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Genetic and Cellular Mechanisms Regulating Anterior Foregut and Esophageal Development

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Abstract

Separation of the single anterior foregut tube into the esophagus and trachea involves cell proliferation and differentiation, as well as dynamic changes in cell-cell adhesion and migration. These biological processes are regulated and coordinated at multiple levels through the interplay of the epithelium and mesenchyme. Genetic studies and in vitro modeling have shed light on relevant regulatory networks that include a number of transcription factors and signaling pathways. These signaling molecules exhibit unique expression patterns and play specific functions in their respective territories before the separation process occurs. Disruption of regulatory networks inevitably leads to defective separation and malformation of the trachea and esophagus and results in the formation of a relatively common birth defect, esophageal atresia with or without tracheoesophageal fistula (EA/TEF). Significantly, some of the signaling pathways and transcription factors involved in anterior foregut separation continue to play important roles in the morphogenesis of the individual organs. In this review, we will focus on new findings related to these different developmental processes and discuss them in the context of developmental disorders (or birth defects) commonly seen in clinics.

Keywords

EA/TEF; Sox2; BMP; Esophageal Muscle; Myenteric System

The newborn infant quickly needs to fill its lungs with air and later, its stomach with food. The two tubes that enable these substances to enter the body - the trachea and the esophagus - are both derived from a developmental intermediate called the anterior foregut. Separation of this single tube into two separate tubes requires coordinated cellular and molecular events that are orchestrated by multiple signaling pathways and their downstream effectors, including several transcription factors. Abnormalities in these regulatory networks lead to defective separation processes resulting in birth defects such as esophageal atresia with or without tracheoesophageal fistula (EA/TEF), a condition commonly seen in clinics (de Jong et al., 2010; Williamson et al., 2006). We have previously summarized the mutations in genes encoding components of multiple signaling pathways that lead to the formation of EA/TEF in both human patients and mouse models (Que et al., 2006). In recent years, an array

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of new findings has been added to the list and new pathways involved in the regulation of foregut morphogenesis have been identified (Table 1). Significantly, recent studies have shown that signaling pathways that regulate anterior foregut separation continue to play essential roles in the subsequent organogenesis of the trachea and the esophagus. In this review, we will focus first on recent advances that further our understanding of how the dorsal-ventral patterning of transcription factors and signaling molecules regulates the separation process. We will then review how these regulatory elements participate in the subsequent development of the two tubes, with a focus on the esophagus. In addition, we will also discuss new findings related to the innervation of the esophagus.

Overview of the Separation of the Anterior Foregut Tube into the Trachea and Esophagus

Much of our understanding of foregut separation comes from experimental and genetic manipulations that have revealed the importance of reciprocal signaling between the endoderm and mesoderm during early development (Grapin-Botton and Melton, 2000; Lewis and Tam, 2006; Morrisey and Hogan, 2010). The foregut tube is derived from the ventral folding of the endodermal epithelial sheet during gastrulation at about embryonic (E) day 8.0 in the mouse (Sherwood et al., 2009; Wells and Melton, 1999). This folding is accompanied at around E9.0 by a process in which the rod-like notochord delaminates from the endoderm and becomes more closely associated with the neural tube (Jurand, 1974). Signals emanating from the notochord are essential for the dorsal-ventral patterning of the neural tube and its subsequent tissue morphogenesis (Chamberlain et al., 2008). In recent years, studies have established that once the foregut tube has formed it also exhibits dorsal-ventral patterning of signaling molecules and transcription factors in both the epithelium and the surrounding mesenchyme (Figure 1A). This dorsal-ventral expression pattern is required for normal anterior foregut morphogenesis (E9.5 –E11.5), in which the dorsal region of the tube gives rise to the esophagus and the ventral region forms the trachea and lung buds.

Dorsal-Ventral Patterning of the Transcription Factors Sox2 and Nkx2.1 in the Early Foregut

Sox2 is a member of the Sox family of conserved transcription factors, which are characterized by an Sry-related high mobility group (HMG) box. Sox2 is important for the development of multiple organs including the tongue, retina, hair follicles and inner ear and is also required for the self-renewal of embryonic stem (ES) cells (Driskell et al., 2009; Kiernan et al., 2005; Okubo et al., 2006; Taranova et al., 2006; Ura et al., 2011). Sox2 is preferentially expressed in the dorsal epithelial cells of the unseparated foregut tube at E9.5 (Figure 1B) in direct contrast to the transcription factor Nkx2.1 (also known as TTF1), which is expressed predominantly in the ventral epithelium (Harris-Johnson et al., 2009; Que et al., 2007). Proper dorsal-ventral patterning of these two transcription factors proves to be a central requirement for foregut morphogenesis. Significant downregulation of Sox2 protein to near 5% of the wildtype level leads to the formation of EA/TEF in *Sox2^{GFP/COND}* hypomorphic mutants (Que et al., 2007). By contrast, deletion of *Nkx2.1* also results in defects in foregut separation and the formation of EA/TEF with high Sox2 expression in the epithelium of the TEF (Minoo et al., 1999; Que et al., 2007). Conversely, the epithelial cells in the fistula of *Sox2^{GFP/COND}* hypomorphic mutants express high levels of Nkx2.1 suggesting that in the absence of a sufficiently high level of Sox2, Nkx2.1 expression expands dorsally and reprograms the dorsal epithelium to a respiratory fate (Que et al., 2007). These findings suggest that the dorsal-ventral arrangement of Sox2 and Nkx2.1 is required for foregut separation and the subsequent differentiation of epithelial progenitor cells into esophageal and tracheal epithelium, respectively.

SOX2 has been shown to bind the promoter region of the *NKX2.1* gene and inhibit its transcription in human embryonic stem cells (Boyer et al., 2005). Nevertheless, it remains to be determined if a similar regulatory mechanism is active during foregut morphogenesis. We have previously shown using *in vitro* organ culture that Fgf10 inhibits *Sox2* expression in the mouse foregut, but it remains to be determined whether this inhibition is mediated by Nkx2.1 (Que et al., 2007). Fgf10 is enriched in the mesenchyme of the ventral foregut before separation occurs (Figure 1A). However, foregut separation proceeds normally in *Fgf10* null mutants despite a lack of lung morphogenesis, suggesting that there are other signaling molecules involved in the regulation of Sox2/Nkx2.1 patterning in the early foregut (Min et al., 1998; Que et al., 2007).

