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Epigenetic Regulation of Surfactant Protein A Gene (*SP-A*) Expression in Fetal Lung Reveals a Critical Role for Suv39h Methyltransferases during Development and Hypoxia[▽]

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***SP-A* gene expression is developmentally regulated in fetal lung. Cyclic AMP (cAMP) induction of *SP-A* expression in human fetal lung type II cells is O_2 dependent and is mediated by increased binding of TTF-1/Nkx2.1 and NF-κB to a critical response element (TBE). This is associated with increased acetylation and decreased methylation of H3K9 at the TBE. Using chromatin immunoprecipitation analysis of fetal lung between 15.5 and 19.0 days of gestation, we observed that the developmental induction of *SP-A* was associated with increased recruitment of TTF-1, NF-κB, PCAF, and CBP, as well as enhanced acetylation and decreased methylation of histone H3K9 at the TBE. Importantly, expression and TBE binding of the H3K9 methyltransferases, Suv39h1 and Suv39h2, was inversely correlated with the developmental upregulation of *SP-A*. In human fetal lung epithelial cells, Suv39H1 and Suv39H2 mRNA levels declined with cAMP induction of *SP-A*. Moreover, hypoxia, which inhibits cAMP stimulation of *SP-A*, markedly increased Suv39h1 and Suv39h2 binding to the TBE. Finally, short hairpin RNA knockdown of Suv39H1 or Suv39H2 in fetal lung epithelial cells repressed H3K9 methylation and greatly enhanced *SP-A* expression. Collectively, our findings suggest that Suv39H1 and Suv39H2 are key hypoxia-induced methyltransferases; their decline in fetal lung during late gestation is critical for epigenetic changes resulting in the developmental induction of *SP-A*.**

Developmental and hormonal regulation of eukaryotic gene expression is mediated by changes in chromatin structure that occur in a tissue- and cell-specific manner. Nucleosomes, the basic building blocks of chromatin (31), are composed of DNA wrapped nearly twice around two copies each of histones H2A, H2B, H3, and H4 (19). Histones are subject to a number of posttranslational modifications, including acetylation, methylation, phosphorylation, ubiquitylation, and ADP-ribosylation (17, 21), which occur primarily on their N-terminal tails. It has been suggested that the combinatorial nature of covalent modifications of the histone tails reveals a "histone code," which provides a unique regulatory system that dictates the transition between transcriptionally silent (heterochromatin) and active (euchromatin) chromatin (17).

Acetylation of histones on specific lysine residues (e.g., K9 and K14 of H3) by histone acetyltransferases (HATs) generally provides an "activating" mark, while histone methylation can either repress or activate gene expression, depending upon the gene-specific context, residue that is methylated and the degree of methylation (e.g., lysines can be mono-, di-, or trimethylated). Histone methylation, which occurs on the ε-amino group of lysine and the guanidine-ε group of arginine residues in the N-terminal histone tails, is catalyzed by two divergent families: the histone lysine methyltransferases (HMTs) and the protein arginine methyltransferases. Arginine methylation is generally an "activating" mark (36), while lysine methylation can either be inhibitory (e.g., H3K9, H3K27, and H4K20) or activating (e.g., H3K4) (17). Methylation of H3K9 plays a role in transcriptional silencing (17) by forming a binding site for heterochromatin protein 1 (HP1); this subsequently mediates the formation of condensed heterochromatin (10, 20).

To study the ontogeny, properties, and control of histone modifications associated with developmental, hormonal, and oxygen regulation of eukaryotic gene expression, we focused on surfactant protein A (SP-A), the major protein of pulmonary surfactant. The *SP-A* gene is silent throughout most of gestation; its transcription is dramatically induced in lung type II cells after ~85% of gestation is complete in association with increased surfactant glycerophospholipid synthesis (7). SP-A is a C-type lectin that serves important roles in innate immunity by facilitating the uptake of microorganisms by lung alveolar macrophages (39) and in surfactant homeostasis (6). *SP-A* expression in cultured human fetal lung type II cells is stimulated by cyclic AMP (cAMP) and interleukin-1 (IL-1) (14, 30). In studies using transgenic mice and transfected type II cells, we identified an ~300-bp region upstream of the rabbit (3, 12, 13, 27) and human (14, 23, 25, 42, 43) *SP-A* genes that is critical for lung cell-specific, developmental, and cAMP induction of expression. This genomic region serves as an "enhanceosome" through which basal and cAMP induction of *hSP-A* promoter activity are mediated by the cooperative interaction of transcription factors bound to a number of key response elements (26). One of these elements, termed the TBE (TTF-1-binding element) binds the homeodomain transcription factor, thyroid

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transcription factor 1 (TTF-1/Nkx2.1/Tebp) and NF-κB in a cAMP-responsive manner (14, 23).

cAMP and IL-1 induction of *SP-A* expression in cultured human fetal lung type II cells is dependent upon a critical atmospheric O_2 tension (10 to 20%) (1) and is blocked when cells are cultured in a hypoxic environment. When type II cells were cultured in 20% O_2, cAMP and IL-1 stimulated the recruitment of TTF-1, NF-κB p65, and the HAT coactivators, CBP and SRC-1, to the TBE region of the *SP-A* promoter. This was associated with increased local acetylation of histone H3 (K9,14); these effects were prevented when the cells were cultured in a hypoxic environment (15). Hypoxia markedly reduced the global levels of CBP and acetylated histone H3 and increased the expression of histone deacetylases. Furthermore, hypoxia caused an increased association of H3K9me_2 bound to the *hSP-A* promoter (15). These collective findings suggest that increased O_2 availability to type II cells late in gestation causes changes in chromatin structure that permit access of TTF-1 and NF-κB to the *SP-A* promoter.

An objective of the present study was to define temporal changes in the *in vivo* binding of critical transcription factors and histone-modifying enzymes to the *SP-A* promoter during mouse fetal lung development and associate these with alterations in posttranslational modification of the core histone H3 at key residues. We observed that the developmental induction of *SP-A* expression was associated with increased *in vivo* recruitment of TTF-1, NF-κB, and the HATs CBP and PCAF to the TBE region. This, in turn, occurred with a profound increase in H3K9,14 acetylation and a decline in H3K9 methylation. Importantly, the decrease in H3K9 methylation was coupled with a selective decline in expression and recruitment of the histone methyltransferases Suv39h1 and Suv39h2 to the TBE region. In studies with cultured human fetal lung type II cells, we observed that TBE binding of Suv39H1 and Suv39H2 was upregulated by hypoxia and markedly diminished with induction of *SP-A* expression during type II cell differentiation in a 20% O_2 environment. The finding that knockdown of Suv39H1 and Suv39H2 caused a pronounced upregulation of *SP-A* gene expression in fetal lung epithelial cells cultured in a hypoxic environment suggests the importance of a decline in these methyltransferases in the developmental induction of *SP-A* gene expression.

MATERIALS AND METHODS

Mice. All animal studies were approved by the Institutional Animal Care and Use Committee of University of Texas Southwestern Medical Center. Timed-pregnant mice (ICR/CD1) were purchased from Harlan. The animals were housed in the Animal Resources Center starting from 14 days postcoitus (dpc). At 15.5 to 19 dpc, the mice were euthanized by isoflurane inhalation and cervical dislocation. The fetuses were removed from their amniotic sacs and subjected to hypothermia for several minutes before decapitation. The fetal lungs were harvested and either frozen in liquid nitrogen for subsequent immunoblotting and quantitative reverse transcription-PCR (qRT-PCR) analysis or incubated in formalin for chromatin immunoprecipitation (ChIP) (see below).

