Down-regulation of Retinoic Acid Receptor α Signaling Is Required for Sacculation and Type I Cell Formation in the Developing Lung

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Abstract

Although retinoic acid (RA) has been shown to be critical for lung development, little is known about when RA is required and the role of individual RA receptors (RAR) in this process. Previously reported data from an RA responsive element RARE-lacZ reporter mouse show that when epithelial tubules are branching and differentiating RA signaling becomes markedly down-regulated in the epithelium. It is unclear why this down-regulation occurs and what role it might play in the developing lung. Here we analyze the effects of preventing potential progenitors of the distal lung from turning off RA signaling by locally expressing constitutively activated RARα or RARβ chimeric receptors (RARVP16) in branching airways of transgenic mice. Continued RA activation resulted in lung immaturity in both cases, but the phenotypes were remarkably different. RARαVP16 lungs did not expand to form saccules or morphologically identifiable type I cells. High levels of surfactant protein C (Sp-C), thyroid transcription factor-1 (Ttf1), and Gata6, but not Sp-A or Sp-B in the epithelium at birth suggested that in these lungs differentiation was arrested at an early stage. These alterations were not observed in RARβVP16 lungs, which showed relatively less severe changes. Our data suggest a model in which activation of RAR signaling at the onset of lung development establishes an initial program that assigns distal cell fate to the prospective lung epithelium. Down-regulation of RA signaling, however, is required to allow completion of later steps of this differentiation program that ultimately form mature type I and II cells.

Retinoids play an important role in development and homeostasis of a variety of organs, including the lung (1,2,3). Retinoic acid (RA), the active form of retinoids, binds to its nuclear receptors RARs and RXRs (isotypes α, β, and γ), which heterodimerize to form the functional unit that transduces RA signaling (4). In the lung these receptors are expressed from the earliest developmental stages throughout embryonic and postnatal life. While RARβ and RARγ transcripts are regionally and dynamically distributed in the developing lung, expression of RARα and all RXRs is ubiquitous and does not change in time or space. At the onset of lung development (E9.5) these receptors are present in the foregut endoderm in largely overlapping...
domains. During branching and until birth, however, epithelial expression of RARβ in the lung is excluded from the distal buds and is maintained in proximal and medium size airways. The distal epithelium expresses RARγ in addition to the ubiquitously expressed RARα (5-8).

The role of these receptors in organogenesis has been studied by genetic inactivation of RAR/RXRs individually or in combination in mice. Dramatic abnormalities reported in RARα/RARβ double knockout mice, which include unilateral agenesis and contralateral lung hypoplasia, strongly support a role for these receptors in lung morphogenesis (3). Nevertheless, it is still unclear which function individual RARs might have, since single knockout mice show few or no lung abnormalities, presumably due to functional redundancy (4). As suggested by in vitro studies, this redundancy does not necessarily demonstrate that one RAR can substitute for another under normal wild-type conditions (9).

Another issue that remains unclear relates to when lung epithelial cells require retinoids to grow and differentiate. The presence of high levels of the RA synthesizing enzyme retinaldehyde dehydrogenase-2 (Raldh2) and RARElacZ reporter gene expression in the E9.5 lung suggests that RA is required at the onset of lung development (6). Severe disruption of RA signaling in mice lacking Raldh2 or in vitamin A-deficient rats results in early embryonic lethality or in lung agenesis, conditions that do not allow the evaluation of retinoid-dependent events in the lung at later stages (10,2). A number of studies in lung cell and organ cultures implicate RA in branching morphogenesis and lung epithelial differentiation (11,12,13). Nevertheless, the role of endogenous retinoids in these events in vivo has been challenged by the observation that RA signaling is markedly down-regulated in epithelial tubules when branching and differentiation are taking place in the embryo (6). Here we investigated the role of this down-regulation and how growth and differentiation of the distal lung epithelium are influenced by RA signaling in vivo.

To address these issues we tested the effect of maintaining RA signaling activated solely in the lung epithelium and in an isotype-specific fashion throughout embryonic lung development. We generated transgenic mice expressing ligand-independent, constitutively active RARs under the control of the lung epithelial-specific surfactant protein C (Sp-C) promoter (14). We used chimeric receptors in which the C terminus of RARα or β is fused to the acidic activation domain (AAD) of the herpes simplex viral protein VP16. Analyses of the transactivation properties of these receptors show that, when co-transfected with a reporter gene containing an RA responsive element, the RARVP16 differentially activates gene expression in P19 and CV1 cells. The chimeras rely on endogenous retinoid receptors for heterodimerization and promoter recognition and appear to mimic many of the functions of the endogenous receptors (15,16).

Here we report important functional differences between RARα and β not revealed by former genetic approaches. αVP16 lungs did not expand to form saccules or morphologically identifiable type I cells; distal identity was maintained, but differentiation was arrested at a stage reminiscent of that of an early pseudoglandular lung. These alterations were not observed in β/VP16 lungs, which underwent later stages of differentiation and showed relatively less severe changes. Our data support the idea that distal lung differentiation cannot occur in the presence of activated RA signaling. They also indicate that putative precursors of the distal lung in branching tubules selectively respond to RARα activation by maintaining a distal developmental program characteristic of early stages. We propose a model in which RA signaling, via RARα, primes early lung bud cells to become distal, being subsequently down-regulated to allow further steps of the distal differentiation program.
MATERIALS AND METHODS

