Stage-specific Effects of cAMP Signaling during Distal Lung Epithelial Development*

Received for publication, October 3, 2006 Published, JBC Papers in Press, October 3, 2006, DOI 10.1074/jbc.M609339200

Jingsong Xu1, Jun Tian, Sandra M. Grumelli, Kathleen J. Haley, and Steven D. Shapiro
From the Division of Pulmonary and Critical Care Medicine, Brigham and Women’s Hospital at Harvard Medical School, Boston, Massachusetts 02115


cAMP signaling is postulated to play a role in distal lung epithelial differentiation based on several observations. First, it enhances fibroblast growth factor-induced transdifferentiation of early tracheal epithelium into respiratory epithelium. Second, there are cAMP-responsive elements in the heterologous promoters of SftpB and SftpA genes. Third, cAMP augments the effect of dexamethasone in maintaining differentiation of human fetal type II pneumocyte culture. However, this concept has not been thoroughly tested in vivo. In the current study, we modulated cAMP signaling in developing distal lung epithelium in vivo using an inducible transgenic system that expressed a mutant form of Goa (Goα2Q227L). We failed to demonstrate the ability of cAMP to promote distal epithelial maturation during embryonic stages. The results argue against its physiological role in this process. In addition, induction of cAMP signaling at the late pseudoglandular stage but not during the canalicular or saccular stage surprisingly delayed distal differentiation by suppressing the expression of Sftpc, SftpA, and Aquaporin 5 as well as the formation of lamellar bodies. This stage-specific inhibitory effect was observed in the absence of cellular toxicity or changes in branching. Transgenic lungs did not show significant changes in the known pathways that are important for distal differentiation. Therefore, we propose the existence of yet-to-be identified CAMP-sensitive novel regulators of early distal lung epithelial differentiation. Although the delay of differentiation seemed to be reversible at later stages, it still led to pronounced permanent postnatal airspace enlargement due to impaired paracrine function of distal epithelium in regulating alveolar myofibroblast development.

Development of the murine lung is initiated at embryonic day 9.5 (E9.5),2 followed by the pseudoglandular (E10.5–16.5), canalicular (E16.5–17.5), saccular (E17.5–P5), and alveolar stages (P5–35) (1). Three morphogenic events at the pseudoglandular stage are important for distal lung epithelial development. First, through branching morphogenesis, the lung primordium evolves into an extensive network of tubular epithelium composed of eight generations of branches. This process requires a complex interaction between several signaling molecules, such as FGF10 (fibroblast growth factor 10), SHH (sonic hedgehog), and BMP4 (bone morphogenetic protein 4) (2–6). Second, distal-proximal polarity of the branching epithelial tree is established at the same time that branching takes place. As early as E10.5–11.5, the expression of many genes is spatially restricted to the distal end of the branches. Shh, Wnt7b, Id2, thrombospondin, transforming growth factor-β, Etv5/Pea3, and Bmp4 are among the genes showing this pattern of expression (7). Distal fate specification requires the BMP4, FGFs, and β-catenin pathways. Mice deficient in either the BMP4 or β-catenin pathways showed complete trans-formation of distal epithelium into proximal epithelium with little sign of type I or type II alveolar cell development (8, 9). Recently published data suggest that β-catenin achieves this function by acting as a transcriptional factor downstream of Wnt signaling (10). FGF10 and FGF7 expressed in the distal mesenchyme are also proposed to regulate distal fate, because in a mesenchyme-free tracheal transdifferentiation assay, FGFs are the most potent distalizing signals (11).

The third event in distal lung epithelial development is the initiation of differentiation of epithelial cells at the end of the pseudoglandular stage. At E12.5, the distal epithelium starts to express differentiation-associated genes, such as SftpC. At E13, glycogen accumulation (which is required for synthesis of phospholipids, a key component of surfactant) is evident in the distal epithelium. At E15, immature lamellar bodies (the precursors of lamellar bodies) can be visualized under electron microscopy (EM). At E16–16.5, immature lamellar bodies are present in the distal cells (13). Very little is known about the genetic regulation of the initiation phase of distal epithelial differentiation, since most trans- genetic models with distal differentiation defects manifested their phenotype at the canalicular or early saccular stage. However, one study did indicate roles for transcription factors Foxa1 and Foxa2 in this early phase of differentiation (14).

Differentiation of distal epithelium is accelerated at the canalicular and saccular stages. During E17 and E18, SftpC and...
Sftpα mRNA expression is increased severalfold, more mature lamellar bodies are generated in type II cells, and the processing of pro-SP-C and pro-SP-B is also greatly increased (13). Type I cells start to emerge at E17.5 and can be identified by expression of aquaporin 5, a type I cell-specific water channel. Type I and Type II cell differentiation at these stages requires the function of transcription factors TTF-1 (15), Etv5/Pea3 (16), GATA6 (17, 18), hypoxia-induced transcriptional factor 2α (19), and Foxa1 and Foxa2 (14, 20) as well as signaling mediated by glucocorticoids (21, 22).

In the context of a complex regulatory program for proximal-distal fate determination and differentiation, substantial published data suggest that cAMP plays a role in promoting distal differentiation. cAMP-generating pharmacologic agents can greatly potentiate FGF-induced transdifferentiation of embryonic tracheal epithelium into respiratory epithelium in explant organ cultures (11). Pharmacologic activation of cAMP synthesis also enhances the differentiated phenotype in human embryonic type II cell primary culture (23, 24). Moreover, the heterologous Sftpα and Sftpβ promoters can be activated by cAMP signaling in vitro (25, 26).

The current study examined the effect of cAMP on distal lung development using two novel approaches. Ex vivo we used lung bud organ culture. In vivo we generated a transgenic line that promotes cAMP production in the distal lung epithelium in a doxycycline-controlled fashion. Surprisingly, we consistently observed both in vivo and ex vivo the suppression of early distal differentiation by cAMP. However, at later stages, cAMP production had no significant influence on distal epithelial gene expression in vivo. In addition, the temporally restricted inhibition of distal epithelial differentiation and lack of detectable changes in known epithelial regulators of distal lung development suggest novel regulators at the late pseudoglandular stage.

