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The esophageal mucosa and submucosa: immunohistology in GERD and Barrett's esophagus

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This paper presents commentaries on the microscopic morphology of esophageal squamous epithelium; the frequency of duplication of the muscularis mucosae (MM) in Barrett's esophagus (BE); the significance of multilayered epithelium; whether cells in the lamina propria reflect those in the epithelium; how stem cells are identified in the squamous esophagus; dilated intercellular spaces; the metastasizing potential of early carcinoma-dependent, molecular or immunohistochemical tests that improve diagnosis; the role of immunohistochemistry IHC in grading of neoplasia in Barrett's esophagus and defining the risk of progression to adenocarcinoma; the roles of CDX1 and CDX2 in squamous and cardiac mucosa; and the role of desmosomal cadherins and lectins in squamous and cardiac mucosa.

Keywords: eosinophilic esophagitis; muscularis mucosae; multilayered epithelium; lamina propria; dilated intercellular spaces; markers; transcription factors

Concise summaries

- The embryonic esophagus is lined by two distinct types of columnar epithelium before squamous epithelium develops, which means that esophageal squamous epithelium is basically a metaplasia that develops in columnar epithelia that are maintained by fish, amphibians, and some reptiles. It has common patterns of reaction to different injuries. This supposedly resistant epithelium is subject to a variety of insults which it never was intended to confront, the most common of which is reflux. In the normal esophagus, the intracellular spaces are difficult to identify, but they become very apparent in severe reflux injury, in many infections, and eosinophilic esophagitis (EoE) may induce the most intense changes in these spaces. The recently described lymphocytic esophagitis also has dilated spaces. It is an embryonic metaplasia.
- Duplication of the muscularis mucosae (MM) is not always present underlying Barrett's esophagus (BE) and, if it is present, may be so focal that the finding cannot be expected to be present in every slide.
- Multilayered epithelium is the most recent term for a distinct subtype of epithelium at the gastro-esophageal junction (GEJ) displaying criteria of columnar and squamous epithelium. The clinical significance of multilayered epithelium rises and falls with a possible connection or precursorship to BE. Detailed studies on cytokeratins and mucins expressed by multilayered epithelium and the surrounding epithelia were also interpreted as indications that multilayered epithelium is a precursor

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lesion of BE based on the findings that cytokeratin-14 was virtually missing in multilayered epithelium and CDX-2 and mucin 2 was detected in almost half of the cases, whereas the presence of cytokeratin 19 was interpreted as possible squamous stem cell marker but with much lesser evidence for a cardia stem cell source. After careful analysis of cytokeratins and mucins, multilayered epithelium needs to be seen as metaplastic epithelium sharing characteristics mainly with bronchial epithelium.

- We know little about the normal lamina propria. Normal lamina propria contains endothelial cells lining vessels, fibroblasts, and/or myofibroblasts. Mucus gland cells and their ducts are present especially distally, and the ducts of submucosal glands also pass through the lamina propria. The lamina propria is virtually always ignored when the main epithelial diseases, with which we deal, including neoplasms or inflammation, are discussed. Substantial information on lamina propria in EoE, reflux disease, or BE is still lacking today.
- Support for the presence of adult stem cell populations in the human esophageal ducts and submucosal glands comes from clinical and experimental investigations. Provocative experiments provide new evidence for the important role of p63, both a suppressor and a transcriptional activator of groups of genes involved in the program of epithelial development, in maintaining squamous epithelia, and for the possible substitution of an alternative source of cell renewal of an adult tissue if its usual stem cell population is inactivated or injured. Given important anatomical and physiological differences between the animal and human esophagus, additional work is needed to refine our understanding of possible stem cell populations in the esophagus, and their role in the renewal of normal squamous epithelium.
- Change in commitment of multipotent stem cells not only explains the variety of cellular phenotypes in BE, as well as the regeneration of squamous epithelium after endoscopic removal of Barrett's mucosa, but also offers a possible key mechanism for the inherent cancer risk of BE. The exact location of the stem cells in the esophagus remains a topic of discussion: interpapillary basal layer cells demonstrate higher proliferation and clonogenic capability, but only display very low expression of β1-integrin, which is not in line with multipotent *stemness,* as undifferentiated cells lose β1-integrin expression when they commit to terminal differentiation. Other groups have located the esophageal stem cells outside the epithelium. In this perspective, and similar to the bulge region of hair follicles, the submucosal gland ducts may be very important. Of interest, it has also been suggested that BE could be the result of bone marrow–derived stem cells.
- Dilated intercellular spaces (DIS), an increase in the space between squamous cells, were first reported in human esophageal epithelia of patients with symptoms of esophagitis, and the current hypothesis is that they induce higher activation of the sensory nerve endings. They are associated with symptoms of reflux, even more so than other histologic parameters, and seem to disappear with resolution of symptoms after treatment.
- Epithelial damage brought on by even stress alone can dilate the intercellular spaces. They are also observed in EoE, but with a different pattern. However, the presence of DIS, when taken in context including clinical presentation and other histologic features, is a sensitive marker of GERD.
- MM duplication is overall very frequent in BE, but it can be patchy. In Barrett's adenocarcinoma, the exact depth of invasion into the duplicated MM neither influences lymph node metastasis nor does it appear to affect survival. The main reason to recognize MM duplication vis-à-vis esophageal adenocarcinoma (EAC) is to avoid the trap of overstaging.
- For the diagnosis of BE, finding goblet cells on hematoxylin and eosin (H&E) is pretty easy, because in this stain, goblet cells are gray or faintly blue compared to the gastric type columnar cells, which are pale pink. A mucin stain, such as an Alcian blue, will stain the goblet cells an intense blue, but this stain also uncovers cells that might be misinterpreted as goblet cells. As for Barrett's complications, the criteria for the dysplasias are not clear-cut, so reproducibility is poor, especially at the lower end of the dysplasia spectrum. Several markers

have been studied in dysplasias including α-methylacyl coenzyme A racemase (AMACR). However, the diagnosis of low-grade dysplasia by itself is not as important as the diagnosis of low-grade dysplasia accompanied by positive p53 as an indication of progression risk. The marker studies are based on diagnoses made on slides stained with H&E. Because the diagnoses were already known, it is obvious that the markers offered no better diagnostic information, possibly except for p53 staining of H&E diagnosed low-grade dysplasia. At this moment, there are no molecular or immunohistochemical tests that improve diagnosis and management compared with H&E-stained biopsies and surveillance.

- Potentially useful antibodies that might improve sensitivity and specificity of detection of intraepithelial neoplasia (dysplasia) and adenocarcinoma include those recognizing p53, p16INK4A, c-erbB2, cyclin D1, p27KiP1 EGFR, COX-2, β-catenin, Rab11a, CD1a, HER2, EGFR, SMAD4, IMP3, Ki-67, Serpins, and AMACR. However, evidence to support consistently positive and negative predictive values of any potentially useful marker awaits larger series, and several fundamental challenges limit clinical applicability of currently reported immunohistochemical markers. Currently, no single marker or panel is clearly predictive of risk to progression to EAC, and it remains unclear if immunohistochemical staining adds clinical value over the gold standard of histological grading and review by a second experienced pathologist.
- Given the fact that Barrett mucosa is *intestinalized*, investigators correctly anticipated relevance of the transcription factors (TFs) CDX1 and CDX2, which participate in intestinal development and maintain the intestinal phenotype. Several groups have shown CDX2 protein expression, as detected by immunohistochemistry (IHC), in virtually all biopsies of Barrett epithelium (i.e., columnar epithelium with goblet cells proximal to the anatomic GEJ). Different bile acids may variably activate the *CDX2* promoter in cell lines, with dehydrocholic acid (DHCA) and cholic acid (CA) achieving the greatest induction. However, Cdx2 expression alone is insufficient to produce the full Barrett phenotype owing, in part, to epigenetic regulation of the adult genome. Furthermore, although Cdx2 expression results in reduced proliferation, this feature must be overcome by some other factors, as BE, even without dysplasia, is proliferative. Expression microarrays have identified hundreds of genes differentially expressed in BE relative to esophageal squamous epithelium. These include three HOXB family TFs, which, in transfection experiments, are also able to induce the expression of intestine-specific proteins. Increased expression of these HOXs occurs in the setting of activating histone modifications and chromatin decompaction.
- Changes in desmosomal cadherins, the adhesion molecules of the adhesive intercellular junction proteins, contribute to the pathogenesis of GERD in the cardia mucosa. Lectins are a structurally heterogeneous group of carbohydrate-binding proteins that are present in normal, inflamed, and neoplastic mucosa, but composition may change according to the underlying condition. As Lectin UEA-I-binding proteins were specifically increased in the squamous epithelium of patients with BE, some authors concluded that it may serve as a potential marker for BE, especially in patients with short-segment BE. However, even if various studies have shown that the expression of cadherins and lectins is altered in GERD and BE, these markers are currently only of scientific interest.

1. The microscopic morphology of esophageal squamous epithelium

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Because this topic is part of a symposium devoted to esophageal tissue resistance, I assume that this discussion relates to how the squamous epithelium of the esophagus resists injury, or how it fails to do

so. The normal squamous mucosa has thick layers of superficial squamous cells full of glycogen which make the cytoplasm look clear. There are thin layers of basal cells and short lamina propria papillae surrounded by basal cells. Stratified squamous epithelium is well suited to those areas of the body subject to constant abrasion because it is thick, and layers can be sequentially sloughed off superficially and replaced before the deeper tissues are exposed.

This is great information, but how did our esophagus get its squamous lining? During embryonic development, the esophagus is initially lined by a thin layer of stratified columnar epithelium, which proliferates to almost include the lumen.[1–3] At approximately 6–7 weeks gestation, the lumen is reformed as a result of vacuoles forming in these columnar cells. As early as 8 weeks, beginning in the middle third, ciliated cells appear and extend proximally and distally to almost cover the entire stratified columnar epithelium. At about 5 months, stratified squamous epithelium initially appears, also starting in the middle third, and this epithelium extends proximally and distally, replacing the ciliated epithelium. Therefore, the embryonic esophagus is lined by two distinct types of columnar epithelium before squamous epithelium develops, which means that esophageal squamous epithelium is basically a metaplasia. What were the stimuli that produced the squamous metaplasia and why did the squamous epithelium replace the two columnar types? None of the sources tell us what was wrong with the columnar epithelium. Teleologically, it is said that stratified squamous epithelium lines the esophagus because it is very protective, especially as we eat crusty and hot foods. However, this epithelium is a problem for maintenance. These are nonkeratinized surfaces, in contrast to the skin, and they must be kept moist by bodily secretions to prevent them from drying and dying. Presumably, that is why we have the submucosal and lamina propria mucus glands.

We know quite a lot about the esophageal epithelium in more primitive vertebrates.[4] Eels, perch, sharks, rays, and skates have ciliated epithelia. The most advanced sport and commercial fish have columnar epithelia with a few mucus cells. Freshwater fish have stratified squamous epithelia with numerous mucus cells. Amphibians have ciliated epithelia, and esophageal epithelia in reptiles vary from group to group. For instance, lizards have ciliated columnar epithelia with goblet cells whereas turtles have heavily keratinized squamous epithelia, which presumably protect the mucosa from the turtle's abrasive diet that includes spiculated sponges and jellyfish. Birds have stratified squamous epithelia. They also have an abrasive diet that is heavy in roughage.

