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REVIEW

The development and stem cells of the esophagus

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ABSTRACT

The esophagus is derived from the anterior portion of the foregut endoderm, which also gives rise to the respiratory system. As it develops, the esophageal lining is transformed from a simple columnar epithelium into a stratified squamous cell layer, accompanied by the replacement of unspecified mesenchyme with layers of muscle cells. Studies in animal models have provided significant insights into the roles of various signaling pathways in esophageal development. More recent studies using human pluripotent stem cells (hPSCs) further demonstrate that some of these signaling pathways are conserved in human esophageal development. In addition, a combination of mouse genetics and hPSC differentiation approaches have uncovered new players that control esophageal morphogenesis. In this Review, we summarize these new findings and discuss how the esophagus is established and matures throughout different stages, including its initial specification, respiratory-esophageal separation, epithelial morphogenesis and maintenance. We also discuss esophageal muscular development and enteric nervous system innervation, which are essential for esophageal structure and function.

KEY WORDS: Esophageal development, Esophageal atresia, Tracheoesophageal separation, Human pluripotent stem cells, Enteric nervous system

Introduction

The esophageal lumen, lined by a stratified squamous epithelium and ensheathed by layers of striated and smooth muscle, is essential for the passage of food from the oropharynx to the stomach. However, compared with other organs, little is known about the development of the esophagus. Initial insights into esophageal development were extrapolated from principles of epidermal skin development, given that both organs include a stratified squamous epithelium (Fuchs, 2007). However, unlike the skin, the esophageal epithelium is originally pseudostratified columnar when it is first established from the early foregut, and it only transitions into a stratified layer later in development, suggesting it forms via a distinct morphogenetic process. Moreover, the esophagus arises from the anterior foregut, which also gives rise to respiratory organs (i.e. the lung and trachea), but understanding the mechanism by which it is specified and separates off from the respiratory system (i.e. in the process of respiratory-esophageal separation, RES) has been a challenge for the field.

Recent studies using a range of model organisms have revealed that a number of signaling pathways and transcription factors are involved in RES and continue to play essential roles during the morphogenesis of the esophageal epithelium. Furthermore, with the use of human pluripotent stem cells (hPSCs), the conserved mechanisms regulating esophageal development have begun to be addressed. In this Review, we summarize these studies and discuss: (1) the cellular and molecular mechanisms regulating RES; (2) epithelial morphogenesis in the esophagus, and conservation between the murine and human esophagus; and (3) the role of epithelial stem cells in esophageal maintenance and the pathogenesis of esophageal diseases. In particular, we focus on stem cell heterogeneity, signaling molecules and transcription factors that have recently been shown to regulate esophageal stem cell homeostasis and disease. Finally, we summarize current understanding of the development of the muscular and enteric nervous system components of the esophagus.

Esophageal specification and RES

The esophagus and the respiratory organs (i.e. the lungs and trachea) are specified from the dorsal and ventral aspects of the anterior foregut, respectively. Distal-proximal invagination in the midline splits the anterior foregut into the esophagus and the respiratory system at approximately embryonic day (E) 9.5-11.0 in mice and weeks 4-6 of human gestation (Fig. 1). Failed RES underlies the pathogenesis of the relatively common congenital anomaly esophageal atresia with or without tracheoesophageal fistula (EA/TEF), which has a prevalence of 1/3000-1/4000 in newborns (Houben and Curry, 2008). Although several hypothetical models (e.g. the 'Septum Model' and the 'Splitting and Extension Model') have been proposed to describe the RES process (reviewed by Que, 2015; Zhang et al., 2017), the exact cellular and molecular mechanisms regulating the separation process remain to be determined. Nonetheless, several genetic studies have demonstrated that multiple signaling pathways, including the Sonic Hedgehog (SHH), bone morphogenetic protein (BMP) and WNT pathways, and transcription factors (e.g. Sox2, NKX2.1 and Isl1) play crucial roles in foregut specification and RES (Que et al., 2006; Que, 2015; Kim et al., 2019) (Fig. 1).

The role of coordinated SHH/GLI and retinoic acid signaling

Shh is enriched in the ventral foregut endoderm, whereas the SHH pathway downstream targets, Gli2 and Gli3, are present in the surrounding mesenchymal compartment before RES. *Shh* null or *Gli2*$^{-/-}$;*Gli3*$^{+/-}$ mutants display the formation of EA/TEF (Litingtung et al., 1998; Motoyama et al., 1998; Pepicelli et al., 1998). The resulting TEFs also show tracheal/bronchial characteristics, e.g. simple columnar epithelium and cartilages (Litingtung et al., 1998). Further studies have demonstrated that disruption of *Gli2* and *Gli3* blocks the expression of Wnt2, Wnt2b and BMP4 in the mesenchyme, causing failed lung specification

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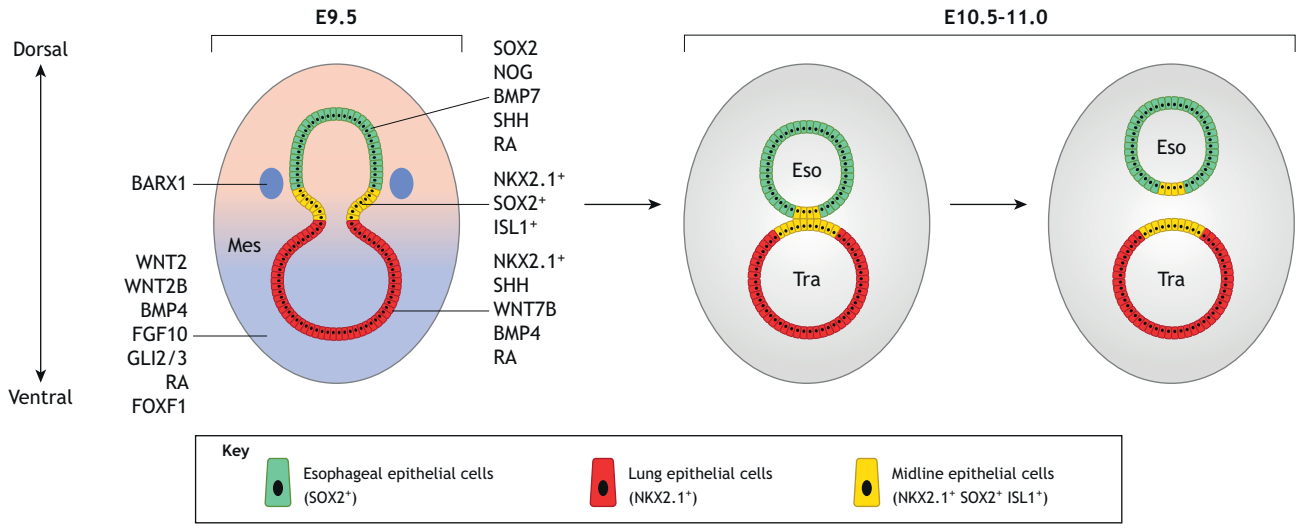

