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RESEARCH REPORT

Wnt/Fgf crosstalk is required for the specification of basal cells in the mouse trachea

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ABSTRACT

Basal progenitor cells are crucial for the establishment and maintenance of the tracheal epithelium. However, it remains unclear how these progenitor cells are specified during foregut development. Here, we found that ablation of the Wnt chaperone protein Gpr177 (also known as Wntless) in mouse tracheal epithelium causes a significant reduction in the number of basal progenitor cells accompanied by cartilage loss in *Shh-Cre;Gpr177$^{loxp/loxp}$* mutants. Consistent with the association between cartilage and basal cell development, Nkx2.1^{+}p63^{+} basal cells are co-present with cartilage nodules in *Shh-Cre;Ctnnb1$^{DM/loxp}$* mutants, which maintain partial cell-cell adhesion but not the transcription regulation function of β-catenin. More importantly, deletion of *Ctnnb1* in the mesenchyme leads to the loss of basal cells and cartilage, concomitant with reduced transcript levels of Fgf10 in *Dermo1-Cre;Ctnnb1$^{loxp/loxp}$* mutants. Furthermore, deletion of Fgf receptor 2 (*Fgfr2*) in the epithelium also leads to significantly reduced numbers of basal cells, supporting the importance of Wnt/Fgf crosstalk in early tracheal development.

KEY WORDS: Fgf, Wnt/β-catenin, Wntless, Airway, Basal progenitor, Cartilage, Mouse

INTRODUCTION

Basal cells are multipotent progenitor cells responsible for generation of the airway epithelium during development and injury repair (Hong et al., 2004; Que et al., 2009; Rock et al., 2009; Yang et al., 2018). Previous studies indicate that the epithelial-mesenchymal interactions are crucial for basal cell development although the underlying molecular mechanism remains largely unknown (Hines et al., 2013; Volckaert et al., 2013). It has been shown that the numbers of basal cells are positively correlated with the differentiation of mesenchymal cells into cartilage at the early stage of tracheal development (Hines et al., 2013). Prior to the separation of the trachea from the anterior foregut, the growth factor Fgf10 is enriched in the ventral mesenchyme where cartilage progenitor cells arise (Que et al., 2007). Interestingly, ubiquitous Fgf10 overexpression promotes basal cell lineage commitment and suppresses ciliated cell differentiation (Volckaert et al., 2013). Conversely, deletion of *Fgf10* or its receptor *Fgfr2* results in the loss of basal cells (Balasooriya et al., 2017; Volckaert et al., 2013). In addition, ubiquitous overexpression of the Wnt inhibitor Dkk1 at embryonic day (E) 10.5 but not E12.5 also leads to increased numbers of basal cells (Volckaert et al., 2013). Wnt7b is able to induce Fgf10 expression during airway epithelial regeneration (Volckaert et al., 2011, 2017). Wnt signaling is also essential for initial specification of respiratory cells from the early foregut. Loss of Wnt2/2b, which are enriched in the ventral foregut mesenchyme, results in failed specification of respiratory progenitor cells (Nkx2.1^{+}) (Goss et al., 2009). Consistent with this, deletion of the canonical Wnt signaling mediator β-catenin also leads to lung and tracheal agenesis, and the anterior foregut becomes an esophageal-like tube lined with stratified squamous epithelium underlined by extensive basal progenitor cells (Goss et al., 2009; Harris-Johnson et al., 2009).

We and others previously showed that respiratory cell fate is specified properly despite severe vasculature abnormalities following deletion of the Wnt chaperon protein Gpr177 (also known as Wntless or Wls) in *Shh-Cre;Gpr177$^{loxp/loxp}$* mutants. Interestingly, in this study we found a significant loss of basal progenitor and cartilage cells in these mutants. Deletion of *Ctnnb1* (encoding β-catenin) in the mesenchyme also leads to the loss of basal progenitor cells and cartilage concomitant with reduced levels of Fgf10 in the trachea of *Dermo1-Cre;Ctnnb1$^{loxp/loxp}$* mutants. Moreover, the numbers of basal progenitor cells are also significantly reduced when the Fgf10 receptor *Fgfr2* is deleted in the epithelium. Together, these findings support the suggestion that in the developing trachea epithelial Wnts activate β-catenin in the mesenchyme to modulate Fgf10 levels, which in turn regulate basal cell specification through epithelial Fgfr2.

