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Original Article

Stage-dependent responses of the developing lung to retinoic acid signaling

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ABSTRACT Morphological analysis of vitamin A-deficient rat fetuses and of retinoic acid receptor (RAR and RXR) mutant mice have demonstrated that retinoic acid (RA) is essential for lung development. To gain further insight into RA signaling pathways during primary lung bud formation and lung branching, we have investigated the effects of RA and of a pan-RAR antagonist in cultures of whole embryos and lung explants. Treatment of E8.0 embryos with the pan-RAR antagonist inhibits the formation of the primitive respiratory system. On the other hand, treatment of E11.75 and E12.5 lung explants with RA inhibits branching morphogenesis, whereas treatment with the pan-RAR antagonist at the same developmental stages stimulates formation of distal buds. The inhibitory effect of RA on branching is strongly decreased in RARβ null lungs, while enhancement of budding by the pan-RAR antagonist is not affected by an RARγ null mutation. Additionally, cellular retinol binding protein one (CRBPI) null lungs are more sensitive than wild type lungs to the pan-RAR antagonist-induced stimulation of branching. These data indicate that retinoid signaling is indispensable for the formation of primary lung buds and the oesophagotracheal septum from the primitive foregut. They also suggest that at the pseudoglandular stage, RA signaling through RARβ, but not RARγ, inhibits distal bud formation thereby promoting the formation of conducting airways. Moreover, the level of CRBPI in the pseudoglandular lung appears to participate in the control of branching morphogenesis.

KEY WORDS: *lung development, organ culture, embryo culture, retinoic acid receptors, cellular retinol binding protein one.*

Introduction

Vitamin A (retinol) exerts multiple effects upon vertebrate development through binding of active metabolites, retinoic acids (RA), to two families of nuclear receptors, the retinoic acid receptors (RAR isotypes α, β and γ and their isoforms) and the retinoid X receptors (RXR isotypes α, β and γ and their isoforms) (reviewed in Kastner *et al.*, 1995; and Chambon, 1996). RARs bind all-*trans* RA (*t*-RA) and 9-*cis* RA (9*c*-RA), whereas RXRs bind only 9*c*-RA. RAR/RXR heterodimers regulate transcription of RA target genes through their activation function domains (AF1 and AF2) and their binding to conserved cis-acting RA response elements (RAREs; reviewed in Mangelsdorf *et al.*, 1995 and Chambon, 1996). Retinoid metabolism might be controlled by cytoplasmic binding proteins such as the cellular retinol binding proteins (CRBPI and II) and cellular retinoic acid binding proteins (CRABPI and II) (Napoli, 1999).

Lung development includes: (i) evagination of the primitive lung and tracheal buds from the embryonic foregut, (ii) branching morphogenesis, which essentially takes place during the pseudoglandular stage and establishes the primitive conducting airways and presumptive terminal sacs, and (iii) alveologenesis, characterized by septation of the terminal sacs to form definitive alveoli (Hogan and Yingling, 1998 and references therein). The importance of RA signaling for prenatal lung development was first established with the finding that vitamin A-deficient (VAD) rat fetuses often display, among other malformations, severe bilateral lung hypoplasia, left lung agenesis and agenesis of the oesophagotracheal septum (Warkany *et al.*, 1948; Wilson *et al.*, 1953). VAD-related congenital lung and tracheal malformations are also observed in RARα$^{-/-}$/RARβ$^{-/-}$, RARα$^{-/-}$/RARβ2$^{-/-}$, RARα$^{-/-}$/RXRα$^{-/-}$ and RARα$^{-/-}$/RXRαAF2° fetuses (Lohnes *et al.*, 1994; Mendelsohn *et al.*, 1994; Kastner *et al.*, 1994, 1997; Ghyselinck *et al.*, 1997; Mascrez *et al.*, 1998).

Abbreviations used in this paper: RAR and RXR, retinoic acid receptors; RA, retinoic acid, ATBN, average terminal bud number; CRBPI, cellular retinol binding protein one; ISH, *In Situ* Hybridization.

