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Up-Regulation of Fetal Rat Lung Parathyroid Hormone-Related Protein Gene Regulatory Network Down-Regulates the Sonic Hedgehog/Wnt/βcatenin Gene Regulatory Network

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ABSTRACT: Lung development depends on endodermal Sonic Hedgehog (Shh) signaling to mesodermal Wingless/int/beta catenin (Wnt/βcatenin), followed by parathyroid hormone–related protein (PTHrP) signaling from endoderm to mesoderm. Fluid distension of fetal rat lung explants up-regulates PTHrP signaling and down-regulates Shh/Wnt/βcatenin signaling, marked by decreases in Patched, Gli, Frizzled, and Dishevelled, inducing fibroblast triglyceride uptake, type II cell saturated phosphatidylcholine, and surfactant protein-B expression. Bumetanide, which inhibits fluid distension, blocked down-regulation of the Shh/Wnt/βcatenin pathway and up-regulation of the PTHrP pathway, whereas PTHrP (1–34, 5×10^{-7} M) treatment overcame bumetanide inhibition, and the PTHrP receptor antagonist PTHrP (7–34) amide (5×10^{-6} M) mimicked bumetanide, indicating that PTHrP signaling mediates fluid distension-induced alveolar differentiation. Fetal rat lung explant automaturation was characterized by decreased Wnt/βcatenin signaling and increased PTHrP/PTHrP receptor signaling, up-regulating fibroblast-specific adipocyte differentiation related protein (ADRP) and peroxisome proliferator–activated receptor gamma. Wnt/βcatenin agonists (LiCl or SB415268) maintained Shh/Wnt/βcatenin signaling, blocking spontaneous up-regulation of the PTHrP pathway, whereas PTHrP or cAMP down-regulated Shh/Wnt/βcatenin signaling and stimulated PTHrP signaling for fibroblast and type II cell differentiation. This is the first evidence that alveolar fluid distension is an organizing principle for PTHrP signaling down-regulation of the Shh/Wnt/βcatenin pathway. **(*Pediatr Res* 60: 382–388, 2006)**

Lung development is characterized by sequential endodermal Shh signaling to the mesodermal Wnt/βcatenin pathway (1), followed by PTHrP endodermal-to-mesodermal signaling that culminates in mature AIF and type II cells (2–4). The formation of the alveolar bed during the canalicular and saccular phases of lung development is determined by genetic and epigenetic GRN (5). The plasticity of the alveolar interstitium derives from both the fibroblasts, which are not terminally differentiated (6), and the epithelial cells, which differentiate into type II cells (7) and can differentiate into type-I cells (8). These phenotypes are affected by various extrinsic factors, including stretch (9), endocrine hormones (10,11), cytokines (3), oxygen (12,13), nutrition (14), nicotine (15), and inflammation (16).

PTHrP is a stretch-regulated paracrine factor expressed by type II cells that is necessary for the formation of alveoli (17). It signals neighboring mesodermal fibroblasts to differentiate by stimulating cAMP (18). The cAMP-dependent PKA pathway stimulates AIF differentiation by up-regulating PPARγ (15) and the expression of the downstream phenotypic genes for fibroblast and type II cell differentiation. Decreased PTHrP signaling that occurs due to prematurity (19), lung injury (15,20), maternal smoking (21), or infection (S. Dargan-Batra *et al.*, American Thoracic Society, May 20–25, San Diego, CA) can lead to myofibroblast transdifferentiation (20,22). AIF phenotypes are critical for alveolarization (20). Proliferation of alveolar myofibroblasts can lead to either the formation of alveolar septa (22) or to granulomatous scar tissue (23), depending on the cellular environment (20).

We wanted to test the paracrine interaction between the Shh/Wnt/βcatenin and PTHrP signaling pathways in an intact model of lung cell development that would mature without extrinsic factors, under the influence of fluid distension generated actively by the lung, not passively and nonphysiologically by ligation, which does not faithfully reproduce the "normal" lung phenotype (24). Therefore, we used bumetanide, a chloride channel blocker that inhibits lung fluid formation (25) to test this hypothesis in explant culture.

To determine whether PTHrP GRN up-regulation is necessary for down-regulation of the Wnt/βcatenin GRN, we chose to study automaturing fetal lung explants in serum- and hormone-free culture (26), as depicted schematically in Figure 1 by steps 1–14.

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Abbreviations: AIF, alveolar interstitial fibroblasts; **Gli,** Glioblastoma; **GRN,** gene regulatory networks; **PKA,** protein kinase A; **PPARγ,** peroxisome proliferator–activated receptor gamma; **Ptc,** Patched; **PTHrP,** parathyroid hormone–related protein; **Shh,** Sonic Hedgehog; **SP-B,** surfactant protein-B; **Wnt/βcatenin,** Wingless/int/beta catenin

