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Pivotal role of NF- κ B in cellular senescence of experimental pituitary tumours

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Abstract

The molecular mechanisms underlying the capability of pituitary tumours to avoid unregulated cell proliferation are still not well understood. However, the NF- κ B transcription factor, which is able to modulate not only cellular senescence but also tumour progression, has emerged as a targeted candidate. This work was focused on the NF- κ B role in cellular senescence during the progression of experimental pituitary tumours. Also, the contribution of the signalling pathways in senescence-associated NF- κ B activation and the senescence-associated secretory phenotype (SASP) and pro-survival-NF- κ B target genes transcription were analysed. A robust NF- κ B activation was seen at E20–E40 of tumour development accompanied by a marked SA- β -Gal co-reactivity in the tumour pituitary parenchyma. The induction of *TNF α* and *IL1- β* as specific SASP-related NF- κ B target genes as well as *Bcl-2* and *Bcl-xl* pro-survival genes was shown to be accompanied by increases in the p-p38 MAPK protein levels, starting at the E20 stage and strengthening from 40 to 60 days of tumour growth. It is noteworthy that p-JNK displayed a similar pattern of activation during pituitary tumour development, while p-AKT and p-ERK1/2 were downregulated. By employing a pharmacological strategy to abrogate NF- κ B activity, we demonstrated a marked reduction in SA- β -Gal activity and a slight decrease in Ki67 immunopositive cells after NF- κ B blockade. These results suggest a central role for NF- κ B in the regulation of the cellular senescence programme, leading to the strikingly benign intrinsic nature of pituitary adenomas.

Key Words

- senescence
- pituitary tumour
- NF- κ B
- SA- β -gal
- proliferation

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Introduction

The pituitary gland, the master regulator of homeostatic balance, responds in a dynamic manner to constantly changing hormonal and metabolic environments. However, an imbalance in the response to endogenous and exogenous signals can lead to hyperplasia and an excess

in hormonal secretion, usually associated with adenoma formation. Pituitary tumours are usually benign neoplasms (Maiza & Caron 2009) and rarely undergo malignant transformation, with the latter occurring in around 0.2% or less of all clinical cases (Scheithauer *et al.* 2006).

Relevant evidence associated premature cell senescence mechanisms with the features of pituitary adenomas (Chesnokova *et al.* 2007, 2008), a process conceived as a tumour suppressive mechanism (Melmed 2011, Campisi & Robert 2014).

Cellular senescence is a programme activated in response to damage and stress (Lopez-Otin *et al.* 2013), being characterized by high levels of senescence associated β -galactosidase activity (SA- β -Gal) (Lee *et al.* 2006) and p16^{INK4A} and p21^{CIP1} expression (Kuilman *et al.* 2010). Furthermore, this process is also associated with a robust pro-inflammatory secretome (Chien *et al.* 2011, Hernandez-Segura *et al.* 2018), the so-called SASP (Coppe *et al.* 2008, Kuilman and Peeper 2009). In this scenario, the nuclear factor kappa B (NF- κ B) signalling pathway has been proposed to control SASP production (Freund *et al.* 2011, Salminen *et al.* 2012), with a causal role suggested in the promotion of cellular senescence (Chien *et al.* 2011). The contribution of NF- κ B in cellular senescence has been previously demonstrated (Rovillain *et al.* 2011, Liu *et al.* 2012), and MEK/ERK signalling has been linked to NF- κ B activation and pro-inflammatory cytokine expression during this cellular process (Acosta *et al.* 2008). Related to this, p38 MAPK activation is sufficient to induce the SASP phenotype and promote cell growth arrest by activating p53 and p16 (Wang *et al.* 2002, Kwong *et al.* 2009, Freund *et al.* 2011). Moreover, c-Jun N-terminal kinase (JNK) signalling is strongly activated by components of senescence secretome, such as TNF α and IL-1, and is also required for p53 activation in response to oncogenic stress (Schramek *et al.* 2011). In addition, the PI3K/Akt pathway can also stimulate NF- κ B activation through the IKK complex (Ozes *et al.* 1999). However, the role of NF- κ B as a tumour suppressor or promoter is still unclear. Although the NF- κ B-driven cytokines may reinforce senescent cell-cycle arrest (Acosta *et al.* 2008, Rovillain *et al.* 2011), they have been reported to be associated with cell proliferation and tumour progression (Pikarsky *et al.* 2004, Karin 2006). Moreover, even though, NF- κ B was found to be up-regulated in human pituitary adenomas (Chen *et al.* 2015), with its pharmacological inhibition reducing cell viability in the GH3 pituitary cell line (Vender *et al.* 2008), the role of this key transcription factor in pituitary cellular senescence has not yet been elucidated. Thus, considering the controversial role of the NF- κ B pathway in tumorigenesis, this study was focussed on the contribution of NF- κ B activation in cellular senescence during *in vivo* experimental pituitary tumour development.

