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Cooperative role between $p21^{cip1/waf1}$ and $p27^{kip1}$ in premature senescence in glandular proliferative lesions in mice

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Summary. Cellular senescence has been considered a novel target for cancer therapy. It has also been pointed out that $p21^{cip1/waf1}$ and $p27^{kip1}$ cyclin-dependent kinase inhibitors (CKIs) play a role in cellular senescence in some tumor types. Therefore, in order to address the possibility of a cooperative role between p21 and p27 proteins in senescence *in vivo* we analyzed cellular senescence in spontaneous glandular proliferative lesions (adrenal, thyroid and pituitary glands) in a double-KO mice model, using γH2AX, p53, p16, PTEN and Ki67 as senescence markers. The results obtained showed that p21p27 double-null mice had the lowest number of γH2AX positive cells in glandular hyperplasias and benign tumors. Also, in this group, Ki67 proliferation index correlated with a lower immunohistochemical expression of γH2AX and p53. The expression of p16 and PTEN do not seem to cause synergism of senescence in the benign lesions analyzed in p21p27 double-KO mice. These observations suggest an intrinsic cooperation between p21 and p27 CKIs in the activation of stress-induced cellular senescence and tumor progression *in vivo*, which would be a physiological mechanism to prevent tumor cell proliferation.

Key words: Animal model, Hyperplasia, $p21^{Cip1/Waf1}$, $p27^{Kip1}$, Premature senescence, Tumor

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Introduction

In 1961, Hayflick and Moorhead defined senescence as an irreversible loss of proliferative capacity of human fibroblasts *in vitro* after a certain number of population doublings. Senescent cells are recognized by morphological features (large, flattened cells) and functional and gene expression pattern changes (Shelton et al., 1999; Collado and Serrano, 2010). Nowadays it is known that the senescence observed by Hayflick and Moorhead is caused by telomere shortening, continuous loss of telomere sequences during cell divisions, or loss of telomere function (Harley et al., 1990; Campisi, 2005; Zang, 2007). Critically shortened or uncapped telomeres induce a DNA damage response (DDR), which is characterized by the activation of Ataxia Telangiectasia Mutated (ATM) and RAD-3 related kinase (ATR) (Rouse and Jackson, 2002). This mechanism induces phosphorylation of Serine-139 in histone H2AX molecules (γH2AX) which triggers the recruitment of multiple DNA repair factors such as 53BP1and NBS1, and also promotes p53 activation (Smogorzewska and de Lange, 2002; Sherr and McCormick, 2002; D'Adda di Fagagna et al., 2003).

Senescence can also be induced by multiple mechanisms regardless of the telomeric process, which lead to cellular stress, such as double strand breaks in DNA that elicit DDR, derepression of the *INK4/ARF* locus, multiple oncogenes such as $HRAS^{G12V}$ or $Braf^{V600E}$ (oncogene-induced senescence, OIS), over-expression of tumor suppressors, mainly p53 and Rb (retinoblastoma tumor suppressor protein) and various others. This type of senescence is known as "stress-

induced premature senescence" (SIPS) (Serrano el al., 1997; Lowe et al., 2004; Zang, 2007; Campisi et al., 2007, ; Collado et al., 2007).

The first evidence of premature senescence induced by tumor supressors involved Pten, where the deletion of conditional *Pten* in mice prostate cells resulted in the development of high-grade PIN which had features of senescence (Chen et al., 2005). Later, it was demonstrated that the ectopic expression of PTEN or suppression of PI3K also causes senescence (Courtois-Cox et al, 2006). Senescence induced by the loss of *Pten* can be enhanced by inactivation of the E3 ubiquitin ligase *Skp2* (Lin et al., 2010). Recently, reduction of PTEN has been proposed as a mechanism to establish a prosenescence therapy in cancer (Alimonti et al., 2010).

Tumor suppressor p53 is a canonical inducer of cellular senescence that activates transcription of $p21^{cip1/waf1}$ (p21) (Vogelstein et al., 2000; Vousden and Prives, 2009). Previous studies using a conditional mouse model in which p53 could be switched on and off, demonstrated that when p53 tumour supressor was restored, both senescence and regression of some tumor types ocurred (Ventura et al., 2007). It was recently reported that p53 activation may both cause senescence or quiescence (reversible cell cycle arrest) depending on the intensity of the p53 response in some cancer cell lines; in particular, moderate activation of p53 does not inhibit mTOR activity and therefore induces senescence, whereas high activation of p53 not only inhibits the cell cycle but also mTOR, inducing quiescence (Leontieva et al., 2010; Demidenko et al., 2010).

Cyclin-depedent kinase inhibitors (CKIs) $p16^{Ink4a}$ (p16), p21 and $p27^{Kip1}$(p27) play an important role in SIPS. p16 (inhibitor of CyclinD/Cdk 4,6 activity) leads to inhibition of Rb and is commonly induced in senescent cells *in vitro* (Serrano et al., 1997; Campisi, 2005). In tumourogenesis of humans melanomas, has been pointed that p16 seems to play a role in senescence (Gruis et al., 1995; Michaloglou et al., 2005). In mouse melanomas driven by BrafV600E, the most common *BRAF* mutant found in the human disease, the tumor suppressor p16 is not required to induce melanocyte senescence and that its loss is not required for tumor progression (Dhomen et al 2009).

p21, the main target of p53 that mediates cell cycle arrest, is an inhibitor of CyclinE/Cdk2 and leads to a common inhibitory response of Rb (Sherr and Roberts, 1999; Collado et al., 2007; Takeuchi et al., 2010). The induction of senescence by p16 and p21 was described in human fibroblast lines (Vogt et al., 1998). Ectopic expression of p21 and p16 enforce the senescence growth arrest (Rodier et al., 2009). Recently, an intrinsic cooperation between p16 and p21 was observed in cellular senescence and tumor suppression in mice skin cancer (Takeuchi et al., 2010).

