Dickkopf-1 (DKK1) reveals that fibronectin is a major target of Wnt signaling in branching morphogenesis of the mouse embryonic lung

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Abstract

Members of the Dickkopf (Dkk) family of secreted proteins are potent inhibitors of Wnt/\(\beta\)-catenin signaling. In this study we show that Dkk1, -2, and -3 are expressed distally in the epithelium, while Kremen1, the needed co-receptor, is expressed throughout the epithelium of the developing lung. Using TOPGAL mice [DasGupta, R., Fuchs, E., 1999. Multiple roles for activated LEF/TCF transcription complexes during hair follicle development and differentiation. Development 126, 4557–4568] to monitor the Wnt pathway, we show that canonical Wnt signaling is dynamic in the developing lung and is active throughout the epithelium and in the proximal smooth muscle cells (SMC) until E12.5. However, from E13.5 onwards, TOPGAL activity is absent in the SMC and is markedly reduced in the distal epithelium coinciding with the onset of Dkk-1 expression in the distal epithelium. To determine the role of Wnt signaling in early lung development, E11.5 organ cultures were treated with recombinant DKK1. Treated lungs display impaired branching, characterized by failed cleft formation and enlarged terminal buds, and show decreased \(\alpha\)-smooth muscle actin (\(\alpha\)-SMA) expression as well as defects in the formation of the pulmonary vasculature. These defects coincide with a pattern of decreased fibronectin (FN) deposition. DKK1-induced morphogenetic defects can be mimicked by inhibition of FN and overcome by addition of exogenous FN, suggesting an involvement of FN in Wnt-regulated morphogenetic processes.

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Introduction

Early embryonic lung development is characterized by a succession of cycles of bud initiation, bud outgrowth, and bud arrest. Lung formation starts at E9.5, with an out-pocketing of the endoderm from the ventral foregut. Initially, the two primary buds undergo repetitive outgrowth and branch laterally and distally in a highly reproducible pattern. They develop asymmetrically, so that in the mouse the right bud gives rise to four lobes, whereas the left bud gives rise to only one.

The differentiation process of the epithelium and mesenchyme is tightly coordinated with the branching process. The most distal epithelial and mesenchymal cells are essentially undifferentiated and as they leave the distal domain, they differentiate progressively (Hogan, 1999). The differentiation process is dependent upon the combination of signals received. Experimental evidence is rapidly emerging that the balance between activating and inhibitory signals is a mechanism of fine regulation in development. For
example, Sonic hedgehog activity is modulated by Hedgehog Interacting Protein (HIP), which binds to SHH and prevents its binding to its receptor, Patched (PTC) (Chuang et al., 2003). Similar inhibitory mechanisms exist between Bone Morphogenetic Protein 4 and extracellular modulators like Noggin, Gremlin, or Chordin (reviewed by Balemans and Van Hul, 2002). In the Wnt pathway, secreted Frizzled-related proteins (sFRP) and more recently Dickkopf (DKK) are secreted molecules shown to inhibit Wnt signaling (reviewed by Kawano and Kypta, 2003).

This work focuses on the role of DKK1 during lung development. Through our study of DKK1, our goal is also to investigate the role of the Wnt pathway during this process. The Wnt growth factor family in the mouse is comprised of 19 different secreted ligands that interact with 10 known seven-transmembrane receptors of the Frizzled (Fz) gene family and either one of two single-span transmembrane proteins, low-density-lipoprotein-receptor-related proteins (LRP-5 and LRP-6) (Pinson et al., 2000; Tamai et al., 2000; Wehrli et al., 2000). Historically, Wnt proteins have been grouped into two classes, canonical and noncanonical. Canonical Wnts bind to frizzled receptors, inhibiting glycogen-synthase kinase-3β (GSK-3β)-mediated phosphorylation of β-catenin. Hypophosphorylated β-catenin accumulates in the cytoplasm, after which it translocates to the nucleus, where it heterodimerizes with members of the TCF/LEF transcription factor family to activate the transcription of TCF/LEF target genes (reviewed by Seto and Bellen, 2004). Noncanonical Wnts activate other Wnt signaling pathways, such as the planar-cell-polarity (PCP)-like pathway that guides cell movements during gastrulation (Heisenberg et al., 2000). Activation of the PCP pathway can antagonize the canonical pathway intracellularly (Ishitani et al., 2003; Kuhl et al., 2001; Torres et al., 1996). Secreted Wnt antagonists can be divided into two functional classes, the secreted Frizzled-related protein (sFRP) class and the Dickkopf class. Members of the sFRP class bind directly to Wnts, thereby altering their ability to bind to the Wnt receptor complex, while members of the Dickkopf class inhibit Wnt signaling by binding to the LRP5/LRP6 component of the Wnt receptor complex. In addition to LRP5/6, DKK1 interacts with the high-affinity DKK1 co-receptors Kremen1 (Krm1) or Kremen2 (Krm2), which functionally cooperate with Dkk1 to block Wnt/beta-catenin signaling (Mao et al., 2002 and reviewed by Kawano and Kypta, 2003). DKK1, DKK3, and DKK4 act as inhibitors of canonical Wnt signaling, while DKK2 can function both as an inhibitor in the presence of Kremen2 or as an activator in its absence (Hoang et al., 2004; Mao and Niehrs, 2003).

