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Oral-specific ablation of *Klf4* disrupts epithelial terminal differentiation and increases premalignant lesions and carcinomas upon chemical carcinogenesis

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BACKGROUND: Squamous cell carcinoma (SSC) of the head and neck is the sixth most common cancer and is rarely diagnosed in early stages. The transcription factor Krüppel-like factor 4 (*Klf4*) suppresses cell proliferation and promotes differentiation. Inducible mice carrying an oral-specific ablation of *Klf4* (K14-CreER^{tam}/Klf4$^{flox/flox}$) develop mild dysplastic lesions and abnormal differentiation in the tongue. Aiming to analyze whether *Klf4* cooperate in oral chemical carcinogenesis,we applied 4-nitroquinoline 1-oxide (4NQO), a tobacco surrogate, to this conditional *Klf4* knockout mice.

METHODS: K14-CreER^{tam}/Klf4$^{flox/flox}$ and control mice were treated with 4NQO for 16 weeks and monitored until week 30. Histopathological samples were used for diagnostic purposes and immunofluorescence detection of epithelial differentiation markers.

RESULTS: 4NQO-treated K14-CreER^{tam}/Klf4$^{flox/flox}$ mice (*Klf4* KO 4NQO) showed a significant weight loss and developed more severe dysplastic lesions than control mice with 4NQO ($P < 0.005$). The *Klf4* KO 4NQO showed a tendency to higher incidence of oral SCC and a marked keratinization pattern in dysplasias, *in situ* carcinomas and SCC. Also, tongues derived from *Klf4* KO 4NQO mice exhibited reduced terminal differentiation as judged by cytokeratin 1 staining when compared with 4NQO-treated controls.

CONCLUSIONS: *Klf4* ablation results in more severe dysplastic lesions in oral mucosa, with a tendency to higher incidence of SCC, after chemical carcinogenesis. We show here, in a context similar to the human carcinogenesis, that absence of *Klf4* accelerates carcinogenesis and correlates with the absence of cytokeratin 1 expression. These results suggest a potential role for KLF4 as a tumor suppressor gene for the tongue epithelium.

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Introduction

Squamous cell carcinoma is the most common histologic type of head and neck cancer, and it is the sixth most common cancer in the world (1, 2). Squamous cell carcinoma of head and neck (HNSCC) is rarely diagnosed in the early stages, and it remains a significant cause of morbidity and mortality worldwide (1–3). Like most epithelial neoplasia, HNSCC arises from the accumulation of genetic and epigenetic alterations in tumor suppressor genes and oncogenes (4). The development of oral carcinoma is well-defined at histopathological level, including changes such as dysplasia, *in situ* carcinoma, malignant transformation into squamous cell carcinoma (SCC) and cancer progression. In spite of the advances in our understanding of the pathogenesis of HNSCC tumorigenesis, only 40–50% of patients with HNSCC will survive >5 years (3). This fact is attributable, at least in part, to our limited knowledge of the molecular pathways that promote HNSCC pathogenesis and the restricted number of targets available for developing new therapies that interfere with the progression of this type of cancer. In particular, the early events in HNSCC carcinogenesis have been less studied and still poorly understood. In this regard, we have recently characterized the development of oral premalignant lesions in the tongue after conditional ablation of the transcription factor Krüppel-like factor 4 (5). Krüppel-like factor 4 (KLF4, also called gut-enriched KLF) is highly expressed in terminally differentiated post-mitotic epithelial cells in organs such as skin and gastrointestinal tract (6, 7). This transcription factor suppresses cell proliferation and promotes differentiation and thus helps to maintain homeostasis in epithelial cells (7, 8). We have found a clear expression of

Klf4 in upper layers of the murine tongue epithelium, and we have described tongue epithelial dysplastic changes upon loss of *Klf4* (5). These alterations reveal an important role of this transcription factor in the differentiation process of the tongue epithelium. Accordingly, Klf4 decrease will trigger a process of unscheduled cell proliferation in oral epithelial cells as shown by neoplastic transformation of dysplastic lesions into oral carcinomas upon K-*ras* activation (5).

However, dysplastic lesions can progress to malignant ones even in absence of K-*ras* activation and chemical carcinogens can introduce multiple mutagenic hits and recapitulate the HNSCC heterogeneity in mice. To extend our understanding of the role of Klf4 in oral carcinogenesis progression, we assessed the effect of Klf4 dysfunction on the oral mucosa subjected to chemical carcinogenesis with a tobacco surrogate such as 4-nitroquinoline 1-oxide (4NQO). This well-known carcinogen has been shown in previous studies to cause oral-specific carcinogenesis in mice or rats (9–11) altering several signaling pathways controlling cell growth and survival (9, 10, 12). In addition, it produces similar histological as well as molecular changes as seen in human oral carcinogenesis (9, 12). The sequential stages of carcinogenesis such as hyperplasia, dysplasia, *in situ* carcinoma, and SCC are induced by 4NQO (9). We show here that tongues lacking Klf4 develop more severe dysplastic lesions when subjected to chemical carcinogenesis than the control group. In addition, *Klf4* conditional knockout mice under 4NQO treatment exhibit a tendency to a higher incidence of oral SCC and a different pattern of keratinization when compared with wild-type mice subjected to the same treatment.

