

Evidence of cellular senescence during the development of estrogen-induced pituitary tumors

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Abstract

Although pituitary adenomas represent 25% of intracranial tumors, they are usually benign, with the mechanisms by which these tumors usually avoid an invasive profile and metastatic growth development still remaining unclear. In this context, cellular senescence might constitute a plausible explanation for the benign nature of pituitary adenomas. In this study, we investigated the emergence of cellular senescence as a growth control mechanism during the progression of estrogen-induced pituitary tumors. The quantification of Ki67-immunopositive cells in the pituitaries of estrogenized male rats after 10, 20, 40, and 60 days revealed that the mitogenic potential rate was not sustained for the whole period analyzed and successively decreased after 10 days of estrogen exposure. In addition, the expression of cellular senescence features, such as the progressive rise in the enzymatic senescence-associated β -galactosidase (SA- β -gal) activity, IL6, IL1 β , and TGF β expression, was observed throughout pituitary tumor development. Furthermore, tumoral pituitary cells also displayed nuclear pATM expression, indicating activated DNA damage signaling, with a significant increase in p21 expression also being detected. The associations among DNA damage signaling activation, SA- β -gal expression, and p21 may provide a reliable combination of senescence-associated markers for *in vivo* pituitary senescence detection. These results suggest a role for this cellular process in the regulation of pituitary cell growth. Thus, cellular senescence should be conceived as a contributing component to the benign nature of pituitary adenomas, thereby influencing the capability of the pituitary gland to avoid unregulated cell proliferation.

Key Words

- pituitary
- estrogen
- immunohistochemistry
- tumor
- cellular senescence

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Introduction

The pituitary gland is a principal controller of endocrine homeostasis. Through the release of specific hormones synthesized by five highly differentiated cells, this gland

responds to central and peripheral signals. In spite of presenting low mitogenic and apoptotic rates, the gland exhibits dynamic and plastic fluctuations in reaction to

exogenous and endogenous stimuli in order to constantly adapt the hormonal and metabolic responses to changing environments (Levy 2002). Nevertheless, several alterations in cell growth can lead to pituitary hyperplasia and high levels of hormone production, frequently related to adenoma formation (Asa & Ezzat 2009).

Pituitary adenomas represent 25% of intracranial tumors, with their prevalence rising with age, and both sexes being similarly affected (Asa & Ezzat 2009). However, despite their relatively common incidence, pituitary tumors are usually benign (Maiza & Caron 2009). The mechanisms by which these tumors may rarely acquire invasive profile and metastatic growth are unclear, even when oncogenic pathways are triggered or tumor suppressor pathways are inactivated. These particularities of pituitary adenomas have led to the hypothesis that there may be an additional process that acts as a protective anti-proliferative mechanism.

Cellular senescence can be interpreted as a spontaneous initial barrier in tumorigenesis, because senescent cells are abundant within pre-malignant lesions, whereas they rarely appear in malignant tumors (Collado et al. 2005). In this way, senescence renders an impediment against oncogenic stimulation and prevents cellular transformation (Collado & Serrano 2010). *In vitro* studies have revealed that certain oncogenic signaling, depending on the context, can paradoxically provoke growth arrest along with a cellular senescent phenotype (Michaloglou et al. 2005). Moreover, emergent evidence has described *in vivo* premature cellular senescence in the early stages of tumor growth, suggesting that this process could act as a potent anti-tumor protective mechanism that must be overcome for tumor progression (Braig et al. 2005, Collado et al. 2005).

Senescence is considered to be a stress response characterized by permanent cell cycle arrest, which can be triggered by different factors including DNA damage, oxidative stress, telomere dysfunction, aberrant proliferative signals of oncogenes, and the inactivation of tumor suppressor genes (Campisi & d'Adda di Fagagna 2007). Consistent with cellular arrest, the senescence phenotype is accompanied by the upregulation of different cell-cycle regulators such as the CDK inhibitors p16^{INK4A}, p15^{INK4B}, p21^{CIP1}, among others (Roninson 2002, Holst et al. 2003). Because several senescence-inducing stimuli provoke epigenomic disruption or genomic damage, many senescent cells harbor a persistent DNA damage response (DDR) signaling, such as the activation of the kinase ataxia telangiectasia mutated (ATM) (Di Micco et al. 2006), and the involvement of p53 activation (Rodier et al. 2007). In addition, senescent cells show higher levels of detectable

senescence-associated β -galactosidase (SA- β -gal; Dimri et al. 1995, Lee et al. 2006), which can also include compromised mitochondrial dynamics and metabolism (Moiseeva et al. 2009), with unbalanced mitochondrial fusion and fission processes (Lee et al. 2007). In fact, senescent cells undergo extensive gene expression changes that emulate the inflammatory response, thereby developing a complex senescence-associated secretory phenotype (SASP), *in vitro* and *in vivo*, associated with the activation of nuclear factor kappa B (NFkB) or C/EBPB (Coppe et al. 2008). SASP proteins include a wide range of growth factors, proteases, chemokines, and cytokines including interleukin 6 (IL6), IL1, transforming growth factor beta (TGFb), IL8, among others, which participate in senescence intercellular signaling, and interact at, or in the immediate vicinity, of the plasma membrane (Coppe et al. 2008, Kuilman et al. 2008, Young & Narita 2009).

Cellular senescence may constitute a plausible explanation for the benign nature of pituitary adenomas through the existence of an intrinsic predisposition of pituitary cells to limit uncontrolled proliferation through this cellular program. Some recent studies on pituitary tumorigenesis in transgenic mice (Chesnokova et al. 2007, 2008) and SA- β -gal in human pituitary tumor (Chesnokova et al. 2011, Alexandraki et al. 2012) have suggested that cellular senescence could be a significant mediator of growth cessation in these tumors. However, it still remains uncertain whether this cellular response takes place in the absence of direct genetic manipulation or during the development of pituitary proliferative injuries, indicating a possible physiological role for cellular senescence in the modulation of pituitary cell growth.

Appropriate *in vivo* models are required to achieve a better understanding of senescence during the development of tumors. In this study, we used a previously developed pituitary tumor experimental model, which consists of long-term estrogen administration in rats, and leads to the formation of prolactin (PRL) secretory tumors presenting hyperplasia/hypertrophy, greater vascularity, and the disruption of the reticulin network (Mukdsi et al. 2006, Palmeri et al. 2009). Considering that it has been postulated that estrogen-induced proliferative lesions could increase the ability of cells to generate prime tumorigenic changes (Melmed 2003), our experimental conditions may represent an accurate *in vivo* model for studying the outcome of pituitary senescence. In this context, the aim of this study was to investigate the emergence of cellular senescence as a growth control mechanism during the progression of estrogen-induced pituitary tumors.

Materials and methods

Animals and experimental models

Three-month-old Wistar strain male rats were maintained at controlled temperature ($21 \pm 3^\circ\text{C}$) and lighting conditions (14 h light:10 h darkness cycle), with free access to commercial laboratory chow and tap water. Taking into account that exogenous estrogen excess induces pituitary tumors (Asa & Ezzat 2002, Mukdsi et al. 2004), intact animals were treated with estradiol benzoate (Sigma–Aldrich) for 10, 20, 40, and 60 days (E10, E20, E40, and E60). Estrogen was implanted subcutaneously in slow-releasing silastic brand capsules (Dow Corning, Medical grade, Midland, MI, USA) filled with 10 mg of estrogen crystals and sealed with silastic cement. The control group was implanted with empty capsules. The rats were decapitated within 10 s after being removed from their cage, thus avoiding any stress or external stimuli. Fresh pituitary glands were photographed *in situ* and weighed immediately after collection. The animals were kept in accordance with the Guide for the Care and Use of Laboratory Animals, published by the United States National Institutes of Health (1996), and the experiments were approved by the Institutional Animal Care Committee of the School of Medicine, National University of Córdoba.

Circulating estrogen levels

Serum estrogen levels were measured by an electrochemiluminescence immunoassay from Roche Diagnostics GmbH, using the commercial Elecsys System 2010 (Elecsys Corporation, Lenexa, KS, USA). For statistical purposes, the serum analyzed was obtained from six rats for each experimental group.

Pituitary cell dissociation

The protocol for pituitary cell dissociation has been previously described (De Paul et al. 1997). Briefly, rat pituitary glands were placed in the minimal essential medium, and then minced and digested with 0.4% w/v trypsin at constant slow agitation at 37°C . The cell suspension was gently dispersed using flame-rounded Pasteur pipettes. Cell viability, tested with Trypan blue exclusion, was always better than 90%. Cell dispersions were transferred to 1.5 ml Eppendorf tubes and centrifuged for 5 min at 400 g.

Cell culture

The rat somatotroph pituitary adenoma GH3B6 cell line was cultured in HAM–F12 medium (Sigma–Aldrich)

supplemented with 5% v/v FCS and 12% v/v horse serum (Invitrogen), as previously described (Petiti et al. 2010). After 3 days of culture and 70% of confluence in order to recreate estrogenic doses administration at E40–E60, cells were exposed to 17 β -estradiol (E_2 ; 4 nM; Sigma–Aldrich) or vehicle for 120 h and then submitted to the cytochemical detection of SA-b-gal staining.

