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Endodermal Wnt signaling is required for tracheal cartilage formation

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Abstract

Tracheobronchomalacia is a common congenital defect in which the walls of the trachea and bronchi lack of adequate cartilage required for support of the airways. Deletion of *Wls*, a cargo receptor mediating Wnt ligand secretion, in the embryonic endoderm using *ShhCre* mice inhibited formation of tracheal-bronchial cartilaginous rings. The normal dorsal-ventral patterning of tracheal mesenchyme was lost. Smooth muscle cells, identified by *Acta2* staining, were aberrantly located in ventral mesenchyme of the trachea, normally the region of *Sox9* expression in cartilage progenitors. Wnt/ β -catenin activity, indicated by *Axin2 LacZ* reporter, was decreased in tracheal mesenchyme of *Wls^{flf};Shh^{Cre/+}* embryos. Proliferation of chondroblasts was decreased and reciprocally, proliferation of smooth muscle cells was increased in *Wls^{flf};Shh^{Cre/+}* tracheal tissue. Expression of *Tbx4*, *Tbx5*, *Msx1* and *Msx2*, known to mediate cartilage and muscle patterning, were decreased in tracheal mesenchyme of *Wls^{flf};Shh^{Cre/+}* embryos. *Ex vivo* studies demonstrated that Wnt7b and Wnt5a, expressed by the epithelium of developing trachea, and active Wnt/ β -catenin signaling are required for tracheal chondrogenesis before formation of mesenchymal condensations. In conclusion, Wnt ligands produced by the tracheal epithelium pattern the tracheal mesenchyme via modulation of gene expression and cell proliferation required for proper tracheal cartilage and smooth muscle differentiation.

Keywords

Tracheobronchomalacia; cartilage; airways; Wls; Sox 9; α SMA

Introduction

The ventral region of the mammalian trachea is supported by incomplete circumferential cartilaginous rings and dorsally by the *trachealis* smooth muscle. Dorsal-ventral patterning of cartilage and muscle protects the upper airways from injury and provides flexibility

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during ventilation and cough, preventing airway collapse during the respiratory cycle. Tracheobronchomalacia is a congenital malformation in which the walls of the conducting airways lack adequate cartilage. While tracheobronchomalacia is a common disorder (1:3000/live births) (Boogaard et al., 2005; Kenny AP, 2013), its etiology is poorly understood. Tracheal and bronchial structures forming the airway are derived from the anterior foregut endoderm and its interactions with surrounding mesenchyme, as the airways separate from the esophagus during the embryonic period at approximately week 12 of gestation in humans (Fausett and Klingensmith, 2012). Given the prevalence of tracheobronchomalacia and the challenges associated with corrective surgery, understanding the mechanisms regulating the formation of the tracheal cartilage will provide the framework to prevent and treat this condition (Carden et al., 2005; Majid et al., 2010).

A recent study proposed that the precise apposition of chondroblasts and myoblasts establishes the boundaries between cartilage and muscle, thus generating a structure needed for rigidity and flexibility characteristic of the trachea (Hines et al 2013). It is presently unclear how dorsal-ventral patterning of developing tracheal mesenchyme is established. Cartilage development is initiated by the commitment of subsets of mesenchymal cells surrounding the developing trachea, towards a chondrogenic lineage (McAteer, 1984). Mesenchymal cells derived from the splanchnic mesoderm, positioned ventral to the developing tracheal tube express the transcription factor *Sox9* as early as E9 (Elluru and Whitsett, 2004; Hines et al., 2013). *Sox9* is a key regulator of cartilage and bone development (Akiyama, 2008; Akiyama et al., 2002) and its role in tracheal cartilage has been demonstrated (Arora et al., 2012; Elluru and Whitsett, 2004; Hines et al., 2013; Park et al., 2010; Turcatel et al., 2013). Signaling mechanisms controlling the initial specification of tracheal cartilage progenitors are poorly defined; however, a number of transcription factors, receptors and ligands that influence *Sox9* expression in the mesenchyme of the trachea have been identified. The transcription factors *Tbx4* and *Tbx5*, expressed by the tracheal mesenchyme promote *Sox9* expression and mesenchymal condensations required for tracheal cartilage formation (Arora et al., 2012). *Shh*, a signaling molecule expressed by the tracheal epithelium, is required for expression of *Sox9* in the ventral tracheal mesenchyme by a mechanism that involves modulation of *Bmp4* signaling (Miller et al., 2004; Park et al., 2010). Epithelial transcription factor *Sox2* is required for tracheal cartilage formation via induction of *Shh* signaling (Que et al., 2009; Tompkins et al., 2009). The calcium channel *Cav3.2*, present in both the epithelium and the mesenchyme of the trachea promotes *Sox9* expression via the activation of the NFAT signaling pathway (Lin et al., 2014). The kinases *Mek1* and *Mek2*, present in both epithelium and mesenchyme of developing trachea, induce *Sox9* expression via activation of ERK/MAPK signaling (Boucherat et al., 2014). Mesenchymal *Fgf10* and its epithelial receptor *Fgfr2* mediate the periodic pattern of expression of *Shh* that influences the formation of cartilaginous rings (Sala et al., 2011). *Fgf18*, expressed by the tracheal mesenchyme, promotes proliferation of tracheal chondrocytes by inducing *Sox9* expression (Elluru et al, 2009). Thus, epithelial-mesenchymal crosstalk is required for specification and differentiation of tracheal cartilage, resembling the mesenchymal-epithelial interactions that govern cell differentiation in developing lung (Shannon and Hyatt, 2004).

Wnt signaling plays a critical role in specification and early patterning of respiratory tract (Goss et al., 2009; Harris-Johnson et al., 2009; Miller et al., 2012). Germ line deletion of *Wnt5a*, which is expressed in the mesenchyme and epithelium of E11.5 developing trachea, disrupted cartilage rings and caused shortening of the trachea (Li et al., 2002). Likewise, ablation of the receptor ROR2, which mediates Wnt5a-induced Wnt signaling, caused miss patterning of cartilage and shortening of the trachea (Oishi et al., 2003). Deletion of β -catenin from the tracheal epithelium caused incomplete cartilaginous rings, while deletion of β -catenin from the tracheal mesenchyme produced a shorter trachea that underwent degeneration by E18.5 (Bell et al., 2008; De Langhe et al., 2008; Yin et al., 2008). Deletion of *Wnt7b*, expressed by the respiratory epithelium and known to mediate Wnt/ β -catenin signaling, caused incomplete cartilaginous rings (Rajagopal et al., 2008). Wnt signaling is also critical for proliferation and differentiation of pulmonary muscle cells (Cohen et al., 2009; De Langhe et al., 2008; Goss et al., 2011; Shu et al., 2002; Yin et al., 2008). Wnt7b promotes vascular smooth muscle differentiation via a mechanism that involves PDGF signaling (Cohen et al., 2009), while Wnt2, produced by the mesenchyme, promotes differentiation of airway smooth muscle by inducing the expression of the ligand *Fgf10* and *Myocd* (*myocardin*), a key regulator of smooth muscle differentiation (Goss et al., 2011). While the genetic data support a role for Wnt signaling in tracheal cartilage formation and suggest a putative role in tracheal muscle differentiation, precise mechanisms mediating these processes remain unclear.

To define the role of Wnt signaling in dorsal-ventral patterning of developing trachea, we generated a mouse model wherein *Wntless* (*Wls*), a cargo receptor that mediates Wnt ligand secretion (Banziger et al., 2006; Goodman et al., 2006), was conditionally deleted either in epithelial cells of the foregut endoderm or mesenchyme of the developing respiratory tract. Epithelial *Wls* was required for activation of canonical Wnt signaling in the tracheal mesenchyme. Wnt ligands produced by epithelial cells of the conducting airways were required for proper specification of the tracheal bronchial cartilage. Deletion of *Wls* from the tracheal epithelium increased proliferation of smooth muscle cells causing thickening and expansion of the muscle layer into the ventral region of the trachea at sites where cartilage is normally formed. Thus, epithelial Wnt signaling promotes dorsal-ventral patterning of the trachea, influencing differentiation and proliferation of tracheal cartilage and smooth muscle cells.