Wnt/ β -catenin Signaling Regulates Foregut Morphogenesis

Wnt signaling is highly conserved from nematodes to humans and is a critical mediator of cell-cell signaling events during embryogenesis. Depending on the ligand engagement, Wnt signaling can be transduced through either the canonical Wnt/ β -catenin pathway or non-canonical β -catenin independent pathways. In the canonical pathway, Wnt ligands (e.g. Wnt3a, Wnt7b) bind to receptors Frizzled (Fzd) and LDL-receptor-related proteins (Lrp) -5 or -6 and inhibit the phosphorylation of β -catenin by glycogen synthase kinase 3 (GSK3) and casein kinase 1 (CK1). This stabilization of β -catenin promotes its accumulation and subsequent translocation into the nucleus where it interacts with members of the T-cell factor/lymphoid enhancer factor (TCF/LEF) family to activate the transcription of target genes (Angers and Moon, 2009; Logan and Nusse, 2004). These genes are important regulators of diverse cellular processes including differentiation, proliferation and adhesion. By contrast, the non-canonical pathways are important in regulating cell polarity and asymmetric cell division. These pathways are mediated by the binding of Wnt ligands (e.g. Wnt5a, Wnt11) to Fzd or alternative receptors (e.g. Ror2) to activate the β -catenin-independent Wnt/PKC/Ca²⁺ and Wnt/JNK polarity pathways (Oishi et al., 2003; Yamamoto et al., 2007; Yamanaka et al., 2002). While recent studies have begun to shed light on the function of canonical Wnt/ β -catenin signaling in foregut morphogenesis, non-canonical Wnt signaling remains largely unstudied in this context.

Canonical Wnt/ β -catenin signaling activity exhibits a dynamic pattern in the anterior foregut region before and after separation processes occur (Figure 2A–D). At E9.5, Wnt signaling is active in the ventral side of the unseparated foregut tube, where Wnt ligands Wnt2 and Wnt2b proteins are enriched (Figure 1A) (Goss et al., 2009; Harris-Johnson et al., 2009). Interestingly, *Wnt2* and *2b* are expressed in the mesenchyme of the ventral foregut while X-gal positive staining in the *BAT-Gal* canonical Wnt signaling reporter mouse line is limited to the epithelium (Figure 2A). This suggests that the Wnt signal receiving cells are located in the epithelium. In line with this notion, deletion of *β -catenin* in the epithelium using *Shh-Cre* results in abnormal separation of the foregut tube and complete lung agenesis (Goss et al., 2009; Harris-Johnson et al., 2009). It has further been shown that at the cellular level, Wnt/ β -catenin abrogation reduces cell proliferation by diminishing Cyclin D1 protein levels (Goss et al., 2009). Moreover, dorsal-ventral patterning of Sox2/Nkx2.1 is disrupted in *Shh-Cre; β -catenin^{loxP/loxP}* mutants in which high levels of Sox2 protein are expressed in the ventral region at the expense of the Nkx2.1+ve domain. Accordingly, the resulting fistula expresses high levels of Sox2 (Harris-Johnson et al., 2009). Consistent with the importance of mesenchymal *Wnt* expression in foregut separation, the combined deletion of *Wnt2/2b* results in similar phenotypic changes (Goss et al., 2009). Notably, *Wnt7b* is expressed in the endoderm of the early foregut and its deletion results in irregular lung branching morphogenesis and vasculature development but does not disrupt foregut separation (Shu et al., 2002).

The homeobox gene *Barx1* is highly expressed in the mesenchyme adjacent to the groove where the future trachea and esophagus split (Figure 1A). Genetic evidence suggests that *Barx1* functions to suppress Wnt signaling activity in this region and limits it to the ventral territory prior to the separation of the trachea and esophagus. Deletion of *Barx1* leads to a dorsal shift of the domain of Wnt activity accompanied by dorsal expansion of *Nkx2.1*, resulting in separation defects (Woo et al., 2011).

Foregut separation requires coordinated cytoskeletal rearrangement and cell shape changes and culminates with the division of a single lumen tube into two. Non-canonical Wnt signaling is known to be an important regulator of these cellular processes (Gros et al., 2009; Roszko et al., 2009). However, single deletion of *Wnt5a* or *Wnt11* has no reported foregut separation defects, possibly due to functional redundancy between these genes (Li et al., 2002; Majumdar et al., 2003). It will be interesting to determine if a combined deletion of *Wnt5a* and *Wnt11* induces defects in the separation process. It is noteworthy that *Rhou*, a Cdc42-related atypical Rho GTPase, has recently been identified as an upstream regulator of the *Wnt5a/JNK/PCP* pathway (Loebel et al., 2011). In the early foregut, *Rhou* expression is limited to the ventral and lateral foregut endoderm. *In vitro* knockdown of *Rhou* disrupts the differentiation and morphogenesis of foregut derivatives in cultured embryos. Reduced *Rhou* activity also attenuates the apical accumulation of F-actin and affects cellular morphology and cytoskeletal organization, disrupting the normal conversion of simple columnar epithelium into pseudostratified epithelium during foregut morphogenesis (Loebel et al., 2011). Interestingly, this epithelial conversion also occurs during foregut separation when the ventral side of the tube develops into the lung and trachea [(Loebel et al., 2011) and Que J unpublished observation]. The effect of *Rhou* deletion on the separation process *in vivo* remains to be determined.

New Findings on the Roles of Bmp Signaling in Foregut Morphogenesis

We have previously shown that Bmp signaling is required for foregut separation. In the unseparated foregut tube, the Bmp ligand *Bmp4* is preferentially expressed in the ventral mesenchyme while *Bmp7* and the Bmp inhibitor *Noggin* are enriched in the dorsal endoderm (Que et al., 2006). Consistent with this dorsal-ventral patterning scheme, Bmp signaling activity is limited to the ventral side of the foregut in the *BRE-LacZ* (Bmp signaling reporter) embryos (Figure 2E). Disruption of dorsal-ventral patterning by *Noggin* deletion leads to the formation of EA/TEF in ~70% of the mutants. Similar to the developing skeleton and heart (Brunet et al., 1998; Choi et al., 2007), *Noggin* deletion leads to increased Bmp signaling in the foregut. Removal of one copy of *Bmp4* or *Bmp7* in the *Noggin* null background rescues separation defects (Li et al., 2007; Que et al., 2006). In addition, *Noggin* deletion also induces abnormal delamination of the notochord from the early definite endoderm epithelial sheet, resulting in epithelial cells of endodermal origin being present in the kinky notochord (Li et al., 2007). These findings suggest an abnormal notochord has a two-fold contribution to defective foregut separation: (1) Abnormal delamination diminishes the quantity of endodermal cells to the point where there are not enough cells to form an intact esophagus and (2) The deformed notochord is unable to provide sufficient signaling to support tissue morphogenesis.

