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WNT2 Signaling in Lung Development

Abstract

Development of the vertebrate lung is a complex process involving the input of signaling pathways to coordinate the specification and differentiation of multiple cell types. The Wnt signaling pathway plays a critical role in the development of many vertebrate tissue types, including the lung. However, the functions of individual Wnt ligands during the specification and development of respiratory lineages have not been fully addressed. In this dissertation, I combine mouse genetic models and ex vivo tissue culture assays to determine the function of Wnt2 signaling in lung development. These studies reveal critical roles for Wnt2 signaling during the specification of lung progenitors in the foregut endoderm, and during airway smooth muscle development. These findings provide new insight into the position and contributions of Wnt signaling within the hierarchy of signaling pathways governing lung morphogenesis.

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WNT2 SIGNALING IN LUNG DEVELOPMENT

Ashley M. Goss

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IN

CELL AND MOLECULAR BIOLOGY

Presented to the Faculties of the University of Pennsylvania

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ABSTRACT
WNT2 SIGNALING IN LUNG DEVELOPMENT

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Development of the vertebrate lung is a complex process involving the input of signaling pathways to coordinate the specification and differentiation of multiple cell types. The Wnt signaling pathway plays a critical role in the development of many vertebrate tissue types, including the lung. However, the functions of individual Wnt ligands during the specification and development of respiratory lineages have not been fully addressed. In this dissertation, I combine mouse genetic models and ex vivo tissue culture assays to determine the function of Wnt2 signaling in lung development. These studies reveal critical roles for Wnt2 signaling during the specification of lung progenitors in the foregut endoderm, and during airway smooth muscle development. These findings provide new insight into the position and contributions of Wnt signaling within the hierarchy of signaling pathways governing lung morphogenesis.

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List of abbreviations

AEC	Alveolar cell type
A-P	Anterior-posterior
APC	Adenomatosis polyposis coli
ASM	Airway smooth muscle
BMP	Bone Morphogenetic Protein
CamKII	Calmodulin-dependent protein kinase II
COPD	Chronic obstructive pulmonary disease
Dkk1	Dkkopf1
DKO	Double knockout
Dsh	Disheveled
EA	Esophageal atresia
EMT	Epithelial-mesenchymal transition
FGF	Fibroblast growth factor
Fzd	Frizzled
GSK3β	Glycogen synthase kinase 3 beta
JNK	Jun N-terminal kinase
Lef	Lymphoid enhancer binding factor
Lrp	Lipoprotein receptor related
Tcf	Transcription factor 1
MRTFB	Myocardin-related transcription factor B
PDGFR	Platelet-derived growth factor receptor

PECAM	Platelet endothelial cell adhesion molecule
PKC	Protein kinase C
Ptc2	Patched 2
PAH	Pulmonary arterial hypertension
ROK	Rho-associated kinase
Shh	Sonic hedgehog
SM	Smooth muscle
SMC	Smooth muscle cell
SM22-α	Smooth muscle protein 22- α
SMA	Smooth muscle α -actin
SRF	Serum Response Factor
TEF	Tracheoesophageal fistula
TGF-β	Transforming growth factor beta
VEGF	Vascular endothelial growth factor
VSM	Vascular smooth muscle

Chapter 1: Introduction

Summary

The evolutionarily conserved Wnt signaling pathway regulates numerous processes during embryonic development, including patterning of the foregut endoderm from which the lung arises. Genetic studies in the mouse highlight the contributions of Wnt signaling to the specification and morphogenesis of not only the lung, but also other foregut-derived organs, such as the liver and pancreas. However, there is little data describing the molecular mechanisms and targets of Wnt signaling in the developing lung. This introductory chapter will summarize the current understanding of Wnt signaling in mouse foregut development, with emphasis placed on identifying critical questions concerning the function of Wnt signaling in lung development.

The role of signaling pathways in lung development and disease

The adult lung is an incredibly complex organ comprised of more than forty different cell types with cells serving both respiratory and nonrespiratory functions¹. The primary function of the lung is to enable gas exchange, and the principle components contributing to this function are a network of branched airway tubules juxtaposed with a similarly branched vascular system. Furthermore, specialized cell types within the airway epithelium carry out additional functions, including surfactant secretion, mucus clearance, fluid transport, and immune surveillance and response²⁻⁶.

Not surprisingly, defects in the signaling pathways that guide the growth and differentiation of these lung cell types can present serious risks for human development.

Mutations in several signaling pathway components are implicated in a broad spectrum of human birth defects referred to as esophageal atresia (EA) and tracheal esophageal fistula (TEF)⁷⁻¹⁰. Moreover, abnormal expression of developmental signaling pathways in the adult lung is correlated to certain diseases including fibrosis¹¹, emphysema^{12, 13}, and cancer¹⁴⁻¹⁶.

Wnt proteins are critical intercellular transducing signals expressed during lung development that are often aberrantly expressed in lung diseases. Several Wnt ligands and components of the Wnt signaling pathway are mutated in many types of lung cancers¹⁷⁻¹⁹. Direct evidence for Wnt signaling and cancer incidence is demonstrated by dominant activation of Wnt signaling in the adult mouse lung epithelium, which results in higher incidence of pulmonary adenomas²⁰. Activated Wnt signaling has also been identified in fibroproliferative diseases such as asthma, pulmonary arterial hypertension (PAH), and chronic obstructive pulmonary disease (COPD)^{21, 22}. Altogether, the emerging data point to a contributing role for Wnt signaling in lung disease. A more thorough understanding of the mechanisms of Wnt signaling in lung development will help to inform therapeutic strategies and clinical care for both congenital and adult lung diseased states.

Overview of the Wnt signaling pathway

Wnt proteins are cysteine-rich secreted glycoproteins that signal through at least three known pathways (outlined in Figure 1.1). Pathway activation involves binding of Wnt ligands to a receptor complex comprised of the Frizzled (Fzd) family of seven-pass transmembrane receptors and members of the family of lipoprotein receptor related 5/6

proteins (Lrp5/6). Currently, 19 Wnt proteins, 10 Fzd receptors, and two Lrp co-receptors have been identified in mammals²³. Adding further complexity to the pathway, studies have demonstrated that individual Wnt proteins can activate several pathways in a cell-type and context-dependent manner²⁴⁻²⁶. Therefore, the particular pathway a Wnt ligand signals through will depend on specific Wnt-Fzd combinations and the presence or levels of intracellular factors that can prevent signaling of one pathway over another^{27,28}.

Wnt proteins mediate many of their effects through the canonical Wnt signaling pathway²⁹ (Figure 1.1). Activation of the canonical pathway begins with Wnt ligand binding to the Fzd-Lrp5/6 co-receptor complex and activation of the intracellular effector protein Disheveled (Dvl), which subsequently inactivates a complex of proteins including the constitutively active serine-threonine kinase glycogen synthase kinase 3 β (Gsk3 β), the scaffolding proteins Axin, and adenomatosis polyposis coli (APC). In the absence of Wnt stimulation, this complex phosphorylates β -catenin, thereby targeting it for proteosomal degradation. Upon Wnt pathway activation, inhibition of this β -catenin degradation complex allows β -catenin to accumulate at high levels and translocate into the nucleus where it complexes with the LEF/TCF family of transcription factors to activate target gene expression²⁸.

Some Wnt ligands also signal through β -catenin independent pathways (Figure 1.1). The best characterized of these non-canonical Wnt pathways are the Ca²⁺/protein kinase C (PKC) and RhoA/ Jun-N-Terminal Kinase (JNK) pathways³⁰. In Ca²⁺/PKC signaling, Wnt binding activates a G-protein dependent activity of Fzd receptors that in turn activates intracellular calcium signaling and calcium dependent protein kinases, including PKC and calmodulin-dependent protein kinase II (CaMKII)^{31,32}. In RhoA/JNK

signaling, Dvl activates the Rho family of GTPases (RhoA, Rac, and Cdc42) and their downstream effectors including Rho Associated Kinase (ROK) and JNK³³. Although these pathways mediate the majority of β -catenin independent Wnt signaling, it remains unclear whether these pathways are truly distinct from one another. Furthermore, recent studies have identified increasing examples of interplay between canonical and non-canonical pathways, as activated non-canonical signaling often attenuates canonical signaling^{34,35}. This is suggestive of a broader Wnt signaling network characterized by distinct combinations of effectors being activated for different environmental contexts and cell-types.

The Wnt ligands and their downstream signaling pathways are evolutionarily conserved mediators of numerous developmental processes including cell fate specification, proliferation, migration, and adhesion³⁶⁻⁴⁰. A significant amount of data describing the role of Wnts in vertebrate development exists in the literature, however more evidence is needed to fully understand the contributions of specific Wnt ligands and their associated downstream signaling mechanisms to vertebrate development and organogenesis.

Vertebrate development and organogenesis

In the early vertebrate embryo, cells of the totipotent epiblast segregate into three embryonic lineages or germ layers — the mesoderm, which forms muscle, blood, and bone; the ectoderm, which yields the skin and nervous system; and the endoderm, which gives rise to the lining of the respiratory and digestive tracts. Segregation of these primordial lineages occurs during gastrulation (E6.0-E7.5 in the mouse), whereby the

totipotent cells of the epiblast divide, rearrange, and involute to set up the vertebrate body plan⁴¹. At the end of gastrulation, the early endoderm consists of a sheet of cells extending along the anterior-posterior axis (A-P) of the embryo⁴². Over a twenty-four hour period, morphogenetic movements transform the endoderm sheet into the primitive gut tube, from which endoderm-derived organs will bud⁴³⁻⁴⁵ (Figure 1.2A). The endoderm of the primitive gut tube is divided into three broad sections-the foregut, which is the most anterior portion; the midgut; and the hindgut. In the mouse, the foregut endoderm gives rise to organs including the thyroid, lungs, liver, ventral pancreas, and stomach. The midgut and hindgut give rise to the stomach, dorsal pancreas, and intestines⁴⁶ (Figure 1.2B).

Prior to organ budding (E8.0-9.5), the early endoderm is a morphologically homogenous germ layer, however signaling pathways have already imposed molecular differences along its A-P axis to demarcate prospective organ primordia through specification of progenitor cell types⁴². Numerous transcription factors are expressed along the A-P axis of the endoderm prior to the onset of organ budding, and identify subgroups of endodermal progenitors populating the organ anlage (as shown in Figure 1.2A). A critical question in the field of developmental biology has focused on what signaling molecules and pathways act upon the multipotent cells of the endoderm to establish discrete A-P zones of competent organ progenitors. The Wnt signaling pathway has been heavily implicated in endoderm patterning^{47, 48}.

In the literature, there is a paucity of evidence describing the specific contributions of Wnt signaling in the developing mouse endoderm, and specifically the foregut endoderm⁴⁹. Additional research is needed to elucidate the mechanisms of Wnt

signaling that potentially coordinate foregut development and organogenesis. Moreover, studies will need to identify direct transcriptional targets the Wnt signaling pathway activates to pattern and instruct organ fate in the multipotent mouse endoderm.

Epithelial-mesenchymal interactions pattern the vertebrate foregut endoderm

The A-P patterning of the vertebrate endoderm occurs in large part via soluble signals derived from adjacent mesodermal tissues⁵⁰. These instructive signals can take the form of hormones, growth factors, cytokines, or extracellular matrix molecules, and they can activate sets of organ-specific genes^{51,52}. Several studies have demonstrated that the splanchnic mesoderm adjacent to the foregut endoderm is necessary for the specification of endodermal progenitors giving rise to foregut-derived organs⁵³⁻⁵⁵. For example, mesoderm adjacent to the ventral foregut is critical for liver induction and budding in the mouse embryo^{43,56,57}. Moreover, tissue recombination experiments demonstrate that a bipotential liver and pancreas precursor in the foregut endoderm is directly influenced by the presence or absence of local mesenchymal cues, indicating that the presence of local signals emitted from the mesoderm underpin endodermal progenitor specification⁵⁸.

Recent studies demonstrate that Wnt ligands produced in the splanchnic mesoderm are crucial in coordinating the development of vertebrate endoderm derivatives in a tightly regulated temporal fashion. Several Wnts are expressed in the mesoderm surrounding the foregut and midgut^{59,60}, and misexpression of Wnt1 and Wnt5a in the mouse embryo strongly suggests that paracrine Wnt signaling patterns the adjacent foregut and midgut during pancreas progenitor specification and differentiation⁶¹. In zebrafish, *wnt2b* expression in the lateral plate mesoderm is required

for liver progenitor specification, and loss of *wnt2b* expression results in liver agenesis⁴⁸. In the invertebrate system of drosophila, Wnts expressed in the lateral mesoderm have been shown to regulate patterning of the gut endoderm⁶². These data indicate that Wnt signaling is an evolutionarily conserved paracrine signaling mechanism coordinating the development of endoderm-derived tissues.

Studies carried out in chick and *Xenopus* models also demonstrate that Wnts expressed in the mesoderm pattern the endoderm. Wnt signaling in conjunction with Fibroblast growth factor (FGF) signaling suppresses foregut fate in endoderm progenitors and concomitantly promotes hindgut fate^{47, 50, 63}. Conversely, inhibiting Wnt/ β -catenin activity in the *Xenopus* posterior endoderm results in the appearance of ectopic liver progenitors and activation of liver-specific genes in the presumptive intestine. Surprisingly, slightly later in development, Wnt and FGF signaling promote the development of foregut derivatives⁴⁷. Thus, the spatial and temporal characteristics of Wnt paracrine signaling occurring between the vertebrate splanchnic mesoderm and foregut endoderm is dynamic and appears to directly impact the sequence and location of organ formation.

Altogether, the published data indicate that different Wnt receptor-ligand combinations and their accompanying levels of expression are likely to create distinct patterns and thresholds of Wnt activity that will coordinate foregut patterning through the specification of endodermal progenitors. Further investigations into the role of Wnt paracrine signaling between the splanchnic mesoderm and adjacent foregut endoderm will be critical for a complete understanding of the role of this pathway in mouse foregut progenitor specification and organogenesis.

Epithelial-mesenchymal interactions regulate the development of lung progenitors in the foregut endoderm

In agreement with the studies examining liver and pancreas progenitor development, several lines of *in vivo* and *in vitro* experimental evidence suggest that lung progenitor specification and bud outgrowth from the foregut endoderm also rely on non-cell autonomous paracrine signaling with the adjacent splanchnic mesoderm. Studies in which embryonic lung mesoderm is grafted onto heterotypic epithelial cells and tissue demonstrate the ability of early embryonic lung mesoderm to induce robust branching and de novo expression of lung-specific genes⁶⁴⁻⁶⁶. Patterning of the foregut region at the site of the presumptive tracheoesophageal bifurcation depends in part on Transforming growth factor β (TGF- β) signals secreted from the adjacent mesoderm^{10, 67}.

Gene targeting experiments have demonstrated that FGF-10 signaling in the splanchnic mesoderm is absolutely necessary for inducing lung bud development in the foregut endoderm. Mice homozygous mutant for either *Fgf10* or its cognate endodermally expressed receptor *Fgfr2* exhibit severely disrupted lung development with no main-stem bronchial development⁶⁸⁻⁷⁰. Interestingly, the levels and spatial characteristics of FGF and FGF receptor (FGFR) expression in both the splanchnic mesoderm and ventral foregut endoderm direct specification of either liver or lung progenitors⁵⁴. This finding suggests that specification of foregut endoderm progenitors is significantly influenced by the presence of local mesodermal paracrine signaling factors in conjunction with the expression of distinct types and combinations of cell surface receptors in the endoderm.

Crosstalk between Sonic hedgehog (Shh) signaling expressed in the endoderm and its targets in the adjacent splanchnic mesoderm also regulate early lung endoderm development. Mice that are *Shh*^{-/-} homozygous mutant form a trachea, but lung branching is severely disrupted⁷¹. Additionally, loss of expression of the *Shh*-responsive transcription factors *Gli2* and *Gli3* in the embryonic mesoderm yields embryos with severe defects throughout lateral mesoderm-derived structures including loss of the esophagus, trachea, and lungs⁷².

Activation of a transcriptional network establishes lung endoderm progenitor identity in the foregut

The paracrine signaling occurring between the splanchnic mesoderm and foregut endoderm regulates the specification of lung endoderm progenitors through upregulation of a transcriptional network governing lung endoderm progenitor identity. Various classes of transcription factors — including the GATA-family members (GATA6) and Forkheads (Foxa1 and Foxa2) — are expressed in the presumptive lung primordium of the foregut endoderm and regulate the development of early lung progenitors^{73, 74}. Compound mutant mouse models abolishing Foxa1 and Foxa2 function indicate that these two genes cooperate to establish competency of the foregut endoderm to give rise to its derivatives including the lung⁷⁵. GATA6 is expressed in lung epithelial progenitors and is required for their survival at the onset of branching morphogenesis^{74, 76}.

The transcription factor Nkx2.1 is the earliest known marker of lung progenitor cells distinguishing the primitive tracheal and lung endoderm field in the anterior foregut⁴⁴ (Figure 1.3A). Of note is that Nkx2.1 is expressed in thyroid precursors during

organogenesis, and therefore Nkx2.1 is not a lung progenitor-specific transcription factor⁷⁷. Targeted disruption of the *Nkx2.1* gene has demonstrated that it is not required for lung specification, suggesting that Nkx2.1 expression is activated by other unidentified primary signals that induce lung specification^{44, 77}.

What factor(s) mediate lung progenitor specification in the foregut endoderm?

The specific signaling events and tissue interactions that commit the multipotent foregut endoderm to a lung cell fate prior to the onset of morphogenesis are under increasing scrutiny in large part due to new and better mouse models. Overall, the evidence published thus far demonstrates a prominent role for the splanchnic mesoderm as a critical signaling center for the specification and maintenance of lung endoderm progenitors in the adjacent foregut. However, the question still remains whether a single gene contributes to the establishment of the lung endoderm primordium and whether there is a lung specific transcription factor mediating the entire lung morphogenesis program.

Despite the drastic disruptions to the lung morphogenesis program in the aforementioned genetic models, with the exception of the *Gli2/3* compound mutants, specification of the early lung and tracheal endoderm still occurs. The presence of Nkx2.1-expressing lung progenitor cells in the absence of these factors begs the question of what other signal(s) direct specification of lung progenitors in the foregut, and are those signals coming directly from the adjacent mesoderm? The Wnt/ β -catenin signaling pathway represents an intriguing and plausible candidate in early lung specification and morphogenesis, given its critical role in the establishment of other foregut derived tissues.

Several Wnt ligands are expressed in the splanchnic mesoderm flanking the ventral foregut endoderm at the appropriate locations and developmental timepoints to mediate lung progenitor specification and development. However, the answer as to whether Wnt ligands regulate these processes awaits new genetic mouse models to address this hypothesis.

Development of the mouse lung primordia

Lung morphogenesis in mice has been categorized into five developmental time periods corresponding to histological and structural progressions⁷⁸ (Table 1.1). For such an elaborate vital organ, the lung has modest beginnings: it develops initially from the tracheal outpocketing of the anterior foregut endoderm (outlined in Figure 1.3). Specification of lung progenitors in the mouse foregut endoderm occurs around E9.0. Lung progenitors can be detected by in situ hybridization as a population of *Nkx2.1*-expressing cells in the ventral aspect of the laryngotracheal groove of the foregut endoderm⁴⁴. The dorsal aspect of the anterior foregut serves as the esophagus primordium and is denoted by robust expression of the transcription factor *Sox2*⁷⁹ (Figure 1.3A).

After specification, the ventral *Nkx2.1*-positive portion of the endoderm tube undergoes morphogenesis to bifurcate and from the trachea, and by E11.5 the septation is complete with a defined trachea and esophagus (Figure 1.3B). At E10.5, the two primitive lung buds are comprised of three cell layers: the inner epithelium (derived from the endoderm), the surrounding mesenchymal stroma, and a thin outer mesothelium⁸⁰. Mesenchymal cells encompass the primitive lung epithelium as it undergoes repetitive terminal and lateral branching during branching morphogenesis invading the splanchnic

mesenchyme surrounding the foregut to give rise to an arborized network of airways⁸¹ (Figure 1.3C). In vivo cell lineage analysis using an inducible cre recombinase mouse model has shown that there are two distinct non-overlapping lung progenitor cell populations in the foregut endoderm, which separately contribute to the proximal conducting airway epithelium and distal airway epithelium⁸². The tracheal endoderm continues to express Nkx2.1 throughout embryonic development⁸³.

Epithelial-mesenchymal signaling drives lung branching morphogenesis

Branching morphogenesis in the mouse lung continues after primary budding through E17.0, resulting in the formation of the bronchi, bronchioles, and distal alveolated airways⁸⁴. Reciprocal mesenchymal-epithelial interactions are absolutely essential to promote and sustain this process for appropriate proximal-distal growth and cell differentiation^{80, 85-88}. The primary driver of the lung branching morphogenesis program is localized expression of the chemoattractant factor FGF-10 in the mesenchyme adjacent to the branching epithelial tubules, which express FGFR-2^{89, 90}. Feedback signaling mediated by Sprouty2, an FGF-10-FGFR-2 inhibitor expressed in the tips of the branching epithelium, controls the size and shape of the epithelial bud^{91, 92}. Experiments conducted in lung organ cultures also suggest that Shh and Bone morphogenetic protein (BMP) co-expression in the distal epithelial tips serves to progressively down-regulate FGF-10 expression to limit bud outgrowth⁹³⁻⁹⁵.

In the current model of lung epithelial branching and patterning, there is a distinct compartment of proliferative multipotent cells located at the distal epithelial tips expressing the transcription factor Id2, which is only expressed in embryonic distal

epithelial cell populations⁹⁶. The maintenance and proliferation of these multipotent cells is coordinated by crosstalk between several pathway molecules expressed in both the branching epithelial bud and adjacent distal mesenchyme, including Wnts, FGFs, Shh, and BMP/TGF- β members^{95, 97-99}. The resulting gradient and spatial organization of molecules generated as the tips elongate restricts multipotency to the distal tip cells. As the epithelial tubules elongate and branch, the progeny of the tip cells continue to divide and undergo differentiation to give rise to all of the cell types of the bronchi and bronchioles⁹⁶.

The primitive lung epithelium differentiates into multiple cell types

Coincident with lung outgrowth and branching morphogenesis is proximal-distal patterning of the developing airway epithelium. In the proximal conducting airways, the epithelium differentiates into various cell lineages including those of the secretory cell types (Clara cells and goblet), ciliated cells, and neuroendocrine cells¹. In the distal airways, the terminal epithelial buds branch and dilate to form the presumptive alveolar acini (Figure 1.3C). These distal epithelial cells differentiate into either the gas-exchange alveolar cell type I, or the surfactant secreting alveolar cell type II¹⁰⁰ (AECI/II). Differentiation of epithelial cells occurs in a proximal-distal direction with the secretory Clara and ciliated cell types appearing around E14.5-E15.5 in the proximal airways. In the distal airways, the alveolar epithelial cell types AECI and AECII differentiate perinatally and through the first week of postnatal life¹⁰¹.

Many of the transcription factors expressed in lung endoderm progenitors and required for their survival and differentiation are also required in the latter stages of

epithelial development. Genetic deletion of *Nkx2.1* and *Foxa2* after the onset of branching morphogenesis inhibits maturation of peripheral airways leading to respiratory failure at birth^{102, 103}. Genetic deletion of *Gata6* or expression of a dominant-negative GATA6 fusion protein in the developing airway epithelium demonstrates that GATA6 is also required in the distal compartment during late alveolar epithelial differentiation^{76, 104}. Notably, branching morphogenesis is not significantly impaired in these mutant models, indicating that in later stages of development, the role of these transcription factors is restricted primarily to airway epithelial cell differentiation.

Mesenchymal signaling influences proximal-distal patterning of the airway epithelium

The concurrent growth and branching of the epithelium in juxtaposition with the developing mesenchyme establishes signaling centers along the A-P axis that act to pattern both tissue compartments in a reciprocal fashion^{80, 90}. Tissue recombination studies first indicated the necessity of epithelial-mesenchymal tissue interactions to promote lung epithelial patterning. In culture, embryonic lung epithelium is unable to survive more than seventy-two hours without the presence of peripheral mesenchyme. This suggests that the cells in the distal lung mesenchyme are releasing soluble factors required by the epithelium for growth and differentiation^{105, 106}. Further investigations into this observation revealed that FGF members can replace the cytodifferentiation-inducing function of the distal mesenchyme in recombination experiments^{107, 108}.

Tissue recombination experiments reinforce the existence of molecular A-P differences in the mesenchyme along the developing respiratory tract. In vitro studies indicate that epithelial progenitors are capable of differentiating into alternative airway

epithelial cell types in response to molecular cues originating from different sections of dissected lung mesenchyme^{106, 109}. These varying sections influence proximal versus distal identity in the airway epithelium^{65, 109, 110}. One identified factor expressed in the mesenchyme that mediates distal identity of epithelial progenitors is Wnt5a. Genetic studies in the mouse lung demonstrate that *Wnt5a* misexpression disrupts key patterning factors in the distal signaling center including Shh, FGF-10, and BMP-4, leading to airway branching and differentiation defects^{97, 98}. These results illustrate the interconnectedness of the signaling interactions between the developing lung epithelium and mesenchyme, and also highlight a critical role for paracrine Wnt signaling in development of the lung epithelium.

The role of Wnt/ β -catenin signaling in patterning the developing lung epithelium

Several Wnt ligands and signaling components of the β -catenin pathway are expressed in embryonic lung epithelial and mesenchymal cell types^{111, 112}, and the importance of epithelial-mesenchymal paracrine signaling suggests that the Wnt/ β -catenin signaling pathway contributes a critical role in these signaling interactions. Transgenic mouse models including *BAT-GAL* and *TOP-GAL* demonstrate canonical Wnt/ β -catenin signaling activity in the developing lung epithelium from E9.5-E11.5, with sporadic activity in a subpopulation of distal airway epithelial cells at E18.5¹¹³⁻¹¹⁵. These observations demonstrate that canonical Wnt/ β -catenin is active during early lung development and may be influencing epithelial differentiation.

The contributions of Wnt/ β -catenin signaling activity to epithelial patterning have been investigated through genetic deletion or inhibition of *β -catenin* in the developing

airway epithelium and mesenchyme. The loss or suppression of epithelial β -catenin signaling leads to the loss of peripheral epithelial progenitors and dysregulation of proximal-distal patterning of the airway epithelium^{115, 116}. Conversely, the expression of a constitutively active β -catenin-Lef1 fusion protein in early lung epithelial cell types leads to hyperplasia of the airway epithelium with a parallel loss of fully differentiated airway cell types¹¹⁴. Interestingly, activated β -catenin signaling also induces a switch in cell lineage commitment with distal lung epithelial cells expressing intestinal markers. This observation suggests that persistently high levels of β -catenin signaling can induce transdetermination of lung epithelial progenitor cell types, or perhaps induce dedifferentiation back to an early type of embryonic endodermal progenitor. Finally, genetic deletion of β -catenin throughout the developing mesenchyme disrupts epithelial cell patterning, indicating that mesenchymal Wnt/ β -catenin signaling also regulates epithelial differentiation¹¹⁷.

Collectively, these results demonstrate a critical role for Wnt/ β -catenin signaling in coordinating lung proximal-distal patterning. Given that there are multiple Wnts expressed in the lung, and β -catenin is expressed in multiple cell types of the lung, this raises the possibility for numerous types of signaling interactions between the epithelium and mesenchyme both cell autonomously and non-autonomously. Therefore, the precise influence and mechanisms of Wnt/ β -catenin signaling on the lung epithelium is likely to be complex and comprise distinct temporal and spatial signaling interactions along the A-P axis.

Development of mesenchymal-derived components of the lung

In the early lung bud, the condensing lung mesenchyme will go on to differentiate into several tissue types including smooth muscle (SM), and vascular and lymphatic vessels. Cell types comprising these lineages include airway and vascular smooth muscle cells, endothelial cells, myofibroblasts, lipofibroblasts, and stromal fibroblasts¹¹⁸⁻¹²¹. SM development is one of the notable patterning events occurring in lung mesenchymal development. At the level of the mainstem bronchi, SM develops partially around the airway on the dorsal surface with cartilaginous rings forming on the ventral surface. Slightly more posterior in the remaining proximal airways, SM forms around the entire epithelium, and in the more distal airways SM is absent. In the developing pulmonary vasculature, SM surrounds the larger blood vessels¹²².

Airway smooth muscle development (ASM) closely succeeds the growth and differentiation of the airway epithelium temporally and spatially. At E11.5 in the mouse, a SM layer envelops the trachea and primary lung buds, and SM continues to develop around the proximal epithelial tubules¹²³. In the current model, one source of ASM originates from a distal pool of *Fgf10*-expressing mesenchymal cells (Figure 1.4). These distal cells proliferate and passively translocate (or perhaps actively migrate) to surround the proximal airways by continued epithelial tube outgrowth and branching. The *Fgf10*-expressing cells now in close proximity to the airway tubules are exposed to pro-myogenic signals diffusing from the distal bud epithelium, which activate SM differentiation^{95, 124, 125}. Continued exposure to these pro-myogenic signals from the adjacent epithelium as the cells consolidate around the airways in conjunction with downregulation of FGF-10 signaling promotes maturation of the ASM¹²⁶.