RESULTS AND DISCUSSION

Blocking Wnt secretion from the epithelium leads to a reduced number of basal progenitor cells and cartilage defects in the trachea of *Shh-Cre;Gpr177$^{loxp/loxp}$* mutants

We previously showed that deletion of *Gpr177* in the epithelium results in abnormal differentiation and proliferation of vascular smooth muscle cells in the developing lung, and that the mutants succumb at birth as a result of severe pulmonary hemorrhage (Jiang et al., 2013). A recent study demonstrated that deletion of *Gpr177* also leads to tracheal cartilage defects in these mutants (Snowball et al., 2015). We investigated whether basal cell specification is affected upon *Gpr177* deletion given the correlation of cartilage and basal cell numbers (Hines et al., 2013). Consistent with previous findings (Snowball et al., 2015), *Gpr177* deletion resulted in the loss of cartilage progenitor cells (Sox9^{+}) whereas smooth muscle cells (SMA^{+}) were expanded in the trachea of *Shh-Cre;Gpr177$^{loxp/loxp}$* mutants (Fig. 1A,B). Intriguingly, basal cells (p63^{+}) were rarely detected in the trachea of *Shh-Cre;Gpr177$^{loxp/loxp}$* mutants at the different developing stages examined (Fig. 1A,B; *n*=3 for each stage). These results suggest that Wnts from the epithelium act in both autocrine and paracrine manners to regulate tracheal development. Notably, *Gpr177* deletion did not appear to affect the specification of

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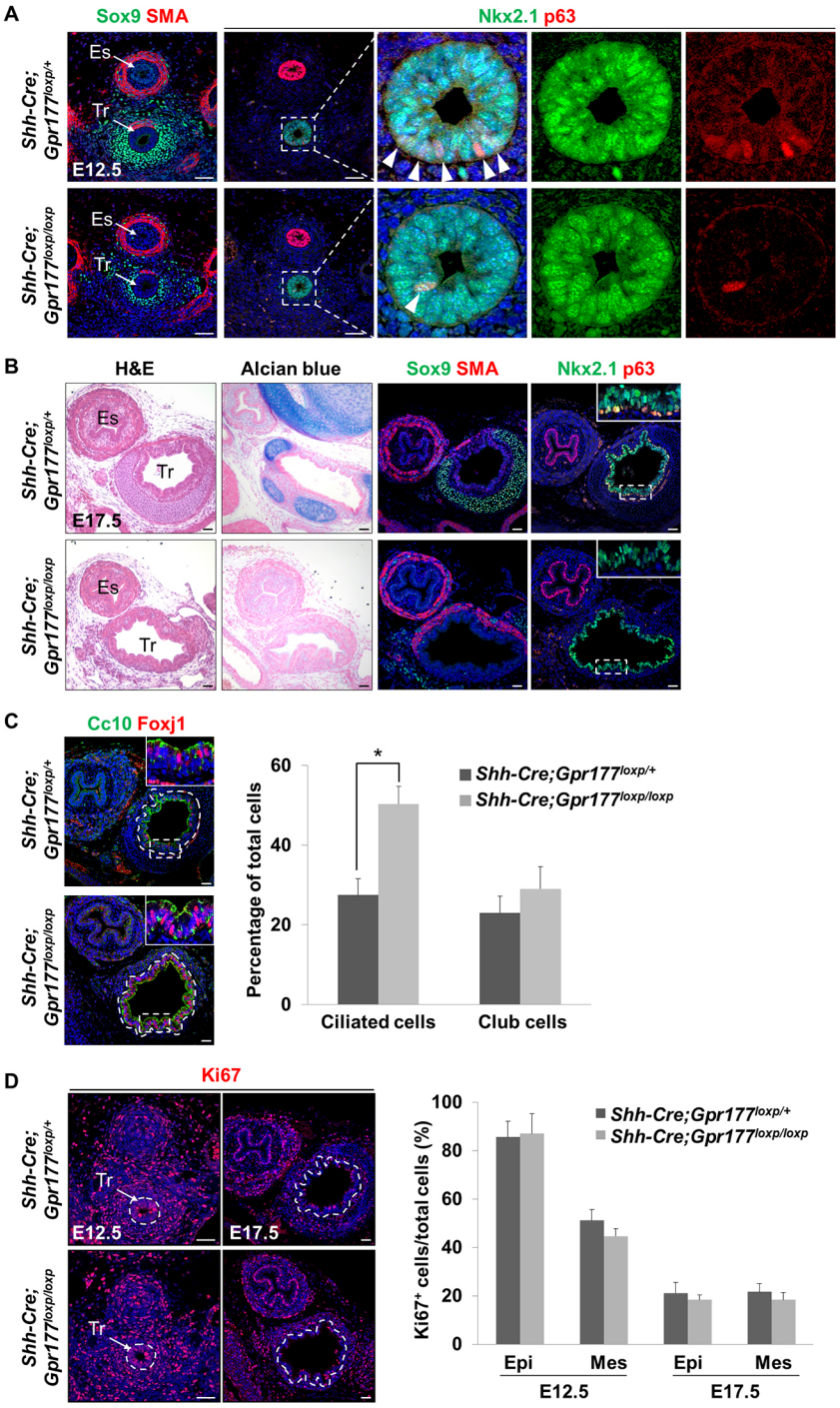

