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RESEARCH ARTICLE | *Translational Human Pathophysiology*

Barrett's metaplasia develops from cellular reprograming of esophageal squamous epithelium due to gastroesophageal reflux

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Minacapelli CD, Bajpai M, Geng X, Cheng CL, Chouthai AA, Souza R, Spechler SJ, Das KM. Barrett's metaplasia develops from cellular reprograming of esophageal squamous epithelium due to gastroesophageal reflux. *Am J Physiol Gastrointest Liver Physiol* 312: G615–G622, 2017. First published March 23, 2017; doi:10.1152/ajpgi.00268.2016.—Gastroesophageal reflux disease (GERD) clinically predisposes to columnar Barrett's metaplasia (BM) in the distal esophagus. We demonstrate evidence supporting the cellular origin of BM from reprograming or transcommitment of resident normal esophageal squamous (NES) epithelial cells in response to acid and bile (A + B) exposure using an in vitro cell culture model. The hTERT-immortalized NES cell line NES-B10T was exposed 5 min/day to an A + B mixture for 30 wk. Morphological changes, mRNA, and protein expression levels for the inflammatory marker cyclooxygenase-2; the lineage-determining transcription factors TAp63 (squamous), CDX2, and SOX9 (both columnar); and the columnar lineage markers Villin, Muc-2, CK8, and mAb Das-1 (incomplete phenotype of intestinal metaplasia) were assessed every 10 wk. Markers of columnar lineage and inflammation increased progressively, while squamous lineage-determining transcriptional factors were significantly decreased both at the mRNA and/or protein level in the NES-B10T cells at/after A + B treatment for 30 wk. Distinct modifications in morphological features were only observed at/after 30 wk of A + B exposure. These changes acquired by the NES-B10T 30-wk cells were retained even after cessation of A + B exposure for at least 3 wk. This study provides evidence that chronic exposure to the physiological components of gastric refluxate leads to repression of the discernable squamous transcriptional factors and activation of latent columnar transcriptional factors. This reflects the alteration in lineage commitment of the precursor-like biphenotypic, NES-B10T cells in response to A + B exposure as the possible origin of BM from the resident NES cells.

NEW & NOTEWORTHY This study provides evidence of the origins of Barrett's metaplasia from lineage transcommitment of resident esophageal cells after chronic exposure to gastroesophageal refluxate. The preterminal progenitor-like squamous cells alter their differentiation and develop biphenotypic characteristics, expressing markers of incomplete-type columnar metaplasia. Development of these biphenotypic precursors in vitro is a unique model to study pathogenesis of Barrett's metaplasia and esophageal adenocarcinoma.

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Barrett's metaplasia; biphenotypic; GERD; reprogramming; transcommitment

CHRONIC GASTROESOPHAGEAL REFLUX DISEASE (GERD) affects more than 20% of the adult population in the United States (35) and causes reflux esophagitis or inflammation in the distal portion of the esophagus (38). The GERD-damaged squamous epithelial cells in this part of the esophagus are eventually replaced by intestinal-type columnar cells through a process called metaplasia (38, 41), Barrett's metaplasia (BM), or Barrett's esophagus (BE). BM is suggested to be either a healing process (36) or a premalignant condition that confers a predisposition to dysplasia and esophageal adenocarcinoma (EAC) (27, 40). EAC is highly aggressive and has increased in incidence by a factor of more than 7 during the past four decades in the United States (13, 27, 30, 33, 39, 40), carrying a very poor prognosis with 5-yr survival rates $< 20\%$ (5).

Many studies have focused on the progression of BM to EAC; however, the cellular origin of BM is unknown and the factors leading to the development of intestinal metaplasia in the esophagus are unclear (25, 36, 45, 46). Chronic inflammation has been implicated in deregulation of "master switches" or lineage-determining transcription factors (TFs), leading to reprograming or transcommitment in the native epithelium of the gastrointestinal tract (25, 29). Indirect evidence from human esophageal biopsy specimens points toward three core TFs: TAp63 (TA isotype of p63 that retains the NH_2-terminal activation domain), CDX2 (caudal-type homeobox 2), and SOX9 (SRY or sex-determining region Y box 9) involved in squamous lineage determination during embryonic esophageal development (6, 14, 28, 32, 34, 42, 47, 49). The intrinsic balance of these TFs in the adult normal esophageal squamous (NES) epithelium is altered during development of BM. The TAp63 TF that shares high resemblance to the tumor suppressor gene p53 (6, 47) is required for development and maintenance of the esophageal squamous stratified epithelium (28, 32, 47) in the adult esophagus. CDX2, the caudal related homeobox TF, is involved in embryonic development and axial patterning of the alimentary tract and regulates the early stages of intestinal differentiation and maintenance. CDX2 is not expressed in the normal adult esophageal squamous epithelium but is expressed at significantly higher levels in BM (34, 42, 49). SOX9 is also essential in the development of embryonic columnar-lined esophagus but is switched off in postnatal life

and is reexpressed again in BM (14, 28, 49). Expression of cyclooxygenase-2 (COX-2) is upregulated and plays has a key role in the development of BM, possibly by disrupting key signaling pathways that maintain homeostasis in the NES epithelium (22).

The monoclonal antibody mAb Das-1 (also referred as 7E12H12, IgM isotype) is a sensitive and specific biomarker of incomplete-type columnar metaplasia (specific for colon epithelium) developed earlier by our group (9). The mAb Das-1 antibody recognizes a high-molecular-weight colonic epithelial protein (CEP) that is not expressed in any other part of the gastrointestinal tract including the small intestinal epithelium, the squamous esophageal epithelium, or the gastroesophageal junction mucosa (1, 9, 12, 18). It has been previously reported that mAb Das-1 reactivity is present in the entire human fetal gastrointestinal tract but is gradually lost in all other parts except the colon in postnatal life (1). Esophageal tissue shows distinct reappearance of mAb Das-1 reactivity in BM at the esophagogastric junction although the adult squamous epithelium does not react with the antibody (3, 9–12, 18, 31). This pattern of expression resembles the previously described embryonic TFs with lineage-determining potential, e.g., TAp63 associated with squamous lineage in the esophagus and CDX2 associated with columnar lineage in the intestines. The mAb Das-1 can identify BM as well as adenocarcinoma arising from BM with 97% sensitivity and 100% specificity, suggesting that BM is indeed a metaplasia of colonic or incomplete intestinal phenotype (8, 10–12, 18, 31). Furthermore, in patients with chronic GERD, mAb Das-1 can detect colonic metaplasia even before the histological appearance of intestinal metaplasia with goblet cells, suggesting the existence of a "pre-Barrett's" stage (10, 11).

Thus we hypothesize that the physiological reflux components (i.e., acid and bile) can cause chronic stress that directly modulates the TFs leading to reprogramming or transcommitment of resident pre-Barrett's squamous esophageal epithelial cells into columnar lineage during BM development. Our goal was to *1*) investigate cellular transcommitment as the possible origin of BM and *2*) develop an in vitro model to study molecular changes associated with GERD leading to BM. This study demonstrates reprogramming or transcommitment of the precursor-like NES cells (NES-B10T) into the columnar phenotype (metaplasia) in vitro after chronic exposure to physiologic stressors present in the gastric refluxate.

