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Inhibition of Cardiomyocyte Hypertrophy by Protein Arginine Methyltransferase 5*

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Background: Protein arginine methyltransferase 5 (PRMT5) is a type II protein arginine methyltransferase that catalyzes the symmetrical dimethylation of arginine residues within target proteins.
Results: PRMT5 interacts with and methylates GATA4 in cardiomyocytes.
Conclusion: PRMT5 suppresses hypertrophic responses in cardiomyocytes by attenuating GATA4 transcriptional activity.
Significance: Targeting PRMT5 may represent a novel therapeutic strategy for preventing cardiac hypertrophy and heart failure.

Protein arginine methyltransferase 5 (PRMT5), a protein arginine methyltransferase that catalyzes the symmetrical dimethylation of arginine residues within target proteins, has been implicated in many essential cellular processes ranging from the regulation of gene expression to cell proliferation and differentiation. PRMT5 is highly expressed in the heart; the functional role of PRMT5 in the heart, however, remains largely elusive. In the present study, we show that PRMT5 specifically interacts with GATA4 in both co-transfected HEK293T cells and neonatal rat cardiomyocytes by co-immunoprecipitation. Importantly, this interaction leads to the arginine methylation of GATA4 at positions of 229, 265, and 317, which leads to an inhibition of the GATA4 transcriptional activity, predominantly through blocking the p300-mediated acetylation of GATA4 in cardiomyocytes. Moreover, overexpression of PRMT5 substantially inhibited the acetylation of GATA4 and cardiac hypertrophic responses in phenylephrine-stimulated cardiomyocytes, whereas knockdown of PRMT5 induced GATA4 activation and cardiomyocyte hypertrophy. Furthermore, in response to phenylephrine stimulation, PRMT5 translocates into the cytoplasm, thus relieving its repression on GATA4 activity in the nucleus and leading to hypertrophic gene expression in cardiomyocytes. These findings indicate that PRMT5 is an essential regulator of myocardial hypertrophic signaling and suggest that strategies aimed at activating PRMT5 in the heart may represent a potential therapeutic approach for the prevention of cardiac hypertrophy and heart failure.

Cardiac hypertrophy, an adaptive process in response to various physiologic or pathologic stimuli, is an early milestone of many heart diseases (1). Although initially adaptive, persistent hypertrophy induced by pathologic conditions such as hypertension has detrimental consequences, eventually contributing to the development of heart failure (2). At the molecular and cellular levels, cardiac hypertrophy is characterized by increased protein synthesis, cellular hypertrophy, and the reactivation of fetal genes, such as atrial natriuretic factor (ANF),[2] B-type natriuretic peptide (BNP), and β-myosin heavy chain (3, 4), which promote pathological cell growth. Accumulating evidence indicates that cardiac transcription factors, such as GATA family transcription factors, nuclear factor of activated T cells (NFAT), and myocyte enhancer factor 2 (MEF2), are critically important in reactivating the fetal growth programs that drive hypertrophic events (4–6).

GATA family transcription factors encode zinc finger DNA-binding proteins that regulate gene expression by binding to the specific consensus DNA sequence (A/T)GATA(A/G)GATA (3). Among six GATA transcription factors in vertebrates, GATA4, GATA5, and GATA6 are expressed in the heart and regulate expression of a wide range of cardiac-specific genes, such as α-myosin heavy chain, cardiac troponin C, cardiac troponin I, atrial natriuretic peptide (ANP), and brain natriuretic peptide (3, 4). In particular, GATA4 has been shown to be very important in promoting the development of cardiac hypertrophy. For example, overexpression of GATA4 induces hypertrophy in both cultured cardiomyocytes and in the hearts of mice (7), whereas inhibition of GATA4 activity by expressing a dominant-negative protein leads to inhibition of agonist-induced protein synthesis and expression of hypertrophic genes in cardiomyocytes (8). Because of its essential role in the heart, GATA4 transcriptional activity is dynamically controlled, and its regulation occurs through diverse posttranslational mechanisms. In addition, phosphorylation of GATA4 by extracellular signal-regulated kinase and p38 MAPK has been shown to increase its activity, thus promoting the expression of hyper-

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[2] The abbreviations used are: ANF, atrial natriuretic factor; ANP, atrial natriuretic peptide; BNP, brain natriuretic peptide; NFAT, nuclear factor of activated T cells; PRMT5, protein arginine methyltransferase 5; GATA, GATA-binding protein; PE, phenylephrine; PRC2, polycomb-repressive complex 2; NRVM, neonatal rat ventricular myocyte; BNP-Luc, BNP promoter-driven luciferase; qRT, quantitative real time; TRITC, tetramethylrhodamine isothiocyanate; m.o.i., multiplicity of infection.

trophic programs (7). In contrast, phosphorylation of GATA4 by glycogen synthase kinase 3β leads to nuclear exit of GATA4, thus suppressing GATA4 transcriptional activity and induction of cardiac hypertrophy (9). GATA4 activity is also influenced by other post-translational modifications, such as acetylation (10), sumoylation (11), and methylation (12). Specifically, p300 has been shown to interact with GATA4 and potentiate its transcriptional activity through acetylation (10). Relevant to this, polycomb-repressive complex 2 (PRC2), a histone methyltransferase, has been shown to directly methylate GATA4 at lysine 299, which results in an inhibition of GATA4 transcriptional activity (12). Taken together, these studies underscore the complexity and critical importance of post-translational modifications in the regulation of GATA4 activity in the heart.

PRMT5 is a type II protein arginine methyltransferase that catalyzes the symmetrical dimethylation of arginine residues within target proteins (13). In mammalian cells, PRMT5 localizes to both the cytoplasm and the nucleus and methylates multiple histone and non-histone proteins, thus exerting diverse biological effects (14–17). For example, methylation of histone protein by PRMT5 can regulate DNA damage repairing, gene expression, and RNA splicing (14). Methylation of non-histone proteins, such as p53, HOXA9, NF-κB, and E2F-1, by PRMT5 has been implicated in the regulation of cell growth, apoptosis, and inflammation (15–17). PRMT5 is widely expressed in different human tissues, with high expression levels observed in heart, skeletal muscle, and testis (18, 19). Interestingly, the expression of PRMT5 in the heart is substantially reduced in aged rats (19), thus implicating a potential role of PRMT5 in age-related heart diseases, such as cardiac hypertrophy and heart failure (20, 21). In the present study, we found that PRMT5 specifically interacts with GATA4 and negatively regulates cardiomyocyte hypertrophy, at least in part, through a novel mechanism involving arginine methylation of GATA4.

EXPERIMENTAL PROCEDURES

Cell Culture—Human embryonic kidney cells HEK293T, HEK293, and Ad293 cells (ATCC) were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS (Invitrogen), 1% penicillin/streptomycin (Invitrogen) in a humidified atmosphere of 5% CO_2 at 37 °C.

Primary Culture of Neonatal Rat Ventricular Myocytes (NRVMs)—We obtained ventricles from 1-day-old Sprague-Dawley rats and isolated cardiac myocytes through digestion with trypsin-EDTA and type2 collagenase. The protocol was approved by the Institutional Animal Care and Use Committee of the Thomas Jefferson University. Briefly, the tissues were cut into small pieces and digested by 0.25% trypsin at 4 °C overnight. Collagenase (Worthington, Lakewood, NJ; 1 mg/ml in HBSS) was used to further digest tissues in a shaking bath at 37 °C for 20 min. The cell suspension was centrifuged at 1000 rpm for 5 min and resuspended in 10% FBS DMEM with 1 g/liter glucose and 10% FBS. Cells were cultured for 2 h to allow fibroblast cells to attach to the flask. NRVMs were collected from the supernatants and cultured with DMEM containing 1 g/liter glucose plus 10% FBS and 1% penicillin/streptomycin.

