Senescence Mediates Pituitary Hypoplasia and Restrains Pituitary Tumor Growth

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Abstract
Understanding factors subserving pituitary cell proliferation enables understanding mechanisms underlying uniquely benign pituitary tumors. Pituitary tumor-transforming gene (Pttg) deletion results in pituitary hypoplasia, low pituitary cell proliferation rates, and rescue of pituitary tumor development in Rb+/- mice. Pttg-/- pituitary glands exhibit ARF/p53/p21-dependent senescence pathway activation evidenced by up-regulated p19, cyclin D1, and Bel-2 protein levels and p53 stabilization. High pituitary p21 levels in the absence of PTTG were associated with suppressed cyclin-D1 levels and p53 stabilization. Although senesence-associated β-galactosidase was enhanced in Pttg-deficient pituitary glands, telomere lengths were increased. DNA damage signaling pathways were activated and aneuploidy was evident in the Pttg-deficient pituitary, triggering senescence-associated genes. To confirm the p21 dependency of decreased proliferation and senescence in the Pttg-null pituitary, mouse embryonic fibroblast (MEF) colony formation was tested in wild-type, Pttg +/-, Rb +/-, Rb +/- Pttg +/-, and Rb +/- Pttg +/- p21 +/- cells. Rb +/- Pttg +/- MEFs, unlike Rb +/- cells, failed to produce colonies and exhibited high levels of senescence. p21 deletion from Rb +/- Pttg +/- MEFs enhanced anchorage-independent cell growth, accompanied by a marked decrease in senescence. As cell proliferation assessed by bromodeoxyuridine incorporation was higher in Rb +/- Pttg +/- p21 +/- relative to Rb +/- Pttg +/- pituitary glands, p21-dependent senescence provoked by Pttg deletion may underlie pituitary hypoplasia and decreased tumor development in Rb +/- Pttg +/- mice. [Cancer Res 2007;67(21):10564–72]

Introduction
After highly specific embryonic commitment, hormone-secreting anterior pituitary cells replicate slowly with slow turnover rates (1, 2). Changes in pituitary size are determined throughout the life span by both extrinsic and intrinsic factors, and pituitary size generally shrinks with age (3). Pituitary hypoplasia is encountered with developmental defects, including transcription factor mutations (e.g., Pit-1). Pituitary hyperplasia may be caused by several factors, including loss of negative feedback by peripheral organ failure (e.g., thyroid), pregnancy, and estrogen treatment (3, 4) and also by hypothalamic hormone excess (5). Pituitary adenomas are common but are usually not associated with apparent hyperplasia (4). In mice, adenomas are preceded by pituitary hyperplasia (6). Mitotic activity is relatively low in aggressive pituitary adenomas compared with tumors arising from more rapidly replicating tissues (3, 7). Uniquely, pituitary carcinoma is exceedingly rare, and the commonly encountered pituitary neoplasms are invariably adenomas. Therefore, understanding mechanisms subserving pituitary cell proliferation should provide insight into the unique pathogenesis of pituitary growth disorders.

Pituitary tumor-transforming gene (Pttg) initially isolated from pituitary tumor cells (8) is overexpressed in pituitary tumors (9, 10). Pttg behaves as a mammalian securin homologue facilitating sister chromatid separation during metaphase (8, 11). Pttg overexpression causes cell transformation, induces aneuploidy (12, 13), promotes tumor formation in nude mice, induces basic fibroblast growth factor, and activates angiogenesis (14, 15). Mice lacking Pttg are viable and fertile and exhibit pituitary hypoplasia (16), whereas pituitary-directed transgenic Pttg overexpression results in focal pituitary hyperplasia and adenoma formation (6). Pttg deletion leads to pituitary p21<sup>Cip1</sup> induction with slowing of young pituitary gland proliferation. Rb +/- mice develop pituitary tumors with ~100% penetrance, whereas only 30% of doubly mutant Rb +/- mice with deleted Pttg develop delayed pituitary tumors (16). Here, we examine mechanisms underlying Pttg-/- pituitary hypoplasia and rescue of pituitary tumor development in Rb +/- Pttg +/- mice.

Apolipoprotein and senescence both control aberrant tissue and organ growth and contribute to tumor suppressor activity (17). Irreversible cell growth arrest associated with cellular senescence is induced by multiple stimuli, including age-linked telomere shortening, DNA damage, oxidative stress, chemotherapies, and oncogene activation (18–20). Cellular senescence is characterized by up-regulation of cell cycle progression inhibitors, including p19<sup>ΔN</sup> (p19), p21<sup>Cip1</sup> (p21), and p16<sup>Ink4a</sup> (p16). In vivo senescent markers are expressed in benign adenomas but not in malignant adenocarcinomas (21).