Another CKI of the Cip/Kip family is p27 (through the inhibition of the activity of the CDK2-cyclin E complexes) that is increased in senescent cells as a result of the inhibition of the PTEN-PI3K signaling pathway (Bringold and Serrano, 2000). Ectopic expression of p27 can induce permanent cell cycle arrest and a senescence-like phenotype in wild-type mouse embryo fibroblasts (Collado et al., 2000).

Senescence has been reported in hyperplasias and pre-malignant stages of tumorigenesis in human and animal models, but was absent in the corresponding malignant tumors, suggesting that senescence may be an endogenous barrier against malignant transformation and, therefore, could play an important role against tumor progression (Braig et al., 2005; Chen et al., 2005; Michaloglou et al., 2005; Collado et al., 2005; Bauer et al., 2007; Acosta et al., 2008; Takeuchi et al., 2010; Mirzayans et al., 2012).

It is known that cell cycle inhibitors p21 and p27 are frequently downregulated in many human cancers and are correlated with poor prognosis (Slirgerland and Pagano, 2000; Mitomi et al., 2005; Ping et al., 2006; Liu et al., 2008). We previously showed that combined deficiency of p21 and p27 proteins in mice was linked to more aggressive spontaneous tumorigenesis resulting in a decreased lifespan (Garcia-Fernandez et al., 2011). The tumors that developed in p21p27 double-null mice were predominantly glandular neoplasms, with higher incidence of pituitary adenomas, pheochromocytomas and thyroid adenomas.

The present study investigates the potential cooperation of p21 and p27 in induced premature senescence, by analyzing hyperplasias and neoplasms in glandular tissues (thyroid, pituitary and adrenal glands) in a double-KO mice model, keeping in mind that senescence is an endogenous barrier against malignant tumor progression.

Materials and methods

Mice generation

The generation of double knockout mice for p21 and p27 proteins was carried out by breeding p27 knockout male mice with p21 knockout females in a C57BL/6J homogeneous genetic background. The animals were housed in a pathogen-free barrier zone at the Barcelona Biomedical Research Park (PRBB), an AAALAC International Accredited Unit. The study was carried out in p21-/-p27-/- mice (n=28), p21+/+ p27-/- mice (n=28), p21-/-p27+/+ mice (n=27) and C57BL/6J wild-type mice (n=30). The animal care and experimental protocols were in accordance with current European (Federation of European Laboratory Animal Science Associations) and International (International Council for Laboratory Animal Science) regulations.

PCR genotyping

Isolation of genomic DNA from the tail was taken from all mice for genotyping by PCR. Detection of p21 wild-type allele and p21-null allele used a common sense primer (5'-AAGCCTTGATTCTGATGTGGGC-

3'). The antisense primers used were specific for the p21 wild-type allele (5'-TGACGAAGTCAAAGTT CCACCG-3') and the p21 knockout allele (5'-GCTATCAGGACATAGCGTTGGC-3'). In the case of p27, the sense primers used were (5'-TGTCAAACGT GAGAGTGTCTAACGG-3') for the wild-type allele and (5'-TGGAACCCTGTGCCATCTCTAT-3') for the p27-null allele. The antisense primers were (5'-AACCCAGCCTGATTGTCTGACGAG-3') for the wild-type and (5'-CCTTCTATCGCCTTCTTGACG-3') for p27-null allele. Detailed PCR methods were previously described (Garcia-Fernandez et al., 2011).

Collection samples and pathological analysis

After necropsy, mouse tissue samples were fixed overnight in 10% buffered formalin, dehydrated in increasing concentrations of ethanol, embedded in paraffin, sectioned 4 μm thick and stained with hematoxylin and eosin (H&E).

Immunohistochemistry was performed on 4 μm de-waxed tissue sections using the Streptavidin-biotin-peroxidase complex method. Sections were immersed in 10 mM citrate buffer (pH 6.0) and heated in a microwave oven (100°C, 15 min) for antigen retrieval. Endogenous peroxidase activity was inactivated by incubation with 3% hydrogen peroxide in methanol (15 min, room temperature). Tissue sections were incubated in a humidified chamber (overnight, 4°C) using the following antibodies: rabbit monoclonal anti-γH2AX (Ser 139) (#9718; 1/250; Cell Signaling, Heart, UK); rabbit anti-Ki67 (NCL-Ki67p1/800; Novocastra, Newcastle Upon Tyne, UK); rabbit anti- p53 protein (CM5) (NCL-p53-CM5p, 1/50; Novocastra, Newcastle Upon Tyne, UK); mouse monoclonal antibody anti-p16 (sc-1661; 1/200; Santa Cruz Biotechnology, Inc. UK) and rabbit monoclonal anti-PTEN(#9559, 1/100 Cell Signaling, Heart, UK).

