Original Article
Notch receptor inhibition reveals the importance of cyclin D1 and Wnt signaling in invasive esophageal squamous cell carcinoma

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Abstract: Esophageal squamous cell carcinoma (ESCC) is one of the most aggressive forms of squamous cell carcinomas. Common genetic lesions in ESCC include p53 mutations and EGFR overexpression, both of which have been implicated in negative regulation of Notch signaling. In addition, cyclin D1 is overexpressed in ESCC and can be activated via EGFR, Notch and Wnt signaling. To elucidate how these genetic lesions may interact during the development and progression of ESCC, we tested a panel of genetically engineered human esophageal cells (keratinocytes) in organotypic 3D culture (OTC), a form of human tissue engineering. Notch signaling was suppressed in culture and mice by dominant negative Mastermind-like1 (DNMAML1), a genetic pan-Notch inhibitor. DNMAML1 mice were subjected to 4-Nitroquinoline 1-oxide-induced oral-esophageal carcinogenesis. Highly invasive characteristics of primary human ESCC were recapitulated in OTC as well as DNMAML1 mice. In OTC, cyclin D1 overexpression induced squamous hyperplasia. Concurrent EGFR overexpression and mutant p53 resulted in transformation and invasive growth. Interestingly, cell proliferation appeared to be regulated differentially between those committed to squamous-cell differentiation and those invading into the stroma. Invasive cells exhibited Notch-independent activation of cyclin D1 and Wnt signaling. Within the oral-esophageal squamous epithelia, Notch signaling regulated squamous-cell differentiation to maintain epithelial integrity, and thus may act as a tumor suppressor by preventing the development of a tumor-promoting inflammatory microenvironment.

Keywords: Esophageal squamous cell carcinoma, organotypic 3D culture, EGFR, P53, cyclin D1, Wnt, Notch, squamous-cell differentiation, invasion, 4-Nitroquinoline 1-oxide

Introduction

Esophageal squamous cell carcinoma (ESCC) is one of the most aggressive forms of squamous cell carcinomas (SCCs) [1, 2] and defined as a malignant epithelial tumor with variable squamous-cell differentiation [3]. Common genetic lesions in ESCC include inactivation of the p53, p16INK4A and p120catenin tumor suppressor genes and overexpression of the cyclin D1 and EGFR oncogenes [4, 5]. EGFR and cyclin D1

are upregulated and p53 mutations are also found in premalignant ESCC lesions, including squamous dysplasia [6-9]. In addition to mouse models, genetically engineered human esophageal cells (keratinocytes) have served as a platform to investigate esophageal tumor biology [10-12]. In particular, organotypic 3D culture (OTC) is a powerful type of human tissue engineering that facilitates esophageal epithelia reconstitution *in vitro* with recapitulation of human pathology [13], revealing, for example,

highly invasive characteristics of transformed human esophageal cells associated with epithelial mesenchymal transition (EMT) [11, 12, 14, 15].

Cell proliferation and differentiation within the stratified squamous epithelia are regulated by Notch signaling [16-18]. Notch ligands stimulate Notch receptor molecules on adjacent signaling receiving cells through cell-cell contact. Ligand binding triggers a series of enzymatic cleavages of the Notch receptor, leading to generation and nuclear translocation of the intracellular domain of Notch (ICN). ICN forms a transcriptional activation complex containing a transcription factor CSL—CBF-1/RBP-jκ, Su(H), *Lag-1*—and the coactivator Mastermind-like (MAML) [19]. Notch target genes include the HES/HEY family of transcription factors.

In the normal esophagus, Notch regulates squamous-cell differentiation [18]. Downregulation of Notch signaling may lead to attenuation of squamous-cell differentiation and enhancement of an invasive subset of ESCC cells [20]. In genetically engineered mouse models, loss of Notch receptor family members or expression of dominant negative MAML1 (DNMAML1), a genetic pan-Notch inhibitor, promotes cutaneous SCC [21-24]. Loss-of-function somatic mutations of Notch receptor paralogs have recently been found in primary SCCs [25-27]. Thus, Notch may act as a tumor suppressor in SCCs.

Functional interplay between Notch and various oncogenes and tumor suppressor genes is implicated in a variety of cancers. In particular, the p53 tumor suppressor protein transactivates Notch1 directly, and thus, p53 dysfunction may lead to the inactivation of Notch signaling [22, 28]. EGFR may suppress p53-mediated transcription of Notch1 [29] in epidermal keratinocytes and SCC cells. By contrast, EGFR activation in cutaneous SCC is induced by loss of Notch signaling in mice [30, 31]. Both EGFR and Notch signaling may activate the transcription of *cyclin D1* gene [32-34].

Herein, we carried out functional studies in OTC with a panel of genetically engineered human esophageal cells in the presence or absence of DNMAML1. Additionally, mice carrying *DNMAML1* targeted to oral, esophageal and forestomach squamous epithelia were exposed to 4-Nitroquinoline 1-oxide (4-NQO), a chemical carcinogen. These model systems reveal activation of Wnt signaling and cyclin D1 upregulation in invasive cells through functional interplay between EGFR and mutant p53. Furthermore, there is evidence for a tumor suppressor role for canonical Notch signaling in regard to maintenance of squamous-cell differentiation and epithelial integrity, providing novel mechanistic insights into the pathogenesis of ESCC.

Materials and methods

Tissue samples

Primary ESCC and adjacent normal tissues were procured via surgery from informed-consent patients in accordance with Institutional Review Board standards and guidelines as described previously [35].

Cell cultures and treatment

EPC2-hTERT, established by immortalizing primary normal human esophageal epithelial cells, and derivatives were grown and subjected to OTC as described [10-12, 15, 18, 36, 37] ([38] for detailed protocols). In brief, 0.5×10^6 of epithelial cells were seeded on top of the collagen/Matrigel matrices containing FEF3 human fetal esophageal fibroblasts, and grown in submerged conditions for 4 days. Cultures were then raised to the air-liquid interface for additional 4 days and harvested for morphological assessment. Each OTC experiment was performed in triplicate. Cells were treated with 10 μM IWR-endo (Santa Cruz Biotechnology, Santa Cruz, CA), a pharmacological Wnt inhibitor and 10 μM IWR-exo (Santa Cruz), an inactive control agent [39]. Doxycycline (Dox) was used at 2 μg/ml in the tetracycline-regulatable (Tet-Off) system.

Retrovirus-mediated gene transfer

Retroviral vectors expressing EGFR (pFB-Neo), $p53^{R175H}$ (pBABE-puro or pBABE-zeo^{loxP}), cyclin D1 (pBABE-bla or pBPSTR-D1 [40]), DNMAML1 (MigRI) were stably transduced into EPC2-hTERT cells. Cells were treated with 300 μg/ml of G418 (Invitrogen), 1 μg/ml of Puromycin (Invitrogen), 5 μg/ml Blasticidin S (Invitrogen) or 0.5 μg/ml zeocin (Invitrogen) for selection as described [11, 12, 18, 36, 38]. Cells transduced with MigRI were subjected to flow sorting to collect cells expressing the brightest level

(top 20%) of green fluorescent protein (GFP) as described [18, 37].

Real-time reverse-transcription polymerase chain reactions (RT-PCR)

Real-time RT-PCR assays were done using TaqMan® Gene Expression Assay (Applied Bio-systems) for cyclin D1 (CCND1, Hs00765553_m1) as described [11, 18].

Western blotting

Western blotting for EGFR, p53, cyclin D1, GFP (for DNMAML1 expressed as a GFP-fusion protein) and β-actin (loading control) was done as described [11, 18, 37].

K14Cre;DNMAML1 Mice and 4-NQO treatment

K14Cre;DNMAML1 mice were described previously [18]. In brief, mice carrying *Lox-STOP-Lox DNMAML1* [41] were intercrossed with *K14Cre* transgenic mice [42], targeting *DNMAML1* into the basal cell layer of the stratified squamous epithelia. 4-7 month-old *K14Cre;DNMAML1* and control littermates were treated with 100 μg/ml 4-NQO (Sigma Aldrich) or 2% propylene glycol (MP Biomedicals, Solon, OH)(vehicle control) in drinking water for up to 18 days and followed up for 8 weeks after 4-NQO withdrawal. All experiments were done under approved protocols from the University of Pennsylvania Institutional Animal Care and Use Committee and NIH guidelines.

