Esophageal development and epithelial homeostasis

Sanne L. Rosekrans, Bart Baan, Vanesa Muncan, and Gijs R. van den Brink
Tytgat Institute for Liver and Intestinal Research and Department of Gastroenterology and Hepatology, Academic Medical Center, Amsterdam, the Netherlands
Submitted 18 March 2015; accepted in final form 25 June 2015

Rosekrans SL, Baan B, Muncan V, van den Brink GR. Esophageal development and epithelial homeostasis. Am J Physiol Gastrointest Liver Physiol 309: G216–G228, 2015. First published July 2, 2015; doi:10.1152/ajpgi.00088.2015.—The esophagus is a relatively simple organ that evolved to transport food and liquids through the thoracic cavity. It is the only part of the gastrointestinal tract that lacks any metabolic, digestive, or absorptive function. The mucosa of the adult esophagus is covered by a multilayered squamous epithelium with a remarkable similarity to the epithelium of the skin despite the fact that these tissues originate from two different germ layers. Here we review the developmental pathways involved in the establishment of the esophagus and the way these pathways regulate gut-airway separation. We summarize current knowledge of the mechanisms that maintain homeostasis in esophageal epithelial renewal in the adult and the molecular mechanism of the development of Barrett’s metaplasia, the precursor lesion to esophageal adenocarcinoma. Finally, we examine the ongoing debate on the hierarchy of esophageal epithelial precursor cells and on the presence or absence of a specific esophageal stem cell population. Together the recent insights into esophageal development and homeostasis suggest that the pathways that establish the esophagus during development also play a role in the maintenance of the adult epithelium. We are beginning to understand how reflux of gastric content and the resulting chronic inflammation can transform the squamous esophageal epithelium to columnar intestinal type metaplasia in Barrett’s esophagus.

esophagus; development; homeostasis; stem cell; endoderm

THE WORD ESOPHAGUS IS DERIVED from the Greek words οὐσεῖν (oisein, to carry) and φαγεῖν (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 pharyngeoesophageal 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 pharyngeoesophageal 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 toward the stomach and to prevent reflux of gastric content into the esophagus. The relatively simple histology of the esophageal epithelium corresponds 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 with 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 given that 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 (Fig. 1). The respiratory appendage consists of a central ventral tracheal bud and two adjacent ventrolateral lung buds (12, 57, 82). 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 (20).

When the mouse esophagus has been clearly established by E11.5, the epithelium consists of a single keratin (K)8-positive cuboidal epithelial layer (Fig. 2) (100). In the following days (E13.5–E17.5) the epithelium gradually becomes more layered until there are around four layers of epithelial cells (71, 100). During development there is a gradual conversion of a K8-positive cuboidal epithelium to a K14-positive squamous epithelium. This process begins at the basal layer around day E17.5 and the basal layer is mostly K14 positive at birth (100). The suprabasal layers will subsequently gradually lose K8 expression postnatally (100). 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 K10 starts at postnatal day (P)1 (100). During development the epithelium contains numerous ciliated cells, which

Fig. 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.

Fig. 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. (100).
have almost completely disappeared by P4 (52, 71). In the adult mouse the epithelium will undergo a process of keratinization; this does not occur in humans.

Recently, Wang et al. (93) proposed a model in which the embryonic cuboidal epithelium is displaced by an undermining population of p63-positive squamous cell precursors that migrate under the cuboidal epithelial cells from the proximal to distal esophagus. 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 vs. 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 (87). Actual lineage tracing experiments are thus required to further examine the interesting hypothesis by Wang et al.

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 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 cells 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 (26). Four families of morphogenetic pathways can roughly be distinguished: Wnt, Hedgehog (Hh), Tgfβ, and Sox2. Each family 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 vs. paracrine signaling. For instance, 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 also below in the Regulators of Esophageal Epithelial Proliferation and Differentiation section) (89). 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 (11).

As will be shown 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 (Fig. 1). One is the improper separation of the esophagus and trachea, leading to the development of tracheoesophageal fistula (TEF). 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 (esophageal atresia; see Table 1 for an overview of the mouse mutants discussed below).

