Growth hormone is a cellular senescence target in pituitary and nonpituitary cells

Vera Chesnokova, Cuiqi Zhou, Anat Ben-Shlomo, Svetlana Zonis, Yuji Tani, Song-Guang Ren, and Shlomo Melmed[1]

Pituitary Center, Department of Medicine, Cedars-Sinai Medical Center, Los Angeles, CA 90048

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Premature proliferative arrest in benign or early-stage tumors induced by oncoproteins, chromosomal instability, or DNA damage is associated with p53/p21 activation, culminating in either senescence or apoptosis, depending on cell context. Growth hormone (GH) elicits direct peripheral metabolic actions as well as growth effects mediated by insulin-like growth factor 1 (IGF1). Locally produced peripheral tissue GH, in contrast to circulating pituitary-derived endocrine GH, has been proposed to be both proapoptotic and prooncogenic. Pituitary adenomas expressing and secreting GH are invariably benign and exhibit DNA damage and a senescent phenotype. We therefore tested effects of nutlin-induced p53-mediated senescence in rat and human pituitary cells. We show that DNA damage senescence induced by nutlin triggers the p53/p21 senescent pathway, with subsequent marked induction of intracellular pituitary GH in vitro. In contrast, GH is not induced in cells devoid of p53. Furthermore we show that p53 binds specific GH promoter motifs and enhances GH transcription and secretion in senescent pituitary adenoma cells and also in nonpituitary (human breast and colon) cells. In vivo, treatment with nutlin results in up-regulation of both p53 and GH in the pituitary gland, as well as increased GH expression in nonpituitary tissues (lung and liver). Intracrine GH acts in pituitary cells as an apoptosis switch for p53-mediated senescence, likely protecting the pituitary adenoma from progression to malignancy. Unlike in the pituitary, in nonpituitary cells GH exerts antiapoptotic properties. Thus, the results show that GH is a direct p53 transcriptional target and fulfills criteria as a p53 target gene. Induced GH is a readily measurable cell marker for p53-mediated cellular senescence.

pituitary hormone | acromegaly

Premature proliferative arrest or cellular senescence occurs in slowly growing benign or early-stage tumors (including skin nevi or polyps), whereas later-stage or malignant tumors seem to escape senescence (1, 2). Tumor-associated senescence induced by oncoproteins is characterized by an initial burst of cell proliferation followed by replication stress and proliferation arrest and can also be triggered by chromosomal instability and DNA damage (3, 4). Consistent with this notion, cells with a senescent phenotype are usually not apoptotic and remain viable and differentiated (5).

Pituitary adenomas exhibit stable or very indolent growth, often during decades of observation (6). Although they exhibit chromosome instability, aneuploidy, and epigenetic changes (7, 8), these tumors very rarely progress to become true metastatic carcinoma (9–12). Mechanisms underlying the unique indolent growth of these invariably benign adenomas remain obscure, and common cancer-associated oncogene mutations are rarely observed (9). Low pituitary tumor proliferative activity may in fact be reflective of proliferative arrest: we found that both nontumorous and tumorous murine and human pituitary cells are prone to senescence (13, 14). Human growth hormone (GH)-secreting pituitary tumors overexpress pituitary tumor transforming gene (PTTG1), initially permissive for pituitary tumor formation (14–17), subsequently leading to defective DNA replication, aneuploidy (16), and proliferative restraint (14). Several lines of evidence support the observed pituitary adenoma senescent phenotype including increased senescence-associated β-galactosidase (SA-β-galactosidase) activity and chromosome condensation into distinct heterochromatic foci (14, 17). In GH-secreting pituitary adenomas, DNA damage and p53-mediated induction of the cyclin-dependent kinase inhibitor p21 blocks Rb phosphorylation, leading to tumor cell senescence, whereas p21 is lost in very rarely occurring pituitary carcinomas (14). Given these observations in the face of heterogenous pituitary adenoma p53 immunoreactivity (18–20) as well as the absence of pituitary tumor-associated p53 mutations (6, 10, 21), we were prompted to study p53-mediated pituitary tumor signaling pathways.

p53 is activated after DNA damage, followed by a cell-specific phenotypic switch to either apoptosis or survival determined by p53 target genes (e.g., Bax is proapoptotic, whereas p21 promotes senescence) (22, 23). p53 is constitutively transcribed and translated but rapidly targeted for degradation by autoregulatory E3 ubiquitin ligase activity of mouse double minute 2 homolog (MDM2). Additionally, MDM2 also blocks the N-terminal p53 transactivation domain. Disrupted posttranslational p53 and MDM2 interactions stabilize and activate p53 (22, 23). Nutlin-3 (nutlin), a small-molecule MDM2 antagonist, inactivates MDM2 to trigger the p53/p21 DNA damage pathway, leading to cell cycle arrest and proliferative senescence (24); we therefore used nutlin to induce cellular senescence.

GH may act in an autocrine fashion or may elicit paracrine effects on surrounding cells (25–27). Endocrine GH secreted by the pituitary exhibits abundant secretion patterns, which are pulsatile (28–30). Circulating GH targets include liver, skeletal, muscle, and adipose tissues (28, 31). In contrast, intracrine GH is expressed intracellularly at relatively low continuous levels, leading to sustained intracellular GH receptor (GHR) signaling

Significance

Growth hormone (GH) is secreted to the circulation by anterior pituitary somatotroph cells and is also expressed locally in peripheral tissues. This study identifies GH as a target for p53-induced senescence in both pituitary and nonpituitary cells. We show marked induction of intracellular GH expression and secretion in response to activation of the p53 DNA damage pathway. GH is shown to be a direct transcriptional target for p53, and up-regulated GH protects pituitary tumor cells from apoptosis. The results demonstrate a novel mechanism determining the pathophysiology of GH-secreting pituitary tumors and elucidate a basis for the invariably benign nature of these adenomas. Our findings show that induced GH can serve as a readily measurable marker for p53-induced senescence.

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[1]To whom correspondence should be addressed. E-mail: melmed@csmc.edu.

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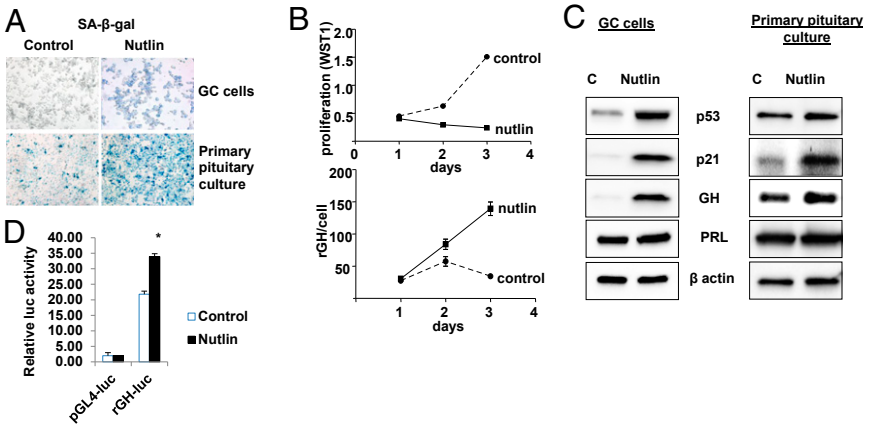

Fig. 1. Senescence induces pituitary cell GH expression. (*A*) SA-β-galactosidase activity in GC and rat primary pituitary cells treated with 7 μM Nutlin 3 (nutlin) or DMSO (control) for 48 h. (*B*) GC cell proliferation rates and GH secretion measured by RIA after 3 μM nutlin for 72 h. GH secretion is normalized to cell number. (*C*) Western blot analysis of GC and primary rat pituitary cells treated with 7 μM nutlin for 48 h. (*D*) GC cells were transfected with pGL4-luc or pGL4-rGH luc reporter plasmids and treated with DMSO or 7 μM nutlin for 48 h. Results were normalized to cotransfected Renilla control reporter vector for transfection efficiency and expressed as mean ± SE calculated from triplicate assays, and experiments repeated three times with similar results. Results of a representative experiment are shown; $*P < 0.05$.