Histology, immunohistochemistry (IHC) and immunofluorescence (IF)

Hematoxylin and Eosin (H&E) staining, IHC and IF were performed as described [18, 35-37]. Sections were incubated with anti-Ki-67 polyclonal antibody (1:1,500; Novocastra, Bannockburn, IL), anti-cyclin D1 monoclonal antibody (1:700; Thermo Scientific, Rockford, IL), anti-cytokeratin 14 (CK14) polyclonal antibody (1:5000; Covance, Princeton, NJ), anti-Involucrin (IVL) monoclonal antibody (Sigma, St Louis, MO) or anti β-catenin polyclonal antibody (1:300; Cell Signaling, Danvers, MA) overnight at 4°C. For IHC, sections were further incubated with biotinylated secondary IgG and subjected to signal development using the DAB peroxidase substrate with the VECTASTAIN® ABC kit (Vector Laboratories, Burlingame, CA). For IF, sections were incubated with an appropriate Cy2- or Cy3-conjugated secondary antibody (1:400; Jackson ImmunoResearch, West Grove, PA) for 30 min at room temperature. Nuclei were counter-stained by 4', 6-diamidino-2-phenylindole (DAPI) (1:10,000; Invitrogen). Stained slides were examined with a Nikon Microphot microscope and imaged with a digital camera at specific magnifications. Labeling indices (LI) for Ki-67 and cyclin D1 were determined by counting at least 600 cells per x200 microscopic field. For OTC, five to seven pictures were taken for each sample to determine average epithelial thickness. Cell invasion was assessed by counting all invasive nests per slide on at least 5 sections.

Statistical analysis

Data from real-time RT-PCR assays and morphological assessment of OTC including epithelial thickness, invasiveness, Ki-67 and cyclin D1 LI were presented as mean ± SE and were analyzed by two-tailed Student's *t* test. Fisher's exact test was done to assess whether inflammation and dysplasia were associated with genotype and/or phenotype in mice by location. *P* <0.05 was considered to be statistically significant.

Results

Generation of a panel of genetically engineered human esophageal epithelial cells

We have demonstrated previously that both *EGFR* and *p53^R175H* are necessary and sufficient for malignant transformation of human esophageal cells EPC2-hTERT [12] and for enrichment of EMT-competent cells capable of invasive growth [11, 15]. DNMAML1-mediated inhibition of Notch signaling sharply suppressed squamous-cell differentiation in EPC2-hTERT and its transformed derivative EPC2T which carries *cyclin D1*, *EGFR* and *p53^R175H* transgenes concurrently [18, 37]. Moreover, DNMAML1 facilitated EMT and invasive growth of EPC2T cells [37]. To better define the individual contribution of these genetic factors to esophageal carcinogenesis, we created a panel of genetically engineered EPC2-hTERT derivatives carrying *cyclin D1*, *EGFR* and *p53^R175H*, either alone or in combinations, both in the presence and absence of *DNMAML1* (**Figure 1** and data not shown). EPC2-hTERT and EPC2T cells with or without DNMAML1 [18, 37] served as
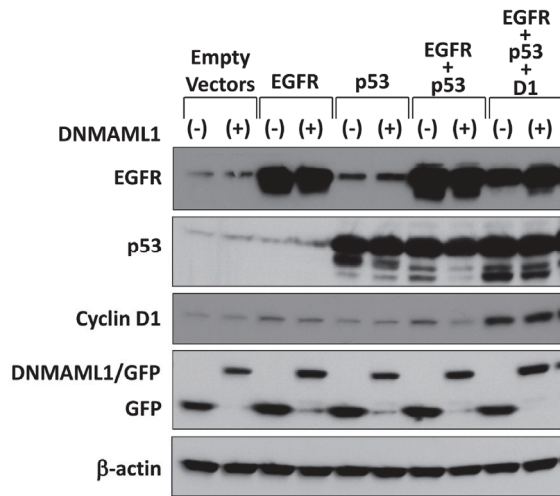
Figure 1. Generation of a panel of genetically engineered human esophageal epithelial cell lines with or without DNMAML1. Derivatives of EPC2-hTERT [10] expressing empty vectors only (parental), EGFR only (EGFR), $p53^{R175H}$ only (p53), EGFR and $p53^{R175H}$ (EGFR + p53) have been described [11]. These cell lines were stably transduced with either GFP (control) or DNMAML1 as previously described to generate EPC2-T cells (EGFR + p53 + D1) expressing either GFP or DNMAML1 [18, 37]. Western blotting documented indicated molecules with β-actin as a loading control. Empty vectors, parental EPC2-hTERT cells with empty vectors only; EGFR, EGFR overexpression; p53, $p53^{R175H}$; and D1, cyclin D1 overexpression.

controls.

Cyclin D1 overexpression induces squamous hyperplasia in OTC

In OTC, we first evaluated reconstituted epithelia formed in the absence of DNMAML1 expression. Cyclin D1 overexpression alone resulted in squamous hyperplasia with increased epithelial thickness (**Figure 2A** and **2B**) and cell proliferation as indicated by an increase in overall and basal cell-specific Ki-67 LI (**Figure 3A-C**). We determined basal cell-specific Ki-67 LI since Ki-67 positive cells were typically localized within the basal cell layer (**Figure 3A**), suggesting that the total number of Ki-67 negative cells may reduce the overall Ki-67 LI in the epithelia demonstrating greater stratification due to increased suprabasal cell layers. Unlike *cyclin D1*, neither *EGFR* alone nor *p53^{R175H}* alone increased epithelial thickness (**Figure 2A** and **2B**) or basal cell-specific Ki-67 LI (**Figure 3C**). Moreover, addition of either *EGFR* or *p53^{R175H}* into the cells overexpressing cyclin D1 did not in-

crease epithelial thickness (**Figure 2A** and **2B**) and basal cell-specific Ki-67 LI (**Figure 3C**), suggesting that cyclin D1 may have a central role in stimulating basal cell proliferation. Such a premise was corroborated in the tetracycline regulatable (Tet-off) system where repression of *cyclin D1* transgene resulted in a decrease in the epithelial thickness as well as in the number of cyclin D1 and Ki-67 positive cells (**Figure 4**). In addition, cyclin D1 overexpression increased cell layers expressing CK14, a marker of basal keratinocytes (**Figures 4B** and **Figure 5**). These data suggest that cyclin D1 stimulates basal cell proliferation and delays terminal differentiation in the squamous epithelium.

Transformed cells with concurrent EGFR overexpression and p53 mutation display invasive growth without requiring ectopically expressed cyclin D1

When OTC was performed with transformed cells carrying *EGFR* and *p53^{R175H}* concurrently, epithelial thickness was suppressed greatly (**Figure 2A** and **2B**). Instead, cell invasion into the stromal compartment was enhanced (**Figure 2A** and **2C**) as described previously [12, 15]. Addition of ectopic *cyclin D1* transgene to this genotype increased epithelial thickness and basal-cell specific Ki-67 LI only modestly (**Figure 2A** and **2B; Figure 3A** and **3C**). Interestingly, overall Ki-67 LI was found to be greatest in cells carrying both *EGFR* and *p53^{R175H}* transgenes, with or without *cyclin D1* transgene (**Figure 3B**). We suspected that cell proliferation may be augmented in the invasive cells within the stromal compartment. When we assessed Ki-67 LI in a compartment-specific manner (**Figure 3D**), cells invading into underlying stroma exhibited significantly higher Ki-67 LI than those within the epithelial compartment. Moreover, ectopically expressed cyclin D1 influenced Ki-67 LI only minimally in the stromal compartment. These findings suggest that transformed esophageal cells may contain a subset of cells that is more committed to downward invasive growth and capable of proliferating actively without requiring ectopically expressed cyclin D1.