This supposedly resistant epithelium is subject to a variety of insults, which it never was intended to confront, the most common of which is reflux. Studies on *in vivo* perfusion of the esophagus with acid or acid and bile acids suggest that there is dilatation of the intracellular spaces in the squamous epithelium, something also referred to as spongiosis in the skin. We can actually see this in biopsies.[5–8] In the normal esophagus, the intracellular spaces are difficult to identify, but they become very apparent in severe reflux injury and in many infections, and EoE may induce the most intense changes in these spaces. The recently described lymphocytic esophagitis also has dilated spaces.

Another common response to injury is expansion of the proliferative zone, the basal cells, and this expansion can be identified as piling up of basal cells and/or lengthening of the papillae, which carry with them multiple layers of basal cells higher into the superficial squamous epithelium.

In summary, the microscopic morphology of the esophageal squamous epithelium seems to relate to what bangs against it. It is well suited to mucosa subject to constant abrasion. It has evolutionary overtones, namely, it is present in some fish but not amphibians, some but not all reptiles, all birds and all mammals. It is an embryonic metaplasia that develops in columnar epithelia that are maintained by fish, amphibians, and some reptiles. It has common patterns of reaction to different injuries that were probably never supposed to bother it. Regardless, it seems to be a good epithelium for people in general, and especially for gastroenterologists and pathologists who deal with it on a daily basis.

2. Frequency of duplication of the MM in BE

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Are there always two layers in BE? If not, how do we know when we are definitively in the submucosa? The presence of dual layers of MM underlying segments of BE has been recognized since the

1980s,[9–12] however, the majority of papers noting this change focus on the issues arising from the presence of a duplicated MM (dMM) rather than the incidence of the change. The few papers that do note the rate of finding these dual layers show considerable variability.[10–18] In papers looking at esophagectomy specimens, dMM is found in 46–100% of specimens[10–16]; a rough average from these patients gives 79% of specimens containing dMM. The rare papers on mucosal resection specimens (EMRs) show a lesser proportion with dMM, with 46% and 66% containing dMM.[17,18] A review of 106 recent EMR cases from 30 patients in my center identified at least focal dMM in 101 (95%) of cases. This focality raises a second point: several studies have noted that, even though dMM is present at some point along the segment of BE, this may be quite patchy. In esophagectomy specimens, the proportion of the BE segment showing dMM ranged from 5% to 90% (mean of 44%)[14] in one study, and 70% to 90% in another.[12] In EMR specimens, extensive duplication was present in 38% of the specimens, moderate in 33%, and minimal in 29%.[18] Another EMR study showed 46% of specimens had focal or extensive dMM (10–100%), wheras 54% had minimal (<10%) or absent dMM.[17] These results demonstrate that dMM is not always present, and if it is present may be so focal that the finding cannot be expected to be present in every slide. This is further complicated by reports of occasional triplication of the MM and cases with thickened single layers of MM.[14] Considering this, how can we be sure that we are looking at submucosal tissues? The presence of submucosal glands is, by definition, indicative of the presence of submucosal tissue. However, these glands are scattered along the esophageal length and are not present in every slide. Also, EMR specimens often have a considerable curvature from muscle contracture, which can make the plane between the deepest layer of the MM and the submucosa difficult to evaluate even when submucosal glands are present. Hahn *et al.*[19] noted that the tissue between the dMM layers had characteristics of the BE lamina propria, with delicate, thin-walled blood vessels. The submucosa, in contrast, characteristically has robust, thick-walled muscular arteries,[16] which are often easily identified on slides, even in EMR specimens (Fig. 1). We also tend to assume that early carcinomas of the upper gastrointestinal tract do not induce stromal desmoplasia until they invade into the submucosa; this is poorly documented, but there are at least anecdotal cases of intramucosal carcinomas with stromal desmoplasia.[14,16]

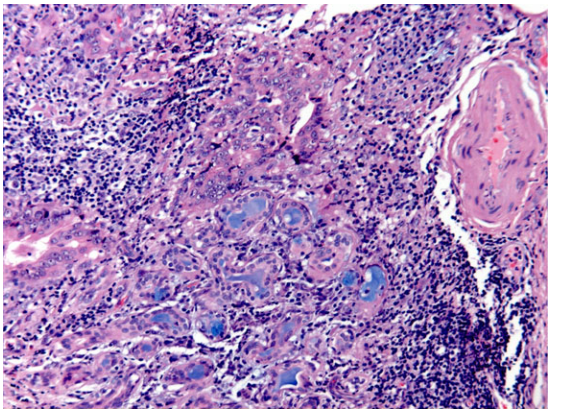

Figure 1. Adenocarcinoma invasive into the submucosa adjacent to a submucosal gland and a thick-walled artery. Early changes of stromal desmoplasia are present.

Overall, dMM is not always present underlying BE. This can make detection of the submucosal tissues difficult: the presence of submucosal glands and thick-walled muscular vessels are the most definitive indicators of the presence of the submucosa, while the presence of desmoplasia may also be of use but requires further study.

3. What is the significance of multilayered epithelium?

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Background

Multilayered epithelium is the most recent term for a distinct subtype of epithelium at the GEJ displaying criteria of columnar and squamous epithelium. In the literature and daily routine practice there are some synonyms available for so-called multilayered epithelium: pseudostratified metaplasia, hybrid epithelium. Without any doubt, squamous metaplasia with reserve cell hyperplasia,[20] multilayered epithelium,[21,22] and pseudostratified metaplasia[23] are good expressions in terms of self-explanatory terms. The first description is from 1981.[20] In 1993 the findings of a distinctive esophageal surface cell at the GEJ were confirmed by electron microscopy.[24] Because multilayered epithelium was found in patients with BE, authors[21,22,24] speculated whether multilayered epithelium is a precursor for BE. This

is in accordance with another publication[25] showing that multilayered epithelium is believed to represent at least a histological marker of reflux disease.

Available data

The clinical significance of multilayered epithelium rises and falls with a possible connection or precursorship to BE because BE is a precancerous condition and may need additional clinical follow-up.

Multilayered epithelium was described in detail in 2001 by Glickman *et al.*[22] as four to eight layers of cells with nuclear pseudostratification, mean length of 40 μm (20–100 μm), with suprabasal and superficial layers being more columnar, and most importantly, missing intercellular spaces/bridges that are found in normal squamous epithelium. The majority is commonly found in surface epithelium (76%). Adjacent epithelium was described as cardia in 77%, goblet cell–containing epithelium in 23%, and intestinal-type epithelium elsewhere in 100%. The two latter are the reasons that a discussion was started seeing multilayered epithelium as a precursor of goblet cell–containing Barrett's epithelium.

Quite often (76%), cilia can be identified at the surface layer of multilayered epithelium. The first descriptions are found by Okuda *et al.* in 1976, Torikata *et al.* in 1989, and Rubio *et al.* in 1990.[26–28]

Detailed studies on cytokeratins[22] and mucins[25] expressed by multilayered epithelium and the surrounding epithelia were also interpreted as indication that multilayered epithelium is a precursor lesion of BE based on the findings that cytokeratin-14 was virtually missing in multilayered epithelium and CDX2 and mucin 2 was detected in almost half of the cases, whereas the presence of cytokeratin-19 was interpreted as possible squamous stem cell marker but due to the missing cytokeratin-14 with much lesser evidence for a cardia stem cell source.

On the other hand, Takubo *et al.*[23] published a detailed analysis of cytokeratins and found CK10, CK14, CK20 negative and CK4, CK7, CK8, CK13, CK18, and tubulin positive. These findings after cytokeratin analysis were interpreted as multilayered epithelium being a type of metaplasia that shares a lot with bronchial epithelium but being no distinct precursor lesion of Barrett's. The prevalence of multilayered epithelium in this study was given as 49%. Thirty-one percent of the cases showed ciliated cells. Shields and Glickman[21,22] found a much smaller prevalence of multilayered epithelium in their series (13–24%).

The frequency of multilayered epithelium in a normal population is still under discussion. Our unpublished data show an average of 5% in patients with and without reflux disease and with and without Barrett's epithelium. If this distribution is indeed representative, this has to be considered as a strong argument against multilayered epithelium being a precursor lesion of BE. This is also in line with a publication from Takubo *et al.*,[20] who first described multilayered epithelium as a kind of ciliated squamous epithelium. It has to be noticed that Barrett's epithelium and reflux disease are rarely found in Asian populations.

Conclusion

In conclusion, multilayered epithelium seems not to represent a precursor of BE, and its prevalence is much smaller than previously anticipated. After careful analysis of cytokeratins and mucins, multilayered epithelium needs to be seen as metaplastic epithelium sharing characteristics mainly with bronchial epithelium.

4. Do cells in the lamina propria reflect those in the epithelium?

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In order to answer this question, we have to know what cells are in both normal and abnormal epithelium and lamina propria. In the normal esophagus, the squamous epithelium and the lamina propria are separate, discrete layers. We know little about the normal lamina propria. The biopsies we receive in routine practice generally capture very little of it. Resected specimens have esophageal diseases, so the resected lamina propria may not be a good source of normal. Normal lamina propria contains endothelial cells lining vessels, fibroblasts, and/or myofibroblasts. Mucus gland cells and their ducts are present especially distally, and the ducts of submucosal glands also pass through the lamina propria. Information from three textbooks, two from 2007 and one from 2008, suggests that the normal lamina propria contains scattered inflammatory cells, mostly T lymphocytes, some plasma cells, mostly IgA-producing and occasional lymphoid follicles around ducts or where ducts were obliterated.[1–3]

The cells in the normal squamous epithelium, also as defined in three recent textbooks, include T lymphocytes, Langerhans cells, and in one reference, a few neutrophils and basophils.

What happens to the cell make-up of the lamina propria in diseases?

The diseases with which we deal are mainly epithelial, including neoplasms and inflammation, including those that are reflux induced and infectious. Peculiarly, in the textbooks and in the literature, the lamina propria is virtually always ignored when these diseases are discussed. For instance in candidiasis, the fungi invade the epithelium from the lumen and often cause striking inflammatory changes in the epithelium including neutrophils, even with small abscesses. In contrast, Herpes virus, which effects squamous epithelium from inside, induces very little intraepithelial inflammation. There is remarkably little information about what happens to the lamina propria in these infections except perhaps a passing mention of a few neutrophils. It is virtually impossible to find an illustration of the lamina propria in these infections in any textbooks. Cytomegalovirus involves endothelial cells of lamina propria so the textbooks hone in on that, probably because the infected cells with the nuclear and cytoplasmic inclusions are so photogenic. But there is also accompanying inflammation, and hardly any textbooks pay attention to that inflammation. Peculiar macrophages in the lamina propria were identified in both herpesvirus and cytomegalovirus infections.

How about EoE? Most of the biopsies that we see in this condition have no lamina propria, only epithelium. However, when we do get lamina propria it tends to be full of collagen and the cells that form it. Fibrosis is a common complication of EoE, so presumably these collagen-forming cells in the lamina propria reflect the eosinophils in the epithelium.

How about reflux disease? There are likely to be more lymphocytes and scattered eosinophils and neutrophils in the epithelium, but the only information in textbooks and the literature about lamina propria changes in reflux have to do with ulcers and squamous changes at the margins. Yet there are likely to be changes of chronicity with plasma cells in the lamina propria at the base and edges of some of these ulcers.