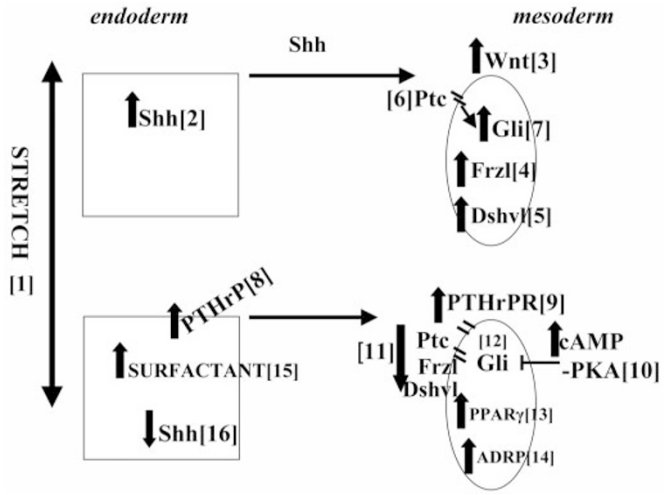

Figure 1. Schematic for automaturation of the alveolar acinus (with references to literature citations). During early embryonic lung development (*upper panel*) endodermal Shh (2) signals to the mesodermal Wnt/Ptc/Gli pathway (3–7). Maturation of the interstitium is driven by alveolar fluid distension (1), which up-regulates the PTHrP signaling pathway between the endoderm and mesoderm (8–16), down-regulating the Wnt pathway by inhibiting Gli (12) and up-regulating PPARγ (13) and ADRP (14). Differentiation of the lipofibroblast stimulates differentiation of alveolar type II cell surfactant synthesis (15) and inhibition of Shh (16) expression.

MATERIALS AND METHODS

Animals. Time-mated Sprague-Dawley rats (e0 = day of mating) were obtained from Charles River Breeders (Holister, CA). The experiments described were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by the Los Angeles Biomedical Research Institute Animal Care Committee.

Reagents. Rp-cAMPS, a specific PKA inhibitor, SB-415286, a sensitive and specific GSK3β inhibitor (which stimulates the Wnt/βcatenin pathway), lithium chloride, and 8-bromo-cAMP were purchased from Sigma Chemical Co. (St. Louis, MO).

Explant culture. Lung tissue was cultured as previously described (27). Explants derived from three to five litters of rats were used for each experiment during the course of these studies.

Competitive RT-PCR. Total cellular RNA was isolated as previously described (15). The appropriate cDNA fragments were amplified using 400 ng of total RNA from lung explants, avian myeloblastosis virus reverse transcriptase, and random hexamers and deoxyribonucleotides. The following primers and conditions have previously been used in our laboratory for the RT-PCR assays (3,4,9,15): PTHrP (sense, 5′TGGACACCAGCATCTACGTCAG; antisense, 3′GACATGGAGTATCCCACGGTGT); PTHrP receptor (sense, 5′CCTCTTTGGCGTCCACTACATTG; antisense, 3′TTGAGGAACCCATCGTCCTTG; ADRP (sense, 5′ACAGCTCACTTATGGTCGTGCC; antisense, 3′AAACAGTGATGAAGCCTGCTCG); SP-B, (sense, 5′TACACAGTACTTCTACTAGATG; antisense, 3′ATAGGCTGTTCACTGGTGTTCC); PPAR((sense, 5′ATGCTTTATCCCCACAGACTC; antisense, 3′GTTGACACAGAGATGCCATTC. The following primers and conditions were used for the indicated RNA determinations: Shh (sense, 5′CACAAGAAACTCCGAACGATT; antisense, 3′ATGCGAGCTTTGGATTCATAG); Ptc (sense, 5′AAACAGGCATAGGCAAGCATC; antisense, 3′TTGGCAGGAGGAGTTGATTGT); Gli (sense, 5′GACTTCCGACAGCCTTCAAAC; antisense, 3′GGACATGTCTAGCCCCAACTC); Frizzled 1 (sense, 5′CAGTTCACTTCCGACAAAGG; antisense, 5′AGGTAGGAAGGCACCCTGAG); Frizzled 3 (sense, 5′ TTTTCCATGGGCGTAGGA; antisense, 5′TAACACGGTTCATGCTGGTG); Frizzled 4 (sense, 5′ACAACCACATGTGCATGGAA; antisense, 5′TCCTTAGCTGAGCGGCTGTA); Frizzled 5 (sense, 5′GACGCCGAGGTTCTGTGTAT; antisense, 5′TGCGCACCTTGTTGTAGAGT); Frizzled 6 (sense, 5′CCCTCGTAAGAGGACACAGC; antisense, 5′TTGCAAGATGCAGAAAGTGC); Disheveled 1 (sense, 5′GGGGGTAGTGGCAGTGAA; antisense, 5′ACCTGTAAGTTCTGGAGGGACA); Dishevelled 2 (sense, 5′GCAGTGGCAGTGAGTCAGAA; antisense, 5′TCATGGGGTTATAGGGGAGAG); Dishevelled 3 (sense, 5′CAAGGAGAAGGACCCAAAAG; antisense, 5′ATCGGGGGACCATAGAGAG); 18s (sense, 5′TTAAGCCATGCATGTCTAAGTAC; antisense, 3′TGTTATTTTTCGTCACTACCTCC). cDNA was synthesized from 1 μg total RNA by RT using 100 U Superscript reverse transcriptase II (Invitrogen, Carlsbad, CA) and random primers (Invitrogen) in a 20 μL reaction containing 1× Superscript buffer (Invitrogen), 1 mM deoxy-NTP mix, 10 mM DTT, and 40 U ribonuclease inhibitor. Total RNA and random primers were incubated at 65°C for 5 min followed by 42°C for 50 min. A denaturing enzyme at 70°C for 15 min terminated the reaction. For PCR amplification, 1 μL cDNA was added to 25 μL of a reaction mix containing 0.2 μM of each primer, 0.2 mM deoxy-NTP mix, 0.5U AccuPrime *Taq* DNA Polymerase (Invitrogen), and 1× reaction buffer. PCR was performed in a RoboCycler (Stratagene, La Jolla, CA). The PCR temperature between 50° and 58°C and cycle number between 27 and 38 were selected for optimal absorbance OD for each individual marker studied. The PCR products were visualized on 2% agarose gels by ethidium bromide staining, and gels were photographed under UV lights. Bands densities were quantified using the Eagle Eye II System (Stratagene). The mRNA levels were normalized to 18s mRNA levels.

