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Concerted loss of TGFβ-mediated proliferation control and E-cadherin disrupts epithelial homeostasis and causes oral squamous cell carcinoma

Thomas Andl[†], Grégoire F.Le Bras[1,†], Nicole F.Richards[1], Gillian L.Allison[1], Holli A.Loomans[2], M. Kay Washington[3,4,5], Frank Revetta[3,4,5], Rebecca K.Lee[1], Chase Taylor[1], Harold L.Moses[2,3] and Claudia D.Andl[1,2,3,4,*]

Division of Dermatology, Department of Medicine, [1]Department of Surgery, [2]Department of Cancer Biology, [3]Vanderbilt Ingram Cancer Center, [4]Vanderbilt Digestive Disease Center and [5]Department of Pathology, Vanderbilt University Medical Center, Nashville, TN 37232-6840, USA

*To whom correspondence should be addressed. Vanderbilt University Medical Center, 2213 Garland Avenue, 10445 MRB IV, Nashville, TN 37232, USA. Tel: +1 615 322 0376; Fax: +1 615 322 6174;
Email: Claudia.andl@vanderbilt.edu

Although the etiology of squamous cell carcinomas of the oral mucosa is well understood, the cellular origin and the exact molecular mechanisms leading to their formation are not. Previously, we observed the coordinated loss of E-cadherin (CDH1) and transforming growth factor beta receptor II (TGFBR2) in esophageal squamous tumors. To investigate if the coordinated loss of *Cdh1* and *Tgfbr2* is sufficient to induce tumorigenesis *in vivo*, we developed two mouse models targeting ablation of both genes constitutively or inducibly in the oral–esophageal epithelium. We show that the loss of both *Cdh1* and *Tgfbr2* in both models is sufficient to induce squamous cell carcinomas with animals succumbing to the invasive disease by 18 months of age. Advanced tumors have the ability to invade regional lymph nodes and to establish distant pulmonary metastasis. The mouse tumors showed molecular characteristics of human tumors such as overexpression of Cyclin D1. We addressed the question whether TGFβ signaling may target known stem cell markers and thereby influence tumorigenesis. From our mouse and human models, we conclude that TGFβ signaling regulates key aspects of stemness and quiescence *in vitro* and *in vivo*. This provides a new explanation for the importance of TGFβ in mucosal homeostasis.

Introduction

The treatment of squamous cell cancers (SCC) of the upper aero-digestive tract has not significantly improved despite great strides to better understand the disease. Their resistance to radio-chemotherapy, high recurrence rate, and metastatic behavior are challenges that require novel models and approaches. The aggressive progression of these tumors is associated with increased cell invasion and metastasis due to the loss of cell–cell-adhesion regulated by E-cadherin and transforming growth factor beta (TGFβ) signaling. E-cadherin has emerged as one of the 'caretakers' of the epithelial phenotype (1). A direct role for E-cadherin in suppression of tumor invasion has been demonstrated by the reversion of undifferentiated, invasive tumors to a differentiated phenotype after transfection of E-cadherin cDNA in cell culture (2). Furthermore, E-cadherin is a powerful prognostic marker for oral squamous cell carcinomas (OSCCs) showing an inverse relationship with survival (3).

Despite the clear link of reduced levels of E-cadherin with invasive behavior and poor patient survival, the precise mechanisms underlying E-cadherin's function in the context of metastasis or therapy-resistance are not well established. One possibility is that the adhesive function of E-cadherin prevents cells from dissociating from each other and migrating into adjacent tissues (4). Alternatively, E-cadherin binds and sequesters β-catenin to the membrane, which regulates the cytoplasmic pool of β-catenin and therefore, its role as an essential intracellular mediator of the Wnt signaling pathway (5).

This hypothesis is supported by studies in *Drosophila* and *Xenopus* embryos (6,7). Mice lacking E-cadherin in the skin and other squamous epithelia, including the esophagus, either die perinatally due to water barrier defects of the epidermis (8) or show hyperproliferative and degenerative responses, but are viable (9). In the mouse model generated by Tinkle *et al*. (8), adhesion complexes are unaffected by the loss of E-cadherin through compensation by P-cadherin.

Alternatively, E-cadherin can be viewed as an inhibitor of epithelial–mesenchymal transition (EMT) and by extension of the establishment of highly therapy-resistant cancer stem cells (CSCs).

The role of TGFβ signaling in EMT is well established (10). However, TGFβ exhibits context–dependent effects on keratinocytes ranging from cell cycle arrest to EMT (11–13).

Loss of *Tgfbr2* has been modeled in mice through a dominant-negative approach in skin and mammary glands, and by the conditional knock-out of *Tgfbr2* (14,15). In these models, tumors develop with a high potential for metastasis, thereby supporting the tumor-suppressive function of *Tgfbr2* and intact TGFβ signaling. Hypermethylation of *TGFBR1* and *TGFBR2* has been reported for high-grade dysplasia and squamous cell carcinoma of the esophagus (16). Moreover restoration of wild-type TGFBR2 expression in colon and breast carcinoma cell lines that lack a functional *TGFBR2* allele (17) and overexpression in thyroid carcinomas reinforces this hypothesis (18). Indeed, re-expression of TGFBR2 conferred growth inhibition, suppressed anchorage independence, and abolished tumor formation in nude mice (17).

We previously observed that 70% of human esophageal tumors demonstrated coordinated loss of E-cadherin and TGFBR2 (19). When grown in three-dimensional organotypic cultures, cells with impaired E-cadherin and TGFBR2 function demonstrate fibroblast-dependent invasion into the underlying matrix (20). When analyzing publicly available databases for *CDH1* and *TGFBR2* expression in head-and-neck squamous cell carcinoma, we observed direct correlations of *CDH1* and *TGFBR2* status with disease recurrence and overall patient survival. Therefore, we hypothesized that coordinated loss of E-cadherin, *Cdh1,* and transforming growth factor beta receptor II, *Tgfbr2,* will lead to tumorigenesis *in vivo*. We developed a mouse model targeting *Cdh1* and *Tgfbr2* loss in the oral–esophageal epithelium using the Epstein–Barr virus L2 promoter, *ED-L2*. For spatio-temporal control, we also generated an inducible mouse model using *Cre-ERT* under the control of the *keratin 14*-promoter. We show that the loss of *Cdh1* and *Tgfbr2* in the absence of carcinogen treatment is sufficient to induce invasive OSCC, although single knock-out mice show no or rare events of tumorigenesis. These double knock-out tumors showed upregulation of stem cell markers, which are known TGFβ targets. Analysis of mouse and human models indicates that TGFβ signaling plays a crucial role in the regulation of proliferation and stemness and eventually tumorigenesis.

