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MicroRNA 26b Encoded by the Intron of Small CTD Phosphatase (SCP) 1 Has an Antagonistic Effect on Its Host Gene

Naoya Sowa,[1] Takahiro Horie,[1] Yasuhide Kuwabara,[1] Osamu Baba,[1] Shin Watanabe,[1] Hitoo Nishi,[1] Minako Kinoshita,[1] Rieko Takanabe-Mori,[2] Hiromichi Wada,[2] Akira Shimatsu,[2] Koji Hasegawa,[2] Takeshi Kimura,[1] and Koh Ono[1*]

[1]*Department of Cardiovascular Medicine, Graduate School of Medicine, Kyoto University, Kyoto 606-8507, Japan*
[2]*Division of Translational Research, Kyoto Medical Center, National Hospital Organization, Kyoto 612-8555, Japan*

ABSTRACT

Tissue-specific patterns of gene expression play an important role in the distinctive features of each organ. Small CTD phosphatases (SCPs) 1–3 are recruited by repressor element 1 (RE-1)-silencing transcription factor/neuron-restrictive silencer factor (REST/NRSF) to neuronal genes that contain RE-1 elements, leading to neuronal gene silencing in non-neuronal cells. SCPs are highly expressed in the heart and contain microRNAs (miR)-26b, 26a-2, and 26a-1 with the same seed sequence in their introns. Therefore, we tried to investigate the roles of miR-26b and its host gene in neonatal rat cardiomyocytes. Overexpression of miR-26b suppressed the mRNA expression levels of ANF, βMHC, and ACTA1 and reduced the cell surface area in cardiomyocytes. We confirmed that miR-26b targets the 3′ untranslated region (3′UTR) of GATA4 and canonical transient receptor potential channel (TRPC) 3. Conversely, silencing of the endogenous miR-26b family enhanced the expression levels of TRPC3 and GATA4. On the other hand, overexpression of SCP1 induced the mRNA expression of ANF and βMHC and increased the cell surface area in cardiomyocytes. Next, we compared the effect of overexpression of SCP1 with its introns and SCP1 cDNA to observe the net function of SCP1 expression on cardiac hypertrophy. When the expression levels of SCP1 were the same, the overexpression of SCP1 cDNA had a greater effect at inducing cardiac hypertrophy than SCP1 cDNA with its intron. In conclusion, SCP1 itself has the potential to induce cardiac hypertrophy; however, the effect is suppressed by intronic miR-26b in cardiomyocytes. miR-26b has an antagonistic effect on its host gene SCP1. J. Cell. Biochem. 113: 3455–3465, 2012.

KEY WORDS: CARDIAC HYPERTROPHY; microRNA; INTRON

MicroRNAs (miRNAs) are endogenous, single-stranded, small, ~22-nucleotide noncoding RNAs. miRNAs are generally considered to negatively regulate gene expression by inhibiting translation and/or promoting mRNA degradation by base-pairing to complementary sequences within the 3′ untranslated region (3′UTR) of protein-coding mRNA transcripts [Bagga et al., 2005; Humphreys et al., 2005; Kiriakidou et al., 2007]. Each of the over 1,400 known human miRNAs is likely to regulate hundreds of mRNAs and thus has the potential to affect many biological processes (miRBase; http://www.mirbase.org/). Several miRNAs are expressed ubiquitously, while others display tissue-specific expression patterns, which suggests that they have unique functions, such as organ development, cell proliferation, cell differentiation, and apoptosis, within specific tissues. The expression of a family of class-C RNA polymerase II (RNAPII) carboxyl-terminal domain (CTD) phosphatases [small CTD phosphatases (SCPs) 1–3] is restricted to non-neuronal tissues. SCPs 1–3 are recruited by the repressor element 1 (RE-1)-silencing transcription factor/neuron-restrictive silencer factor (REST/NRSF) to neuronal genes that contain RE-1 elements, leading to neuronal gene-silencing in non-

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*Correspondence to: Koh Ono, MD, PhD, Department of Cardiovascular Medicine, Kyoto University, 54 Shogoin-Kawaharacho, Sakyo-ku, Kyoto 606-8507, Japan. E-mail: kohono@kuhp.kyoto-u.ac.jp
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neuronal cells [Chong et al., 1995; Schoenherr and Anderson, 1995; Chen et al., 1998]. SCPs are highly expressed in the heart and contain miRNAs (miR)-26b, 26a-2, and 26a-1 in their introns (miRBase). Therefore, these miRNAs may have specific functions in the heart.

Gene expression-modulating miRNAs are encoded in diverse genomic locations, including intergenic regions, introns of protein-coding genes, and introns/exons of non-coding RNA genes [Griffiths-Jones et al., 2008]. While most human miRNAs lie between protein-coding genes, about one-third are within the introns of annotated mRNAs [Rodriguez et al., 2004; Saini et al., 2007]. These intronic miRNAs have three unique features that are different from the intergenic miRNAs in the process of biogenesis. First, they are derived from the processing of pre-mRNA introns, and are spliced out of the transcript of the encoded gene. Thus, the intronic miRNAs share the same promoter as the encoded gene products. Second, the generation of these miRNAs requires Pol-II and RNA splicing components during their biogenesis [Lin et al., 2003; Ying and Lin, 2004]. Third, nearly 30% of the spliced introns are exported out of the nucleus, and have a moderate half-life in the cytoplasm [Clement et al., 1999], suggesting that these introns function as miRNAs in cells [Cullen, 2004].

The coregulation of a miRNA with its host gene typically exhibits one of two main functions: (i) an antagonistic effect by the miRNA-mediated knock-down of genes with perturbing effects on a pathway or a biological process activated by the host gene, or (ii) a synergistic effect by the miRNA-mediated fine tuning of a target gene generating a positive effect on the host gene. The present study sought to determine the function of miR-26b in cardiomyocytes and to compare the functions of miR-26b and its host gene, SCP1. It has been reported that many miRNAs contribute to cardiac hypertrophy [Gladka et al., 2012]. It is difficult to address the effect of miR-26b with other miRNAs. However, miR-26b is expressed with its host gene in parallel; it may modulate the effect of SCP1 in cardiomyocytes.

Transgenic mice that expressed cardiac-specific canonical transient receptor potential channel (TRPC) 3 clearly showed an association between store-operated Ca^{2+} entry and calcineurin-nuclear factor of activated T cells (NFAT) 3 activation [Nakayama et al., 2006]. NF-AT3 interacts with the cardiac zinc finger transcription factor GATA4, resulting in the synergistic activation of cardiac transcription for hypertrophy, including ANF [Molkentin et al., 1998]. Transgenic mice that express TRPC3, activated forms of calcineurin or NF-AT3, and GATA4 in the heart develop cardiac hypertrophy [Molkentin et al., 1998; Liang et al., 2001; Nakayama et al., 2006]. We confirmed that miR-26b targets the 3′UTR of GATA4 and TRPC3. Since miR-26b expression is reduced in the mouse model of cardiac hypertrophy, it is possible that TRPC3 and GATA4 protein can be up-regulated as a result of miR-26-dependent post-transcriptional regulation.