Fig. 1. Endodermal specification and respiratory-esophageal separation. At E9.5 in mice, the foregut endoderm is specified into dorsal SOX2$^+$ esophageal epithelial cells (green) and ventral NKX2.1$^+$ precursors (red). Although the dorsal esophageal epithelial cells also express Noggin, Bmp7 and Shh, the ventral epithelial cells are enriched with Shh, Wnt7b and Bmp4. The foregut endoderm shows active retinoic acid (RA) signaling. Note that midline epithelial cells (MECs, yellow) co-express SOX2, NKX2.1 and ISL1. At this time, Barx1 is expressed in the dorsal mesenchyme, whereas Wnt2, Wnt2B, Bmp4, Fgf10, Gli2/3, retinoic acid and Foxf1 are enriched in the ventral mesenchyme. Separation of the esophagus and trachea (respiratory-esophageal separation; RES) then occurs between E9.5 and E11.0. Note that MECs contribute to the epithelium in both the ventral esophagus and the dorsal trachea. Eso, esophagus; Mes, mesenchyme; Tra, trachea.

and RES (Rankin et al., 2016). In addition, the transcription factor Foxf1 is regulated by Shh/Gli signaling, and some mutants heterozygous for the *Foxf1* allele (*Foxf1*$^{+/-}$) in a CD1 genetic background display EA/TEF (Mahlapuu et al., 2001). *Shh*$^{-/-}$ and *Gli2*$^{-/-}$;*Gli3*$^{-/-}$ mutants also show reduced Foxf1$^+$ mesenchymal cells in the anterior foregut (Nasr et al., 2019). On the other hand, ablating retinoic acid (RA) signaling in the foregut endoderm leads to a loss of Shh expression in the foregut endoderm and impaired lung epithelial specification (Desai et al., 2004; Wang et al., 2006; Rankin et al., 2016). Blocking RA signaling specifically in the mesoderm also represses the expression of Wnt2, Wnt2b, Bmp4 and Foxf1 (Rankin et al., 2016). These findings suggest that RA signaling is required for the differentiation of both the epithelial and mesenchymal compartments in the early foregut.

BMP signaling during esophageal specification

Balanced BMP signaling activity is essential for specifying lung versus esophageal cell fate. Analysis of the BMP reporter mouse line *BRE-gal* demonstrates high BMP activity in the ventral foregut (Zhang et al., 2018). Consistently, the ligand Bmp4 is expressed in the mesenchyme of the ventral foregut at E9.5 (Que et al., 2006; Li et al., 2008). Although *Bmp4* deletion does not alter initial lung specification, the proliferation of epithelial and mesenchymal cells is reduced, leading to lung hypoplasia and absence of the trachea (Li et al., 2008). However, ablation of BMP receptors (*Bmpr1a;b*) in the foregut results in loss of phosphorylated SMAD1/5/8, which are downstream effectors in the BMP pathway, and a complete absence of the lung progenitor marker Nkx2.1 (Domyan et al., 2011). In addition, the anterior foregut remains as a single tube containing only cells positive for Sox2, which marks esophageal progenitor cells at an early stage. Further studies have shown that SMAD1/5/8 directly inhibit Sox2 expression and that *Sox2* deletion rescues RES in *Bmpr1a;b*$^{-/-}$ mutants (Domyan et al., 2011). These studies indicate that BMP signaling promotes lung epithelial specification while suppressing esophageal cell fate through Sox2 repression.

In contrast to the ventrally enriched pattern of BMP4 expression, expression of the BMP antagonist Noggin (Nog) is limited to the dorsal epithelium of the anterior foregut and the notochord (Que et al., 2006; Li et al., 2007; Rodriguez et al., 2010). *Nog* deletion disrupts RES and leads to the formation of EA/TEF in ∼70% of mutants (Que et al., 2006; Li et al., 2007). Notably, the epithelial cells in the TEFs connecting the trachea and stomach exhibit Nkx2.1 expression at E14.5 (Que et al., 2006). Deletion of *Bmp4* or *Bmp7* rescues RES in *Nog*$^{-/-}$ mutants (Que et al., 2006; Li et al., 2007), further confirming that balanced BMP signaling is crucial for early foregut morphogenesis. It is worth pointing out that deletion of the chromosomal region spanning the *NOG* locus is found in patients with EA/TEF (Marsh et al., 2000).

WNT signaling in the developing esophagus

WNT signaling also promotes lung development while inhibiting esophageal cell fate. The Wnt ligands Wnt2 and Wnt2b are enriched in the mesenchyme surrounding the ventral foregut endoderm before RES. The foregut endoderm of *Wnt2*$^{-/-}$;*Wnt2b*$^{-/-}$ mutants fails to differentiate into lung progenitors and the mutants display complete lung agenesis (Goss et al., 2009). In line with this finding, conditional deletion of the β-catenin gene (*Ctnnb1*) with *Shh-Cre* also results in the loss of lung differentiation, and the single anterior foregut is lined with Sox2$^+$ esophageal progenitor cells (Goss et al., 2009; Harris-Johnson et al., 2009; Hou et al., 2019). By contrast, β-catenin gain-of-function leads to the presence of Nkx2.1$^+$ lung progenitor cells in the esophageal and stomach domains (Goss et al., 2009; Harris-Johnson et al., 2009). In a separate study, deletion of the transcription factor Barx1, which is a putative downstream target of WNT signaling, was also shown to cause failed RES. Barx1 is enriched in the midline mesenchyme in which RES occurs. In *Barx1*$^{-/-}$ mutants, the esophagus fails to separate from the respiratory system, and the ventral part of the resulting TEF is lined with Nkx2.1$^+$ respiratory progenitor cells (Woo et al., 2011). Further analysis suggests that *Barx1* deletion reduces expression of the WNT signaling inhibitors sFRP1 and sFRP2 in the mesenchyme,

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promoting ectopic activation of canonical WNT signaling activities in the dorsal foregut endoderm. This expansion of WNT signaling appears to re-specify the dorsal foregut endoderm into respiratory cells (Woo et al., 2011).

Antagonizing roles between Sox2 and Nkx2.1

Before RES, Sox2 and Nkx2.1 are enriched in the dorsal and ventral foregut endoderm respectively (Fig. 1). These two transcription factors are individually crucial for the development of respiratory and esophageal epithelium. Conditional deletion of *Sox2* (using tamoxifen induction) at E6.5 causes a complete loss of the esophagus in $Foxa2^{CreER};Sox2^{loxp/loxp}$ mice, confirming the importance of Sox2 in initial esophageal specification (Trisno et al., 2018). By contrast, when tamoxifen is administered at E7.0 and E8.0, bronchiole-like tubular tissues develop in the TEFs of $Foxa2^{CreER};Sox2^{loxp/loxp}$ mutants (Teramoto et al., 2020). Notably, although patients heterozygous for *SOX2* develop EA/TEF, as part of Anophthalmia-Esophageal-Genital (AEG) syndrome (OMIM 206900) (Williamson et al., 2006), $Sox2^{+/-}$ mouse mutants show normal RES. However, when Sox2 is further reduced to 30% of normal levels, about 60% of *Sox2* hypomorphic mouse mutants ($Sox2^{EGFP/COND}$) display EA/TEF. The resulting TEFs are also lined with $Nkx2.1^{+}$ lung progenitor cells (Que et al., 2007). $Nkx2.1^{-/-}$ mouse mutants also develop EA/TEF, and the resulting anterior foregut tube demonstrates esophageal phenotypes with layers of surrounding smooth muscles (Minoo et al., 1999; Que et al., 2007). Chromatin immunoprecipitation followed by sequencing (ChIP-seq) has revealed that Nkx2.1 can directly bind to the *Sox2* promoter and suppress transcription of *Sox2*. Nkx2.1 also directly represses the expression of multiple esophageal genes including *Klf5* (Kuwahara et al., 2020). In addition, the primary lung buds that form in $Nkx2.1^{-/-}$ mutants fail to differentiate into mature lung epithelial cells, suggesting the requirement of Nkx2.1 for lung development (Minoo et al., 1999; Que et al., 2007).

