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Localized *Fgf10* expression is not required for lung branching morphogenesis but prevents differentiation of epithelial progenitors

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SUMMARY

Localized *Fgf10* expression in the distal mesenchyme adjacent to sites of lung bud formation has long been thought to drive stereotypic branching morphogenesis even though isolated lung epithelium branches in the presence of non-directional exogenous Fgf10 in Matrigel. Here, we show that lung agenesis in *Fgf10* knockout mice can be rescued by ubiquitous overexpression of *Fgf10*, indicating that precisely localized *Fgf10* expression is not required for lung branching morphogenesis *in vivo*. *Fgf10* expression in the mesenchyme itself is regulated by Wnt signaling. Nevertheless, we found that during lung initiation simultaneous overexpression of *Fgf10* is not sufficient to rescue the absence of primary lung field specification in embryos overexpressing *Dkk1*, a secreted inhibitor of Wnt signaling. However, after lung initiation, simultaneous overexpression of *Fgf10* in lungs overexpressing *Dkk1* is able to rescue defects in branching and proximal-distal differentiation. We also show that Fgf10 prevents the differentiation of distal epithelial progenitors into Sox2-expressing airway epithelial cells in part by activating epithelial β-catenin signaling, which negatively regulates Sox2 expression. As such, these findings support a model in which the main function of Fgf10 during lung development is to regulate proximal-distal differentiation. As the lung buds grow out, proximal epithelial cells become further and further displaced from the distal source of Fgf10 and differentiate into bronchial epithelial cells. Interestingly, our data presented here show that once epithelial cells are committed to the Sox2-positive airway epithelial cell fate, Fgf10 prevents ciliated cell differentiation and promotes basal cell differentiation.

KEY WORDS: Basal cells, Branching, Dkk1, Fgf10, Lung development, Wnt signaling, Mouse

INTRODUCTION

Lung development in the mouse initiates at embryonic day (E)9-9.5 with the specification of the primary lung field and formation of lung buds, an outpocketing of endodermal respiratory progenitor cells from the ventral wall of the anterior foregut. *Nkx2.1* is one of the earliest markers of the lung endoderm and is expressed in the ventral foregut at E9.5 (Lazzaro et al., 1991; Kimura et al., 1996; Minoo et al., 1999). Wnt2a/b signaling in the foregut endoderm is required for *Nkx2.1* expression and maintenance of the respiratory fate (Monkley et al., 1996; Zakin et al., 1998; Goss et al., 2009; Harris-Johnson et al., 2009). Inactivation of *Ctnnb1* (which encodes β-catenin) in endoderm, or inactivation of *Wnt2a* and *Wnt2b*, which are expressed in the mesoderm, results in complete absence of lung and trachea development (Goss et al., 2009; Harris-Johnson et al., 2009). An important role for Wnt2a/b is to regulate *Fgf10* expression in the mesenchyme adjacent to the sites of lung bud formation (De Langhe et al., 2008; Yin et al., 2008; Goss et al., 2009; Goss et al., 2011). Interestingly, although knockout mice for *Fgf10* (*Fgf10*$^{-/-}$) or its receptor *Fgfr2b* (*Fgfr2b*$^{-/-}$) do not develop a lung, these mice do not exhibit a defect in the initial specification of the primary lung field as the trachea does develop in the absence of Fgf10 signaling (Bellusci et al., 1997; Min et al., 1998; Sekine et al., 1999; De Moerlooze et al., 2000; Weaver et al., 2000; Abler et al., 2009). However, following formation of the primary lung buds, Fgf10 is absolutely required for their initial outgrowth and survival of lung epithelial progenitors.

Retinoic acid (RA) signaling is also essential for lung bud formation (Desai et al., 2004; Desai et al., 2006) by indirectly regulating *Fgf10* expression through Wnt and Tgfβ signaling pathways. The Wnt antagonist *Dkk1* is expressed throughout the foregut, but is excluded from the primary lung fields where RA signaling functions to suppress *Dkk1* expression. This allows for increased Wnt2a/b signaling and upregulation of *Fgf10* expression (Chen et al., 2007; Chen et al., 2010). RA signaling also induces *Fgf10* expression by suppressing Tgfβ signaling in the lung mesenchyme (Chen et al., 2010).

Following primary lung bud outgrowth, lung development ensues by branching laterally and distally in a highly reproducible and repetitive pattern (Metzger et al., 2008). The specific molecules that pattern branching have not been identified. *Fgf10* is dynamically localized in the mesenchyme surrounding prospective epithelial buds and has been shown *in vitro* to act as a chemoattractant on nearby epithelial cells (Bellusci et al., 1997; Park et al., 1998; Weaver et al., 2000). These data have led to the current prevailing theory that directional bud outgrowth is dependent on the precisely localized expression of *Fgf10* in mesenchyme distal to the branch point, which regulates the formation of the initial domain branches and all subsequent branches (Bellusci et al., 1997; Warburton, 2008; Ornitz and Yin, 2012).

Fgf10 acts on the distal lung epithelium via its receptor Fgfr2b, which activates β-catenin signaling and prevents the distal epithelial

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progenitors from differentiating into airway epithelial cells by initially inhibiting Sox2 expression (Park et al., 1998; Que et al., 2007; Ramasamy et al., 2007; Nyeng et al., 2008; Hashimoto et al., 2012). β-Catenin is not only a downstream transcriptional target of epithelial Fgf10 signaling (Lü et al., 2005), but increasing data also indicate that Fgf10 is able to increase nuclear β-catenin directly, via phosphorylation of β-catenin on Ser552 and inhibition of Gsk3β, through the PI3K/AKT pathway (He et al., 2007; Ramasamy et al., 2007; Volckaert et al., 2011). In addition, FGF signaling via Erk/MAPK phosphorylates the Wnt co-receptor Lrp6 on Ser1490 and Thr1572 and phosphorylates β-catenin directly on Tyr142, thereby releasing it from cadherin complexes (Krejci et al., 2012). In turn, epithelial β-catenin activation participates in the induction of *Fgfr2b* expression to increase Fgf10 signaling further (Shu et al., 2005). Epithelial β-catenin signaling, mediated primarily through Fgf10 signaling, is a regulator of branching morphogenesis and functions to maintain the distal epithelial progenitor cells in an undifferentiated state by inhibiting Sox2 expression (Mucenski et al., 2003; De Langhe et al., 2005; Shu et al., 2005; Hashimoto et al., 2012).

Here, we show that lung agenesis in *Fgf10*$^{-/-}$ mice can be rescued by ubiquitous overexpression of *Fgf10*, suggesting that epithelial branching morphogenesis is not dependent on exact *Fgf10* localization. We also report that overexpression of the canonical Wnt inhibitor *Dkk1* from E8.0 onwards using *Rosa26-rtTa;Tet-Dkk1* mice prevents the specification of the primary lung field in the ventral foregut, confirming previous reports on the role of Wnt signaling in primary lung field specification (Cohen et al., 2009; Goss et al., 2009; Harris-Johnson et al., 2009; Chen et al., 2010). However, we found that specification of the primary lung field could not be rescued by simultaneous overexpression of *Fgf10* using *Rosa26-rtTa;Tet-Dkk1;Tet-Fgf10* mice.

Instead, our data show that after lung initiation, from E10.5 onwards, downregulation of *Fgf10* expression is the primary cause for decreased branching and proximalization of lungs overexpressing *Dkk1*, as simultaneous overexpression of *Fgf10* and *Dkk1* rescues these defects in branching and proximal-distal differentiation. We therefore propose a model in which localized expression of *Fgf10* is not required for stereotypic branching morphogenesis and suggest that Fgf10 signaling functions primarily as a permissive factor.

Interestingly, we found that in the trachea, *Fgf10* plays an additional role in directing the differentiation of Sox2-expressing cells into the basal cell lineage and that overexpression of *Fgf10* at later stages of lung development directs the differentiation of Sox2-expressing proximal airway epithelium to a basal cell fate while blocking the ciliated cell fate.

MATERIALS AND METHODS

Transgenic embryos

Tet-Dkk1 and *Rosa26-rtTa* mice were described previously (Volckaert et al., 2011). *Rosa26-rtTA* mice were crossed with *Tet-sFgfr2b* (Hokuto et al., 2003), *Tet-Fgf10* (Clark et al., 2001) and *Tet-Dkk1* mice to generate double transgenic embryos allowing for inducible expression by simply feeding pregnant females with doxycycline (dox)-containing food (rodent diet with 625 mg/kg doxycycline, Harlan Teklad TD.09761). TOPGAL mice were a generous gift from Dr Elaine Fuchs (DasGupta and Fuchs, 1999) and *Fgf10*^{LacZ} mice were a generous gift from Robert Kelly (Kelly et al., 2001).

β-Galactosidase staining

Embryos and embryonic lungs containing *TOPGAL* or *Fgf10*^{LacZ} alleles were stained as described previously (Volckaert et al., 2011).

