Akt Activation Synergizes with Trp53 Loss in Oral Epithelium to Produce a Novel Mouse Model for Head and Neck Squamous Cell Carcinoma

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Abstract

Head and neck squamous cell carcinoma (HNSCC) is a common human neoplasia with poor prognosis and survival that frequently displays Akt overactivation. Here we show that mice displaying constitutive Akt activity (myrAkt) in combination with Trp53 loss in stratified epithelia develop oral cavity tumors that phenocopy human HNSCC. The myrAkt mice develop oral lesions, making it a possible model of human oral dysplasia. The malignant conversion of these lesions, which is hampered due to the induction of premature senescence, is achieved by the subsequent ablation of Trp53 gene in the same cells in vivo. Importantly, mouse oral tumors can be followed by in vivo imaging, show metastatic spreading to regional lymph nodes, and display activation of nuclear factor-κB and signal transducer and activator of transcription-3 pathways and decreased transforming growth factor-β type II receptor expression, thus resembling human counterparts. In addition, malignant conversion is associated with increased number of putative tumor stem cells. These data identify activation of Akt and p53 loss as a major mechanism of oral tumorigenesis in vivo and suggest that blocking these signaling pathways could have therapeutic implications for the management of HNSCC. [Cancer Res 2009;69(3):1099–108] 

Introduction

Head and neck squamous cell carcinoma (HNSCC) is the sixth most common type of cancer worldwide. Although new therapeutic approaches, including fractionated radiotherapy, targeted chemotherapeutic agents, and concurrent radiotherapy and chemotherapy (1–4), have been recently evaluated, the improvement in overall survival in patients with HNSCC is still low. The term HNSCC comprises epithelial tumors that arise in the oral cavity, pharynx, larynx, and nasal cavity, with the main risk factors being alcohol and/or tobacco abuse (5). HNSCC results from the accumulation of numerous genetic and epigenetic alterations that occur in a multistep process. The molecular alterations displayed by human HNSCC affect several pathways that influence cell proliferation, apoptosis, differentiation, angiogenesis, inflammation, immune surveillance, and metastasis. The major pathways involved in HNSCC development include the pRb- and p53-dependent pathways, epidermal growth factor receptor (EGFR), signal transducer and activator of transcription 3 (Stat3), nuclear factor-κB (NFκB), and transforming growth factor β (TGFβ); reviewed in refs. 6, 7). Moreover, the initiation, growth, recurrence, and metastasis of HNSCC, as in many other solid epithelial cancers, have been related to the behavior of a small subpopulation of “tumor-initiating” or cancer stem cells (8, 9). In spite of the fact that the molecular mechanisms of HNSCC are not completely understood, several candidate genes of potential therapeutic relevance are now being validated through in vitro analyses (6, 10, 11); however, these studies cannot recapitulate the complex nature of HNSCC tumors in vivo. Thus, animal models of HNSCC will become essential tools, providing relevant insights of the molecular perturbations of these tumors. Nonetheless, there are few suitable genetically defined mouse models in which to study the progression of this type of tumor under preclinical settings (6) and that fully recapitulate the molecular characteristics of human HNSCC. Here we present a new HNSCC transgenic mouse model based on the expression of constitutively active Akt kinase combined with the ablation of Trp53 gene in stratified epithelia, which phenocopies the molecular alterations previously found in human HNSCC. The characteristics described here make this model an excellent and unique preclinical tool for the therapeutic management of HNSCC at different steps.

Materials and Methods

Mice and histologic procedures. The generation of Bk5myrAkt and Trp53f/f;K14cre mice and the protocols for genotyping have been previously described (12–15). These mice were in an immunocompetent mixed C57/B6×DBA/2×FVB/n background. All the animal experiments were approved by the Animal Ethical Committee (CEEA) and conducted in compliance with Centro de Investigaciones Energéticas, Medioambientales y Sostenibles (CIEMAT), Madrid; Avenida Complutense 22, E-28040 Madrid, Spain. Phone: 34-914962517; Fax: 34-913466484; E-mail: jesus.paramio@ciemat.es or John DiGiovanni, Department of Carcinogenesis, Science Park-Research Division, University of Texas M. D. Anderson Cancer Center, Smithville, TX 78957. E-mail: jdigiova@mdanderson.org. 