The antibodies were diluted in Tris-buffered saline (TBS). For the negative controls, the primary antibodies were replaced by non-immune serum. After three rinses in TBS (5 min each), samples were incubated with biotinylated goat anti-rabbit IgG (1:400; Vector Laboratories, Burlingame, CA). After 30 min incubation with the secondary antibody, tissue sections were washed in TBS (3 times 5 min each) and immediately incubated for 30 min with streptavidin-peroxidase complex diluted 1:400 in TBS (Zymed Laboratories, Invitrogen, CA, USA). The chromogen used was 3-3'-diaminobenzidine (DAB Peroxidase Substrate Kit, Vector Laboratories, Burlingame, CA). Nuclei were counterstained with Harris hematoxylin for 1 min.

γH2AX immunostaining cells were considered positive when at least one positive focus per nucleus was observed at 40x objective in an Olympus VANOX AHBS3 microscope. For quantitative evaluation of each antibody, 5 fields of 100 cells per high power field of each sample section were scored per condition.

Statistical analysis

γH2AX, Ki67, p53, p16 and PTEN positive cell countings were analyzed using two way-analysis of variance (ANOVA) with SPSS Statistics 19 Software (IBM). *Post hoc* analyses were performed to study significant differences. Pearson's correlation coefficient was carried out to measure the linear correlation between variables. Results are expressed as mean ± standard error of mean (s.e.m) and statistical significance was accepted for $P<0.05$.

Results

Spontaneous proliferative lesions

We analyzed pituitary gland, thyroid, and adrenal gland which frequently contained spontaneous proliferative lesions in p21p27 double-KO mice. In order to assess positive γH2AX foci in normal tissue, thyroid gland (n=3), pituitary gland (n=3) and adrenal gland (n=3) were immunostained using anti-γH2AX antibody. Positive staining was less than 0.5% in all the analyzed samples. Spontaneous hyperplasias and neoplastic lesions from these glands were selected for histopathology and classified according to current histological criteria (Mohr, 2001). Hyperplastic lesions within adrenal glands and thyroid were detected in p21p27 double-KO mice (n=5), p27-null mice (n=6), p21-deficient mice (n=5) and wild-type mice (n=6). Benign tumors in the adrenal gland, thyroid and pituitary gland were observed in p21p27 double-KO mice (n=7), p27-null mice (n=7), p21-deficient mice (n=6) and wild-type mice (n=6); and malignant neoplasms in the adrenal gland and thyroid gland developed in p21p27 double-KO mice (n=6), p27-null mice (n=5), p21-KO mice (n=5) and wild-type mice (n=5).

The average life span for p21p27 double-null mice with proliferative lesions was 8 months, slightly less than for p27 null-mice (10,2 months), p21 KO-mice (14,2 months), and wild-type mice (12,8 months).

DNA damage foci in epithelial hyperplasias and neoplasias

Analysis of hyperplasias

γH2AX was always detected in the nucleus. The number of γH2AX foci of positive cells in glandular hyperplasias (Figs. 1, 2) showed significant differences among groups of mice ($P<0.001$). The highest values were found in hyperplasias developing in wild-type mice (8.2±0.5), followed by those developing in p21-null mice (5.2±0.7), and p27-KO mice (3.9±0.2). The lowest values were found in hyperplasic lesions originating in double-null mice for p21 and p27 (1.3±0.4) (Fig. 2).

The *post hoc* analysis showed that hyperplasias coming from double-KO mice had significantly lower

