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Fibroblast Growth Factor 9 signaling inhibits airway smooth muscle differentiation in mouse lung

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Abstract

In mammalian lungs, airway smooth muscle cells (airway SMCs) are present in the proximal lung adjacent to bronchi and bronchioles, but are absent in the distal lung adjacent to terminal sacs that expand during gas exchange. Evidence suggests that this distribution is essential for the formation of a functional respiratory tree, but the underlying genetic mechanism has not been elucidated. In this study, we test the hypothesis that fibroblast growth factor 9 (*Fgf9*) signaling is essential to restrict SMC differentiation to the proximal lung. We show that loss of *Fgf9* or conditional inactivation of Fgf receptors (Fgfr) 1 and 2 in mouse lung mesenchyme results in ectopic SMCs. Our data support a model where FGF9 maintains a SMC progenitor population by suppressing differentiation and promoting growth. This model also represents our findings on the genetic relationship between FGF9 and sonic hedgehog (SHH) in the establishment of airway SMC pattern.

Keywords

fibroblast growth factor 9 (*Fgf9*); lung; airway smooth muscle cells

Introduction

In mammalian lung, airway smooth muscle cells (SMCs) are one of several types of SMCs or smooth muscle-like cells, together with vascular SMCs and myofibroblasts (Owens, 1995; Sparrow and Lamb, 2003). Unlike vascular SMCs that reside adjacent to the pulmonary vasculature and myofibroblasts that reside in the distal alveoli, airway SMCs as their name implies surround the airway epithelium, and thus are also termed parabronchial SMCs. Increases in airway SMC number and size are frequently documented in asthmatic lungs and may exacerbate airway constriction (Lazaar and Panettieri, 2003). Little is known regarding the cellular and molecular basis of this pathology.

In the developing lung, airway SMCs form adjacent to the proximal but not distal epithelium (Tollet et al., 2001). Multiple lines of evidence suggest that this precise arrangement of airway SMCs is essential for fetal lung development (Nakamura and McCray, 2000; Sparrow and Lamb, 2003; Kim and Vu, 2006). For example, epithelial branching is reduced in mutants with ectopic SMCs that cap the distal epithelial tips (Zhou et al., 1996; Weaver et al., 1999), raising the possibility that aberrant distal localization of SMCs may influence branching (Zhou et al., 1996). Furthermore, experiments from a variety of species show that

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in the fluid-filled embryonic lung, SMCs contract rhythmically, leading to a wave of airway narrowing and “squeezing” of the fluid in a proximal-to-distal direction (Vilos and Liggins, 1982; Schittny et al., 2000). As distal epithelium is not constricted by smooth muscles, fluid pressure would lead to distension of the epithelial tips. Experimental increase of the pressure leads to hyperplastic lungs while decrease of the pressure leads to hypoplastic lungs (Hooper et al., 1993; Blewett et al., 1996), suggesting that pressure created by properly positioned SMCs is essential for lung growth. In addition, it has been shown that distension of epithelial tips leads to an increase in parathyroid hormone-like peptide production. This in turn stimulates alveoli formation in preparation for gas exchange (Torday and Rehan, 2006).

In this study, we focus on the role of Fibroblast growth factor (FGF) signaling in airway SMC development. There are twenty-two genes encoding FGFs and four genes encoding high affinity FGF receptors (FGFRs) in the mouse genome (Itoh and Ornitz, 2004). For *Fgfr1*, *Fgfr2* and *Fgfr3*, two major splice variants (IIIb and IIIc) are transcribed from each gene. These variants encode isoforms that differ in the C-terminal half of the third Immunoglobulin-like domain and display divergent ligand binding specificity (Mohammadi et al., 1997). Gene expression and mitogenic activity assays show that the IIIb isoform is mainly expressed in the epithelium and preferentially binds to ligands secreted from mesenchymal tissues. In contrast, the IIIc isoform is mainly expressed in the mesenchyme and preferentially binds to ligands secreted from epithelial tissues (Orr-Urtreger et al., 1991; Peters et al., 1992; Orr-Urtreger et al., 1993; Zhang et al., 2006b). Through this paracrine mechanism, FGF signaling plays essential roles in epithelial-mesenchymal interaction during organ formation.

Distinct FGFs and FGFRs are involved in different aspects of lung development. Gain-of-function experiments have implicated FGF9 as the principal factor involved in airway SMC development (Colvin et al., 2001; Weaver et al., 2003; Mailleux et al., 2005; Del Moral et al., 2006; White et al., 2006; Ramasamy et al., 2007). FGF9 is expressed in the epithelium and the mesothelium (the pleura), and signals to the mesenchyme through the IIIc isoform of FGFRs (Colvin et al., 1999; Weaver et al., 2003; Del Moral et al., 2006). When added in culture or overexpressed in transgenic lungs, FGF9 leads to a reduction in airway SMCs, suggesting that it is sufficient to inhibit SMC differentiation (Weaver et al., 2000; Del Moral et al., 2006; White et al., 2006). Whether FGF9 signaling is essential for restricting airway SMC to the proximal lung has not been determined.

Another key FGF that functions in lung development is FGF10. *Fgf10* expression marks the progenitors for airway SMCs in the distal lung mesenchyme (Mailleux et al., 2005). However, as FGF10 preferentially binds to FGFR2-IIIb expressed in the lung epithelium, its primary role is to drive the outgrowth and branching of the epithelium (Bellusci et al., 1997; Min et al., 1998; Sekine et al., 1999; De Moerlooze et al., 2000; Weaver et al., 2000; Ramasamy et al., 2007).

In airway SMC development, FGF9 functions closely with the sonic hedgehog (SHH) pathway (Weaver et al., 2003). Opposite to the role of FGF9, SHH induces SMC differentiation (Pepicelli et al., 1998; Li et al., 2004; Miller et al., 2004). It is proposed that the balance of activities between FGF9, secreted primarily from the mesothelium, and SHH, secreted from the epithelium, dictates SMC differentiation in the mesenchyme sandwiched between the two signaling sources (Weaver et al., 2003). This model (termed the Sandwich Model) provides a spatial guide to understanding the complex control of airway SMC differentiation.

In this study, we used a loss-of-function approach to investigate the requirement of FGF9 signaling in pulmonary SMC development and to test the Sandwich model. Previous studies

show that *Fgfr1* and *Fgfr2* are the principal Fgfrs expressed in embryonic lung mesenchyme and are the likely mediators of FGF9 signaling (Orr-Urtreger et al., 1991; Peters et al., 1992; Orr-Urtreger et al., 1993; White et al., 2006; White et al., 2007). We show that knockout of *Fgf9* or conditional inactivation of both *Fgfr1*and *Fgfr2* in lung mesenchyme leads to ectopic SMCs in the distal lung. We explore the characteristics of these SMCs, their origin and potential molecular mediators of FGF pathway function in this process. Our data leads to a model of airway SMC development by integrating two distinct roles of FGF9 in this process. Finally, we use a combination of genetic and pharmacological approaches to demonstrate that FGF9 and SHH signaling regulate airway SMC development independently rather than through a common genetic pathway.

Results

Cre-mediated Fgfr inactivation in lung mesenchyme leads to reduced mesenchyme thickness

To address Fgfr function in lung mesenchyme, we used the *Brachyury* (*T*)-Cre transgenic line to conditionally inactivate Fgfrs (Perantoni et al., 2005). T-Cre is first active at Embryonic day (E) 7.5 in the primitive streak and later in most of the mesodermal lineages at the level of and posterior to the heart. This line has been useful in studying gene function during organ development because cre recombines targeted floxed genes in all mesodermally-derived tissue of a given organ (Perantoni et al., 2005). To examine whether T-Cre activity would result in recombination in the developing lung, we mated T-Cre transgenic mice to *Gt(ROSA)26Sor*^{tm1sor} (*R26R*) mice that report Cre activity through β-galactosidase (β-gal) expression (Soriano, 1999), generating T-Cre;*R26R* embryos. We observed β-gal activity in the entire lung mesenchyme and mesothelium at E10.0, shortly after the formation of primary buds (Fig. 1A,B). This result demonstrates that T-Cre has been active in the lineage that gives rise to all lung mesenchymal and mesothelial cells at the beginning of lung development.

