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Impairing squamous differentiation by *Klf4* deletion is sufficient to initiate tongue carcinoma development upon K-*Ras* activation in mice

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Oral squamous cell carcinoma (SCC) is among the most prevalent cancers in the world and is characterized by high morbidity and few therapeutic options. Like most cancers, oral SCC arises from a multistep process involving alterations of genes responsible for balancing proliferation and differentiation. Among these, Krüppel-like factor 4 (*Klf4*) suppresses cell proliferation and promotes differentiation and thus helps to maintain epithelial homeostasis. However, the prevailing role of Klf4 in maintenance of normal homeostasis in oral epithelium has not been established *in vivo*. Here, we used an inducible oral-specific mice model to selectively ablate *Klf4* in the oral cavity. We generated K14-CreER^{Tam}/*Klf4*$^{f/f}$ mice that survived to adulthood and did not present overt phenotype. However, histologically these mice showed dysplastic lesions, increased cell proliferation and abnormal differentiation in the tongue 4 months after induction, supporting a homeostatic role of Klf4 in the oral epithelia. Furthermore, by breeding these mutants with a transgenic line expressing at endogenous levels K-*ras*^{G12D}, we assessed the role of disrupting differentiation gene programs to the carcinogenesis process. The K14-CreER^{TAM}/K-*ras*^{G12D}/*Klf4*$^{-/-}$ mice rapidly develop oral SCC in the tongue. Thus, our findings support the emerging notion that activation of differentiating gene programs may represent a barrier preventing carcinogenesis in epithelial cells harboring oncogenic mutations, and thus that molecules acting upstream and downstream of Klf4 may represent components of a novel tumor-suppressive pathway.

Introduction

Squamous cell carcinoma (SCC) is the most common histologic type of cancer arising from different regions within the head and neck and it represents at least 90% of all oral malignancies. With over 600 000 new cases annually diagnosed, SCC of head and neck (HNSCC) is the sixth most common cancer in the world (1–3). Therapy for HNSCC is limited by anatomic location of the tumors, which may involve vital structures such as the mouth, the nasal cavity and paranasal sinuses, or those necessary for speech and swallowing. This disease is associated with quite significant mortality and morbidity rates and in spite of the vast amount of research and advances accomplished in the field of oncology and surgery, only 40–50% of patients with HNSCC will survive >5 years (1).

It is well known that HNSCC arises from a process that involves the sequential acquisition of genetic and epigenetic alterations in tumor suppressor genes and oncogenes. Among them, the most frequently reported alterations include loss of heterozygosity and promoter silencing of the *p16^{INK4}* gene, inactivating mutations in the *p53* tumor suppressor gene, and aberrant activity of signaling networks such as the epidermal growth factor receptor, Ras, transforming growth factor-β and PI3K-AKT-mTOR signaling pathways (4). Recently, mutations of genes in the Notch pathway have been reported (5,6). Notch often controls stem cell fate decisions in multiple cell types, including the immune system (7,8). Activating mutations of Notch have been reported in chronic lymphocytic leukemia (9) and in large B-cell lymphoma (10), suggesting that persistent activation of Notch can enhance cell growth and survival. However, most mutations in Notch in HNSCC are inactivating, suggesting that Notch can act as a potential tumor suppressor gene in this cancer type (5,6). In this case, Notch may contribute to epithelial cell differentiation (11,12), and its dysfunction may limit the ability of cells to initiate cell differentiation programs.

In this regard, a balance between cellular proliferation and differentiation is crucial for maintaining tissue homeostasis. A substantial body of literature has primarily focused on the deregulation of genes related to the proliferation side of this balance in the carcinogenic process. However, the role of an altered differentiation in cancer has been much less explored, and this process may provide new insights regarding the understanding of cancer development and progression in epithelial tissues. Of interest, Krüppel-like factors are a family of transcription factors that play an important role in many fundamental biologic processes including proliferation, apoptosis and differentiation (13–15). Among these, Krüppel-like factor 4 (KLF4, also called gut-enriched KLF) is highly expressed in terminally differentiated postmitotic epithelial cells in organs such as skin and gastrointestinal tract (16). KLF4 is also expressed in the tongue epithelium, tooth bud and palate (17,18). Although Klf4 has been used as a reprogramming factor to reprogram mouse fibroblasts into inducible pluripotent stem cells (14,19), in the skin, Klf4 plays a key role in the terminal differentiation of the stratified epithelia (18,20). This transcription factor suppresses cell proliferation and promotes differentiation and thus helps to maintain homeostasis in epithelial cells (14,21). Consistently, *Klf4*-knockout (KO) mice exhibit impaired terminal differentiation and abnormal permeability of the epidermal barrier supporting the role of Klf4 in skin differentiation (18). The ability of KLF4 to affect barrier function in the skin is probably due to its ability to regulate gene clusters of the Sprr and keratin families (22,23), which play a key role in maintaining epithelial barrier integrity. However, the prevailing role of Klf4—as a proliferation or differentiation factor—in the maintenance of normal homeostasis in oral epithelium has not yet been established *in vivo*.

Here, we took advantage of a transgenic mice model specific for oral cavity that we have previously developed (24) to selectively ablate this transcription factor in the oral cavity where it is normally expressed (18). The driver line of this system, K14-CreER^{tam}, specifically expresses the Cre recombinase enzyme in the oral cavity under the control of the cytokeratin 14 (K14) promoter, whereas CreER^{tam} activity is regulated by tamoxifen administration in the above-mentioned tissues. Even the K14 promoter targets other tissues besides the oral and tongue epithelium, in this transgenic system Cre-mediated recombination is highly restricted to the oral cavity, as shown before (24). The ability to generate oral-specific *Klf4* conditional KO mice enabled us to document that this transcription factor contributes to epithelial cell differentiation in this cellular compartment. Furthermore, by breeding the conditional *Klf4* KO mice with a transgenic mice expressing at endogenous levels oncogenic K-*ras*^{G12D}, we were able to assess the role of disrupting differentiation gene programs to the carcinogenesis process. Indeed, while *ras* activation alone induces

Abbreviations: HNSCC, squamous cell carcinoma of head and neck; Klf4, Krüppel-like factor 4; KO, knockout; PCNA, proliferating cell nuclear antigen; SCC, squamous cell carcinoma.

