p21^{Cip1} restrains pituitary tumor growth

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As commonly encountered, pituitary adenomas are invariably benign. We therefore studied protective pituitary proliferative mechanisms. Pituitary tumor transforming gene (*Pttg*) deletion results in pituitary p21 induction and abrogates tumor development in *Rb*$^{+/-}$*Pttg*$^{-/-}$ mice. p21 disruption restores attenuated *Rb*$^{+/-}$*Pttg*$^{-/-}$ pituitary proliferation rates and enables high penetrance of pituitary, but not thyroid, tumor growth in triple mutant animals (88% of *Rb*$^{+/-}$ and 72% of *Rb*$^{+/-}$*Pttg*$^{-/-}$*p21*$^{-/-}$ vs. 30% of *Rb*$^{+/-}$*Pttg*$^{-/-}$ mice developed pituitary tumors, $P < 0.001$). p21 deletion also accelerated S-phase entry and enhanced transformation rates in triple mutant MEFs. Intranuclear p21 accumulates in *Pttg*-null aneuploid GH-secreting cells, and GH_3 rat pituitary tumor cells overexpressing PTTG also exhibited increased levels of mRNA for both p21 (18-fold, $P < 0.01$) and ATM (9-fold, $P < 0.01$). PTTG is abundantly expressed in human pituitary tumors, and in 23 of 26 GH-producing pituitary adenomas with high PTTG levels, senescence was evidenced by increased p21 and SA-β-galactosidase. Thus, either deletion or overexpression of *Pttg* promotes pituitary cell aneuploidy and p53/p21-dependent senescence, particularly in GH-secreting cells. Aneuploid pituitary cell p21 may constrain pituitary tumor growth, thus accounting for the very low incidence of pituitary carcinomas.

acromegaly | nonfunctioning adenoma | pituitary adenoma | PTTG

Perturbed cell proliferation subserves pituitary trophic disorders that lead to pituitary hypoplasia, hyperplasia, or adenoma formation (1, 2). These conditions, associated with either hormonal hyposecretion or hypersecretion, are characterized by several unique features, including reversibility as exemplified by pregnancy-associated hyperplasia (3), transcription-factor- or aging-associated hypoplasia, and a compelling resistance to adenomatous malignant transformation. Pituitary tumors, commonly encountered as intracranial neoplasms, are invariably benign, but cause significant morbidity through mass effects and/or the inappropriate secretion of pituitary hormones (4, 5). Changes leading to pituitary tumorigenesis involve both intrinsic pituicyte dysfunction and altered availability of paracrine or endocrine factors that regulate hormone secretion and cell growth (6–9). Mitotic activity is relatively low, even in aggressive pituitary adenomas, in contrast to tumors arising from more rapidly replicating tissues (2). To gain insight into the unique pathogenesis of disordered pituitary growth, we examined mechanisms subserving pituitary cell proliferation.

Pituitary tumor transforming gene (*Pttg*) (10), a mammalian securin that facilitates sister-chromatid separation during metaphase (11), exhibits oncogene properties as overexpression causes cell transformation, induces aneuploidy (12, 13), and promotes tumor formation (14). PTTG also regulates transcription of several genes (15), including those encoding G1-S-phase proteins (16, 17). *Pttg* is overexpressed in pituitary tumors (18), and abundance of the protein correlates with pituitary, thyroid, colon, and uterine tumor invasiveness (19). Equilibrium of intracellular PTTG levels is important for maintaining chromosomal stability, as both high or low *Pttg* expression induce genetic instability (20, 21), and cells overexpressing *Pttg* (22) and *Pttg*-null cells (23) both exhibit aneuploidy. Enhanced chromosomal rearrangements and chromatid breaks are observed after DNA damage in *Pttg*$^{-/-}$ HCT116 cells, highlighting the securin requirement for the maintenance of chromosomal stability after genotoxic stress (24).

Pituitary-directed transgenic *Pttg* overexpression results in focal pituitary hyperplasia and small but functional adenoma formation (14). In contrast, mice lacking *Pttg* exhibit selective endocrine cell hypoplasia (25). The permissive role of PTTG for pituitary adenoma development was further confirmed by the observation that *Pttg* deletion from the *Rb*$^{+/-}$ background results in p53/p21 senescence pathway activation, associated with pituitary hypoplasia and attenuated adenoma formation (23, 26).

p21$^{Cip/Kip}$ (p21), a transcriptional target of p53, is induced in response to a spectrum of cellular stresses and acts to constrain the cell cycle. p21 may also be induced as a result of DNA damage or oncogene expression, triggering irreversible cell cycle arrest (i.e., senescence), which facilitates tumor growth arrest (27–30). Mechanisms effecting cell senescence act to buffer the cell from proproliferative signals, and in vivo senescent markers are predominantly expressed in benign but not in malignant lesions (31). p21 may mediate either suppression or promotion of cell proliferation depending on intracellular localization of the protein (32), and intranuclear p21 acts to arrest proliferation of unstable or aneuploid cells (33, 34). Activating oncogene functions may also switch to growth restraint, depending on the cellular genetic context and functional p21 status (35).

To delineate mechanisms underlying the protective role of p21 in pituitary tumor growth, we generated triple mutant *Rb*$^{+/-}$*Pttg*$^{-/-}$*p21*$^{-/-}$ mice and show that p21 deficiency rescues pituitary cell proliferation and restores abrogated pituitary tumor formation in *Rb*$^{+/-}$*Pttg*$^{-/-}$ mice. Paradoxically, p21 is also induced in *Pttg*-transfected GH_3 pituitary cells and in human GH-secreting pituitary adenomas exhibiting high PTTG levels. The results indicate that both *Pttg* deletion and *Pttg* overexpression trigger pituitary cell aneuploidy and p21 induction. High pituitary p21 levels appear to promote senescence and restrain pituitary tumor growth and may underlie the failure of invariably benign pituitary tumors to progress to true malignancy.

