The molecular features of tongue epithelium treated with the carcinogen 4-nitroquinoline-1-oxide and alcohol as a model for HNSCC

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Head and neck squamous cell carcinoma (HNSCC) is the sixth most common type of cancer affecting humans worldwide. To determine the potential mechanisms by which chronic tobacco and alcohol abuse lead to HNSCC of the oral cavity, we have used both the 4-nitroquinoline-1-oxide (4-NQO) murine oral carcinogenesis and the Meadows–Cook alcohol models. In this study, we treated mice with 4-NQO in drinking water for 10 weeks and then administered 20% (w:v) ethanol (EtOH) for another 10 weeks. We observed increased levels and/or activation of signaling proteins [p38 mitogen-activated protein kinase (MAPK), β-catenin and Erk 1/2] that are typically altered during HNSCC initiation in humans. We found that EtOH administration alone increased the expression of p38 MAPK but not Erk 1/2 MAPK. Total β-catenin levels in the tongues increased by 2- to 3-fold after 4-NQO treatment, with or without EtOH. However, EtOH combined with 4-NQO reduced phosphorylated β-catenin levels, whereas 4-NQO treatment alone did not. These data implicate EtOH as a regulator of β-catenin signaling in this HNSCC model. We also utilized K14-CreERTM; ROSA26 mice to mark permanently stem/progenitor cells in the tongue epithelia. We found that 4-NQO alone and 4-NQO plus EtOH treatment resulted in massive, horizontal expansion of stem/progenitor cell populations arising from single stem cells in the basal layer of the epithelia. This expansion is consistent with carcinogen-associated, symmetric division of stem/progenitor cells. Our data suggest that specific therapeutic targets for prevention of HNSCC of the oral cavity associated with both alcohol and tobacco use are p38 MAPK and β-catenin.

Introduction

Head and neck squamous cell carcinoma (HNSCC), which can be subdivided into oral cavity cancer and oropharyngeal cancer, is the sixth most common malignancy in the world today, with ~600,000 cases diagnosed per year (1). Despite improvements in diagnosis and treatment, there is a high rate of tumor recurrence, with several factors that contribute to this poor outcome (2,3). First, HNSCC is often diagnosed at an advanced stage (4). Second, field carcinization (the presence of one or more mucosal areas consisting of tumorigenic cells that have acquired molecular or genetic alterations) may contribute to the development of multiple primary tumors correlated with increased survival rates (5). Approximately 50% of HNSCC patients will develop a disease recurrence within 5 years after treatment, which is typically a combination of chemotherapy and radiation, and this recurrence rate has remained unchanged for the past three decades (6). Third, HNSCC is often associated with tobacco use, alcohol consumption, an unhealthy diet, an inactive lifestyle and poor oral hygiene (7). Tobacco abuse and alcohol consumption are independent risk factors; however, these two agents may synergize to increase the risk of developing HNSCC (8).

Epidemiological studies using Caucasian males found that the relative risk of HNSCC in chronic smokers and drinkers is 34:1, whereas the relative risk for people who only abuse tobacco or only alcohol are 7:1 and 3:1, respectively, compared with those who consume neither tobacco nor alcohol (8,9). Chemicals found in tobacco products, such as dibenz[a,j]anthracene, benzo-(a)-pyrene, 4-amino-1-phenyl, acetaldheyde, catechol and benzene, have been shown to be carcinogenic (10). However, there are still many unanswered questions regarding the role of ethanol (EtOH) in the initiating steps of HNSCC.

