

# Epigenetic Mechanisms Modulate Thyroid Transcription Factor 1-mediated Transcription of the Surfactant Protein B Gene<sup>\*§</sup>

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Epigenetic regulation of transcription plays an important role in cell-specific gene expression by altering chromatin structure and access of transcriptional regulators to DNA binding sites. Surfactant protein B (Sftpb) is a developmentally regulated lung epithelial gene critical for lung function. Thyroid transcription factor 1 (Nkx2-1) regulates Sftpb gene expression in various species. We show that Nkx2-1 binds to the mouse Sftpb (mSftpb) promoter in the lung. In a mouse lung epithelial cell line (MLE-15), Nkx2-1 knockdown reduces Sftpb expression, and mutation of Nkx2-1 cis-elements significantly reduces mSftpb promoter activity. Whether chromatin structure modulates Nkx2-1 regulation of Sftpb transcription is unknown. We found that DNA methylation of the mSftpb promoter inversely correlates with known patterns of Sftpb expression *in vivo*. The mSftpb promoter activity can be manipulated by altering its cytosine methylation status *in vitro*. Nkx2-1 activation of the mSftpb promoter is impaired by DNA methylation. The unmethylated Sftpb promoter shows an active chromatin structure enriched in the histone modification H3K4me3 (histone 3-lysine 4 trimethylated). The ATP-dependent chromatin remodeling protein Brg1 is recruited to the Sftpb promoter in Sftpb-expressing, but not in non-expressing tissues and cell lines. Brg1 knockdown in MLE-15 cells greatly decreases H3K4me3 levels at the Sftpb promoter region and expression of the Sftpb gene. Brg1 can be co-immunoprecipitated with Nkx2-1 protein. Last, Nkx2-1 and Brg1 with intact ATPase activity are required for mSftpb promoter activation *in vitro*. Our findings suggest that DNA methylation and chromatin modifications cooperate with Nkx2-1 to regulate Sftpb gene cell specific expression.

Surfactant protein B (Sftpb)<sup>2</sup> is a gene critical for the function of pulmonary surfactant, a surface-active material that lines the lung alveoli (1–3). Mice deficient in Sftpb gene die at birth due

to respiratory failure (4). In humans, inherited Sftpb deficiency is associated with the development of newborn respiratory distress syndrome (5), the major cause of neonatal morbidity and mortality in developed nations. Sftpb levels are also reduced in a variety of lung diseases such as adult respiratory distress syndrome (6), familial interstitial lung disease (7), respiratory syncytial virus infection (8), and inflammatory diseases of the lung (9, 10). The Sftpb gene is expressed in the developing lung epithelium and in alveolar type II and bronchiolar Clara cells in the adult lung; this gene is regulated in a multifactorial manner by hormones, growth factors, and cytokines (11).

An enhancer region located at –439 to –331 bp of the human Sftpb 5'-flanking regulatory region (12) has been identified in the mouse Sftpb gene. Among the factors that bind to this enhancer are Nkx2-1, RAR/RXR, STAT3, and nuclear receptor coactivators such as SRC-1, ACTR, TIF2, and CBP/p300. These proteins form a complex, called the enhanceosome, that has intrinsic histone acetyltransferase activity (13–15). Nkx2-1 plays a central role in formation of the enhanceosome complex (13, 14, 16) and in the regulation of the Sftpb gene. Transgenic mice carrying mutations in the Nkx2-1 binding site or deletion of the enhancer region in the 1.5-kb hSftpb promoter LacZ construct show abolished or significantly reduced LacZ reporter gene expression in the lung (16). However, during mouse lung development, Sftpb gene expression can be detected at gestational days 11–12 (17), significantly lagging behind the Nkx2-1 expression, which appears as early as gestational day 8.5–9 (18, 19). Also, Nkx2-1 is expressed in thyroid and brain where Sftpb is not expressed. These observations suggest that Nkx2-1 alone is not sufficient for cell type-specific activation of Sftpb gene transcription.

Heritable patterns of DNA methylation repress transcription by promoting the formation of an inactive chromatin structure and hindering the binding of transcription factors (20). Methylation and demethylation of the DNA occur during development, establishing distinctive DNA methylation patterns in different cell types (21). Although DNA methylation has been regarded as a relatively stable chromatin mark, a recent report shows that upon activation of ER $\alpha$  target genes, CpG dinucleotides of the target promoters undergo cyclical demethylation and remethylation with a frequency of ~2 h (22, 23), suggesting that this epigenetic mechanism could be involved in dynamic changes in gene expression. Other chromatin modifications can repress or activate specific genes by changing the structure

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<sup>2</sup> The abbreviations used are: Sftpb, surfactant protein B; mSftpb, mouse Sftpb; Brm, Brahma; Brg1, Brahma-related gene-1; RT, reverse transcription; qRT, real time RT; 5-azadC, 5-aza-2'-deoxycytidine; GFP, green fluorescent protein; shRNA, short hairpin RNA; ChIP, chromatin immunoprecipitation assay.

of the chromatin and altering the accessibility of the transcriptional machinery to specific loci. SWI/SNF, ISWI, NuRD, and INO80 complexes (24, 25) use the energy derived from ATP hydrolysis to remodel the chromatin architecture of target promoters (26, 27). Brahma (Brm) and Brahma-related gene-1 (Brg1) are the central catalytic subunits of numerous chromatin remodeling complexes. Brg1 has been implicated in the control of gene transcription, for example, in nuclear receptor-mediated transcriptional activation (28). The temporal recruitment of transcription factors and chromatin-remodeling complexes to specific loci seems to be gene-specific and dictated by the interplay between transcriptional regulators and local chromatin structure (29, 30). Multiple mechanisms have been proposed to explain targeting of remodeling complexes to specific regulatory sequences, including interactions with RNA polymerase II holoenzyme (31), binding of bromodomains in the chromatin remodeling proteins to acetylated histones or other proteins (32, 33), and recruitment by sequence-specific transcriptional regulators (34–37).

Epigenetic regulation of gene expression has been implicated in cell differentiation and transcriptional control of tissue-specific genes in many organs (38–41), but very few studies have linked these mechanisms to the regulation of genes in the lung (42–44). We have recently determined that chromatin remodeling genes, including ATP-dependent chromatin remodeling complex members, polycomb genes, and DNA methyltransferases, change their levels of expression during initiation of mouse lung organogenesis (19). We have also shown that methylation of the proximal promoter of the T1 $\alpha$  (podoplanin) gene silences transcription of this gene in lung alveolar type II cells and is demethylated as cells differentiate into type I cells *in vitro* (42). These findings prompted us to hypothesize that epigenetic mechanisms cooperate with transcription factors and play an important role in the regulation of lung gene expression. To test this hypothesis, we evaluated the role of DNA methylation and chromatin modifications in the Nkx2-1-mediated transcription of the mouse Sftpb gene.

## EXPERIMENTAL PROCEDURES

**Cell Lines and Tissues**—E10 cells, kindly provided by Dr. A. Malkinson (University of Colorado) and Dr. Randall J. Ruch (University of Toledo), are spontaneously immortalized adult epithelial cells isolated from mouse lung derived originally by Dr. A. Lykke (University of New South Wales) (45). Cells were cultured in CMRL 1066 medium, 10% fetal bovine serum, 0.5 mM glutamine, 100 units/ml penicillin, and 100  $\mu$ g/ml streptomycin. MLE-15 is a murine lung epithelial cell line provided by Dr. Jeffrey A. Whitsett (Cincinnati Children's Hospital Medical Center). These cells were immortalized *in vivo* by expression of a Sftpc-driven SV40 T antigen (46) and were cultured in modified conditions as described previously (47). SW-13, obtained from ATCC (Manassas, VA), is a human adrenal small cell carcinoma cell line. These cells were grown in Leibovitz's L-15 medium (ATCC) containing 10% fetal bovine serum, 100 units/ml penicillin, and 100  $\mu$ g/ml streptomycin. All cell culture materials were obtained from Invitrogen. Adult mouse tissues were dissected from FVB wild type mice (Charles River Laboratories).

**Isolation and Culture of Murine Alveolar Type II Cells**—Cells were isolated by a previously described method (48). Briefly, 6-week-old C57BL6 male and female mice were anesthetized, and the trachea was exposed and cannulated with a 20-gauge luer stub adapter. Lungs were perfused with 10–20 ml of 0.9% saline via the pulmonary artery, and 3 ml of dispase (50 units/ml, BD Biosciences) was rapidly instilled through the tracheal cannula followed by 0.5 ml of agarose solution warmed at 45 °C. Lungs were immediately covered with ice for 2 min to gel the agarose, removed from the animals, and incubated in 1 ml of dispase for 45 min at room temperature. After this incubation, lungs were transferred to HEPES-buffered Dulbecco's modified Eagle's medium containing 100 units/ml DNase I, and lobes were gently separated from the bronchi. Cells in suspension were subsequently filtered through 100-, 40-, and 20- $\mu$ m nylon mesh, centrifuged at 130  $\times$  g for 8 min at 4 °C, and incubated for 1–2 h at 37 °C on tissue culture plates coated with CD45 and CD32. After incubation, type II cells were gently panned from the plate and centrifuged. Type II cells were resuspended in culture media for day 0 cells and cultured on tissue culture plastic dishes for 6 days to become type I-like cells (48).

**RNA Purification, RT-PCR, and Real Time RT (qRT)-PCR**—Total RNA was isolated from mouse lung and cell lines with TRIZOL reagent (Invitrogen) as described previously (42) and was treated with DNase using DNA-free kit (Ambion, Texas). Isolated RNA (0.5–1  $\mu$ g) was reverse-transcribed (RT) using avian myeloblastosis virus reverse transcriptase (Promega) or TaqMan reverse transcription reagents (Applied Biosystems Inc.) following the manufacturer's protocols. RT-PCR reactions were performed with 2  $\mu$ l of the RT product, 2.5 mM MgCl<sub>2</sub>, 0.4 mM dNTP, 4 ng/ $\mu$ l primers, 0.5  $\mu$ l of polymerase, and Q solution (Qiagen) in a total volume of 50  $\mu$ l. Primer sets for each gene are shown in [supplemental Table S2](#). cDNA samples were also amplified using  $\beta$ -actin gene primers described previously (49) as controls. The PCR products were electrophoresed on 1.2% agarose gels following standard methods. qRT-PCR analyses of Brg1, Brm, Nkx2-1, and Sftpb mRNA were performed in an ABI7000 system (Applied Biosystems). For Brg1 and Brm mRNA analyses, we used SYBR Green Master Mix (Applied Biosystems). Primers sequences were: Brg1, 5'-CGAACCAA-AGCAACCATCG-3' (forward) and 5'-TCTCTTCGCATGCACACCA-3' (reverse); Brm, 5'-GGTCAGGTTTGGTAGCA-GTTTG-3' (forward) and 5'-CAGTGGCTTTGAATGGTTCCT-3' (reverse). A calibration curve was generated for each gene using mouse total lung cDNA. Data were normalized to  $\beta$ -actin. TaqMan gene expression assays were used for Nkx2-1 (Mm00447558\_ml, Applied Biosystems) and Sftpb (Mm00455681\_ml, Applied Biosystems) mRNA analyses. Reactions were performed with TaqMan PCR Master Mix (Applied Biosystems). Relative amounts of Nkx2-1 and Sftpb mRNAs were determined using comparative C<sub>T</sub> and normalized to  $\beta$ -actin.

