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# Oral carcinogenesis induced by 4-nitroquinoline 1-oxide in lecithin:retinol acyltransferase gene knockout mice

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**Abstract**

Lecithin:retinol acyltransferase (*LRAT*) regulates retinol (vitamin A) metabolism by esterifying retinol. *LRAT* expression is decreased in cultured human squamous cell carcinoma cells of the head and neck relative to normal epithelial cells. We investigated whether the carcinogen 4-nitroquinoline 1-oxide (4-NQO) induced a higher incidence of oral cancer in *LRAT* knockout ($LRAT^{-/-}$) than in wild-type (Wt) mice. We also investigated retinol deprivation during 4-NQO treatment in $LRAT^{-/-}$ mice as a model for rapid retinol deficiency. We observed higher levels of *secreted frizzled-related protein* (*Sfrp*) 2, an inhibitor of WNT signaling, in tongue tumors in $LRAT^{-/-}$ versus Wt. $LRAT^{-/-}$ embryonic stem cells also expressed higher *Sfrp2* transcripts, indicating an interaction between retinol and WNT signaling. *Cox-2*, *Cyclin D1*, *p21*, *Trop2* and $RAR\beta_2$ were not differentially expressed in Wt versus $LRAT^{-/-}$ tongue tumors. Wt and $LRAT^{-/-}$ mice fed a retinol-sufficient diet showed the same oral tumor incidence after 4-NQO treatment. In contrast, tongue tumors developed in 60% of Wt mice and in 100% of $LRAT^{-/-}$ mice fed a retinol-deficient diet during 4-NQO treatment ($P=.22$); moreover, the bromodeoxyuridine labeling index was 21.0±2.4% in $LRAT^{-/-}$ normal tongue epithelium as compared to 9.9±0.8% in Wt normal tongue epithelium ($P<.001$). Thus, partial retinol deficiency during carcinogen treatment (achieved in $LRAT^{-/-}$) resulted in more proliferating cells in tongue epithelia from $LRAT^{-/-}$ mice and, ultimately, a greater probability of carcinogenesis.
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*Keywords:* Stem cell; Squamous cell carcinoma of the head and neck; Retinol; Cancer prevention; Retinoic acid; Nutrition

## 1. Introduction

Head and neck cancer is an important health threat worldwide [1,2]. According to the National Cancer Institute, over 22,000 new oral cancer cases are diagnosed annually in the United States (http://www.cancer.gov/cancertopics/types/oral/), and they account for approximately 3–5% of all cancers. Retinoids are structurally related compounds that are derived from vitamin A (retinol). Retinoids regulate cell proliferation and differentiation [3]. All-*trans* retinoic acid (RA), the most biologically active metabolite of retinol, acts by binding and activating its nuclear receptors, which are transcription factors that directly regulate the transcription of certain "target" genes [4]. Two families of nuclear receptors mediate the effects of retinoids. RA binds and activates the retinoic acid receptor (RAR) family (α, β and γ), while 9-*cis* RA binds to both RARs and the retinoid X receptor family (α, β and γ) [3–5]. Studies have shown that alterations in retinoid signaling occur during the malignant transformation of oral epithelia [6–9].

Abnormal metabolism of retinoids and reduced expression of $RAR\beta_2$ have been observed in many types of malignant cells, as well as in the transition from premalignant lesions to aggressive carcinomas, including oral cancers [6,8–13]. RA treatment can up-regulate $RAR\beta_2$ gene expression in normal epithelial cells and can also partially restore the reduced $RAR\beta_2$ expression in certain carcinoma cells [14,15]. For these reasons, retinoids and drugs that modulate the functions of RARs are useful therapeutic and cancer chemopreventive agents for many types of cancers [16–19].

Lecithin:retinol acyltransferase (*LRAT*) catalyzes the esterification of retinol and functions as one of the enzymes that regulate the levels of tissue retinoids. Retinyl esters are stored in a variety of tissues and can be hydrolyzed to retinol by retinyl ester hydrolases [20,21]. High *LRAT* activity is found in the liver [22–25], lung [26,27] and small intestine [28,29], but expression is also detected in the eye [30–32], testis [33], skin [34,35], mammary gland [34,36], prostate epithelium [37] and oral cavity epithelial cells [34]. *LRAT* gene knockout mice do not store retinyl esters in the liver and other tissues; therefore, they are more susceptible to retinol deficiency [26,38,39]. *LRAT* also plays a role in the homeostasis of retinol in both mouse embryos and adult mice as the dietary retinol level fluctuates [40,41].

*Abbreviations:* BrdU, bromodeoxyuridine; EB, embryoid body; ES, embryonic stem; *LRAT*, lecithin:retinol acyltransferase; 4-NQO, 4-nitroquinoline 1-oxide; RA, all-*trans* retinoic acid; RAR, retinoic acid receptor; Wt, wild type; *Sfrp*, *secreted frizzled-related protein*.

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Since our previous research has shown that the expression of *LRAT* is much higher in normal cultured human oral epithelial cells than in oral carcinoma cell lines [34], we wanted to determine whether low or absent *LRAT* activity increased the incidence or severity of carcinogen-induced oral carcinogenesis. We developed a mouse model of carcinogenesis in which the carcinogen 4-nitroquinoline 1-oxide (4-NQO) induces both tongue and, at a lower incidence, esophageal squamous cell carcinoma (SCC) [42]. In this model, 4-NQO is added to drinking water for several weeks. Then, 4-NQO is removed and, over the next several weeks, hyperplasias, dysplasias, papillomas and SCCs develop specifically on the tongue and, at a lower frequency, in the esophagus [42]. This murine oral carcinogenesis model is based on the 4-NQO rat model used previously by many researchers (e.g., Makita et al. [43] and Tanaka et al. [44]). One advantage of the murine model over the rat model is that various transgenic and knockout animals can be tested in the murine model. In this study, we investigated whether the carcinogen 4-NQO induced a higher incidence of oral cancer in mice in which the *LRAT* gene is disrupted by homologous recombination [26].

## 2. Materials and methods

### 2.1. Animals

*LRAT* gene knockout ($LRAT^{-/-}$) mice were produced as previously described [26] and bred into a C57BL/6 background. Genotypes were determined by polymerase chain reaction (PCR) from tail genomic DNA [26]. All studies were performed with the approval of the Research Animal Resource Center of Weill Cornell Medical College.