Immunohistochemistry

All immunostaining was performed on paraffin sections of formalin-fixed lungs and embryos with the following primary antibodies: Nkx2.1 (1:200; Neomarkers), keratin 5 (1:200; Neomarkers), FoxA2 (1:2000; Seven Hills Bioreagents), Scgb1A1 (1:500; Seven Hills Bioreagents), Pro-Sftpc (1:500; Seven Hills Bioreagents), β-tubulin (1:500; Seven Hills Bioreagents), Sox2 (1:1000; Seven Hills Bioreagents), CGRP (1:5000; Sigma-Aldrich), Cy3-conjugated mouse anti-α-smooth muscle actin (1:200; Sigma-Aldrich), phospho-Histone-H3 (Ser10) (1:200; Cell Signaling Technology), P-β-catenin Ser552 (1:200; Cell Signaling Technology), P-Akt Ser473 (1:100; Cell Signaling Technology), fibronectin (1:500; a kind gift of Dr Kenneth Yamada, National Institute of Dental and Craniofacial Research, NIH, Bethesda, MD, USA), PDPN (1:50; Developmental Studies Hybridoma Bank), Scgb1A1 (1:200; Santa Cruz), p63 (1:200; Santa Cruz), Fgfr2 (1:200; Santa Cruz), CD31 (PECAM) (1:50; BD Biosciences), E-cadherin (1:200; BD Biosciences), Sox9 (1:100; Millipore). All fluorescent staining (except the Cy3-conjugated α-SMA) was performed with Cy3- and FITC-conjugated secondary antibodies from Jackson ImmunoResearch and mounted using Vectashield with DAPI (Vector Laboratories). Photographs were taken with a Zeiss AxioImager and Axiovision software.

Quantification of P-Akt Ser473- and P-β-catenin Ser552-positive epithelial cells

Epithelial P-Akt Ser473 quantification was performed by measuring mean gray value over mean epithelial surface area using ImageJ software. P-β-catenin Ser552 quantification was performed by determining the ratio of P-β-catenin Ser552-labeled epithelial cells over the total number of epithelial cells per field. The percentage of P-β-catenin Ser552-labeled cells is reported. Statistical analysis was performed using Student's *t*-test.

Quantitative real-time PCR

RNA was isolated from whole lungs or tracheas using RNALater (Ambion) and Total RNA Kit I (Omega Biotek) according to the manufacturer's instructions. RNA concentration was determined by spectrophotometry. cDNA was generated using SuperScript III First-Strand Synthesis System (Invitrogen) according to the manufacturer's instructions. Comparative real-time PCR was performed for β-glucuronidase (Mm00446953_m1), *Dkk1* (Mm00438422_m1), *Fgfr2b* (Mm01269938_m1), *Fgf10* (Mm01297079_m1), *Nmyc* (*Mycn*) (Mm00476449_m1), *Sftpc* (Mm00488144_m1), fibronectin (Mm01256744_m1), *Scgb1a1* (Mm00442046_m1), *Foxj1* (Mm00807215_m1), *Cgrp* (*Calca*) (Mm00801463_g1), *Acta2* (SMA) (Mm00725412_s1), *Spry2* (Mm00442344_m1), keratin 5 (*Krt5*) (Mm01305291_g1), *p63* (*Trp63*) (Mm00495788_m1) Taqman Gene Expression Assays (Applied Biosystems) using a StepOne Plus system (Applied Biosystems). β-Glucuronidase was used as a reference control to normalize equal loading of template cDNA.

In situ hybridization

Whole-mount *in situ* hybridization was performed as previously described (De Langhe et al., 2005). Sections from paraffin-embedded embryonic lungs were hybridized as previously described (De Langhe et al., 2008; De Langhe et al., 2005). The following mouse cDNAs were used as templates for the synthesis of digoxigenin-labeled riboprobes: a 584-bp fragment of *Fgf10* (Bellusci et al., 1997), a 948-bp full-length *Spry2* cDNA and a 162-bp fragment of *Fgfr2IIIb*.

Quantification of branching

Peripheral lung buds were counted and statistical analysis was performed using Student's *t*-test.

RESULTS

Fgf10 overexpression partially rescues lung and limb agenesis in *Fgf10*$^{-/-}$ mice

The importance of Fgf10 in lung morphogenesis has been illustrated by the drastic phenotype in mice lacking *Fgf10* or its receptor *Fgfr2b*. The trachea still develops in these mice, but the lung fails to form, revealing an essential function for Fgf10 signaling in lung formation (Min et al., 1998; Arman et al., 1999; Sekine et al., 1999; De Moerlooze et al., 2000). Similarly, the lack of limbs in these mice illustrates the important role of Fgf10 signaling in limb formation.

The current prevailing theory is that directional lung bud outgrowth is dependent on the precisely localized, directional expression of *Fgf10* in the lung mesenchyme distal to the branch point, which regulates the formation of the initial domain branches and all subsequent branches (Warburton, 2008; Ornitz and Yin, 2012). However, the observation that isolated lung epithelium branches when grown in Matrigel in the presence of recombinant Fgf10 (Bellusci et al., 1997; Park et al., 1998; Ohtsuka et al., 2001) contradicts this model for branching morphogenesis.

To investigate the importance of localized *Fgf10* expression for branching morphogenesis *in vivo*, we generated *Fgf10*$^{-/-}$*;Rosa26-rtTa;Tet-Fgf10* mice in which we can induce *Fgf10* expression ubiquitously. Although *Fgf10*$^{-/-}$ mice have no limbs (Fig. 1C) and show complete lung agenesis (Fig. 1F), ubiquitous overexpression of *Fgf10* in a *Fgf10*$^{-/-}$ background from E9.5 onwards rescued lung development with a close to normal lobulation and epithelial branching pattern (Fig. 1E) compared with wild-type lungs (Fig. 1D). In addition, our data demonstrate that ubiquitous overexpression of *Fgf10* partially rescues limb formation, with budding of both front and hind limbs at the correct position, similar to control embryos (Fig. 1A,B). This is interesting considering the observation that in chicken ectopic Fgf10 signaling results in the formation of ectopic limbs (Ohuchi et al., 1997).

To determine whether the ubiquitously overexpressed *Fgf10* in our system is not post-transcriptionally regulated to allow localized expression and branching morphogenesis to occur, we performed *in situ* hybridization for *Fgf10* on E12.5 wild-type and *Fgf10*$^{-/-}$*;Rosa26-rtTa;Tet-Fgf10*-rescued lung sections. Fig. 1G illustrates that in E12.5 wild-type lungs, *Fgf10* expression is limited to the distal mesenchyme whereas low ubiquitous levels of *Fgf10* expression can be found throughout *Fgf10*$^{-/-}$*;Rosa26-rtTa;Tet-Fgf10* lungs in both the mesenchyme and epithelium (Fig. 1H). We found that low levels of *Fgf10* were essential for proper lung development whereas higher levels perturbed the branching pattern. Thus, to demonstrate that *Fgf10* is indeed ubiquitously expressed in our model, we also performed whole-mount *in situ* hybridization for *Fgf10* on E13.5 wild-type (wt) (Fig. 1I) and *Rosa26-rtTa;Tet-Fgf10* (Fig. 1L) lungs induced from E10.5. To further show that Fgf10 protein does not act locally in *Fgf10*$^{-/-}$*;Rosa26-rtTa;Tet-Fgf10*-rescued lungs, we performed *in situ* hybridization for *Spry2* expression. In the wt lung, *Spry2* expression is regulated by Fgf10 and is restricted to the epithelium of the distal branching tips (Fig. 1J), whereas in *Fgf10*$^{-/-}$*;Rosa26-rtTa;Tet-Fgf10*-rescued lungs *Spry2* is expressed at low levels throughout the entire lung epithelium (Fig. 1K).

In contrast to the current dogma, which ascribes a pivotal role to localized *Fgf10* expression in directing lung epithelial bud and limb outgrowth, our data reveal that the spatial distribution of *Fgf10* is less important for the process of epithelial bud outgrowth and stereotypic branching than was previously thought. Therefore, we speculate that localized *Fgf10* expression might be important primarily for proximal-distal patterning by keeping the distal epithelium in an undifferentiated state.

Dkk1 overexpression effectively abrogates the initiation of the respiratory lineage, which cannot be rescued by simultaneous overexpression of *Fgf10*

Wnt/β-catenin signaling has proven to be essential in specifying lung progenitor cells in the developing foregut (Goss et al., 2009; Harris-Johnson et al., 2009). Recently, an elegantly performed series of foregut explant experiments revealed that RA is a major regulator of Wnt and Tgfβ pathways during the formation of the lung primordium (Chen et al., 2010). The authors proposed a mechanism through which RA activates canonical Wnt signaling by inhibiting its negative regulator *Dkk1*. Dkk1 binds to the Wnt co-receptor Lrp5/6, making it inaccessible to Wnt ligands (Bao et al., 2012). Previous experimental set-ups have shown that Dkk1 effectively inhibits Wnt/β-catenin signaling in the lung (De Langhe et al., 2005; Shu et al., 2005; Chen et al., 2010).