**DNA Extraction, Modification with Bisulfite, and Sequencing**—Genomic DNA was isolated from E10 and MLE-15 cell lines, primary type II cells, and type II cells cultured on plastic dishes for 6 days and adult organs including liver, heart, kidney, thyroid, and lung by previously described methods (42). DNA (0.5  $\mu$ g) was chemically modified using the CpGenome DNA

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modification kit (Chemicon, CA). Bisulfite-treated DNA (5 of 25  $\mu$ l) was then amplified. The primers used for amplification of bisulfite-modified DNA targeting the Sftpb gene promoter were: Sftpb F1, 5'-GTGAGTGGTGGAGGATTTTTTGG-3'; Sftpb R1, 5'-CTCCTACTTCAACATCCATCCA-3'; Sftpb F2, 5'-AGAGGATTTTAAAGTAAGGG-3'; Sftpb R2, 5'-CTCACCTACACCTAAACAAC-3'. Conditions were 94 °C for 3 min, 30 cycles at 94 °C for 45 s, 55 °C for 45 s, and 72 °C for 2 min, ending with 72 °C for 7 min. The PCR mixture contained 1  $\times$  PCR buffer, Q solution, 2.5 mM MgCl<sub>2</sub>, 0.4 mM dNTP, 0.5  $\mu$ l of polymerase, and 4 ng/ $\mu$ l primers Sftpb F1 and Sftpb R1. Amplicons were purified with a PCR purification kit (Qiagen). Secondary PCR was performed with the purified PCR products using primers Sftpb F2 and Sftpb R2 and the PCR conditions described above. PCR products were electrophoresed and purified using a gel isolation kit (Qiagen) and sequenced at the Boston University Core Sequencing Facility.

**Southern Blot Hybridization**—High molecular weight genomic DNAs were prepared from E10 and MLE-15 cell lines, liver, and lung as described above. Ten micrograms of genomic DNA were digested with HpaII and RsaI restriction enzymes, resolved on a 1.5% agarose gel, and transferred to nylon membranes (Amersham Biosciences). For the probe, a KpnI and SacI fragment of the 5' region of Sftpb gene was digested from the -1176bpSftpbLuc plasmid and labeled with [ $\alpha$ -<sup>32</sup>P]dCTP. The blot was prehybridized, hybridized, and washed as described previously (49).

**5-Aza-2'-deoxycytidine (5-azadC) Treatment**—E10 cells were treated with 1  $\mu$ M 5-azadC (Sigma) dissolved in 1  $\times$  phosphate-buffered saline and compared with untreated cells as described previously (42). After 8 days, cells were harvested for RT-PCR analyses.

**Plasmid Constructions and Site-directed Mutagenesis**—Genomic fragments containing -1176, -400, -297, -170, and -134 bp of the 5' end region of the mouse Sftpb gene were generated by PCR amplification. The following oligonucleotides were used: sense primers 5'-AGGGTACCAGACAGTGGTGGTGCATGCT-3', which corresponds to nucleotides -1176 to -1157, 5'-AGGGTACCCTAAGTGTGCC-3', which corresponds to nucleotides -400 to -387, 5'-AGGGTACCTAGAGAGGCTCATTCTG-3', which corresponds to nucleotides -297 to -280, 5'-AGGGTACCTAGGAACCGACATCG-3', which corresponds to nucleotides -170 to -154, 5'-AGGGTACAGGGGTGTCC-3', which corresponds to nucleotides -134 to -124 of the Sftpb promoter region, and antisense primer 5'-CGAGCTCAGTAGCCACTGCAGTAGGTGC-3', which corresponds to nucleotides +48 to +27 of the Sftpb 5' untranslated region. Fragments were cloned into KpnI and SacI sites of pGL3 basic vector (0Luc, Promega). Constructs were verified by sequencing. For methylation studies the wild type luciferase cDNA was replaced with a CpG-free synthetic luciferase. CpG-free luciferase cDNA was amplified by using sense primer 5'-GTCCAAGCTTCTCACTATAGGAGG-3' and antisense primer 5'-GCTCTAGATTATTTGCCACCCTTCTTGG-3' and pMOD-lucSh vector (InvivoGen) as template. This fragment was subsequently cloned in the HindIII and XbaI sites of different deletions of Sftpb promoter pGL3 luciferase constructs to replace the wild type luciferase cDNA. These con-

structs were named -1176Luc, -400Luc, -297Luc, -170Luc, and -134Luc. The CpG-free luciferase cDNA was verified by DNA sequencing. For chromatin remodeling studies, -1176 bp of the mouse Sftpb promoter was linked to the episomal pREP4 luciferase reporter and named -1176bpSftpbpREP4Luc. To generate this construct, a KpnI and HindIII fragment obtained from the -1176Luc was subcloned into the KpnI and HindIII site of pREP4Luc vector (kindly provided by Dr. Keji Zhao, National Institutes of Health) (50). The -1176bpSftpbLuc construct containing the mouse Sftpb promoter fragment -1176 to +48 bp served as the template for mutation of single Nkx2-1 putative binding sites by QuikChange II site-directed mutagenesis kit (Stratagene, CA) following the manufacturer's protocols. The sequences of primers for introducing mutations into the Sftpb promoter are listed in [supplemental Table S3](#). The single, double, or triple Nkx2-1 binding site mutants served as template for mutation of double, triple, and quadruple Nkx2-1 binding sites, respectively, in the -1176 bp of the 5' end region of the mouse Sftpb gene. Mutations were confirmed by sequencing. The pBJ5 empty vector, pBJ5Brg1, and pBJ5mBrg1 (hBrg1 K798R) expression constructs were kindly provided by Drs. Weidong Wang (National Institute on Aging Laboratory of Genetics) and Gerald R. Crabtree (Stanford University) (51). pCMV-Nkx2-1 is a rat Nkx2-1 expression construct provided by Dr. Roberto Di Lauro (Stazione Zoologica A. Dohrn, Napoli, Italy). Rat Nkx2-1 protein sequence is >98% identical to mouse Nkx2-1 and regulates mouse target genes (52, 53).

**Retrovirus and Lentivirus Production and Transductions**—To knock down Brg1 and Brm gene expression, a replication incompetent, VSV-G pseudotyped retroviral vector was employed containing a GFP tracking reporter gene along with either a non-targeting control shRNA or an shRNA targeting a mRNA sequence common to both Brg1 and Brm (vector kindly provided by Dr. Stephen Smale, UCLA) (54). Retroviruses were packaged by triple transfection of the 293T cell line with Trans-IT 293 (Mirus Bio) and plasmids encoding the retroviral backbone as well as gag/pol and VSV-G viral genes. Viral supernatants were concentrated ~100-fold by ultracentrifugation for 90 min at 48,960  $\times$  g on a Beckman SW28 rotor. Titers were performed through infection of FG-293 cells and assessment of GFP reporter gene expression by flow cytometry; viral titers of ~3  $\times$  10<sup>8</sup>–2  $\times$  10<sup>9</sup> transduction units/ml were typically achieved with this protocol. Infection of MLE-15 cells with retroviral vectors was performed for 16 h in the presence of Polybrene (5  $\mu$ g/ml) at a multiplicity of infection of 35. Cells were harvested 48 h after infection and analyzed for transduction efficiency and viability by flow cytometry analysis of GFP reporter gene expression and propidium iodide exclusion, respectively. Knockdown of Brg1 and Brm in the infected population (without cell sorting) was quantified at the mRNA level by qRT-PCR and at the protein level by Western blot. To knock down Nkx2-1 gene expression, we used a mix of three shRNAs cloned in the pLKO.1 lentivirus vector (individual clones TRCN0000020449, TRCN0000020450, and TRCN0000086264) targeting Nkx2-1 mRNA of mouse, rat, and human origin (contained in shRNA sets RHS4533 and RMM4534, Open Biosystems). This combination of shRNAs gave us the greatest down-regulation of Nkx2-1 mRNA. The

pLKO.1 lentivirus empty vector (RHS4080, Open Biosystems) was used as non-silencing control. Enhanced GFP shRNA-positive control (RHS4459, Open Biosystems) was used to generate viral particles and calculate infection efficiency and transduction units in MLE-15 cells. To produce the lentiviral particles, we cotransfected the pLKO.1 vectors with packaging plasmids in the Trans-Lentiviral packaging mix (Open Biosystems) in the TLA-HEK293T packaging cell line following the manufacturer's protocols. For the experiments, MLE-15 cells were seeded at 40–50% confluence in P100 tissue culture plates. The mixture, including 80  $\mu$ l of the viral stock (at  $6 \times 10^5$  transduction units/ml), 20  $\mu$ l of fetal bovine serum, 32  $\mu$ l of 8  $\mu$ g/ml Polybrene, and 868  $\mu$ l of serum-free media, was added to 2 ml of serum-free media and incubated at room temperature for 20 min. The viral mixture was added to the cells and incubated at 37 °C for 3–4 h. Then, we removed the transduction viral mix and added Dulbecco's modified Eagle's medium with 10% fetal bovine serum, 100 units/ml penicillin, and 100  $\mu$ g/ml streptomycin for at least 48 h to allow cells to reach confluence. Cells were trypsinized and cultured in the same media containing 5  $\mu$ g/ml puromycin dihydrochloride (Sigma) for 48 h to select for transduced cells. Knockdown of Nkx2-1 in MLE-15 cells was quantified at protein levels by Western blot and at mRNA levels by qRT-PCR.