(26). Surprisingly, we observed that senescence-induced p53 was associated with markedly increased intracrine GH synthesis and secretion in rat and human pituitary in vitro and in vivo, and in breast and colon cells. We show here that as a p53 target gene, intracrine GH exhibits tissue-specific effects in the pituitary as an antiapoptotic switch for p53-mediated senescence. The results indicate a unique mechanism for pathogenesis of GH-secreting tumors, whereby tumor cell senescence enables a phenotypic change from unrestrained proliferation to enhanced differentiated GH expression. Discovery of GH as a p53 target gene enables application of a unique and readily measurable cell marker for target-based screening for p53 activity and cellular senescence.

Results

Senescence Induces GH Expression in Pituitary and Nonpituitary Cells. Nutlin treatment of GC cells resulted in strong senescence features, as evidenced by SA-β-galactosidase activity with decreased proliferation as assessed by WST1 assay. Specifically, GH secretion was induced ~fourfold by nutlin ($P < 0.001$) (Fig. 1 *A* and *B*). Western blot analysis showed that in GC and in primary rat pituitary cells 48 h nutlin treatment increased p53 levels with subsequent induction of p21, a transcriptional target for p53, which arrests cell proliferation. Furthermore, in both GC and primary pituitary cells nutlin induced GH peptide expression (Fig. 1*C*). Moreover, when GC cells were transfected with the constructs pGL4-luc or PGL4-luc-rGH, nutlin-induced senescence increased rat GH promoter activity, as detected by luciferase reporter expression (Fig. 1*D*). In contrast, nutlin did not alter prolactin (PRL) expression, indicating specific effects on GH (Fig. 1*C*). Taken together, these results indicate that nutlin-induced pituitary cell senescence is associated with increased p53 and triggers GH synthesis and secretion.

To study p53-regulated intracellular GH, we selected human breast (MCF7) and colon (HCT116) carcinoma cells, as well as colon cells (hPCC) because they have intact p53 status (21, 32, 33), and all are known to express GH (25, 34). hPCC seemed to be most sensitive to nutlin because senescence was observed in these primary cells after 48 h, whereas the cancer cells required longer drug exposure times. MCF7 cells became senescent after 72 h and HCT116 after 96 h of treatment (Fig. 2*A*). All three senescent cell types, MCF7, HCT116, and hPCC, exhibited increased p53 and p21 expression, and the treatment evoked appearance of an additional p53 isoform band (Fig. 2 *B* and *C*). These truncated isoforms [47 kDa (p53β) or 48 kDa (p53γ)] previously observed in human cells complex with p53 to regulate DNA binding, apoptosis, and senescence (35). Although baseline intracrine GH expression was very low in these nonpituitary cells, GH expression increased markedly after nutlin treatment, whereas PRL expression (for MCF7 and HCT116 cells) was unchanged (Fig. 2*B*). GH secretion measured using a biotin-labeled antibody array of culture medium (RayBio Biotin Label-based Human Antibody Array Kit) was shown to be induced 5.7-fold in MCF7 and fourfold in HCT116 cells by nutlin (Fig. 2*D*), further demonstrating that senescence-mediated GH induction is not limited to pituitary cells.

Effects of Caylin and Etoposide on Pituitary Cell GH Expression. We tested effects of caylin, a modified nutlin analog (36), to verify

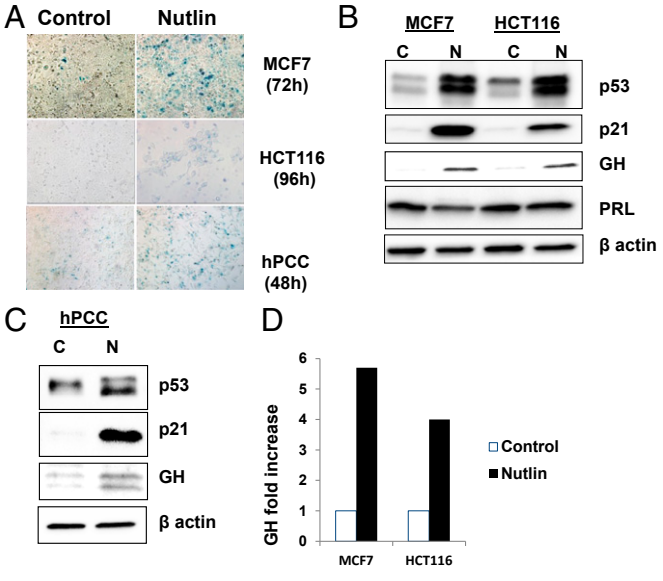

Fig. 2. Senescence induces GH expression in nonpituitary cells. (*A*) SA-β-galactosidase activity in MCF7, HT116, and hPCC cells. MCF7 and HCT116 cells were treated with 7 μM nutlin for 72 and 96 h, respectively; hPCC cells were treated with 3 μM nutlin for 48 h. (*B* and *C*) Western blot analysis of (*B*) MCF7 and HCT116 cells and (*C*) hPCCs treated with nutlin. (*D*) human antibody array showing medium GH derived from MCF7 and HCT116 cells treated with nutlin.

that activation of GH transcription and secretion is indeed senescence-mediated rather than nutlin-specific. Caylin also evoked senescence, p53/p21, induced GH expression (Fig. S1 *A* and *B*), and enhanced medium GH secretion threefold (Fig. S1*C*). Etoposide, a chemotherapeutic agent that activates p53-dependent DNA damage signaling, similarly induced p53 and GH abundance (Fig. S1*D*).

p53 Activates GH Transcription in Senescent GC Cells. To assess the p53 requirement for the observed increased GH expression and secretion, GC cells were infected with lentiviral particles expressing rat MDM2 shRNA. As expected, MDM2 was down-regulated, whereas p53 was induced, likely owing to enhanced stability (22), thus leading to increased p21 expression. In these cells, GH was also markedly induced, confirming the key p53 requirement for this differentiated pituitary cell function (Fig. 3*A*).

