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ORIGINAL ARTICLE

Retinoic acid regulates avian lung branching through a molecular network

Hugo Fernandes-Silva[1,2] · Patrícia Vaz-Cunha[1,2] · Violina Baranauskaite Barbosa[1,2] · Carla Silva-Gonçalves[1,2] · Jorge Correia-Pinto[1,2,3] · Rute Silva Moura[1,2,4]

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Abstract Retinoic acid (RA) is of major importance during vertebrate embryonic development and its levels need to be strictly regulated otherwise congenital malformations will develop. Through the action of specific nuclear receptors, named RAR/RXR, RA regulates the expression of genes that eventually influence proliferation and tissue patterning. RA has been described as crucial for different stages of mammalian lung morphogenesis, and as part of a complex molecular network that contributes to precise organogenesis; nonetheless, nothing is known about its role in avian lung development. The current report characterizes, for the first time, the expression pattern of RA signaling members (*stra6*, *raldh2*, *raldh3*, *cyp26a1*, *rarα*, and *rarβ*) and potential RA downstream targets (*sox2*, *sox9*, *meis1*, *meis2*, *tgfβ2*, and *id2*) by in situ hybridization. In the attempt of unveiling the role of RA in chick lung branching, in vitro lung explants were performed. Supplementation studies revealed that RA stimulates lung branching in a dose-dependent manner. Moreover, the expression levels of *cyp26a1*, *sox2*, *sox9*, *rarβ*, *meis2*, *hoxb5*, *tgfβ2*, *id2*, *fgf10*, *fgfr2*, and *shh* were evaluated after RA treatment to disclose a putative molecular network underlying RA effect. In situ hybridization analysis showed that RA is able to alter *cyp26a1*, *sox9*, *tgfβ2*, and *id2* spatial distribution; to increase *rarβ*, *meis2*, and *hoxb5* expression levels; and has a very modest effect on *sox2*, *fgf10*, *fgfr2*, and *shh* expression levels. Overall, these findings support a role for RA in the proximal–distal patterning and branching morphogenesis of the avian lung and reveal intricate molecular interactions that ultimately orchestrate branching morphogenesis.

Keywords Chick lung · Pulmonary development · Branching morphogenesis · Signaling pathways · *sox2* · *sox9*

Introduction

The avian respiratory system is constituted by the parabronchial lung that is involved in gas exchange and air sacs which control air movements. In the chicken embryo, *Gallus gallus*, the embryonic lung originates from the primitive foregut around embryonic day 3 (E3) [1]. Subsequent airway branching leads to the formation of the primary bronchus (mesobronchus) and, by lateral sprouting, secondary bronchi emerge from the dorsal surface of the mesobronchus. This lateral (or monopodial) branching is similar to the domain branching observed during mammalian lung development [2]. In addition to these morphological resemblances, the molecular mechanisms underlying chick lung branching appear to be highly conserved. For instance, it has been shown that FGF (fibroblast growth factor) signaling is important for the events that control primary bud formation [3]. Moreover, it is also essential for pulmonary morphogenesis since in vitro FGF receptor

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✉ Rute Silva Moura
rutemoura@med.uminho.pt

1 Life and Health Sciences Research Institute (ICVS), School of Medicine, University of Minho, 4710-057 Braga, Portugal

2 ICVS/3B's, PT Government Associate Laboratory, 4710-057 Braga/Guimarães, Portugal

3 Department of Pediatric Surgery, Hospital de Braga, 4710-243 Braga, Portugal

4 Biology Department, School of Sciences, University of Minho, 4710-057 Braga, Portugal

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inhibition impairs lung branching [4]. Similarly, canonical WNT (wingless-related integration site) signaling is active in early stages of chick lung branching morphogenesis [5], whereas non-canonical WNT signaling (via WNT-5a) has been implicated in pulmonary distal airway and vasculature development [6]. Likewise, SHH (sonic hedgehog) signaling members are expressed in chick embryonic lung [7] and their importance in chick lung branching was revealed in *talpid*^{3} mutants that display abnormal (hypoplastic) lung phenotype due to a defective SHH signaling [8]. Other signaling pathways such as microRNAs [9] and TGFβ-BMP (transforming growth factor β-bone morphogenetic protein) signaling [10] have also been characterized in the chick developing lung. Overall these signaling pathways behave similarly to their mammalian counterparts (reviewed in [11]). Nonetheless, there are many other molecular players, already identified in mammalian lung branching, that have not been described in the chick lung, as for instance retinoic acid signaling [12] among many others [13, 14].

Retinoic acid (RA), the active metabolite of vitamin A, is vital for vertebrate embryonic development, namely in somitogenesis, neurogenesis, and organogenesis (reviewed in [15, 16]). Nonetheless, abnormal levels of RA due to metabolic or signaling alterations elicit congenital malformations (reviewed in [17]). Retinol, the dietary form of retinoic acid, enters the cell via transmembrane protein STRA6 (stimulated by retinoic acid 6) [18]. Once in the cytoplasm, retinol undergoes a series of intracellular oxidative reactions until it is transformed into retinal by retinol dehydrogenase; eventually, retinal is oxidized to RA by retinal dehydrogenase (RALDH1-3). RA intracellular levels must be sharply regulated at the tissue level which is achieved by the action of enzymes belonging to the cytochrome P450 26 subfamily (CYP26A1, B1, and C1) (reviewed in [19]). These enzymes degrade RA and together with synthesizing enzymes ultimately regulate the availability of this molecule for signaling. RA is then transferred to the nucleus bound to the cellular retinoic acid-binding transporter where it binds to specific retinoic acid nuclear receptors (RARs). There are three subtypes (RARα, RARβ, and RARγ) that bind to retinoic X receptor (RXR) to form a heterodimer that recognizes specific DNA sequences called RARE (retinoic acid response element) in the promoter region of retinoid-target genes. In the presence of RA, conformational changes occur that facilitate transcription of specific genes that convey the appropriate cellular responses (reviewed in [20]). Molecular targets of RA signaling pathway include transcription factors such as nuclear receptors (namely *rarβ*), homeodomain genes, genes coding for retinoic acid signaling members (for instance, *cyp26a1*), and genes belonging to other signaling pathways as for instance *fgf10*, *shh* or *tgfβ* [15].

Lung development is a complex process deeply dependent on interactions between signaling pathways that promote both spatial and temporal synchronization of pulmonary morphogenesis. RA is fundamental for proper lung patterning, and pulmonary morphogenesis depends on the timely expression of RA signaling genes. In mammals, maternal vitamin A deficiency causes severe lung abnormalities [21, 22] that are replicated with the ablation of either RARs or RALHD, namely lung agenesis and lung hypoplasia [23–25]. It has been demonstrated that RA plays a crucial role in the events underlying primary lung formation; RA-deficient endoderm fails to develop the lung bud primordium due to the disruption of the RA downstream molecular network that involves FGF, SHH, WNT signaling, and *hox* genes [26–28]. During branching morphogenesis, RA signaling activity seems to be restricted to the proximal-most region of the lung which support's the establishment of proximal airways phenotype, most likely through *rarβ* receptor [29]. On its turn, downregulation of RA signaling in the distal lung enables the expression of specific genes that facilitate budding of the pulmonary tree [12]. In general, RA is involved in proximal–distal patterning of the developing lung. RA is also required for smooth muscle cell differentiation of the developing airways [30] and alveologenesis (reviewed in [31]).

The importance of RA signaling in mammalian lung development is quite well explored; however, nothing is known about its function in the chick lung. Accordingly, to gain new insights into the role of retinoids in the chick developing lung, we examined the spatial distribution of key members of this signaling pathway, specifically *stra6*, *raldh2*, *raldh3*, *cyp26a1*, *rarα*, and *rarβ*. In situ hybridization revealed a common mesenchymal expression for all the studied signaling members. Furthermore, we analyzed the expression pattern of some putative RA downstream targets selected according to their association with mammalian lung, such is the case of *sox2*, *sox9*, *tfgβ2*, and *id2*, or chick limb development, namely *meis1* and *meis2*. To determine RA function in early organogenesis of the avian lung, in vitro lung explants were supplemented with increasing doses of RA and its impact on lung branching and gene expression (*cyp26a1*, *sox2*, *sox9*, *rarβ*, *meis2*, *hoxb5*, *tgfβ2*, *id2*, *fgf10*, *fgfr2*, and *shh*) was evaluated. With this study, we demonstrated the importance of retinoic acid signaling in early stages of chick lung morphogenesis and unveil a retinoic acid-dependent molecular network that most likely contributes to appropriate lung branching and patterning.

Materials and methods

Ethical statement

This work, performed at early stages of chick development, does not need ethical approval from review board institution or ethical commission.

Eggs and embryos

Fertilized chicken eggs, *Gallus gallus*, were incubated between 4.5 and 5.5 days in a 49% humidified atmosphere at 37 °C. Embryonic lungs were dissected under a stereomicroscope (Olympus SZX16, Japan) and staged in b1, b2, and b3 according to the number of secondary buds formed: 1, 2 or 3, respectively [4]. Dissected lungs were processed for in situ hybridization or for in vitro lung explant culture.

In vitro lung explant culture

After dissection in PBS, stage b2 lungs were transferred to nucleopore polycarbonate membranes with 8 µm pore size (Whatman, USA) and incubated in 24-well culture plates. Membranes were presoaked in 400 µL of Medium 199 (Sigma, USA) for 1 h before the lungs were placed on them. Lungs were randomly assigned one of four experimental groups ($n \geq 15$ per condition): 0.1, 1, 10 µM of retinoic acid (R2625, Sigma) or DMSO (1 µL/mL) as control (since retinoic acid was resuspended in this solvent). Chick lung explants were kept in culture for 48 h as previously described [4]. At the end of the culture, explants were washed in PBS, fixed in a 4% formaldehyde solution, and stored at 4 °C, overnight. Lastly, explants were processed for in situ hybridization.

Morphometric analysis

Lung branching was monitored daily by photographing the explants. At D0 (0 h) and D2 (48 h) of culture, the total number of peripheral airway buds was determined. For the morphometric analysis, the internal perimeter of the lung (epithelium) and the outer perimeter of the lung (mesenchyme) were assessed at D0 and D2 using AxionVision Rel. 4.3 (Carl Zeiss GmbH, Germany). The results of branching and morphometric analysis were expressed as D2/D0 ratio. All quantitative morphometric data are presented as mean ± SEM. Statistical analysis was performed using SigmaStat 3.5 (Systat Software Inc., USA). Since normality test failed in branching analysis, Kruskal–Wallis one-way analysis of variance was used followed by a Dunn's method for pairwise multiple comparisons. Regarding morphometric analysis, one-way ANOVA was used followed by a Holm–Sidak method for pairwise multiple comparisons.

RNA probes

Antisense digoxigenin-labeled RNA probes were produced as previously described: *stra6* [32], *raldh2* [33], *raldh3* [34], *rarα* and *rarβ* [35], *cyp26a1* [36], *meis1* and *meis2* [37], *hoxb5* [38], *tgfβ2* [39], *l-cam* and *sox2* [40], *sox9* [41], *fgf10* [5], *fgfr2* [42], *shh* [43], and *id2* [44]. Probes were obtained by in vitro transcription reaction using Dig RNA labeling mix (Roche Applied Sciences, Germany) according to the manufacturer's protocol.