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

Published OnlineFirst January 27, 2009; DOI: 10.1158/0008-5472.CAN-08-3240

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pellet was resuspended in high-calcium medium and left overnight at 37°C. Trypsin (5 min, 37°C) digestion of mice of the different genotypes were digested with 1 unit/mL dispase for each genotype (only nonlesional oral epithelium was observed in control samples from myrAkt compared with nontumoral tissue (Fig. 2A,B)). Cells were then washed in FACS buffer (PBS-1% fetal bovine serum, 0.09% azide) and incubated for 20 min with the indicated primary antibodies or the appropriate control antibodies. When fluorochrome-conjugated antibodies were used, cells were then washed and fixed in 2% paraformaldehyde. Otherwise, cells were washed, incubated with the appropriate fluorochrome-conjugated secondary antibody, and then fixed. Cells were subsequently analyzed in an EPICS XL flow cytometer (Coulter Electronics). Primary antibodies used were rat mAbs anti-CD34 and anti-CD44 (Pharmingen), rabbit polyclonals anti-CD133 (Abcam), and anti-iNp63 (Abcam).

**Results**

The Akt activation has been previously involved in the development and progression of HNSCC (20–23). To further confirm this, the expression of phosphorylated Akt was studied in tissue microarrays containing 84 HNSCC and 59 oral dysplasias from human patients (not shown; examples are provided in Supplementary Fig. S1). We found a significant number of dysplasias (42 of 59) and tumors (41 of 84) showing the increased Akt activity, suggesting that Akt activation is an early event during human HNSCC development.

To analyze the functions of deregulated Akt activity in vivo, we have recently generated transgenic mice expressing myrAkt in the basal layer of stratified epithelia (12, 13). Several founders and all mice corresponding to one of the established myrAkt lines, all of them characterized by displaying the highest levels of Akt activity (13), developed oral cavity lesions that were easily detectable as oral leukoplaikia and erythroplakia in the palate, cheeks, and lips (arrows in Fig. 1A). Microscopic analyses showed hyperplasia of the tongue and palate glands with signs of adenocarcinomatous conversion (Fig. 1A, compare with control in Fig. 1A), in situ carcinomas of the oral mucosa (Fig. 1A), and lip trichoepithelioma (Fig. 1A). We confirmed the expression of the transgene and phosphorylated Akt, indicative of increased Akt activity, in the basal layer of the nonlesional oral epithelia of myrAkt mice (Fig. 1B), which remains in oral dysplasias (Fig. 1B), trichoepithelioma (Fig. 1B), and oral tumors (Fig. 1B). BrdUrd incorporation revealed a mild increase in cell proliferation of myrAkt nontumoral oral epithelia compared with nontransgenic mice (Fig. 2A and B), but we did not find further increase in dysplasias and tumor samples from myrAkt compared with nontumoral tissue (Fig. 2A and B). With respect to the process of epithelial differentiation,
Because deregulated Akt activity may lead to premature senescence (25, 26), we analyzed whether such mechanism might explain the lack of malignant conversion in myrAkt mice. We observed that the areas of malignant tissue display a strong senescence-associated $\beta$-galactosidase activity (SA-$\beta$gal; “I” in Fig. 3A, $n = 10$), suggestive that in these regions premature senescence occurred, whereas the hyperplastic and dysplastic areas were predominantly SA-$\beta$gal negative (“hyp/dysp” in Fig. 3A). In primary oral keratinocyte cultures, a significant number of enlarged (Fig. 3B) and SA-$\beta$gal–positive cells (Fig. 3B) were detected in myrAkt keratinocytes compared with control (Fig. 3B), and myrAkt cultures also displayed a reduced growth rate (Fig. 3C). Akt-induced senescence has been previously associated with the activation of p53-dependent mechanisms (26, 27); in agreement, Western blot and luciferase assays (Fig. 3D) revealed the increased expression of p53 and p53-dependent genes in primary oral myrAkt keratinocytes in parallel with the phosphorylation of Akt and Foxo3a and increased expression of $\beta$-catenin and $\Delta$Np63 (Fig. 3D). The mechanisms leading to premature senescence and thus p53 activation may involve overexpression of $\beta$-catenin (28), the induction of Pml (29–31), and/or the production of reactive oxygen species due to reduced expression and/or inhibition of Foxo3a (32). Analysis of reactive oxygen species by FACS revealed no major differences between control and myrAkt oral keratinocytes (data not shown). On the other hand, Western blot analysis showed that the increased Akt activity of myrAkt cells is associated with the up-regulation of Pml and $\beta$-catenin expression (Fig. 3D), thus suggesting that, in this system, Akt can mediate a premature senescence that is associated with the increased expression of these two proteins.

