Gpr177 regulates pulmonary vasculature development

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SUMMARY
Establishment of the functional pulmonary vasculature requires intimate interaction between the epithelium and mesenchyme. Previous genetic studies have led to inconsistent conclusions about the contribution of epithelial Wnts to pulmonary vasculature development. This discrepancy is possibly due to the functional redundancy among different Wnts. Here, we use Shh-Cre to conditionally delete Gpr177 (the mouse ortholog of Drosophila Wntless, Wls), a chaperon protein important for the sorting and secretion of Wnt proteins. Deletion of epithelial Gpr177 reduces Wnt signaling activity in both the epithelium and mesenchyme, resulting in severe hemorrhage and abnormal vasculature, accompanied by branching defects and abnormal epithelial differentiation. We then used multiple mouse models to demonstrate that Wnt/β-catenin signaling is not only required for the proliferation and differentiation of mesenchyme, but also is important for the maintenance of smooth muscle cells through the regulation of the transcription factor Kruppel-like factor 2 (Klf2). Together, our studies define a novel mechanism by which epithelial Wnts regulate the normal development and maintenance of pulmonary vasculature. These findings provide insight into the pathobiology of congenital lung diseases, such as alveolar capillary dysplasia (ACD), that have abnormal alveolar development and dysmorphic pulmonary vasculature.

KEY WORDS: Wntless, Wnt, Klf2, Lung morphogenesis, Hemorrhage, Mouse, Wls

INTRODUCTION
Generation of a functional pulmonary vasculature requires close interplay of epithelium and mesenchyme. Previous studies have shown that epithelial signaling molecules, including sonic hedgehog and vascular endothelial growth factors (VEGFs), regulate the proliferation and differentiation of mesenchymal cells (Healy et al., 2000; Miller et al., 2004; Del Moral et al., 2006; White et al., 2006; Chen et al., 2010). However, recent studies regarding the role of epithelial Wnts in vasculature development generate inconsistent conclusions (Shu et al., 2002; Rajagopal et al., 2008). In one study, deletion of Wnt7b leads to hemorrhage and abnormal vasculature (Shu et al., 2002). In another study, where multiple Wnt7b mutant alleles have been used, Wnt7b disruption has minimal effects on the formation of the pulmonary vasculature (Rajagopal et al., 2008). We reason that compensation among multiple Wnt proteins present in the epithelium in different genetic backgrounds may underlie this discrepancy. To circumvent this issue, we used a mouse line harboring floxed Gpr177 (Wls – Mouse Genome Informatics) the mouse ortholog of Drosophila Wntless, which regulates the sorting and secretion of Wnt proteins (Bänziger et al., 2006; Bartscherer et al., 2006; Fu et al., 2009). Conditional deletion of Gpr177 in the epithelium results in poorly developed vasculature with reduced proliferation and differentiation of mesenchyme. Further analysis revealed that Wnt/β-catenin is required for the maintenance of pulmonary vascular smooth muscle cells through the transcription factor Kruppel-like factor 2 (Klf2).

MATERIALS AND METHODS

Mice
The Shh-Cre, Deromo1-Cre, smooth muscle myosin heavy chain (SMMHC)-CreER (MHC-CreER), Gpr177loxp/loxp, β-Cateninloxp/loxp and Axin2-lacZ mouse strains, and genotyping methods have been reported previously (Brault et al., 2001; Yu et al., 2003; Harle et al., 2004; Yu et al., 2005; Wirth et al., 2008; Fu et al., 2011). All mouse experiments were approved by the IACUC at the University of Rochester.

Tissue preparation, histology, immunostaining and X-gal staining
The immunostaining and histochemistry were performed as previously described (Que et al., 2009; Rodriguez et al., 2010; Liu et al., 2013). The antibodies used for immunohistochemistry include: rabbit anti-Sox2 (Seven Hills), rabbit anti-Sox9 (Millipore), rabbit anti-cleaved caspase 3 (Cell Signaling), rat anti-Sgcb1a1 (R&D), mouse anti-a-tubulin (Sigma), rabbit anti-β-Catenin (Sigma), rabbit anti-α-smooth muscle actin (Sigma), goat anti-Klf2 (Santa Cruz) and mouse anti-smooth muscle actin (Sigma). The secondary antibodies were either fluorescence- or DAB-conjugated, and images were acquired with a Leica SP1 confocal microscope. Whole-mount X-gal staining was performed as previously described (Rodriguez et al., 2010; Chen et al., 2012).

