

## Regulation of retinoic acid signaling during lung morphogenesis

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### SUMMARY

Little is known about how retinoic acid (RA) synthesis, utilization and metabolism are regulated in the embryonic lung and how these activities relate to lung pattern formation. Here we report that early lung bud formation and subsequent branching morphogenesis are characterized by distinct stages of RA signaling. At the onset of lung development RA signaling is ubiquitously activated in primary buds, as shown by expression of the major RA-synthesizing enzyme, *RALDH-2* and activation of a *RARE-lacZ* transgene. Nevertheless, further airway branching appears to require downregulation of RA pathways by decreased synthesis, increased RA degradation in the epithelium via P450RAI-mediated metabolism, and inhibition of RA signaling in the mesenchyme by COUP-TF-II expression. These mechanisms controlling local RA signaling may be critical for normal branching, since we show that manipulating RA

levels in vitro to maintain RA signaling activated as in the initial stage, leads to an immature lung phenotype characterized by failure to form typical distal buds. We show that this phenotype likely results from RA interfering with the establishment of a distal signaling center, altering levels and distribution of *Fgf10* and *Bmp4*, genes that are essential for distal lung formation. Furthermore, RA upregulates *P450RAI* expression, suggesting the presence of feedback mechanisms controlling RA availability. Our study illustrates the importance of regional mechanisms that control RA availability and utilization for correct expression of pattern regulators and normal morphogenesis during lung development.

Key words: Lung development, Retinoic acid, Branching morphogenesis, Mouse

### INTRODUCTION

Although several studies have shown that retinoids are important for proper lung morphogenesis and for differentiation of the respiratory epithelium, little is known about the ontogeny of retinoic acid (RA) signaling in the lung and the role of RA in lung pattern formation. Maternal administration of teratogenic doses of retinoids as well as retinoid deprivation cause marked lung dysmorphogenesis in embryos (Wilson et al., 1953; Shenefelt et al., 1972; Dickman et al., 1997). Dramatic abnormalities that resemble those observed in retinoid-deficient conditions are found in compound RA receptor (RAR) null mutant mice. However, the distribution of these malformations in the lung and other organs does not correlate well with the sites where RARs are normally expressed (Mendelsohn et al., 1994). While this discrepancy could result from disruption of RA-dependent events outside the lung, it also indicates that, besides distribution of retinoid receptors, other factors such as ligand bioavailability and the presence of RA activators or repressors also control RA signaling in the lung.

RA is generated by a series of oxidative reactions that convert retinol to retinaldehyde and ultimately to the active form retinoic acid (Zhao et al., 1996). While several enzymes have been shown to catalyze these reactions (Vonesch et al.,

1994; Zhao et al., 1996; Romert et al., 1998), retinaldehyde dehydrogenases V1 and V2/*RALDH-2* have a prominent role in generating RA. *RALDH-2* expression is developmentally regulated in many organs (Niederreither et al., 1997; Moss et al., 1998; McCaffery et al., 1999). *RALDH-2*<sup>-/-</sup> mice in a *RARE-lacZ* transgene background (Rossant et al., 1991) do not produce sufficient RA to activate *lacZ* expression anywhere in the body, other than in the eye (Niederreither et al., 1999). The early death and the major defects observed in these mutants not only confirm the importance of retinoid signaling in organogenesis, but they also suggest that *RALDH-2* is a critical enzyme in the RA synthetic pathway.

A balance between retinoid synthesis and degradation determines the RA concentration in target tissues. RA degradation in target cells may serve as a mechanism to protect RA-sensitive tissues from high RA levels. P450RAI (CYP26) is an RA-inducible RA-metabolizing enzyme of the cytochrome P450 family. P450RAI specifically converts RA into several hydroxylated products, limiting retinoid signaling in target cells or generating metabolites which differentially affect morphogenesis and regeneration (White et al., 1996; Fujii et al., 1997; Moss et al., 1998; Iulianella et al., 1999). Retinoid signaling can also be controlled by inhibition of receptor function. The Chicken Ovalbumin Upstream Promoter-Transcription Factors (COUP-TFs) are nuclear

receptors that are believed to interfere with RA-mediated transactivation by sequestering RXRs. Because RXRs heterodimerize with members of the steroid receptor superfamily, including RARs, COUP-TFs act as inhibitors of RA actions (reviewed by Tsai and Tsai, 1997). *COUP-TFs* are expressed in a wide and partially overlapping domains during organogenesis. In the lung, only *COUP-TFII* has been reported (Jonk et al., 1994), and its role in lung development is still unclear. Targeted disruption of *COUP-TFII* gene leads to embryonic lethality by day 9-9.5 from defects in angiogenesis and heart development (Pereira et al., 1999).

Here we perform an integrative study to understand how RA signaling is regulated during lung morphogenesis and how RA influences lung pattern formation. By mapping sites of retinoid synthesis (*RALDH-2* expression), utilization (activation of a *RARE-lacZ* transgene) and degradation (*P450RAI* expression) and sites of expression of an RA signaling antagonist (*COUP-TFII*) in the embryonic lung, we identify distinct stages of RA signaling. We found that branching morphogenesis appears to require downregulation of RA signaling to allow proper expression of genes involved in distal lung formation, such as *Fgf10* and *Bmp4*. Maintaining a status of RA activation characteristic of the early lung in organ cultures results in an immature lung phenotype characterized by failure to form typical distal buds. Our study illustrates the importance of regional mechanisms that regulate RA availability and utilization for correct expression of gene regulators of pattern and normal lung morphogenesis.

## MATERIALS AND METHODS

### Embryonic lung cultures

Day-11.5 lungs isolated from CD-1 mouse embryos were cultured in BGJb control medium or in media containing all-trans retinoic acid (Sigma) at a final concentration of  $10^{-7}$  to  $10^{-5}$  M (Cardoso et al., 1995, 1996). Late exposure experiments consisted of culturing lungs in control medium for 3 days followed by another 3-day treatment with all-trans RA ( $10^{-5}$  M). Lung cultures were harvested at days 1, 3 and 6 and fixed in 4% paraformaldehyde. In some experiments, ion-exchange beads AG1-X2 (BioRad, Hercules, CA) soaked in RA solution (30  $\mu$ M), or heparin beads soaked in FGF10 solution (human recombinant, 100  $\mu$ g/ml, R&D) were grafted onto day-11.5 lung explants and cultured for 24-72 hours (Eichele et al., 1985; Park et al., 1998). Controls consisted of beads soaked in PBS grafted in contralateral lungs.

### In situ hybridization analysis

Isotopic ( $^{35}$ S-labeled riboprobes) and non-isotopic in situ hybridizations were performed on paraffin sections (6-12  $\mu$ m) of embryonic mouse lungs, as described by Cardoso et al. (1996) and Mendelsohn et al. (1999). Whole-mount in situ hybridization was performed in embryonic lungs (gestation days 9.5 to 18) and lung cultures (control, RA-treated explants) using digoxigenin-labeled riboprobes (Maxiscript, Promega), as formerly described (Wilkinson et al., 1992; Lebeche et al., 1999). Probes were generated from plasmids carrying cDNAs for *RALDH-2* (Zhao et al., 1996), *P450RAI* (Iulianella et al., 1999), *Fgf10* (Park et al., 1998), *Bmp4* (Lebeche et al., 1999), *COUP-TFII* (Jonk et al., 1994) and nuclear receptors RXRs and RARs (Mendelsohn et al., 1999). Expression patterns of *RALDH-2* and *Fgf10* were compared in day 10 to day 12.5 lungs from the same litter and with a similar pattern of airway branching. Results were confirmed in at least 3 specimens.