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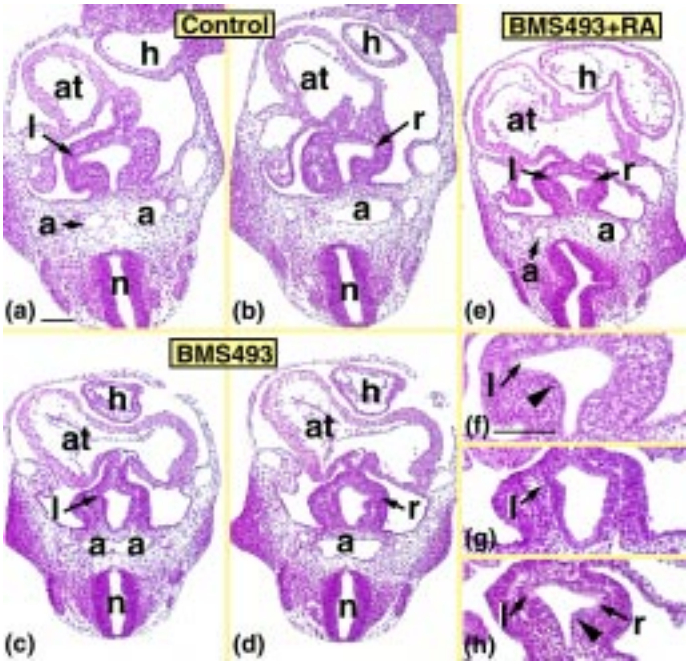

Fig. 1. Effects of an RAR antagonist in whole embryo cultures. *Representative transverse histological sections through the primary left and right lung buds of three E8.0 embryos cultured for 48 hours in:* **(a,b,f)** *vehicle (ethanol) alone,* **(c,d,g)** *10^{-6} M BMS493 and* **(e,h)** *10^{-6} M BMS493 and 10^{-7} M RA. (f,g,h) represent high power views of (a,c,e), respectively. a, dorsal aortas; at, primitive atrium; h, heart outflow tract; l, left lung bud; n, neural tube; r, right lung bud; the arrow heads indicate oesophagotracheal folds. Scale bars = 65 μm.*

The addition of RA to growth-arrested prenatal lung explants provided evidence that RA signaling could be involved in the stimulation of lung branching (Schuger *et al.*, 1993). However, independent studies have shown an inhibitory effect of RA treatment upon branching morphogenesis in lung cultures (Cardoso *et al.*, 1995). To explore RA signaling pathways during primary lung bud formation and subsequently during branching morphogenesis, we have used: (i) lung explant and whole embryo culture systems, (ii) RA and a specific pan-RAR antagonist and (iii) wild type and RARβ, RARγ and CRBPI null mutant embryos.

Results

RA signaling is required for primary lung bud formation in whole embryo cultures

Embryos were collected at E8.0 (i.e. 36 hours prior to the formation of primary lung buds and tracheal diverticulum; Kaufman, 1992), and cultured for 48 hours (i.e. until the equivalent of E9.5 *in vivo*) either in the presence of the pan-RAR antagonist, BMS493 (Chazaud *et al.*, 1999; Wendling *et al.*, 2000), or in the presence of the retinoid vehicle (i.e. ethanol alone). Treatment with 10^{-6}M BMS493, inhibited primary lung bud outgrowth (Fig. 1a-d; l and r) and caused a failure of oesophagotracheal fold formation (arrowheads in Fig. 1f compare with 1g). These defects were partially prevented by the simultaneous addition to the culture medium of 10^{-6}M BMS493 and 10^{-7}M RA (Fig. 1e,h; l, r and arrowhead). Therefore, a block in RA signal transduction before and at the onset of lung bud appearance inhibits the formation of the respiratory system from the primitive foregut.

In vitro lung branching morphogenesis is inhibited by RA and stimulated by a pan-RAR antagonist

Lung explants from E11.75 or E12.5 embryos were cultured for 4 days, unless otherwise indicated. Control explants were cultured in the presence of the retinoid vehicle alone. RA at final concentrations of 10^{-7}M and 10^{-6}M significantly decreased average terminal bud number (ATBN) in a dose-dependent fashion, while 10^{-8}M was ineffective (Fig. 2a). In contrast, BMS493 at final concentrations of 10^{-6}M and $2x10^{-6}$M significantly increased ATBN in a dose-dependent manner, while 10^{-7}M and 10^{-8}M were ineffective (Fig. 2b,c). Retinoid-induced changes in ATBN were prevented when 10^{-6}M RA and 10^{-6}M BMS493 were simultaneously added to the culture medium, indicating that the effects of BMS493 are caused by a specific inhibition of RA-signaling (Fig. 2a,c). Altogether, these data suggest that retinoid signaling decreases branching during the pseudoglandular stage of lung morphogenesis.