Materials and Methods

Mouse Breeding and Genotyping: Animals were housed in pathogen-free conditions and handled according to protocols approved by CCHMC Institutional Animal Care and Use Committee (Cincinnati, OH USA). Generation of the *Wntless* (*Wls*) conditional knockout (CKO) mouse has been described (Carpenter et al., 2010). *Wls^{ff};Shh^{Cre/+}* embryos were obtained by breeding *Wls^{ff}* mice with *Shh^{Cre}* mice and breeding the resulting mice with *Wls^{ff}* mice. *Wls^{ff};Dermo1^{Cre/+}* mutants were generated by breeding *Wls^{ff}* mice with *Dermo1^{Cre}* mice (Sosic et al., 2003) and breeding resultant mice with *Wls^{ff}*. *Wls^{ff};Shh^{Cre/+};Axin2^{LacZ}* mice were obtained by mating *Wls^{ff/+};Shh^{Cre/+}* with *Axin2^{LacZ}* (Joeng et al., 2011; Lustig et al., 2002). *Wls^{ff};Shh^{Cre/+};Rosa mT/mG* mice were generated by breeding *Wls^{ff/wt} Shh^{Cre+/-}*; with *Rosa mT/mG* mice (Muzumdar et al., 2007). *Wls^{ff};Col2a1^{Cre/+}* mutants were generated

by breeding *Wls^{ff}* mice with *Col2a1Cre* mice (Ovchinnikov et al., 2000) and breeding resultant mice with *Wls^{ff}* mice. *Sox9^{ff};Col2a1^{Cre/+}* and *Sox9^{ff};Dermo1^{Cre/+}* embryos were obtained by crossing *Sox9^{ff}* with *Col2a1Cre* or *Dermo1Cre* and mating resulting mice to *Sox9^{ff}* mice. Genotypes of transgenic mice were determined by PCR using genomic DNA isolated from mouse tail or embryonic tissue. Primers utilized for genotyping have been provided as supplementary material (Table S1).

Histology, Immunohistochemistry and Immunofluorescence Staining: Embryonic tissue was fixed and embedded in paraffin or frozen using OCT. Sections (6µm) were processed for H&E or DAB staining as described (Mucenski et al., 2003). For immunofluorescence, antigen retrieval was performed, when necessary, using 10 mM citrate buffer, pH 6, before slides were blocked for 2 hours in TBS containing 10% Donkey serum and 1% BSA. Following blocking, slides were incubated, overnight, at 4° in primary antibody diluted accordingly in blocking solution. After removal of unbound primary antibody, slides were incubated with secondary antibody at a dilution of 1:200 for one hour. Slides were then washed and coverslip using Vecta Shield mounting media with or without Dapi. Fluorescent staining was visualized and photographed using an automated fluorescence microscope (Zeiss). Source and dilution of primary antibodies utilized for this study has been provided as Supplementary data (Table S2).

Whole Mount X-galactosidase Staining

Embryonic lungs were dissected and fixed in 4% paraformaldehyde (PFA) in PBS for 30 minutes, then washed in PBS and stained two to three hours in X-gal staining solution. To stop the reaction, explants were washed in 3% dimethyl sulfoxide-PBS, rinsed in PBS, washed and stored in 70% ethanol. Explants were processed for paraffin embedding and sectioning. Slides were deparaffinized and counterstained with fast red.

In-Situ Hybridization

Procedure was performed according to a protocol developed by Advanced Cell Diagnostics (ACD) (Wang et al., 2012). In situ probes were designed by ACD. In brief, slides were baked and deparaffinized. In situ probes were added to the slides and hybridization was performed for 2 hours at 40°C followed by several rounds of amplification steps. Signal was detected by chromogenic reaction using DAB and slides were counter stained with Hematoxylin. All experiments were run simultaneously with a positive control probe recognizing transcripts for the housekeeping gene *Ppib* (which encodes for peptidylpropyl isomerase B, an enzyme found in the endoplasmic reticulum) as well as a negative control probe that recognizes a bacterial transcript (*Dapb*). After mounting with permanent mounting media, slides were photographed using a wide field Nikon i90 microscope.

Lectin Staining

Samples were fixed overnight in PFA and rinsed multiple times in PBS. Afterwards, samples were incubated for 1 hour in blocking buffer comprised of 2% Goat Serum, 1% BSA, .3% Triton X and PBS. After removing blocking buffer, lectin PNA solution (in 10% normal goat serum and PBS) was added to the samples at a final concentration of 10 µg/ml. The samples were then covered in aluminum foil and incubated overnight at 4C. Finally,

explants were rinsed with PBS multiple times and photographed using a Leica fluorescence dissecting microscope.

Whole Mount Immunofluorescence Staining and Confocal Microscopy. Samples were staining as previously described (Ahnfelt-Ronne et al., 2007). E11.5 and E13.5 tracheal lung explants were isolated and fixed overnight in 4% PFA and stored in methanol 100%. Samples were permeabilized using Dent's Bleach and hydrated in a graded series of methanol. Explants were blocked using a commercial reagent (Invitrogen TSA kit blocking reagent). Primary antibody was diluted in blocking solution and samples were incubated overnight. After several washes, secondary antibody was applied for two hours at a 1:500 dilution. After washing, samples were dehydrated and stored in Methanol. Samples were cleared with Murray's clear before imaging (Jahrling et al., 2009; Ott, 2008). Pictures were obtained using a confocal microscope (NikonA1Rsi). Image processing was performed using IMARIS software (BITPLANE Scientific Software).

Cell Proliferation and Cell Death: E11.5 pregnant mice were injected with BrdU at concentration 100ug/g of body weight. Embryos were isolated at day E12.5 or E13.5. Embryonic sections were labeled with BrdU, Nkx2.1, Sox9 and SMA antibodies to determine mitotic cells in tracheal epithelium and mesenchyme. Labeled cells and total cells were counted per each field photograph at 20X, and ratios of proliferating cells to total cells, defined as number of Sox9 and α SMA stained cells, were calculated. Average mitotic index was determined for five different samples. To determine cell death, TUNEL assay was performed on sections of E12.5, E13.5 and E14.5 embryos using a commercially available detection kit (Roche).

Embryonic Whole Tracheal Explant Culture: Embryonic tracheas were harvested at E11.5 and cultured at air-liquid interphase as described (Hyatt et al., 2004). Wnt5a, Wnt3a (200ng/ml), XAV-393 (1uM) or Bmp-2 (100ng/ml) were added to the media one hour after initiation of culture. Explants were harvested and processed for RNA isolation after 72 hrs. in culture.

Micromass Culture

Primary MEF cells were obtained from ATCC and cultured in Dulbecco's modified Eagle's medium (GIBCO) supplemented with 20% non-Heat inactivated fetal bovine serum (ATCC) along with 1% Penicillin/Streptomycin. Cells were plated into 12-well plates in micro-masses consisting of 125,000 cells/10ul. After three hours, cells were flooded with culture media. Wnt 3a, Wnt5a, Bmp-2 and LiCL were added to the media. Cells were harvested after 6 days of culture and process for RNA isolation.

RNA Extraction and RT-PCR. Gene expression was determined by quantitative RT-PCR. RNA was isolated from embryonic tracheal explants using a commercially available kit (RNAeasy mini kit or micro kit, Qiagen-Promega). Reverse transcription was performed according to manufacturer instructions (Verso Fisher Sci), and Taqman probes were utilized to detect differential expression using a StepOnePlus RT-PCR System.

Statistics: Quantitative data were presented as mean \pm standard error. Experiments were repeated at least twice with a minimum of three biological replicates for each group. Statistically significant differences were determined by paired T-test or one-way ANOVA followed by post hoc pairwise multiple comparison procedures (Bonferroni method). Significance was set at $P < 0.05$.