Materials and methods

Transgenic mice

The K14-CreER^{tam} and *Klf4*$^{flox/flox}$ mouse strains used in the work have been described before (5, 13). Briefly, their genetic backgrounds were as follows: K14-CreER^{tam}, FVB/N; *Klf4*$^{flox/flox}$ C57BL/6. K14-CreER^{tam} mice were crossed with Klf4$^{flox/flox}$ mice to generate K14-CreER^{tam}/Klf4$^{flox/+}$ line. These mice were further crossed with *Klf4* $^{flox/flox}$ to generate K14-CreER^{tam}/Klf4$^{flox/flox}$ line (hereafter: KO). K14-CreER^{tam} mice were used as hemizygotes in all the established lines. All mice studied were on a mixed FVB/N–C57BL/6 genetic background. The inducible K14-CreER^{tam} oral-specific system was activated by oral administration of tamoxifen to 1-month-old animals, 1 mg per mouse per day for five consecutive days (13). All mice from the control groups also received tamoxifen as control (Fig. 1A).

Genotyping was performed on tail biopsies by PCR using specific primers (5, 13). 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 local 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/

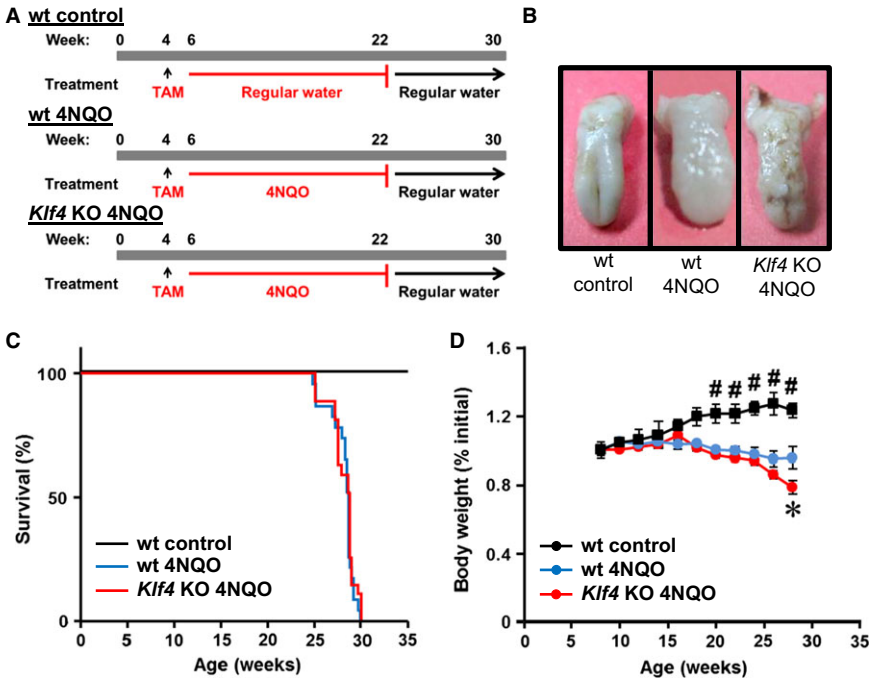

Figure 1 (A) Scheme of experimental groups and timeline used in the study. (B) Gross appearance of tongues from wild-type control group, wt 4NQO group, *Klf4* KO 4NQO group from animals sacrificed at 30 weeks. (C) Kaplan–Meier survival curves for the experimental groups (wt control $n = 9$, wt 4NQO $n = 27$, *Klf4* KO 4NQO $n = 23$). Statistically significant decreased survival rate was obtained for 4NQO-treated groups compared with the wt control. Mice from the *Klf4* KO 4NQO group did not show differences in survival rate compared with wt 4NQO group. *$P \leq 0.0001$ Long Rank test. (D) Time course of body weight of wt control group and both experimental groups along different treatment stages. Two-way ANOVA interaction $P < 0.05$, followed by Newman–Keuls *post hoc* test: #$P < 0.005$ vs. wt control group, and *$P < 0.05$ KO 4NQO vs. wt 4NQO group. Each point represents mean ± SEM.

12). All efforts were made to minimize the number of animals used and their suffering.

Protocol 4NQO

4NQO obtained from Sigma-Aldrich was dissolved in propylene glycol (Sigma-Aldrich, St. Louis, MO, USA) as stock solution (4 mg/ml), stored at 4°C, and diluted in the drinking water to a final concentration of 50 μg/ml on the day of use. Drinking water was changed weekly. Mice were divided in three groups as follows: wild-type control group (wt control), wild-type 4NQO group (wt 4NQO), and *Klf4* KO 4NQO group (*Klf4* KO 4NQO). The experimental groups received 4NQO in the drinking water for 16 weeks starting at 6 weeks old, after which all animal cages were reverted to regular water, and mice were monitored until week 30. This schedule was selected based on preliminary studies and previously reported schedules for 4NQO oral carcinogenesis (14). All animals underwent weekly full oral cavity examination under inhalatory anesthesia, and any observed pathologic changes were documented. Animals were euthanized on week 30 for tissue collection and further analysis.