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benign dysplastic lesions, here we provide evidence that the concomitant *Klf4* deletion and K-*ras* activation, when targeted specifically to a sensitive cell compartment within the epithelium of the tongue, is sufficient to promote the development of malignant oral SCC lesions with a very short latency. Thus, our findings support the emerging notion that the activation of differentiating gene programs may represent a natural barrier preventing cancer progression in stratified epithelial cells harboring oncogenic mutations, and hence that molecules acting upstream and downstream of Klf4 may represent components of a novel tumor-suppressive pathway.

Materials and methods

Transgenic mice

The K14-CreER^{tam}, LSL-K-*ras*^{G12D} and *Klf4*$^{flox/flox}$ mouse strains have been described before (24,25). Their genetic backgrounds were as follows: K14-CreER^{tam} and LSL-K-*ras*^{G12D}, FVB/N; *Klf4*$^{flox/flox}$ C57BL/6.

K14-CreER^{tam} mice were crossed with *Klf4*$^{flox/flox}$ mice to generate K14-CreER^{tam}/*Klf4*$^{flox/flox}$ line and subsequently bred with LSL-K-*ras*$^{G12D/+}$ mice to generate K14-CreER^{TAM}/K-*ras*$^{G12D/+}$/*Klf4*$^{flox/+}$ mice. These mice were further crossed with *Klf4*$^{flox/flox}$ to generate K14-CreER^{TAM}/K-*ras*$^{G12D/+}$/*Klf4*$^{flox/flox}$ line. K14-CreER^{tam} mice were used as hemizygotes in all the lines established. All mice used for the experiments were on a mixed FVB/N–C57BL/6 genetic background. Tamoxifen was administered to 1-month-old animals, 1 mg per mouse per day orally, for five consecutive days. All mice were examined daily. Control mice received only the tamoxifen solvent (sunflower oil) with the same schedule. Genotyping was performed on tail biopsies by PCR using specific primers (24,25). Cre-mediated recombination of the *Klf4*-floxed allele was confirmed as described previously (25) using genomic DNA purified from tissues of wild-type and the K14-CreER^{tam}/*Klf4*$^{flox/flox}$ mice treated with or without tamoxifen.

All experimental procedures were in accordance with institutional (Institutional Animal Care and Use Committee of the Oncology Institute Angel H. Roffo, University of Buenos Aires) and government regulations (Servicio Nacional de Sanidad y Calidad Agroalimentaria, RS617/2002, Argentina). This study was specifically approved by the Institutional Animal Care and Use Committee of the Oncology Institute Angel H. Roffo, University of Buenos Aires (RS933/2012, protocol 2012/12). All efforts were made to minimize the number of animals used and their suffering.

Tissue preparation, histology, immunohistochemistry and immunofluorescence

All tissues were dissected, fixed overnight in buffered 4% paraformaldehyde at room temperature, dehydrated and embedded in paraffin. Hematoxylin and eosin-stained sections were used for diagnostic purposes, and unstained serial sections for immunostaining analysis. High-magnification pictures were obtained from representatives areas avoiding twisted or folded histological samples. Periodic acid–Schiff staining was used in carcinomas with the purpose of highlighting the loss of integrity in the basement membrane in a microinvasive area. Immunohistochemistry was performed as described previously (26). Antibodies used include cytokeratin 14 (MK14; Covance) at dilution 1:400; antibody for proliferating cell nuclear antigen (PCNA; Zymed) at dilution 1:100; Cyclin D1 (92G2; Cell Signaling Technology) at dilution 1:100; p53 (NCL-p53-CM5p; Novocastra, Newcastle, UK) at dilution 1:500. We determined the number of PCNA-positive cells in 6–7 high power fields of each tongue related to the total number of cells for each field.

The labeling index was calculated as the number of labeled cells over the total number of cells in the selected area (magnification: ×40), employing the image analysis software Image J to distinguish between positive nuclei (with 3,3′-diaminobenzidine deposits) and hematoxylin-stained nuclei.

Klf4 immunostaining was performed on cryostat section from snap-frozen optimal cutting temperature-embedded tongue samples and sectioned 8–10 µm thick. Sections were fixed in cold acetone 10 min at room temperature. The antibody for Klf4 was kindly provided by Dr J.Segre (National Human Genome Research Institute, National Institutes of Health, Bethesda, MD) (18). Tissues samples for immunofluorescence were also snap frozen in optimal cutting temperature. Sections were fixed in 3.6% buffered formaldehyde/10% methanol for 5 min, followed by fixation in ethanol–acetic acid for 5 minutes at room temperature and incubated with the antibodies mentioned above and antibodies for detection of cytokeratin 1 and 6. K1 (MK1, 1:500) and K6 (MK6, 1:400) antibodies were from Covance. Alexa 488 (Invitrogen) or Cy3 (Jackson Immunoresearch)-conjugated secondary antibodies were used for detection. The slides were counterstained with 4′,6-diamidino-2-phenylindole and mounted with Vectashield.

Histological slides were scanned using an Aperio T3 Scanscope (Aperio Technologies, Vista, CA) in order to obtain panoramic low-magnification views of tissues. The pictures presented at higher magnification were taken with a Nikon TE2000-S microscope and images were acquired with a digital camera Nikon FDX-35 and postacquisition processed with the Micrometrics SE Premium software. High-resolution immunofluorescence images were obtained on an Olympus Fluoview FV1000 confocal laser scanning microscope.

Western blot

Western blots were performed on lysates from frozen tissues. Protein concentration was determined, and 50 µg of total proteins were separated on sodium dodecyl sulfate–polyacrylamide gel electrophoresis, transferred to nitrocellulose membranes and blocked with 5% dry milk. Antibodies used include cyclin D1 (bcl-1; Thermo Scientific); p21 (sc-397), p27 (sc-528) and actin from Santa Cruz Biotechnology.

Statistical analysis

Kaplan–Meier survival curve and its statistical analysis, as well as log-rank test followed by a *post hoc* comparison (Holm–Sidak test), were performed using the SigmaStat software package.