We report here that Pttg deletion results in pituitary-specific senescent features, including increased levels of p33 and the cyclin-dependent kinase (Cdk) inhibitors, p19 and p21, overexpression of cyclin D1, apoptosis block, and elevated senescence-associated β-galactosidase (SA-β-gal) expression. Decreased pituitary Cdk2 activity, Rb phosphorylation, and cyclin A levels all contribute to attenuated pituitary cell proliferation. In primary mouse embryonic fibroblasts (MEF), Pttg deletion results in p21 induction resembling the pituitary phenotype. Rb +/- Pttg +/- MEFs failed to produce anchorage-independent colonies and also exhibited enhanced senescence. In contrast, deletion of p21 from the Rb +/- Pttg +/- background enhanced anchorage-independent cell growth, accompanied by a marked decrease in the number of senescent MEFs.
Rb<sup>+/−</sup>/Pttg<sup>+/−</sup>/p21<sup>+/−</sup> mice also showed increased pituitary cell proliferation. Thus, senescence provoked by Pttg deletion represents a mechanism underlying low pituitary cell proliferation and preventing pituitary tumor development in Rb<sup>+/−</sup>/Pttg<sup>+/−</sup>/mice. Pttg-null mice are therefore reflective of a robust in vivo model for pituitary hypoplasia and premature senescence. Pituitary senescence should be considered as a contributing factor for the overwhelmingly benign nature of pituitary tumors.

Materials and Methods

Animals. Experiments were approved by the Institutional Animal Care and Use Committee. Because of multiple crossbreedings, we estimated approximate genetic background of experimental animals as follows. Ptg<sup>−/−</sup> mice were generated on a B6;129Sv genetic background and backcrossed to C57BL/6 (B6) parental strain four times. Thus, Ptg<sup>−/−</sup> mice used for the study carried ~99% of the B6 genotype. Rb<sup>+/−</sup>/ mice on a 129/Sv genetic background were purchased from The Jackson Laboratory and crossbred with Ptg<sup>−/−</sup> mice for at least five times. Therefore, more than 98% of Rb<sup>−/−</sup> genetic material was derived from the Ptg<sup>−/−</sup> background. Compound Rb<sup>−/−</sup>/Pttg<sup>−/−</sup>/ mice were bred by crossing Rb<sup>−/−</sup>/Pttg<sup>−/−</sup>/ females and males ("double knockout breeding"). Four genotypes were obtained from the same breeding: Rb<sup>−/−</sup>/Pttg<sup>−/−</sup>/, Rb<sup>−/−</sup>/Pttg<sup>−/−</sup>/p21<sup>+/−</sup>/, Rb<sup>−/−</sup>/Pttg<sup>−/−</sup>/p21<sup>+/−</sup>/ and Rb<sup>−/−</sup>/Pttg<sup>−/−</sup>/p21<sup>+/−</sup>/mice and MEFs were obtained from the same breeding.

Therefore, we estimate that the overall genetic backgrounds of Rb<sup>−/−</sup>/Pttg<sup>−/−</sup>/ and Rb<sup>−/−</sup>/Pttg<sup>−/−</sup>/p21<sup>+/−</sup>/mice from double knockout breeding are very close, whereas triply mutant Rb<sup>−/−</sup>/Pttg<sup>−/−</sup>/p21<sup>+/−</sup>/ mice have a slightly higher percentage of 129Sv genetic material.

Protein analysis. Pituitary tissues were processed (Immunoprecipitation kit, Roche) for Western blot analysis (16), and proteins were separated by SDS-PAGE, electroblotted onto Millipore membrane, and incubated with p21 (1:300; BD Pharmingen); p30, p19, p16, cyclin D1, Mlh1, Mlh3, Sumo1, hypoxia-inducible factor-1α (HIF-1α), Bcl-2 (all 1:200), MDM2, cyclin A, cyclin D1, Mlh1, Mlh3, and P21 (1:1,000), growth hormone (GH; 1:800), thyroid-stimulating hormone (TSH; 1:400), insulin (10 mmol/L sodium citrate (98.9%)) and 30% (2 mol/L) trehalose. Pituitary tissues were processed (Immunoprecipitation kit, Roche) for Western blot analysis (16), and proteins were separated by SDS-PAGE, electroblotted onto Millipore membrane, and incubated with p21 (1:300; BD Pharmingen); p30, p19, p16, cyclin D1, Mlh1, Mlh3, Sumo1, hypoxia-inducible factor-1α (HIF-1α), Bcl-2 (all 1:200), MDM2, cyclin A, cyclin D1, Mlh1, Mlh3, and P21 (1:1,000), growth hormone (GH; 1:800), thyroid-stimulating hormone (TSH; 1:400), insulin (10 mmol/L sodium citrate (98.9%)) and 30% (2 mol/L) trehalose.

Pituitary Gland Senescence

Telomere length and telomerase activity. Terminal restriction fragment lengths were analyzed by Southern blotting (TelomAGG Telomere Length Assay, Roche). Pituitary gland genomic DNA was isolated by Purigene genomic DNA extraction (Gentra). DNA (5 μg) was digested with Bsal and HinfI (New England Biolabs), resolved on 0.8% agarose gel, transferred, and hybridized with a telomeric TTAGG repeat probe. Telomere length was calculated according to the following formula: mean telomere length = Σ(OD1) / Σ(OD2/L), where OD1 is the chemiluminescent signal and L is the length of the fragment at position i.

Telomerase activity was detected with the TRAPEze Telomerase Detection kit (Chemicon International). Protein extracts were incubated in the presence of specific oligonucleotides, serving as telomerase substrates for addition of telomeric repeats. Substrate oligonucleotides were labeled with [γ-32P]ATP (10 μCi/μL) using T4 polynucleotide kinase and, after subsequent PCRs, separated on 12% nondenaturing acrylamide gel.