Plasmid Constructs—To generate Myc-tagged expression vectors, human GATA4 fragments were amplified by PCR using the following primers: L1–440, 5′-GAGACTCGAGATGTACCAAAGCCTGGCTATGG-3′ and 5′-GAGATCTAGATTACGCGGTGATTATGTCCCCATG-3′; L1–329, 5′-GAGACTCGAGATGTACCAAAGCCTGGCTATGG-3′ and 5′-GAGATCTAGATGTCTTAGATTTATTCAGATTC-3′; L1–294, 5′-GAGACTCGAGATGTACCAAAGCCTGGCTATGG-3′ and 5′-GAGATCTAGAGCAGGCATTGCAAAGAGGCT-3′; L1–215, 5′-GAGACTCGAGATGTACCAAAGCCTGGCTATGG-3′ and 5′-GAGATCTAGACTCTCTGCCTTCTGAGAAGTCAT-3′; L240–440, 5′-GAGACTCGAGTGTGGCCTCTACCACAAGATGAA-3′ and 5′-GAGATCTAGATTACGCGGTGATTATGTCCCCATG-3′; L294–440, 5′-GAGACTCGAGTGCGGCCTCTACATGAAGCTCC-3′ and 5′-GAGATCTAGATTACGCGGTGATTATGTCCCCATG-3′. To generate the GST-tagged expression vectors, human GATA4 were amplified by PCR using the following primers containing EcoRI and XhoI sites: 5′-GAGAGAATTCATGTACCAAAGCCTGGCTATGG-3′ and 5′-GAGACTCGAGTTACGCGGTGATTATGTCCCCATG-3′. GATA4 was subcloned into pGEX-5x-1 vector. pFLAG-CMV-PRMT5 mutants (G367A/R368A) were constructed using QuikChange II site-directed mutagenesis kit (Agilent Technologies) and PCR primers as following: 5′-GTGCTGGGAGCAGCAGCGGGACCCCTGGTG-3′ and 5′-CACCAGGGGTCCCGCTGCTGCTCCCAGCAC-3′.

The PCR primers for mutation of GATA4 at R229W, R265W, and R317W were as follows: R229W, 5′-CCACTCTGGAGGTGGGATGGGACAGGA-3′ and 5′-TCCTGACCCATCCCACCTCCAGAGTGG-3′; R265W, 5′-TCTGCCTCCCGCTGGGTAGGCCTCTCC-3′ and 5′-GGAGAGGCCTACCCAGCGGGAGGCAGA-3′; R317W, 5′-GGGATTCAAACCTGGAAACGGAAGCCC-3′ and 5′-GGGCTTCCATTTCCAGGTTTGAATCCC-3′.

Adenovirus Construction—Adenoviruses harboring wild-type FLAG-tagged PRMT5 (Ad-PRMT5) was made using AdMax (Microbix) as described previously (22). The viruses were made and propagated in Ad293 cells and purified using $CsCl_2$ banding followed by dialysis against 20 mmol/liter Tris-buffered saline with 10% glycerol. Titering was performed on Ad293 cells using Adeno-X Rapid Titer kit (Clontech) according to the manufacturer's instruction.

Purification of Protein and in Vitro Methylation Assay—GATA4 protein was expressed in bacteria as recombinant glutathione *S*-transferase (GST) fusion protein and eluted from glutathione-agarose beads with 20 mM glutathione. Wild-type and FLAG-tagged PRMT5 mutants were expressed in HEK293 cells and immunoprecipitated with anti-flag M2 agarose (Sigma). 10 μg of GST-GATA4 were incubated with immunoprecipitated wild-type FLAG-PRMT5 or FLAG-PRMT5 mutants, and 2 μl of *S*-adenosylmethionine (PerkinElmer Life Sciences) in 80 μl of methyltransferase reaction buffer (20 mM Tris-HCl, pH 8.0, 5 mM $MgCl_2$, 4 mM DTT) at 37 °C for 90 min. Reactions were stopped by adding 20 μl of 4× SDS-PAGE loading buffer followed by heating at 95 °C for 5 min. Samples were loaded on SDS-PAGE and analyzed by Coomassie Blue staining and Western blotting. Methylation of GST-GATA4 was detected by anti-dimethyl-arginine symmetric antibody (Millipore).

Immunoprecipitation—Cell lysates were prepared and precleared for 1 h at 4 °C with protein A/G-agarose (Sigma). Anti-

GATA4 or anti-FLAG M2-agarose (Sigma) were incubated with cell lysates. Immune complexes were collected after overnight incubation at 4 °C. Three times after washing with the lysis buffer, immune complexes were resolved by SDS-PAGE and transferred to nitrocellulose membranes (Bio-Rad).

Immunoblotting—Cell lysates were made using radioimmune precipitation assay buffer (Thermo Scientific) containing 25 mM Tris-HCl, pH 7.6, 150 mM NaCl, 1% Nonidet P-40, 1% sodium deoxycholate, 0.1% SDS, and proteinase inhibitor mixture containing 2 mM PMSF, 20 μg/ml aprotinin, 10 μg/ml leupeptin. After a 1-h extraction at 4 °C with rocking, insoluble material was removed by centrifugation. Supernatants were resolved by SDS-PAGE and transferred to nitrocellulose (Bio-Rad). Blots were blocked with 5% nonfat milk in PBS with 0.1% Tween 20 (PBST) and then developed with diluted antibodies. Blots were visualized on an Odyssey Imaging System (LI-COR). The intensity of the bands was quantified by using the Odyssey software.

Transient Transfection and Luciferase Assay—HEK293 cells seeded in 6- or 24-well plates were transfected with GATA4, p300, and PRMT5 plasmids combined with BNP-Luc using FuGENE 6 transfection reagent. 48 h after transfection cells were lysed, and the reporter activity was determined with a luminescence counter (PerkinElmer Life Sciences) using the Dual-Luciferase Reporter Assay System (Promega) according to the instructions of the manufacturer. Firefly luciferase activity was normalized for transfection efficiency by corresponding Renilla luciferase activity. All transfection experiments were performed at least five times in duplicate.

Knockdown of PRMT5 by Small Interfering RNA (siRNA)—One pair of siRNA oligonucleotides for rat PRMT5 (5′-GAUUGCAGUAGCUCUUGAA-3′ and 5′-UUCAAGAGCUACUGCAAUC-3′), and a negative control siRNA (MISSION siRNA Universal Negative Control; Sigma) were used for the transfection of NRVMs with Lipofectamine RNAiMAX® transfecting reagent (Invitrogen) in serum-free medium according to the manufacturer's recommendation.

Quantitative Real Time-PCR (qRT-PCR)—Total RNA was extracted from cardiomyocytes and treated with DNase I using the RNeasy® Micro kit (Qiagen). qRT-PCR analysis was performed as described previously (23). Briefly, 200 ng of total RNA was used for reverse transcription using the iScrip cDNA synthesis kit (Bio-Rad). Messenger RNA levels of ANF and BNP were determined by qRT-PCR using cDNA obtained from the reverse transcription reactions as template, with MyiQ™ Single-Color Real-Time PCR Detection System (Bio-Rad) and HotStart-IT SYBR Green One-Step qRT-PCR Master Mix Kit (Affymetrix). The primer sequences are described as follows: ANF forward (5′-TGGGCTCCTTCTCCATCACC-3′) and reverse (5′-GCCAAAAGGCCAGGAAGAGG-3′); BNP, forward (5′-CAGAACAATCCACGATGCAG-3′) and reverse (5′-GCTGTCTCTGAGCCATTTCC-3′); PRMT5, forward (5′-TGAGGCCCAGTTTGAGATGCCTTA-3′) and reverse (5′-CCAAGGTGCAATAGCGGTTGTTGT-3′); 18 S RNA forward (5′-TCAAGAACGAAAGTCGGAGG-3′) and reverse (5′-GGACATCTAAGGGCATCAC-3′). The expression levels of ANF and BNP relative to 18 S RNA were determined using the $2^{-\Delta\Delta Ct}$ method.

Determination of Protein/DNA Ratio in Cardiomyocytes—Myocytes were seeded in 6-well plates and then transduced with either Ad-LacZ or Ad-PRMT5 for 48 h. 48 h after treatment with or without PE, the pellet was suspended in the saline, sodium citrate buffer plus 0.25% SDS. Samples were then split into aliquots for protein and DNA measurements as described previously (24). Protein was measured using a total protein assay kit (Lorry method, Sigma). DNA was measured using Quant-iT™ dsDNA high sensitivity assay kit (Invitrogen). Fluorescence was measured at excitation and emission wavelengths of 510 and 527 nm.