Results

Excision of Klf4 *gene alters the homeostasis of the tongue squamous epithelium*

In order to explore the consequences of *Klf4* deletion in the squamous epithelium of the tongue, we bred *Klf4*-floxed mice with a K14-CreER^{tam} inducible and tissue-specific mouse line (24). Up to 2 months after the tamoxifen induction, K14-CreER^{tam}/*Klf4*$^{flox/flox}$ mice did not present any overt phenotype. At necropsy, no obvious changes were seen in the oral mucosa, tongue or skin. However, histological evaluation revealed that the majority of tongues of K14-CreER^{tam}/*Klf4*$^{flox/flox}$ mice showed focal acanthosis compared with control littermates (Figure 1A). These morphological changes in the KO tongue were more evident at 4 months after the induction of the system. The tongue of these mice exhibited marked acanthosis and hyperorthokeratosis (Figure 1B) (four out of four mice). After 6 months of evolution, K14-CreER^{tam}/*Klf4*$^{flox/flox}$ mice still appeared healthy in spite of displaying more pronounced tongue phenotypes. Histologically, these tongues showed hyperplasia and dysplastic areas, which range from mild to moderate dysplasia (nine out of nine mice) (Figure 1C and D). Examination of older mice (9 months, four out of four mice) did not show further exacerbation of the mentioned phenotype. Control K14-CreER^{tam}/*Klf4*$^{flox/flox}$ animals treated with solvent alone did not develop lesions or alterations in normal histology neither exhibit any differences in survival rate (Figure 3B).

We analyzed by immunohistochemistry the expression of Klf4 in the tongue of the K14-CreER^{tam}/*Klf4*$^{flox/flox}$ mice using a specific antibody (18) to confirm Klf4 expression in the tongue and that our transgenic mouse system leads to Klf4 deletion in the tongue. The wild-type tongue exhibits conspicuous expression of Klf4 in all cells in the differentiating upper layers of the stratified epithelium, with labeling localized in cell nuclei (Figure 1E). Contrastingly, KO tongues exhibit a patchy Klf4 expression, in areas with no detectable expression of this protein spanning from the basal to the cornified stratum (Figure 1F and G). We observed that these patched areas lacking Klf4 expression were intermingled with areas retaining normal Klf4 expression and distribution. This arrangement of knocked out Klf4 expression is in concordance with the expected recombination pattern produced by K14-CreER^{tam} system as reported before (24). We have shown previously that upon tamoxifen induction the K14-CreER^{tam} driver line targets sporadic cells within the oral cavity and the epithelium lining the tongue, likely involving a subset of epithelial stem cells and their progeny within these tissues (24). We also confirmed that tamoxifen treatment leads to CreER^{tam}-dependent recombination of the *Klf4*-floxed alleles by PCR analysis using genomic DNA from the KO tongue (Supplementary Figure 1, available at *Carcinogenesis* Online). No recombination was observed in the absence of CreER^{tam} expression (liver, spleen) or tamoxifen induction (tongue, liver, spleen without tamoxifen) instead PCR analysis detected a null band corresponding to Cre-mediated recombination of *Klf4* alleles in the tongue of K14-CreER^{tam}/*Klf4*$^{flox/flox}$ mice treated with tamoxifen (Supplementary Figure 1, available at *Carcinogenesis* Online).