Microarray analysis. Total pituitary mRNA derived from 2-month WT and Pttg<sup>−/−</sup>/male mice and prepared with Trizol reagents (Invitrogen) was processed with the Oligo GEArray Mouse DNA Damage Signaling Pathway (SuperArray, Inc.). RNA was labeled (TrueLabeling-AMP 2.0 kit) with biotin-16-dUTP, and cRNA probe was synthesized using a thermal cycler. The probe was added to prehybridized GEArray membranes. Fifty-four of 113 genes were expressed, and densitometry measurements were normalized to both background and 8 housekeeping genes and analyzed using GEArray Expression Analysis Suite software.

Chromatin immunoprecipitation assay. Chromatin immunoprecipitation (ChIP) was done using the ChiP-IT kit (Active Motif) using AtT20 pituitary corticotroph cells that exhibit abundant PTTG expression. Cells were cross-linked with formaldehyde, harvested, and sonicated, and ChiP was done with polyclonal PTG1 antibody (Zymed) as well as nonspecific mouse IgG. DNA released from precipitated complexes was amplified by PCR with specific primers spanning 120 nucleotides upstream from the murine p21 transcription start site. Each ChiP sample was also subjected to PCR with primers spanning ~468 to ~765 nucleotides upstream from the transcription start site (negative control), and products were resolved in 1% agarose gel and visualized by ethidium bromide.

Electrophoretic mobility shift assay. A DNA fragment corresponding to +1 to 120 bp of the mouse p21 promoter and containing a putative PTTG-binding element was used as an electrophoretic mobility shift assay (EMSA) probe. The DNA fragment was digested with EcoRI (New England Biolabs) to generate 5’ overhangs and labeled with 20 μCi [α-32P]dATP (300 Ci/mmol, NEN-DuPont). Nuclear extracts (400 ng) derived from AtT20 cells were incubated with radiolabeled probe (100,000 cpm) in DNA-binding buffer (50 mmol/LTris-HCl, 1 mmol/LMgCl2, 1 mmol/LNaCl, 0.5% Nonidet P-40, 0.1 mmol/LEDTA, and 10 μg/mLpoly(dI·dC). Dye was added and electrophoresed on a 5% polyacrylamide gel.

Determination of telomeric DNA terminal length. Tissue DNA was isolated by centrifugation, and the telomere length was determined by the Southern blotting technique utilizing a telomeric repeat (TTAGGG) probe (11). Probes were prepared by the random priming method. PTTG expression was determined (Chip-IT kit, Active Motif) using AtT20 pituitary corticotroph cells that exhibit abundant PTGG expression. Cells were cross-linked with formaldehyde, harvested, and sonicated, and ChiP was done with polyclonal PTG antibody (Zymed) as well as nonspecific mouse IgG. DNA released from precipitated complexes was amplified by PCR with specific primers spanning 120 nucleotides upstream from the murine p21 transcription start site. Each ChiP sample was also subjected to PCR with primers spanning ~468 to ~765 nucleotides upstream from the transcription start site (negative control), and products were resolved in 1% agarose gel and visualized by ethidium bromide.

Senescence-associated β-galactosidase. SA-β-gal enzymatic activity was detected in pituitary cryosections (10 μm) using a β-gal staining kit (Senescence Cell Staining kit, Sigma-Aldrich). Sections were washed with PBS (pH 6.0), fixed overnight, washed in PBS, and stained with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) overnight at 37°C. At pH 6.0, only senescent cells are stained.

MEF preparation and culturing. MEFs were prepared from an embryonic day 13.5 mouse embryo and cultured in the DMEM supplemented with 2 mmol/L glutamine, 100 U/mL penicillin and streptomycin, and 10% Fetal bovine serum (FBS; Invitrogen) as described (23). For colony formation in soft agar, MEFs in the third passage were trypsinized and counted and 1 × 10<sup>5</sup> cells were inoculated in 12-well plates in triplicate overnight, fixed, and assayed for β-gal.

Bromodeoxuryridine incorporation. One-month Rb<sup>−/−</sup>/Pttg<sup>−/−</sup>/p21<sup>+/−</sup> and Rb<sup>−/−</sup>/Pttg<sup>−/−</sup>/p21<sup>+/−</sup>/ mice were injected i.p. with bromodeoxuryridine
(BrdUrd; 50 μg/g body weight; Sigma-Aldrich) thrice 2 h apart and sacrificed 24 h after the first injection. Pituitary sections were stained for BrdUrd (5-BrdUrd Labeling and Detection kit, Roche). One thousand cells were analyzed per field, three randomly chosen visual fields per section were counted, and three sections per animal derived from three animals of each genotype were analyzed.

Statistical analysis. The BrdUrd incorporation and MEF colony formation were analyzed using unpaired t test with a probability of P < 0.05 considered significant.

Results

Pttg deletion results in pituitary gland senescence. The ABF/p53/p21 senescence pathway is activated in the Pttg−/− pituitary gland as evidenced by several support of WT. Pituitary p53 levels are low, whereas p53 protein expression was up-regulated in 2-month Pttg−/− and Rb+/−Pttg−/− mice, and pituitary p21 induction was correlated with increased p53 levels. Both p21 and cyclin D1 protein levels were induced in Pttg-deficient pituitary glands (Fig. 1A).