Fig. 1. Loss of epithelial Wnt secretion leads to abnormal development of tracheal cartilage and basal cells. (A) Loss of epithelial *Gpr177* causes a dramatic reduction in the numbers of cartilage progenitor cells (Sox9$^+$) and basal cells (p63$^+$) in the trachea of *Shh-Cre; Gpr177$^{loxp/loxp}$* mutants at E12.5. Arrowheads indicate the basal cells. (B) Loss of epithelial *Gpr177* leads to the loss of cartilage (Alcian Blue$^+$ Sox9$^+$) and a reduction in the number of basal cells at E17.5. (C) Loss of epithelial *Gpr177* increases the number of ciliated cells (Foxj1$^+$) in the mutant trachea (*P<0.05, n=3 for each). Cc10 (Scgb1a1) marks club cells. (D) Proliferation (determined by Ki67 immunohistochemistry) of both epithelial and mesenchymal cells is slightly but not significantly decreased in the mutant trachea (P>0.05, n=3 for each). Data are represented as mean±s.e.m. Es, esophagus; H&E, Hematoxylin & Eosin; Tr, trachea. Dashed lines outline the basement membranes. Scale bars: 50 µm.

respiratory cells from the early foregut, and all the epithelial cells express Nkx2.1 (Fig. 1A,B). Increased differentiation of ciliated cells (Foxj1$^+$) was also observed in the tracheal epithelium at E18.5 (Fig. 1C; *P<0.05, n=3 for each). In addition, although loss of *Gpr177* led to the reduced proliferation of both epithelial and mesenchymal cells, the difference between mutants and wild-type controls was not significant (Fig. 1D; P>0.05, n=3 for each).

Tracheal basal cells (Nkx2.1$^+$p63$^+$) and cartilage nodules are present in the unseparated foregut of *Shh-Cre;Ctnnb1$^{DM/loxp}$* mutants

β-Catenin has two major roles, mediating Wnt-activated transcription regulation and cell-cell adhesion functions (Heuberger and Birchmeier, 2010). Thus far, genetic studies assessing the role of β-catenin in the developing lung have relied on the *Ctnnb1^{loxp}* allele, which ablates both transcription regulation and cell-cell adhesion functions upon Cre-mediated recombination (Brault et al., 2001; De Langhe et al., 2008; Goss et al., 2009; Stenman et al., 2008). Although many of the phenotypic changes seem to recapitulate observations in mutants lacking Wnt ligands (Goss et al., 2009; Stenman et al., 2008), it is unclear whether the cell-cell adhesive function of β-catenin contributes to lung and tracheal development.

Another mouse line containing a mutated *Ctnnb1* allele (*Ctnnb1* double mutant; hereafter referred as *Ctnnb1^{DM}*) was recently established (Valenta et al., 2011). This mutant form of β-catenin includes a single amino acid change (aspartic acid mutated to