Results

p21 Deletion Restores Pituitary Tumor Development in Rb$^{+/-}$Pttg$^{-/-}$ Mice. We hypothesized that high pituitary p21 contributed to the protective effect of *Pttg* deletion on pituitary tumor development in *Rb*$^{+/-}$ mice (23). We therefore deleted p21 from the *Rb*$^{+/-}$*Pttg*$^{-/-}$ background and generated triple mutant *Rb*$^{+/-}$*Pttg*$^{-/-}$*p21*$^{-/-}$ mice. Spontaneous WT pituitary tumors were observed in 6 of 85 animals

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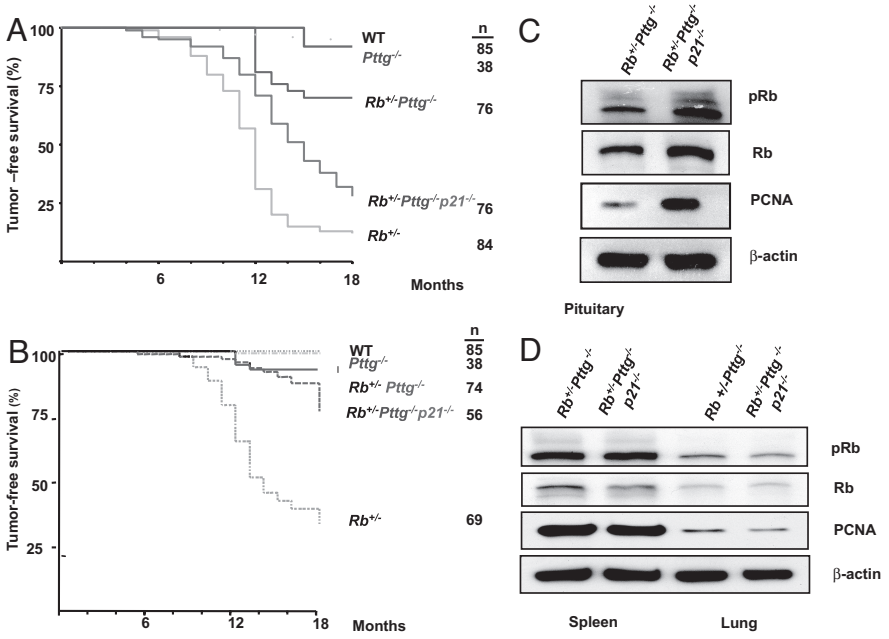

Fig. 1. p21 deletion from the $Rb^{+/-}Pttg^{-/-}$ background restores pituitary but not thyroid tumor development in mice. (*A*) Kaplan–Meier survival analysis (log-rank test) of the time of death with evidence of pituitary tumor showed significant differences between $Rb^{+/-}Pttg^{-/-}$ and $Rb^{+/-}Pttg^{+/+}$ ($P < 0.001$), between $Rb^{+/-}Pttg^{-/-}$ and $Rb^{+/+}Pttg^{-/-}$ ($P < 0.05$), and between a $Rb^{+/-}Pttg^{-/-}$ and $Rb^{+/-}Pttg^{-/-}p21^{-/-}$ mice ($P = 0.007$). (*B*) Kaplan–Meier survival analysis (log-rank test) with evidence of thyroid tumors in the different genotypes showed significant differences between $Rb^{+/-}Pttg^{-/-}$ and $Rb^{+/-}Pttg^{+/+}$ ($P < 0.001$), and between $Rb^{+/-}Pttg^{+/+}$ and $Rb^{+/-}Pttg^{-/-}p21^{-/-}$ mice ($P < 0.001$). No differences were observed between $Rb^{+/-}Pttg^{-/-}$ and $Rb^{+/-}Pttg^{-/-}p21^{-/-}$ mice. *n*, number of animals killed. Western blot analysis of proliferation markers in (*C*) pituitary and (*D*) spleen and lung lysates derived from $Rb^{+/-}$ $Pttg^{-/-}$ and $Rb^{+/-}$ $Pttg^{-/-}$ $p21^{-/-}$ mice.

(5%) starting at 9 months of age. Of 38 $Rb^{+/+}Pttg^{-/-}$ mice, 3 (8%) harbored pituitary tumors by 18 months. *Rb* heterozygous mice die mostly from pituitary tumors at 8–15 months depending on their genetic backgrounds (36, 37). Mice developed pituitary tumors starting from 4 months of age, and by 18 months, 74 of 84 (88%) $Rb^{+/-}Pttg^{+/+}$ mice harbored confirmed tumors. *Pttg* deletion delayed the appearance of pituitary tumors; of 78 double mutant $Rb^{+/-}Pttg^{-/-}$ mice, only 30% harbored tumors by 18 months. p21 removal from the $Rb^{+/-}Pttg^{-/-}$ background (triple mutant $Rb^{+/-}Pttg^{-/-}p21^{-/-}$ mice) restored tumor formation almost to levels observed in single mutant $Rb^{+/-}$ mice; of 76 such triple mutant mice, 63 (72%) developed pituitary tumors (Fig. 1*A*).

$Rb^{+/-}Pttg^{+/+}$, $Rb^{+/-}Pttg^{-/-}$, and $Rb^{+/-}Pttg^{-/-}p21^{-/-}$ mice developed intermediate and anterior lobe tumors, with some animals developing tumors in both pituitary sites. Fewer triple mutant animals simultaneously developed both intermediate and anterior lobe tumors (supporting information (SI) Fig. S1).