Triolein uptake assay. Triolein uptake assay was performed as previously described (14).

Choline incorporation assay. Choline incorporation assay was performed as previously described (2).

Statistical analyses. Experiments were repeated three or more times to confirm the validity of observations. The significance of differences within groups was determined by one-way ANOVA with Newman-Keuls posthoc test. Statistical analyses of the relative levels of specific mRNA were performed after arc sine transformation of the ratio data. The null hypothesis was rejected when $p < 0.05$ was obtained.

RESULTS

Effects of inhibitors of fluid distension or Wnt signaling on PTHrP-mediated fetal rat lung explant automaturation. We cultured e14 fetal rat lung tissue in explant culture for up to 5 d to determine whether fluid distension induced the fibroblast lipogenic phenotype, characterized by triglyceride uptake. Triglyceride uptake increased more after 5 d in culture (Fig. 2), a lipofibroblast-specific mechanism mediated by PTHrP up-regulation of ADRP. This effect was blocked by incubation of the explants with bumetanide (2×10^{-4} M), an inhibitor of fluid secretion by fetal lung explants. To determine whether PTHrP mediated this effect, we co-incubated the explants with bumetanide and PTHrP (5×10^{-5} M, 5×10^{-7}

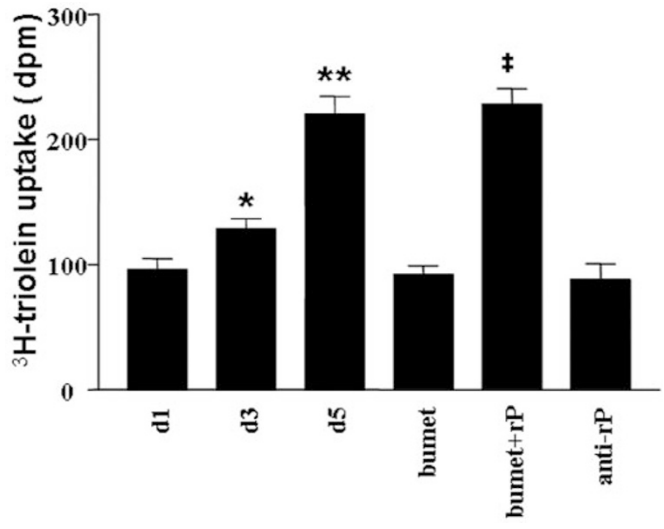

Figure 2. Fluid distension causes PTHrP-mediated lung maturation *in vitro*. Fetal rat lung explants were treated with bumetanide (*bumet*, 2×10^{-4} M), PTHrP (*rP*, 5×10^{-7} M), PTHrP (7–34) amide (*anti-rP*, 5×10^{-6} M), as indicated, from d 0 to 5 in culture. On d 1, 3, and 5, the explants were incubated with ^{3}H-triolein. Each bar represents the mean ± SD, $n = 5$. *$p <$ 0.05, **$p < 0.01$, d 3, d 5 *vs* d1; ‡$p < 0.0001$, bumet *vs* bumet + rP, by one-way ANOVA.

M, respectively), and found that PTHrP restored the bumetanide-inhibition of triglyceride uptake; incubation of the explants with the specific PTHrP receptor antagonist PTHrP (7–34) amide (5×10^{-6} M) blocked the increase in triglyceride uptake, verifying that endogenous PTHrP mediated the increase in triglyceride uptake by the maturing explants. Bumetanide did not affect basal triglyceride uptake, or PTHrP and PTHrP receptor mRNA levels, indicating that it specifically blocked fluid distension without affecting PTHrP signaling itself. Incubation of the explants with the GSK-3β inhibitor SB415286 (5×10^{-7} M), a Wnt pathway agonist, also blocked the spontaneous increase in triglyceride uptake, an effect overridden by co-incubation with PTHrP (1–34) (5×10^{-7} M), suggesting that PTHrP stimulates fibroblast differentiation by down-regulating the Wnt pathway. To test this hypothetical mechanism we performed the following series of experiments.