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alanine, D164A) in the first Armadillo repeat of β-catenin, which prevents TCF-dependent transcription regulation while maintaining the ability to mediate cellular adhesion (Valenta et al., 2011). Notably, *Ctnnb1*$^{DM/DM}$ mutants die at E10.5 (Gay et al., 2015). We combined this allele with the *Ctnnb1*^{loxp} allele to address whether β-catenin acts in the epithelium to regulate basal cell development. As expected, β-catenin protein was retained in the epithelial junction of *Shh-Cre;Ctnnb1*$^{DM/loxp}$ but not *Shh-Cre;Ctnnb1*$^{loxp/loxp}$ mutants (Fig. S1). Similar to *Shh-Cre;Ctnnb1*$^{loxp/loxp}$ mutants, the anterior foregut remained a single-lumen tube in *Shh-Cre;Ctnnb1*$^{DM/loxp}$ embryos (Fig. 2A). Consistent with previous findings (Goss et al., 2009; Harris-Johnson et al., 2009), the unseparated foregut was specified as an esophageal-like muscular tube lined by squamous basal cells (Nkx2.1^{-}p63^{+}) in *Shh-Cre;Ctnnb1*$^{loxp/loxp}$ mutants (Fig. 2B). By contrast, the ventral side of the unseparated foregut in *Shh-Cre;Ctnnb1*$^{DM/loxp}$ mutants demonstrated respiratory cell differentiation (Nkx2.1^{+}) underlined by cartilage nodules (Sox9^{+}) (Fig. 2C). These ventral epithelial cells expressed the columnar cell marker Krt8, but not the squamous cell marker Krt13 (Fig. S2B). More importantly, tracheal basal cells (Nkx2.1^{+}p63^{+}) were present in the proximity of the cartilage nodules, confirming the association of cartilage and basal cell development (Hines et al., 2013). Of note is that some residual ciliated and club cells were also present in the ventral foregut of *Shh-Cre;Ctnnb1*$^{DM/loxp}$ mutants (Fig. S2C). Consistent with previous findings, deletion of *Ctnnb1* dramatically reduced the mRNA levels of two canonical Wnt signaling targets, Axin2 and Lef1, in both *Shh-Cre;Ctnnb1*$^{loxp/loxp}$ and *Shh-Cre;Ctnnb1*$^{DM/loxp}$ mutants (Fig. S3). Taken together, these results suggest that the cellular adhesion function of β-catenin plays roles in tracheal development. That being said, we cannot rule out the possibility that some residual β-catenin-mediated transcription regulation activities remain present in *Shh-Cre;Ctnnb1*$^{DM/loxp}$ mutants, even though TCF-transactivation-dependent Wnt signaling is ablated in various studies using the *Ctnnb1*^{DM} mouse line (Azim et al., 2014; Gay et al., 2015; Valenta et al., 2016, 2011).

Epithelial Wnts regulate basal cell and cartilage development through mesenchymal β-catenin

Loss of epithelial Wnts inhibits the development of tracheal cartilage and basal cells in *Shh-Cre;Gpr177*$^{loxp/loxp}$ mutants. We investigated whether epithelial Wnts directly regulate basal cell and cartilage development through β-catenin in the mesenchyme. We deleted *Ctnnb1* with *Dermo1-Cre*, which is active in mesenchymal progenitor cells as early as E10.5 (Hines et al., 2013; Sala et al., 2011). As previously described, the trachea is separated from the early foregut but is shortened, accompanied by simplified lung branching morphogenesis in *Dermo1-Cre;Ctnnb1*$^{loxp/loxp}$ mutants (De Langhe et al., 2008). Interestingly, cartilage progenitor cells (Sox9^{+}) were absent in the trachea of mutants at E11.5 (Fig. 3A). Sox9 immunostaining further confirmed the loss of cartilage at E14.5 (Fig. 3A). By contrast, smooth muscle cells (SMA^{+}) extended to the ventral side of the trachea (Fig. 3A). Loss of cartilage was accompanied by a significant reduction in the number of basal progenitor cells, which were barely detected in the trachea at E11.5 (n=3) and E14.5 (n=5) (Fig. 3B). Notably, p63^{+} cells were also barely detected in the ventral foregut epithelium prior to the separation of the trachea from the foregut (Fig. 3B). To test whether Wnts secreted by the mesenchyme are required for cartilage and basal cell development, we generated *Dermo1-Cre;Gpr177*$^{loxp/loxp}$ mutants. Deletion of *Gpr177* in the mesenchyme did not appear to