*In Vitro DNA Methylation, Transient Transfections, and Cotransfections*—Different deletions of mSftpb promoter-CpG-free luciferase reporter constructs were divided into two aliquots. One aliquot (15–20  $\mu$ g of DNA) was methylated *in vitro* with 60 units/ml SssI methylase and 320 mM S-adenosylmethionine (New England Biolabs) for 6 h at 37 °C. This enzyme methylates CpG dinucleotides in both strands (55). A second aliquot was incubated similarly but without the SssI methylase (control). Complete methylation was verified by digestion with the methylation-sensitive restriction enzyme HpaII. The methylated and control plasmid DNA were purified by phenol/chloroform extraction and ethanol precipitation. Methylated and control constructs were used for transfection. The mSftpb promoter-luciferase reporter constructs (control or methylated, 2  $\mu$ g) and pCMV $\beta$ -gal plasmid (0.5  $\mu$ g) were transfected into MLE-15 cells or cotransfected with various concentrations of pCMV-Nkx2-1 expression construct into E10 cells with the DEAE-dextran/chloroquine method (42). For the above experiments, MLE-15 cells were cultured at an initial density of  $1 \times 10^6$  and E10 cells at  $0.8 \times 10^5$  per well in 6-well plates. Luciferase activity was analyzed at 48 h after transfection. LacZ activity was used to normalize for transfection efficiency. The mouse –1176bpSftpbpREP4luc and *Renilla* constructs were cotransfected into SW-13 cells with empty vectors, pBJ5Brg1, pBJ5mBrg1 (hBrg1 K789R), pCMV-Nkx2-1, combined pBJ5Brg1 and pCMV-Nkx2-1, or pBJ5mBrg1 and pCMV-Nkx2-1 expression vectors with Lipofectamine 2000 (Invitrogen) as described previously (56). SW-13 cells were cultured at an initial density of  $0.3 \times 10^6$  cells per well in 6-well plates. Luciferase and *Renilla* activities were analyzed after 48 h with the dual luciferase system (Promega) as described previously (56). Expression of Brg1 and mBrg1 in SW-13 cells was quantified at the mRNA level by qRT-PCR using oligonucleotides described above, and expression of Nkx2-1 in MLE-15,

E10, and SW-13 cells was analyzed at the protein level by Western blot using  $\alpha$ -Nkx2-1 (ab76013, Abcam or 07-601, Upstate).

*Co-immunoprecipitation Assays and Western Blots*—Nuclear proteins were extracted from MLE-15 cells by a previously described method (57). 300  $\mu$ g of nuclear extracts were diluted in 10 volumes of  $1 \times$  phosphate-buffered saline containing  $1 \times$  protease inhibitor mixture and  $1 \times$  phosphatase inhibitor mixture (Pierce) and incubated on a rotator with 5  $\mu$ l of rabbit IgG (Santa Cruz Biotechnology) at 4 °C for 30 min. 50  $\mu$ l of protein A/G beads (Santa Cruz Biotechnology) were added, and samples were incubated for an additional 20 min and centrifuged at 10,000 rpm for 2 min. 500  $\mu$ l of the supernatant containing  $\sim 300 \mu$ g of total nuclear proteins were incubated overnight on a rotator at 4 °C with 10  $\mu$ l of  $\alpha$ -Nkx2-1 (07-601, Upstate) or  $\alpha$ -Brg1 (sc-10768, Santa Cruz Biotechnology) antibodies or rabbit IgG, then 50  $\mu$ l of pre-washed protein A/G beads (Santa Cruz Biotechnology) were added, and samples were incubated at 4 °C for 2 h. The beads were washed 3 times with 1.0 ml of wash buffer (10 mM Tris, pH 7.5, 1 mM EDTA, 100 mM NaCl, and 0.1% Nonidet P-40) and boiled in 30  $\mu$ l of  $1 \times$  Laemmli SDS buffer (Boston Bioproducts Inc.). Co-immunoprecipitated proteins released from the beads were separated by electrophoresis under reducing conditions and transferred to polyvinylidene difluoride membranes (Millipore). The blots were blocked with 5% nonfat dry milk in TBST (10 mM Tris, pH 8, 150 mM NaCl, 0.1% Tween 20) and incubated with  $\alpha$ -Brg1 antibody (Santa Cruz Biotechnology) followed by horseradish peroxidase-conjugated goat  $\alpha$ -rabbit IgG (Vector). Western blots were developed with the enhanced chemiluminescent substrate (Pierce) and exposed to Eastman Kodak BioMax XAR film.

*Chromatin Immunoprecipitation Assays (ChIPs)*—MLE-15 cells, E10 cells, and mouse tissues were fixed in 1% formaldehyde in  $1 \times$  phosphate-buffered saline at room temperature for 10–15 min. After washing with  $1 \times$  phosphate-buffered saline, cells were resuspended in lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl, pH 8, and  $1 \times$  protease inhibitor mixture (Pierce)) and sonicated by using a Branson 450 dismembrator to achieve a chromatin fragment size of 500–2000 bp. We immunoprecipitated chromatin fragments by incubating the samples (equal amount of DNA) at 4 °C overnight with 5  $\mu$ g of  $\alpha$ -Brg1 (sc-10768, Santa Cruz Biotechnology),  $\alpha$ -H3K4me3 (histone 3-lysine 4 trimethylated) (05-745, Upstate),  $\alpha$ -H3K9me3/K27me3 (histone 3-lysine 9 trimethylated or lysine 27 trimethylated) (ab8898, Abcam, MA), and 10  $\mu$ l of  $\alpha$ -Nkx2-1 (07-601, Upstate) antibodies. Control experiments were performed with the corresponding IgG isotype (Santa Cruz Biotechnology) to determine nonspecific binding. Pre-absorbed protein A/G beads (Santa Cruz Biotechnology) were used to immunoprecipitate chromatin-antibody complexes. Equal volumes of immunoprecipitated DNA solution, 10% of the input DNA fragments, and genomic DNA were amplified by PCR using oligonucleotides spanning  $\sim 300$  bp of the proximal promoters of Sftpb (sense primer 5'-GTGTCAGCTGAATCC-CATCTC-3'; antisense primer 5'-CAGGCTTCTCAATCCT-CTACTC-3'), thyroglobulin (sense primer 5'-GGTACAGAA-CCCTGGCATT-3'; antisense primer 5'-TGTCCCTACT-GGGATGAAG-3'), –1176bpSftpbLuc constructs (sense primer 5'-GCAGGTGCCAGAACATTTCTC-3'; antisense

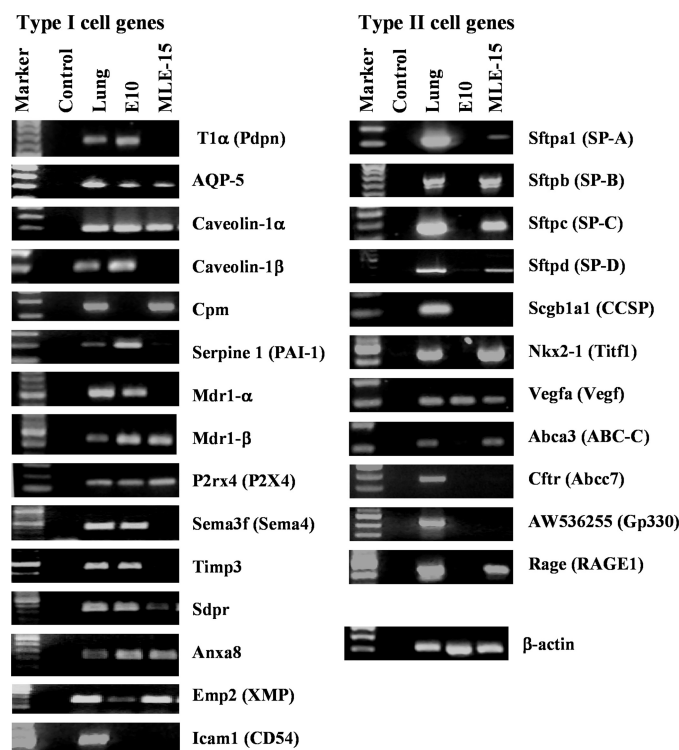
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primer 5'-GCTCTTGACGCAGGGTAAGTG-3'), and the  $\beta$ -actin coding region as the negative control. Binding of Nkx2-1 to the Sftpb promoter was also quantified by qPCR in a StepOnePlus equipment using SYBR Green Master Mix (Applied Biosystems) and oligonucleotides Sftpb 5'-TCAAGA-GCCAGGAAGGAAGCT-3' (forward) and 5'-CCCAGGCTT-CTCAATCCTCTACT-3' (reverse). Equal volumes of immunoprecipitated DNA solutions from ChIPs were used for amplification. A calibration curve was generated using mouse lung genomic DNA. Data were normalized to IgG control.

### RESULTS

**Characterization of Lung Epithelial Cell Lines**—Studies of pulmonary biology, especially at the molecular level, are difficult to carry out in the whole organ due to the large number and variety of cell types that form the lung. Many molecular studies can be performed in isolated primary lung cells. A complementary approach for studies of gene regulation is to identify immortalized or transformed cell lines that express genes characteristic of the normal cell *in vivo*. To evaluate chromatin modifications on the promoters of lung expressed genes, we sought to use lung epithelial cell lines that mimic some of the features of alveolar type II or type I cells. Based on expression patterns in normal adult lung, there are now a small group of genes specific to type I cells that are not expressed in type II cells; likewise, there are a number of genes expressed by type II cells and not by type I cells. The MLE-15 cell line has served as a model of the alveolar type II cell phenotype in a number of studies (58, 59). E10 cells are a spontaneously immortalized cell line derived from cultured adult mouse lung that express many of the type I cell-specific markers such as T1 $\alpha$  (podoplanin) and caveolin-1 (60). To extend the characterization of the molecular phenotypes of E10 and MLE-15 cell lines, we used RT-PCR to assess expression of mRNAs for alveolar epithelial differentiation genes. [Supplemental Table S1](#) lists the genes selected for the study, their reported cell specificities, and references. Primer sets for each gene are shown in [supplemental Table S2](#). Fig. 1 shows the patterns of amplification of 27 genes including  $\beta$ -actin. These results indicate that of the 26 type I and type II cell genes tested ~60% (15/26 in E10 and 17/26 in MLE-15 cells) exhibit patterns of expression that match the pattern that these genes display *in vivo*, either present or absent. E10 cells match the patterns of gene expression of type I cells, whereas MLE-15 cells match the type II cell pattern as previously reported (61). MLE-15 cells expressing Sftpb and Nkx2-1, and the E10 cells not expressing either were used in our *in vitro* studies to evaluate regulation of mouse Sftpb gene expression by Nkx2-1 and epigenetic mechanisms.