To functionally show that p53-mediated senescence was responsible for the reduced malignant conversion of oral lesions in myrAkt mice, we bred them with a mouse line bearing p53 loss in stratified epithelial tissues ($Trp53^{F/F};K14Cre$; refs. 14, 15). Whereas somatic ablation of the $Trp53$ gene in stratified epithelia leads to spontaneous tumor development (15) affecting primarily the epidermis and the oral cavity to a much lesser extent (14 of 57), the $Trp53^{F/F};K14Cre;myrAkt$ mice displayed overt malignant tumors in the oral cavity (Fig. 4A), which also affected the palate (Fig. 4A), the ventral side of the tongue (Fig. 4A), and the external and internal sides of the lips (Fig. 4A). Tumor incidence studied in a large cohort of $Trp53^{F/F};myrAkt$ (n = 33), $Trp53^{F/F};K14Cre;myrAkt$ (n = 20), and $Trp53^{F/F};K14Cre;myrAkt$ mice (n = 29) revealed that the complete absence of $Trp53$ leads also to earlier tumor appearance (Fig. 4B).

The use of mouse models as preclinical tools to assess the efficiency of targeted therapies requires that the tumors generated can be followed by in vivo imaging in individualized manner. This is of a particular relevance in the case of tumors arising in places where they can only be detected after necropsy, such as the internal parts of the oral cavity. Moreover, this may allow a more defined quantification of the tumor growth properties and the presence of secondary tumors and/or metastasis. We thus studied whether the $Trp53^{F/F};K14Cre;myrAkt$ mouse tumors can be monitored by positron emission tomography (PET) imaging. On FDG administration, tumors can be easily detected in oral area (arrow in Fig. 4C) and, importantly, the tumor progression at different time points can also be followed in vivo in the same animals (Fig. 4C). In addition, in vivo PET imaging studies also revealed the development of secondary proximal (denoted by yellow arrows in Fig. 4C) and distant tumors (denoted by white arrow in Fig. 4C) in mice.
with time. Histopathologic examination revealed that these distant tumors corresponded to lymph node metastases (Supplementary Fig. S2). These metastases can be detected only when the primary tumors display undifferentiated highly aggressive characteristics (Supplementary Fig. S2A) at advanced stages. The metastatic cells, detected by the expression of keratin K5, affected primarily the proximal lymph nodes located at the submandibular region (Supplementary Fig. S2B–D). In addition, K5-positive cells were also detected infiltrating distal mesenteric lymph nodes (Supplementary Fig. S2E) and forming micrometastatic nodules in the lungs (Supplementary Fig. S2F–F').