Materials and methods

Dataset analysis

To determine the clinical correlation of *TGFBR2* and *CDH1*, a comprehensive analysis of the Human Genome Atlas HNSCC data set was performed via cBio Portal (21,22). A total of 279 HNSCC patients were used in the analysis. Briefly, de-identified patient data, including gene expression and clinical data, were extracted from individual records available on cBio Portal. The collected information from each patient was analyzed in Prism version 6.00 for Mac

Abbreviations: CDH1, E-cadherin; CSC, cancer stem cell; DAPI, 4′,6-diamidino-2-phenylindole; dKO, double knockout mice; EMT, epithelial–mesenchymal transition; KO, single knockout mice; OSCC, oral squamous cell carcinoma; PDPN, podoplanin; SCC, squamous cell cancer; TGFBR2, transforming growth factor beta receptor II.

[†]These authors contributed equally to this work.

(GraphPad software, La Jolla, CA, www.graphpad.com). Survival analysis using the Kaplan–Meier method was performed using the Mantel-Cox log-rank test and log rank test for trend. Contingency table analysis of *TGFBR2* and *CDH1* differential expression and prognosis and survival was performed using Fisher's exact test. Relative risk (RR) and the corresponding 95% confidence interval (95% CI) are presented. Statistical significance was defined as $P < 0.05$.

Breeding and genotyping of mice

All animal studies were approved by the Institutional Animal Care and Use Committee at Vanderbilt University. The Epstein–Barr virus promoter *ED-L2* was used to target Cre activity to the oral–esophageal squamous epithelium as described previously (23); these mice were a gift from Dr. Jonathan Katz (University of Pennsylvania). ED-L2-Cre transgenic mice on a C57BL/6J background were crossed with loxP-flanked E-cadherin, *Cdh1*, mice (Jackson Laboratory, Bar Harbor, Maine) and/or with TGFβ receptor II, *Tgfbr2*$^{flox/flox}$ mice, both on a C57BL/6J background. We bred *ED-L2-Cre* (23) and *Cdh1*$^{flox/flox}$ mice as well as *ED-L2-Cre* and *Tgfbr2*$^{flox/flox}$ mice to generate single knockout mice (KO), and double knockout (dKO) mice, *L2-Cre*/*Cdh1*$^{flox/flox}$/*Tgfbr2*$^{flox/flox}$, subsequently referred to as L2/dKO. The *Cdh1*$^{flox/flox}$ mice were genotyped according to instruction on http://jaxmice.jax.org/protocolsdb/f?p=116:1:2821520305073265. *Tgfbr2*$^{flox/flox}$ mice were genotyped as described previously (24). A second colony was generated using *K14-Cre-ERT* mice (CD1 background, Jackson Laboratory, Bar Harbor, ME) to generate conditional single and double knock-out mice *K14-Cre-ERT*/*Cdh1*$^{flox/flox}$/*Tgfbr2*$^{flox/flox}$, hereafter K14/dcKO, for spatio-temporal control of gene knockout using oral gavage or intraperitoneal injection of tamoxifen.

Tamoxifen induction

Tamoxifen (Sigma, St Louis, MO) was diluted in peanut oil (Sigma) and administered three times at a concentration of 1 mg/day on alternating days by intraperitoneal injection. Alternatively, (Z)-4-hydroxytamoxifen (Sigma) was administered by oral gavage using a feeding needle. After initial Cre-mediated recombination resulting in the knock-out of *Cdh1* and *Tgfbr2*, mice received a «booster» shot once a month for the first 3 months.

Histology

After sacrificing the mice, tongue, esophagus and forestomach were removed and processed for histology. Additionally, buccal mucosa, lymph nodes, lung, liver and tumors were collected in diseased mice. Briefly, tissues were fixed in 10% formalin and embedded in paraffin. Five micron sections were applied to Probe-on Superfrost Plus slides (Fisher Scientific, Pittsburgh, PA). Slides were stained with hematoxylin and eosin, and images were captured on a Zeiss microscope with a Zeiss Hrc5 camera (Carl Zeiss Microscopy, Thornwood, NY). Matched littermate controls and mutant mice were examined histologically by review of slides in a blinded fashion by our pathologist.

Antibodies

Antibodies against E-cadherin, β-catenin, p120 and integrin β1 (ITGB1) were purchased from BD Biosciences (Franklin Lakes, NJ). Anti-pSMAD2, MECP2, Ki67 (clones 8D5 and D3B5), and YAP/TAZ (clone D24E4) were from Cell Signaling Technology (Danvers, MA). Anticytokeratin K14 was from Thermo Fisher Scientific (Fremont, CA). p63 and α-tubulin antibodies were purchased from Abcam (San Francisco, CA); and Ki67 from Vector Laboratories (Burlingame, CA). Anti-MYC, Cyclin D1, PML and TGFBR2 (clone L21) were from Santa Cruz Biotechnologies (Santa Cruz, CA). Podoplanin (PDPN) was from eBioscience (San Diego, CA), and SOX2 from EMD Millipore (Billerica, MA).

Immunohistochemistry and immunofluorescence

Immunohistochemistry was performed with the Vecta Elite kit (Vector Laboratories, Burlingame, CA) following the manufacturer's protocol using their reagents. Antigen retrieval was performed by heating paraffin sections in a pressure cooker for 12 min followed by a one-hour incubation in the pressure cooker. Primary antibody was incubated overnight at 4°C and secondary antibody for 30 min at 37°C. Then the signal was developed using the diaminobenzidene substrate kit for peroxidase. For immunofluoresence staining, primary antibody was incubated overnight at 4°C and detected using Texas-Red or fluorescein isothiocyanate-labeled secondary antibody (Vector Laboratories, Burlingame, CA). Stained sections were mounted with 4′,6-diamidino-2-phenylindole (DAPI)-containing mounting medium (Vector Laboratories, Burlingame, CA).

Western blotting

Western blots were performed as described previously (20). Experiments were repeated at least twice.

Results

Coordinated loss of Cdh1 *and* Tgfbr2 *results in squamous cell carcinoma*

We have demonstrated previously that the coordinated loss of E-cadherin (*CDH1*) and TGFβ receptor II (*TGFBR2*) is frequently detected in upper aero-digestive tract cancer (19). We queried the HNSCC dataset of the Cancer Genome Atlas, through the use of cBio Portal (21,22) to correlate *CDH1* and *TGFBR2* expression with patient disease recurrence and overall survival. HNSCC patients with a homozygous deletion or mutation in *TGFBR2* exhibited lower survival compared with patients without homozygous deletion or mutation of *TGFBR2* (Mantel-Cox log-rank $P = 0.71$; log-rank test for trend $P = 0.09$) (Figure 1A). We next examined E-cadherin protein expression, as examined by reverse phase protein array, and observed no overall survival differences between patients with downregulated E-cadherin, compared with those without downregulated E-cadherin ($P = 0.58$) (Figure 1B). The lack of statistical significance within these two analyses is probably due to low patient numbers following subgroup stratification. Additionally, we looked at the synergistic effect of *TGFBR2* and *CDH1* on disease recurrence and overall survival. Though not statistically significant, patients with either or both *TGFBR2* or *CDH1* altered had higher proportions of disease recurrence or death compared with patients without *TGFBR2* or *CDH1* altered (Figure 1C and D). The compiled data indicate a negative impact on HNSCC patient prognosis when *TGFBR2* and *CDH1* are disregulated.

