Mechanical Stretch Up-regulates MicroRNA-26a and Induces Human Airway Smooth Muscle Hypertrophy by Suppressing Glycogen Synthase Kinase-3β*

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Airway smooth muscle hypertrophy is one of the hallmarks of airway remodeling in severe asthma. Several human diseases have been now associated with dysregulated microRNA (miRNA) expression. miRNAs are a class of small non-coding RNAs, which negatively regulate gene expression at the post-transcriptional level. Here, we identify miR-26a as a hypertrophic miRNA of human airway smooth muscle cells (HASMCs). We show that stretch selectively induces the transcription of miR-26a located in the locus 3p21.3 of human chromosome 3. The transcription factor C/EBP enhancer-binding protein α (C/EBPα) directly activates miR-26a expression through the transcriptional machinery upon stretch. Furthermore, stretch or enforced expression of miR-26a induces HASMC hypertrophy, and miR-26a knockdown reverses this effect, suggesting that miR-26a is a hypertrophic gene. We identify glycogen synthase kinase-3β (GSK-3β), an anti-hypertrophic protein, as a target gene of miR-26a. Luciferase reporter assays demonstrate that miR-26a directly binds to the proximal 3′-untranslated region of the GSK-3β mRNA. Stretch or enforced expression of miR-26a attenuates the endogenous GSK-3β protein levels followed by the induction of HASMC hypertrophy. miR-26a knockdown reverses this effect, suggesting that miR-26a-induced hypertrophy occurs via its target gene GSK-3β. Overall, as a first time, our study unveils that miR-26a is a mechano-sensitive gene, and it plays an important role in the regulation of HASMC hypertrophy.

MicroRNAs (miRNAs)2 are an evolutionarily conserved novel class of small non-coding RNAs that have achieved status as potent regulators of gene expression. According to their genomic location relative to protein-coding gene locus, miRNAs can be divided into intergenic miRNAs and intronic miRNAs. The former have independent transcriptional units, including promoter, transcript sequence, and terminator; therefore, they do not overlap with other genes (1, 2). The later are found in the introns of protein-coding host genes, and they generally share the same regulatory motifs with their host genes (3–5). Most of the miRNAs are transcribed by RNA polymerase II as primary miRNAs (1, 2) and processed by the RNase III enzymes Drosha and Dicer to produce 21- to 23-nucleotide double-stranded RNA duplexes (6, 7). These smaller RNAs are then exported to the cytoplasm by Exportin 5 (8, 9), where they are subsequently processed into mature miRNAs by Dicer. The mature miRNAs are loaded into the miRNA-induced silencing complex (7), where they recognize their target protein-coding miRNAs to inhibit mostly mRNA translation or degradation (10) by base pairing to complementary sequences within the 3′-untranslated region (3′ UTR). With respect to miRNAs functions, they play pivotal roles in the pathophysiological processes such as apoptosis, cell differentiation, cell proliferation, and organ development (4, 11). Functionally speaking, several human diseases have now been associated with dysregulated miRNAs expression.

Airway remodeling is a characteristic feature observed in the airways of patients having severe asthma and chronic obstructive pulmonary disease. Clinical studies have shown that both hypertrophy and hyperplasia of human airway smooth muscle cells (HASMCs) play key roles in airway remodeling (12–16). Many lines of evidence have demonstrated that hypertension in cardiomyocytes, skeletal myotubes, and smooth muscle cells is induced by various hypertrophic stimuli, including mechanical stretch (17–27). Recently, it has been shown that miRNAs play important roles in the induction of cardiac hypertrophy as well as response to hypertrophic stimuli (28–32). However, the role of miRNAs in the regulation of smooth muscle hypertrophy is completely unknown. Furthermore, the induction of specific miRNAs in response to hypertrophic stimuli, including stretch, is also lacking in these cells.

The present study was aimed to investigate whether stretch can induce miRNAs expression, and whether miRNAs are involved in the regulation of airway smooth muscle hypertrophy. Our results show that stretch induces HASMC hypertrophy through miR-26a up-regulation. Promoter analysis of the miR-26a gene reveals that C/EBPα directly binds to the promoter of miR-26a and activates its expression. In addition, we identify glycogen synthase kinase-3β (GSK-3β) as a target gene of miR-26a. Luciferase reporter assay demonstrate that miR-
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**TABLE 1**