We found that Cre-mediated recombination also occurred in a few isolated cells in the epithelium of T-Cre;*R26R* lungs. Detection of this epithelial activity led us to consider the possibility that conditional inactivation of *Fgfr1* and *Fgfr2* (hereafter referred to as *Fgfr1;r2*) by T-Cre in a few cells in the epithelium may complicate the interpretation of their role in lung mesenchyme. Even though *Fgfr1-IIIb* variant is not required for normal lung epithelium development, the *Fgfr2-IIIb* variant is essential in the epithelium for lung initiation (Partanen et al., 1998; De Moerlooze et al., 2000). To circumvent this potential complication, we generated T-Cre;*Fgfr1*$^{del/co}$;*Fgfr2c*$^{-/-}$ (hereafter referred to as *Fgfr1;2-DKO* for double knockout) embryos. In these embryos, *Fgfr1* is inactivated by T-Cre-mediated recombination of the *Fgfr1*^{co} allele starting at E10.5 (Fig. 1C,D) (Xu et al., 1999), and *Fgfr2* activity is absent in lung mesenchyme due to the use of a null allele of *Fgfr2-IIIc*, the *Fgfr2* variant that is specifically expressed in mesoderm-derived mesenchymal tissues in the embryo (Orr-Urtreger et al., 1991; Peters et al., 1992; Orr-Urtreger et al., 1993; Eswarakumar et al., 2002). The *Fgfr1;2-DKO* mutant embryos die at E14.5 with truncation of the anterior-posterior body axis. To address the possibility that potential defects in surrounding body wall structures may interfere with lung development in these mutant embryos, we examined the chest cavity in transverse sections. We found that the mutant chest cavity remains normal in size (Fig. 1E,F). Furthermore, mutant lungs initiate normally (data not shown), suggesting that the mesodermal lineage for the lung is properly established.

Close examination of the mutant lungs showed that the earliest phenotype is detected at E11.5 when portions of the mesenchyme are thinner than normal (Fig. 1G,H). At this stage, the epithelial branching pattern is normal. At E12.5 however, epithelial branching is reduced

in *Fgfr1;2-DKO* lungs and thinning of the mesenchyme is more apparent (Fig. 1I,J). The gross morphological phenotype of *Fgfr1;2-DKO* lungs is similar to that of a previous *Fgfr* mutant, and of the *Fgf9* null mutant (Colvin et al., 2001; White et al., 2006), suggesting that FGFR1 and FGFR2 are the main receptors that mediate FGF9 signaling to lung mesenchyme. This is further supported by our finding that the *Fgfr1;2-DKO* mutant lungs can no longer respond to exogenous FGF9 in its activity to promote mesenchymal growth (Supplementary Fig. 1).

FGF9 signaling through FGFR1;R2 is essential for suppressing SMC differentiation in distal lung mesenchyme

To address the extent that FGF signaling is reduced in *Fgfr1;2-DKO* lungs, we assayed for the expression of genes that are regulated by FGF activity in lung mesenchyme (Minowada et al., 1999; Liu et al., 2003). We found that the expression of *Pea3* and *Spry4* is reduced but not absent (Fig. 2A–D), suggesting that FGF signaling is downregulated but not abolished in lung mesenchyme.

To address if FGF signaling plays a role in airway SMC development, we assayed for the expression of smooth muscle actin (SMA), an early SMC marker (Fig. 2E–J). In E12.5 *Fgfr1;2-DKO* lungs, we found that in the proximal mesenchyme, SMA expression remains restricted to the parabronchial mesenchyme as normal (Fig. 2E,H). However, in the distal mesenchyme, either separate or continuous with the proximal domain, ectopic SMA-positive cells are detected subjacent to epithelial tips (Fig. 2E–J and 7B,D). Consistent with this result, we found that the expression of *Prdm6*, a transcription factor present in SMCs (Davis et al., 2006), is extended to the distal mesenchyme in *Fgfr1;2-DKO* lungs (Supplementary Fig. 2). In support of the proposal that FGFR1 and FGFR2 are the main receptors that mediate FGF9 signaling in lung mesenchyme, we detected ectopic SMA-positive cells in the distal mesenchyme of *Fgf9* mutant lungs (Fig. 2K–N). In both *Fgfr1;2-DKO* and *Fgf9* mutants, the ectopic SMA expression is most pronounced around posterior branch tips (Fig. 2J,N), although isolated SMA-positive cells are also found adjacent to anterior branch tips (arrowheads in Fig. 2I and data not shown). Together these results indicate that FGF9 signaling through FGFR1 and FGFR2 is essential for preventing the presence of SMA-expressing cells in distal mesenchyme.

As SMA is the earliest marker expressed in the course of SMC differentiation (Owens, 1995), we analyzed the expression of additional genes to determine whether ectopic SMA-positive cells have fully differentiated into mature SMCs. In E12.5 *Fgfr1;2-DKO* lungs, using an antibody against smooth muscle myosin (SMM), a marker for mature SMCs, we detected SMM-positive cells in the distal mesenchyme (Fig. 2O,P), suggesting that a subset of the ectopic SMA-positive cells have differentiated into mature SMCs.

A current model proposes that airway SMCs arise from distal mesenchyme, and are translocated to the more proximal parabronchial region as epithelial tips advance into the distal mesenchyme (Weaver et al., 2003; Mailleux et al., 2005). This model raises the possibility that the presence of SMC in the distal mesenchyme of *Fgfr1;2-DKO* lungs may be secondary to reduced epithelial growth into the mesenchyme. To address this possibility, we analyzed SMA expression at E11.75. This time point is chosen because branching morphology in the *Fgfr1;2-DKO* mutant remains normal at this stage (Fig. 3A,C). We also examined the rate of cell proliferation in control and *Fgfr1;2-DKO* lungs and found no statistically significant difference in the proliferation rate of either epithelial or mesenchymal cells, as assayed by percentage BrdU-positive nuclei (Fig. 3E–G, for epithelium, mutant 45.6±5.4% vs control 49.7±8.2%, p=0.17; for mesenchyme, mutant 45.7±5.2% vs. control 45.2±4.2%, p=0.79, n=12 sections from 3 mutant lungs and 12 sections from 5 control lungs). We detected ectopic SMA-positive cells in the distal

mesenchyme at this stage (Fig. 3A–D), suggesting that their presence is not secondary to a defect in epithelial proliferation and morphogenesis.

As SMCs are normally detected adjacent to proximal airways, another possible explanation for their presence in the distal mesenchyme is that they formed in response to signals from a proximalized distal epithelium. To address whether distal epithelium has lost its normal character in the *Fgfr1;2-DKO* lungs, we assayed for the expression of Surfactant Protein C (SP-C). We found that SP-C is expressed in the distal epithelium of mutant lungs at E14.5, suggesting that the distal epithelium has maintained its characteristics (Fig. 3H,I). Furthermore, ectopic SMA and SMM expression is detected adjacent to SP-C expressing epithelial cells, suggesting that the presence of ectopic SMCs cannot be explained by prior proximalization of the epithelium (Fig. 3I, K).

To further address whether there is a more global change of proximal-distal (P-D) patterning that led to the formation of SMCs in the distal mesenchyme, we assayed for additional markers for either the proximal or distal portion of the lung (Fig. 3L–S, Supplementary Fig. 3). With the exception of markers for the SMC, all other genes with P-D restricted expression are detected in a similar pattern in the *Fgfr1;2-DKO* mutant compared to control lungs. These data together suggest that the ectopic SMCs did not arise due to a defect in global P-D patterning of the lung. Instead, they formed likely due to a defect in local control of SMC differentiation in the distal mesenchyme.

