the transient nature of small interfering RNA transfection and suboptimal transfection and/or RNAi efficiency in CD44H cells. Thus, future experiments will require utilization of a system allowing complete loss of SOD2 expression to delineate better the functional roles of SOD2 in EMT, generation and maintenance of CD44H cells both *in vitro* and *in vivo*.

In addition to providing insight into the functional role of SOD2 in EMT, this study offers EPC2T cells as a tool for investigation of the dynamics underlying EMT. Interestingly, CD44L and CD44H subpopulations isolated from EPC2T (that is EPC2-hTERT-EGFR-p53^{R175H}-cyclin D1) display limited spontaneous CD44L to CD44H and CD44H to CD44L conversion as compared with EPC2-hTERT-EGFR-p53^{R175H} and OKF6-hTERT-EGFR-p53^{R175H} (Supplementary Figure S1), suggesting a potential negative role for cyclin D1 in facilitating keratinocyte plasticity. Despite well-established oncogenic functions, the role of cyclin D1 as it relates to EMT and CSCs remains unclear. For instance, a recent study in prostate cancer reports that cyclin D1 signaling anti-correlates with EMT-associated gene expression while also driving the expansion of an existing prostate stem cell pool.[51] Evaluation of characteristics associated with stemness and tumorigenicity in CD44L and CD44H subpopulations isolated from EPC2-hTERT-EGFR-p53^{R175H} cells with and without ectopic cyclin D1 expression are ongoing, and have the potential to provide further insight into the role of cyclin D1 in epithelial cell fate decisions. Furthermore, the differential repopulation abilities of genetically engineered transformed esophageal keratinocytes in presence or absence of ectopic cyclin D1 expression provide a tractable system for monitoring both EMT and the reverse process of mesenchymal–epithelial transition.

MATERIALS AND METHODS

Cell culture, treatment and morphological assessment

EPC2-hTERT (telomerase-immortalized normal human esophageal keratinocytes), OKF6-TERT-2 (telomerase-immortalized normal human oral keratinocytes) and transformed derivatives (EPC2-hTERT-neo-puro, EPC2-hTERT-EGFR-p53^{R175H}, EPC2T and OKF6-hTERT-EGFR-p53^{R175H}) were established and grown in keratinocyte-serum-free medium (Life Technologies, Carlsbad, CA, USA) at 37 °C in a 5% CO_2 atmosphere as described previously.[20,52] Countess Automated Cell Counter (Life Technologies) was used to count cells with 0.2% Trypan blue dye to exclude dead cells. Cells were treated with 5 ng/ml of recombinant human TGF-β1 (R&D Systems, Minneapolis, MN, USA) reconstituted in 4 mM HCl containing 0.1% bovine serum albumin. A cell-permeable IKK-2 Inhibitor IV (Cat. No. 401481, EMD Millipore, Billerica, MA, USA) was supplied in dimethyl sulfoxide (vehicle) and used to inhibit NF-κB activity at 20 nM. Phase-contrast images were acquired using a Nikon Eclipse TS100 microscope (Nikon, Tokyo, Japan). Spindle-shaped cells were scored by counting at least 100 cells per high-power field ($n=6$) under light microscopy. Senescence-associated β-galactosidase-expressing cells were scored by counting at least 100 cells high-power field ($n=6$) under light microscopy as described.[18,20]

RNAi and transfection

Small interfering RNA sequences directed against SOD2 (Silencer Selects s13268 and s13269, Life Technologies; SOD2-1 and SOD2-2), ZEB1 s229970 and s229971 (Life Technologies; ZEB1-1 and ZEB1-2), ZEB2 (s19032 and 19033, Life Technologies; ZEB2-1 and ZEB2-2), NF-κB p65 (Silencer Selects s11915, Life Technologies) or a non-silencing control sequence (Silencer Select Negative Control #1) (10 nM) (Life Technologies) were transfected transiently with Lipofectamine RNAi Max reagent (Life Technologies), following the manufacturer's instructions. Sixteen hours after the transfection, cells were exposed to hypoxia or normoxia, or treated with or without H_2O_2 for 48 h. Transient transfection of reporter plasmids and luciferase assays were performed as described previously.[18] Briefly, 200 ng of pSODLUC-3340 (a gift of Dr Burgering, University Medical Center Utrecht), P7/pGL3 or I2E-P7/pGL3[29–31] was transfected. Mean firefly luciferase activity was normalized to co-transfected renilla luciferase activity. Transfection was carried out at least three times, and variation between experiments was $<15\%$.