$Rb^{+/-}$ mice also spontaneously develop medullary thyroid (C-cell) carcinoma (36). Sixty-three percent of $Rb^{+/-}$ mice (44 of 69 animals) developed medullary C-cell carcinoma by 18 months. Although deletion of *Pttg* from the $Rb^{+/-}$ background abrogated thyroid tumor development and the incidence of tumors dropped from 57 to 7% in $Rb^{+/-}Pttg^{-/-}$ animals (5 of 74 mice), p21 ablation did not restore thyroid tumor development in $Rb^{+/-}Pttg^{-/-}$ mice. Of 56 $Rb^{+/-}Pttg^{-/-}p21^{-/-}$ animals, only 9 (16%) developed medullary thyroid carcinoma (Fig. 1*B*).

p21 Deletion Enhances Pituitary Cell Proliferation. p21 deletion from the $Rb^{+/-}Pttg^{-/-}$ background enhances pituitary Rb levels and Rb phosphorylation at Ser-807/811, a residue preferentially phosphorylated by cyclin D-Cdk2 complexes (38) (Fig. 1*C*), and specifically increases pituitary PCNA levels in triple mutant $Rb^{+/-}Pttg^{-/-}p21^{-/-}$ mice. In contrast, spleen and lung Rb, phosphoRb, and PCNA levels were unchanged (Fig. 1*D*).

p21 Expression in the Pttg-Null Pituitary Gland. Growth inhibiting p21 activity correlates with nuclear localization of the protein (32). Strong intranuclear p21 expression was evident in $Pttg^{-/-}$ anterior and, to a much lesser extent, in intermediate lobe pituitary cells, but not in WT pituitary nuclei (Fig. 2 *A–D*). Importantly, multiple GH-secreting cells exhibit aneuploidy evidenced by the presence of macronuclei, and these readily expressed p21 (Fig. 2*E*). Of 1,078 oddly shaped aneuploid nuclei, 452 cells (≈42%) expressed high to moderate levels of intranuclear p21, whereas intranuclear p21 was not detected in TSH-, FSH-, LH- (data not shown and ref. 23), or ACTH-secreting $Pttg^{-/-}$ cells (Fig. 2*F*). Accordingly, 20% of GH-producing cells, ≈7% of PRL-producing cells, and 1.5% of MSH-producing cells coexpressed p21 (Fig. 2*G*).

Pttg-Null MEFs Exhibit Chromosomal Instability, DNA Damage, and p21-Induced Senescence. We tested mechanisms underlying p21-mediated pituitary senescence, using mouse embryonic fibroblasts

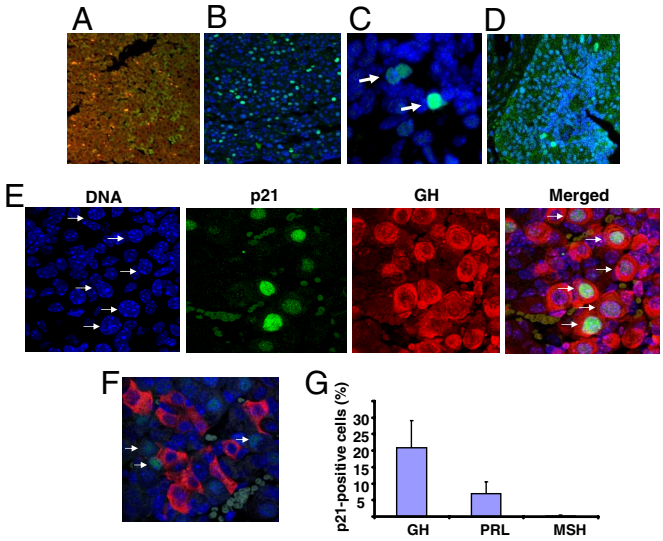

Fig. 2. Pituitary p21 expression. Fluorescence immunohistochemistry of p21 expression in WT (*A*) and $Pttg^{-/-}$ (*B*) pituitary glands. High resolution (×100) confocal image shows intranuclear p21 expression in $Pttg^{-/-}$ pituitary anterior (*C*) and intermediate (*D*) lobes. Paraffin slides were labeled with p21 antibody (green). Here and below slides were counterstained with DNA-specific dye ToPro3 (blue). (*E*) *Pttg* deletion evokes aneuploidy and enhances p21 expression in GH-producing pituitary cells. Confocal image of *Pttg*-null pituitary tissue labeled with p21 antibody (green) and GH- antibody (red). (*F*) The same as *E* but stained with ACTH antibody (red). In *E* and *F*, arrows indicate aneuploid nuclei expressing p21. (*G*) Percent of hormone-producing cells coexpressing intra-nuclear p21 in $Pttg^{-/-}$ glands.