We have also generated two other transgenic mouse models. The first one expresses myrAkt and is deficient for pRb in stratified epithelial tissues (RbF/F;K14Cre; ref. 18). The somatic loss of Rb1 in stratified epithelia does not lead to spontaneous tumor formation (18, 33). Neither the pathologic characteristics of the oral lesions that developed in myrAkt mice nor the tumor development rate was affected in the absence of pRb in myrAkt mice (Supplementary Fig. S3). The second model bears the simultaneous ablation of Pten (34) and Trp53 suppressor genes in stratified epithelia (PtenF/F;Trp53F/F;K14Cre). In contrast to the corresponding parentals, mice lacking both Pten and p53 displayed a progressive weakening and died of yet unknown causes or had to be sacrificed due to ethical reasons (Supplementary Fig. S4A). However, all these animals developed tumors in the oral cavity or lips with complete penetrance by 18 weeks of age (Supplementary Fig. S4B). In some instances, only sporadic tumors, primarily localized in the mouth margins, were observed in mice lacking Pten or p53 (Supplementary Fig. S4C and D). Overall, the gross appearance (Supplementary Fig. S4E), localization, and histologic characteristics of the tumors (Supplementary Fig. S4F–H) in double-deficient mice were indistinguishable from those observed in Trp53F/F;K14Cre;myrAkt mice. These results show that the activation of Akt, either by myrAkt expression or by Pten ablation, in conjunction with Trp53 (but not Rb1) loss, leads to the development of malignant carcinomas in the oral region in mice.

We next studied whether p53 loss can overcome the myrAkt-induced premature senescence. The expression of SA-β-gal was almost negligible in tumors from Trp53F/F;K14Cre;myrAkt mice (n = 15; Fig. 5A) compared with the expression of this senescence marker in myrAkt tumors (see Fig. 3). Moreover, the proliferative arrest observed in myrAkt keratinocytes did not take place in Trp53F/F;K14Cre;myrAkt cells (Supplementary Fig. S5). In agreement, proliferation was increased in Trp53F/F;K14Cre;myrAkt compared with myrAkt oral tumors (Fig. 5A; Supplementary Fig. S6), and Trp53F/F;K14Cre;myrAkt primary oral keratinocytes did not display significant SA-β-gal staining (Fig. 5B) or reduce growth rate (not shown). Luciferase experiments also showed that, as expected, the induction of p53-dependent targets was negligible in Trp53F/F;K14Cre;myrAkt keratinocytes (Supplementary Fig. S7). These data also reinforce the suggestion that deregulated Akt contributes to a premature senescence that prevents further malignant conversion and increased proliferation, and that this process can be evaded by the loss of p53.

Because deregulated Akt activity in keratinocytes can also modulate angiogenesis resulting in increased epithelial tumorigenicity (19), we have also analyzed this process in normal oral mucosa, dysplasia, and tumor samples in myrAkt and Trp53F/F;K14Cre;myrAkt mice. CD31 staining of normal oral epithelia of control and myrAkt mice revealed increased formation of blood vessels by the expression of constitutively active Akt (Supplementary Fig. S8 and data not shown). CD31 staining also revealed that
the number blood vessel was enhanced in dysplasias and tumors compared with normal oral epithelia in myrAkt mice (Supplementary Fig. S8 and data not shown). However, we did not detect significant differences in the number of blood vessels in Trp53F/F; K14CremyrAkt compared with myrAkt samples (Supplementary Fig. S8 and data not shown). Similarly, the altered differentiation of oral epithelia promoted by myrAkt expression (Fig. 2C) was not further aggravated by the subsequent p53 loss (Supplementary Fig. S9). These results suggest that the augmented tumor susceptibility and malignancy mediated by ablation of the Trp53 gene in myrAkt mice is not primarily mediated by altered differentiation, nor did it augment angiogenesis.

