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Growth hormone is permissive for neoplastic colon growth

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Growth hormone (GH) excess in acromegaly is associated with increased precancerous colon polyps and soft tissue adenomas, whereas short-stature humans harboring an inactivating GH receptor mutation do not develop cancer. We show that locally expressed colon GH is abundant in conditions predisposing to colon cancer and in colon adenocarcinoma-associated stromal fibroblasts. Administration of a GH receptor (GHR) blocker in acromegaly patients induced colon p53 and adenomatous polyposis coli (APC), reversing progrowth GH signals. p53 was also induced in skin fibroblasts derived from short-statured humans with mutant GHR. GH-deficient prophet of pituitary-specific positive transcription factor 1 (*Prop1*)^{-/-} mice exhibited induced colon p53 levels, and cross-breeding them with *ApC*^{min+/-} mice that normally develop intestinal and colon tumors resulted in GH-deficient double mutants with markedly decreased tumor number and size. We also demonstrate that GH suppresses p53 and reduces apoptosis in human colon cell lines as well as in induced human pluripotent stem cell-derived intestinal organoids, and confirm in vivo that GH suppresses colon mucosal p53/p21. GH excess leads to decreased colon cell phosphatase and tensin homolog deleted on chromosome 10 (PTEN), increased cell survival with down-regulated APC, nuclear β -catenin accumulation, and increased epithelial-mesenchymal transition factors and colon cell motility. We propose that GH is a molecular component of the “field change” milieu permissive for neoplastic colon growth.

growth hormone | acromegaly | colon | growth hormone deficiency

The pituitary gland secretes growth hormone (GH), which acts as an endocrine regulator by signaling through membrane-associated GH receptors (GHR) to elicit direct peripheral actions as well as to induce insulin growth factor (IGF1) production (1–4). Most growth-promoting endocrine actions of GH are mediated by IGF1; however, GH also acts independently of IGF1 to regulate muscle, bone, and adipose tissue functions (5–7).

Local GH (structurally identical to pituitary GH) is expressed in nonpituitary tissues, including the colon, prostate, and breast (8–10), where it similarly binds the GHR to signal in a paracrine/autocrine fashion (11, 12). Intracellular GH also acts within cells in an intracrine fashion, directly targeting intracellular GHR to regulate nuclear genes (9, 11, 12).

GH deficiency appears to confer protection against development of malignancies. Abrogating GH signaling by inducing GH deficiency as seen in Ames [prophet of pituitary-specific positive transcription factor 1 (*Prop1*)^{-/-}] mice, or by disrupting GHR, as in *GHR*^{-/-} dwarf mutant mice, is protective of cancer development (13, 14). Indeed, 20 y of follow-up have shown that individuals who harbor inactivating GHR mutations do not develop cancer, whereas unaffected relatives develop cancer at rates similar to those in the general population (15). These clinical observations are buttressed by animal studies showing that GHR inhibition suppresses colon carcinoma xenograft growth in nude mice (16) and reduces susceptibility to induced colon cancer in GH-deficient rats (17).

In contrast, transgenic mice expressing universally high circulating and tissue GH exhibit an increased incidence of soft tissue tumors (18–21), and mice overexpressing bovine GH exhibit preneoplastic liver lesions. The latter are believed to be a consequence of the direct effect of GH on the liver rather than mediated by IGF1, as transgenic mice overexpressing IGF1 do not exhibit similar liver pathology (22, 23). Furthermore, acromegaly patients with excess systemic GH elaborated by a GH secreting pituitary tumor have increased prevalence of colon polyps (24–26) as well as increased colon length with prominent mucosal folds and overgrowth (27), and also exhibit fourfold increased rates of colon adenocarcinoma (28–31).

Colorectal cancer results from inactivating mutations of tumor-suppressor genes, such as adenomatous polyposis coli (APC), p53, deleted in colorectal cancer (DCC), deleted in pancreatic cancer locus 4 (DPC4), and Kristen rat sarcoma viral oncogene homolog (K-ras), as well as DNA damage-repair abnormalities and chromosomal instability. Many of these genomic events target the transition from normal mucosa to small adenomas, then to large adenomas, and ultimately to carcinomas (32–34). Furthermore, the surrounding milieu for colon tumor development includes the extracellular matrix, cancer-associated fibroblasts (CAFs), vascular endothelial and smooth muscle cells, and immune responses (35). For example, ulcerative colitis (UC) is associated with increased rates of colon adenocarcinoma, and

Significance

Growth hormone (GH) excess in acromegaly is associated with increased colon polyps and cancer, whereas short-stature humans harboring a GH receptor mutation do not develop cancer. Administration of a GH receptor blocker in acromegaly patients induced colon p53. In contrast, p53 is suppressed by GH in colon cells, in vivo in colon tissue, and in induced pluripotent stem cell-derived intestinal organoids. GH excess leads to cell survival with downregulated adenomatous polyposis coli, nuclear β -catenin accumulation, and increased epithelial-mesenchymal transition factors. Because locally expressed GH is abundant in conditions predisposing to colon cancer, GH appears to be a molecular component of the milieu permissive for neoplastic colon growth. These results explain the protective effects of GH deficiency against development of neoplasms.