Epithelial signaling influences lung mesenchymal development

In vitro studies demonstrate that cultured embryonic lung mesenchyme fails to develop and differentiate in the absence of lung epithelium^{127, 128}. Subsequent studies reveal that the two epithelial compartments of the lung — the inner endoderm and outer mesothelium — both signal to and coordinate the development of the lung mesenchyme and its derivatives.

Genetic evidence demonstrates that Shh expressed from the developing lung epithelium is a critical epithelial factor signaling to the adjacent mesenchyme to regulate gene expression through its receptor Patched (Ptc) and downstream transcriptional activators Gli2 and Gli3^{129, 130}. *Shh*^{-/-} null mutant lungs are hypoplastic with significantly decreased proliferation and downregulation of critical factors in the mesenchymal compartment⁷¹. Combinatorial genetic loss of the transcriptional activator molecules *Gli2* and *Gli3* also results in a significant loss of lung mesenchymal tissues^{72, 85, 131}. Interestingly, transgenic overexpression of *Shh* in the lung epithelium leads to increased proliferation in the adjacent mesenchyme and upregulates expression of mesenchymal targets including *Ptc* and *Bmp4*, indicating that Shh is a broad regulator of signaling activities in the mesenchymal compartment^{80, 93}.

The outer mesothelium layer expresses FGF-9 and recent investigations demonstrate that FGF-9 signaling will inhibit the response of the mesenchyme to Shh expressed from the endoderm. Loss of FGF-9 signaling in the mesothelium leads to decreased stromal proliferation, ultimately yielding a lung with significantly reduced mesenchymal mass^{132, 133}. Cumulatively, these gain- and loss-of-function experiments highlight the significance of the signaling interplay between both epithelial compartments

and the mesenchymal compartment during lung morphogenesis. The reciprocal paracrine signaling between all three tissues appears to directly impact the size and fate of a mesenchymal progenitor pool in the distal lung.

Paracrine signaling between the developing epithelium and mesenchyme is critical for SM development in the lung. The distal tips of the epithelial buds secrete pro-myogenic signals including Shh and BMP-4 to the adjacent mesenchyme during branching morphogenesis to promote ASM development^{80, 95, 124}. Misexpression of these signaling molecules often impairs the differentiation of adjacent immature smooth muscle cells (SMCs). In addition to the loss of proliferation and target gene expression in the mesenchyme of *Shh*^{-/-} null mutant lungs, there is also a loss of SM development^{71, 93}. *Fgf10* hypomorphic mice demonstrate that epithelial BMP-4 expression is a target of FGF-10 signaling from the adjacent mesenchyme, and the subsequent activation of BMP-4 signaling is critical for ASM differentiation¹²⁵.

The role of Wnt/ β -catenin signaling in patterning lung smooth muscle

Recent studies indicate that Wnt/ β -catenin signaling is active and plays a role during SM development in the lung. From E14.5 to E18.5, transgenic mouse models show Wnt/ β -catenin signaling activity in the developing ASM¹¹⁵. Canonical Wnt/ β -catenin signaling from the lung epithelium appears to be critical for SM development in the lung. Loss of the epithelial-expressed *Wnt7b* gene leads to fatal interruptions in lung vascular smooth muscle (VSM) development¹³⁴. Furthermore, treating lung explants with the canonical Wnt signaling inhibitor Dickkopf-1 (Dkk-1) impairs SM gene expression¹³⁵.

More recent studies demonstrate that genetic deletion of β -catenin in the mesenchymal compartment impairs the amplification of *Fgf10*-expressing cells and results in a significant loss of ASM¹³⁶. Moreover, genetic deletion of β -catenin in early lung SMCs also results in a significant loss mature SM²². Interestingly, Cohen et al. showed that in utero administration of the activating Wnt/ β -catenin signaling pharmacological agent LiCl enhanced SM development in wild-type embryos, suggesting that Wnt/ β -catenin signaling is sufficient to promote SM development in the lung²². Together, these studies demonstrate that Wnt/ β -catenin signaling is critical for SM development in the lung.

Despite the evidence in the literature implicating several signaling pathways in SM differentiation, there is still a considerable lack of information on the development of early SMCs in the lung. The Wnt/ β -catenin signaling pathway represents an appealing candidate pathway regulating early-stage SMCs because of its temporospatial patterns of expression in the embryonic lung. Currently in the lung field, Wnt7b is the only canonical Wnt ligand reported to play a critical role in SM development, and Wnt7b paracrine signaling specifically contributes to VSM development¹³⁴. The loss of ASM in β -catenin loss-of-function models suggests that other Wnt ligands expressed in the lung are contributing to SM development. Therefore, the generation and examination of additional loss-of-function mouse models for candidate Wnt ligands expressed in the lung may reveal additional roles for Wnt signaling in SM development.

Wnt ligands expressed in the mouse lung

Several Wnt ligand-encoding genes are expressed in the developing mouse lung including *Wnt2*, *Wnt2b*, *Wnt5a*, *Wnt7b*, and *Wnt11*. *Wnt7b* is a canonical Wnt ligand expressed at high levels in the developing airway epithelium¹³⁴. *Wnt5a* is expressed in both the developing mesenchyme and epithelium at later stages of lung development, with higher levels of transcript expression observed in and around the distal branching airway epithelium⁹⁷. *Wnt11* is expressed diffusely at low levels throughout the embryonic lung epithelium and mesenchyme^{112, 137}.

Genetic knockout mouse models carrying targeted disruptions of Wnt ligand-encoding genes expressed in the lung have been generated and many are reported to have defects. *Wnt5a* knockout mice die shortly after birth from respiratory failure, and exhibit late stage lung branching defects with a parallel loss of maturation of distal lung epithelial and mesenchymal cell types⁹⁷. *Wnt11* knockout mice also suffer from perinatal lethality and display kidney branching defects with no reported lung phenotype¹³⁸, suggesting other Wnts in the lung may function redundantly with *Wnt11*. *Wnt7b* knockout mice die perinatally and exhibit severely hypoplastic lungs with decreased epithelial and mesenchymal proliferation in addition to VSM defects^{134, 139}.

Expression of Wnt2 and Wnt2b in the mouse lung

The *Wnt2* and *Wnt2b* ligands are expressed throughout lung organogenesis and more recently have come under scrutiny in our laboratory as compelling candidates for the regulation of lung morphogenesis. *Wnt2b* is a second member of the *Wnt2* family, sharing approximately 70% amino acid identity with *Wnt2*¹⁴⁰. Both are expressed in overlapping spatiotemporal patterns in the splanchnic mesoderm and lung mesenchyme

during lung development. *Wnt2* and *Wnt2b* are initially expressed around E7.5-E9.5 in the splanchnic mesoderm surrounding the foregut involution^{140, 141}. The two genes are later expressed throughout the developing lung mesenchyme around E11.5, and at sites within the heart and vasculature¹⁴². We have observed that *Wnt2* is expressed at higher levels than *Wnt2b* in the lung mesenchyme, and the robust expression of *Wnt2* persists to the perinatal stages of embryonic development, while *Wnt2b* expression levels remain much lower¹⁴³.

Unfortunately, previously generated mouse models carrying null alleles of *Wnt2* and *Wnt2b* have not revealed clear roles for Wnt2 and Wnt2b signaling in lung development. Monkley et al. generated a *Wnt2* knockout mouse model, and *Wnt2*^{-/-} homozygous mutant mice display runting in the litters and placental defects, with no observable lung phenotype¹⁴¹. RT-PCR performed on lung and heart tissue from these *Wnt2* knockout mice indicated that this model was a hypomorph due to the presence of residual *Wnt2* transcripts. With respect to Wnt2b, our laboratory has analyzed a collaborator's *Wnt2b* knockout mouse model, and these mice do not exhibit any lung developmental defects¹⁴³. One interpretation for the lack of lung defects in these knockout models is that Wnt2 and Wnt2b are functioning redundantly during lung development. However, the lack of a lung phenotype in the reported *Wnt2* knockout mouse model is also likely to reflect the fact that the mutant allele is a hypomorph.

Potential functions of Wnt2 and Wnt2b signaling in the lung

These confounding factors have left many unanswered questions concerning the role of the Wnt2 signaling lung development, and this provided the impetus for my

investigations into the contributions of the *Wnt2* and *Wnt2b* ligands to lung development. Several unanswered questions remain regarding their function in lung development. First, what are the roles of the *Wnt2* and *Wnt2b* ligands in the splanchnic mesoderm and developing lung mesenchyme? Second, are *Wnt2* and *Wnt2b* functioning redundantly in these tissues? Third, are these ligands signaling canonically through β -catenin, or non-canonically in their target tissues? And finally, are *Wnt2* and *Wnt2b* signaling in a paracrine and/or autocrine manner, and what are the downstream targets? In order to address these questions, our lab recently generated a new *Wnt2* knockout mouse model, which exhibits a complete loss of *Wnt2* expression at the transcript and protein level. Additionally, we were granted permission to use and investigate a *Wnt2b* knockout model from a collaborating lab. The resulting analysis of the *Wnt2* and *Wnt2b* knockout models, which is described in this thesis, provides novel insight into the contributions of these ligands during lung specification and morphogenesis.

Table 1.1: Progression of lung development in mice.

Period	Developmental Timepoints	Description
Embryonic	E9.5-E11.5	Septation of the trachea and esophagus, formation of primitive lung buds, first round of branching morphogenesis to yield primary bronchi
Pseudoglandular	E11.5-E16.6	Continued branching morphogenesis giving rise to network of bronchiolar airway tubules and buds; vasculogenesis and innervation commence
Canalicular	E16.6-E17.4	Formation of the distal pulmonary acinus (sacculles) and associated vascular network; epithelial differentiation commences
Terminal saccular	E17.4-P5	Dilation of the distal airway space, distal epithelial differentiation, surfactant synthesis
Alveolar	P5-P30	Alveolar growth and septation, maturation of pulmonary vasculature

Figure 1.1: The Wnt signaling pathway.

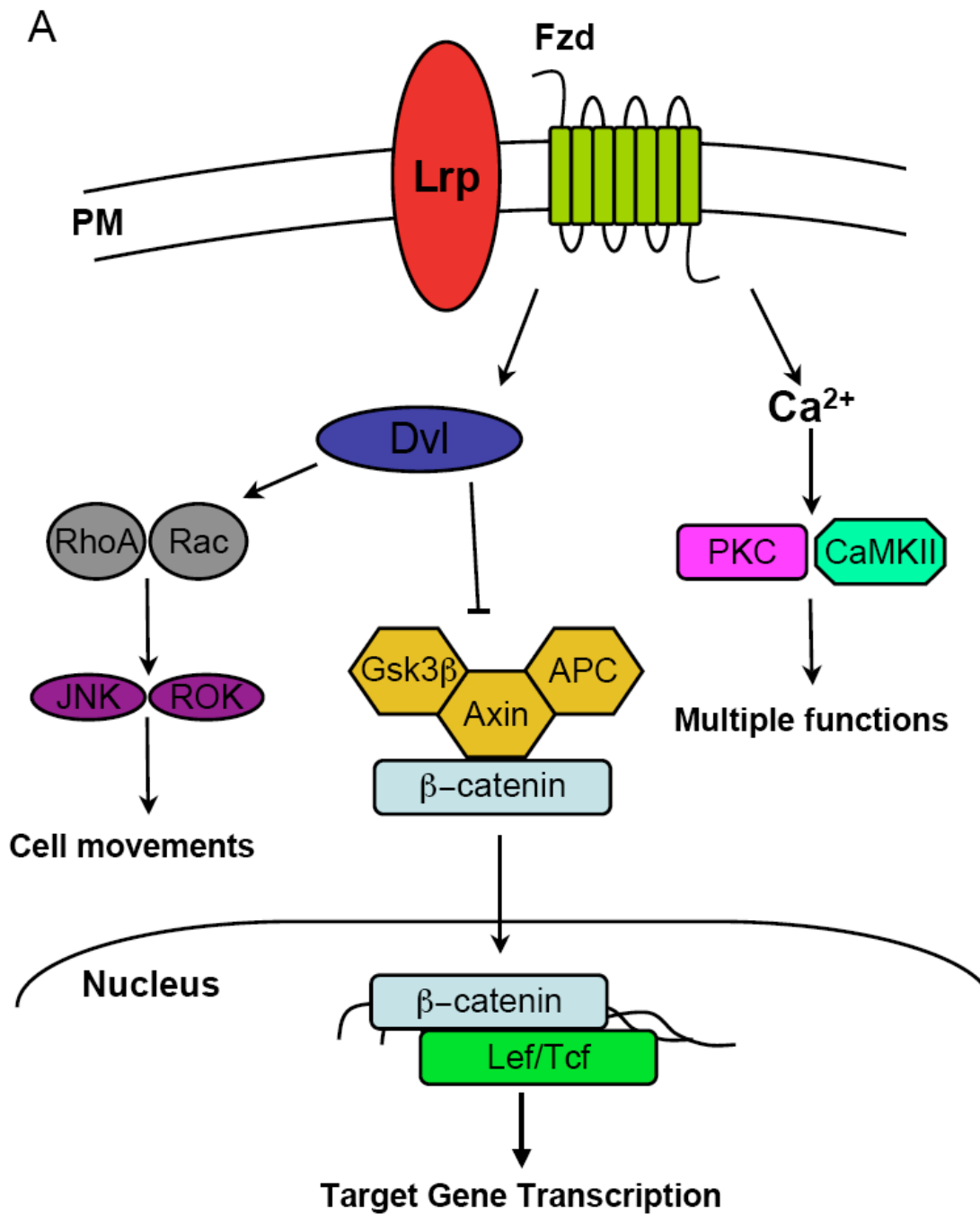


Figure 1.1: The Wnt signaling pathway.

(A) Overview of the Wnt signaling pathway illustrates that Wnt ligands can activate at least three distinct downstream signaling pathways. In the canonical β -catenin pathway, Wnt ligand binding to a Fzd/Lrp co-receptor complex activates the intracellular protein Disheveled (Dvl), which leads to the inhibition of the β -catenin degradation complex (composed of Gsk3 β , Axin2, and APC) and accumulation of β -catenin in the cytoplasm. β -catenin then enters the nucleus to interact with members of the Lef/Tcf family of transcription factors to activate target gene transcription. In β -catenin independent pathways, Wnt ligand binding to a Fzd receptor can activate either intracellular calcium signaling, which influences a variety of cellular processes, or activate RhoA/JNK signaling to mediate cell movement.

Figure 1.2: Endoderm development in the mouse.

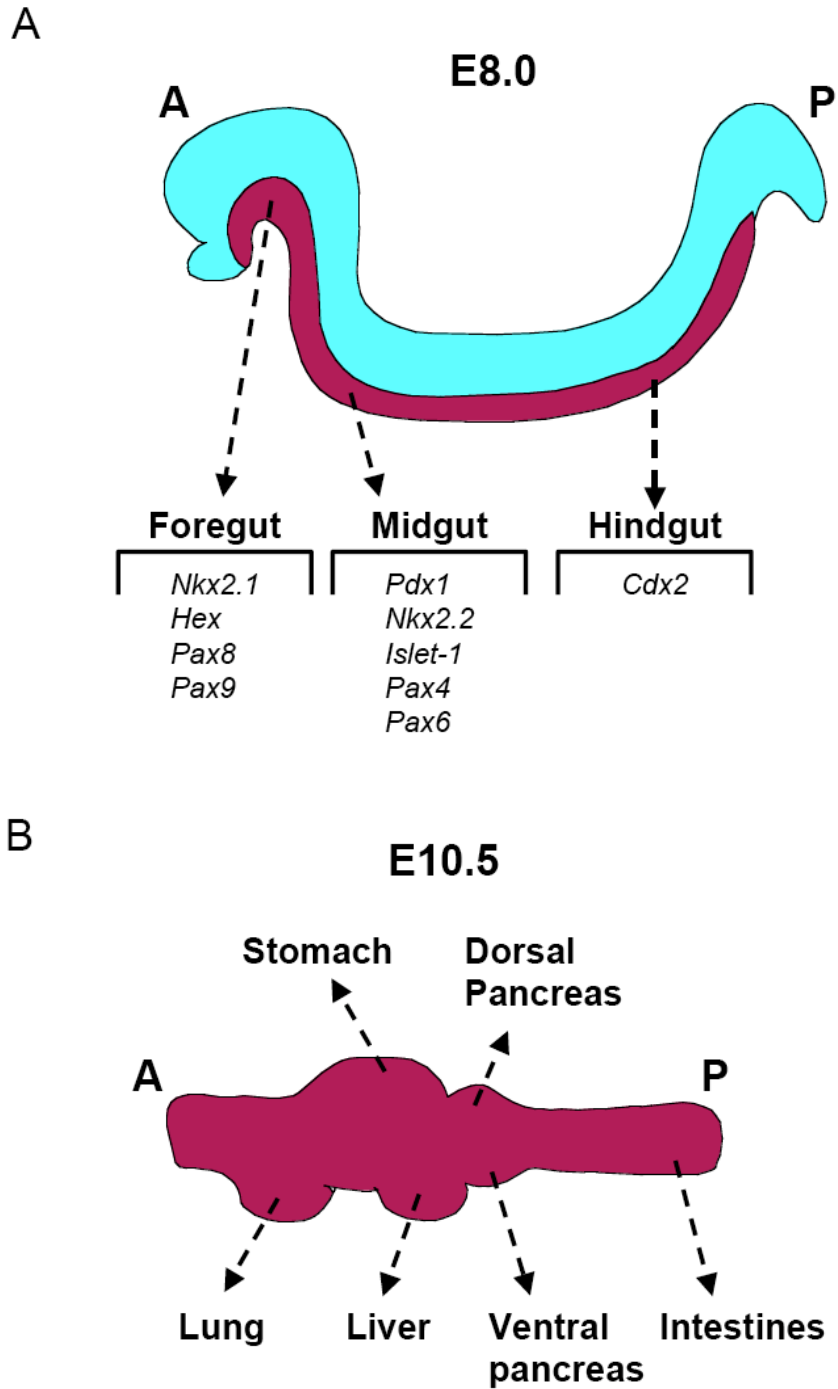


Figure 1.2: Endoderm development in the mouse.

(A) Schematic of the developing mouse embryo and endoderm at E8.0 after gastrulation occurs (E6.0-E7.5). At the end of gastrulation, the primitive endoderm germ layer exists as a sheet of cells that quickly undergoes a series of morphogenetic movements to form the primitive endoderm tube (purple). The primitive endoderm is divided into three anatomical sections: the anterior foregut, midgut, and posterior hindgut. Despite its homogenous appearance along the A-P axis of the developing embryo, molecular differences already denote primitive organ domains. In the foregut, transcription factors including the homeodomain genes *Nkx2.1* [also known as thyroid transcription factor 1 (*Ttf1*)]¹⁴⁴ and *Hex* (hematopoietically expressed homeobox gene)¹⁴⁵, as well as the paired box (Pax) genes *Pax8*¹⁴⁶ and *Pax9*^{146, 147}, demarcate the thyroid/parathyroid, lung, and liver domains, respectively. In the midgut, the transcription factors *Pax4*¹⁴⁸ and *Pax6*¹⁴⁹, *Nkx2.2*¹⁵⁰, *Islet-1*¹⁵¹, and *Pdx1*^{152, 153} are expressed in the presumptive stomach, pancreas, and duodenum domains, respectively. *Cdx2*, a *caudal* homologue and member of the axial patterning *Protohox* gene cluster, is expressed in the hindgut¹⁵⁴. (B) By E10.5, primitive organs have begun to bud and develop from the endoderm. The foregut endoderm gives rise to the lung, stomach, liver, and ventral pancreas. The midgut endoderm also contributes to the stomach and dorsal pancreas and duodenum of the intestines. The remaining hindgut gives rise to the intestines.

Figure 1.3: Lung organogenesis in the mouse.

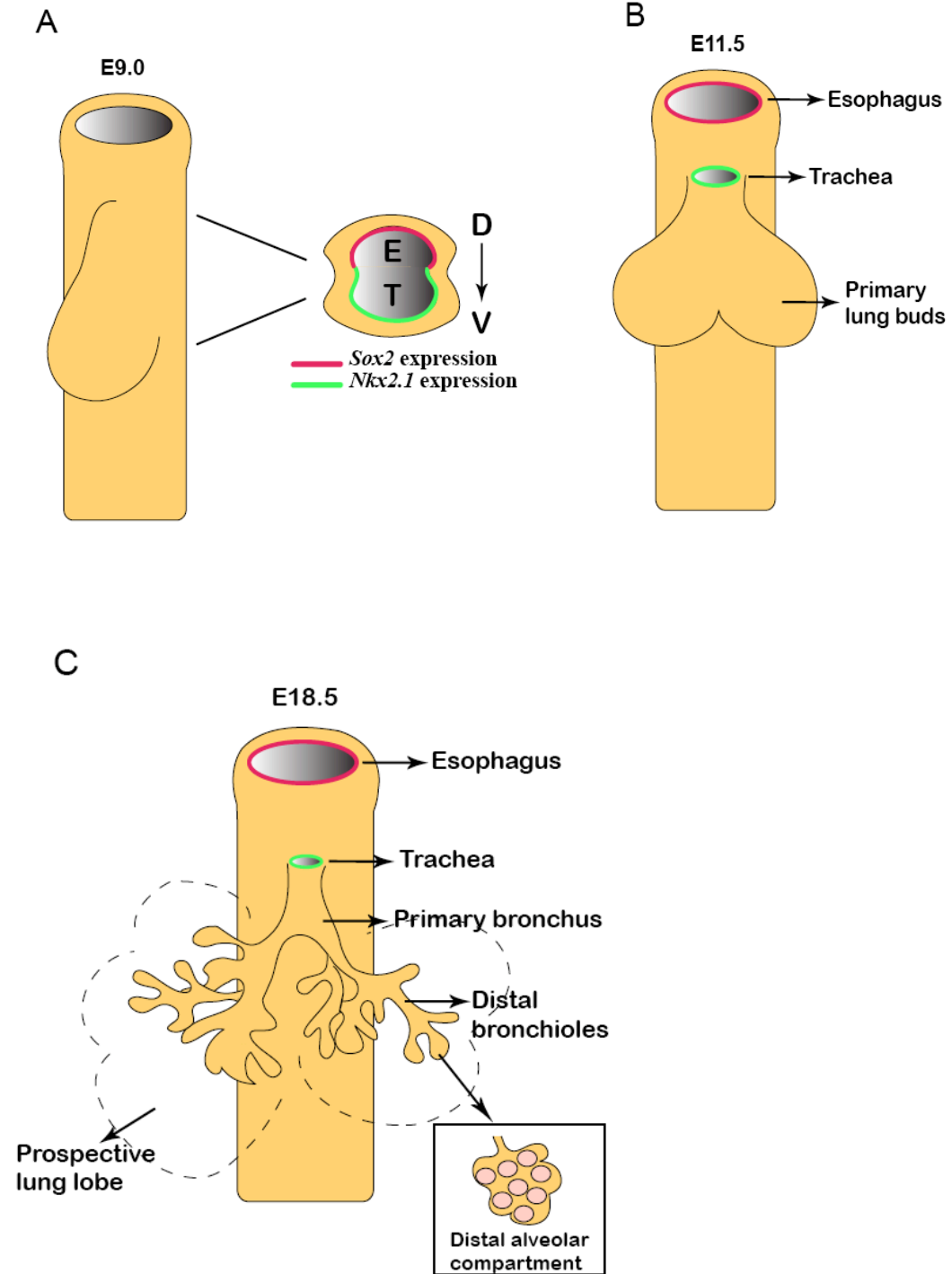
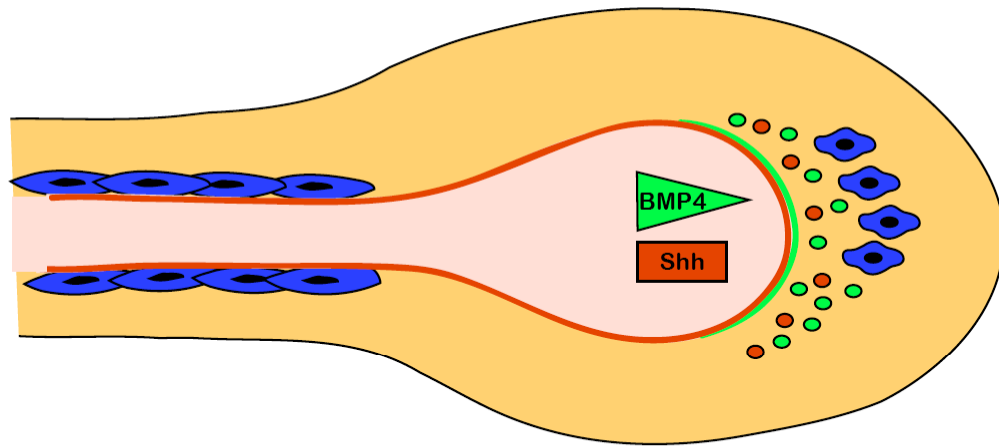


Figure 1.3: Lung organogenesis in the mouse.

(A-C) Schematic of lung development. (A) Around E9.0, tracheal/lung progenitors are specified in the ventral foregut endoderm as denoted by the expression of Nkx2.1, the earliest marker of lung identity. Subsequently, morphogenetic movements initiate the bifurcation of the tracheal endoderm (Nkx2.1 positive) and the esophageal endoderm (Sox2 positive). (B) By E11.5, tracheoesophageal septation has completed and lung morphogenesis commences with endoderm outgrowth to yield two primary lung buds. Branching morphogenesis promotes repetitive outgrowth and branching of the lung buds to yield an arborized network of airways (C). The proximal airways comprise the primary bronchi and distal bronchioles, while the distal alveolar compartment comprises the distal airways.

Figure 1.4: Airway smooth muscle development in the lung.



$SM22\alpha^+$
 SMA^+



$FGF-10^+$
 $SM22\alpha^+$

$FGF-10^-$

SMA^-

Figure 1.4: Airway smooth muscle development in the lung.

Overview of smooth muscle (SM) development in the mouse lung. A sub-mesothelial pool of proliferating immature SMCs expressing FGF-10 and SM22 α are apparent in the lung bud by day E11.5. As the lung bud undergoes branching morphogenesis, the mesenchyme condenses to bring the FGF-10⁺/SM22 α ⁺ SMCs in close proximity to the branching epithelial bud, which is secreting pro-myogenic factors including Shh and BMP-4. The cells coalesce around the airways and continue to differentiate in response to epithelial signaling, flattening to resemble fibroblast-like mature airway smooth muscle (ASM). During this process, the cells continue to express SM22 α and now SMA, a mature ASM marker. Down-regulation of FGF-10 is thought to propel full differentiation of ASM.

Chapter 2: A role for Wnt2 in lung airway smooth muscle development

A majority of the data presented in this chapter is part of a forthcoming manuscript. The data describing the *Wnt2* expression patterns, *Wnt2* knockout mouse model, and *Wnt2*^{-/-} null mutant hypoplasia phenotype are published in Goss et al., 2009.

Summary

In Chapter 1, I gave an overview of the current concept of airway smooth muscle (ASM) development in the mouse embryonic lung. Although several signaling pathways have been identified as important for smooth muscle (SM) development and maturation after the onset of branching morphogenesis, there is less data describing the pathways regulating the early stages of SM development in the lung mesenchyme. In this chapter, I identify the Wnt2 ligand as a critical regulator of SM development. I show that the loss of Wnt2 leads to the down-regulation of a transcriptional and signaling network governing early SM development. This loss results in impaired differentiation of the primitive lung mesenchyme into an immature SM lineage that gives rise to ASM.

Introduction

Disruptions to SM development in humans can lead to chronic health complications including asthma and pulmonary hypertension^{155, 156}. Understanding the signaling pathways coordinating the various specification and differentiation processes occurring during SM development are critical to a complete understanding of lung development as well as pathogenesis in the post-natal lung. Aberrant Wnt signaling is

implicated in several lung diseases including asthma¹⁵⁷, and therefore deciphering the contributions of Wnt signaling during lung development may offer compelling correlates to its contributions to the diseased state.

As I introduced in Chapter 1, the primitive lung mesenchyme gives rise to several cell lineages including smooth muscle cells (SMCs), which will go on to form a sheath around the airways and vasculature in the maturing embryonic lung. SM development is an intriguing patterning event occurring in the mouse lung because the origins of SMC types are still unclear and the pathways regulating proliferation, migration, and differentiation of primitive SMCs are complex and unresolved. Currently, there are no markers distinguishing the origins of airway and vascular smooth muscle (ASM and VSM), thus the identity of a SMC progenitor giving rise to ASM and/or VSM remains elusive. There are data suggesting that two subpopulations of lung mesenchyme give rise to SMC lineages that contribute separately to ASM and VSM. Genetic fatemapping experiments indicate that a subpopulation of mesothelial cells in the developing lung pleura contribute to VSM in the mouse lung, and not ASM¹⁵⁸. In contrast, an enhancer trap transgenic mouse line expressing lacZ from the *Fgf10* locus identified a subpopulation of *Fgf10*-expressing distal mesenchymal cells that condense around bronchial airways and give rise to ASM¹²⁵.