MATERIALS AND METHODS

Cell culture. NES-B10T is a nonneoplastic, telomerase-immortalized, normal esophageal squamous epithelial (NES) cell line. It is derived from endoscopic biopsy tissue of the esophageal squamous epithelium obtained 3–4 cm proximal to the squamo-columnar junction of a patient with GERD and BM (37, 48). These cells express squamous cell markers (cytokeratin 13 and 4), demonstrate contact inhibition, and do not exhibit anchorage-independent growth in soft agar. Cells were maintained at 37°C in a 5% CO_2 incubator in the appropriate cell culture medium (37, 48). The transcript levels and protein expression characteristics of the NES-B10T 30-wk-treated cells were compared with a telomerase-immortalized, nonneoplastic, human BE cell line (BAR-T) (17).

Acid and bile exposure of NES-B10T cells. Hydrochloric acid was used to adjust the pH of the culture medium to experimental conditions. The bile acid β-glycochenodeoxycholic acid (Sigma, St. Louis, MO), was diluted to optimum working concentrations of 200 μM with the culture medium adjusted to pH 4 immediately before exposure to cells. For chronic exposure, 0.1×10^6 cells growing on six-well plates were incubated with acid and bile (A + B, pH 4) for 5 min per day for up to 30 wk. The cells were harvested 24 h after the last A + B treatment and rinsed with 1× PBS, supplemented with growth medium, and returned to the incubator. No A + B treatment was done on the day the cells were passed. The exposure time was optimized from similar studies showing that 5 min was sufficient for induction of signal transduction pathways regulating cellular machinery without causing cell death (2, 7, 15, 16). The untreated control NES-B10T cells were grown in parallel without A + B exposure. Reproducibility of experiments was observed by taking 20-wk cells exposed to A + B and treating them further for 10 wk along parallel untreated controls.

Morphological changes. NES-B10T cells were exposed to A + B for 30 wk of treatment, and morphological changes, such as shape, size, and distribution on cell culture petri dishes, were examined weekly. Cells were observed using the Venus USB 2.0 digital camera at ×10 magnification on the Olympus CK40 microscope during the 30-wk A + B-exposure period and for an additional 3 wk after cessation of treatment.

RT-PCR analysis. Total RNA was extracted using the Qiagen RNAse Mini kit per manufacturer's protocol (Qiagen). The RNase-Free DNase set (Qiagen) was used to remove any genomic DNA contamination. cDNA was generated using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems) on the Gene Amp PCR System 9700 (Applied Biosystems).

Real-time RT-PCR underwent 40 cycles of denaturing at 95°C for 15 s, annealing at 60°C for 20 s, and extending at 72°C for 20 s on the Roche Light Cycler 2.0 Instrument using the QuantiTect SYBR green PCR kit (Qiagen). Expression of target mRNA was normalized with respect to β-actin using the ΔΔ-CT method. Primers were as follows: COX-2: forward (F)-5′-CTC AGG CAG AGA TGA TCT ACC C-3′ and reverse (R)-5′-GTC TGG AAC AAC TGC TCA TCA C-3′; CDX2: F-5′CCC GAA CAG GGA CTT GTT TA-3′ and R-5′-AGA CCA ACA ACC CAA ACA GC-3′; SOX9: F-5′-TTT CCA AGA CAC AAA CAT GA-3′ and R-5′-AAA GTC CAG TTT CTC GTT GA-3′; TAp63: F-5′-TGT ATC CGC ATG CAG GAC T-3′ and R-5′-CTG TGT TAT AGG GAC TGG TGGA C-3′; Muc-2: F-5′-GAA GGT GCT GTC CTT TCT ACT G-3′ and R- 5′-CCT GGC ACT TGG AGG AAT AAA-3′; Villin: F-5′-CTG CTA CAT CAT CCT GGC TAT C-3′ and R-5′-GTC ATC CAT CTG TGT GGT GTA G-3; and CK8: F-5′-GCAGAACAAGATGCTGGAGA-3′ and R-5′-CCGCCTAAGGTTGTTGATGTA-3′.

Western blot analysis. Thirty micrograms of total cell extracts for NES-B10T cells were resolved on 4–20% gradient SDS-polyacrylamide gel (Invitrogen). The blots were then incubated with the respective primary antibodies against COX-2, CDX2, CK8, Muc-2, α-tubulin (Abcam), mAb Das-1 (developed in our laboratory available at EMD Millipore), and TAp63 (antibody was a kind gift from Dr. Borivoj Vojtesek) and subsequently with horseradish peroxidase-conjugated anti-mouse IgM or IgG secondary antibody (Santa Cruz Biotechnology). Blots were then developed by ECL reagent (Perkin-Elmer). The same blots were also incubated with an horseradish peroxidase-conjugated anti-tubulin antibody (Sigma) to determine amount of protein loading. Target protein was quantified by ImageJ (National Institutes of Health public domain) processing software using tubulin as the internal control.

Indirect immunofluorescence and flow cytometry. Confluent (5×10^6) cells were fixed and permeabilized with Cytofix/Cytoperm solution (BD Biosciences) at room temperature for 10 min. Cells were incubated with primary antibody (mAb Das-1, CK8, or CK4) at 1:100 dilution overnight at 4°C (12). Cells were then incubated at room temperature for 1 h with 1:200 secondary antibody and conjugated with fluorescent dye (Alexa Fluor 488-conjugated goat anti-mouse IgM or IgG; Invitrogen), followed by 3× PBS washes. DAPI (50 μl/ml; Life Technologies) was used for nuclear staining. Cells were

mounted on glass slide and visualized by ultraviolet microscopy (Zeis-Axiophot microscope) for direct immunofluorescence. The same cells were also analyzed by fluorescence-activated cell sorting (FACS) at Rutgers University Core Facility (Cancer Institute of New Jersey) utilizing the Beckman-Coulter Cytomics FC500 instrument. Isotype control monoclonal antibodies (MOPC IgG or IgM) in equal concentrations was utilized to account for nonspecific binding or background. The change in fluorescence among the control untreated NES-B10T, NES-B10T 30-wk A + B, and BAR-T cells was estimated by the Overton method of cumulative histogram subtraction (26). Coimmunofluorescence staining FACS was performed with CK4 (IgG) and mAb Das-1(IgM) primary antibodies followed by secondary antibody conjugated with fluorescent dye (Alexa Fluor 488 goat anti-mouse IgG or R-phycoerythrin goat anti-mouse IgM, respectively; Abcam) and analyzed with Kaluza Analysis Software version 1.5 at the Flow Cytometry/Cell Sorting and Confocal Microscopy Core Facility (Rutgers, Piscataway, NJ).

