Multiple Roles of Epithelial Signaling During Craniofacial and Foregut Morphogenesis

by

Katherine Kretovich Billmyre

Department of Cell Biology
Duke University

Date:_______________________

Approved:

___________________________
John Klingensmith, Supervisor

___________________________
Blanche Capel, Chair

___________________________
Amy Bejsovec

___________________________
Mary Hutson

___________________________
Terry Lechler

Dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, in the Department of Cell Biology in the Graduate School of Duke University

2015
ABSTRACT

Multiple Roles of Epithelial Signaling During Craniofacial and Foregut Morphogenesis

by

Katherine Kretovich Billmyre

Department of Cell Biology
Duke University

Date:_______________________

Approved:

___________________________
John Klingensmith, Supervisor

___________________________
Blanche Capel, Chair

___________________________
Amy Bejsovec

___________________________
Mary Hutson

___________________________
Terry Lechler

An abstract of a dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Cell Biology in the Graduate School of Duke University

2015
Abstract

During embryonic development many structures crucial for breathing and eating arise from the pharyngeal and anterior foregut epithelium (FGE), which contains the oral ectoderm and the foregut endoderm. Proper differentiation and signaling within and from this epithelial tissue is necessary for the development of the mandible, the esophagus, and the trachea. Many birth defects occur in these structures that greatly disrupt the ability of affected infants to breathe and eat. This dissertation investigates the importance of the pharyngeal and anterior FGE in mandible and foregut development.

The most rostral portion of the pharyngeal epithelium contributes to the development of the mandible. At embryonic day 10.5 the mandible is a bud structure, composed of neural crest-derived mesenchyme and core mesoderm surrounded by pharyngeal epithelium. The mesenchyme needs to receive Hedgehog signaling for mandible development, but the epithelial tissue that signals to the mesenchyme has not been identified in mammals. Data presented in Chapter 2 show that Sonic Hedgehog is necessary at two distinct stages of mandible development by using a tissue specific genetic ablation to remove Sonic Hedgehog from the pharyngeal endoderm. First, we show that Sonic Hedgehog promotes cell survival prior to cartilage differentiation through immunostaining for Caspase-3, an apoptosis marker. Second, a rescue of early
cell death with the p53 inhibitor pifithrin-α shows that Sonic Hedgehog is necessary for
cartilage condensation and differentiation later in development. Without cartilage
differentiation the mandible is unable to elongate properly and hypoplasia occurs.

Caudal to the pharyngeal epithelium is the anterior FGE, which develops into the
larynx, esophagus and trachea. The anterior FGE is a single endodermal tube at E9.5 and
by E11.5 compartmentalizes into two distinct tubes: the esophagus and trachea. While
the signaling pathways involved in proper compartmentalization of the foregut are well
studied, nothing is known about the cellular behaviors that drive this complex event.
One important event during foregut compartmentalization is the establishment of dorso-
ventral patterning, which is necessary for separation to occur. To elucidate the
importance of dorso-ventral patterning, we take advantage of two genetic mouse models
with disrupted patterning, an activation of and a removal of β-catenin in the ventral
foregut endoderm. Data presented in Chapter 3 show that β-catenin is important for
epithelial pseudostratification and the establishment of a region of double-positive cells
at the dorso-ventral midline through close examination of epithelial morphogenesis at
E10.5 prior to compartmentalization. This data has established two mouse models for
studying changes in epithelial morphology during foregut compartmentalization. In
total, this body of work details how signals originating in the pharyngeal and anterior
foregut epithelium regulate both mesenchymal and epithelial behaviors during
mandible and foregut development.
Dedication

This is dedicated to my parents who instilled in me a desire to learn at a young age. Also to all the teachers and mentors I have had throughout my life that have helped me get here today. And lastly to my husband whose support and patience throughout graduate school has been paramount to my success.
Contents

Abstract ........................................................................................................................................iv

List of Tables ................................................................................................................................xii

List of Figures .................................................................................................................................xiii

1. Introduction .................................................................................................................................. 1

1.1 Morphogenesis of the mammalian foregut epithelium ................................................................. 1

1.1.1 Anterior foregut epithelium origins and development ................................................................. 1

1.1.2 Hedgehog signaling .................................................................................................................. 2

1.1.3 Canonical WNT Signaling ......................................................................................................... 5

1.2 Signaling and morphogenesis during mandible development ....................................................... 7

1.2.1 Anatomy of the mammalian mandible .................................................................................... 7

1.2.2 Etiologies of micrognathia ....................................................................................................... 10

1.3 Signaling and morphogenesis during foregut development ......................................................... 13

1.3.1 Anatomy of the mammalian foregut ....................................................................................... 13

1.3.2 Models of tracheoesophageal compartmentalization and lung budding ................................. 16

1.3.3 Genes and pathways linked to human EA/TEF ......................................................................... 20

1.3.4 Murine models of anterior foregut defects ............................................................................. 24

1.3.4.1 Improper notochord resolution can cause EA/TEF .............................................................. 26

1.3.4.2 Proper dorso-ventral patterning is necessary for compartmentalization .......................... 29

1.3.5 Dorso-ventral patterning during foregut compartmentalization ............................................. 29

1.3.5.1 Regulation of dorso-ventral patterning ............................................................................. 32
1.3.6 Cellular behaviors during compartmentalization ........................................ 35
  1.3.6.1 Programmed cell death ........................................................................ 35
  1.3.6.2 Proliferation ....................................................................................... 36
  1.4 Cre-LoxP technology .................................................................................. 37

2. Sonic Hedgehog from pharyngeal arch 1 epithelium is necessary for early mandibular arch cell survival and later cartilage condensation differentiation ........................................ 40
  2.1 Introduction ............................................................................................... 40
  2.2 Methods .................................................................................................... 44
    2.2.1 Mice ...................................................................................................... 44
    2.2.2 Dissections, processing and histology ................................................ 45
    2.2.3 Immunohistochemistry and cell death analysis ................................ 45
    2.2.4 Skeletal preparations ........................................................................... 45
    2.2.5 RNA collection and qPCR .................................................................... 46
    2.2.6 In situ preparation ................................................................................ 47
    2.2.7 Quantification of proliferation and cell death ....................................... 47
    2.2.8 Pharmacological inhibition of p53 ....................................................... 47
  2.3 Results ....................................................................................................... 48
    2.3.1 Nkx2.5Cre expression overlaps with Shh expression in both the oral ectoderm and pharyngeal endoderm ................................................................. 48
    2.3.2 Loss of epithelial Shh results in micrognathia starting at embryonic day 11.5 50
    2.3.3 A loss of epithelial Shh from PA1 results in a complete loss of Meckel’s cartilage and tongue ....................................................................................... 53
2.3.4 Shh from PA1 epithelium is required for cartilage condensation formation in pharyngeal arch one................................................................................................. 56

2.3.5 PA1 proliferation is unchanged in Nkx2.5Cre;Shh^fx/embryos prior to cartilage initiation.................................................................................................................. 58

2.3.6 Increased apoptosis in Nkx2.5Cre;Shh^fx/embryos at E10.5 results in decreased mandibular bud size ........................................................................................................... 58

2.3.7 Epithelial Shh is important for development of distal PA1 at E10.5 ............... 61

2.3.8 Pharmacological inhibition of p53 rescues PA1 cell survival but not Meckel’s cartilage outgrowth in Nkx2.5Cre;Shh^fx/embryos .......................................................... 66

2.3.9 Epithelial Shh regulates differentiation factors during cartilage condensation development................................................................................................................. 69

2.4 Discussion............................................................................................................. 72

2.4.1 Epithelial Shh acts as a PA1 survival signal...................................................... 76

2.4.2 Localization of Dlx2, Dlx5 and Fgf8 is regulated by epithelial Shh ............... 78

2.4.3 Shh is necessary for Meckel’s cartilage differentiation .............................. 80

3. Wnt signaling regulates development of the dorso-ventral midline epithelium during foregut compartmentalization ............................................................... 84

3.1 Introduction........................................................................................................... 84

3.2 Methods .............................................................................................................. 88

3.2.1 Mice .............................................................................................................. 88

3.2.2 Dissections, processing and histology .......................................................... 89

3.2.3 Immunohistochemistry and cell death analysis ........................................... 89

3.2.4 Skeletal preparations .................................................................................... 90

3.2.5 RNA collection and qPCR .......................................................................... 90
3.3 Results .......................................................................................................................... 90

3.3.1 The foregut epithelium undergoes a remodeling event prior to compartmentalization .............................................................................................................. 90

3.3.2 The ventral foregut epithelium has actin localized apically and in filopodia during compartmentalization but no decrease in E-cadherin ........................................................................... 93

3.3.3 The ventral foregut epithelium is pseudostratified by E10.5............................................ 98

3.3.4 Ablation and activation of \( \beta\)-Catenin in the ventral foregut result in two unique models to study dorso-ventral patterning ........................................................................................................ 102

3.3.5 \( \beta\)-catenin\textsuperscript{ACT} is a novel model of compartmentalization defects ............ 106

3.3.6 \( \beta\)-catenin is necessary for pseudostratification of the ventral foregut ............... 109

3.3.7 Nkx2-1 is not necessary for pseudostratification of the ventral foregut prior to compartmentalization ........................................................................................................... 111

3.3.8 Dorso-ventral patterning does not regulate ephrin-B2 gene expression.............. 113

3.3.9 Barx1 gene expression is regulated by \( \beta\)-catenin in the foregut ............................. 117

3.4 Discussion ..................................................................................................................... 120

4. Conclusions ..................................................................................................................... 124

4.1 Discussion of Results .................................................................................................. 124

4.1.1 Epithelial Shh is necessary for cell survival and cartilage initiation during mandible development ........................................................................................................ 124

4.1.2 \( \beta\)-catenin regulates pseudostratification in the foregut epithelium through a mechanism independent of ventral patterning ......................................................... 126

4.1.3 The dorso-ventral midline consists of a population of SOX2/NKX2-1 double-positive cells which are important in compartmentalization ........................................ 127

4.2 Future Studies ......................................................................................................... 128
4.2.1 How is Shh regulating cartilage development and is it through an Fgf8 dependent mechanism? ................................................................. 128

4.2.2 How is β-catenin regulating pseudostratification? ................................................................. 129

4.2.3 Are NKX2-1/SOX2 double-positive midline cells regulating midline specific gene expression? ........................................................................ 131

4.2.4 Can modeling be used to predict which cellular behaviors are necessary for compartmentalization? ........................................................................ 135

4.2.5 Using chicken as a model may answer questions which mice cannot ........ 136

Appendix ......................................................................................................................... 140

References ......................................................................................................................... 144

Biography ......................................................................................................................... 161
List of Tables

Table 1: Human Diseases with Micrognathia ................................................................. 12

Table 2: Table of genetic mouse models with foregut defects and the causative mechanism ............................................................................................................ 25
List of Figures

Figure 1: Shh Signaling........................................................................................................4
Figure 2: WNT Signaling......................................................................................................6
Figure 3: Mandible development occurs in multiple stages.................................................9
Figure 4: The foregut compartmentalizes between E10 and E11.5.......................................15
Figure 5: Models of foregut compartmentalization.............................................................19
Figure 6: Disruptions in foregut compartmentalization can result in a variety of defects of the esophagus and trachea.................................................................23
Figure 7: Adriamycin treatment of wildtype mice can result in EA ..................................28
Figure 8: A signaling network of specific genes is necessary to establish proper Nkx2-1 and Sox2 expression, leading in turn to either respiratory fate or esophageal fate .......31
Figure 9: Cre-LoxP Technology..........................................................................................39
Figure 10: Nkx2.5Cre expression overlaps with Shh expression in both the oral ectoderm and pharyngeal endoderm.................................................................49
Figure 11: Loss of epithelial Shh results in micrognathia starting at embryonic day 11.5 52
Figure 12: Shh is necessary for Meckel’s cartilage formation. ........................................55
Figure 13 Cartilage condensations do not form in Nkx2.5Cre;Shh<sup>fx/fx</sup>-embryos........57
Figure 14: SHH acts as a survival signal in PA1 at E10.5..................................................60
Figure 15: Epithelial Shh is important for development of distal PA1 at E10.5.................65
Figure 16: Pharmacological inhibition of p53 rescues PA1 cell survival but not Meckel’s cartilage outgrowth in Nkx2.5Cre;Shh<sup>fx/fx</sup> embryos........................................68
Figure 17: Epithelial Shh regulates differentiation factors during cartilage condensation development ...........................................................................................................71
Figure 18: Epithelial Shh is necessary for early PA1 mesenchymal cell survival and for regulation of differentiation factors during mandibular cartilage condensation development. .................................................................75

Figure 19: The foregut epithelium remodels between E9.5 and E11.5 ...................... 92

Figure 20: E-cadherin protein expression is not lost in epithelial cells at the point of compartmentalization ................................................................................................................................. 95

Figure 21: Actin-rich protrusions are present at the point of foregut compartmentalization ................................................................................................................................. 97

Figure 22: The foregut epithelium is pseudostratified at E10.5............................... 101

Figure 23: β-catenin is necessary and sufficient for Nkx2-1 expression ............... 105

Figure 24: Ventral activation of β-catenin causes foregut compartmentalization defects without disrupting dorso-ventral differentiation ................................................................. 108

Figure 25: Removal of ventral β-catenin disrupts pseudostratification................... 110

Figure 26: Loss of Nkx2-1 does not disrupt pseudostratification of the ventral foregut 112

Figure 27: Expression of Eph-ephrins during foregut compartmentalization .......... 116

Figure 28: Barx1 is upregulated when ventral β-catenin is absent.......................... 118

Figure 29: Regulation of foregut compartmentalization by dorso-ventral patterning... 119

Figure 30: Organ culture of dissected foreguts.......................................................... 134

Figure 31: Chicken as a model system for studying foregut development ............. 139

Figure 32: Ventral foregut Shh is not necessary for dorso-ventral patterning or pseudostratification ................................................................................................................................. 143
1. Introduction

Embryonic development is a tightly regulated process that depends on proper signaling and cellular processes to create the highly complex structures that are present in the adult. Even small disruptions in the processes occurring during development can result in malformations in the body plan. In humans, congenital malformations occur in 1 in 33 live births and defects can range from mild and cosmetic to serious and life-threatening (CDC.gov). Furthermore, more than 20% of infant deaths are due to birth defects (CDC.gov), the majority of which have no known etiology. To gain better insight into the mechanisms underlying human birth defects we must strive to understand how embryonic development occurs normally. My dissertation work focuses on the development of two embryonic structures that are common sites of congenital malformations: the mandible and the foregut. Work presented here will investigate the importance of epithelial signaling and morphogenesis in the normal development of these structures.

1.1 Morphogenesis of the mammalian foregut epithelium

1.1.1 Anterior foregut epithelium origins and development

After gastrulation the early mouse embryo consists of the three germ layers: endoderm, mesoderm and ectoderm, which give rise to the structures of the embryo. Prior to E10.5 the endodermal precursor to the anterior foregut epithelium is a single
epithelial sheet from which the notochord resolves via an epithelial-mesenchymal transition (Jurand 1974). After notochord resolution the endodermal layer folds to form an epithelial tube, which along with the mesenchyme, makes up the undivided anterior foregut. The oral cavity, which forms the future mouth, is the most rostral structure of the anterior foregut while the future stomach is the most caudal structure. In between these two structures the larynx, esophagus, trachea and lungs will develop, along with other budding organs.

At E10.5 the anterior-ventral region of the embryo consists of pharyngeal arches that are neural crest-derived mesenchymal pouches, which are covered by pharyngeal epithelium. Interactions between the mesenchymal NCC and pharyngeal epithelium, consisting of the oral ectoderm and foregut endoderm, are necessary for proper patterning and differentiation of pharyngeal arch derivatives (Couly et al. 2002). The first pharyngeal arch will develop into the mandible while lower arches will contribute to the larynx and otic structures. Caudal to the pharyngeal arches, the foregut endoderm will give rise to the esophagus and trachea. Depending on the structure, the foregut endoderm will differentiate into different types of epithelia.

1.1.2 Hedgehog signaling

Hedgehog signaling, one of the key developmental signaling pathways, is implicated in the development of structures throughout the embryo. Hedgehog
signaling occurs through three secreted ligands: sonic hedgehog, indian hedgehog and desert hedgehog. This dissertation will focus on sonic hedgehog (Shh) signaling as it is the relevant Hh ligand during mandible outgrowth and foregut compartmentalization.

*Shh* is a secreted protein (Figure 1) which signals through a transmembrane receptor, *Patched (Ptch)*. When *Shh* is not present *Ptch* inhibits another membrane bound protein, *Smoothened (Smo)* (Figure 1). *Shh* binding results in the activation of *Smo* by releasing *Ptch* inhibition. *Smo* then activates cytoplasmic Gli transcription factors, which in turn activate target genes in the nucleus (Figure 1). *Shh* is expressed in the anterior foregut epithelium prior to and during mandible and foregut development (Litingtung et al. 1998).
Figure 1: Shh Signaling

When no Shh ligand is present Patched (Ptch) inhibits Smoothend (Smo) resulting in no activation of the Gli transcription factors. However, when Shh is present it binds the Ptch receptor relieving inhibition on Smo resulting in activation of the Gli factors and thereby expression of target genes.
1.1.3 Canonical WNT Signaling

Similar to Hedgehog signaling, WNT signaling is an intracellular signaling pathway, and is involved in many developmental and patterning events. There are multiple Wnt ligands that signal through a transmembrane receptor, Frizzled (Fzd) (Figure 2). Additionally, there are many Fzd receptors, which can bind not only Wnt ligands but other ligands as well. When a Wnt ligand is not present, cytoplasmic β-catenin is bound by a destruction complex (Axin, GSK3 and APC), which results in the degradation of β-catenin. Activation of a Fzd receptor results in the stabilization of β-catenin, which then travels to the nucleus and through interaction with TCF regulates transcription of target genes (Figure 2). In addition to a signaling role, β-catenin is a structural component of epithelial adherens junctions. Therefore, the dual roles of β-catenin must be considered in evaluating any phenotypes caused by genetic disruptions of β-catenin. In the anterior foregut epithelium WNT signaling is active in the ventral domain (AM Goss et al. 2009).
Figure 2: WNT Signaling

Activation of the WNT signaling pathway occurs when a Wnt ligand binds one of the Frizzled receptors. When Frizzled is bound, β-catenin is freed from a destruction complex (APC, GSK3 and Axin) and allowed to travel to the nucleus. Once in the nucleus, β-catenin activates transcription of target genes through an interaction with TCF. When Wnt ligands are not present the destruction complex results in the degradation of β-catenin and therefore no transcriptional regulation of target genes by β-catenin.
1.2  **Signaling and morphogenesis during mandible development**

1.2.1  **Anatomy of the mammalian mandible**

The adult mandible is composed of many tissue types including muscle, cartilage, bone, and specialized epithelia. These tissue types are all necessary for a fully functional mandible. Mandible development begins with the migration of the cranial neural crest cells (CNCC) from the dorsal rhombomeres to the pharyngeal arches (Figure 3A) (Bronner-Fraser 1994). This migration event results in the population of the arches with neural crest-derived mesenchyme in addition to a small amount of mesodermal-derived mesenchyme that was present prior to migration (Figure 3A). Lineage tracing experiments have shown that the majority of the mandibular structures are neural crest derived (Chai et al. 2000; Jeong et al. 2004).

The developing first arch is composed of two principle parts, an upper maxillary bud and a lower mandibular bud. The maxillary bud develops into the palate and upper craniofacial structures, while the mandibular bud develops into some otic components, dentary bone, supporting cartilages, and muscles (Chai & Maxson Jr. 2006). Meckel’s cartilage, a key structure during the development of the mandible, is a scaffold for the ossification of the dentary bone (Radlanski & Renz 2006). The development of Meckel’s cartilage begins in PA1 around E11.5 as a cluster of ectomesenchyme (Figure 3B), also described as a pre-cartilage condensation (Chai & Maxson Jr. 2006; Chai et al. 2000).
Condensations rapidly develop and go through three stages: initiation, adhesion and differentiation (Hall & Miyake 2000). This condensation then elongates to form a complete Meckel’s cartilage rod, composed of both NCC and non-neural crest cells (Figure 3C) (Chai et al. 2000; Ito et al. 2002). While Meckel’s cartilage elongates, the dentary bone ossifies and surrounds the cartilage rod (Figure 3D,E). In addition to Meckel’s cartilage and the dentary bone, the mandible has three other proximal structures: the coronoid, condylar and angular cartilages (Figure 3E). These structures are important for the temporomandibular joint to function correctly. The posterior region of the mandibular arch develops into some of the otic components. Other important structures such as the tongue, larynx and the rest of the otic components develop from the second pharyngeal arch.
Figure 3: Mandible development occurs in multiple stages

A) The mandible develops from the lower region of the first pharyngeal arch, which is populated by neural crest cells shown in red. B-D) After neural crest migration, a cartilage condensation (blue) forms from neural crest cells and elongates to form Meckel’s cartilage. D-E) The dentary bone (shown in purple) ossifies around the elongating Meckel’s cartilage to form the complete mandible structure.
1.2.2 Etiologies of micrognathia

The majority of human mandible defects are characterized by a decrease in mandible size, called micrognathia or mandibular hypoplasia. There are multiple etiologies that are responsible for this type of defect: disruption in NCC migration, increased cell death, loss of proliferation and defective differentiation.

