SOX5 is a transcription factor with homology to the high mobility group box region of the testis-determining factor, SRY. Both the mouse and human SOX5 genes encode a 48-kDa SOX5 protein (S-SOX5) that is only present in tissues containing cells with motile cilia/flagella. The mammalian sperm-associated antigen 6 gene (SPAG6) encodes an axonemal central apparatus protein. Because human and mouse SPAG6 gene promoters contain multiple potential binding sites for SOX5, SPAG6 gene regulation by S-SOX5 was investigated in BEAS-2B cells, a line derived from human bronchial cells. Like FOXJ1, a transcription factor known to be essential for motile ciliogenesis, S-SOX5 stimulates mouse and human SPAG6 promoter activity in BEAS-2B cells, but the effect was abrogated when the SOX5 binding sites were mutated or deleted. S-SOX5 and FOXJ1 function cooperatively in stimulating SPAG6 promoter activity. The SPAG6 message was up-regulated when S-SOX5 was over-expressed in BEAS-2B cells, and silencing of S-SOX5 by RNA interference down-regulated SPAG6 transcripts. Chromatin immunoprecipitation and EMSA experiments demonstrated that S-SOX5 associates with the SPAG6 promoter directly. The present study demonstrates that SPAG6 is a S-SOX5 target gene, indicating a key role for S-SOX5 in the formation and function of motile cilia.

Hair follicle, gut, B cell, and cartilage development (6–13). SOX genes are expressed in many tissues and are implicated in the etiology of many diseases (14).

SOX5 is a member of the SOXD group, which includes three genes, SOX5, SOX6, and SOX13 (15). Mouse Sox5 is expressed as a short transcript (2 kb) in adult testis (16) and a longer transcript (6 kb) in other tissues (17). The short transcript encodes a 48-kDa protein isoform that lacks the N-terminal half of the larger protein encoded by the 6-kb transcript. The former protein was the first to be discovered and was named SOX5 (called S-SOX5 in this paper). The longer SOX5 isoform was originally named L-SOX5 (18), but most authors refer to this isoform as SOX5. L-SOX5 is highly expressed in chondrocytes and striated muscles, indicating a likely role in human cartilage and muscle development (19, 20). The phenotype of L-Sox5 knock-out mice demonstrated that L-SOX5 is essential for cilia formation and notochord extracellular matrix sheath formation, notochord cell survival, and development of the nucleus pulposus of intervertebral discs (21–23).

The different tissue distribution of S-SOX5 suggests that it plays roles that are distinct from those of L-SOX5. It has been reported that S-SOX5 regulates genes expressed in the testis including the testicular isoform of hormone-sensitive lipase, IkBβ, and ZNF230 (24–26). Besides the testis, S-SOX5 has been reported to be highly expressed in human brain (27). The high abundance of S-SOX5 in testis and brain, tissues that contain motile cilia/flagella, suggests that S-SOX5 might play a role in ciliogenesis and/or spermatogenesis. To test this idea, we selected a target gene, sperm-associated antigen 6 (SPAG6), that is representative of 9+2 cilia, encoding a protein in the axoneme central pair.

Mammalian sperm-associated antigen 6 is the orthologue of Chlamydomonas PF16. SPAG6 was first identified in a human testis cDNA expression library (28). Mouse Spag6 was subsequently cloned from a library generated from mixed germ cells (29). Both human and mouse SPAG6 genes each encode 1.8- and 2.8-kb mRNAs highly expressed in testis. In sperm, SPAG6 protein is present in the sperm tail, and immunoelectron microscopy demonstrated that SPAG6 is localized to the central apparatus of the axoneme, consistent with its localization in the Chlamydomonas flagella (30). In addition to testis and sperm, SPAG6 is also expressed in tissues that contain cells bearing 9+2 motile cilia, such as brain and lung (28, 31–33). As
SPAG6 protein contains eight contiguous armadillo repeats, which places it in a family of proteins known to mediate protein-protein interactions. It has been shown that SPAG6 associates with SPAG16L and SPAG17, two other proteins localized to the axoneme central apparatus (32, 39, 40). In Spag6 mutant mice, both SPAG16L and SPAG17 were missing from sperm (32, 39, 40). These data imply that SPAG6 is a critical protein in either the assembly or structural integrity of the sperm tail axoneme.

Using a bioinformatics approach, we identified potential transcription factors that might bind to the mouse and human SPAG6 proximal promoter regions and regulate SPAG6 gene expression. This analysis revealed multiple potential binding sites for SOX5, a transcription factor that has not been previously implicated in the regulation of genes encoding ciliary/flagellar proteins. We therefore investigated the role of S-SOX5 in the regulation SPAG6 gene expression.

EXPERIMENTAL PROCEDURES

5’-Rapid Amplification of cDNA Ends

5’-Rapid amplification of cDNA ends was conducted to define the 5’-untranslated region (UTR) of SPAG6 mRNA and transcriptional start sites using a Human Marathon cDNA Amplification kit (Clontech) according to the manufacturer’s instructions.

