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Eosinophil depletion protects mice from tongue squamous cell carcinoma induced by 4-nitroquinoline-1-oxide

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Summary. Aims: Tumor-associated tissue eosinophilia (TATE) has been correlated with prognosis in oral squamous cell carcinoma (OSCC). This study aimed to investigate whether eosinophils depletion affects experimental oral carcinogenesis.

Methods and Results: BALB/c (wild type - WT) and eosinophil-deficient (*Δdb*/GATA-1) mice were treated with the carcinogen 4-nitroquinoline-1-oxide (4NQO) in drinking water for 28 weeks. Tongues were collected for histopathological and immunohistochemical analysis, as well as for the evaluation of cytokines/chemokines by ELISA. The tongue SCC induced by 4NQO was associated with a rise in eosinophil numbers. WT-treated group showed a significantly increased incidence of SCC, with higher cytological atypia, in comparison with *Δdb*/GATA-1 mice. Consistently, the proliferative index was higher in WT compared to the *Δdb*/GATA-1/GATA-1-treated group. No significant changes in the concentration of CCL3, CCL11 and TNF-α were detected for both groups after 4NQO treatment. Conclusions: These results suggest that eosinophils might be responsible for the deleterious outcome of experimental tongue carcinogenesis, given that their ablation protects mice from OSCC.

Key words: Eosinophil, Squamous cell carcinoma, Tonge, GATA, 4NQO

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Introduction

Eosinophils are considered multifunctional leucocytes that participate in innate and adaptive immune responses (Hothenberg and Hogan, 2006; Gatault et al., 2012). After different stimuli, including carcinogens, eosinophils are recruited to inflammatory sites, releasing a variety of cytotoxic proteins, interleukins (e.g. TNF-α) and chemokines (e.g. CCL5 and CCL11) (Hothenberg and Hogan, 2006; Martinelli-Kläy et al., 2009). Some of these molecules induce pro-inflammatory effects, such as upregulation of adhesion mechanisms, cellular trafficking and vascular permeability, which may promote angiogenesis and, thus, favor tumor growth (Munitz and Schaffer-Levi, 2004; Hothenberg and Hogan, 2006; Martinelli-Kläy et al., 2009).

The increased number of infiltrated eosinophils in tumors (also called Tumor-Associated Tissue Eosinophilia-TATE) has been implicated as a prognostic factor in human cancers (Munitz and Schaffer-Levi, 2004; Oliveira et al., 2011; Gatault et al., 2012). However, the exact role of eosinophils in malignant tumors is controversial (Martinelli-Kläy et al., 2009; Pereira et al., 2010; Gatault et al., 2012). While some authors suggested that TATE may be correlated with better prognosis (Goldsmith et al., 1992; Dorta et al., 2002), others reported their association with worse tumor evolution (Wong et al., 1999; Alrawi et al., 2005; Said et al., 2005; Oliveira et al., 2011) or even no effect (Oliveira et al., 2009; Tadbir et al., 2009). In part, these controversies could be attributed to some limitations associated to human studies, such as differences in

genetic background, tumor staging, and also in the criteria used to assess the density of eosinophils (Dorta et al., 2002; Alkhabuli and High, 2006; Falconieri et al., 2008).

In this regard, the use of mouse models of squamous cell carcinoma (SCC) could be an alternative to reduce some variables associated with clinical samples. The tongue SCC model induced by 4-nitroquinoline-1-oxide (4NQO) administration in drinking water is characterized by significant DNA damage and dysplastic changes in the tongue epithelium (Tang et al., 2004; Lu et al., 2006). This process culminates in neoplastic transformation, mimicking several parameters of the human condition (Tang et al., 2004; Kanojia and Vaidya, 2006; Carvalho et al., 2012; Moon et al., 2012).

In the current study the involvement of eosinophils in SCC was investigated employing an experimental model of tongue SCC induced by 4NQO in eosinophil-depleted mice (*Δdb*/GATA-1 deficient-mice).

Materials and methods

Mice

Twenty-five *Δdb*/GATA-1 (eosinophil-deficient mice) (donated by Dr. A. Humbles, Harvard, Boston, MA) (Humbles et al., 2004) and BALB/c (wild type – WT) male mice were bred in the animal facility of Universidade Federal de Minas Gerais, Brazil. *Δdb*/GATA-1 and BALB/c mice present the same genetic background (Humbles et al., 2004; Jackson laboratory, available at http://jaxmice.jax.org/strain/005653.html). Mice were maintained under standard conditions with a 12 h light/dark cycle, controlled temperature (24±2°C) and had free access to commercial chow and drinking water. All experimental procedures described in the current study were approved by the institutional Ethics Committee (CETEA/UFMG-protocol number 12/2011). The mice were weighed weekly and no changes in their weight were observed during the experimental period.

Induction of SCC by 4NQO

The mouse model of tongue carcinogenesis used in the current study was adapted from the protocol described by Tang et al. (2004). Briefly, 4-Nitroquinoline-1-oxide (4NQO), obtained as a powder (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in ethylene glycol (Sigma-Aldrich) to a final concentration of 50 μg/ml and stored at 4°C. The stock solution was prepared weekly and diluted in the drinking water of mice.