RA-induced inhibition of lung branching is mediated by RARβ

During lung development, RARβ is expressed at high levels in the epithelium and mesenchyme of proximal primary and secondary bronchi (Dollé *et al.*, 1990; Ghyselinck *et al.*, 1998 and see below), which correspond to areas of morphogenetic stability when compared to the morphogenetically active distal buds (Hilfer *et al.*, 1985; Mollard and Dziadek, 1998).

The expression pattern of RARβ transcripts in E11.75 lung explants cultured for 24 hours in the absence of retinoids was

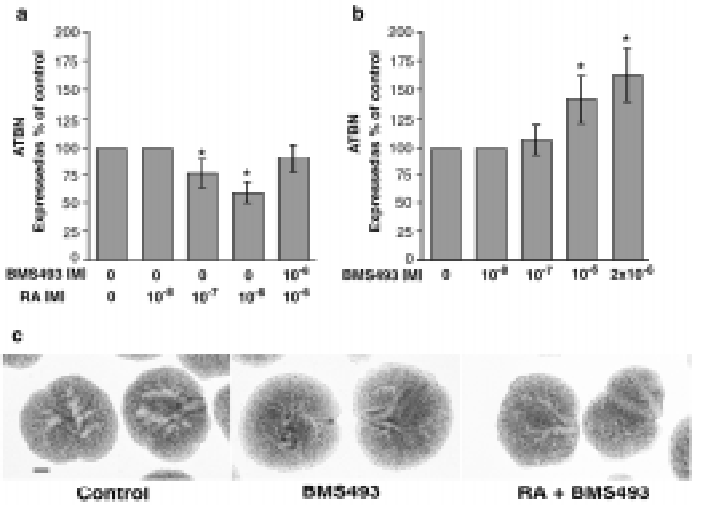

Fig. 2. Effects of RAR agonists and antagonists on E11.75 wild type lungs after four days in culture. (a,b) *average terminal bud number (ATBN) versus treatment. Values are calculated from the average percentages with respect to control ATBN from 21 individual experiments each containing ten explants per treatment group. Treatment with 10^{-6} M and 10^{-7} M RA always resulted in a significant decrease in ATBN, an effect negated by the concomitant presence of 10^{-6} M BMS493. BMS493 treatment alone at either 10^{-6} M or $2x10^{-6}$ M resulted in significant increases in ATBN.* **(c)** *Increased explant size and terminal bud numbers of explants treated with 10^{-6} M BMS493 are reduced by the concomitant addition of 10^{-6} M RA. * $p<0.05$ relative to controls. Error bars represent +/- standard deviation. Scale bar = 200 μm.*