Further support for the importance of dorsal-ventral Bmp signaling comes from findings from the tissue specific ablation of *Bmp4*. Deletion of *Bmp4* using *Foxg1-Cre* results in tracheal agenesis accompanied by reduced cellular proliferation in both the epithelial and mesenchymal compartments. Significantly, although the trachea does not separate from the foregut, expression of the tracheal lineage marker *Nkx2.1* is preserved in the ventral endodermal epithelium, suggesting that *Bmp4*-mediated signaling is required for separation but not for the initial specification of the tracheal epithelium (Li et al., 2008). In line with these findings, deletion of *Bmp receptors 1a* and *1b* in *Shh^{cre/+}; Bmpr1a^{fl/-}; Bmpr1b^{-/-}*

compound mutants also leads to tracheal agenesis, reduction of *Nkx2.1* and ventral expansion of *Sox2*, which is associated with the abrogation of Bmp signaling. Interestingly, Wnt signaling in the foregut of these mutants is not altered, indicating that Wnt signaling does not operate downstream of Bmp during foregut separation. A genetic complementation study showed that removal of the *Sox2* gene in a *Shh^{Cre/+}; Bmpr1a^{fl/-}; Bmpr1b^{-/-}* background rescues the separation defect, further emphasizing that a dorsal-ventral distribution of signaling and transcription factors is required for foregut separation (Domyan et al., 2011).

Smad proteins, including Smad1/5/8 and Smad4, are key mediators of Bmp signaling. Upon Bmp ligand engagement, Smad1/5/8 are phosphorylated and associate with Smad4, followed by nuclear translocation and activation of downstream target gene transcription (Conidi et al., 2011). Significantly, no foregut separation defects result from the deletion of *Smad4* using *Nkx2.5-Cre*, which is active at ~E9.5 in both the mesenchyme and epithelium in the ventral foregut [(Que et al., 2009) and Que J unpublished observation]. This could be due to the fact that Smad4 mediates both Bmp and Tgf β signaling, suggesting that simultaneous loss of these two signals rescues foregut separation defects. In this vein, it will be interesting to determine how Tgf β signaling is involved in foregut morphogenesis.

Summary of Foregut Tube Separation

Sox2 and *Nkx2.1* maintain reciprocal domains of expression in the early foregut endoderm prior to separation. This patterning is controlled by a signaling network consisting of the Fgf, Wnt and Bmp pathways (Figure 1A). The disruption of signaling networks or transcription factor distribution affects cellular proliferation, differentiation and cytoskeletal rearrangement and results in separation abnormalities. Thus far, we have gained considerable insight into the patterning of the ventral side of the foregut, which is characterized by active *Nkx2.1* expression and suppressed *Sox2* expression. By contrast, we know little about the signaling pathways mediating epithelial-mesenchymal interaction in the dorsal region. This discrepancy is due in part to a deeper understanding of the respiratory derivatives of the ventral foregut than of the development the dorsal foregut derivative, the esophagus. Therefore, more studies in esophageal development are necessary to broaden our understanding of the signaling pathways that act on the dorsal foregut during separation.

Overview of Esophageal Development

Once the esophagus separates from the foregut, it undergoes extensive morphogenesis to become a functional tube that is ensheathed by layers of muscle and lined with stratified squamous epithelium. At the time of foregut separation (~E11.0) the lumen is comprised of a ciliated simple columnar epithelium. It is then gradually replaced by a stratified squamous epithelium that consists of an undifferentiated basal progenitor layer and several differentiated suprabasal layers (Figure 3B)(Yu et al., 2005). Meanwhile, the mesenchymal cells surrounding the nascent esophagus proliferate and differentiate into multiple layers of muscle cells. Although our understanding of esophageal morphogenesis comes primarily from studies of mouse models, there are some important structural differences between the human and mouse esophagi (Table 2). It is noteworthy that in mice the epithelium of both the anterior stomach (also known as the forestomach/proximal stomach) and the esophagus is stratified and keratinized. Recent studies have shown that transcription factors and signaling pathways that are important for foregut separation continue to be critical in the subsequent esophageal epithelial morphogenesis (Table 3). Moreover, new genetic tools have revealed surprising findings about the mechanism by which the mesenchyme develops into muscle cells and their connection to neural networks, and these findings will be reviewed in the context of human esophageal diseases.

Transcription Factors Sox2, p63 and Nrf2 Regulate Epithelial Morphogenesis in the Developing Esophagus

Sox2 remains highly expressed in the epithelial cells of the esophagus and the forestomach after foregut separation is completed (Figure 3A). Genetic evidence has shown that Sox2 is required for the stratification and lineage differentiation of progenitor cells of both during their development. Reduced Sox2 protein levels in *Sox2^{GFP/COND}* hypomorphic mutants blocks the formation of a stratified squamous epithelium, resulting in simple columnar epithelial cells that secrete a large amount of mucin (Que et al., 2007). These phenotypic changes resemble mucous metaplasia in the lower esophagus, a pathological condition commonly seen in clinics. This metaplasia partially recaptures phenotypic changes in Barrett's esophagus (BE, also called intestinal metaplasia), in which simple columnar intestinal-like cells replace the stratified squamous epithelium of the esophagus (Souza et al., 2011). Of note is that Sox2 protein levels are dramatically decreased or completely lost in human BE biopsies (Chen et al., 2008). Moreover, recent studies have shown that *SOX2* gene amplification and increased SOX2 protein levels are associated with esophageal squamous cancer (Bass et al., 2009; Gen et al., 2010). *Sox2* is exclusively expressed in the basal progenitor cells of the adult esophagus (Arnold et al., 2011), but whether the gain- or loss- of *-Sox2* function initiates the pathological conditions remains to be determined.