The identity of ectopic SMCs

In an early developing lung, there are two types of SMCs, airway SMCs (parabronchial) and vascular SMCs (subjacent to major blood vessels) (Owens, 1995; Sparrow and Lamb, 2003). These SMC types participate in distinct physiological processes, yet little is known regarding how they arise in relationship to each other. To address whether the ectopic SMCs that form following inactivation of FGF9 signaling are specified to be either vascular or airway SMCs, we assayed for the expression of *Heyl* (Leimeister et al., 2000), a vascular SMC marker, and *Noggin*, an airway SMC marker (Weaver et al., 2003). We found that *Heyl* expression in *Fgfr1;2-DKO* lungs remains normal, and is not detected in the distal mesenchyme, suggesting that the ectopic SMA-positive cells detected in these mutant lungs are not vascular SMCs (Fig. 4A–D). To effectively assay for *Noggin* expression, we used the $Noggin^{lacZ}$ allele, a β-gal reporter of its expression (McMahon et al., 1998). We found that in $Noggin^{lacZ/+}$ lungs that are otherwise normal, β-gal activity is detected in parabronchial mesenchymal cells, consistent with it being an airway SMC marker (Fig. 4E,F) (Weaver et al., 2003). However in $Noggin^{lacZ/+}$ lungs where FGF9 signaling is inactivated (in $Fgf9^{-/-}$; $Noggin^{lacZ/+}$ mutant), in addition to the normal parabronchial pattern, β-gal positive cells are also detected in the distal mesenchyme (Fig. 4G,H), consistent with the possibility that at least a subset of the ectopic SMCs are airway SMCs in character. Taken together, our data suggest that FGF9 signaling through FGFR1;R2 is necessary to inhibit differentiation of airway SMCs in the distal lung mesenchyme.

The origin of ectopic SMCs

Ectopic SMCs may arise from precocious differentiation of airway SMC progenitors, or from transdifferentiation of other cell types present in the distal mesenchyme. Evidence from cell culture suggests that endothelial cells can give rise to SMCs (Arciniegas et al., 1992; Zhang et al., 2006a). Consistent with previous observations (White et al., 2007), we found that endothelial cells are reduced in *Fgfr1;2-DKO* distal mesenchyme (Fig. 5A–D), raising the possibility that ectopic SMCs may arise from transdifferentiation of endothelial cells.

To address this possibility, we first genetically-labeled the endothelial lineage by mating Tek-Cre mice (Koni et al., 2001) to *Gt(ROSA)26Sor*^{tm2Sho} (*Rosa-Gfp*) Cre reporter mice (Mao et al., 2001) to generate Tek-Cre;*Rosa-Gfp* embryos. In these embryos, Tek-Cre leads to reporter recombination in endothelial cells, permanently marking these cells and their descendants with *Gfp* expression. To inactivate FGF signaling in Tek-Cre;*Rosa-Gfp* lungs, we cultured them in the presence of SU5402, a pharmacological antagonist of FGF signaling (Mohammadi et al., 1997). We found that SU5402 treatment leads to ectopic SMCs and reduced endothelial marker expression in the distal mesenchyme (Fig. 5F–I and data not shown), suggesting that SU5402 can mimic the SMC defect observed in *Fgfr1;2-DKO* and *Fgf9* mutant lungs. The ectopic SMCs in these treated lungs do not express GFP (n=5/5 lungs), indicating that they did not originate from endothelial cells. This finding suggests that following inactivation of FGF signaling, ectopic airway SMCs develop either from other specified cell types or prematurely from progenitors residing in the distal mesenchyme.

If ectopic SMCs arise from precocious differentiation of SMC progenitors, one would predict a reduction in the number of progenitors that remain. To address this possibility, we examined the expression of *Fgf10*, a marker for SMC progenitors in the distal lung mesenchyme (Mailleux et al., 2005). By using RNA in situ hybridization and real-time RT-PCR analysis, we found that *Fgf10* is downregulated in *Fgfr1;2-DKO* lung compared to control (Fig. 5J–M, mutant 66.6±1.3% vs. control 100±11.1%, n=3 each, p=0.035). To determine whether this reduction is solely due to an overall reduction in the lung mesenchyme population, we examined the expression of *Tbx4*, a gene expressed in the mesenchyme. We found that although *Tbx4* expression is slightly reduced in *Fgfr1;2-DKO* lungs (Fig. 5N–P, mutant 96.1±25.5% vs. control 100±24.5%, n=3 each, p=0.858), possibly reflecting thinning of the mesenchyme, *Fgf10* expression is significantly downregulated in the mutant. These results suggest that in addition to its function in maintaining mesenchyme cell proliferation (White et al., 2006), signaling through FGFR1;R2 also specifically maintains the size of the SMC progenitor population. We postulate that this role is achieved by inhibiting progenitor differentiation into SMCs. When *Fgfrs* are inactivated, SMC progenitors differentiate prematurely, leaving fewer *Fgf10*-positive cells in the distal mesenchyme.

FGF regulates *Myocardin* expression

A recent study shows that WNT activity is reduced in *Fgf9* mutant lungs and in mutant lungs where *Fgfr1;2* are inactivated by *Dermo1-cre* (Yin et al., 2008). Similarly in our *Fgfr1;2-DKO* lungs where *Fgfr1;2* are inactivated by T-cre, we observed a reduction in the expression of *Wnt2* and *Lef1*, a downstream readout of WNT signaling (Fig. 6A–D). This result was confirmed by real-time RT-PCR data (for *Wnt2*, mutant 41.1±7.2% vs control 100±17.4%, n=3 each, p=0.012; for *Lef1*, mutant 46.5±18.3% vs. control 100±9.4%, n=3 each, p=0.020). These findings raised the possibility that FGF9 may function through WNT to control SMC differentiation. However, inactivation of β-Catenin, a key mediator of WNT signaling in lung mesenchyme does not lead to ectopic SMCs in the distal mesenchyme (Yin et al., 2008), suggesting that FGF9 signaling controls SMC differentiation through additional pathways.

Previous studies show that *Myocardin* is a key transcription factor necessary and sufficient for SMC differentiation (Wang and Olson, 2004). It acts by binding to *Sma* and *Smm* promoters, activating their expression. In *Fgfr1;2-DKO* lungs at E12.5, we found that *Myocardin* is ectopically expressed in the posterior tip of the left lobe and right caudal lobe where ectopic airway SMC differentiation is most frequently detected (Fig. 6E,F and data not shown). These results suggest that FGF9 signaling may inhibit SMC differentiation partly through repressing *Myocardin* expression.

FGF9 and SHH pathways function in parallel to control SMC differentiation

A current model proposes that airway SMC pattern is achieved through balancing the opposing roles of FGF9 and SHH signaling in SMC differentiation (Weaver et al., 2003). These pathways may act in a linear manner, where one suppresses the function of the other (Fig. 7A, mechanisms 1, 2). Alternatively, they may control airway SMC differentiation independently (Fig. 7A, mechanism 3). We distinguish among the three possible mechanisms using a combination of pharmacological and genetic approaches.

Mechanism 1—To address if SHH promotes SMC differentiation solely through repressing FGF9 function (Fig. 7A, Mechanism 1), we investigated whether downregulation of SHH signaling would reverse the ectopic SMC phenotype in *Fgfr1;2-DKO* lungs. We reasoned that a persistence of ectopic SMCs would support Mechanism 1, while a loss of these cells would argue against this mechanism.

To suppress SHH signaling, we used cyclopamine, a small molecule inhibitor of SHH receptor Smoothened (Incardona et al., 1998). In lung explant cultures, we performed dose-titration experiments and determined that the minimal concentration of cyclopamine (1μM) that is needed to suppress SHH signaling (Supplementary Fig. 4). In control experiments, we found that control lungs cultured in the presence of 1μM cyclopamine show a reduced domain of SMA expression (n=5/5) compared to control lungs cultured without cyclopamine (n=8) (arrowheads in Fig. 7B,C). This reduction is not likely due to cell death, as we observed no increase in cell death in lungs treated with this concentration of cyclopamine (n=3/3) (data not shown). These data indicate that inhibition of SHH signaling by cyclopamine leads to decreased SMC differentiation in control lungs in culture.

To test Mechanism 1, we cultured *Fgfr1;2-DKO* lungs in the presence of cyclopamine. We found that cyclopamine treatment leads to a drastic reduction in SMA expression in *Fgfr1;2-DKO* lungs (n=2/2) compared to vehicle-treated controls (n=2) (arrowheads in Fig. 7D,E). This reduction is similar to the effect seen in control lungs. These data suggest that in the absence of FGF9 signaling, the lung is still sensitive to changes in SHH pathway activity. This result argues against the possibility that SHH signaling impacts SMC differentiation solely by repressing FGF9 function.