The majority of mesenchymal cells in the pharyngeal arches are derived from NCC, which migrate from the dorsal rhombomeres. This is a coordinated migration event involving an epithelial to mesenchymal transition (EMT) (Bronner-Fraser 1994). There are multiple stages that are necessary and disruption during them can result in decreased numbers of NCC populating the pharyngeal arches. First, if the EMT event is disrupted the NCC will not delaminate from the neural ectoderm and migrate. The delamination event is regulated by a multitude of signaling pathways that control the levels of cell adhesion molecules, such as E-cadherin and N-cadherin (Van de Putte et al. 2003; Nakagawa & Takeichi 1998). If cadherins are not downregulated in NCC, delamination from the neural tube and subsequent migration to the pharyngeal arches does not occur. After becoming migratory, the NCC navigate to the pharyngeal arches through distinct migratory streams.

Many micrognathia phenotypes occur after the pharyngeal arches are populated with NCC. First, if proliferation or survival of the NCC derived mesenchyme is
disrupted, a loss of mandible tissue occurs due to low numbers of cells. Many signaling pathways have been shown to be important in the survival of NCC including Shh (Brito et al. 2006), BMP (Tang et al. 2010), and FGF (Abu-Issa et al. 2002) signaling.

Lastly, proper mandible outgrowth depends on chondrogenesis of Meckel’s cartilage and ossification of the dentary bone. Once differentiation of the cartilage and bone has begun, elongation of these two structures occurs rapidly over a few days of embryonic development in the mouse. Commonly, hypoplasia occurs when the differentiation of Meckel’s cartilage is interrupted, impairing ossification of the dentary bone (Radlanski & Renz 2006). However, mandibular hypoplasia can also occur as a result of defective ossification independent of a cartilage defect (Barron et al. 2011).

The etiologies responsible for micrognathia are based on disruptions in basic cellular behaviors that are necessary for the development of most embryonic structures, making the mandible a good model for furthering our understanding of the complex regulation of many cellular processes. In addition, many human craniofacial syndromes result from defects in NCC development. These human syndromes are often linked to specific genes, which are also present in mice, allowing for reverse genetics studies regarding their mechanistic role in the development of micrognathia (Table 1).
Table 1: Human diseases with micrognathia

<table>
<thead>
<tr>
<th>Disease</th>
<th>Genetic Disruption in Human Cases</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cri du Chat</td>
<td>Sp- (small deletion on chromosome 5)</td>
<td>(Mainardi et al 2001)</td>
</tr>
<tr>
<td>Hallermann’s</td>
<td>FGFR2</td>
<td>(Galvin et al 1996)</td>
</tr>
<tr>
<td>Pierre Robin</td>
<td>SOX9</td>
<td>(Jakobsen et al 2007)</td>
</tr>
<tr>
<td>Progeria</td>
<td>LMNA</td>
<td>(Eriksson et al 2003)</td>
</tr>
<tr>
<td>Russell-Silver</td>
<td>Multifactorial</td>
<td>(Gicquel et al 2005)</td>
</tr>
<tr>
<td>Treacher-Collins</td>
<td>TCOF1</td>
<td>(Dauwerse et al 2010)</td>
</tr>
</tbody>
</table>
1.3 **Signaling and morphogenesis during foregut development**

1.3.1 **Anatomy of the mammalian foregut**

The anterior foregut tube is initially derived from the embryonic endoderm, an epithelial sheet that folds ventrally to form a single endodermal tube around embryonic day 8 (E8.0) in the mouse. This tube is sandwiched between the neural tube (dorsal) and the developing heart loop (ventral). Once the tube is formed, the most dorsal cells resolve from the endodermal tube to establish the notochord (Tremblay & Zaret 2005; Franklin et al. 2008; Aoto et al. 2009). The process of notochord resolution in mouse, which occurs between E8.25 and E9.5 (days of embryonic development since fertilization), has been described in detail (Jurand 1974). During development, the notochord acts as a signaling center that secretes signals important for the proper patterning of the foregut, neural tube and surrounding tissues (Chamberlain et al. 2008).

After notochord resolution the single common foregut tube compartmentalizes into the trachea and esophagus by an undefined set of cellular behaviors. The compartmentalization process begins around E9.5 with the formation of lung buds from the ventral foregut endoderm (FGE) at the level of the 6th pharyngeal arch (Cardoso & Lü 2006). Starting at the site of lung bud formation, the common foregut primordium will then separate into two tubes, the esophagus on the dorsal side and the trachea on
the ventral side (Fig1 A-C) (Zaw-Tun 1982; O’Rahilly & Muller 1984).

Compartmentalization appears to occur at the dorso-vental midpoint of the lateral walls of the common foregut tube (Figure 4B”). For the purpose of this thesis we refer to the nexus of the dorsal and ventral regions of the lateral wall as the dorso-ventral midline (Figure 4B’). The compartmentalized region forms in a caudal to rostral direction extending to the future larynx. Once the esophageal and tracheal tubes are established, they differentiate into structurally and functionally distinct organs. By the time of birth, the esophageal epithelium consists of stratified squamous cells and the esophageal mesenchyme develops into concentric rings of smooth muscle (Yu et al. 2005), aiding in the passage of ingested materials to the stomach. On the other hand, the tracheal epithelium differentiates into a pseudostratified epithelium, and the mesenchyme develops C-shaped cartilage rings ventrally and the trachealis muscle dorsally (Perl et al. 2002; McAteer 1984), providing a conduit for air exchange with the lungs. Without proper patterning and differentiation, the esophagus and trachea will not function correctly upon birth. Much work has focused on the differentiation of the respiratory and digestive systems after compartmentalization but many questions remain as to how separation occurs.
Figure 4: The foregut compartmentalizes between E10 and E11.5

A-C) The anterior foregut compartmentalizes into the esophagus (marked in pink) and trachea (marked in green) between embryonic day 10.0 (28 somites) and E11.5. Prior to compartmentalization the foregut tube is a single cell layer thick, shown in panel A’. During compartmentalization the ventral foregut endoderm becomes pseudostratified (panel B’). The area we refer to as the dorso-ventral midline (ie. the lateral midpoint of the foregut wall) is marked with a square (panel B’). The endoderm then comes together at the dorso-ventral midline (panel B”) prior to resolving into two separate tubes (panel C’). It is important to remember that this process is occurring in three dimensions and that cellular rearrangements are occurring throughout the endoderm.
1.3.2 Models of tracheoesophageal compartmentalization and lung budding

Three different models have been proposed to explain the division of the common foregut tube into the esophagus and trachea: 1) the outgrowth model (Zaw-Tun 1982; O’Rahilly & Muller 1984), 2) the watershed model (Sasaki et al. 2001) and 3) the septation model (Figure 5) (Qi & Beasley 2000; Sutliff & Hutchins 1994) (Figure 5).

The outgrowth model hypothesizes that the trachea grows out of the common foregut tube like an elongating bud (Figure 5, see arrow), while the common foregut tube per se differentiates into the esophagus (Zaw-Tun 1982; O’Rahilly & Muller 1984). This model is not supported by experimental results reported in the literature. For example, increased levels of proliferation specifically in the region of the ventral primordium at the point where compartmentalization begins are necessary correlates of this mechanism, but have not been detected (Ioannides et al. 2010). Additionally, this explanation is unlikely because the single undivided foregut tube contains two domains which express either respiratory or esophageal markers (Figure 4A) (Aubin et al. 1997).

Alternatively, the watershed model suggests that both the trachea and esophagus are elongating while separated by a mesenchymal septum that blocks elongation of the dorso-ventral midline of the lateral wall (Figure 5) (Sasaki et al. 2001a). The watershed model proposes that the mesenchyme and epithelium are both active in separating the common foregut tube. This requires the existence of increased proliferation in the dorsal
and ventral regions compared to the midline of the lateral walls (Figure 5, see arrows).

As noted previously, increased levels of proliferation in these regions have not been reported. Additionally, this model depends on the presence of a mesenchymal septum. It is possible that a mesenchymal septum does exist and moves rostrally as the compartmentalization event occurs. However, this hypothesis remains to be tested.

Both the watershed model and the outgrowth model suggest that the common undivided foregut tube does not change in length over the course of compartmentalization (Figure 5). To test this hypothesis, Ioannadies, Copp and colleagues (Ioannides et al. 2010) measured the length of the common foregut tube before and after compartmentalization. They found that, counter to predictions from these models, the common foregut tube actually decreases in length (Figure 5). This evidence suggests that the outgrowth and watershed models are incorrect, because these potential mechanisms of compartmentalization would result in no change in the length of the uncompartmentalized foregut tube.

Finally, the septation model, is based on the concept that “lateral edges” (Figure 4B”) occur at the dorso-ventral midline of the foregut tube (Figure 5) (Keith & Spicer 1906; Qi & Beasley 2000). These ridges are thought to be regions of the epithelium that thicken at the dorso-ventral midline, make contact across the lumen and fuse. The point of contact then moves rostrally to separate the common foregut tube into the esophagus
and trachea. In this model, the epithelium actively fuses to separate the common foregut tube into two tubes (Figure 4C’, Figure 5). The septation model has been widely accepted in the field as the model most consistent with experimental evidence (Qi & Beasley 2000). However, based on three-dimensional reconstructions of wild type mouse foreguts and scanning electron microscopy of wild type chicken foreguts, there is little evidence that overt lateral ridges or a septum exist (Sasaki et al. 2001a; Metzger et al. 2011). Interestingly, Metzger et al. described the region of the epithelium where the trachea and esophagus are separating as a “saddle”. While there is no evidence of distinct lateral ridges or an epithelium septum, it is possible that there is an epithelial “saddle” which develops when the lung buds form and moves rostrally to separate the two tubes. This type of separation event would require apical constriction and collective migration of epithelial cells through the “saddle” region as it moves.

Without live imaging of the active compartmentalization event, it is unlikely that any model will be proven correct. The morphogenetic mechanism likely involves a combination of mesenchymal and epithelial cell movements and rearrangements that occur at the D/V midline, separating the common primordium into two tubes. Until there is a much deeper understanding of the cellular behaviors occurring during compartmentalization, the exact mechanism underlying foregut compartmentalization will remain a mystery.
Figure 5: Models of foregut compartmentalization

Prior to compartmentalization the E10.0 undivided foregut has a dorsal (pink) and ventral (green) domain. There are three main models of foregut compartmentalization: Outgrowth model, Watershed model and the Septation model. The compartmentalization event starts at the level of the lung buds (marked with asterisk). The future location of this level of tissue is marked with asterisks in the models showing compartmentalization occurring. In the outgrowth and watershed models, arrows showing the direction of growth mark regions of outgrowth/proliferation. In the septation model an arrow demonstrates where the septation event is moving rostrally along the undivided foregut tube.
1.3.3 Genes and pathways linked to human EA/TEF

Defects in the development of the respiratory and digestive systems are especially detrimental to survival. Consequently, the mortality rate of infants born with foregut abnormalities was 100% until the 1940s (Choudhury et al. 1999). However, with new surgical interventions the mortality rate is currently below 10%. In humans a range of foregut phenotypes can occur depending on the region of the foregut that is affected. Most common is the presence of an esophageal atresia (EA) with or without a tracheoesophageal fistula (TEF). EA/TEF is characterized by an esophagus ending in a blind pouch and a fistula connecting the stomach to the trachea, usually around the level of the lungs. EA’s occur in about 1 in 3,500 live births and are classified into four types, labeled A-D (Torfs et al. 1995; Gross 1957). The most common of these is Type C (Figure 6B), which is the presence of both an EA and a TEF. These EA/TEFs can be corrected surgically, however, treatment requires multiple surgeries and typically results in a diminished quality of life for the patient (Kovesi & Rubin 2004).

A better understanding of the underlying causes of foregut defects may enable development of earlier and better treatments. To complicate matters, 48% of the time human foregut defects occur along with a host of other developmental abnormalities, making it difficult to determine the underlying cause of the phenotype (Holder et al. 1964). The most common of these are congenital heart defects and defects in other
endodermal organ systems. For example, often patients diagnosed with VACTER/VACTERL syndrome, will have EA/TEFs along with either vertebral, anal, cardiac or renal defects. Many of these defects result from improper morphogenesis of an endodermal tissue, suggesting that there is a common developmental mechanism between the organ systems. VACTER/VACTERL syndrome is tentatively linked to changes in multiple genes including \textit{HOXD13, ZIC3, PTEN} and \textit{FOX} genes (Shaw-Smith 2010). However, any role of these genes in the development of EA/TEF has yet to be determined. Studying common features of this group of defects instead of focusing on one organ system at a time may lead to more information about the development of EA/TEF and VACTER/VACTERL syndrome.

Interestingly, several patients with EA/TEF have deletions spanning the chromosomal region which contains \textit{NOGGIN} (Puusepp et al. 2009). This finding in conjunction with \textit{Noggin} mouse mutant studies suggested that mutations in \textit{NOGGIN} could cause human foregut defects. However, when the coding region of \textit{NOGGIN} was examined for point mutations in 50 patients with EA/TEF, only one patient had a mutation within the coding region, and this resulted in a predicted conservative amino acid change (Murphy et al. 2012). Such data suggest that mutations in the coding region of \textit{NOGGIN} are not major factors in the molecular etiology of human EA/TEF. To
determine if NOGGIN is involved human EA/TEF, the regulatory region of the gene will have to be examined further.

Mutations in other genes, including MYCN (van Bokhoven et al. 2005) and SOX2 (Williamson et al. 2006), are linked to the development of EA/TEF in human patients independently of VACTER/VATERL defects. While the links between these genes and patient pathology are not well studied or understood, Sox2 mutations in mice suggest that the loss of Sox2 causes foregut defects by disrupting dorsal patterning (Que et al. 2007). Using experimental mouse models to further investigate the roles of these genes may help us gain a better understanding of the morphogenic mechanisms underlying this spectrum of defects.
Figure 6: Disruptions in foregut compartmentalization can result in a variety of defects of the esophagus and trachea

A) The foregut normally separates into an esophagus (in pink) and a trachea with lungs budding off (in green). B) Esophageal atresia with tracheoesophageal fistula (EA/TEF) involves the esophagus ending in a blind pouch, and an open passage (fistula) connecting the trachea to the stomach. The fistula often contains both esophageal and tracheal characteristics (marked in yellow). This type of defect is found in several genetic mouse models and a pharmacological model (Adriamycin). While Shh mutants do have EA/TEF, their phenotype is often more complex and may be caused by different underlying mechanisms. C) Another common foregut defect is tracheal agenesis, in which the ventral foregut does not form a trachea but differentiates into an esophagus instead. This defect appears to be caused by a loss of ventral patterning, marked by Nkx2-1 expression. D) A more severe form of tracheal agenesis can occur when in addition to a loss of ventral fate, lung agenesis occurs as well. This defect has been found in mouse mutants where ventral WNT signaling has been completely removed. E) A final potential class of foregut defect is when compartmentalization appears to have stalled and the single primordium does not separate into two tubes. In these mutants dorso-ventral patterning remains intact but separation does not occur. While Ephrin-B2 and RAR mutants seem likely to have normal dorso-ventral patterning, the expression of Sox2 and Nkx2-1 have not been examined in these models.
1.3.4 Murine models of anterior foregut defects

Currently, our knowledge of foregut compartmentalization comes primarily from genetic manipulation in mouse targeted gene mutation models (Table 2). These mutants have been used to probe the molecular genetics and morphogenic regulation of foregut compartmentalization, as well as the later differentiation of the tracheal and esophageal tubes. These genetic mouse models have also helped elucidate the underlying causes of murine foregut phenotypes (Table 2), and therefore are helpful in thinking about the pathogenesis of the corresponding human malformations. The two main etiologies that have been shown to result in foregut defects are notochord resolution defects (Qi & Beasley 1999; Possoegel et al. 1999; Gillick et al. 2003; Mortell et al. 2004; Fausett et al. 2014) and dorsal-ventral patterning (D/V patterning) defects (Minoo et al. 1999; Que et al. 2007).
Table 2: Table of genetic mouse models with foregut defects and the causative mechanism

<table>
<thead>
<tr>
<th>Mouse Gene</th>
<th>Malformation</th>
<th>D/V patterning</th>
<th>Mechanism</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nkx2.1⁻/⁻</td>
<td>TEF, small lungs</td>
<td>Loss of ventral Nkx2.1</td>
<td>Loss of D/V</td>
</tr>
<tr>
<td>Sox2²/GFP/cond</td>
<td>EA/TEF</td>
<td>Moderate loss of sox2</td>
<td>Loss of D/V</td>
</tr>
<tr>
<td>BMP4⁻/⁻</td>
<td>TEF; tracheal agenesis</td>
<td>Loss of ventral Nkx2.1</td>
<td>Loss of D/V</td>
</tr>
<tr>
<td>β-catenin²/⁻</td>
<td>TEF; tracheal agenesis</td>
<td>Loss of ventral Nkx2.1</td>
<td>Loss of D/V</td>
</tr>
<tr>
<td>Wnt2/2b⁻/⁻</td>
<td>TEF; tracheal agenesis</td>
<td>Loss of ventral Nkx2.1</td>
<td>Loss of D/V</td>
</tr>
<tr>
<td>Noggin⁻/⁻</td>
<td>EA/TEF</td>
<td>established</td>
<td>Notochord resolution</td>
</tr>
<tr>
<td>Adriamycin Treated</td>
<td>EA/TEF</td>
<td>established</td>
<td>Notochord resolution</td>
</tr>
<tr>
<td>Shh⁻/⁻</td>
<td>EA/TEF</td>
<td>established</td>
<td>Unknown</td>
</tr>
<tr>
<td>Gli2⁻/⁻;Gli3⁺/⁺</td>
<td>No E,T or lungs</td>
<td>unknown</td>
<td>Unknown</td>
</tr>
<tr>
<td>EphrinB2²/²;²/±</td>
<td>TEF</td>
<td>unknown</td>
<td>Unknown</td>
</tr>
<tr>
<td>Foxf1⁻/⁻</td>
<td>TEF, narrow E, small lungs</td>
<td>unknown</td>
<td>Unknown</td>
</tr>
<tr>
<td>β-catenin³/⁻</td>
<td>TEF</td>
<td>present</td>
<td>Unknown</td>
</tr>
<tr>
<td>RAR mutants</td>
<td>TEF; tracheal agenesis</td>
<td>unknown</td>
<td>Unknown</td>
</tr>
<tr>
<td>Barx1⁻/⁻</td>
<td>TEF</td>
<td>established</td>
<td>Unknown</td>
</tr>
</tbody>
</table>
1.3.4.1 Improper notochord resolution can cause EA/TEF

When defects in notochord resolution occur, the foregut typically displays EA/TEF (Figure 6B). This phenotype is thought to result from excess dorsal foregut endodermal tissue remaining attached to the notochord during resolution (Li et al. 2007; Fausett et al. 2014). The improper resolution event leaves insufficient dorsal foregut endoderm (dFGE) behind to form the esophagus during compartmentalization. EA/TEF has been found in mutant mice lacking Noggin, a BMP signaling inhibitor (Fausett et al. 2014; Li et al. 2007). The phenotype found in Noggin−/− mice closely resembles Type C human EA/TEF (Fausett et al. 2014; Li et al. 2007). Noggin mutants have notochord abnormalities caused by defective notochord resolution prior to foregut compartmentalization.

Another model of EA/TEF is the Adriamycin rodent model. Adriamycin is an anthracycline antibiotic and chemotherapeutic. VACTERL-like phenotypes occur in a fraction of offspring when Adriamycin is injected into pregnant wild-type mice and rats, as long as the injection occurs prior to foregut compartmentalization (Figure 6B, Figure 7) (Ioannides et al. 2010; Qi & Beasley 1999). This system has been extensively studied as a model for VACTERL defects. A recent study suggests that the notochord defects detected in embryos from Adriamycin treated dams were due to a delayed down-regulation of adhesion markers during notochord resolution, resulting in a loss of dorsal
foregut endoderm (Hajduk et al. 2012). This defect in notochord resolution closely mirrors the defect found in Noggin mutants with EA/TEF (Fausett et al. 2014).
Figure 7: Adriamycin treatment of wildtype mice can result in EA

ICR mice injected with Saline daily from E6.5-E9.5 develop a normal esophagus and trachea by E15.5 (A). However, daily injection of Adriamycin from E6.5-E9.5 results in EA/TEF in about 50% of embryos. Only the loss of the esophagus, not the tracheo-esophageal fistula, is shown in panel B. Scale bar= 2mm.
1.3.4.2 Proper dorso-ventral patterning is necessary for compartmentalization

The second type of defect that leads to a failure of compartmentalization is loss of either dorsal or ventral patterning. The result is esophageal agenesis (Figure 6C), where the esophagus does not form when dorsal patterning is lost, or tracheal agenesis, where trachea does not form when ventral patterning is lost. This defect is hypothesized to be caused by a lack of differentiation of either the esophagus or trachea (Que et al. 2007; Minoo et al. 1999). Multiple mouse models (Figure 6C-D) exhibit this type of defect and we will discuss them in more detail below, in the section on dorso-ventral patterning.

