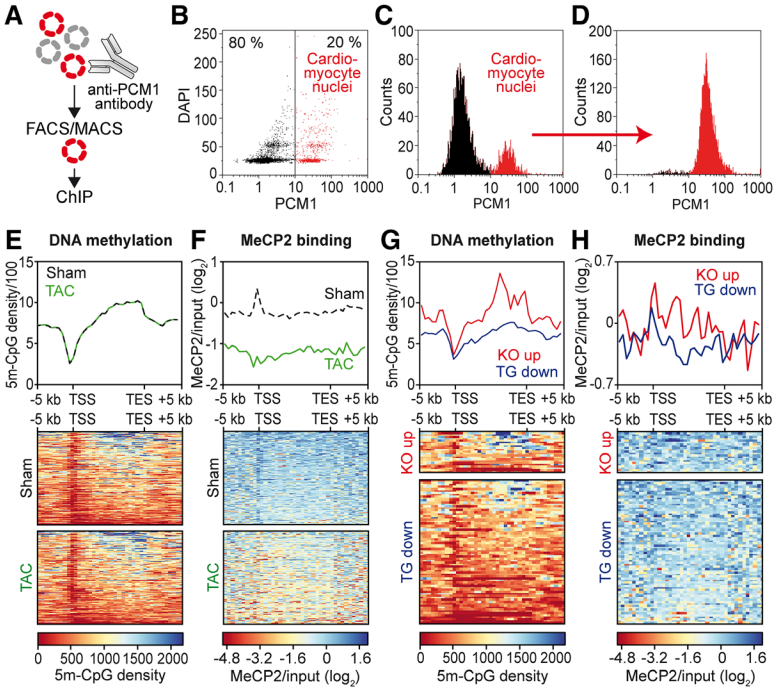

Figure 7. DNA methylation and methyl-CpG–binding protein 2 (MeCP2) binding to its target genes in purified mouse cardiomyocyte nuclei. **A**, Schematic illustration of cardiac nuclei isolation, staining of cardiomyocyte nuclei by anti-pericentriolar material 1 (PCM1) antibody and purification by magnetic-assisted sorting (MACS). **B–D**, Nuclei from male wild-type (WT) C57BL/6N mouse hearts after TAC were stained with DAPI or anti-PCM1 and analyzed by flow cytometry. Cardiomyocyte nuclei are indicated in red color. **E** and **F**, DNA methylation density (**E**, data derived from the study by Gilsbach et al[14]) and MeCP2 binding to its target genes in WT mice under sham and TAC conditions as determined by chromatin immunoprecipitation followed by next generation sequencing (ChIP-seq). Average levels (upper diagrams) and individual genes (heat maps) displaying CpG methylation (**E**) and MeCP2 ChIP-seq (**F**) of gene profiles which were differentially expressed in MeCP2-TG vs WT hearts after TAC. **G** and **H**, DNA methylation density (**G**, data derived from the study by Gilsbach et al[14]) and MeCP2 binding in WT hearts to genes which were upregulated in MeCP2-KO or downregulated in MeCP2-TG hearts. FACS indicates fluorescence-assisted sorting.

cardiac tissue sections revealed redistribution of cardiac mitochondria in MeCP2-TG hearts from interfibrillar and subsarcolemmal locations to the perinuclear region (Figure 6C and 6D). Respiratory measurements in cardiac fibers or in isolated mitochondria revealed a reduced maximal oxygen consumption in the presence of palmitoylcarnitine and malate as substrates but not with glutamate and malate (Figure 6E, 6F, 6H, and 6I). In parallel, tissue ATP levels were significantly lower in MeCP2-TG fibers incubated in the presence of palmitoylcarnitine but were not altered with glutamate (Figure 6G and 6J). These functional results are consistent with repression of genes involved in fatty acid metabolism by MeCP2 (Online Figure VIIE).