Mechanism 2—To address if FGF9 inhibits SMC differentiation solely through inhibiting SHH function (Fig. 7A, Mechanism 2), we first assayed for the expression of *Ptch1*, an indicator of SHH activity. We show that in *Fgfr1;2*-DKO lungs, *Ptch1* expression is slightly downregulated (Fig. 7F,G), similar to the observation in *Fgf9* mutant lungs (White et al., 2006). This downregulation suggests that FGF9 signaling positively regulates SHH activity and argues against Mechanism 2. To more rigorously address this mechanism using a functional test, we investigated whether downregulation of FGF signaling would rescue the absence of SMCs in *Shh* null mutant lungs (Pepicelli et al., 1998;Miller et al., 2004). We reasoned that a continued absence of SMCs would support Mechanism 2, while a rescue of SMCs would argue against this mechanism.

We cultured *Shh* mutant lungs in the presence of SU5402, the FGF antagonist that leads to ectopic SMCs when added to control lung culture (Fig. 5H). We found that in *Shh* mutant lungs, SU5402 treatment leads to an increase in SMA expression compared to vehicle-treated control (Normalized relative areas of SMA/E-Cadherin expression, mutant 321±90.9% vs control 100±34.7%, n=5 each, $p < 0.005$) (Fig. 7H,I). These data suggest that in the absence of SHH signaling, the lung is still sensitive to changes in FGF pathway activity. This result argues against the possibility that FGF9 signaling impacts SMC differentiation solely through regulating SHH function in this process.

Mechanism 3—Our data from pharmacological experiments using mutant lungs are consistent with the possibility that FGF9 and SHH function in parallel to control airway SMC differentiation (Fig. 7A, Mechanism 3). To further test all three possible mechanisms using a genetic approach, we generated *Fgf9* and *Shh* double homozygous mutant embryos (*Fgf9*$^{-/-}$;*Shh*$^{-/-}$, hereafter referred to as *Fgf9;Shh* mutant) and examined SMA expression in these lungs. Each of the three possible mechanisms predicts a different SMC pattern in double mutant lungs. Mechanism 1 predicts a presence of SMCs not just in the proximal, but also in the distal mesenchyme, similar to the phenotype in *Fgf9* single mutant lungs (see Fig. 2M, 7K). Mechanism 2 predicts an absence of SMCs similar to the phenotype in *Shh* mutant lungs (Fig. 7L). Mechanism 3 predicts an intermediate phenotype, where SMCs are present, but not as wide-spread as in *Fgf9* single mutant lungs.

Our data from the *Fgf9;Shh* mutant lungs (n=3) show that while SMA-positive cells are rescued, they are not as extensive as in *Fgfr1;2-DKO* lungs and *Fgf9* mutant lungs (Fig. 7J–M). Thus, consistent with data from pharmacological experiments, SMA pattern in *Fgf9;Shh* mutant lungs suggests that FGF9 and SHH function in parallel to control SMC differentiation (Mechanism 3).

Discussion

In this study, we provide loss-of-function genetic evidence demonstrating that FGF9 signaling through FGFR1 and FGFR2 plays an essential role in generating the proper airway SMC pattern by inhibiting SMC differentiation in distal lung mesenchyme. Furthermore, genetic and pharmacological data demonstrate that FGF9 and SHH function through parallel mechanisms to control SMC differentiation. These findings led us to propose a model of FGF9 function in airway SMC development.

A model: FGF9 maintains a SMC progenitor population in the developing lung

We determined the mechanism underlying FGF9 control of SMC development by testing possible cause of the ectopic SMC phenotype in *Fgfr1;2-DKO* and *Fgf9* mutant lungs: transdifferentiation from other mesenchymal cell types, increase in SMC progenitor number or premature differentiation of SMC progenitors. Our result from the lineage-labeling experiment suggests that the ectopic SMCs did not arise from transdifferentiation of endothelial cells (Fig. 5E–I). Furthermore, reduction of SMC progenitor marker *Fgf10* expression in *Fgfr1;2-DKO* (Fig. 5J–M) and *Fgf9* mutant lungs (Colvin et al., 2001) suggests a decrease, rather than an increase of progenitor cells. Thus we argue that the most parsimonious explanation for the decrease in SMC progenitors and concomitant increase in differentiated SMCs is that inactivation of FGF9 signaling leads to premature differentiation of SMC progenitors. Thus in a normal lung, FGF9 inhibits differentiation of SMC progenitors.

Based on these data and previous findings, we propose a model of FGF9 function in lung SMC development (Fig. 8). We propose that FGF9, secreted by the mesothelium and distal epithelium, maintains a SMC progenitor population in the distal mesenchyme by performing two distinct roles. First, FGF9 signaling prevents the progenitors from responding to SMC-inducing signals, thus holding them in an undifferentiated state (Fig. 2E–P, 7J,K) (Weaver et al., 2003). Second, FGF9 promotes growth of the distal mesenchyme (Fig. 1G–J) (Colvin et al., 1999;Del Moral et al., 2006;White et al., 2006), thus replenishing the progenitor population. We postulate that the size of this population is limited by the range of FGF9 signaling. As the progenitors proliferate, some would translocate proximally out of the range of FGF9 signaling. Once they do, they can then respond to SMC-inducing signals and differentiate. Thus, FGF9 maintenance of the progenitors provides a critical mechanism to ensure a steady supply of SMCs that will progressively line the elongating airways.

This model of FGF9 function in airway SMC development shares similarities with aspects of a recently proposed model of limb development (Tabin and Wolpert, 2007). In a limb bud, FGF9 is one of several FGFs expressed from the apical ectodermal ridge (AER) that signals to the underlying limb bud mesenchyme to direct limb patterning. It was postulated that AER-FGFs maintain a pool of progenitors in the distal limb bud mesenchyme. Furthermore, AER-FGFs play a role in limb bud growth (Sun et al., 2002; Yu and Ornitz, 2008). These similarities between the developing limb and lung suggest that FGF9 signaling may act in multiple developmental settings to influence the balance between progenitor renewal and differentiation.

FGF9 inhibits SMC differentiation by overriding multiple SMC-inducing cues

Our model explains why SHH, expressed at a higher level in the distal epithelium than in the proximal epithelium, is only able to induce SMC proximally. We postulate that in the distal mesenchyme, FGF9 prevents SHH from promoting SMC differentiation. This is supported by results from mesenchymal cell culture where exogenous FGF9 protein can suppress SMC differentiation in cells pre-treated with recombinant SHH (Weaver et al., 2003). In addition, overexpression of *Fgf9* in lung epithelium prevents SMC differentiation in the presence of SHH (White et al., 2006).

In addition to SHH, other SMC-inducing signals such as TGFβ are present in the developing lung (Zhou et al., 1996). These signals may promote SMC differentiation in the absence of SHH. At first glance, this possibility is not supported by the observation that no SMCs develop in $Shh^{-/-}$ mutant. However, we argue that unlike in wild-type lungs, FGF9 signaling may inhibit SMC differentiation in the entire mesenchyme of the $Shh^{-/-}$ lung because it is hypoplastic. This may explain why other positive regulators of SMC differentiation fail to promote SMC fate in $Shh^{-/-}$ lungs. Supporting this explanation, when FGF signaling is inactivated in $Shh^{-/-}$ mutant lungs (in either *Fgf9;Shh* double mutant or by SU5402 treatment, Fig. 7I,M), some SMCs are present. These data are consistent with the premise of our model that in a normal developing lung, cells are competent to respond to SMC-inducing signals only after they exit FGF influence.

FGF9 signaling performs distinct roles on different cell types present in the distal mesenchyme

The ability of FGF9 signaling to suppress differentiation in lung appears to be restricted to the SMC lineage. In *Fgfr1;2-DKO lungs* and in *Fgf9* mutant lungs, unlike SMCs, endothelial cells are decreased rather than increased (Fig. 5A–D) (White et al., 2007), suggesting that FGF9 promotes the endothelial lineage. The different effects on the cellular level are carried out by different mediators of FGF9 function in these lineages. While previous analyses show that FGF9 stimulation of pulmonary capillary development is mediated by *Vegfa* (White et al., 2007), we show that FGF9 inhibition of SMC differentiation is likely mediated through its repression of *Myocardin* expression (Fig. 5E,F).