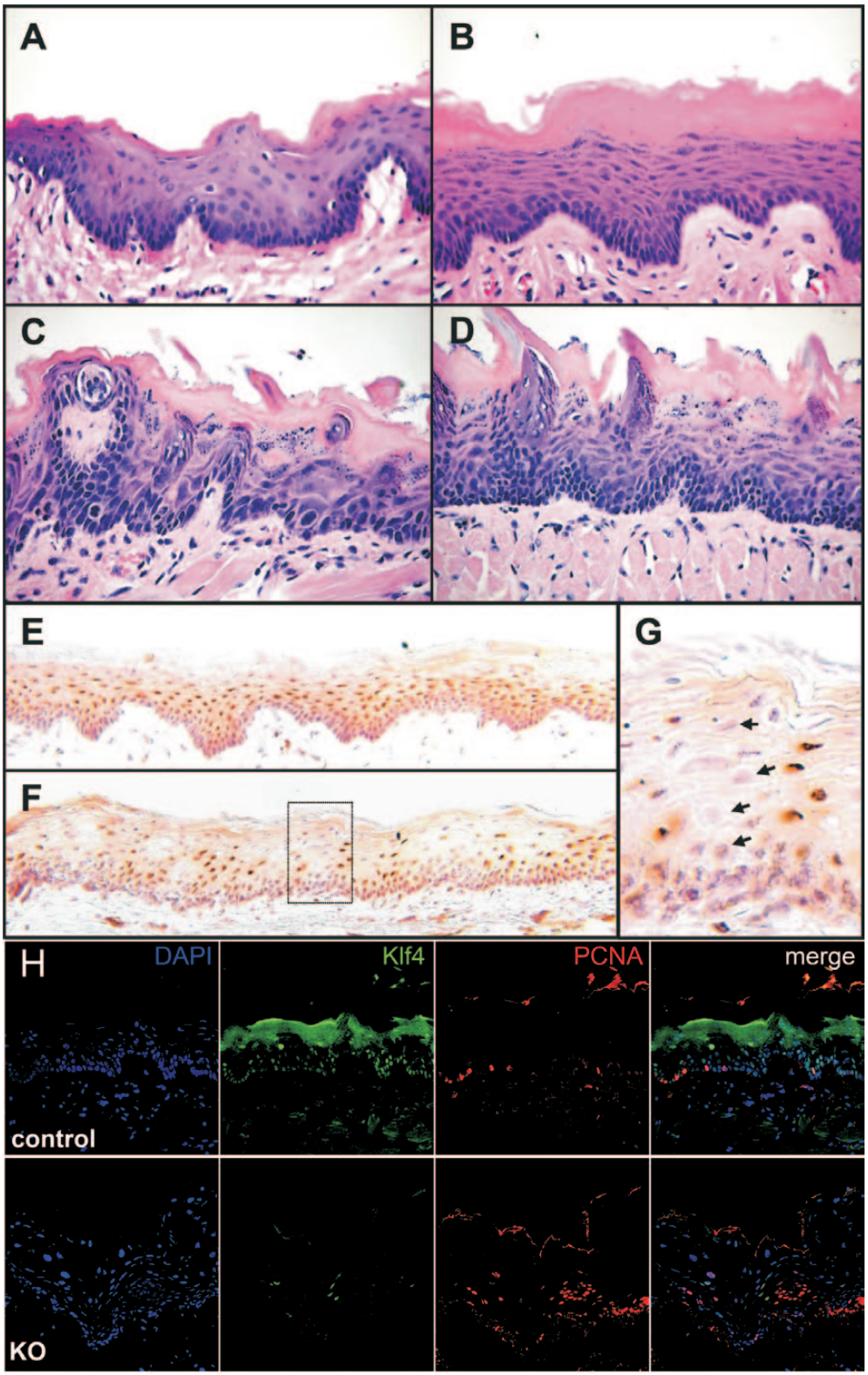

Fig. 1. Morphological changes in the Klf4 KO tongue. (**A**) The tongue mucosa presented epithelial acanthosis and a duplicated basal layer. (**B**) Tongue epithelium shows hyperorthokeratosis and basal layer stratification. (**C** and **D**) Tongue mucosa with moderate epithelial dysplasia. (**C**) Notice at cellular level, the mitotic figures, (**D**) the cellular and nuclear pleomorphism and the nuclear hyperchromasia. (**E**) Immunostaining for Klf4 in the wild-type tongue. Nuclear staining for Klf4 in the upper stratum of the epithelium. (**F**) Tongue from K14-CreER^{tam}/*Klf4*$^{flox/flox}$ mice exhibits a patchy Klf4 expression with clear columns of cells from the basal to the cornified stratum of no detectable expression of this protein. (**G**) Detail of F at higher magnification. Notice the absence of Klf4 expression from basal to upper layer (arrows) intermingled with an area with expression of the protein (right side). Original magnifications: ×5, ×10 and ×40. (**H**) Immunofluorescence labeling of Klf4 and PCNA of tongue from wild-type (control) and KO mice. Sections were labeled with antibodies as indicated and color coded on each frame. All tissues were counterstained with 4′,6-diamidino-2-phenylindole (DAPI) for nuclear localization. The upper layers of the control tongue presented nuclear staining for Klf4 (green fluorescence) and few scattered PCNA-positive cells were seen in the basal layer (red fluorescence). The

To explore the differentiation status of the KO tongues, we studied the pattern of expression of K14 that is typically expressed in the basal layer of normal squamous epithelium of the tongue (24,27). We observed changes in the expression pattern of K14 from localized expression in the basal layer in control tongues to a generalized expression spanning the upper layers in the Klf4 KO tongues (Figure 2A and B, Supplementary Figure 2, available at *Carcinogenesis* Online), in concordance with the uncompartmentalized pattern of expression of the cell proliferation marker, PCNA (see below and Supplementary Figure 2, available at *Carcinogenesis* Online). These changes were similar for K14-CreER^{tam}/*Klf4*$^{flox/flox}$ mice of 4 and 6 months of age. To further characterize the differentiation status of