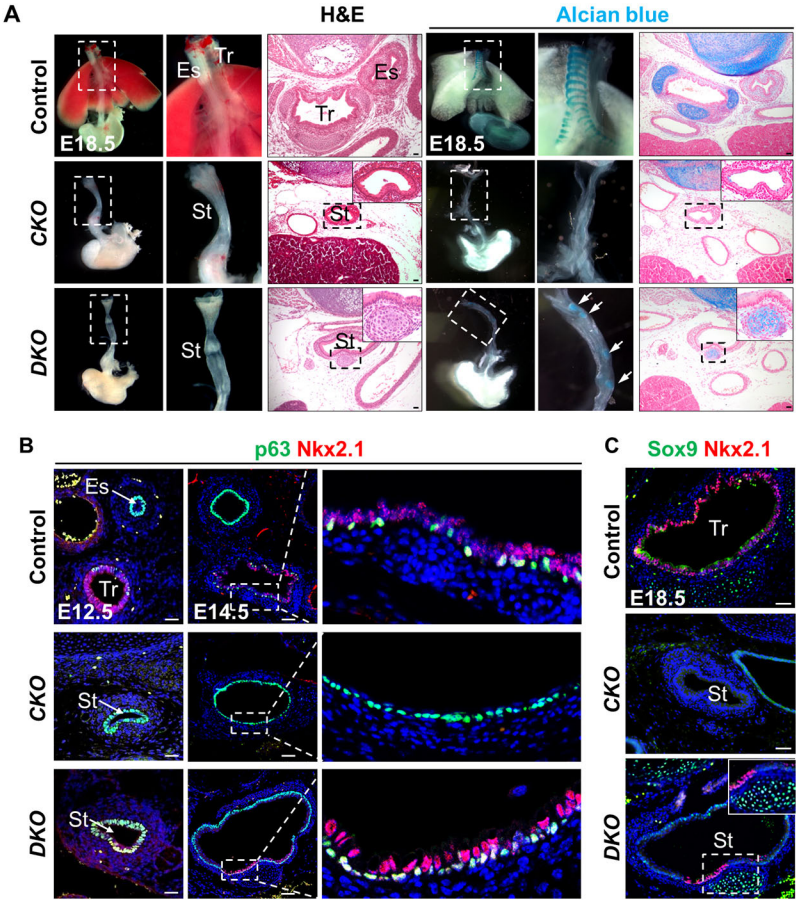

Fig. 2. Residual tracheal basal cells and cartilage nodules in *Shh-Cre;Gpr177*$^{DM/loxp}$ mutants. (A) Failed separation of the trachea and esophagus in *Shh-Cre;Gpr177*$^{loxp/loxp}$ (*Ctnnb1*^{CKO}) and *Shh-Cre;Gpr177*$^{DM/loxp}$ (*Ctnnb1*^{DKO}) mutants. Note the stratified squamous epithelium lining the muscular tube in *Ctnnb1*^{CKO} mutants and residual cartilage nodules (Alcian Blue^{+}) in *Ctnnb1*^{DKO} mutants. (B) Tracheal basal cells (Nkx2.1^{+} p63^{+}) are present in the ventral side of the unseparated foregut in *Ctnnb1*^{DKO} but not *Ctnnb1*^{CKO} mutants. (C) Residual respiratory epithelium (Nkx2.1^{+}) is underlined by cartilage nodules (Sox9^{+}) in *Ctnnb1*^{DKO} mutants. Es, esophagus; H&E, Hematoxylin & Eosin; St, single lumen tube; Tr, trachea. Scale bars: 50 µm.

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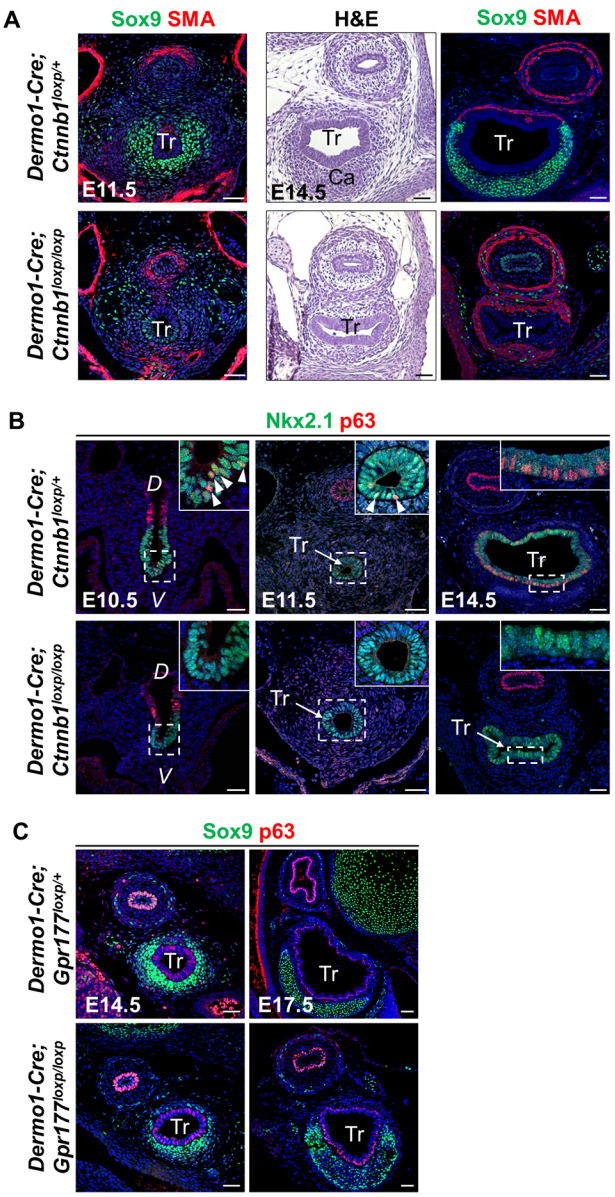

