Basal stem cells contribute to squamous cell carcinomas in the oral cavity

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The cells of origin of oral cavity squamous cell carcinoma (OCSCC) are unknown. We used a cell lineage tracing approach (adult K14-CreERTAM; ROSA26 mice transiently treated with tamoxifen) to identify and track normal epithelial stem cells (SCs) in mouse tongues by X-gal staining and to determine if these cells become neoplastically transformed by treatment with a carcinogen, 4-nitroquinoline 1-oxide (4-NQO). Here, we show that in normal tongue epithelia, X-gal(+)-cells formed thin columns throughout the entire epithelium 12 weeks after tamoxifen treatment, indicating that the basal layer contains long-lived SCs that produce progeny by asymmetric division to maintain homeostasis. Carcinogen treatment results in a ~10-fold reduction in the total number of X-gal(+) clonal cell populations and horizontal expansion of X-gal(+) clonal cell columns, a pattern consistent with symmetric division of some SCs. Finally, X-gal(+) SCs are present in papillomas and invasive OCSCCs, and these long-lived X-gal(+) SCs are the cells of origin of these tumors. Moreover, the resulting 4-NQO-induced tumors are multiclonal. These findings provide insights into the identity of the initiating cells of oral cancer.

Introduction

Oral cavity squamous cell carcinoma (OCSCC) is one of the most common human cancers in the world, with 25280 new cases diagnosed and 5470 deaths in the USA in 2011 (1). The two major etiological factors in OCSCC are tobacco and alcohol (2,3). The recurrence of this cancer and the development of metastases indicate that some cancer cells, possibly including cancer stem cells (SCs), are either inherently resistant or acquire resistance to cancer treatment (4,5). Cancer SCs have been identified as a potential contributor to head and neck malignancies (6–11). However, the cells of origin of OCSCC, i.e. the normal cells initially neoplastically transformed during carcinogenesis, are not known.

Both the tongue and the skin are composed of stratified, squamous epithelia (12,13). Studies on skin cancer in mice have shown that neoplastic transformation of different normal epidermal cell populations in the differentiation lineage hierarchy results in tumors with different degrees of malignancy (14–16) and that the transformation of epidermal SCs, not transient amplifying cells, results in SCC of the skin (17,18). SCs in normal mouse tongue epithelium have been identified in the basal layer, and in a manner similar to what occurs in the skin, the differentiating progeny of SCs move up to the surface of tongues and eventually are shed (13). Here, we address whether the epithelial basal layer cells serve as the SCs in the normal oral cavity, and additionally we determine the cells of origin of OCSCC.

OCSCCs induced in mice by the carcinogen 4-nitroquinoline 1-oxide (4-NQO) added to the drinking water demonstrate similarities to human oral tumors in terms of their morphological, histopathological and molecular characteristics (19–21). 4-NQO treatment causes the loss of p16, elevation of epidermal growth factor receptor protein levels in mouse tongue epithelia and the development of papillomas and OCSCCs in mouse tongues (20). Here, we use an inducible cell lineage tracing approach to characterize the location and function of epithelial SCs in the normal tongue and in oral cancer in mice induced by 4-NQO.

Materials and methods

Tamoxifen treatment and β-galactosidase assays for K14-CreERTAM; ROSA26 mice

K14-CreERTAM (Cat# 56822) double-positive transgenic mice and ROSA26 floxed STOP-LacZ double-positive transgenic mice (Cat# 003747), purchased from the Jackson Laboratory (Bar Harbor, ME), were bred to obtain K14-CreERTAM; ROSA26 mice. The K14-CreERTAM; ROSA26 mice received tamoxifen treatment (4 mg/mouse/day) by intraperitoneal injections on two consecutive days. At various time points (n = 2 per time point) after tamoxifen treatment, mouse ears and tongues were harvested for β-galactosidase activity assays (X-gal staining). The details are described in the Supplementary data, available at Carcinogenesis Online. The care and use of animals in this study were approved by the Institutional Animal Care and Use Committee (IACUC) of Weill Cornell Medical College.

