Bile Acids Initiate Lineage-Addicted Gastroesophageal Tumorigenesis by Suppressing the EGF Receptor-AKT Axis

Li Gong, M.D., Ph.D.^{1,a}, Philip R. Debruyne, M.D., Ph.D.^{1,b}, Matthew Witek, M.S., M.D.¹, Karl Nielsen, M.S.¹, Adam Snook, Ph.D.¹, Jieru E. Lin, Ph.D.¹, Alessandro Bombonati, M.D.², Juan Palazzo, M.D.², Stephanie Schulz, Ph.D.¹ and Scott A. Waldman, M.D., Ph.D.¹

Abstract

While bile acids are a risk factor for tumorigenesis induced by reflux disease, the mechanisms by which they contribute to neoplasia remain undefined. Here, we reveal that in gastroesophageal junction (GEJ) cells bile acids activate a tissue-specific developmental program defining the intestinal epithelial cell phenotype characterizing GEJ metaplasia. Deoxycholic acid (DCA) inhibited phosphorylation of EGF receptors (EGFRs) suppressing the proto-oncogene AKT. Suppression of EGFRs and AKT by DCA actuated an intestine-specific cascade in which NF- κ B transactivated the tissue-specific transcription factor CDX2. In turn, CDX2 orchestrated a lineage-specific differentiation program encompassing genes characterizing intestinal epithelial cells. Conversely, progression from metaplasia to invasive carcinoma in patients, universally associated with autonomous activation of EGFRs and/or AKT, was coupled with loss of this intestinal program. Thus, bile acids induce intestinal metaplasia at the GEJ by activating the lineage-specific differentiation program involving suppression of EGFR and AKT, activating the NF- κ B-CDX2 axis. Induction of this axis provides the context for lineage-addicted tumorigenesis, in which autonomous activation of AKT corrupts adaptive intestinal NF- κ B signaling, amplifying tumorigenic programs.

Keywords: bile acids, deoxycholic acid, EGFR phosphorylation, NF- κ B, CDX2, GUCY2C, intestinal metaplasia, neoplastic transformation, gastroesophageal junction, esophageal adenocarcinoma, gastric adenocarcinoma, lineage-addicted tumorigenesis

Introduction

Adenocarcinomas of the gastroesophageal junction (GEJ) are principal causes of cancer and cancer-related mortality worldwide.^{1,2} The incidence of adenocarcinoma of the esophagus is rising in both the United States and Europe, and in white males in the United States has become the solid tumor with one of the most rapidly increasing incidences over the past 30 years.^{1,2} Rates of adenocarcinoma below the GEJ in the gastric cardia also have increased during the last 30 years.^{1,2} Virtually all adenocarcinomas of the esophagus arise from Barrett's metaplasia, in which normal squamous epithelial cells of the esophagus are replaced by intestinal epithelial and goblet cells, associated with gastroesophageal reflux disease (GERD).^{3,4} Most adenocarcinomas of the stomach arise from intestinal metaplasia of gastric epithelium.⁴

Transformation at the GEJ, in part, reflects GERD.^{3,5} There is a strong association between the concentration of bile acids in refluxate and the degree of GERD, and bile reflux is a main predisposing factor for Barrett's esophagus.^{6,7} Similarly, the risk of intestinal metaplasia in stomach is related to the concentration of bile acid in gastric juice.⁸ Moreover, bile acid reflux promotes intestinal metaplasia, establishing the context for progression to neoplasia in the upper gastrointestinal (GI) tract in rodents.^{9,10}

Although important etiologic factors associated with transformation at the GEJ have been identified, the mechanisms by which bile acids induce neoplasia remain undefined.^{3,4} The current model of tumorigenesis in most tissues suggests that accumulation of sequential mutations in proto-oncogenes and tumor suppressors produces genomic instability corrupting homeostatic programs, including the cell cycle, metabolism, DNA repair, and apoptosis, conferring an evolutionary survival advantage, a hallmark of therapy-resistant cancer.^{11,12} While these processes characterize the distal transformation continuum, the specific pathophysiological events that initiate this oncogenic cascade, and which represent the greatest opportunity for cancer prevention, remain elusive.¹² An evolving paradigm expanding this genetic view of cancer¹² suggests that primary survival circuits

common to all cells are subordinate to, and hierarchically organized by, lineage-dependent developmental programs that define the repertoire of mechanisms maintaining cellular homeostasis.¹³ In this model, unique patterns of neoplasia characterizing different tissues reflect tumor initiation through mechanisms that disrupt lineage-restricted executive programs followed by propagation and amplification by deregulated subordinate survival circuits.