Experimental mice (9 WT and 6 *Δdb*/GATA-1) received treatment with 4NQO daily for 28 weeks. The control groups (n=5 per group) received drinking water without 4NQO during the same period. The mean intake of water was recorded, but there was no difference between the groups. After 28 weeks of carcinogen treatment, mice were euthanized and tongue, cervical lymph nodes, liver, stomach, duodenum, jejunum, ileum and large intestine were collected for microscopic analysis.

Light microscopy

Tongue, cervical lymph nodes, liver, stomach, duodenum, jejunum, ileum and large intestine tissues were fixed in 10% buffered formalin, embedded in paraffin wax and cut longitudinally (3 μm). The slides were deparaffinized, rehydrated, and stained with H&E. The tongue lesions were classified using the following score (adapted from Barnes et al., 2005): 0 - normal, 1 - mild dysplasia (changes limited at basal third of the lining epithelium), 2 - moderate (when the changes represented two-thirds of the lining epithelium), 3 - severe (more than two-thirds of the epithelium affected), 4 - carcinoma in situ (full thickness of the lining epithelium, but without involvement of the connective tissue) and 5 - invasive carcinoma (carcinomatous islands into the connective tissue). For microscopic analysis, 20 consecutive fields were evaluated by two examiners (S.J.M and S.T.A) blinded to the group status. To validate the reliability of the inter- and intra-examiner evaluations, the Intraclass Correlation Coefficient test was performed and there were significant positive correlations ($p<0.001$). The other organs were also evaluated for general histopathological analysis by a pathologist (R.M.A).

Eosinophils staining technique

For the eosinophil staining, the Sirius Red staining protocol was followed (Meyerholz et al., 2009), adapted from the original method published by Llewellyn (1970) with elimination of the sodium chloride step (Llewellyn, 1970). Slides were incubated in Harris hematoxylin (two minutes) and rinsed in tap water followed by a rinse in 100% ethanol. The slides were then immersed in an alkaline (pH 8-9) Sirius Red solution (Sigma-Aldrich, CI 35780) for two hours and rinsed in tap water. Stained eosinophils were counted in 20 consecutive fields of the epithelium lining area (including a third of lamina propria, under epithelial layer), at x 400 magnification. Results were expressed as a total number of eosinophils per sample.

Ki67 and PCNA immunohistochemistry and cell counting

Immunohistochemistry was performed using the streptavidin-biotin method. Briefly, serial sections of tongue tumors were deparaffinized, rehydrated and rinsed in distilled water. They were then incubated with 0.3% hydrogen peroxide twice for 15 min. For antigen retrieval, the slides were incubated in citric acid buffer (pH=6.0) at 96°C for 20 min. The slides were incubated at 4°C overnight with the monoclonal mouse anti-human Ki67 antibody (clone MM1; Novocastra, Newcastle, UK) at 1:50 or the monoclonal mouse anti-human PCNA

antibody (clone 124; Dako, Glostrup, Denmark) at 1:800. The immunolabeling was visualized through incubation in 3,3-diaminobenzidine (DAB) solution (Dako). Finally, the sections were stained with Mayer's hematoxylin and covered. Negative controls were obtained by omission of the primary antibody, which was substituted by 1% PBS-BSA.

The immunostained cells were analyzed by light microscopy (Axioskop 40 ZEISS; Carl Zeiss, Gottingen, Germany) at 1.000x magnification and counted in the basal and suprabasal epithelial layers in the total area of 20 consecutive fields in two sections. A proliferative index of Ki67 and PCNA positive cells was obtained by adding the results of basal and suprabasal epithelium layers.

ELISA and MPO activity

Tongue lesional samples were also collected for immunoenzimatic assays. The samples were weighed and homogenized in phosphate buffered saline (0.4 mM NaCl and 10 mM $NaPO_4$) containing protease inhibitors (0.1 mM PMSF, 0.1 mM benzethonium chloride, 10 mM EDTA, and 0.01 mg/mL aprotinin A) and 0.05% Tween-20 at 100 mg/mL. The homogenate was centrifuged (8.946 x g) at 4°C for 10 min. The supernatant was then collected and stored at -70°C until further analysis. The concentration of CCL3, CCL5, CCL11 and TNF-α was measured using commercially available enzyme-linked immunosorbent assay (ELISA) kits, according to the manufacturer's instructions (R&D Systems, Minneapolis, MN, USA). The results were expressed as picograms of cytokines (± S.E.M.) normalized for 100 mg tissue.

The MPO activity, a neutrophil enzyme marker, was also evaluated in homogenized tongue tissues by enzymatic reaction, as previously described (Queiroz-Junior et al., 2009). The MPO contents were expressed as relative units calculated from standard curves based on the MPO activity from 5% casein peritoneal-induced neutrophils.