In this study, we compared the function of miR-26b and its host gene to determine the functional relationship between them. Our data indicated that SCP1 itself has the potential to induce cardiac hypertrophy; however, the effect is suppressed by its intronic miR-26b in cardiomyocytes.

METHODS

PLASMIDS

Expression vectors for the negative control and miR-26b were generated using BLOCK-iTTM PolIImiR RNAi Expression Vector Kits in accordance with the manufacturer's protocol (Invitrogen). The following primers were used to amplify and clone part of the 3′UTR of rat TRPC3 and GATA4 into a pMIR-REPORT^{TM} luciferase vector at the SpeI/HindIII sites in accordance with the manufacturer's instructions:

Rat TRPC3 3′UTR forward 5′-ACTAGTCAATAATATTCCTAAGTATGAAATACTTGAAAAACCGTTGTGTAAAAACTCAAG-3′, reverse 5′-AAGCTTGAGTTTTTACACAACGGTTTTTCAAGTATTTCATACTTAGGAATATTATTGACT-3′;

Rat GATA4 3′UTR forward 5′-CTAGTTGAAGAACAACTGGTAGAACTTGAAGTTGTTGACAATCACTTAGGGATAAGGGTA-3′, reverse 5′-CGCGTACCCTTATCCCTAAGTGATTGTCAACAACTTCAAGTTCTACCAGTTGTTCTTCAA-3′.

To create an anti-miR-26b (decoy) vector, the luciferase 3′UTR was modified to include six tandem sequences that were complementary to miR-26b and separated by three nucleotide spaces. Rat SCP1 was amplified from rat heart cDNA (BD Biosciences Clontech) using iProof DNA polymerase (Bio-Rad Laboratories, Inc.) and cloned into pcDNA3.1 (Invitrogen). A fragment of genomic SCP1 was amplified from rat genomic DNA using the same primers. The sequences of all constructs were analyzed using an ABI 3100 genetic analyzer. All of these constructs were correctly inserted into a pLenti6/V5-D-TOPO vector (Invitrogen) driven by a CMV promoter to stably express genes in neonatal rat cardiac myocytes (NRCMs) [Nishi et al., 2010].

ISOLATION OF NEONATAL RAT CARDIAC MYOCYTES AND GENERATION OF A MOUSE MODEL OF CARDIAC HYPERTROPHY

The investigation conformed to the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) and was approved by the Kyoto University Ethics Review Board (Med Kyo 09280). NRCMs were prepared as described previously [Horie et al., 2008]. Male 10-week-old C57BL/6 mice were treated with a transverse aortic constriction (TAC) procedure as described previously [Rockman et al., 1991].

WESTERN BLOTTING

Cell lysates were prepared as described previously and subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis followed by standard Western blotting procedures [Horie et al., 2008]. The primary antibodies used were: anti TRPC3 (ACC-016; Alomone), 1:1,000; anti-GATA4 (SC-1237; Santa Cruz Biotechnology), 1:1,000; heavy chain cardiac myosin antibody (ab15; Abcam), 1:100; anti-SCP1 (NBP-55978; Novus Biologicals), 1:1,000; anti-b-actin (A5441; Sigma–Aldrich), 1:1,000; and anti-GAPDH (#2118; Cell Signaling Technology), 1:1,000. As secondary antibodies, anti-rabbit (NA934; GE Healthcare), and anti-goat IgG (305-035-003; Jackson Immuno Research Laboratories) were used at a dilution of

1:2,000 and 1:40, respectively. Immunoblots were detected using an LAS-1000 system (FUJI FILM).

QUANTITATIVE REAL-TIME (q-RT) PCR FOR MRNA

Total RNA was isolated using TRIzol® reagent (Invitrogen) and treated with RNase-free DNase I (Takara). cDNA was synthesized using SuperScript II reverse transcriptase (Invitrogen) and the PCR reaction was performed on an ABI PRISM® 7900HT (Applied Biosystems) with a SYBR Green PCR master mix (Applied Biosystems), normalized with GAPDH. We used the delta–delta cycle threshold method for the quantification of gene expression. The efficiency of amplification was checked in all of the primer pairs. Gene-specific primers were as follows:

ANF sense, 5′-CGTGCCCCGACCCACGCCAGCATGGGCTCC-3′
ANF antisense, 5′-GGCTCCGAGGGCCAGCGAGCAGAGCCCTCA-3′;
β-MHC sense, 5′-GGAGCTCACCTACCAGACAGA-3′;
β-MHC antisense, 5′-CTCAGGGCTTCACAGGCATCC-3′;
ACTA1 sense, 5′-TCCCTGGAAAAGAGCTATGAGCT-3′;
ACTA1 antisense, 5′-GATCCCGCAGACTCCAT-3′;
TRPC3 sense, 5′-TCTATGCTTACGACGAGGA-3′;
TRPC3 antisense, 5′-CCTTGTAGGCATTGATCCTA -3′;
GATA4 sense, 5′-TCTCACTATGGGCACAGCAG -3′;
GATA4 antisense, 5′-GCGATGTCTGAGTGACAGGA -3′;
SCP1 sense, 5′-CCCCATGGACAGCTCGGCCGTCATTACT-3′;
SCP1 antisense, 5′-CTAGCTCCCGGGCCTAGGCTGTCT-3′;
GAPDH sense, 5′-TTGCCATCAACGACCCCTTC-3′;
and GAPDH antisense, 5′-TTGTCATGGATGACCTTGGC-3′

We used TaqMan MicroRNA Assays™ (Applied Biosystems) to determine the expression levels of miR-26b in accordance with the manufacturer's instructions.

LENTIVIRUS PRODUCTION AND DNA TRANSDUCTION

As described previously, lentiviral stocks were produced in 293FT cells in accordance with the manufacturer's protocol (Invitrogen) [Horie et al., 2008, 2010]. In brief, virus-containing medium was collected 48 h post-transfection and filtered through a 0.45-μm filter. One round of lentiviral infection was performed by replacing the medium with virus-containing medium (containing 8 μg of Polybrene® per ml), followed by centrifugation at 2,500 rpm for 30 min at 32°C. Cells were used for analysis 2 or 3 days after DNA transduction.