MeCP2 Is Bound to Methylated Genes in Cardiomyocytes

To directly assess MeCP2 binding to its target genes, cardiomyocyte nuclei were isolated and purified from sham- and TAC-operated wild-type mouse hearts (Figure 7A–7D). Cardiomyocyte nuclei were identified by anti-PCM1 staining (Figure 7A)[14] and nuclei were isolated with >98% purity by fluorescence- or magnetic-assisted sorting (Figure 7B–7D). Purified cardiomyocyte nuclei were then used to determine MeCP2 binding by chromatin immunoprecipitation followed by next generation sequencing. DNA methylation profiles were reanalyzed from our previously published data.[14] First, genes that were differentially regulated by MeCP2 between sham and TAC conditions were analyzed (Figure 7E and 7F). Although DNA methylation density of these genes did not differ between sham and TAC (Figure 7E), MeCP2 binding to these genes was greatly reduced in response to TAC (Figure 7F). Loss of MeCP2 from its target genes after TAC is consistent with MeCP2 repression, which was observed after pressure overload (Figures 1 and 2). To determine whether MeCP2 target genes showed particular DNA methylation profiles, genes which were repressed in MeCP2-TG hearts and those which were upregulated in MeCP2-knockout hearts were further analyzed (Figure 7G and 7H). Interestingly, genes that were repressed in MeCP2-TG cardiomyocytes showed significantly lower DNA methylation density than genes that were upregulated after MeCP2 ablation (Figure 7G). Furthermore, MeCP2 binding levels correlated well with DNA methylation density of upregulated versus downregulated genes (Figure 7H). Taken together, these data demonstrate that MeCP2 is bound to its target genes in a DNA methylation–dependent manner and is lost from these target genes after TAC.

MeCP2 Directly Represses *Ppargc1a*

To further study the effect of MeCP2 expression levels on mitochondrial gene expression, the nuclear coregulators, Pgc1α (gene symbol *Ppargc1a*) and Pgc1β (gene symbol *Ppargc1b*), and mitochondrial genes were investigated in more detail. *Ppargc1a* and *Ppargc1b* were repressed in transgenic mice with MeCP2 expression under control of the α-myosin heavy chain gene promoter (Online Figure VIIIA), in doxycycline-regulatable MeCP2-TG mice (Online Figure VIIIB) and in cardiomyocytes, which were transduced with an adenovirus expressing MeCP2 (Online Figure VIIIC). In contrast, siRNA targeting *MeCP2* led to increased expression of *Ppargc1a* and *Ppargc1b* in myocytes in vitro (Online Figure VIC). Several mitochondrial genes involved in fatty acid metabolism showed a similar pattern of regulation with repression by adenoviral MeCP2 expression and induction by *MeCP2* siRNA (Online Figure VIIID).

To further characterize the effect of MeCP2 on the *Ppargc1a* gene, chromatin immunoprecipitation experiments were performed in cardiomyocytes (Figure 8A–8D).