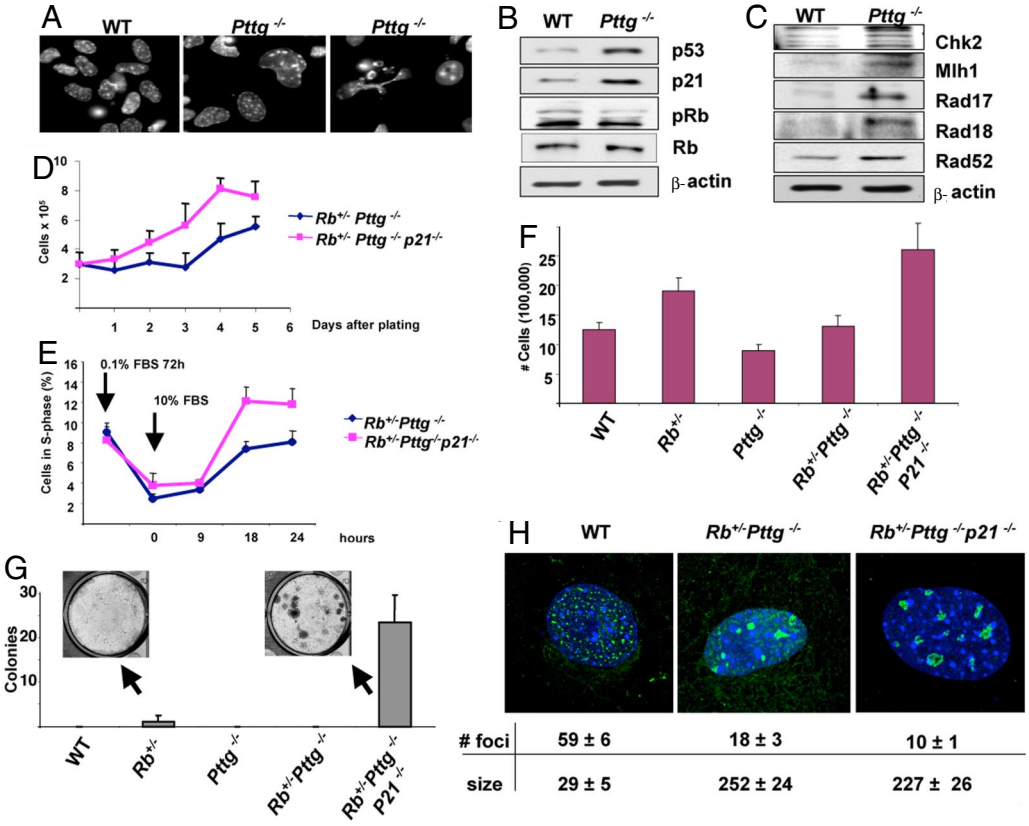

Fig. 3. (*A–C*) *Pttg*$^{-/-}$ MEFs exhibit abnormal nuclear morphology, DNA damage, and activation of the p53/p21 senescence pathway. (*A*) Hematoxylin and eosin staining of 1st passage WT and *Pttg*$^{-/-}$ MEFs. (*B*) Western blot analysis of p53/p21 senescence pathway markers in 10th passage MEFs. (*C*) Western blot analysis of DNA damage/repair proteins. Experiments were repeated 3 times with similar results, and representative blots are shown. (*D–F*) p21 deletion results in increased cell proliferation, accelerated G1-S phase transition, and enhanced transformation. (*D*)12th passage MEFs (3×10^5) were plated in duplicate 10-cm dishes and cells counted daily for 5 days. (*E*) 8th passage MEFs were synchronized in 0.1% FBS for 72 h and then cultured in 10% FBS. At the indicated times, duplicate samples were pulsed with BrdU for 30 min and analyzed by flow cytometry and S-phase cells were identified by staining with BrdU antibodies. (*F*) Proliferation of 12th passage MEFs from 5 experimental genotypes. In *D–F*, the cell number at each time-point represents the average of duplicate plates ± range. These experiments were performed with 2 independent MEF preparations with similar results, and a single experiment is depicted. (*G*) 4th passage MEFs derived from the indicated genotypes were infected with retrovirus encoding Ras+T-Ag and cultured in 6-well plates in triplicate for 21 days, and the number of colonies per well was counted. Numbers of colonies are expressed as mean ± SE of 3 independent MEF preparations. (*H*) γ-foci in 10th passage MEF nuclei. Depicted are single representative high-resolution confocal images of asynchronous and untreated mouse MEFs fixed and labeled with anti γ-H2AX antibody. Foci were counted and measured in 30 to 40 nuclei of each genotype, and focus number and size were determined by using ImageJ Software (http://rsb.info.nih.gov/ij).

(MEFs) prepared from 13.5 embryonic day embryos. We first tested whether DNA damage-associated senescence underlies the restraining effect of *Pttg* deletion on cell growth. First passage *Pttg*$^{-/-}$ but not WT cells exhibit genetic instability, with macronuclei, micronuclei, and bizarre-looking nuclei, all signs of aneuploidy (Fig. 3*A*).

Pttg deletion resulted in enhanced p53 and p21 expression with no changes in total Rb, but decreased Rb phosphorylation, reflective of decreased *Pttg*$^{-/-}$ MEF proliferation capacity (Fig. 3*B*). Levels of Chk2 kinase, a critical checkpoint for cellular responses to DNA damage (39), were increased in *Pttg*-null MEFs, and further evidence for DNA damage was observed by up-regulated DNA repair proteins including Rad17, Rad18, Rad52, and Mlh1 (Fig. 3*B*). Thus, *Pttg*-deficient MEFs exhibit p21-dependent senescent features similar to those observed in *Pttg*-null pituitary tissues (23).

p21 Deletion Increases Growth and Transformation of Rb$^{+/-}$Pttg$^{-/-}$ MEFs. To assess the role of p21 in restraining tumorigenic properties of *Rb*$^{+/-}$*Pttg*$^{-/-}$ cells, we examined effects of p21 disruption on MEF growth and transformation potential. For these experiments MEFs were derived from WT, *Pttg*$^{-/-}$, *Rb*$^{+/-}$, and *Rb*$^{+/-}$*Pttg*$^{-/-}$ genotypes with and without p21 deletion from embryos obtained from intercross-breeding of triply mutant *Rb*$^{+/-}$*Pttg*$^{+/-}$*p21*$^{+/-}$mice. Growth properties of fibroblasts did not differ for up to 12 passages, at which time *Rb*$^{+/-}$*Pttg*$^{-/-}$*p21*$^{-/-}$ MEFs continue to grow while *Rb*$^{+/-}$*Pttg*$^{-/-}$ cell growth was attenuated (Fig. 3 *F* and *D*).

