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Cdx genes, inflammation, and the pathogenesis of intestinal metaplasia

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Abstract

Intestinal metaplasia is a biologically interesting and clinically relevant condition in which one differentiated type of epithelium is replaced by another that is morphologically similar to normal intestinal epithelium. Two classic examples of this are gastric intestinal metaplasia and Barrett's esophagus. In both, a chronic inflammatory microenvironment, provoked either by *Helicobacter pylori* infection of the stomach or acid and bile reflux into the esophagus, precedes the metaplasia. The *Caudal*-related homeodomain transcription factors Cdx1 and Cdx2 are critical regulators of the normal intestinal epithelial cell phenotype. Ectopic expression of Cdx1 and Cdx2 occurs in both gastric intestinal metaplasia as well as in Barrett's esophagus. This expression precedes the onset of the metaplasia and implies a causal role for these factors in this process. We will review the observations regarding the role of chronic inflammation and the Cdx transcription factors in the pathogenesis of gastric intestinal metaplasia and Barrett's esophagus.

Keywords

Cdx1; Cdx2; Barrett's metplasia; intestinal metaplasia; gastroesophageal reflux; NFKB

I. Introduction: Metaplasia, dysplasia, and transdifferentiation

The epithelium lining the gastrointestinal (GI) tract is remarkably evolved in form in order to provide for specific functions. Though embryologically derived from the same endodermal cell layer, markedly different epithelial structures emerge in each GI organ in order to provide certain critical features or properties specific to that epithelium [1]. In the small intestine and colon, a simple columnar epithelium emerges expressing a variety of cell adhesion and membrane transport proteins in order to maximize absorptive capacity while

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still serving as a competent barrier to invasive organisms and noxious external environments [2]. In the stomach, where digestion of food begins, the simple epithelium is structured to enhance the secretion of acid and pepsin for digestion of ingested nutrients while maintaining a barrier to prevent autodigestion of the organism. Lastly, the esophagus is lined by a multilayered squamous epithelium, designed to withstand the friction and injury from rapidly propelling food boluses from the mouth to the stomach. Highly regulated development patterns direct emergence of these specialized epithelial structures. However, it is well known that these patterns can be disrupted in adults. These alterations, typically called metaplasias and dysplasias, occur with regularity and contribute to significant human morbidity and mortality. They are therefore an important area for research and clinical investigation.

The National Cancer Institute defines metaplasia as a “change of cells to a form that does not normally occur in the tissue in which it is found.” The term is used to describe the replacement of one differentiated tissue type with another, fully-differentiated type (Figure 1)[3]. It is from the Greek word *metaplassein* meaning ‘mold into a new form’. Human pathological metaplasia is generally thought to occur in settings where the normal cellular environment has been changed or altered. This new environment is stressful to the cells and frequently provokes an inflammatory response. It is not presently clear how metaplastic cells emerge, or in many instances what cell they emerge from, but the new cell type is better suited to withstand the altered environment. The metaplastic change can be reversed if the normal cellular environment is restored, but this does not happen frequently in humans, and thus the changes are often permanent [4, 5]. Metaplastic cells and tissue are not thought to be directly carcinogenic, though their presence has been associated with an increased risk for neoplasia in many organ systems. This includes the bladder, lungs, ovary, and most prominently, the stomach and esophagus (Table 1) [6–8].

The term metaplasia is not synonymous with the term dysplasia, which specifically refers to abnormally developed cells with neoplastic morphology. Dysplastic cells are in fact the earliest form of pre-cancerous cells that are histologically identifiable. Metaplastic cells can be dysplastic and progress to cancer, but so can normal non-metaplastic cells. Metaplasia is also distinct from the term transdifferentiation, which is a specific form of metaplasia. Transdifferentiation refers to a differentiated cell type transforming into another differentiated cell type [3]. It is a biological term, implying a significant change in a cell’s developmental program, whereas metaplasia is a pathological description based on tissue morphology without any specific mechanistic implications. Since the molecular basis for human metaplasia of the gastrointestinal tract remains unknown, we will exclusively use this term as we explore the contribution of the homeodomain transcription factors Cdx1 and Cdx2 to intestinal metaplasia of the esophagus and stomach.

II. The CDX genes and their roles during normal development

Structure

Homeobox transcription factors (Hox) are frequently involved in patterning of multicellular organisms. The homeobox is comprised of a basic helix-loop-helix domain that binds DNA. This family of genes is expressed during embryonic and fetal development and determines

cell fate and the patterning of the organism [9]. At parturition, Hox family gene expression decreases significantly with the exception of a few members which remain active. One set of Hox genes expressed in the adult is the *Caudal*-related homeobox genes, or *Cdx* genes [10, 11]. Originally discovered in *Drosophila*, the *Cdx* genes have been identified in a wide variety of organisms [12–15]. There are three *Cdx* genes in mouse and humans, *Cdx1*, *Cdx2*, and *Cdx4* [10, 11, 16]. During embryogenesis they are vital in the regulation of the anterior-posterior patterning. Of these, *Cdx1* and *Cdx2* are the most important for gastrointestinal tract development [16].

The human *CDX1* gene is located on chromosome 5 at position 5q31-33. It contains three exons, spans 40 kb and encodes a 265 amino acid protein [17–19]. The *CDX2* gene is located on chromosome 13 at position 13q12. It contains three exons, spans 7 kb and encodes a 313 amino acid protein [13, 20–22]. *CDX1* and *CDX2* share 92% identity in the homeobox domain (55 of 60 aa) with less similarity outside this region. Structurally, CDX proteins have several identifiable domains; the homeodomain, a transcriptional activation domain, and A, B, and C boxes (Figure 2) [23]. Little is known about the A, B, and C domains, but all are located amino-terminal to the homeodomain. The A domain contains a DxE motif that may be involved in subcellular localization, while the B domain is localized within the transactivation domain of the CDX genes.

Expression

The alimentary canal develops from the primitive gut tube, with the epithelium arising from the endoderm. While both *Cdx1* and *Cdx2* are expressed in several developing tissues during early embryonic development, their expression in the endoderm of the developing gut commences at about day E12.5 in the mouse. *Cdx1* expression at E12.5 occurs in the distal intestine and expression subsequently expands to encompass the entire length of the intestine by E14.5, and its expression remains throughout adulthood [16, 18, 24]. *Cdx2* expression can be observed in the posterior gut by E8.5, but becomes limited to the endoderm by E12.5 [12, 16]. As with *Cdx1*, its expression is maintained throughout adulthood. Both *Cdx1* and *Cdx2* expression reach their peak during late embryogenesis, when the visceral endoderm is developing into a simple columnar epithelium [11].

Expression patterns of *Cdx1* and *Cdx2* in the adult intestine are similar but distinct. *Cdx1* gene expression is evident in the duodenum of the small intestine [16, 25]. Its expression steadily increases in the posterior direction throughout the small intestine and colon. Along the crypt-villus axis, *Cdx1* expression is highest in the crypt with significantly decreasing expression as cells exit the crypt and begin to differentiate toward the villus tip. Like *Cdx1*, the anterior limit of *Cdx2* expression is the beginning of the duodenum. Expression of *Cdx2* also steadily increases throughout the small intestine; however, *Cdx2* reaches its peak expression in the cecum of the proximal colon. *Cdx2* levels then steadily decline in the distal colon and rectum [16]. Unlike *Cdx1*, *Cdx2* is expressed throughout the entire crypt-villus axis. However, *Cdx2* is potentially regulated post-translationally along this axis. Phosphorylation of *Cdx2* at Serine 60 diminishes as cells differentiate and migrate to the villus tip [26]. This phosphorylation event negatively regulates *Cdx2* transcriptional activity, and therefore, *Cdx2* is likely to become more transcriptionally active as cells migrate up the

villi. Cdx2 is also phosphorylated at serine 281 by cyclin-dependent kinase 2 (Cdk2) and shunted toward the proteosome for degradation [27, 28]. This reduces Cdx2 protein levels in colon cancer cells and in the intestinal crypt compartment, likely further diminishing Cdx2 activity.