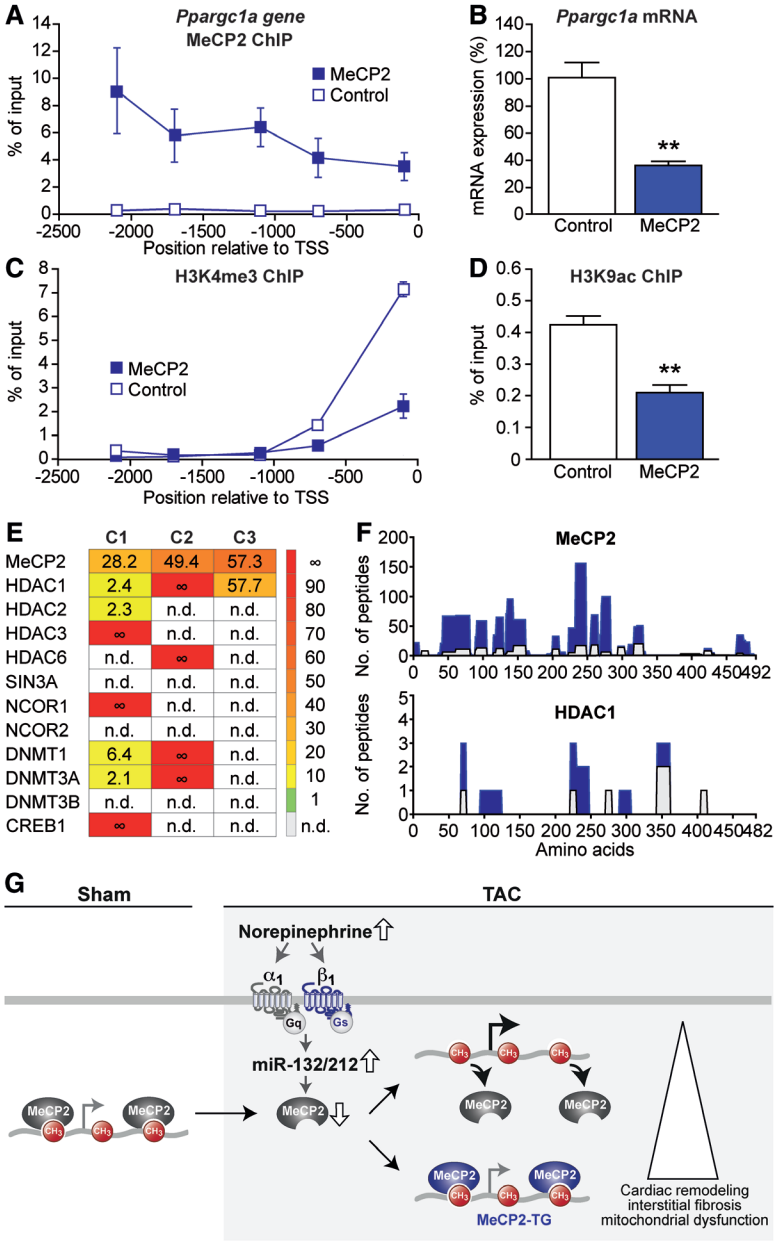

Figure 8. Binding of methyl-CpG–binding protein 2 (MeCP2) and repression of *Ppargc1a* gene expression. A–D, *Ppargc1a* repression and chromatin remodeling in response to adenoviral expression of MeCP2 in rat neonatal cardiomyocytes. **A**, Chromatin immunoprecipitation (ChIP) followed by polymerase chain reaction analysis revealed enrichment of MeCP2 in the *Ppargc1a* promoter region (n=3). **B**, MeCP2-repressed *Ppargc1a* mRNA levels (n=4 experiments performed in triplicate). **C** and **D**, ChIP signals for active chromatin marks H3K4me3 (**C**) and H3K9ac (**D**) were significantly lower in MeCP2-transduced cardiomyocytes when compared with native cells (**C**, experiment performed in triplicate and **D**, n=4 experiments performed in triplicate). Mean±SEM; $^{**}P<0.01$. **E** and **F**, Proteomic analysis identifying interaction partners of MeCP2 at 3 different lysis conditions (C1, C2, and C3, for details see Methods section of this article). Shown are ratios of protein intensities from neonatal rat cardiomyocytes with adenoviral expression of MeCP2 and β-galactosidase as control (∞ indicates not detected in control; and n.d., not detected; **E**, n=1 per condition). Peptide coverage of MeCP2, histone deacetylase 1 (HDAC1) in neonatal rat cardiac myocytes with adenoviral expression of MeCP2 (**F**, blue columns) or β-galactosidase as control (**F**, white columns) (summarized results from n=3 conditions). All 3 immunoprecipitation conditions resulted in pull down of HDAC1 indicating an interaction with MeCP2. **G**, Overview of the pathway leading to MeCP2 repression after activation of α_1- and β_1-adrenoceptors in cardiomyocytes. Induction of *miR-212/132* contributes to downregulation of MeCP2, which is removed from its target genes and thus allows for cardiac adaptation during chronic pressure overload. Prevention of MeCP2 repression in transgenic (MeCP2-TG) mice aggravated cardiac remodeling, interstitial fibrosis, and mitochondrial dysfunction.

Binding of MeCP2 could be identified in the promoter region of *Ppargc1a* (Figure 8A). MeCP2 binding repressed *Ppargc1a* mRNA (Figure 8B) and led to reduction of active chromatin marks, including H3K4me3 and H3K9ac (Figure 8C and 8D) without alterations in the total amount of histone H3, which could be immunoprecipitated (data not shown).