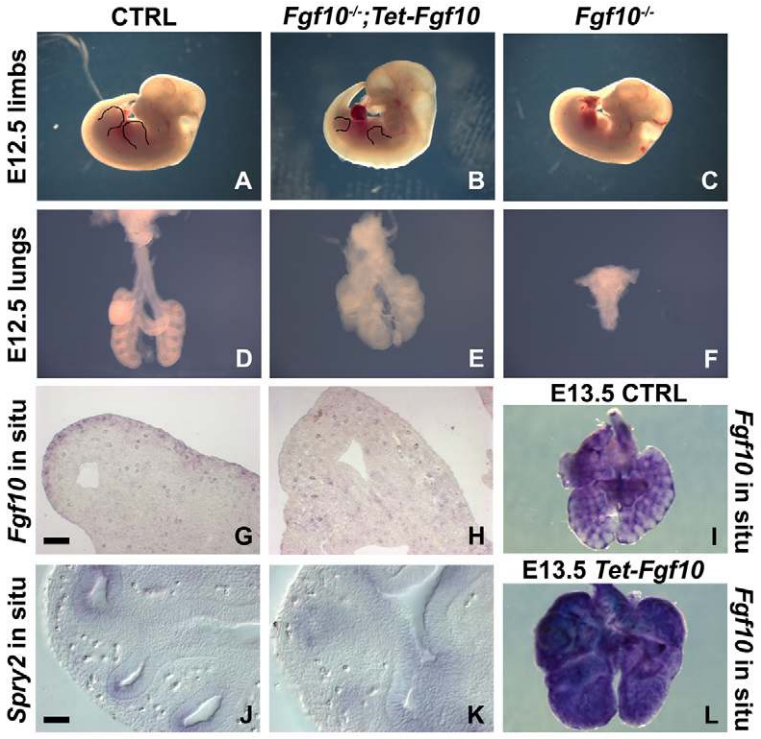

Fig. 1. *Fgf10* overexpression partially rescues lung and limb agenesis in *Fgf10*$^{-/-}$ mice. (**A-C**) E12.5 wild-type (A), *Fgf10*$^{-/-}$*;Rosa26-rtTA;Tet-Fgf10* dox-induced at E9.5 (B) and *Fgf10*$^{-/-}$ (C) embryos. Black outlines visualize front and hind limbs. (**D-F**) E12.5 lungs from wild-type (D), *Fgf10*$^{-/-}$*;Rosa26-rtTA;Tet-Fgf10* dox-induced at E9.5 (E) and *Fgf10*$^{-/-}$ (F) mice. (**G**,**H**) *Fgf10 in situ* hybridization on sections from E12.5 wild-type lung (G) and *Fgf10*$^{-/-}$*;Rosa26-rtTA;Tet-Fgf10* lung dox-induced at E9.5 (H). (**J**,**K**) Vibratome sections from whole-mount *Spry2 in situ* hybridization on E12.5 wild-type lung (J) and *Fgf10*$^{-/-}$*;Rosa26-rtTA;Tet-Fgf10* lung dox-induced at E9.5 (K). (**I**,**L**) Whole-mount *Fgf10 in situ* hybridization on E13.5 wild-type lungs (I) and *Rosa26-rtTA;Tet-Fgf10* lungs dox-induced at E10.5 (L). $n \geq 3$. Scale bars: 50 µm (G,H,J,K).

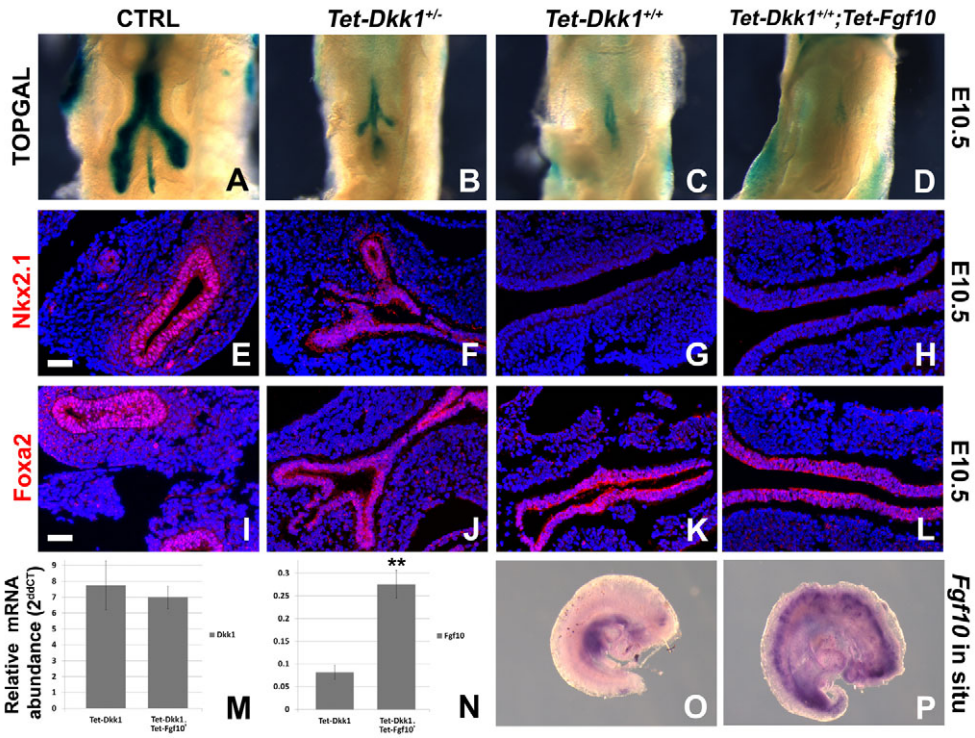

Fig. 2. *Dkk1* overexpression abrogates initial lung formation. (**A-D**) β-Gal staining on E10.5 control (ctrl) *TOPGAL* (A), *Rosa26-rtTA;Tet-Dkk1*$^{+/-}$*;TOPGAL* (B), *Rosa26-rtTA;Tet-Dkk1*$^{+/+}$*;TOPGAL* (C) and *Rosa26-rtTA;Tet-Dkk1*$^{+/+}$*;Tet-Fgf10;TOPGAL* (D) lungs/foreguts dox-induced at E8.0. (**E-H**) Nkx2.1 immunostaining on E10.5 ctrl (E), *Rosa26-rtTA;Tet-Dkk1*$^{+/-}$ (F), *Rosa26-rtTA;Tet-Dkk1*$^{+/+}$ (G) and *Rosa26-rtTA;Tet-Dkk1*$^{+/+}$*;Tet-Fgf10* (H) lungs/foreguts dox-induced at E8.0. (**I-L**) Foxa2 immunostaining on E10.5 ctrl (I), *Rosa26-rtTA;Tet-Dkk1*$^{+/-}$ (J), *Rosa26-rtTA;Tet-Dkk1*$^{+/+}$ (K) and *Rosa26-rtTA;Tet-Dkk1*$^{+/+}$*;Tet-Fgf10* (L) lungs/foreguts dox-induced at E8.0. (**M**,**N**) qPCR analysis of relative *Dkk1* (M) and *Fgf10* (N) mRNA abundance on E10.5 *Rosa26-rtTA;Tet-Dkk1*$^{+/+}$ and *Rosa26-rtTA;Tet-Dkk1*$^{+/+}$*;Tet-Fgf10* embryos dox-induced at E8.0. $^{**}P<0.01$ (Student's *t*-test); $n \geq 3$. Error bars represent standard error. (**O**,**P**) Whole-mount *Fgf10 in situ* hybridization on an E10.5 *Rosa26-rtTA;Tet-Dkk1*$^{+/+}$ embryo (O) and a *Rosa26-rtTA;Tet-Dkk1*$^{+/+}$*;Tet-Fgf10* embryo dox-induced from E10.5 onwards (P). $n \geq 3$. Scale bars: 50 μm (E-L).

Based on this model, we hypothesized that ubiquitous overexpression of *Dkk1* would override the effect of endogenous RA *in vivo* and result in failure of initiation of the respiratory lineage. Indeed, by analyzing expression of Nkx2.1, the earliest known marker for both lung and trachea (Kimura et al., 1996), and Foxa2, an endoderm marker (Ang et al., 1993), we found that overexpression of *Dkk1* prior to lung initiation affects primary lung field specification in a dose-dependent manner (Fig. 2E-G,I-K). Mice homozygous for *Dkk1* fail to specify the primary lung field illustrated by the lack of Nkx2.1 expression (Fig. 2G) and formation of lung branches (Fig. 2C,G,K), whereas heterozygous mice show proper specification of the primary lung field (Fig. 2F) but form smaller lung branches (Fig. 2B,F,J) compared with control lungs (Fig. 2A,E,I).

Through regulation of Wnt2a/b and Tgfβ signaling, RA also regulates mesenchymal *Fgf10* expression around this stage of lung development (Chen et al., 2010). However, as specification of respiratory lineages in the foregut does occur in *Fgf10*$^{-/-}$ mice, Fgf10 is not thought to be essential for the initial specification of respiratory progenitors (Sekine et al., 1999). To investigate whether Fgf10 could be the main driver of β-catenin signaling in the epithelium at this early stage and as such could rescue lung agenesis, we simultaneously overexpressed *Fgf10* and *Dkk1 in vivo* from E8.0 onwards using *Rosa26-rtTa;Tet-Dkk1;Tet-Fgf10* mice (Fig. 2D,H,L-P). However, we found that lung formation was still abrogated in these embryos (Fig. 2D,H,L). This indicates that even though Fgf10 can activate/enhance epithelial β-catenin signaling at later stages of lung development (see below), Fgf10 alone is not sufficient to specify the lung progenitors in the developing foregut in the absence of Wnt ligand-mediated epithelial β-catenin signaling. Our findings, obtained using a novel model of Wnt signaling inhibition, emphasize the importance of canonical Wnt2a/b signaling in the initiation and specification of the respiratory cell lineage (Goss et al., 2009).