The GEJ exemplifies tissues whose pathophysiological transformation along the neoplastic continuum depends on execution of lineage-specific developmental programs coordinating key survival circuits. Chronic inflammation, produced by bile acid-containing refluxate, induces intestinal metaplasia in which normal epithelium transdifferentiates into polarized columnar epithelium with Goblet cells.^{3,4} Execution of intestinal differentiation programs mediating metaplasia in response to inflammatory insult is restricted to stomach and esophagus, reflecting canonical developmental programs imprinted on the GI tract during ontogeny. Induction of these lineage-specific programs activates expression of CDX2, a member of the homeobox family of transcription factors, important in establishing and maintaining the intestinal epithelium by regulating enterocyte-specific transcription.¹⁴ Although CDX2 is not expressed in normal gastric or esophageal epithelia, it is ectopically expressed in intestinal metaplasia suggesting an important role in initiating the oncogenic cascade at the GEJ.^{8,15,16} This causal relationship is underscored by the development of intestinal metaplasia in transgenic mice that express ectopic CDX2 in the foregut.^{17,18} Indeed, CDX2 is one of the most likely contributing factors to the initiation of intestinal metaplasia in the upper GI tract.⁴

While key components associated with the induction of intestinal differentiation programs have been identified, the mechanisms mediating metaplasia and its subsequent progression to invasive carcinoma remain to be defined. Recent studies revealed that in human GEJ cells, bile acids induce ectopic expression of

¹Departments of Pharmacology and Experimental Therapeutics and ²Pathology, Anatomy, and Cell Biology, Thomas Jefferson University, Philadelphia, Pennsylvania, USA. Current affiliations: ^aDivision of Bioequivalence, Food and Drug Administration, Washington, DC, USA; ^bDepartment of Oncology, AZ Groeninge Hospital, Kortrijk, Belgium.

Correspondence: SA Waldman (scott.waldman@jefferson.edu)

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CDX2 and downstream lineage-specific differentiation programs by activating nuclear factor kappa B (NF- κ B).¹⁹ Indeed, bile acids specifically activate and induce nuclear translocation of the p50, but not the p65, subunit of NF- κ B. In the nucleus, p50 homodimers bind to consensus sites in the promoter of CDX2, inducing its transactivation and expression which, in turn, initiates intestinal transdifferentiation. Of significance, this cascade recapitulates a developmental program that defines lineage-specification in epithelial cells in the intestine.^{20,21} Further, this signaling mechanism regulates CDX2 expression through AKT, a proto-oncogene that modulates the balance of proliferation and differentiation along the crypt-villus in intestine. Indeed, inhibition of AKT stimulates binding of p50 homodimers to the NF- κ B binding sites of the CDX2 promoter in intestinal cells, inducing expression of CDX2, inhibiting proliferation and promoting differentiation.^{20,21} The ability of bile acids to activate NF- κ B and CDX2 in cells through a canonical tissue-specific circuit defining intestinal epithelial development underscores the critical role of lineage-addiction in initiating the transformation continuum at the GEJ.

While induction of intestine-specific programs through CDX2 transactivation are central to intestinal metaplasia at the GEJ, the mechanisms by which bile acids activate NF- κ B and downstream developmental circuits remain undefined. Here, we reveal that bile acids activate NF- κ B, CDX2 transactivation, and downstream intestinal programs in human GEJ cells by inhibiting AKT phosphorylation and activation, recapitulating lineage-specific circuits regulating differentiation in intestinal epithelium. Regulation of AKT activity controlling NF- κ B and downstream intestine-specific differentiation programs reflect modulation of epidermal growth factor receptor (EGFR) dephosphorylation and inactivation by bile acids. Importantly, the transition from intestinal metaplasia to adenocarcinoma, in which >80% of tumors exhibit gain-of-function alterations in EGF receptor and/or AKT signaling,²²⁻²⁵ was associated with a loss of downstream intestinal differentiation programs. In the context of the absence of NF- κ B expression in normal GEJ cells,¹⁹ these observations suggest a previously unappreciated lineage-addiction of tumorigenesis at the GEJ. Thus, chronic exposure to bile acids induces lineage-specific programs mediating intestinal transdifferentiation, characterized by ectopic expression of NF- κ B and suppression of AKT activation. Sequential development of autonomy in the AKT signaling axis, characterizing >80% of GEJ tumors,^{22,23,25} corrupts ectopic lineage-specific NF- κ B signaling, disrupting intestinal differentiation programs and reciprocally inducing cell survival circuits providing a competitive advantage to evolving cancer cells.