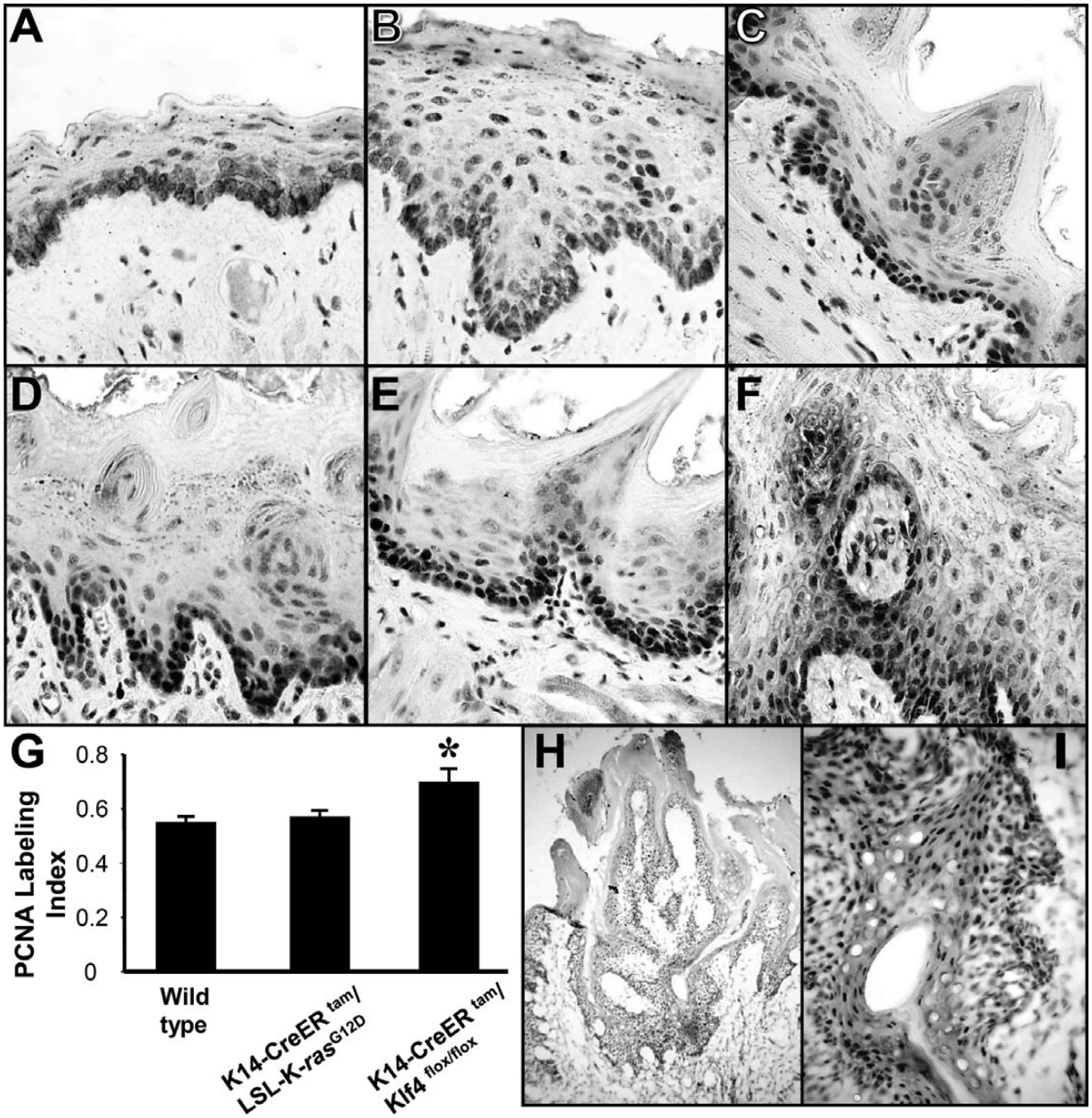

Fig. 2. Changes in the pattern of expression of K14 in tongues from K14-CreER^{tam}/*Klf4*$^{flox/flox}$ mice. (**A**) In control tongue, K14 is expressed in the basal layer; (**B**) however, the Klf4 expression in KO tongue shows staining in the upper layers. Increased PCNA expression in KO tongues for Klf4. (**C**) PCNA immunostaining in the basal layer of control tongue. (**D**) PCNA expression in the basal layer and immediately upper layer in tongue from K14-CreER^{tam}/LSL-K-*ras*$^{G12D/+}$ mice. (**E**–**G**) PCNA staining in KO tongues for Klf4. (**E**) Expression of PCNA in basal and upper layers in a hyperplastic area. (**F**) PCNA immunostaining from basal to suprabasal layers in a dysplastic area. (**G**) PCNA-labeling index showed statistically significant increase of the proliferative capacity in the K14-CreER^{tam}/*Klf4*$^{flox/flox}$ tongues compared with the other two groups (K14-CreER^{tam}/LSL-K-*ras*$^{G12D/+}$, wild-type), analysis of variance $P \leq 0.001$ *post hoc* Holm–Sidak, $*P \leq 0.005$. (**H** and **I**) Immunostaining for Klf4 in an oral papilloma from K14-CreER^{tam}/LSL-K-*ras*$^{G12D/+}$ mice. Detail of H at higher magnification, notice the nuclear staining for Klf4 in the upper startum of the epithelium. (**I**) Original magnifications: ×2.5, ×25 and ×40.

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single color images were merged to determine coexpression of Klf4 and PCNA. The control tongue did not show coexpression of Klf4 and PCNA coincident with a compartimentalized distribution of them. No immunostaining with Klf4 is evident in this area of the KO tongue and the labeling for PCNA identifies the proliferating cells from the basal and suprabasal layers (red fluorescence). The merged image showed clusters of PCNA-labeled cells in suprabasal layers that coincide with regions of Klf4 deletion. Original magnifications: ×5, ×10 and ×40. Confocal images, original magnification, ×40.

the tongues, we analyzed the pattern of expression of cytokeratin 1, a marker of keratinocyte differentiation, in control and KO samples. Control tongues showed K1-positive cells only in the upper layers of the epithelium, whereas KO tongues did not exhibit labeled cells (Supplementary Figure 2, available at *Carcinogenesis* Online). These changes in the pattern of expression of cytokeratins are consistent with abnormalities in the normal differentiation and keratinocyte maturation of the tongue. We also studied the expression pattern of cytokeratin 6, which appears constitutively expressed throughout the epithelium of the control tongue (28). K6 was poorly expressed in the KO tongue, different from its conspicuous expression in control tongue (Supplementary Figure 2, available at *Carcinogenesis* Online).

To gain a major insight into the proliferative changes underlying the growth of the epithelium in the tongues from K14-CreER^{tam}/*Klf4*$^{flox/flox}$ mice, we performed PCNA immunostaining and calculated the nuclear labeling index (Figure 2). Furthermore, the proliferative status of the KO tongues were compared with hyperplastic lesions produced by K-*ras*^{G12D} expression driven by the same promoter (24). Although in normal tissues cell proliferation is strictly restricted to the basal layer of the epithelium, we observed an increased proliferative capacity of the K14-CreER^{tam}/*Klf4*$^{flox/flox}$ tongues, with PCNA-positive cells both in the basal and suprabasal layer, resembling the staining of the K14-CreER^{tam}/LSL-K-*ras*$^{G12D/+}$ tongues (Figure 2D and E). As we have described above, the *Klf4* KO tongues showed areas of hyperplasia as well as areas with dysplastic changes in different proportion for each mouse (Figure 2E and F). KO tongues presented a marked shift of positive PCNA-immunolabeled cells toward suprabasal layers (Figures 1H and Figure 2E and F). This suggests that restricting the differentiating potential of epithelial cells by *Klf4* gene deletion is sufficient to enable the proliferation of suprabasal cells, which may contribute to the progressive accumulation of dysplastic changes.

To quantify the proliferative status of each histological area, we selected representative areas of hyperplasia and dysplasia from tongues of K14-CreER^{tam}/*Klf4*$^{flox/flox}$ mice of 4 and 6 months after the induction of the system. We found a statistically significant increase in the proliferative capacity of the K14-CreER^{tam}/*Klf4*$^{flox/flox}$ tongues compared with the other two groups (K14-CreER^{tam}/LSL-K-*ras*$^{G12D/+}$ and wild-type mice) (Figure 2G). Proliferation in the epithelium of the tongues from K14-CreER^{tam}/LSL-K-*ras*$^{G12D/+}$ was not statistically significantly different from that of wild-type mice, suggesting that Ras alone does not increase cell proliferation when a single mutant copy is driven by its own promoter, as we previously reported (24) (Figure 2G).

To further understand whether the increased proliferative status of the KO tongues was due to the loss of Klf4, we have analyzed the expression pattern of Klf4 and PCNA by double immunofluorescence staining. We found that accumulated PCNA-positive cells in suprabasal layers coincide with patched areas lacking Klf4 expression (Figure 1H). In this regard, we also performed double immunostaining for each of the cytokeratins presented above together with PCNA labeling. We observed areas of a clear colocalization of PCNA- and K14-positive cells (Supplementary Figure 2, available at *Carcinogenesis* Online) and areas of basal and suprabasal PCNA-positive cells that did not show K1 and K6 immunostaining in the KO tongues (Supplementary Figure 2, available at *Carcinogenesis* Online).

Thus, the increase in PCNA labeling found in the *Klf4* KO tongues may reflect abnormalities in the switch from proliferation to differentiation in which Klf4 is implicated. Indeed, homeostasis of the tongue epithelium seems to be disrupted upon Klf4 deletion. We found increased cell proliferation not restricted to the basal layer and concomitant changes in the expression pattern of K14, K1 and K6.