Immunofluorescent Staining—NRVMs were fixed in 4% formaldehyde in PBS. To visual the sarcomere reorganization, fixed NRVMs were incubated with Alexa Fluor 546 phalloidin. To determine the colocalization of PRMT5 and GATA4 in cardiomyocytes, fixed NRVMs were incubated with anti-PRMT5 antibody (1:100 dilution; Cell Signaling) and anti-GATA4 antibody (1:100 dilution; Santa Cruz) followed by fluorescein-5-isothiocyanate (FITC)-conjugated secondary antibodies (1:250 dilution, Invitrogen) and TRITC-conjugated secondary antibodies (1:300 dilution; Invitrogen). Cell nuclei were stained with DRAQ5. To examine the expression of ANP in cardiomyocytes, fixed NRVMs were incubated with anti-ANP antibody (1:100 dilution; Santa Cruz) and followed by FITC-conjugated secondary antibodies (1:250 dilution, Invitrogen). Cell nuclei were stained with DAPI. Images were visualized using an Olympus confocal microscope (Olympus, Tokyo Japan).

Subcellular Fractionation and Electrophoretic Mobility Shift Assay (EMSA)—Subcellular fractions were prepared by differential centrifugation of cell homogenates as described previously (22). The oligonucleotides corresponding to the binding sequence of GATA4 (5′-CACTTGATAACAGAAAGTGATAACTCT-3′) were synthesized and labeled with IRDye 700 (IDT). EMSA was performed according to the protocol provided by LI-COR.

Statistical Analyses—Data are expressed as the means ± S.E. The statistical significance of differences was assessed by Student's *t* tests or analysis of variance as appropriate; a value of $p < 0.05$ was considered statistically significant.

RESULTS

PRMT5 Interacts with GATA4—To assess the functional role of PRMT5 in the heart, we evaluated whether PRMT5 interacts with GATA4. To this end, co-immunoprecipitation experiments were performed in HEK293T cells that were co-transfected with PRMT5 and GATA4 expression vectors. Immunoprecipitation of GATA4 led to co-immunoprecipitation of FLAG-tagged PRMT5 when both proteins were cotransfected (Fig. 1*A*). As expected, the anti-GATA4 antibody did not immunoprecipitate FLAG-tagged PRMT5 in the absence of GATA4. Similarly, immunoprecipitation of FLAG-PRMT5 resulted in coimmunoprecipitation of GATA4, whereas the anti-FLAG antibody did not immunoprecipitate GATA4 in the absence of FLAG-tagged PRMT5 (Fig. 1*B*). These results indicate that PRMT5 and GATA4 co-exist in the same complex in mammalian cells. Furthermore, PRMT5 was found to directly associate with GATA4 in a GST pulldown assay (Fig. 1*C*). To further confirm this physical interaction between GATA4 and

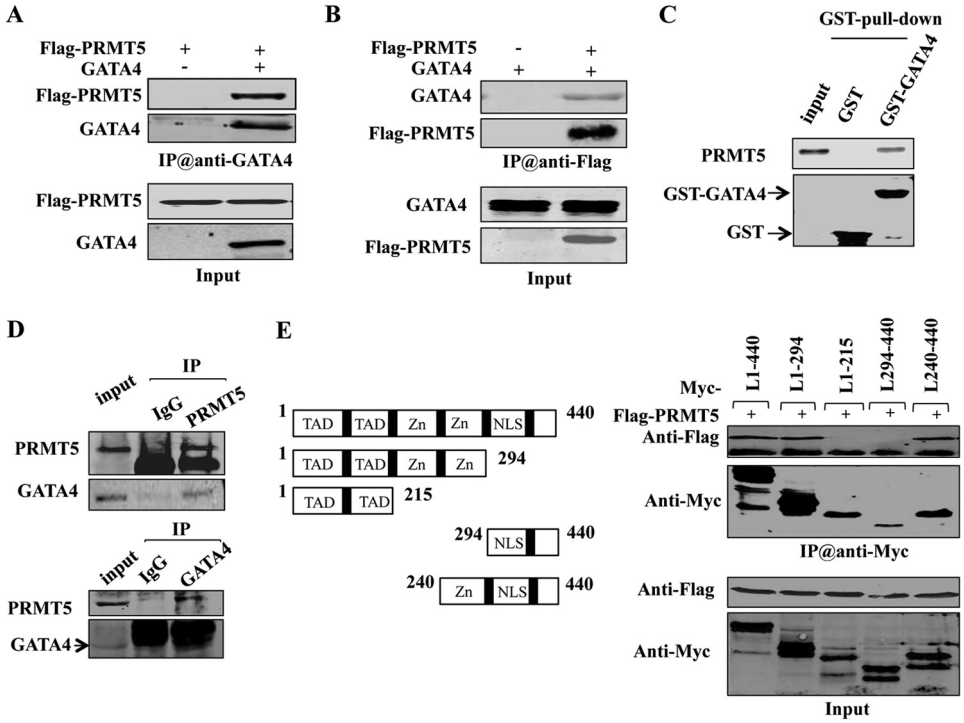

FIGURE 1. **PRMT5 interacts with GATA4.** *A*, FLAG-tagged PRMT5 expression vector in combination with either empty vector or pcDNA-GATA4 was co-transfected into HEK293T cells. Extracted proteins were precipitated (*IP*) by anti-GATA4 antibody and then separated by 10% SDS-PAGE. The transferred membrane was immunoblotted with either anti-PRMT5 or anti-GATA4 antibody. *B*, FLAG-tagged PRMT5 expression vector in combination with either empty vector or pcDNA-GATA4 was co-transfected into HEK293T cells. Extracted proteins were precipitated by anti-FLAG antibody and then separated by 10% SDS-PAGE. The transferred membrane was immunoblotted with either anti-PRMT5 or anti-GATA4 antibody. *C*, GST pulldown assays were performed using purified FLAG-PRMT5 with recombinant GST or GST-GATA4 and then detected with the indicated antibodies. *D*, cell lysates obtained from neonatal rat cardiomyocytes were immunoprecipitated with anti-GATA4 antibody or control IgG and then separated by 10% SDS-PAGE. Transferred membrane was immunoblotted with either anti-PRMT5 or GATA4 antibody. *E*, FLAG-PRMT5 expression vector in combination with either empty vector or expression vectors of Myc-GATA4 mutants were co-transfected into HEK293 cells. Extracted proteins were precipitated by anti-Myc antibody and then separated by 10% SDS-PAGE. The transferred membrane was immunoblotted with either anti-FLAG or anti-Myc antibody. *TAD*, transcriptional activation domain; NLS, nuclear localization sequence.

PRMT5, co-immunoprecipitation was performed with cell lysates obtained from NRVMs. As shown in Fig. 1*D*, GATA4 co-precipitated with endogenous PRMT5 in NRVMs. Taken together, these results indicate that protein-protein interactions exists between PRMT5 and GATA4 in cardiomyocytes under physiological conditions.

GATA4 comprises the N-terminal transcriptional activation domain, zinc finger domain, and C-terminal nuclear localization sequence (25). To map the binding region of PRMT5 in GATA4, we constructed a series of myc-tagged GATA4 deletion mutants (Fig. 1*E*) and co-expressed these proteins with FLAG-tagged PRMT5 in HEK293 cells. Cell lysates from transfected HEK293 cells were then immunoprecipitated with anti-myc antibody and analyzed by Western blot. We found that deletion of the C-terminal region of GATA4 residues 295–440 (L1–294) and the N-terminal region up to residues 239 (L240–440) had no effect on its binding to PRMT5. However, deletion of the zinc finger domain (L1–215 or L294–440) of GATA4 completely abolished its interaction with PRMT5 (Fig. 1*E*). Thus, these findings indicate that the zinc finger domain of GATA4, particularly the second zinc finger domain, is critically important for its binding to PRMT5.

