Chick pulmonary Wnt5a directs airway and vascular tubulogenesis

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Wnt5a is an important factor patterning many aspects of early development, including the lung. We find pulmonary non-canonical Wnt5a uses Ror2 to control patterning of both distal air and vascular tubulogenesis (alveolarization). Lungs with mis/overexpressed Wnt5a develop with severe pulmonary hypoplasia associated with altered expression patterns of Shh, L-CAM, fibronectin, VEGF and Flk1. This hypoplastic phenotype is rescued by either replacement of the Shh protein or inhibition of fibronectin function. We find that the effect of Wnt5a on vascular patterning is likely to be through fibronectin-mediated VEGF signaling. These results demonstrate the pivotal role of Wnt5a in directing the essential coordinated development of pulmonary airway and vasculature, by affecting fibronectin levels directly, and by affecting the fibronectin pattern of expression through its regulation of Shh. Data herein suggest that Wnt5a functions in mid-pulmonary patterning (during alveolarization), and is distinct from the Wnt canonical pathway which is more important in earlier lung development.

KEY WORDS: Lung development, Wnt5a, Shh, Fibronectin, Ror2, VEGF, Flk1

INTRODUCTION

Pulmonary development involves coordinated patterning of both the airway and vascular systems (Cardoso and Lu, 2006). Their synchrony is necessary for effective gas transfer, and maldevelopment of either is frequently lethal (Berrocal et al., 2004; Cullinane et al., 1992; deMello, 2004; Galambos and deMello, 2007). Although there have been many studies of airway development (for reviews, see Cardoso, 2001; Costa et al., 2001; Kimura and Deutsch, 2007), relatively little is known about pulmonary vascular pattern formation or the coordinated development of the two.

Although the anatomy of the avian lung differs from that of the mammalian lung, both develop similarly and have anatomical functional equivalents. The avian lung forms by a series of closed circular buds arising from the main airway branches, which differs from the dichotomous branching morphogenesis in mammalian lung development. In contrast to the mammalian lung, which terminates in alveoli, the avian lung forms a looping anastomotic network of air-vascular surfaces (parabronchi) that end in terminal air buds and air capillaries. For all vertebrates, pulmonary vascular patterning continues in coordination with airway development. In the chick embryo, vasculogenesis is the main process by which the pulmonary vasculature initially forms, being guided by the budding airways (Anderson-Berry et al., 2005; Hislop, 2005). This interstitial microvasculature connects the large pulmonary vessels to the terminal buds of the airways and the capillaries via two mechanisms, sprouting and intussusceptive angiogenesis (Makanya et al., 2007). Sprouting angiogenesis predominates from early to mid-gestation (E15), and is the major mechanism for setting up the basic hexagonal interstitial vascular pattern seen in avians. Intussusceptive angiogenesis, a novel process involving endothelial cell extension into the lumen of a vessel (splitting the vessel), predominates from ~E15, producing the massive expansion of the vasculature that is necessary for vital gas exchange (alveologenesis). Intussusceptive angiogenesis occurs simultaneously with the rapid growth of the airway epithelium into the mesenchyme to create the huge surface area of the mature lung. After E18, the air capillaries and the blood capillaries are in close proximity, forming the functional equivalent of the mammalian alveolus (for a review, see Maina, 2006). The chick embryo is fully air breathing by E18, 2-3 days before hatching. Airway and vascular channels, epithelial and endothelial tubules, are the fundamental structural units of the lung (Cardoso and Lu, 2006), and develop in response to morphogenetic growth factors, transcription factors, extracellular matrix proteins and their receptors (Hogan and Kolodziej, 2002). Although much is known about the factors regulating airway development, those controlling this coordinated air and vascular patterning are unknown.

The Wnt family of secreted signaling molecules functions in numerous key developmental events (Wodarz and Nusse, 1998). Wnts are broadly categorized into two groups based on their signal transduction pathway. Canonical Wnts transduce their signals through receptors of the frizzled (Fz) family by a β-catenin-dependent pathway (Wodarz and Nusse, 1998). Non-canonical Wnt signal via either the planar cell polarity (PCP) pathway or the Wnt/Ca++ pathway (Veeman et al., 2003; Widelitz, 2005), and may use Fz receptors or other receptors, including the orphan tyrosine kinase Ror2 (Keeble et al., 2006; Oishi et al., 2003). The Wnt signaling pathway has been well described and includes numerous regulators (Pandur et al., 2002; Widelitz, 2005). Most described Wnt antagonists interfere with the canonical pathway, such as the Dickkopf (Dkk) proteins, of which Dkk1 specifically interrupts only the canonical-Wnt function (Glinka et al., 1998; Kawano and Kypta, 2003; Niehrs, 2006).

Whereas canonical Wnt signaling is known to regulate lung development early in branching morphogenesis (Dean et al., 2005; Mucenski et al., 2005; Okubo and Hogan, 2004), the non-canonical

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Wnts (such as Wnt5a) appear to act later (Li et al., 2005). Wnt5a moderates many cellular events, including cell adhesion (Jonsson and Andersson, 2001; Torres et al., 1996; Toyofuku et al., 2000; Weeraratna et al., 2002), migration (Jonsson and Andersson, 2001), proliferation (Li et al., 2003; Yamaguchi et al., 1999) and differentiation (He et al., 1997; Kuhl et al., 2001; Kuhl et al., 2000; Sheldahl et al., 1999). These findings suggest that Wnt5a is a good candidate for coordinating pulmonary air and vascular pattern formation.