Ras *activation cooperates with* Klf4 *deletion to induce in situ carcinomas and SCC in the tongue*

We have shown previously that K-*ras*^{G12D} produces tumoral oral lesions and marked tongue hyperplasia when its expression is directed by the same Cre driver line, K14-CreER^{tam}, than we used in the current study (24). Interestingly, in these lesions cell proliferation was coupled to cell differentiation, resulting only in well-differentiated hyperplastic benign lesions (24). In this regard, to confirm Klf4 expression in the oral lesions from the K14-CreER^{tam}/LSL-K-*ras*$^{G12D/+}$ mice, we performed immunohistochemistry and found expression of Klf4 (Figure 2H and I). Thus, to analyze whether activation of K-*Ras* can cooperate with *Klf4* dysfunction and the consequent decreased cell differentiation in oral carcinogenesis, we bred K14-CreER^{tam}/*Klf4*$^{flox/flox}$ mice with LSL-K-*ras*$^{G12D/+}$ mice in order to express the mutated form of *ras* specifically in cells lacking Klf4.

Of note, these novel compound mice (K14-CreER^{tam}/LSL-K-*ras*$^{G12D/+}$/*Klf4*$^{flox/flox}$) exhibited a clear oral phenotype after only few days of tamoxifen administration (survival time, 23.3 ± 0.9 days after tamoxifen induction) as well as some mild skin lesions (Figure 3). All of the 19 compound mice developed oral papillomas and some of them showed skin lesions in the ventral neck as well as papillomas and crusted lesions in the skin of the fore- and hindlimbs (Figure 3A and data not shown). At necropsy, the tongue looked abnormal with verrucous lesions (Figure 3A). Additionally, 21% of the compound mice developed carcinomas (4 out of 19 mice) (Figure 3D and E).

The tongues from K14-CreER^{tam}/LSL-K-*ras*$^{G12D/+}$/*Klf4*$^{flox/flox}$ mice were affected in all cases, displaying a wide range of pathological alterations between animals as well as within regions within each tongue area. The histopathological changes ranged from mild to severe dysplasia concomitant with *in situ* carcinomas in different areas of the epithelium (Supplementary Figure 3A–D, available at *Carcinogenesis* Online). The tumors were characterized as well differentiated squamous cell carcinomas, verrucous carcinomas-like and presented keratin pearls, dyskeratosis near to the basal stratum and moderate cell atypia (Figure 3D and E and Supplementary Figure 3E and F, available at *Carcinogenesis* Online). Some of them showed progression into invasive carcinomas infiltrating the surrounding (adjacent) muscle fibers (Supplementary Figures 3F and 4A, available at *Carcinogenesis* Online). The compound mice also developed squamous cell papillomas within the oral mucosa. These papillomas presented dysplastic changes, cellular atypia in the basal stratum and mitotic figures in the upper stratum (Supplementary Figure 3G and H, available at *Carcinogenesis* Online).

Cell cycle-related genes regulated by Klf4 *contribute to tongue carcinoma development*

As expected, the carcinomas showed an elevated proliferative capacity, as evidenced by PCNA and cyclin D1 immunostaining (Figure 4A–D). Klf4 has a growth inhibitory role by regulating expression of key cell cycle genes, including activation of *CDKN1A* (p21) and inhibition of cyclin D1 expression (29,30). To understand how *Klf4* absence affects cell cycle progression in the described oral lesions in the K14-CreER^{tam}/LSL-K-*ras*$^{G12D/+}$/Klf4$^{flox/flox}$ mice, we measured p21 and cyclin D1 levels of expression. We did not observe expression of p21 in lysates from oral papillomas and carcinomas (Figure 4E) as expected for the antiproliferative role of Klf4 described above. Analysis of cyclin D1 levels by western blot revealed a clear expression in carcinomas and papillomas with a tendency toward higher levels in carcinomas (Figure 4E). Furthermore, expression analysis of cyclin-dependent kinase p27 (*KIP1*), other cell cycle-related gene positively regulated by KLF4 (31), showed decreased levels in both lesions with a more pronounced effect in the carcinomas (Figure 4E). We next analyzed the status of p53 in our animal system. p53 staining was negative in dysplasias and tumors from the compound mice as well as in the KO tongues, suggesting that alterations leading to the accumulation of this gene product may not be part of the mechanisms of carcinogenesis in this animal model (Supplementary Figure 4B, available at *Carcinogenesis* Online). Thus, the ablation of *Klf4* correlates with the absence of p21 and a decrease in p27 expression together with augmented expression of cyclin D1 in papillomas and carcinomas. These results suggest that *Klf4* acts as a tumor suppressor gene upon K-*Ras* activation in tongue epithelium.