Human fetal lung explants. Lung tissues from midtrimester human abortuses (16 to 20 weeks gestation) were obtained from Advanced Bioscience Resources ABR, Inc. (Alameda, CA), a nonprofit company, in accordance with the Donors Anatomical Gift Act of the State of Texas. The protocols were approved by the Institutional Review Board of the University of Texas Southwestern Medical Center at Dallas. The tissues were minced and rinsed several times with Waymouth's MB752/1 medium (Invitrogen catalog no. 11220), placed on filter paper supported by wire mesh grids, and cultured at an air-liquid interface in serum-free Waymouth's medium with or without dibutyryl cAMP (Bt_2cAMP) (1 mM) containing 3% antibiotics or antimycotics. The media were changed daily. Explants were harvested every 24 h over a 48-h period and then frozen in liquid nitrogen for qRT-PCR.

Isolation of epithelial cells and type II cells from human fetal lung. We have developed a method for isolation and culture of epithelial cells from freshly obtained human fetal lung tissue. The method is based on one that we established for the isolation of human fetal type II cells from lung explants after 5 days of culture in medium containing Bt_2cAMP (4). Briefly, freshly obtained human fetal lung was minced into 1- to 2-mm^3 cubes and dissociated by collagenase digestion (0.5 mg/ml). The cell suspension was then incubated with DEAE-dextran (250 μg/ml) for 45 min at 37°C, which selectively eliminates fibroblasts; this results in an enriched epithelial cell preparation. The epithelial cell suspension was further depleted of any remaining fibroblasts by layering onto a two-step (60%/90%) Percoll gradient, followed by centrifugation for 30 min at 1,000 × *g*. Cells at the 60/90 interface were collected, washed, and plated onto culture dishes that were coated with extracellular matrix (ECM) from confluent monolayers of Madin-Darby canine kidney (MDCK) cells. The ECM-coated dishes were prepared by treatment of MDCK monolayers with sodium deoxycholate (1%) (4). When cultured in medium containing Bt_2cAMP, the epithelial cells isolated from the midgestation fetal lung tissues develop the capacity to express SP-A, whereas cells cultured in the absence of Bt_2cAMP do not. The same procedure was followed for isolation of differentiated type II cells from human fetal lung explants after organ culture for 5 days in medium containing Bt_2cAMP (4).

Isolation of epithelial and fibroblast cell populations from lung tissues of 15.5- to 19.0-dpc fetal and neonatal mice. Mouse fetal lung tissues were minced and dissociated by collagenase digestion, as described above. The dissociated cells were washed, resuspended in Waymouth's medium containing 10% fetal bovine serum, and plated onto tissue culture dishes. After 10 min of culture at 37°C, the medium (containing suspended epithelial cells and some fibroblasts) was removed; these were plated onto a second set of culture dishes. The cells that adhered to the first set of dishes during the 10-min culture period were considered to be a relatively "pure" fibroblast population. These were flash-frozen for subsequent RNA and protein analysis or incubated in formalin (0.4% final concentration) for ChIP analysis. The cells plated onto the second set of culture dishes were incubated at 37°C for an additional 35 min. The cells that adhered to these dishes (a mixture of fibroblasts and epithelial cells) were discarded, while the nonadherent cells in the supernatant were considered to be a relatively "pure" population of epithelial cells. These cells were centrifuged at 1,000 × *g* for 5 min, and the cell pellet was flash frozen for subsequent RNA and protein analysis or incubated in formalin for ChIP analysis.

Immunoblot analysis. Nuclear extracts were prepared after homogenization of the mouse fetal lung tissues on ice in NE1 lysis buffer (10 mM HEPES [pH 7.5], 10 mM $MgCl_2$, 5 mM KCl, 0.1% Triton X-100, protease inhibitor, 1 mM phenylmethylsulfonyl fluoride [PMSF]) and centrifugation at 7,000 × *g* for 10 min at 4°C. The supernatants were harvested as cytoplasmic fractions. The nuclear pellets were resuspended in NE2 buffer (25% glycerol, 20 mM HEPES [pH 7.9], 500 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA [pH 8.0]), vortexed thoroughly, and incubated on ice for 30 min with vortexing every 5 min. The suspended pellets were centrifuged at 14,000 × *g* for 10 min at 4°C; supernatants were harvested as the nuclear extracts and stored at −80°C.

Whole-cell extracts of fetal mouse lung tissues were prepared after homogenization on ice in radioimmunoprecipitation assay buffer (25 mM Tris-HCl [pH 7.6], 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate, protease inhibitor, 1 mM PMSF) using a Dounce homogenizer. The homogenates were sonicated three times for 10 s each time at a 50% amplitude and centrifuged at 14,000 × *g* for 15 min at 4°C. The supernatant fractions were stored at −80°C.

qRT-PCR. Total RNA was isolated from fetal lung tissues using TRizol (Invitrogen catalog no. 15596-018) and treated with DNase to remove contaminant DNA; 2 μg of RNA was reverse transcribed using random primers and Superscript II reverse transcriptase (Invitrogen catalog no. 18064). SYBR green or TaqMan primer sets directed against mouse Suv39H1 and Suv39H2 mRNAs (Table 1) were used for relative quantification of mRNA levels.

ChIP. Mouse fetal lung tissues isolated at 15.5 to 19 dpc were finely minced, immediately placed in 1% formalin, and incubated with rocking for 20 min at room temperature. Cross-linking was terminated by addition of glycine (0.125 M final concentration). The tissues were lysed and sonicated on ice to produce sheared soluble chromatin, which was incubated at 4°C overnight with antibodies (2 μg) against TTF-1 (23), NF-κB p65 (Santa Cruz catalog no. sc-372X), PCAF (Santa Cruz catalog no. sc-8999X), CREB-binding protein (CBP; Santa Cruz catalog no. sc-583X), Suv39h1 and Suv39h2 (Abgent), or modified histones associated with "active" chromatin (acetyl-H3K9,14) and "inactive" chromatin

TABLE 1. Primers designed for quantitative real-time RT-PCR of genes and ChIP qPCR

Analysis and gene	Primer	
	Orientation[b]	Sequence (5′–3′)[c]
qRT-PCR[a]		
mG9aL	F	CAA GGA TGG CGA GGT TTA CTG
	R	TGC GTG GGA ACC GTA GAT CT
mSuv39h1	F	CGG ATC ACC GTG GAG AAT G
	R	CAC TCA CAG CCA ACA GCT ACC T
hSuv39H1	F	ATC CGC GAA CAG GAA TAT TAC C
	R	GAG GAT ACG CAC ACA CTT GAG ATT
mSuv39h2	F	CTG ATG AGT TCA CAG TGG ATG CA
	R	TTT GGG TCA CAA CTA TGA TTC ACA
hSuv39H2	F	CCC AAA TCT TCA GGT GTT CAA TG
	R	GGT TCT TGT GGA AAA CAA TGC TAT T
mESET	F	TCA CGG AAA CAA GTA GCC AAG A
	R	GCC AAA GGT GAC CGA TAT GTC T
mGLP/EuM	F	TTC AGG CGG GTG CCA ATA T
	R	CTG CGT CCT TCG GAT CCA
TaqMan assay		
mSuv39h1		Mm00468952_m1
hSuv39H1		Hs00162471_m1
mSuv39h2		Mm00469689_m1
mSuv39H2		Hs00226596_m1
ChIP: *mSP-A*	F	CAC TTG GGA GTT TGC CTT CTG
	R	AGG AAG CAG CCA TCG TGT GTA

[a] The primers were designed to determine specific expression using SYBR green qRT-PCR.
[b] F, forward; R, reverse.
[c] For the four TaqMan assay primers, the Applied Biosystems reference numbers are given.

