CDX2 ALONE PLAYS AN IMPORTANT ROLE IN EPITHELIAL PHENOTYPIC CHANGES IN BARRETT'S METAPLASIA IN THE ESOPHAGUS, AND ESOPHAGEAL SUBMUCOSAL GLANDS SEEM TO BE THE PREDOMINANT CELL OF ORIGIN FOR BARRETT'S ESOPHAGUS

By

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As members of the Master's Committee, we certify that we have read the thesis prepared by *Alma Banuelos*, titled *CDX2 ALONE PLAYS AN IMPORTANT ROLE IN EPITHELIAL PHENOTYPIC CHANGES IN BARRETT'S METAPLASIA IN THE ESOPHAGUS, AND ESOPHAGEAL SUBMUCOSAL GLANDS SEEM TO BE THE PREDOMINANT CELL OF ORIGIN FOR BARRETT'S ESOPHAGUS* and recommend that it be accepted as fulfilling the thesis requirement for the Master's Degree.

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Final approval and acceptance of this thesis is contingent upon the candidate's submission of the final copies of the thesis to the Graduate College.

We hereby certify that we have read this thesis prepared under our direction and recommend that it be accepted as fulfilling the Master's requirement.

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Abstract

Gastroesophageal Reflux Disease (GERD) is a long-known disorder caused by back flow of acid (also known as acid reflux) from the stomach into the esophagus. A patient with GERD may develop Barrett's Esophagus and have increased risk of developing esophageal adenocarcinoma, a potentially fatal cancer of the esophagus with a low chance of survival. In esophageal research, there are two fields of study regarding Barrett's esophagus. There is the field intent on understanding the pathogenesis of the change from the normal squamous lining of the esophagus to Barrett's esophagus and the second field concerns the transition from Barrett's esophagus to adenocarcinoma. This thesis explores the first question. Upon evaluation of the current literature, the cell-of-origin and transcription factors involved highly support the hypothesis that a submucosal cell in the esophagus and CDX2 an intestinal transcription factor alone are responsible for Barrett's disease. CDX2 is normally present in intestinal epithelium that plays a role in maintaining an intestinal epithelial phenotype. By improving treatment options, therapeutic treatments or surveillance for patients (similar to colorectal cancer), we hope to be able to lower the incidence of Barrett's or adenocarcinoma.

Introduction

Patients with chronic GERD symptoms often present with a “burning sensation in the chest” and “difficulty swallowing” that results from the regurgitation of food. This burning sensation and regurgitation of food masks a chronic inflammation in the esophagus that often leads to the development Barrett’s Esophagus, in which the typical stratified squamous epithelium is replaced with simple columnar epithelium. The molecular mechanisms underlying the metaplastic change in the epithelial phenotype in Barrett’s esophagus, and the identity of the cell type that gives rise to the columnar cells in Barrett’s esophagus are a topic of active investigation. Esophageal research has focused on understanding the molecular pathways and transcription factors, including CDX1 and CDX2, that are responsible for the development of Barrett’s. In addition, research has attempted to identify the cell-of-origin from which cells are re-programmed to cause squamous epithelium to change into intestinal columnar cells. Four theories for the cell of origin have been proposed including native squamous cells of the esophagus, submucosal glands of the esophagus, circulating bone marrow-derived cells, and direct extension of gastric epithelial cells. Most recent data support an innate progenitor cell unique to the squamocolumnar junction that can expand into metaplastic glands. In order to understand this disease, we must first understand the histology and anatomy of the gastrointestinal tract that includes the esophagus (stratified squamous epithelium), stomach consisting of the gastroesophageal junction (gastric pits with columnar epithelium), and small intestine (columnar intestinal epithelium) that contains CDX2 in the villus differentiated cells. Upon understanding the normal anatomy, we can compare it to pathological findings in Barrett’s esophagus where the epithelium resembles the small intestine.

Anatomy of the Upper Gastrointestinal Tract

Esophagus

The esophagus is part of the digestive system that transports food and water but does not have any absorptive or digestive function. The esophagus originates in the neck region at the hypopharynx and passes through the chest cavity to the stomach. The esophagus is made of four distinctive layers as illustrated in Figure 1.

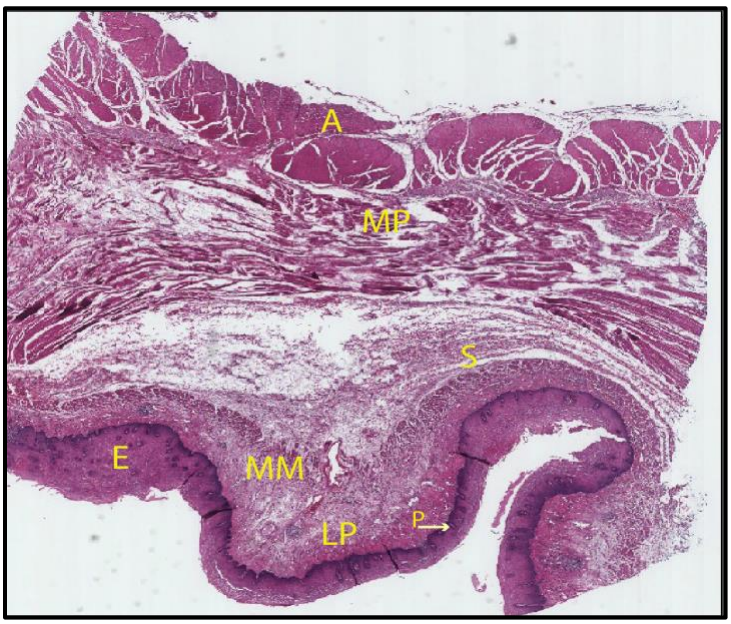


***Figure 1*. The 4 Layers of the Esophagus.** The mucosa [including the Epithelium (E), Lamina Propria (LP), Papilla (P) and Muscularis Mucosa (MM)], Submucosa (S), Muscularis Propria (MP), & Adventitia (A). Adapted from the University of Arizona Pathology Image Collection by Aperio.

Starting from the lumen, the first layer is called the mucosa, which is composed of a nonkeratinized stratified squamous epithelium (Fig. 1) supported by the lamina propria that comes into epithelium to form a papilla in basal layer (Fig.1P & Fig.2P), made up of connective tissue, and the muscularis mucosae (MM), a thin muscular layer at the base of the mucosa. According to

Histology: A Text and Atlas: with correlated Cell and Molecular Biology, the basal layer of the epithelium contains basal cells with high nuclear-cytoplasmic ratio. Lamina muscularis mucosae (MM) separates the mucosa from the submucosa and contains smooth muscle. The submucosa (S) consists of dense irregular connective tissue, lymphatic vessels containing focal lymphocytes, nerve fibers, seromucous glands and ganglion cells. The muscularis externa or muscularis propria follows, consisting of two muscle layers, an inner circular layer and outer longitudinal layer that serve an important role by responding to the enteric nervous system to produce contractions known as peristalsis (Ross & Pawlina, 2016). The muscularis externa in the esophagus is different than the rest of the digestive system in that it is divided into the upper, middle, and lower portions. The upper segment is made of skeletal muscle (voluntary contractions associated with swallowing), the middle is mixed smooth and skeletal muscle, and lower is made entirely of smooth muscle (involuntary contractions of peristalsis to move food into the stomach). Finally, the outermost layer of the esophagus is the adventitia (loose connective tissue) that contains no mesothelial covering until it passes below the diaphragm, where it becomes a serosa (smooth mesothelium).

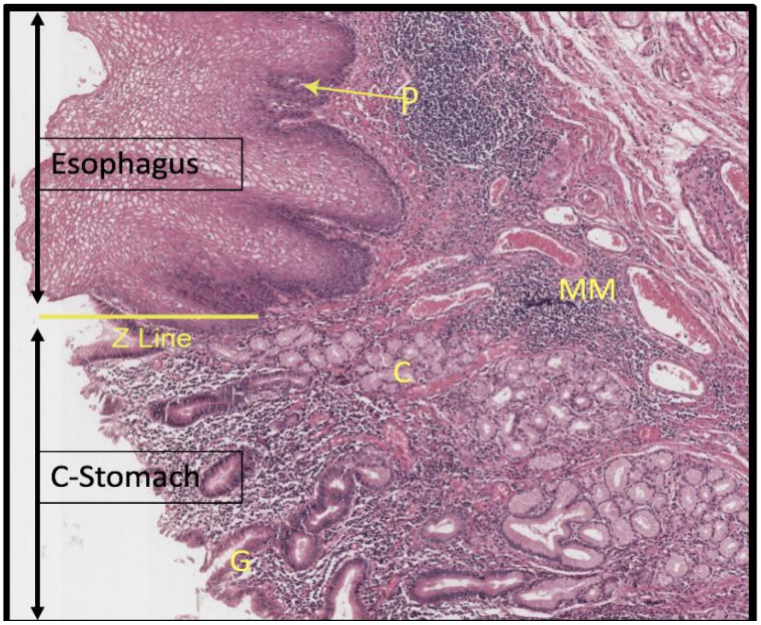


***Figure 2*. Gastroesophageal Junction.** Normal histology illustrating the transition zone also known as the Z line, Labels: Cardiac of the Stomach (C-Stomach), Gastric glands (G), Papilla (P), Muscularis Mucosea (MM), Cardiac Glands (C). Adapted from the University of Arizona Pathology Image Collection by Aperio.

At the distal end of the healthy esophagus, there is a transition zone from stratified squamous epithelium to columnar gastric mucosa. This transition zone is known as the Z line, which overlaps with the gastroesophageal junction (GEJ) in the healthy gut (Figure 2). The Z line is easily identified by color change from pale to deep red and texture change from smooth to rugose (Floch & Netter, 2010). Contractions of muscles around the GEJ prevent retrograde flow of gastric contents into the lower esophagus while allowing deposition of the food bolus from the esophagus into the stomach. These muscles typically serve as a functional, but not an anatomically definitive sphincter (*e.g.*, not like the pyloric sphincter), known as the lower esophageal sphincter (LES). Their contraction creates a pressure differential between the esophagus and stomach that prevents reflux of gastric contents (Boeckxstaens, 2005). The muscular sphincter components function with coordinated relaxation and contraction. This action of the sphincter can be observed during

swallowing as it relaxes and then tonically closes to prevent symptoms of reflux and regurgitation. Failure of this sphincter-like mechanism results in the symptoms of GERD. According to *Netter's Gastroenterology*, the anti-reflux mechanism depends on the proper function of the esophageal and diaphragm muscle combination that makes up the LES, and the stomach. Reflux develops when LES pressure drops, as with enlargement of the stomach, which shortens the LES length. Medical treatment cannot (at this time) resolve abnormal LES function. Therefore, medical treatment of GERD centers on suppression of acid secretion in the stomach (Floch & Netter, 2010).

Stomach

The stomach is part of the digestive system that functions to digest food through the secretion of acid, producing a pulpy fluid mix called chyme (Ross & Pawlina, 2016). Similar to more distal parts of the digestive tract, from the lumen out it contains a mucosa made of simple columnar epithelial cells with gastric glands (G), lamina propria (LP), and muscularis mucosea (MM) as shown in Figure 2. The stomach is divided into three regions based on the type of glands that it contains; these regions are called the cardiac, fundus, and pylorus. As shown in Figure 2, the first part of the stomach is the cardiac region; it contains cardiac glands that contain mucus-secreting cells. It also contains gastric glands (that contain large pits that secrete mucus), parietal cells, and chief cells. Parietal cells secrete hydrochloric acid (HCL) whereas chief cells secrete digestive enzymes such as pepsin.

Small Intestine

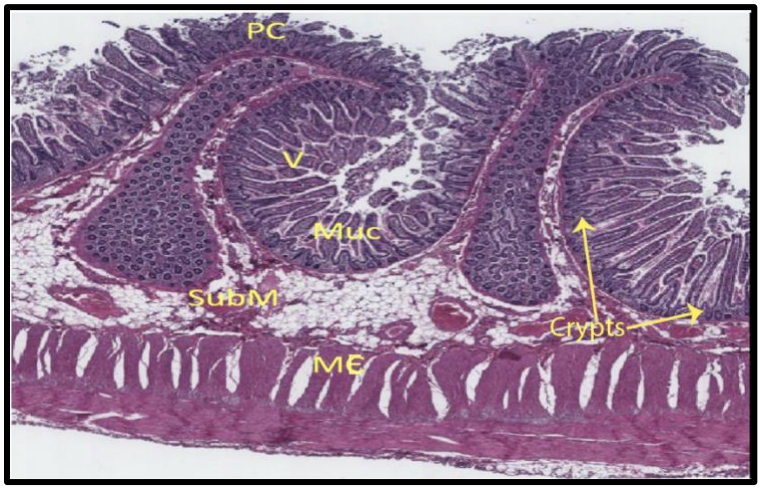


***Figure 3*. Section of small intestine of the mouse**. Labels: PC (plicea circularies), mucosa (Muc), villi (V), submucosa (Subm), crypts of Lieberkuhn (Crypts). Adapted from the University of Arizona Pathology Image Collection by Aperio.