Wnt signaling plays an important role in lung development. Conditional inactivation of the β-catenin gene in the epithelium of the developing mouse lung leads to neonatal death resulting from severe lung defects (Mucenski et al., 2003); branching of secondary bronchi is altered and the number of small peripheral alveolar ducts and terminal sacculles is markedly reduced. In addition, the epithelium fails to undergo proper distal differentiation, lacking the expression of pro-SP-C protein and Vascular endothelial growth factor-A (Vegf-A), the latter correlating with a reduction in alveolar capillaries. So far, inactivation of only two WNT ligands has resulted in lung defects. Wnt7b, a canonical Wnt, is expressed in epithelial cells of the peripheral lung. Wnt7b−/− mice exhibit perinatal death due to respiratory failure. Defects were observed in proliferation of the lung mesenchyme resulting in lung hypoplasia. In addition, Wnt7b−/− embryos and newborn mice exhibit severe defects in the smooth muscle component of the major pulmonary vessels, with increased apoptosis of the vascular smooth muscle cells (VSMCs), resulting in rupture of the major blood vessels and hemorrhages in the lungs after birth (Shu et al., 2002). Wnt5a, a noncanonical Wnt, which is able to inhibit canonical Wnt signaling (Topol et al., 2003; Westfall et al., 2003), is expressed at high levels in the distal lung mesenchyme. Wnt5a−/− mice die perinatally from lung defects including truncation of the trachea, overexpansion of the peripheral airways, and delayed lung maturation. Absence of Wnt5a activity in mutant lungs leads to increased cell proliferation and up-regulation of the expression of Fgf10, Bmp4, Shh, and Ptc (Li et al., 2002).

To determine the role of canonical Wnt signaling in early lung development, we treated E11.5 lung explants with recombinant Dickkopf-1 (DKK1), a potent and specific diffusible inhibitor of Wnt action that is also endogenously secreted by the distal lung epithelium. We show that organ cultures treated with DKK1 display impaired branching, characterized by failed cleft formation and enlarged terminal buds. DKK1-treated lung explants further show reduced α-smooth muscle actin (α-SMA) expression and defects in the formation of the pulmonary vascular network. These defects coincide with a pattern of decreased fibronectin (FN) deposition and Platelet-derived growth factor-A (Pdgf-a) expression. DKK1-induced morphogenetic defects can be mimicked by inhibition of FN and overcome by addition of exogenous FN. Our findings suggest an intersection between the Wnt and FN-integrin pathways and an involvement of FN in Wnt-regulated morphogenetic processes.

Materials and methods

Transgenic embryos

TOPGAL and Sp-C-GFP mice were a generous gift of Dr. Elaine Fuchs and Dr. Jonathan Heath, respectively. Flk1LacZ mice were obtained from Jackson Laboratories. All mice were bred in the C57BL6 background. Lungs were isolated from embryonic day 11.5 (E11.5) embryos.

β-galactosidase staining

TOPGAL or Flk1LacZ lungs at different developmental stages (E11.5–E14.5) were dissected and fixed in 4%
paraformaldehyde in PBS at 4°C for 10 min with rocking, washed twice for 10 min in PBS at 4°C, transferred into freshly prepared X-gal solution, and stained at 37°C until a clear precipitate formed (Hogan et al., 1994). After rinsing with PBS, lungs were post-fixed in 4% paraformaldehyde in PBS. For vibratome sections, samples were embedded in an albumin (300 mg/ml)–gelatin (5 mg/ml) mix, cross-linked with glutaraldehyde (0.6%), and sectioned at 30 μm. For micromtome sections lungs were fixed in 4% paraformaldehyde, washed in PBS, dehydrated, and paraffin embedded. Sections were stained with eosin for 2 s.

In situ hybridization

Microdissected and cultured lungs were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 2 h. They were washed twice with PBS, dehydrated overnight in 70% ethanol at 4°C, and stored in absolute ethanol at −20°C. The whole-mount in situ hybridization protocol used was based on a previously described method (Winnier et al., 1995). The following mouse cDNAs were used as templates for the synthesis of digoxigenin-labeled riboprobes: a 1.5-kb full-length mouse Bmp-4 (Winnier et al., 1995), a 818-bp full-length Dkk1 and 1.4-kb Kremen1 and -2 probes (kindly provided by Dr. Christopher Niehrs), a 991-bp Dkk2 fragment (cloned by RT-PCR using primers Dkk2-F agttcttaagagtcccgagt and Dkk2-R cgtcctctgaggtgaacctggc), a 301-bp Dkk3 fragment (cloned by RT-PCR using primers Dkk3-F egctctegaggtgaacctggc and Dkk3-R gttcgggtgcatagcatctgc), a 800-bp Fz7, a 1-kb Fz8 (kindly provided by Dr. Jeremy Nathan), a 360-bp Lef1 probe (kindly provided by Dr. Rudolf Grosschedl), a 642-bp Shh, a 630-bp Wnt-2A, a 360-bp Wnt5a, a 300-bp Wnt7b (kindly provided by Dr. Andrew McMahon), a 900-bp Pdgf-a fragment (kindly provided by Dr. Linda Karlsson), a 584-bp fragment of Fgf10 (Bellusci et al., 1997), and a 2-kb LacZ probe (kindly provided by Dr. Robert Kelly).