Materials and methods

Animals and experimental model

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. As exogenous estrogen excess induces pituitary tumours (Asa & Ezzat 2009, Sabatino *et al.* 2015, 2018), animals were treated with estradiol benzoate (EB, 10 mg) (Sigma Aldrich) for 10, 20, 40, and 60 days (E10, E20, E40, and E60) with EB being implanted subcutaneously in slow releasing silastic brand capsules (Dow Corning, Medical grade, Midland, MI, USA). Control group were implanted with empty capsules. Animals belonging to the different experimental groups were killed by decapitation, and fresh pituitary samples were collected and processed for the different experimental approaches. 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), with the experimental designs being approved by the Institutional Animal Care Committee of the School of Medicine, National University of Córdoba.

Pyrrolidinedithiocarbamate administration protocol

To assess the role of NF- κ B on pituitary tumour development, the potent NF- κ B inhibitor pyrrolidinedithiocarbamate was used (PDTC; Sigma-Aldrich) (Liu *et al.* 1999, Cuzzocrea *et al.* 2002, Miao *et al.* 2017, Yang *et al.* 2017) according to the following experimental design. Animals were randomly divided into four experimental groups ($n=5$), referred to as control, E20, PDTC, and PDTC-E20. Rats from E20 and PDTC-E20 were implanted subcutaneously with 10 mg EB for 20 days as described above, with animals from the control and PDTC groups being implanted with empty capsules. At the same time, PDTC and PDTC-E20 received daily intraperitoneal injections of the NF- κ B inhibitor (50 mg/kg) for 20 days, while control and E20 received sterile saline injections. Body weight, water, and food intake were periodically measured. At the end of treatments, animals from all groups were simultaneously killed, and fresh pituitaries were photographed *in situ*, weighed after collection, and processed for the different experiments.

Cellular fractionation

Subcellular fractionation was performed as previously described (Mongi-Bragato *et al.* 2016). Briefly, pituitaries were

homogenized in cytoplasmic extract buffer (10 mM HEPES pH=7.5, 60 mM KCl, 1 mM EDTA, 1 mM dithiothreitol (DTT), 1 mM NaVO₄, 1 mM phenylmethanesulfonyl fluoride (PMSF), 1 μ g/mL aprotinin, 1 μ g/mL leupeptin, 1 μ g/mL pepstatin, and 0.075% NP40 v/v) and centrifuged at 2000 *g* for 5 min at 4°C to pellet the nuclear fraction. Supernatants were centrifuged at 10,000 *g* for 10 min at 4°C, and the resulting supernatants were used as cytoplasmic extracts. Then, the nuclear pellets were resuspended in nuclear extract buffer (20 mM TrisHCl pH=8, 420 mM NaCl, 60 mM KCl, 1 mM EDTA, 1 mM NaVO₄, 1 mM PMSF, 25% glycerol v/v, 1 μ g/mL aprotinin, 1 μ g/mL leupeptin, and 1 μ g/mL pepstatin), and centrifuged at 10,000 *g* for 10 min at 4°C. The resulting supernatants were used as nuclear extracts, with aliquots of each sample being used for total protein quantification according to the Bradford method (Bio-Rad Protein Assay, Bio-Rad).

Western blotting analysis

Protein (40 μ g) extracts were separated by SDS-PAGE and transferred to nitrocellulose membranes. Blocking was performed using 5% BSA (w/v) in T-TBS, and membranes were incubated overnight at 4°C with the corresponding primary antibody: anti-NF- κ B p65 (RelA; 1:700), anti-total (t)-ERK (1:2000), anti-I κ B α (1:300), or anti-p53 (1:200) (Santa Cruz Biotechnology); anti-phosphorylated (p)-RelA S536 (1/500), anti-p-ERK 1/2 (1:700), anti-p-Akt (1:500), anti-t-Akt (1:2000), anti-p-JNK (1:1000), anti-p-IKK α S176/ β S180 (1:300), anti-Lamin B (1:500), or anti-phosphorylated Histone H2AX (p- γ H2AX) (1/2000) (Cell Signaling); anti-p-p38 MAPK (1:10,000, Millipore), anti-t-p38 MAPK (1:10,000), anti-t-JNK (1:5000), or anti- α -Tubulin (1:2000) (Sigma-Aldrich). After washing, membranes were incubated with a peroxidase-conjugated (HRP) goat anti-rabbit (1:5000; Bio-Rad), goat anti-mouse (1:3000; Jackson Laboratories), or mouse anti-goat (1:2000, Santa Cruz Biotechnology) and signals revealed with an ECL detection system (Amersham Biosciences). Emitted light was captured on Hyperfilm (Amersham Pharmacia Biotech). The α -Tubulin and Lamin B antibodies were used as appropriate loading controls from cytosolic and nuclear extracts, respectively, and values were expressed as the percentage of the control group. Semi-quantitative signals were derived from the densitometric analysis using Image J software (v1.51; National Institutes of Health).