Next we tested effects of p53 on endogenous GH expression. GC cells transiently transfected with pCMV-rp53 exhibited marked induction of GH and p21 (Fig. 3*B*). Medium GH also increased from 20 ± 2.8 to 35 ± 3.1 ng/mL (Fig. 3*C*). Transient transfection of HCT116 cells with pcDNA-hp53 resulted in up-regulation of p21 and GH (Fig. 3*D*). To further confirm the p53 dependency of senescence-associated GH induction, we transfected GC cells with specific rat sip53 RNA and cotreated the cells with increasing concentrations of nutlin. GH was dose-dependently induced 24 h after nutlin treatment, whereas the drug failed to induce GH in cells where p53 was suppressed (Fig. 3*E*). We also used $p53^{-/-}$ HCT116 cells (37) (no pituitary p53-null cells are available) and showed that nutlin induced GH in p53-replete cells but not in nonpituitary cells devoid of p53 (Fig. 3*F*).

Scanning the upstream rat GH promoter region (GeneQuest 7.1, DNAStar), we identified two potential p53 binding sites, one located at −1154 to −1144 bp, and the other at −2047 to −2037 bp upstream of the transcription start site (TSS). We therefore tested p53 recruitment to the endogenous GH promoter by chromatin immunoprecipitation assay. GC cells transfected with plasmid expressing rat p53 were fixed, sonicated, and equal amounts of chromatin DNA (normalized inputs) incubated with negative IgG control or polyclonal p53 antibodies. Precipitated chromatin DNA was then PCR amplified with rGH promoter primers. Compared with normalized negative IgG controls, p53 was shown to bind at −1118 to −680 bp upstream of the GH TSS, bound less at −199 to +165 and −2040 to −1823 bp, and did not bind the −3084 to −2934 bp GH promoter region (Fig. 4*A*). Enrichment of specific −1118 to −680 bp GH promoter sequences in the precipitate resulted in p53 association with the GH promoter in vivo. The results are consistent with location of p53 binding sites on the GH promoter, as we had determined by computer analysis.

To test whether endogenous p53 binds the GH promoter, GC cells were treated with 7 μM nutlin for 48 h and processed as above. Similar to results observed for exogenous p53 (Fig. 4*B*), enrichment of specific −1118 to −680 bp GH promoter sequences was enhanced, indicating the association of endogenous p53 with the GH promoter (Fig. 4*B*). In contrast, binding was not seen at the −199 to +165 or −2040 to −1823 bp sequences.

EMSA results further confirmed p53 binding to the rat GH promoter. According to results of the ChIP assay (Fig. 4*C*), two oligonucleotides [−1154/−1144 (probe 1) and −2047/−2037 bp (probe 2)] consistent with two potential p53 binding motifs on the rat GH promoter were synthesized. We examined the binding of p53 in the GC nuclear extract to radiolabeled probes 1 and 2. As shown in Fig. 4*C*, incubation of labeled probe 1 and GC nuclear extract resulted in the appearance of a DNA–protein complex (lane 2); binding was abolished by adding cold competitive probe 1 (lane 4) or cold p53 consensus oligonucleotides (lane 5). We also used labeled p53 consensus oligonucleotides as positive controls, showing a similar sized DNA–protein interaction band (lane 6). Addition of a specific p53 antibody resulted in markedly decreased binding intensity with labeled probe 1 (lane 3), as well as with labeled p53 consensus oligonucleotides (lane 7). No supershifted band was visible after addition of the p53 antibody; however, partial loss of the band complex suggests that p53 specifically binds the −1154/−1144 site of GH promoter. Labeled oligonucleotide 2 exhibited similar specific p53 binding (Fig. S2).

When GC cells were cotransfected with pGL4-rGH luc reporter plasmid and pcDNA3-hp53, luciferase was induced ~twofold (Fig. 4*D*), indicating p53 activation of the GH promoter.

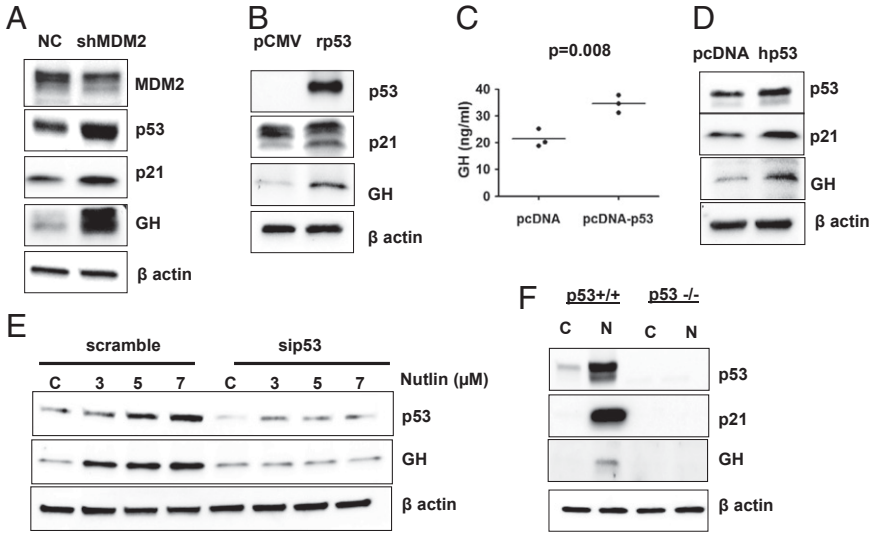

Fig. 3. p53 activates GH transcription in senescent cells. Western blot analysis of (*A*) GC cells infected with lentiviral particles expressing rat MDM2 shRNA. (*B*) GC cells transiently transfected with pCMV or pCMV-rp53 (p53). (*C*) GC cells were plated in triplicate wells, transiently transfected with pCMV or pCMV-rp53 for 48 h, culture medium collected, and GH measured by RIA. (*D* and *E*) Western blot analysis of (*D*) HCT116 cells transiently transfected with pcDNA of pcDNA-hp53 (p53) and (*E*) GC cells transfected with scrambled siRNA or rat sip53 for 24 h, followed by indicated nutlin doses for 48 h. (*F*) Western blot analysis of HCT116 cells with or without p53 expression treated with 7 μM nutlin for 96 h. All experiments were repeated two times, and representative results are shown.

