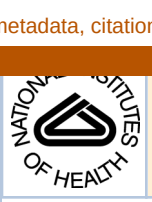

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Molecular Mechanism of Barrett's Esophagus

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Abstract

Barrett's esophagus (BE) is defined as metaplastic conversion of esophageal squamous epithelium to intestinalized columnar epithelium. As a premalignant lesion of esophageal adenocarcinoma (EAC), it develops as a result of chronic gastroesophageal reflux disease (GERD). Many studies have been conducted to undertand the molecular mechanism of this disease. This review summarizes recent results of involving squamous transcription factors, intestinal transcription factors, signaling pathways, stromal factors, microRNAs, and other factors in the development of BE. A conceptual framework is proposed to guide future studies. We expect elucidation of the molecular mechanism of BE will help us develop proper management of GERD, BE, and EAC.

Keywords

Barrett's esophagus; transcription factor; signaling pathway

Introduction

There are two major histological types of esophageal cancer, squamous cell carcinoma (ESCC) and adenocarcinoma (EAC). ESCC arises in squamous epithelial cells that line the esophagus, and it usually occurs in the upper and middle part of the esophagus. It is known that ESCC develops as a result of carcinogen exposure in a pathological sequence of hyperplasia, dysplasia and carcinoma. In contrast, EAC arises from metaplastic glandular tissue in the lower third of the esophagus. It is thought to develop as a result of long-term gastroesophageal reflux in a pathological sequence of reflux esophagitis (GERD), Barrett's esophagus (BE), dysplasia and adenocarcinoma (1).

Baseline risk for EAC is quite low in the general population. It is 30–125 times higher in patients with BE (2, 3). Although there is still debate regarding whether BE is always needed for the development of EAC, recent studies have suggested that most, if not all, EAC develops from existing BE (4). On the other hand, BE is a common clinical identity, with as many as 3 million Americans harboring this lesion (5). The yearly risk for EAC in non-dysplastic BE is approximately 0.5% per person-year (6, 7), and the majority of subjects with this condition never progress to EAC. However, in the minority that do progress to cancer, EAC has a poor prognosis with a five-year survival rate of less than 15%, according

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to data from the NCI Surveillance Epidemiology and End Results. Our poor understanding of the pathogenesis of BE and EAC has limited our ability to stratify risk for BE among the enormous numbers of subjects with GERD, and treatments for the condition are limited. Therefore, it is very important to study the pathogenesis and molecular mechanism of BE in order to design evidence-based and scientifically sound interventions to prevent EAC.

Conceptual framework

In theory, BE, the metaplastic conversion of esophageal squamous epithelium to intestinalized columnar epithelium, may develop by two distinct mechanisms (8). One possibility is direct conversion of differentiated cells, a process called *transdifferentiation*. Alternatively, it may develop from altered differentiation of *stem cells*. In general, the stem cell theory is favored by most researchers, although there is no solid experimental evidence to exclude the possibility of transdifferentiation.

There are four potential cellular origins of BE in human esophagus, each supported by experimental data (9, 10). *1) Stem cells of squamous epithelium* in the basal cell layer may undergo *de novo* metaplasia. The resulting metaplastic change produces stem cells for future BE. *2) Cells at the gastroesophageal junction* or transitional zone may colonize the gastric cardia or distal esophagus in response to noxious luminal contents. *3) Stem cells in the neck of esophageal submucosal gland duct* may colonize the esophagus when mucosal damage of the squamous epithelium takes place. *4) Bone marrow-derived stem cells* may colonize the esophagus when there is inflammation or damage, and undergo metaplasia (11–13).

At the cellular level, four major cell lineages are present in BE, columnar epithelial cells, Paneth cells, enteroendocrine cells, and goblet cells. Each individual cell lineage has a unique differentiation program as we have learned from intestinal development. These cells are expected to interact with each other and other cells in the stroma, such as inflammatory cells, fibroblasts.