There are several routes by which EtOH may function to enhance oral carcinogenesis (11). Alcohol may function as a solvent that facilitates the uptake of other carcinogenic compounds, especially those found in tobacco smoke, into the oral mucosa (11). Also, acetaldehyde (AA), an EtOH metabolite, may behave as a co-carcinogen by promoting the formation of DNA adducts. Higher incidences of HNSCC have been observed in people harboring a single-nucleotide polymorphism, demonstrated as a lysine substitution for a glutamine at amino acid residue 487 in aldehyde dehydrogenase 2, the key enzyme responsible for metabolizing AA into acetate (12). This mutation results in reduced aldehyde dehydrogenase 2 activity, suggesting that AA could have carcinogenic properties (12,13). In addition to single-nucleotide polymorphisms found in aldehyde dehydrogenase 2, polymorphisms of alcohol dehydrogenase (ADH) 1B and ADH1C, which metabolize alcohol to AA, have been observed in patients with HNSCC (13). More specifically, the ADH1B*2 allele encodes an enzyme that is 40 times more active than the enzyme encoded by the ADH1B*1 allele, and the enzyme encoded by ADH1C*1 is 2.5 times more active than the one encoded by the ADH1C*2 (13). Additionally, EtOH has been reported to reduce levels of retinoic acid, a metabolite of retinol (vitamin A), by competitively inhibiting retinol oxidation by CYP2E1, as well as by promoting increased metabolism of retinoid acid by CYP2E1 (14,15). The reduction in retinol oxidation by EtOH could alter retinoid signaling events, for example, epigenetic alterations such as the acetylation and methylation of histones and hypomethylation of DNA, which could have a role in tumorigenesis (16).

The 4-nitroquinoline-1-oxide (4-NQO) murine model of oral carcinogenesis that we developed (17) has been used extensively to identify how chronic tobacco abuse contributes to human oral cancers and how therapeutic treatments can reduce or prevent these malignancies (17–22). The 4-NQO is a synthetic, water soluble carcinogen that mimics the effects of chronic tobacco consumption by promoting DNA adduct formation (23), A-G nucleotide substitution (24) and intracellular oxidative stress (25), resulting in histological and molecular alterations very similar to those found in human oral carcinogenesis (17,26). The Meadows–Cook model (27) mimics chronic human alcohol abuse by allowing direct contact of the oral mucosa with alcohol.

In this study, we combined the Meadows–Cook and 4-NQO carcinogenesis models to represent human alcohol and tobacco abuse in order to determine the molecular alterations associated with the pretumor environment of HNSCC of the oral cavity. Additionally, we wanted to understand the effects of carcinogen and/or alcohol treatment on the stem/progenitor cell population of the tongue epithelium. Here, we present some of the molecular features of the initiation of oral cancer, increased cell proliferation, increased oncogenic signaling, modification of cell–cell adhesion properties and increased stem cell/progenitor proliferation.

Materials and methods

Animals and treatments

Eight-week-old female wild-type C57BL/6 mice (Jackson Laboratory, Bar Harbor, ME) were housed five per cage in a 12 h light/dark cycle. After a week of acclimation, mice were separated into five separate experimental groups.

Abbreviations: AA, acetaldehyde; ADH, alcohol dehydrogenase; EtOH, ethanol; H&E, hematoxylin and eosin; HNSCC, head and neck squamous cell carcinoma; IHC, immunohistochemistry; MAPK, mitogen-activated protein kinase; NQO, 4-nitroquinoline-1-oxide; Untr., untreated; VC, vehicle control.

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Each of the experimental groups was treated with either propylene glycol (vehicle control, VC) or 100 μg/ml 4-NQO (Cat# N8141; Sigma, St Louis, MO) for 10 weeks (as described in ref. 17) and then treated with normal drinking water (untreated, Untr.) or drinking water containing 20% (w/v) EtOH for 10 weeks (Figure 1). EtOH (95%) was prepared weekly to a 20% (w/v) final concentration and, after a 1 week graded increase in EtOH concentration (5%, 10% and 20%), was administered to the mice. Propylene glycol and the normal drinking water are the vehicle and untreated control for 4-NQO and EtOH administration, respectively. The experiment groups were designated as follows: VC/Untr. (Group 1), VC/EtOH (Group 2), 4-NQO/Untr. (Group 3), 4-NQO/EtOH (Group 4) and 4-NQO/EtOH(s) (Group 5) (Figure 1). The 4-NQO/EtOH(s) group differs from the 4-NQO/EtOH in that the former group received EtOH administration during the last week of 4-NQO treatment. The body weight, food intake and EtOH or water intake were monitored weekly for the duration of the experiment (Supplementary Figures S1 and S2, available at Carcinogenesis Online). All procedures involving the use of mice were performed in accordance with the National Institutes of Health and approved by the Weill Cornell Medical College Institutional Animal Use and Care Committee.