**EXPERIMENTAL PROCEDURES**

**Lung Bud Culture**—Swiss Webster mice (Jackson Laboratory, ME) were set up for timed mating. On the day when a vaginal plug was identified, the embryos were considered 0.5 days old. Lung buds were microdissected from E11.5 embryos and cultured in Costar Transwells (Corning Glass). The growth medium was Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% fetal bovine serum (Sigma) and antibiotics. Medium was placed only at the bottom of the wells so that organ cultures could be maintained at the air-liquid interface. After an initial growth of 12 h in regular medium, tissues were transferred to fresh medium that contained various treatment reagents and allowed to grow for up to 5 days. Each treatment group included three lung buds. For Northern blot analysis each RNA sample was generated from pooled lysate of three lung buds in the same experimental group.

**Transgenic Mice**—Using a PCR-based method, the Q227L point mutation was engineered in the IMAGE clone 3154475 (purchased from ATCC (Manassas, VA)), which carries a full-length mouse Gαs cDNA. This mutation was first introduced into a 280-base pair EcoRI-BamHI fragment on the IMAGE clone 3154475 was then replaced with this PCR fragment to generate a full-length mutant cDNA. In the second step, an Nhel-SspI IRES-EGFP cassette from pIRE-EGFP vector (Clontech, Mountain View, CA) was cloned into a modified pTRE2 vector (Clontech) between the Nhel and EcoRV sites. The resulting vector is called pTRE2-IRES/EGFP. Then, in the last step, an EcoRI-ClaI GsαQ227L cDNA fragment was ligated into the pTRE2-IRES/EGFP vector. The resulting transgene was then released from the vector as a 3.5-kb NotI fragment, purified, and microinjected for transgenic animal production.

The tet-O-GαsQ227L transgenic line was generated in a C3HxC57/B6 hybrid background. The previously published hSPC-rtTA transgenic line was in an FVB/N background (27). After both hSPC-rtTA and tet-O-GαsQ227L transgenes were bred into the C57/B6 background for four generations, two transgenic lines were crossed to generate double transgenic hSPC-rtTA+/−, GαsQ227L+/− mice and single transgenic GαsQ227L+/+ mice. Matings were then set up between these two groups of mice to produce timed pregnancy. Genotyping of hSPC-rtTA transgene was performed as previously described (27). The tet-O-GαsQ227L transgene was identified through PCR detection of GFP coding sequence using forward primer ccgatggtgccacaattcag and reverse primer atgccggctctctctgctagg. For transgene induction, 1 mg/ml doxycycline (Sigma) and 5% sucrose was added to the drinking water.

**In Situ Hybridization**—In situ hybridization was performed with a simplified version of a previously published protocol (28). IMAGE clone 1278132 (GenBank™ accession number AA882457) (ATCC, Manassas, VA) was used as the template for generating the digoxigenin-labeled SP-C probe. Probe synthesis was performed with digoxigenin RNA labeling mix from Roche Applied Science. Slides were processed as previously published and hybridized at 62 °C with 1.6 mg/ml probe in a buffer that consists 50% formamide, 5× SSC, and 40 mg/ml herring sperm DNA. After 16–20 h of incubation, slides were washed twice in 2× SSC at 15 min each at room temperature, followed by a wash in 2× SSC at 60 °C for 1 h and a wash in 0.2× SSC for 30 min at 60 °C and incubated with an alkaline phosphatase-conjugated anti-digoxigenin antibody (Roche Applied Science). SP-C-expressing cells were visualized with alkaline phosphatase substrates nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate.

**Real Time RT-PCR**—cDNA was synthesized with Amersham Biosciences RT-PCR beads. Real time RT-PCRs were carried out on a GeneAmp system 9600 with a GeneAmp 5700 sequence detector (Applied Biosystems). PCR products were quantified by the amount of binding and fluorescence emission of SYBR green dye. Expression level was calculated with the formula 2^[(Ct(cyclophilin)−Ct(gene))/100] × 100, as published previously. The expression of the gene of interest is normalized with that of the housekeeping gene Ppiα (cyclophilin), which was defined as 100 arbitrary expression units (29). Primers are listed in Table 1.

**Immunohistochemistry**—Staining was performed with 5-µm paraffin sections from either embryonic or P7 lungs, which were fixed either in 4% paraformaldehyde overnight (embryonic) or in 10% formalin for 2 days (P7 lungs). Immunostaining of PDGFRα and CC-10 was performed with the avidin-biotin
TABLE 1
Gene-specific primers for real time PCR assays

| Gene       | Forward primer | Reverse primer |
|------------|----------------|----------------|
| CyclinH5   | GGGGCGAGCTTATACAGCCA | TCCCTCTAAGTGAAACCTGCG |
| Spf5c      | GAAGCTCTTCTCTTCTGATGTT | GCAAGCTGCAATATTCCCTGTT |
| Sfp5a      | AAGGCGCGCCCTGTTGCTGAA | GAGTATGGTCCTGTGCTGAA |
| Aquaporin5  | GCTGCTAACTACACAAAGG | GCTTACACCTTCTCTTGTT |
| Ssh        | GCTGCTTCTACAACATGCGG | GCTGCTAGCTTCTCTTGTT |
| Ets5       | GAGGCCTCTTCTTCTGATT | GCCACCTTTCCTGAATTC |
| Id2        | CCTCTACAAAGACGACTGA | TCTCTTACAGGATCTCAG |
| Sox9       | ACCCGCTGATGACGGACGAC | TCCCTTTACAGCTCTGTT |
| Gata6      | GAGCTCTCTTCAAGTAGCAGG | CCTGGTCTCAAGCCTCTGTT |
| Abca3      | TGAGCGCTGACCTTCAGAGG | CCTGTTGGCCAGAGACCT |
| Fgr3       | AGGGACCTACTACGCGAAC | CACCTTTACAGCTCTGCGC |
| Fgr4       | CTACACGCGCTGGATGGAC | TGACAGTATTCCCGGCAGG |

horseradish peroxidase method (Vector Laboratories, Burlingame, CA), using 3,3′-diaminobenzidine as the chromogenic substrate. Immunostaining of phospho-CREB was performed with the tyramide signal amplification biotin system (PerkinElmer Life Sciences) also using 3,3′-diaminobenzidine as the substrate. Antibody against phospho-CREB was purchased from Cell Signaling Technology (Danvers, MA). Antibodies against PDGFRα and CC-10 were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Immunostaining of α-SMA was performed with an alkaline phosphatase-conjugated monoclonal antibody (Sigma) using Vector Red as the substrate (Vector Laboratories). A terminal deoxynucleotidyltransferase-mediated dUTP nick end-labeling assay was performed with an ApopTag®Plus peroxidase in situ apoptosis detection kit (Chemicon International, Temecula, CA). Anti-BrdUrd immunohistochemistry was performed as described previously (28). BrdUrd incorporation into embryonic lungs was achieved via intraperitoneal injection of 100 mg/kg BrdUrd into pregnant females 1 h before harvesting the embryos. In all of the above procedures, slides were counterstained with methyl green.