### *RARE-lacZ* transgenic mice, $\beta$ -galactosidase staining and immunohistochemistry

To determine sites of RA utilization, we used *RARE-lacZ* transgenic mice (Rossant et al., 1991). These animals carry the bacterial *lacZ* gene under the control of a heat shock promoter (hsp 68) and the RA-responsive element from the *RAR- $\beta$*  promoter. *lacZ* is expressed at sites where RA activates RARs.  $\beta$ -galactosidase ( $\beta$ -gal) staining was performed in glutaraldehyde-fixed lungs using X-gal as substrate (Cheng et al., 1993). Specificity was confirmed by the presence of  $\beta$ -gal signals in structures such as eye (not shown) and atrial wall, and absence in ventricle in day 9.5 embryos, as previously reported (Fig. 3A; Rossant et al., 1991; Moss et al., 1998).  $\beta$ -gal staining was also performed in lung organ cultures. Specimens were processed for histological analysis in 6  $\mu$ m sections counterstained with Nuclear Fast Red or Hematoxylin (Sigma). To ensure that  $\beta$ -gal staining was not limited by inadequate diffusion of the substrate to the inner layers of the lung, we tested three different conditions. First, we examined sections of day-12 lungs overstained by overnight incubation with X-gal. We also performed immunohistochemistry for  $\beta$ -gal in sections of *RARE-lacZ* embryos (below). In addition, we performed X-gal staining in *RARE-lacZ* lungs cultured for up to 6 days in control medium (described above), and we compared epithelial staining of these cultures with that observed in vivo at equivalent time points. The relatively flat morphology of these cultures allows accessibility of reagents throughout all layers and easy visualization of expression patterns (Cardoso et al., 1995; Meneghetti et al., 1996). For immunohistochemistry, sections were incubated overnight with rabbit polyclonal  $\beta$ -gal antibody (Eppendorf 5 prime, Inc), diluted at 1:1000. Signal was amplified using horseradish peroxidase-labeled anti-rabbit antibody (Vectastain ABC kit) according to the manufacturer's instructions.

### Gene expression in primary lung mesenchymal cell cultures

Embryonic lungs were isolated at gestation day 16.5. Mesenchymal cells were separated from the epithelium by differential adherence, as described by Lebeche et al. (1999) and cultured in the presence of all-trans RA ( $10^{-7}$ ,  $10^{-5}$  M) for 24 hours. Total RNA was extracted using Trizol Reagent® (GIBCO-BRL). Purity of mesenchymal cell preparations was assessed in parallel by immunocytochemical detection of vimentin (Sigma). Contamination with non-expressing cells represented less than 1% of the monolayer. Reverse transcription reaction was carried in 500 ng of total RNA and equal amounts of DNA were loaded for semiquantitative PCR. *Fgf10* (Park et al., 1998) and  *$\beta$ -actin* (Cardoso et al., 1995) amplifications were performed as described by Lebeche et al. (1999). Experiments were repeated at least three times.

## RESULTS

### *RALDH-2* expression and its relationship to *Fgf10* in the embryonic lung

To map sites of RA synthesis in the lung, we assessed *RALDH-2* expression by whole-mount in situ hybridization. High levels of *RALDH-2* are present in the dorsal wall of the coelomic cavity, including the presumptive area of the nascent lung bud (Zhao et al., 1996; Niederreither et al., 1997). At embryonic day 9.5, we detected strong expression of *RALDH-2* along the anterior foregut, in mesenchyme of the prospective trachea and lung primordia (Fig. 1A). Lung mesothelial surfaces are labeled while respiratory epithelium is negative (Fig. 1A-C). At day 10, high *RALDH-2* levels are maintained in trachea (mesenchyme) and proximal lung (pleura), but intensity of signals decline in a gradient fashion towards the distal lung (Fig. 1D,E).

Within subsequent days, *RALDH-2* signals become unevenly distributed along the mesothelial surfaces of the lung. Local levels change as airways branch, concentrating at sites of less branching activity (Figs 1F, 2C). This dynamic pattern of *RALDH-2* expression suggests that local control of RA synthesis influences lung patterning. Isotopic in situ hybridization of lung sections shows that *RALDH-2* signals are restricted to the parietal and visceral pleura (Fig. 1G), although by this method regional differences in expression are less clear.

From these observations, we hypothesized that during early stages of branching, sites of RA synthesis inversely correlate with sites of prospective budding. We used expression of *Fgf10* as an early marker of site-specific lung bud induction. FGF10 has been shown to act as a bud inducer and a chemoattractant factor for the lung epithelium during branching morphogenesis (Bellusci et al., 1997a; Park et al., 1998). Time-lapse study of *Fgf10* expression in the embryonic lung suggests that its regional induction in the mesenchyme precedes bud formation (Bellusci et al., 1997a).

Comparison of the patterns of *Fgf10* and *RALDH-2* expression in the early lung reveals that areas where *Fgf10* is expressed are low in *RALDH-2* signals (Fig. 2A-F). For instance, in the right lung at day 11, while strong *RALDH-2* signals localize to basal regions of the lobes (arrowheads in Fig. 2C), *Fgf10* concentrates at the apexes (Fig. 2D). At day 11.5, *RALDH-2* is expressed along the medial and cranial surfaces of the left lung, while *Fgf10* is abundant laterally, in the caudal tip and cranially in a region that seems to only partially overlap with *RALDH-2* (Fig. 2E,F). This complementary pattern of expression suggests that RA signaling influences early lung pattern formation by suppressing local expression of *Fgf10* (see below).

As the branching pattern becomes more complex, *RALDH-2* distribution no longer correlates well with *Fgf10* (Fig. 2G,H), although differences in regional (Fig. 2G) or cellular (Fig. 1I) distribution can still be observed. The overall levels of *RALDH-2* in the lung appear to decrease toward late gestation. These observations suggest that RA is synthesized during all stages of embryonic development and that the pleura is the source of an RA gradient, acting as a signaling center for RA-dependent control of gene expression.

Expression of *RALDH-2* in the trachea also changes with time. High levels are found in the tracheal mesenchyme at early stages (days 9.5-11), but expression progressively decreases and is undetectable by day 14 (Fig. 1H).

### ***RARE-lacZ* transgene identifies distinct stages of lung morphogenesis**

Embryonic lungs from *RARE-lacZ* transgenic mice were used to localize sites of RA utilization. At day 9.5, prominent *RARE-lacZ* signals are detected throughout all layers of the foregut where trachea and lung primordia are forming (Fig. 3A). Ubiquitous signals in the primordial lung is consistent with the idea that RA is activating widely expressed retinoid receptors. The patterns of *RALDH-2* and *lacZ* expression suggest that RA diffuses from mesothelial cells or mesenchyme to activate RARs in all layers, including the epithelium.

At day 10, shortly before the appearance of secondary branches, *lacZ* activity remains strong throughout tracheal and lung epithelia but decreases considerably in the subepithelial mesenchyme (Fig. 3B,C), particularly at sites of prospective

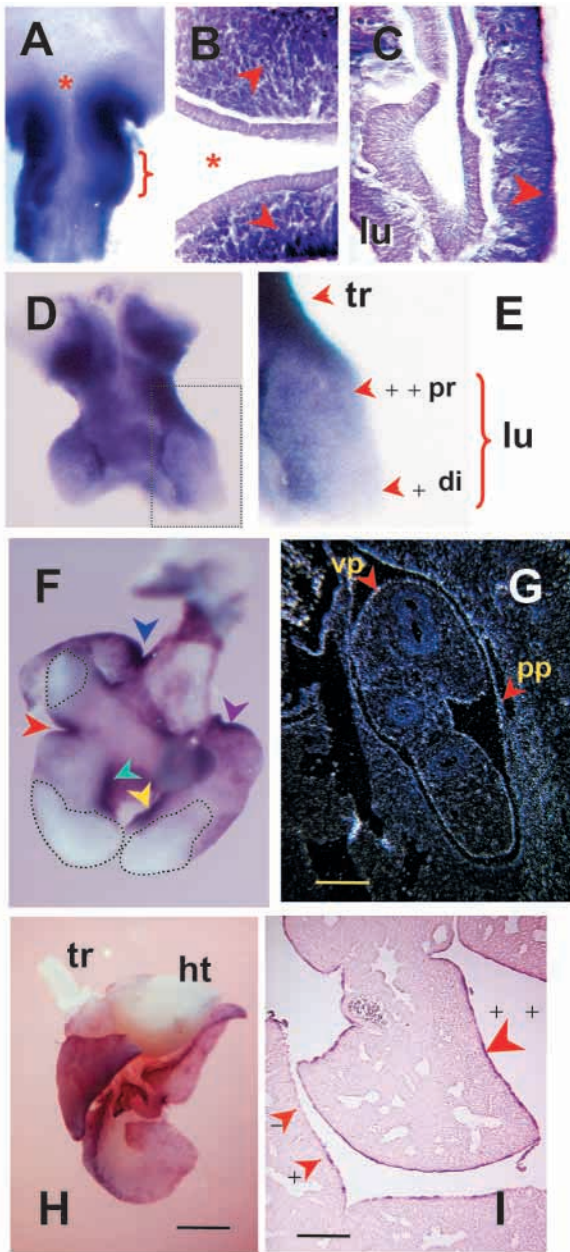
budding (arrowheads in Fig. 3B). At day 11, when large areas of the lung mesenchyme become *lacZ*-negative, strong *lacZ* expression is seen in the tracheal mesenchyme, where *RALDH-2* is also expressed at high levels (compare staining in Fig. 2C and Fig. 3D).