A recent study using hPSC-derived organoids demonstrated that SOX2 is also required for the specification of human esophageal cells (Trisno et al., 2018). SOX2 knockdown results in increased NKX2.1 expression during the specification of human foregut progenitors into esophageal cell lineages. By contrast, SOX2 overexpression in human lung progenitor cells downregulates NKX2.1 levels. Further analyses revealed that SOX2 inhibits canonical WNT signaling, an essential pathway for lung cell fate specification, by promoting expression of WNT inhibitors such as *SFRP1*, *SFRP2* and *DKK1*. In line with this finding, it was found that transcript levels of the canonical WNT signaling downstream target *Axin2* are upregulated in the dorsal foregut endoderm of *Sox2*-deletion mouse mutants. These findings together support the notion that SOX2 promotes initial specification towards an esophageal fate at early stages of human foregut development (Trisno et al., 2018). In the future, it will be of interest to use a similar strategy to study the role of NKX2.1 in human foregut development; the establishment of NKX2.1 loss- and gain-of-function hPSC lines will therefore be essential.

The role of Isl1 in midline epithelial cells during RES

RES involves dynamic cellular movements at the dorsal-ventral boundary of the foregut (Que et al., 2006; Fausett et al., 2014). A recent study identified a group of epithelial cells located at this boundary, termed midline epithelial cells (MECs), that contributes to both the tracheal and esophageal epithelium (Kim et al., 2019). MECs co-express Nkx2.1 and Sox2. Surprisingly, *Nkx2.1-CreER*-labeled cells incorporate into the ventral epithelium of the esophagus before RES (Kim et al., 2019). A morpholino oligo screen to knock down gene expression in *Xenopus laevis* embryos identified Isl1 as a novel transcription factor involved in RES, demonstrating that *Isl1* mutant *Xenopus* embryos exhibit abnormal RES. Moreover, the role played by Isl1 in foregut development appears to be conserved: ∼50% of $Shh\text{-}Cre;Isl1^{loxp/loxp}$ mouse mutants display EA/TEF, with lung lobe fusion observed in all mutants (Kim et al., 2019). Notably, Isl1 is co-expressed with Nkx2.1 and Sox2 in MECs in addition to its enrichment in the ventral respiratory epithelium and mesenchyme. When *Sox2-CreER* is used to delete *Isl1* before RES in mice, 100% of mutants display EA/TEF, highlighting the importance of Isl1-expressing MECs in RES. These findings also suggest that *Shh-Cre* does not efficiently target MECs. Notably, Shh is expressed in the ventral foregut epithelium at E9.5-E10.5, and then shifts to the dorsal epithelium at E11.5 (Rodriguez et al., 2010). Therefore, it is possible only a subpopulation of MECs is targeted by *Shh-Cre* during RES.

Isl1 has been shown to regulate the expression of Nkx2.1 in MECs (Kim et al., 2019). It is noteworthy that some patients with EA/TEF exhibit deletion of the 5q11.2 chromosomal region covering the *ISL1* gene (de Jong et al., 2010), supporting the idea that ISL1 plays a similar role in human RES. In addition, lung lobation defects (e.g. horseshoe lung) are often concomitant with EA/TEF in humans and in mouse genetic models (e.g. $Nog^{-/-}$, $Shh^{-/-}$). It is therefore tempting to speculate that the mechanisms modulating lung lobation and RES overlap, likely involving cell-cell adhesion and migration. To address this, a recent study used *Xenopus* embryos to study the cellular mechanisms underlying RES (Nasr et al., 2019), revealing that $Foxf1^{+}$ mesenchyme surrounding the foregut epithelium is required for forming the midline constriction at the dorsal-ventral boundary. Following constriction, the polarized midline epithelium from both lateral sides intercalates to form a transient septum in the center. During RES, the polarity protein aPKC is removed from the apical surface of the septum epithelium by Rab11-mediated endocytosis (Nasr et al., 2019). Accordingly, when the dynamin inhibitor dynasore is applied to block endocytosis, the expression of aPKC is maintained on the apical surface, concomitant with failed RES (Nasr et al., 2019). Consistently, Cas9-mediated deletion or antisense morpholino knockdown of *Rab11a* causes abnormal formation of the constricted epithelial septum and failed RES. These findings demonstrate that, in *Xenopus*, endocytosis is required for the formation of the septum during RES in a Rab11-dependent manner. Although it remains to be confirmed, this study provides a cellular explanation for the previous mouse models of RES separation. It will be intriguing to assess whether perturbation of Rab-mediated endocytosis influences RES in rodents via genetic approaches.

Epithelial morphogenesis in the developing esophagus

The epithelium in the adult esophagus is stratified squamous. However, earlier in development (following RES), the epithelium lining the nascent esophagus is composed of pseudostratified columnar cells (Fig. 2). These nascent esophageal progenitor cells (EPCs) express Trp63 and high levels of the columnar cell marker Krt8 (Fig. 2), the expression of which is maintained until late gestation (∼E18.0 in mice). By contrast, Krt7 is expressed only briefly in EPCs (at ∼E13.0 in mice) and its expression is accompanied by stratification of the epithelium, which generates basal ($Trp63^{+}$ $Krt5^{+}$ $Krt14^{+}$) cells and differentiating squamous suprabasal ($Krt4^{+}$ $Krt13^{+}$) cells (Yu et al., 2005; Jiang et al., 2017; Zhang et al., 2017, 2018) (Fig. 2). During stratification, multi-ciliated cells are occasionally observed on the surface layer of the

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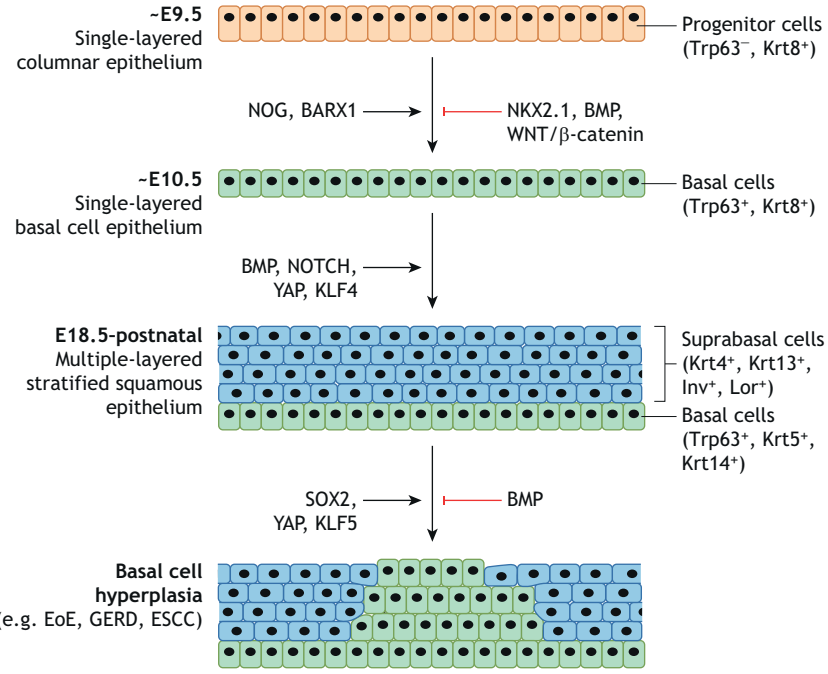