Generation of Transgenic Mice

The chimeric receptors used in our study have been previously described and tested in cell lines by Underhill et al. (16). They consisted of the C terminus of the human RARα or the mouse RARβ2 genes fused to the acidic activation domain of the herpes simplex virus protein VP16. RARαVP16 (1282 bp) and RARβ2VP16 (1516 bp) constructs were cloned into the HindIII-SpeI sites of a pGL3 vector (Promega) containing a 3.7-kb fragment of the human surfactant protein C (Sp-C) promoter. This promoter has been shown to direct transgene expression specifically to the lung distal epithelium, with increasing activity toward birth (14). The resulting Sp-C-RARVP16 constructs (Fig. 1A) were digested with AatII and NotI, purified and injected in FVB mouse fertilized eggs, and subsequently transplanted into pseudopregnant mothers. A diagram summarizing the expression pattern of endogenous and transgenic RARs is presented in Fig. 1B. Transgenic mice were identified by Southern blot analysis of tail genomic DNA (10 μg) using a 300-bp BglII-BamHI VP16 fragment as a probe (16). A calibration curve with serial dilutions of the digested plasmid was run in parallel using 10 μg of wild-type genomic DNA as a carrier to estimate number of copies of transgene inserted per genome. To check whether RA signaling was activated by transgene expression in RARVP16 expressing lungs, we crossed Sp-C-RARβ-VP16 mice with a well characterized reporter mouse in which lacZ is under the control of a RA response element (RARE)-hsp68 promoter (17). β-galactosidase staining of Sp-C-RARβ/RARE-lacZ lungs was performed at E18.5 as described in Malpel et al. (6).

Morphology/Transgene Expression

For all procedures lungs were fixed with 4% paraformaldehyde overnight at 4 °C and processed for paraffin sections (5 μm). Histological analysis was performed in hematoxylin-eosin (H&E)-stained sections. Sites of transgene expression were determined by in situ hybridization and immunohistochemistry using RAR isotype-specific reagents and anti VP16 antibody (see below).

In Situ Hybridization

We performed isotopic in situ hybridization in lung sections, as previously described (18), using 35S-labeled ribo-probes synthesized from cDNA clones of Shh, Ptc, Bmp4 (gifts from Dr. Andrew P. McMahon, Harvard Biolabs), Tgfβ1 (gift from Dr. Parviz Minoo, University of Southern California), RARα and β (gifts from Dr. Cathy Mendelsohn, Columbia University), Fgf10 (gift from Dr. Nobuyuki Itoh, Kyoto University), Gata6 (gift from Dr. Michael Parmacek, University of Pennsylvania), Sp-A, Sp-B, and Sp-C (12,18). Briefly, sections were deparaffinized and re-hydrated. Pre-hybridization included treatment of sections with 4% paraformaldehyde, digestion with proteinase K (20 μg/ml), and acetylation with 0.25% acetic anhydride/1.4% triethanolamine. Sections were hybridized overnight at 50 °C and subsequently washed with 5× SSC-50% formamide, dehydrated with graded alcohols/0.3 M ammonium acetate, and dried at room temperature for autoradiography. Sections were dipped in photographic emulsion (Kodak, NTB-2), incubated at 4 °C for 1–3 weeks, and developed in Kodak D-19 developer.

Antibodies/Immunohistochemistry

Sections were deparaffinized, rehydrated, and washed in PBS. Endogenous peroxidase activity was quenched with 0.3% hydrogen peroxide in methanol. Sections were preincubated with the appropriate preimmune serum (rabbit or goat), and then incubated with primary antibody overnight at 4 °C. The antibodies used in this investigation were: Clara cell secretory protein (Ccsp, goat, polyclonal anti-rabbit, gift from Drs. Singh and Katal, University of Pittsburgh),
T1α (mAB 8.1.1, Developmental Studies Hybridoma Bank, University of Iowa), VP16 (rabbit, polyclonal anti-mouse, Clontech), RARα and β (rabbit, polyclonal anti-mouse, gifts from Dr. Pierre Chambon). Immunostaining was performed using the anti-rabbit and anti-goat IgG Vectastain and the DAB staining kits from Vector Laboratories, according to the manufacturers' protocol. For counterstaining, methyl green was used.

Assessment of Cell Proliferation/Death

Changes in cell proliferation were assessed by PCNA staining (cell proliferation kit, Zymed Laboratories Inc.). Cell death was investigated by TUNEL assays (Apoptag kit, Intergen). Immunostaining was performed as above or according to manufacturer’s specifications.

Morphometric Analyses

We performed a computer-assisted morphometric analysis of T1α-stained sections to estimate the area occupied by labeled (distal epithelium) versus unlabeled (mesenchyme) cells in control and βVP16 transgenic lungs. T1α specifically labels type I cells, which encompass the vast majority of the epithelial surface of the distal lung (19). Digital images from 10 random peripheral fields were acquired at 40× magnification and analyzed using OpenLab software (Improvision, Inc.) with the density slice module and advanced measurement settings. Optical densities correspondent to epithelium (T1α positive) or mesenchyme (non-positive tissue) were estimated. Results were normalized as percent of total tissue to correct for differences in inflation of the lungs. In addition, we used TUNEL- or PCNA-stained sections to estimate the number of cells undergoing apoptosis or proliferation in wild-type, α, and β VP16 mice. For this we counted labeled and unlabeled nuclei in epithelium and mesenchyme in 6–8 random peripheral fields at 40× magnification (~250 cells per field). Results were expressed as percent of total cells.

Periodic Acid Schiff (PAS) Staining

We used the PAS-staining kit (Sigma) to detect glycogen in lung sections. Briefly, after being deparaffinized and rehydrated, sections were preincubated with either buffer (PBS) solution or diastase solution, for 10 min. After washing with PBS both groups were treated with 0.5% periodic acid solution for 5 min and then with Schiff's reagent for 1 min. Sections were counterstained with hematoxylin. Specificity of staining for glycogen was confirmed by loss of PAS signals when sections were pretreated with diastase.