One mechanism by which Wnt5a might regulate these cellular events is by affecting the extracellular matrix, a component of which is fibronectin. Fibronectin has been implicated in branching morphogenesis (Roman and McDonald, 1992; Sakai et al., 2003), vasculogenesis (Bull et al., 1993; Hall et al., 2000; Jozaki et al., 1990) and intussusceptive angiogenesis (Makanya et al., 2007), and it interacts with VEGF (Vascular Endothelial Growth Factor) (Goerges and Nugent, 2004), which is also implicated in lung vascular and airway branching morphogenesis (Warburton et al., 2005). Although it is known that canonical Wnt signaling decreases fibronectin expression in murine lung development (Warburton et al., 2005; Gradv et al., 1999), Wnt5a signaling in fibronectin regulation has not been well studied. We hypothesized that Wnt5a affects pulmonary vascular tubulogenesis through interactions with fibronectin and the VEGF pathway.

Here, we show that mis/overexpressed Wnt5a in the developing avian lung acts non-ordinarily to induce marked pulmonary hypoplasia with dramatic alterations of vascular pattern. Wnt5a controls aspects of both distal airway and vascular tubulogenesis at mid-developmental stages via interactions with Shh, VEGF, Fk5/1 and fibronectin, differing from the canonical Wnt pathway. Understanding the role of Wnt5a in pulmonary air and vascular tubulogenesis may help to explain the pathogenesis of pulmonary malformations that often involve developmental errors or arrest, in both systems of the lung, as is seen in pulmonary hypoplasia with its associated pulmonary hypertension.

MATERIALS AND METHODS

Embryos

Timed, fertilized, white leghorn eggs (SPAFAS, CT) were incubated in a humidified incubator (Kuhl, NJ) at 38°C until used experimentally. Embryos were staged (st) according to Hamburger and Hamilton (Hamburger and Hamilton, 1951) or by embryonic day (E).

In ovo viral infection

We used replication-competent avian specific retrovirus (RCAS; both A and B coats) expressing murine Wnt5a, chick Shh (Riddle et al., 1993), chick Wnt3a (Stevens et al., 2003) or a dominant-negative construct of murine Ror2. The Ror2-DAF-GPI (dnRor2) construct is composed of the extracellular domain of murine Ror2 fused in frame with the membrane anchoring GPI domain (Mikels and Nusse, 2006). All were synthesized using established techniques (Logan and Tabin, 1998; Morgan and Fekete, 1996), and were grown and harvested in DF1 cells (Cepek, 1991). Control infections were performed using RCAS-A or B constructed with green fluorescent protein (GFP).

Embryos were injected, in ovo, at E2 (st11-15) in the right anterior-lateral region targeting the pre-lung field, following a published fate map (Matsushita, 1995), with approximately 1 μl of freshly defrosted virus. Injections were carried out under a Nikon SMZ800 dissection microscope, using a Hamilton syringe fitted with pulled glass micropipette needles (Morgan and Fekete, 1996). When two viruses were used (made with different coats of RCAS to ensure coordinated infection), equal aliquots from each were mixed before injection. Eggs were then sealed and returned to the incubator until harvesting. More than 35 dozen embryos were injected with RCAS-Wnt5a, and 20 dozen embryos were injected with either RCAS-Wnt3a or RCAS-dnROR2. Controls consisted of both uninjected and RCAS-GFP-injected embryos; these controls showed normal lung development in all cases. Injections were verified either by tissue or whole-mount analysis using standard in situ hybridization techniques and published riboprobes (Riddle et al., 1993; Roberts et al., 1995); immunohistochemistry was used to detect expression of Rcs protein (Riddle et al., 1993; Roberts et al., 1995) and fluorescence microscopy for GFP.

Organ Culture

Lungs explants were isolated from wild-type and injected/injected embryos at E8, placed in 24-mm Transwell-clear permeable support plates (Corning) and incubated for 4 days in BGB1 (Gibco) medium containing 0.2 mg/ml ascorbic acid (Sigma), 50 units/ml penicillin and 50 units/ml streptomycin (Sigma) at 37°C with 5% CO2. Experiments involved the addition of 1 mg/ml H-Arg-Gly-Asp-Ser-OH (RGDS; Calbiochem) (Pozzetto et al., 2005), 250 ng/ml murine recombinant Dkk1 protein (R&D Systems), 1 μg/ml recombinant mouse Shh (R&D Systems) or 4 μg/ml cycloheximide (Toronto Research Chemical) (Li et al., 2004). As a control, we incubated RCAS-Wnt5a (injected in ovo) and wild-type explants with non-treated medium. RCAS-dnRor2(A) was injected into E8 lungs either alone or in combination with RCAS(A)-Wnt5a or RCAS(B)-Wnt5a. Harvested lungs were then incubated for 4 days in treated or non-treated medium. Controls were injected with RCAS-GFP using the same protocol. To study the interactions between fibronectin and Shh, we cultured E8 wild-type or Wnt5a-overexpressing lung explants in cycloheximide-treated medium for 2 days, followed by cycloheximide and RGDS combined medium for 2 days. Control lungs were incubated with cycloheximide or RGDS alone for 4 days. When possible, as an internal control, the contralateral lungs (completely untreated) were cultured in wells adjacent to the experimental lung.