Single-nucleotide polymorphism genotyping. We utilized the single-nucleotide polymorphism (SNP) trace panel assay for cell authentication. This assay generates a unique genomic profile of any human cell line as an effective and sensitive authentication method comparable with short tandem repeat profiling (20). Cell line (NES-B10T and NES-B10T 30-wk A + B) authentication was completed at RUCDR Infinite Biologics (Piscataway, NJ). A similar analysis for the BAR-T cell line was performed previously (7).

Statistical analysis. The results were compared between A + B-treated and untreated NES-B10T cells and an authenticated BE cell line BAR-T, using the Student's *t*-test. $P < 0.05$ was considered statistically significant for observed changes.

RESULTS

Molecular changes. Chronic acid (A; pH 4) and bile acid (B) treatment for 30 wk significantly induced mRNA expression of the inflammatory marker COX-2 (480-fold increase compared with untreated cells, $P = 0.005$). Simultaneously, significant induction of mRNA levels of the columnar epithelium-specific TFs CDX2 (15-fold increase, $P = 0.038$) and SOX9 (45-fold increase, $P = 0.001$) and the columnar differentiation markers CK8 (3.3-fold increase, $P < 0.05$), Muc-2 (14-fold increase, $P = 0.04$), and Villin (5-fold increase, $P = 0.04$) was observed (Fig. 1). These changes in mRNA expression progressively increased from 0 to 10, 20, and 30 wk of treatment (Fig. 1). Intriguingly, inhibition of mRNA expression of the squamous TF TAp63 was observed throughout the 0, 10, and 20 wk of treatment while reaching a significant inhibition by 30 wk (12-fold inhibition, $P = 0.002$). These changes observed in NES-B10T 30-wk A + B cells followed the same profile as the human Barrett's epithelial cell line BAR-T that has a significantly ($P = 0.01–0.05$) different expression profile compared with the control NES-B10T cells (Fig. 1). When allowed to grow without any A + B exposure for another 3 wk beyond the 30-wk A + B exposure, the NES-B10T 30-wk A + B cells retained the altered gene expression characteristics and did not revert to the basal levels seen in the control untreated cells. No significant cell death or

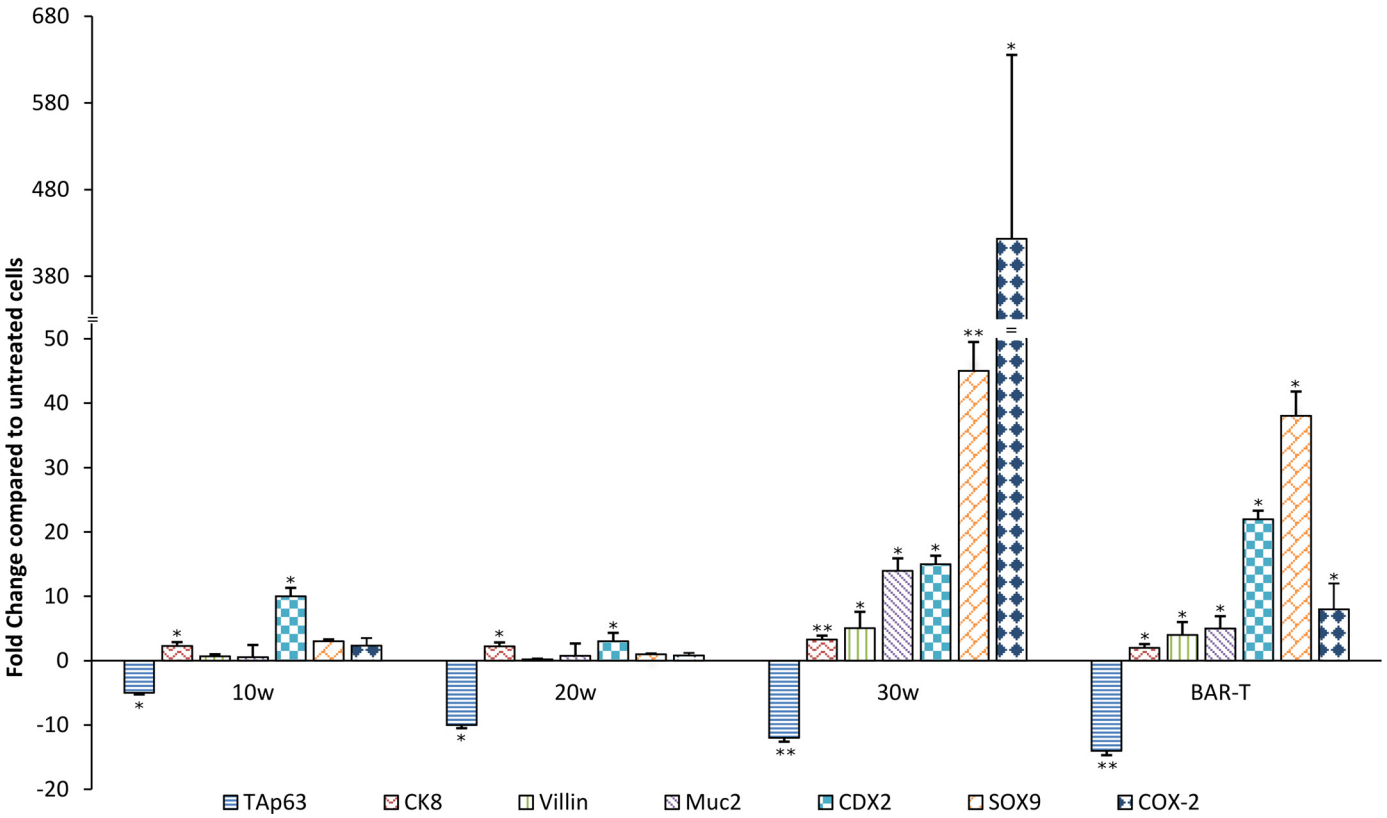

Fig. 1. Fold change in gene transcript levels of TAp63, CK8, Villin, Muc-2, CDX2, SOX9, and inflammatory cyclooxygenase 2 (COX-2) in NES-B10T cells exposed to 10, 20, and 30 wk of daily acid (pH 4) and bile (A +B) treatment compared with untreated control NES-B10T and the Barrett's cell line BAR-T. Expression of the squamous transcriptional factor TAp63 decreases as the columnar transcription factors CDX2 and SOX9; the columnar differentiation markers CK8, Villin, and Muc-2; and inflammatory COX-2 significantly increases with daily A + B treatment of the NES-B10T cells. The changes are progressive and are most significant at 30 wk. Gene expression profiles of NES-B10T 30-wk A + B cells and the Barrett's cell line BAR-T are similar and significantly different ($n = 3$; $*P \leq 0.05$, $**P \leq 0.01$) from the untreated control NES-B10T.

change in proliferation was observed in the NES-B10T cells as a result of A + B exposure.