SIRNA-MEDIATED KNOCK-DOWN OF SCP1

The oligonucleotides used for siRNA of SCP1 were as follows: SCP1 siRNA-1: sense, (GATCCGCCGTCATTACTCAGATCATTCAAGAGA-TGATCTGAGTAATGACGGCTTTTTTAT) and antisense, (CGATAA-AAAAGCCGTCATTACTCAGATCATCTCTTGAATGATCTGAGTAAT-GACGGCG). SCP1 siRNA-2: sense, (GATCCGGTTTCTCTGCTGC-CAAATTTCAAGAGAATTTGGCAGCAGAGAAACCTTTTTTAT) and antisense, (CGATAAAAAAGGTTTCTCTGCTGCCAAATTCTCTTGAAA-TTTGGCAGCAGAGAAACCG).

A randomly shuffled form of siRNA-1 was used as a control. Every siRNA construct was made by the use of a pSINsi-mU6 vector (Takara Bio Inc.), and the siRNA-producing constructs were introduced into lenti-virus vector plasmid and transduced to cardiac myocytes. Two days after transduction, cells were used for analysis.

DUAL-LUCIFERASE ASSAYS AND LIPOFECTION

For atrial natriuretic factor (ANF) promoter assays, a pGL3-basic (Promega, Madison, WI) reporter plasmid containing the rat ANF gene promoter (3.4 kb nucleotides) was used [Kaburagi et al., 1999]. Cells were plated in 24-well plates and transiently transfected with firefly luciferase reporter plasmid (0.1 μg) and expression vectors (0.5 μg) using Lipofectamine™ Reagent (Invitrogen). A fixed amount (0.01 μg) of internal control reporter, Renilla reniformis luciferase, driven by the thymidine kinase (TK) promoter (pRL-TK: Promega) was also co-transfected to normalize the transfection efficiency. At 48 h after transfection, luciferase activities were measured using a dual luciferase kit (PicaGene® dual kit, Toyo Ink Co.). The relative luciferase activity of each construct (arbitrary units) was reported as the fold induction.

IMMUNOCYTOCHEMISTRY AND MEASUREMENT OF CELL SIZE

The investigation conformed to the *Guide for the Care and Use of Laboratory Animals*, published by the National Institutes of Health (NIH Publication No. 85-23, Revised 1996), and was approved by the Institutional Animal Research Committee of Kyoto University. Primary neonatal rat ventricular cardiac myocytes were prepared from Sprague–Dawley rats as described previously (9). The cardiac myocytes were grown on flask-style chambers with glass slides (Nalgen Nunc, Naperville, IL). The cells were then fixed with 3% formaldehyde in phosphate-buffered saline (PBS) for 15 min at room temperature. Immunocytochemical staining for β-myosin heavy chain (MHC) was performed using the indirect immunoperoxidase method. The anti-cardiac β-MHC polyclonal antibody (Novo-Castra, Newcastle, UK) was used at a dilution of 1:50. Sheep anti-mouse IgG, horseradish peroxidase conjugate (NA931) was used as a secondary antibody at a dilution of 1:200 (GE Healthcare). A total of 50 myocardial fibers were selected randomly from cardiac myocytes stained with anti-β-MHC antibody, and the surface areas of these cells were measured semiautomatically with the aid of an image analyzer (Luzex 3U; Nikon, Tokyo, Japan) as described previously [Abe et al., 2007].

STATISTICS

Data are presented as means ± SE. Statistical comparisons were performed using unpaired two-tailed Student's *t*-tests or one-way analysis of variance with Bonferonni's post hoc test where appropriate, with a probability value of <0.05 taken to indicate significance.

RESULTS

OVEREXPRESSION OF miR-26b REDUCES CARDIOMYOCYTE CELL SURFACE AREA AND THE EXPRESSION LEVELS OF HYPERTROPHY-RELATED GENES

To study the impact of miR-26b on cardiomyocytes, we over-expressed miR-26b in NRCMs (Supplemental Fig. 1A). The cell

surface area was significantly reduced by the transduction of miR-26b (Fig. 1A,B). Figure 1C–E shows changes in the expression levels of hypertrophy-related genes such as ANF, β-MHC, and skeletal muscle actin (ACTA1). Since ANF luciferase activity is also reduced by the transduction of miR-26b, it may target transcription factors that bind to the ANF gene promoter or upstream molecules of those transcription factors (Fig. 1F). These results were also confirmed by phenylephrine-induced hypertrophy in neonatal cardiomyocytes (Supplemental Fig. 1B).

miR-26b TARGETS TRPC3 AND GATA4 IN NRCMs

The computational miRNA target prediction algorithm showed that TRPC3 and GATA4 are the targets of miR-26b (TargetScan; http://www.targetscan.org/, Fig. 2A,B). Overexpression of miR-26b reduced the mRNA and protein levels of TRPC3 and GATA4 (Fig. 2C,D). Moreover, expression of miR-26b reduced the expression of the luciferase reporter gene fused with TRPC3 and GATA4 3′UTR sequences (Fig. 2E,F).

REDUCTION OF ENDOGENOUS miR-26b USING THE FORCED EXPRESSION OF A "DECOY" GENE

To assess the functional consequences of silencing endogenous miR-26b and miRNAs with the same seed sequence in vitro, we used NRCMs infected with a lenti-virus vector in which a 3′UTR with six tandem sequences complementary to miR-26b were linked to the luciferase reporter gene (miR-26b decoy, Fig. 3A).