The transcription factor Trp-63 (p63) is a member of the p53 family, which also includes p73. p63 has two isoforms, TAp63 and Δ Np63 which are transcribed from different promoters and have distinct properties and expression patterns (Candi et al., 2007). While TAp63 is highly expressed in oocytes and is considered a "guardian of the female germline" (Laurikkala et al., 2006), Δ Np63 is the main isoform in the stratified epithelium and is critical to stratification processes (Shalom-Feuerstein et al., 2011). Deletion of the *p63* gene affects all stratified epithelia, including the skin and esophagus (Daniely et al., 2004; Mills et al., 1999; Yang et al., 1999). In mutants, the esophageal epithelium fails to stratify and remains simple-columnar with multiple cilia on the apical surface (Daniely et al., 2004). Interestingly, epithelial cells in the forestomach switch on genes normally expressed by mucous-producing cells, suggesting that *p63* deletion not only abrogates stratification but also affects epithelial differentiation (Wang et al., 2011). Pertinent to these findings, *p63* expression has been shown to be low or completely absent in Barrett's esophagus (Daniely et al., 2004; Wang et al., 2011).

The transcription factor nuclear factor erythroid 2-related factor 2 (Nrf2) is pivotal for mounting cellular defense against oxidative stress via the induction of cytoprotective proteins including NAD(P)H quinone oxidoreductase 1 (Nqo1) and glutathione S-transferase (GST) family members (Nguyen et al., 2009). Nrf2 is targeted for degradation by Cullin-3 (Cul-3) based ubiquitin E3 ligase through the substrate adaptor Keap1 (Kobayashi et al., 2004). The cytoprotective function of Nrf2 protein in the developing esophagus is revealed by the study of mutants lacking *Keap1*, which die at weaning from occlusion of the upper digestive tract by keratin overproduction (Chen et al., 2012; Wakabayashi et al., 2003). The mutants have increased expression of the differentiation markers Involucrin and Loricrin despite no changes in epithelial proliferation. In addition, high levels of Nrf2 protein accumulate in the nuclei of *Keap1* mutants and initiate the transcription of *Nqo1* and *GSTs*. Deletion of *Nrf2* in a *Keap1* null background rescues the hyperkeratosis phenotype (Wakabayashi et al., 2003). These studies provide novel links between the Nrf2/Keap1 pathway and esophageal development but leave open questions of how this pathway or oxidative stress regulates progenitor cell differentiation in the esophagus at the cellular and molecular levels.

Dual Roles of Bmp Signaling for Epithelial Morphogenesis in the Mouse Esophagus and Forestomach

Bmp signaling activity displays a dynamic pattern in the developing esophagus and forestomach. From E11.0~E14.5 the highly proliferative stratifying epithelium remains negative for Bmp activity. However, at E15.0 Bmp signaling is detected in the esophageal suprabasal cells of Bmp reporter (BRE) embryos (Rodriguez et al., 2010). This two-stage presentation of Bmp signaling correlates with Bmp function in the regulation of epithelial development (Figure 3B). In *Noggin* null mutants, Bmp signaling is ectopically activated during the first stage and results in a simple columnar epithelium that contains a decreased number of p63 positive cells and forms convoluted glandular mucin-secreting pits. In the second stage, activation of Bmp signaling in the suprabasal cells is accompanied by epithelial differentiation. Deletion of *Bmp receptor 1A* with *Shh-Cre* perturbs lineage differentiation, resulting in Sox2 and p63 expression in the top layers of the epithelium (Rodriguez et al., 2010). During foregut separation Bmp negatively regulates Sox2 transcription (Domyan et al., 2011). It will be interesting to determine whether a similar regulatory mechanism exists in the developing esophagus.

Mesenchymal Differentiation into Muscle Cells Involves the Regulatory Roles of Myogenic Regulatory Factors and Homeobox Genes *Foxp1* and *Foxp2*

Although it has been known for some time that muscle is the major mesenchymal derivative in the developing esophagus, the mechanisms regulating its differentiation are just beginning to be unraveled. The adult mouse esophagus has three muscle layers: the muscularis mucosae (smooth muscle) and two muscularis externae layers (longitudinal and circumferential skeletal muscle in the thoracic segment) (Samarasinghe, 1972; Sang and Young, 1997) (Table 2). In the developing esophagus the outer two muscle layers are composed entirely of smooth muscle cells that are subsequently converted to striated muscle in a craniocaudal direction from E15.5-P21 (Kablar et al., 2000). Earlier studies regarding the formation of the muscle layers have been contradictory, but the introduction of genetic tracing tools has provided new insight into this process (Rishniw et al., 2003).

Initial immunostaining studies showed that markers for smooth muscle (MLCK) and skeletal muscle (MHC) were transiently co-localized in single cells of the esophageal mesenchyme at E15.5 (Patapoutian et al., 1995). Therefore, it was thought that skeletal muscle is derived from the transdifferentiation of the two outer layers of smooth muscle cells (Kablar et al., 2000; Patapoutian et al., 1995; Sang and Young, 1997). Recently, however, genetic lineage-tracing studies using smooth muscle myosin heavy chain-Cre (*SmmMHC-Cre*) mice revealed that transdifferentiation of smooth muscle cells into skeletal muscle cells does not occur in the developing esophagus. Rather, the adult skeletal muscularis externae (longitudinal and circumferential layers) and the initial outer layers of embryonic smooth muscle cells have distinct precursor origins (Rishniw et al., 2003). These findings were further confirmed by a selective gene deletion strategy in which *SmmMHC-Cre* was used to delete loxP-flanked *myogenin*, a gene essential for striated myogenesis. Despite robust *SmmMHC-Cre* expression in all smooth muscles of the embryonic esophagus, striated myogenesis progresses normally in the esophagus of *SmmMHC-Cre; myogenin^{loxP/loxP}* mutants (Rishniw et al., 2011).