Among the most common alterations found in human HNSCC are the activation of EGFR and the subsequent activation of the NFκB and Stat3 pathways (35–37). We found by Western blot analysis (Fig. 5C) a mild increase in EGFR expression in myrAkt and Trp53F/F;K14CremyrAkt keratinocytes compared with control cells. On the other hand, activation of EGFR (as observed by Tyr-phosphorylated EGFR; Fig. 5C) was only observed in transgenic samples, although no significant differences were found between myrAkt and Trp53F/F;K14CremyrAkt cells. With regard to the NFκB pathway, we observed decreased expression of IκBα and increased expression of IκB kinase (IKK)-γ, IKKα, p50, and p65/RelA, suggesting increased NFκB activity. This was further corroborated by luciferase experiments (Fig. 5C). With respect to Stat3 activation, no significant changes were observed in Tyr-phosphorylated, active Stat3 in myrAkt oral keratinocytes compared with controls or in luciferase experiments (Fig. 5C). In contrast, Trp53F/F;K14CremyrAkt keratinocytes showed a remarkable increase in both NFκB and Stat3 pathways (Fig. 5C). Indeed, the expression of IκBα was further decreased, whereas the expression of p50, p65/relA, phosphorylated p65, and Tyr-phosphorylated

Figure 3. Premature senescence induction by deregulated Akt activity. A, examples of H&E and SA-β-gal stainings of oral squamous cell carcinoma in myrAkt transgenic mice showing the presence of SA-β-gal–positive areas in tumoral (T) but not in hyperplastic or dysplastic (hyp/dysp) parts. Bar, 100 μm. B, primary oral keratinocyte cultures from control and myrAkt transgenic mice show the presence of enlarged flattened cells in transgenic cultures. SA-β-gal staining shows increased number of senescent cells in transgenic compared with control cultures. C, doubling population of primary keratinocyte cultures derived from control or myrAkt transgenic mice. D, Western blot analysis of primary keratinocytes showing the increased expression of β-catenin, ΔNp63, p53, Puma, Pml, p16Ink4a, and phosphorylated Foxo3a and reduced total Foxo3a in parallel with increased phosphorylation of Akt. Of note, no changes were observed in p19Arf; tubulin was used as a loading control. Luciferase experiments in oral primary keratinocytes showing increased transcription from p21, Noxa, Puma, and mdm2 promoters together with increased activity of p53-responding element.
Stat3 were increased in Trp53<sup>F/F</sup>;K14Cre;myrAkt compared with myrAkt cells (Fig. 5C). As above, these findings were further corroborated by luciferase experiments in primary oral keratinocytes using the same Stat3 and NF-kB responding elements (Fig. 5C). Together, these data indicated that activation of NF-kB and Stat3 takes place in the Trp53<sup>F/F</sup>;K14Cre;myrAkt cells independently of EGFR activation, which showed similar levels in Trp53<sup>F/F</sup>;K14Cre;myrAkt and Trp53<sup>F/F</sup>;K14Cre;myrAkt keratinocytes.

Another common alteration of human HNSCC is the reduced expression of TGFβ type II receptor (TGFβRII; ref. 38). We also observed by Western blot a reduction in TGFβRII expression in myrAkt and Trp53<sup>F/F</sup>;K14Cre;myrAkt oral keratinocytes (Fig. 5C). However, when tumors were studied by immunohistochemistry, we observed that the expression of TGFβRII was reduced in Trp53<sup>F/F</sup>;K14Cre;myrAkt compared with myrAkt oral tumors (Fig. 5D). Finally, we have previously shown that human HNSCC displaying increased Akt activation are characterized by several alterations such as increased expression of cyclin D1, c-myc, β-catenin, and ΔNp63 and reduced Foxo3a expression (22). Western blot analysis of primary keratinocytes (Fig. 5C), luciferase experiments, and immunohistochemistry of tumor samples (Supplementary Fig. S10) confirmed these alterations in Trp53<sup>F/F</sup>;K14Cre;myrAkt tumors and keratinocytes, although no major differences were found between Trp53<sup>F/F</sup>;K14Cre;myrAkt and myrAkt samples. Collectively, these data show that mouse Trp53<sup>F/F</sup>;K14Cre;myrAkt tumors and primary cells recapitulate and phenocopy most of the molecular alterations found in human HNSCC.