The small intestine is the main site for digestion and absorption of food. It is the longest component of the digestive system measuring over 6m in humans (Ross & Pawlina, 2016). It is divided into three segments: duodenum, jejunum, and ileum. Figure 3 shows a micrograph from a section of slide of the jejunum. The jejunum is the principal site of absorption of nutrients in the small intestine. It consists of villi (V) that are finger-like projections that are covered with largely-absorptive columnar epithelial cells (enterocytes) and goblet cells. The circular folds are called plicae circularies (PC) that contains the mucosa (MuC) and submucosa (SubM) layers. The crypts of Lieberkuhn are tubular glands composed of paneth cells, stem cells, and endocrine cells. The tissue on the lower edge (in this image) is the muscularis externa (ME).

Symptoms of GERD

Typical symptoms of GERD include epigastric abdominal pain, dysphagia, nausea and bloating. At least 24% of the adult population in the United States has heartburn or regurgitation at least once a week (Malfertheiner & Hallerback, 2005). According to the American Gastroenterological Association, the average person who presents with GERD are over the age of 50, from Caucasian genetics, and often overweight (Spechler et al., 2011). When GERD is chronic, complications may include peptic esophageal erosion, ulceration, strictures (narrowing of the lumen), Barrett esophagus and esophageal adenocarcinoma (Philip et al., 2013). Endoscopy is the preferred method to diagnose reflux and to obtain a biopsy sample of the esophagus to rule out or diagnose Barrett's esophagus or cancer.

Finding the Molecular Pathogenesis of Barrett's Esophagus

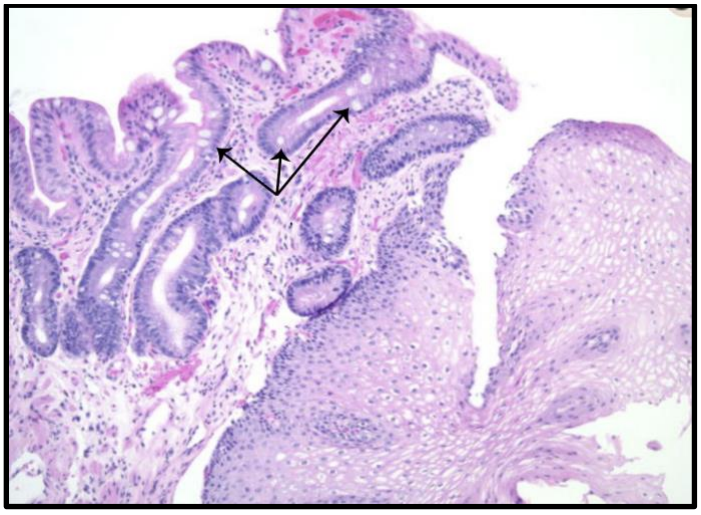


Figure 4. **Nondysplastic Barrett's esophagus.** Arrows: Goblet cells present in esophagus with intestinal columnar epithelium. Reprinted from *Techniques in Gastrointestinal Endoscopy*, *12*, Atkinson and Amitabh, Screening for Barrett's esophagus, 62-66, Copyright (2010), with permission from Elsevier.

Barrett's esophagus was named after Norman Barrett, a surgeon who noticed a change in the lining of the esophagus that appeared as a salmon color (Barrett, 1950). In Barrett's esophagus the Z line is no longer in the same area as the GEJ, rather it has moved up the esophagus while in comparison GEJ remains in the same area. According to the American College of Gastroenterology, Barrett's esophagus is defined as a columnar epithelium with goblet cells extending ≥1cm above the top of the gastric folds (Shaheen et al., 2016). Goblet cells have distinct mucous vacuoles that stain pale blue by H&E (*Robbins Basic Pathology*; Fig 4). "All four intestinal cell types; enterocytes, goblet cells, paneth cells and enteroendocrine cells; can be present in BM" (Colleypriest, Palmer, Ward, & Tosh, 2009, p.313). Barrett's esophagus treatment consists of proton pump therapy and endoscopic eradication therapy resection for Barrett's esophagus with dysplasia (Naini et al., 2016). Dysplasia is an abnormal growth of cells that have not passed the basement membrane. Dysplasia in the esophagus can be classified as low- or high-grade dysplasia similar to inflammatory bowel disease classification system of epithelial abnormalities (Riddell et al., 1983). The classification system in low-grade dysplasia, the crypt architecture is intact consisting of enlargement of nuclei, with crowding of cells, and usually low numbers of goblet cells (Haggitt, 1994). In high grade dysplasia, there is loss of normal crypt architecture with irregular shapes of the crypts, crowding "back-to-back," and loss of cell polarity with loss of goblet and mucus cells (Burke, Sobin, Shekitka, & Helwig, 1991). High grade dysplasia increases the risk of adenocarcinoma. Patients with esophageal adenocarcinoma have a 5-year overall survival rate of less than 20% (Le Bras, Farooq, Falk, & Andl, 2016). In summary, the area of esophageal research specifically relating to Barrett's esophagus is complex and therefore understanding the pathogenesis of Barrett's could help create better therapeutic drugs for patients, increase their survival rate and prevent progression into adenocarcinoma.

Transdifferentiation vs Transcommitment

The progression to Barrett's esophagus develops through the process of metaplasia, where one differentiated tissue type replaces another due to chronic GERD, although the molecular events that take place are still unclear. According to Jankowski, Harrison, Perry, Balkwill, and Tselepsis (2000), the ultimate drivers of Barrett's metaplasia are likely to be transcription factors, which alter the differentiation of stem cells in the normal esophageal epithelium via transcommitment, cellular reprogramming of stem cells; or transdifferentiation a conversion of one differentiated cell type to another without undergoing mitosis (Shen, Burke, & Tosh, 2004).

Transdifferentiation is the circumstance where there is a direct conversion from squamous to intestinal cell types. Reasons to support this argument include the observation of a multilayered epithelium first hypothesized by Shields, et al. (1993). They saw a transition zone epithelium in patients with Barrett's esophagus that contained both squamous and columnar epithelial features with at least four to eight layers. According to *Blaustein's Pathology of the Female Genital Tract:* these cell layers were similar to features seen in the cervical transformation zone where cells are columnar and change into squamous cell metaplasia (Kurman, 1987). Similarly, Glickman et al. (2001) obtained esophageal mucosal biopsies containing multilayered epithelia with Barrett's esophagus to understand the origin of multilayered epithelium by analyzing mucin histochemical and immunophenotypic characteristics. Glickman et al. (2001) observed mature columnar epithelium with cytoskeletal structural proteins cytokeratin 8/8(CK8/18) by immunohistochemical staining in the superficial layers. These mature columnar epithelial cells contained characteristics similar to the columnar epithelium in Barrett's esophagus while the basal cells contained mature squamous cytokeratin (CK13). This illustrates that the cells did not fully transdifferentiate into intestinal cell types, but rather they might have been in an intermediate stage of conversion to a

columnar phenotype (Glickman et al., 2001). Although a multilayered epithelium has been observed in patients with Barrett's esophagus, there has been no full phenotypic conversion of squamous to intestinal-type columnar epithelium as seen in Barrett's esophagus in cultured cells *in vitro.* The multilayered epithelium might contribute to the pathogenesis in Barrett's but is not the main factor because they represent cells that are likely undergoing transdifferentiation. This may be an intermediate step in the conversion to Barrett's metaplasia but the mature Barrett's does not display multilayered cells. According to Jankowski et al. (2000), cells lining the esophagus will die shortly (about 11 days), including the multilayered cells, leaving only squamous progenitor cells which should make more squamous cells. This is a drawback of transdifferentation. In order to maintain Barrett's epithelium, the squamous basal cell progenitors would have to switch from generating one cell type to another and this switching of progenitors is what is found in transcommitment.

In contrast to transdifferentiation, transcommitment has stronger evidence for playing a role in the development of Barrett's esophagus. Transcommitment is the idea that for cells to change phenotype, they must go through cellular reprogramming and cell division (*e.g.* from stem cells/progenitor cells). If transcommitment is the mechanism, then one of the big questions in esophageal research is where these cells are coming from that undergo these cellular changes in patients with GERD. Four general hypotheses for Barrett's esophagus cell of origin have been proposed. Figure 5, summarizes the research indicating the proposed cell-of-origin coming from: 1) the native esophageal squamous epithelium (Yu et al., 2005; Giroux et al., 2017); 2) native epithelium from esophageal submucosal glands, or their ducts (Gillen, Keeling, Bryne, West, & Hennessy, 1988; Owen et al., 2018); 3) epithelium found at the squamocolumnar junction that can migrate (from Wang et al., 2011; Quante et al., 2012; Jiang et al., 2017); 4) and circulating bone

marrow cells (Epperly et al., 2004).

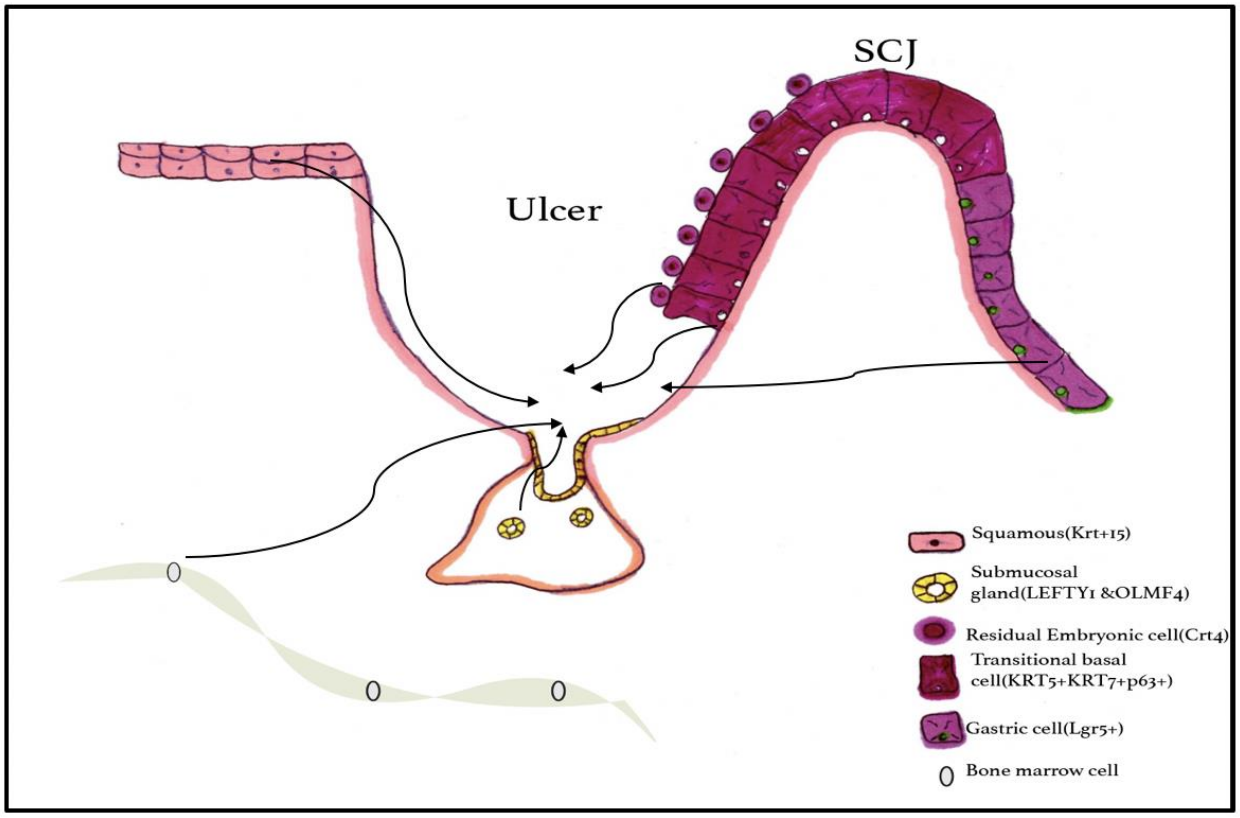


Figure 5. **Cells of Origin.** Chronic injury results in ulceration of esophageal epithelium. The four hypothesis for the cell-of-origin include: bone marrow stem cells, esophageal squamous cells, esophageal submucosal gland cells, and gastroesophageal junction/gastric cells that include the residual embryonic stem cell and transitional basal cells migrating to the area of injury to induce a change in the phenotype from squamous epithelium to intestinal metaplasia. Original drawing by Banuelos.