| Primer name | Sequence 5’-3’ | Underline Purpose |
|-------------|----------------|-------------------|
| miR-26a-C-F | GAATATGGATAGATGCTGATCCACAGGAG | BamHI Cloning |
| miR-26a-C-R | TGGCTGATTGCAAGAGCTGAGA | HindIII Cloning |
| GSK-3β-F1 | ACCCGTATGCTGGAAGAGGAG | Cloning |
| GSK-3β-R1 | TCTAGAGATGGTGATGGGATT | Cloning |
| GSK-3β-F1 | ACCCGTATGCTGGAAGAGGAG | Cloning |
| GSK-3β-R2 | TCTAGAGATGGTGATGGGATT | Cloning |
| GSK-3β-3’UTR-F | AGCTCTGGCTCAGAGAGGAGGAC | SacI Cloning |
| GSK-3β-3’UTR-R | AGCTCTGGCTCAGAGAGGAGGAC | Cloning |
| miR-26a 5’UTR-F | AGACTGAGGAAGAGAGGAGGAC | KpnI Cloning |
| miR-26a 5’UTR-R1 | AGACTGAGGAAGAGAGGAGGAC | KpnI Cloning |
| miR-26a 5’UTR-R2 | AGACTGAGGAAGAGAGGAGGAC | Cloning |
| miR-26a 5’UTR-R | AGACTGAGGAAGAGAGGAGGAC | qPCR |
| Chip-F1000 | CTGTGTGCTCCCTGAGGCC | qPCR |
| Chip-R1000 | CTGTGTGCTCCCTGAGGCC | qPCR |
| Chip-R750 | CTGTGTGCTCCCTGAGGCC | qPCR |
| CTDSP1-F | GGTTTGGCCTCGCCTGCTG | qPCR |
| CTDSP1-R | GGTTTGGCCTCGCCTGCTG | qPCR |
| CTDSP2-F | GGTTTGGCCTCGCCTGCTG | qPCR |
| CTDSP2-R | GGTTTGGCCTCGCCTGCTG | qPCR |
| GSK-3β-F2 | GAGAGAGGAGGACCTCCAGTCCTG | qPCR |
| GSK-3β-R3 | GAGAGAGGAGGACCTCCAGTCCTG | qPCR |
| GAPDH-F | GGGTAGAAGATGGAGTGA | qPCR |
| GAPDH-R | GGGTAGAAGATGGAGTGA | qPCR |

26a directly interacts with the 3’UTR of the GSK-3β mRNA. Consequently, *miR-26a* is able to convey the hypertrophic signal by suppressing the translation of GSK-3β mRNA. These findings reveal that *miR-26a* is a novel regulator in airway smooth muscle hypertrophy.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Stretch**—Primary HASMCs (obtained from Lonza, Walkersville, MD) were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 1× non-essential amino acids, 2 mM l-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin in a humidified incubator containing 5% CO2 at 37 °C. Prior to experiments, 2×105 cells at passage 7 were plated on type I collagen-coated Bioflex 6-well plates or normal cell culture 6-well plates in the above growth medium. All experiments were conducted after the cells were serum-deprived for 24 h. For stretch stimulation, cells grown on Bioflex plates were stimulated with a 1-h cyclic strain at 1 Hz (0.5 s of deformation alternating with 0.5 s of relaxation) for every 12 h or unless otherwise stated using a computer-controlled vacuum strain apparatus (Flexercell Strain Unit, FlexCell International, Hillsborough, NC) with a vacuum pressure that is sufficient to generate 12% strain. Cells grown in normal cell culture plates were used for non-stretch experiments.

**miRNA Microarray Analysis**—After stretch, total RNA samples were isolated by TRIzol reagent according to the manufacturer’s protocol (Invitrogen). Ten micrograms of total RNA was size-fractionated with YM-10 Microcon centrifugal filter (Millipore, Billerica, MA), and then used for miRNA expression analysis with miRNA microarray (LC Sciences, Houston, TX).

**Construction of Expression Plasmids—miR-26a** precursor DNA containing 77-bp stem-loop sequence and 100-bp native flanking sequence to both upstream and downstream of the stem loop (as shown in Fig. 4A) was synthesized with *miR-26a*-C-F and *miR-26a*-C-R primers, and cloned into pSilencer 4.1-CMV vector (Ambion, Austin, TX) according to the manufacturer’s instructions. GSK-3β cDNA with or without 3’UTR was synthesized and cloned into pcDNA 3.1D/V5-His-TOPO vector (Invitrogen) according to the manufacturer’s instructions. GSK-3β-F1 and GSK-3β-R1 primers were used to synthesize GSK-3β cDNA with 3’UTR. GSK-3β cDNA without the 3’UTR was synthesized using GSK-3β-F1 and GSK-3β-R2 primer sets. To generate reporter vector bearing *miR-23a*-binding sites, a 650-bp human GSK-3β 3’UTR sequence was synthesized and cloned into pmirGLO vector (Promega, Madison, WI) according to the manufacturer’s instructions. GSK-3β-3’UTR-F and GSK-3β-3’UTR-R primers were used. To generate reporter vector bearing C/EBPα-binding elements, a 750-bp and a 1000-bp human *CTDSPL/miR-26a* 5’UTR DNA (as shown in Fig. 4) were synthesized and cloned into pGL4.1 luciferase reporter vector (Promega). The following primer pairs were used: *miR-26a* 5’UTR-F1 and *miR-26a* 5’UTR-R1 (for 1000 bp), and *miR-26a* 5’UTR-F2 and *miR-26a* 5’UTR-R2 (for 750 bp). PCRs were performed to synthesis inserts with AccuPrime Pfx DNA polymerase according to the manufacturer’s protocols (Invitrogen). Primers information was detailed in Table 1. Constructs were sequenced by the DNA Sequence Core Facility of Baylor College of Medicine to verify insert identities.