Myogenin belongs to the myogenic regulatory factor (MRF) family, which also includes Myf5, MyoD and MRF4. MRFs are a group of basic helix-loop-helix (bHLH) transcription factors that play essential regulatory functions in the development of skeletal muscle in multiple tissues, including the esophagus (Kablar et al., 2000; Kassam-Duchossoy et al., 2004; Rudnicki et al., 1993; Valdez et al., 2000). Loss of *Myf5* but not *MyoD* in the E17.5 esophagus results in the loss of skeletal muscle and outer muscle layers that remain positive for smooth muscle actin (Kablar et al., 2000). Shh and its downstream transcription factor

Gli have been shown to directly regulate *Myf5* transcription (Borello et al., 2006; Gustafsson et al., 2002). Consistently, severe defects of myotomal components within the somite have been reported in *Shh*^{-/-} mutants along with decreased expression of *Myf5* and *MyoD* (Chiang et al., 1996). It remains to be investigated whether *Shh* regulates skeletal development in the esophagus through similar interactions. Due to the formation of EA/TEF in *Shh*^{-/-} null mutants (Litingtung et al., 1998), a tissue specific *Shh* ablation will be needed.

A recent genetic study showed that the homeobox genes *Foxp1* and *Foxp2* are also required for the differentiation of the mesenchyme into the skeletal muscle of the esophagus (Shu et al., 2007). *Foxp1* and *Foxp2* are both expressed in the muscular component of the E14.5 esophagus while *Foxp1* is also expressed in the epithelium. *Foxp1*^{+/-}; *Foxp2*^{-/-} mutants die at birth and have a complete absence of esophageal skeletal muscle. Interestingly, these mutants have only one outer layer of muscle, which remains as smooth muscle at E18.5 (Shu et al., 2007), suggesting that *Foxp1* and *Foxp2* cooperatively regulate the specification of skeletal muscle.

The Neuronal Innervation of Muscles Requires Close Interaction of Neural Progenitor Cells with Microenvironmental Factors

The striated muscle in the adult esophagus is innervated by both intrinsic and extrinsic neurons (Neuhuber et al., 2006; Sang and Young, 1998), whereas the smooth muscle in the muscularis mucosae is directly innervated mostly by intrinsic neurons (Kamikawa and Shimo, 1979; Storr et al., 2001; Worl et al., 2002). The extrinsic neurons include the vagal nerve which contains sensory and motor fibers (Chang et al., 2003; Powley and Phillips, 2002) and mediates communication between the central and the intrinsic nervous systems (enteric nervous system, ENS), including the myenteric and the submucosal plexi (Aziz and Thompson, 1998). Previous dye-labeling lineage tracing experiments identified vagal sensory fibers innervating the developing mouse esophagus at around E12.0 (Ratcliffe et al., 2006). By contrast, ENS development in the esophagus initiates at E9.0 and proceeds postnatally until around two weeks after birth as a result of extensive proliferation and differentiation of precursor cells migrating from the neural crest (Breuer et al., 2004; Durbec et al., 1996; Sang and Young, 1997; Taraviras and Pachnis, 1999). Along the route of travel the crest-derived precursor cells interact closely with microenvironmental signaling factors including growth factors and extracellular matrix components to sequentially switch on genes necessary for ENS development.

The transcription factor *Sox10* is expressed in the pre-migratory neural crest cells destined to colonize the esophagus and other parts of the gut. The role of *Sox10* in ENS development was identified by positional cloning of the *Dominant megacolon (Dom)* locus of mice. Animals homozygous for this mutation (*Dom/Dom*) die during embryogenesis (60% die at E12–E13) and lack enteric neurons and their precursors from the entire length of the esophagus and gastrointestinal tract (Kapur, 1999). Prior to entry into the foregut, crest-derived progenitor cells start to express c-*Ret*, a receptor for glial cell line-derived neurotrophic factor (*Gdnf*) (Taraviras et al., 1999; Taraviras and Pachnis, 1999). *Gdnf* is enriched in the gut muscle and serves as a potent chemo-attractant for the migratory crest progenitor cells. c-*Ret*, a member of receptor tyrosine kinase, regulates the survival, proliferation and differentiation of progenitor cells during early stages of ENS development. Disruption of *Gdnf/Ret* signaling in *Ret* null mutants reduces the number of enteric neurons in the esophagus and forestomach and leads to aganglionosis in other parts of gastrointestinal tract (Durbec et al., 1996; Yan et al., 2004). Consistently, combined deletion of *Sulf1* and *Sulf2*, sulfotransferases that modify the binding of *Gdnf* to extracellular matrix and c-*Ret*, also leads to reduced esophageal innervation (Ai et al., 2007). Shortly after the expression of c-*Ret* the crest progenitor cells start to express *Mash1*, a basic helix-loop-helix

(b-HLH) transcription factor. Mash1 is critical for the generation of sublineages of enteric neurons. Deletion of Mash1 leads to the loss of serotonin- and nitric oxide synthase (NOS)-containing neurons (Blaugrund et al., 1996; Guillemot et al., 1993; Sang et al., 1999). Interestingly, while Sox10 is not required for the expression of Mash1 in neural crest progenitor cells in vitro, it regulates Mash1 induction in vivo and Mash1 expression is lost in *Dom/Dom* mutants (Kim et al., 2003). Mash1 induction by Sox10 imparts multipotent neural crest progenitors with neural differentiation capability. However, once neural differentiation is initiated, Mash1 attenuates Sox10 expression, supporting a negative-feedback loop in ENS development (Kim et al., 2003).

A functional extrinsic nervous system is critical for the generation of peristaltic movement in the striated muscle portion of the esophagus. By contrast, the intrinsic nervous system fine-tunes peristalsis by regulating the contractility of smooth muscle cells in response to inputs from the extrinsic system. In humans, the ENS also modulates the activity of the submucosal glands in the esophagus. Neural innervation defects can lead to neonatal death (*Mash1*^{-/-}) and esophageal disorders, such as megaesophagus (*Sulf1*^{-/-}; *Sulf2*^{-/-}) (Guillemot et al., 1993; Neuhuber et al., 2006; van der Weyden et al., 2009). Defects in the generation of distal esophageal inhibitory neurons (NOS+ve) can also lead to achalasia, a motility disorder in which the lower esophageal sphincter (LES) fails to relax (Francis and Katzka, 2010). Mutant mice deficient in *neuronal NOS* (nNOS), *Lsc/p115* (Rho guanine nucleotide exchange factor 1) or *Rassf1* (Ras association family member 1) have impaired relaxation of the LES (Goyal and Chaudhury, 2010; Sivarao et al., 2001; van der Weyden et al., 2009; Zizer et al., 2010). Interestingly, although it has been proposed that the inhibitory neurons mediate LES relaxation through intramuscular interstitial cells of Cajal (ICC-IM), achalasia is not observed in *W/W^v* mutants in which ICC-IM cells are ablated, which suggests that this unique cell population is not directly involved in controlling muscle tone in the esophagus (Sivarao et al., 2001).