1.3.5 Dorso-ventral patterning during foregut compartmentalization

A significant body of research exists on the necessity of dorso-ventral (D/V) patterning within the foregut endoderm. The dorsal foregut endoderm (dFGE) is marked by expression of the HMG-domain transcription regulator Sox2 (Que et al. 2007), whereas the ventral FGE is marked by Nkx2-1 (Minoo et al. 1999), another transcription regulator (Figure 8). Data suggest that both of these factors must be expressed in their proper domains for compartmentalization to occur (Que et al. 2007; Minoo et al. 1999).

In the ventral domain, Nkx2-1 regulates multiple respiratory specific genes and is necessary for differentiation of trachea and lung specific cell types (Bohinski et al. 1994; Ray et al. 1996). In Nkx2-1 null mice, the foregut endoderm is dorsalized and Sox2 is
expressed throughout the entire foregut tube (Figure 6C) (Minoo et al. 1999). $Nkx2-1$ foreguts do not compartmentalize and resemble a human defect called tracheal agenesis, where the differentiated foregut is mainly esophageal in character with circumferential smooth muscle rings surrounding the foregut tube. In tracheal agenesis the foregut is a single tube that connects the oral cavity with the lungs and stomach.

While $Nkx2-1$ expression is necessary to establish the ventral foregut domain, $Sox2$ is necessary to establish the dorsal foregut domain. Until recently, little was known about the role of $Sox2$ in foregut compartmentalization because a complete deletion of $Sox2$ in mice results in death prior to gastrulation. However, a hypomorphic allele of $Sox2$ shows that a reduction in $Sox2$ levels does result in an EA/TEF phenotype 60% of the time (Figure 6B) (Que et al. 2007). Furthermore, conditional removal of $Sox2$ in the ventral endoderm results in EA/TEF in 10% of embryos and a shortened trachea in 60% of embryos (Que et al. 2007). While both $Nkx2-1$ and $Sox2$ are required for the development of an esophagus and trachea, it is unknown if they play a role in the compartmentalization event itself or if they are simply necessary to pattern the tissue domains to set up the cellular machinery of compartmentalization.
Figure 8: A signaling network of specific genes is necessary to establish proper *Nkx2-1* and *Sox2* expression, leading in turn to either respiratory fate or esophageal fate.

The expression of *Sox2* and *Nkx2-1* are regulated by a complex set of mesenchymal (blue) and endodermal (dorsal marked by pink and ventral marked by green) signaling events involving BMP, Eph-ephrin, FGF and WNT signaling. When any steps in this pathway are disrupted, a loss of dorso-ventral patterning can occur, leading to foregut compartmentalization defects.
1.3.5.1 Regulation of dorso-ventral patterning

Multiple signaling pathways are responsible for the proper localization of $Nkx2-1$ and $Sox2$ within the foregut endoderm (Figure 8). Both $Nkx2-1$ and $Sox2$ must be properly localized for proper foregut development, so understanding the signaling network that regulates them could shed light on the early stages of compartmentalization.

The ventral establishment of $Nkx2-1$ depends on both BMP and WNT signaling from the surrounding mesenchyme and vFGE (Figure 8) (Goss et al. 2009; Harris-Johnson et al. 2009; Domyan et al. 2011; Li et al. 2008). A ligand for the BMP pathway, $BMP4$, is present in the ventral mesenchyme and signals via BMP receptors 1A and 1B ($Bmpr1a;b$), present in the ventral endoderm (Figure 8) (Domyan & Sun 2011; Rodriguez et al. 2010; Li et al. 2008). In the absence of mesenchymal $BMP4$ or endodermal $Bmpr1a;b$, $Nkx2-1$ protein expression is lost and tracheal agenesis occurs (Li et al. 2008; Domyan et al. 2011). When ventral $Nkx2-1$ expression is lost, dorsal $Sox2$ expression expands into the ventral domain resulting in an esophageal fate (Figure 6C). Interestingly, in mice with $Bmpr1a;b$ conditionally removed from the ventral endoderm, a reduction in $Sox2$ can rescue both $Nkx2-1$ expression and the tracheal agenesis phenotype (Domyan et al. 2011). These data suggest that BMP signaling is necessary for repressing ventral $Sox2$ expression, not for inducing ventral $Nkx2-1$ expression (Figure 8).
Defining the role of BMP signaling in the foregut is complicated by the fact that secreted BMP agonists are present in and around the foregut endoderm. *Noggin*, a secreted BMP agonist, is expressed in the dorsal foregut and the notochord (Figure 8), suppressing BMP signaling in the dorsal foregut region (Stottmann et al. 2001); reducing the genetic dosage of *BMP4* (by half) (Que et al. 2007) or of *BMP7* (entirely) (Li et al. 2007) rescues the *Noggin* mutant compartmentalization defect completely. Repression of dorsal BMP signaling by *Noggin* allows *Sox2* to be expressed in the dorsal region (Figure 8). In *Noggin*<sup>−/−</sup> mice, the foregut has normal dorso-ventral patterning, suggesting that inhibition of BMP signaling is not necessary for establishment or maintenance of D/V patterning (Fausett et al. 2014). Instead, research suggests that *Noggin* is necessary for proper resolution of the notochord from the foregut endoderm prior to compartmentalization (Li et al. 2007; Fausett et al. 2014).

While BMP signaling is important in repressing ventral *Sox2*, WNT signaling is necessary for establishing ventral *Nkx2-1* (Figure 8). The WNT ligands important in foregut compartmentalization are *Wnt2* and *Wnt2b*, which are both found in the ventral foregut mesenchyme (Figure 8) (Goss et al. 2009). When both *Wnt2/2b* are removed from the ventral mesenchyme, endodermal *Nkx2-1* is not established and respiratory agenesis occurs (Figure 6D) (Goss et al. 2009). In addition, when β-*Catenin* (also known as *Ctnnb1*), encoding the downstream target of canonical WNT signaling, is conditionally
removed from the foregut endoderm, the result is a phenocopy of the Wnt2/2b mutant mouse (Figure 6C) (Harris-Johnson et al. 2009). WNT signaling is also sufficient to establish Nkx2-1 expression in certain contexts. Conditional activation of β-Catenin in the ventral foregut and anterior stomach endoderm results in ectopic Nkx2-1 expression in the stomach (Figure 6E) (Harris-Johnson et al. 2009; Goss et al. 2009). However, Domyan and colleagues (Domyan et al. 2011) activated β-Catenin in the endoderm of mice without endodermal Bmpr1a;b and found that activated β-Catenin is not sufficient to rescue Nkx2-1 expression when BMP signaling is not present (Figure 8). Therefore, BMP signaling is not acting downstream of β-Catenin in the ventral foregut. Furthermore, when β-Catenin was conditionally removed from the ventral foregut endoderm there was no change in BMP signaling levels (Domyan et al. 2011). These two sets of data support a model in which BMP signaling is functioning independently of WNT signaling in establishing ventral foregut identity (Figure 8).

While the establishment of the ventral endoderm identity has been well studied, it is less clear how Sox2 expression in the dorsal FGE is established and regulated. It appears that Sox2 is the default state of the early foregut endoderm, with all cells expressing Sox2 at E8.5. Although Sox2 seems to be turned on throughout the epithelium by default, multiple pathways repress Sox2 in the ventral FGE (Figure 8). This repression results in a gradient of Sox2 expression with Sox2-Hi cells located in the dorsal FGE and
Sox2-Lo cells found in part of the ventral FGE. Sox2 repression and Nkx2-1 activation, results in the establishment of a very clear Sox2-Nkx2-1 pattern by E9.5. As was previously discussed, active endodermal BMP signaling represses Sox2 in the ventral FGE (Domyan et al. 2011).

In addition to BMP signaling, fibroblast growth factor (FGF) signaling is active in the ventral foregut mesenchyme. For example, the FGF ligand Fgf10 is present in the ventral foregut mesenchyme (Figure 8) (Min et al. 1998). When it is genetically ablated, the foregut separates normally, but lung development is impaired (Min et al. 1998). Even though loss of Fgf10 does not result in compartmentalization defects, in foreguts cultured ex vivo, FGF10 is able to inhibit Sox2 expression (Que et al. 2007). This suggests that Fgf10 may play a role in repressing ventral Sox2 expression.

1.3.6 Cellular behaviors during compartmentalization

1.3.6.1 Programmed cell death

Programmed cell death has long been thought to be necessary for foregut compartmentalization. Excess cell death is present at the point of compartmentalization, and reduced levels of cell death occur in Adriamycin treated mice with EA/TEF (Orford et al. 2001; Qi & Beasley 2000; Williams et al. 2000; Zhou et al. 1999; Ioannides et al. 2010). To determine the exact role of cell death during compartmentalization, Ioannides
et al. 2010 (Ioannides et al. 2010) quantified the percentage of dying cells present during the process of foregut compartmentalization. In fact, there is more cell death present (up to 35% of epithelial cells undergoing PCD) at the D/V boundary than the dorsal or ventral domains of the foregut in the common foregut tube at the point of compartmentalization (Ioannides et al. 2010). However, the foregut develops normally in Apaf1−/− mice, which lack programmed cell death (Ioannides et al. 2010). If the foregut epithelium were undergoing a fusion event, as suggested by the septation model, it would be expected that excess cell death would be occurring at the point of fusion. The cells would need to be eliminated, but would not be required for the dorso-ventral midline fusion event itself. This suggests that the excess apoptosis occurring at the point of compartmentalization is likely a byproduct of the separation event, and is not necessary for compartmentalization to occur.

1.3.6.2 Proliferation

Both the outgrowth model and the watershed model hypothesize that the foregut epithelium is growing rapidly to form the esophagus and trachea (O’Rahilly & Muller 1984; Zaw-Tun 1982). Higher levels of proliferation found specifically in the growing trachea or esophagus would support the outgrowth and/or watershed models, depending on the localization of the dividing cells. However, no research has shown a presence of excess proliferation in the dorsal or ventral regions of the epithelium, or at
the D/V midline (Ioannides et al. 2010). While many organs do bud and grow out from the foregut endoderm during development (Slack 1995; Fagman et al. 2006; Tremblay 2011), this does not seem to be the case with the development of the trachea and esophagus.

1.4 Cre-LoxP technology

The work presented in Chapters 2 and 3 relies heavily on the use of tissue-specific genetic manipulations to determine the roles of Shh and β-Catenin during mandible and foregut development. In order to perform these genetic manipulations a Cre-LoxP approach was utilized to specifically disrupt signaling in and from the anterior foregut endoderm.

Cre-Lox recombination is based on the system discovered in P1 bacteriophage (Sauer & Henderson 1988; Sternberg & Hamilton 1981). The Cre-Lox system is dependent on two main components: Cre recombinase and LoxP sites. In mice this is accomplished by having strain of mice with a Cre-recombinase expressed under a tissue-specific promoter and a second strain with a gene of interest flanked by LoxP sites in the same orientation under an endogenous promoter (Figure 9). When a target gene is flanked by LoxP sites it is referred to as a floxed allele. Upon crossing a Cre strain with a floxed strain, cells that have the Cre-recombinase will mediate removal of the region
between the two LoxP sites. The location of the LoxP sites within the target gene will determine which portion of the gene is removed (Figure 9). This usually results in a loss of function of the target gene, but strategic placement of the LoxP sites can also result in a gain of function allele if a critical regulatory region is removed. The implementation of Cre-LoxP technology in mice revolutionized mouse genetic studies. Prior to the advent of Cre-LoxP technology it was difficult to target distinct expression domains in mice and determine their necessity.

While Cre-LoxP technology has helped make significant advances in our understanding of many genetic pathways in mice it does have some caveats. Often the promoters driving expression of Cre-recombinase can be “leaky” or not specific to the tissue where it is supposed to be driving expression. This can result in the deletion of target genes ectopically, which confounds the results when this occurs. Alternatively, recombination of the target gene can be incomplete allowing partial expression of the target gene when it should be completely removed. To determine that the system is working appropriately, any new Cre expressing line must be crossed to a reporter line, such as R26R\textsuperscript{lacZ}, to determine that Cre expression and recombination is complete in the correct tissue.
Figure 9: Cre-LoxP Technology

To ablate a gene in a specific tissue, two components are necessary: Cre recombinase and a floxed allele. Cre recombinase is expressed under a tissue-specific promoter limiting its expression to the tissue of interest. The floxed allele, present throughout the mouse, is composed of two loxP sites flanking the target gene. When these two components are present in the same cell, the Cre recombinase cuts at the loxP sites removing the target gene. The rest of the cells, which do not have Cre expression, do not have excision of the target gene and therefore have normal expression of the target gene.
2. Sonic Hedgehog from pharyngeal arch 1 epithelium is necessary for early mandibular arch cell survival and later cartilage condensation differentiation

Work presented in this chapter is adapted from the following publication:

Billmyre, K.K., Klingensmith, J., 2015. Sonic hedgehog from pharyngeal arch 1 epithelium is necessary for early mandibular arch cell survival and later cartilage condensation differentiation. Dev. Dyn. doi:10.1002/dvdy.24256

2.1 Introduction

Neural crest cells (NCC) are a set of migratory, multipotent cells that originate in the dorsal neural folds (Gammill & Bronner-Fraser 2003) and are capable of producing a multitude of differentiated tissue types, including bone, cartilage, neurons and connective tissue (Schneider & Helms 2003; Santagati & Rijli 2003). With the exception of some posterior bones derived from mesoderm, the majority of the craniofacial skeleton, is derived from NCC (Jeong et al. 2004). In mammals, patterning and morphogenesis of the mandible, a major craniofacial structure, begins with the migration of cranial NCC (NCC) into pharyngeal arch one (PA1); in the mouse, this migration starts at embryonic day 8.0 (E8.0) and finishes by E9.5. After migration, the NCC populate a portion of the pharyngeal arch mesenchyme, the ectomesenchyme, which is distinct from the mesodermal mesenchyme. Further development of the ectomesenchyme depends on multiple signals from non-neural crest components, the overlying epithelium and
mesodermal mesenchyme (Ferguson et al. 2000; Trainor et al. 2002; Trainor & Krumlauf 2000; Couly et al. 2002; Hu et al. 2003).

Deficient development of the mandible results in a common birth defect, micrognathia, where the lower jaw is truncated. Micrognathia often occurs in conjunction with human cases of holoprosencephaly (HPE), which is a loss of rostroventral midline patterning and a truncation of craniofacial structures. One gene that has been linked to human HPE is Sonic Hedgehog (\textit{SHH}) (Roessler et al. 1996). In the mouse, \textit{Shh} is expressed in three epithelia near the developing pharyngeal arch: the pharyngeal endoderm, the oral ectoderm and the ventral forebrain neuroepithelium (Jeong et al. 2004). The genetic ablation of all \textit{Shh} sources in mouse leads to severe holoprosencephaly, including a truncation of the lower jaw (Chiang et al. 1996).

Unfortunately, the severity of these early craniofacial defects in the \textit{Shh\textsuperscript{-/-}} mutant mouse model has complicated direct investigation into later roles of \textit{Shh} in murine mandible development. Chick has proven an amenable model to investigate the role of \textit{Shh} in mandibular development through gain and loss of function experiments, which have suggested that epithelial \textit{Shh} is necessary and sufficient for the survival and proliferation of the ectomesenchyme within PA1 (Hu & Helms 1999; Ahlgren & Bronner-Fraser 1999). More specifically, removal of the chick pharyngeal endoderm resulted in a loss of mandibular outgrowth that could be rescued by application of SHH.
protein (Couly et al. 2002; Haworth et al. 2007; Brito et al. 2006). Furthermore, addition of SHH to PA1 induced the development of ectopic Meckel’s cartilage (Brito et al. 2008).

While the work in chick supports a role for Shh from the pharyngeal endoderm in mandible outgrowth, much of this work has been done with the addition of external sources of SHH. The transient and indirect nature of such experiments limits the ability to interpret the direct effect of endogenous epithelial Shh on PA1 development. This is further obscured by the expression of Shh at the same time period from other nearby sources, including the notochord and floorplate.

An important insight into the roles of Shh signaling stemmed from the ablation of the Shh receptor specifically in NCC. Jeong and colleagues (2004) used a NCC-specific Cre, Wnt1Cre, (Danielian et al. 1998) to ablate Smoothened (Smo), an obligate receptor for hedgehog family ligands, and thereby inhibit all hedgehog (Hh) signaling in the NCC. The resulting Wnt1cre;Smo–/– embryos develop a severely hypoplastic mandible along with other craniofacial and laryngeal defects (Jeong et al. 2004). However, there are multiple hedgehog signaling ligands, including desert, indian and sonic hedgehog, which can all signal through Smo. In mouse, the deletion of either Ihh (St-Jacques et al. 1999) or Shh (Chiang et al. 1996) results in craniofacial defects. However, Ihh is not present in the mandibular arch until E12.5, which is after the initial growth of the mandible. Therefore the defects resulting from the loss of Hh signaling in NCC are likely
due to signaling by *Shh*. This result supports previous chick research showing the *Shh* is necessary for lower jaw development, but none of this work addresses which specific domain(s) of *Shh* is necessary in the mouse.

Based on previous experiments disrupting Hh signaling in the chick and mouse, a major role for *Shh* in mandible development may be to promote early survival of pharyngeal NCC (Jeong et al. 2004; Brito et al. 2006; Couly et al. 2002; Haworth et al. 2007; Benouaiche et al. 2008). An alternate mechanism is that endogenous *Shh* regulates NCC development to result in cartilage growth and differentiation. In *Shh*-/- mice, Meckel’s cartilage condensations were reportedly seen in the mandible region at E13.5 (Melnick et al. 2005). However, these condensations did not appear to grow and differentiate in the mutants (Melnick et al. 2005). When wild type Meckel’s cartilage was cultured *ex vivo* and treated with cyclopamine, a SHH antagonist, chondrocyte differentiation was lost (Melnick et al. 2005). In addition, SHH has been shown to be important for the chondrogenesis of cranial NCC derivatives in zebrafish (Wada et al. 2005a). Together these studies suggests that SHH is indeed important for mandibular cartilage differentiation, but the mechanism remains unknown (Chai & Maxson Jr. 2006).

In sum, though it has been established that *Shh* is required for PA1 development, the cellular role(s) of *Shh* and the endogenous source(s) of such SHH in mandibular
development remain unclear. In this study, we use a genetic ablation approach in mouse to elucidate the domain of Shh expression that is necessary for mammalian mandible development, and then to analyze its cellular role. Our results establish a dual role for Shh during mandibular development: Shh secreted from the pharyngeal epithelia is necessary in the mouse for early PA1 mesenchymal cell survival, and also for later differentiation of cartilage condensations in the developing mandibular arch.

2.2 Methods

2.2.1 Mice

Nkx2.5Cre;Shh+/– males (Moses et al. 2001) were mated with Shh+/– females to generate Nkx2.5Cre;Shhfx/– embryos. All mice were maintained on an ICR outcrossed background. Embryos were genotyped for Cre (Meyers et al. 1998) and for the presence of Shh. Embryonic day (E) is defined as E0.5 on day of vaginal plug. Wnt1Cre;Smo+/– mice were generated using Wnt1Cre;Smo+/– males (Danielian et al. 1998; Zhang et al. 2001) crossed with Smoflox/flox females (Long et al. 2001). All mouse work was in accordance with protocols approved by the Duke University Animal Care and Use Committee.
2.2.2 Dissections, processing and histology

Embryos were dissected in phosphate-buffered saline (PBS). Fixation was in 4% paraformaldehyde (PFA) overnight at 4°C for paraffin embedded samples and 2 hours at 4°C for frozen embedded samples. Mutant embryos E11.5 and earlier were somite matched to wild type littermates. Embryos were dehydrated and cleared with xylene for paraffin processing using standard procedures (Hogan 1994) and for frozen processing were passed through a sucrose gradient and embedded in 3:1 OCT and 15% Sucrose. Hemotoxylin and Eosin (H&E) staining and Alician Blue were performed using standard procedures.

2.2.3 Immunohistochemistry and cell death analysis

Primary antibodies were used at the following concentrations: Rabbit anti-Sox9 (1:1000, Millipore), Rabbit anti-Caspase-3 (1:250, Cell Signaling), mouse anti-PHH3 (1:750, Cell Signaling). All immunohistochemistry results are representative from at least three mutants and three controls.