To elucidate the functional consequences of the simultaneous E-cadherin and TGFBR2 loss *in vivo*, we generated mice with targeted deletion of the E-cadherin gene, *Cdh1*, and *Tgfbr2* in the oral cavity, esophagus and forestomach using the Epstein–Barr virus promoter *ED-L2*. Additionally, we established a tamoxifen-inducible colony using *K14-Cre-ERT* mice.

The observed ablation of *Cdh1* and *Tgfbr2* expression was restricted to focal areas and presented in a mosaic knock-out. This pattern resulted in focal SCC formation of the head-and-neck area (Figure 1) as well as forestomach (Supplementary Figure S1, available at *Carcinogenesis* Online) and death by the age of 18 months or older. Histological analysis of tissues with constitutive and inducible loss of both, Cdh1 and Tgfbr2 in *L2/dKO* or *K14/dcKO,* earlier identified a atrophic epithelium in targeted areas of the tongue, oral cavity and esophagus (Figure 2A; Supplementary Figures S2A and S3, available at *Carcinogenesis* Online). We also observed hyperplasia of selected minor salivary glands of the tongue (data not shown).

Invasive OSCCs were present in 74% and 77% of the constitutive and induced dKO mice, respectively (Table I). SCCs of the tongue could be detected as early as 10 months of age in dKO animals, but not in animals with deletion of a single gene, or in non-induced *K14/dcKO* mice. Some tumors progressed to grow into the skin exhibiting their invasive nature (Figure 1E and F) but no multifocal disease was observed. Anatomical subsites of the tumors were the buccal mucosa with involvement of the facial skin (Figure 1E and F), the oropharynx and the apex and dorsal and ventral tongue (Figure 1G, site-specific focal invasion in one area of the ventral tongue, rectangle; H for normal non-induced control animal; 1G, I; representative images of focal advanced disease). Furthermore, in the forestomach, an extension of the squamous esophageal epithelium, tumorigenesis occurred at the squamous-gastric junction and was characterized by well-differentiated areas with keratin-pearl formation (Supplementary Figure S1A–D, available at *Carcinogenesis* Online).

Mosaic ablation of Cdh1 *and* Tgfbr2 *in the tongue, esophagus and forestomach*

To verify loss of *Cdh1* and *Tgfbr2*, we performed immunofluorescence stainings with antibodies against E-cadherin (Figure 2A and B) and pSmad2 (Figure 2C and D), a downstream target of TGFβ signaling. In *K14/dcKO* mice, tamoxifen-induction led to the mosaic loss of E-cadherin and pSmad2 (Figure 2A and C). Non-induced control *K14/dcKO* littermates or *dKO* mice lacking the Cre-recombinase