Protein analysis. Western blot analysis demonstrated a significant increase in the protein expression of the inflammatory marker COX-2 (1.5-fold), the columnar TF CDX2 (2.3-fold), the columnar differentiation markers CK8 (4-fold) and Muc-2 (1.75-fold), and colonic mAb Das-1 (incomplete intestinal phenotype: 4-fold) and a simultaneous decrease of TF TAp63 (2.4-fold) in the NES-B10T 30-wk A + B cells (Fig. 2, *A* and *B*) compared with untreated parallel controls. Indirect immunofluorescence staining was performed and analyzed by FACS (Fig. 3, *Ai*, *Bi*, and *Ci*; $n = 3$) to confirm overexpression of the columnar markers mAb Das-1 (2-fold, $P = 0.004$; Fig. 3, *Ai* and *Aii*) and CK8 (5-fold, $P = 0.004$; Fig. 3, *Bi* and *Bii*) and simultaneously reduction of the squamous marker CK4 (4-fold reduction, $P = 0.007$; Fig. 3, *Ci* and *Cii*). The pattern of higher levels of columnar markers (mAb Das-1 and CK8) and lower levels of the squamous marker CK4 was similar between NES-B10T 30-wk A + B and the BAR-T cells. The expression profiles of mAb Das-1, CK8, and CK4 in BAR-T are distinctly ($P = 0.02–0.05$) different from the levels of these markers in the NES-B10T control cells (Fig. 3, *Ai*, *Bi*, *Ci*, *Ei*, *Eii*, and *Eiii*). The coimmunofluorescence staining with CK4 and mAb Das-1 antibodies analyzed by FACS identified 2.85% of the control NES-B10T cells to be biphenotypic (i.e., staining positive for both CK4 and mAb Das-1) and the rest were CK4+ cells. The NES-B10T 30-wk A + B-treated cells were found to have a higher percentage of biphenotypic cells (10.42%) (Fig. 3, *Di* and *Dii*). This 3.6-fold increase ($P < 0.02$) in the biphenotypic cell population was concurrent with reduction of the pure $CK4^+$ squamous population ($P < 0.05$). A pure mAb $Das\text{-}1^+$ population of cells was, however, not identified.

Morphological changes. A + B exposure induced morphological changes in NES-B10T cells at 30 wk. These cells grew round or oval and tended to form clumps (Fig. 4), unlike untreated control cells that grew evenly dispersed, maintaining an elongated shape on the culture plate. No such morphological changes were observed at 10 or 20 wk of treatment. NES-B10T 30-wk A + B cells retained the morphological characteristics even without further A + B exposure for at least the 3 wk studied.

SNP genotyping. Authentication by SNP trace panel assay of NES-B10T cells post-A + B treatment was completed at RUCDR Infinite Biologics, and the results confirmed authenticity of NES-B10T A + B-treated for 30 wk to that of NES-B10T baseline profile.

Changes in NES-B10T 30-wk A + B cells are reproducible and sustained without further A + B exposure. Reproducibility of observations at the gene and morphological levels was confirmed by treating already exposed 20 A + B cells to an additional 10 wk of A + B treatment. This exposure produced the same changes observed at 30-wk A + B. Interestingly, the NES-B10T-treated cells for 30 wk maintained morphological characteristic as well as molecular changes when studied for at least an additional 3 wk after cessation of 30 wk of A + B exposure.

DISCUSSION

The incidence of EAC has increased significantly in the last 40 yr, particularly in the United States (35). Most cases of EAC are presumed to arise from the BM epithelium; however, clinical cancer risk stratification and prediction are limited due to lack of evidence in the literature (35, 36, 38, 41). Identifying the mechanisms underlying the origin and development of BM is of critical importance because such molecular processes may identify the biological markers of disease initiation that perhaps may be targeted for effective prevention of BM and possibly EAC and may ultimately improve a patients' outcome.

In vivo BM appears to result from chronic, repeated injury of the esophageal squamous epithelium by the gastric refluxate

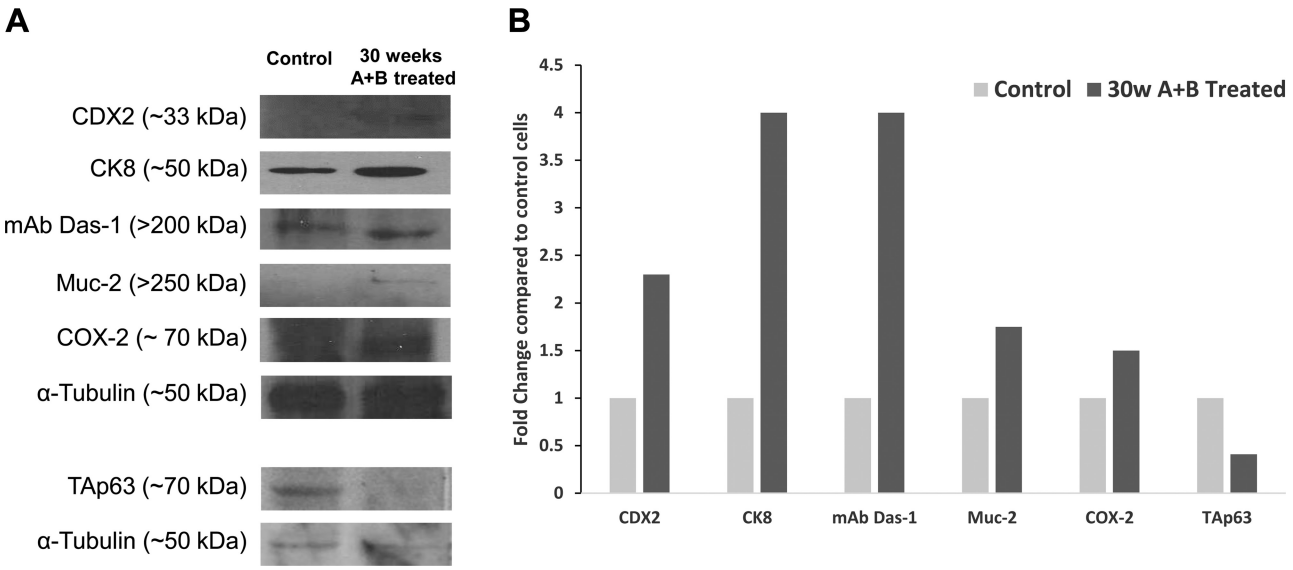

Fig. 2. *A*: representative Western blot of CDX2 (2.3-fold increase), CK8 (4-fold increase), colonic marker mAb Das-1 (4-fold increase), Muc-2 (1.75-fold increase), COX-2 (1.5-fold increase), and TAp63 (2.4-fold decrease) in NES-B10T cells exposed to 30 wk of daily acid (pH 4) and bile (A + B) treatment, compared with untreated control cells. *B*: band intensity of the different markers obtained by Western blot was estimated by ImageJ analysis ($n = 2$). All samples were derived at the same time and processed in parallel.