Dkk1 overexpression reduces *Fgf10* expression and prevents amplification of a pool of *Fgf10*-expressing parabronchial smooth muscle progenitor cells

The distal lung comprises two pools of mesenchymal cell populations: sub-epithelial and sub-mesothelial (White et al., 2006). Whereas the former responds to epithelial-derived Shh, which inhibits *Fgf10* expression (Pepicelli et al., 1998), we have previously shown that the sub-mesothelial cells are parabronchial smooth muscle cell (PSMC) progenitors (Mailleux et al., 2005) expressing high levels of *Fgf10*. Through mesenchymal-specific deletion of *Ctnnb1* (the gene encoding β-catenin), we previously showed that Wnt/β-catenin signaling regulates *Fgf10* expression and controls the amplification of these PSMC progenitors (De Langhe et al., 2008). Furthermore, we have previously shown that treatment of embryonic lung explants with Dkk1 prevents the differentiation of PSMCs (De Langhe et al., 2005). Since then, a specific role for Wnt ligands Wnt7b and Wnt2a

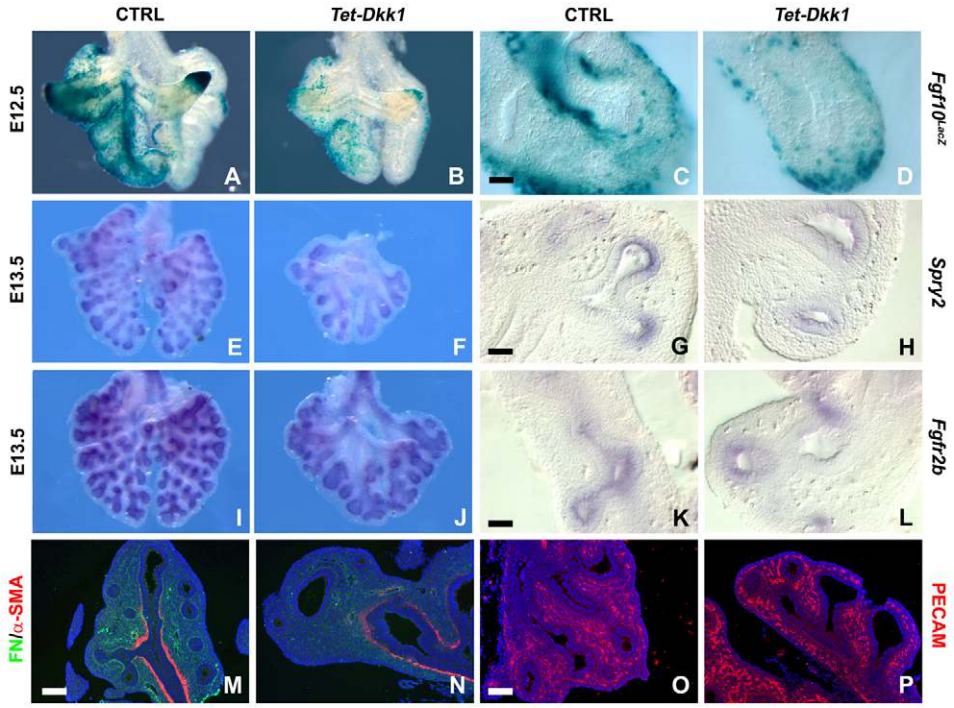

Fig. 3. *Dkk1* overexpression reduces *Fgf10* expression and prevents amplification of distal parabronchial smooth muscle cell progenitors. (**A-D**) β-Gal-stained lungs (A,B) and corresponding vibratome sections through the medial lobes (C,D) of E12.5 control (ctrl) *Fgf10*^{LacZ} (A,C) and *Rosa26-rtTA;Tet-Dkk1;Fgf10*^{LacZ} lungs (B,D) dox-induced at E10.5. (**E-L**) Whole-mount *in situ* hybridization (E,F,I,J) and corresponding vibratome sections (G,H,K,L) for *Spry2* (E-H) and *Fgfr2b* (I-L) on E13.5 ctrl (E,G,I,K) and *Rosa26-rtTA;Tet-Dkk1* lungs (F,H,J,L) dox-induced at E10.5. (**M**,**N**) Immunostaining for fibronectin (FN) and α-SMA on E13.5 ctrl (M) and *Rosa26-rtTA;Tet-Dkk1* (N) lungs dox-induced at E10.5. (**O**,**P**) Immunostaining for Pecam on E13.5 ctrl (O) and *Rosa26-rtTA;Tet-Dkk1* (P) lungs dox-induced at E10.5. *n*≥3. Scale bars: 50 μm (C,D,G,H,K,L); 100 μm (M-P).

in airway smooth muscle precursor development and *Fgf10* expression has been described (Rajagopal et al., 2008; Cohen et al., 2009; Goss et al., 2011).

To broaden our understanding of the role of canonical Wnt signaling and its interplay with Fgf10 during early branching morphogenesis, we induced *Rosa26-rtTa;Tet-Dkk1* mice from E10.5 onwards. At this stage, the lung is composed of the trachea and two growing lung buds. Using *Fgf10*$^{LacZ+}$ reporter mice, which express *lacZ* under the transcriptional control of *Fgf10* regulatory sequences, we confirmed that *Fgf10* expression and the pool of PSMC progenitors in the distal mesenchyme is significantly reduced in E12.5 *Rosa26-rtTa;Tet-Dkk1;Fgf10*$^{LacZ+}$ lungs (Fig. 3A,B; Fig. 4K) and that fewer β-galactosidase (β-gal)-positive PSMCs could be found around the bronchi (Fig. 3A-D). This also resulted in decreased epithelial *Fgf10* signaling in *Rosa26-rtTa;Tet-Dkk1* lungs, demonstrated by reduced and more 'distalized' epithelial expression of *Spry2* (Fig. 3E-H; Fig. 4K) and *Fgfr2b* (Fig. 3I-L; Fig. 4K), two Fgf10 target genes (Mailleux et al., 2001; Shu et al., 2005). The reduction of PSMC progenitors in lungs overexpressing *Dkk1* resulted in a discontinuous, thinner PSMC layer (Fig. 3N; Fig. 4K) compared with control lungs (Fig. 3M; Fig. 4K). Our findings recapitulate previously reported defects in PSMC formation after inhibition of mesenchymal β-catenin signaling (De Langhe et al., 2005; De Langhe et al., 2008; Cohen et al., 2009; Goss et al., 2011). Interestingly, endothelial cell differentiation visualized by Pecam immunostaining is normal in *Rosa26-rtTa;Tet-Dkk1* lungs (Fig. 3O,P), in contrast to what we observed after mesenchymal-specific deletion of *Ctnnb1* (De Langhe et al., 2008). This is possibly due to the fact that Dkk1 does not inhibit Fgf9-mediated activation of β-catenin signaling (Yin et al., 2008; Yin et al., 2011). Lastly, we found that fibronectin (FN) deposition was reduced in lungs overexpressing *Dkk1* (Fig. 3N; Fig. 4K) compared with control lungs (Fig. 3M; Fig. 4K), confirming our previous observation in lung explants grown in the presence of Dkk1 (De Langhe et al., 2005).

Fgf10 positively regulates β-catenin signaling through activation of Akt and prevents differentiation of the distal epithelial progenitors

Fgf10 has been shown to regulate epithelial β-catenin signaling during lung development as well as in the adult lung (Lü et al., 2005; Ramasamy et al., 2007; Volckaert et al., 2011). We therefore examined the status of epithelial Wnt/β-catenin signaling in lungs overexpressing *Fgf10*. To do this, we introduced the TOPGAL reporter allele into *Rosa26-rtTa;Tet-Fgf10* mice and analyzed TOPGAL activity by β-gal staining. E13.5 *Rosa26-rtTa;Tet-Fgf10;TOPGAL* lungs induced from E10.5 showed a notable increase in epithelial TOPGAL activity compared with control TOPGAL lungs (Fig. 4A,B). In addition, we quantified activation of epithelial Wnt signaling by quantitative real-time PCR (qPCR) for *Nmyc*, an epithelial-specific Wnt target gene (Fig. 4L) (Shu et al., 2005), confirming our TOPGAL readout.

Fgf10 is thought to directly activate β-catenin signaling by activating the PI3K-AKT pathway (Volckaert et al., 2011). Phosphorylated Akt (P-Akt) acts to prevent the degradation of β-catenin by inhibiting its negative regulator Gsk3β and to help drive β-catenin to the nucleus by phosphorylating it directly on Ser552 (He et al., 2007). To elucidate whether Fgf10 directly activates β-catenin signaling through this mechanism, we performed immunostaining for P-Akt or P-β-cat-Ser552 together with E-cadherin (cadherin 1), to outline the epithelium on E13.5 control and *Rosa26-rtTa;Tet-Fgf10* lungs induced from E10.5 (Fig. 4C-F). We found a 17±1.2% (mean±standard error; *n*≥4; *P*=0.017) increase in P-Akt phosphorylation and a 167±34% (*n*≥4; *P*=0.000021) increase in β-cat-Ser552 phosphorylation in the epithelium of lungs overexpressing *Fgf10* versus wild-type lungs. Our results therefore indicate that Fgf10 signaling activates epithelial β-catenin signaling by activating Akt.