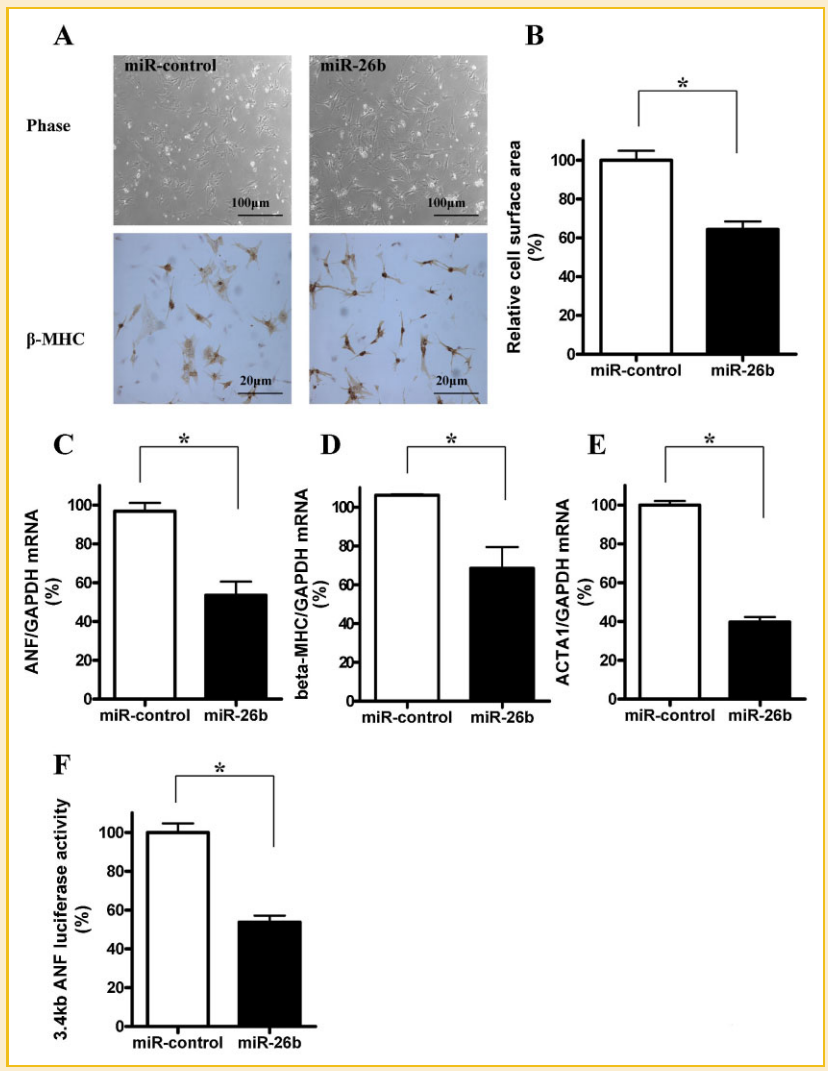

Fig. 1. Overexpression of miR-26b reduces the cardiomyocyte cell surface area and the expression levels of hypertrophy-related genes. A: Cardiomyocytes transduced with miR-26b or control genes. The primary antibody against β-MHC was stained with a secondary antibody conjugated with peroxidase (brown signals). B: Overexpression of miR-26b reduces the cardiomyocyte surface area. Values are the means ± SE (n = 6 each). The mean surface area of miR-control-transfected cardiomyocytes is 714 μm^2. $^*P < 0.05$ versus control. C–E: Overexpression of miR-26b reduces the expression levels of hypertrophy-related genes. Values are the means ± SE (n = 6 each). $^*P < 0.05$ versus control. F: NRCMs were transfected with a luciferase expression vector driven by the 3.4 kb wild-type ANF promoter in the presence or absence of miR-27b. Values are the means ± SE (n = 6 each). $^*P < 0.05$ versus control.

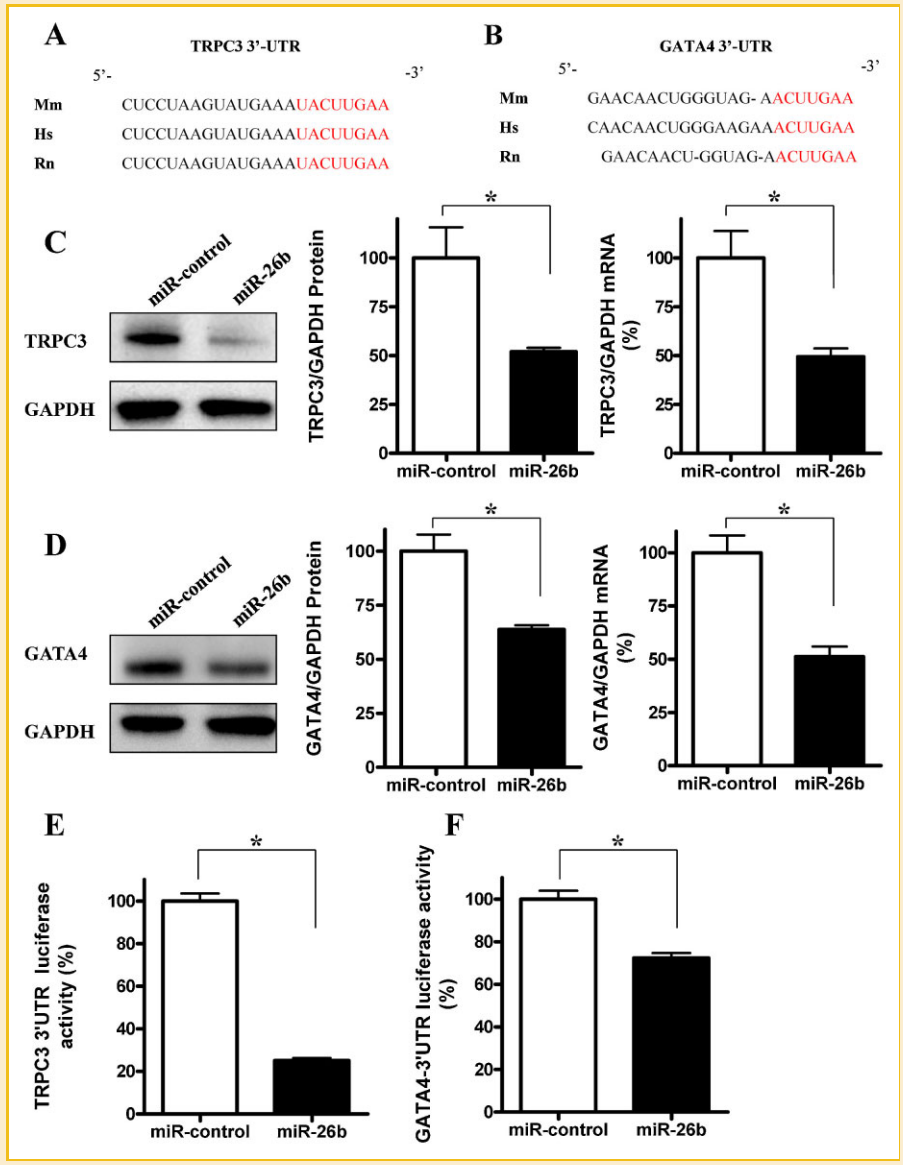

Fig. 2. miR-26b targets TRPC3 and GATA4 in NRCMs. A: Potential binding sites of miR-26b in the 3′UTR of TRPC3 (Mm, mouse; Hs, human; Rn, rat). B: Potential binding sites of miR-26b in the 3′UTR of GATA4 (Mm, mouse; Hs, human; Rn, rat). C: Changes in protein and mRNA levels of TRPC3 after transfection of miR-26b. Values are the means ± SE (n = 6 each). $^{*}P < 0.05$ versus control. D: Changes in protein and mRNA levels of GATA4 after transfection of miR-26b. Values are the means ± SE (n = 6 each). $^{*}P < 0.05$ versus control. E: Changes in TRPC3 3′UTR luciferase activity by the transduction of miR-26b in NRCMs. Values are the means ± SE (n = 6 each). $^{*}P < 0.05$ versus control. F: Changes in GATA4 3′UTR luciferase activity by the transduction of miR-26b in NRCMs. Values are the means ± SE (n = 6 each). $^{*}P < 0.05$ versus control.