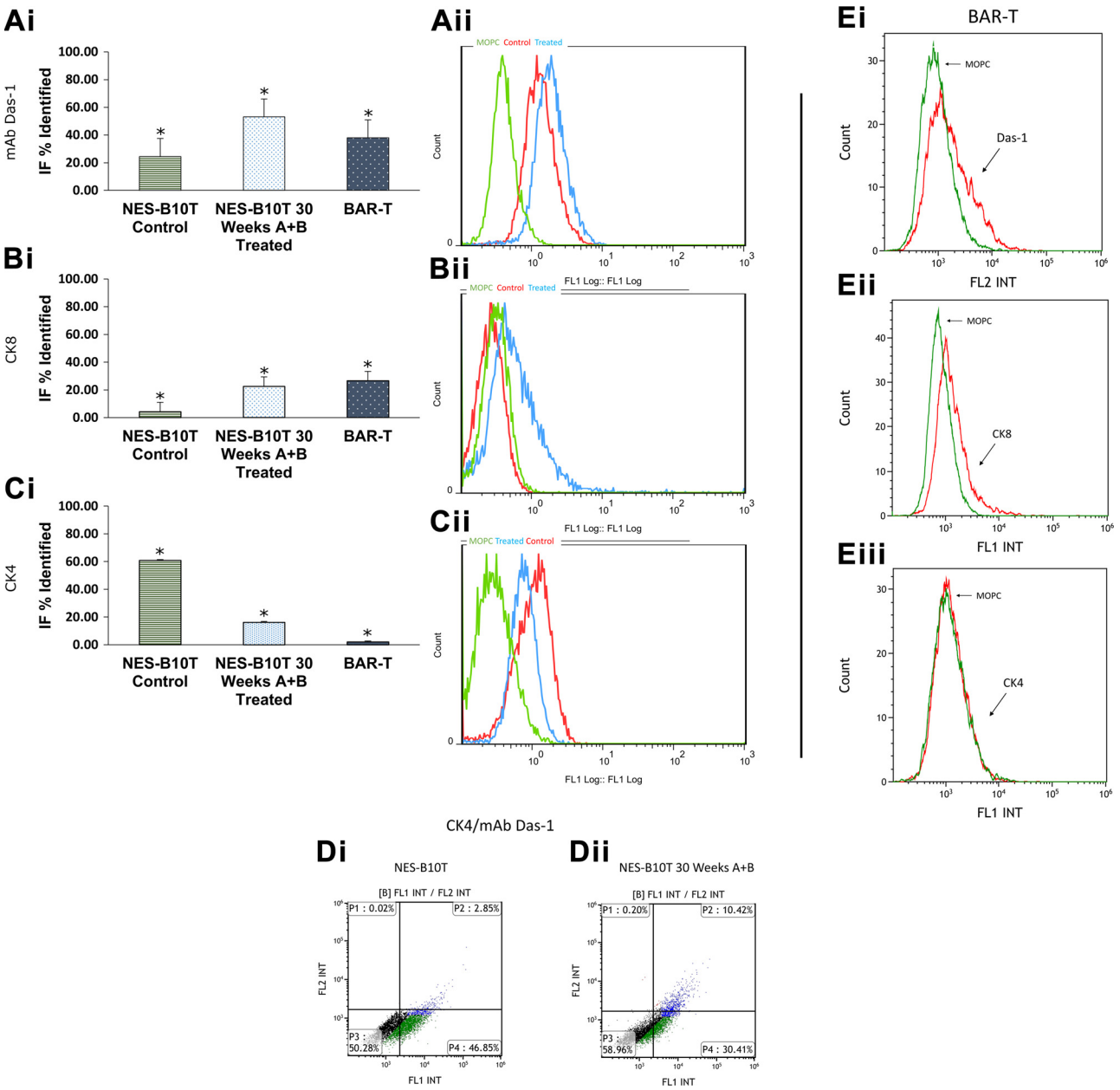

Fig. 3. Indirect immunofluorescence (IF) analysis of epithelial markers expressed by NES-B10T 30-wk acid and base (A + B) cells compared with untreated control and the Barrett's cell line BAR-T. There is a significant increase in the columnar markers mAb Das-1 (*Ai*) and CK8 (*Bi*), while the squamous marker CK4 (*Ci*) is significantly decreased after 30-wk of A + B treatment of NES-B10T cells ($n = 3$, $^*P < 0.01$). The representative graphs show results of FACS analysis after indirect IF (*Aii*, *Bii*, and *Cii*). The distributions of mAb Das-1, CK8, and CK4 staining in NES-B10T 30-wk A + B cells and BAR-T cells (*Ei*, *Eii*, and *Eiii*, respectively) are similar and significantly different from the control NES-B10T cells ($P < 0.05$); MOPC, isotype control monoclonal antibody. Coimmunofluorescence staining of control NES-B10T (*Di*) vs. NES-B10T 30-wk A + B (*Dii*) cells demonstrate a higher percentage of biphenotypic cells ($P < 0.02$) with a reduction of the pure $CK4^+$ squamous population after chronic A + B treatment ($P < 0.05$). No distinct pure mAb Das-1 population was identified.

(36, 38). It has been postulated that the continuous cycle of injury and repair in the distal esophagus may direct the precursor cells from the NES epithelium to revert to their embryological columnar phenotype (38). This process involving alterations in the pattern of "master switches" is referred to as reprogramming or transcommitment, based on observations from animal models and genetic manipulation of cell lines (25, 29, 36, 38, 45, 46). The appearance of columnar metaplasia in the NES epithelium was also referred to as transdifferentiation by some investigators, considering that both cell types involved in switching phenotypes were mature/terminally differentiated (36, 38, 41). Since the NES-B10T cell line was derived from a biopsy specimen of a patient with GERD and BM (37, 48), these cells may be derived from preterminal parent cells that retain the proliferative capacity and, therefore, may not represent true "fully terminally differentiated" epithelial cells (37, 48). They may also represent pre-Barrett's cells that are histologically squamous but also express markers of undifferentiated-type colonic metaplasia (44). Consequently, the proliferative, biphenotypic NES-B10T cells modify their

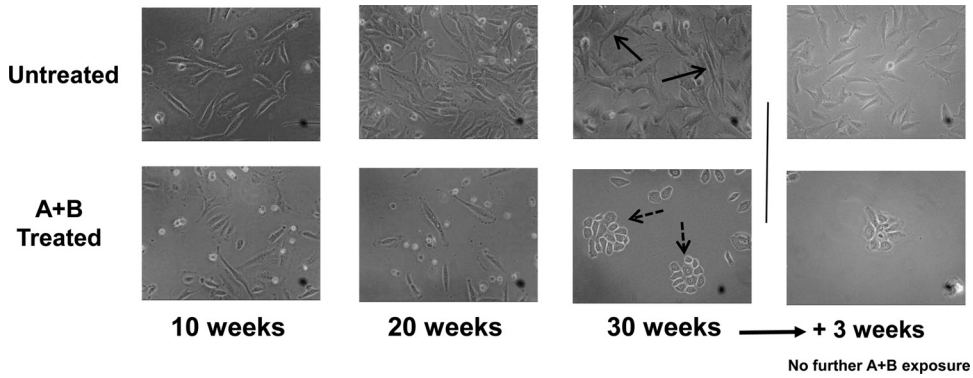

Fig. 4. Morphological changes appear in NES-B10T cells after 30 wk of daily acid (pH 4) and bile (A + B) treatment. The cells grew round or oval in clumps in A + B-treated group (dashed arrows). The parallel control untreated cells grew dispersed and maintained an elongated shape similar to the parent cell line (solid arrows). NES-B10T 30-wk A + B cells retained the morphological characteristics even without further A + B exposure for at least the 3 wk studied.

predetermined squamous commitment and differentiate into a columnar phenotype under the influence of the noxious physiologic environment created by gastric refluxate. Considering their precursor-like properties, this behavior is more synonymous with reprogramming or transcommitment rather than transdifferentiation.