Role of Cdx expression during intestinal development

Given their intestinal epithelium-specific expression patterns, Cdx1 and Cdx2 were long thought to play a critical role in intestinal epithelial development and differentiation. This was first borne out by studies establishing a critical role for these factors in directing the expression of a number of intestine-specific genes. Sucrase isomaltase (SI) was the first intestinal gene identified as a Cdx2 transcriptional target, and it remains one of the best characterized [29–31]. Subsequently, other intestine-specific genes have been found to rely upon Cdx1 and/or Cdx2 for their developmental and tissue-specific expression [32–42]. In addition to this role, Cdx2 is also an important regulator of intestinal cell proliferation, apoptosis, chromosomal stability, migration, polarity, cell adhesion and columnar shape (Figure 3) [11, 43–49]. Three cell adhesion genes, *E-cadherin*, *Claudin-2*, and *Desmocollin-2* are transcriptional targets of Cdx2 [36, 48, 50]. More significantly, in addition to enhancing cell to cell adhesion in this manner, Cdx1 or Cdx2 expression induces cell polarity and a columnar morphogenesis in several cell lines [45, 51, 52].

Germline null mice for Cdx1 and Cdx2 have been generated and analyzed [53, 54]. Cdx1-deficient mice are viable [53]. Analysis of these mice reveals a change in the patterning of the axial skeleton such that the cervical and thoracic vertebrae and ribs exhibit features of bones normally found more anterior than their native position. This is called an anterior homeotic shift or transformation. Analysis of the small intestine and colon of these mice did not reveal any gut abnormalities. Using the murine *Villin* promoter, Cdx1 was ectopically expressed in the intestinal villi and overexpressed in the intestinal crypt compartments [49]. While these mice had no overt phenotype, there was a reduction in endogenous *Cdx2* gene expression, which may be a compensatory event. Moreover, these mice were less susceptible to tumor development after treatment with Dextran Sodium Sulfate and Azoxymethane, suggesting Cdx1 may have an anti-tumor effect in the colon [49].

Homozygosity for the *Cdx2* null allele, on the other hand, results in embryonic lethality [54]. This is due to a defect in trophoblast differentiation, and therefore, a failure of the blastocyst to implant. Analysis of skeletal abnormalities *Cdx2*^{+/-} heterozygous mice, which are viable, reveals an anterior homeotic shift similar to that observed in Cdx1 null mice, suggesting an overlapping role in the development of the embryonic skeleton. Unlike *Cdx1* null mice, however, *Cdx2* heterozygous mice do have defects in the gut. These mice develop regions of squamous differentiation focally in the ileum, cecum and proximal colon. Surrounding these regions of stratified squamous tissues is the presence of gastric epithelia that is in turn surrounded by small intestinal cells contained within the normal colonic epithelium. These lesions demonstrate that haploinsufficient expression of Cdx2 leads to an anterior homeotic shift in intestinal development similar to that seen in skeletal development. Conditional gene ablation of Cdx2 using the Cre-LoxP system further substantiated the homeotic role of Cdx2 in the intestinal epithelium. Foxa3-Cre mice were

bred with Cdx2 loxP mice to disrupt the Cdx2 gene prior to development of the intestinal epithelium [55]. Loss of Cdx2 in this context results in the replacement of normal simple columnar intestinal cells with a stratified squamous epithelium in the proximal intestine, consisting of keratinocytes due to aberrant expression of esophageal transcription factors including Sox2 [55]. Interestingly, when Cdx2 is deleted in the fetal intestine after it has been specified using the Villin Cre transgene, epithelial cell polarity is lost in intestinal epithelial cells (Gao and Kaestner, Genes Dev, 24: 1295).

Taken together, these data illustrate the importance of Cdx gene expression in the proper patterning of the developing gut and the differentiation of the intestinal epithelium. It is now recognized that Cdx1 and Cdx2 expression is tissue-specific, that they are necessary for the expression of intestine-specific genes, and that they can induce a mature polarized columnar morphology. Moreover, these observations establish that Cdx2 is both necessary and sufficient for driving the more posterior cell fate in the gut, while Cdx1 is not absolutely required for gut development.

III. Intestinal metaplasia is a pathological condition in humans

The characteristic intestinal-type epithelium is normally confined to the small intestine and colon. Intestinal metaplasia (IM), where intestinal-type epithelium is observed ectopically in other tissues, has been reported in a number of gastrointestinal and non-GI tissues including the esophagus, stomach, pancreas, liver, gall bladder, bile ducts, the bladder and urinary tract, ovary, endometrium, and sinonasal epithelium, among others. Cdx gene expression has been detected in nearly all these instances [56–58]. Indeed, the Cdx genes remain one of the most likely candidates for transcription factors that induce intestinal metaplasia, as will be discussed in detail below. Definitions of IM vary somewhat between tissues, but in general they focus on the replacement of normal epithelium with an epithelium that resembles the intestine (Figure 4). Generally, these definitions include the presence of intestinal mucin-secreting goblet cells [59, 60], though there are incomplete forms of IM [60] and a current debate as to whether goblet cells are in fact required for the definition in some tissues [61].

Although intestinal metaplasia is considered benign and is typically asymptomatic by itself, IM is a clinically relevant lesion. In the esophagus and stomach, IM occurs in the setting of chronic inflammation in response to either bile and acid reflux (esophagus) or *Helicobacter pylori* infection (stomach). IM is one of several clinical consequences from these processes, the others being epithelial ulcerations, esophageal strictures, gastric atrophy, and SPEM. Of greater clinical concern, however, is that most, if not all forms of intestinal metaplasia have been associated with adenocarcinomas in humans, and the presence of IM is considered to be an important risk factor for adenocarcinoma development [6, 62, 63]. This association with neoplasia remains incompletely explained, but current hypotheses suggest that the cellular environment that provokes IM also likely induces mutations within metaplastic cells. It is these genetic mutations, though not obvious histologically in the benign-appearing IM, that contribute to transformation and neoplasia of these tissues.

V. Chronic gastric inflammation gives rise to gastric intestinal metaplasia (GIM)

Worldwide, gastric intestinal metaplasia and gastric adenocarcinoma are far more prevalent than Barrett's esophagus and the associated esophageal adenocarcinoma. GIM occurs in the setting of chronic inflammatory conditions of the stomach, primarily from *Helicobacter pylori* infection and, to a lesser extent, autoimmune conditions [6, 64]. Both etiologies give rise to two types of gastric metaplasia, pseudopyloric metaplasia and intestinal metaplasia [65]. In autoimmune metaplastic atrophic gastritis (AMAG), there is an immune response directed against parietal cells and intrinsic factor in the oxyntic mucosa [64]. The resulting gastritis involves the gastric body and fundus, but spares the antrum. In late stages of the disease, the oxyntic glands are nearly entirely replaced by metaplastic glands.

H. pylori-induced GIM, also known as environmental metaplastic atrophic gastritis (EMAG) is the more common condition. Chronic infection by the Gram-negative bacillus *H. pylori* is quite high in non-Western countries, with infection rates starting at 50% of the population, with higher rates in certain subpopulations [6, 66]. EMAG differs from AMAG in several important ways; 1) EMAG involves the whole gastric epithelium, including the antrum; 2) gastric acid production is diminished but not lost entirely as can be seen with AMAG; 3) parietal cell and intrinsic factor antibodies are not present in EMAG; and 4) there is increased risk for gastric ulcers and gastric adenocarcinomas in EMAG compared to AMAG [66].