Several signaling pathways are known to be critical in lung SM development. FGF-10 signaling is important for ASM development and mediates this process in part, through signaling interactions with the epithelium. FGF-10 signaling from the mesenchyme activates a gene expression program in the adjacent developing epithelium, which signals in a reciprocal fashion back to the mesenchyme to direct SMC

differentiation¹²⁵. The pro-myogenic signals expressed in the developing airway epithelium include Shh and BMP-4, and these factors direct SMC differentiation along the branching epithelial tubule as *Fgf10*-expressing cells condense around the primitive airway^{159,160} (see Figure 1.4). FGF-9 signaling from the mesothelium is also reported to mediate the development of immature SMCs in the distal mesenchyme by limiting the differentiation response of the mesenchyme to Shh signaling¹³³.

Published data demonstrate that canonical Wnt/ β -catenin signaling contributes to SM development in the lung. Transgenic canonical Wnt/ β -catenin reporter mice show β -catenin signaling activity in developing ASM cells, and this activity colocalizes with the mature SMC marker smooth muscle α -actin (SMA)¹¹⁵. Genetic deletion of β -catenin in primitive SMCs leads to a significant loss of mature SM in the lung²². Wnt7b is a canonical Wnt ligand expressed in the developing epithelium, and *Wnt7b* knockout mice display perinatal pulmonary hemorrhaging owing to disrupted VSM development¹³⁴. Further investigation into the mechanisms of Wnt7b signaling revealed that Wnt7b broadly regulates the development of SMCs in the early lung bud mesenchyme²². The effects on the early SMC population at-large are intriguing in context with the observed perinatal VSM defects. These data suggest that epithelial-based Wnt signaling preferentially directs VSM development.

Recently published data also implicates a role for autocrine canonical Wnt/ β -catenin signaling in lung SM development. Genetic deletion of β -catenin in the developing lung mesenchyme using a pan-mesenchymal *Dermo1-cre* driver leads to disrupted ASM development¹³⁶. These results differ somewhat from the disrupted SM

patterning in *Wnt7b*^{-/-} null mutants, suggesting that Wnt7b may also signal through a β -catenin independent pathway to regulate VSM.

Wnt2 is a ligand expressed throughout the developing lung mesenchyme. The expression pattern of Wnt2 suggests that it may play a role in the development of mesenchymal-derived lineages. In this chapter, I examine a recently generated mouse line from our laboratory carrying a *Wnt2* null allele. Examination of *Wnt2*^{-/-} mice reveals a dramatic loss in ASM perinatally, stemming from decreased proliferation and differentiation of immature SMCs in the early lung bud. These results suggest that Wnt2 signaling plays an important role in the development of SM in the mouse lung.

Results

Expression of Wnt2 during lung development

Previously published data on *Wnt2* expression in the embryonic mouse lung indicated that this ligand is expressed throughout lung organogenesis¹⁴¹. We confirmed this using a *Wnt2*-specific probe for in situ hybridizations on foregut endoderm and lung tissue throughout embryonic development. *Wnt2* is first expressed in the mesoderm surrounding the ventral aspect of the anterior foregut endoderm from E9.0-E9.5 during the period when the lung is specified (Figure 2.1A, B), and continues throughout the developing lung mesenchyme with expression persisting up to perinatal timepoints^{140, 141}.

The expression pattern of *Wnt2* suggests that the gene plays a critical role in the development of mesenchymal lineages in the lung. To further investigate the role of Wnt2 function in lung development, our laboratory generated mice carrying a null allele of the *Wnt2* gene using homologous recombination in embryonic stem (ES) cells (Figure

2.2). Heterozygous mutant *Wnt2*^{+/-} mice were interbred to generate *Wnt2*^{-/-} null mutant embryos, which were examined for possible lung developmental defects. The majority of *Wnt2*^{-/-} null mutants live to E18.5, but die within a few minutes of birth due to respiratory and cardiovascular defects¹⁶¹ (Table 2.1).

Loss of Wnt2 leads to lung hypoplasia and down-regulation of pathways required for lung development.

To explore the reason for the perinatal lethality in *Wnt2*^{-/-} null mutants, histological analysis was performed on embryos from E11.5-E18.5. These examinations reveal lung hypoplasia in *Wnt2*^{-/-} null mutants that is apparent throughout the course of embryogenesis and postnatally (Figure 2.3A-J). The developing lung mesenchyme is also noticeably thinned in *Wnt2*^{-/-} null mutants (Figure 2.3G-J). Despite the hypoplasia, wholemount immunostaining with the pan-epithelial marker E-cadherin shows that branching of the terminal airways appears grossly normal in E14.5 *Wnt2*^{-/-} null lungs (Figure 2.3K-N).

To better understand the cause for the lung hypoplasia, cell proliferation analysis was undertaken using a Ki67 antibody to mark proliferating cells. Ki67 immunostaining reveals decreased proliferation in both the epithelial and mesenchymal cell compartments in *Wnt2*^{-/-} null lungs (Figure 2.3O-Q). Additionally, several signaling pathways and transcription factors known to be important for lung growth and differentiation including *Fgf10*, *Nkx2.1*, *Bmp4*, *N-myc*, and *cyclin D1* are reduced in *Wnt2*^{-/-} null lungs (Figure 2.3R)^{70, 144, 162-164}. In contrast, proximal-distal patterning of the epithelium is unperturbed in *Wnt2*^{-/-} null lungs, as denoted by expression of SP-C⁸², a marker of distal alveolar

epithelial cells, and CC10¹⁶⁵, a marker of proximal bronchiolar epithelial cells (Figure 2.3S-V). Together, these data suggest that Wnt2 signaling is important for lung development and that loss of Wnt2 signaling disrupts proliferation and expression of genes known to regulate lung development, and in particular, disrupts the development of the lung mesenchyme.

Wnt2 signaling through β -catenin regulates airway smooth muscle development in the lung mesenchyme

The appearance of a thin mesenchyme in *Wnt2*^{-/-} null lungs suggests possible defects in mesenchymal cell development and differentiation. Closer examination of *Wnt2*^{-/-} null lungs at E18.5 reveals a reduction in SM surrounding the bronchiolar airways as demonstrated by the loss in expression of the mature SM marker, SM22 α (Figure 2.4A-D). At high magnification, SM22 α -expressing cells are sporadic around the airways, producing significant gaps in the SM layer (Figure 2.4C, D). Interestingly, SM22 α expression in the SM layer of adjacent blood vessels appears unaltered (Figure 2.4A, B), suggesting no impairment to VSM development.

The Wnt/ β -catenin transgenic reporter mice *BAT-GAL* and *TOP-GAL* show Wnt/ β -catenin signaling activity in the developing lung SM^{114, 115}, and Wnt2 is reported to signal canonically through the Wnt/ β -catenin pathway in the lung^{159, 166}. To determine if Wnt2 signals through β -catenin in the developing ASM, *Wnt2*^{+/-} heterozygous mutant mice were crossed into the transgenic *BAT-GAL* reporter background to yield *Wnt2*^{-/-}:*BAT-GAL* embryos. In E14.5 control *BAT-GAL* embryos carrying wild-type *Wnt2* alleles, lacZ-positive cells are evident underlying the developing airway epithelium

(Figure 2.4E). In the absence of *Wnt2*, there is a reduction in the number of lacZ-positive cells in the presumptive ASM layer, indicating a loss of Wnt/ β -catenin activity (Figure 2.4F). Additionally, expression of the Wnt/ β -catenin transcriptional target *Axin2* is reduced in the primitive ASM layer of E14.5 *Wnt2*^{-/-} null lungs (Figure 2.4H). These data suggest that *Wnt2* signals through β -catenin in the lung mesenchyme to mediate proper ASM development. However, this does not preclude a potential role for *Wnt2* paracrine signaling to the adjacent epithelium.

Wnt2 regulates development of the primitive lung mesenchyme and immature smooth muscle

The loss of mature ASM suggests that there is an earlier defect in SMC development in the primitive mesenchyme. The reduced proliferation index of the mesenchymal compartment in *Wnt2*^{-/-} null lungs (Figure 2.3 O-Q) is likely one contributing factor to a loss in SMC development. However, the loss may also reflect disruption to the differentiation of immature SMCs in *Wnt2*^{-/-} null lungs that give rise to ASM.

SM22 α is a highly useful SM marker because it is expressed early in SMC differentiation¹⁶⁷. In E11.5 wild-type embryos, SM22 α expression is distinct in a population of cells forming around the developing proximal airway (Figure 2.5A). In *Wnt2*^{-/-} null lungs, this expression is reduced (Figure 2.5B), indicating that there is a loss of SMC differentiation in the absence of *Wnt2* signaling. Quantitative PCR on E11.5 wild-type and *Wnt2*^{-/-} null lung buds confirms a significant reduction in SM22 α expression (Figure 2.5L).

PDGFR α and PDGFR β are receptor tyrosine kinases through which members of the platelet-derived growth factor family (PDGF) of ligands signal in embryonic mesenchyme to regulate proliferation, migration, and differentiation¹⁶⁸. PDGFR α and PDGFR β are broadly expressed in the multipotent mesenchymal cells of the early lung bud, and have been shown to be important in the development of SMCs from the lung primitive mesenchyme^{118, 169}. Both receptors continue to be expressed in mature SM, with PDGFR β expressed more strongly in VSM^{22, 170}. In E11.5 wild-type embryos, expression of PDGFR β is observed in mesenchymal cells that are coalescing around the developing airways and vasculature (Figure 2.5C), however in *Wnt2*^{-/-} null lungs, there is reduced PDGFR β expression in the mesenchyme (Figure 2.5D). Quantitative PCR on E11.5 wild-type and *Wnt2*^{-/-} null lung buds demonstrates a significant reduction in *Pdgfr β* expression (Figure 2.5K). Overall, these data suggest that loss of *Wnt2* function disrupts the development of immature SMCs in the early lung mesenchyme.

Wnt2 signaling is necessary for smooth muscle gene expression in the lung

To assess whether the earliest stages of SMC differentiation are activated in *Wnt2*^{-/-} null lungs, expression levels of the transcription factors *myocardin* and *MRTFB* were examined. Myocardin is a member of the Myocardin family of Serum Response Factor (SRF) co-factors, expressed in the developing and mature SM of several tissues including the lung¹⁷¹. Myocardin is a critical upstream regulator of myogenesis and in primitive SMCs, myocardin transactivates numerous SM differentiation genes including *SM22 α* and *SMA*^{172, 173}. Two other family members expressed in embryonic and adult SMCs that potentially confer transcriptional activity to SRF to mediate myogenesis are the

myocardin-related transcription factors A and B (MRTFA, MRTFB)¹⁷⁴. MRTFA is broadly expressed throughout the developing mouse embryo in multiple tissue types¹⁷⁵, whereas MRTFB exhibits a more cell-lineage restricted expression pattern including developing cardiac and immature SMCs¹⁷⁶.

At E11.5, in situ hybridizations on wild-type lung tissue show *myocardin* expression around the proximal airway of the primary lung bud (Figure 2.5E). In *Wnt2*^{-/-} null lung tissue, there is a reduction in expression of *myocardin* in the developing ASM (Figure 2.5F). Slightly later at E14.5, *myocardin* expression levels remain lower in the ASM of *Wnt2*^{-/-} null lungs than in wild-type lungs (Figure 2.5G, H). A significant reduction in *myocardin* expression levels is confirmed by quantitative PCR on E11.5 wild-type and *Wnt2*^{-/-} null lung bud tissue (Figure 2.5I). Quantitative PCR also demonstrates that *MRTFB* expression is significantly reduced in *Wnt2*^{-/-} null lung buds (Figure 2.5J). The significant reductions in *myocardin* and *MRTFB* expression in *Wnt2*^{-/-} null lung buds indicates the down-regulation of a myogenic transcriptional program, suggesting that there is reduced SMC differentiation in the absence of Wnt2 signaling.

Wnt2 signaling is sufficient to promote smooth muscle development in the lung

The loss of early SMC gene expression suggests that Wnt2 signaling regulates SMC differentiation from the multipotent mesenchyme, however it is unclear whether Wnt2 signaling is sufficient to promote SMC development in the lung mesenchyme. To better understand the effects of Wnt2 signaling on mesenchymal cells, a recombinant Wnt2 protein (rWnt2) was used to treat 10T1/2 cells, an immortalized multipotent fibroblast-like mouse cell line¹⁷⁷. Culturing 10T1/2 cells in the presence of rWnt2 for 48

hours leads to increased expression of several SMC genes including *SM22 α* , *SMA*, *myocardin*, *Pdgfra*, *Pdgfr β* , and *Fgf10*, as assessed by quantitative PCR (Figure 2.6A). Expression of the canonical Wnt transcriptional target gene *Axin2* is significantly upregulated, indicating that the rWnt2 protein is activating Wnt/ β -catenin signaling in a mesenchymal cell type.

To further investigate the in vivo effects of rWnt2 on SMC development, E11.5 wild-type lung buds were dissected and cultured for 48 hours with either control PBS or rWnt2 in a lung explant assay, which closely mimics in vivo development¹⁷⁸. Explants were collected to examine for changes in SMC development by histological methods and quantitative PCR. In control lung bud explants, SM22 α immunostaining shows expression in the presumptive ASM layer (Figure 2.6C). In lung bud explants cultured in the presence of rWnt2, SM22 α expression is increased around the airways (Figure 2.6D). The appearance of increased ASM is complemented by increased expression of additional SMC marker genes including *SM22 α* , *myocardin*, *MRTFB*, *Fgf10*, *Pdgfr β* , and *Pdgfra*. The mature SM marker *SMA* is also increased in the presence of rWnt2, although not to the extent of other early SMC markers, suggesting that Wnt2 signaling promotes the differentiation and expansion of immature SMCs in the lung mesenchyme, rather than full differentiation to a more mature SMC type.

Other mesenchymal-derived lineages in *Wnt2*^{-/-} null lungs

Other cell types in the mouse lung are derived from the developing mesenchyme including endothelial and lymphatic cell lineages¹⁷⁹. The overall poor mesenchymal development observed *Wnt2*^{-/-} null lungs could signify disruption to the development of

multiple mesenchymal lineages, and therefore *Wnt2*^{-/-} null lungs were examined for defects in endothelial and lymphatic cell lineages.

At E18.5, compared to wild-type lung tissue, *Wnt2*^{-/-} null lungs do not exhibit any alteration in the levels or localization of the endothelial cell marker PECAM (Figure 2.7A, B). Quantitative PCR performed on E12.5 wild-type and *Wnt2*^{-/-} null lung buds tissue reveals only slight reductions in the expression of the endothelial marker genes *Pecam1* and *Flk1* (Figure 2.7C, D). Members of the VEGF family of molecules are important for regulating endothelial and lymphatic development in multiple tissue types¹⁸⁰. Several of these members were also analyzed by quantitative PCR on E12.5 wild-type and *Wnt2*^{-/-} null lung buds. VEGFA, VEGFC, and VEGFD are expressed in the lung during development¹⁸¹, and quantitative PCR demonstrates comparable expression levels of *VegfA* and *VegfD* between wild-type and *Wnt2*^{-/-} null lung bud tissue (Figure 2.7E). Interestingly, *VegfC* expression is significantly reduced in the absence of Wnt2 function (Figure 2.7G). Together, these data indicate that the loss of Wnt2 in the embryonic lung affects other mesenchymal-derived lineages at the level of gene expression, during early developmental timepoints.

Expression of signaling pathways important for smooth muscle development in *Wnt2*^{-/-} null lungs

Several signaling pathways have been identified to be critical for SM development in the mouse lung including the Shh, BMP, Wnt7b, and FGF-10 signaling pathways. *Wnt2* is expressed early in the primitive mesenchyme, and Wnt2 signaling may lie upstream of these pathways and regulate pathway members to mediate SM

development. At E10.5, in situ hybridizations reveal that *Wnt7b* expression is reduced in *Wnt2*^{-/-} null mutant foreguts (Figure 2.8A, B). Quantitative PCR on E11.5 wild-type and *Wnt2*^{-/-} null lung buds demonstrates that *Wnt7b* expression levels are approximately fifty percent lower in *Wnt2*^{-/-} null lungs than wild-type lungs (Figure 2.8I). These data suggest that Wnt2 may be signaling in a paracrine manner to regulate *Wnt7b* expression.

Shh and BMP-4 are well established as critical epithelial-mesenchymal regulators of SM development at the lung bud tip during branching morphogenesis^{80, 93, 95, 159}. In situ hybridization (Figure 2.8E, F) and quantitative PCR (Figure 2.8K) on E11.5 *Wnt2*^{-/-} null lung buds indicates that *Shh* expression levels are similar to wild-type expression levels in the primitive lung endoderm. The expression levels of *Bmp4* expression levels are also comparable between wild-type and *Wnt2*^{-/-} null lung buds as assessed by in situ hybridization (Figure 2.8G, H), and quantitative PCR (Figure 2.8L).

Shh signals from the lung epithelium to its cognate receptor Patched2 (*Ptc2*), which is expressed in the mesenchyme⁹³. Interestingly, quantitative PCR demonstrates that in E11.5 *Wnt2*^{-/-} null lung buds, *Ptc2* expression is significantly reduced (Figure 2.8M). These data indicate that Wnt2 signaling is directly or indirectly regulating the responsiveness of the mesenchyme to Shh signaling, which could lead to SM differentiation defects¹⁸².

FGF signaling in *Wnt2*^{-/-} null lungs

FGF signaling is another pathway critical for proper lung SM development¹⁸³. In situ hybridization shows reduced *Fgf10* expression in the primitive lung mesenchyme of *Wnt2*^{-/-} null mutant foreguts at E10.5 (Figure 2.8C, D). Quantitative PCR performed on

E11.5 lung buds also indicates that *Fgf10* expression levels are approximately fifty percent lower in *Wnt2*^{-/-} null lung buds than expression levels in wild-type lung buds (Figure 2.8J). These data suggest that Wnt2 signaling is upstream of *Fgf10* expression in the primitive lung mesenchyme, and the absence of Wnt2 signaling leads to a significant loss in *Fgf10* expression that impairs development of SMCs into ASM.

FGF-10 signaling regulates smooth muscle gene expression in the lung

Genetic models strongly suggest that FGF-10 signaling is necessary for ASM development in the lung^{125, 183}. To test whether FGF-10 signaling acts upstream of Wnt2 expression, E11.5 wild-type lung bud explants were treated with either control PBS or rFgf10. Expression of *Wnt2* was analyzed by quantitative PCR in rFGF-10-treated explants to ascertain whether FGF-10 signaling also modulates Wnt2 signaling (Figure 2.9A). Interestingly, *Wnt2* gene expression levels are unaffected by exogenous rFGF-10, suggesting that FGF-10 signaling neither activates nor inhibits Wnt2 signaling in a feedback loop.

Treatment of lung explants with rFGF-10 results in a significant upregulation of several mature SM markers including *SM22α*, *SMA*, and *Pdgfrα* (Figure 2.9A). The upregulation in SM gene expression is also evident in histological sections of rFGF-10-treated wild-type explants. SM22α immunostaining shows an increased layer of staining in the developing ASM layer compared to untreated explants (Figure 2.9B, C).

The expression levels of a few SM genes were not affected by treatment with rFGF-10, including *MRTFB* and *myocardin* (Figure 2.9A). This could suggest that FGF-10 signaling promotes SMC development by enhancing the transcriptional activity of the

myocardin/MRTFB/SRF co-factors, rather than by upregulating *myocardin* and *MRTFB* expression. Interestingly, *Pdgfr β* expression levels were unchanged in the presence of rFGF-10. In mature SM of the embryonic lung, *Pdgfr β* is highly expressed in the VSM¹⁶⁹, and *Fgf10*-expressing cells are not reported contribute to the VSM¹²⁵. The increase in *Pdgfr α* expression in parallel with the lack of *Pdgfr β* upregulation in rFGF-10-treated lung explants is intriguing, and could reflect differential upregulation of genes that promote differentiation of immature SMCs into ASM cell populations versus VSM cell populations.

Wnt2 regulates smooth muscle development through activation of downstream FGF-10 signaling

The data thus far suggest that Wnt2 signaling mediates ASM development in part, through downstream activation of FGF-10 signaling. To further investigate the relationship between Wnt2 and FGF-10 signaling, lung buds were dissected from *Wnt2*^{+/-} heterozygous mutant crosses for lung explant rescue assays with rFGF-10 to determine if activated FGF-10 signaling can rescue Wnt2 loss-of-function. Lung bud explants were individually cultured in the presence of either control PBS or rFGF-10 for 48 hours, and subsequently harvested for histological sectioning and quantitative PCR.

Quantitative PCR assessment of SM gene expression in control *Wnt2*^{-/-} null lung bud explants treated with PBS reiterates previous results showing decreased expression of SM genes including *SM22 α* , *SMA*, *myocardin*, and *Pdgfr α* (Figure 2.10A). In the presence of rFGF-10, there is partial rescue of SM gene expression levels. Compared to untreated *Wnt2*^{-/-} null lung bud explants, rFGF-10-treated *Wnt2*^{-/-} null lung bud explants

exhibit significant upregulation of *SM22α* and *SMA* expression. *Myocardin* and *Pdgfra* expression show gross upregulation in rFGF-10-treated *Wnt2^{-/-}* null lung bud explants, however the changes are not statistically significant compared to untreated *Wnt2^{-/-}* lung bud explants. *SM22α* immunostaining on histological cross-sections shows reduced expression around the developing airways in *Wnt2^{-/-}* null lung bud explants as expected (Figure 2.10C). In the presence of rFGF-10, *SM22α* expression is increased and is localized around the airways in *Wnt2^{-/-}* null lung bud explants as in wild-type explants (Figure 2.10B, D). Overall, these data suggest that rFGF-10 can promote the expansion and differentiation of a reduced immature SMC population in *Wnt2^{-/-}* null lung bud explants. The upregulation of several SM genes in the presence of rFgf10 is not statistically significant enough to deem a full rescue. However, there is an overall trend towards upregulated SM gene expression and the histological data also support a partial rescue of the *Wnt2^{-/-}* loss-of-function ASM phenotype.

Taken together, these data suggest a model in which a Wnt2-FGF-10 signaling axis regulates the proliferation and differentiation of ASM from primitive mesenchymal cells in the lung (Figure 2.11). Wnt2 signaling promotes the differentiation of an immature pool of SMCs through direct or indirect activation of a myogenic regulatory network including *myocardin*, *SM22α*, and *MRTFB*. Wnt2 also activates FGF-10 signaling in the mesenchyme, which further drives proliferation and mediates continued SMC differentiation into mature ASM expressing *SM22α*, *SMA*, and *Pdgfra*.

Discussion

Recent genetic evidence demonstrates that Wnt/ β -catenin signaling lies upstream of signals governing SM development in the mouse lung. Loss of the canonical ligand Wnt7b in the embryonic lung epithelium leads to severe VSM defects, demonstrating a critical role for paracrine Wnt/ β -catenin signaling between the developing epithelium and mesenchyme¹³⁴. Additional studies demonstrate that genetic deletion of β -catenin in the primitive lung mesenchyme also disrupts ASM development¹³⁶, implicating a potential function for other canonical Wnt ligands expressed in the lung signaling in mesenchymal cells to give rise to ASM. In this study, I identify Wnt2 as a canonical Wnt ligand required in the lung mesenchyme to mediate ASM development. This study provides the first evidence of a role for Wnt2 signaling in lung SM development.

One of the striking aspects of our *Wnt2*^{-/-} null mutant phenotype is the appearance of a hypoplastic lung, which is evident from early stages of lung bud outgrowth through perinatal timepoints. One contributing factor to this hypoplasia is the reduction in proliferation in both the epithelial and mesenchymal compartment, suggesting that Wnt2 is signaling in both an autocrine and paracrine manner to regulate the development of both tissue compartments. This observation agrees with the well-established role for Wnts in tissue growth and a previously described role for Wnt signaling in early lung development regulating overall proliferation¹³⁹. Interestingly, proximal-distal patterning of the epithelium is unaltered in *Wnt2*^{-/-} null mutants, suggesting that Wnt2 paracrine signaling does not regulate epithelial cell identity.

Canonical Wnt/ β -catenin signaling is active during early lung development¹⁸⁴, and the data presented here demonstrate that Wnt2 signals canonically through β -catenin

in the lung. This is supported by the finding of reduced Wnt/ β -catenin activity in the developing ASM of *Wnt2*^{-/-} null mutants. However this does not rule out the loss of Wnt/ β -catenin activity in other cells types that are not resolved in the *BAT-GAL* transgenic model.

Interestingly, defects in ASM are observed *Wnt2*^{-/-} null mutants, whereas the VSM appears grossly normal. Wnt7b signaling from the lung epithelium regulates VSM development¹³⁴, and analysis of *Wnt2*^{-/-} null mutant lungs demonstrates that Wnt7b is expressed in *Wnt2*^{-/-} null mutants, albeit at reduced levels. Additionally, *Wnt2* expression is reduced in *Wnt7b*^{-/-} null lungs (E. Cohen, personal communication), suggesting there is signaling interplay between epithelial- and mesenchymal-expressed Wnt ligands in the lung. If Wnt2 signaling is important for VSM development, the potential effects from loss of Wnt2 signaling may be compensated by the presence of Wnt7b signaling. Furthermore, the additional Wnt2-family member, Wnt2b, is also expressed in the lung mesenchyme and could function redundantly with Wnt2 signaling (see Chapter 3)¹⁴³.

A recent study describing conditional mutants in β -catenin signaling in the developing lung mesenchyme also suggests a role for Wnt/ β -catenin in the differentiation of the endothelial lineage¹³⁶, and *Wnt2*^{-/-} null embryoid bodies are unable to differentiate into endothelial cells¹⁴². In contrast to the published data, the current evidence does not indicate a significant defect in endothelial development in *Wnt2*^{-/-} null lungs. Despite slight reductions in the early gene expression of select endothelial and lymphatic markers, perinatally, the vasculature and lymphatic vessels appear histologically normal. This does not, however, obviate persistent molecular deficiencies in the development of these tissues that could affect the physiology and respiration of the neonate. The lack of a

prominent embryonic phenotype could be due to compensation by other Wnt ligands including Wnt2b and Wnt7b, or other factors signaling through β -catenin to regulate endothelial development. Additional studies will be undertaken to address whether there are any disruptions in endothelial and development throughout the course of embryonic lung development. The significant reduction in *VegfC* expression hints at possible lymphatic defects¹⁸⁵, suggesting that further analysis of lymphatic markers including LYVE-1¹⁸⁶ and Prox1¹⁸⁷ is warranted.

Wnt2 is expressed during the earliest stages of lung morphogenesis, and therefore the perinatal ASM deficiency could be the result of an early defect in the development of SMCs in the distal mesenchyme. Analysis of *Wnt2*^{-/-} null mutants during the early stages of myogenesis indicates reduced expression of the genes regulating the earliest aspects of SMC differentiation from the primitive mesenchyme including *myocardin*, *MRTFB*, and *Pdgfr β* . These results suggest that Wnt2 signaling is regulating the development of the multipotent mesenchymal compartment and differentiation of immature SMCs in the mouse lung.

The reduced *Pdgfr β* expression might be affecting the development of immature SMCs that give rise to the VSM, however the VSM appears histologically normal in *Wnt2*^{-/-} null lungs. Wnt7b paracrine signaling also regulates SMC development in the primitive mesenchyme, and loss of Wnt7b function decreases the expression of *Pdgfr α* and *Pdgfr β* ²². Yet perinatally, *Wnt7b*^{-/-} mutants display VSM defects¹³⁴, a cell lineage predominantly expressing *Pdgfr β* ¹⁷⁰. Interestingly, the data presented here show that activation of FGF-10 signaling — a pathway regulating ASM development — leads to the upregulation of *Pdgfr α* expression, and not *Pdgfr β* expression. Additional analyses will

need to be undertaken to better understand whether there are differences in expression levels between *Pdgfr α* and *Pdgfr β* in *Wnt2^{-/-}* null mutants at discrete embryonic timepoints. The embryonic origins of ASM and VSM are unclear, and currently there are no known genes expressed preferentially in ASM or VSM progenitor populations. The disparate SM phenotypes between *Wnt7b^{-/-}* null mutants and *Wnt2^{-/-}* null mutants may suggest that these signaling pathways are acting on two distinct, as-of-yet described immature SMC subpopulations, or it may suggest differential downstream signaling effects (e.g. activation of FGF-10) from Wnt ligands that are expressed from different tissue compartments.