2.2.4 Skeletal preparations

The skeletons of wild type and Nkx2.5Cre;Shh<sup>fl</sup> embryos were collected at E17.5 and stained with Alizarin Red and Alcian Blue as previously described (Hogan 1994).
2.2.5 RNA collection and qPCR

RNA was purified from E10.5 mandibles from wild type and mutant embryos using Trizol reagents (Invitrogen). Following RNA extraction, cDNA was synthesized using iScript™ cDNA Synthesis Kit (BIO RAD). Quantitative PCR amplifications were performed in a StepOnePlus real-time PCR machine (Applied Biosystems) using the SensiMix™ SYBR & Fluorescein Kit (Bioline). For each gene, the PCR reaction was carried out in triplicates and the relative levels of mRNAs were normalized to that of HPRT. Statistical tests were performed using the REST program and *P*-value less than 0.05 was considered statistically significant. Primers used: p53 F ‘GGT ACC TTA TGA GCC ACC C’, p53 R ‘GAA GGT TCC CAC TGG AGT C’, p21 F ‘TTG CAC TCT GGT GTC TGA G’, p21 R ‘GTG ATA GAA ATC TGT CAG GCT G’ (Gubelmann et al. 2011)

2.2.6 *In situ* preparation

Whole-mount *in situ* hybridization with Digoxygenin-labeled antisense riboprobes has previously been described (Neubuser et al. 1997). All riboprobes have been previously reported: Fgf8 (Crossley & Martin 1995), Msx1 (Helms Lab), Dlx2 (Porteus et al. 1992), Dlx5 (Liu et al. 1997).

2.2.7 Quantification of proliferation and cell death

Serial sections were taken through whole mandibular arches in control and mutant embryos at E10.5. The number of proliferating and apoptotic cells was counted and normalized to the volume of tissue. Total nuclei were counted in one mutant and one control to make sure there were no differences in cell density. Statistics were performed using a Student’s T-test.

2.2.8 Pharmacological inhibition of p53

Pregnant female mice were injected with pifithrin-α (Sigma) at 2.2 mg per kg (body weight) in PBS by intraperitoneal injection. Female mice were treated at E7.5 for 10 days, and embryos were subsequently analyzed E16.5 (Jones et al., 2008).
2.3 Results

2.3.1 Nkx2.5Cre expression overlaps with Shh expression in both the oral ectoderm and pharyngeal endoderm

To investigate the role of Shh during mandible formation, a conditional tissue specific gene ablation approach was used to isolate the active sources of Shh present at the time of mandibular development. At embryonic day 10.5 (E10.5) Shh is expressed in epithelial domains adjacent to the PA1 mesenchyme: the pharyngeal endoderm and oral ectoderm (Figure 10A). At the same time, Shh is also expressed in the notochord, the floorplate of the neural tube; these domains are adjacent to the streams of migrating NCCs that eventually form much of the mandibular mesenchyme. To isolate this epithelial source of SHH from the axial sources, we used Nkx2.5Cre, expression of which overlaps with Shh in the oral ectoderm and pharyngeal endoderm (Figure 10B) (Goddeeris et al. 2007; Moses et al. 2001). When Nkx2.5Cre;Shh+/− mice are crossed to mice bearing a Shh+/− allele, Shh is specifically ablated in these domains in the mutant, Nkx2.5Cre;Shh+/− (Goddeeris et al. 2007). This ablation results in a complete loss of Shh signaling in PA1, as shown by a loss of Ptc1 expression (a transcriptional target of Hh signaling) by in situ and Lac-Z reporter expression (Goddeeris et al. 2007). Although these embryos died at birth as a result of cardiac defects (Goddeeris et al. 2007), we were able to examine the development of the craniofacial structures prior to birth.
Figure 10: Nkx2.5Cre expression overlaps with Shh expression in both the oral ectoderm and pharyngeal endoderm.

A) ShhGFPCre+/R26R:YFP lineage traces Shh expression in the oral ectoderm (oe) and pharyngeal endoderm (pe) at E10.5. Nkx2.5Cre is expressed in the oral ectoderm, pharyngeal endoderm and the mesodermal core (mc) at E10.5 B. Scale bar= .1mm
2.3.2 Loss of epithelial Shh results in micrognathia starting at embryonic day 11.5

The portion of the craniofacial skeleton derived from maxillary PA1 appears to be relatively normal (Figure 11A-F) with the exception of the basisphenoid and palatine bone, which both appeared to be slightly hypoplastic when compared to littermate controls (Figure 11G-H). Furthermore, the mesoderm-derived proximal skull, such as the parietal, interparietal, basioccipital and supraoccipital bones, all developed normally (Figure 11A,B). The loss of epithelial Shh expression resulted in malformations of the middle ear but not in the otic capsule (Figure 11I-J). The ear is missing the tympanic ring, incus and malleus, which are all derived from mandibular PA1 mesenchyme (Figure 11I-J). Additionally, the stapes, a second arch derivative, is missing in mutant embryos (Figure 11I-J). Most significantly for our study, in 100% of late gestation Nkx2.5Cre;Shh<sup>fx/fx</sup> embryos (N=15), the mandible was hypoplastic (Figure 11K-L). These results indicate that the development of multiple pharyngeal arch skeletal derivatives, particularly the mandibular component of PA1, requires epithelial Shh expression.

Nkx2.5Cre;Shh<sup>fx/fx</sup> mutant embryos exhibited a range of mandibular defects (Figure 11K-L). All mutants had hypoplastic dentary bones, but still developed secondary structures including the coronoid, condylar and angular processes (Figure 11K-L). At E17.5 the mutant mandibles were 47% shorter in length than those of wild type littermates, when normalized against total craniofacial length (N=15). When we
examined earlier stages, we found that the hypoplastic mandible phenotype could be
detected starting at E11.0 (Figure 11O-P), but outgrowth did not appear defective at
earlier stages (Figure 11M-N). The clear reduction in mandibular bud size after E11
could be the result of decreased NCC migration. In previous studies, when E9.0-10.0
Nkx2.5Cre;Shh<sup>fx</sup> embryos were stained with AP2, a NCC migration marker, there was
normal staining of the streams of migratory NCC, when compared to the control
samples (Goddeeris et al. 2007). These earlier results show that NCC migration is not
detectably impaired by a loss of epithelial Shh from PA1, consistent with the apparently
normal outgrowth of the mandibular bud prior to E11.0 (Figure 11M-P).
Figure 11: Loss of epithelial Shh results in micrognathia starting at embryonic day 11.5

Littermate controls (A, C, E, G, I, K) and Nkx2.5Cre;Shh^{fl/+} (B, D, F, H, J, L) embryos stained with alizarin red marking bones and Alcian Blue marking cartilage at E17.5 (N=10). A-B) Lateral view of skull showing the supraoccipital (so), interparietal (ip), parietal (pa), frontal (fn) and nasal bone (na). C-D) Skull bases showing the zygomatic process of maxilla (mxzp) and palatal process of maxilla (mxpp) with dentary bones (db) removed. E-F) Basioccipital (bo) G-H) Basisphenoid (bs) and palatine (pl) bones. I-J) Otic capsules (oc) with associated structures including incus (in), malleus (ma), stapes (st), styloid process (sp) and tympanic ring (ty). K-L) Dentary bone with Meckel’s cartilage (mc) and secondary processes: condylar (cdp), coronoid (crd) and angular (ap) M-N) Whole mount images of control and mutant mandibular arches at E10.5 show no visual difference in size. O-P) Whole mount E11.5 mandibular arches show drastic size increase in Nkx2.5Cre;Shh^{fl/+} mandibular arches between E10.5 and E11.5 (M,O) but very little size change between mutant mandibular arches from E10.5 to E11.5 (N=20). Scale bar= 1mm
2.3.3 A loss of epithelial Shh from PA1 results in a complete loss of Meckel’s cartilage and tongue

The defects observed in Nkx2.5Cre;Shhflox mutants suggest that in the mouse, epithelial Shh is necessary at post-migratory stages for NCC survival, establishment of cartilage condensations and/or differentiation of Meckel’s cartilage. Because there was no morphologically visible Meckel’s cartilage in late-stage embryos (Figure 11K-L), we first explored the possibility that Shh is necessary for the formation of Meckel’s cartilage. Nkx2.5Cre;Shhflox embryos were stained with Alcian Blue (AB), a marker for differentiated cartilage, to see if Meckel’s cartilage was present at any point during development. When coronal sections of E16.5 embryos were examined, an identifiable dentary bone was present in both mutant and wildtype littermates (Figure 12A-B) but Meckel’s cartilage and the tongue were absent. To determine if Meckel’s cartilage was present initially but was then lost, whole mount AB staining was preformed on E14.5 mandibles. In control embryos but in none of the mutant littermates, it was possible to visualize the rod of Meckel’s cartilage running through the developing dentary bone, as early as E14.5 (Figure 12C-D). Together, our data suggest that the primary defect in Meckel’s cartilage formation occurs at or before the time of cartilage condensation development.

The complete lack of Meckel’s cartilage in Nkx2.5cre;Shhflox mutants when the Shh PA1 epithelial expression domain is ablated allows for a comparison to the phenotype resulting from the ablation of the Shh receptor in NCC. It has been shown previously
that a loss of the Shh receptor, smoothened (Smo), in NCC results in results in micrognathia (Jeong et al. 2004). Therefore, it was important for us to determine if a loss of Smo in NCC resulted in the same mandibular phenotype as a loss of Shh in PA1 epithelia. When Nkx2.5Cre;Shh^{fx/-} embryos were compared with Wnt1Cre;Smo^{fx/-} embryos, in which Smo is ablated from the NCC, their mandibular buds were very similar in size and shape (data not shown). The only gross morphological difference was that Wnt1Cre;Smo^{fx/-} embryos had a truncated Meckel’s cartilage (Jeong et al. 2004), while Nkx2.5Cre;Shh^{fx/-} embryos had no Meckel’s cartilage (Figure 12A-D). This comparison suggests that in addition to the PA1 ectomesenchymal NCC derivatives, another population of cells must also respond to epithelial Shh to promote mandibular development.
Figure 12: *Shh* is necessary for Meckel’s cartilage formation.

A-B) H&E with AB staining of E16.5 coronal sections show the presence of the dentary bone (db) in both control and mutant samples but the loss of Meckel’s cartilage (mc) in the mutant embryos. C-D) Whole mount Alcian Blue staining of control and Nkx2.5Cre;Shh<sup>−/−</sup> embryos at E14.5 shows a complete loss of Meckel’s cartilage (mc). Panel D is at a higher magnification than C to emphasize the loss of cartilage staining. (N=10) Scale bars=1mm
2.3.4 *Shh* from PA1 epithelium is required for cartilage condensation formation in pharyngeal arch one

Previously it had been hypothesized that in *Shh* null mutants, the precursor to Meckel’s cartilage, a cartilage condensation, forms but does not grow (Melnick et al. 2005). Thus we examined the formation of cartilage condensations in the mandibular arch of *Nkx2.5Cre;Shh*<sup>−/−</sup> embryos at E11.5. In wild type embryos, condensations were clearly visible at this stage as clusters of cells that were more densely packed than the neighboring mesenchyme (Figure 13A,C). In mutant embryos no cartilage condensation was visible (Figure 13 compare C to D). The failure of cartilage condensation formation was confirmed by antibody staining for the early chondrocyte marker, SOX9. In the mandibular bud of control embryos, SOX9 protein was detected in a condensed rod shape at E11.5 (Figure 13E). In contrast, no SOX9-positive cells were detected in the mandibular bud of mutant embryos (Figure 13F). Therefore, in the mouse, epithelial *SHH* is required for the initiation of mandibular cartilage condensations.
Figure 13 Cartilage condensations do not form in Nkx2.5Cre;Shh<sup>fl/fl</sup> embryos.

A-D) Coronal sections through control and Nkx2.5Cre;Shh<sup>fl/fl</sup> embryos at E11.5 stained with H&E. Control embryos have a region of cells which are more densely packed than the surrounding mesenchyme which is characteristic of a cartilage condensation marked MC. Nkx2.5Cre;Shh<sup>fl/fl</sup> embryos do not have the same condensed structure present. E-F) Serial sections stained with anti-SOX9 (green), a chondrocyte marker, shows a loss of chondrocytes in Nkx2.5Cre;Shh<sup>fl/fl</sup> embryos at E11.5 (DAPI in blue). (N=10) Scale bar= 0.1mm
2.3.5 PA1 proliferation is unchanged in \textit{Nkx2.5}^{\text{Cre}};\textit{Shh}^{\text{fx/-}}\textit{embryos prior to cartilage initiation}

During development and cancer progression, \textit{Shh} can act as a mitogen stimulating increased cell division (Rowitch et al. 1999; Kenney et al. 2003; Bhatia et al. 2011). To determine whether hypoplasia of mandibular PA1 could be due to a loss of proliferating post-migratory NCC, E10.5 embryos were stained using phospho-histone H3 (pHH3), a marker for dividing cells. Quantification of pHH3-positive cells throughout the entire PA1 showed no change in the amount of labeled cells in the \textit{Nkx2.5}^{\text{Cre}};\textit{Shh}^{\text{fx/-}} mutant PA1 in comparison to wild type controls (Figure 14A-B, E). These results indicate that cell proliferation in PA1 is not significantly changed by the loss of PA1 epithelial \textit{Shh} expression, suggesting that \textit{SHH} does not act as a mitogen during early mandible development.

2.3.6 Increased apoptosis in \textit{Nkx2.5}^{\text{Cre}};\textit{Shh}^{\text{fx/-}}\textit{embryos at E10.5 results in decreased mandibular bud size}

\textit{Shh} has been shown to be a survival signal in many developmental contexts (Ahlgren & Bronner-Fraser 1999; Hu & Helms 1999). Therefore, decreased cell survival in PA1 prior to cartilage condensation formation could be responsible for the micrognathia phenotype of \textit{Nkx2.5}^{\text{Cre}};\textit{Shh}^{\text{fx/-}} mice. We quantified the amount of apoptosis at E10.5 throughout the entire PA1 of both control and mutant embryos using an apoptosis marker, anti-cleaved caspase-3. In contrast to the unchanged proportion of
proliferating cells, there was a 75% increase in apoptosis in PA1 in mutant embryos (N=4) when compared to control littermate embryos (N=5) (Figure 14C-D, E). This increase in apoptotic cells was largely localized to the lateral mesenchymal regions of PA1. These data suggest that the reduction in mandible size is due to increased cell death in the mesenchyme prior to Meckel’s cartilage development.
Figure 14: SHH acts as a survival signal in PA1 at E10.5

A-B) Coronal sections through PA1 of wildtype and mutant embryos show similar numbers of dividing cells, marked by anti-phosphohistone H3 (pHH3). C-D) Mutant PA1 mesenchyme has increased regions of apoptosis, marked by caspase-3, when compared to littermate controls. E-F) Increased magnification show regions of apoptosis (red) and DAPI (blue). G) Quantification of the total number of dividing and apoptotic cells (throughout the entire PA1 and normalized to area) showed no significant change in the numbers of dividing cells (p=.12) but a large increase in the number of apoptotic cells (p<.0001). (N=4) Scale bar= 0.05mm
2.3.7 Epithelial Shh is important for development of distal PA1 at E10.5

To determine if Shh is involved in regulating downstream differentiation factors, we assayed the expression patterns and levels of well-established mandible patterning genes, via in situ hybridization and qPCR in both control and mutant embryos at E10.5. We wanted to examine both regional patterning markers as well as three critical signaling pathways which are known to function during mandible development: BMP signaling, FGF signaling and Endothelin-1 signaling.

The first set of genes examined were members of the Dlx family, which are important for establishing the regional identity of PA1 (Beverdam et al. 2002; Jeong et al. 2008; Depew et al. 2002). By in situ hybridization, no change in the proximal mesenchymal localization of Dlx2 was visible in PA1 at E10.5 (Figure 15A-D). However, Dlx2 is lost in the ectodermal domain where Shh has been removed (Figure 15A,C). On the other hand, Dlx5 expression in the mesenchyme near the oral ectoderm appears to be expanded distally (Figure 15E-H). Dlx5 is normally expressed in the proximal mesenchyme of PA1. In addition, there was no detectable change in the spatial expression of Dlx2/Dlx5 in the maxillary domain of PA1 (Figure 15A-H).

Next, we wanted to determine if Shh was regulating expression of Msx1, a downstream target of BMP signaling, which is known to be important in the development of cranial neural crest derived structures (Vainio et al. 1993). In embryos
without Msx1 there is impaired neural crest migration and increased apoptosis in the pharyngeal arches (Ishii et al. 2005). Furthermore, Msx genes have been shown to regulate apoptosis downstream of Shh signaling during limb development (Lallemand et al. 2009). These previous findings made Msx1 an attractive candidate to examine in Nkx2.5Cre;Shh<sup>fx/-</sup> mice. However, there was no change in the localization or expression levels of Msx1 RNA in mutant E10.5 PA1 when compared to control samples (Figure 15-I, L, U). Furthermore, the expression level of Gli3 RNA, a target of Msx and Shh signaling, was unchanged (Figure 15U). This was not surprising, as a loss of Gli3 expression alone does not result in mandibular defects (Mo et al. 1997). In addition to working downstream of Hh signaling in the limb, the Msx genes are established targets of BMP4 signaling. At this stage, Bmp4 is expressed in the endodermal and ectodermal layer and removing Bmp4 from the ectoderm results in a range of hypoplastic mandibular phenotypes (Liu et al. 2005). These phenotypes result from excess apoptosis in the E10.5 pharyngeal arch. However, Nkx2.5Cre;Shh<sup>fx/-</sup> mice had no change in the amount of Bmp4 expression when quantified by qPCR (Figure 15U).

We also assessed whether the loss of Shh from the PA1 epithelium resulted in changes in Fgf8 localization or expression. Fgf8 is downstream of both BMP4 and Shh signaling during mandible formation. When ectodermal Bmp4 is removed, proximal ectodermal Fgf8 expression is lost (Liu et al. 2005), while in Shh<sup>fv</sup> embryos, Fgf8
expression is down-regulated in the pharyngeal arches (Yamagishi et al. 2006).

Furthermore, exogenous FGF8 seems to rescue Meckel’s cartilage chondrogenesis in wildtype mandibles treated with the Shh antagonist cyclopamine in culture (Melnick et al. 2005). When Fgf8 expression was assessed by in situ, it was lost specifically in the mandibular arch of PA1 but not the maxillary region in Nkx2.5Cre;Shhfloxed embryos at E10.5 (Figure 15M-P, U). This result suggests that in mammals, reducing epithelial Shh in PA1 reduces ectodermal expression of Fgf8.

Lastly, we examined expression of Barx1, a downstream target of Endothelin-1 (Edn1) signaling in craniofacial development (Clouthier & Schilling 2004; Walker et al. 2006). Edn1 signaling has been shown repeatedly to be important in specification of proximal-distal patterning in the developing lower jaw. Interestingly, genetic ablation of Edn1 signaling in PA1 results in a hypoplastic Meckel’s cartilage (Tavares et al. 2012). We evaluated Edn1 signaling indirectly by examining Barx1 localization, because Barx1 is important in the proliferation of the ectomesenchyme, regulates Col2a1 and is found in mesenchymal condensations throughout the embryo (Tissier-Seta et al. 1995; Sperber & Dawid 2008; Barlow et al. 1999; Jones et al. 1997). When examined by in situ, Barx1 was localized to the proximal domains of the developing PA1 in both control and mutant samples (Figure 15Q-T). However, it appeared that there was a loss of the most distal region of Barx1 expression in the mutant mandibular arch (Figure 15S). This result in
combination with the distal shift in Dlx5 localization, suggests that distal patterning of PA1 is disrupted by a loss of Shh.
Figure 15: Epithelial Shh is important for development of distal PA1 at E10.5.

*In situ* hybridization of control (A, B, E, F, I, J, M, N, Q, R) and Nkx2.5Cre;Shh<sup>fl/fl</sup> (C, D, G, H, K, L, O, P, S, T) embryos (N>6). A-D) Dlx2 shows a loss of ectodermal Dlx2 expression (arrow) in mutant embryos E-H) Dlx5 expression shows a reduction in the size of the distal region of PA1 (bracket and arrow) in mutant PA1 I-L) Epithelial Fgf8 expression is lost in mutant PA1 compared to control (arrows) M-P) Msx1 expression is similar in control and mutant embryos Q-T) Barx1 expression appears reduced in mutant embryos U) qPCR for Bmp4 pathway, Shh pathway, Fgf8, and apoptosis genes show no significant changes in expression levels, with the exception of *Ptc*, a reporter of Shh signaling, which was significantly downregulated (p<.0001) (N=5). Scale bar=1mm
2.3.8 Pharmacological inhibition of p53 rescues PA1 cell survival but not Meckel’s cartilage outgrowth in Nkx2.5Cre;Shh\textsuperscript{fx/} embryos

In the Nkx2.5Cre;Shh\textsuperscript{fx/} model studied here, as well as in some other mouse models of mandibular hypoplasia (Jeong et al. 2004; Liu et al. 2005), increased apoptosis in the pharyngeal mesenchyme around E10 appears to be the main cellular behavior driving a hypoplasia event. However, little is known about the apoptotic pathways that are activated in this developmental context. In other instances, such as cerebellar development, retinal development, and multiple cancers, it has been shown that Shh signaling protects against the apoptosis triggered by P53 activation (Abe et al. 2008; Prykhozhij 2010; Malek et al. 2011). Therefore we hypothesized that the loss of Shh could result in activation of P53 in Nkx2.5Cre;Shh\textsuperscript{fx/} embryos.