Fig. 3. Deletion of *Ctnnb1* in the mesenchyme causes the loss of tracheal cartilage and basal cells in *Dermo1-Cre;Ctnnb1*$^{loxp/loxp}$ mutants. (A) Tracheal cartilage is absent in *Dermo1-Cre;Ctnnb1*$^{loxp/loxp}$ mutants. Note some neuronal-like cells (Sox9^{+}) in the mutant trachea. (B) Basal cells are barely detected in both ventral and dorsal sides of the mutant trachea at different developmental stages. Arrowheads indicate Nkx2.1^{+} p63^{+} basal cells. (C) Deletion of *Gpr177* in the mesenchyme does not disrupt cartilage and basal cell development in the trachea of *Dermo1-Cre;Gpr177*$^{loxp/loxp}$ mutants. Ca, cartilage; D, dorsal; Tr, trachea; V, ventral. Scale bars: 50 µm.

disrupt basal cell and cartilage formation, supporting the suggestion that mesenchymal Wnts are not needed for tracheal development (Fig. 3C). Together, these data demonstrate that β-catenin in the mesenchyme is a crucial mediator for regulation by epithelial Wnts of cartilage and basal cell specification in the developing trachea.

Mesenchymal β-catenin regulates basal cell specification through crosstalk with Fgf10/Fgfr2 signaling

Previous studies have shown that Fgf10 overexpression leads to an increased number of basal cells in the airways (Volckaert et al., 2013). Additionally, loss of Fgf10 or Fgfr2 impairs basal cell maintenance in adult airways (Balasooriya et al., 2017; Volckaert et al., 2013). We therefore investigated whether the transcript levels of Fgf10 are downregulated in *Dermo1-Cre;Ctnnb1*$^{loxp/loxp}$ mutants at E11.5. Consistent with mitigated Wnt activities, the transcript levels of the Wnt/β-catenin downstream targets Axin2 and Lef1 were significantly decreased (Fig. 4A). Interestingly, we also observed a dramatic reduction in the transcript levels of Fgf10 upon *Ctnnb1* deletion in the mesenchyme (Fig. 4A), which is consistent with the previous finding of Fgf10 downregulation in the sub-mesothelial mesenchyme in *Dermo1-Cre;Ctnnb1*$^{loxp/loxp}$ mutants (De Langhe et al., 2008). Previously, we and others have shown that Fgf10 is expressed in the ventral mesenchyme of the foregut prior to tracheal-esophageal separation and then restricted to the inter-cartilage compartment after tracheal cartilage condensation occurs (Que et al., 2007; Sala et al., 2011). By contrast, the Fgf10 receptor Fgfr2 is uniformly expressed in the epithelium (Sala et al., 2011). We hypothesized that decreased Fgf signaling contributes to the loss of basal cells in *Dermo1-Cre;Ctnnb1*$^{loxp/loxp}$ mutants. We therefore deleted *Fgfr2* in the early foregut epithelium using *Shh-Cre*. Consistent with previous findings, loss of *Fgfr2* led to lung agenesis and truncated trachea (Sala et al., 2011). Conditional loss of *Fgfr2* also led to less condensed cartilage, although the alternative pattern of smooth muscle and cartilage seemed not to be perturbed (Fig. 4B,C). Importantly, similar to what has been observed in *Dermo1-Cre;Ctnnb1*$^{loxp/loxp}$ mutants, the number of basal cells was significantly reduced in the trachea of *Shh-Cre;Fgfr2*$^{loxp/loxp}$ mutants (Fig. 4D). These findings support a model whereby Fgf10 from the mesenchyme under the control of β-catenin is required for the specification of basal cells in the developing trachea. Crosstalk between Hippo signaling and Wnt7b-induced Fgf10 expression has been shown to regulate basal cell-fueled epithelial regeneration in the adult trachea (Volckaert et al., 2011, 2017). Here, our findings support the hypothesis that the β-catenin/Fgf10/Fgfr2 axis plays an important role in basal cell specification during early tracheal development.

In summary, our study revealed that Wnt proteins secreted from the respiratory epithelium are crucial for both tracheal cartilage and basal cell development. We found that residual β-catenin, presumably mediating cell-cell adhesion function, is crucial for the specification of tracheal epithelium and cartilage development in *Shh-Cre;Ctnnb1*$^{DM/loxp}$ mutants. β-Catenin in the mesenchyme plays significant roles in the specification of basal progenitor cells and cartilage. Our further genetic studies suggest that mesenchymal β-catenin regulates Fgf10, which relays to its receptor Fgfr2 in the epithelium to regulate basal cell specification (Fig. 4D).