**Western Blot Analysis**—E15.5 lungs were homogenized in a buffer that contained 25 mM Tris-HCl, pH 7.5, 50 mM NaCl, 1 mM EDTA, 1 mM EGTA, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, and a complete proteinase inhibitor mixture (Roche Applied Science). For further protein extraction, the homogenates were supplemented with 1% Triton X-100 and rocked at room temperature for 1 h. Insoluble fractions were then removed by a 15-min centrifugation at 13,000 × g. The resulting protein lysates were resolved on an SDS-polycrylamide gel and transferred to Immobilon™ membrane (Millipore Corp., Bedford, MA). After incubation of the membrane with horseradish peroxidase-conjugated secondary antibody, followed by chemiluminescent substrates (Amersham Biosciences), the target proteins were visualized by x-ray autoradiograph. Anti-phospho-CREB, anti-CREB, and anti-phospho-Smad1/5/8 antibodies were purchased from Cell Signalling Technology. Anti-α-tubulin antibody was purchased from Sigma.

**Transmission Electron Microscopy**—Accessory lobes from E17 lungs were first fixed in Karnovsky’s solution and then postfixed in osmium tetroxide. Thin sections were stained with uranyl acetate and lead citrate. Lamellar bodies were monitored under JEOL 1200EX transmission EM at the Harvard Cell Biology Core facility for EM analysis.

**RESULTS**

**Lung Bud Culture**—We first examined the effect of cAMP on lung development using an in vitro lung bud organ culture system. Timed matings were set up as described under “Experimental Procedures.” When embryos were 11.5 days old, lung buds were microdissected and cultured on microporous membranes at an air-liquid interface in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum. The explants continued to branch for up to 5 days. They not only recapitulated branching morphogenesis in culture but also demonstrated time-dependent maturation of many features of distal differentiation. For example, SP-B was expressed only in either the propeptide form or the partially processed form on days 1 and 2 by Western blot analysis (data not shown). The completely processed mature form was not evident until days 3 and 4, and it became much more abundant at day 5 (data not shown). After the initial 12 h of culture in regular medium, lung buds were transferred to fresh medium supplemented with either vehicle (Me2SO) or 5 μM forskolin and 5 μM 3-isobutyl-1-methylxanthine (IBMX). Forskolin directly activates adenylyl cyclases, whereas IBMX inhibits all of the phosphodiesterases that can degrade cAMP. The combination of these two reagents has been used routinely to achieve sustained intracellular cAMP signaling (31, 32).

Forskolin and IBMX had at least four effects on lung bud culture. First, forskolin and IBMX exposure induced distal epithelial dilation (Fig. 1B, black arrows) compared with the vehicle control group (Fig. 1A). Second, after 3 days of culture, forskolin and IBMX significantly decreased the number of branches in the treatment groups (Fig. 1C), which was 43.7 ± 3.19 (n = 3) compared with 58.7 ± 3.1 (n = 3) in the control group (p = 0.004). Third, these two agents induced thinning of the mesenchyme, which was often evident on a whole mount image (Fig. 1B, red arrows). Histological analysis of the midtransverse sec-
cAMP Signaling in Lung Development

The suppression was gradually reversed after prolonged exposure (4–5 days), suggesting that this effect is stage-specific. The suppression was gradually reversed after prolonged exposure (4–5 days), suggesting that this effect is stage-specific. The delayed onset of expression was an unexpected observation, challenging a positive role of cAMP in promoting distal epithelial differentiation during development. To address the in vivo relevance of these findings, we designed a transgenic model to examine the role of cAMP signaling in lung epithelial development in vivo. Although we chose to focus on the effect of cAMP signaling on epithelial differentiation in the rest of this study, there are ongoing efforts investigating its effects on branching morphogenesis and lung mesenchyme development.

**Generation of gaQ227L Transgenic Mice**—To achieve ectopic cAMP synthesis in vivo, we expressed a constitutively active mutant form of Gaq, which carries a glutamine-to-leucine substitution (GaQ227L) and was initially identified as a somatic mutation in patients with pituitary or thyroid adenomas (33). Because this mutation disrupts GTPase activity, GaQ227L stimulates adenylyl cyclases constitutively in the absence of receptor ligation (Fig. 2A). To avoid unwanted negative effects of prolonged constitutive expression, we employed a bitransgenic system that is pharmacologically regulated by either tetracycline or its analog doxycycline. Similar transgenic systems have been used in several studies to modulate in vivo gene function in the murine lung (9, 14, 27, 34). Fig. 2B schematically illustrates this system. In short, the gaQ227L transgene is under the control of a cytomegalovirus minimum promoter that harbors seven tetO sites, serving as cis-binding elements for the heterologous transcription factor rtTA, which is expressed from a separate line of transgene that is driven by the 3.7-kb human surfactant-associated protein C promoter, as published previously (27). Because binding of rtTA to tetO requires tetracycline or its analog doxycycline, the GaQ227L transgene is expected to be inactive until the animals are exposed to doxycycline.