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 vs. airway endoderm. Sox2 marks the endodermal cells that will form the esophagus and is expressed throughout the esophageal epithelium in the adult (69). Nkx2.1 identifies the endodermal cells from which the respiratory tract will form and is expressed in alveolar epithelial cells in the adult (36). These transcription factors not only are useful markers to identify esophageal vs. 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 (36, 56). 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 (69). Mice that lack Nkx2.1 have an undivided foregut tube that connects the pharynx to the stomach (56). 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 (69). The lung buds form but fail to undergo branching morphogenesis and form cystic structures that fail to express markers of lung differentiation (56). 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
Table 1. Mouse mutants

<table>
<thead>
<tr>
<th>Mouse Model</th>
<th>For gut phenotype</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nkx2.1+/−</td>
<td>undivided foregut tube connecting the pharynx to the stomach, impaired lung bud development</td>
<td>56</td>
</tr>
<tr>
<td>Sox22GFPCOND</td>
<td>majority develops TEF/EA</td>
<td>69, 95</td>
</tr>
<tr>
<td>p63+/−</td>
<td>hypoplastic proximal esophagus and septation defects</td>
<td>14, 99</td>
</tr>
<tr>
<td>Shh+/−</td>
<td>hypoplastic proximal esophagus and septation defects</td>
<td>42, 65</td>
</tr>
<tr>
<td>Gli2+/−</td>
<td>small esophageal lumen with poorly developed mesenchyme</td>
<td>58</td>
</tr>
<tr>
<td>Gli2+/−/Gli3+/−</td>
<td>hypoplastic foregut and absent trachea and lung appendages</td>
<td>58</td>
</tr>
<tr>
<td>Fosf1+/−</td>
<td>TEF, narrow esophagus, and lung hypoplasia</td>
<td>46</td>
</tr>
<tr>
<td>Noggin−/−</td>
<td>TEF/EA, with intact airway differentiation</td>
<td>41, 68</td>
</tr>
<tr>
<td>Bmp4+/−</td>
<td>single tube connecting the pharynx to the stomach and hypoplastic lungs</td>
<td>40</td>
</tr>
<tr>
<td>Bmp7+/−</td>
<td>no TEF/EA was seen</td>
<td>41, 68</td>
</tr>
<tr>
<td>Fgf10−/−</td>
<td>lung agenesis</td>
<td>16</td>
</tr>
<tr>
<td>Fgf2/IIIc−/−</td>
<td>lung agenesis, with complete loss of trachea and lungs</td>
<td>24</td>
</tr>
<tr>
<td>Wnt2/2b−/−</td>
<td>lung hypoplasia</td>
<td>77</td>
</tr>
<tr>
<td>Wnt7b−/−</td>
<td>septation defects</td>
<td>97</td>
</tr>
<tr>
<td>Barx1−/−</td>
<td>septation defects and hypoplastic lungs</td>
<td>53</td>
</tr>
</tbody>
</table>

TEF, tracheoesophageal fistula; EA, esophageal atresia.

The trachea, for branching organogenesis, and for 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 (2), Que et al. (69) used a hypomorphic Sox2-mutant mice to study the role of Sox2 in esophageal development. 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 (9, 95). 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 vs. airway specification that are expressed in nonoverlapping 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 (13, 99). p63 plays a critical role in maintaining homeostatic proliferation of basal cells as p63 expression specifically marks the basal layer of squamous tissues (98) and p63−/− mice completely lack stratified squamous epithelial tissues at birth. 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 (14, 99). 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. We will focus on phenotypes in mouse models because these are often more insightful than mutations in humans, in whom the many important mutations are missed because of embryonic lethality, and patients are rare and difficult to characterize at the molecular level because of the inability to obtain tissue. For an excellent overview of the human mutations that result in foregut phenotypes we refer to recent reviews (8, 10).

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 (42, 70). 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 (28). 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 (42, 65). 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−/− 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−/− Gli3+/− mutant mice, the foregut is hypoplastic and fails to form the appendages for the trachea and lungs at E9.5 (58). In Gli2−/− Gli3+/− 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 because 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 (46) 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. The Foxf1 homozygous mutation leads to early embryonal lethality and cannot be evaluated for a foregut phenotype. However, Foxf1+/−-mutant mice have a clear foregut phenotype that is very similar to Shh- and Gli-mutant mice. In Foxf1+/− mice the esophagus is poorly developed and fails to separate properly from the trachea, the lungs are hypoplastic, and branching morphogenesis is reduced (46). 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 (40, 72). These Bmps display a nonoverlapping 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 (72) and immunohistochemical localization of the phosphorylated form of Smads1, 5, and 8 (40), 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 (40). This tube shows esophageal-type differentiation since 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 with 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 since Bmp4 is one of the key mesenchymal targets of Hedgehog-Foxf1/Foxf2 signaling in the esophagus (42) and the intestine (11).