In order to better assess the possible involvement of P53 downstream of Shh during PA1 development, we tested whether decreasing P53 activity could rescue the micrognathia phenotype. The P53 pharmacological inhibitor pifithrin-\(\alpha\) has been shown to rescue diverse craniofacial defects caused by increased P53 activity when injected into pregnant female mice (Jones et al. 2008). To test whether pifithrin-\(\alpha\) could rescue the micrognathia present in Nkx2.5Cre;Shh\textsuperscript{fx/} embryos, pregnant females were injected daily with pifithrin-\(\alpha\) from E7.5 until embryo collection at either E10.5 or E13.5. To assess whether the inhibition of P53 resulted in decreased apoptosis, embryos were collected at E10.5 and stained with anti-caspase-3. Upon quantification, we observed that
Nkx2.5Cre;Shh<sup>fx</sup>-<sup>-</sup> embryos treated with pifithrin-α had the same levels of apoptosis as wildtype littermates (Figure 16A-C). Thus, pifithrin-α was able to rescue the increased PA1 apoptosis in Nkx2.5Cre;Shh<sup>fx</sup>-<sup>-</sup> embryos, reducing apoptosis to the basal level normally found in wildtype littermates.

After establishing that pifithrin-α was capable of rescuing the increased apoptosis phenotype, we assessed later structural phenotypes. Embryos were collected at E13.5 after 6 days of injections to determine the jaw phenotype. Surprisingly, these embryos still had the same micrognathia phenotype as observed in untreated embryos (Figure 16D,E). When the mandibular buds of the control and mutant embryos from dams treated with pifithrin-α were stained with the pre-cartilage marker Sox9, the control embryos had a normal Sox9 positive Meckel’s cartilage, while the mutant embryos showed no Sox9 positive cells (Figure 16F,G). Thus the inhibition of P53 by pifithrin-α could rescue the increased apoptosis yet was not able to restore cartilage differentiation. These results show that Shh is important in multiple stages of mandible development: first in neural crest cell survival and later in early cartilage development.
Figure 16: Pharmacological inhibition of p53 rescues PA1 cell survival but not Meckel’s cartilage outgrowth in Nkx2.5Cre;Shh\textsuperscript{fx/} embryos

A-C) Embryos collected at E10.5 after 3 days of pifithrin-α injections show a significant decrease in apoptosis by quantified cleaved caspase 3 staining (green). White arrow marks apoptosis occurring in heart tissue outside of PA1. D-G) Embryos collected at E13.5 after 6 days of injections show no rescues of Meckel’s cartilage. Nkx2.5Cre;Shh\textsuperscript{fx/} embryos have micrognathia (red bracket) and no Meckel’s cartilage by SOX9 (green) antibody staining while the littermate control embryo has a normal mandible and Meckel’s cartilage.
2.3.9 Epithelial Shh regulates differentiation factors during cartilage condensation development

Since rescuing PA1 cell death did not rescue cartilage formation, we wanted to determine whether Shh is directly regulating cartilage condensation initiation, adhesion, or differentiation (Hall & Miyake 2000). Therefore gene expression levels of key markers of each of these stages were examined to determine if they are regulated by Shh signaling. RNA was collected from isolated E11.5 mandibles of embryos from both PBS and pifithrin-α injected dams, to determine whether rescuing the cell death phenotype (Figure 18C) affected condensation development.

Our results suggest that Shh is involved in the initiation of cartilage condensations, since condensations were missing in the developing mandibles of both PBS injected and pifithrin-α injected mutant embryos. The first gene examined was Tgfβ-1, which in Shh-independent contexts is important for condensation formation and mandible development (Ito et al. 2002; Dudas & Kaartinen 2005). Expression levels of Tgfβ-1 were unchanged between control and mutant embryos (PBS and pifithrin-α injected) (Figure 17). This result suggests that changes in condensation markers seen in Nkx2.5Cre;Shh<sup>fl/fl</sup> mutants are independent of changes in Tgfβ-1 expression levels, despite evidence that Tgfβ-1 is a major regulator of condensation formation in other contexts. The next gene examined was N-CAM, which is important for initiation and adhesion within a developing cartilage condensation (Chimal-Monroy & Diaz de Leon 1999).
There were no changes in *N-CAM* gene expression between control and mutant embryo samples (Figure 17). Together, these results suggest that there are appropriate expression levels of important genes for cartilage condensation, initiation and adhesion during Meckel’s cartilage development in *Nkx2.5Cre;Shh* embryos.

This left as most likely the possibility that *Shh* is important specifically in cartilage differentiation. Two differentiation markers, Collagen-II (*Col2a*) and *Sox9*, are regulated by *Shh* in the context of tracheal cartilage development (Park et al. 2010). In mutant mandibles from pifithrin-α injected dams, *Col2a* gene expression levels were reduced by 40% in comparison with wildtype control samples (Figure 17). *Sox9*, a differentiation marker usually found co-expressed with *Col2a*, was significantly downregulated (by 60%) in both PBS and pifithrin-α injected mutant mandibles when compared to control samples (Figure 17). Finally, we looked at the expression of Collagen-I (*Col1a*), which is often highly expressed prior to cartilage differentiation but decreases at the time of differentiation (Castagnola et al. 1988). Interestingly *Col1a* expression was 2.75 fold higher in PBS and pifithrin-α injected mutant mandibles when compared to wildtype mandibles (Figure 17). Together these results support a role for *Shh* in the regulation of cartilage differentiation through regulation of *Collagen-I*, *Collagen-II* and *Sox9* gene expression (Figure 18 C,D).
Figure 17: Epithelial Shh regulates differentiation factors during cartilage condensation development

qPCR was performed using E11.5 mandibles from PBS treated and pifithrin-α treated control and Nkx2.5Cre;Shh^{flx} mutant embryos (N=4 for each group). Tgfβ-1 and NCAM showed no change between groups while Col2a (p=.005) was downregulated in pifithrin-α treated mutants (PIF-MT) but not in PBS injected mutants (PBS-MT). Lastly, Sox9 (p=.0165) was down significantly and Col1a (p=.04) was up significantly in both PBS-MT and Pif-MT when compared to control samples.
2.4 Discussion

This study has shown that in mammalian development, Sonic Hedgehog from the pharyngeal epithelium is necessary for proper formation of the mandible. These results support previous studies showing that hedgehog signaling is necessary for cranial NCC development (Jeong et al. 2004; Brito et al. 2006; Couly et al. 2002; Trainor & Krumlauf 2000; Haworth et al. 2007; Wada et al. 2005b). The results presented here show definitively that Shh is the critical hedgehog signaling protein for mandibular development, and that the relevant source of Shh is the PA1 epithelium rather than the axial midline domains (Fig 9).

Embryological experiments in chick have suggested that Shh from the pharyngeal endoderm is both sufficient and necessary for PA1 development. Our study extends these findings from an avian model into mammals as well as to a genetic loss-of-function analysis, by specifically disrupting Shh in the epithelium of PA1 using a spatiotemporal gene ablation approach. Further, we definitively show that mammalian Shh, produced in the PA1 epithelium, is an essential signal to the PA1 mesenchyme during mandible development.

In contrast to previous work that studied the consequences of disrupted Hh signaling specifically in CNCC (Jeong et al., 2004), the mutant model here directly addressed the effect of epithelial Shh expression on the entire PA1 epithelium and

72
mesenchyme, including both NCC and mesodermal derivatives. The comparison of mutant phenotypes resulting from a loss of Hh signal reception by NCC, versus the loss of Shh emanating from the pharyngeal epithelium, indicates a more severe hypoplasia of the mandible when the epithelial source of Shh is absent. This suggests that there is another population of cells, in addition to the NCC derived mesenchyme, which needs to receive Shh signaling for the mandible to develop properly. This population of cells may be the epithelium itself and/or the non-NCC derived mesodermal core of PA1.

Through detailed analysis of the craniofacial skeleton of Nkx2.5Cre;Shh\textsuperscript{fl/fl} embryos, we determined that PA1 derivatives were disrupted, including multiple skeletal and cartilage elements. The palatine, basisphenoid and dentary bone were hypoplastic while other elements were missing completely in Nkx2.5Cre;Shh\textsuperscript{fl/fl} embryos, including otic components (tympanic ring, incus, malleus, stapes) and Meckel’s cartilage. The rest of the craniofacial skeleton appears to have developed normally in the absence of this source of Shh. While this study did not investigate the development of otic structures in detail, these results show that epithelial Shh plays an important role in PA1 and PA2 derived ear elements.
Figure 18: Epithelial Shh is necessary for early PA1 mesenchymal cell survival and for regulation of differentiation factors during mandibular cartilage condensation development.

There are two main stages where epithelial signaling is necessary for mandibular development: A-B) cell survival at E10.5, and C-D) cartilage condensation differentiation at E11.5. A) When epithelial (dark blue) Shh is present neural crest derived mesenchymal cells (light blue) are protected against apoptosis. At E10.5 the majority of PA1 is made up of NCC but there are some non-neural crest mesenchymal cells present. B) However, when Shh is removed from the epithelial the mesenchymal cells undergo increased cell death (red cells) due to p53 activation. The loss of Shh results in increased cell death supporting an early cell survival role of Shh. C) Later in development Shh from the epithelium is important in regulating expression of cartilage differentiation factors Sox9 and Col2a in the condensing mesenchymal cells (light green). D) Interestingly, a rescue of the cell death phenotype in Nkx2.5Cre;Shh<sup>fl</sup> mutants does not rescue the cartilage development phenotype suggesting that Shh pays a later role in cartilage development. Based on qPCR data, it appears that Shh signaling is important in regulating cartilage differentiation but not initiation or adhesion.
2.4.1 Epithelial Shh acts as a PA1 survival signal

Previously studies indicated no apparent defect in the ability of neural crest cells to migrate and populate PA1 properly in Nkx2.5Cre;Shh<sup>flox</sup>-<sup>flox</sup> mice (Goddeeris et al. 2007). Size differences of mandibular PA1 between Nkx2.5Cre;Shh<sup>flox</sup>-<sup>flox</sup> embryos and control embryos did not appear until E11.5, consistent with the hypothesis that Shh signals from the PA1 epithelium are not necessary for NCC migration. We investigated if Shh was necessary for proliferation of mesenchymal cells in PA1, as Shh acts as a mitogen in other contexts. However, when levels of proliferation were assessed in mutant versus control embryos, no change was found. Therefore, Shh is not acting as a mitogen in this context.

By contrast, Shh signaling is necessary for survival of a subset of PA1 mesenchymal cells, as there was markedly increased lateral apoptosis at E10.5, after NCC migration was completed, in Nkx2.5Cre;Shh<sup>flox</sup>-<sup>flox</sup> embryos. However, at E9.5 there was no apparent increase in apoptosis in mutant embryos (data not shown). By comparison, the conditional removal of Smo in the NCC resulted in increased midline apoptosis at E9.5, and at E10.5 and increase in both midline and lateral apoptosis (Jeong et al. 2004). The two different patterns in the distribution of apoptotic cells reveal differences in the functional significance of loss of epithelial Shh versus loss of Hh responsiveness in the NCCs. One relevant point that may explain this difference is that the loss of Smo via Wnt1Cre means a loss of Hh signal responsiveness from the earliest stages of NCC
migration. In contrast, loss of Shh expression in the PA1 epithelium is a later event, which would affect NCC largely after they migrate into PA1 and with spatial specificity. A second point is that NCCs lacking Smo are not only unable to respond to Shh, but also to benefit from any other functions of Smo, such as response to other Hh ligands. In any event, the levels and distribution of apoptosis we detected in embryos lacking Shh expression in PA1 suggest that most PA1 mesenchymal cells that contribute to the mandible survive even without epithelial Shh. This further suggests that apoptosis is only part of the pathological problem underlying defective mandibular development in embryos devoid of PA1 Shh signals.

Based on the role that Shh plays in the development of multiple cancers, we suspected that Shh was regulating P53 in mandibular arch development. When P53 was pharmacologically inhibited in Nkx2.5Cre;Shh<sup>fx/-</sup> embryos, early cell survival was rescued, supporting an inhibitory role of SHH on P53 activity in this developmental context. Although we did not investigate the biochemical mechanism underlying this effect, previous work suggests Hh signaling is necessary for MDM2 to ubiquinate and degrade p53 (Malek et al. 2011); thus when a required Hh signal is absent, MDM2 does not cause degradation of p53. Regardless of mechanism, this study demonstrates the rescue of a Shh-related apoptosis defect <i>in utero</i>, thus presenting a potential mechanism for studying other defects associated with increased apoptosis due to loss of Shh signaling.
2.4.2 Localization of Dlx2, Dlx5 and Fgf8 is regulated by epithelial Shh

Patterning of the mandibular arch is strictly controlled, involving a multitude of genes. Many are expressed in one of two domains, proximal (lateral) or distal (medial) (Ferguson et al. 2000; Mina et al. 2002; Thomas et al. 1998). The distal domain is the major regulator of mandibular development (McGonnell et al. 1998; Mina et al. 2002; Thomas et al. 1998). Interestingly, when we examined the localization of two markers of proximal gene expression, Dlx2 and Dlx5, we found that a loss of epithelial Shh resulted in altered Dlx2/5 localization. In Nkx2.5Cre;Shh<sup>fx</sup> embryos, Dlx2 was lost in the distal ectoderm, but not the proximal mesenchyme which is interesting since epithelial Dlx2 is established by epithelial BMP4 signaling (Thomas et al. 2000). The loss of Dlx2 in the epithelium suggests that BMP signaling may be decreased.

On the other hand, proximal Dlx5 expression was expanded to the midline domain of PA1 in Nkx2.5Cre;Shh<sup>fx</sup> embryos. The altered Dlx5 expression could be due to a loss of the distal domain of PA1 or an expansion of the proximal domain. Mice with conditional ablation of Hand2 in NCC have expanded Dlx5 expression distally, resulting in a loss of the tongue and a hypoplastic dentary bone (Barron et al. 2011). These phenotypes are reminiscent to the loss of tongue and hypoplastic mandible seen with a loss of epithelial Shh. The Barron study did not examine Meckel’s cartilage, but it appears to be absent. Dlx5 expression induces Runx2 expression, required for
osteogenesis, and increased distal Dlx5 can cause premature ossification of the dentary bone (Barron et al. 2011). It is possible that Shh is either directly or indirectly regulating a network of gene expression that protects the mandible from premature ossification.

In addition to the disruption of Dlx2 and Dlx5 expression, the loss of epithelial Shh results in a loss of ectodermal Fgf8 specifically in the proximal epithelium of PA1. Shh has been shown to regulate PA1 Fgf8 expression in Shh−/− mice (Yamagishi et al. 2006; Moore-Scott & Manley 2005; Aoto et al. 2009). Fgf8 is important in mandible development at E10.5 for PA1 cell survival (Trumpp et al. 1999; Tucker et al. 1999) and is necessary later in mandible development for cartilage differentiation (Abzhanov & Tabin 2004; Melnick et al. 2005). Our results show that in the mouse, epithelial Shh is necessary for Fgf8 expression, which in turn is likely important for cell survival and establishment of Meckel’s cartilage.

A key difference between the conditional removal of Smo in NCC and the removal of Shh from pharyngeal epithelia is the disruption of epithelial Fgf8. While Fgf8 expression was not directly examined in the Smocko mutants, the normal expression of Spry suggests that Fgf8 signaling is intact (Jeong et al. 2004). It is possible that the loss of Fgf8, which is expressed proximally, is responsible for the increased apoptosis seen laterally in our mutants. Further work will need to establish the temporal necessity of Fgf8 expression for Meckel’s cartilage formation in mouse.
Lastly, we observed a slight loss of the most distal domain of Barx1 in mutant embryos. Barx1 is a downstream target of Edn1 signaling but is also regulated by other signaling pathways, including Fgf8 (Trumpp et al. 1999). Therefore, the reduction in Barx1 is likely due to the loss of epithelial Fgf8. Furthermore, Barx1 is important for the differentiation of cells into cartilage, and the loss of Barx1 results in loss of mandibular cartilage (Nichols et al. 2013). The connection between Barx1 expression and Shh needs to be investigated further to determine if Shh directly regulates Barx1.

2.4.3 **Shh is necessary for Meckel’s cartilage differentiation**

Previous work in chick has shown that Shh is important for Meckel’s cartilage formation, but the mechanism has not been investigated. Our results support a role for Shh in the establishment of Meckel’s cartilage condensations prior to outgrowth however a previous study identified areas of dense cells in Shh<sup>−/−</sup> mandibular arches, thought to be cartilage condensations (Melnick et al. 2005). It is possible the condensations seen in the earlier study are not, in fact, Meckel’s cartilage (Melnick et al. 2005). Additionally, when Smoothened was removed from the NCC prior to mandible development, a small Meckel’s cartilage and dentary bone did form (Jeong et al. 2004). These results differ from our results that show a complete loss of Meckel’s cartilage when epithelial Shh is removed. The disparity is likely due to the total loss of Shh ligand exposure in PA1 epithelium and mesenchyme of Nkx2.5Cre;Shh<sup>−/−</sup> mice, compared to a
specific loss in ectomesenchyme in the Wnt1Cre;Smo\textsuperscript{fl/fl} mice. In this latter mutant, epithelial and mesenchymal non-NCC cells would still be able to receive and respond to Shh signals, which would be absent in the Nkx2.5Cre;Shh\textsuperscript{fl/fl} mutants. The differences in phenotype between these two crosses imply that Shh signaling is important in the development of Meckel’s cartilage through signaling in other cell populations besides neural crest derived mesenchyme.

Our results show that Shh has at least two roles during mandibular development: 1) promoting survival of PA1 mesenchymal cells (NCC and non-NCC); and 2) regulating the differentiation of cartilage condensations. Treating Nkx2.5Cre;Shh\textsuperscript{fl/+} mice with a P53 pharmacological inhibitor has allowed for separation of these two potential roles of Shh. If Shh signaling is only needed as a survival signal during PA1 development, inhibition of apoptosis should have resulted in normal cartilage development (Figure 18 C,D). However our results showed that inhibition of apoptosis rescued PA1 cell survival, but not cartilage formation (Figure 18 C,D). Furthermore, we found no evidence that Shh is necessary at the stage of cartilage initiation and adhesion when markers, Tgfβ-1 and NCAM, were examined by qPCR. It is possible that these genes are not sufficiently expressed in the control tissue for us to see small changes in gene expression. Alternatively, Shh may not be necessary for the initiation of cartilage condensations. We
do not have a way to visualize condensation prior to the adhesion/differentiation stage and therefore cannot assess if they are present in our Nkx2.5Cre;Shh<sup>fx/-</sup> embryos.

While Shh does not appear to be necessary for cartilage initiation, we did find that in the later context of cartilage condensation development, Shh regulates Col1a, Sox9 and Col2a expression. The loss of Sox9 and Col2a gene expression in pifithrin-α Nkx2.5Cre;Shh<sup>fx/-</sup> embryos supports Shh signaling as necessary for cartilage differentiation. Interestingly, we found an increase in Col1a gene expression in Nkx2.5Cre;Shh<sup>fx/-</sup> embryos. Collagen-I is normally involved in promoting bone development (Grant et al. 1996), suggesting that the excess Collagen-I expression in mutant embryos may result in premature bone formation. It is possible that the increased expression of Col1a is responsible for the small dentary bone developing without Meckel’s cartilage present. Our data suggest a novel role of Shh signaling as a transcriptional regulator of Col1a expression in mandible condensation development.

In other developmental contexts Shh is a regulator of chondrogenesis, and our results further support the role of Shh in chondrogenesis during mandible development. The mechanism behind chondrogenesis of Meckel’s cartilage should be investigated further, as gaining an understanding of the role of Shh signaling during chondrogenesis will provide information about the normal development of many cartilages, not just Meckel’s cartilage. In this regard, our study demonstrates it is possible to bypass early
cell death defects due to a loss of Shh to gain a better understanding of Shh later in pharyngeal arch development.

Mammals exhibit enormous diversity of craniofacial structures, especially of the viscerocranium, the skeleton surrounding the mouth (Fish et al. 2014). Our results suggest that variability in the level of Shh in PA1 of various mammals, or to Shh responsiveness in CNCC, could play a key role in controlling the size and patterning of Meckel's cartilage. Differences in Meckel's cartilage might in turn result in corresponding variability in the size and shape of the mandible, and partially account for species-specific variation in lower jaw development. In the future, investigating the levels of Shh signaling in Meckel's cartilage formation in various species may provide interesting insights into the role of Shh in evolution as well as in congenital disease.
3. Wnt signaling regulates development of the dorso-ventral midline epithelium during foregut compartmentalization

3.1 Introduction

In Chapter 2, we investigated the role of signaling from the rostral foregut epithelium during the development of the mandible. In addition to being a signaling center during organogenesis, the foregut epithelium undergoes changes in morphology that are crucial for proper development. Caudal to the pharyngeal arches the foregut epithelium is a highly dynamic tissue that compartmentalizes into the larynx, trachea, and esophagus, in addition to budding to form the lungs, liver and pancreas. In this Chapter, we investigate the changes in cellular morphology that are occurring during organogenesis of the foregut by taking advantage of genetic mouse models with defects in foregut compartmentalization due to alterations in dorso-ventral patterning.