**Transfection and Luciferase Assays**—Cells were grown in Opti-MEM I medium (Invitrogen) for 24 h before transfection. Cells were transfected with 2.2 μg of expression vector bearing has-**miR-26a** precursor, GSK-3β cDNA, or GSK-3β cDNA without 3’UTR, or 2.4 μg of reporter vector bearing GSK-3β 3’UTR or C/EBPα-binding elements by using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocols. The *Renilla* luciferase vector pGL4.74 was co-transfected with firefly luciferase vectors as a normalizer. For *miR-26a* inhibitor assays, cells were transfected with 400 ng of has-*miR-26a* miRCURY LNA knockdown probe (antagomir) or scrambled probe (Exiqon, Woburn, MA). For small interference RNA-mediated knockdown studies, 500 pmol of small interference RNA specific for human C/EBPα or nonspecific small
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miR-26a is representative of three separate experiments. Each mM EDTA/150 mM NaCl/20 mM Tris, pH 8.1/1 qPCR to assay the expression of debris was removed by centrifugation, and the chromatin solution (polyethylene glycol) and sonicated four times for a 30-s interval of

dotTaq (Roche Applied Science, Indianapolis, IN)/1 mg/ml 4-(2-aminoethyl)benzenesulfonic acid (Ambion) as a sample normalizer were used in each reaction. RT-qPCRs for mRNA expression were performed by using the following method. One microgram of total RNA was reverse-transcribed by using SuperScript III First-Strand Synthesis Super Mix according to the manufacturer’s protocol (Invitrogen). RT-qPCRs were performed by using Brilliant II SYBR Green QPCR Master Mix (Stratagene, La Jolla, CA) according to the manufacturer’s instructions. The following primer sets were used in PCRs: human GAPDH primers were used as a sample normalizer. RT-qPCRs were performed on an Mx 3005p Real Time PCR system (Stratagene). The temperature cycle profile for the qPCR reactions was 95 °C for 15 min and 40 cycles of 94 °C for 15 s, 55 °C for 30 s, and 72 °C for 30 s. Melting curve analysis was also included at one cycle of 95 °C for 1 min, 55 °C for 30 s, and 95 °C for 30 s to verify the specificity of the amplified PCR products. The amount of amplified transcripts (2 − ΔCT) was estimated by the comparative CT (ΔCT) method and normalized to an endogenous reference (GAPDH) relative to a calibrator. All PCR products were verified on agarose gel stained with ethidium bromide to discriminate between the correct amplification products and the potential primer dimers.

Western Blot—Cell lysates were isolated by using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Pierce) according to the manufacturer’s instructions. Forty micrograms of proteins was resolved by SDS-PAGE and transferred to a nitrocellulose membrane. Membrane was blocked with 5% fat-free milk for 1 h and probed with mouse anti-α-smooth muscle (SM) actin, anti-SM22, anti-smMHC (myosin heavy chain), anti-GSK-3β (glycogen synthase kinase-3β), or anti-tubulin. Antibody binding was detected with a peroxidase-conjugated goat anti-mouse IgG and chemiluminescence (Pierce).

DNA and Protein Synthesis Analyses—We used Click-iT EdU (5-ethyl-2′-deoxyuridine, a thymidine analog) and Click-iT HPG (l-homopropargylglycine, a glycine analog) according to the manufacturer’s instructions (Invitrogen). Briefly, after treatments, cells were incubated with 10 μM EdU or HPG for the indicated periods, harvested, washed, and fixed with Click-iT fixative for 15 min. Cells were permeabilized with 1× saponin-based permeabilization and wash buffer for 30 min.
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Cell Size Analysis—Cells were stained for α-actin fibers, and cell size was determined by computer-assisted planimetry. 100–200 cells in 20–30 fields were examined in each experiment.

Statistical analysis—The results are expressed as means ± S.E. of at least three independent experiments. The comparison among different groups was performed by one-way analysis of variance followed by Bonferroni test using SigmaStat 3.5 software. Paired data were evaluated by Student’s t test. p < 0.05 was considered statistically significant.