Carcinogenesis in the oral cavity induced by the carcinogen 4-NQO, tissue dissection and immunostaining

Four weeks after tamoxifen injection, K14-CreERTAM; ROSA26 mice (~10 week old) were treated with vehicle as a negative control (n = 20) or 100 μg/ml 4-NQO (Sigma, St Louis, MO) (n = 40) for 10 weeks, as described previously (19,20), and mouse ears and tongues were harvested when visible tumors were developed after the termination of the 4-NQO treatment. Also, mouse ears and tongues (n = 3 per treatment and per time point) were harvested at various time points during the 4-NQO treatment. The tongues of mice were dissected immediately after cervical dislocation and gross lesions were identified and photographed. The histological diagnosis of squamous neoplasia was performed by a board certified pathologist (T.S.) on hematoxylin and eosin (H&E)-stained tissue samples that had been stained previously with X-gal (19,20). Paraform-embedded sections from X-gal-stained mouse tongues were stained with a Ki67 antibody and an E-cadherin antibody (Supplementary data, available at Carcinogenesis Online).

Terminal deoxynucleotidyl transferase dUTP nick-end labeling assays

Terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assays were performed on paraffin-embedded mouse tongue sections by using a TUNEL assay kit from Millipore, as described in Supplementary data, available at Carcinogenesis Online.

Statistical analyses

We performed statistical analyses by Student’s t-test or one-way analysis of variance and subsequently the Bonferroni test or the Tukey test for multiple comparisons. Differences with a P < 0.05 (two-tailed test) were considered statistically significant.

Results

LacZ expression after tamoxifen treatment marks cells in normal tongue epithelium

We exposed K14-CreERTAM; ROSA26 mice to a brief, 2 day tamoxifen treatment to mark permanently cells that express keratin 14 at the time of tamoxifen treatment and examined the X-gal(+) cells in tongues and in ears, which served as the positive control for X-gal staining (22), at different time points thereafter. SCs in normal tongue epithelium express keratin 14 (13), and this truncated keratin 14 promoter that drives the expression of CreERTAM is active only in the basal layer of normal tongue and skin stratified squamous epithelia (23). There is a floxed STOP cassette immediately upstream of the reporter gene lacZ in ROSA26 mice. Tamoxifen treatment transiently activates CreERTAM, resulting in excision of the floxed STOP cassette and permanent, continuous LacZ expression in the basal layer of mouse tongue and skin epithelia, detected as a blue signal by X-gal staining.

Abbreviations: 4-NQO, 4-nitroquinoline 1-oxide; H&E, hematoxylin and eosin; OCSCC, oral cavity squamous cell carcinoma; SC, stem cell; TUNEL, terminal deoxynucleotidyl transferase dUTP nick-end labeling.

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(Figure 1A and B). Tamoxifen treatment did not activate CreER<sup>TAM</sup> in all basal cells in K14-CreER<sup>TAM</sup>; ROSA26 tongues because we did not observe all epithelial basal cells marked with X-gal, as expected from other published work using this approach (24). In addition, higher tamoxifen concentrations resulted in a greater number of X-gal-stained cells in the basal layer (Supplementary Figure S1, available at Carcinogenesis Online), indicating that the cells are equivalent and that the CreER<sup>TAM</sup> is not 100% efficient. We conclude that our cell lineage tracing approach marks a small proportion of normal basal cells in mouse tongues and that these marked cells are representative of the entire basal cell population. Because the marked, individual cells are sufficiently distant from each other, we can perform clonal analysis and follow the fate of individual, marked cells.