DNA Constructs

Human SPAG6 Promoter Constructs—1.5 kb of the human SPAG6 promoter that contains multiple putative SOX5 binding sites was amplified and cloned into KpnI/NheI sites of the PGL3 basic vector. A 500-bp SPAG6 promoter sequence containing one putative SOX5 binding site was also cloned into KpnI/NheI sites of the PGL3 basic vector.

Mouse Spag6 Promoter Constructs—A 1.5-kb mouse Spag6 promoter fragment was cloned in PGL3 basic vector as previously described (31). A 250-bp mouse Spag6 promoter sequence containing two putative SOX5 binding sites, including the transcriptional start site, was cloned into KpnI/Xhol sites of the PGL3 basic vector.

Site-directed Mutagenesis of SOX5 Binding Sites in SPAG6 Promoter Constructs—SOX5 binding sites in the 500-bp human and the 250-bp mouse SPAG6 promoter constructs were mutated or deleted using a QuikChange XL Site-directed Mutagenesis kit (Stratagene, La Jolla, CA), and the mutations/deletions were confirmed by DNA sequencing.

Mouse and Human S-SOX5 Expression Constructs—Full-length mouse S-Sox5 cDNA was amplified and cloned into the BamHI/Sall site of the pTarget (pT) vector (Promega). S-Sox5/pTarget plasmid was digested with KpnI and Xhol restriction enzymes, and the S-Sox5 cDNA was subcloned to the KpnI/Xhol site of the pcDNA3 mammalian expression vector (Invitrogen). pcDNA3 carries a neomycin resistance cassette, which was used for G418 selection to generate stable BEAS-2B cell lines for use in subsequent experiments. A mouse S-Sox5 expression plasmid with a FLAG tag fusion in the N terminus was also generated. The S-Sox5 cDNA was cloned into the Sall/KpnI sites of the pRK-7 vector (kindly provided by Dr. Wenhui Hu, Department of Physiology, Virginia Commonwealth University). A Foxj1 expression plasmid in pcDNA3 was previously described (41).

To create a human S-SOX5/pTarget construct, full-length human S-SOX5 cDNA was amplified from human testis cDNA (Clontech) and subcloned into the BamHI/NotI sites of pTarget vector.

Human SOX5 RNAi Constructs—RNAi constructs targeting human SOX5 transcript were generated with the iLenti™ siRNA Expression System (Capital Biosciences, Inc., Washington, D. C.).
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The target sequences were identified through BLOCK-iT™ RNAi Designer (Invitrogen). Three sets of oligonucleotides were designed according to the user’s manual. Double-stranded oligonucleotides (see supplementary Table 1) were generated, phosphorylated, and then ligated to linearized iLenti™ vector. The ligation products were used for transformation, and positive clones were identified by restriction digestion and sequencing.

Western Blot Analysis

Equal amounts of protein (50 µg/lane) were heated to 95 °C for 10 min in 4× sample buffer, loaded onto 10% SDS-polyacrylamide gels, electrophoretically separated, and transferred to polyvinylidene difluoride membranes. The membranes were blocked and then incubated with antibodies against SOX5 (Aviva Systems Biology, San Diego, CA) or GAPDH (Santa Cruz Biotechnology, Santa Cruz, CA) overnight at 4 °C. After washing, the blots were incubated with an anti-rabbit immunoglobulin conjugated to horseradish peroxidase for 1 h at room temperature. SOX5 or GAPDH protein was detected with the Super Signal Pico Chemiluminescent or Femto Maximum Sensitive system (Pierce).

Immunofluorescence Staining

Human bronchial BEAS-2B cells (American Type Culture Collection (ATCC), Rockville, MD) were seeded into two-chamber slides (BD Biosciences) and transfected with pTarget (control) and S-SOX5/pTarget. Two days after transfection, cells were washed with PBS and fixed in 100% methanol at −80 °C for 7 min. Cells were blocked in PBS containing 10% goat serum for 30 min at room temperature followed by overnight incubation with the polyclonal anti-rabbit SOX5 primary antibody at 4 °C. After washing cells three times with PBS, the secondary antibody, Alexa Fluor 488 anti-rabbit IgG (Invitrogen), was applied in 10% goat serum for 1 h at room temperature. After washing three more times with PBS, the slides were mounted and sealed with nail polish. SOX5 staining was visualized using a Nikon Eclipse TS100 phase contrast/fluorescent microscope; images were obtained with a Nikon Coolpix 4500 digital camera.

Generation of S-SOX5 Stable Cell Lines

BEAS-2B cells were seeded at 1.5 × 10⁶ cells/100-mm dish and the next day transfected with 6 µg/dish pcDNA3 or S-SOX5/pcDNA3 plasmids. 24 h after transfection, the cells were supplied with fresh medium containing 1.5 mg/ml G418. Resistant clones were selected within 15–20 days and expanded. The G418 concentration was maintained at 1 mg/ml in the cell culture medium (42).