### 2.2. 4-NQO treatment

The carcinogen 4-NQO (Sigma, St. Louis, MO) was used to induce oral carcinogenesis, as previously described by this laboratory [42]. Briefly, freshly prepared 4-NQO stock solution (5 mg/ml in propylene glycol) was added to drinking water at 60 μg/ml, and treatment was started in mice at 6 weeks of age. The 4-NQO-treated group contained 42 mice, including 20 wild-type (Wt) mice (18 males and 2 females) and 22 $LRAT^{-/-}$ mice (19 males and 3 females). The control (not treated with 4-NQO) group contained 15 mice, including 8 Wt mice (6 males and 2 females) and 7 $LRAT^{-/-}$ mice (4 males and 3 females). Only propylene glycol was added to the drinking water for the control group; no 4-NQO was added. The drinking water was freely accessible at all times. After the 8-week 4-NQO treatment had ended, the mice were maintained on regular water (without carcinogen) for another 16 weeks. All of these mice were maintained on standard laboratory chow, which contains 25 IU of retinyl palmitate per gram, throughout the entire experiment. The mice were then sacrificed, and lesions on the tongues were assessed by gross and histological examinations. Three mice, including one Wt male, one $LRAT^{-/-}$ male and one $LRAT^{-/-}$ female, unexpectedly died during the 4-NQO treatment, and they were not included in further data analysis.

In an additional treatment group set up to test the effects of vitamin A deficiency, five Wt mice (two males and three females) and five $LRAT^{-/-}$ mice (three males and two females) were fed a vitamin-A-deficient diet (TD 88407; Harlan Teklad) for 6 weeks during the 4-NQO treatment. Serum retinol levels declined by greater than 90% in the $LRAT^{-/-}$ mice within 2–3 weeks on this diet [26]. In contrast, there was no decline in serum retinol levels in Wt mice during the 6 weeks that they were on this vitamin-A-deficient diet [26]. We purposely used a higher 4-NQO dose and a shorter treatment period (6 weeks) so that the $LRAT^{-/-}$ mice would be only partially vitamin-A-deficient during the 4-NQO treatment. The concentration of 4-NQO in the drinking water for this group of mice was 100 μg/ml, and the duration of 4-NQO treatment was 6 weeks. Normal drinking water and regular vitamin-A-sufficient diet (25 IU/g retinyl palmitate) were resumed after 6 weeks of 4-NQO treatment. After another 16 weeks, the oral tumors in this group were examined as described above.

### 2.3. Bromodeoxyuridine (BrdU) incorporation

At 5 h before sacrifice, five male mice (two Wt and three $LRAT^{-/-}$) that had been fed the vitamin-A-deficient diet for 6 weeks during 4-NQO treatment were injected intraperitoneally with BrdU (cat no. 00-0103; Zymed, South San Francisco, CA) at 1 ml/100 g body weight. Their tongues were dissected and fixed in 4% paraformaldehyde. Tissue slides were then prepared and analyzed for BrdU incorporation using an immunohistochemical detection kit (cat no. 93-3943; Zymed), as previously described [42]. The number of BrdU-positive cells was determined by counting in four to five high-power fields (200×) of each sample. Data from these counts were pooled, and BrdU labeling indices were calculated as the percentage of BrdU-labeled cell nuclei over a total of 300 epithelial cell nuclei in each area.

### 2.4. Semiquantitative and quantitative real-time reverse transcription (RT) PCR

The tongues were dissected and temporarily stored in RNA*later* (Ambion, Austin, TX). When a tumor was present, the entire tumor or part of the tumor, along with adjacent nontumor tongue tissue, was harvested. Total RNA was then extracted from the tissue samples using Trizol reagent (Invitrogen, Carlsbad, CA), and 1 μg of each sample of total RNA was used for RT in a 20-μl reaction using SuperScript II Reverse Transcriptase (Invitrogen). The cDNA produced from RT was diluted 1:5, and 1 μl of the diluted cDNA was used in PCR. All PCRs were performed three times.

The gene-specific primers for semiquantitative RT-PCR are listed as follows: for *LRAT* (GenBank accession number AF255061), the 5′ primer 5-CTGACCAATGACAAGGAACGCACTC-3, the 3′ primer 5-CTAATCCCAAGACAGCCGAAGCAAGAC-3 and 34 cycles were used, and a 370-bp product was expected; for *secreted frizzled-related protein* (*Sfrp*) 2 (GenBank accession number NM_009144), the 5′ primer 5-CAACCTGCTGGGCCACGAGACC-3, the 3′ primer 5-GCTTGCGGATGCTGCGGGAGAT-3 and 40 cycles were used, and a 695-bp product was expected; for *Sfrp4* (GenBank accession number NM_016687), the 5′ primer 5-TCCCTCGAACACAAGTCCCTCTCA-3, the 3′ primer 5-TGCGGCTGGCTATCTGCTTCTTGT-3 and 35 cycles were used, and a 234-bp product was expected; for *Frzb* (GenBank accession number NM_011356), the 5′ primer 5-CGACTTCCAGCACGAGCCCATCA-3, the 3′ primer 5-GCTCTGACAGGCTTACATTTG-3 and 35 cycles were used, and a 261-bp product was expected. The primers were designed around introns to avoid detection of any signal from potential genomic DNA contamination. *36B4* (5′ primer, 5-AGAACAACCCAGCTCTGGAGAAA-3; 3′ primer, 5-ACACCCTCCAGAAAGCGAGAGT-3) was used as control for both semiquantitative RT-PCR (28 cycles were used and a 448-bp product was expected) and quantitative real-time PCR [45]. Semiquantitative RT-PCR was performed using the following conditions: 94°C for 30 s, 58°C for 30 s and 72°C for 1 min, with a final extension at 72°C for 10 min. *Taq* polymerase was purchased from Invitrogen (cat no. 18038-042). PCR products were subjected to 1.2% agarose gel electrophoresis. Gel images, stained with ethidium bromide, were recorded and quantitated with a FluorChem 8800 system (Alpha Innotech, San Leandro, CA).

For quantitative real-time RT-PCR, the reactions were performed in 20 μl, and a Bio-Rad thermal cycler (MyiQ Single-Color Real-Time PCR Detection System) was used. Samples were denatured initially at 95°C for 3 min, and then 46 cycles were performed using the following conditions: 94°C for 15 s, 58°C for 30 s and 72°C for 45 s. SYBR Green fluorescence emissions were recorded at 80°C after each cycle. PCRs were performed in triplicate. Quantitative results were calculated using MyIQ software (Bio-Rad) and normalized to the level of *36B4* mRNA. The primers used in the PCRs are listed as follows: for *Cox-2* (GenBank accession number NM_011198), the 5′ primer 5-GCCCAGCACTTCACCCATCAG-3 and the 3′ primer 5-ATCATCAGACCAGGCACCAGACC-3; for *Cyclin D1* (GenBank accession number NM_007631), the 5′ primer 5-AAGTGCGTGCAGAAGGAGATTGT-3 and the 3′ primer 5-GGATAGAGTTGTCAGTGTAGATGC-3; for *p21* (GenBank accession number NM_007669), the 5′ primer 5-AGGCCCAGTACTTCCTCTGC-3 and the 3′ primer 5-CAATCTGCGCTTGGAGTGATA-3; for *Trop2* (GenBank accession number NM_020047), the 5′ primer 5-CCTGCGCTGCGACGAAGTGGTG-3 and the 3′ primer 5-TCTGCCGAAGCTCTATCTGAATGG-3; for *RARβ* (GenBank accession number NM_011243), the 5′ primer 5-GTCATCGGGCTACCACTAT-3 and the 3′ primer 5-GCTCCGCTGTCATCTCAT-3. These primers detect all *RARβ* transcripts.