A Wnt-FGF regulatory network is thought to govern development of the lung mesenchyme¹¹⁷, and mice carrying hypomorphic or null alleles of *Fgf10* and *Fgfr2*, the cognate receptor of FGF-10, exhibit ASM deficiencies^{183, 188}. In *Wnt2^{-/-}* null mutant lungs, both *Fgf10* expression and *Fgfr2* expression are reduced, implicating a Wnt2-FGF-10 signaling network in the coordination of ASM development. Using a lung explant assay, I demonstrate that FGF-10 signaling promotes ASM development through upregulation of several mature SM markers. These findings would suggest that FGF-10 signaling mediates the expansion and differentiation of immature SMCs towards a more mature ASM lineage.

The upstream signals governing *Fgf10* expression are poorly understood. Recently published data from our laboratory examining β -catenin mutant hearts demonstrate that Wnt/ β -catenin signaling regulates *Fgf10* expression in the developing cardiac mesoderm¹⁸⁹. Furthermore, during late stages of lung development, *Fgf10* expression is regulated by Wnt5a⁹⁸. In wild-type lung bud explants treated with rWnt2,

Fgf10 expression is significantly upregulated, suggesting that FGF-10 signaling is downstream of *Wnt2* signaling. However, ectopic FGF-10 expression in lung bud explants does not alter *Wnt2* expression, suggesting there is no feedback signaling occurring between FGF-10 and *Wnt2*.

Wnt2 may be regulating FGF-10 signaling directly or indirectly in the lung mesenchyme. ASM development relies upon signaling interactions between FGF-10 in the mesenchyme and *Shh* in the epithelium⁹⁴. Loss of *Shh* expression in the lung endoderm, however, does not lead to reduction in *Fgf10* expression⁷¹, suggesting that other factors expressed in the mesenchyme are regulating FGF-10 signaling. One possible factor that may regulate *Fgf10* expression in the lung could be *Ptc2*, a mesenchymal *Shh* target. In *Wnt2*^{-/-} null lungs, there is a significant decrease in *Ptc2* expression, indicating a reduced responsiveness of the lung mesenchyme to pro-myogenic signaling.

In summary, the studies I have described demonstrate that *Wnt2* signaling is necessary for ASM development in the mouse lung. *Wnt2* expression during early lung morphogenesis directs the proliferation and differentiation of ASM from the primitive lung mesenchyme. *Wnt2* signaling coordinates ASM development through direct or indirect upregulation of several well-established myogenic transcription factors and FGF-10 signaling, which promotes further commitment of immature SMCs towards the ASM lineage. In the absence of *Wnt2* signaling, ectopic FGF-10 can partially rescue ASM development. These results define a *Wnt2*-FGF-10 signaling axis governing early ASM development in the lung.

Materials and Methods

Mice

Wnt2 mutant mice were generated using recombineering techniques to replace a portion of the coding region of the first exon with the reverse tet-activator cDNA (rtta). Correctly targeted ES cells were used to generate chimeric mice that were bred to transmit these mutant alleles through the germline. Generation and genotyping of the BAT-GAL mouse line has been previously described¹¹³. The University of Pennsylvania Institutional Animal Care and Use Committee approved all animal protocols. Genotyping primer sequences for the Wnt2 mice are as follows:

Wnt2^{rtta}:

Wnt2Forward (5' TGAGTCTCACCCTAGCCGCA 3')

Wnt2Reverse (5' ACTGGGAATCAGCCAGGGAGGGT 3')

Wnt2rttaReverse (5' TCTCAATGGCTAAGGCGTCG 3')

Histology

Embryos were fixed in 4% paraformaldehyde for 24 hours, dehydrated in a series of ethanol washes, and then embedded in paraffin for tissue sectioning. Dissected embryonic lung buds and foreguts were fixed in 4% paraformaldehyde overnight, dehydrated in a series of methanol washes and blocked a 10% normal goat serum/PBS solution before wholemount immunostaining. Radioactive in situ hybridization and immunohistochemistry were performed as previously described¹⁹⁰. Tissue sections were stained with the following antibodies and dilutions: anti-Axin2 (1:100), anti-CC10 (Santa Cruz; 1:20), anti-E Cadherin (Sigma, 1:50), anti-Ki67 (Vector Laboratories, 1:50), anti-Pdgfr β (Cell Signaling Technology Inc.; 1:50), anti-PECAM (PharMingen; 1:500), anti-SPC (Chemicon; 1:500), and anti-SM22 α (Abcam; 1:500). Quantification of Ki67

positive cell populations was performed by using at least three different tissue sections from at least three different embryos of the same genotype. β -galactosidase histochemical staining of embryos was performed as previously described by our laboratory¹³⁴.

Quantitative RT-PCR

Total RNA was isolated from lung tissue at the indicated time points using Trizol reagent, reverse transcribed using SuperScript First Strand Synthesis System (Invitrogen), and used in quantitative real time PCR analysis using the oligonucleotides listed below. RNA was isolated from at least 5 lung bud tissue samples corresponding to each genotype.

Axin2

F (5' CAGCCCTTGTGGTTCAACCT 3')

R (5' GGTAGATTCCTGATGGCCGTAGT 3')

Bmp4

F (5' CCCTTTCCACTGGCTGATCA 3')

R (5' GGGACACAACAGGCCTTAGG 3')

CC10

F (5' ATCCTAACAAGTCCTCTGTGTAAGCA 3')

R (5' GGAGACACAGGGCAGTGACA 3')

Cyclin D1

F (5' GATGTGAGGGAAGAGGTGAAGGT 3')

R (5' CAATGAGAATCTGGTTCTGAACGT 3')

Fgf9

F (5' TTCATGCGGTGGGTTCTTATT 3')

R (5' TCCTCATCCAAGCTTCCATCA 3')

Fgf10

F (5' CAGTAAGACACGCAAGCATTACTG 3')

R (5' AATCTGATCCAATTCTTCCATGGT 3')

Fgfr2

F (5' GCTTCTCAGTGAGTTTTAATAACAGCTT 3')

R (5' GAATGATGCTGGGCTTTTGC 3')

Flk1

F (5' AGAGCGATGTGTGGTCTTTCG 3')

R (5' TCTCCTACAAAATTCTTCATCAATCTTG 3')

MRTFB

F (5' CAACATGGAGTGGCTAGACATTACC 3')

R (5' GGTCCAGAAAATCAGCAGAGAAC 3')

Myocardin

F (5' CAACACCTTGCCCAGTTATCAG 3')

R (5' GCAGCGGACAAGTCAGATGA 3')

Nkx2.1

F (5' CTTCATCTTTCCCCCTTCCT 3')

R (5' GGTTGAATTTGCTTGGCTGTTT 3')

N-myc

F (5' TGTGTTGACATTAAGAATGTTGGTTTAC 3')

R (5' TTTCCAAGGTCATGGCAGAAC 3')

Pdgfra

F (5' TGCAGTTGCCTTACGACTCCAGAT 3')

R (5' AGCCACCTTCATTACAGGTTGGGA 3')

Pdgfrβ

F (5' ACTACATCTCCAAAGGCAGCACCT 3')

R (5' TGTAGAACTGGTCGTTTCATGGGCA 3')

Pecam1

F (5' ATTCCTCAGGCTCGGGTCTT 3')

R (5' CATGCACCTTCACCTCGTA 3')

Ptc2

F (5' AACATGGTCGCCTTTTTTCATG 3')

R (5' GCATTACCGCTGCAAAGTTG 3')

Shh

F (5' ACCCAACTCCGATGTGTTCCGTTA 3')

R (5' TATATAACCTTGCCTGCCGCTGCT 3')

rSMA (these sequences are based on rat sequence, but cross-react with mouse transcripts)

F (5' GCTCCTCCAGAACGCAAATATT 3')

R (5' TCGTCATACTCCTGTTTGCTGATC 3')

SP-C

F (5' CCCTCCACACCCACCTCTAA 3')

R (5' CACAGCAAGGCCTAGGAAAGC 3')

VegfA

F (5' GTACCTCCACCATGCCAAGTG 3')

R (5' AGCTTCGCTGGTAGACATCCA 3')

VegfC

F (5' AGAAGACCGTGTGCGAATCG 3')

R (5' AGATGTGGCCTTTTCCAATACG 3')

VegfD

F (5' CAGGAGAACCCTTGATTCAACTTC 3')

R (5' GGGTAGTGGGCAACAGTGACA 3')

Wnt2

F (5' TCTTGAAACAAGAATGCAAGTGTC 3')

R (5' GAGATAGTCGCCTGTTTTCTGAA 3')

Wnt7b

F (5' GGATGCCCGTGAGATCAAAA 3')

R (5' CACACCGTGACACTTACATTCCA 3')

Lung explant cultures

Lung buds were dissected from E11.5 embryos and cultured in BGjb media (Invitrogen) supplemented with .1mg/ml ascorbic acid and anti/anti for 48 hours as previously described¹⁰⁴. Explants were cultured on a .4µm membrane filter (BD Falcon). Embryos were collected from wild-type females or generated from Wnt2 heterozygous mutant crosses. Exogenous recombinant human Wnt2 protein (Novus Biologicals) was added to the lung explant media at a concentration of .15ug/ml. Exogenous recombinant human Fgf10 (R & D Systems) was added to the lung explant media at a concentration of 200ug/ml. At least 8 explants were used for each experimental condition and treatment.

Cell Culture

10T1/2 cells were cultured in Eagle's Basal medium with Earle's BSS, 2 mM L-glutamine, 1.5 g/L sodium bicarbonate, and 10% heat-inactivated fetal bovine serum as suggested by ATCC. Recombinant human Wnt2 protein was added to the 10T1/2 media at a concentration of .15ug/ml, and cultured for 48 hours before harvesting for Q-PCR analysis.

Table 2.1: Survival ratios of Wnt2 mutants.

Genotypes of offspring from crosses between $Wnt2^{+/-}$ heterozygous mice (expect 1:2:1 ratio of $Wnt2^{+/+}$: $Wnt2^{+/-}$: $Wnt2^{-/-}$).

Genotype	E12.5-E14.5	E18.5	3 weeks
+/+	23 (25%)	18 (24%)	53 (30.5%)
+/-	48 (52%)	41 (54%)	114 (66%)
-/-	21 (23%)	17 (22%)	7 (4%)

Figure 2.1: Expression pattern of Wnt2 during lung development.

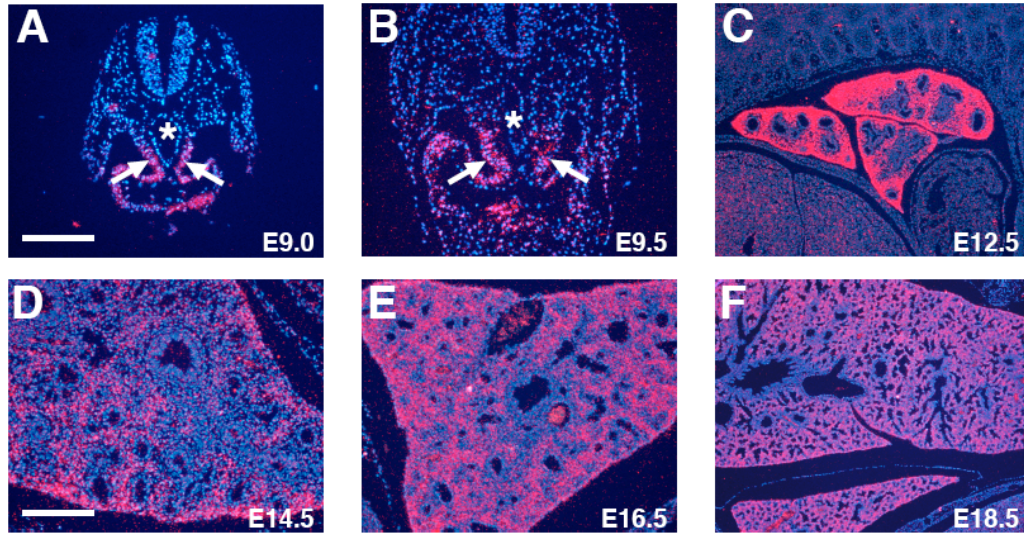


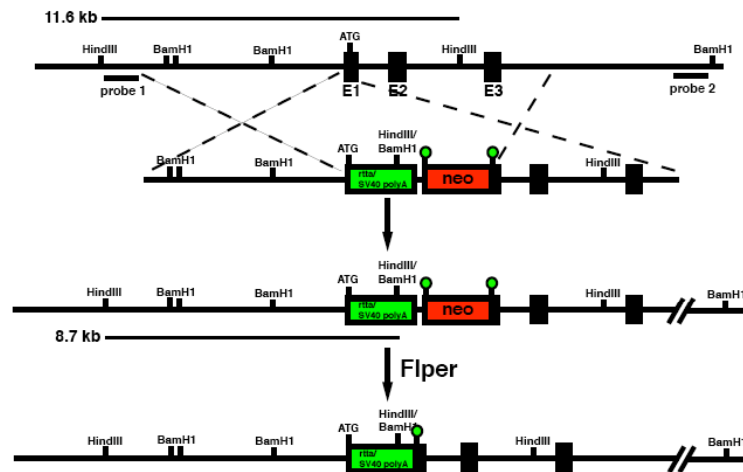
Figure 2.1. Expression pattern of Wnt2 during lung development.

(A-F) In situ hybridization for *Wnt2* expression on wild-type embryo cross-sections. (A, B) *Wnt2* is expressed in the splanchnic mesoderm surrounding the ventral aspect of the anterior foregut from E9.0-E9.5. (C-F) From E12.5-E18.5, *Wnt2* is expressed in the developing mesenchyme with higher levels surrounding the distal regions of the branching airways. Scale bars=200 μm (A, B), 600 μm (C, D, E), 800 μm (F).

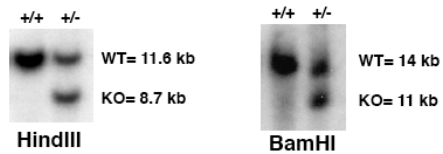
Figure 2.2: Wnt2 gene targeting strategy.

A

Wnt2



B



C

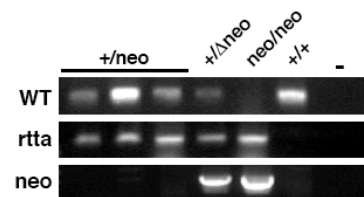


Figure 2.2: Wnt2 gene targeting strategy.

(A) Schematic of the *Wnt2* gene targeting strategy with a representative Southern blot using the indicated probe1/ HindIII and probe 2/BamHI digests (B). The reverse tet-activator (rtta) cDNA was used to replace the coding region of exon 1 of *Wnt2*. The neomycin cassette was removed using Flper mice (flp sites=green circles). (C) PCR was used to verify its loss (+/neo=*Wnt2*^{+/-} with neo cassette, +/ Δ neo=*Wnt2*^{+/-} without neo cassette, neo/neo=*Wnt2*^{-/-} with neo cassette, +/+ =wild type littermate, - = water PCR control). For unknown reasons, the rtta is not active in this animal model.

Figure 2.3: Loss of Wnt2 leads to lung hypoplasia and down-regulation of pathways required for lung development.

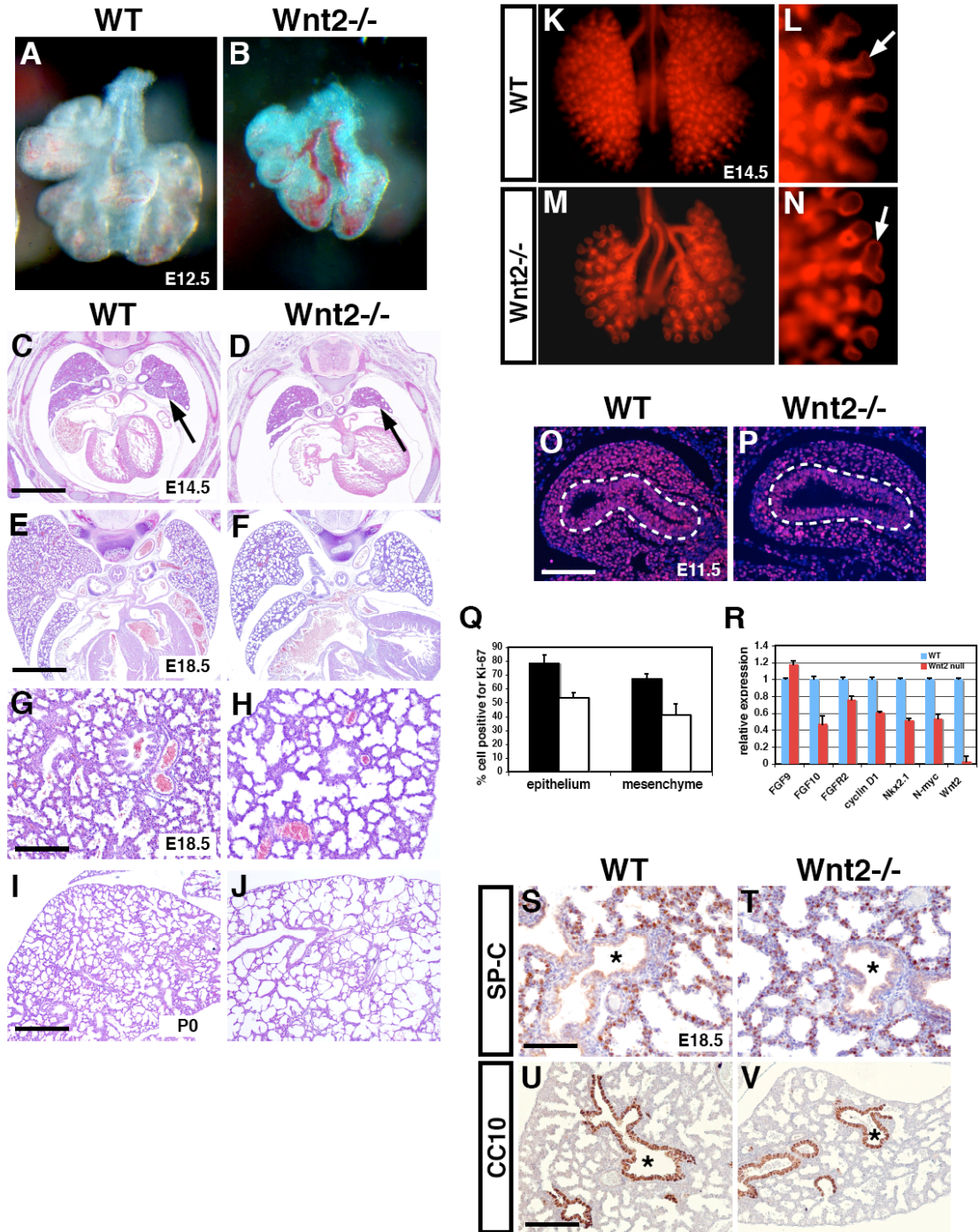


Figure 2.3: Loss of Wnt2 leads to lung hypoplasia and down-regulation of pathways required for lung development.

Wnt2^{-/-} null mutants exhibit lung hypoplasia as shown by whole mount comparison (A, B) and H+E staining of histological cross-sections from E14.5 and E18.5 *Wnt2*^{-/-} null embryos (C-H) and P0 lungs (I, J). Dilation of alveolar sacs and thinned mesenchyme is observed at E18.5 (G, H) and at P0 (I, J) in *Wnt2*^{-/-} null lungs. (K-N) Whole mount immunostaining of E14.5 lung buds with E-cadherin shows relatively normal distal branching of airways in *Wnt2*^{-/-} null lungs (M, N). Ki67 immunostaining indicates a significant decrease in proliferation in both the lung endoderm and mesenchyme of *Wnt2*^{-/-} null mutants (O, P), and this is confirmed by quantification of proliferation in both compartments (Q). (R) Quantitative PCR on E11.5 *Wnt2*^{-/-} null lungs indicates decreased expression of genes critical for lung development. (S-V) Normal proximal-distal patterning occurs in *Wnt2*^{-/-} null lungs as assessed by SP-C (S, T) and CC10 (U, V) immunostaining. Error bars indicate one standard deviation. Airways denoted by (*) symbol on histological sections. Scale bars=800 μm (C, D, E, F), 200 μm (G, H, S, T), 400 μm (U, V), 600 μm (I, J), 100 μm (O, P).

Figure 2.4: $Wnt2^{-/-}$ null lungs exhibit airway smooth muscle defects.

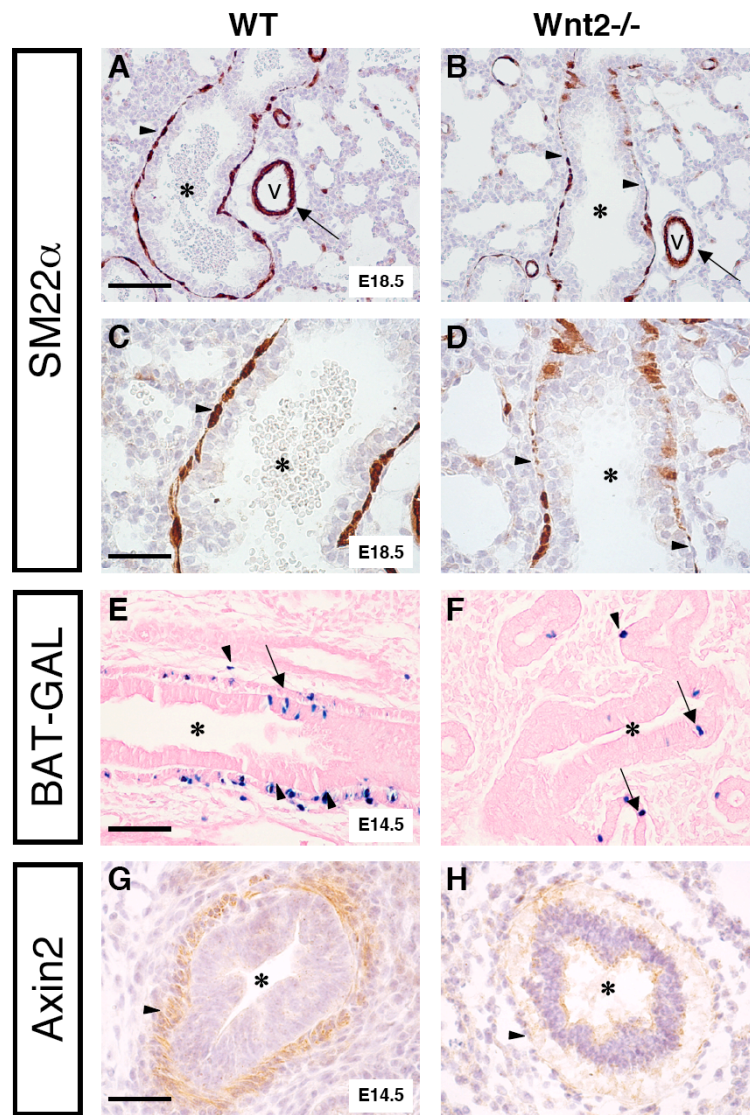


Figure 2.4: $Wnt2^{-/-}$ null lungs exhibit airway smooth muscle defects.

Histological cross-sections of E18.5 wildtype (A, C) and $Wnt2^{-/-}$ null mutant embryos (B, D). (A-D) SM22 α immunostaining demonstrates reduced SM around the distal airways (B, arrowheads) in $Wnt2^{-/-}$ null lungs. Higher magnification (C, D) reveals the lack of a contiguous layer of SM surrounding the airway epithelium in $Wnt2^{-/-}$ null lungs (D, arrowheads). The SM layer surrounding adjacent blood vessels does not appear to be reduced (arrows in A, B). (E, F) Histological cross-sections of lacZ-stained E14.5 wild-type *BAT-GAL* and $Wnt2^{-/-}$:*BAT-GAL* mutant embryos shows lacZ expression in wild-type epithelium (E, arrows) and primitive ASM cells (E, arrowheads). Loss of *Wnt2* in the *BAT-GAL* background results in decreased lacZ expression in SMCs (F, arrowhead), while epithelial lacZ expression is retained (F, arrows). (G, H) Axin2 immunostaining on E14.5 wild-type lung cross-sections demonstrates robust expression in the developing ASM (G, arrowhead), and this expression is reduced in $Wnt2^{-/-}$ null mutant lungs (H, arrowhead). Airways denoted by (*) symbol on histological sections. Scale bars=200 μm (A, B, E, F), 100 μm (C, D, G, H).

Figure 2.5: Loss of Wnt2 disrupts development of the lung mesenchyme and immature smooth muscle.

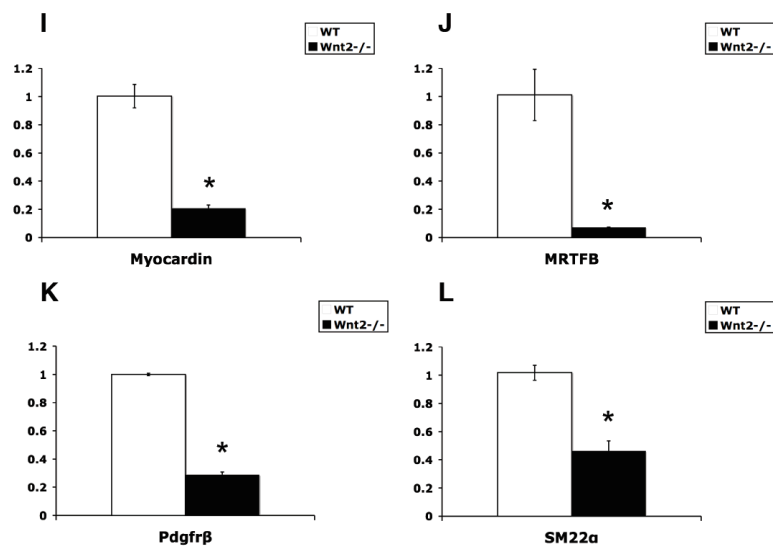
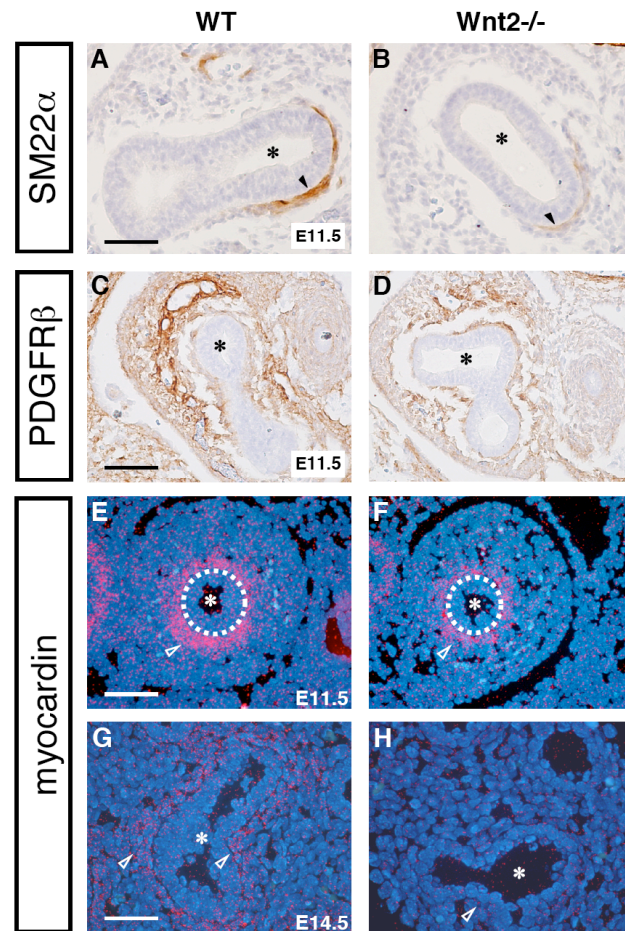


Figure 2.5: Loss of Wnt2 disrupts development of the lung mesenchyme and immature smooth muscle.

Histological cross-sections of E11.5 and E14.5 wild-type (A, C, E, G) and *Wnt2*^{-/-} null mutant embryos (B, D, F, H). (A, B) At E11.5, SM22 α immunostaining demonstrates expression in the primitive ASM layer (A, arrowhead), and this expression is reduced in *Wnt2*^{-/-} null lungs (B, arrowhead). (C, D) PDGFR β immunostaining shows expression throughout the lung mesenchyme in E11.5 wild-type lung tissue (C), however this mesenchymal expression is reduced in *Wnt2*^{-/-} null lungs (D). (E-H) In situ hybridizations for *myocardin* show decreased expression in the developing SM surrounding the airways at E11.5 (arrowheads in E, F) and later at E14.5 (arrowheads in G, H). (I-L) Assessment of mesenchymal and immature smooth muscle gene expression by quantitative PCR on E11.5 lung buds demonstrates significantly reduced levels of *myocardin*, *MRTFB*, *Pdgfr β* , and *SM22 α* expression in *Wnt2*^{-/-} null lung buds. Error bars indicate one standard deviation. *P < 0.05. N=5 lung buds. Airways denoted by (*) symbol on histological sections. Scale bars=200 μ m (A-F), 400 μ m (G, H).

