Homeostasis of the esophageal epithelium: A quest for the stem cell
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Chapter 2

Esophageal development and epithelial homeostasis

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INTRODUCTION

The word esophagus is derived from the Greek words οἰσέμεν (oisein, to carry) and φαγείν (phagein, to eat). This description fits well with the functional role of the esophagus that mainly serves to “carry food” into the stomach. From the pharyngoesophageal junction, the esophagus passes through the mediastinum and diaphragm and connects to the cardia of the stomach at the gastroesophageal junction or Z-line. The pharyngoesophageal and gastroesophageal junctions anatomically overlap with the upper and lower esophageal sphincters. Both sphincters are closed except during swallowing to assure a unidirectional flow of esophageal content towards the stomach and to prevent reflux of gastric content into the esophagus. The relatively simple histology of the esophageal epithelium fits well with the fact that the esophagus has no role other than to pass food through the thorax to the stomach. It does not play a known digestive, endocrine or metabolic role and the epithelium consists of a simple stratified squamous epithelium, which provides a good protective layer against the unmodified food stream on its way to the stomach. Despite the perhaps somewhat prosaic functional role of the esophagus compared to other organs in the body, we feel that it is essential to gain a better understanding of the mechanisms that regulate normal esophageal homeostasis. Esophageal cancer is a disease with a dismal prognosis as the incidence rate in the USA is 4.6/100.000 whereas the mortality rate is 4.4/100.000 indicating a mortality of around 95% for the disease. A better understanding of the pathways that maintain esophageal epithelial homeostasis and the way these pathways are deregulated during oncogenesis may provide novel approaches to treatment of esophageal cancer. In this review we aim to give an overview of the current understanding of the mechanisms involved in esophageal development and homeostasis.

DEVELOPMENT OF THE ESOPHAGUS

Normal esophageal morphogenesis and endodermal differentiation

The esophagus develops from the foregut. A critical phase of esophageal morphogenesis is when the respiratory appendage starts to form from the foregut tube at mouse embryonic day (E)9.5 and human E26 (Figure 1). The respiratory appendage consists of a central ventral tracheal bud and two adjacent ventrolateral lung buds. The airways will separate from the esophagus by a process of elongation and septation, a process that is completed by mouse E11.5. The esophagus and trachea show distinct endodermal and mesenchymal development. The trachea will develop pseudostratified columnar epithelium and is enveloped in cartilage rings ventrally. The esophagus will form multilayered squamous epithelium and the esophageal mesenchyme develops the
smooth muscle layer required for esophageal motility and propulsion of food. The exact mechanism of foregut separation into esophagus and trachea has not been examined by in vivo imaging and remains a matter of debate.\textsuperscript{4}

When the mouse esophagus has been clearly established by E11.5 the epithelium consists of a single keratin (K)\textsubscript{8} positive cuboidal epithelial layer.\textsuperscript{5} In the following days (E13.5-E17.5) the epithelium gradually becomes more layered until there are around four layers of epithelial cells.\textsuperscript{5,6} During development there is a gradual conversion of a K\textsubscript{8} positive cuboidal epithelium to a K\textsubscript{14} positive squamous epithelium. This process begins at the basal layer around day E17.5 and the basal layer is mostly K\textsubscript{14} positive at birth.\textsuperscript{5} The suprabasal layers will subsequently gradually lose K\textsubscript{8} expression postnatally.\textsuperscript{5} The onset of squamous cell differentiation can also be observed at the suprabasal layers which start to express the squamous cell differentiation marker involucrin around E15.5. Expression of the late differentiation marker K\textsubscript{10} starts at postnatal day (P)1.\textsuperscript{5} During development the epithelium contains numerous ciliated cells, which have almost completely disappeared by P4.\textsuperscript{6,7} In the adult mouse the epithelium will undergo a process of keratinization, this does not occur in humans (Figure 2).

Recently, Wang et al. proposed a model in which the embryonic cuboidal epithelium is displaced by an undermining population of p63 positive squamous cell precursors that migrates under the cuboidal epithelial cells from the proximal to distal esophagus.\textsuperscript{8} Wang et al. propose that the squamous cell progenitors thus outcompete cuboidal progenitors by displacing them from access to the basement membrane. This is an interesting hypothesis but so far the evidence is circumstantial. Wang et al. used terms such as tracing and tracking for their study of the behavior of the squamous versus cuboidal cells it should be stressed that no actual lineage tracing was performed of either population. In lineage tracing experiments a defined population of cells is genetically irreversibly marked so that the fate of the cells can be traced irrespective of changes in the phenotype. In the experiments of Wang et al. squamous cells and cuboidal cells were examined with cell lineage markers by immunofluorescence at different time points of development only. Such experiments do not demonstrate that a cell that expresses a cuboidal cell marker at one point in development was not at the basal layer expressing a squamous cell marker the day before. The experiments therefore by no means excluded a scenario that cuboidal cells actually transdifferentiate to a squamous cell fate in a proximal-to-distal wave. By comparison, such a wave of differentiation is known to transform the intestinal epithelium from a cuboidal to a columnar epithelium along the proximodistal axis.\textsuperscript{9} Actual lineage tracing experiments are thus required to further examine the interesting hypothesis by Wang et al.
Figure 1 | Esophageal and airway development from the endoderm. (A-C) The esophagus will develop from the dorsal part and the respiratory tract from the counterpart localized at the ventral side. (D-H) Common types of esophageal atresia and/or tracheoesophageal fistula. (D) Esophageal atresia. (E) Tracheoesophageal fistula. (F) Esophageal atresia with distal tracheoesophageal fistula. (G) Esophageal atresia with proximal tracheoesophageal fistula. (H) Esophageal atresia with double tracheoesophageal fistula.