The initiation, growth, recurrence, and metastasis of solid tumors, including HNSCC, have been related to the behavior of a small subpopulation of tumor-initiating or cancer stem cells. In human HNSCC, these cells are characterized by the expression of specific markers such as CD44 and CD133 (8, 9). On the other hand, we have recently shown that myrAkt expression leads to an increased number of adult epidermal stem cells of the hair bulge characterized by K15 and CD34 expression (13). Consequently, we monitored whether the expression of myrAkt may affect stem cell behavior in the tumors and how this may be modulated by subsequent p53 loss. We observed increased expression of K15 and CD34 in parallel with Akt activity (Fig. 6A) in tumors derived from Trp53<sup>F/F</sup>;K14Cre;myrAkt mice compared with myrAkt samples (Fig. 6A). We also noticed that the population expressing ΔNp63 in myrAkt tumors (Fig. 6A) is increased due to the expansion into the suprabasal-like compartments in Trp53<sup>F/F</sup>;K14Cre;myrAkt samples (Fig. 6A). Finally, we also observed CD44 and CD133 expression in myrAkt and Trp53<sup>F/F</sup>;K14Cre;myrAkt tumors (Fig. 6A). As in the case of ΔNp63, the number of cells expressing these cancer stem cell markers is increased in Trp53<sup>F/F</sup>;K14Cre;myrAkt samples compared with myrAkt tumors due to the expansion of positive population into suprabasal-like compartments (Fig. 6A, insets). To further corroborate these observations, we also carried out FACS analysis of primary oral keratinocytes. These showed an increase in the population of cells expressing CD34, ΔNp63, and CD44 (Fig. 6B) in myrAkt cultures, which in the case of CD34 is strikingly increased in Trp53<sup>F/F</sup>;K14Cre;myrAkt cells (Fig. 6B). Nonetheless,
Figure 5. Trp53F/F;K14Cre;myrAkt tumors bypass senescence and phenocopy human HNSCC alterations. A, examples of H&E and SA-β-gal stainings of oral squamous cell carcinoma in Trp53F/F;K14Cre;myrAkt mouse showing the almost complete absence of SA-β-gal staining in tumoral (T) and hyperplastic-dysplastic (hyp/dysp) areas. Bar, 100 μm. Bottom, summary of proliferation analysis measured in nonlesional, dysplastic, and tumoral areas of the oral epithelia formed in control (black columns), myrAkt (blue columns), and Trp53F/F;K14Cre;myrAkt (red columns) mice (*, P < 0.01; **, P < 0.005). B, quantitative analysis of SA-β-gal–positive cells in primary oral cultures derived from mice of the quoted genotypes. C, Western blot analysis of primary oral keratinocytes showing the expression of the quoted proteins. Bottom, luciferase activity showing the increased activation of NFκB and Stat3 reporters in Trp53F/F;K14Cre;myrAkt oral keratinocytes. D, immunohistochemistry analysis of TGFβRII expression in myrAkt and Trp53F/F;K14Cre;myrAkt oral tumors showing the reduced expression in Trp53F/F;K14Cre;myrAkt sample. Squared areas, higher magnification of tumoral areas. Bar, 150 μm (D).
no significant changes were observed in the population of cells expressing CD44 or ΔNp63 between myrAkt and Trp53<sup>F/F</sup>;K14Cre;myrAkt keratinocyte cultures (Fig. 6B), indicating that, in both cases, the observed increase in tumors (Fig. 6B) is limited to the in vivo situation. With respect to CD133, we observed, in agreement with previous data of human HNSCC lines (8, 9), that the number of cells expressing this cancer stem cell marker is very low, although it is augmented in myrAkt (4-fold) and further increased (10-fold) in Trp53<sup>F/F</sup>;K14Cre;myrAkt keratinocytes (Fig. 6B). Collectively, these data indicate that in our myrAkt mouse models there is an increase in the population of cells bearing characteristics of epithelial and tumor stem cells, especially in the case of Trp53<sup>F/F</sup>;K14Cre;myrAkt mouse.