Real-time reverse transcription–PCR

RNA isolation, cDNA synthesis and real-time reverse transcription–PCR were performed using TaqMan Gene Expression Assays (Life Technologies) for SOD2 (Hs00167309_m1), catalase (Hs00156308_m1), GPX2 (Hs00702173_s1), GPX7 (Hs00210410_m1), *CDH1* (Hs00170423_m1), *CDH2* (Hs00983062_m1), *ZEB1* (Hs00232783_m1), *ZEB2* (Hs00207691_m1) as described.[20] SYBR green (Life Technologies) was used to quantitate mRNA for *β-actin* as described previously.[18] SYBR green was also used to quantitate mRNA for NF-κB p65, IL6 and IL8 with paired forward and reverse primers NF-κB p65-F (5′-CTCCGCGGGCAGCAT-3′) and NF-κB p65-R (5′-TCCTGTGTAGCCATTGATCTTGAT-3′); IL-6-F (5′-GCAGAAAAAGGCAAAGAATC-3′) and IL-6-R (5′-CTACATTTGCCGAAGAGC-3′); and IL-8-F (5′-CACCGGAAGGAACCATCTCA-3′) and IL-8-R (5′-TGGCAAAACTGCACCTTCACA-3′). Primer pairs specific to the 3′-untranslated region of the 1.5- and 4.2-kb *MnSOD* transcripts were used to determine their levels as described.[28] Relative level of each mRNA was normalized to β-actin, which serves as an internal control.

Western blotting

Whole-cell lysates were prepared as described previously.[18] Twenty μg of denatured protein was fractionated on a NuPAGE Bis-Tris 4–12% gel (Life Technologies). Following electrotransfer, Immobilon-P membranes (Millipore) were incubated with primary antibodies for NF-κB p65 (D14E12 XP Rabbit mAb #8242, Cell Signaling Technology, Beverly, MA, USA) at 1:1000, phospho-NF-κB p65^{Ser536} (93H1 Rabbit mAb, #3033, Cell Signaling) at 1:1000, SOD2 (ab13534, Abcam, Cambridge, UK) at 1:1000, GPX1 (#3206, Cell Signaling) at 1:1000 or catalase (#8841, Cell Signaling) at 1:1000 and then with the appropriate horseradish peroxidase-conjugated secondary antibody (GE Healthcare, Piscataway, NJ, USA). E-Cadherin, N-cadherin, ZEB1, ZEB2 and β-actin (a loading control) were detected as described previously.[20]

Immunofluorescence

Cells grown on glass coverslips precoated with bovine collagen (1 μg/ml; Organogenesis, Canton, MA, USA) were fixed in 3% formaldehyde for 20 min, permeabilized with 0.1% Triton X-100 in phosphate-buffered saline and blocked with 5% bovine serum albumin for 1 h. Cells were incubated with anti-SOD2 (1:100; ab13534, Abcam) overnight at 4 °C, and then with Rabbit-Cy2-conjugated secondary antibody (1:600; Jackson ImmunoResearch, West Grove, PA, USA) for 1 h at room temperature. Nuclei were counterstained using 4′,6-diamidino-2-phenylindole (1:10 000; Life Technologies). Stained objects were imaged with a Leica TCS SP8 confocal microscope using LAS software (Leica Microsystems, Buffalo Grove, IL, USA).

Flow cytometry and FACS

Flow cytometry and FACS were performed as described previously.[37] To determine CD44^{high}-CD24$^{low/-}$ cells (CD44H) and CD44$^{low/-}$-CD24$^{low/-}$ cells (CD44L) subpopulations, cells were suspended in Hank's balanced salt solution (Life Technologies) containing 1% bovine serum albumin (Sigma-Aldrich, St Louis, MO, USA) and stained with PE/Cy7-anti-CD24 at 1:10 (BioLegend, San Diego, CA, USA) and APC-anti-CD44 at 1:20 (BD Biosciences, San Jose, CA, USA) on ice for 30 min. FACS Vantage SE (BD Biosciences) was used to isolate CD44L and CD44H cells. Flow cytometry was repeated for each genotype and condition at least three times.

DCF assay

ROS were determined by flow cytometry using DCF dye (Life Technologies) as described previously.[37] In brief, cells were incubated with 10 μM DCF at 37 °C for 30 min and further cultured for up to 3 h prior to flow cytometry. Cells were incubated with or without the antioxidant *N*-acetyl-L-cysteine (Sigma-Aldrich) at 10 or 1 mM as indicated for 1 h.