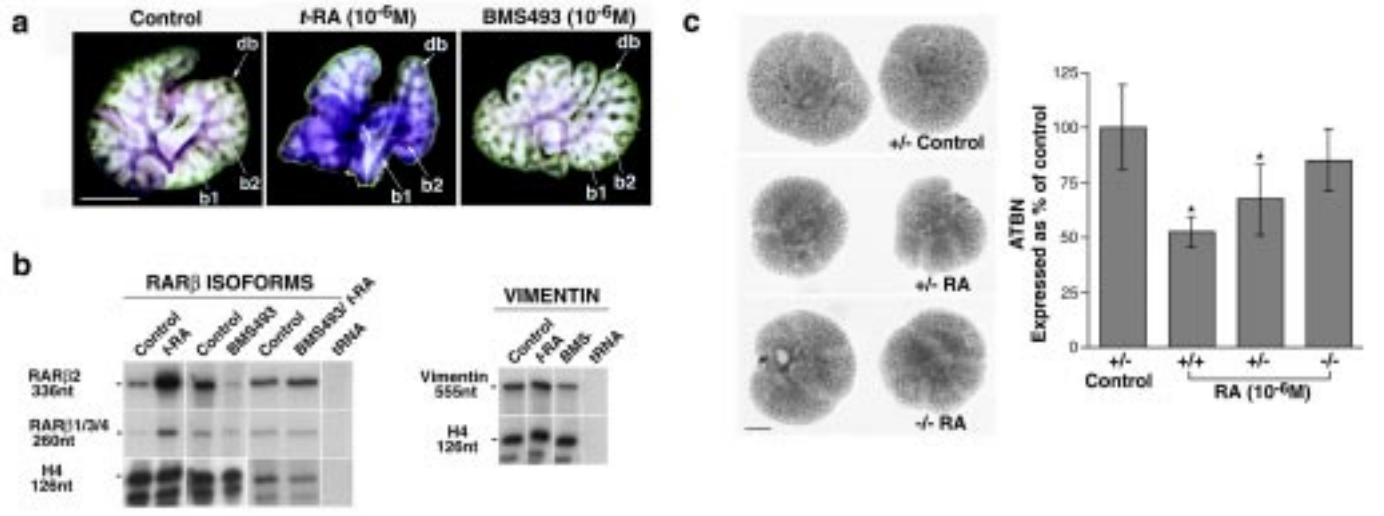

Fig. 3. RARβ expression during branching morphogenesis and effects of RA treatment on RARβ$^{-/-}$ lungs. **(a)** *Whole-mount ISH for the detection of RARβ transcripts following 24 hours of exposure of E11.75 lung explants to either retinoid vehicle alone, 10^{-6} M RA or 10^{-6} M BMS493.* **(b)** *RNAse protection analyses of RARβ1/3/4, RARβ2 and vimentin transcripts in lungs explanted at E11.75 and cultured for four days in the presence of retinoids or ethanol; the explants were analyzed four hours after the last addition of the retinoids and/or ethanol to the culture media (2 μg RNA per track; exposure times, four to 24 hours).* **(c)** *Appearance of representative E12.5 RARβ, heterozygote (+/-) and null (-/-) mutant lungs from RARβ$^{+/-}$/RARβ$^{+/-}$ crosses, cultured for 24 hours in the absence of retinoids and then 48 hours in the presence of either vehicle alone or 10^{-6} M RA. Note that the explants were first cultured for 24 hours in the absence of added retinoid to permit lung bund count; no significant difference was found between wild type and RARβ null lung explants at the onset of RA treatment. The accompanying histogram depicts ATBN versus treatment at 72 hours of culture; each group consists of seven explants and is representative of two individual experiments. b1, primary bronchi; b2, secondary bronchi; db, distal bud; m, mesenchyme; * p<0.01 relative to control. Error bars represent +/- standard deviation. Scale bars = 100 μm.*

similar to that observed *in vivo* at E13.5 (Fig. 3a). RA treatment at 10^{-6}M induced the expression of RARβ throughout the pulmonary tree, including the distal buds, whereas treatment with BMS493 (10^{-6}M) decreased RARβ expression in secondary bronchi (Fig. 3a,b). Retinoid-induced changes in RARβ expression were prevented when 10^{-6}M RA and 10^{-6}M BMS493 were simultaneously added to the culture medium (Fig. 3b), indicating that competition between RA and BMS493 modified transcription in explanted lungs, as previously observed in cultured cells (unpublished results from our group). It is also noteworthy that only limited changes in the expression of vimentin, a specific mesenchymal marker, occurred following retinoid treatment (Fig. 3b). Therefore, a modification in the epithelial to mesenchymal ratio cannot account for the alterations in RARβ transcription levels. Altogether, these results suggest that RARβ could mediate the observed RA-induced inhibition of lung branching. In order to investigate this possibility, we compared the effects of 10^{-6}M RA upon ATBN in explants from RARβ null embryos (Ghyselinck *et al.*, 1997), heterozygotes and wild type littermates. Wild type and RARβ null lung explants were cultured for 24 hours without added retinoid, and then treated for 48 hours with 10^{-6}M RA (Fig. 3c). Both wild type and RARβ$^{+/-}$ RA-treated explants exhibited a significant reduction in ATBN, when compared to controls (i.e. RARβ$^{+/-}$ explants cultured with ethanol alone). In contrast, there was no significant difference in ATBN between RA-treated RARβ null explants and controls (Fig. 3c). These data indicate that RARβ is involved in RA-induced inhibition of branching.