Figure 2.6: Wnt2 signaling is sufficient to promote smooth muscle development in mesenchymal cell types.

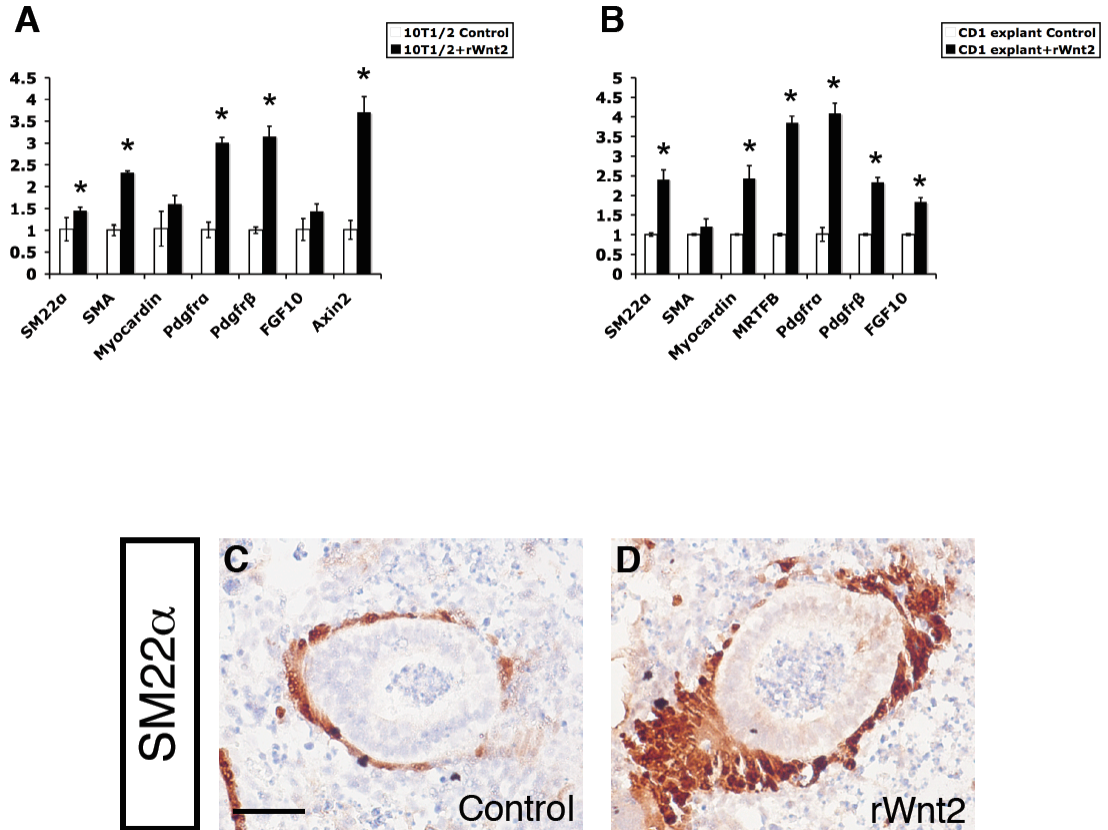


Figure 2.6: Wnt2 signaling is sufficient to promote smooth muscle development in mesenchymal cell types.

Assessment of SM gene expression by quantitative PCR in 10T1/2 cells (A) and wild-type E11.5 lung bud explants (B) cultured in the absence (control) or presence of rWnt2 protein. (A) Treating immortalized multipotent fibroblast-like 10T1/2 cells for 48 hours with rWnt2 leads to increased expression of SM genes including *SM22 α* , *SMA*, *myocardin*, *Pdgfra*, *Pdgfr β* , and *Fgf10*. Treatment of wild-type lung bud explants with rWnt2 also leads to increased SM gene expression (B). (C, D) SM22 α immunostaining on histological cross-sections of the cultured lung bud explants shows increased expression in the developing ASM in the presence of rWnt2 (D). Error bars indicate one standard deviation. *P < 0.05. N=5 lung bud explants. Scale bars=200 μ m (C, D).

Figure 2.7: Development of other mesenchymal-derived lineages in $Wnt2^{-/-}$ null lungs.

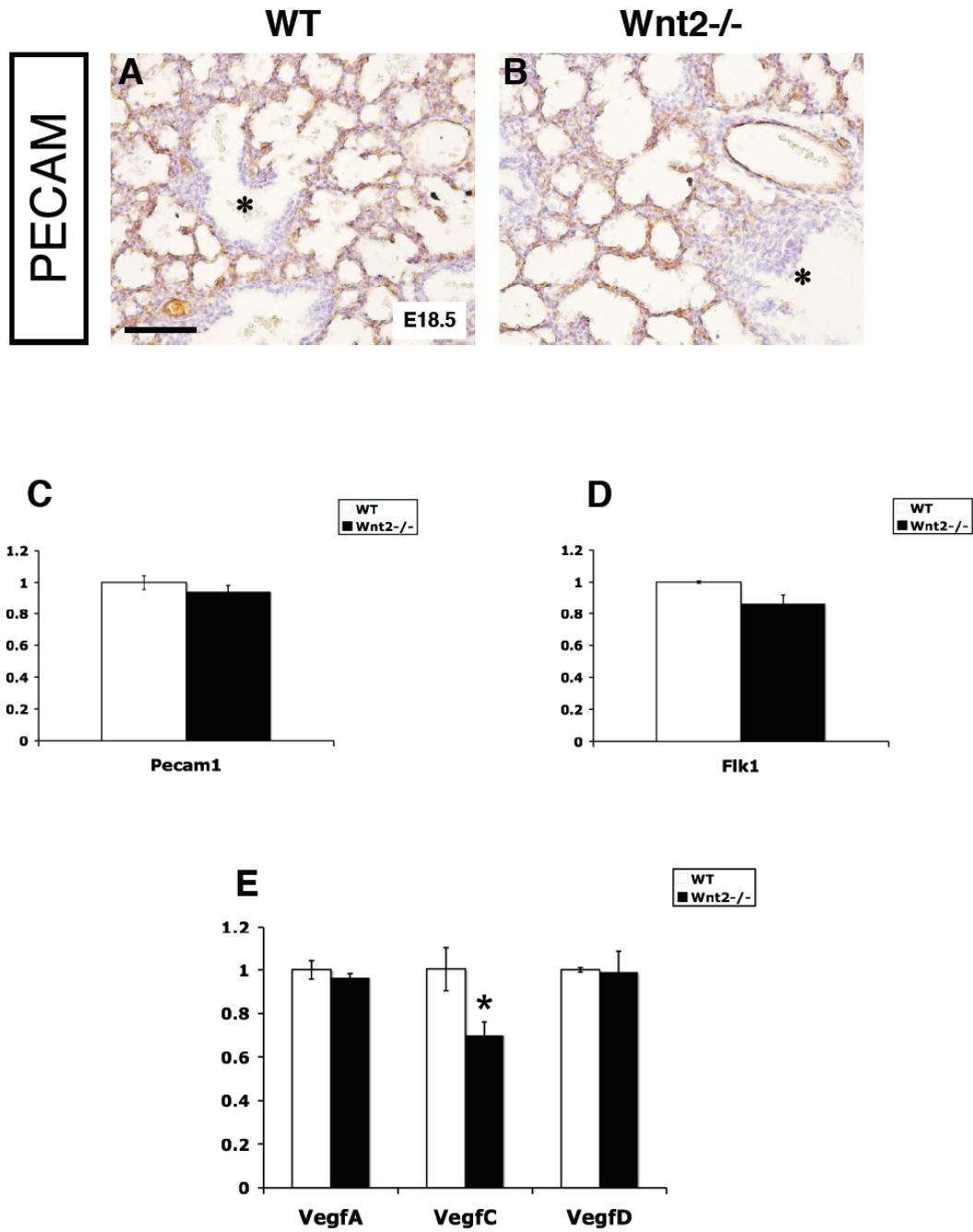


Figure 2.7: Development of other mesenchymal-derived lineages in $Wnt2^{-/-}$ null lungs.

Histological cross-sections of E18.5 wild-type (A) and $Wnt2^{-/-}$ null mutant embryos (B). PECAM immunostaining shows no change in expression levels between wild-type (A) and $Wnt2^{-/-}$ null lung tissue (B). (C-E) Quantitative PCR on E12.5 lung bud tissue shows no significant changes in expression levels of *Pecam1* (C) and *Flk1* (D) between wild-type and $Wnt2^{-/-}$ null mutant lung buds. (E) Analysis of Vegf family members expressed in the lung indicates that *VegfC* expression is significantly reduced in $Wnt2^{-/-}$ null mutant lung buds. Error bars indicate one standard deviation. * $P < 0.05$. N=5 lung buds. Airways denoted by (*) symbol on histological sections. Scale bars=600 μm (A, B).

Figure 2.8: Evaluation of signaling factors important for smooth muscle development in *Wnt2*^{-/-} null lungs.

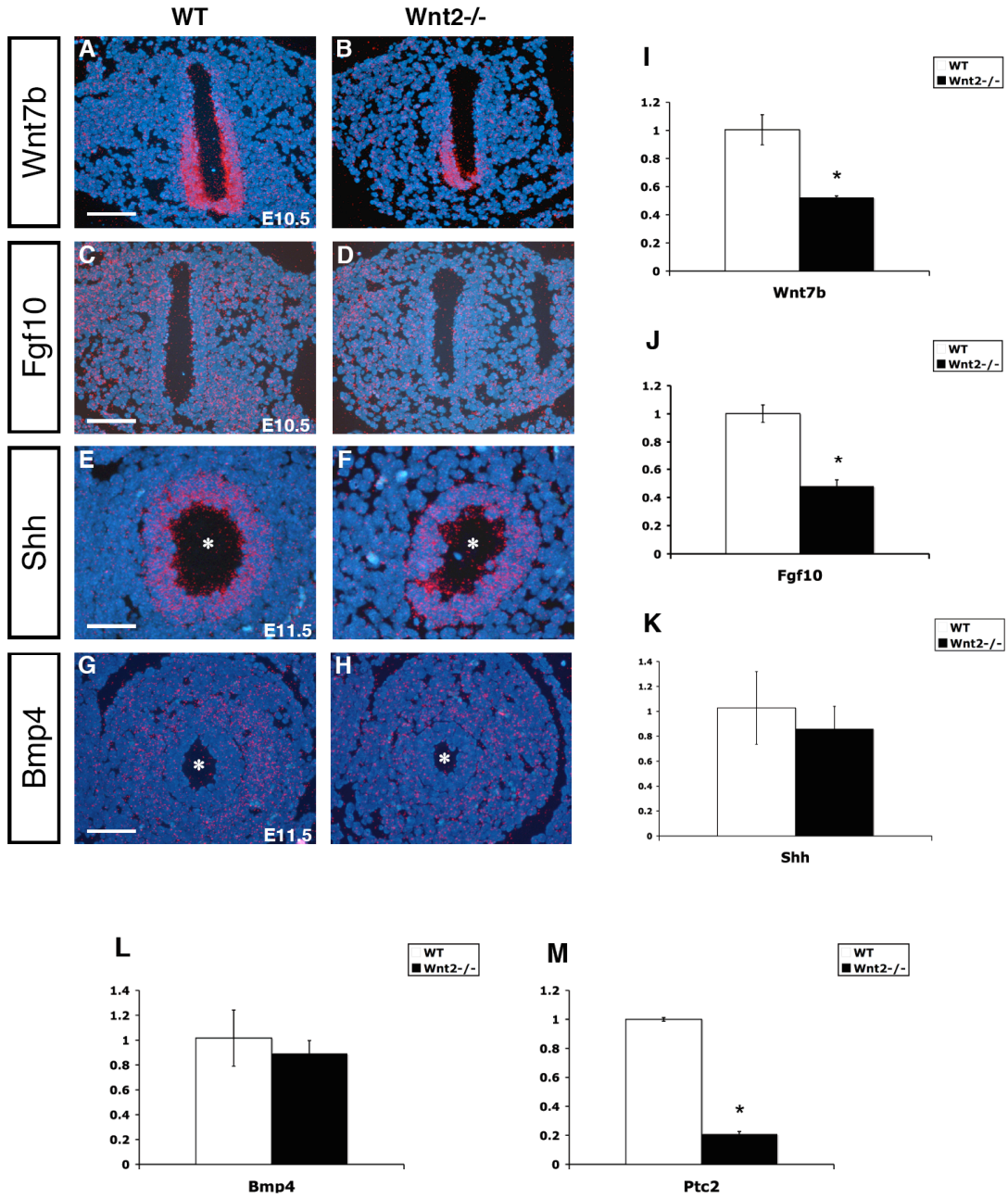


Figure 2.8: Evaluation of signaling factors important for smooth muscle development in *Wnt2*^{-/-} null lungs.

Histological cross-sections of wild-type (A, C, E, G) and *Wnt2*^{-/-} null mutant embryos (B, D, F, H). (A, B) At E10.5, in situ hybridization for *Wnt7b* shows reduced expression in the primitive lung endoderm of *Wnt2*^{-/-} null tissue. (C, D) In the adjacent mesenchyme, in situ hybridization shows that *Fgf10* expression is also reduced in E10.5 *Wnt2*^{-/-} null mutant tissue. (E, F) At E11.5, in situ hybridization for *Shh* shows similar levels of expression in the primitive lung epithelium of wild-type and *Wnt2*^{-/-} null tissue. (G, H) At E11.5, in situ hybridizations indicate no changes in *Bmp4* expression levels in the lung mesenchyme and epithelium between wild-type and *Wnt2*^{-/-} null mutant tissue. (I-M) Assessment by quantitative PCR on E11.5 lung buds demonstrates that *Wnt7b* (I), *Fgf10* (J), and *Ptc2* (M) expression levels are significantly reduced in *Wnt2*^{-/-} null lung buds, whereas the expression levels of *Shh* (K) and *Bmp4* (L) are unchanged. Error bars indicate one standard deviation. *P < 0.05. N=5 lung buds. Primitive airway denoted by (*) symbol on histological sections. Scale bars=100 μm (A-H).

Figure 2.9: FGF-10 signaling regulates smooth muscle gene expression and development in lung buds.

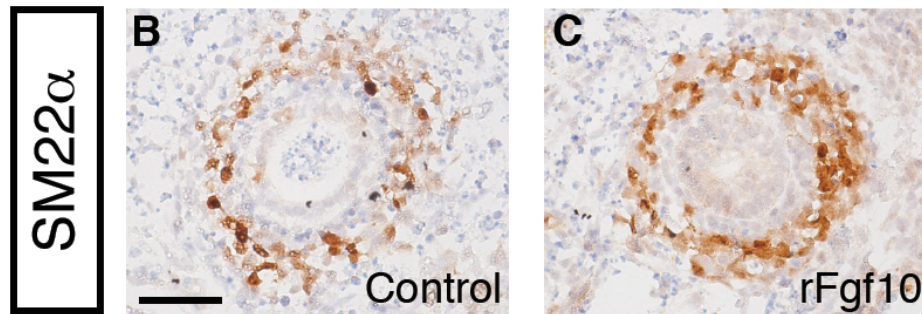
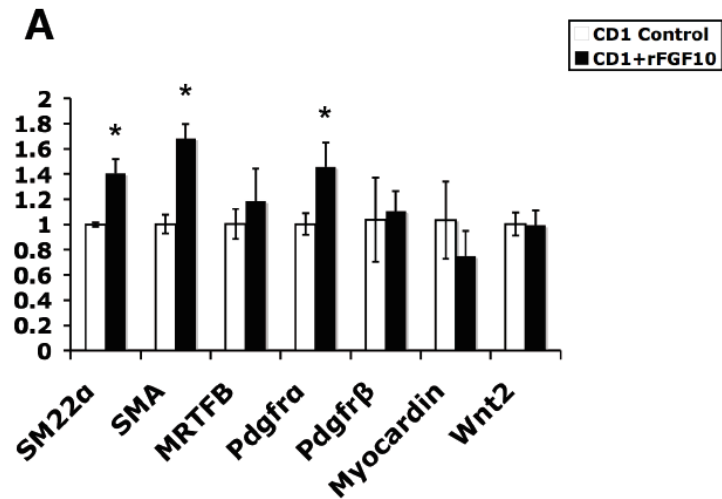


Figure 2.9: FGF-10 signaling regulates smooth muscle gene expression and development in lung buds.

(A) Assessment of SM gene expression by quantitative PCR in wild-type lung bud explants treated with either PBS (control) or rFGF-10. Treating wild-type lung bud explants with rFGF-10 leads to increased expression of SM genes including *SM22 α* , *SMA*, and *Pdgfra*. (B, C) *SM22 α* immunostaining on histological cross-sections of lung bud explants treated with rFGF-10 shows increased expression in the developing SM around primitive airways (C). Error bars indicate one standard deviation. *P < 0.05. N=5 lung bud explants. Scale bars=100 μ m (B, C).

Figure 2.10: Wnt2 regulates smooth muscle development through activation of downstream FGF-10 signaling.

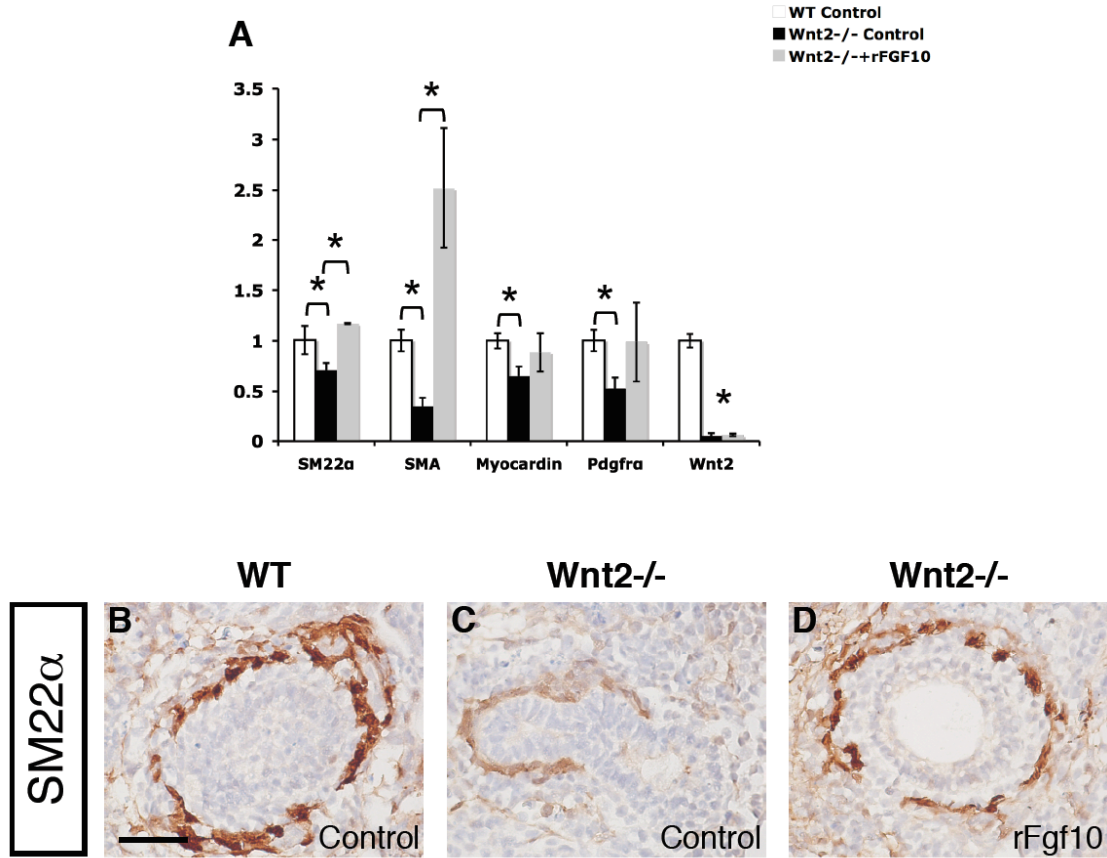


Figure 2.10: Wnt2 regulates smooth muscle development through activation of downstream FGF-10 signaling.

(A) Assessment of SM gene expression by quantitative PCR in cultured E11.5 *Wnt2*^{+/+} wildtype and *Wnt2*^{-/-} null lung bud explants. In the presence of rFGF-10, the expression levels of *SM22α* and *SMA* are significantly upregulated compared to untreated *Wnt2*^{-/-} null lung bud explants. The expression levels of *myocardin* and *Pdgfra* are increased, however the increase is not statistically significant compared to expression levels in untreated *Wnt2*^{-/-} null lung bud explants. *Wnt2* expression levels were also measured to confirm the genotyping of lung bud explant populations. (B-D) SM22α immunostaining on histological cross-sections of control *Wnt2*^{-/-} null lung bud explants shows reduced expression in the primitive ASM (C). In the presence of rFGF-10, there is increased SM22α expression in the SM layer surrounding the airways, and this expression pattern appears similar to that in *Wnt2*^{+/+} wildtype lung bud explants (compare B and D). Error bar indicates one standard deviation. *P < 0.05. N=8 lung bud explants. Scale bars=100 μm (B-D).

Figure 2.11: Model of Wnt2 signaling in lung airway smooth muscle development.

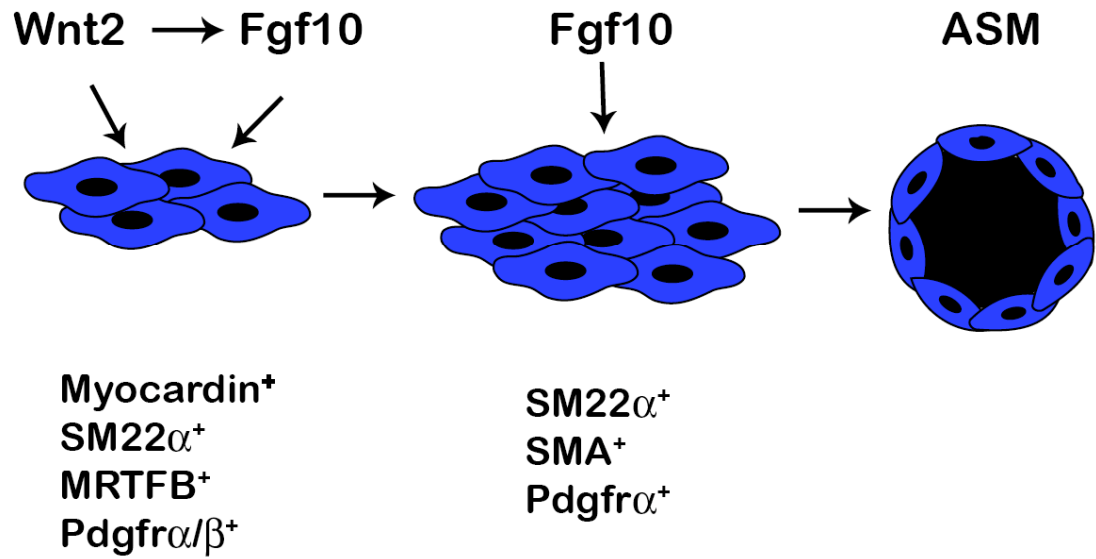


Figure 2.11: Model of Wnt2 signaling in lung airway smooth muscle development.

Wnt2 signaling in the primitive lung mesenchyme promotes the proliferation and differentiation of the PDGFR α / β -expressing multipotent mesenchyme into early-stage SMCs in part, by activating a myogenic transcriptional network including myocardin, SM22 α , and MRTFB. Wnt2 signaling also activates FGF-10 signaling, which further promotes amplification and differentiation of immature SMCs into an intermediate SMC type expressing mature ASM markers including SM22 α , SMA, and PDGFR α .

Chapter 3: Wnt2/2b and β -catenin signaling are required for lung organogenesis

This chapter has been published in *Developmental Cell* (Goss et al., 2009).

Summary

In Chapter 1, I gave an overview of mouse endoderm development and how progenitor specification within the foregut endoderm precedes organogenesis. However the molecular pathways that specify foregut endoderm progenitors in the mouse are poorly understood. The Wnt family of ligands comprises plausible candidates for mediating mouse foregut endoderm identity because of its prominent role in the development of multiple tissue types and spatiotemporal patterns of expression during foregut endoderm development. In this chapter, I show that Wnt2/2b signaling is required to specify lung endoderm progenitors within the anterior foregut. I also show that this phenotype is recapitulated by an endoderm restricted deletion of β -catenin, demonstrating that Wnt2/2b signaling through the canonical Wnt pathway is required to specify lung endoderm progenitors within the foregut. And finally, I show that activation of canonical Wnt/ β -catenin signaling results in reprogramming of esophagus and stomach endoderm to a lung endoderm progenitor fate. Together, these data reveal that canonical Wnt2/2b signaling is uniquely required for specification of lung endoderm progenitors in the developing foregut.

Introduction

As mentioned in Chapter 1, the vertebrate gut tube is patterned such that organs are specified in a precise spatial location along the anterior-posterior axis of the developing embryo. Signaling molecules expressed in the surrounding splanchnic mesoderm are thought to promote development and patterning of these organs including the thyroid, lung, liver, and pancreas, in part through proper specification of endoderm progenitors⁴². Although several important signaling pathways have been implicated in the regulation of foregut endoderm development, the pathways that uniquely specify the lung within the anterior foregut are unknown.

Wnt signaling is one such pathway known to be important for early tissue morphogenesis. Multiple roles for β -catenin in cell proliferation and differentiation have been reported in the endodermal components of several tissues including the liver, pancreas, and lung¹⁹¹⁻¹⁹⁶. However, whether Wnt signaling plays a role in the specification of foregut derived tissues remains unclear.

In this chapter, I show that Wnt2 and Wnt2b play an essential and cooperative role in specifying lung endoderm progenitors within the anterior foregut without affecting the specification of other foregut derived tissues. Moreover, I show that activation of Wnt/ β -catenin signaling can reprogram posterior endoderm to a lung progenitor fate indicating the potent role of Wnt signaling in specifying early lung endoderm progenitors. These studies reveal a unique role for Wnt/ β -catenin signaling in promoting lung endoderm specification in the foregut.

Results

Expression of *Wnt2* and *Wnt2b* during foregut development

Previous studies have reported the expression of several Wnt ligands in the lung including *Wnt2*, *Wnt5a*, *Wnt7b*, and *Wnt11*^{97, 197}. Given the importance of the Wnt pathway in endoderm development and regulation of tissue specific progenitors, the expression pattern of candidate Wnt ligands were explored to determine whether any were expressed in the appropriate spatial and temporal pattern to regulate development of lung endoderm progenitors in the foregut. The analysis revealed that from E9.0-E10.5, *Wnt2* and *Wnt2b* are expressed in a highly restricted domain of the splanchnic mesoderm surrounding the ventral aspect of the anterior foregut when the lung is specified (Figure 2.1A, B; Figure 3.1A, B). The *Wnt2* expression pattern was described in Chapter 2 — briefly, *Wnt2* is expressed in the splanchnic mesoderm at E9.0, and later at E11.5 throughout the developing lung mesenchyme up through birth¹⁴¹.

The expression of *Wnt2b*, like *Wnt2*, is upregulated at E11.5 in the developing lung mesenchyme (Figure 3.1C, D), although at lower levels and for a shorter duration than *Wnt2* (compare Figure 2.1C, D with Figure 3.1C, D). Notably, the expression patterns of *Wnt2/2b* overlap spatially and temporally in the splanchnic mesoderm and lung mesenchyme (compare Figure 2.1A, B and Figure 3.1A, B), suggesting that both ligands function redundantly in these tissues. The spatial and temporal expression of *Wnt2/2b* in the splanchnic mesoderm also suggests that Wnt2/2b signaling regulates lung specification and development.

Loss of Wnt2 function leads to lung developmental defects and loss of Wnt2b function does not appear to impact lung development

In Chapter 2, I described investigations examining the role of Wnt2 signaling in lung development using a *Wnt2* loss-of-function knockout mouse model. Those results revealed that loss of Wnt2 function impaired proper lung development and led to perinatal mortality. To understand the functions of Wnt2b during lung development, *Wnt2b*^{-/-} null mutant mice were obtained from a collaborating laboratory. These mice were generated using homologous recombination to insert loxP sites flanking exons 2 and 3 at the *Wnt2b* locus (Figure 3.2). To generate *Wnt2b*^{-/-} null mutant mice, *Wnt2b* conditional mutant mice were bred to the transgenic *CMV-cre* mouse line, which expresses a tissue wide cre recombinase. Interestingly *Wnt2b*^{-/-} null mutants are viable with no discernable lung phenotype, suggesting that Wnt2 is compensating and functioning redundantly with Wnt2b.

