Published in final edited form as:

Cancer Res. 2007 November 1; 67(21): 10564–10572. doi:10.1158/0008-5472.CAN-07-0974.

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 Bcl-2 protein levels and p53 stabilization. High pituitary p21 levels in the absence of PTTG were associated with suppressed cyclin-dependent kinase 2 activity, Rb phosphorylation, and cyclin A expression, all required for cell cycle progression. Although senescence-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.

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

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Note: Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

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^{Cip1} 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.

Apoptosis 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, chemotherapy, and oncogene activation (18–20). Cellular senescence is characterized by up-regulation of cell cycle progression inhibitors, including p19^{ARF} (p19), p21^{Cip1} (p21), and p16^{INK4A} (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 p53 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*^{+/-}*Pttg*^{-/-}p21^{-/-} 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*^{+/-}*Pttg*^{-/-} 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. *Pttg*^{-/-} mice were generated on a B6;129Sv genetic background and backcrossed to C57BL/6 (B6) parental strain four times. Thus, *Pttg*-null mice used for the study carried ~99% of the B6 genotype. *Rb*^{+/-} mice on a 129/Sv genetic background were purchased from The Jackson Laboratory and crossbred with *Pttg*^{-/-} mice for at least five times. Therefore, more than 98% of *Rb*^{+/-} genetic material was derived from the *Pttg*^{-/-} background. Compound *Rb*^{+/-}*Pttg*^{-/-} mice were bred by crossing *Rb*^{+/-}*Pttg*^{+/-} females and males (“double knockout

breeding"). Four genotypes were obtained from the same breeding: $Rb^{+/-}Pttg^{-/-}$, $Rb^{+/-}Pttg^{+/+}$, $Rb^{+/+}Pttg^{-/-}$, and $Rb^{+/+}Pttg^{+/+}$ [wild-type (WT)].

$p21^{-/-}$ (B6;129S2-Cdkn1a^{tm1Tyj}) was purchased from The Jackson Laboratory, backcrossed to B6 WT mice twice at which point animals had 96.9% of B6 genetic material, and then bred by crossing $Rb^{+/-}Pttg^{+/-}p21^{+/-}$ females and males ("triple knockout breeding"). $Rb^{+/-}Pttg^{-/-}p21^{+/+}$ and $Rb^{+/-}Pttg^{-/-}p21^{-/-}$ mice and MEFs were obtained from the same breeding.

Therefore, we estimate that the overall genetic backgrounds of $Pttg^{-/-}$ and $Rb^{+/-}Pttg^{-/-}$ mice from double knockout breeding are very close, whereas triply mutant $Rb^{+/-}Pttg^{-/-}p21^{-/-}$ 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); p53 (1:300), p19, p16, cyclin A, cyclin D1, Mlh1, Mlh3, Sumo1, hypoxia-inducible factor-1 α (HIF-1 α), Bcl-2 (all 1:200), MDM2 (1:100; Santa Cruz Biotechnology), phosphorylated Rb (Ser^{807/811}), and phosphorylated Cdk2 (Thr¹⁶⁰; both 1:1,000; Cell Signaling Technology) antibodies overnight followed by corresponding secondary antibodies (1:1,000; Sigma-Aldrich).

For measurement of kinase activity of Cdk2, whole pituitary cell lysate (1 μ g protein) was precleared with equilibrated G beads (Immunoprecipitation kit) and incubated with Cdk2 antibodies (1:200; Santa Cruz Biotechnology) for 4 h. Immunocomplexes were precipitated with protein G beads and incubated in kinase buffer containing 20 μ Ci [γ -³²P]ATP (NEN-DuPont) and 2 μ g histone H substrate (Upstate) for 30 min at 30°C. The reaction mixture was resolved on SDS-PAGE gel and autoradiographed.

For histologic analyses, tissues were fixed and paraffin embedded and sections were stained with H&E and periodic acid-Schiff. The streptavidin-biotin-peroxidase complex technique was used with rabbit polyclonal Bcl-2 antibodies (1:50) followed by goat anti-mouse (22).