For observing changes in epithelial stem cells in the tongue caused by 4-NQO and/or EtOH treatment, K14-CreERTM double-positive transgenic mice (Cat# 56822) and ROSA26 floxed STOP-LacZ double-positive transgenic mice (Cat# 003474) were generated, as described in ref. (28). The K14-CreERTM; ROSA26 mice received ~4 mg tamoxifen (Cat# T5648; Sigma) treatment each day by intraperitoneal injections for two consecutive days. Four weeks after tamoxifen treatment, the K14-CreERTM; ROSA26 mice were subjected to 4-NQO and/or EtOH administration, as described above. After the termination of 4-NQO and/or EtOH treatment, mouse tongues and ears were harvested for β-galactosidase activity assays (X-gal staining). X-gal staining was performed on frozen sections and on whole mounts, as described in ref. (28).

Measurement of blood alcohol content
At weeks 4 and 8, serum from two randomly selected mice was extracted from their tails 1.5 h into the dark cycle of the mouse housing facility. Blood alcohol content was determined by using the Alcohol Reagent Set (Cat# A7504; Pointe Scientific, Canton, MI), as described by the manufacturer.

Tissue processing and histopathological analysis
Immediately after cervical dislocation, the tongues were harvested, examined for the presence of gross lesions and photographed. Longitudinally cut tongues (dorsal/ventral) were fixed overnight in 4% paraformaldehyde at 4°C, transferred into 70% EtOH, processed, embedded in paraffin and sectioned into 7 μm sections. Following hematoxylin and eosin (H&E) staining, histopathological analysis was performed by a trained pathologist blinded

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**Fig. 1.** Experimental outline of the combined Cook–Meadows and 4-NQO murine carcinogenesis models and histopathological analysis of cellular modifications in the tongue epithelia. (A) C57BL/6 wild-type and K14-CreERTM; ROSA26 mice were divided into five experimental groups. For the first 10 weeks, mice were treated with either propylene glycol (VC) or 100 μg/ml 4-NQO; then for the next 10 weeks, mice were treated with either normal drinking water (Untr.) or 20% (w/v) EtOH after 1 week of a graded increase in EtOH concentration. The experimental groups are as follows: VC/Untr. (group 1), VC/EtOH (group 2), 4-NQO/Untr. (group 3), 4-NQO/EtOH (group 4) and 4-NQO/EtOH(s) (group 5). The VC and Untr. conditions are represented by the solid white blocks; the 4-NQO treatments are represented by the solid black blocks; the EtOH treatments are represented by the gray blocks; the graded EtOH concentrations are represented by the horizontal alternating white and gray blocks. For the 4-NQO-EtOH(s) group, the horizontal black, white and gray blocks indicate that the EtOH treatment began during the last week of 4-NQO administration. (B) Representative H&E-stained images of the entire tongue epithelium analyzed by a board-certified pathologist showing cellular modifications of the tongue epithelia: normal squamous mucosa (i, ii), squamous mucosa showing mild dysplasia (iii), squamous mucosa with moderate-to-severe dysplasia (iv) and squamous mucosa with severe dysplasia and focus of superficially invasive squamous cell carcinoma (v).
to the identities of the slides. The lesions observed were classified into three major categories: hyperplasia, dysplasia (mild, moderate and severe) and squamous cell carcinoma.

**Immunohistochemical analysis**

Paraffin-embedded sections, prepared at 7 µm thickness, from at least three mice per treatment group were deparaffinized and rehydrated in graded EtOH concentrations and distilled water. For antigen retrieval, slides were immersed in diluted (3:320) citrate-based antigen unmasking solution (Cat# H-3300; Vector Laboratories, Burlingame, CA) and the slides were treated with 3% hydrogen peroxide prepared in methanol. After blocking with mouse anti-IgG blocking solution (Cat# MKB-2213; Vector Laboratories), rat anti-IgG blocking solution (Cat# CT ST005; R&D Systems, Minneapolis, MN) or phosphate-buffered saline containing 10% goat serum and 0.1% Tween for 1 h at 4°C (for rabbit primary antibodies), the sections were incubated with the following antibodies overnight at 4°C: mouse monoclonal anti-E-cadherin (1:2000; Cat# ab58033; Abcam, Cambridge, MA), rat polyclonal anti-β-catenin (1:300; Cat# M7249; Dako, Carpentaria, CA), rabbit polyclonal anti-Erk 1/2 (1:250; Cat# 9111; Cell Signaling, Danvers, MA) and rabbit polyclonal anti-β-actin (1:500; Cat# 6302; Abcam). After incubation with the primary antibodies, the slides were treated with secondary antibodies supplied in the Mouse on Mouse (Cat# MBB-2213; Vector Laboratories), SuperPicture HRP Polymer Conjugate (Cat# 87–8963; Life Technologies) or Rat Cell & Tissue Staining HRP-Dab (Cat# CT ST005; R&D Systems) kits for mouse, rabbit and rat primary antibodies, respectively. Antibody signals were visualized by peroxidase reaction using 3,3-diaminobenzidine as a chromogen. As a negative control, tissue sections were incubated in the absence of primary antibody to ensure specificity of the primary antibody.