RARα1, RARα2 and RARγ2 transcripts are expressed ubiquitously in E13.5 lungs *in vivo* (our unpublished data). Thus, aside from RARβ isoforms (Ghyselinck *et al.*, 1998), RARγ1 is the only RAR isoform displaying a restricted pattern of expression in the developing lung, being expressed preferentially within the distal bud epithelium (Fig. 4a,b). In order to investigate whether RARγ could be involved in lung branching, RARγ null lungs (Lohnes *et al.*, 1993) were cultured in the presence of the pan-RAR antagonist. Wild type, RARγ$^{+/-}$ and RARγ null lungs treated with 10^{-6}M BMS493 all responded with a similar increase in bud formation when compared to ethanol-treated RARγ$^{+/-}$ controls (Fig. 4c). Thus, RARγ is clearly not involved in transducing the RA signal which inhibits distal lung bud formation.

CRBPI is involved in the regulation of lung branching by retinoids

The lung at the pseudoglandular stage is a major site of expression of CRBPI in the embryo (Dollé *et al.*, 1990). In cultured E12.5 wild type lungs, CRBPI expression was increased in the presence of 10^{-6}M RA and decreased by 10^{-6}M BMS493 (Fig. 5a). Thus, increased and decreased expression of CRBPI are correlated with retinoid-induced inhibition and stimulation of branching, respectively. To further test an involvement of CRBPI in lung morphogenesis, explants from E12.5 CRBPI null mutants (Ghyselinck *et al.*, 1999) were cultured in the presence of various concentrations of the pan-RAR antagonist. In CRBPI null lungs, but not wild type lungs, a concentration of BMS493 as low as 10^{-7}M induced a significant increase in ATBN (Fig. 5b), indicating that CRBPI null lungs displayed a higher sensitivity to RAR antagonism.

Discussion

RA is instrumental for alveolar septation as its administration to newborn rats increases the number of alveoli and restores alveolar

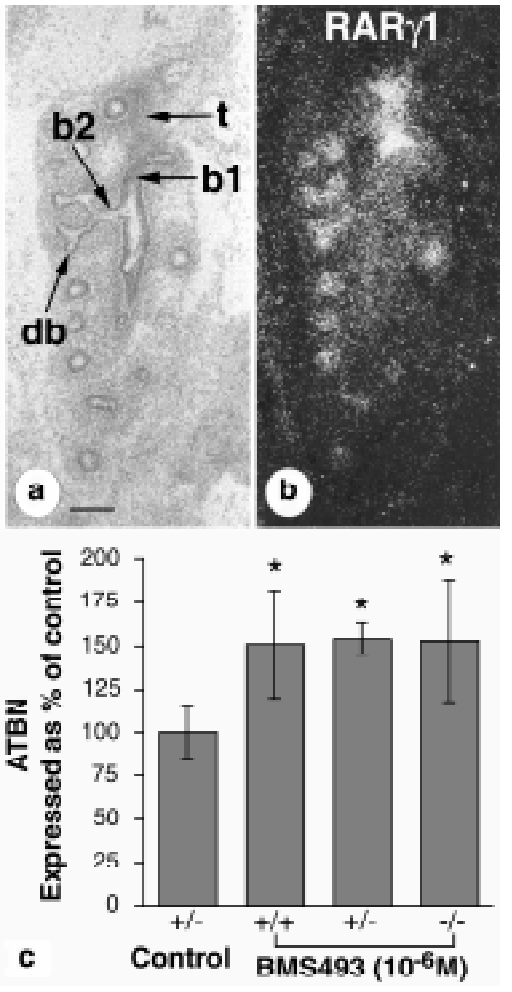

Fig. 4. RARγ1 transcript localization and effect of BMS493 treatment on RARγ$^{-/-}$ lungs. *Light field* **(a)** *and corresponding dark field* **(b)** *photomicrographs of longitudinal sections from E13.5 lung demonstrates that RARγ1 transcripts preferentially localize to the distal budding epithelium (db) and tracheal mesenchyme (t) whereas the regions of the primary (b1) and secondary (b2) bronchi only show a weak ISH signal.* **(c)** *E12.5 RARγ wild type (+/+), heterozygote (+/-) and null (-/-) mutant lungs from RARγ$^{+/-}$/RARγ$^{+/-}$ heterozygote crosses were cultured for 24 hours in the absence of retinoids and then for 72 hours in the presence of 10^{-6} M BMS493 (seven explants per group, two individual experiments). No significant differences in the stimulation of ATBN were observed between BMS493 treated groups. * p<0.05 relative to control. Error bars represent +/- standard deviation. Scale bar = 100 μm.*