(di- and trimethyl-H3K9; Upstate) or with nonimmune IgG. Immune complexes were collected on protein A/G agarose and eluted. Cross-linking of immunoprecipitated chromatin complexes and of "input controls" was reversed by heating at 65°C for 4 h, followed by proteinase K (Invitrogen) treatment. DNA was purified and analyzed by qPCR using forward and reverse primers that amplify an ~75-bp region encompassing the TBE of the mouse or human *SP-A* genes (Table 1).

For ChIP analysis of type II cells or epithelial cells isolated from human fetal lung, formalin (0.4% final concentration) was added to 100-mm dishes of cells that had been cultured with or without Bt_2cAMP (two dishes per treatment) for various periods. After formalin addition, the dishes were incubated on an orbital shaker for 10 min at room temperature. Cross-linking was terminated by the addition of glycine (0.125 M final concentration). Cells were washed with ice-cold phosphate-buffered saline, harvested, and sonicated on ice to produce sheared soluble chromatin. The sheared chromatin was diluted with ChIP dilution buffer (Upstate catalog no. 17-295) in a 3-ml total volume. Aliquots (1 ml) of diluted lysates were incubated with or without antibodies against Suv39H1 and Suv39H2 (Abgent catalog numbers AP1190a and AP1281a, respectively) or with nonimmune IgG and incubated at 4°C overnight. Immune complexes were collected, and cross-linking was reversed. DNA was purified and analyzed by qPCR using forward and reverse primers that amplify the region encompassing the TBE of the human *SP-A2* gene (Table 1) (15), as described above. The qPCR results were calculated by using $\Delta\Delta C_T$ method and expressed as the fold enrichment over input and IgG. The data shown are representative of three independent experiments with comparable results and represent means ± the standard errors of the mean (SEM) of three determinations.

Lentivirus shRNA production and transduction into human fetal lung epithelial cells. The GIPZ lentivirus expressing short hairpin interference RNA (shRNAmir) targeting either the methyltransferases Suv39H1 or Suv39H2 and shRNAmir nontargeting shRNA were generated from shRNAmir library purchased from Open Biosystems using HEK293T cells. Human fetal lung primary epithelial cells were seeded at a density of 4×10^6 cells/60-mm culture dish in complete medium containing 3% antibiotic-antimycotic and 10% fetal calf serum and incubated overnight at 37°C in an atmosphere of 95% air and 5% CO_2. The next day, the medium was replaced with serum-free/antibiotic-free medium containing 8 μg of Polybrene/ml. The cells were then transduced with the recombinant lentiviruses at a multiplicity of infection of 0.5 and placed in an atmosphere of 2% O_2, 93% N_2, and 5% CO_2. At 24 h posttransduction, the culture medium was changed to medium containing 3% antibiotic-antimycotic and Bt_2cAMP. Cells were then harvested at 24, 48, and 72 h posttransduction; mRNA and protein levels were analyzed by qRT-PCR and immunoblotting, respectively.

RESULTS

***In vivo* binding of TTF-1 and NF-κB (p65) to the TBE during mouse lung development increases with induction of *SP-A* gene expression and correlates with increased recruitment of the coregulators PCAF and CBP and local changes in H3K9 acetylation.** Previous work from our laboratory using cultured type II cells revealed that TTF-1 and NF-κB cooperatively interact at the TBE to mediate cAMP and IL-1 induction of *SP-A* gene expression (14). However, the roles of these endogenous transcription factors in developmental regulation of *SP-A* gene expression in fetal lung remains to be defined. To analyze gestational changes in TBE binding of endogenous TTF-1 and NF-κB (p65), we carried out ChIP analysis of mouse fetal lung tissue between 15.5 and 19 dpc and correlated these findings with the developmental changes in *SP-A* mRNA levels in the same tissues analyzed by qRT-PCR. As found previously (8, 18), SP-A mRNA levels were virtually undetectable in mouse fetal lung at 15.5 dpc, increased modestly at 16.5 dpc, and rose dramatically thereafter toward term (19.0 dpc) (Fig. 1A). This increase in SP-A mRNA was not reflective of a global increase in gene transcription, since the expression of key globally expressed genes (e.g., the TATA-binding protein [TBP] gene) did not change during development (data not shown). Using ChIP, we observed that TBE binding of TTF-1 remained relatively low through 16.5 dpc, increased at 17.5 dpc, and rose dramatically to term (Fig. 1B) in association with the developmental induction of *SP-A* expression. A highly similar pattern of TBE binding of endogenous p65 also was observed (Fig. 1E). Importantly, nuclear levels of TTF-1 protein remained relatively constant throughout late gestation, while nuclear p65 levels increased during the same period (Fig. 1G). The increased binding of endogenous TTF-1 to the *SP-A* promoter may be caused by changes in its posttranslational modification (23), while the developmental increase in binding of NF-κB to the *SP-A* promoter is likely due to its activation and nuclear translocation.

In previous studies with cultured human fetal type II cells, we observed that cAMP and IL-1 induction of TTF-1 and NF-κB binding to the TBE were associated with increased local levels of H3K9,14 acetylation and increased recruitment of the HATs SRC-1 and CBP (15). To analyze developmental changes in levels of acetylated H3K9,14 associated with the TBE, in the present study, we carried out ChIP analysis of mouse fetal lung tissues between 15.5 and 19.0 dpc. Coordinately, we used ChIP to analyze *in vivo* binding of two HATs with specificity for H3K9 and K14: PCAF and CBP (21, 37), which are known to interact *in vivo* (40). We found that the level of acetylated H3K9 at the TBE remained relatively low through 17.5 dpc, increased markedly at 18.5 dpc, and even further at term (19 dpc) (Fig. 1D). Importantly, global levels of acetylated H3K9 were not gestationally altered (Fig. 1G). This indicates that the local increase in H3K9,14 acetylation at the *SP-A* promoter is due to the selective recruitment of HATs to the TBE-containing region. Accordingly, we found that temporal changes in