Generation of Adenovirus-expressing S-SOX5

An adenovirus (Ad)³-expressing mouse S-SOX5 (AdS-SOX5) was generated with AdEasy™ Adenoviral Vector system (catalogue no. 240010; Stratagene) following the instruction manual. Briefly, mouse S-Sox5 cDNA was subcloned into adenovirus shuttle vector, pShuttle-CMV, and the cDNA was transferred into the pAdEasy-1 virus genome by homologous recombination in Ad packaging HEK-293 cells. The control virus was made with empty pShuttle-CMV plasmid. The expression of S-SOX5 was tested by Western blot analysis of protein from COS-1 cells and BEAS-2B cells infected with AdS-SOX5 or control Ad virus. The AdS-SOX5 and control Ad virus were amplified in the Virus Facility of Virginia Commonwealth University.

RT-PCR and real-time PCR

Total RNA was isolated from cultured cells and indicated tissues with TRIZol (Invitrogen), the RNA was reversed transcribed, and the cDNAs were used for RT-PCR or real-time PCR. For real-time PCR, the cDNAs from BEAS-2B cells transfected with pcDNA3 or S-Sox5/pcDNA3 plasmids, or SOX5 RNAi plasmid DNA, infected with AdS-SOX5 or control Ad virus, were utilized for PCR. 18 S rRNA was amplified simultaneously to normalize the expression level of target genes. The primers were synthesized according to the published sequences (43). Real-time PCR was carried out using 2× SYBR Green master mix (Bio-Rad).

Transient Transfection and Promoter Activity Assays

The promoter activity was assessed using BEAS-2B human bronchial epithelial cells and MDCK cells (ATCC). Cells were maintained in BEBM (Cambrex, for BEAS-2B cells) or DMEM with 10% fetal calf serum (for MDCK cells). 24 h prior to transfection, 3 × 10⁴ cells were plated in 12-well plates. Triplicate wells were transfected with indicated plasmids using FuGENE 6 (Roche Diagnostics). In addition, 25 ng of the Renilla luciferase plasmid was transfected into each well to evaluate transfection efficiency. 48 h after transfection, the cells were harvested, and the promoter activity was measured with the Dual Luciferase Reporter Assay system (Promega).

Chromatin Immunoprecipitation (ChIP) Assay

ChIP assay was performed using a ChIP assay kit (Upstate Millipore) following the manufacturer’s instructions. Briefly,
the BEAS-2B cells were infected with AdS-SOX5. 48 h after infection, the proteins bound to DNA were cross-linked with 1% formaldehyde; cells were subsequently resuspended in the lysis buffer, followed by sonication. After preclearing with salmon sperm DNA/protein A-agarose (50% slurry), the protein extracts were subjected to immunoprecipitation with antibody against SOX5 or normal rabbit IgG. Immunocomplexes were extensively washed, and the DNA was recovered and resuspended in 50 μl of 10 mM Tris-Cl (pH 8.5). The DNA was used as template for PCR amplification with three sets of primers flanking SOX5 binding sites. As a negative control, PCR was carried out with a primer set 3.5 kb upstream of the transcription start site where no SOX5 binding site is nearby. The experiment was repeated three times. The primer sequences are seen in the supplementary Table 1.

FIGURE 3. Structures of human SOX5 variants and analysis of SOX5 expression in BEAS-2B cells. A, structures of human SOX5 variants. The translational start sites and primers for amplifying individual transcript are indicated. B, RT-PCR amplification of the three SOX5 transcripts with specific primers shown in A with RNA isolated from BEAS-2B cells. C, Western blot analysis of S-SOX5 protein expression in mouse testis and BEAS-2B cells. Note that only 48-kDa S-SOX5 protein was detected when the Femto system was used. D, RT-PCR amplification of SOX6 transcripts in BEAS-2B cells and human testis. Con, no template control.

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To purify FLAG-tagged S-SOX5 protein, the plasmid containing FLAG-S-SOX5 cDNA was transfected into 293T cells, 48 h after transfection, the protein was purified with EZviewRed Anti-FLAG M2 Affinity Gel system (Sigma-Aldrich) following the manufacturer’s manual. For EMSA experiments, six double-stranded synthetic oligonucleotides were made; three contained putative SOX5 binding sites, and the other three had the same sequences as the previous ones except that five nucleotides in the SOX5 binding sites were mutated. The double-stranded synthetic oligonucleotides were labeled with T4 polynucleotide kinase and [γ-32P]ATP. The EMSA binding reaction was mixed in 2× binding buffer (Promega) with up to 4 μg of purified S-SOX5 protein and 1 × 105 cpm of 32P-labeled double-stranded oligonucleotide probes (1 ng). Reaction mixtures were incu-
bated at room temperature for 30 min and then subjected to an 8% polyacrylamide gel at 250 V for 3 h. The dried gels were then exposed to x-ray films.

RESULTS

Identification of the 5′-UTR of Human SPAG6 Gene—To define the 5′-UTR of human SPAG6 mRNA and identify transcriptional start sites, 5′-rapid amplification of cDNA ends was performed with a reverse primer located close to the 5′ end of human SPAG6 mRNA. PCR products were amplified and subcloned into the pCR2.1 TOPO TA vector, and then 15 clones were subjected to DNA sequencing. The 5′-UTR sequence was submitted to GenBank (EF432114). The 5′-UTR is GC-rich, and four potential transcription start sites were identified based on the sequencing results as noted in GenBank.