At the molecular level, metaplasia is believed to be mediated by activation or inactivation of transcription factors (14). Therefore, transcription factors that regulate expression of target genes specific for certain cellular functions may be regarded as “drivers”. Genes involved in other functions such as celluar structures, metabolism and defense, are likely to be “passengers”. Through analysis of gene expression data some of these drivers and passengers can be fished out. The main challenge is to verify the true drivers. Because of the complexity and heterogeneity of the Barrett’s phenotype, we would expect: (1) multiple drivers may participate in the development of BE, yet some drivers may be essential and others dispensable; (2) drivers may regulate expression of themselves. To verify drivers, we would expect *in vitro* experiments help us distinguish drivers from passengers with gene expression, cellular morphology and functions as the end points. *In vivo* experiments are needed to verify true drivers with histopathology, gene expression and tissue functions as the end points. Besides the individual genes, several signaling pathways are known to be involved in BE. Linking signaling pathways and drivers into a molecular network is challenging, but making these connections will explain the molecular mechanism of BE.

With this conceptual framework in mind, we first analyzed microarray and SAGE datasets in the public databases with bioinformatics tools to identify potential drivers, passengers, and signaling pathways involved in the development of BE (15). Out of 14 published studies in the literature, three microarray datasets (two cDNA arrays and one oligo array) and one SAGE dataset met our criteria of data inclusion. SAM is used for identifying differentially expressed individual genes, SAGE (Poisson) for analysis of SAGE data, and GSEA for identifying an *a priori* defined set of genes differentially expressed in BE. These gene sets are either grouped according to a certain signaling pathway (GSEA curated), or the presence

of a consensus binding sequence for a known transcripton factor in the promoter regions (GSEA motif). Fifty-five BE genes and thirteen NE (normal esophagus) genes were identified as differentially expressed genes (cutoff >4 fold) by both SAM and SAGE (Poisson). Using immunohistochemical staining, we further narrowed this list to four categories of genes: Category I contains 25 genes expressed in BE only; Category II contains 5 genes expressed in NE only; Category III contains 12 genes expressed more in BE than in NE; and Category IV contains 2 genes expressed more in NE than in BE. Furthermore, with immunohistochemical staining of human BE tissues, we confirmed loss or downregulation of transcription factors related to development of esophageal squamous epithelium (e.g., P63, Sox2, Pax9) and overexpression of transcription factors related to intestinal development (e.g., Cdx1, Cdx2, HNF1α, HNF3α, HNF3β, HNF3γ, HNF4α, GATA4, GATA6, Sox9, Math1). Besides individual genes, we also identified the TGFβ/BMP signaling pathway as an active pathway in BE.

Our data suggest that when esophageal epithelial stem cells are stimulated by gastroesophageal reflux, the squamous differentiation program may be inactivated through loss of expression of squamous transcription factors. Meanwhile, the columnar differentiation program may be activated through expression of intestinal transcription factors. These molecular events may lead to inactivation of squamous differentiation and activation of columnar differentiation, which produces four major cell lineages in BE: columnar epithelial cells, Paneth cells, enteroendocrine cells, and goblet cells (16). Since differentiation of each of these cell lineages and squamous epithelial cells may require different sets of drivers, the overall molecular mechanism is expected to be very complex, even before considering interactions among these cell lineages and the stroma.

1. Squamous transcription factors: P63, Sox2 and Pax9

P63 is a critical initiator of epithelial stratification and a key regulator of cell adhesion and survival of progenitor cells in squamous epithelium (17–19). In mouse esophagus, when basal cells become differentiated, p63 is down-regulated and eventually turned off in terminally differentiated superficial cells (20, 21). In p63-deficient mice, embryonic esophageal epithelium appears columnar containing both ciliated and goblet-like cells (22). In human esophagus, p63 is expressed in the basal layer, suprabasal cell layer and submucosal glands of esophageal squamous epithelium (20, 23). When esophageal squamous epithelial cells are exposed to bile and acid, p63 becomes down-regulated (24), explaining why p63 is lost in BE (25).