Effect of Wnt inhibitors on fibroblast and type II cell maturation in PTHrP-dependent automaturing fetal rat lung explant cultures. First, we determined the mRNA levels of Wnt signaling genes during automaturation of the fetal rat lung cultures. As can be seen in the left panel of Figure 3, Frizzled 1, 3, 4, and 5 mRNA levels markedly decreased (50, 86, 37, 76%, respectively) between e14 and e19, whereas Frizzled 6 increased by 44% during this same period; and, as can be seen in the right panel of Figure 3, Disheveled 2 and 3 mRNA levels decreased by 30 and 35%, respectively, whereas Disheveled 1 increased 109%. We then examined the mRNA levels of PTHrP and its signaling genes for fibroblast maturation, namely the PTHrP receptor, ADRP, and PPARγ, during the process of lung maturation *in vitro* (Fig. 4). We observed a progressive increase in the expression of PTHrP during the 5-d culture period paralleled by concomitant increases in the expression of the PTHrP receptor, ADRP, and PPARγ. We then probed for the expression of SP-B and Shh as functional markers for type II cell differentiation and probed for the fibroblast Shh/Wnt pathway-regulated genes, Gli and Ptc. There was a progressive increase in SP-B expression over the 5-d incubation period (Fig. 5), indicative of type II cell maturation, and a concomitant, progressive decrease in the expression of Shh, Ptc, and Gli.

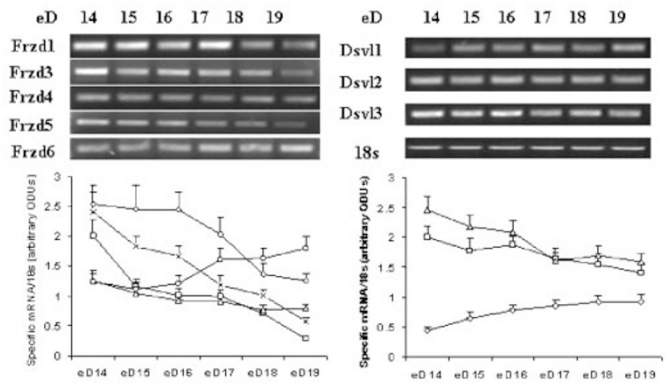

Figure 3. Fetal rat lung explant automaturation down-regulates Wnt pathway signaling. Fetal rat lung explants were cultured for up to 5 d and were harvested daily from d 0 to 5, equal to e14–19 *ex vivo*. RT-PCR of Frizzled and Dishevelled was used to monitor the pattern of Wnt signaling. Frizzled 1 (–◇–), 3 (–□–), 4 (–△–), 5 (–×–), 6 (–○–); Disheveled 1 (–◇–), 2 (–□–), 3 (–△–). Values are means ± SD, $n = 5$.

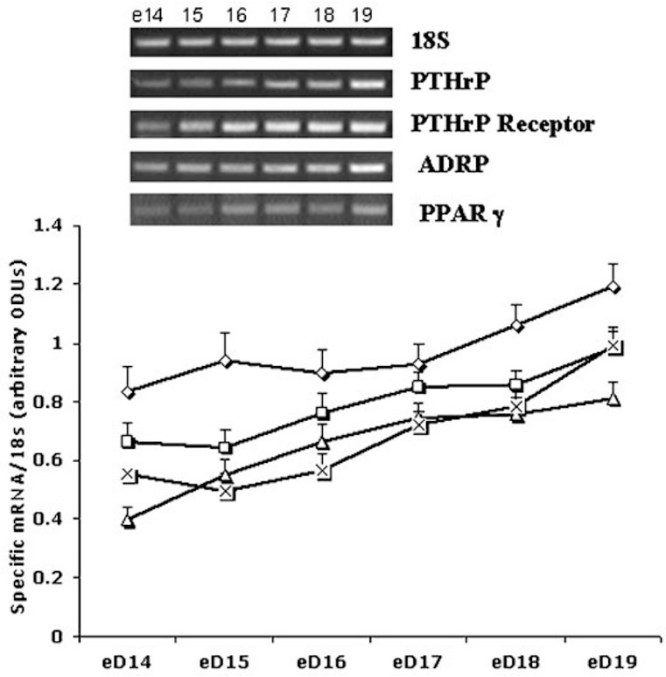

Figure 4. Fetal rat lung explant automaturation coordinately up-regulates PTHrP signaling between type II cells and fibroblasts. Fetal rat lung explants were cultured for up to 5 d and were harvested daily from d 0 to 5, equal to e14–19 *ex vivo*. RT-PCR of PTHrP expression was used to monitor type II cell differentiation; RT-PCR of PTHrP receptor, ADRP, and PPARγ was used to monitor maturation of PTHrP signaling to fibroblasts. Note the patterns of coordinate up-regulation of PTHrP (–×–) in type II cells with up-regulation of PTHrP receptor (–△–), ADRP (–◇–), and PPARγ (–□–) in fibroblasts (see graph below representative gel). Values are means ± SD, $n = 5$.