In situ hybridization
In situ hybridization analysis was performed as described previously (Que et al., 2006; Fu et al., 2009).

Cell culture, gene knockdown and luciferase assay
Rat lung vascular smooth muscle cell line PAC1 was maintained in 10% FBS DMEM medium. Klf2 shRNA were purchased from Sigma-Aldrich. shRNA-mediated knockdown was performed as previously described (Liu et al., 2013). To test whether the Klf2 promoter is regulated by Wnt/β-catenin signaling, a 3 kb promoter region of the Klf2 gene was cloned into the pGL3 luciferase report vector, and then co-transfected into PAC1 cells with CatcLEF or Wnt3a plasmids. Point mutation of the potential binding sites was generated with QuickChange II XL Site-Directed Mutagenesis kit (Aligent). Luciferase activity was determined 24-48 hours after transfection using a dual reporter luciferase kit (Promega).

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Accepted 26 June 2013
Reverse transcription PCR
RNA extraction and cDNA synthesis were performed as previously described (Rodriguez et al., 2010). The primer sequences are provided in the supplementary material Table S1.

Western blot analysis
Primary antibodies were Ki62 (Chemicon) and β-actin (Abcam); the blots were processed as previously described (Que et al., 2007).

Statistical analysis
For the measurement of the proliferation index, five different high-magnification views for each tissue section and total 20 sections were examined for both mutant and wild-type lungs at E12.5 and E14.5. Data are expressed as mean±s.e.m. Differences between two samples were analyzed by Student’s t-test. P-values of 0.05 or less were considered statistically significant.

RESULTS AND DISCUSSION
Deletion of epithelial Gpr177 in Shh-Cre;Gpr177lox/-lox mutants leads to neonatal lethality and severe pulmonary hemorrhage
Wnt signaling is crucial for the initiation of lung morphogenesis (Goss et al., 2009; Harris-Johnson et al., 2009; Chen et al., 2010). Previous characterization studies have shown that lung epithelial cells express Wnt7b and Wnt11 (Lako et al., 1998; Cohen et al., 2009). To obtain a comprehensive picture of Wnts expressed in the early lung, we first performed RT-PCR and found that Wnt2, Wnt2b, Wnt3a, Wnt5a, Wnt5b, Wnt6, Wnt7b, Wnt8a, Wnt9a, Wnt11 and Wnt16 are expressed in the epithelium at this stage (supplementary material Fig. S1A). We observed that Wnt3a, Wnt5a, Wnt6, Wnt7b, Wnt8a, Wnt9a, Wnt11 and Wnt16 are present at E11.5, whereas Wnt1, Wnt3, Wnt4, Wnt7a, Wnt8b, Wnt9b, Wnt10a and Wnt10b fall below detectable levels at this stage (supplementary material Fig. S1A). We further observed that Wnt3a, Wnt5a, Wnt6, Wnt7b, Wnt11 and Wnt16 are expressed in the epithelium at this stage (supplementary material Fig. S1A).

Gpr177 is ubiquitously expressed in the E9.5 lung (supplementary material Fig. S1B), and we used Shh-Cre to delete Gpr177 in the epithelium (Gpr177Δ/Δ). The separation of the trachea and esophagus is completed, and the lung budding is also normal. These findings are different from the Shh-Cre;β-cateninlox/lox mutants, which lack the trachea and lung (Harris-Johnson et al., 2009), supporting the hypothesis that mesenchymal Wnts (Wnt2/2b) are important for the specification of respiratory cells in the early foregut (Goss et al., 2009). Consistent with the role of Gpr177 in the regulation of Wnt secretion, but not production, the transcript levels of epithelial Wnts, including Wnt6, Wnt7b and Wnt11 are not altered significantly after Gpr177 deletion at E11.5 (P>0.05, supplementary material Fig. S1C). By contrast, canonical Wnt signaling is decreased in both the epithelium and mesenchyme of the E12.5 Gpr177Δ/Δ, Axin2-lacZ lung (Fig. 1A,B). Immunostaining of activated β-catenin confirms the decrease of signals in the epithelium and mesenchyme (supplementary material Fig. S1D,E). These findings were further supported by the decrease in the transcript levels of Wnt/β-catenin downstream targets Axin2 and Lef1 (supplementary material Fig. S1F).