Author contributions: V.C., C.Z., A.B.-S., M.U., and S.M. designed research; V.C., S.Z., C.Z., M.V.R., T.A., R.B., M.W., K.W., and V.A.L. performed research; V.C., S.Z., C.Z., M.V.R., A.B.-S., M.U., and S.M. analyzed data; and V.C. and S.M. wrote the paper.

The authors declare no conflict of interest.

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Data deposition: Information on the pegvisomant clinical trial protocol is available at <https://clinicaltrials.gov/ct2/show/NCT01261000>.

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mucosal mapping indicates that chronically inflamed colonic mucosa undergoes a “field change” of cancer-associated molecular alterations before histological evidence of dysplasia (36). Multiple factors sustain colon proliferative signaling and enable resistance to cell death and evasion of growth suppressors. CAF-derived growth factors act through MAPK and PI3K mammalian target of rapamycin (mTOR) to mediate cell proliferation, survival, cytoskeletal rearrangement, and invasion (37–39), enabling normal mucosa to undergo premalignant changes within a pro-growth milieu. Thus, colon polyp recurrence is common in UC, presumably because of an underlying field effect (36).

Here, we present evidence supporting a novel mechanism whereby GH mediates the colon microenvironment by suppressing p53. This mechanism appears to underlie the linkage between GH and colon cell proliferative control. As GH appears to potentiate colon tissue growth (27), we treated acromegaly patients with the GHR inhibitor pegvisomant and observed induced p53 and APC expression in colon tissue biopsy specimens. Furthermore, using skin fibroblasts derived from short-stature human subjects harboring an inactivating *GHR* mutation (40) and insensitive to GH (41, 42), we show high p53 levels, as well as enhanced p53/p21 induction in response to DNA damage. Consistent with these clinical findings, we show that *Prop1*^{-/-} mutant mice devoid of GH exhibit strong colon p53 expression. Because APC-deficient *Apc*^{min/+} mice develop multiple intestinal tumors (43), we generated doubly mutant *Apc*^{min/+}*Prop1*^{-/-} mice, and observed markedly diminished tumor incidence. We elucidate mechanisms for these observations and show that GH acts to attenuate p53 stabilization, as well as suppress APC, phosphatase and tensin homolog deleted on chromosome 10 (PTEN), and apoptosis, while inducing cell motility. In intestinal organoids generated from induced human pluripotent stem cells (iPSCs), GH treatment suppressed p53, APC, and E-cadherin expression. Finally, in vivo, high GH resulted in murine colon tissue p53 and p21 suppression. These findings represent a novel concept that protective effects of GH

deficiency against tumor growth are mediated by p53 and that GH excess enables a protumorigenic cell phenotype by inhibiting tumor suppressors.

Results

Deficient GH Signaling Is Protective for Colon Neoplasia Growth.

Disrupted human GH signaling is associated with elevated p53 levels. We first tested mechanisms underlying the protective effects of GH signaling deficiency. We examined primary skin fibroblasts derived from short-stature *GHR*^{mut} Laron syndrome subjects harboring an inactivating *GHR* mutation, and which are defective in GH signaling (44). When actively proliferating asynchronous cells were pulsed with BrdU, incorporation was 40% lower in mutated than in WT fibroblasts (Fig. S1A), indicating decreased proliferation in cells lacking a functional GHR. Higher baseline p53 expression was observed in mutated compared with normal sibling age-, sex-, and passage-matched fibroblasts (Fig. 1A). p21, a cell cycle inhibitor, was also increased, and was associated with decreased Ki67 and proliferating cell nuclear antigen (PCNA) proliferation markers (Fig. 1A). Observed changes in p53 and p21 proteins reflect increased levels of both p53 and p21 mRNAs in mutant cells (Fig. S1B).