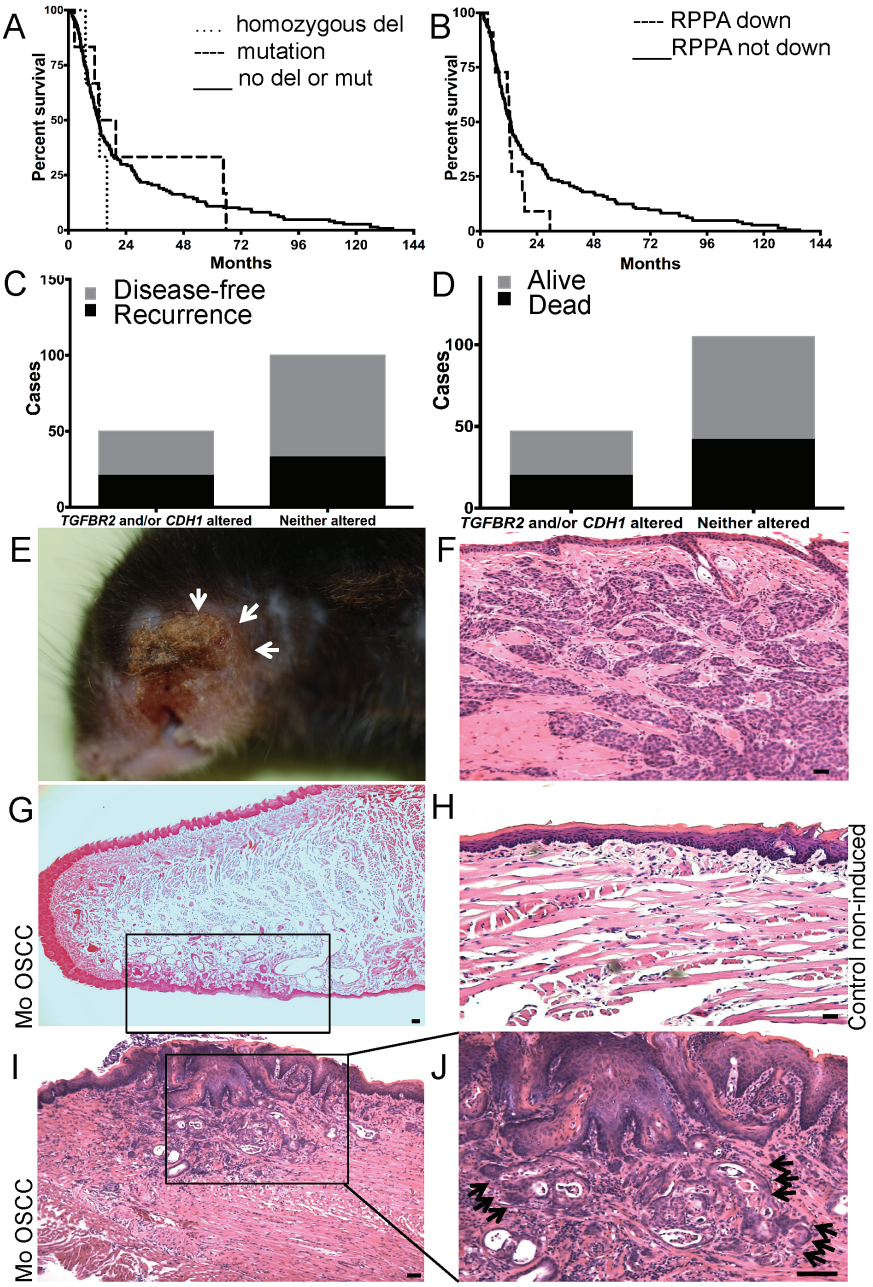

Fig. 1. Altered *TGFBR2* and *CDH1* in patient prognosis and mouse model. (**A**) Overall survival of HNSCC patients when stratifying by *TGFBR2* status. Patients with homozygous deletion or mutation of *TGFBR2* showed lower survival compared with patients without a homozygous deletion or mutation of *TGFBR2*. ($P = 0.71$). (**B**) Overall survival of HNSCC patients when stratifying by CDH1 reverse phase protein array expression. Patients with downregulation of CDH1 protein expression showed lower survival compared with patients with unaltered CDH1 protein expression. ($P = 0.58$). (**C**) Contingency analysis of *TGFBR2* and/or *CDH1* expression and disease-free survival. Patients with altered *TGFBR2* and/or altered *CDH1* had a higher proportion of disease recurrence than patients with unaltered *TGFBR2* or *CDH1* (RR = 1.27, 95% CI = 0.83, 1.96). (**D**) Contingency analysis of *TGFBR2* and/or *CDH1* expression and overall survival. Patients with altered *TGFBR2* and/or altered *CDH1* had no proportional difference in overall survival compared with patients with unaltered *TGFBR2* or *CDH1* (RR = 1.06, 95% CI = 0.71, 1.60). (**E**) *Cdh1/Tgfbr2* double knockout (dcKO) mice present with lesions that can involve the facial skin, in this case the whisker pad, due to their invasive nature (arrows). (**F**) Histological analysis of a OSCCs originating in the oral cavity extending to the buccal mucosa and involving the surface of the skin. (**G**) Low magnification of a representative tongue with focal tumor lesion on the ventral side (rectangle). (**H**) Normal mucosa of control animal. (**I**) Invasive SCC of the tongue (**J** higher magnification) invading into the muscle (black arrows). Scale bar, 50 micron.

gene displayed membranous E-cadherin staining similar to the unaffected adjacent mucosa of experimental mice or nuclear pSmad2 protein expression (Figure 2B and D). We showed loss of E-cadherin and pSmad2 in the invading tissues (Figure 2A and C, white dashed circles, Supplementary Figure S2, available at *Carcinogenesis* Online), which was of squamous origin as shown by the K14-positive staining (Figure 2G, serial section of a representative area of focal expression loss).

E-cadherin is a key component of adherens junctions and binds β-catenin and p120 through its cytoplasmic tail. Loss of E-cadherin was accompanied by the loss of β-catenin expression in the tongue, esophagus and forestomach (Figures 2E; SupplementaryFigure S2, available at *Carcinogenesis* Online). Non-induced control animals did not exhibit the loss of β-catenin (Figure 2F). The areas of E-cadherin and β-catenin loss were still positive for K14 staining, demonstrating the squamous origin of the invasive tissue lesion (Figure 2G).

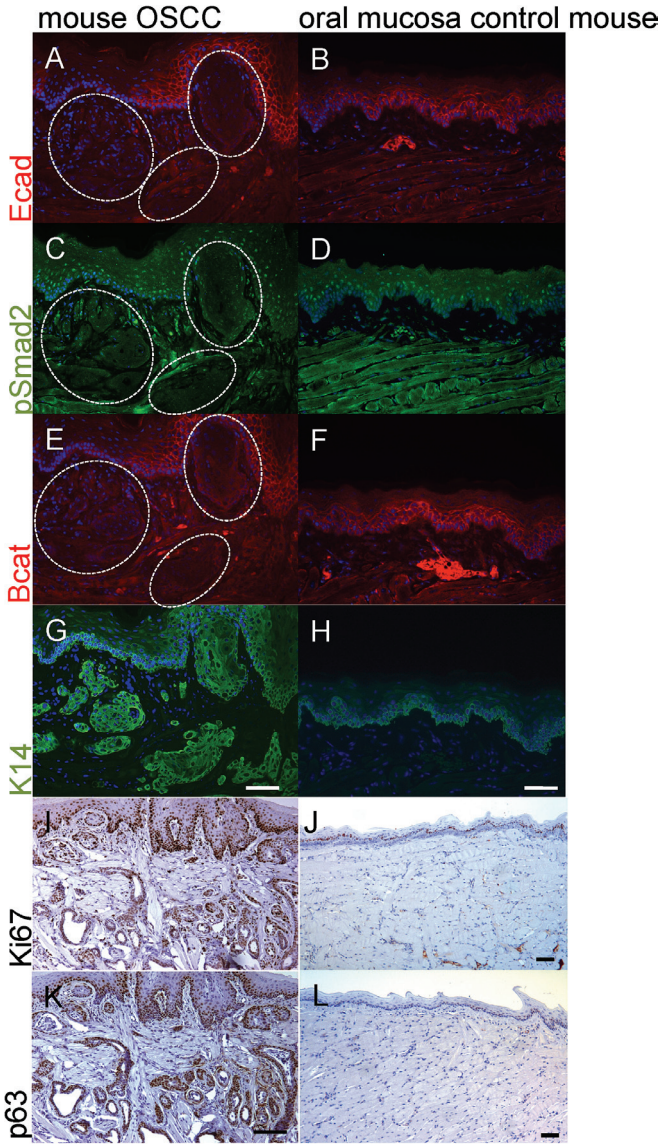

Fig. 2. Tumors exhibit disruption of adherens junctions and increased proliferation. Focal invasive areas (dashed white circles) of a tongue SCC are negative for E-cadherin (**A**, red) compared with the expression pattern of the normal tongue epithelium (**B**). (**C**) Nuclear pSmad2 (green) signal is lost in invasive areas (dashed white circles). (**D**) Normal mouse mucosa has positive pSmad2 signal throughout. B-catenin, red, is lost in these invasive cells (dashed white circles (**E**)), while (**G**) K14, green, stains positive. The signal is increased compared with the normal tongue epithelium (**H**) of a non-induced control animal. The tumor cells invading into the center of the tongue are strongly positive for Ki67 (**I**) and p63 (**K**) compared with normal tongue, (**J**) and (**L**), respectively. Scale bars, 50 micron.

Table I. Overview of tumor incidence in mice

Genotype	Tumor incidence (number of animals)
L2/dKO	74% (17/23)
L2/KO	8% (3/36)
K14/dcKO, induced	77% (27/35)
K14/dcKO, non-induced	6% (2/30)
K14/cKO, induced	10% (3/30)

Number of double knock-out (dKO) presenting with tumors compared with single knock-out (KO) animals. Knock-out in L2-driven animals is constitutive, K14 is a conditional knock-out (cKO) and tamoxifen-induced.

