Type II cell differentiation and expression of the major surfactant protein, SP-A, in mid-gestation human fetal lung (HFL) are induced by cAMP and inhibited by TGF-β. cAMP induction of SP-A promoter activity is mediated by increased phosphorylation and DNA binding of thyroid transcription factor-1 (TTF-1/Nkx2.1), a master regulator of lung development. To further define mechanisms for developmental induction of surfactant synthesis in HFL, herein, we investigated the potential roles of microRNAs (miRNAs, miRs). To identify and characterize differentially regulated miRNAs in mid-gestation HFL explants during type II pneumocyte differentiation in culture, we performed miRNA microarray of RNA from epithelial cells isolated from mid-gestation HFL explants before and after culture with or without Bt$_2$cAMP. Interestingly, the miR-200 family was significantly up-regulated during type II cell differentiation; miR-200 induction was inversely correlated with expression of known targets, transcription factors ZEB1/2 and TGF-β2. miR-200 antagonists inhibited TTF-1 and surfactant proteins and up-regulated TGF-β2 and ZEB1 expression in type II cells. Overexpression of ZEB1 in type II cells decreased DNA binding of endogenous TTF-1, blocked cAMP stimulation of surfactant proteins, and inhibited miR-200 expression, whereas cAMP markedly inhibited ZEB1/2 and TGF-β. Importantly, overexpression of ZEB1 or miR-200 antagonists in HFL type II cells also inhibited LPCAT1 and ABCA3, enzymes involved in surfactant phospholipid synthesis and trafficking, and blocked lamellar body biogenesis. Our findings suggest that the miR-200 family and ZEB1, which exist in a double-negative feedback loop regulated by TGF-β, serve important roles in the developmental regulation of type II cell differentiation and function in HFL.

Developmental induction of surfactant protein A (SP-A)$^2$ expression in fetal lung provides a marker for differentiation of type II cells and their capacity to synthesize surfactant, a phospholipid-rich lipoprotein, which is essential for air breathing. SP-A, the major surfactant protein and a component of the innate immune system, binds to and activates alveolar macrophages to facilitate pathogen clearance within the lung alveoli (1). Moreover, the induction of SP-A in the maturing fetal lung and its secretion into amniotic fluid near term serves as a signal for the initiation of labor (2–4). SP-A expression by HFL epithelial cells is stimulated by cAMP and IL-1, which enhance recruitment to the hSP-A promoter of the critical transcription factors, thyroid transcription factor 1 (TTF-1/Nkx2.1) and nuclear factor κB (NF-κB), and histone-modifying cofactors, which promote permissive changes in chromatin structure (5, 6). By contrast, cAMP induction of hSP-A expression is inhibited by glucocorticoids (7–9) and TGF-β (10, 11) and is blocked by hypoxia (6, 12). Notably, TGF-β mediates inhibitory effects of hypoxia on lung alveolar development in neonatal mice (13) and down-regulates TTF-1 expression in lung adenocarcinoma cells (14).

To further define mechanisms for type II cell differentiation and developmental induction of hSP-A expression, we have investigated the potential role of miRNAs, evolutionarily conserved, potent regulators of gene expression that are important in lung organogenesis (15–19), carcinogenesis (20), and O$_2$/hypoxia regulation of gene expression (21, 22). miRNAs inhibit gene expression by binding through imperfect base pairing via their “seed sequences” (nucleotides 2–8 at their 5'-ends) to

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The microRNA microarray data reported in this paper have been deposited in the Gene Expression Omnibus (GEO) database, http://www.ncbi.nlm.nih.gov/geo (accession number GSE61183).

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2 The abbreviations used are: SP-A, surfactant protein A; ABCA3, ATP-binding cassette, subfamily A, member 3; HFL, human fetal lung; miRNA or miR, microRNA; Bt$_2$cAMP, dibutyryl-cAMP; TTF-1/Nkx2.1, thyroid transcription factor 1; ZEB, zinc finger E-box-binding homeobox; EMT, epithelial to mesenchymal transition; LPCAT1, lysophosphatidylcholine acyltransferase 1; TGF-βRI, TGF-β type I receptor; RT-qPCR, reverse transcription-quantitative PCR; TBE, TTF-1-binding element.
complementary sites, typically in the 3′-untranslated regions of target mRNAs. This results in inhibition of mRNA translation and/or increased mRNA degradation (23, 24). Approximately 1,000 miRNAs are encoded by the human genome; these regulate ~30% of expressed genes (25). A single miRNA can downregulate a sizable number of functionally related mRNAs; thus, miRNAs may target gene networks. Little is known of the roles of miRNAs in type II cell differentiation and surfactant production.

To identify miRNAs that are differentially expressed during type II cell differentiation and the induction of SP-A expression, we conducted miRNA microarray analysis of RNA from epithelial cells isolated from mid-gestation HFL explants before and after culture with Bt2cAMP. Previously, we observed that upon culture of HFL explants in serum-free medium, type II cells differentiate spontaneously within the prealveolar ducts and develop the capacity to produce surfactant (26). Moreover, cAMP enhances type II cell differentiation and induction of SP-A gene expression (27). Notably, we observed that several members of the miR-200 family were significantly up-regulated in concert with type II cell differentiation. The miR-200 family consists of five members, which exist in two conserved clusters in the human genome on chromosome 1 (which contains miR-429/200a/200b) and 12 (which contains miR-200c/141) (28). Up-regulation of miR-200 family members observed during type II cell differentiation was associated with decreased expression of known targets, ZEB1, ZEB2 (29–31), and TGF-β (32, 33). ZEB1/2 (zinc finger E-box-binding homeobox 1 and 2) are E-box-binding transcription factors induced by TGF-β signaling that promote epithelial to mesenchymal transition (EMT) and increase metastasis of cancer cells (31, 34, 35). ZEB1/2 and miR-200s exist in a double-negative feedback loop, whereby ZEBs negatively regulate miR-200 transcription and miR-200s down-regulate ZEB expression via direct targeting (36, 37).

