IKKβ overexpression together with a lack of tumour suppressor genes causes ameloblastic odontomas in mice

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Odontogenic tumours are a heterogeneous group of lesions that develop in the oral cavity region and are characterized by the formation of tumoural structures that differentiate as teeth. Due to the diversity of their histopathological characteristics and clinical behaviour, the classification of these tumours is still under debate. Alterations in morphogenesis pathways such as the Hedgehog, MAPK and WNT/β-catenin pathways are implicated in the formation of odontogenic lesions, but the molecular bases of many of these lesions are still unknown. In this study, we used genetically modified mice to study the role of IKKβ (a fundamental regulator of NF-κB activity and many other proteins) in oral epithelial cells and odontogenic tissues. Transgenic mice overexpressing IKKβ in oral epithelial cells show a significant increase in immune cells in both the oral epithelia and oral submucosa. They also show changes in the expression of several proteins and miRNAs that are important for cancer development. Interestingly, we found that overactivity of IKKβ in oral epithelia and odontogenic tissues, in conjunction with the loss of tumour suppressor proteins (p53, p16 and p19), leads to the appearance of odontogenic tumours that can be classified as ameloblastic odontomas, sometimes accompanied by foci of secondary ameloblastic carcinomas. These tumours show NF-κB activation and increased β-catenin activity. These findings may help to elucidate the molecular determinants of odontogenic tumourigenesis and the role of IKKβ in the homoeostasis and tumoral transformation of oral and odontogenic epithelia.

International Journal of Oral Science (2020) 12:1; https://doi.org/10.1038/s41368-019-0067-9

INTRODUCTION

The IKK complex is responsible for the regulation of the NF-κB family of transcription factors, which regulates genes associated with cell survival and increased proliferation. Deregulated NF-κB activation underlies disease states in many organs, including chronic inflammation and cancer. The IKK complex is formed by two catalytic subunits with kinase activity (IKKa and IKKB) and one regulatory subunit (IKKy or NEMO).1 In addition to the role of IKKβ as a positive regulator of NF-κB activity, it interacts with, phosphorylates, and thereby modifies the activity of a plethora of proteins implicated in a number of functions.2,3 Furthermore, it has recently been reported that the IKK complex acts as a general regulator of gene expression by modifying mRNA stability.5 Thus, IKKβ is able to regulate cellular physiology in different ways and, not surprisingly, changes in the activity of IKKβ are associated with cancer in several cell types. Interestingly, IKKβ can either promote or prevent tumour development, depending on the cell type and other circumstances that are not yet well understood, probably due to the large number of proteins regulated by this kinase.4

Previously, we generated transgenic mice overexpressing IKKβ in basal cells of the stratified epithelia and in exocrine glands. In addition to other phenotypes, IKKβ overexpression in these mice led to greater numbers of CD45+ haematopoietic cells as well as granulocytes (Gr-1+), macrophages (F4/80+) and B cells (B220+) in the forestomach epithelium and to the development of supernumerary teeth due to reduced apoptosis and upregulation of the WNT signalling pathway in the embryonic incisor bud epithelium of KS-IKKβ mice.6 Carcinogenesis experiments performed in a genetic background prone to tumour development through the expression of an active form of RAS revealed that KS-IKKβ mice are resistant to skin cancer,7 but they develop more malignant tumours than control littermates in the forestomach and the palate.8 In the course of these experiments, we crossed the KS-IKKβ transgene into backgrounds lacking p53 in epithelial cells (p53KO/KS-IKKβ mice) or lacking p16 and p19 in every cell (Ink4a/Arf KO/KS-IKKβ). Surprisingly, a high percentage of these mice developed spontaneous odontogenic tumours.

Odontogenic tumours are a heterogeneous group of lesions of the oral cavity that result in the formation of tumoural structures that differentiate as teeth. Their classification is still under debate and has been recently modified by the World Health Organization.9 These lesions are currently classified as odontogenic cysts and odontogenic tumours (benign or malignant), and each of these categories is in turn classified into a number of

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Received: 24 May 2019 Accepted: 14 October 2019
Published online: 02 January 2020
Additionally, we found a modest but statistically non-significant increase in these cell types in the oral submucosa (Fig. 1b). Immunohistochemical staining of palate sections verified the overexpression of IKKβ (Fig. 1c, g; arrowheads) and the presence of more CD45+ and CD11b+ cells in transgenic samples (Fig. 1d, e, h, i; arrows).