All of the Gpr177Δ/Δ mutants (n=23) die at neonatal stage with reduced lung sizes (7.1±3.6 mg versus 25.4±2.9 mg (P<0.01), and the lung branching seems delayed in the mutants (Fig. 1C; supplementary material Fig. S1G). More importantly, severe hemorrhaging is present throughout the interstitial space, apparently more severe than previously reported in the Wnt7b-null mutants (Shu et al., 2002) (Fig. 1D,E). Gpr177 was also deleted in the mesenchyme with Dermo1-Cre (Dermo1-Cre), which is activated at ~E10.5 (De Langhe et al., 2008), and the mutants die between E15.5 and 17.5 without hemorrhage or visible vasculature defects (supplementary material Fig. S1H,J). In both Gpr177Δ/Δ and Dermo1-Cre;Gpr177lox/-lox mutants, the airways are enlarged, which is more pronounced in the former ones (supplementary material Fig. S1H,J). Previous studies have shown that the only function for Gpr177 is to regulate the sorting and secretion of Wnt proteins (Bánziger et al., 2006; Port and Basler, 2010; Fu and Hsu, 2013). Therefore, these findings suggest that the Gpr177-mediated production of epithelial Wnts is important for pulmonary vasculature development, and multiple Wnts in the epithelium possibly compensate for the loss of Wnt7b (Shu et al., 2002; Rajagopal et al., 2008).

Limited alveolar development in the lungs of Shh-Cre;Gpr177lox/-lox mutants
We next asked whether there are defects in the differentiation of the epithelium. The proximal-distal patterning of the epithelial progenitor cells seems unaffected, as shown by normal distribution of Sox2+ and Sox9+ cells in the E14.5 mutant lungs (Que et al., 2009; Rawlins, 2011). Similarly, Sox2 expression is maintained in the proximal airways, while Sox9 is lost in the distal lung in both mutants and controls at E18.5 (Fig. 2B). All five
mutant lungs. The insets are the high magnification view of the boxed region. Scale bars: 50 μm.

Fig. 2. Loss of epithelial Gpr177 leads to limited alveolar development in the E18.5 lung. (A) Proximal-distal specification of epithelial progenitor cells is unaffected in the E14.5 mutant lung. Proximal and distal epithelial progenitor cells are labeled with Sox2 and Sox9, respectively. (B) Sox2 is maintained in the proximal airways of the normal and mutant lungs at E18.5. (C,D) Clara cells (Scgb1a1*) and ciliated cells (α-acetylated tubulin*) are present in the proximal airways of the normal and mutant lungs. (E-G) The numbers of type I cells (T1α, arrowheads) and type II cells (SpC* and ABCA3*, arrowheads) are reduced in the mutant lungs. The insets are the high magnification view of the boxed region. Scale bars: 50 μm.

epithelial types (Clara, ciliated, neuroendocrine, type I and II cells) are present in the mutants (Fig. 2C-G). However, the numbers of type I and type II alveolar cells are reduced in the mutants. Although Clara [Cc10 (Scgb1a1*)], neuroendocrine (CGRP*) and ciliated cells (α-acetylated tubulin*) are lined along the bronchiolar and bronchioalveolar airways in the proximity of type I (T1α) and type II cells (SpC* and ABCA3*) in the control lungs (Fig. 2C-G and data not shown), the enlarged airways in the central region of the mutant lungs are packed with Clara and ciliated cells (Fig. 2C,D), and only a small number of type I and type II cells are present at the peripheral region (Fig. 2E-G). A previous study showed that deletion of the β-catenin gene in the Spc-rtTA;tetO7CMV-Cre;β-catenin<sup>-loxp/loxp</sup> mutants also affects the differentiation of the epithelium (Mucenski et al., 2003). Our findings therefore support the observation that epithelial Wnts control epithelial morphogenesis in an autocrine fashion. However, it is also possible that the mesenchymal defects seen in the Gpr177<sup>−/−</sup> mutants impact epithelial development and contribute to the abnormal phenotypes. Previous studies have shown that signaling molecules (e.g. Fgf10) from the mesenchyme regulate lung morphogenesis (Weaver et al., 2000; Volckaert et al., 2011). Moreover, heterozygous loss of Foxf1, a transcription factor enriched in the mesenchyme, also leads to abnormal vasculature development and lung branching defects (Lim et al., 2002; Stankiewicz et al., 2009). Intriguingly, the transcript levels of Foxf1 are reduced in the Gpr177<sup>−/−</sup> mutants (supplementary material Fig. S1K). In future studies it will be interesting to determine whether the reduced levels of Foxf1 also contribute to the abnormal lung morphogenesis in the Gpr177<sup>−/−</sup> mutants. Notably, heterozygous loss-of-function FOXF1 mutations have been associated with the pathobiology of alveolar capillary dysplasia (ACD), which is characterized by the dramatically reduced numbers of capillary vessels and poor alveolar development (Bishop et al., 2011; Mestan and Steinhome, 2011).