Statistical analyses

Data from experiments are presented as mean ± s.e. ($n=3$) and were analyzed by a two-tailed Student's *t*-test. $P<0.05$ was considered significant.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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ABBREVIATIONS

CSC, cancer stem cell; EMT, epithelial–mesenchymal transition; FACS, fluorescence-activated cell sorting; RNAi, RNA interference; ROS, reactive oxygen species; SOD2, superoxide dismutase 2; TGF-β, transforming growth factor-β

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Inhibition of Notch signaling enhances transdifferentiation of the esophageal squamous epithelium towards a Barrett's-like metaplasia via KLF4

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Short Title: Notch signaling regulates Barrett's-like metaplasia via KLF4

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Abbreviations: BE, Barrett's esophagus; CDX1, caudal type homeobox 1; dnMAML, dominant-negative Mastermind-like; DSC1, desmocollin 1; DSC3, desmocollin 3; EAC, esophageal adenocarcinoma; EGJ: esophago-gastric junction; ESCC, esophageal squamous cell carcinoma; GERD, gastroesophageal reflux disease; GSI, gamma-secretase inhibitor; HES1, hairy and enhancer of split-1; ICN1, intracellular Notch 1; KLF4, Krüppel-like factor 4; TMA, tissue microarray.

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Abstract

Background and Aims: Barrett's esophagus (BE) is defined as an incomplete intestinal metaplasia generally characterized by the presence of columnar and goblet cells in the formerly stratified squamous epithelium of the esophagus that can lead to esophageal adenocarcinoma. Currently, there is no clearly identified cell of origin for human BE. Therefore, we investigated the role of Notch signaling in the development of BE metaplasia.

Methods: Human BE samples were stained for Notch receptors and downstream targets. Notch signaling was inhibited in human esophageal epithelial cells by expression of dominant-negative-Mastermind-like (dnMAML) and cell transdifferentiation was assessed by 3D organotypic culture and by BE-lineage specific gene expression measured by QPCR. Finally, KLF4 knockdown was performed to rescue the phenotype observed following Notch signaling inhibition.

Results: RNA microarray revealed that BE samples expressed lower levels of Notch receptors (NOTCH2 and NOTCH3) and the ligand (JAG1). Furthermore, BE tissue microarray showed decreased NOTCH1 expression and Notch signaling downstream target HES1. Expression of dnMAML promoted transdifferentiation of esophageal epithelial cells towards columnar-like cells as demonstrated by higher expression of columnar keratins (K8, K18, K19, K20) and glandular mucins (MUC2, MUC3B, MUC5B, MUC17) and lower expression of squamous keratins (K5, K13, K14). In 3D culture, elongated-shaped cells were observed in the basal layer of the epithelium formed by cells expressing dnMAML. Importantly, knockdown of KLF4 reversed the effects of Notch inhibition on BE-like transdifferentiation.

Conclusions: Notch signaling inhibition promotes transdifferentiation of esophageal cells towards BE-like metaplasia in part via KLF4. These results suggest that transdifferentiation of the esophageal epithelium could participate in the evolution to BE.

Keywords: Barrett's esophagus; KLF4; Notch signaling; transdifferentiation

Introduction

Barrett's esophagus (BE) is defined as an incomplete intestinal metaplasia of the esophagus. BE is characterized classically by the presence of differentiated intestinal columnar cells and post-mitotic mucin-producing goblet cells, and is estimated to have a prevalence of 5-6% in the US.[1,2] Gastroesophageal reflux disease (GERD), abdominal obesity, smoking and *Helicobacter pylori* infection eradication have been linked as factors associated with the development of BE. The local pro-inflammatory microenvironment is critical to the nurturing and maintenance of BE. [1,3] BE can progress to low-grade dysplasia, high-grade dysplasia and culminate in the development of esophageal adenocarcinoma (EAC). It is estimated that 0.5% of BE patients will develop EAC, and this increases to 10% for BE patients with high-grade dysplasia.[2]

In 2013, an estimated 17,990 patients were diagnosed in the US with esophageal cancer, both EAC and ESCC (esophageal squamous cell carcinoma). Esophageal cancer is one of the deadliest cancers in the US with a 5-year survival rate of 17.3% in 2013.[4] Therefore, studying the molecular mechanisms underlying the pathogenesis of BE could provide novel information to be used in the clinical outcomes of both BE and EAC patients. The study of BE has been historically limited to human biopsy samples, which have been used to analyze histopathological and genetic changes. Some of the known changes occurring in BE are the methylation and LOH of p16 and LOH of p53.[5] Mosaic pattern of genetic alterations can be found in biopsy samples complicating the identification of the initiating genetic changes that lead to BE. There have several hypothesis proposed for the cell of origin of BE.[6] These include: (1) transdifferentiation of cells from the esophageal basal layer or from the ducts of the esophageal submucosal glands, (2) migration of cells located at the esophago-gastro junction or of the gastric cardia cells [7,8]and (4) bone marrow derived stem cells.[9]