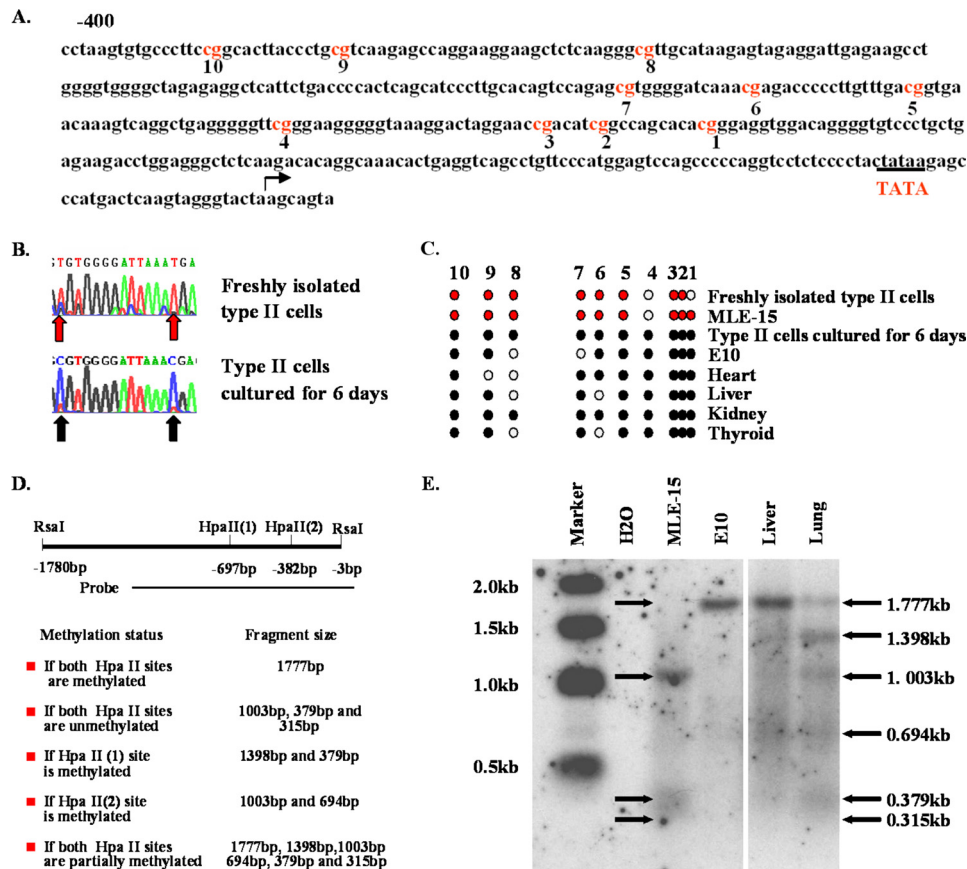
**Methylation Status of the Sftpb Gene Promoter**—To evaluate whether the methylation status of the mouse Sftpb promoter correlated with the known adult patterns of Sftpb expression (62), we first mapped the CpG dinucleotide distribution within ~400 bp 5' to the transcription initiation site in the mouse Sftpb gene (63). This region, the focus of our studies, contains functional domains that are known to drive expression of the mouse and also the human Sftpb gene (63). Within this 400-bp fragment we identified 10 CpG sites (Fig. 2A). In addition, this



**FIGURE 1. RT-PCR analysis of type I and type II cell marker genes in mouse lung epithelial E10 and MLE-15 cell lines.** Each gel shows DNA molecular weight markers, a control with no cDNA template, and amplified products from adult mouse lung, E10, and MLE-15 cells. The genes are organized into two groups identified as *Type I cell genes* or *Type II cell genes*. Experiments were performed three times.

region contains cis-elements for several transcription factors, including two potential Nkx2-1 binding sites identified using TESS (Transcription Element Search Software) (64) (see Fig. 5A). The upstream region of the mouse Sftpb promoter included in some of our assays (–400 to –1176 bp) is less conserved between mice and humans and contains additional putative methylation sites and 2 additional potential Nkx2-1 sites identified using TESS (64) (Fig. 5A). Genomic DNA from MLE-15, E10 cells, primary type II cells, and type II cells cultured on plastic dishes for 6 days, thyroid, liver, heart, and kidney was extracted and treated with sodium bisulfite, which converted cytosines to uracils, whereas methylated cytosines remained unmodified. DNA was amplified by PCR and sequenced. This method allowed evaluation of the overall methylation state of each site in the Sftpb promoter in the population of cells under study. We found that the Sftpb promoter was unmethylated in MLE-15 cells and primary type II cells, where high levels of Sftpb expression are detected, whereas these sites were methylated in E10, type II cells cultured for 6 days, thyroid, liver, heart, and kidney, where Sftpb is not expressed (62) (Fig. 2, B and C). To determine Sftpb promoter methylation status in total mouse lung, where Sftpb is expressed in alveolar type II and bronchiolar Clara epithelial cells but not in other cell types, we performed Southern blot hybridization analyses of genomic DNA digested with methylation-sensitive HpaII and insensitive RsaI restriction enzymes. Maps of digestion and the probe used for the analysis are shown in Fig. 2D. Sftpb gene promoter was found to be unmethylated in MLE-15 cells and

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**FIGURE 2. Methylation status of CpGs in the proximal 5'-region of the mouse Sftpb gene.** *A*, nucleotide sequence and location of potential methylation sequences are shown in red and numbered. The transcriptional initiation site is indicated by an arrow. *B*, examples of primary sequence data obtained by bisulfite genomic sequencing of the proximal Sftpb promoter in freshly isolated type II cells and type II cells cultured on tissue culture plastic dishes for 6 days. Methylated cytosines are indicated by black arrows, and unmethylated cytosines are indicated by red arrows. *C*, shown is the methylation status of each CpG in the proximal 5'-region of the Sftpb gene, analyzed by sodium bisulfite genomic sequencing method. Colored circles represent the results of two or three independent experiments. Red, white, and black circles indicate unmethylated, partially methylated, and methylated cytosine residues, respectively. The locations of CpG sites are indicated by numbers. *D*, shown is the prediction of Sftpb promoter DNA fragmentation by restriction enzymes RsaI and HpaII, depending on the DNA methylation patterns. *E*, genomic DNA from E10 and MLE-15 cell lines, adult mouse liver, and lung were cut with RsaI + HpaII. Restriction fragments were separated by gel electrophoresis, blotted, and probed with a [ $\alpha$ - $^{32}$ P]-labeled Sftpb promoter fragment. Experiments were performed three times.

completely methylated in E10 cells and adult liver. These data were consistent with bisulfite genomic sequencing. In the lung, where there is a mixture of expressing and non-expressing cells, we found a mixture of methylated and unmethylated sites within the Sftpb proximal promoter (Fig. 2E). These data indicate an inverse correlation between methylation status of the Sftpb gene promoter and Sftpb expression.

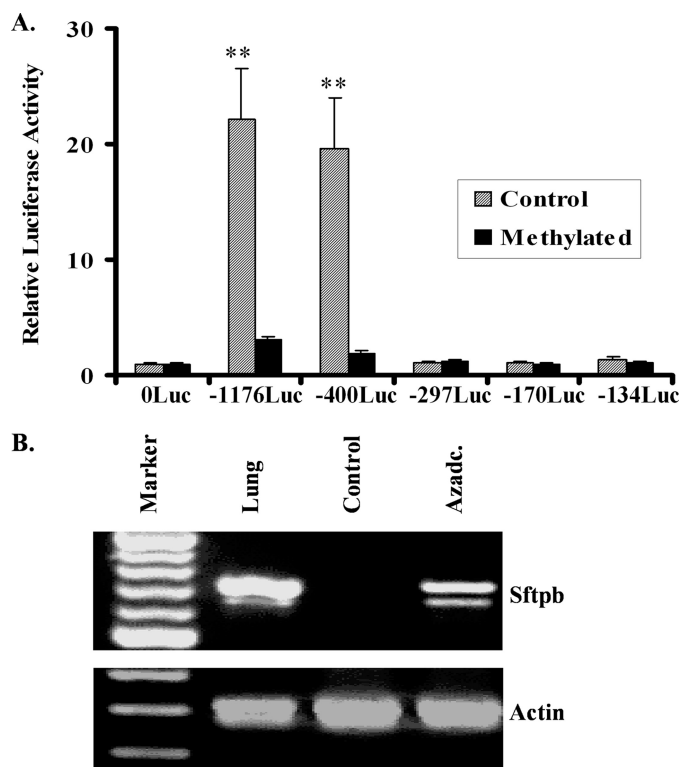
**In Vitro Methylation of the Sftpb Promoter Region Represses Its Transcriptional Activity, and Sftpb Expression Is Moderately Activated after 5-azadC Treatment**—To examine whether the mouse Sftpb promoter activity can be repressed by CpG methylation, CpG-free luciferase reporter constructs containing -1176, -400, -297, -170, and -134 bp of 5'-regulatory sequences of the Sftpb gene were treated *in vitro* with (methylated) or without (control) SssI methylase and subsequently transfected into MLE-15 cells. Because MLE-15 cells express marker genes for the type II cell phenotype, they likely contain most transcription factors necessary for Sftpb gene activation.

The full methylation of the -1176 and -400 bp of mSftpb promoter-luciferase constructs reduced about 10-fold the transcriptional activity of the promoter compared with mock-methylated constructs (Fig. 3A). This repression is not due to an effect of methylation on the luciferase reporter gene because in the CpG-free luciferase reporter constructs, the luciferase cDNA does not contain CpG dinucleotides. The -297-, -170-, and -134-bp fragments of mSftpb promoter have very low promoter activity, so the effect of methylation is not evident. These results indicate that methylation of the CpG sites in the mSftpb promoter is sufficient to significantly down-regulate mSftpb promoter transcriptional activity. To investigate whether DNA methylation has an effect on Sftpb expression, E10 cells that do not express Sftpb were treated with 1  $\mu$ M 5-azadC under the conditions described under "Experimental Procedures." This treatment moderately activated the transcription of the Sftpb gene, as the message can only be detected by RT-PCR (Fig. 3B) and not by Northern blot (data not shown). This result may have multiple interpretations. However, this study in conjunction with the differential methylation status of the Sftpb gene and the *in vitro* promoter methylation experiments highlights the importance of this

mechanism in regulation of Sftpb transcription. In addition to DNA methylation, other factors might be necessary to achieve high levels of Sftpb expression.