Both Human and Mouse SPAG6 Promoters Contain Multiple Potential SOX5 Binding Sites—To study transcription regulation of SPAG6 gene expression further, putative transcription factor binding sites in the proximal promoters of the mouse (AY792595) and human (EF432114) genes were analyzed using ConSite, open source software that determines high probability binding sites for known transcription factors. Multiple putative binding sites for SOX5, an SRY-related transcription factor with a high mobility group DNA binding domain, were identified in both promoter sequences with the default setting, the TF score cutoff being 80% (Fig. 1), these are putative SOX binding sites, and it must be recognized that the program cannot identify those sites which uniquely bind SOX5.

S-Sox5 Transcripts Are Abundant in Mouse Tissues Containing Cells with Motile Cilia—Three mouse Sox5 transcript variants were identified in GenBank. Transcript variant 1 has 15 exons, and transcript variant 2 is almost identical to transcript variant 1, except that it lacks exon 9 of transcript variant 1. These
two transcripts translate the L-SOX5 protein. The third transcript variant has only eight exons, the first of which is not present in transcript variants 1 and 2, whereas exons 2–8 are identical to exons 8–14 of transcript variant 2 (Fig. 2A). This third variant has been reported to be highly expressed in the testis (16) and translates a truncated 48-kDa SOX5 protein (S-SOX5). The unique exon 1 of this variant allowed us to design a specific primer set to amplify this variant. RT-PCR result revealed that this transcript (“testis variant”) is only expressed in the testis, lung, and brain (Fig. 2B, top panel), all tissues that contain motile cilia.

The tissue distribution of Sox5 encoding L-SOX5 protein was also examined with specific primers (Fig. 2B). This transcript is present in heart, brain, kidney, skeletal muscle, uterus, oviduct and ovary, but not in the testis and lung (Fig. 2B, middle panel). Western blot analysis was conducted with an antibody that recognizes both SOX5 proteins. The 48-kDa S-SOX5 is expressed in the testis, brain, and lung. The 84-kDa L-SOX5 is present in heart, brain, kidney, and skeletal muscle (Fig. 2C). Besides the 48-kDa and 84-kDa proteins, the antibody also recognized a 25-kDa protein in some tissues, including liver, spleen, and testis (Fig. 2C), which probably represents the translated protein from an additional Sox5 transcript that was reported in the Ensembl database (ENSUST0000011746).

S-SOX5 Is Expressed in BEAS-2B Cells—Multiple human SOX5 transcript variants were identified in databases (Fig. 3A). Transcript variants 1 (NM_006940) and 2 (NM_152989) translate into full-length L-SOX5. Transcript variant 3 (NM_178010) translates into a truncated SOX5 protein, and this transcript variant is similar to the mouse S-Sox5 transcript.

RT-PCR and Western blot analysis were used to investigate endogenous expression of SOX5 in BEAS-2B cells. A single PCR product was amplified with the primer set specific for human S-SOX5 cDNA (transcript variant 3, Fig. 3B). However, no PCR product was amplified with primer sets that are specific for L-SOX5 messages (transcript variants 1 and 2, Fig. 3B), indicating that S-SOX5 message is the predominant isoform in BEAS-2B cells. Western blot analysis was conducted with total lysates from BEAS-2B cells. The 48-kDa S-SOX5 was detected with the Femto Maximum Sensitive substrate system (Fig. 3C). The expression of SOX6, another member in SOXD group, in BEAS-2B cells was also investigated. Even though SOX6 is in the same group as SOX5, and like S-SOX5, it is expressed in the testis, the message was not detected in BEAS-2B cells (Fig. 3D).

S-Sox5 Is Localized in the Nuclei in Transfected BEAS-2B Cells—To study the function of S-SOX5 protein, full-length mouse Sox5 cDNA was subcloned into the mammalian expression vector pTarget. To test the construct, S-Sox5/pTarget or empty vector was transfected into COS-1 cells, and Western blot analysis was performed. The 48-kDa S-SOX5 protein was detected only in the cells transfected with S-Sox5/pTarget, but not cells transfected with control empty pTarget plasmid when the Pico Chemiluminescent substrate system was used (supplemental Fig. 1A). Cellular localization of S-SOX5 following S-Sox5/pTarget transfection into BEAS-2B cells was investigated using immunofluorescent staining with a specific antibody against S-SOX5. S-SOX5 was detected only in the nuclei, whereas normal rabbit IgG showed no signal (supplemental Fig. 1B).