Results

Deletion of *Wls* from the respiratory tract epithelium caused tracheobronchomalacia

Wls is expressed in both epithelium and mesenchyme of developing trachea as determined by in situ hybridization (Fig.1 A). To define the role of Wnt signaling in upper airway differentiation *Wls* was deleted from the epithelial cells of the embryonic foregut endoderm by breeding *Wls* *CKO* mice (Carpenter et al, 2010) to the *Shh* *Cre* mice. Efficient and specific Cre mediated recombination in the trachea was demonstrated by detection of GFP in the epithelium of the respiratory tract of embryos at E11.5 after breeding the *Shh**Cre* mice to the *Rosa* *mT/mG* reporter mice (Fig.1 B). Deletion of *Wls* was confirmed by PCR using primers that recognize the mutant allele in tracheal tissue (Supp. Fig.1). Lungs of *Wls*^{*ff*};*Shh*^{*Cre/+*} mice were hypoplastic (Fig.1 F,G compare to C,D) (Cornett et al., 2013). Tracheal and bronchial, but not laryngeal cartilage was absent as determined by Alcian blue (Fig.1 C,F) and H&E staining at E18.5 (Fig.1 D, E, G, H, E' and H'). Cross sections of E18.5 *Wls*^{*ff*};*Shh*^{*Cre/+*} embryos stained with H&E confirmed the lack of cartilage in the trachea and the presence of a distinct esophageal tube. Taken together, these data demonstrate that deletion of *Wls* from the epithelium of the developing trachea caused agenesis of the tracheal and bronchial cartilage, findings consistent with defects associated with tracheobronchomalacia.

Canonical Wnt signaling is active in tracheal mesenchyme and is severely impaired after epithelial deletion of *Wls*

Wls is a cargo receptor mediating secretion of Wnt ligands from the producing cells (Banziger et al., 2006). However, it remains unclear whether *Wls* differentially promotes secretion of Wnt ligands eliciting β -catenin dependent or independent signaling. To assess whether Wnt ligands produced by the tracheal epithelium induce canonical Wnt response, *Wls*^{*ff*};*Shh*^{*Cre/+*} mice were crossed to *Axin2**Lac-Z* mice. Axin promoter activity was decreased after deletion of *Wls* from the epithelium. X-gal staining was present in the subepithelial mesenchymal cells of conducting airways and lung periphery of E12.5 control embryos. In contrast, no staining was detected in *Wls*^{*ff*};*Shh*^{*Cre/+*} embryos (Fig.2 A,B). At E14.5, X-gal staining was detected in the mesenchyme of the trachea resembling the pattern of cartilaginous rings (Fig.2C). Alcian blue staining and *Axin2* promoter activity were present in the tracheal mesenchyme at sites of chondrogenesis (Fig.2D); however, *Axin2* LacZ staining was excluded from mesenchymal condensations and was confined to the subepithelial mesenchyme of developing trachea. Similarly to findings at E12.5, *Axin2* promoter activity was not detected in tracheas of E14.5 *Wls*^{*ff*};*Shh*^{*Cre/+*} embryos (Fig. C).

While the expression pattern of Wnt ligands in developing lung is well established, less is known about the temporal-spatial expression of Wnt ligands in the trachea. At E11.5 *Wnt5a*

was expressed in both epithelium and mesenchyme of the trachea. *Wnt7b* was expressed in a circumferential pattern in tracheal epithelium of E11.5 and E13.5 embryos. In contrast, by E13.5 *Wnt5a* was expressed predominantly in ventral mesenchyme of the trachea, but it was also detected to lesser degree in the epithelium of the trachea (Fig.2G). At E13.5 *Wnt2* and *Wnt2b* were present in tracheal mesenchyme. *Wnt2b* was enriched in the dorsal and lateral regions of the tracheal mesenchyme. *Wnt2* and *Wnt2b* were also detected in the mesenchyme of the developing distal lung (Supp. Fig.2). *Wnt4* and *Wnt11* were detected at low levels in the epithelium and mesenchyme of the trachea respectively (Fig.2H). *Wnt11* was also detected in ventral body wall confirming our previous studies (Supp. Fig.2) (Snowball et al., 2015). In situ probes recognizing a housekeeper gene *Ppib* and a bacterial gene *Dapb*, were run *in parallel* and served as a positive and negative experimental control (Suppl. Fig2). Data presented demonstrate that epithelial *Wls* is required for Wnt/ β -catenin signaling in developing tracheal mesenchyme and that *Wnt7b* and *Wnt5a* are abundantly expressed in the E11.5 tracheal epithelium where they are likely to participate in mesenchymal tracheal patterning downstream of *Wls*.

Epithelial *Wls* regulates dorsal ventral patterning of the tracheal mesenchyme

Since epithelial deletion of *Wls* drastically impaired cartilage at E18.5, we sought to identify the timing of cartilage and smooth muscle patterning at earliest stages. In the mouse, tracheal cartilage is well formed by E15 (Park et al., 2010; Tiozzo et al., 2009). At E13.5 in *Wls^{ff};Shh^{Cre/+}* embryos smooth muscle layer, detected by α SMA staining, was expanded into the ventral aspect of the trachea and cartilage was absent (Fig.3 A and B). At E14.5, the precise patterning of the normal trachea in control mice was evidenced by a dorsal stripe of muscle. Cartilage was present as partial circumferential rings on the ventral side of the trachea. In contrast, in tracheas of *Wls^{ff};Shh^{Cre/+}* mice, α SMA staining was expanded to the ventral side of the trachea (Fig.3 C, D, E, F). Epithelial cells lining the trachea stained for TTF-1 in *Wls^{ff};Shh^{Cre/+}* embryos, demonstrating maintenance of respiratory epithelial cell identity. In *Wls^{ff};Shh^{Cre/+}* embryos, the orientation of muscle fibers was disrupted and organized parallel to the longitudinal axis of the trachea. In contrast, in control embryos, muscle fibers of the *trachealis* muscle were oriented transversally to the longitudinal axis of the trachea (Fig.3 E' versus F'). Few Sox9 stained cells were observed randomly scattered throughout the dorsal mesenchyme of the *Wls^{ff};Shh^{Cre/+}* tracheas (Fig.3F'). These cells were also positive for Sox10 (data not shown). At E18.5, virtually no Sox9 positive cells were present in the tracheal mesenchyme (Supp. Fig.3). *Sox9* transcripts were diminished while *Acta2* transcripts were increased in tracheal tissue isolated from *Wls^{ff};Shh^{Cre/+}* embryos (Fig.3 G). These findings are in agreement with the previously described expression profile of Sox9 and α SMA in *Wls^{ff};Shh^{Cre/+}* tracheas. These data support the concept that Wnt ligands, dependent upon *Wls* secretion from endodermally derived cells, promote cartilage formation and may repress smooth muscle on the ventral side of the trachea, as well as influence the arrangement of tracheal smooth muscle.

Epithelial Wnt signaling regulates Sox9, mesenchymal cell proliferation and mesenchymal cells condensations

A critical event during cartilage formation is the condensation of mesenchymal cells into chondrogenic nodules (Bi et al., 1999). In developing trachea, condensations are established

by E13.5. Whole mount tracheal lung explants were stained with PNA (Peanut Agglutinin Lectin) to label condensing cells (Gillotte et al., 2003). At E13.5, a clear pattern of condensed mesenchymal cells resembling cartilaginous rings was readily distinguished (Fig. 4 A, arrow), while in *Wls^{ff};Shh^{Cre/+}* tracheas condensations were seldom detected (Fig.4 B, arrow). These data were supported by whole mount staining of control tracheal lung explants wherein Sox9 was strongly expressed in mesenchymal condensations (Fig.4 C, arrow). In contrast, in tracheas of *Wls^{ff};Shh^{Cre/+}* embryos Sox9 staining was weakly detected in subepithelial mesenchyme (Fig.4 D arrow). Sox9 was strongly expressed in the respiratory epithelium of the lung (arrow head in Fig.4 E and F). These data indicate that epithelial *Wls* is required for signaling to tracheal mesenchymal cells to influence Sox9 expression and to form mesenchymal condensations on the ventral side of the trachea.