The esophagus, during human embryogenesis, is initially lined by ciliated columnar cells (1). As maturation proceeds, these cells are replaced by stratified squamous cells that become the resident cell type in postnatal life (1, 6, 41). In adult epithelial tissues, key lineage TFs work in an orchestrated manner to maintain cellular homeostasis (29). This process in the adult NES epithelium is mainly controlled by the master TF TAp63, the NH_2-terminal transactivation domain TA isotype of p63 that also regulates the proliferative potential in the mature epithelium (32, 43, 47). Experiments with p63-deficient mice showed the loss of p63 isoforms, including TAp63, prevented conversion of the columnar epithelium into the squamous type during embryonic development and forced the NES epithelial cells toward intestinal metaplasia (6).

Earlier studies in human BE tissues demonstrated that the loss or downregulation of p63 and its isoforms was accompanied by overexpression of intestinal-columnar TFs, such as CDX2, SOX9, and CEP, the marker for colonic/columnar (incomplete) metaplasia (1, 2, 4, 7, 8, 10, 19, 24). This information indirectly suggested the key roles of the master switches TAp63, CDX2, and SOX9 in governing the reprogramming or transcommitment process of resident NES cells into an intestinal-type columnar lineage. The gastroesophageal refluxate is implicated in disruption of the cell lineage-determining factor(s) causing decreased levels of TAp63 in the adult esophagus thus allowing reexpression of the latent embryological columnar lineage-determining signals CDX2 and SOX9 (15, 38, 43) consistent with precursor-like biphenotypic characteristics.

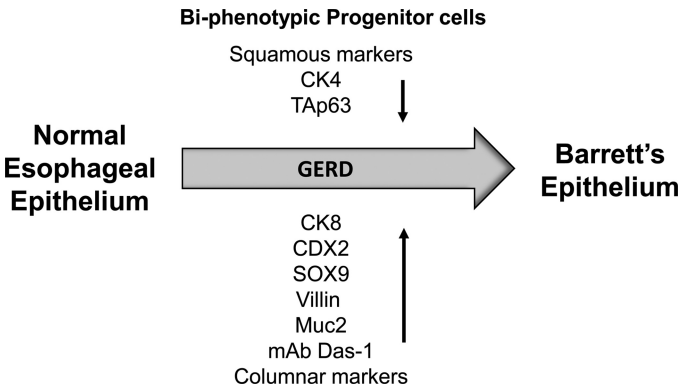

Fig. 5. Schematic of the proposed origin of Barrett's metaplasia from reprogramming or transcommitment of preterminal normal esophageal squamous epithelial cells due to chronic gastroesophageal reflux disease (GERD). Those esophageal squamous epithelial cells that may reprogram their differentiation process under the influence of gastric reflux components form biphenotypic progenitor like cells that express markers of both squamous and columnar lineage. These biphenotypic progenitors may be the precursors for the Barrett's columnar epithelium.

Longitudinal observations from this in vitro study highlight that the BM directly originates from the resident NES epithelial cells due to chronic exposure to physiologic components found in GERD refluxate in the absence of any stem cells or artificial genetic manipulation (21, 23). Our data support that chronic A + B induce downregulation of TAp63 and overexpression of CDX2, SOX9, and mAb Das-1, thus driving the squamous cells toward progenitor-like biphenotypic cells that may be the precursors of the colonic or incomplete type of columnar metaplasia (8, 10, 11, 38).

The molecular changes in the NES-B10T cells were not evident at 10 or 20 wk of A + B exposure but only at and after 30 wk, indicating that chronicity (duration) of exposure is important in propagating reprogramming or transcommitment. It is also important to note that after switching their commitment due to the exposure to A + B for 30 wk, the NES-B10T cells seem to retain the molecular and morphological changes without further treatment for a period of 3 wk (Fig. 5).

In conclusion, the physiological reflux components, i.e., A + B, caused chronic stress that modulated the levels of the transcriptional factors TAp63, CDX2, and SOX9 leading to reprogramming or transcommitment of normal esophageal squamous epithelial cells into expressing markers of columnar lineage in vitro. The NES-B10T 30-wk A + B-exposed cell culture model is a physiological, reliable, and reproducible tool to study the molecular mechanisms involved during transcommitment. This unique model may contribute to a better understanding of the molecular mechanisms underlying BM origins and pathogenesis and may also possibly be utilized for future chemoprevention studies to prevent the incidence of EAC arising from BM.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

C.D.M., M.B., and K.M.D. conceived and designed research; C.D.M., M.B., X.G., C.L.C., and A.A.C. performed experiments; C.D.M., M.B., R.F.S., S.J.S., and K.M.D. analyzed data; C.D.M., M.B., and K.M.D. interpreted results of experiments; C.D.M. and M.B. prepared figures; C.D.M., M.B., and K.M.D. drafted manuscript; C.D.M., M.B., R.F.S., and S.J.S. edited and revised manuscript; C.D.M., M.B., and K.M.D. approved final version of manuscript.