The embryonic precursor to the trachea and esophagus is a single epithelial tube called the foregut. During mouse development this single tube compartmentalizes into the trachea and esophagus over a period of about twenty-four hours from embryonic day E10.0 to E11.0. This event requires extensive remodeling of the epithelium and surrounding mesenchyme. When the foregut does not separate appropriately a severe birth defect can occur, called esophageal atresia and tracheoesophageal fistula (EA/TEF).
This defect results in the esophagus ending in a blind pouch while the trachea is connected to the stomach through an esophageal-like fistula.

Multiple genetic murine models have established two main etiologies that cause defects in foregut compartmentalization. These two etiologies are improper notochord resolution and disrupted dorso-ventral patterning (Fausett et al. 2014; Li et al. 2007; Qi & Beasley 1999; Que et al. 2007; Minoo et al. 1999). Notochord resolution occurs prior to foregut compartmentalization around E8.25-E9.5 (Jurand 1974). Previous work suggests when this process is disrupted, extra endoderm cells are incorporated into the notochord, resulting in a reduction in the amount of foregut endoderm present (Fausett et al. 2014; Li et al. 2007). Consequently, when foregut separation begins there is insufficient endoderm for both the trachea and esophagus to develop. Additionally, dorso-ventral patterning is established in the foregut epithelium after closure of the single foregut tube. The dorsal region of the foregut will develop into the esophagus and the ventral region into the trachea. Many publications have shown that if one of these regions is not specified, the foregut will develop into a single tube that is either esophageal or tracheal in nature, depending on the region lost (Que et al. 2007; Minoo et al. 1999; Rodriguez et al. 2010). While both of these classes of defect are medically relevant, neither of them appears to be the result of a disruption in the actual process of
compartmentalization. As a result, we currently know little about what drives separation of the foregut tube endoderm or what cellular behaviors are involved.

Previous studies have observed that the epithelium at the dorso-ventral midline contains multiple cell layers at E10.5 just prior to compartmentalization (Jacobs et al. 2012; Ioannides et al. 2010). It has been hypothesized that this multilayered epithelium is required for the compartmentalization of the foregut (Ioannides et al. 2010). This previous work led us to hypothesize that since epithelial dynamics including polarity, actin dynamics, and differential adhesion play a substantial role in embryonic development and they could be important in the epithelial changes occurring prior to and during compartmentalization. In addition, as it is known the dorso-ventral patterning must be established for compartmentalization, it seemed likely that the dorso-ventral midline was where a majority of epithelial movements were occurring during compartmentalization.

The establishment of the dorso-ventral midline boundary is well studied and it has been shown that two transcriptions factors, Nkx2-1 and Sox2, mark the ventral and dorsal foregut domains, respectively. We now know that a complex network of signaling pathways regulate proper dorso-ventral patterning (Figure 8). An especially interesting aspect of Sox2/Nkx2-1 regulation is the reciprocal inhibition that occurs between Sox2 and Nkx2. In stem cells, Sox2 has been shown to bind the promoter region
of Nkx2-1 and inhibit its transcription (Boyer et al. 2005). Furthermore, genetic manipulations have suggested that Sox2 and Nkx2-1 mutually repress each other to maintain dorso-ventral patterning (Que et al. 2007; Minoo et al. 1999; Harris-Johnson et al. 2009; Goss et al. 2009; Domyan et al. 2011). This mutual repression results in predominantly dorsal expression of Sox2 and predominantly ventral expression of Nkx2-1 in the foregut endoderm. The role of Sox2 and Nkx2-1 in establishing a differentiation program of either esophageal or tracheal fate is well understood. However, if their role were as simple as controlling differentiation then embryos which have both dorsal Sox2 and ventral Nkx2-1 expression, such as crosses with a ventral activation of β-Catenin (Goss et al. 2009; Harris-Johnson et al. 2009), should in theory compartmentalize normally. It is likely that the boundary of Sox2 and Nkx2-1 expression is necessary for maintaining important positional cues at the dorso-ventral midline, which in turn regulate specific cellular behaviors. Yet, very few genes are expressed specifically in the midline region of the epithelium or the mesenchyme.

While genetic studies in mouse models have elucidated many of the key signaling pathways and proteins necessary for proper foregut compartmentalization; little is known about the epithelial cell behaviors occurring. The process of compartmentalization likely depends on many different cellular behaviors and collective movement events. To address this, we examined wildtype mice to carefully characterize
the changes in cellular morphology of the foregut epithelium for the first time and identify target processes to investigate further. Additionally, we hypothesized that crucial cellular processes were occurring at the dorso-ventral midline, which we tested using genetic mouse models with defects in dorso-ventral patterning. The resulting data showed for the first time that $\beta$-Catenin is necessary for pseudostratification of the ventral foregut through an Nkx2-1 independent mechanism, and established a novel mouse model with subtle defects in dorso-ventral midline patterning that can be used to further investigate epithelial morphology at the dorso-ventral midline.

3.2 Methods

3.2.1 Mice

Mice carrying the $Shh^{GFPCre/+}$ allele were mated to mice carrying a conditional loss-of-function allele of $\beta$-Catenin ($Ctnnb1^{E2-6}$) or a conditional gain of function allele of $\beta$-Catenin ($Ctnnb1^{deltaE3}$) to generate either $Shh^{GFPCre/+}; Ctnnb1^{E2-6/E2-6}$ ($\beta$-cateninCKO) or $Shh^{GFPCre/+};Ctnnb1^{deltaE3/+}$ ($\beta$-cateninACT) embryos (Harfe et al. 2004; Harada et al. 1999; Brault et al. 2001). For single cell labeling, $Shh^{CreER/+}$ males were mated with $R26^{R2YFP/YFP}$ females and injected with 200 uL of 20 mg/ml tamoxifen dissolved in corn oil (Harfe et al. 2004; Srinivas et al. 2001). $Nkx2-1^{-/-}$ embryos were a generous gift from the Rubenstein Lab (Minoo et al. 1999).
3.2.2 Dissections, processing and histology

Embryos were dissected in phosphate-buffered saline (PBS). Fixation was in 4% paraformaldehyde (PFA) overnight at 4°C for paraffin embedded samples and 2 hours at 4°C for frozen embedded samples. Mutant embryos E11.5 and earlier were somite matched to wild type littermates. Embryos were dehydrated and cleared with xylene for paraffin processing using standard procedures (Hogan 1994) and for frozen processing were passed through a sucrose gradient and embedded in 3:1 OCT and 15% Sucrose. Hemotoxylin and Eosin (H&E) staining and Alcian Blue were performed using standard procedures.

3.2.3 Immunohistochemistry and cell death analysis

Primary antibodies were used at the following concentrations: Rabbit anti-SOX9 (1:1000, Millipore), goat anti-SOX2 (1:500, Neuromics), mouse anti-NKX2-1 (1:100, ThermoScientific), chick anti-GFP (1:5000, Abcam), rat anti-E-CADHERIN (1:1000, Invitrogen), rabbit anti-Laminin (1:1000, Lightner et al. 1989), filamentous actin was visualized using Alexa Fluor®-488 conjugated phalloidin at 1:200. Nuclei were visualized using DAPI. All immunohistochemistry results are representative from at least three mutants and three controls.
3.2.4 Skeletal preparations

The skeletons of wild type and mutant embryos were collected at E17.5 and stained with Alizarin Red and Alcian Blue as previously described (Hogan 1994).

3.2.5 RNA collection and qPCR

RNA was purified from E10.5 mandibles from wild type and mutant embryos using Trizol reagents (Invitrogen). Following RNA extraction, cDNA was synthesized using the iScript™ cDNA Synthesis Kit (BIO RAD). Quantitative PCR amplifications were performed in a StepOnePlus real-time PCR machine (Applied Biosystems) using the SensiMix™ SYBR & Fluorescein Kit (Bioline). For each gene, the PCR reaction was carried out in triplicate and the relative levels of mRNAs were normalized to that of HPRT. Statistical tests were performed using the REST program and P-value less than 0.05 was considered statistically significant.

3.3 Results

3.3.1 The foregut epithelium undergoes a remodeling event prior to compartmentalization

To determine what cellular behaviors are occurring at the dorso-ventral midline, we needed to identify cellular behaviors or changes in epithelial morphology that could be important in the compartmentalization process. We decided to characterize changes in the foregut epithelium at E9.5 (prior to foregut compartmentalization), E10.5 (during
compartmentalization), and E11.5 (after completion of compartmentalization). By staining the foregut epithelium with E-cadherin, an adherens junction marker, and laminin, a basement membrane marker, it was possible to examine the appearance of the foregut epithelium temporally throughout compartmentalization. The epithelium went through a dramatic thickening between E9.5 and E10.5 and completely resolved into an esophagus and trachea by E11.5 (Figure 19). Furthermore, between E9.5 and E10.5 the foregut epithelium appeared to be pinching together at the dorso-ventral midline causing the shape of the foregut lumen to change from an oval to a “figure eight” (Figure 19). Based on the changes in the appearance of the epithelium, three cellular behaviors were identified as potentially mechanistically important during compartmentalization. These behaviors, described in more detail below, are epithelial-mesenchymal plasticity (EMP), actin constriction and pseudostratification.
Figure 19: The foregut epithelium remodels between E9.5 and E11.5

A) The foregut epithelium is a single cell layer thick at 27 somites prior to compartmentalization when visualized using E-cadherin (pink), DAPI (white) and Laminin (blue). B) During active compartmentalization at 32 somites the foregut epithelium has undergone a transformation and is multiple nuclei thick. C) By E11.0 the epithelium resolves into a single cell layered esophageal epithelium and a pseudostratified tracheal epithelium. Scale Bar: .1mm
3.3.2 The ventral foregut epithelium has actin localized apically and in filopodia during compartmentalization but no decrease in E-cadherin

The first behavior we investigated was epithelial-mesenchymal plasticity (EMP).

In this Chapter, EMP is being used to describe a weak and transient epithelial mesenchymal transition (EMT) that would allow cells more motility for active epithelial morphogenesis. Based on the previously described septation model of foregut compartmentalization, cells at the point of dorso-ventral midline fusion could be exhibiting EMP. There is evidence for this from previous work showing that during compartmentalization, the dorso-ventral midline of the foregut epithelium has increased expression of WT1 (Que et al. 2007), a transcription factor involved in EMT (Miller-Hodges & Hohenstein 2012), and decreased expression of p63, an inhibitor of EMT (Que et al. 2007; Lindsay et al. 2011). These data suggest that expression of EMP-related genes may be occurring in the foregut dorso-ventral midline epithelium during compartmentalization. If the hypothesis of a “saddle” (Metzger et al. 2011) moving rostrally to separate the common foregut is correct, then the epithelium would decrease cell adhesion allowing cells to move through the “saddle” region, before reforming into the esophagus and trachea. To address this possibility, the compartmentalizing foregut was stained with E-cadherin to examine cell-cell junctions. Serial sections through the point of compartmentalization did not show a reduction in E-cadherin protein levels in any region of the foregut epithelium (Figure 20). These data suggest that if EMP is
occurring, it is not through a reduction in E-cadherin. It is important to note that though E-cadherin protein expression is not dynamic during this event, EMP may still be occurring through changes in different adhesion molecules.
Figure 20: E-cadherin protein expression is not lost in epithelial cells at the point of compartmentalization

A-E) Transverse sections through a compartmentalizing foregut stained with E-cadherin (green) and laminin (red) show cell shape changes during compartmentalization. C’, E’ Magnified sections show the levels of E-cadherin just above the point of compartmentalization and in the middle of the “saddle” region. Scale Bar= .1mm
We next examined actin dynamics. It seemed likely based on the pinching of the foregut tube at the dorso-ventral midline prior to compartmentalization, that actin was involved in compartmentalization, similar to its role in neural tube closure and lung budding (Kim et al. 2013; Brouns et al. 2000). For example, the lateral epithelia of the neural tube develop actin-rich filopodia, which reach out across the luminal space and may help bring the two sides together (Brouns et al. 2000). These filopodial structures are not normally made by epithelia, but are commonly found on migrating cells. When the dividing foregut was stained using Phalloidin, an actin stain, we found that actin-rich cellular projections (filopodia-like structures) appeared to be present at the point of compartmentalization (Figure 21). In addition to filopodia, actin could be involved in increasing apical constriction along the dorsal and ventral sides of the foregut, forcing the epithelium together at the dorso-ventral midline. It is plausible that actin constriction is necessary to push the epithelium together at the dorso-ventral midline, where a subsequent epithelial fusion event occurs. This constriction event would be a more complex version of the apical constriction that occurs during lung budding (Kim et al. 2013). The apical surface of the dividing foregut epithelium had high levels of actin (Figure 21), however further work will need to investigate if constriction is occurring.
Figure 21: Actin-rich protrusions are present at the point of foregut compartmentalization

At E10.5 (32s) the foregut is in the process of compartmentalizing and has apical actin present throughout the epithelium, as visualized by Phalloidin staining. A) The epithelium has a layer of apical actin in the uncompartmentalized foregut but does not have obvious actin protrusions. B) Where the epithelium is about to undergo the compartmentalization event, a large amount of apical actin is present and when examined at a higher magnification (Panel D), actin-rich protrusions are present at the D/V midline (white arrow). C) After compartmentalization apical actin is still present in the esophagus and trachea. Scale Bar=.1 mm
3.3.3 The ventral foregut epithelium is pseudostratified by E10.5

Finally, it was necessary to determine if pseudostratification or stratification of the epithelium is responsible for the thickening of the epithelium between E9.5 and E10.5. Pseudostratified and stratified epithelia have different characteristics that could affect the cellular behaviors occurring at the point of compartmentalization. There is evidence that pseudostratification is important in many epithelial morphogenesis events such as neural tube closure (Miyata 2008), villus formation in the intestine (Grosse et al. 2011), and retinal development (Zwaan et al. 1969). In these cases, it is thought that pseudostratified epithelia are able to undergo cell shape and polarity changes quickly to drive morphogenesis (Langman et al. 1966; Eiraku et al. 2011). In the foregut, the epithelium must undergo a reorganization event that results in a single tube of epithelial cells potentially moving through a “saddle” region and separating into two tubes. Pseudostratification is thought to contribute to the rapid elongation of epithelial tubes and in the case of the foregut, pseudostratification of the epithelium could aid a similar epithelial reorganization event. Pseudostratification is the result of a special type of cell division called interkinetic nuclear migration (INM) during which the nuclei of dividing cells move to the apical surface to divide. The movement of the nuclei throughout the cell cycle is responsible for the multiple layers of nuclei present throughout the epithelium (FC Sauer 1936). To determine if the foregut epithelium became
pseudostratified prior to compartmentalization, a single cell labeling approach was used. $Shh^{CreER^{+}}$ mice were crossed with $R26R^{^{YFP/yFP}}$ mice and injected with tamoxifen 24 hours prior to sacrifice. This short tamoxifen exposure results in a low number of GFP+ cells, allowing for analysis of cell shape and location of single cells within the epithelium (Figure 22A). Thick sections were taken through the undivided foregut and confocal stacks were made, allowing for the reconstruction of entire cells to quantify the variety of cell shapes. Based on their apico-basal location within the epithelium, cells were categorized into four different classes: apical only, basal only, apical to basal (i.e. spanning the entire epithelium), or NA (not quantifiable). If the foregut epithelium were stratified we would expect to see labeled cells either apically or basally localized but not spanning the whole epithelial layer (apical to basal). However, if the epithelium were pseudostratified the labeled cells would only span the apico-basal axis of the epithelium and not be found basally or apically. Quantification of four embryos showed that the majority (78%) of cells span the epithelial layer while a smaller percentage are localized only at the apical or basal surface (Figure 22B). These data suggest that the foregut epithelium becomes pseudostratified as opposed to stratified prior to compartmentalization. Since pseudostratification could contribute to compartmentalization, and since compartmentalization does not occur in mutant foreguts lacking dorso-ventral patterning, we wanted to determine the relationships
between dorso-ventral patterning, pseudostratification and foregut compartmentalization.
Figure 22: The foregut epithelium is pseudostratified at E10.5

A) *Shh*<sup>CreER<sup>T<sup>26R<sup>YFP</sup> mice are treated with tamoxifen for 24 hours and than collected and stained with GFP (green), E-cadherin(red), Laminin (white) and DAPI (blue) to visualize location of cells within the epithelium. B) Shape of cells was quantified into four classes regarding their position within the epithelium: apical-basal, apical only, basal only, and undetermined (N/A). Scale bar=.1mm
3.3.4 Ablation and activation of $\beta$-Catenin in the ventral foregut result in two unique models to study dorso-ventral patterning

The first step in determining which epithelial cell behaviors might be regulated by dorso-ventral patterning was to identify a mouse model with quantifiable alterations in dorso-ventral patterning. We carefully characterized the dorso-ventral patterning defects in two different mouse models: an ablation and an activation of $\beta$-catenin specifically in the ventral foregut. Previous studies have shown that $\beta$-catenin is both necessary and sufficient to establish NKX2-1 protein expression, a crucial marker of ventral identity (A M Goss et al. 2009; Harris-Johnson et al. 2009). In addition, both ablation and activation of $\beta$-catenin in the ventral foregut cause a loss of foregut compartmentalization (A M Goss et al. 2009; Harris-Johnson et al. 2009). While these previous studies establish that alterations, whether ablation or activation, in ventral foregut $\beta$-catenin expression result in a loss of compartmentalization, they do little to identify the etiologies behind these defects and instead focus on the defects in lung development that occur in these embryos (A M Goss et al. 2009; Harris-Johnson et al. 2009). Therefore we wanted to examine these two mouse models to determine if we could use them to study cellular behaviors at the dorso-ventral midline when dorso-ventral patterning was disrupted.

To both ablate and activate $\beta$-catenin in the ventral foregut we used $Shh^{GFPCre+}$, which at E10.5 is expressed in the ventral foregut. To ablate $\beta$-catenin, we used an allele
in which exons 2 through 6 are flanked by Loxp sites. Thus, Cre recombination generates a null allele in tissues expressing Shh ($\beta$-catenin$^{\text{Cre}}$). Alternatively we activated $\beta$-catenin by using an allele in which only exon 3 of $\beta$-catenin in flanked by Loxp sites. Exon 3 contains the phosphorylation domain without which the protein is incapable of being degraded ($\beta$-catenin$^{\text{ACT}}$). Previous work showed that removal of $\beta$-catenin results in a loss of Nkx2-1 while an activation of $\beta$-catenin does not appear to disrupt dorso-ventral patterning (Harris-Johnson et al. 2009). To establish that the dorso-ventral patterning defects were as reported, we examined the pattern of SOX2 (a dorsal marker) and Nkx2-1 (a ventral marker) using antibody staining at E10.5. In wildtype embryos the ventral and dorsal epithelia are clearly marked by anti-Nkx2-1 and anti-SOX2 (Figure 23A). Upon close examination, it was clear that the dorso-ventral midline region is composed of Nkx2-1 /SOX2 double-positive cells. Intriguingly, this double-positive region of cells has not been previously characterized or its importance investigated.