RESULTS

Sp-C-RARVP16 Regulation by RA

We performed a preliminary study in the mouse lung epithelial cell MLE 15 cells to check whether the 3.7kb Sp-C promoter fragment was itself regulated by RA. MLE 15 are immortalized type II epithelial cells originated from lung tumors of transgenic mice expressing the SV40 large T antigen under the control of the same 3.7-kb Sp-C promoter used here (20). We treated MLE15 cells with RA (10^-9-10^-5 M) for 24 h and assessed expression of SV40 large T antigen by semiquantitative PCR. Fig. 1C shows that levels of SV40T were not significantly changed by RA at any of the concentrations tested. Thus, RA signaling induced by the Sp-C-RARVP16 constructs is not expected to interfere with its own transcription.

RARα and β Activation Differentially Affects Survival

To investigate viability at birth and to obtain F0 founders, pregnancies were allowed to reach term and pups were observed during their initial 24 h of life. While 2 of 4 pups identified as βVP16 transgenics survived and reached adulthood, no αVP16 newborn from three independent injections lived for more than 10 min. At least 4 αVP16 pups died at birth; 3 were
found partially cannibalized by the mother. An additional injection of the αVP16 construct was performed and embryos were dissected at E18.5; one was identified as transgenic. In all transgenics lung morphology was grossly preserved; proximal and distal lung formed and the number of lobes was normal. Collapse or uneven inflation was common in both α and β transgenics at birth. Transgenic lungs were not markedly different in size from wild type, although αVP16 mice were slightly smaller than the other groups at E18.5 and at birth, as illustrated in Fig. 1, D and E. βVP16 mice that reached adulthood were apparently normal and fertile; however, one of the lines developed lung tumors within the first year of age. In the present study we analyzed the lung phenotype of transient transgenics αVP16 or βVP16 that died at birth.

RARBVP16 Activates RARE-lacZ Reporter Gene

Because some βVP16 pups survived and their lungs appeared grossly normal, we tested whether the βVP16 construct efficiently activated RA signaling in the lung. We crossed mice from a surviving line (RARβVP16, copy number = 5) into a background of a RARE-lacZ transgenic reporter mouse previously shown to express lacZ at sites where RARs are activated (17). Xgal staining of Sp-C-RARβVP16/RARE-lacZ lungs at E18.5 confirmed lacZ expression in the distal lung epithelium (Fig. 1G) where we expected to find RARBVP16 transcripts. This pattern of staining and the absence of lacZ signals in lungs of wild-type littermates (Fig. 1F) indicated that RA signaling, normally inactive at this stage (6), was activated by the βVP16 transgene in the distal lung.

Expression of Endogenous RARs and RARVP16 Transgenes in the Lung

To determine sites of transgene expression we first performed immunohistochemistry of lung sections using a polyclonal antibody raised against the VP16 epitope. Fig. 2 (A–C) shows specific VP16 staining in distal lung epithelium of both transgenics, not observed in wild type. We then assessed RAR expression by in situ hybridization using isotype-specific RAR probes that recognized both endogenous and chimeric receptors. At birth wild-type lungs express ubiquitous endogenous RARα and no RARβ signals in the distal epithelium (Fig 2, D and G and Ref. 5). αVP16 transgene expression was readily identified by the strong distal signals, in sharp contrast to the low levels of endogenous RARα (Fig. 2E). This is also illustrated in βVP16 lungs, where high levels of RARβ expression in type II cells and epithelial cells occupying the most distal portions of terminal bronchioles (Fig. 2I, red arrowhead) contrast with the low endogenous signals in proximal airways (Fig. 2G, yellow arrowhead). Although transgenic mice had different gene copy numbers (αVP16 n = 1, βVP16 n = 20), the chimeric receptors appeared to be expressed in the distal lung epithelium at equivalent high levels in a per cell basis. No significant difference in the intensity of RAR signals in the distal epithelium was observed by in situ hybridization (Fig. 2, E and I) or immunostaining (Fig. 2J) when sections of αVP16 and βVP16 lungs were processed in parallel. Because of the consistent lethal phenotype at birth, we could not generate a line of αVP16 mice in RARElacZ background as we did with βVP16 mice. However, the presence of endogenous RARB ectopically induced in distal epithelial tubules of αVP16 lungs indicated that RA signaling was efficiently activated (Fig. 2H, red arrowhead). Together the data suggest that the difference in the severity of the α or βVP16 phenotypes is likely related to the type of RAR being activated rather than to a difference in levels of RAR expression of each transgenic construct.

Constitutively Activated RARα, but Not β Disrupts Type I Cell Development and Sacclulation

At birth the distal lung of a wild type mouse consists of saccular structures lined by flat type I cells, and by cuboidal surfactant-producing type II cells (Fig. 3A). Expression of αVP16 transgene dramatically altered the architecture of the distal lung (Fig 3B). Formation of typical saccules was impaired and instead, tubule-like structures lined by a low columnar epithelium
that lacked flat type I-like cells were present. Consistent with disruption of type I cell development was the nearly absent expression of the marker T1α in these tubules (Fig. 3E). Mature type I cells characteristically express T1α, aquaporin 5, caveolin 1, and ICAM 1 (19). Genetic studies, however, show that among these markers only T1α loss affects type I cell formation when its expression is disrupted in mice (21). Overall, αVP16 expressing structures resemble immature epithelial tubules normally seen in the lung at the early pseudoglandular stage (∼E10-11). The phenotype was consistently seen in αVP16 lungs at E18.5 and at birth. By contrast, none of these changes were observed in βVP16 lungs, which showed saccular structures with morphologic features compatible with typical type I and II epithelial cells using T1α staining (Fig. 3, C and F). Expression of α or βVP16 transgenes did not disrupt proximal epithelial differentiation as seen by the proper expression of Clara cell secretory protein (Ccsp) and location of ciliated cells (Fig. 3, G–I). Moreover, blood vessel formation was not grossly disrupted in any of these mice as indicated by morphology, PECAM staining of endothelial cells, or epithelial expression of the angiogenic factor VEGF (data not shown).