Although miR-200 family members have been described previously as suppressors of proliferation and metastasis of lung adenocarcinoma (38) and other cancer cell types (39), their roles in normal epithelial cell differentiation within the developing fetal lung have not been investigated. In this study, we conducted miRNA microarray analysis of RNA from epithelial cells isolated from mid-gestation HFL explants before and after 48 and 96 h of culture with Bt2cAMP, as described above. RNA was extracted from the isolated epithelial cells using an miRNeasy kit (Qiagen). After passing a quality control test, the RNA was sent to LC Sciences (Houston, TX) for miRNA microarray analysis of the 894 mature human miRNAs known at that time, using quadruple replicates of the “before culture” and the 48- and 96-h incubation time points.

**Experimental Procedures**

**Isolation and Culture of HFL Explants and Type II Cells**—Mid-gestation HFL tissues were obtained from Advanced Biosciences Resources (Alameda, CA). Because we had no access to identifiers for human subjects, our research was classified as “exempt” by the Internal Review Board of the University of Texas Southwestern Medical Center. Human fetal lung explants and type II pneumocytes were isolated and cultured as described in detail previously (26, 40). Briefly, fetal lung tissues were minced and rinsed in serum-free Waymouth’s MB752/1 medium (Gibco). Lung explants were placed in 35-mm sterile culture dishes on lens paper supported by stainless steel grids. The explants were cultured in serum-free Waymouth’s medium containing Bt2cAMP (1 mM; Sigma) for 5 days to enrich the population of differentiated type II cells. Cells were dispersed from the explants by digestion with collagenases type I (0.5 mg/ml; Sigma) and type IA (0.5 mg/ml; Sigma). The resulting cell suspension was depleted of fibroblasts by incubation with diethylaminoethyl-dextran (250 µg/ml) for 30 min at 37 °C, followed by centrifugation at 400 × g for 5 min. The cell pellet was resuspended in Waymouth’s MB752/1 medium containing 10% (v/v) fetal bovine serum (FBS) and loaded onto a 90–60% Percoll gradient. The type II cells were collected and plated onto 60-mm tissue culture dishes coated with extracellular matrix prepared from Madin-Darby canine kidney cells (40) and incubated overnight. Cells were then washed three times with medium to eliminate dead and nonadherent cells and cultured in Waymouth’s medium without FBS. The plating density of the cells after overnight incubation was ~50–60%.

**Isolation and Culture of HFL Epithelial Cells**—The method used for isolation and culture of undifferentiated epithelial cells (41) is based on that developed for differentiated type II cells (40). Epithelial cells were directly isolated from freshly obtained HFL by collagenase digestion, followed by diethylaminoethyl-dextran treatment and Percoll gradient centrifugation without prior explant culture.

**A549 Cells**—The human lung adenocarcinoma cell line, A549 (ATCC CCL 185) was maintained in Waymouth’s MB752/1 medium containing FBS (10% v/v). Experiments were conducted when the cells reached ~80% confluence.

**Culture Conditions**—After isolation, 3 × 10⁶ HFL epithelial or type II cells were plated onto 60-mm dishes and cultured overnight in Waymouth’s medium containing 10% FBS. The next day, cells were washed three times in serum-free Waymouth’s medium and cultured in serum-free medium with or without Bt2cAMP (1 mM) and with or without recombinant human TGF-β1 (10 or 100 ng/ml; Biolegend, 580706), alone or in combination for up to 72 h. Cells were harvested at different time points for extraction of RNA and protein and for morphological analysis.

**Microarray Analysis**—Epithelial cells were isolated from mid-gestation HFL explants before and after 48 and 96 h of culture in the presence of Bt2cAMP, as described above. RNA was extracted from the isolated epithelial cells using an miRNeo kit (Qiagen). After passing a quality control test, the RNA was sent to LC Sciences (Houston, TX) for miRNA microarray analysis of the 894 mature human miRNAs known at that time, using quadruple replicates of the “before culture” and the 48- and 96-h incubation time points.

**Reverse Transcription-Quantitative PCR (RT-qPCR)**—Total RNA was isolated from HFL type II cells, isolated epithelial cells, and HFL explants cultured with or without Bt2cAMP by the one-step method (TRizol, Invitrogen). RNA was treated
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Recombinant Adenovirus Transductions—For ZEB1 overexpression experiments, HFL type II cells were transduced with recombinant adenoviruses expressing CMOV expression vectors for β-galactosidase (β-gal), as control, and ZEB1 or ZEB2 (28) at a multiplicity of infection of 100 pfu/cell. Cells were collected 48 h postinfection for mRNA and protein extraction. To assess transduction efficiency, β-gal staining (β-gal staining kit, Invitrogen) was performed on cells 72 h after transduction with the β-gal recombinant adenovirus (42).