Fig. 2. Signaling pathways and transcription factors involved in formation of the stratified squamous epithelium lining the mouse esophagus. At ∼E9.5, the epithelium lining the dorsal anterior foregut tube is composed of a single layer of columnar progenitor cells expressing Krt8 but not Trp63. By ∼E10.5, Trp63 is expressed in esophageal progenitor cells. Noggin and Barx1 promote specification towards esophageal lineage, whereas NKX2.1, BMP and Wnt/β-catenin signaling inhibit esophageal commitment. The esophageal epithelium then undergoes squamous stratification, with basal cells (Trp63$^+$) in the bottom layer and suprabasal cells (Krt4$^+$ Krt13$^+$ Inv$^+$ Lor$^+$) in the apical layer. The key signaling pathways (e.g. BMP and NOTCH) and transcription factors (e.g. Yap and Klf4) that regulate stratification are highlighted. Note that dysregulation of these factors can cause basal cell hyperplasia, as observed in eosinophilic esophagitis (EoE), gastroesophageal reflux disease (GERD) and esophageal squamous cell carcinoma (ESCC). Sox2, Yap and Klf5 promote basal cell hyperplasia whereas BMP activation represses this process.

fetal esophagus (Menard, 1995; Daniely et al., 2004). The apical layers of epithelium then continue to mature, expressing terminal differentiation markers such as Loricrin (Lor) and Involucrin (Inv) (Fig. 2). Notably, a thick keratin layer begins to form shortly after birth in the mouse but not in the human esophagus; this is just one of many differences between the human and mouse esophagus (see Box 1). A number of genetic studies in mice have uncovered the key signaling pathways and transcription factors that play significant roles in esophageal epithelial development. With the ability to differentiate hPSCs into esophageal cells *in vitro* (see Box 2 and Fig. 3), we can now begin to address whether these mechanisms are conserved in human esophageal morphogenesis.

Box 1. Differences between the mouse and human esophagus

Although the overall structure of the mouse and human esophagus is similar, several distinct characteristics exist between them in adults. First, the human esophagus is composed of more layers of both basal and suprabasal cells. In the adult mouse esophagus, the epithelium contains four to six layers of cells, whereas there are 20-30 layers in the human esophagus (Treuting, 2012; Zhang et al., 2017). Second, the human esophagus contains extensive submucosal glands that produce mucins and bicarbonate ions to facilitate food transportation and provide epithelial protection through neutralizing refluxate such as pepsin and bile acid from the stomach (Long and Orlando, 1999; Orlando, 2006). In addition, the submucosal glands can produce growth factors such as EGF to promote epithelial growth (Orlando, 2006). Third, the human esophagus contains specialized structures including papillae, which are not apparent in the mouse esophagus (Seery and Watt, 2000; Barbera et al., 2015). Fourth, the mouse esophagus is covered with a layer of acellular keratin, which is absent in the human esophagus. It is also worth mentioning that the proximal portion of the rodent stomach (the forestomach) is also lined by a stratified and keratinized squamous epithelium (two to four layers) (Liu et al., 2013). Finally, when the musculature of the esophagus develops in mice, most of the smooth muscle in the outer layer is later replaced by striated muscle (SM), except for within the lower esophageal sphincter and distally. By contrast, in the human esophagus, only the upper third is composed of SM; the middle third is a mix of both striated and smooth muscle, and the lower third consists of only smooth muscle (Kuo and Urma, 2006).

Signaling pathways regulating epithelial stratification and differentiation

BMP signaling plays an important role in esophageal morphogenesis. Following RES, expression of the BMP antagonist Nog is maintained in the epithelium throughout the nascent esophagus (Zhang et al., 2018). Suppression of BMP signaling is important for the specification of EPCs. Loss of Nog leads to the formation of glandular units lined by simple columnar cells in the esophagus of ∼40% of *Nog*$^{-/-}$ mutants that do not have EA/TEF. However, following stratification, BMP signaling activity is detected in the apical layers of the epithelium, suggesting that it is involved in the squamous differentiation of EPCs. Consistently, deletion of BMP receptor 1a (i.e. in *Shh-Cre;Bmpr1a*$^{loxp/loxp}$ mutants) disrupts the differentiation process, resulting in the expansion of Trp63$^+$ EPCs (Rodriguez et al., 2010). On the other hand, ectopic BMP activation (using a constitutively activated *Bmpr1a* allele) causes premature differentiation of EPCs and enhances esophageal epithelial differentiation and squamous stratification (Rodriguez et al., 2010).

NOTCH signaling also regulates esophageal morphogenesis in both mouse and humans. Signaling through the pathway is initiated by the binding of Notch ligands (JAG1/2 or DLL1/3/4) to Notch receptors (NOTCH1-4) on neighboring cells. This binding leads to cleavage of the NOTCH intracellular domain (NICD) by γ-secretase, followed by translocation of NICD into the nucleus where it works with the transcription factor RBPJκ to regulate gene transcription (Kopan and Ilagan, 2009). In contrast to *DLL1/3/4*, which are expressed at low levels, *JAG1/2* are highly expressed in the mouse and human fetal esophagus (Zhang et al., 2018). The receptors *NOTCH1/3/4* are also highly expressed in the developing epithelium. Deletion of *Jag1/2* in mice leads to reduced epithelial stratification and thin Krt13$^+$ suprabasal layers (Zhang et al., 2018). Epithelial stratification is also impaired in *Shh-Cre;RBPJκ*$^{loxp/loxp}$ mutants, confirming the importance of Notch signaling in esophageal development. Furthermore, upon treatment with the γ-secretase inhibitor DAPT, KRT13-expressing suprabasal cells are

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Box 2. The generation of esophageal epithelial cells from stem cells

Human pluripotent stem cells (hPSCs), including human embryonic stem cells and induced pluripotent stem cells, can theoretically be induced into all cell types through a step-wise induction protocol (see Fig. 3). In the case of hPSC differentiation towards endoderm and then foregut endoderm, Activin A appears to play a crucial role in driving initial endoderm derivation (Yiangou et al., 2018). Upon BMP and Wnt activation, the foregut endoderm progenitors give rise to lung cell lineages (Green et al., 2011; Longmire et al., 2012; Huang et al., 2014; McCauley et al., 2017). Building on these pioneering studies, a protocol to derive esophageal progenitor cells (EPCs) from hPSCs has been developed (Que et al., 2006; Li et al., 2008; Rodriguez et al., 2010; Mou et al., 2016; Zhang et al., 2018). This involves applying Noggin and SB431542 to block BMP and TGFβ signaling, leading to the generation of EPCs from hPSC-derived foregut endoderm (Zhang et al., 2018). These EPCs are able to reconstitute a stratified esophageal epithelium similar to that of the human fetal esophagus in 3D organoids, air-liquid interface culture and *in vivo* transplantation assays (Zhang et al., 2018). A 3D organoid-based culture approach to generate EPCs from hPSCs has also been developed (Trisno et al., 2018). In this approach, endodermal cells are first cultured into anterior foregut organoids, which in turn are induced into esophageal organoids consisting of stratified epithelium following l-2 months of culture. Notably, the addition of EGF and FGF10 appears to improve growth of the esophageal organoids but not specification towards EPCs (Trisno et al., 2018; Zhang et al., 2018; Bailey et al., 2019). Successful derivation of EPCs from hPSCs not only allows for an unlimited supply of EPCs but also offers a tool to investigate the mechanisms controlling human esophageal development. It should be also noted, however, that the main products of current *in vitro* hPSC differentiation systems are esophageal epithelial cells; other esophageal components such as muscles and enteric neurons are missing. To fully recapitulate *in vivo* esophageal development, future endeavors should thus focus on generating the various cell types present in the esophagus and integrating them into a complex 3D structure.

dramatically reduced during the differentiation of hPSC-derived EPCs. These findings suggest a conserved role for NOTCH signaling in the morphogenesis of the esophageal epithelium. Notably, a previous study has shown that NOTCH inhibition also impairs the differentiation and stratification of immortalized human esophageal epithelium in an organotypic 3D culture (Ohashi et al., 2010).