At E11.5, Sox9 staining was present in the tracheal mesenchyme of control and reduced in *Wls* deficient embryos (Fig.5 A). Since Wnt signaling modulates cell proliferation, we reasoned that the lack of Sox9 positive cells could be related to decreased mesenchymal cell proliferation or increased cell death. At E12.5, cell proliferation was decreased in the mesenchyme of the *Wls^{ff};Shh^{Cre/+}* tracheas (Fig.5 B and C). To test whether loss of epithelial *Wls* mediated signaling differentially affected proliferation of chondroblasts or myoblasts, we quantified cells expressing Sox9 or α SMA in cross sections of tracheas from E13.5 embryos. BrdU labeling demonstrated increased numbers of α SMA but decrease numbers of Sox9 proliferating cells. Likewise, numbers of Sox9 stained cells were decreased and those staining for α SMA stained cells were increased (Fig.5 D and E). Moreover, the number of Sox9 cells in close apposition with the tracheal epithelium was decreased and α SMA stained mesenchymal cells in close proximity to the tracheal epithelium were increased in *Wls^{ff};Shh^{Cre/+}* embryos, demonstrating the malpositioning of muscle cells on the ventral side of the trachea (Fig.5 F). TUNEL studies did not detect differences in cell death between controls and *Wls^{ff};Shh^{Cre/+}* tracheal tissue (data not shown). Thus, these data demonstrate that epithelial *Wls* controls mesenchymal cell proliferation, modulating proliferation and numbers of chondrogenic and myogenic cells in the embryonic tracheal mesenchyme.

Mesenchymal deletion of Sox9 is not sufficient to promote expansion of tracheal smooth muscle

To assess whether *Wls* effects on tracheal mesenchyme patterning depend on *Sox9*, we deleted *Sox9* from the developing tracheal mesenchyme using *Dermo1Cre* and *Col2a1Cre* mice. Deletion of *Sox9* from the mesenchyme of the trachea using *Dermo1Cre* mice, reduced the number and altered the shape of cartilaginous rings, resulting in a shorter trachea, findings resembling the *Wnt5a* loss of function (Li et al., 2002) (Supp. Fig. 4 H). *Col2a1* is a direct target of *Sox9* and it is expressed in developing trachea at E10.5 in a similar distribution to that of *Sox9* (Elluru et al., 2009; Elluru and Whitsett, 2004). We utilized *Col2a1Cre* mice to delete *Sox9* in chondrocyte progenitors. While cartilage formation was severely impaired, deletion of *Sox9* did not cause robust expansion of the muscle layer into the ventral side of the trachea (Fig. 6 B,D, F, H and Supp. Fig.4 F), findings contrasting the phenotype observed in *Wls^{ff};Shh^{Cre/+}* embryos (Fig.3B). We also determined that *Acta2* levels were not altered in tracheas of E14.5 *Sox9^{ff};Col2a1^{Cre/+}*

embryos (Fig.6 I). These data demonstrate that while *Sox9* is required for tracheal cartilage development, deletion of *Sox9* did not cause expansion of the muscle layer into the ventral side of the trachea as seen in the *Wls^{f/f};Shh^{Cre/+}* embryos.

To test whether mesenchymal *Wls* influenced tracheal patterning, *Wls* was conditionally deleted using *Dermo1Cre* mice. *Dermo1Cre* efficiently targets the mesenchyme surrounding the E10.5 foregut as well as mesenchyme of the E12.5 developing trachea, as verified by GFP staining in *Dermo1-Cre;Rosa mT/mG* mice (Supp. Fig. 5A). Neither *Sox9* mRNA nor staining were altered by mesenchymal deletion of *Wls* (Supp Fig.5). Expression levels and pattern of α SMA staining were unaltered. Deletion of *Wls* from chondrocyte precursors, using *Col2a1Cre* mice did not affect cartilage formation or tracheal patterning (Supp. Fig5). These data suggest that secretion of mesenchymal Wnt ligands may play a redundant role in regards to tracheal patterning, supporting the concept that the epithelial *Wls* mediated signaling directs mesenchymal patterning of the trachea.

Canonical Wnt signaling modulates *Sox9* in prechondrogenic tracheal explants

It is established that canonical Wnt signaling modulates *Sox9* activity during endochondral bone formation (Akiyama et al., 2004; Day and Yang, 2008; Hill et al., 2005). To test the hypothesis that epithelial Wnt signaling regulates *Sox9* expression to induce tracheal chondrogenesis, we utilized *ex vivo* tracheal explants cultures. We tested whether *Wls^{f/f};Shh^{Cre/+}* tracheal explants isolated at E11.5 and incubated for 72hrs. were responsive to Bmp-2, a chondrogenic signal (Lengner et al., 2004; Pan et al., 2008). Bmp-2 induced *Sox9* and *Col2a1* expression. Neither addition of Wnt3a (a Wnt ligand that similarly to Wnt7b typically induces Wnt/ β -catenin signaling in respiratory tract) nor Wnt5a alone induced *Sox9* in tracheal explants (Fig.7A); however, addition of both ligands to the culture media robustly induced *Sox9* and to lesser degree *Col2a1* (Fig.7B). *Acta2* mRNA was not significantly changed by addition of Wnt3a or Wnt5a (Fig.7A and B). When E11.5 control tracheal explants were incubated in the presence of Wnt canonical signaling inhibitor XAV 939 (Huang et al., 2009), *Sox9* expression was modestly decreased and *Col2a1* was sharply decreased (Fig.7C). To test if Wnt signaling modulates *Sox9* expression after formation of mesenchymal condensations, MEF (mouse embryonic fibroblasts) cells were seeded in micromasses and treated with Bmp-2 to induced *Sox9* (Lengner et al., 2004). In this system, incubation in presence of LiCl, an activator of Wnt/ β -catenin signaling (Klein and Melton, 1996), repressed *Sox9* and *Col2a1* and did not alter *Acta2* (Fig. 7D). Similar results were obtained when tracheal explants isolated at E13.5 (stage when mesenchymal condensations are formed), were treated with LiCl resulting in decreased *Sox9* and *Col2a1* mRNAs (data not shown). These data suggest that Wnt/ β -catenin signaling plays differential roles during tracheal chondrogenesis, and that Wnt signaling is necessary to promote *Sox9* and *Col2a1* in prechondrogenic tracheal mesenchyme *ex vivo*.

Epithelial *Wls* mediated signaling is necessary for expression of factors required for tracheal patterning of cartilage and muscle

Since addition of Bmp-2 induced *Sox9* and *Col2a1* in explants and in micromass cultures, we asked whether BMP signaling was altered in tracheas of *Wls^{f/f};Shh^{Cre/+}* embryos. *Bmp4* and *Bmp6* mRNA levels were modestly decreased in the mesenchyme of developing

tracheas of E11.5 and E13.5 *Wls^{ff};Shh^{Cre/+}* as determined by quantitative RT-PCR and in situ hybridization (Fig.8A and B). Furthermore, treatment of tracheal explants from *Wls^{ff};Shh^{Cre/+}* embryos with Bmp-2, strongly induced *Sox9* expression (Fig.7B). *Msx1*, *Msx2*, *Tbx4* and *Tbx5*, transcription factors known to influence cartilage and smooth muscle development, were reduced in *Wls^{ff};Shh^{Cre/+}* tracheal tissue (Fig.8A and B). At E13.5 *Wls* mRNA was decreased in the tracheal mesenchyme of *Wls^{ff};Shh^{Cre/+}* as determined by in situ hybridization, suggesting that *Wls* may influence its own expression (Fig.8B). To test whether Wnt/ β -catenin signaling modulated the expression of genes regulating chondrogenesis *ex vivo*, control tracheal explants isolated at E11.5 were incubated with XAV 939 for 72 hrs. resulting in decreased expression of *Msx2*, *Tbx4* and *Tbx5* (Fig.8 C). Taken together, these data support the concept that epithelial *Wls* influences expression of genes required for cartilage differentiation at least in part, via canonical Wnt signaling.