Materials and Methods

Tissues

Formalin-fixed, paraffin embedded blocks from 75 patients who underwent endoscopic biopsy for GERD were obtained from pathology under an IRB-approved protocol at Thomas Jefferson University Hospital. All biopsies were designated as esophageal origin by the endoscopist. The specimens consisted of 26 cases with Barrett's esophagus; 15 cases of Barrett's esophagus with low-grade dysplasia; 8 cases of Barrett's esophagus with high-grade dysplasia; and 10 cases of adenocarcinomas arising in Barrett's esophagus. Four blocks with adenocarcinoma of the colon served as positive controls and 6 blocks with normal GEJ and 6 blocks with cardiac mucosa of the stomach without intestinal metaplasia served as negative controls. Sections

(5 μ m) were immunostained with a polyclonal antibody to human guanylyl cyclase C (GUCY2C) as described previously.²⁶ Only cells exhibiting distinct apical membrane staining, independently of the level of expression, were considered positive for GUCY2C expression. Focal expression was defined as positivity of staining in less than 10% of specimens.

Cell cultures, treatments, and viral infections

OE19 human GEJ adenocarcinoma cells were maintained as described previously.¹⁹ In some experiments, cells were grown in medium containing 1% fetal bovine serum for 24 hours prior to EGF pre-treatment or deoxycholic acid (DCA) or AG1478 treatments. Stock solutions of DCA were dissolved in phosphate-buffered saline (PBS) while AG1478 was dissolved in dimethyl sulfoxide. Final concentrations of dimethyl sulfoxide as a vehicle in experiments never exceeded 0.1% (vol/vol). Adenovirus was amplified in HEK293 cells, and titers were quantified with the Adeno-X Rapid Titer Kit (Clontech, Mountain View, CA, USA). Cells were transduced with adenovirus for 48 hours before initiating treatments. In other experiments, OE19 cells stably expressing either HER-CD533, a dominant negative form of the EGFR, or pIkBa, a hydrolysis- and phosphorylation-resistant form of IkB were produced by transduction with pMSCV2.2-based vectors.²⁷ After 48 hours, cultures were selected for 1 to 2 weeks in 2.5 μ g/mL puromycin (Sigma, St. Louis, MO, USA) and then pooled for immunoblot analysis.

Reagents, antibodies, and immunoblot analyses

The M2 Flag antibody was obtained from Sigma, and AG1478 from Calbiochem (San Diego, CA, USA). The phospho-EGFR, EGFR, and actin antibodies were from Santa Cruz Biotechnologies, Inc. (Santa Cruz, CA, USA) and the phospho-Akt and Akt antibodies were from Cell Signaling Technologies (Danvers, MA, USA). Other reagents were obtained, and immunoblot analyses conducted, as described previously.¹⁹

Transcriptional reporter assay

Transfection, luciferase assays, and the generation of reporter gene constructs were described previously.¹⁹ A pGL-3-Basic Luciferase Vector (Promega, Madison, WI, USA; negative control) and a pGL3 construct containing fragment -835 to +117 of the GUCY2C promoter (Nde) or -908 to +119 of the CDX2 promoter were used. In some experiments, the firefly luciferase reporter gene pNF- κ B-Luc (PathDetect[®] cis-reporting systems; Stratagene, La Jolla, CA, USA) was used. For the luciferase assay, OE19 cells plated 24 hours prior to transfection at 2×10^5 cells/well in 24-well plates were co-transfected with 0.5 μ g of the firefly luciferase reporter plasmid and 5 ng of the *Renilla* luciferase reporter plasmid pRL-TK (Promega) using Effectene according to the manufacturer's instruction (Qiagen, Valencia, CA). After 24 hours, cells were starved in low serum media containing 1% FBS for 24 hours, followed by treatments with varying concentrations of DCA or AG1478, and then lysed in buffer supplied by the manufacturer. The firefly and *Renilla* luciferase activities were measured on a luminometer (Turner Designs, Sunnyville, CA, USA). The relative firefly luciferase activities were calculated by normalizing transfection efficiencies using *Renilla* luciferase activities.