Initially, we tested the functionality of the tetO-gaQ227L transgene in an in vitro transfection assay. COS7 cells were transfected with cytomegalovirus-rtTA (an expression vector with a viral promoter) driving a luciferase reporter that contains cAMP-responsive elements in its promoter and the tetO-gaQ227L transgene. Exposure of transfecants to doxycycline resulted in a 3-fold increase in luciferase activity (Fig. 2C). Subsequently, several transgenic lines in a C3HxC57BL/6 hybrid background were established. Offspring of these lines were set up for timed mating with hSPC-rtTA mice. After pregnant females were exposed to doxycycline from E13.5 to E16.5, embryonic lungs were harvested for RNA isolation. Northern blot analysis of one gaQ227L line showed doxycycline-dependent expression of a transcript with the predicted size of 2.9 kb at E16.5 (Fig. 2D). However, the transgene transcript is expressed at a level much lower than the 2.1 and 3.1-kb endogenous transcripts (Fig. 2D). We bred the gaQ227L transgene to homozygosity in order to enhance the level of expression. Most of the analyses presented in this study were performed using the mating scheme shown in Fig. 3A, in which 50% of the offspring are double transgenic for both hSPC-rtTA and tetO-gaQ227L.
The other 50% are single-transgenic for tetO-gαsQ227L and serve as experimental controls for the double transgenic mice. All animals in this mating scheme, including both parents and their offspring, carried the gαsQ227L transgene in a homozygous configuration. Because the transgene is only expected to express in a small fraction of cells in the lung, total tissue cAMP quantification is not a sensitive assay for transgene function. Instead, we monitored phosphorylation of CREB as a read-out for cAMP signaling. Immunohistochemical staining using an antibody that binds specifically to the phosphorylated form of CREB identified its activation in the bitransgenic distal epithelium (asterisks in Fig. 2G), whereas single transgenic controls showed little CREB phosphorylation in the distal epithelium (asterisks in Fig. 2F). It is interesting that control lungs showed striking CREB phosphorylation in the proximal epithelium, suggesting a role for CREB in regulating proximal epithelial development. Ectopic CREB phosphorylation was not detected in the vasculature or other mesenchymal cells; nor was there an obvious increase in endogenous CREB phosphorylation in the proximal epithelium. Western blot analysis of total lung lysates isolated from E15.5 mice exposed to doxycycline for 4 days also showed increased phosphorylation of CREB (Fig. 2E). In summary, the bitransgenic system was able to activate cAMP signaling in a distal epithelium-specific fashion. This system was regulatable by doxycycline and demonstrated high stringency with almost no base-line leakage of expression of the mutant gαs, in the absence of doxycycline exposure (Figs. 3B and 6, A–C).

**Suppression of Distal Differentiation at the Late Pseudoglandular Stage**—The effect of ectopic cAMP signaling on distal lung epithelial differentiation was first tested at the late pseudoglandular stage. gαsQ227L transgene expression was induced by doxycycline from E14.5 to E16.5. Using RNA samples isolated from E16.5 lungs, the expression of the transgene and distal epithelial markers was quantified by real time RT-PCR. To ensure that in utero doxycycline exposure was sufficient and the transgene was induced, every embryonic litter used in this study was first tested for the level of transgene expression prior to further phenotypic analysis.

Since the transgene is transcribed into a bicistronic mRNA that contains both gαsQ227L and GFP coding sequences (Fig. 2B), detection of GFP-encoding cDNA by real time PCR was used as a surrogate read-out of transgene expression. As expected, in all cases, double transgenic lungs showed robust expression of GFP-encoding cDNA (Figs. 3B and 6). We first examined three distal differentiation markers, SftpC, SftpA, and Aquaporin5. The expression levels of these genes were normalized to the level of the housekeeping gene cyclophilin, in which cyclophilin expression level was defined as 100 arbitrary units, as published previously (29) (see “Experimental Procedures” for the calculation formula). Double transgenic lungs showed 79% suppression of SftpC expression (p < 0.0001) and a 94% suppression of SftpA expression (p = 0.011) compared with their littermate controls (Fig. 3B). Although Aquaporin5 expression was not detectable at E16.5 by either in situ hybridization or immunohistochemistry, it was detectable at a low level by real time RT-PCR. Again, double transgenic lungs showed a 63% reduction of Aquaporin5 expression (p = 0.022) (Fig. 3B). Moreover, gαsQ227L expression from E14.5 to E17 also inhibited lamellar body biosynthesis according to TEM studies (Fig. 3, F and G). Since E16.5 is early for detecting mature lamellar bodies, embryos were exposed to doxycycline for an additional half day and harvested at E17. Three single transgenic and three double transgenic lungs were included in TEM analysis. From
epithelial cells labeled by BrdUrd, which was 59.2 ± 4.1% (n = 3) in single transgenic lungs and 56.1 ± 3.2% (n = 4) in double transgenic lungs (p = 0.53) (Fig. 3E). We also found that the single and double transgenic lungs exposed to doxycycline from E14.5 to E16.5 did not show any significant difference in either the number of acinar tubules per ×100 field, 51.7 ± 5.1 (n = 3) versus 50 ± 4.3 (n = 5) (p = 0.64), or the lung weight/body weight ratio, 0.040 ± 0.003 (n = 3) versus 0.040 ± 0.001 (n = 5) (p = 0.96) (Fig. 3H), indicating no defects in branching morphogenesis. Together these data indicated that the transgene did not cause the phenotype at the late pseudoglandular stage by mechanisms that could have limited the proper growth of the distal epithelial compartment, such as inhibiting cell proliferation, hampering branching, or triggering cytotoxicity. Normal expression of several other distal markers, such as Id2 (Fig. 5), Sox9, Gata6, and Abca3 (Table 2) in the double transgenic embryonic lungs further supports this conclusion.

As discussed in the Introduction, loss of distal differentiation in some transgenic models was coupled to complete proximalization of the distal compartment (8, 9). However, results from several experiments indicated that this was not the case in GαsQ227L-expressing embryonic lungs. First, the transgenic distal epithelium remained cuboidal and therefore morphologically distal. Second, there was no evidence of increased Scgb1a1 and Foxj1 expression as assessed by real time PCR (data not shown), which otherwise would indicate an increase in the number of proximal epithelial cells. Last, the normal expression pattern of CC10 and α-smooth muscle actin (a marker of proximal mesenchyme) as assessed by immunohistochemistry (Fig. 4) further argued against fate changes in GαsQ227L-expressing lungs. Therefore, we concluded that the loss of features of distal lung epithelial differentiation was not caused by proximalization of the distal lung epithelial compartment either.