Proper lung morphogenesis is dependent on a tightly regulated balance between progenitor cell expansion and differentiation. This is reflected in the temporal and spatial expression of multiple genes

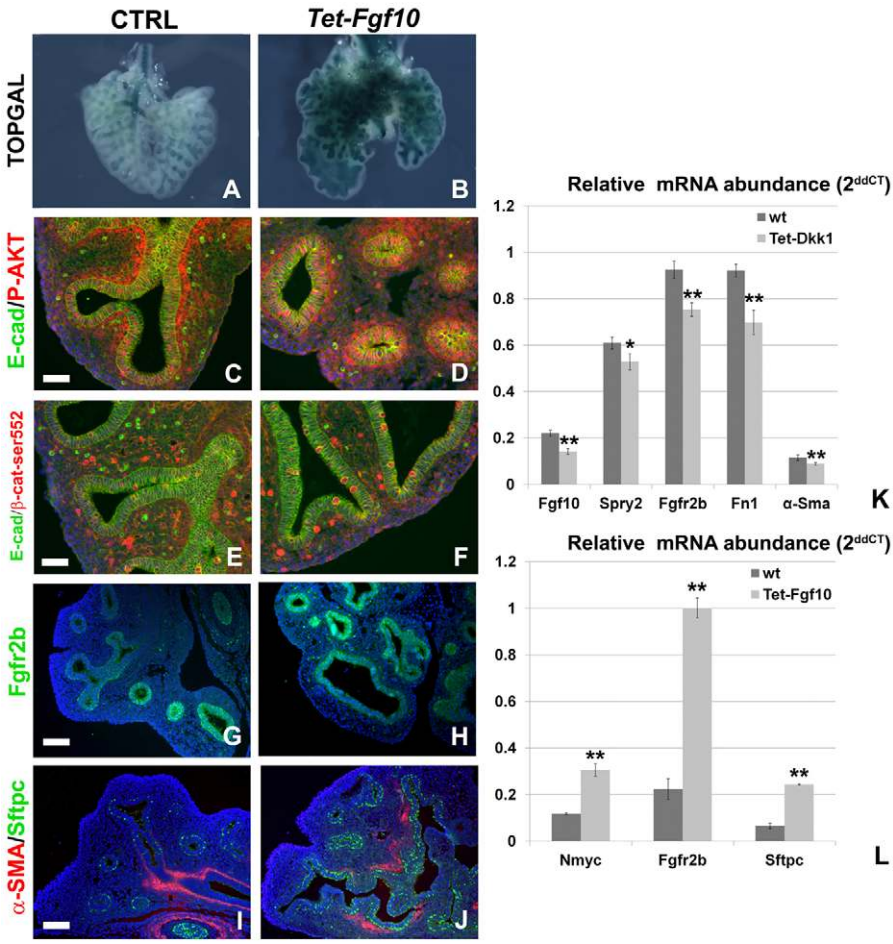

Fig. 4. Fgf10 signaling prevents differentiation of distal epithelial progenitors by positively regulating β-catenin signaling. (**A**,**B**) β-Gal staining on E13.5 control (ctrl) TOPGAL (A) and *Rosa26-rtTA;Tet-Fgf10;TOPGAL* (B) lungs dox-induced at E10.5. (**C-F**) Immunostaining for E-cadherin and p-Akt-Ser473 (C,D) or β-catenin-Ser552 (E,F) on E13.5 ctrl (C,E) and *Rosa26-rtTA;Tet-Fgf10* (D,F) lungs dox-induced at E10.5. (**G**,**H**) Immunostaining for Fgfr2b on E13.5 ctrl (G) and *Rosa26-rtTA;Tet-Fgf10* (H) lungs dox-induced at E10.5. (**I**,**J**) Immunostaining for α-SMA and Sftpc on E13.5 ctrl (I) and *Rosa26-rtTA;Tet-Fgf10* (J) lungs dox-induced at E10.5. (**K**) qPCR analysis of relative *Fgf10*, *Spry2*, *Fgfr2b*, *α-Sma* (*Acta2*) and *Fn1* mRNA abundance on E11.5 (*Fgf10*, *Spry2*) or E12.5 (*Fgfr2b*, *α-Sma*, *Fn1*) ctrl and *Rosa26-rtTA;Tet-Dkk1*$^{+/+}$ lungs dox-induced at E10.5. ***P*<0.01, **P*<0.05 (Student's *t*-test); *n*≥3. (To allow for better visualization, *Fn1* expression levels presented in the graph are reduced by a factor of 2). (**L**) qPCR analysis of relative *Nmyc*, *Fgfr2b* and *Sftpc* mRNA abundance on E12.5 ctrl and *Rosa26-rtTA;Tet-Fgf10* lungs dox-induced at E10.5. ***P*<0.01 (Student's *t*-test); *n*≥3. Error bars represent standard error. Scale bars: 50 μm (C-F); 100 μm (G,H); 75 μm (I,J).

involved in proximal-distal patterning in the developing lung, including Wnts, *Shh*, *Nmyc*, *Fgf10* and *Bmp4*. Consequently, perturbations in these signaling systems can shift the balance either towards maintaining a distal population of undifferentiated, proliferating progenitor cells or towards differentiation into conducting airway epithelial cells (Rawlins et al., 2009a; Domyan and Sun, 2011).

Previous work has revealed an important function for Wnt/β-catenin signaling in the regulation of proximal-distal differentiation in the developing airway epithelium (Mucenski et al., 2003; Okubo and Hogan, 2004; De Langhe et al., 2005; Shu et al., 2005; Hashimoto et al., 2012). This led to a model in which Wnt/β-catenin signaling is crucial in maintaining the reservoir of epithelial and mesenchymal progenitor cells residing in the distal tips of the embryonic lung. Loss of Wnt signaling can therefore shift the balance from progenitor cell maintenance to differentiation. Our data show that ubiquitous overexpression of *Fgf10* from E10.5 to E13.5, possibly by increasing epithelial β-catenin signaling, prevents the differentiation of distal epithelial progenitors, which is illustrated by the maintenance of Fgfr2b (Fig. 4G,H,L) and Sftpc (Fig. 4I,J,L) in more proximal epithelium.

Downregulation of *Fgf10* expression is the primary cause for decreased branching and proximalization of lungs overexpressing *Dkk1*

To examine the status of epithelial Wnt/β-catenin signaling in lungs overexpressing *Dkk1*, we crossed the TOPGAL reporter allele with the *Rosa26-rtTa;Tet-Dkk1* line and analyzed β-catenin signaling activity by β-gal staining. E11.5 *Rosa26-rtTa;Tet-Dkk1;TOPGAL* lungs induced from E10.5 showed a notable reduction in epithelial TOPGAL activity compared with control TOPGAL lungs (Fig. 5A,B). To analyze whether the reduction in Fgf10 signaling is the primary reason for reduced epithelial β-catenin signaling in lungs overexpressing *Dkk1*, we simultaneously overexpressed *Fgf10* and found that epithelial Wnt/β-catenin signaling was rescued (Fig. 5A-C). Activation of epithelial Wnt signaling in these different lungs was further quantified by qPCR for *Nmyc*, an epithelial-specific Wnt target gene (Fig. 5D) (Shu et al., 2005), confirming our TOPGAL readout. Together, our data indicate that Fgf10 is able to activate epithelial Wnt signaling, probably independently of Wnt ligands or enhanced Wnt-mediated signaling, a mechanism that is also observed in the adult lung airway epithelium after injury (Volckaert et al., 2011).

We found that decreased *Fgf10* expression is the primary cause for decreased branching and the premature differentiation of distal Sox9-expressing epithelial progenitors into Sox2-expressing conducting airway epithelial cells in lungs overexpressing *Dkk1* as both branching and proximal-distal differentiation could be rescued by simultaneous overexpression of *Fgf10* (Fig. 5E-G,I-K). As such, branching in E11.5 lungs overexpressing *Dkk1* was reduced by 25±2.2% (*n*≥4; *P*=0.00004) compared with wild-type lungs, whereas simultaneous overexpression of *Fgf10* with *Dkk1* partially rescued branching morphogenesis by increasing branching by 12±0.8% (*n*≥4; *P*=0.008) compared with lungs overexpressing *Dkk1* alone.