Cell cycle analysis

Dispersed pituitary cells from control and estrogenized animals were fixed in 70% v/v ice-cold ethanol by gentle vortexing. After centrifugation (400 g for 5 min), pellets were washed with citrate–phosphate buffer of pH 7.8. Finally, the cells were incubated with RNase (ribonuclease A 0.7 mM; Sigma–Aldrich) at 37°C for 30 min, and stained with propidium iodide (75 μM ; Sigma–Aldrich) for 1 h. The fluorescence intensity was analyzed by flow cytometry using a BD FACS Canto II, and cell cycle analysis of the DNA content was performed using ModFit LT Program (Verity Software, Verity Software House, Topsham, ME, USA). For statistical purposes, cellular dispersions from nine animals of each experimental group were analyzed.

Cytochemical detection of SA-b-gal staining

SA-b-gal-positive cells were detected by using a b-gal staining kit (Senescence Cell Staining Kit, Sigma–Aldrich), one of the most extended staining methods for cellular senescence mentioned in the bibliography (Dimri et al. 1995, Lee et al. 2006). Briefly, pituitary cryosections (8 μm thickness) were fixed in a mixture containing 2% v/v formaldehyde and 0.2% v/v glutaraldehyde in PBS for 7 min. After washing, the staining solution was added according to the manufacturer's instructions and cryosections were incubated at 37°C in a warm room for 20 h. Pituitary sections were mounted and examined under light microscope and photographed at $100\times$, with only senescent cells staining in blue at a pH of 6.0. In order to determine the senescent cell index, dispersed pituitary cells from control and estrogenized animals, as well as control and estrogen-stimulated tumoral GH3B6 cells, were subjected to the same SA-b-gal staining procedure and counted in a Neubauer chamber. Rat kidney was used as positive control and the same reaction, but omitting the substrate, served as negative controls. For statistical purposes, randomly chosen visual fields photographed at $400\times$ and 2000 cells were counted for each cell dispersion for all experimental groups in triplicate.

Also, with the purpose of determining the double localization of SA-b-gal/Ki67 and SA-b-gal/PRL into the pituitary parenchyma, after performing the cytochemical detection of SA-b-gal staining, the same pituitary cryosections were processed by immunocytochemistry with monoclonal anti-Ki67 antibody (1:50; BD Pharmingen, Oxford, UK) or rabbit polyclonal anti-PRL (1:4000; NIH Hormone Program, Bethesda, MD, USA, USA), as referred to in the next section.

Immunocytochemistry

Paraffin-embedded pituitary glands were processed by immunocytochemistry for phosphorylated ATM (pATM), p53, and Ki67. The sections (3 μ m thickness) were treated with 3% v/v hydrogen peroxide in methanol for 30 min to inhibit endogenous peroxidase, followed by an antigen retrieval method using a hot antigen unmasking solution (0.01 M citrate buffer; pH 6.0). Then, sections were incubated for 30 min in 10% v/v normal serum (Sigma–Aldrich) to block nonspecific binding, which was followed by overnight incubation with anti-Ki67 (1:50), rabbit polyclonal anti-p53 (1:200; Santa Cruz), or mouse monoclonal anti-pATM (1:750; Abcam, Cambridge, UK) at 4 °C in a humidifier chamber. The sections were then incubated with biotinylated secondary antibodies and ABC complex, with staining carried out using 3,3'-diaminobenzidine tetrahydrochloride as a chromogen, according to the manufacturer's instructions (Sigma–Aldrich). Diaminobenzidine (Sigma–Aldrich) was utilized as the chromogenic substrate for 10 min at room temperature, and the sections were rinsed in running water, with hematoxylin being used as the counterstain (Sigma–Aldrich). Controls were performed by applying the same protocol, but omitting primary antibodies. The slides from three animals of each experimental condition were photographed in randomly chosen fields at 400 \times , with more than 2000-immunoreactive cells being examined. The Ki67, p53, and pATM labeling indexes were determined using NIH Image J Software (National Institutes of Health, Bethesda, MD, USA), and the number of positively stained nuclei was recorded along with the total nuclei.

Also, to assess colocalization of PRL/p21, PRL/Ki67, PRL/p53, and PRL/pATM, additional sets of slides from different experimental conditions were processed for confocal laser scanning microscopy. Paraffin-embedded pituitary sections were processed as mentioned before, but including incubation for 1 h in 70% v/v ethanol supplemented with 0.25% v/v NH_3 , while rehydrating the deparaffinized sections in graded alcohol in order to reduce autofluorescence (Baschong *et al.* 2001). Then, pituitary sections were

incubated with anti-PRL (1:4000), anti-Ki67 (1:50), anti-pATM (1:750), anti-p21 (1:50; Santa Cruz), or p53 (1:300) for 1 h. These slices were washed, and further incubated with Alexa 647 anti-rabbit or Alexa 594 anti-mouse secondary antibodies (1:1000; Invitrogen) for 1 h. The images were then obtained using an inverted FluoView FV 1000 confocal laser scanning microscope (Olympus) and were collected with a 60 \times objective. The analysis of confocal microscopy images was carried out using FV10-ASW 1.6 Viewer Software (Olympus Inc., Olympus, Hamburg, Germany).

Ultrastructural immunogold labeling

The subcellular localization of p21 and pATM in pituitaries was determined by a immuno-electron-microscopy post-embedding protocol as previously reported (Sabatino *et al.* 2013). In brief, pituitary glands were embedded in LR-White (London Resin Corporation, Berkshire, UK), and thin sections were incubated overnight on a drop of anti-p21 or anti-pATM antiserum, which was diluted 1:100. Immunoreactive sites were labeled with anti-mouse secondary antibody conjugated to 15 nm colloidal gold particles (1:30; Electron Microscopy Sciences, Hatfield, PA, USA). Then, to validate the specificity of the immunostaining, negative controls were performed, and the primary antibodies were replaced with either 1% w/v BSA in PBS or diluted preimmune serum followed by the corresponding secondary antibody. Finally, sections were stained with an aqueous uranyl acetate saturate solution, and examined under a Zeiss LEO 906-E electron microscope and photographed using a Megaview III camera.

Also, the effect of estrogen as an inducer of pituitary tumor development was documented in semithin sections from epoxy resin-embedded glands by high-resolution light microscopy. With this aim, pituitaries from control and E60 were fixed in a mixture of 4% v/v formaldehyde and 2% v/v glutaraldehyde in 0.1 M cacodylate buffer for 2 h and then treated with 1% OsO_4 before being block-stained with 1% v/v uranyl acetate, as previously described (De Paul *et al.* 2009). After dehydration with a series of graded cold acetones, glands were embedded in Araldite. The semithin sections (200 nm) were cut using a JEOL ultramicrotome with a diamond knife, stained with Toluidine blue, and examined using a Zeiss Axiostar Plus light microscope (Oberkochen, Germany).

Subcellular fractionation

The nuclear and cytoplasmic extracts from control and estrogen-treated rat pituitary glands were prepared

according to the method of Sugawara *et al.* (1993) with modifications (Mukdsi *et al.* 2004). Briefly, three pituitaries per experimental group (from a total of three independent experiments) were minced and homogenized (4 °C) with a teflon glass Potter Elvehjem tissue grinder in 2 ml 0.3 M sucrose, 1 mM EDTA, 1 mM phenylmethylsulphonyl fluoride, and 10 mM HEPES/KOH (pH 7.5). The homogenate was centrifuged at 1000 *g* for 10 min, and the supernatant was aliquoted and stored at −20 °C until required for cytoplasmic protein quantifications. The pellet containing the nuclear fraction was resuspended in 2 ml solution of 0.3 M sucrose, 1 mM CaCl₂, 10 mM Tris–HCl (pH 7.5), 1 mM dithiothreitol (DTT), and protease inhibitors. Then, 75 µl of 10% v/v Igepal CA-630 were added, and the solution was incubated at 4 °C for 10 min and centrifuged at 250 *g* for 5 min. After washing the pellet once with the same buffer, it was resuspended in two volumes of 20 mM HEPES (pH 7.8), 0.6 M KCl, 0.02 mM ZnCl₂, 0.2 mM EGTA, 0.5 mM DTT, and protease inhibitors, and incubated at 4 °C for 30 min. The suspension was centrifuged at 20 000 *g* at 4 °C for 30 min, and the aliquots were stored frozen until they were used in western blotting assays.

Western blotting analysis

The total protein concentration was measured using a Bio-Rad Kit (Bio-Rad Protein Assay, Bio-Rad) and subcellular fractions were run in 12% v/v acrylamide gel. To estimate the corresponding molecular weights, the Full Range Rainbow molecular weight marker was used (Amersham). The membranes were incubated overnight at 4 °C with the corresponding primary antibody: rabbit polyclonal anti-mitofusin 1 (MFN1) (1:750), rabbit polyclonal anti-dynamin-related protein 1 (DRP1) (1:500), rabbit polyclonal anti-rat sarcoma (RAS) (1:200), rabbit polyclonal anti-TGFβ1 (1:300), mouse monoclonal anti-IL6 (1:200), mouse monoclonal anti-p21 (1:200), rabbit polyclonal anti-p53 (1:200), rabbit anti-total ERK1/2 (1:300) (Santa Cruz), rabbit polyclonal anti-NFκB (1:1000), rabbit polyclonal anti-IL1β (1:300) (Abcam), rabbit polyclonal anti-cytochrome *c* oxidase subunit 4 (COX4) (1:100), rabbit polyclonal anti-pERK1/2 (1:700) (Cell Signaling Technology, Inc., Boston, MA, USA), mouse monoclonal anti-β-actin (1:1000), or rabbit polyclonal anti-HDAC1 (1:500; Sigma–Aldrich). After washing, blots were incubated with a peroxidase-conjugated (HRP) goat anti-rabbit (1:2500) or goat anti-mouse (1:2500) diluted in blocking buffer, rinsed in PBS/0.1% v/v Tween 20, and revealed with an ECL detection system (Amersham Biosciences). Emitted light

was captured on Hyperfilm (Amersham Pharmacia Biotech), and the β-actin, COX4, and HDAC1 antibodies were used as loading controls.