To further determine a rate-limiting p21 function in the transition between quiescence and proliferation, we compared cell cycle entry of *Rb*$^{+/-}$*Pttg*$^{-/-}$ and *Rb*$^{+/-}$*Pttg*$^{-/-}$*p21*$^{-/-}$ quiescent 8th passage MEFs. Cells grown in 0.1% FBS for 72 h were stimulated by the addition of 10% FBS. Flow cytometry of cells pulsed with BrdU demonstrated that by 18 h more triple mutant *Rb*$^{+/-}$*Pttg*$^{-/-}$*p21*$^{-/-}$ MEFs began to enter S-phase, whereas *Rb*$^{+/-}$*Pttg*$^{-/-}$ MEFs lagged. Thus, the absence of p21 accelerates G_0-S transition (Fig. 3*E*).

To test the effects of p21 deletion on cell transformation, MEFs were infected with retrovirus bearing Ras+T-Ag oncogenes. *Rb*$^{+/-}$ MEFs were readily transformed and exhibited enhanced cell growth and small colony formation. However, *Rb*$^{+/-}$*Pttg*$^{-/-}$ MEFs did not form colonies consistent with a protective effect of *Pttg* deletion on cell transformation. Deletion of p21 enhanced proliferation while markedly increasing *Rb*$^{+/-}$*Pttg*$^{-/-}$*p21*$^{-/-}$ MEF colony number and size (Fig. 3*G*).

Accumulation of γ-H2AX foci (γ –foci) in cells is reflective of double-strand DNA breaks (40) and γ-focus size depends on the severity of DNA damage. In agreement with others (41), a multitude of small foci were observed in 8th passage WT MEFs. The number of foci were decreased with markedly increased *Rb*$^{+/-}$*Pttg*$^{-/-}$ and *Rb*$^{+/-}$*Pttg*$^{-/-}$*p21*$^{-/-}$ MEF focus size, confirming accumulation of DNA damage in these cells. Foci number and size were similar in the 2 genotypes (Fig. 3*H*).

p21 Is Induced in GH$_3$ Cells Overexpressing *Pttg*. Aneuploidy induces cellular p21 levels (33, 34), and pituitary aneuploidy caused by *Pttg* deletion resulted in p21 induction mostly in GH-producing pituitary cells (Fig. 2*E*). We therefore tested effects of *Pttg* overexpression and silencing on pituitary cell p21 levels. We transiently transfected rat GH$_3$ pituitary cells with plasmids expressing EGFP-PTTG, and immunocytochemistry showed enhanced p21 protein expression in these cells as compared with vector-transfected cells (Fig. 4*A*). Transfected cells were sorted by flow cytometry, and increased p21 mRNA levels were observed in EGFP-PTTG-positive GH$_3$ (Fig.

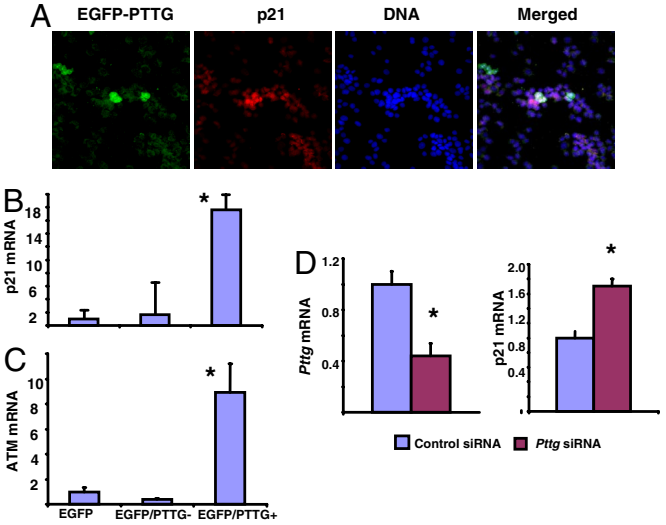

Fig. 4. Both *Pttg* overexpression and silencing induces p21 in GH3 cells. (*A*) Confocal image of double fluorescence immunohistochemistry of rat GH-producing GH_3 cells transfected with EGFP or EGFP-PTTG. Cells were fixed, labeled with EGFP antibody (green), p21 antibody (red). Cells coexpressing EGFP-PTTG and p21 appeared white. (*B* and *C*) *Pttg* overexpression induced p21 mRNA and ATM mRNA levels in GH_3 cells. Cells were transfected with plasmids expressing EGFP or EGFP-PTTG and sorted by flow cytometry, and real-time PCR of p21 mRNA and ATM mRNA was performed. (*D*) *Pttg* silencing by *Pttg* siRNA induces p21 mRNA levels detected by real-time PCR. Values are expressed as mean ± SE of triplicate measurements for each experimental group. All experiments were repeated twice, and representative experiments are shown. *, $P < 0.05$ vs. control.