Histologically, GIM is characterized by the presence of intestinal cell types including goblet, Paneth, and absorptive cells. The metaplastic glands show reorganization of the whole crypt, with displacement of the proliferative zone from the neck region to the base of the crypt, and modification of the stroma that surrounds the metaplastic glands [67–70]. GIM can be divided histologically into two major types: a complete or fully intestinalized type, and an incomplete type with both gastric-and-intestinal (GI) mixed glands. Complete IM is characterized by loss of gastric mucin markers (MUC1, MUC5AC, MUC6) and gain of the intestinal mucin MUC2, whereas incomplete GIM is characterized by the maintenance of gastric mucin markers, together with the gain of the intestinal mucin MUC2 [71]. An earlier method of classification based on cell morphology and classical mucin staining methods such as periodic acid-Schiff (PAS), Alcian blue (AB), and high iron diamine (HID) identified three distinct grades of GIM: type I, containing absorptive cells, Paneth cells and goblet cells secreting sialomucins; Type II with goblet and columnar cells secreting sialomucins; and Type III, with goblet and columnar cells secreting sulfomucins. Type I corresponds to the complete type and types II and III to the incomplete type in the newer classification scheme.

The clinical importance of the distinction between complete and incomplete GIM remains unclear. In animal models for GIM, the incomplete type was found often found to appear first, followed by the Type I or complete GIM. This suggested that GIM might be caused by the gradual intestinalization of the glands from the incomplete to the complete type [68, 69]. However, some (but not all) human studies suggest the Type III GIM has the highest risk for progression to gastric adenocarcinoma [72, 73]. This has led to the speculation that

incomplete GIM types in humans, rather than representing an early GIM stage, may instead signify a late development as the tissue progresses toward dysplasia and cancer. GIM is recognized as a preneoplastic condition [6, 74]. Development of gastric cancer in humans is recognized as a step-wise process, with chronic gastritis as the initial step, followed by atrophic gastritis and GIM, then dysplasia and cancer [75]. Gastric cancer remains an important cause of morbidity and mortality worldwide, with 870,000 new cases each year, resulting in 650,000 deaths [76, 77]. It is therefore hoped that research into understanding the mechanisms that give rise to GIM will lead to better prevention strategies and reduced deaths from gastric adenocarcinoma.

IV. Chronic acid and bile reflux in the esophagus give rise to Barrett's esophagus

Chest pain, regurgitation, water brash, and heartburn are among the more common symptoms associated with gastroesophageal reflux disease (GERD). GERD is a common condition in the US, causing pain and discomfort in 40% of the US population [78]. Many have infrequent episodes of GERD managed with antacids. However, a number of patients will have symptoms several times a week to daily and require chronic acid-suppressive medical therapy for symptom relief. These patients in particular are at risk for the complications of chronic GERD which include dysphagia (difficulty swallowing), odynophagia (painful swallowing), stricture formation, and intestinal metaplasia of the esophagus, also known as Barrett's esophagus.

Traditionally, three types of metaplastic columnar epithelium have been described in the esophagus: gastric cardiac epithelium, gastric fundic-type epithelium, and specialized intestinal metaplasia, or Barrett's Esophagus (BE) [59]. BE occurs at the gastroesophageal junction and is visible endoscopically as a salmon colored patch of epithelium extending into the esophagus from the gastro-esophageal junction (Figure 5). BE is characterized not only by the morphological intestinalization that is apparent histologically (Figure 4), but by changes in gene expression patterns as well. Intestinal proteins such as villin, sucrase isomaltase, acidic mucins/MUC2, and the intestine-specific transcription factors Cdx1 and Cdx2 are commonly detected in human BE tissues [79]. However, the *sine qua non* for the diagnosis of Barrett's metaplasia has been the presence of mucin-producing goblet cells, typically demonstrated by Alcian blue staining (Figure 4) [80].

The most current hypothesis regarding the pathogenesis of BE is that it arises in the setting of chronic acid and bile reflux that results in chronic injury to and inflammation of the esophageal epithelium. There are an estimated two million individuals in the U.S. with BE, based on population studies [81]. The mean age at diagnosis is 55, and males are affected at twice the rate of females [82]. Barrett's esophagus is also seen more commonly in Caucasians and Hispanics compared to Asian and African Americans. While heartburn and other GERD symptoms are common in BE patients, a third or more of BE patients display none of these symptoms [83]. Most of these patients will, however, have asymptomatic reflux disease as the cause of their Barrett's esophagus. GERD symptoms are, however, predictive of the length of the esophagus that is involved with the Barrett's Epithelium.

Those patients having significant GERD symptoms are much more likely to have long-segment BE (length ≥ 3 cm), while those with asymptomatic GERD are more likely to have only short-segment BE [84, 85].

Unless it is associated with symptomatic gastroesophageal reflux disease (GERD), Barrett's esophagus itself is typically asymptomatic. However, the presence of BE has been implicated as an important risk factor for esophageal adenocarcinoma (EAC) (Figure 6). The rate of BE progression to EAC has been estimated to be 0.5 to 1% per year [86], with an incidence of 6-7 per 1,000 person years [87]. Thus, only a small portion of patients with BE will progress to EAC. While, in general, death from metastatic EAC is rare among all BE patients, EAC patients have a dismal 5 year survival rate of 15-20%. In addition, the incidence of EAC has been steadily increasing in the US and other Western nations [87], which is why prevention and early detection of neoplasia are clinically important. This is why endoscopy is employed to both screen for BE and survey periodically for evidence of progression to dysplasia and carcinoma. Dysplasia is recognized as a critical predictor of progression to EAC when found in BE, and has been used clinically as an indication for surgical and, more recently, endoscopic resection [81, 87]. A number of other genetic abnormalities have been associated with the development of EAC as well [79, 88].

VI. Intestinal Metaplasia and Inflammation

It is evident from research into BE and GIM that inflammation plays a critical role in disease progression, both in the development of intestinal metaplasia as well as its progression to adenocarcinoma. Recent research has focused on understanding the role of cytokines secreted into the microenvironment, as well as signaling events induced in the epithelium in response to these cytokines. We will explore these processes now in greater detail in the setting of GERD and BE, but similar mechanisms are involved in the setting of *Helicobacter pylori* infection, chronic gastritis, and GIM.

Cytokines and Eicosanoids in Barrett's Esophagus

The exposure of esophageal epithelia to acid and bile salts as a result of gastro-esophageal reflux is an important factor in the pathogenesis of Barrett's esophagus. Acid exposure of esophageal epithelia results in at least a partial loss of barrier function [89]. This increased permeability may be responsible for the initial inflammatory response observed in reflux disease. A robust inflammatory response is observed with reflux esophagitis, Barrett's esophagus, and esophageal adenocarcinoma. Interestingly, based on the cytokines present in the microenvironment of BE (IL1 β , IL4, IL6, IL8, TNF α and IFN γ), the immune response is mixed with a slight predominance of Th2 components [90]. This type of pro-inflammatory cytokine response has been previously associated with tumor formation and progression, in part by recruiting immature myeloid cells, tumor-associated macrophages, and neutrophils to foster tumor formation [91–95]. This type of immune response may trigger signaling events that result in metaplasia of the epithelium prior to the onset of carcinogenesis.

Eicosanoids are oxygenated lipids that, in the setting of inflammation, activate signaling events that lead to tissue repair responses. There are two main types of eicosanoids, prostaglandins and leukotrienes. Eicosanoids in general cause vasodilation and increase

blood vessel's permeabilization, which induces edema and facilitates the extravasation of immune cells. Cyclooxygenases (Cox1 and Cox2) are the rate-limiting enzymes in prostaglandin biosynthesis and as such their expression is tightly regulated [96, 97]. Cox proteins initiate the conversion of arachidonic acid to prostaglandin. There are several different prostanoid products that can be synthesized. The type produced depends upon the expression of tissue-specific isomerases [98].