To further confirm that deficient GH signaling results in p53 accumulation, we transfected *GHR*^{mut} human fibroblasts with increasing amounts of pcDNA3.1hGHR plasmid using electroporation. Reinstatement of WT GHR in these cells led to dose-dependent suppression of baseline p53 abundance (Fig. 1B). When GHR expression was attenuated by transfecting WT human skin fibroblasts with specific *GHR* siRNA, p53 levels were also induced (Fig. 1C). Decreased GHR levels were concordant with down-regulation of the proliferation markers Ki67 and PCNA (Fig. S1C), similar to that observed in *GHR* mutated cells (Fig. 1A). Moreover, transfection of WT fibroblasts with siGHR RNAi decreased BrdU incorporation by 37% (Fig. S1D). These results

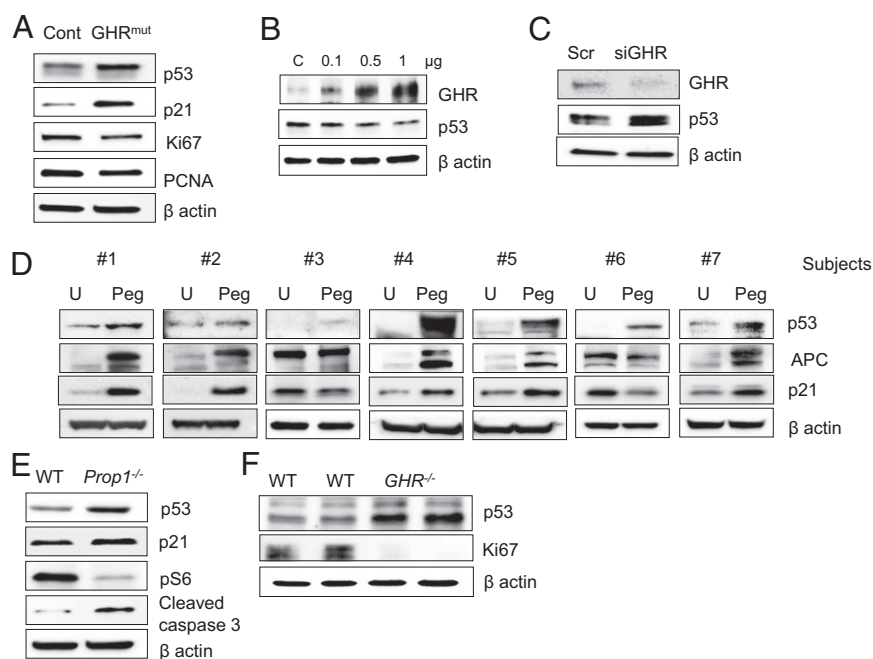


Fig. 1. Disrupted GH signaling is associated with p53 accumulation. Western blot analyses of (A) normal human skin fibroblasts and fibroblasts derived from a short-stature subject with a *GHR* mutation (*GHR*^{mut}); (B) *GHR*^{mut} fibroblasts transfected with pcDNA3.1 (control, C) or increasing amounts of pcDNA3.1hGHR DNA (total amount of transfected DNA was 1 μg in all samples) and harvested 48 h later; (C) p53 in normal human skin fibroblasts transfected with 10 pM hGHR siRNA and harvested 48 h later. Experiments were performed three times and representative results are shown. (D) Western blot analysis of p53, p21, and APC in colon mucosal biopsies derived from seven human subjects at baseline before (untreated, U) and after 8 wk treatment with pegvisomant (Peg). Western blot analyses of colon tissue derived from (E) WT and *Prop1*^{-/-} mice and (F) *GHR*^{-/-} mice. In each experiment three to six mice were used.

show that disrupted human GH signaling induces p53 abundance and decreases normal skin fibroblast proliferation.

Acromegaly patients with high levels of circulating GH exhibit increased incidence of colon adenomas (45). Pegvisomant, a GHR antagonist, prevents GH binding by disrupting internal rotation and activation of downstream GH signaling (46–48). To examine whether clinical inhibition of GH signaling alters colon tissue p53 expression, seven acromegaly subjects were prospectively enrolled to undergo colon mucosal biopsy before and after 8 wk of pegvisomant treatment (Table S1). The efficacy of pegvisomant treatment was confirmed by observed decreased serum IGF1 levels in all subjects (Table S1). GHR blockade resulted in induced colon tissue p53 expression in all seven subjects, which was accompanied by APC induction in five of seven subject specimens (Fig. 1D). p21, a proximal marker of proliferation, was induced in these same subjects concordantly with p53 induction. The remaining two subjects (#3 and #6) showed no change or decrease in APC as well as reduced p21 expression.