### 2.5. Mouse embryonic stem (ES) cells and embryoid bodies (EBs)

The mouse $LRAT^{-/-}$ ES cell line was generated from $LRAT^{+/-}$ ES cells using a method reported previously [26,46]. The $LRAT^{+/-}$ ES cells had been produced previously in this laboratory to make the $LRAT^{-/-}$ mice [26]. The Wt and $LRAT^{-/-}$ ES cell lines were maintained as monolayer cultures in embryonic stem cell culture medium (ESCM) [Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 2 mM glutamine, 1× penicillin/streptomycin (cat no. 10378-016; Invitrogen), 1× nonessential amino acids (cat no. 11140-019; Invitrogen), 1 mM sodium pyruvate (cat no. 11360-070; Invitrogen), 0.1 mM β-mercaptoethanol and leukemia-inhibitory factor (LIF) ($10^3$ U/ml ESGRO; Invitrogen)]. When grown in suspension without LIF, ES cells are able to form spheroid aggregates termed EBs [47–49]. ES cells within developing EBs are able to differentiate into various committed cell types derived from all three primitive layers: endoderm, mesoderm and ectoderm [50,51]. For the formation of EBs, ES cells were trypsinized and seeded at $1\times10^6$ cells per 100-mm ultralow culture dish (Corning, New York) in the ESCM without LIF. The EBs were maintained as a suspension culture in the ESCM without LIF, and the culture medium was refreshed every 48 h. The EBs were harvested at the designated times for gene expression analyses.

### 2.6. Statistical methods

Fisher's Exact Test was employed to compare the incidence of carcinogenesis, and chi-square test was used to compare BrdU incorporation between Wt and $LRAT^{-/-}$ mice. A difference with a $P$ value of <.05 was considered statistically significant. We

Table 1
Oral carcinogenesis after 4-NQO treatment while on a vitamin-A-sufficient diet

| | Incidence[a] (%) | Number of tumors | | | | Size of tumors (mm)[b] | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | 0 | 1 | 2 | ≥3 | <1 | ≥1 | ≥2 | ≥3 |
| Wt ($n$=19) | 73.7 | 5 | 5 | 5 | 4 | 4 | 3 | 4 | 3 |
| *LRAT*$^{-/-}$ ($n$=20) | 80.0 | 4 | 8 | 4 | 4 | 3 | 4 | 4 | 5 |

[a] No statistically different incidence in oral tumors was detected between the Wt mice and the *LRAT*$^{-/-}$ mice ($P$=.716, Fisher's Exact Test). The incidence of tumors was determined by gross examination of the tongues. The samples with visible tumors, regardless of the number and size of the tumors, were counted as one positive case.

[b] If a sample contained more than one tumor, the size of the tumor was indicated by the largest mass on the tongue.

consulted with Dr. Kathy Zhou (WCMC Biostatistics Core Facility) for statistical analysis of our data.

## 3. Results

### 3.1. Effects of 4-NQO on oral carcinogenesis in Wt and *LRAT*$^{-/-}$ mice fed a diet containing a standard vitamin A level (vitamin-A-sufficient diet)

Our studies reported here primarily focused on the effects of the lack of *LRAT* activity in 4-NQO-induced carcinogenesis in animals that had *sufficient* levels of vitamin A in their diets. These *LRAT*$^{-/-}$ mice have diet-derived retinol in their tissues, but they are unable to store retinol as retinyl esters in tissues. The carcinogen 4-NQO induced visible tumors on the surfaces of tongues in both Wt and *LRAT*$^{-/-}$ mice. The overall incidence of oral tumors, including both male and female mice, was 73.7% in Wt mice (14 of 19) and 80.0% in *LRAT*$^{-/-}$ mice (16 of 20). No statistically significant difference was detected between the Wt mice and the *LRAT*$^{-/-}$ mice ($P$=.716, Fisher's Exact Test). Among the male mice, tumors were found on tongues in 70.6% (12 of 17) of Wt mice and in 77.8% (14 of 18) of *LRAT*$^{-/-}$ mice. No significant difference was detected between the Wt mice and the *LRAT*$^{-/-}$ male mice in terms of percentages of mice with oral tumors ($P$=.711, Fisher's Exact Test). Tongue tumors were found in two Wt female (2 of 2) and two *LRAT*$^{-/-}$ female mice (2 of 2). These results are summarized in Table 1. No tumor was found on the tongues of control Wt or *LRAT*$^{-/-}$ mice not treated with carcinogen (0 of 15).

On gross examination, the tumors appeared as single or multiple masses of various sizes, ranging from tiny lesions (diameter <0.1 mm) to large masses (>2.0 mm). The large masses had either a narrow base or a wide base. On gross examination, no metastasis was observed in the liver, lungs and kidneys. However, at these time points, micrometastases, such as the migration of tumor progenitor cells to these organs, cannot be ruled out.

All tongue samples were subjected to further histological examination. The tongues from mice that did not develop visible tumors after 4-NQO treatment appeared normal or mildly hyperplastic. Pathological diagnoses were made in 17 mice that developed tongue abnormalities, including nine samples from Wt mice and eight samples from *LRAT*$^{-/-}$ mice. Among the nine Wt mice, hyperplasia was seen in two cases (2 of 9), dysplasia in two cases (2 of 9), SCC *in situ* in three cases (3 of 9) and invasive SCC in two cases (2 of 9). The *LRAT*$^{-/-}$ mice showed a similar range of pathological changes. Among eight tongue samples from *LRAT*$^{-/-}$ mice, hyperplasia was seen in two cases (2 of 8), dysplasia in three cases (3 of 8), SCC *in situ* in two cases (2 of 8) and invasive SCC in one case (1 of 8). More details of the gross and histological examinations are provided (Fig. 1 and Table 2). Thus, we conclude that in *LRAT*$^{-/-}$ mice fed a diet with adequate vitamin A levels, the inability to store retinol as retinyl esters does not lead to a greater incidence of oral tumors after 4-NQO treatment.