The complementary sequences act as a decoy, sequestering endogenous miR-26b and miRNAs that have the same seed sequence [Nishi et al., 2010]. When the miR-26b decoy was transduced into NRCMs, miR-26b levels were significantly reduced (Fig. 3B). Moreover, ANF promoter activity significantly increased by the expression of miR-26b decoy (Fig. 3C). The expression of miR-26b decoy enhanced the protein level of TRPC3 and GATA4 in NRCMs (Fig. 3D,E).

OVEREXPRESSION OF SCP1 INDUCES INCREASED CARDIOMYOCYTE CELL SURFACE AREA AND THE EXPRESSION LEVELS OF HYPERTROPHY-RELATED GENES, AND siRNA AGAINST SCP1 REVERSED THESE EFFECTS ON NRCMs

To determine the role of the SCP1 gene in the heart, we overexpressed it in NRCMs. The overexpression of SCP1 was confirmed by western blotting (Fig. 4A) It significantly increased the cell surface area of NRCMs (Fig. 4B,C). We also confirmed that

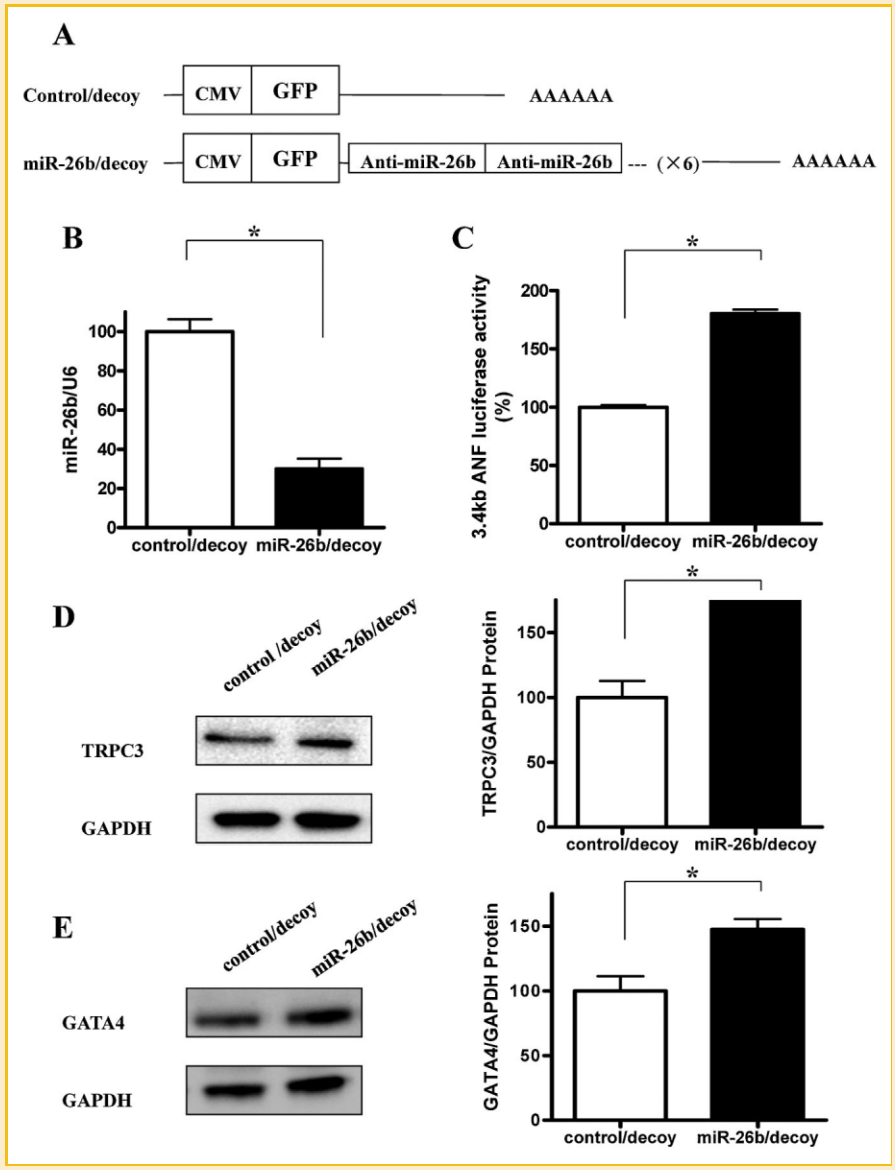

Fig. 3. Reduction of endogenous miR-26b using the forced expression of a "decoy" gene reverses the effect of miR-26b. A: Schematic diagram of the construction of the miR-26b decoy gene. Six tandem antisense sequences of miR-26b were inserted after GFP in a miR-26b decoy-expressing plasmid. B: Changes in the levels of endogenous miR-26b after overexpression of the miR-26b decoy gene. Values are the means ± SE (n = 6 each). $^{*}P < 0.05$ versus control. C: Changes in the levels of wild-type 3.4 kb ANF promoter activity after overexpression of the miR-26b decoy gene. Values are the means ± SE (n = 6 each). $^{*}P < 0.05$ versus control. D: Changes in the protein levels of TRPC3 after overexpression of the miR-26b decoy gene. Values are the means ± SE (n = 6 each). $^{*}P < 0.05$ versus control. E: Changes in the protein levels of GATA4 after overexpression of the miR-26b decoy gene. Values are the means ± SE (n = 6 each). $^{*}P < 0.05$ versus control.

ANF, β-MHC, and ACTA1 levels were significantly increased (Fig. 4D–F). We generated siRNA against SCP1. RNAi-1 and 2 significantly reduced the mRNA expression levels in NRCMs (Supplemental Fig. 1C). These siRNAs against SCP1 significantly reduced the cell surface area of NRCMs (Fig. 4G). These findings suggest that miR-26b has an antagonistic effect on its host gene SCP1 in NRCMs.

COMPARISON OF THE EFFECTS OF THE OVEREXPRESSION OF FRAGMENTS OF SCP1 cDNA OR GENOMIC DNA THAT COVER THE 5′ END OF EXON 1 AND THE 3′ END OF EXON 7

Since the expression of SCP1 induces a cardiac hypertrophic phenotype and the expression of its intronic miR-26b has the opposite effect, we compared the effect of the overexpression of SCP1 with its introns and SCP1 cDNA to observe the net function of