Discussion

In the present study, we demonstrated that endodermal *Wls* mediated signaling is required for tracheal cartilage development. Epithelial *Wls* influenced the expression of pro-chondrogenic genes in part by activation of Wnt/ β -catenin signaling in tracheal mesenchyme. Epithelial *Wls* mediated signaling limited the proliferation of muscle layer into the ventral and lateral regions of the trachea. Therefore, precise regulation of Wnt ligand secretion from the tracheal bronchial epithelium is required for normal patterning of tracheal- bronchial cartilage.

Initiation of the chondrogenic program in developing trachea requires Wnt signaling. While commitment of tracheal mesenchymal cells towards the chondrogenic lineage is defined by the expression of *Sox9*, the events upstream of *Sox9* expression are less well understood. Present data demonstrated that epithelial *Wls* is required for *Sox9* expression and formation of tracheal cartilage (Fig. 1 and 3). We determined that Wnt/ β -catenin signaling was operative in the sub-epithelial mesenchyme of the trachea of control embryos, and that epithelial deletion of *Wls* severely impaired Wnt/ β -catenin activity in the tracheal mesenchyme of *Wls^{ff};Shh^{Cre/+}* embryos (Fig.2).

Our data support the concept that epithelial *Wls* influences gene expression required for chondrogenesis. Expression of *Bmp4* and *Bmp6* was reduced, to some extent, in *Wls^{ff};Shh^{Cre/+}* embryos. It has been shown that BMP signaling plays a critical role in cartilage formation during limb development (Barna and Niswander, 2007; Duprez et al., 1996; Karamboulas et al., 2010). At early stages of respiratory tract development, *Bmp4* is expressed in the anterior foregut and it is required for outgrowth of the trachea (Li et al., 2008). Moreover, *Bmp4* is expressed in the ventral region of the prechondrogenic tracheal mesenchyme (this study and (Park et al., 2010), where cartilage will be formed. Our studies showed that Bmp-2 (a ligand with 80% homology to Bmp-4) induced *Sox9* expression in tracheal explants of control and *Wls^{ff};Shh^{Cre/+}* embryos (Fig.7 A). Present data also suggest that in tracheal development, both Bmp and Wnt signaling influence the differentiation of tracheal mesenchyme into cartilage (Fig. 7 and Fig. 8). These data are supported by recent studies demonstrating that the interplay between Bmp and Wnt signaling determines the sites of expression of *Sox9* during patterning of digits (Raspopovic et al., 2014). Our data

also demonstrated that epithelial *Wls* is necessary for *Tbx4* and *Tbx5* expression. *Tbx4* and *Tbx5*, are known to promote tracheal cartilage development by inducing *Sox9* expression as well as promoting mesenchymal condensations (Arora et al., 2012). It is unclear whether *Tbx4* and *Tbx5* are direct targets of Wnt signaling. Our *in vitro* studies showed that pharmacological inhibition of Wnt/ β -catenin signaling in prechondrogenic control tracheal explants decreased the mRNA levels of *Sox9*, *Col2a1*, *Tbx4* and *Tbx5* thus confirming the requirement of canonical Wnt signaling in the tracheal mesenchyme for establishment of the chondrogenic program (Fig.8). These data are in agreement with the concept that Wnt signaling plays a dynamic role in cartilage development and both anomalously increased or diminished levels of Wnt signaling will disrupt chondrogenesis (Ling et al., 2009; Liu et al., 2008). Previous *in vivo* and *in vitro* studies suggest that β -catenin activity fluctuates during chondrogenesis, being higher in prechondrogenic mesenchyme, and at lower levels during chondrocyte maturation (Gaur et al., 2006; Ling et al., 2009).

Wnt ligands act synergistically to induce tracheal cartilage

Wls is required for secretion and activity of all lipid modified Wnt ligands (Najdi et al., 2012). Our studies shown that *Wnt7b*, *Wnt5a* and to lesser degree *Wnt4* transcripts were found in a circumferential expression pattern in the tracheal epithelium at E11.5 (Fig.2). Deletion of *Wnt7b* was associated with abnormal cartilaginous rings (Rajagopal et al., 2008). Mice carrying a null allele of *Wnt5a* developed a short trachea with anomalous, discontinuous cartilaginous rings (Li et al., 2002), resembling the tracheal phenotype observed in *Sox9^{ff};Dermo1^{Cre/+}* mice. It is presently unknown whether *Wnt4* participates in tracheal patterning, however mutations in *WNT4* are associated with lung hypoplasia (Mandel et al., 2008). Thus, the lack of cartilage observed in tracheas of *Wls^{ff};Shh^{Cre/+}* embryos may result from the combined loss of *Wnt7b* and *Wnt5a* signaling, a model supported by our *in vitro* data, wherein addition of both *Wnt3a* and *Wnt5a* induced expression of *Sox9* and *Col2a1* in *Wls^{ff};Shh^{Cre/+}* tracheal explants. Thus Wnt/ β -catenin activity induced by epithelial Wnt ligands is required, at least in part, for commitment of mesenchymal cells towards the chondrogenic lineage (Fig. 7 and Fig. 8).

Wnt signaling is required for differentiation and patterning of the trachealis muscle It is unknown how the muscle lineage is initially specified in the tracheal mesenchyme. In peripheral lung, *Wnt7b* and *Wnt2* play essential roles in establishment of vascular and airway smooth muscle (Cohen et al., 2009; Goss et al., 2011). In our model, deletion of *Wls* from the epithelium of the trachea resulted in expansion of muscle layer into the ventral aspect of the trachea findings not seen in other experimental models of tracheomalacia (Hines et al., 2013; Turcatel et al., 2013). Our studies showed that deletion of *Sox9* from tracheal mesenchyme using the *Col2a1Cre* mice caused severe loss of cartilage; however, expansion of α SMA staining into the ventral side of the trachea was not observed (Fig.6). Epithelial deletion of *Wls* caused a sharp change in proliferation profile, resulting in increased proliferation of α SMA stained cells (myoblasts) accompanied by decreased proliferation of *Sox9* stained cells (chondroblasts). This proliferation profile contrasts with that observed in control developing tracheas, wherein *Sox9* stained cells (chondroblasts) proliferation is higher when compared to α SMA stained cells (myoblasts) proliferation (Fig.

5). Thus, epithelial *Wls* influences mesenchymal cell proliferation to balance myoblast and chondroblast growth in developing trachea.

Wnt signaling may pattern tracheal muscle by inducing genes that antagonize muscle development in the ventral mesenchyme of the trachea. The transcription factors *Msx1* and *Msx2*, targets of BMP signaling and known to play many roles in embryonic development, were expressed in the ventral aspect of the trachea (Alappat et al., 2003; Ishii et al., 2005). *Msx1* and *Msx2* were decreased in *Wls^{fl/fl};Shh^{Cre/+}* tracheal tissue. *Msx2* mRNA was decreased in control prechondrogenic tracheal explants incubated with the canonical Wnt inhibitor XAV 939 (Fig.8). Studies on vascular development and disease have shown that *Msx1* and *Msx2* actively repressed smooth muscle differentiation (Cheng et al., 2014; Hayashi et al., 2006; Lallemand et al., 2005). Thus, these data suggest that *Msx1* and *Msx2* may antagonize the formation of smooth muscle in the ventral mesenchyme of the trachea.

Besides effects in tracheal muscle patterning, deletion of epithelial *Wls* changed the orientation of the muscle fibers of the *trachealis* muscle, being oriented parallel to the longitudinal tracheal axis, rather than the transverse orientation observed in *trachealis* muscle of control embryos (Fig.3). This abnormal arrangement of the muscle layer resembles the phenotype previously described by deletion of the transmembrane protein *Tmem 16*; however, in the latter model of tracheomalacia, expansion of muscle into the ventral aspect of the trachea was not described (Rock et al., 2008). Thus, Wnt signaling from the epithelium plays a dual role in *trachealis* muscle development: preventing muscle differentiation in the ventral tracheal mesenchyme and being required for normal morphology of the *trachealis* muscle fibers.