Figure 2 | Development of the esophageal epithelium. By embryonic day (E)11.5 the epithelium consists of a single cuboidal epithelial layer. All cells are positive for K8 (depicted in pink). Around E17.5 cells start to lose expression of K8 (orange reflects K8 negative cells). Gradually basal cells start to express K14 (purple). Based on the model described in Yu et al.\textsuperscript{5}
Signaling pathways involved in esophageal morphogenesis

Patterning of cellular fate during development is dependent on positional information that couples the position of a cell to its function. Spatial information is laid down in a tissue by the formation of gradients of extracellular signals or so called morphogens. Receptive cells will respond to the morphogen in a concentration dependent manner, resulting in the expression and activation of different transcription factors. The combined activity of these transcriptional regulators is one of the most important determinants of cellular phenotype. Thus a cellular function will depend on a cell’s position in the concentration gradient. In each tissue multiple gradients exist of different morphogens and their antagonists, allowing formation of intricately patterned tissues.

A limited number of morphogenetic signaling families are used in different constellations throughout development, this has been aptly termed the morphogenetic code. Four families of morphogenetic pathways can roughly be distinguished: Wnt, Hedgehog (Hh), Tgf-β families and a large group of receptor tyrosine kinases such as fibroblast growth factor, platelet-derived growth factor and epidermal growth factor, which share similar intracellular signaling pathways. The incredible level of variation in tissue patterning stems from the sheer infinite variation in which these pathways are modified by gradients of various agonists and antagonists, differences in the expression of intracellular downstream regulators of signaling output and for example differences in autocrine versus paracrine signaling. For example, the Hedgehog signaling pathway is a mitogenic pathway that is involved in oncogenesis of the skin and brain where it acts in an autocrine fashion on the affected cells. Similarly, in the adult esophagus a hedgehog ligand is expressed in the basal layer and signaling acts in a autocrine fashion on basal cells and stimulates their proliferation (see below). In contrast, in the intestine signaling is uniquely from the differentiated epithelial cells to the underlying mesenchyme. Here hedgehog signaling regulates survival and expansion of the mesenchyme and in fact negatively regulates epithelial precursor cell proliferation.

As will be seen below these morphogenetic pathways are critical regulators of esophageal development. The function of the different regulators of esophageal development identified to date has been revealed by foregut abnormalities observed in mouse mutants and in humans with congenital abnormalities. Three different important gross structural abnormalities can be observed in the various mutants. One is the improper separation of the esophagus and trachea, leading to the development of tracheoesophageal fistula. Another is defective outgrowth of the airways resulting in a hypoplastic respiratory system. Finally some mutants fail to maintain the esophageal tube resulting in hypoplasia or atresia of the esophagus (Figure 1).
**Sox2 and Nkx2.1 are tissue specific transcriptional regulators of gut-airway separation**

Two tissue specific transcriptional regulators have been identified that are specific markers of esophageal versus airway endoderm. Sox2 marks the endodermal cells that will form the esophagus and is expressed throughout the esophageal epithelium in the adult.\(^1\) Nkx2.1 identifies the endodermal cells from which the respiratory tract will form and is expressed in alveolar epithelial cells in the adult.\(^1\) These transcription factors are not only useful markers to identify esophageal versus respiratory differentiation, but also play a key role in the establishment of the respective organs.

Nkx2.1 expression marks a population of cells in the anterior foregut at E9.0 just before the formation of the respiratory primordium.\(^1\),\(^1\) Hereafter Nkx2.1 will be expressed only in the respiratory primordium and in the developing airways and is excluded from the endoderm of the dorsal foregut tube. This dorsal foregut region is now marked by exclusive expression of Sox2 and will develop into the esophagus.\(^1\) Mice that lack Nkx2.1 have an undivided foregut tube that connects the pharynx to the stomach.\(^1\) The most proximal part of this Nkx2.1 knockout foregut is enclosed by a few poorly developed cartilage rings but the remainder of the mesenchyme is characterized by smooth muscle development as typical for the esophagus. In accordance, the endoderm expresses markers of esophageal differentiation such as Sox2 and p63.\(^1\) The lung buds form but fail to undergo branching morphogenesis and form cystic structures that fail to express markers of lung differentiation.\(^1\) Thus Nkx2.1 marks the endodermal cells that will form the respiratory primordium. Furthermore Nkx2.1 is required for the proper elongation and separation of the trachea, for branching organogenesis and differentiation of lung endoderm.

Sox2 marks the prospective esophageal foregut cells. As an alternative for Sox2\(^{-/-}\) mice, which are embryonic lethal at the blastocyst stage,\(^1\) Que et al. used a hypomorphic Sox2 mutant mice to study the role of Sox2 in esophageal development.\(^1\) They reported that a large proportion of Sox2 hypomorphic mice display fusion of the esophageal lumen with the tracheal lumen (tracheoesophageal fistula) combined with loss of the proximal esophagus (atresia). In Sox2 hypomorphic animals with an intact esophagus the esophageal diameter was diminished. At E18.5 the remaining esophagus was covered with columnar epithelium and lacked expression of esophageal maturation markers, such as p63 and keratin 14. In contrast, the epithelium is strongly positive for Nkx2.1 and expresses markers of airway differentiation. The importance of Sox2 in esophageal development is underscored by the fact that Sox2 mutations cause tracheoesophageal fistula and esophageal atresia in humans.\(^1\),\(^1\) This phenotype indicates that Sox2 is required to maintain the esophageal endoderm during development and represses Nkx2.1 mediated airway-type maturation.
Thus, Sox2 and Nkx2.1 are critical regulators of esophageal versus airway specification, that are expressed in non-overlapping patterns and repress each other's expression and activity.