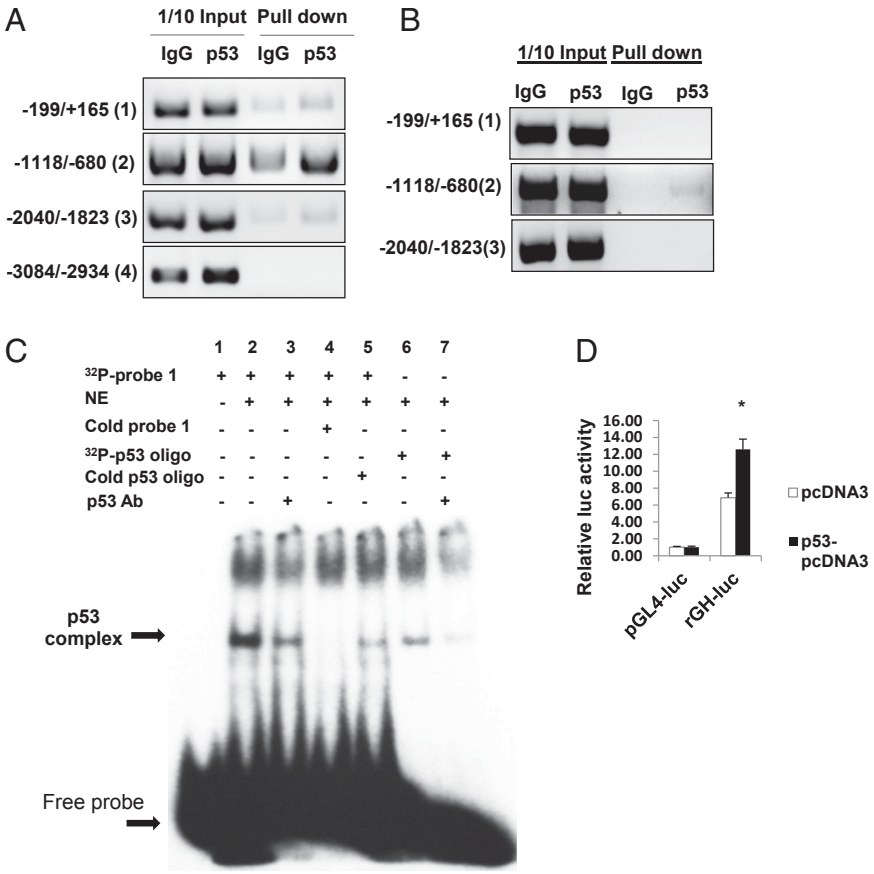

Fig. 4. p53 binds to and activates GH promoter. (*A*) ChIP assay showing specific p53 binding to the rat GH promoter. GC cells were transfected with pCMV-rp53 and chromatin immunoprecipitated with anti-p53 or control IgG antibody as indicated. GH promoter fragments were detected by PCR analysis using four primer sets. Enrichment of specific GH promoter sequences was obtained with primer sets 1, 2, and 3 but not with set 4 or with IgG antibody. The experiment was repeated twice, and results of a representative assay shown. (*B*) ChiP assay showing specific endogenous p53 binding to the rat GH promoter. GC cells were treated with 7 μM nutlin for 48 h and chromatin immunoprecipitated with anti-p53 or control IgG antibody as indicated. GH promoter fragments were detected by PCR analysis using three primer sets. Enrichment of specific GH promoter sequences was obtained with primer set 2 but not with set 1 or 3 or with IgG antibody. The experiment was repeated twice, and results of a representative assay shown. (*C*) Representative EMSA shows binding of GH nuclear extract (NE) p53 with probe 1(−1154/−1144 bp upstream from the GH TSS), corresponding to putative p53 binding sites on GH promoter. Competition assays were performed using a 100-fold excess of cold probe 1 (lane 4) and unlabeled p53 consensus oligos (lane 5). Labeled p53 consensus oligos were used as positive control, showing DNA–protein complex of the same size (lane 6). Addition of anti-p53 antibodies (Ab) markedly decreased p53 complexing with labeled probe 1 (lane 3) and with labeled p53 consensus oligos (lane 7), indicating the specificity of p53 binding to GH promoter. (*D*) GC cells were transfected with pGL4-luc or pGL4-rGH luc reporter plasmids and cotransfected with pcDNA3 expressing vector, or pcDNA3-hp53; cells were harvested after 24 h, assayed for luciferase, and results normalized to cotrasfected Renilla reporter vector for transfection efficiency. Results are expressed as mean ± SE from triplicate assays, and experiments were repeated three times with similar results. Results of a representative experiment are shown. $*P < 0.05$.

GH-Secreting Human Pituitary Adenomas Are Senescent and Respond to Nutlin with Reinforced Senescence and GH Induction. We next tested whether senescence induces GH expression in human pituitary adenomas. In our earlier studies we showed that GH-secreting adenomas exhibit increased SA-β-galactosidase enzymatic activity (14). Because β-galactosidase protein levels correlate with its enzymatic activity (38), we tested both β-galactosidase and GH immunofluorescence in tissue arrays derived from 41 GH-secreting human pituitary adenomas. In 3 of 41 tumors, where GH immunoreactivity was undetectable (i.e., patients had elevated circulating GH levels and acromegaly), β-galactosidase immunostaining was similarly undetectable; 10 tumors expressed medium (<30% cells per slide) GH and β-galactosidase abundance, whereas the remaining tumors expressed both GH and β-galactosidase abundantly. Thus, levels of β-galactosidase and GH correlated (Fig. 5 *A*–*C*). We next treated primary cultures derived from three GH-secreting pituitary adenomas with 5 μM nutlin for 48 h. In all three separate tumor cultures, nutlin treatment further induced GH expression and secretion with induced p53 and p21. PRL secretion was not altered in two pituitary cultures and were undetectable in the third culture (Fig. 5 *D* and *E*). These ex vivo results using human tumor tissue support our in vitro observations and underscore the close relationship between pituitary tumor senescence and GH abundance.

Nutlin Induces GH Expression in Vivo. Effects of nutlin on GH expression in vivo were analyzed in C57BL/6 mice treated with 40 mg/kg body weight nutlin every 2 d, for a total of six injections. Control animals were treated with DMSO vehicle. GH mRNA levels, analyzed by real-time PCR, were shown to be induced in pituitary, lung, and liver tissues after nutlin treatment (Fig. 6*A*). Because GH expression in nonpituitary tissues is low, only protein derived from pituitary tissue was analyzed by Western blot. Nutlin treatment resulted in marked up-regulation of pituitary p53, indicative of DNA damage senescence pathway activation, and increased GH expression. Pituitary PRL abundance was not altered (Fig. 6*B*). Thus, nutlin triggers pituitary and extrapituitary p53 and GH accumulation in vivo.

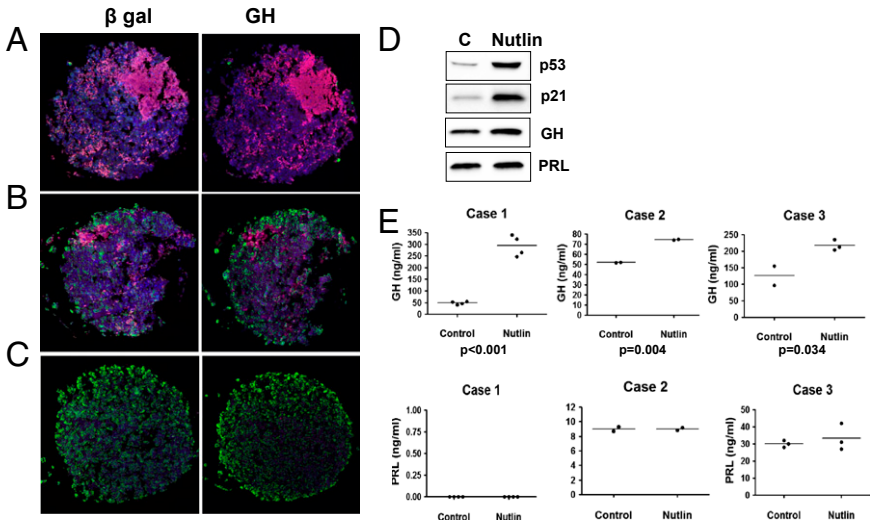

Fig. 5. GH–secreting human pituitary adenomas are senescent, and senescence further induces GH expression and secretion. (*A*–*C*) Confocal image of three different human GH-secreting adenoma specimens labeled with GH or β-galactosidase (both green). Nuclei are counterstained with DNA-specific dye DAPI (blue). Blood appears pink. Both proteins are similarly distributed in pituitary adenoma. Primary GH cell adenoma cells were plated in duplicate wells, treated with 5 μM nutlin for 48 h. (*D*) Cells were collected and Western blot analysis performed. The experiments were performed in cultured cells derived from three GH-secreting adenomas with similar results, and the result of a representative experiment is shown. PRL expression served as loading control. (*E*) Culture medium from three cases of GH-secreting adenoma cells was collected, and GH and PRL measured by ELISA.