When $\beta$-catenin is removed, there is a complete loss of Nkx2-1 expression (Figure 23B). Lastly, activation of $\beta$-catenin resulted in what appeared to be normal ventral Nkx2-1 expression and dorsal SOX2 expression (Figure 23C). However, when the dorso-ventral midline was examined, there were no double-positive cells present in $\beta$-catenin$^{\text{ACT}}$ embryos (Figure 23D). This interesting result suggests that the double-positive cell
population may be interesting, as no cause for the loss of foregut compartmentalization has been established in the $\beta$-catenin$^{ACT}$ model.
Figure 23: β-catenin is necessary and sufficient for Nkx2-1 expression

A) Wildtype embryos stained with dorsal marker SOX2 (pink) and ventral marker NKX2-1 (green) have three regions of cells Sox2+ only, Nkx2-1+ only and double+ cells. B) Loss of ventral β-catenin results in a complete loss of Nkx2-1 expression throughout the foregut. C) Over activation of β-catenin expands the Nkx2-1+ only domain and results in almost no double+ cells. D) Quantification of Sox2+ and Nkx2-1+ cells throughout the undivided foregut tube shows a significant loss of double+ cells in activated β-catenin mutants. Scale bar=.1mm
3.3.5 **β-catenin**\textsuperscript{ACT} is a novel model of compartmentalization defects

To further characterize the β-catenin models, we wanted to establish whether their uncompartmentalized foregut tubes were esophageal or tracheal in nature. To accomplish this, E17.5 whole foreguts were stained with Alcian Blue to examine the laryngeal and trachea cartilage. Additionally, foregut sections were stained with the cartilage marker, anti-SOX9, and the smooth muscle marker, anti-aSMA. In control foreguts, the laryngeal cartilage and the regularly spaced ventral trachea cartilage rings formed normally (Figure 24A,D). Furthermore, aSMA marked the trachealis muscle along the dorsal aspect of the trachea as well as well-organized smooth muscle rings around the esophagus. In β-catenin\textsuperscript{CKO} embryos, the laryngeal cartilages appeared relatively normal, but 100% of the trachea cartilage rings were lost (Figure 24B,E). Furthermore, when sectioned the single tube appeared to be esophageal in nature based on the concentric rings of esophageal muscle. In contrast, activation of β-catenin in the ventral foregut resulted in a larger single tube that appeared to have both esophageal and trachea characteristics as seen by the presence of ventral SOX9 and dorsal aSMA (Figure 24F). In whole mount, cartilage rings were still present but not nicely organized as in the control embryos suggesting that ventral patterning is present but not normal (Figure 24C). This is the first report of a single uncompartmentalized foregut with both esophageal and tracheal differentiated cell types, localized relatively normally, late in
development in the same tube. This makes this model even more intriguing, as it suggests that the double-positive cells at the dorso-ventral midline may be necessary for proper compartmentalization.
Figure 24: Ventral activation of $\beta$-catenin causes foregut compartmentalization defects without disrupting dorso-ventral differentiation

A-C) Bone preparations of E18.5 foreguts show cartilage in blue and bone in red. A) At E18.5 a well developed larynx and tracheal cartilage rings are present. B) Removal of $\beta$-catenin in the ventral foregut endoderm results in slight laryngeal defects and complete loss of tracheal cartilage. C) Ectopic expression of $\beta$-catenin in the ventral foregut causes slight laryngeal defects along with disrupted tracheal cartilage patterning (arrow). D-F) Transverse sections through E15.5 wild type (D), $\beta$-catenin$^{Cko}$ (E), $\beta$-catenin$^{ACT}$ (F), foreguts stained with smooth muscle marker, alpha-SMA (red) and cartilage marker, SOX9 (green). Scale bar=2mm
3.3.6 \textit{β-catenin} is necessary for pseudostratification of the ventral foregut

After establishing that the loss of double-positive cells at the dorso-ventral midline occurred alongside the loss of foregut compartmentalization, we wanted to determine the relationship between the double-positive cells and pseudostratification. To determine if the dorso-ventral midline was important in the transition from columnar to pseudostratified, we compared the undivided foreguts of wildtype, \textit{β-catenin}^{CKO} (no D/V patterning), and \textit{β-catenin}^{ACT} (no double+ cells) embryos at E10.0. Foreguts were stained with antibodies against NKX2-1, SOX2, and E-Cadherin. Wildtype foreguts had a clear region of Nkx2-1+ Sox2+ cells at the midline and multiple layers of nuclei were visible (Figure 25A,D). Furthermore, when visualized using E-cadherin the epithelium appeared pseudostratified as the morphology of the cells was thin in width and elongated in height. (Figure 25G). In contrast, \textit{β-catenin}^{CKO} embryos had no Nkx2-1+ cells while E-cadherin and DAPI staining showed a single cell layer of nuclei and a visually columnar epithelium (Figure 25B,E,H). Lastly, \textit{β-catenin}^{ACT} embryos had a clear loss of double-positive Nkx2-1 and Sox2 cells, but no change in epithelial height compared to control samples and visually appeared to be pseudostratified based on epithelial morphology (Figure 25C,F,I). This very interesting result suggested ventral patterning might be necessary for pseudostratification.
Figure 25: Removal of ventral $\beta$-catenin disrupts pseudostratification

A-F) Visualization of the dorso-ventral midline epithelium using DAPI shows wildtype, and $\beta$-catenin$^{ACT}$ embryos have multiple layers of nuclei compared to the single cell layer in mice with $\beta$-catenin removed. G-I) E-cadherin staining in the same region of foregut epithelium suggests the epithelium in the $\beta$-catenin$^{CKO}$ mice is columnar instead of pseudostratified. Scale bar=.05mm
3.3.7 *Nkx2-1* is not necessary for pseudostratification of the ventral foregut prior to compartmentalization

Based on the apparent loss of pseudostratification resulting from an absence of ventral \( \beta\)-catenin, we hypothesized that ventral patterning per se is necessary for pseudostratification, rather than \( \beta\)-catenin itself. To determine if this was true, *Nkx2-1* null embryos were obtained from the Rubenstein lab (Minoo et al. 1999). These embryos were examined for \( \beta\)-Catenin, NKX2-1, SOX2, and E-cadherin expression to address the importance of *Nkx2-1* in pseudostratification. Control embryos had normal dorso-ventral patterning and epithelial morphology based on \( \beta\)-catenin and E-cadherin protein localization (Figure 26A-D). Surprisingly, *Nkx2-1*\( ^{-/-}\) foreguts visually appeared to be pseudostratified based on epithelial morphology with normal E-cadherin and \( \beta\)-catenin localization (Figure 26E-H). This ruled out *Nkx2-1* as a regulator of pseudostratification and suggested that pseudostratification is not directly necessary for foregut compartmentalization.
**Figure 26: Loss of Nkx2-1 does not disrupt pseudostratification of the ventral foregut**

A & E) Nkx2-1\(^{-/-}\) mice have no detectable NKX2-1 (green) protein and expanded region of SOX2 (pink) when compared to control embryos. B & F) Loss of Nkx2-1 does not disrupt β-catenin (green) protein expression in the foregut. C-D & G-H) Nkx2-1\(^{-/-}\) embryos stained with E-cadherin (pink) Laminin (white) and nuclear stain, DAPI (blue), show no loss of pseudostratification when compared to control samples. Scale bar=1mm
3.3.8 Dorso-ventral patterning does not regulate ephrin-B2 gene expression

While it was not possible to identify any cellular behaviors clearly regulated by dorso-ventral patterning, it seemed likely that expression of genes known to be necessary for foregut compartmentalization and expressed in a midline specific pattern might be regulated by D/V patterning. Two candidate genes stood out as potentially important based on previous work: ephrin-B2 and Barx1.

A recent study has shown that Eph-ephrin signaling appears to be active specifically at the dorso-ventral midline of the foregut (Dravis & Henkemeyer 2011). Eph-ephrin signaling is a complex receptor tyrosine kinase signaling pathway that is often active in the development of boundaries during morphogenesis (Himanen et al. 1998). This signaling pathway has historically been studied largely in the development of the brain and nervous system, but recently has been implicated in multiple compartmentalization events, including uro-genital compartmentalization, palate shelf closure, body-wall closure and foregut compartmentalization (Dravis et al. 2004; Dravis & Henkemeyer 2011). The Eph-ephrin pathway is comprised of 13 members, which are separated into two classes: an A-subclass and a B-subclass (Toth et al. 2001; Himanen et al. 1998). These two classes are distinguished by the structure of their ligands. The A-subclass ligands are extracellular and attached to the cell membrane by a glycosylphosphatidylinositol anchor (GPI). The B-subclass ligands span the membrane
and have a cytoplasmic tail, giving the ligand the ability to reverse signal to their own cell when attached to a receptor (Holland et al. 1996; Brückner et al. 1997). Therefore we decided to determine which Eph-ephrin family members were present in the foregut during foregut compartmentalization. E10.5 foregut RNA was collected and QPCR for different Eph-ephrin family members was performed. When expression levels of the different family members were normalized to the entire E10.5 embryo, receptors EphB3 and EphB4 were enriched in the foregut while ephrinB2 was the only ligand significantly enriched (Figure 27A). This supported the previous work showing compartmentalization defects occurred when ephrin-B2 reverse signaling was specifically disrupted by replacing the cytoplasmic signaling domain with a LacZ domain (Dravis et al. 2004). As a result, foregut defects occurred in ephrin-B2LacZ/LacZ embryos at an incidence rate of 47% of null embryos (Dravis & Henkemeyer 2011). Additionally, the foregut expression pattern of EphB3 is especially interesting because it is found specifically at the foregut midline prior to and during compartmentalization (Dravis & Henkemeyer 2011). Because a loss of reverse signaling results in a loss of foregut compartmentalization, the localization of the active ligand is crucial to determining the role of Eph-ephrin signaling in the foregut. Active ephrin-B ligand (ephrin-B1-3) is found throughout the foregut epithelium and the mesenchyme at the dorso-ventral midline via staining with a phospho-antibody (Dravis & Henkemeyer
2011). These data together suggest that Eph-ephrin signaling is active at the midline region during compartmentalization and is at least partially necessary for compartmentalization. Furthermore, Eph-ephrin signaling is an interesting candidate to regulate key cell behaviors at the dorso-ventral midline because in other developmental contexts it regulates processes such as boundary formation, guided migration and cytoskeletal dynamics, which could be important for foregut compartmentalization (Kullander & Klein 2002).

In addition to evidence supporting ephrin-B2 signaling as necessary for foregut compartmentalization, canonical Wnt signaling has been shown to regulate localization of EphB-ephrinB expression in the intestine (Clevers and Battle 2006). To determine if β-catenin, or dorso-ventral patterning, was regulating ephrinB2 expression levels during foregut compartmentalization, qPCR was preformed on E10.0 un-compartmentalized foreguts from wildtype, β-catenin<sup>CKO</sup> and β-catenin<sup>ACT</sup> embryos. No significant changes in ephrinB2 expression were found in either mutant (Figure 27B). However, it is possible the dorso-ventral patterning is regulating localization of EPHRINB2 rather than its expression levels. Unfortunately, there are few tools available to address EPHRINB2 localization at this time. Furthermore, it is unknown whether D/V patterning is established in ephrin-B2 mutants. If so, then this would be a very attractive model to directly examine the cellular behaviors involved in foregut compartmentalization.
Figure 27: Expression of Eph-ephrins during foregut compartmentalization

A) qPCR of Eph-ephrin pathway members showed two receptors EphB3 and EphB4 and one ligand ephrin-B2 were enriched in the foregut at E10.5 when compared to the whole embryo. B) qPCR of ephrin-B2 in wild type, β-catenin<sup>CKO</sup>, β-catenin<sup>ACT</sup> embryos showed no change in ephrin-B2 expression levels with disruptions in dorso-ventral patterning.
3.3.9 *Barx1* gene expression is regulated by *β-catenin* in the foregut

The second candidate we chose to examine was the indirect *Wnt* inhibitor, *Barx1*. Embryos lacking *Barx1* appear to have almost normal dorso-ventral patterning based on SOX2 and NKX2-1 expression, yet the foregut fails to compartmentalize (Woo et al. 2011). This makes *Barx1* an attractive downstream target of dorso-ventral patterning. During stomach and foregut development, *Barx1* is found in the mesenchyme underlying the endoderm (Kim et al. 2005). It may be required in this context for the expression of the secreted *Wnt* agonists, secreted frizzled-related proteins (sFRPs) 1 and 2, which block *Wnt* signaling within the overlying dorsal endoderm (Woo et al. 2011; Kim et al. 2005). In the foregut, *Barx1* is found specifically in the dorsal mesenchyme, and *Barx1*−/− embryos appear to have a similar TEF-like foregut defect to *β-cateninACT* embryos (Harris-Johnson et al. 2009; AM Goss et al. 2009; Woo et al. 2011). While *Barx1* is not expressed specifically at the D/V midline, it is one of the only known models that display almost normal dorso-ventral patterning and TEF. We examined *Barx1* expression by QPCR in wildtype, *β-cateninCKO* and *β-cateninACT* E10.5 foreguts. Excitingly, we found a significant increase in *Barx1* in *β-cateninCKO* foreguts compared to wildtype and *β-cateninACT* foreguts (Figure 28). Because *Barx1* is normally found in the dorsal mesenchyme, it seems possible that a loss of ventral patterning actually results in ventral expansion of *Barx1* expression (Figure 29).
Figure 28: *Barx1* is upregulated when ventral $\beta$-catenin is absent

qPCR of *Barx1* in wild type, $\beta$-catenin$^{\text{CKO}}$, $\beta$-catenin$^{\text{ACT}}$ embryos showed no change in *Barx1* expression levels when there was a loss of double-positive cells at the dorso-ventral midline but there was a significant increase in *Barx1* expression when ventral patterning was lost.
Figure 29: Regulation of foregut compartmentalization by dorso-ventral patterning

Work in this chapter has shown the ventral $\beta$-catenin expression regulates pseudostratification independent of ventral patterning. Furthermore, $\beta$-catenin$^{ACT}$ embryos have a specific loss of midline NKX2-1/SOX2 double-positive cells that may be important for foregut compartmentalization. This model will allow for future investigation into cellular behaviors occurring at the dorso-ventral midline. Lastly, gene expression changes in $\beta$-catenin$^{CKO}$ and $\beta$-catenin$^{ACT}$ embryos suggest that Barx1 expression may be regulated by dorso-ventral patterning.
3.4 Discussion

Previous work has established that dorso-ventral patterning and notochord resolution are both necessary for foregut compartmentalization defining the roles of many major signaling pathways, however, it did not determine the actual cellular mechanism behind foregut organogenesis. Additionally, the importance of the dorso-ventral boundary has never been investigated even though it is where the actual separation of the esophagus and trachea occurs. In this study, we carefully document normal changes in epithelial morphology during compartmentalization and investigate the importance of those changes at the dorso-ventral midline using two mouse models with different severities of disrupted dorso-ventral patterning.

Through examination of wildtype foreguts prior to and during compartmentalization, we identified that E-cadherin protein expression was not visibly changed at the point of compartmentalization. While E-cadherin expression is a master regulator of “epithelial-ness”, the epithelium could still be in a state of EMP at the point of compartmentalization. One potential explanation is that the E-cadherin present, is actually more dynamic within the cell-cell junctions resulting in weak cell-cell interactions and more movement within the epithelial layer. During zebrafish pharyngeal pouch formation, live imaging of tagged E-cadherin has shown that junction proteins move in and out of junctions more rapidly in regions of the epithelium.
undergoing rearrangements (Choe et al. 2013). Determining if a situation like this is occurring during foregut compartmentalization will require the establishment of a live imaging system for mouse foregut compartmentalization. In addition, our examination of actin localization during foregut compartmentalization has shown that apical actin and filopodia are present at the point of compartmentalization. However, while Phalloidin staining is not sufficient to determine if a constriction event is actually occurring, yet it seems unlikely that constriction is occurring since lateral ridges are not present when examined by SEM (Metzger et al. 2011). In the future, computer modeling of the compartmentalization event could predict the amount of actin constriction necessary for two separate tubes to form (Kim et al. 2013).

The most interesting finding was that $\beta$-catenin$^{\text{CKO}}$ embryos appeared to lack pseudostratification not due to a loss of ventral $Nkx2-1$ (Figure 29). This data suggests that $\beta$-catenin itself is regulating pseudostratification of the foregut epithelium. This needs to be investigated further as $\beta$-catenin could be playing either a structural or signaling role in the regulation of pseudostratification. To dissect the two functions in the mouse embryonic foregut it would be necessary to ablate each function individually using conditional knockout mice (Valenta et al. 2011). Very little is known about the regulation of pseudostratification and dissecting the importance of $\beta$-catenin in this event would be very interesting and novel.
While this study was not able to test the necessity of EMP, actin or pseudostratification, we were able to determine that a special set of NKX2-1/SOX2 double-positive cells are likely important for compartmentalization. By characterizing the $\beta$-catenin$^{ACT}$ more thoroughly, we identified that dorso-ventral patterning is not in fact normal, and there is a population of NKX2-1/SOX2 double-positive cells missing (Figure 29). When late stage un-compartmentalized foreguts were examined for differentiation markers of the esophagus and trachea, we found that $\beta$-catenin$^{ACT}$ embryos had markers from both. This is in comparison to the complete loss of ventral cartilage in $\beta$-catenin$^{CKO}$ embryos. In the future it will be necessary to test what is special about this population of cells and confirm that the compartmentalization defect is a direct result of these cells missing not the activation of $\beta$-catenin. As this will be difficult to test in vivo, it is likely this work will need to be completed in culture.

Lastly, we were able to show by qPCR that Barx1 gene expression is increased when ventral patterning is lost. This is significant because very little is known about what role Barx1 is playing in the foregut and how it is regulated. Previous work suggested that mesenchymal Barx1 represses Wnt signaling in the dorsal foregut endoderm (Woo et al. 2011). However, it is possible that Barx1 localization within the mesenchyme is regulated by the ventral endoderm through an unknown signal (Figure 29). In the stomach mesencymal Barx1 is down-regulated after epithelial specification by
two microRNAs miR-7a and miR203 (Kim et al. 2011). We attempted to examine Barx1 expression localization by in situ and protein localization by immunofluorescence however, neither of these techniques worked specifically in the foregut. Due to our lack of methods to visualize Barx1 in our system, we cannot conclude that the increase in Barx1 expression levels is due to an expansion in the Barx1 domain. In addition, there is a very promising cellular mechanism for which Barx1 may regulate foregut compartmentalization. Based on work in the stomach, it is possible that Barx1 is necessary for Wt1 expression, a transcription factor involved in EMT (Kim et al. 2007). In the foregut Wt1 is localized at the dorso-ventral midline prior to compartmentalization (Que et al. 2007). Further work will be needed to determine if Barx1 in the foregut is regulating Wt1 and potentially an EMT-like event.

In conclusion, this study has been able to characterize some key cellular behaviors during foregut compartmentalization and open new avenues to be investigated. However, there is still much to learn about the cellular behaviors necessary during foregut compartmentalization. In addition to the characterization of foregut behaviors, we have established β-catenin\textsuperscript{ACT} embryos as a novel model to guide future work regarding the importance of NKX2.1/SOX2 double-positive cells at the dorso-ventral midline.
4. Conclusions

4.1 Discussion of Results

4.1.1 Epithelial Shh is necessary for cell survival and cartilage initiation during mandible development

Prior to this study, most of the knowledge regarding Shh signaling during mandible outgrowth was based on tissue ablation and ectopic bead experiments in chick (Brito et al. 2008; Brito et al. 2006). This work established a paradigm for Hh signaling as important for both NCC cell survival and chondrogenesis. Work in chick set a foundation for the identifying the role of Shh in the mammalian development. However, due to the severe midline defects in Shh-/- mice, it was previously difficult to isolate a specific function of Shh in the mandible. To build on these findings, work from Chapter 2 shows that in a mammalian system Shh plays a role in survival and differentiation of post-migratory NCC and therefore mandible outgrowth. Using a tissue specific ablation, this study is the first to show in mammals that the pharyngeal epithelium is the source of Shh necessary for proper mandible development. Additionally, this is the first work to separate the roles of Shh in cell survival and chondrogenesis in vivo.

Our model of micrognathia is unique, as previous work in mouse and chick has not ablated Shh specifically in the PA1 epithelium. This approach allowed us to examine the specific role of Shh without confounding factors from altered signaling within the NCC or the addition of transient protein. Additionally, our loss of Meckel’s cartilage
phenotype is different from previous work showing that the removal of Smo, a Shh mediator, specifically in the NCC caused a hypoplastic mandible but not a total loss of Meckel’s cartilage (Jeong et al. 2004). This difference in phenotype led us to hypothesize the Shh was important specifically in chondrogenesis and not just cell survival. To address this, we treated pregnant females with pifithrin-α, a p53 inhibitor, to bypass the early cell death phenotype. Originally, we anticipated that this would fully rescue the mandible outgrowth defect; however, the cell death phenotype was rescued but cartilage initiation was not. This exciting result supported previous working implicating Shh in chondrogenesis of various cartilages (Park et al. 2010). When RNA expression levels of cartilage specific genes were examined, there was no evidence that Shh was regulating cartilage initiation, as no significant changes in Tgfβ-1 and NCAM expression levels were observed. However, a loss of Sox9 and Col2a gene expression in pifithrin-α treated Nkx2.5Cre;Shh<sup>fx</sup> embryos supported a role for Shh signaling in later stages of cartilage differentiation. Most interesting is that our data suggest a novel role of Shh signaling as a transcriptional repressor of Col1a in the mandible. Col1a is normally involved in promoting bone development (Grant et al. 1996) and usually seen downregulated in tissues undergoing chondrogenesis.

The pharyngeal and foregut epithelia are important signaling centers for the development of many structures, including the mandible. For example, Shh has been
shown to be important in tracheal cartilage patterning (Park et al. 2010). Therefore, the
information we have gained regarding Shh in the chondrogenesis of the mandible can be
applied to other systems such as the foregut. Investigating the relationship between
epithelial signals and their targets is necessary to gain a better understanding of many
developmental events as the underlying mechanism is often conserved.