Adjacent oral mucosa (Figure 2A and E) still showed expression similar to normal mucosa in non-induced control mice (Figure 2B and F) suggesting a coordinate loss and disruption of adherens junction function. Similarly, the loss of E-cadherin binding partners β-catenin and p120 was observed in the forestomachs, which exhibit mosaic loss of expression for E-cadherin and pSmad2 (Supplementary Figure S2A, available at *Carcinogenesis* Online) compared with control animals (Supplementary Figure S2C, available at *Carcinogenesis* Online) and develop tumors (Supplementary Figure S2C and D, available at *Carcinogenesis* Online).

E-cadherin can regulate cell growth, and the loss of contact inhibition, which increases cell proliferation. Therefore, we analyzed the mouse tumors for their proliferation potential. Tumors of the oral cavity and the forestomach were strongly positive for both the proliferation marker Ki67 and p63, a squamous lineage marker (Figure 2I and K; Supplementary Figure S1E and G, available at *Carcinogenesis* Online), when compared with normal mucosa (Figure 2J and L; Supplementary Figure S1F and H, available at *Carcinogenesis* Online) in control animals.

Interestingly, although we confirmed knockout of E-cadherin and concomitant loss of β-catenin and concomitant loss of pSmad2 expression, the esophagus of *L2/dKO* and *K14/dcKO* displayed no evidence of tumor initiation (Supplementary Figure S3A–F, available at *Carcinogenesis* Online). We observed increased infiltration of immune cells in the submucosa suggesting an inflammatory microenvironment (Supplementary Figure S3A and B, available at *Carcinogenesis* Online), which appears to lack the ability to promote tumorigenesis.

Occurrence of regional and distant metastasis in dKO mice

The ability of the tumors in the *L2/dKO* mice *and K14/dcKO* mice to metastasize to regional lymph nodes and lungs mimicked the metastatic spread of the human tumors and corroborate their similarities to the human disease (Figure 3). K14-positive cell clusters were identified in regional lymph nodes and the lung (Figure 3, lymph node involvement in A–C and pulmonary metastasis in D–F). Local metastasis to the lymph nodes appears to precede pulmonary metastasis, as not all animals in that we observed K14-postitive clusters in the lymph nodes showed positive lungs. In contrast, we identified positive lymph nodes in all animals with K14-positive cells spread to the lungs. The identified lung metastases were highly proliferative and of epithelial origin as shown by Ki67 immunohistochemistry staining and strong positivity for p63, respectively (Figure 3G and H).

Upregulation of c-Myc, Cyclin D1 and Yap1 phenocopies human OSCC

The murine OSCCs demonstrated upregulation of the oncogene Cyclin D1 (Figure 4A–C), which is seen in human SCCs. This observation further highlighted the similarity with human OSCCs. Additionally, the expression of c-Myc and Yap1, another squamous oncogene (25,26), was upregulated in mouse tumors (Figure 4D–I). Interestingly, nuclear localization for c-Myc was similar in all areas of the tumor tissues (Figure 4D and E) but absent in normal control animals (Figure 4F), Cyclin D1 overexpression was restricted to areas of early invasion (Figure 4A and B; C control tongue from normal animal shows unspecific staining in the keratinized layer). Neither c-Myc nor Cyclin D1 was expressed at detectable levels in the mouse tongue epithelium of non-induced control animals. Although Yap1 was highly expressed and localized to the nucleus in the tumor samples (Figure 4G and H), its expression was restricted to the cytoplasm of the basal layer in normal controls (Figure 4I). The upregulation of c-Myc and Cyclin D1 upon loss of E-cadherin and TGFβ signaling was also observed in the squamous tumors of the forestomach (Supplementary Figure S2E–H, available at *Carcinogenesis* Online).

A quiescent basal cell layer in human oral mucosa and its loss during tumorigenesis

TGFβ signaling and E-cadherin are important regulators of keratinocyte proliferation (12,13,27,28). Furthermore, ITGB1 is a known TGFβ1 target and has been described as a stem cell marker (29,30).