Senescence-Induced GH Protects Pituitary Cells from Apoptosis but Enables Apoptosis in Nonpituitary Cells. Because senescence is usually associated with apoptotic block (2), we tested the role of intracellular GH in apoptosis. GC and HCT116 cells were transfected with rGH-IRES2-ZSGreen1 and hGH-IRES2-ZSGreen1 plasmids, respectively, and cleaved caspase-3 levels were determined 48 h after transfection. In GC cells, GH overexpression resulted in a decrease in the levels of cleaved caspase-3, whereas in HCT116 cells high GH triggered cleaved caspase-3 induction, indicative of increased apoptosis (Fig. 7*A*). Thus, GH overexpression exerts opposing effects on apoptosis in pituitary and nonpituitary cells.

Next, GC cells were transfected with scrambled RNA or specific rat GH siRNA and 12 h later subsequently treated with nutlin for up to 12 h. In nutlin-treated GC cells suppressed GH resulted in activation of apoptotic markers including cleaved caspase-3, cleaved poly ADP ribose polymerase (PARP) (39, 40), and up-regulated antiapoptotic protein Bcl-W (Fig. 7*B*). In contrast, when GC cells were transfected with rGH-IRES2-ZSGreen1 for 12 h and subsequently treated with nutlin for 3–12 h, these markers were attenuated compared with control pIRES2-ZSGreen1–transfected cells. Pituitary GH overexpression thus enables a senescence-mediated antiapoptotic function with decreased levels of cleaved caspase-3, cleaved PARP, and induced Bcl-W expression (Fig. 7*C*). Thus, p53-induced GH seems to protect pituitary cells from apoptosis.

Untransfected nonpituitary HCT116 cells exhibit induced GH 96 h after treatment with 10 μM nutlin, but when transfected with human siGH RNA, these cells become more sensitive to nutlin. Therefore, HCT116 cells were treated with a lower dose (5 μM) of nutlin for 48 h to induce GH and then cotreated with scrambled or siGH RNA for a further 24 and 48 h, still in the presence of nutlin. In contrast to pituitary cells, in HCT116 cells undergoing senescence, suppression of endogenous GH resulted in a concomitant attenuation of apoptotic markers, including cleaved caspase-3 and cleaved PARP (Fig. 7*D*).

Of note, unlike untransfected HCT116 cells, HCT116 transfectants overexpressing hGH and treated with 5 μM nutlin exhibited prominent apoptosis, suggesting that high intracellular GH reinforces apoptosis in these nonpituitary cells. Thus, GH effects on apoptosis seem to be tissue specific.

Discussion

We had shown previously that GH-secreting pituitary adenomas exhibit PTTG-provoked aneuploidy and DNA damage and abundantly express p21 (14). We therefore hypothesized that p21-

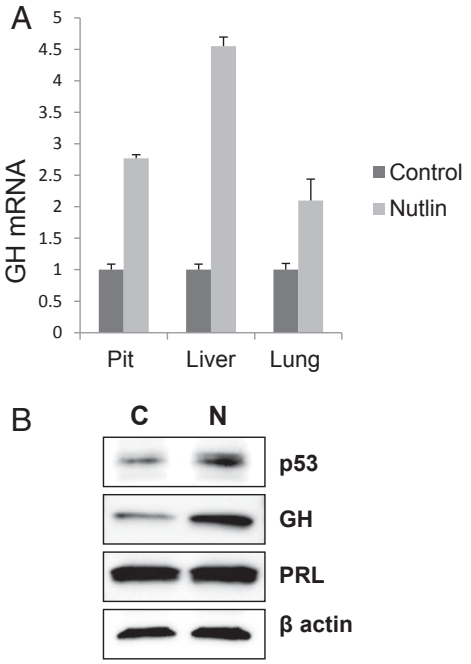

Fig. 6. Senescence induces GH expression in vivo. Mice were injected with 40 mg/kg body weight nutlin (N) or DMSO (C) i.p. every 2 d (six doses). (*A*) Fold induction of tissue GH expression detected by real-time PCR (mean ± SD, n = 3). Respective tissue GH expression in vehicle-treated mice was normalized to 1. Pit, pituitary. (*B*) Western blot analysis of p53, GH. and PRL expression in two pooled pituitary gland extracts.

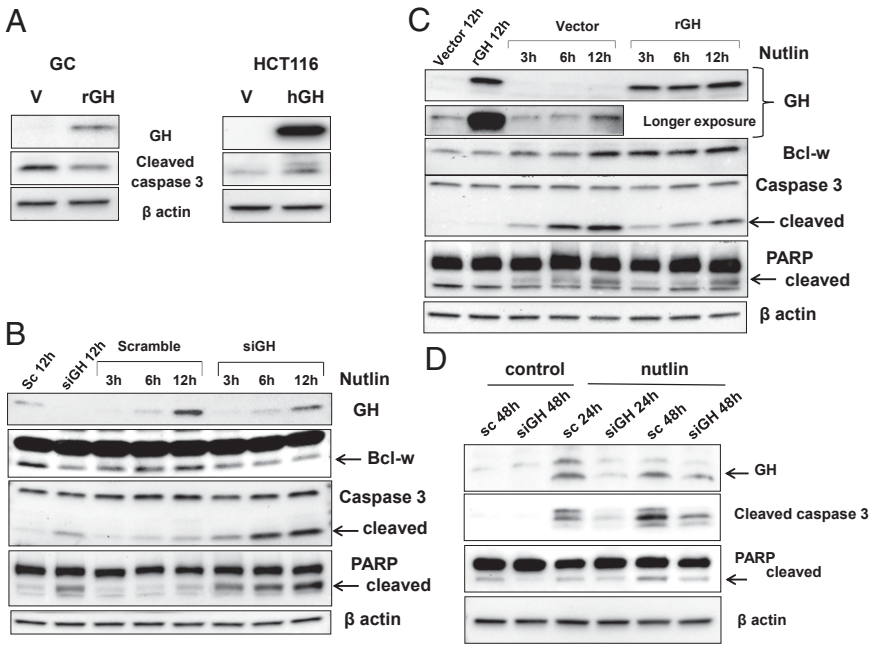

Fig. 7. Induced GH protects GC cells from apoptosis and promotes apoptosis in HCT116 cells. Western blot analysis for GH and apoptotic markers. (*A*) GC and HCT116 cells were transfected with pIRES2-ZsGreen1 (V), rGH-IRES2-ZSGreen1 (rGH), or hGH-IRES2-ZSGreen1(hGH) and collected 48 h later. (*B*) GC cells were transfected with 5 μM rat siGH or scramble siRNA for 12 h and then treated with 7 μM nutlin for the indicated times. (*C*) GC cells were transfected with pIRES2-ZsGreen1 or rGH-pIRES2-ZsGreen1 plasmids for 12 h and treated with 7 μM nutlin for the indicated times. (*D*) HCT116 cells were treated with 5 μM nutlin for 48 h, then were cotreated with scramble (sc) or siGH RNA for another 24 or 48 h. Experiments were repeated twice, and representative blots are shown.

induced proliferative cell cycle arrest and senescence underlie the benign nature of these commonly encountered endocrine neoplasms. Here we show that senescence stimulates GH expression in vitro and in vivo, and in both normal and tumorous pituitary cells senescence-induced p53 mediates GH transcription. Furthermore, GH is also induced in senescent nonpituitary human cancer cells and in primary human colon cells. Up-regulated intracellular GH protects senescent pituitary tumor cells from apoptosis and promotes apoptosis in nonpituitary cells.