Recent studies have undertaken a genetic approach to investigate the initiating events that lead to the development of Barrett's esophagus. Through this approach, two genes, CDX1 and MYC (c-Myc), have been identified by us to have a role in the development of BE.[10] CDX1 is part of the caudal homeobox family of transcription factors (CDX1 and CDX2), which are important in the development and differentiation of the small intestine and colon. [11] In fact, intestinal conditional knockout of CDX2 in the mouse results in squamous metaplasia. [12] MYC is a transcription factor known to bind to E-box sequences and can activate 15% of all genes in the human genome.[13,14] MYC is overexpressed in many cancers, including amplification in

EAC.[15,16] MYC is involved in the activation and the regulation of a variety of cellular processes such as cell cycle progression, cell differentiation, energy metabolism, angiogenesis and DNA damage repair.[14] In our original microarray analysis of BE samples, the negative regulators of MYC, MXI1 and MXD1, were found to be downregulated in BE human tissue while MYC target genes (ODC1, CA2) were rather increased.[10] This suggested that MYC was active in Barrett's esophagus. In addition, the microarray data showed increased CDX1 and CDX2 expression in BE.[17] Our previous studies using a human esophageal epithelial cell line immortalized with hTERT (EPC2-hTERT) have allowed us to study human esophageal biology.[18]. Our previous data have shown that CDX1 overexpression together with MYC in the EPC2-hTERT cells can lead to a partial change towards Barrett's esophagus.[10]

Interestingly, loss of Notch signaling is required for the goblet cell lineage in the small intestine.[19,20] In addition, inhibition of Notch signaling in the small intestine by either γ-secretase inhibitor (GSI), or conditional knockout of CSL, can lead to a goblet cell hyperplasia.[19,21,22] Notch signaling pathway comprises four homologous transmembrane Notch receptors (NOTCH1-NOTCH4) that can be activated by transmembrane ligands Delta or Jagged generally expressed by neighboring cells allowing a juxtacrine signaling.[23,24] Upon binding of the ligand, the Notch receptors undergo protease-mediated cleavage by ADAM-family metalloproteases at the extracellular domain and by γ-secretase at the intracellular domain.[23] These events lead to the release of the intracellular domain (ICN) allowing its nuclear translocation, binding to CSL and activation of Notch target genes, such as HES1 and HES5.[23,25] Interestingly, Notch is known to be involved in the cell fate decision of several cell types including lymphocytes, neurons, skin and others.[25]

Notch signaling can be a negative regulator of Krüppel-like factor 4 (KLF4) expression in the context of the intestine. Indeed, it has been demonstrated previously that KLF4 promoter contains ICN responsive elements, through which Notch can inhibit its expression.[26,27] KLF4 is part of a family of DNA-binding transcription factors that have been shown to play a role in multiple processes from proliferation, cell differentiation, inflammation to pluripotency.[28] Recently, strong KLF4 expression was reported in human BE biopsies but also that its promoter was activated by bile acid.[29] In addition, KLF4 can increase the transcriptional activity of MUC2 and CDX2, suggesting a potential role in BE development.[29]

As a result, we utilize an innovative three-dimensional (3D) organotypic culture model system to demonstrate that the cooperation of MYC, CDX1, and inhibition of Notch signaling

results in a switch of cell identity and lineage specification from the normal esophageal squamous epithelium to a BE-like metaplasia. This is mediated through KLF4. These novel data suggest a new paradigm in which transdifferentiation of the esophagaeal basal cells could lead to the initation of BE.

Materials and Methods

Further information about microarray, viral production, shRNA construction, RNA extraction and qPCR, Luciferase assay and Western Blotting are available in Supplemental Materials and Methods.

Cell lines

EPC2-hTERT-MYC-CDX1 cells and their derivatives: EPC2-hTERT-MYC-CDX1-dnMAML, EPC2-hTERT-MYC-CDX1-dnMAML-shKLF4,EPC2-hTERT-MYC-CDX1-dnMAML-shScramble, EPC2-hTERT-MYC-CDX1-shHES1 and EPC2-hTERT-MYC-CDX1-shScramble were grown in KSFM (Keratinocyte Serum Free Medium, Invitrogen) with Ca^{2++} and supplements: BPE (bovine pituitary extract), EGF and 1% Penicillin Streptomycin (Invitrogen), as described previously. [17] Cells were treated with 0.06 mmol/L calcium chloride (Ca^{2++}) to promote squamous differentiation for 48 hrs before harvesting RNA. Phoenix A cells were grown in DMEM (Invitrogen) with 10% FBS (Sigma) and 1% Penicillin Streptomycin. FEF3 (fetal embryonic fibroblasts) were grown in DMEM supplemented with 10% HyClone FBS (GE Healthcare Life Sciences) and 1% Penicillin Streptomycin, as described previously.[30]

Stable transduction

The MYC-CDX1 cells were transduced with pBabe-puro or pBabe-dnMAML-GFP-puro or pBabe-zeo, pBabe-dnMAML-GFP-zeo. The MYC-CDX1 cells were also transduced with pLKO.1 shScramble-puro or pLKO.1 TRC puro-shHES1 and the MYC-CDX1-dnMAML (zeo) cells were transduced with pLKO.1 shScramble-puro or pLKO.1 shKLF4. Transduced cells were selected with 1ug/ml puromycin or 10ug/ml zeocin for 7 days.