Notch inhibition impairs epithelial stratification and facilitates invasive growth in transformed cells with concurrent EGFR overexpression and p53 mutation

Activation of Notch signaling occurs at the onset of squamous-cell differentiation in normal eso-
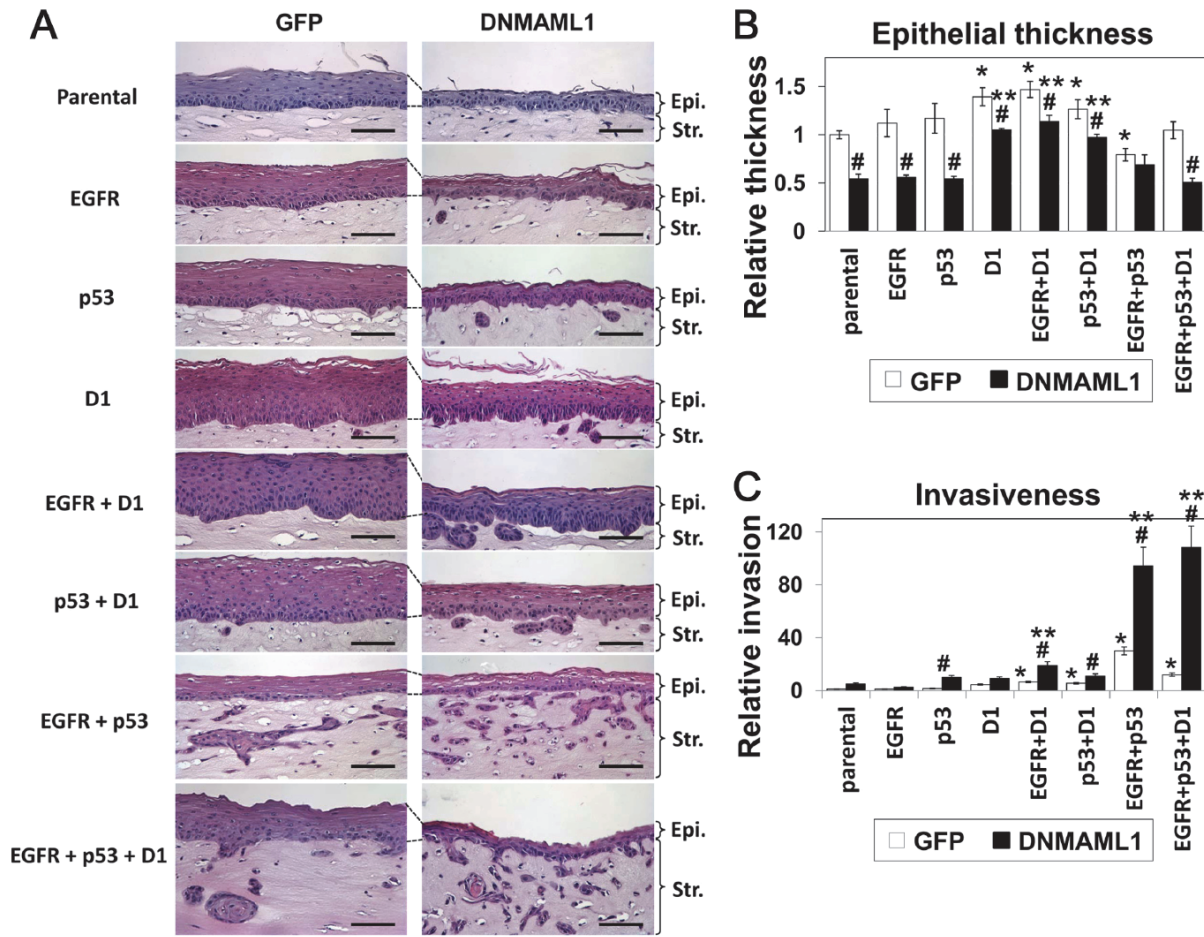
Figure 2. Morphology of OTC with genetically engineered human esophageal epithelial cell lines with or without DNMAML1. Cell lines with indicated genotypes expressing either DNMAML1 or GFP (control) were grown in OTC to reconstitute stratified squamous epithelia. Sections from the paraffin embedded end products were subjected to H&E staining (A) to assess epithelial thickness (B) and cell invasion (C). Note that DNMAML1 suppressed epithelial thickness and stimulated cell invasion into the stroma, in particular in the cells carrying *EGFR* and *p53*[R175H] concurrently. Parental, EPC2-hTERT with empty vectors only; EGFR, EGFR overexpression; p53, p53[R175H]; and D1, cyclin D1 overexpression. Epi, epithelium; Str, stroma; Scale bar, 100 μm. #, $P < 0.05$ vs. GFP, n=5. *, $P < 0.05$ vs. Parental + GFP, n=5. **, $P < 0.01$ vs. Parental + DNMAML1, n=5.

phageal epithelial cells [18]. We next determined the impact of genetic pan-Notch inhibition on reconstituted epithelium. DNMAML1 suppressed epithelial stratification in all genotypes (**Figure 2A**). This was partially antagonized by ectopically expressed cyclin D1 in nontransformed cells (**Figure 2A** and **2B**, see "D1", "EGFR+D1" and "p53+D1" under the column for DNMAML1 in **A**) with increased CK14-positive basal and parabasal cell layers in the presence of DNMAML1 (**Figure 5**). Thus, cyclin D1 overexpression may increase a pool of basal keratinocytes that cannot undergo terminal differentiation due to Notch inhibition by

DNMAML1; however, basal and parabasal cell layers were not extended when DNMAML1 was expressed in transformed cells carrying transgenic *EGFR* and *p53*[R175H] and *cyclin D1* (i.e. EPC2T) (**Figure 2A,** see "EGFR+p53+D1" under the column for DNMAML1 in **A**). Therefore, cyclin D1 may fail to expand basal and parabasal cell layers once cells are fully transformed to gain invasive characteristics.