As our earlier findings showed that Pttg-null mice exhibit pituitary, splenic hypoplasia, and thymic hyperplasia (24), we examined p21 expression in these tissues. Unlike the pituitary, changes in p21 protein levels were not observed in spleen or thymus, indicating a pituitary-selective effect of Pttg deletion.

The Cdk inhibitors p19 and p16 are both associated with cell cycle restraint. p16 protein levels did not differ between genotypes (data not shown), whereas p19 was up-regulated in the Pttg−/− pituitary gland (Fig. 1B). p19 also prevents p53 degradation (25, 26) and restrains MDM2, a negative regulator of p53 (27). In Pttg−/− mice, pituitary MDM2 protein levels were markedly decreased (Fig. 1C).

High-pH SA-β-gal activity, an in vivo marker of senescence (28), was expressed in 2- and 6-month pituitary glands derived from both Pttg−/− and WT mice (Fig. 1D). Quantification showed significantly higher number of SA-β-gal–positive cells in Pttg-null pituitary gland of both ages (Supplementary Fig. S1A). SA-β-gal activity was not detected in lung, ovary, muscle, or liver tissues, whereas SA-β-gal–positive cells were evident in WT skin (Supplementary Fig. S1B).

Intranuclear p21 is expressed in the Pttg-null pituitary intermediate lobe, colocalizing with MSH expression. In the anterior lobe, p21 is abundantly coexpressed in GH-producing cells and in prolactin-producing cells. p21 did not colocalize with ACTH, LH, or TSH in the Pttg−/− anterior pituitary (Supplementary Fig. S2). As shown earlier (16), p21 expression was very low in the pituitary gland of WT animals.

Pttg deletion is associated with increased pituitary Bcl-2 expression and slow cell cycle progression. As cellular senescence is often associated with apoptosis block, we analyzed pituitary expression of Bcl-2, which acts downstream of p53 and suppresses apoptosis (29, 30). Pituitary Bcl-2 protein levels were elevated in both Pttg−/− and doubly mutant Rb+/−Pttg−/− animals as measured by both pituitary immunostaining and Western blot analysis (Fig. 2A and B).

p21 restrains Cdk2 activity, leading to decreased Rb phosphorylation (29, 30), subsequently slowing cell cycle progression. Pttg deletion results in decreased pituitary Cdk2 activity (Fig. 2C) and cyclin A protein levels, associated with reduced Rb phosphorylation at Ser807/811, a residue preferentially phosphorylated by cyclin E-Cdk2 complexes (Fig. 2D; ref. 31). These disruptions are likely responsible for the low pituitary cell proliferation rates seen in animals lacking Pttg (16).

Pttg-dependent pituitary senescence is not associated with telomere shortening. Pituitary glands derived from 2-month Pttg−/− mice exhibited longer telomeres than WT (Fig. 3A). Average WT telomere length was ~40 kb, and ~50 kb in Pttg-deficient pituitaries, consistent with observed elevated telomerase activity. In pituitary extracts derived from 2- and 12-month-old Pttg−/− animals, telomerase added more TTAGGG telomeric repeats to substrate nucleotide than WT (Fig. 3B). In contrast, differences between genotypes were not noted in splenic tissue. The observed pituitary senescence pathway is likely not characterized by premature aging.

Pttg deletion is associated with pituitary aneuploidy and activation of DNA damage signaling pathways. Activation of the p53/p21 pathway often indicative of the senescent state may initially occur as a result of DNA damage or aneuploidy (32, 33). Because Pttg−/− cells exhibit genetic instability (24, 34), we examined 2-month mouse pituitary nuclear morphology by confocal microscopy. In WT animals, cell nuclei were evenly sized and shaped, with only occasional large nuclei and no micronuclei observed. In similarly aged Pttg−/− pituitary glands, however,

Figure 1. Pituitary p21, p53, and p19 expression are induced in the 2-mo Pttg−/− pituitary gland, indicating premature senescence. A to C, Western blot analysis of pituitary lysates derived from WT, Rb+/−, Pttg−/−, and Rb+/− Pttg−/− mice. p19 antibodies recognize both p1935S and p1967NS. ARF/INK4A locus alternative splicing products. Experiments were repeated thrice with similar results.

C, cryosections of pituitary glands derived from 2- and 6-mo Pttg−/− and WT mice and stained with X-Gal. Three pituitary glands from each group were stained. Representative sections are shown.
Figure 2. Pttg deletion is associated with increased Bcl-2 and decreased pituitary cell cycle progression. A, immunohistochemistry of pituitary sections derived from Pttg+/− and Pttg−/− mice and immunostained for Bcl-2. Two mice per genotype, three sections per mouse, were analyzed, and representative sections were shown. Western blot analysis of pituitary Bcl-2 protein levels (B) and Cdk2 kinase activity (C) in pituitary lysates derived from WT and Pttg−/− mice. D, Western blot analysis of pituitary lysates derived from WT, Rb+/−, Pttg−/−, and Rb−/− Pttg−/− mice. Experiments were repeated thrice with similar results.