BMP4 and FGF/Evt5 Signaling in the Transgenic Lungs—Since BMP4 signaling plays an essential role in distal fate specification, we interrogated potential changes in BMP4 signaling in the lungs of double transgenic embryos by examining two molecular events that can be activated by BMP4. First, we per-
The real time RT-PCR analysis showed similar levels of gene encoding inhibitory DNA-binding protein 2 (Id2), which is known to be a transcriptional target of BMP4 signaling (36). This assay did not detect any significant loss of Smad1/5/8 phosphorylation in double transgenic lungs (Fig. 5 B). After one additional day of exposure to doxycycline, the expression levels of these genes in double transgenic E18.5 lungs further recovered and were similar to their baseline levels in the control group (p = 0.80, 0.20, and 0.78, respectively) (Fig. 6 C). Consistent with this, doxycycline exposure during the canalicular and the early saccular stages from E16.5 to E18.5 failed to produce inhibitory effect on Shh and Aquaporin5 mRNA expression (p = 0.026, 0.86, and 0.67, respectively; Fig. 6 A). In situ hybridization also showed similar pattern and level of SftpC mRNA expression in the single and double transgenic lungs from this group of embryos (Fig. 6 D and E). Neither the recovery nor the lack of effect at later stages was due to the lack of transgene activation, because in all cases robust levels of GFP-encoding transcript were detected (Fig. 6 A–C). Taken together, all in vivo gene expression data were consistent with the findings observed in the lung bud culture system and strongly supported a stage-specific inhibitory effect of cAMP signaling.

**Expression of GaQ,227L at the Late Pseudoglandular Stage**

**Impairs Alveogenesis**—To assess the long term impact of GaQ,227L expression at the pseudoglandular stage, two litters of embryos were allowed to develop to term after exposure to doxycycline from E14.5 to E16.5. At postnatal day 50, lungs were harvested from these animals and subjected to morpho-

**TABLE 2**

Summary of additional real time PCR data

Expression level is relative, normalized to that of cyclophilin and presented in arbitrary units (see “Experimental Procedures” for calculation formula). S.D. values are shown.

| Gene          | Single transgenic | Double transgenic | p value |
|---------------|-------------------|-------------------|---------|
| Sox9<sup>a</sup> | 1.37 ± 0.58       | 1.59 ± 0.31       | 0.92    |
| Gata6<sup>a</sup> | 0.56 ± 0.17       | 0.74 ± 0.23       | 0.17    |
| Abca3<sup>a</sup> | 2.28 ± 0.86       | 2.04 ± 0.81       | 0.71    |
| Fgf5<sup>a</sup>    | 0.90 ± 0.24       | 0.73 ± 0.24       | 0.32    |
| Fgf4<sup>a</sup>    | 1.22 ± 0.34       | 1.44 ± 0.32       | 0.36    |

* RNA samples from P7 lungs exposed to doxycycline from E14.5 to E16.5. The single and double transgenic groups represent four and five embryos, respectively.

* RNA samples from E16.5 lungs exposed to doxycycline from E14.5 to E16.5. The single and double transgenic groups represent four and five embryos, respectively.

expression was due to altered FGF signaling in distal epithelium.

**Distal Gene Expression Recovered from Early Inhibition at the Canalicular and Saccular Stages**—Although the loss of distal epithelial marker expression at the end of the pseudoglandular stage was quite striking, such a loss was not sustained at later stages even in the presence of continued doxycycline exposure. For example, after doxycycline exposure from E14.5 to 17.5, SftpC, SftpA, and Aquaporin5 mRNA levels in double transgenic lungs were 78, 69, and 75% of the levels detected in their single transgenic littermate controls, respectively (Fig. 6 B). Although these decreases were all statistically significant (p = 0.0063, 0.043, and 0.047, respectively), the magnitude of these changes was much lower when compared with those 1 day earlier at E16.5 (Fig. 5 B). After one additional day of exposure to doxycycline, the expression levels of these genes in double transgenic E18.5 lungs further recovered and were similar to their baseline levels in the control group (p = 0.80, 0.20, and 0.78, respectively) (Fig. 6 C). Consistent with this, doxycycline exposure during the canalicular and the early saccular stages from E16.5 to E18.5 failed to produce inhibitory effect on Shh and Aquaporin5 mRNA expression (p = 0.026, 0.86, and 0.67, respectively; Fig. 6 A). In situ hybridization also showed similar pattern and level of SftpC mRNA expression in the single and double transgenic lungs from this group of embryos (Fig. 6 D and E). Neither the recovery nor the lack of effect at later stages was due to the lack of transgene activation, because in all cases robust levels of GFP-encoding transcript were detected (Fig. 6 A–C). Taken together, all in vivo gene expression data were consistent with the findings observed in the lung bud culture system and strongly supported a stage-specific inhibitory effect of cAMP signaling.

**FIGURE 4.** GaQ,227L expression does not alter distal-proximal polarity. Genotypes are indicated in each panel. A and B, immunohistochemical analysis of CC10 expression after Dox exposure from E14.5 to E17.5. C and D, in situ hybridization also showed similar pattern and level of SftpC mRNA expression in the single and double transgenic lungs from this group of embryos (Fig. 6 D and E). Neither the recovery nor the lack of effect at later stages was due to the lack of transgene activation, because in all cases robust levels of GFP-encoding transcript were detected (Fig. 6 A–C). Taken together, all in vivo gene expression data were consistent with the findings observed in the lung bud culture system and strongly supported a stage-specific inhibitory effect of cAMP signaling.

**FIGURE 5.** GaQ,227L expression does not alter downstream BMP4 and FGF signaling. A, real time RT-PCR analysis of Id2, Etv5, and Shh expression in single (open bar, n = 4) and double (filled bar, n = 4) transgenic lungs exposed to doxycycline from E14.5 to E16.5. B, Western blot analysis of Smad1/5/8 phosphorylation in lungs exposed to doxycycline from E13.5 to E15.5.

formed Western blot analysis of lung homogenates from embryos exposed to doxycycline from E14.5 to E16.5 for Smad1/5/8 phosphorylation (35). This assay did not detect any significant loss of Smad1/5/8 phosphorylation in double transgenic lungs (Fig. 5 B). Second, we examined expression of the gene encoding inhibitory DNA-binding protein 2 (Id2), which is known to be a transcriptional target of BMP4 signaling (36). The real time RT-PCR analysis showed similar levels of Id2 mRNA expression between single and double transgenic littermate controls (p = 0.25, Fig. 5 A). Taken together, these data indicated that cAMP did not delay distal epithelial differentiation via an effect on BMP4 signaling.