3D Organotypic culture

EPC2-hTERT-MYC-CDX1 cells and its derivatives were grown using the 3D organotypic culture system as described previously.[31] Cultures were fixed overnight in 10% buffered formalin phosphate (Fisher) before paraffin embedding and sectioning.

Histology and Immunohistochemistry

Hematoxylin and eosin (H&E) staining as well as immunohistochemistry (IHC) were performed as described previously.[10] The following antibodies were used for IHC: K13 (Abcam) 1:500, K19 (BioLegend) 1:100, HES1 (Abcam) 1:500, NOTCH1 (Epitomics) 1:100 and ICN1 (Cell Signaling) 1:200. Biotinylated secondary antibodies (Jackson Immunoresearch Laboratories Inc.) and ABC avidin-biotin-DAB detection kit (Vector Labs) were used for detection and visualization according to supplier's protocol.

Cell height measurement

Quantification of cell height at the basal layer of MYC-CDX1 and MYC-CDX1-dnMAML cells grown in 3D organotypic cultures was performed by measuring 15 cells, per HPF (high power field) of H&E, 360 total cells per sample. We measured 4 independent 3D organotypic cultures for each cell line. Statistical analysis for significance was determined by student t-test with $p<0.05$ as statistically significant.

Tissue Microarray

Tissue microarray (TMA) of human biopsies of Barrett's esophagus (n=15-23), normal esophagus (n=25-27) and liver control were stained for status of Notch signaling. IHC staining of TMA was performed using the following antibodies: HES1, NOTCH1 and ICN1. Scoring for positive staining of HES1, ICN1 and NOTCH1 was analyzed by quantitative evaluation of staining intensity with a scale of 0-2 (0=none to 2=strong), by pathologist (AJK-S) in a blind manner.

Statistical Analysis

For gene expression changes in qPCR studies, statistical significance of comparisons between the MYC-CDX1 and MYC-CDX1-dnMAML cells and between the MYC-CDX1-dnMAML-shScramble and MYC-CDX1-dnMAML-shKLF4 cells were determined by the student *t-test* with $p<0.05$ as statistically significant. Error bars represent the mean ± SEM (Standard Error of the

Mean) from at least triplicate experiments. Scoring data of TMA was analyzed for statistical significance by Fisher's Exact Test with $p<0.05$ as statistically significant.

Results

Notch signaling is downregulated in human Barrett's esophagus

In order to investigate the status of Notch signaling in Barrett's esophagus (BE), we performed a RNA microarray on human BE biopsies compared to adjacent normal squamous esophagus (GEO accession # GSE 13083).[10] A significant decrease of NOTCH2 and NOTCH3 receptors by 4- and 2-fold respectively, was observed in BE versus paired normal squamous esophagus. Expression of the Notch ligand JAG1 was also decreased by 3-fold in BE biopsies, thereby suggesting a downregulation of Notch signaling in BE (Figure 1A). We next performed IHC of human BE (n=15-23) and normal esophagus (n=25-27) tissue microarray (TMA) for the NOTCH1 receptor, the active form of NOTCH1, ICN1 (Intracellular NOTCH1) as well as one of its key downstream targets, namely the transcription factor HES1 (Figure 1B-C). We observed positive nuclear staining for HES1 and NOTCH1 restricted to the basal layer of the normal esophageal epithelium, whereas ICN1 showed more diffused staining (Figure 1B). In BE, we observed a loss of nuclear staining for HES1 and loss of nuclear and membrane NOTCH1, and decreased staining for ICN1 (Figure 1B). The TMA was scored for intensity of staining (Supplemental Table 1). HES1, NOTCH1 and ICN1 protein expression is significantly decreased in BE when compared to the normal esophagus (Figure 1C). Both the RNA microarray and TMA data suggest that Notch signaling is downregulated in BE compared to the normal esophageal epithelium thus Notch signaling may be necessary for the maintenance of the normal esophageal squamous epithelium.

Inhibition of Notch signaling induces morphological changes in esophageal epithelial cells.