Each of the known prostanoids binds different cellular receptors and provokes different cellular responses. PGE₂ is the prostanoid most abundant in Barrett's esophagus. It stimulates angiogenesis, increases cell proliferation and migration, and inhibits apoptosis [99–102]. Cox2 expression appears to be directly related to acid reflux as its expression level positively correlates with the amount of acid reflux [103]. Additionally, Cox2 levels increase with the evolution of Barrett's esophagus to dysplasia and to adenocarcinoma [104]. This increase in Cox expression has led to the notion that Cox inhibitors may be therapeutic in esophageal adenocarcinomas. In fact, Cox inhibition by a selective Cox inhibitor in a surgical rat model of esophageal adenocarcinoma decreased tumor formation [105]. Additionally, anti-inflammatory drugs have been associated epidemiologically with inhibiting tumor formation, possibly through their ability to inhibit Cox activity [106, 107]. However, at present, no clear clinical data supports their routine use to prevent Barrett's esophagus progression to dysplasia and cancer.

NFκB in Barrett's Esophagus

Cytokine signaling in esophageal epithelial cells may play a critical role in the initial steps of the transdifferentiation process of Barrett's esophagus. NFκB is important for Cdx1 and Cdx2 transcriptional induction [108, 109]. Induction of NFκB by cytokine stimulation (and prostaglandin activity) has been established in many cell types and has pleiotrophic effects on the cell, including the induction of cell survival pathways as well as transcriptional activation of target genes. Recent reports suggest that NFκB transcriptional activity may play a causal role in BE. Acid and bile salts can induce Cdx1 and Cdx2 protein in esophageal cells and in esophageal adenocarcinoma cells [108, 110, 111]. This induction is dependent upon NFκB activity and may be regulated by PI3K signaling [58]. Interestingly, two NFκB binding sites have been identified in the promoter of Cdx2, suggesting a potential for NFκB in directly linking inflammation with Cdx2 expression [108]. However, the complete mechanism by which acids, bile, and PI3K signaling regulate NFκB and Cdx gene expression is not fully understood.

Chronic Inflammatory microenvironment in Gastric Intestinal Metaplasia

In the stomach, gastritis is frequently caused by *H. pylori* infection [112–116]. Several *H. pylori* proteins have been associated with gastritis including vacuolating cytotoxin (VacA) and cytotoxin-associated antigen A (CagA) [117, 118]. VacA induces vacuolization of the epithelium and subsequently apoptosis, contributing to the formation of gastric ulcers [117]. In addition to its effects on the epithelium, VacA also has effects on the immune system. VacA can alter B-cell antigen presentation and T-cell mediated cytokine responses, resulting in the inhibition of T-cell activation and a lack of proliferation of both B- and T-cells [119–

121]. This inhibition suppresses proper immune surveillance and allows *H. pylori* strains expressing VacA to establish chronic infections [121].

Another *H. pylori* virulence factor, the CagA protein, is injected into gastric epithelial cells directly by the bacteria using a type IV secretion method [118, 122]. Once in the epithelial cell, CagA is phosphorylated by Src kinases and subsequently interacts with Shp2 [118]. This results in dysregulated tyrosine phosphatase activity within the cell and may potentiate a tissue response to the infection and inflammation as a result of *H. pylori* infection. These complex immunological and cell signaling events associated with *H. pylori* infection remain an important focus of ongoing research activity.

VII. DNA damage from chronic inflammation promotes metaplasia and carcinogenesis

One aspect of immune system activation with important implications for the induction of both metaplasia and cancer is the increased production of highly reactive free-radical species such as reactive oxygen species (ROS) and reactive nitrogen species (RNS). These agents contribute to oxidative stress in the microenvironment. ROS and RNS are generated in mitochondria and peroxisomes of inflammatory and epithelial cells [123]. ROS include hydrogen peroxide, superoxide, and hydroxyl radicals, while RNS are frequently found in the form of peroxynitrite [124, 125]. Interestingly, the bile salts deoxycholate and chenodeoxycholate can increase ROS with activation of PI3K and MAPK signaling and the activation of COX2 [126, 127].

The balance between the production of ROS and RNS and the scavenging of these harmful molecules determines the oxidative stress level. Several genes function to protect cells from reactive species, including superoxide dismutase, catalase, glutathione peroxidase and glutathione-S-transferase [128]. However, some of these genes are downregulated in Barrett's esophagus. In contrast, other aspects of ROS and RNS production are increased. Enhanced TNF- α and IL-1 levels with chronic GERD induce the expression of inducible nitric oxide synthase (iNOS), which catalyzes nitric oxide (NO) production, an important contributor to RNS [129–131]. Normally, ROS and RNS are produced for short periods in response to pro-inflammatory cytokines and are useful in combating infections [123]. In the setting of chronic inflammation, prolonged exposure to these free radicals can damage proteins, lipids, and DNA. It is speculated that the DNA damage, if not repaired by the cell, can lead to altered gene expression patterns, the induction of metaplasia, the transformation of the epithelium and, ultimately, the emergence of esophageal adenocarcinoma (Figure 7) [132].

Reactive oxygen and nitrogen species can alter DNA in several ways. DNA nucleotides are very stable chemically, but free-radicals can induce DNA single and double-stranded breaks as well as modifications of purine and pyrimidine bases. As an example, NO and superoxide react to produce peroxynitrite [133, 134]. This can then react with guanine to generate 8-nitroguanine which is a mutagenic base as it mispairs with adenosine rather than cytosine. If not excised prior to DNA replication, the 8-nitroguanine will induce a G to T point mutation. This type of mutation, when occurring within gene coding regions, can lead to missense and

nonsense mutations that activate oncogenes like Ras, or inactivate tumor suppressors like p53 [135, 136].

Modifications to lipids can also occur with oxidative stress. Lipid hydroperoxidases can occur from either the interaction of ROS with polyunsaturated fatty acids, or as a product of eicosanoid biosynthesis [123, 137]. One such lipid is malondialdehyde (MDA). MDA is formed by the natural breakdown of prostaglandin H2 (PGH2), a product of Cox2 activity. MDA is a potent mutagen as it can react with DNA nucleosides to form DNA adducts, leading to DNA damage [138, 139]. 4-hydroxy-2(E)-nonenal or HNE is abundantly produced under oxidative stress conditions [123]. HNE can be synthesized by the conversion of linoleic acid into several different types of hyperperoxyoctadecadenoic acids (HPODEs) [137, 140]. These acids can decompose into the genotoxic lipids HNE and 4-oxo-2(E)-nonenal. At low levels, these lipids enhance cell proliferation but at higher levels, they can lead to DNA adducts with similar DNA damaging effects as the better known MDA and ROS.

In summary, the immune system and inflammatory microenvironment can have profound effects on the epithelium. In the esophagus, acid and bile salt exposure induces a chronic inflammatory response by injury to the epithelium as well as induction of Cox2. In the stomach, it is the infection by *Helicobacter pylori* that induces a chronic inflammatory environment. The presence of immune cells such as macrophages and neutrophils can have pleiotropic effects (Figure 7), including the induction of epithelial proliferation, activation of stromal fibroblasts, tissue neovascularization, and the generation of ROS and RNS that cause DNA damage, and many of these effects are likely to be causative agents in the development of both intestinal metaplasia and adenocarcinomas in the esophagus and stomach.

XIII. The roles of Cdx1 and Cdx2 in *H. pylori* induced Gastric Intestinal Metaplasia (GIM)

Ectopic CDX1 and CDX2 expression in GIM

Neither CDX1 nor CDX2 are expressed in the normal gastric epithelium, however both are highly expressed in nearly all intestinal metaplasias of the stomach. CDX1 and CDX2 mRNAs are widely present in the human intestinal and colonic mucosae. By Northern blot hybridization and reverse-transcriptase polymerase chain reaction (RT-PCR), both CDX1 and CDX2 mRNAs were detected in the mild and severe types of GIM but not in normal gastric mucosa without IM [141, 142]. The highest levels of these mRNAs were observed in GIM associated with chronic atrophic gastritis. Moreover, CDX2 mRNA can be detected in chronic gastritis absent evidence for GIM, and this expression precedes that for CDX1 and other markers of intestinalization [143]. These findings imply that the expression of CDX2 may be the critical trigger for the subsequent initiation and development of intestinal metaplasia.