Detection of apoptosis by DNA nick-end labeling

Nick-end labeling was detected using the TUNEL technique, as previously reported (Palmeri *et al.* 2009). Pituitary sections fixed on glass slides were processed as stated in the manufacturer's protocol (*In Situ* Cell Death Detection Kit; Roche). As positive control, the reaction was carried out on DNase recombinant I (6500 U/ml, Invitrogen)-treated sections to induce DNA strand breaks before the labeling procedure. The slides were observed using a Zeiss Axiostar plus microscope at 400×.

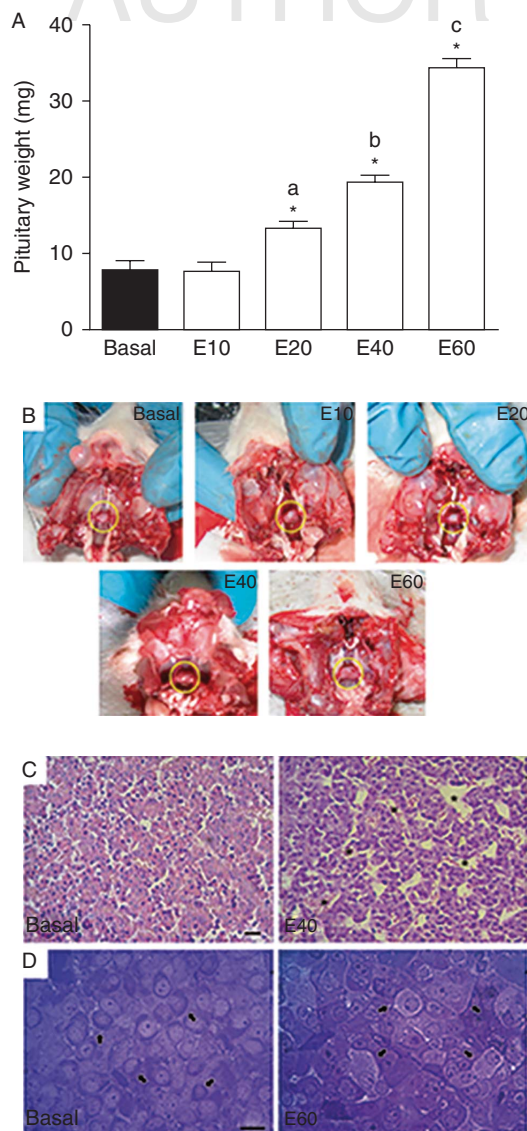
Statistical analysis

The statistical analysis was carried out using an ANOVA with Fisher's post-test (InfoStat version 2004; Grupo InfoStat, Facultad de Ciencias Agropecuarias, Universidad Nacional de Córdoba, Córdoba, Argentina). In order to analyze the binomial proportion data resulting from counting the data of the cytochemical reactions, a generalized linear mixed modeling procedure of InfoStat Software was utilized with a model that included the fixed effect of treatment (with the logit link function being the default for binomial proportion data). The results were given as the means ± S.E.M., and the data were obtained from three replicates measured for three independent experiments. The significance levels were chosen at $P < 0.05$.

Results

Estrogen treatment induced pituitary tumor development

For the purpose of evaluating the cellular senescence during the progression of the experimental pituitary tumors, proliferative lesions were induced in Wistar male rats as previously reported (Mukdsi *et al.* 2006, Sabatino *et al.* 2013). For all the periods analyzed: E10, E20, E40, and E60, the mean values of the serum E₂ levels were above 3.7 nM (4.00 ± 0.18 nM in E40), representing 100 times more than those achieved in control rats (0.18 ± 0.01 nM). The effect of prolonged estrogen administration on pituitary mass expansion was examined and a significant and progressive increase was observed, with the highest values being obtained at E60 (Fig. 1A). This was also corroborated by the macroscopic observation in sellae,

**Figure 1**

Rat pituitary expansion after estrogen administration. (A) Pituitary weight (mg) from control (basal) and estrogen-treated rats ($n=12/\text{group}$). Results are expressed as means \pm s.e.m.; Fisher's test ($*P<0.05$ vs basal; means with different letters are statistically different). (B) Photographs of representative in sellae pituitary glands derived from male rats submitted to different periods of tumor development. The pituitary gland sizes are depicted by a circle. (C) Paraffin-embedded adenohypophysis sections from control and estrogen-induced tumoral pituitaries (E40). The parenchyma is surrounded by a network of vascular channels (*), with a marked development in models of sustained stimulation with estradiol benzoate, mainly starting from 40 days of hormone treatment. Staining: H/E. Scale bar: 10 μm . (D) Micrographs of semithin sections (200 nm) of Araldite-embedded pituitary gland from control and E60 stained with Toluidine blue. The sustained estrogen exposition provoked a streaking hypertrophy of endocrine cells and hormonal release, as revealed by the lower Toluidine blue staining in the cytoplasm in E60 compared with control cells (arrow). Scale bar: 10 μm . A full colour version of this figure is available at <http://dx.doi.org/10.1530/ERC-14-0333>.

which revealed a notable size growth compared with normal pituitaries (Fig. 1B). The increase in the pituitary weight was also accompanied by vascular channel enlargement (Fig. 1C), with noticeable cellular hypertrophy being observed by high-resolution light microscopy (Fig. 1D).

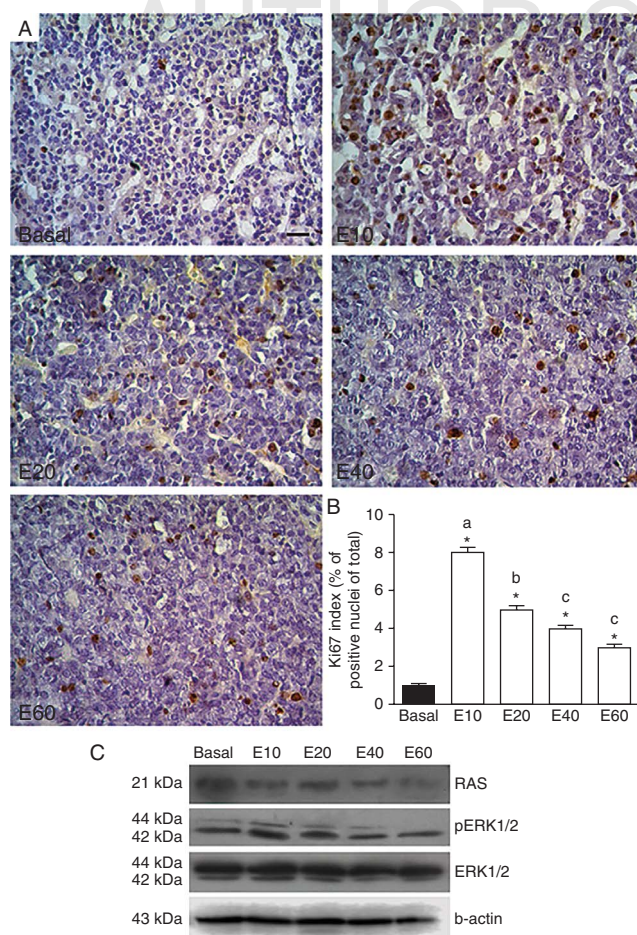
Next, we analyzed the time-course changes during the pituitary tumor development by determining the Ki67 labeling index. The quantification of Ki67-immunopositive cells revealed a significant increase in cell proliferation for all time periods analyzed compared with controls, with a peak reached of approximately eightfold higher at E10. In spite of the noteworthy pituitary proliferative response, the estrogen-induced mitogenic potential rate was not sustained for the whole period analyzed and successively decreased after the E10 condition. Interestingly at E60, the proliferative rate exhibited a progressive reduction compared with that attained at E10, with minimal changes observed from E40, which might indicate a plateau phase, although this index still continued to be higher, reaching threefold higher than the control values (Fig. 2A and B).

RAS-deregulated oncogenic signaling induces cellular senescence (Braig *et al.* 2005, Chen *et al.* 2005, Collado *et al.* 2005, Michaloglou *et al.* 2005). Thus, to determine the contribution of RAS/ERK1/2 signaling during the estrogen-induced pituitary tumor development, their expression levels were analyzed by western blotting. As shown in Fig. 2C, RAS signaling was progressively suppressed, with its expression being less evident at E40–E60 than that at baseline conditions of normal pituitaries. When the pERK1/2 protein was analyzed, a similar pattern expression was observed for its phosphorylated form during the development of experimental pituitary tumors.