Organ culture

Isolated E11.5 lung explants were removed on day 11.5 (E11.5) of gestation were placed on Nuclepore filters, which were then laid on the surface of 800 μl DMEM/F12 medium containing 50 units/ml penicillin and 50 μg/ml streptomycin and 10% heat-inactivated fetal calf serum in Nunclon dishes (technique adapted from Lebeche et al., 1999). For some experiments illustrated in Figs. 9–11, organ cultures were carried out in the absence of serum. No difference was seen in overall growth, budding, or differentiation of the lungs compared to lungs grown with serum. We investigated the effects of DKK1 on these cultures by adding 200 ng/ml of exogenous recombinant human DKK1 (R&D Systems). Dose response experiments (25, 50, 100, 200, and 400 ng/ml) were carried out. No response could be seen at concentrations of DKK1 lower that 200 ng/ml. No differences were observed between 200 and 400 ng/ml. The biological effect of an initial dose of DKK1 (added at t = 0 h) on lung goes down with time. A peak in the phenotype (essentially decreased cleft formation) is obtained at 48 h and then the treated lung resumes a development that is similar to the nontreated lung. After 96 h of culture, the treated and untreated lungs have the same phenotype. However, a clear phenotype can be seen when the medium gets refreshed with DKK1 (200 ng/ml) every 24 h. This suggests that recombinant DKK1 protein is very unstable. Fibronectin was inhibited by adding anti-FN antibody R745 diluted 1:50 to cultured E11.5 lung explants. Rescue experiments were performed by adding human plasma FN (2 mg/ml) dialyzed against DMEM/F12 to DKK1 (200 ng/ml) treated cultures. Explants were cultured for 3 days. Due to the limited stability of DKK1, fresh DKK1 was added every day.

Immunohistochemistry

Cultured lungs (72 h) were fixed in 4% paraformaldehyde for 20 min, washed in PBS, dehydrated, and paraffin embedded. Sections were treated with a monoclonal anti-α-smooth muscle actin antibody, clone 1A4, Cy3 conjugated (from Sigma®) at 1:200 and/or polyclonal anti-β-galactosidase antibody (from US Biological) at 1:200. Anti Sp-C polyclonal antibody was used at dilution 1:1000 (a kind gift of Dr. Jeffrey Whitsett). All slides were mounted with DAPI containing Vectashield® and photomicrographs were taken.

RT-PCR

RNA was isolated from individual lung cultures grown in the presence or absence of DKK1, using Trizol reagent (Invitrogen) according to the manufacturer’s instructions. PCR reactions were performed with a minimum number of cycles. RNA concentration was determined by spectrophotometry. cDNA was generated using Superscript first-strand synthesis system for RT-PCR (Invitrogen) according to the manufacturer’s instructions. Competitive RT-PCR was performed for β-actin, Vegf-A, and Bmp4 as previously described (Zhao et al., 1998) to determine the effects of treatment on mRNA levels in the lungs. The same primers were used to amplify both cDNA and competitor for each gene of interest. A known amount of competitor was added to products of reverse transcription. Competitive PCR, using β-actin, was performed with all samples to equalize the concentration of cDNA in different samples.

Semiquantitative RT-PCR was used for the other genes of interest. The number of cycles for the different PCR reactions was chosen to be in the linear amplification phase. Oligonucleotides used were Shh-F gtcttacagctgag, Shh-R ctgcgagtaccagtggat (32 cycles); Fgf10-F tgttttggctctctgtgagg, Fgf10-R ggatactgacacattgctgag
Results

Wnt signaling in early lung development

Whole-mount RNA in situ hybridizations for Wnt ligands, receptors, and the Tef/Lef1 transcription factors were performed to determine the expression of some of the mediators of Wnt signaling during lung development. In the early mouse lung (at E12.5), Wnt7b is expressed in the lung epithelium with a more intense expression distally (Fig. 1A and Shu et al., 2002), Wnt2a is highly expressed in the distal mesenchyme (Fig. 1B) and Wnt5a shows low expression in the mesenchyme and epithelium and is highest around the trachea and pharynx (Fig. 1C and Belluscì et al., 1996). Lef1 (Lymphoid enhancer factor 1) is highly expressed in the distal mesenchyme and at lower levels throughout the mesenchyme adjacent to the lung epithelium (Fig. 1D and Tebar et al., 2001). Fz7 is expressed in the mesenchyme with higher expression distally and shows weaker expression in the epithelium (Fig. 1E and data not shown). Fz8 is highly expressed throughout the epithelium (Fig. 1F). Fz2, 3, and 6 are expressed both in the epithelium and mesenchyme (data not shown). These expression patterns suggest that Wnt signaling can originate from the epithelium and mesenchyme and can target both tissues in an autocrine and/or paracrine fashion.