Importantly, GH induction is shown to be p53 dependent: p53 suppression abrogates nutlin-stumulated GH induction in both pituitary and nonpituitary cells. In contrast, p53 accumulation after MDM2 shRNA treatment results in increased intracellular GH. Forced p53 overexpression in pituitary GC cells and in nonpituitary HCT116 cells enhances GH abundance, and GH secretion was also increased. Moreover, p53 binds to the rat GH promoter inducing GH transcription. Relatively little information is available on regulation of the distal rat GH promoter (9, 41–44), although a remote locus control region located 14.5 Kb 5′ to the human GH promoter determines human GH gene activation (45). Absence of this presumptive distant locus in our rat constructs might explain the relatively moderate GH promoter activity induction by p53 we observed.

The concordant link between the senescence phenotype and GH expression is apparent in GH-secreting pituitary tumors, because abundance and distribution of β-galactosidase expression correlate with GH in these adenomas. Although pituitary GH-secreting adenoma cells usually express high GH levels, treatment with nutlin further enhances p53 and p21 and further induces GH expression and secretion. Uniquely, p53/p21 pituitary senescence is lineage-specific, because it is observed only in GH-secreting tumors but not in other pituitary tumor types, which preferentially exhibit p15/p16-mediated senescence (46). This proposed mechanism accounts for the known clinical phenotypes for acromegaly (GH-secreting pituitary tumor); that is, densely granulated GH-secreting adenomas that secrete higher GH levels are smaller and less invasive, whereas sparsely granulated adenomas secreting less GH are larger and grow more aggressively, presumably not having acquired a senescent phenotype (47–51) or having evaded senescence. The few available pituitary GH-secreting carcinomas examined were in fact devoid of p21 immunoreactivity (14). These observations and experiments showing induced GH in the tissues of nutlin-treated mice underscore a strong association between proliferative senescence and differentiated GH abundance in vivo.

GH secreted by the pituitary gland acts as an endocrine regulator in multiple tissues (52–55) and elicits peripheral IGF1 by signaling through the membrane-associated GHR linked to Jak2 (56–60). It was shown that GH is also expressed locally in nonpituitary cells (including colon, prostate, breast, and brain), where it feeds back to membrane-associated GHR to signal in an autocrine fashion via Jak2 (61, 62). In addition, intracrine GH is synthesized and acts within cells via intracellular GHRs to directly regulate nuclear genes, and ∼50% of cellular Jak2 is localized within the nucleus (63). GH can also act within the cell to promote a transformed phenotype. Because of the sustained nature of intracrine GH signaling, it may locally promote cell proliferation and transformation (25). It is not known whether intracellular GH also triggers local IGF1 production or whether this action differs in pituitary and nonpituitary cells.

Endocrine GH functions are clearly distinct from those of autocrine/intracrine GH. Intracrine (i.e., intracellular-derived) GH signaling within the same cell acts to elicit specific cell responses. Thus, distinctive cell growth gene expression profiles for exogenous vs. intracellular GH are remarkably different (64). Differences in cell cycle targeting by circulating vs. intracellular GH are exemplified by observations that patients with long-standing uncontrolled acromegaly develop benign colon polyps, or prostate hypertrophy, but do not exhibit an apparent increased incidence of colon, prostate, or breast cancer (65). In contrast, enhanced autocrine/intracrine GH induces oncogenic transformation of human mammary epithelial cells by enabling

epithelial mesenchymal transition (66–68). Local GH is also expressed in lung, gastric, and prostate malignancies (27). Because senescent cells not only express but also secrete GH, this hormone is here defined as a component of the senescence-associated secretory phenotype (SASP) (5). Some soluble SASP factors, when chronically released, may also promote deleterious phenotypes in adjacent premalignant cells (69, 70).

Distinctive features of pituitary adenomas are likely determined by the unique properties of highly differentiated pituitary cells. These cells proliferate slowly, and microadenomas do not invariably progress to macroadenomas; macroadenomas are stable or exhibit very slow growth. Oncogene mutations commonly encountered in nonendocrine neoplasms (e.g., ras and p53) are not generally present in pituitary adenomas (9). Intriguingly, despite sustained high levels of autocrine/intracrine GH, normal pituitary somatotrophs and human GH-secreting adenoma cells rarely, if ever, undergo GH-induced transformation observed in mammary epithelial cells (65). It is thus apparent that in the pituitary, mechanisms buffering the master endocrine gland from oncogenic effects of GH are in place. These unique protective pathways allowing highly differentiated pituitary cells to defuse the pro-oncogenic properties of intracrine GH likely include intact p53/p21, as also exemplified by our earlier observations (13, 14).

Because our results show that up-regulated GH protects senescent pituitary cells from apoptosis, we hypothesize that GH switches pituitary cells from p53-dependent apoptosis to enable cell-cycle arrest. Somatotroph cells require preservation to maintain vital hormone function, especially during the developmental surge of GH, or with development of somatotroph tumors. This notion is consistent with findings that the pituitary gland is prone to senescence, and senescent pituitary cells express high levels of GH, whereas baseline apoptosis levels are low in normal pituitary and in human pituitary adenomas, including GH-secreting tumors (71, 72). In contrast, proapoptotic actions of senescence-induced GH in nonpituitary cells are consistent with a recent report showing proapoptotic effects of autocrine but not endocrine GH in prostate cancer LNCaP cells (73). Proapoptotic effects of p53-induced GH likely reflect senescence evasion in nonpituitary tumor cells, in agreement with proposed oncogenic GH properties observed in breast cancers (27). Opposing effects of intracellular GH on pituitary and nonpituitary cell viability could be attributed to the presence of tissue-specific dimeric GH isoforms, which could act as partial agonists or antagonists of monomeric classic 22-kDa GH in nonpituitary human cells and tissues, as recently proposed (74).

Cell-specific mechanisms underlying effects of induced endogenous GH may determine distinctive attributes of highly differentiated and slowly renewing (e.g., pituitary) vs. rapidly proliferating (e.g., colon) tissues and their respective responses to DNA damage. Although apoptosis permanently eliminates cells, proliferation arrest allows for DNA damage repair. In the pituitary, a gland critical for homeostatic function but exhibiting low regenerative capacity, senescence seems to be a more appropriate mechanism for tumor suppression than apoptosis. Conversely, in tissues with strong regenerative capacity, apoptosis would be the preferred antitumor mechanism preventing clonal outgrowth of mutated cells (75). Nonpituitary cells undergoing DNA damage with induced GH may release a senescence secretome, including GH, affecting surrounding cells.