Figure 3. Pttg deletion does not result in telomere shortening. A, Southern blot-based telomere restriction fragment length analysis in genomic DNA derived from the pituitary gland of WT (lane 1) and Pttg−/− (lane 2) mice. B, telomeric repeat amplification assay for pituitary telomerase activity measurement in WT and Pttg−/− mice. Lane 1, spleen extract from WT; lane 2, spleen extract from Pttg−/− 2-mo mice; lane 3, pituitary extract from WT; lane 4, pituitary extract from Pttg−/− 12-mo mice; lane 5, pituitary extract from WT; lane 6, pituitary extract from Pttg−/− 2-mo mice; lane 7, telomerase-positive cell extract (positive kit control); lane 8, heat-treated telomerase-positive cell extract (negative control); lane 9, control template (1 μL); lane 10, control template (2 μL). Five pituitaries per sample were pooled for both assays. Experiments were repeated twice with similar results.

several cells had very large macronuclei and few micronuclei (Fig. 4A). Quantification showed an average of 3 WT pituitary cells with macronuclei per high-power field, and 26 (1.4%) such cells in a total of 1,876 pituitary cells were assessed. In Pttg−/− animals, an average of 23 cells containing macronuclei was detected per high-power field, and 140 (10%) such cells were found in a total of 1,404 cells counted. This observation provides further evidence that Pttg deficiency triggers aneuploid features in pituitary cells.

Of 113 genes included in the DNA damage signaling pathway array, pituitary expression of 54 was detected in both genotypes in two separate experiments. The hybridized signal was normalized to background and the average signal was obtained from eight housekeeping genes on the same array. Expression changes were not evident for 43 genes, 2 were decreased, and expression of 9 increased >30% in Pttg-deficient pituitary tissue relative to WT (Fig. 4B; Supplementary Table S1).

To confirm these genotypic differences, Western blot analysis was done and showed up-regulated Mlh3, Mlh1, and Sumo1 in Pttg−/− animals (Fig. 4C).

Hypoxic stress can trigger DNA damage signaling, and HIF-1α transcription factor is induced in the Pttg-null pituitary gland (Fig. 4D). DNA damage signaling activates p53/p21 levels, and these results show that Pttg deletion also activates p53/p21-dependent pathways for pituitary gland senescence.

Pttg binds to the p21 promoter via Sp1. As PTTG suppresses p21 promoter activity (16), we explored whether PTTG is recruited to the endogenous p21 promoter. Lysates derived from AtT20 pituitary corticotrophs expressing high Pttg levels were isolated, and ChiP assays were done with a polyclonal PTTG1 antibody. PTTG was recruited to the p21 promoter region, spanning the first 120 nucleotides upstream from the transcription start site (Fig. 5A), but did not bind to the −468 to −765 promoter region (data not shown). Enrichment of specific +1 to −120 p21 promoter sequences in the precipitated indicated PTTG association with the p21 promoter in vivo. DNA identity of extracted PTTG-DNA complexes obtained by ChiP was confirmed by sequencing using primers spanning the first 120 nucleotides upstream from the transcription start site.

We then used EMSA to test whether PTTG binds the p21 promoter consensus sequence in untreated AtT20 cells. Addition of the probe generated by PCR using the same set of primers used for the ChiP assay to AtT20 nuclear fractions resulted in a specific band that was competed by 50- and 500-fold excess competitor. Addition of two concentrations of PTTG antibody dose dependently abolished PTTG-p21 binding (Fig. 5B). The absence of the specific band shift indicates that antibody binding interferes with formation of protein-DNA complexes (Methods in molecular biology. Transcription factor protocols, Humana Press, 2000). Nonspecific DNA-binding protein (Fig. 5B, lanes 5 and 6) did not interfere with DNA probe binding.

p21 deletion increases proliferation in Rb+/−/Pttg−/− pituitary gland. To confirm the effects of p21 deletion on in vivo proliferation of pituitary cells, we compared pituitary proliferation rates in doubly mutant Rb+/−/Pttg−/− and triply mutant
$Rb^+/+ /Pttg^{−/−} /p21^{−/−}$ mice obtained from intercrossbreeding of $Rb^+/+ /Pttg^{−/−}$ animals. We assessed BrdUrd incorporation in the pituitary anterior and intermediate lobe of experimental animals in vivo. In the anterior $Rb^+/+ /Pttg^{−/−}$ pituitary gland, 8.1 ± 0.9% of cells were positive for BrdUrd, whereas in $Rb^+/+ /Pttg^{−/−} /p21^{−/−}$ pituitary gland 11.1 ± 1.2% of cells were positive. However, differences in intermediate lobe BrdUrd incorporation were more pronounced. Cells (2.05 ± 0.8%) were positive in $Rb^+/+ /Pttg^{−/−}$ versus 6.8 ± 1.4% positive cells in $Rb^+/+ /Pttg^{−/−} /p21^{−/−}$ animals ($P < 0.05$; Fig. 5C). Thus, p21 deletion resulted in increased intermediate pituitary lobe proliferation in $Rb^+/+ /Pttg^{−/−}$ mice.