We subsequently determined the effects of LiCl and SB415286, which stimulate the Wnt pathway by inhibiting GSK-3β, on the expression of Shh, which regulates the Shh pathway intermediates Gli and Ptc (Fig. 6). Note the dose-dependent effect of graded amounts of LiCl (2 and 5 mM) and SB415286 (10, 20, and 50 μM), on the incremental increases in Shh, Gli, and Ptc. Treatment of the lung explants with 8-bromo-cAMP (5×10^{-5} M), a PTHrP/PKA pathway agonist, completely inhibited the expression of these Shh pathway-regulatory genes.

We then determined the effects of the Wnt and PKA pathways on fibroblast functional differentiation based on triglyceride uptake as an end-point (Fig. 7). cAMP treatment of d 3 explants (10^{-5} M/24 h) stimulated triglyceride uptake by 25%; conversely, the GSK-3β inhibitor LiCl inhibited triglyceride uptake in a dose-dependent manner at 2 and 5 mM, inhibiting triglyceride uptake by 20 and 35%, respectively. The PKA agonist cAMP "normalized" the LiCl effect on triglyceride uptake.

To test the hypothesized role of the PTHrP cAMP-dependent PKA pathway in the down-regulation of the Wnt pathway, we treated d 3 fetal rat lung explants with PTHrP or 8-bromo-cAMP for 24 h in culture and determined their effects on the expression of epithelial SP-B and Shh, and fibroblast Gli and Ptc (Fig. 8). Both PTHrP and cAMP up-

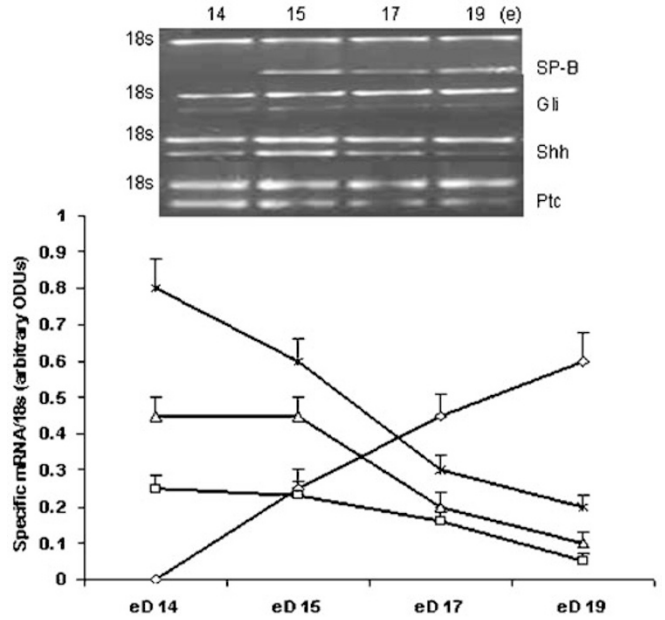

Figure 5. Fetal rat lung explant automaturation coordinately up-regulates maturation of type II cells and down-regulates Shh signaling by fibroblasts. Fetal rat lung explants were cultured for up to 5 d, and were harvested 0, 1, 3, and 5 d postculture (equal to e15, 17, and 19 *ex vivo*). SP-B and Shh expression by RT-PCR were used to monitor type II cell differentiation; Gli and Ptc were monitored by RT-PCR for changes in fibroblast Wnt signaling. Note the patterns of spontaneous down-regulation of the fibroblast Shh signaling pathway members Gli (–□–) and Ptc (–×–) in association with down-regulation of Shh (–△–) in type II cells, and up-regulation of type II cell SP-B (–◇–) (see graphic quantitation below representative gel). Values are means ± SD, $n = 5$.

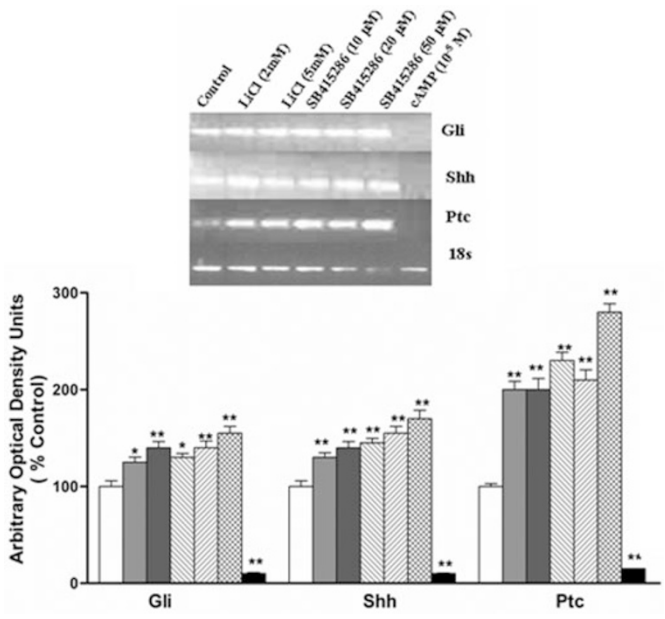

Figure 6. Dose-dependent inhibition of GSK-3β prevents down-regulation of the Wnt pathway. Treatment of d 5 (equal to e19) explants with either LiCl (2 mM = *light gray*, 5 mM = *dark gray*) or the specific GSK-3β inhibitor SB415286 (10 μM = *cross hatched right to left*, 20 μM = *cross hatched left to right*, 50 μM = *cross hatched*) prevents the spontaneous decrease in expression of Gli, Shh, and Ptc, whereas cAMP (1×10^{-5} M = *black*) completely inhibited the expression of these Shh pathway-regulatory genes. Each bar represents the mean ± SD, $n = 5$. $^{*}p < 0.05$, $^{**}p < 0.01$, control *vs* treated by one-way ANOVA.