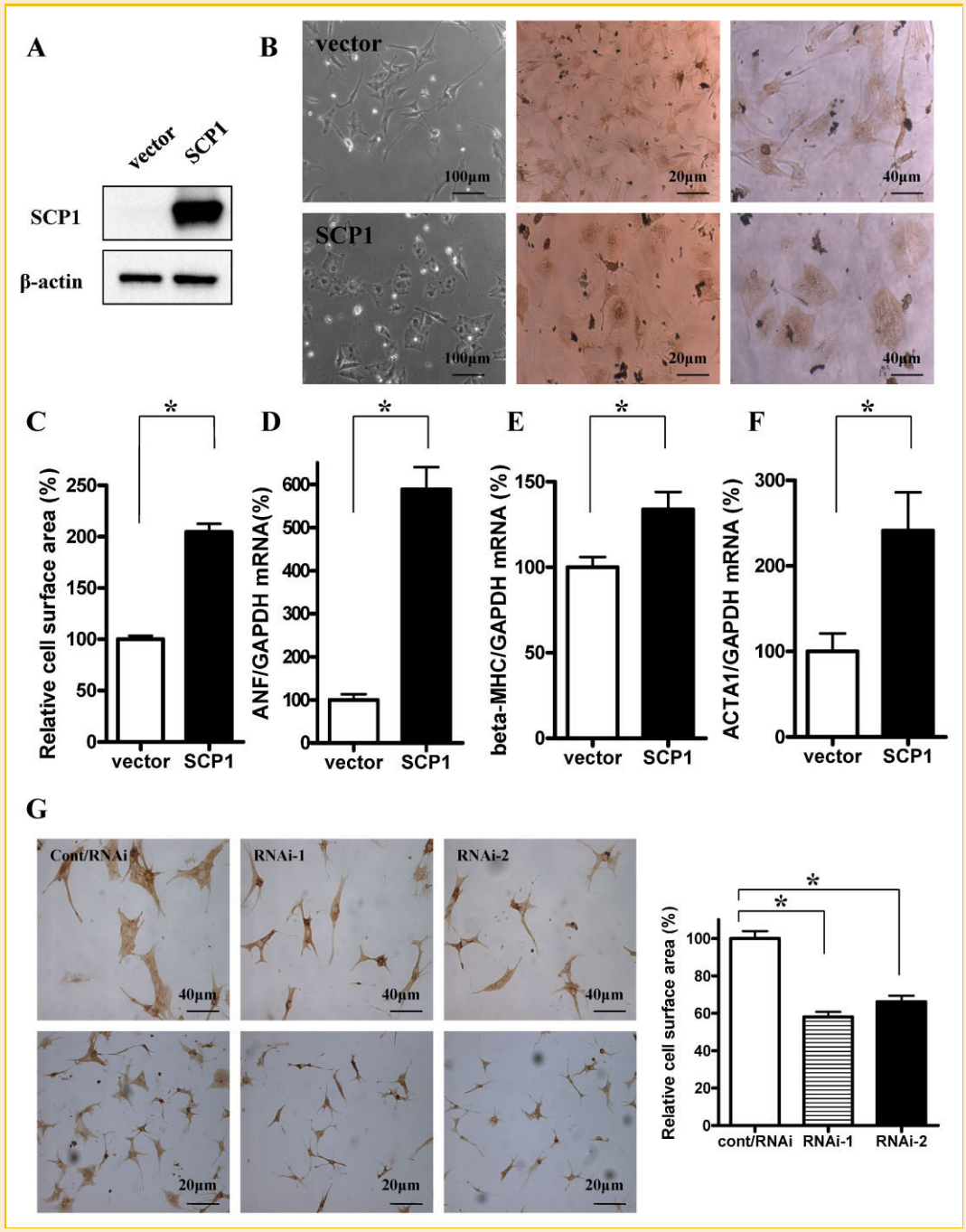

Fig. 4. Changes in cardiomyocyte cell surface area and the expression levels of hypertrophy-related genes after transfection of SCP1. A: Cardiomyocytes transduced with SCP1 or control genes. B: Cardiomyocytes transduced with SCP1 gene or control vector. The primary antibody against β-MHC staining indicates cardiomyocytes (brown signals). C: Changes in cardiomyocyte cell surface area after transfection of SCP1. The mean surface area of vector transfected cardiomyocytes is 642 μm^2. $^*P < 0.05$ versus control. D–F: Changes in hypertrophy-related genes after transfection of SCP1. Values are the means ± SE (n = 6 each). $^*P < 0.05$ versus control. G: The effect of RNAi against SCP1. Values are the means ± SE (n = 6 each). The mean surface area of control RNAi-transfected cardiomyocytes is 612 μm^2. $^*P < 0.05$ versus control. H: Cardiomyocytes transduced with SCP1 RNAi-1 and -2. β-MHC staining indicates cardiomyocytes.

SCP1 expression on cardiac hypertrophy in vitro. To overexpress SCP1 with its introns, we cloned a DNA fragment from the first ATG to the stop codon of SCP1 from mouse genome DNA (gDNA) and compared its function to that of SCP1 cDNA (cDNA; Fig. 5A). As shown in Figure 5B, miR-26b levels were significantly elevated in NRCMs with gDNA compared with vector- or cDNA-transduced NRCMs. Figure 5C indicates that the expression levels of SCP1 mRNA were the same in both cDNA and gDNA transduced cells.