In addition to CDX mRNA expression, strong nuclear immunoreactivity for CDX2 has also been demonstrated in human gastric intestinal metaplasia across several studies [142, 144]. In an early study, positive staining for CDX2 was detected in 85% (41 of 48) of GIM

samples tested [142]. No positive staining was observed in normal gastric epithelia controls. CDX2 staining in GIM was also noted to be much higher than that observed in carcinomas, suggesting CDX2 levels may change during neoplastic progression [142, 145]. In a second study, nuclear staining for CDX2 was observed in 17/18 foci of IM lesions and in 54% of gastric carcinoma cases by immunohistochemistry [144]. In a much larger study, 131 samples (89.7%) stained positive for nuclear CDX2-expressing cells among the 146 cases of intestinal metaplasia tested. Once again, it was reported that the CDX2-expressing cells in intestinal metaplasia were more prevalent than in dysplasia and carcinoma samples [146]. All of these findings show that aberrant expression of CDX2 is consistently observed in GIM and in a subset of gastric carcinomas, and indirectly implies that CDX2 expression may promote the intestinal differentiation characteristic of GIM.

Murine models of GIM by ectopic Cdx1 or Cdx2 expression

In addition to this clinical association of Cdx expression with GIM, the ectopic expression of Cdx1 and Cdx2 has been shown to cause gastric intestinal metaplasia experimentally. Several transgenic mouse models have been reported in which ectopic Cdx1 or Cdx2 expression was directed to gastric epithelium [147–149]. In all three cases, the gastric epithelium was intestinalized to some extent. In the first published report, *cis*-regulatory elements of Foxa3 (*Hnf3γ*) were used to direct Cdx2 expression in the gastric mucosa of transgenic mice [147]. Histologic analysis showed the presence of alcian blue-positive intestinal-type goblet cells in the gastric mucosa of the Foxa3/Cdx2 mice but not wild-type littermates. Moreover, in Foxa3/Cdx2 transgenic mice, the intestinal-type goblet cells produced sulfated mucins, which is consistent with a type III GIM. The expression of a number of intestine-specific genes was also detected in the gastric mucosa of the Foxa3/Cdx2 mice including alkaline phosphatase, villin, Muc2, and trefoil family factor 3 (Tff3).

In a second study, Cdx2 expression was directed to the gastric mucosa of transgenic mice using the stomach-specific H^+/K^+ -ATPase promoter [148]. Ectopic Cdx2 expression specifically induced GIM in the parietal cell lineage. For the first 18 days after birth, the structure and number of parietal cells in the gastric mucosa of transgenic mice were the same as their normal littermates. However, starting day 19, parietal cells began to disappear and with their loss the proliferating zone moved from the isthmus to the base of the glands. Thereafter, parietal cells decreased gradually and disappeared completely by day 37. All of the gastric mucosal cells, except for enterochromaffin-like (ECL) cells, were completely replaced by an intestinal metaplasia consisting of goblet cells, enteroendocrine cells, and absorptive cells expressing alkaline phosphatase. Pseudopyloric gland metaplasia was observed with this model. In contrast, H^+/K^+ -ATPase/Cdx1 transgenic mice also develop an intestinal metaplastic mucosa complete with Paneth cells (which had not been previously reported for the Cdx2 transgenic lines) but no pseudopyloric gland metaplasia was observed [149].

One limitation for the H^+/K^+ -ATPase models, however, is that the Cdx1 or Cdx2 proteins are expressed only in the zymogenic zone, and not in the antrum. In addition, the loss of parietal cells raises concerns that some of the effects are from parietal cell loss and not Cdx gene expression directly. However, further study of the H^+/K^+ -ATPase/Cdx2 mice revealed that

they spontaneously developed gastric intestinal-type adenocarcinoma [150]. This phenotype was 100% penetrant at 100 weeks of age, and was not typically detected until 80 weeks of age. These tumors were characterized by high levels of intranuclear p53 and β -catenin protein. Mutations in *p53* and *APC* genes were identified in many of the tumors to explain the nuclear localization of these factors. As a proof of the proposed model, crossing the H^{+}/K^{+} -ATPase /Cdx2 mice with transgenic mouse lines that harbored mutations in the *p53* or *APC* genes led to accelerated gastric tumorigenesis by 30 weeks of age. In contrast to these neoplasia studies, it should be noted that Foxa3/Cdx2 did not develop neoplasia even when crossed onto the same C57/BL6 background as the H^{+}/K^{+} -ATPase /Cdx2 mice, nor when these mice were fed a high-salt diet that has been associated with gastric adenocarcinoma in some human populations [151]. Despite these limitations, together these findings suggest ectopic Cdx1 or Cdx2 expression in gastric epithelium is sufficient to induce gastric intestinal metaplasia and adenocarcinoma in murine model systems.

Regulation of Cdx2 expression in GIM

The molecular mechanisms regulating normal *CDX2* gene expression in the intestine and during development are imperfectly understood. Yet despite these limitations, there has been some advance in our understanding of mechanisms regulating *CDX2* gene expression in GIM. A single factor, BMP4, has been identified as a possible inducer of ectopic *CDX2* gene expression. BMP4 is an extracellular signaling peptide with roles in a number of developmental systems [152]. Of interest, BMP4 has an important role in promoting differentiation of intestinal epithelial cells, and mutations in members of this pathway have been associated with juvenile polyposis syndrome, which is a hamartomatous syndrome of the intestine and colon [153, 154].

In studies of human biopsy specimens, BMP2 and 4, as well as the cell-surface receptor BMPRII, and the BMP intracellular signaling factor SMAD4, were all found to be elevated in 56% of GIM foci examined [69]. To establish a functional role for BMPs in the induction of GIM, studies were performed in AGS cells, a human gastric adenocarcinoma cell line that developed in the setting of GIM. When BMP2 and BMP4 were added to the AGS cells in culture, CDX2 expression levels were increased. Moreover, when SMAD4 levels were experimentally reduced in these cells, CDX2 levels were similarly reduced. Studies with the CDX2 promoter demonstrated it could be transactivated by SMAD4 and SMAD1. Based on these observations, it was concluded that the BMP pathway is active in intestinal metaplasia and that BMP2 and BMP4 regulate CDX2 expression and promote intestinal differentiation through the canonical SMAD signal transducers [69].

In contrast to BMP4 induction of CDX2 gene expression, several other factors have been identified that normally suppress CDX2 in the gastric epithelium. These factors are generally lost or disrupted in GIM, and this loss coincides with CDX2 induction. SOX2 is a developmentally important transcription factor suggested to promote normal gastric epithelial development and inhibit GIM. In one study, SOX2 levels gradually diminished from gastritis through GIM and inversely correlated with CDX2 levels [155]. Whether loss of SOX2 in GIM is an etiologic cause for ectopic CDX2 gene expression will need to be clarified in future studies.

The data are stronger regarding Sonic hedgehog (Shh). Shh and its receptor Patched (Ptch) control gastrointestinal patterning in general, and gastric gland differentiation in particular [156]. Shh binds to its receptor, relieving Ptch repression of Smoothened (Smo). Smo then induces stabilization of the transcription factor Gli by its dissociation from a SuFu-containing protein complex, which in the uninduced state targets Gli for degradation. Stabilized Gli then translocates to the nucleus and induces target gene transcription. High levels of Shh are found in parietal cells, while Ptc is expressed in the chief cells of the fundic glands. Atrophic changes in the gastric epithelium, specifically the loss of parietal cells, leads to the loss of Shh expression and signaling, and is associated with ectopic expression of CDX2. This relationship between Shh and Cdx2 is further supported by studies with Shh-null mice. These mice exhibit changes in their gastric epithelium that is reminiscent of an intestinal phenotype [157]. Together, these findings implicate Shh as an essential signal for fundic gland differentiation that also prevents GIM. In its absence, such as in the setting of atrophic gastritis, CDX2 can be ectopically expressed and promote intestinalization of the involved gastric epithelium.