Chromatin Immunoprecipitation—HFL epithelial cells were cultured as described above and then cross-linked for 10 min using 0.4% formaldehyde. Glycine (0.125 m) was added to stop cross-linking. Cells were washed in 1× PBS and lysed with lysis buffer containing 1 mM PMSF and 1× protease inhibitor. The chromatin was sheared by sonication into fragments ranging from 500 bp to 1 kb. The lysate was diluted and subjected to immunoprecipitation with preimmune IgG, as negative control, and with specific antibody against TTF-1 (Santa Cruz Biotechnology, Inc., SC-13040). The immunoprecipitated complexes were collected using agarose beads (Upstate Biotechnology, 17-295) and subjected to heating at 65 °C to reverse cross-linking. The isolated DNA was quantified by qPCR using primers that flanked the TTF-1-binding element (Table 1) and the ΔΔCt method.

Immunoblotting—Whole cell, nuclear, and cytoplasmic extracts (Thermo Scientific, 78835) were prepared from HFL epithelial cells, type II cells, and explants before and after culture. Proteins were separated by SDS-PAGE and transferred to PVDF membranes. The membranes were probed using antibodies specific for SP-A (27), ZEB1 (generously provided by Dr. Douglas Darling), ZEB2 (Santa Cruz Biotechnology, sc-48789), and TGF-β2 (Abcam, ab66045).

Transient Transfection of hSP-A-Luciferase Reporter Vectors—1 day before transfection, A549 cells were plated in 24-well culture dishes (1 × 10^5 cells/well) in Waymouth’s MB752/1 medium containing 10% FBS. After 24 h, the cells were co-transfected with 0.6 µg of the hSP-A-Luciferase reporter construct and either 0.2 µg of pCMV4/TTF-1 or an empty expression vector (pCMV4) in the absence or presence of 0.3 µg of pcDNA3.1/ZEB1 or with compensatory amounts of pcDNA3.1 empty vector. All cells were cotransfected with 0.1 µg of Renilla expression vector (pRhl-TK) as an internal control to assess transfection efficiency. The plasmids were combined with 4 µl of Fugene HD (Promega, E2311) in Opti-MEM medium (Gibco) and incubated at room temperature for 15 min. The cells were incubated with reporter and expression plasmid/Fugene HD mixtures in 500 µl of Waymouth’s MB752/1 medium containing 2% FBS for 48 h at 37 °C. The cells were then lysed, and lysates were assayed for luciferase activity by using a Dual-Glo luciferase assay kit (Promega), using a 7715 Microplate luminometer (Cambridge Technology, Cambridge, UK).

Results

Microarray Analysis Reveals That miR-200c and miR-200b Are Significantly Up-regulated during HFL Type II Cell Differentiation—To identify and characterize differentially regulated miRNAs during differentiation of mid-gestation HFL

| TABLE 1
| Primers used
| Transcript | Primer sequence (5’–3’)
| SYBR Green* | Human SP-A Forward GGGCAGTTGAATGACAGAA Reverse CTAAGCAGCATACAGATCC | ZEB1 Forward GATGATGATGTCAGCTGCAGA Reverse CTTGCCCTCTTTCTAAGGCZEB2 Forward AACAAGGATATCCTAAGACCTC Reverse TGCCCTCCTCCAGGTTTTCC | TGF-β1 Forward ACTCTCCACACTGGAGCTTC Reverse GCATCATAGGGTGTCCA | TGF-β2 Forward CAGACACTCTGAGATGACCA Reverse CCTCTGCTCAGATAGCTCT | TGF-β3 Forward ACTTCGACCACTTGGAGCTTC Reverse GCTCTCCTCAGGTTGCTCA | E-cadherin Forward CCAACACACTTCAAGAAGTC Reverse CGGCTGAATGCGGGCGGATC | Vimentin Forward TTTGAGAAGACTCTCAGAGAGGA Reverse CCACACATGGATCTGGATC | h185 Forward ACCACGACTAGAGATAGTGGGA Reverse GCCCTAGTCTCGAAACACA | ChIP* hSP-A Forward TTTTCTTTCCTACGAGGGCTGCTC Reverse CAACTCTTCCTCGGACACTCT | "Primer sequences used to analyze expression of SP-A, miR-200 targets, and EMT markers by quantitative RT-PCR using the SYBR Green assay.

| TABLE 2
| Assay IDs for TaqMan primers used to analyze the expression of miR-200 family
| TaqMan assay | Assay ID |
| hsa-miR-200c | 002300 |
| hsa-miR-200b | 002251 |
| hsa-miR-200a | 000502 |
| hsa-miR-141 | 000463 |
| hsa-miR-429 | 001024 |
| U6snRNA | U6snRNA |
| Pri-miR-200c | Hs 0303157 pri |

with deoxyribonuclease to remove any contaminating DNA; 1 µg was reverse-transcribed using random primers and oligo(dT) blend in the iScript™ cDNA synthesis kit (170-8891, Bio-Rad). Validated primer sets directed against mRNA for the target genes (Table 1) (28) were used for quantitative PCR amplification. Expression of miRNAs was determined using TaqMan primers (ABI) (Table 2). The Bio-Rad CFX 384 real-time system was utilized for the quantitative detection of PCR products using SYBR Green dye and TaqMan probes. Relative gene expression was calculated using the comparative cycle threshold (ΔΔCt) method.

miRNA Inhibitor Transfections—HFL type II cells and epithelial cells were cultured in Waymouth’s MB752/1 medium (Gibco). For miRNA antagonist studies, the cells were transfected with 60 nM scramble control or miCURY LNA miRNA family inhibitor for miR-200 (Exiqon) using HiPerFect transfection reagent (Qiagen), as described previously (28). The cells were then treated with or without Bt2cAMP. The cells were harvested for mRNA and protein analysis 48 and 72 h after transfection with miRNA inhibitor.
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A. Before Culture  
Human Fetal Lung Explants  
Human Fetal Lung Explants  
Isolated Undifferentiated Fetal Lung Epithelial Cells  
Isolated 48 h differentated Fetal lung Epithelial Cells  
Isolated 96 h differentiated Fetal lung Epithelial Cells  
miRNA microarray analysis 894 miRNAs