4.1.2 β-catenin regulates pseudostratification in the foregut
epithelium through a mechanism independent of ventral patterning

While data from Chapter 2 show the importance of signaling from the epithelium
in driving mesencymal cell behaviors, experiments in Chapter 3 attempt to investigate
the importance of epithelial morphology during compartmentalization. One of the
behaviors that is plausibly involved in the compartmentalization event is
pseudostratification. Currently, very little is known about the importance and regulation
of pseudostratification, which is interesting as it is occurs in multiple developmental and
adult epithelia (Grosse et al. 2011; F. C. Sauer 1936; Zwaan et al. 1969). Most research
focusing on pseudostratification has been centered on the mechanics of the cell division
event that results in pseudostratification. This previous work establishes both actin and
microtubule dynamics as necessary for the movement of the nucleus during the cell
cycle (Spear & Erickson 2012). Results presented in Chapter 3 are the first to demonstrate
that β-catenin, directly or indirectly, regulates pseudostratification in any epithelia tissue.
Furthermore, we show that the β-catenin dependent loss of pseudostratification is not
just a downstream effect of losing ventral patterning, as pseudostratification is normal in \(Nkx2-1^{-/-}\) embryos. While these experiments do not test if pseudostratification is directly necessary for foregut compartmentalization, the work presented in Chapter 3 establishes a new model to study the genetics behind pseudostratification.

### 4.1.3 The dorso-ventral midline consists of a population of SOX2/NKX2-1 double-positive cells which are important in compartmentalization

The past 2 to 3 decades have resulted in the advent of many genetic mouse models that have well characterized foregut defects. However, establishing the gene network involved in notochord resolution and dorso-ventral patterning has provided little to no information regarding the mechanism by which the single foregut tube compartmentalizes into two distinct tubes. As a result, we still know almost nothing about the cellular behaviors occurring during compartmentalization. Previous work has established the necessity of proper dorso-ventral patterning and notochord resolution (Fausett et al. 2014; Minoo et al. 1999; Que et al. 2006) but no models have been established which have compartmentalization defects but normal dorso-ventral patterning. The work presented in Chapter 3 establishes mice with an activation of \(\beta\)-catenin in the ventral foregut, as a novel model of foregut defects. This model currently has no known etiology regarding the loss of compartmentalization as dorso-ventral patterning is relatively normal and no notochord defects have been observed. The
defining feature of this new class of defect is a specific loss of NKX2-1/SOX2 double-positive cells at the dorso-ventral midline but no complete loss of either dorsal or ventral patterning. Further investigation into the phenotype at later stages showed that the uncompartmentalized foregut tube was properly patterned with differentiated smooth muscle and cartilage, even though compartmentalization does not occur. This is the first example of an uncompartmentalized foregut with both esophageal and tracheal characteristics and, presumably, without notochord defects. However, there is the caveat that this model is created by an activation of β-catenin, which could cause defects that may confound any results regarding normal cell behaviors during compartmentalization. Yet even with that caveat, previous work has shown no increases in proliferation in these mutants, one of the major concerns when activating β-catenin (Harris-Johnson et al 2009). In conclusion, this is the first mouse model that will potentially allow investigation into the cellular behaviors occurring at the dorso-ventral midline during compartmentalization.

4.2 Future Studies

4.2.1 How is Shh regulating cartilage development and is it through an Fgf8 dependent mechanism?

One of the major remaining questions from our data in Chapter 2 is by what mechanism does Shh regulate cartilage development in the mandible? In the trachea, Shh is necessary for Sox9 expression through a BMP/Noggin dependent mechanism (Park et
al 2010). However, in the mandible Fgf8 is capable of rescuing loss of cartilage *ex vivo* after treatment with the Shh antagonist cyclopamine (Melnick et al 2005). In our model, we see a complete loss of PA1 Fgf8 by *in situ* and would predict that Shh is signaling through Fgf8 during chondrogenesis. Future work will need to address if chondrogenesis in our mutants can be rescued by treatment with FGF8 in culture. Furthermore, treatment with FGF8 in culture will determine if Fgf8 regulates Sox9, Col1a and Col2a or if the changes we saw in their expression levels were Shh specific.

Additionally, the absence of Shh in the ventral foregut epithelium results in laryngeal and otic defects, which are derived from the second pharyngeal arch. In the future it would be interesting to establish if these defects result from a loss of cell survival and cartilage differentiation, as in the mandible.

**4.2.2 How is β-catenin regulating pseudostratification?**

Our finding that β-catenin is necessary for pseudostratification of the foregut epithelium is the first time a signaling pathway has been connected with foregut pseudostratification. However, there are two important questions which our work was unable to address: 1) Is β-catenin sufficient for pseudostratification and 2) Which functional role of β-catenin is necessary for pseudostratification?

To address the first question, β-catenin must be activated in a tissue that is not normally pseudostratified, ideally the dorsal foregut epithelium. While we had an allele
that conditionally activates $\beta$-catenin, there unfortunately was no Cre specific to the dorsal foregut epithelium when this experiment was underway. However, now there is a Sox2$^{CreER^{+}}$ mouse (Arnold et al. 2011), which could potentially activate $\beta$-catenin specifically in the dorsal foregut. Furthermore, this Cre would allow for temporal control of $\beta$-catenin activation, which would bypass any early defects due to increased canonical Wnt signaling. Activation of $\beta$-catenin in the dorsal foregut would determine if $\beta$-catenin is sufficient to induce pseudostratification of a simple columnar epithelium. In addition to this experiment, it would be important to see if the paradigm of $\beta$-catenin regulation of pseudostratification holds true in other epithelia.

While establishing the sufficiency of $\beta$-catenin in pseudostratification is important, identifying the mechanism by which $\beta$-catenin regulates this behavior is potentially a more important question. There are two functional roles of $\beta$-catenin that could be responsible for pseudostratification, a cell-cell junction role and Wnt signaling transduction role (Daugherty & Gottardi 2007). $\beta$-catenin is a structural component of Adherens junctions, which connect epithelial cells. Within the junctional complex, $\beta$-catenin connects cadherins to the actin cytoskeleton. Pseudostratification is known to utilize the cytoskeleton to move the nucleus during cell division and it is possible that the structural function of $\beta$-catenin is necessary for this event. The other alternative is that canonical Wnt signaling is important in activating an unknown set of genes.
necessary for pseudostratification. While we were unable to identify which role of β-
catenin is important for this phenotype, it is now possible to do so as a mouse has been
developed which specifically ablates each function of β-catenin individually (Valenta et
al 2011). By using these two new alleles to individually disrupt β-catenin function in the
ventral foregut and examining the pseudostratification of the foregut, this question can
be answered. Once it is known which function of β-catenin is necessary for
pseudostratification there are many questions regarding regulation of
pseudostratification in the foregut that will be able to be addressed.

4.2.3 Are NKX2-1/SOX2 double-positive midline cells regulating
midline specific gene expression?

Data presented in Chapter 3 established a new mouse model that suggested to us
that the NKX2-1/SOX2 double-positive cells at the dorso-ventral midline were
potentially important for compartmentalization. One of the caveats previously
mentioned is that this model is the result of an activation of β-catenin. To further prove
that the loss of compartmentalization in this model is due to the absence of the double-
positive cells and not increased β-catenin signaling, a different model will need to be
used. Currently, there are no genetic tools available in mice to ablate the double-positive
region of cells. Fortunately, the mouse foregut is amenable to culturing ex vivo. The
uncompartmentalized foregut will develop normally in culture for up to 72 hours and
separate into an esophagus and trachea (Figure 30). It is possible to overexpress Nkx2-1
in the midline epithelium by electroporation of Nkx2-1 into the double-positive cell region prior to foregut compartmentalization and allowing the foreguts to develop in culture. Overexpression of Nkx2-1 would result in a loss of double-positive cells by repressing Sox2 expression, a similar mechanism to that which occurs in the β-catenin^{ACT} mice. While this technique is not as precise as an in vivo approach, it would allow us to exclude extopic β-catenin expression as the cause for the loss of compartmentalization in the β-catenin^{ACT} mice.

Additionally, an ex vivo culture approach would allow for more direct examination of the midline genes necessary for compartmentalization. Using cultured foreguts, one lateral wall of the foregut can be manipulated while using the other side as a control. With this experimental model, we can potentially identify midline genes that are regulated by changes in dorso-ventral patterning since we can compare changes within a single sample.

One of the experiments we were unable to complete was to show Barx1 localization in the foreguts of control and β-catenin^{Cre} embryos. Previous work shows that Barx1^{+} embryos have normal dorso-ventral patterning (Woo et al. 2011), therefore we can assume that Barx1 does not regulate dorso-ventral patterning but instead is potentially regulated by it. To support this, our expression data suggest an increase in Barx1 occurs when ventral patterning is lost. At this time there are no working
antibodies or in situ probes available, so any experiments regarding the localization of Barx1 cannot be conducted. However, by using an ex vivo approach it is possible to address if Barx1 needs to be restricted to the dorsal mesenchyme for compartmentalization to occur. This could be accomplished by placing Barx1 coated beads in the ventral mesenchyme, allowing development to occur ex vivo, and then checking for compartmentalization defects.

The second question that we were unable to address is how does loss of reverse ephrin-B2 signaling result in an uncompartmentalized foregut tube. From previous work on the ephrin-B2^{LacZ/LacZ} mouse, it is known that reverse ephrin-B2 signaling is likely taking place in the foregut epithelium at the dorso-ventral midline (Dravis & Henkemeyer 2011). However, the ephrin-B2^{LacZ/+} mice have severe breeding problems making them a difficult model to use. An alternative would be to electroporate a dominant negative form of ephrin-B2, which disrupts reverse signaling, into the foregut epithelium prior to compartmentalization. This approach would allow us to gain better insight into the function of ephrin-B2 during foregut compartmentalization and potentially identify some important cell behaviors without the difficulties present in the mouse model.
Foreguts can be dissected out of E10.0 embryos (28s) and cultured in liquid media for over 72 hours. When the foregut is removed from the embryo the mesenchyme directly attached to the epithelium is retained to help support the epithelium. Cultured foreguts develop slightly slower than they do in vivo but do separate into a normal esophagus and trachea in addition to forming lung buds. A-C) Foreguts that have developed in vivo and have been dissected and stained with E-cadherin, an epithelial marker, compartmentalize between 28 and 35 somites. D-F) Foreguts developed in culture, starting at 28s, diving normally by 72 hours. This work was done in collaboration with Sarah Fausett.

Figure 30: Organ culture of dissected foreguts
4.2.4 Can modeling be used to predict which cellular behaviors are necessary for compartmentalization?

Traditionally, approaches of genetic ablations and visualization using antibodies have proved to be unsuccessful at determining the cellular behaviors occurring during foregut compartmentalization. Data shown in Chapter 3 identified two cellular behaviors occurring during compartmentalization, apical actin and pseudostratification. However, it is difficult to say how necessary either of those behaviors is during the compartmentalization event. Additionally, the presence of apical actin does not mean that apical constriction is occurring. While it is possible to disrupt actin constriction and pseudostratification with pharmacological treatments in culture, the drugs that are used to accomplish this disrupt many other processes, confounding the results. Recently, computer modeling of lung bud formation predicted that apical constriction is sufficient to initialize lung budding in chicken development (Kim et al. 2013). A similar approach using computer modeling could be used to answer many of the questions that still exist about foregut compartmentalization.

As previously discussed, there are multiple cellular behaviors whose necessity during compartmentalization are not easily addressed experimentally. During foregut compartmentalization, there is a substantial amount of apical actin and, potentially, constriction occurring, but without live imaging or modeling we cannot assess the role of apical constriction. Furthermore, the mesenchyme is likely playing an active role by
pushing on the epithelium to shape the foregut, yet without a way to visualize this event it is difficult to determine the role of the mesenchyme. In addition to the dynamics of the actin cytoskeleton and mesenchyme, it is likely that there is some type of migration event happening within the epithelium. It is possible that this event could be a slight decrease in the adhesiveness between cells within the epithelium or it could be a much larger collective migration of cells through the “saddle” region of the compartmentalizing foregut. Without a way to visualize and model these behaviors, it is almost impossible to assess the morphogenic movements with the genetic tools in the field. Using genetically modified mice with relevant tissue domains labeled with GFP should allow the tracking of movements of cells over time with the proper imaging tools. In the future, utilizing computer modeling, in conjunction with ex vivo culture, will aid in the advancement in our understanding of the cellular behaviors occurring during foregut compartmentalization.

4.2.5 Using chicken as a model may answer questions which mice cannot

Another embryological model system that should be useful for studying foregut compartmentalization is chicken (Metzger et al. 2011). This is often overlooked because there are numerous genetic mouse models with foregut defects. Yet we have found that the chicken foregut develops in a very similar manner to that of the mouse, starting as a single endodermal tube and separating into an esophagus and trachea (Figure 31A-B).
Many of the same signaling pathway genes involved in early mouse foregut development are conserved and similarly expressed, including components and targets of FGF, BMP, and Shh signaling (Morrisey & Hogan 2010; Metzger & Krasnow 1999; Affolter et al. 2003). In addition, it is likely that similar physical mechanisms are working to shape the developing epithelium (Morrisey & Hogan 2010; Davies 2002). To examine the normal cellular behaviors occurring during foregut development, the chicken may be a superior model to the mouse. For example, in ovo manipulations of chicken embryos are substantially more facile, either by electroporation (Figure 31C) or treatment with pharmaceuticals, than live manipulations of mouse embryos. The chicken model allows for more directed alterations of wild type behaviors by injecting plasmids or drugs straight into the foregut tube and allowing the embryo to develop in ovo (Figure 31C).

Furthermore, the chicken has been published as a model of Adriamycin induced foregut defects (Naito et al. 2009). This was accomplished by injecting Adriamycin into a developing egg prior to compartmentalization and allowing the chicken to develop. In our own hands this approach resulted in caudal regression, a loss of posterior structures, which is often associated with VACTERL syndrome (Figure 31D-E). However, in our hands, such embryos do not display EA/TEF, which suggests this may not be the most sensitive phenotype to Adriamycin exposure. Nonetheless, this example establishes a method for potentially altering many different signaling pathways and cellular
behaviors by drug treatment \textit{in ovo}. To do these same experiments in mouse, the foregut must be cultured \textit{ex vivo} leading to confounding factors from the loss of surrounding structures and signals. More research will need to be done to confirm that chicken foreguts have a dorso-ventral patterning network similar to that in mice. Once that point is established, electroporation can be used to disrupt dorso-ventral specific genes identified from mouse studies to examine which cellular behaviors are important.

Furthermore, live imaging of foregut compartmentalization could be accomplished in chicken embryos. The uncompartmentalized foregut endoderm can be labeled using DiI and the labeled cells tracked through compartmentalization. By tracking the cells from multiple regions of the foregut, we could determine what types of movements are occurring during compartmentalization. In the future, it will be necessary to use alternative models to investigate the complex movements that are occurring in both the epithelium and mesenchyme during foregut compartmentalization. We are currently limited by the lack of live imaging to visualize changes in epithelial morphology and ability to interrupt specific behaviors to determine their importance.
A) At stage 20 in chicken the foregut starts as a single tube (FG) \((A')\) with lung buds (LB) \((A'')\) starting to develop. B) By stage 25 the chicken foregut has compartmentalized \((B')\) into an esophagus (E) and trachea (T). Caudal to the separated esophagus and trachea, \((B'')\) the trachea ends in two lung buds. C-D) The chicken is a system that is amenable to electroporation of plasmids directly into the foregut lumen of an embryo in ova prior to foregut compartmentalization. The embryo can then develop in ova until after foregut compartmentalization. The electroporation can be targeted to either the dorsal or ventral side of the foregut, visualized by inclusion of a marker such as GFP. C) GFP targeted to the ventral domain of the foregut. The electroporation occurred prior to compartmentalization and the foregut was dissected after compartmentalization had occurred (stage 25). D-E) Treatment of chicken embryos in ova with Adriamycin at stage 11 results in caudal regression (white arrows compare posterior structures), a loss of the posterior structures of the embryo. Caudal regression is seen in Noggin mutants and Adriamycin treated embryos. (D-E. Scale Bar = 1mm)
Many developmental pathways have been found to be necessary for proper foregut development, either during compartmentalization or lung bud outgrowth (Figure 8). While the basic signaling network regulating these events has been established, there are still some pathways where the mechanisms behind the foregut defect are unknown. One of the major unanswered questions in the field of foregut development is the importance of Sonic Hedgehog (Shh) during compartmentalization. Litingtung et al first described the foregut defects present in the Shh−/− mouse in 1998. The set of defects which occur in the Shh−/− embryos consist of a single undefined foregut tube with a complex esophageal atresia and trachea-esophageal fistula (Litingtung et al. 1998). The defects present in these embryos have been difficult to characterize due to how highly disorganized nature of the single foregut tube. Over the past decade since the Shh−/− foregut defect was first described, many studies have attempted to isolate the domain of Shh expression necessary for foregut compartmentalization. After much work it seemed likely that Shh was actually necessary early in the development of the prechordal plate, an endodermal signaling center which is present around E7 (Aoto et al. 2009). However, work from a previous lab member identified the ventral foregut domain of Shh between E8.5 and E9.5 as necessary for compartmentalization, while excluding the floorplate, notochord and later foregut expression domains (Davenport,
2013, Figure 32A-E). This was a novel finding as it narrowed down the important domain of Shh expression for foregut compartmentalization. However, this work left many open questions regarding the mechanism behind Shh foregut defects. To address these questions, we characterized the foregut phenotype resulting from the removal of ventral foregut Shh by examining dorso-ventral patterning and epithelial morphology at the dorso-ventral midline.

Due to the ventral localization of Shh expression prior to foregut compartmentalization, it seemed likely that Shh could be important for establishing ventral patterning or Nkx2-1 expression. To assess this, E10.5 foreguts were stained with NKX2-1 and SOX2 antibodies. We observed normal ventral and dorsal expression of both NKX2-1 and SOX2 along with the presence of NKX2-1/SOX2 double-positive cells at the dorso-ventral midline (Figure 32F-G). In addition, we assessed the epithelial morphology at the midline for pseudostratification using an E-cadherin antibody (Figure 32H). Visually the epithelial layer appeared to be pseudostratified and of normal thickness. While these results suggest that Shh does not appear to be necessary for dorso-ventral patterning, more embryos will need to be examined to confirm this phenotype. Furthermore, we have not examined enough late stage embryos to confirm that the loss of compartmentalization phenotype is fully penetrant.
If future work shows that loss of compartmentalization is fully penetrant at late stages and confirms that dorso-ventral patterning is normal; determining the mechanism behind the loss of compartmentalization in \( \text{Sox17Cre;Shh}^{\beta/-} \) embryos will be crucial as it likely represents a novel class of foregut defect. Comparing this model to \( \beta\text{-catenin}^{ACT} \) embryos, will allow for a greater investigation into the necessity and importance of the double-positive cell population at the midline, as both have compartmentalization defects and but different phenotypes in the double-positive cell population. Developing \( \text{Sox17Cre;Shh}^{\beta/-} \) embryos as a model to study the cellular behaviors and movements responsible for foregut compartmentalization should be a priority in the future as there is a lot of information to be gained from understanding \( \text{Shh} \) related birth defects.
Figure 32: Ventral foregut Shh is not necessary for dorso-ventral patterning or pseudostratification

A-B) The expression patterns of both Sox17Cre and Foxa2NFPCre are shown using LacZ staining at E10.5. There is slight ectopic recombination in the notochords of Sox17Cre;R26RlacZ embryos. To show that the notochord domain is not responsible for the foregut defects seen in Sox17Cre;Shhfl/fox2NFPCre, specific to the notochord and floorplate, was used to rule out any involvement of these domains. C-E) At E17.5 control and Foxa2NFPCre;Shhfl/foreguts have separated into a distinct esophagus and trachea while Sox17Cre;Shhfl/embryos have a single uncompartmentalized tube. F-H) Close examination of the foregut epithelium at E10.5 prior to compartmentalization shows that the epithelium has multiple layers of nuclei (visualized using DAPI), normal dorso-ventral patterning with NKX2-1/SOX2 double positive cells (anti-Nkx2-1 in green, anti-SOX2 in red) and pseudostratified epithelial morphology (anti-E-cadherin in white). Work presented in panels A-E are reproductions of data produced by Chandra Davenport. Scale bar=.05mm
References


Biography

Katie Elisabeth Kretovich Billmyre was born in Newton, Massachusetts in 1987. Katie grew up in Southeast Michigan and, as the daughter of two Finance professors, spent considerable time in the classroom. In 2009, Katie received her B.S in Cellular Molecular Biology from the University of Michigan. Next she moved to Durham, NC to pursue her PhD in Cell Biology at Duke in the lab of Dr. John Klingensmith. During her graduate career Katie earned a Certificate for College Teaching and a Certificate in Cell and Molecular Biology. She participated in Preparing Future Faculty, a Team-based learning fellowship, and received a Bass fellowship to develop and teach her own course. Her graduate work has resulted in the publication of a review and a manuscript. At the time of her defense she has another manuscript in preparation.

Katherine Kretovich Billmyre and John Klingensmith. “β-catenin controls Barx1 expression by regulating dorso-ventral patterning during foregut compartmentalization”

Katherine Kretovich Billmyre and John Klingensmith. “Sonic Hedgehog from pharyngeal arch 1 epithelium is necessary for early pharyngeal arch cell survival and later cartilage differentiation during mandibular development” Developmental Dynamics. In Press 2015