Alterations in the expression of tumour suppressor proteins and microRNAs in K5-IKKβ oral keratinocytes. We next studied the expression levels of several genes and microRNAs (miRNAs) that are important for tumour development in cultured oral epithelial cells from wt and tg mice with IKKβ overexpression. K5-IKKβ oral keratinocytes showed increased phosphorylation of Ser256 of p65, indicative of NF-κB activation (Fig. 1k). Interestingly, in contrast to the increased expression of p16, p19 and p53 found in murine skin keratinocytes overexpressing IKKβ,7 oral keratinocytes overexpressing IKKβ expressed lower levels of these tumour suppressor proteins (Fig. 1k), suggesting that the pathways modified by IKKβ in oral and skin keratinocytes are at least partially different. Reduced expression was confirmed at the mRNA level for p19, but not for p16 and p53, suggesting the involvement of some mechanism of posttranscriptional regulation (in fact, we observed an increase in the level of p53 mRNA; Fig. 1l). Immunohistochemical studies confirmed the lower expression of p19 in K5-IKKβ oral epithelial cells in vivo, where most of the nuclei of the basal layers were negative for p19 staining (Fig. 1f, j). In summary, we have found that overexpression of IKKβ in oral epithelial cells leads to decreased expression of several tumour suppressor proteins.

To characterize the expression of other important players in tumour transformation, we studied the expression of several microRNAs previously described to be altered in cancer (Fig. 1m). K5-IKKβ oral keratinocytes expressed substantially lower amounts of several members of the let-7 group, miR-125b, miR-21 and miR-34c than wt oral keratinocytes. Interestingly, let-7 family members, miR-125b and miR-34 tend to be downregulated in tumour samples, including those from the oral cavity.15–16 In summary, K5-IKKβ oral epithelial cells presented lower levels of a number of miRNAs that are usually downregulated in transformed states. In contrast, we did not observe a decrease in miR-203, which is another microRNA whose downregulation is usually associated with tumour transformation, nor a significant difference for miR-205 (Fig. 1m).

Loss of tumour suppressor proteins led to spontaneous odontogenic tumours in K5-IKKβ transgenic mice. In the context of our study on the role of IKKβ in skin cancer, we generated cohorts of K5-IKKβ transgenic mice that also lacked either p53 in stratified epithelial tissues (p53ΔE contemplate/K5-IKKβ mice) or p16 and p19 in every cell (Ink4a/Arf KO/K5-IKKβ mice). Interestingly, in addition to an Ink4a/Arf-dependent antitumour role of IKKβ in skin cancer,7 we observed that overexpression of IKKβ in the absence of either epithelial p53 or both p16 and p19 led to the appearance of maxillary tumours (usually monolateral) that caused facial distortion and swelling in some mice (Fig. 2a, b). These tumours appeared as radiopaque protuberant masses that expanded past the natural snout line and were associated with bone loss (Fig. 2c, d). We did not observe any odontogenic tumours in mice lacking the K5-IKKβ transgene, independently of the p53 or p16/p19 genetic background, or in K5-IKKβ mice bearing wt copies of p53 and Ink4a/Arf (Fig. 2e, f). By contrast, p53ΔE contemplate/K5-IKKβ and Ink4a/Arf KO/K5-IKKβ mice developed similar odontogenic tumours at frequencies of 13.6% and 34.4%, respectively (Table 1). These differences in the distribution of odontogenic tumours in mice bearing and lacking the K5-IKKβ transgene were statistically significant (P < 0.0001, Fisher exact test). We did not observe differences between the sexes in the...
incidence of odontogenic tumours in either p53EKO/K5-IKKβ or Ink4a/Arf KO/K5-IKKβ mice.