In Barrett's mucosa, the lamina propria and the epithelial structures are mixed, which means the lamina propria always is present in a biopsy. Barrett's mucosa almost always has some lamina propria plasma cells, in other words, chronic inflammation, and the number varies widely from patient to patient. But the textbooks and literature ignore the lamina propria to concentrate on the epithelium. One study, published only in abstract form, suggested that lamina propria inflammation was due to reflux, but as far as I can tell, the results of this study were never published.

In summary, the answer to the question, "Do cells in the lamina propria reflect those in the epithelium?" is that there is just not enough published information to answer this question. Maybe it is time to do a definitive study on the lamina propria in esophageal diseases. We eagerly await the results of that study.

5. How are stem cells identified in the squamous esophagus?

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What is a stem cell?

Stem cells are defined as unspecialized cells that are self-renewing, able to generate an amplified population with asymmetric cell division and slow-cycling growth. Other essential properties include the ability to retain telomere length, resist apoptosis, and serve as a cell-population source for renewal of a tissue over the lifetime of an organism. Stem cells are capable of dividing and renewing themselves for long periods, and through asymmetric cell division they may give rise to specialized cells that differentiate. Embryonic stem cells can give rise to all three types of adult tissues, whereas adult stem cells are thought to give rise to tissues where they reside.[29,30]

What are the putative sources of esophageal stem cells?

Possible candidates include the basal zone of the squamous epithelium, submucosal gland ducts, submucosal glands, and circulating stem cells of bone marrow origin. This review will focus on putative adult stem cells located in the esophagus itself, not addressing a potential role of circulating bone marrow stem cells.

In experimental models, stem cells have the ability to exclude Hoechst 33342 DNA-binding

dye mediated by the ABCG2 transporter and to retain tritiated-thymidine labeling after bromodeoxyuridine (BRDU) treatment, indicative of slow cycling. Rustgi *et al.* detected a cell population with these characteristics in the basal epithelium of the squamous epithelium in a mouse model.[30] Unlike humans, however, mice lack esophageal submucosal glands. Therefore, while providing evidence for a possible adult stem cell population in the basal zone of the squamous epithelium, a mouse model cannot address whether there are stem cell populations in ducts and submucosal glands of the human esophagus.

Nonetheless, because the esophageal squamous lining is an analog of the skin, and because submucosal ducts have some similarity to the sweat glands and hair follicles of the skin, it is reasonable to review the results from Elaine Fuchs and team regarding skin renewal and stem cells in a murine model. Different populations of cells that meet the criteria for adult stem cells were detected in the basal zone of the epidermis, at the isthmus of the hair follicle, and at the hair follicle bulge.[30,31] These locations are anatomically similar to the squamous basal zone of the esophagus, esophageal duct, and submucosal gland; therefore, similar separate adult stem cell populations may be present in these analogous regions in the human esophagus. In addition, there is evidence for the migratory capability of hair follicle and bulge cells, and for their contribution to epidermal healing following injury.[32] Human esophageal duct and submucosal gland populations might have similar capabilities to migrate and repair the esophageal mucosa.

Support for the presence of adult stem cell populations in the human esophageal ducts and submucosal glands comes from clinical and experimental investigations. During endoscopy, squamous islands are commonly observed in the midst of columnar-lined esophagus; histological examination confirms the location of these squamous islands in association with ducts from submucosal glands.[33] Two studies from the laboratory of Nicholas Wright provide experimental evidence of stem cell populations in the ducts and submucosal glands. They used laser-capture microdissection and loss-of-heterozygosity studies to identify the same somatic mutation in a clone of BE and in the adjacent normal squamous duct.[34] Later, they demonstrated that neosquamous islands comprised clonal patches of cells sharing the same mitochondrial DNA mutation as adjacent Barrett epithelial glands and the underlying duct cells.[35]

Finally, a p63 knockout mouse model has also provided new insights into esophageal renewal and putative stem cells. *p63* is a homolog of the *p53* tumor suppressor gene, expressed in stem cells of skin, breast, and prostate, and involved in morphogenesis and maintenance of all stratified epithelia. p63 appears to be both a suppressor and a transcriptional activator of groups of genes involved in the program of epithelial development. Although it is essential for stem cell regeneration in stratified squamous epithelia, it is not necessary for lineage differentiation in a nonsquamous direction. Mice in which *p63* has been knocked out lack continuous epidermis and exhibit disrupted development of squamous epithelia. They also have thymic hypoplasia. Epithelial cells in culture that lack p63 are deficient in their ability to grow in clones.[36]

Examination of the esophagi from *p63* knockout mice revealed a diminution of $p63^{+}$ cells in the basal zone of the squamous epithelium. Following programmed damage to the squamous-lined esophagus, residual putative embryonic stem cells at the EGJ migrated into the distal esophagus, accompanied by the development of columnar metaplasia of the distal esophagus.[37] *p63* null mice also failed to undergo the normal transition in embryo from columnar-lined to squamous-lined esophagus. These provocative experiments provide new evidence for the important role of p63 in maintaining squamous epithelia and for the possible substitution of an alternative source of cell renewal of an adult tissue if its usual stem cell population is inactivated or injured. They also raise new ideas about the role of the GEJ reserve or stem cell population in BE, and for the important developmental role of p63 in orchestrating developmental changes in esophageal development. Given the absence of submucosal glands in mice, however, there remain important unanswered questions about the role of stem cells (and of p63) in submucosal glands and ducts in humans in the setting of chronic reflux, esophageal injury, and repair.

In conclusion, how are stem cells identified in the squamous esophagus?

Research efforts have used animal models, including the p63 knockout model, cell-culture models, and

laser-dissection mapping of patches of epithelium that share common mitochondrial DNA mutations. Given important anatomical and physiological differences between animal and human esophagi, additional work is needed to refine our understanding of possible stem cell populations in the esophagus, their role in renewal of normal squamous epithelium, and the ways that normal renewal is disrupted in association with acid and bile reflux, or in embryonic development, to result in columnar metaplasia and BE.

6. Can stem cells be identified in Barrett's dysplasia?[37–45]

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The human esophagus is lined by a nonkeratinizing stratified squamous epithelium. In response to chronic GERD, this multilayered epithelium can be replaced by a single layer of columnar, usually mucus-secreting, epithelium (a process called Barrett's metaplasia). Differentiation of the Barrett's metaplastic epithelium may be of either gastric or intestinal phenotype, and is therefore histologically subclassified into these two subtypes, although internationally the definition of BE tends to be restricted to the intestinal phenotype (with presence of goblet cells). BE is a predominant risk factor for the development of adenocarcinoma (30- to 50-fold increased risk in comparison with the general public). This has major clinical implications, as esophageal cancer is a common cause of malignancy-related death, accounting for more than 500,000 deaths per year worldwide.

Initially, it was believed that Barrett's metaplasia was the result of upward migration from transitional zone cells of the GEJ,[38] but this could not explain the development of columnar epithelium in artificially defective mucosa above a squamous barrier in animal models. A second hypothesis, a metaplastic conversion from fully differentiated squamous epithelium into a columnar epithelium (so-called transdifferentation[40]) was dismissed by the observation that squamous epithelium may redevelop after endoscopic removal of Barrett's epithelium. Hence, a third hypothesis recently gained a lot of interest, being that BE is the consequence of change in commitment of multipotent stem cells. This not only explains the variety of cellular phenotypes in Barrett's epithelium and the regeneration of squamous epithelium after endoscopic removal of Barrett's mucosa, but also offers a possible key mechanism for the inherent cancer risk of BE, as multipotent stem cells have been implicated in carcinogenesis in other organs (e.g., colon).[41]

However, the exact location of the stem cells in the esophagus remains a topic of discussion. Most evidently, and in analogy to the epidermis of the skin, one would expect the stem cells to be located in the basal layer of the epithelium. This is in line with observations in mouse models, where a $CD34^+$ stem cell population was identified in the esophageal basal layer[43] (although the histology of human esophageal epithelium is remarkably different from that of mouse, with the latter lacking papillae). Data on the exact location of basal layer stem cells (at the top of papillae vs. interpapillary) are conflicting:[41,42] interpapillary basal layer cells demonstrate higher proliferation and clonogenic capability (making them excellent stem cell candidates), but only display very low expression of β1-integrin, which is not in line with mutipotent stemness, as undifferentiated cells lose β1-integrin expression when they commit to terminal differentiation.

Other groups have located the esophageal stem cells outside the epithelium. In this perspective, and similar to the bulge region of hair follicles, the submucosal gland ducts may be very important. Because their proximal two-thirds are lined by a columnar epithelium, and their distal third by a squamous epithelium, the glandular neck region of these glands has been suggested as the location for Barrett's stem cells.[43] This *gland duct theory* is supported by several findings:[44] (1) histological evaluation revealed that gland ducts frequently open on to the surface of Barrett's epithelium; (2) immunohistochemical similarities have been found between submucosal glands and Barrett's epithelium in pig tissues and cell cultures; (3) a *p16* mutation was found in common between submucosal glands and adjacent Barrett's epithelium; and (4) islands of neo-squamous epithelium were found to be wild type at loci containing mutations within the adjacent Barrett's epithelium. More recently, using nonpathogenic mitochondrial DNA mutations as clonal markers, it was found that Barrett's metaplastic glands are indeed clonal, contain multiple stem cells, and share a common squamous progenitor.[37]

Of interest, it has also been suggested that BE could be the result of bone marrow–derived stem cells.[45]

7. Are DIS normal?

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DIS were first reported in human esophageal epithelia of patients with symptoms of esophagitis.[46,47] They have subsequently been shown in *in vitro* experimental and *in vivo* esophageal perfusion studies. They were detected on transmission electron microscopy (TEM) with or without computer-assisted morphometry,[48] as well as on light microscopy (LM).[49] A summary of the findings of DIC and its association with nonerosive and erosive reflux disease in the literature is shown in Table 1.[8] In most studies, a statistically significant difference was found in intercellular spaces as measured by TEM between patients with reflux disease and control subjects.[50] DIS can be experimentally induced in humans by acid or acid–pepsin[51] and in animals (mostly rabbits) by acid or acid–pepsin, bile acid regardless of pH,[52] hypertonic solutions, or stress with or without acid–pepsin.[8] The current hypothesis is that DIS induces higher activation of the sensory nerve endings. Symptom score has been found to be most strongly associated with DIS of all the histological parameters (DIS, basal cell hyperplasia, papillary elongation, necrosis/erosion, eosinophils, and neutrophils) of GERD.[53] A few studies have shown a response (reversibility) to treatment for GERD.[54]

In summary, DIS is nonspecific and is seen in up to 30% of asymptomatic subjects and in nonreflux esophagitis patients. However, DIS is seen in much higher rates, up to 94%, of patients with GERD. An association has been found between DIS and exposure to acid, acid–pepsin, bile, and stress (both experimentally and empirically). It appears to be associated with symptoms of reflux, even more so than other histologic parameters, and appears to disappear with resolution of symptoms after treatment.

8. Why and when do DIS occur?

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GER is common in healthy subjects with no symptoms. GERD, a common disorder in the Western world, occurs when reflux lasts more than a few minutes, and disrupts the physiologic circadian rhythm[55] causing symptoms and (or) complications. Some patients experience heartburn and esophageal erosions, but for others the disease may get complicated with esophageal strictures, Barrett's metaplasia, and adenocarcinoma.

