The Role of Ephrins-B1 and -B2 During Fetal Rat Lung Development

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Eppri-B1, -B2 and receptor EphB4 expression pattern was assessed in fetal rat lungs between 15.5 and 21.5 days post-conception, by immunohistochemistry. Fetal rat lungs were harvested at 13.5 dpc, cultured during 4 days and treated with increasing doses of ephrins-B1 and -B2 and the activity of key signaling pathways was assessed. Results: Ephrin-B1 presents mesenchymal expression, whereas ephrin-B2 and its receptor EphB4 were expressed by the epithelium. Both ephrins stimulated pulmonary branching. Moreover, while ephrin-B1 did not affect the pathways studied, ephrin-B2 supplementation decreased activity of JNK, ERK and STAT. This study characterizes the expression pattern of ephrins-B1, -B2 and EphB4 receptor throughout rat lung development. Conclusion: Our data highlight a possible role of ephrins as molecular stimulators of lung morphogenesis. Moreover, it supports the idea that classical vascular factors might play a role as airway growth promoters.

Key Words
Ephrin-B1 • Ephrin-B2 • EphB4 • Fetal lung development • Branching
Introduction

Lung morphogenesis is a very complex and tightly regulated phenomenon. Transcription factors, extracellular matrix molecules, intercellular adhesion molecules and soluble growth factors have been identified as controlling factors of pulmonary development. Together, these molecular players act along the proximal-distal axis of the respiratory tract and influence local expression of specific genes that ultimately play a preponderant role on the development of the immature lung [1].

The Eph receptors and their ligands, ephrins, constitute the largest subgroup of the family of receptors tyrosine kinase (RTKs). This family of receptors is divided into two subgroups based on the similarity of their extracellular domain sequences, whereas ephrins are classified according to their structure and their affinity for the correspondent receptor subgroup [2]. Regarding ephrins-B, Eph/ephrin signaling can be bi-directional, inducing intracellular pathways downstream of both the Eph receptor and the ephrin ligand. The latter is defined as reverse signaling [3]. Although ephrins-A preferably bind to EphA and ephrins-B to EphB receptors, ephrins are also able to bind and activate Eph receptors from the opposite subgroup [4]. Nevertheless, some ephrins seem to have higher affinity for specific receptors as reported for ephrin-B2, which mainly binds to EphB4 receptor [5, 6].

The roles of ephrins in cell adhesion, vascular development, cell migration and tissue-border maintenance highlight a potential role in lung branching morphogenesis [3, 7]. In fact, ephrins expression has been described in other organs that, as the lung, develop through branching processes. For instance, the expression of ephrins-B1 and -B2, and EphB4 (ephrin-B2 cognate receptor) has been confirmed in adult mouse mammary gland and fetal mouse pancreas [8-10]. The expression of ephrin-B2 and its receptor has also been detected in fetal mouse kidney [11]. It is noteworthy that ephrin-B1 mutations have been found in nearly 87% of craniofrontonasal syndrome cases. Some of these cases exhibit diaphragmatic defects and consequently lung defects [12-16]. Moreover, another study described ephrin-B1 gene (EFNB1) duplications in fetuses that died from CDH and concomitant pulmonary hypoplasia and pulmonary hypertension [17]. On the other hand, ephrin-B2 and EphB4 receptor have been widely described as arterial and venous markers, respectively, and implicated in the development of the vascular system during embryogenesis [5, 6, 18, 19]. Ephrin-B2, ephrin-B1 and EphB4 receptor RNA was identified in sorted endothelial cells from mouse adult lung [20].

There is extensive evidence for a direct synergism between pulmonary vascular and airway development. Indeed, vascular regulatory factors play a crucial role in airway and alveolar morphogenic processes, and growth factors produced in the airway epithelium can also regulate vasculature formation [21-24].

Ephrins have been described as crucial for microvascular maturation mainly during septum maturation and capillary remodeling from double into a single capillary layer. They are also known to be involved in the process of alveologenesis, contributing for the establishment of the secondary septa and for alveolar formation [25, 26]. In fact, ephrin-B2 has been described as a contributary to normal postnatal alveolar development as its inhibition leads to arrested alveolar and abrogated lung vascular growth. Moreover, in a setting where ephrin-B2 is downregulated, ephrin-B2 treatment preserves alveolar and lung vascular growth attenuating pulmonary hypertension caused by O\textsubscript{2}−-induced arrested alveolar growth [27, 28]. Ephrin-B1 -B2 and EphB4 knockout mice die during embryogenesis and displayed several defects including angiogenesis impairment. In published data with Ephrin-B2 or EphB4 knockout mice, stained vessels were completely absent in knockouts [28, 29]. Thus, ephrin-B1, ephrin-B2 and its main receptor appear as potential key players in fetal lung development. Nonetheless, very little is known regarding ephrins-B1 and -B2 function during fetal lung development. Hence, in the present work we aimed to disclose novel roles of such proteins in normal lung development.
Materials and Methods