number in animal models of emphysema (Massaro and Massaro, 1996, 1997). Alveolar septation is a late developmental event, as it is initiated only at the end of the fetal period, and essentially takes place during early post-natal life in rodents (reviewed in Hogan and Yingling, 1998). In the present study, we have analyzed the role of RA during the embryonic and pseudoglandular stages of prenatal lung development.

Retinoid signaling is indispensable for the formation of primary lung buds and the oesophagotracheal septum from the primitive foregut

The inhibition of primary lung bud and oesophagotracheal fold formation induced in cultured embryos by the pan-RAR antagonist and observed at a stage equivalent to E9.5 *in vivo*, indicates that RA is normally required for the appearance of these structures. This observation also indicates that the severe lung hypoplasia (or agenesis) and absence of the oesophagotracheal septum previously described at fetal stages in retinoic acid receptor compound mutant mice, as well as in VAD rats, are determined prior and/or during the embryonic stage of lung development. In keeping with this idea, a recent analysis of RARα$^{-/-}$/RARβ$^{-/-}$ embryos shows that the left primitive lung bud and left oesophagotracheal fold are markedly hypoplastic or absent at E9.5, i.e. at the earliest developmental stage when the primary lung buds and tracheal diverticulum can be identified morphologically (our unpublished results). Our genetic dissection of the retinoid signaling pathway strongly suggests that the functional heterodimers involved in the primary lung and tracheal bud formation are RARα/RXRα (Kastner *et al.*, 1997; Mascrez *et al.*, 1998).

In transfected cells, the pan-RAR antagonist BMS493 stabilizes the interaction of RAR/RXR heterodimers with co-repressors (our unpublished results). Therefore, the possibility exists that in addition to blocking endogenous retinoid signaling, BMS493 may also act to silence basal transcription of developmental genes in our culture systems. However, this appears unlikely because the lung and tracheal defects reported here are similar to those exhibited by E9.5 embryos lacking RARα and RARβ (see above).

Signaling through RARβ exerts an inhibitory effect upon budding at the pseudoglandular stage

At the pseudoglandular stage of lung development, RARβ is preferentially expressed in the proximal clefts of the pulmonary tree (Dollé *et al.*, 1990; Ghyselinck *et al.*, 1998). Proximal clefts correspond to areas of morphogenetic stability when compared to the distal tips of the pulmonary tree, which are the major sites of budding (Hilfer *et al.*, 1985; Mollard and Dziadek, 1998). The present data demonstrate that, during the pseudoglandular stage of lung development, a block in RA signaling increases formation

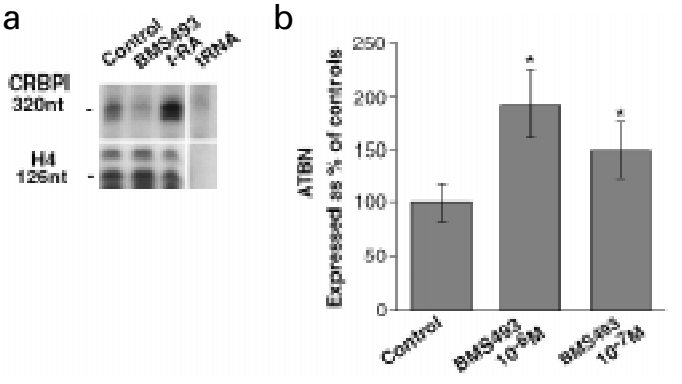

Fig. 5. A role for CRBPI during branching morphogenesis. (a) *RNAse protection analysis of CRBPI transcripts in E12.5 wild type lungs cultured for four days in the presence of retinoids or ethanol. The explants were analyzed four hours after the last addition of the retinoids and/or ethanol to the culture media (2 μg RNA per track; exposure times, four to 24 hours). Six experiments per group, 10 lungs per experiment.* **(b)** *ATBN in CRBPI null mutant lungs cultured for four days in the presence of either ethanol alone (control), or 10^{-6} M or 10^{-7} M BMS493. Six explants per group, three individual experiments; * p<0.01 relative to control. Error bars represent +/- standard deviation. Note that the responses to retinoids were identical in the CRBPI and CD1 genetic backgrounds (data not shown).*