Three non-contiguous areas from the anterior and posterior portions of each section were photographed for analysis, and three sections from three different mice were measured. The representative photomicros used in this study were matched to ones taken by a trained pathologist blinded to the identities of the samples. The average staining index for Ki67 was calculated as the number of positive β-catenin cells divided by the total number of cells in each field, and the averages of these numbers were used as the labeling index.

**Western blotting analysis**

Mouse tongues were homogenized, lysed in protein extraction buffer (0.5M Tris-HCl, pH 6.8, 10% sodium dodecyl sulfate and 1% sodium dodecyl sulfate) containing 1X of Halt Protease (Cat# 87786) and phosphatase inhibitors (Cat# 78420; ThermoScientific, Rockford, IL) and denatured by boiling. Total protein lysates (30 µg) were resolved on 10% SDS-PAGE and transferred (Cat# 78420; ThermoScientific, Rockford, IL) and denatured by boiling. The following antibodies were used: mouse monoclonal anti-β-catenin (1:1000; Cat# 53050; Abcam), rabbit polyclonal anti-β-catenin (1:1000; Cat# 9911; Cell Signaling), rabbit polyclonal anti-β-catenin (1:4000; Cat# 6302; Abcam), rabbit polyclonal anti-α-tubulin (1:1000; Cat# 2128; Cell Signaling), rabbit polyclonal anti-phospho (Ser33/37/Thr42) β-catenin (1:1000; Cat# 9811; Cell Signaling), rabbit polyclonal anti-β-actin (1:1000; Cat# 3700; Cell Signaling) and rabbit polyclonal anti-β-catenin (1:4000; Cat# 6302; Abcam). Where indicated, the primary antibody, are also shown (Figure 3a-e), β-catenin (Figure 3f-j) and Erk 1/2 (Figure 3k-o) in the basal and suprabasal layers of the 4-NQO/Untr., 4-NQO/EtOH and 4-NQO/EtOH(s) experimental groups and compared with those of the VC/Untr. and VC/EtOH groups. Compared with those of the VC/Untr. and VC/EtOH experimental groups, we observed a greater expansion of E-cadherin (Figure 3a-e), β-catenin (Figure 3f-j) and Erk 1/2 (Figure 3k-o) in the suprabasal layers of the 4-NQO/Untr., 4-NQO/EtOH and 4-NQO/EtOH(s) experimental groups. Negatives sections, which were only stained with a secondary antibody, are also shown (Supplementary Figure S3, available at Carcinogenesis Online).

**Assessing the alterations in the stem/progenitor cell population in the tongue epithelium using a K14-CreERT2™; ROSA26 murine line**

Using K14-CreERT2™; ROSA26 double-positive mice, we have developed a method to determine the effects of 4-NQO and/or EtOH administration on the stem/progenitor cell profile in the tongue epithelium (28). The K14-CreERT2 transgene insert has a Cre recombinase and a mutant form of the mouse estrogen receptor ligand-binding domain, both of which are driven by a truncated human keratin 14 promoter. The truncated K14 promoter drives expression of the transgene in the basal layer of the epidermis, primarily in the tongue and skin (29). The ROSA26 line contains a floxed stop cassette that is located immediately upstream of the reporter gene, LacZ. Thus, K14-CreERT2™; ROSA26 double-positive mice, after a brief treatment with tamoxifen (a synthetic ligand for the mutant estrogen receptor), will permanently exhibit blue signals in the tongue epithelial basal layer cells and their progeny after X-gal staining (Figure 4A and B) (28). Of note, tamoxifen treatment did not activate CreERT2™ in all of the basal cells in the tongue epithelia of these mice, as we did not observe X-gal signals in all epithelial basal cells—an observation that has been made previously by others as well (30). In addition, higher concentrations of tamoxifen resulted in greater proportions of X-gal-labeled cells. Therefore, our cell lineage tracing approach permanently marks a small population of normal basal cells in the tongue, and these marked cells are representative of the entire population of basal cells (28,30).