Whole mount in situ hybridization

Dissected lungs were fixed in a 4% formaldehyde solution with 2 mM EGTA in PBS, pH 7.5, at 4 °C overnight. Afterward, lungs were dehydrated through a methanol series and stored at −20 °C. Whole mount in situ hybridization ($n \geq 9$ per stage/gene for whole lungs and $n \geq 4$ per gene/condition for lung explants) was performed as previously described [45]. Each group of lungs/probes was processed simultaneously and developed for the same amount of time. All the lungs were photographed with an Olympus U-LH100HG camera coupled to a stereomicroscope (Olympus SZX16).

Cross section preparation

Hybridized chicken lungs were dehydrated through an ethanol series, embedded in 2-hydroxyethyl methacrylate (Heraeus Kulzer, Germany) and sectioned in 25-µm-thick slides by a rotatory microtome (Leica RM 2155, Germany). All lung sections were photographed using a camera (Olympus DP70) coupled to a microscope (Olympus BX61). Afterwards, slide sections were stained with hematoxylin–eosin as previously described [9] and photographed again at the same magnification.

Results

Expression analysis of retinoic acid signaling pathway

To study the expression pattern of the retinoic acid signaling members and potential targets of the pathway, embryonic chick lungs were analyzed by in situ hybridization to characterize the expression pattern of *stra6*, *raldh2*, *raldh3*, *cyp26a1*, *rarα*, *rarβ*, *sox2*, *sox9*, *meis1*, *meis2*, and *tfgβ2*. Representative lungs of the three stages studied were then processed for histological sectioning. Additionally, to clearly identify the epithelial and mesenchymal compartment, and thus facilitate image analysis, slide sections were stained with hematoxylin–eosin (Figs. S1 and S2).

stra6 expression seems to be present in the most proximal region of the lung, namely in the trachea (Fig. 1a, dark arrowhead) and until the first secondary bud formed. On the other hand, it is absent from both distal epithelium and mesenchyme (Fig. 1c, dagger), and also from secondary bronchi (Fig. 1b, asterisk). This expression pattern

is constant for the three stages studied. Histological sectioning of hybridized lungs clearly showed that *stra6* is expressed in the periepithelial mesenchyme surrounding the main bronchus (Fig. 1d, double dagger) and absent in the lung epithelium (Fig. 1d, asterisk and black arrow).

raldh2 is expressed in the ventral mesenchyme (Fig. 1e, open arrowhead) and in the outermost region of the dorsal mesenchyme (Fig. 1f, dashed arrow). On its turn, it is not expressed in the tracheal region (Fig. 1g, dark arrowhead), in the epithelium of the main bronchus (Fig. 1f, black arrow), and in the distal–dorsal mesenchyme (Fig. 1g, dagger). This pattern is consistent in the three stages studied. Histological sections confirmed that *raldh2* transcript is exclusively located in the cells that outline the embryonic lung, the mesothelium (Fig. 1h, section sign), and in the ventral mesenchyme. Furthermore, it is not present in the secondary buds (Fig. 1h, asterisk). Additionally, *raldh3* expression pattern was also assessed but it was not detected in the embryonic chick lung in the three stages studied (data not shown).

cyp26a1 is present in the mesenchyme of the trachea region (Fig. 1i, dark arrowhead), in the dorsal mesenchyme adjacent to the uppermost secondary bud (Fig. 1k, dashed arrow), and in the ventral mesenchyme (Fig. 1k, open arrowhead). The distal region of the embryonic lung, mesenchyme, and epithelium, does not express *cyp26a1*

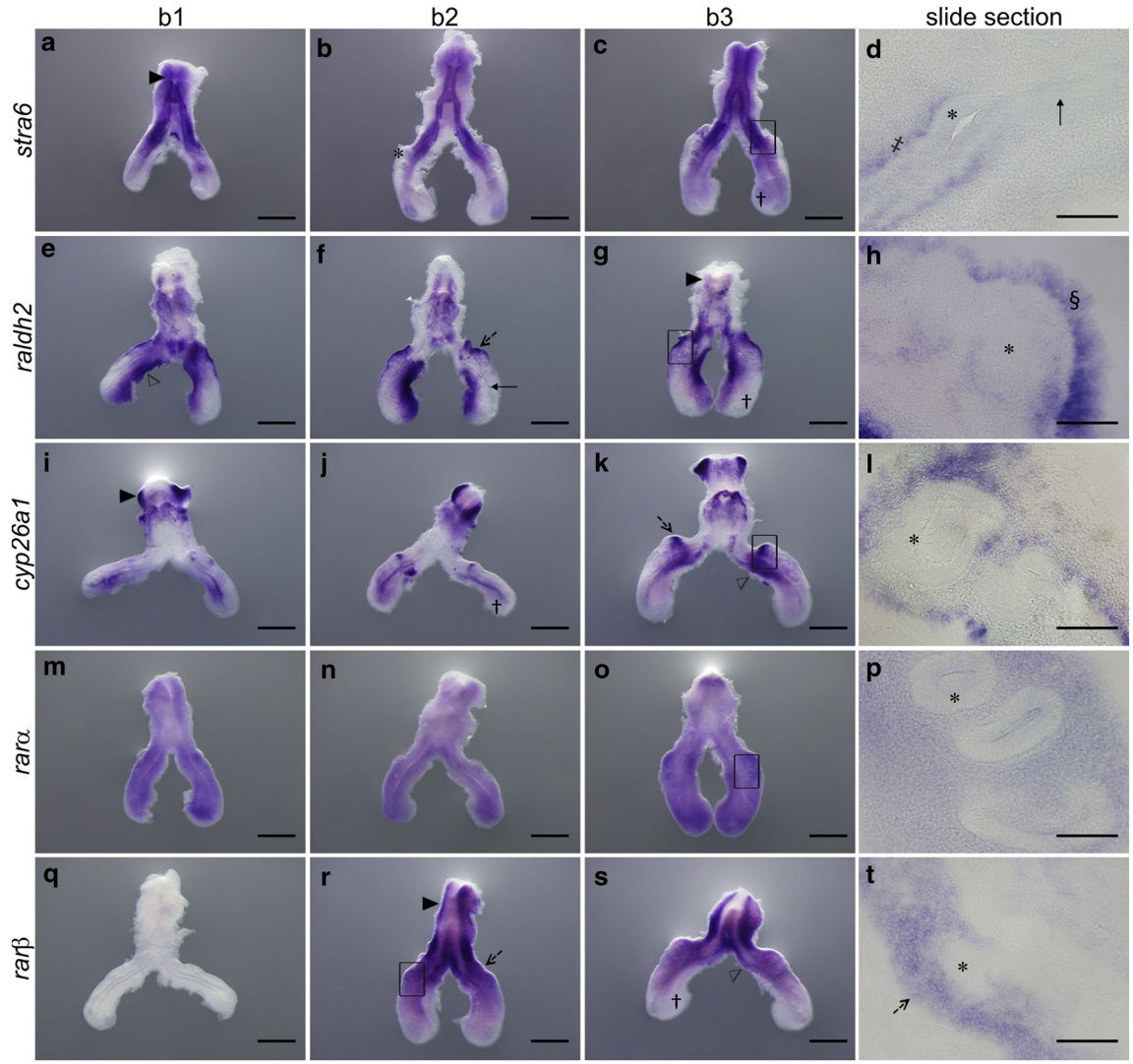

Fig. 1 Retinoic acid signaling core elements' expression pattern at early stages of chick lung development. Representative examples of in situ hybridization of stages b1, b2, and b3 lungs for: *stra6* (**a**–**d**), *raldh2* (**e**–**h**), *cyp26a1* (**i**–**l**), *rarα* (**m**–**p**), and *rarβ* (**q**–**t**), $n \geq 9$ per stage. *Scale bar* whole mount, 500 µm; slide sections, 100 µm. *Black rectangles* in images **c**, **g**, **k**, **o**, and **r** indicate the region shown in corresponding slide section. *Asterisk* secondary bronchi. *Black arrow* main bronchus epithelium. *Dagger* distal mesenchyme. *Dark arrowhead* trachea region. *Dashed arrow* dorsal mesenchyme. *Double dagger* periepithelial mesenchyme. *Open arrowhead* ventral mesenchyme. *Section sign* mesothelium

(Fig. 1j, dagger). There are no differences between the three stages studied. Slide sectioning confirmed that expression is not present in the entire epithelium, namely main bronchi and secondary bronchi (Fig. 1l, asterisk).

rarα is ubiquitously expressed throughout all pulmonary mesenchyme of the chick lung, in the three stages studied (Fig. 1m–o). Histological sections revealed that this gene is absent from the epithelial compartment of the main bronchi and secondary bronchi (Fig. 1p, asterisk).

rarβ expression is evident in the mesenchyme of the tracheal region (Fig. 1r, dark arrowhead) and also in the dorsal (Fig. 1r, dashed arrow) and ventral mesenchyme (Fig. 1s, open arrowhead). Moreover, it is absent from the distal region of the main bronchi (Fig. 1s, dagger). *rarβ* expression seems to be stage dependent since it is not detected in stage b1 lungs (Fig. 1q) but it is highly expressed in stages b2 and b3 (Fig. 1r and s, respectively). Sectioning of the hybridized lungs clearly showed that *rarβ* is not expressed in the epithelium of secondary buds (Fig. 1t, asterisk) but in the surrounding (dorsal) mesenchyme (Fig. 1t, dashed arrow).

sox2 transcript is present in the epithelium of the main bronchi (Fig. 2a, black arrow), except its distal most region (Fig. 2b, bracket), and its expression appears to be more intense in the trachea region (Fig. 3c, dark arrowhead). Additionally, *sox2* is absent from the secondary bronchi (Fig. 2b, asterisk). Histological sectioning of hybridized lungs confirms that *sox2* is exclusively expressed in the epithelial compartment (Fig. 2d, black arrow); as secondary bronchi start to elongate, it is possible to observe *sox2* expression in the region where they emerge from the main bronchus, whereas in the distal region it is still absent (Fig. 2d, asterisk). The expression pattern of secondary bronchi mimics the pattern of the main bronchus.

sox9 mRNA is absent from the epithelial compartment (Fig. 2e, black arrow) except in the distal tip of the main bronchus (Fig. 2e, bracket) and in the secondary bronchi (Fig. 2f, asterisk). Moreover, *sox9* is present in the mesenchyme of the trachea region (Fig. 3g, dark arrowhead). There seems to be a slight decrease in *sox9* expression levels in a stage-dependent manner (from b1 to b3). Slide sectioning revealed that *sox9* expression is restricted to the distal tip of the secondary bronchi (Fig. 2f, h, asterisk) while the region where they emerge from the main bronchus lacks *sox9*. Additionally, *sox9* is expressed in the surrounding mesenchyme.

meis1 is expressed in the mesenchyme, namely in the trachea (Fig. 2i, dark arrowhead), in the ventral mesenchyme (Fig. 2k, open arrowhead), and proximal–dorsal region of the lung (Fig. 2i, dashed arrow). In the three stages studied, *meis1* is not expressed throughout all the epithelium of the main bronchi (Fig. 2j, k, black arrow) and secondary bronchi (Fig. 2j, asterisk). Slide sections confirmed the absence of epithelial expression in both the main bronchi and in secondary bronchi (Fig. 2l, black arrow and asterisk, respectively).

meis2 transcript is present all over the mesenchymal compartment (Fig. 2m–o) and absent from the epithelial compartment (Fig. 2m–o, black arrow and asterisk). This expression pattern was corroborated in the slide sections that undoubtedly show the lack of expression in the epithelium of the main bronchus and secondary bronchi (Fig. 2p, black arrow and asterisk, respectively), and the abundant expression in the surrounding mesenchyme (Fig. 2p, dashed arrow).

tgfβ2 mRNA is present in the dorsal mesenchyme neighboring the secondary buds (Fig. 2q, dashed arrow) and also in the distal ventral mesenchyme (Fig. 2r, dagger). In opposition, *tgfβ2* has no expression in the epithelium (Fig. 2r, black arrow). Sectioning of selected hybridized lungs illustrated the nonexistence of expression in the main bronchi epithelium (Fig. 2t, black arrow) and in the secondary bronchi (Fig. 2t, asterisk). Dorsal mesenchyme was identified as mesothelium in the slide sections (Fig. 2t, section sign).