For immunofluorescence analysis, p21 polyclonal antibody (1:30) conjugated with Alexa Fluor 488 fluorescent dye or with Alexa Fluor 568 fluorescent dye (both from Molecular Probes) was used. Antigen retrieval was done in 10 mmol/L sodium citrate (98°C for 40 min and cooling down for 20 min). Melanocyte-stimulating hormone (MSH) antibody (1:200) was purchased from Phoenix Pharmaceutical. Adrenocorticotropic hormone (ACTH; 1:1,000), growth hormone (GH; 1:800), thyroid-stimulating hormone (TSH; 1:200), prolactin (1:200), and luteinizing hormone (LH; 1:200) antibody were obtained from the National Hormone and Peptide Program, National Institutes of Diabetes, Digestive and Kidney Diseases (Bethesda, MD) and Dr. Parlow (Harbor-UCLA Medical Center, Torrance, CA). Control reactions lacked primary antibodies or were stained with blocking antibodies.

Telomere length and telomerase activity

Terminal restriction fragment lengths were analyzed by Southern blotting (TeloTAGGG Telomere Length Assay, Roche). Pituitary gland genomic DNA was isolated by Purigene genomic DNA extraction (Gentra). DNA (5 μ g) was digested with *RsaI* and *HinfI* (New England Biolabs), resolved on 0.8% agarose gel, transferred, and hybridized with a telomeric TTAGGG repeat probe. Telomere length was calculated according to the following formula: mean telomere length = $\Sigma(OD_i) / \Sigma(OD_i/L_i)$, where OD_i is the chemiluminescent signal and L_i 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 - 32 P]ATP (10 mCi/mL) 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*^{-/-} 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 (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 PTTG1 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 murine 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 *Eco*RI (New England Biolabs) to generate 5' overhanging end and labeled with 20 μ Ci [α - 32 P]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 (Gel Shift Assay Core System, Promega). For competition studies, unlabeled DNA fragments were added 1 h before addition of labeled probe. For supershift experiments, antibodies to PTTG1 or nonspecific antibodies to AP2 (Promega) were added to the binding mixture 1 h before addition of labeled DNA probe. Protein-DNA complexes were resolved by gel electrophoresis on nondenaturing 4% PAGE.

Senescence-associated β -galactosidase

SA- β -gal enzymatic activity was detected in pituitary cryosections (10 μ m) 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 2×10^4 cells were inoculated in 12-well plates in noble agar in DMEM supplemented with 20% FBS. Colonies were scored 14 days later. For senescence assays, MEFs of the fourth passage were trypsinized and counted and 1×10^5 cells were plated in six-well plates in triplicate overnight, fixed, and assayed for β -gal.

Bromodeoxyuridine incorporation

One-month $Rb^{+/-}Pttg^{-/-}p21^{+/+}$ and $Rb^{+/-}Pttg^{-/-}p21^{-/-}$ mice were injected i.p. with bromodeoxyuridine (BrdUrd; 50 μ g/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 ARF/p53/p21 senescence pathway is activated in the $Pttg^{-/-}$ pituitary gland as evidenced by several lines of support. 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 Ser^{807/811}, 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 pathway**

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, 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-1 α 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 precipitate

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 $Rb^{+/-}Pttg^{+/-}p21^{+/-}$ animals. We assessed BrdUrd incorporation in the pituitary anterior and intermediate lobe of experimental animals *in vivo*. In the anterior $Rb^{+/-}Pttg^{-/-}$ pituitary gland, $8.1 \pm 0.98\%$ of cells were positive for BrdUrd, whereas in $Rb^{+/-}Pttg^{-/-}p21^{-/-}$ pituitary gland $11.1 \pm 1.2\%$ of cells were positive. However, differences in intermediate lobe BrdUrd incorporation were more pronounced. Cells ($2.05 \pm 0.8\%$) were positive in $Rb^{+/-}Pttg^{-/-}$ versus $6.8 \pm 1.4\%$ 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 $Rb^{+/-}Pttg^{-/-}$ 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 (28). 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.