Sox2 is a member of the *Sry*-like high mobility group domain protein family. It is expressed in the pharynx, esophagus, and stomach of chicken gut, but not in the lower gastrointestinal tract, where Cdx1 and Cdx2 are present (26, 27). Sox2 mutations are associated with esophageal atresia in anophthalmia-esophageal-genital syndrome (28). Sox2 is an amplified lineage-survival oncogene in lung and esophageal squamous cell carcinoma (29). Its down-regulation is associated with intestinal metaplasia in the stomach (30, 31). Mice with hypomorphic Sox2 developed mucus-producing cells in the esophagus, and had fewer p63-expressing cells. Interestingly, esophagus with a hypomorphic Sox2 expressed genes normally expressed in glandular stomach and intestine (TTF1, TFF1, TFF2, Agr2, etc.) (32). In fact, these genes which are negatively regulated by Sox2 were found to be overexpressed in human BE. These data suggest a potential role of loss of Sox2 in the development of BE. Indeed, Sox2 is not expressed in human BE or in a rat model (25). Although a repressive interaction between Cdx2 and Sox2 was found to occur at the prospective stomach-intestine border during development (33), Sox2 expression was upregulated in the intestinal metaplastic mucosa of Cdx2-transgenic mice (34). This study suggested that Cdx2 may

mediate intestinal metaplasia by overriding the function of Sox2 without inactivating its expression.

Pax9 belongs to a group of nine transcription factors characterized by the presence of a DNA-binding "paired" domain. It is critical for development of mouse thymus, parathyroid, limbs, palate, teeth and vertebral column. In adult mice, its expression is confined to the tongue, esophagus, salivary glands and thymus (35–37). Loss of Pax9 impairs terminal differentiation of squamous epithelial cells of mouse tongue (35). In humans, Pax9 is significantly reduced in dysplasia and squamous cell carcinoma, suggesting a role for Pax9 in the normal differentiation process of esophageal squamous epithelium (38). In human BE, Pax9 expression is significantly reduced (15), but it is unclear how down-regulation of Pax9 may contribute to BE.

2. Intestinal transcription factors

a. Caudal-related homeobox gene (Cdx1 and Cdx2)

As members of the Caudal-related homeobox gene family, Cdx1 and Cdx2 are critical for intestinal development. They have mostly overlapping, but also certain distinct, functions in intestinal development (39, 40). Relatively speaking, Cdx2 may play a dominant role in regulating intestinal cell differentiation (41). However, Cdx1 and Cdx2 may regulate expression of each other in a reciprocal manner (42).

Cdx2 plays an important regulatory role in the development of intestinal metaplasia in the foregut, and in colon cancer development (39, 43). Two independent studies have shown that stomach-specific Cdx2 transgenic overexpression induced intestinal metaplasia in the mouse stomach within weeks after birth (44, 45). Homozygous knockout of Cdx2 is embryonically lethal, and heterozygous knockout produces colonic harmatomas with squamous epithelium appearing in the colon (46). These studies suggest that Cdx2 might be a pivotal switch between intestinal columnar epithelium and squamous epithelium in the gastrointestinal tract.

Several lines of evidence have suggested an important role of Cdx2 in the development of human BE: 1) In normal intestinal epithelium, Cdx2 is expressed in most cell lineages with Paneth cells having a lower level of expression than other cells (39). Squamous epithelial cells of normal human esophagus do not express Cdx2, while submucosal glands weakly express Cdx2 in the cytoplasm. In human BE, Cdx2 is expressed in both goblet and non-goblet cells (47). Dysplasia and adenocarcinoma may have decreased levels of Cdx2, or even lose Cdx2 expression. In EAC, a high level of Cdx2 expression is usually associated with well or moderately differentiated tumors (47–53).

2) A low level of Cdx2 mRNA was detectable by RT-PCR in biopsy samples of squamous epithelium of GERD patients, even before the appearance of Cdx2 protein, other marker genes, or histological metaplasia (48, 53).