Phosphorylated Cdk2, a marker of cell proliferation, was induced in the pituitary but not in spleen of triply mutant $Rb^+/+ /Pttg^{−/−} /p21^{−/−}$ relative to $Rb^+/+ /Pttg^{−/−}$ animals (Fig. 5D).

**p21 deletion increases transformation potential and decreases senescence in $Rb^+/+ /Pttg^{−/−}$ MEFs.** The in vivo results suggest that p53/p21 senescence pathway activation as a consequence of $Pttg$ deletion leads to pituitary tumor growth restraint in $Rb^+/+ /Pttg^{−/−}$ mice. To clarify the role of p21-induced senescence in this process, we examined the effect of p21 disruption on MEF growth, transformation potential, and senescence. MEFs were derived from WT, $Pttg^{−/−}$, $Rb^+/+$, and $Rb^+/+ /Pttg^{−/−}$ genotypes with and without p21 deletion from embryos obtained from intercrossbreeding of $Rb^+/+ /Pttg^{−/−} /p21^{−/−}$ mice.

Pttg deletion results in p21 protein up-regulation compared with $Rb^+/+$ and even more so with WT mice, similar to pituitary p21 induction seen in Pttg-null animals (Fig. 6C). We therefore assessed whether these cells retain the ability to arrest under nonadherent conditions (35). MEFs derived from two different embryos of each genotype were assayed for anchorage-independent growth in soft agar. Of the p21-positive cells, only $Rb^+/+$ MEFs were capable of forming moderate numbers of small colonies, whereas WT, $Pttg^{−/−}$, and $Rb^+/+ /Pttg^{−/−}$ failed to produce colonies. p21 deletion from the $Rb^+/+ /Pttg^{−/−}$ background markedly increased the number of colonies (from 3.25 ± 0.9 in $Rb^+/+ /Pttg^{−/−} /p21^{−/−}$ to 42.8 ± 8 in $Rb^+/+ /Pttg^{−/−} /p21^{−/−}$; $P < 0.01$; Fig. 6A). Thus, increased anchorage-independent MEF growth indicates increased transforming potentials of $Rb^+/+ /Pttg^{−/−} /p21^{−/−}$ compared with $Rb^+/+ /Pttg^{−/−} /p21^{−/−}$ cells.

The number of colonies correlates inversely with the number of SA-β-gal–positive senescent MEF cells in the fourth passage ($r = −0.68; P < 0.05$). $Pttg^{−/−}$ and $Rb^+/+ /Pttg^{−/−}$ cells show the highest percentage of senescent cells, whereas p21 deletion from the $Rb^+/+ /Pttg^{−/−}$ background decreased the number of SA-β-gal–positive cells (from 23.4 ± 7.1 in $Rb^+/+ /Pttg^{−/−} /p21^{−/−}$ to 8.25 ± 2.2 in $Rb^+/+ /Pttg^{−/−} /p21^{−/−}$ cells; $P < 0.05$; Fig. 6B) Supplementary Fig. S3). Thus, induced p21 is required for the increased senescence observed in Pttg-deficient primary cells.

To further investigate mechanisms leading to induced transformation in $Rb^+/+ /Pttg^{−/−} /p21^{−/−}$ cells, we compared expression of proteins that regulate cellular proliferation in $Rb^+/+ /Pttg^{−/−}$ and $Rb^+/+ /Pttg^{−/−} /p21^{−/−}$ MEFs during the fourth passage. Up-regulated levels of phosphorylated Rb observed in $Rb^+/+ /Pttg^{−/−} /p21^{−/−}$ MEFs are likely a result of increased levels of phosphorylated Cdk2 and also reflect increased proliferation of these cells. Concomitantly, the Cdk inhibitor p16 is induced in $Rb^+/+ /Pttg^{−/−} /p21^{−/−}$ cells lacking p21 (Fig. 6D). Induction of proteins restraining cell cycle progression could account for the moderate senescence observed in $Rb^+/+ /Pttg^{−/−} /p21^{−/−}$ MEFs. Thus, the requirement of p21 for increased senescence evident in Pttg-deficient primary MEF cells is not absolute.
Discussion

The results show that pituitary senescence observed as early as 2 months is a mechanism for restraining pituitary tumor development in Pttg-null mice. Age-related telomere shortening triggers entry into replicative senescence; however, a similar end point, premature senescence, can be achieved in response to activated oncogenes, oxidative stress, radiation, chemical drugs, and DNA damage (19). Replicative senescence is associated with telomere shortening (17) and senescent features were also observed in benign tumors (21). However, whether premature senescence occurs in vivo in nontumorous tissue has been controversial. We show here that pituitary glands derived from young Pttg−/− mice exhibit senescence not associated with telomere shortening and premature aging. The observed changes include p53 accumulation, induced p21, apoptosis block, and increased SA-β-gal expression. These features are consistent with the small pituitary size, decreased pituitary cell proliferation of Pttg−/− mice, and marked decline in pituitary tumor development in compound Rb+/− Pttg−/− mice relative to singly mutant Rb−/− animals (16).

p53 is normally ubiquitinated by MDM2 (36), and when the p53-p21 senescence pathway is activated, p19 (ARF) is induced, protecting p53 from ubiquitination by disrupting MDM2 activity (27). Our results show increased pituitary p19 and decreased MDM2 protein levels in Pttg-null mice, associated with p53 accumulation. p21 is a transcription target for p53, and activated p53 induces pituitary p21 expression, leading to inhibition of Cdk2 activity and decreased Rb phosphorylation and ultimately restraint of pituitary cellular proliferation. p21 binding to proliferating cell nuclear antigen could be another mechanism by which p21 attenuates pituitary cell growth in these animals (37). Enhanced pituitary cyclin D1 expression is associated with senescence and may occur due to protection of cyclin D-Cdk4/6 complexes from degradation by induced p21 (30).