The combined loss of Wnt2 and Wnt2b signaling leads to lung agenesis

To address the combined role of Wnt2/2b signaling in lung development, the *Wnt2* and *Wnt2b* knockout mice were interbred to generate *Wnt2/2b* double knockout (DKO) mutants. Histological examination of *Wnt2/2b* DKO mutants at E14.5 reveals complete lung agenesis with no signs of tracheal development (Figure 3.3D-F). In the absence of lung tissue, the atria of the developing heart fill the chest cavity. Interestingly, other foregut-derived tissues appear unperturbed in *Wnt2/2b* DKO mutants (Figure 3.3G-Q). Histological sections indicate the presence of stomach, liver, pancreas, gut, and kidneys in *Wnt2/2b* DKO mutants (Figure 3.3N-Q) that are structurally comparable to

their counterparts in wild-type embryos (Figure 3.3J-M). These results demonstrate that *Wnt2* and *Wnt2b* signal cooperatively in the splanchnic mesoderm to specifically mediate the development of the lung, but not the development of other foregut-derived organs.

To understand whether an initial lung bud developed in *Wnt2/2b* DKO mutants, embryos were collected at earlier timepoints during lung development. At E11.5, while the esophagus and trachea are readily apparent in wild-type embryos (Figure 3.4A-C), neither lung nor tracheal development is observed in *Wnt2/2b* DKO mutants (Figure 3.4D-F). Additionally, immunostaining of dissected foreguts with a pan-epithelial E-cadherin antibody three dimensionally resolves the complete lack of tracheal budding in *Wnt2/2b* DKO mutants (Figure 3.4G, H). The whole mount immunostained tissue demonstrates that *Wnt2/2b* DKO mutants possess only a single endoderm tube, with no detectable ventral budding from the foregut.

Loss of *Wnt2/2b* leads to the loss of lung endoderm progenitors

The absence of tracheal budding suggests that there is a loss of lung progenitors in the ventral foregut endoderm of *Wnt2/2b* DKO mutants. To determine whether the lung endoderm lineage is specified in *Wnt2/2b* DKO mutants, embryos were collected at E9.5 when the lung primordia is established in the foregut. Histological sections reveal that there is no detectable budding of the ventral endoderm in *Wnt2/2b* DKO mutants (Figure 3.5A, E). Specification of the lung in the foregut was examined by immunostaining for *Nkx2.1* expression in *Wnt2/2b* DKO mutant tissue. Recall from Chapter 1 that *Nkx2.1* is a transcription factor that is the earliest known marker of the developing lung endoderm, and also denotes the thyroid primordia^{77, 198}. *Nkx2.1* expression is first observed by

immunohistochemistry and in situ hybridization at E9.5 in the ventral aspect of the foregut, demarcating where the trachea will bud from the anterior foregut (Figure 3.5B)^{54, 144, 199}. Surprisingly, Nkx2.1 expression is absent in the anterior foregut region of *Wnt2/2b* DKO mutants (Figure 3.5F), confirming the loss of tracheal and lung development. Examining for Nkx2.1 expression along the anterior-posterior axis of *Wnt2/2b* DKO mutants demonstrates a specific loss of Nkx2.1 expression at the level of the presumptive tracheal bifurcation (Figure 2.3I-P).

The thyroid primordium, which is anterior to the lung primordium, retains expression of Nkx2.1 (Figure 3.5C, G), indicating that *Wnt2/2b* signaling is not required for thyroid specification. The single endoderm tube in *Wnt2/2b* DKO mutants expresses the esophageal epithelial marker p63 (Figure 3.5D, H), demonstrating that the endoderm tube retains esophageal identity in the absence of tracheal and lung specification. Overall, these results suggest that *Wnt2/2b* signaling regulates the specification of lung progenitors in the foregut endoderm.

Loss of lung progenitors is not due to aberrant cell proliferation or cell death in *Wnt2/2b* DKO mutant foreguts

The loss of lung progenitors in the foregut endoderm could be attributed to cell proliferation defects and/or increased cell apoptosis. Ki67 immunostaining was performed in order to analyze the proliferation index of wild-type and *Wnt2/2b* DKO mutant foregut endoderm. Compared to wild-type foregut tissue (Figure 3.6A), there is no quantitative difference in proliferation in *Wnt2/2b* DKO mutants (Figure 3.6B, C). Additionally, TUNEL staining demonstrates that there is no increase in apoptosis in

Wnt2/2b DKO mutant foreguts over wild-type foreguts (Figure 3.6D, E). Therefore, the loss of lung endoderm progenitors in *Wnt2/2b* DKO mutants is not due to either decreased cell proliferation or increased cell death, suggesting that the loss stems directly from the absence of a specification event.

Loss of lung gene expression in *Wnt2/2b* DKO mutants

Several genes are reported to play critical roles in the development of foregut endoderm progenitors, with some specifically regulating lung progenitor development. Quantitative PCR on dissected foreguts from E10.5 embryos (Figure 3.6F) confirms the loss of *Nkx2.1* expression in *Wnt2/2b* DKO foreguts. The minimal expression of *Nkx2.1* in *Wnt2/2b* DKO mutant foreguts is likely due to the presence of contaminating thyroid cells, which retain *Nkx2.1* expression (Figure 3.5G). *Foxa2* is a transcription factor expressed throughout the foregut endoderm, which contributes to foregut progenitor competence²⁰⁰. In situ hybridization for *Foxa2* reveals expression in the foreguts of wild-type tissue and *Wnt2/2b* DKO tissue (Figure 3.6G, H), indicating that foregut endoderm identity is not lost in the absence of *Wnt2/2b* signaling.

The expression of *Wnt7b*, an additional marker of early lung endoderm progenitors in the anterior foregut¹³⁴, is also lost in *Wnt2/2b* DKO mutants. In situ hybridization demonstrates an absence of *Wnt7b* expression in the ventral foregut (Figure 3.6I, J), and quantitative PCR also confirms this loss (Figure 3.6F). The residual *Wnt7b* expression in the *Wnt2/2b* DKO foreguts is likely due to other contaminating tissue types (e.g. the pancreas primordium)⁶¹.

Wnt2/2b DKO phenotype is distinct from other lung agenesis phenotypes

The phenotype of *Wnt2/2b* DKO mutant embryos is not mimetic to other lung hypoplasia phenotypes, including the loss of *Fgf10* and loss of *Gli2/Gli3* expression, in that the lung is uniquely affected and specification is completely lost. *Fgf10*^{-/-} null mutants form a trachea which does not branch, indicating that the lung endoderm lineage is specified but fails to grow and branch^{68,70}. *Gli2/Gli3* double null mutants fail to form a lung, but other aspects of foregut development are severely affected, including the loss of the esophagus⁷². To develop a hierarchical model of lung specification, expression of *Fgf10*, *Gli2*, and *Gli3* was assessed to determine whether their expression was affected by the loss of Wnt2/2b signaling.

In situ hybridization on *Wnt2/2b* DKO mutant tissue reveals reduced expression of *Fgf10* (Figure 3.6K, L), suggesting that *Fgf10* expression is downstream of Wnt signaling in the anterior foregut. Although our laboratory has demonstrated that *Fgf10* is a direct target of Wnt/ β -catenin signaling in cardiac mesoderm¹⁸⁹, it remains possible that loss of *Fgf10* expression is secondary to a loss of lung specification. In contrast to the loss of *Fgf10* expression, *Gli2* and *Gli3* expression were unchanged in the anterior foregut region of *Wnt2/2b* DKO mutants. In situ hybridization shows comparable *Gli3* expression levels between wild-type and *Wnt2/2b* DKO mutant tissue (Figure 3.6M, N), and quantitative PCR indicates no change in *Gli2* or *Gli3* expression in *Wnt2/2b* DKO mutant foreguts (Figure 3.6F). Together, these data suggest that Wnt2/2b signaling acts upstream of *Fgf10*, but not *Gli2/Gli3* in the regulation of lung specification.

Wnt2 and Wnt2b signal through the β -catenin-dependent canonical pathway in the primitive lung endoderm

Wnt ligands can signal through several distinct pathways to regulate cell specification and tissue development. The best understood of these is the β -catenin dependent canonical pathway, which has been demonstrated to regulate development and differentiation of several tissues including hair follicles, intestinal epithelium, and the heart^{189, 201, 202}. To assess whether the canonical Wnt pathway was affected by the loss of *Wnt2/2b*, we crossed the *BAT-GAL* canonical Wnt reporter line¹¹³ to *Wnt2/2b* DKO mutants and performed lacZ staining in wild-type *BAT-GAL* embryos, *Wnt2^{-/-}:BAT-GAL*, *Wnt2b^{-/-}:BAT-GAL*, and *Wnt2/2b:BAT-GAL* DKO null mutants.

LacZ expression from the *BAT-GAL* Wnt reporter line is reduced in *Wnt2^{-/-}* null mutant foreguts (Figure 3.7A-D), suggesting that Wnt2 signals through β -catenin in the foregut endoderm. These data complement the *BAT-GAL* data presented in Chapter 2 (Figure 2.4E, F), which demonstrated that Wnt2 signals through β -catenin in the lung mesenchyme at later developmental stages. LacZ expression is also reduced in *Wnt2b^{-/-}* null mutants (Figure 3.7E), and is absent in the anterior foregut endoderm in *Wnt2/2b* DKO mutants (Figure 3.7F). The loss of β -catenin activity in the foregut endoderm upon the combined loss of *Wnt2* and *Wnt2b* suggests that Wnt2/2b signal through β -catenin in the foregut. Therefore, these data placed into context with the *Wnt2/2b* DKO lung agenesis phenotype suggest a critical role for β -catenin signaling in mediating lung progenitor development in the foregut.

To further address whether canonical Wnt/ β -catenin signaling is necessary in the developing foregut endoderm for lung specification, I utilized a commercially available

mouse line that expresses cre recombinase throughout the foregut endoderm to modulate β -catenin signaling activity. The mouse line harbors a gene encoding a gfp-cre recombinase fusion protein, which has been inserted at the Sonic hedgehog (*Shh*) locus through homologous recombination²⁰³. As a knock-in model, cre recombinase expression should faithfully recapitulate the temporospatial dynamics of *Shh* promoter activity in the developing endoderm. To confirm the fidelity of the mouse model as a tool, *Shh-cre* heterozygous mice were bred to the cre-dependent lacZ mouse reporter strain, *ROSA26*²⁰⁴. Lineage mapping in *Shh-cre:R26R* embryos demonstrates cre activity in the foregut endoderm at E8.75 (Figure 3.7G), just prior to lung specification, and at E9.25 in the tracheal bifurcation at the onset of lung specification (Figure 3.7H, I-K). Slightly later at E11.5, there is cre activity in the tracheal endoderm that extends throughout the primitive endoderm of the lung, stomach, and gut (Figure 3.7L-O).

β -catenin signaling is necessary for lung progenitor specification in the foregut endoderm

To test whether β -catenin signaling activity contributes to lung progenitor development, the β -catenin gene (*Ctnnb1*) was genetically deleted using the *Shh-cre* mouse line in conjunction with a mouse line carrying conditional alleles of β -catenin²⁰⁵. Immunostaining for β -catenin protein expression demonstrates β -catenin gene excision as denoted by the absence of β -catenin protein in E9.5 *Ctnnb1^{lox/lox}.Shh-cre* mutant foreguts (Figure 3.8I, J). Interestingly, *Ctnnb1^{lox/lox}.Shh-cre* mutants exhibit a histological phenotype identical to *Wnt2/2b* DKO mutants. Histological sections demonstrate a complete lack of tracheal budding in *Ctnnb1^{lox/lox}.Shh-cre* mutants (Figure

3.8E-G). Whole mount E-cadherin immunostaining confirms the loss of tracheal and lung bud formation in *Ctnnb1^{lox/lox};**Shh-cre* mutants (Figure 3.8O, P).

Additionally, there is a complete loss of Nkx2.1 expression in the ventral foregut at the site of the presumptive tracheal bifurcation (Figure 3.8H). However, specification of other gut-derived tissues including the esophagus and thyroid is unaffected (Figure 3.8M, N). Immunostaining for p63 expression indicates that the single endoderm tube in *Ctnnb1^{lox/lox};**Shh-cre* mutants retains esophageal identity (Figure 3.8K, L). Overall, these data demonstrate that β -catenin signaling in the foregut endoderm is required to specify lung endoderm progenitors.

Activation of Wnt/ β -catenin signaling leads to reprogramming of the posterior foregut endoderm to a lung endoderm fate

The potent role for Wnt/ β -catenin signaling in specifying lung endoderm progenitors suggested that ectopic activation of this pathway might promote expansion of lung endoderm progenitor identity outside the normal region in the foregut. To test this hypothesis, the *Shh-cre* mouse line was crossed with the *Ctnnb1^{(ex3)lox}* mouse line to generate *Ctnnb1^{(ex3)lox};**Shh-cre* mutants. These mutants will express the stabilized form of β -catenin lacking the phosphorylation sites required for β -catenin degradation, thereby leading to strong activation of Wnt/ β -catenin signaling²⁰⁶. At E10.5, *Ctnnb1^{(ex3)lox};**Shh-cre* mutants display defects in tracheal-esophageal septation (Figure 3.9E, F), and immunostaining reveals expansion of Nkx2.1 positive lung progenitors into the hindgut region, corresponding to the primitive stomach endoderm (Figure 3.9H). This expansion

is also evident at E11.5 in *Ctnnb1^{(ex3)lox}·Shh-cre* mutants, where the esophagus and proximal stomach are populated with Nkx2.1 positive lung progenitors (Figure 3.9O, P).

To determine whether this expansion of Nkx2.1 lung progenitors represents a reprogramming of foregut endoderm or an increase in Nkx2.1 expression in esophagus and stomach endoderm, p63 immunohistochemistry was performed on E11.5 wild-type and *Ctnnb1^{(ex3)lox}·Shh-cre* mutants. These data show that p63 expression is lost in the esophagus and proximal stomach of *Ctnnb1^{(ex3)lox}·Shh-cre* mutants (Figure 3.9R, T). Moreover, co-immunostaining for p63 and Nkx2.1 on *Ctnnb1^{(ex3)lox}·Shh-cre* mutant foregut sections demonstrates the lack of co-expression of Nkx2.1 and p63 in esophageal endoderm cells (Figure 3.9V), suggesting that activation of Wnt/ β -catenin signaling reprograms esophagus and stomach endoderm to a lung endoderm progenitor fate. Altogether, these data indicate that activation of Wnt/ β -catenin signaling reprograms posterior regions of the foregut endoderm to a lung endoderm progenitor fate, suggesting that activation of this pathway drives lung endoderm specification in a dominant manner.

In conclusion, this work describes a model of Wnt2/ β -catenin dependent activity as the required signal for specification of lung endoderm progenitors within the foregut (Figure 3.10). Loss of *Wnt2/2b* expression, or β -catenin signaling, disrupts lung progenitor specification leading to lung agenesis in the mouse. Conversely, dominant activation of β -catenin signaling promotes the expansion of lung progenitor specification outside of the normal foregut endoderm domain.

Discussion

Mutations in several genes have resulted in either a severe truncation in lung development (i.e. *Fgf10*^{-/-} null mutants), or defects in the splanchnic mesoderm leading to severe foregut agenesis including the lung and esophagus (i.e. *Gli2/Gli3* double mutants)^{70, 72}. In this chapter, I show that Wnt2/2b are distinct in their ability to specify lung progenitors within the developing foregut, whereas they are expendable in the specification of other organs including the thyroid, esophagus, liver, and pancreas. Furthermore, I show that activation of canonical Wnt signaling can reprogram esophagus and stomach endoderm to a lung progenitor fate. These data support the importance of mesoderm to endoderm signaling that promotes development of foregut derived tissues, and extends these findings to provide a molecular hierarchy of foregut endoderm specification.

Previous reports have elucidated additional roles for Wnt signaling in the developing lung. Loss of β -catenin or expression of the Wnt inhibitor dkkopf1 (*Dkk1*) in lung epithelium after lung specification leads to decreased distal airway epithelial development and an overall proximalization of the lung^{191, 192}. A *Dermo1-cre* mesenchymal specific loss of β -catenin in the lung leads to defective lung mesenchymal proliferation and development^{117, 207}. A previous report on a different Wnt2 allele did not report a lung phenotype although approximately 50% of null animals died by birth. This could be explained by the presence of significant levels of a truncated Wnt2 mRNA species observed in this previous allele¹⁴¹.

Expression of several other Wnt ligands besides Wnt2 and Wnt2b has been reported in the lung including Wnt7b and Wnt5a^{97, 197}. Wnt7b has been shown to

regulate mesenchymal proliferation as well as epithelial proliferation and maturation^{134, 139}. Wnt5a is expressed initially in both the mesenchyme and distal epithelium of the developing lung, and loss of Wnt5a leads to increased mesenchymal proliferation and a loss in late airway maturation⁹⁷. Since Wnt5a has been reported to act in the non-canonical Wnt pathway²⁰⁸, which can antagonize β -catenin dependent canonical signaling, the increased proliferation observed in the lung mesenchyme of Wnt5a mutants could be due to increased canonical Wnt signaling in this tissue. The present study shows that in addition to regulation of lung development and growth, Wnt signaling through Wnt2/2b is essential for specification of lung endoderm progenitors in the foregut.

In contrast to previous studies in zebrafish which demonstrated an important role for *wnt2b* in liver development and specification as well as fin development, these data show that Wnt2/2b are not required for mammalian liver specification^{48, 209}. The studies described here suggest that the role for Wnt/ β -catenin signaling along the anterior-posterior axis of the foregut varies between species, which may have occurred as Wnt2/2b and the canonical Wnt pathway were co-opted during evolution to specify the lung during the vertebrate expansion into the terrestrial environment. The specificity for Wnt signaling, in particular Wnt2 and Wnt2b, in regulating specification of the lung is interesting in light of previous reports showing an important role for this pathway in pancreas and liver development¹⁹³⁻¹⁹⁶. This may be due to the precise expression pattern of these two Wnt ligands or to an important sensitivity of lung endoderm progenitors to canonical Wnt signaling. Moreover, the phenotype in *Ctnnb1^{(ex3)lox};**Shh-cre* mutants is likely due to the timing and specificity of the *Shh-cre* line since we do not observe early activity in the liver (Figure 3.7O).

It is also important to note that since *Fgf10* is a direct target of Wnt/ β -catenin signaling¹⁸⁹, the ability of Wnt2/2b to regulate its expression in the mesoderm surrounding the anterior foregut in a cell autonomous manner could affect other pathways important for mesoderm-endoderm signaling during lung development. Given the critical importance of Wnt2/2b signaling in lung endoderm specification, it will be interesting in future studies to determine whether simple activation of Wnt signaling can rescue the Wnt2/2b phenotype in foregut endoderm. Previous reports have shown that Wnt/ β -catenin signaling is also important in adult lung progenitor expansion after injury^{164,210}. Thus, Wnt signaling plays a key role in embryonic as well as adult lung endoderm progenitor development, which reinforces the importance of understanding critical developmental pathways that are recapitulated upon injury and repair.

Wnt/ β -catenin signaling is a critical developmental pathway considered to be important for both self-renewal and differentiation of stem/progenitor cells. With vigorous efforts underway to determine whether agonists or antagonists can be used to manipulate this pathway for therapeutic purposes, our findings that Wnt signaling is central to lung endoderm specification and has the ability to reprogram foregut endoderm to a lung endoderm fate provides important information for investigating lung regeneration. In summary, these data provide a molecular hierarchy of foregut endoderm progenitor specification with Wnt2/2b signaling acting dominantly to specify lung endoderm progenitors in the anterior foregut.

Materials and Methods

Mice

The generation of the *Wnt2* mutant mice was described in Chapter 2. *Wnt2b* mutant mice were generated using recombineering techniques to insert loxP sites flanking exons 2 and 3. Successfully targeted ES cells were used to generate chimeric mice that were bred to transmit these mutant alleles through the germline. *Wnt2b* mutants were crossed to *CMV-cre* mice to delete exons 2 and 3 and generate a null allele. The *Wnt2b* locus still carries the neomycin selection gene cDNA from the original targeted ES cell. Both lines were maintained on a C57BL/6:129SVJ mixed background. The generation and genotyping of *Shh-cre*, *Ctnnb1^{lox/lox}*, *BAT-GAL*, and *Ctnnb1^{ex3lox}* mice have been previously described^{113, 203, 205, 206}. The University of Pennsylvania Institutional Animal Care and Use Committee approved all animal protocols. Genotyping primer sequences are as follows:

Wnt2b^{neo}:

*Wnt2b*Forward (5' AGCTCTTTGTCGGTGGAGGTAAAGG 3')

*Wnt2b*Reverse (5'CGGCTGATGGGTAGAACCATTACCT 3')

*Wnt2b*NeoReverse (5'ATCAGCAGCCTCTGTTCCACATAC 3')

Histology

Embryos were fixed in 4% paraformaldehyde for 24 hours, dehydrated in a series of ethanol washes, and then embedded in paraffin for tissue sectioning. Dissected embryonic lung buds and foreguts were fixed in 4% paraformaldehyde overnight, dehydrated in a series of methanol washes, and blocked in a 10% normal goat serum/PBS solution before whole mount immunostaining. In situ hybridization and

immunohistochemistry was performed as previously described¹⁹⁰. Tissue sections were stained with the following antibodies and dilutions: anti- β -catenin (BD Transduction, 1:100), anti-E Cadherin (Sigma, 1:50), anti-Ki67 (Vector Laboratories, 1:50), anti-Nkx2.1 (Santa Cruz, 1:50), anti-p63 (Santa Cruz, 1:50). Quantification of positive cell populations was performed using at least three different tissue sections from at least three different embryos of the same genotype. LacZ histochemical staining of embryos was performed as previously described¹³⁴. TUNEL staining was performed as previously described²¹¹.

Quantitative RT-PCR

Total RNA was isolated from lung tissue at the indicated time points using Trizol reagent, reverse transcribed using SuperScript First Strand Synthesis System (Invitrogen), and used in quantitative real time PCR analysis using the oligonucleotides listed below.

Gli2

F (5' CGGAAGGTTGAAGGCATTGA 3')

R (5' GTTCCACATGCCATTTCTTATCTG 3')

Gli3

F (5' CCACTGTCAGATCATAGCTTCGA 3')

R (5' CTTGCCGATAAGTGCCCATAG 3')

Nkx2.1

F (5' CTTTCATCTTTCCCCCTTCCT 3')

R (5' GGTTGAATTTGCTTGGCTGTTT 3')

Wnt7b

F (5' GGATGCCCGTGAGATCAAAA 3')

R (5' CACACCGTGACACTTACATTCCA 3')

Figure 3.1: Expression pattern of Wnt2b during lung development.

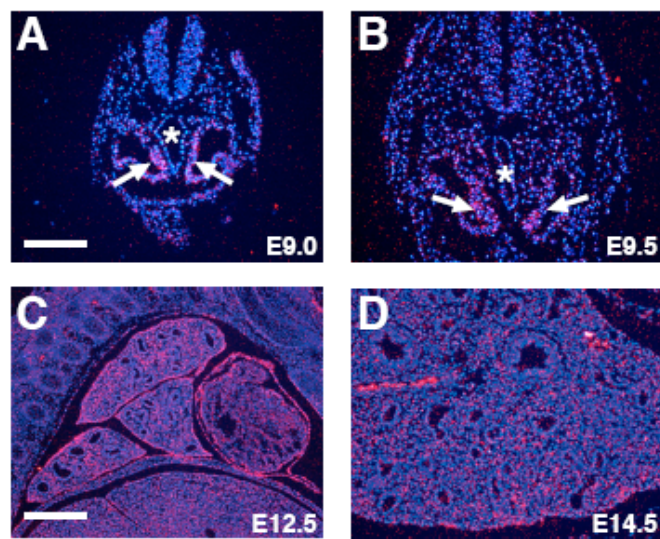


Figure 3.1: Expression pattern of *Wnt2b* during lung development.

(A-D) In situ hybridization for *Wnt2b* expression on wild-type embryo cross-sections. (A, B) *Wnt2b* is expressed in the splanchnic mesoderm flanking the ventral domain of the mouse foregut endoderm from E9.0-E9.5. (C, D) From E12.5-E14.5, *Wnt2b* expression is observed in the mesothelium encasing the lung and at lower levels in the distal mesenchyme. After E14.5, *Wnt2b* expression is downregulated. Both *Wnt2* and *Wnt2b* exhibit overlapping spatiotemporal patterns of expression (see Figure 2.1 for *Wnt2* expression pattern). Scale bars=200 μm (A, B), 600 μm (C, D).

Figure 3.2: Wnt2b gene targeting strategy.

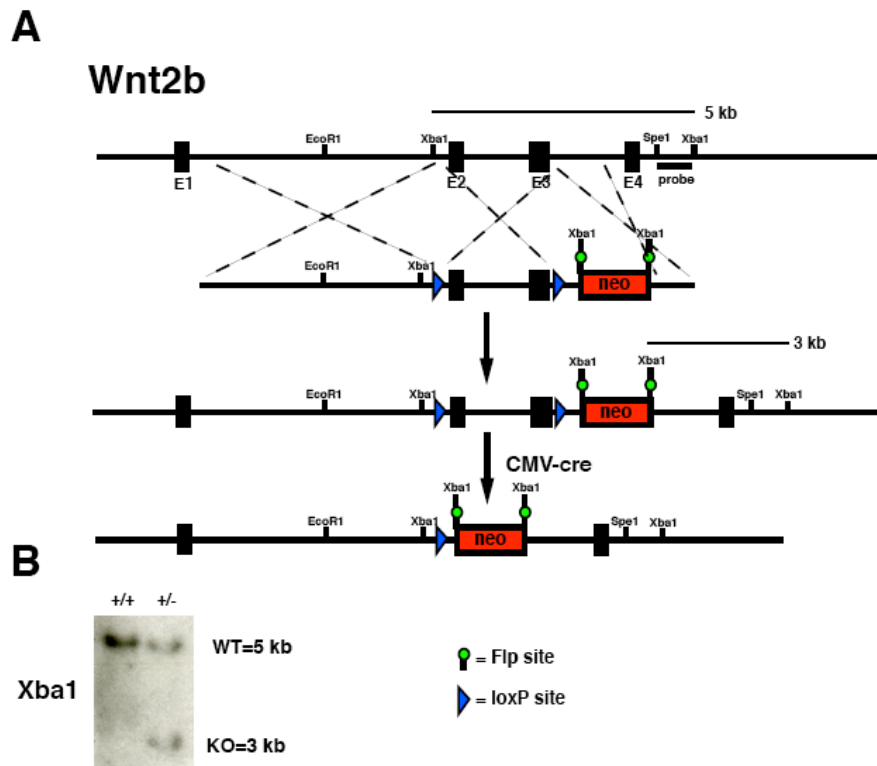


Figure 3.2: Wnt2b gene targeting strategy.

(A) Schematic of the *Wnt2b* gene targeting strategy with a representative Southern blot using the indicated probe and an Xba1 digest (B). A conditional knockout of the *Wnt2b* gene was generated by homologous recombination to insert loxP sites flanking exons 2 and 3. *Wnt2b* mutant alleles ($Wnt2b^{\Delta\Delta}$) were generated by mating the *Wnt2b* conditional mutants to transgenic mice expressing a tissue-wide cre recombinase under a minimal promoter (CMV-cre).

Figure 3.3: Loss of both Wnt2 and Wnt2b results in lung agenesis.