### 3.2. Expression of Cox-2, Cyclin D1, p21, RARβ and Trop2 in the tongue tissue of 4-NQO-treated Wt and *LRAT*$^{-/-}$ mice fed a vitamin-A-sufficient diet

Previous reports have suggested that aberrant expression of the *Cox-2* [52,53], *Cyclin D1* [54–56], *p21* [57], *Trop2* [58] and *RARβ* [6,9] genes was involved in the process of head and neck carcinogenesis. To determine the expression of these genes in the tongues of Wt and *LRAT*$^{-/-}$ mice after 4-NQO treatment while on a vitamin-A-sufficient diet, we examined the mRNA levels of these genes in the mice that developed tumors on their tongues (seven tumor samples from 4-NQO-treated Wt mice and seven tumor samples from 4-NQO-treated *LRAT*$^{-/-}$ mice, all while on a vitamin-A-sufficient diet) using quantitative real-time RT-PCR. Transcripts of *Cox-2*, *Cyclin D1*, *p21* and *Trop2* were detected from all samples (7 of 7 in Wt mice and 7 of 7 in *LRAT*$^{-/-}$ mice). No consistently different pattern was detected between the 4-NQO-treated Wt tissue samples and the *LRAT*$^{-/-}$ tissue samples. The level of *RARβ* mRNA was very low in all of the tissue samples. The fluorescent signal for *RARβ* mRNA passed the threshold of detection in three Wt samples (3 of 7) and three *LRAT*$^{-/-}$ samples (3 of 7). The real-time

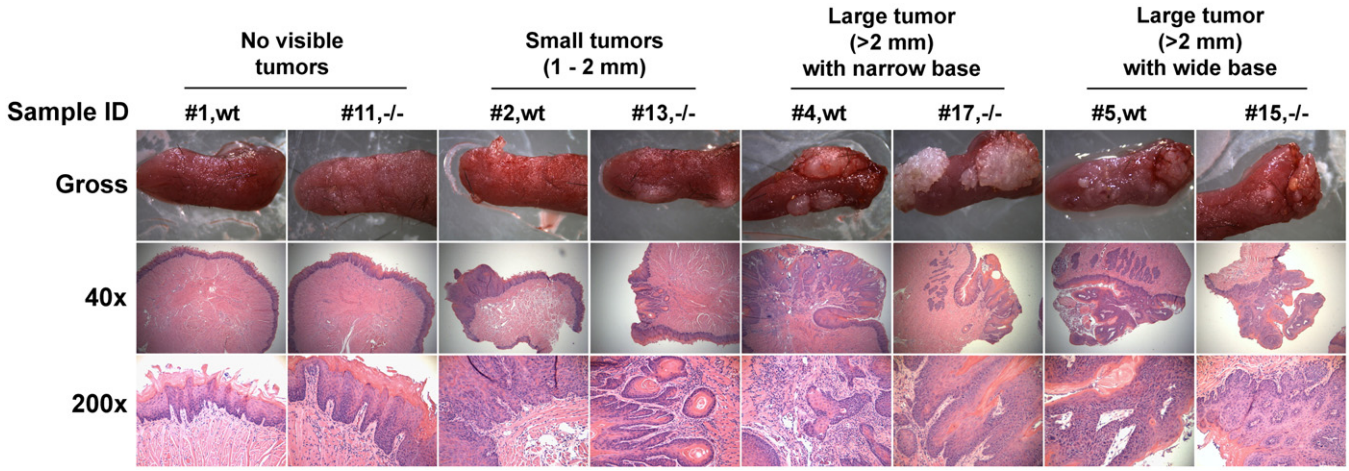


Fig. 1. Gross and histological morphologies of mouse tongues after 4-NQO treatment while on a vitamin-A-sufficient diet. Eight tongue samples from Wt and *LRAT*$^{-/-}$ male mice that were treated with 4-NQO. The genotypes and pathological diagnoses are as follows: Sample 1, Wt, normal epithelium; Sample 11, *LRAT*$^{-/-}$, hyperplasia; Sample 2, Wt, SCC *in situ*; Sample 13, *LRAT*$^{-/-}$, invasive SCC; Sample 4, Wt, invasive SCC; Sample 17, *LRAT*$^{-/-}$, moderate dysplasia; Sample 5, Wt, SCC *in situ*; Sample 15, *LRAT*$^{-/-}$, moderate dysplasia. More details are shown in Table 2. (−/−) *LRAT*$^{-/-}$.

Table 2
Examination of the tongues after 8 weeks of 4-NQO treatment, followed by 16 weeks without 4-NQO[a]

| Sample ID[b] | Gender | Genotype[c] | Gross examination | Pathological diagnosis |
|---|---|---|---|---|
| 1 | Male | +/+ | No visible lesions | Normal |
| 2 | Male | +/+ | 1.0×1.0 mm×2/dorsal | *In situ* SCC |
| 3 | Male | +/+ | 2.0×2.0 and 1.0×1.0 mm/dorsal | Mild dysplasia |
| 4 | Male | +/+ | 2.5×2.5 mm/dorsal; 1.0×1.0 mm/ventral | Invasive SCC |
| 5 | Male | +/+ | 2.5×2.5 mm and two tiny lesions/dorsal | *In situ* SCC |
| 6 | Male | +/+ | 2.0×3.0 mm/dorsal | *In situ* SCC |
| 7 | Male | +/+ | 2.5×3.5 and 1.0×1.0 mm/dorsal | Invasive SCC |
| 8 | Male | +/+ | 3.5×3.5 mm/dorsal | Moderate dysplasia |
| 9 | Female | +/+ | 1.5×1.5 mm/dorsal | Hyperplasia |
| 10 | Female | +/+ | Multiple tiny lesions/dorsal and ventral | Hyperplasia |
| 11 | Male | −/− | No visible lesions | Hyperplasia |
| 12 | Male | −/− | 1.5×1.5 mm and multiple tiny lesions/dorsal | Mild dysplasia |
| 13 | Male | −/− | 1.0×2.0 mm/lateral | Invasive SCC |
| 14 | Male | −/− | 2.5×2.5 mm/dorsal | Hyperplasia |
| 15 | Male | −/− | 2.5×3.5 and 1.0×1.0 mm/dorsal | Moderate dysplasia |
| 16 | Male | −/− | 2.5×3.5 mm/dorsal; 1.5×1.5 mm/ventral | *In situ* SCC |
| 17 | Male | −/− | 3.0×3.5 and 2.5×2.5 mm/dorsal | Moderate dysplasia |
| 18 | Female | −/− | 1.5×1.5 mm/dorsal | Hyperplasia |
| 19 | Female | −/− | 5.0×5.0 mm/dorsal | *In situ* SCC |

[a] All mice were on a vitamin-A-sufficient diet throughout the experiment. The controls (Wt and *LRAT*$^{-/-}$ mice NOT treated for 8 weeks with 4-NQO but on a vitamin-A-sufficient diet throughout the experiment) were uniformly negative for tumors. The samples without pathological diagnoses are not included in this table.
[b] The sample IDs used in this table also apply to Figs. 1, 3 and 4.
[c] (+/+) Wt; (−/−) *LRAT* null mice.