Murine GH deficiency increases colon p53 in vivo. Because GH signaling deficiency appears to protect against tumor formation, we tested colon tissue derived from *Prop1*^{-/-} mice devoid of pituitary GH (49) (Fig. S2A). We observed markedly induced colon p53 and p21 levels, and cleaved caspase 3, a marker of apoptosis, was also induced, whereas pS6 abundance, an end-point marker of mTORC1 activity, was attenuated (Fig. 1E). Basal colon GH mRNA levels, already low in WT mice (32 ± 0.8 Ct), were almost undetectable in colon tissue derived from *Prop1*^{-/-} mice (36.2 ± 0.9 Ct) (Fig. S2B).

Mice lacking GHR (*GHR*^{-/-}) also exhibited high colon p53 expression (Fig. 1F), supporting the hypothesis that GH signaling deficiency is associated with p53 induction. Similar to our observations in GHR-mutated and WT fibroblasts where GHR was suppressed (Fig. 1A and Fig. S1C), *GHR*^{-/-} mice exhibit markedly

decreased colon Ki67, indicative of decreased proliferation in colon tissue devoid of GHR (Fig. 1F).

Murine GH deficiency attenuates intestinal tumor development. We next tested whether enhanced colon p53 abundance could account for protection of GH-deficient mice from tumor formation. Homozygous *APC*^{min/-} mice are not viable, but *APC*^{min+/-} mice develop multiple small intestine tumors by 9 mo with 100% penetrance (43). These mice also develop colon tumors when kept on a high-fat diet (50). As *Prop1*^{-/-} mice are not fertile, *Prop1*^{+/-} mice were cross-bred with *APC*^{min+/-} mice, and tumor development examined in doubly mutant *APC*^{min+/-}*Prop1*^{-/-} animals, all maintained on high-fat diets.

All 30 male and all 22 female *APC*^{min+/-} mice, as well as 10 of 16 male and 1 of 8 female *APC*^{min+/-}*Prop1*^{-/-} mice, developed small intestine tumors by 9 mo. Both tumor size and number were strikingly decreased in doubly mutant animals (Fig. 2A and B). By this age, 67% (20 of 30) of *APC*^{min+/-} males also developed colon adenomas (one to two tumors each), whereas a single colon tumor was observed in 12.5% (2 of 16) of double mutant male mice (Fig. 2C). Colon tumors were not observed in female mice of either genotype. Thus, GH deficiency constrains both the size and number of intestinal tumors in these compound transgenic male mice.

We then analyzed colon tissue, carefully dissecting nontumorous colons of 7-mo-old *APC*^{min+/-} and *APC*^{min+/-}*Prop1*^{-/-} male mice. Colon p53 was induced in doubly mutant compared with *APC*^{min+/-} mice (Fig. 2D). Immunofluorescence analysis indicated that *APC*^{min+/-} mice exhibit high GH expression in colon tumor stromal cells, as well as in epithelial tumor cells (Fig. 2E). In contrast, GH was not detected in tumor-free *APC*^{min+/-}*Prop1*^{-/-} colon tissue (Fig. 2E).

To directly study effects of GH deficiency on colon cells, we used HCT116 colon cancer cells harboring WT p53 and primary nontumorous human colon cells (hNCC). These cells are stable for up to 10–12 population doublings; only cells from passage 4

