Published in final edited form as:

Ann N Y Acad Sci. 2011 September ; 1232: 309–315. doi:10.1111/j.1749-6632.2011.06071.x.

Molecular aspects of esophageal development

Mark Rishniw¹, Pavel Rodriguez², Jianwen Que^{2,3}, Zoe D. Burke⁴, David Tosh⁴, Hao Chen⁵, and Xiaoxin Chen⁵

¹College of Veterinary Medicine, Department of Biomedical Sciences, Cornell University, Ithaca, New York.

²Department of Cell Biology, Duke University Medical Center, Durham, North Carolina.

³Department of Biomedical Genetics, University of Rochester, Rochester, New York.

⁴Centre for Regenerative Medicine, Department of Biology and Biochemistry, University of Bath, Bath, United Kingdom.

⁵Julius L. Chambers Biomedical/Biotechnology Research Institute, North Carolina Central University, Durham, North Carolina.

Abstract

The following on molecular aspects of esophageal development contains commentaries on esophageal striated myogenesis and transdifferentiation; conversion from columnar into stratified squamous epithelium in the mouse esophagus; the roles for BMP signaling in the developing esophagus and forestomach; and evidence of a direct conversion from columnar to stratified squamous cells in the developing esophagus.

Keywords

BMP signaling; esophagus; forestomach; stratification; differentiation; myogenesis; transdifferentiation; fate mapping; embryonic esophageal culture model; columnar epithelium; stratified squamous epithelium

1. Esophageal striated myogenesis: transdifferentiation or not?

The adult esophagus is composed of several muscle layers—a smooth muscle *muscularis mucosae* layer and two *muscularis externae* layers (longitudinal and circumferential). The *muscularis externae* layers are variably composed of striated and smooth muscle elements, with substantial differences existing between species, for example, they are composed almost entirely of striated muscle in the adult mouse and are completely smooth muscled in birds. Embryologically, however, the esophageal musculature begins as a smooth muscle-lined tube in all species, with subsequent conversion to varying proportions of striated muscle in a craniocaudal direction.

This conversion process has been the focus of several studies over the past 15 years, beginning with an observation by Patapoutian *et al.* that smooth muscle cells directly

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Mark Rishniw mr89@cornell.edu Pavel Rodriguez and Jianwen Que jianwen_Que@urmc.rochester.edu Zoe D. Burke and David Tosh d.tosh@bath.ac.uk Hao Chen and Xiaoxin Chen lchen@nccu.edu.

Conflicts of interest

The authors declare no conflicts of interest.

transdifferentiated into striated muscle cells.¹ Using mice as a model of esophageal myogenesis, where the conversion from one cell type to the other is virtually 100%, these authors showed coexpression of smooth muscle and striated muscle markers in *muscularis externae* myocytes by immunohistochemical labeling, and suggested that smooth muscle cells were able to switch phenotype well after commitment and differentiation. These findings were both supported by other investigators and challenged by other investigators.² Thus, the mechanism of esophageal striated myogenesis remained unresolved. We performed a series of experiments to determine whether transdifferentiation accounts for esophageal muscle phenotype switching.^{3–5}

In the first experiment, we fate mapped smooth muscle cells by crossing a mouse expressing eGFP and Cre-recombinase under the control of the smooth muscle myosin heavy chain (SmMHC) promoter (so-called SMCG2 mice) with R26R mice.³ The expression of eGFP and Cre-recombinase is strictly confined to smooth muscle cells in the SMCG2 mouse. R26R mice (also known as Rosa26R mice) have a constitutively expressed β -lactamase gene (under the control of the Rosa promoter), which is inhibited from expression by a lox-P flanked stop codon. Removal of the stop codon by Cre-recombinase results in constitutive β -lactamase expression. Thus, by crossing the SMCG2 and R26R mice, we could selectively activate β -lactamase expression in any cell that has expressed SmMHC (i.e., smooth muscle cells). Examination of E15 mouse embryos showed robust eGFP and Cre-recombinase expression (and β -lactamase expression) along the full length of the esophagus. However, P1 and P15 esophagi showed expression of these smooth muscle elements to more caudal portions of the esophagus. Importantly, no striated muscles in the *muscularis externae* showed β -lactamase expression (as evidenced by X-gal staining). This would suggest that these cells had never activated the SmMHC promoter to express Cre-recombinase and, therefore, did not derive from a smooth muscle cell precursor cell.