Mice in which both Bmp receptor 1a and 1b were specifically deleted from the endoderm by 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 (72). Of those, Noggin is likely the most relevant Bmp antagonist during foregut development, since Noggin-mutant mice have a clear foregut phenotype (41, 68). 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 (68). This suggests not only that Bmp signaling is 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 (68). The importance of the reciprocal regulation of Bmp signaling between airway and esophagus was explained with an elegant experiment by Domyan et al. (18) since 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 (18). Thus Bmp signaling in the airway endoderm is required to repress Sox2 expression, 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. Fgf acts 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 (61). This splicing event gives rise to epithelial “b” isoforms (FGFR1b to FGFRIIIb) and mesenchymal “c” isoforms (FGFR1c 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 (55, 76). 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 (16). 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 (69). 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 Bmp’s 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 (24). Wnt7b is expressed in the ventral endoderm at the same time in development (77). Wnt2b double-mutant mice fail to induce expression of Nkx2.1 and display complete lung and tracheal agenesis with intact esophageal development (24). Wnt2b mutant embryos show a much less dramatic phenotype with modest lung hypoplasia. Airway development requires canonical Wnt signaling because Shh-Cre-Ctnnb1floXmice in which β-catenin is specifically deleted from the early endoderm are a phenocopy of Wnt2b/2b-mutant mice (24). The esophagus develops normally in Shh-Cre-Ctnnb1floXmice, indicating that canonical Wnt signaling is not involved in normal esophageal development (24). In Shh-Cre-Ctnnb1floXmice, 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 to allow normal esophageal development is Barx1 (97). 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 (97).

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 (summarized in Fig. 3).

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 (Fig. 2). 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 to the basement membrane, the so-called basal layer (37, 49). 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 with the nuclei in the basal layer. Advancing upward, toward 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 (34). 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 (96), 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 (94). 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 (90). Differentiating cells start to degrade their nucleus and other organelles and make keratohyalin granules, which contain profilaggrin (45, 47). This is the precursor to filaggrin (33), which will aggregate keratins into tight bundles, resulting in the typical flattened shape of differentiated esophageal epithelium.
lial cells. In addition, cells will start to synthesize specialized proteins such as involucrin (3) and loricrin (51), 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 vs. 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 (Fig. 4). First of all, the human esophageal epithelium contains more cell layers and it is folded along papillae. Proliferation and mitosis in the mouse is limited to basal cells (37, 49). In humans this is extended to the fifth to sixth suprabasal layers (4). Unlike the murine esophageal epithelium, the human esophageal epithelium is nonkeratinized and cells retain their nucleus (27). 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 turnover of epithelial cells. Esophageal submucosal glands (44) are present in human but not in mice and may play an important protective role in humans.

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 (see Fig. 3) (88). 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 (1). 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 (1). Reciprocally, overexpression of Sox2 leads to an increase in epithelial progenitor cells and loss of differentiated features (43). 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 squamous cell carcinoma (ESCC) cell lines suggests that p63 may be required for epithelial proliferation (85). Although the in vivo role of p63 in adult esophageal epithelium has not been addressed, genetic deletion of p63 in organotypic culture derived from adult esophageal epithelium indeed inhibited their self-renewal (30).

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 (29, 89). Shh is expressed by epithelial cells of the basal layer in the adult esophagus. In contrast to the exclusively paracrine Hedgehog signaling the cuboidal epithelium during development, we found that Hedgehog signaling is also autocrine in the adult squamous epithelium (similar to the skin). Using in situ hybridization we found that the cells in the basal layer expressed both the Hedgehog receptor Smo and transcription factor Gli-1 and basal cells were marked by LacZ expression in Gli1-LacZ reporter mice (89). We examined the role of Shh by activating the Hedgehog (Hh) pathway using two mouse models: one in which the inhibitory receptor Pch1 can be conditionally deleted and another in which Hh pathway transcription factor 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 (89).