Our previous data have shown that CDX1 overexpression together with MYC in the human immortalized esophageal epithelial EPC2-hTERT cells can initiate changes towards Barrett's esophagus.[10] Yet, the changes observed in that study only showed a partial transdifferentiation, which suggested the need to explore additional genetic alteration in the context of MYC and CDX1 overexpression. Given our RNA microarray and human TMA data, we inhibited Notch signaling in the context of MYC and CDX1. We infected EPC2-hTERT-MYC-CDX1 (MYC-CDX1) cells with a construct encoding for dominant-negative Mastermind-like

(dnMAML) to inhibit Notch signaling (MYC-CDX1-dnMAML cells). We confirmed expression of dnMAML-GFP tagged protein by western blotting using a GFP specific antibody (Figure 2A). Furthermore, we confirmed that dnMAML expression can inhibit Notch signaling by a luciferase reporter assay. We used a 8X-CSL-Luciferase reporter construct, which upon expression of ICN1 can activate luciferase expression, to verify Notch signaling abolition following dnMAML overexpression. We observed activation of the luciferase reporter in the MYC-CDX1-ICN1 cells; however, we observed a significant inhibition of 9.98-fold in presence of dnMAML (MYC-CDX1-dnMAML-ICN1 cells) (Figure 2B). We also confirmed downregulation of Notch target genes expression by dnMAML via quantitative PCR (qPCR). Indeed, we observed a significant decrease of HES1 (3.3-fold) and HES5 (12.5-fold) expression in the MYC-CDX1-dnMAML when compared to MYC-CDX1 cells (Figure 2C). Those results support that dnMAML overexpression is sufficient to inhibit Notch signaling.

We next used 3D organotypic cultures to analyze for changes in cell differentiation and morphology.[31] We observed that MYC-CDX1-dnMAML cells formed a thinner stratified epithelium than MYC-CDX1 cells, suggesting disruption of normal differentiation. We also noted that the MYC-CDX1-dnMAML 3D cultures showed an altered cell morphology in the basal layer (Figure 2D), when compared to the MYC-CDX1 cells. In order to characterize further these changes in the basal layer, we performed electron microscopy of the MYC-CDX1 and MYC-CDX1-dnMAML cultures (Figure 2E). We observed an elongation of the MYC-CDX1-dnMAML basal cells when compared to the MYC-CDX1 cells, suggesting these cells are starting to acquire columnar-like morphological characteristics. Indeed, the height of the basal cells was significantly higher (1.4-fold) in 3D cultures overexpressing dnMAML (Figure 2F). These changes in cell morphology in the basal layer in the MYC-CDX1-dnMAML cells suggest that the inhibition of Notch signaling promotes the transdifferentiation of the normal esophageal epithelium towards a more columnar-like epithelium.

Inhibition of Notch signaling induces a switch from squamous markers to columnar markers gene expression

In order to investigate if the morphological changes observed in MYC-CDX1-dnMAML cells reflect changes in lineages, we further analyzed our 3D cells regarding squamous and BE lineage gene expression. We stained sections of our 3D cultures for the squamous keratin 13

(K13). In MYC-CDX1 cells, we observed strong staining for K13 in the suprabasal region; interestingly staining was reduced substantially in MYC-CDX1-dnMAML cells. Furthermore, we studied the columnar keratin 19 (K19). In MYC-CDX1-dnMAML cells, we observe increased positive staining in the basal as well as in the suprabasal compartments (Figure 3B).

Furthermore, we quantified the gene expression of other squamous and columnar lineage keratins in MYC-CDX1-dnMAML in comparison to MYC-CDX1 cells. Cells were grown in the presence of calcium (0.6 mmol/L) for 48 hrs to allow squamous differentiation, before analyzing changes in gene expression.[32] We observed that MYC-CDX1-dnMAML cells expressed reduced levels of squamous keratins: K5 (5-fold) K13 (16.6-fold) and K14 (5-fold) in qPCR (Figure 4A). Furthermore, the MYC-CDX1-dnMAML cells expressed higher levels of columnar keratins: K8 (2.2-fold), K18 (2.8-fold), K19 (1.9-fold) and K20 (2.8-fold) compared to the MYC-CDX1 cells (Figure 4B). These results suggest that inhibition of Notch signaling via dnMAML is promoting a switch in gene expression from squamous to columnar keratins. Furthermore, since BE is often characterized by the presence of goblet cells in the esophageal epithelium, we investigated the expression of mucins, the major protein family secreted by this cell type. Interestingly, we observed increased levels of MUC2 (10.4-fold), MUC3B (21.5-fold), MUC5B (305-fold) and MUC17 (116.3-fold) in MYC-CDX1-dnMAML cells when compared to MYC-CDX1 cells (Figure 4C). Thus, inhibition of Notch signaling is promoting expression of goblet cell lineage genes. Finally, we quantified the expression of squamous differentiation genes desmocollin1 and desmocollin3 (DSC1, DSC3). We observed that dnMAML overexpression in MYC-CDX1 cells decreased significantly DSC1 (20-fold) and DSC3 (3.7-fold) expression (Figure 4D). Taken together, these data support the premise that inhibition of Notch signaling in cooperation with MYC and CDX1 orchestrates a genetic switch from a squamous cell lineage to an intestinal columnar cell lineage.