These results are in line with a model in which Fgf10 signaling localized distally, prevents the differentiation of the distal epithelial

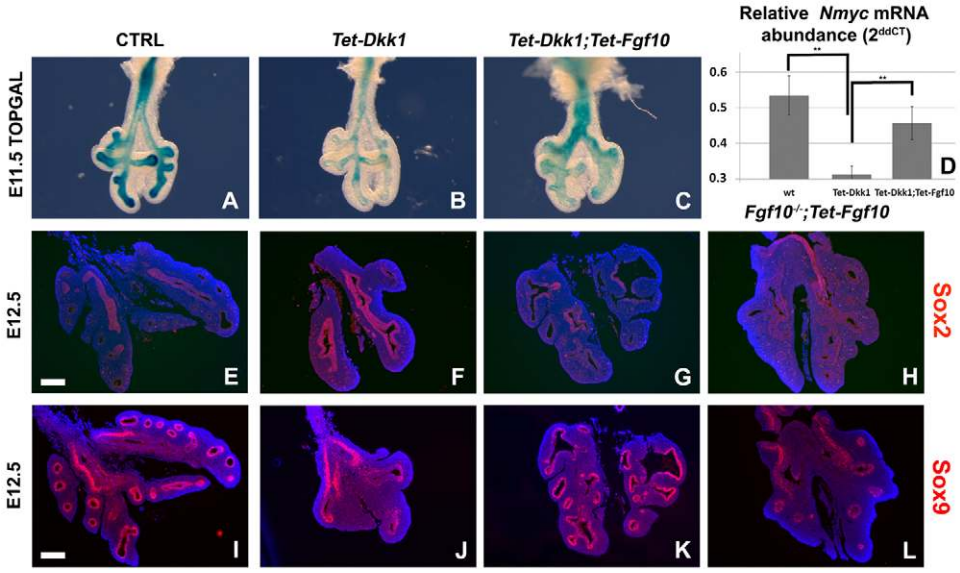

Fig. 5. Reduced epithelial Wnt signaling in lungs overexpressing *Dkk1* results in proximal-distal differentiation defects that can be rescued by overexpressing *Fgf10*. (**A-C**) β-Gal staining on E11.5 control (ctrl) TOPGAL (A), *Rosa26-rtTA;Tet-Dkk1;TOPGAL* (B) *Rosa26-rtTA;Tet-Dkk1;Tet-Fgf10;TOPGAL* (C) lungs dox-induced at E10.5. (**D**) qPCR analysis of relative *Nmyc* mRNA abundance on E11.5 ctrl, *Rosa26-rtTA;Tet-Dkk1*$^{+/+}$ and *Rosa26-rtTA;Tet-Dkk1*$^{+/+}$*;Tet-Fgf10* lungs dox-induced at E10.5. ***P*<0.01 (Student's *t*-test); *n*≥3. Error bars represent standard error. (**E-L**) Immunostaining for Sox2 (E-H) and Sox9 (I-L) on E12.5 ctrl (E,I), *Rosa26-rtTA;Tet-Dkk1* (F,J), *Rosa26-rtTA;Tet-Dkk1;Tet-Fgf10* (G,K) and *Fgf10*$^{-/-}$*;Rosa26-rtTA;Tet-Fgf10* (H,L) lungs dox-induced at E10.5. *n*≥3. Scale bars: 200 μm (E-L).

progenitors into airway epithelial cells by activating epithelial β-catenin signaling and inhibiting Sox2 expression (Hashimoto et al., 2012). Interestingly, a proximal-distal axis of differentiation is still present in *Fgf10*$^{-/-}$ lungs rescued by overexpression of *Fgf10* ubiquitously (Fig. 5H,L), indicating that other factors besides *Fgf10* play a role in distal epithelial progenitor maintenance.

Fgf10 inhibits Sox2 expression and proximal epithelial differentiation prior to E12.5 but promotes basal cell differentiation/amplification afterwards

We next determined the exact window of opportunity for Fgf10 to inhibit *Sox2* expression and thus prevent the differentiation of distal epithelial progenitors into airway epithelial cells. To do this, we induced *Rosa26-rtTa;Tet-Fgf10* lungs from either E11.5, E12.5 or E13.5 until E18.5 and monitored Sox2 (proximal epithelium), Sox9 (distal epithelial progenitors), p63 and K5 (basal cells), Scgb1a1 (Clara cells) and Sftpc [alveolar type II (ATII) or distal epithelial progenitor cells] markers of differentiation. We found that only overexpression of *Fgf10* from E11.5 onwards was able to prevent the differentiation of almost all distal Sox9-expressing epithelial progenitors into Sox2-expressing airway epithelial cells (Fig. 6B,F,J,N). Overexpression of *Fgf10* from E12.5 onwards did not prevent the differentiation of all distal epithelial progenitors as some had already differentiated into Sox2-expressing airway epithelial cells by this time (Fig. 6C,G,K,O). *Fgf10* overexpression

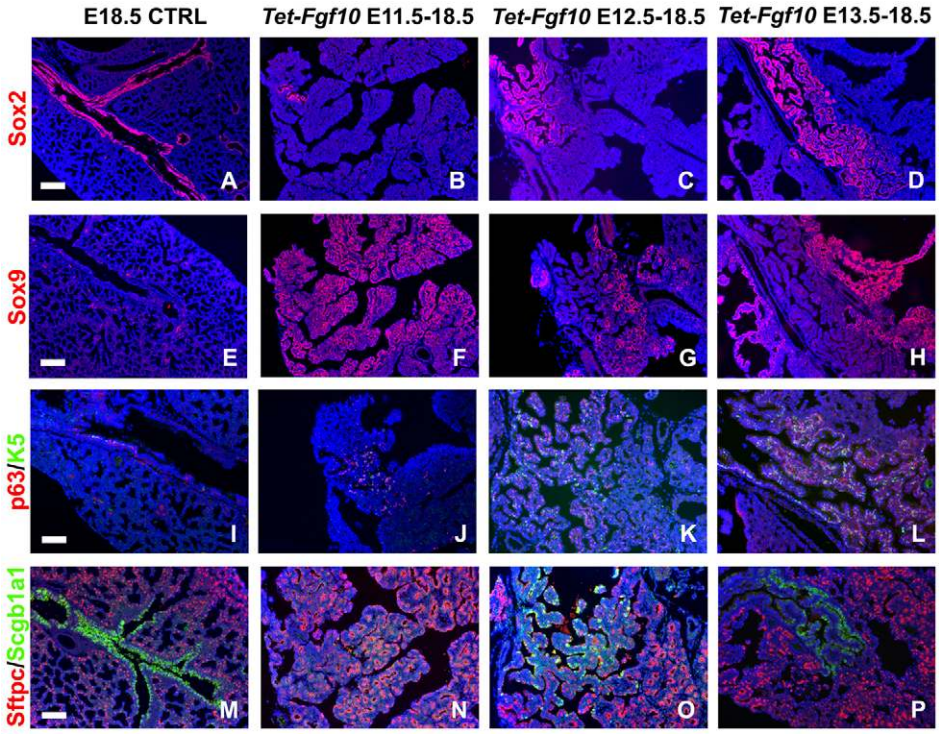

Fig. 6. Early *Fgf10* overexpression prevents proximal differentiation whereas *Fgf10* overexpression after E12.5 promotes basal cell differentiation. (**A-D**) Immunostaining for Sox2 on E18.5 control (ctrl) (A) and *Rosa26-rtTA;Tet-Fgf10* lungs induced from E11.5 (B), E12.5 (C) and E13.5 (D). (**E-H**) Immunostaining for Sox9 on E18.5 ctrl (E) and *Rosa26-rtTA;Tet-Fgf10* lungs induced from E11.5 (F), E12.5 (G) and E13.5 (H). (**I-L**) Immunostaining for p63 and K5 on E18.5 ctrl (I) and *Rosa26-rtTA;Tet-Fgf10* lungs induced from E11.5 (J), E12.5 (K) and E13.5 (L). (**M-P**) Immunostaining for Sftpc and Scgb1a1 on E18.5 ctrl (M) and *Rosa26-rtTA;Tet-Fgf10* lungs induced from E11.5 (N), E12.5 (O) and E13.5 (P). *n*≥3. Scale bars: 200 μm (A-H); 100 μm (I-P).

from E12.5 or E13.5 onwards did, however, prevent further differentiation of the remaining distal Sox9-expressing epithelial progenitors. This was evidenced by the persistent expression of Sox9 and Sftpc in distal epithelial cells in these lungs at E18.5 (Fig. 6G,H,O,P) as opposed to control lungs in which the distal epithelium had differentiated into ATII cells expressing Sftpc but not Sox9 (Fig. 6E,M). Interestingly, *Fgf10* overexpression from E12.5 or E13.5 onwards also had an unexpected effect on Sox2-expressing airway epithelial cells, preventing neuroendocrine and ciliated cell differentiation (data not shown) and promoting differentiation of a subset of Sox2-positive cells into p63-positive basal cells (Fig. 6K,L). In addition, Fgf10 induced proliferation in Clara cells and ectopic basal cells (supplementary material Fig. S1B,C,E,F). Interestingly, we found that Fgf10 also induced expression of p63 in some Sox9-positive cells at the transition zone between Sox2- and Sox9-positive cells, suggesting that a subset of Sox9-positive cells can become basal cells or that at the transition zone some cells are triple positive, expressing Sox2, Sox9 and p63 simultaneously (supplementary material Fig. S1A,D).

Opposing and similar effects of Dkk1 and Fgf10 on lung epithelial differentiation

Rosa26-rtTa;Tet-Dkk1 mice induced from E10.5 survive to E18.5 and demonstrate a more drastic phenotype than previously reported for *Sftpc-rtTa;Tet-Dkk1* mice (Shu et al., 2005). This is likely to be due to higher and more ubiquitous levels of *Dkk1* expression. E18.5 *Rosa26-rtTa;Tet-Dkk1* lungs are dramatically 'proximalized' with the formation of very few primitive alveolar sacs, containing few alveolar type I (ATI) (Pdpn+) and II (ATII) (Sftpc+) cells (Fig. 7B,E) compared with control lungs (Fig. 7A,D). By contrast, induction of *Fgf10* expression from E15.5 onwards prevented the conclusion of the pseudoglandular stage in *Rosa26-rtTa;Tet-Fgf10* lungs (Fig. 7C,F). As such, *Rosa26-rtTa;Tet-Fgf10* lungs induced from E15.5 failed to transition out of the branching or pseudoglandular stage into the canalicular and saccular stages. However, differentiation of ATI cells did occur based on podoplanin (Pdpn; also known as T1α) expression (Fig. 7F).