MATERIALS AND METHODS

Mice

The *Shh-Cre*, *Dermo1-Cre*, *Gpr177*$^{loxp/loxp}$, *Ctnnb1*$^{loxp/loxp}$ and *Fgfr2*$^{loxp/loxp}$ mouse strains and genotyping methods have been reported previously (Brault et al., 2001; Fu et al., 2009; Harfe et al., 2004; Yu et al., 2003). *Ctnnb1*$^{DM/+}$ mice were kindly provided by Dr Konrad Basler of University of Zurich (Valenta et al., 2011). All mice are kept on a mixed genetic background comprising C57BL/6 and 129SvEv substrains. All mice were maintained in the University's animal facility according to institutional guidelines. All mouse experiments were conducted in accordance with procedures approved by the Institutional Animal Care and Use Committee.

Tissue processing, histology and immunostaining

For paraffin sections, tissues were fixed in 4% paraformaldehyde (PFA) overnight and processed as previously described (Jiang et al., 2017). For cryosections, tissues were fixed in 4% PFA in PBS at 4°C overnight, placed in 30% sucrose in PBS, and embedded in OCT. The primary antibodies used for immunostaining analysis were: rabbit anti-Nkx2.1 (1:500, ab76013, Abcam); mouse anti-p63 (1:500, CM163, Biocare); rabbit anti-Sox9 (1:1000,

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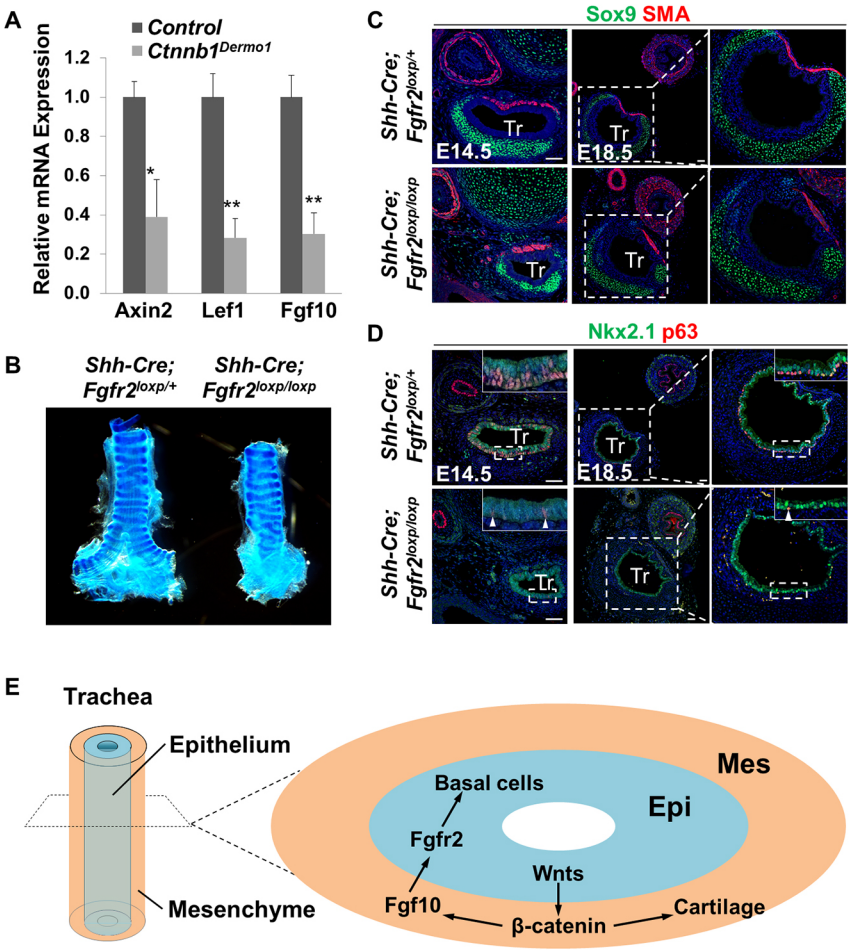