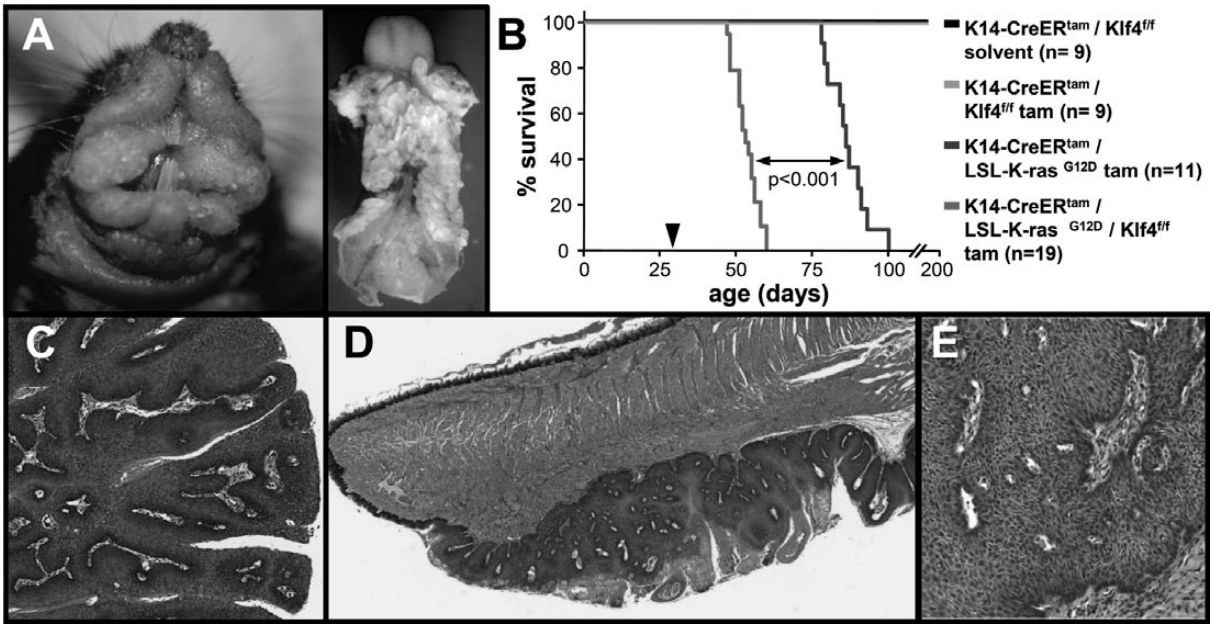

Fig. 3. Tongue transformation upon *Klf4* deletion and Ras activation. (**A**) K14-CreER^{tam}/LSL-K-*ras*$^{G12D/+}$/*Klf4*$^{flox/flox}$ mice developed squamous papillomas in the oral mucosa and tongue carcinomas upon tamoxifen induction. Gross appearance of a carcinoma of the tongue from a compound mouse after tamoxifen treatment. (A, right side) The verrucous lesions of the ventral area of the tongue correspond to the carcinoma. (**B**) Kaplan–Meier survival curve of the indicated mice lines treated (tam) or not (solvent) with tamoxifen. The compound mice (K14-CreER^{tam}/LSL-K-*ras*$^{G12D/+}$/*Klf4*$^{flox/flox}$) resulted in a statistically significant decreased survival rate when compared with mice expressing an activated ras allele alone (K14-CreER^{tam}/LSL-K-*ras*$^{G12D/+}$ mice), $P \leq 0.001$. The K14-CreER^{tam}/LSL-K-*ras*$^{G12D/+}$/*Klf4*$^{flox/flox}$ mice appears healthy after 200 days of evolution. K14-CreER^{tam}/*Klf4*$^{flox/flox}$ animals treated with solvent alone did not develop any lesions or exhibit any differences in survival rate. The arrowhead indicates the initiation of the tamoxifen induction. Log rank test, $P \leq 0.001$. (**C**) Oral squamous papilloma from a K14-CreER^{tam}/LSL-K-*ras*$^{G12D/+}$/*Klf4*$^{flox/flox}$ mice. The hyperplastic epithelium is evident even at this low magnification. (**D**) Histopathology of a tongue verrucous carcinoma from a compound mice. The carcinoma is located in the ventral tongue. (**E**) Depicts a detail of an area of the carcinoma from D showing the exophytic and infiltrative growth of this carcinoma. Scanscope original magnifications: ×1.5, ×4, ×10.

Discussion

The histology of the adult normal oral mucosa is maintained by a strict balance between cellular proliferation and differentiation, with cells proliferating only in the basal layer and progressively differentiating to form the stratified layers that characterize the squamous epithelium of the oral mucosa. We show here that specific inactivation of Klf4 in the tongue results in hyperplasia and dysplastic lesions by 6 months of age. We monitored proliferation and differentiation in these Klf4 KO lesions and showed that they present increased basal cell proliferations that is not restricted to the basal layer, and marked changes in the pattern of expression of basal cytokeratin 14, which is expressed throughout the epithelium. In this regard, K14 was expressed poorly in the basal layer but strongly in the upper layers of the dysplastic lesions, with an inverted distribution when compared with normal epithelium, resembling the changes described in chemically induced skin papillomas and tongue carcinomas (27,32). Furthermore, K6 was poorly expressed in the KO tongue and a suprabasal cytokeratin, as K1, was absent from the same samples. Thus, the homeostasis in the tongue epithelium seems to be disrupted upon *Klf4* deletion. The dysplastic lesions reported may result from abnormalities in the switch from proliferation to differentiation in which KLF4 is implicated. These dysplastic changes upon loss of KLF4 may represent a relevant role of this transcription factor in the differentiation process of the tongue epithelium, and its loss appear to trigger a process of unscheduled cell proliferation in epithelial cells. Indeed, the analysis of dual immunostaining for PCNA and Klf4 showed that areas of increased PCNA staining in suprabasal cells coincide with areas of no expression for Klf4.

However, in other system, it has been reported that loss of Klf4 in the tongue results in hypertrophy when targeted non-specifically the tongue epithelium with an ED-L2 promoter (33). Different transgenic systems are not always entirely consistent, and often these discrepancies are based in the cellular compartment that is targeted by the driver mouse line used. The K14-CreER^{TAM} system causes the inactivation of Klf4 in a cellular compartment that includes the oral epithelial stem cells (24). This system allowed us target a relevant compartment of cells to study the role of Klf4 in the homeostasis of the tongue.

In this regard, the increased cell proliferation and the abnormal expression of cytokeratins reflect a disruption in the normal balance between cell proliferation and differentiation and might therefore pave the way for malignant conversion of the squamous epithelium of the tongue.

Indeed, when a mutated K-*ras* gene was conditionally expressed concomitantly with *Klf4* deletion it induced *in situ* carcinomas and carcinomas instead of causing only oral papillomas and tongue hyperplasia. Here, we demonstrate that the combined inactivation of KLF4 and the activation of Ras cooperate to cause oral carcinogenesis resulting in the development of an array of premalignant and malignant lesions in the tongue. These results are consistent with the idea that KLF4 plays critical roles in differentiation and maintenance of cell cycle checkpoint functions and it is logical to assume that KLF4 would function as a tumor suppressor molecule. In this regard, numerous studies have reported loss of KLF4 expression in human tumors, including colorectal, stomach, esophageal and skin (14,21,29,34). Prior animal models, including *Klf4*-KO mice, have shown defects in skin differentiation (18) as well as a reduced number of secretory goblet cells in the colon (25), indicating that KLF4 functions as a proliferation–differentiation switch. In these models is accepted that loss of KLF4 mediate terminal differentiation. Indeed, defects in this switch are often associated with cancer (21). However, ectopic expression of human KLF4 in basal keratinocytes of the skin was reported to induce squamous epithelial dysplasia in a transgenic mouse model (35). In this regard, several reports derived from genetic screens identify KLF4 as a putative oncogene rather than a tumor suppressor (36,37). Therefore, accumulating evidence suggests that the role of KLF4 may depend on the genetic context and its interaction with tissue-specific proliferation/differentiation