PRMT5 Methylates GATA4—Because PRMT5 is a methyltransferase, the interaction of PRMT5 with GATA4 prompted us to evaluate whether GATA4 is a substrate of PRMT5. To test this hypothesis, we performed an *in vitro* methylation assay using GST-GATA4 fusion protein as a substrate. GST-GATA4 was incubated with either FLAG-tagged wild-type or PRMT5 mutant (G367A/R368A), a loss of function mutation lacking methyltransferase activity (26), in the presence *S*-adenosylmethionine. The methylation of GATA4 was further detected by Western blot using a specific anti-dimethylation symmetric antibody. As shown in Fig. 2*A*, GATA4 was robustly methylated by wild-type PRMT5, but not by PRMT5 mutant, indicating that GATA4 is in fact a good substrate for PRMT5. Previous studies have shown that arginine residues in the R*X*G motif and the sequences containing proline at either or both of these positions *X*(P)R*XXX*(P) were significantly favored to be methylated by arginine methyltransferase (27, 28). To pinpoint the PRMT5-mediated methylation sites in GATA4, we examined the protein sequence of GATA4 and identified the arginine residues at positions of 229 (Arg-229), 265 (Arg-265), and 317 (Arg-317) as potential sites (Fig. 2*B*). To test whether these sites are methylated by PRMT5, we generated GATA4 single or triple mutants (R229W, R265W, R317W, and R229W/R265W/R317W). FLAG-tagged PRMT5 together with either wild-type GATA4 or GATA4 mutants were co-transfected into HEK293 cells. The methylation of GATA4 was detected by immunoprecipitation followed by Western blot using the specific anti-dimethylation symmetric antibody. As shown in Fig. 2, *B* and *C*,

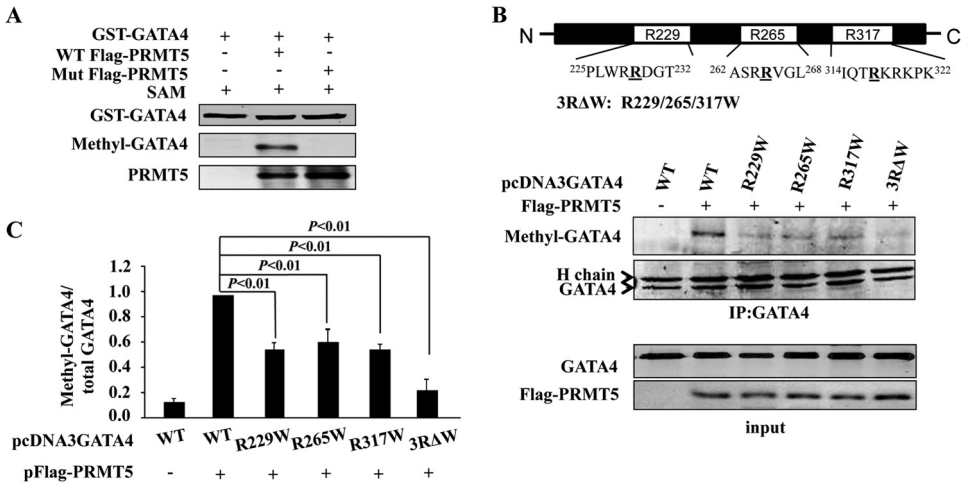

FIGURE 2. **PRMT5 directly methylates GATA4.** *A*, *in vitro* methylation assay was performed using recombinant GST-GATA4 together immunoprecipitated with either wild-type FLAG-PRMT5 (*WT FLAG-PRMT5*) or mutant FLAG-PRMT5 (*Mut FLAG-PRMT5*) in the presence of S-adenosylmethionine (*SAM*). The proteins were separated by SDS-PAGE, and detected with anti-dimethyl-arginine symmetric antibody. *B*, *in vivo* methylation assays were performed with wild-type GATA4 or GATA4 mutants together with FLAG-PRMT5. Wild-type GATA4 or GATA4 mutant expression vectors were co-transfected with PRMT5 expression vector into HEK293 cells. 48 h after transfection GATA4 was immunoprecipitated (*IP*) with anti-GATA4 antibody, and the methylation of GATA4 was determined by using anti-dimethyl-arginine symmetric antibody. *C*, densitometric analysis of GATA4 methylation as shown in the *panel B*.

single mutation of GATA4 at the positions of 229, 265, and 317 resulted in a significant reduction of GATA4 methylation, approximately by 30% reduction from each location, whereas simultaneous mutation of all three sites markedly attenuated PRMT5-dependent GATA4 methylation by ~80%. Taken together, these results indicate that GATA4 is a substrate for PRMT5 and that GATA4 methylation most likely occurs at multiple sites, including Arg-229, Arg-265, and Arg-317.

PRMT5 Negatively Regulates GATA4 Activity—GATA4 has been shown to regulate expression of various cardiac genes, such as ANF and BNP, and its activity can be potentiated by p300 mediated acetylation (10). Thus, we sought to determine whether PRMT5 affects the transcriptional activity of GATA4. To end this we performed a BNP promoter-driven luciferase (BNP-Luc) assay in HEK293 cells. PRMT5 together with either wild-type or GATA4 mutant, p300, and BNP-Luc plasmids were co-transfected into HEK293 cells. As shown in Fig. 3*A*, overexpression of GATA4 alone or in combination with p300 substantially increased the BNP promoter activity, which was markedly inhibited, when PRMT5 was co-expressed. In contrast, the expression of PRMT5 had no effect on the BNP-Luc activity in the absence of GATA4. To further examine the inhibitory effect of PRMT5 on GATA4 DNA binding activity, we performed EMSA. As shown in Fig. 3, *B* and *C*, overexpression of PRMT5 markedly attenuated the GATA4 DNA binding activity. Consistent with previously reported studies (10), ectopic expression of p300 significantly increased the GATA4 DNA binding activity. However, this increased activity was markedly attenuated in the presence of PRMT5. The specificity of GATA4 binding activity was confirmed by both competition assays with unlabeled oligonucleotides and supershift assays using anti-GATA4 antibody. Therefore, our results strongly suggest that the interaction of PRMT5 with GATA4 negatively regulates GATA4 DNA binding activity. Interestingly, we also found that mutation of GATA4 at either R229W or R265W did not significantly affect the inhibition of p300-induced GATA4 transcriptional activity by PRMT5. However, when GATA4 was mutated at R317W, the inhibitory effect of PRMT5 on p300-induced GATA4 transcriptional activity was completely abolished (Fig. 3*D*), hence showing that the arginine methylation of GATA4 at position 317 is required for the PRMT5-mediated inhibition of GATA4 activity.

To further determine the mechanism underlying the inhibition of GATA4 transcriptional activity by PRMT5, we examined whether methylation of GATA4 by PRMT5 inhibits its acetylation by p300. In this regard, we co-expressed p300 and FLAG-PRMT5 together with either wild-type GATA4 or GATA4 mutants in HEK293 cells. Nuclear extracts from these cells were subjected to immunoprecipitation with anti-GATA4 antibody followed by Western blot with anti-symmetric dimethylation, anti-acetylated lysine, anti-p300, and anti-FLAG antibodies, respectively. As shown in Fig. 3*E*, GATA4 was robustly acetylated in HEK293 cells when p300 was co-expressed. However, in the presence of PRMT5, the p300-mediated acetylation of GATA4 was substantially attenuated. Likewise, mutation of Arg-229 and Arg-265 to Trp-229 and Trp-265 in GATA4 had no effect on the PRMT5-mediated inhibition of GATA 4 acetylation by p300, albeit the methylation of GATA4 was significantly attenuated. In contrast, mutation of Arg-317 to Trp-317 in GATA4 completely abolished the inhibition of PRMT5 on p300-mediated GATA4 acetylation, further suggesting that arginine methylation of GATA4 at position 317 is required for the inhibition of p300-mediated GATA4 acetylation by PRMT5 (Fig. 3, *E* and *F*).