RT-PCR results from eight 4-NQO-treated tissue samples, including two tumor-negative tongue samples and six tumor-positive tongue samples, are shown (Fig. 2). These tongue tumor samples were selected for analysis based on their comparable gross morphology and *36B4* mRNA levels.

### 3.3. Expression of transcripts of the Sfrp family in tongue tumors from 4-NQO-treated Wt and *LRAT*$^{-/-}$ mice fed a vitamin-A-sufficient diet

Additional genes, including *LRAT* and members of the *Sfrp* gene family, were examined by semiquantitative RT-PCR. Under the PCR conditions indicated in Materials and Methods, *LRAT* mRNA was detected in all seven tongue samples of the Wt mice that developed tongue tumors (7 of 7). No *LRAT* mRNA was detected from *LRAT*$^{-/-}$ samples, as expected (Fig. 3A).

The *Sfrp* gene family encodes antagonists of the WNT pathway [59,60]. Some members of the *Sfrp* gene family are silenced in human SCC of the head and neck [61,62]. *Sfrp2* mRNA was detected in three tongue tumor samples from Wt mice (3 of 7) and in four tongue tumor samples from *LRAT*$^{-/-}$ mice (4 of 7). The *Sfrp2* mRNA levels detected in the tumor samples from *LRAT*$^{-/-}$ mice were consistently higher than those in the tumor samples from Wt mice. The semiquantitative RT-PCR results from representative samples are shown (Fig. 3A).

We also assayed mRNA levels of *Sfrp1*, *Sfrp4* and *Frzb*, which are other members of the *Sfrp* gene family. *Frzb* mRNA was detected in one tongue sample (moderate dysplasia) from 4-NQO-treated Wt mice (1 of 7). In the 4-NQO-treated *LRAT*$^{-/-}$ mice, *Frzb* transcripts were detected in three tongue samples (3 of 7) that showed lesions with inflammation (Sample 25) or moderate dysplasia (Samples 15 and 17) (Fig. 3B). *Sfrp1* and *Sfrp4* transcripts were detected in tongue tumor samples from both Wt and *LRAT*$^{-/-}$ mice (3 of 3 in Wt mice and 3 of 3 in *LRAT*$^{-/-}$ mice), but no difference in *Sfrp1* or *Sfrp4* expression was detected between the Wt tissue samples and the *LRAT*$^{-/-}$ tissue samples (data not shown).

In order to determine how the expression of these *Sfrp* family members correlates with the function of the WNT pathway during oral carcinogenesis, we examined the mRNA levels of all 19 murine *WNT* members in the tongue samples from Wt and *LRAT*$^{-/-}$ mice. The following *WNT* members were also examined by semiquantitative PCR: *WNT1, WNT2, WNT2b, WNT3, WNT3a, WNT4, WNT5a, WNT5b, WNT6, WNT7a, WNT7b, WNT8a, WNT8b, WNT9a, WNT9b, WNT10a, WNT10b, WNT11* and *WNT16*. We did not find any difference in the mRNA levels of these *Wnt* genes between Wt and *LRAT* gene knockout mice (data not shown).

### 3.4. Expression of Sfrp2, Sfrp4 and Frzb transcripts in mouse ES cells and EBs

To assess the physiological significance of the changes in gene expression, we assessed the levels of the *Sfrp2*, *Sfrp4* and *Frzb* gene transcripts in undifferentiated mouse Wt and *LRAT*$^{-/-}$ ES cells and in differentiated cells within EBs (Days 6 and 12). In undifferentiated ES cells (Day 0), the level of *Sfrp2* mRNA in Wt ES cells was lower than the level of *Sfrp2* mRNA in *LRAT*$^{-/-}$ ES cells. However, upon EB differentiation (Day 12), the *Sfrp2* mRNA level in Wt EBs was higher than that in *LRAT*$^{-/-}$ EBs (Fig. 3C). The highest level of *Sfrp4* mRNA was detected in undifferentiated ES cells (Day 0). *Sfrp4* mRNA levels decreased in both Wt and *LRAT*$^{-/-}$ EBs, but a greater decrease was observed in *LRAT*$^{-/-}$ EBs as compared to Wt EBs (Day 12) (Fig. 3C). No *Frzb* mRNA was detected in undifferentiated Wt or *LRAT*$^{-/-}$ ES cells (Day 0). The *Frzb* mRNA level increased in both Wt and *LRAT*$^{-/-}$ EBs, but a more marked increase was detected in *LRAT*$^{-/-}$ EBs (Day 12) (Fig. 3C). These results suggest that changes in the levels of expression of *Sfrp* gene family members occur during the differentiation of both Wt and *LRAT*$^{-/-}$ ES cells, and that the patterns of these changes are different between the Wt cells and the *LRAT*$^{-/-}$ cells.

### 3.5. Effects of a vitamin-A-deficient diet on 4-NQO-induced carcinogenesis and cell proliferation

Five Wt mice (two males and three females) and five *LRAT*$^{-/-}$ mice (three males and two females) were fed a vitamin-A-deficient diet during the entire 6-week 4-NQO treatment. On this diet, only *LRAT*$^{-/-}$ mice — and not the Wt mice — showed a large (>90%) decrease in serum vitamin A levels [26]. Tumors were found on the tongues of three Wt mice (60%) (2 of 2 of the males; 1 of 3 of the females) and on the tongues of all five *LRAT*$^{-/-}$ mice (100%) (3 of 3 of the males; 2 of 2 of the females) ($P$=.22, Fisher's Exact Test). Although the difference between the Wt mice and the *LRAT*$^{-/-}$ mice is not statistically significant because of small sample groups, there is a trend showing that partial retinol deficiency makes mice more sensitive to carcinogen-induced tumorigenesis.