TOPGAL mice, which carry a β-galactosidase gene under the control of a LEF/TCF and β-catenin inducible promoter (DasGupta and Fuchs, 1999), were used to investigate where LEF/TCF transcription factor complexes were activated during early lung development. From E10.5 until E12.5, TOPGAL activation occurred throughout the epithelium and in the mesenchyme adjacent to the proximal airways where the bronchial smooth muscle cells (SMC) arise (Figs. 1G–J and Maretto et al., 2003). At E13.5 (data not shown) and E14.5 (Figs. 1K,L), TOPGAL activity was no longer detected in the mesenchyme (data not shown) and the activity in the epithelium was now significantly reduced distally concomitant with the onset of expression of Dkk1 in the distal epithelium (Figs. 2A,B), Dkk1 (Figs. 2A,B) and Dkk3 (Fig. 2E) expression in the distal epithelium starts at E13.5 and E12.5, respectively, while Dkk2 (Figs. 2C,D) expression in the epithelium starts as early as E11.5 and is up-regulated distally at later stages. The expression of high-affinity DKK1 co-receptors Kremen1 (Fig. 2F) and Kremen2 (expressed at lower level, data not shown), which functionally cooperate with Dkk1 to block Wnt/β-catenin signaling, is detected between E11.5 and E14.5 throughout the epithelium.

DKK1 treatment inhibits canonical WNT signaling

TOPGAL transgenic mice were also used to test whether exogenously supplied recombinant DKK1 protein in lung explant cultures of E11.5 lungs would result in the inhibition of canonical Wnt signaling. TOPGAL activity as shown by
β-gal staining after 72 h of culture was notably reduced in the distal epithelium of DKK1 treated organ cultures (Figs. 3B,D; n = 5) compared to controls (Figs. 3A,C). The detection of remaining TOPGAL activity in the more proximal epithelium of DKK1 treated organ cultures (Fig. 3B) is likely the result of the high stability of the β-galactosidase protein. In support of this conclusion, whole-mount in situ hybridization for LacZ was performed on lung explants cultured for 72 h in the presence or absence of DKK1. The expression of LacZ was not detected after 72 h of culture in lung explants treated with DKK1 (Fig. 3F) while control explants still showed expression throughout the epithelium and in the proximal mesenchyme (Fig. 3E). This data was confirmed by semiquantitative RT-PCR for LacZ on RNA isolated from DKK1 treated and untreated lung explants grown for 72 h (Fig. 7M).

**DKK1 treatment of lung explant cultures disrupts branching**

Transgenic mice expressing green fluorescent protein (GFP) under the control of the Sp-C (surfactant protein-C) promoter (which at early developmental stages is uniformly expressed in the distal epithelium) were used to study the effect of exogenously supplied recombinant DKK1 protein on branching morphogenesis in explant cultures of E11.5 lungs. The expression of GFP in the epithelium allowed us to follow the growth and branching of the lung during culture (Fig. 4). E11.5 Sp-C-GFP lung explants were cultured for 72 h in the presence or absence of 200 ng/ml of recombinant DKK1. DKK1-treated lungs were not significantly different in overall size compared to controls (compare Figs. 4A,E,I,M with B,F,J,N). DKK1 treatment resulted in decreased branching characterized by a defect in cleft formation and...
enlarged terminal buds compared to controls. DKK1-treated lungs had an average of 24.4 ± 3.2 peripheral buds compared to control lungs which had 34.4 ± 3.1 (P ≤ 0.0001, n = 5), resulting in a decrease in branching of 29%. Defects in branching started to appear after 48 h (compare Figs. 4I,K with J,L) in culture and were very obvious after 72 h of culture in the presence of DKK1 (Figs. 4N,P) as compared to control lungs (Figs. 4M,O). Interestingly, these defects in branching morphogenesis are not associated with defects in proliferation. Proliferation rates, assessed by BrdU incorporation, in the epithelium of DKK1-treated lungs vs. control lungs, were not significantly different (36 ± 9% and 40 ± 9%, respectively, n = 5, P = 0.2). At the same time, no significant difference in apoptosis could be seen between lung explants treated with DKK1 vs. control lungs (6.9 ± 1% and 5.3 ± 1%, respectively, n = 7, P = 0.05).