Lastly, while epigenetic regulation of CDX2 gene occurs in several different contexts [158–161], it does not appear to be involved in the induction of GIM. Methylation-specific PCR analysis found no significant *CDX2* promoter methylation in both normal and GIM gastric tissues [162]. Therefore CDX2 expression in intestinal metaplasia is not attributable to demethylation, but rather to aberrant transcriptional activation. In summary, GIM is a pre-neoplastic lesion consisting of the transdifferentiation of the gastric mucosa to an intestinal phenotype. Both CDX1 and CDX2 are ectopically expressed in human GIM, and forced ectopic Cdx expression has been shown to be sufficient to induce extensive GIM development in mouse transgenic models. Together, these findings establish a causative role for the CDX transcription factors in human GIM.

IX. The Roles of Cdx1 and Cdx2 in GERD-induced Barrett's Esophagus

The Pattern of CDX1 and CDX2 Expression Observed in GERD and in Barrett's Esophagus

The differentiation of an intestinal-type epithelium in the esophagus is thought to depend on the expression of the master regulator of intestinal epithelial development, Cdx2 [55]. The human *CDX2* gene is aberrantly expressed in metaplastic lesions of the esophagus, as it is in other examples of intestinal metaplasia like the liver and gall bladder [163–165]. Several studies have demonstrated CDX2 expression by immunohistochemistry in biopsy specimens in 100% of the BE samples analyzed [165, 166]. These included BE without dysplasia, BE with dysplasia, and EAC biopsies. Expression of the CDX proteins were detected in all BE cells, including goblet cells. In fact, both CDX1 and CDX2 expression can be detected in chronic esophagitis without intestinal metaplasia as well as Barrett's esophagus samples. CDX1 expression has been detected in the reactive, inflamed stratified squamous epithelium adjacent to Barrett's esophagus epithelium [109, 167]. Likewise, CDX2 expression has been detected in inflamed squamous epithelium [165, 166, 168]. Examination of 19 squamous epithelium biopsy specimens from patients with Barrett's esophagus revealed expression of CDX2 in 6 of the samples [169]. Interestingly, the expression of CDX2 in reactive squamous epithelia precedes the expression of sucrose-isomaltase, villin, mucin 2, alkaline

phosphatase, guanylyl cyclase C, and defensins which are additional markers of Barrett's esophagus (Figure 8) [168, 170–172]. These data demonstrate that induction of CDX genes precedes the morphologic changes that define Barrett's esophagus and suggest that CDX1 and/or CDX2 may play a significant role in the pathogenesis of BE. Lastly, although CDX2 expression can easily be detected in well or moderately well differentiated EAC, expression is decreased and can even be lost in poorly differentiated EAC [173, 174]. This suggests that while CDX expression is likely to play a causative role in the development of BE, it may also have an inhibitory role in the progression of esophageal adenocarcinoma.

Modeling BE using *in vitro* and *in vivo* systems

The first step in understanding the development of Barrett's esophagus is to know its cell of origin. Unfortunately, the cell of origin in human BE is not known. Most agree that stem cells from one of four tissue compartments are the most likely source for this cell, but there is as yet no consensus as to which it is. Currently, it is believed that the BE cell of origin emerges from 1) the esophageal squamous epithelium, 2) the distal esophagus submucosal gland epithelium, 3) the proximally-migrating gastric cardia epithelium, or 4) infiltrating bone marrow stem cells [80, 175].

Strong support exists for stem cells from the stratified squamous epithelium as the likely cell of origin for BE [176–178]. These are the most abundant stem cell type where the BE emerges. Furthermore, they are directly injured by the acid and bile refluxate. It is possible this refluxate, and the inflammatory microenvironment that develops in its response, can reprogram these stem cells to adopt an intestinal cell fate. Support for this hypothesis comes from several sources. We know from studies of esophageal development that the esophageal tube is initially lined with a simple columnar epithelium [179]. At 18 weeks of age, however, this columnar epithelium transforms into that of a squamous epithelium. This event appears to be independent of significant proliferation within the squamous cells or apoptosis of the columnar cells. This suggests the committed columnar cells can differentiate into squamous cells. This is, by definition, a transdifferentiation process. If indeed the embryonic esophageal epithelium can undergo a transdifferentiation event, it might be possible that the mature squamous epithelium under conditions of acid, bile salts and inflammation may undergo a similar process, only in reverse. This is especially intriguing in light of newly discovered approaches to derive induced pluripotent stem cells (iPS) from differentiated fibroblasts and other cell types [180]. A cell's differentiation state may thus be more plastic than previously believed.

However, other candidate sources for the BE progenitor cell are supported by studies as well. Stem cells contained within the submucosal glands of the distal esophagus may be altered by the refluxate and inflammatory microenvironment. Due to epithelial erosion of the squamous epithelium in response to acid and bile reflux, the glandular cells may proliferate as a repair response and replace squamous keratinocytes [181]. Similarly, it is possible that bone marrow derived stem cells (BMDC) may be the BE cell of origin [182]. It is known that BMDCs can be identified in many adult organs and in some circumstances contribute to tissue repair following injury. This is supported by studies in a mouse model for GIM, in which BMDCs are found in areas of chronic inflammation and intestinal metaplasia [183].

Lastly, gastric cardia cells migrating proximally after GERD-associated tissue injury have also been proposed as the BE cell of origin [80, 175]. In all these proposed mechanisms, ectopic Cdx1 and Cdx2 expression must be elicited to induce the intestinal cell morphology and gene expression patterns associated with Barrett's Epithelium, as none of these proposed cells of origin normally express these transcription factors in their native environments.

Despite not being certain about the cell of origin for BE, several groups have tried to model BE development in tissue culture systems *ex vivo*. These studies have focused exclusively on determining if esophageal squamous keratinocytes can be induced to differentiate into BE epithelial cells, since cell lines for the other proposed progenitor compartments do not presently exist. In an early paper on this subject, chronic acid and bile acid treatment of rat and murine esophageal keratinocytes induced low but detectable levels of Cdx2 expression [184, 185]. However, these cells retained their keratinocyte morphology as an intestinal cell phenotype was not induced. Subsequently, it was demonstrated that primary human esophageal keratinocytes treated with BMP-4 begin expressing BE-associated cytokeratins and a more intestinalized pattern of gene expression [186]. Cdx expression was not, however, induced by BMP4 treatment.

Several studies have utilized an immortalized human esophageal keratinocyte cell line called HET1A. Treatment of HET-1A cells with acid, bile, DNA methyltransferase inhibitors like 5-azacytidine, or the growth factor HB-EGF, all induced ectopic CDX2 mRNA and protein expression [161, 187]. More specifically, forced ectopic CDX2 expression in these HET-1A cells was associated with increased cell proliferation and the induction of intestinal genes including Guanyl Cyclase-C, Villin, cytokeratin 20, and MUC2 [161]. One limitation of these studies, however, is that HET-1A cells are immortalized by ectopic expression of the SV40 T antigen, a viral oncoprotein which inactivates p53 and the retinoblastoma protein (pRb), among other targets [188]. Since inactivating p53 mutations are acquired late in Barrett's esophagus [189], HET-1A cells may therefore not be an ideal cell line in which to study CDX2-mediated intestinalization.

This concern has been substantiated by studies using a normal human esophageal keratinocyte cell line known as EPC-hTERT. EPC-hTERT cells are immortalized only by ectopic expression of human telomerase protein (hTERT) [190]. hTERT expression is not known to alter a cell's pattern of proliferation or differentiation, nor are apoptotic and senescence pathways affected. Forced Cdx2 expression in these cells severely inhibited cell proliferation and did not induce intestinalization [191, 192]. EPC-hTERT cells would not stably express Cdx2 unless cell proliferation was enhanced by the overexpression of cyclin D1. Moreover, in those cells engineered to stably express both cyclin D1 and Cdx2, Cdx2 expression was not sufficient to induce significant intestinalization. However, agents that altered DNA methylation patterns and promoted chromatin remodeling synergized with Cdx2 to enhance the expression of intestine-specific and BE-associated genes including Villin, Carbonic Anhydrase I, Na-H Exchanger 2, cytokeratin 20, and SLC26A3 [191, 192]. Considering the difference in response between the HET-1A and EPC-hTERT cells to Cdx2 expression, it suggests the choice of cells can influence experimental outcomes.