Deletion of epithelial Gpr177 affects vasculature development and reduces the proliferation of mesenchymal cells

The severe hemorrhaging in the Gpr177 mutant lung prompted us to examine pulmonary vasculature development. The numbers of blood vessels (Pecam1*) in the E11.5 mutant lung are decreased when compared with the control (supplementary material Fig. S2A), and the difference becomes more prominent at E14.5 (Fig. 3A). Similarly, the numbers of smooth muscle cells (SMA*) surrounding blood vessels and airways are reduced at E12.5 and E14.5 (Fig. 3B; supplementary material Fig. S2B), and the transcript levels of Pecam1 and SMA are also reduced in the E12.5 mutant lung (supplementary material Fig. S2C). In addition, deletion of Gpr177 results in significant decrease in the proliferation of mesenchymal cells at E12.5 and E14.5 (<0.05, Fig. 3C,D; supplementary material Fig. S2D,E), whereas the proliferation of epithelial cells is slightly increased (Fig. 3C,D; supplementary material Fig. S2D,E). Moreover, the proliferation of smooth muscle cells and endothelium is also significantly reduced at E14.5 (<0.05, supplementary material Fig. S2F,G).

Loss of Gpr177 reduces the levels of basement membrane proteins laminin and tenasin C (Tnc) in the mutant lung at E14.5 (Fig. 3E,F). Electronic microscopy (EM) further shows thinning of the basement membrane and disjointed endothelial junctions at E16.5 (supplementary material Fig. S3A). TnC has previously been identified as a downstream player in the Wnt/β-catenin pathway (Cohen et al., 2009), suggesting that Gpr177 in the epithelium modulates mesenchymal differentiation in a canonical Wnt signaling-dependent manner. Consistently, deletion of β-catenin in the mesenchyme with Derm1-Cre leads to reduced numbers of blood vessels (supplementary material Fig. S3B) (De Langhe et al., 2008). Through examining the expression of genes that are known
to regulate vasculature development, we determined that VEGF-c is reduced in the E16.5 Gpr177^Δ/Δ mutant lung (supplementary material Fig. S3C). Intriguingly, both the transcript and protein levels of Kruppel-like factor 2 (Klf2) are also consistently reduced in the mutant lungs at E12.5, E14.5, E16.5 and E18.5 (Fig. 3G,H; supplementary material Fig. S3D). Klf2 is a zinc-finger transcription factor that has been shown to regulate the proliferation and migration of vascular smooth muscle cells (Wu et al., 2008). Klf2 deletion leads to destabilized blood vessels and severe intra-embryonic and intra-amniotic hemorrhage (Kuo et al., 1997). Taken together, these findings indicate epithelial Gpr177 regulates pulmonary vasculature development through modulating canonical Wnt signaling in the mesenchyme, and they also suggest that Klf2 is a Wnt signaling mediator.

**Wnt/β-catenin regulates the proliferation of vascular smooth muscles through Klf2**

A stabilized and mature vessel wall is required for maintaining the integrity of blood vessels. We asked whether canonical Wnt signaling is required for the maintenance of the vascular smooth muscle cells lining the vessel walls. To address this issue, we deleted β-catenin in the smooth muscle cells using the SMMHC-CreER mouse line (Wirth et al., 2008; Gomez et al., 2013). Tamoxifen was injected into the mothers that harbor E11.5 SMMHC-CreER;β-catenin^loxP/loxP^ and control SMMHC-CreER;β-catenin^loxP/loxP^ embryos, and the lungs were examined at E14.5 (Fig. 4A). Removal of β-catenin leads to the reduced number of smooth muscle cells surrounding both the bronchiolo and blood vessels (Fig. 4B-C’), accompanied by reduced expression of Klf2 proteins (Fig. 4D,E).