B. -2.5  
Before Culture  
48 h  
96 h  
hsa-miR-29a  
hsa-miR-200c  
hsa-miR-200b  
hsa-miR-21  
hsa-let-7i

C. miRNAs  
Known targets

- hsa-miR-200c  
- ZEB1, ZEB2, TGF-β2, TGF-βRI, SMAD2

- hsa-miR-200b  
- ZEB1, ZEB2, TGF-β2, TGF-βRI, SMAD2

FIGURE 1. miRNA microarray analysis of RNA of epithelial cells isolated from HFL explants before and after differentiation in culture. A, mid-gestation HFL tissues were harvested before and after 48 and 96 h of culture in the presence of Bt2cAMP in serum-free medium. At each time point, epithelial cells were isolated, and RNA was processed and subjected to miRNA microarray analysis using a commercial vendor (LC Sciences), which carried out miRNA microarray for 894 mature miRNAs, using quadruple replicates of the “before culture” and 48- and 96-h incubation time points. B, heat map of five miRNAs that were up-regulated ≥2-fold at both 48 and 96 h, as compared with the tissue before culture. Two of these miRNAs, miR-200b and miR-200c, are members of the miR-200 family. We focused our studies on miR-200 family members because of the potential importance of their known targets (C) to epithelial cell differentiation. C, confirmed targets for the miR-200 family: ZEB1/2, TGF-β2, TGF-βRI, and SMAD2.

In explants in culture, we performed miRNA microarray analysis of RNA from epithelial cells isolated from the HFL explants before culture and after 48 and 96 h of culture in the presence of Bt2cAMP (Fig. 1A). Previously, we observed that upon culture of HFL explants in serum-free medium, type II cells differentiate spontaneously within the prealveolar ducts and develop the capacity to produce surfactant (26). Moreover, cAMP enhances the rate of type II cell differentiation and induction of SP-A gene expression (27). Upon miRNA microarray of 894 mature human miRNAs in the HFL epithelial cells “before culture” and after 48 and 96 h of culture in the presence of Bt2cAMP, five miRNAs were up-regulated ∼2-fold at both the 48- and 96-h time points, as compared with the HFL epithelial cells before culture (Fig. 1B). Interestingly, two of these are members of the miR-200 family, miR-200b and miR-200c, which exist in two different clusters in the human genome on chromosome 1 (which contains miR-429, -200a, and -200b) and on chromosome 12 (which contains miR-200c and -141) (28). Also shown are the confirmed targets of these miRNAs (Fig. 1C). These include transcription factors ZEB1/2, which are known to regulate epithelial to mesenchymal transition (29, 37) and to repress E-cadherin and other markers of epithelial differentiation (43, 44) as well as TGF-β2 (32, 33), TGF-β receptor I (TGF-βRI), and Smad2 (32). As mentioned, TGF-β family members suppress SP-A expression in lung type II cells (10, 11). This suggests that miR-200 may play an important role in HFL epithelial cell differentiation through targeting ZEB1/TGF-β signaling.

miR-200 Family Expression Increases, whereas Expression of ZEB1/2 and TGF-β1, -2, and -3 Decreases during HFL Epithelial Cell Differentiation—To analyze the temporal induction of miR-200 family members and their confirmed targets, ZEB1/2, in mid-gestation HFL explants before and after type II cell differentiation in culture for 24–72 h in cAMP-containing medium, we used Taqman-based and SYBR Green quantitative RT-PCR, respectively. We observed that preceding the temporal induction of SP-A mRNA in HFL explants cultured in cAMP containing medium (Fig. 2A), expression of miR-200b and miR-200c significantly increased (Fig. 2B), whereas expression of their targets ZEB1/2 declined markedly, both at the mRNA (Fig. 2C) and protein (Fig. 2E) levels. Expression of TGF-β1, -2, and -3, which exist in a double-negative feedback loop with miR-200 (29, 37) and positively regulate ZEB1/2 expression (37), were analyzed in the same samples and found to be significantly down-regulated during HFL differentiation (Fig. 2D). To confirm that these events occurred within the epithelium, we cultured epithelial cells isolated from mid-gestation HFL on extracellular matrix-coated dishes in medium containing cAMP. Using this culture system, we observed that cAMP induction of SP-A mRNA (Fig. 3A) was associated with a pronounced increase in expression of miR-200c and a modest increase in miR-200b (Fig. 3B). This was associated with a marked repression of ZEB1 and ZEB1/2 mRNA and protein (Fig. 3, C and D) and TGF-β1, -2, and -3 mRNA (Fig. 3E). The EMT marker vimentin also declined, whereas E-cadherin, an index of epithelial cell differentiation, increased with the temporal induction of SP-A mRNA (Fig. 3F). As mentioned, TGF-β signaling has been reported to down-regulate SP-A expression and inhibit type II cell differentiation (10, 45). Notably, the mechanism(s) that mediates these inhibitory effects of TGF-β is not known. We suggest that early in lung development, increased expression of TGF-β family members activates their downstream targets, ZEB1 and -2, resulting in enhanced EMT and branching morphogenesis and inhibition of epithelial cell differentiation. The subsequent decline in TGF-β signaling and decrease in ZEB1/2 is permissive for an increase in miR-200 family expression, leading to enhanced type II cell differentiation and induction of SP-A expression.