Finally, to examine whether this decrease in cell proliferation could be due to apoptosis, we also determined the biochemical features of this type of cell death in pituitary cells by the TUNEL method. As shown in Fig. 3, for all experimental groups, the signs of apoptotic nuclei were practically absent with no significant changes in the number of TUNEL-positive cells being detected, thus suggesting that the modifications observed in the growth rate could not have been associated with apoptosis.

Tumoral pituitary cells acquired features of the cellular senescence phenotype

To try to determine whether cellular senescence takes place during estrogen-induced pituitary tumor development, we turned our attention to the detection of SA-b-gal,

**Figure 2**

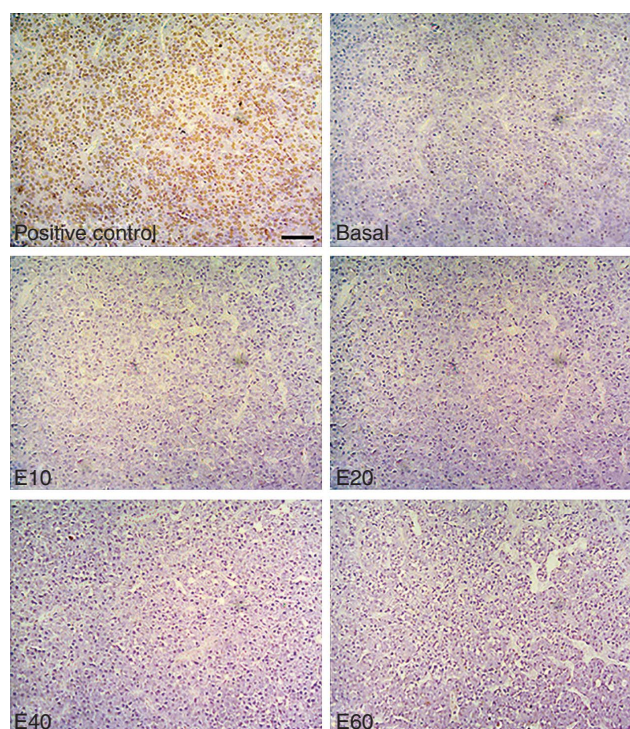
Ki67 immunolabeling in the estrogen-induced pituitary tumors. (A) Immunocytochemical detection of Ki67 in sections of pituitaries from control and estrogenized rats. A remarkable increase in Ki67 reactivity (brown signal) was seen at the beginning of the proliferative injuries. Representative fields of anterior pituitary sections from a representative experiment from a total of three independent experiments with similar results are shown. Scale bar = 20 μ m. (B) The Ki67-positive nuclei staining is expressed as a percentage of the total nuclei counted. Data are expressed as means \pm S.E.M. using the Fisher's test (* P < 0.05 vs basal; means with different letters are statistically different). (C) RAS/pERK protein expression during the development of experimental pituitary tumors. Analysis of RAS and pERK1/2 (total fraction) from control and tumoral pituitary cells by western blotting revealed a RAS/pERK gradual suppression during pituitary tumor development. The b-actin and total ERK1/2 protein expression confirmed equal loading occurring in all lanes. Data are from a representative experiment from a total of three independent experiments with similar results. A full colour version of this figure is available at <http://dx.doi.org/10.1530/ERC-14-0333>.

and as shown in Fig. 4A a progressive rise in the enzymatic activity was observed. In order to perform a morphometric analysis of these modifications, the SA-b-gal reaction was also developed on dispersed pituitary cells. The SA-b-gal-positive cell index revealed a baseline expression at

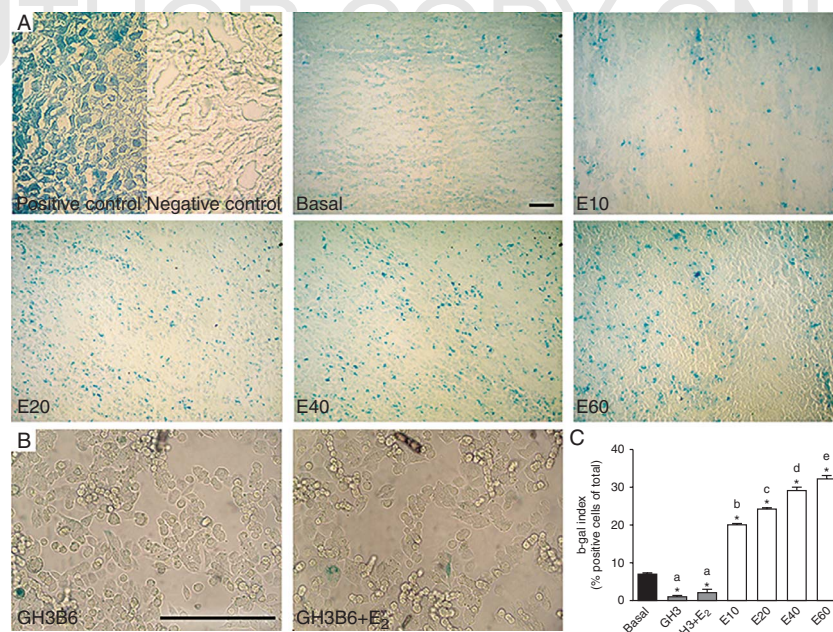
around 7% of the total pituitary cells, whereas in pituitary tumors a significantly higher number of SA-b-gal-positive cells was observed, with values ranging from almost threefold in the earliest days of the proliferative injury and rising over time until a fivefold increase was recorded at E60 (Fig. 4C).

As it has been previously described that cellular senescence might be overcome in order to develop unregulated cell proliferation (Campisi 2005, Collado & Serrano 2010), we next evaluated SA-b-gal reactivity in the established GH3B6 pituitary tumor cell line under baseline conditions as well as after sustained estrogen stimulation. As expected, positive cells were scarce, hardly exceeding 1–2%, a value far below those obtained in tumoral pituitaries (Fig. 4B and C).

Hence, during estrogen-induced pituitary tumor development, a cellular senescence process might be emerging, while GH3B6 pituitary tumoral cells rarely become senescent.

**Figure 3**

TUNEL assay on normal and experimental pituitary tumors. No significant detection of biochemical features of apoptosis (brown signal) was detected in histological samples from each experimental condition. Positive control: pituitary gland section treated with DNAase I. Data from a representative experiment for a total of three independent experiments with similar results from each experimental group. Scale bar = 100 μ m. A full colour version of this figure is available at <http://dx.doi.org/10.1530/ERC-14-0333>.

**Figure 4**

Senescence features are expressed during pituitary tumor development. (A) Cryosections from normal and tumoral pituitaries showing increases in SA-b-gal reactivity during pituitary tumor development, and GH3B6 cells presenting a low detection of SA-b-gal. The SA-b-gal enzymatic activity is dyed in blue. Positive control, rat kidney and negative control, rat kidney omitting the substrate. Results of a representative experiment are shown. Scale bar = 100 μ m. (B) SA-b-gal reaction in E₂-stimulated GH3B6 cells. The cells were cultured with a sustained E₂ exposition (120 h; 4 nM).

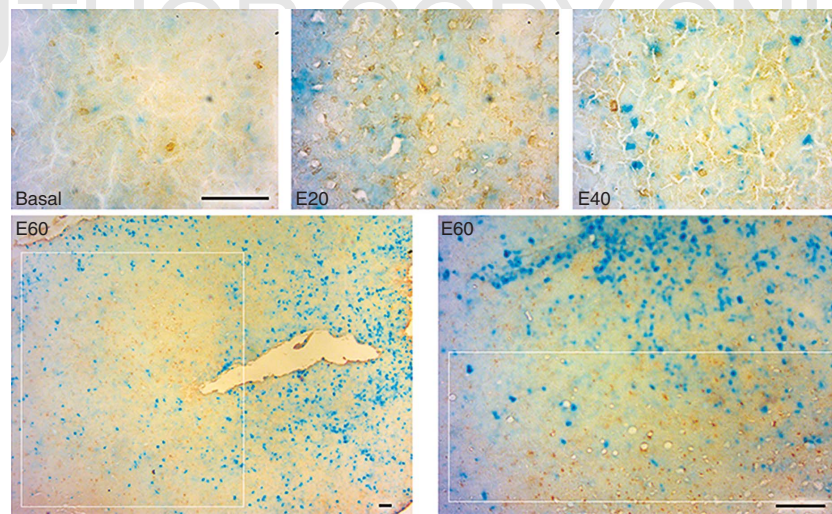
(C) The number of SA-b-gal-positive cells stained in blue was evaluated and related to the total number of pituitary cells counted for each experimental group. Also, SA-b-gal index from control and estrogen-induced GH3B6 pituitary tumoral cell line was determined. Data were statistically analyzed by the Fisher's test, and the results are expressed as means \pm S.E.M. (* P < 0.05 vs basal). A full colour version of this figure is available at <http://dx.doi.org/10.1530/ERC-14-0333>.