The progressive loss of mesenchymal  $\beta$ -gal staining in the lung precedes the appearance of the secondary airway branches. Interestingly, secondary bud formation is accompanied by loss of epithelial *lacZ* expression (asterisk in Fig. 3D,E). After day 11.5, staining is largely absent from the epithelium (Fig. 3F; also compare *lacZ* expression in lung sections in Fig. 3C and 3H). As described in Materials and Methods, we checked for an artifactual lack of penetration of substrate in the specimens. By overstaining day-12 lungs (Fig. 3G,H), we observed a clear pattern of *lacZ* expression restricted to the outer layers of the lung (mesothelium and subpleural mesenchyme). The subepithelial mesenchyme and epithelium were both negative, with the exception of scattered *lacZ*-positive epithelial cells, more frequently found at proximal sites (Fig. 3G). Evidence of overstaining and adequate diffusion of reagents was provided by detection of labeled blood cells in subepithelial capillaries (arrowhead in Fig. 3H), which are not labeled under standard conditions. Immunohistochemistry (Fig. 3L) and X-gal staining of lung cultures (Fig. 6C) confirmed the lack of epithelial staining.

Although negative in the epithelium, *RARE-lacZ* expression remains at diminished levels in pleura and peripheral mesenchyme after day 12. At day 14.5,  $\beta$ -gal staining seems to delimit lobular structures, following the pattern of presumptive connective tissue septae (Fig. 3I). Histological inspection of these areas reveals *lacZ* signals in cells adjacent to indentations of the pleura, suggesting that they are setting the pattern of presumptive lobular septae (Fig. 3J). By day 16, *lacZ* expression is almost undetectable in the lung even by immunohistochemical methods (Fig. 3K,L).

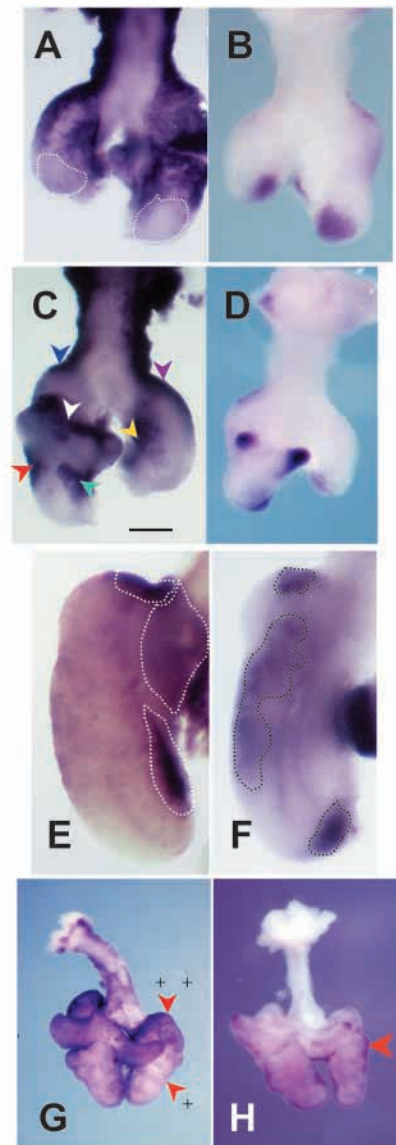
### **Loss of epithelial $\beta$ -gal staining is not associated with loss of retinoid receptor expression during branching**

Since *RALDH-2* levels remained high as epithelial *lacZ* expression became undetectable, we checked whether RA responsiveness in *RARE-lacZ* transgenes decreased due to an altered pattern of retinoid receptor expression in the epithelium. Although the developmental pattern of RARs and RXRs has been reported (Dollé et al., 1990, 1994), specific information about patterns in the lung between day 11-12 is not available. By using non-isotopic in situ hybridization, we found that at day 11 and 12, the lung epithelium expresses *RAR $\alpha$* , *RXR $\alpha$*  and *RXR $\beta$*  at both proximal and distal sites (data not shown). Expression of these receptors in all layers of the lung at all times confirms the ubiquitous pattern previously published (Dollé et al., 1990, 1994). Epithelial expression of *RAR $\beta$*  (Fig. 4A,B) is found only at proximal sites and this pattern remains unchanged from day 11 to 12 (in Fig. 4, red and blue arrowheads indicate epithelial and mesenchymal expression, respectively). We also found that epithelial expression of *RAR $\gamma$*  at day 11 is very low and restricted to distal tubules (Fig. 4C, see the high levels in proximal mesenchyme and absence of signals in proximal epithelium), but becomes strong in both proximal and distal tubules at day 12 (Fig. 4D). Our analysis shows that distal budding is not



**Fig. 1.** *RALDH-2* expression in mouse embryonic lung as seen by whole-mount in situ hybridization. (A) Ventral view of the anterior foregut (\*, branchial arches at the top) and lung primordium (bracket) at approximately day 9.5. (B,C) Sections showing that while all epithelia are negative, high levels of *RALDH-2* (arrowheads) are present in mesenchyme of prospective trachea (\*), pleura and subpleural mesenchyme of the lung (lu). (D,E) Dorsal view of a day-10 lung showing *RALDH-2* gradient (++/+), with highest levels in trachea (tr) and proximal lung (pr) and low levels distally (di) (E, right lung, boxed area on D). During branching morphogenesis (day 11.5; F,G) *RALDH-2* expression concentrates in areas of low branching activity (colored arrowheads depict site-specific expression, same sites are shown 0.5 day earlier on Fig. 2C). Isotopic in situ hybridization (G) shows *RALDH-2* restricted to the visceral (vp) and parietal pleura (pp). At later time points (day 14; H), *RALDH-2* becomes diffuse in the lung and negative in trachea and heart (ht). (I) Section of day-16.5 lung shows that mesothelium continues to express *RALDH-2* at variable levels. Scale bars: G, 110  $\mu$ m; H, 1400  $\mu$ m; I, 140  $\mu$ m.