3) Many "marker" genes of BE, such as villin, GCC, SI, are known to be transcriptionally regulated by Cdx2 (54–58). The presence of goblet cells is diagnostic of human BE, and Cdx2 regulates expression of critical genes of goblet cell differentiation, such as Muc2 and TFF3 (59, 60). More importantly Cdx2 regulates expression of many transcription factors essential for intestinal development, such as Cdx1, Cdx2, Math1, Hox genes, KLF4, HNF1 β, HNF3β, HNF4α, GATA5, GATA6, and Hes1 (61). For example, Math1 (also called Atoh1 or Hath1) is a transcriptional target of Cdx2 (60), and plays a critical role in the development of goblet cells: Cdx2 transfection into IEC6 cells and human esophageal squamous epithelial cells upregulates Math1 (59, 62). Knockout of Hes1, a direct upstream

negative regulator of Math1, leads to an increased number of goblet cells in mouse small intestine (63), whereas loss of Math1 completely prevents the development of goblet cells in mouse intestine. Development of Paneth cell and enteroendocrine cells is also suppressed (64).

4) Treatment of human and rodent esophageal squamous epithelial cells with either acid or bile acids, which mimics gastroesophageal reflux, induces expression of Cdx2 (59, 65, 66).

5) Transfection of Cdx2 into human esophageal squamous epithelial cells induces metaplastic changes in morphology and gene expression (59). HET1A cells with stable transfection of human Cdx2 form crypt-like structures *in vitro*. Microarray analysis and quantitative real-time PCR showed that stable transfection of Cdx2 up-regulated differentiation markers of intestinal columnar epithelial cells and goblet cells in HET1A cells. This may be partially due to modulation of Notch signaling pathway.

6) Transgenic overexpression of Cdx2 in mouse esophagus interferes with differentiation of normal esophageal squamous epithelial cells, and induces a potentially transitional cell type between squamous epithelial cells and columar epithelial cells (67).

Apart from Cdx2, Cdx1 is also involved in the development of human BE (68, 69), although Cdx2 expression precedes Cdx1 expression (70). Similar to Cdx2, transgenic overexpression of Cdx1 induces intestinal metaplasia in the stomach. However the Cdx1 phenotype is not exactly the same as Cdx2 phenotype (71). Cdx1 mRNA and protein are universally expressed in BE, but not in normal esophageal squamous epithelium. This tissue-specific expression is attributable to the methylation status of the Cdx1 promoter (68, 69).

Expression of Cdx1 and Cdx2 is regulated by promoter methylation and single nucleotide polymorphisms (SNPs):

1. Promoter methylation is a well-known mechanism for gene silencing or activation. Embryonic mouse esophagus has the keratin 8 promoter methylated when columnar epithelium transdifferentiates into squamous epithelium during embryogeneis (72). Many genes on the TGFβ and WNT signaling pathways are frequently hyper- or hypomethylated in BE. Cdx1 and Cdx2, as well as GATA4, GATA6, Muc2, are all regulated by promoter methylation. When human esophageal epithelial cells are exposed to acid and/or bile treatment, activation of gene expression is commonly associated with reduced level of promoter methylation (59, 69). Even in normal esophagus, treatment with 5-aza-2′-deoxycytidine upregulates Cdx2 expression (67). Interestingly, loss of promoter methylation and activation of NFκB pathway synergize in inducing Cdx1 expression in human esophageal epithelial cells, when they are exposed to bile and proinflammatory cytokines (69).
2. SNPs of Cdx1 and Cdx2 genes have not yet been well characterized. Two previous studies failed to associate several SNPs of Cdx2 with colon cancer (73, 74). These SNPs (IVS1+1020, IVS1+1476, IVS1−1575, IVS1−1042, IVS1−34, IVS1−33, IVS2−429, Exon3+877, and 3UTR+10) were mostly located in a short noncoding region. However, it is known that a roughly 9.5-kb 5′-flanking region from the human Cdx2 gene contained key *cis* elements for regulating transcription in colon cancer cells (75). Wong *et al.* sequenced a 400-bp region upstream of exon 1 and all three exons of Cdx1 in 37 colon cancer cell lines. Three SNPs were identified with one each one in the promoter, 5′UTR and coding region of exon 1. None of these were associated with Cdx1 expression in these cells (76). In fact, the major regulatory region of human Cdx1 gene is far upstream. Transgenic expression of

the nucleotides −15,601 to +68 of the Cdx1 gene was restricted to the intestinal epithelium, which was identical to endogenous Cdx1 gene expression. DNase I hypersensitivity assays further narrowed two active chromatin regions at approximately −5.8 and −6.8 kb upstream of the Cdx1 gene, respectively (77, 78). Therefore, we believe that in order to find functionally important SNPs of Cdx1 and Cdx2 genes, larger areas in the regulatory regions need to be examined.