Impact of retinoic acid supplementation in lung branching and gene expression

With the purpose of studying the role of retinoic acid in the embryonic chick lung, b2 lung explants were cultured, in vitro for 48 h, and supplemented with three different doses of RA, selected according to the literature: 0.1, 1, and 10 μM RA. Additionally, a set of b2 lungs was supplemented with DMSO (control group). Lung explants were morphometrically analyzed and probed for selected genes: *l-cam*, *cyp26a1*, *sox2*, *sox9*, *rarβ*, *meis2*, *hoxb5*, *tfgβ2*, *id2*, *fgf10*, *fgfr2*, and *shh*.

Retinoic acid-treated lungs presented an increase in the number of peripheral airway buds when compared with DMSO-treated explants (Fig. 3b, e, h, k). Nonetheless, to provide clear evidence of the increase of lung branching after RA treatment, in situ hybridization for the epithelial marker E-cadherin (L-CAM, in the chick) was performed. In whole mount lungs, *l-cam* is exclusively expressed in the pulmonary epithelium and the secondary bronchi can be easily recognized (Fig. S3). This experimental approach allows an unambiguous visualization of the epithelial tips, demonstrating that there is an increase in the number of secondary buds formed in a dose-dependent manner (Fig. 3c, f, i, l). Morphometric analysis revealed that RA supplementation elicits an increase in branching, indicated by the ratio between the number of secondary buds at D2 and D0, in a dose-dependent manner when compared to DMSO-treated explants (Fig. 4a). Furthermore, 1 and 10 μM RA doses induced a statistically significant

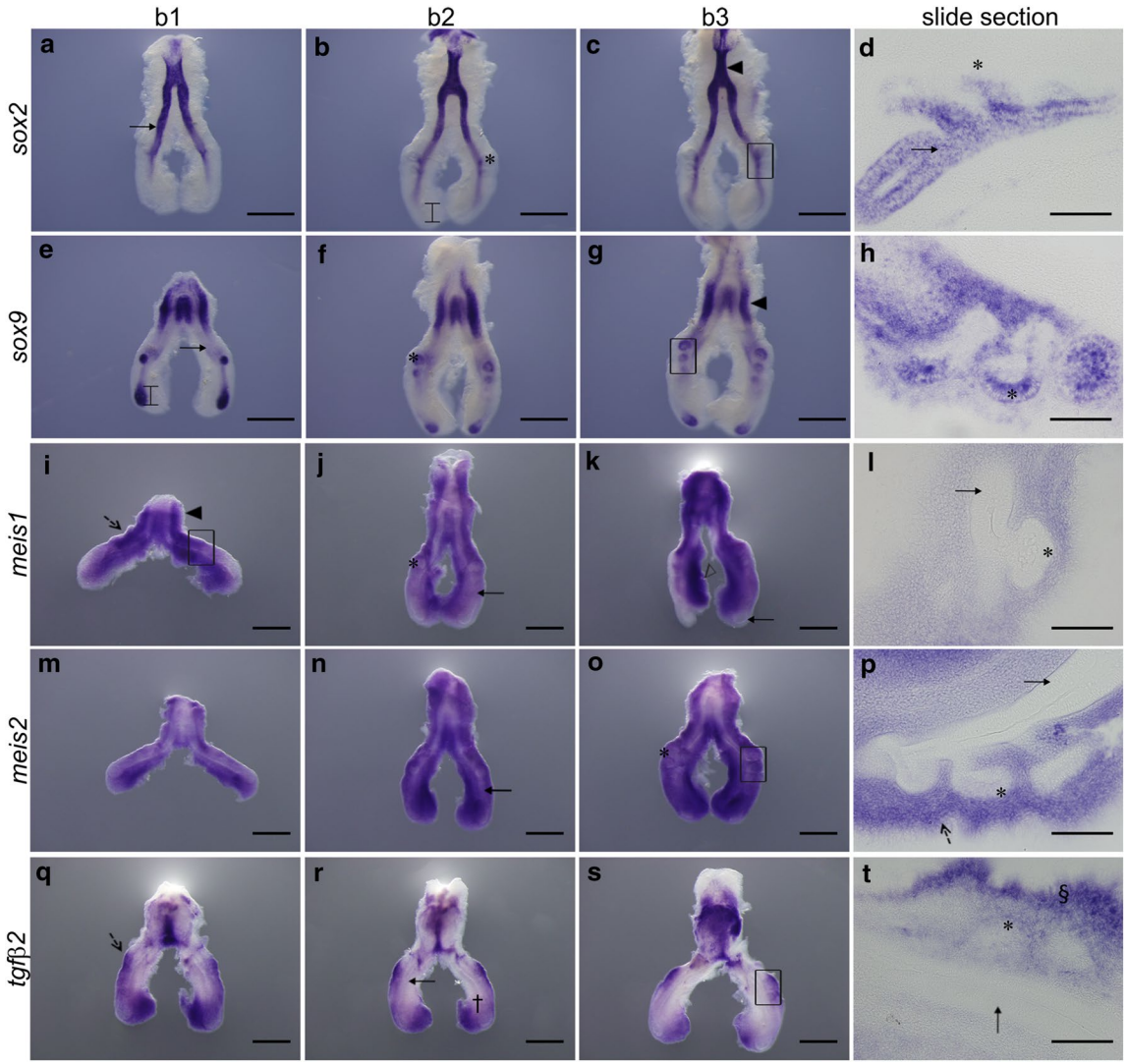

Fig. 2 Retinoic acid pathway potential targets expression pattern at early stages of chick lung development. Representative examples of in situ hybridization of stages b1, b2, and b3 lungs for: *sox2* (**a**–**d**), *sox9* (**e**–**h**), *meis1* (**i**–**l**), *meis2* (**m**–**p**), and *tgfβ2* (**q**–**t**), $n \geq 9$ per stage. *Scale bar* whole mount, 500 µm; slide sections, 100 µm. *Black rectangles* in images **c**, **g**, **i**, **o**, and **s** indicate the region shown in corresponding slide section. *Asterisk* secondary bronchi. *Black arrow* main bronchus epithelium. *Dagger* distal mesenchyme. *Dark arrowhead* trachea region. *Dashed arrow* dorsal mesenchyme. *Open arrowhead* ventral mesenchyme. *Section sign* mesothelium. *Error bar* distal region

increase in lung branching when compared to DMSO-treated explants. On the other hand, there is no statistically significant difference between the lowest RA dose (0.1 µM) and DMSO, or between the two highest doses (Fig. 4a). In addition, the D2/D0 ratio of the epithelial and mesenchymal perimeter was determined (Fig. 4b). RA treatment induced a statistically significant increase in the epithelial perimeter of 1- and 10-µM-treated lungs when compared to control. On the other hand, mesenchymal perimeter did not vary between the different conditions.

To confirm that, after RA supplementation, this signaling pathway was indeed affected, lung explants were probed with *cyp26a1*, a downstream target and a regulator of RA intracellular levels. Retinoic acid supplementation leads to an increase of *cyp26a1* expression levels in a dose-dependent manner (Fig. 5). This increase is evident in the explants treated with 1 µM RA when compared with DMSO (Fig. 5i and c, respectively), and even more clear in the 10-µM-treated explants when compared with control (Fig. 5l and c, respectively). On the other hand, 0.1-µM-treated explants are similar to control explants. This comparison is possible since all explants were developed for the same amount of time. Moreover, *cyp26a1* expression pattern remains unaltered after culture (proximal

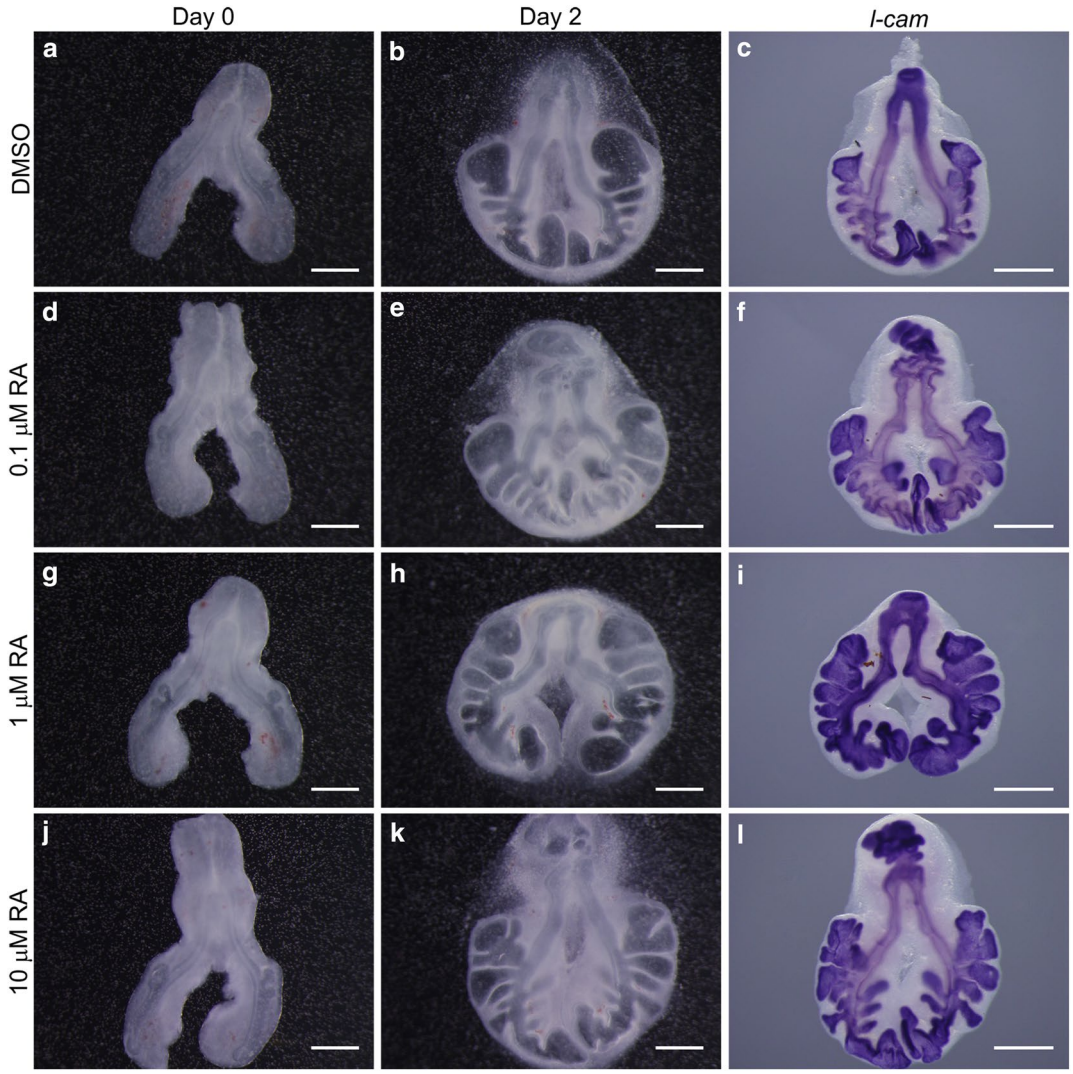

Fig. 3 In vitro RA supplementation of chick lung explants and *l-cam* expression analysis. Representative examples of stage b2 lung explant culture at D0 (**a**, **d**, **g**, **j**) and D2 (**b**, **e**, **h**, **k**), treated with DMSO (**a**, **b**), 0.1 µM RA (**d**, **e**), 1 µM RA (**g**, **h**) or 10 µM RA (**j**, **k**), and probed with *l-cam* (**c**, **f**, **i**, **l**) ($n \geq 4$); *scale bar* 500 µm

and dorsal mesenchyme); nonetheless, it extends to all mesenchymal compartments in the highest dose tested (Fig. 5l).