FGF9 pathway shares different relationships with the SHH pathway in their control of lung mesenchyme growth, vascular formation and SMC development. In controlling mesenchyme growth, both signals stimulate cell proliferation. It is proposed that FGF9 indirectly promotes proliferation of subepithelial mesenchyme by maintaining SHH activity (White et al., 2006). In vascular development, however, FGF9 and SHH act independently to promote capillary formation (White et al., 2007). Our data in this study suggest that FGF9 and SHH controls SMC development also through parallel pathways, although their effect in this process are antagonistic rather than cooperative.

Multiple other signals, including WNT, TGFβ, PDGF and BMP (Noggin) pathways have been implicated in the development of lung mesenchyme (Zhou et al., 1996; Lindahl et al., 1997; Weaver et al., 1999; Shu et al., 2005; Kim and Vu, 2006; Ramasamy et al., 2007; De Langhe et al., 2008). It is important to address how these signals function in concert to sculpt the stereotypical pattern of SMCs. Our study demonstrates that a combination of pharmacological and genetic analyses offer an effective approach in dissecting signal interactions during lung development.

Understanding airway SMC pathology: significance beyond development

Studies of airway SMC development in embryonic lungs will contribute to our understanding of airway SMC homeostasis in adult lungs. Hyperplasia of SMCs is found in some asthma patients and may affect disease severity (Lazaar and Panettieri, 2003). It is interesting to note that factors that play a role in SMC formation in the embryonic lung, such as TGFβ (Zhou et al., 1996)q have also been implicated as a link between inflammation and SMC hyperplasia in adult asthmatic lungs (Vicencio et al., 2004; Sheppard, 2006). These data raise the possibility that a cohort of signaling molecules and transcription factors may perform similar roles in SMC development in embryonic lung and SMC homeostasis in adult lung. We propose that approaches similar to these used in this study can be adapted to investigate the cellular origins and molecular mechanisms underlying airway SMC pathology in lung diseases such as asthma.

Experimental Procedures

Generation of mutant embryos

Embryos were dissected from time-mated mice, counting noon on the day when the vaginal plug was found as embryonic day (E) 0.5. To generate *Brachyury* (*T*)-Cre*; Fgfr1* $^{del/co}$*; Fgfr2IIIc* $^{-/-}$ mutant embryos, males carrying a T-Cre allele (Perantoni et al., 2005), one *Fgfr1*^{del} allele (Xu et al., 1999) and one *Fgfr2IIIc*$^{-}$ allele (Eswarakumar et al., 2002) were mated to females carrying two conditional *Fgfr1* alleles (*Fgfr1*$^{co/co}$) (Xu et al., 1999) and one *Fgfr2IIIc*$^{-}$ allele. To generate *Shh* and *Fgf9* double null mutant embryos, we intercrossed mice doubly heterozygous for null alleles of *Fgf9* (Colvin et al., 2001) and *Shh* (Harfe et al., 2004). To generate Tek-Cre; *Gt(ROSA)26Sor*^{tm2Sho} (*Rosa-Gfp*) embryos, Tek-Cre (Koni et al., 2001) males were mated to *Rosa-Gfp* females (Mao et al., 2001). To generate *Fgf9* mutant embryos containing a *Noggin*^{lacZ} reporter allele (McMahon et al., 1998), males doubly heterozygous for a null allele of *Fgf9* and the *Noggin*^{lacZ} allele were mated to females carrying one copy of the *Fgf9* null allele.

RNA in situ hybridization

RNA whole-mount in situ hybridization was performed as previously described (Neubuser et al., 1997). A probe that specifically recognizes the *Fgfr1-IIIc* isoform was generated by RT-PCR of *Fgfr1* exon 9 using the primers: 5′-TCTGGAAGCCCTGGAAGAGAGA-3′ and 5′-TGCGCAGAGGGATGCTCTTG-3′. Because exon 9 is within the region that is flanked by *loxP* sites in the *Fgfr1*^{co} allele (Xu et al., 1999), it is deleted by Cre and therefore the mRNA generated by the Cre-modified allele cannot be detected with this probe.

β-galactosidase (β-gal) staining and histology

To assay Cre activity, the *Gt(ROSA)26Sor*^{tm1sor} (*R26R)* reporter line (Soriano, 1999) was mated to T-Cre mice (Perantoni et al., 2005). β-gal activity was assayed by a standard protocol. β-gal stained lung buds were fixed in 4% paraformaldehyde and embedded in JB-4 plastic resin (Polysciences) following manufacturer's protocol. Sections were cut at 10 μm

and counterstained with 1% eosin. To assay for *Noggin*^{lacZ} expression, lungs were fixed and stained by a standard protocol.

Immunohistochemistry and immunofluorescent staining

For wholemount staining, lung tissue samples were fixed in 4% paraformaldehyde overnight, dehydrated and stored in 100% methanol at −20°C. For cryosections, samples were fixed in 4% paraformaldehyde overnight, equilibrated into 30% sucrose in PBS, then frozen in OCT and sectioned at 14 μm thickness. For antibody staining, samples were rehydrated in PBS, blocked with either milk diluent/blocking solution (1:10) (KPL, Inc.) in sterile water or 5% NGS in PBS with 0.1% Tween for 1 hour, then stained in primary antibody in the same blocking solution at 4°C overnight. Samples were washed in blocking solution three times for 30 minutes each, and stained in secondary antibody in blocking solution at 4°C overnight. Samples were then washed in PBS with 0.1% Tween three times for 30 minutes each time, and were mounted in Vectashield (Vector Laboratories, Inc.). For immunohistochemistry, a DAB substrate kit (Vector Laboratories, Inc.) was used for peroxidase color reaction. The primary antibodies used were: rabbit anti-SP-C (Chemicon, used at 1:1,000 dilution), mouse anti-BrdU (also termed Ab-2, Calbiochem, used at 1:50 dilution), rat anti-PECAM (also termed CD31, BD Sciences, used at 1:50 dilution), Cy3-conjugated mouse anti-Smooth Muscle Actin (Sigma, used at 1:1000 dilution), rat anti-E-Cadherin (Sigma, used at 1:1,000 dilution), rabbit anti-GFP (Synaptic Systems, used at 1:1,000 dilution), and rabbit anti-Smooth Muscle Myosin (Biomedical Tech, used at 1:100 dilution). The secondary antibodies used were: FITC-conjugated goat anti-rat (Jackson ImmunoResearch Labs, used at 1:100 dilution), FITC-conjugated goat anti-rabbit (Jackson ImmunoResearch Labs, used at 1:200 dilution), Cy3-conjugated goat anti-mouse (Jackson ImmunoResearch Labs, used at 1:500 dilution), and HRP-conjugated goat anti-rat (KPL, used at 1:100 dilution).

Cell proliferation assay

Pregnant females received an intraperitoneal injection of 100μg BrdU (Sigma) per gram bodyweight 1 hr. prior to sacrifice. Lung tissue samples were processed for cryosectioning and stained as described above, with the exception that sections were pretreated with 500u/mL DNAse I (Roche) for 10 min. for antigen unmasking before staining. After immunostaining, slides were counterstained with DAPI. For each 20x field of view, the number of BrdU+ nuclei relative to the number of total nuclei in the epithelium and mesenchyme was calculated. The percentage BrdU+ nuclei between control (5 lungs, n=12 20× fields of view) and *Fgfr1;2-DKO* (3 lungs, n=12 20× fields of view) was compared using Student's t-test. Results were considered statistically significant if $p \leq 0.05$.