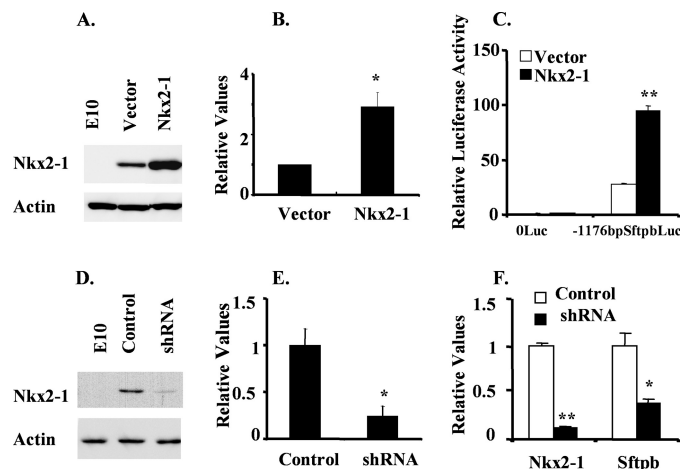
**Nkx2-1 Regulates Sftpb Gene Transcription**—Expression of the Sftpb gene in mice depends on the transcription factor Nkx2-1. This is indirectly illustrated by the observation that Sftpb gene expression is entirely lacking in Nkx2-1<sup>-/-</sup> embryonic lung (65, 66), and it is increased in transgenic mice overexpressing Nkx2-1 (52). Nkx2-1 also enhances human and mouse Sftpb promoter activity in cotransfection experiments (14, 67). To confirm that Nkx2-1 is important for mouse Sftpb gene expression, transient cotransfections with an Nkx2-1-expressing vector and the mouse -1176bpSftpbLuc construct were performed in MLE-15 cells. Western blot assays showed that Nkx2-1 protein levels were significantly increased by the cotransfection with an Nkx2-1-expressing vector compared with the empty vector (Fig. 4, A and B). Nkx2-1 significantly enhanced mSftpb promoter activity by 3.42-fold over the normal activity of that promoter fragment in MLE-15 cells (Fig.

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**FIGURE 3.** A, repression of mSftpb promoter-driven luciferase transcription by CpG methylation is shown. Different deletions of mSftpb promoter-CpG-free luciferase reporter constructs were methylated with SssI methylase (methylated) or without SssI methylase (control) *in vitro* and were transfected into MLE-15 cells. Results are given as luciferase activity normalized to  $\beta$ -galactosidase activity from cotransfected pCMV-LacZ. Data represent the mean and S.E. of three or four independent experiments (\*\*,  $p < 0.005$  for methylated versus control). B, 5-azadC treatment turns on Sftpb expression in E10 cells. E10 cells were treated with 1  $\mu$ M 5-azadC. Total RNA was purified from 5-azadC-treated or untreated (Control) E10 cells and analyzed for Sftpb and  $\beta$ -actin expression by RT-PCR. PCR products were visualized in 1.2% agarose gel. Experiments were performed three times.

4C). In addition, MLE-15 cells that express endogenous Nkx2-1 and Sftpb were transduced with a shRNA targeting the Nkx2-1 gene or non-silencing shRNA. Fig. 4, D–F, shows that Nkx2-1 protein is significantly knocked down by specific Nkx2-1 shRNAs. Nkx2-1 and Sftpb mRNA levels are significantly reduced compared with control (non-targeting shRNA). To further confirm that putative Nkx2-1 binding sites in the mouse Sftpb promoter mediated transactivation, we constructed mSftpb promoter mutants harboring single, double, triple, and quadruple mutations of putative Nkx2-1 binding sites in  $-1176$  bp of the 5' end region of the mouse mSftpb promoter. The mSftpb promoter mutants were transiently transfected into MLE-15 cells, and promoter activity was determined. As illustrated in Fig. 5A, site-specific mutations in the  $-1176$ bpLuc constructs decreased promoter activity. Mutation at positions  $-348$  to  $-340$  and  $-106$  to  $-97$  decreased promoter activity most significantly. Mutations at positions  $-1094$  to  $-1086$  and  $-695$  to  $-687$  did not impair promoter activity. These data suggest that the two proximal Nkx2-1 binding sites are more important for Sftpb promoter activity than the more distal sites. These results are consistent with the Sftpb promoter deletion analyses in Fig. 3A. To determine whether mutation of all four potential Nkx2-1 binding sites within the

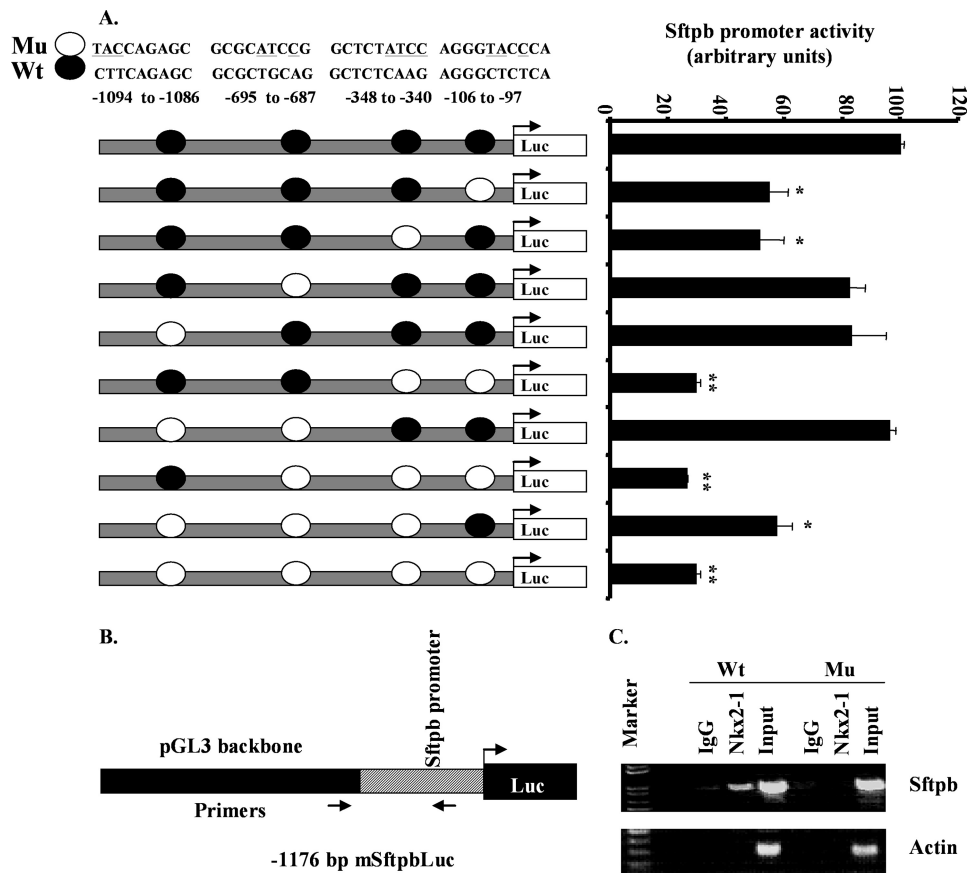


**FIGURE 4.** Regulation of the mouse Sftpb gene transcription by Nkx2-1. A–C,  $-1176$ bpSftpbLuc or 0Luc constructs were cotransfected with pCMV-Nkx2-1 or empty vector in MLE-15 cells. A, shown are Western blot analyses of Nkx2-1 protein in MLE-15 cells 48 h after cotransfection. Non-expressing E10 cells were used as the negative control. Representative immunoblots with  $\alpha$ -Nkx2-1 monoclonal antibody (ab76013, Abcam) (upper row) and  $\alpha$ - $\beta$ -actin antibody (lower row) are shown. B, chemiluminescence intensity of the Nkx2-1 bands normalized to actin is expressed as relative values that reflect the mean and S.E. of three independent experiments (\*,  $p < 0.05$  for pCMV-Nkx2-1 expression versus empty vector). C, luciferase activity was measured in cotransfected MLE-15 cells after 48 h. Results are given as relative luciferase activity normalized to  $\beta$ -galactosidase activity from cotransfected pCMV-LacZ. Data represent the mean and S.E. of two independent experiments (\*\*,  $p < 0.005$  for pCMV-Nkx2-1 expression versus empty vector). D, shown are Western blot analyses of Nkx2-1 expression in MLE-15 cells transduced with shRNAs targeting Nkx2-1 (shRNA) compared with non-silencing shRNA control (Control).  $\beta$ -Actin was used as the control. E, chemiluminescence intensity of the Nkx2-1 bands normalized to actin is expressed as relative values that reflect the mean and S.E. of three independent experiments (\*,  $p < 0.05$  for shRNA versus control). F, shRNA down-regulation of Nkx2-1 significantly inhibits transcription of the Sftpb gene in MLE-15 cells. qRT-PCR analysis of Nkx2-1 and Sftpb mRNA levels normalized to  $\beta$ -actin in Nkx2-1 shRNA-transduced cells versus control (\*,  $p < 0.05$ ; \*\*,  $p < 0.005$  for shRNA versus control) is shown. Data represent the mean and S.E. of two independent experiments.

$-1176$ -bp fragment interferes with Nkx2-1 binding, luciferase reporters driven by wild type or the quadruple Nkx2-1 site mutant were transduced into MLE-15 cells. Binding of endogenous Nkx2-1 to these constructs was assessed by ChIP assays. Fig. 5B shows the primer locations used to amplify DNA after immunoprecipitation. Nkx2-1 is bound to the wild type Sftpb promoter (wild type), but mutation of four potential Nkx2-1 binding sites (mutant) appreciably reduced binding of Nkx2-1 to the same region (Fig. 5C).