Since Etv5/Pea3 acting downstream of FGF is also believed to be important for distal lung epithelial development (16), we performed real time RT-PCR to monitor Etv5 expression. We did not detect a significant decrease in Etv5 RNA level compared with controls (p = 0.19, Fig. 5 A). In addition, another downstream target of FGF signaling, Shh (3), showed no significant loss of mRNA expression either (p = 0.36, Fig. 5 B). Therefore, it is unlikely that the phenotype induced by GaQ,227L expression was due to altered FGF signaling in distal epithelium.

**cAMP Signaling in Lung Development**
double transgenic lungs at postnatal day 7 (Table 2), and elastin staining also appeared to be normal (data not shown). Defective alveogenesis may also occur as a result of improper alveolar myofibroblast development (39). These α-SMA-expressing cells are located at the tip of septation during alveogenesis and are thought to play an essential role in the initiation of secondary septa. We performed anti-α-SMA immunohistochemistry to quantify the number of alveolar myofibroblasts in postnatal day 7 lungs, which was significantly decreased from 24.2 ± 2.9 in single transgenic lungs (n = 4) to 16.2 ± 3.2 in double transgenic lungs (n = 4) (p < 0.0001) (Fig. 7, J–L). Consistent with this finding, we also found that after doxycycline exposure from E14.5 to E16.5, double transgenic lungs developed fewer PDGF-positive cells (p < 0.0001) (Fig. 7, G–I) at E16.5, which, at this stage of development, are considered to be the progenitor cells for alveolar myofibroblasts (40). The number of cells per ×200 high power field was 65.3 ± 8.5 in single transgenic lungs (n = 3) versus 4.14 ± 7.3 (n = 4) in double transgenic lungs. Since short-term transgene activation did not alter cellular proliferation and branching morphogenesis (Fig. 3) and there was no obvious evidence of an increase in inflammatory cells on the HE staining of either P7 (not shown) or P50 (Fig. 7) lungs, defective alveolar myofibroblast development was likely to be the primary cause of failed alveogenesis.

Expression of GαsQ227L at the Early Pseudoglandular Stage

Impairs Branching and Cellular Proliferation—We have shown earlier that pharmacological activation of cAMP signaling hampered branching in lung bud culture. However, in vivo activation of cAMP signaling by GαsQ227L at the late pseudoglandular stage did not show such an effect. We suspect that this difference was due to stage-specific sensitivity of branching morphogenesis to cAMP signaling. To test this, we exposed embryos to doxycycline from E11.5 to E15.5. Double transgenic lungs at E15.5 showed a significant decrease in the number of distal tubules per ×100 high power field (Fig. 8C), which was 46.6 ± 2.6 (n = 4) in double transgenic lungs versus 63.0 ± 3.2
cAMP Signaling in Lung Development

**FIGURE 7.** G_{o,Q227L} expression at the late pseudoglandular stage causes abnormal alveolar smooth muscle cell development and defective alveogenesis. In C, F, I, and L, the open bars represent single transgenic animals, and the filled bars represent double transgenic animals. A–C, representative hematoxylin/eosin staining (A and B) and mean cord length value (C) of 50-day-old single (A) and double (B) transgenic lungs exposed to doxycycline from E14.5 to E16.5. D–F, representative hematoxylin/eosin staining (D and E) and mean cord length value (F) of 50-day-old single (D) or double (E) transgenic lungs exposed to doxycycline from E16.5 to E18.5. Note exposure at the pseudoglandular stage but not in the canalicular and early saccular stages can cause permanent airspace enlargement. G–I, immunohistochemical analysis showed decreased number of PDGFRα-positive alveolar myofibroblast cells in double transgenic lungs (H) compared with single transgenic controls (G) after exposure to doxycycline from E14.5 to E16.5. J–L, immunohistochemical analysis showed a decreased number of α-SMA-positive alveolar myofibroblast cells (arrowheads) in double transgenic lungs (K) compared with single transgenic controls (J) at postnatal day 7 after prenatal doxycycline exposure from E14.5 to E16.5.

To evaluate the long term effect of G_{o,Q227L} expression at the early pseudoglandular stage, two litters of embryos exposed to doxycycline from E11.5 to E15.5 were allowed to develop to term and were harvested at postnatal day 50. Airspace enlargement was also evident and appeared even more severe in the double transgenic group (data not shown). Although the double transgenic animals in this experimental group were also present in normal Mendelian ratio (8 of 16) at the time of weaning, three double transgenic animals subsequently died before reaching adulthood. All remaining five double transgenic animals developed fluid-filled cystic structures at the pleural surface of their lungs at the time when they were harvested. These cysts can be easily identified on an hematoxylin/eosin-stained section (asterisk in Fig. 8B), and some of them were also located inside the lobe (data not shown). We speculate that the three double transgenic animals that did not survive could have died from pneumothorax resulting from severe forms of cystic malformations.

**DISCUSSION**

Knowledge of pathways that promote distal lung epithelial maturation is required both to understand normal lung development and to develop therapeutic strategies for both neonatal and adult lung disease. Promoting lung maturity and surfactant biosynthesis is particularly important in neonatal patients with bronchopulmonary dysplasia (BPD), a pathological condition primarily driven by lung immaturity combined with environmental insults, such as hyperoxia, mechanical ventilation-induced lung injury, and infection (41, 42). Our current clinical approach mainly relies on the use of glucocorticoids to promote lung maturation. Although the use of glucocorticoids can reduce the incidence of RDS by 50%, there remain additional patients who do not benefit, and glucocorticoids also present several unwanted side effects, such as neurotoxicity and defective alveogenesis (41, 43). To enhance lung maturation, several candidate pathways have been investigated, including the VEGF, retinoic acid, nitric oxide, and cAMP pathways.