Histological examination of these tumours revealed characteristics of ameloblastic odontomas (or odontoameloblastomas) that agreed with the international classification of dental tumours in mice. The ameloblastic odontomas of both p53EKO/K5-IKKβ and Ink4a/Arf KO/K5-IKKβ mice appeared as well-circumscribed tumours that showed differentiation into hard dental tissues, normally located in the central areas of the tumour, and soft tissues composed of proliferating ameloblastic epithelium, generally located at the periphery of the tumour (Fig. 2g–j). The hard tissues of the tumours showed different degrees of morphologic differentiation of tooth structures, ranging from primitive tooth buds (denticles; open arrows in Fig. 2g, h) to mature teeth with distinct ameloblasts, odontoblasts, dentin-like and enamel-like material and cementum (Fig. 2g, open arrowhead). The inner areas of these tooth structures usually contained cells that resembled the stellate reticulum.
It was not rare to find multifocal areas of haemorrhage (Fig. 2g, asterisks), necrosis, inflammation and poorly calcified new bone formation (Fig. 2g, h, j, yellow arrowheads). Although ameloblastic odontomas usually do not metastasise,17 the soft ameloblastic epithelium of the periphery of the tumours that developed in both p53EKO/K5- IKKB and Ink4a/ArfKO/K5- IKKB mice tended to form foci of highly proliferative and hyperchromatic solid cellular masses (Fig. 2i, j, white arrowheads) showing severe pleomorphism and abundant mitosis, suggestive of the development of secondary ameloblastic carcinoma (Fig. 2j, inset); these foci displayed local infiltration and destruction of the alveolar bone (Fig. 2i, j, yellow arrows) and subcutaneous tissues, even leading to infiltration and ulceration of the skin.
Odontogenic tumours derived from IKKβ-expressing cells showed activation of proliferative pathways

We next immunohistochemically studied the expression of epidermal and mesenchymal markers in odontogenic tumours as well as the expression of proteins that are important in the transduction of prosurvival and proliferation signals that are frequently activated in cancer. The ameloblastic odontomas of mice overexpressing IKKβ maintained the epithelial differentiation of the soft ameloblastic epithelium, as it was mainly positive for staining with an antibody specific for keratin K5 (Fig. 3a). The ameloblastic regions of the tumours that expressed keratin K5 at higher levels also showed detectable expression of IKKβ (Fig. 3b) and increased activation of NF-κB, measured as staining for phospho-p65 (Fig. 3c). Notably, the hard and internal portions of the denticles (dentin, odontoblasts and stellate reticular cells) did not express keratin K5 (Fig. 3a, d) or p63 (Fig. 3e) but were positive for vimentin staining (Fig. 3f, arrowheads), indicating the mesenchymal differentiation of these cells, as occurs in normal teeth. The ameloblastic odontomas showed activation of the canonical WNT/β-catenin, STAT3 and AKT signalling pathways in the ameloblastic epithelium, as they exhibited nuclear β-catenin and STAT3 staining, especially in the foci of secondary ameloblastic carcinoma (insets in Fig. 3g, h), which were also positive for staining with an antibody specific for phospho-AKT (Ser473), an active form of AKT (Fig. 3i).

These results were confirmed by western blot analysis of protein extracts from several odontogenic tumours. We analysed three ameloblastic odontomas from Ink4a/Arf KO/K5-IKKβ and two from the p53EKO/K5-IKKβ background as well as non-tumoural oral and odontogenic tissues from the genotypes indicated in Fig. 4. All the tumours (lanes 1–5) expressed higher levels of IKKβ than the non-tumoural oral and dental tissues of K5-IKKβ (lanes 6–8) or wild-type mice (lane 9). In addition, tumoural samples showed increased levels of phospho-p65 and phospho-IκBα, indicative of NF-κB activation, and increased expression (and, to a lesser extent, phosphorylation) of AKT and STAT3 than non-tumoural samples. Odontogenic tumours showed increased total and active β-catenin as well. Interestingly, the tumoural samples also expressed more MMP2; as MMP2 degrades type IV collagen, the most abundant component of the basement membrane, its overexpression could contribute to the aggressive local behaviour and metastatic potential (see below) observed in the foci of secondary ameloblastic carcinoma that developed in the pre-existing ameloblastic odontomas. Notably, we only detected p19

Table 1. Spontaneous ameloblastic odontomas and other epithelial tumours in p53EKO/K5-IKKβ, p53EKO, Ink4a/Arf KO/K5-IKKβ, and Ink4a/Arf KO mice.