To test, whether all mitochondrial effects of MeCP2 were downstream of *Ppargc1a* and *Ppargc1b* repression, *Ppargc1a,b* expression was reduced by siRNAs and the effect of MeCP2 on fatty acid metabolism genes was evaluated (Online Figure VIIIE and VIIIF). Although part of the mitochondrial genes were no longer repressed by MeCP2 in cells with *Ppargc1a,b* downregulation, several other genes were still responsive to MeCP2, including *Acadm, Fabp3*, and *Ppara* (Online Figure VIIIF).

MeCP2 Interaction Partners

Several mechanisms have been proposed how MeCP2 may modulate gene expression by association with repressor or activator proteins.[15–17,28] To identify which proteins associate with MeCP2 specifically in cardiomyocytes, immunoprecipitation experiments followed by mass spectrometry were performed in neonatal rat cardiomyocytes (Figure 8E and 8F). Three conditions applying different lysis buffers (C1–C3) were used for immunoprecipitation (Figure 8E). cAMP response element-binding protein 1 that was shown to act as a transcriptional activator when bound to MeCP2 in neurons[17] was only enriched under mild (C1) but not under more stringent conditions (C2 and C3; Online Table II). In contrast, histone deacetylase 1 (HDAC1) was enriched under all 3 conditions when compared with control-transduced myocytes (Figure 8E and 8F; Online Table II)

suggesting an interaction between MECP2 and HDAC1 in cardiomyocyte nuclei.

Discussion

In this study, we have identified a novel signaling pathway linking adrenergic activation in cardiomyocytes with microRNA-mediated repression of the epigenetic reader MeCP2. Our data show that MeCP2 is an important modulator of cardiac gene expression during the development of and recovery from cardiac hypertrophy and failure. Prevention of MeCP2 repression in mice after chronic cardiac pressure overload aggravated cardiac hypertrophy, mitochondrial and contractile dysfunction. In contrast, ablation of MeCP2 expression in cardiac myocytes in vivo facilitated functional recovery from cardiac failure by mechanical unloading of the heart. We have identified target genes of MeCP2 and show that repression of MeCP2 coincides with loss of MeCP2 binding from its target genes, whereas DNA methylation remains stable at these genes. The results suggest that modulation of the epigenetic reader MeCP2 in cardiomyocytes is important for adaptation during cardiac hypertrophy and failure.

We have identified microRNAs that contribute to repression of cardiac MeCP2 in response to chronic pressure overload. *miR-212/132* were upregulated by activation of α_1- and β_1-adrenoceptors. This extends previous studies that have demonstrated that *miR-212/132* are induced in response to cardiac damage or by angiotensin II signaling.[20,29] *MiR-212/132* can target additional mRNAs beyond *MeCP2*, including *FOXO3* which is involved in the control of cardiac autophagy.[20] This example illustrates that the same miRs may initiate protective (eg, repression of MeCP2) as well as pathological signals (eg, downregulation of FOXO3) within the same cell type. Further studies will be important to identify whether different pathologies may shift the balance between these opposing microRNA functions.

MeCP2 is part of a large family of reader proteins, which recognize and bind to methylated cytosines within DNA CpG dinucleotides.[16,30] Interestingly, pressure overload led to loss of MeCP2 binding from its target genes, which is consistent with decreased MeCP2 protein abundance under this condition. In other systems, multiple posttranslational modifications, including phosphorylation of MeCP2, have been identified to affect MeCP2 binding to cofactors and methylated DNA and thus modulate MeCP2's effect on gene expression.[31]

The repressive function of MeCP2 in cardiomyocytes was functionally relevant because MeCP2-TG mice showed aggravated cardiac hypertrophy and dysfunction after pressure overload. The predominance of mitochondrial genes among MeCP2-repressed genes may be a consequence of the fact that MeCP2 directly targeted both, nuclear transcription (co)factors and mitochondrial genes. Chromatin immunoprecipitation revealed that MeCP2 was enriched at the promoters and gene bodies of several mitochondrial genes, which are involved in fatty acid metabolism but also at the promoters of nuclear (co)factors regulating mitochondrial gene expression. MeCP2 binding to the promoter of the *Ppargc1a* gene was associated with inhibition of *Ppargc1a* expression and reduction of active chromatin marks H3K4me3 and H3K9ac. Interestingly, repression of *Ppargc1a* by MeCP2 was also observed in mice with neuron-specific overexpression of MeCP2,[17] suggesting a similar mechanism in neurons and cardiomyocytes. Decreased fatty acid oxidation is a general feature of metabolic remodeling in heart failure and several underlying signaling pathways have been characterized in detail.[32,33] Interestingly, prevention of FAO dysfunction in a gene-targeted mouse model led to increased contractile function, attenuation of hypertrophy and reduction of fibrosis after cardiac pressure overload.[34]