Statistical analysis

Results were expressed as the mean ± standard error mean (SEM). Statistical analysis was performed using the software GraphPad Prism 5.0. The non-parametric unpaired Student t test and analysis of variance (ANOVA) followed by *Student-Newman-Keuls post hoc* analysis were performed. Results with $p<0.05$ were considered statistically significant.

Results

4NQO treatment increases the number of eosinophils in tongue lesions

The first step of the current study was to investigate whether the induction of SCC by 4NQO was associated with the presence of eosinophils in tongue lesions. Indeed, the number of Sirius Red-stained eosinophils was significantly increased in tongue samples of WT-treated mice compared to their respective non-treated controls ($p<0.05$) (Fig. 1). No eosinophils have been detected in tongue samples of *Δdb*/GATA-1 mice.

Histopathological investigation of tongue SCC and metastatization after 4NQO treatment

After detecting increased numbers of eosinophils in tongue lesions induced by 4NQO, potential differences between WT and eosinophil-deficient (*Δdb*/GATA-1) mice were evaluated. The histopathological analysis of tongue samples showed that WT mice treated with 4NQO for 28 weeks (Fig. 2D-E) presented pronounced cytological atypia (pleomorphism, atypical mitosis, hyperchromatism) and islands of squamous cells invading the connective tissue, in comparison with the control (Fig. 2B), in which no changes in epithelium architecture have been observed. *Δdb*/GATA-1 mice also presented significant changes in tongue epithelium after treatment with 4NQO as indicated by the histological score (Fig. 2A), but these alterations were less

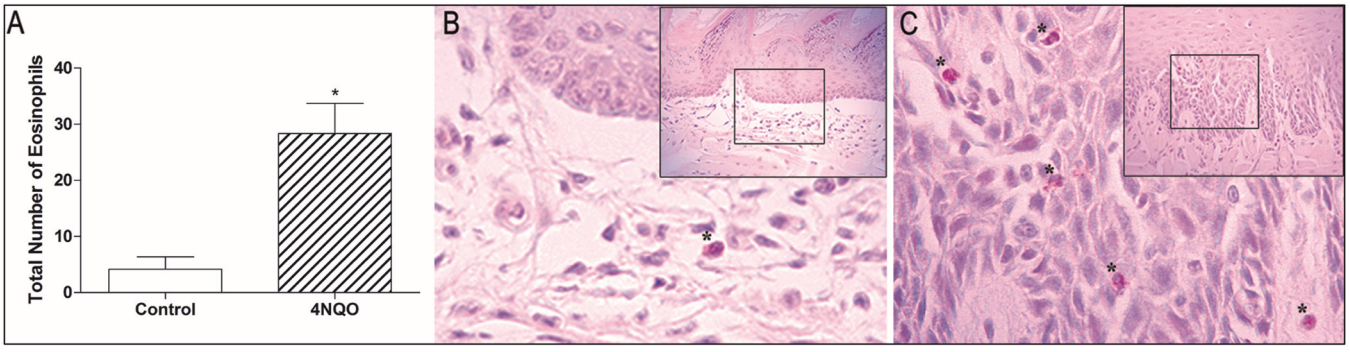

Fig. 1. Detection of eosinophils in tongue SCC induced by 4NQO. **A.** Total number of Sirius Red-stained eosinophils in tongue samples of WT control and 4NQO-treated mice. * $p<0.05$ in relation to control; unpaired Student's t test; **B and C.** Representative photomicrographs of WT control and 4NQO-treated tongue samples, respectively. Asterisks indicate Sirius Red-stained eosinophils.