LacZ marks SCs in normal tongue epithelium

We postulated that if lacZ marks oral cavity epithelial SCs, which by definition means that the cells have a long life span, these cells and their progeny would be stained by X-gal both at early and later time points after tamoxifen treatment. On the contrary, if the lacZ-labeled cells are not long-lived SCs, the X-gal-stained cells would only be detected at early time points after tamoxifen administration and these cells would disappear at later time points because they would differentiate and eventually die. We treated K14-CreER<sup>TAM</sup>; ROSA26 mice with tamoxifen for 2 days and performed whole-mount X-gal staining of tongues at different time points thereafter. One and 2 days after tamoxifen injection, some X-gal(+) cells (blue cells) were detected in the tongues (Figure 1A, 1 and 2 days) and ears (data not shown) by whole-mount X-gal staining. One and 2 weeks after tamoxifen treatment, increased numbers of X-gal(+) cells were present in the tongues. Compared with the 1 and 2 week time points, fewer X-gal(+) labeled cells were detected at 4, 8 and 12 weeks after tamoxifen treatment (Figure 1A, 1, 2, 4, 8 and 12 weeks). The fact that we detected X-gal(+) cells 12 weeks after tamoxifen treatment indicates that X-gal(+)-labeled cells constitute some of the long-lived epithelial cells in tongues. We detected very few X-gal(+) cells in tongues 12 weeks after oil (vehicle for tamoxifen) injection, indicating that the ROSA26 floxed STOP-LacZ transgene was not leaky (Figure 1A, no Tam).

We then examined H&E-stained sections of paraffin-embedded X-gal-stained tissues (shown in Figure 1A) to determine the locations of these X-gal(+) cells in tongue epithelia (Figure 1B). Scattered X-gal(+) cells were detected in the epithelia of tongues 1 and 2 days after tamoxifen treatment, with the majority of X-gal(+) cells residing in the basal layer (Figure 1B, 1 and 2 days), including some single basal cells (Supplementary Figure S2, available at Carcinogenesis Online). We found that as early as 2 days after tamoxifen administration, X-gal(+) cells were present in the suprabasal layers, including some almost at the superficial layers (Figure 1B, 2 days, white arrow). Since the life span of tongue epithelia suprabasal cells is short (~5–7 days) (13), we postulated that these X-gal(+) cells in suprabasal layers would move to the surface and would then be shed. One and 2 weeks after tamoxifen treatment, a greater number of X-gal(+) cells appeared in the suprabasal layers of the tongues, whereas a large number of X-gal(+) cells were also present in the basal layer (Figure 1B, 1 and 2 weeks). By 4–12 weeks after tamoxifen treatment, the majority of X-gal(+) cells were located in the epithelial basal layer, including a few single X-gal(+) basal cells (Supplementary Figure S2, available at Carcinogenesis Online). Approximately 30% of the total number of X-gal(+) cells were present in thin columns that spanned the entire epithelium (Figure 1B, 4, 8 and 12 weeks, white arrows; Supplementary Figure S3, available at Carcinogenesis Online). As a negative control, 12 weeks after oil (vehicle for tamoxifen) injection, we detected almost no X-gal(+) cells in the H&E-stained sections (Figure 1B, 12 weeks, no Tam). We conclude that the X-gal(+) cells in the basal layer comprise some of the SCs in tongue epithelia, that these SCs have a long life span since we detect them at 12 week post-tamoxifen treatment and that these normal SCs produce X-gal(+) cells that differentiate and move to the surface as columns of cells directly above the basal SCs. Our data indicate that under normal conditions, SCs in the tongue divide asymmetrically to generate more differentiated daughter cells that move up through the epithelium as they differentiate (Supplementary Figure S2, available at Carcinogenesis Online). We did not see a statistically significant expansion in size of these clonal X-gal(+) populations even after 50 weeks, indicating a lack of or very limited symmetric division of the lacZ-labeled basal cells along the basement membrane during normal tongue epithelial homeostasis (Supplementary Figure S4, available at Carcinogenesis Online).

Carcinogen treatment results in fewer, larger X-gal(+)-stained regions in tongue epithelia