Fibronectin deposition is decreased in DKK1-treated lung explants

In order to better define the observed defects in branching morphogenesis, we analyzed the deposition of FN, a protein previously described to be downstream of Wnt signaling in Xenopus (Gradvil et al., 1999) and the perturbation of which leads to defects in branching in embryonic salivary glands, lung, and kidney (Sakai et al., 2003). The deposition of FN was therefore investigated by immunohistochemistry on DKK1-treated and control lung explants and was found to be markedly decreased in both the epithelial basement membrane and the mesenchyme of the DKK1-treated lung explants after 72 h of culture (Fig. 5, n = 4).

DKK1 treatment perturbs differentiation of the mesenchyme

At E12.5, TOPGAL activity is present in the mesenchyme around the bronchi (Fig. 6A). These cells are also co-expressing α-SMA (Fig. 6B) and therefore represent proximal bronchial SMC. These observations led us to investigate whether DKK1 treatment of E11.5 lung explants would result in aberrant smooth muscle cell differentiation. E11.5 lung explants were cultured up to 72 h in the presence or absence of DKK1. In control lungs, α-SMA is detected at high levels around the bronchi and
is excluded from the tip of the buds (Figs. 6C,E). In DKK1-treated lung explants, further distal expression of α-SMA was severely impaired (Figs. 6D,F; \( n = 10 \)). α-SMA expression was mostly detected proximally in DKK1-treated lung explants as early as 24 h after treatment (data not shown). Interestingly, no differences could be found in Sp-C expression in the epithelium in untreated vs. DKK1-treated lung explants (data not shown, \( n = 4 \)). This result suggests that in our experimental conditions, the proximal–distal differentiation of the lung epithelium is not dramatically affected.

**DKK1 treatment results in a decrease in Pdgf-a and Wnt2a expression**

Because of the observed defects in branching, whole-mount in situ hybridization after 72 h of culturing in the presence or absence of DKK1 was carried out to determine the expression of key regulatory molecules in lung morphogenesis. The expression pattern of *Pdgf-a* was studied because of its known role in myofibroblast progenitor proliferation and differentiation (Lindahl et al., 1997). *Pdgf-a* expression was markedly reduced in lung explants grown in the presence of DKK1 (Fig. 7B) compared to control lungs (Fig. 7A, \( n = 3 \)). Further, to test whether DKK1 could be involved in a feedback loop with canonical Wnts expressed distally in the lung, the expression pattern of *Wnt2a* was studied by whole-mount in situ hybridization. *Wnt2a* was markedly reduced in the distal mesenchyme in lung explants grown in the presence of DKK1 (Fig. 7D) compared to control lungs (Fig. 7C; \( n = 3 \)). No differences could be detected in the expression of *Fgf10* (Figs. 7F,E), *Bmp4* (Figs. 7H,G), or *Shh* (Figs. 7J,I). This data was confirmed by competitive and/or semiquantitative RT-PCR on RNA isolated from DKK1-treated and untreated lung explants grown for 72 h (Fig. 7M).
DKK1 perturbs vascular development

To study the effect of DKK1 on vasculogenesis in the lung, lungs isolated from E11.5 Flk1LacZ/+ mice were cultured in the presence or absence of exogenous recombinant DKK1. Flk1 LacZ/+ mice exhibit β-galactosidase expression in endothelial cells under the control of the Flk1 promoter (Shalaby et al., 1995). β-gal staining illustrates abnormal vessel formation in DKK1 treated explants: Blood vessels are thinner, show reduced sprouting from preexisting vessels (angiogenesis), and impaired formation of large vessels and establishment of interconnections (Figs. 8C,D), compared to untreated lung explants (Figs. 8A,B; n = 3). Quantification of the surface corresponding to the β-galactosidase staining, reported in percentage of the total lung surface, shows a significant difference between control and DKK1-treated lung explants (3.3 ± 1.1% vs. 1.5 ± 0.7%, respectively, P < 0.05). This defect occurs without a decrease in Vegf-a expression based on competitive RT-PCR results (Fig. 7M).

Inhibition of FN activity recapitulates DKK1 defects in the lung

A polyclonal anti-FN antibody was used to inhibit cleft formation and branching in the lung as described by Sakai et al. (2003). Treatment with an anti-FN antibody significantly inhibited branching (Figs. 9A,B) of the lung explants. Lung explants treated with an anti-FN antibody had an average of 15.6 ± 4.6 peripheral buds compared to control lungs which had an average of 35.4 ± 2.4 (P ≤ 0.0001, n = 7) peripheral lung buds, resulting in a
Fig. 5. Fibronectin expression is decreased in DKK1-treated lungs. Lungs isolated from E11.5 wild-type mice where grown in culture for 72 h in the presence (B, D) or absence (A, C) of recombinant DKK1 (200 ng/ml). Fibronectin expression was analyzed by immunohistochemistry on sections. (A, C) Fibronectin is present in the basement membrane around the epithelium (arrowheads) and in the mesenchyme at sites of cleft formation (arrows). (B, D) Fibronectin deposition by the epithelium (arrowheads) into the basement membrane and at sites of cleft formation (arrows) is reduced upon DKK1 treatment. Experimental conditions and photography (exposure time) were identical between control and DKK1-treated lungs to allow for direct comparison of FN expression. The exposure time was chosen so that the maximum signal (i.e., for CTRL) was below the saturation point of the camera.