Importantly, EAC incidence has increased in the past 20 years,[56] yet most patients with GERD have no symptoms and endoscopy is often negative.[57] Given that the risk of esophageal damage and BE increases with higher reflux levels, pathology is often used to evaluate patients. Before 1979, the histology

Table 1. Summary of the presence of DIS and its association with nonerosive (NERD) and erosive (ERD) reflux disease

Author, year	Method	NERD	ERD	Control
Solcia, 2000	LM & EM	68%	90%	8%
Villanacci, 2001	LM	71%	100%	–
Vieth, 2004	LM	56%	Red streak: 91%	–
Bove, 2005	LM-before (B) versus after (A) acid perfusion	B: 80% A: 70%	B: 86% A: 86%	B: 22% A: 44%
Zentilin, 2005	LM	pH+: 83% pH–: 67%	94%	30%
Takubo, 2005	LM	E Ca: 33%	48%	21% Autop: 0%
Ravelli, 2006	LM (% area)	2.21 + 2.60% (5% in nonreflux esophagitis)		0.44 + 0.13%

LM, light microscopy; EM, electron microscopy; E Ca, esophageal cancer; Autop, autopsy.

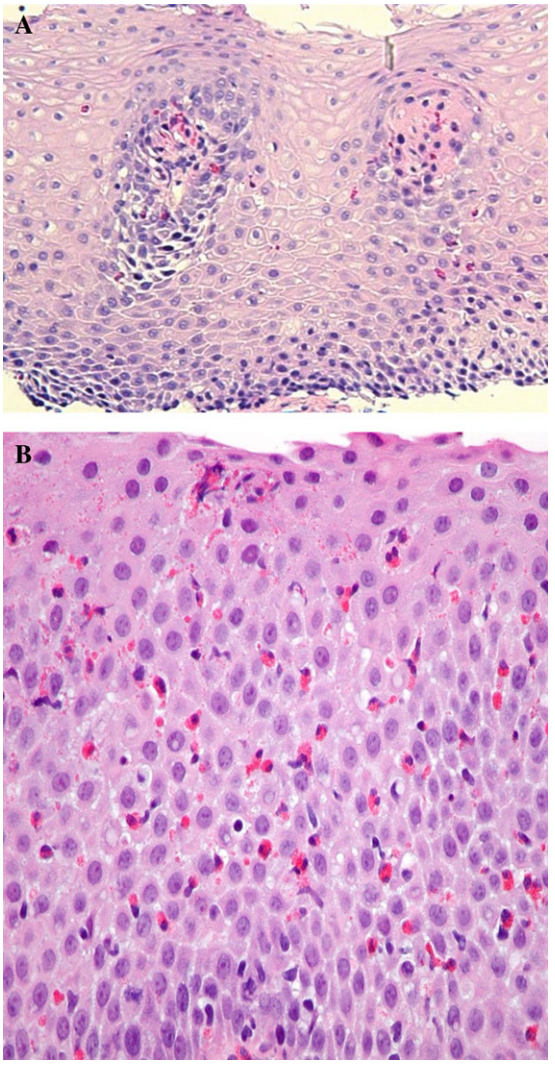

Figure 2. (A) GERD, the DIS are mainly basal, H&E 10×. (B) Markedly DIS in all three layers are more often noted with EoE, H&E 10×.

features most often used included basal cell hyperplasia, papillae elongation, and any inflammation (eosinophils, lymphocytes, and neutrophils); these features have limited sensitivity and specificity.[58]

DIS are considered as a more sensitive marker of GERD (Fig. 2A).[59] Hopwood *et al.* first defined it using electron microscopy[50] as greater than 0.47–2.4 mm, mainly in the basal layer. Today, DIS are diagnosed at LM when round dilations or diffuse widening of intercellular spaces are easily observed using a 40× objective.[60]

DIS are not specific for GERD. Epithelial damage brought on by even stress alone[61] can dilate the intercellular spaces. As Souza *et al.* suggested, refluxed gastric juice can stimulate esophageal epithelial cells to produce chemokines that eventually damage the esophageal tissue;[62] it is possible a similar mechanism exists with other irritants.

DIS are also observed in EoE. The pattern is nonetheless different. In GERD, the DIS are mainly basal (Fig. 2A).[59] In contrast, marked DIS in all three layers are more often noted with EoE (Fig. 2B).[63]

All tests have significant limits. Today, there is no gold standard method for the diagnosis of GERD. The presence of DIS, when taken in context including clinical presentation, DIS pattern, and other histologic features, are a more sensitive marker of GERD.[59]

9. Is the metastasizing potential of early carcinoma dependent on extent of spread through the MM?

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The anatomy of BE is unique. Unlike mucosal metaplasias occurring elsewhere in the gastrointestinal tract, the metaplasia of BE involves both epithelium and stroma. Stromal alterations include duplication of the esophageal MM, hypertrophy and splaying of a single MM, extension of MM bundles into the lamina propria, lamina propria fibrosis, and benign entrapment of metaplastic ± dysplastic glands within smooth muscle and fibrous tissue.[10–12,64] Recognition of stromal alterations in BE began over three decades ago, but only recently has attention focused on how these might affect the diagnosis, staging, and prognosis of early EACs. These issues are made more important by the increasing incidence of EAC—reported to be the fastest rising malignancy in the United States—and by the widespread use of endoluminal therapies such as endoscopic mucosal resection for accurate staging and treatment of intramucosal adenocarcinomas.

The first description of MM duplication in the English literature appeared in 1981, in the case report of a 71-year-old Japanese man with two EACs and a double MM beneath columnar-lined mucosa.[9] The authors of that report surmised that the deeper or outer MM represented original esophageal MM, but that the inner layer arose from

gastric MM. Two separate case reports mentioning MM duplication in Japanese patients followed. In 1991, Takubo *et al.* published the first study of MM duplication in BE, showing that it was both frequent (present in seven of eight cases with BE) and highly specific to BE (present in none of 352 cases without BE).[11] In a follow-up study from 1994, Nishimaki *et al.* confirmed the high prevalence of MM duplication in resection specimens with BE (14 of 15 cases, 93%) and asserted that dual MM—along with specialized metaplastic columnar epithelium—was "part of the specific histological changes characteristic of Barrett's esophagus."[12] Meanwhile, studies from Canada and Europe by Rubio and colleagues in 1988 and 1991 emphasized other stromal changes in BE—termed the *musculo–fibrous anomaly*—which included hypertrophy and fibrosis of the MM, fibrosis of the lamina propria and submucosa, smooth muscle ingrowth into the lamina propria, and subsequent entrapment, distortion, and cystic dilatation of columnar-lined glands.[10,64] These features were felt to be morphologically akin to the mucosal prolapse changes of solitary rectal ulcer syndrome and could frequently result in difficulties differentiating true invasion from benign glandular entrapment in BE; however, MM duplication was not at that time a recognized component of the musculo–fibrous anomaly. Despite these multiple studies from the 1980s and 1990s of stromal alterations in BE, this phenomenon actually received little notice in histology textbooks or in clinical practice.

Depth of invasion is an important prognostic factor in early EAC. Depth of invasion correlates strongly with the risk of lymph node metastasis and therefore is essential to the determination of therapy (i.e., esophagectomy with lymph node dissection versus endoscopic mucosal resection without lymph node removal). In a study from the University of Texas M.D. Anderson Cancer Center, for example, Liu *et al.* found lymph node metastases in 0% of adenocarcinomas limited to the lamina propria, 12% of those in the MM, 8% in the superficial submucosa, and 36% of the deep submucosa.[65] But can refinement of staging—that is, giving a precise depth of invasion into lamina propria, inner MM, space between dMM layers, or outer MM—yield even more refined prognostic information?

This question is difficult to address because of several factors. First, even though MM duplication is overall very frequent in BE, it can be patchy. We found that even among the 92% of BE surgical resections and 66% of BE endoscopic mucosal resections that contained MM duplication, the linear extent of duplication varied widely, from $<5\%$ to $>90\%$, and with means of 48% and 43%, respectively.[14,18] Therefore, studied cases have to include not only T1 adenocarcinomas, but those that occur in an area of MM duplication. Second, determination of exact depth of invasion (and even the presence of invasion versus entrapment) can be difficult in cases with marked MM hypertrophy or fibrous obliteration. Finally, because of the relatively low risks of lymph node metastases, distant metastases, and death from T1 adenocarcinomas, large numbers of cases are needed to produce adequate events for statistical analysis.

Nevertheless, three studies from the U.S. population including 30 patients with invasion into dMM,[14] 82 patients with invasion into dMM,[18] and 41 patients with invasion into dMM,[16] have addressed this question and the results—singly or in combination—are clear (Table 2). Exact depth of invasion into the dMM neither influences lymph node metastasis nor does it appear to affect survival. A theoretical basis for this finding is also supported by studies that have shown similar densities of lymphatic/vascular channels in the lamina propria and in the space between dMM. The major cutoffs in risk for T1 adenocarcinomas occur between intramucosal invasion (including dMM) and submucosal invasion, and between superficial submucosal invasion and deep submucosal invasion, but not within intramucosal carcinoma itself.

In summary, the main reason to recognize MM duplication vis-à-vis EAC is to avoid the trap of overstaging: Do not misinterpret invasion through inner MM as submucosal invasion, and (worse yet) do not misinterpret invasion into outer MM or into thickened MM as invasion into muscularis propria.

10. Are there molecular or immunohistochemical tests, either singly or in combination, that improve diagnosis and management compared with H&E-stained biopsies and surveillance?

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Table 2. Risk of lymph node metastasis in relation to depth of invasion into duplicated MM in the United States

Study	No. cases	No. invading into duplicated MM	Lymph node metastasis			Other findings
			Depth of invasion	+ Lymph nodes		
Abraham *et al.*[14]	30	30	Inner MM	1/10	10%	–
			Between MM layers	1/12	8%	
			Outer MM	1/8	12%	
Kaneshiro *et al.*[15]	185	82	Lamina propria	1/68	1.5%	Depth of invasion did not predict survival
			Inner MM	0/38	0%	
			Between MM layers	0/11	0%	
			Outer MM	0/33	0%	
			Submucosa (inner third)	3/35	9%	
Estrella *et al.*[16]	99	41	Lamina propria or inner MM	1/28	4%	In multivariate analysis, only lymphovascular invasion predicted recurrence-free survival
			"Duplicated MM space"	0/41	0%	
			Submucosa	10/30	33%	

MM, muscularis mucosae.

This question is directed at two problems, the diagnosis of Barrett's mucosa and the diagnosis of its neoplastic complications.

Is there a better way to diagnose Barrett's mucosa than using H&E? Currently, the histologic diagnosis of Barrett's varies with where one lives. In all places, there must be endoscopic evidence of Barrett's, so nothing has replaced the eyeballs of the endoscopist for this part of the diagnosis. Also, regardless of where one lives, biopsies from the endoscopic Barrett's must have columnar mucosa. Pathologists have no problem identifying columnar mucosa. So nothing has replaced the eyeballs of the pathologist. If you live in the United Kingdom and some other countries, columnar mucosa of any type is needed for the Barrett's diagnosis, as long as the endoscopic requirement is satisfied. If you live in the United States and some other countries, this columnar mucosa must have goblet cells to satisfy the Barrett's diagnosis. A mucin stain such as an Alcian blue will bring out goblet cells, but finding them on H&E is pretty easy, because in this stain, goblet cells are gray or faintly blue compared with the gastric-type columnar cells, which are pale pink. A mucin stain, such as an Alcian blue, will stain the goblet cells an intense blue, but this stain also uncovers cells that might be misinterpreted as goblet cells. Specifically, Barrett's mucosae have columnar cells that contain mucin in their apical cytoplasm that stains blue with the Alcian blue. These are not goblet cells but peculiar columnar cells. These have even been given the name of *columnar blues*, and so far, they have not been considered to be a marker for intestinal metaplasia.