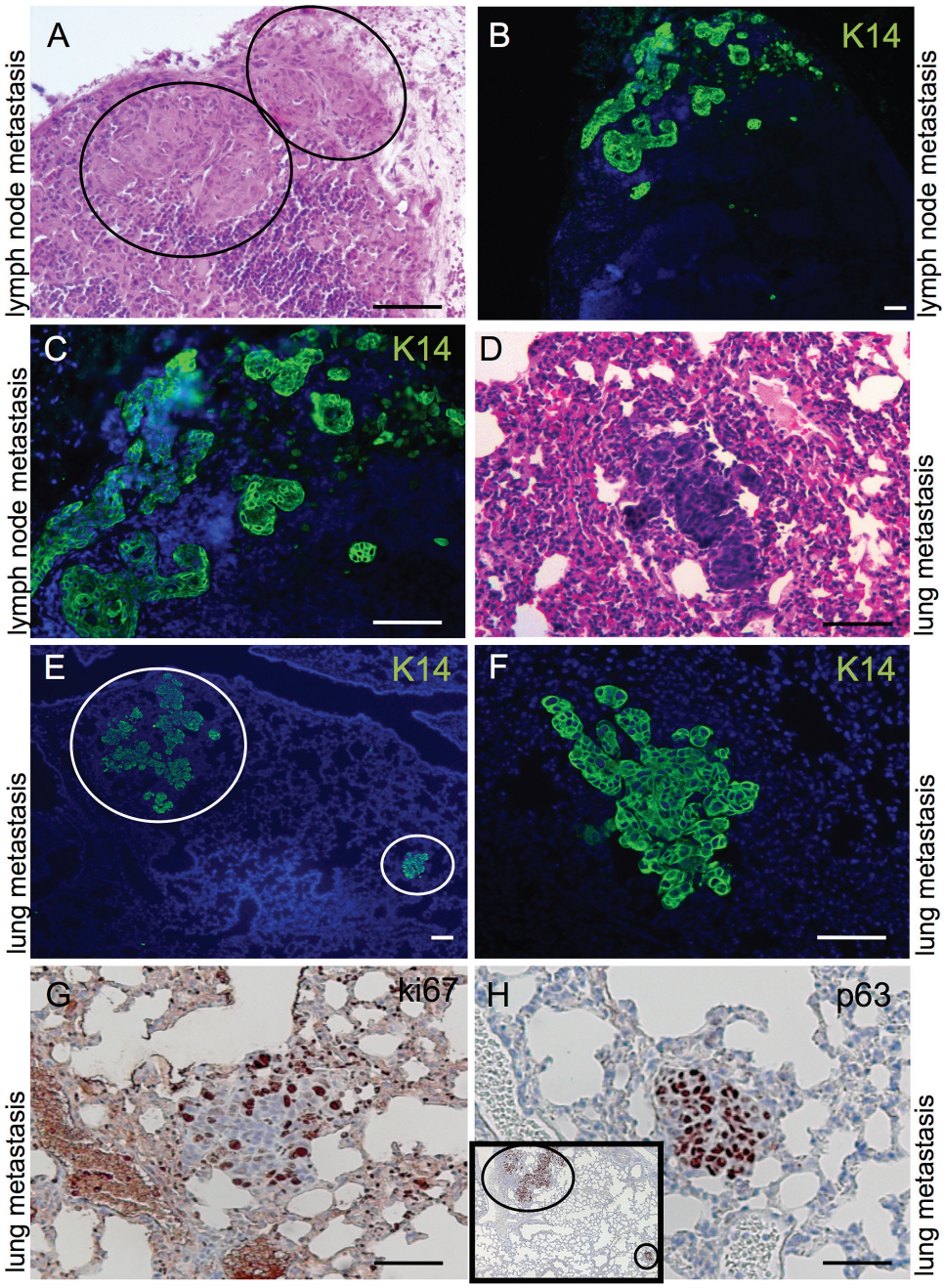

Fig. 3. OSCCs in *Cdh1/Tgfbr2* knockout mice are able to metastasize regionally and distantly. Tumor cell nests could be detected with keratin 14, K14, staining in regional lymph nodes (**A**, hematoxylin and eosin; **B** and **C**, K14 immunofluorescence). Tumors can spread to the lung as seen on lung sections stained with hematoxylin and eosin (**D**), and anti-K14 antibody in (**E**, **F** higher magnification). Lung metastases are Ki67- and p63-positive (**G**–**H**). Scale bars, 50 micron.

In an attempt to determine whether stemness and proliferation are affected by the coordinated loss of E-cadherin and TβRII in the mouse model, we analyzed ITGB1 (Figure 5A–D), PML, a regulator of TGFβ signaling (31,32, Figure 5E–H) and MECP2, which has functions in chromatin organization. All of them were restricted to the basal and quiescent cells in the normal human epithelium (Figure 5I–L). For this analysis we performed immunofluorescence staining of normal human tongue, human OSCC tissues, organotypic three-dimensional reconstructs of human keratinocytes expressing dominant-negative mutants of E-cadherin and TGFBR2 to mimic the coordinated loss established *in vivo* (20) and compared them with the mouse tumors. We showed that the expression of several stem cell markers is restricted to the basal layer in the human oral mucosa (Figure 5A, E and I, dashed line demarcates the basal membrane). These markers were upregulated in the human (Figure 5B, F and J) and mouse OSCCs (Figure 5D, H and L) and their spatial restriction to the basal layer disrupted in human organotypic reconstruct cultures (Figure 5C, G and K, dashed line demarcates the basal membrane, circles the invasive areas).

Western blot analysis using paired normal control and tumor samples from the two dKO mouse models (Figure 5M) confirmed lower expression levels of Cdh1, Tgfbr2 and lower level activation of the TGFβ signaling as measured by pSmad2 in the tumor samples. Using antibodies against additional putative stem cell markers such as Pdpn, Myc as well as the transcription factor Sox2, which is involved in the determination of cell fate and stem cell maintenance, we observed elevated protein expression in the mouse tumor samples (Figure 5M). That upregulation of PDPN occurs in human OSCC was shown

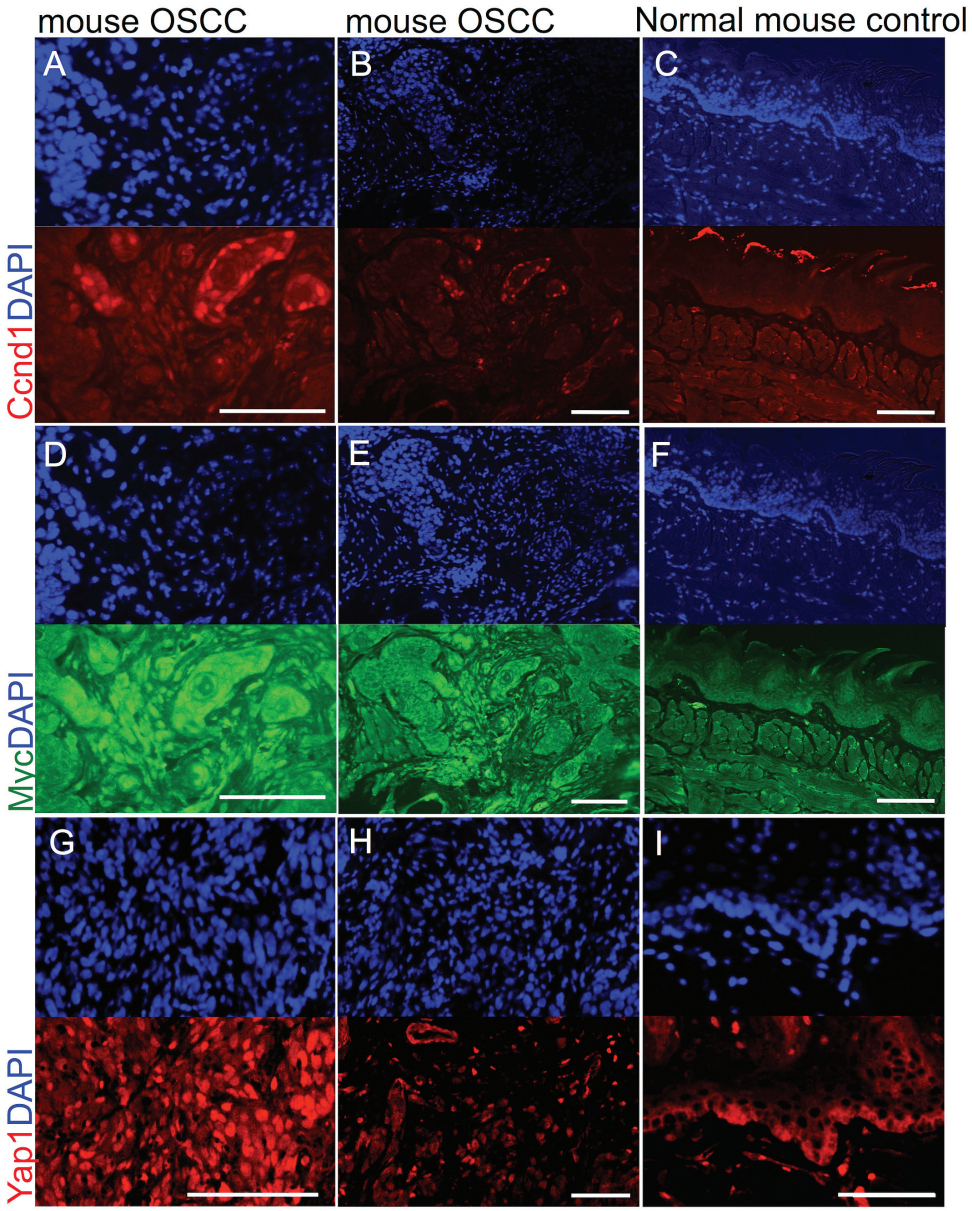

Fig. 4. Upregulation of Cyclin D1 (Ccdn1), c-Myc and Yap1 in mouse OSCCs. Mouse tumors are positive for Ccdn1 (**A**, **B**; red, DAPI blue), Myc (**D**, **E**; green, DAPI blue), and Yap1 (**G**, **H**; red, DAPI blue). Ccdn1 and Myc are up-regulation compared with normal mucosa (**C** and **F**, respectively). Yap1 (**G**, **H**) is localized to the nucleus in tumor tissues compared with cytoplasmic localization in normal oral mucosa (**I**). Scale bars, 50 micron.