SP-A and miR-200 Are Highly Expressed in Differentiated Type II Cells Compared with Adenocarcinoma Cell Line A549; Expression Is Inversely Correlated with ZEB1 and TGF-β1—ZEB1/2 are known to be up-regulated in cancer and to promote EMT (46). In this study, we compared expression of SP-A mRNA, miR-200 family, ZEB1, and ZEB2 in differentiated HFL type II cells and A549 cells, a lung adenocarcinoma cell line of presumed type II cell origin. SP-A and miR-200 family members were expressed at relatively high levels in the differentiated type II cells but were barely detectable in the A549 cell line (Fig. 4,
and C). On the other hand, ZEB1 and TGF-β1, were expressed at relatively low levels in the differentiated HFL type II cells, whereas their expression in the A549 cells was significantly elevated (Fig. 4, B and D). Interestingly, ZEB2 and TGF-β isoforms 2 and 3 were significantly reduced in A549 cells, as compared with type II cells. In addition, the epithelial cell maker E-cadherin was highly expressed in type II cells compared with A549 cells (Fig. 4E); however, no significant differences were observed in vimentin expression (Fig. 4F). These findings suggest that in A549 cells, increased ZEB1 is driven by elevated expression of TGF-β1 and decreased miR-200 expression. Thus, silencing of SP-A, miR-200, and E-cadherin gene expression in A549 cells may be mediated, in part, by enhanced ZEB1 and TGF-β1 expression.

**cAMP Inhibition of ZEB1/2 and TGF-β Expression and Signaling Are Inversely Correlated with SP-A Expression in HFL Epithelial Cells**—Previously, we demonstrated that cAMP increases the rate of type II cell differentiation and enhances SP-A gene expression in HFL explants (27). However, the mechanisms whereby cAMP enhances type II cell differentiation are incompletely defined. In the present study, we analyzed the effects of cAMP on expression and signaling of ZEB1/2 and TGF-β in association with the induction of SP-A expression (Fig. 5A) in HFL epithelial cells in primary culture. The epithelial cells were cultured with or without Bt2cAMP for 48–72 h. Using RT-qPCR and immunoblotting, we found that ZEB1/2 mRNA and protein expression were profoundly decreased by cAMP treatment (Fig. 5B and D). This effect of cAMP on ZEB1/2 expression could either be direct or indirect. It is known that ZEB1/2 expression increases in response to TGF-β and hedgehog signaling (47). In addition, in other systems, it was observed that ZEB1 and TGF-β1 exist in a positive feedback loop (32). Thus, we investigated effects of cAMP on TGF-β expression in HFL epithelial cells. Using RT-qPCR, we found that mRNA expression of all TGF-β isoforms, TGF-β1, TGF-β2, and TGF-β3, were decreased significantly by cAMP treatment (Fig. 5C). Importantly, nuclear levels of phospho-Smad2, a downstream transcriptional mediator of the TGF-β signaling pathway, and C-terminal binding protein 1 (CtBP1),
Figure 3. miR-200b/c expression increases in HFL epithelial cells during cAMP-induced differentiation in culture in association with epithelial cell marker, E-cadherin, whereas expression of ZEB1/2, TGF-β family members, and mesenchymal marker, vimentin, decline. HFL epithelial cells were cultured for up to 72 h in Bt2cAMP-containing medium. RNA was isolated at each time point and analyzed for SP-A (A); miR-200b and miR-200c (B); ZEB1/2 mRNA (C) and protein (D); TGF-β1, -2, and -3 (E); and E-cadherin and vimentin (F) mRNA using RT-qPCR. Expression of each miRNA and mRNA was normalized to U6 and h18S, respectively. Values are presented relative to the before culture (BC) sample. Data are the mean ± S.E. (error bars) of values from triplicate samples of a representative experiment repeated at least three times with comparable results. For each gene, the mean values indicated by different lowercase letters are significantly different (p < 0.05) from one another. Lowercase letters shared among treatment groups indicate no significant difference (p > 0.05) in mean values. In the immunoblot shown in panel D, nuclear extracts from HFL epithelial cells cultured with or without Bt2cAMP were analyzed by immunoblotting for ZEB1 and ZEB2 proteins. β-actin is used as a loading control.

Figure 4. SP-A and miR-200 are highly expressed in differentiated type II cells compared with A549 adenocarcinoma cells and are inversely correlated with ZEB1 and TGF-β1. Human fetal lung type II cells and A549 cells were cultured for 48 h. mRNA was extracted from each cell type and analyzed by RT-qPCR for the expression of SP-A (A); ZEB1 and ZEB2 (B); miR-200 (C); TGF-β1, -2, -3 (D); E-cadherin (E); and vimentin (F). Data are the mean ± S.E. (error bars) of triplicate samples, plotted relative to values in type II cells. **, p ≤ 0.01, significantly different compared with the type II cells; ns, p > 0.05, not significantly different from type II cell values.
known to interact with ZEB1 at target gene promoters and to enhance its repressive activity (48, 49), were also reduced by cAMP treatment (Fig. 5D). Thus, cAMP enhances type II cell differentiation by inhibiting ZEB1/2 expression and function, at least in part, through inhibition of TGF-β signaling.

miR-200 and ZEB1 Regulate SP-A, SP-B, and SP-C Gene Expression and Lamellar Body Accumulation in HFL Type II Cells—As shown in Fig. 3, miR-200 expression increased with cAMP-induced lung epithelial cell differentiation, whereas its known target, ZEB1, declined markedly. To determine whether endogenous miR-200 and ZEB are involved in the regulation of expression of all of the surfactant proteins, HFL type II cells were transfected with miR-200 LNA inhibitors (anti-miR-200). RT-qPCR was performed to confirm down-regulation of all miR-200 family members (miR-200a/b/c, miR-141, and miR-429) in type II cells after transfection with miR-200 antagonist (Fig. 6B). As shown in the immunoblot in Fig. 6A, when the type II cells were transfected with anti-miR-200, protein levels of SP-A as well as the hydrophobic surfactant proteins SP-B and SP-C were markedly reduced. By contrast, SP-D was not significantly affected (data not shown). It was reported previously in other systems that ZEB1 and TGF-β2 are known direct targets of miR-200 and that TGF-β1 is down-regulated by miR-200 (32). As compared with the LNA control, transfection of anti-miR-200 manifested a pronounced decrease in OsO4-stained inclusion bodies within 48 h, indicating a reduction in surfactant lipoprotein synthesis (Fig. 6C).