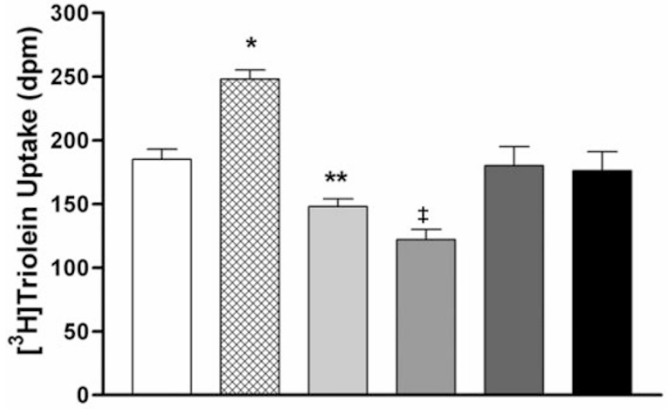

Figure 7. PKA agonists stimulate and GSK inhibitors block triglyceride uptake through a functionally common pathway. Treatment of d 4 fetal rat lung explants with the GSK-3β inhibitor LiCl decreases triglyceride uptake in a dose-dependent manner. Each bar represents the mean ± SD, $n = 5$. $^{*}p < 0.02$; $‡p < 0.01$, control *vs* LiCl treatment, by one-way ANOVA. cAMP (1×10^{-5} M = *cross hatched*) alone significantly increases triglyceride uptake ($^{*}p < 0.001$ control *vs* cAMP treatment, by one-way ANOVA). LiCl (2 mM = *light gray*; 5 mM = *medium gray*) inhibits triglyceride uptake in a dose-dependent manner, and cAMP normalizes the LiCl effect (2 mM = *dark gray*; 5 mM = *black*).

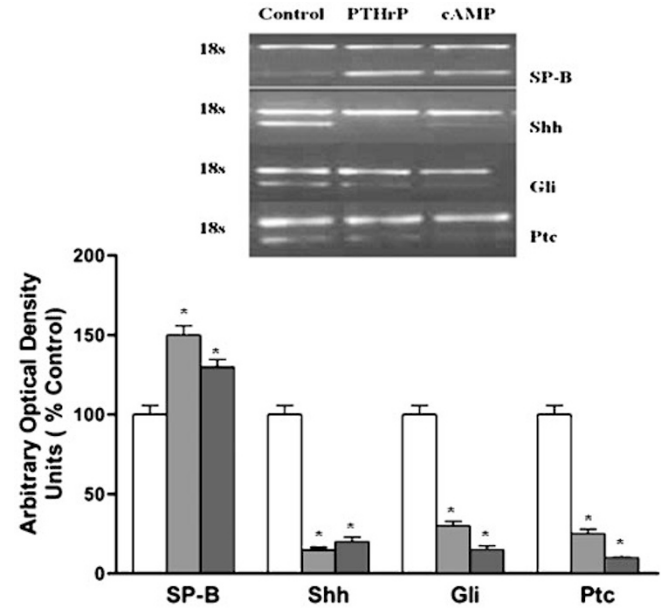

Figure 8. PKA agonists reciprocally up-regulate type II cell differentiation and down-regulate Wnt pathway expression. Day 3 (equal to e17) fetal rat lung explants were treated with PTHrP (5×10^{-7} M = *light gray*) or cAMP (5×10^{-6} M = *dark gray*) for 24 h and were subsequently analyzed for expression of SP-B, Gli, Shh, and Ptc expression by RT-PCR normalized to 18s mRNA. Note the increase in SP-B and the concomitant decreases in Gli, Shh, and Ptc. Each bar represents the mean ± SD, $n = 5$ ($^{**}p < 0.01$; $^{***}p < 0.001$; treated *vs* control, by one-way ANOVA).

regulated SP-B expression and down-regulated Shh, Gli, and Ptc. To quantitate this effect of the PKA and Wnt pathways on type II cell differentiation, we treated d 4 fetal rat lung explants with cAMP and/or LiCl and assayed for saturated phosphatidylcholine (satPC) synthesis (Fig. 9). cAMP itself stimulated the rate of satPC synthesis 38%, whereas LiCl had a dose-dependent inhibitory effect on satPC synthesis, which was partially overridden by 8-bromo-cAMP. We further ex-