Two recent interesting observations deserve further studies. 1) Acid and bile treatment induced Cdx2 expression in esophageal squamous epithelial cells from patients with BE, but not from GERD patients without BE. This observation suggests that fine tuning of Cdx2 regulation in the esophagus might determine gene expression, and even susceptibility to BE (79). 2) miR-9 was found to regulate Cdx2 expression in gastric cancer cells (80), suggesting microRNAs may regulate, or be regulated by, Cdx2. Involvement of miRNAs addes a player of complexity to our understanding of the role of Cdx2 in BE.

b. Hepatocyte nuclear factors (HNFs)

HNFs are liver-enriched homeodomain-containing transcription factors that regulates many genes in the liver, pancreas and intestines (81–83). HNF1α and 1β control terminal differentiation and cell fate commitment in the intestine (84). GATA4, HNF1α, and Cdx2 have been shown to play cooperative roles in regulating expression of marker genes in intestinal epithelium and human BE (55, 82, 85, 86). Exposure of esophageal epithelial cells to bile induced expression of Muc4 through HNF1α (87). HNF1α regulates expression of differentiation markers of columnar epithelial cells, such as FABP1 and lactase-phlorizine hydrolase (88–91).

HNF3α is known to be amplified and overexpressed in human EAC (92). HNF3β plays a critical role in lung development (93). Knockout of HNF3β in the lung suppressed alveolarization and induced goblet cell hyperplasia (94). In the intestine, HNF3α and HNF3β regulate expression of Agr2 and Muc2, markers of goblet cells (95–97).

HNF4α is essential for embryonic development as well as homeostasis and function of adult intestine (98). HNF4α knockout mice failed to form normal colonic crypts due to suppressed epithelial cell proliferation. Goblet cell maturation and expression of a set of genes were also perturbed (99, 100). HNF4α is regulated by HNF1α/β and GATA6 (101). In return, HNF1α expression is activated by HNF4α and HNF4β, which may mediate the stimulating effects of TGFβ pathway (102, 103).

c. GATA4 and GATA6

GATA4 and GATA6 belong to a subfamily of the GATA transcription factor family involved in differentiation of mesoderm and endoderm-derived tissues. They are expressed in the stomach and small intestine, but not in the esophagus (104, 105). GATA4 and GATA6 regulate expression of intestinal differential markers alone or in concert with other transcriptional factors such as HNFs, Cdx2, TTF1 (89, 106–111). GATA4 regulates expression of HNF4 and is reciprocally regulated by TGFβ pathway (112, 113). GATA4 and the TGFβ signaling pathway cooperate in regulating intestinal epithelial gene expression (114), and GATA4/GATA6 activate BMP4 at the transcriptional level during early stages of embryogenesis (115). Normal esophagus does not express GATA4 and GATA6 (116). However, both are expressed in human BE and a rat model (15, 25). Aberrant promoter methylation and gene amplification of GATA4 and GATA6 have been reported in human EAC (117–119).

3. Signaling pathways

a. TGFβ and BMP signaling pathway

An important recent finding is the increased expression of BMP4 and activation of its signaling pathway in BE and GERD. Treatment of primary squamous cell with BMP4 induced squamous dedifferentiation and columnar differentiation (120, 121). In the normal adult esophagus, BMP4 is not expressed. When stimulated by reflux, the stromal cells may produce inductive factors such as BMP4, which impact development and homeostasis of the overlying epithelium. This hypothesis is in agreement with the role of BMP signaling pathway in intestinal and esophageal development (122–124).