Summary of the Genetic and Cellular Mechanisms Underlying Esophageal Development

Conversion of the simple columnar epithelium of the embryonic esophagus into a stratified squamous epithelium requires the continued participation of Sox2 and Bmp signaling. p63 also plays an important role in epithelial morphogenesis and is essential for epithelial stratification. In future studies, it will be interesting to examine the regulatory relationship between these transcription factors. While Wnt signaling is known to be active in the esophagus after its separation from the foregut (Figure 4)(Chen et al., 2012), its role in esophageal development remains to be discerned. In addition, the transformation of the thin mesenchyme into multiple layers of muscle infiltrated by nerves and blood vessels requires microenvironmental factors including growth factors and elements of the extracellular matrix.

Conclusion and Future Directions

The separation of the anterior foregut into the trachea and esophagus and the subsequent development of the esophagus involve reciprocal interactions between the epithelium and the mesenchyme that are mediated by signaling molecules and transcription factors. Still, how morphogenetic processes such as cell proliferation, differentiation and migration are controlled and regulated at the cellular level remains largely unexplored. Gaining a greater understanding of these processes will require more genetic and molecular studies using new mouse lines for gain and loss-of-function experiments. Biochemical tools in conjunction with *in vivo* genetic manipulation will be helpful in providing further insights into pertinent molecular mechanisms. Techniques including chromatin immunoprecipitation (ChIP) and ChIP-sequencing that have been instrumental in studying the function of transcription factors in the development of other tissues will be especially important. One issue that has

not been well-studied is the morphogenesis of the submucosal glands, whose secretory products are critical for esophageal luminal clearance and tissue resistance (Long and Orlando, 1999). In addition, the expansion of gland ductal cells has been associated with re-epithelialization in Barrett's esophagus patients after laser and photodynamic therapy (Biddlestone et al., 1998). However, we know little about the morphogenesis of these unique glands due to the lack of proper animal models. We don't even know when exactly they are generated or whether they are generated from ingrowths of surface squamous epithelium or as direct extensions of the oropharyngeal minor salivary glands (Johns, 1952; Krause et al., 1976). The recently introduced Zinc-finger nuclease (ZFN) technology will enable genetic engineering in species other than mouse and will likely provide answers to these outstanding questions through studies in species that do have submucosal glands, such as swine and opossum (Carroll, 2011; Watanabe et al., 2010). This new tool will be particularly useful in targeting candidate genes that may potentially regulate gland initiation and the specification of progenitor cells into different lineages within glands (Abdulnour-Nakhoul et al., 2007; Long and Orlando, 1999; Watanabe et al., 2010).

Advances in the understanding of morphogenetic processes during embryonic development will promote greater insights into the pathophysiology of esophageal diseases. We and others have shown that abnormal levels of Sox2 and Bmps are associated with esophageal diseases including Barrett's esophagus and cancers (Bass et al., 2009; Chen et al., 2008; Milano et al., 2007; Que et al., 2007). A better characterization of relevant mechanisms will help in devising therapeutic strategies for targeting these diseases. Finally, we expect that better knowledge of esophageal development will lend support to future mechanistic studies of foregut separation as a whole.

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Highlights

- An updated overview of foregut morphogenesis and esophageal development.
- Dorsal-ventral patterning of signaling and transcription factors in early foregut.
- Disruption of D-V patterning affects the separation of the trachea and esophagus.
- Same molecules are essential for subsequent esophageal development.
- Overview of muscle development and innervation in the esophagus.

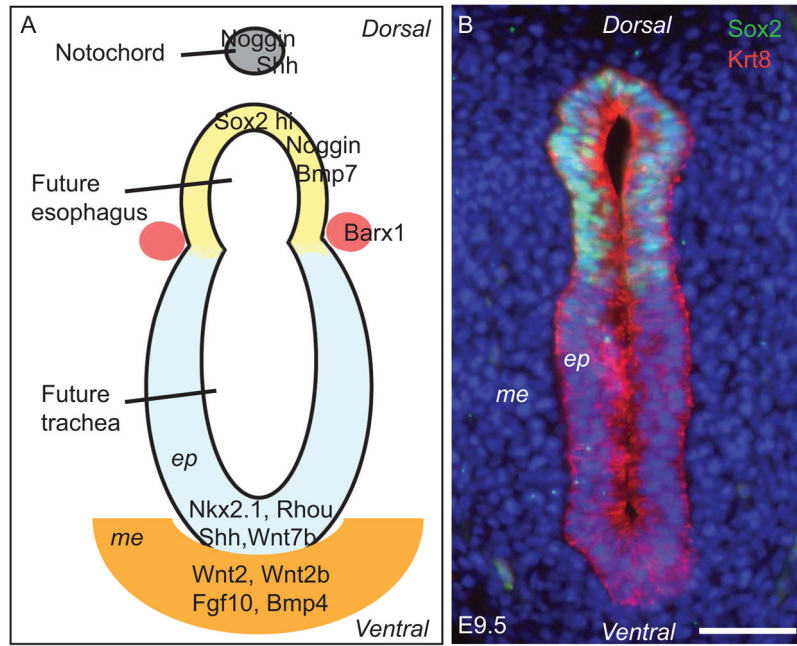


Figure 1.

Dorsal-ventral patterning of the E9.5 anterior foregut. (A) Schematic section through the unseparated anterior foregut tube showing high levels of *Sox2*, *Noggin*, *Bmp7* in the dorsal epithelium, which will give rise to the esophagus. Conversely, the transcription factor *Nkx2.1* and signaling molecules *Shh* and *Wnt7b*, along with the Rho GTPase family member *Rho*, are highly expressed in the ventral epithelium, which will contribute to the formation of the trachea. The homeobox gene *Barx-1* is expressed predominantly in the mesenchyme demarcating the separation site of the dorsal and ventral foregut. *Wnt2*, *Wnt2b*, *Fgf10* and *Bmp4* are enriched in the ventral mesenchyme and are important for gene expression in the underlying epithelium. Mutation of *Sox2*, *Nkx2.1* or *Rho* or defects in the *Shh*, *Wnt* or *Bmp* signaling pathways leads to abnormal foregut development, including the formation of esophageal atresia with/without tracheoesophageal fistula (EA/TEF). (B) Immunostained section through the E9.5 foregut tube showing high levels of *Sox2* protein in the dorsal epithelium. The cytoskeleton protein Keratin 8 (*Krt8*) is expressed in both the dorsal and ventral pseudostratified epithelium. Nuclei are counterstained with DAPI. Scale bar: 50 μ m. ep, epithelium; me, mesenchyme.