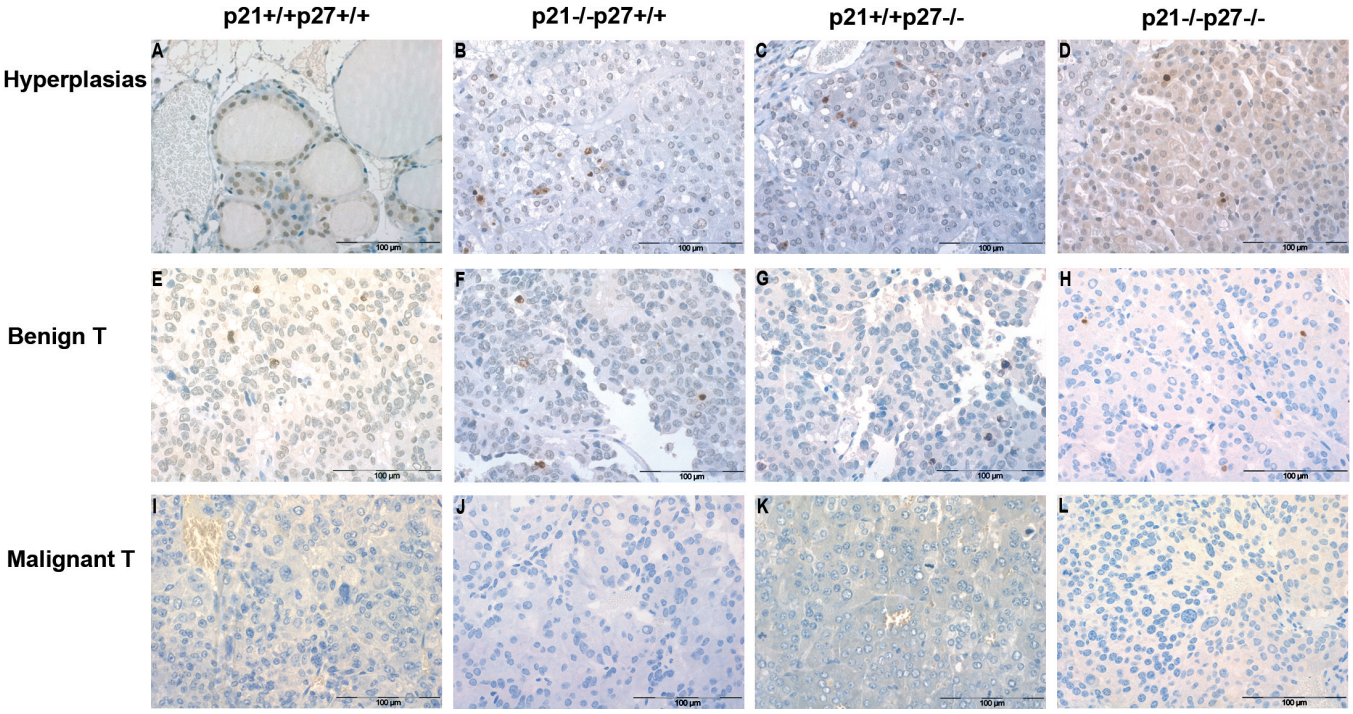

Fig. 1. Representative images showing γH2AX immunoexpression in spontaneous hyperplasias, benign and malignant tumors in p21+/+p27+/+, p21-/-p27+/+, p21+/+p27-/- and p21-/-p27-/- mice. **A.** Thyroid gland. **B-D, H-L.** Adrenal gland. **E-G.** Pituitary gland. Regardless of the group, the number of positive cells was higher in hyperplasias than in neoplasias, this difference was clearly evident in malignant tumors. **T**, tumor. Streptavidin biotin peroxidase complex method. Bar: 100 μm.

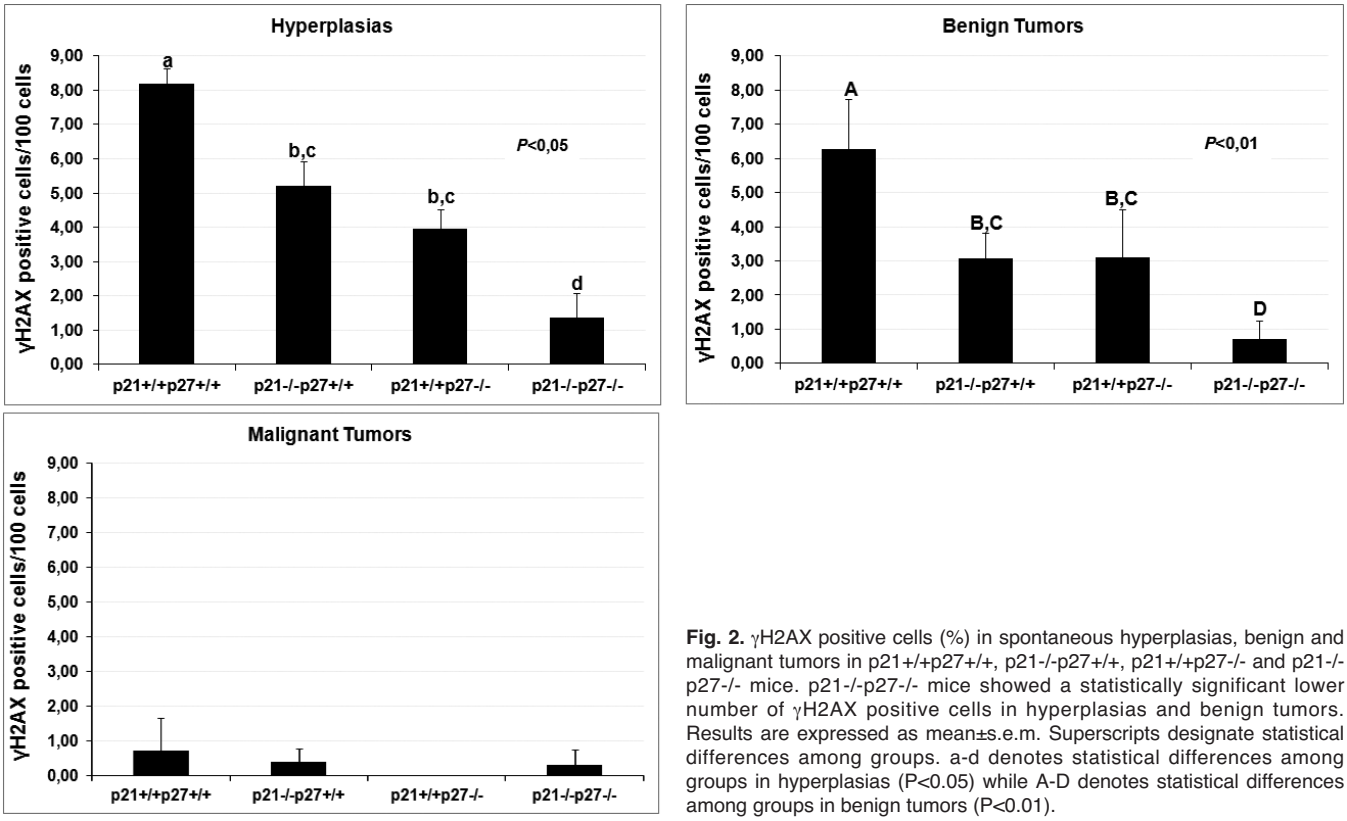

Fig. 2. γH2AX positive cells (%) in spontaneous hyperplasias, benign and malignant tumors in p21+/+p27+/+, p21-/-p27+/+, p21+/+p27-/- and p21-/-p27-/- mice. p21-/-p27-/- mice showed a statistically significant lower number of γH2AX positive cells in hyperplasias and benign tumors. Results are expressed as mean±s.e.m. Superscripts designate statistical differences among groups. a-d denotes statistical differences among groups in hyperplasias (P<0.05) while A-D denotes statistical differences among groups in benign tumors (P<0.01).

numbers of γH2AX positive cells when compared to each of the other groups (P<0.001) whereas hyperplasic lesions in wild-type mice showed significantly higher values when compared to the rest of the groups (P<0.001). However, differences between hyperplasias in p21-null animals and p27-KO mice were evident but not statistically significant (P=0.05) (Fig. 2).