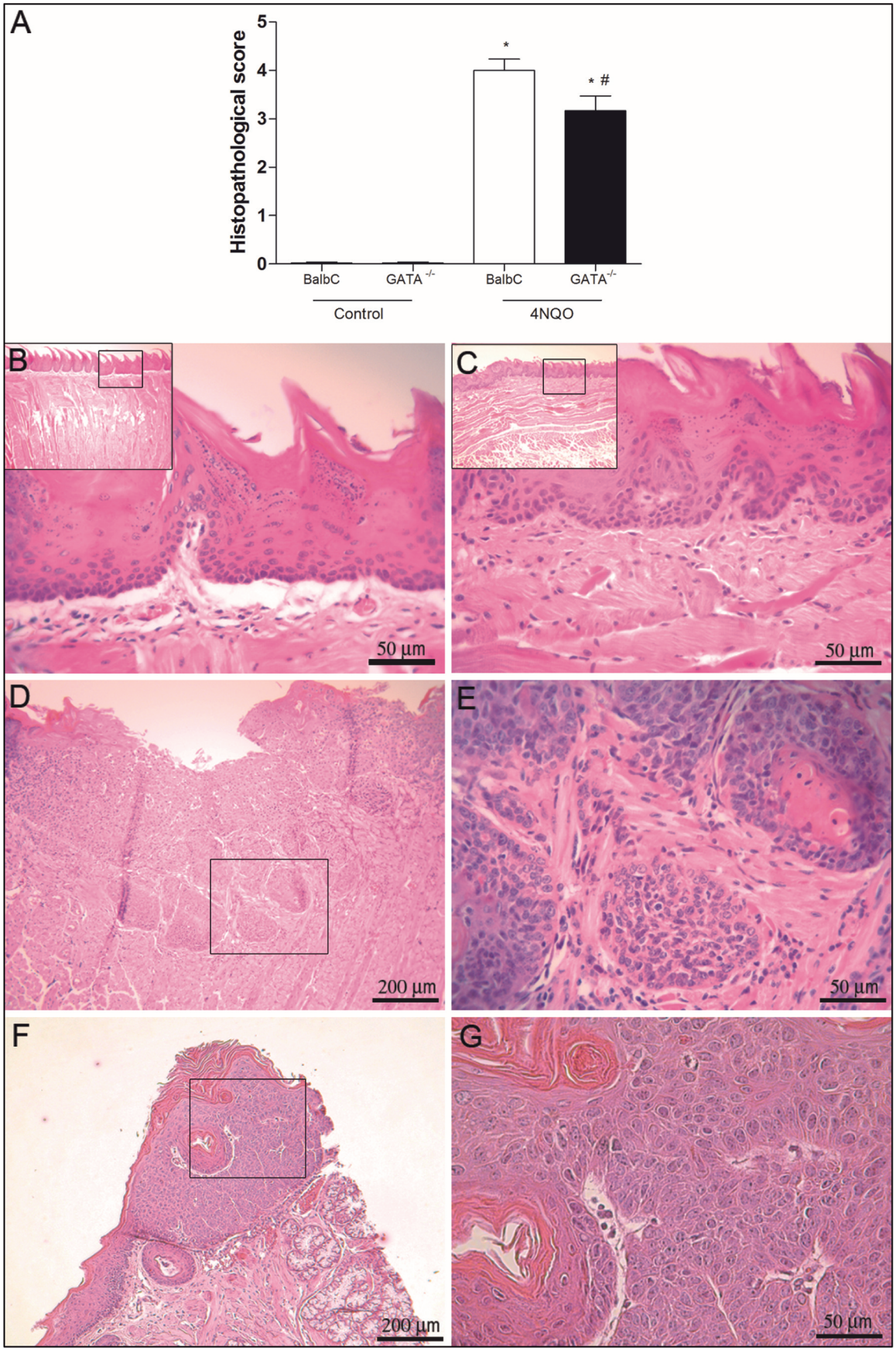

Fig. 2. Histopathological evaluation of tongue samples of control and experimental mice. **A.** Histopathological scores of tongue samples. * indicates $p<0.05$ versus the respective control group. # indicates $p<0.05$ versus WT-treated group. **B and C** show representative photomicrographs of WT and *Δdb*/GATA-1control tongue samples, respectively. **D and F.** Representative photomicrographs of WT and *Δdb*/GATA-1 tongue samples of mice treated with 4NQO for 28 weeks. **E and G** correspond to the higher increase of the squares indicated in D and F, respectively.