Tissue processing

Lungs were dissected and fixed [with 4% paraformaldehyde (PFA) or 10% formalin in RNase-free PBS] for 2 hours at room temperature. Fixed embryonic lungs were washed in PBS with 0.1% Tween 20 (PBT) and either taken through a graded series of methanol/PBT washes or kept at −20°C in methanol until used for expression studies. Hematoxylin and Eosin (H&E) or periodic acid-Schiff (PAS) stains were prepared using standard protocols (Branco and Stevens, 1990) on 5-μm paraffin sections.

In situ hybridization

In situ hybridization, on 5-μm sections from paraffin-embedded tissues, was performed using digoxigenin-labeled riboprobes generated from chick Wnt5a [a gift of Andrew McMahon (Dealy et al., 1993)], Nks2.1 [provided by Michael Kessler (Pera and Kessel, 1998)], Shh, Bmp4, Fgfo1, Rcs, Wnt5a [provided by Cliff Tabin (Riddle et al., 1993; Roberts et al., 1995; Roberts et al., 1998)] and quail VEGF [from E. Laufer and C. Tabin (Flamme et al., 1995)], using techniques minimally altered from those published (Riddle et al., 1993) and developed using BM Purple AP Substrate (Roche), per the manufacturer’s instructions.

Immunohistochemistry

Immunohistochemical staining on paraffin sections was performed using standard techniques. For antigen retrieval, sections were heat treated in a microwave for 20 minutes at medium power in 0.01 M sodium citrate buffer (pH 6). Before antibody incubation, the peroxidase was quenched with H2O2. Biotinylated secondary antibody (Vector Laboratories) was used to localize antibody-antigen complexes in tissue, using the ABCComplex/HRP Detection System (Dakocytomation Antibodies), following the manufacturer’s directions. Antigen detection was enhanced with 3,3’-Diaminobenzidine (DAB). The following antibodies were used in the study: mouse anti-active-β-catenin (1:150; Upstate), rabbit anti-Ror2 antibody (1:50; Cell Signaling), chick anti-L-CAM (no dilutions; Developmental Studies Hybridoma Bank), rabbit anti-sheep Surfactant Protein B (SP-B; 1:50; US Biological), mouse anti-PCNA (1:150; NeoMarkers), rabbit anti-rat Clara Cell (CC16; 1:250; US Biological), mouse anti-actin smooth muscle Ab-1 (1:300; NeoMarkers, clone 1A4), mouse anti-fibronectin (1:500; BD transduction laboratories) and mouse Flk1 A-3 (1:1000; Santa Cruz Biotechnology). Fluorescein elderberry bark lectin (E. lectin; Fluorescein labeled Sambucus Nigra Lectin; 1:400; Vector Laboratories)
was used to mark endothelial cells. Sections were permeabilized using 0.1% Triton X-100 for one hour at room temperature. Lectin was incubated overnight (~18 hours).

RESULTS

Wnt5a is expressed in the developing chick lung

In the early developmental stages of the lung, Wnt5a is not expressed (Fig. 1A), but Wnt3a is expressed in the epithelium (Fig. 1B). By E11, Wnt5a expression was strongly detected both in epithelium and in the immediately subjacent mesenchyme, but it was not detected in interstitial blood vessels (Fig. 1C). This pattern of expression differs from that of Wnt3a, which is found in the epithelium and interstitial vessels, but not in the mesenchyme (Fig. 1D). Later in chick lung morphogenesis, Wnt5a expression declined in the mesenchyme, becoming exclusively epithelial by E15 (Fig. 1E). It continued to be expressed to near hatching (Fig. 1G), and was restricted to the proximal epithelium and the distal-most luminal epithelium (arrows, Fig. 1G). The expression of Wnt3a declined after E14; it was detected only in the budding epithelium at E15 (arrows, Fig. 1F) and was not detected after E16 (Fig. 1H; data not shown). We could detect only weak expression of Wnt5a in the interstitial vessels at E11 (Fig. 1E), a slightly stronger expression at E15, and no detectable expression after E16. By contrast, Wnt3a was strongly expressed in the interstitial vessels at E12 (Fig. 1D) but expression diminished rapidly afterwards (from E14; data not shown; weakly present at E15, Fig. 1F).

Mis/overexpression of Wnt5a disrupts lung growth and vascular pattern

From E10 onwards, Wnt5a mis/overexpression produced a striking pulmonary hypoplastic phenotype (Fig. 2B). RCAS-Wnt5a expression was detected from the first lung buds, at E3, and continued throughout development, with strong expression observed primarily in the epithelium and endothelium, in the atria muscle subjacent to the epithelium, and in the vascular muscle walls (data not shown). Control injected embryos (sham injections and RCAS-GFP injections) showed no phenotype (despite strong GFP expression being observed following the RCAS-GFP injections; data not shown).

Histological features of Wnt5a mis/overexpressing lungs included a decreased numbers of airways, dilatation of the airways, and a thinner epithelial-vascular layer in the mature parabronchi and terminal bud with less complex budding (Fig. 2E). By E20, the infected lungs showed pulmonary lymphangiectasia and many showed pulmonary airway proteinaceous secretions (Fig. 2I; data not shown). Wnt5a mis/overexpressing parabronchial epithelium, although much thinner than wild type, differentiates normally into Clara cells (CC16 positive) and type 2 pneumocytes (surfactant B positive), and expresses the epithelial lung marker Nkx2.1 (see Fig. S1 in the supplementary material). There was no demonstrable difference in the number and distribution of these specific cell types.