We next investigated the effects of *Dkk1* and late-stage *Fgf10* overexpression on the differentiation of conducting airway epithelial cells. We found that overexpression of *Dkk1* resulted in smaller

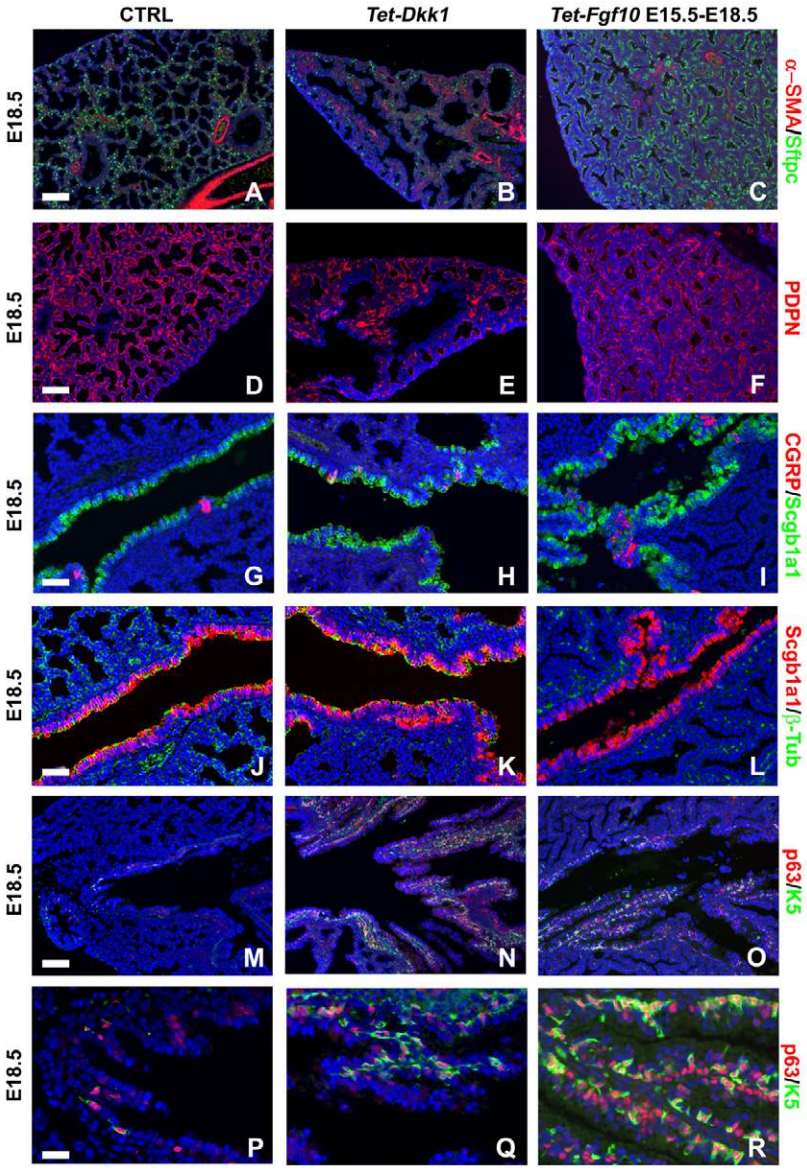

Fig. 7. Opposing and similar effects of Dkk1 and Fgf10 on lung epithelial differentiation. (**A-F**) Immunostaining for α-SMA and Sftpc (A-C) or for Pdpn (D-F), on E18.5 control (ctrl) lungs (A,D), E18.5 *Rosa26-rtTA;Tet-Dkk1* lungs dox-induced from E10.5 onwards (B,E) and E18.5 *Rosa26-rtTA;Tet-Fgf10* (C,F) lungs dox-induced from E15.5 onwards. (**G-I**) Immunostaining for Scgb1a1 and Cgrp on E18.5 ctrl (G), *Rosa26-rtTA;Tet-Dkk1* (H) and *Rosa26-rtTA;Tet-Fgf10* (I) lungs induced from E10.5 and E15.5, respectively. (**J-L**) Immunostaining for Scgb1a1 and β-tubulin on E18.5 ctrl (J), *Rosa26-rtTA;Tet-Dkk1* (K) and *Rosa26-rtTA;Tet-Fgf10* (L) lungs induced from E10.5 and E15.5, respectively. (**M-R**) Immunostaining for keratin5 and p63 on E18.5 ctrl (M,P), *Rosa26-rtTA;Tet-Dkk1* (N,Q) and *Rosa26-rtTA;Tet-Fgf10* (O,R) lungs induced from E10.5 and E15.5, respectively. P-R are higher magnifications of M-O, respectively. $n \geq 3$. Scale bars: 100 μm (A-F,M-O); 50 μm (G-L); 25 μm (P-R).

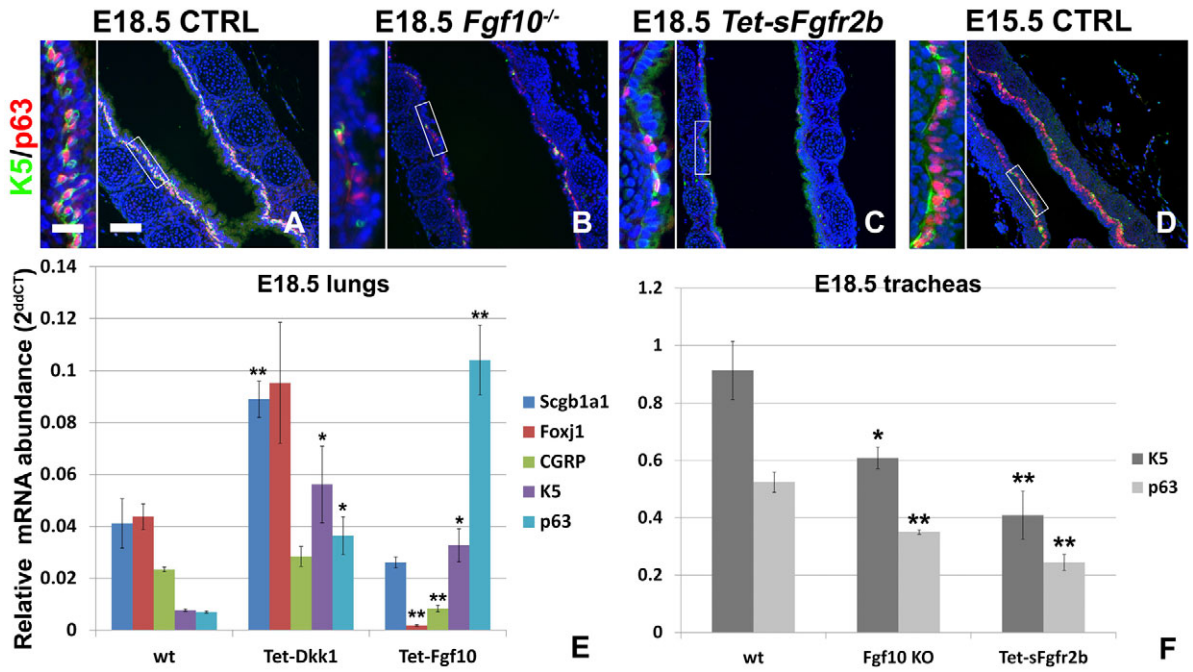

Fig. 8. Fgf(10) signaling regulates basal cell differentiation in the trachea. (**A-D**) Immunostaining for p63 and K5 on E18.5 wild-type (A), *Fgf10*$^{-/-}$ (B), *Rosa26-rtTA;Tet-sFgfr2b* induced from E15.5 (C) and E15.5 wild-type (D) tracheas. Insets show high magnification images of boxed areas. (**E**) qPCR analysis of relative *Scgb1a1*, *FoxJ1*, *Cgrp*, keratin 5 and *p63* mRNA abundance on E18.5 lungs from wild-type mice, *Rosa26-rtTa;Tet-Dkk1* mice induced from E10.5 and *Rosa26-rtTA-Tet-Fgf10* mice induced from E15.5. (**F**) qPCR analysis of relative keratin 5 and *p63* mRNA abundance on E18.5 tracheas from wild-type mice, *Fgf10*$^{-/-}$ mice and *Rosa26-rtTA-Tet-sFgfr2b* mice induced from E15.5. ***P*<0.01, **P*<0.05 (Student's *t*-test). $n \geq 3$. (To allow for better visualization *Scgb1a1*, *Foxj1* and *Cgrp* expression levels presented in the graph are reduced by a factor of 1500, 10 and 5, respectively.) Error bars represent standard error. Scale bars: 100 µm (A-D); 25 µm (insets in A-D).

neuroendocrine bodies (Fig. 7H) than were observed in control lungs (Fig. 7G), whereas overexpression of *Fgf10* had the opposite effect (Fig. 7I). However, when quantified by qPCR, *Rosa26-rtTA;Tet-Dkk1* lungs showed similar amounts of *Cgrp* expression as in control lungs whereas *Rosa26-rtTA;Tet-Fgf10* lungs showed a reduction in overall *Cgrp* expression (Fig. 8E). This discrepancy between immunostaining and whole lung mRNA expression levels is likely to be due to overall changes in the ratio of cells present in these lungs. Alternatively, it is possible that *Fgf10* overexpression promoted the amplification of neuroendocrine cells that had already differentiated by E15.5 but prevented further differentiation of new neuroendocrine cells. Interestingly, we found that overexpression of *Fgf10* from E15.5 onwards was still able to completely block ciliated cell differentiation (Fig. 7L) compared with control lungs and lungs overexpressing *Dkk1*, showing a 'salt and pepper'-like pattern of Clara (Scgb1a1) and ciliated (β-tubulin) cells (Fig. 7J,K). Changes in Clara versus ciliated cell distribution were further quantified by qPCR for *Scgb1a1* and *Foxj1* (ciliated cells) (Fig. 8E) and the absence of ciliated cells in *Rosa26-rtTA;Tet-Fgf10* lungs was confirmed.