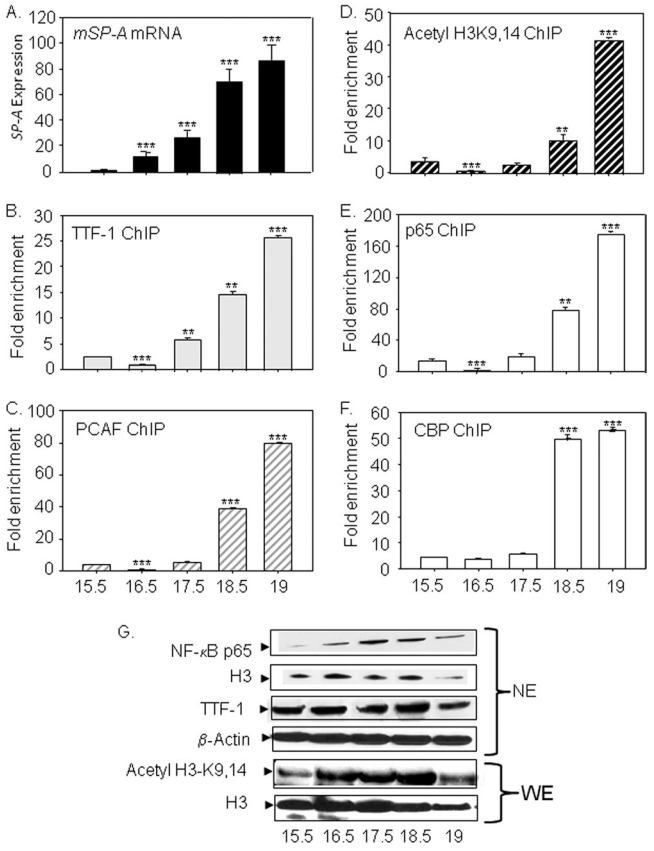

FIG. 1. Developmental induction of SP-A mRNA in mouse fetal lung is associated with increased recruitment of TTF-1, NF-κB p65, and coactivators PCAF and CBP and increased local levels of acetylated H3K9,14 within the genomic region surrounding the TBE. (A) SP-A mRNA in mouse fetal lung at 15.5 to 19 (term) days gestation was analyzed by qRT-PCR using 36B4 as the reference gene. Lung tissues from four individual fetuses were analyzed from four different pregnant mice at each gestational age. (B to F) ChIP was performed in mouse fetal lung at 15.5 to 19.0 dpc to assess the binding of endogenous transcription factors and coactivators using antibodies to TTF-1 (B), PCAF (C), acetyl H3K9,14 (D), p65 (E), and CBP (F). The immunoprecipitated DNA fragments were analyzed by qPCR using primers that amplify an ~75-bp region surrounding the TBE (Table 1). The binding of endogenous transcription factors, coregulators, and modified histones is expressed relative to the level of input and IgG. The data shown are representative of one of three independent experiments with comparable results and represent the means ± the SEM of triplicate analyses. In panels A to F, the data represent the means ± the SEM at each time point. Asterisks indicate significant differences from values at 15.5 dpc (***, $P \leq 0.01$; **, $P \leq 0.05$). (G) Nuclear extracts (NE) and whole-tissue extracts (WE) from mouse fetal lung isolated at 15.5 to 19 dpc were analyzed by immunoblotting to assess the protein levels of TTF-1 and NF-κB p65 and the global levels of acetylated histone H3-K9,14.

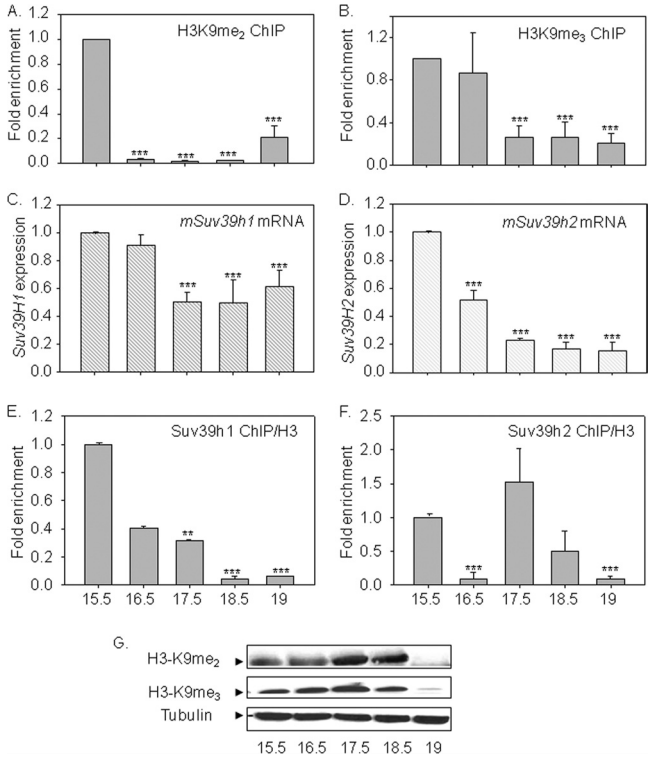

FIG. 2. Expression and recruitment to the *SP-A* promoter of the H3K9 methyltransferases Suv39h1 and Suv39h2 are associated with local changes in H3K9 methylation and inversely correlated with the developmental induction of SP-A mRNA levels in mouse fetal lung. Binding of endogenous $H3K9me_2$ (A) and $H3K9me_3$ (B) to the *SP-A* promoter in mouse fetal lung at different gestational ages was determined by ChIP analysis. The data represent means ± the SEM of three independent experiments performed in triplicate. qRT-PCR was performed to assess mRNA expression of the H3K9 methyltransferases, Suv39h1 (C) and Suv39h2 (D), in mouse fetal lung at 15.5 to 19.0 dpc. The data are means ± the SEM of fetal lung tissues from four pregnant mice at each gestational age. (E and F) ChIP analysis of the recruitment of endogenous Suv39h1 (E) and Suv39h2 (F) to the TBE region of the *SP-A* promoter in mouse fetal lung isolated at 15.5 to 19.0 dpc. The data were normalized to total histone H3. The data are means ± the SEM from a representative experiment conducted in triplicate that was repeated three times with similar results. Asterisks in panels A to F represent significant differences from 15 dpc values (**, $P < 0.01$; ***, $P \leq 0.001$). (G) The global levels of $H3K9me_2$ and $H3K9me_3$ in mouse fetal lung at each gestational time point were determined by immunoblot analysis of whole protein extracts.

binding of PCAF (Fig. 1C) and CBP (Fig. 1F) to the TBE region were highly similar to those for acetylated H3K9,14; TBE binding of these HATs remained low through 17.5 dpc, with a pronounced increase in binding at 18.5 dpc. The similarity in the developmental kinetics of their binding to the *SP-A* promoter suggests their cooperative interaction as part of a complex recruited to the TBE.