Tissue preparation, histology, and immunofluorescence

All tissues were dissected, fixed overnight in buffered 4% paraformaldehyde at room temperature, dehydrated, and embedded in paraffin. H&E-stained sections were used for diagnostic purposes.

Immunofluorescence was performed as previously described (5) on cryostat sections from snap-frozen OCT-embedded tongue samples. Antibodies for detection of cytokeratin 1 (MK1, 1:500) and 14 (MK14, 1:400) were from Covance, and the antibody for Klf4 was kindly provided by Dr. J. Segre (National Human Genome Research Institute, NIH, Bethesda, MD, USA) (15). Alexa 488 (Invitrogen, Waltham, MA, USA)-conjugated secondary antibody was used for detection. The slides were counterstained with 4′, 6-diamidino-2-phenyllindole (DAPI) and mounted with Vectashield.

The pictures were taken with a Nikon TE2000-S microscope (Tokyo, Japan), and images were acquired with a digital camera Nikon FDX-35. High-resolution immunofluorescence images were obtained on an Olympus Fluoview FV1000 (Center Valley, PA, USA) confocal laser scanning microscope.

Data analysis and 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 (Systat Software Inc., San Jose, CA, USA). The statistical analysis of body weight was performed by two-way ANOVA.

H&E-stained histological samples of transversal sections of tongues from all animals groups were blindly inspected by a trained pathologist with diagnostic purpose. To quantify the maximal dysplasia level in each animal, the pathologist performed a detailed analysis of the tongue epithelium to classified three degrees of dysplasia. Degrees of dysplasia: mild (the architectural disturbances are limited to the lower third of the epithelium with minimal cytological atypia), moderate (the architectural disturbances extend into the middle third of the epithelium), and severe (the architectural disturbances are recognized in more than two-thirds of the epithelium with associated cytological atypia). As different dysplasia levels co-exist in a given animal, tongues were classified according to their severest degree of dysplasia (maximal dysplasia level). Statistical analysis of proportions in multiple groups was analyzed using chi-squared test of independence.

Results

Klf4 deletion enhances the chemical carcinogenesis of the tongue

To analyze whether *Klf4* dysfunction can cooperate with chemical carcinogenesis progression, we applied a protocol of 4NQO carcinogen to K14-CreER^{tam}/*Klf4* $^{flox/flox}$ mice (5). Mice were divided in three groups (Fig. 1A). Wild-type mice received 4NQO in the drinking water for 16 weeks (wt 4NQO group) as well as the K14-CreER^{tam}/*Klf4*$^{flox/flox}$ mice (*Klf4* KO 4NQO group). Treatment started after tamoxifen induction and continues until week 22 when the 4NQO delivery was reverted to regular water and mice were monitored until week 30 as indicated in Fig. 1A. The third group, wild-type mice, was provided only with regular water along the whole experiment (wt control group). As expected, all mice of the wt control group survived the 30 weeks without developing lesions. Mice of both experimental groups presented visible oral lesions after 10 weeks of 4NQO treatment. At 12 weeks, animals of both experimental groups displayed lesions in the mucosa of the tongue. These lesions appeared as white-whitish patches or plaques located on the dorsal and ventral part of the tongue and on the lateral border of the tongue. These white patches resemble human oral leukoplakia. Of interest, approximately 50% of the *Klf4* KO mice under 4NQO treatment showed in addition some red spots or ulcerated lesions between white patches by 14 weeks; however, the mice of wt 4NQO group did not present that kind of lesions at the same time. Also, some animals of both experimental groups presented exophytic lesions with wartlike appearance or flat white patches in the oral mucosa. The number and size of tongue lesions continued to increase progressively even after switching to regular water without 4NQO in all the experimental mice. Indeed, all mice developed a wide range of oral lesions in the tongue by the end of the study (Fig. 1B).

As expected from previous studies (9, 14, 16), both 4NQO-treated groups displayed significantly shorter survival times and weight loss than solvent-treated group (Fig. 1C,D). The *Klf4* KO 4NQO group did not show differences in survival rate compared with control 4NQO group (Fig. 1C). However, *Klf4* KO mice under 4NQO treatment showed a significant higher weight loss compared with wt 4NQO animals (Fig. 1D). Of note, previously we have shown that *Klf4* KO mice did not present an overt phenotype along the whole experiment timeframe reported, and all animals appeared healthy without body weight changes by the end of the follow-up period (9 months) (5).