of distal buds in wild type lungs and that RA reduces branching much less efficiently in RARβ null lungs than in wild type lungs. Moreover, the branching inhibition induced by RA treatment is correlated with ectopic expression of RARβ in distal buds, whereas the increase in distal bud number caused by a block in RA signaling is correlated with a decrease of RARβ expression in the pulmonary tree. Collectively, these findings provide evidence that activation of RARβ by RA favors morphogenetic stabilization over *de novo* budding during formation of the pulmonary tree.

At the pseudoglandular stage of lung development, the RARγ1 isoform is preferentially expressed in the distal buds. The findings that (i) RARγ null lungs respond to a block in RA signaling similarly to wild type lungs, and (ii) that RARβ null lungs, which still express RARγ, are refractory to RA-induced branching inhibition altogether suggest that RARγ is dispensable for the transduction of RA-mediated patterning cues during lung branching.

A role for CRBPI in the control of lung branching morphogenesis by RA

It has been proposed that CRBPI could play a role in RA synthesis (Napoli, 1999). The observation that CRBPI null lungs are approximately 10-fold more sensitive than wild type lungs to the stimulatory effect of the pan-RAR antagonist upon distal bud formation suggests indeed that the CRBPI present in the lung could be involved in the production of RA in this developing organ. Thus, it is conceivable that the actual level of CRBPI in the lung at the pseudoglandular stage could participate in the control of branching morphogenesis.

Material and Methods

Lung and whole embryo culture

The mouse lines carrying the RARβ, RARγ and CRBPI null mutations and their genotyping protocols have been described previously (Lohnes *et al.*, 1993; Ghyselinck *et al.*, 1997; Ghyselinck *et al.*, 1999). The morning of appearance of the vaginal plug was designated as E0.5. For explant culture, E11.75 and E12.5 lungs from $RAR\beta^{+/-}$ x $RAR\beta^{+/-}$, $RAR\gamma^{+/-}$ x $RAR\gamma^{+/-}$, $CRBPI^{-/-}$ x $CRBPI^{-/-}$, $CRBPI^{+/+}$ x $CRBPI^{+/+}$ and wild type CD1 crosses were dissected in PBS and incubated in BGJB medium (Fitton-Jackson modified, Gibco BRL), 5% de-lipidated fetal calf serum (FCS, Gibco BRL) and 180 mg/l vitamin C (Sigma) on Millipore filters (Gibco BRL), at 37°C, in the presence of 5% CO_2. Half of the media, supplemented with fresh retinoids (see below), was changed daily. The cultured lung buds reproducibly grew and branched for at least seven days in culture (data not shown). Average terminal bud number (ATBN) was calculated after counting every bud in each explant at 0, 24, 48, 72 and 96 hours after the commencement of culture. Significant differences in ATBN between different groups were determined by ANOVA and Newman-Keuls multiple comparison tests according to previously described methods (Motulsky, 1995). The synthetic retinoid BMS493 [a specific pan RAR (α, β and γ) antagonist (Bristol-Myers-Squibb, NJ); Chazaud *et al.*, 1999; Wendling *et al.*, 2000] and RA (Sigma) were diluted in ethanol and added to the culture medium at a final ethanol concentration of 0.1% at the beginning of culture and every subsequent 24 hours.

Whole embryos from CD1 x CD1 crosses were collected at E8.0, staged and cultured according to previously described methods in the presence of retinoids or ethanol vehicle alone for 48 hours (New, 1990; Wendling *et al.*, 2000). For morphological assessment, serial transverse histological sections were stained with hematoxylin and eosin.

RNAse protection and in situ *hybridization*

Total RNA preparation, RNAse protection assays and *in situ* hybridizations (ISH) were performed as previously described (Chirgwin *et al.*, 1979; Décimo *et al.*, 1995; Mollard and Dziadek, 1997; Ghyselinck *et al.*, 1997). CRBPI, H4, RARβ and vimentin cDNAs have been previously described (Ghyselinck *et al.*, 1997; Mollard and Dziadek, 1997).

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