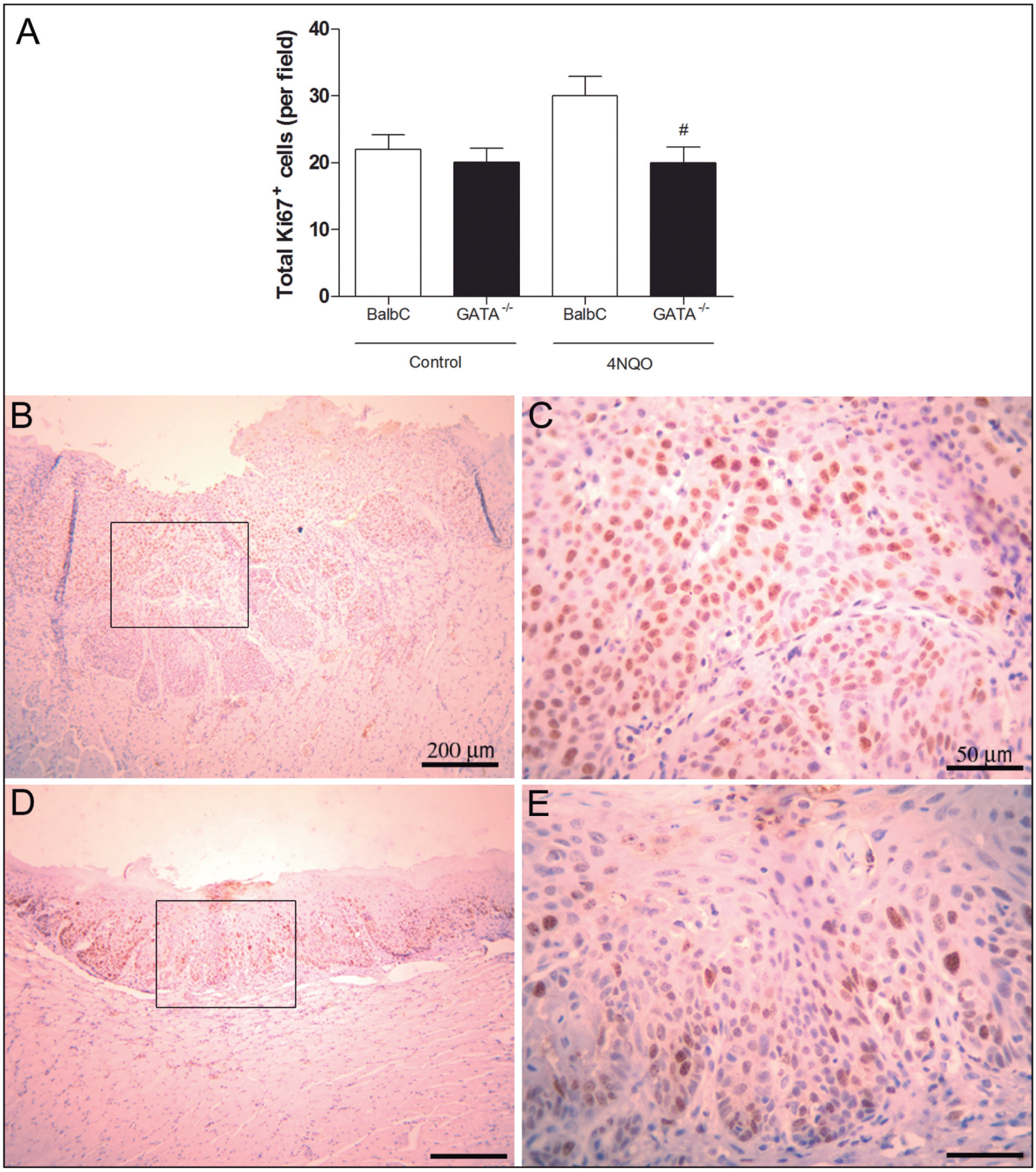

Fig. 3. Immunohistochemical expression of Ki67 in the tongue of mice. **A.** Quantification of total Ki67 positive cells in control and experimental groups. # p<0.05 when compared to WT-treated mice. **B and D** represent WT and *Δdbl*/GATA-1 tongue samples of mice treated with 4NQO for 28 weeks. **C and E** correspond to the higher view of the squares in panels B and D, respectively.

pronounced in comparison to WT mice (Fig. 2F-G). WT-treated mice had 78% of lesions graduated with scores 4 and 5, while *Δdb*/GATA-1-treated mice presented better histopathological scores (67% scores 2 and 3). Tongue samples of WT and *Δdb*/GATA-1 treated mice were also graduated histopathologicaly until the twentieth week, but there were no differences between these groups (data not shown).

Consistently with these findings, the immuno-expression of Ki67, a proliferative marker, in the epithelial cells was significantly increased after 4NQO-treatment in WT when compared to *Δdb*/GATA-1 mice ($p<0.05$) (Fig. 3A-E). The number of PCNA immunostained cells was also significantly increased in WT-treated compared to *Δdb*/GATA-1-treated group, (Fig. 4).

The histopathological analysis of cervical lymph nodes, liver, stomach, duodenum, jejunum, ileum and large intestine of WT and *Δdb*/GATA-1 mice revealed no occurrence of metastasis. The liver samples of WT-treated mice presented variable degrees of hepatocyte tumefaction, steatosis and hemorrhage. Intestine samples presented variable degrees of inflammation in the lamina propria. These histopathological alterations were less pronounced in *Δdb*/GATA-1-treated mice (data not shown). Analysis of the stomach of WT-treated mice (n=2) showed squamous cell carcinoma in the aglandular portion of the organ (Fig. 5). In contrast, no histopathological alterations have been detected in the stomach of *Δdb*/GATA-1-treated mice (Fig. 5).

Inflammatory parameters in 4NQO-induced tongue SCC

In addition to the evaluation of microscopic aspects of the lesions induced by 4NQO, some inflammatory parameters were also investigated. The MPO activity (a neutrophil-enzyme marker) in tongue samples was marginal and there were no differences when comparing the experimental and control groups (Fig. 6).

The concentrations of the chemokines CCL3, CCL5, and CCL11 in tongue samples did not differ between WT control and WT-treated mice. Nevertheless, the levels of CCL3 and CCL11 were significantly decreased in *Δdb*/GATA-1-treated mice in comparison to the WT-treated group (Fig. 7A and B, respectively). The levels of CCL5 were significantly smaller in *Δdb*/GATA-1-treated mice than the respective control (Fig. 7C). The expression of TNF-α was also reduced in the *Δdb*/GATA-1-treated group when compared to the WT-treated mice, but this result reached no statistical significance (Fig. 7D).

Discussion

The major findings of the current study can be summarized as follows: i) the treatment of mice with 4NQO induced invasive tongue SCC, which was associated with increased numbers of eosinophils; ii) mice lacking eosinophils (*Δdb*/GATA-1) had OSCC with better histopathological scores and lower proliferative indexes than the respective WT mice.