Importantly, histologic evaluation also revealed differences between *Klf4* KO mice under 4NQO treatment and animals from the group wt 4NQO. Similar to human oral

carcinogenesis, all mice from both experimental groups presented clear precancerous lesions—potentially malignant disorders—(different grades of dysplasia classified into mild, moderate, and severe) and *in situ* carcinomas distributed along the tongue by the end of the 4NQO treatment. Mice from the wt 4NQO group develop a sequence of histopathological lesions ranged from hyperplasia to different grades of dysplasia, conversion to carcinoma *in situ*, and eventually, squamous cell carcinoma as previously characterized for this model (9, 11) (Fig. 2A,B and data not shown). However, detailed histologic analysis of hyperplasias, dysplasias, and *in situ* carcinomas from the tongues of *Klf4* KO 4NQO group revealed a marked keratinization pattern in contrast with the wt 4NQO group (Fig. 2C,D). Mice of both experimental groups developed squamous cell carcinomas of the tongue. Notably, 22% of the *Klf4* KO mice under carcinogen treatment developed squamous cell carcinomas (5 of 23 mice) vs. 7% of the wt 4NQO group (2 of 27 mice). The SCCs exhibited by the *Klf4* KO 4NQO mice were invasive and with a marked keratinization (Fig. 3). Furthermore, quantitative differences were observed regarding the severity of premalignant lesions in mutants treated with the carcinogen.

Quantitatively analysis revealed that more tongues from *Klf4* KO 4NQO mice were diagnosed with severe dysplasia than from the other two groups together. The KO tongues for Klf4 under 4NQO treatment developed severe dysplasia in more than 50% of the cases, and the remaining dysplastic lesions were mild (Fig. 4). In clear contrast, the tongues from *Klf4* KO mice receiving vehicle alone showed 75% of mild dysplasia and 25% of moderate dysplasia (Fig. 4). Finally, wt 4NQO group developed all different grades

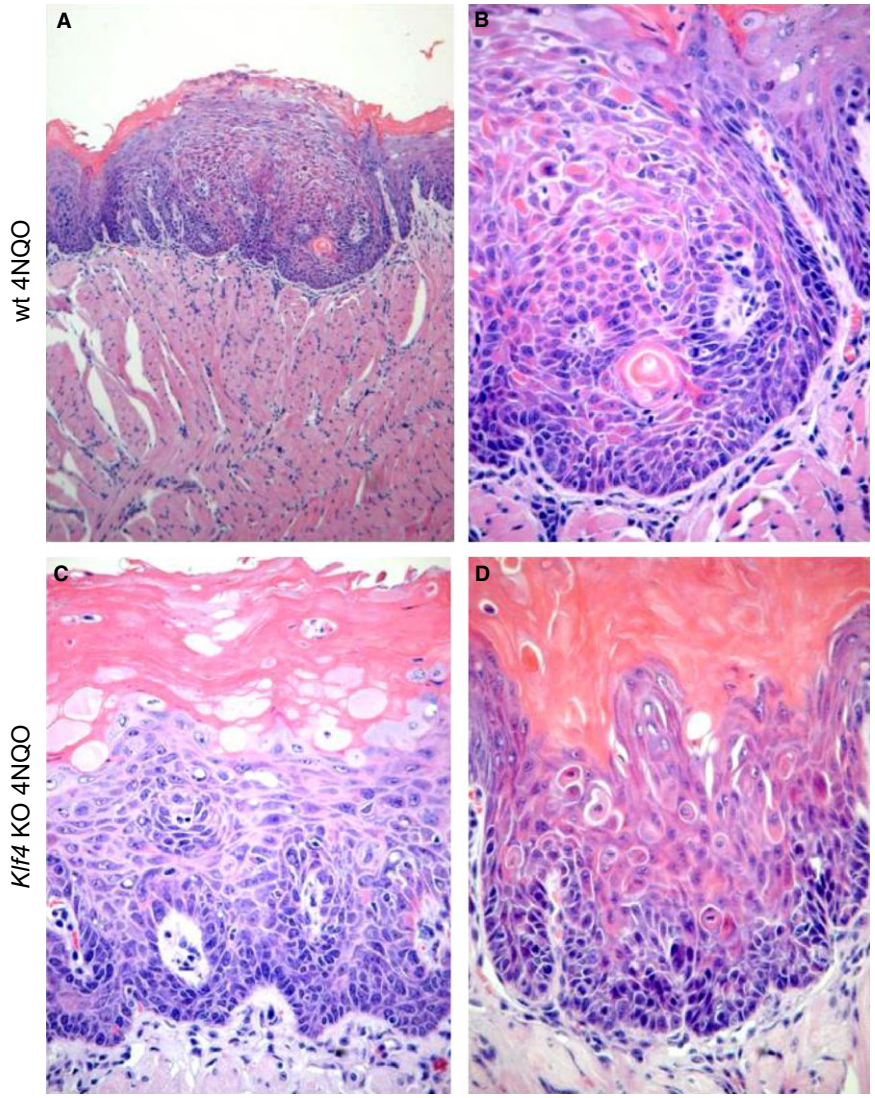

Figure 2 Representative histologic pictures of tongues from both experimental groups of mice. (A) Tongue mucosa from a mouse of the wt 4NQO group. *In situ* carcinoma in the tongue mucosa with parakeratotic layer on the surface and marked acanthosis. (B) Detail of the *in situ* carcinoma from A, notice the drop-shaped rete ridges, showing marked cellular atypia with dyskeratosis and keratin pearls within rete ridges. (C) Tongue mucosa from a mouse of the *Klf4* KO 4NQO group. Severe dysplasia with marked keratinization on the surface. (D) Tongue mucosa from a *Klf4* KO mouse under 4NQO treatment. *In situ* carcinoma with marked keratinization on the surface, note the irregular epithelial stratification with dyskeratosis and cellular and nuclear pleomorphism with nuclear hyperchromasia. Original magnifications: ×40.