Activation of RARα but Not β Prevents Expression of Sp-B and Sp-A

To investigate the differentiation status of the RARVP16 epithelia we assessed expression of early (Sp-C) and late (Sp-B, Sp-A) markers of lung type II cells (22). During normal development Sp-C expression has been reported in the lung by in situ hybridization at E10–10.5, but signals can be detected by PCR in the foregut endoderm as early as E9–9.5 (Refs. 14 and 23).2 Fig. 4A illustrates the restricted expression of Sp-C to type II cells in wild-type neonatal lungs. Strong Sp-C signals were found in the majority of the cells lining distal epithelial tubules of αVP16 lungs (Fig. 4B). These tubules, however, showed virtually no expression of Sp-B and Sp-A, markers that, unlike Sp-C, normally appear in the distal epithelium only by E14 and E16, respectively (Fig. 4, D, E, G, and H; Ref. 22). These and the results from the former section indicate that αVP16 epithelial cells are distal in fate, but are likely immature. Moreover, they suggest that both programs of type I and type II cell differentiation, characteristic of later stages of development, are disrupted by constitutive activation of RARα. By contrast strong Sp-C, Sp-B, and Sp-A signals were detected in the epithelium of βVP16 mice, as seen in wild type (Fig. 4, C, F, and I).

Lung Cell Proliferation and Cell Death Are Altered by Constitutively Activated RARs

PCNA immunostaining of wild-type newborn lungs showed few scattered labeled cells in the distal lung, confirming previous reports that distal cell proliferation is minimal at birth (Fig. 5A and Ref. 8). By contrast αVP16 lungs showed a dramatic increase in number of PCNA-labeled nuclei (Fig. 5, B and D). Labeling was predominantly epithelial (∼80% of total labeled cells) but was also present in the mesenchyme (20%). Since the αVP16 lungs are not bigger than wild type lungs, we reasoned that the increase in proliferation should be accompanied by transgene-induced increase in cell death. Thus, we estimated the number of apoptotic nuclei in these lungs by TUNEL assay. In wild-type TUNEL-labeled nuclei were infrequent; most of the cells counted in the distal fields (∼90%) were unlabeled (Fig. 5, E and H). In αVP16 lungs, however, TUNEL-positive cells averaged 40% of total cells; 70% of the labeled cells were epithelial (Fig. 5, F and H). The increased cell proliferation and cell death in the peripheral lung suggest that rapid turnover was taking place at sites of αVP16 transgene expression. The changes described above were also found, but to a lesser extent, in βVP16 lungs (Fig. 5, C and G). It is likely, however, that cell proliferation and cell death are stimulated at comparable levels by both α and β transgenes. Differences in PCNA labeling depicted in Fig. 5D possibly reflect the fact that αVP16-expressing cells, unlike the βVP16, do not undergo type I cell differentiation, and thus continue to proliferate.

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2M. Ramirez, personal communication.
Ttf1 and Fgf10 Are Up-regulated in αVP16 Lungs

To gain insights into how the RARVP16 phenotypes originated, we investigated expression of thyroid transcription factor-1 (Ttf1 or Nkx 2.1), and fibroblast growth factor Fgf10, known regulators of early distal lung development. No distal lung structures form in mutant mice lacking these genes (24-28). At the onset of lung development Fgf10 and Ttf1 are present at high levels in the mesenchyme and epithelium of the lung primordia, respectively. Both are expressed in the distal lung throughout branching morphogenesis; however, around birth, signals become less prominent and more diffuse (24-26). Our in situ hybridization revealed strong Ttf1 signals in the distal epithelium of αVP16 neonatal lungs more typical of earlier developmental stages. This contrasted with the low level signals seen in βVP16 and wild-type littermates (Fig. 6, A–C). High levels of Fgf10 were detected in the mesenchyme associated with αVP16 tubules, which was not seen in βVP16 and wild-type lungs (Fig. 6, D–F). This finding suggests that epithelial activation of RARα indirectly influences mesenchymal gene expression (see discussion).

Gata6 Is Differentially Up-regulated in αVP16 Epithelium

There is strong evidence implicating the transcription factor Gata6 in acquisition of late features of differentiation in the developing lung (29-33). We investigated Gata6 expression in αVP16 lungs because of its essential role in type I cell formation (31-33) and because Gata factors are RA targets (34). During normal development, Gata6 expression initiates in the lung epithelium at E10.5 and continues up to E17.5 being subsequently down-regulated once sacculation commences (30,33). Here, we found that at birth αVP16 epithelial tubules maintained high levels of Gata6 expression typically seen in immature lungs at the pseudoglandular stage. This contrasted with wild-type and βVP16 tubules in which only low levels of Gata6 signals were observed (Fig. 6, G–I). This suggested that proper regulation of Gata6 expression cannot occur in the presence of continued activation of RARα signaling.

βVP16 Lungs Have Late Features of Differentiation but Are Less Mature than Wild-type Lungs

As described above, βVP16 lungs dramatically differed from the αVP16 lungs in which they undertook further steps toward distal differentiation. Nevertheless, these lungs had features of immaturity not observed in wild type. Histological analysis showed that airspaces were separated by a thick mesenchymal layer, inappropriate for gas exchange (Fig. 7A and Fig. 3C). An increased proportion of mesenchyme to epithelium in βVP16 lungs was inferred by a decrease in the area occupied by type I cells in distal fields, as assessed by morphometric analysis of T1α-stained sections (see “Materials and Methods”) (Fig. 7A, graph). We also found that type II cells of βVP16 lungs preserved high content of glycogen in their cytoplasm at birth. Periodic acid-Schiff (PAS) staining of βVP16 lungs showed intense labeling of the distal epithelium similarly to that seen in a wild-type E16 lung (Fig. 7, B–D). PAS-positive glycogen vacuoles have been described in the normal distal epithelium at around E14.5; however, further maturation of type II cells is characterized by progressive loss of glycogen and appearance of surfactant inclusions (35). No PAS labeling was detected in the αVP16 expressing epithelium, consistent with the idea that these lungs are even less mature than their βVP16 counterpart (data not shown). How βVP16 expression influenced this phenotype is still obscure. βVP16 mice that reached adulthood looked normal.