Fig. 4. Mesenchymal β-catenin regulates the specification of basal cells through Fgf10/Fgfr2 signaling. (A) Reduced transcript levels of the Wnt/β-catenin downstream targets Axin2, Lef1 and Fgf10 in the trachea of *Dermo1-Cre;Ctnnb1*$^{loxp/loxp}$ mutants (*P<0.05, **P<0.01; n=3 independent experiments). (B) Deletion of *Fgfr2* does not perturb the pattern of tracheal cartilage rings in *Shh-Cre;Fgfr2*$^{loxp/loxp}$ mutants as shown by Alcian Blue staining. (C) Deletion of *Fgfr2* leads to less-condensed cartilage. (D) Loss of *Fgfr2* causes significant reduction in the number of basal cells (arrowheads). (E) Diagram depicts that the epithelial-mesenchymal interaction mediated by Wnt/β-catenin signaling regulates tracheal basal cell and cartilage development. Epithelial Wnt proteins directly activate mesenchymal β-catenin to promote the expression of Fgf10, which in turn activates epithelial Fgfr2 to modulate basal cell specification. Epi, epithelium; Mes, mesenchyme; Tr, trachea. Scale bars: 50 µm.

AB5535, Millipore); mouse anti-smooth muscle actin (SMA) (1:2000, A2547, Sigma); chicken anti-KRT8 (1:1000, ab107115, Abcam); rabbit anti-Krt13 (1:1000, ab92551, Abcam); rabbit anti-β-catenin (1:200, 8480S, Cell Signaling Technology); rabbit anti-Cc10 (1:500, 06-263, Millipore); mouse anti-Foxj1 (1:200, 14-9965-82, eBioscience); mouse anti-Ki67 (1:500, 550609, BD Biosciences). Fluorescent secondary antibodies (donkey anti-rabbit IgG Alexa Fluor 488, A21206; donkey anti-rabbit IgG Alexa Fluor 555, A31572; donkey anti-mouse IgG Alexa Fluor 555, A31570; goat anti-chicken IgY Alexa Fluor 555, A21437; all from Invitrogen) were used for detection and visualization. Images were obtained using a Nikon SMZ1500 Inverted microscope (Nikon). Confocal images were obtained with a Zeiss LSM T-PMT confocal laser-scanning microscope (Carl Zeiss).

Alcian Blue staining

Whole lungs were dissected in PBS solution and fixed in 95% ethanol. Alcian Blue staining was performed as previously described (Jiang et al., 2017). Briefly, whole lungs and sections were treated with 3% acetic acid solution for 3 min, then stained in Alcian Blue (A3157, Sigma) for 5 min and counterstained with Nuclear Fast Red (N8002, Sigma).

RNAscope *in situ* hybridization

Samples were dissected and fixed in fresh 4% PFA at 4°C for 24 h, dehydrated in serial ethanol and embedded in paraffin. Sections were cut and *in situ* hybridization of Axin2 (probe-400331, Advanced Cell Diagnostics) was performed with the RNAscope 2.5 HD Assay-Red kit (322360, Advanced Cell Diagnostics) according to the manufacturer's instruction.

Reverse transcription and real-time PCR

RNA extraction and reverse transcription was performed using the Super-Script III First-Strand SuperMix (Invitrogen) according to the manufacturer's instructions. cDNA was subjected to quantitative real-time PCR using the StepOnePlus Real-Time PCR Detection System (Applied Biosystems) and iTaq Universal SYBR Green Supermix (Bio-Rad). All real-time quantitative PCR experiments were performed in triplicate. The prime sequences were as follows: β-actin forward 5′-CGGCCAGGTCATCACTATTGGCAAC-3′ and reverse 5′-GCCACAGGATTCCATACCCAA-3′; Axin2 forward 5′-CAGCCCTTGTGGTTCAAGCT-3′ and reverse 5′-GGTAGATTCCTGATGGCCGTAGT-3′; Lef1 forward 5′-GCAGCTATCAACCAGATCC-3′ and 5′-GATGTAGGCAGCTGTCATTC-3′; Fgf10 forward 5′-CGGGACCAAGAATGAAGACT-3′ and reverse 5′-AGTTGCTGTTGATGGCTTTG-3′.

Statistical analysis

Student's t-test was used for statistical analysis. Data are presented as mean± s.e.m.; P<0.05 was considered statistically significant.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: J.Q., M.J.; Methodology: Z.H., Y.Z., M.J.; Validation: Q.W., X.S., Y.L., M.J.; Formal analysis: J.Q., M.J.; Investigation: Z.H., Q.W., X.S., H.C., Y.Z., Y.Y., J.Q., M.J.; Resources: Y.L., Y.Z., M.M., Y.Y., J.Q.; Data curation: Z.H., M.J.; Writing - original draft: J.Q., M.J.; Writing - review & editing: J.Q., M.J.; Visualization: H.C.; Supervision: J.Q., M.J.; Project administration: J.Q.; Funding acquisition: J.Q.

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Supplementary information

Supplementary information available online at
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