| Items                                      | p53EKO/K5-IKKβ | p53EKO | Ink4a/Arf KO/K5-IKKβ | Ink4a/Arf KO |
|--------------------------------------------|----------------|--------|---------------------|--------------|
| Mice analysed                              | 59             | 55     | 61                  | 43           |
| Mice with tumours                          | 26             | 31     | 28                  | 9            |
| Number of epithelial tumours               | 35             | 46     | 28                  | 2            |
| Mice with ameloblastic odontomas           | 8              | 0      | 21                  | 0            |
| Number of ameloblastic odontomas           | 10             | 0      | 21                  | 0            |
| Ameloblastic odontomas vs. epithelial tumours/ % | 28.6          | 0      | 75.0                | 0            |
| Mice with ameloblastic odontomas/ %        | 13.6           | 0      | 34.4                | 0            |
| Age of odontogenic tumour appearance/months | 5–12           | –      | 6–10                | –            |

Fig. 3 Immunohistochemical analyses of ameloblastic odontomas. a–i Immunohistochemical staining for keratin K5 (a, d), IKKβ (b), phospho-p65 (c), p63 (e), vimentin (f), β-catenin (g), STAT3 (h) and phospho-AKT (i) are shown. Am ep, ameloblastic epithelia; De, dentin; Od, odontoblasts. Bar: 100 μm, except in the insets in g and h (50 μm).
in tumoural samples from animals with a wild-type Ink4a/Arf locus (lanes 4–5).

In summary, the analyses of odontogenic tumours and non-tumoural samples by immunohistochemistry and western blotting indicated activation of the NF-κB, AKT, STAT3 and WNT/β-catenin pathways in tumoural samples, independent of their genetic background.

Odontogenic tumours in K5-IKKβ transgenic mice can metastasize. Ameloblastic odontomas are considered benign or low-grade malignant neoplasias that usually do not metastasize but behave as locally aggressive growths invading and destroying surrounding tissues, including bone. By contrast, the tumoural lesions observed in K5-IKKβ mice simultaneously lacking p53 or p16 and p19 were able to metastasize (Fig. 5). We observed metastasis to a cervical lymph node, which was filled with cells similar to stellate reticulum cells (Fig. 5a) in an Ink4a/ArfKO/K5-IKKβ mouse. In another Ink4a/ArfKO/K5-IKKβ mouse, we found an ameloblastic odontoma with foci of secondary ameloblastic carcinoma around the denticles (Fig. 5b, arrowheads), which developed microscopic metastatic foci in the lung, showing a cellular pattern similar to secondary ameloblastic carcinoma with abundant areas of squamous differentiation (Fig. 5c, arrows). The metastatic nature of this lesion was confirmed by its positive immunohistochemical staining for keratin K5, IKKβ and p63 (arrowheads in Fig. 5d–f). From these results, we conclude that the ameloblastic odontomas developed as a consequence of IKKβ overexpression in the context of a lack of tumour suppressor proteins are able to evolve into secondary ameloblastic carcinomas and metastasize to regional lymph nodes and the lung.

DISCUSSION

IKKβ exerts its multiple functions by positively regulating the activity of NF-κB but also via the phosphorylation and, hence, regulation of a number of other substrates that are important in multiple pathways affecting cell physiology. The role of IKKβ in cancer is consequently complex, showing both tumour-promoting and tumour-repressing activities, depending on the tissue and the cellular context.

NF-κB drives a proinflammatory pathway that is constitutively activated and promotes invasion in oral squamous cell carcinomas (SCC). In this report, we have characterized the frequency of infiltrating haematopoietic cells in the oral epithelia of both wild-
type and K5-IKKβ transgenic mice. The presence of immune cells in the oral mucosa is not surprising, as the oral cavity represents a gate for the entry of microorganisms into the body. Notably, approximately 10% of the cells in the oral epithelium of wild-type mice are of immune origin (i.e., CD45+ cells). In addition, IKKβ overexpression triggers a strong oral inflammatory response, as the percentage of immune cells increases up to more than 50% of the total cells in the oral epithelia. Accordingly, in the underlying submucosa, there is also a marked inflammatory response, although it is not as strong as in the oral epithelium. Some ameloblastic carcinomas in humans have been proposed to develop from ameloblastomas. Our results suggest that increased IKKβ expression provokes NF-κB activation and a strong inflammatory response that could contribute to the tumoural transformation of the odontogenic epithelium through the uncontrolled release of inflammatory cytokines and facilitate the development of a secondary ameloblastic carcinoma in a pre-existing ameloblastic odontoma, although the existence of other driving factors cannot be ruled out.