Statistics

All data represent at least three independent experiments performed in duplicate and are presented as mean $\pm$ SEM.

Promoter activities were analyzed utilizing the nonparametric Mann–Whitney *U* test. Intensities of bands from immunoblotting experiments were analyzed using the unpaired *t*-test. Statistical values of $p < 0.05$ were considered significant. Statistical analyses were performed with GraphPad Prism® software (GraphPad Software, Inc., San Diego, CA, USA).

Results

DCA induces intestinal differentiation programs in GEJ cells through NF- κ B

The hydrophobic bile acid and tumor promoter DCA induced tissue-specific programs in OE19 cells by transactivating the intestinal transcription factor CDX2 (Figure 1A1), reflected by an increase in CDX2 protein (Figure 1B). In turn, overexpression of CDX2 activated lineage-specific programs, reflected by transactivation (Figure 1A2) and overexpression (Figure 1B) of the intestinal epithelial cell differentiation marker GUCY2C. Activation of these lineage-specific programs in OE19 cells *in vitro* correlated closely with ectopic induction of these programs producing overexpression of GUCY2C in Barrett's metaplasia (Figure 1C). Moreover, induction of CDX2 expression and downstream lineage-specific programs reflected by GUCY2C in OE19 cells was mediated by ectopic expression of NF- κ B, which is not normally produced by GEJ cells²⁸ but contributes to tissue-specific activation of CDX2 expression in intestinal epithelial cells.^{20,21} Expression of a dominant negative mutant of I κ B α , producing constitutive inhibition of NF- κ B, prevented

transactivation of CDX2 and induction of GUCY2C by DCA (Figure 1D).

DCA regulates intestinal differentiation programs in GEJ cells through AKT

In intestinal cells, regulation of NF- κ B and downstream signaling by CDX2 is mediated, in part, by suppressing the proto-oncogene AKT. Inhibition of AKT induces the nuclear translocation of p50 NF- κ B homodimers which, in turn, bind to consensus sites in the CDX2 promoter, resulting in its transactivation.^{20,21} In OE19 cells, DCA inhibited the phosphorylation and activation of AKT (Figure 2A, 2B). Further, overexpression of constitutively activated myristolated AKT (myrAKT)²⁹ inhibited basal and DCA-mediated CDX2 transactivation (Figure 2C) suggesting that DCA induces metaplasia by engaging lineage-specific circuits, including inhibition of AKT, producing downstream NF- κ B, CDX2 and GUCY2C signaling.

DCA inhibits EGFR that induces intestinal differentiation programs

While the role of the AKT-NF- κ B-CDX2 signaling axis is established in lineage specification in intestinal epithelial cells,^{20,21} upstream modulators of this axis remain undefined. AKT is a downstream effector for the EGFR, which modulates cell growth, survival, differentiation, and metastasis of tumors, including those arising from the GI tract.^{30,31} Here, DCA inhibited the phosphorylation and activation of EGFR in OE19 cells

(Figure 3A). Indeed, inhibition of EGFR by DCA was similar to that induced by an inhibitor of the EGFR tyrosine kinase, AG1478,³² (Figure 3B) and by a dominant negative truncation mutant of the EGFR, HER-CD533³³ (Figure 3C, 3D). Further, AG1478 and HER-CD533 mimicked the effects of DCA on suppression of phosphorylation and activation of AKT (Figure 4A, 4B). Suppression of AKT reflecting inhibition of EGFR phosphorylation by AG1478 and HER-CD533 recapitulated the effects of DCA on downstream intestinal programs, inducing transactivation (Figure 4C) and expression (Figure 4D) of CDX2 and GUCY2C. Moreover, like DCA, induction of intestinal programs by AG1478 and HER-CD533 was mediated by NF- κ B (Figure 5). Indeed, AG1478 and HER-CD533 induced transactivation of the NF- κ B promoter (Figure 5A). Similarly, the ability of EGFR inhibition by AG1478 or HER-CD533 to induce CDX2 and GUCY2C transactivation was eliminated by the dominant negative mutant of I κ B, which constitutively inhibits NF- κ B (Figure 5B, 5C). Thus, DCA induction of intestinal differentiation programs through the AKT-NF- κ B-CDX2 axis recapitulates