DISCUSSION

Distal Lung Development in the Presence of Continued Activation of RARs in the Epithelium

Here we provide in vivo evidence that distal lung forms but proper differentiation cannot occur if RA signaling is maintained active in branching epithelial tubules. Our model is fundamentally different from in vivo or in vitro systems in which exogenous RA is ubiquitously
available to all cells. In previous studies where we reported disruption of distal morphogenesis by all-trans RA in lung organ cultures, the mesenchymal effects could not be distinguished from those in the epithelium because the whole explant was exposed to RA. We ascribed the RA effects to the inhibition of mesenchymal Fgf10 expression and downstream events (12, 18, 6). By expressing ligand independent constitutively activated RARs in the distal epithelium: 1) we maintained a status of RA activation characteristic of the early lung throughout late developmental stages, 2) we prevented potential progenitors of the developing distal lung epithelium from turning off RA signaling, 3) we avoided the direct effects of RA in the mesenchyme and we could study the primary effects of RA in the lung epithelium. Previously reported phenotypes of RAR/RXR double knockouts already suggested that distal lung formation is more critically affected by lack of RARα than by RARβ signaling (36). In the present work a gain of function model was designed to study distal lung development under activation of a particular RAR when endogenous RA signaling by all receptors is down-regulated. We focused on RARα and β because of the unique lung defects reported in the double knockout mice (3). The left lung agenesis and right lung hypoplasia described in these mutants is difficult to interpret in light of our results and expression pattern studies and likely results from disruption of early RA-dependent events such as left-right body plan specification (37, 38). Although not defining RAR specific function, these studies point to a potential role of RA in regulating the appearance of progenitors of the prospective lung in the primitive foregut. A recent report demonstrates that progenitors of the distal lung are specified well before the onset of lung development (23) at stages that overlap with the establishment of RA signaling in the anterior foregut (17,10).

RARα Regulates a Distal Program of Differentiation Characteristic of That of an Early Lung

A prominent feature of the αVP16 transgene at birth is the presence of Sp-C, Ttf1 and Gata6 in immature and highly proliferative epithelial tubules. During normal development these features are first seen together when secondary buds are forming (E10.5), coincidently with the down-regulation of RA signaling in the epithelium (6,14,26,30). There is evidence suggesting that at least in structures such as the anterior foregut, RA may synergize with resident signaling molecules and initiate a Gata-dependent developmental program (34). Whether in the lung RARα activity in primary buds serves to initiate Gata6 expression in secondary buds, we cannot determine. In our model αVP16 epithelial cells expanded and preserved their distal identity but they did not undergo further steps toward distal differentiation. Constitutive RARα activation interfered with the proper temporal expression of Gata6, a gene that is critical to regulate surfactant protein expression in branching epithelial tubules and to establish the mature type II and type I cell phenotype (30-33). The αVP16 phenotype shared some of the features previously described in genetic models in which Gata6 expression is altered. For example, when perinatal high levels of Gata6 are maintained in the distal lung by expression of a Sp-C-Gata6 transgene, Ttf1 levels are increased, and terminal differentiation of type II and type I cells is blocked (32). In the Xenopus developing heart down-regulation of Gata6 is critical for progression of the cardiomyogenic differentiation program; like in our model, this differentiation program can be disrupted by artificially maintaining high levels of Gata6 in heart cells (39). Although in our study Gata6 and Ttf1 disregulation represents an important component of the αVP16 phenotype, the overall changes reflect disruption of a broader mechanism controlled by RA that is similarly found in other developing systems. In structures such as the limb bud, attenuation of RAR mediated signaling is critical for skeletal development. While required for early limb development, continued RA signaling at later stages interferes with the differentiation of chondroprogenitors (40). The above examples underscore a more general role of retinoids in controlling timing of progenitor cell differentiation in various systems.
**RARα Regulation of Epithelial-Mesenchymal Interactions**

Another interesting feature of αVP16 lungs was the presence of strong Fgf10 signals in distal mesenchyme (Fig. 6D). Since in this model activation of RA signaling is restricted to the epithelium (as demonstrated by epithelial up-regulation of endogenous RARβ, Fig. 2H), it is likely that epithelial RARα indirectly controls expression of a diffusible signal that regulates Fgf10 expression in the mesenchyme. A potential candidate could be sonic hedgehog (Shh), an epithelial target of RA known to inhibit Fgf10 in lung organ cultures (18,24). We found low levels of expression of Shh and its receptor Patched (Ptc) in αVP16 lungs (data not shown). The relevance of this observation for the αVP16 phenotype, however, was difficult to evaluate, since at this stage Shh signals were also low in βVP16 and wild type. For the same reason we could not conclude that bone morphogenetic protein 4 (Bmp4), a gene whose expression in branching epithelial tubules is activated by Fgf10 (41), was critically affected by RARVP16. Low levels of Bmp4 were similarly present in the distal epithelium of all lungs perinatally (data not shown). Although αVP16 lungs were slightly smaller than wild type, epithelial activation of the FGF pathway and branching did not seem to be greatly affected by αVP16. Similar Sp-C driven transgenic models that target components of the Fgf pathway to the distal epithelium show dramatic disruption of branching morphogenesis not observed in our transgenes (42-44).