BrdU immunostaining was performed on the tongue tissue sections of Wt and *LRAT*$^{-/-}$ mice that were fed the vitamin-A-deficient diet during the 6 weeks of 4-NQO treatment and subsequently developed tumors. The regions of the tongues that appeared grossly normal were subjected to BrdU staining. Three tongue tissue samples from control Wt mice that were fed the regular vitamin-A-sufficient diet and were not treated with 4-NQO were stained as controls. After BrdU injection, cells are expected to incorporate BrdU in S-phase, so this assay is a measure of cell proliferation [63]. The BrdU labeling index was calculated and expressed as the percentage of BrdU-positive nuclei divided by the total nuclei counted under a microscope [42]. As compared to the BrdU labeling index (7.1±0.9%, mean±S.E.M.) calculated from the control Wt mice (not treated with 4-NQO) on a vitamin-A-sufficient diet, treatment with 4-NQO while on a vitamin-A-deficient diet

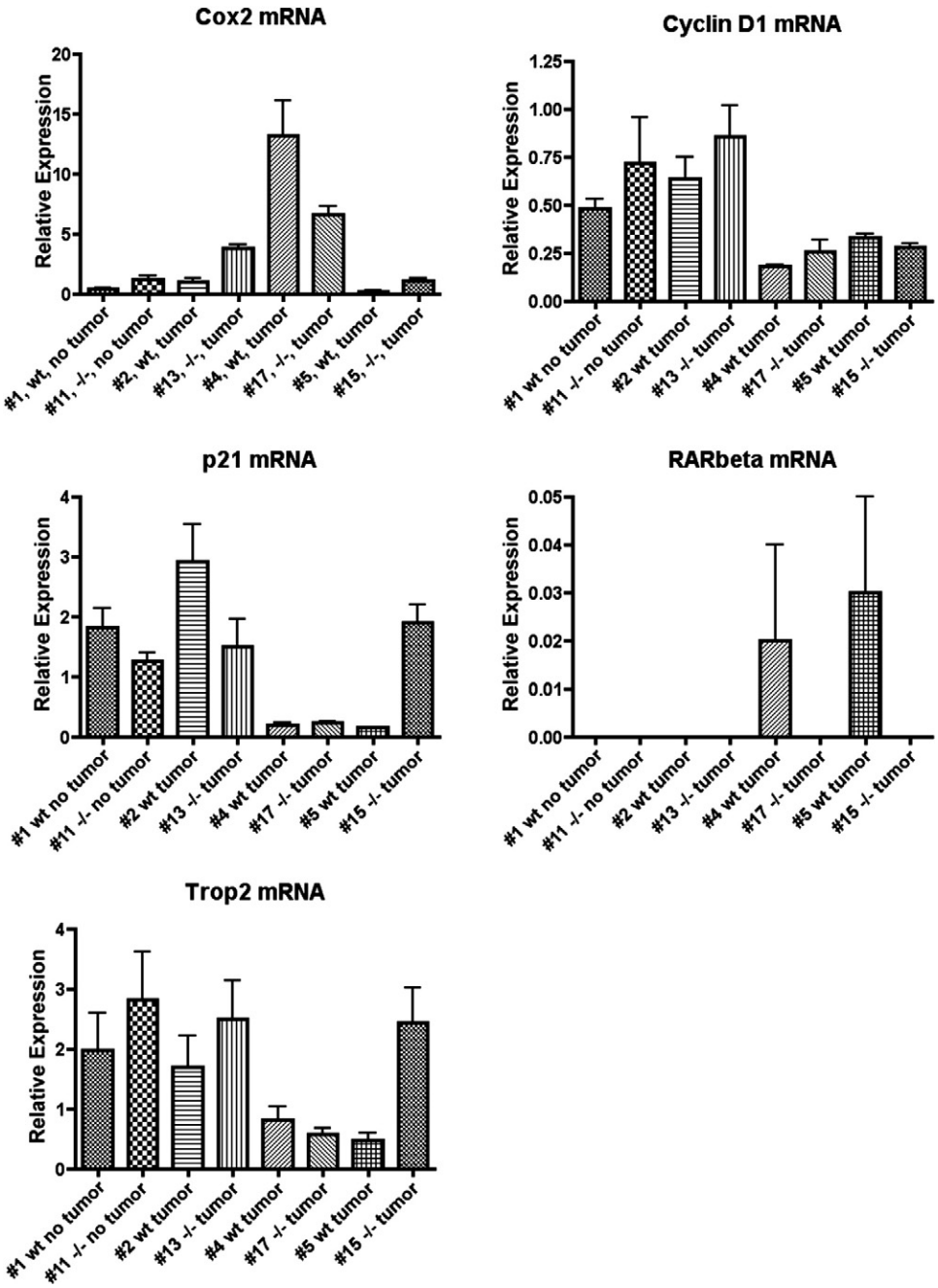


Fig. 2. Examination of *Cox-2*, *Cyclin D1*, *p21*, *RARβ* and *Trop2* mRNA levels by real-time quantitative RT-PCR. All mice were on a vitamin-A-sufficient diet through the experiment. Total RNA was isolated from the samples of tongues that developed visible tumors (Wt: Samples 2, 4 and 5; $LRAT^{-/-}$: Samples 13, 17 and 15) versus those without visible tumors (Wt: Sample 1; $LRAT^{-/-}$: Sample 11) after 4-NQO treatment. Each real-time PCR was performed in triplicate. Means and standard deviations were calculated and normalized to the level of *36B4* mRNA, and graphs were plotted. The gross morphology of each sample can be found in Table 2. (−/−) $LRAT^{-/-}$.

resulted in a statistically significant increase in the BrdU labeling index in the tongue tissue sections of only the $LRAT^{-/-}$ mice. The BrdU labeling index of the tongue tissues from $LRAT^{-/-}$ mice (21.0±2.4%) was significantly higher than the BrdU labeling index of the tongue tissues from Wt mice (9.9±0.8%) after 4-NQO treatment while on a vitamin-A-deficient diet ($P<.001$, chi-square test) (Fig. 4). This result indicates that a greater proportion of proliferating cells was detected in the tongue epithelial tissues from the $LRAT^{-/-}$ mice than in the tongue epithelial tissues from the Wt mice. As discussed above, the serum retinol in Wt mice did not decrease over the 6-week treatment while on the vitamin-A-deficient diet; thus, the fact that the BrdU labeling index in these tissues was not different from the BrdU labeling index in the negative control Wt mice (7.1±0.9%) was not surprising. The representative fields of BrdU-immunostained tissues that correspond to the grossly normal tongue tissues adjacent to the tumors are shown (Fig. 4).