To examine the functional roles of ZEB1 in the regulation of SP-A, as well as hydrophobic surfactant proteins, HFL type II cells cultured in the presence of cAMP were infected with recombinant adenoviruses expressing either ZEB1 or β-galactosidase/LacZ, as control. β-Galactosidase staining was performed on HFL type II cells infected with adenovirus expressing β-gal to assess the transduction efficiency, which was close to 100% (Fig. 7A). ZEB1 overexpression markedly inhibited cAMP induction of SP-A, SP-B, and SP-C in the cultured type II cells (Fig. 7B); this was associated with an inhibition of pri-miR-200c expression (Fig. 7B). In addition, when A549 cells were cotransfected with a luciferase reporter construct, hSP-A_{-291}:LUC (comprising 291 bp of hSP-A 5’-flanking DNA, containing a TTF-1 response element (5)) and a ZEB1 expression vector,
basal and TTF-1 induction of SP-A promoter activity were significantly decreased (Fig. 7C). This suggests that ZEB1 represses SP-A expression at the transcriptional level. Using chromatin immunoprecipitation (ChIP), we found that ZEB1 overexpression significantly decreased binding of endogenous TTF-1 to the SP-A promoter (Fig. 7D), whereas TTF-1 protein levels were not significantly altered (Fig. 7D). This suggests that ZEB1 negatively regulates SP-A expression in alveolar type II cells by inhibiting TTF-1 binding and transcriptional activity at the SP-A promoter. As was observed with anti-miR-200 overexpression, HFL type II cells infected with recombinant adenoviruses expressing ZEB1 manifested a profound decrease in osmiophilic lamellar bodies (Fig. 7E). This decrease in lamellar bodies is consistent with the finding that ZEB1 overexpression significantly decreases expression of lysophosphatidylcholine acyltransferase 1 (LPCAT1) (Fig. 7B), an enzyme that catalyzes synthesis of disaturated phosphatidylcholine, the major surface-active component of lung surfactant (4, 50, 51), and decreased expression of the phospholipid transporter, ATP-binding cassette, subfamily A, member 3 (ABCA3), which plays an essential role in lamellar body biogenesis in type II cells (52). Thus, increased ZEB1 and inhibition of miR-200 expression decrease type II cell differentiation and surfactant synthesis in HFL type II cells.

**TGF-β1 Inhibition of SP-A Expression in HFL Epithelial Cells Is Associated with Increased ZEB1 Expression and Decreased Recruitment of TTF-1 to the SP-A Promoter**—TGF-β promotes EMT, in part, through induction of ZEB1/2 transcription factors, which inhibit E-cadherin expression (35, 46). As mentioned, it was observed previously that TGF-β and ZEB exist in a positive feedback loop (37). Moreover, miR-200s and ZEBs negatively regulate one another’s expression (28, 29, 32),
whereas TGF-β2 (53), TGF-βRI, and Smad2 (33) are direct targets of miR-200. Thus, the TGF-β/ZEB-positive feedback loop is negatively impacted by miR-200. As mentioned, TGF-β has been observed to inhibit type II cell differentiation and down-regulate SP-A gene expression (10, 11, 54); however, the underlying mechanisms are not well understood. To analyze the effects of TGF-β on ZEB1 protein in these cells is shown. A549 cells were co-transfected the hSP-A LUC reporter construct and TTF-1 and/or ZEB1 expression plasmids. Data are the mean ± S.E. of three independent experiments, each performed in quadruplicate. The mean values with different lowercase letters are significantly different from one another (p < 0.01). D, effects of ZEB1 overexpression on TTF-1 recruitment to the TBE (TTF-1 response element) containing region of the hSP-A promoter in cultured HFL type II cells were analyzed using ChIP-qPCR; data are the mean ± S.E. of two independent experiments. The values were normalized to the input; TTF-1 binding is expressed relative to the IgG control and presented as cAMP-induced binding of endogenous TTF-1 relative to control/untreated cells (**, p ≤ 0.01). An immunoblot of nuclear TTF-1 levels in cells infected with β-gal- and ZEB1-expressing adenoviruses is shown. E, HFL type II cells were transduced either with β-gal- or ZEB1-overexpressing adenoviruses and stained with osmium tetroxide to reveal phospholipid-containing lamellar inclusions. Images were captured at ×100 under phase contrast.
cells co-treated with TGF-β (Fig. 8E). This was associated with TGF-β repression of SP-A expression. These collective findings suggest that TGF-β inhibits SP-A expression through induction of ZEB1 and decreased TTF-1 recruitment to the hSP-A promoter.