Fig. 6. DKK1 reduces SMA expression in the mesenchyme. (A) β-gal (green) immunohistochemistry on sections of E12.5 TOPGAL lungs show TOPGAL activity in the epithelium and the proximal SMC (arrows). (B) Co-immunostaining of β-gal (green) and SMA (red) (arrows) (on sections) in the proximal SMC around the upper respiratory airways. Lungs isolated from E11.5 WT mice were grown in culture for 72 h in the presence (D, F) or absence (C, E) of recombinant DKK1 (200 ng/ml). α-Smooth muscle actin (SMA) expression was analyzed by immunohistochemistry (red); nuclei were stained with DAPI (blue). (C, E) In the untreated lung explants, the expression of SMA is found in the bronchial SMC around the airways. (D, F) In DKK1-treated lung explants, SMA expression did not expand in spite of lung growth, and very little SMA expression can be detected more distally around the airways. Experimental conditions and photography (exposure time) were identical between control and DKK1 treated lungs to allow for direct comparison of α-SMA expression. The exposure time was chosen so that the maximum signal (i.e., for CTRL) was below the saturation point of the camera.
increase in branching of 56%. Treatment with an anti-FN antibody further reduced smooth muscle differentiation around the airways (Figs. 9C,D) and perturbed proper lung vascular network formation (Figs. 8E,F), recapitulating the defects seen after treatment with DKK1. Quantification of the surface corresponding to the β-galactosidase staining shows a significant difference between control and lung explants treated with antibodies against FN (3.3 ± 1.1% vs. 1.0 ± 0.2%, respectively, P < 0.05). However, Pdgf-a expression was unchanged in lung explants grown in the presence of an anti-FN antibody (Fig. 7L) compared to control lungs (Fig. 7K, n = 3).

Exogenously supplied FN rescues the DKK1 phenotype

Exogenous FN (2 mg/ml) (Sakai et al., 2003) was added to DKK1-treated organ cultures to replace the FN depleted by DKK1 treatment. Experimental supplementation of FN successfully rescued branching morphogenesis (Figs. 10L,J) compared to organ cultures treated with DKK1 alone (Figs. 10E–H) and control lungs (Figs. 10A–D). While DKK1-treated lungs responded as previously described (Fig. 4), no significant difference in number of peripheral buds could be found between control lung explants (29 ± 2.4) and lung explants treated with DKK1+FN (29.4 ± 2.5) (P = 0.3786, n = 5). Addition of exogenous FN to DKK1-treated lung explants did not rescue the loss of TOPGAL (Fig. 10L) activity compared to control lungs (Fig. 10K), indicating that the inhibitory effect of DKK1 on canonical Wnt signaling was not affected and that FN is acting downstream of DKK1. Further, exogenously supplied FN not only rescued but enhanced α-SMA expression distally in DKK1-treated lung explants (Figs. 11E,F, n = 3) compared to, respectively, lung explants treated with DKK1 alone (Figs. 11C,D), or untreated control explants (Figs. 11A,B). Close examination of the distal portion of the lungs showed that, while in control lungs, the expression of α-SMA is found in the SMC around the bronchi but excluded from the tip (Fig. 11B); FN treatment (in presence of DKK1) resulted in an expansion of the α-SMA expression domain more distally (Fig. 11F). Application of exogenous FN to DKK1-treated lung explants also partially rescued vascular network defects (Figs. 8G,H; n = 3). Quantification of the surface corresponding to the β-galactosidase staining shows no significant difference between control and lung explants treated simultaneously with DKK1 and FN (3.3 ± 1.1% vs. 3.3 ± 0.6%, respectively, P < 0.05).

Discussion

Careful analysis of TOPGAL mice showed that canonical Wnt signaling in the lung is dynamic. From E10.5 until E12.5, TOPGAL activation occurred throughout the epithelium and in the proximal SMC. However, from E13.5 onwards, TOPGAL activity was only detected in the epithelium but was reduced distally, concomitant with the onset of expression of Dkk1 in the distal epithelium. To determine the role of canonical Wnt signaling in early lung development, we treated E11.5 lung explants in vitro with recombinant DKK1, a potent and specific diffusible inhibitor of Wnt action that is also endogenously secreted by the lung epithelium. We used TOPGAL, Sp-C-GFP and Flk1LacZ/ mice, respectively, to show that DKK1 treatment of lung explants inhibited canonical Wnt signaling, impaired branching and resulted in defects in the formation of the pulmonary vascular network. The DKK1-treated lungs further displayed a reduction in α-SMA expression around the bronchi. These defects coincided with a pattern of decreased FN deposition. We further observed a reduction in Wnt2a and Pdgf-a expression. However, no difference in expression could be detected for the major lung morphogens Fgf10, Shh, and Bmp4, nor Vegf, and no significant differences in proliferation of the epithelium could be found. Inhibition of FN activity with an anti-FN antibody recapitulated the DKK1 imposed morphogenetic defects in the lung whereas exogenously supplied FN rescued the DKK1 phenotype.