Noggin, a BMP antagonist, is expressed in BE at a low level. It has been shown that 70% of homozygous noggin knockout mice developed esophageal atresia. Interestingly, reducing the gene dosage of BMP4 by 50% rescued the Noggin null phenotype (125). This study suggests that fine tuning of BMP4 expression has a critical role on esophageal morphogenesis. When BMP4 signaling is inhibited in the mouse intestine by transgenic overexpression of Noggin, villus morphogenesis is suppressed in the small intestine, and crypts become highly proliferative. Nevertheless, goblet cells, enteroendocrine cells and Paneth cells are normally differentiated (126).

Although EAC tends to have decreased levels of TGFβRII, Smad2 and Smad4 through promoter methylation (127, 128), it may not be the same at the stage of BE. Cdx2 was found to interact with SMAD3 independent of SMAD4, resulting stimulation of SMAD3 transcriptional activity. Cdx1 also interacts with SMAD3 by inhibiting the SMAD3/SMAD4-dependent transcription (129).

b. WNT signaling pathway

The WNT signaling pathway is well known for its critical role in gastrointestinal development and cancer development (130–132). Many studies have clearly shown than the WNT signaling pathway and its key component β-catenin are involved in the development of EAC. Targeting this pathway may have therapeutic effects on EAC (133). Although mutations of APC and β-catenin are rare in EAC, promoter hypermethylation (APC, SFRP1), downregulation of E-cadherin, nuclear translocation of β-catenin and WNT2 overexpression, are commonly seen in EAC (134–136).

Cdx1 and Cdx2 are downstream effectors of the activated WNT pathway that mediate intestinal metaplasia. Cdx1 is transcriptionally regulated by the WNT pathway (137, 138). In the stomach, CagA physically interacts with E-cadherin and impairs the complex formation between E-cadherin and β-catenin, causing cytoplasmic and nuclear accumulation of β-catenin. As a result, Cdx1 and a goblet cell marker (Muc2) are activated (139). In endometrial cancer cell lines, overexpression of an active form β-catenin resulted in a significant increase in endogenous Cdx2 expression, independent of TCF4. Cells overexpressing exogenous Cdx2 may inhibit β-catenin/TCF4-mediated transcriptional activation of target genes probably through indirect mechanisms (140). In the lung, transgenic overexpression of β-catenin disturbed normal development, and induced strong expression of Cdx1, Math1 and other genes characteristic of intestinal Paneth, goblet cells, and non-lung secretary cell types (141).

As a downstream target of WNT signaling pathway, Sox9 exerts negative feedback on the WNT pathway and Cdx2 (142–144). Sox9 is known to be expressed in the intestinal epithelium, and specifically in stem cells and Paneth cells. Knockout of Sox9 affected differentiation throughout the intestinal epithelium with a disappearance of Paneth cells. Other cell lineages (goblet, enteroendocrine, columnar cells) were more or less affected

(142, 145). Sox9 expression is also modulated by the TGFβ pathway (146), the NFκB pathway (147), and GATA4/FOG2 complex (148). A recent study has clearly shown a potential role of Sox9 in the development of BE (149).

SAM pointed domain-containing Ets transcription factor (Spdef) is a transcription factor responsive to Wnt signaling that has been found to regulate goblet cell differentiation in the airway (150, 151). A recent study on Spdef knockout mice demonstrated its involvement in maturation of goblet and Paneth cells in the intestine (152). In fact, Spdef was found to be overexpressed in BE as well (unpublished data). These data suggested that Wnt signaling plays a potentially critical role in the development of human BE through downstream effectors many of which are transcription factors.

c. NFκB signaling pathway

Corresponding to increased levels of IL8 and IL1β, there is a step-wise increase of NFκB activity in human GERD, BE, dysplasia and EAC (153). As a consequence, many downstream genes may be activated and contribute to BE (154, 155). Gastroesophageal reflux is known to activate NFκB pathway and induce proinflammatory cytokines in esophageal epithelial cells (156). Accumulating evidence has shown that NFkB, when activated, upregulates Cdx1 and Cdx2 expression. Two independent studies have shown that induction of Cdx2 expression in esophageal epithelial cells by bile was mediated by binding sites in the proximal promoter for NFκB (66, 79, 157). Not only Cdx2, but also Cdx1 is regulated in a similar way by NFκB pathway in the esophageal epithelial cells when stimulated by gastroesophageal reflux (69). Similar to regulation in the esophagus, bacterial components may upregulate Cdx2 and Muc2 expression in rat biliary epithelial cells through Toll-like receptors and the NFkB pathway. This mechanism may contribute to induction of intestinal metaplasia in the bile duct by bacterial infection (158).