Is there a better way to diagnose dysplasias and carcinoma than by H&E-stained sections? First, the criteria for the dysplasias are not clear-cut, so reproducibility is poor, especially at the lower end of the dysplasia spectrum. The diagnosis becomes more reproducible at the higher end. We pathologists are not so great in diagnosing dysplasias, especially low grade, so we need help. This is where markers might be helpful. Several markers have been studied in dysplasias including AMACR.[67–69] This is expressed in prostate and colon cancers. There are a few published studies about this marker in Barrett's mucosa. In one study, 11% of low-grade dysplasias, 64% of high-grade dysplasias, and 75% of carcinomas stained positively. In another study, there was staining even in 27% of those biopsies labeled as indefinite for dysplasia (IND), with 90% in low-grade and 96% in high-grade dysplasia and

also in 96% of carcinomas. The results of the third study were between those other two. Therefore, it is clear that these three studies used different diagnostic criteria for the different categories. The first study upgraded the diagnoses whereas the third one downgraded them. Furthermore, in these studies, different specimens were used. Two of the studies used biopsies and/or resections whereas the third study used only resections. Diagnosing dysplasias in resected specimens is not the important issue, because the esophagus has been resected. The important dysplasia diagnoses are in biopsies, and none of these studies give us much help with biopsies. *p53* is a tumor suppressor gene, a TF important in cell cycle regulation. Its protein is nuclear, which makes immunostains easy to interpret. In two studies, $p53^+$ low-grade dysplasia was much more likely to progress to higher grade dysplasia and carcinoma than $p53^-$ low-grade dysplasia.[69,70] This suggests that the diagnosis of low-grade dysplasia by itself is not as important as the diagnosis of low-grade dysplasia accompanied by positive *p53* as an indication of progression risk.

Other markers have been tested including *c-myc* amplification, which was found only in high-grade dysplasia and carcinoma, two of the easier diagnoses by H&E.[71] MUC2 was found in nondysplastic mucosa whereas dysplasias were negative. MUC1 was found in half the carcinomas.[72] APC and p16 hypermethylation staining increased from negative to low-grade dysplasia to high-grade and carcinoma, and hypermethylation of both seemed to predict progression.[73] A number of other markers studies have been published, all of which conclude that there are differences in expression in various types of neoplastic and nonneoplastic Barrett's mucosa. A problem with all of these studies is the diagnostic criteria used for dysplasias. Many referred to a 1983 paper by Riddell *et al.* dealing with dysplasias in inflammatory bowel disease, but not in Barrett's mucosa. In some studies, the authors referred to the use of "standard criteria" for dysplasias, but there are no standard criteria. Remarkably, in some of the marker studies, no histologic criteria were given for the diagnoses. Regardless, in all these studies, the diagnoses were made on H&E-stained sections and the markers were related to those H&E-section diagnoses.

In conclusion, the marker studies are based on diagnoses made on slides stained with H&E. Because the diagnoses were already known, it is obvious that the markers offered no better diagnostic information, possibly except for p53 staining of H&E-diagnosed low-grade dysplasia. So, is there a better way to manage Barrett's than by diagnoses based on H&E and appropriate endoscopic surveillance? Some markers seem to correlate with progression, but in order to determine if markers are valid determinants of surveillance or treatment, long-term studies are needed basing follow-up only on the markers, not on diagnoses made by H&E. We eagerly await such studies. At this moment, there are no molecular or immunohistochemical tests that improve diagnosis and management compared with H&E-stained biopsies and surveillance.

11. Does IHC have a role in: (1) grading of neoplasia in Barrett's esophagus? or (2) defining the risk of progression to adenocarcinoma?

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Many investigators have looked for immunohistochemical markers that might improve the sensitivity and specificity of detection of intraepithelial neoplasia (dysplasia) and adenocarcinoma over routine light microscopic examination of esophageal biopsies in patients with BE. Potentially useful antibodies include those recognizing p53, $p16^{INK4A}$, c-erbB2, cyclin D1, $p27^{Kip1}$ EGFR, COX-2, β-catenin, Rab11a, CD1a, HER2, EGFR, SMAD4, IMP3, Ki-67, Serpins, and AMACR.[74] Although individual studies have reported favorable results in distinguishing low-grade (LGD) from high-grade dysplasia (HGD), or HGD from EAC,[75] reproducibility of staining results has not been confirmed when studied in multiple laboratories. Although there is evidence that p53 might be helpful to predict patients who may progress to HGD,[75] it is reported to be expressed in approximately 5% of nondysplastic BE. Therefore, although the rate of p53 expression is higher in dysplasia than in metaplasia, it is not specific for dysplasia. In addition, evidence to support consistently positive and negative predictive values of any potentially useful marker awaits larger series. Therefore, at the current time, few laboratories are routinely applying IHC in the evaluation of esophageal biopsies for neoplasia in BE.

Table 3. AMACR immunohistochemistry: summary of four studies[66–68,77] showing percentage of cases expressing AMACR and the sensitivity of the stain in each study by histology category

Grade	% Positive	% Sensitivity	% Specificity
BE no dysplasia	0		100
Indefinite for dysplasia	0, 22, 21, 27	22	
Low-grade dysplasia	11, 18, 38, 90	38, 91	
High-grade dysplasia	60, 64, 81, 96	64, 81, 96	100
EAC	67, 72, 75, 96	72, 96	100

AMACR, α-methylacyl coenzyme A racemase; BE, Barrett esophagus; EAC, esophageal adenocarcinoma.

Does a menu of stains offer increased value over use of a single immunohistochemical stain? Van Dekken *et al.* applied a panel including epidermal growth factor receptor (EGFR), ERBB2 (HER2/neu), MYC, CDKN2A (p16), SMAD4, MET, CCND1 (cyclin D1), CTNNB1 (β-catenin), and TP53 (p53), comparing expression in 86 cases along the spectrum from BE to EAC. Among antibodies with statistically significant results ($P < 0.001$), β-catenin distinguished BE from LGD, and both cyclin D1 and p53 distinguished LGD from HGD. Despite this degree of significance, fewer than 25% of LGD cases showed intense staining for β-catenin, and only 45% of cases of HGD expressed cyclin D1.[76]

One of the better-studied markers, AMACR, is a mitochondrial peroxisomal enzyme expressed in colonic adenocarcinomas but not in normal colonic epithelium. Esophageal biopsies from patients with BE have been stained for AMACR by several groups, who examined cases of nondysplastic BE, IND, LGD, HGD, and EAC. Table 3 summarizes four studies and shows the percentage of cases in each histological category that express AMACR and also the sensitivity and specificity, when reported. Of note, all groups reported uniform absence of reactivity for BE without dysplasia; therefore, AMACR staining may be helpful in cases concerning possible dysplasia. Although a definite trend was seen in all studies, there was considerable variation in staining in each neoplastic category; the percentage of IND cases that expressed AMACR ranged from 0% to 29%. This may reflect differences in histological classification of the study set, in terms of the investigators' thresholds for assigning grade, as well as differences in thresholds for positive results.[67–69,77] IND cases with AMACR expression were more likely to progress to EAC than negative IND cases, but the positive predictive value was only modest (0.44).[77]

Most of the above stains are expressed in neoplasia but not in nonneoplastic BE. In contrast, Beclin-1 has a central role in autophagy and is upregulated with bile acid exposure and BE, but downregulated with neoplastic progression. Of note, bile acid reflux is reported in 22% of patients with esophagitis but is more common in BE, affecting 54% of patients with BE and 76% of patients with EAC. Loss of nuclear expression of Beclin-1 is seen in LGD, HGD, and in EAC, with statistically significant differences in staining intensity; however, there is some overlap in RNA levels of expression between nonneoplastic BE and EAC.[78] Beclin-1 may be useful for inclusion in a staining menu, as it is desirable for a panel to combine markers that are expected to be positive with markers that are typically negative.

Several fundamental challenges limit clinical applicability of currently reported immunohistochemical markers. First, each study used a set of cases that were classified histologically; however, histological classification is imperfect, given interobserver variability.[77] As seen with AMACR, the sensitivity and specificity of a marker varies among studies, and this variation may reflect differences in interpretation and classification of the study set, rather than marker utility. Different studies also used different thresholds for positive results, with no consensus standard. Case numbers are relatively small in most studies. There is no single pathway from normal to cancer. It is even controversial whether HGD evolves from LGD, or whether it represents a distinctive form of intraepithelial neoplasia, with potentially distinctive etiology and expression profiles. It is also well known that even within a single histological category or grade, individual neoplasms often have different expression profiles. In Barrett's neoplasia each group (LGD, HGD, intramucosal adenocarcinoma, and

adenocarcinoma that has invaded the submucosa) is heterogeneous, comprising a range of histological patterns and cytological phenotypes, with different genetic signatures and transcription profiles. In the vast majority of studies, there is no long-term follow-up to correlate clinical outcomes with immunohistochemical results. Validation of markers for utility in diagnosis and prognosis remains to be done. And finally, cost-effectiveness studies are needed to define if addition of IHC in the grading of Barrett neoplasia adds value over review by a second experienced pathologist, which is the current American College of Gastroenterology recommendation.

In conclusion, clear differences in immunohistochemical expression of specific antigens are seen in different grades in some, but not in all, cases of Barrett's neoplasia. These findings are useful and important contributions to our understanding of the process of neoplastic development and associated altered protein expression. Applying these stains in a research setting will continue to inform our understanding of the biology of Barrett's neoplasia. It remains unclear if immunohistochemical staining adds clinical value over the gold standard of histological grading and review by a second experienced pathologist. Currently, no single marker or panel is clearly predictive of risk to progression to EAC. Larger multicenter studies including a range of cases are needed to provide definitive evidence for the utility and cost-effectiveness of immunohistochemical staining in routine clinical diagnostic work.

12. What are the roles of CDX1 and CDX2 in squamous and cardiac mucosa?

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Introduction

The TFs *CDX1* and *CDX2*, human homologues of the *Drosophila* homeobox gene *Caudal*, participate in intestinal development and patterning and maintain the intestinal phenotype. They are normally expressed in the tubal gut distal to the stomach and direct their effects through numerous target genes. Given the fact that Barrett mucosa is *intestinalized*, investigators correctly anticipated CDX1/2's relevance. This brief commentary here will discuss (1) knockout and transgenic mouse models that highlight the critical functions of CDX, (2) expression of these TFs in human esophageal biopsy material, and (3) experiments in cell cultures and cell lines in which conditions mimicking GERD are established or CDX1 or CDX2 are transfected. It will conclude with reference to more recent studies suggesting that, although CDX expression appears critical in the establishment and maintenance of BE, it is not the sole determinant.

Knockout and transgenic mouse models

The $Cdx1^{-/-}$ mouse has no gut phenotype, attributed to the protein's functional redundancy. $Cdx2^{-/-}$ embryos fail to implant, as Cdx2 is critical to the developing trophectoderm. The $Cdx2^{+/-}$ mouse develops metaplastic, squamous-lined polyps in the ileocecal region.[79] The adjacent mucosa is characterized by the sequential interposition of cardiac, cardio-oxyntic, oxyntic, and small intestinal mucosa. The polyps occur in areas of haploinsufficiency, with the gut reverting to the default foregut (squamous) differentiation program. In the developing embryo, a morphogenic milieu exists to direct the intercalary growth described earlier. A more recent *Cdx2* conditional knockout overcomes the implantation block.[80] The tubal gut ends as a blind pouch at the cecum and is lined by squamous epithelium.