By whole mount X-gal staining and by H&E staining of portions and sections of the tongue, respectively, we detected the presence of an X-gal(+) cell population in the tongue epithelia of VC/Untr. mice even 24 weeks after tamoxifen treatment (Figures 4 and 5). These X-gal(+) populations were characterized by X-gal(+) cells that span the entire epithelium from the basal layer through the suprabasal layers, suggesting that these X-gal(+) basal cells constitute some of the long-lived epithelial stem cells in the tongue epithelium.
Additionally, these epithelial stem cells can divide asymmetrically to produce progeny that differentiate and move toward the surface of the tongue in columns in the suprabasal layers to maintain homeostasis (Figures 4 and 5).

To evaluate the effects of administration of 4-NQO alone, administration of EtOH alone and the combined administration of 4-NQO and EtOH, we assessed the numbers and sizes of X-gal(+) cell clusters in K14-CreERT2; ROSA26 mice treated with 4-NQO and/or EtOH for 10 weeks (4 weeks post tamoxifen treatment) (Figure 4A and B). No pathologic abnormalities were found within the X-gal(+) cell clusters shown in the representative sections (Figure 4B); however, we did observe pathologic abnormalities in other regions of the tongues of mice from the 4-NQO/Untr. and 4-NQO/EtOH groups (data not shown). Here, we found that the numbers of X-gal(+) cell clusters from the 4-NQO/Untr. group [range: 1–22 X-gal(+) cells; n = 10] and the 4-NQO/EtOH group (range: 1–10; n = 10) were lower than those of the VC/Untr. group (range: 21–54; n = 5; P = 0.02 and 0.016, respectively) (Figure 4Ci). Mice of the VC/EtOH group had the highest overall numbers of X-gal(+) cells/slide, although this did not reach statistical significance compared with the VC/Untr. group (range: 24–196; n = 5) (Figure 4Ci).

Additionally, we observed an inverse relationship between the numbers of X-gal(+) cells/field and the sizes of X-gal(+) cell clusters when we compared the 4-NQO and 4-NQO/EtOH groups to the Untr. and EtOH alone groups (Figure 4C). The average sizes of X-gal(+) cell clusters/field were as follows: VC/Untr., 1414.4 pixels²; VC/EtOH, 1678.6 pixels²; 4-NQO/Untr., 11105.9 pixels² and 4-NQO/EtOH, 7302.1 pixels² (P = 0.02) (Figure 4Ci). Thus, we show that 4-NQO treatment and 4-NQO combined with EtOH lead to an expansion in

Fig. 2. IHC and quantitative analysis of Ki67 demonstrate increased cellular proliferation in mice treated with 4-NQO and 4-NQO plus EtOH. (A) IHC analysis was conducted to observe Ki67 staining in five treatment groups: (i) VC/Untr., (ii) VC/EtOH, (iii) 4-NQO/Untr., (iv) 4-NQO/EtOH and (v) 4-NQO-EtOH(s). All IHC observations were made by using photomicrographs taken from three non-contiguous fields of the anterior and the posterior regions of the tongue. Also, the representative IHC photomicrographs presented in this study were matched to those taken by a trained pathologist, blinded to this study. (B) The staining index of Ki67 is represented by the percentage of stained cells versus the total number of cells in three non-contiguous fields from three separate mice. Analysis of variation combined with the Tukey test determined statistical significance, where **P < 0.01.
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Finally, the fold changes in Erk 1/2 protein levels, compared with that of the VC/Untr. group, were as follows: VC/EtOH (~1.5); 4-NQO/Untr. (~3.2; \( P < 0.01 \)); 4-NQO/EtOH (~2.0; \( P < 0.05 \)) and 4-NQO/EtOH(s) (~4.0; \( P < 0.01 \)) (Figure 6B). These data show that both 4-NQO administration and 4-NQO with EtOH administration can increase the levels of Erk 1/2 in the tongue.