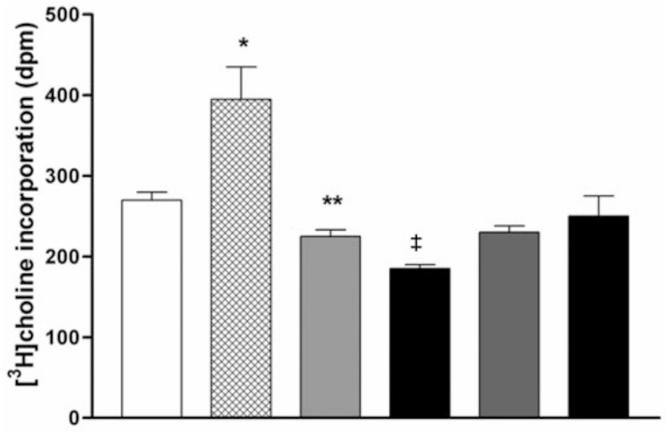

Figure 9. PKA agonists stimulate and GSK inhibitors block surfactant phospholipid synthesis through a functionally common pathway. Treatment of d 4 fetal rat lung explants with the GSK-3β inhibitor LiCl decreases surfactant phospholipid synthesis in a dose-dependent manner. Each bar represents the mean ± SD, $n = 5$ (**$p < 0.02$; ‡ $p < 0.01$, control *vs* $LiCl_2$ treatment, by one-way ANOVA). cAMP alone (*cross hatched*) significantly increases surfactant phospholipid synthesis (*$p < 0.001$ control *vs* treated by one-way ANOVA). LiCl (2 mM = *light gray*; 5 mM = *black*) inhibits triglyceride uptake in a dose-dependent manner, and cAMP normalizes the LiCl effect (2 mM = *dark gray*; 5 mM = *black, far right*), suggesting that the cAMP and LiCl act on type II cell differentiation through a common mechanism.

plored the interrelationship between the PKA and Wnt pathways (Fig. 10) by treating d 4 fetal rat lung explants with a specific PKA antagonist (Rp-cAMP, 1×10^{-8} M), PTHrP (5×10^{-7} M), 8-bromo-cAMP (5×10^{-5} M), or the GSK-3β inhibitory Wnt pathway agonist SB415286 (1×10^{-8} M). After 24 h in culture, the PKA antagonist inhibited satPC synthesis by 25%, whereas both PTHrP and cAMP stimulated satPC synthesis by 50%. The Wnt agonist inhibited satPC by 42%. There was a 30% automaturational increase in satPC

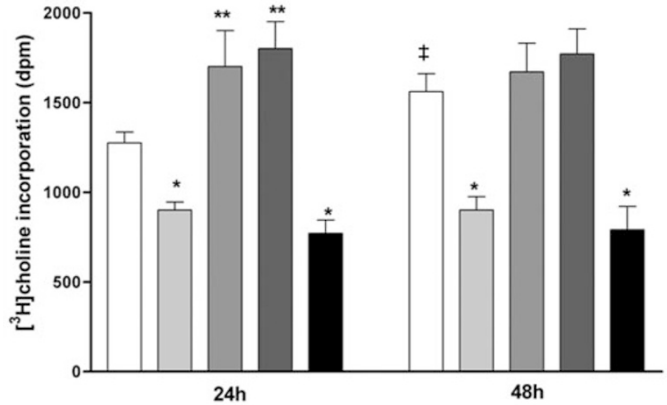

Figure 10. Inhibition of the PKA pathway inhibits surfactant phospholipid synthesis; inhibition of the Wnt Pathway stimulates surfactant phospholipid synthesis. There was a 30% automaturational increase in surfactant phospholipid synthesis at 48 h (‡$p < 0.05$, 24 h control *vs* 48 h control = *open bars*). Treatment of d 19 fetal rat lung explants with either a PKA antagonist (Rp-cAMP, 10^{-8} M = *light gray*) or a GSK inhibitor (SB415286, 10^{-8} M = black) inhibited surfactant phospholipid synthesis at both 24 and 48 h. Each bar represents the mean ± SD, $n = 5$ (*$p < 0.01$, control *vs* treated, by one-way ANOVA), whereas PTHrP (*medium gray*) and cAMP (*dark gray*) stimulated it (**$p < 0.01$, control *vs* treated by one-way ANOVA), consistent with the downstream effect of fibroblast differentiation on type II cell differentiation.

synthesis between 24 and 48 h in culture, which was inhibited 40% by the PKA antagonist. At this stage of automaturation, PTHrP and cAMP were unable to further stimulate satPC synthesis; the Wnt pathway agonist blocked the automaturational increase in satPC synthesis.

DISCUSSION

The present series of studies was designed to determine the functional interrelationship between the up-regulation of PTHrP expression by type II cells in response to fluid distension, its sequential up-regulation of fibroblast cAMP-dependent PKA through its interaction with the PTHrP receptor, and the concomitant down-regulation of the Wnt/βcatenin GRN. We had previously shown that PTHrP stimulates cAMP expression by the fetal rat lung fibroblast (18) and surfactant synthesis by type II cells (2–4,9,15,20,21), but the intermediate steps in the pathway that coordinated these mesenchymal and epithelial effects were not determined, though we had provided experimental evidence that it was paracrine in nature (18). We subsequently showed that PTHrP up-regulated the expression of leptin by the immature lung fibroblast, and that leptin bound to its receptor on the epithelial type II cell, stimulating epithelial cell surfactant phospholipid synthesis (2) and surfactant protein expression (4), thus resolving the paracrine mechanism for PTHrP-stimulated lung maturation. However, we wanted to determine whether this mechanism could be observed in intact lung tissue, solely under the influence of alveolar fluid distension. McCray *et al.* (25) had previously shown that fetal rat lung explants secrete lung liquid within the developing alveoli, offering a model to study the functional interrelationship between alveolar fluid stretch, up-regulation of PTHrP expression by type II cells, and stimulation of its paracrine targets. Because PKA down-regulates the fibroblast Wnt/βcatenin pathway by inhibiting Gli (28), which is the only Gli family member that is Shh regulated (29), we hypothesized that PTHrP signaling is necessary for the down-regulation of the Wnt/βcatenin GRN and up-regulation of the PKA GRN for the maturation of the lipofibroblast (30), and indirectly for the maturation of the epithelial type II cell by means of this paracrine loop (2).