DNMAML1 greatly augmented invasive growth of cells transformed by EGFR and p53[R175H], but did not require ectopic cyclin D1 expression (**Figure 2A** and **2C**). Given the possibility of
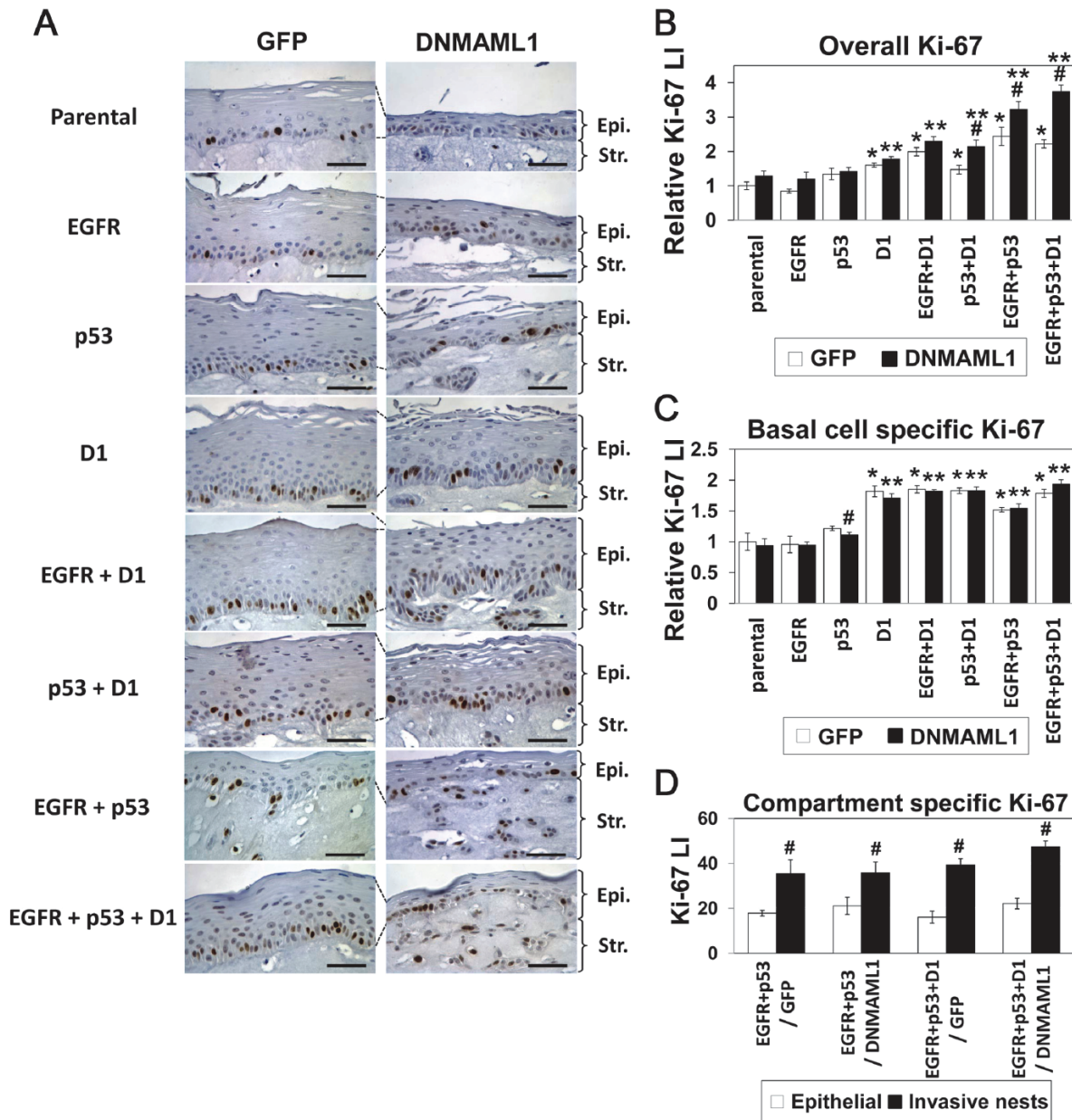
Figure 3. Assessment of cell proliferation in OTC with genetically engineered human esophageal epithelial cell lines with or without DNMAML1. Cell lines with indicated genotypes expressing either DNMAML1 or GFP (control) were grown in OTC to reconstitute stratified squamous epithelia. Serial sections from the paraffin embedded end products from Figure 2A were subjected to IHC for Ki-67 (A), to determine overall Ki-67 LI (B), basal cell-specific Ki-67 LI (C) and compartment-specific Ki-67 LI (D). Parental, EPC2-hTERT with empty vectors only; EGFR, EGFR overexpression; p53, p53R175H; and D1, cyclin D1 overexpression. Epi, epithelium; Str, stroma; Scale bar, 50 μm. #, P< 0.05 vs. GFP (B, C); n=5. #, P< 0.05 vs. epithelial compartment (D); n=5. *, P< 0.05 vs. Parental + GFP, n=5. **, P< 0.01 vs. Parental+DNMAML1, n=5.

negative and positive regulation of Notch1 by EGFR [29] and wild-type p53 [22, 28], respectively, EGFR overexpression and p53 dysfunction may lead to impaired squamous-cell differentiation without DNMAML1; however, neither *EGFR* or *p53R175H* alone nor combination of the two, inhibited squamous-cell differentiation significantly compared with DNMAML1 (**Figure 5**). In all genotypes tested, DNMAML1 affected basal cell-specific Ki-67 LI only minimally, if at
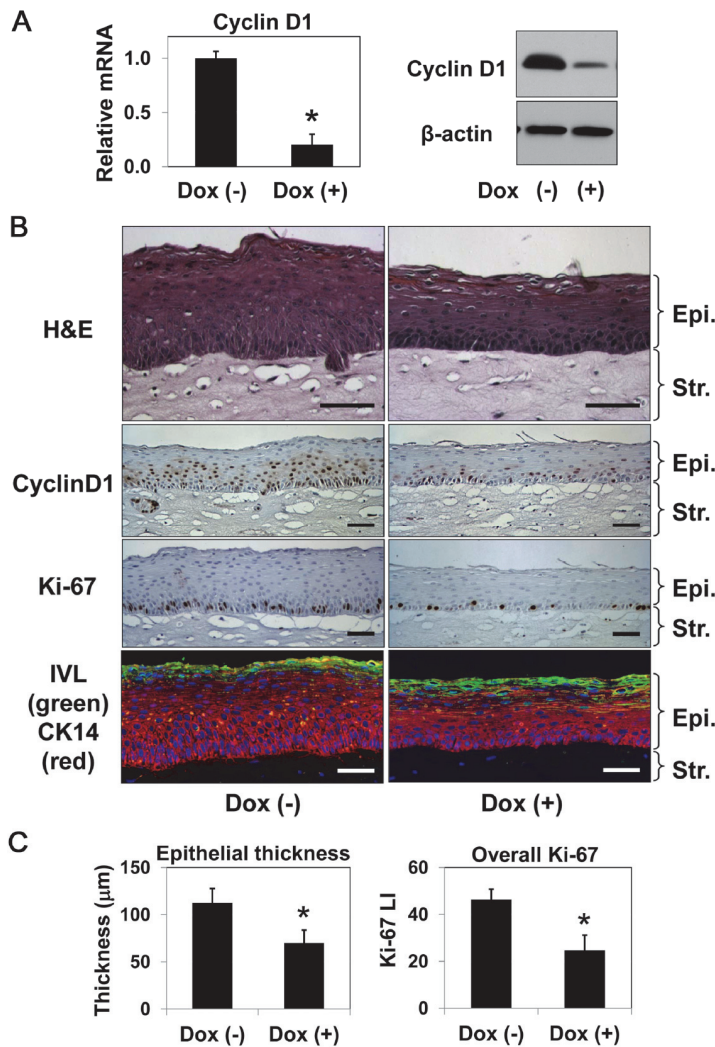
Figure 4. Cyclin D1 induces squamous hyperplasia in OTC. EPC2-hTERT cells with tetracycline-regulatable (Tet-Off) cyclin D1 were grown in the absence [Dox (-)] or presence of 2 μg/ml Doxycycline [Dox (+)] in monolayer culture or OTC. In (A), cyclin D1 was determined by real-time RT-PCR (left) with β-actin as an internal control and Western blotting with β-actin as a loading control (right). *, $P<0.01$, vs. Dox (-); n=3. In (B), serial sections from the paraffin embedded end products of OTC were subjected to H&E staining, IHC for cyclin D1 and Ki-67, and IF for CK14 (red) and IVL (green) with DAPI (blue) to assess morphology, cyclin D1 expression, proliferation and differentiation markers, respectively in the reconstituted stratified squamous epithelia. Epi, epithelium; Str, stroma; Scale bar, 50 μm. In (C), epithelial thickness and overall Ki-67 LI were determined using the H&E and Ki-67 stained samples from (B). *, $P<0.05$ vs. Dox (-); n=5.

all, when compared to GFP only-expressing controls (**Figure 3C**); however, DNMAML1 increased the overall Ki-67LI in the presence of both *EGFR* and *p53*[R175H] (**Figure 3B**). This was accounted

for by a significantly increased Ki-67 LI found specifically within invasive cells (**Figure 3D**) as DNMAML1 enhanced cell invasion greatly (**Figure 2A** and **2C**).

These findings indicate that squamous-cell differentiation requires canonical Notch signaling. Once Notch signaling is disrupted, cells fail to undergo squamous-cell differentiation. Under such conditions, ectopically overexpressed cyclin D1 may increase proliferative basal keratinocytes in nontransformed cells; however, Notch inhibition in transformed cells facilitates invasive growth in which cell proliferation may be increased without requiring ectopic cyclin D1.