We then used the SMCG2 mouse to examine coexpression of eGFP or Cre-recombinase (which is localized to nuclei in smooth muscle cells) and the striated muscle transcription factor, myogenin.⁴ We could find no instances of coexpression of these two markers (one smooth, one striated) in any cells within the developing *muscularis externae*.

Finally, we used a selective deletion strategy to determine whether smooth muscle cells transdifferentiate into striated muscles.⁵ We used the SMCG2 mouse to selectively delete the myogenin gene from smooth muscle cells by crossing it with a mouse that had a lox-P-flanked myogenin gene. Since myogenin is essential for striated myogenesis, we hypothesized that, if smooth muscle cells transdifferentiated into striated muscle cells within the esophagus, deletion of myogenin in these cells prior to the transdifferentiation process would prevent this event, resulting in a myodysplasia, characterized by persistence of smooth muscle along the length of the esophagus. However, despite robust Cre-recombinase expression (and, presumably, efficient myogenin deletion), esophageal myogenesis progressed in these mice in a normal manner.

Taken together, our data convincingly suggest that esophageal striated myogenesis does not occur via a process of transdifferentiation, but that striated and smooth muscle cells arise from distinct precursor populations.

2. Conversion from simple columnar into stratified squamous epithelium in the mouse esophagus and forestomach is regulated by BMP signaling

The epithelium lining of the adult esophagus is squamous and stratified. However, in the early embryo when the esophagus is separated from the anterior foregut tube the epithelium is simple columnar.^{6,7} Our laboratory is interested in molecular mechanisms underlying the

conversion of the epithelium using the mouse as a model organism. We include the forestomach in our studies as the epithelium lining this tissue also goes through similar morphological and histological transitions. We previously showed that BMP signaling plays important roles in the early foregut development,⁸ and here we further explored its role in the development of the esophagus and forestomach using several genetically engineered mouse lines.

We first used a BMP reporter mouse line harboring a *BRE-lacZ* allele along with *in situ* hybridization to localize transcripts for BMP signaling components, including different antagonists. We demonstrated that when epithelium stratifies between embryonic day (E) 10.5 and E14.5, BMP signaling is not activated in the epithelium of the esophagus and forestomach, correlating with the presence of the BMP antagonist Noggin. However, activation of BMP signaling after E14.5 at the top layers of the epithelium is correlated with differentiation of the epithelium (Fig. 1). We further showed that deletion of the *Noggin* gene stalls the conversion of simple columnar into stratified epithelium. We then exploited a *Shh-Cre* allele that drives recombination in the embryonic foregut epithelium to generate either gain-or-loss-of-function models for the *Bmpr1a* (Alk3) receptor.

In gain-of-function embryos (*Shh-Cre; Rosa26^{CAG-loxstoplox}-caBmpRIa*), high levels of ectopic BMP signaling also prevents the transition from simple columnar to multilayered undifferentiated epithelium in the esophagus and forestomach. In loss-of-function experiments, conditional deletion of the BMP receptor in *Shh-Cre;Bmpr1a^{flx/flx}* embryos allows the formation of a multilayered squamous epithelium, but which fails to differentiate, as shown by the absence of expression of the suprabasal markers, loricrin, and involucrin.

Together, these findings suggest multiple roles for BMP signaling in the developing esophagus and forestomach.⁹

3. Conversion of columnar to stratified squamous epithelium in the developing esophagus

The mammalian esophagus exhibits a remarkable change in epithelial organization during the transition from embryonic to adult tissue. Initially, the esophagus is lined by a simple columnar epithelial layer, which is gradually replaced by a stratified squamous tissue comprised a clearly distinct basal layer surrounded by the spinous, granulated, and cornified suprabasal layers.