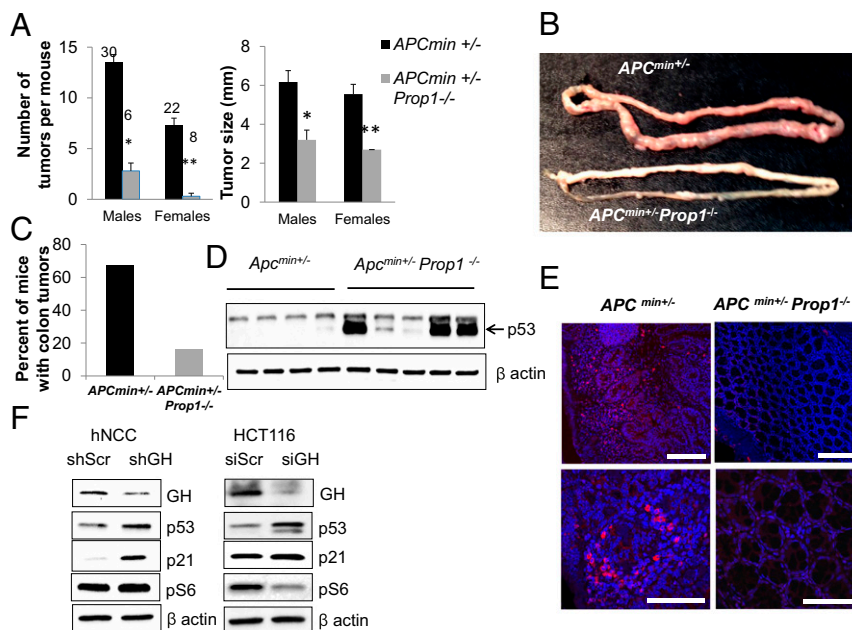


Fig. 2. GH deficiency attenuates tumor development by inducing p53. (A) Number of small intestine tumors per mouse and average tumor size (mm); * $P < 0.01$, ** $P < 0.001$. Results were analyzed by ANOVA followed by Tukey test. Numbers above the columns indicate number of mice analyzed. (B) Small intestine of *APC*^{min+/-} and *APC*^{min+/-}*Prop1*^{-/-} mice. (C) Percent of mice bearing colon tumors in 9-mo-old *APC*^{min+/-} and *APC*^{min+/-}*Prop1*^{-/-} mice. (D) Western blot analysis of colon p53 in tissue derived from pretumorous 7-mo-old *APC*^{min+/-} and *APC*^{min+/-}*Prop1*^{-/-} mice. (E) Immunofluorescent confocal images of GH immunoreactivity in colon tumor of *APC*^{min+/-} and *APC*^{min+/-}*Prop1*^{-/-} mice. Both *APC*^{min+/-} tumor stroma and tumor epithelium are immunopositive for GH (red), whereas no GH is detected in the colon of *APC*^{min+/-}*Prop1*^{-/-} mice. (Upper) (Magnification, 20 \times .) (Scale bar, 200 μ m.) (Lower) (Magnification, 63 \times .) (Scale bar, 100 μ m.) (F) Western blot analysis of hNCC stably infected with lentivirus expressing nonspecific scramble (Scr) or GH shRNA and HCT116 cells transfected with nonspecific scramble or *hGH* siRNA for 48 h. Experiments were each performed at least three times, and representative results are depicted.

were used. We previously detected immunoreactive GH in both cell lines (51). Nontransformed hNCC were stably infected with lentivirus expressing human shGH RNA, whereas HCT116 cells were transfected with siGH RNAi. In both cell lines, GH suppression resulted in p53 up-regulation, with increased levels of p21, a transcriptional target for p53 (52), and S6 phosphorylation was suppressed in malignant HCT116 cells but not in non-tumorous colon cells (Fig. 2F), confirming our *in vivo* observations (Fig. 1E).

GH Is a Component of the Field Change Enhancing Progression of Colon Epithelial Cell Neoplasia. In contrast to GH signaling deficiency, GH excess is associated with increased incidence of colon tumors. We therefore hypothesized that GH participates in the field change enabling neoplastic colon growth, and tested mechanisms underlying colon GH action.

GH expression in human colon tissue. Immunofluorescent staining and confocal microscopy of human colon tissue arrays show that normal colon GH expression is low. Because patients with UC exhibit higher rates of colon adenocarcinomas (36), we tested GH expression in inflamed human colon specimens. Although GH is undetectable in stromal cells in normal colon tissue, GH expression was enhanced in 11 of 20 (55%) colon specimens derived from patients with UC (Fig. 3A). In these specimens, GH colocalizes with IgA, a plasma cell marker (Fig. 3B). Similar stromal expression patterns were also observed in benign colon adenomas (Fig. 3C). In human colon adenocarcinoma specimens, GH, although not expressed within epithelial tumor cells, was expressed in fibroblasts surrounding malignant colon carcinoma and colocalized with α -SMA (smooth muscle actin), a marker for CAFs (Fig. 3D and E). Eight of 32 (25%) adenocarcinoma specimens and 12 of 32 (38%) colon adenocarcinoma metastases analyzed exhibited high (>11% positive cells) fibroblast GH expression. GH immunopositivity was not detected in normal colon mucosa fibroblasts. We found that the GHR is expressed in both colon epithelial and stromal cells, and that receptor expression was markedly induced in 25 of 70 (35%)

analyzed epithelial colon adenocarcinoma cells compared with normal mucosa (Fig. 3F).

GH suppresses p53 expression. We tested endocrine effects of GH by treating colon cells with increasing doses of GH for 24 h. Although p53 mRNA levels were not altered (Fig. 4A), we observed dose-dependent p53 protein attenuation in both hNCC and HCT116 cell lines (Fig. 4B). Accordingly, p21 was also dose-dependently reduced (Fig. 4B). Consistent with previous findings (2), GH treatment of hNCC resulted in early increase in STAT5 phosphorylation (for up to 3 h), indicative of GHR activation, and phospho STAT5 levels were normalized 24 h later (Fig. S3A).