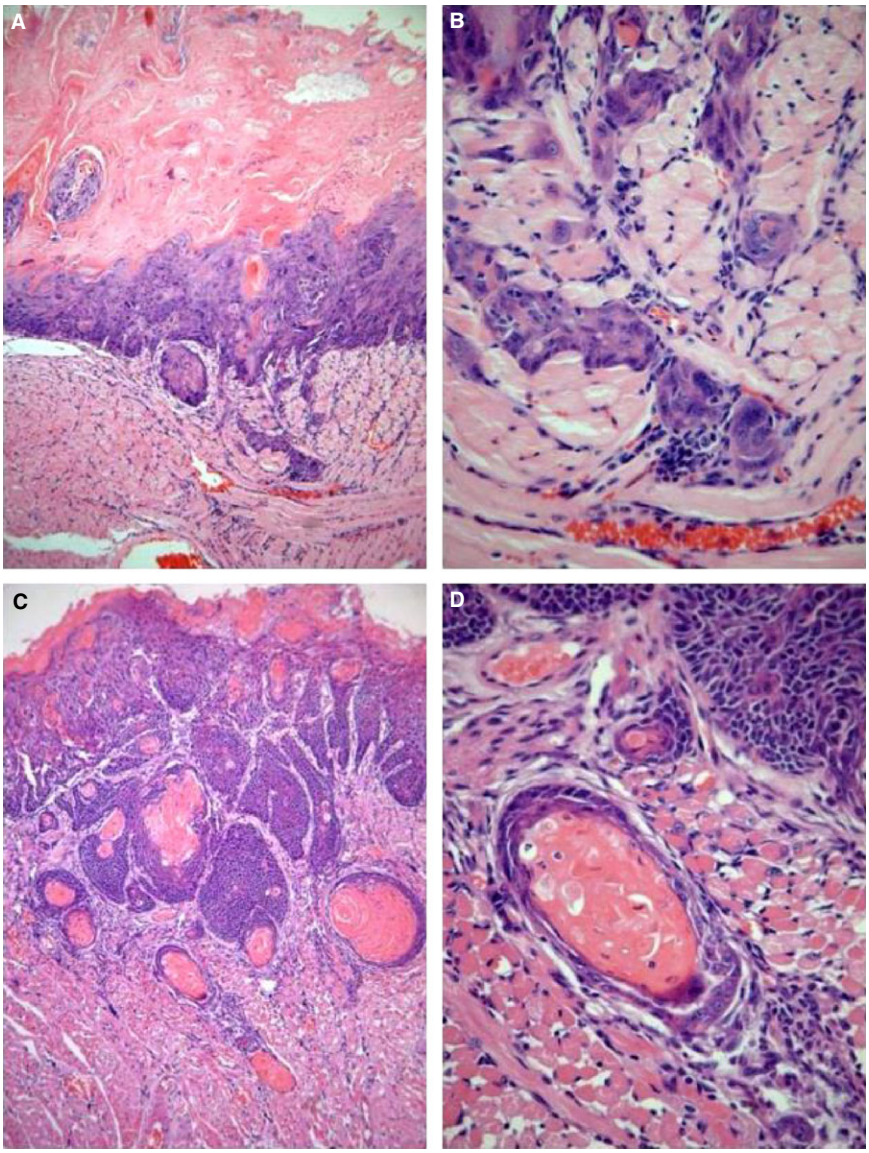

Figure 3 Squamous cell carcinomas in the *Klf4* KO mice under 4NQO treatment. (A) Microinvasive SCC of the tongue, note the marked surface keratinization. (B) Tumor infiltration between the muscle fibers of the tongue. (C) Invasive SCC of the tongue, note the marked keratinization with formation of keratin pearls, and tumor nests invading muscle tissue. (D) Tumor nests with keratinization between muscle fibers. Original magnifications: ×10, ×40.