The present series of experiments supports this sequence of cell-mediated endodermal-mesodermal interactions, which would account for the well-documented fluid stretch-regulation of the epithelial and mesenchymal components of the alveolar interstitium during fetal lung development (31,32). We used specific agonists and antagonists for the Wnt/βcatenin and PTHrP signaling pathways to test this hypothesis, and targeted cell-type specific genes in each pathway that we had observed empirically increasing or decreasing during the spontaneous maturation of the lung explants. Although there is evidence for cAMP stimulation of type II cell surfactant synthesis, we chose a stage in lung development when cAMP would not have had such an effect, *i.e.* cAMP stimulates surfactant synthesis (33) but does not do so directly on immature type II cells (34). Similarly, Wnt/βcatenin signaling is observed in both the mesoderm and endoderm of the developing lung, but the endodermal expression occurs at a

much earlier stage of lung development (1) and would not be affected by Wnt/βcatenin pathway agonists at e18–19, as in the present experiments. Regarding the potential toxic effects of the cell-type and pathway-specific antagonists we used, we selectively monitored the stimulation of paracrine-dependent differentiation markers in the complementary tissue compartment, *e.g.* monitoring endodermal markers when mesodermal cells were inhibited, and vice versa, to ensure viability of the epithelial-mesenchymal system.

Immature mesodermal cells are dominated by the Wnt/βcatenin pathway (35), which confers the myogenic fibroblast phenotype. The developing epithelium expresses Shh, which stimulates mesodermal Wnt/βcatenin through its receptor-mediated down-stream interactions with Ptc and Gli (35), actively promoting the myogenic fibroblast phenotype. Descriptively, as the endoderm and mesoderm of the alveolar interstitium mature, endodermal Shh signaling through the mesodermal Wnt/βcatenin pathway decreases as endodermal PTHrP signaling to the mesodermal PTHrP receptor signaling pathway is concomitantly up-regulated (36). We have exploited the stretch-regulation of PTHrP (2,3) to test the hypothesis that fetal lung fluid stretches the alveolar interstitium and stimulates PTHrP signaling, which down-regulates the mesodermal Wnt/βcatenin pathway through cAMP-dependent PKA inhibition of Gli (28), up-regulating the PTHrP signaling pathway, inducing the lipofibroblast phenotype (30,37). The mature lipofibroblast produces leptin (4), which induces endodermal type II cell differentiation. The down-regulation of endodermal Shh expression by the mature epithelial type II cells ensures constitutive down-regulation of the Shh/Wnt/βcatenin GRN, molecularly stabilizing these key alveolar interstitial phenotypes.

In support of a functional genomic interrelationship between the Wnt/βcatenin and PTHrP GRN during lung development, Bellusci *et al.* (1) have shown that overexpression of Shh by type II cells causes a stage-specific delay in mesenchymal and epithelial development beginning at e16.5 in the mouse, characterized by increased mesodermal Ptc expression, at the stage in lung development when PTHrP signaling is up-regulated (38). Importantly, the PTHrP knockout mouse lung exhibits the same epithelial and mesodermal developmental characteristics as does Shh overexpression (1), and in both cases the resulting lung immaturity leads to a respiratory death at the time of birth. The same stage-specific effects of PTHrP deficiency causing failed lung development in both of these models is striking and may reflect the necessity for up-regulating PTHrP signaling to down-regulate the Wnt/βcatenin pathway.

A wide variety of lung cell types emerge from epithelial-mesenchymal interactions mediated by specific soluble paracrine growth factors and their cognate receptors residing on neighboring cells of different germ line origins (39). A variety of experiments have demonstrated that the distension of the alveolus determines its cytoarchitecture (2,3,31,32). Conversely, chronic lung disease is characterized by simplification and/or loss of alveoli, which may reflect the failure of the mechanotransduction-induced mechanism of lung development described herein (20). We had previously postulated that lung development and remodeling represent a mechanistic continuum, based upon experimental observations consistent with this working hypothesis (40). The present experiments provide an integrated, cell/molecular mechanism that can generate either homeostasis or disease, depending upon the dominant cellular cross-talk.

This series of experiments has shown that alveolar fluid distension acts as an organizing principle to up-regulate the PTHrP GRN and actively down-regulate the Shh/Wnt/βcatenin pathway in a cell-specific, spatiotemporal pattern.

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