YAP, a central transcriptional mediator in the Hippo signaling pathway, has also been implicated in esophageal epithelial development. *YAP* (also known as *Yap1*) deletion leads to reduced stratification of the esophageal epithelium in *Shh-Cre;Yap*$^{loxp/loxp}$ mutants (Bailey et al., 2019). Further studies have also revealed that YAP signaling is required for the proliferation of EPCs (Bailey et al., 2019). By contrast, increased YAP signaling activity, induced by using a constitutive allele (*YAP*^{5SA}), causes abnormal expansion of esophageal basal cells and thickening of the epithelium (Bailey et al., 2019). In hPSC-derived EPC organoids, YAP inhibition with the inhibitor Verteporfin or via siRNA-mediated knockdown also reduces the proliferation of EPCs and results in thinning of the organoid epithelium (Bailey et al., 2019), suggesting a conserved role for YAP signaling in the regulation of esophageal development. In the future, it will be interesting to further determine whether YAP upstream regulators such as MST1/2 and LATS1/2 also play such roles in the morphogenesis of the esophageal epithelium.

Transcription factors involved in epithelial morphogenesis

Three transcription factors have thus far been shown to regulate morphogenesis of the esophageal epithelium: Trp63, and the Krüppel-like factors Klf4 and Klf5. Trp63 is a member of the p53 family of transcription factors, which also includes Trp53 and Trp73. Depending on the transcription initiation sites used, the *Trp63* gene produces two major classes of Trp63 transcripts that either contain (TAp63) or lack (ΔNp63) the transactivation domain. These isoforms can be further divided into three alternatively spliced forms: α, β and γ (Murray-Zmijewski et al., 2006). The ΔNp63 isoforms are believed to function as dominant-negative forms that inhibit Trp63 proteins. In the developing esophagus, these ΔNTrp63 isoforms are the predominant *Trp63* transcripts. Deletion of *Trp63* causes failed conversion of columnar cells into a stratified squamous epithelium (Mills et al., 1999; Yang et al., 1999; Yu et al., 2005). *ΔNTrp63* deletion also consistently results in stratification defects (Romano et al., 2012; Pignon et al., 2013). The resulting epithelium lining the esophagus includes numerous multi-ciliated columnar cells (Daniely et al., 2004), which express high levels of Krt8 but lack expression of Krt5 and Krt14 (Mills et al., 1999; Yang et al., 1999; Daniely et al., 2004; Rosekrans et al., 2015). Despite these findings, it remains unclear how Trp63 regulates the squamous differentiation program at the cellular and molecular level.

Klf4 and Klf5 are zinc-finger proteins belonging to a relatively large family of KLF transcription factors that includes at least 17 members. Klf4 and Klf5 exhibit a reciprocal expression pattern in the esophagus, with Klf5 enriched in basal cells and Klf4 enriched in suprabasal cells. Ectopic overexpression of Klf5 promotes the proliferation of basal cells (Goldstein et al., 2007). Further studies have revealed that Klf5 increases cell proliferation by upregulating the expression of EGFR, which in turn activates MEK/ERK signaling (Yang et al., 2007). Conversely, overexpression of Klf4 reduces proliferation while enhancing squamous differentiation in the epithelium, in part by reducing Klf5 expression (Goldstein et al., 2007). In a separate study, deletion of *Klf4* was shown to lead to increased basal cell proliferation and hyperplasia (Tetreault et al., 2010).

In summary, multiple signaling pathways and transcription factors play crucial roles in epithelial morphogenesis. However, it remains unclear whether and how these signaling pathways regulate the transcription factors. In addition, although excessive mucous cells are present in the esophagus of *Sox2* hypomorphic mutants (Que et al., 2007), how Sox2 influences epithelial morphogenesis is yet to be determined. Furthermore, the role of the epithelium in the lengthening of the esophagus has not been studied. A recent study showed that Wnt5a-Ror2 signaling promotes esophageal elongation by synchronizing the radial polarity of smooth muscle cells (Kishimoto et al., 2018). In the future, it will be interesting to explore how the epithelium coordinates its morphogenesis with that of the mesenchyme to achieve growth in length.

Basal cells in the adult esophagus: heterogeneous versus homogeneous

The epithelium of the adult esophagus turns over every ∼3.5 days in mice and every ∼11 days in humans. This relatively quick turnover rate requires the coordinated proliferation and differentiation of basal cells (Marques-Pereira and Leblond, 1965; Doupe et al., 2012; Pan et al., 2013). In the mouse esophagus, proliferating cells are limited to a single layer, in which basal cells are located (Jiang et al., 2015). By contrast, the human esophagus contains approximately two to four layers of basal cells, with cells in the bottom-most layer rarely proliferating, whereas the other layers show extensive proliferation (Seery and Watt, 2000). However, in patients with eosinophilic esophagitis (EoE) and gastroesophageal reflux disease (GERD),

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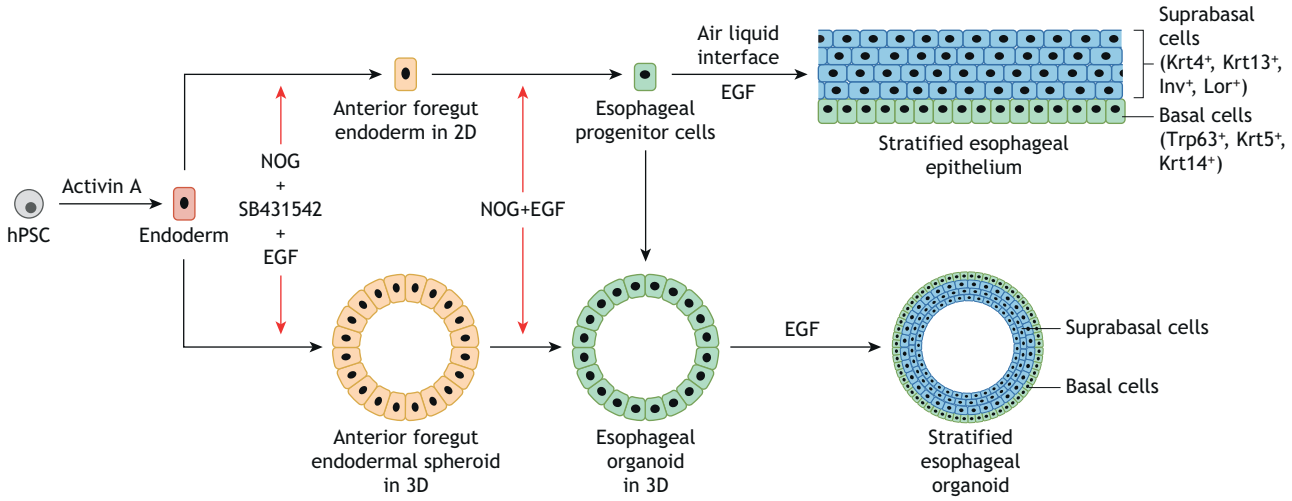