Wnt signaling in early lung development

In the embryonic lung, Wnt ligands, Wnt receptors, and associated Wnt transcription factors are detected from very early stages of development in the epithelium and mesenchyme, suggesting that Wnt signaling can occur in both tissue compartments. However, our results using TOPGAL mice show that Wnt signaling occurs mostly in the epithelium and additionally in the mesenchymal cells directly apposed to the proximal bronchi (Fig. 1 and Maretto et al., 2003). The mesenchymal TOPGAL-positive cells in the lung also express α-SMA, indicating that differentiated SMC around the proximal airways are responding to Wnt signaling (Fig. 6). TOPGAL mice have previously been shown to be excellent reporters for Wnt signaling (DasGupta and Fuchs, 1999) and in vivo overexpression in the distal lung epithelium of the noncanonical Wnt5a, which blocks canonical Wnt signaling, leads to a decrease in TOPGAL activity (Dr. C. Li, personal communication).

Wnt signaling is modulated by secreted antagonists, like secreted Fzr-related proteins (sFrp) and members of the Dickkopf family. We show herein that the expression of Dickkopf-1, -2, and -3 in the distal epithelium of the lung starts from E13.5, E11.5, and E12.5, respectively (Fig. 2). The onset of Dkk1 expression correlates with the reduction of TOPGAL activity in the distal epithelium from E13.5 onwards. Interestingly, Dkk1−/− mice have no lung phenotype (Mukhopadhyay et al., 2001 and Westphal personal communication). This suggests that the expression of Dkk1, Dkk2, and Dkk3 (Fig. 2) in the distal epithelium is redundant.

Transgenic experiments in mouse have shown that Dkk1 overexpression inhibits canonical Wnt signaling.
Ectopically expressed $Dkk1$ under the control of a keratin-14 promoter, which directs transgene expression to the surface epithelium and to the basal cells of the epidermis during embryogenesis, resulted in a complete failure of hair follicle placode formation and blocked tooth and mammary gland development before the bud stage (Andl et al., 2002). Transgenic mice ectopically expressing $Dkk1$ in the gut epithelium showed a loss of intestinal crypts, and disrupted intestinal homeostasis (Pinto et al., 2003). Adenoviral expression of $Dkk1$ in both the small intestine
and colon of adult mice markedly inhibited proliferation, accompanied by progressive architectural degeneration with the loss of crypts, villi, and glandular structure (Kuhnert et al., 2004). Here, we treated lung explants from TOPGAL embryos with recombinant DKK1 protein and our results show a marked decrease in TOPGAL activity, indicating that we could inhibit canonical Wnt signaling in the lung (Fig. 3).

Decreased FN deposition perturbs branching, smooth muscle differentiation, and formation of the lung vasculature

The extracellular matrix (ECM) protein FN has been shown to be essential for cleft formation during the initiation of epithelial branching in several organ systems including the lung (Gill et al., 2003; Sakai et al., 2003). Fibronectin is secreted by the lung epithelium as part of the basement membrane and has been shown to be a Wnt target gene in Xenopus (Gradal et al., 1999). Our results suggest that the decrease in FN deposition by the lung epithelium in response to DKK1 treatment (Fig. 5) is the primary cause for decreased branching of the epithelium (Fig. 4). Interestingly, the observed defect in branching seemed mainly to be associated with the ECM defect as no differences in epithelial proliferation could be found (data not shown), as well as no changes in the expression of key morphogenetic genes such as Fgf10, Shh, and Bmp4 could be detected (Fig. 7). Moreover, the branching defects induced by DKK1 could be recapitulated by treating lung explants with a blocking anti-FN antibody (Fig. 9) and could be rescued by co-treatment with exogenous FN (Fig. 10).

We also demonstrated that DKK1 treatment perturbed expression of α-SMA, a terminal marker of smooth muscle differentiation. This result also was associated with the decrease in FN expression, as treatment of lung explants with a blocking anti-FN antibody reduced α-SMA expression around the bronchi (Fig. 9). Previously, it has been shown that the differentiation of lung embryonic mesenchymal cells into SMC is triggered by their spreading along the airway basement membrane. Blocking of basement membrane assembly prevented mesenchymal cell spreading and smooth muscle differentiation (Yang et al., 1998). Further evidence for this was found when we studied α-SMA expression in DKK1 treated lung explants that were co-treated with exogenous FN. Treatment with exogenous FN led to an increase in α-SMA expression around the bronchi, which was even further expanded distally compared to control lung explants (Fig. 11), suggesting premature differentiation of the SMC.