Analysis of benign tumors

In benign tumors (Figs. 1, 2) the highest positivity for γH2AX was observed in neoplasias developing in wild-type mice (6.3±1.4) and the lowest in adenomas coming from p21p27 double-KO mice (0.7±0.4), with significant differences when compared to benign tumors in p27-KO mice (P<0.005), p21-KO mice (P<0.001) and wild-type mice (P<0.001).

When comparing the γH2AX foci-positive cells in benign neoplasias arising in p27-KO mice and those in p21-null mice, no significant differences were observed (Fig. 2). On the other hand, adenomas from wild-type mice showed significant differences when compared to benign neoplasms in p27-KO mice (P<0.05) and p21-null mice (P<0.01) (Fig. 2).

Analysis of malignant tumors

As expected, no significant differences were found among malignant epithelial tumors developing in the different groups of mice (Fig. 2) and all carcinomas analyzed were practically devoid of γH2AX-positive nuclei (Fig. 1).

Proliferation index

Ki-67 positivity was detected in cell nuclei. In glandular hyperplasias and benign tumors, when comparing Ki67 proliferation index among groups, no statistical differences were found (Fig. 3). The highest number of Ki67 positive cells was noted in proliferative benign lesions developing in p27-null mice and the lowest positivity was noted in hyperplasias and benign tumors in double-KO mice.

On the other hand, Ki67 proliferative index was significantly higher in carcinomas developing in p21p27 double-KO when compared to similar tumors in p21-KO (P<0.01) (Fig. 3). The highest positivity was found in malignant tumors in p21p27 double-null mice (35.3±5.5), followed by carcinomas arising in wild-type mice (25.5±8.5) and p27-KO mice (23.4±7.4), while p21-KO mice carcinomas showed the lowest Ki67 positivity (20.3±6.2) (Fig. 4).

Analysis of p53

p53 positivity was detected in cell nuclei. In glandular hyperplasias (Fig. 4), p53 positive cells were significantly lower in p21-KO mice when compared to hyperplasias developing in p21p27 double-null mice (P<0.001) and wild-type mice (P<0.01), but no significant differences were found with glandular hyperplasias in p27-KO mice (Table 1). However, when benign and malignant neoplasias were analyzed, no significant differences were evident among groups (Table 1).

Analysis of p16

p16 positivity was detected in cell nuclei (Fig. 5). When comparing p16 immunoexpression values among

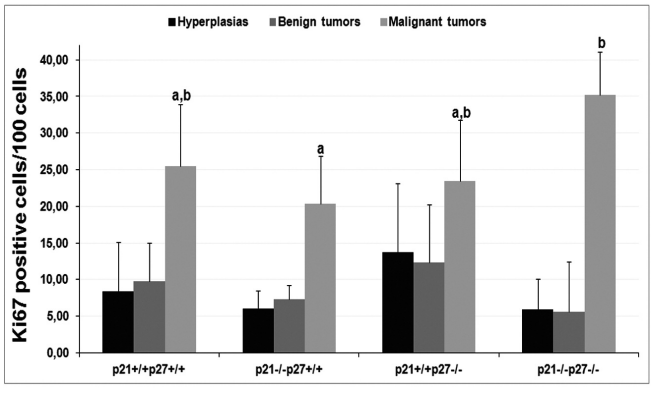

Fig. 3. Ki67 positive cells (%) in spontaneous hyperplasias, benign and malignant tumors in p21+/+p27+/+, p21-/-p27+/+, p21+/+p27-/- and p21-/-p27-/- mice. Ki67 positive cells were more frequent in malignant tumors than in benign neoplasias and hyperplasias. In malignant tumors, p21-/-p27-/- mice showed the highest number of Ki67 positive cells, though only statistically significant when compared to p21-/-p27+/+ mice (P<0.01). Data are presented as mean±s.e.m. Superscripts (a-c) designate significant differences among groups.

Table 1. p53 positive cells (%) in spontaneous glandular hyperplasias and tumors.

Mice Groups	Hyperplasias	Benign Tumors	Malignant Tumors
p21+/+p27+/+	1.4[a]	1.0	0.8
p21-/-p27+/+	0.2[a,b]	1.1	0.2
p21+/+p27-/-	0.9	1.6	0.1
p21-/-p27-/-	1.5[b]	1.3	0.6

[a]: significant differences were found between p21+/+p27+/+ mice when compared to p21-/-p27+/+ mice (P<0.01) [b]: significant differences between p21-/-p27+/+ mice and p21-/-p27-/- mice (P<0.001).

Table 2. p16 positive cells (%) in spontaneous glandular hyperplasias and tumors.

Mice Groups	Hyperplasias	Benign Tumors	Malignant Tumors
p21+/+p27+/+	7,6	2,5	0,1
p21-/-p27+/+	7,5	2,6	0,1
p21+/+p27-/-	7,6	2,7	0,1
p21-/-p27-/-	7,2	2.2	0,1

groups with similar pathology, no significant differences were found (Table 2). The highest positivity for p16 was observed in hyperplasias, 7,2±0,4 in p21p27-null mice, 7,6±1,2 in p27-KO mice, 7,5±0,7 in p21-KO mice and 7,6±0,7 in wild-type mice and the lowest was found in malignant tumors (Fig. 5).