S-SOX5 Stimulates Both Human and Mouse SPAG6 Promoter Function—To study the effect of S-SOX5 on SPAG6 gene transcription, the 1.5-kb human SPAG6 gene promoter, including the transcription start site, was amplified and subcloned into PGL3 basic vector. The S-SOX5 expression plasmids and human or mouse SPAG6 promoter constructs were co-transfected into bronchial epithelial cell-derived BEAS-2B or renal epithelial cell-derived MDCK cells, and relative luciferase activity was analyzed. In BEAS-2B cells, both the human and mouse SPAG6 promoters displayed basal activity, which was about
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**FIGURE 8.** S-SOX5 directly binds to a probe that contains a SOX5 binding site in the human SPAG6 proximal promoter. A, schematic representation of the human SPAG6 promoter showing the location of the probe containing a SOX5 binding site used in this study. The sequences of the putative SOX5 binding sites are indicated in supplemental Table 1. B, EMSA using purified S-SOX5 protein and a probe containing SOX5 binding site (WT Probe) or SOX5 binding site mutated probe (MT Probe). L, 1 μg of S-SOX5 protein; H, 4 μg of S-SOX5 protein. The purified S-SOX5 protein was incubated with the indicated labeled double-stranded oligonucleotide probes, then subsequently applied to 8% polyacrylamide gels. C, competition of labeled WT probe with unlabeled WT and unlabeled MT probes. L, 50-fold unlabeled probe; H, 250-fold unlabeled probe.

4-fold higher than the empty PGL3 basic vector. Co-transfection of S-SOX5, increased relative luciferase activity an additional 3-fold only in BEAS-2B cells (Fig. 4). In MDCK cells, even though both human and mouse Spag6 promoter activity was detected, expression of S-SOX5 had no effect on the function of both promoters (Fig. 4).

FOXJ1, a transcription factor that is known to be essential for motile ciliogenesis, also activated SPAG6 promoter function in BEAS-2B cells. The relative luciferase activity was 7-fold greater compared with control (baseline SPAG6 promoter activity). The combination of S-SOX5 and FOXJ1 resulted in a 13-fold relative luciferase activity in BEAS-2B cells. There was no additional effect of co-transfection of S-SOX5 and FOXJ1 in MDCK cells.

**Forced Expression of S-SOX5 Increases SPAG6 mRNA Expression in BEAS-2B Cells**—S-SOX5 adenovirus and BEAS-2B cells stably expressing S-SOX5 were generated, and the effect of S-SOX5 on SPAG6 mRNA expression was investigated. To confirm S-SOX5 protein expression in the BEAS-2B/S-SOX5 stable cell line or BEAS-2B cells infected with Ads-SOX5, Western analysis was performed. The 48-kDa S-SOX5 protein was highly expressed in both cell types, but it was not detected in the control cells with the Pico system (Fig. 5A). Real-time PCR was conducted to compare relative SPAG6 mRNA abundance in the same cells. As expected, in both cell types, relative mRNA levels were also reduced in the two specific RNAi cell lines. SPAG6 mRNA levels were correspondingly reduced in the stable cell lines with the two specific RNAi constructs (Fig. 6C).

**SPAG6 mRNA level was significantly higher than in controls (Fig. 5B).**

**Knockdown of S-SOX5 in BEAS-2B Cells Decreases SPAG6 mRNA Expression**—To study further the regulation of SPAG6 by S-SOX5, three human SOX5 RNAi plasmid constructs were generated; the targeting positions were 167–188, 225–246, and 1109–1130 of SOX5 transcript variant 3 (NM_178010), respectively. To test the efficiency of these constructs, COS-1 cells were co-transfected with these constructs and S-Sox5/pTarget and subjected to Western blot analysis. All three of the RNAi constructs effectively reduced S-SOX5 protein expression, especially the constructs targeting 225–246 and 1109–1130 (Fig. 6A). BEAS-2B stable cell lines were generated with these RNAi and control constructs, and real-time PCR was conducted to analyze S-SOX5 and SPAG6 mRNA expression. The S-SOX5 protein expression was reduced in the BEAS-2B cells stably expressing the RNAi plasmids (Fig. 6B), and the level of reduction was consistent with the reduction of protein level in COS-1 cells (Fig. 6A). S-SOX5 mRNA levels were also reduced in the two specific RNAi cell lines.

**SPAG6 mRNA level was significantly higher than in controls (Fig. 5B).**

**Knockdown of S-SOX5 in BEAS-2B Cells Decreases SPAG6 mRNA Expression**—To study further the regulation of SPAG6 by S-SOX5, three human SOX5 RNAi plasmid constructs were generated; the targeting positions were 167–188, 225–246, and 1109–1130 of SOX5 transcript variant 3 (NM_178010), respectively. To test the efficiency of these constructs, COS-1 cells were co-transfected with these constructs and S-Sox5/pTarget and subjected to Western blot analysis. All three of the RNAi constructs effectively reduced S-SOX5 protein expression, especially the constructs targeting 225–246 and 1109–1130 (Fig. 6A). BEAS-2B stable cell lines were generated with these RNAi and control constructs, and real-time PCR was conducted to analyze S-SOX5 and SPAG6 mRNA expression. The S-SOX5 protein expression was reduced in the BEAS-2B cells stably expressing the RNAi plasmids (Fig. 6B), and the level of reduction was consistent with the reduction of protein level in COS-1 cells (Fig. 6A). S-SOX5 mRNA levels were also reduced in the two specific RNAi cell lines. SPAG6 mRNA levels were correspondingly reduced in the stable cell lines with the two specific RNAi constructs (Fig. 6C).