Taking into consideration that the lowest dose tested (0.1 µM) did not exhibit alterations in *cyp26a1* expression levels (Fig. 5f) and branching (Fig. 4) when compared to control explants, the subsequent studies were performed using only 1 µM and 10 µM RA doses. The expression levels and spatial distribution of several potential downstream targets of retinoic acid signaling (*sox2*, *sox9*, *rarβ*, *meis2*, *hoxb5*, *tgfβ2*, *id2*, *fgf10*, *fgfr2*, and *shh*) were assessed in RA- and DMSO-treated b2 lung explants by in situ hybridization. From this point onwards, only the images of the in situ hybridization are represented because D0 and D2 images for DMSO, 1, and 10 µM are identical in all cases.

After RA supplementation, *sox2* expression levels remain virtually unaltered (Fig. 6a–c). On the other hand, *sox9* expression levels decrease in a dose-dependent manner (Fig. 6d–f); moreover, a progressive loss of the distal expression is also perceived (Fig. 6f).

RA-treated lungs showed an evident increase in *rarβ* expression (Fig. 7b, c) when compared to controls (Fig. 7a) in the tracheal region of the lung. Supplementation studies revealed that only the highest dose tested (10 µM) leads to an increase in *meis2* expression levels (Fig. 7f) when compared to DMSO-treated explants (Fig. 7d). There seem to be no differences between DMSO and the lowest dose of RA tested. Regarding *hoxb5*, RA-treated lungs present higher expression levels than control explants (Fig. 7g). However, the *hoxb5* expression is not

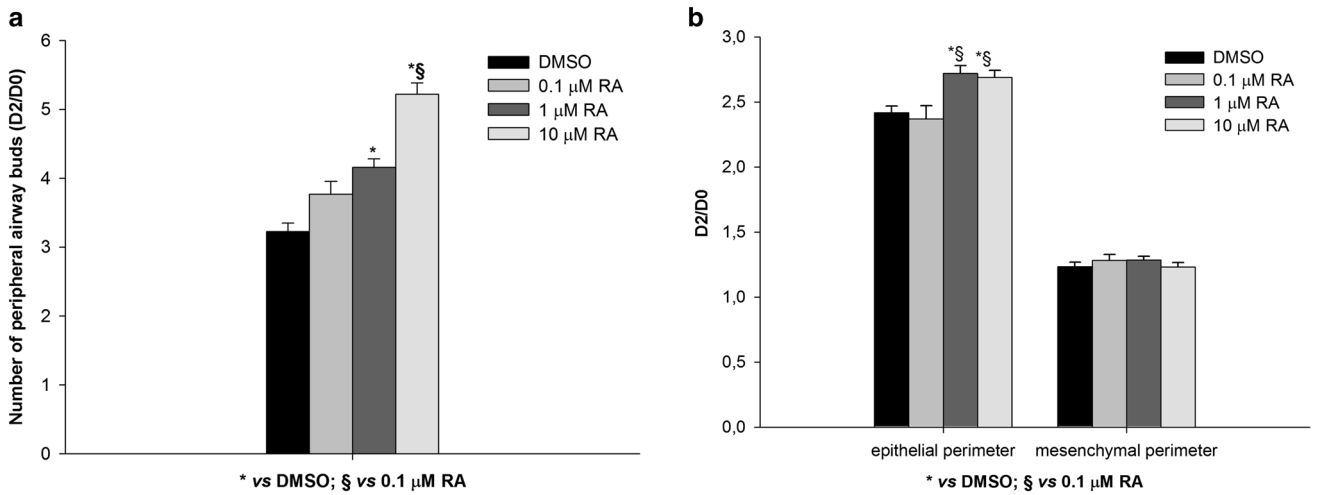

Fig. 4 Morphometric analysis of stage b2 lung explants treated with DMSO, 0.1, 1, and 10 μM of RA ($n \geq 15$/condition). Branching (**a**) and perimeter (**b**) results are expressed as D2/D0 ratio, and represented as mean ± SEM. $p \leq 0.001$: * vs. DMSO; § vs. 0.1 μM of RA

dose dependent, since 1- and 10-μM-treated explants display similar expression levels (Fig. 7h, i). In chick lung explants, this gene is expressed mainly in the ventral mesenchyme. RA supplementation caused an increase in *tgfβ2* expression levels (Fig. 7k, l) when compared to control explants (Fig. 7j), and there seem to be no differences between the two doses tested. In addition, *tgfβ2* expression was expanded to the mesenchymal compartment of the lung. As regards *id2*, after RA supplementation, lung explants displayed an increase in its expression levels (Fig. 7n, o) when compared to DMSO (Fig. 7m); furthermore, in RA-treated lungs, *id2* dorsal expression widened to the distal region of the lung, whereas in DMSO-treated lungs it is constrained to the region adjoining the proximal secondary buds (Fig. 7m). *id2* expression pattern in whole mount lungs is described in Fig. S4.

After RA treatment it is possible to observe a slight decrease in *fgf10* expression levels (Fig. 8b, c) when compared to DMSO-treated explants (Fig. 8a). Likewise, *fgfr2* expression levels display a similar trend in RA-treated lungs (Fig. 8d–f). On its turn, RA supplementation caused a slight increase in *shh* expression levels (Fig. 8h, i) when compared to control explants (Fig. 8g).

Discussion

Retinoic acid plays an essential role during vertebrate embryogenesis, and its distribution and levels must be strictly regulated throughout development. RA signaling is involved in numerous processes such as proliferation, differentiation, and morphogenesis of several organs, namely the lung. Indeed, RA is essential for growth and differentiation of the mammalian lung [12, 46]. Pulmonary development, specifically branching morphogenesis, also depends on the activity of other signaling pathways as, for instance, FGF, WNT or SHH just to name a few [47]. All the signaling pathways involved in this embryonic event regulate and interact with each other, and this finely tuned molecular network leads to the formation of a healthy and fully functional lung. FGF, WNT, and SHH signaling pathways have already been described in the chick embryonic lung, and it has been demonstrated that they interact with each other [4, 5, 7]. Conversely, retinoic acid signaling has not been described hitherto. In this report, we intended to uncover the RA molecular network underlying chick lung branching and to characterize, for the first time, the expression pattern of several RA signaling members in the chick developing lung. For this purpose, we analyzed embryonic chick lungs that display similar anatomic features with mammalian fetal lung; in these early stages, the lateral chick lung branching pattern is similar to one of the mammalian branching subroutines (described by [2]). In this study, we characterized, by in situ hybridization, the expression pattern of genes involved in RA transport (*stra6*), biosynthesis (*raldh2* and *raldh3*), degradation (*cyp26a1*), and signaling regulation (*rarα* and *rarβ*). Moreover, the localization of putative RA downstream targets such as *sox2*, *sox9*, *meis1*, *meis2*, *tfgβ2*, and *id2* (Fig. S4) was also described. Furthermore, the influence of RA on branching was evaluated in vitro, in lung explant cultures supplemented with increasing doses of RA; lung explants were then assessed for gene expression of *l-cam*, *sox2*, *sox9*, *cyp26a1*, *rarβ*, *meis2*, *hoxb5*, *tfgβ2*, *id2*, *fgf10*, *fgfr2*, and *shh* to disclose the molecular network that contributes to RA response.

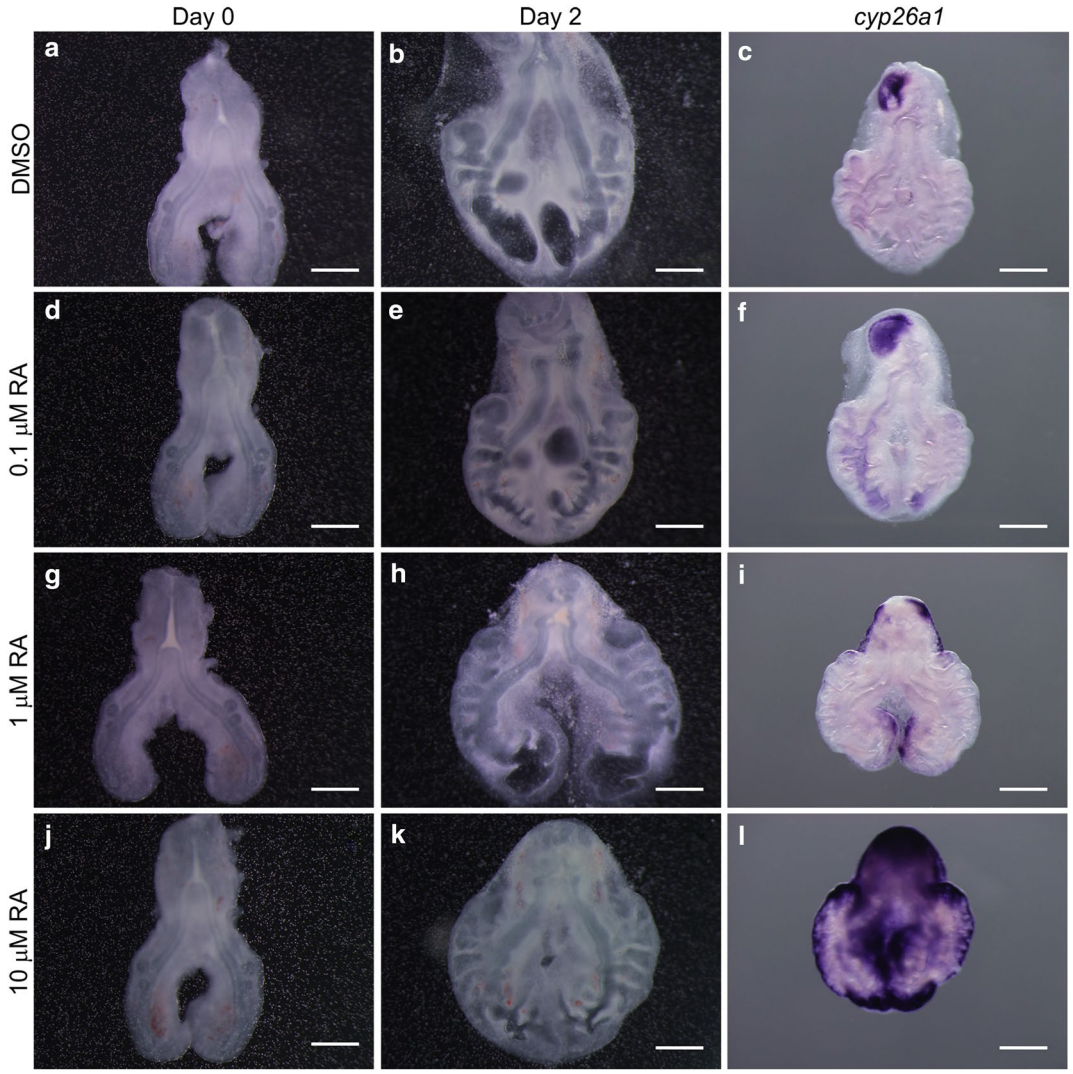

Fig. 5 In vitro RA supplementation of chick lung explants and *cyp26a1* expression analysis. Representative examples of stage b2 lung explant culture at D0 (**a**, **d**, **g**, **j**) and D2 (**b**, **e**, **h**, **k**), treated with DMSO (**a**, **b**), 0.1 µM RA (**d**, **e**), 1 µM RA (**g**, **h**) or 10 µM RA (**j**, **k**), and probed with *cyp26a1* (**c**, **f**, **i**, **l**) ($n \geq 4$); *scale bar* 500 µm. The signal observed in the most proximal region of the lung (**c**, **f**) is due to the accumulation of developing solution