Figure 1. Induction of CDX2 and GUCY2C by DCA in GEJ cells, mediated by NF- κ B, recapitulates Barrett's esophagus. (A) DCA increased CDX2 (A1) and GUCY2C (A2) promoter transactivation in OE19 cells. (B) DCA induced CDX2 and GUCY2C protein expression in OE19 cells. (C) GUCY2C is ectopically expressed in GEJ biopsies with Barrett's esophagus, but not in normal GEJ biopsies (magnification, 20 $\times$). (D) Dominant negative I κ B mutant (IkBM) blocked the effects of DCA on CDX2 (D1) and GUCY2C (D2) promoter transactivation in OE19 cells. * $p < 0.05$; *** $p < 0.001$; NS = not significant.

Figure 2. AKT mediates the effects of DCA on CDX2 activity in GEJ cells. DCA inhibited phosphorylation of AKT (A) and decreased the ratio of phosphorylated to total AKT (B) in OE19 cells. (C) Myristolated AKT (myrAKT) blocked the effects of DCA on CDX2 promoter transactivation in OE19 cells. * $p < 0.05$; ** $p < 0.01$.

the effects of inhibitors of EGFRs in GEJ cells. These observations suggest that DCA inhibition of EGFRs induces intestinal differentiation programs ectopically activated by bile acids at the GEJ.

EGFRs mediate DCA induction of intestinal differentiation programs in GEJ cells

Constitutive inhibition of EGFRs by HER-CD533 mimicked the effects of DCA on EGFR phosphorylation and activation and CDX2 transactivation in OE19 cells (Figure 6A, 6B). Further, HER-CD533 eliminated the ability of DCA to inhibit EGFR phosphorylation and transactivation of CDX2 (Figure 6A, 6B). Conversely, agonist activation of EGFRs induced receptor phosphorylation and inhibited transactivation of CDX2 (Figure 6C, 6D). Moreover, agonist activation of EGFRs eliminated DCA inhibition of receptor phosphorylation and CDX2 transactivation (Figure 6C, 6D). Thus, DCA induces intestinal differentiation programs in GEJ cells by suppressing EGFR phosphorylation which, in turn, induces downstream activation of CDX2 expression.

Attenuated lineage-specific programming along the metaplasia-carcinoma continuum

While induction of metaplasia by bile acids reflects inhibition of EGFRs and suppression of survival circuits, there is an established relationship between progression along the transformation continuum and activation of EGFRs and AKT. Indeed, more than 80% of GEJ tumors exhibit upregulation of the proto-oncogene AKT and downstream signaling contributing to tumorigenesis.^{22–25,34} These observations predict that, in the context of progressive upregulation of EGFR and AKT signaling, there should be an associated suppression of lineage-specific programs, with loss of markers of intestinal differentiation along the transformation continuum. Indeed, while 100% of specimens

from patients with Barrett's esophagus or low-grade dysplasia homogeneously expressed GUCY2, this marker was expressed heterogeneously in 50%, and absent in 37.5%, of patients with high-grade dysplasia (Figure 7). Moreover, GUCY2C was expressed heterogeneously in 20%, and absent in 60% of patients with adenocarcinoma (Figure 7). Progressive loss of expression of GUCY2C along the transformation continuum, in conjunction with the established gain of function in EGFR-AKT signaling along that continuum,^{22–25} supports the pathophysiological model in which the EGFR-AKT-NF- κ B-CDX2 axis mediates lineage-specific tumorigenesis, balancing adaptive metaplasia, and malignant neoplasia at the GEJ.