Immunohistochemistry

To assess SA- β -Gal detection as well as RelA and Ki67 staining, pituitaries were placed in Crioplast (Biopack,

Buenos Aires, Argentina), immersed in liquid nitrogen, and stored at -80°C. Sections were cut in a cryostat (CM1510S, Leica Microsystems, Wetzlar, Germany) and mounted on positively charged microscope slides. Then, pituitary cryosections (7 μ m) from control, tumour, and PDTc-treated animals were processed for RelA and Ki67 detection. Briefly, pituitary sections were fixed in absolute methanol, treated with 3% v/v hydrogen peroxide, blocked for 30 min in 5% BSA (w/v), and incubated at 4°C with goat anti-RelA (1:100) or mouse anti-Ki67 (1:50; BD Pharmingen). Next, the sections were incubated with biotinylated secondary antibodies and ABC complex, using DAB as a chromogen (Sigma-Aldrich). Controls were performed by applying the same protocol, but omitting primary antibodies. Samples from four animals of each experimental condition were photographed at 40 $\times$, with more than 1000-immunoreactive cells being examined. The Ki67 index was determined using NIH Image J Software (National Institutes of Health), and the number of positively stained nuclei was recorded along with the total nuclei.

Cytochemical detection of SA- β -Gal staining and double SA- β -Gal/RelA immunolabelling

SA- β -gal-positive cells were detected by using the β -Gal staining kit (Senescence Cell Staining Kit, Sigma-Aldrich), as previously described (Sabatino *et al.* 2015). Sections were photographed at 100 $\times$, with only senescent cells being stained in blue at a pH=6.0. After performing the SA- β -Gal staining, the same cryosection was processed by immunocytochemistry for the purpose of determining the SA- β -Gal/RelA double localization, by applying the anti-RelA antibody as described in the previous section. Slides with samples from four animals of each experimental condition were photographed in randomly chosen fields at 400 $\times$, with more than 1000-immunoreactive cells being examined.

Gene expression analysis by qPCR

Total RNA was purified using the Direct-zol RNA miniprep kit (Zymo Research, Irvine, CA, USA) according to manufacturer's instruction. Thereafter, 2 μ g of total RNA from each condition were reverse transcribed using Reverse Transcriptase M-MLV (Promega) with random hexamer primers (Thermo Fisher Scientific). Real-time PCR was used to evaluate the quantitative expression of mRNA for *Bcl-2*, *Bcl-xl*, *TNF α* , *IL-1 β* , and *HPRT* as the control. The cDNA was analysed for control and tumour

Table 1 Primer sequences for gene expression.

Gene	Accession number	Note	Sequence	Amplicom (bp)
<i>Bcl-xl</i>	NM 001033672.1	Gene expression	(F) 5'GCTGGTGGTTGACTTTCTCTCC 3' (R) 5'GGCTTCAGTCTGTTCTCTTCG 3'	46
<i>Bcl-2</i>	L14680	Gene expression	(F) 5'ATCGCTCTGTGGATGACTGAGTAC 3' (R) 5'AGAGACAGCCAGGAGAAATCAAAC 3'	86
<i>TNFα</i>	NM 012675.3	Gene expression	(F) 5'AGCACGGAAAGCATGATCCG 3' (R) 5'CGAGCAGGAATGAGAAGAGG 3'	78
<i>IL-1β</i>	NM 031512	Gene expression	(F) 5'GACAAGAGCTTCAGGAAGGC 3' (R) 5'GCAAGACATAGGTAGCTGCC 3'	32
<i>HPRT</i>	NM012583.2	Gene expression	(F) 5'CTCGAAGTGTGGATACAGGC 3' (R) 5'CTTGGCTTTTCCACTTTCGC 3'	98

conditions using real-time PCR SYBR Green Master Mix (Thermo Fisher Scientific) single stranded cDNA (100 ng of the input total RNA equivalent), and 800 nM of amplification primer were used in a reaction volume of 15 μ L. Runs were performed on a standard 7500 system (Applied Biosystems), and the running condition was 95°C for 10 min, 40 cycles at 95°C for 15 s, and 60°C for 1 min. Gene expression was normalized to the housekeeping gene (HPRT) and calculated using the comparative $2^{-\Delta\Delta C_t}$ method. Melting curve analyses were performed to ensure the specificity of the qPCR product. These assays were performed four times in duplicate. All primers were from Thermo Fisher and the gene-specific primer sets are described in Table 1.

Statistical analysis

The statistical analysis was carried out using the Statistica 7.1 program (Statsoft, Tulsa, OK, USA). Data were analysed using a one-way ANOVA, and the main significant effects indicated by the ANOVA were further analysed through Fisher's least significant difference *post hoc* test.

Results

NF- κ B signalling is activated during the experimental pituitary tumour development.