Several studies have shown that DNA methylation is changing during cardiomyocyte development and disease.[12–14] Recently, we have described the DNA methylome of purified cardiomyocyte nuclei at basepair resolution during mouse development and after cardiac pressure overload.[14] Specific changes in DNA methylation could be identified in failing versus nonfailing myocytes, but these differentially methylated regions could not be directly associated with enhancers or gene bodies.[14] This study suggests that DNA methylation is rather stable at MeCP2 target genes in failing cardiomyocytes.

Previous studies in other cell types and tissues have identified multiple proteins, which may associate with MeCP2 leading to modulation of gene expression.[35] Although MeCP2 has been mostly characterized as a repressor, it may also activate gene expression, for example, by recruting cAMP response element-binding protein.[17] We have found several HDACs in MeCP2 immunoprecipitation assays. HDACs 1, 2, and 3 that were pulled down together with MeCP2 from cardiomyocytes belong to class I HDACs, which remove acetyl groups from histone tails and thus contribute to gene repression.[36] Further studies will be required to demonstrate direct association of MeCP2 with HDACs at target genes and to test whether HDAC subtype binding to MeCP2 changes during development and in heart failure. Previous studies have provided further links between adrenergic (and calcium) signaling and HDACs.[37,38] Class IIa HDACs are responsive to protein kinases A and D, as well as calcium/calmodulin-dependent kinase to regulate shuttling of these HDACs between the nucleus and the cytosol.[37,38]

Taken together, this study shows that reversible repression of the DNA reader protein MeCP2 is essential to facilitate adaptation of the heart during the development of and recovery from cardiac hypertrophy and failure. Future studies will be essential to determine how adrenergic and other pathological signals may converge in cardiomyocyte nuclei at MeCP2, HDACs, and other epigenetic factors to control pathological gene expression.

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Disclosures

None.

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Novelty and Significance

What Is Known?

- Activation of the adrenergic system facilitates cardiac remodeling and dysfunction in heart failure.
- Epigenetic mechanisms, including microRNAs and DNA methylation have been implicated in the development of heart failure.

What New Information Does This Article Contribute?

- This study links activation of adrenergic signaling in cardiac myocytes with induction of 2 microRNAs, which repress methyl-CpG–binding protein 2 (MeCP2).
- Prevention of MeCP2 repression in a transgenic mouse model aggravated cardiac remodeling and dysfunction.
- MeCP2 was bound to its target genes in a DNA methylation–dependent manner.
- In failing cardiac myocytes, MeCP2 binding to its target genes was reduced, but DNA methylation of these loci remained constant.

Adrenergic activation is an essential part of neuroendocrine activation, which has been well characterized in development and progression of heart failure. The aim of this study was to identify a link between adrenergic signaling and DNA methylation and its alteration in the failing heart. Activation of α_1- and β_1-adrenergic receptors induced expression of *miR-212/132*. Upregulation of these microRNAs reduced expression of MeCP2. In biopsies from failing mouse and human hearts MeCP2 expression was reduced when compared with native hearts. Prevention of MeCP2 repression in a transgenic mouse model aggravated cardiac hypertrophy and dysfunction after transverse aortic constriction. MeCP2 target genes were identified by transcriptome, chromatin immunoprecipitation, and DNA methylation analyses. In heart failure, MeCP2 was removed from its target genes, but DNA methylation of these genes remained constant. This study demonstrates that repression of the methyl-DNA reader protein MeCP2 facilitates adaptation of the heart during the development and regression of cardiac hypertrophy and failure.