Retinoic acid signaling members are expressed at early stages of chick lung development

Retinoic acid must go through a series of cellular/enzymatic steps before it reaches the nucleus where it binds to its nuclear receptors. The gateway of RA pathway is STRA6, a transmembrane protein responsible for the main entry of retinol inside the cell, and that influences RA intracellular levels since it can regulate the uptake of vitamin A [18]. Nonetheless, and due to its lipophilic nature, RA can also cross the cellular membrane without the need of a membrane receptor [48]. During chick lung development, *stra6* gene is expressed in the periepithelial mesenchyme surrounding the epithelium of the main bronchus until the first secondary bud (Fig. 1a–d). Furthermore, *stra6* mRNA is absent from the entire lung epithelium, and its expression is restricted to proximal mesenchyme, in the three stages studied. This expression pattern is in agreement with what has been described in the mouse lung; in fact, at embryonic day (E) 13.5, *stra6* is present in the mesenchyme surrounding the bronchi [49]. It has been reported that homozygous mutations in this gene cause diverse congenital defects, among them lung hypoplasia, congenital diaphragmatic hernia (CDH), and alveolar capillary dysplasia [50, 51], proving that *stra6* plays a key role in lung morphogenesis by mediating retinol influx and thus contributing to the maintenance of RA levels. It is likely that, also in the chick lung, *stra6* has a similar function.

raldh2 codes for a key oxidative enzyme of RA pathway and it is mainly expressed in the ventral lung mesenchyme

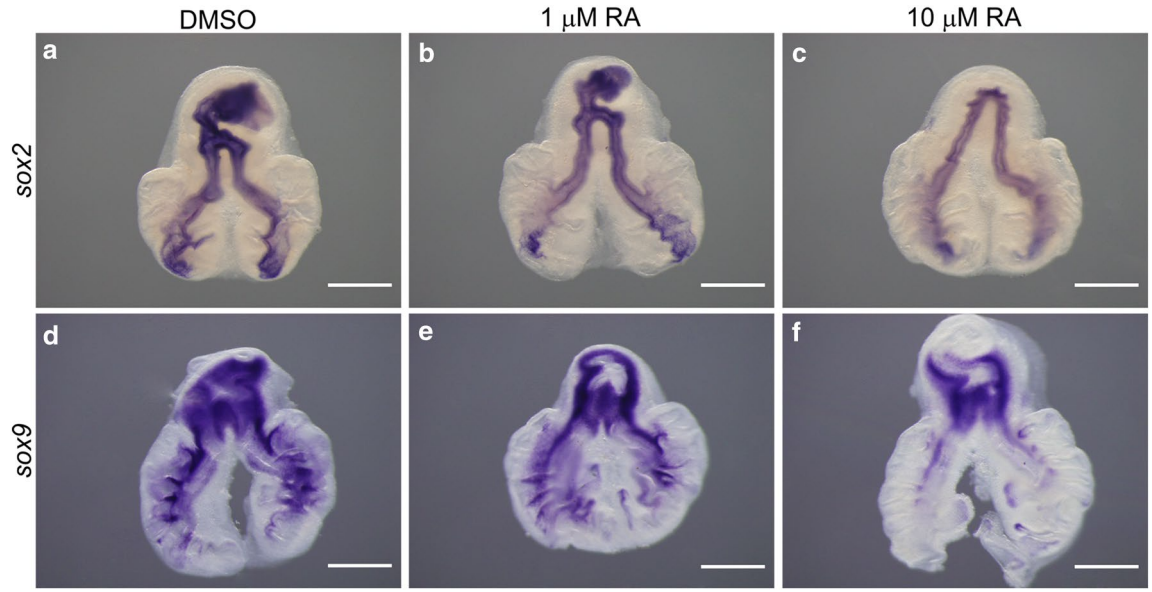

Fig. 6 In vitro RA supplementation of chick lung explants followed by *sox2* and *sox9* expression analysis. Representative examples of stage b2 lung explant culture at D2 (48 h), treated with DMSO (**a**, **d**), 1 μM RA (**b**, **e**) or 10 μM RA (**c**, **f**), and probed *with sox2* (**a**–**c**) and *sox9* (**d**–**f**) ($n \geq 5$); *scale bar* 500 μm

of the chick lung (Fig. 1e, open arrowhead) and in the outermost region of the lung, the mesothelium (Fig. 1h, section sign). This expression pattern remains constant in the three stages studied. In the mouse model, *raldh2* is expressed in the lung primordium at E9.5 which suggests that RA signaling is ubiquitously activated at the onset of pulmonary development; actually, *raldh2(−/−)* knockouts fail to develop lungs [26]. Throughout development, in the early pseudoglandular stage, *radlh2* expression becomes more restricted to mesothelial cells and decays towards the distal region of the lung [12]. This feature is also perceived in the embryonic chick lung: there is a decreasing gradient of *raldh2* expression at the surface of the avian lung from the proximal–dorsal to the distal–dorsal region. The spatial distribution of this enzyme seems to point to a role of RA in pleura development. In addition, *raldh2* expression prevails in areas of low branching activity and presents a complementary pattern to *fgf10*. Likewise, in the chick lung, this complementary expression pattern is conserved. *raldh2* is expressed in the ventral region of the lung, whereas *fgf10* is expressed in the dorsal region [4]. In the chick lung, it has been demonstrated that FGF signaling is of utmost importance in the branching process [4] and that the cyst–branch differences are due to regional (ventral–dorsal) alterations of *fgf10* levels [52]. It seems likely that retinoic acid signaling might hamper *fgf10* expression levels or interfere with extracellular matrix remodeling, namely heparan sulfate proteoglycan levels and, therefore, contribute to the accurate patterning of the lung. In summary, *raldh2*'s presence indicates that RA is being synthesized in chick lung mesenchyme and that, most probably, this signaling pathway is active in this cellular compartment. Nonetheless, there are other factors that might influence the activity of this pathway, namely the existence of degradative enzymes (like CYP26) that contribute to the regulation of RA intracellular levels and the accessibility of retinoic nuclear receptors (RARs).

On the other hand, *raldh3* expression was exceptionally faint (data not shown) even after a longer developing process (36 h). In the mammalian lung, *raldh3* has been also described as weakly expressed in the lung epithelium of the main bronchi at E12.5–E14.5 [25]. *raldh3* knockout mouse displays newborn ocular and nasal defects but does not exhibit lung abnormalities, suggesting that this gene is not relevant for pulmonary morphogenesis [53]. The absence of *raldh3* in early stages of chick lung development implies that, perhaps, it may also not be required for lung organogenesis.

Retinoic acid levels must be sharply regulated to maintain adequate signaling activity. Biosynthetic enzymes, such as RALDH2, or degradative enzymes, such as CYP26 (that belong to the cytochrome P450 family), are involved in this regulatory mechanism. *cyp26a1* has a very particular expression pattern in the mesenchyme of tracheal region (Fig. 1i, dark arrowhead). In the mouse fetal lung, at the early pseudoglandular stage (E11.5) *cyp26a1* is absent from the tracheal region and confined to the epithelial compartment [12]. Nonetheless, from E14.5 onwards, *cyp26a1* expression is detected in both epithelium and mesenchyme, and later (E16.5) it is associated with cartilage rings.