In order to analyse the molecular link between NF- κ B activation and pituitary senescence, the cytoplasmic and nuclear RelA protein content and SA- β -Gal activity were assessed during the tumour development. As shown in Fig. 1A and I, a marked increase in the RelA protein level was detected in the cytosolic compartment, starting from the earliest stages of tumour induction. In addition, a significant NF- κ B activation, as revealed by its increase in

the nuclear compartment, was observed mainly after 20 and 40 days of tumour development (Fig. 1B and H), thereby demonstrating NF- κ B nuclear translocation. Furthermore, an increase in the p-RelA S536 protein levels was detected over tumour development, with a marked enhancement occurring at the E40 endpoint (Fig. 1C and I), thus revealing that NF- κ B is transcriptionally active under this tumoral context. In line with this, immunohistochemistry analysis revealed a marked RelA nuclear localization in the majority of pituitary cells derived from E20 and E40 conditions. Furthermore, the double SA- β -Gal and RelA staining in the same cryosection revealed the co-existence of senescent regions with nuclear translocation of NF- κ B for the tumour conditions examined (Fig. 1J). Therefore, with the aim of investigating the induction of the NF- κ B canonical pathway, we evaluated IKK α/β phosphorylation (p-IKK α S176/ β S180) and the I κ B α protein levels during the pituitary tumour development. Figure 1I, shows a representative image of the p-IKK α S176/ β S180 protein level detected in the cytosolic compartment after 20 days of tumour induction, concurring with earliest signs of NF- κ B activation. Increasing I κ B α protein levels were also observed, starting at E20 and continuing up to the advanced stage of tumour growth (Fig. 1D and I). In addition, in order to evaluate the contribution of p53 and p- γ H2AX in senescence and NF- κ B activation, their protein levels were determined at different points of the pituitary tumour progression. As shown in Fig. 1E and I, an increase in p53 protein levels was detected from E20 in the cytosolic compartment of pituitary cells. Moreover, although an initial increase in nuclear p53 expression was observed at the E10 endpoint, this signal was no longer observed from E20 (Fig. 1F and H). In parallel, p- γ H2AX expression revealed a gradual increase from the start of tumoral development, but this recovered to normal levels at E60 (Fig. 1G and H).

Figure 1

NF- κ B activation during the pituitary tumour development. Densitometric analysis of (A) cytosolic RelA, (B) nuclear RelA, (C) cytosolic p-RelA S536, (D) cytosolic I κ B α , (E) cytosolic p53, (F) nuclear p53, and (G) p- γ -H2AX protein expression levels during the development of pituitary tumours. The RelA/ α -tubulin, p-RelA S536/ α -tubulin, I κ B α / α -tubulin, p53/ α -tubulin ratios and RelA/lamin B, p53/lamin B, and p- γ -H2AX/lamin B ratios are represented as a percentage relative to the control (control data were set to 100%). (H) Representative Western blot analysis of RelA, p53, and p- γ -H2AX nuclear protein levels at the different stages of tumour induction. (I) Representative Western blot analysis of RelA, p-RelA S536, p-IKK α S176/ β S180, I κ B α , and p53 cytosolic protein levels during the development of pituitary tumours. Lamin B and α -tubulin signals were used to confirm equivalent total nuclear and cytosolic protein loading, respectively. The data from five independent experiments were evaluated by the ANOVA-Fisher test and expressed as mean $\pm$ s.e. normalised to the control group. * P < 0.05 vs control. (J) RelA and SA- β -Gal staining in cryosections from normal and estrogen-induced pituitary tumours. Immunohistochemistry was performed using the Avidin-Biotin peroxidase method (brown signal for RelA) and an enzymatic assay (SA- β -Gal enzymatic activity dyed blue). The RelA-positive nuclei staining can be seen in the areas labelled for SA- β -gal in the pituitary parenchyma (inset). However, single cells expressing SA- β -Gal or RelA can also be discriminated (arrows). Representative fields of anterior pituitary sections of a representative experiment from a total of three independent experiments with similar results are shown. Scale bar: 20 μ m. A full colour version of this figure is available at <https://doi.org/10.1530/JOE-19-0506>.

ERK1/2 and Akt activation are suppressed concomitantly with increased p38 MAPK and JNK phosphorylation in the course of pituitary tumour development

It is known that NF- κ B is an important target for Ras-activated effectors, including ERK1/2 and Akt proteins, with Ras being linked with NF- κ B activation and the expression of certain pro-inflammatory cytokines in senescent cells (Courtois-Cox *et al.* 2006). In this context, and to elucidate the signalling pathways involved in pituitary tumour growth and the possible crosstalk with NF- κ B activation, we next analysed the ERK1/2 and Akt protein levels. Figure 2A and E show a significant increase in p-ERK1/2 at E10 followed by a marked suppression of this signal at the prolonged stages of tumour induction. Figure 2B and E show a different phosphorylation profile

for Akt. The phosphorylation of this protein was inhibited at all stages of the treatment, except at E40 where it remained at levels similar to those observed at baseline conditions.

The involvement of JNK and p38 MAPK signalling pathways in controlling NF- κ B activation in the senescence programme has also been previously demonstrated (Freund *et al.* 2011, Schramek *et al.* 2011). To try to further characterise the NF- κ B activation mechanisms involved in pituitary tumour cell growth, the p-p38 MAPK and p-JNK protein content was examined. Figure 2C and F show a significant increase in p-p38 starting at the E20 stage, which strengthened over time until 60 days of tumour development. Additionally, a time-dependent increased p-JNK ratio was revealed, starting from E10 and with a marked enhancement occurring at E60 (Fig. 2D and F).