Circulating Bone Marrow-Derived Stem Cells

One potential cell-of-origin for Barrett's esophagus is a circulating bone marrow-derived stem cell. In the study by Sarosi et al. (2008), bone marrow-derived cells played an important role in the formation of esophageal columnar metaplasia. Their experiment consisted of inducing reflux esophagitis in female rats by esophagojejunostomy and then doing whole body irradiation. Once

the female rats had no bone marrow cells they were then tail-vein injected with bone marrow cells from male rats. After eight weeks, it was observed that female rats that received bone marrow transplants contained Y chromosomes observed through fluorescence *in situ* hybridization (FISH) in both the squamous cells and metaplastic columnar cells of the esophagus. This experiment supported the notion of circulating bone marrow stem cells are sufficient to produce metaplasia because the female rats had Y chromosome in cells in the esophagus. Similarly, Hutchinson, et al. (2010), observed that a male patient with acute myeloid leukemia who received a bone marrow transplant from his sister had developed esophageal carcinoma that showed (through FISH) XX carcinoma cells. This study showed that a male patient had been carrying two X chromosomes that had come from his female donor (origin from bone marrow stem cells) suggesting that these cells may be playing an important role in esophageal adenocarcinoma development, presumably from Barrett's esophagus. From these observations it can be inferred that bone marrow stem cells might be a cell of origin for the columnar epithelium. But there is an argument against this hypothesis; bone marrow stem cells did not give rise to complete glands such as those present in Barrett's esophagus (Wang & Souza, 2016). The bone marrow derived stem cells were not completely present in all of the glands of Barrett's metaplasia only in some glands. In summary, the patient and animal models both show that they exhausted the esophageal cells or nearby resident cells therefore needed to send out signals to repair the site of injury therefore recruiting bone marrow derived stem cells from his sister.

A Native Esophageal Progenitor Cell

The native esophageal progenitor cell is also a candidate for the cell of origin in the development of Barrett's esophagus. The esophagus starts off during the first seven weeks in humans as pseudostratified columnar epithelium (De Hertogh et al., 2005). By 8 weeks gestational

age, the esophageal epithelium has become ciliated and simple columnar. In the 5th month of gestation the epithelium changes from columnar to squamous near mid-esophagus. Following this (around 5 months), the esophagus is stratified squamous epithelium (Denardi & Riddell, 1991). This developmental argument supports the notion that the full phenotype of squamous in adults can be reprogrammed back to its original esophageal stem cell as seen in the fetus since they are presumed to be from the same cell lineage and as fetal cells they were capable of forming columnar epithelial cells. Therefore, adult stem cells may regain this capability given the appropriate environment.

Similarly, in cultured mouse esophagus there is also a transition from simple columnar in basal layer to stratified squamous (Yu, Slack, & Tosh, 2005). This is illustrated in Figure 6, where cytokeratin 8 and 18 (K8, K18), type I acidic proteins that form keratin intermediate filaments, were markers for stratified columnar epithelium and were highly expressed at the beginning of culture and then decreased over next several days (Yu et al., 2005). After a few days (day 5 to day 9 of culture), cytokeratin 14 (K14) was observed in the basal layer. At approximately 15 days of culture K8 was no longer present in the basal nor suprabasal layers of the epithelium consisting only of stratified squamous epithelium expressing K14. In comparison to *in vitro* experiments, the two-month mouse *in vivo* also did not have expression of K8 in neither the basal nor suprabasal layers of the epithelium. Thus by using cytokeratins as markers, they were able to demonstrate that this process happened through transdifferentiation, changing stratified columnar into squamous epithelium. It was also noted that these cells did not undergo programmed cell death and cell division inhibitors did not affect the cell division process, therefore, cell proliferation did not affect the conversion process (Yu et al., 2005). This study illustrates that stratified columnar epithelium is able to undergo direct conversion from columnar to stratified squamous epithelium, providing

inductive support for the hypothesis that esophageal progenitor cells can be the cell of origin in Barrett's esophagus by transdifferentation. Nevertheless, it can be argued that this is a maturation or differentiation event where columnar cells are the precursors of squamous cells rather than transdifferentation actually occurring (Yu et al., 2005). This argument of being part of the maturation process rather than transdifferentation weakens the support of esophageal progenitor stem cells in adults being the cell-of-origin as it is being compared to fetal development where the molecular mechanisms might not be the same. Adult stem cells in the basal layer of the esophagus can be assumed to be able to revert back to their ability to produce cells as seen during development, but it cannot be presumed, that adult stem cells will revert back to their original state in the same way that fetal stem cells do just because an *in vitro* experiment produced cells that had a change in appearance.

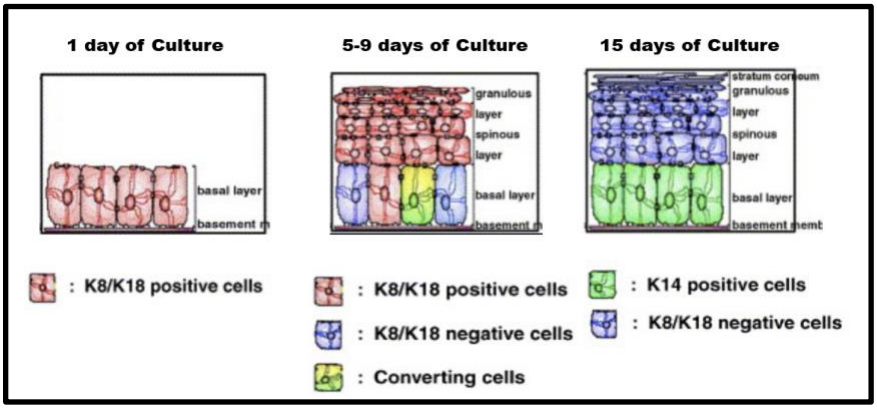


Figure 6. **Results from Exp on stratified squamous in vitro culture from day 1 to 15.** Day 1 of culture with one cell layer thick columnar epithelium with markers (K8, K18) to 15 days of culture with fully differentiated stratified squamous cells expressing markers (K14). Adapted from *Developmental Biology*, *1*, Yu et al, Conversion of columnar to stratified squamous epithelium in the developing mouse oesophagus, p.156-170, Copyright (2005), with permission from Elsevier.

Figure 6. Illustrates the basal cells playing an important role in the maturation or transdifferentation into columnar epithelium cells. The basal cells differentiate and contribute to

epithelial renewal as well as "migrate" to the luminal surface (Giroux et al., 2017). In these basal cells it has been observed through genetic *in vivo* lineage tracing of a long-lived progenitor cell that keratin 15 (*KRT15*) expression in basal cells is important for their proliferation and self-renewal. Keratin 15, was first observed in hair follicles that played an important role in wound repair and basal cell carcinoma (Ito et al., 2005). It also been observed at high levels of expression in *KRT15+* ureter cell carcinomas (Tai et al., 2013). In contrast, in the study by Giroux (2017), *KRT15*$^{-}$ basal cells from the esophagus of mice were reduced resulting in decreased proliferation, and atrophy of the esophageal epithelium. Thus, *KRT15* is a necessary cell in self-renewal as well as in tissue regeneration due to radioresistant-induced injury. Although *KRT15+* cells were expressed there could also be other progenitor or stem cell population in the basal layers with self-renewal capabilities that can be compared to the small intestine having more than one progenitor stem cell population (Giroux et al., 2017). Lastly, further investigations are needed to understand how KRT15+ basal cells play a role in the development of Barrett's esophagus through the process of transcommitment or differentiation as there has been no recent literature regarding *KRT15+* basal cells in acid induced injury.

From these observations we can conclude that the basal cells of the esophageal stratified squamous epithelium contain precursor progenitor cells seen in the development of murine esophagus through the expression of cytokeratin markers CK14 and KRT15+. Basal cells can also "migrate" towards the luminal surface and differentiate. Similarly, in the human esophagus, progenitor cells were not limited to the basal layer but rather were widespread with the same phenotype (Barbera et al., 2015). This illustrates that there is cell renewal of progenitor cells. The native esophageal squamous basal progenitor cell type is a candidate for the development of Barrett's esophagus. However, in these studies esophageal submucosal glands were not included,

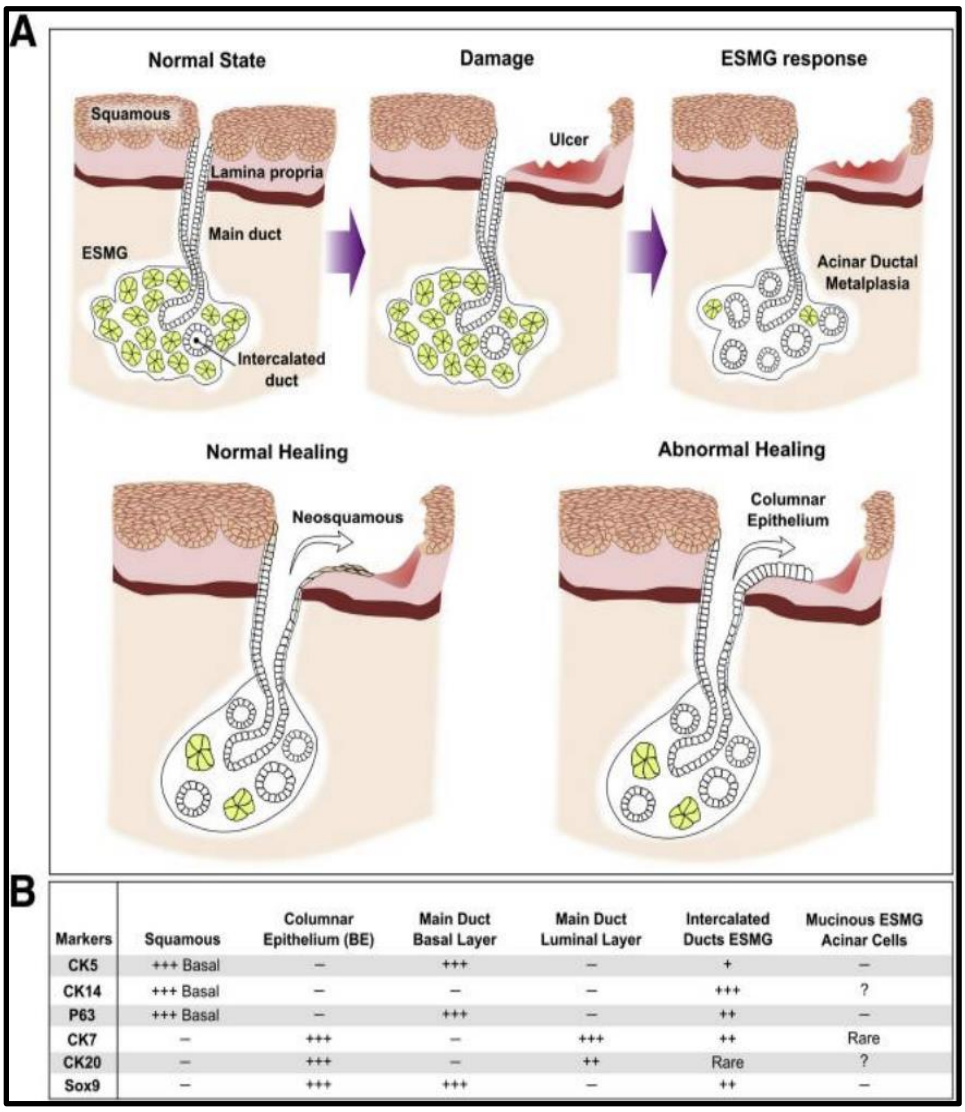
because rodents do not have esophageal submucosal glands, therefore leaving open the possibility that they are also a source for the cell-of-origin for the columnar epithelial cells of Barrett's esophagus.

Submucosal Gland/ Duct *vs.* Gastric Columnar Epithelial Cells

The submucosal gland may also be the origin for Barrett's esophagus. The submucosal glands are located underneath the muscularis mucosa in the esophagus. They are made of clusters of cells known as acini, containing mucous cells, serous cells, and oncotic cells (cell containing many mitochondria). Each acinus is surrounded by a basement membrane and a myoepithelial cell layer (Al Yassin, 1977). Their function is to secrete mucus, growth factors, and bicarbonate (Al Yassin, 1977). Acinar cells can become proliferative and change phenotype in ductal metaplasia due to injury (Garman et al., 2015).