**p63 is a critical regulator of esophageal squamous epithelial differentiation**

The p53 homologue p63 is a critical transcriptional regulator of squamous epithelial cell fate.\(^{19,20}\) p63 plays a critical role in maintaining homeostatic proliferation of basal cells as p63 expression specifically marks the basal layer of squamous tissues\(^{21}\) and \(p63^{-/-}\) mice completely lack stratified squamous epithelial tissues at birth.\(^{20}\) The esophagus in p63 mutant mice has a pseudostratified columnar epithelium that shows signs of respiratory maturation, such as presence of ciliated and goblet cells.\(^{22,23}\) Since the columnar epithelium observed in Sox2 hypomorphic mice was devoid of p63 expression. This most likely indicates that Sox2 functions upstream of p63 in the induction of a squamous phenotype in the esophageal endoderm.

**The morphogenetic signaling network responsible for gut-airway separation**

As mentioned above, a limited number of morphogenetic signaling pathways is used in patterning the tissues of our body. The major known morphogenetic pathways are all involved in endodermal-mesenchymal interactions during esophageal development. As we will review below there is a noticeable difference in the role of the various signaling pathways between the developing esophagus and airways and many signaling defects in the pathways discussed below lead either to a preferential esophageal or a predominant airway phenotype. For clarity we will discuss the pathway separately but try to indicate interactions between the different pathways where these are known.

**Sonic Hedgehog signaling.** The first morphogen identified as a critical regulator of gut-airway separation was Sonic Hedgehog. Sonic Hedgehog (\(Shh\)) is initially expressed throughout the anterior endoderm, but is restricted to the distal esophagus at later stages.\(^{24,25}\) \(Gli\) transcription factors, which mediate Hedgehog signaling, are selectively expressed in the mesoderm indicating that Hedgehog signals exclusively in a paracrine manner from endoderm to mesoderm.\(^{26}\) The critical role of Shh signaling in foregut development was revealed in Shh mutant mice. The phenotype of \(Shh^{-/-}\) mice is remarkably similar to Sox2 mutant mice. At E17.5 the proximal \(Shh^{-/-}\) esophagus is hypoplastic and the developing trachea and lungs fail to separate correctly from the gut. More distally (where Shh expression is highest in normal mice) there is no discernible remaining esophagus in \(Shh^{-/-}\) mice at this point in development.\(^{24,27}\) This suggests that paracrine Shh signaling to the mesenchyme is required to allow the proper elongation and survival of esophageal tissue and to maintain a mesenchymal barrier between the developing tubes of the esophagus and airway.
This important role of Hedgehog signaling in normal esophageal development was confirmed in mice with mutations in the Gli transcription factors. In Gli2\textsuperscript{−/−} mice, the esophagus has a very small lumen surrounded by a poorly developed mesenchymal layer that fails to develop an αSma positive smooth muscle layer. In Gli2\textsuperscript{−/−} Gli3\textsuperscript{+/-} mutant mice, the foregut is hypoplastic and fails to form the appendages for the trachea and lungs at E9.5.\textsuperscript{28} In Gli2\textsuperscript{−/−} Gli3\textsuperscript{−/−} mice that survive until later stages of development only a very small proximal esophageal remnant can be observed and the mice lack both trachea and lungs.

The striking similarity between Shh and Sox2 mutant mice suggests that these factors are functionally related. It is unlikely that Sox2 acts directly downstream of Shh as Shh signaling is uniquely to the mesenchyme. There are two alternative options that are not mutually exclusive. First endodermally expressed Sox2 could act upstream of Shh as a critical transcription factor required for Shh expression. Alternatively, endodermal Sox2 expression could depend on mesenchymally expressed factors that are controlled by Shh signaling.

The transcription factor Foxf1 is probably the key mesenchymal target of Shh signaling. Mahlapuu and colleagues have demonstrated that mesenchymal expression of Foxf1 can be induced by ectopic expression of Shh and that Shh mutant mice lack mesenchymal Foxf1 expression in the foregut.\textsuperscript{29} The Foxf1 homozygous mutation leads to early embryonal lethality and cannot be evaluated for a foregut phenotype. However, Foxf1\textsuperscript{+/-} mutant mice have a clear foregut phenotype that is very similar to Shh and Gli mutant mice. In Foxf1\textsuperscript{+/-} mice the esophagus is poorly developed and fails to separate properly from the trachea, the lungs are hypoplastic and branching morphogenesis is reduced.\textsuperscript{29} Thus Sox2/Shh signaling induces mesenchymal Foxf1 expression which is required to allow the mesenchymal cells to support esophageal elongation and survival and the appropriate separation of the esophagus and airways.