Furthermore, we determined the SA-b-gal reactivity and Ki67 staining in the same cryosection for each experimental condition. As shown in Fig. 5, for all examined conditions, the cellular senescence response was noticeably profuse regarding cell proliferation. By analyzing the joint progression of both biomarkers during the tumoral development, we found that, toward E40 and E60, it was possible to discriminate pituitary parenchyma differential areas in which Ki67-positive cells were predominant, and adjacent zones bore a higher proportion of cells exhibiting the SA-b-gal stain.

To investigate the SASP and involvement of NFkB expression (Kuilman *et al.* 2008, Freund *et al.* 2010), next we evaluated IL6, IL1b, TGFb1, and NFkB protein expression levels in pituitary extracts by western blotting. As shown in Fig. 6A, a remarkable increase in IL6 expression levels toward E40 and E60 was detected. In agreement with this result, we also showed increases in IL1b and TGFb1 protein expression levels from E20 to E60 (Fig. 6A). These responses were also accompanied

by a rise in both the cytoplasmic and nuclear NFkB expression, indicating the latter's activation in the context of an experimental pituitary tumor (Fig. 6B). These data indicate that under the present experimental context, inflammatory network activation was triggered for this context together with cellular senescence response.

In order to further characterize the senescence phenotype achieved by tumoral pituitaries, we were also interested in determining whether the mitochondrial dynamics has been compromised, because it was previously reported that mitochondrial dysfunction may facilitate cellular senescence emergence (Moiseeva *et al.* 2009). Thus, by western blotting, the expression of MFN1 and DRP1 were assessed as indicators of fusion and fission processes respectively. As shown in Fig. 6C, both proteins were affected during the development of experimental pituitary tumors, while MFN1 protein expression was progressively upregulated; the DRP1 expression was notably decreased during *in vivo* pituitary tumor development.

**Figure 5**

SA-b-gal and Ki67 staining in cryosections from normal and tumoral pituitaries. For all examined conditions, proliferating cells (white rectangle) did not show any SA-b-gal expression. Results of a

representative experiment are shown. Scale bar = 20 μ m. A full colour version of this figure is available at <http://dx.doi.org/10.1530/ERC-14-0333>.

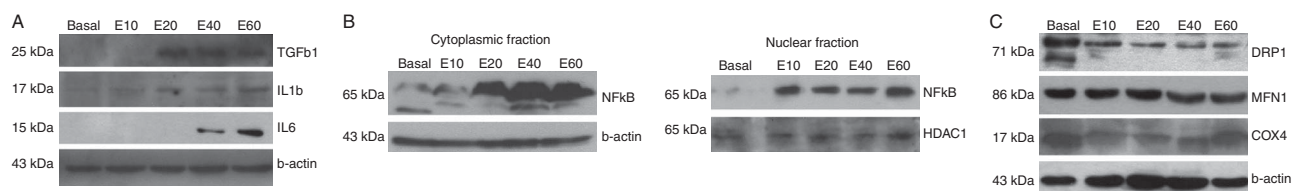
Activated DNA damage signaling, p21 accumulation, and an abnormal cell cycle profile are displayed in experimental pituitary tumors

The pattern of expression of several key cycle regulators involved in the senescence pathways was examined. Considering that the DDR plays an essential role in the senescence pathways (Di Micco *et al.* 2006), we aimed to find out if SA-b-gal expression was accompanied by DDR activation.

By immunocytochemistry, a significant increase in the number of pituitary cells displaying nuclear pATM expression was detected in experimental pituitary tumors, reflecting the activation of DNA damage signaling. The pATM signal showed a sustained increase from E10 to E60, with a significant peak of nuclear pATM-positive cells of around 30% at E40 (Fig. 7A and B). The pATM subcellular

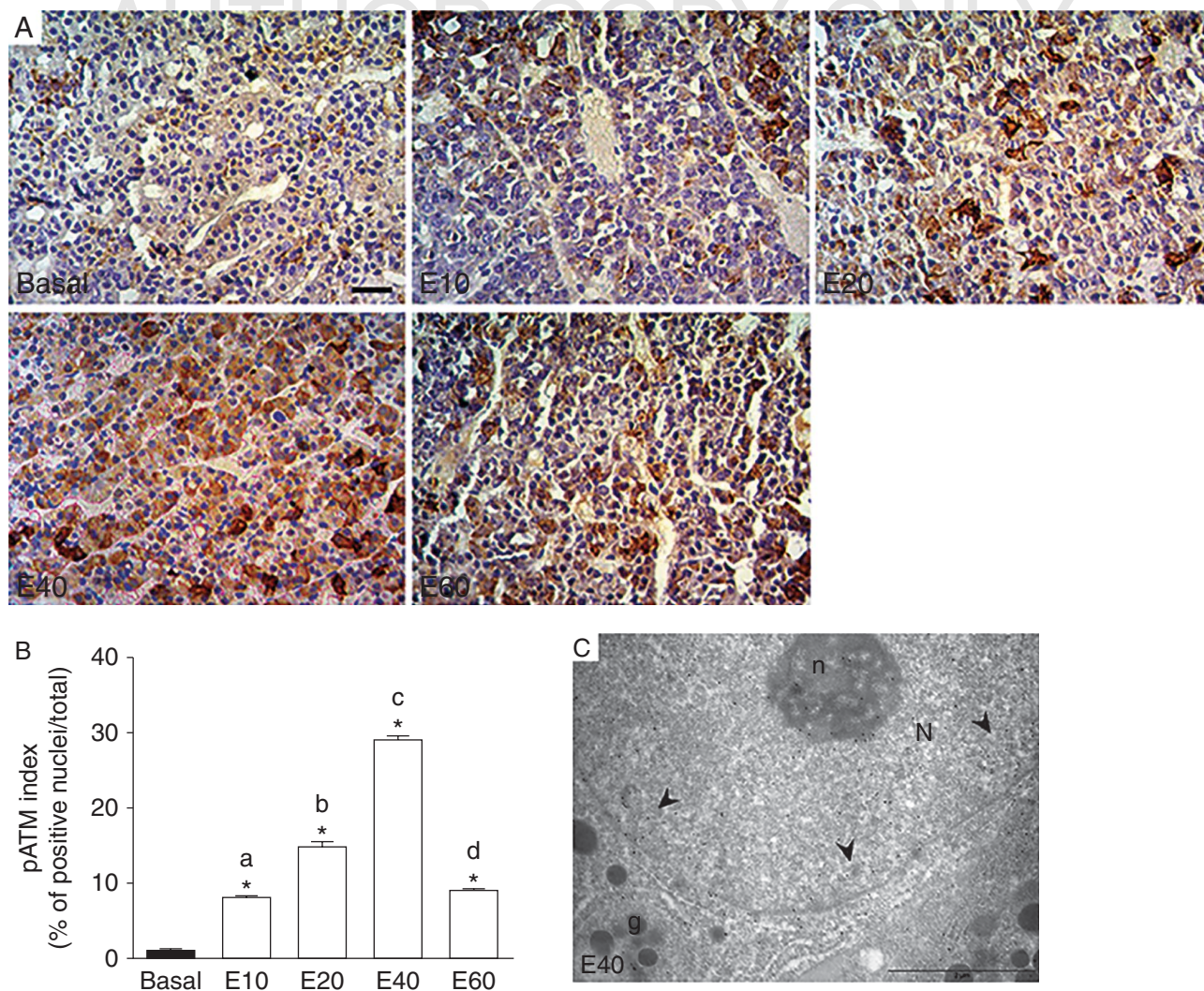
localization was corroborated by electron microscopy immunogold labeling, which revealed both nuclear and cytoplasmic immunoreactivity in tumoral pituitaries taking place at E40 (Fig. 7C).

The p53 protein, one of the major regulators of cell cycle progression in response to DNA damage or arrest of DNA synthesis, is considered to be an ATM target (Pluquet & Hainaut 2001). Using immunocytochemistry, an increase in p53 expression was detected for E10 and E20, whereas for E40 this signal was no longer observed (Fig. 8A). Considering that the p53 function is related to its intracellular localization, we also analyzed p53 subcellular expression by western blotting from nuclear and cytoplasmic pituitary extracts. An important increase in p53 signaling was immunodetected from E20 in the cytosolic compartment of pituitary cells, while a decrease

**Figure 6**

Senescence features are expressed during pituitary tumor development. Western blotting expression of IL6, IL1b, and TGFb1 (A); NFkB (cytoplasmic and nuclear fractions) (B) and DRP1; MFN1 (total extracts) (C) and from control and tumoral pituitary glands revealed signs associated with

inflammatory network activation and mitochondrial fusion. The b-actin, COX4, and HDAC1 protein expressions were used as loading controls. Data are from a representative experiment from a total of three independent experiments with similar results.