Discussion

The combination of the Meadows–Cook and 4-NQO murine carcinogenesis models can be used to assess cellular events prior to HNSCC. The 4-NQO murine carcinogenesis model has been used extensively to determine the prognostic factors (32), to expand on new therapeutic strategies (33) and to elucidate various signaling pathways (21,34–37) involved in the initiation and progression of oral cancers. In this study, we supplemented the 4-NQO murine model for oral carcinogenesis by combining it with the Meadows–Cook model to determine the cellular and molecular changes induced by the administration of alcohol and the carcinogen, 4-NQO. The combination of these two models is useful for the following reasons: (i) there is limited information concerning the molecular role that EtOH may have in initiating and enhancing oral carcinogenesis and (ii) the combination of carcinogen and alcohol administration closely represents human behavior with respect to chronic alcohol and tobacco use because both of the substances are in direct contact with the oral mucosa.

In this study, we performed experiments to examine the early molecular alterations that occur before the development of frank HNSCC of the oral cavity. A major obstacle in treating HNSCC of the oral cavity is the limited number of molecular markers to detect this disease at early stages (6). Thus, we have a very compelling model in which to observe early stages of carcinogenesis associated with alcohol and/or tobacco consumption.

Increased cellular proliferation in the epithelial layer of the tongue occurs at early stages of carcinogenesis.

We assessed the expression of Ki67 by IHC to determine changes in cellular proliferation in the tongue epithelia (Figure 2). Using this model, we found that Ki67 staining was only observed in the basal layer of the tongue is maintained even after 4-NQO treatment. The loss of stem cells; thus, an adequate stem cell pool in the epithelial basal layer of the tongue is maintained even after 4-NQO treatment.

Effects of 4-NQO and/or EtOH administration on the expression of β-catenin, phosphorylated β-catenin, p38 and Erk 1/2

We then searched for potential changes in molecular markers associated with Wnt/β-catenin signaling, inflammatory signaling and mitogen-activated protein kinase (MAPK) signaling. Compared with the VC/Untr. group, the fold change levels of phosphorylated β-catenin were as follows: 1 (VC/Untr.); 1.1 (VC/EtOH); 1.6 (4-NQO/Untr.); 0.58 (\( P < 0.01 \)) (4-NQO/EtOH) and 0.47 (\( P < 0.01 \)) [4-NQO/ EtOH(s)] (Figure 6A). Interestingly, these results indicate that a decrease in phosphorylated β-catenin occurs only after 4-NQO plus EtOH exposure and not after 4-NQO alone (4-NQO/Untr.).

The fold changes in the total β-catenin levels in the experimental group, compared with that of the VC/Untr. group, treated with 4-NQO were as follows: 4-NQO/Untr. (2.72; \( P < 0.01 \)); 4-NQO/EtOH (3.7; \( P < 0.01 \)) and 4-NQO/EtOH(s) (5.4; \( P < 0.001 \)) (Figure 6A). Thus, 4-NQO treatment is associated with an increase in total β-catenin levels. In contrast, the fold change for total β-catenin for the VC/EtOH experimental group compared with the VC/Untr. group was small, just 1.5-fold (\( P > 0.05 \)) (Figure 6B).

Furthermore, our quantitative western blotting analysis suggests that p38 MAPK may be a target of EtOH, since there was an increase in p38 levels in all of the EtOH-treated groups: VC/EtOH 2.3-fold increase (\( P < 0.01 \)); 4-NQO/EtOH 2.7-fold increase (\( P < 0.01 \)) and 4-NQO/EtOH(s) 4.4-fold increase (\( P < 0.01 \)) (Figure 6B). In contrast, the 4-NQO/Untr. samples did not show a significant difference in p38 levels relative to the VC/Untr. samples (Figure 6B).

Finally, the fold changes in Erk 1/2 protein levels, compared with that of the VC/Untr. group, were as follows: VC/EtOH (~1.5); 4-NQO/Untr. (~3.2; \( P < 0.01 \)); 4-NQO/EtOH (~2.0; \( P < 0.05 \)) and 4-NQO/EtOH(s) (~4.0; \( P < 0.01 \)) (Figure 6B). These data show that both 4-NQO administration and 4-NQO with EtOH administration can increase the levels of Erk 1/2 in the tongue.
layers of tongues that were harvested from the VC/Untr. and VC/EtOH treated groups (Figure 2A). However, in the 4-NQO/Untr., 4-NQO/EtOH and 4-NQO/EtOH(s), we found increased Ki67 staining in the basal layer and the suprabasal layers and in dysplastic and invasive areas (Figure 2A and B).