d. Hedgehog signaling pathway

Hedgehog signaling pathway was initially found to play a critical role in foregut development (159, 160). It was then found to be involved in upper gastrointestinal cancer (161, 162). A recent study has shown that Hedgehog ligand expression can be induced by acid or bile exposure in BE epithelium. Transgenic expression of Sonic hedgehog in mouse esophageal epithelium induces expression of stromal Bmp4, epithelial Sox9, and columnar cytokeratins (149). An independent study has verified an association among Sonic hedgehog, BMP4, and Cdx2 in human BE tissues (163).

e. Notch signaling pathway

Notch signaling pathway is known to play a critical role in intestinal development. Genetic disruption of Notch signaling results in increased number of goblet cells in mouse small intestine (63, 164). Similary, blocking Notch signaling pathway with γ-secretase inhibitors enhances the development of goblet cells in the intestine (165, 166). This effect depends on the presence of Math1 (167). Ectopic Notch signaling in adult intestinal progenitor cells leads to bias against secretory fates (168).

In the esophagus, Notch1 and Notch3 coordinated esophageal squamous differentiation (169). Its interaction with p63 suggests that Notch signling pathway might promote squamous epithelial cell differentiation, while p63 maintains the progenitor cell population (170). In our previous study, a set of genes on the Notch signaling pathway were modulated by Cdx2 transfection into HET1A cells, with JAG1, Notch4, Notch3, PSEN2, MSI1, Math1, and DF up-regulated and Hes1 downregulated (59).Treatment with a bile acid was found to inhibit Notch signaling in human EAC cells (171). Immunohistochemistry confirmed the presence of an intact and activated Notch signaling pathway in metaplastic BE epithelium,

but not in the normal human esophagus. Treatment of a rat model of reflux-induced BE with a γ-secretase inhibitor converted the proliferative BE epithelial cells into terminally differentiated goblet cells, whereas the squamous epithelium remained intact (172). These data suggest that inhibition of Notch signaling pathways favors goblet cell differentiation and potentially promote BE.

4. Stromal factors

Epithelial-mesenchymal interactions play a critical role in epithelial cell differentiation. Manipulation of stromal factors has been implicated in the development of BE (173, 174) and EAC (175). One of the most well-studied aspects is the association between inflammation and BE (176, 177). Pro-inflammatory cytokines are associated with the development of human BE (153, 178, 179). A recent study on the surgical rat model showed that inflammatory changes preceded histopathology of caustic damage in reflux esophagitis. Chemokines like IL8 and IL1β were secreted by esophageal squamous epithelial cells to mediate esophagitis (180). This observation is consistent with a recent study showing transgenic overexpression of IL1β in the squamous epithelium of mouse esophagus and forestomach causes spontaneous inflammation and intestinal metaplasia at the squamocolumnar junction in the stomach. Intestinal metaplasia was characterized by expression of TFF2 and Cdx2, and promoted by exposure to bile acid (181).

Extracellular matrix exerts a significant impact on differentiation of esophageal epithelial cells. Squamous esophageal epithelial cells cultured on connective tissue from the skin failed to show the expected pattern of differentiation (182). Furthermore, conversion of embryonic stem cells to columnar or squamous epithelia has been shown to depend on the components of the extracellular matrix (183). Cdx2 expression in colon cancer cells is adaptable and strongly dependent on the surrounding extracellular matrix (184, 185).