**RARβ Has a Less Defined Role in Distal Development**

In our model maintained activation of RARβ signaling in distal tubules did not prevent the appearance of type I cells and allowed late features of type II cells to develop. Since both transgenes were efficiently expressed in each model, the likely explanation for the difference in phenotype is that RARβ targets are not directly involved in regulation of distal epithelial development. There is evidence that RARβ mediates the inhibitory effect of exogenous RA in distal bud morphogenesis in vitro (12,18). Mollard et al. (45) have shown that this effect is markedly attenuated in lungs from RARβ−/−, but not from α or γ null mutant mice. They postulate that RARβ activation in airways favors morphogenetic stabilization as opposed to budding, and, thus, it is more likely to be involved in formation of proximal, conductive airways (45). Although we do not have evidence that RARβ targets act in distal development, it is possible that, when expression of RARα is disrupted, RARβ signaling may serve as a backup system to rescue RARα function. Both receptors have been reported in largely overlapping domains in the foregut endoderm at the onset of lung development (7).

**Concluding Remarks**

Our data summarized in Fig. 8 provide genetic evidence that RARα and β have nonoverlapping targets in the developing lung epithelium and supports the idea of stage-specific requirement for retinoids in the embryonic lung. We propose a model in which activation of RARα signaling at the onset of lung development establishes an initial program that either assigns distal cell fate to the prospective lung epithelium or, alternatively, contributes to expand an initial pool of distal progenitor cells that will subsequently differentiate into the distal lung. Down-regulation of RA signaling, however, is required to allow completion of later steps of this differentiation program and, ultimately, to form mature type I and II cells. Based on this and other reports (6,46), the model predicts that a window of RA requirement for this process occurs in the lung during its initial stages of embryonic development and lasts up to when branching initiates. A number of studies indicate that during neonatal life RA is again required to regulate morphogenetic processes, in this case, formation of the definitive alveoli. These reports support the idea that, during alveolization, RARs also activate retinoid-dependent cellular events in a time and isotype-specific fashion (46-48).
Acknowledgments

We thank Jeffrey Whitsett for providing us with the Sp-C promoter construct used to generate the transgenes and Janet Rossant and Nadia Rosenthal for providing us with the RARE-lacZ mouse line. We thank Andrew McMahon, Parviz Minoo, Cathy Mendelsohn, Nobuyuki Itoh, and Michael Parmacek for cDNA clones; and Pierre Chambon, Sikandar Katyal, and Michael Parmacek for antibodies. We thank Jining Lu, Jerome Brody, Mary Williams, and Cathy Mendelsohn for critical reading of the manuscript. We are also grateful to Anne Hinds, Guetchyn Millien, Xiaoqing Qi, Jun Qian, and Renee Anderson for excellent technical assistance. We would like to thank Hou-Xiang Xie and Robin McDonald from the Boston University Transgenic Core Facility.

REFERENCES

1. Wilson JG, Roth CB, Warkany J. Am. J. Anat 1953;92:189–217. [PubMed: 13030424]
2. Dickman ED, Thaller C, Smith SM. Development 1997;124:3111–3121. [PubMed: 9272952]
3. Mendelsohn C, Lohnes D, Decimo D, Lufkin T, LeMeur M, Chambon P, Mark M. Development 1994;120:2749–2771. [PubMed: 7607068]
4. Chambon P. FASEB J 1996;10:940–954. [PubMed: 8801176]
5. Dolle P, Ruberte E, Leroy P, Morriss-Kay G, Chambon P. Development 1990;110:1133–1151. [PubMed: 1966045]
6. Malpel SM, Mendelsohn C, Cardoso WV. Development 2000;127:3057–3067. [PubMed: 10862743]
7. Mollard R, Viville S, Ward SJ, Decimo D, Chambon P, Dolle P. Mech. Dev 2000;94:223–232. [PubMed: 10842077]
8. Hind M, Corcoran J, Maden M. Am. J. Physiol. Lung Cell Mol. Physiol 2001;282:L468–L476. [PubMed: 11839540]
9. Taneja R, Roy B, Plassat JL, Zusi CF, Ostrowski J, Reczek PR, Chambon P. Proc. Natl. Acad. Sci. U. S. A. 1996;93:6197–6202. [PubMed: 8650243]
10. Niederreither K, Subbarayan V, Dolle P, Chambon P. Nat. Genet 1999;21:444–448. [PubMed: 10192400]
11. Schuger L, Varani J, Mitra R Jr. Gilbride K. Dev. Biol 1993;159:462–473. [PubMed: 8405671]
12. Cardoso WV, Williams MC, Mitsialis SA, Joyce-Brady M, Rishi AK, Brody JS. Am. J. Respir. Cell Mol. Biol 1995;12:464–476. [PubMed: 7742011]
13. Metzler MD, Snyder JM. Endocrinology 1993;133:1990–1998. [PubMed: 8404646]
14. Wert S, Glasser SW, Korfhagen TR, Whitsett JA. Dev. Biol 1993;156:426–443. [PubMed: 8462742]
15. Colbert MC, Hall DG, Kimball TR, Witt SA, Lorenz JN, Kirby ML, Hewett TE, Klevitsky R, Robbins J. J. Clin. Investig 1997;100:1958–1968. [PubMed: 9329959]
16. Underhill TM, Cash DE, Linney E. Mol. Endocrinol 1994;8:274–285. [PubMed: 8015546]
17. Rossant J, Zirngibl R, Cado D, Giguere V. Genes Dev 1991;5:1333–1344. [PubMed: 1907940]
18. Cardoso WV, Mitsialis SA, Brody JS, Williams MC. Dev. Dyn 1996;207:47–59. [PubMed: 8875075]
19. Williams MC. Annu. Rev. Physiol 2003;65:669–695. [PubMed: 12428023]
20. Ikeda K, Clark JC, Bachurski CJ, Wikenheiser KA, Cuppoletti J, Mohanti S, Morris RE, Whitsett JA. Am. J. Physiol 1994;267:L309–17. [PubMed: 7524342]
21. Ramirez MI, Millien G, Hinds A, Cao Y, Seldin DC, Williams MC. Dev. Biol 2003;256:61–72. [PubMed: 12654292]
22. Mendelson CR. Annu. Rev. Physiol 2000;62:875–915. [PubMed: 10845115]
23. Perl A, Wert S, Nagy A, Lobe C, Whitsett J. Proc. Natl. Acad. Sci. U. S. A. 2002;99:10482–10487. [PubMed: 12145322]
24. Bellusci S, Grindley J, Emoto H, Itoh N, Hogan BL. Development 1997;124:4867–4878. [PubMed: 9428423]
25. Park WY, Miranda B, Lebeche D, Hashimoto G, Cardoso WV. Dev. Biol 1998;201:125–134. [PubMed: 9740653]
26. Kimura S, Hara Y, Pineau T, Fernandez-Salguero P, Fox CH, Ward JM, Gonzalez FJ. Genes Dev 1996;10:60–69. [PubMed: 8557195]
27. Sekine K, Ohuchi H, Fujiwara M, Yamasaki M, Yoshizawa T, Sato T, Yagishita N, Matsui D, Koga Y, Itoh N, Kato S. Nat. Genet 1999;21:138–141. [PubMed: 9916808]