In conclusion, we propose a model wherein epithelial Wnt signaling from endodermal progenitors of the respiratory tract mediates dorsal ventral patterning of the trachea. Epithelial Wnt ligands including Wnt7b and Wnt5a activate Wnt/ β -catenin in the mesenchyme of the developing trachea to influence expression of chondrogenic factors including *Tbx4*, *Tbx5*, *Msx1*, *Msx2*, *Sox9* and *Col2a1*, genes required for initiation of chondrogenesis in the ventral region of the trachea. Epithelial *Wls* also differentially promotes cell proliferation of mesenchymal cells and prevents the anomalous proliferation of muscle cells into the ventral region of the trachea. By influencing differentiation of prechondrogenic mesenchyme and cell proliferation, epithelial Wnt ligands direct dorsal ventral patterning of developing trachea. Taken together, abnormalities in secretion of Wnt ligands produced by the epithelial cells of the upper airways, provides a mechanism underlying congenital tracheobronchomalacia.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- Wls is required for dorsal-ventral patterning of the developing trachea.
- Wnt ligands from tracheal epithelium promote proliferation of tracheal mesenchyme.
- Wnt/ β catenin signaling is necessary for tracheal chondrogenesis.
- Epithelial Wnt ligands are necessary for *trachealis* muscle formation and patterning

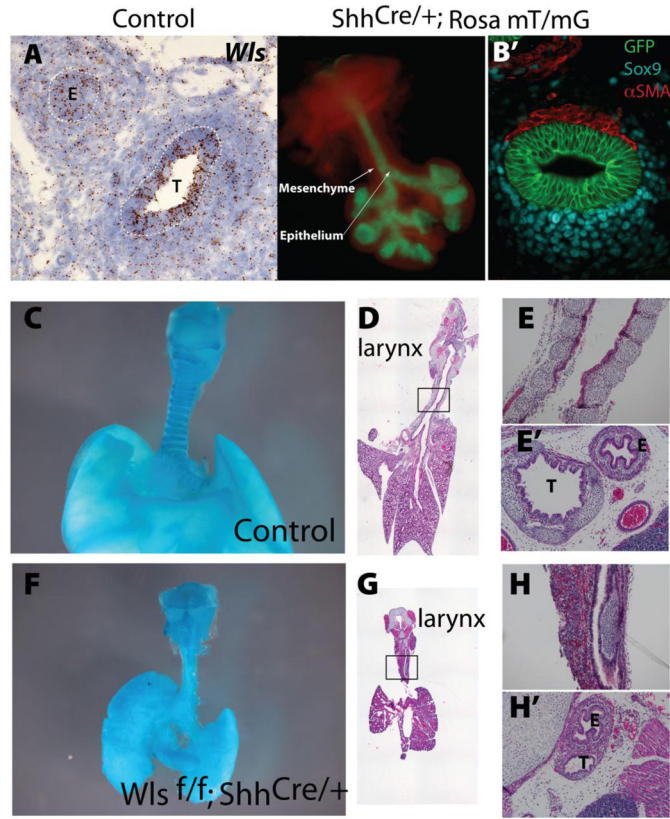


Figure 1. Deletion of *Wls* from the embryonic foregut endoderm causes tracheal- bronchial cartilage agenesis

In situ hybridization was performed on cross sections of E13.5 embryos. Representative image depicting expression of *Wls* in trachea and esophagus is shown. Dotted lines demark the tracheal and esophageal epithelium (A). Whole mount of tracheal lung explant depicting epithelial GFP expression driven by Cre mediated recombinase in *Wls^{f/f}; Shh^{Cre/+}; Rosa mT/mG* mice is shown. Cross section of the tracheal explant demonstrates the circumferential expression of GFP indicating sites of Cre mediated recombination (B). Alcian blue (C,F) and H & E (D,E,G,H) stainings of whole mounts and tracheal/lung sections demonstrate the near absence of cartilage in *Wls^{f/f}; Shh^{Cre/+}* trachea and bronchi. Higher magnifications of regions in squares are shown in E and H. Cross sections of control (E') and *Wls^{f/f}; Shh^{Cre/+}* (H') E18.5 embryos depict esophagus and trachea. Note the lack of cartilage in *Wls^{f/f}; Shh^{Cre/+}* embryos and the position of trachea in close proximity to the esophagus.

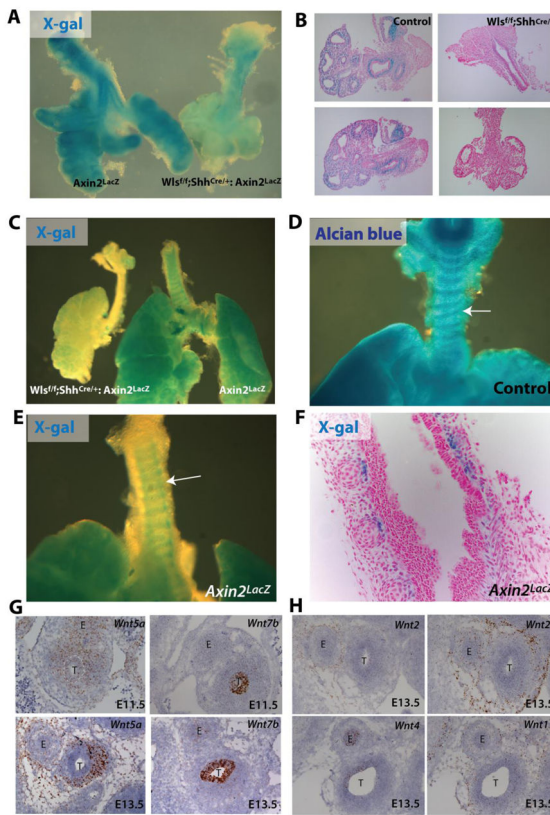


Figure 2. Decreased mesenchymal canonical Wnt signaling after deletion of epithelial *Wls*
 X-gal staining was performed on trachea-lung explants of E12.5 *Axin2^{LacZ}* and *Wls^{ff};Shh^{Cre/+};Axin2^{LacZ}* mice. X-gal staining was primarily observed in mesenchyme of *Axin2^{LacZ}* mice, while staining was nearly absent in tissue of *Wls^{ff};Shh^{Cre/+};Axin2^{LacZ}* mice (A,B). At E14.5, X-gal staining was detected in the tracheal mesenchyme of control embryos in a pattern similar to that of the cartilaginous rings. In contrast, no staining was detected in the trachea of *Wls^{ff};Shh^{Cre/+}* embryos (C). E14.5 control tracheal explants were stained with Alcian blue to determine sites of chondrogenesis. The staining pattern is similar to the X-gal staining of *Axin2^{LacZ}* mice (D). X-gal staining was restricted to the periphery of tracheal mesenchymal condensations (E, F). In situ hybridization was performed to determine expression pattern of several Wnt ligand mRNA. At E11.5 *Wnt5a* was detected in both epithelium and mesenchyme of developing trachea. At E13.5 *Wnt5a* RNA was enriched in the ventral mesenchyme of developing trachea, while *Wnt7b* was restricted to the epithelium of E11.5 and E13.5 trachea (G). *Wnt2* and *Wnt2b* mRNA were detected in dorsal and lateral mesenchyme surrounding the trachea. *Wnt4* mRNA was detected at low levels in the tracheal epithelium, while *Wnt11* was present in tracheal mesenchyme of E13.5 embryos (H).