Recently it was reported that BMP signaling (and antagonism) is also involved in homeostasis of the adult esophageal epithelium (31). In contrast to their nonoverlapping expression in development, in the adult mouse esophagus, both BMP4 and 7 are expressed in the epithelial basal layer and signal toward the suprabasal epithelium. Simultaneously, the expression of the BMP antagonists follistatin and gremlin2 limit BMP signaling in the basal layer itself and the underlining mesenchyme, respectively. Genetic hyperactivation of BMP signaling via epithelial overexpression of a constitutively active BMP receptor 1 results in large-scale differentiation of esophageal epithelial progenitor cells (31).

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 (Klf) family also play a role in homeostasis of the esophageal epithelium in the adult animal. Expression of Klf5 is restricted to the basal layer and seems to regulate proliferative capacity. Transgenic overexpression of Klf5 in the esophageal epithelium results in a twofold increase in proliferation rate, without further abnormalities in esophageal epithelial homeostasis (23). In contrast to Klf5, Klf4 is expressed in the suprabasal layer. Klf4 plays a critical role in normal esophageal epithelial differentiation. Klf4-deficient mice show impaired differentiation and hyperproliferation, resulting in epithelial dysplasia (84).

One of the major cell-to-cell signaling pathways with a role in esophageal homeostasis is the Notch signaling pathway. Ohashi et al. (59) have shown that Notch signaling through the transcription factor CSL is required for human esophageal epithelial differentiation in organotypic cultures in vitro and in the mouse esophageal epithelium in vivo. 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 (21).

Interestingly, we found upregulation of Notch pathway components (Dll3, Jag2, and Hes5) in a mouse model that leads to esophageal precursor cell differentiation (73). In this model we chemically induced endoplasmic reticulum (ER) stress and subsequent unfolded protein response (UPR) activation via thapsigargin treatment. Thapsigargin is a plant-derived inhibitor of ER Ca2+-ATPases that induces ER stress by Ca2+-depletion of the ER. In vivo, thapsigargin treatment led 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, Ah1Cre-Rosa26-LacZ-Grp78−/− mice (73). 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 nonrecombined wild-type cells, as was found in intestinal epithelium (25). This suggests that also in the esophageal
epithelium the UPR may serve as a quality control mechanism that forces progenitor cells with accumulated unfolded proteins to initiate differentiation.

**Esophageal Epithelial Pathophysiology, the Development of Barrett’s Esophagus**

Although the focus of this review is on the pathways that maintain esophageal epithelial homeostasis and we have limited insight into how these pathways are deregulated during carcinogenesis, we will briefly review the available data on how the signaling pathways discussed above have been implicated in the development of Barrett’s esophagus, the precursor to esophageal adenocarcinoma (64, 74). For a broader review on Barrett’s esophagus we refer to recently published reviews (15, 80).

The development of esophageal adenocarcinoma occurs in a stepwise manner that is relatively well characterized. The major risk factor for the development of esophageal adenocarcinoma is gastroesophageal reflux disease (GERD). In GERD, the lower esophageal sphincter fails to prevent reflux of gastric content, thereby leading to exposure of the esophageal epithelium to gastric acid and bile acids. This can eventually lead to the development of chronic esophageal inflammation and ulceration. In a subset of patients with esophagitis, the stratified squamous epithelium at the gastroesophageal junction is converted to intestinal-type columnar epithelium, a process called metaplasia. This intestinal metaplasia is called Barrett’s esophagus and is a precursor lesion for the development of adenocarcinoma. Population-based studies have shown that Barrett’s esophagus is present in 0.5–1.5% of the Western population (15). The progression of Barrett’s epithelium to adenocarcinoma again occurs in a well-established stepwise fashion in which the epithelium will first develop areas of low-grade dysplasia, which progresses to high-grade dysplasia and cancer. The risk of developing esophageal adenocarcinoma is very low [between 0.05–0.5% per year for nondysplastic metaplasia (15)] but increases steeply as the epithelium progresses from normal epithelium to low-grade and high-grade dysplasia.