Discussion

While adenocarcinoma of the GEJ remains a leading cause of cancer-related morbidity and mortality worldwide,^{1,2} the mechanisms underlying its pathogenesis remain incompletely defined.^{3,4} At the GEJ, neoplasia is preceded by metaplasia in which the normal epithelium is replaced by cells with a morphology and gene expression profile characteristic of differentiated intestinal epithelial cells.^{3,4} With an incidence of approximately 0.5%–2% of the population of Western countries, intestinal metaplasia places patients at 10%–15% lifetime risk of developing adenocarcinoma.³ In turn, metaplasia at the GEJ reflects GERD, the most common medical condition in Western countries, affecting approximately 30% of resident adults.³

Bile acids in refluxate are associated with intestinal metaplasia and neoplastic transformation. The severity of GERD is related to the concentration of bile acids in refluxate, and these components are principal risk factors for metaplasia at the GEJ.^{6–8} Also, bile acids induce metaplasia and progression to neoplasia in the upper GI tract in experimental animals.^{9,10} Moreover, there is a direct relationship between bile acid exposure and risk of progression to adenocarcinoma.^{3,5–10} Although bile acids play a role in the pathogenesis of neoplasia at the GEJ, the mechanisms by which they induce initiation and progression of transformation remain unknown.^{3,4,35} The present study reveals a previously unappreciated signaling paradigm mediating the earliest stages of transformation wherein bile acids promote intestinal transdifferentiation of GEJ cells by suppressing EGFRs. Inhibition of those receptors suppresses AKT phosphorylation and activation that engages a canonical intestinal differentiation program mediated by the NF- κ B-CDX2 axis. Ectopic expression of the tissue-specific transcription factor CDX2 in GEJ cells induces a lineage-specific program of gene expression, including GUCY2C, which in part, defines the differentiated intestinal epithelial cell phenotype.

There is an established role for EGFR signaling in regulating intestinal epithelial cell proliferation and differentiation. Indeed, EGFR null mice, which die shortly after birth, exhibit severe defects in intestinal cell proliferation and organization.^{36,37} Further, mutation or aberrant expression of EGFRs is associated with transformation at the GEJ, including the induction of Barrett's metaplasia and esophageal cancer.^{22–25,34} An emerging paradigm suggests that bile acids, especially hydrophobic counterparts like DCA, modulate receptor tyrosine kinases, including EGFRs, by remodeling cholesterol and protein components of plasma membrane lipid rafts.³⁸ Interestingly, acute (minutes) exposure to bile acids activates EGFRs through ligand-dependent and independent mechanisms in cells and tissues.^{39,40} Conversely, the present study revealed that protracted (hours) incubation with bile acids, reflecting an exposure that more closely approximates

Figure 3. DCA inhibited EGFR phosphorylation in GEJ cells. (A) DCA induced dephosphorylation of EGFRs, and decreased the ratio of phosphorylated to total EGFRs, in OE19 cells. (B) AG1478 inhibited EGFR phosphorylation in OE19 cells in a dose-dependent fashion. (C) Dominant negative EGFR (HER-CD533) is stably expressed in pMSCV2.2-transfected OE19 cells (OE19-HER-CD533 cells). (D) HER-CD533 inhibited EGFR phosphorylation, and decreased the ratio of phosphorylated to total EGFRs, in OE19 cells. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

chronic inflammation induced by GERD associated with metaplasia,⁴¹ suppressed EGFR phosphorylation, and activation in GEJ cells. Suppression of EGFR by DCA, and the associated activation of canonical programs underlying intestinal lineage specification in GEJ cells, suggests that, in part, bile acids contribute to the induction of metaplasia through inhibition, rather than activation, of EGFRs.

EGFR phosphorylation is linked to survival programs in normal tissues and tumors through the proto-oncogene AKT.^{30,31,42} In that context, there is a canonical signaling pathway contributing to spatiotemporal organization of the intestinal crypt-surface axis in which suppression of AKT induces downstream p50 NF- κ B signaling.²¹ In turn, p50 NF- κ B specifically translocates to the nucleus, inducing expression of the tissue-specific transcription factor CDX2, which orchestrates expression of genes, in part, defining the intestinal epithelial cell phenotype.^{20,21} Previous studies revealed that induction of intestinal programs in GEJ cells by bile acids activates p50 NF- κ B signaling and transactivation of

CDX2, suggesting that this lineage-specific pathway contributes to the development of metaplasia characterizing Barrett's esophagus.¹⁹ The present studies support this model of intestinal transdifferentiation at the GEJ, demonstrating the canonical contribution of AKT suppression to the induction of the p50 NF- κ B-CDX2 signal axis produced by bile acids.²¹ These observations support a model in which metaplasia is induced by bile acid exposure at the GEJ through suppression of EGFRs and downstream AKT signaling, which activates the NF- κ B-CDX2 signal axis regulating programs defining intestinal epithelial cell differentiation.