Activation of the canonical Wnt signaling occurs before the detection of frank HNSCC of the oral cavity

Abnormal activation of the Wnt/β-catenin signaling pathway has been associated with the development and progression of human HNSCC through increased expression of the ligands (Wnt proteins) and receptors (Frizzled proteins) in this pathway (38,39). Activation of the canonical Wnt signaling pathway is marked by the sequestering of glycogen synthase kinase 3B by Dishevelled (Dsh), preventing the phosphorylation of glycogen synthase kinase 3B and the subsequent degradation of β-catenin. Increased β-catenin levels in the nucleus allow β-catenin to bind to members of the T-cell factor/lymphoid-enhancer transcription factor family, thus initiating transcription of target genes (38). Activation of the canonical Wnt pathway results in increased cellular proliferation (40) and activation or expression of several genes associated with human HNSCC, including c-Myc, cyclin D1 and cyclooxygenase 2 (38). Additional studies of HNSCC have shown alterations in canonical Wnt signaling implicated in increased invasive properties leading to tumorigenesis (41).

We also observed increased expression of β-catenin in the suprabasal layers (Figure 3f–j), increased total β-catenin (Figure 6Aiii and 6Bii) and decreased levels of phosphorylated β-catenin in 4-NQO treated mice (Figure 6Ai and 6Bi). Although there was an increase in β-catenin expression in the suprabasal layers, this could be a secondary effect of the increased cellular proliferation induced by 4-NQO treatment. However, Yu et al (42) determined that β-catenin, through its interactions with E-cadherin at the cell membrane, could function as an adhesion molecule in patients diagnosed with HNSCC. Interestingly, we detected a 2-fold decrease in the phosphorylated β-catenin level in the tongues of mice from the 4-NQO/EtOH and 4-NQO/EtOH(s) experimental groups but not in mice treated with 4-NQO alone (Figure 6Bii). This result suggests that EtOH, in cooperation with the carcinogen, 4-NQO, contributes to a reduction in the degradation of β-catenin. These data enhance the development of early molecular markers of HNSCC of the oral cavity because our reported changes in Wnt signaling occur before frank tumor formation. Unlike some of the previously mentioned studies (39,41,43,44), our analyses were performed using an in vivo murine model with high similarity to the human oral cavity. How EtOH plus 4-NQO treatment

Fig. 4. 4-NQO and 4-NQO with EtOH administration induce changes in stem/progenitor cells of the tongue epithelium. (A) Representative photomicrographs of whole mount X-gal-stained tongues taken from VC/Untr. (i), VC/EtOH (ii), 4-NQO/Untr. (iii) and 4-NQO/EtOH (iv) experimental groups of the K14-CreER<sup>TA</sup>M; ROSA26 murine line to determine β-galactosidase activity as a permanent marker of a representative population of stem cells; magnification, ×4. (B) H&E-stained sections of the X-gal-stained tongues from the four experimental groups (as described in Figure 4A); magnification, ×200. (C) Quantification of the numbers of X-gal-stained spots (i) and the areas of X-gal stained spots (ii) from whole mount X-gal-stained tongues.
increases β-catenin expression in the suprabasal layers and inhibits the phosphorylation of β-catenin is currently under investigation.

*Increased activation of the p38 kinase and Erk MAPK signaling pathways is associated with early-stage carcinogenesis*

The MAPK signaling pathways play a critical role in cell growth, proliferation, differentiation, migration and apoptosis (45). Erk, p38 kinase and c-jun N-terminal kinase are the best-characterized members of the MAPKs and deregulation of these kinases can lead to HNSCC (45). Here, we observed increased levels of Erk 1/2 in the 4-NQO/Untr., 4-NQO/EtOH and 4-NQO/EtOH(s) experimental groups (Figure 6Avi and Bvi). Although preliminary, we have observed an increase in expression of cyclin A2, cyclin B2, cyclin E1 (downstream effectors of the MAPK/extracellular signal-regulated kinase signaling pathway) by RNAseq analysis for the 4-NQO/Untr. and 4-NQO/EtOH experimental groups (data not shown).