The molecular mechanism of the conversion of normal esophageal epithelium to intestinal metaplasia is incompletely understood and it is not known whether the metaplastic epithelium is derived from the normal esophageal epithelium or arises from gastric cardia stem cells (66) or from remnant embryonic epithelial cells that persist at the gastroesophageal junction, as has also been proposed (93). Below we will discuss some of the pathways that may play a role in the development of Barrett’s esophagus.

**Genetic predisposition.** Genomewide association studies (GWAS) that examined genetic predisposition to the development of Barrett’s esophagus have implicated several pathways with a role in the development of the esophagus (38, 62, 81). One of the most significant association with the development of Barrett’s esophagus found in the first large GWAS is a single-nucleotide polymorphism (SNP) that is very close to FOXF1 (81), the mesenchymally expressed Hedgehog target that acts upstream of BMP4 during development (see above). Although, it was described that several transcription factors that regulate expression of FOXF1 bind in the region that is in linkage disequilibrium with the associated SNP, it is not yet clear how the risk allele affects FOXF1 expression. Given the role of BMP4 in the development of Barrett’s esophagus described below it could be speculated that the FOXF1 risk allele may act to increase stromal BMP4 expression; however, this has not yet been investigated.

A subsequent large GWAS identified a further two transcriptional regulators with a key role in development in the risk to develop Barrett’s esophagus (38). The first is FOXP1. Foxp1 is expressed by the esophageal epithelium and muscle layer during development (78) and it has been shown that Foxp1 cooperates with Foxp2 in esophageal development. Foxp2−/− Foxp1+− double mutant had a defect in the development of the esophageal muscle wall. However, the role of Foxp1 in the esophageal epithelium is not known and it is not known which of the morphogenetic pathways discussed above interact with FOXP1 in the context of the esophagus. Intriguingly, however, FOXP1 is overexpressed in diffuse large B cell lymphoma, where it enhances Wnt signaling (91). Increased Wnt signaling could play a role in the development of intestinal metaplasia since Wnt signaling needs to be repressed to allow the development of normal esophageal epithelium and the Wnt pathway is the major pathway in the specification of the intestinal stem cell phenotype. The potential for interaction with the Wnt signaling pathway is something FOXP1 has in common with the second transcription factor with an association with Barrett’s esophagus identified in the same GWAS (38).

**BARX1.** BARX1 is an important repressor of the Wnt signaling pathway during development (discussed above). In addition, TBX5, a transcription factor that was identified in the largest GWAS to date (62), is a key regulator of limb development through the regulation of the expression of Wnt ligands (83). The third GWAS also identified a locus near GDF7, a member of the BMP pathway that is interesting in the light of the potential role of BMP signaling in the development of Barrett’s esophagus discussed below. In conclusion, although it is not always easy to interpret the data from a GWAS and the functional consequences of the different alleles that have now been associated with Barrett’s have yet to be elucidated, it seems that most factors have a likely association with the Wnt signaling pathway, a key pathway for intestinal stem cell specification, or the BMP pathway, which has also been implicated in functional experiments discussed below.

**Functional experiments.** It has been shown that Barrett’s epithelium gains the expression of key transcriptional regulators of intestinal epithelial cell fate such as CDX1 and CDX2, and it has been reported that both of these factors are induced by acid and bile (80). Transgenic ectopic expression of Cdx2 can convert gastric epithelium to intestinal metaplasia in mice (79). However, a similar approach has failed to induce metaplasia in the esophagus (35), indicating that expression of CDX2 alone is insufficient to cause Barrett’s esophagus. One of the factors that could play a role in the development of metaplasias in general is the ectopic and aberrant expression of morphogens in immune cells and/or mesenchymal cells due to the chronic inflammatory infiltrate. Indeed, in Barrett’s esophagus it was found that BMP4 is overexpressed in the stroma and that treatment of esophageal cells with BMP4 induces columnar morphology (54). This suggests that stromal BMP4 expression may play an important role in the development of intestinal metaplasia. However, treatment with BMP4 alone was insufficient to cause intestinal metaplasia (54). Other investigators have later found that Hedgehog ligands Shh and Ihh are overexpressed in Barrett’s esophagus vs. normal epi-
In conclusion, although much remains to be investigated regarding the molecular mechanism of the development of Barrett's esophagus, it seems that induction of expression of CDX1/2 by gastric content in combination with epithelial expression of BMP4 signaling is sufficient to induce intestinal metaplasia in esophageal cells, suggesting that these two factors combined may play a critical role in the process (48).