The replacement of one epithelium with another during esophageal development has been documented in both mouse and human, and in 1993, Thorey *et al.* proposed that the basal layer of the stratified squamous epithelium is derived from the columnar epithelium.^{10–12} However, direct evidence for this conversion was lacking due to the availability of suitable model systems.

We have developed an *in vitro* model for studying esophageal development based on the culture of esophageal explants from embryonic day 11.5 (E11.5) mouse embryos. This culture model recapitulates the *in vivo* development of the esophagus.⁷ Using immunohistochemical analysis of sections from developing mouse embryos, we initially demonstrated that the transition from columnar to stratified squamous epithelium is accompanied by a change in expression of the intermediate filament proteins cytokeratin 8 (K8, a marker of columnar epithelium) and 14 (K14, a marker for the basal epithelial layer). At E11.5 all the cells of the esophageal epithelium express K8 but not K14. Gradually, between E15.5 and E17.5, K8 expression is lost in the basal layer but is retained in the suprabasal layers until postnatal day 3 when it is completely lost. In contrast, K14 is absent

in the esophageal epithelium at E11.5 but begins to be expressed around E17.5 in the basal cells, which are no longer expressing K8.

The explant culture model was established by placing esophageal tissue dissected from E11.5 embryos onto fibronectin-coated glass coverslips. Within 24 h, the explants attached and flattened onto the fibronectin substratum at which stage they are composed of a tube of epithelium surrounded by mesenchyme. The explants remain viable in culture for up to 20 days and differentiate as stratified squamous epithelium, exhibiting the same switch in expression of K8 to K14 as observed *in vivo*.

To determine the origin of the stratified squamous epithelium, we used our explant model to explore the possibility that basal cells arise through programmed cell death of the columnar cells, selective overgrowth of the squamous epithelium, or through a direct conversion of one cell type to another.

To distinguish the first of these possibilities, we separated the cells after 7, 11, or 15 days of culture by flow cytometry and then immunostained for either K8 or K14 and propidium iodide to identify dead cells. If the switch in cell type was due to a preferential death of the columnar cell population, the number of dead cells would be higher in the K8-expressing population. However, there was no observable difference in the proportion of dead cells in the K8- and K14-positive populations indicating that programmed cell death does not play a role in the conversion from columnar to stratified squamous epithelium.

To determine whether the switch from columnar to stratified squamous epithelium was due to selective overgrowth of the squamous epithelium we immunostained for the proliferation marker Ki-67. The embryonic esophageal epithelium in culture is highly proliferative up until the culture differentiates into multilayers after which time the majority of the proliferating cells become localized to the K14-positive cells of the basal layer. In order to determine whether cell division was required for conversion, we treated esophageal explants with mitomycin C, a DNA crosslinking agent that prevents the cells from entering the cell cycle. If the cell conversion is dependent on cell division, then we predicted it would not be able to occur in the presence of a cell cycle inhibitor. However, the transition from K8-positive columnar layer to the K14-expressing cells continued to occur in the presence of mitomycin C indicating that cell proliferation does not play a role in the epithelial cell conversion.

The possibility that the cells of the basal layer arise directly from the columnar epithelium was also investigated. Upon costaining for K8 and K14, we found some cells coexpressing both markers suggesting that K14-positive cells arose directly from K8-positive cells. To trace the lineage of the stratified squamous basal cells, we electroporated a reporter plasmid, the K14 promoter driving GFP expression, into esophageal explants obtained from E15.5 embryos. This timepoint was chosen since the basal phenotype starts to appear between E15.5 and E17.5. Costaining for GFP and K8 revealed that at least some of the cells that have an activated K14 promoter (GFP-positive) also express K8, indicating that the basal cells of the squamous epithelium arise through a direct conversion from K8-positive columnar cells.