To further confirm that the GH effects on p53 occur through GHR activation, we pretreated hNCC with 20 μ g/mL pegvisomant for 1 h, treated cells with 500 ng/mL GH, and harvested them 24 h later. Suppressing effects of GH on p53 and p21 were abolished and both proteins were up-regulated when GHR was blocked with pegvisomant (Fig. S3B), indicating that GH effects on p53 are GHR-mediated.

We did not observe IGF1 changes in the two cell lines after GH treatment (Fig. S4A). Treatment with increasing doses of IGF1 did not suppress p53 protein expression (Fig. S4B). Moreover, IGF1 suppression with lentivirus expressing shIGF-1 RNA did not affect GH-induced decreases in p53 (Fig. S4C). The results suggest that GH action on colon cell p53 may be IGF1-independent.

Mechanisms for p53 suppression by GH. p53 half-life is short and, in unstressed cells, the protein is maintained at low levels by ubiquitination and subsequent degradation (53). We explored mechanisms for GH-mediated p53 suppression by treating hNCC with MG-132, a proteasome inhibitor that blocks degradation of ubiquitin-conjugated proteins, in the presence of GH. In cells not treated with MG-132, GH suppressed p53, as expected, whereas p53 levels accumulated in transfectants treated with MG-132 despite the presence of GH (Fig. 4C). Because GH enhances p53 binding to ubiquitin, we immunoprecipitated protein lysates derived from hNCC treated with IgG or antiubiquitin antibodies, and

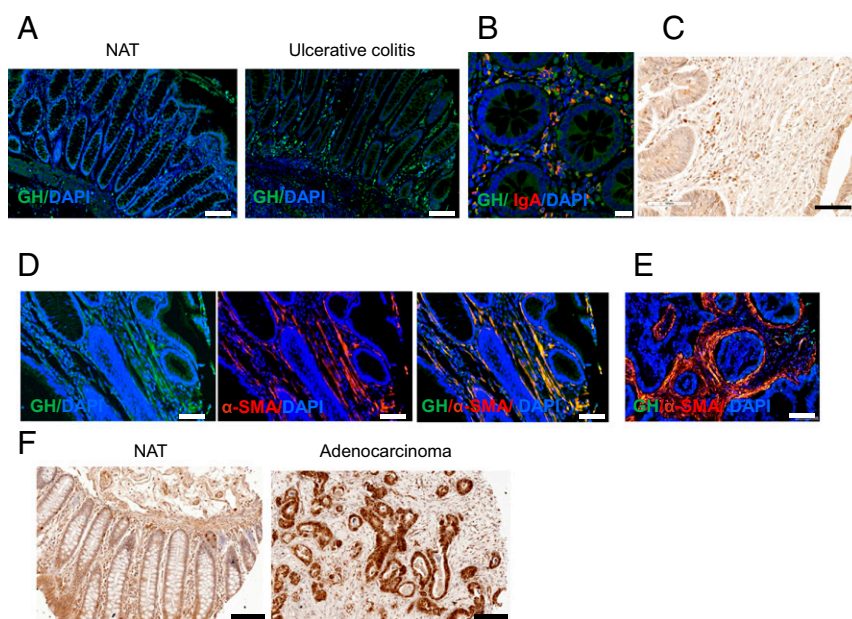


Fig. 3. GH expression in human colon tissue. Human colon tissue arrays comprising specimens from normal colon tissue, ulcerative colitis, colon adenocarcinoma, and colon adenocarcinoma metastasis. Representative images are shown. (A) GH (green) induced in colon of patients with ulcerative colitis vs. normal adjacent tissue (NAT). (Scale bar, 100 μ m.) (B) GH (green) coexpressed in stromal cells with IgA (red), a marker for plasma cells. (Scale bar, 25 μ m.) (C) GH (brown) expressed in benign colon adenoma stromal tissue. (Scale bar, 100 μ m.) GH (green) and α -SMA (red) expressed in CAFs in (D) adenocarcinoma and in (E) metastasis. (Scale bars, 100 μ m.) (F) GHR (brown) in normal adjacent tissue (NAT) and in colon adenocarcinoma. (Scale bar, 100 μ m.)