As has been discussed previously, the current model for BE pathogenesis holds that acid and bile reflux into the esophagus can induce an inflammatory response and tissue injury that results in BE. Rodent surgical models appear to verify this hypothesis. Rats have historically been used for surgical anastomosis of the esophagus to the small intestine, either joining to the duodenum (EDA or EGDA models) or jejunum (EJA) [193–195]. These surgeries induce reflux into the esophagus in both models, but the EJA only allows for the refluxate to contain bile salts, while the EDA and EGDA surgeries contain both acid and bile salts. Both types of surgeries result in the development of a BE-like epithelium and esophageal adenocarcinoma in rats. *Cdx2* expression is detected in both the resulting glandular metaplasia as well as some of the surrounding reactive and proliferative squamous epithelium [158, 196, 197]. *Cdx* protein was also less frequently detected in the resulting adenocarcinomas that arose in a portion of the animals undergoing the surgery, which is consistent with the pattern for *CDX2* in human BE and esophageal adenocarcinomas [11]. Attempts have been made to perform the EJA surgery in mice. Thus far, success has been limited due to anatomy (this is a difficult surgery in mice since they are much smaller than rats) and outcomes (less frequent intestinal metaplasia than with rats) [198].

As with any system, there are limitations of these models that may effect the experimental interpretations. These models use a reflux system to induce changes in the distal esophagus, but the nature of the reflux is important. The timing and duration of reflux appears to be critical in the development of BE. Reflux patients appear to develop BE when the reflux is long in duration rather than being brief and pulsatile in nature. Additionally, these models never address the cell of origin. The intestinal columnar epithelium is surgically placed immediately adjacent to the squamous epithelium of the esophagus, and intestinalization is seen in the esophagus. One concern is that this may not be a true metaplastic process at all. Rather, it may simply be an expansion of the intestinal epithelium into the lumen of the esophagus to repair the esophageal mucosa that is ulcerated and eroded by the bile and acid refluxate. This is a very important issue that will need to be addressed to validate the rat surgical model for continued experimental use.

Signaling Pathways Regulating *Cdx* Expression in BE

It is clear that many factors such as acid, bile salts, and inflammation within the microenvironment of the esophageal epithelium contribute to the morphogenesis of BE. These have all been shown to induce expression of the *CDX2* gene [161, 168, 184, 199]. What has been less well defined are the intracellular signaling events that control this process. Many inflammatory and acid or bile acid-response pathways converge on NF κ B signaling [185, 200]. The *CDX2* gene is a well-established target gene of NF κ B [58]. Moreover, NF κ B appears to be a critical regulator of *CDX2* expression in Barrett's esophagus [185, 200]. In a recently reported study, *CDX2* gene expression was induced in human esophageal keratinocytes that were exposed to acid and bile salts. This induction could be blocked by inhibition of NF κ B. An unusual aspect of this study was the finding that keratinocytes from BE patients responded differently than keratinocytes from non-BE patients to acid and bile salts. Specifically, the p50 subunit of NF κ B, which was required for induction of the *CDX2* promoter, was activated only in keratinocytes from BE patients. The reason for this difference in response was not immediately clear, but suggests that some

patients may be predisposed to develop BE if they activate the NF κ B p50 subunit in response to acid and bile reflux. Further research into differences between BE patients and GERD patients without BE appears warranted.

Additional signaling pathways that may induce CDX expression are retinoic acid and hedgehog signaling. Retinoic acid plays an important role in the proper differentiation program of stratified squamous epithelium. Modulation of RA signaling has been implicated in BE pathogenesis [201]. Lithocholic acid, a bile acid, can compete with RA for binding to the Retinoid X Receptor, RXR. Additionally, RA synthesis is increased in BE and is necessary for normal columnar cell differentiation. Treatment of explant cultures of normal human esophagi with all-trans retinoic acid induces ulceration and sloughing of the stratified epithelial cells with concomitant proliferation of submucosal glands. These submucosal glands replace the epithelium at the surface of the explants. These data are intriguing in that they support the submucosal gland theory as a site of origin for the BE cells. However, it is unclear if these cells change into a fully differentiated BE epithelium after RA treatment. Nonetheless, it establishes RA signaling as a potential inducer of BE. RA signaling also induces PI3K expression and activation which has been linked to NF κ B induction and therefore may induce CDX2 transcription via this pathway.

Embryologic observations, as well as data from GIM, suggest a role for the BMP4 and Hedgehog pathways in BE development. Sonic hedgehog (Shh) signaling is involved in the embryonic development of the esophagus and trachea. In the embryonic esophagus, Shh expression can be detected in esophageal endoderm [202, 203]. This expression induces Hedgehog signaling in mesenchymal cells. These activated mesenchymal cells are then induced to express BMP-4, a Gli transcriptional target [204]. This epithelial-mesenchymal interaction appears to be important to maintain the columnar cell fate of the early esophagus. At the time of the replacement of the columnar cells with a squamous epithelium, a BMP antagonist, noggin, is expressed in the epithelium and inhibits the BMP4 signal from the mesenchymal cells and thereby induces a squamous cell fate [205].

Shh is not expressed in adult esophageal tissue, despite the early embryologic expression. A recent publication explored the reestablishment of Shh signaling as a potential cause in the metaplastic process of BE [204]. It was demonstrated that both Shh and the related Indian Hedgehog (Ihh) factors were induced in murine esophageal and HET-1A cells in response to acid and bile acid exposure. In response, Ptc-expressing mesenchymal cells were induced to express BMP-4. BMP-4, in turn, stimulated esophageal squamous cells to express the transcription factor SOX9, which is known to induce the expression of keratins 8/18 and DMBT1 in columnar cells. These findings are consistent with an earlier report implicating BMP4 in Barrett's pathogenesis [186] and with CDX2 expression in GIM [69]. Together these findings establish Hedgehog and BMP4 signaling as important regulatory pathways that contribute to the development of BE in patients with reflux disease.

Another transcription factor that is important for the development of BE is MYC. Cytokine signaling may be important in MYC induction, which appears to be downstream of TNF α signaling [206]. IHC staining has demonstrated that MYC is expressed in BE [167]. Interestingly, MYC expression increases in dysplastic lesions and reaches its highest levels

in EAC. Recently, MYC activation has also been implicated in BE pathogenesis by microarray analysis [167]. While MYC mRNA levels were not significantly changed in BE tissues compared to normal controls, the levels of several negative regulators of MYC, i.e. MXI1 and MXD1, are down-regulated. Consistent with this finding, MYC RNA levels did not change in BE, but MYC protein levels were increased. Moreover, the MYC targets ODC1 and CA2 were both induced in BE epithelium compared to normal controls, further supporting an increase in signaling along the MYC axis. A role for MYC in BE pathogenesis was further substantiated by co-expression of MYC with Cdx1 in EPC-hTERT cells. This co-expression results in the induction of a gene expression pattern that is consistent with that observed in BE. When these co-expressing cells were cultured in a 3-dimensional culture system, they remained morphologically as stratified squamous epithelium but interspersed within the differentiating cell layer were mucin-expressing cells reminiscent of goblet cells, suggesting a partial shift in cell differentiation away from the keratinocyte lineage. Other critical signaling pathways may synergize with C1 and MYC to shift further these cells toward a BE cell fate.