**Nkx2-1 Activation of the Sftpb Promoter Is Inhibited by CpG Methylation**—To evaluate whether activation of the mSftpb promoter by Nkx2-1 could be inhibited by promoter methylation, we cotransfected  $-1176$ Luc or methylated  $-1176$ Luc with either an empty vector or different doses of a rat Nkx2-1 expression construct into E10 cells that lack Nkx2-1. Fig. 6A shows that Nkx2-1 significantly increases reporter activity from the  $-1176$ Luc but has no effect on methylated  $-1176$ Luc. These findings indicate that methylation of the Sftpb promoter can interfere with the Nkx2-1-mediated activation of Sftpb transcription. Activity of the mSftpb promoter in E10 cells and dose-dependent induction by Nkx2-1 is moderate but statistically significant, suggesting that this cell line lacks other factors necessary to achieve maximal activation of Sftpb by Nkx2-1. Fig. 6B shows Western blot analyses of expressed Nkx2-1 pro-

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**FIGURE 5. Mutational analysis of Nkx2-1 binding sites in the mSftpb promoter.** A, putative Nkx2-1 binding sites were altered by site-directed mutagenesis (left) as described under "Experimental Procedures." Wild type and mutant mSftpb promoter-Luciferase constructs were transiently transfected in MLE-15 cells (right). Luciferase activity of mutant mSftpb promoter constructs was normalized to  $\beta$ -galactosidase activity from cotransfected pCMV-LacZ and is expressed relative to wild type (Wt) ( $p < 0.05$  (\*) and  $p < 0.005$  (\*\*)) for mutant versus wild type promoter. Location and sequence of four potential Nkx2-1 binding sites in mSftpb promoter (wild type) and their corresponding mutations (Mu) are indicated above the promoter map. Mutated nucleotides are underlined. White and black ovals indicate mutated or wild type Nkx2-1 binding sites, respectively; the arrow indicates the transcription start site. B and C, mutation of four potential Nkx2-1 binding sites in the Sftpb promoter blocks binding of Nkx2-1 in MLE-15 cells. B, shown is a schematic representation of the location of the primers used to amplify the mSftpb gene promoter. Sense primer for -1176bpSftpbLuc construct is located in the backbone of the vector. C, MLE-15 cells were transfected with wild type or mutant containing mutations of four potential Nkx2-1 binding sites for 48 h, and ChIP assays were performed with the indicated antibody and its corresponding IgG. DNA from immunoprecipitation assays were amplified by PCR with primer locations indicated in B.

tein in E10 cells 48 h after cotransfection. We observed an increased signal in E10 cells transfected with different amounts of Nkx2-1 expression vector using two different  $\alpha$ -Nkx2-1 antibodies;  $\alpha$ -Nkx2-1 polyclonal antibody (Fig. 6B, middle row) is more sensitive but shows some background signal in untransfected E10 cells that do not express Nkx2-1 mRNA. We also include Western blot analyses of Nkx2-1 expression with a monoclonal  $\alpha$ -Nkx2-1 antibody (upper row). This antibody is more specific but less sensitive; we only observed signal in cells transfected with 1  $\mu$ g of Nkx2-1 expression vector.

**Methylation of the Sftpb Promoter Inversely Correlates with Nkx2-1 Binding in Vivo**—Cytosine methylation in the promoter region could potentially interfere with binding of specific transcription factors. Sftpb is expressed in the lung but not in the thyroid, where Nkx2-1 is also expressed (68). Interestingly, we now show that the mouse Sftpb promoter is methylated in the thyroid. To determine whether methylation of Sftpb promoter inversely correlates with Nkx2-1 binding *in vivo*, we per-

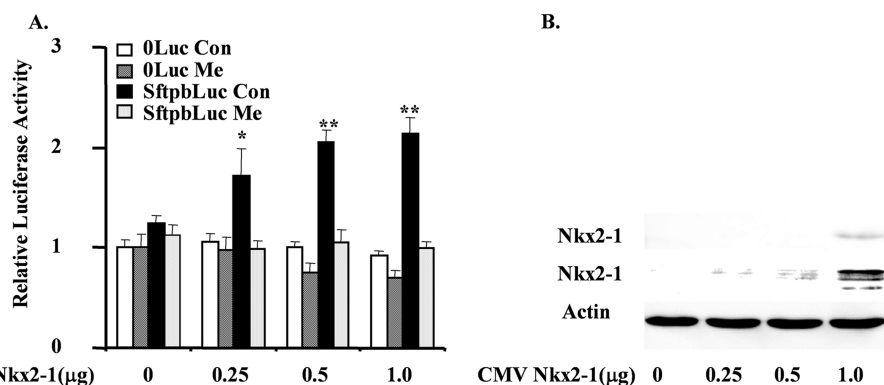
formed ChIP assays to examine Nkx2-1 binding to the Sftpb promoter in lung versus thyroid. Fig. 7A shows that Nkx2-1 is enriched at Sftpb proximal promoter in the lung but not in the thyroid. qPCR shows that enrichment in the lung versus thyroid is 3.67-fold (Fig. 7B). As a control, we show that Nkx2-1 is enriched at the thyroglobulin gene promoter in the thyroid. These data suggest that recruitment of Nkx2-1 to the Sftpb proximal promoter may depend on its methylation status.

**Brg1-containing Complexes Are Bound to the Sftpb Promoter in Sftpb-expressing Cells and Are Required for Efficient Sftpb Gene Expression**—The Brg1 and Brm ATPases form several kinds of chromatin remodeling complexes. Some of these complexes activate, whereas others repress gene expression by changing the structure of the chromatin. To test whether the endogenous mouse Sftpb gene is regulated by Brg1 or Brm remodeling complexes, we evaluated binding of these proteins to the Sftpb promoter in Sftpb-expressing and -non-expressing cells. ChIP analyses were performed with specific antibodies in MLE-15 cells and mouse lung, where Sftpb is highly expressed or E10 cells and liver where Sftpb is not expressed. Immunoprecipitated DNA was subjected to PCR analysis with primers designed for the promoter region of

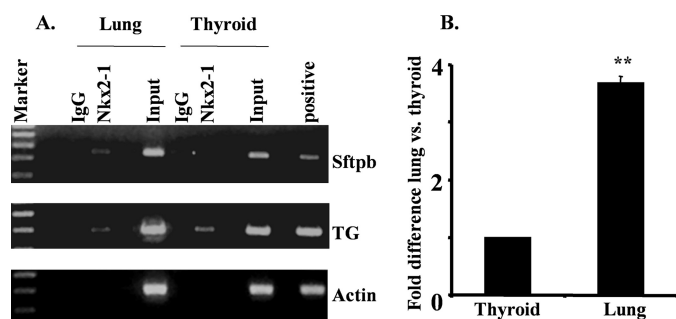
the Sftpb gene. Relative to Brg1, we detected very low levels of Brm mRNA by real time RT-PCR in MLE-15 cells, did not detect Brm protein by Western blot, and could not detect binding of Brm to the Sftpb promoter in ChIP assays (data not shown). Brg1, in contrast, is enriched at the Sftpb promoter in MLE-15 cells and lung but is not in E10 cells and liver (Fig. 8). The difference in Brg1 levels bound at the Sftpb promoter in MLE-15 and E10 cells is not due to differences in Brg1 expression in these cell types, as Western blot analysis of cell extracts from MLE-15 and E10 cells showed similar levels of Brg1 protein in these cells (data not shown). The enrichment of Brg1 at the Sftpb promoter correlates with the transcriptional activation of the Sftpb gene. Based on the role of Brg1 in regulation of gene expression by chromatin remodeling, we hypothesized that Brg1 is required for Sftpb promoter remodeling and gene activation. To address this issue, MLE-15 cells were transduced with retroviral vectors expressing a GFP reporter gene along with either a non-silencing control shRNA (control) or shRNA



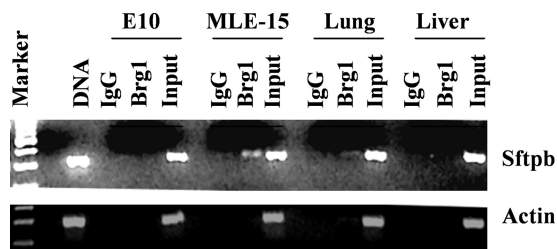
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**FIGURE 6. Nkx2-1 activation of the mSftpb gene promoter is inhibited by mSftpb promoter methylation.** A, E10 cells, which do not express endogenous Nkx2-1 or Sftpb, were transfected with mock-methylated CpG-free luciferase reporter vector (*0Luc Con*), methylated CpG-free luciferase reporter vector (*0Luc Me*), mock-methylated –1176Luc construct (*SftpbLuc Con*), and methylated –1176Luc construct (*SftpbLuc Me*) together with increasing amounts of pCMV-Nkx2-1. Results are given as relative luciferase activity normalized to  $\beta$ -galactosidase activity from cotransfected pCMV-LacZ. Data represent the mean and S.E. of two independent experiments ( $p < 0.05$  (\*) and  $p < 0.005$  (\*\*)) for pCMV-Nkx2-1 expression versus empty vector. B, Western blot analysis of overexpressed Nkx2-1 protein in E10 cells 48 h after cotransfection. Representative immunoblots with  $\alpha$ -Nkx2-1 monoclonal antibody (ab76013, Abcam, upper row),  $\alpha$ -Nkx2-1 polyclonal antibody (07-601, Upstate, middle row), and anti- $\beta$ -actin antibody (lower row) are shown. Data represent two independent experiments. CMV, cytomegalovirus.



**FIGURE 7. Differential binding of Nkx2-1 to the Sftpb proximal promoter in mouse lung and thyroid.** A, ChIP analyses of the mouse Sftpb gene were performed using indicated antibodies and their corresponding IgGs. 10% unprecipitated genomic DNA (*Input*) and mouse lung genomic DNA (*positive*) were amplified as controls. Primers for Sftpb gene are located in the mSftpb promoter (–624 to –304 bp). Thyroglobulin promoter (*TG*) and  $\beta$ -actin coding regions (*Actin*) were used as control. Data represent three independent experiments. B, a ChIP assay followed by qPCR shows a 3.67-fold greater association of Nkx2-1 to the Sftpb promoter in mouse lung versus thyroid. The -fold difference value compares the lung to thyroid binding after normalization to IgG control. Data represent the mean of two independent immunoprecipitations (\*\*,  $p < 0.005$  for lung versus thyroid).