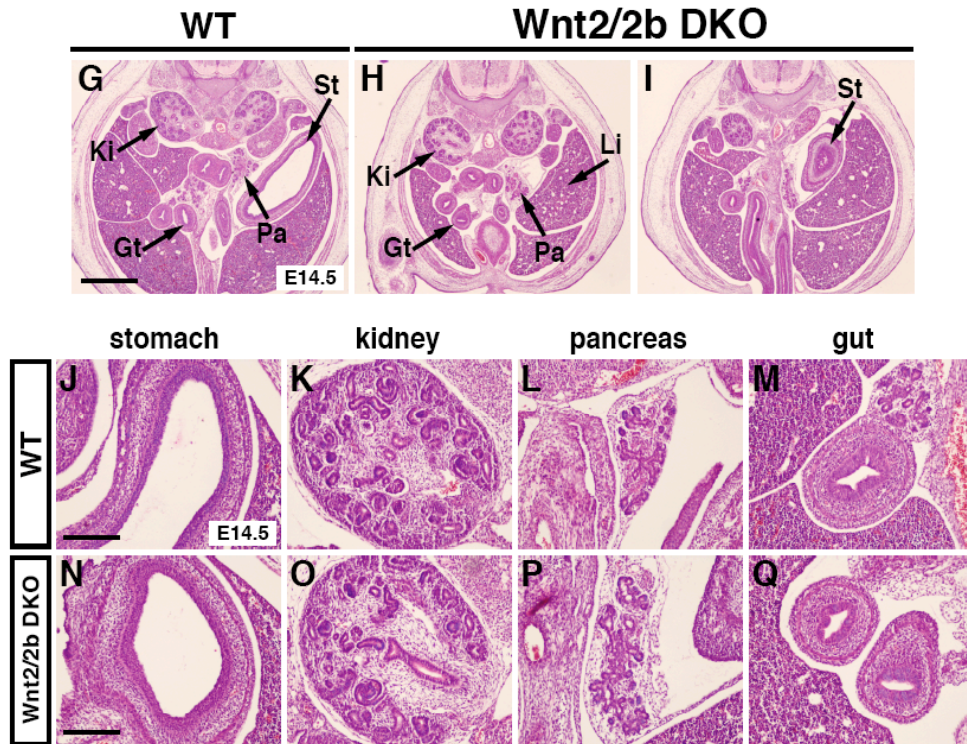
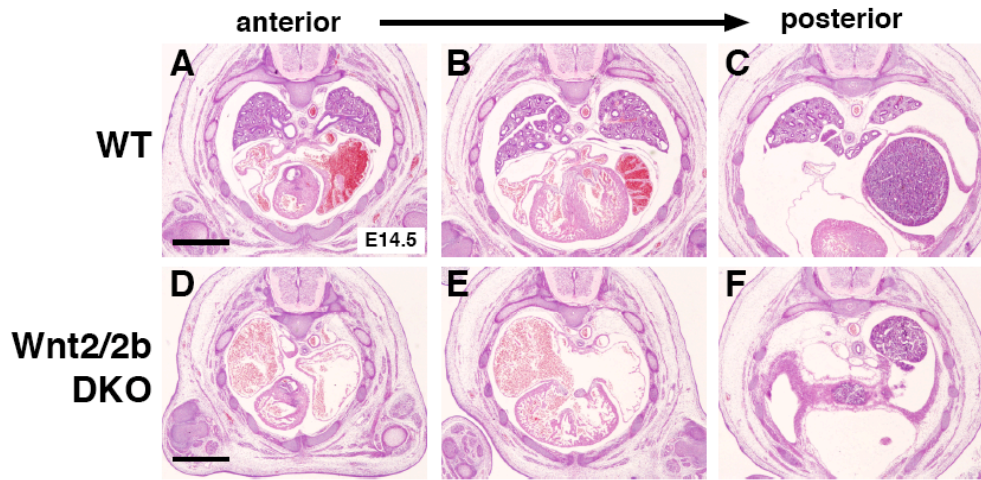


Figure 3.3: Loss of both *Wnt2* and *Wnt2b* results in lung agenesis.

H+E staining of histological cross-sections from E14.5 wild-type (A-C, G, J-M) and *Wnt2/2b* double knockout (DKO) mutant embryos (D-F, H, I, N-Q). (D-F) *Wnt2/2b* DKO mutants exhibit lung agenesis, however, specification of other foregut-derived organs is not affected in *Wnt2/2b* DKO mutants (H, I). As with wild-type histological cross-sections E14.5 (G), *Wnt2/2b* DKO mutants develop a stomach (St), liver (Li), pancreas (Pa), kidney (Ki), and intestine (Gt) (H and I). Higher magnification pictures show relatively normal architecture of these and other gut-derived organs in both wild-type (J-M) and *Wnt2/2b* DKO mutants (N-Q). Scale bars=800 μm (A-I), 400 μm (J-Q).

Figure 3.4: Loss of Wnt2 and Wnt2b leads to tracheoesophageal defects and loss of tracheal budding.

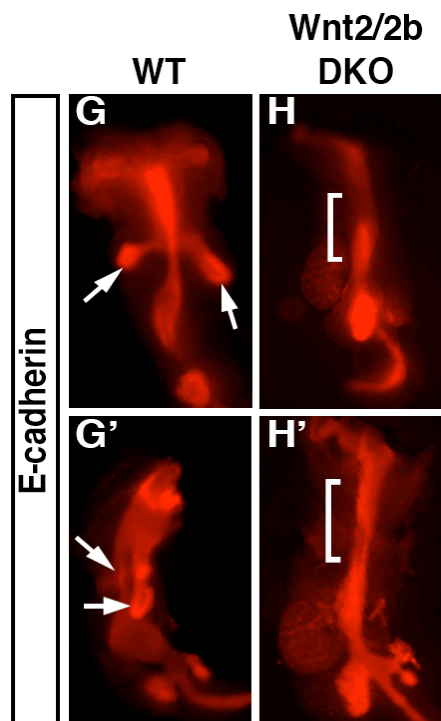
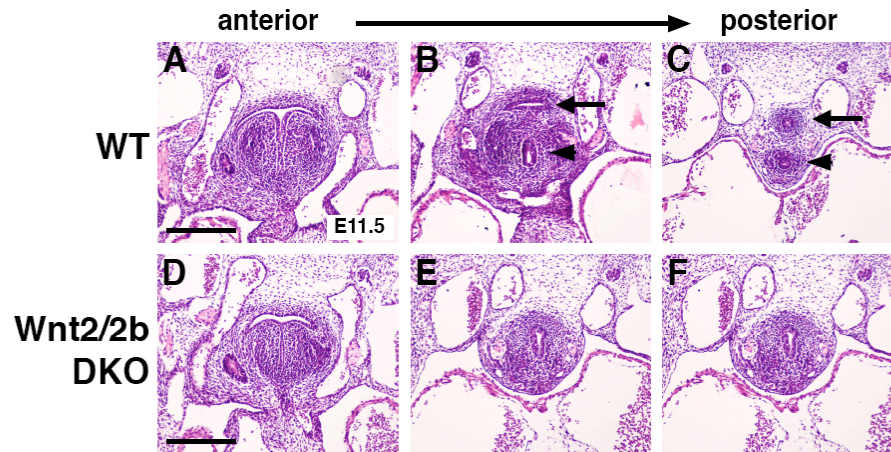


Figure 3.4: Loss of *Wnt2* and *Wnt2b* leads to tracheoesophageal defects and loss of tracheal budding.

H+E staining of histological cross-sections from E11.5 wild-type (A-C) and *Wnt2/2b* DKO mutant embryos (D-F). Posterior to the laryngotracheal groove (pictured in A and D), *Wnt2/2b* DKO mutants do not exhibit septation of the trachea and esophagus (E, F). In contrast, a clear separation of the esophagus (B and C, arrow) from the trachea (B and C, arrowhead) is observed in wild-type embryos. (G, H) E-cadherin whole mount immunostaining of endoderm dissected from wild-type (G) and *Wnt2/2b* DKO (H) shows lack of tracheal budding from the foregut in *Wnt2/2b* DKO mutants (G and H, arrows and brackets). Scale bars=200 μ m (A-F).

Figure 3.5: Loss of Wnt2 and Wnt2b results in the loss of lung progenitor specification.

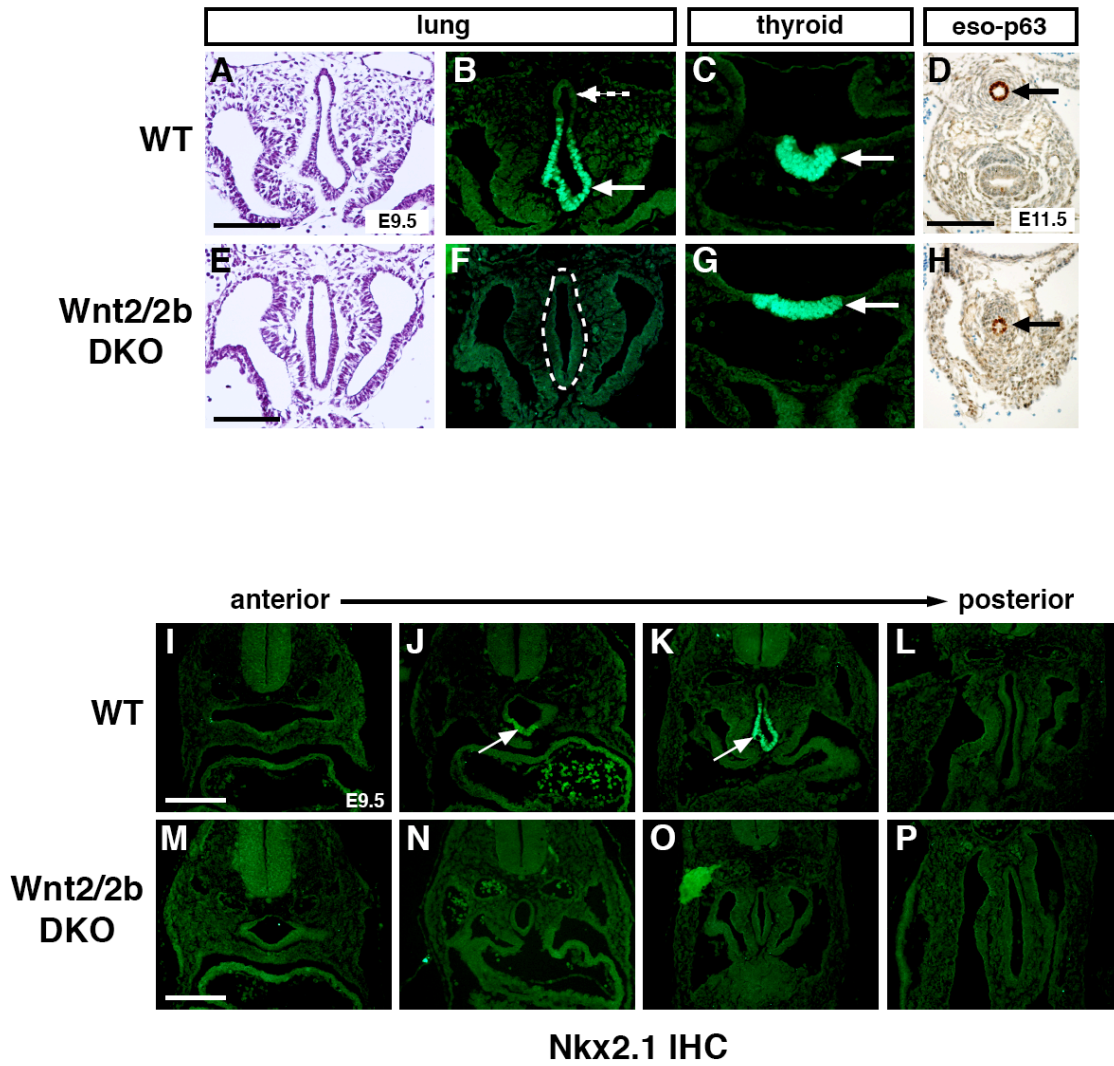


Figure 3.5: Loss of *Wnt2* and *Wnt2b* results in the loss of lung progenitor specification.

Histological cross-sections of E9.5 wild-type (A-C) and *Wnt2/2b* DKO mutant embryos (E-G). (A, E) At E9.5, when the lung is initially specified, there is no detectable budding of the trachea from the anterior foregut in *Wnt2/2b* DKO mutants. (B, C, F, G) Immunostaining for Nkx2.1 shows that wild-type embryos express Nkx2.1 in the region where the trachea will bud from the foregut (B), however expression is not observed in *Wnt2/2b* DKO mutants (F, outline). Nkx2.1 expression is observed in both wild-type (C) and *Wnt2/2b* DKO mutants (G) in the thyroid primordium. Immunostaining for the esophagus epithelial marker p63 on E11.5 wild-type (D) and *Wnt2/2b* DKO mutant embryos (H) demonstrates expression in the single gut tube in *Wnt2/2b* DKO mutants at E11.5 (D and H, arrows). Serial cross-sections through E9.5 wild-type (I-L) and *Wnt2/2b* DKO mutant embryos (M-P) confirm lack of Nkx2.1 expression in the foregut endoderm of *Wnt2/2b* DKO mutants. Panels K and O correspond to panels B and F, respectively. Scale bars=100 μm (A-H), 250 μm (I-P).

Figure 3.6: Cell proliferation, apoptosis, and lung gene expression in *Wnt2/2b* DKO mutant foreguts.

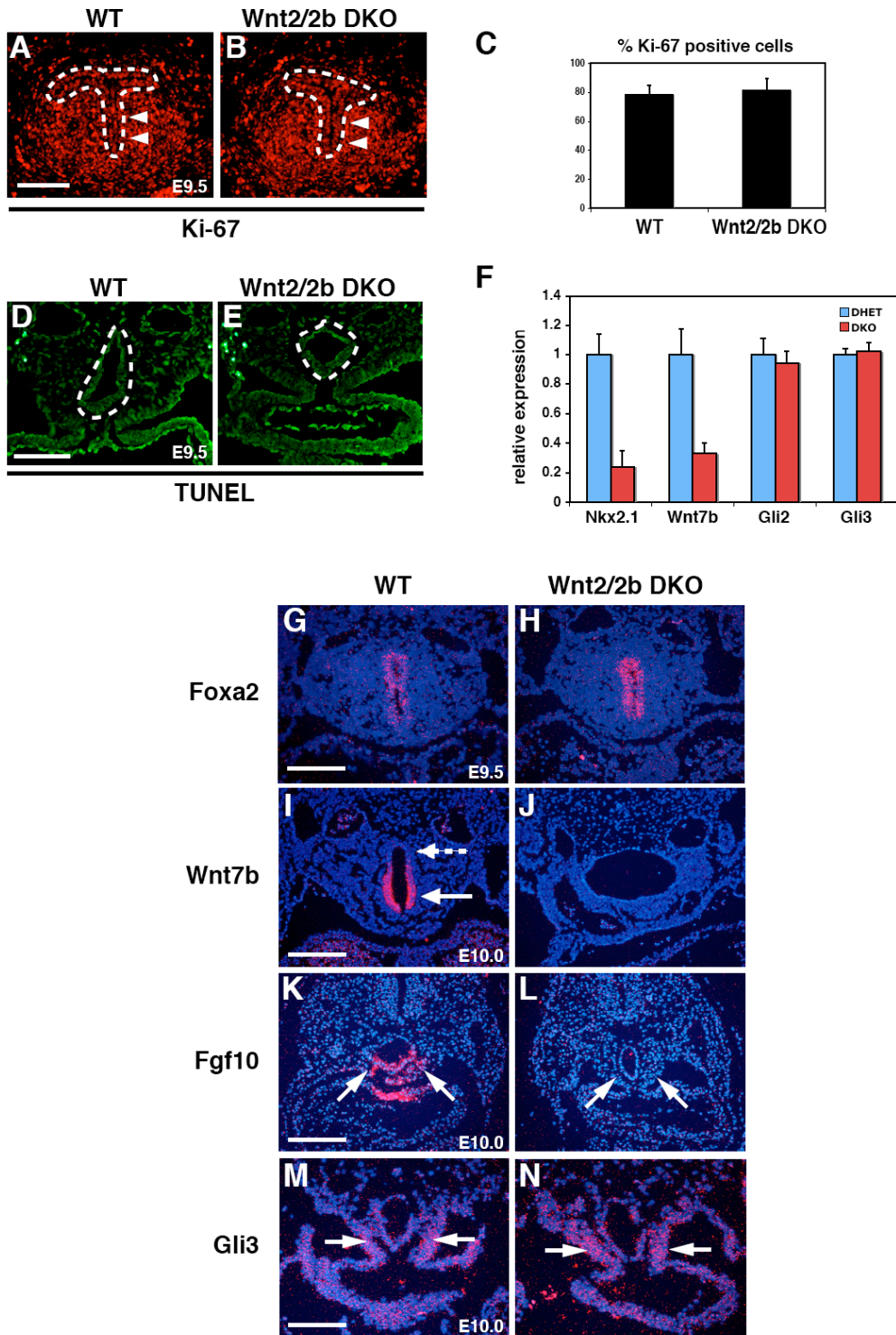


Figure 3.6: Cell proliferation, apoptosis, and lung gene expression in *Wnt2/2b* DKO mutant foreguts.

Histological cross-sections of E9.5 wild-type (A, D) and *Wnt2/2b* DKO mutant embryos (B, E). (A-C) Proliferation as measured by Ki-67 immunostaining is unchanged in the foregut endoderm of E9.5 *Wnt2/2b* DKO mutants (outlined region). (D, E) TUNEL staining does not indicate an increase in apoptotic cells in *Wnt2/2b* DKO mutant foreguts (outlined region). (F) Quantitative PCR performed on dissected foreguts of *Wnt2/2b* double heterozygous (DHET) and *Wnt2/2b* DKO mutants for *Nkx2.1*, *Wnt7b*, *Gli2*, and *Gli3* expression. *Wnt2/2b* double heterozygous mutants were used as controls since they were the only non-DKO controls obtained in all three litters used to generate this tissue. (G-N) In situ hybridization on wild-type and *Wnt2/2b* DKO mutant embryo cross-sections at the denoted embryonic timepoints. (G, H) *Foxa2* expression is unchanged in *Wnt2/2b* DKO mutant embryos. (I, J) *Wnt7b* expression, which marks early lung endoderm progenitors in the ventral aspect of the foregut (arrow) versus the dorsal aspect (dashed arrow), is lost in E10.0 *Wnt2/2b* DKO foregut endoderm. (K, L) *Fgf10* expression is reduced in the ventral mesoderm surrounding the foregut in *Wnt2/2b* DKO mutants. (M, N) *Gli3* expression is unchanged in *Wnt2/2b* DKO mutants. Error bars indicate one standard deviation. Scale bars=200 μm (A, B, D, E, G, H, K, L), 100 μm (I, J, M, N).

Figure 3.7: Wnt2/2b signal through the β -catenin-dependent canonical pathway in the primitive lung endoderm.

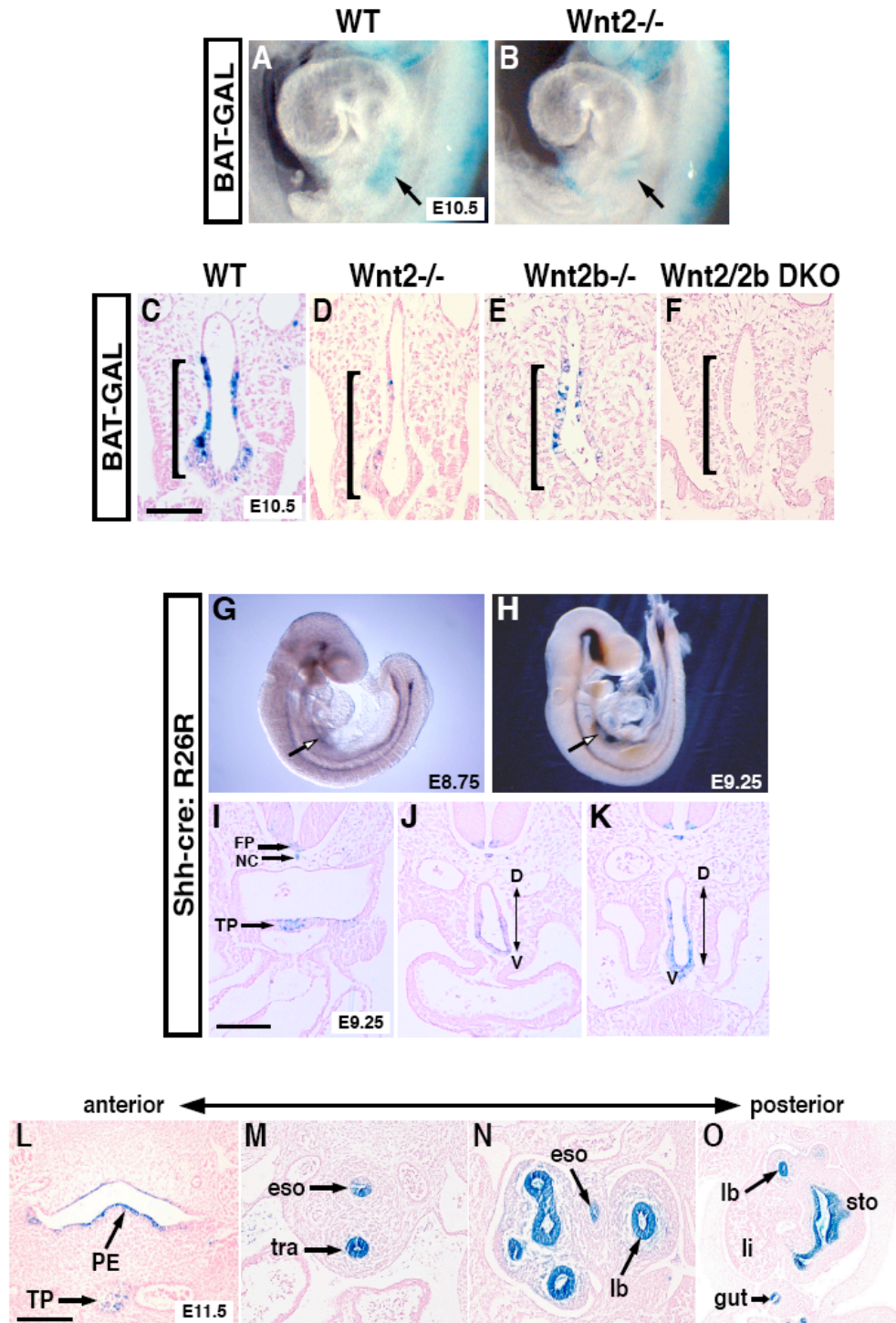


Figure 3.7: Wnt2/2b signal through the β -catenin-dependent canonical pathway in the primitive lung endoderm.

(A, B) Whole mount lacZ staining of *Wnt2^{-/-}:BAT-GAL* embryos shows a decrease in canonical Wnt signaling in the anterior foregut at E10.5 (A and B, arrows). (C-F) Histological cross-sections of lacZ-stained wild-type *BAT-GAL*, *Wnt2^{-/-}:BAT-GAL*, *Wnt2b^{-/-}:BAT-GAL*, and *Wnt2/2b:BAT-GAL* DKO mutant embryos show a loss of lacZ expression in the ventral aspect of the foregut endoderm in the region where the trachea is specified (brackets). (G-O) Lineage tracing of the *Shh-cre* line. *Shh-cre:R26R* embryos were stained at E8.75 (G) and E9.25 (H) to show cre recombinase activity in the anterior foregut (arrows). (I-K) Cross-sections of E9.25 *Shh-cre:R26R* embryos shows lacZ expression in the thyroid primordium (TP), floor plate (FP), notochord (NC), and the ventral aspect of the foregut endoderm. (L-O) Cross-sections of an E11.5 *Shh-cre:R26R* embryo shows lacZ expression in the thyroid primordium (TP), pharyngeal endoderm (PE), esophagus (eso), trachea (tra), endoderm of the lung buds (lb), and the endoderm of the stomach (sto) and hindgut (gut). LacZ expression is absent in the early liver (li) (O). D=dorsal, V=ventral. Scale bars=100 μ m (C-F, I-N), 200 μ m (O).

Figure 3.8: Wnt2/2b signal through β -catenin-dependent pathways to specify lung progenitors in the foregut endoderm.

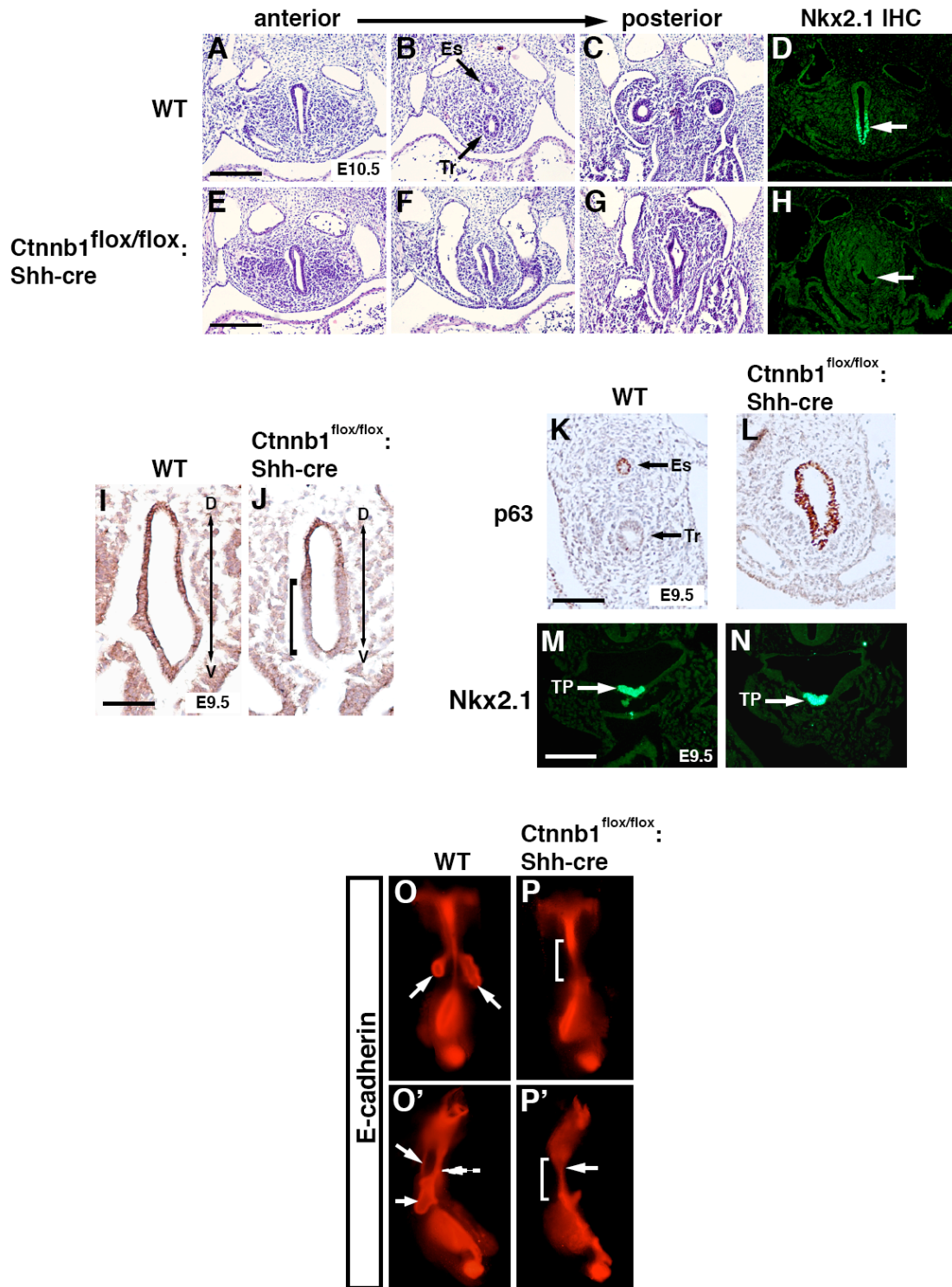


Figure 3.8: Wnt2/2b signal through β -catenin-dependent pathways to specify lung progenitors in the foregut endoderm.

(A-H) Histological cross-sections from E10.5 wild-type (A-D) and *Ctnnb1^{flox/flox};**Shh-cre* mutant embryos (E-H). H+E stained cross-sections show that genetic deletion of foregut endoderm β -catenin expression leads to tracheoesophageal defects and absence of lung development in *Ctnnb1^{flox/flox};**Shh-cre* mutants (E-G). (D, H) Nkx2.1 immunostaining demonstrates expression in the ventral foregut endoderm of wild-type embryos (D), but Nkx2.1 expression is lost in *Ctnnb1^{flox/flox};**Shh-cre* mutants (H), indicating a loss of lung specification in these mutants. (I, J) Immunostaining for β -catenin expression in *Ctnnb1^{flox/flox};**Shh-cre* mutants at E9.5 shows reduced expression of β -catenin in the ventral portion (V) of the anterior foregut (J), confirming *β -catenin* gene excision. (K, L) p63 immunostaining shows that the foregut tube in *Ctnnb1^{flox/flox};**Shh-cre* mutants (L) retains esophagus identity at E9.5. (M, N) Nkx2.1 immunostaining at E9.5 shows that thyroid (TP) specification is retained in *Ctnnb1^{flox/flox};**Shh-cre* mutants (N) in tissue anterior to the presumptive site of the tracheal bifurcation. (O, P) E-cadherin whole mount immunostaining shows normal tracheal budding in wild-type embryos (O, arrow) and lack of tracheal budding in *Ctnnb1^{flox/flox};**Shh-cre* mutants (P, arrow). The esophagus is still present in the *Ctnnb1^{flox/flox};**Shh-cre* mutants (P', bracket). D=dorsal, Es=esophagus, Tr=trachea, TP=thyroid primordium, V=ventral. Scale bars=200 μ m (A-H), 100 μ m (I-K), 50 μ m (M, N).

Figure 3.9: Activation of Wnt/ β -catenin signaling leads to expansion of lung endoderm progenitors into the stomach.