In wt lungs, K5^{+}/p63^{+} basal cells can be found only in the main stem bronchi and trachea (Fig. 7M,P). Surprisingly, analysis of E18.5 *Rosa26-rtTa;Tet-Dkk1* lungs induced from E10.5 revealed K5^{+}/p63^{+} basal cells lining the entire airway epithelium (Fig. 7N,Q). This indicates that inhibition of Wnt signaling promotes the differentiation of basal cells probably through premature airway epithelial cell differentiation and increased *Sox2* expression. This would be consistent with a recent report showing that β-catenin negatively regulates Sox2, whereas Sox2 regulates p63, a transcription factor essential for basal cell differentiation (Koster et al., 2004; Hashimoto et al., 2012). Interestingly, we found that overexpression of *Dkk1* after E12.5 had no effect on basal cell amplification (data not shown). By contrast, we found that overexpression of *Fgf10* from E15.5 onwards still promotes basal cell differentiation of a subset of Sox2-positive airway epithelial cells (Fig. 7O,R). qPCR analysis of *K5* and *p63* confirms that E18.5 *Rosa26-rtTa;Tet-Dkk1* lungs induced from E10.5 show an approximately sevenfold increase in *K5* and an approximately fivefold increase in *p63* expression (Fig. 8E) compared with E18.5 wild-type lungs, whereas E18.5 *Rosa26-rtTa;Tet-Fgf10* lungs induced from E15.5 show an approximately fourfold increase in *K5* and ~15-fold increase in *p63* expression (Fig. 8E).

It is tempting to speculate that the reason why basal cells can only be found in the main stem bronchi and trachea in wild-type mice is the presence of *Fgf10* expression in the mesenchyme between the cartilaginous rings (Tiozzo et al., 2009) but not in the airway smooth muscle cells that line the bronchi (Mailleux et al., 2005).

Fgf(10) signaling regulates basal cell differentiation in the trachea

To investigate whether FGF signaling is important for basal cell differentiation or their maintenance in the trachea, we analyzed E18.5 *Fgf10*$^{-/-}$ tracheas and found that basal cells were still present in the upper trachea (data not shown) but significantly reduced in the lower trachea (Fig. 8B) compared with wild-type tracheas (Fig. 8A). This was confirmed by qPCR analysis in which we found a 33% reduction in *K5* and *p63* expression compared with wild-type tracheas (Fig. 8F). It is possible that other Fgfs such as Fgf7, which is highly expressed in the trachea (Finch et al., 1995) and acts via the same Fgfr2b receptor, might also play a role in basal cell

differentiation in the trachea. To test this, we also analyzed E18.5 *Rosa26-rtTa;Tet-sFgfr2b* tracheas induced from E15.5, which overexpress a secreted dominant-negative receptor for Fgf10 and Fgf7, and found them to be almost devoid of basal cells (Fig. 8C). This is in line with a 54% reduction in *K5* and *p63* expression compared with wild-type tracheas as shown by qPCR analysis (Fig. 8F). Interestingly, we found that at E15.5, the trachea is already lined with immature p63/K5-positive basal cells (Fig. 8D), therefore attributing a potentially crucial role for FGF signaling in basal cell maintenance.

DISCUSSION

We have shown here that localized expression of *Fgf10* is not required to direct stereotypic branching and that the role of Fgf10 signaling during early lung development lies primarily in preventing distal epithelial cell differentiation, in part by activating epithelial β-catenin signaling. Our data demonstrating a crucial role for Fgf10/Fgfr2b signaling in regulating proximal-distal patterning of the developing airway epithelium is in accordance with several previous reports. Hypomorphic *Fgf10* mutant lungs show a reduction in distal epithelial markers and decreased epithelial β-catenin signaling (Ramasamy et al., 2007). Conditional inactivation of *Fgf10* in the mesenchyme or its receptor *Fgfr2b* in the epithelium leads to downregulation of the distal epithelial marker Sox9, and the expansion of the proximal marker Sox2 (Abler et al., 2009). Finally, *Fgf10* overexpression has been shown to inhibit terminal differentiation of the lung epithelium causing these cells to adopt a distal fate (Nyeng et al., 2008) or induction of adenomatous malformations (Clark et al., 2001). Our current data confirm and expand on some of these earlier findings.

The lung has a complex three-dimensional structure that features major differences along its proximodistal axis in terms of the composition of the endoderm-derived epithelium. The trachea and primary lung buds arise by different morphogenetic processes from contiguous regions of the embryonic foregut (Cardoso and Lü, 2006). A distinguishing feature of the adult mouse cartilaginous airways (i.e. trachea and primary bronchi) is that *Fgf10* is expressed in the mesenchyme between the cartilage rings (Tiozzo et al., 2009; Sala et al., 2011) and that they contain a discontinuous population of basal stem cells that express p63 and specific keratins (K14 and K5) (Rock et al., 2010). The more distal airways (small bronchi and bronchioles) lack basal cells and are surrounded by airway smooth muscle, which does not express *Fgf10* during normal homeostasis (Mailleux et al., 2005). In the cartilaginous airways, basal cells are considered to be on top of the stem cell hierarchy and are able to self renew and give rise to Clara cells, goblet cells and ciliated cells (Rock et al., 2009). Clara cells themselves are also considered to be stem cells and during normal homeostasis they can give rise to new Clara cells and terminally differentiated ciliated cells (Evans et al., 1976; Rawlins et al., 2009b). Interestingly, p63 is a master regulator required for the development of basal cells (Daniely et al., 2004) and it induces a basal cell phenotype and squamous metaplasia when ectopically expressed in Clara cells (Koster et al., 2004). This form of Clara cell reprogramming may occur to some extent after airway epithelial injury, as Clara cells under such conditions have been shown to be able to give rise to basal cells (Rawlins et al., 2009b).

Very little is known about how basal cells arise during lung development. Our data indicate that blocking Wnt signaling during the very early stages of lung development results in a profound 'proximalization' of the lung epithelium and increased basal cell differentiation. Blocking Wnt signaling is likely to result in increased Sox2 expression, which is a positive regulator of p63 expression (Hashimoto et al., 2012).

In addition, we have discovered a new important role for Fgf10 signaling in the differentiation of Sox2-expressing airway epithelial cells, by preventing ciliated cell differentiation and promoting basal cell differentiation. Fgf10 probably prevents ciliated cell differentiation through activation of the Notch pathway in Clara cells (Guseh et al., 2009; Tsao et al., 2009; Morimoto et al., 2010; Morimoto et al., 2012), which prevents ciliated cell differentiation, similar to our observations in the adult lung (Volckaert et al., 2011). The mechanism through which Fgf10 promotes basal cell differentiation is still unclear.

These findings have potentially important consequences with regard to the role of basal cells and Fgf10 signaling in adult lung injury and repair. Basal cells are not present during normal homeostasis in the adult mouse distal conducting airway epithelium, but can be found only in the trachea and main stem bronchi (Rock et al., 2010). However, after H1N1 infection, 'distal airway stem cells' (DASCs) with basal cell characteristics can be found in the distal mouse airway and have been implicated in the regeneration of the alveolar epithelium (Kumar et al., 2011). Interestingly, a recent study using direct lineage tracing of ATII cells after bleomycin-induced lung injury provides evidence that the majority of the newly generated ATII cells are not derived from the pre-existing ATII cells (Chapman et al., 2011). In support of this, it was shown by lineage tracing that distal airway Clara stem cells can regenerate alveolar epithelium after catastrophic bleomycin- and H1N1-mediated injury, giving rise to (most of the) newly generated ATII cells (Rock et al., 2011; Chen et al., 2012; Zheng et al., 2012). We have previously demonstrated an important role for Fgf10 secreted by airway smooth muscle cells in the regeneration of the airway epithelium after naphthalene-mediated epithelial injury (Volckaert et al., 2011), suggesting that Fgf10 secreted from airway smooth muscle after injury might promote basal cell differentiation/amplification after injury and thus generate the stem cells required to regenerate the injured lungs. Future experiments will be required to test this hypothesis.

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Competing interests statement

The authors declare no competing financial interests.

Author contributions

This project was conceived and executed by S.D.L. Experiments were designed by T.V. and S.D.L. T.V. and S.D.L. wrote the manuscript. T.V., C.L., P.M. and S.D.L. edited the manuscript. T.V., A.C., E.D. and S.D.L. performed all experiments and characterizations of the phenotypes. C.L., P.M. and S.D.L. generated *Tet-Dkk1* mice.

Supplementary material

Supplementary material available online at
http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.096560/-/DC1

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