It is noteworthy that while intestinal transdifferentiation programs were engaged in Barrett's metaplasia and low-grade dysplasia, there was progressive restriction of the intestinal phenotype, reflected by loss of GUCY2C, in high-grade dysplasia and adenocarcinoma (Figure 7). These results compare closely with studies of CDX2 expression, in which this tissue-specific transcription factor is similarly lost in a graded fashion along the transformation continuum.^{8,15,16} Of significance, aberrant activation of EGFRs and AKT commonly contributes to tumorigenesis in the distal GI tract, associated with sporadic cancer in colon and rectum.⁴³ In the context of lineage-specific tumorigenesis, EGFR and AKT recapitulate that role in the proximal GI tract, and their aberrant signaling is associated with progression along the transformation continuum to adenocarcinoma at the GEJ.^{22–25,34} These observations suggest a model in which intestinal metaplasia is an adaptive response to chronic refluxate exposure in the context of GERD³⁵ wherein bile acids, including DCA, suppress EGFRs and AKT, inducing the canonical NF- κ B-CDX2 axis and

intestinal transdifferentiation.^{19,21} Further exposure to refluxate and chronic inflammation subsequently induces autonomous activation of EGFRs and AKT, through mutation and/or gene amplification, which are hallmarks of adenocarcinoma at the GEJ.^{22–25,34} Autonomous signaling through the EGFR-AKT axis permits escape from adaptive intestinal transdifferentiation and the development of progressive dysplasia and carcinoma.

The prevailing oncogenomic view of cancer suggests that tumors arise as a result of sequential accumulation of mutations in tumor suppressors and proto-oncogenes.¹² Beyond this oncogene-addicted model of neoplasia, there is an emerging paradigm suggesting that tumorigenesis is a hierarchical process involving executive developmental programs, indelibly imprinted on tissues, that regulate lineage-specific homeostatic circuits mediated by tumor suppressors and proto-oncogenes.¹³ In this model of lineage-addicted tumorigenesis, corruption of developmentally restricted programs organizing homeostatic processes, in turn, defines the set of subordinate tumor suppressors and oncogenes

Figure 4. Inhibitors of EGFR phosphorylation mimic the effects of DCA on AKT, CDX2 and GUCY2C in GEJ cells. (A) AG1478 inhibited AKT phosphorylation in a dose-dependent fashion in OE19 cells. (B) HER-CD533 inhibited AKT phosphorylation in OE19 cells. AG1478 (C1, D1) and HER-CD533 (C2, D2) increased CDX2 and GUCY2C promoter transactivation (C) and protein expression (D) in OE19 cells. * $p < 0.05$; ** $p < 0.01$.

Figure 5. Inhibitors of EGFR phosphorylation regulate CDX2 and GUCY2C through NF-κB in GEJ cells. AG1478 (A1) and HER-CD533 (A2) increased transactivation of an NF-κB reporter construct in OE19 cells. Dominant negative IκB mutant blocked the effects of AG1478 (B) and HER-CD533 (C) on CDX2 (B1, C1) and GUCY2C (B2, C2) promoter transactivation in OE19 cells. * $p < 0.05$; ** $p < 0.01$; NS = not significant.

specifying patterns of transformation that uniquely characterize individual tissues.¹³ In that context, the present observations suggest a model of lineage-addicted tumorigenesis at the GEJ. Chronic exposure to bile acids³⁵ suppresses EGFR and AKT signaling, inducing NF-κB expression.^{19,21} It is noteworthy that normal GEJ mucosa is devoid of NF-κB expression²⁸ and this ectopic expression represents one critical limb of a lineage-specific differentiation program characteristic of intestinal epithelium²¹ and central to adaptive intestinal metaplasia.^{19,35} However, the inevitable development of autonomy in the EGFR and/or AKT pathways^{22–25,34} suppresses adaptive signaling mediating intestinal transdifferentiation, and induces maladaptive signaling by ectopically expressed NF-κB contributing to tumor-promoting survival circuits that are the hallmarks of cancer.^{21,44,45} Thus, ectopic expression of intestine-specific NF-κB-dependent transcriptional programs induced in GEJ cells by chronic exposure to bile acids establishes the context for and enables the evolution of lineage-addicted tumorigenesis upon subsequent changes in EGFR and AKT signaling.