Analysis of PTEN

PTEN antibody stained positive in cell nuclei with immunohistochemistry. All the glandular lesions arising in double-KO mice and p27-KO mice were negative (Fig. 5). In p21-KO mice one pituitary adenoma (16% of benign tumors) showed 10% of positive cells. In wild-type group mice one thyroid adenoma (16% of benign tumors) showed 7% of positive cells.

Correlation among variables

In all groups, regardless of the type of lesion analyzed, high immunohistochemical expression of Ki67 proliferative index was correlated with low immunohistochemical expression of γH2AX (r=-0.429; P<0.001) and p53 protein (r=-0.324; P<0.005).

Discussion

In this study we evaluated the role of p21 and p27 proteins inducing premature senescence in spontaneous proliferative lesions arising from the adrenal, thyroid and

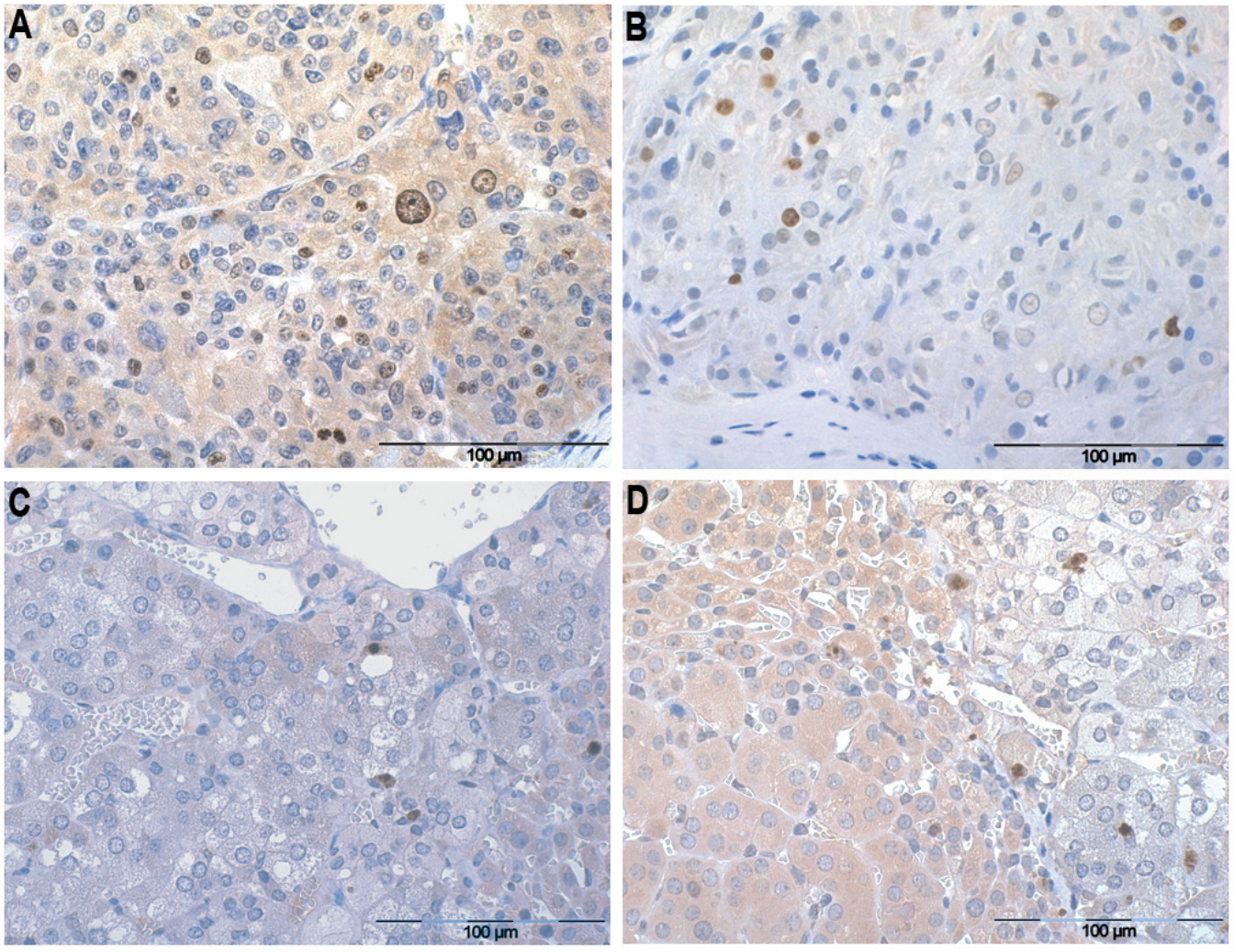

Fig. 4. Representative images showing Ki67 and p53 immunoreactivity. The highest positivity of Ki67 was found in malignant tumors in p21-/-p27-/- mice (**A**, adrenal gland, malignant pheochromocytoma), which showed statistically significant differences when compared with p21-/-p27+/+ mice (**B**, thyroid gland carcinoma) (P<0.01). Regarding p53, differences among groups were more significant in hyperplasias than in neoplasias; in this way p21-/-p27-/- mice showed a significantly higher staining (**C**, adrenal gland) when compared to p21-/-p27+/+ animals (**D**, adrenal gland) (P<0.001). Streptavidin biotin peroxidase complex method. Bar: 100 μm.

pituitary glands in a double-knockout model mouse generated on a C57BL/6J background. Our observations showed cooperation between p21 and p27 proteins in SIPS. This finding is supported by the lower senescence observed in glandular hyperplasias and adenomas in double deficient mice when compared to similar lesions in mice lacking either p21 or p27.