A transgenic mouse model in which *Cdx2* is placed under the H^+/K^+-*ATPase* β subunit promoter (normally expressed in gastric parietal cells) results in complete intestinalization of the stomach.[81] Although morphologically normal at birth, intestinalized crypts are detected at day 19, and at day 37 the oxyntic mucosa is largely replaced by crypts composed of goblet, absorptive, and enteroendocrine cells. This result was independently validated by a group who placed *Cdx2* under the *Foxa3* promoter, and, although the *Cdx1* knockout mouse has no intestinal phenotype, forced *Cdx1* expression in the stomach also leads to profound intestinalization.

CDX expression in esophageal biopsy material

As first reported by Phillips *et al.* in 2003, several groups have shown CDX2 protein expression, as detected by IHC, in virtually all biopsies of Barrett's epithelium (i.e., columnar epithelium with goblet cells proximal to the anatomic GEJ) (Fig. 3).[82] CDX2 expression was also found in 30% (20/62) of esophageal cardia-type mucosal samples (i.e., without goblet cells), suggesting that that epithelium is at

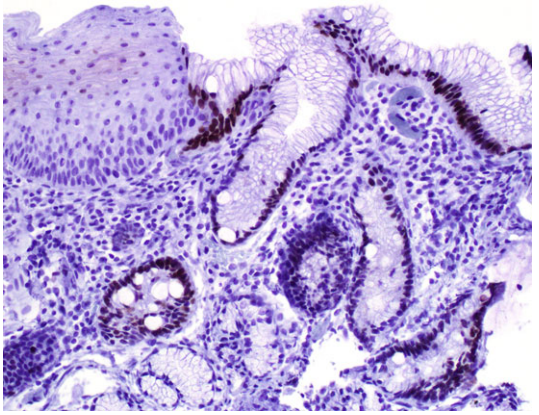

Figure 3. CDX2 expression in BE: CDX2 is expressed by the columnar epithelium in virtually all Barrett biopsies (note the goblet cells in this example) and 30–40% of biopsies of esophageal metaplastic columnar epithelium without goblet cells (immunoperoxidase, original magnification 200×).

least partially intestinalized. CDX2 expression is not detected in cardia-type epithelium in the stomach.

Utilizing qualitative reverse transcriptase–polymerase chain reaction (RT–PCR), Eda *et al.* found CDX2 mRNA in squamous mucosal biopsies from 10 of 15 (67%) GERD patients with mucosal breaks and zero of five normal controls; CDX1 mRNA was not detected.[83] Although Moons and colleagues did not detect CDX2 protein expression (by IHC) in 40 biopsies of esophageal squamous mucosa taken from GERD patients, they did find small amounts of CDX2 mRNA in 6 of 19 (33%) biopsies taken from 5 cm above the neo-squamocolumnar junction in Barrett's patients.[84]

Experiments in cell lines and keratinocyte cell cultures

BE arises in the setting of gastroduodenal reflux and inflammation. Investigators have attempted to model BE by exposing cell lines and cell cultures to low pH, bile acids, and/or inflammatory cytokines, which have been found to induce the expression of CDX1/2. In addition, the more direct effects of CDX1/2 are assessed in transfection experiments. This line of investigation has also served to highlight the importance of upstream NF-κB signaling and epigenetic transcriptional regulation.

In one example, Kazumori *et al.* showed that different bile acids variably activated the *CDX2* promoter in cell lines, with DHCA and CA achieving the greatest induction.[85] The effect was abrogated by mutation of NF-κB–binding sites in the *CDX2* promoter. When primary rat esophageal keratinocyte cell cultures were incubated with either DHCA or CA, Cdx2 expression was induced in a dose-dependent manner. Finally, Cdx2 transfection of the cell culture resulted in expression of the intestine-specific apomucin MUC2.

Wong and colleagues performed a similar series of experiments, with their key contribution being the demonstration of the importance of the methylation status of gene promoters.[86] Using bisulfite sequencing, they showed that although the *CDX1* promoter was completely methylated in normal esophageal squamous and gastric corpus mucosa, it was unmethylated in normal colonic mucosa and demethylated in BE. Although the inflammatory cytokine TNF-α had no effect on CDX1 mRNA expression in cell lines with a normally methylated *CDX1* promoter, treatment with the demethylating agent 5-aza-2′-deoxycytidine rendered them responsive.

CDX expression alone is insufficient to account for BE

Given the striking phenotype of the transgenic mouse models in which Cdx2 is expressed in the stomach, one might assume that a transgenic mouse in which Cdx2 is expressed in the esophagus might provide an excellent model of BE. John Lynch's group at the University of Pennsylvania has produced such a mouse, in which Cdx2 is expressed in squamous epithelium using the keratin-14 promoter.[87] The mouse expresses Cdx2 in basal keratinocytes, which is associated with reduced proliferation, formation of DIS, reduced cell–cell adhesion, and, ultrastructurally, assumption of a more secretory-like phenotype (reduced keratin bundles, increased endoplasmic reticulum). Goblet cells are not induced, though, nor are Cdx2 targets like Muc2, sucrase isomaltase, and alkaline phosphatase. Although treatment with 5′-azacytadine did not alter the model's morphology, it increased Cdx2 mRNA levels 250-fold and resulted in expression of the Cdx2 targets Cdx1, keratin-18, and SLC26A3/DRA. The authors concluded that Cdx2 expression alone is insufficient to produce the full Barrett's phenotype and that this is in part due to epigenetic regulation of the adult genome. Furthermore, although Cdx2 expression results in reduced proliferation, that phenotype must be overcome by some other factor(s), as BE, even without dysplasia, is proliferative.

Gene expression microarrays have identified hundreds of genes (including *CDX1*) differentially expressed in BE compared to squamous-lined esophagus. Rebecca Fitzgerald's group has recently focused on the *HOXB* gene cluster (like CDXs, these TFs also contain the homeobox domain).[88] HOXB5, HOXB6, and HOXB7 are upregulated in BE, and transfection experiments involving a human esophagus–derived cell line demonstrated upregulation of the intestinal markers keratin 20, MUC2, and villin (but not CDX2). They also found that expression of these HOXs was related to specific activating histone modifications in their gene promoters and to chromatin decompaction.

Summary

BE is characterized by the replacement of a differentiated squamous epithelium with a columnar epithelium that is intestinalized. CDX2, a TF that is both a marker of intestinal differentiation and a significant player in directing the intestinal differentiation program, is expressed in virtually all Barrett's biopsies. In cell lines and keratinocyte cell cultures, experimental conditions mimicking GERD promote CDX1 and CDX2 expression and transfection experiments induce the production of CDX targets. This research has also highlighted the importance of upstream NF-κB signaling and epigenetic regulation of these TFs by promoter methylation.

Although transgenic mouse models in which Cdx1 or Cdx2 are constitutively expressed in the stomach demonstrate profound intestinalization, a mouse in which Cdx2 expression is placed under the keratin-14 promoter fails to fully recapitulate the Barrett's phenotype. Treatment of the mouse with a demethylating agent advances the columnar phenotype, again highlighting the importance of epigenetic regulation. Expression microarrays have identified hundreds of genes differentially expressed in BE relative to esophageal squamous epithelium. These include three *HOXB* family TFs, which, in transfection experiments, are also able to induce the expression of intestine-specific proteins. Increased expression of these HOXs occurs in the setting of activating histone modifications and chromatin decompaction.

A model has emerged in which gastroduodenal reflux and inflammatory cytokines participate in creating an environment favorable to the expression of key TFs, including but not limited to CDX1/2, which direct intestinal differentiation. Key features of that favorable environment include NF-κB signaling, promoter demethylation, histone modification, and chromatin decompaction.

13. What is the role of desmosomal cadherins and lectins in squamous and cardiac mucosa?

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Desmosomes represent adhesive intercellular junction proteins. Their adhesion molecules are the desmosomal cadherins desmoglein and desmocollin. Both adhesion molecules are widely expressed in the tissue, including the stratified squamous epithelium, the skin, salivary glands, hair follicles, prostate, and testis. Moreover, it was shown that desmosomal cadherins are involved in various pathological processes like pemphigus foliaceous, mucocutaneous pemphigus vulgaris, pemphigus vulgaris, and paraneoplastic pemphigus.[89]

Focusing on esophageal squamous epithelium, desmoglein 1 and desmocollin 1 are mainly expressed in the superficial parts, whereas desmoglein 2 and desmocollin 2 are only found in the basal cell layer. Contrary, the expression of desmoglein 3 and desmocollin 3 is mostly restricted to the basal epithelial layer while there is only a weak expression of these desmosomal cadherins in the superficial layers of esophageal squamous epithelium. One recent study evaluated the gene expression of desmoglein 1, desmoglein 2, and desmoglein 3 in the esophageal mucosa.[90] The authors described a uniform upregulation of desmosomal genes in the esophageal mucosa of patients with GERD, thereby supporting the concept of changes in the desmosomal compartment in the pathogenesis of GERD. The same group assessed different desmosomal components in cardia mucosa in relation to GERD and *Helicobacter pylori* infection and found an upregulation of desmoglein 2 in cardia mucosa of patients with GERD.[91] They concluded that these findings underline the concept that changes in the desmosomal compartment contribute to the pathogenesis of GERD in the cardia mucosa.

Lectins are a structurally heterogeneous group of carbohydrate-binding proteins, which were found to be highly specific for their respective sugar

moieties. Lectins have a distinct role in biological recognition phenomena including regulation of cell adhesion.[92] Based on their binding specificity, five distinct classes of lectins can be differentiated. Various studies have already shown that lectins are present in normal, inflamed, and neoplastic mucosa, but composition may change according to the underlying condition.

In 1999, Poorkhalkali and coworkers analyzed 12 different lectins to investigate the differences in glycoconjugate production among different mammalian species.[93] In general, the strongest lectin staining was found in the stratum superficiale and the weakest staining in the stratum germinativum. Interestingly, superficial damage to the rabbit esophagus epithelium after exposure to pepsin/HCl produced a considerable decrease in electrical resistance and a decreased staining of the esophageal epithelium with selected lectins. For example, this effect was shown for the lectins WGA and UEA-II. Pretreatment of the esophageal mucosa with a compound with protective properties (sucrose octasulfate) prevented to some extent the decrease in resistance and lectin staining. Recently, lectin-binding patterns were evaluated in patients with GERD and *Heliobacter pylori* infection.[94] Therefore, 88 patients were included and lectin-binding patterns were examined immunohistochemically at the squamocolumnar junction and in squamous and columnar-lined epithelium. Lectin binding was significantly reduced for the lectins UEA-I, DBA, and PNA in columnar-lined epithelium, and for DBA in the squamous epithelium of patients with GERD, respectively. *H. pylori* infection was associated with reduced PNA and DBA binding to the deep glandular mucosa of columnar-lined epithelium and surface squamous epithelium, respectively. More recently, the same group analyzed the binding pattern of different lectins at the GEJ in patients with NERD, ERD, and BE.[95] One hundred and twenty-two patients were included, and staining patterns of lectins were semiquantitatively evaluated using an immunohistochemical score. It was found that in patients with BE, lectin binding of UEA-I and DBA were significantly decreased at the superficial and deep glandular body. Comparisons of lectin-staining scores between GERD and BE revealed significant increases of UEA-I in both the stratum superficiale and stratum spinosum of squamous epithelium in patients with BE. No difference was observed between patients with GERD and controls. As lectin–UEA-I–binding proteins were specifically increased in the squamous epithelium of patients with BE, the authors concluded that UEA-I may serve as a potential marker for BE, especially in patients with short-segment BE. Nevertheless, further validation studies of this promising approach are still anticipated. Very recently, it was shown that cell-surface glycans are altered in the progression from BE to adenocarcinoma and lead to specific changes in lectin-binding patterns.[96] The binding of wheat germ agglutinin to human tissue was determined to be specific. By using molecular imaging, this specific binding was validated by successful visualization of high-grade dysplastic lesions in BE, which were previously not detectable by conventional endoscopy.