In the current study, 4NQO, a water soluble chemical carcinogen, was used to induce SCC in the tongue of mice. The classical advantages of employing this carcinogen are its solubility and its ability to induce genetic damage (DNA adduct formation and DNA strand breaks) that culminate in neoplastic transformation similar to humans (Vered et al., 2003; Ribeiro et al., 2004; Tang et al., 2004). It also causes damage similar to tobacco (Kanojia and Vaidya, 2006; Lu et al., 2006), one of the major risk factors for SCC (Massano et al., 2006). Herein, 4NQO treatment in drinking water induced histopathological SCC features in the tongue epithelium of WT mice in a time-dependent manner, given that the histopathological scores found after 28 weeks were worse than those observed after 20 weeks of treatment (data not shown). Indeed, some authors support that chemical carcinogenesis is a multi-step process, which involves the long-term exposure to one or more chemical carcinogens (Boyd and Reade, 1988). A previous report has shown that 4NQO induces clinical signs of SCC slowly (Gannot et al., 2004). In the current study, mice did not exhibit significant clinical changes in their tongue surfaces and in the mean body weight after 4NQO treatment. Furthermore, no evidence of metastasis has been detected as previously described (Tang et al., 2004). In line with this, a recent study demonstrated lymph node metastasis in this model, but the dose used was 4 times greater than that employed in the current study (Li et al., 2012). Despite these clinical data, mice exhibited pronounced epithelial atypia and unfavorable histopathological scores, consistent with SCC. Accordingly, the immunoexpression of the proliferative markers Ki67 and PCNA was significantly

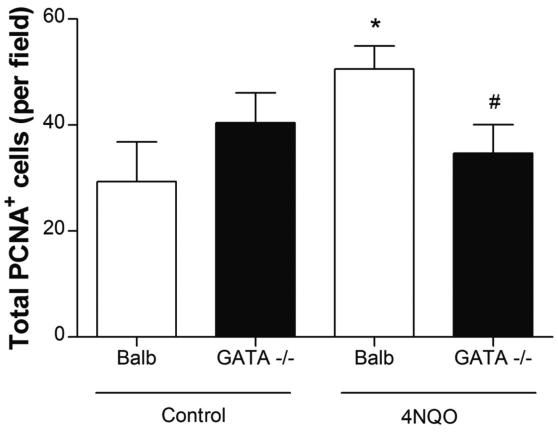

Fig. 4. Immunohistochemical expression of PCNA in the tongue of mice. Quantification of total PCNA positive cells in WT and *Δdb*/GATA-1 tongue samples of mice treated or not with 4NQO for 28 weeks. * $p<0.05$ when comparing the WT-treated mice to the respective control. # $p<0.05$ when compared to WT-treated mice.

increased in WT-treated mice. The expression of these markers is strongly related to increased cellular proliferation and neoplastic conversion during SCC development (Silva et al., 2007; Fracalossi et al., 2011).

All these histopathological parameters induced by the treatment with 4NQO were associated with increased numbers of eosinophils, when compared with the control. These findings suggested for the first time that eosinophils could be associated with the development of tongue SCC in this experimental model.

The presence of eosinophils in human cancers has been reported in different anatomical sites (Fernándes-Aceñero et al., 2000; Cuschieri et al., 2002; Spiegel et al., 2002; Tadbir et al., 2009). Nevertheless, the precise role of TATE in SCC is controversial. Several studies have shown that TATE may be correlated with better (Goldsmith et al., 1992; Dorta et al., 2002), worse (Wong et al., 1999; Alrawi et al., 2005; Said et al., 2005) or even may have no influence on SCC prognosis (Oliveira et al., 2009; Tadbir et al., 2009). Here, after detecting increased numbers of eosinophils in tongue lesions induced by 4NQO, the involvement of this cell type in tumor development was explored using *Δdb*/GATA-1 mice. This mouse lineage is known to be devoid of eosinophils due to an engineered deletion of a palindromic double-enhancer binding site for GATA proteins in the gene encoding GATA-1 (Humbles et al., 2004). The data showed that the treatment of these eosinophil-deficient mice with 4NQO induced histopathological signs of SCC in their tongue, but these signs were significantly decreased when compared with WT mice. The lack of eosinophils was associated with a