by immunofluorescence staining compared with normal tongue (Figure 5N, co-staining with the proliferation marker Ki67).

Together, these data indicate a role for TGFβ-mediated regulation of stemness and suggest a mechanism how loss of this signaling pathway could result in tumorigenesis.

Discussion

The nature of the oral mucosal cells that are prone to initiate cancer is still unknown. Important events in this progression are the loss of normal proliferation control and the ability to invade locally and into the lymphatic system to advance to metastatic disease. We believe that TGFβ signaling and the cell adhesion protein E-cadherin play major roles in these processes. However, their contributions are multifaceted, complex and at times contradictory.

For instance, TGFβ signaling has been described as a tumor suppressor during early stages and as a tumor-promoter in later stages of tumorigenesis (33). However, inactivation of TGFβ signaling through mutations of TGFβ receptors and of their downstream effector molecules, the Smads, is associated with OSCC (34,35). The tissue-specific knockout of components of the TGFβ signaling pathway such as TGFBR1, TGFBR2 and Smad4 has been shown to result in tumor formation providing overwhelming evidence for the tumor suppressive function of this signaling pathway.

TGFβ and inhibition of proliferation in stem cells

TGFβ signaling has several context-dependent effects on keratinocytes with growth inhibition being the primary outcome. Additionally, TGFβ signaling is a potent inducer of EMT. These two major aspects of TGFβ are now frequently discussed in the context of cancer stem cells and normal stemness.

Many normal stem cell populations rely on TGFβ signaling for their quiescence control and their slow-cycling properties (36–39). We speculated that the tumorigenic advantages of elimination of TGFβ signaling are based on relaxing growth control in the stem cell compartment.

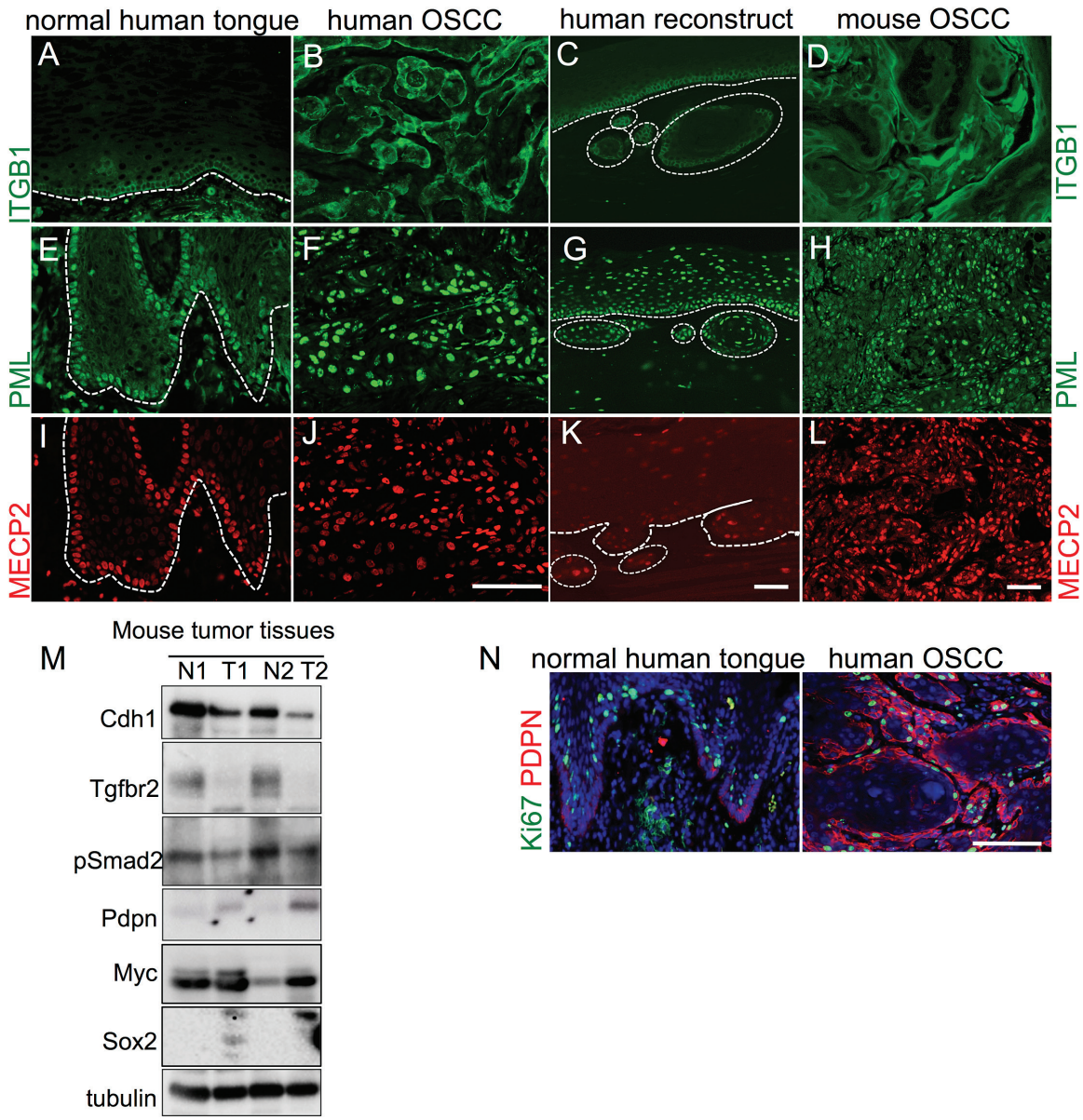

Fig. 5. Changes in putative stem cell marker expression during tumor progression. The expression of ITGB1 (**A**, green) was restricted to the basal layer in the normal human epithelium compared with human OSCC tissues (**B**), organotypic reconstructs with functional loss of E-cadherin and TGFBR2 (**C**) or mouse OSCCs (**D**). Similarly, PML (green) outlines the basal cell layer in normal human oral mucosa (**E**), but its signal increases in human (**F**) and mouse OSCCs (**H**) as well as loses it restriction to the basal layer in organotypic reconstructs (**G**). The expression of MECP2 mirrors PML expression and localization (**I**–**L**). Scale bars, 50 micron. Western blot analysis (**M**) of two paired mouse tissues, normal N versus tumor T shows the upregulation of Pdpn, Myc and Sox2. Upregulation of PDPN (red) in human OSCC is shown by immunofluorescence compared with normal human tongue (**N**, Ki67 is green). Western blots shown in this figure were cropped.