Figure 2 MAPKs and Akt signalling activation during pituitary tumour development. (A) Western blotting expression levels of p-ERK1/2 from cytosolic extracts of normal and tumour pituitaries. An early p-ERK activation was detected at E10, followed by its suppression in the latter stages of tumour development. (B) Western blotting expression levels of p-Akt during tumour progression. (C) Graph panel showing p-p38 protein levels in control and tumour pituitaries. An increase in p-p38 levels was detected from the E20 stage of tumour development. (D) p-JNK protein levels during pituitary tumour development. A progressive increase in p-JNK expression was observed throughout pituitary tumour progression, with a marked rise detected at E60 (E), and (F) representative images of Western blot showing p-ERK, p-Akt, p-p38, and p-JNK signals are presented. Expression of α -tubulin was used as the loading control and confirmed that equal loading occurred in all lines. Data from five independent experiments were evaluated by the ANOVA-Fisher test and represent the mean $\pm$ s.e. normalised to the control group. * $P < 0.05$ vs control; # $P < 0.05$ vs E10.

SASP and pro-survival NF- κ B target gene expression is activated during the development of pituitary tumours

The balance between cell proliferation and senescence mechanisms, which is linked to NF- κ B activation, finally defines the tumour growth. Having demonstrated that NF- κ B activation in senescent cells (Fig. 1E) is also accompanied by p38 MAPK activation (Fig. 2C and F), we examined the transcriptional role of different NF- κ B-target genes implicated in the SASP phenotype. A significantly increased *TNF α* expression was detected at the E20 and E40 stages, which returned to the basal level by the end of tumour induction (Fig. 3A). Interestingly, a remarkable enhancement of the IL1 β expression levels was observed at stages E40 and E60 (Fig. 3B).

In order to explore NF- κ B-regulated pro-survival gene expression, the *Bcl-2* and *Bcl-xl* gene expression was assessed. Figure 3C shows increased *Bcl-2* expression at the beginning of tumour development (E10 and E20), with these values gradually returning to the basal condition. However, in the case of *Bcl-xl*, its expression was not induced until the E20 and E40 stages of tumour induction (Fig. 3D).

NF- κ B inhibition attenuated the emergence of cellular senescence in pituitary tumours

It has been demonstrated that NF- κ B genetic or pharmacological inhibition abrogates cellular senescence by reducing growth arrest and SASP expression (Tilstra *et al.* 2012, Moiseeva *et al.* 2013). Considering the substantial NF- κ B activation observed at the specific

Figure 3

SASP and pro-survival NF- κ B target gene expression during pituitary tumour progression. (A) TNF α and (B) IL-1 β gene expression levels in normal and tumour pituitaries. The significant increase in both NF- κ B-controlled cytokines detected at the advanced stages of tumour induction indicates NF- κ B control on the transcriptional programme mediating cellular senescence. (C) Bcl-2 mRNA expression levels were augmented at the initial steps of tumour development (E10 and E20), returning progressively to basal levels as the tumour developed. (D) Bcl-xl gene expression levels during pituitary tumour development. HPRT was amplified as a housekeeping gene and showed no changes during the experiments. Data were evaluated by the ANOVA-Fisher test and are expressed as mean $\pm$ s.e. of tumour/control pituitary ratio ($n = 4$ per group). * $P < 0.05$ vs control.

end-points of pituitary tumour development (Fig. 1A and B), and also the up-regulation of the NF- κ B-downstream target genes associated with the senescence secretome and proliferation control at the same stages of tumour induction (Fig. 3), the next step taken was to try to corroborate these findings under *in vivo* NF- κ B inhibition. Since simultaneous NF- κ B nuclear translocation and IKK activation were detected at E20, NF- κ B signalling was then pharmacologically inhibited by daily PDTC administration for 20 days (PDTC-E20 group). PDTC treatment did not induce any changes in body weight, food, water intake, or behaviour (data not shown). Figure 4A shows that, as expected, PDTC administration inhibited NF- κ B nuclear translocation after 20 days of pituitary lesion progression.

Figure 4B reveals that the pharmacological NF- κ B blockage slightly, but significantly, reduced the pituitary tumour weight at PDTC-E20 when compared to the E20 endpoint, with macroscopic observation *in sellae* of the pituitaries for the different experimental conditions also confirming this weight reduction (Fig. 4C). To determine the effect of NF- κ B inhibition on cellular senescence, the SA- β -Gal enzymatic activity and cell proliferation were also measured under PDTC treatment. As shown in Fig. 4D and E, PDCT was able to reduce SA- β -Gal reactivity at the E20 stage of tumour development. In addition, the

quantification of Ki67-immunopositive cells revealed a significant decrease of cellular growth in animals receiving the PDTC inhibitor compared to the E20 group (Fig. 4F and G).

Discussion

In the present study, we have demonstrated the significant role exerted by NF- κ B on the pituitary cellular senescence process during the development of *in vivo* experimental tumours. Moreover, our findings have delineated the possible signalling pathways involved in the control of NF- κ B activation, which includes the induction of specific SASP-related NF- κ B target genes. We have also shown the attenuation of SA- β -Gal expression through an *in vivo* NF- κ B inhibition approach. In addition to its contribution in the pituitary cell growth control via senescence, NF- κ B also stimulated pituitary cell proliferation, with this finding being correlated by up-regulation of relevant pro-survival NF- κ B target genes, thus demonstrating the coexistence of both cellular processes during the development of pituitary tumours.