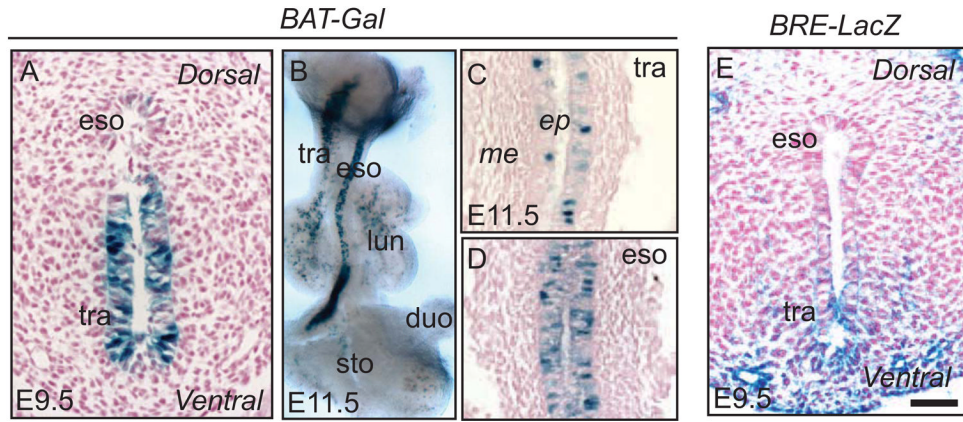


Figure 2.

Dynamic signaling activities in the early anterior foregut tube before and after separation. (A–D) Wnt signaling indicated by the *BAT-Gal* reporter line. (A) Wnt activity is high in the ventral foregut epithelium at E9.5 as shown by X-gal staining. (B–D) Wnt signaling activities are observed in the epithelium lining both tubes after the anterior foregut separates into the trachea and esophagus at E11.5. (C,D) Sagittal sections. (E) Bmp signaling activity (as reported by Bmp reporter line *BRE-LacZ*) is high in the epithelium and mesenchyme of the ventral side of the unseparated foregut, consistent with the presence of high levels of Bmp4 and absence of the antagonist Noggin, as shown in Figure 1A. Scale bar: 50 μ m. eso, esophagus; tra, trachea; lun, lung; duo, duodenum; sto, stomach; ep, epithelium; me, mesenchyme.

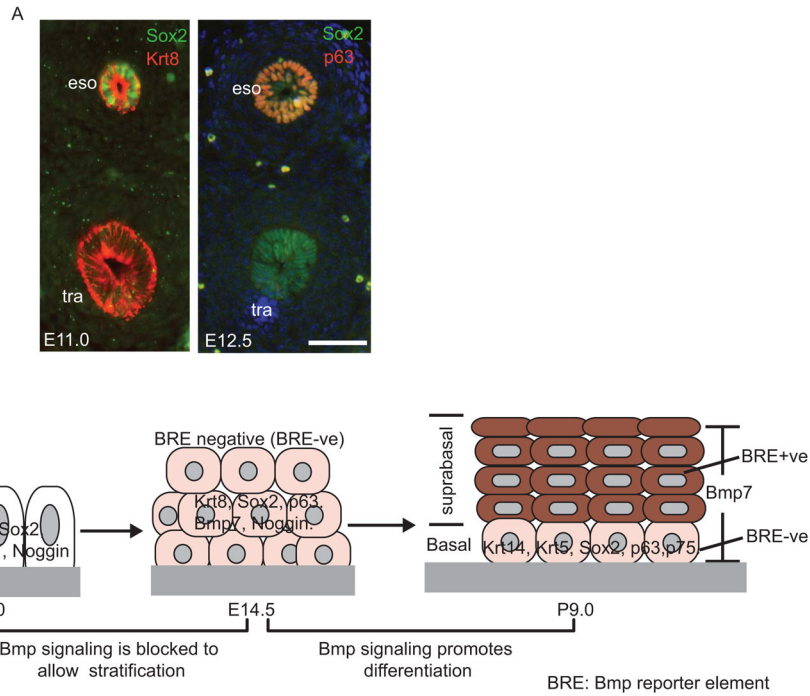


Figure 3.

Conversion of simple columnar to stratified squamous epithelium in the esophagus involving dynamic Bmp signaling activities. (A) Immunostained cross sections of E11.0 and E12.5 trachea and esophagus. Krt8 is expressed in the epithelium of both the esophagus and trachea after their formation from the foregut. Sox2 remains highly expressed in the epithelium of the dorsal, foregut-derived esophagus at E11.0 and E12.5. Note the conversion from single to multi-layered epithelium in the esophagus from E11.0 to E12.5, shown by co-immunostaining with p63 and Sox2 antibodies. (B) Schematic of the stratification and differentiation of esophageal epithelium through a two-stage Bmp signaling pattern. Stratification of the epithelium from E11.0 to E14.5 correlates with Noggin-mediated suppression of Bmp signaling. Ectopic Bmp activity in *Noggin* null and *Shh-Cre; Rosa26^{ca}Bmpr1a* mutants inhibits the stratification process. Differentiation of the top layers of epithelium at E14.5-P9.0 requires activation of the Bmp signaling pathway while basal progenitor cells remain negative for Bmp signaling. Deletion of *Bmpr1a* in *Shh-Cre; Bmpr1a^{loxp/loxp}* mutants inhibits the differentiation of suprabasal cells. Scale bar: 50 μ m. eso, esophagus; tra, trachea.