Overexpression of PRMT5 Attenuates PE-induced Cardiomyocyte Hypertrophy—Because GATA4 is critically involved in cardiac hypertrophy (4, 8, 9), we then investigated the effect of PRMT5 on PE-induced hypertrophy in cultured neonatal rat cardiomyocytes. As shown in Fig. 4, *A–C*, PE stimulation resulted in sarcomere organization and a marked increase of cell surface area and protein/DNA ratio; however, all these parameters were substantially inhibited in cardiomyocytes pretransduced with adenovirus bearing PRMT5 (Ad-PRMT5). Similarly, as shown in Fig. 4*D*, overexpression of PRMT5 mark-

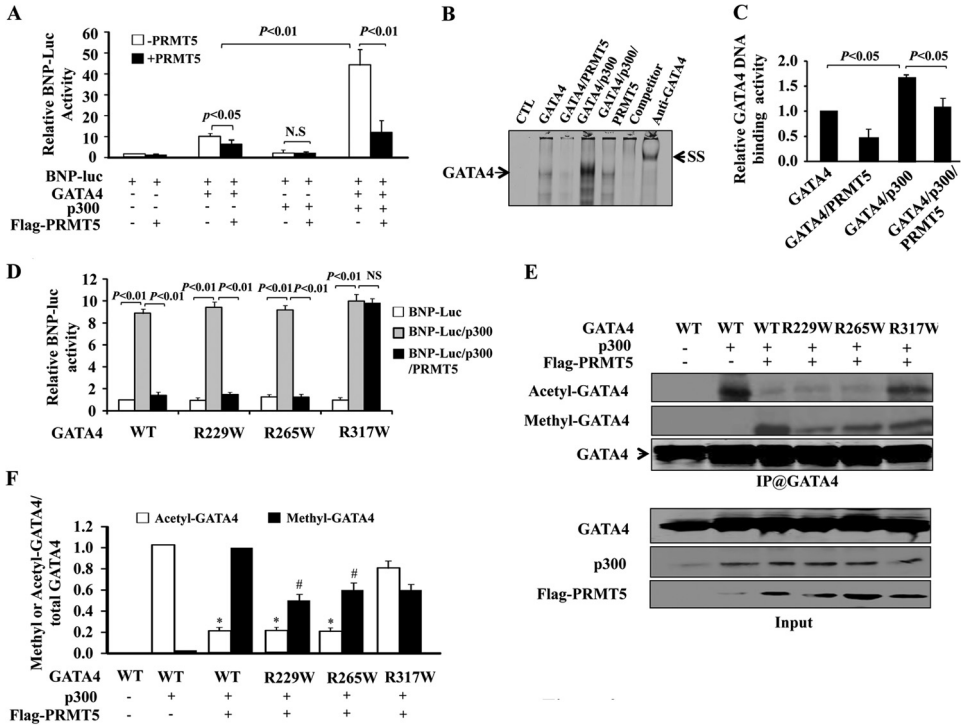

FIGURE 3. **PRMT5 inhibits GATA4 activation.** *A*, HEK293 cells cultured in 6-well plates were co-transfected with 300 ng of BNP-Luc, 100 ng of pcDNA-GATA4, 100 ng of pCMV-p300, and 300 ng of pFLAG-PRMT5 as indicated, and 50 ng RL-SV40 was used as a control. The total DNA content was equalized in each well. The relative promoter activities were calculated from the ratio of firefly to Renilla luciferase activities. 36 h after transfection, luciferase assays were performed. *N.S.*, not significant. *B* and *C*, HEK293T cells were transfected with pcDNA-GATA4, pCMV-p300, and pCMV-PRMT5 as indicated. 48 h after transfection, nuclear protein was extracted, and EMSA was performed. The GATA4 complex was partially supershifted by anti-GATA4 antibody and blocked by 200× unlabeled oligonucleotides. For the supershift (*SS*) assay 5 μl of GATA4 antibody was added to the binding reaction. Quantitative data from three independent experiments are shown in *C*. *D*, HEK293 cells in 24-well plates were co-transfected with 100 ng of BNP-Luc, 100 ng of wild-type pcDNA-GATA4 (WT), or 100 ng of mutant GATA4 (R226W, R265W, and R317W), 100 ng of pCMV-p300, and 100 ng of pFLAG-PRMT5 as indicated. 50 ng of RL-SV40 was used as a control. 36 h after transfection, luciferase assays were performed. *E* and *F*, methylation of GATA4 at R317 blocks GATA4 acetylation. HEK293 cells were co-transfected with pCMV-p300, pFLAG-PRMT5, wild-type pcDNA-GATA4 (*WT*), or GATA4 mutants as indicated. 48 h after transfection, nuclear extracts were immunoprecipitated (*IP*) with anti-GATA4 antibody and subjected to immunoblotting sequentially with anti-acetylated-lysine and anti-dimethyl-arginine symmetric antibody. The amount of methylation and acetylation of GATA4 was quantified in *F* (*, $p < 0.01$ compared with WT GATA4 + p300 group; #, $p < 0.01$ compared with WT GATA4 + p300 +PRMT5 group).

edly inhibited both basal and PE-induced expression of ANF and BNP in cardiomyocytes as determined by qRT-PCR. In contrast, transduction of cardiac cells with adenovirus bearing LacZ (Ad-LacZ) had no effect on the expression of ANF and BNP. The effect of PRMT5 on PE-induced ANF expression was further confirmed by immunostaining using anti-ANP antibody in cardiomyocytes (Fig. 4*E*). Taken together, our findings indicate that overexpression of PRMT5 inhibits PE-induced hypertrophic responses in NRVMs.

PRMT5 Inhibits GATA4 Transcriptional Activity by Blocking Its Acetylation in NRVMs—To further delineate the molecular mechanism by which PRMT5 inhibits PE-induced cardiomyocyte hypertrophy, we examined the functional role of PRMT5 interaction with GATA4 in NRVMs. The effect of PRMT5 on GATA4 transcriptional activity in cardiac cells was determined by measuring BNP promoter-driven luciferase activity. As shown in Fig. 5*A*, transfection of PRMT5 substantially inhibited GATA4-dependent and p300-augmented GATA4 transcriptional activity in the absence and presence of PE stimulation. Similarly, as shown in Fig. 5*B*, treatment of cardiomyocytes with PE markedly increased the GATA4 DNA binding activity in Ad-LacZ-transduced cardiomyocytes, as determined by EMSA. Moreover, overexpression of PRMT5 in cardiomyocytes substantially inhibited the PE-induced GATA4 DNA binding activity, which is consistent with our results obtained in HEK293 cells (Fig. 3*B*). In addition, binding of GATA4 to BNP promoter was significantly increased by PE stimulation; however, this effect was dramatically attenuated by overexpression of PRMT5 (Fig. 5*C*). To further investigate the mechanism(s) responsible for the inhibitory effect of PRMT5 on PE-induced cardiomyocyte hypertrophy, we examined whether PRMT5 had an effect on the acetylation of GATA4 in cardiomyocytes. To this end cardiomyocytes were transduced with either Ad-PRMT5 or Ad-LacZ, and the nuclear extracts were then subjected to immunoprecipitation with anti-GATA4 antibody followed by Western blot to detect the acetylation and methylation of GATA4 in the presence or absence of PE stimulation. As shown in Fig. 5*D*, in cardiomyocytes transduced with Ad-LacZ, PE stimulation had no effect on PRMT5 expression but significantly inhibited the interaction of PRMT5 with GATA4 and GATA4 methylation, which is accompanied by a robust increase in GATA4 acetylation. Overexpression of PRMT5 further increased the interaction of PRMT5 with GATA4 and methylation of GATA4, both of which, however, were markedly inhibited after PE stimulation. Accordingly, the acetylation of GATA4 was concomitantly increased but still much lower than that in cardiomyocytes transduced with Ad-LacZ. Taken together, these results suggest that methylation of GATA4 by