Senescence has been proposed as a defence mechanism underlying the failure of benign pituitary tumours to progress to true malignancy (Chesnokova *et al.* 2008, Melmed 2015). Regarding this, human tumours with high levels of SA- β -Gal activity express elevated p21 levels, but, this cyclin-dependent kinase inhibitor was not detected in human pituitary carcinomas (Chesnokova *et al.* 2008). Accordingly, several *in vivo* studies have demonstrated that senescence is indeed a contributing factor to the benign nature of pituitary tumours (Donangelo *et al.* 2006, Chesnokova *et al.* 2008). In line, we have previously demonstrated clear hallmarks of cellular senescence as a potent tumour suppressor process in experimental pituitary tumours, indicating a role for this mechanism in the regulation of pituitary cell growth (Sabatino *et al.* 2015).

Nevertheless, the factors and molecular mechanisms behind the capability of the pituitary gland to avoid unregulated cell proliferation and exactly how the cellular signalling networks are integrated are still not well understood. One targeted candidate is the NF- κ B transcription factor, with there being evidence indicating that this transcription factor is substantially increased in the cytoplasm and nucleus in human pituitary adenomas (Chen *et al.* 2015). In agreement, by using experimental estrogen-induced pituitary tumours that constituted a faithful *in vivo* model of senescence, we demonstrated

Figure 4

NF- κ B inhibition attenuates the emergence of the cellular senescence and proliferation processes during *in vivo* pituitary tumour development. (A) Representative Western blot analysis of RelA nuclear protein expression. PDTC treatment (50 mg/kg, i.p.; 20 days) was able to block NF- κ B nuclear translocation in *in vivo* estrogen-induced tumours. Expression of lamin B confirmed equal loading occurring in all lanes. (B) Pituitary weight (mg) after estrogen and/or PDTC (50 mg/kg) administered daily for 20 days. The data were evaluated by the ANOVA-Fisher test and are expressed as mean $\pm$ s.e. ($n = 5$ per group). * $P < 0.05$ vs control # $P < 0.05$ vs E20. (C) Photographs of representative *in sellae* pituitaries from normal ones and those submitted to 20 days of tumour development, with or without PDTC treatment (50 mg/kg/i.p.). Pituitary gland sizes are depicted by a circle. (D) Pituitary cryosections stained with SA- β -Gal (blue signal) from control and E20 tumour glands from animals treated with vehicle or PDTC. Scale bar: 60 μ m. Pituitary tumour from animals treated with the NF- κ B inhibitor PDTC showed a reduced SA- β -gal reactivity at E20 compared to E20 without PDTC. (E) The SA- β -gal index from the different experimental conditions. (F) Ki67 immunolabelling (brown signal) in cryosections of normal glands and E20 pituitary tumours from animals treated with PDTC or vehicle. A reduction in Ki67 immunoreactivity was seen after daily PDTC injection for 20 days of evolution of proliferative injury. Scale bar: 20 μ m. (G) Ki67 index for the different experimental conditions. Data were statistically analysed by the ANOVA-Fisher's test, and the results are expressed mean $\pm$ s.e. ($n = 4$ per group). * $P < 0.05$ vs control # $P < 0.05$ vs E20 group. A full colour version of this figure is available at <https://doi.org/10.1530/JOE-19-0506>.

an increase in cytosolic NF- κ B levels during pituitary tumour development, accompanied by NF- κ B nuclear translocation at the specific endpoints E20 and E40, which may suggest that there exists a limited time period of transcriptional activity by NF- κ B over the course of tumour progression.

The NF- κ B canonical pathway activation classically involves phosphorylation and proteasomal-mediated degradation of the inhibitory protein I κ B α . Interestingly, increased I κ B α protein levels were found as the pituitary tumour advanced, with the first sign of an I κ B α increase being detected at the tumour stages where NF- κ B nuclear translocation was observed. Regarding this, several authors have pointed out that NF- κ B stimuli appear to trigger negative feedback pathways in some cell types, such as the hepatoma cell line and human colon cell line, which terminate the NF- κ B response by increasing the stability of newly synthesized I κ B α (Place *et al.* 2001, 2003,

Werner *et al.* 2005, 2008, Moss *et al.* 2012). Thus, the rise in I κ B α protein levels detected followed by NF- κ B nuclear translocation may suggest that a regulatory mechanism was triggered in order to modulate NF- κ B activation in experimental pituitary tumours. Thus, in this context, the absence of NF- κ B nuclear translocation at E60 stage could be a result of the negative feedback from I κ B α .

The activity of NF- κ B is also regulated by a plethora of pathways, whose activation depends on the cellular context. The PI3K/Akt and MEK/ERK signalling are critical for inducing the cellular senescence phenotype (Satyanarayana *et al.* 2004, Kim *et al.* 2017), with both these pathways being associated with IKK/NF- κ B complex activation in a tumour context (Tyagi *et al.* 2014). Despite these findings, the p-IKK/NF- κ B nuclear accumulation detected at the E20 and E40 stages appeared to be ERK and Akt-independent, since both pathways were mainly suppressed during pituitary tumour development.