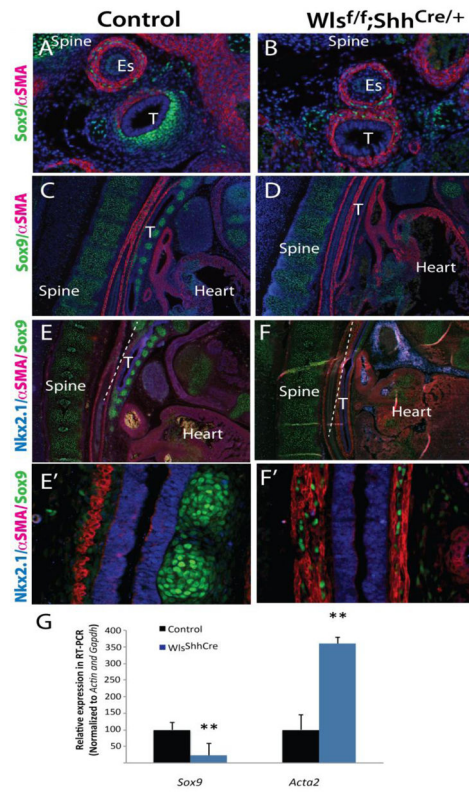


Figure 3. Loss of epithelial Wls-mediated signaling disrupts dorsal-ventral patterning of the trachea

Longitudinal and cross sections of E13.5 (A,B) and E14.5 (C,D,E,F) embryos were stained with Sox9, αSMA and Nkx2.1 antibodies. DAPI was utilized to visualize cell nuclei. Sox9 was absent in the tracheal mesenchyme but expressed in vertebral cartilage of *Wls^{fl/fl};Shh^{Cre/+}* embryos. αSMA staining was expanded into the ventral region of the trachea of *Wls^{fl/fl};Shh^{Cre/+}* embryos (B,D,F). In developing trachea of *Wls^{fl/fl};Shh^{Cre/+}* embryos, muscle fibers run parallel to the longitudinal axis of the trachea in contrast to the transversal arrangements of the fibers observed in control trachea (E', F'). Sox9 was decreased and *Acta2* mRNA increased in tracheal explants of *Wls^{fl/fl};Shh^{Cre/+}* embryos at E14.5 (G). N=4, **p<0.01, T-trachea Es= esophagus.

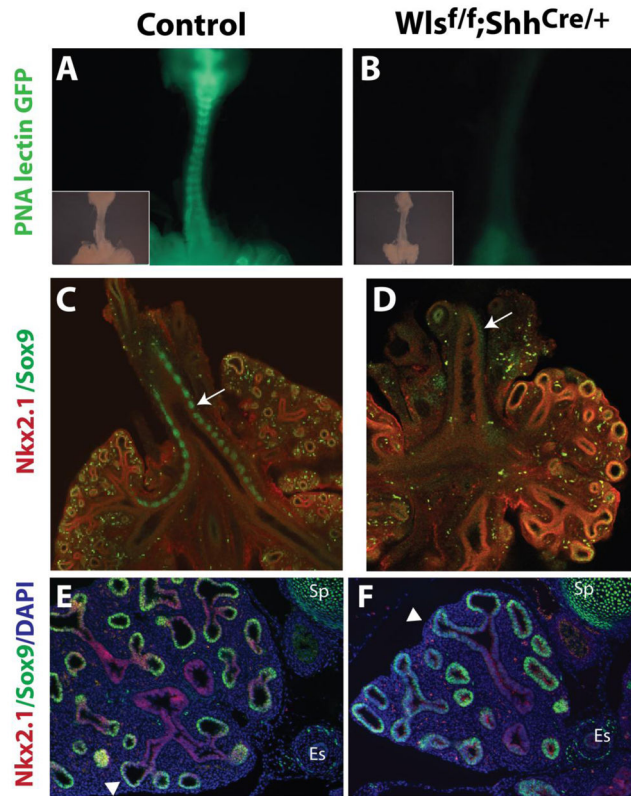


Figure 4. Epithelial *Wls* is required for prechondrogenic mesenchymal condensations
 Tracheal lung explants of E13.5 embryos were stained with PNA lectin. Lack of staining demonstrates absence of mesenchymal condensations in tracheas of *Wls^{ff};Shh^{Cre/+}* embryos (arrow B vs. arrow A). Insets in A and B correspond to bright field images of whole mounts stained with PNA lectin. Confocal images of E13.5 whole mounts show decreased Sox9 staining in tracheal mesenchyme (arrow in C, and D). Sox9 staining in cells of the peripheral lung overlapped with Nkx2.1 in both control and *Wls^{ff};Shh^{Cre/+}* pulmonary tissue (arrowhead in G and H). Es= esophagus, Br=Bronchus, Sp=spine.

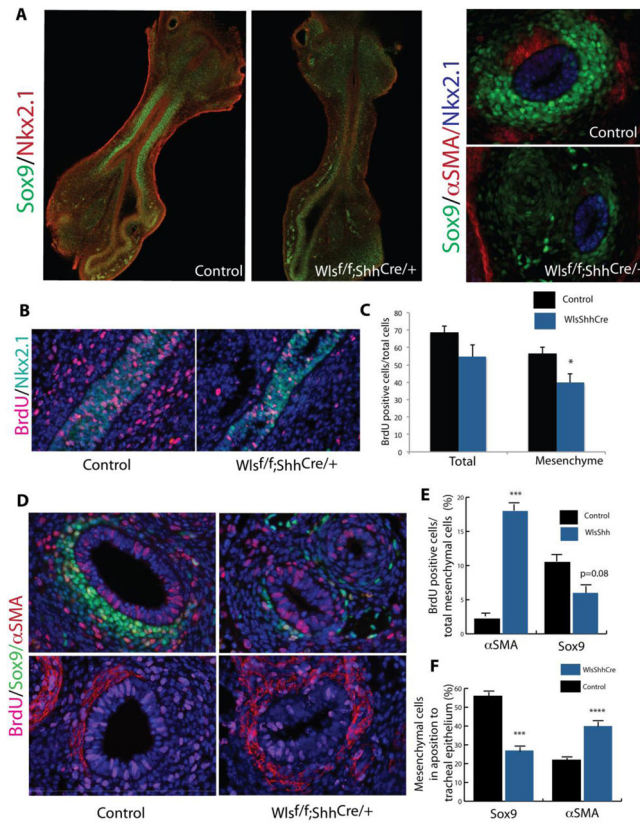


Figure 5. Epithelial Wnt ligands modulate mesenchymal cell proliferation

Confocal images of E11.5 tracheal lung explants stained for Sox9 and Nkx2.1 are shown. Sox9 staining was nearly absent in the tracheal mesenchyme of *Wls^{f/f};Shh^{Cre/+}* mice. Transverse sections of tracheal tissue depict the extent of Sox9 expression and the lack of αSMA staining in *Wls^{f/f};Shh^{Cre/+}* at E11.5. Dams were injected at E11.5 with BrdU to determine cell proliferation. Longitudinal sections of E12.5 tracheas were stained for BrdU and Nkx2.1; proliferation was decreased in mesenchyme of *Wls^{f/f};Shh^{Cre/+}* embryos (B,C). Smooth muscle cell proliferation was increased at E13.5, while proliferation of Sox9 stained cells was reduced in the tracheal mesenchyme of *Wls^{f/f};Shh^{Cre/+}* embryos (D, E). Increased numbers of αSMA stained cells were found in direct apposition to the tracheal epithelium. Numbers of Sox9 stained cells in direct contact with the tracheal epithelium were significantly reduced (F).

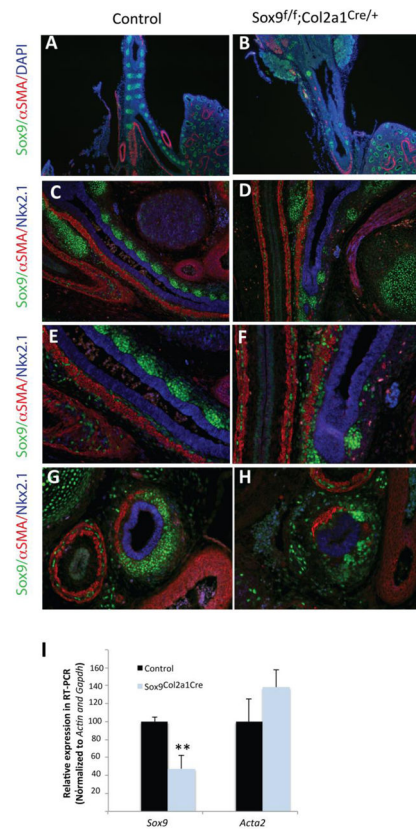


Figure 6. Mesenchymal deletion of Sox9 from developing trachea does not promote expansion of tracheal muscle layer

Sox9 was deleted using *Col2a1Cre* mice, resulting in tracheal cartilage agenesis (B,D,F). No expansion of muscle into the ventral mesenchyme of the trachea was observed (D,F). RT-PCR analysis demonstrates that *Sox9* mRNA is decreased while *Acta2* mRNA was not significantly changed in E14.5 *Sox9^{ff};Col2a1^{Cre/+}* trachea (G). N=4 **p<0.01.