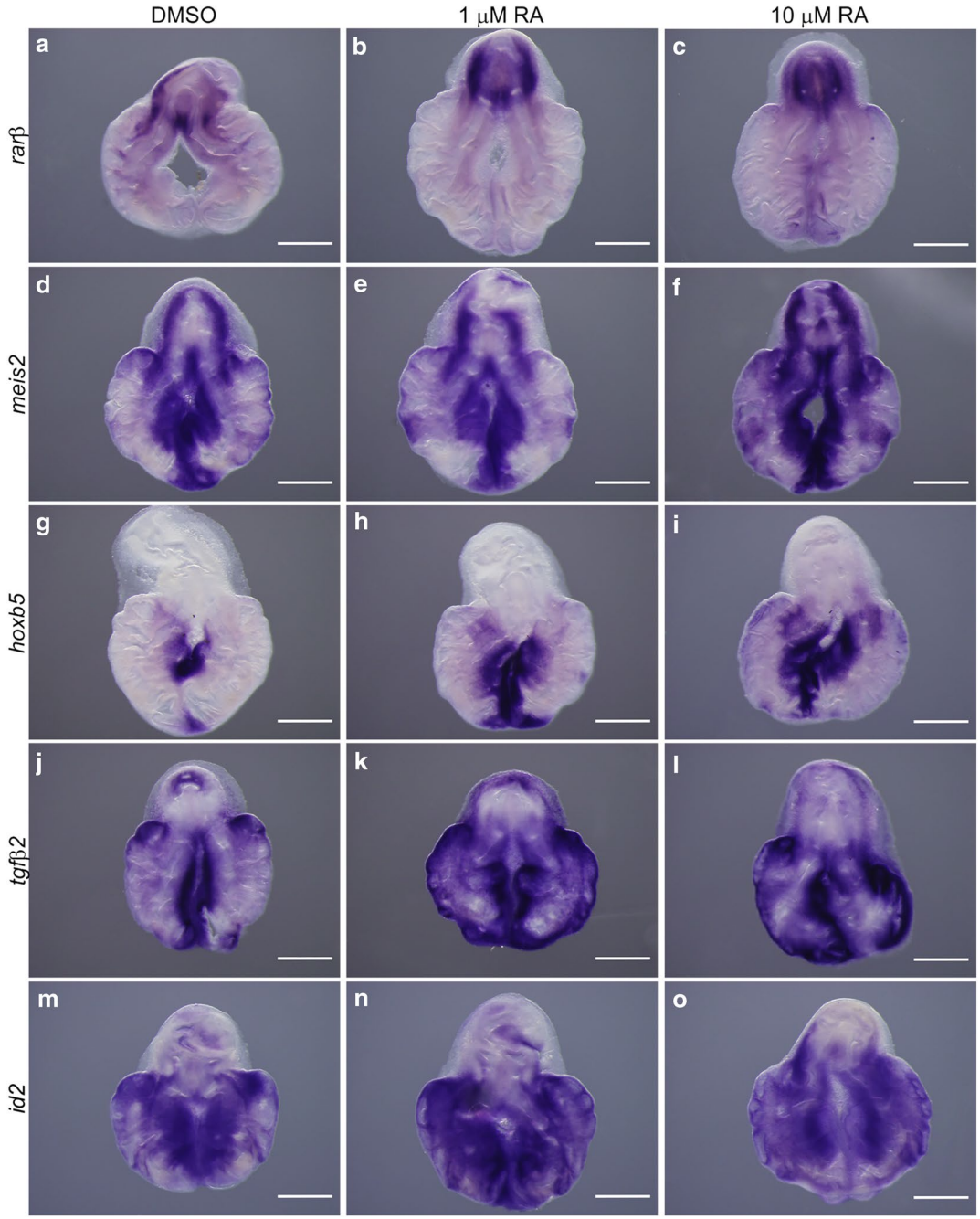

Fig. 7 In vitro RA supplementation of chick lung explants followed by *rarβ*, *meis2*, *hoxb5*, *tgfβ2*, and *id2* expression analysis. Representative examples of stage b2 lung explant culture at D2 (48 h), treated with DMSO (**a**, **d**, **g**, **j**, **m**), 1 μM RA (**b**, **e**, **h**, **k**, **n**) or 10 μM RA (**c**, **f**, **i**, **l**, **o**), and probed with *rarβ* (**a**–**c**), *meis2* (**d**–**f**), *hoxb5* (**g**–**i**), *tgfβ2* (**j**–**l**) and *id2* (**m**–**o**) ($n \geq 4$); *scale bar* 500 μm

Throughout development *cyp26a1* progressively expands proximally. In the chick lung, in the three stages studied (that match early mouse pseudoglandular stage), *cyp26a1* expression is observed in the lung's proximal region, similar to later stages of mouse fetal lung, and it is perhaps involved in the mechanisms underlying trachea development. Furthermore, it is present in the dorsal mesenchyme surrounding the proximal-most bud (Fig. 1k, dashed arrow) and in the ventral mesenchyme bordering the epithelium; interestingly, *raldh2* is expressed in the most peripheral layers of the mesenchyme, in the same regions. This sequential spatial expression of *raldh2* and *cyp26a1* (from the periphery to the interior of the mesenchymal compartment) most likely regulates the availability of retinoic acid by creating a gradient, due to local degradation of RA by *cypa26a1*, therefore, affecting the pathway activity and influencing

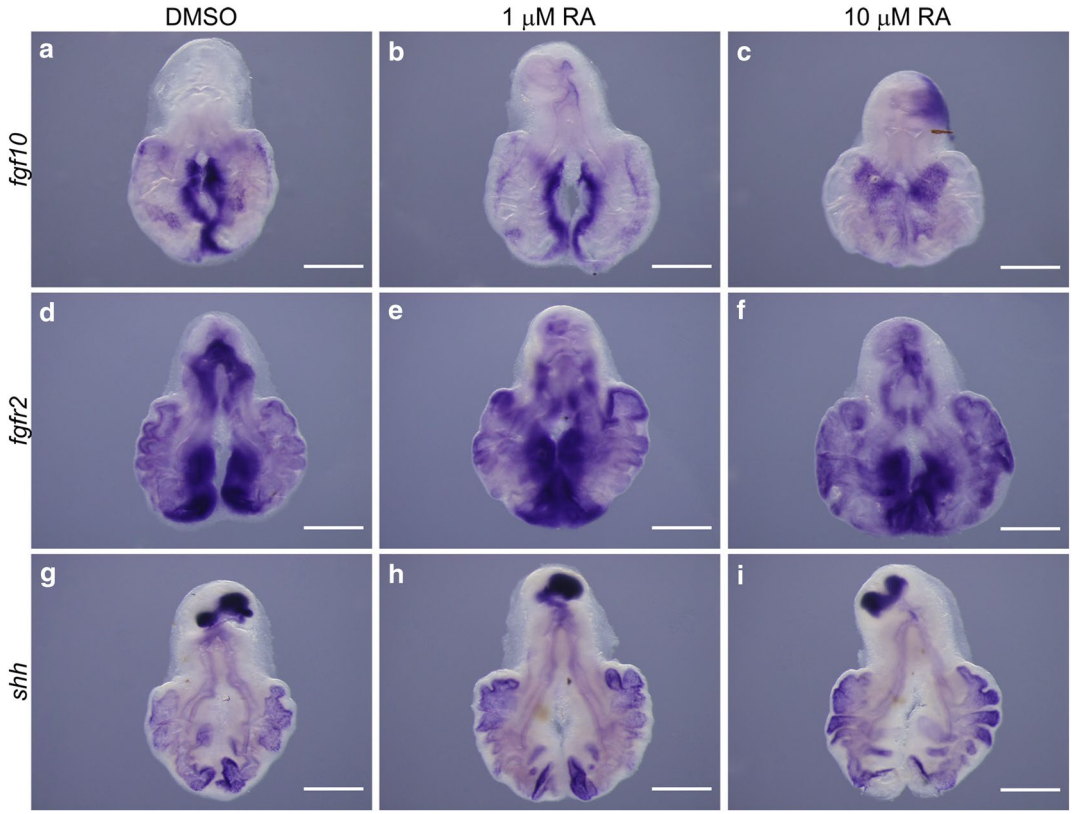

Fig. 8 In vitro RA supplementation of chick lung explants followed by *fgf10*, *fgfr2* and *shh* expression analysis. Representative examples of stage b2 lung explant culture at D2 (48 h), treated with DMSO (**a**, **d**, **g**), 1 μM RA (**b**, **e**, **h**) or 10 μM RA (**c**, **f**, **i**), and probed with *fgf10* (**a**–**c**), *fgfr2* (**d**–**f**) and *shh* (**g**–**i**) ($n \geq 4$); *scale bar* 500 μm

mesenchymal and epithelial morphogenesis. In the dorsal mesenchyme, *raldh2* and *cyp26a1* expressions seem to act as if it were pinpointing the location of the proximal-most branch.

The retinoic acid signaling response is transduced by nuclear retinoic acid receptors (RARα, β, and γ, each having several isoforms) that activate transcription of specific genes by forming a heterodimer with RXR. The vital function of these receptors was disclosed when single and double knockout mice were produced. Mice lacking simply one RAR isoform appeared phenotypically normal [54, 55], suggesting a functional redundancy mechanism among RAR family. Conversely, double null receptor mutations are lethal in utero or shortly after birth [56] and develop several respiratory tract abnormalities, among them: lung agenesis, lung hypoplasia, and lack of esophageal–tracheal septum [23]. Furthermore, double knockouts with different combinations of RAR subtypes yield different lung phenotypes indicating that RARs (α, β, and γ) have multiple developmental roles in pulmonary morphogenesis [23, 55]. *rarα* is ubiquitously expressed in the mesenchyme of the entire chick lung in the three stages studied (Fig. 1m–p). During mammalian embryogenesis, *rarα* transcripts are universally expressed at all stages and do not vary temporally or spatially [57]; the same is true in the epithelium of the developing lung [12, 58]. On the other hand, in the chick lung, *rarβ* exhibits a stage-dependent expression: it is completely absent from stage b1 lungs (Fig. 1q); however, in stages b2 and b3 it is detected in the mesenchymal compartment of the most proximal region (Fig. 1r, s). In the mouse, *rarβ2* is expressed as early as E9.5, prior to lung bud formation, and continues in tracheal and lung bud epithelium. By E11-12.5, *rarβ2* is found in the epithelium of the proximal bronchi, in the tracheal region (both epithelium and mesenchyme), and it is excluded from the distal part of the lung [12, 57]. In both chick and mouse developing lung, *rarβ* expression is circumscribed to the proximal region of the lung which may point towards a similar role for *rarβ* in the morphogenesis of that area. Nevertheless, the cellular compartment is not the same: chick mesenchyme vs. mouse epithelium. In fact, *rarα* expression pattern presents the same difference. Indeed, all the members of retinoic acid signaling characterized in this study are expressed in the mesenchymal compartment and completely absent the lung epithelium. This feature sustains the hypothesis that, in the chick lung, retinoic acid availability may have a

direct effect on mesenchyme morphogenesis while its putative effect in the epithelium must be through the crosstalk with other signaling pathways. In the mouse model, some members of the retinoic acid signaling are expressed in the mesenchyme (*stra6*, *raldh2*, and *cypa26a1*) while others in the epithelium (*rarα* and *rarβ*), which is a clear example of epithelial–mesenchyme interactions. It is worth mentioning the lack of *rarβ* expression in stage b1 lungs. This experiment was repeated several times to confirm this result. Currently we do not have a plausible explanation for this finding; eventually, it might be related to the *rarβ* isoform that was analyzed since different isoforms may present tissue-specific expression [59].

Retinoic acid signaling potential targets are expressed in the embryonic chick lung

In the present study, several RA pathway putative downstream targets were also examined in the chick lung, for the first time by in situ hybridization. These genes were selected based on the current knowledge of RA signaling in processes such as limb development (*meis1* and *meis2*) [60] and also on the available data from mammalian lung development (*tfgβ2*) [61]. Additionally, *sox2* and *sox9* (and *id2*) were also characterized since they are differentially expressed in the mammalian lung establishing the proximal vs. distal fate of the respiratory tree, respectively [62, 63], and because RA signaling regulates lung patterning [12].

sox2 and *sox9* belong to the SRY-related HMG-box family of transcription factors that play numerous functions during embryonic development, among them, regulation of cell specification and differentiation (reviewed by [64]). In the pulmonary context, *sox2* and *sox9* are particularly important for the proliferation and differentiation of upper and lower branching epithelium, respectively (reviewed by [65]). In the chick lung, *sox2* is exclusively expressed in the epithelium of the main bronchus, except its distal tip (Fig. 2a–d). This expression pattern is very similar to the mammalian lung [62] and it is in agreement with a previous study in the avian lung [66]. *sox2* is expressed in the foregut endoderm and it has been implicated in trachea and esophagus morphogenesis [67, 68]. As lung development proceeds, during branching morphogenesis, *sox2* is restricted to pulmonary epithelium but it is not detectable at the tips of the emerging secondary bronchi budding out from the primary bronchus. In fact, it has been shown that loss of *sox2* at branching sites is required for branching morphogenesis to occur; additionally, *sox2* is also necessary for the timely differentiation and proliferation of the proximal airway epithelium [62, 69]. It is possible that, also in the chick lung, *sox2* plays a similar function considering that its expression is equivalent to the mammalian lung.

sox9 is expressed in the epithelial tip of the main bronchi and secondary bronchi, and in the mesenchyme surrounding the proximal region of the lung (Fig. 2e–h). This expression pattern matches its mammalian counterpart [70]. In the mouse lung, the absence of proximal mesenchymal *sox9* expression impairs cartilage development [71, 72] and proper tracheal epithelium differentiation [73]. At the epithelial level, *sox9* (and *id2*)-expressing cells delimit the distal branching epithelium and originate distal pulmonary cell lineages [63]. Recently, it has been demonstrated that SOX9 is crucial for the regulation of branching morphogenesis by controlling not only proliferation and differentiation but also extracellular matrix composition [63]. Taking into account that *sox9* spatial distribution in the avian lung is comparable to the mouse lung, it is reasonable to assume that it might have a similar role. It is worth mentioning that *id2* (inhibitor of differentiation 2) expression is not an absolute match to its mammalian counterpart (Fig. S4). In fact, in the mouse lung, *id2* is exclusively transcribed in the epithelial tip cells of the developing lung. Additionally, it has been demonstrated that Sox9+/Id2+ cells are initially capable of generating both airway and alveolar epithelium until E13.5; after this time point, these progenitors can only generate alveolar cells [74]. In the chick lung, *id2* is widely expressed in the mesenchyme although it is also present in the epithelial tips. This spatial distribution may point toward other roles for *id2* in chick lung development.