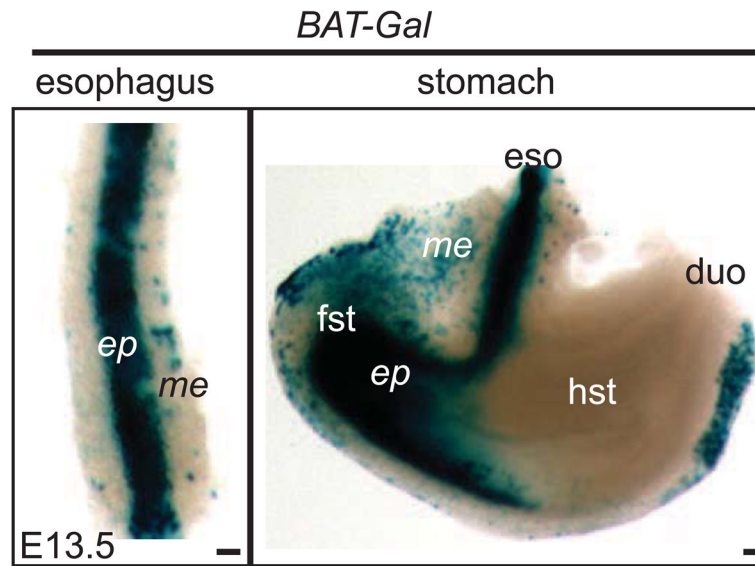


Figure 4.

Wnt signaling activity as shown by the *BAT-Gal* reporter in the E13.5 esophagus and stomach. The stratified epithelium in the esophagus and forestomach is strongly positive for X-gal staining. Sporadic X-gal positive cells are also present in the mesenchyme of both the esophagus and forestomach. Note that part of the mesenchyme in the hindstomach is also positive for X-gal staining. Scale bar: 50 μ m. *ep*, epithelium; *me*, mesenchyme; *fst*, forestomach; *eso*, esophagus; *hst*, hindstomach; *duo*, duodenum.

Table 1

Genes Associated with Defects in Tracheoesophageal Development in Mouse and Human.

Mouse gene	Foregut malformations (mouse)	EA/TEF (human)	Reference
<i>Shh</i> ^{-/-}	EA/TEF, rudimentary lung buds	EA/TEF in some <i>SHH</i> ^{+/-} patients	(Litingtung et al., 1998; Spilde et al., 2003)
<i>Gli2</i> ^{-/-} ; <i>Gli3</i> ^{+/-}	EA/TEF, abnormal lungs	EA/TEF in some patients with <i>GLI3</i> mutation	(Johnston et al., 2005; Motoyama et al., 1998)
<i>Gli2</i> ^{-/-} ; <i>Gli3</i> ^{-/-}	No esophagus, trachea and lungs		
<i>Foxf1</i> ^{+/-}	Narrow esophagus or TEF, lung hypoplasia	EA/TEF with the deletion of locus containing <i>FOXF1</i> gene	(Mahlpuu et al., 2001; Stankiewicz et al., 2009)
<i>RARα</i> ^{-/-} ; <i>RARβ</i> ^{-/-}	EA/TEF, lung hypoplasia or agenesis	Unknown	(Luo et al., 1996) (Kastner et al., 1997)
<i>RARα1</i> ^{-/-} ; <i>RARβ</i> ^{-/-}			(Luo et al., 1996)
<i>Nkx2.1</i> ^{-/-}	TEF, rudimentary lung buds	Unknown	(Minoo et al., 1999)
<i>Sox2</i> ^{GFP/COND} hypomorph	EA/TEF, abnormal lung epithelial differentiation	EA/TEF in <i>SOX2</i> ^{+/-} patients	(Que et al., 2007; Williamson et al., 2006)
<i>Noggin</i> ^{-/-}	EA/TEF	EA/TEF with the deletion of locus containing <i>NOG</i> gene	(Li et al., 2007; Marsh et al., 2000; Que et al., 2006)
<i>HoxC4</i> ^{-/-}	Blocked esophageal lumen with abnormal musculature	Unknown	(Boulet and Capecchi, 1996)
<i>PCSK5</i> ^{Vcc/Vcc*}	TEF, lung hypoplasia	Unknown	(Szumska et al., 2008)

Mutations of *MYCN*, *CHD7*, and *MID1.2* have been implicated in the cause of syndromic EA/TEF in humans. However, mouse genetic deletion models reveal no EA/TEF.

* Vcc is an Ethylnitrosourea (ENU)-induced mouse mutation which predicts a C470R amino acid change. (Adapted from (de Jong et al., 2010; Que et al., 2006))

Abbreviation: EA, esophageal atresia; TEF, tracheoesophageal fistula.

Table 2

Comparison of the Human and Mouse Esophagus.

	Mouse	Human
Length	Adult 1.0–1.5 cm	Adult 18–26cm
Keratin layer	Yes	No
Thickness of epithelium	3–5 cells	20–30 cells
Muscularis externa	Cervical and majority of thoracic segments are striated. The lower thoracic and distal segments are smooth muscle.	Cervical region (upper third): skeletal muscle Thoracic region (middle third): skeletal and smooth muscle Abdominal region (lower third): smooth muscle
Submucosal glands	No	Yes
Epithelium in the forestomach	Non-glandular, stratified squamous epithelium	Simple columnar glandular epithelium

Table 3

Transcription Factors Relevant to the Development of Epithelium and Mesenchyme in the Esophagus.

Gene	Expression Pattern	Esophageal Phenotype of Deletion Mutant	Reference
<i>Sox2</i>	Epithelium	Mucous metaplasia in the esophagus and forestomach, and the epithelial stratification is disrupted.	(Que et al., 2007)
<i>P63</i>	Epithelium	Mucous metaplasia in the esophagus and forestomach, and the epithelial stratification is disrupted.	(Daniely et al., 2004; Wang et al., 2011)
<i>Keap1</i>	Epithelium	Thickened cornification (keratin layer) in the esophagus and forestomach.	(Wakabayashi et al., 2003)
<i>Foxp1/Foxp2</i>	Foxp1 (epithelium and mesenchyme); Foxp2 (mesenchyme)	<i>Foxp1</i> ^{+/-} ; <i>Foxp2</i> ^{+/-} mutants have a complete absence of esophageal skeletal muscle.	(Shu et al., 2007)
<i>HoxC4</i>	Mesenchyme	Disorganized musculature. Complete esophageal blockage due to epithelial proliferation.	(Boulet and Capecchi, 1996)