**Figure 7**

DNA damage in estrogen-induced pituitary tumors. (A) DNA damage signaling labeled by intranuclear accumulation for pATM immunoreactivity (brown signal) in normal and tumoral pituitaries. Representative sections from normal and estrogen-treated anterior pituitary glands are shown. Scale bar = 20 μ m. (B) The pATM-positive nuclei as a proportion of the total nuclei in pituitary sections are shown and results are expressed as means \pm s.e.m.; Fisher's test (* P < 0.05 vs basal; means with different letters are

statistically different). (C) Electron micrograph of pituitary cells from E40 expressing pATM label by using secondary antibody conjugated with 15 nm gold particles. Black arrows indicate pATM signaling accumulated in the cell compartments. N, nucleus; g, hormone granules; m, mitochondrion. Scale bar = 2 μ m. A full colour version of this figure is available at <http://dx.doi.org/10.1530/ERC-14-0333>.

in p53 nuclear fraction levels starting at E20 was also observed (Fig. 8B). Therefore, we next evaluated the expression of the CDK inhibitor p21 protein, a transcriptional target for p53 that is involved in cell cycle responses to DNA damage (Besson *et al.* 2008). By western blotting analysis, an increase in the nuclear p21 was observed, starting from E20 and strengthened over time from 40 to 60 days of tumor development (Fig. 9A). The subcellular localization of p21 analyzed by electron microscopy immunogold labeling for E40 pituitary glands revealed a streaking immunoreactivity in the nuclear and

cytoplasmic compartments of pituitary cells (Fig. 9B), a result that might be related to the lower pituitary cell proliferation detected at E40 (Fig. 2A and B). Moreover, p21 cytoplasmic accumulation was also observed for E20 and E40. However, an additional evaluation performed on GH3B6 tumoral pituitary cells showed that these cells did not express p21 (Fig. 9C), which could be associated with the low SA-b-gal reactivity observed in this cell type (Fig. 4B and C).

Alterations in the pituitary cell cycle profile were analyzed by FACS, and in order to try to discriminate

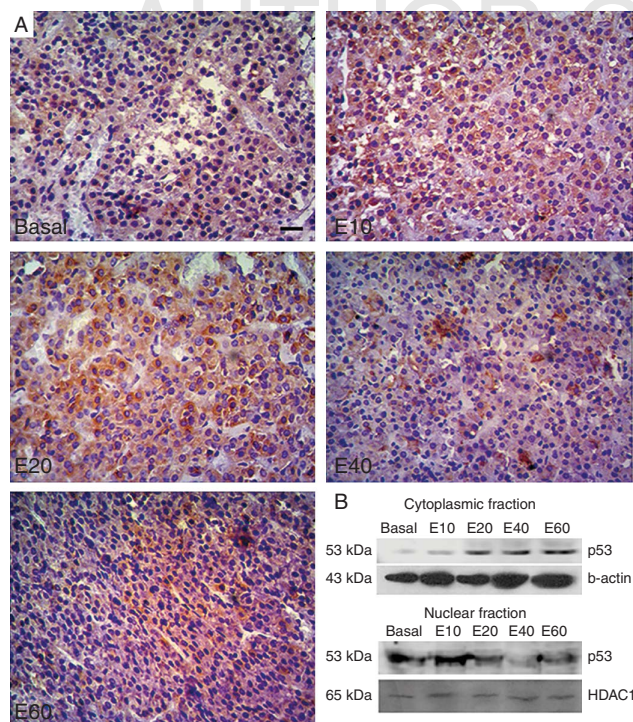


Figure 8
p53 expression during pituitary tumor development. (A) p53 immunoreactivity (brown signal) in normal and tumoral pituitary sections. An increase in p53 detection is observed at E10 and E20. Representative fields of anterior pituitary sections are also shown. Scale bar = 20 μ m. (B) The p53 expression showed a cytoplasmic redistribution during the tumor development as revealed by western blotting in fractionated pituitary extracts. Expression of b-actin and HDAC1 was used as loading controls. Representative images of three independent experiments are presented. A full colour version of this figure is available at <http://dx.doi.org/10.1530/ERC-14-0333>.

between the cycling population and S-phase arrest, the S-phase was compared with the G2/M population, with the result being expressed as a ratio. As shown in Fig. 9D, a significant increase in the S-phase:G2/M ratio was detected for E40 compared with the control ratio (57.63 ± 14.87 vs 12.59 ± 10.86 respectively; $P < 0.05$). These results together with the DDR activation and p21 upregulation reinforce the possibility of a delay occurring in the cell cycle progression, which became more evident toward 40 days of tumor progression.

Senescence markers are not restricted to PRL cells

As most pituitary tumors induced by estrogen in experimental animals are PRL-secreting tumors (De Nicola et al. 1978, Wiklund et al. 1981, Sadoul et al. 1992, Mukdsi et al. 2004), we attempted to determine whether lactotroph specific cell lineage was involved in the pituitary senescent

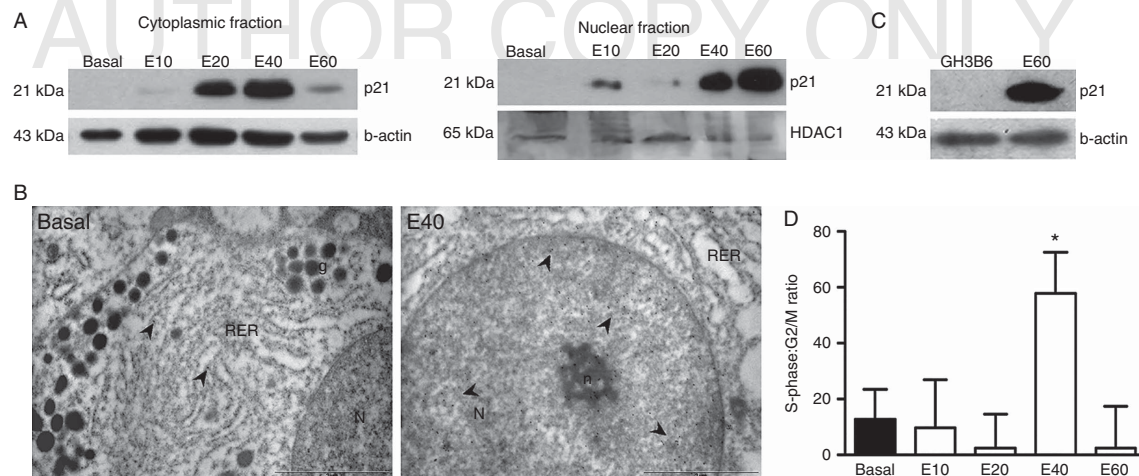
response. The colocalization between PRL/Ki67, PRL/p21, PRL/p53, PRL/pATM, and PRL/Sa-b-gal proteins was carried out by dual immunolabeling for different experimental conditions. As shown in Fig. 10, for all the combination of markers analyzed, it was possible to detect double-stained cells, indicative of lactotrophs, which might possibly be senescent as shown by SA-b-gal, DDR activation by pATM or p21 accumulation indicating cellular arrest. However, along with double-stained cells, it was also possible to observe single-stained cells, representative of either non-senescent lactotrophs or nonlactotroph cells undergoing senescence. Therefore, none of the marker expressions examined exclusively colocalized with PRL-positive cells, suggesting that the senescence phenomena may not be restricted to lactotroph populations.

Discussion

In this study, we have demonstrated clear evidence of pituitary senescence taking place during *in vivo* estrogen-induced pituitary tumor development. The cell proliferation reduction and expression of cellular senescence features such as SA-b-gal and IL6 expression, as well as DNA damage signaling activation and p21 expression, indicate a role for senescence in the regulation of pituitary cell growth.

The particularities of benign pituitary tumors have led to the appearance of senescence being considered, which has been proposed as an intrinsic barrier for tumor development that emerges at the early stages of neoplastic growth (Priour & Peeper 2008). This pituitary cellular response might be beneficial for organisms, because it allows proliferation to be arrested while maintaining its physiological functions (Adams 2009), and therefore, hormones may continue to be secreted (Mooi 2009).

The tumor suppression role of cellular senescence has been convincingly established *in vitro* (Bringold & Serrano 2000, Campisi 2001, Mooi & Peeper 2006), but further evidence is still needed to demonstrate the relationship between *in vivo* senescence and proliferation control (Mooi & Peeper 2006, Collado & Serrano 2010). In this context, benign pituitary microadenomas are faithful models of *in vivo* senescence (Mooi 2009, Chesnokova & Melmed 2010). Previous studies have already reported that murine pituitary adenomas with altered PTTG and Rb expression trigger cellular senescence (Chesnokova et al. 2007, 2008). These authors also correlated the high levels of growth hormone (GH) with the senescence-associated markers in adenomas, and suggested that cellular senescence is activated in a lineage-specific fashion

**Figure 9**

The expression of CDK inhibitor p21 was increased in tumoral pituitary glands. (A) The analysis of the p21 protein expression levels by western blotting revealed an increase in its expression in both nuclear and cytosolic extracts during tumor development. (B) The ultrastructural localization of p21 was examined in control (basal) and E40 pituitary glands by electron microscopy immunogold labeling. Black arrows indicate p21 signaling mainly expressed at the nuclear cell compartment. N, nucleus; n, nucleolus; RER, rough endoplasmic reticulum. Scale bar = 2 μ m. (C) Total extract of established GH3B6 tumoral cells did not express p21. The expression of

b-actin and HDAC1 was used as loading controls. Representative images of three independent experiments are shown. (D) S-phase:G2/M ratio analysis during experimental pituitary tumor development. The DNA content was determined by flow cytometry in order to evaluate modifications in the pituitary cell cycle profile of normal and tumoral pituitary cells. A notable increase in the S-phase:G2/M ratio is observed for E40, when compared with control ratio. The means \pm S.E.M. of the percentages of cells at different cell cycle stages are presented Fisher's test (* $P < 0.05$ vs basal).