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28. Minoo P, Su G, Drum H, Bringas P, Kimura S. Dev. Biol 1999;209:60–71. [PubMed: 10208743]
29. Morrissey E, Ip H, Lu M, Parmacek M. Dev. Bio 1996;177:309–322. [PubMed: 8660897]
30. Keijzer R, van Tuyl M, Meijers C, Post M, Tibboel D, Grosveld F, Koutsourakis M. Development 2001;128:503–511. [PubMed: 11171334]
31. Yang H, Lu MM, Zhang L, Whitsett JA, Morrissey EE. Development 2002;129:2233–2246. [PubMed: 11959831]
32. Koutsourakis M, Keijzer R, Visser P, Post M, Tibboel D, Grosveld F. Mech. Dev 2001;105:105–114. [PubMed: 11429286]
33. Liu C, Morrissey E, Whitsett JA. Am. J. Physiol. Lung Cell Mol. Physiol 2002;283:L468–L475. [PubMed: 12114210]
34. Ghatpande S, Ghatpande A, Zile M, Evans T. Dev. Biol 2000;219:59–70. [PubMed: 10677255]
35. Young SL, Fram EK, Spain CL, Larson EW. Am. J. Physiol. Lung Cell Mol. Physiol 1991;260:L113–L122.
36. Kastner P, Messaddeq N, Mark M, Wendling O, Grondona JM, Ward S, Ghyselinck N, Chambon P. Development 1997;124:4749–4758. [PubMed: 9428411]
37. Tsukui T, Capdevila J, Tamura K, Ruiz-Lozano P, Rodriguez-Esteban C, Yonei-Tamura S, Magallon J, Chandraratna RA, Chien K, Blumberg B, Evans RM, Belmonte JC. Proc. Natl. Acad. Sci. U. S. A 1999;96:11376–11381. [PubMed: 10500184]
38. Chazaud C, Chambon P, Dolle P. Development 1999;126:2589–2596. [PubMed: 1031971]
39. Gove C, Walmsley M, Nijjar S, Bertwistle D, Guille M, Partington G, Bomford A, Patient R. EMBO J 1997;16:355–368. [PubMed: 9029155]
40. Weston AD, Rosen V, Chandraratna RA, Underhill TM. J. Cell Biol 2000;148:679–690. [PubMed: 10684250]
41. Weaver M, Dunn NR, Hogan BL. Development 2000;127:2695–2704. [PubMed: 10821767]
42. Celli G, LaRochelle WJ, Mackem S, Sharp R, Merlino G. EMBO J 1998;17:1630–1655.
43. Perl AKT, Hokuto I, Impagnatiello MA, Christofori G, Whitsett JA. Dev. Biol 2003;258:154–168. [PubMed: 12781690]
44. Peters K, Werner S, Liao X, Wert S, Whitsett J, Williams L. EMBO J 1994;13:3296–3301. [PubMed: 8045260]
45. Mollard R, Ghyselinck NB, Wendling O, Chambon P, Mark M. Int. J. Dev. Bio 2000;44:457–462. [PubMed: 11032179]
46. Massaro D, Massaro G. Am. J. Physiol. Lung Cell Mol. Physiol 2002;282:L345–L358. [PubMed: 11839527]
47. Massaro GD, Massaro D, Chan WY, Clerch LB, Ghyselinck N, Chambon P, Chandraratna RA. Physiol. Genomics 2000;4:51–57. [PubMed: 11074013]
48. Massaro GD, Massaro D, Chambon P. Am. J. Physiol. Lung Cell Mol. Physiol 2003;284:L431–L433. [PubMed: 12533315]
**Fig. 1. Constitutive active RARs**

A, Sp-C-RARαVP16 and Sp-C-RARβVP16; a 3.7-kb fragment of the Sp-C promoter was used to drive expression of RARα or RARβ fused to the VP16 transactivation domain and a SV40 poly(A) sequence. B, diagram representing expression of endogenous RARα and β (WT, left) and chimeric receptors (transgenics, right) in the lung; note that only epithelial expression is represented and RARγ is excluded from the analysis. C, Northern blot analysis of MLE cells (derived from Sp-C-SV40T transgenic mice) treated with all-trans RA (10^{-5} to 10^{-9} M) for 24 h; levels of SV40T are not changed by RA suggesting that the Sp-C promoter is not regulated by RA. D and E, freshly isolated lungs of WT and RARαVP16 transgenic mice at E18.5 (medial, accessory lobes) and at birth (P0) demonstrate smaller size of transgenics. F and G, RARE-lacZ and βVP16 mice were crossed, and X-gal staining was performed in E18.5 lungs; βVP16 activates RA signaling in the distal lung of Sp-C-βVP16/RARE-lacZ mice (G); no lacZ expression is found in WT/RARE-lacZ littermate (F). Histological section shows X-gal staining in type II (t2) cells (G, right). lu, lung; tr, trachea.
Fig. 2. RARVP16 transgene expression