## *RALDH-2* *Fgf10*



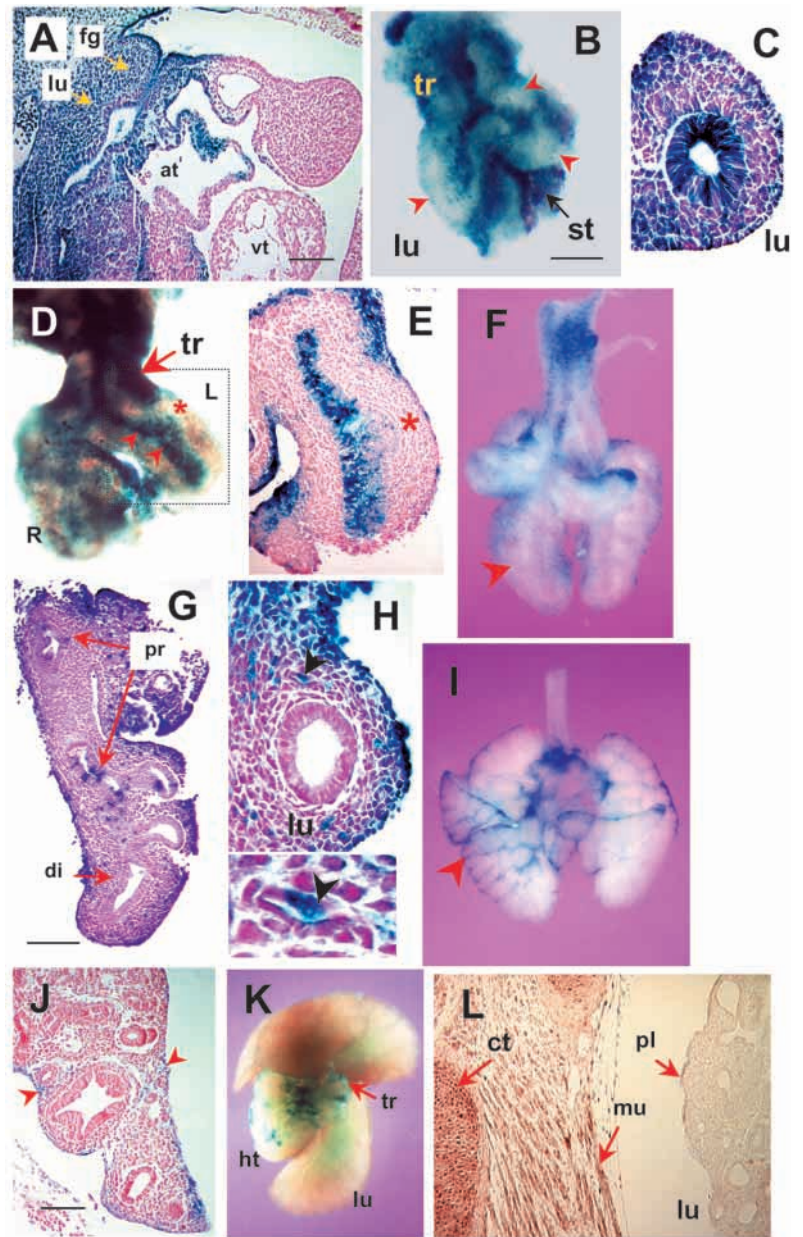
**Fig. 2.** Whole-mount in situ hybridization shows that patterns of *RALDH-2* and *Fgf10* (early marker of distal budding) are dynamic and largely complementary during early branching morphogenesis. (A-D) Day-11 lung, dorsal (A,B) and ventral (C,D) views. (E,F) Day-11.5 left lung (for details see Results). (G,H) By day 12.5, *RALDH-2* distribution is still uneven with highest levels cranially (++), nevertheless expression no longer correlates with *Fgf10*.

associated with loss of any of the known retinoid receptors. The presence of RA synthesizing enzyme and receptors, but no *lacZ* activation suggests the presence of additional mechanisms of RA regulation during branching morphogenesis.

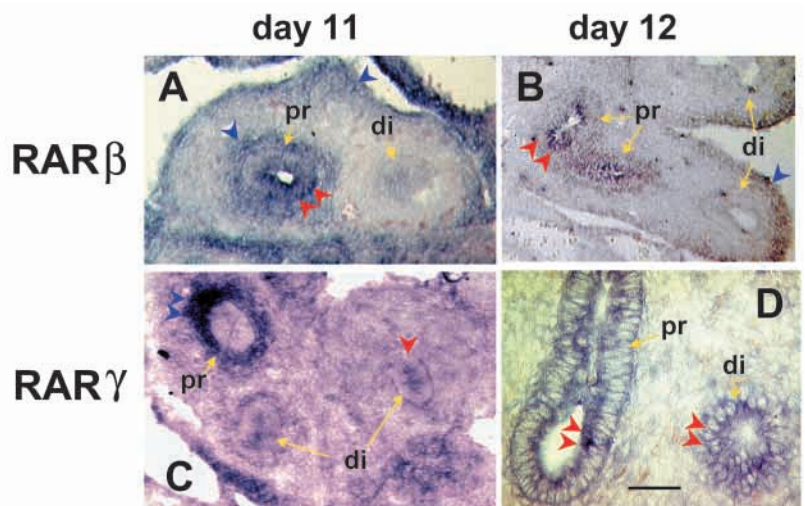
### ***P450RAI* and the control of RA levels in the developing lung epithelium**

Because our data suggested the presence of a mechanism controlling *lacZ* activation at specific sites and time, we explored the possibility that negative regulators of RA signaling could be expressed during lung branching

**Fig. 3.** Expression of *RARE-lacZ* transgene (whole mounts and sections of X-gal- stained specimens) in the developing mouse lung. (A) Section of a day-9.5 embryo shows ubiquitous *lacZ* transgene expression in anterior foregut (fg) destined to become prospective trachea and lung (lu) primordium. (B,C) At day 10, epithelial expression remains strong in trachea, lung and esophagus/stomach (st), but decreases in subepithelial mesenchyme, particularly at sites of prospective budding (arrowheads). (D,E) At day 11, a gradient of *lacZ* expression is evident with higher levels in tracheal mesenchyme; tracheal and bronchial (double arrowhead) epithelia strongly express *lacZ*, however secondary epithelial buds (\*) become  $\beta$ -gal-negative (E, section of area indicated in D). (F-H) At day 12, epithelia are largely  $\beta$ -gal-negative (arrowhead in F); expression is limited to a few epithelial cells in trachea and proximal (pr) airways, pleura and subpleural mesenchyme. Overstained specimens showing  $\beta$ -gal-labeled blood cells (arrowhead in H, inset) in negative areas of distal (di) mesenchyme suggest proper diffusion of reagents (see Methods). (I,J) At day 14.5, *lacZ* expression localizes to pleural and mesenchymal cells in prospective lobular septae. (K,L) At day 16, expression is almost undetectable in lung or trachea; immunohistochemistry (L) shows *lacZ* expression in cartilage (ct) and muscle cells (mu) of the thoracic wall, but not in lung parenchyma or pleura (pl). Scale bars: A, 190  $\mu$ m; B, 280  $\mu$ m; G, 150  $\mu$ m; J, 100  $\mu$ m. at, atrium; vt, ventricle; ht, heart; R, L, right and left lungs.



**Fig. 4.** RAR expression in the day-11 to -12 embryonic lung (non-isotopic in situ hybridization). Red and blue arrowheads indicate epithelial and mesenchymal signals, respectively; number of arrowheads represent relative intensity of signals. (A,B) *RAR $\beta$*  is expressed in proximal (pr) but not in distal (di) epithelia at day 11 (A) and 12 (B); *RAR $\beta$*  is also found in the subepithelial mesenchyme of proximal airways at day 11 and in subpleural mesenchyme at both stages. (C,D) *RAR $\gamma$*  expression at day 11 (C) is undetectable in proximal epithelium and is weak in distal epithelium, but it is strong in subepithelial regions of proximal mesenchyme. At day 12 (D), *RAR $\gamma$*  signals in epithelia increase at both proximal and distal sites (D). Scale bar, 80  $\mu$ m.



morphogenesis. The cytochrome P450RAI (CYP26) oxidase has been shown to catabolize RA, and to be expressed in a developmental fashion, presumably controlling RA levels in embryonic tissues (Fujii et al., 1997; White et al., 1996). We determined the developmental pattern of *P450RAI* expression in the lung as an indication for local RA degradation.

From day 9.5-10, when *RARE-lacZ* is diffusely activated, no *P450RAI* is found in the lung primordia (Fig. 5A). *P450RAI* expression is first seen in the lung at day 10.5 throughout the lung bud epithelium (Fig. 5B). Its onset of expression coincides with the appearance of lateral (secondary) buds and local extinction of epithelial *RARE-lacZ* transgene expression. By day 12-14.5 (Fig. 5C-E), a proximal-distal gradient is established with higher levels in distal buds. Although not easily visualized, weak signals are also seen in the lung mesenchyme in between distal buds (Fig. 5E). From day 14.5 onwards, *P450RAI* expression expands proximally to include the tracheal epithelium and tracheal mesenchyme. Mesenchymal expression localizes to prospective tracheal rings (arrowhead in Fig. 5E). By day 16.5, *P450RAI* expression has shifted from distal to proximal sites and becomes restricted to the main bronchi and trachea. Restriction of *P450RAI* expression to proximal sites was confirmed by in situ hybridization in lung organ cultures (Fig. 5G).