**Ethics Statement**

Animal experiments were performed according to the Portuguese law for animal welfare (‘Diário da República, Portaria 1005/92’) and the protocol was approved by the Committee on the Ethics of Animal Experiments of the Life and Health Sciences Research Institute of the University of Minho (DGVCV 022162 - 520/000/000/2006). Animals were housed in an accredited house and treated as specified by the recommendations of the ‘Guide for the Care and Use of Laboratory Animals’ published by the US National Institutes of Health (NIH Publication No.85-23, revised 1996). Moreover, all efforts were made to minimize animal suffering.

**Experimental Design and Animal Model**

Sprague-Dawley female rats (225g, Charles-River) were maintained in appropriate cages under controlled conditions and fed with commercial solid food. The rats were mated and checked daily for vaginal plug. The day of plugging was defined as gestational day 0.5 for time dating purposes. Fetuses were removed by caesarean section at 13.5, 15.5, 17.5, 19.5 and 21.5 days post-conception (dpc). Fetuses were sacrificed by decapitation, its lungs dissected under a binocular surgical microscope (Leica MZFLIII, Leica Microsystems GmbH) and processed either for immunohistochemistry (IHC) or western blot studies. 13.5 dpc fetal lungs were also collected to perform explant culture.

**Immunohistochemistry**

IHC was performed on paraformaldehyde-fixed and paraffin-embedded lungs. Sections (4μm) were then placed on SuperFrost®Plus slides (Menzel-Glaser, J1800AMNZ). After 15’ at 65°C, slides were dewaxed in xylene and rehydrated in ethanol. Antigen retrieval was achieved by boiling the slides in 1 mM citrate buffer (Thermo Scientific, AP-9003-125) followed by cooling down at room temperature. Incubation with 3% hydrogen peroxide (Sigma, 95313-500 ml) in distilled water for 20’ allowed quenching endogenous peroxidase. Incubation with primary antibodies occurred at 4°C overnight. The primary antibodies used were a polyclonal goat anti-ephrin-B1 in a 25μg/mL concentration (R&D Systems, Inc., AF473), a polyclonal rabbit anti-ephrin-B2 (Santa Cruz Biotechnology Inc., sc-15397) in a 1:25 dilution and a polyclonal rabbit anti-EphB4 in a 1:100 dilution (Santa Cruz Biotechnology, Inc., sc-5536). Negative control reactions included omission of the primary antibody. The incubation with the corresponding secondary antibodies occurred at room temperature and was carried according to manufacturer’s instructions. Ephrin-B1: goat ImmunoCruz™ Staining System (Santa Cruz Biotechnology Inc., sc-2023). Ephrin-B2 and EphB4: UltraVision detection system anti-polyvalent horseradish peroxidase (Thermo Scientific, TP-125-H2). To visualize peroxidase activity. 3,3’-diaminobenzidine tetrahydrochloride (Dako, K3468) was used. Sections were counterstained with 50% hematoxylin.

**Fetal lung explant cultures**

After harvesting and dissection, 13.5 dpc lungs were transferred to 8 μm Isopore membranes (Millipore, TETPO1300) previously soaked in DMEM (Lonza, 12-604F) for 1 hour. Cultures were then incubated in 200μL of complex medium, 50% Dulbecco’s modified eagle medium (DMEM), 50% nutrient mixture F-12 (Invitrogen, Carlsbad, CA, USA) supplemented with 100 mg/mL streptomycin, 100 units/mL penicillin (Invitrogen), 0.25 mg/mL ascorbic acid (Sigma–Aldrich, St Louis, MO, USA) and 10% fetal bovine serum (Invitrogen) [30]. After 1 hour of incubation, recombinant ephrin-B1 (R&D Systems Inc., 473-EB-200) and ephrin-B2 (R&D Systems Inc., 496-EB-200) were added to lung explants in order to achieve a final concentration of 0.01, 0.1 and 1μg/mL. The recombinant ephrins were daily added. The four experimental groups (control and three doses for each protein) were all constituted by n=12 lungs. The explants were incubated in a 5% CO₂ incubator, at 37°C, during 96 hours. The medium was replaced at 48 hours. The explants were daily photographed to monitor branching morphogenesis. At day 0 (D₀: 0 hours) and day 4 (D₄: 96 hours) of culture, the total number of peripheral airway buds (branching), the epithelial perimeter and area were determined for all lung explants using the ImageJ 1.44 software (National Institutes of Health, USA). The results of branching, epithelial perimeter and area were expressed as D₄/D₀ ratio. At the end of the incubation time, explants were washed in PBS and stored at -80°C until use.
Western blot