Our results showing high intracellular GH levels with DNA damage-induced senescence suggest that abundant local intracrine GH is sustained, and in nonpituitary cells may play a protumorigenic role, consistent with observed autocrine GH actions in breast tissues (27, 67) and with protumorigenic properties of the senescence-associated secretome (76).

Materials and Methods

Human Tissue. Cedars Sinai Institutional Review Board protocol was approved, and informed consent for the use of tissue for research was obtained from each patient before surgery. Pituitary tumors were freshly collected at transphenoidal surgery according to the protocol. Samples were harvested and cultured, or formalin-fixed and paraffin-embedded for immunohistochemistry. Diagnosis of individual tumors was established on the basis of clinical features, histology, and pituitary hormone immunohistochemistry. Human GH–cell adenoma arrays were generated at Yale Cancer Center/Pathology Tissue Microarray Facility (http://medicine.yale.edu/pathology/researchprograms/tissueservices) with samples obtained from verified pituitary tumor specimens.

Cells. Rat somatotroph GC, human breast carcinoma MCF7, and human colon carcinoma HCT116 cells were obtained from American Type Culture Collection, and $p53^{+/+}$ and $p53^{-/-}$ HCT116 cells were generous gifts from Bert Vogelstein (Johns Hopkins University, Baltimore, MD). GC cells were cultured in DMEM with sodium pyruvate and 2 mM glutamine (Invitrogen), and 10% (vol/vol) FBS (Gemini Bioproducts). HCT116 cells were cultured in McCoy's 5A medium (Invitrogen) and 10% (vol/vol) FBS. MCF7 cells were cultured in high glucose DMEM (Invitrogen), supplemented with 0.01 mg/mL human insulin (SAFCBiosciences) and 10% (vol/vol) FBS.

Primary pituitary cultures were derived from five freshly isolated rat pituitary glands, or from human GH-secreting adenoma specimens. Tissue was chopped with a sterile scalpel into ~1- to 2-mm fragments, rinsed, and digested with DMEM containing 0.3% BSA, 0.35% collagenase, and 0.15% hyaluronidase (all from Sigma-Aldrich) at 37 °C for 30 min. The mixture was centrifuged at 350 × *g* for 5 min at 4 °C, and cell pellets resuspended and cultured in NeuroCult NS-A basal Medium supplemented with NeuroCult NS-A Proliferation Supplement (Stem Cell Technologies) in 48-well plates. Plates were pretreated with ECL Matrix (Millipore). Cells were treated as described below. Forty-eight hours after plating cells were harvested for protein isolation.

hPCCs were purchased (Applied Biological Materials) and cultured in plates pretreated with Applied Cell Extracellular Matrix in PriGrow III Media (both from Applied Biological Materials) supplemented with 5% FBS and prenicillin/streptomycin. Cells were treated at the sixth passage.

In Vitro Treatments. Nutlin 3 (Sigma-Aldrich) and Caylin 1 (Cayman Chemical Company) were prepared as 20 mM DMSO stock solutions and cells treated with indicated amounts of either drug for the indicated times (48–96 h). Etoposide (Sigma-Aldrich) was prepared as a 10-mM DMSO stock solution, cells treated with 1 μM etoposide overnight, media changed, and cells collected 24 h later. Control cells were treated with appropriate concentrations of DMSO vehicle.

In Vivo Treatments. Nutlin-3 (Calbiochem) was dissolved in DMSO, and three C57BL/6 mice were injected with nutlin i.p. at a dose of 40 mg/kg body weight in 100 μL DMSO every 2 d for a total of six injections, as previously described (77). Three control animals received DMSO. Mice were killed, and RNA and protein isolated from the pituitary, lung, and liver. Because this treatment resulted in visible colon changes, colon tissue was not processed for assays.

Constructs and Transfections. Lentiviral particles expressing rat MDM2 shRNA or nontargeted shRNA control (GFP Control Lentiviral Particles) (both from Santa Cruz Biotechnology) were received as stock solutions (10^6 IU/200 μM in DMEM). Cells were infected with 5 multiplicity of infection, and 5 μg/mL polybrene added to the cultures. After overnight culturing medium was changed, cells were split 48 h later, and grown thereafter in 4 μg/mL puromycin for selection of infected cells. At the third passage, ~50% MDM silencing was achieved, and cells were collected and tested. Human pcDNA3.1-p53 were a generous gift from P. Koeffler (Cedars-Sinai Medical Center, West Hollywood, CA). Rat pCMV-p53 was purchased from OriGene. Rat siGH and human siGH1 RNAs, and rat sip53 RNA were purchased from Sigma-Aldrich. The rat GH promoter fragment (–4192/+167) was synthesized (Genewiz) and subcloned to pGL4.10 luciferase reporter vector (Promega Biosciences). Rat GH and human GH1 were synthesized (Genewiz) and subcloned to pIRES2-ZsGreen1 vector (Clontech). All plasmids were verified by DNA sequencing. Cells were plated 1 d before transfection or treatment. Transient transfection was carried out using 5 nM siRNA or 1 μg/mL plasmid DNA and 2.5 μL/mL Lipofectamine 2000 according to the manufacturer protocol (Invitrogen).

Protein Analysis. Pituitary tissues or cells were lysed in RIPA buffer (Sigma-Aldrich) with 10 μM protein inhibitors (Sigma-Aldrich) for Western blot

analysis, proteins separated by SDS/PAGE, electroblotted onto Trans-Blot Turbo Transfer Pack 0.2-μm PVDF membrane (BioRad), and incubated overnight with antibodies, followed by corresponding secondary antibodies (Sigma-Aldrich). Antibodies used were: goat polyclonal p53 (R&D Systems) and mouse monoclonal p21 (BD Pharmigen). Mouse monoclonal β-actin was purchased from Millipore. Goat polyclonal rat PRL, rabbit polyclonal rat GH, and human GH1 antibodies were obtained from A. Parlow (National Hormone and Peptide Program, Harbor-UCLA Medical Center, Torrance, CA), or human polyclonal GH1 antibodies from R&D Systems were used.

For immunofluorescence analysis of human tissue we used antibodies to GH1 (obtained as above) and rabbit polyclonal β-galactosidase (Abcam), followed by secondary antibodies conjugated with goat anti-rabbit Alexa-Fluor 488 (Invitrogen). Antigen retrieval was performed in 10 mM sodium citrate, and control reactions were devoid of primary antibodies or stained with blocking antibodies. Samples were imaged with a Leica TCS/SP spectral confocal scanner (Leica Microsystems) in dual emission mode to distinguish autofluorescence from specific staining.