Pulmonary hypoplasia also occurred following Wnt3a mis/overexpression (Fig. 2C), but it was not as striking as that produced by Wnt5a mis/overexpression (Fig. 2B). The histology was different as well, with less parabronchi and more terminal budding, in addition the parabronchi formed were abnormally shaped (Fig. 2F).

In the mis/overexpressing Wnt5a lung, there was a slight increase in the number of muscularized arterioles expressing smooth muscle actin; however, they were thinner and less muscularized than their wild-type counterparts (see Fig. S1 in the supplementary material). The increase in number of the smaller caliber vessels was more dramatic, as demonstrated by endothelial-specific elderberry bark lectin staining (Hagedorn et al., 2004) (Fig. 2K,N, compare with 2J,M). Pulmonary interstitial vessels typically cluster centrally, between the airways, forming a distinct hexagonal pattern (Fig. 2I,M); this pattern was lost in the Wnt5a mis/overexpressing lungs, where vessels were densely clustered subjacent to the parabronchial airways (Fig. 2K,N). The Wnt5a mis/overexpressed vascular phenotype was distinct from that of RCAS-Wnt3a infected lungs, which developed with the larger, earlier-formed, interstitial blood vessels, but not in the mesenchyme (Fig. 2A). This pattern of expression differs from that of Wnt3a, which is found in the epithelium and interstitial vessels, but not in the mesenchyme (Fig. 2D). Later in chick lung morphogenesis, Wnt5a expression declined in the mesenchyme, becoming exclusively epithelial by E15 (Fig. 2E). It continued to be expressed to near hatching (Fig. 2G), and was restricted to the proximal epithelium and the distal-most luminal epithelium (arrows, Fig. 2G). The expression of Wnt3a declined after E14; it was detected only in the budding epithelium at E15 (arrows, Fig. 2F) and was not detected after E16 (Fig. 2H; data not shown). We could detect only weak expression of Wnt5a in the interstitial vessels at E11 (Fig. 2E), a slightly stronger expression at E15, and no detectable expression after E16. By contrast, Wnt3a was strongly expressed in the interstitial vessels at E12 (Fig. 2D) but expression diminished rapidly afterwards (from E14; data not shown; weakly present at E15, Fig. 1F).

Fig. 1. The expression patterns of Wnt5a and Wnt3a are spatially and temporally distinct. (A,B) Whole-mount in situ hybridization at E7. No pulmonary expression is detected for Wnt5a (A, positive expression in esophagus, white arrow), whereas expression is detected for Wnt3a (white arrows, B). (C) Wnt5a expression is observed in the mesenchyme adjacent to the distal epithelium (black arrowheads) and weakly within the distal epithelium (arrow) at E11. (E) By E15, Wnt5a is expressed strongly in the epithelium (both luminal and budding, arrows). (G) At E20, Wnt5a is expressed in the epithelium surrounding the parabronchial lumen (black arrow), and in the proximal-most epithelium (red arrow). Interstitial vascular expression for Wnt5a is weakly present from E11 (red arrowhead, C); this expression is slightly stronger at E15 (red arrowhead, E; data not shown) but is not detectable at E20 (red arrowhead, G). (D) Wnt3a is strongly expressed in the interstitial vasculature (red arrowhead) and the distal epithelium (arrow) at E12. (F,H) Expression of Wnt3a is restricted to the budding epithelium (arrows, F) by E15, and is not detected after E16 (E20 section shown in H); red arrowheads in G and H highlight large interstitial vessels. P, parabronchi (distal airway); SB, secondary bronchi.
vessels normally patterned but dysfunctional, with extravasated red blood cells spilling into the mesenchyme surrounding them (Fig. 2F; data not shown). The smaller, later-formed angiogenic interstitial blood vessels were more numerous than in wild type (Fig. 2L,O), but remained in the interstitium, without clustering subepithelia (Fig. 2L,O; Fig. 3C,F).

**Fig. 2. Wnt5a mis/overexpressed lungs show pulmonary hypoplasia with abnormal air and vascular tubulogenesis.** (A-C) Whole lungs dissected from wild-type (WT, A), RCAS-Wnt5a-infected (B), and RCAS-Wnt3a-infected (C) embryos at E14, showing pulmonary hypoplasia of the infected lungs. (D-I) H&E-stained lungs showing dramatic thinning of the airway epithelium by mis/overexpression of Wnt5a (compare brackets in E with D) but not following RCAS-Wnt3a infection (compare brackets in G with F). RCAS-Wnt5a mis/overexpressed lungs at E15 have more normal appearing airways and airway epithelial thickness (compare G with F) but develop with abnormal interstitial muscularized vessels, often with extravasated red blood cells (arrows and insert, G). At E20, Wnt5a mis/overexpressing lungs often show pulmonary lymphangiectasia (arrows in I) and disordered larger interstitial vessels (arrowheads, I, compare with wild-type lungs, H). (J-L) At E14, normal developing pulmonary endothelium is localized between airways or parabronchi, as Elderberry bark lectin shows (red arrow, J). With RCAS-Wnt5a infection, the developing vasculature clusters subepithelial to the parabronchi (red arrow, K). The number of elderberry bark lectin-stained interstitial vessels are increased by Wnt3a mis/overexpression but these vessels are localized normally in the interstitium (L), not clustered subepithelial as seen with Wnt5a mis/overexpression (K). Nuclei were counterstained with DAPI (blue). (M,N,O) Cartoon tracings of J,K,L make pattern changes of vasculature clearer. Epithelial cells are orange; endothelial cells, green.
**Wnt5a affects the VEGF pathway**