To begin to define the underlying mechanisms for TGF-β inhibition of SP-A expression, HFL epithelial cells were cultured with or without Bt2cAMP and with or without TGF-β1 in the absence or presence of LY364947, an ATP-competitive inhibitor of TGF-βRI, which inhibits phosphorylation of
Smad2/3 (57). We found that TGF-β inhibition of cAMP-induced SP-A expression was prevented by LY364947 (Fig. 8F), indicating that TGF-β down-regulates SP-A expression through a Smad2/3-dependent pathway. This effect of LY364947 to restore cAMP induction of SP-A in the presence of TGF-β was associated with decreased ZEB1 expression (Fig. 8F), suggesting that TGF-β1 inhibition of SP-A expression may be through a pathway involving ZEB1 and Smad2.

Knockdown of ZEB1 in HFL Epithelial Cells Reduces the Inhibitory Effects of TGF-β1 on SP-A Expression.—To determine whether the inhibitory effects of TGF-β1 on SP-A expression are mediated through ZEB1, we infected HFL epithelial cells with lentiviruses expressing ZEB1 shRNA or non-targeting shRNA as control. After 48 h, cells were treated for 4 h with or without TGF-β1 and with or without Bt2cAMP. In the absence of TGF-β treatment, knockdown of ZEB1 further enhanced cAMP stimulation of SP-A expression, both at the mRNA (Fig. 8G) and protein (Fig. 8H) levels, indicating that endogenous ZEB1 has a repressive effect on SP-A expression. Moreover, ZEB1 knockdown greatly attenuated the inhibitory effect of TGF-β1 on SP-A mRNA expression (Fig. 8G) and blocked the inhibitory effect of TGF-β1 on SP-A protein (Fig. 8H). This suggests that the suppressive effect of TGF-β1 on SP-A expression is mediated, at least in part, through ZEB1.

Discussion

MicroRNAs are evolutionarily conserved, potent regulators of gene expression that serve important roles in development and disease (15–19). However, their functional roles in the regulation of type II cell differentiation during fetal lung development have not been elucidated. In the present study, we utilized miRNA microarray of RNA from epithelial cells isolated from mid-gestation HFL explants before and after culture in the presence of cAMP to identify miRNAs that are differentially expressed during type II cell differentiation and with induction of SP-A expression. We observed that members of the miR-200 family were significantly up-regulated with type II cell differentiation and induction of SP-A and E-cadherin expression, whereas the known miR-200 targets ZEB1, ZEB2 (36), and TGF-β2 (53), as well as other TGF-β family members, were coordinately down-regulated.

ZEB1 and -2, which exist in a double-negative feedback loop with miR-200 family members (29, 32, 37), are induced by TGF-β and serve critical roles in EMT (14). E-cadherin, which promotes polarity and adhesion of epithelial cells, is transcriptionally repressed by ZEB1/2 and related EMT factors (30). Repression of E-cadherin is considered to be a critical step in the transition of epithelial cells to an invasive, undifferentiated phenotype (30, 31, 44). Members of the miR-200 family are key inducers of epithelial cell differentiation and inhibitors of tumor progression and stem cell self-renewal (58, 59). Their actions are mediated by targeting ZEB1/2 (36), TGF-β2 (53), TGF-βR1, and Smad2 (33), as well as mRNA transcripts of genes involved in cell cycle progression, cell proliferation, and self-renewal. These include Bmi1 and Suz12 components of the PRC1 and PRC2 polycomb repressor complexes, which suppress cell differentiation pathways, as well as the stem cell transcription factor KLF4 (59). Through repression of ZEB1/2, miR-200s evoke up-regulation of E-cadherin and increase epithelial cell polarity (30, 36).

Using HFL type II cells in primary culture, we demonstrated for the first time that miR-200 family antagonists (anti-miRs) caused increased expression of ZEB1, TGF-β2, and phospho-Smad2 and inhibited expression of SP-A and the hydrophobic surfactant proteins, SP-B and SP-C. These changes in gene expression were associated with a marked decrease in accumulation of osmiophilic lamellar bodies, the storage form of surfactant, as compared with cells transfected with non-targeting siRNAs. This strongly suggests that miR-200s serve a key role in regulating fetal lung type II cell differentiation and function through suppression of ZEB1 and TGF-β signaling. This conjecture was further supported by our findings that overexpression of ZEB1 or treatment with TGF-β caused a pronounced inhibition of SP-A, SP-B, and SP-C expression by cultured HFL type II cells. Moreover, ZEB1 overexpression also markedly suppressed accumulation of lamellar bodies in the cultured cells and decreased expression of LPCAT1 and ABCA3, enzymes involved in surfactant disaturated phosphatidylcholine synthesis and trafficking to lamellar bodies. Whether the inhibitory effect of ZEB1 on SP-A expression is direct or mediated through increased TGF-β signaling is not clear. However, the finding that ZEB1 knockdown caused a marked up-regulation of SP-A in the absence or presence of TGF-β treatment suggests that TGF-β inhibition of SP-A expression is mediated by ZEB1.

ZEB1/2 are zinc finger transcription factors that can repress gene transcription by forming a complex with histone deacetylases (60) and the transcriptional repressors, CtBP1 and/or CtBP2 (48). CtBPs also have been proposed to serve as redox/NADPH sensors that are induced by hypoxia, mediate E-cadherin suppression, and enhance lung cancer cell migration and invasion under hypoxic stress (61). Like ZEB1/2, CtBP2 is a direct target of the miR-200 family (62). Mass spectrometric analysis of proteins associated with CtBP1 in HeLa cells revealed a “repressive” complex containing histone deacetylases 1 and 2, CtBP2, histone H3 lysine 9 (H3K9) histone methyltransferases, and chromodomain proteins, which can promote local formation of a repressive chromatin structure (63). We suggest that ZEB1 may inhibit SP-A expression by forming a repressive complex at the SP-A promoter containing CtBPs and histone deacetylases, which catalyze the deacylation and subsequent methylation of H3K9, resulting in chromatin compaction.