Developmental induction of *SP-A* during mouse fetal lung development is inversely correlated with H3K9 di- and tri-methylation and with expression and recruitment of the methyltransferases Suv39h1 and Suv39h2. As mentioned above, histone H3K9 acetylation is a chromatin mark associated with gene activation, whereas methylation of this residue is linked to gene repression. H3K9 acetylation and methylation are known to be reciprocally regulated. Using ChIP, we found that at 15.5 dpc, the levels of $H3K9me_2$ and $H3K9me_3$ associated with the TBE region were relatively high (Fig. 2A and B). Interestingly, $H3K9me_2$ declined to nearly undetectable levels by 16.5 dpc and remained low throughout the remainder of gestation (Fig. 2A). $H3K9me_3$ levels at the TBE declined more modestly at 16.5 dpc, reaching a nadir by 17.5 dpc (Fig. 2B). On the other hand, the global nuclear levels of $H3K9me_2$ and $H3K9me_3$ remained elevated until 19.0 dpc, at which time they

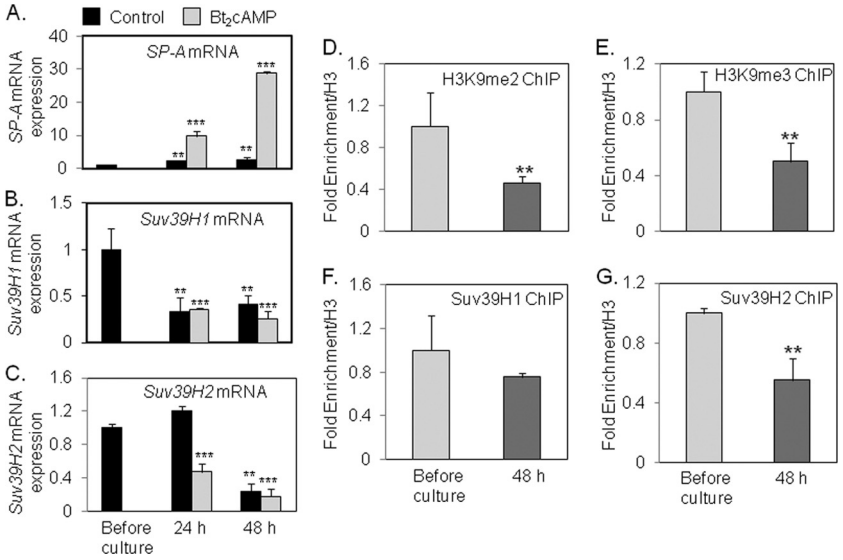

FIG. 3. Downregulated expression and binding of Suv39H1 and Suv39H2 to the TBE region of the *SP-A* promoter in human fetal lung explants during differentiation in culture are associated with decreased binding of methylated H3K9 and increased *SP-A* expression. SP-A (A), Suv39H1 (B), and Suv39H2 (C) mRNA levels were analyzed using qRT-PCR in midgestation human fetal lung explants before and after culture for 24 to 48 h in serum-free medium in the absence (control) or presence of Bt_2cAMP. Analysis of h18S RNA was used as an internal control. The data are means ± the SEM of triplicate culture dishes. Control (**) or Bt_2cAMP-treated (***) values significantly different from those of fetal lung before culture ($P \leq 0.001$) are indicated by asterisks. Binding of endogenous H3K9me$_2$ (D), H3K9me$_3$ (E), Suv39H1 (F), and Suv39H2 (G) to the TBE region of the *hSP-A* promoter was analyzed by ChIP in midgestation human fetal lung tissue before and after 48 h of culture in Bt_2cAMP-containing medium. The data are means ± the SEM of triplicate samples normalized to the total histone H3. **, significantly different from fetal lung before culture ($P \leq 0.03$).

declined markedly (Fig. 2G). Thus, the levels of methylated H3K9 bound to the *mSP-A* promoter are selectively altered and not reflective of the global levels of histone methylation.

H3K9 methylation is dynamic and regulated by a number of methyltransferases. To determine whether the decline in H3K9 methylation was due to a decrease in the expression of one or more H3K9 methyltransferases, we analyzed developmental changes in expression of the H3K9 methyltransferases Suv39h1 and Suv39h2, G9a, ESET, and GLP/EuM in fetal mouse lung as a function of gestational age. The mRNA levels of Suv39h1 and Suv39h2, which catalyze H3K9 di- and trimethylation, were significantly decreased in mouse fetal lung after 16.5 dpc (Fig. 2C and D). The decrease in Suv39h1 and h2 mRNA was associated with a developmental decline in nuclear protein levels (data not shown). The developmental decline in Suv39h1 and Suv39h2 mRNA expression occurred both in epithelial cells and in fibroblasts isolated from the fetal mouse lung during gestation (data not shown). This indicates that the changes in mRNA expression were not reflective of a decline in the proportion of fibroblasts relative to epithelial cells during development. ChIP was utilized to analyze binding of endogenous Suv39h1 and Suv39h2 to the TBE region of the *SP-A* promoter between 15.5 and 19.0 dpc. Interestingly, *in vivo* binding of Suv39h1 to the TBE region of the *mSP-A* promoter was highest at 15.5 dpc, declined by ~60% at 16.5 dpc, and declined more significantly thereafter. In contrast, Suv39h2 binding was transiently and profoundly decreased at 16.5 dpc, increased at 17.5 dpc, and then declined thereafter (Fig. 2E and F). In contrast to Suv39h1 and h2, G9a, ESET, and GLP/EuM mRNA did not manifest any change in expression between 15.5 and 19.0 dpc (data not shown). However, ChIP analysis revealed that *in vivo* binding of G9a to the mouse *SP-A* promoter declined markedly at 16.5 dpc and then increased at 17.5 dpc and remained elevated to term (data not shown). In this regard, the temporal binding pattern of G9a was similar to that of Suv39h2 (Fig. 2F). The decline in binding of Suv39h1, Suv39h2, and G9a to the *SP-A* promoter at 16.5 dpc was clearly associated with a pronounced decrease in dimethylation of H3K9 and with the initiation of *SP-A* expression in fetal mouse lung.

Suv39H2 and Suv39H1 mRNA expression decreases during differentiation of human fetal lung explants in culture. Explants from midgestation human fetal lung differentiate spontaneously in organ culture in serum-free medium and develop the capacity for surfactant phospholipid synthesis (35) and *SP-A* expression (30). The rate of type II cell differentiation and of *SP-A* expression is increased by cAMP treatment (30). To further investigate whether the decrease in mRNA levels of Suv39h1 and Suv39h2 methyltransferases observed in developing mouse fetal lung also occurs during differentiation of the human fetal lung, we analyzed the mRNA levels for *SP-A*, *Suv39H1*, and *Suv39H2* before and after culture of midgestation human fetal lung explants for 24 and 48 h in serum-free medium, with or without Bt_2cAMP. As observed previously (30), *SP-A* mRNA was essentially undetectable in the human fetal lung tissue before culture, significantly increased in untreated explants and even more markedly with Bt_2cAMP treatment after 24 h and was increased ~10-fold by Bt_2cAMP after 48 h of culture (Fig. 3A). Suv39H1 mRNA levels were elevated in the fetal lung tissue before culture, declined significantly after 24 h of culture in the absence or presence of Bt_2cAMP, and remained relatively low after 48 h (Fig. 3B) in association