of dysplasia almost equally distributed (Fig. 4). The differences described for the incidence of different grades of premalignant lesions in the tongues from the *Klf4* KO 4NQO group may help to explain why this group developed more SCC than the wt 4NQO animals. To further understand whether the increased incidence of severe dysplasia described in the epithelium of tongues from *Klf4* KO mice under 4NQO treatment was due to the loss of Klf4, we compared its expression in the tongues of animals from all groups. As we reported before, the wild-type tongues exhibit widespread expression of Klf4 in all cells in differentiating upper layers of the stratified epithelium, with labeling localized in cell nuclei (Fig. 5). Importantly, the normal pattern of Klf4 observed in water-treated wild-type mice was preserved in the epithelium of wild-type tongue under the treatment with 4NQO (Fig. 5). Contrastingly, *Klf4* KO tongues with 4NQO carcinogenesis exhibit a patchy Klf4 expression, in areas with no detectable expression of this transcription factor spanning from the basal to the cornified stratum (Fig. 5). This arrangement of knocked out Klf4 expression is in concordance with our previous publications and related to the expected recombination pattern produced by K14-CreER^{TAM} system as reported before (13). We confirmed that our transgenic mouse system leads to *Klf4* deletion in the tongues of *Klf4* KO mice under 4NQO treatment and that precancerous lesions—potentially malignant disorders—of both experimental groups differ in Klf4 expression.

Excision of Klf4 gene alters the pattern of expression of cytokeratin 1

We have next analyzed the expression pattern of cytokeratins 1 and 14, by immunofluorescence staining, with the purpose of studying the differentiation status of tongue

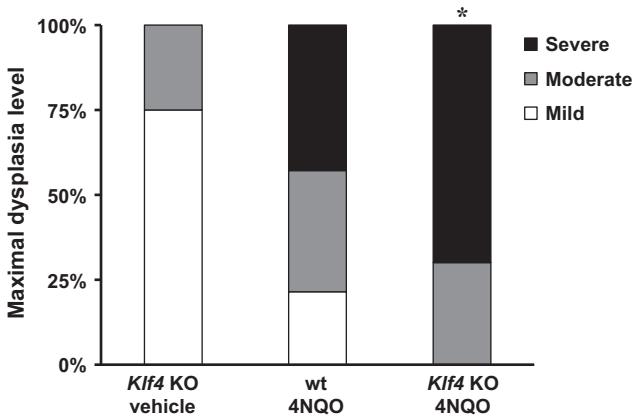

Figure 4 The maximal dysplasia level is depicted for each experimental group and the *Klf4* KO mice. Tongues were classified according to their severest degree of dysplasia. Data are presented as percentage of total cases per group. Chi-squared test of independence $\chi^2_4 = 14.9$, $^*P < 0.005$ vs. *Klf4* KO vehicle and wt 4NQO groups.

lesions of both experimental groups. Coinciding with previous reports, 4NQO treatment resulted in a marked increase in K1 expression level as well as a generalized spanning of the staining through the upper layers (12). In contrast, we found that tongues from the *Klf4* KO 4NQO group exhibited fewer K1-labeled cells when compared with tongues from the wt 4NQO group (Fig. 6). These results are in concordance with our previous report where tongues from *Klf4* KO mice did not exhibit K1-labeled cells in contrast with wild-type tissue (5). Then, we explored the pattern of expression of K14 that is typically expressed in the basal layer of normal squamous epithelium of the tongue (12, 13) as a marker for undifferentiated cells. K14 expression in the control tongues showed clear localized expression in all cells of the basal layer in contrast with the generalized K14 labeling observed in the tongues from the basal to the upper layers in both experimental groups (Fig. 6). Thus, the absence of increased K1 labeling found in the *Klf4* KO tongues under 4NQO treatment may reflect the modulation of the chemical carcinogenesis process upon *Klf4* deletion. Indeed, we have shown before that homeostasis of the tongue epithelium seems to be disrupted upon Klf4 deletion (5).

Discussion

We show here that the oral mucosa lacking *Klf4* transcription factor is more susceptible to chemical carcinogenesis, exhibiting increased incidence of severe dysplastic lesions as well as oral SCC. Besides that, a different pattern of keratinization and cytokeratin 1 expression was observed reinforcing the notion that the process of tumorigenesis was different between wild type and Klf4 KO mice under 4NQO treatment.

We have previously found that specific inactivation of Klf4 in the tongue produces hyperplastic and mild dysplastic lesions (5). Thus, the tongue carcinogenic process with 4NQO appears to be enhanced together with the specific genetic condition of *Klf4* ablation. In this regard, we found differences in severity of dysplasia levels in tongue from *Klf4* KO mice treated with 4NQO compared with wild-type mice also under the carcinogen treatment. Tongue chemical carcinogenesis upon 4NQO treatment is a well-characterized process (9, 11, 12). Although the experimental protocols used in different studies vary in relation to duration of carcinogen application, sacrifice time points, and mice strains, the histological changes produced by 4NQO are a common feature of the model (9, 10). The incidence of SCC reported in the present study is lower than the one reported for the same 4NQO protocol applied to others mouse strains such as CBA or C57 BL/6 (9, 10). In this