**Forced Expression of S-SOX5 Increases SPAG6 mRNA Expression in BEAS-2B Cells**—S-SOX5 adenovirus and BEAS-2B cells stably expressing S-SOX5 were generated, and the effect of S-SOX5 on SPAG6 mRNA expression was investigated. To confirm S-SOX5 protein expression in the BEAS-2B/S-SOX5 stable cell line or BEAS-2B cells infected with Ads-SOX5, Western analysis was performed. The 48-kDa S-SOX5 protein was highly expressed in both cell types, but it was not detected in the control cells with the Pico system (Fig. 5A). Real-time PCR was conducted to compare relative SPAG6 mRNA abundance in the same cells. As expected, in both cell types, relative mRNA levels were also reduced in the two specific RNAi cell lines. SPAG6 mRNA levels were correspondingly reduced in the stable cell lines with the two specific RNAi constructs (Fig. 6C).

**SPAG6 mRNA level was significantly higher than in controls (Fig. 5B).**
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as revealed by the indicated shifted bands in Figs. 8B and 9B. The shifted signals represent specific binding of S-SOX5 and the probes because no shifted bands were observed when the probes were incubated with BSA or when the S-SOX5 protein was incubated with the probes with SOX5 binding sites mutated (Figs. 8B and 9B). The specific signals were eliminated by unlabeled probes in both the mouse and human proximal promoters. Besides the three binding sites, S-SOX5 also binds to the two site flanked by the ChIP assay primers (supplemental Fig. 3), but S-SOX5 does not bind the probe (Fig. 7Aa sites) that does not contain SOX5 binding site (data not shown).

S-SOX5 Fails to Activate SPAG6 Promoter Activity When SOX5 Binding Sites Are Mutated or Deleted—To investigate further whether S-SOX5 activates SPAG6 promoters through binding to SOX5 binding sites, a 500-bp human SPAG6 promoter construct containing one SOX5 binding site and a 250-bp Spag6 promoter construct containing two SOX5 binding sites were generated. Like the 1.5-kb SPAG6 promoter construct, S-SOX5 stimulated both promoters. When the SOX5 binding sites were mutated or deleted, the stimulating effect was ablated (Fig. 10 and supplemental Fig. 4).

DISCUSSION

The SPAG6 gene has been conserved during evolution. The protein plays an indispensable role in the integrity of the axoneme and flagella motility. To study transcriptional regulation of the gene, we characterized the mouse and human SPAG6 proximal promoters and identified transcription factors that might regulate SPAG6 gene expression (31). To search for potential transcription factor binding sites, the promoter sequences were analyzed with the ConSite program. It was found that both the human and mouse SPAG6 promoters have similar potential transcription factor binding sites, including binding sites for CREB/CREM, SOX17, SPZ1, forkhead transcription factors, and SOX5. Thus, both mouse and human SPAG6 promoter regions contain putative binding sites for similar transcription factors, which strongly suggests that both human and mouse SPAG6 genes are regulated by similar mechanisms. In this study, we focused on the study of regulation of mammalian SPAG6 genes by SOX5.

The SOX5 gene encodes two major proteins, the full-length 84-kDa L-SOX5 (SOX5) and the 48-kDa SOX5 (S-SOX5). The SOX5 protein, but not the L-SOX5 protein, is expressed in tissues with motile cilia (Fig. 2), suggesting a role of this transcription factor in motile ciliogenesis. The presence of potential binding sites for SOX5 in both the human and mouse SPAG6 gene promoters and the fact that the Spag6 message is also abundant in mouse tissues with motile cilia, paralleling the tissue distribution of S-SOX5, suggested that S-SOX5 regulates Spag6 gene transcription. To explore this role in vitro, we examined the promoter in the context of BEAS-2B cells, a line derived from viral transformed human bronchial epithelial cells. BEAS-2B cells express endogenous S-SOX5 (Fig. 3), indicating that this cell line is suitable for the study of genes transcriptionally regulated by SOX5. Immunofluorescence staining revealed that expressed S-SOX5 protein was located in the nuclei (supplemental Fig. 1), supporting the notion that SOX5 functions as a transcription factor.

S-SOX5 stimulated SPAG6 promoter function in BEAS-2B cells, but not in MDCK cells (Fig. 4), and other cell types, including COS-1 cells and CHO cells (data not shown), that are derived from tissues not bearing motile cilia, implying that S-SOX5 can regulate SPAG6 gene expression only in the con-
S-SOX5 Regulates SPAG6 Gene Expression

FIGURE 10. Functional analyses of SOX5 binding sites in human and mouse SPAG6 promoters. A, effect of S-SOX5 on human SPAG6 promoter activity. Left, maps of the wild type human SPAG6 promoter construct containing one SOX5 binding site and the construct with mutation of the SOX5 binding site. Right, effect of S-SOX5 on the wild type and mutated SPAG6 promoter function. Values are means ± S.E. (error bars) from three separate experiments. B, effect of S-SOX5 on mouse Spag6 promoter activity. Left, maps of the mouse Spag6 promoter constructs that contains two SOX5 binding sites and constructs with series mutation of the SOX5 binding sites. Right, effect of S-SOX5 on the wild type and mutated Spag6 promoter function. Values are means ± S.E. from three separate experiments. *, p < 0.05 compared with controls (without S-SOX5 stimulation).