Three different pooled samples of fetal lungs for each gestational age (13.5 – 21.5 dpc) (obtained from different pregnant female rats), and cultured lungs (treated with ephrin-B1 at 1 μg/mL, ephrin-B2 at 0.01 μg/mL and non-treated) were processed for western blot analysis. Proteins were obtained according to Kling et al. [31]. Twenty-five or five micrograms (for explants) of protein were loaded in 12% acrylamide mini gel, electrophoresed and then transferred to nitrocellulose membranes (HybondTM -C Extra, GE Healthcare Life Sciences, 25800047). Blots were probed with polyclonal goat anti-ephrin-B1 (1:750; R&D Systems, Inc., AF473), polyclonal rabbit anti-ephrin-B2 (1:2000; Santa Cruz Biotechnology Inc., sc-15397), polyclonal rabbit anti-EphB4 (1:200, Abcam, ab123791), and with antibodies to non-phosphorylated and phosphorylated forms of p38 (8690, 4511), p44/42 (ERK1/2) (4695, 4370), JNK (9258, 4671), Akt (4685, 13038) and STAT3 (12640, 9131) (1:1000; Cell Signaling Technology Inc.) according to manufacturer’s instructions. Activity of intracellular signaling pathways from lung explants was measured by the ratio between phosphorylated protein and total protein amount. The data was also normalized against the control group. For loading control, blots were probed with β-tubulin (1:200000; Abcam, ab6046).

Blots were incubated with a secondary anti-rabbit horseradish peroxidase conjugate (Santa Cruz Biotechnology Inc., sc-2004), developed with Super Signal West Femto Substrate (Pierce Biotechnology Inc., 34095) and the chemiluminescent signal was captured using the Chemidoc XRS (Bio-Rad). Quantitative analysis was performed with Quantity One 4.6.5 1-D Analysis Software (Bio-Rad Laboratories Ltd).

Statistical Analysis

Data are presented as mean ± SEM. Statistical analysis was performed using the statistical software GraphPad Prism (version 5; GraphPad Software Inc., USA). Statistical comparison of experimental groups was achieved by t-test and one-way ANOVA on ranks. The Student-Newman-Keuls test was used for post-test analysis. Statistical significance was set at p<0.05.

Results

Pulmonary expression pattern of ephrin-B1, ephrin-B2 and EphB4 receptor

In order to determine ephrin-B1, ephrin-B2 and its receptor EphB4 expression pattern during fetal lung development, immunohistochemical analysis was performed for five different gestational ages: 13.5, 15.5, 17.5, 19.5 and 21.5 dpc. Ephrin-B1 exhibits strong mesenchymal expression observed in all gestational ages (Fig. 1A). Faint epithelial expression is detected at 15.5 and 17.5 dpc. Nonetheless, from 19.5 dpc on, the epithelial expression is absent, as well as endothelial expression.

Remarkably, ephrin-B2 expression pattern is highly distinct from ephrin-B1 (Fig. 1B). Ephrin-B2 is predominantly expressed in the epithelial compartment, and it is also observed in the cells surrounding the epithelium at 13.5 and 15.5 dpc. Additionally, ephrin-B2 is not detected in vascular smooth muscle cells neither in endothelial cells. At 21.5 dpc, ephrin-B2 expression appears to be restricted only to epithelial cells from terminal and respiratory bronchioles.

EphB4 expression pattern (Fig. 1C) is very similar to its cognate ligand: mainly detected in epithelial structures throughout all gestational ages. Interestingly, at 13.5 and 15.5 dpc, EphB4 receptor is also weakly expressed in potentially less differentiated cells surrounding the epithelium. This expression seems to diminish as the fetal lung matures, becoming progressively specific to the epithelium. On the other hand, again no expression was detected in vascular structures, namely smooth muscle or endothelial cells from both arterial and venous structures.