5. microRNAs

Several studies have been published to show changes in the microRNA profile of human BE and EAC (186–194). Although the major goal of these studies was to identify biomarkers and mechanism of EAC, some data showed an involvement of microRNAs in the development of BE. Certain microRNAs (e.g., miR-203) are known to modulate expression of critical genes associated with BE (e.g., p63). On the other hand, transcription factors including p63 are known to modulate processing of microRNAs such as miR-21 (195, 196). It would be interesting to determine whether microRNAs are drivers or passengers in the development of BE.

6. Other factors

Retinoic acid is required for diverse developmental programs (197). Retinoic acid alone or in synergy with WNT signaling activates Cdx1 (198, 199), HNF1α and HNF4α (200), GATA4 (201), and Sox9 (202). Interestingly, the level of retinoic acid increased in BE tissue compared to normal esophagus, and then decreased in EAC (203, 204). Expression of retinoic acid receptors and retinoid X receptors was also altered in BE compared to normal esophagus (205, 206). More importantly, bile treatment increases retinoic acid receptor activity, which further induces columnar differentiation in esophageal epithelial cells (207, 208).

Runt-related transcriptional factor gene 3 (RUNX3) belongs to the runt domain family of transcriptional factors that plays an important role in celluar differentiation of the esophagus (209). Frequent silencing of RUNX3 by promoter methylation has been reported in both BE and EAC (210–212). As in the esophagus, RUNX3 also plays an important role in the

development of the stomach (213). Interestingly, RUNX3 attenuated Wnt signaling in the intestine and interacted with TGFβ signaling (214, 215). RUNX3 is frequently silenced in gastric cancer through promoter methylation or protein mislocalization (216, 217), and loss of RUNX3 allows gastric epithelial cells to differentiate into intestinal cell types (218, 219), suggesting that loss of RUNX3 may play a similar role in the development of BE.

Krüppel-like factor 4 and 5 (KLF4 and KLF5) are zinc-finger transcription factors that are expressed in the epithelial tissues. KLF4 is required for terminal differentiation of goblet cells in the colon (220). It is known to be regulated by transcription factors (TCF4 and Sox9) and the WNT signaling pathway in the intestine (221). The Notch signaling pathway negatively regulates KLF4 expression, and Notch inhibition increases KLF4 expression and goblet cell differentiation in the intestine (222, 223). KLF4 was recently found to be involved in TGFβ signaling as well (224). In the esophagus, KLF4 is essential for squamous epithelial differentiation and interacts with KLF5 to maintain normal epithelial homeostasis (225, 226). In a recent study, KLF4 was found to be expressed in Barrett's epithelium, and its expression was shown to respond to bile acids through the NFκB signaling pathway. More interestingly, KLF4 interacted with Cdx2 through transcriptional regulation (227).

Summary

In summary, development of BE is complex at the molecular level. As we have discussed above, this process involves squamous transcription factors, intestinal transcription factors, signaling pathways, microRNAs, SNPs, epigenetics, and many other factors. It is challenging to distinguish drivers from passengers, and although a molecular network has started to emerge, we do not yet fully understand how gastroesophageal reflux may induce intestinal metaplasia in the esophagus.

In vivo animal models are essential for validation of drivers of BE. K14-Cdx2 transgenic mice failed to generate Barrett's phenotype in mouse esophagus (67). Several reasons may explain this observation. Expression level of the transgene may not be optimal due to chromatin remodeling and promoter strength. Cdx2 overexpression alone may not be able to drive intestinal metaplasia. Cooperation with other factors (e.g., loss of p63) may be needed. K14 promoter is only active in squamous epithelial cells, and therefore may not be able to drive the whole process of intestinal metaplasia. Finally, rodent esophagus is covered by keratinized squamous epithelium, which is different from the non-keratinized squamous epithelium of human esophagus. It is unclear how such a difference between species may impact the value and use of animal models in BE.

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Abbreviations

BE	Barrett's esophagus
BMP	bone morphogenetic protein
EAC	esophageal adenocarcinoma
GERD	gastroesophageal reflux disease
HNF	hepatocyte nuclear factor
KLF	Krüppel-like factor

RUNX3	runt-related transcriptional factor 3
SNP	single nucleotide polymorphism
Spdef	SAM pointed domain-containing Ets transcription factor
TGF	transforming growth factor

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