Wnt signaling may regulate proliferation and invasion within the invasive compartment

We explored further how cell proliferation may be stimulated in the invasive cells with concurrent *EGFR* and *p53*[R175H]. *Cyclin D1* is a Notch target gene [33]; however, Notch1-mediated cyclin D1 induction was impaired by DNMAML1 in EPC2-hTERT cells (data not shown) along with other Notch target genes as described previously [18]. Thus, it is unlikely that canonical Notch signaling stimulates cell proliferation via cyclin D1 expression in the presence of DNMAML1. We hypothesized that the Wnt pathway may be activated to induce cyclin D1 in invasive cells. When we examined EPC2-hTERT cells expressing EGFR and p53[R175H] concurrently, β-catenin was localized to the cell membrane within the epithelial compartment (**Figure 6A**). By contrast, β-catenin was mainly localized to the cytoplasm of invasive cells within the stromal compartment (**Figure 6B**), suggesting activation of Wnt signaling. A similar pattern of β-catenin expression was observed in transformed cells with DNMAML1 (data not shown). These findings were further corroborated by restoration of β-catenin localization to the cell
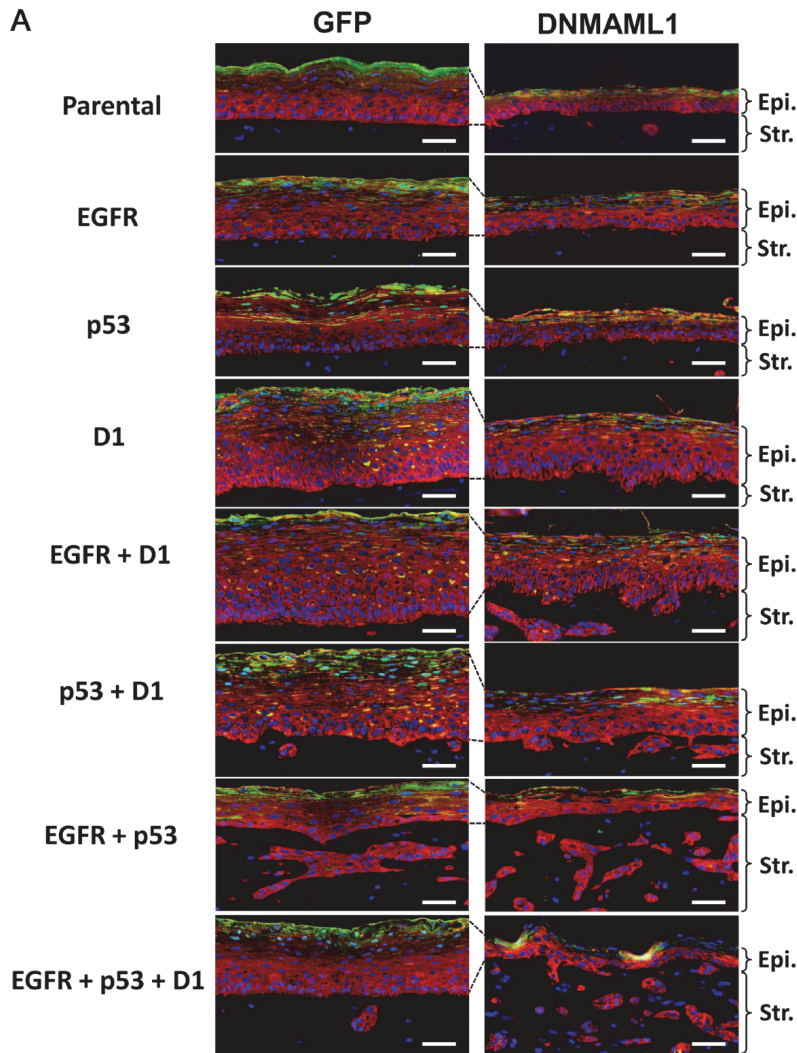
A

Figure 5. Assessment of squamous-cell differentiation in OTC with genetically engineered human esophageal epithelial cell lines with or without DNMAML1. Cell lines with indicated genotypes expressing either DNMAML1 or GFP (control) were grown in OTC to reconstitute stratified squamous epithelia. Serial sections from the paraffin embedded end products from Figure 2A were subjected to IF for CK14 (red) and IVL (green) with DAPI (blue). Parental, EPC2-hTERT with empty vectors only; EGFR, EGFR overexpression; p53, $p53^{R175H}$; and D1, cyclin D1 overexpression. Epi, epithelium; Str, stroma; Scale bar, 50 μm.

membrane when OTC was carried out in the presence of IWR-1-endo, a pharmacological Wnt inhibitor, but not with IWR-exo, an inactive control agent (**Figure 6C** and **6D**). Wnt inhibition reduced cell invasion and proliferation, albeit to a modest extent (**Figures 7** and **8**). Cyclin D1 was found to be upregulated in invasive cells with concurrent EGFR and $p53^{R175H}$ with or without DNMAML1 (**Figure 9A** and **9C**). Wnt signal-

ing may transcriptionally activate cyclin D1; however, IWR-1-endo did not reduce cyclin D1 expression (**Figure 9B** and **9D**). Therefore, cyclin D1 expression in the invasive cells may be regulated via differential mechanisms.

4-NQO induces invasive ESCC with Wnt activation in mice carrying DNMAML1 targeted to the squamous epithelia

To delineate how Notch inhibition may impact upon carcinogenesis in mice, *DNMAML1* was targeted to the oral and esophageal squamous epithelia as described previously [18]. Mice expressing DNMAML1 and control littermates were subjected to 4-NQO administration, which was initially planned for 8 weeks as done in wild-type mice [43]. All control mice tolerated 4-NQO treatment well. Unexpectedly, 4-NQO-treated DNMAML1 mice suffered from weight loss and severe dehydration, which may be associated with inflammation observed in the tongue (**Table 1** and data not shown), preventing mice from drinking. 4-NQO treatment was suspended at day 18 for all mice since 8 out of 9 (89%) 4-NQO-treated DNMAML1 mice were found dead or euthanized during 4-NQO administration or within a week after 4-NQO withdrawal. Histology was available for 4 euthanized mice, all of which (100%) displayed squamous dysplasia in the tongue despite a short period of 4-NQO administration (**Table 2** and data not shown). Three out of four 4-NQO-treated DNMAML1 mice (75%) exhibited esophageal squamous dysplasia. One of the 4-NQO-treated DNMAML1 mice recovered from dehydration after 4-NQO withdrawal and was sacrificed 8 weeks later along with control littermates. Histology of this mouse revealed highly invasive
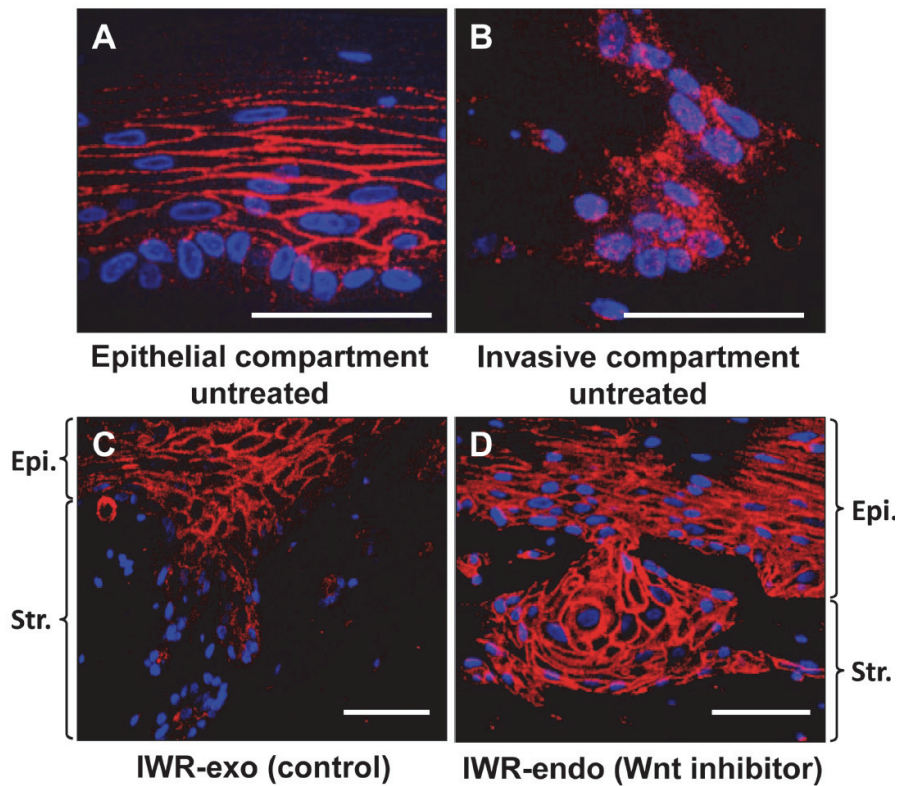
Figure 6. OTC reveals Wnt activation in the invasive fronts. EPC2-hTERT-EGFR-p53^{R175H} cells [11, 12] with GFP (EGFR + p53/GFP) (see Figure 1 for expression) were grown in OTC in the presence or absence (untreated) of either 10 μM IWR-endo (Wnt inhibitor) or 10 μM IWR-exo (control agent) and subjected to IF for β-catenin (red) with DAPI (blue) in (A-D). (A), epithelial compartment (untreated); (B), invasive compartment (untreated); and (C), IWR-exo; (D), IWR-endo. Scale bar, 50 μm.