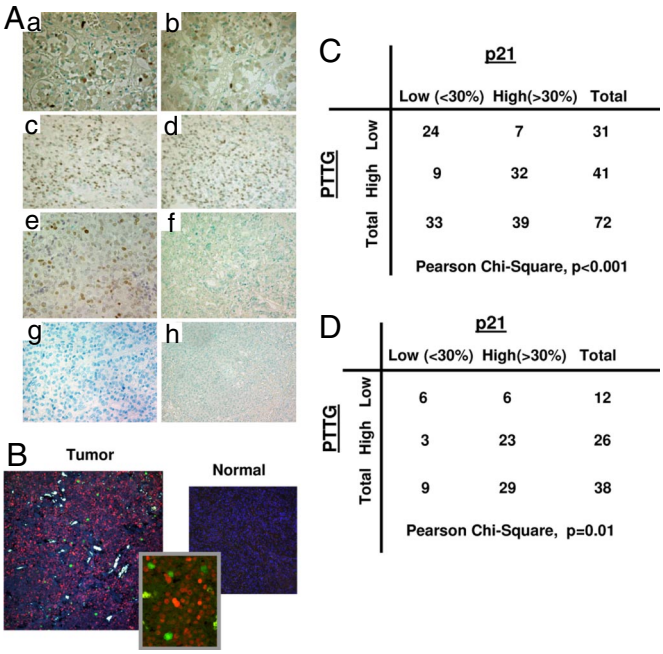

Fig. 5. p21 is induced in human GH-producing pituitary adenomas. (*A*) (*a–e*) Immunohistochemistry of pituitary adenoma sections stained for p21 (brown). (*f*) Normal pituitary. (*g*) Breast carcinoma. (*h*) Pituitary carcinoma. (*B*) Confocal image of fluorescence immunohistochemistry for p21 and PTTG expression. Specimen was labeled with p21 antibody (red) and PTTG antibody (green). (*C* and *D*) Reciprocal p21 and PTTG expression in all pituitary tumor types (*C*) and in GH-secreting adenomas (*D*).

4*B*). To confirm the aneuploid state and DNA damage in these cells, we measured the protein kinase mutated in ataxia telangiectasia (ATM), essential in sensing aneuploidy and DNA damage. ATM and p21 cooperate to impede aneuploidy and maintain chromosomal stability (33). In EGFP-PTTG-positive GH_3 cells, ATM mRNA levels were induced 9-fold as compared with EGFP-only transfected cells (Fig. 4*C*). Silencing of *Pttg* in GH_3 cells with *Pttg* siRNA also enhanced p21 mRNA levels (Fig. 4*D*), thus confirming our in vivo observation showing p21 up-regulation in the *Pttg*-null pituitary gland.

p21 Is Induced in Human Pituitary Tumors. Seventy-nine human pituitary adenomas of various phenotypes, and seven normal pituitaries were analyzed for p21 expression. Normal pituitary tissues exhibit weak p21 immunoreactivity. In nonsecreting pituitary oncocytomas (21 samples) and null cell adenomas (7 samples) p21 immunoreactivity was not detected. In ACTH-producing tumors (8 samples), 3 were weakly positive and 5 were negative for p21. Of 5 gonadotropin-producing tumors, 1 was strongly positive, 1 weakly positive, and 3 were negative. However, in GH-producing adenomas (38 samples), 29 tested strongly positive and 9 weakly positive for p21 immunoreactivity. In contrast, p21 was not detected in 4 human GH-producing pituitary carcinomas, nor in high grade human breast carcinoma specimens (Fig. 5*A*).

Seventy-two pituitary adenomas of various phenotypes were analyzed for both p21 and PTTG expression, and in 56 such tumors strong positive correlation was observed between PTTG and p21 expression ($P < 0.01$) (Fig. 5 *B* and *C*). Twenty-three of twenty-six GH-producing pituitary adenomas (88%) express both high p21 and PTTG levels ($P = 0.025$) (Fig. 5*D*).

GH-secreting pituitary adenomas expressed markers of senescence. Senescence-associated β-galactosidase activity (SA-β-gal) (42) was analyzed in cryosections, and β-galactosidase protein levels in paraffin sections of surgically removed normal pituitary tissue and in GH-secreting adenomas. Levels of β-galactosidase protein correlate positively with its enzymatic activity (43). Unlike nontumorous pituitary tissue, GH-secreting adenomas were strongly positive for this senescence marker. Tumors with high levels of SA-β-gal activity express high p21 (Fig. 6*A*). To confirm that p21 is expressed in senescent cells, we double-labeled GH-secreting adenoma specimens with antibodies to both p21 and β-galactosidase. All 15 p21-expressing adenomas examined were positive for β-galactosidase, and p21 colocalized with β-galactosidase in adenomatose tissue, whereas adjacent normal tissue did not express either of these proteins (Fig. 6*B* and Fig. S2).

Discussion

Both pituitary hypoplasia and pituitary adenomas exhibit features of aneuploidy and p21 induction (23, 44). p21 is up-regulated and contributes to restraining cell cycle progression in the *Pttg*-null hypoplastic pituitary gland (23, 26). Intranuclear p21 is also observed in established GH-secreting pituitary adenomas, and when *Pttg* was either overexpressed or knocked down in GH_3 cells, p21 levels were further enhanced.

Pttg-deficient pituitary glands are hypoplastic, and *Pttg*-null multipotent adult progenitor cells (45) and pituitary cells (23, 26) exhibit decreased cell proliferation and premature senescence associated with p53/p21 activation. Abundant p21 expression underlies decreased pituitary tumor development in *Pttg*-deficient $Rb^{+/-}$ mice (23). Indeed, when p21 was deleted, the number of $Rb^{+/-}Pttg^{-/-}p21^{-/-}$ animals developing pituitary tumors reverted to the high tumor penetrance observed in $Rb^{+/-}$ animals. While *Pttg* ablation protects $Rb^{+/-}$ mice from developing thyroid tumors, p21 deletion, unlike in the pituitary, did not increase the incidence of these tumors in $Rb^{+/-}Pttg^{-/-}$ mice. This finding is not surprising given that thyroid p21 levels are not elevated in *Pttg*-null mice (not shown). Thus, removal of p21 from the $Rb^{+/-}Pttg^{-/-}$ background appears to be a selective determinant of pituitary tumorigenesis.