Coincident *P450RAI* expression and RA clearance in embryonic tissues has been previously reported (Moss et al., 1998; Iulianella et al., 1999). Our data supports the idea that loss of *lacZ* expression in branching airways is due to the presence of P450RAI in the lung epithelium. This enzyme may be critical for local regulation of RA levels in the lung epithelium, determining whether RARs will be activated in proximal or distal sites. Thus, RA degradation likely represents a major regulatory mechanism controlling RA bioavailability to receptors during lung epithelial branching.

### COUP-TFII, an antagonist of RA signaling in the lung mesenchyme

While the loss of *lacZ* expression in the epithelium could result from the presence of P450RAI, it was unclear why *lacZ* activation was absent in large areas of the mesenchyme from mid to late developmental stages. The developing lung expresses the RA-signaling inhibitor COUP-TFII (Jonk et al., 1994). We found that in the day-11 to -11.5 lung COUP-TFII is expressed in the mesenchyme at slightly higher levels distally, but is not seen in the epithelium (Fig. 5I). At day 14.5, mesenchymal levels markedly increase and distribution of signals is homogenous (Fig. 5J). At day 16.5, signals are still strong between buds (double arrowheads in Fig. 5K); nevertheless, they appear to be less concentrated in subpleural areas (arrowhead). By day 18, signals are not detected in any layer of the lung (not shown).

*COUP-TFII* is also found in the epithelium of trachea and extrapulmonary airways. This pattern is already seen at day 11.5 (Fig. 5H); signals become stronger in the bronchial epithelium at day 14.5 (Fig. 5J). Distal epithelial tubules are not labeled at any time. COUP-TFII is a strong candidate for modulating RA signaling in the developing lung mesenchyme. During branching morphogenesis, COUP-TFII might prevent activation of RA target genes through RARs that are present in the mesenchyme. In addition, by inhibiting RA signaling activation in the proximal epithelium, COUP-TFII might

contribute to inactivating *RARE-lacZ* expression in the epithelium.

### Exogenous RA activates retinoid signaling in proximal lung and disrupts morphogenesis

Our data show that distal lung formation is associated with local restriction of RA synthesis and utilization in vivo. We have previously reported that RA treatment of embryonic lungs in culture fosters growth of an immature proximal-like phenotype and prevents normal distal development (Cardoso et al., 1995). We tested the possibility that this effect could be related to an abnormal pattern of RAR activation due to excess and diffuse availability of ligand.

Lungs from *RARE-lacZ* transgenes cultured in control conditions express *lacZ* in a pattern that mimics that seen in vivo. As shown in Fig. 6C, after 24 hours in culture, *lacZ* is undetectable in epithelial tubules, but is present in mesothelial and mesenchymal cells at the sites previously described. In contrast, RA-treated cultures ( $10^{-5}$  M) show *lacZ* expression in the epithelium within the initial 24 hours (Fig. 6D). Staining is prominent at proximal sites in both epithelial and mesenchymal layers and is minimal distally. Interestingly, while the pattern of *lacZ* expression of a 24-hour control culture is reminiscent of that found in a day-12 to -12.5 lung in vivo, the *lacZ* pattern of RA-treated cultures resembles rather the pattern seen in the day-9.5 to -11 lung in vivo. Cultures exposed to RA ( $10^{-6}$ - $10^{-5}$  M) for 3-6 days have reduced number of terminal branches and show immature proximal-like epithelial tubules without typical distal acinar structures at the tips (Fig. 6A,B; Cardoso et al., 1995).

The ability of RA to alter lung pattern depends on the time at which the embryonic lung is treated with RA (Cardoso et al., 1995). While RA treatment at earlier stages (day 11-12) induces this phenotype, late exposure to RA (see Methods) still induces proximal *lacZ* activation, but has no effect on pattern (Fig. 6E). Thus, control of RA signaling appears to be critical for correct branching morphogenesis at around day 11-12, when typical distal epithelial structures are forming. Interestingly, this time window coincides with the stage when we found major changes in the pattern of *RALDH-2* and *lacZ* expression in vivo. Collectively, our in vivo and in vitro observations strongly support the idea that RA is involved in proximal-distal patterning of the developing lung.

### RA signaling controls expression of distal regulators of pattern

We looked for a molecular mechanism to explain how RA signaling could be influencing lung pattern formation. As suggested by assessment of *RALDH-2* and *Fgf10* in vivo, RA and FGF10 signaling are tightly connected during the initial stages of lung morphogenesis. Moreover, northern analysis has shown that RA treatment of lung cultures ( $10^{-6}$  M) inhibits *Fgf10* expression (Bellusci et al., 1997a). Since budding depends on regional expression of *Fgf10*, we performed whole-mount in situ hybridization in RA-treated cultures to examine how the spatial distribution and overall levels of *Fgf10* were affected by RA.

RA downregulates *Fgf10* within 24 hours. As shown in Fig. 7A, peripheral expression of *Fgf10* is markedly reduced and there is expansion of *Fgf10* non-expressing areas of the subepithelial mesenchyme (compare levels near bud 1 in

control and RA panels, also compare positive areas in between buds 4 and 5). Expansion of negative areas almost extinguishes FGF10 expression between buds (compare yellow arrows in both panels) and does not result from enlargement of the distal epithelial bud (changes in bud morphology are evident only after 48-72 hours). A potential mechanism for RA-induced disruption of budding is the restriction of *Fgf10* expression to sites less accessible to the epithelium (for instance, most peripheral layers far from the tip bud), thus interfering with FGF10-mediated chemotaxis and bud induction (Park et al., 1998; Bellusci et al., 1997a).

To determine whether the RA effect on *Fgf10* required the presence of the epithelium, embryonic lungs were dissociated; mesenchymal cells were isolated from the epithelium by differential adherence and cultured in RA ( $10^{-7}$ - $10^{-5}$  M, see Methods). Assessment of gene expression by semiquantitative PCR showed that RA inhibited *Fgf10* within 24 hours (Fig. 7A). A mechanism for RA-FGF10 interaction is proposed in the Discussion and illustrated in Fig. 8B.

In addition, we investigated RA regulation of *Bmp4*, a gene that has a critical role in specification of proximal-distal lung cell fate (Weaver et al., 1999), and interacts with FGF10 during branching morphogenesis (Lebeche et al., 1999). In control 24-hour cultures, we detected strong *Bmp4* signals restricted to the tip bud epithelium. In contrast, *Bmp4* expression in RA-treated cultures becomes progressively diffuse and low at the tips (Fig. 7B, compare levels in buds 1, 5 and 6 in control and RA). Within 72 hours (Fig. 7C), while overall *Bmp4* levels decline in controls (arrowhead), RA treatment nearly abolishes *Bmp4* expression in these explants (arrow).

We have previously shown that FGF10 upregulates *Bmp4* expression in the lung (Lebeche et al., 1999). To test whether the inhibitory effect of RA on *Bmp4* was mediated by a decrease in the levels of FGF10 in the mesenchyme, we cultured lungs in RA medium and FGF10 was locally supplied by introducing an FGF10-loaded heparin bead (Park et al., 1998). By 72 hours, we found *Fgf10* (not shown) and *Bmp4* (Fig. 7C, yellow arrow) expression markedly inhibited in these explants. Nevertheless *Bmp4* levels were maintained high in the epithelium surrounding the FGF10 bead even after a 3-day RA treatment (arrowhead in right panel Fig. 7C). These results show that expression of *Fgf10* and *Bmp4* are tightly connected and can be modulated by RA during branching morphogenesis in vitro. They also show that the dramatic changes in pattern induced by RA in the lung are preceded by changes in the levels and distribution of these pattern regulators.