KLF4 knockdown reverses the morphological and transcriptional changes following Notch signaling inhibition

Classically, Notch signaling leads to activation of transcriptional factors such as HES1 and HES5. In the intestine, HES1 has been shown to be a negative regulator of HATH1, thereby promoting the absorptive cell fate over the secretory cell fate.[33,34] Therefore, we investigated if downregulation of HES1 could mimic the results obtained with dnMAML overexpression in

MYC-CDX1 cells. We performed stable knockdown of HES1 with two independent shRNA constructs (shHES1#1 and shHES1 #2) in MYC-CDX1 cells (Supplemental Figure 1A). We did not observe any changes on squamous and columnar keratins or on mucin genes expression upon knockdown of HES1 suggesting that HES1 alone is not sufficient to transdifferentiate the esophageal epithelial cells (Supplemental Figure 1B-D). We observed a trend of upregulation of HATH1 expression validating the functional HES1 knockdown (Supplementary Figure 1E). Thus, HES1 knockdown could not recapitulate the effects of Notch signaling inhibition suggesting that Notch might act via other downstream targets to regulate transdifferentiation to BE.

Active Notch signaling mediated by ICN1 downregulates KLF4 expression.[27] Conversely, inhibition of Notch signaling via GSI can cause upregulation of KLF4 expression.[26] Interestingly, KLF4 expression is increased in MYC-CDX1-dnMAML cells when compared to MYC-CDX1 cells (Figure 5A, 5B). Therefore, we investigated if KLF4 knockdown in MYC-CDX1-dnMAML cells could reverse the genetic changes observed following dnMAML overexpression. Using a stable lentiviral infection, we achieved significant knockdown of KLF4 using 2 independent shRNA sequences in MYC-CDX1-dnMAML cells (Figure 5C, 5D). We observed the highest degree of KLF4 knockdown in the shKLF4 #3 (3.3-fold in RNA and 25-fold in protein). Stable KLF4 knockdown results in a decrease in elongated (columnar-like) cells observed with inhibition of Notch signaling in 3D culture (Figure 5E). Furthermore, the cells at the basal layer of the epithelium have a more cuboidal shape, suggesting the inhibition of KLF4 in the MYC-CDX1-dnMAML cells can reverse the morphological changes observed with inhibition of Notch signaling.

Analysis of columnar keratins showed significant decreased K8 and K20 expression, but no changes in K18 and K19 suggesting that MYC-CDX1-dnMAML-shKLF4 (Figure 6A). We also observed a significant decrease in MUC2 and MUC5B expression, but no changes in MUC3B and MUC17 (Figure 6B). Furthermore, we evaluated the expression of the squamous keratins K5, K13 and K14 in MYC-CDX1-dnMAML-shKLF4 cells and MYC-CDX1-dnMAML-shScramble cells. KLF4 knockdown significantly increased the expression of K5, K13 and K14; supporting the premise that KLF4 knockdown can partially reverse the switch from squamous to columnar keratins observed with Notch signaling inhibition (Figure 6C). Moreover, expression of the squamous differentiation marker DSC1 is increased significantly in the MYC-CDX1-dnMAML-shKLF4 cells (Figure 6D). Overall, these results demonstrate that KLF4 mediates in

part dnMAML-induced transdifferentiation. Thus, we find a novel function for Notch signaling in BE development mediated by KLF4. In addition, we provide new evidence to supporting the model that esophageal epithelial basal cells might serve as a potential cell of origin for Barrett's esophagus metaplasia.

Discussion

Barrett's esophagus (BE) is defined as an incomplete intestinal metaplasia of the esophagus and the biological mechanisms underlying its development remain to be clarified. Herein, we demonstrate that Notch signaling is downregulated in human BE suggesting that the development of intestinal metaplasia in the esophagus could require inhibition of Notch signaling. Indeed, inhibition of Notch promotes transdifferentiation in our model system. First, there is the appearance of columnar-like elongated cells in the basal layer of 3D organotypic cultures in response to Notch inhibition. Second, there is a switch in genes that denote the squamous versus BE lineages, characterized by a robust diminution of squamous keratins and differentiation markers in favor of a strong induction of columnar keratins and mucins. Therefore, our results suggest that the combination of Notch inhibition and MYC and CDX1 overexpression promotes transdifferentiation of esophageal epithelial cells towards a BE metaplasia-like state.