ESCC containing spindle-shaped tumor cells reminiscent of EMT (**Figure 10**), in addition to dysplasia in the tongue and esophagus. IHC documented cyclin D1 overexpression and β-catenin cytoplasmic localization in the invasive fronts of ESCC lesions (**Figure 10**). Amongst 4-NQO-treated control mice (without DNMAML1), four out of 34 (12%) developed squamous dysplasia in the tongue (n=1) or the esophagus (n=3), but not cancer. These results suggest that Notch inhibition by DNMAML1 promoted 4-NQO-mediated oral and esophageal carcinogenesis. Moreover, Notch-independent cyclin D1 upregulation and Wnt activation may contribute to invasive growth of ESCC in mice.

Cyclin D1 is overexpressed in invasive lesions of primary ESCC with cytoplasmic stabilization of β-catenin

Finally, we performed IHC for cyclin D1 and β-catenin on primary ESCC tissue samples (n=12). Cyclin D1 was overexpressed in 9 out of 12 human ESCC samples (75%) in agreement with our previous observations [44]. Cyclin D1 was not expressed constitutively as cyclin D1 positive tumor cells were typically found in the invasive fronts (**Figure 11**) where tumors cells are often poorly-differentiated, displaying spindle-shaped morphology reminiscent of EMT [37]. β-catenin was expressed on the cell membrane of well-differentiated ESCC cells and normal keratinocytes within the adjacent squamous epithelia (data not shown). By contrast, in 10 out of 12 cases (83%), invasive ESCC cells expressed β-catenin in the cytoplasm (**Figure 11**) with occasional nuclear localization (data not shown), suggesting activation of the Wnt pathway in the invasive fronts. These findings implicate the roles of the tumor microenvironment in regulation of cyclin D1 expression and Wnt signaling.

Discussion

Notch inhibition reveals differential regulation of proliferation and differentiation between squamous epithelial cells and invasive cells within the stroma

This study aimed at analyzing the complex interplay between cyclin D1, EGFR and mutant p53 during the development and progression of ESCC. In OTC, the Notch and Wnt pathways
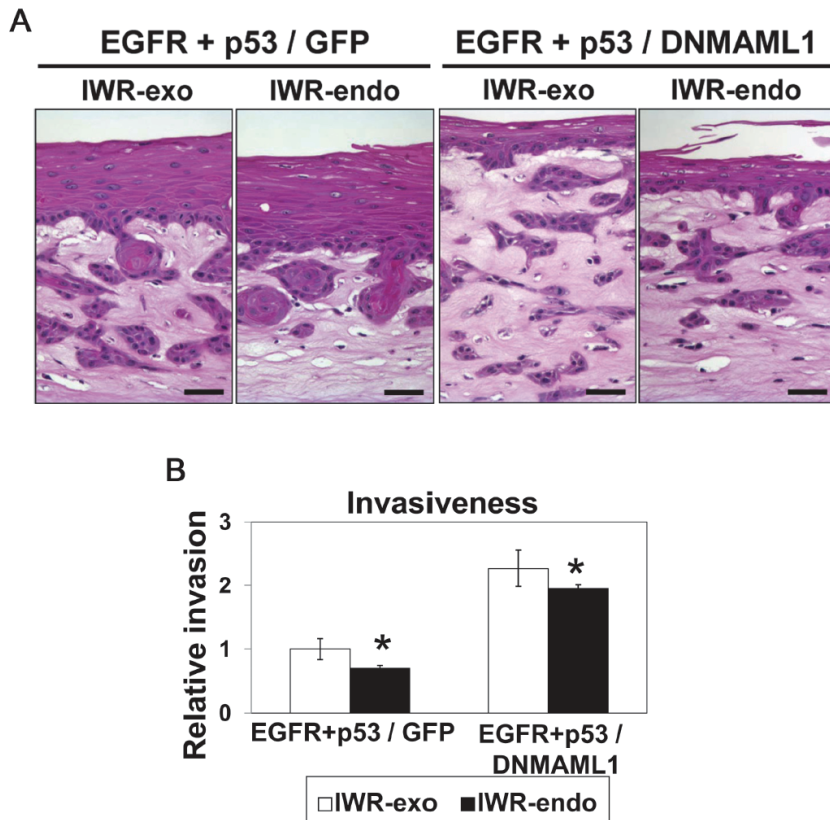
Figure 7. Wnt inhibition reduces invasion in OTC. EPC2-hTERT-EGFR-p53R175H cells [11, 12] with GFP (EGFR + p53/GFP) or DNMAML1 (EGFR + p53/DNMAML1) (see Figure 1 for expression) were grown in OTC in the presence or absence (untreated) of either 10 μM IWR-endo (Wnt inhibitor) or 10 μM IWR-exo (control agent) and subjected to H&E staining (A) to assess invasiveness as quantitated in (B). EGFR, EGFR over-expression; p53, p53R175H. Scale bar, 50 μm. *, P< 0.05 vs. IWR-exo; n=6.
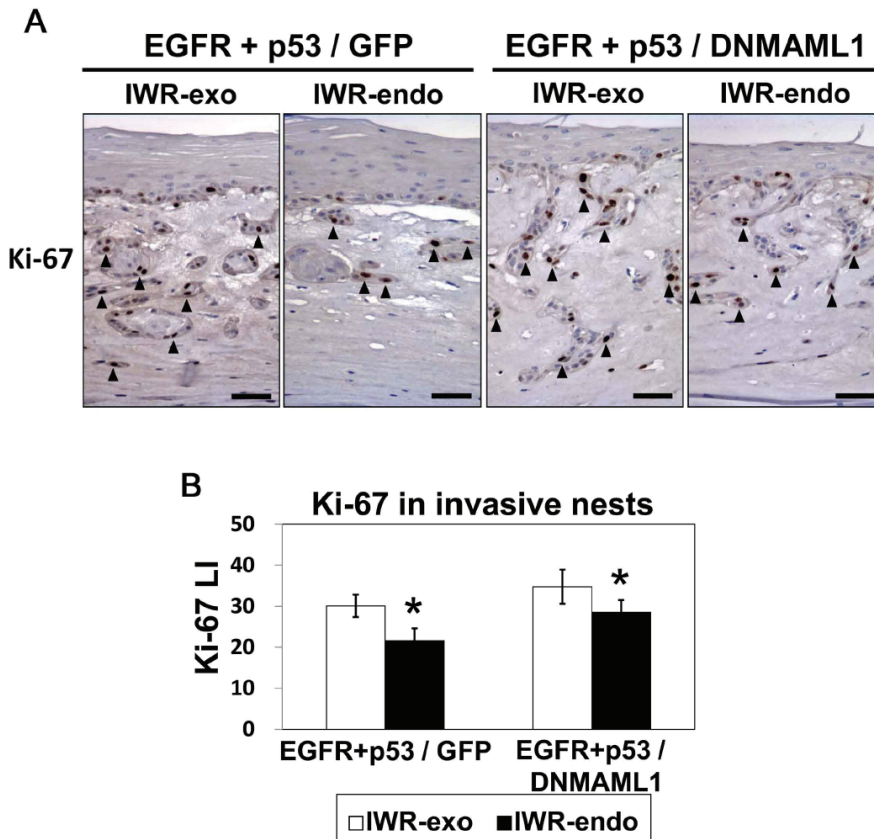