## 4. Discussion

The process of the malignant transformation of the human oral epithelium is frequently accompanied by a reduced expression of *LRAT* [34]. Although most retinol is converted into retinyl esters in the liver and lung, *LRAT* functions in many human epithelial tissues,

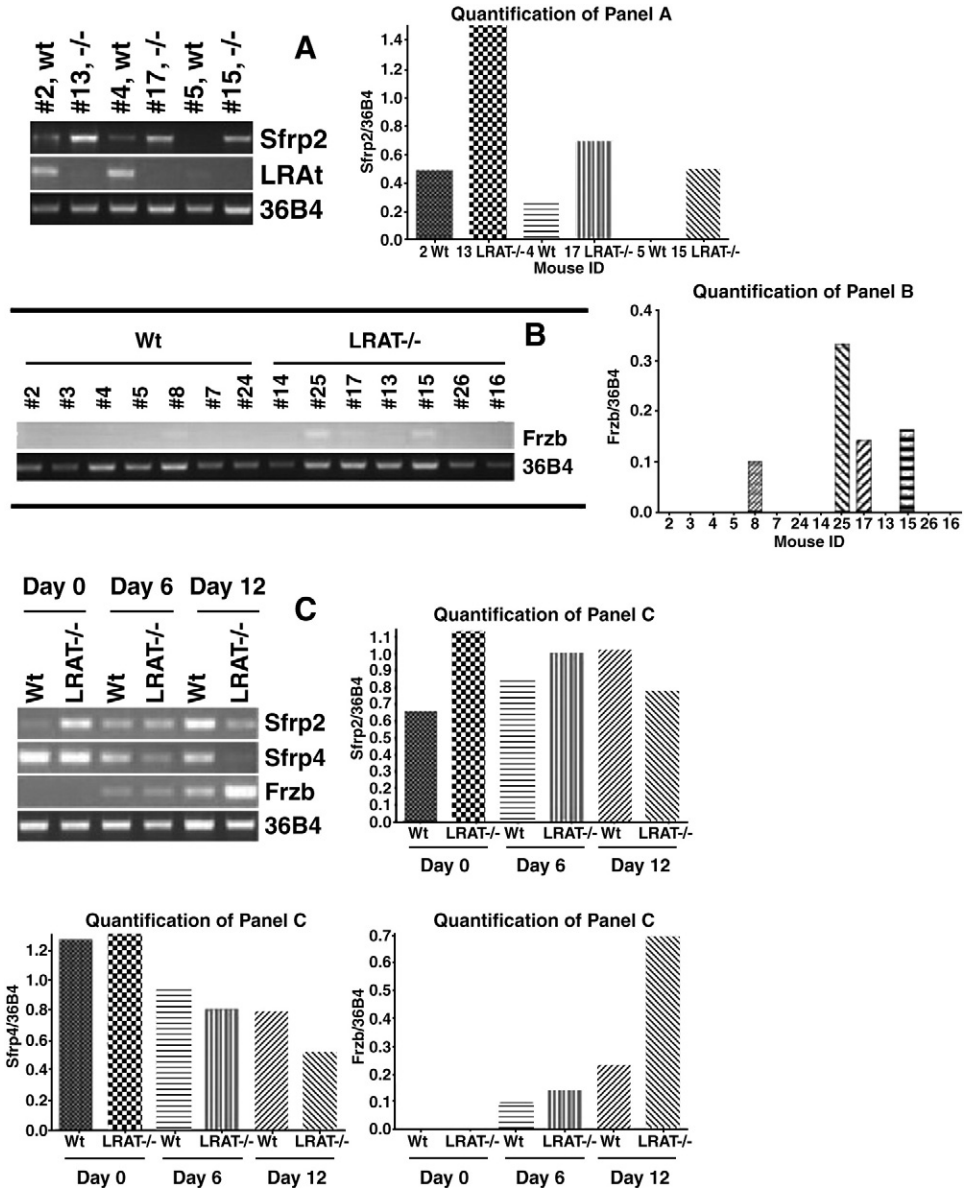


Fig. 3. Examination of gene expression in tongue tumors and EBs by semiquantitative RT-PCR. All mice were on a vitamin-A-sufficient diet throughout the experiment. (A) The level of *LRAT* and *Sfrp2* mRNA in six representative tongue tumor samples (Wt: Samples 2, 4 and 5; $LRAT^{-/-}$: Samples 13, 17 and 15). Higher levels of *Sfrp2* mRNA were detected in the tongue tumors of $LRAT^{-/-}$ mice (Samples 13, 17 and 15) as compared to the tongue tumors of Wt mice (Samples 2 and 4). No *Sfrp2* mRNA was detected from Sample 5 (Wt). *LRAT* mRNA was detected in all samples from Wt mice (Samples 2, 4 and 5), but not in samples from $LRAT^{-/-}$ mice. Sample 5 showed an *LRAT* band with a greater number of RT-PCR cycles; a faint band can be seen in (A). (−/−) $LRAT^{-/-}$. (B) The level of *Frzb* mRNA in tongue samples. *Frzb* mRNA was detected in one tongue sample (Sample 8) from Wt mice and in three tongue samples (Samples 15, 17 and 25) from $LRAT^{-/-}$ mice. The pathological changes in these tongue samples are as follows: Sample 8, moderate dysplasia; Sample 15, moderate dysplasia; Sample 17, moderate dysplasia; Sample 25, inflammation. (C) Measurement of *Sfrp2*, *Sfrp4* and *Frzb* mRNA levels in cultured undifferentiated murine Wt and *LRAT*-null ES cells and EBs. Wt and $LRAT^{-/-}$ ES cells (Day 0) and EBs (Days 6 and 12) were harvested. Total RNA was extracted, and the mRNA levels of *Sfrp2*, *Sfrp4* and *Frzb* were examined by RT-PCR. Quantitation of RT-PCR blots in (A)–(C) is shown to the right of each panel. Quantitation was performed using Alpha Innotech FluorChem software, and data were normalized to *36B4* mRNA levels.

including the oral epithelium [34]. We also showed that *LRAT* plays a role in retinol uptake by epithelial cells [34]. Therefore, it is important to determine whether the loss of *LRAT* expression, via knockout of the *LRAT* gene in all tissues of the mice by homologous recombination [26], contributes directly to the process of malignant transformation.