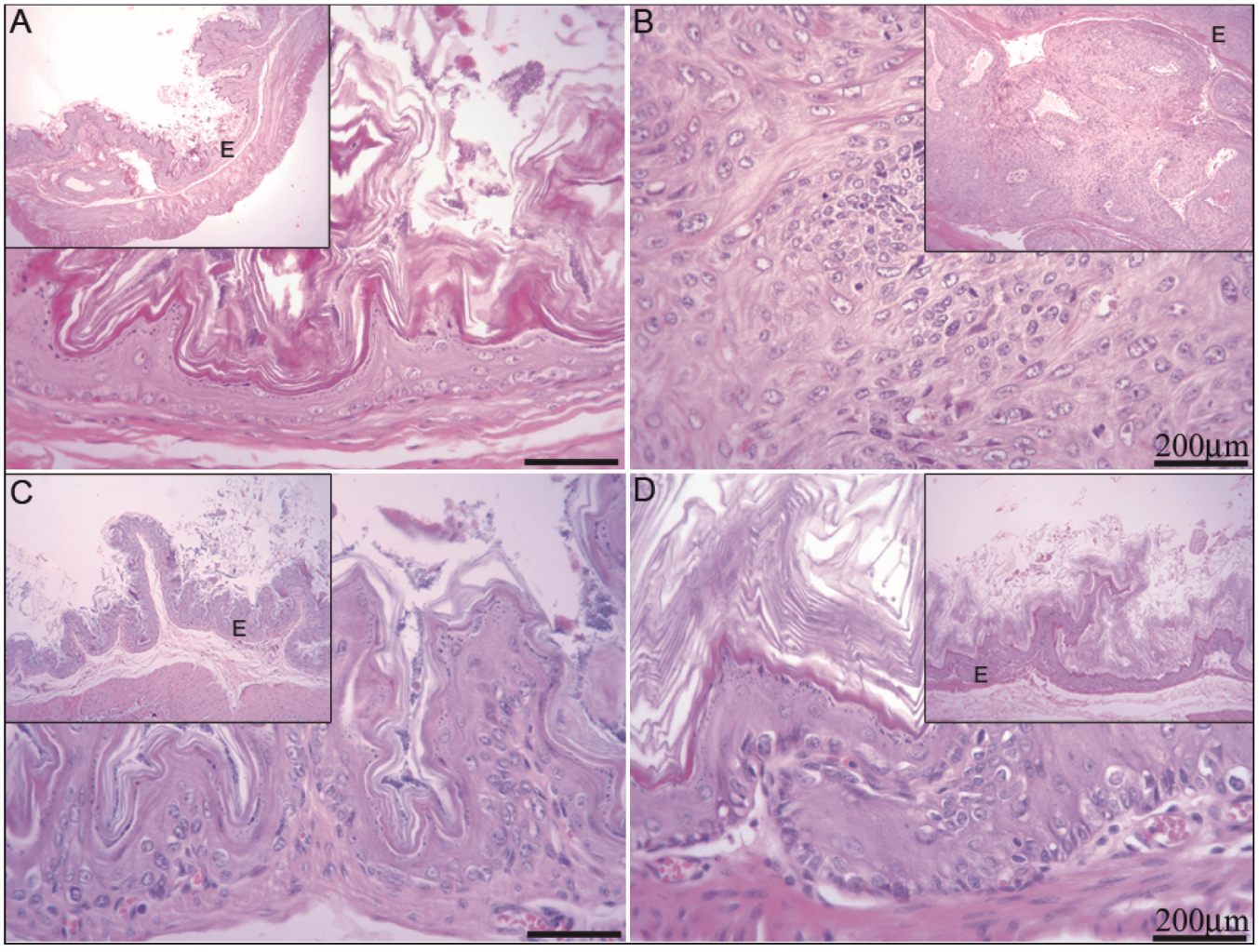

Fig. 5. Histopathological analysis of stomach of mice after 4NQO treatment. **A.** Microscopical appearance of stomach of WT. **B.** Histopathological analysis of the stomach of WT-treated mice showing squamous cell carcinoma in the aglandular portion of the organ. **C.** *Δdb*/GATA-1 stomach sample of control mice. **D**. No evidence of SCC was seen in *Δdb*/GATA-1-treated mice. E: epithelium stomach surface. x 400; insets, x 100

better status of SCC after 28 weeks of 4NQO treatment. *Δdb*/GATA-1 mice also seemed to present protection against potential systemic commitment induced by 4NQO, i.e. these mice did not present alterations in other evaluated organs, except for the tongue, in contrast to WT mice, which presented malignant transformation of the stomach epithelium in some cases. These findings are in line with the study of Wong and colleagues (1999), who found that the ablation of TATE by administration of anti-interleukin-5 monoclonal antibody in hamsters induced smaller tumor burden and delayed the onset of SCC development in these animals (Wong et al., 1999).

Eosinophils are multifunctional leucocytes involved in cytotoxicity, inflammatory processes, tissue remodeling and modulation of immune responses (Gatault et al., 2012). Moreover, eosinophils are considered to be active components of peritumoral and intratumoral inflammatory infiltrate (Dorta et al., 2002; Hogan, 2007; Martinelli-Kläy et al., 2009). Under specific stimuli, eosinophils are able to promote directly or indirectly the release of several inflammatory mediators, including cytokines and chemokines (CCL2, CCL3, CCL5 and CCL11) that may recruit inflammatory cells to the tumor environment and allow the modulation of the immune responses (Hothenberg and Hogan, 2006; Gatault et al., 2012). In this regard, the induction of SCC lesions by 4NQO did not trigger an increase in the expression of CCL3, CCL5, CCL11 and TNF-α in the tongue of mice in the evaluated time-point. These data are in agreement with some evidence from the literature, indicating that the experimental model of 4NQO-induced tongue is not associated with marked expression of inflammatory mediators in tumor sites (Gannot et al.,