We next investigated whether a 10 week 4-NQO treatment affected the number and location of the X-gal(+) cells in the tongue epithelia. We treated K14-CreER<sup>TAM</sup>; ROSA26 mice with 4-NQO starting 4 weeks after the 2 day tamoxifen treatment, a time when some of the basal cells were permanently marked with lacZ. We examined the X-gal(+)-labeled cells in tongues at different time points during and after the carcinogen treatment by whole-mount X-gal staining. One week of carcinogen treatment did not cause obvious quantitative changes in the distribution and sizes of X-gal(+) cell populations (Figure 2A, 1 week, Con, 4-NQO; Figure 2Cii). Two weeks of 4-NQO treatment did not affect the numbers of X-gal(+) cell populations on the tongue dorsal surface (Figure 2Cii), but did increase the sizes of some X-gal(+) areas on the tongue dorsal surface by 2-fold (P < 0.05) (Figure 2Ci). However, starting at 6 weeks of carcinogen treatment, 4-NQO-treated tongues displayed an ~10-fold increase in the number of X-gal(+)-labeled cells on the tongue dorsal surface, as compared...
with controls (Figure 2A, 10 weeks, Con, 4-NQO; Figure 2Ci, 6 and 10 weeks). Many of the X-gal(+) regions were 3–7-fold larger in size than the X-gal(+) regions in control tongues, especially at the 6 and 10 week time points (Figure 2A, 10 weeks, Con, 4-NQO; Figure 2Ci, 6 and 10 weeks). Thirty-six weeks after the termination of 4-NQO treatment (46 weeks after the start of 4-NQO), X-gal(+) cell populations were even larger (Figure 2A, 46 weeks, 4-NQO). We conclude that 4-NQO caused a decrease in the total number of X-gal(+) clonal cell populations and an increase in the sizes of X-gal(+) cell populations.

We also examined H&E-stained sections from these 4-NQO-treated tongues (Figure 2B). Compared with control tongues, 1 and 2 weeks of carcinogen treatment did not cause obvious changes in the distribution and sizes of X-gal(+) regions (Figure 2B, 1 week, Con, 4-NQO; Figure 2Ciii, 1 and 2 weeks); however, 6 and 10 weeks of 4-NQO treatment resulted in 4.1 ± 2.4- and 4.6 ± 2.1-fold increases in the sizes of X-gal(+) cell populations in both basal and suprabasal layers (Figure 2B, 10 weeks, Con, 4-NQO; Figure 2Ciii, 6 and 10 weeks), respectively. Also, 36 weeks after the termination of 4-NQO (46 weeks after the start of 4-NQO), X-gal(+) clonal

Fig. 2. The carcinogen, 4-NQO, reduces the numbers of X-gal(+) cell populations and increases the size of some X-gal(+) cell populations in tongues. X-gal staining is indicated by an arrow. In (A) and (B), Con, control; 4-NQO, 4-NQO treated. (A) Whole-mount X-gal staining on K14-CreER<sup>TM1</sup>, ROSA26 mouse tongues at various time points after the start of 4-NQO administration (×8, the tip portions of mouse tongues are shown here though we measured the dorsal surfaces of the entire tongues). For 1 and 10 weeks, n = 3 per treatment; for 46 weeks, n = 5 for control and n = 22 for 4-NQO. (B) H&E-stained sections from X-gal-stained tongues at various time points after the start of 4-NQO administration (×200; scale bars, 50 μm). (C) quantification of the numbers and areas of X-gal(+) cell populations. (i) The numbers of X-gal(+) cells on the dorsal side of tongues, (ii) the sizes of the biggest X-gal(+) cell populations on the dorsal side of mouse tongues and (iii) the sizes of the biggest X-gal(+) cell groups in H&E-stained sections (three mice at each time point and six to eight sections from each mouse). In each panel, C: control; N: 4-NQO treated. (D) Quantification of Ki67 labeling indices in tongue epithelia. Differences with a P value of <0.05 (marked with an asterisk), P < 0.01 (marked with two asterisks) and P < 0.001 (marked with three asterisks) after one-way analysis of variance and subsequently the Bonferroni test for multiple comparisons (two-tailed test) were considered to be statistically significant.
cell populations were even larger (Figure 2B, 46 weeks, 4-NQO). Thus, a 10 week 4-NQO treatment resulted in fewer X-gal(+) clonal cell populations and some of the X-gal(+) clonal cell populations dramatically increased in size. We interpret these data to indicate that some of the X-gal(+) basal SCs are killed by the 4-NQO treatment and other SCs divide symmetrically to maintain an adequate number of SCs in the basal layer. This interpretation is supported by our TUNEL assay data (Supplementary Figure S5, available at Carcinogenesis Online). In control (not treated with 4-NQO) tongue epithelium, we detected apoptotic suprabasal layer cells but no or very few apoptotic basal layer cells (Supplementary Figure S5, available at Carcinogenesis Online). On the contrary, tongue epithelia during 4-NQO treatment displayed a greater number of apoptotic cells in the suprabasal layers, and more importantly, many basal layer cells were also undergoing apoptosis (Supplementary Figure S5, available at Carcinogenesis Online). Even after the termination of 4-NQO treatment, tongue epithelia still showed a greater number of basal layer cells undergoing apoptosis than tongue epithelia not treated with carcinogen (Supplementary Figure S5, available at Carcinogenesis Online), suggesting that 4-NQO had a long-term impact on some basal layer cells.