Lastly, unlike with GIM, epigenetic modifications of DNA occur in BE and appear to be a critical contributor both to BE pathogenesis as well as the progression to EAC. Methylation patterns have been studied for numerous genes in BE, and many genes have altered expression based on changes in the methylation of their promoters [207–212]. One report identified six genes out of a panel of twenty with significantly increased DNA methylation in the Barrett's tissue when compared to normal esophagus and stomach. This methylation typically targets known tumor-suppressors such as p16INK4, p14ARF, and AKAP12, and silences their expression [207, 213, 214]. This methylation was found to be progressive, increasing with each step in the sequence through dysplasia to esophageal adenocarcinoma.

Also among the genes whose expression is regulated by methylation in BE are *CDX1* and *CDX2* [109, 159, 161]. However, unlike p16INK4 and other tumor suppressor genes, the *CDX1* and *CDX2* genes appear to be demethylated in BE cells compared to normal squamous esophagus. A study of CDX1 expression in endoscopic biopsy samples was performed by bisulfate sequencing and demonstrated that most samples had demethylated promoter sequences [109]. In fact, CDX1 induction by TNF α stimulation of NF κ B- is entirely dependent on the methylation status of the CDX1 promoter. The promoter must be nearly devoid of methylated cytosines for efficient induction by TNF α stimulation of cells. This links CDX1 expression to inflammatory stimulation, but the question remains as to how the gene is selected for demethylation in BE. CDX2 expression appears to be sensitive to methylation as well. In human biopsy specimens of normal esophagus, EAC, and esophageal squamous cell carcinomas (ESCC), the CDX2 gene was demethylated only in the EAC tissues [159]. Treatment of ESSC cell lines with 5-aza-2'-deoxycytidine (DAC) induced CDX2 expression in 65% of the cell lines tested. In another series of studies, treatment of HET-1A and SEG1 cells with acid, bile acids, or DAC demethylated the CDX2 promoter and induced CDX2 expression [161]. Together these observations establish that changes in epigenetic gene regulation contribute significantly to the processes that gives rise to Barrett's esophagus and esophageal adenocarcinoma in humans.

X. Summary and Conclusions

Intestinal metaplasia of the stomach and esophagus are interesting and clinically relevant lesions in humans. They arise in the setting of a chronic inflammatory condition, and are associated with an increased risk for the subsequent development of adenocarcinomas. The complete pathogenesis for these conditions remains to be established, however studies over the last two decades now allow us to postulate possible mechanisms. In the case of Barrett's esophagus, one current model suggests that inflammation from chronic acid and bile reflux induces changes in the expression of transcription factors that results in the reversion of keratinocytes into a more embryologically primitive columnar cell type (Figure 9). Coincident with this is the induction of the transcription factors CDX1 and CDX2. This appears to drive the expression of an intestinal cell phenotype in these cells. Future studies will need to more completely catalogue gene expression changes and chromatin remodeling events occurring prior to and with the onset of intestinal metaplasia, establish the "cell of origin" for the metaplasia, and develop novel animal and cell culture models if we are to better our understanding of these important diseases in order to improve patient care and prevent the associated cancers.

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Figure 1.

Example of the considerable morphologic changes that can occur in tissues with the development of a metaplasia. In this example, a multi-layer squamous epithelium is converted to a simple columnar type epithelium. This type of metaplastic change is classically observed in Barrett's esophagus.

Figure 2.

Schematic of the domains critical for Cdx2 transcriptional activity including the DNA binding homeodomain and putative transactivating domain. Cdx1 has a similar structure (not shown). Phosphorylation sites at Serine 60 and Serine 281 are also indicated.

Phosphorylation of Cdx2 at these sites reduces Cdx2 transcriptional activity (Serine 60) and protein stability (Serine 281).

Figure 3.

The transcription factor Cdx2 regulates a number of functions critical to intestinal epithelial development, differentiation, and morphology. Best studied are Cdx2's role in promoting the intestinal cell differentiation and the intestine-specific expression of genes whose products support intestinal epithelial cell functions. Also well studied is the critical role played by Cdx2 at promoting cell to cell adhesion and the adoption of a polarized, columnar cell morphology. Less well understood are Cdx2's regulation of intestinal cell apoptosis, proliferation, and chromosomal stability.

Figure 4.

Histologic changes associated with metaplasia in Barrett's esophagus. **A.** Hematoxylin & Eosin staining of normal esophagus demonstrating a uniform multilayered squamous epithelium. **B.** Hematoxylin & Eosin staining of tissue demonstrating intersection of Barrett's esophagus with normal squamous epithelium. **C.** Higher-powered magnification of the Barrett's epithelium stained with Hematoxylin & Eosin. Goblet cells are visible with blue inclusions. **D.** Alcian blue staining of Barrett's esophagus tissue reveals expression of intestinal mucins.

Figure 5. Diagram illustrating anatomic location of Barrett's esophagus. Endoscopic views of the gastroesophageal junction in a normal individual and a patient with Barrett's esophagus. Endoscopic photos kindly provided by Nuzhat Ahmad M.D.

Figure 6.

Endoscopic views of esophageal adenocarcinomas occurring at the gastroesophageal junction. Hematoxylin & Eosin staining of an esophageal adenocarcinoma (EAC) arising in the setting of Barrett's esophagus (BE). Endoscopic photos kindly provided by Nuzhat Ahmad M.D.

Figure 7.

Inflammatory microenvironment provokes metaplasia in Barrett's esophagus by several mechanisms. Gastric acid and bile acid reflux in GERD injures the esophageal epithelium, inducing an inflammatory response. Cytokines expressed by both injured epithelial cells and infiltrating inflammatory cells contribute to metaplasia in a number of ways. 1. Induce cell proliferation and inhibit the tumor-suppressor apoptosis pathways in injured and damaged epithelial cells. 2. Proinflammatory cytokines stimulate ROS and eicosanoid production by phagocytic and non-phagocytic cells (including epithelial cells), increasing oxidative stresses and DNA mutagenesis. 3. Cytokines and chemokines are chemotactic and provoke inflammatory cell migration into the involved tissue. Moreover, cytokines influence the differentiation and activity of dendritic, macrophage, lymphocytic, and NK cells. 4. Cytokines signal to endothelial cells and stimulate angiogenesis. Within the epithelial cells, the combination of ROS damage, alterations in DNA methylation patterns, and activation of the NFκB signaling pathway (p50 subunit is especially important) by cytokines and the acid/bile refluxate contribute to activation of targets genes that further amplify the inflammation and ROS (Cox-2) and promote intestinal differentiation (Cdx2).

Figure 8.

Histology of Barrett's esophagus. **A.** Hematoxylin & Eosin staining of Barrett's esophagus demonstrates a uniform columnar morphology with the presence of occasional goblet cells. **B.** Immunohistochemistry (IHC) staining for Keratin 20 labels all Barrett's cells. **C.** IHC for Muc5AC labels both columnar and goblet cells. **D.** IHC for Muc2 localizes to the goblet cells. **E.** IHC for Cdx1 labels Barrett's epithelium and not the adjacent squamous epithelium and localizes to the nucleus. **F.** IHC for Cdx2 labels the nuclei of Barrett's cells.

Figure 9.

Schematic model for the induction of metaplasia in Barrett's esophagus, based on the ectopic expression of a number of developmentally important transcription factors that occurs in the setting of chronic inflammation due to acid and bile reflux into the esophagus in GERD patients.

Table 1

Examples of metaplasias in human tissues.

Tissue	Normal Epithelium	Metaplastic Epithelium	Provoking Stimulus
Airways	Columnar epithelium	Squamous epithelium	Cigarette smoke
Urinary bladder	Transitional epithelium	Squamous epithelium	Bladder stone
Sinonasal	Columnar epithelium	Intestinal columnar epithelium	Wood dust
Gall Bladder	Columnar epithelium	Squamous epithelium	Gall stones
Esophagus	Squamous epithelium	Intestinal columnar epithelium	Gastro-esophageal reflux
Stomach	Gastric columnar epithelium	Intestinal columnar epithelium	Chronic <i>Helicobacter pylori</i> infection