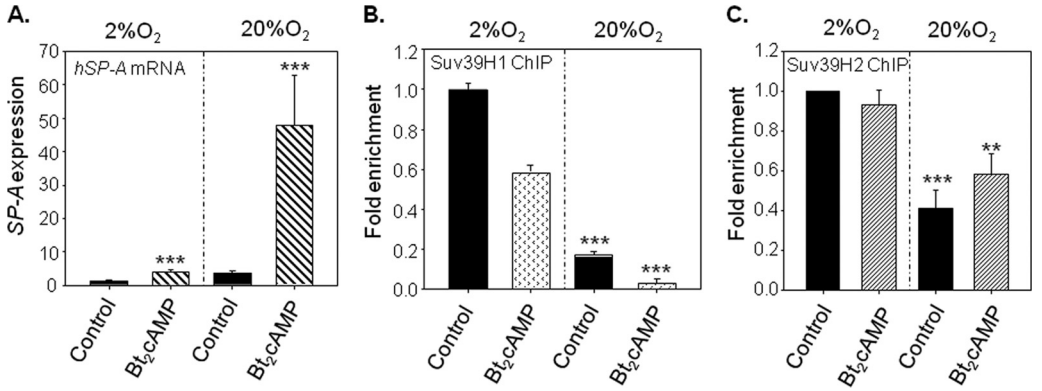

FIG. 4. Suv39H1 and Suv39H2 recruitment to the TBE region of the *hSP-A* promoter is upregulated by hypoxia. Human fetal lung type II cells were cultured for 48 h in the absence or presence of Bt_2cAMP in an atmosphere of 2 or 20% O_2. (A) SP-A mRNA levels were assessed by qRT-PCR. The data are means ± the SEM for control ($n = 5$) and Bt_2cAMP-treated ($n = 6$) dishes of cells. Binding of endogenous Suv39H1 (B) and Suv39H2 (C) to the TBE region of the *hSP-A* promoter was analyzed by ChIP. The data are means ± the SEM of triplicate samples from an experiment that was repeated at least three times with similar results. Values are expressed relative to those of cells cultured in control medium in a 2% O_2 environment. Results significantly different from the respective treatment group in 2% O_2 versus 20% O_2 environments are indicated by asterisks (**, $P < 0.01$; ***, $P < 0.001$).

with the induction of *SP-A* expression. Suv39H2 mRNA levels remained elevated in untreated fetal lung explants after 24 h but were significantly reduced by Bt_2cAMP treatment; Suv39H2 mRNA levels were reduced in cAMP-treated and control explants at 48 h (Fig. 3C). These findings further suggest that the decrease in Suv39H1 and Suv39H2 expression is strongly associated with type II cell differentiation and increased *SP-A* gene expression and supports the important role of Suv39H1 and Suv39H2 methyltransferases in the repression of *SP-A* expression in fetal lung. Thus, elevated expression of these two methyltransferases in fetal lung in early gestation to midgestation likely maintains a repressive chromatin structure that blocks *SP-A* gene expression. ChIP was conducted to assess binding of di- and trimethylated H3K9 and of Suv39H1 and H2 to the *hSP-A* promoter in the midgestation human fetal lung tissue before culture and after 48 h of incubation in Bt_2cAMP-containing medium. As seen in Fig. 3D and E, binding of H3K9me$_2$ and H3K9me$_3$ was significantly decreased after 48 h of culture in the presence of Bt_2cAMP compared to the fetal lung tissue prior to culture. This decline was associated with a significant decrease in the levels of bound Suv39H2 (Fig. 3G). Although Suv39H1 binding also declined during this period, the decrease did not reach statistical significance (Fig. 3F).

Suv39H2 and Suv39H1 recruitment to the *SP-A* TBE region in cultured human fetal lung type II cells is increased by hypoxia. In studies using cultured human fetal lung type II cells, we previously observed that cAMP and IL-1 induction of *SP-A* expression was prevented when the cells were maintained in a hypoxic (2% O_2) environment (15). The inhibitory effects of hypoxia were mediated, in part, by decreased recruitment of endogenous TTF-1, NF-κB, and HATs to the TBE with reduced levels of acetylated H3K9,14 associated with this genomic region. Importantly, hypoxia also caused a global increase in levels of histone H3 dimethylated on K9 (H3K9me$_2$) and increased association of H3K9me$_2$ with the *SP-A* promoter (15). To determine whether O_2 tension alters the expression and recruitment of the histone methyltransferases Suv39H1 and Suv39H2, human fetal lung type II cells were cultured for 48 h in a 2 or 20% O_2 environment in control medium and in medium containing Bt_2cAMP. SP-A mRNA levels were determined by using qRT-PCR. As reported previously (2, 15), SP-A mRNA expression was reduced in type II cells cultured in 2% O_2 and was only modestly inducible by Bt_2cAMP compared to the marked induction by cAMP treatment in explants cultured in 20% O_2 (Fig. 4A). Moreover, we found that mRNA expression of Suv39H1 and Suv39H2 was not affected by oxygen tension but was significantly reduced by Bt_2cAMP treatment (data not shown). However, using ChIP, we observed that Suv39H1 and Suv39H2 binding to the *hSP-A* TBE region was significantly increased in cells cultured in 2% O_2, compared to cells cultured in 20% O_2 (Fig. 4B and C). This suggests that the reduced level of *SP-A* expression in hypoxia is due, in part, to increased binding of endogenous Suv39H1 and Suv39H2 to the *hSP-A* promoter, resulting in a repressed chromatin state. Thus, low oxygen tension favors a repressive chromatin state at the *SP-A* promoter by increasing binding of the methyltransferases Suv39H1 and Suv39H2. It should be noted that in "control" type II cells, *in vivo* binding of Suv39H1 (Fig. 4B) and H2 (Fig. 4C) was significantly reduced in 20% versus 2% O_2; however, SP-A mRNA levels changed very modestly (Fig. 4A). This is in contrast to what was observed in Bt_2cAMP-treated tissues. We believe that this is reflective of the fact that the decline in Suv39H1/2 binding is permissive for induction of *hSP*-A promoter activity. cAMP stimulation of *hSP-A* expression in a 20% O_2 environment also requires the recruitment and binding of stimulatory transcription factors, such as TTF-1, NF-κB (14, 15), and estrogen-related receptor α (ERRα) (24, 25).

Suv39H2 and Suv39H1 mediate repression of *SP-A* gene expression in hypoxia. Our findings thus far indicate that expression of Suv39H1 and H2 methyltransferases and levels of H3K9me$_2$ and H3K9me$_3$ bound to the *SP-A* promoter temporally decline in mouse fetal lung with the developmental in-