Previously, we observed that expression and SP-A promoter binding of the H3K9 histone methyltransferases, Suv39H1 and Suv39H2, and of the repressive chromatin mark, trimethylated H3K9, declined in HFL type II cells with cAMP induction of SP-A expression (41). Moreover, hypoxia, which blocks cAMP stimulation of SP-A (12), markedly increased TBE binding of endogenous Suv39H1, Suv39H2, and trimethylated H3K9 (41). Notably, TGF-β inhibition of IL-2 gene expression in T-cells was reported to be mediated by Smad2/3 interaction with NF-κB and recruitment of Suv39H1 to the IL-2 promoter (64). In H441 lung adenocarcinoma cells, TGF-β inhibition of SP-B expression was associated with Smad3 interaction with the transcriptional activators, TTF-1/Nkx2.1, HNF-3, and FoxA1.
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(65, 66). Thus, TGF-β inhibition of SP-A expression in HFL type II cells may be mediated, in part, by Smad2/3 interaction with both TTF-1 and NF-κB, which we found to cooperatively interact at a composite response element (TBE) in the SP-A promoter to enhance its expression (5).

Cyclic AMP stimulation of HFL type II cell differentiation and SP-A gene expression is mediated by enhanced TTF-1/NKx2.1 phosphorylation (55), acetylation (56), and binding together with NF-κB to the SP-A promoter (5). In the present study, we demonstrated for the first time that cAMP profoundly inhibited ZEB1/2 and TGF-β1/2/3 expression. Conversely, overexpression of ZEB1 or ZEB2 or treatment with TGF-β markedly attenuated cAMP stimulation of SP-A expression in cultured HFL type II cells. Moreover, using ChIP, we observed that ZEB1 overexpression inhibited binding of endogenous TTF-1 to the TBE region of the hSP-A promoter in cultured HFL type II cells and suppressed TTF-1 induction of hSP-A promoter activity (Fig. 7). This is in accordance with studies of vascular smooth muscle cells, where ZEB1 repression of the procollagen gene, pro-collA2, was caused by its competition with the related homeobox protein, Nkx2.5, for binding to an upstream overlapping DNA binding site (67). Because TTF-1 is essential for fetal lung development, our findings reveal an important mechanism by which TGF-β acting via ZEB1 inhibits type II cell differentiation and SP-A expression.

Cyclic AMP, which inhibits expression of TGF-β and EMT factors (Fig. 5), is anti-fibrotic. This anti-fibrotic action is exemplified by cAMP inhibition of fibroblast proliferation, extracellular matrix production, and EMT; mediated by the cAMP effector, Epac (exchange protein activated by cAMP) and by protein kinase A (PKA) (68). PKA regulatory subunits have been reported to interact with Smad3 (69), and cAMP acting via PKA inhibits Smad-mediated transcription (70). Thus, during HFL differentiation, increased cAMP may enhance TTF-1 transcriptional activity and attenuate TGF-β induction of ZEB expression through inhibition of the Smad signaling pathway. This, in turn, will increase miR-200 expression, which targets and down-regulates various components of the TGF-β/ZEB signaling pathway.

A549, a lung adenocarcinoma cell line of presumed type II cell origin (71), lacks endogenous TTF-1 (55). In the present study, we observed that SP-A, E-cadherin, and miR-200 family members, which were highly expressed in primary cultures of HFL type II cells, were virtually undetectable in A549 cells. Moreover, ZEB1 and TGF-β1, the TGF-β isof orm that normally is expressed in lung mesenchyme, were much more highly expressed in A549 cells as compared with the HFL type II cells (Fig. 4, B and D). Interestingly, TGF-β2 (which typically is expressed in the distal lung epithelium) and ZEB2 were expressed at significantly higher levels in HFL type II cells than in A549 cells (Fig. 4, B and D). Unlike differentiated HFL type II cells, A549 cells are PKA-deficient (55). Thus, decreased PKA signaling may promote increased TGF-β1 expression and signaling and increased ZEB1, which, in turn, inhibit E-cadherin and SP-A expression.

In conclusion, our findings reveal for the first time that the miR-200 family and its targets, ZEB1/2, serve key roles in differentiation and function of the surfactant-producing cell in fetal lung, the type II cell. Moreover, cAMP promotes type II cell differentiation and surfactant protein gene expression in HFL by its marked effect to repress ZEB1 and TGF-β expression and signaling, resulting in increased TTF-1 transcriptional activity. Cyclic AMP induction of type II cell differentiation is dependent upon a critical O2 tension (12). Thus, we propose that during early to mid-gestation when the fetal lung is relatively hypoxic, cAMP signaling is inhibited, allowing increased expression of TGF-β and ZEB1/2, which exist in a double-positive feedback loop (Fig. 9). This maintains suppression of miR-200s and permits further up-regulation of ZEB1/2 and TGF-β family members. The increased TGF-β and ZEB1/2 suppress TTF-1 transcriptional activity and promote increased plasticity of epithelial cells and their capacity to acquire invasive/mesenchymal properties without entirely committing to a mesenchymal phenotype. This process is probably critical for branching morphogenesis (72) and inhibition of epithelial cell differentiation. During the third trimester of gestation, increased vascularization of the fetal lung and elevated local O2 tension promote increased cAMP signaling. This causes suppression of TGF-β and ZEB1 and increased TTF-1 binding and transcription of genes involved in lung epithelial cell differentiation (Fig. 9).

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