RESULTS

Mechanical Stretch Up-regulates miRNAs Expression—Others and we have previously shown that stretch can induce HASMCs gene expressions through mechanosignal transduction pathways (33–38). To explore whether stretch can induce the expression of miRNAs in HASMCs, we performed a microRNA array screen using total RNA isolated from HASMCs 12 h after stretch. Among 837 mRNAs, the array uncovered the induction of 50 differentially regulated mechanosensitive miRNAs, and this was based on a p value of 0.01. Among those, 28 miRNAs were up-regulated, including the highly up-regulated miRNAs miR-16, miR-26a, and miR-140 (Fig. 1A), and 22 miRNAs were down-regulated. To confirm the validity of miR-16, miR-26a, and miR-140 up-regulations by stretch, a portion of the RNA used for the microarray was converted into cDNA and subjected to qPCR or solution hybridization analysis. Consistent with the microarray findings, miR-16, miR-26a, and miR-140 were strongly up-regulated by stretch (Fig. 1B and C). The small nuclear RNA U6, a control and normalizer for miRNAs, was relatively unchanged by stretch. These results indicate that HASMCs respond to stretch by strongly up-regulating miR-16, miR-26a, and miR-140, and the genes that transcribe these miRNAs are mechanosensitive.

Stretch Induces HASMCs Hypertrophy—Accumulating evidences have demonstrated that stretch can induce cardiac, skeletal, and smooth muscle cell hypertrophy (17–27). To test this in HASMCs, we stimulated the cells with a 1-h stretch for every 12 h up to 3 days. Our data showed that cells stimulated with stretch displayed an increase in cell size (Fig. 2A). Changes in cell size were accompanied by an increase in protein synthesis, because the HPG incorporation was enhanced. Stretching of HASMCs also increased DNA synthesis as evidenced by higher incorporation of EdU (Fig. 2B and C). Twelve hours after stretch, the incorporation of EdU or HPG was detected by using Click-iT Cell Reaction Buffer Kit (Invitrogen) according to the manufacturer’s instructions. Flow cytometry was used to estimate the fluorescence intensity of Alexa Fluor-488 bound EdU or HPG.

FIGURE 2. Stretch induces HASMC hypertrophy and hyperplasia. HASMCs were stimulated with a 1-h stretch for every 12-h period, and then cell size, DNA synthesis, protein synthesis (A), protein/DNA ratio (B), and cell count (C) were analyzed for the indicated periods. *, p < 0.05 versus control without stretch (white bar) for the indicated time point. Each bar indicates mean ± S.E. (n = 3).

FIGURE 3. Stretch-induced miR-26a is involved in initiating hypertrophy. HASMCs were transfected with anti-miR-26a, anti-miR-16, anti-miR-140, or nonspecific miRNA (NS-miR). Twenty-four hours after transfection cells were stimulated with a 1-h stretch for every 12 h. A, cell size, DNA synthesis, and protein synthesis were analyzed after 72 h. B, a portion of cells used in A was analyzed to show contractile proteins such as α-actin, smooth muscle 22 (SM22), and myosin heavy chain (MHC) expressions by Western blot. C, cells were transfected with anti-miR-26a, anti-miR-16, anti-miR-140, or NS-miR for 24 h followed by a 1-h stretch. Total RNA was isolated after 36 h, and the levels of miRNAs were quantified by RT-qPCR. Gel pictures are representative of three separate experiments. *, p < 0.05 versus stretch. Each bar indicates mean ± S.E. (n = 3).
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To find out the molecular target of miR-26a, we searched for predicted miR-26a targets. GSK-3β is a target of miR-26a—Recent studies have shown that miRNAs play important roles in cardiac hypertrophy (28–32). Induction of miRNAs and hypertrophy by stretch led us to consider whether the above stretch-induced miRNAs may participate in conveying the hypertrophic effect of stretch. To this end, we used miR-16, miR-26a, and miR-140 antagonists (anti-miR) to knock down their endogenous expressions in the stretch-induced hypertrophic cascade. Interestingly, miR-26a knockdown attenuated the stretch-induced hypertrophic responses as evidenced by decrease in cell size, HPG incorporation (Fig. 3A), and contractile proteins such as α-smooth muscle actin, SM22, and smooth muscle MHC expressions (Fig. 3B). In contrast, neither miR-16 nor miR-140 had a role in any of the above stretch-induced hypertrophic responses. These results indicate that miR-26a unlike miR-16 and miR-140 is involved in mediating the hypertrophic effects of stretch. In addition, miR-26a, miR-16, and miR-140 did not attenuate the EdU incorporation, which was increased by stretch (Fig. 3A), suggesting that these miRNAs are unable to inhibit the stretch-induced hyperplasia. We tested whether transfection of HASMCs with the antagonimer of miR-16, miR-26a, or miR-140 could influence their endogenous levels of expression. As expected, miR-16, miR-26a, and miR-140 antagonimers inhibited their endogenous levels of expressions, suggesting the specificity of the antagonimers (Fig. 3C).