Adherens junctions in tumorigenesis beyond cell adhesion

Even in established cancer cell lines, the loss of E-cadherin can be accompanied by the expansion of CSC populations, EMT, and increased colony-forming capacity and tumorigenesis (40). Of interest, CSC populations seem characterized by low levels of E-cadherin (41) and purified squamous stem cells frequently have a low proliferation rate (41,42).

We have shown previously that E-cadherin and TGFBR2 physically interact and that E-cadherin can be an important determinant of TGFBR2 levels (19). Therefore, we speculated that the disruption of both genes may have synergistic effects in regard to malignant transformation in the mucosa. Indeed, in our model we detected an increase in tumor incidence in double compared with single KO mice.

The *ED-L2*-Cre mouse model has been successfully used to abolish the expression of another cell–cell adhesion molecule, p120, exemplifying that the disruption of adherens junctions is truly a hallmark of epithelial cancers. L2-Cre/p120$^{flox/flox}$ mice presented with a similar phenotype of invasive squamous tumors in the oral cavity and forestomach. However, esophageal SCCs were described (43), which we do not observe in our dKO systems. Interestingly, the p120 model is associated with increased numbers of immature myeloid cells, inflammation and desmoplasia in the tumor microenvironment. Analysis of esophageal tissues from our two models showed infiltrating immune cells in some cases, but no signs of esophageal tumorigenesis. Interestingly, the oral tissues in the mouse seem to be more sensitive to aberrations in TGFβ signaling when compared with the esophagus since treatment with an anti-TGFβ antibody results in oral and to a lesser extent esophageal lesions (44). Similarly, conditional inactivation of *Tgfbr2* in mouse fibroblasts induced SCCs in adjacent epithelia including the forestomach, an extension of the squamous esophageal epithelium, but no tumors were observed in the esophagus itself (45).

Synergy between TGFβ signaling and other signaling pathways in oral carcinogenesis

Furthermore, the function of adherens junctions and TGFβ signaling intersect at the process of EMT. However, the overall trend appears to favor a loss of TGFβ signaling in squamous carcinogenesis. For example, TGFBR2 and SMAD4 have been shown to be significantly down-regulated in OSCC tumor tissues compared with normal oral epithelium (46). *Smad4−/−* mice develop OSCC supporting the importance of the TGFβ pathway in the regulation of epithelial homeostasis (47). Similarly, coordinated Krt5-Cre mediated deletion of *Smad4* and *Pten* showed accelerated tumor formation in the forestomach with a corresponding increase in proliferation in the squamous epithelium of the forestomach and esophagus. Expression of cell cycle regulators such as p21, p16 and p27 was downregulated and Cyclin D1 upregulated (48). Combined loss of *Pten* and *Tgfbr1* also promotes OSCCs with upregulation of putative stem cell markers such as CD44, CD133, Sox2 and Oct4 (49). Interestingly, double knockdown of *Pten* and *Tgfbr1* resulted in activation of the mTOR pathway and increased levels of survivin (50). Therefore, treatment with rapamycin (50) or the mTOR inhibitor PF-04691502 (51) suppressed onset and progression of tumorigenesis. We tested the contributions of the PTEN/Akt pathways in our model without detecting any differential expression or activation in the tumor tissues compared with normal adjacent tissues samples (data not shown).

Tgfbr2 loss alone is not sufficient to induce OSCCs in our model. K14-mediated knock-out of *Tgfbr2* alone resulted in spontaneous anal and genital squamous cell carcinoma in a study by Guasch *et al*. (52). Four major pathways have been identified driving OSCC: mitogenic signaling, NOTCH, cell cycle and TP53 (53). Amongst the prominent driver genes were CCND1 and MYC. TGFβ antimitotic function involves cyclin-dependent kinase inhibitor gene responses and the downregulation of c-Myc for activation of the p15 arrest pathways (54). Given the considerable overlap of the pathways, we analyzed the head-and-neck squamous cell carcinoma TCGA provisional set for alterations in those pathways: p15 and p16 were altered in 39% of cases, most having homozygous deletion of the proteins. Twenty-two percent of the cases show MYC amplification or mRNA upregulation. Crosstalk between TGFβ and Notch-signaling has been described to dependent on ZEB1 and ZEB2 (55). Genetic inhibition of the Notch-mediated transcriptional activity prevented squamous differentiation and enriched for EMT-competent cells. Data from the head-and-neck squamous cell carcinoma TCGA data set show Notch alterations with an overall occurrence in 21% of the cases.

Key targets of the Hippo pathway, YAP and WWTR1, can interact with Smad molecules (56). However, the outcome of this interaction depends largely on the cell context. The YAP/Smad complex can promote EMT (57), and has been shown to induce a pro-invasive and metastatic phenotype in breast cancer (58). The Crumbs polarity complex interacts with YAP, which relays cell density information by promoting cytoplasmic retention of YAP resulting in suppressed TGF-β signaling (59). Interestingly, YAP can mediate cytoplasmic retention of phosphorylated SMAD3 thereby suppressing its function (60). Silencing of both YAP1 and IGF2BP3 can restore TGF-β signaling and also inhibited pluripotent gene expression and tumorigenesis. The adherens junction component α-catenin is also implicated in the regulation of cell density and tumor suppression, and has been shown to be a negative regulator of Yap1 (61). Furthermore, this study showed that activation of Yap1 lead to epidermal stem cell expansion and squamous-cell carcinoma-like tumors. We hypothesize that *Cdh1* and *Tgfbr2* knockout could initiate cancer initiating cell and CSC generation.

This leads to a novel hypothesis that the combined loss of Cdh1 and Tgfbr2 may in fact initiate tumorigenesis with long latency, since it provides an increased proliferation rate for cells, potentially stem cells, which are prone to malignant transformation over time. Future studies will have to show whether this is a mechanism that underlies early steps of squamous epithelial carcinogenesis and coins the molecular make-up and frequency of CSCs.

Supplementary material

Supplementary Figures S1–S3 can be found at http://carcin.oxfordjournals.org/

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