**Bone Morphogenetic Protein signaling.** Members of the Bone Morphogenetic Protein (Bmp) signaling pathway play a critical role in foregut development. Bmp4 and Bmp7 are the major Bmp ligands expressed during foregut development.\textsuperscript{30,31} These Bmps display a non-overlapping expression pattern, with expression of Bmp4 being restricted to the mesenchyme ventral to the developing trachea and Bmp7 expression in the epithelium of the developing esophagus and its surrounding posterior mesenchyme. Given the strong similarity between Bmp4 mutant and Bmp receptor mutant mice (see below) it seems that the mesenchymally expressed Bmp4 is the key Bmp ligand during gut-airway development. Signaling by the Bmp pathway seems to occur mainly in the posterior foregut endoderm and mesenchyme. This was assessed by LacZ staining in a BRE-LacZ Bmp signaling reporter mouse\textsuperscript{31} and immunohistochemical localization of the phosphorylated form of Smads1,5 and 8,\textsuperscript{30} the Smads that mediate Bmp signaling.
Consistent with expression of Bmp4 in the ventral mesoderm surrounding the developing trachea, Bmp4 plays a critical role in tracheal development. At E11.5 *Bmp4* conditional knockout animals in which Bmp4 is deleted from the foregut endoderm and mesoderm show a clear failure of foregut separation with a single tube connecting the pharynx to the stomach and hypoplastic lungs.\(^{30}\) This tube shows esophageal type differentiation as it is positive for esophageal endodermal marker *Pax9*, negative for the tracheal endoderm marker *Nkx2.1* and negative for the tracheal mesenchyme marker *Col2a*. The authors of this study found that Bmp4 signaling is not required for specification of the tracheal primordium which formed normally at E9.25. However, the tracheal primordium was reduced in size compared to wild type mice at E9.5. Thus, Bmp4 is required for proper airway development after the initial specification of the tracheal primordium.

The importance of Bmp4 in airway development may explain why the airways fail to develop properly in Shh mutant mice as Bmp4 is one of the key mesenchymal targets of Hedgehog-Foxf1/Foxf2 signaling in the esophagus\(^ {24}\) and the intestine.\(^ {12}\)

Mice in which both Bmp receptor 1a and 1b were specifically deleted from the endoderm using a *ShhCre* showed a similar phenotype as the *Bmp4* conditional mutant mice. The *Bmpr1a/b* double mutant mice developed a single foregut tube that was positive for esophageal endodermal marker Sox2 and negative for the airway endodermal marker *Nkx2.1*. Thus again showing that Bmp signaling is required to induce and/or maintain a specific airway endodermal phenotype.

Conversely, actively antagonizing Bmp signaling has also been shown to play a role in protecting the esophageal endoderm against the airway phenotype inducing influence of the Bmp signaling pathway. Several Bmp antagonists are expressed in the developing foregut.\(^ {31}\) Of those, Noggin is likely the most relevant Bmp antagonist during foregut development, as *Noggin* mutant mice have a clear foregut phenotype.\(^ {32,33}\) Noggin is expressed in the dorsal foregut endoderm and lung mesenchyme from E10.5-11.5 and at later stages (E14.5) is confined to the developing esophageal smooth muscle layer. Loss of Noggin expression in *Nog^−/−* mutant mice showed an opposite phenotype of the Bmp4 and Bmpr1a/b mutant mice with intact airway differentiation in a single foregut tube connecting the airways to the stomach.\(^ {33}\) This suggests that Bmp signaling is not only required for proper airway differentiation but that suppression of Bmp signaling in the posterior foregut is equally important to allow proper esophageal development. The reciprocal nature of Noggin and Bmp4 signaling was clearly demonstrated by the fact that the phenotype of the *Nog^−/−* mice could be rescued by reducing the gene dose of *Bmp4* in *Nog^−/−/Bmp4^+/−* mutant mice.\(^ {33}\) The importance of the reciprocal regulation of Bmp signaling between airway and esophagus was explained with an elegant experiment by Domyan et al.\(^ {34}\) as these authors showed that the Bmpr1a/b airway phenotype can be rescued by deletion of Sox2 in *Bmpr1a/b Sox2* double mutant animals. Domyan and colleagues found that Bmp signaling directly represses the Sox2 promoter.\(^ {34}\) Thus, Bmp
signaling in the airway endoderm is required to repress Sox2 expression, thus allowing the airway endodermal phenotype to be expressed. Conversely, Noggin mediated repression of Bmp signaling allows the proper development of the esophageal endoderm by protecting Sox2 expression against the repressive influence of the Bmp pathway.

In conclusion, an endodermal-mesenchymal signaling network has been discovered in which endodermally expressed Shh induces Bmp4 in the mesenchyme via the Foxf1 transcription factor. This Bmp4 signals reciprocally to the endoderm of the developing airways to repress endodermal Sox2 expression and allow proper airway differentiation. The esophageal endoderm is protected from this mesenchymal Bmp4 signal by secreting the Bmp antagonist Noggin.