### P450RAI inducibility by RA in the lung

The developmental profile of P450RAI led us to propose that its increased expression in the distal lung epithelium could be part of a mechanism to prevent epithelial *lacZ* activation and thus allow proper distal bud formation. Because *P450RAI* is RA-inducible in F-9 teratocarcinoma cells (Abu-Abed et al., 1998), we anticipated that in lungs cultured with RA, *P450RAI* levels should be upregulated at distal sites. We tested this hypothesis by assessing *P450RAI* in RA-treated lungs at  $10^{-7}$  and  $10^{-5}$  M (Fig. 6F-H). Surprisingly, upregulation of *P450RAI* was not observed in the distal lung under these conditions. Distal signals in RA-treated cultures were actually lower than in controls. Instead, *P450RAI* upregulation was found in proximal airway epithelium in explants treated with  $10^{-7}$  M RA

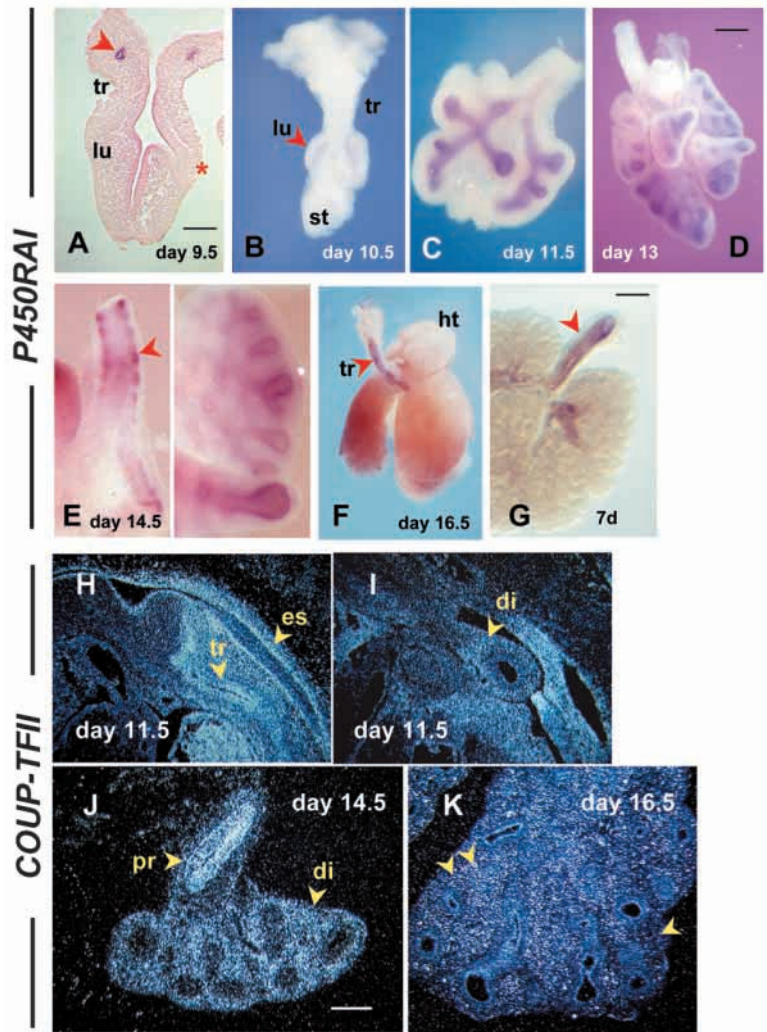
within 24 hours (Fig. 6G). At  $10^{-5}$  M, we observed dramatic induction of *P450RAI* in proximal mesenchyme (Fig. 6H). *P450RAI* was also upregulated to a lesser degree in proximal epithelium and in the mesenchyme of the accessory lobe. *P450RAI* induction was confirmed when local concentrations of RA were increased by the addition of RA-soaked beads to lung explants for 48 hours. *P450RAI* expression was upregulated in the proximal airway at the side containing the RA bead, but not where a control (PBS) bead was present (Fig. 6I). As shown in Fig. 6D,H, sites of increased expression of *P450RAI* in the mesenchyme corresponded to areas where *lacZ* activation was more prominent in RA-treated *RARE-lacZ* lungs. Conversely, distal epithelial tubules of RA-treated lungs expressed little *lacZ* and *P450RAI*. This observation is consistent with the idea that P450RAI induction in the lung is mediated by retinoid receptor activation. It is unclear however, why RA treatment failed to induce distal expression of *lacZ* and *P450RAI*. Perhaps RA induces an inhibitor of retinoid receptor function in the distal epithelium that interferes with RA induction of target genes, including *P450RAI*. Although COUP-TFII could be this inhibitor, we were unable to consistently show *COUP-TFII* upregulation by RA in lung explants due to variability of expression within RA-treated cultures (data not shown). This perhaps reflects the fact that, as levels of COUP-TFII expression increase, receptor function is impaired and further upregulation is limited. Variability could result from different stages of this process occurring at the same time in the explant.

### DISCUSSION

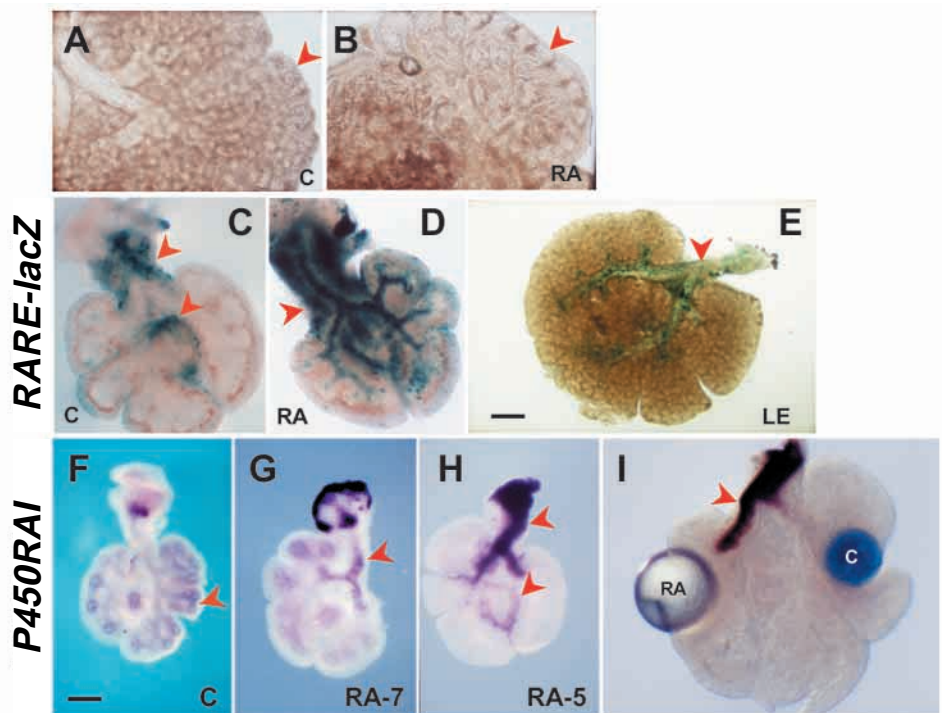
We investigated the mechanisms by which the RA pathway is regulated in the developing lung and how RA signaling influences specific developmental events during lung morphogenesis.