A study by Gillen et al. (1988), proposed that the esophageal mucosal glands were the cell-of-origin and argued against the proposed cell-of-origin being the gastric columnar epithelial cells. They induced reflux in canine esophagus after they surgically removed two regions. First, 2 cm of squamous epithelium in the lower esophagus next to the squamocolumnar junction (Z-line) was removed. Next to the excised region they left a 2cm wide ring of normal squamous epithelium followed by another excision of squamous epithelium. If the gastric columnar epithelial cells were just creeping up into the esophagus, then the lower excised region should fill in and the upper region would serve as a negative control. After healing time, in both excised areas there was re-epithelization of columnar epithelium while the non-excised squamous region stayed the same. This study suggests that that columnar epithelium may develop from submucosal glands (Figure 8A). This study failed to support the hypothesis of "creeping substitution" of gastric columnar epithelium since the excised area proximal to the squamous band in the upper esophagus contained

columnar epithelial cells and this could not easily be explained by migration of cells from the stomach.



Markers	Squamous	Columnar Epithelium (BE)	Main Duct Basal Layer	Main Duct Luminal Layer	Intercalated Ducts ESMG	Mucinous ESMG Acinar Cells
CK5	+++ Basal	–	+++	–	+	–
CK14	+++ Basal	–	–	–	+++	?
P63	+++ Basal	–	+++	–	++	–
CK7	–	+++	–	+++	++	Rare
CK20	–	+++	–	++	Rare	?
Sox9	–	+++	+++	–	++	–

Figure 7. **Esophageal submucosal glands**. (A) Top row shows normal state of epithelium with esophageal submucosal glands (ESMG) below, followed by ulceration at the area of damage resulting in acinar ductal metaplasia within the ESMG. Bottom row: Normal healing where the ESMGs remain normal mucinous acini with neosquamous epithelium. Far right shows abnormal healing with acinar ductal metaplasia in ESMGs and columnar epithelium in the esophagus. (B) Esophageal and Submucosal glands: comparison of shared markers. Reproduced from "Proposed role of ESMGs in normal and abnormal healing of the esophagus, and the markers use to distinguish

The esophageal submucosal glands (ESMG) are shown in Figure 7. This illustrates a proposed role for the ESMG during the development of Barrett's esophagus. In the normal state, the ESMG contain mostly acinar cells with squamous-lined ducts that connect to the squamous epithelium, with only a few intercalated ducts connecting the two. Below the figure lies an illustration of normal *vs* abnormal healing. In normal healing there is continuity of ESMG within the basal layer of esophageal squamous cells where they change from cuboidal ductal cells into a neosquamous epithelium. In contrast, in abnormal healing such as in the case of reflux esophagitis, there is continuity of ESMG ducts to columnar epithelium thus supporting the notion of a ductal source of origin (Garman, 2017). Similarly, Coad et al. (2005), observed in human tissue that esophageal gland ducts released their products onto the lumen of esophageal cells and underneath the squamous islands there was ESMG continuous with the squamous epithelium. In Barrett' esophagus, the markers as shown in Figure 7.B, CK7, CK20 and SOX9 (a columnar epithelial transcription factor), are present in the epithelial layer as well as in the luminal layer and intercalated ducts of ESMGs show similar molecular markers that support the argument of ESMGs being the cell-of-origin. Patients with acinar ductal metaplasia will regenerate the ulcerated surface in the esophagus. The submucosal ductal cells will start producing columnar cells with increased expression of transcription factors associated with intestinal cells fates, therefore leading to Barrett's esophagus.

Garman et al. (2015), obtained samples from patients with high-grade dysplasia (HGD) or esophageal adenocarcinoma cells (EAC). They quantitatively studied the types of acinar and ductal cells in esophageal submucosal glands. They observed that patients with HGD or EAC had more

ductal metaplasia (based on a scoring system that used inflammation and histologic analysis of ductal-type epithelium with a dilated lumen), as compared to their normal mucinous state which has pale and glandular cells. They specifically looked at the ESMGs that changed over time in patients with HGD or EAC, compared to controls. This is the first experiment looking specifically at the ESMGs themselves. However, this experiment's purpose was not to make an association between ESMGs and EAC, but rather to observe histological differences between ESMGs of individuals with HGD or EAC and patients without HGD or EAC. Their findings support that persistent inflammation will allow for surface esophageal cells and ESMGs to undergo metaplasia therefore resulting in EAC. Although their experiment did not try to make an association between the two, it has been previously supported that ESMGs and surface esophageal cells are in connection with each other (Glickman et al., 2001). This connection allows ESMGs to be the cell-of-origin during ulceration of the esophagus allowing esophageal cells to undergo a change in phenotype due a change in environment.

Recently a study by Owen et al. (2018), used single-cell RNA-sequencing (RNA-seq) to generate gene expression profiles from whole tissue biopsy samples from the esophagus, duodenum, and stomach. They hypothesized that by using single-cell RNA-seq they would be able to better understand relationships between cells of the gastrointestinal tract, as well as normal tissue *vs* Barrett's esophagus tissue. They observed that submucosal gland cells and Barrett's esophagus cells obtained from patient samples had similar expression levels of olfactomedin 4 (OLFM4), a stem cell marker in the crypts of the small intestine and colon (Kosinki et al., 2007). OLFM4 is known to play an important role along with LGR5, a leucine-rich orphan G protein-coupled receptor. LGR5+ cells are present in the gastric cardia but not in the normal esophagus (Barker et al., 2007). In the study by Van Der Flier et al. (2009), stem cell marker OLFM4 was

heavily increased in cells of colorectal carcinoma (observed through *in situ* hybridization). Similarly, in the esophageal submucosal glands there was increase expression of OLFM4 where these progenitor cells contain alkaline secretions that try to protect the esophagus from acid, bile, and pepsin damage (Owen et al., 2018). Owen and colleagues observed that LEFTY1, a developmental gene, part of the transforming growth factor beta (TGF-B) superfamily, was highly expressed in esophageal mucosal glands but not in gastric or duodenal cells. Both LEFTY1 and OLFM4 were expressed in Barrett's esophagus cells that contained non-goblet cells. In the goblet cells there was no expression of LEFTY1, only MUC2, an intestinal mucin contained in the goblet cells. The non-goblet cells containing LEFTY1 expression illustrate that goblet cell presence is not needed for the presence of Barrett's esophagus. In the United Kingdom, the presence of goblet cells is not needed for diagnosis of Barrett's while in United States goblet cells must be present (Salimian et al., 2018). By the use of single RNA-seq they support the argument that Barrett's esophagus might be originating from esophageal submucosal glands in that OLFM4 and LEFTY1, upon exposure to acid and bile, will try to protect the area of injury as well as repair the loss of esophageal cells.

Gastroesophageal Junction/Gastric Cardia

In contrast to submucosal glands, recently a new proposed cell that may contribute to the origin of Barrett's columnar epithelium has been identified in the transition zone at the gastroesophageal junction (GEJ). Jiang et al. (2017) proposed that a new potential cell-of-origin is located within the transitional epithelium with distinct basal progenitor cells (p63+KRT5+KRT7+) as shown in Figure 6. According to Glickman et al. (2001), p63 is a homolog of p53 (tumor suppressor gene) that plays an important role in how the cells proliferate and differentiate. In the mammary glands, p63 is important for self-renewal of stem cells (Yang et al., 1998). The

cytoplasmic intermediate filament Keratin 7 (KRT7) is normally present in the columnar epithelium, while Keratin 5 (KRT5) is normally present in squamous epithelium (Lersch, Stellmach, Stocks, Giudice & Fuchs, 1989; Lloyd et al., 1995; Rock et al., 2009). This study consisted of using different mouse models and human samples. They used lineage tracing with green fluorescent protein (GFP) to identify basal stem cells. They also did *in vitro* 2D and 3D cultures from mouse and humans to determine how cells self-renew. They used an inducible vector system to overexpress CDX2 to induce intestinal metaplasia. They demonstrated that p63 in basal cells was decreased when CDX2 was upregulated. In contrast, there was multilayered epithelium when p63 was still present. Multilayered epithelium had p63 expression, where it was believed to play an important role in the onset of Barrett's, in contrast to advancing disease to full intestinal metaplasia and p63 expression is absent (Glickman et al., 2001). Jiang et al. (2017), used human cultures and concluded that humans also have the same basal progenitor cells in the transition zone. By the use of multiple models, they conclude that the cell-of-origin is from the transitional epithelium and might be a new therapeutic target. In this experiment, most of their data was focused at the transitional epithelium changing into intestinal metaplasia but not the proximal esophageal ulceration. Therefore, further investigations are needed to demonstrate transitional epithelia as the cell-of-origin in comparison to residual embryonic cells or gastric cardia cells.

Residual embryonic stem cells located proximal to the transitional epithelium have previously been identified. Wang et al. (2011), used a p63-knockout mouse that did not contain squamous epithelia in the esophagus although they contained carbonic anhydrase (CAR4+) normally expressed in development, and (KRT7) expression. They observed migration of CAR4 residual embryonic stem cells in the injured area after treatment with diphtheria toxin. These cells were columnar but did not develop into intestinal metaplasia. They argue against

transdifferentiation since esophageal cells were not present and able to repopulate the area of damage tissue, therefore invoking a role for residual embryonic cells. The squamous cells were not present, therefore could not be reprogrammed to become intestinal cells. Arguments against residual embryonic cells are that there have been no observations that they serve any function in adults *in situ* or that they are responsible for actually inducing Barrett's metaplasia due to GERD (Jiang et al., 2017). Wang et al. (2011) argued that Barrett's may develop by competitive cell lineages rather than genetic alterations due to the environment the cells are in.

From the squamo-columnar junction of the esophagus, the gastric cardia follows in the upper gastrointestinal tract of the human. The gastric cardia might be a potential origin for the columnar epithelial cells found in Barrett's esophagus. This hypothesis is supported by mouse models that overexpress interleukin 1 beta (IL-1β), along with the addition of bile acids, leads to Barrett's esophagus (Quante et al., 2012). In mice that developed Barrett's esophagus there was high expression of LGR5, a leucine-rich orphan G protein-coupled receptor that normally labels active stem cells in the small intestine and is found in the gastric cardia but is not normally present in normal esophagus (Barker et al., 2007). In this experiment, through lineage tracing, they concluded that metaplastic columnar epithelium in the esophagus develops from LGR5+ progenitor cells located in the gastric cardia. This implies that the LGR5+ cells moved from gastric cardia into the esophagus. This would be an example of transmigration. Similarly, Jang, Lee, and Kim (2015) investigated the presence of LGR5+ cells in normal and pathological gastric mucosa in patients. They concluded that LGR5+ cells play an important role in gastric intestinal metaplasia and speculated that it might function similarly in stem cells that differentiate into intestinal cells in Barrett's Esophagus. If LGR5+ cells are the stem cell in Barrett's esophagus, then they will contribute to Barrett's epithelial cells to become intestinal-like. Although LGR5 is a promising

stem cell marker in the gastric cardia (part of the stomach that is adjacent to the esophagus), some arguments against this transmigration model are that there are other potential stem cells markers expressed in the cell-of-origin contributing to Barrett's esophagus that are not expressed in the gastric cardia epithelial stem cells. This is part of the controversy in terms of the cell-of-origin being transmigrating gastric progenitors, progenitors being present in squamous epithelium cells, or submucosal glands; there are conflicting data using different models.

Progenitor Cell of Origin to Development of GERD

Direct Acid Burn Injury to Cytokine Mediated Inflammation

Constant gastric acid refluxed into the esophagus, also known as GERD, causes reflux esophagitis. The gastric juice contains hydrochloric acid, bile from the small intestine and the digestive enzyme, pepsin (Collins, Crothers, Mcfarland, & Love, 1985). Since 1935, the traditional concept proposed by Dr. Asher Winskelstein, an American gastroenterologist, has been that reflux esophagitis starts when refluxed acid and pepsin damage the proteins of junctional complexes that are keeping the esophageal epithelial cells together (Ismail-Beigi, Horton, & Pope, 1970; Souza, 2017). This is a direct chemical injury that causes death of surface epithelial cells then progresses through the epithelium, lamina propria, muscularis mucosa and then into submucosa as illustrated in Figure 8A. According to several researchers (Frierson, 1990; Orlando, 2008; Souza, 2017), this direct damage causes neutrophils and eosinophils to infiltrate and in turn induce proliferation of esophageal basal cells and papillary hyperplasia (the papilla is elongated). Figure 2 shows normal papilla layer in the lower layers of the esophagus.