Figure 8. Wnt inhibition reduces cell proliferation in the invasive fronts. EPC2-hTERT-EGFR-p53R175H cells [11, 12] with GFP (EGFR + p53/GFP) or DNMAML1 (EGFR + p53/DNMAML1) (see Figure 1 for expression) were grown in OTC in the presence of either 10 μM IWR-endo (Wnt inhibitor) or 10 μM IWR-exo (control agent). Serial sections from the paraffin embedded end products from Figure 7A were subjected to IHC for Ki-67 (A) to determine Ki-67 LI in the invasive nests (B). EGFR, EGFR overexpression; p53, p53R175H. Scale bar, 50 μm. *, P< 0.05 vs. IWR-exo (control agent), n=6. Arrow heads indicate Ki-67 positive cells.
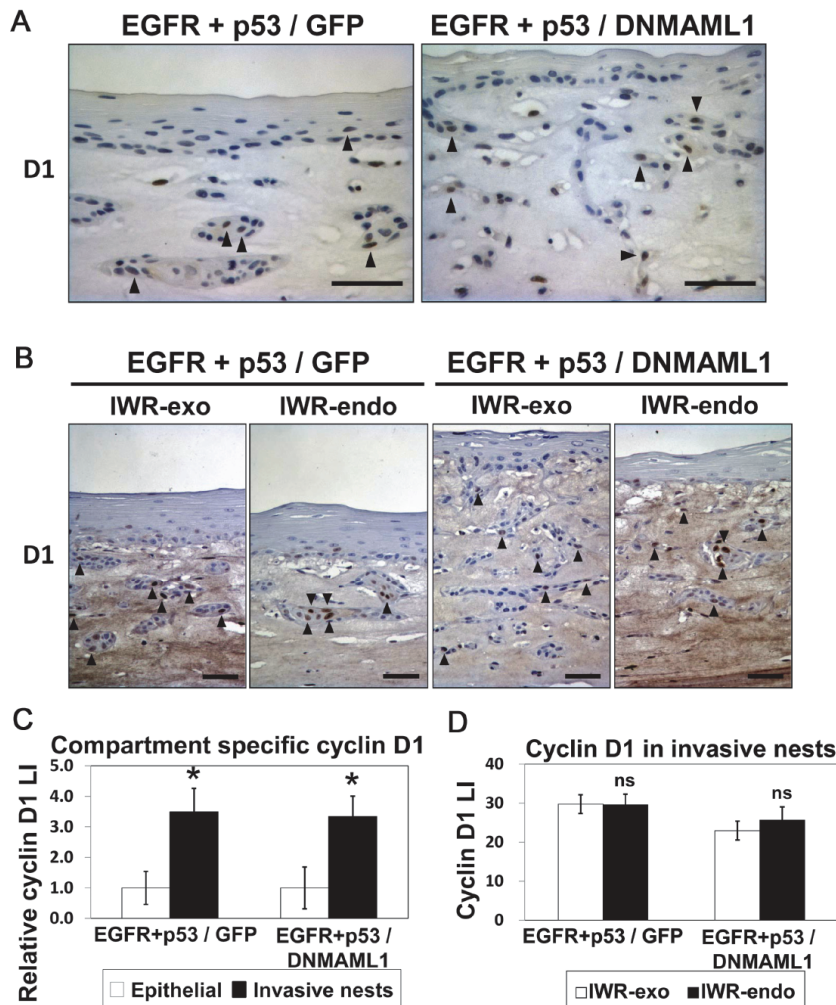
Figure 9. Wnt inhibition does not affect cyclin D1 expression in the invasive fronts. Cell lines with indicated genotypes expressing DNMAML1 or GFP (control) were grown in OTC in the presence or absence (untreated) of either 10 μM IWR-endo (Wnt inhibitor) or 10 μM IWR-exo (control agent) and subjected to IHC for cyclin D1 (A and B) to determine compartment-specific cyclin D1 LI (C) as well as the impact of Wnt inhibition upon cyclin D1 LI within the invasive nests (D). EGFR, EGFR overexpression; p53, p53^{R175H}. Scale bar, 50 μm. *, $P< 0.01$ vs. epithelial compartment, n=6; *ns*, vs. IWR-exo (control agent), n=6. D1, cyclin D1. Arrow heads indicate cyclin D1 positive cells.

Table 1. 4-NQO and DNMAML1 induce inflammation in mice.

genotype	4NQO treatment	n	inflammation			
			skin	tongue	esophagus	forestomach
DNMAML1 (-)	(-)	4	0 (0%)	0 (0%)	0 (0%)	0 (0%)
	(+)	12	1 (8.3%)	3 (25%)	4 (33%)	1 (8.3%)
DNMAML1 (+)	(-)	5	5 (100%)*	0 (0%)	1 (20%)	0 (0%)
	(+)	5^a	5 (100%)*	4 (80%)**	1 (20%)	1 (20%)

^{a} Histology was available for 5 euthanized mice only due to early death in 8 mice out of 9. Note that DNMAML1 caused skin inflammation in mice with or without 4-NQO treatment [*, $P< 0.01$ vs. DNMAML1 (-)]. 4-NQO induced tongue inflammation significantly more in the presence of DNMAML1 [**, $P< 0.05$ vs. DNMAML (+) and 4-NQO (-)].

were interrogated using genetic and pharmacological inhibitors, respectively. Our foremost novel finding is that cell proliferation may be regulated differentially between the squamous-epithelial compartment and the underlying stroma. Within the squamous epithelium, non-

transformed and transformed esophageal cells were found to depend upon canonical Notch signaling to undergo squamous-cell differentiation (**Figure 5**). By contrast, transformed cells exhibited invasive growth in a Notch-independent manner that is enhanced by

Table 2. 4-NQO and DNMAML1 induce dysplasia in mice

genotype	4NQO treat-ment	n	dysplasia			
			skin	tongue	esophagus	forestomach
DNMAML1	(-)	4	0 (0%)	0 (0%)	0 (0%)	0 (0%)
(-)	(+)	12	1 (8.3%)	0 (0%)	2 (17%)	0 (0%)
DNMAML1	(-)	5	4 (80%)*	2 (40%)	0 (0%)	0 (0%)
(+)	(+)	5[a]	3 (60%)*	5 (100%)[#]	3 (60%)[b]	0 (0%)

[a] Histology was available for 5 euthanized mice only due to early death in 8 mice out of 9; [b] One of 3 mice with esophageal dysplasia had ESCC in the lower esophagus.
Note that DNMAML1 caused skin dysplasia in mice with or without 4-NQO treatment
[*, P< 0.01 vs. DNMAML1 (-)]. 4-NQO induced tongue dysplasia significantly more in the presence of DNMAML1 [**, P< 0.05 vs. DNMAML (+) and 4-NQO (-)].
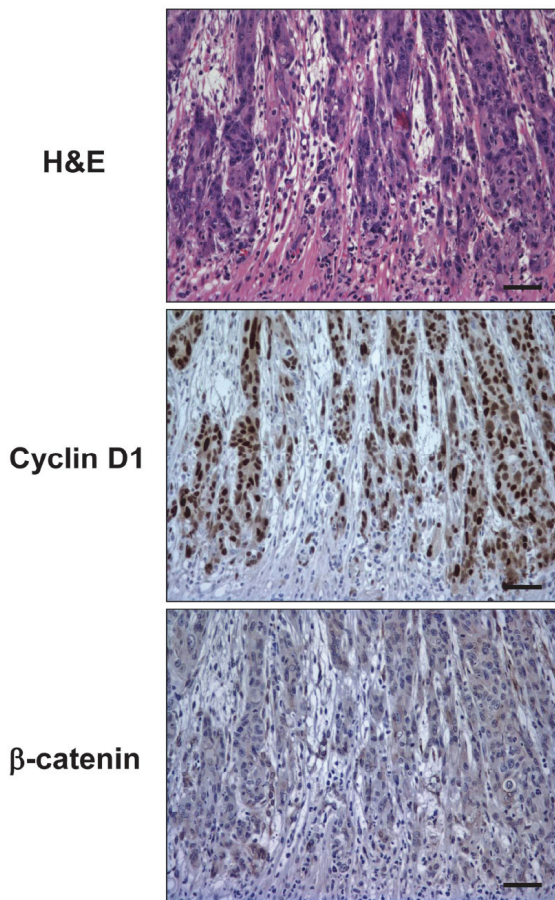