miRNAs are small non-coding RNAs that regulate gene expression by modifying the stability and transcription rate of target messenger RNAs. Each miRNA usually regulates a number of different targets and, hence, shows pleiotropic effects. Not surprisingly, miRNA deregulation is an important player in cancer pathogenesis, including oral SCC and head and neck cancer. The miRNAs that were downregulated by IKKβ overexpression included let-7b, let-7c, miR-125b, miR-21 and miR-34c; interestingly, all of these miRNAs are functionally related to tumoural transformation. miRNAs of the let-7 family promote cellular differentiation and are considered tumour suppressors. These miRNAs are frequently expressed at low levels in human cancers and cancer stem cells. Among their targets, there are several cancer genes, such as RAS, MYC, HMG2 and STAT3, and a negative feedback loop between let-7 members and β-cat is has been described. Therefore, the low levels observed for let-7b and let-7c could be important in the development of ameloblastic odontomas and secondary ameloblastic carcinomas in K5-IKKβ mice or, alternatively, could be secondary to β-cat overactivity.

Although the function of the miR-125 family is somewhat controversial, its members are known to target several oncogenes, transcription factors and growth factors, and its function is tumour suppressive in a number of organs and tissues, including ectodermal derivatives. miR-125b is downregulated in oral SCC, and low expression of miR-125b contributes to head and neck cancer development; moreover, the restoration of miR-125b expression in oral SCC cell lines reduces the proliferation of these cells.

miR-34c belongs to a family of microRNAs that are induced by p53 and cause cell cycle arrest and apoptosis, partially by targeting the expression of c-MYC. This miRNA also regulates multiple components of the WNT pathway, including β-cat, and acts as a tumour suppressor miRNA in many cell types. Restoration of miR-34c expression leads to a decrease in the migration, invasion and metastatic potential of nasopharyngeal carcinoma cell lines. Notably, miR-34c is expressed in oral keratinocytes at much higher levels than in skin keratinocytes (more than 150 times; results not shown). This difference indicates that the functions exerted by this specific microRNA could be more important in oral epithelia than in skin. More studies are needed to determine whether the different effects of IKKβ on tumoural transformation in oral and skin keratinocytes are mediated by miR-34c.

The reduced level of miR-21 found in K5-IKKβ oral keratinocytes is surprising, as this miRNA is considered to be oncogenic and is overexpressed in many types of tumours. miR-203 is differentially expressed in the epithelium and mesenchyme of dental primordia, and hence, it could be important in tooth organogenesis. Thus, the overexpression of miR-203 found in K5-IKKβ oral keratinocytes could contribute to the development of ameloblastic odontomas and secondary ameloblastic carcinomas observed in p53Exo/K5-IKKβ and Ink4a/ArfKO/K5-IKKβ mice. Under treatment with oral carcinogens, IKKβ promotes the appearance of more oral tumoural lesions (and more malignant lesions) in the palate and forestomach in a genetic background prone to the development of epithelial cancers (TgAC mice). Interestingly, these mice do not develop odontogenic tumours. By contrast, the cooperation of IKKβ overexpression with the lack of tumour suppressor proteins (p53 or p16 and p19) leads to the appearance of spontaneous ameloblastic odontomas in ~25% of animals, indicating that although the overexpression of IKKβ makes odontogenic epithelia prone to tumoural transformation, a concomitant lack of tumour suppressor proteins is needed for odontogenic tumour formation.

There are no published data regarding the genomic alterations present in ameloblastic tumours in repositories of genomic data for human cancers such as TCGA. Nevertheless, data available at cBioportal indicate that the most mutated genes in head and neck SCC include TP53 (71.5%) and CDKN2A (the human locus that encodes p16 and p14, which is the human equivalent of murine p19; 22.1%). The implication of CDKN2A in the pathogenesis of odontogenic and other head and neck cancers is reinforced by the characterization of CDKN2A as a susceptibility locus for nasopharyngeal carcinoma in a genome-wide association study performed in a Chinese population; in addition, it has been suggested that the methylation of the CDKN2A locus is an important mechanism of odontogenic tumourigenesis, and loss of heterozygosity is observed for both TP53 and 9p22-p21 (the genomic region where the CDKN2A locus occurs) in odontogenic tumours.