While actuation of tissue-specific developmental programs underlies lineage-addicted tumorigenesis, these same programs offer a unique opportunity for managing patients at risk for adenocarcinoma at the GEJ. Ectopic induction of the intestine-specific transcription factor CDX2 by bile acids, in turn, transactivates genes characteristic of the differentiated enterocyte phenotype.¹⁶ Normally absent in the upper GI tract, CDX2 is nearly ubiquitously expressed in cells undergoing intestinal metaplasia at the GEJ.^{15,16,20,46} CDX2 regulates the expression of genes characterizing the intestinal epithelial cell phenotype, which in addition to GUCY2C includes sucrase isomaltase, mucin type 2, and LI-cadherin, all of which have been identified in cells undergoing intestinal metaplasia at the GEJ.¹⁶ Indeed, these genes may serve as markers of transformation at the GEJ, and may be useful for detecting occult metaplasia, malignancies, and occult metastases.⁴⁷

Beyond its utility as a marker, ectopic expression of GUCY2C may provide unique approaches to cancer prevention at the GEJ. GUCY2C is the intestinal receptor for the paracrine hormones guanylin and uroguanylin, the most commonly lost gene products in colorectal tumorigenesis.⁴⁸ GUCY2C has emerged as a component of programs organizing spatiotemporal patterning along the crypt-surface axis whose silencing, reflecting loss of the paracrine ligands, promotes hyperproliferation and genetic instability underlying intestinal neoplasia.^{49,50} Of significance, GUCY2C contributes to crypt-villus homeostasis and opposes tumorigenesis, in part, by suppressing AKT and its downstream signaling.⁴⁸ The present studies highlight the

Figure 6. DCA induces CXD2 activity through inhibition of EGFRs in GEJ cells. HER-CD533 blocks DCA-dependent EGFR dephosphorylation (A) and CDX2 promoter transactivation (B) in OE19 cells. EGF (10 ng/mL) eliminates DCA-induced EGFR dephosphorylation (C) and CDX2 promoter transactivation (D) in OE19 cells. * $p < 0.05$; NS = not significant.

reciprocal role of AKT in esophageal transformation. On one hand, AKT suppression drives adaptive intestinal metaplasia. On the other hand, autonomous AKT activation contributes to escape from adaptive metaplasia and progression to invasive carcinoma.^{22,25} In that context, the universal ectopic overexpression of GUCY2C by cells undergoing Barrett's metaplasia^{19,26} offers an opportunity to prevent progression from metaplasia to dysplasia and carcinoma by GUCY2C ligand supplementation. This lineage-specific model of chemoprevention exploits the ectopic expression of GUCY2C induced by bile acids in GEJ cells to target oral supplementation with its endogenous paracrine ligands to suppress AKT signaling, preventing metaplastic escape and neoplasia.⁴⁸

In summary, this study suggests that the EGFR-AKT-NF- κ B-CDX2 signaling axis is central to the induction of intestinal metaplasia by bile acids at the GEJ. Bile acid-induced inhibition of EGFR phosphorylation initiates a tissue-specific program in which downstream suppression of AKT actuates an intestinal epithelial cell

Figure 7. Intestinal differentiation programs, reflected by GUCY2C expression, are attenuated across the continuum of transformation at the GEJ. GUCY2C is expressed in GEJ biopsies with Barrett's esophagus and low-grade dysplasia, but only focally in some patients with high-grade dysplasia, and not in some adenocarcinomas. Magnification, 40 $\times$.

signaling sequence mediated by p50 NF- κ B and ectopic expression of the tissue-specific transcription factor CDX2. In turn, CDX2 transactivates critical genes defining the intestinal epithelial cell phenotype characterizing metaplasia. In the context of the near-universal activation of EGFRs and/or AKT during progression from metaplasia to carcinoma,^{22–25,34} the present results highlight the contribution of this intestinal differentiation program to lineage-addicted tumorigenesis¹³ at the GEJ. Moreover, the tissue-specific molecular mechanism revealed here underscores a previously unappreciated therapeutic opportunity for chemoprevention of GEJ cancer, exploiting the ectopic expression of GUCY2C and its ability to suppress oncogenic AKT signaling by oral paracrine ligand supplementation.⁴⁸

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