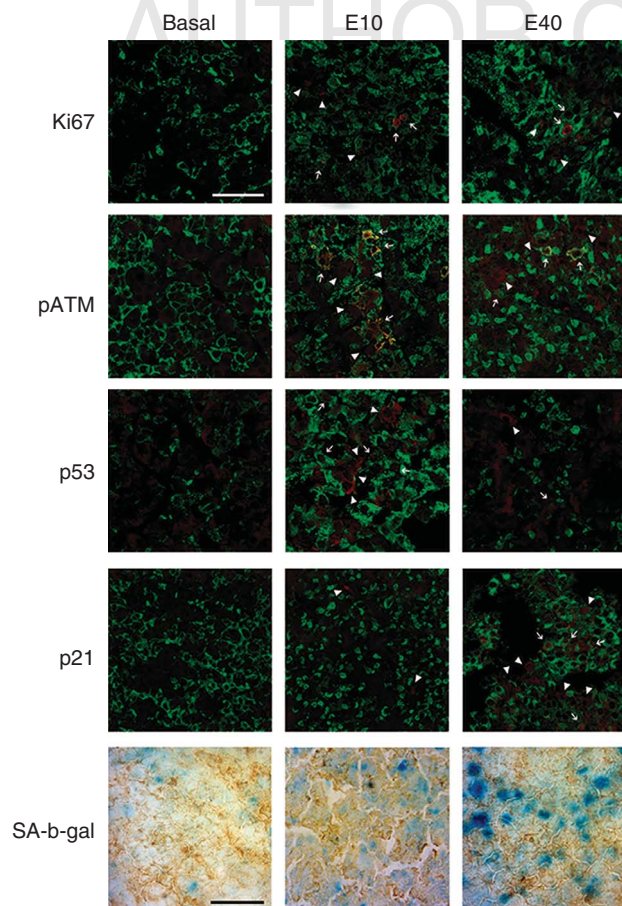
(Chesnokova *et al.* 2008, 2011, 2013). However, to date, no attempt has been made to analyze the emergence of cellular senescence with pituitary tumor progression in the absence of genetic manipulation or specific senescence-inducing stimuli. In this study, we have demonstrated that both senescence markers and DNA damage signaling were expressed during the development of estrogen-induced pituitary tumors. This present *in vivo* approach allows us to suggest a role of cellular senescence as a pituitary intrinsic response to chronic cellular over-activation.

Although the pituitary cells of adult rats are characterized by low proliferation rates (0.7–0.8%; McNicol & Carbajo-Perez 1999), we have observed a strong stimulation of cell proliferation accompanied by hypertrophy and vascular network development after the start of estrogen treatment, with all of these parameters contributing to the significant mass increase revealed by assessing the wet pituitary weight data. Although the proliferation index still presents significantly higher values at E60 than those detected in controls, the initial increase in cell proliferation lost potency at advanced endpoints, indicating a plateau phase in the cell proliferation rate until the end of stimulation, as shown by Ki67 immunoreactivity. Consequently, this biphasic pattern might be distinct from the exponential proliferation

typically associated with aggressive tumoral progression (Hanahan & Weinberg 2000).

Furthermore, GH3B6 represents an equivalent for estrogen-induced pituitary tumor cells, but as an established and autonomous pituitary tumoral stage, showing a basal proliferation rate of around 25–30% of total cells (Giles *et al.* 2010, Petiti *et al.* 2015), while normal pituitary cells showed 1% of proliferation, with the replication index of estrogen-induced pituitary tumoral cells reaching a maximum of 8%. The use of this tumoral cell line permitted us to analyze the presence of cellular senescence in a cell population, which might represent a clone in the heterotypic tumor complexity that has acquired growth signaling autonomy and may have possibly lost the ability to respond to some cellular arrest signals.

Although intrinsic tumoral suppression has been classically associated with apoptosis induction (Lowe *et al.* 2004), we detected very few apoptosis events throughout the adenoma development, in agreement with previous findings (Spady *et al.* 1999, Palmeri *et al.* 2009). Earlier reports have demonstrated that estrogen administration enhances cell survival in the anterior pituitary gland by inhibiting apoptosis (Spady *et al.* 1999). Therefore, an apoptotic block manifested during an estrogen-induced tumor development may support

**Figure 10**

Ki67, pATM, p21, p53, and SA-b-gal expression in lactotroph cells. Confocal laser scanning microscopy of pituitary paraffin-embedded sections from control, E10, and E40 glands co-stained for PRL (green) with Ki67, pATM, p21, or p53 (red). Single-stained lactotrophs, single-stained non-PRL cells (arrowheads), and double-stained lactotrophs (arrows) are seen. Also, immunolabeling for PRL (brown) and histochemistry for SA-b-gal (blue) were performed on the same cryosection. Data are from a representative experiment from a total of three independent experiments with similar results. Scale bar = 20 μ m. A full colour version of this figure is available at <http://dx.doi.org/10.1530/ERC-14-0333>.

pituitary senescence being a cytological stress response (Marcotte *et al.* 2004). In this way, senescence might be a more appropriate mechanism for pituitary tumor suppression, taking into account that the pituitary gland is critical for the homeostatic function, but which exhibits a regenerative capacity (Chesnokova *et al.* 2013).

It is known that multiple processes might take place in the gland during tumor development. In fact, it is the balance between proliferation with death or senescence that determines the growth rate of a particular tumor (Lowe *et al.* 2004). The determination of double SA-b-gal/Ki67-positive cells allowed us to confirm the idea that senescence is an important outcome in estrogen-induced

pituitary tumors, as it was observed to be profuse regarding cell proliferation. In addition, the nonoverlapped distribution of Ki67 and SA-b-gal-positive cells observed at E60 supported the hypothesis of heterotypic cell populations that coexist during tumor development. Hence, it is possible to associate this coexistence of different kinds of responses to the fact that the five cell types constitute the pituitary cell population. Thus, specific diverse cell-type trophic pathways may be activated in reaction to long-term estrogen treatment.

Considering that the senescent cell phenotype still lacks specific markers, in this study several senescence-associated features were evaluated in order to investigate this cellular mechanism. The strong association between the SASP pattern and the other senescence biomarkers (p21, pATM, and Sa-b-gal) led us to connect these increased cytokine expressions with emerging pituitary senescence within estrogen-induced pituitary tumors. A putative role for IL6 in pituitary senescence induction has been previously suggested (Fuertes *et al.* 2010), with TGF β 1 and IL1 β also having a known impact on pituitary tissue homeostasis (Haedo *et al.* 2009). We have recently demonstrated somatolactotroph GH3B6 tumoral cells exhibiting a refractory behavior to the TGF β 1 inhibitory effects on cell cycle progression, as a consequence of the crosstalk with proliferative pathways that counterbalances the TGF β 1 signal (Petiti *et al.* 2015). Considering the specific activities implied in the secretion of each cytokine, while IL6 is considered to be a critical controller of the SASP and seems to have a more significant role in autocrine senescence (Kuilman *et al.* 2008), the TGF β 1 and IL1 as SASP factors are able to trigger senescence by paracrine actions (Hubackova *et al.* 2012).

In this regard, it is known that the proteins encoded by NF κ B target genes contribute in multiple ways to the biological effects of NF κ B, which include inflammation response, apoptosis, mitogenic activity, implications in the evolution of neoplastic injuries, cellular senescence, and DNA repair (Hayden & Ghosh 2004, Pikarsky & Ben-Neriah 2006). Furthermore, NF κ B operates as a molecular switch that conveys signals from an intricate signaling network, thereby regulating essential processes through the gene transcription regulation.

Regarding the focus of our study, NF κ B cooperates through feedback regulation within the senescence process, because it can trigger SASP in response to persistent DNA damage signaling (Freund *et al.* 2010). Therefore, we focused on NF κ B activity as an accompaniment of the SASP activation evidence, rather than the association with growth regulation pathways in which NF κ B activity may

also surely participate (Freund *et al.* 2010). In this context, several reports have implied that SASP induction is an NFkB-dependent mechanism (Acosta *et al.* 2008, Chien *et al.* 2011, Salminen *et al.* 2012), suggesting that the SASP is caused primarily by genotoxic stress rather than by proliferative arrest *per se* (Freund *et al.* 2010). From our data, the notable increase in SASP component expression with NFkB activation suggests that the inflammatory network was triggered during pituitary tumor development. Under the present experimental context, the expression of these cytokines collectively reinforces the role of SASP in ensuring an efficient growth arrest, as was also proposed by others (Yang *et al.* 2006, Acosta *et al.* 2008, Kuilman *et al.* 2008, Wajapeyee *et al.* 2008).

The mitochondrial metabolism is also compromised during cellular senescence, because DNA damage can be powered by oxidative stress triggering premature senescence (Moiseeva *et al.* 2009). In our study, we detected a decreased DRP1 together with increased MFN1 expression, suggestive of a misbalance between mitochondrial fission toward fusion processes in favor of an enlargement of the organelle. Therefore, the alteration of the mitochondrial dynamics equilibrium toward the prevalence of fusion processes might indicate an adaptation of the functionality of this organelle during pituitary tumor development, occurring together with cellular senescence. Regarding this, an ineffective mitochondrial fission triggering cellular senescence has been previously described (Lee *et al.* 2007).