Carcinogen treatment causes proliferation of cells in tongue epithelia

Since we observed increases in the sizes of clonal X-gal(+) cell populations after 4-NQO treatment (Figure 2), we tested if the 4-NQO treatments affected the proliferation of tongue epithelial cells [both X-gal(+) and X-gal(−) cells] by immunostaining with an antibody to Ki67, a marker of cell proliferation (25). We found that in the control (non-carcinogen treated) tongue epithelium, 24.7 ± 9% of the cells were Ki67 positive, and these cells were normal based on maturation without cytologic or architectural atypia (Supplementary Figure S6, available at Carcinogenesis Online), and that 1 week of 4-NQO did not significantly change the Ki67 labeling index (30.2 ± 9%) (Figure 2D). However, a 10 week 4-NQO treatment resulted in an approximately 2-fold increase in the Ki67 labeling index (47.3 ± 12%) in tongue epithelium, almost all in the basal layer (Supplementary Figure S6, available at Carcinogenesis Online and Figure 2D, P < 0.05). Even 36 weeks after the termination of the 10 week 4-NQO treatment (46 weeks after the start of 4-NQO), in regions without visible tumors, tongue epithelium showed a labeling index of 47.8 ± 14%, mainly in the basal layer, a significantly greater labeling index than that of tongues of control animals of the same age (P < 0.01) (Figure 2D). We conclude that a 10 week 4-NQO treatment induced an increase in basal cell proliferation for a long period after the end of the 4-NQO treatment. We observed no differences in Ki67 labeling between X-gal(+) and X-gal(−) cells (data not shown). Basal SCs induced in mice by 4-NQO show similarities to human oral tumors in terms of their morphological, histopathological and molecular characteristics (19–21).

Normal tongue epithelia contain SCs that reside in the basal layer

A X-gal(+)-basal cell and another X-gal(+)-cell directly above it, presumably the progeny of the basal X-gal(+) cell (Supplementary Figure S2, available at Carcinogenesis Online, 1 day), and the columns of X-gal(+) cells in tongue epithelia we observed, containing basal layer cells and their progeny in the suprabasal compartment (Figure 1B, 4, 8, and 12 weeks), suggest that under normal conditions, the basal SCs divide asymmetrically to produce daughter cells that differentiate and move to the epithelial surface directly above the SC. Our data are not consistent with the clonal epidermal proliferative unit model of the skin epithelia in which a slow-cycling SC at the center of each epidermal proliferative unit and this SC produces an adjacent cluster of short lived, differentiating cells that move to the surface (Figure 5). Since we observed increases in the sizes of clonal X-gal(+) cell populations after 4-NQO treatment (Figure 2), we tested if the 4-NQO treatments affected the proliferation of tongue epithelial cells [both X-gal(+) and X-gal(−) cells] by immunostaining with an antibody to Ki67, a marker of cell proliferation (25). We found that in the control (non-carcinogen treated) tongue epithelium, 24.7 ± 9% of the cells were Ki67 positive, and these cells were normal based on maturation without cytologic or architectural atypia (Supplementary Figure S6, available at Carcinogenesis Online), and that 1 week of 4-NQO did not significantly change the Ki67 labeling index (30.2 ± 9%) (Figure 2D). However, a 10 week 4-NQO treatment resulted in an approximately 2-fold increase in the Ki67 labeling index (47.3 ± 12%) in tongue epithelium, almost all in the basal layer (Supplementary Figure S6, available at Carcinogenesis Online and Figure 2D, P < 0.05). Even 36 weeks after the termination of the 10 week 4-NQO treatment (46 weeks after the start of 4-NQO), in regions without visible tumors, tongue epithelium showed a labeling index of 47.8 ± 14%, mainly in the basal layer, a significantly greater labeling index than that of tongues of control animals of the same age (P < 0.01) (Figure 2D). We conclude that a 10 week 4-NQO treatment induced an increase in basal cell proliferation for a long period after the end of the 4-NQO treatment. We observed no differences in Ki67 labeling between X-gal(+) and X-gal(−) cells (data not shown). Basal SCs induced in mice by 4-NQO show similarities to human oral tumors in terms of their morphological, histopathological and molecular characteristics (19–21).