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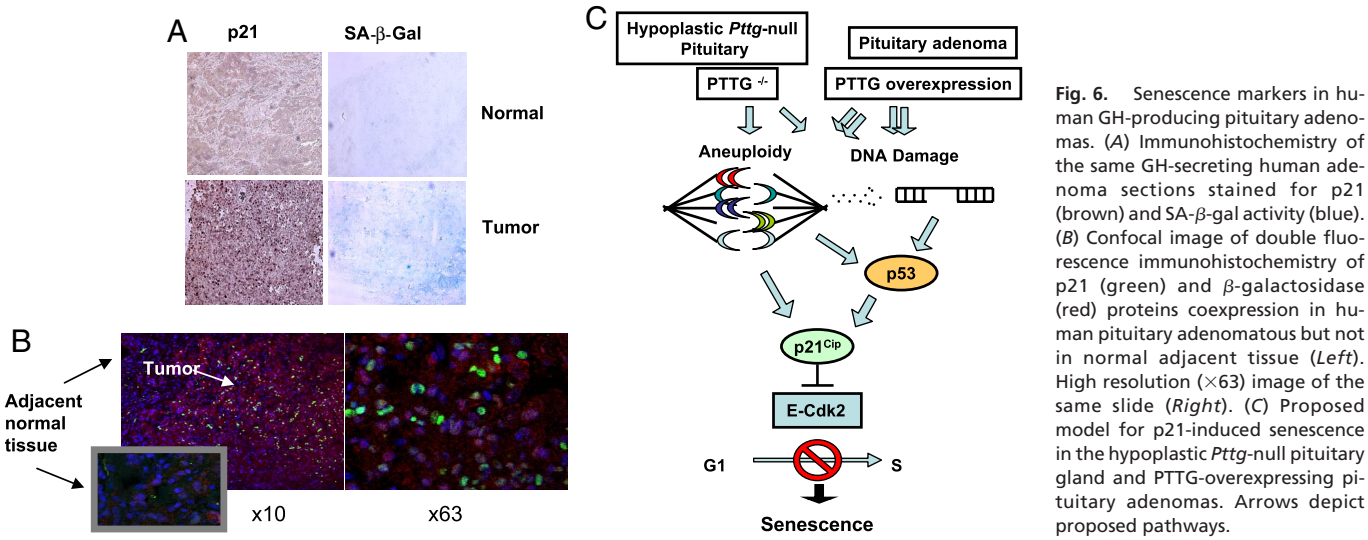

Fig. 6. Senescence markers in human GH-producing pituitary adenomas. (*A*) Immunohistochemistry of the same GH-secreting human adenoma sections stained for p21 (brown) and SA-β-gal activity (blue). (*B*) Confocal image of double fluorescence immunohistochemistry of p21 (green) and β-galactosidase (red) proteins coexpression in human pituitary adenomatous but not in normal adjacent tissue (*Left*). High resolution (×63) image of the same slide (*Right*). (*C*) Proposed model for p21-induced senescence in the hypoplastic *Pttg*-null pituitary gland and PTTG-overexpressing pituitary adenomas. Arrows depict proposed pathways.

p21 deletion enhanced $Rb^{+/-}Pttg^{-/-}$ pituitary phosphoRb and PCNA, both indicative of accelerated pituitary cell replication. Surprisingly, p21 absence did not affect spleen or lung Rb and PCNA protein levels, further highlighting a pituitary-selective role for p21 in these animals. p21 tissue specificity has been reported, and Rb induces the p21 promoter in epithelial cells, but not in fibroblasts (46). Thus, *Pttg* deletion resulted in pituitary, but not thyroid, thymic, or splenic p21 induction (23). Effects of PTTG perturbation on p21 expression and cell cycle arrest may therefore be pituitary-selective.

p21 deletion or mutation is not commonly encountered in human tumors, and mice lacking p21 exhibit moderately increased spontaneous tumor development only after 16 months (47). p21 may exert a cell- or tissue-specific tumor-suppressing function, unmasked under conditions where other genetic alterations or stresses are present. Thus, p21 deficiency accelerated Ras-dependent oncogenesis in MMTV/v-Ha-ras mice (48), and we show that high p21 levels are associated with restrained pituitary tumor formation in mutant $Rb^{+/-}Pttg^{-/-}$ mice.

Surveillance mechanisms protecting genome integrity maintain the status of chromosomal DNA (39). In addition to direct repair of DNA breaks, cells respond to DNA damage by halting cell cycle progression, or undergoing programmed cell death, with both processes requiring p53 and p21 (32). Enhanced apoptosis was not evident in the $Pttg^{-/-}$ pituitary (26) while proteins associated with DNA repair are induced. Chk2 is constitutively elevated in senescent cells and also contributes to cell cycle restraint through p21 up-regulation (39), and Chk2 is activated in primary $Pttg^{-/-}$ MEFs. p21 directly inhibits Cdk2, thereby limiting cell cycle progression, also evident by observed decreased Rb phosphorylation.

MEFs are often reflective of in vivo processes and are useful to study cell tumorigenic potentials. p21 deletion enhanced transformation of $Rb^{+/-}Pttg^{-/-}$ primary fibroblasts. These results are concordant with increased anchorage-independent growth observed in $Rb^{+/-}Pttg^{-/-}p21^{-/-}$ MEFs (23) and indicate that these primary cells exhibit tumorigenic potential.