**Discussion**

The recent improvements of therapies, using fractionated radiotherapy, targeted chemotherapy, and concurrent radiotherapy and chemotherapy (1–4), have led only to a moderate increase in the overall survival in HNSCC patients. Molecularly targeted therapies are promising in HNSCC management, and several candidate molecules of potential therapeutic relevance are now being validated through in vitro analyses (6, 10, 11). Therefore, in vivo systems aimed to the analysis of these therapies are necessary. However, these approaches have been hindered by a lack of appropriate animal models mimicking these tumors at both the pathologic and molecular levels. In recent years, several possible mouse models for HNSCC have been described. Nevertheless, the
On the transcriptional activity of sustained in Pml(29–31) are likely candidates. Of note, the expression of Pml is involved in premature senescence. Although the mechanism by which Akt activity induces premature senescence in oral lesions is unknown, the mechanism is involved in the induction of p53-dependent radioresistance, tumor cell proliferation, and hypoxia, the three major features of head and neck squamous cell carcinomas (42), indicating that progression to cancers occurs rapidly when the TGFβRII-null epithelial tissues are exposed to activated oncogenes and/or loss of additional tumor suppressors.

Here we present a novel and plausible mouse model of human HNSCC to study the consequences of its activation in initiation and progression, which will allow us to address questions like the role of Akt in the development of different types of cancer. In this mouse model, constitutively active Akt leads to rapid progression towards cancers (42), indicating that progression to cancers occurs rapidly when the TGFβRII-null epithelial tissues are exposed to activated oncogenes and/or loss of additional tumor suppressors.

The Akt activator has been shown to be an effective antitumor agent in several preclinical studies. In this study, we demonstrate that Akt activation in epithelial basal cells induces spontaneous tumors in the oral cavity, which results in anal and genital squamous cell carcinomas (42), indicating that progression to cancers occurs rapidly when the TGFβRII-null epithelial tissues are exposed to activated oncogenes and/or loss of additional tumor suppressors.

We thank Drs. A. Berns and P. Krimpenfort (Netherlands Cancer Institute (NKI), Amsterdam, the Netherlands) for providing additional mouse strains, Jesus Martinez and the personnel of the animal facility of CIEMAT for the excellent care of the animals, and Pilár Hernández (CIEMAT) for the histologic preparations.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

Received 8/21/2008; revised 10/15/2008; accepted 11/8/2008.

Grant support: Ministerio de Educación y Ciencia grant SAF 2005-0003, Comunidad Autónoma de Madrid Oncocyte Program grant S2006/BIO-0232, Ministerio de Ciencia e Innovación grant PT-09/0106/2006-3, and Ministerio de Sanidad y Consumo grant ISCIII-RETIC RD06/0020 (J.M. Paramio) and NIH grant CA57111, National Institute of Environmental Health Sciences Center grant ES00784, and Cancer Center Support Grant CA16672 (J. DiGiovanni) and Ministerio de Educación y Ciencia grant SAF2007-66227 (M. Garín).

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We thank Drs. A. Berns and P. Krimpenfort (Netherlands Cancer Institute (NKI), Amsterdam, the Netherlands) for providing additional mouse strains, Jesus Martinez and the personnel of the animal facility of CIEMAT for the excellent care of the animals, and Pilár Hernández (CIEMAT) for the histologic preparations.

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