Regarding Akt signalling, the reestablishment of p-Akt to control levels at E40 stage might be a response to extracellular events occurring within the tumour setting (Sabatino *et al.* 2018). Moreover, the ERK activation detected at the earlier stages of tumour induction was correlated with the initial burst of proliferation, which was followed by cellular senescence (Sabatino *et al.* 2015). Furthermore, these results are consistent with previous evidence showing that, after a transient activation of downstream Ras effectors, there was a shutdown of this signalling as a negative feedback underlying cellular senescence (Courtois-Cox *et al.* 2006, Hayes *et al.* 2016).

The involvement of additional signalling pathways that control NF- κ B and the senescence programme has also been demonstrated (Freund *et al.* 2011). This mechanism can be triggered by the JNK signalling activation through p53 regulation (Schramek *et al.* 2011), and an intrinsic crosstalk between NF- κ B and JNK has been reported (Bubici *et al.* 2004). Furthermore, p38 MAPK regulates SASP mainly through NF- κ B transcriptional activity, which is required for the expression of most SASP factors (Salminen *et al.* 2012). Supporting these evidences, under the present experimental conditions, we detected sustained p38 MAPK and JNK activation from E20 stage, which corresponded with IKK phosphorylation and NF- κ B nuclear translocation at the same endpoint of tumour induction. Moreover, the SASP components evaluated in our study were detected concomitantly with p38 MAPK/NF- κ B induction, possibly indicating that this pathway is linked to the senescence secretome expression during pituitary tumour progression.

It has been reported that the p38 MAPK/NF- κ B pathway is a necessary and sufficient DNA damage response (DDR)-independent regulator of the senescence secretome (Freund *et al.* 2011). Nevertheless, DDR could also be triggered and orchestrated in cooperation with NF- κ B, the senescence machinery in pituitary tumours (Sabatino *et al.* 2015). Interestingly, we found increased p53 activation occurring only at the early stages of tumour development and with p- γ H2AX induction starting at E20, as previously reported in our laboratory (Sabatino *et al.* 2015, 2018). The cytoplasmic p53 retention observed from E20 might indicate its incapability to exert its canonical functions, so it is possible that p53 is able to initiate senescence arrest. Interestingly, NF- κ B activation was detected after the reduction in p53 nuclear levels. In agreement, it has been previously described that NF- κ B and p53 engage in reciprocal negative regulation (Huang *et al.* 2007, Xia *et al.* 2009). Thus, it is possible that NF- κ B takes control of cellular senescence machinery after the E20 endpoint in a p53-independent fashion.

In order to address a possible relationship between NF- κ B activation and pituitary senescence, we first analysed the joint expression of this transcription factor and SA- β -Gal. A co-expression of SA- β -Gal and RelA was detected at the E20 stage of tumour growth, revealing NF- κ B activation upon pituitary senescence. Also, *in vivo* exposition to the NF- κ B inhibitor PDTC dramatically suppressed the emergence of cellular senescence at the E20 endpoint, as evidenced by reduction in SA- β -Gal activity.

Although several *in vitro* studies have demonstrated the preponderant role exerted by NF- κ B on cellular senescence and its influence in tumour initiation and progression (Coppe *et al.* 2010, Rovillain *et al.* 2011), few studies have extended these observations to the *in vivo* model, while other studies have produced some conflicting results. For example, NF- κ B activation was reported to promote senescence and SASP induction together with suppressed proliferation (Liu *et al.* 2012, Xu *et al.* 2018), whereas in other investigations, its blockade promoted senescence and replicative arrest of tumour cells (Nogueira *et al.* 2011). Thus, the putative dual role of NF- κ B in tumour promotion or suppression may depend on the biological setting in which this transcription factor is activated. Accordingly, during pituitary tumour development, NF- κ B appears to be implicated in both senescence and proliferative responses. This notion is supported by the enhancement detected in mRNA levels of several pro-survival factors, such as *Bcl-2* and *Bcl-xl* (transcriptionally controlled by NF- κ B), at those stages of tumour development where this transcription factor was mainly detected in the nuclear compartment. Moreover, at the same time, the *TNFA* and *IL-1 β* mRNA levels were also increased.