**Fibroblast Growth Factor signaling.** The Fibroblast Growth Factors (Fgfs) are a large group of morphogens with an important role in endodermal development. During gut-airway development this pathway provides a critical signal from the mesenchyme to the overlying endoderm. Fibroblast growth factors (Fgfs) act through tyrosine kinase transmembrane receptors. Thus far, four Fgf receptors have been identified. The tissue-specific alternative splicing of the FGF receptors I-III is the main mechanism by which FGF-FGFR binding specificity is regulated.\(^ {35}\) This splicing event gives rise to epithelial “b” isoforms (FGFRIb to FGFRIIib) and mesenchymal “c” isoforms (FGFRIc to FGFRIIIc), which differ in the binding specificity profiles for the many different Fgfs. The key Fgf with an established role in foregut development is Fgf10. At E10.5 Fgf10 is expressed in the anterior mesenchyme surrounding the prospective trachea. The importance of this mesenchymal Fgf10 expression is underscored by the fact that Fgf10 mutant mice develop a trachea, but completely lack further development of the lung buds.\(^ {36,37}\) Fgf10 in the mesenchyme signals to a specific IIIb isoform of the Fgfr2 (the main receptor for Fgf10) expressed in the epithelium. Indeed, mice that specifically lack the Fgfr2 IIIb isoform display lung agenesis similar to Fgf10 mutant mice.\(^ {38}\) The role of mesenchymal-to-epithelial Fgf10-Fgfr2 IIIb signaling seems to lie in the reciprocal regulation of Nkx2.1/Sox2 expression. Fgf10 promotes an airway phenotype by positively regulating Nkx2.1 expression and repression of the expression of Sox2.\(^ {13}\) It has not been examined how the mesenchymal-endodermal Fgf10-Fgfr2 IIIb signaling axis relates to the Shh-Bmp4 signaling interactions in the foregut that have been mentioned above.

**Wnt signaling.** Similar to the Bmps and Fgfs, Wnts play a key role in airway development. Wnt2 and Wnt2b are expressed in the ventral mesoderm that surrounds the endoderm of the prospective airways around E9.0-10.5.\(^ {39}\) Wnt7b is expressed in the ventral endoderm at the same time in development.\(^ {40}\) Wnt2/2b double mutant mice fail to induce expression of Nkx2.1 and display complete lung and tracheal agenesis with intact esophageal development.\(^ {39}\) Wnt7b mutant embryos show a much less dramatic
phenotype with modest lung hypoplasia. Airway development requires canonical Wnt signaling as *Shh-Cre-Ctnnb1* mutants in which b-catenin is specifically deleted from the early endoderm are a phenocopy of *Wnt2/2b* mutant mice. The esophagus develops normally in *Shh-Cre-Ctnnb1* mutants indicating that canonical Wnt signaling is not involved in normal esophageal development. In *Shh-Cre-Ctnnb1(ex3)fl/wt* mice, in which β-catenin is constitutively activated in the early endoderm, an induction of Nkx2.1 positive cells is observed in the developing esophagus with concomitant loss of p63 expression. This firmly establishes the important role of canonical Wnt signaling in airway epithelial specification. One of the transcription factors that is required to repress Wnt signaling in the developing esophagus in order to allow normal esophageal development is Barx1. Barx1 is expressed in the mesoderm in between the developing esophagus and trachea and it has been suggested that Barx1 negatively regulates Wnt signaling through the regulation of secreted frizzled related proteins. As *Wnt2b* mutant mice showed complete loss of *Fgf10* expression it seems that canonical Wnt acts upstream of *Fgf10* and Nkx2.1 as one of the key drivers of airway epithelial specification.

**Figure 3** | Schematic overview of genes expressed during development in the esophageal and respiratory endoderm and mesoderm.
### Table | Knock-out mice

<table>
<thead>
<tr>
<th>Mouse model model</th>
<th>Foregut phenotype</th>
<th>Reference</th>
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</thead>
<tbody>
<tr>
<td><em>Nkx2.1&lt;sup&gt;-/-&lt;/sup&gt;</em></td>
<td>undivided foregut tube connecting the pharynx to the stomach, impaired lung bud development</td>
<td>15</td>
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<tr>
<td><em>Sox2&lt;sup&gt;2EGFP/COND&lt;/sup&gt;</em></td>
<td>majority develops TEF/EA</td>
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<td><em>p63&lt;sup&gt;−/−&lt;/sup&gt;</em></td>
<td>esophagus with pseudostratified columnar epithelium that shows signs of respiratory maturation</td>
<td>22, 23</td>
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<tr>
<td><em>Shh&lt;sup&gt;−/−&lt;/sup&gt;</em></td>
<td>hypoplastic proximal esophagus and septation defects</td>
<td>24, 27</td>
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<tr>
<td><em>Gli2&lt;sup&gt;−/−&lt;/sup&gt;</em></td>
<td>small esophageal lumen with poorly developed mesenchyme</td>
<td>28</td>
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<tr>
<td><em>Gli2&lt;sup&gt;−/−&lt;/sup&gt;/Gli3&lt;sup&gt;−/−&lt;/sup&gt;</em></td>
<td>hypoplastic foregut and absent trachea and lung appendages</td>
<td>28</td>
</tr>
<tr>
<td><em>Foxf1&lt;sup&gt;−/−&lt;/sup&gt;</em></td>
<td>TEF, narrow esophagus and lung hypoplasia</td>
<td>29</td>
</tr>
<tr>
<td><em>Noggin&lt;sup&gt;−/−&lt;/sup&gt;</em></td>
<td>TEF/EA, with intact airway differentiation</td>
<td>32, 33</td>
</tr>
<tr>
<td><em>Bmp4&lt;sup&gt;−/−&lt;/sup&gt;</em></td>
<td>single tube connecting the pharynx to the stomach and hypoplastic lungs</td>
<td>30</td>
</tr>
<tr>
<td><em>Bmp4&lt;sup&gt;−/+&lt;/sup&gt;</em></td>
<td>no TEF/EA was seen</td>
<td>32, 33</td>
</tr>
<tr>
<td><em>Bmp7&lt;sup&gt;−/−&lt;/sup&gt;</em></td>
<td>no TEF/EA was seen</td>
<td>32, 33</td>
</tr>
<tr>
<td><em>Fgf10&lt;sup&gt;−/−&lt;/sup&gt;</em></td>
<td>Lung agenesis</td>
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<tr>
<td><em>Fgfr2 IIIb&lt;sup&gt;−/−&lt;/sup&gt;</em></td>
<td>Lung agenesis, with complete loss of trachea and lungs</td>
<td>39</td>
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<tr>
<td><em>Wnt2/2b&lt;sup&gt;−/−&lt;/sup&gt;</em></td>
<td>lung hypoplasia</td>
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<td>lung hypoplasia</td>
<td>40</td>
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<tr>
<td><em>Barx1&lt;sup&gt;−/−&lt;/sup&gt;</em></td>
<td>septation defects</td>
<td>41</td>
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</tbody>
</table>