Reduced retinoid signaling has been implicated in the malignant transformation of human oral epithelia [6–9], and the cancer chemopreventive effects of RA have also been observed in animal experiments and in humans [15,64,65]. Our results show that loss of *LRAT* expression does not directly affect the process of carcinogenesis when mice are given adequate retinol in their diet. However, the absence of the *LRAT* gene causes mice to be much more susceptible to vitamin A (retinol) deficiency [26,38,39]. Although inactivation of the *LRAT* gene itself did not result in a statistically significant increase in oral cancer incidence in $LRAT^{-/-}$ mice fed a vitamin-A-sufficient diet as compared to Wt mice fed a vitamin-A-sufficient diet (Tables 1 and 2), our results show a statistically significant increase in the number of proliferating tongue epithelial cells in $LRAT^{-/-}$ mice that were fed a vitamin-A-deficient diet during the 4-NQO treatment, as compared to Wt mice fed the same diet during 4-NQO treatment. This is striking because this BrdU measurement was performed 16 weeks after the diet had been changed back to a vitamin-A-sufficient diet (Fig. 4). Thus, secondary vitamin A deficiency resulting from the lack of the *LRAT* gene in *LRAT* gene knockout mice may lead to a reprogramming of basal epithelial cells that lasts for many weeks and results in increased proliferation and hyperplasia. This hypothesis is also

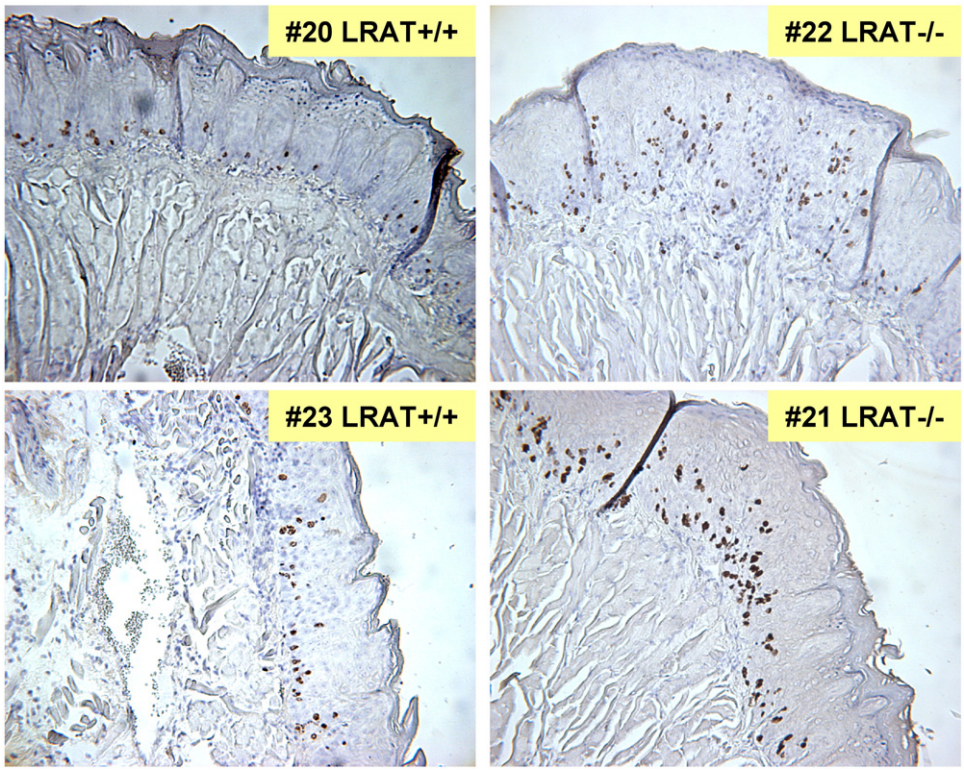


Fig. 4. BrdU staining of tongue tissue sections from Wt and *LRAT*$^{-/-}$ mice. The levels of BrdU in tongue epithelia were analyzed by immunohistochemical staining. Two samples from Wt mice (Samples 20 and 23) and two samples from *LRAT*$^{-/-}$ mice (Samples 21 and 22) are shown. The Wt and *LRAT*$^{-/-}$ mice were fed a vitamin-A-deficient diet during 4-NQO treatment for 6 weeks and then maintained on a vitamin-A-sufficient diet and regular drinking water for another 16 weeks. BrdU was injected intraperitoneally at 5 h before sacrifice. The images represent tongue tissues near the tumor. The number of BrdU-positive cells was determined by counting in four to five high-power fields (200×) of each sample. Data from these counts were pooled, and BrdU labeling indices were calculated as the percentage of BrdU-labeled cell nuclei over a total of 300 epithelial cell nuclei in each area. Chi-square test was used to compare BrdU incorporation between Wt and *LRAT*$^{-/-}$ mice.

supported by our data showing a trend toward a higher oral cancer incidence in *LRAT*$^{-/-}$ mice (5 of 5) as compared to Wt mice (3 of 5) fed a vitamin-A-deficient diet during the 4-NQO treatment.

To identify the molecular regulatory mechanisms underlying the carcinogenesis process in Wt and *LRAT*$^{-/-}$ mice, we examined the mRNA levels of many genes that have been reported to play a role in the process of head and neck carcinogenesis. The patterns of *Cox-2*, *Cyclin D1*, *p21*, *Trop2* and *RARβ*$_2$ gene expression indicate that these genes are not expressed differently in carcinogen-treated Wt versus *LRAT*$^{-/-}$ mice, consistent with the lack of any difference in tumor incidence between Wt and *LRAT*$^{-/-}$ mice when both are on a retinol-sufficient diet.

In contrast, the levels of *Sfrp2* mRNA were higher in *LRAT*$^{-/-}$ tumor tissues than in Wt tumor tissues from mice on the normal vitamin-A-sufficient diet (Fig. 3A). Increased WNT signaling that activates oncogene pathways through β-catenin is seen in many cancer tissues, including cancer of the oral cavity [66,67]. Silencing of the *Sfrp2* gene via epigenetic inactivation by promoter hypermethylation is frequently observed in human oral SCCs [61,62]. We found that *Sfrp2* transcripts are higher in tongue tumors from *LRAT*$^{-/-}$ mice than in tongue tumors from Wt mice (Fig. 3A), suggesting that expression of genes that encode some of the antagonists of the WNT pathway is inversely correlated with *LRAT* gene expression. The pattern of *Sfrp2* mRNA expression in tongue tumors from Wt versus *LRAT*$^{-/-}$ mice resembles the pattern of *Sfrp2* mRNA expression in cultured Wt versus *LRAT*$^{-/-}$ ES mouse cells (Fig. 3A and C). The *Sfrp2* gene is transcriptionally activated by RA via RARγ in cultured F9 teratocarcinoma cells; since *Sfrp2* mRNA increases in response to RA in F9 Wt cells even in the presence of the protein synthesis inhibitor cycloheximide, *Sfrp2* is a direct target of RA [68,69]. These data from our laboratory provide another link between *Sfrp2*, WNT signaling and retinoid signaling.

The pattern of *Frzb* transcript expression in tongue samples also suggests that WNT signaling is differentially regulated in Wt and *LRAT*$^{-/-}$ mice. Our results suggest a cross-talk between vitamin A signaling (via the level of *LRAT*) and the WNT signaling pathway during the process of malignant transformation of the oral cavity.

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