Apoptosis block is a characteristic of senescent cells (20), and high p21 levels arrest p53-dependent and p53-independent apoptosis. Up-regulation of the antiapoptotic factor Bcl-2 supports the absence of apoptosis in Pttg−/− pituitary glands, shown also by terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling assay of pituitary and pancreatic tissues (data not shown; ref. 38). Thus, in the pituitary of Pttg-null mice, the ARF/p53/p21 senescence pathway is activated, likely providing a mechanism for the observed pituitary hypoplasia.

SA-β-gal, an in vivo marker of senescence (28), is expressed only in skin samples, concordant with observations in human dermal fibroblasts and keratinocytes (29). Surprisingly, the normal pituitary also age dependently expresses SA-β-gal, which may reflect low levels of endogenous pituitary cell proliferation. Nevertheless, Pttg deletion strongly exacerbates these senescent features.

Cells that expressed high levels of p21 undergo senescence. p21 protein is abundantly expressed in anterior lobe GH- and prolactin-producing cells and is also expressed in the intermediate lobe of Pttg-deficient pituitary gland, whereas very little p21 expression was noted in WT pituitary (16). In our experiments, Rb+/− Pttg−/− mice with a prevalence of B6 genetic background develop both intermediate and anterior lobe pituitary tumors (data not shown) as it was shown previously (39). Up-regulated p21 likely restrains both intermediate and anterior lobe pituitary tumor growth in Rb+/− Pttg−/− mice.

![Figure 5. PTTG binds to the Sp1 site on the p21 promoter.](image)

A. ChiP assay was done in nuclear fractions derived from AtT20 cell lysates. Top, schematic of the approximate location of primers used in the PCR. IgG, nonspecific antibody; PTTG, specific antibody. Experiment was repeated four times. Representative assay is shown. B. EMSA analysis of PTTG binding using 32P-labeled dsDNA probe spanning 120 nucleotides upstream from the p21 promoter transcription start site. Lane 1, free probe; lane 2, nuclear extract only; lane 3, 50-fold; lane 4, 500-fold excess of unlabeled specific competitive oligonucleotide (p21 promoter); lane 5, 50-fold; lane 6, 500-fold excess of unlabeled nonspecific oligonucleotide (AP2); lane 7, 1:100; lane 8, 1:1 PTTG antibody; lane 9, 1:100; lane 10, 1:1 nonspecific IgG. Experiment was repeated thrice with similar results. C. p21 deletion increased pituitary cell proliferation in Rb+/− Pttg−/− mice. BrdUrd-positive pituitary cells in 4-wk Rb+/− Pttg−/− and Rb−/− Pttg−/− pituitary glands. IL, intermediate lobe; PL, posterior lobe. Percentage of BrdUrd-positive cells is shown below. Each value represents mean percentage of positive cells ± SE (1,000 cells per field, three fields per section, three sections per animal, n = 3 animals per genotype analyzed). D. Western blot analysis of phosphorylated Cdk2 (pCdk2) protein levels in the pituitary and spleen of Rb+/− Pttg−/− and Rb−/− Pttg−/− mice. Experiment was done twice with similar results.
Telomere length may function as a “mitotic clock” to sense cell aging, and telomere shortening signals replicative senescence (17). Increased telomere lengths observed in the Pttg-null pituitary indicate that senescence is not associated with exhausting replicative potentials of pituitary cells, consistent with increased telomerase activity. Unlike in human cells, telomerase activity is detected in adult murine tissues and telomerase also acts independently of telomere lengthening (17). The basis for the increased telomerase activity observed in the Pttg−/− pituitary is presently not clear.

Senescence-associated changes are likely provoked by activation of DNA damage signaling pathways observed in Pttg−/− pituitary glands. Surveillance mechanisms protecting genome integrity maintain the status and quality of chromosomal DNA and delay or arrest cell cycle progression in response to DNA damage signaling pathway activation (40). In response to DNA damage, p53 levels increase (30), whereas disrupted p53 leads to extension or arrest cell cycle progression in response to DNA damage maintaining the status and quality of chromosomal DNA and delaying senescence in triply mutant mice (24). The results of this study provide further in vivo evidence for aneuploidy in the pituitary gland of Pttg−/− mice, including the presence of both macronuclei and micronuclei. Whether aneuploidy is the cause of DNA damage signaling pathway activation remains unproven, but both partially activate similar genes. Thus, aneuploidy also triggers ATM and p21 expression, and p21 safeguards against further genome instability by suppressing proliferation of affected cells (33). This hypothesis is supported by findings that increased aneuploidy inhibits tumor formation (44).