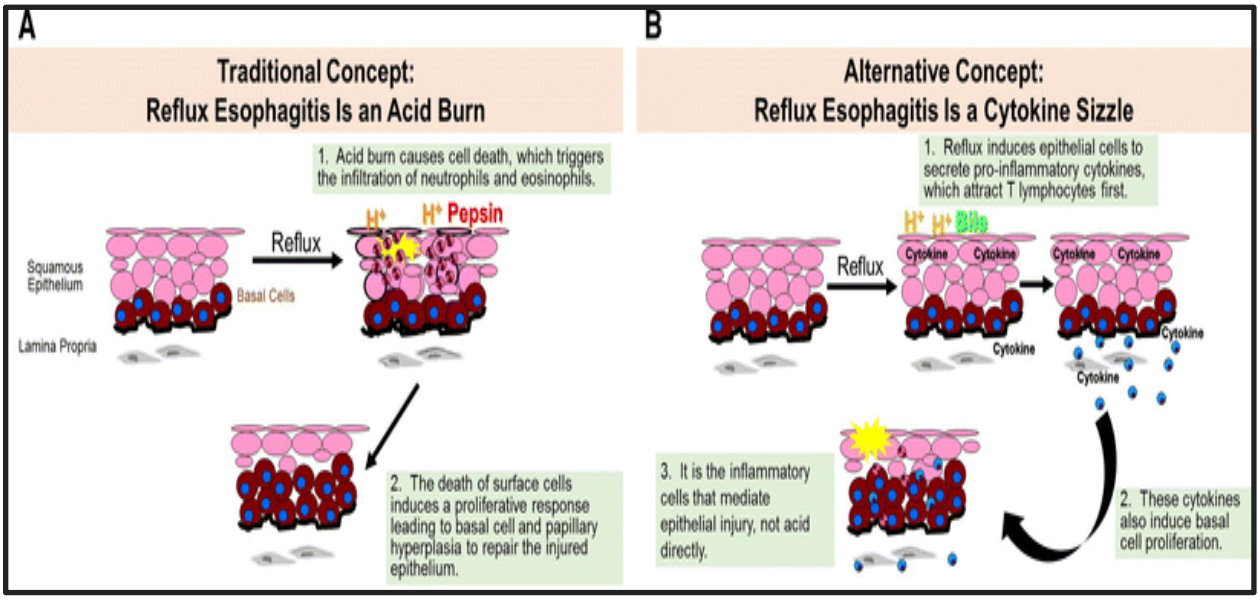


Figure 8. **Traditional vs. Alternative Concept in Reflux Esophagitis.** (A) Traditional Concept of Reflux esophagitis known as direct acid burn injury leading to basal cell and papillary hyperplasia to repair the site of injury versus (B) Alternative Concept of inflammatory cells (cytokines) inducing epithelial injury. Reproduced from "Reflux esophagitis and its role in the pathogenesis of Barrett's metaplasia" by Souza, 2017, *Journal of Gastroenterology*, 7, pp. 767-776. Copyright 2017 Japanese Society of Gastroenterology. Reprinted by permission from Springer Nature.

A different paradigm for GERD pathogenesis suggests that refluxed gastric juice does not kill esophageal epithelial cells directly but rather stimulates a cytokine-mediated response that attracts T lymphocytes and other inflammatory cells that damage the mucosa (Figure 8B). A variety of immune cells are present in the esophagus, including T lymphocytes, regulatory T lymphocytes, B lymphocytes, plasma cells, mast cells, and dendritic cells (Aceves, Hirano, Furuta, and Collins, 2012; Fuentebella et al., 2010; Vicario et al., 2010). The cellular mechanism by which this model works is that acid and bile salts activate NADPH oxidase, which forms an oxygen radical and creates reactive oxygen species (ROS) as shown in Figure 9.

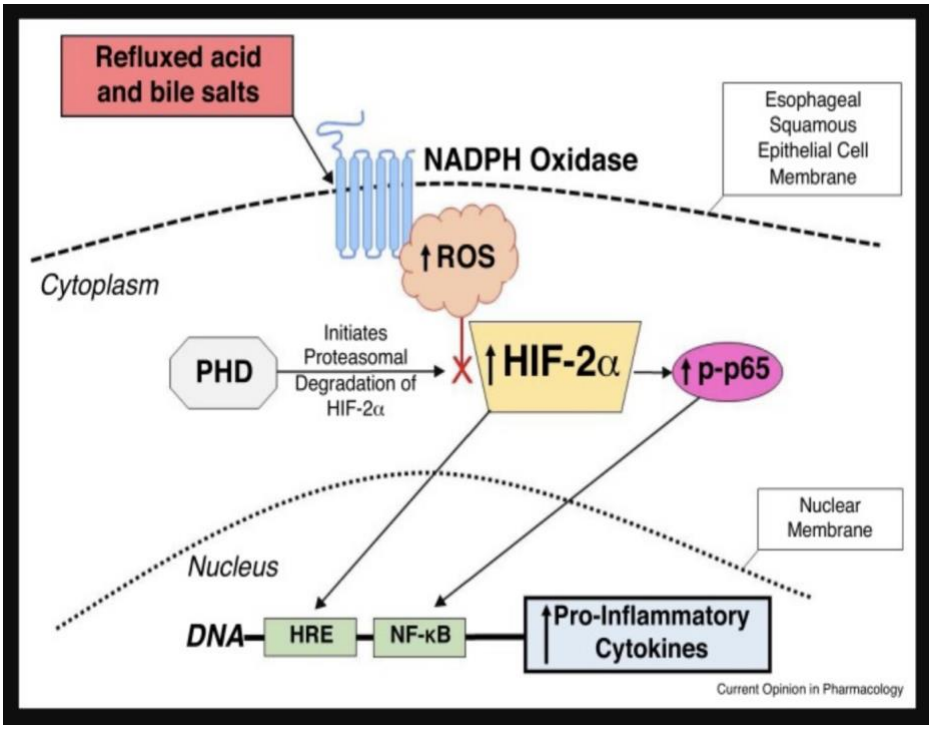


Figure 9. **Cytokine-mediated inflammation mechanism.** NADPH oxidase creates reactive oxygen species (ROS), decreasing the activity of (PHD), an enzyme that initiates proteasomal degradation of HIF-2α. HIF-2α is a transcription factor that enables cells to respond to hypoxic stress and can mediate inflammatory processes. In normal oxygen conditions HIF is inactive because prolyl hydroxylases cause HIFs to be degraded in the proteasome. Reprinted from *Current Opinion in Pharmacology*, 37, Souza et al., 2017, 37, A new paradigm for GERD pathogenesis. Not acid injury, but cytokine-mediated inflammation driven by HIF-2α: a potential role for targeting HIF-2α to prevent and treat reflux esophagitis, 93-99. Copyright (2017), with permission from Elsevier.

Recent studies by Huo and colleagues (2017), found that stopping proton pump inhibitors in patients resulted in the rapid development of acute reflux esophagitis associated with increased HIF-2α in the esophageal squamous epithelium. This is consistent with the model shown in Figure 9. This increase in epithelial HIF-2α is associated with increased epithelial NF-κB/p65 activity and increased mRNA expression of pro-inflammatory molecules IL-8, IL-1β, cycologenase-2 (COX-2), tumor necrosis factor (TNF-α), intercellular adhesion molecule (ICAM-1), and interferon-γ (IFN-γ). These pro-inflammatory cytokines will attract T lymphocytes and other inflammatory

cells to damage the esophagus (Fitzgerald et al, 2002; Isomoto et al., 2003; Huo et al., 2017). Targeting HIF-2 α can thus provide novel therapies by inhibiting production of inflammatory cytokines (Huo et al., 2017). Therefore, blocking these inflammatory cytokines should produce relief from damage due to refluxed acids and bile salts typically associated with Barrett's metaplasia.

The hypothesis of the cytokine-mediated response arose from rats studies in 2008 where the esophagus and the duodenum were connected (esophagoduodenostomy) in order to induce reflux esophagitis. On postoperative day 3 *in vivo*, the rat models showed esophageal inflammation in the submucosa with only T lymphocytes; no neutrophils were seen until day 7 and the esophageal mucosa was intact. By postoperative week 1, the lymphocytic inflammation reached the lamina propria basal cells and induced hyperplasia and by week 2, the epithelial layer was inflamed (Souza et al., 2009). Finally, during week 4, the death of surface squamous epithelial cells was observed. Souza and colleagues also used cultures of esophageal squamous cells from GERD patients and found that the cells secreted interleukin IL-8 and IL-Iβ, which are pro-inflammatory cytokines (Souza et al., 2009). When exposed to acidic bile salts these squamous cells did not die. This study illustrated for the first time that reflux acid does not cause direct caustic injury but rather acts through a cytokine mediated response via increased expression levels of IL-8 and IL-Iβ causing injury due to prolonged inflammation leading to ulceration and mucosal edema.

Similarly, in 2016 Dunbar and colleagues did a study on patients with GERD in which they stopped Proton Pump Inhibitor (PPI) treatment in these patients to induce reflux esophagitis. These patients had increased T lymphocyte-predominant esophageal inflammation, and basal cell and papillary hyperplasia without loss of surface esophageal squamous cells. This study further

supported that reflux esophagitis is cytokine-mediated. The human esophagus one week after PPI discontinuation had predominant T lymphocyte infiltration of the mucosa. Chronic injury causes the epithelium to undergo severe reactive changes such as basal zone (progenitor cell zone) thickening due to increased basal cell proliferation (Ismail-Beigi, Horton, & Pope, 1970). It has been observed that patients with GERD will develop esophageal ulcers, which are distinct breaks in the margin of the esophageal mucosa due to constant injury that result in necrosis (cell death) of surface layers of the esophageal mucosa (Frierson as cited in Dunbar et al., 2016). In general, ulcers can result from tissue necrosis triggered by mucosal ischemia (lack of blood supply), free radical formation, and cessation of oxygen and nutrient delivery (Tarnawski et al., 2000). According to Tarnwaski and Ahluwalia (2012), "the cellular and molecular mechanisms and factors that induce and mediate the esophageal-epithelial proliferative response to ulceration and regulate esophageal ulcer (wound) healing have not been established" (p.23).

Wound Healing After Ulceration

Wound healing is the process by which the tissue repairs itself through four different stages. The first stage of wound healing due to injury is the slowing of blood flow also known as hemostasis. Inflammation follows, succeeded by proliferation (rapid increase in cells making new collagen and other components of the extracellular matrix), and lastly maturation (remodeling state). In the stomach, a gastric ulcer during healing undergoes the following: hemostasis, inflammation, cell proliferation, epithelial regeneration, gland reconstruction, formation of granulation tissue, new blood vessel formation, interactions between various cells and the matrix and tissue remodeling, resulting in scar formation (Tarnawski, 2005). Gastric ulcer healing can be compared to esophageal ulcer healing with epithelial proliferation, but keratinocyte growth factor

(KGF; also known as fibroblast growth factor 7) FGF7 and its receptor are the key players in the regeneration of the epithelium of an ulceration caused by acetic acid in animal models (Baatar et. al, 2002). The application of acetic acid in animal models is used in a few studies to understand wound healing of an esophageal ulceration as compared to wound healing of a stomach ulceration.

New insights into wound healing were reported by Agoston et al. (2018), who used mouse models. They reported that epithelial-mesenchymal-transition (EMT) might be a contributing factor to progression of Barrett's esophagus. They did an investigation of the early histologic events in the development of a columnar-lined esophagus in rats after connecting esophagus to jejunum (esophagojejunostomy; Agoston et al., 2018). The rats developed ulceration in the squamous-lined distal esophagus starting at the anastomotic site and progressing proximally up the esophagus, with subsequent progressive re-epithelialization of the distal portion of the ulcer bed (adjacent to jejunum) by an intestinal type of columnar epithelium (Agoston et al., 2018) as illustrated in Figure 10.