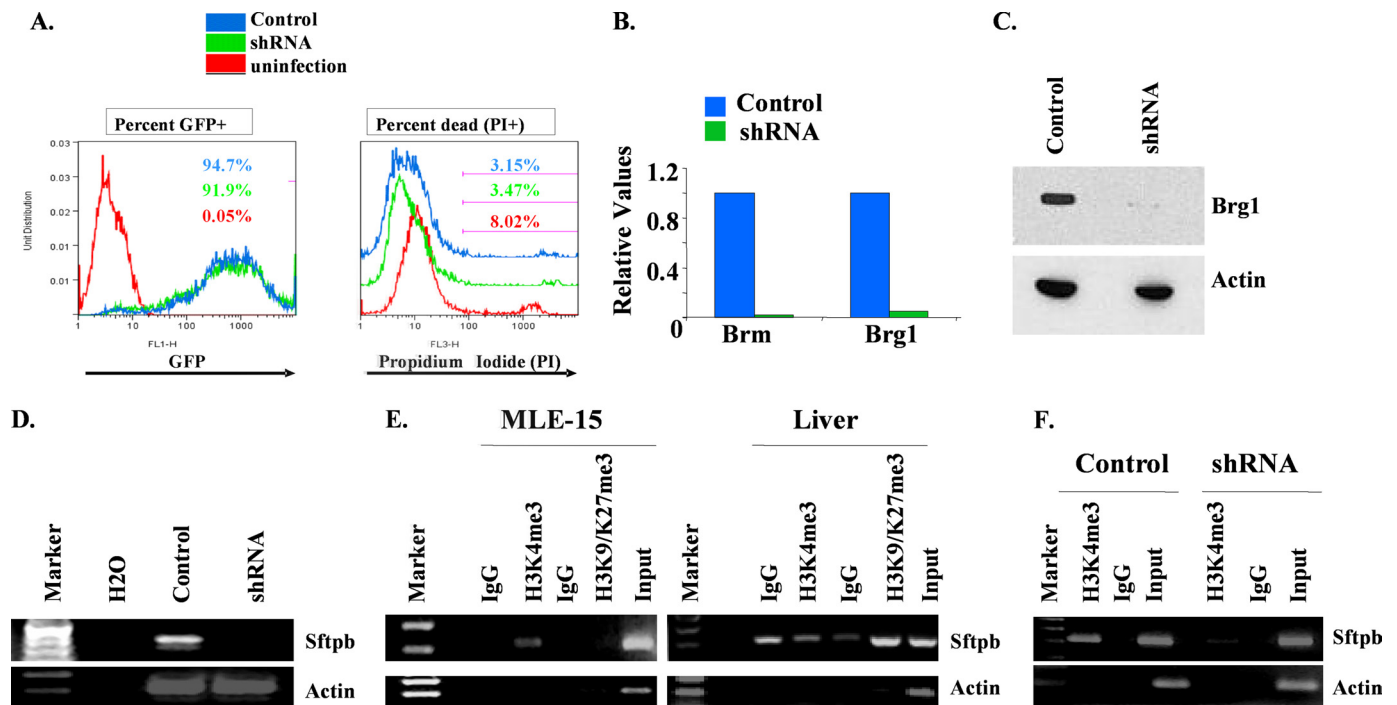
**FIGURE 8. Brg1 binds to the Sftpb gene promoter in mouse lung and MLE-15 cells that express Sftpb but not in mouse liver and E10 cells that do not express Sftpb.** In these ChIP analyses DNA purified from immunoprecipitates with  $\alpha$ -Brg1 antibody or its corresponding IgG was analyzed with primers covering the Sftpb promoter (–624 to –304 bp) and  $\beta$ -actin as a control. The input lane represents PCR using 10% genomic DNA before immunoprecipitation. Data represent two independent experiments.

targeting an mRNA sequence common to Brg1 and Brm coding regions (54). As shown in Fig. 9A, both vectors (control and shRNA) were robustly transduced in MLE-15 cells with similar efficiency. Real time RT-PCR analyses confirmed a significant reduction of Brg1 and Brm relative mRNA levels, and immunoblot assays showed that Brg1 protein levels were significantly knocked down by the specific Brg1/Brm shRNA-expressing vector compared with the control (non-targeting) shRNA-expressing vector (Fig. 9, B and C). Fig. 9D shows that knockdown of Brg1/Brm by shRNA greatly inhibits Sftpb gene transcription. Because Brg1 binds to the Sftpb promoter and knockdown of Brg1 reduces Sftpb expression, we conclude that the

endogenous Sftpb gene is regulated by Brg1-containing complexes.

Chromatin structure can be altered by ATP-dependent sliding or exchange of nucleosomes or by covalent modification of histone tails like histone acetylation and methylation. Whether Brg1 containing complexes can directly or indirectly affect histone methylation is not clear. To address this question, we performed ChIP analyses using antibodies against H3K4me3, H3K9me3/K27me3, or control IgG in MLE-15 cells and liver as control. We found enrichment of H3K4me3, a mark of active chromatin, at the Sftpb promoter in MLE-15 cells, in contrast to enrichment of H3K9me3/K27me3, a mark of silent chromatin, at the Sftpb promoter in liver (Fig. 9E). These data confirm that the Sftpb promoter is in an active conformation in MLE-15 cells and in a silent conformation in liver. Next, we investigated whether knockdown of Brg1/Brm by shRNA affects H3K4me3 content at the Sftpb promoter. We found a decrease in the enrichment of H3K4me3 at the Sftpb gene promoter by Brg1/Brm shRNA knockdown compared with a non-silencing shRNA (Fig. 9F). These results suggest that interactions between chromatin remodeling complexes and histone modification enzymes contribute to the dynamic changes in chromatin structure that control transcription of the Sftpb gene.

**Brg1 Protein Interacts with Nkx2-1, and the ATPase Activity of Brg1 Is Required to Activate the Sftpb Promoter**—To further examine the mechanism of Brg1 recruitment to the mouse Sftpb promoter, we investigated whether endogenous Nkx2-1 protein can interact with Brg1. Immunoprecipitation with an antibody to endogenous Nkx2-1 co-immunoprecipitated Brg1 in MLE-15 cell extracts (Fig. 10A). To determine whether the interaction between Brg1 and Nkx2-1 is functionally relevant for Sftpb gene transcription, we cloned –1176 bp of the Sftpb promoter, which contains multiple Nkx2-1 binding sites, into the episomal pREP4-luc reporter construct that self-assembles into nucleosomes and can be remodeled by the Brg1-containing SWI-SNF complex (50). SW-13 cells were used to study the



**FIGURE 9. *Brg1* and *Brm* knockdown by shRNA.** *A*, MLE-15 cells were transduced with retroviral vectors expressing a GFP reporter gene along with either a non-silencing control shRNA (*Control*) or shRNA targeting an mRNA sequence common to *Brg1* and *Brm* coding regions (*shRNA*). *B*, qRT-PCR analysis of *Brg1* and *Brm* mRNA levels normalized to actin in shRNA-transduced cells versus non-silencing shRNA control. *C*, Western blot analysis of *Brg1* expression in the shRNA-transduced MLE-15 cells compared with control is shown.  $\beta$ -Actin was used as the control. Data are representative of three experiments. *D*, down-regulation of *Brg1* and *Brm* reduces *Sftpb* mRNA expression in MLE-15 cells. *Sftpb* expression was analyzed by RT-PCR in *Brg1* and *Brm* shRNA-transduced cells and non-silencing control.  $\beta$ -Actin was used as the control. *E*, shown is the histone methylation pattern of the endogenous *Sftpb* gene promoter in MLE-15 cells and liver. ChIP analyses of the *Sftpb* gene promoter (*Sftpb*) were performed by using  $\alpha$ -H3K4me3 or  $\alpha$ -H3K9me3/H3K27me3 antibodies or their corresponding IgG. The input lane represents PCR using 10% genomic DNA without immunoprecipitation.  $\beta$ -Actin coding region (*Actin*) was used as the control. *F*, down-regulation of *Brm* and *Brg1* by shRNA changes histone methylation of the endogenous *Sftpb* gene promoter. ChIP analyses of *Sftpb* gene promoter were performed with  $\alpha$ -H3K4me3 antibody or its corresponding IgG. The  $\beta$ -actin coding region was used as a control. Data represent two or three independent experiments.

effect of *Brg1* and *Nkx2-1* on *Sftpb* transcription. These cells have a nonfunctional SWI/SNF complex due to the lack of *Brg1* and *Brm* subunits but are capable of restoring SWI/SNF complex function upon *Brg1* expression (69). These cells also have very low or no-expression of endogenous *Nkx2-1* (50). SW-13 cells were cotransfected with  $-1176\text{bpSftpbpREP4Luc}$  and either empty vectors or *Nkx2-1* or the wild type *Brg1* or *Brg1* containing a K798R mutation that inactivates the ATPase activity (51) or combinations of *Nkx2-1* and *Brg1* or *Nkx2-1* and mutant *Brg1* expression constructs. As shown in Fig. 10*B*, expression of wild type *Nkx2-1*, *Brg1*, and mutant *Brg1* alone had no effect on m*Sftpb* promoter activity. However, m*Sftpb* promoter activation was increased more than ~5-fold with co-expression of *Nkx2-1* and wild type *Brg1*. In contrast, co-expression of *Nkx2-1* with mutant *Brg1* failed to activate the m*Sftpb* promoter. Therefore, the results indicate that endogenous *Nkx2-1* directly or indirectly interacts with *Brg1* in solution and that *Brg1* ATPase activity is required for *Nkx2-1*-mediated *Sftpb* transactivation. Fig. 10, *C* and *D*, show that expression levels of *Nkx2-1* and *Brg1* are similar in all conditions tested. It is known that *Brg1* protein contains a bromodomain that binds acetylated histones and other acetylated proteins (32, 33) and that *Nkx2-1* recruits nuclear-receptor coactivators that acetylate histones (70). Therefore, *Brg1* may be recruited to the *Sftpb* promoter either by interactions with *Nkx2-1*-containing complexes or with acetylated histones in

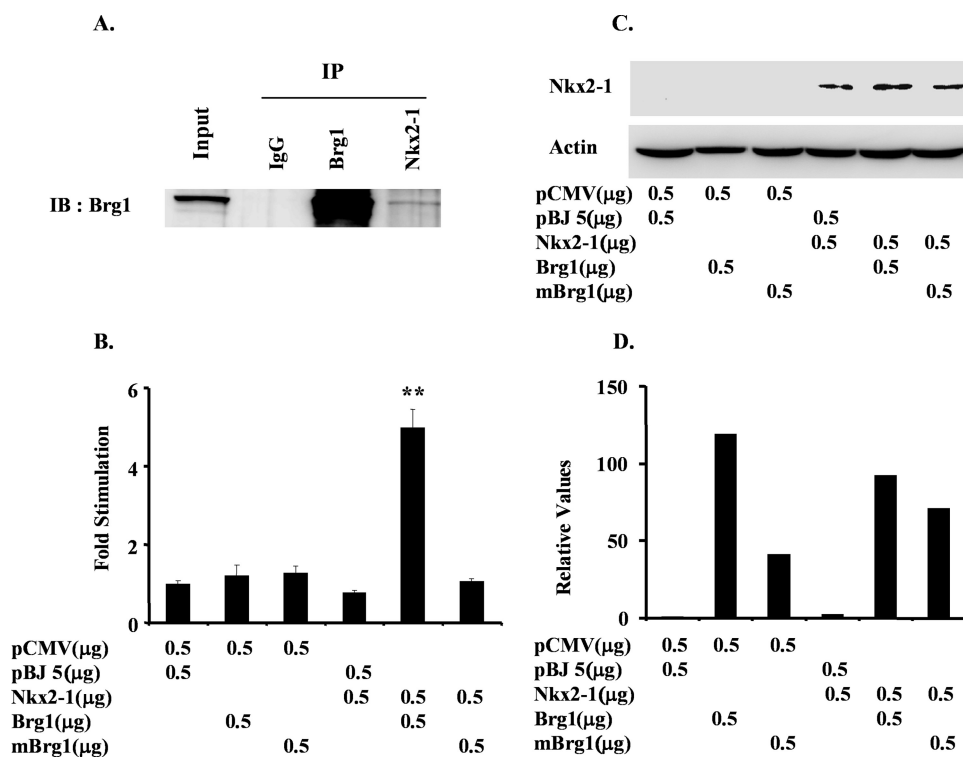
neighboring nucleosomes. However, *Brg1* recruitment is necessary to increase *Nkx2-1*-mediated activation of *Sftpb* transcription.