In conclusion, proteins within the intercellular spaces can be further characterized (e.g., cadherins, lectins). Various studies have shown that the expression of cadherins and lectins is altered in GERD and BE. Currently, these markers are only of scientific interest, but further characterization will lead to a better understanding of early damage due to reflux disease.

Conflicts of interest

The authors declare no conflicts of interest.

References

1. Takubo, K. 2007. *Pathology of the Esophagus.* 2nd ed. Tokyo: Springer.
2. DeNardi, F.G. & R.H. Riddell. 2007. "Esophagus," Chapter 22 In *Histology for Pathologists.* 3rd ed., S.E. Mills, Ed. Philadelphia: Lippincott Williams & Wilkins.
3. Fenoglio-Preiser, F. *et al.* 2008. "The nonneplastic esophagus." In *Gastrointestinal Pathology: An Atlas and Text.* 3rd ed. Philadelphia: Lippincott Williams & Wilkins.
4. Stevens, C.E. & I.D. Hume. 2004. *Comparative Physiology of the Vertebrate Digestive System.* Cambridge, UK: Cambridge University Press.
5. Vieth, M., R. Fiocca, J. Haringsma, *et al.* 2004. Radial distribution of dilated intercellular spaces of the esophageal squamous epithelium in patients with reflux disease exhibiting discrete endoscopic lesions. *Dig. Dis.* **22:** 206–212.
6. Orlando, L.A. & R.C. Orlando. 2009. Dilated intercellular speces as a marker of GERD. *Curr. Gastroenterol. Rep.* **11:** 190–194.
7. Farré, R., F. Fornari, K. Blondeau, *et al.* 2010. Acid and weakly acidic solutions impair mucosal integrity of distal esposed and proximal non-exposed human oesophagus. *Gut* **59:** 164–169.
8. Van Malenstein, H., F. Ricard & D. Sifrim. 2008. Esophageal dilated intercellular spaces (DIS) and nonerosive reflux disease. *Am. J. Gastroenterol.* **103:** 1021–1028.

9. Kato, H., T. Iizuka & H. Watanabe. 1981. Double adenocarcinoma in Barrett's Esophagus. *Jpn. J. Clin. Oncol.* **11:** 523–530.
10. Rubio, C.A. & R. Riddell. 1988. Musculo-fibrous anomaly in Barrett's mucosa with dysplasia. *Am. J. Surg. Pathol.* **12:** 885–889.
11. Takubo, K., K. Sasajima, K. Yamashita, *et al.* 1991. Double muscularis mucosae in Barrett's esophagus. *Hum. Pathol.* **22:** 1158–1161.
12. Nishimaki, T., A.H. Holscher, M. Schuler, *et al.* 1994. Chronic esophagitis and subsequent morphological changes of the esophageal mucosa in Barrett's esophagus: a histological study of esophagectomy specimens. *Surg. Today* **24:** 203–209.
13. Barbour, A.P., M. Jones, I. Brown, *et al.* 2010. Risk stratification for early esophageal adenocarcinoma: analysis of lymphatic spread and prognostic factors. *Ann. Surg. Oncol.* **17:** 2494–2502.
14. Abraham, S.C., A.M. Kraskinas, A.M. Correa, *et al.* 2007. Duplication of the muscularis mucosae in Barrett esophagus: an underrecognized feature and its implication for staging of adenocarcinoma. *Am. J. Surg. Pathol.* **31:** 1719–1725.
15. Kaneshiro, D.K., J.C. Post, L. Rybicki, *et al.* 2011. Clinical significance of the duplicated muscularis mucosae in Barrett esophagus-related superficial adenocarcinoma. *Am. J. Surg. Pathol.***35:** 697–700.
16. Estrella, J.S., W.L. Hofstetter, A.M. Correa, *et al.* 2011. Duplicated muscularis mucosae invasion has similar risk of lymph node metastasis and recurrence-free survival as intramucosal esophageal adenocarcinoma. *Am. J. Surg. Pathol.* **35:** 1045–1053.
17. Mandal, R.V., G.F. Forcione, W.R. Brugge, *et al.* 2009. Effect of tumor characteristics and duplication of the muscularis mucosae on the endoscopic staging of superficial Barrett esophagus-related neoplasia. *Am. J. Surg. Pathol.* **33:** 620–625.
18. Lewis, J.T., K.K. Wang & S.C. Abraham. 2008. Muscularis mucosae duplication and the musculo-fibrous anomaly in endoscopic mucosal resections for barrett esophagus: implications for staging of adenocarcinoma. *Am. J. Surg. Pathol.* **32:** 566–571.
19. Hahn, H.P., A.Shahsafaei & R.D. Odze. 2008. Vascular and lymphatic properties of the superficial and deep lamina propria in Barrett esophagus. *Am. J. Surg. Pathol.* **32:** 1454–1461.
20. Takubo, K. 1981. Squamous metaplasia with reserve cell hyperplasia in the esophagogastric junction zone. *Acta Pathol. Jpn.* **31:** 349–359.
21. Shields, H.M., S.J. Rosenberg, F.R. Zwas, *et al.* 2001. Prospective evaluation multilayered epithelium in Barrett's esophagus. *Am. J. Gastroenterol.* **96:** 3268–3273.
22. Glickman, J.N., Y. Chen, D. Antonioli, *et al.* 2001. Characterization of a distinctive multilayered epithelium associated with Barrett's esophagus. *Am. J. Surg. Pathol.* **25:** 569–578.
23. Takubo, K., M. Vieth, N. Honma, *et al.* 2005. Ciliated surface in the esophagogastric junction zone: a precursor of Barrett's mucosa or ciliated pseudostratified metaplasia? *Am. J. Surg. Pathol.* **29:** 211–217.
24. Shields, H.M., F. Zwas, D.A. Antonioli, *et al.* 1993. Detection by scanning electron microscopy of a distinctive esophageal surface cell at the junction of squamous and Barrett's epithelium. *Dig. Dis. Sci.* **38:** 97–108.
25. Glickman, J.N., S.J. Spechler, R.F. Souza, *et al.* 2009. Multilayered epithelium in mucosal biopsy specimens from the gastroesophageal junction region is a histologic marker of gastroesophageal reflux disease. *Am. J. Surg. Pathol.* **33:** 818–825.
26. Okuda, T. & T. Ogata. 1976. An electron microscopic study of the ciliated cells in the human gastric mucosa. *Arch. Histol. Jpn.* **39:** 149–156.
27. Torikata, C. *et al.* 1989. Ultrastructure of metaplastic ciliated cells in human stomach. *Virchows Arch A Pathol. Anat.* **414:** 113–119.
28. Rubio, C., T. Hayashi & G. Stemmermann. 1990. Ciliated gastric cells: a study of their phenotypic characteristics. *Mod. Pathol.* **3:** 720–723.
29. Blanpain, C. & E. Fuchs. 2009. Epidermal homeostasis: a balancing act of stem cells in the skin. *Nat. Rev. Mol. Cell Biol.* **10:** 207–217.
30. Lander, A.D., J. Kimble, H. Clevers, *et al.* 2012. What does the concept of the stem cell niche really mean today? *BMC Biol.* **10:** 19.
31. Kalabis, J., K. Oyama, T. Okawa, *et al.* 2008. A subpopulation of mouse esophageal basal cells has properties of stem cells with the capacity for self-renewal and lineage specification. *J. Clin. Invest.* **118:** 3860–3869.
32. Lu, C.P., L. Polak, A.S. Rocha, *et al.* 2012. Identification of stem cell populations in sweat glands and ducts reveals roles in homeostasis and wound repair. *Cell* **150:** 136–150.
33. Coad, R.A., A.C. Woodman, P.J. Warner, *et al.* 2005. On the histogenesis of Barrett's oesophagus and its associated squamous islands: a three-dimensional study of their morphological relationship with native oesophageal gland ducts. *J. Pathol.* **206:** 388–394.
34. Leedham, S.J., S.L. Preston & S.A. McDonald. 2008. Individual crypt genetic heterogeneity and the origin of metaplastic glandular epithelium in human Barrett's oesophagus. *Gut* **57:** 1041–1048.
35. Nicholson, A.M., T.A. Graham, A. Simpson, *et al.* 2012. Barrett's metaplasia glands are clonal, contain multiple stem cells and share a common squamous progenitor. *Gut* **61:** 1380–1389.
36. Senoo, M., F. Pinta, C.P. Crum, *et al.* 2007. p63 is essential for the proliferative potential of stem cells in stratified epithelia. *Cell* **129:** 523–536.
37. Wang, X., H. Ouyang, Y. Yamamoto, *et al.* 2011. Residual embryonic cells as precursors of a Barrett's-like metaplasia. *Cell* **145:** 1023–1035.
38. Hamilton, S.R. & J.H. Yardley. 1977. Regeneration of cardiac type mucosa and acquisition of Barrett mucosa after esophagogastrostomy. *Gastroenterology* **72:** 669–675.
39. Tosh, D. & J.M. Slack. 2002. How cells change their phenotype. *Nat. Rev. Mol. Cell Biol.* **3:** 187–194.
40. Morrison, S.J. & A.C. Spradling. 2008. Stem cells and niches: mechanisms that promote stem cells maintenance throughout life. *Cell* **132:** 598–611.
41. Seery, J.P. 2002. Stem cells of the oesophageal epithelium. *J. Cell Sci.* **115:** 1783–1789.