**TEF**: tracheoesophageal fistula  
**EA**: esophageal atresia

In conclusion, the tissue specific transcription factors and morphogenetic pathways that regulate esophagus-airway separation and differentiation have partially been resolved. Sox2 and Nkx2.1 are the key endodermal transcriptional regulators of esophageal and airway fate, respectively. Development of the esophagus critically depends on Hedgehog signaling and actively suppressing BMP-signaling. In contrast, the Fgf, Bmp and Wnt signaling pathways are key regulators of airway development.

### ESOPHAGEAL EPITHELIAL HOMEOSTASIS IN THE ADULT

**Murine esophageal epithelium**

In contrast to the rest of the gastrointestinal tract, which is covered with a single layer of columnar epithelium, the esophagus is lined with a multilayered squamous epithelium (Figure 4). This epithelial phenotype is reflective of its role to transport rather than modify and absorb luminal content. The esophageal epithelium in the mouse is constantly renewed from a population of cells that are neatly organized with their nuclei perpendicular
Esophageal development and epithelial homeostasis

As differentiating cells leave the basal layer, they change their shape and orientation to become larger, flattened and aligned parallel to the basement membrane. These cells have a large cytoplasm, causing the enlarged nuclei to be spread further apart from each other, compared to the nuclei in the basal layer. Advancing upwards, towards the lumen, nuclei are degraded and cells develop keratohyalin granules, which can be identified as basophilic small round structures. The surface layer of the murine esophagus is keratinized, possibly to form a strong protective layer against abrasive food components. The rate of proliferation of the cells in the basal layer is tightly coupled to the rate at which differentiating cells are lost in the esophageal lumen. The mechanisms that regulate homeostasis in this dynamic equilibrium have not been described.

**Keratins as markers of esophageal epithelial differentiation**

Keratins are the building blocks of intermediate filaments that form part of the cells cytoskeleton. Keratins are expressed in a highly cell type and maturation state specific manner and several keratins are useful markers of the differentiation state of esophageal epithelial cells. In esophageal basal cells three keratins are present. Keratin 14 is paired with keratin 5 and both are expressed in all basal layer cells in the adult squamous epithelium. Keratin 15 is the third keratin member expressed specifically in the basal layer. As esophageal epithelial cells leave the basal layer and start to differentiate they shut down expression of keratins 5, 14 and 15 and induce the expression of keratin 4 and its partner keratin 13. Differentiating cells start to degrade their nucleus and other organelles and make keratohyalin granules which contain profilagrin. This is the precursor to fillagrin which will aggregate keratins into tight bundles resulting in the typical flattened shape of differentiated esophageal epithelial cells. In addition cells will start to synthesize specialized proteins such as involucrin and loricrin which form the cornified cell envelope just beneath the plasma membrane, a structure with a key role in epithelial barrier formation in keratinized epithelia.

**Human versus murine esophagus**

Most studies on dynamics of epithelial homeostasis are performed in rodents. It is however important to note that there are key differences between the murine and human esophageal epithelium. First of all, the human esophageal epithelium contains more cell layers and it is folded along papillae (Figure 4). Proliferation and mitosis in the mouse is limited to basal cells. In humans this is extended to the 5th-6th suprabasal layers. Unlike the murine esophageal epithelium, the human esophageal epithelium is non-keratinized and cells retain their nucleus. Therefore, keratohyalin granules are rare. In rodents keratinization of the esophageal epithelium may serve to protect against abrasive dietary components. The human esophageal epithelium is exposed to harmful
dietary substances as well. The main mechanisms by which the esophageal epithelium copes with this is high turn-over of epithelial cells. Esophageal submucosal glands\textsuperscript{55} are present in human but not in mice and may play an important protective role in humans.

![Figure 4](image-url)  

**Figure 4** | Adult murine esophageal epithelium versus adult human esophageal epithelium.

### Regulators of esophageal epithelial proliferation and differentiation

The mechanisms by which esophageal epithelial homeostasis is regulated are relatively poorly characterized. It is becoming clear that many of the same pathways that regulate morphogenesis of an epithelium during development are often also critical to regulate epithelial homeostasis in the adult epithelium.\textsuperscript{56} This notion seems to be valid for the esophageal epithelium. For example, as described above, Sox2 is the key tissue specific transcriptional regulator that defines the esophageal epithelial phenotype during development. In the adult epithelium Sox2 is expressed in virtually all cells of the basal layer. Lineage tracing of Sox2+ cells in the adult esophageal epithelium showed that Sox2+ cells can generate long lived clones of cells that persist in the esophageal epithelium.\textsuperscript{57} In transgenic mice that express the thymidine kinase gene under control of the Sox2 promoter, treatment with ganciclovir causes ablation of Sox2-expressing cells and this results in complete loss of basal cells.\textsuperscript{57} Reciprocally, overexpression of Sox2 leads to an increase in epithelial progenitor cells and loss of differentiated features.\textsuperscript{58} Together these data support the notion that the key role of Sox2 as a tissue specific transcription factor in development is maintained in the adult esophageal epithelium.