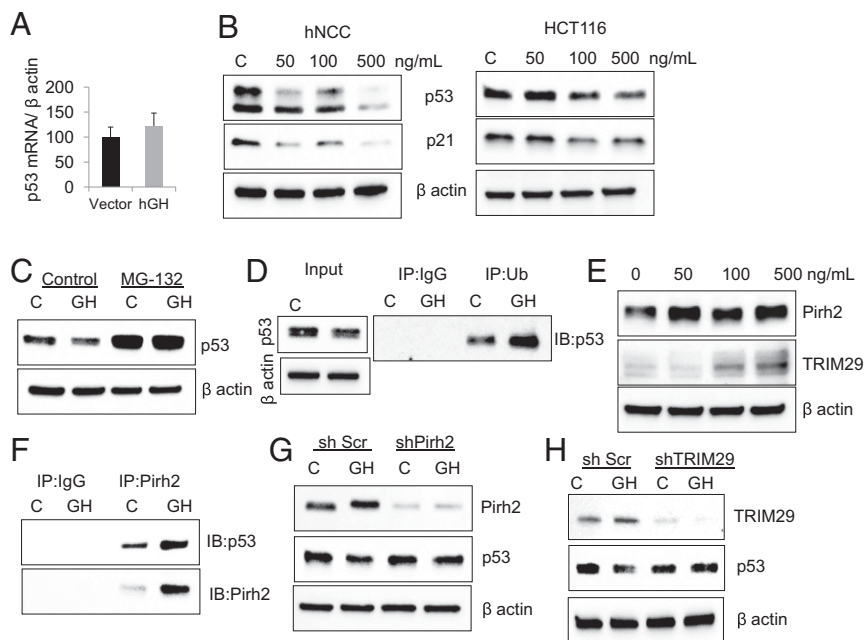


Fig. 4. GH suppresses p53, inducing ubiquitination. (A) Real-time PCR of *p53* mRNA levels in hNCC treated with 500 ng/mL GH. Normalized PCR results are expressed as fold-change vs. control (100%). Results are depicted as mean \pm SEM of triplicate measurements. Two independent experiments were performed, and representative results are shown. (B) Western blot analysis of hNCC and HCT116 cells treated with indicated doses of GH for 24 h. Experiments were performed at least three times and representative results are depicted. (C) Western blot analysis of p53 in hNCC untreated (C) or treated with GH for 24 h, and then treated with control (C) or 0.5 μ M MG-132 for an additional 24 h. (D) p53 and ubiquitin interactions analyzed in hNCC untreated (C) or treated with GH (500 ng/mL) for 6 h. Cell lysates were immunoprecipitated (IP) with IgG or anti-ubiquitin antibodies and immunoblotted for p53. (E) Western blot analysis of Pirh2 and TRIM29 in hNCC treated with indicated doses of GH. (F) p53 and Pirh2 interactions analyzed in hNCC untreated (C) or treated with GH (500 ng/mL) for 6 h. Cell lysates were immunoprecipitated (IP) with IgG and Pirh2, antibodies and immunoblotted for p53 and Pirh2 (for input, see D). (G) Western blot analysis of p53 in hNCC stably expressing scramble (Scr) shRNA or *shPirh2* RNA and treated with GH for 24 h. (H) Western blot analysis of hNCC stably expressing scramble (Scr) shRNA or *TRIM29* shRNA and treated with GH (500 ng/mL) for 24 h. Experiments were each performed at least three times and representative results depicted.

immunoblotting showed that GH enhanced p53 degradation by increasing ubiquitin binding (Fig. 4D).

Ubiquitin-dependent p53 degradation is primarily mediated by ubiquitin E3 ligase MDM2 (53). GH treatment did not alter either total or pMDM2 colon cell levels (Fig. S3B); however, GH enhanced expression of Pirh2, another p53-specific E3 ligase, likely responsible for accelerated p53 ubiquitination. (Fig. 4E). Indeed, GH treatment of hNCC for 6 h increased p53 binding to Pirh2 (Fig. 4F). When Pirh2 was suppressed in hNCC stably expressing *shPirh2*, high GH was unable to fully suppress p53 protein (Fig. 4G). GH-induced Pirh2 up-regulation was abolished in cells pretreated with pegvisomant (Fig. S3C), further supporting a strong association between GHR activation and p53 degradation.

Tripartite motif-containing 29 (TRIM29), another E3 ligase (54), binds p53, resulting in nuclear sequestration, rendering p53 susceptible to ubiquitination (55). Because GH treatment enhanced TRIM29 levels in hNCC (Fig. 4E), we treated hNCC cells stably expressing *TRIM29* shRNA with GH for 6 h. p53 was not decreased by GH in the presence of suppressed TRIM29 (Fig. 4H), indicating that p53 degradation in response to GH treatment is mediated, at least in part, by Pirh2 and TRIM29.