**Enforced Expression of miR-26a Induces Hypertrophy**—We overexpressed miR-26a in HASMCs to evaluate whether miR-26a itself induces hypertrophy in the absence of stretch. We engineered an miR-26a-expressing construct in an expression vector (Fig. 4A). Expression of the pre-miR-26a and mature miR-26a were observed in

**FIGURE 4.** *Enforced expression of miR-26a induces hypertrophy.* A, structure of pSilencer-miR-26a construct contains pre-miR-26a, cytomegalovirus promoter (CMV), simian virus 40 (SV40), and neomycin. The blue letters represent two restriction sites BamHI and HindIII. The red letters indicate 22 bases of mature miR-26a sequence. The underlined letters represent 77-bp stem-loop sequence, and the green letters indicate 100-bp native flank sequence to both upstream and downstream of the stem-loop sequence. B–E, cells were transfected with pSilencer or pSilencer-miR-26a and/or anti-miR-26a for 24 h followed by a 6-h stretch for every 12 h. B, 36 h after transfection, the overexpression of pre-miR-26a and mature miR-26a was confirmed by RT-qPCR (upper panel) and solution hybridization methods (lower panel), respectively. Cell size, DNA synthesis, protein synthesis, and cell count (C), and α-actin, SM22, and MHC expressions (D) were analyzed 72 h after transfection. Protein/DNA ratio (E) was also analyzed. β-Actin served as a loading control. Gel pictures are representative of three separate experiments, *p < 0.05 versus control and *, p < 0.05 versus pSilencer-miR-26a. Each bar indicates mean ± S.E. (n = 3).

2A). The increase in DNA synthesis was accompanied by an increase in proliferation (Fig. 2B). The protein synthesis was much higher than the DNA synthesis as evidenced by the increased protein/DNA ratio (Fig. 2C). All these changes were effective from 48 h after initiation of stretch. These data provide experimental evidences demonstrating that stretch can induce both hypertrophy and hyperplasia of HASMCs, but the former is more dominant response than the later one.

**Stretch-induced miR-26a Participates in Initiating Hypertrophy**—Recent studies have shown that miRNAs play important roles in cardiac hypertrophy (28–32). Induction of miRNAs and hypertrophy by stretch led us to consider whether the above stretch-induced miRNAs may participate in conveying the hypertrophic effect of stretch. To this end, HASMCs stably transfected with the construct (Fig. 4B). Our results showed that miR-26a alone could induce HASMC hypertrophy as evidenced by increased cell size, HPG incorporation (Fig. 4C), contractile proteins expressions (Fig. 4D), and protein-DNA ratio (Fig. 4E). Introduction of anti-miR-26a blocked the miR-26a-induced hypertrophic effects. Surprisingly, miR-26a inhibited cell proliferation, because the Edu incorporation and total number of cells were reduced (Fig. 4C). Overall, regardless of stretch, miR-26a up-regulation induces HASMC hypertrophy. Furthermore, miR-26a inhibits the proliferation of HASMCs, and such inhibitory effect occurs only in the absence of stretch.
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gets, focusing our attention on the regulators of hypertrophy. In agreement with this, the public data base of animal miRNA miRGen (available on-line) has listed GSK-3β as one of the potential targets of miR-26a. GSK-3β has a conservative miR-26a seed sequence in its 3’ UTR (Fig. 5A). Recent studies have shown that GSK-3β is an anti-hypertrophic molecule and negatively regulates hypertrophy in airway smooth muscle, cardiac, and skeletal muscles (39–47). These data provided a strong rationale to test our hypothesis of whether GSK-3β is functionally a downstream target of miR-26a in the hypertrophic pathway of HASMCs. First, we detected the expression levels of GSK-3β in the hypertrophic model of stretch. Both total and phosphorylated GSK-3β protein levels were decreased in a time-dependent manner upon stretch (Fig. 5B). Second, we tested whether miR-26a transcriptionally or post-transcriptionally suppresses the endogenous GSK-3β expression. To test this, we overexpressed miR-26a in HASMCs using the pSilencer-miR-26a construct. The enforced expression of miR-26a significantly decreased GSK-3β protein but not mRNA levels, (Fig. 5C), suggesting that miR-26a predominantly suppresses GSK-3β translation. To confirm whether miR-26a influences the protein translation of GSK-3β, we analyzed the protein levels of GSK-3β in cells that overexpress both miR-26a and GSK-3β. We cloned GSK-3β full-length cDNA in the mammalian expression vector pcDNA 3.1. Expression of GSK-3β mRNA and protein were observed in HASMCs stably transfected with the construct (Fig. 5D). GSK-3β expression was suppressed by miR-26a in a dose-dependent manner (Fig. 5E).