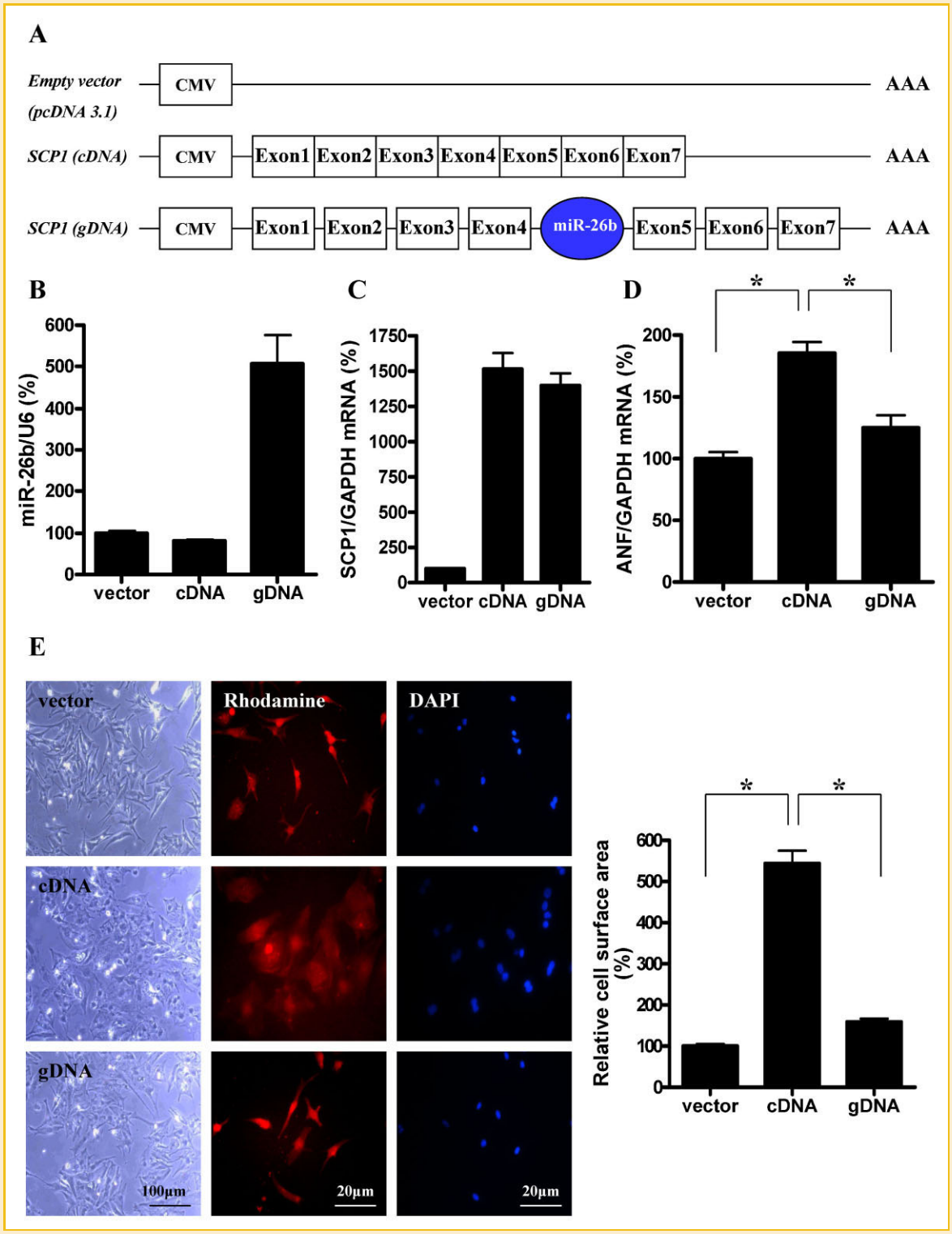

Fig. 5. Comparison of the effect of overexpression of fragments of SCP1 cDNA or genomic DNA that cover the 5′-end of exon 1 and the 3′-end of exon 7. A: Schematic diagram of the construction of fragments of SCP1 cDNA or genomic DNA that cover the 5′-end of exon 1 and the 3′-end of exon 7. B: miR-26b levels after transduction of SCP1 gDNA. Values are the means ± SE (n = 5 each). $^{*}P < 0.05$ versus control. C: SCP1 levels after transduction of SCP1 cDNA and gDNA. Values are the means ± SE (n = 4 each). D: ANF levels after transduction of SCP1 cDNA and gDNA. Values are the means ± SE (n = 5 each). $^{*}P < 0.05$. E: Changes in cell surface area after transduction of SCP1 cDNA and gDNA. Values are the means ± SE (n = 5 each). The mean surface area of vector-transfected cardiomyocytes is 610 μm2. $^{*}P < 0.05$.

cDNA significantly increased ANF gene expression and the cardiomyocyte cell surface area in NRCMs compared with cells transduced with gDNA (Fig. 5D,E). Moreover, miR-26b target genes TRPC3 and GATA4 are downregulated by gDNA compared to cDNA (Supplemental Fig. 2A,B). Therefore, SCP1, which is known to be recruited by REST/NRSF to neuronal genes that contain RE-1 elements and leads to neuronal gene silencing, potentially induces cardiac hypertrophy, which is negatively regulated by its intronic miR-26b.

EXPRESSION OF SCP1 AND miR-26b IN A MOUSE MODEL OF CARDIAC HYPERTROPHY

To elucidate the pathologic roles of miR-26b in vivo, gene expression of ANF, ACTA1, SCP1, and miR-26b was examined in the hearts of mice that had been subjected to TAC, which results in afterload-induced cardiac hypertrophy. As a result, ANF and ACTA1 gene expression was up-regulated and SCP1 mRNA and miR-26b expression was down-regulated during the 4 weeks of TAC (Fig. 6A–D). On the other hand, TRPC3 and GATA4 gene expression was enhanced (Fig. 6E,F). These findings suggested that miR-26b could be associated with the development of cardiac hypertrophy in terms of the upregulation of TRPC3 and GATA4 via a reduction in miR-26b in vivo.

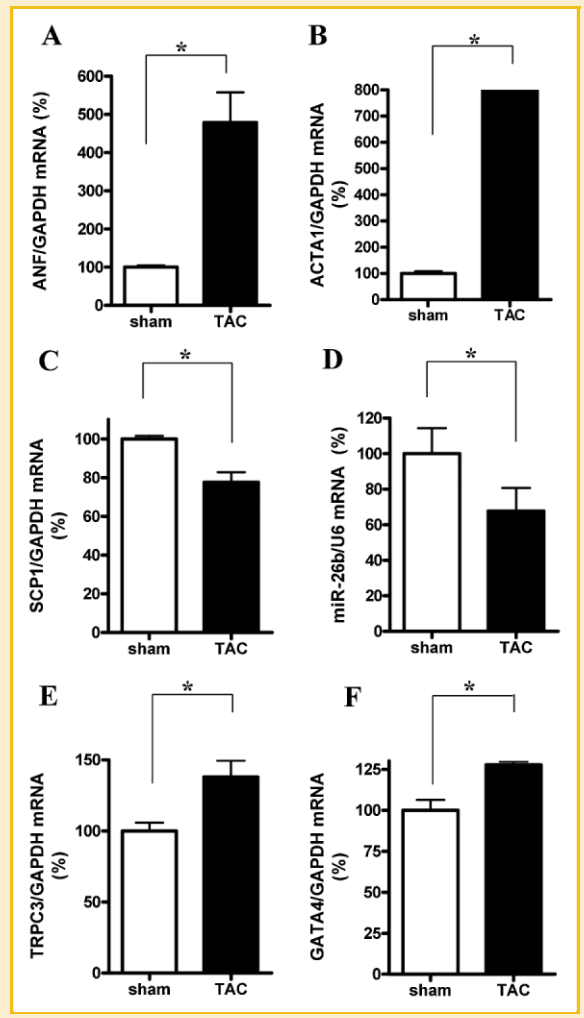

Fig. 6. Analysis of a mouse model of cardiac hypertrophy. A: Changes in the ANF mRNA levels after TAC. Values are the means ± SE (n = 5 each). *$P < 0.05$. B: Changes in the ACTA1 mRNA levels after TAC. Values are the means ± SE (n = 5 each). *$P < 0.05$. C: Changes in the SCP1 mRNA levels after TAC. Values are the means ± SE (n = 5 each). *$P < 0.05$. D: Changes in the miR-26b levels after TAC. Values are the means ± SE (n = 5 each). *$P < 0.05$. E: Changes in the TRPC3 mRNA levels after TAC. Values are the means ± SE (n = 5 each). *$P < 0.05$. F: Changes in the GATA4 mRNA levels after TAC. Values are the means ± SE (n = 5 each). *$P < 0.05$.