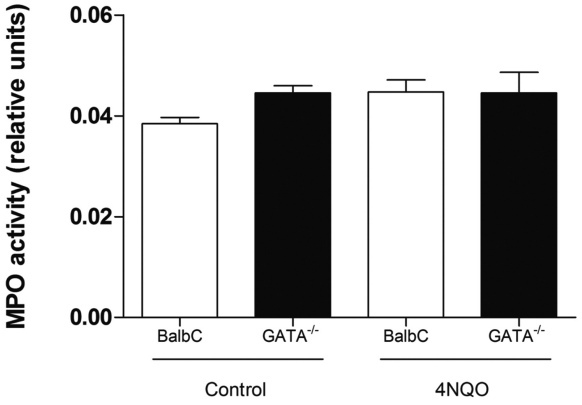

Fig. 6. Myeloperoxidase activity in tongue samples of mice. The presence of neutrophils was indirectly measured by MPO activity. The MPO expression in both experimental and control groups was marginal and there were no differences among the groups.

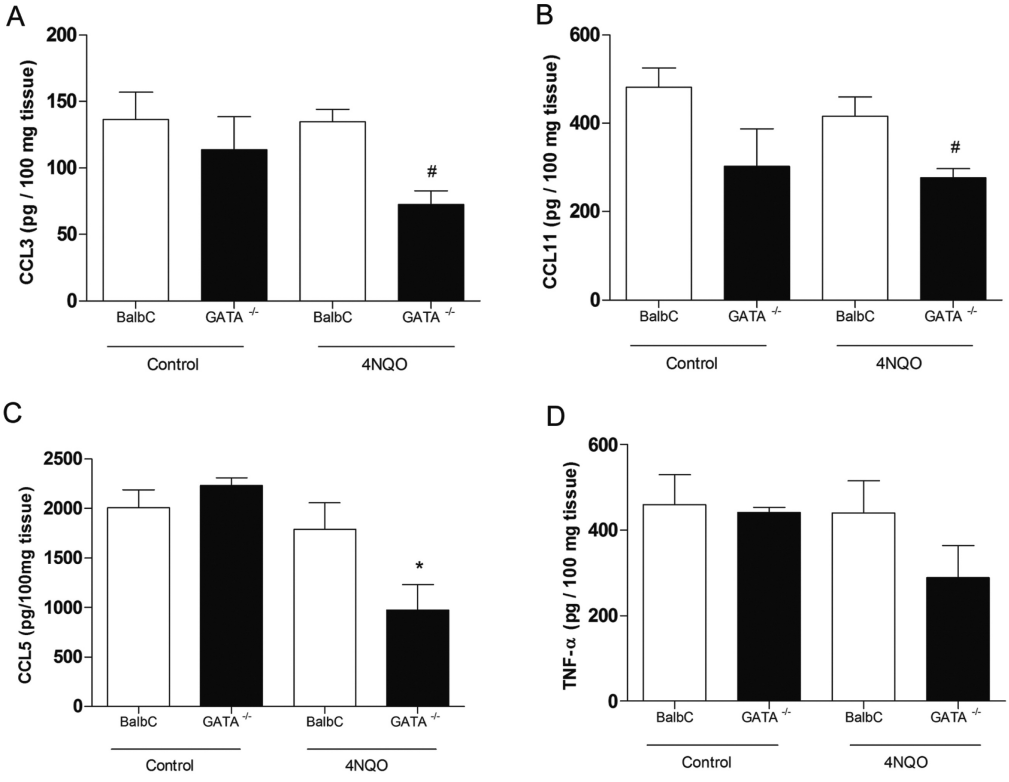

Fig. 7. Inflammatory mediators induced by 4NQO in tongue samples. **A-D.** Concentration of CCL3, CCL11, CCL5 and TNF-α in control and experimental WT and *Δdb*/GATA-1 mice, respectively. *p <0.05 when compared to the respective control; # p<0.05 when comparing WT- and *Δdb*/GATA-1-treated mice.

2004; Tang et al., 2004; Schoop et al., 2009). One hypothesis is that the expression of such mediators could be increased in the earlier stages of tumor development, although this remains to be addressed in further studies. Nevertheless, the levels of the CC chemokines 3 and 11 were significantly decreased in tongue lesional samples of *Δdb*/GATA-1-treated mice versus WT-treated mice. Moreover, the 4NQO-treatment was associated with reduced expression of CCL5 in *Δdb*/GATA-1-treated mice in relation to the respective control. These results may suggest that the recruitment of inflammatory cells may be partially impaired in *Δdb*/GATA-1-treated mice during carcinogenesis. However, no changes in MPO were observed after 4NQO treatment between the groups. In view of the non-mechanistic nature of the current data, these findings deserve further investigation.

Taken together the current results indicate that eosinophils may participate in tongue carcinogenesis, given that their ablation protected mice from SCC. Studies using mouse models can be helpful in the clarification of the roles of specific cell types in tumoral biology and can provide a better understanding about their involvement in tumor development.

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