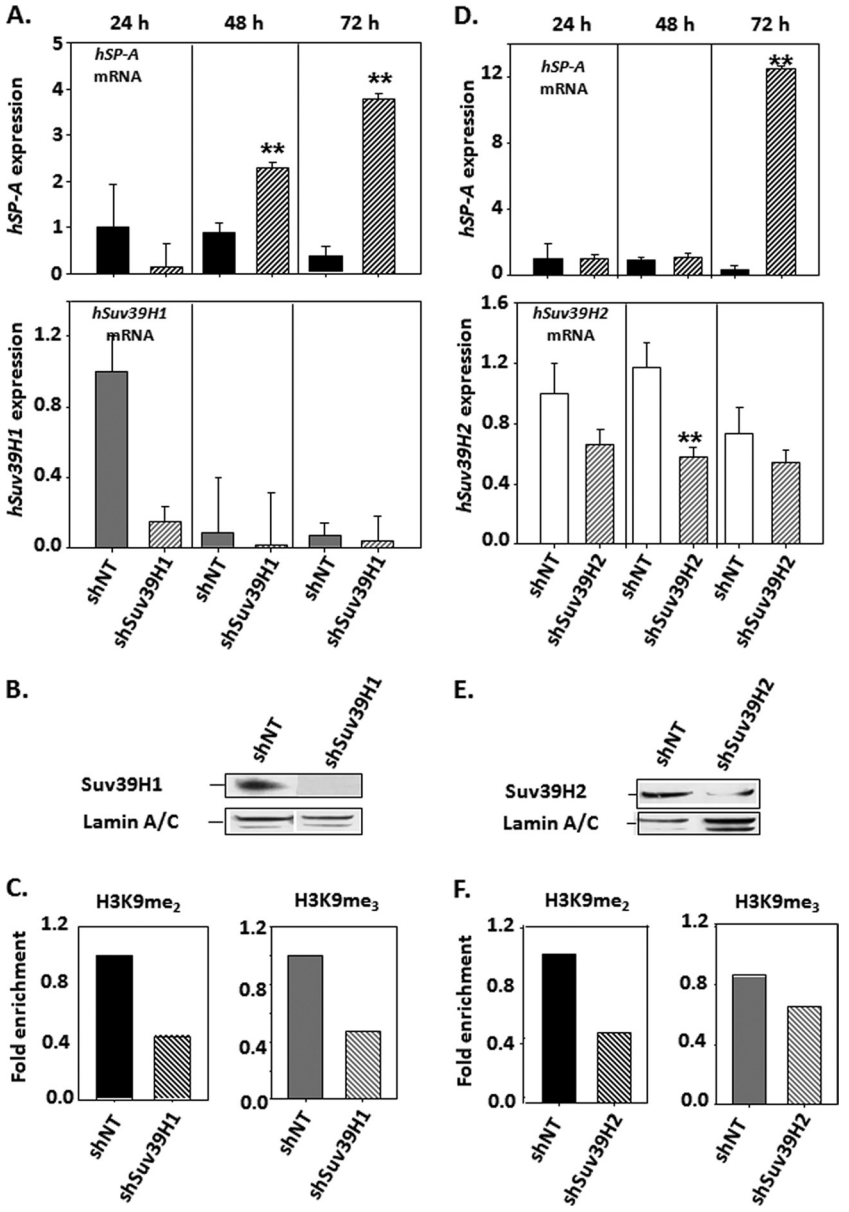

FIG. 5. shRNA knockdown of Suv39H1 and Suv39H2 methyltransferases derepresses *SP-A* gene expression in human fetal lung epithelial cells cultured in a hypoxic environment. Epithelial cells isolated from midgestation human fetal lung were transduced with lentiviruses expressing nontargeting shRNA (shNT) or shRNA targeting Suv39H1 (shSuv39H1) or Suv39H2 (shSuv39H2) and cultured for 24 to 72 h in the presence of Bt_2cAMP in a 2% O_2 environment. The mRNA levels for SP-A and Suv39H1 (A) and for SP-A and Suv39H2 (D) were analyzed by qRT-PCR in the respective knockdown experiments using h18S RNA as an internal standard. Asterisks in panels A and D indicate significant differences (**, $P < 0.01$) from values in cells transduced with nontargeting control shRNA at that time point. Immunoblot analysis of Suv39H1 (B) and Suv39H2 (E) in human fetal lung epithelial cells cultured for 48 h under the conditions described above was performed; lamin A/C was used as a loading control. ChIP analysis of the levels of endogenous $H3K9me_2$ and $H3K9me_3$ (C and F) bound to the TBE region of the *SP-A* promoter at the 72-h time point was performed. The data are means ± the SEM of duplicate samples from a representative experiment.

duction of *SP-A* expression. Furthermore, increased binding of Suv39H1 and H2 to the *hSP-A* promoter in human fetal lung type II cells by hypoxia prevents cAMP induction of *SP-A* expression. Interestingly, cAMP treatment clearly downregulates mRNA levels of both Suv39H1 and Suv39H2 independently of oxygen tension (data not shown). To functionally define the roles of these methyltransferases in the regulation of *SP-A* expression, we amplified lentiviruses expressing shRNA targeting either Suv39H1 or Suv39H2 in HEK293T cells. Lentiviruses expressing shRNAs specific for Suv39H1 and Suv39H2 were transduced into human fetal lung epithelial cells cultured in a hypoxic (2% O_2) environment in medium containing Bt_2cAMP. Parallel dishes of cells were transduced with nontargeting shRNA, as a control. The lentiviral constructs also express green fluorescent protein, which facilitates analysis of the transduction efficiency, which was ~75%. To conduct these studies, we utilized epithelial cells freshly isolated from midgestation human fetal lung because SP-A mRNA levels are

low to undetectable in these cells before culture. The epithelial cells were cultured in a 2% O_2 environment, which prevents marked induction of *SP-A* expression when the cells are cultured with Bt_2cAMP (Fig. 4A).

As can be seen in Fig. 5, after 24 h, transduction of shRNA for Suv39H1 (Fig. 5A, lower panel) and Suv39H2 (Fig. 5D, lower panel) caused a pronounced reduction in the respective mRNAs compared to cells transduced with nontargeting shRNA. In the case of Suv39H1, after 48 h of incubation, the levels of endogenous Suv39H1 mRNA declined markedly so that an effect of the Suv39H1 shRNA could no longer be ascertained. Nonetheless, at the 48-h time point, >95% knockdown of Suv39H1 and ~75% knockdown of Suv39H2 protein levels (Fig. 5B and E) was evident using the targeting compared to nontargeting shRNA. In the case of Suv39H1 knockdown, this was associated with a pronounced upregulation of *SP-A* mRNA levels after 48 and 72 h (Fig. 5A). On the other hand, a marked induction of SP-A mRNA was observed only after 72 h after knockdown of Suv39H2 (Fig. 5D). The reason for this temporal difference in the induction of *SP-A* expression is not understood but could possibly be due to differences in the relative levels of expression of Suv39H1 and H2 in the human fetal lung tissue.

To assess whether derepression of *SP-A* gene expression following Suv39H1 and Suv39H2 knockdown was associated with decreased binding of these methyltransferases and of methylated H3K9 at the *hSP-A* promoter, we performed ChIP 72 h after lentiviral transduction. A decrease in H3K9me_2 levels at the TBE was observed with Suv39H1 (Fig. 5C) and Suv39H2 (Fig. 5F) knockdown. H3K9me_3 binding also was significantly decreased following Suv39H1 knockdown (Fig. 5C) but was not significantly affected by Suv39H2 knockdown (Fig. 5F). Similar results also were obtained by siRNA knockdown of the mRNAs encoding the two histone methyltransferases (data not shown). shRNA-mediated knockdown of each of the methyltransferases caused a selective decrease in their binding to the TBE region of the *hSP-A* promoter (data not shown). Collectively, these findings suggest that increased promoter binding of Suv39H1 and Suv39H2 by hypoxia mediates repression of *SP-A* expression in human fetal lung epithelial cells.

DISCUSSION

The gene encoding the major surfactant protein, SP-A, is silent in fetal lung throughout ~85% of gestation and thereafter is dramatically upregulated to term. As described above, we previously observed that lung cell-specific, developmental, and cAMP induction of *SP-A* gene expression are mediated by the binding of transcription factors and coregulators to response elements within an ~300-bp region immediately upstream of the *SP-A* gene. Moreover, cAMP and IL-1 stimulation of *SP-A* expression in human fetal lung type II cells is dependent upon a critical O_2 tension. One of the response elements essential for cAMP/IL-1 induction of *SP-A* expression in human fetal type II cells, the TBE, binds TTF-1, NF-κB, and HAT coactivators in an O_2-dependent manner (15). This is associated with increased local acetylation of histone H3K9 (15) and of TTF-1 (41). When human fetal lung type II cells were cultured in a hypoxic environment, there was reduced recruitment to the TBE of HAT coactivators, decreased local levels of H3K9ac and increased expression of histone deacetylases (HDACs). Furthermore, hypoxia increased levels and association of methylated H3K9 with the *SP-A* promoter (15).