We focused our study of hyperplastic lesions and tumors arising in the pituitary, thyroid and adrenal glands since these are the most frequent sites for spontaneous proliferative lesions in p21p27 double-KO mice and p27-null mice (Fero et al., 1996; Nakayama et al., 1996; Kiyokawa et al., 1996; Besson et al., 2007; Garcia-Fernandez et al., 2011). Although hematopoietic tumors are the most common type of neoplasm found in p21-KO mice, epithelial proliferative lesions have also been described (Martin-Caballero et al., 2001; Garcia-Fernandez et al., 2011). The mean age of p21p27 double-KO mice analyzed in our study was 8 months, which is considered middle-age for the mouse lifespan and most biomarkers have not changed yet (Flurkey et al., 2007). Moreover, the analysis of γH2AX positive foci in normal glands was similarly low in all cases. These results agree with a previous report describing lower frequency of γH2AX foci-positive cells in 12 month old mice (Wang et al., 2009), which suggested that cellular senescence was associated with SIPS rather than with aging of the mouse.

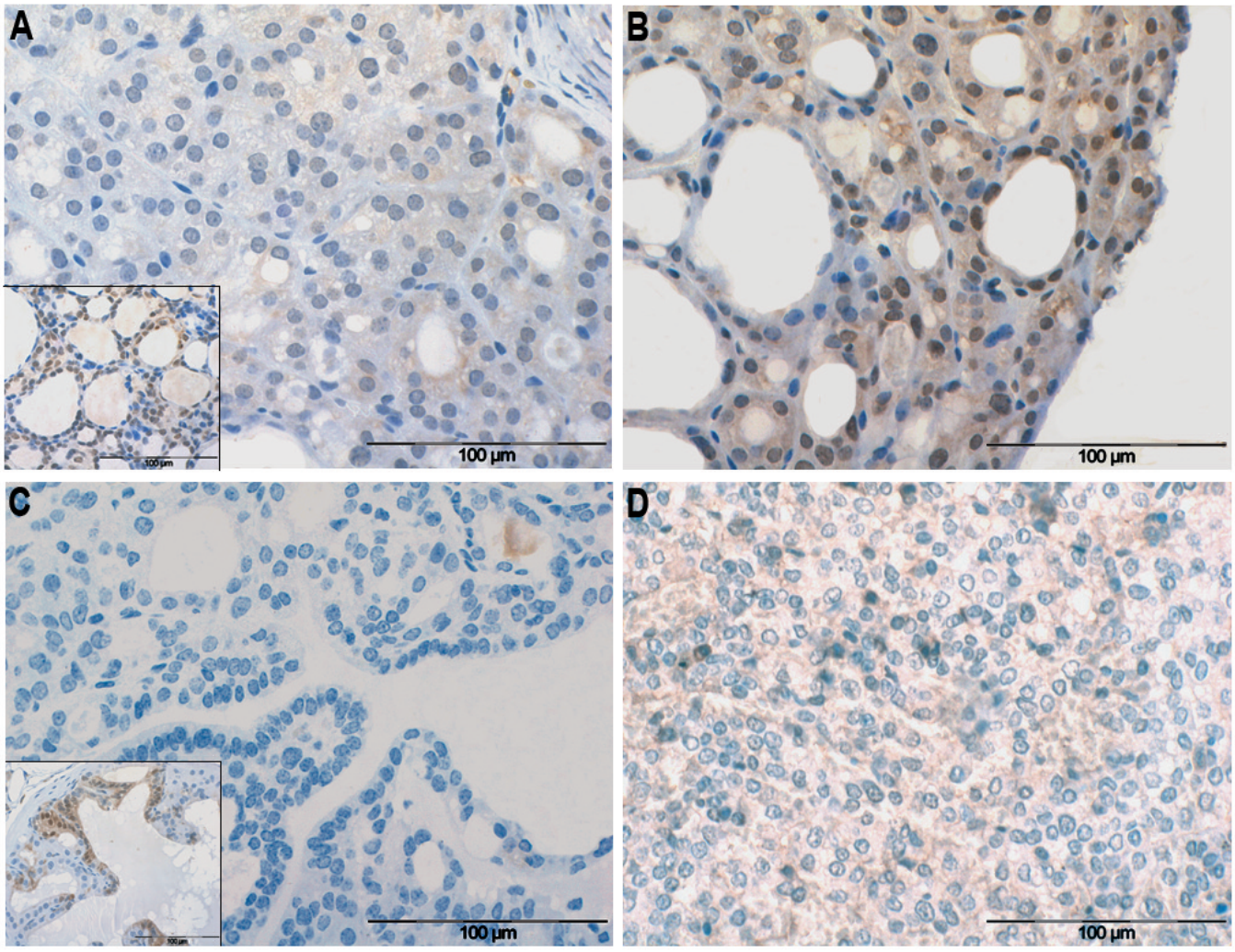

Fig. 5. Representative images showing p16 and PTEN immunoreactivity. As shown, p16 immunopositivity decreased in neoplasias when compared with hyperplasias: p21-/-p27-/- mouse, pituitary gland adenoma **(A)**, p21+/+p27+/+ mouse, thyroid gland hyperplasia **(B)**; a positive control for p16 immunopositivity has been included in the inset in **A** (normal thyroid gland, mouse). On the other hand, most of the tumors were negative when stained against PTEN (**C**, p21-/-p27-/- mouse, thyroid gland adenoma). **D.** Only one benign pituitary tumor in a p21 KO animal were slightly positive; in the inset in **(C)** a positive control for PTEN has been included (PIN, prostate gland, mouse). Streptavidin biotin peroxidase complex method. Bar: 100 *µ*m.