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 of the human cell replicative life span. In this study, DNA damage signaling pathways in *Pttg*-null pituitary glands induced multiple changes, all involved in preserving or stabilizing p53. Thus, ataxia-telangiectasia mutated (ATM), which senses DNA damage, is induced in the *Pttg*-null pituitary gland. Mlh1 and Mlh3 augment p53 phosphorylation by ATM and stimulate ATM-dependent p53 activation (27). Sumo1 may also reinforce p53 stability and activity (41), leading to p21 induction and restrained pituitary cell proliferation in *Pttg*-deficient mice.

It is not clear how DNA damage pathways are activated. PTTG is a mammalian securin homologue, which protects chromosomal stability and mediates faithful chromatid separation (42). *In vitro*, both *Pttg* deletion and overexpression result in aneuploidy and genetic instability (13,43). We showed earlier using MEF metaphase karyotyping the presence of binucleated and multinucleated cells, aberrant chromosomal morphology, quadriradial and triradial chromosomes, chromosome breaks, and premature centromere division resulting from *Pttg* deficiency (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 ARF/p53/p21 pathway may also be activated by hypoxic stress, evident by up-regulation of HIF-1 α in *Pttg*-null pituitary cells. HIF-1 α transcription factor prevents p53 from degradation (45). The basis for *Pttg*^{-/-} pituitary HIF-1 α activation is not known, but similarly increased baseline HIF-1 α 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 anchorageindependent growth in *Rb*^{+/-}*Pttg*^{-/-} MEFs. Anchorageindependent 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 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 $Rb^{+/-}Pttg^{-/-}$ 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 absence also directly activates pituitary p21 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. PTTG1 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 $Rb^{+/-}Pttg^{-/-}p21^{-/-}$ 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 of 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 $Rb^{+/-}Pttg^{-/-}$ 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Grant support: NIH grant CA75979 (S. Melmed) and The Doris Factor Molecular Endocrinology Laboratory.

We thank Fabio Redondo for excellent technical assistance and the staff of the Comparative Medicine Department for exemplary animal care.

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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 p19^{ARF} and p16^{INK4A}, ARF/INK4A locus alternative splicing products. Experiments were repeated thrice with similar results. D, 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. *pRb*, phosphorylated Rb.

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 μ L); *lane 10*, control template (2 μ L). Five pituitaries per sample were pooled for both assays. Experiments were repeated twice with similar results.

Figure 4.

Pttg^{-/-} mice exhibit abnormal nuclear morphology and up-regulated DNA damage signaling pathway. A, H&E pituitary staining of a 2-mo WT (left) and *Pttg*^{-/-} (middle) male mouse. Confocal micrograph of 2-mo *Pttg*^{-/-} male mouse pituitary. Right, nuclei were stained with ToPro3. Magnification of left and middle panels is similar; right panel is at a higher magnification to illustrate micronuclei. Dark arrow, macronuclei; white arrow, micronuclei. B, comparison of hybridization results of DNA damage signaling pathway gene arrays. Four pituitary glands per genotype were pooled. Two separate experiments were done, and one is shown. Solid circles, genes up-regulated in *Pttg*^{-/-} mice: 1G-ATM, 6B-Mlh1, 6C-Mlh3, G10-Rad 52, F11-Rev3l, B12-Sumo1, F12-Tlk1, G12-Tlk2, and E13-Ube2b; dashed circles, genes up-regulated in WT mice: G2-Cspg6 and D4-G22p1. Western blot analysis of Mlh1, Mlh3, and Sumo1 (C) and HIF-1α (D) protein levels in WT and *Pttg*^{-/-} pituitary gland. Each experiment was repeated thrice.

Figure 5.

PTTG binds to the Sp1 site on the p21 promoter. **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× PTTG antibody; *lane 9*, 1:100; *lane 10*, 1× nonspecific IgG. Experiment was repeated thrice with similar results. **C**, p21 deletion increased pituitary cell proliferation in $Rb^{+/-}Pttg^{-/-}p21^{-/-}$ mice. BrdUrd-positive pituitary cells in 4-wk $Rb^{+/-}Pttg^{-/-}p21^{+/+}$ and $Rb^{+/-}Pttg^{-/-}p21^{-/-}$ 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 $Rb^{+/-}Pttg^{-/-}p21^{+/+}$ and $Rb^{+/-}Pttg^{-/-}p21^{-/-}$ mice. Experiment was done twice with similar results.

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.