Ephrins-B1 and -B2, and EphB4 receptor protein expression levels were assessed by western blot analysis (Fig. 2). For the gestational ages studied, EphB4 receptor, ephrins-B1 and -B2 expression was detected at relatively constant levels. Nevertheless, despite the higher variation in ephrin-B1 expression levels throughout all gestational ages, no statistical significant differences were found.
Aiming to unveil ephrins-B1 and -B2 potential role in fetal lung development, functional studies were performed using lung explant cultures. Explants were daily supplemented with three different doses of either ephrin-B1 or ephrin-B2 recombinant protein (selected according to literature). Morphometric analysis revealed a statistically significant effect of both ligands on lung morphogenesis. Recombinant ephrin-B1 promoted an increase in the number of peripheral airway buds regardless of the dose tested (Fig. 3B). On its turn, ephrin-B2 lowest dose (0.01 μg/mL) significantly increased lung branching and the internal epithelial perimeter (Fig. 4B and C, respectively). Moreover, ephrin-B2 treated explants present an increase in total lung area regardless of the dose studied (Fig. 4D).

*Ephrin-B1 and ephrin-B2 affect fetal lung morphogenesis in rat*

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Ephrin-B2 influences the phosphorylated form of ERK, JNK and STAT3

The signaling pathways that act downstream ephrin-B1 and ephrin-B2, specifically in lung morphogenesis, are poorly understood. In order to further investigate the effect of ephrins on fetal rat lung growth, treated lung explants were assessed for intracellular signaling pathways known to regulate lung growth and development.

Pooled samples of lung explants treated with 0.01μg/mL of recombinant ephrin-B1 or 1μg/mL of recombinant ephrin-B2 (selected according to its maximal effect on lung explants growth) were evaluated for modulation of p38, p44/42 (ERK1/2), JNK, Akt and STAT3 pathways (Fig. 5). Ephrin-B1 treatment did not influence the phosphorylation levels of the analyzed pathways. Strikingly, recombinant ephrin-B2 administration promoted the inactivation of ERK, JNK and STAT signaling pathways in fetal lung explants.
Fig. 4. Ephrin-B2 supplementation induces growth of rat fetal lung explants. (A) Representative examples of fetal lung explants cultured with three different doses of ephrin-B2 recombinant protein (0.01, 0.1 and 1μg/mL) at day zero (D₀) and day 4 (D₄). Original magnification x25. Scale bar = 6349 µm (all images at same magnification). Morphometric analysis of branching (B), internal perimeter (C), area (D), and external perimeter (E). A significant increase in the area of the explants was observed for all tested doses. The lowest dose significantly stimulated both branching and internal perimeter. All data was normalized with the control group (C=1; dashed lines). Results are expressed as D₄/D₀ ratio. p< 0.05: * vs. control group.

Discussion

There is growing evidence for the involvement of ephrins in numerous biological processes. Clearly, this family of proteins is implicated in regulation of key steps of embryonic development. This study demonstrates that two ephrin ligands - B1 and B2 - and one well-established ephrin-B2 receptor - EphB4 - are constitutively expressed during fetal lung development. Two recent studies presented strong evidence for the role of ephrin-B2 in lung morphogenesis and repair [27, 32]. Our findings not only provide further proof of ephrin-B2 function but also show how yet another ephrin ligand promotes branching of the rat fetal lung.

The constitutive and stable protein expression levels of ephrins-B1 and -B2, and EphB4 receptor particularly during early gestational stages, which are characterized by
intense ramification and growth phenomena, underscore them as potential players in fetal lung branching. On the other hand, the distinct expression patterns presented by the two ligands suggest distinct or, perhaps, synergistic functions. Ephrin-B1 expression was
mainly detected in mesenchymal tissue, whereas strong epithelial expression was found for ephrin-B2 and the receptor EphB4. Interactions between epithelial structures and the surrounding mesenchyme are known to dictate and to be essential for proper branching of the lung epithelium [33-35]. Therefore, our results suggest a so far undervalued contribution from this RTK subfamily of ligands to such mesenchymal-epithelial interactions that guide lung branching morphogenesis.