RCAS-Wnt5a infection did not affect epithelial VEGF expression (black arrows in insets, Fig. 3A,B), but did affect its vascular expression. Wnt5a mis/overexpression resulted in a marked decrease of detectable expression of VEGF in the interstitial vessels at E14 (compare red arrowheads in Fig. 3B and 3A). This was in contrast to the dramatic increase in protein levels of its receptor Flk1 (Vascular Endothelial Growth Factor Receptor 2) (Saha et al., 2004), and the altered placement of the Flk1-expressing cells (black arrows, Fig. 3C,D,E). Flk1 was expressed in wild-type airway epithelium but not in RCAS-Wnt5a-infected lungs (compare Fig. 3D and 3E). These findings were present from E12 to E16, afterwards expression of both VEGF and Flk1 decreased (data not shown).

Wnt5a mis/overexpression did not appreciably affect VEGF expression (Fig. 3C), or the pattern or levels of Flk1 expression, although there were more Flk1-expressing interstitial vessels (Fig. 3G).

**Wnt5a overexpression affects cell proliferation in epithelial and mesenchymal cells differently than in endothelial cells**

The abnormally increased and malpatterned interstitial blood vessels and the thinned epithelium in the Wnt5a-mis/overexpressing lungs were examined with proliferation and apoptosis markers. PCNA expression tends to cluster in the normal parabronchial epithelium and the subepithelial mesenchyme at E14 (see Fig. S2 in the supplementary material). In the Wnt5a-mis/overexpressing lungs, proliferation was found to be reduced in epithelial cells and overall (i.e. total number of cells), but increased in the subepithelial mesenchyme-endothelial cell compartment (see Fig. S2 in the supplementary material). By E20, PCNA expression was absent in the Wnt5a-mis/overexpressing distal epithelium, but remained strong in the proximal epithelium (see Fig. S2 in the supplementary material). These differences are statistically significant. There was no difference in apoptotic markers between wild-type and Wnt5a mis/overexpressing lungs in any compartment (epithelial, stromal or vascular; data not shown), and there was no histologically demonstrable necrosis.

**Wnt5a in the developing avian lung appears to act through Ror2 to activate a non-canonical Wnt pathway**

On sections of E14 wild-type lungs, activated β-catenin was expressed in vessels (red arrowheads, Fig. 4A) and in airway epithelium with a distinct epithelial pattern: weak/absent in the luminal airway epithelium (black arrow in Fig. 4A) but strong in the apical/budding epithelium of the budding terminal airways (red arrows in inset, Fig. 4A). In Wnt5a-mis/overexpressed lungs, β-catenin staining was weaker overall than in wild type, with almost no vascular staining (red arrowheads, Fig. 4E) and with an epithelial staining pattern that was markedly different from wild type. We detected the strongest staining in the luminal epithelium (black arrow, Fig. 4F), with much weaker staining in the budding apical epithelium (red arrows in inset, Fig. 4F), the inverse of wild type.

No upregulation of activated β-catenin levels was detected with Wnt5a mis/overexpression, suggesting that a non-canonical pathway must be used. To investigate which pathway Wnt5a uses in the lung, we preferentially inhibited the canonical pathway with soluble Dkk1 protein (Glinka et al., 1998; Niehrs, 2006). In organ culture, Dkk1 exposure resulted in mild pulmonary hypoplasia (compare Fig. 4B and 4C) but, importantly, failed to rescue the Wnt5a-mis/overexpression phenotype (compare Fig. 4G and 4H). When we used a dominant-negative construct of Ror2 (RCAS-dnRor2) to inhibit the non-canonical pathway (Mikels and Nusse, 2006), explants developed enlarged, hyperplastic lungs that were in most part due to relatively normally formed, but dilated airways (Fig. 4D). Co-expression of dnRor2 and Wnt5a (via RCAS) resulted in a partial rescue of the hypoplastic phenotype (Fig. 4H, compare with 4G).
Ror2 is normally expressed in the large muscular (proximal) pulmonary interstitial vessels (red arrowheads, Fig. 5A,B) and in the early pulmonary epithelium, prominently in the luminal epithelium (red arrow, Fig. 5A) and weakly in the budding terminal epithelium (red arrow, Fig. 5A). Expression continues in the large vessels (red arrowhead, Fig. 5B), and becomes restricted to the budding epithelium (red arrow, Fig. 5B) later in development.

Mis/overexpression of \( \text{dnRor2} \) in the lungs, which were allowed to develop in ovo, resulted in large lungs (pulmonary hyperplasia) with a relatively normal airway pattern (Fig. 5D, compare with 5C), but with a markedly decreased number of interstitial blood vessels (compare Fig. 5F with 5E). RCAS-\( \text{dnRor2} \) infection appears to limit the vascular pattern to the interstitium as no E. Lectin was detected either within or below the epithelium (compare Fig. 5F with 5E).