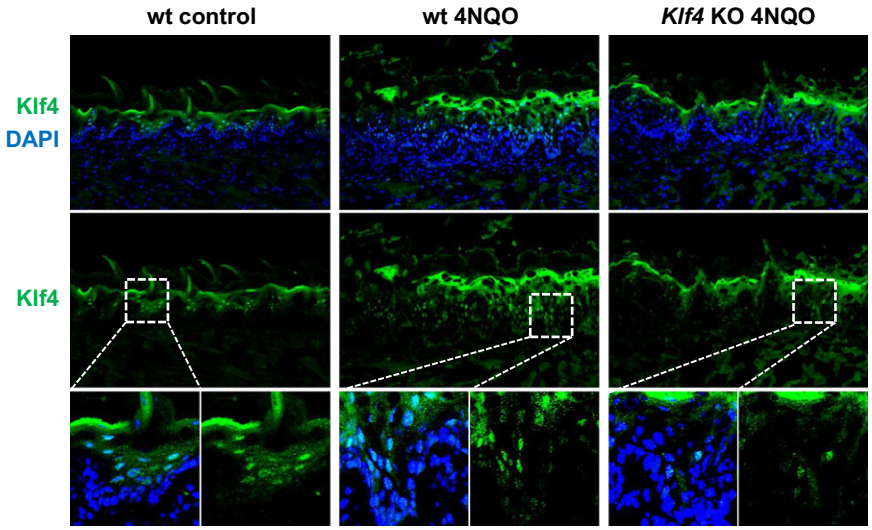

Figure 5 Immunofluorescence labeling of Klf4 of tongue from wild-type (wt control), wt 4NQO, and *Klf4* KO 4NQO mice. Sections were labeled with antibodies as indicated and color coded on each frame. All tissues were counterstained with 4′,6-diamidino-2-phenylindole (DAPI) for nuclear localization. wt control and wt 4NQO groups: the upper layers of the tongue epithelium presented nuclear staining for Klf4 (green fluorescence). The magnification, notice the nuclear staining for Klf4 in the upper stratum of the epithelium. *Klf4* KO 4NQO: No immunostaining with Klf4 is evident in this area of the *Klf4* KO tongue. Confocal images, original magnification, ×20, ×40.