Our data reveal a dynamic pattern of expression of components of the RA pathway that can be summarized into three phases. Phase 1: during the initial stage of lung formation there is highly active and ubiquitous retinoid signaling. RA is abundant, and the synthetic pathway is not counterbalanced by expression of the degrading enzyme. Phase 2: this starts when lateral buds appear and is characterized by a proximal-distal gradient of RA activation, with less RA response in the distal mesenchyme near sites of budding. This decreased response at distal sites can be ascribed to local low levels of RALDH-2, limiting ligand availability and expression of COUP-TFII, antagonizing retinoid signaling. Progression of branching morphogenesis is accompanied by suppression of RAR activation in the epithelium, where local levels of RA are then controlled by P450RAI-mediated RA degradation. Furthermore, during the initial stages of branching morphogenesis, levels of RALDH-2 are regionally controlled so that its expression concentrates in areas of less branching activity. Phase 3: at the end of the pseudoglandular period, this phase is characterized by continued RA synthesis but little evidence of RA usage. RA signaling is negatively regulated in the distal lung mesenchyme by COUP-TFII, while RA levels are controlled in proximal airways by P450RAI. The presence of RA pathway components in different cell layers of the developing lung suggests the presence of tissue-specific

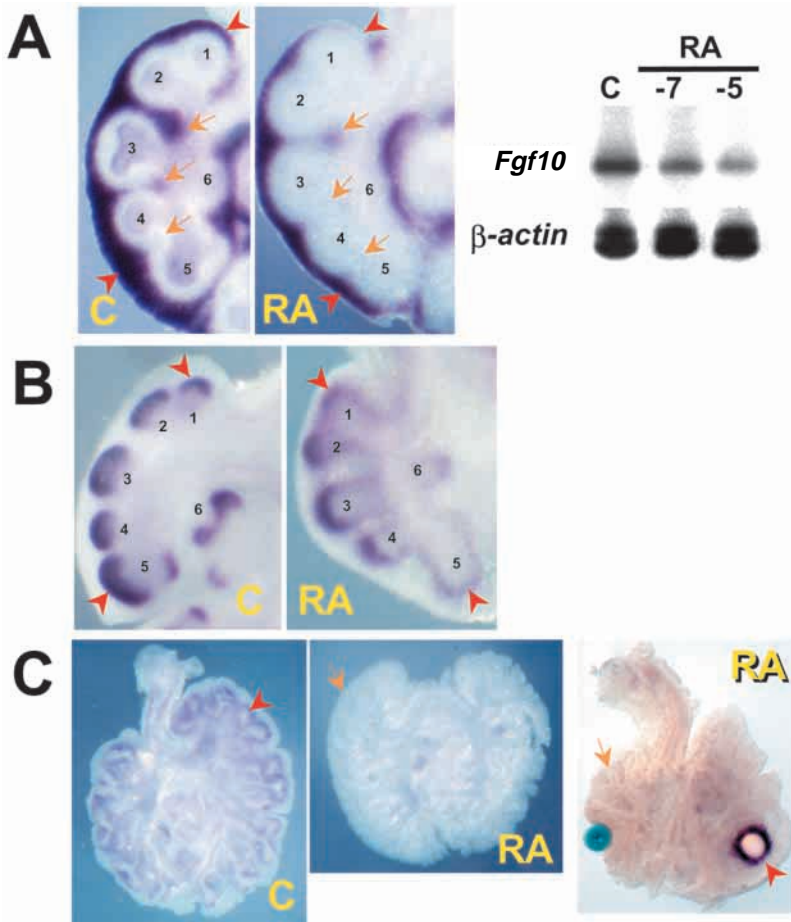
**Fig. 5.** Developmental expression of *P450RAI* (A-G) and *COUP-TFII* (H-K) in embryonic lung by in situ hybridization. (A) Section of foregut with tracheal and lung primordia (asterisks) at day 9.5 shows that *P450RAI* (arrowhead) is not expressed in the respiratory tract. (B) Weak signals are first detected at day 10.5 throughout the lung epithelium, but not in trachea or alimentary tract (st, stomach). (C-E) A gradient is established from day 11.5 to day 14.5 in the lung epithelium with highest levels in distal buds. (E) At day 14.5, expression extends proximally to encompass the trachea in both epithelium and mesenchyme. In trachea and main bronchi, mesenchymal *P450RAI* appears to delineate prospective tracheal rings (arrowhead in E). (F) At day 16.5, expression is restricted to proximal sites and is hardly detected in distal lung. (G) At late stages, proximal *P450RAI* expression (arrowhead) is confirmed in lung explants cultured for 7 days in control conditions. (H,I) At day 11.5 *COUP-TFII* (arrowhead) is expressed in trachea (epithelium and mesenchyme) and lung (mesenchyme only, slightly higher levels distally). (J) At day 14.5, epithelium of extrapulmonary airways (pr, proximal) and lung mesenchyme are heavily labeled while no expression is found in lung epithelial tubules. (K) At day 16.5, stronger *COUP-TFII* signals are found in the mesenchyme between buds (double arrowhead) than in subpleural mesenchyme (single arrowhead). es, esophagus; tr, trachea; di, distal lung. Scale bars: A, 100  $\mu$ m; D, 800  $\mu$ m; G, 120  $\mu$ m; J, 180  $\mu$ m.



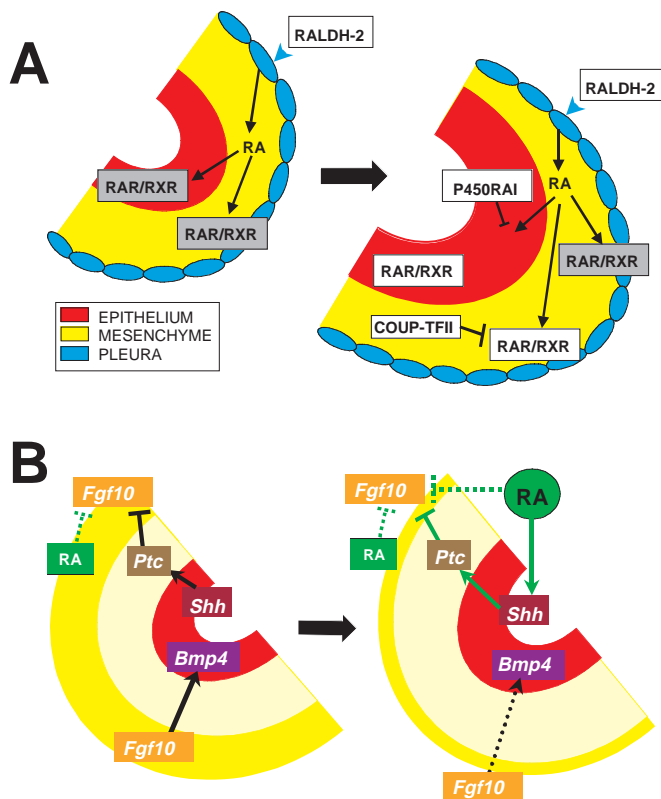
**Fig. 6.** RA effects on lung pattern formation (A,B; whole mounts), *lacZ* (C-E; X-gal staining) and *P450RAI* (F-I; whole-mount in situ hybridization) expression. (A,B) Day-11.5 lungs cultured in control and RA media for 6 days; RA  $10^{-5}$  M induces growth of immature proximal-like airways (arrowhead in B) and blocks typical distal lung development (arrowhead in A). (C) Control RARE-*lacZ* lungs cultured for 24 hours express *lacZ* (arrowhead) in mesenchyme (proximal and accessory lobe), but not in epithelium. (D) RA induces high levels of *lacZ* expression in proximal mesenchyme and epithelium at 24 hours before morphological changes are observed. (E) Late exposure to RA (LE, day 6; see Methods) activates *lacZ* in proximal epithelium (arrowhead), but has no effect on lung patterning. (F-H) *P450RAI* expression in control and RA-treated lungs at 24 hours. In controls (F), *P450RAI* is found throughout the epithelium with highest levels in distal buds. At  $10^{-7}$  M (G), *P450RAI* is upregulated in proximal epithelium. At  $10^{-5}$  M (H), expression is markedly increased in proximal mesenchyme and epithelium and slightly increased in the mesenchyme of the accessory lobe. (I) Lung explants grafted with RA and PBS (control; c) beads; *P450RAI* is upregulated on the side containing the RA bead. Scale bar: E, 100  $\mu$ m; F, 150  $\mu$ m.