Wnt5a regulates epithelial cadherins and mesenchymal fibronectin

Budding morphogenesis in other organs uses cadherins as cues for patterning (Meyer et al., 2004), and, of the many members of the cadherin family, E-cadherin is most predominantly expressed in epithelial tissues. L-CAM is its avian homolog (Gallin et al., 1987; Pecina-Slaus, 2003), and we find its expression exclusively in the epithelium of the chick lung (Fig. 6A). With mis/overexpression of \( \text{Wnt5a} \), L-CAM expression was reduced in distal (red arrowhead, inset, Fig. 6B) but not proximal (red arrow, Fig. 6B) epithelium. In fact, the epithelial bud expression is diminished and nearly absent in \( \text{Wnt5a} \)-mis/overexpressing lungs (compare red arrowheads, insets, Fig. 6A,B).

Fibronectin is normally concentrated adjacent to the airway epithelium in the subepithelium of the terminal airways (red arrow, Fig. 5A) and weakly in the budding terminal epithelium (red arrow, Fig. 5A). Expression continues in the large vessels (red arrowhead, Fig. 5B), and becomes restricted to the budding epithelium (red arrow, Fig. 5B) later in development. Mis/overexpression of \( \text{dnRor2} \) in the lungs, which were allowed to develop in ovo, resulted in large lungs (pulmonary hyperplasia) with a relatively normal airway pattern (Fig. 5D, compare with 5C), but with a markedly decreased number of interstitial blood vessels (compare Fig. 5F with 5E). RCAS-\( \text{dnRor2} \) infection appears to limit the vascular pattern to the interstitium as no E. Lectin was detected either within or below the epithelium (compare Fig. 5F with 5E).

**Fig. 4.** \( \text{Wnt5a} \) acts in the non-canonical pathway via \( \text{Ror2} \) in lung development. (A,E) In wild-type lungs at E14, high levels of active β-catenin are detected in a patchy distribution in the buds of the most distal epithelium (black arrow, A and red arrows, inset A), whereas levels in the \( \text{Wnt5a} \)-mis/overexpressed lung are decreased (black arrow, E and red arrows, inset E). Active β-catenin is expressed in large interstitial vessels in wild type (red arrowheads, A) but is not detected in RCAS-\( \text{Wnt5a} \)-infected lungs (red arrowheads, E). (B-D,F-H) Explants grown for 5 days in organ culture demonstrate complex budding and overall organ growth in wild type (B), but lungs cultured after infection with RCAS-\( \text{Wnt5a} \) are hypoplastic with airway budding disarray and simplification (F). Treatment with the recombinant Dickkopf protein (Dkk1) impaired lung budding and growth in wild type (C) and did not rescue the \( \text{Wnt5a} \) phenotype (G, compare with F). The \( \text{Wnt5a} \)-mis/overexpressed hypoplastic phenotype (F) is partially rescued when embryos are co-infected with \( \text{dnRor2} \) (H). Infection with the RCAS-\( \text{dnRor2} \) construct alone showed dilated airways and mild overgrowth (hyperplasia; D).

**Fig. 5.** Inhibition of \( \text{Wnt5a} \) by \( \text{dnRor2} \) affects pulmonary development. (A) \( \text{Ror2} \) immunohistochemistry demonstrates expression at E14 in the epithelium (red arrow) and interstitial vessels (red arrowhead) of wild-type lungs. (B) At E18, \( \text{Ror2} \) is expressed in the interstitial large muscularized vessel (red arrowhead) and surrounding the luminal epithelium (red arrow and inset). (C,D) H&E staining of RCAS-\( \text{dnRor2} \)-infected lungs (D) shows an increase in size compared with wild type (C). (E,F) Elderberry bark lectin staining highlights the decrease in endothelium with \( \text{dnRor2} \) mis/overexpression.
Fig. 6. Wnt5a affects the expression of adhesion factors. Lungs imaged in A-G are all from in-ovo experiments; H-I are from explant (Exp) experiments. (A) L-CAM antibody localizes in the epithelium in wild-type lungs at E14 (red arrowhead, inset); arrow indicates the distal airway magnified in inset. (B) In Wnt5a-mis/overexpressing lungs, L-CAM expression is observed in the proximal epithelium (red arrow), but is reduced in the most-distant budding epithelium (red arrowhead, inset). (C) At E14 in wild-type lungs, fibronectin is principally expressed subepithelially (arrow, inset) and in the interstitial vasculature (red arrowhead, inset). (D) At this stage, fibronectin levels are increased diffusely in RCAS-Wnt5a-injected lungs as compared with wild type. (E,F) The fibronectin levels remain altered through to E20, when fibronectin is normally localized around the parabronchi lumen, surrounding the atrial muscle (red arrow, inset, E), in few distal luminal epithelial cells, and in the interstitial vasculature (red arrowhead, E). With Wnt5a mis/overexpression (F), fibronectin is overexpressed diffusely (red arrow shows a similar pattern surrounding the atrial muscle as in E, but note the diffuse expression throughout epithelium); however, almost no expression of fibronectin can be detected clustered in the interstitium between parabronchi (red arrowhead). (G) At E14, Wnt3a mis/overexpression does not significantly affect the level or pattern of fibronectin expression (compare with wild type in C). (H) In wild-type explants, normal fibronectin expression is observed subepithelially (red arrow) and in the interstitial vasculature (red arrowhead). (I) dnRor2 mis/overexpression decreases fibronectin expression overall and in the subepithelium (red arrow) and the interstitial vessels (red arrowhead). (J) Explants incubated with Dkk1 recombinant protein fail to develop vessels but show near normal subepithelial fibronectin expression (red arrow, J; compare with H).