Fig. 3. The differentiation of human pluripotent stem cells into esophageal cells. Human pluripotent stem cells (hPSCs) are first differentiated into endodermal cells (via treatment with Activin A) and are then cultured in either 2D (top) or 3D (bottom) in the presence of Noggin (NOG), SB431542 (a TGFβ inhibitor) and EGF to generate anterior foregut endodermal cells. These cells are further differentiated into esophageal progenitor cells by culturing them in the presence of Noggin and EGF. The esophageal progenitor cells are then cultured further, either in an air-liquid interface to form a stratified epithelium or as 3D esophageal organoids that become stratified (i.e. with Trp63^{+} basal cells and Krt13^{+} suprabasal cells located on the outside and inside of the organoids, respectively).

extensive proliferation of basal cells occurs throughout all of the basal layers (Jiang et al., 2015). In an EoE mouse model, increased levels of the BMP inhibitor Follistatin have been shown to promote basal cell hyperplasia (Jiang et al., 2015). Notably, although BMP7 is expressed throughout the epithelium, BMP4 is only expressed in subpopulations of basal cells in the mouse esophagus (Jiang et al., 2015). This raises an important question (Fig. 4): are basal cells are a heterogeneous or a homogeneous population?

Evidence for heterogeneous basal cells

It is widely accepted that homeostasis in some tissues, such as the hematopoietic system and the intestinal epithelium, is maintained by a rare population of slow-cycling stem cells and transit-amplifying (TA) cells (Li and Clevers, 2010). Similarly, early studies suggest that the epidermis is maintained by coordinated activities of stem cells and TA cells (Potten, 1975). A number of studies have tried to assess whether similar cells exist in the esophagus. One such study demonstrated that Itga6^{hi} CD71^{lo} murine esophageal epithelial cells are enriched for bromodeoxyuridine (BrdU) label-retaining cells (LRCs), and are therefore considered to be slow-cycling stem cells (Croagh et al., 2007). By contrast, Itga6^{hi} CD71^{hi} cells divide actively and represent TA cells. Another study demonstrated that murine LRCs are enriched in a CD34^{hi} side population of cells that harbor the ability to exclude Hoechst dye (Kalabis et al., 2008). This purified side population can be serially passaged in 2D cell culture and maintains the capacity to form a stratified squamous epithelium in 3D organotypic culture. Furthermore, the *in vitro*-expanded side population is able to repair an epithelium upon transplantation into an injured esophagus (Kalabis et al., 2008). Several other studies provide additional evidence that esophageal basal cells are heterogeneous. For example, Sox2^{GFP+} basal cells in mice can be divided into multiple populations based on their expression of Itgb4, Itga6 and CD73. Amongst them, Sox2^{+}Itgb4^{hi}Itga6^{hi}CD73^{hi} cells exhibit high organoid forming efficiency in 3D culture, suggesting that this population possesses stem cell potential (DeWard et al., 2014). Moreover, lineage tracing and *in vitro* culture suggest that Krt15^{+} basal cells in mice represent a subpopulation of long-term stem cells. This basal cell subpopulation is resistant to radiation and serves as a major cell source for epithelial regeneration (Giroux et al., 2017).

Basal cells in the human esophagus also appear to be heterogeneous. For example, the neurotrophin receptor p75^{NTR} is expressed at different levels in basal cells and has been used as a surface marker to isolate slow cycling stem cells (Okumura et al., 2003). In line with this, LRCs have been identified in the human esophageal epithelium via 5-iodo-2′-deoxyuridine (IdU) labeling. These labeled cells retain IdU for at least 67 days following administration of IdU through intravenous infusion. Notably, co-labeling of IdU and Ki-67 (Mki67) is rarely observed. This study also demonstrates that LRCs are significantly more abundant in the basal layer of the papillae (the papillary basal layer, PBL), which is formed by the invagination of connective tissue into the esophageal epithelium, than in the interpapillary basal layer (IBL) (Pan et al., 2013) (Fig. 4). This is in contrast with a previous study that reported enrichment of slow-cycling basal cells (i.e. LRCs) in the IBL, with TA cells being more frequently observed among parabasal/epibasal cells and in the PBL (Seery and Watt, 2000). Moreover, in this context, the IBL was shown to contain cells that are more clonogenic and prone to forming large colonies. Notably, this study also showed that invaginating papillae form when esophageal epithelial cells are co-cultured with esophagus- but not skin-originated stromal tissue in organotypic cultures, suggesting that tissue-specific cues induce papillae formation (Seery and Watt, 2000). It will be intriguing to further identify these cues, which may be cytokines or growth factors that can alter stem cell proliferation or symmetric versus asymmetric division.

Homogenous basal cells

Although some genes (e.g. *BMP4*) are only expressed in subpopulations of basal cells (Jiang et al., 2015), other genes including *Trp63* and *Sox2* are expressed in all basal cells, suggesting that basal cells in the mouse esophagus might in fact be a homogenous population, with a single progenitor maintaining epithelial homeostasis. By leveraging models established to study the epidermis, a number of studies have attempted to address this (Doupe et al., 2012; Alcolea et al., 2014; Piedrafita et al., 2020).

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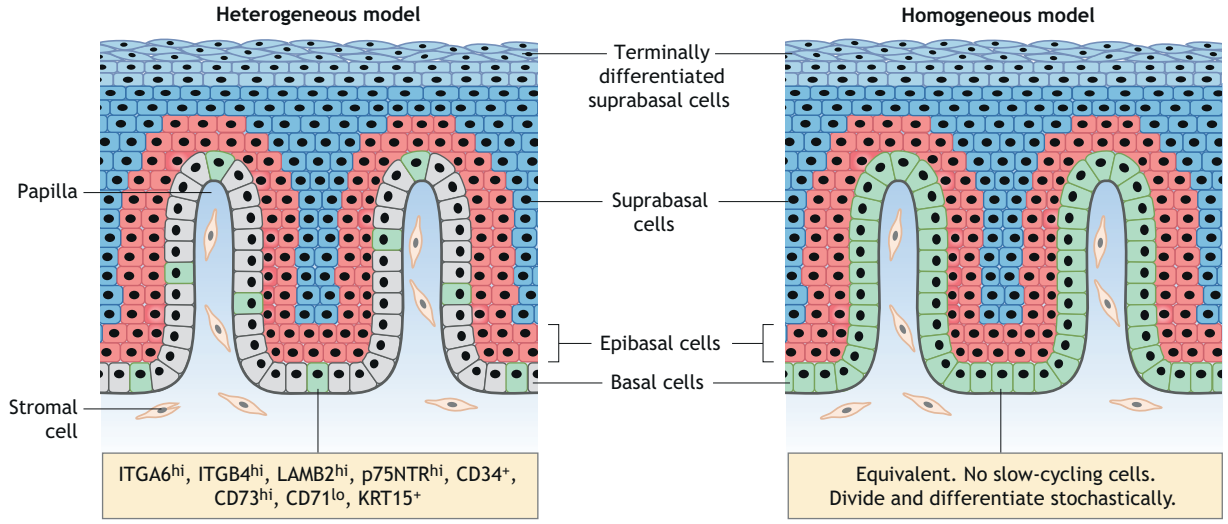

Fig. 4. Heterogeneous versus homogeneous models of stem cells in the adult esophagus. In the heterogeneous model (left), populations of heterogeneous esophageal stem cells are distinguished by the expression of individual markers including ITGA6^{hi}, ITGB4^{hi}, LAMB2^{hi}, p75NTR^{hi}, CD34^{+}, CD73^{hi}, CD71^{lo} and KRT15^{+}. Note that these markers are not necessarily expressed in the same cells. In the homogeneous model (right), the stratified squamous epithelium is maintained by a single progenitor cell, i.e. the basal cells are homogeneous.