We then asked whether canonical Wnt signaling directly regulates the transcription of Klf2. Three potential β-catenin-binding sites were identified in the 3 kb promoter region of the Klf2 gene, and this 3 kb DNA was cloned into the pGL3 luciferase reporter construct (Fig. 4F). Upon co-transfection with a dominant Wnt signaling activator, CatcLEF (Fu et al., 2009), or of Wnt3a plasmids into PAC1 cells, the Klf2-driven luciferase reporter signal is increased by 2.4-fold and 1.6-fold, respectively (Fig. 4F).

Furthermore, point mutation analysis revealed that the two distal binding sites, 408 bp and 1533 bp, upstream of the transcription start site are crucial for the functional regulation of Klf2 by β-catenin (Fig. 4G), supporting the observation that Klf2 is a direct Wnt/β-catenin downstream target. We next tested whether Klf2 is required for the proliferation of PAC1 cells by using virus-mediated shRNA to knockdown Klf2. The knockdown efficiency is ~70% for both shRNA constructs that target two individual regions of the Klf2 mRNA sequence (supplementary material Fig. S3E). Significantly, knockdown of Klf2 leads to a 57% decrease in the proliferation of PAC1 cells (Fig. 4H). Together, these findings suggest that the canonical Wnt/β-catenin signaling is crucial for the expansion and maintenance of vascular smooth muscle cells, and this function is in part mediated through Klf2.

In summary, we use multiple mouse models to demonstrate that Gpr177 regulation of Wnt secretion from the lung epithelium is important for both epithelial and mesenchymal development. These findings are consistent with the crucial roles of Wnt signaling in lung development and maintenance (Morrisey and Hogan, 2010; Giangreco et al., 2012). Conditional deletion of epithelial Gpr177 affects lung branching, epithelial differentiation and pulmonary vasculature morphogenesis. Our further analysis revealed that Wnt/β-catenin is required for the maintenance of vascular smooth muscle cells through Klf2. Together, these findings not only provide important information about the normal mechanism by which...
epithelial Wnts control vasculature development, but provide important insights into birth defects, including alveolar capillary dysplasia, that present with abnormal alveologenesis and dysmorphic vasculature (Bishop et al., 2011; Mestan and Steinhorn, 2011).

Acknowledgements
We are thankful for the stimulating discussions with Drs Brigid Hogan and Barry Stripp (Duke University, Durham NC, USA), and Drs Michael O’Reilly and Thomas Mariani (University of Rochester, NY, USA). We are grateful that Dr Gary K. Owens and Laura Shankman (Cardiovascular Research Center of the University of Virginia, Charlottesville, USA) shared with us the mouse lung primordium. Barry Stripp (Duke University, Durham NC, USA), and Drs Michael O’Reilly and Thomas Mariani (University of Rochester, NY, USA) are thankful for the stimulating discussions with Drs Brigid Hogan and Barry Stripp (Duke University, Durham NC, USA), and Drs Michael O’Reilly and Thomas Mariani (University of Rochester, NY, USA).

Funding
This research is supported by the March of Dimes Basil O’Connor Starter Scholar Research Award (to J.Q.).

Competing interests statement
The authors declare no competing financial interests.

Author contributions
M.J. and J.Q. designed the experiments. M.J., W.K. and J.F. performed the experiments. S.O. provided the embryos. and their littermates, and the embryos were harvested at E14.5. Loss of β-catenin leads to decreased number of smooth muscle cells surrounding the blood vessels and airways in the SMMPHC-CreER β-catenin<sup>+</sup> mutants. There is a discontinuous smooth muscle ring surrounding the blood vessels (arrowheads) and airways (arrows). Asterisks indicate blood vessels.

(B)–(D) β-Catenin deletion leads to reduced levels of Klf2 in the smooth muscle cells of the blood vessels (arrowheads) and airways (arrows). Klf2 expression is maintained in a subpopulation of mesenchymal cells (SMAs negative). Inactivation of the beta-catenin gene by Wnt1-cre-mediated deletion results in dramatic brain malformation and failure of craniofacial development. Development 128: 1253-1264.