Inhibition of fibronectin function rescues Wnt5a-induced branching abnormalities in the lung

We used the soluble peptide RGDS to inhibit fibronectin function (Yamada and Kennedy, 1984). Although RGDS markedly inhibits lung growth and airway development (compare Fig. 7A with 7B), the RCAS-Wnt5a-injected lung explant phenotype (Fig. 7D) was effectively rescued with RGDS treatment (Fig. 7E). The Wnt5a-mis/overexpressing lung explants, which show hypoplasia with a more normal airway development (Fig. 7C), were not rescued by RGDS (Fig. 7E).

Wnt5a mis/overexpression affects other known pulmonary pattern formation signaling pathways

We studied whether the mis/overexpression of Wnt5a affects other known developmentally important pathways and factors in the lung. At E10, Shh expression, which is normally strongly detected in the proximal and distal epithelium, was decreased in Wnt5a-mis/overexpressed lungs (Fig. 8A,B). Epithelial Bmp4 and Fgf10 expression were also markedly reduced by Wnt5a mis/overexpression, but no changes in the expression of Nkx2.1 were detected (see Fig. S3 in the supplementary material).

In lung explants, we found that the addition of exogenous Shh protein in wild-type lungs resulted in pulmonary hyperplasia (enlarged lungs, Fig. 8D), mainly due to a marked expansion of mesenchyme (asterisk in 8J). The airway and vascular pattern, where present, was normal, with preservation of the hexagonal vascular pattern (upper half of explant in Fig. 8J, magnified in 8P). Cycloamine inhibition resulted in marked pulmonary hypoplasia (Fig. 8E), with an abnormal collection of interstitial vessels (black arrows in Fig. 8K,Q). RCAS-Wnt5a-infected explants showed, as
expected, marked pulmonary hypoplasia with an abnormal vascular and airway pattern. The collection of misplaced vessels, unassociated with airways, was prominent and similar to that seen in cyclopamine-treated explants (see black arrows in Fig. 8K,L). Exogenous Shh protein rescues the Wnt5a-mis/overexpression pulmonary hypoplasia (Fig. 8G, compare with 8F), mainly because of an increase in the interstitial mesenchyme (asterisks, Fig. 8M), but the hexagonal vascular pattern is only focally restored (black arrows, Fig. 8M; arrowheads, 8S). Inhibition of the Hh pathway by cyclopamine in the RCAS-Wnt5a-infected explants resulted in an exacerbation of the hyperplastic phenotype (Fig. 8H), drastically altering the airway pattern (Fig. 8N,T). The abnormal collection of small vessels present in the Wnt5a-mis/overexpressing explants was rescued by Shh and worsened by cyclopamine (black arrows in Fig. 8L,M,N).

**Wnt5a increases fibronectin levels directly and appears to affect its pattern of expression through Shh**

Increasing levels of Shh did not affect fibronectin levels or the pattern of perivascular and subepithelial expression (Fig. 8V), whereas Wnt5a mis/overexpression affected both (Fig. 8X). The addition of Shh protein to Wnt5a-mis/overexpressing lung explants restored the normal pattern of fibronectin expression without decreasing its elevated level (note strong expression surrounding the airway epithelium; arrows, Fig. 8Y). Cyclopamine inhibition of Shh function did not affect fibronectin levels but did decrease the strong peri-airway expression (red arrow, Fig. 8W). Wnt5a-mis/overexpressing explants cultured in the presence of cyclopamine failed to restore the fibronectin pattern or to decrease the elevated level of expression (Fig. 8Z). These data suggest that Wnt5a affects the levels of fibronectin directly, and pattern of expression indirectly, through its inhibition of Shh.

**DISCUSSION**

Lung development is directed by intrinsic epithelial-mesenchymal regulatory factors functioning in a temporal, spatial and cell type-specific manner, the context of which ensures proper coordinated epithelial airway and pulmonary vascular tubulogenesis. We studied the role of Wnt5a in avian lung development and describe findings that suggest it has a key regulatory function in both these processes.

Avian pulmonary expression of Wnt5a is present in the epithelial and subepithelial tissues, and in the interstitial vasculature, but becomes exclusively epithelial by late stages. The expression is strongest in mid-embryonic stages when it includes all airway epithelium – parabronchi to terminal buds. By near hatching stages, the expression is patchy and present only in the few epithelial cells near and at the apical lumen of the distal airways. The significance of the discontinuous epithelial expression pattern is unknown; it does not appear to be related to the expression of the epithelial cell type markers SpB and CC16. However, the apical bias in expression may be important in regulating other factors.

In the chick lung, once the branching process is complete (by E14), the epithelium from the parabronchi bud into the surrounding mesenchyme, becoming adjacent to the distal pulmonary vasculature to form the gas-exchange surface. Our results show that the over/misexpression of Wnt5a results in budding dysfunction, which is likely to be due to altered extracellular matrix proteins and abnormal cell adhesion. We find an increase in fibronectin levels and a decrease in L-CAM levels following Wnt5a mis/overexpression. Spatially controlled fibronectin levels are clearly necessary for normal pulmonary development, as its inhibition in wild-type lungs results in dramatic branching dysfunction. This role appears to be conserved in the mammalian lung, as fibronectin is strongly expressed and its expression is maximal where branching morphogenesis occurs (Roman and McDonald, 1992). De Langhe and colleagues (De Langhe et al., 2005) showed that canonical Wnt signaling in mouse affects (at least) the proximal larger pulmonary vasculature by the downregulation of fibronectin. As both the canonical and non-canonical pathway target fibronectin, it must be an important protein to be regulated for normal pulmonary development.