The p38 kinase signaling pathway activation is directly involved in the production of cytokines, such as interleukin-6 and interleukin-8, and the expression of both genes increases in malignancies that display inflammatory properties, like HNSCC (46). Using the Meadows–Cook and 4-NQO murine carcinogenesis models, Guo et al (21) demonstrated that EtOH administration promoted inflammatory properties in HNSCC through increased expression of 5-lipoxygenase and cyclooxygenase 2. Increased p38 kinase expression and activation have been associated with HNSCC in prior studies (45,46), and we observed increased p38 kinase levels in treated mice from the VC/EtOH, 4-NQO/EtOH and 4-NQO/EtOH(s) experimental groups (Figure 6Avi and Biii). Prior studies have shown that following EtOH treatment or abuse, there is increased p38 kinase expression and activation in the liver (47) and in the sera of patients diagnosed with HNSCC, which are reduced after radiation therapy (48). Since we observed 2- to 3-fold increases in p38 kinase levels after EtOH treatment (Figure 6Biii), with or without 4-NQO administration, we are currently testing if the downstream members of the p38 kinase pathway are also activated by EtOH, independently of the Erk 1/2 and c-jun N-terminal kinase pathways. Our current study supports a role for the increased activity of p38 kinase in the initiation of HNSCC and targets inhibition of p38 kinase as a potential treatment for HNSCC.

*The stem cell component of the tongue epithelium is altered after 4-NQO treatment*

We show that 4-NQO and 4-NQO plus EtOH treatments result in a reduction in the numbers of X-gal(+) cell populations and an increase in the sizes of the X-gal(+) cell populations (Figures 4 and 5). 4-NQO can induce cell death, in addition to neoplastic transformation, depending on the dose (31,49). As we addressed above, the X-gal(+) basal cells in the tongue epithelium are representative of the epithelial stem/progenitor cell population that maintains the cellular homeostasis of tongue epithelium by producing progeny (also X-gal(+)) which differentiate over time (28). Here, we have evaluated the effects of 4-NQO on these X-gal(+) stem/progenitor cell populations. It has been reported that 4-NQO causes cell death and neoplastic transformation of cultured oral keratinocytes (31). In this study, we show that 4-NQO treatment causes a decrease in the numbers of X-gal(+) clonal cell populations, concomitant with an expansion in the sizes of X-gal(+) clonal cell populations (Figures 4 and 5). These data suggest that during and after 4-NQO treatment, some stem/progenitor cells die and that the surviving, neighboring basal stem/progenitor cells compensate for the 4-NQO induced stem cell loss by symmetric stem/progenitor cell self renewal, resulting in the progressive expansion of some X-gal(+) clonal cell populations along the basal layer.

The clonal expansion of stem/progenitor cells can have major dire consequences, as all of the X-gal(+) stem/progenitor cells in each of these large clonal populations (Figure 4A and B) are likely to be derived from one stem cell. If one of these ‘founder’ stem cells of the clone, upon treatment with 4-NQO, develops a mutation that initiates the neoplastic transformation process, then all of the stem cells derived from this initial cell will possess this first mutation. This situation creates a potentially large population of stem cells primed for further mutational hits that could result in frank squamous cell carcinoma.

**Fig. 5.** 4-NQO treatment induces cellular proliferation within X-gal(+) positive regions of the tongue epithelium. Representative micrographs of whole mount X-gal-stained tongues, followed by Ki67 staining of the same sections of VC/Untr. (A and C) and 4-NQO/Untr. (B and D) treatment groups. Panels A and B are ×200 magnification and panels C and D are ×600 magnification.
In conclusion, we have shown that the combination of the Meadows–Cook and 4-NQO murine carcinogenesis models is useful for determining the initial steps leading to neoplastic transformation in the oral cavity. Because of the similarities between human and murine oral mucosa, we can use this model to identify novel biomarkers, to further analyze molecular markers and to determine the efficacy of drugs for treatment of EtOH- and tobacco-induced oral carcinogenesis in humans. Additionally, using our K14-CreER\textsuperscript{TA}, ROSA26 murine line, we have observed a carcinogen-associated modification of the stem cell population in the tongue epithelium that may increase our understanding of potential tumor-initiating cells in HNSCC.

Supplementary material

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