The text of the appropriate cells that may contain programs to support production of axonemes with a 9 + 2 structure, such as male germ cells, ependymal cells, and tracheal and bronchial epithelial cells. This cell-specific function is also consistent with the tissue distribution of S-SOX5.

The effect of S-SOX5 on SPAG6 transcription was confirmed by overexpression of S-SOX5 and silencing SOX5 in BEAS-2B cells. Overexpression of S-SOX5 significantly increased SPAG6 mRNA expression. Conversely, SPAG6 mRNA expression was dramatically decreased when SOX5 mRNA was knocked down by RNA interference. Given that S-SOX5 is the predominant SOX5 isoform in BEAS-2B cells, it is unlikely that the down-regulation of SPAG6 mRNA was the result of knockdown other SOX5 isoforms.

The fact that S-SOX5 RNA knockdown reduces SPAG6 gene expression, whereas forced expression of S-SOX5 increases endogenous SPAG6 levels, indicates that the abundance of SOX5 in cells is a determinant of the level of SPAG6 gene transcription. BEAS-2B cells studied under our culture conditions evidently have a steady-state level of S-SOX5 that is sufficient to drive some SPAG6 transcription, but not at the maximal potential rate because additional S-SOX5 augments SPAG6 above the basal state.

S-SOX5 associates directly with multiple SOX5 binding sites of the SPAG6 promoter. This conclusion was supported by the facts that DNA can be amplified by several primer sets that flank SOX5 binding sites (Fig. 7). The EMSA studies indicated that S-SOX5 binds specifically to these cis-elements (Figs. 8 and 9 and supplemental Fig. 3). These binding sites have functional significance because S-SOX5 could not stimulate SPAG6 promoter function when SOX5 binding sites were mutated or deleted (Fig. 10 and supplemental Fig. 4).

Given that there are multiple putative SOX5 binding sites in the promoter region, a comprehensive analysis of all putative sites was not carried out. We cannot quantify the relative contribution of each cis-element. Thus, what we can conclude is that S-SOX5 is an important transcription factor controlling the SPAG6 promoter.

It has been reported that other transcription factors, including FOXJ1 and RFX, regulate ciliogenesis. FOXJ1 is a forkhead/winged-helix transcription factor family. It is expressed in embryonic node, adult lung, choroid plexus, testis, and oviduct (44, 45). Targeted deletion of the Foxj1 gene resulted in defective ciliogenesis in airway epithelial cells and randomized left-right asymmetry. Half of the mice had situs inversus (46). FOXJ1 is also required for epithelial cell ciliogenesis in the oviduct (47). Microarray analysis indicates that Xenopus Foxj1 induces the formation of cilia by up-regulating the expression of motile cilia genes, which includes the Spag6 gene (48). Thus, FOXJ1 plays an essential role in the formation of motile cilia (38). Our studies revealed that FOXJ1 stimulates SPAG6 promoter function in BEAS-2B and MDCK cells. The stimulatory effect is greater than S-SOX5, suggesting that FOXJ1 might play a dominant role in the regulation of the SPAG6 gene. Interestingly, in the presence of the combination of S-SOX5 and FOXJ1, significantly higher promoter activity was obtained, suggesting that the two transcription factors function cooperatively to stimulate SPAG6 promoter function.

Cells with 9 + 2 cilia/flagella presumably express a factor(s) that allows S-SOX5 to transactivate the SPAG6 gene promoter. Notably, we have previously cloned the promoters of the genes encoding SPAG16L and SPAG17, two other central apparatus proteins. Both of these promoters also contain putative SOX5 binding (supplemental Figs. 5 and 6), and S-SOX5 can also activate these promoters in BEAS-2B cells (data not shown), raising the possibility that the function of S-SOX5 is to regulate formation of the central apparatus through transcriptional control of a suite of genes encoding key components of the central pair of microtubules.

In summary, our study reveals that S-SOX5 regulates SPAG6 gene expression, acting in concert with FOXJ1. These observations provide the first evidence of S-SOX5 involvement in...
motile cilia/flagella through control of expression of an axo-