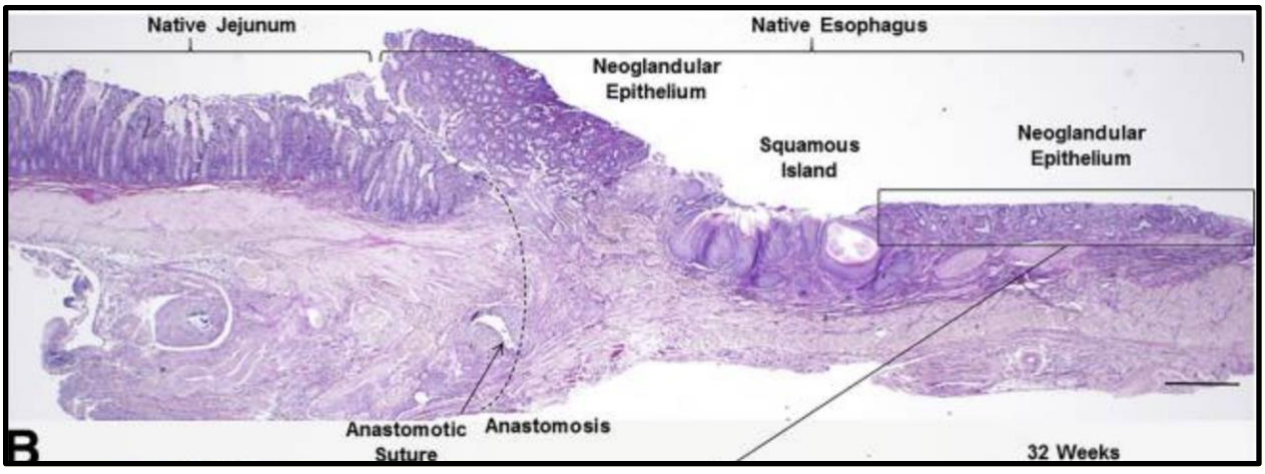


Figure 10. **Anastomosis of jejunum to the esophagus.** Arrow: Anastomotic suture. Neo-glandular epithelium arising proximal to anastomosis and in proximal esophagus with squamous islands in between. Reproduced from

As illustrated in Figure 10, at the anastomotic site there was neo-glandular epithelium that contained enlarged nuclei, prominent nucleoli, and increased mitosis (Agoston et al., 2018). Next to the neo-glandular epithelium was an island of squamous epithelium and then far distally from the jejunum this squamous epithelium was followed by additional neo-glandular epithelium. Similarly, in previous study done by Takubo et al. (2003), patient biopsies contained islands of squamous epithelium with intestinal metaplasia and columnar-lined mucosa around the islands. These were formed when the squamous epithelia were destroyed by the reflux, which then caused the ulcer. The ulcer was then filled in with columnar cells that got there via EMT that had originated in the jejunum. EMT was seen during the wound healing processes triggered by inflammatory cytokines during tissue injury, scarring and fibrosis (Stone et al., 2016). It was observed that there was decreased expression of E-cadherin, which is required for normal cell-to-cell adhesion in epithelia. Thus, with down-regulation, EMT occurs. The neoglandular epithelium also contained a population of spindle-shaped, mesenchymal-appearing cells that showed expression of the EMT transcription factor (TWIST1). Overexpression of TWIST1 also induces EMT; this is a key process in metastases formation in cancer (Zhu, Ma, Wang, Song, & Lv, 2016). Esophagojejunostomy resulted in the growth of immature-appearing glands that express both TWIST1 and E-cadherin into the deep mesenchyme of the ulcerated distal esophagus, and the apparent extension of neoglandular epithelium around squamous islands, all suggesting an important role for EMT in this esophageal wound healing process (Agoston et al., 2018). Lastly, Agoston and coworkers also noted that there were severe reactive changes in the squamous epithelium, bordering the wound (or ulceration), including basal cell hyperplasia, papillary

hyperplasia, abnormal accumulation of fluid in the epidermis, and surface erosions (2018). The neoglandular epithelium at the anastomosis also expressed PDX-1 (a foregut transcription factor present in the duodenum, jejunum, and pancreas) similar to that of adjacent jejunum. This supports the idea that the glandular cells migrated from the jejunum due an environmental advantage in comparison to squamous cells without being genetically reprogrammed. Few research studies have been done on the cellular and molecular mechanisms of wound healing in the esophagus as compared to gastric ulcer healing. Agoston et al. (2018) noted that the first columnar cells get to the esophagus via wound healing and EMT and not through cellular reprogramming. In the case of human Barrett's esophagus, they hypothesized that nearby gastric progenitor cells, or transitional basal cells located at the squamocolumnar junction help initiate the process of metaplasia in conjunction with the wound healing process in order to change the epithelial phenotype in the esophagus to columnar cells with gastric features. Later on, in the setting of ongoing GERD, these cells might undergo genetic reprogramming to produce a columnar epithelium with intestinal features (O'Riordan, 2004). This experiment supports the idea that in order to repair the area of injury that nearby cells with mesenchymal appearance migrate in to heal the wound; the cell present in the esophagus had markers of epithelial cells, E-cadherin, and TWIST1 markers showing EMT. In this case, the area was repopulated with jejunum cells. However, in humans, the cell that repopulates can be from esophageal submucosal cells, bone marrow cells, esophageal basal cells, GEJ, or gastric cardia mesenchymal cells. The columnar cells present in the esophagus will continue to experience acid and reflux, allowing cellular reprogramming of these columnar cells to change into columnar cells with intestinal features, as seen in Barrett's esophagus.

Cellular Reprogramming of Epithelial Cells

Transcription Factors

After wound healing, chronic inflammation may persist due to GERD and lead esophageal cells to undergo cellular reprogramming. Figure 11 illustrates cellular reprogramming with squamous cells turning into columnar cells and then into either intestinal or goblet cells, both types are found in Barrett's esophagus. The specific transcription factors that have been shown to be involved in phenotype change from squamous to columnar are: an increased concentration of sex determining region 9 (SOX9), and a decrease in SOX2 and a decrease in tumor protein p63. The change in phenotype from columnar to intestinal is controlled by two caudal homeobox gene family transcription factors (CDX1 and CDX2; Silberg, Swain, Suh, & Taber, 2000). In addition, an increase in transcription factor forkhead box protein A2 (FOXA2) can change the columnar cell to a goblet cell. Figure 11 shows a model of known transcription factors but their regulation by acid, bile injury, and other components of reflux is the hypothesis.

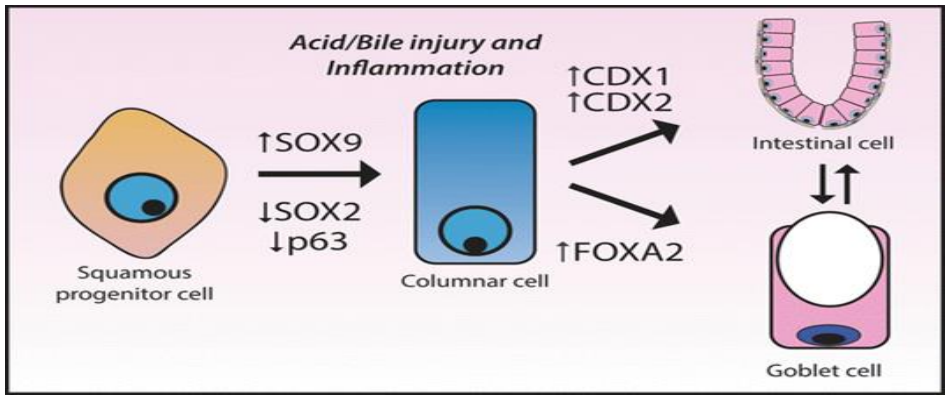


Figure 11. **A hypothesis of how acid, bile, and other components regulate known transcription factors in the pathogenesis of Barrett's esophagus.** Specific transcription factors involved in phenotype change from squamous to columnar are: increased concentration of sex determining region 9 (SOX9), and a decrease in SOX2 and a

decrease in tumor protein p63. The phenotype change from columnar to intestinal is controlled by two caudal homeobox gene family transcription factors (CDX1 and CDX2). In addition, an increase in transcription factor forkhead box protein A2 (FOXA2). Reproduced from "Transcommitment model of Barrett's esophagus" by Wang and Souza, 2016, *Advances in Experimental Medicine and Biology*, 908, p.182-212. Copyright (2016) Springer Nature. Reprinted with permission.

SOX9, was expressed in esophageal squamous cells in an *in vivo* reconstitution model, and it alone induced formation of a columnar-like epithelium (Clemons et al., 2012). SOX9 is known to play an important role in normal gut development and was upregulated through the Hedgehog (Hh) signaling pathway by activating the pathway in conditional mouse models that resulted in upregulation of FOXA2, followed by intestinal mucin (MUC2) expression found in Barrett's intestinal metaplasia (Wang et al., 2014; Van Der Sluis et al., 2008). In the study by Agoston and colleagues (2018), they also observed increased proliferation markers (Ki-67) and SOX9 expression to try to heal the area near the ulcer, yet the cells still retained a squamous phenotype, therefore it could not be considered a metaplastic process. One reason they might not have seen the columnar phenotype is because the duration of their study was not extended. Therefore, they could not assess whether SOX9 expression in their squamous cells eventually contributed to a metaplastic columnar-lined esophagus (Agoston et al., 2018). They observed EMT during initial wound healing and noted later during persistent chronic GERD, there can be overexpression of SOX9 that changes the epithelium phenotype. In contrast, in this same study the stratified squamous epithelium had SOX9 upregulation and SOX2 downregulation illustrating that it was undergoing cellular reprogramming, yet maintained a squamous phenotype. This illustrates the importance of upregulation of SOX9 to induce columnar epithelia. Minacapelli et al. (2017) showed that Barrett's metaplasia can develop from cellular reprogramming of normal esophageal squamous epithelial cells by GERD. GERD causes downregulation of a homolog of tumor

suppressor p53, TAp63, (a TA isotype that retains the NH2-terminal activation domain; Yang et al., 1998). In this experiment they used *in vitro* cell culture of normal esophageal squamous epithelial cells and exposed them to acid and bile. This resulted in a change of transcription factor levels. Minacapelli et al. (2017), further supported the notion of transcommitment by their observation of TAp63 and its important role in Barrett's esophagus.

Similar to TAp63, SOX2, is a transcription factor that is normally found in embryonic esophagus and regulates endoderm differentiation into stratified epithelium (Que et al., 2007). It was observed by Asanuma et al. (2016) that SOX2 expression decreased in esophageal squamous cells from Barrett's patients in culture that were exposed to nitric oxide (NO) and was lower than the values for cells exposed to acid and bile salts. The molecular mechanism by which this occurs is illustrated in Figure 13: NO disrupts Akt signaling. This involves the Protein Kinase B pathway, and in esophageal squamous cells NO causes S-nitrosylation (SNO) of Akt protein. S-nitrosylation is the covalent binding of NO to protein cysteine residues, forming S-nitrosothiols that can interfere with protein function through their effects on protein conformation (Asanuma et al., 2016). Blocking the activation of Akt and Rictor-mTOR (a mechanistic target of rapamycin, which is a large protein kinase that phosphorylates AKT-Ser473 (located in the hydrophobic motif of the protein's C-terminal tail), leads to decreased expression of SOX2 mRNA and thus inhibits squamous esophageal differentiation (Fig. 12). In addition, NO decreases activity of p63, a transcription factor that is a homolog of tumor suppressor p53 that is important in the development of stratified epithelial tissues such as the epidermis, breast, and prostate (Fig. 11 and 12; Barbieri & Pietenpol, 2006). Lastly, in p63 null embryos, stratified epithelial tissue was gradually destroyed and there were changes to the that, in the mouse, resembled Barrett's esophagus. (Senoo, Pinto, Crum & Mckeon, 2007; Yang et al., 1999). This shows that limiting p63 is sufficient to convert

squamous cells to columnar (see Fig 11. and 12) and may indicate that neither downregulating SOX2 nor upregulating SOX9 is necessary for this transformation.