L-CAM and β-catenin expression patterns are altered by Wnt5a mis/overexpression; their general expression levels decreased and the new ‘invading’ epithelial buds have lost nearly all expression. This location, at the interface of where the migrating and budding epithelium mingles with the migrating endothelium, must affect the coordination that needs to occur to form a normally functioning air capillary unit. The reduction of β-catenin and L-CAM in the epithelial buds may also affect the epithelium in an autocrine manner, as we find the airbud epithelium to be decreased (thinned) in the Wnt5a-mis/overexpressing lungs. The interference with these extracellular proteins mediating epithelial and endothelial growth and migration processes, has been shown in other systems as well (Smith et al., 2006).

Our results point to an important role of Wnt5a in directing pulmonary vascular development. The number of vascular spaces is increased in Wnt5a-mis/overexpressing lungs, suggesting an angiogenic effect of Wnt5a, a property that has been suggested by others as well (Cheng et al., 2008; Masckauchan et al., 2006; Zhang et al., 2006), but we also show that the affected vessels are malpatterened. Results presented herein suggest that sprouting
Wnt5a and air/vascular tubulogenesis

angiogenesis (responsible for the hexagonal pattern) is interrupted and intussusceptive angiogenesis (important in expansion and ‘alveolarization’) is stimulated. This appears to be a Wnt5a/non-canonical pathway-specific function, as neither of these vascular patterns/growths is affected by our manipulations of the canonical pathway. Wnt5a may direct pulmonary vascular patterning by regulating the VEGF pathway via its effect on fibronectin, as fibronectin contains VEGF-binding domains (Flamme et al., 1995).
Wnt5a mis/overexpression results in a near loss of detectable VEGF expression in interstitial vessels that is associated with strongly upregulated Flk1 in the vessels, which are malpositioned subepithelially (adjacent to the VEGF-expressing epithelium and where the highest concentration of fibronectin is present). Also, we show that Wnt5a inhibits Shh, which is known to regulate VEGFA (White et al., 2007).

Data herein implicates the non-canonical pathway, through Ror2, as the pathway used by Wnt5a in lung development. By inhibiting Ror2, we can rescue much of the phenotype of Wnt5a mis/overexpression in both the airway and vascular patterning; this is not seen with inhibition of the canonical pathway. The activation of the canonical pathway by mis/overexpression of Wnt5a affects early proximal airway branching with relatively normal terminal budding, and also disrupts the early, larger interstitial vessels. Therefore, it is possible that the disruption of early airway branching or budding morphogenesis would affect, secondarily, vascular patterning. This reiterates the importance of canonical Wnt signaling in early, proximal airway development (distinct from the role of Wnt5a in mid-to-late gestational stages of airway and vascular patterning), specifically in the alveolar stages where airway budding and intussusceptive angiogenesis are so important.

Many systems important in body pattern formation are also known to play roles in pulmonary development, including the Hedgehog, Bmp, Nk and Fgf pathways (Belluscì et al., 1997a; Belluscì et al., 1997b; Belluscì et al., 1996; Pepicelli et al., 1998; Yuan et al., 2000). The interaction of these principal events, however, varies in a tissue-specific manner. Our results show that non-canonical Wnt signaling, by Wnt5a, functions as an inhibitor of these pathways. These results are supported by the results of others. Mice null for Wnt5a develop hyperplastic lungs in which Shh, Bmp4 and Fgf10 are upregulated (Li et al., 2002). Overexpressing Wnt5a with the surfactant-B promoter demonstrated that Wnt5a acts upstream of Fgf10, even though it acts as an activator of Fgf10 expression (Li et al., 2005). The difference in these and our results is likely to be due to the timing of the mis/overexpression; the surfactant B promoter directs expression very late in lung development. Thus, our results support a function of Wnt5a as an important upstream modulator of the secreted morphogens known to be involved in early-mid lung development. The interaction of Wnt5a, Shh and fibronectin shown here is intriguing, Shh, by affecting specifically the pattern and not the level of fibronectin, rescues the vascular part of the Wnt5a-mis/overexpressing phenotype. These results show the importance of the spatial pattern of expression of fibronectin in directing the normal vascular pattern in the lung. The specific pathway (Wnt5a-Shh-fibronectin) is a novel and important finding.

We also describe for the first time, to our knowledge, a pulmonary hyperplastic phenotype in explants incubated with excess Shh, and in those in which the non-canonical pathway is inhibited via Rcan-DnRor2. This phenotype is reminiscent of the human congenital cystic adenomatoid malformation (CCAM) (Wilson et al., 1992). Our study suggests that some of the molecular factors that may be involved in generating pulmonary hypoplasia are likely to affect vascular development, exacerbating clinical hypoxemia, the lethal common denominator in this disorder. Other human congenital malformations of the lung that involve pulmonary vascular anomalies (for example alveolar-capillary dysplasia) (Cullinan et al., 1992) are poorly understood molecularly (Groenman et al., 2005). The findings described here may provide an insight into the etiology of these often lethal, congenital diseases. In addition, they emphasize the complex relationships of pathways during lung development, in which survival requires interactions that are carefully choreographed by factors such as Wnt5a.

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Supplementary material
Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/135/7/1365/DC1

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