In vitro lung culture

Freshly dissected E11.5 or E11.75 lungs were placed on a Nuclepore Track-Etch membrane (Whatman, 8μm) and cultured in 4-well culture dish (Nalge Nunc International) in GIBCO™ BGJb media (Invitrogen). Lungs were cultured with or without either 200ng/ml FGF9 (Peprotech), 1μM cyclopamine (Toronto research chemical Inc.), 2.5μM SU5402 (*Shh* mutant lung culture) or 10μM SU5402 (Tek-Cre;Rosa-Gfp lung culture) (Pfizer). Approximately 0.5 ml of medium was added to each well, with the lung placed on the membrane at the air and medium interface. The cultures were maintained in 100% humidity with an atmosphere of 95% air and 5% CO_2 for either 24 hours (cyclopamine and SU5402 cultures) or 48 hours (FGF9 cultures). For 48 hour-cultures, medium was changed once after 24 hours.

Real-time RT-PCR

Total RNA was extracted from individual control and *Fgfr1;2-DKO* E12.5 lungs using Trizol reagent (Invitrogen) according to manufacturer's instructions. Total RNA (1μg per sample) was reverse-transcribed using the Superscript-II first strand synthesis system (Invitrogen). The cDNA prepared from 5 ng of RNA was PCR amplified and quantified using SYBRgreen (Applied Biosystems) following the manufacturer's protocol. The primers used were: *Fgf10 (*5′-TCCGTACAGTGTCCTGGAGAT-3′ and 5′-CCCCTTCTTGTTCATGGCTA-3′); *Lef1* (5′-AGCACGGAAAGAGAGACAGC-3′ and 5′-CCTGAAGTCGACTCCTGTAGC-3′); *Tbx4* (5′-AGAGGGACTCCAGCCTCTTC-3′ and 5′-TTTCCAGGTAGGATCGCTTG-3′); *Wnt2* (5′-GCAACACCCTGGACAGAGAT-3′ and 5′-ACAACGCCAGCTGAAGAGAT-3′); and *β-actin* (5′-CGGCCAGGTCATCACTATTGGCAAC-3′ and 5′-GCCACAGGATTCCATACCCAAGAAG-3′). Reactions were performed in duplicate. Expression values were normalized using *β-actin* as loading control, and compared using Student's t-test. The results were considered statistically significant if $p \leq 0.05$.

Quantitative Morphometric Analysis of *Shh* mutant lungs

The area of SMA and E-Cadherin expression in *Shh* mutant lungs cultured in DMSO (n=5) or SU5402 (n=5) was quantified using MetaMorph software (Molecular Devices). SMA expression values were normalized using E-Cadherin as a control for lung size and compared using a Student's t-test. Results were considered statistically significant if $p \leq 0.05$.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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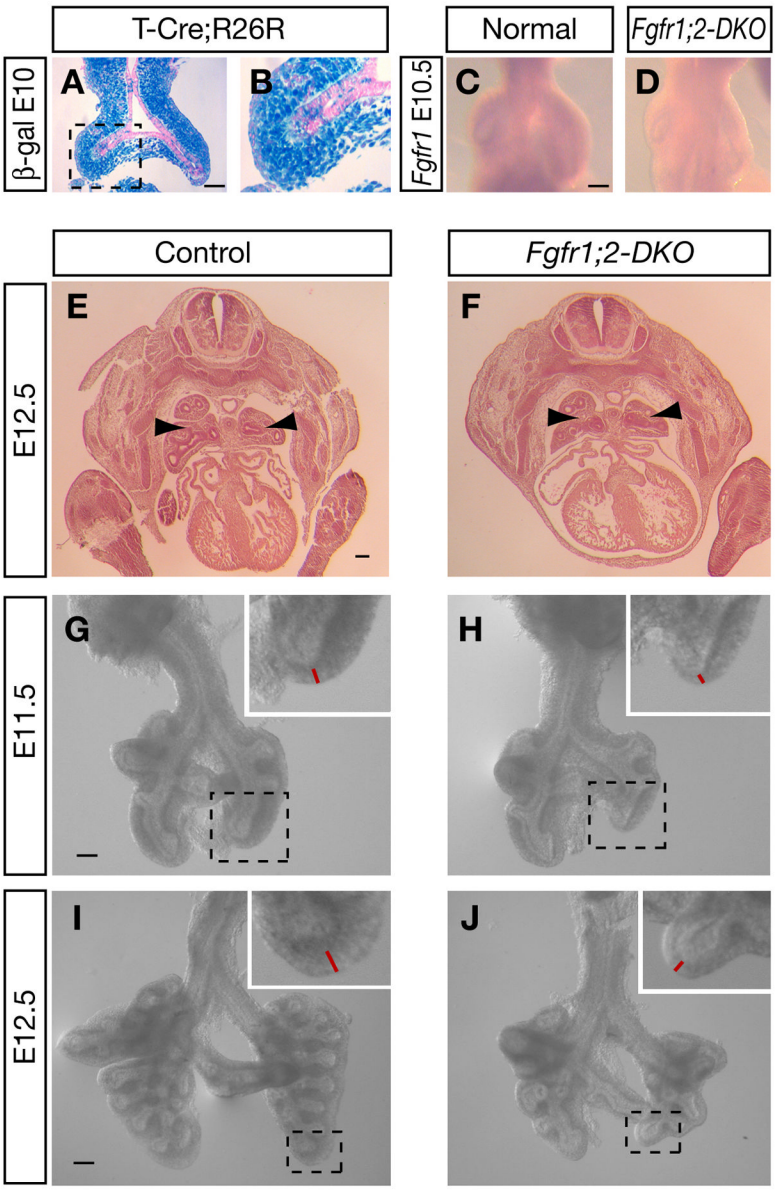

Figure 1. Inactivation *Fgfr1;2* in lung mesenchyme results in a reduction in mesenchyme thickness

(A,B) Section of β-gal stained T-Cre;R26R lung at Embryonic day (E) 10.0 shortly after lung initiation. Boxed area in A is magnified in B. Blue-stained mesenchymal and mesothelial cells encase the single layer columnar epithelium that contains a few blue cells. (C,D) *Fgfr1* expression as indicated by RNA in situ hybridization in E10.5 lungs using a probe that hybridizes to the portion of the transcript that is deleted by Cre-mediated recombination (Xu et al., 1999). *Fgfr1* expression is detected in the mesenchyme of the control lung (C), but is absent in the *Fgfr1;2-DKO* lungs (D). (E,F) Transverse sections of E12.5 embryos showing that the chest cavity of the *Fgfr1;2-DKO* mutant is similar in size compared to control. Arrowheads indicate lungs. (G–J) Brightfield images of lungs. Boxed regions are magnified in the insets. Red lines indicate the mesenchymal thickness at the caudal tip of the left lobe which is reduced in *Fgfr1;2-DKO* lungs. Epithelial branch number in the *Fgfr1;2-DKO* lung is comparable to that of normal at E11.5, but is reduced at E12.5.

In all figures, solid horizontal lines at the bottom of representative panels indicate relative scales of samples shown. Each mutant lung is shown at the same magnification as corresponding normal control.

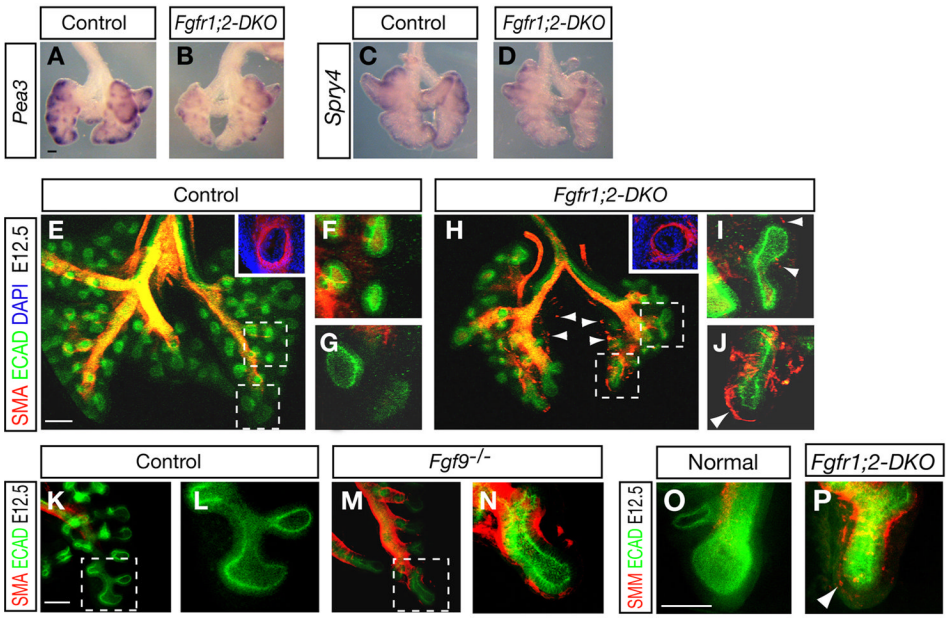

Figure 2. Inactivation of FGF9 signaling in lung mesenchyme leads to ectopic SMA-expressing cells