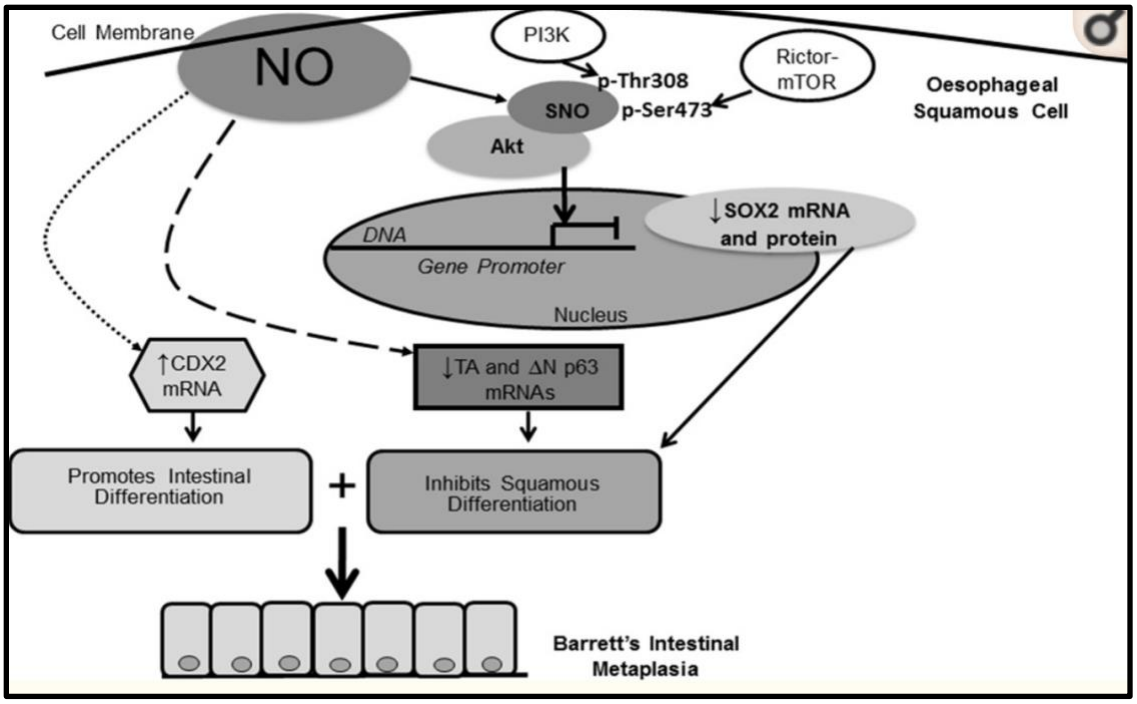


***Figure 12*. Protein Kinase B pathway.** Nitric Oxide (NO) causes AKT pathway S-nitrosylation(SNO) therefore blocking PI3K and MTOR activation. This causes downregulation of SOX2 mRNA. NO exposure also results in upregulation of CDX2 mRNA Reproduced from [In Oesophageal Squamous Cells, Nitric Oxide Causes S-Nitrosylation of Akt and Blocks SOX2 (Sex Determining Region Y-Box 2) Expression, Asunuma et al., 65, 1416-1426, Copyright 2016] with permission from BMJ Publishing Group Ltd.

CDX1 & CDX2 Involvement

Caudal homeobox gene family transcription factors, CDX1 and CDX2, are important in the change from columnar phenotype to intestinal metaplasia (Silberg et al., 1997). CDX1 is normally found in the proliferative crypts of the intestines, and it is found in Barrett's metaplastic tissue but not in normal esophageal squamous tissue (Silberg et al., 1997). Similarly, cells of the stomach do not express CDX1. However, Mutoh et al. (2004), demonstrated that the gastric epithelium had intestinal features by ectopic expression of CDX1 in mice models. According to

Wong et al. (2005), NF-κB signaling plays a key role in activating CDX1 expression only if the CDX1 promoter is unmethylated or partially methylated. CDX1 is also expressed through TNF-alpha and IL-1β similar to IL-1β found in reflux esophagitis during cytokine-mediated inflammation. In spite of all of the emphasis on CDX1, the association of CDX1 and CDX2 is seen in intestinal metaplasia where CDX2 is needed in order for CDX1 to be upregulated (Eda et al., 2002).

CDX2 is normally found in the differentiated villus compartment of the intestine in humans (Silberg et al., 2000). It may also be involved in reprogramming esophageal progenitor cells in those patients with GERD that develop Barrett's esophagus. CDX2 has been found in patients with GERD and their esophageal epithelial cells respond differently to acid and bile salt exposure by upregulating CDX2 (Souza et al., 2017). Minacapelli and colleagues (2017), in addition to noticing downregulation of Tp63, noted a normal esophageal squamous cell lines to have high expression levels of SOX9, CDX2, and other columnar markers such as MUC2 and CK8.

The precise mechanism by which CDX2 is regulated is not well understood and it is hypothesized to be regulated by Hh signaling. Recently, Huang et al. (2019), studied the role of -proton-pump inhibitors (PPIs) that suppress acid in the stomach, in the setting of levels of bile acids which could play a role in the regulation of SOX2 and CDX2 as part of Hh signaling pathway. This idea came from a previous study by Huo et al. (2014), where they showed that PPIs not only aid in stomach acid suppression but also inhibit IL-8 expression. The known mechanism of action for PPIs are to inhibit gastric acid secretion by the potassium/ hydrogen pump (K+/H+ATPase) in the stomach (Lindberg, Nordberg, Alminger, Braendstroen, & Wallmark, 1986). Similarly, Huang and colleagues noted that omeprazole, a PPI downregulated SOX9 and

CDX2 mediated by Hh signaling in Barrett's esophageal cells (2019). Huang et al. (2019), also showed that SOX9 might be regulated through different mechanisms while they confirmed that CDX2 is downstream mediator of Hh signaling. The studies by Huo et al. (2014) and Huang et al. (2019), illustrate that PPIs such as omeprazole can help in preventing Barrett's esophagus progression through different mechanisms other than the classic mechanism of action by blocking the Hydrogen/Potassium ATPase enzyme important for gastric acid secretion (Lindberg et al., 1986). Studying the effects of omeprazole in patients with Barrett's esophagus can allow us to identify ways in which therapeutic drugs currently used can be improved while also allowing us to understand cellular and molecular mechanisms involved in Barrett's disease. In summary, cellular reprogramming is supported by the regulation of specific transcription factors such as SOX9, SOX2, p63, CDX1, and CDX2, which seem play important roles in changing the phenotype of esophageal epithelial cells. Although there might be other transcription factors that are yet to be identified, most studies on esophageal epithelium and intestine have shown these transcription factors play an important role in the development of Barrett's esophagus.

Discussion

Barrett's esophageal research is an interesting field of study. Barrett's esophagus is caused by failure of the esophageal sphincter located at the gastroesophageal junction to properly close, resulting in reflux of acid and bile into the esophagus. Acid and bile are mediators of esophageal epithelial injury. The injury of epithelial cells does not occur directly but rather through cytokine-mediated inflammation (Souza et al., 2009). This area of esophageal research is interesting in that it was previously thought that esophageal cells were directly damaged by acid similar to any other form of chemical injury to organs such as the skin. The idea of esophageal cells not being damaged

directly was observed by Dunbar and colleagues (2016) in patients with GERD. They report that there was initially no loss of surface esophageal squamous cells, but rather there was continuous inflammation in the deeper layers of the esophagus as shown in Figure 13. This suggests that the initiating process in the development of Barrett's esophagus is continuous damage to the squamous epithelial lining due to leukocyte infiltration.

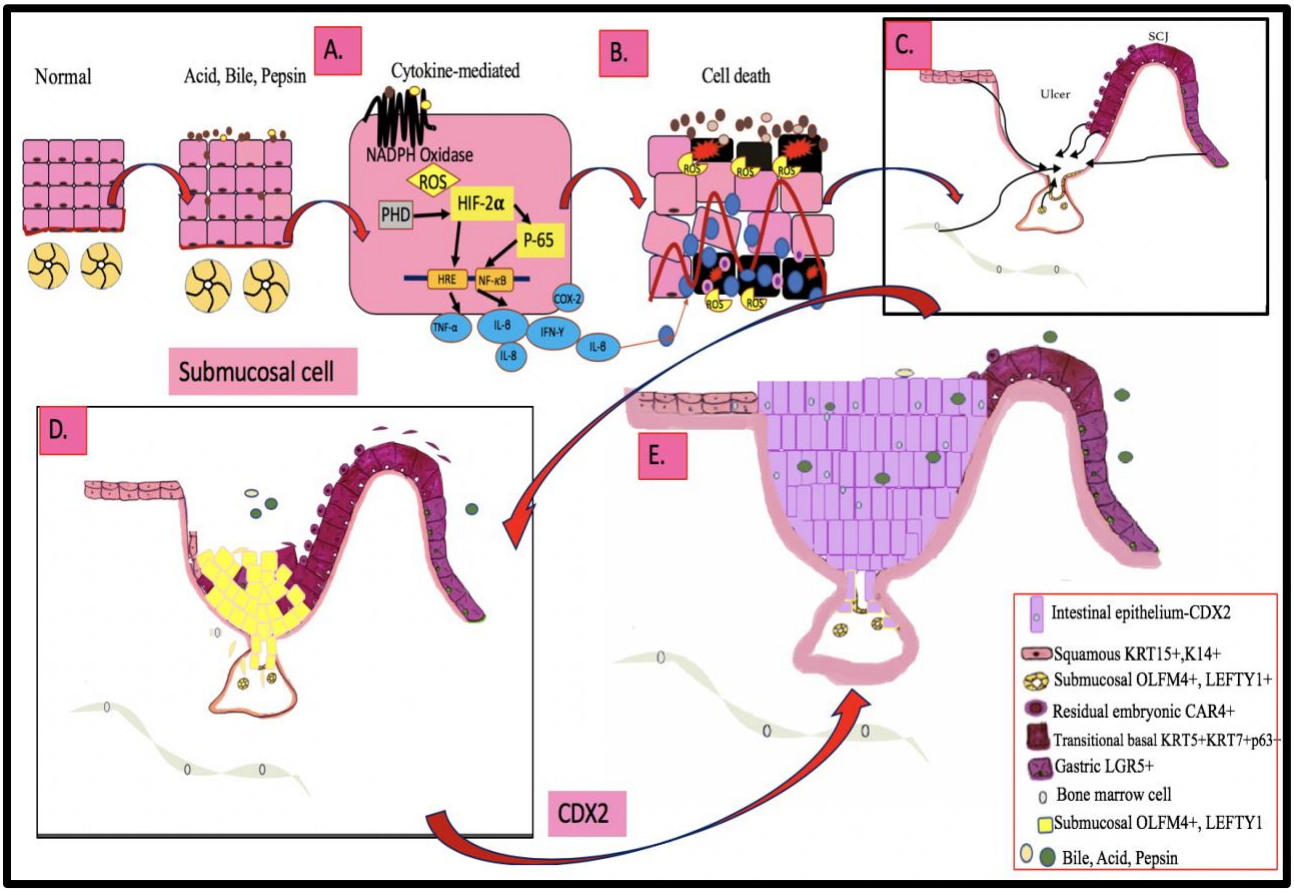


Figure 13. **Proposed pathogenesis of Barrett's esophagus**. During chemical injury of the outer-skin surface there is direct damage to the cells. (A) In contrast, esophageal cell injury is regulated through cytokine-mediated inflammation by NADPH oxidase and HIF-2α. (B) Without resolution this eventually results in necrosis of surface layers and breaks in the surface lining of the esophagus also known as ulceration (commonly referred as erosive esophagitis). (C) This ulcer that is now present in the esophagus will allow for wound healing to proceed via migration of circulating bone marrow stem cells, esophageal progenitor cells, submucosal glands of esophagus, and the gastroesophageal junction region containing residual embryonic stem cells, transitional basal cells, or cardia

stem cells. (D) The submucosal glands will be the predominant cell-of-origin via EMT with regeneration of columnar epithelia at the ulceration site. (E) CDX2 expression alone without CDX1 will allow for development of Barrett's intestinal metaplasia.

According to Agoston et al. (2018), during the wound healing process, cells start changing into columnar epithelial cells with the help of neighboring cells that include jejunum cells. In their study, the induction of acid, bile, and noxious material from the anastomosis of the jejunum to the esophagus quickly induced injury resulting in ulceration of the esophagus. To fill the ulcerated space, jejunum cells migrated to the area of injury to help in re-epithelization. The epithelium then becomes neoglandular with PDX1 expression in both esophagus and jejunum cells. PDX1 is an important transcription factor normally present in intestinal cells such as the duodenum and jejunum. In this particular study PDX1 and MUC2 were the intestinal markers present in the neoglandular epithelium of the esophagus. The jejunum cells migrated via EMT into the esophagus. This was observed through high power microscopy with focal nuclear staining showing spindle-shaped mesenchymal-appearing cells with markers of epithelial cadherin (E-cadherin) and mesenchymal (TWIST) cells. These cells exhibited a columnar phenotype within the neoglandular region although they are not considered reprogrammed since they moved from the jejunum into the esophagus. The proximal ulcer edge next to the neoglandular tissue of the jejunum was squamous with increased expression of SOX9 and loss of SOX2 expression and increase in proliferation marker (Ki-67) normally expressed in wound healing. These cells maintained their squamous phenotype even with increases in SOX9, illustrating that cellular reprogramming had not occurred. According to Slack (2007), in order for metaplasia to occur there needs to be increased expression of molecular markers along with change in the phenotype of the cell. Jejunal cells migration with upregulation of EMT markers supports the idea of EMT. The neoglandular epithelium stained positive for MUC2, CDX2, and other intestinal markers although it did not have

all the markers for Barett's metaplasia such as intestinal marker of goblet cells DAS-1, (Agoston et al., 2018). This supports the notion of wound healing and not quite cellular reprogramming. This experiment for the first time illustrates wound healing as part of the pathophysiology in development of Barrett's esophagus. This is similar to the skin during acute inflammation where healing involves EMT. Chronic inflammation can also lead to fibrosis, and fibrotic strictures are seen in Barrett's esophagus. Fully developed intestinal metaplasia was not achieved in this study as it is in Barrett's esophagus, as they were only able observe reflux esophagitis on the squamous side of the ulcer border, and only some intestinal markers were present; Das-1(intestinal marker of goblet cells) was not present, illustrating that this is still part of wound healing process. They concluded that if their experiment was extended they would have had a better understanding of the pathogenesis of Barrett's esophagus. There has not been additional extensive research in understanding specifically the wound healing repair process in the esophagus. In summary, the study by Agoston and colleagues (2018) illustrates a possible mechanism in the development of Barrett's esophagus after ulceration through repair of the area of injury (due to elevated acidic and bile salts) via wound healing.