## DISCUSSION

Epigenetic mechanisms cooperate with DNA binding transcription factors to control patterns of gene expression in normal and diseased tissues (39, 41, 71, 72). To understand the contribution of these mechanisms to the regulation of lung-specific gene expression, we characterized novel interactions between chromatin modifications and the transcription factor *Nkx2-1* in the regulation of the expression of the lung-specific gene *Sftpb* in mice. We elected to evaluate the roles of DNA methylation and of the ATP-dependent chromatin-remodeling protein *Brg1* because these mechanisms are known to interact with tissue-specific transcription factors in other systems (71, 73). Our data show that unmethylated CpG sites at the *Sftpb* promoter and binding of *Brg1* with an intact ATPase catalytic domain are necessary for *Sftpb* promoter activation by *Nkx2-1*.

Changes in DNA methylation can selectively activate or inactivate genes and determine when and where a gene is expressed. *Sftpb* expression is initiated in the mouse lung epithelium at embryonic day 11, and later on it is restricted to alveolar epithelial type II cells and bronchiolar epithelial Clara cells (17). We show herein that *in vivo* there is an inverse correlation between *Sftpb* gene promoter methylation status and known

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**FIGURE 10. Brg1 co-immunoprecipitates with Nkx2-1.** *A*, MLE-15 cell lysates (*Input*) and immunoprecipitates (*IP*) obtained with rabbit IgG control,  $\alpha$ -Brg1 antibody, or  $\alpha$ -Nkx2-1 antibody were analyzed by Western blot with  $\alpha$ -Brg1 antibody (immunoblot (*IB*): Brg1). Data represent three independent experiments. *B*, activation of mouse Sftpb promoter requires Nkx2-1 and Brg1 with intact ATPase activity. The  $-1176\text{bpSftpbpREP4Luc}$  construct was cotransfected with empty vectors, Nkx2-1, mutant Brg1, combined Nkx2-1 and Brg1, or Nkx2-1 and mutant Brg1 expression constructs into SW-13 cells. Luciferase activity was analyzed after 48 h. Data represent the mean and S.E. of three independent experiments (\*\*,  $p < 0.005$  for Nkx2-1 and Brg1 versus empty vectors). *C*, Western blot analysis of Nkx2-1 protein levels in SW-13 cells 48 h after cotransfection is shown. Data represent two independent experiments. Representative immunoblots with  $\alpha$ -Nkx2-1 antibody (07-601, Upstate, upper row) and  $\alpha$ - $\beta$ -actin antibody (lower row) are shown. *D*, qRT-PCR analysis of Brg1 mRNA levels in SW-13 cells 48 h after cotransfection is shown. Brg1 mRNA levels are normalized to actin and compared with empty vector. Data represent two independent experiments.

Sftpb gene expression patterns. *In vitro* methylation of CpGs in the promoter region of the Sftpb gene significantly suppressed the transcriptional potential of this promoter, whereas demethylation with 5-azadC activated Sftpb expression. These results meet two accepted criteria for the role of DNA methylation in cell type-specific gene repression; (i) the promoter of Sftpb is methylated in non-expressing cells, and (ii) induced demethylation results in activation of Sftpb. Notably, although Nkx2-1 plays a critical role in activation of Sftpb gene, Sftpb is not transcribed in the thyroid where Nkx2-1 is transcriptionally active. The methylation status of the Sftpb promoter we observed in the thyroid likely silences Sftpb expression by blocking Nkx2-1 binding to the promoter. This supports a role of DNA methylation *in vivo* in preventing misexpression of this lung gene and indicates that the presence of Nkx2-1 is not enough to turn on the expression of Sftpb.

In addition to the role of DNA methylation in defining normal patterns of gene expression in different tissues, we evaluated its role in regulating gene expression during alveolar epithelial cell differentiation. In the distal lung Sftpb is expressed in alveolar type II cells. Lung injury often causes the death and desquamation of alveolar type I cells, leading to an accelerated differentiation of post-mitotic type II cells into flattened alveolar type I cells. Elucidating the molecular events that switch

these cells between the two phenotypes will facilitate the search for new ways of repairing the lung. These two phenotypes can be partially reversed *in vitro* by altering culture conditions (74), suggesting that some changes in gene regulation, perhaps simple ones, occur rapidly and affect a group of genes. We evaluated whether DNA methylation could be involved in this process. Previously, we have reported that cytosine methylation of Sp1 sites in the T1 $\alpha$  (podoplanin) gene promoter may contribute to changes in expression patterns of this type I cell marker gene (42). In the present study, we showed that 10 CpG sites in the Sftpb proximal promoter region were unmethylated in freshly isolated type II cells where Sftpb was highly expressed. More interesting, Sftpb proximal promoter was partially *de novo*-methylated in type II cells cultured on plastic dishes for 6 days. These data suggest that the change in type II to type I cell phenotype in culture may be affected by changes in methylation patterns. These findings support a role of DNA methylation in controlling lung cell phenotypes, as has been shown in T cells and embryonic stem cells (38, 73, 75–77).

We also showed *in vivo* that the ATP-dependent chromatin remodeling protein Brg1 is recruited to the Sftpb promoter. In cell lines, expression of the Sftpb gene is impaired by Brg1 knock-down. These results, thus, demonstrate that Brg1-mediated chromatin remodeling activity is a critical component of Sftpb activation. Consistent with the present observations, Brg1 has been reported to modulate the expression of a subset of genes through interactions with specific transcription factors (34, 35, 78). For example, Brg1 interacts with Smad3 to selectively increase the expression of a subset of TGF- $\beta$ -inducible genes (34) and is a critical factor for the functional activity of glucocorticoid receptor (79). However, the mechanisms involved in the recruitment of Brg1-containing complexes to particular regions in the genome still are not fully understood. It has been shown that Nkx2-1 can recruit SRC-1, ACTR, TIF2, and CBP/p300 coactivators, which catalyze the acetylation of core histones (13, 15, 70) and Nkx2-1 itself (16). A bromodomain found in the Brg1 protein recognizes acetylated residues in histones and other proteins (32, 33). Our current study suggests that Nkx2-1 may directly or indirectly recruit Brg1 remodeling complexes to the Sftpb gene promoter, perhaps by interactions with acetylated residues, and that Sftpb promoter activity requires Nkx2-1 and Brg1 with intact ATPase activity. The nature of the Brg1 complexes bound to the Sftpb

promoter will require further evaluation as Brg1 is found in several types of chromatin remodeling complexes (80).

Sftpb mRNA levels vary among different cell types in the lung and among individuals and are differentially regulated in lung diseases (5–8, 14). In particular, reduced Sftpb levels are correlated with surfactant dysfunction and susceptibility to lung injury. Genetic variation may account for some of the differences but not for all. For example, some patients with Sftpb deficiency or reduced Sftpb levels have no detectable Sftpb mutations in coding and regulatory regions (81). Notably, Sftpb gene expression can be inhibited by decreased binding of Nkx2-1 to the Sftpb promoter without alteration of the nuclear levels of Nkx2-1 protein (11). In the present study we observed that methylation of the Sftpb gene promoter can inhibit Nkx2-1 activation of the Sftpb promoter. In contrast, Brg1-containing chromatin remodeling complexes are required for activation of Sftpb transcription by Nkx2-1. It will be interesting to evaluate in the future whether hormones and cytokines can induce epigenetic changes on the Sftpb promoter similar to the regulation of many inflammatory and immune genes (40, 72) and whether epigenetic modifications can directly affect the transcription of Sftpb in lung disease and repair.

Based on our results and previous literature, we propose that in Sftpb-expressing cells, Nkx2-1 and/or other transcription factors recruit cofactors such as nuclear receptor coactivators with intrinsic acetyltransferase activity to acetylate histone tails at the Sftpb gene promoter (13, 15, 70) and/or acetylate Nkx2-1 (16). Acetylated Nkx2-1 and/or histones may recruit Brg1 complexes (32, 33) to the Sftpb promoter that further open the chromatin structure around the Sftpb transcriptional start site. On the other hand, methylation of Sftpb promoter prevents transcription factor binding including Nkx2-1, leading to gene silencing in non-expressing cells. However, the mechanism of Sftpb promoter demethylation, opening of the chromatin structure by Brg1-containing complexes, and interactions with Nkx2-1 at the Sftpb locus will require further elucidation. Nkx2-1 controls other lung-specific genes, such as Sftpc, Sftpa, and Abca3; future studies will focus on regulation of other Nkx2-1 lung target genes to determine whether control of gene expression by Nkx2-1 in cooperation with DNA methylation and Brg1 chromatin remodeling complexes is a more general mechanism of gene regulation in the lung.

Epigenetic regulation of gene expression is proving to play a key role in many organs during development and disease (36, 37, 71, 72, 82). There are few studies, however, on epigenetic regulation of lung gene expression (43). We present herein the first report that the transcription factor Nkx2-1 regulates a downstream lung target gene through cooperation with promoter methylation and the ATPase Brg1. The study also highlights the role of epigenetic mechanisms in the regulation of a surfactant protein gene. Because decreased Sftpb expression has been shown in a variety of lung diseases (6–10), epigenetic modifications of the Sftpb gene may contribute to the pathogenesis of those diseases.

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