Figure 10. Cyclin D1 overexpression and Wnt activation in the invasive fronts of primary ESCC developed in a DNMAML1 mouse. Primary ESCC developed in the lower esophagus of a 4-NQO-treated DNMAML1 mouse was subjected to H&E staining, IHC for cyclin D1 and β-catenin.
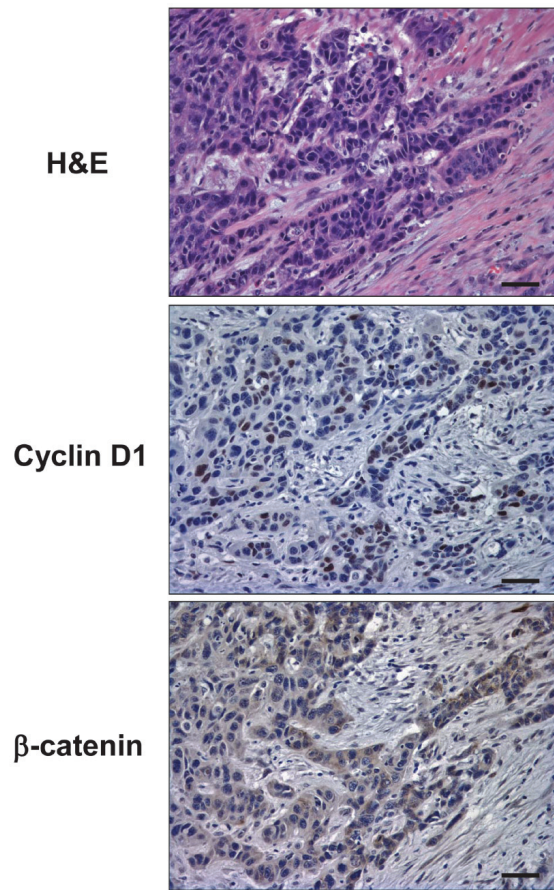

Figure 11. Cyclin D1 overexpression and Wnt activation in the invasive fronts of primary ESCC in human. Serial sections from a representative primary ESCC tumor were subjected to H&E staining, IHC for cyclin D1 as well as β-catenin.

DNMAML1 (**Figures 2** and **3**), and involves cyclin D1 and Wnt signaling (**Figures 6-9**). Aberrant expression of cyclin D1 and β-catenin stabiliza-

tion were observed in the invasive fronts of primary human ESCC (**Figure 11**) as well as mouse ESCC induced by 4-NQO in the presence of DNMAML1 (**Figure 10**), recapitulating the find-

ings in the OTC model (**Figure 1-5**).

Our data also showed that cyclin D1 overexpression stimulates cell proliferation within the squamous epithelium (**Figures 2-4**). As a mitogenic sensor and a regulator in cell cycle control [45], cyclin D1 is often overexpressed in ESCC via gene amplification or EGFR-mediated transcriptional activation [32, 44] and is associated with lymphatic invasion and lymph node metastasis [46], culminating in poor prognosis [47]. Although Notch may transcriptionally activate cyclin D1 [33, 34], our data with DNMAML1 indicate that canonical Notch signaling may be dispensable for cyclin D1 induction in invasive ESCC cells.

Notch inhibition by DNMAML1 may impair epithelial integrity to facilitate oral-esophageal squamous-cell carcinogenesis

We confirmed that Notch inhibition by DNMAML1 is not sufficient for non-transformed cells (e.g. EGFR overexpression alone and p53^{R175H} alone) to undergo robust invasion. Moreover, potential Notch inhibitory effects by EGFR overexpression [29] or impaired p53-mediated transactivation of Notch1 [22, 28] did not appear to have a significant negative impact upon squamous-cell differentiation in the absence of DNMAML1 (**Figure 5**). By contrast, malignant transformation by concurrent EGFR and p53^{R175H} expression appeared to be necessary and sufficient for invasive growth, although DNMAML1 enhanced both cell proliferation and invasion in this context. This agrees with our previous observations that transformed esophageal cells underwent EMT during invasive growth when Notch-dependent squamous-cell differentiation was suppressed [15, 37].

We demonstrated for the first time that Notch inhibition promotes oral and esophageal carcinogenesis in a genetically engineered mouse model. Notch may act as a tumor suppressor in the skin by maintaining the skin-barrier functions to prevent emergence of a tumor-promoting inflammatory microenvironment [22-24, 30, 48, 49]. Consistent with such a premise, we observed induction of severe dehydration and early death in response to 4-NQO administration in most mice expressing DNMAML1 under CK14 promoter-driven Cre, causing substantive inflammation in the skin and the tongue (**Table 1**). Given the fact that 4-NQO is

an ultraviolet mimetic agent [50] and that DNMAML1 affects esophageal squamous cell differentiation [18], 4-NQO and DNMAML1 may cooperate to promote oral-esophageal carcinogenesis by impairing epithelial barrier functions to generate an inflammatory microenvironment. Since cutaneous inflammation may have contributed to 4-NQO-induced dehydration, preventing us to complete scheduled 4-NQO administration, future studies should aim at targeting DNMAML1 specifically to the oral-esophageal squamous epithelia [51] to minimize skin-related phenotypes.

Wnt signaling and cyclin D1 may be activated in the invasive fronts of ESCC

Our data suggest that Wnt signaling may facilitate ESCC progression. IWR-endo antagonizes Wnt signaling by stabilizing Axin [39] and decreasing the cytoplasmic pool of β-catenin as well as cell proliferation and invasion (**Figures 6-8**). Although somatic mutations have not been found in the Wnt pathway, Wnt activation has been implicated in a small subset (8-15%) of primary ESCC [52, 53]. Axin is downregulated during invasive growth of primary ESCC and inversely correlated with depth of invasion, lymphatic invasion and lymph node metastasis [54]. Wnt inhibitory factor 1 is frequently silenced epigenetically in ESCC [55]. Wnt1 can activate the Wnt pathway in ESCC cells [56]. Cancer-associated fibroblasts also secrete Wnt2 to promote esophageal cancer progression [57].

Cyclin D1 is an essential direct target gene for the Wnt pathway [58, 59]. Moreover, Wnt1 and Wnt5a can transactivate EGFR to induce cyclin D1 expression [60]. Wnt activation and cyclin D1 overexpression were observed in SCC developed in the mouse epidermis with DNMAML1 targeted by the SM22α promoter at midgestation [24]; however, Wnt inhibition did not reduce cyclin D1 expression (**Figure 9**), implying additional regulatory mechanisms for cyclin D1 expression. Amongst possible explanations are the non-soluble factors such as collagen and fibronectin in the extracellular matrix that are known to activate cyclin D1 via focal adhesion kinase-mediated pathway [61].

In sum, we have validated OTC as an excellent *in vitro* model system that can recapitulate many pathological characteristics of ESCC observed in both genetic mouse models and hu-

man patients. Notch inhibition in OTC and a genetic mouse model not only vindicated the tumor suppressor roles of Notch signaling, but also revealed Notch-independent induction of cyclin D1 and activation of Wnt signaling in the invasive fronts where the tumor microenvironment may influence the gene expression and cell signaling in transformed human esophageal cells.

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