Transdifferentiation may be viewed as reprogramming and involves the replacement of one cell type into another cell type. The cell of origin for the development of BE remains the subject of investigation and much debate, but transdifferentiation of the esophageal stratified epithelium is one of the proposed models. [6] One study of human BE biopsies has revealed that esophageal cells undergo a transition of expression of intestinal markers like CDX2 and MUC5AC.[35] Interestingly, NOTCH1 activation is observed at the onset of squamous differentiation of the esophagus. NOTCH1 and NOTCH3 orchestrate the transcriptional regulation of early differentiation markers in a CSL-dependent manner.[36] Perturbation of esophageal squamous differentiation is notably observed following loss of Notch signaling in the esophagus epithelium.[36] Notch signaling orchestrates cell differentiation in several tissues. Loss of Notch signaling is required for the differentiated goblet cells and other secretory cell lineages in the small intestine.[19,20]

We show that the combination of Notch inhibition with MYC and CDX1 overexpression leads to characteristic features of BE, namely the production of mucin by goblet-like cells and the presence of columnar-like cells. It was suggested previously that HATH1 induction by Notch inhibition induces MUC2 via CDX2 expression in esophageal cancer cell lines. [37] Moreover, goblet cell differentiation was induced by Notch inhibition in the L2-IL-1β Barrett's-like metaplasia mouse model.[8] Hence, Notch signaling inhibition could be necessary for the initiation

of the BE metaplasia program by orchestrating the transcriptional regulation of key genes implicated in goblet cell terminal differentiation.

KLF4 can be regulated negatively by Notch signaling.[26] Herein, we demonstrate that inhibition of Notch signaling causes an activation of KLF4 expression and that knockdown of KLF4 could reverse some of the genetic and morphological changes induced by Notch signaling inhibition. These data support KLF4 as one of the potential drivers of the activation of intestinal cell lineage genes upon inhibition of Notch signaling, suggesting a new mechanism through which Notch signaling could participate in BE initiation. Interestingly, KLF4 is strongly expressed in Barrett's esophagus and its expression is induced in response to bile acids. KLF4 and CDX2 also cooperate to induce the production of MUC2. Interestingly, KLF4 has been linked to other models of transdifferentiation as conversion of smooth muscle cells into osteogenic cells in the context of hyperphosphatemia or conversion of fibroblasts into neural progenitors or cardiomyocytes.[38-40] KLF4 is one of the key reprogramming factors (OCT4, SOX2, MYC, NANOG and KLF4) that can reprogram the fate of somatic cells into inducible pluripotent stem cells (iPSC). KLF4 is also recognized for its capacity to maintain the pluripotent state of embryonic stem cells (ESC).[28] Therefore, KLF4 activation in response to Notch inhibition could facilitate the transdifferentiation of the esophageal squamous cells into intestinal-like cells by binding to the promoter of columnar keratins and mucin genes to promote their expression (Figure 7).

Herein, we provide evidence to support the notion that esophageal basal cells might serve as potential cells of origin for BE. Several models of BE cells of origin are proposed and they are not necessarily mutually exclusive and may be contextual dependent. One model suggests that cells may migrate from the esophago-gastric junction (EGJ) or from the gastric cardia (Ref). By lineage-labeling gastric cardia stem cells (LGR5+) cells in L2-IL-1β transgenic mice that develop BE and EAC, it was demonstrated that migration of gastric cardia cells give rise to BE tissue. [8] It is also possible that bone marrow derived stem cells give rise to BE. Indeed, male to female bone marrow transplants in a severe reflux esophagitis rat model, showed that the developing BE epithelium was of male origin.[9] Suggesting that stem cells originating from the bone marrow can contribute to BE development.

Metaplasia may represent an adaptive response to a stressful local environment, and involve a complex interplay with epigenetic and genetic factors or alterations. It can occur in several tissue including esophagus (BE), stomach, pancreas, lung, cervix and skin. Metaplasia

may be reversible or irreversible, and may progress to dysplasia and cancer. For example, BE can progress to low-grade and high-grade dysplasia and culminate into esophageal adenocarcinoma (EAC). Herein, we observe an initiation of transdifferentiation of esophageal epithelial cells to a BE-like metaplasia, involving a change in cell identity and adoption of a BE-like lineage. Our studies suggest that Notch signaling and pivotal transcription factors- MYC, CDX1 and KLF4 can promote a partial reprogramming of the esophageal cells toward BE. However, the complete emergence of BE, and certainly progression to a dysplastic state and EA involves additional mechanisms, such as inflammation and activation of Hedgehog signaling and Wnt signaling.[41-43] Our studies may provide a platform for the utilization of some new biomarkers for early detection of metaplastic BE.