DISCUSSION

The functional regulation of SCP1 has been well studied and documented; however, the molecular function of miR-26b in intron 4 has not been well established. In this study, we demonstrated that miR-26b derived from intron 4 of SCP1 inhibited TRPC3 and GATA4 expression at both the mRNA and protein levels in NRCMs. The antihypertrophic effect of miR-26b was attenuated by overexpression of the antisense sequence of miR-26b. When the function of miR-26b was assessed in the context of its host gene, SCP1, miR-26b suppressed the hypertrophic effect of SCP1 in cardiomyocytes. Overexpression of SCP1 increases cell surface area with the enhanced expressions of ANF, β-MHC, and ACTA1 but not with GATA4 (Fig. 4B–F and Supplemental Fig. 2A,B). However, it is not strange because increase in GATA4 expression is not always observed in cardiac hypertrophy in vitro and in vivo [Morimoto et al., 2008]. Intronic miRNAs have been shown to be critically involved in the regulation of their host genes as well as other genes, and thus important cellular events and pathological processes [Barik, 2008; Ronchetti et al., 2008]. This is further supported by the finding in the present study that intronic miRNA suppressed cellular events induced by the host gene.

In response to stress signals, the mammalian heart increases in size [Swynghedauw, 1999]. This is largely accomplished by an increase in the size of myocytes (hypertrophy) rather than by an increase in their number (hyperplasia). The function of GATA4 and TRPC3 during cardiac hypertrophy is well-known. Ca^{2+}-dependent signaling effectors are present in cardiac myocytes, where they influence the cardiac hypertrophic response [Heineke and Molkentin, 2006]. It has been hypothesized that Ca^{2+}-activated signaling effectors are compartmentalized in membrane microdomains in direct proximity or even attached to Ca^{2+} influx channels, and the induction of TRPC channel activity during cardiac hypertrophy is hypothesized to generate a distinct Ca^{2+} signaling microdomain that can impact calcineurin signaling and the hypertrophic response directly [Watanabe et al., 2009]. Overexpression of TRPC3 has been reported to induce cardiac hypertrophy through calcineurin/nuclear factor of activated T cells (NFAT) 3 signaling in transgenic (TG) mice [Bush et al., 2006; Nakayama et al., 2006]. Moreover, TG mice showed increased cardiac hypertrophy after stimulation with a neuroendocrine agonist and pressure overload [Nakayama et al., 2006]. Dominant-negative TRPC3 TG mice were protected from loss of cardiac functional performance following long-term pressure-overload

stimulation [Wu et al., 2010]. NF-AT3 interacts with the cardiac zinc finger transcription factor GATA4, resulting in synergistic activation of cardiac transcription for hypertrophy, including ANF [Molkentin et al., 1998]. Transgenic mice that express TRPC3, activated forms of calcineurin or NF-AT3, and GATA4 in the heart all develop cardiac hypertrophy in a similar way, which suggests that these molecules can be targets for the treatment of cardiac hypertrophy [Molkentin et al., 1998; Liang et al., 2001; Nakayama et al., 2006]. In our experiment, miR-26b expression was reduced during the development of hypertrophy in TAC heart. We also examined whether miR-26b can reduce phenylephrine-induced cardiac hypertrophy. Phenylephrine enhanced the expressions of ANF, ACTA1, and TRPC3 and overexpression of miR-26b significantly reduced these expressions (Supplemental Fig. 2B). Therefore, the reduction of miR-26b might be associated with the development of cardiac hypertrophy through the upregulation of TRPC3 and GATA4 in vivo.

GATA4 protein levels are higher in neonatal and hypertrophied hearts, where it plays an essential role in development and hypertrophy. While homozygous GATA4 null mice were developmentally arrested at E7-E10.5 due to defective cardiac looping [Kuo et al., 1997; Molkentin et al., 1997], heterozygous mice survived normally [Pu et al., 2004]. However, the latter had mild systolic and diastolic dysfunction with a reduced myocyte number [Bisping et al., 2006]. Furthermore, when pressure overload was applied to the heart, it induced eccentric hypertrophy, apoptosis, fibrosis, and cardiac failure. On the other hand, the overexpression of dominant negative Trpc3 in the heart does not greatly affect development, but does inhibit pathological hypertrophy induced by neuroendocrine stimulation or pressure overload. Therefore, miR-26b may have a greater effect on pathological hypertrophy in the adult phase.

The 3′UTR of GATA4 is known to be a ''hot spot'' for mutations associated with congenital heart diseases, most of which have been predicted to result in misfolding of the first 500 bases of the 3′UTR, which encompasses the miR-26b target site [Reamon-Buettner et al., 2007]. This supports a post-transcriptional mode of regulation involving the 3′UTR. Although mutations in the binding site of miR-26b may enhance the hypertrophic effect of SCP1, no single nucleotide polymorphism has been found in the potential miR-26b binding site (dbSNP http://www.ncbi.nlm.nih.gov/projects/SNP/).

There have been no reports on the miRNA-mediated regulation of TRPC3. Since TRPC3 has been recognized to be a molecular hub that links extracellular signals to a Ca^{2+}-dependent hypertrophy-inducing pathway, it would be interesting to determine the effect of the miR-26b-family on cardiac hypertrophy in vivo.

In this article, we compared the effects of the functions of intronic miR-26b and its host gene in cardiomyocytes. In general, intronic miRNAs are expressed from the host gene promoter and require RNA splicing machinery. However, intronic miRNA may have its own transcriptional regulation. A previous article indicated that ~35% of intronic miRNAs can be transcribed from Pol II or Pol III intron-resident promoters, independent of the host gene promoter by a ChIP-on-chip analysis or ChIP-on-chip combined with nucleosome patterning [Ozsolak et al., 2008; Wang et al., 2009; Monteys et al., 2010]. Moreover, it is conceivable that different post-transcriptional regulatory mechanisms could control miRNA processing, depending on whether the transcript originated from the host gene or an intronic promoter/enhancer. The presence of antisense transcripts, which can form RNA/RNA duplexes of complementary transcripts, can make it even more difficult to predict the functional amount of host gene and its intronic miRNAs, especially in vivo [Stark et al., 2008]. In any case, miR-26b has the potential to reduce cardiac hypertrophy and the augmentation of miR-26b expression may help to reduce cardiac hypertrophy.

In summary, this study showed that intronic miR-26b derived from intron 4 functioned as a negative regulator of its host gene SCP1 in cardiomyocytes. GATA4 and TRPC3 play key roles in the function of miR-26b and the simultaneous inhibition of these molecules may further enhance its anti-hypertrophic activity. Since GATA4 and TRPC3 are associated with hypertrophic signals specifically only in cardiomyocytes, miR-26b may have different functions in other cells and tissues. Our study revealed a novel pathway that was critical in the regulation of SCP1 function in cardiomyocytes.

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