42. Seery, J.P. & F.M. Watt. 2000. Asymmetric stem-cell divisions define the architecture of human oesophageal epithelium. *Curr. Biol.* **10:** 1447–1450.
43. Ahnen, D.J., R. Poulsom, G.W. Stamp, *et al.* 1994. The ulceration-associated cell lineage (UACL) reiterates the Brunner's gland differentiation program but acquires the proliferative organization of the gastric gland. *J. Pathol.* **173:** 317–326.
44. Barbera, M. & R.C. Fitzgerald. 2010. Cellular origin of Barrett's metaplasia and oesophageal stem cells. *Biochem. Soc. Trans.* **38:** 370–373.
45. Sarosi, G., G. Brown & K. Jaiswal. Bone marrow progenitor cells contribute to esophageal regeneration and metaplasia in a rat model of Barrett's esophagus. *Dis. Esophagus* **21:** 43–50.
46. Hopwood, D., G. Milne & K.R. Logan. 1979. Electron microscopic changes in human oesophageal epithelium in oesophagitis. *J. Pathol.* **129:** 161–167.
47. Pope, C.E.I. 1978. Gastroesophageal reflux disease (reflux esophagitis). In *Gastrointestinal Disease: Pathophysiology, Diagnosis, Management.* M.H. Sleisenger & J.S. Fordtran, Eds.: 541–568. 2nd ed. Philadelphia: Saunders.
48. Tobey, N.A., J.L. Carson, R.A. Alkiek & R.C. Orlando. 1996. Dilated intercellular spaces: a morphological feature of acid reflux–damaged human esophageal epithelium. *Gastroenterology* **111:** 1200–1205.
49. Zentilin, P., V. Savarino, L. Mastracci, *et al.* 2005. Reassessment of the diagnostic value of histology in patients with GERD, using multiple biopsy sites and an appropriate control group. *Am. J. Gastroenterol.* **100:** 2299–306.
50. Dent, J. 2007. Microscopic esophageal mucosal injury in nonerosive reflux disease. *Clin. Gastroenterol. Hepatol.* **5:** 4–16.
51. Bove, M., M. Vieth, F. Dombrowski, *et al.* 2005. Acid challenge to the human esophageal mucosa: effects on epithelial architecture in health and disease. *Dig. Dis. Sci.* **50:** 1488–1496.
52. Farré, R., H. van Malenstein, R. De Vos, *et al.* 2008. Short exposure of oesophageal mucosa to bile acids, both in acidic and weakly acidic conditions, can impair mucosal integrity and provoke dilated intercellular spaces. *Gut* **57:** 1366–1374.
53. Tadiparthi, R.A., A. Bansal, S. Wani, *et al.* 2011. Dilated intercellular spaces and lymphocytes on biopsy relate to symptoms in erosive GERD but not NERD. *Aliment Pharmacol. Ther.* **33:** 1202–1208.
54. Vaezi, M.F., J.C. Slaughter, B.S. Smith, *et al.* 2010. Dilated intercellular space in chronic laryngitis and gastro-oesophageal reflux disease: at baseline and post-lansoprazole therapy. *Aliment Pharmacol. Ther.* **32:** 916–924.
55. Konturek, P.C., T. Brzozowski & S.J. Konturek. 2011. Gut clock: implication of circadian rhythms in the gastrointestinal tract. *J. Physiol. Pharmacol.: Off. J. Polish Physiol. Soc.* **62:** 139–150.
56. Pohl, H. & H.G. Welch. 2005. The role of overdiagnosis and reclassification in the marked increase of esophageal adenocarcinoma incidence. *J. Natl. Cancer Inst.* **97:** 142–146.
57. Patrick, L. 2011. Gastroesophageal reflux disease (GERD): a review of conventional and alternative treatments. *Altern. Med. Rev.* **16:** 116–133.
58. Allende, D.S. & L.M. Yerian. 2009. Diagnosing gastroesophageal reflux disease: the pathologist's perspective. *Adv. Anat. Pathol.* **16:** 161–165.
59. Neumann, H. *et al.* 2011. Intercellular space volume is mainly increased in the basal layer of esophageal squamous epithelium in patients with GERD. *Dig. Dis. Sci.* **56:** 1404–1411.
60. Yerian, L. *et al.* 2011. Refinement and reproducibility of histologic criteria for the assessment of microscopic lesions in patients with gastroesophageal reflux disease: the Esohisto Project. *Dig. Dis. Sci.* **56:** 2656–2665.
61. Farre, R. *et al.* 2007. Critical role of stress in increased oesophageal mucosa permeability and dilated intercellular spaces. *Gut* **56:** 1191–1197.
62. Souza, R.F. *et al.* 2009. Gastroesophageal reflux might cause esophagitis through a cytokine-mediated mechanism rather than caustic acid injury. *Gastroenterology* **137:** 1776–1784.
63. Sridhara, S. *et al.* 2012. Increased numbers of eosinophils, rather than only etiology, predict histologic changes in patients with esophageal eosinophilia. *Clin. Gastroenterol. Hepatol.* **10:** 735–741.
64. Rubio, C.A. & B. Aberg. 1991. Further studies on the musculo-fibrous anomaly of the Barrett's mucosa in esophageal carcinomas. *Pathol. Res. Pract.* **187:** 1009–1013.
65. Liu, L., W.L. Hofstetter, A. Rashid, *et al.* 2005. Significance of the depth of tumor invasion and lymph node metastasis in superficially invasive (T1) esophageal adenocarcinoma. *Am. J. Surg. Pathol.* **29:** 1079–1085.
66. Dorer, R. & R.D. Odze. 2006. AMACR immunostaining is useful in detecting dysplastic epithelium in Barrett's esophagus, ulcerative colitis, and Crohn's disease. *Am. J. Surg. Pathol.* **30:** 871–877.
67. Lisovsky, M., O. Falkowski & T. Bhuiya. 2006. Expression of alpha-methyl-coenzyme A racemose in dysplastic Barrett's epithelium. *Hum. Pathol.* **37:** 1601–1606.
68. Scheil-Bertram, S., D. Lorenz, C. Ell, *et al.* 2008. Expression of α-methylacyl coenzyme A racemose in the dysplasia carcinoma sequence associated with Barrett's esophagus. *Mod. Pathol.* **21:** 961–967.
69. Skacel, M., R.E. Petras, L.A. Rybicki, *et al.* 2002. p53 expression in low grade dysplasia in Barrett's esophagus: correlation with interobserver agreement and disease progression. *Am. J. Gastroenterol.* **97:** 2508–2513.
70. Kaye, P.V., S.A. Haider, M. Ilyas, *et al.* 2009. Barrett's dysplasia and the Vienna classification: reproducibility, prediction of progression and impact of consensus reporting and p53 immunohistochemistry. *Histopathology* **54:** 699–712.
71. Sarbia, M., J. Arjumand, M. Wolter, *et al.* 2001. Frequent c-myc amplification in high-grade dysplasia and adenocarcinoma in Barrett esophagus. *Am. J. Clin. Pathol.* **115:** 835–840.
72. Chinyama, C.N., R.E.K. Marshall, W.J. Owen, *et al.* 1999. Expression on MUC1 and MUC2 mucin gene products in Barrett's metaplasia, dysplasia and adenocarcinoma: an immunopathological study with clinical correlation. *Histopathology* **35:** 517–524.

73. Wang, J.S., M. Guo, E.A. Montgomery, *et al.* 2009. DNA promoter hypermethylation of p16 and APC predicts neoplastic progression in Barrett's esophagus. *Am. J. Gastroenterol.* **104:** 2153–2160.
74. Flejou, J.-F. 2005. Barrett's oesophagus: from metaplasia to dysplasia and cancer. *Gut* **54**(Suppl I): i6–i12.
75. Younes, M., R.M. Lebovitz, L.V. Lechago, *et al.* 1993. p53 protein accumulation in Barrett's metaplasia, dysplasia, and carcinoma: a follow-up study. *Gastroenterology* **105:** 1637–1642.
76. van Dekken, H., W.C. Hop, H.W. Tilanus, *et al.* 2008. Immunohistochemical evaluation of a panel of tumor cell markers during malignant progression in Barrett esophagus. *Am. J. Clin. Pathol.* **130:** 745–753.
77. Sonwalkar, S.A., O. Rotimi, N. Scott, *et al.* 2010. A study of indefinite for dysplasia in Barrett's oesophagus: reproducibility of diagnosis, clinical outcomes and predicting progression with AMACR (alpha-methylacyl-CoA-racemase). *Histopathology* **56:** 900–907.
78. Roesly, H.B., M.R. Khan, H.D. Chen, *et al.* 2012. The decreased expression of Beclin-1 correlates with progression to esophageal adenocarcinoma: the role of deoxycholic acid. *Am. J. Physiol. Gastrointest. Liver Physiol.* **302:** G864–G872.
79. Chawengsaksophak, K., R. James, V.E. Hammond, *et al.* 1997. Homeosis and intestinal tumours in Cdx2 mutant mice. *Nature* **386:** 84–87.
80. Gao, N., P. White & K.H. Kaestner. 2009. Establishment of intestinal identity and epithelial-mesenchymal signaling by Cdx2. *Development. Cell* **16:** 588–599.
81. Mutoh, H., Y. Hakamata, K. Sato, *et al.* 2002. Conversion of gastric mucosa to intestinal metaplasia in Cdx2-expressing transgenic mice. *Biochem. Biophys. Res. Commun.* **294:** 470–479.
82. Phillips, R.W., H.F. Frierson, Jr., C.A. Moskaluk. 2003. Cdx2 as a marker of epithelial intestinal differentiation in the esophagus. *Am. J. Surg. Pathol.* **27:** 1442–1447.
83. Eda, A., H. Osawa, K. Satoh, *et al.* 2003. Aberrant expression of CDX2 in Barrett's epithelium and inflammatory esophageal mucosa. *J. Gastroenterol.* **38:** 14–22.
84. Moons, L.M., D.A. Bax, E.J. Kuipers, *et al.* 2004. The homeodomain protein CDX2 is an early marker of Barrett's oesophagus. *J. Clin. Pathol.* **57:** 1063–1068.
85. Kazumori, H., S. Ishihara, M.A. Rumi, *et al.* 2006. Bile acids directly augment caudal related homeobox gene Cdx2 expression in oesophageal keratinocytes in Barrett's epithelium. *Gut* **55:** 16–25.
86. Wong, N.A., J. Wilding, S. Bartlett, *et al.* 2005. CDX1 is an important molecular mediator of Barrett's metaplasia. *Proc. Natl. Acad. Sci. USA* **102:** 7565–7570.
87. Kong, J., M.A. Crissey, S. Funakoshi, *et al.* 2011. Ectopic Cdx2 expression in murine esophagus models an intermediate stage in the emergence of Barrett's esophagus. *PLoS One.* **6:** e18280.
88. di Pietro, M., P. Lao-Sirieix, S. Boyle, *et al.* 2012. Evidence for a functional role of epigenetically regulated midcluster HOXB genes in the development of Barrett esophagus. *Proc. Natl. Acad. Sci. USA* **109:** 9077–9082.
89. Chidgey, M.A. 1997. Desmosomes and disease. *Histol. Histopathol.* **12:** 1159–1168.
90. Wex, T., K. Mönkemüller, A. Stahr, *et al.* 2012. Gastro-oesophageal reflux disease is associated with up-regulation of desmosomal components in oesophageal mucosa. *Histopathology.* **60:** 405–415.
91. Wex, T., D. Kuester, K. Mönkemüller, *et al.* 2012. Assessment of desmosomal components (desmoglein 1–3, plakoglobin) in cardia mucosa in relation to gastroesophageal reflux disease and *Helicobacter pylori* infection. *Hum. Pathol.* **43:** 1745–1754.
92. Kilpatrick, D.C. 2002. Animal lectins: a historical introduction and overview. *Biochim. Biophys. Acta.* **1572:** 187–197.
93. Poorkhalkali, N., I. Jacobson & H.F. Helander. 1999. Lectin histochemistry of the esophagus in several mammalian species. *Anat. Embryol. (Berl.).* **200:** 541–549.
94. Neumann, H., T. Wex, M. Vieth, *et al.* 2007. Gastroesophageal reflux disease leads to major alterations in lectin-binding in the columnar epithelium of the gastroesophageal junction. *Scand. J. Gastroenterol.* **42:** 791–798.
95. Neumann, H., T. Wex, K. Mönkemüller, *et al.* 2008. Lectin UEA-I-binding proteins are specifically increased in the squamous epithelium of patients with Barrett's esophagus. *Digestion* **78:** 201–207.
96. Bird-Lieberman, E.L., A.A. Neves, P. Lao-Sirieix, *et al.* 2012. Molecular imaging using fluorescent lectins permits rapid endoscopic identification of dysplasia in Barrett's esophagus. *Nat. Med.* **18:** 315–321.