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REFERENCES

1. Degnan, B. M., Vervoort, M., Larroux, C., and Richards, G. S. (2009) Carv.
   Opin. Genet. Dev. 19, 591–599
2. Phochanukul, N., and Russell, S. (2010) Int. J. Biochem. Cell. Biol. 42, 453–464
3. Chew, L. J., and Gallo, V. (2009) J. Neurosci. Res. 87, 3277–3287
4. Reiprich, S., Kriesch, J., Schreiner, S., and Wegner, M. (2010) J. Neuro-
   chem. 112, 744–754
5. Horowitz, E., Azuma, M., Iwata, Y., Nakamura, K., and Ikegawa, S. (2002)
   EMBO J. 21, 130, 615–625
6. Sapiro, R., Tarantini, L. M., Velazquez, F., Kiriakidou, M., Hecht, N. B.,
   Buican, M., and Strauss, J. F., III (2002) Mol. Cell. Biol. 22, 6298–6305
7. Horowitz, E., Zhang, Z., Jones, B. H., Moss, S. B., Ho, C., Wood, I. R.,
   Wang, X., Sammel, M. D., and Strauss, J. F., III (2005) Mol. Hum. Repro.
   11, 307–317
8. Christiano, A. M. (2008) Nat. Genet. 40, 394–407
9. Kanai-Azuma, M., Kanai, Y., Gad, J. M., Yonekawa, H., Yazaki, K., Tam, P. P.,
   and Hayashi, Y. (2002) J. Clin. Invest. 110, 2197–2205
10. Budde, L. M., Wu, C., Tilman, C., Douglas, L., and Ghosh, S. (2002) Mol.
    Biol. Cell 13, 4179–4194
11. Xu, W., Zhang, S., Qiu, W., He, G., Liu, Y., Sun, Y., Ma, Y., Dong, J.,
    and Zhang, W. (2009) Mol. Biol. Rep. 36, 1187–1193
12. Wunderle, V. M., Critcher, R., Ashworth, A., and Goodfellow, P. N. (1996)
    Genomics 36, 354–358
13. Bonnefond, A., Zirngibl, R. A., Jurdic, P., and Aubin, J. E. (2007)
    Mol. Cell. Biol. 27, 7993–8004
14. You, Y., Huang, T., Richer, E. J., Schmidt, J. E., Zabner, J., Borok, Z., and
    Brody, S. L. (2007) Am. J. Physiol. Lung Cell. Mol. Physiol. 293, L650–L657
15. Zhang, Z., Tang, W., Zhou, R., Shen, X., Wei, Z., Patel, A. M., Powell, J.
    T., Bennett, J., and Strauss, J. F., III (2007) Cell. Motil. Cytoskeleton 64, 360–376
16. Sonic, K. M., Chari, R., Deleuwe, R. J., Shadoe, A., Chi, B., Tsao, M. S.,
    Jones, S., Marra, M., Ling, V., Ng, R., Macaulay, C., Lam, S., and Lam, W. L.
    (2006) Am. J. Respir. Cell Mol. Biol. 35, 651–661
17. Smith, E. F., and Lefebvre, P. A. (1996) J. Cell Biol. 132, 359–370
18. Smith, E. F., and Lefebvre, P. A. (2000) Cell Motil. Cytoskeleton 46, 157–165
19. Ralston, K. S., Lerner, A. G., Diener, D. R., and Hill, K. L. (2006) Eukaryot.
    Cell 5, 696–711
20. Branche, C., Kohl, L., Toutirais, G., Buisson, J., Cosson, J., and Bastin, P.
    (2006) J. Cell Sci. 119, 3434–3455
21. Yu, X., Ng, C. P., Habacher, H., and Roy, S. (2008) Nat. Genet. 40, 1445–1453
22. Zhang, Z., Sapiro, R., Kafiphamer, D., Buican, M., Bray, J., Chen, K.,
    Kiriakidou, M., McNama, M., Curtis, A., Zhang, M., Blanchette-Mackie, E. J.,
    and Strauss, J. F., III (2002) Mol. Cell. Biol. 22, 7993–8004
23. Zhang, Z., Jones, B. H., Tang, W., Moss, S. B., Wei, Z., Ho, C., Pollack, M.,
    Horowitz, E., Bennett, J., Baker, M. E., and Strauss, J. F., III (2005) Mol. Cell.
    Proteomics 4, 914–923
24. You, Y., Huang, T., Richer, E. J., Schmidt, J. E., Zabner, J., Borok, Z., and
    Brody, S. L. (2004) Am. J. Physiol. Lung Cell. Mol. Physiol. 286, 1650–1657
25. Zhang, Z., Austin, S. C., and Smyth, E. M. (2001) Mol. Pharmacol. 60, 480–487
26. Gonzalez, I. L., and Schmickl, R. D. (1986) Am. J. Hum. Genet. 38, 419–427
27. Brody, S. L., Hackett, B. P., and White, R. A. (1997) Genomics 45, 509–518
28. Blatt, E. N., Yang, X. H., Wuerffel, M. K., Hamilos, D. L., and Brody, S. L.
    (1999) Am. J. Respir. Cell Mol. Biol. 21, 168–176
29. Brody, S. L., Yang, X. H., Wuerffel, M. K., Song, S. K., and Shapiro, S. D.
    (2000) Am. J. Respir. Cell Mol. Biol. 23, 45–51
30. Okada, A., Hotta, Y., Sato, S., Ito, K., Iwata, Y., Nakamura, K., and Ikegawa, S.
    (2002) Gene 298, 59–68
31. Kou, I., and Ikegawa, S. (2004) J. Biol. Chem. 279, 50942–50948
32. Smits, P., Li, P., Mandel, J., Zhang, Z., Deng, J. M., Behringer, R. R., de
    Croomebregue, B., and Lefebvre, V. (2001) Dev. Cell 1, 277–290
33. Smits, P., and Lefebvre, V. (2003) Development 130, 1135–1148
34. Dy, P., Han, Y., and Lefebvre, V. (2008) Genesis 46, 294–299
35. Blaise, R., Grober, J., Rouet, P., Tavernier, G., Daegelen, D., and Langin, D.
    (1999) J. Biol. Chem. 274, 9327–9334