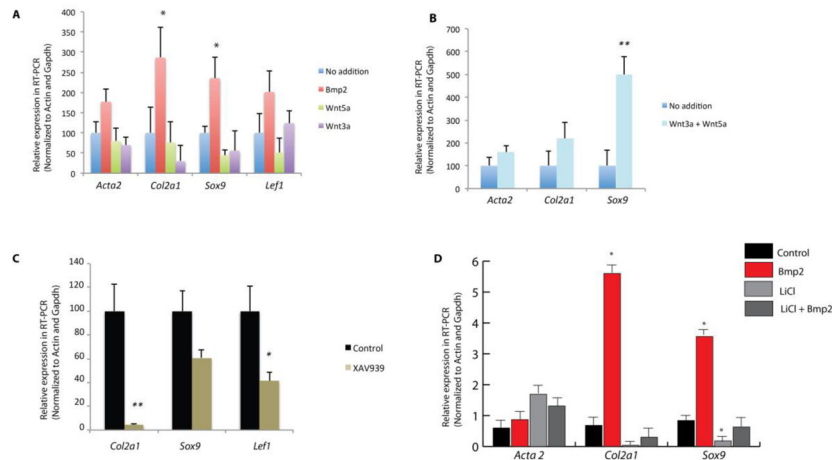


Figure 7. Canonical Wnt signaling modulates expression of Sox9 ex vivo and in vitro Bmp-2 (100ng/ml) induced *Sox9* expression in *Wls^{fl/fl};Shh^{Cre/+}* tracheal explants, while Wnt3a or Wnt5a (200 ng/ml) did not induce *Sox9* in tracheal explants. (A) Addition of Wnt3a and Wnt5a to culture media of *Wls^{fl/fl};Shh^{Cre/+}* explants increased *Sox9* and *Col2a1* mRNA (B). Control tracheal explants were cultured in the presence of the canonical Wnt antagonist XAV-939. Addition of XAV-939 (1uM) reduced expression of *Col2a1*, *Lef1* (a direct canonical Wnt target) and to lesser degree *Sox9* (C). In MEF micromass cultures, addition of Bmp-2 robustly induced *Sox9* and *Col2a1*. Addition of Bmp-2 and LiCl repressed Bmp-2 induced *Sox9* in MEF micromasses (D). N=5 * p<0.05 ** p<0.02 vs. C.

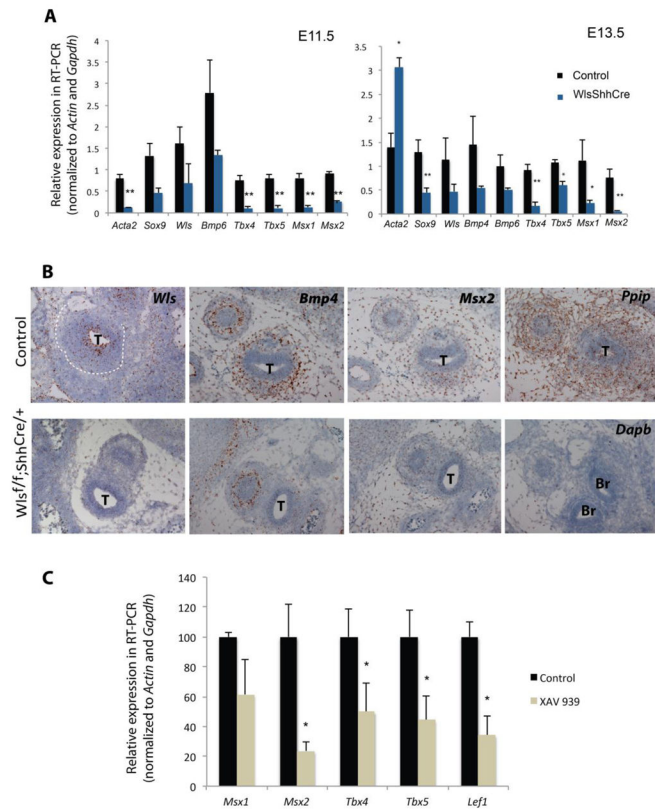


Figure 8. Canonical Wnt signaling influences expression of genes controlling cartilage differentiation

Quantitative RT-PCR performed on E11.5 and E13.5 tracheas of control and *Wls^{ff};Shh^{Cre/+}* embryos demonstrated decreased expression of genes required for cartilage patterning, as well as increased levels of *Acta2* at E13.5, N= 3 **p*<0.05, ** *p*<0.01 (A). Analysis of *Wls*, *Bmp4* and *Msx2* mRNA by in situ hybridization is shown. *Bmp4* and *Msx2* localized to the ventral region of the trachea where cartilage will be formed. Epithelial deletion of *Wls* decreased *Bmp4* and *Msx2* mRNA in ventral tracheal mesenchyme. *Wls* RNA was decreased in tracheal epithelial cells of *Wls^{ff};Shh^{Cre/+}* mice. *Ppib* transcripts, positive control, were detected throughout the section while *Dapb* transcripts, negative control, were not detected (B). Incubation of tracheal explants with XAV-939 decreased *Msx2*, *Tbx4* and *Tbx5* and *Lef1* mRNAs. Representative experiments of explant cultures are shown. N=3 **p*<0.05 (C). T= Trachea, Br= Bronchus.

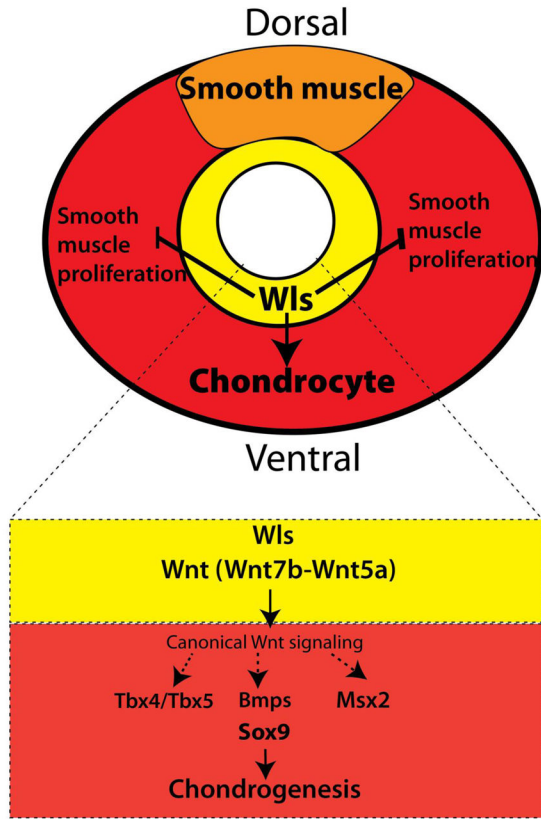


Figure 9. Model for dorsal ventral patterning of developing trachea
 Epithelial Wls promotes cartilage development and prevents the expansion of muscle cells into the ventral side of the trachea. Wnt ligands produced by the epithelium promote canonical Wnt signaling in the tracheal mesenchyme, influencing the expression of transcription factors required for differentiation of mesenchymal cells into cartilage. Epithelial Wls regulates mesenchymal proliferation and modulates proliferation of smooth muscle cells. Thus, by promoting cartilage differentiation and modulating smooth muscle cell proliferation, epithelial Wnt signaling directs the patterning of the tracheal mesenchyme.