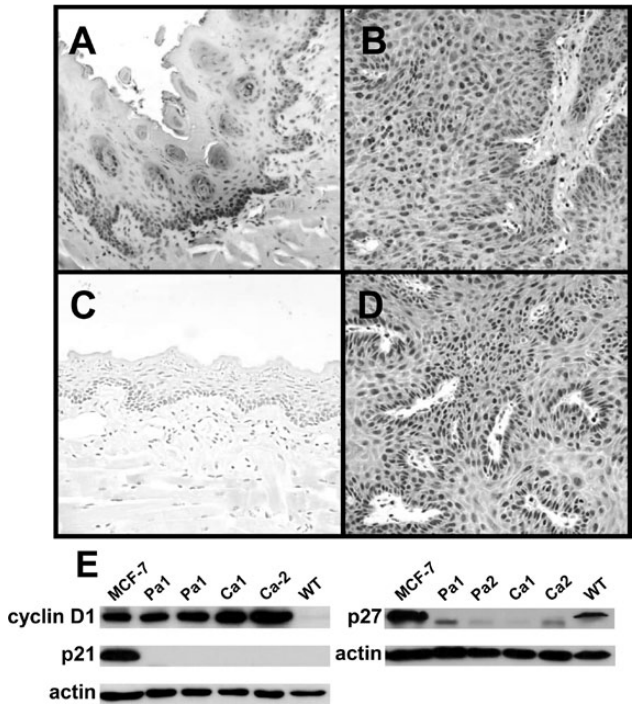

Fig. 4. Immunohistochemical expression of PCNA and cyclin D1 from KO tongues for Klf4. (**A**) PCNA staining in a dysplastic area showing positive reaction in the basal layer and in some areas of the upper layer. (**B**) The carcinoma shows increased mitotic activity reflected by a higher number of PCNA-positive cells across the epithelium. (**C**) No evident staining for cyclin D1 is observed in the wild-type tongue. (**D**) Cyclin D1-positive cells appear randomly distributed at all levels of the carcinoma parenchyma. Original magnifications: ×10, ×40. (**E**) Western blot analysis of p21, cyclin D1, p27 in oral lesions from the compound mice. Samples of total cell lysates from papillomas (Pa) and carcinomas (Ca) from K14-CreER^{tam}/LSL-K-*ras*$^{G12D/+}$/*Klf4*$^{flox/flox}$ mice, respectively, were used. Oral mucosa from wild-type mice were used as normal control and MCF-7 cells were used as positive control for p21. Both Pa and Ca do not show expression of p21 but they show cyclin D1 expression. Both Pa and Ca show decreased levels of p27. Actin was used as loading control.

pathways, including p53/p21 signaling. Our results confirm Klf4 as a critical molecule in the proliferation–differentiation equilibrium as proposed before in other organs (33,38) and support its role as a tumor suppressor gene for the tongue epithelium. Since the role of *Klf4* as a tumor suppressor gene may critically depend on other transcription factors and KLF4 activates the promoter of *p21*$^{WAF1/Cip1}$ gene in a p53-dependent manner (39,40), we have analyzed the status of p53 in our mouse model. The lack of immunohistochemical-labeled cells in preneoplastic and neoplastic lesions of our model can be associated with the lack of p53 accumulation. This suggests that mutations affecting the activity or stabilization of this tumor suppressor gene product may not be present in our mouse system. However, we cannot assure that p53 function may be altered, for instance, by epigenetic events related to enhancing its degradation or its transcriptional activity. In this scenario, we speculate that the absence of Klf4 is relevant determining the lack of p21 expression by a mechanism independent of p53 accumulation in our mice. Further studies will be necessary to assure this possibility.

Besides that, the potential role of terminal differentiation of keratinocytes in skin and mucosal epithelia has been recently highlighted as a key barrier for cancer initiation/progression in skin cancer and HNSCC (5,41). As mentioned before KLF4 is highly expressed in differentiated, postmitotic epithelial cells of different organs (16,17). Ectopic expression of KLF4 results in cell cycle arrest (16,22), and its transcriptional targets, including *CDKN1A*, are involved in differentiation and cell cycle inhibition (22). In this regard, p21 seems to have an important role in determining the outcome of KLF4 signaling. Indeed, we did not observe expression of p21 in carcinomas and oral papillomas from K14-CreER^{tam}/LSL-K-*ras*$^{G12D/+}$/*Klf4*$^{flox/flox}$ animals. In addition, we found increased expression of cyclin D1, which is negatively regulated by KLF4, supporting the idea that the described oral lesions in the compound mice are triggered by the absence of KLF4 concomitant to K-ras activation. In this regard, we cannot assure that the increase in cyclin D1 expression is only a result of KLF4 ablation. Cyclin D1 constitutes a primary downstream effector of Ras and exerts various promitogenic functions. However, it has been suggested that the activation of the Ras–cyclin D1 pathway might represent another means of overcoming KLF4's antiproliferative effect (36).

In addition, we analyzed the expression of another cell cycle negative regulator such as p27. The expression levels of p27 were decreased in the oral papillomas and carcinomas studied, with a more pronounced effect in the carcinomas. In this regard, the cyclin-dependent kinase p27 is positively regulated by KLF4. Recently it has been shown by chromatin immunoprecipitation analysis physical interaction between KLF4 protein and a specific motif in *p27*^{Kip1} promoter (42) in human cervical cancer where KLF4 may also function as a tumor suppressor by inhibiting cell growth and tumor formation.

Either by the lack of expression of p21 or the reduction of p27 levels we can explain the abnormal regulation in the cell cycle progression and the consequent malignant conversion upon K-Ras activation in the oral phenotype of the K14-CreER^{tam}/LSL-K-*ras*$^{G12D/+}$/*Klf4*$^{flox/flox}$ mice.

These results, together with the effect shown by the knock down of KLF4 in the tongue, point this transcription factor as a tumor suppressor gene for the squamous epithelium of the tongue, and we present a system where K-Ras cooperates with a differentiation-specific tumor suppressor gene in a two-hit fashion way to transform the oral mucosae. In particular, we found that KLF4 is relevant to maintain tongue epithelium homeostasis and when its deletion is targeted specifically to the epithelium of the tongue in addition to K-Ras activation can trigger a series of events that are sufficient for full oral carcinogenesis.

Supplementary material

Supplementary Figures 1–4 (reference 43 is cited in Supplementary Figure 4) can be found at http://carcin.oxfordjournals.org/

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