Subsequently, we analyzed the effect of miR-26a on GSK-3β translation. To do this, a reporter construct containing the luciferase gene fused to the GSK-3β-3’UTR (luc-GSK-3β-3’UTR) was transfected into HASMCs with or without miR-26a overexpression vector. As shown in Fig. 6A, cells transfected with luc-GSK-3β-3’UTR alone had luciferase activity, and cells co-transfected with pSilencer-miR-26a significantly reduced the luciferase activity. Introduction of miR-26a antagonim was reverted to the inhibitory activity of miR-26a. SC-miR-26a antagonim was also able to rescue luc-GSK-3β-3’UTR luciferase activity (Fig. 6A). Finally, we tested whether miR-26a suppresses GSK-3β by base pairing on the 3’UTR of GSK-3β mRNA. To do this, we cloned GSK-β cDNA without 3’UTR in the pcDNA 3.1 vector, and the expression of GSK-3β mRNA without the 3’UTR in HASMCs stably transfected with the construct was confirmed by RT-qPCR and Western blot (Fig. 6B). As shown in Fig. 6C, miR-26a was unable to influence the expression of GSK-3β without its 3’UTR, suggesting that the GSK-3β 3’UTR contains an active seed of miR-26a. Overall, these data provided experimental evidences demonstrating that GSK-3β is a target gene of miR-26a.

To understand if enforced expression of GSK-3β can influence the hypertrophic cascades induced by stretch or miR-26a we used cells that overexpress GSK-3β from Fig. 5D. Transfection of miR-26a induced HASMC hypertrophy, and such hypertrophy was inhibited by the ectopic expression of GSK-3β (Fig. 6D). Ectopic expression of GSK-3β also was able to attenuate the stretch-induced hypertrophy (Fig. 6E). These data suggest that miR-26a and GSK-3β are both involved in the regulation of HASMC hypertrophy. Overall, our data support a model
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in which miR-26a participates in HASMC hypertrophy by suppressing GSK-3β protein expression, which in turn triggers the expression of smooth muscle-specific markers and hypertrophy (see Fig. 10).

Stretch Selectively Transcribes miR-26a—Two loci align with miR-26a in the human genome (48). miR-26a-1 is located in an intron of C-terminal domain RNA polymerase II polypeptide A small phosphatase-like (CTDSPL) at chromosome 3 (Fig. 7A), and miR-26a-2 is located in an intron of C-terminal domain RNA polymerase II polypeptide A small phosphatase 2 (CTDSPL2) at chromosome 12 (figure not shown). To understand whether the stretch-induced miR-26a transcription occurs via the activation of CTDSPL and/or CTDSPL2 gene(s) we performed qPCR with primers specific for CTDSPL and CTDSPL2. Surprisingly, stretch increased the mRNA levels of CTDSPL (Fig. 7B), but not CTDSPL2 (Fig. 7C), in a time-dependent manner. More precisely, CTDSPL mRNA was detectable at 1 h, elevated at 12 h, suddenly reduced at 24 h, and was reduced at 48 h after stretch. To monitor the kinetics of miR-26a induction, mature miR-26a was assayed over a 48-h time course after stretch. miR-26a induction by stretch followed a similar pattern of expression as CTDSPL, reaching its highest levels at 12 h and slowly decreasing at 24 and 48 h after stretch (Fig. 7D). In these experiments, the levels of U6 and GAPDH, a control and normalizer for miR-26a and CTDSPL, respectively, were relatively unchanged by stretch. These findings provide evidence that 1) human CTDSPL is expressed at lower levels, and its levels increases by stretch, 2) most of the stretch-induced miR-26a transcribes from the miR located on chromosome 3, and 3) the expression of miR-26a is not correlated with the expression of CTDSPL2 in response to stretch.

miR-26a Is under the Control of C/EBPα in Stretch-induced Hypertrophy—To determine the specific transcription factor by which primary miR-26a was induced by stretch we first analyzed the miR-26a promoter region using the public software PATCH (available online), focusing on mechanosensitive AP-1 and C/EBPα transcription factors that are activated by stretch in HASMCs (34). A scan of 1.8 kb genomic sequence located upstream of the ATG of CTDSPL gene identified eight putative AP-1 and three C/EBPα consensus binding sites in the first 750 bp. In addition to this, two AP-1 and C/EBPα-binding sites were identified between 750 and 1800 bp upstream of the ATG (Fig. 8A). The presence of AP-1 and C/EBPα binding elements on the CTDSPL 5′ UTR (miR-26a promoter) led us to consider whether miR-26a is a transcriptional target of AP-1 and/or C/EBPα. First, using ChIP assays, we tested whether these transcription factors could directly bind to the promoter region of the miR-26a. As shown in Fig. 8 (B and C), activation of miR-26a promoter by stretch was completely dependent on the C/EBPα-binding sites as evidenced by qPCR (Fig. 8B) and the visualization of PCR products on 1% agarose gel (Fig. 8C). In contrast, stretch had no effect on c-fos activity on the miR-26a promoter. Second, we tested whether C/EBPα could influence the promoter activity of miR-26a. We generated two promoter constructs by cloning the first 750 bp, and a 1000 bp region (from −800 to −1800) upstream of the ATG (Fig. 8D) into
luciferase reporter plasmid, named pGL750 and pGL1000, and tested them in a luciferase reporter assay 48 h after transfection. Cells transfected with the pGL1000 construct had higher luciferase activities than control cells upon stretch (Fig. 8E). In contrast, stretch did not activate the luciferase gene in cells transfected with the pGL750 construct. Finally, we tested whether the endogenous C/EBPα/H9251 is able to influence the promoter activity of miR-26a. To achieve this, C/EBPα RNAi was produced, and they could decrease their endogenous expression levels both in total and phosphorylated states (Fig. 9A). The promoter of miR-26a was activated upon stretch, but knockdown of C/EBPα by RNAi abolished the stretch-induced miR-26 promoter activation (Fig. 9B). We next explored the relationship between C/EBPα and miR-26a in the hypertrophic cascades of stretch. To do this, we carried out the above RNAi strategy. Knockdown of C/EBPα inhibited the hypertrophic responses (Fig. 9C) of stretch. Taken together, these data suggest that miR-26a is a direct transcriptional target of C/EBPα during stretch-induced HASMC hypertrophy.