Regarding the factors in the inflammatory infiltrate that promote the development of metaplasia and dysplasia in the intestinal type epithelium, important recent work has shown that IL-1 and IL-6 are important contributors. In concordance with both the skin epidermis and the gastrointestinal 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 (6, 7, 22, 67), 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 (37, 49) in the rat esophagus showed with the use of [3H]thymidine pulse-chase experiments that proliferation is restricted to the basal layer. 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” (37).

The current debate focuses on the question whether indeed all basal cells have an equal capacity for self-renewal (5) or whether the basal layer is organized in a stem cell-transit amplifying (TA) cell hierarchy (32) 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. We will restrict ourselves to the potential presence of stem cell population(s) during homeostasis since even less is known about the behavior of potential stem cells during wound repair and carcinogenesis.

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., has self-renewal capacity, and is capable of generating all the cell types present in a tissue, i.e., has 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 h and can be considered fast cycling (7). In contrast, the term stem cell has also been used for slow-cycling, “label-retaining cells” (LRCs) that contribute to epithelial homeostasis after damage (39). 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. (19) showed that there are low numbers of LRCs found in the esophagus basal layer epithelium, as was previously described (32). However, Doupe et al. found that these cells are not of epithelial origin since these cells expressed the hematopoietic lineage marker CD45. This suggests that a population of label-retaining stem cells may indeed be absent from the murine esophageal epithelium. In humans such a LRC might be present, since one study described the identification of LRCs after 5-iodo-2′-deoxyuridine labeling in patients undergoing esophagectomy (63).

Importantly, the presence (or absence) of a pool of label retaining (reserve stem) cells does not exclude the existence of faster-cycling dedicated stem cells in the esophagus. To further address this question, Doupe et al. (19) examined the fate of single-cell-derived clones in the basal layer in a lineage trace experiment, using a Ah-CreERT2*LSL-eYFP-reporter mouse. Mathematical modeling of the results led them to conclude that the majority (65%) of the basal layer consists of esophageal progenitors (EPs). These EPs divide on average ~2x/wk and for every division “esophageal progenitors are functionally equivalent” (19). This concept is very similar to the interpretation of previous research by Leblond and colleagues (49).

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. (32) isolated a subpopulation of cells from the esophageal epithelium on the basis of a dye-exclusion method and provided evidence for high clonogenic potential within this subpopulation of cells, suggesting that they might be stem cells. In addition, several other studies have used different cell surface markers to isolate distinct subpopulations from the murine (17, 30, 32) and also human (4, 30, 60, 75) esophageal epithelium via FACS sorting. With one exception (4), the investigators report considerable differences in self-renewal capacity between the different epithelial populations. These data provide compelling evidence for the existence of phenotypically and functionally distinct epithelial cell populations within the basal layer of mice and humans, suggestive of a more hierarchical stem cell-transit amplifying cell organization similar to most other epithelial tissues.

Additionally, methods have now been established for both mouse and human 3D organotypic esophageal epithelial cultures (17, 30, 69). Culturing of such esophagospheres will provide an easy platform to advance our insights into the regulation of the esophageal stem/progenitor cell populations and epithelial homeostasis. In addition, such cultures can be used to model esophageal carcinogenesis, similar to the organoid cultures that are being used to model colorectal carcinogenesis (50, 86).
summary, the existence of a specific esophageal stem cell population is still a matter of debate although several lines of evidence indicate that there may be considerable heterogeneity of self-renewal potential in the basal layer of the esophagus. Further use of the established tools to culture primary esophageal cell in vitro and lineage tracing in vivo will be required to shed more light on this important issue.

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 the mechanisms involved in tissue repair, the development of Barrett’s esophagus, and carcinogenesis. The existence of specific stem cell populations that drive esophageal renewal needs further experimental evidence, especially given an importance of stem cells as the most likely cell of origin during oncogenic transformation.

GRANTS

G. van den Brink acknowledges financial support from the Netherlands Organization of Scientific Research (NWO; VIDI Grant).

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


REFERENCES


Review

G228
ESOPHAGEAL DEVELOPMENT AND EPITHELIAL HOMEOSTASIS