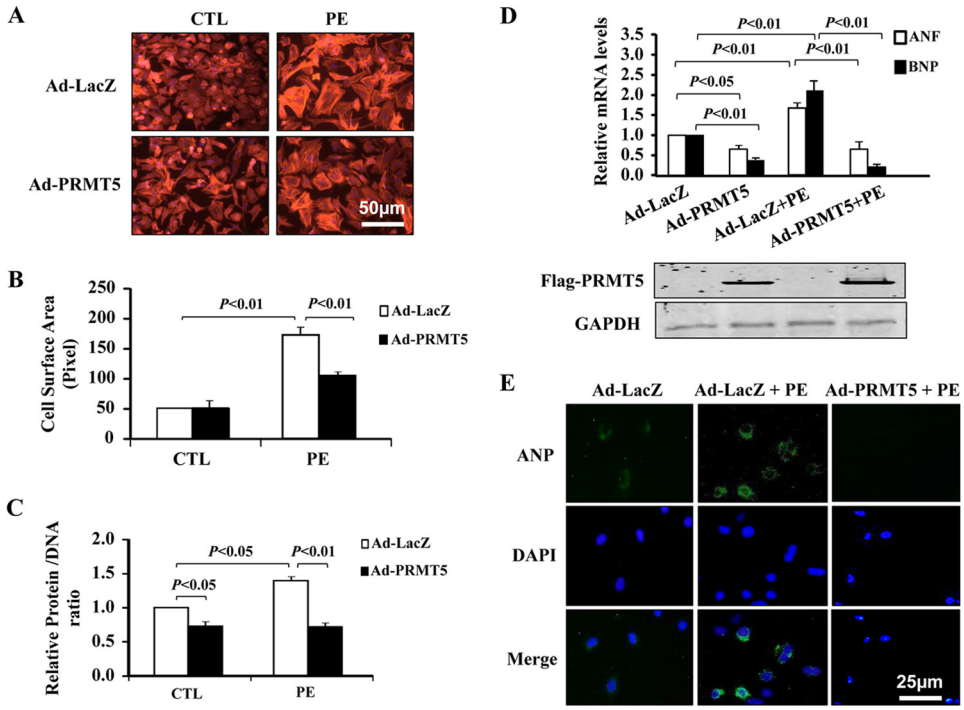

FIGURE 4. **Overexpression of PRMT5 blocks PE-induced hypertrophy in NRVMs.** *A*, myocytes cultured in 6-well plates were infected with either Ad-PRMT5 or Ad-LacZ (50 m.o.i.) and stimulated with PE (50 μM) for 48 h. The cells were washed, fixed, and stained with Alexa Fluor® 546 phalloidin to show the sarcomere organization (*red*). DAPI staining was done to visualize the nuclei (*blue*). *Scale bar*, 50 μm. *B*, mean myocyte surface area was obtained from 200 myocytes per well. Each *column* represents the mean of three wells obtained from three preparations. *C*, myocytes were seed in 6-well plates and then transduced with either Ad-Lac Z or Ad-PRMT5 (50 m.o.i.) for 48 h. After 48 h of treatment with or without 50 μM PE, the pellet was suspended in the saline-sodium citrate buffer plus 0.25% SDS. Samples were then split into aliquots for protein and DNA measurements. *D*, ANF and BNP transcripts were examined by qRT-PCR. The expression of FLAG-tagged PRMT5 was determined by Western blot (*low panel*). *E*, cardiomyocytes were transduced with Ad-PRMT5 or Ad-LacZ (50 m.o.i.). 24 h after transduction, myocytes were cultured in serum-free medium for 24 h before the stimulation with PE (50 μM) for 48 h. The expression of ANP was determined by immunofluorescent staining using anti-ANP antibody. *Scale bar*, 25 μm.

PRMT5 reversibly regulates the acetylation of GATA4 in cardiomyocytes.

Accumulating evidence suggests that the functional role of PRMT5 is regulated by its subcellular localization (29–33). Disruption of PRMT5 interaction with GATA4 by PE stimulation prompted us to speculate that PE stimulation may regulate the subcellular localization of PRMT5 in cardiomyocytes. As shown in Fig. 6*A*, under unstimulated conditions, GATA4 is colocalized with PRMT5 primarily in the nucleus of cardiomyocytes. However, after PE stimulation, PRMT5 is redistributed to the cytoplasm of cardiomyocytes, as demonstrated by both immunostaining and Western blotting analysis (Fig. 6, *A* and *B*).

Knockdown of PRMT5 Induces Cardiomyocyte Hypertrophy—To further substantiate the functional significance of endogenous PRMT5 in cardiomyocytes, we performed loss-of-function studies using RNA interference technique. As shown in Fig. 7*A*, transfection of cardiomyocytes with PRMT5 siRNA markedly inhibited expression of PRMT5 by ~80%, as determined by both qRT-PCR and Western blot analysis. As expected, PE stimulation resulted in hypertrophic responses, as demonstrated by an increase in stress fiber formation, cell surface area, and protein/DNA ratio in control siRNA-transfected cardiomyocytes (Fig. 7, *B--D*). Remarkably, knockdown of PRMT5 resulted in a potent hypertrophic response, as reflected by increased cell surface area and protein/DNA ratio. Furthermore, PE-induced hypertrophic responses were further enhanced in PRMT5 knockdown cardiomyocytes. Likewise, knockdown of PRMT5 markedly increased the base-line expression of ANF and BNP, as determined by qRT-PCR (Fig. 7*E*), whereas the expression of ANF and BNP in PE-stimulated cardiac myocytes was barely affected. Moreover, both BNP promoter activity and GATA4 DNA binding activity were markedly increased in PRMT5 knockdown cardiomyocytes (Fig. 7, *F* and *G*). Collectively, these results suggest that PRMT5 is a critical regulator in controlling cardiomyocyte hypertrophy.

DISCUSSION

In the present study we identify PRMT5, a protein arginine methyltransferase, as a novel negative regulator of cardiomyocyte growth. We demonstrate that ectopic expression of PRMT5 potently inhibits PE-induced increases in protein synthesis, cell size, and expression of hypertrophic genes, whereas knockdown of PRMT5 induces hypertrophic genetic programs, thus implicating PRMT5 as a critical regulator of cardiomyocyte remodeling. Importantly, we demonstrate that PRMT5 specifically interacts with GATA4 and methylates GATA4 primarily at Arg-229, Arg-265, and Arg-317, hence leading to an inhibition of p300-induced GATA4 acetylation and transcriptional activation. Mechanistically, we demonstrate that both GATA4 interaction with PRMT5 and its arginine methylation are substantially disrupted in cardiomyocytes by PE stimulation, which in turn leads to an increased GATA4 acetylation and a subsequent induction of cardiomyocyte hypertrophy. Thus, we for the first time provide compelling evidence sug-