Discussion

We used a cell lineage tracing approach to characterize the epithelial SCs in the normal tongue and in mouse papillomas and OCSCCs induced by 4-NQO. The OCSCCs induced in mice by 4-NQO show similarities to human oral tumors in terms of their morphological, histopathological and molecular characteristics (19–21).

X-gal(+) cells are present in tongue invasive SCs

We next assessed if X-gal(+) cells were present in the invasive SCC induced by 4-NQO (20). Among 40 mice treated with 4-NQO, OCSCCs from 19 mice contained X-gal(+) cells (Figure 4A, arrow). These data indicate that X-gal(+) cells are present in the invasive SCC. We also detected dual-labeled X-gal(+)/Ki67(+) cells in tongue OCSCCs (Figure 4B, arrow), suggesting that these cells are proliferating, as were a similar percentage of X-gal(−) cells (data not shown). These X-gal(+) cells in OCSCCs must have originated from the basal X-gal(+) SCs we observed prior to 4-NQO treatment (Figure 1B), indicating that long-lived SCs become neoplastically transformed in the oral cavity. Indeed, these X-gal(+) cells were malignantly transformed cells, characterized by migration across the basement membrane, nuclear atypia and increased mitoses (Figure 4B, arrow). In addition, the X-gal(+) cells (circled) (Supplementary Figure S7B, available at Carcinogenesis Online) in a X-gal(+) dysplastic region connected to an invasive cell carcinoma exhibited lower E-cadherin protein levels than control epithelial cells (Supplementary Figure S7A, available at Carcinogenesis Online). Since all of the OCSCCs we examined contained both X-gal(+) and X-gal(−) cells, the OCSCCs are polyclonal in that they originated from more than one basal SC.
a progressive increase in sizes of neighboring clones over time, i.e. the proportion of clones of size $n$ at time $t$ will be the same as that of clones of size $2n$ at time $2t$ (27,28). However, over a long time period (e.g. 50 weeks after tamoxifen injection), we did not detect horizontal expansion of X-gal(+) clonal regions in the whole-mount X-gal staining in control tongues (Figure 2A, 46 weeks, Con; Supplementary Figure S4, available at Carcinogenesis Online) or of the column-like X-gal(+) clonal cell clusters in control tongue epithelia (Figure 2B, 46 weeks, Con; Figure 2Ci). Very few basal cells in the normal epithelium undergo random apoptosis (Supplementary Figure S5A, available at Carcinogenesis Online). Thus, we reason that unlike the SCs in the mouse interfollicle epidermis (27,28), the epithelial SCs in normal tongue epithelia do not often detach from the basal layer and eventually die; therefore, there is little or no need for compensation by the proliferation of neighboring SCs to maintain the SC pool in the basal layer and thus, little or no expansion of clonal sizes (in our study, the sizes of X-gal(+) cell populations, Figure 2, panels A and B, 46 weeks, Con; Figure 2Ci, ii; Supplementary Figure S4, available at Carcinogenesis Online). Therefore, our lineage tracing data in the normal tongue epithelium are not consistent with the scaling behavior expected by the ‘random fate’ model (28).