Homeobox genes, *meis1* and *meis2*, are downstream targets of retinoic acid signaling and have been directly implicated in proximal–distal limb patterning [60]. Likewise, RA is an upstream activator of *meis2* during telencephalon development, namely in the dorsoventral patterning of neural progenitor cells [75]. However, the presence of these genes during lung development has not been described up to now. *meis1* and *meis2* display a similar expression pattern. They are ubiquitously and exclusively expressed in the mesenchymal compartment and absent from lung epithelium (Fig. 2i–p). In the chick embryo, *meis* genes are limited to the proximal region of the limb [60, 76] and confined to the intermediate subdivision of the telencephalon [75]; in both cases, they were associated with axis patterning. Considering the widespread expression of *meis* in the chick lung, contrasting with the aforementioned events, it is probable that *meis* genes may perform another type of function in lung morphogenesis.

The transforming growth factor-β (TGFβ) superfamily of secreted cytokines has three subtypes. In this report, the expression pattern of *tgfβ2*, which has been associated with mammalian lung development [60], was investigated. In the chick developing lung, *tgfβ2* mRNA is present in the mesenchyme surrounding the secondary bronchi (Fig. 2q, dashed arrow), in the mesothelium layer (Fig. 2t, section sign) and, to a lesser extent, in the distal ventral

mesenchyme (Fig. 2r, dagger). This expression pattern is quite different from its mammalian counterpart that is mainly expressed in the distal epithelium and absent from the proximal region of the lung [77, 78]. Remarkably, *tgfβ2* expression pattern in the chick lung resembles *tgfβ3* expression in the mouse lung, at E11.5. *tgfβ3* is detected in the proximal mesenchyme and, in addition, the pulmonary mesothelium [78]. A previous study had already reported the presence of *tgfβ2* in the mesenchyme and pleura of stage 27 chick lungs and correlated this expression with *tgfβ1* spatial localization [39]. In the chick developing lung, *tgfβ2* expression is different but still comparable to a different mammalian isoform.

To conclude, all the genes belonging to the RA machinery characterized in this report share a common characteristic: they are all solely expressed in the mesenchymal compartment, although differently distributed along the proximal–distal and dorsoventral axis of the developing chick lung. This feature seems to be species specific but, nonetheless, it is reasonable to believe that retinoic acid signaling most probably influences proliferation and differentiation and hence contributes to chick lung branching morphogenesis.

Retinoic acid signaling and chick lung branching and patterning

Retinoic acid versatility extends from embryonic development to the adult stage [20, 79]; however, regardless of the context, RA intracellular levels must be finely regulated to assure the appropriate signaling activity. In the mammalian lung, RA is of paramount importance during primary bud formation, branching morphogenesis, and alveologenesis (reviewed in [80]). Once we have shown that all the retinoic signaling machinery is present in the chick lung, we asked what would be the impact of RA supplementation in chick lung branching, in vitro. For this purpose, b2 chick lung explants were supplemented with three different doses of RA selected according to the literature [81]; additionally, lung explants were also treated with DMSO as it is a RA solvent.

Morphometric analysis, measured as the D2/D0 ratio of the number of peripheral buds, revealed that RA supplementation increases chick lung branching in a dose-dependent manner (1 and 10 μM) when compared to DMSO (Fig. 4a). Furthermore, 0.1 μM RA-treated explants were comparable to controls. Representative explants of the four experimental conditions were probed with *l-cam* (an epithelial marker) to reinforce the data of the morphometric analysis. With this approach, it is visually evident that the number of secondary buds formed augments in a dose-dependent manner (Fig. 3c, f, i, l). Additionally, RA treatment triggers an increase in the D2/D0 ratio of the epithelial perimeter corroborating the data regarding the number of secondary buds formed in 1- and 10-μM-treated lungs; nonetheless, the mesenchymal perimeter remains unaltered (Fig. 4b). Similar results were obtained following fetal rat lung explants' treatment with exogenous 1 μM RA, for 96 h; an increase in the number of peripheral buds, epithelium perimeter, and total area of the lung was reported [82]. A previous study, using fetal mouse lung, described that 1 μM RA treatment for 48 h promotes branching; however, the new distal buds formed look like proximal branches [83]. Actually, several reports have revealed that RA treatment, in vitro, decreases the average terminal bud number and that the new terminal buds formed present a "proximal-like" aspect [58, 81]. It has been demonstrated that the expression levels and, importantly, the spatial distribution of distal markers of differentiation, such as surfactant proteins, are impaired [64, 67]. Moreover, it has been shown that the expression of genes involved in organ patterning, as for instance *hox* genes (among many others), is compromised when lungs are supplemented with RA, which may explain the impairment in the proximal–distal organization of the lung [84, 85]. Overall, these experiments indicate a clear role for retinoic acid signaling in the proximal–distal patterning of the early mammalian lung, most likely by interfering with the expression of genes involved in cell fate identity.

To confirm that the observed results were indeed a result of the activation of RA signaling, chick lung explants were probed with *cyp26a1*, a downstream target of RA signaling and part of a feedback mechanism controlling RA availability [12]. This enzyme can oxidize RA and, consequently, regulate its intracellular levels and limit RA signaling activity (since high levels may have a detrimental effect). RA supplementation increased *cyp26a1* expression level in a dose-dependent manner (Fig. 5) indicating an increase in signaling activity. This comparison is possible since all explants were developed for the same amount of time. It seems like DMSO and 0.1-μM-treated explants (Fig. 5c and f, respectively) do not express *cyp26a1*. This is probably due to the fact that the developing reaction had to be stopped to prevent further signal in 10-μM-treated explants (Fig. 5l), and it is not an effect of the in vitro culture system or a real lack of *cyp26a1* expression. In 1- and 10-μM-treated explants, *cyp26a1* is expressed in the proximal and dorsal mesenchyme, similar to whole mount lungs. Nonetheless, the highest RA dose triggers an ectopic expression of this gene in the mesenchymal compartment, surrounding branching and non-branching regions. With this experiment, we can assure that, in the chick lung, *cyp26a1* is also a target of retinoic acid signaling pathway since its expression is upregulated with RA supplementation. Hence, it is reasonable to assume that the

observed increase in branching is a result of RA signaling activation. Moreover, the loss of *cyp26a1* proximal–distal spatial distribution probably will interfere with lung patterning.

In the embryonic chick lung, retinoic acid supplementation causes an evident augment in branching (Figs. 3, 4). To clarify if this increase was accompanied by an alteration in the proximal–distal patterning of the chick lung, as it occurs in the mammalian lung, the expression levels of early molecular markers of proximal (*sox2*) and distal (*sox9*) airway progenitors were evaluated (Fig. 6). Retinoic acid had no impact on *sox2* expression levels which may well indicate that proximal epithelial fate and proximal structures, such as the conducting airways, would develop normally. Indeed, *sox2* overexpression in the lung epithelium impairs epithelial differentiation and prematurely commits cells to a specific differentiation program [62], while *sox2* deletion diminishes the mature secretory and ciliated lineages in the respiratory tree [69]. Conversely, *sox9* spatial distribution and expression levels were affected by RA supplementation. It has been demonstrated that epithelial-specific loss of *sox9* triggers the formation of cyst-like structures at the distal epithelial branch tips with concomitant defects in the differentiation of distal progenitor cells [63, 86], whereas *sox9* overexpression blocks the differentiation of distal epithelial progenitors [63]. In the particular case of the chick lung, the decrease in *sox9* expression levels may lead to a decrease in distal epithelial multipotent progenitors and induce a proximal differentiation program. Simultaneously, *sox9* spatial distribution was altered by RA and it was progressively excluded from the distal regions of the lung but maintained in the proximal mesenchyme; this change in the expression pattern may, likewise, contribute to the disproportion of the proximal vs. distal cell phenotype. Taken together, these results seem to point to an alteration in the fate of distal cells, while proximal cells will probably not be affected, even though new distal branches do not exhibit a proximal appearance. Overall, these results imply that retinoic acid may be a regulator of the proximal–distal patterning of the embryonic chick lung, as it occurs in the mammalian lung and that it is involved in the epithelial–mesenchymal interactions that guide avian pulmonary morphogenesis.

Retinoic acid signaling molecular network

Retinoic acid signaling pathway conveys its cellular response by up/down regulating the transcription of several target genes, which regulate biological processes such as proliferation, differentiation, and patterning, through the action of retinoic acid nuclear receptors. Taking this into consideration, we sought to unveil the molecular network that might be orchestrating retinoic acid action during chick lung branching, in vitro. In this sense, explants treated with 1 and 10 μM were evaluated for the expression levels of selected genes. Since 0.1 μM dose did not display differences in both branching and *cypa26a1* expression levels, we opted to exclude it from the subsequent studies.

From the RA signaling components characterized in the previous sections, we decided to analyze *rarβ* expression levels since it had already been shown that RARβ protein levels [82] and *rarβ* expression levels [58] increased after RA supplementation. Data regarding *rarα* were not so evident and, therefore, we selected only one nuclear receptor. *raldh2* was not assessed for two main reasons: on the one hand, the oxidative reaction catalyzed by RALDH2 is surpassed in our in vitro experimental setup because we supplemented directly with RA, the end product of this reaction; furthermore, the reaction catalyzed by this enzyme is irreversible implicating that the interconversion of RA to retinal is not feasible, which means that RA levels must be regulated by the action of CYP26 enzymes (as it is observed in Fig. 5). STRA6 transporter facilitates the entrance of retinol into the embryonic cells when attached to a retinol-binding protein (RBP4); since the in vitro experimental setup used lacks both retinol and RBP4, the predominant method for RA entrance is membrane diffusion. For this reason, *stra6* was not evaluated as a target.

rarβ is one of the transducers of RA cellular response since it behaves as a final decision maker by interacting with the promoter region of target genes. RA supplementation increased *rarβ* expression levels (Fig. 7a–c) and its spatial distribution was similar to the proximal/tracheal localization observed in whole mount lungs (Fig. 1r, s). It has been demonstrated, in the rat model, that explants treated with 1 μM RA present a decrease in RARα and RARγ and an increase in RARβ protein levels revealing a direct regulation of RAR by RA [82]. A previous study also demonstrated the interplay between RA and its own receptor [58]. Mollard and coworkers showed that mouse fetal lung explants supplemented with 1 μM RA display an obvious increase in *rarβ* expression levels; nonetheless, the spatial distribution is quite altered [58]. *rarβ* is ectopically expressed in the distal buds as a result of RA signaling activation, whereas RA signaling inhibition leads to a decrease in *rarβ* expression levels in the proximal region. These data prompt the authors to propose that RARβ favors morphogenetic stabilization over de novo budding during formation of the pulmonary tree since they observed a proximalization of the distal region of the lung following RA treatment. Moreover, it has been proved that *rarβ2*, together with *rarα*, is crucial for the formation of the mouse tracheal–esophageal septum, among other events. In double αβ2 mutants, the separation between the esophagus and the trachea fails to occur [23]. Our results suggest that, as it occurs in the mammalian lung, *rarβ* is a target of RA signaling in the

chick lung and imply that, at least in the proximal region, it might be implicated in the cellular response conveyed by RA and probably also implicated tracheal morphogenesis. Since we did not observe an expansion of the *rarβ* expression domain after RA supplementation, it is probably not directly related to the proximal–distal cell fate decision.