Unlike the glucocorticoid, VEGF, and retinoic acid pathways, the concept that enhanced cAMP signaling in respiratory epi-

(n = 3) in single transgenic lungs (p < 0.001). This indicated that cAMP signaling could inhibit branching morphogenesis in vivo if activated at the early pseudoglandular stage. We also noticed a decrease in the percentage of distal epithelial cells labeled by BrdUrd (Fig. 8D): from 58.3 ± 4.5% in single transgenic lungs (n = 3) to 44.0 ± 3.7% in double transgenic lungs (n = 4) (p = 0.006). This is in clear contrast to the lack of effect of G_{o,Q227L} on cell proliferation at the late pseudoglandular stage. Consistent with the changes in branching and cellular proliferation, doxycycline exposure also caused a decrease in lung weight/body weight ratio (Fig. 8C), which was 0.030 ± 0.002 in the double transgenic group (n = 4) compared with 0.040 ± 0.002 in the single transgenic group (n = 3) (p = 0.001).
cAMP Signaling in Lung Development

thelium may promote lung maturation is primarily supported by in vitro observations (11, 23–26). The transgenic system we developed allowed us to investigate this concept in vivo. Since the current system allows activation of cAMP signaling in a distal epithelial-specific fashion, it is more specific than the previously used pharmacological methods for promoting cAMP signaling in vivo. The lack of substantial activation of Sftpc, Sftpa, Aquaporin5, and Abca3 expression at the canalicular and sacular stages in transgenic lungs with induced cAMP signaling argues against a physiological role for cAMP in promoting distal epithelial maturation.

Given these findings, how do we reconcile the difference between observations in vivo versus in vitro? CAMP is known to promote cell adhesion and polarity (44, 45), two cellular features that are often compromised or lost under in vitro conditions. Therefore, one possible mechanism by which cAMP enhances differentiation in vitro is to promote cell adhesion and/or polarity. If cell adhesion and polarity are maintained in vivo by pathways other than cAMP, ectopic cAMP is not required to influence distal differentiation in vivo. Despite the fact that we were unable to detect a positive contribution of cAMP signaling in normal lung development, cAMP may help to restore distal lung epithelial function in disease states, particularly in those with cell injury that is associated with loss of adhesion or polarity. The transgenic model developed in the current study certainly presents a unique opportunity to test the role of cAMP signaling in acute lung injury in future investigations.

The fact that enhanced cAMP signaling only suppresses differentiation at the pseudoglandular stage, but not at the canalicular and early saccular stages, suggests that some regulators of the early phase of differentiation have pseudoglandular stage-specific roles. In other words, the transcriptional regulation of distal markers may employ different mechanisms at different developmental stages. We also provided evidence that the cAMP-sensitive early regulators are not in the known pathways for distal differentiation, such as BMP4 and FGF signaling pathways. Although we did not provide a molecular analysis of β-catenin or GATA6 signaling, the phenotype caused by GaqQ227L does not resemble those caused by deficit in either β-catenin or GATA6 signaling. For example, an epithelium-specific β-catenin deficit caused proximalization of the distal epithelial fate (9), which was not observed in GaqQ227L-expressing lungs. Suppression of GATA6 function led to substantial loss of Aquaporin5 expression at E18.5 (18), whereas in GaqQ227L-expressing lungs Aquaporin5 expression was recovered by E18.5 following early suppression. N-myc is another transcription factor known to be involved in distal epithelial development (46). Loss of N-myc function causes a drastic loss of Sox9 expression (46). Since Sox9 expression was normal in GaqQ227L-expressing lungs (Table 2), the phenotype is unlikely linked to changes in N-myc function either. Therefore, based on molecular and phenotypic comparisons, we postulate the existence of yet-to-be-identified cAMP-sensitive novel pathway of early distal epithelial development.

Epithelium-specific GaqQ227L expression at the end of pseudoglandular stage had an indirect inhibitory effect on myofibroblast progenitor lineage development, causing a reduced production of alveolar myofibroblasts, consequently decreased postnatal secondary septation, and eventually airspace enlargement. This observation suggests that differentiating distal lung epithelium at the end of the pseudoglandular stage provides a paracrine signal that promotes myofibroblast development. And production of this paracrine signal is sensitive to the inhibition by cAMP signaling.

Findings in the current report also indicated that developmental defects occurred during early lung development could have a long term structure consequence without causing mortality or morbidity by early adulthood. One may speculate that if similar defects take place in human subjects, then they may be unnoticed and undiagnosed until later in life, when injury or diminished reserve capacity unmasks subtle abnormalities in lung development. Therefore, we believe that the potential etiologic link between subtle developmental defects and a subset of adult pulmonary diseases is worth future investigation in human subjects.

Acknowledgments—We thank Ron McCarthy (Washington University Transgenic Core Facility) for generating the transgenic lines, Louise M. Trakimas (Harvard Cell Biology Core Facility) for EM analysis, Yolanda Porrata for anti-phospho-CREB immunohistochemistry, and Ruiyang Tian for performing some of the real time PCR assays. We thank Caroline Owen for critical reading of the manuscript.

REFERENCES

1. Ten Have-Opbroek, A. A. (1991) Exp. Lung Res. 17, 111–130
2. Warburton, D., Schwarz, M., Tefft, D., Flores-Delgado, G., Anderson, K. D., and Cardoso, W. V. (2000) Mech. Dev. 92, 55–81
cAMP Signaling in Lung Development