Although ephrin-B2 and its receptor have been widely described as angiogenic factors [5, 6, 18, 19], epithelial expression of ephrin-B2 has been observed in the epithelium. However, at 18 dpc ephrin-B2 was expressed in some endothelial cells and, more weakly, in non-endothelial cells with epithelial morphology [36]. In human fetal lung, ephrin-B2 is expressed in the pulmonary arterial endothelium and EphB4 expressed in endothelial cells of the pulmonary vein, primary capillary plexus and pulmonary artery [37]. In rat lung, endothelial staining was absent for the three proteins studied. These results are in agreement with observations from a recent knock-in mouse model in which the intracellular domain of ephrin-B2 was replaced by bacterial β-gal sequences. The same study showed how ephrin-B2 reverse signaling blockage results in pulmonary hypoplasia and decreased lung compliance [32]. Finally, in the adult mouse lung, both ephrins are enriched in the endothelial compartment [20]. The different observations in fetal lung might be due to the chosen study model and technical approach. Nevertheless, all together these observations suggest an intense endothelial-epithelial crosstalk played by these proteins since early stages of the fetal development to the adult organism. Indeed, vascular regulatory factors are crucial for airway and alveolar morphogenic processes. In fact, growth factors produced in the airway epithelium are able to regulate vasculature formation [38, 39]. Taking all this into account, we hypothesized that both ephrins-B1 and -B2 would affect fetal lung growth and branching morphogenesis.

In order to evaluate ephrins-B1 and -B2 role in lung branching morphogenesis, fetal lung explants were cultured with increasing doses of ephrin-B1 or ephrin-B2. Ephrin-B1 supplementation stimulated the formation of peripheral airway buds. Additionally, ephrin-B2 supplementation increased not only lung branching but also the internal epithelial perimeter and area of the lung explants. These results strongly support a stimulatory role of ephrin-B1 and ephrin-B2 during fetal lung development. The importance of ephrins-B1 and -B2 in different tissues during embryogenesis is highlighted by the lethality of ephrin-B1 knockout mice [29] and angiogenesis impairment caused by ephrin-B2 gene disruption [5]. Also, in a recent study, ephrin-B2 was shown, by means of siRNA mediated gene silencing, to be implicated in normal postnatal alveolar development. Authors observed decreased alveolarization associated with fewer pulmonary vessels [27].

Aiming to identify the signaling pathways that mediate the effect of ephrins-B1 and -B2 in lung growth, the activity of p38, ERK, JNK, Akt and STAT3 proteins was studied. Many factors that regulate fetal lung branching morphogenesis activate signaling pathways that culminate with MAPK, PI3K/Akt and p38 cascades [30, 31, 40-42]. In the present study, ephrin-B1 was found not to modify the phosphorylated levels of these proteins. In a different study it has been already described that ephrin-B1 signaling does not activate the p38 and ERK signaling pathways. However, ephrin-B1 led to the phosphorylation of JNK on a specific residue. Therefore, the putative phosphorylation of JNK in different residues from the ones here studied cannot be rule out [43]. A very recent study has shown that CNK1 (Connector Enhancer of KSR1) is required for the ephrin-B1-dependent JNK phosphorylation [44]. It would be interesting in future studies to access the role of this protein in the context of ephrin-B1 mediated fetal lung branching. Moreover, it would also be interesting to check if Shh, Retinoic Acid, Bmp or Fgf signaling pathways, classical regulators of lung branching, are changed when ephrin-B1 is administrated [45-49].

In contrast, ephrin-B2 stimulation induced a decrease in phosphorylated levels of ERK, JNK and STAT3, indicating a decrease in these intracellular signaling. These results
might reflect regulatory loops in which ephrin-B2, by stimulating other branching-promoter pathways, leads to lower activity of the specific pathways here studied. Regarding ERK, although classically this pathway has been implicated as a promoter of branching morphogenesis, it was already described that ERK pathway can be involved in lung growth inhibition [42, 50]. In a lung explant culture system similar to the one used in the present study, stimulation with leukemia inhibitory factor and concomitant activation of ERK pathway resulted in branching inhibition [50]. Recent published data has also shown that knockdown of ephrin-B2 results in increased phosphorylation levels of several kinases such as ERK, STAT, c-Jun. This indicates that multiple prominent cell signaling pathways are affected by changes of EphB2 activity [51]. The putative involvement of ERK, JNK and STAT3 in lung growth inhibition might still be an indirect or balancing effect of the activation of other pathways or simply context dependent. Further investigation will help to determine whether the inactivation of such pathways is essential for ephrin-B2 function in lung development or if the obtained results reflect crosstalk and modulation by other non-canonical intracellular pathways.

In summary, this study demonstrated that both ephrin-B2 and its cognate receptor EphB4 are constitutively expressed in pulmonary epithelium, whereas ephrin-B1 is mainly expressed in the mesenchyme of the rat fetal lung. It is now clear that both ephrin-B1 and ephrin-B2 significantly influence lung morphogenesis. However, further investigation will be necessary to properly describe the expression pattern and functional contribution of other members of the ephrin-B subfamily of ligands as they might act synergistically. Ultimately, in vivo studies will be crucial to further confirm these factors as promoters of lung branching morphogenesis.

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