Following wound healing of the esophagus, the esophagus now mostly consists of columnar and intestinal-looking cells as shown through histological staining where intestinal transcriptions factors like CDX2 and CDX1 were observed. Similarly, CDX1 and CDX2 are known to play an important role in intestinal cells (Silberg et al., 2004). This illustrates that, after or during wound healing, there can be cellular reprogramming involving important transcription factors. These two transcription factors are CDX2 and CDX1. I propose that CDX2 alone can allow for the conversion to intestinal epithelium without CDX1 as illustrated in Silberg et al. (2002), where CDX2 alone induced intestinal metaplasia in CDX2 transgenic mice using a yeast

artificial chromosome (specialized linear DNA vector) that contained *cis* regulatory element FOXA3. Similarly, it was noted by Simmini and colleagues (2014), that the inactivation of CDX2 in adult intestinal stem cells lead to the formation of gastric stem cells, thus showing the important role of CDX2 as absolute master controller of intestinal epithelium. CDX2 plays an important role in the disruption of the esophagus, stomach, and gut function and phenotype. It is interesting to note that when CDX2 is downregulated in the gut, gastric-looking stem cells appear. In the stomach, CDX2 expression results in appearance of intestinal metaplasia. The intestinal metaplasia is also seen in the esophagus when there is upregulation of CDX2. Therefore, from previous understanding of the role of CDX2 in the gastrointestinal tract, I conclude that CDX2 alone without CDX1 can induce intestinal metaplasia as shown in Figure 13. In contrast, CDX1 works in partnership with c-Myc, a proto-oncogene that codes for transcription factors, to allow for mucin production and transcommittment in Barrett's esophagus of normal esophageal squamous cells (Miller, Thomas, Islam, Muench, & Sedoris, 2012). This contrasts the previous belief that CDX2 and CDX1 are needed for intestinal metaplasia, and rather indicates that there are different mechanisms by which they can be upregulated and one might not need both of them together to induce development of intestinal cells. Transcription factors like CDX1 and CDX2 play an important role in gastrointestinal tract development of Barrett's metplasia. Similarly, these transcription factors were turned on early on by a specific cell that could have been a esophageal cell, submucosal cell, gastric, or circulating stem cells that migrated to the site of injury.

Bone marrow stem cells can be the cell-of-origin as supported by Sarosi et al. (2008). They demonstrated that female rats that received bone marrow cells from male rats had male cells in their columnar esophageal epithelium. However, the cells were not able to produce complete glands like in Barrett's esophagus. This was also observed in the male patient who had bone

marrow transplant from his sister and then developed XX carcinoma cells. Since the patient had previous history of acute myeloid leukemia and then received a bone marrow transplant the tissue resident esophageal or gastric cells nearby might have been exhausted by undergoing so much regenerative cell division therefore needing to recruit bone marrow stem cells. Aikou et al. (2013) opposed bone-marrow derived cells as the cell-of-origin that forms Barrett's esophagus. They did not observe in their anastomosis mouse models followed by transplant with green fluorescent protein (GFP)-expressing donor cells and GFP-positive cells in columnar metaplasia. This illustrates that the theory of bone marrow stem cell to may not be the main cell of origin since they were only able to produce some glands in Barrett's, but can contribute to Barrett's metaplasia when other cells such as esophageal squamous cells are exhausted since they were only able to produce some glands in Barrett's.

In contrast, a native esophageal cell may be a potential as a cell-of-origin for Barrett's Esophagus. There are certain protein markers during embryonic development that indicate a change in phenotype from columnar to squamous epithelial cells. I propose that these developmental switches play an important role throughout life and when there is induced damages of esophageal cells due to acid and bile the cells are reprogrammed. This opposes the idea of Agoston and colleagues (2018), that cellular reprogramming occurs following ulceration of the esophageal lining. Similar to Agoston and colleagues' migration of jejunum cells, in the human it is possible that the cell-of-origin originates from the proximal esophageal basal cells that are K14+ or KRT15+. The present esophageal cells repopulate the surface and revert to their embryonic phenotype to change into columnar epithelial cells as supported by the expression of immature basal squamous cytokeratin (K14) and columnar cytokeratin (K19) secreting cells in the multilayered epithelium observed by (Glickman et al., 2001). However, they showed that

submucosal glands and the multilayered epithelia were similar in terms of having a continuous connection with each other and having low K14 and high K19 columnar expression. This illustrates that that the submucosal glands have more potential to be the cell-of-origin, having columnar cytokeratin expression with continuous induction by acid, bile, and noxious material, simultaneously with cellular reprogramming of the cells changing into intestinal epithelium. This hypothesis of esophageal squamous epithelial cells as the cell-of-origin is promising although there has been no direct evidence of transcommitment or transdifferentiation of stratified squamous cells transforming into glands.

In a previous experiment by Gillen and colleagues (1988), they contradicted the model of cell migration of gastric cardiac cells. In a study using canine esophagus, they surgically removed 2 cm of squamous epithelium in the lower esophagus and upper esophagus. They observed that the excised areas underwent re-epithelization with a columnar epithelium, supporting the idea that submucosal glands, rather than gastric columnar cells, were the source of cells. This experiment did not look at protein markers. It can now be argued that there was wound healing via EMT. The cells migrated to the excised area to repopulate with columnar cells. Most recently, there are proposals regarding migration of the transitional basal cells and residual embryonic stem cells. The progenitor cells (p63+KRT5+KRT7+) are proposed to be the cell-of-origin in the transitional epithelium arguing against gastric cardia cells (LGR5+) as they were not expressed in the experiment (Jiang et al., 2017). Residual embryonic stem cells (CAR4+) contribute to the development of Barrett's esophagus as recently illustrated by Jang and colleagues (2015). All the stem/progenitor cells in the region of gastroesophageal junction and gastric cardiac have been put into a category together since they are in close proximity to each other. There is support that markers of these cells are seen in columnar metaplasia. The mice models, based upon injury, need

to repair the damage site. The GEJ/gastric cardia stem/progenitor cells will repopulate the area due to their environmental advantage caused by bile, acid, and noxious material more than their vulnerable esophageal squamous cell counterparts. These cells within this GEJ region (transitional basal cells and residual embryonic cells) and cardia cells are the candidates of origin. One could argue that are so many different markers without a definitive accurate model to conclude the specific cell of origin. This illustrates the weakness in concluding which is the predominant cell of origin in this region and is currently the controversial topic of debate.

The predominant cell of origin that may contribute to the development of Barrett's esophagus is the esophageal mucosal gland as illustrated in Figure 13. Krüger and colleagues (2017), used a porcine model to induce injury using radiofrequency ablation (RFA) normally used for treatment with patients with Barrett's esophagus. They observed that in the ablation of the esophagus lining after around one week, there is proliferation of squamous epithelium as well as change in esophageal submucosal glands with increased expression of SOX9. The control pig that had only endoscopy showed low amounts of SOX9 in ducts. After RFA was performed, it was noted that there was high expression of SOX9, CK7 (an intestinal phenotype marker) and the ductal cells within esophageal mucosal glands changed phenotype. It can be theorized that SOX9 in esophageal mucosal glands plays an important role in progenitor cells, and when injured they increase in SOX9 expression as well change in phenotype. Owen et al. (2018), showed that the esophageal glands may play a role in development of Barrett's esophagus. The esophageal glands exhibited stem cell crypt marker OLFM4 and developmental marker LEFTY1, however these markers were not observed in gastric cells. The esophageal submucosal cells might have an environmental advantage in migrating to the ulceration as compared to esophageal cells or gastric cells. Similarly, Glickman et al. (2001), showed evidence that mucosal gland ducts and esophageal

cells were related by similar cytokeratin expression (showing low CK14). This illustrates the similarity between Barrett's esophagus cells and submucosal glands. Garman and colleagues (2015) showed that patients with ductal metaplasia also had HGD/esophageal carcinoma. Therefore, I argue for the cell-of-origin to be coming from the submucosal glands. The reason for this is that submucosal glands showed direct connection to surface esophageal squamous cells. This connection allows for regeneration of cells with columnar phenotype from acini ducts to heal the area of injury. The intermittent exposure to acid and bile reflux will upregulate columnar transcription factors like SOX9 and intestinal transcription factor CDX2 that will allow for a change into intestinal metaplasia cells. This can be compared to the mechanism by which the crypts in the intestine contain stem cells that go through a proliferation phase to heal the area of injury and nearby cells (Sturm & Dignass, 2008). The whole intestine contains intestinal epithelia as compared to the esophagus with proximal stratified squamous and gastric columnar epithelia located distally. There are limited ways by which the small intestine and colon are able to regenerate. In closing, the environmental changes due to acid, bile, and noxious material will result in a competition between the surrounding cells in the esophagus to repopulate the injured areas.

Pigs and dogs are good candidates for evaluation in the development of Barrett's esophagus as they have similar anatomy to humans. By performing RFA, Krüger et al. (2017), noticed esophageal submucosal glands as possible cell-of-origin. In comparison, patients post-operatively coming into clinic after RFA were found to have buried glands underneath neosquamous epithelium (Shaheen et al., 2009). This illustrates that submucosal glands in Barrett's esophagus are a source of origin to allow for the recurrence of intestinal epithelial cells. These submucosal glands that produce mucin and are columnar cells will repopulate the area. Using pigs in esophageal research is beneficial in that their anatomy is structurally similar to humans. One

limitation of using pigs when looking for a definitive conclusion about human cells-of-origin being from the submucosal glands are that pigs have a lot of submucosal glands in comparison to humans. In pigs it seems likely that the ductal metaplasia would have a higher chance of occurring since glands are more numerous. Dogs are a good candidate to use for esophageal research, the time it takes for them to develop Barrett's esophagus is around a year as compared to mice with time frame of about three to eight months (Kapoor et al., 2015). Unfortunately, the mouse model has limitations for understanding the pathophysiology of Barrett's disease since their anatomy is not similar to humans. Mice do not have submucosal glands, and therefore cannot support the notion of a submucosal cell being the cell of origin for Barrett's esophagus. It is important to note that anastomosis was performed in mice models. This shows a fault where mice models are surgically induced to have GERD and thereby acquiring other ways to allow for the repopulation in the area of injury such as jejunum that was sutured next to the esophagus. Humans otherwise, do not acquire GERD the same way as mice who were induced with high amounts of bile, acid, and reflux material in shorter time period to reproduce intestinal metaplasia. *According to Comparative Anatomy and* Histology*: A Mouse and Human Atlas:* mice have keratinized epithelium while humans have non-keratinized epithelium. Mice also do not have a Z-line (Treuting, 2011). This illustrates the anatomy differences between humans and mice. Although pigs, dogs, and mice are currently being used it is important to note that they are not perfect models for humans with Barrett's esophagus.

In summary, a patient with persistent GERD, acid and bile reflux, chronic inflammation, and cellular reprogramming over time will increase their chances of developing Barrett's esophagus. Therefore, it is important to understand the pathogenesis of Barrett's esophagus (cell-of-origin, molecular pathways, and reprogramming of cells) to develop effective therapeutics for

patients. There are many hypothesis regarding the cell-of-origin including bone marrow, esophageal, and GEJ/gastric cardia although there are conflicting arguments regarding the cell-of-origin. From the literature, it is plausible to assume that the cell of origin is likely coming from the submucosal glands, going through cellular reprogramming as well as wound healing along with additional re-programming triggered by consistent chronic inflammation. This process eventually upregulates CDX2 in humans to allow for the intestinal phenotype, which predicts a risk for esophageal adenocarcinoma.

Appendix-Acknowledgments:

Reprinted by permission from Springer Nature Customer Service Centre GmbH: Springer Nature*, Stem Cells, Pre-neoplasia, and Early Cancer of the Upper Gastrointestinal Tract. Advances in Experimental Medicine and Biology*, Transcommitment: Paving the Way to Barrett's Metaplasia, Wang & Souza, Springer International Publishing Switzerland (2016)

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