A second transcriptional regulator with a conserved role between development and adult epithelial homeostasis may be p63. Work in esophageal cell carcinoma (ESCC) cell lines suggests that p63 may be required for epithelial proliferation,\textsuperscript{59} however the role of
p63 in adult esophageal epithelium has not been addressed by conditional mutagenesis in the adult esophagus.

Since Shh is a morphogen with a key role in esophageal morphogenesis. We and others have examined the role of Shh signaling in the adult esophagus.\textsuperscript{11,60} Shh is expressed by epithelial cells of the basal layer in the adult esophagus. In contrast to the exclusively paracrine Hedgehog signaling in the cuboidal epithelium during development, we found that Hedgehog signaling is autocrine in the adult squamous epithelium (similar to the skin). Using \textit{in situ} hybridization we found that the cells in the basal layer expressed both the Hedgehog receptor \textit{Smo} and transcription factor \textit{Gli-1} and basal cells were marked by LacZ expression in Gli1-LacZ reporter mice.\textsuperscript{11} We examined the role of Shh by activating the Hedgehog (Hh) pathway using two mouse models: one in which the inhibitory receptor \textit{Ptch1} can be conditionally deleted and another in which Hh pathway transcription factor \textit{Gli1} can be conditionally overexpressed. In these mouse models, which both lead to increased Hh signaling, we observed an expansion of the proliferating cell compartment accompanied with impaired maturation and migration of epithelial cells. This is consistent with an autocrine role for Shh signaling in the epithelial cells of the basal layer of the adult esophagus and indicates that Hh signaling regulates the phenotype of basal cells in the esophageal epithelium and promotes their proliferation.\textsuperscript{11}

In addition to the factors shown to be important in development, several other signaling molecules and mechanisms have been described to regulate adult esophageal homeostasis:

Two transcription factors of the Krüppel-like factor (Kfl) family also play a role in homeostasis of the esophageal epithelium in the adult animal. Expression of \textit{Klf5} is restricted to the basal layer and seems to regulate proliferative capacity. Transgenic overexpression of Klf5 in the esophageal epithelium results in a two-fold increase in proliferation rate, without further abnormalities in esophageal epithelial homeostasis.\textsuperscript{61} In contrast to Klf5, \textit{Klf4} is expressed in the suprabasal layer. Klf4 plays a critical role in normal esophageal epithelial differentiation. \textit{Klf4}-deficient mice show impaired differentiation and hyperproliferation, resulting in epithelial dysplasia.\textsuperscript{62}

One of the major cell-to-cell signaling pathways with a role in esophageal homeostasis is the Notch signaling pathway. Ohashi et al. have shown that Notch signaling through the transcription factor CSL is required for human esophageal epithelial differentiation in organotypic cultures \textit{in vitro} and in the mouse esophageal epithelium \textit{in vivo}.\textsuperscript{63} Their work suggested a key role for the expression and activation of NOTCH1 and NOTCH3. The key role for Notch signaling in esophageal homeostasis is underscored by the finding that Notch pathway genes are frequently mutated in esophageal squamous cell carcinomas.\textsuperscript{64}

Interestingly, we found upregulation of Notch pathway components (\textit{Dll3}, \textit{Jag2} and \textit{Hes5}) in a mouse model that leads to esophageal precursor cell differentiation.\textsuperscript{65} In
this model we chemically induced endoplasmic reticulum (ER) stress and subsequent unfolded protein response (UPR) activation via thapsigargin treatment. This lead to reduced proliferation and increased progenitor differentiation in esophageal epithelium and correlated with increased expression of several Notch signaling components. More evidence for the involvement of the UPR in esophageal epithelial homeostasis came from experiments with a genetic ER-stress model; \textit{Ah1Cre-Rosa26-LacZ-Grp78\textsuperscript{-/-}} mice.\textsuperscript{65} UPR activation in response to conditional deletion of a major ER chaperone Grp78 in esophageal epithelium of these mice resulted in rapid differentiation followed by repopulation of the epithelium from the non-recombined wild type cells, as was found in intestinal epithelium.\textsuperscript{66} This suggests that also in the esophageal epithelium the UPR may serve as a quality control mechanism which forces progenitor cells with accumulated unfolded proteins to initiate differentiation.

**ESOPHAGEAL STEM CELLS**

In concordance with both the skin epidermis and the GI-tract, the esophageal epithelium is constantly renewed. This suggests the presence of an actively proliferating stem cell population to fuel this renewal. However, despite the fact that in the last decade stem cells have been identified in the mouse skin and the other tissues of the murine gastrointestinal tract,\textsuperscript{67-70} to date no esophageal stem cell has been conclusively demonstrated.