A–C, immunostaining of VP16 in WT, αVP16, and βVP16 lungs at birth. A, expression of VP16 in distal epithelium of WT is absent. B and C, strong nuclear staining is seen in distal epithelial cells of αVP16 and βVP16 lungs (arrowheads in B and C). D–F, isotopic in situ hybridization (ISH) of RARα in WT, αVP16, and βVP16 transgenic lungs. E, high levels of RARα (arrowhead) are present in the distal epithelium of αVP16 lungs, which contrasts to the low levels of endogenous RARα seen ubiquitously in WT (D) and βVP16 (F). G–I, isotopic in situ hybridization of RARβ in WT, αVP16, and βVP16 lungs at birth. G, no endogenous RARβ is present in the distal lung of WT and only low levels are seen in more proximal bronchiolar epithelium (yellow arrowhead in G). I, This contrasts with the strong RARβ expression in the distal epithelium of βVP16 transgenics (arrowheads in H and I). H, endogenous RARβ is up-regulated in the distal epithelium of the αVP16 lung (arrowhead in H). J, immunohistochemistry using RAR isotype-specific antibodies shows similar high level signals of RARα and RARβ in the distal epithelium of the respective transgenics. Bars in A and D represent 50 and 160 μm, respectively.
Fig. 3. RARVP16 effects on morphology
A–C, hematoxylin-eosin staining of lung sections from newborn WT, αVP16, and βVP16 mice. A, distal saccules of WT show typical type I (t1) and type II (t2) epithelial cells (bottom panel). B, αVP16 transgenics show tubule-like structures in the distal lung instead of typical saccules. These tubules are lined by a columnar epithelium (arrowhead, and lower panel), and type I flat cells are absent. C, by contrast, expression of βVP16 does not prevent saccluation and both type I and type II cells can be identified. D–F, immunostaining for the type I cell marker T1α shows epithelial expression in WT and βVP16 but not in αVP16 lungs (asterisk in E). G–I, the proximal epithelial marker Ccsp (arrowhead) is not present in αVP16 (asterisk in H) or βVP16 epithelium (I). Bars in A, B, E, and G represent 100, 30, 80, and 70 μm, respectively.
Fig. 4. Surfactant protein gene expression in newborn wild type and transgenic lungs

A–C, Sp-C, the earliest marker of type II cells (arrow in A), is expressed in the epithelium of αVP16 (B) and βVP16 (C) mice, as assessed by non-isotopic ISH. By isotopic ISH, late markers of type II cell differentiation, Sp-B (D–F) and Sp-A (G–I), are strongly expressed in WT and βVP16 mice but are nearly absent in αVP16 epithelium (asterisks in E and H). Blue arrows indicate gene expression in bronchiolar non-ciliated epithelial cells (D–I). Bars in A, E, I represent: 40, 40, and 50 μm, respectively.
Fig. 5. Analysis of cell proliferation (PCNA, A–D) and cell death (TUNEL, E–H)

PCNA or TUNEL-labeled epithelial (LEp) and mesenchymal (LMes), as well as unlabeled cells (NL) were counted in 6–8 random distal fields of WT, αVP16, and βVP16 lungs at 40× magnification. Results were expressed as percent of the total number of cells per field (an average of 250 cells). A, WT lungs show almost no PCNA labeling but some TUNEL-positive cells (E) at birth. Labeling for both markers is dramatically increased in αVP16 (B and F) and to a lesser extent in βVP16 (C and G) lungs. Graphics represent mean (bar, numbers) and S.E. Bars in A and E represent 50 μm.
Fig. 6. Expression of Ttf1, Fgf10, and Gata6 in newborn WT and RARVP16 transgenic lungs by isotopic in situ hybridization

A, Ttf1 expression is increased in epithelial tubules of αVP16 lungs (arrowheads) as compared with βVP16 (B) and wild-type littermates (C). D, high levels of Fgf10 (arrowheads) are present in the distal mesenchyme associated with αVP16 tubules, which contrast with the low level signals in βVP16 (E) and WT (F) lungs. G, strong Gata6 signals are present in the distal epithelium of αVP16 tubules at birth (arrowhead). However, in βVP16 (H) and WT (I), Gata6 is expressed at low levels in the distal epithelium (arrowheads); at this stage most of the signals are present in the blood vessels (bv, arrows) as previously reported. Bars in A, D, F, G, and H represent 25, 70 100, 55, and 120 μm.
Fig. 7. Characterization of lung immaturity in βVP16 mice
A. **T1α immunostaining of WT and βVP16 lungs at birth.** Relative area occupied by type I epithelial (T1α-positive, brown) and mesenchymal (T1α-negative, blue) cells in 10 random distal fields of WT and βVP16 lungs. Graph represents mean and S.E. of measurements, data are expressed as percentage of total cells. An increased proportion of nonstained (mesenchyme) areas relative to stained areas (epithelium) in βVP16 transgenes suggests immaturity. B. **PAS staining of transgenic and WT lungs.** The distal epithelium of βVP16 lungs preserved high levels of glycogen in the cytoplasm (left panel, arrowhead). Glycogen vacuoles are abundant in WT E16 embryonic lungs (right panel) but are rare in WT at birth (PO, middle panel). Bars in A and B represent 150 and 35 μm, respectively.

*J Biol Chem.* Author manuscript; available in PMC 2007 December 28.
Fig. 8. Summary of findings and proposed model
A, gene expression in the distal lung of αVP16, βVP16 and WT mice. B, diagram representing the effect of RARVP16 in differentiation of the lung epithelium at birth: αVP16 maintains the lung in an early immature (pseudoglandular) stage; βVP16 and WT lungs undergo sacculation and form distal type I and type II cells; βVP16 lungs, however, are not fully mature. Black bars represent activated RA signaling.