H2AX is a core histone H2A variant, and H2AX phosphorylation foci (γ-foci) are evident at sites of double-stranded DNA breaks (DSB). Cells normally exhibit abundant small foci, with decreased focus number, but their size increases with DSB accumulation (40, 41). γ-foci were increased in $Rb^{+/-}Pttg^{-/-}$ and $Rb^{+/-}Pttg^{-/-}p21^{-/-}$ MEFs, implying that *Rb* and *Pttg* mutations facilitate DNA damage. Although DSB levels are similar in $Rb^{+/-}Pttg^{-/-}$ and $Rb^{+/-}Pttg^{-/-}p21^{-/-}$ cells, MEFs lacking p21 proliferate faster and are more readily transformed. p21 therefore protects damaged cells from proliferation and transformation.

WT pituitary cells exhibit weak cytoplasmic p21 expression, while *Pttg*-deficient pituitary glands exhibit intranuclear p21 localization, especially in GH- and PRL- and, to a lesser extent, in MSH-producing cells (23). The results showing induced intranuclear p21 in pituitary tumor cells also overexpressing *Pttg* are seemingly inconsistent with the conclusion that *Pttg* absence restrains pituitary tumor development by activating p21 senescent pathways. *Pttg* behaves as an oncogene (19) and is permissive for pituitary tumor formation in mice (14, 26). Both loss of *Pttg* and *Pttg* overexpression result in aneuploidy (here and in refs. 22 and 26), and *Pttg* overexpression triggers genetic instability characterized by DNA breakage (20, 21, 44). Whether aneuploidy is the cause of DNA damage signaling-pathway activation remains unproven, but both processes activate p21 expression (33). p21 is strongly induced in aneuploid cells and functions to preserve chromosomal stability by suppressing cell cycle progression (34). Here we observe high p21 levels in *Pttg*-null anterior pituitary cells and also in GH-secreting GH_3 cells where *Pttg* was either silenced or overexpressed, as well as in human GH-secreting adenomas overexpressing *Pttg*. In the *Pttg*-null hypoplastic pituitary, p21 expression is associated with pituitary cell senescence, and leads to restrained cell growth. Furthermore, in *Pttg*-overexpressing pituitary tumors, p21 acts as a tumor suppressor on the background of genomic instability (33) to restrain proliferation and trigger senescence. Thus, altered PTTG expression results in activation of p53/p21 senescence in both *Pttg*-deficient and *Pttg*-overexpressing pituitary cells (Fig. 6*C*).

Although PTTG and p21 are coexpressed in the same tumor, these proteins do not appear to colocalize in the same cell. PTTG is a cell cycle protein expressed mostly during mitosis and in G2, whereas p21 is overexpressed in G1 to prevent the damaged cell from entering S phase.

We did not observe an inverse correlation between tumor size and levels of p21 expression. Our hypothesis is that PTTG, functioning as an oncogene, acts proximally in tumor formation, and, as a result of aneuploidy and DNA damage, p21 appears later to restrain subsequent tumor growth and malignant transformation. This hypothesis is supported by clinical observations that tumor size and malignancy are not reciprocally correlated. In fact, the overwhelming majority of large invasive pituitary adenomas are not malignant (2).

ACTH-secreting microadenomas are mostly immunonegative for p21, and ≈30% of PRL-secreting microadenomas are positive for p21. FSH-, LH-, null-adenomas, and oncocytomas are mostly

negative for p21 immunoreactivity. However, >70% of GH-secreting adenomas strongly express p21. Although a cell lineage specific effect is evident in pituitary adenomas, p21 expression in the normal non-tumorous pituitary is very low. The results therefore indicate that tumor growth triggers p21 expression predominantly in GH-secreting, and to a lesser extent in PRL-secreting, tumor cells.

Mechanisms underlying specific endocrine cell sensitivity to globally disrupted PTTG levels are unclear. Of note, the evolutionarily conserved GH-IGF1 growth pathway is critical for lifespan regulation, and this axis is attenuated when DNA damage and genomic instability accumulate to induce senescence (49). These observations underscore the sensitivity of GH-secreting cells to PTTG-induced genomic instability, and resultant p21 induction and senescence may constrain tumor growth. This postulate is consistent with experimental and clinical observations that somatotrophs are more sensitive to aging, radiation damage, or compressive damage than other pituitary cell types (50, 51). Our data (not shown) indicate that, unlike rat thyroid FRTL cells, GH_3 cells are extremely sensitive to etoposide-induced DNA damage responding with p21 induction and senescence. Pathways restraining pituitary tumor growth and progression are lineage-specific, as shown in pituitary gonadotrophs, where MEG3 functions as a tumor suppressor gene in a p21-independent manner (7).

The results depict an in vivo model for pituitary senescence, whereby a proto-oncogene switches between oncogenic and tumor-suppressive modes depending on the genetic context. Thus, similar to the KLF4 transcription factor (35), *Pttg* behaves as either a tumor suppressor or oncogene depending on p21 status which couples cell cycle control and maintenance of chromosomal stability (33, 34). High levels of *Pttg* behaving as a securin protein, cause defective metaphase-anaphase progression and chromosomal instability, and promote pituitary tumor formation. Activation of pituitary DNA damage pathways triggers p21, a barrier to tumor growth (52), which in turn may restrain further growth and malignant transformation of pituitary tumors

Methods

Animals. Experiments were approved by the Institutional Animal Care and Use Committee. Pretumorous 2–4 month $Rb^{+/-}Pttg^{-/-}$, $Rb^{+/-}Pttg^{+/+}$, $Rb^{+/+}Pttg^{-/-}$, $Rb^{+/+}Pttg^{+/+}$, and $Rb^{+/-}Pttg^{-/-}p21^{-/-}$ mice and MEFs were used in this study.

Human Tissue Samples. Pituitary tumors were collected at transphenoidal surgery according to an approved Institutional Review Board protocol. Normal anterior pituitary controls were obtained at surgery or from fresh autopsy specimens.

Details for techniques and procedures can be found in *SI Materials and Methods*.

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