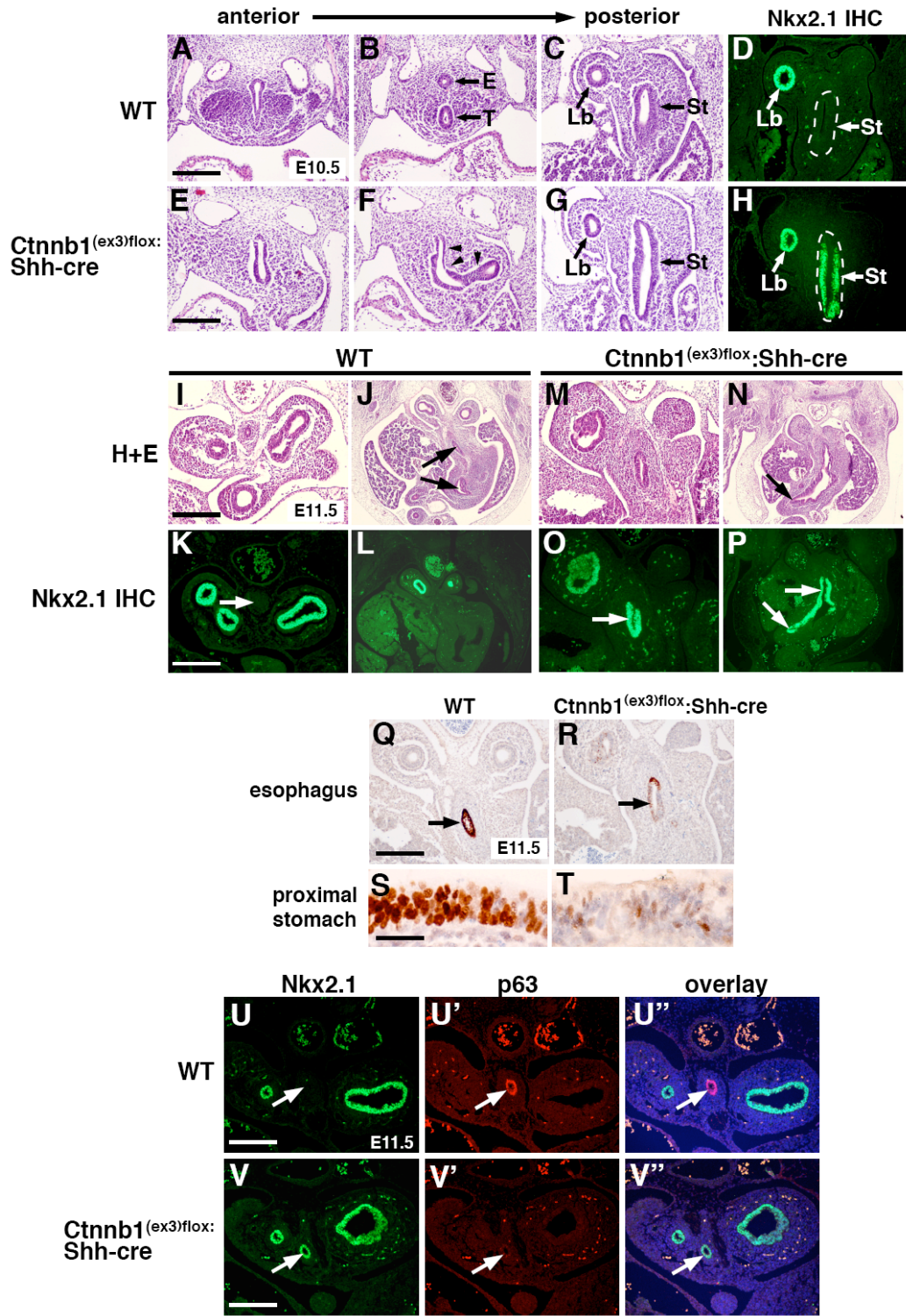


Figure 3.9: Activation of Wnt/ β -catenin signaling leads to expansion of lung endoderm progenitors into the stomach.

Histological cross-sections of E10.5 wild-type (A-D) and *Ctnnb1*^{(ex3)floxed}:*Shh-cre* mutant embryos (E-H). H+E stained cross-sections show that trachea-esophagus septation is disrupted in *Ctnnb1*^{(ex3)floxed}:*Shh-cre* mutants (F, arrowheads). (D, H) Immunostaining for Nkx2.1 expression reveals expansion of Nkx2.1 positive lung endoderm progenitors into the stomach (St, outlined region). (I-N) H+E stained cross-sections from E11.5 wild-type (I and J) and *Ctnnb1*^{(ex3)floxed}:*Shh-cre* (M and N) mutant embryos. (K-P) Immunostaining for Nkx2.1 expression shows expression of Nkx2.1 in the esophagus of E11.5 *Ctnnb1*^{(ex3)floxed}:*Shh-cre* mutants (O, arrow), but not in wild-type embryos (K, arrow). Expression of Nkx2.1 is also extended into the stomach of E11.5 *Ctnnb1*^{(ex3)floxed}:*Shh-cre* mutants (P, arrows). (Q-T) Immunostaining for p63 expression shows reduced expression in the esophagus and stomach endoderm of E11.5 *Ctnnb1*^{(ex3)floxed}:*Shh-cre* mutants (R, T). (U-V) Co-immunostaining for both Nkx2.1 and p63 expression shows that p63-positive endoderm is lost (V') while Nkx2.1-positive endoderm is present in the esophagus (V, arrow). E=esophagus, Lb=lung bud, St=stomach. Scale bar=200 μ m (A-I, M, K, O, Q, R, U-V), 400 μ m (J, N, L, P), 30 μ m (S, T).

Figure 3.10: Model of Wnt2/Wnt2b and β -catenin signaling for lung progenitor specification in the foregut endoderm.

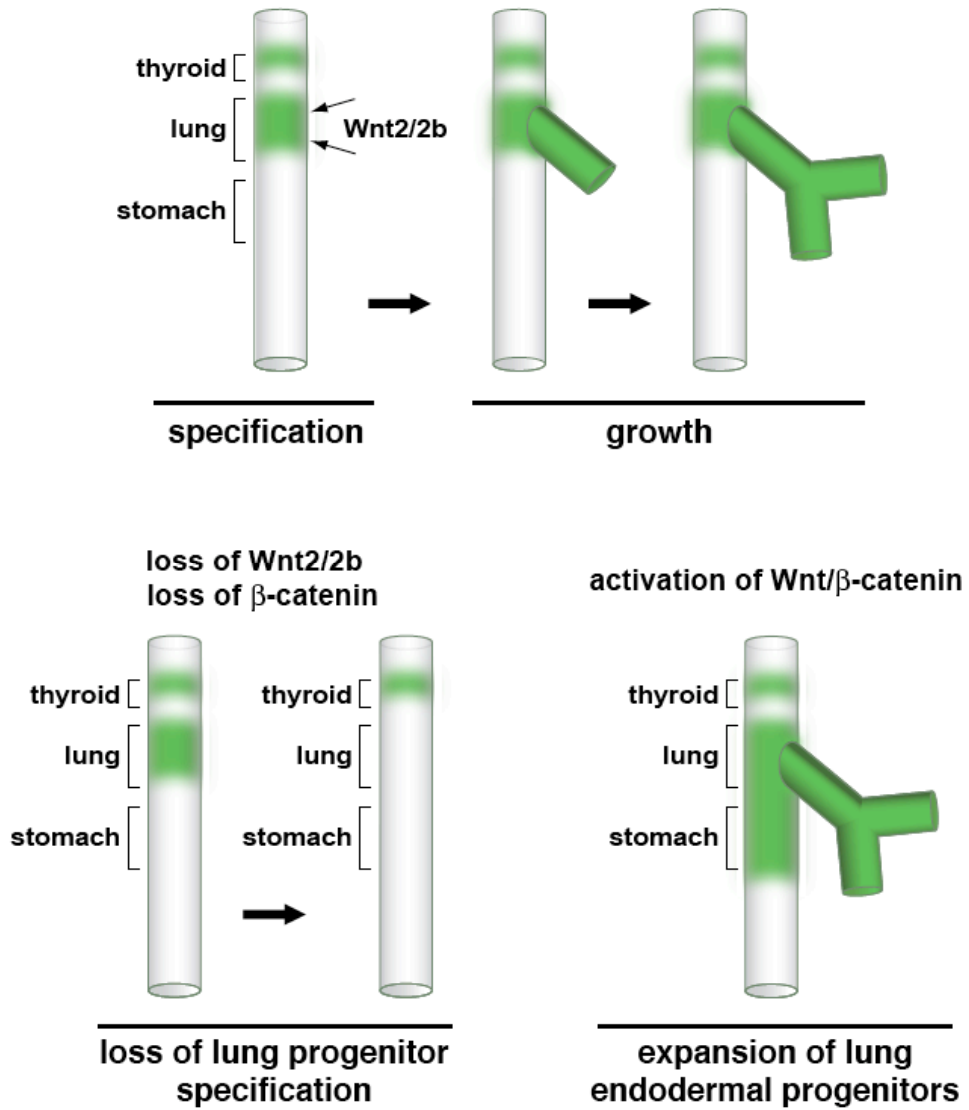


Figure 3.10: Model of Wnt2/Wnt2b and β -catenin signaling for lung progenitor specification in the foregut endoderm.

Model showing necessity and sufficiency of Wnt2/2b and β -catenin signaling for lung progenitor specification in the anterior foregut endoderm. Wnt2 and Wnt2b expression in the splanchnic mesoderm signals in a paracrine manner to the adjacent foregut endoderm activating β -catenin signaling to specify lung endoderm progenitors. Loss of either Wnt2/Wnt2b expression in the mesoderm, or β -catenin signaling in the foregut endoderm, leads to the loss of lung endoderm progenitors and lung agenesis. Moreover, dominant activation of β -catenin signaling in the endoderm leads to the expansion of lung progenitors into the presumptive stomach.

Chapter 4: Conclusions and Future Directions

Summary

Lung development is a complex process involving input from multiple signaling pathways to coordinate specification, morphogenetic, and differentiation events. The Wnt signaling pathway indisputably plays a critical role in lung development, given the reported lung defects in several Wnt knockout and β -catenin conditional mutant mouse models. However, the contributions of individual Wnt ligands and canonical Wnt/ β -catenin signaling to the specification and development of respiratory cell types have not been fully addressed. Wnt ligands are secreted molecules that can activate downstream signaling pathways in cell-autonomous and non-cell autonomous manners. An important theme in the development of organs is the necessity for cross-talk between different tissue types, such as the developing lung mesenchyme and endoderm. Wnts as intercellular ligands can act upon multiple tissues, thereby adding complexity to their functions during the development of organs. The two roles identified for Wnt2 signaling in this thesis reflect the dual autocrine and paracrine signaling capabilities of Wnt ligands.

In this dissertation, I analyze a series of mouse models to determine the function of Wnt2 signaling in lung development. These studies reveal two distinct roles for Wnt2 signaling during lung development: one in the splanchnic mesoderm flanking the site of lung specification in the foregut endoderm, and a second role in the developing lung mesenchyme.

In Chapter 2, I describe a role for Wnt2 signaling in the development of the lung mesenchyme. I show that Wnt2 signaling is required for overall lung growth and development of airway smooth muscle. Additionally, I place Wnt2 signaling as an upstream regulator in a hierarchy of smooth muscle development. I show that Wnt2 signaling promotes the activation of an early smooth muscle transcriptional regulatory network that supports the development of immature smooth muscle. I also show that Wnt2 signaling activates downstream FGF-10 signaling, a pathway well known to promote the development of airway smooth muscle.

In Chapter 3, I elaborate my investigations into the functions of Wnt2 signaling by studying the cooperativity between Wnt2 and its associated family member-Wnt2b, which is co-expressed with Wnt2 in the lung. My studies reveal that both ligands signal cooperatively in the splanchnic mesoderm tissue prior to lung morphogenesis to regulate the specification of lung progenitors in the foregut endoderm. The combined loss of Wnt2/2b function results in the complete loss of the lung, and this loss is tightly restricted to the lung field in the foregut. I also demonstrate that Wnt2 and Wnt2b mediate their effects on the endoderm via activation of β -catenin signaling in the foregut, and that an endoderm-specific loss of β -catenin recapitulates the lung agenesis phenotype.

In contrast to previous reports, these results demonstrate that Wnt2 signaling is critical for lung development. The data collected from these investigations establishes a firm understanding of what processes Wnt2 and Wnt2b coordinate during embryonic development. The data also prompts additional questions concerning the roles of Wnt2 and β -catenin signaling in both the embryonic and adult lung.

Future Directions

What cell lineages are *Wnt2*-expressing cells contributing to during lung development?

The data presented in Chapter 2 on the *Wnt2* loss-of-function model suggest that *Wnt2* signaling is having a critical impact on the development of smooth muscle in the lung. However, this result does not definitely establish whether *Wnt2*-expressing cells are giving rise to smooth muscle lineages only, or to other mesenchymal lineages in the mouse lung.

To better understand the lineage contributions of *Wnt2*-expressing cells, our laboratory has recently generated a tamoxifen-inducible knock-in *Wnt2*^{cre/ERT2} allele (Figure 4.1). This allele carries a tamoxifen-inducible cre recombinase cDNA cassette homologously inserted at the *Wnt2* locus. This allele will permit conditional fatemapping of *Wnt2*-expressing cells during lung development based on the endogenous activity of the *Wnt2* promoter. An initial experiment testing for cre recombinase activity using the *Rosa26* reporter mouse strain indicates that the allele is activated in lung tissue; however, the efficiency of cre recombinase-mediated excision is still unclear and the tamoxifen induction method is being optimized. Use of this genetic tool will enable us to more thoroughly study and define the contribution and behavior of *Wnt2*-expressing cells in the lung.

It will be very interesting to see whether fatemapping in the *Wnt2*^{cre/ERT2} mouse model demonstrates that *Wnt2*-expressing cells contribute to smooth muscle in the lung. Additionally, it will also be of interest to see whether *Wnt2*-expressing cells contribute to

both vascular and smooth muscle cells. Wnt2 signaling is reported to mediate endothelial gene expression in embryoid bodies¹⁴², and there are subtle molecular deficiencies during early endothelial development in *Wnt2*^{-/-} null mutant lungs (see Chapter 2). Therefore it is possible that subpopulations of *Wnt2*-expressing cells contribute to the developing endothelium, and this will be revealed through fatemapping. The fatemapping studies may also reveal additional lineage contributions of *Wnt2*-expressing cells in the mesenchyme. Finally, fatemapping in the *Wnt2*^{cre/ERT2} model may also serve as an additional tool to study the behaviors of *Wnt2*-expressing cells (e.g. migration, EMT) in the context of reduced Wnt2 signaling, other genetic deficiencies, or during lung injury and repair.

The reported *Wnt2* expression data indicates that *Wnt2* is expressed in the developing lung mesenchyme^{141, 143, 159}. However the expression data is based on in situ hybridization experiments, and does not exclude the possibility that *Wnt2* is expressed at low levels in the developing lung epithelium. Preliminary data indicate that *Wnt2* is expressed at low levels in the postnatal airway epithelium (discussed below), and is highly upregulated after airway injury (Figure 4.2). The expression of *Wnt2* in the adult epithelium suggests the possibility for the presence of *Wnt2*-expressing cells in the developing epithelium. Thus, the *Wnt2*^{cre/ERT2} model may reveal a previously unknown site of *Wnt2* expression in the embryonic lung.

A role for Wnt2 signaling in asthma?

Recent studies show upregulation of Wnt/ β -catenin signaling in airway smooth muscle in a mouse model of allergen-induced asthma^{22, 212}. Given the prominent role described in Chapter 2 for Wnt2 signaling in airway smooth muscle development, it will

be interesting to look at whether Wnt2 expression is activated in airway smooth muscle in an asthmatic model. To investigate further and genetically determine whether Wnt2 signaling is activated during asthma, the *Wnt2^{cre/ERT2}* fatemapping model could be utilized in the induced asthma model and analyzed for Wnt2 signaling activity. Additionally, the contribution of *Wnt2*-expressing cells to the asthmatic phenotype can be analyzed, including airway smooth muscle proliferation.

Is Wnt2 mediating its effects via cross-talk with the adjacent epithelium?

Epithelial-mesenchymal interactions are integral to the development of many cell types in the lung. Studies have shown that disruption of Wnt7b signaling leads to defects in smooth muscle development in the lung mesenchyme¹³⁴. Expression of Wnt7b is downregulated in *Wnt2^{-/-}* null mutant lungs, suggesting a possible paracrine mechanism for Wnt2 signaling to the adjacent epithelium to activate Wnt7b expression and coordinate smooth muscle development. Interestingly, the expression of other key signaling factors known to regulate smooth muscle development are unaltered, and therefore the relative contribution of reduced epithelial-mesenchymal signaling to the loss of smooth muscle in *Wnt2^{-/-}* null mutant lungs is uncertain.

To interrogate the contribution of epithelial signaling in *Wnt2^{-/-}* null mutant lungs, it will be interesting to isolate *Wnt2^{-/-}* null mutant lung mesenchyme and try rescuing the loss of smooth muscle gene expression with rWnt2 treatment. If Wnt2 is signaling in an autocrine manner to regulate smooth muscle development, then rWnt2 should fully rescue gene expression. If however, Wnt2 signaling requires the presence of the epithelium to mediate smooth muscle development, the rescue effect may be mild or negligible in the presence of rWnt2. An alternative approach to examine the requirements

for epithelial cross-talk would be to culture isolated wildtype lung mesenchyme in the presence of rWnt2, and examine whether smooth muscle gene expression is significantly upregulated. If Wnt2-mediated epithelial cross-talk is required for coordinating smooth muscle gene expression, I would not predict a dramatic upregulation of gene expression in the presence of rWnt2.

Can ectopic Wnt2 expression impose a lung progenitor identity on early endoderm?

An intriguing concept emerging from the results in Chapter 3 is that of Wnt2 signaling serving as a master regulator of the lung progenitor fate by activating β -catenin signaling in the foregut endoderm. A question left unresolved from the investigations described in Chapter 3 is whether the restricted effects of lung progenitor loss in the Wnt2 signaling loss-of-function model is due to the restricted expression pattern of Wnt2/2b, the presence of other factors expressed along the A-P axis in the mesoderm flanking the endoderm, or intrinsic patterning in the foregut endoderm which limits the responsiveness to Wnt2/2b signaling.

It will be interesting to test whether ectopic Wnt2 signaling can expand lung progenitor identity in isolated endoderm, using published techniques⁵⁰. If rWnt2 can induce Nkx2.1 expression along the endoderm tract beyond the zone of the foregut, this would suggest that the early endoderm is competent to give rise to Nkx2.1-positive progenitors. If rWnt2 is not capable of promoting Nkx2.1 identity beyond the foregut region, this would suggest that intrinsic factors expressed in the endoderm are already acting to restrict the foregut competence. Additionally, if the endoderm is cultured with adjacent mesoderm and rWnt2 is still able to expand the Nkx2.1 expression domain, this would suggest that the restricted pattern of Nkx2.1 expression in wild-type embryos is a

consequence of the restricted Wnt2/2b expression pattern. If, however, rWnt2 is unable to expand Nkx2.1 expression in the presence of mesoderm, this would suggest that other factors expressed in the mesoderm are restricting the activity of Wnt2 signaling.

A role for Wnt2/ β -catenin signaling in lung stem cells?

In Chapter 3, I demonstrated that Wnt2 and β -catenin signaling coordinate the specification of an Nkx2.1-positive lung progenitor cell population in the embryo. This raises the question of whether or not a similar mechanism imposes a respiratory progenitor fate in embryonic stem cells (ESCs) and cells of the adult lung. A significant barrier to the use of stem cell-derived therapies is the lack of knowledge on how to efficiently direct a pluripotent cell type towards a homogenous population of tissue-specific progenitors. Moreover, successful attempts too often rely on the introduction of transgenes to use tissue-specific promoters to drive differentiation^{213, 214}. There are established methods for culturing mouse ESCs into definitive endoderm^{215, 216}, and it will be interesting to test whether adding rWnt2 to ESC-derived endoderm would promote the specification of Nkx2.1-expressing progenitors. Additionally, it will also be interesting to test whether activating β -catenin signaling using the β -catenin agonist lithium chloride would also promote Nkx2.1 expression.

One caveat to this approach is that it will require careful characterization of Nkx2.1 cells to distinguish between thyroid and lung progenitors. Sustained Wnt/ β -catenin signaling will also block differentiation in lung cell types^{114, 217}, therefore persistent exposure to rWnt2 and activated β -catenin signaling may inhibit the differentiation of Nkx2.1-positive cells into mature respiratory lineages.

Is Wnt2 signaling important for mediating the lung injury and repair response?

An emerging paradigm in adult injury/repair and disease mechanisms is the re-expression of critical developmental signaling pathways²¹⁸⁻²²⁰. Wnt ligands are not expressed at high levels in adult tissues; therefore the role for Wnt signaling in adult tissues is relatively unknown. This prompts the question of whether Wnt2 signaling is activated in adult airways during injury and repair processes.

Preliminary data indicate that Wnt2 may play a role in the adult lung during airway repair. In the post-natal mouse lung, *Wnt2* is expressed at low levels (Figure 4.2A-C). However, after naphthalene-induced bronchiolar airway injury, *Wnt2* expression is highly upregulated in both the epithelium and mesenchyme (Figure 4.2D-F). The expression of *Wnt2* in the injured epithelium is intriguing, and could be originating in cells undergoing EMT in the basal lamina of the denuded airway or de novo *Wnt2* expression in the exfoliating airway epithelium. Possible insights into the functions of *Wnt2* re-expression during airway injury will be accomplished by examining the repair response in surviving *Wnt2*^{-/-} null mutants (less than 20% survive after birth) or by examining the response in *Wnt2/2b* heterozygous compound mutants. The injury and repair response will be examined at the cellular level by immunohistochemistry assessing for proliferation or differentiation of repopulating airway cell types in the denuded airways.

If Wnt2-deficient adult lungs lose the ability to repair properly, it would be interesting to test whether a simple injection of lithium chloride would rescue repair by activating β -catenin signaling. Additionally, our laboratory has its own hyperoxic

chamber, which will permit testing for *Wnt2* re-expression in other types of more distal airway injury.

Concluding remarks

In this dissertation I have investigated the function of *Wnt2* and *Wnt*/ β -catenin signaling during lung development. These investigations describe the roles of *Wnt2* and *Wnt*/ β -catenin signaling in lung specification within the mouse foregut endoderm, and in the development of airway smooth muscle. Overall, these results contribute significantly to the body of knowledge for *Wnt* signaling in lung development. In examining these data in conjunction with the published literature, two important themes stand out. First, the activities, interactions with other pathways, and functions of *Wnt* signaling in the lung are dynamic. The requirements for *Wnt* signaling activity appear to occur at discrete timepoints during lung morphogenesis, and in or between discrete tissue and cellular compartments. Secondly, an increasing amount of published data implicates *Wnt* signaling in lung airway repair and pathogenesis. Therefore, it will be exciting to see how these and future findings will transition into investigations on adult pathological processes and regenerative technologies.

Figure 4.1: Schematic of *Wnt2^{cre/ERT2}* knock-in strategy.

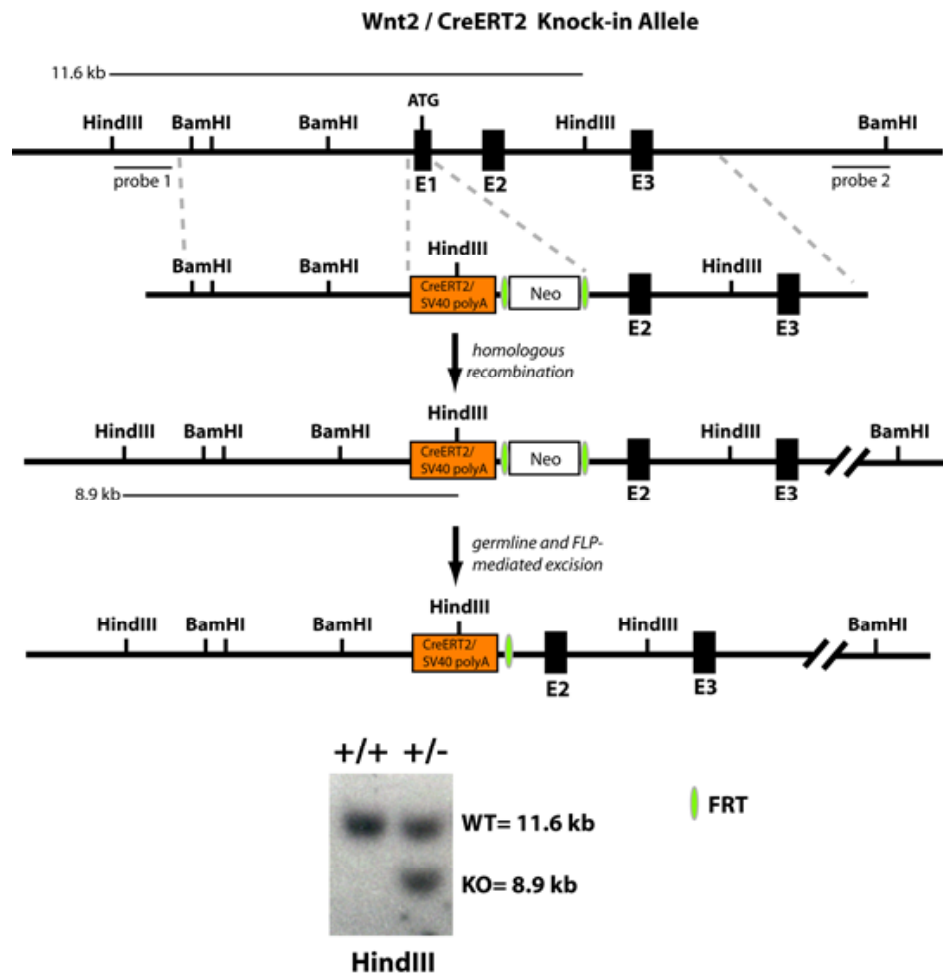


Figure 4.1: Schematic of $Wnt2^{cre/ERT2}$ knock-in strategy.

Diagram of the $Wnt2^{cre/ERT2}$ gene targeting strategy with a representative Southern blot using the indicated probe/ HindIII. A cre recombinase cDNA fused to a mutant form of the human estrogen receptor ligand binding domain (creERT2) was used to replace the coding region of exon 1 of $Wnt2$. The mutant receptor binds only the synthetic ligand 4-hydroxytamoxifen, and in the presence of ligand cre recombinase is expressed in all cells that express the endogenous gene. The neomycin cassette was removed using Flper mice (flp sites=green circles).

Figure 4.2: Upregulation of Wnt2 expression after airway injury.

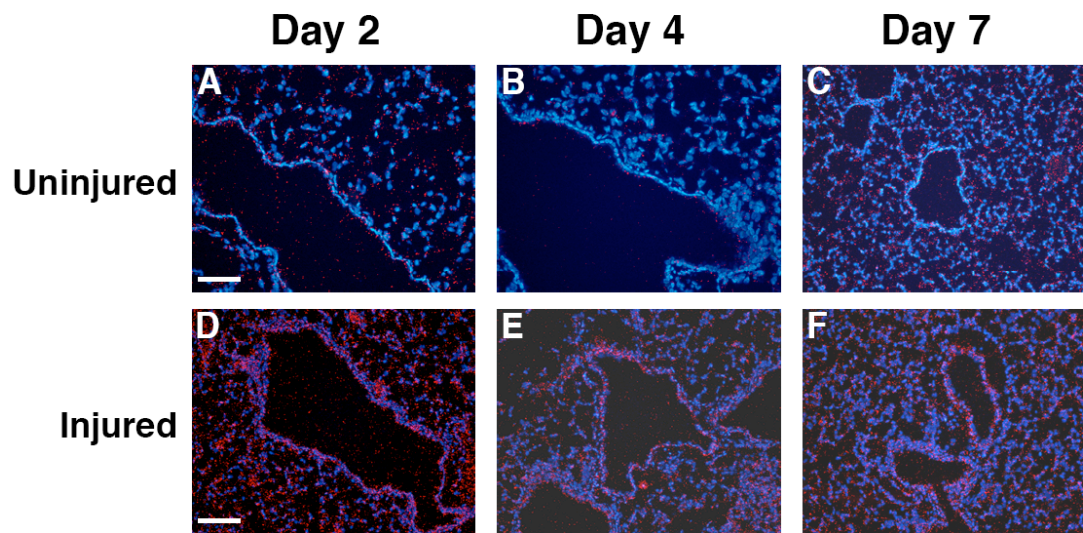


Figure 4.2: Upregulation of Wnt2 expression after airway injury.

Histological cross-sections showing in situ hybridizations for *Wnt2* expression in the postnatal wild-type mouse lung after naphthalene injury at 2, 4, and 7 days post-injection. (A-C) In control animals receiving saline intraperitoneal injections, low levels of *Wnt2* expression are observed in the uninjured lung. However upon intraperitoneal naphthalene injection, *Wnt2* expression is highly upregulated throughout the airway epithelium and mesenchyme at 2 days (C), and *Wnt2* expression levels remain upregulated through to day 7 of injury (E and F). Scale bars=200 μm (A-F).

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