(A–D) In E12.5 *Fgfr1;2-DKO* lungs, the expression of *Pea3* and *Spry4* as detected by RNA in situ is reduced but not abolished. (E–P) Confocal images of antibody stained lungs. Colors are as labeled. Boxed areas in E, H, K, M are magnified in F/G, I/J, L and N, respectively. Insets in E and H are transverse section in the proximal lung showing that in the mutant, SMA-positive cells remain restricted to the parabronchial region, similar to the normal pattern. Arrowheads in J and P indicate ectopic SMA or SMM-positive cells near distal epithelial tips in the posterior mesenchyme, while arrowheads in H and I indicate ectopic SMA-positive cells near distal epithelial tips in other regions of the lung.

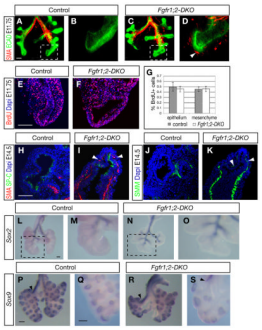

Figure 3. Ectopic SMCs arise independent of P-D patterning defects

(A–D) Confocal images of antibody stained lungs. Colors are as labeled. In the *Fgfr1;2-DKO* lung, ectopic SMA-positive cells are present, and the epithelial branching pattern remains normal. (E, F) BrdU analysis in lung sections. (G) Diagram illustrating that at E11.75 there is no statistically significant difference in cell proliferation in *Fgfr1;2-DKO* lungs compared to control. (H–K) Immunofluorescent staining in lung sections. J and K are adjacent sections of H and I, respectively. SP-C (green) is expressed in the distal epithelium of *Fgfr1;2-DKO* lungs, similar to control lungs. Arrowheads indicate ectopic SMA- or SMM-positive cells adjacent to distal epithelium in *Fgfr1;2-DKO* lungs. (L–S) By RNA in situ hybridization, *Sox2* and *Sox9* are both expressed in a normal pattern in *Fgfr1;2-DKO* lungs as shown in wholemount (L-O,P,R) and in sections (Q,S). Arrowheads indicate the distal extent of the proximal *Sox9* expression domain.

Note that the proximal expression domain of *Sox9* remains in the proximal mesenchyme, while the distal expression domain of Sox9 remains in the distal epithelium.

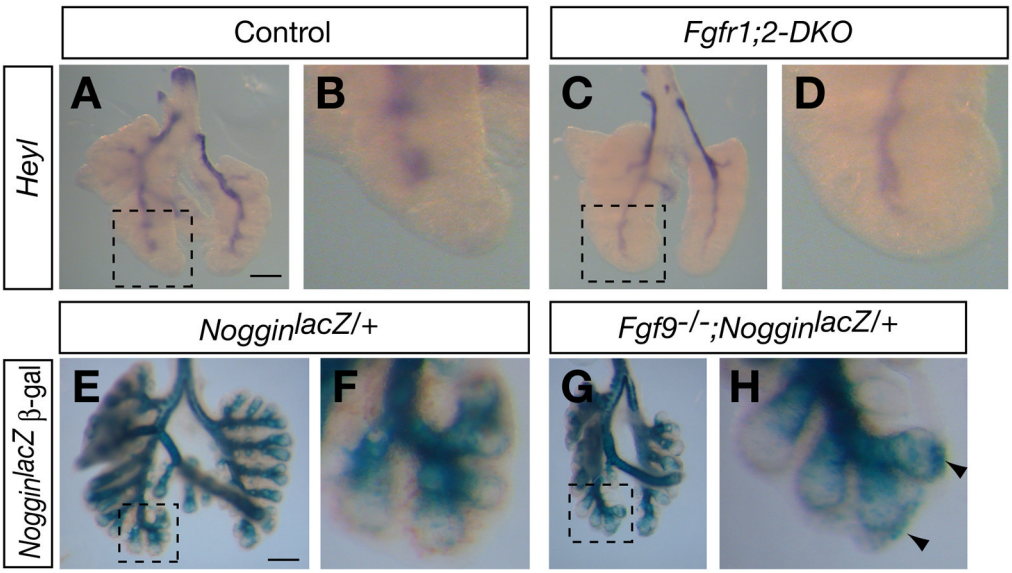

Figure 4. Inactivation of FGF9 signaling leads to ectopic airway SMCs

(A–D) By RNA in situ, in E12.5 *Fgfr1;2-DKO* lungs, *Heyl* is expressed in vascular SMCs, and is absent from the distal mesenchyme, similar to its pattern in control lungs. (E–H) *Noggin* expression as analyzed by β-gal staining in *Noggin*^{lacZ} lungs. In E12.5 *Fgf9*$^{-/-}$;*Noggin*$^{lacZ/+}$ lungs, in addition to the normal pattern in parabronchial mesenchyme, β-gal activity is also detected in the distal mesenchyme (arrowheads in H). Boxed areas in A, C, E and G are magnified in B, D, F and H, respectively.

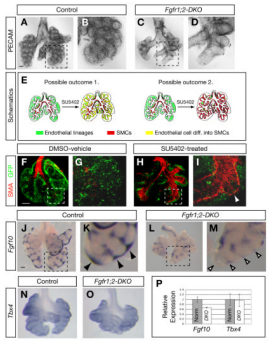

Figure 5. Downregulation of FGF signaling leads to reduced airway SMC progenitors

(A–D) Endothelial cells are labeled by anti-PECAM antibody. Boxed areas in A and C are magnified in B and D, respectively. Endothelial cells are reduced in *Fgfr1;2-DKO* lungs compared to control. (E) Schematics of possible outcomes of ectopic SMC lineage experiment using Tek-Cre;*Rosa-Gfp* lungs. In outcome 1 following SU5402 treatment, there is overlap of endothelial lineage (labeled by GFP, green) and SMA expression (red, with the combination being yellow), suggesting that there is differentiation of endothelial cells into SMCs. In outcome 2 following SU5402 treatment, there is no overlap of green GFP-expressing cells and red SMA-expressing cells, indicating that ectopic SMCs do not arise from the endothelial lineage. (F–I) E11.5 Tek-Cre;*Rosa-Gfp* lungs after 24 hours of culture. Boxed areas in F and H are magnified in G and I, respectively. In SU5402-treated lungs, ectopic SMA-expressing cells in the distal mesenchyme (arrowhead in I) are not GFP-positive. (J–O) *Fgf10* and *Tbx4* expression as assayed by RNA in situ hybridization. Boxed area in J and L are magnified in K and M, respectively. As indicated by arrowheads, *Fgf10* expression is reduced in the mutant. *Tbx4* expression is also slightly reduced in the mutant. (P) Quantification of relative gene expression in E12.5 *Fgfr1;2-DKO* lungs compared to normal control by real-time RT-PCR analysis.