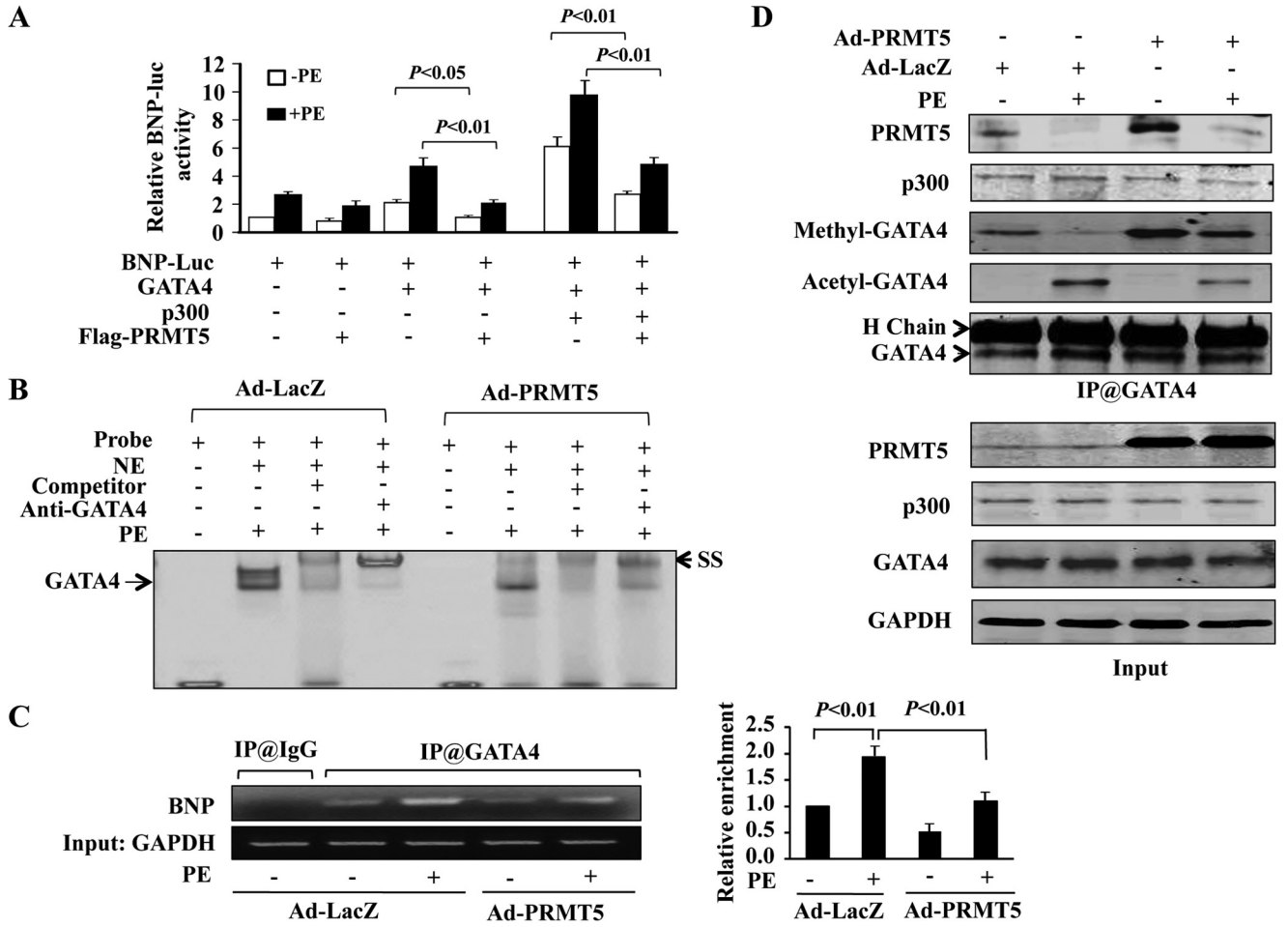

FIGURE 5. **PRMT5 attenuates GATA4 activity by inhibiting its acetylation in NRVMs.** *A*, cardiomyocytes cultured in 24-well plates were transfected with 300 ng of BNP-Luc together with either 100 ng of pcDNA-GATA4, 100 ng of pCMV-p300, 300 ng of pFLAG-PRMT5, or empty vector as indicated and then stimulated with or without PE (50 μM) for 48 h. A luciferase assay was performed. *B*, cardiomyocytes were transduced with Ad-PRMT5 or Ad-LacZ (50 m.o.i.). 24 h after transduction, myocytes were cultured in serum-free medium for 24 h before the stimulation with PE (50 μM) for 48 h. Nuclear protein (*NE*) was extracted, and EMSA was performed. The GATA4 complex was supershifted (*SS*) by anti-GATA4 antibody and blocked by 200 times of unlabeled oligonucleotides. *C*, PRMT5 attenuates the binding activity of GATA4 to BNP promoter. Myocytes were seeded in 6-well plates and then transduced with either Ad-LacZ or Ad-PRMT5 (50 m.o.i.) for 48 h. After 1 h of treatment with or without 50 μM PE, ChIP assays were performed using the ChIP assay. *IP*, immunoprecipitate. *D*, cardiomyocytes were transduced with Ad-PRMT5 or Ad-LacZ (50 m.o.i.) and stimulated with PE (50 μM) for 48 h. Nuclear protein was extracted, and immunoprecipitation was then performed with anti-GATA4 antibody and then separated by 10% SDS-PAGE (total cell lysates were used as input) followed by immunoblotting with anti-acetylated-lysine, anti-dimethyl-arginine symmetric antibody, anti-p300, anti-PRMT5, and anti-GATA4 antibody as indicated.

gesting that PRMT5-dependent arginine methylation of GATA4 is a critical post-translational event required for suppressing cardiomyocyte growth.

GATA transcription factors, particularly GATA4, GATA5, and GATA6, have emerged as important regulators in cardiac development and postnatal heart remodeling (3, 4, 8, 11). Indeed, both loss- and gain-of-function of studies have underscored the critical importance of GATA4 in cardiac remodeling. For instance, selective overexpression of GATA4 in the heart has been shown to result in cardiac hypertrophy, whereas overexpression of dominant negative GATA4 suppresses the hypertrophic responses in cardiomyocytes (8), indicating an essential role of GATA4 in cardiac remodeling under pathophysiological conditions. A growing body of evidence suggests that the GATA4 transcriptional activity is regulated primarily through post-translational modifications, including phosphorylation, acetylation, sumoylation, and protein-protein interactions (10–12). In response to hypertrophic stimuli, such as endothelin-1, PE, and isoproterenol, GATA4 is phosphorylated at Ser-105 by the MAPK pathway, which in turn enhances its transcriptional activity and promotes cardiomyocyte hypertrophy (34). In addition, GATA4 is subjected to the lysine modifications via acetylation and sumoylation (10, 11). Indeed, the transcriptional co-activator p300 interacts with GATA4 and enhances its transcriptional activity by acetylating lysine residues in the C-terminal region (35, 36). Furthermore, cardiac overexpression of intact p300 has been shown to induce acetylation of GATA4 and cardiac hypertrophy and promote left ventricular remodeling after myocardial infarction *in vivo* (37). However, overexpression of mutant p300 lacking HAT (histone acetyltransferase) activity diminishes these effects, suggesting that p300-induced acetylation of GATA4 is required for the development of cardiac hypertrophy (37). At this point the molecular mechanism underlying the regulation of p300-mediated acetylation of GATA4 in the heart has not been clearly elucidated. Recently, arginine methylation of histone and nonhistone proteins has been recognized as an important mechanism involved in regulating cell proliferation, differentiation,

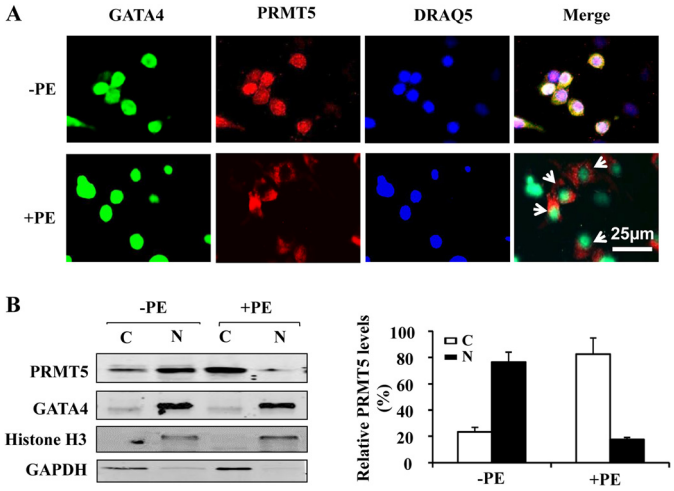

FIGURE 6. **PE stimulation promotes PRMT5 cytoplasmic translocation.** *A*, myocytes cultured in 6-well plates were starved in serum-free medium for 24 h before the stimulation with PE (50 μM) for 4 h. The cells were then washed, fixed, and stained with anti-GATA4 monoclonal antibody and anti-PRMT5 polyclonal antibody (the *white arrow* indicates PRMT5 cytoplasmic translocation). *Scale bar*, 25 μm. *B*, myocytes were starved in serum-free medium for 24 h before the stimulation with PE (50 μM) for 4 h. The cytoplasmic (*C*) and nuclear (*N*) fractions of cardiomyocytes stimulated with or without PE were detected by using anti-PRMT5 and anti-GATA4 antibodies.

and signal transduction (14–17). At this time whether GATA4 is subjected to arginine modification has not been reported. Intriguingly, in two independent studies, both GATA4 and PRMT5 have been shown to be associated with SWI/SNF (hSWI/SNF) complexes (26, 38), which prompted us to speculate that PRMT5 may potentially interact with GATA4. Indeed, in the present study we demonstrate that PRMT5 specifically interacts with the zinc finger domain of GATA4 in both co-transfected HEK293T cells and native cardiomyocytes (Fig. 1). This interaction is functionally important because it promotes GATA4 methylation, which leads to an inhibition of GATA4 transcriptional activity and cardiomyocyte hypertrophy.