Currently, there is a lack of consensus about the presence of dedicated stem cells in the esophagus. The mouse esophageal epithelium is devoid of clearly identifiable structural features such as crypts and glands that serve as stem cell niches in other tissues. However, the region to which proliferation is restricted has been unequivocally pinpointed. Pioneering work performed by Leblond and colleagues in rat esophagi showed with the use of \textsuperscript{3}H-thymidine pulse-chase experiments that proliferation is restricted to the basal layer and this led the authors to conclude that ‘if stem cells are defined as cells which produce cells similar to themselves as well as differentiating cells, the basal cells are the stem cells of the esophageal epithelium’.\textsuperscript{42,43}

The current debate focusses on the question if indeed all basal cells have an equal capacity for self-renewal\textsuperscript{71} or if the basal layer is organized in a stem cell-transit amplifying (TA) cell hierarchy,\textsuperscript{72} as is found in for example the small intestine. In the following paragraphs we will try to summarize the evidence presented for the two hypotheses. To avoid confusion in nomenclature we will use a description to define a dedicated tissue stem cell as follows: A tissue-specific stem cell has the ability to generate new stem cells, i.e. have self-renewal capacity and is capable of generating all the cell types present in a tissue, i.e. have tissue-renewal capacity. Of note, these characteristics are, in our opinion, independent of the cycling time and/or label-retaining characteristics of
these cells. It should be mentioned here that the well characterized Lgr5+ stem cells of the small intestine cycle once every 24 hours and can be considered fast cycling. In contrast, the term stem cell has also been used for slow-cycling, ‘label-retaining cells’ (LRCs) that contribute to epithelial homeostasis after damage. These LRCs are in general cycling only rarely in a homeostatic tissue and can therefore be identified by the prolonged retention of a DNA label after chronic infusion of such a label.

With an elegant in vivo Histone2B-GFP pulse chase labeling experiment, Doupe et al. showed that there are low numbers of LRCs found in the esophagus basal layer epithelium, as was previously described. Doupe et al. showed, however, that these are most probably not of epithelial origin and are positive for hematopoietic lineage marker CD45. This suggests that such a population of LRC cells indeed is absent from the esophageal epithelium. This finding, however, does not exclude the existence of faster-cycling dedicated stem cells.

On the basis of further experimentation using a double-conditional Ah-CreERT2*LSL-eYFP-reporter mouse in a tracing experiment and mathematical modeling of the results Doupe et al. propose that 65% of the basal layer consists of esophageal progenitors (EPs) which on average divide ~2x/week and for every division “esophageal progenitors are functionally equivalent”. As was suggested by Leblond and colleagues.

In contrast, other studies suggest that the mouse basal layer is functionally heterogeneous and provide evidence for a stem cell/TA hierarchy in the esophageal epithelium. Kalabis et al. isolated a ‘side population’ (SP) from the esophageal epithelium on the basis of a dye exclusion method. This method was used for identification of stem cells in several tissues and is based on the finding that stem cells have an increased capacity to pump out DNA binding dye. Characterization of the isolated esophageal ‘SP’ cells with FACSorting and in vitro experimentation provided evidence for high clonogenic potential within this subpopulation of cells, suggesting that they might be stem cells.

Using two cell surface markers, namely transferrin receptor (CD71) and the integrin isoform a6, another study identified three phenotypically and functionally distinct subpopulations from the murine esophageal epithelium. The authors concluded that these corresponded to a stem cell, a TA and a differentiated cell compartment.

Limited studies have been performed on human esophageal material. As described above, the arrangement of the human esophageal epithelium differs from the mouse. The human esophageal basal epithelium is divided into two distinct areas by regular invaginations of the basal layer, termed papillae. The papillary basal layer (PBL) lines the papillae and the flat interpapillary basal layer (IBL) lies in between these structures.

Two studies suggest that proliferation is asymmetrically compartmentalized between the IBL and the PBL, with the IBL containing more proliferative cells. Interestingly, on the basis of further experimentation these studies reach contrasting conclusions.
On the one hand, isolation of PBL and IBL cells using their differential integrin beta1 (Itgb1) hi (PBL) and lo (IBL) staining, showed higher clonal capacity in Itgb1 hi fractions. This led Seery et al. to conclude that the basal layer is heterogeneous and the IBL might harbor a stem cell population.76 In line with this, ex vitro studies, combined with some in vitro experiments, have suggested Neurotrophin receptor p75NTR, and, alternatively, a a6 bright/CD71 dim as potential human esophageal stem cell markers.77,78

On the other hand Barbera et al. used CD34 and Epcam to isolate three subpopulations from human esophagus, with the CD34 hi EpCam lo population representing the IBL cells. Surprisingly, all three subpopulations showed the same clonogenic potential. The authors explain this result, as being due to plasticity of esophageal cells to injury. In vitro cells are no longer attached to the basement membrane which would result in an injury signal, making all cells equally capable of forming colonies.53

Using 5-iodo-2'-deoxyuridine (IdU) labeling in patients undergoing esophagectomy, another paper described the presence of cytokeratin-expressing LRCs in human PBL,79 adding yet another layer of complexity the story.

In summary, to date, very few studies have been performed to directly tackle the question of the existence of dedicated stem cells in the esophagus. In our view, due to differences in methodology/markers used and the extent of the functional assays performed, both in vitro and in vivo, in the different studies, this question is still open.

Concluding remarks
Clearly we still have very limited insight in pathways and genes involved in normal homeostasis of the esophageal epithelium. Enhancing our knowledge about the mechanism of proliferation and pathways driving differentiation is therefore crucial. It would lead to better understanding of carcinogenesis, development of Barrett esophagus and tissue repair. The involvement of stem cells/progenitor cells in esophageal homeostasis needs further experimental evidence, especially given an implication of stem cells in oncogenic transformation and development of esophageal cancer.
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