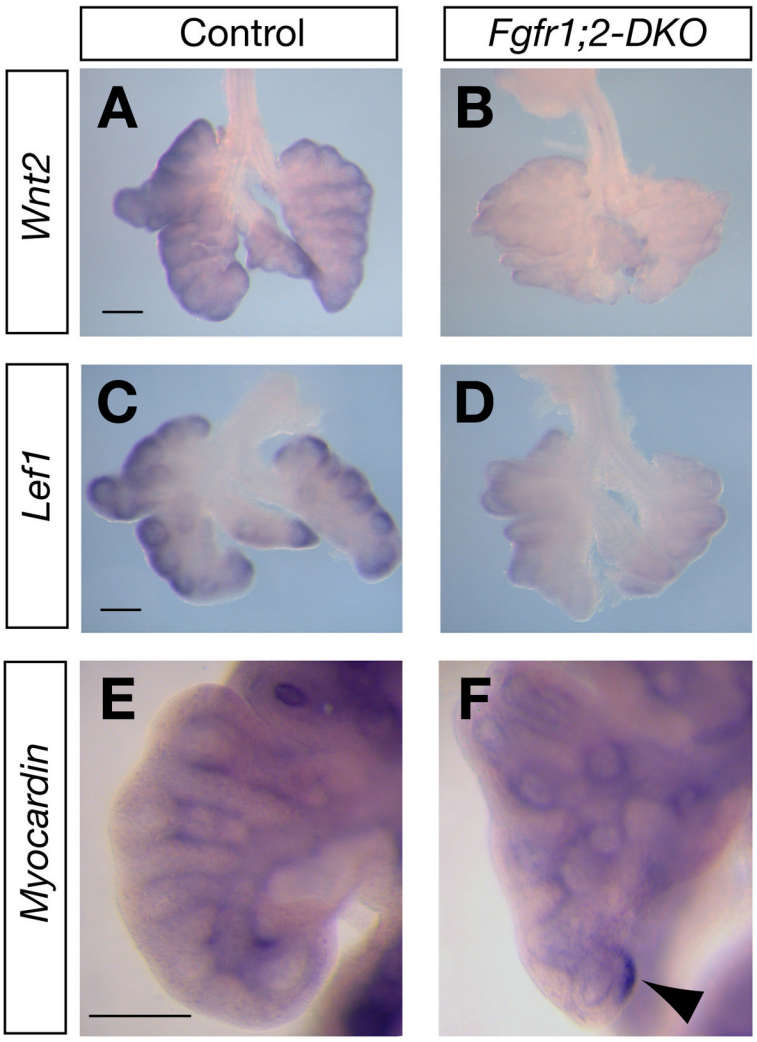

Figure 6. Inactivation of *Fgfr1;2* results in decreased WNT signaling activity and ectopic *Myocardin* expression

(A–F) Gene expression as detected by RNA in situ hybridization in E12.5 lungs. In *Fgfr1;2-DKO* lungs, the expression of *Wnt2* and *Lef1* is reduced. Arrowhead in F indicates ectopic *Myocardin* expression in the posterior distal mesenchyme where ectopic SMA-positive cells are most frequently observed. Ventral views are shown in all panels.

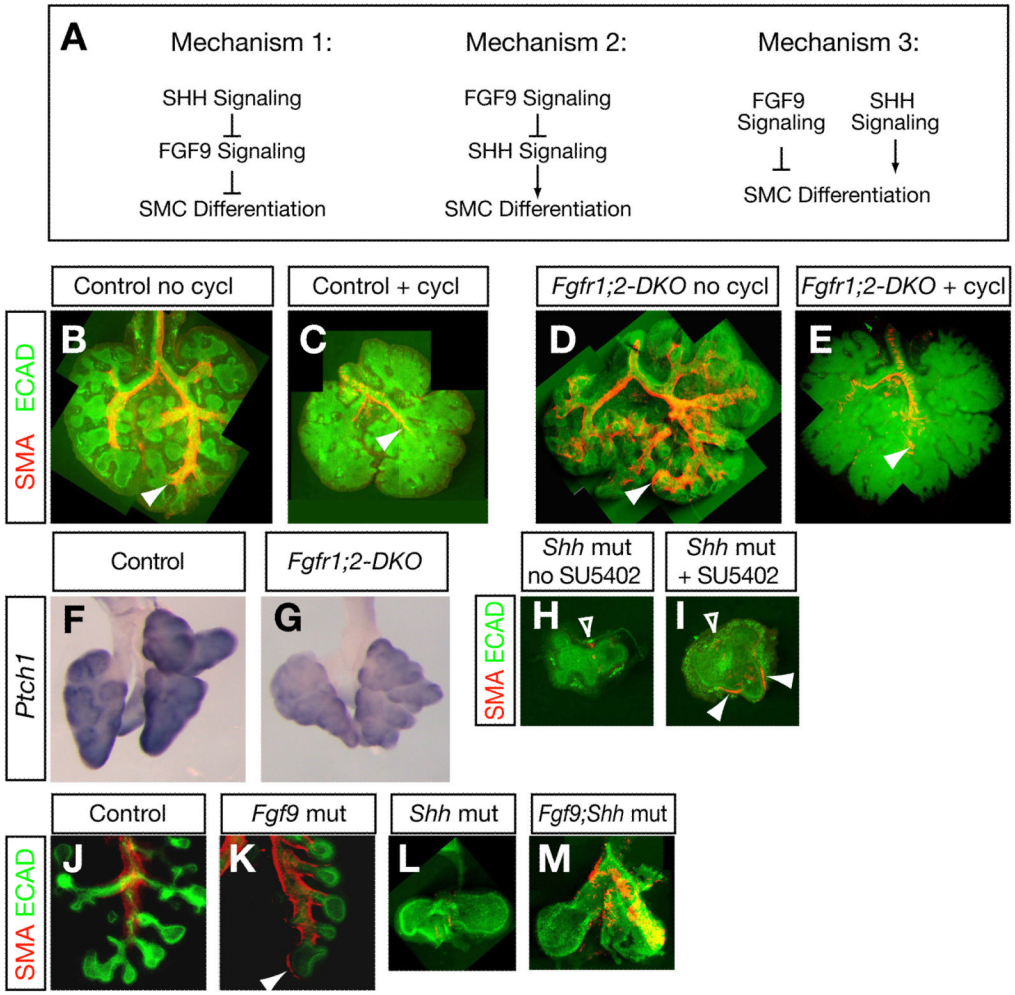

Figure 7. FGF9 and SHH pathways act in parallel to control SMC differentiation

(A) Three possible mechanisms to illustrate how FGF9 and SHH signaling pathways may coordinate to regulate SMC differentiation. (B–E, H–M) Confocal compositions of lungs with SMCs labeled by anti-SMA antibody (red) and epithelium labeled by anti-ECAD antibody (green). (B–E) E11.75 lungs cultured in the presence or absence of cyclopamine (cycl). Arrowheads indicate the distal boundary of SMA expression. Cycl treatment leads to a reduced SMA-positive domain in both control and *Fgfr1;2-DKO* lungs. (F,G) By RNA in situ hybridization, *Ptch1* expression is reduced in the E12.5 *Fgfr1;2-DKO* lung compared to normal control. (H,I) E11.5 $Shh^{-/-}$ mutant lungs cultured in the presence of SU5402 exhibit a larger SMA-positive domain than those cultured in the absence of SU5402. Open arrowheads indicate SMCs that are likely residual from esophageal tissue. Solid arrowheads indicate ectopic SMCs. (J–M) E12.5 lungs of indicated genotypes. Compared to normal control lung, SMCs are present in both the parabronchial and distal mesenchyme of *Fgf9* single mutant lung, absent in the *Shh* single mutant lung, and present in a disorganized pattern in the *Fgf9;Shh* double mutant lung.

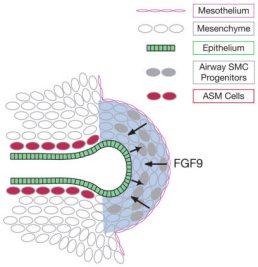

Figure 8. A model of the role of FGF9 signaling in airway SMC development

Diagram of a distal lung unit with one epithelial branch encased in mesenchyme and mesothelium. FGF9 expressed by the mesothelium and distal epithelium signals to FGFRs expressed in the mesenchyme. The range of FGF9 signaling is indicated by the blue shaded region in the distal mesenchyme. Airway SMC progenitors constitute a subset of the cells in the distal mesenchyme. FGF9 signals to distal mesenchymal cells to suppress airway SMC differentiation and promote proliferation. As a consequence of cell proliferation, some mesenchymal cells are translocated out of the FGF9 signaling range. These cells can then differentiate into SMCs in response to inductive cues from the epithelium.