Luciferase Assays. GC cells were transfected with 200 ng pGL4.10 or pGL4-rGH reporter plasmid and cotransfected with 800 ng pcDNA3 or pcDNA3-hp53 in 12-well plates, or treated with nutlin. pGL4.74 hRluc/Tk vector encoding Renilla luciferase was used as an internal control (5 ng per well) to assess transfection efficiency. After 24 h, whole-cell lysates were collected for reporter detection by the dual-luciferase reporter system (Promega) according to the protocol and measured using an Orion Microplate Luminometer (Berthold Detection System). Transfections were performed in triplicate and repeated three times to assure reproducibility. All luciferase reads were normalized to corresponding Renilla value. Relative luciferase activity was calculated using the following formula: relative luciferase levels = Luc-promoter/Luc-pGL4.10.

Chromatin Immunoprecipitation Assay. Using a ChIP kit (Active Motif), approximately 10^7 GC cells treated with nutlin transfected with appropriate plasmid (pCMV6-rp53 or pcDNA3-mC/EBPδ) were cross-linked and lysed. Chromatin was sonicated to ~600- to 1,000-bp length fragments with four rounds of 10-s pulses using 25% power. The sheared chromatin DNA mixture (normalized inputs) was incubated with 2 μg p53 antibody (R&D Systems) overnight at 4 °C. Negative control IgG (2 μg) and positive control RNA pol II (2 μg) antibody plus bridging antibody (ChIP-IT control kit; Active Motif) were added to each ChIP reaction. The endpoint PCR amplification used precipitated immunocomplexes as template and rat GH promoter primers. Four primer pairs were designed in different GH promoter regions, with primer set 1 the closest to ATG and primer set 4 the furthest. Primer set 1 (−199/+165) forward, 5′-GGC GGT GGA AAG GT-3′; reverse, 5′-GGC GGA AGT TGG GAT-3′; primer set 2 (−1118/−680) forward, 5′-CAT CAG TTT ATG CTG CTA TG-3′; reverse, 5′-CTC CTC CTC CTG CTC TT-3′; primer set 3 (−2040/−1823) forward, 5′-CCA CGC CCT GAC TTA C-3′; reverse, 5′-CTT AGA GGC TGC CAA CT-3′; primer set 4 (−3084/−2934) forward, 5′-CAG CCT GCT CTA CAA AGT GAG-3′; reverse, 5′-ACA CCA GTC TCA GCC AGT CT-3′. The positive control was amplified with β-actin primers. PCR products were resolved in 2% agarose gel and visualized by GelRed (Biotium).

Electrophoresis Mobility Shift Assay. Complementary oligonucleotides corresponding to the two different p53 binding motifs in the rat GH promoter [−1097/−1067 (probe 1) and −1989/−1960 (probe 2)] were annealed and labeled with ^{32}P using T4 polynucleotide kinase (Promega). Ten micrograms of GC nuclear extracts were incubated with oligonucleotides in 20 μL of EMSA reaction buffer containing 0.5 μg of poly(dI-dC), 12 mM Hepes (pH 7.9), 60 mM KCl, 0.2 mM EDTA, 2 mM dithiothretitol, and 12% glycerol for 15 min. Competitive assays with unlabeled (cold) probes or unlabeled p53 consensus oligos (Santa Cruz Biotechnology) were performed at 100-fold excess to verify the binding specificity. To perform the supershift assay, nuclear extracts in EMSA reaction buffer were incubated with anti-p53 antibodies (EMD Millipore) for 15 min, at which time probes were then added. The DNA–protein complex was analyzed by 4% DNA retardation gel, and exposure 12 h for imaging.

Oligonucleotides were synthesized as follows: probe 1: 5′-GAGGAACAA-GTCTTCCTTTTCCCAAGACACG-3′ and the complement. Probe 2: 5′-TGAAGC-AAGCTCCTTAAGGACCAAGCCATC-3′ and the complement.

Real-Time PCR. Total mRNA derived from mouse tissues with TRIzol reagents (Invitrogen) was subjected to quantitative PCR. Pituitary, lung, and liver tissues from nutlin-treated or control mice were pooled. TaqMan Gene Expression Assays for murine GH were purchased from Applied Biosystems. Amplicons were detected using the relevant probes tagged with MGB quencher and FAM dye. TaqMan murine actin control Expression Assays with probe tagged with MGB and VIC (Applied Biosystems) were used as reference genes. As per the manufacturer's guidelines, data were expressed as Ct values and used to determine ΔCt values [ΔCt = Ct of the target gene minus Ct of the housekeeping actin gene]. A comparative threshold cycle (C_T) method was used for relative gene expression quantification.

SA-β-Galactosidase Activity. SA-β-galactosidase enzymatic activity was assayed in vitro using a β-galactosidase staining kit (Senescence Cell Staining Kit; Sigma-Aldrich). Briefly, 10,000 cells were plated in 12-well plates, treated for the indicated times, incubated at 37 °C overnight, and washed with PBS (pH 6.0), fixed, and stained with 5-bromo-4- chloro-3-indolyl-h-D-galactopyranoside (X-Gal) overnight at 37 °C. Only senescent cells stain at pH 6.0.

Cell Proliferation Assay. Ten thousand cells per well were plated 1 d before experiments. Cell number was assessed using the WST-1 Cell Proliferation Assay Kit, which marks metabolically viable cells (Clontech), and read at 450-nm absorbance in a Victor 3 1420_015 spectrophotometer (Perkin-Elmer).

Hormone Assays. Cells were plated in triplicate wells, treated with Nutlin 3 or Caylin 1 for 48 h, medium changed for serum-free overnight, collected, centrifuged to remove debris, frozen for later RIA measurements, and cells collected and counted. RIA for rat GH was performed using reagents provided by A. Parlow (National Hormone and Peptide Program, Harbor-UCLA Medical Center, Torrance, CA). Rat GH (5 μg) was iodinated with I-125 (500 μCi; Perkin-Elmer Life & Analytical Sciences) mixed with 0.1 mg Iodo-Gen (Pierce) using 10-mL columns prepared by G-75 Sephadex (Sigma Chemical). Human GH1 was measured by GH Elisa (R&D Systems), and human PRL by immunoradiometric assay (Siemens). The amount of secreted hormone was normalized to cell number or to levels of PRL secretion (for human adenomas).

Antibody Array. MCF7 and HCT116 cells were treated with 7 μM Nutlin 3 for 72 and 96 h, respectively, and medium was collected, filtered, and assessed for secreted GH1 using RayBio Labeled Based Human Antibody Array 1 (RayBiotech, #AAH-BLG-1) according to the manufacturer's instructions. Briefly, samples were dialyzed overnight, biotin-labeled, and glass chip arrays blocked with blocking buffer, biotin-labeled sample added onto preprinted glass chip capture antibodies, and incubated to allow for target protein interactions. Streptavidin-conjugated fluorescent dye (Cy3 equivalent) was then applied to the array, the glass chip dried, and samples shipped to RayBiotech, where laser fluorescence scanning was used to visualize signals, and the results analyzed. After subtracting background signals and normalization to positive controls, signal intensities between array images were compared with determine relative differences in protein expression levels. A 1.5-fold increase in signal intensity for a single analyte between samples or groups was considered a measurable and significant difference in expression, provided that both sets of signals were well above background (mean background +2 SDs, accuracy 95%). Results are presented as fold increase over control DMSO-treated cells.

Statistics. Differences between groups were analyzed using ANOVA followed by nonparametric *t* test (Mann-Whitney) or Student *t* test. Probability of $P < 0.05$ was considered significant.

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