The embryonic esophageal culture model has provided evidence of a direct conversion from columnar to stratified squamous cells in the developing esophagus (Fig. 2). Further analysis of the mechanisms involved in this conversion may also provide important insights underlying the mechanism(s) involved in Barrett's metaplasia in which the reverse conversion from stratified squamous epithelium to columnar epithelium occurs.¹³

4. Understanding Barrett's esophagus from the perspective of development

The developmental process by which a single fertilized egg becomes a complex organism has fascinated people for many years, and continues to interest biologists today. Discoveries in developmental biology constantly generate insights on the molecular basis of many human diseases. Indeed, many disease-related genes and pathways were identified first as critical ones in embryonic development, such as BMP, WNT, Hedgehog.

Barrett's esophagus (BE) is a premalignant lesion of esophageal adenocarcinoma in which the normal squamous epithelium is replaced by intestinalized columnar epithelium. Although it is known to develop as a consequence of chronic gastroesophageal reflux, its molecular aspect is not fully understood. Two lines of evidence suggested that research on esophageal development may shed light on the molecular mechanism of BE. (a) BE may originate from stem cells residing in the esophagus (e.g., interpapillary basal cells, ductal cells of submucosal glands). (b) Human embryonic esophagus is initially lined with a ciliated pseudostratified columnar epithelium containing goblet cells, which is later replaced by a stratified non-keratinized squamous epithelium containing submucosal glands. A question becomes obvious, Is BE an adaptive reversal of embryonic development?

Current status

Human esophagus begins to form during the 4th week of embryonic development, with the formation of the foregut. Up to the 8th week, the esophageal epithelium appears as a pseudostratified columnar epithelium which then becomes ciliated. Starting from the 4th month of gestation, the ciliated epithelium is gradually replaced by a squamous epithelium until a non-keratinized stratified squamous epithelium is fully developed. Residual islands of columnar epithelium remain and down-grow to generate submucosal glands. The ducts are therefore lined partly by squamous epithelial cells. Keratinization does not normally occur in humans, nor in carnivores, although it is normally seen in rodents and ruminants. At about the 6th or 7th week of gestation, the circular muscle coat, ganglion cells of the myenteric plexus, and blood vessels start to develop.¹⁴

However, molecular mechanism of esophageal development is poorly understood. Only several genes and molecular pathways have been reported to be involved in esophageal development. Among them, P63 and Sox2 play key roles in epithelial development, as well as the pathways, such as BMP, Hedgehog, NGF/NGFR and Nrf2/Keap1. As for neural development, SULF1/SULF2 regulate heparan sulfate-mediated GDNF signaling for esophageal innervation.¹⁵ Rassf1a was also found to be involved in development of esophageal nerve, both ganglia and nerve fibres. As for the muscle and blood vessels, FOXM1 was required. Gene methylation also participates in modulating gene expression during esophageal development.¹⁶ A recent microarray study found a group of transcriptional factors exclusively expressed in esophageal progenitor cells, Foxe1, Dlx3, Erf, Nfix, Nrl, Otx1, Pitx1, Tcfab2c, Twist1 and Zfpn1a2, and therefore provided clues to further demonstrate their functions in esophageal development.

Future perspectives

It is obvious that we need more research on esophageal development for the sake of understanding BE and other esophageal diseases as well. Both the epithelium and the mesenchyme may be equally important because of epithelium-mesenchymal interactions in development and the involvement of mesenchymal structures in BE. For example, it is not known how lower esophageal sphincter muscle develop; how motor nerves develop and regulate the function of muscles; how sensory nerves develop and respond to reflux; how submucosal glands develop and whether we may stimulate glandular secretion for antacid

therapy,¹⁷ how the epithelium develop and keratinize (in rodents) and whether we may manipulate this process to enhance epithelial resistance to reflux.