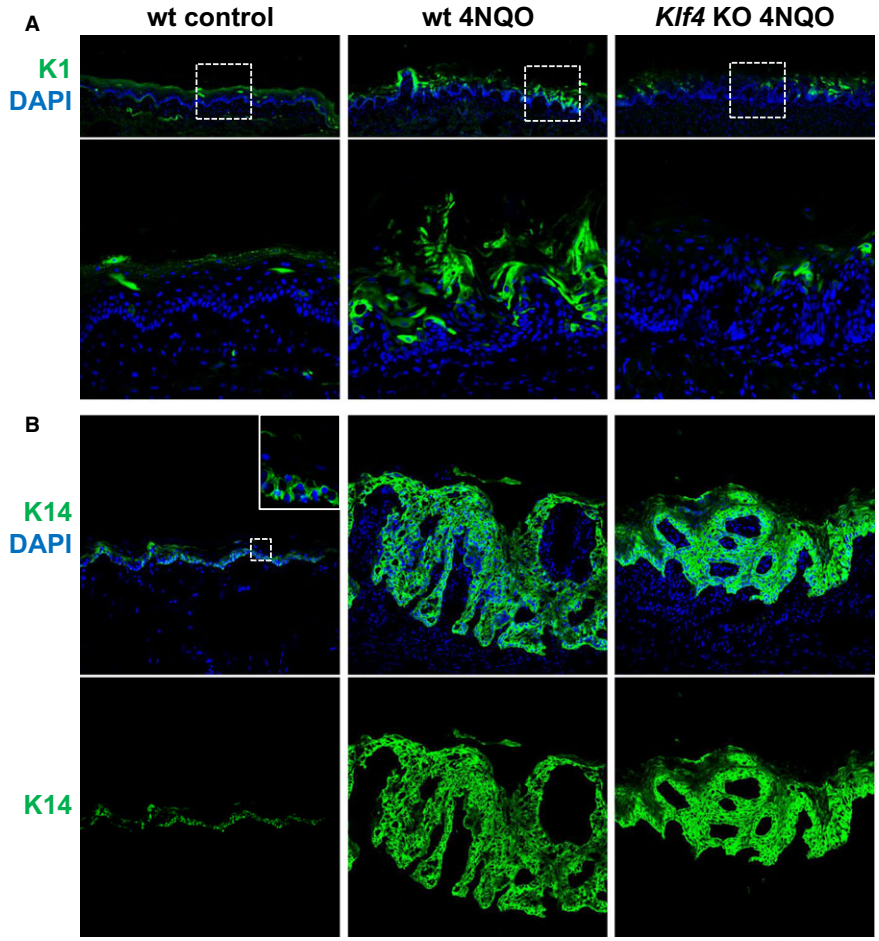

Figure 6 Expression of cytokeratins 1 and 14 in tongues from wild-type control group and from both experimental groups. Sections were labeled with antibodies as indicated and color coded on each frame. All tissues were counterstained with DAPI for nuclear localization. (A) Cytokeratin 1 expression. At lower magnification, the control tongue presented scattered K1 positive cells only in the upper layers of the epithelium (green); the inset indicate an area with few positive cells showed below at higher magnification (left images). The tongue from the wt 4NQO group exhibits areas of conspicuous expression of K1 throughout upper layers of the epithelium. Notice below the detail from one area with a generalized expression of K1 at higher magnification. The tongues from the *Klf4* KO 4NQO group exhibited evident less labeled cells compared with the wt 4NQO group. The *Klf4* KO 4NQO tongue showed presented a poor expression of K1 and in some areas did not exhibit evident labeled cells with K1. The marked inset is presented below at higher magnification showing the detail of few positive cells in the upper layers of the epithelium from a dysplastic area in contrast with the equivalent area from the wt 4NQO group. Confocal images, original magnification, ×10, ×40. (B) Cytokeratin 14 expression. The control tongue showed localized expression of K14 in all cells of the basal layer (green fluorescence), left bottom image. The upper image showed the merge of the K14 immunofluorescence counterstained with DAPI for nuclear localization. Both experimental groups exhibit a generalized K14 labeling in all tongues from the basal to the upper layers (green fluorescence). Confocal images, original magnification, ×40.

work, we used for all the experiments mice on a mixed FVB/N-C57BL/6 genetic background as published previously (5) where we have described the changes developed in the tongue mucosa upon specific *Klf4* deletion. The FVB/N mice strain has been shown to be resistant to the carcinogen effects of 4NQO (10, 17) and may explain the low incidence of SCC in both experimental groups.

We have previously shown that *Klf4* ablation disrupts homeostasis of the tongue epithelium. In the present report, we extended our results providing evidence that absence of Klf4 enhances the process of malignant transformation of the tongue induced in an environmental-defined model of chemical carcinogenesis.

Cytokeratins are epithelial-specific intermediate filament proteins which are expressed in a differentiation-dependent and tissue-specific manner. Alterations in cytokeratins expression have been reported in oral malignancies and are thought to contribute to malignant transformation of stratified epithelial cells (18–20). Upregulation of K1 and K14 has been previously observed in mouse tongue exposed to 4NQO (12). Thus, the increase in level and areas expressing K14 observed in *Klf4* KO mice is expected as a consequence of the carcinogenic process triggered by 4NQO, and it is in concordance with other reports (12) and our previous data (5). However, *Klf4* ablation prevents the upregulation of K1, in agreement with our previous report showing that the *Klf4* KO mice did not express higher levels of K1 (5). K1 is a suprabasal cytokeratin that reflects the differentiated status of the upper layer of the epithelium. In particular, the absence of K1 expression has been reported in human oral severe dysplasia as well as in poorly differentiated carcinomas (21). Therefore, we speculate that

the absence of K1 expression reflects in part the changes in epithelial differentiation and maturation due to Klf4 ablation, and this condition contributes to a faster malignant transformation of the oral epithelium of the tongue under 4NQO treatment. In this regard, it has been shown that KLF4 activates the promoter of keratin 4 in the esophagus epithelia (22). K4 is a marker of keratinocyte differentiation in the stratified epithelia of the human esophagus (23, 24). These studies demonstrate an important aspect of the role of KLF4 in controlling *in vivo* differentiation of specific epithelial functions. Furthermore, we have reported that when a mutated K-*ras* gene was conditionally expressed concomitantly with Klf4 deletion, it induced *in situ* carcinomas and carcinomas instead of causing only tongue hyperplasia and mild dysplasia (5).

Here, we combined a genetically defined animal system to ablate *Klf4* in the oral mucosa together with a protocol of chemical carcinogenesis with a tobacco surrogate. This animal model of oral chemical carcinogenesis with 4NQO produces progressive changes in the oral mucosa that finally turns into oral SCC. The molecular changes underlying the 4NQO carcinogenesis includes decreased expression of tumor suppressor genes, such as *p16* and *p53*, and increased expression of the epidermal growth factor receptor (11, 12, 14). Nowadays, it is well accepted that progressive changes occurring in oral tumoral lesions of the 4NQO mouse model reflect changes that occur in human HNSCC (9). Thus, we have in the present work a different environment to study the effect of *Klf4* ablation in the oral mucosa than the previous one with K-*ras* activation (5). In a context more similar to the human carcinogenesis, the absence of Klf4 accelerates the carcinogenesis and correlates with the absence of K1 expression.

We reported here significant changes in dysplasia levels even when *Klf4* ablation was restricted to a limited number of cells. Of interest, the K14-CreER^{TAM} system used to inactivate Klf4 has been characterized before by our group, and we found that the cellular compartment affected includes the oral epithelial stem cells (5, 13). Therefore, we have targeted a relevant compartment of cells to study the role of Klf4 in the homeostasis of the tongue affecting the balance between cell proliferation and differentiation during chemical carcinogenesis.

Taken together, the results described here point KLF4 as a tumor suppressor gene for the tongue epithelium and reinforce the notion that this transcription factor has an important role in the differentiation process of the squamous epithelium of the tongue. In particular, we show here that tongues lacking Klf4 develop more severe dysplastic lesions when subjected to chemical carcinogenesis and exhibit a tendency to a higher incidence of oral SCC.

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Conflict of interest statement

The authors declare that there are no conflict of interests.