DNA damage foci detection, by means of γH2AX immunohistochemistry, has been proposed as a convenient and accurate marker for the detection of cellular senescence (Celeste et al., 2003; von Zglinicki and Martin-Ruiz, 2005; Wang et al., 2009; Bernardes and Blasco, 2012). Even though in previous studies *in vitro* (Passos et al., 2007) and *in vivo* (Matheu et al., 2009; Wang et al., 2009; Ewald et al., 2010), similar numbers of SA- beta-gal and γH2AX stained cells have been observed, there are certain advantages for the use of γH2AX over other standard markers, such as SA-beta-gal, as γH2AX can be used in archived paraffin-embedded tissues (Ewald et al., 2010).

In our study, cellular senescence has been estimated by combining different biomarkers such as γ-H2AX, p53, p16, PTEN and Ki-67 cell proliferation index. Currently, the dual pattern of detection of γH2AX foci-positive cells and a proliferation marker, such as Ki-67 protein index, provides accurate evidence of cell senescence in pathology studies (Wang et al., 2009; Collado and Serrano, 2010; Ewald et al., 2010).

As previously mentioned, glandular hyperplasias arising in p21p27 double-KO mice showed statistically significant lower values of γH2AX positive cells when compared to the rest of the groups ($P<0.001$). These data suggest that p21 and p27 proteins enhance cellular senescence in pre-tumoral lesions coinciding with the inhibition of cell proliferation, which was confirmed by the low Ki-67 index. We observed that glandular hyperplastic lesions showed a significant decrease of γH2AX positive-cells in p27-KO mice when compared to p21-KO mice ($P<0.05$). The data suggests that the loss of p27 plays a relevant role in reduced cellular senescence in the glandular pre-neoplastic lesions developed in p21p27 double-null mice. These results agree with previous descriptions in human and mouse hyperplasias, where loss of p27 or its downregulation has been associated with an increase in the number of senescent cells (Cordón-Cardo et al., 1998; Choi et al., 2000).

In benign tumors, the analysis of γH2AX-positive cells showed significant differences among groups ($P<0.001$). While benign tumors developed in double-KO mice showed the lowest number of γH2AX-positive cells, neoplasms arising in wild-type mice showed the highest number of γH2AX positive cells. This seems to indicate that both p21 and p27 proteins could be responsible for the phosphorylation of H2AX in the non-malignant glandular tumors analyzed. In our study, benign tumors showed the highest values of p53 expression although no significant differences were observed between groups.

In accordance with our results, p27 expression has been associated with prostatic cellular senescence, and genetic ablation of p27 leads to downregulation of senescence markers and progression to cancer in humans (Choi et al., 2000; Majunder et al., 2008). Furthermore, p27, p21 and Arf4 induction trigger cellular senescence and restrain proliferation in mice with deficiency of the Skp2 in a *Pten* deficient model. This enhanced the development of benign adrenal tumors and restricted the incidence of prostatic PIN (Lin et al., 2010). Similarly, Campaner et al. (2010) pointed out that p21 and p16 trigger cellular senescence in pancreatic ,cells and splenic Bcell, on CDK2-deficient mice with c-Myc oncogenic-stress. Interestingly, although high p21 levels are associated with the development of benign pituitary tumors (Chesnokova et al., 2008), in our study all the pituitary neoplasias that developed in double-KO mice, p21-KO mice and p27-KO mice were adenomas, suggesting a more complex mechanism in pituitary tumor malignancy development.

The malignant tumors analyzed were devoid of γH2AX-positive nuclei in all groups of mice and there was an inverse correlation between γH2AX and the Ki67 proliferation index. This confirms that senescence has not been found in malignant neoplasias (Collado et al., 2005; Chen et al., 2005; Lazzerini et al., 2005; Acosta et al., 2008, Efeyan et al., 2009).

In our study similar values of p53 expression were observed in proliferative glandular lesions analyzed in double-KO, wild-type and p27-null mice; significant differences of p53 protein values were only observed in p21-KO mice glandular hyperplasias. This data suggests that activation of the Arf/p53 pathway does not seem to play a role in SIPS observed in the glandular lesions analyzed on double-KO mice. Interestingly, in our model the downregulation of p27 protein was not related with the deficit of PTEN, as was observed in the proliferative lesions that developed in double-KO mice and p27-KO mice.

The lack of significant differences of p16 expression among mice groups with similar types of lesions in our study suggests that p16 does not play a cooperating role in SIPS in our model, similar to has been reported (Dhomen et al., 2009). In our study benign proliferative lesions arising from all groups of mice showed higher values of p16 expression in agreement whit previous studies (Krimperfort et al., 2001; Michaloglou et al., 2005). Importantly, in this cancer model the results suggest that the expression of p53, p16 and PTEN do not seem to trigger the senescence observed in proliferative benign glandular lesions developed in double-KO mice.

In summary, p21p27 double-null mice showed the lowest number of γH2AX positive cells in spontaneous glandular proliferative lesions, suggesting an intrinsic cooperation between p21 and p27 CKIs in the activation of stress induced cellular senescence and tumor progression *in vivo*, which is considered a physiological mechanism to prevent tumor cell proliferation.

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