Although most work on the molecular aspect of esophageal development was performed in mice,¹⁸ selection of proper animal species in this research area is always a critical issue. Non-keratinization of the esophageal squamous epithelium, presence of submucosal glands, availability of proper transgenic and knockout techniques and tools, cost of maintenance, are all important factors for consideration. Each animal species has its advantages and limitations. Mice remain the first choice in most labs, although they do not have submucosal glands and their epithelium is keratinized. In case a genetic modification is lethal, *ex vivo* and *in vivo* organ culture of embryonic esophagus may still generate useful information. Pig esophagus is very similar to human esophagus in anatomy and physiology. However, cost of maintenance is a major limitation. Recently we have identified a short piece of non-keratinized stratified squamous epithelium in zebrafish pharynx similar to human esophageal epithelium. An obvious advantage of the zebrafish system is that genetic manipulation is more convenient and economic in zebrafish than in other larger animals.

In summary, we believe developmental research can make great contributions to our understanding of the molecular mechanism of BE. Those critical genes and molecular pathways for development may play important roles in BE as well (Figure 3). Take P63 as an example, a translational approach may be adopted for future studies. P63 was first demonstrated as a critical gene in the development of esophageal epithelium in knockout mice. Loss of P63 resulted in a simple columnar epithelium which failed to develop into stratified squamous epithelium in the esophagus. P63 expression was then found to be negative in most cases of human BE. Moreover it was already lost in some cases of multi-layered epithelium, a transitional stage before normal esophagus and BE. Recent *in vitro* cell culture studies confirmed that exposure of esophageal squamous epithelial cells to gastroesophageal refluxate down-regulated P63 expression. Now, there is an obvious need to generate an esophagus-specific conditional P63 knockout model to demonstrate whether loss of P63, either alone or in combination with other genetic factors, may lead to BE *in vivo*.

Acknowledgments

Supported by NIH grants U56 A092077 and P20 MD000175.

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Concise summaries

- Embryologically, the esophageal musculature begins as a smooth muscle-lined tube in all species, with subsequent conversion to varying proportions of striated muscle in a craniocaudal direction.
- Using mice as a model of esophageal myogenesis, where the conversion from one cell type to the other is virtually 100%, coexpression of smooth muscle and striated muscle markers in *muscularis externae* myocytes has been shown by immunohistochemical labeling, suggesting that smooth muscle cells were able to switch phenotype well after commitment and differentiation. Esophageal striated myogenesis does not occur via a process of trans-differentiation; rather, striated and smooth muscle cells arise from distinct precursor populations.
- In the early embryo, when the esophagus is separated from the anterior foregut tube, the epithelium is simple columnar. Using several genetically engineered mouse lines, it has been shown that bone morphogenetic protein (BMP) signaling plays important roles in the early molecular mechanisms underlying the conversion of the epithelium foregut development.
- Immunostaining for the proliferation marker Ki-67 was made to determine whether the switch from columnar to stratified squamous epithelium was due to selective overgrowth of the squamous epithelium.
- The possibility that the cells of the basal layer arise directly from the columnar epithelium has also been investigated.
- The embryonic esophageal culture model has provided evidence of a direct conversion from columnar to stratified squamous cells in the developing esophagus.

Figure 1.

Model proposing that between E10.5 and E13.5, the inhibition of BMP signaling, as mediated by an inhibitor such as Noggin, is necessary to allow the stratification of simple columnar to multilayered epithelium. Then, after E14.5-15.5, active BMP signaling is required for suprabasal differentiation of the epithelium.

Figure 2.

Summary of the conversion of esophageal epithelium from simple columnar to stratified squamous tissue during development. At E11.5 the esophageal epithelium consists of a single layer of K8-expressing cells. Between E15.5 and E17.5, the epithelium starts to become multilayered and the loss of columnar K8 expression from the basal layer coincides with the onset of K14 expression. In postnatal/adult esophagus, K8 expression is not detectable, the basal layer cells express only K14, and the esophagus is fully differentiated as a stratified squamous tissue.

Figure 3. Understanding Barrett's esophagus from the perspective of development. Several developmental genes and pathways are known to play important roles in the development of Barrett's esophagus. Others remain to be identified and studied for their potential contribution to Barrett's esophagus.