**DISCUSSION**

Many lines of evidence demonstrate that physical forces elicit a number of biologically relevant signals in the human body. The best characterized system responses to mechanical stimuli are the cardiovascular (17–21), musculoskeletal (22–24), and pulmonary physiology (25, 33–38). Structural
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FIGURE 8. miR-26a is a C/EBPα-dependent gene. A, schematic representation of the 5′UTR of the human CTDSPL gene (miR-26a promoter). Region between −1800 and +1 bp contains putative binding elements for AP-1 (bottom) and C/EBPα (top). B and C, HASMCs were stimulated with a 1-h stretch, and then the chromatin was isolated and precipitated with anti-c-Fos, anti-C/EBPα, anti-RNA Poly II, or nonspecific IgG. qPCRs were performed with two sets of primers, as shown in A, specific for miR-26a promoter to identify the specific transcription factor and its region of binding to the miR-26a promoter (B) and resolved in 1% agarose gel (C). D and E, a 750-bp (pGL750) and 1000-bp (pGL1000) promoter region was synthesized and linked to luciferase (Luc) reporter gene (D). Cells were transfected with empty vector or the pGL750 or pGL1000 miR-26a promoter region. Forty-eight hours after transfection, cells were stimulated with a 1-h stretch, and then firefly luciferase activities were estimated and normalized to total protein production in a time-dependent manner, suggesting that the stretch-induced hypertrophy in HASMCs could be mediated through the suppression of GSK-3β gene expression. Interestingly, our data show that GSK-3β is a downstream target of miR-26a, and enforced expression of miR-26a in HASMCs inhibited cell proliferation, and then transforms them to hypertrophic phenotype in the absence of stretch. One possible explanation is that some other signaling pathway(s) that might regulate the stretch-induced proliferation may overdominate the inhibitory role of miR-26a in cell proliferation. To our knowledge, the identification of miR-26a as a stretch-responsive or mechanosensitive miRNA in primary HASMCs represents the first evidence linking miRNA to airway smooth muscle hypertrophy.

Like miR-26a, many proteins have emerged to regulate hypertrophy and promote disease upon their dysregulated expression. Among these, one of the potential proteins that regulates hypertrophy is GSK-3β. GSK-3β is constitutively active in unstimulated cells and inactivated upon phosphorylation at Ser9 (62). Although initially described as an inhibitor of glycogen synthesis through phosphorylation of glycogen synthase (63), GSK-3β was later revealed as a key signaling molecule regulating many aspects of cellular function, including protein synthesis, cytoskeletal integrity, and gene expression (64). More importantly, GSK-3β negatively regulates cardiac (39–43), skeletal (44, 45), and airway smooth muscle (44, 47) hypertrophy as evidenced by the finding that GSK-3β overexpression inhibits the hypertrophic phenotype, protein synthesis, and hypertrophic genes expression. In this study, we determined the expression of α-actin, SM22, and smMHC genes as the indicators of airway smooth muscle hypertrophy. Many studies have shown the up-regulation of these proteins occurs when airway smooth muscle cells enter into the hypertrophic cascade upon GSK-3β activation (46–47). Interestingly, our results showed that stretch decreased GSK-3β phosphorylation due to the inhibition of total protein production in a time-dependent manner, suggesting that the stretch-induced hypertrophy in HASMCs could be mediated through the suppression of GSK-3β gene expression. Interestingly, our data show that GSK-3β is a downstream target of miR-26a, and enforced expression of miR-26a in HASMCs inhibited cell proliferation, and then transforms them to hypertrophic phenotype in the absence of stretch. One possible explanation is that some other signaling pathway(s) that might regulate the stretch-induced proliferation may overdominate the inhibitory role of miR-26a in cell proliferation. To our knowledge, the identification of miR-26a as a stretch-responsive or mechanosensitive miRNA in primary HASMCs represents the first evidence linking miRNA to airway smooth muscle hypertrophy.