3. Lebecche, D., Malpel, S., and Cardoso, W. V. (1999) *Mech. Dev.* **86**, 125–136
4. Bellusci, S., Furuta, Y., Rush, M. G., Henderson, R., Winnier, G., and Hogan, B. L. (1997) *Development* **124**, 53–63
5. Bellusci, S., Grindley, J., Emoto, H., Itoh, N., and Hogan, B. L. (1997) *Development* **124**, 4867–4878
6. Chuang, P. T., and McMahon, A. P. (2003) *Trends Cell Biol.* **13**, 86–91
7. Liu, Y., and Hogan, B. L. (2002) *Gene Expr. Patterns* **2**, 229–233
8. Weaver, M., Yingling, J. M., Dunn, N. R., Bellusci, S., and Hogan, B. L. (1999) *Development* **126**, 4005–4015
9. Mucenski, M. L., Wert, S. E., Nation, J. M., Lody, D. E., Huelens, J., Birchmeier, W., Morrissey, E. E., and Whitsett, J. A. (2005) *J. Biol. Chem.* **280**, 40231–40238
10. Shu, W., Guttentag, S., Wang, Z., Andl, T., Ballard, P., Lu, M. M., Piccolo, S., Birchmeier, W., Whitsett, J. A., Millar, S. E., and Morrissey, E. E. (2005) *Dev. Biol.* **283**, 226–239
11. Shannon, J. M., Gebb, S. A., and Nielsen, L. D. (1999) *Development* **126**, 1675–1688
12. Alescio, T., and Dani, A. M. (1972) *J. Embryol. Exp. Morphol.* **27**, 155–162
13. Stahlman, M. T., Gray, M. P., Falconieri, M. W., Whitsett, J. A., and Alescio, T., and Dani, A. M. (1972) *J. Embryol. Exp. Morphol.* **27**, 124, 53–63
14. Wan, H., Dingle, S., Xu, Y., Besnard, V., Kaestner, K. H., Ang, S. L., Wert, S., Stahlman, M. T., and Whitsett, J. A. (2005) *J. Biol. Chem.* **280**, 13809–13816
15. DeFelice, M., Silberschmidt, D., DiLauro, R., Xu, Y., Wert, S. E., Weaver, T. E., Bachurski, C. J., Clark, J. C., and Whitsett, J. A. (2003) *J. Biol. Chem.* **278**, 35574–35583
16. Liu, Y., Jiang, H., Crawford, H. C., and Hogan, B. L. (2003) *Dev. Biol.* **261**, 10–24
17. Liu, C., Morrisey, E. E., and Whitsett, J. A. (2002) *Am. J. Physiol.* **283**, 1468–1475
18. Yang, H., Lu, M. M., Zhang, L., Whitsett, J. A., and Morrisey, E. E. (2002) *Development* **129**, 2233–2246
19. Compernolle, V., Brusselmann, K., Acker, T., Hoet, P., Tjwa, M., Beck, H., Plaisance, S., Dor, Y., Keshet, E., Lupu, F., Nemery, B., Dewerchin, M., Van Veldhoven, P., Plate, K., Moons, L., Collen, D., and Carmeliet, P. (2002) *Nat. Med.* **8**, 702–710
20. Besnard, V., Wert, S. E., Kaestner, K. H., and Whitsett, J. A. (2005) *Am. J. Physiol.* **289**, L750–L759
21. Muglia, L. J., Bae, D. S., Brown, T. T., Vogt, S. K., Alvarez, J. G., Sunday, M. E., and Majzoub, J. A. (1999) *Am. J. Respir. Cell Mol. Biol.* **20**, 181–188
22. Cole, T. J., Solomon, N. M., Van Driel, R., Monk, J. A., Bird, D., Richardson, S. J., Dilley, R. J., and Hooper, S. B. (2004) *Am. J. Respir. Cell Mol. Biol.* **30**, 613–619
23. Alcorn, J. L., Smith, M. E., Smith, J. F., Margraf, L. R., and Mendelson, C. R. (1997) *Am. J. Respir. Cell Mol. Biol.* **17**, 672–682
24. Bates, S. R., Gonzales, L. W., Tao, J. Q., Rueckert, P., Ballard, P. L., and Fisher, A. B. (2002) *Am. J. Physiol.* **282**, L267–L276
25. Li, J., Gao, E., and Mendelson, C. R. (1998) *J. Biol. Chem.* **273**, 4592–4600
26. Liu, C., and Whitsett, J. A. (1997) *J. Biol. Chem.* **272**, 17327–17332
27. Tichelaar, J. W., Lu, W., and Whitsett, J. A. (2000) *J. Biol. Chem.* **275**, 11858–11864
28. Xu, J., Liu, Z., and Ornitz, D. M. (2000) *Development* **127**, 1833–1843
29. Xu, J., Tian, J., and Shapiro, S. D. (2005) *Am. J. Respir. Cell Mol. Biol.* **32**, 381–387
30. Houghton, A. M., Quintero, P. A., Perkins, D. L., Kobayashi, D. K., Kelley, D. G., Marconcini, L. A., Mecham, R. P., Senior, R. M., and Shapiro, S. D. (2006) *J. Clin. Invest.* **116**, 753–759
31. Hoang, T., Fenne, I. S., Cook, C., Borud, B., Bakke, M., Lien, E. A., and Mellgren, G. (2004) *J. Biol. Chem.* **279**, 49120–49130
32. Pearson, G. W., Earnest, S., and Cobb, M. H. (2006) *Mol. Cell. Biol.* **26**, 9039–9047
33. Lania, A., Mantovani, G., and Spada, A. (2001) *Eur. J. Endocrinol.* **145**, 543–559
34. Ray, P., Tang, W., Wang, P., Homer, R., Kuhn, C., Ill, Flavell, R. A., and Elias, J. A. (1997) *J. Clin. Invest.* **100**, 2501–2511
35. Liu, F., Hata, A., Baker, J. C., Doyley, J., Carcamo, J., Harland, R. M., and Massague, J. (1996) *Nature* **381**, 620–623
36. Miyazono, K., and Miyazawa, K. (2002) *Sci. STKE* **2002**, PE40
37. Weinstein, M., Xu, X., Ohyama, K., and Deng, C. X. (1998) *Development* **125**, 3615–3623
38. Yanagisawa, H., Davis, E. C., Starcher, B. C., Ouchi, T., Yanagisawa, M., Richardson, J. A., and Olson, E. N. (2002) *Nature* **415**, 168–171
39. Bostrom, H., Willetts, K., Pekna, M., Leveen, P., Lindahl, P., Hedstrand, H., Pekna, M., Hellstrom, M., Gebre-Medhin, S., Schalling, M., Nilsson, M., Kurland, S., Tornell, J., Heath, J. K., and Betsholtz, C. (1996) *Cell* **85**, 863–873
40. Lindahl, P., Karlsson, L., Hellstrom, M., Gebre-Medhin, S., Willetts, K., Heath, J. K., and Betsholtz, C. (1997) *Development* **124**, 3943–3953
41. Jobj, A. H., and Ileogmai, M. (2000) *Annu. Rev. Physiol.* **62**, 825–846
42. Jobj, A. H., and Ileogmai, M. (2001) *Curr. Opin. Pediatr.* **13**, 124–129
43. Griep, D. G., and Halliday, H. L. (2004) *Treat. Respir. Med.* **3**, 295–306
44. Porter, S. E., Dwyer-Nield, L. D., and Malkinson, A. M. (2001) *Am. J. Physiol.* **280**, L1282–L1289
45. Nishiyama, M., Hoshino, A., Tsai, L., Henley, J. R., Goshima, Y., Tessier-Lavigne, M., Poo, M. M., and Hong, K. (2003) *Nature* **423**, 990–995
46. Okubo, T., Knoepfle, P. S., Eisenman, R. N., and Hogan, B. L. (2005) *Development* **132**, 1363–1374