The AFB/p53/p21 pathway may also be activated by hypoxic stress, evident by up-regulation of HIF-1α in Pttg-null pituitary cells. HIF-1α transcription factor prevents p53 from degradation (45). The basis for Pttg−/− pituitary HIF-1α activation is not known, but similarly increased baseline HIF-1α expression was evident in Pttg−/− bone marrow-derived stem cells, which also exhibit increased hypoxic sensitivity (46).

Experiments with MEFs confirmed the involvement of p21 in restraining pituitary cell proliferation and decreasing tumorigenic properties in Rb+/−Pttg−/− mice. p21 deletion markedly increased cell proliferation, decreased senescence, and increased anchorage-independent growth in Rb+/−Pttg−/− MEFs. Anchorage-independent cell growth correlates with tumorigenicity and p21 deletion increased pituitary cell proliferation in triply mutant Rb+/−Pttg−/−p21−/− mice relative to Rb+/−Pttg−/− animals. Although the genetic background of these triply mutant mice differs slightly from Rb+/−Pttg−/− animals described earlier (16), preliminary results obtained from this breeding show that none of 10 available Rb+/−Pttg−/− mice developed tumors, whereas 89% of 17 Rb+/−Pttg−/−p21−/− mice developed tumors, implying the.

![Figure 6. Pttg deletion results in decreased MEF anchorage-independent growth, increased senescence, and up-regulation of p21 protein levels. A, number of colonies (>2 mm) formed by third-passage MEFs 14 d after plating. MEFs were plated in triplicates. Columns, mean of two independent experiments; bars, SE. B, number of senescent cells in fourth-passage MEFs. Cells were plated in triplicate. Columns, mean of two independent experiments; bars, SE. C, Western blot analysis of p21 protein levels in MEFs. D, Western blot analysis of cell cycle proteins in Rb+/− Pttg−/− p21−/− and Rb+/− Pttg−/− p21−/− MEFs. Experiment was done twice with similar results.](https://www.aacrjournals.org/cancerres/article-pdf/67/21/10570/30000688/cancerres-2007-00608r.pdf)
important role of p21 in restraining pituitary tumor growth. Collectively, these results confirm the role of Pttg in pituitary cell proliferation and tumor development in Rb/C0Pttg/C0 mice and indicate the p21-dependent senescence pathway involvement in this process.

The results shown here provide a postulated mechanism for the observed Pttg+/− pituitary hypoplasia. PTTG deletion may regulate p21 by leading to chromosomal instability, DNA damage signaling pathway activation, and stimulation of pituitary p53/p21-dependent senescence. PTTG also appears to contribute to pituitary cancer expression, as PTTG binding to Sp1 suppresses p21 promoter activity (16). BLAST analysis yielded several binding sites for Sp1 situated within the putative PTTG-binding region on the p21 promoter. PTTG and Sp1 interaction was confirmed by His-tag pull-down assay, and their interaction domains were further mapped using poly-His-tagged PTTG1, Sp1, and their respective fragments (47).

PTTG-p21 interactions are surprisingly prominent in the pituitary. Unlike the pituitary, splenic, or thymic, p21 expression is not induced in the Pttg-null genotype. p21 deletion results in enhanced phosphorylated Cdk2 protein expression in the pituitary gland but not in spleen of Rb/C0Pttg/C0 mice. These findings could be explained by tissue-specific properties of the p21 promoter. For example, Rb stimulates the p21 promoter in epithelial cells but not in fibroblasts (48). Thus, the effect of Pttg deficiency on p21 expression may also be preferential for pituitary tissue. Pttg deletion may also only affect selected organ-specific stem cells. Pttg-null mice develop diabetes mellitus due to decreased pancreatic β cell proliferation (38). Similarly, targeted deletion of the cell cycle protein Cdk4 resulted in organ-specific proliferation defects in pituitary and pancreatic β cells (49). The unique requirement for replicative endocrine tissue for Pttg or Cdk4 implies that slow adaptive cell cycle progression is controlled by mechanisms distinct from the rapid cell cycle of skin or digestive tract regenerative tissues (49). This is consistent with clinical observations that tumors arising from endocrine glands (pituitary and pancreatic islets) rarely exhibit malignant phenotypes (3), whereas carcinomas frequently arise from actively regenerative tissues.

We show here that Pttg deletion facilitates pituitary gland senescence independently of telomere shortening. This animal model constitutes a unique example of in vivo pituitary senescence, protective for pituitary tumor development in the Rb/C0Pttg/C0 mouse and for the exceedingly rarely encountered human malignant pituitary neoplasms. Pttg overexpression is observed in most human pituitary tumors. Pttg behaves as an oncogene (50), and Pttg is permissive for pituitary tumor formation in mice (6). Pttg is also a mammalian securin, and both loss of Pttg as observed in Pttg-null mice and Pttg overexpression result in aneuploidy. Moreover, levels of Pttg expression positively correlate with the levels of aneuploidy in human GH- and prolactin-producing pituitary tumors (51), and we observe high p21 levels in GH- and prolactin-producing Pttg-null anterior pituitary cells. In both cases (Pttg overexpression or absence), the consequences of aneuploidy may be proliferation restraint and senescence.

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References

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