**Fig. 7.** RA regulation of *Fgf10* (A) and *Bmp4* (B,C) in lungs explants (in situ hybridization) and embryonic lung mesenchymal cells (semiquantitative RT/PCR). (A) In 24-hour controls, *Fgf10* is expressed in the mesenchyme peripherally (red arrowheads) and inbetween buds (orange arrows) but not in subepithelial regions (white area immediately around distal buds, numbered 1-6). RA ( $10^{-5}$  M) downregulates *Fgf10* to almost extinguish expression inbetween buds and restrict *Fgf10* to even more peripheral regions. *Fgf10* downregulation is also detected by semiquantitative PCR in embryonic lung mesenchymal cells treated with RA ( $10^{-7}$ - $10^{-5}$  M) for 24 hours. (B) In 24-hour controls, *Bmp4* expression is strong and restricted to the distal lung epithelium, but is downregulated and diffuse in RA-treated cultures (as exemplified in buds 1, 5 and 6). (C) At 72 hours, *Bmp4* signals decline in controls (arrowhead) and are virtually absent (arrow) in RA cultures. Introduction of an FGF10-loaded heparin bead in RA-treated cultures maintains high levels of *Bmp4* in the adjacent epithelium (arrowhead). Downregulation of *Bmp4* (arrow) is found in the other regions of the explant including where a control PBS bead (blue) was grafted.



**Fig. 8.** Models describing regulation of the RA pathway during lung morphogenesis (A) and RA interactions with pattern-related genes (B). (A) The developing lung bud is represented at an initial stage (left) and during branching morphogenesis (right). RA signaling (RAR/RXR in box) is represented in gray when activated, and in white when suppressed. Pleural cells express RALDH-2, the RA synthesizing enzyme, and are a source of RA in the lung. Ligand availability is determined by regional distribution of RALDH-2. When primary buds are forming, RA ubiquitously activates RA signaling in the epithelium (red), mesenchyme (yellow) and mesothelium (pleura, blue). As secondary buds start to form and branching morphogenesis proceeds, RA signaling is suppressed in the epithelium by the appearance of the RA-degrading enzyme P450RAI, limiting ligand availability. RA signaling is also restricted in some areas of the mesenchyme by COUP-TFII, which inhibits RAR/RXR activation of target genes. See text for further explanations. (B) A developing distal bud is represented under normal conditions (left) and in the presence of exogenous RA (right). During branching morphogenesis, an RA gradient from the pleura maintains low levels of RA signaling activated in peripheral mesenchyme, preventing large increases in *Fgf10* expression. Local levels of *Fgf10* are also controlled by the Shh/Ptc pathway from the growing bud. FGF10 induces high levels of *Bmp4* at the tips and establishes a distal signaling center controlling proximal-distal pattern formation (Lebeche et al., 1999; Weaver et al., 1999). Exogenous RA activates RA signaling in the epithelium, upregulates the Shh/Ptc pathway and downregulates *Fgf10*, restricting its expression to most peripheral areas. RA may also act directly on *Fgf10*, although here, the indirect pathway might be preferentially activated (see text). Combined low levels of FGF10 and its restriction to peripheral sites less accessible to the tip bud epithelium, interferes with chemotaxis, disrupts *Bmp4* expression and prevents distal bud formation.

mechanisms to control RA signaling when lung morphogenesis is taking place (see model presented in Fig. 8).

We first describe that while the lung synthesizes RA throughout its embryonic life, branching morphogenesis and differentiation occurs in the near absence of retinoid utilization by the epithelium. This is intriguing since retinoid deficiency or blockage of retinoid signaling has been associated with disruption of lung epithelial morphogenesis. Induction of acute vitamin-A deficiency in rats during a period when the lung bud forms generates severe abnormalities, including lung agenesis (Dickman et al., 1997). This finding and our data showing abundant RA synthesis and utilization in the foregut at the onset of lung development support a direct involvement of RA in primordial lung bud formation. Targeted disruption of *RALDH-2*, the major RA synthesizing enzyme in the early embryo, results in death by gestational day 10.5 (Niederreither et al., 1999). While these mice show several defects including limb agenesis, a lung phenotype has not been reported. Interestingly, the limb defect described in *RALDH-2*<sup>-/-</sup> animals is also seen in *Fgf10*<sup>-/-</sup> mice (Min et al., 1998; Sekine, 1999). Furthermore, in *RALDH-2*<sup>-/-</sup> mutants, *Fgf10* expression is not seen in the region of the prospective limb bud, although it is not affected in other areas (Niederreither et al., 1999). This suggests that *Fgf10* regulation by RA is indirect and depends on other signals present in specific regions of the embryo. Our data are compatible with a model where inactivation of RA signaling in the mesenchyme allows FGF10 expression and bronchial budding to occur.

It is unclear whether the RA effects on *Fgf10* in the lung are direct or involve interaction with other signaling molecules in the epithelium or mesenchyme. We and others have shown that exogenous RA activates *Sonic hedgehog* (*Shh*) signaling in the developing lung epithelium (Cardoso et al., 1996; Bellusci et al., 1997a), and *Shh* inhibits *Fgf10* expression, likely via *Patched* (*Ptc*) in the mesenchyme (Bellusci et al., 1997a,b; Lebeche et al., 1999). Thus, *Shh* signaling may mediate the RA effects on FGF10. Interestingly, we show that in the absence of the epithelium, *Fgf10* is downregulated by RA ( $10^{-7}$ - $10^{-5}$  M) in lung mesenchymal cells within 24 hours (Fig. 7A). These observations support the presence of both direct and indirect (*Shh*-dependent) pathways. The peripheral distribution of *RALDH-2* and *RARE-lacZ* signals and their exclusion from the epithelium of the day-11 to -12 lung suggest that during normal branching morphogenesis RA acts on *Fgf10* independently from the *Shh* pathway. In contrast, when exogenous RA activates *RARE-lacZ* expression in the epithelium and disrupts distal development, upregulation of the *Shh* pathway might play a critical role on *FGF10* downregulation (Fig. 8).

Data from this and our previous studies suggest that lung development is arrested when mechanisms that establish a proximal-distal gradient of RA or prevent RA activation are overridden by excess RA (Cardoso et al., 1995, 1996). Recently, BMP4 signaling has been implicated in specification of the proximal-distal axis of the developing lung. Weaver and collaborators (1999) reported a proximalized phenotype in lungs of transgenic mice expressing a dominant negative BMP4 receptor or a BMP4 antagonist. They propose that a combination of distal signals including FGF10 and BMP4 generates an apical signaling center in the distal lung bud to regulate proximal-distal axis specification. Our results are

consistent with the idea that RA interferes with the establishment of this distal signaling center. By doing so, RA maintains the lung in a proximal-like immature stage and distal development does not proceed. This stage is characterized not only by morphologic criteria, but also by a pattern of gene expression reminiscent of an immature lung (data from here and Cardoso et al., 1995, 1996). The data suggest that during normal development, restriction of RA signaling in time and space is fundamental for correct expression of *Fgf10* and *BMP4* and distal morphogenesis. RA modulates *Fgf10* expression and FGF10 seems to be necessary for inducing and restricting *Bmp4* signals to the distal epithelium. These ideas are summarized in the diagram in Fig. 8.

Although our data show that RA signaling appears to become less important in the lung at later developmental stages, *RALDH-2* expression is maintained at appreciable levels even around birth. As suggested by the low RA utilization and the presence of COUP-TFII, a tight control of RA signaling may still be important at late stages to maintain or inhibit expression of RA targets in the peripheral mesenchyme. It is conceivable that RA plays distinct roles in early and late branching.

Analysis of retinoid receptor double mutants shows that some of the defects found in the lung are likely due to developmental arrest or growth retardation (Mendelsohn et al., 1994). Activation of RARs in the lung epithelium seems to be characteristic of an immature status of differentiation that can be prolonged in culture by high concentrations of RA. Interestingly, as shown in our previous studies, the embryonic lung is rather resistant to low concentrations of exogenous RA (Cardoso et al., 1995, 1996). Here we propose that at least two mechanisms prevent inappropriate activation of retinoid signaling in the developing lung. Under physiological conditions P450RAI seems to control ligand bioavailability in the epithelium, while COUP-TFII inhibits retinoid receptor function in the mesenchyme and possibly in the epithelium of extrapulmonary airways. Over- or misexpression of RA upregulates P450RAI expression, suggesting the presence of feedback systems regulating RA bioavailability. The efficiency of these feedback mechanisms controlling RA signaling may explain why changes in lung pattern formation are seen only at high RA concentrations.

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