The molecular mechanism underlying inhibition of GATA4 transcriptional activity by PRMT5 remains elusive but certainly involves the inhibition of p300-dependent acetylation of GATA4. In HEK293 cells, co-transfection of PRMT5 with GATA4 modestly inhibited GATA4 activity but profoundly inhibited the p300-enhanced GATA4 transcriptional activity. In the present study we demonstrated that PRMT5 directly methylates GATA4, primarily at Arg-229, Arg-265, and Arg-317. Mutation of either Arg-229 or Arg-265 in the zinc finger domain of GATA4 had no effect on PRMT5-mediated inhibition of GATA4 activity. However, mutation of Arg-317 completely abolished the inhibition of GATA4 activity by PRMT5, thus implicating a critical role of PRMT5-mediated GATA4 methylation at Arg-317 in regulating GATA4 transcriptional activity. Indeed, p300 has been to shown to induce lysine acetylation of GATA4 at positions of 311, 318, 320, and 322 (39). Considering the close proximity of Arg-317 to these acetylated residues, it is reasonable to speculate that methylation of Arg-317 in GATA4 might induce a conformational change and create a steric hindrance that blocks the lysine acetylation of GATA4 by p300. In contrast to PRC2-induced methylation of GATA4 at Lys-299 that inhibits the interaction of p300 with GATA4 (12), arginine methylation of GATA4 by PRMT5 marginally affects the interaction of p300 with GATA4 in cardiomyocytes (Fig. 5*D*), further suggesting an allosteric regulation of GATA4 acetylation by methylation at its adjacent arginine residues. Furthermore, we postulate that arginine methylation of GATA4 by PRMT5 may play an important role in regulating other post-translational modifications of GATA4, such as phosphorylation, sumoylation, ubiquitination, and its interaction with other cardiac transcription factors, such as NFAT and GATA6 that are involved in the development of cardiac hypertrophy under certain pathological circumstances. Ongoing studies are currently testing these hypotheses.

In mammalian cells PRMT5 has been shown to localize in both cytoplasm and nucleus and methylate multiple histone and non-histone proteins implicated in diverse cellular and biological processes, including transcriptional regulation, cell cycle progression (15), RNA metabolism (40), ribosome biogenesis (41), and Golgi apparatus structure maintenance (42). In the nucleus, PRMT5 has been found in the SWI/SNF and NURD chromatin-remodeling complexes (26, 38, 43) where it methylates histones as well as transcription factors/regulators, which normally results in a transcriptional repression (15). In the cytoplasm PRMT5 forms a 20 S protein arginine methyltransferase complex consisting of spliceosomal snRNP Sm proteins, PRMT5, pICln, and WD repeat protein (MEP50/WD45) (44–46), which is essentially involved in pre-mRNA splicing. Depending on its cellular localization, PRMT5 has been shown to exert different or even opposite effects. For example, in cytoplasm PRMT5 is required for the growth of prostate cancer cells in a methyltransferase-dependent manner, whereas PRMT5 in the nucleus inhibits prostate cancer cell growth (29). In this study we demonstrate that PE stimulation causes a rapid translocation of PRMT5 from the nucleus to the cytoplasm, which subsequently relieves its repression on GATA4 transcriptional activity, thus leading to an increased expression of hypertrophic genes in cardiomyocytes. In addition to its critical roles in cardiac hypertrophy, GATA4 is also involved in cardiomyocyte survival (47); thus it would be very interesting to investigate whether the PRMT5/GATA4 pathway plays a role in regulating cardiac cell survival and apoptosis under both physiological and pathological conditions, such as in the aging heart. Furthermore, the physiological significance of PRMT5 in exercise-induced hypertrophy and normal cardiovascular development remains unclear but clearly warrants further investigation.

In summary, our data support PRMT5 as an important regulator of cardiomyocyte hypertrophy through its interaction with GATA4. This interaction is functional, as it leads to decreases GATA4 activity, and it is likely physiological, as it suppresses cardiac hypertrophy as a constitutive negative regulator in the unstimulated cardiomyocytes. However, in the presence of hypertrophic stresses, PRMT5 is translocated to the cytoplasm, leading to activation of GATA4 and expression of hypertrophic genes. The mice with PRMT5 gene deficiency are embryonic-lethal due to the abrogation of pluripotent cells in blastocysts (30), which prevents assessing the functional importance of PRMT5 in the heart at this time. Generation of cardiac-specific transgenic or knock-out mice of PRMT5 will

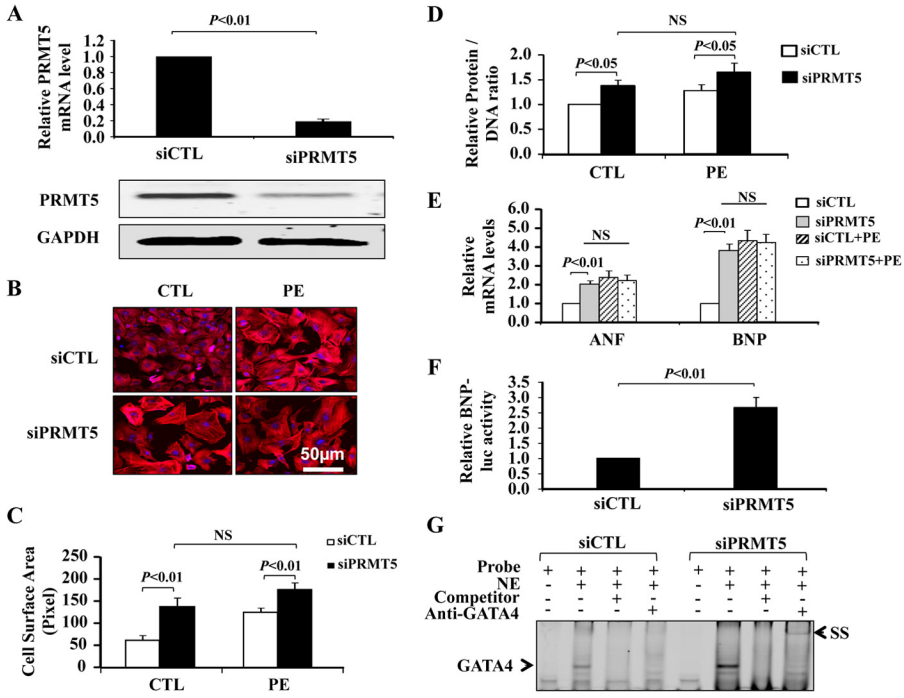

FIGURE 7. **Knockdown of PRMT5 induces cardiomyocyte hypertrophy.** *A*, myocytes were seeded in 6-well plates 72 h after transfection of either PRMT5 siRNA (*siPRMT5*) or control siRNA (*siCTL*), and the expression of PRMT5 in NRVMs was determined by qRT-PCR and Western blot analysis. *B* and *C*, 72 h after transfection of either PRMT5 siRNA or control siRNA, and NRVMs were treated with PE (50 μM) for 48 h. The cells were washed, fixed, and stained with Alexa Fluor® 546 phalloidin to visualize sarcomere organization. Mean myocyte surface area was obtained from 200 myocytes per well. Each column represents mean of three wells obtained from three culture preparations. *Scale bar*, 50 μm. *N.S.*, not significant. *D*, cell pellets were suspended in the saline-sodium citrate buffer plus 0.25% SDS. Samples were then split into aliquots for protein and DNA measurements. *E*, myocytes were seeded in 6-well plates 48 h after transfection of PRMT5 siRNA (*siPRMT5*) or control siRNA (*siCTL*), the cells were stimulated with PE (50 μM) for 48 h, and the expression of ANF and BNP transcripts was examined by real-time PCR. *F*, NRVMs cultured in 24-well plates were transfected with PRMT5 siRNA (*siPRMT5*) or control siRNA (*siCTL*) for 48 h, then transfected with 300 ng of BNP-Luc for 36 h. Luciferase assay was performed. *G*, cardiomyocytes were transfected with PRMT5 siRNA (*siPRMT5*) or control siRNA (*siCTL*). 72 h after transfection nuclear protein was extracted (*NE*), and EMSA was performed. The GATA4 complex was supershifted (*SS*) by anti-GATA4 antibody and blocked by 200× unlabeled oligonucleotides.

be critical for further elucidating the functional significance of PRMT5 *in vivo*. Moreover, identification of relevant physiological substrates of PRMT5 under various biological conditions will provide a clearer mechanistic understanding of its roles in regulating cardiac function.

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