Based on these collective findings, we postulate that during early gestation to midgestation when the developing lung undergoes extensive branching morphogenesis and is poorly vascularized, the relatively low O_2 tension enhances expression and recruitment to the *SP-A* promoter of histone methyltransferases (HMTs) and HDACs. This results in increased methylation and decreased acetylation of key residues (e.g., H3K9) in histone N-terminal tails and a repressed chromatin state. After midgestation, the increase in vascularization and O_2 availability to the ductular epithelium causes decreased expression and recruitment of HDACs and HMTs to the *SP-A* promoter, activation and binding of TTF-1 and NF-κB, which further facilitates the recruitment of HATs, resulting in increased H3K9 acetylation and opening of chromatin structure. We suggest that via its alternative methylation versus acetylation, lysine 9 of histone H3 serves a pivotal role as a molecular switch that alters chromatin structure to silence or activate *SP-A* gene expression.

In the present study, we tested the validity of this hypothesis using two models of lung development/differentiation: mouse fetal lung during 15.5 to 19 days gestation and midgestation human fetal lung explants during differentiation in culture (30, 35). Using ChIP, we observed that the developmental induction of *SP-A* expression in mouse fetal lung at 16.5 dpc was temporally associated with decreased expression and binding of the H3K9 methyltransferases Suv39h1 and Suv39h2 and of H3K9me_2 and H3K9me_3 to the TBE region of the *mSP-A* promoter. Interestingly, increased TBE binding of endogenous key transcription factors TTF-1 and NF-κB, coactivators PCAF and CBP, and acetylated H3K9 was evident by 17.5 dpc and increased progressively to term. These findings suggest that decreased H3K9 methylation may initiate permissive changes in chromatin at the *SP-A* promoter to allow activating transcription factors to bind and recruit HATs, leading to increased H3K9 acetylation, the further opening of chromatin structure and induction of *SP-A* expression.

A similar decline in Suv39H1 and Suv39H2 mRNA levels and binding was observed during type II cell differentiation and induction of *SP-A* expression in human fetal lung explants cultured in Bt_2cAMP-containing medium. Notably, Suv39H-dependent methylation of H3K9 promotes recruitment and binding of the heterochromatic adaptor proteins, HP1α and HP1β (5, 22), which have been implicated in gene silencing. Previously, we observed that glucocorticoid inhibition of *hSP-A* gene expression in human fetal lung type II cells was associated with increased H3K9 methylation in the TBE region, coupled with increased expression and recruitment of HP1α (16). Thus, decreased methylation of H3K9 and recruitment of HP1 proteins to the *SP-A* promoter during type II cell differentiation may provide a conserved mechanism for permissive changes in chromatin structure that facilitate the binding of activating transcription factors and coregulators and induction of *SP-A* gene expression.

Suv39h1 and Suv39h2 are heterochromatin-enriched enzymes that manifest overlapping patterns of expression during

mouse embryogenesis. In adult mice, Suv39h2 is primarily expressed in the testis (29). Mice that are null for both *Suv39h1* and *Suv39h2* genes manifested decreased viability, retarded growth, hypogonadism, and male infertility, as well as chromosomal instability, resulting in an increased propensity to develop B-cell lymphomas (33). In contrast to the histone methyltransferase G9a, which directs mono- and dimethylation of H3K9 in silent euchromatin, Suv39h1 and Suv39h2 predominantly catalyze trimethylation of H3K9 at pericentric heterochromatin (34). However, studies of retinoblastoma (Rb) protein regulation of the *cyclin E* promoter indicate that Suv39H1 also plays a role in repression of euchromatic genes (28). Notably, a role for G9a in pericentric heterochromatin formation also has been described (9).

Enzymatically, Suv39h manifests a preference for mono- and dimethylated H3K9 as a substrate (32), suggesting its possible functional interaction with H3K9 mono- and dimethylases, such as G9a and ESET. Recently, it was reported that Suv39h1 can exist in a "megacomplex" with G9a, ESET, and GLP that promotes H3K9 methylation in both euchromatin and heterochromatin (11). In MEFs that were *Suv39h*$^{-/-}$, G9a, ESET, and GLP methyltransferases were destabilized at the protein but not at the mRNA level (11), suggesting that the integrity of these methyltransferases is interdependent. In the present study, we observed that ESET, G9a, and GLP/EuM mRNA levels remained relatively constant in fetal mouse lung during late gestation (data not shown), while the expression of Suv39h1 and Suv39h2 mRNA declined remarkably. Whereas these findings suggest the particular importance of Suv39h1 and Suv39h2 in catalyzing methylation of H3K9 and silencing the *SP-A* locus during early to midgestation, *in vivo* binding G9a to the TBE region declined markedly at 16.5 dpc (data not shown) and followed a somewhat similar pattern to that of Suv39h2 during fetal lung development. Thus, G9a may be part of the complex of methyltransferases bound to the *SP-A* promoter that maintain gene silencing. *In vivo* binding of the methyltransferases ESET and GLP/EuM, also found to be associated with Suv39h1 and G9a in MEFs, remains to be determined.

In the present study, we also observed that hypoxia enhanced recruitment of endogenous Suv39H1 and Suv39H2 to the *SP-A* TBE region in cultured human fetal lung type II cells in concert with inhibitory effects on basal and cAMP induction of *SP-A* expression. Interestingly, cAMP treatment inhibited Suv39H1 recruitment to the TBE region in both 2 and 20% O_2 environments, while no apparent effect of cAMP on Suv39H2 recruitment was evident. Previously, we found that hypoxia also upregulated the expression of class I, II, and IV HDACs in type II cells and that this induction was inhibited by Bt_2cAMP (15). Notably, sirtuin 1 (SIRT1), an NAD^+-dependent class III histone deacetylase with specificity for H3K9, interacts with and promotes Suv39H1 activation by deacetylating a lysine residue in its SET domain (38). Thus, SIRT1 activation can coordinately increase both H3K9 deacetylation and H3K9 methylation, resulting in gene repression. In light of these findings, it will be of interest to determine whether SIRT1 and other HDACs coordinate pivotal modifications in H3K9 methylation versus acetylation during type II cell differentiation in developing fetal lung.

Finally, we made the striking observation that shRNA-mediated knockdown of Suv39H1 and Suv39H2 caused a pronounced induction of SP-A mRNA expression in human fetal lung epithelial cells cultured in a hypoxic environment. This was associated with decreased levels of $H3K9me_3$ in the genomic region surrounding the TBE. These intriguing findings suggest that the downregulation of the Suv39h1 and h2 histone methyltransferases with increasing O_2 availability to the lung epithelium during late gestation plays a key role in chromatin structure changes that mediate induction of *SP-A* gene expression in the developing fetal lung.

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