Our results indicate that Wnt signaling is not likely to play a direct role in the differentiation of the distal mesenchyme into bronchial SMC, because TOPGAL activity could not be found in the mesenchyme adjacent to the lung epithelium.
to the distal epithelium from E11.5 to E14.5, which is the developmental time frame of our organ cultures. Rather, we hypothesize that blocking Wnt signaling in the distal epithelium results in a decrease of FN deposition by the epithelium. This would lead to a defective epithelial matrix and basement membrane, which is necessary for the...
spreading of the smooth muscle progenitors and their differentiation. Interestingly, increased deposition of FN into the subepithelial space of the airways is observed in all forms of asthma and occurs early in the progression of the disease (Hocking, 2002). Asthma is also characterized by an increase in the number of bronchial SMC. FN is capable of inducing α-SMA expression and promotes the differentiation of SMC in vitro (Thannickal et al., 2003).

Interestingly, no significant defect could be found in differentiation of the proximal SMC (evaluated by maintenance of α-SMA expression) that had already differentiated in E11.5 lungs before the start of DKK1 treatment. TOPGAL activity could be found in the proximal SMC until E12.5 in vivo (Figs. 1 and 6). This raises a question about the role of Wnt signaling in these proximal SMC. One possibility is that Wnt signaling is already switched off at E12.5 and that the presence of TOPGAL activity is due to the high stability of the β-galactosidase protein. This would indicate that Wnt signaling is directly involved in the differentiation of the proximal bronchial SMC. A second, less likely, possibility would be that the Wnt signaling in the proximal SMC is necessary for maintenance of their differentiation. However, we were not able to address this possibility because lungs isolated before E11.5 do not develop well in vitro. Deleting β-catenin specifically in the SMC in vivo should therefore be very informative.

Inhibition of canonical Wnt signaling further perturbed the proper formation of the lung vasculature, probably through the down regulation of FN deposition, as sprouting of blood vessels (angiogenesis) is mediated by α5β1 integrins through their binding to FN (Kim et al., 2000a,b, 2002). Indeed, similar defects in the lung vasculature were obtained after treatment of lung explants with a blocking anti-FN antibody (Fig. 8), and defects induced by treatment with DKK1 could partially be rescued by co-treating lung explants with exogenous FN (Fig. 8). We cannot exclude that there could also be a direct effect of Wnt signaling on the formation of the lung vasculature, since Wnt2a is needed for proper placental vascularization, although Wnt2a−/− mice did not have an abnormal lung phenotype (Monkley et al., 1996).

Possible functions for Wnt signaling in the epithelium

The exact functions of Wnt signaling are still controversial. The present consensus about Wnt signaling during development is that the Wnt pathway is critical for progenitor cell proliferation and/or lineage differentiation and is involved in maintaining progenitor cells in an undifferentiated state and possibly maintaining the differentiation of fully matured cells (Batlle et al., 2002; Huelsken et al., 2001; Merrill et al., 2001; Pinto et al., 2003; Reya et al., 2003, reviewed in Alonso and Fuchs, 2003).

β-catenin inactivation in the distal epithelium leads to a smaller proximalized lung. This indicates that Wnt signaling is probably not critical for the development of the proximal airway, but rather is necessary to prevent premature differentiation of the distal epithelium. Supporting this idea is that inactivating β-catenin in the proximal conducting airways...
using the CCSP promoter (which is active only from E14 onwards) does not perturb lung development (Mucenski et al., 2003). Expression of a constitutively active β-catenin–LEF1 fusion protein in transgenic embryo’s using the lung-endoderm-specific Sp-C promoter lead to an accumulation of proliferating epithelial cells that do not express morphological or molecular features of differentiated lung epithelial lineages. High levels of Wnt signaling in lung epithelium inhibited the terminal differentiation of pulmonary specific epithelial cell types, as judged by cell morphology and gene expression (Okubo and Hogan, 2004).

We hypothesize that the distal portion of the embryonic lung can be considered to be a reservoir of epithelial and mesenchymal progenitor cells. TOPGAL activation from E9.5 up to E12.5 in the most distal part of the lung suggests that Wnt signaling is instrumental to prevent premature differentiation.

Our results further indicate that canonical Wnt signaling is inhibited in the distal epithelium from E13.5 onwards. It is possible that this inhibition coincides with the entry of the progenitor cells into a differentiation pathway. The window of time between E10.5 and E14.5 is also critical for FGF signaling, to allow the survival and expansion of the precursor cells at the tip (Hokuto et al., 2003). It is therefore likely that perturbation of the FGF or Wnt pathway during this time frame will result in a reduction or loss of these progenitors, causing severe defects in subsequent lung development.

In conclusion, our results provide new insights into the function of Wnt signaling during early lung development. Further studies will be needed to elucidate the role of Wnt signaling during later stages of lung development and in tissue repair after injury.

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