4-NQO treatment results in the expansion of the sizes of some X-gal(+) cell clones and a loss of other X-gal(+) cell clones

It has been reported that 4-NQO causes cell death and also neoplastic transformation of cultured oral keratinocytes (29–31). We show here that 4-NQO treatment caused a decrease in the numbers of some X-gal(+) clonal cell populations (Figure 2C) concomitant with an expansion in sizes of some X-gal(+) clonal cell populations (Figure 2A–C) and an increase in cell proliferation (Figure 2D). Moreover, 4-NQO treatment resulted in increased apoptosis in basal cells over the long term (Supplementary Figure S5, available at Carcinogenesis Online). Therefore, our data suggest that during and after 4-NQO treatment, some SCs die and that the surviving, neighboring basal SCs compensate for this 4-NQO-induced SC loss by symmetric SC self-renewal, resulting in the progressive expansion of some X-gal(+) clonal cell populations and the disappearance of others (Figure 5). In addition, 4-NQO induces mutations in cells by forming DNA adducts (31,32), which may contribute to the increased cell proliferation by modifying cell cycle checkpoints. Therefore, if mutations caused by 4-NQO provide some SCs with a proliferative advantage, these SCs would replace their neighboring SCs and we would see an expansion of some X-gal(+) clones after the end of 4-NQO treatment. This is in fact what we observe (Figure 2A and B, 46 weeks). Strikingly, after 4-NQO treatment, we detected some very large X-gal(+) clones (Figure 2A and B, 46 weeks). This SC clonal expansion can have dire consequences, as all of the X-gal(+) cells in these large clonal populations are likely derived from one SC (e.g. Figure 2A and B, 46 weeks, 4-NQO, arrows) and if, hypothetically, that SC has acquired an initial deleterious mutation, all of the SCs in the basal layer that are derived from this SC will also carry this deleterious mutation.

SCs in tongue epithelia are the cells of origin of mouse tongue cancers induced by 4-NQO

We observed X-gal(+) cells in 4-NQO-induced papillomas and in invasive OSCCCs, and some of these X-gal(+) cells were proliferating (Figures 3B and 4B). Previous in vitro studies showed that it takes about 10 passages for normal rat oral keratinocytes to be neoplastic transformed after 4-NQO treatment and that the ability of cells to proliferate is very important for this transformation (29). Studies on cancers from other tissues, such as mouse skin, brain, intestine and blood, show that SCs are more likely to be the cells of origin of cancers in these tissues rather than the SC progeny that do not live long enough to accumulate the mutations leading to the required neoplastic transformation (17,18,33–35). The data reported here show that the papillomas and OSCCCs most likely arise from the tongue epithelial basal SCs, although we cannot completely exclude the possibility of spontaneous transformation of suprabasal cells. The tumors also appear to be multiclonal in origin since we did not see any tumors (out of 40 mice examined) comprised completely of X-gal(+) cells.
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SUPPLEMENTARY MATERIAL

Fig. 4. X-gal (+) cells are present in the invasive SCCs in 4-NQO-treated tongues. Whole-mount X-gal staining was performed on the tongues prior to paraffin embedding. (A) H&E-stained invasive SCCs (40x); scale bar, 200 μm; ×200; scale bar, 50 μm; ×600; scale bar, 20 μm); (B) Ki67 staining (marked by an arrow) in the invasive SCC shown in panel (A). Scale bars are 50 μm for ×200 and 20 μm for ×600.

Fig. 5. Model of homeostasis of tongue epithelia. Under normal conditions, tongue epithelial basal cells undergo ‘asymmetric divisions’ (red arrow) to both self-renew and produce a progeny cell destined for differentiation in the suprabasal layers, and some cells in the superficial layers are shed (asterisks); in 4-NQO-treated tongues, some basal SCs are killed by the carcinogen, and the surviving, neighboring basal SCs undergo ‘symmetric’ divisions (blue arrow) to compensate for the loss of SCs. In addition, they still undergo asymmetric divisions (red arrow) to self-renew and produce differentiated progeny in suprabasal layers. Blue cells, X-gal (+) cells; non-blue cells, X-gal (-) cells.

Our lineage tracing data demonstrate that all tongue epithelial basal cells are SCs. We observe a small percentage of the basal cells labeled with X-gal in tongues of mice not treated with 4-NQO, and we also observe a small percentage of cells in papillomas and in invasive OSCSCs labeled with X-gal. The lineage tracing data are most consistent with asymmetric division of the basal SCs in normal epithelia, but symmetric division of some SCs after treatment of the mice with 4-NQO (see model, Figure 5).

Supplementary data can be found online at http://cancer.oxfordjournals.org/

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