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we found that the miR-26a-GSK-3β-mediated signaling pathway was not involved in the stretch-induced proliferation of HASMCs. This is also consistent with the previous finding demonstrating that GSK-3β does not participate in HASMC hyperplasia (46). Our search for GSK-3β targets provided only five miRNAs (has-miR-199a, -199b, -26a, -26b, and -302a). Because miR-199a, miR-199b, miR-26b, and miR-302a were not induced by stretch we cannot rule out the possibility that these miRNAs are not upstream targets of GSK-3β. It is well recognized that miRNAs may function according to a “combinational circuitry model,” whereby a single miRNA targets multiple miRNAs and several miRNAs may target a single mRNA (65). Thus, further studies are required to elucidate specifically whether hypertrophic stimuli other than stretch can induce the expression of these predicted miRNAs, and whether miR-26a can regulate proteins that are involved in the hypertrophic cascade of HASMCs other than GSK-3β.

Gene expression-modulating miRNAs are encoded in diverse genomic locations, including intergenic regions, introns of protein-coding genes, and introns/exons of noncoding RNA genes (66). Most human miRNAs lie between protein-coding genes, whereas about one-third is within the introns of annotated mRNAs (3, 67). In the human genome, two distinct genes, CTDSPL and CTDSP2, encode different pri-miR-26a (miR-26a-1 and miR-26a-2) leading to the generation of identical mature miR-26a. miR-26a-1 is located in an intron of CTDSPL at chromosome 3, and miR-26a-2 is located in an intron of CTDSP2 at chromosome 12. It is likely that the intronic miRNAs are processed from the same primary transcript as the precursor mRNAs, and thus, their expression levels are regulated by the expression of the host mRNA (68). We examined the correlation between the miR-26a expression profile and the expression profile of the host genes by RT-qPCR in response to stretch. Surprisingly, in a time-dependent manner, stretch increased up-regulation of CTDSPL mRNA levels, but had no effect on CTDSP2 mRNA levels. As anticipated, miR-26a induction by stretch followed a similar pattern of the expression of CTDSPL levels. These results indicate that most of the miR-26a in HASMCs is miR-26a-1 in response to stretch, and is processed from the same primary transcript as its host gene CTDSPL. In contrast, in the absence of stretch, most of the miR-26a transcripts are miR-26a-2 that occurs by the activation of the host gene CTDSP2. Moreover, our study also showed that, in the absence of stretch, the basal CTDSPL mRNA levels were barely detectable, unlike CTDSP2 mRNA levels, and the basal miR-26a levels were high. This suggests the existence of a strong correlation between the expression of miR-26a and CTDSP2 mRNA, consistent with previous finding (48). We also determined the specific transcription factor by which pri-miR-26a is induced by stretch. Using ChIP assay, we found that activation of miR-26a promoter by stretch was completely dependent on the C/EBPα-binding sites. The luciferase reporter assays demonstrated that the stretch-induced activation of miR-26a promoter by C/EBPα was effective only in the presence of the distal miR-26a promoter region (1000 bp), which contains two C/EBPα-binding sites. Moreover, the small interference RNA-mediated knockdown of C/EBPα experiment confirmed the requirement of C/EBPα for the activation
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FIGURE 10. miR-26a pathway in the initiation of HASMC hypertrophy. Up-regulation of miR-26a by stretching of HASMCs or enforced expression of miR-26a suppresses GSK-3β protein expression that triggers global cellular protein synthesis. As a result, smooth muscle hypertrophic marker proteins (α-actin, SM22, and MHC) are up-regulated, which, in turn, initiates hypertrophy.

of miR-26a promoter by stretch. The present study also corroborated the necessity of C/EBPα for the induction of HASMC hypertrophy by stretch. Our study indicates that C/EBPα plays a key role in the induction of HASMC hypertrophy via miR-26a up-regulation in response to stretch.

Taken together, our data demonstrate that miR-26a is a mechanosensitive and hypertrophic miRNA of HASMCs. Stretch activates the miR-26a promoter through C/EBPα transcription factor, which leads to the up-regulation of miR-26a. In addition, stretch selectively induces the transcription of miR-26a located in the locus 3p21.3 of human chromosome 3. Molecular functional analyses indicate that miR-26a-induced hypertrophy is mediated through the suppression of its target gene GSK-3β expression (Fig. 10). We anticipate our study can serve as a model system for studying gene regulation by miRNAs in the development of gene therapy for airway diseases such as asthma and chronic obstructive pulmonary disease.

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