Retinoic acid signaling has been associated with transcriptional regulation of genes crucial for pattern formation and cell fate decision, such is the case of *homeobox* genes that code for a large family of transcription factors. There are 16 major classes of homeobox genes, among them *hox* and *meis* (reviewed in [87]). *hox* temporal–spatial distribution is crucial for providing the correct vertebra identity [88]. In the chick lung, it has been disclosed that *hoxb5* to *hoxb9* expression pattern correlates with the morphological subdivisions of the bronchial tree and the air sacs [89]. Regarding *meis* genes, they have been identified as determinants of proximal limb compartments and telencephalon development [75, 76]. Interfering with retinoic acid signaling alters patterning and it has been described that it alters *hox* [90] and *meis* genes [60, 75]. In this report, we aimed to elucidate if, in the developing chick lung, *hox* and *meis* expressions were also retinoic acid dependent. Since the expression patterns of *meis1* and *meis2* described in this report are similar, we choose to study only *meis2* gene in this context. RA supplementation revealed that only the highest dose tested (10 μM) results in an increase in *meis2* expression levels, especially in the ventral mesenchyme (Fig. 7f), but its distribution is comparable to whole lungs (Fig. 2m–o). This is, to the best of our knowledge, the first evidence in the literature of this association during lung development. As *hoxb5* is concerned, its expression is augmented in RA-treated explants although there are no differences between the two doses tested (Fig. 7g–i). The spatial distribution of this gene is in agreement with the expression pattern described at E5 by others [89]; *hoxb5* is highly expressed in the ventral mesenchyme and, to a much lesser extent, in the dorsal mesenchyme. In the mouse lung, it has been shown that HOXB5 levels increase in the mesenchyme adjacent to the elongated airways that emerge in terminal region of the lung, after RA treatment [85]; based on these findings, Volpe and coworkers suggest that this imbalance might be the cause of loss of distal phenotype in RA-treated lungs, probably through the modulation of extracellular matrix components [85, 91]. In the embryonic chick lung, it seems clear that *hoxb5* is expressed in response to RA; nonetheless, and as it occurs with *meis2*, it maintains its spatial distribution.

Retinoic acid is part of a complex regulatory mechanism that, in the end, leads to primary bud formation. It has been shown that TGFβ inhibition by RA in the developing foregut is required for *fgf10* expression and, consequently, primary bud induction [27, 92]. Furthermore, in the mammalian lung, TGFβ signaling also plays a key role in branching morphogenesis. In general, the levels of TGFβ ligands and their cognate receptors must be finely regulated throughout development to assure that signaling occurs at the right time and place. In fact, TGFβ type II receptor contributes to a distinct TGFβ response in the epithelial and mesenchymal compartments [93]. TGFβ null mutant's display, among other features, abnormal fetal lung development [94]; additionally, in vitro studies have shown that addition of TGFβ1, β2, or β3 decreases branching [78]. TGFβ signaling is also important for chick lung development. Explants treated with TGFβ1 exhibit a decrease in branching in a dose-dependent manner; on the other hand, blocking TGFβ receptor type I enhances branching, even though it decreases lung size [10]. Taking all this into consideration we asked if retinoic acid modulates *tgfβ2* expression during branching, as it occurs in the early foregut. RA supplementation causes an increase in *tgfβ2* expression levels, which is not dose dependent, and an expansion of *tgfβ2* expression domain (Fig. 7j–l), similar to *cyp26a1* (Fig. 5). The widespread expression of both genes after retinoic acid treatment suggests the existence of a regulatory mechanism between RA and TGFβ signaling pathways responsible for maintaining signaling at the right time and place. TGFβ signaling cellular response depends on the existence of receptors that are present in the neighboring cells, in the mesenchymal and/or epithelial compartment. It has been shown that TGFβ signaling is able to alter N-myc epithelial expression [95], cyclin A [96], and mesenchymal α-smooth muscle actin [78].

The Id (inhibitor of DNA binding/differentiation) proteins are a subfamily of the helix–loop–helix transcription factors that play key roles in the regulation of cell cycle progression and cell differentiation [97]. Id factors may be regulated by TGFβ signaling and contribute to coordinate proliferation and differentiation in a cell-specific manner. In RA-treated lungs, *id2* mesenchymal dorsal expression widens in the distal region of the lung (Fig. 7m–o) similar to what is observed with *tgfβ2* spatial distribution, in the same culture conditions (Fig. 7j–l). Indeed, it has been shown that TGFβ2 upregulates Id2 leading to cell death inhibition and ectopic digit formation in the chick limb [44]. Taking this into consideration, it is likely that RA stimulation in the chick lung leads to an increase in *tgfβ* levels/signaling that consequently increases *id2* levels that may alter the proliferation/differentiation state of the distal cells, thus contributing to an increase in branching.

Lung branching morphogenesis is a particularly complex developmental event that relies in the interaction of distinct molecular players, namely FGF10 and its cognate receptor FGFR2 [98, 99] and SHH (reviewed in [100]). Additionally, in the mouse lung, it has been demonstrated that *fgf10* expression is downregulated and limited to the

most peripheral areas of the lung after the exogenous RA activation; moreover, in the same experimental conditions, SHH pathway is upregulated [12, 29]. FGF and SHH signaling are also crucial for avian lung development [4, 5, 8]. Taking this into consideration, we questioned if the expressions of these well-known regulators of pulmonary development were RA dependent in the chick lung. RA supplementation triggered only a mild decrease in *fgf10* and *fgfr2* expression levels (Fig. 8a–f) and a slight increase in *shh* levels of expression in the highest dose tested (Fig. 8g–i). To a certain degree, it is in agreement with what happens in the mammalian lung. It means that, despite the fact that RA machinery is confined to the mesenchymal compartment, it is able to influence the expression of epithelial genes such as *fgfr2* and *shh*, and also *sox9*.

In general, there seems to be regional specificity regarding RA signaling in the chick lung, which defines the proximal and distal areas of the avian lung. Nonetheless, when compared to the mammalian lung, RA treatment elicited differences regarding specific genes which could be explained by the specifics of the avian lung.

Final remarks

Retinoic acid is crucial in different stages of mammalian lung development, and it has been associated with the proximal–distal patterning of the early lung. To date, this signaling pathway had not been characterized in the avian lung. The major findings of this report are summarized in Fig. 9. All the RA signaling members described in this study are exclusively expressed in the mesenchyme, whereas in the mammalian lung, there is a combined expression of mesenchymal and epithelial compartments. From these data, we can speculate that, in the chick lung, RA directly modulates the expression of mesenchymal target genes, via RARs, that will contribute to accurate lung development and, accordingly, to the pathway activity. On the other hand, its effect on the epithelial compartment (*sox9*) is most likely dependent on the interaction with other molecular players known to be involved in epithelial–mesenchymal interactions. Retinoic acid stimulated lung branching when added exogenously and, although apparently it seems not to interfere with the proximal–distal pattern of the new branches

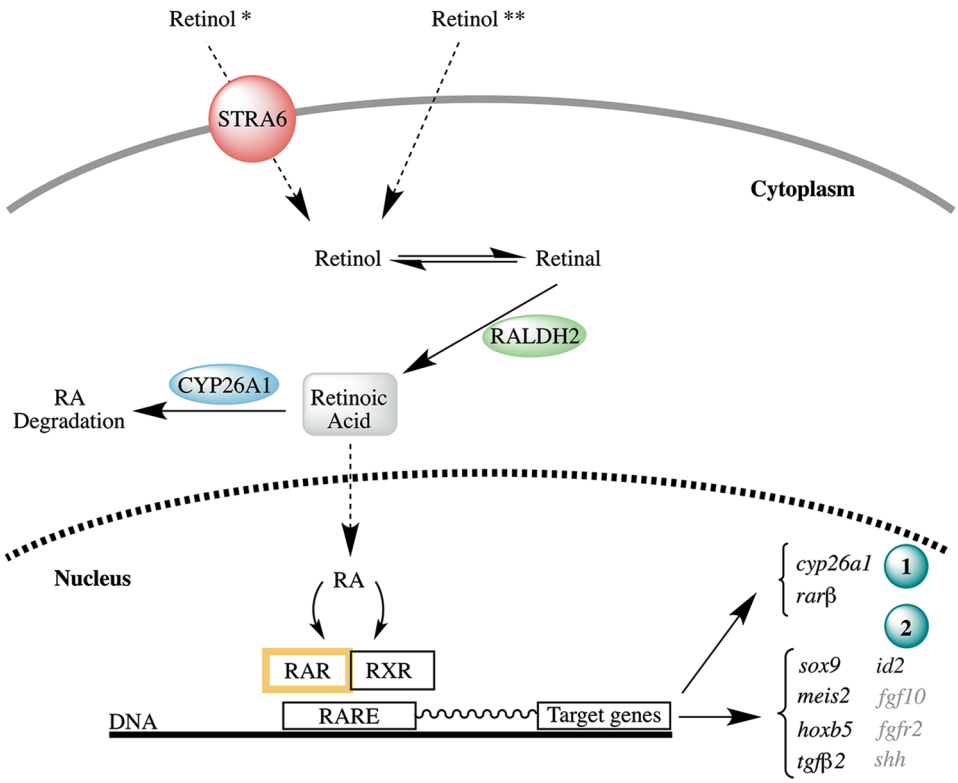

Fig. 9 Retinoic acid signaling pathway in the embryonic chick lung. Retinol enters the cell mainly through STRA6 transmembrane protein in the proximal region of the lung (*single asterisk*) and, eventually, by diffusion in the distal region (*double asterisks*). Cytoplasmic retinol is converted into retinal, which is then oxidized into retinoic acid by RALDH2 enzyme. The intracellular retinoic acid may have two different fates, it can be metabolized by CYP26A1 or transported to the nucleus. In the nucleus, RA interacts with RARs, namely *rarα* and *rarβ*, and RXR; these heterodimers interact with RAREs in the promoter region of RA target genes, thus modulating their transcription. In this report we show two groups of RA target genes: (1) RA machinery genes: *cyp26a1* and *rarβ*; (2) RA interaction with other signaling pathways: *sox9*, *meis2*, *hoxb5*, *tgfβ2*, *id2*, *fgf10*, *fgfr2*, and *shh*

formed, at the molecular level *sox9* expression is impaired which points toward a defective pulmonary patterning. Increased *cyp26a1* expression levels revealed that RA-induced signaling is active in these conditions. Likewise, altered levels of *hoxb5*, *meis2*, *tgfβ2*, *id2*, and *fgf10*, *fgfr2*, and *shh* (only faintly) uncover intricate molecular interactions between different signaling pathways that, eventually, control lung branching morphogenesis. RA seems to regulate patterning and branching in the chick lung by maintaining signaling at the right time and place. This report contributes to the description of the molecular network underlying chick lung development and highlights the species-specific features of the avian lung.

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