For example, using a doxycycline-inducible *Rosa26*$^{M2rtTA/TetO\text{-}HGFP}$ mouse strain to mark all esophageal epithelial cells with the fusion protein Histone-2B EGFP, it was shown that the mouse esophagus does not contain LRCs. Specifically, it was reported that, upon doxycycline withdrawal, GFP expression is diluted by cell division, and none of the epithelial cells expresses GFP after a 4 week chase, indicating that no slow-cycling stem cells exist in the esophagus (Doupe et al., 2012). Further mathematical modeling supported a model in which basal cells are functionally equivalent and divide stochastically to generate proliferating and differentiating cells with equal probability (Doupe et al., 2012; Alcolea et al., 2014; Piedrafita et al., 2020). It is worth mentioning that the mouse esophagus and the epidermis are both maintained by a single progenitor, based on lineage tracing and mathematical modeling (Doupe et al., 2012; Alcolea et al., 2014; Piedrafita et al., 2020).

Recently, human esophageal cells were analyzed and sorted into distinct populations based on the expression of CD34 and EpCAM. These subpopulations demonstrate no difference in self-renewal capacity in 2D culture. Further experiments showed that the determining factor for the success of 3D organoid culture is the number of cells plated, rather than the epithelial subpopulation (Barbera et al., 2015). This study also questions the preferential location of stem cells in the IBL over the PBL, proposing that stem cells are not restricted to a specific cell compartment. That being said, the study suggests the presence of a rare quiescent population of ITGB1^{+}CD34^{+} cells at the tip of papillae (Barbera et al., 2015). Follow-up characterization and functional testing of this population is therefore warranted in the future.

In summary, although marker gene expression suggests that basal cells are heterogeneous, mathematical modeling built on lineage tracing analysis supports the notion of basal cell homogeneity. The key to solving the controversy may depend on the advancement of experimental technology. Along this line, single-cell RNA sequencing technology was recently used to study the human esophagus (Madissoon et al., 2020). Although basal cells were distinguished from other cell types based on transcript profiling, in-depth analysis of basal cell heterogeneity was not pursued in this study (Madissoon et al., 2020). Therefore future endeavors should include single-cell analyses, *in vitro* genetic manipulation and animal modeling, combined with live imaging as used in the study of the epidermis (Mesa et al., 2018), to further address the cellular and molecular mechanisms underlying basal cell heterogeneity versus homogeneity.

Muscle development in the esophagus

Muscle contraction in the esophagus, which is initiated by swallowing, is important for the peristaltic movements of the esophagus that propel food into the stomach. The murine esophagus is ensheathed by the muscularis externa, which initially includes an inner circular layer and an outer longitudinal layer of smooth muscles (McHugh, 1995; Kablar et al., 2000). Smooth muscle cells start to differentiate from the mesenchyme surrounding the esophageal epithelium at ~E11.0.

The inner circular layer of smooth muscle become obvious at E12.5, whereas the outer layer of smooth muscle is not discernible until E14.5 (Kablar et al., 2000). The smooth muscle later becomes replaced with striated muscle (Fig. 5), although there are differences in this process between mice and humans (see Box 1). The outer layer of smooth muscle is not discernible until E14.5.

The development of striated muscle

Early mouse studies suggest that striated muscle is derived from the direct transdifferentiation of smooth muscles (Patapoutian et al., 1995). However, more recent genetic lineage-tracing studies have revealed that striated muscle originates from Isl1^{+} cranial cardiopharyngeal mesoderm (Gopalakrishnan et al., 2015). Isl1^{+} myogenic progenitor cells migrate in an anterior-to-posterior fashion at E12.5 to mix with smooth muscles and differentiate into striated muscle. This replacement process continues until around the first 2 weeks of postnatal age (Patapoutian et al., 1995). Further genetic studies have demonstrated that Tbx1 is required for the colonization of Isl1^{+} progenitors in the esophagus, and that deletion of *Tbx1* disrupts striated muscle formation (Gopalakrishnan et al., 2015). In a separate study, the same group demonstrated that HGF/MET signaling is important for the migration of Isl1^{+} progenitor cells from the anterior to the posterior of the developing esophagus (Comai et al., 2019).

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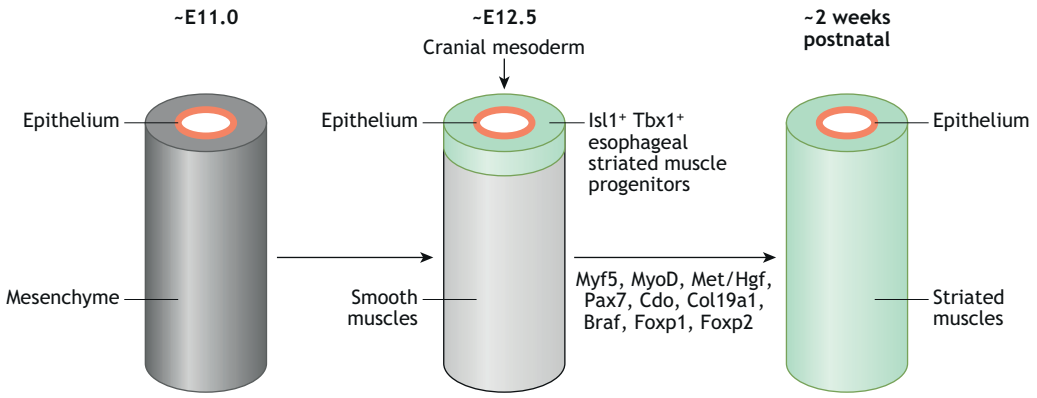

Fig. 5. Musculature development in the mouse esophagus. Between E11.0 and E12.5, smooth muscle cells differentiate from the mesenchyme surrounding the esophageal epithelium. Striated muscle progenitor cells (Isl1$^+$ Tbx1$^+$) then migrate from the cranial mesoderm to the anterior end of the esophagus at ~E12.5. These striated muscle progenitors differentiate to replace smooth muscles under the control of multiple molecules including Myf5, MyoD, Met/Hgf, Pax7, Cdo, Col19a1, Braf, Foxp1 and Foxp2. The replacing process ends at ~2 weeks after birth.