If the senescence phenomenon emerges with the aim to counteract the exacerbated tumour growth by activating the immune response (Acosta *et al.* 2008, Coppe *et al.* 2008, Rovillain *et al.* 2011), the proliferation would be expected to be increased when senescence is inhibited. Nevertheless, several investigations have suggested that SASP from senescent cells can also act extrinsically and promote cell transformation (Coppe *et al.* 2010, Franceschi & Campisi 2014). Moreover, persistent SASP may help tumour cells to migrate and invade (Coppe *et al.* 2008, Tammela *et al.* 2017). It was also demonstrated that endogenous IL-6, as a SASP component, and hormone secretion (Chesnokova *et al.* 2013, Sapochnik *et al.* 2017) underlie the senescence-associated slow proliferation rate and benign nature of pituitary tumours. In contrast, other evidence indicates that senescence and pituitary stem cell associated persistent activation of SASP drive paracrine

tumour formation (Mertens *et al.* 2015, Carreno *et al.* 2017, Gonzalez-Meljem *et al.* 2017, Gonzalez-Meljem & Martinez-Barbera 2018). Thus, our observations showing that, when NF- κ B activation was inhibited, cell senescence was prevented with cell proliferation being only slightly suppressed could be explained by the differential contribution of senescence in benign pituitary tumour evolution. Moreover, given the preponderant role of NF- κ B in proliferation and tumour cell growth (Xia *et al.* 2014), the inhibition of this transcription factor in the context of pituitary tumour may induce a slight reduction in the proliferation.

Although the progression from a benign to an aggressive/malignant character is still a matter of controversy, it possibly results as a consequence of bypassed senescence (Chesnokova *et al.* 2008, Melmed 2011). Furthermore, multiple molecular events may occur to initiate the transformation from benign pituitary adenomas to aggressive and/or malignant ones, including cell cycle gene dysregulation, epigenetic modifications, oncogene activation, and tumour suppressor gene inactivation (Melmed 2011), with some factors also being involved in senescence growth arrest. Also, in the tumour microenvironment, SASP paracrine components such as VEGF (Lloyd *et al.* 1999, Ortiz *et al.* 2012) and matrix metalloproteinase 9 (Turner *et al.* 2000) possibly contribute to the transformation of pituitary adenomas to carcinomas, but the exact mechanism still remains unclear. Moreover, the transformation to pituitary carcinomas might be cell-type specific, mainly occurring in PRL- and ACTH-secreting tumours, possibly because these adenomas are less likely to show markers of senescence, when compared with other adenoma types (Alexandraki *et al.* 2012). In line, it has been hypothesized that disruption of the pituitary tumour transforming gene (PTTG) and p21 pathways that induce cell senescence and thus prevent malignant transformation is involved in the transition of adenomas into carcinomas (Chesnokova *et al.* 2008). In spite of evidence indicating a correlation of senescence secretome components with a transition to pituitary malignancy, a great body of literature shows that senescence is implicated in the benign nature of these lesions, thereby increasing the capability of the pituitary gland to avoid unregulated cell proliferation

The fact that, during the early stages of pituitary tumour development, the proliferation was not completely suppressed, with the senescence being substantially reduced after NF- κ B blockade, may support the hypothesis of a competition occurring between these crucial processes (cell senescence and proliferation) with a

Pituitary Tumour Cell Microenviroment

Figure 5

Model for signalling pathways involved in NF- κ B activation during the development of experimental pituitary tumours. NF- κ B regulates TNF α and IL1- β as specific SASP-related NF- κ B target genes as well as Bcl-2 and Bcl-xl as pro-survival genes at specific stages of tumour progression. Pharmacological NF- κ B blockage (PDTC administration) induced a marked reduction in SA- β -Gal activity and a slight decrease in Ki67 immunopositive cells, thus exposing the pivotal role of NF- κ B in the regulation of pituitary cell growth. A full colour version of this figure is available at <https://doi.org/10.1530/OE-19-0506>.

differential contribution of NF- κ B in order to control the pituitary neoplastic growth (Fig. 5). In line, the double SA- β -Gal/Ki67 positive cells revealed a non-overlapping distribution of pituitary cells (Sabatino *et al.* 2015), which allowed us to reveal differential role of senescence and proliferation in the temporal course of the development of proliferative lesions. These findings demonstrate a key role of NF- κ B in pituitary senescence and also suggest that other NF- κ B-independent mechanisms take control of the cell proliferation process at the first stages of tumour induction. Indeed, ROS and certain oxidative stress biomolecules that remain elevated during pituitary tumour development can also lead to sustained pituitary tumour growth (Sabatino *et al.* 2018).

Collectively, our data may expose the coexistence of different NF- κ B activation pathways that leads to trigger an intricate signalling network tending to suppress mitogenic responses, while at the same time, promoting molecular events that encourage the cellular senescence process.

Thus, activation of this pivotal transcription factor in pituitary tumours could be considered to be a relevant player that may contribute to the slow and benign characteristics of pituitary neoplastic growth.

Given the key role of senescence as a barrier against tumour progression, a better understanding of the ways in which the underlying molecular targets can control the *in vivo* senescence may help in the design of novel compounds that can limit tumour cell growth and hormone secretion or even in the development of preventive strategies for tumour treatment.

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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Author contribution statement

B M B planned and performed the majority of the experiments and wrote the major part of the manuscript. B M B and A D P contributed to study design and planned the experiments. B M B, L d V S, E G, A C V, and N S performed the experiments and collected the study data. B M B, A L, R B L, A I T, S G, and A D P contributed to data analysis and interpretation. All the authors contributed to manuscript writing and read and approved the final manuscript. R B L, A I T, S G, and A D P reviewed the manuscript and contributed to quality control. All authors have reviewed the study and approved the final version.

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