REFERENCES

1. **Badve S, Lôgdberg L, Sokhi R, Sigal SH, Botros N, Chae S, Das KM, Gupta S.** An antigen reacting with das-1 monoclonal antibody is ontogenically regulated in diverse organs including liver and indicates sharing of developmental mechanisms among cell lineages. *Pathobiology* 68: 76–86, 2000. doi:10.1159/000028117.
2. **Bajpai M, Liu J, Geng X, Souza RF, Amenta PS, Das KM.** Repeated exposure to acid and bile selectively induces colonic phenotype expression in a heterogeneous Barrett's epithelial cell line. *Lab Invest* 88: 643–651, 2008. doi:10.1038/labinvest.2008.34.
3. **Barr H, Upton MP, Orlando RC, Armstrong D, Vieth M, Neumann H, Langner C, Wiley EL, Das KM, Pickett-Blakely OE, Bajpai M, Amenta PS, Bennett A, Going JJ, Younes M, Wang HH, Taddei A, Freschi G, Ringressi MN, Degli'innocenti DR, Castiglione F, Bechi P.** Barrett's esophagus: histology and immunohistology. *Ann N Y Acad Sci* 1232: 76–92, 2011. doi:10.1111/j.1749-6632.2011.06046.x.
4. **Chen H, Fang Y, Tevebaugh W, Orlando RC, Shaheen NJ, Chen X.** Molecular mechanisms of Barrett's esophagus. *Dig Dis Sci* 56: 3405–3420, 2011. doi:10.1007/s10620-011-1885-6.
5. **Crane SJ, Locke GR, Harmsen WS, Zinsmeister AR, Romero Y, Talley NJ.** Survival trends in patients with gastric and esophageal adenocarcinomas: a population-based study. *Mayo Clin Proc* 83: 1087–1094, 2008. doi:10.4065/83.10.1087.
6. **Daniely Y, Liao G, Dixon D, Linnoila RI, Lori A, Randell SH, Oren M, Jetten AM.** Critical role of p63 in the development of a normal esophageal and tracheobronchial epithelium. *Am J Physiol Cell Physiol* 287: C171–C181, 2004. doi:10.1152/ajpcell.00226.2003.
7. **Das KM, Kong Y, Bajpai M, Kulkarni D, Geng X, Mishra P, Banerjee D, Hirshfield K.** Transformation of benign Barrett's epithelium by repeated acid and bile exposure over 65 weeks: a novel in vitro model. *Int J Cancer* 128: 274–282, 2011. doi:10.1002/ijc.25343.
8. **Das KM, Prasad I, Garla S, Amenta PS.** Detection of a shared colon epithelial epitope on Barrett epithelium by a novel monoclonal antibody. *Ann Intern Med* 120: 753–756, 1994. doi:10.7326/0003-4819-120-9-199405010-00006.
9. **Das KM, Sakamaki S, Vecchi M, Diamond B.** The production and characterization of monoclonal antibodies to a human colonic antigen associated with ulcerative colitis: cellular localization of the antigen by using the monoclonal antibody. *J Immunol* 139: 77–84, 1987.
10. **Glickman JN, Wang H, Das KM, Goyal RK, Spechler SJ, Antonioli D, Odze RD.** Phenotype of Barrett's esophagus and intestinal metaplasia of the distal esophagus and gastroesophageal junction: an immunohistochemical study of cytokeratins 7 and 20, Das-1 and 45 MI. *Am J Surg Pathol* 25: 87–94, 2001. doi:10.1097/00000478-200101000-00010.
11. **Griffel LH, Amenta PS, Das KM.** Use of a novel monoclonal antibody in diagnosis of Barrett's esophagus. *Dig Dis Sci* 45: 40–48, 2000. doi:10.1023/A:1005449024524.
12. **Halstensen TS, Das KM, Brandtzaeg P.** Epithelial deposits of immunoglobulin G1 and activated complement colocalise with the M(r) 40 kD putative autoantigen in ulcerative colitis. *Gut* 34: 650–657, 1993. doi:10.1136/gut.34.5.650.
13. **Hayeck TJ, Kong CY, Spechler SJ, Gazelle GS, Hur C.** The prevalence of Barrett's esophagus in the US: estimates from a simulation model confirmed by SEER data. *Dis Esophagus* 23: 451–457, 2010. doi:10.1111/j.1442-2050.2010.01054.x.
14. **Herfs M, Hubert P, Delvenne P.** Epithelial metaplasia: adult stem cell reprogramming and (pre)neoplastic transformation mediated by inflammation? *Trends Mol Med* 15: 245–253, 2009. doi:10.1016/j.molmed.2009.04.002.
15. **Huo X, Juergens S, Zhang X, Rezaei D, Yu C, Strauch ED, Wang JY, Cheng E, Meyer F, Wang DH, Zhang Q, Spechler SJ, Souza RF.** Deoxycholic acid causes DNA damage while inducing apoptotic resistance through NF-κB activation in benign Barrett's epithelial cells. *Am J Physiol Gastrointest Liver Physiol* 301: G278–G286, 2011. doi:10.1152/ajpgi.00092.2011.
16. **Huo X, Zhang HY, Zhang XI, Lynch JP, Strauch ED, Wang JY, Melton SD, Genta RM, Wang DH, Spechler SJ, Souza RF.** Acid and bile salt-induced CDX2 expression differs in esophageal squamous cells from patients with and without Barrett's esophagus. *Gastroenterology* 139: 194–203.e1, 2010. doi:10.1053/j.gastro.2010.03.035.
17. **Jaiswal KR, Morales CP, Feagins LA, Gandia KG, Zhang X, Zhang HY, Hormi-Carver K, Shen Y, Elder F, Ramirez RD, Sarosi GA Jr, Spechler SJ, Souza RF.** Characterization of telomerase-immortalized, non-neoplastic, human Barrett's cell line (BAR-T). *Dis Esophagus* 20: 256–264, 2007. doi:10.1111/j.1442-2050.2007.00683.x.
18. **Kesari KV, Yoshizaki N, Geng X, Lin JJ, Das KM.** Externalization of tropomyosin isoform 5 in colon epithelial cells. *Clin Exp Immunol* 118: 219–227, 1999. doi:10.1046/j.1365-2249.1999.01046.x.
19. **Lagorce C, Paraf F, Vidaud D, Couvelard A, Wendum D, Martin A, Fléjou JF.** Cyclooxygenase-2 is expressed frequently and early in Barrett's oesophagus and associated adenocarcinoma. *Histopathology* 42: 457–465, 2003. doi:10.1046/j.1365-2559.2003.01627.x.
20. **Liang-Chu MM, Yu M, Haverty PM, Koeman J, Ziegle J, Lee M, Bourgon R, Neve RM.** Human biosample authentication using the high-throughput, cost-effective SNPtrace(TM) system. *PLoS One* 10: e0116218, 2015. doi:10.1371/journal.pone.0116218.
21. **Liu T, Zhang X, So CK, Wang S, Wang P, Yan L, Myers R, Chen Z, Patterson AP, Yang CS, Chen X.** Regulation of Cdx2 expression by promoter methylation, and effects of Cdx2 transfection on morphology and gene expression of human esophageal epithelial cells. *Carcinogenesis* 28: 488–496, 2007. doi:10.1093/carcin/bgl176.
22. **Lurje G, Vallbohmer D, Collet PH, Xi H, Baldus SE, Brabender J, Metzger R, Heitmann M, Neiss S, Drebber U, Holscher AH, Schneider PM.** COX-2 mRNA expression is significantly increased in acid-exposed compared to nonexposed squamous epithelium in gastroesophageal reflux disease. *J Gastrointest Surg* 11: 1105–1111, 2007. doi:10.1007/s11605-007-0210-3.
23. **Mari L, Milano F, Parikh K, Straub D, Everts V, Hoeben KK, Fockens P, Buttar NS, Krishnadath KK.** A pSMAD/CDX2 complex is essential for the intestinalization of epithelial metaplasia. *Cell Reports* 7: 1197–1210, 2014. doi:10.1016/j.celrep.2014.03.074.
24. **Mirza ZK, Das KK, Slate J, Mapitigama RN, Amenta PS, Griffel LH, Ramsundar L, Watari J, Yokota K, Tanabe H, Sato T, Kohgo Y, Das KM.** Gastric intestinal metaplasia as detected by a monoclonal antibody is highly associated with gastric adenocarcinoma. *Gut* 52: 807–812, 2003. doi:10.1136/gut.52.6.807.
25. **Mutoh H, Hakamata Y, Sato K, Eda A, Yanaka I, Honda S, Osawa H, Kaneko Y, Sugano K.** Conversion of gastric mucosa to intestinal metaplasia in Cdx2-expressing transgenic mice. *Biochem Biophys Res Commun* 294: 470–479, 2002. doi:10.1016/S0006-291X(02)00480-1.
26. **Overton WR.** Modified histogram subtraction technique for analysis of flow cytometry data. *Cytometry* 9: 619–626, 1988. doi:10.1002/cyto.990090617.
27. **Parker SL, Tong T, Bolden S, Wingo PA.** Cancer statistics, 1997. *CA Cancer J Clin* 47: 5–27, 1997. doi:10.3322/canjclin.47.1.5.
28. **Que J, Choi M, Ziel JW, Klingensmith J, Hogan BL.** Morphogenesis of the trachea and esophagus: current players and new roles for noggin and Bmps. *Differentiation* 74: 422–437, 2006. doi:10.1111/j.1432-0436.2006.00096.x.
29. **Regalo G, Leutz A.** Hacking cell differentiation: transcriptional rerouting in reprogramming, lineage infidelity and metaplasia. *EMBO Mol Med* 5: 1154–1164, 2013. doi:10.1002/emmm.201302834.
30. **Rex DK, Cummings OW, Shaw M, Cumings MD, Wong RK, Vasudeva RS, Dunne D, Rahmani EY, Helper DJ.** Screening for Barrett's