In view of the fact that our experimental conditions did not include cytotoxic drugs, irradiation stimuli, or other stimuli strongly associated with DNA damage or senescence induction, the manifestation of senescence signs during the development of the pituitary tumor constitutes a noteworthy finding of our study. Nevertheless, it cannot be ignored that a multiplicity of molecular pathways could be involved in the development of estrogen-induced pituitary tumors (Sarkar 2006). Regarding this, we analyzed RAS/ERK pathway activation due to the fact that abnormally high mitogenic signals such as estrogen-induced RAS activation (Weigel 1996) might function as a prolonged cellular overactivation capable of inducing cellular senescence (Serrano & Blasco 2001). However, this key core element is part of a complex signaling network with many interactions (McKay & Morrison 2007), and there have been extensive attempts to clarify its contribution in the promotion of cell growth, with it being the fine regulation of these pathways which determines cell cycle arrest or malignant transformation (Serrano *et al.* 1997, Roberts & Der 2007). Moreover, although there are some studies

showing increases in RAS/ERK activity in pituitary tumors (Karga *et al.* 1992, Cai *et al.* 1994, Dworakowska *et al.* 2009), its role in these tumors is far from being fully deciphered (Cakir & Grossman 2009). In our study, RAS/pERK signaling was progressively suppressed thereafter, with its expression being less evident at E40–E60 than at baseline conditions. These results imply that RAS/ERK effects were therefore, to a major extent, impaired, and that there might exist a regulation mechanism that could attenuate the abnormal mitogenic signals. Supporting this notion, Courtois-Cox *et al.* (2006) have shown that the suppression of RAS is sufficient to induce cellular senescence, and that this event, on its own, can activate the known downstream mediators of the senescence response through a variety of mechanisms.

The detection of pATM expression demonstrated in our study suggests the possibility of DNA damage during experimental tumoral development, because ATM has been reported to be one of the first proteins that responds to DDR (Di Micco *et al.* 2006). Therefore, the combination of a very high mitogenic signaling, the possibility of chromatin disorder, and a blockage of apoptosis might favor the emergence of cellular senescence as an alternative to control pituitary growth (Di Micco *et al.* 2006, Chesnokova & Melmed 2010).

As senescence pathway involves cell cycle machinery committed to cellular arrest (Kuilman *et al.* 2010), we evaluated pituitary cellular arrest through the analysis of the p53 and p21 restriction-point cell cycle proteins and the cell cycle profile.

It is known that p53 integrates complex signaling networks, increasing lifespan through genome maintenance and triggering various cell fate decisions (Rodier *et al.* 2007). At the early stages of pituitary tumor development, we observed an increase in p53 expression levels, with its intracellular localization being principally detected in the cytoplasm compartment. Furthermore, as it has been described that estrogen induces p53 inactivation through intracellular redistribution toward the cytosol (Molinari *et al.* 2000), our findings could be interpreted as a p53 stabilization mechanism, as previously suggested (Liu *et al.* 2006). Despite having found DDR activation, the cytoplasmic p53 retention might indicate its incapability to exert its canonical functions, thereby triggering a response that either repairs DNA damage or else removes the affected cell from the replicative pool. This indirect evidence may explain the great accumulation of pATM signaling, thus in turn favoring the increase in the senescence signs observed at E40.

Although in most cells, p53 is crucial for a senescence response following DNA damage (Di Leonardo *et al.* 1994),

in others it is only required to induce senescence growth arrest and not necessary for the maintenance of senescence, which subsequently becomes irreversible and p53 independent (Beausejour *et al.* 2003). Thus, cellular senescence might not appear to entail a consistent sustained rise in p53 protein levels (Itahana *et al.* 2001). Under our experimental conditions, it is possible that nuclear p53 initiated both senescence growth arrest and some of the functional aspects of the senescent phenotype by inducing p21. These p21 levels can then be sustained, at least for several weeks, by a p53-independent mechanism (Itahana *et al.* 2001).

CDK inhibitor p21 expression, strongly related to cellular senescence pathways, elicits cell cycle arrest (Roninson 2002). Pituitary growth control by p21 has already been described, indicating its key role in senescence pathways, while it was undetectable in pituitary carcinomas (Chesnokova *et al.* 2007). In this study, the upregulation of nuclear p21 protein expression in close relationship with the highest levels of SA-b-gal reactivity suggests that cell cycle restraint might take place during the development of pituitary tumors. Moreover, in our study, the lack of SA-b-gal and p21 protein expression reactivity in GH3B6 pituitary cells indicates the incapability of these cells to trigger a self-limiting senescence arrest at the tumoral stage acquired in GH3B6 cells. In fact, Chesnokova *et al.* (2008) have already reported that p21 expression underlies decreased pituitary tumor development, and that this CDK inhibitor was undetectable in tested human pituitary carcinomas. Thus, under the present experimental conditions, p21 might constitute another significant mediator in constraining pituitary growth in tumor development. In addition, it has been reported that this CDK inhibitor can function under either p53-dependent or independent conditions (Gartel & Tyner 1999). Considering that in our study no association between the increase in p21 levels with a p53 expression pattern was seen, p21 might therefore be induced in a p53-independent fashion. In agreement with this, p53-independent effects of p21 have also been reported in relationship with cell cycle arrest of senescent cells (Noda *et al.* 1994). Furthermore, some studies have presented evidence of induction of the CDK inhibitor p21 in response to activation of the cytokines IL6 and TGF β signal transduction pathways (Zeng & el-Deiry 1996, Bellido *et al.* 1998, Florenes *et al.* 1999). Thus, SASP induction in experimental pituitary tumors may be responsible for senescence cell growth arrest through p21 induction. Concerning the increase observed in cytoplasmic p21, this data could be interpreted as the

outcome of the increased p21 synthesis or a degradation taking place in the cytoplasm. Nonetheless, it may also be linked to apoptosis delay or inhibition due to pleiotropic context-dependent p21 functions (Arai *et al.* 2006).

The CDK inhibitor p21 is also responsible for inhibiting ongoing DNA synthesis, thus allowing time for DNA repair (Luo *et al.* 1995), and also playing a role in the G2 phase transition (Besson *et al.* 2008). The significant decrease in the G2/M cell proportion observed at E40, concomitant with an increase in p21 expression and DDR activation, may indicate a p21-mediated blockage of ongoing DNA synthesis, which was involved, at least in part, in the declining cell progression from the S phase to G2/M. Considering that S-phase checkpoint activation in response to DNA damage has been previously reported (Ogryzko *et al.* 1997, Pedoux *et al.* 1998, Knudsen *et al.* 2000), our results suggest a possible delay taking place in the cell cycle progression mediated by p21, provoked by the occurrence of high DDR signaling.

Considering that prolactinomas are the most frequently occurring neoplasms in humans and in laboratory animals (Ciccarelli *et al.* 2005), we found that during estrogen-induced pituitary tumor growth, the expression of all the senescence markers used was not confined to the lactotroph population. The direct assumption of the involvement of PRL cells in pituitary senescence may be premature for several reasons. Firstly, alpha and beta estrogen receptors are also present in folliculostellate (Mitchner *et al.* 1998), somatotroph, thyrotroph, and gonadotroph cells (Nishihara *et al.* 2000, Gonzalez *et al.* 2008). Also, compelling reports by Nolan & Levy (2009) in Wistar male rats have shown that approximately two-thirds of anterior pituitary cell divisions after estrogen treatment occur in non-PRL-positive cells, with only one-thirds of the mitotic figures corresponding to lactotroph cells.

PRL cells might represent a phenotype less sensitive to growth inhibition programs. Indeed, other reports have suggested that the senescence contribution to benign pituitary tumor development might be cell-type specific rather than universal. In agreement, Chesnokova *et al.* (2008) have described senescence taking place predominantly in GH-secreting tumors, and to a lesser extent in PRL-secreting tumors. In addition, Alexandraki *et al.* (2012) showed that SA-b-gal is overexpressed only in nonfunctional pituitary adenomas and GH-secreting tumors, and that in the context of senescence, these adenomas behave differently compared with ACTH- and PRL-secreting adenomas. Finally, as the great majority of pituitary carcinomas are derived from these latter types of adenoma, we might speculate that the lack or bypass of

senescence in lactotrophs may permit the occasional prolactinoma to develop into a carcinoma. In this study, we have provided evidence for the involvement of senescence from the early proliferative response of the pituitary gland to estrogen and also we have demonstrated that the changes observed were not associated exclusively with PRL cells.

In summary, in this study, we have demonstrated clear evidence of the emergence of pituitary senescence in the development of tumoral pituitary injuries induced by estrogen. The association between DDR activation, SA- β -gal expression, and p21 involvement constitutes a reliable combination of senescence-associated markers for *in vivo* pituitary senescence detection. Moreover, the participation of cellular senescence should be conceived as a contributing component behind the strikingly benign intrinsic nature of pituitary adenomas, thereby influencing the capability of the pituitary gland to avoid unregulated cell proliferation. Understanding this mechanism might provide significant insights into cancer control networks and lead to new therapeutic strategies. The future recognition of the intracellular targets involved in the responses to anti-mitogenic signals would also help to interpret the abnormalities responsible for their dysfunction in tumorigenesis.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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