At present, it is uncertain which of the proteins encoded by the CDKN2A locus needs to be lost to cooperate with IKKβ overexpression in odontogenic tumour formation. Mice lacking both p16 and p19 develop tumours, mainly sarcomas and lymphomas, but not odontogenic tumours. Mice that are null for p16 (but not for p16), generated by deleting exon E1B, develop tumours similar to those observed in p16 and p19 double-null mice, although a lack of p19 in association with Tax oncogene expression has been implicated in osteosarcoma development. Wild-type keratinocytes of the oral epithelium express p16 at higher levels than skin keratinocytes, suggesting that p16 loss could be important in oral tumourigenesis. Nevertheless, the deletion of either p16 or p19 individually in an animal model overexpressing IKKβ would allow the specific role of these proteins in the development of odontogenic tumours to be discerned.

β-cat expression deserves particular attention as a possible driver of odontoma formation, as WNT overactivity causes the development of supernumerary teeth and mice with increased WNT activity in the oral epithelium develop odontomas. In addition, β-cat expression appears to increase with the aggressiveness of odontogenic lesions, which also increases its nuclear localization. Accordingly, through western blotting and immunohistochemical analyses, we detected increased β-cat levels in the ameloblastic odontomas of our animal models, especially in the areas of secondary ameloblastic carcinoma. Therefore, p53Exo/K5-IKKβ mice and Ink4a/ArfKO/K5-IKKβ mice represent good models for studying the pathogenesis of the progression to secondary ameloblastic carcinoma from pre-existing ameloblastic odontoma and are also very useful for testing the efficacy of IKKβ or WNT/β-cat inhibitors in preventing odontogenic tumour development.

In summary, our results show that IKKβ overactivity in oral and odontogenic epithelia leads to changes in both the cellular composition of the epithelium and the expression of several tumour suppressor proteins and miRNAs that result in a preneoplastic state of the cells. IKKβ overactivity in transgenic mice in conjunction with the absence of tumour suppressor proteins (such as p53, p16 and/or...
p19) leads to the appearance of odontogenic tumours, which show activation of the WNT/β-catenin pathway. Therefore, these results indicate the possibility of treating ameloblastic odontomas with pharmacologic inhibitors of IKKβ or WNT/β-catenin to prevent their evolution to ameloblastic carcinoma.

MATERIALS AND METHODS

Mice and genotyping
All mouse husbandry and experimental procedures were performed according to European and Spanish laws and regulations and approved by the local Animal Ethical Committee and the competent authority (code PROEX 086/15). The genotyping of K5-IKKβ, p53EKO/K5-IKKβ and Ink4α/ArfKO/K5-IKKβ mice was performed by PCR analysis of tail DNA as previously described.35-38 K5-IKKβ mice are available from the European Mouse Mutant Archive (code EM:09179). The expected genotypes were obtained in roughly Mendelian rates in the different crosses.

Histology and immunohistochemistry
Mouse tissues were dissected, immediately fixed in 10% buffered formalin or 70% ethanol, and embedded in paraffin. Five micron thick sections were used for H&E staining or immunohistochemical preparations. The antibodies used are listed in Supplementary Table S1. Secondary antibodies were purchased from the Jackson ImmunoResearch Laboratory. Immunoreactivity was revealed using an ABC-peroxidase system (Vector Laboratories), and the sections were lightly counterstained with haematoxylin. Control experiments omitting the primary antibody produced no signal.

Culture of oral keratinocytes
Epithelial and submucosal cells were harvested from the oral tissue (inner cheek and palate) of 2-day-old K5-IKKβ and control mice as described previously with minor modifications.39 The samples were incubated overnight at 4°C with 1 unit per mL dispase (Sigma, P-3417); then, the oral epithelia were separated and digested with trypsin (Gibco) for 15 min at 37°C. Keratinocytes were seeded in high-calcium medium [Ca2+ and Mg2+ free EMEM (BioWhittaker, Lonza, Switzerland) supplemented with 4% Chelex-treated (Bio-Rad, Hercules, CA) foetal bovine serum (Cultek, Madrid, Spain) and 0.2 mmol·L−1 Ca2+]. After overnight culture for cell attachment, the dishes were washed three times with cold PBS, and growth medium (CnT-24, Cellntec, Switzerland) was added. Then, the keratinocytes were allowed to grow to confluence. The keratinocytes were subjected to consecutive passages at low dilution using high-calcium medium for seeding. Cells that had undergone fewer than five passages were used for western blot analysis.