In addition to colonization, proliferation and differentiation of muscle progenitors are important for building striated muscle. *Pax7* deletion reduces striated muscle proliferation, leading to thinning of the striated muscle layer along the esophagus. Consequently, *Pax7* mutants develop megaesophagus, which is characterized by enlargement of the esophageal tube due to muscle defects (Chihara et al., 2015). In this condition, the esophagus also loses the ability to contract and fails to propel food toward the stomach. Moreover, colonization of the muscle progenitors is accompanied by differentiation into mature striated muscle, and the myogenic regulatory factors Myf5 and MyoD are two factors that are essential for this differentiation process (Kablar et al., 2000). The transcription factors Foxp1 and Foxp2 also play a role. The differentiation of striated muscle is impaired in the esophagus of *Foxp1*$^{+/-}$;*Foxp2*$^{-/-}$ mutants, resulting in the formation of a thin and dilated muscular layer (Shu et al., 2007).

Development of the lower esophageal sphincter

The lower esophageal sphincter (LES) is composed of a bundle of muscles at the distal end of the esophagus. It is normally closed to prevent acid and stomach contents from traveling backwards. During swallowing, however, the LES is relaxed to allow food to pass into the stomach (Kuo and Urma, 2006). The mutation of several genes has been associated with muscle dysfunction that consequently causes abnormal LES development, megaesophagus, or both.

The WNT signaling component Fzd4 is important for striated muscle and LES development. Deletion of the *Fzd4* gene leads to loss of striated muscle in the lower esophagus, including the LES. Consequently, megaesophagus and LES dysfunction occur in *Fzd4* mutants (Wang et al., 2001). The mechanism by which Fzd4 regulates striated muscle development remains to be identified. Notably, the outer layer of smooth muscle remains in the mutant esophagus. Given that Fzd4 is a WNT signaling receptor, it will be of interest to investigate whether WNT signaling is involved in striated muscle development. Deletion of the cell surface receptor gene *Cdo* (*Cdon*) also results in megaesophagus and aberrant patterning of smooth muscles surrounding the LES, which consequently disrupts LES relaxation and causes achalasia, i.e. an inability to open the LES (Romer et al., 2013). Megaesophagus also develops in *Col19a1* mutants, in which nitric oxide-dependent relaxation of the LES is impaired, and most mutants die of malnourishment within 3 weeks after birth (Sumiyoshi et al., 2004). *Braf*^{Q241R} mutants, in which constitutive Braf activation promotes RAS/MAPK signaling, also develop a dilated esophagus concomitant with impaired relaxation of the LES (Inoue et al., 2017). In this case, underdeveloped striated muscle appears to cause motility defects (Inoue et al., 2017). Although these genetic studies have identified several genes involved in striated muscle and LES development, they also raise some important questions. For example, what is the molecular mechanism driving the migration of striated muscle progenitor cells along the smooth muscle scaffold? What are the cellular and molecular mechanisms promoting the differentiation of striated muscle progenitors? Seeking answers to these questions requires a better understanding of the role of specific genes in cell migration, cell-cell interaction and cell fate determination. Muscle cell-specific gene deletion with conditional alleles could offer new insights in this regard.

Innervation of the esophagus

The esophageal striated muscle is innervated by the enteric nervous system (ENS), which plays important roles in controlling the motility, immune response and secretions of the gastrointestinal (GI) tract (Lake and Heuckeroth, 2013). The origin of the esophageal ENS is still controversial. In contrast to the lower GI tract, in which the enteric neurons are derived from the vagal neural crest, esophageal enteric neurons likely have dual origins, including the hindbrain and the anterior trunk-level neural crest (Durbec et al., 1996). However, a recent study has suggested that the esophageal ENS originates from two sources: vagal Schwann cells adjacent to somites 1 and 3, and neural crest cells adjacent to somites 3-7, which give rise to sympathetic ganglia (Espinosa-Medina et al., 2017). Despite the controversy, it is known that neural crest cells migrate into the foregut and innervate the mesenchyme at ~E8.5-E9.5 (Durbec et al., 1996). After colonizing the mesenchyme of the gut, the ENS precursor cells migrate inward to form two layers of ganglia, namely the myenteric plexus and the submucosal plexus (Lake and Heuckeroth, 2013). The myenteric plexus is located between the smooth and striated muscle layers, whereas the submucosal plexus is located between the epithelium and smooth muscle layer (Lake and Heuckeroth, 2013).

Multiple genes control the development and function of the esophageal ENS. Deletion of neuronal nitric oxide synthase (nNOS) in mice impairs ENS function and LES relaxation, causing achalasia (Sivarao et al., 2001). Loss of *Ascl1* leads to loss of nitric oxide synthase (NOS)-containing myenteric enteric neurons in the mouse

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esophagus (Sang et al., 1999). The mutants die at birth owing to failed esophageal peristalsis or achalasia (Sang et al., 1999). In addition, loss of *Ret* leads to reduced numbers of neurons and glial cells in the esophagus, suggesting that Ret is partially required for esophageal ganglia development (Durbec et al., 1996). A recent study demonstrated that Nrg1-ErbB3 signaling is also required for esophageal ENS development: deletion of *Nrg1* or *Erbb3* reduces the numbers of esophageal enteric neurons (Espinosa-Medina et al., 2017). In addition, loss of hepatocyte growth factor-regulated tyrosine kinase substrate (*Hgs*) reduces the innervation of ganglia, causing esophageal dilation (Ye et al., 2016).

Development of the esophageal ENS is also regulated by many other signaling pathways. For example, its development is disrupted in *Sulf1*$^{-/-}$;*Sulf2*$^{-/-}$, *Arhgef1*$^{-/-}$ and *Dominant megacolon* (*Sox10*^{Dom}) mouse mutants (Kapur, 1999; Ai et al., 2007; Zizer et al., 2010). However, although mutations in multiple genes have been associated with abnormal ENS development, the exact cellular and molecular function of the proteins encoded by these genes remains to be investigated. In addition, rigorous lineage tracing is needed to define the cell source(s) contributing to the esophageal ENS. Further studies also need to focus on the mechanisms underlying the migration of neural crest cells into the esophagus and their differentiation and maturation into neurons and glia cells.

Concluding remarks

As we have reviewed here, recent studies indicate that a number of signaling molecules and transcription factors are involved in esophageal development, including in epithelial specification, RES and the subsequent morphogenesis of the esophageal epithelium. Genetic studies have also provided insights into the development of the muscular and nervous system components of the esophagus. Although thus far limited knowledge is available regarding stem cells in the adult esophagus, the key message that emerges is that the pathogenesis of esophageal diseases is often associated or characterized by stem/progenitor cell abnormalities. For example, basal cell hyperplasia is a key phenotype in EoE patients, although it remains unknown whether/which basal cell subpopulations are responsible for the hyperplastic phenotype.

What is clear, however, is that some developmental signaling pathways and factors are re-used during disease progression. For example, suppressed BMP signaling promotes basal cell hyperplasia in EoE (Jiang et al., 2015). Indeed, studies of mouse models suggest that crosstalk between the Th2 cytokine IL13 and BMP signaling drives hyperproliferation of basal cells (Jiang et al., 2015). In addition, *SOX2* gene amplification has been found in ∼30% of patients with esophageal squamous cell carcinoma (Bass et al., 2009). Moreover, mouse models suggest that SOX2 cooperates with activated Stat3 signaling to drive basal cell transformation and cancer initiation (Liu et al., 2013). The re-use/reactivation of developmental signaling thus appears to be associated with disease progression in many tissues. As such, a more comprehensive understanding of the developmental mechanisms regulating esophageal development will not only provide insights into how the esophagus is established in embryonic stages, but will also facilitate the elucidation of esophageal disease mechanisms in adults.

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Competing interests

The authors declare no competing or financial interests.

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