esophagus in colonoscopy patients with and without heartburn. *Gastroenterology* 125: 1670–1677, 2003. doi:10.1053/j.gastro.2003.09.030.

31. **Rogge-Wolf C, Seldenrijk CA, Das KM, Timmer R, Breumelhof R, Smout AJ, Amenta PS, Griffel LH.** Prevalence of mabDAS-1 positivity in biopsy specimens from the esophagogastric junction. *Am J Gastroenterol* 97: 2979–2985, 2002. doi:10.1111/j.1572-0241.2002.07114.x.
32. **Roman S, Pétré A, Thépot A, Hautefeuille A, Scoazec JY, Mion F, Hainaut P.** Downregulation of p63 upon exposure to bile salts and acid in normal and cancer esophageal cells in culture. *Am J Physiol Gastrointest Liver Physiol* 293: G45–G53, 2007. doi:10.1152/ajpgi.00583.2006.
33. **Ronkainen J, Aro P, Storskrubb T, Johansson SE, Lind T, Bolling-Sternevald E, Vieth M, Stolte M, Talley NJ, Agréus L.** Prevalence of Barrett's esophagus in the general population: an endoscopic study. *Gastroenterology* 129: 1825–1831, 2005. doi:10.1053/j.gastro.2005.08.053.
34. **Silberg DG, Swain GP, Suh ER, Traber PG.** Cdx1 and cdx2 expression during intestinal development. *Gastroenterology* 119: 961–971, 2000. doi:10.1053/gast.2000.18142.
35. **Sobieraj DM, Coleman SM, Coleman CI.** US prevalence of upper gastrointestinal symptoms: a systematic literature review. *Am J Manag Care* 17: e449–e458, 2011.
36. **Souza RF, Krishnan K, Spechler SJ.** Acid, bile, and CDX: the ABCs of making Barrett's metaplasia. *Am J Physiol Gastrointest Liver Physiol* 295: G211–G218, 2008. doi:10.1152/ajpgi.90250.2008.
37. **Souza RF, Shewmake KL, Shen Y, Ramirez RD, Bullock JS, Hladik CL, Lee EL, Terada LS, Spechler SJ.** Differences in ERK activation in squamous mucosa in patients who have gastroesophageal reflux disease with and without Barrett's esophagus. *Am J Gastroenterol* 100: 551–559, 2005. doi:10.1111/j.1572-0241.2005.41122.x.
38. **Spechler SJ, Souza RF.** Barrett's esophagus. *N Engl J Med* 371: 836–845, 2014. doi:10.1056/NEJMra1314704.
39. **Spechler SJ.** Barrett esophagus and risk of esophageal cancer: a clinical review. *JAMA* 310: 627–636, 2013. doi:10.1001/jama.2013.226450.
40. **Thrift AP, Pandeya N, Whiteman DC.** Current status and future perspectives on the etiology of esophageal adenocarcinoma. *Front Oncol* 2: 11, 2012. doi:10.3389/fonc.2012.00011.
41. **Tosh D, Slack JM.** How cells change their phenotype. *Nat Rev Mol Cell Biol* 3: 187–194, 2002. doi:10.1038/nrm761.
42. **Vallbohmer D, DeMeester SR, Peters JH, Oh DS, Kuramochi H, Shimizu D, Hagen JA, Danenberg KD, Danenberg PV, DeMeester TR, Chandrasoma PT.** Cdx-2 expression in squamous and metaplastic columnar epithelia of the esophagus. *Dis Esophagus* 19: 260–266, 2006. doi:10.1111/j.1442-2050.2006.00586.x.
43. **von Holzen U, Enders GH.** A surprise cell of origin for Barrett's esophagus. *Cancer Biol Ther* 13: 588–591, 2012. doi:10.4161/cbt.20088.
44. **Wang DH, Souza RF.** Transcommitment: Paving the Way to Barrett's Metaplasia. *Adv Exp Med Biol* 908: 183–212, 2016. doi:10.1007/978-3-319-41388-4_10.
45. **Wang X, Ouyang H, Yamamoto Y, Kumar PA, Wei TS, Dagher R, Vincent M, Lu X, Bellizzi AM, Ho KY, Crum CP, Xian W, McKeon F.** Residual embryonic cells as precursors of a Barrett's-like metaplasia. *Cell* 145: 1023–1035, 2011. doi:10.1016/j.cell.2011.05.026.
46. **Xian W, Ho KY, Crum CP, McKeon F.** Cellular origin of Barrett's esophagus: controversy and therapeutic implications. *Gastroenterology* 142: 1424–1430, 2012. doi:10.1053/j.gastro.2012.04.028.
47. **Zaika AI, El-Rifai W.** The role of p53 protein family in gastrointestinal malignancies. *Cell Death Differ* 13: 935–940, 2006. doi:10.1038/sj.cdd.4401897.
48. **Zhang HY, Zhang X, Chen X, Thomas D, Hormi-Carver K, Elder F, Spechler SJ, Souza RF.** Differences in activity and phosphorylation of MAPK enzymes in esophageal squamous cells of GERD patients with and without Barrett's esophagus. *Am J Physiol Gastrointest Liver Physiol* 295: G470–G478, 2008. doi:10.1152/ajpgi.90262.2008.
49. **Zhang X, Westerhoff M, Hart J.** Expression of SOX9 and CDX2 in nongoblet columnar-lined esophagus predicts the detection of Barrett's esophagus during follow-up. *Mod Pathol* 28: 654–661, 2015. doi:10.1038/modpathol.2014.157.