For proliferation studies, cells were incubated with 10 µmol·L−1 BrdU for 1 h, and BrdU incorporation was detected by immunohistochemistry. The detection of senescence was carried out by using a senescence β-galactosidase staining kit (9600, Cell Signaling). In colony-forming assays, growing colonies were fixed and stained with Coomassie blue, and the number of colonies was counted.

Flow cytometry analysis of cell populations from oral epithelia
Epithelial keratinocytes and submucosal cells were harvested from the oral tissue (hard and soft palate) of 9-week-old K5-IKKβ and control mice and analysed as previously described for skin cells.7

Protein extraction and western blot analysis
Total protein extracts (30 µg) from cultured mouse oral keratinocytes, tumours or tissues were prepared following standard techniques. The antibodies used are listed in Supplementary Table S1.

RNA isolation and real-time PCR
Total RNA, including miRNA, was isolated from cell cultures from oral tissue by using the miRNeasy Mini Kit (Qiagen) according to the manufacturer’s instructions. The reverse transcription reaction was performed by using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) for mRNA and the TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems) for miRNA. Quantitative qRT-PCR was performed in a 7500 Fast Real-Time PCR System using GoTaq qPCR Master Mix (Promega) for mRNA and TaqMan Universal PCR Master Mix (Applied Biosystems) for miRNA. The sequences of the oligonucleotides used are provided in.7 For miRNA, TaqMan probes were used. TBP was used for the normalization of mRNA expression, and SnoRNA202 and SnoRNA234 were used for the normalization of miRNA expression.

CT analysis
CT studies were performed in a small-animal Argus PET-CT scanner (SEDECAL, Madrid, Spain) in mice that were anaesthetized by the inhalation of 2%–2.5% isoflurane in 100% oxygen, with the following acquisition parameters: voltage 45 kV, current 150 µA, 8 shots, 360 projections and standard resolution.

Images were analysed using the image analysis software ITK-SNAP.

Statistical analysis
For mRNA expression, BrdU incorporation, colony formation and SA-β-galactosidase assays, p-values were determined by using the unpaired, two-tailed Student’s t-test. For flow cytometry assays, the Mann-Whitney test was used. All experiments were performed at least three times. P < 0.05 were considered significant, and the data were expressed as the mean ± SEM. For miRNA expression, we employed REST 2009 Software (Qiagen), which calculates statistical significance using bootstrapping and randomization algorithms.

ACKNOWLEDGEMENTS

This work was funded by project PI17/00578, from the “Instituto de Salud Carlos III” (Ministry of Science, Innovation and Universities) and co-funded by the European Regional Development Fund, and approved by the Ethics Committee of our Institution. It has been founded also by projects CB16/12/00228, P116/00161, RD16/0011/0011, RD12/0019/0023 and SAF2017–84248-P. We would like to thank Rebeca Sánchez-Dominguez and Omaira Alberquilla for their help with the flow cytometry studies; Federico Sánchez-Sierra and Pilar Hernández for their excellent histological processing of the samples; and the personnel of the CIEMAT Animal Unit for mouse care. We also thank Manuel Serrano (Institute for Research in Biomedicine, Barcelona, Spain) for his generous gift of Ink4α/ArfKO mice. Thanks also go to Anton Berns (Netherlands Cancer Institute, NKI, The Netherlands) for supplying the p53EKO mice.

AUTHOR CONTRIBUTIONS

Conception and design of the experiments: A.P., M.N. and A.R. Data acquisition: A.P., C.S.C., R.S.B., M.O., M.L.C., M.N. and A.R. Data analysis and interpretation: A.P., A.B., M.O., M.A.M., J.C.S., M.N. and AR. Writing of the paper: A.P., A.B. and A.R. All authors reviewed and provided final approval of the paper.

ADDITIONAL INFORMATION

The online version of this article (https://doi.org/10.1038/s41368-019-0067-9) contains supplementary material, which is available to authorized users.

Competing interests: The authors declare no competing interests.

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