GH inhibition of p53 results in APC suppression. APC, acting as a tumor suppressor, is protective for intestinal tumor development, and APC mutations result in familial adenomatous polyposis in humans and mice (56). We tested GH effects on APC in hNCC and observed dose-dependent attenuation of APC protein levels (Fig. 5A). The APC promoter is activated by caudal-related homeobox transcription factor (CDX2) (57), which is suppressed by phosphorylated extracellular signal-regulated kinase (ERK1/2) (58). Although pERK was dose-dependently induced by GH, CDX2 levels were

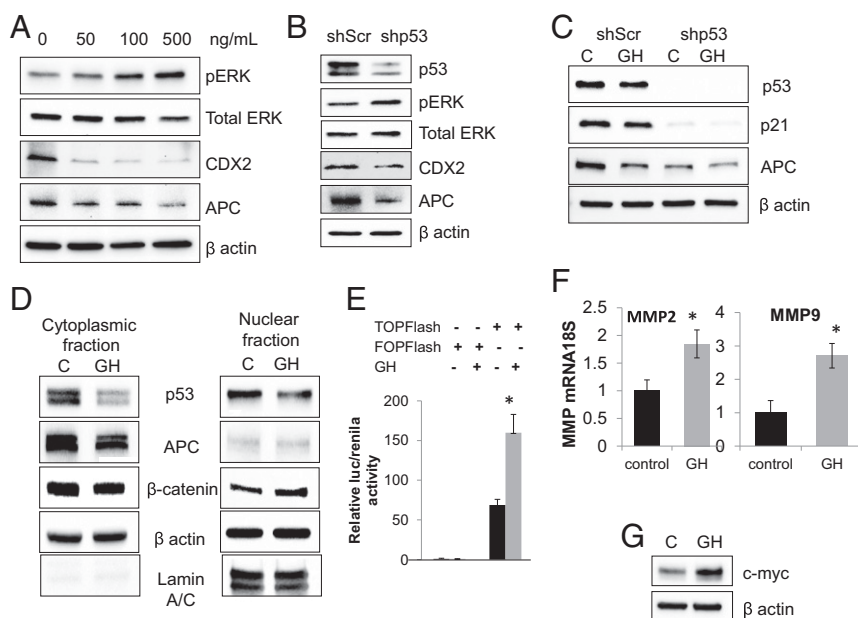
decreased, likely accounting for the observed suppression of APC in GH-treated cells (Fig. 5A).

We assessed whether GH suppression of APC is p53-dependent by generating hNCC stably infected with lentivirus expressing *p53* shRNA. With no GH treatment, these cells exhibited induced ERK phosphorylation, followed by decreased CDX2 and decreased APC expression (Fig. 5B), similar to that observed after treating WT hNCC with GH (Fig. 5A). Furthermore, p53-deficient cells did not respond to GH and expressed very low APC levels, whereas, in control cells stably expressing scramble shRNA, GH significantly attenuated APC levels (Fig. 5C). The results suggest that p53 suppression may be required for GH-dependent APC down-regulation in nontumorous colon cells.

GH suppression of APC induces nuclear β -catenin. When APC is suppressed, β -catenin undergoes nuclear translocation, exerting pro-growth actions (59). We therefore examined whether GH-mediated APC suppression regulates β -catenin localization. Because HCT116 cells express a mutated β -catenin isoform that cannot be phosphorylated and is not degraded (60), we used only hNCC. When treated with GH, both p53 and APC were suppressed in the cytoplasmic fraction, whereas p53 was suppressed in both cytoplasmic and nuclear fractions. β -Catenin abundance was also decreased in cytoplasmic fractions and up-regulated in nuclear fractions (Fig. 5D). Cytoplasmic β -catenin is abundant, and quantification showed cytoplasmic expression decreased \sim 20%, whereas intranuclear β -catenin expression was increased by 67% (Fig. S5).

We next isolated cytoplasmic and nuclear protein fractions from hNCC stably expressing *shp53*. In these cells, GH did not increase nuclear β -catenin, whereas in control hNCC stably expressing scramble shRNA, nuclear β -catenin was increased (Fig. S6), consistent with markedly attenuated APC (Fig. 5D).

Fig. 5. GH suppresses APC and increases β -catenin nuclear translocation. Western blot analysis of ERK/APC pathway in (A) hNCC treated with indicated doses of GH for 24 h and (B) hNCC stably expressing scramble (Scr) *shRNA* or *p53shRNA*. Experiments were repeated twice and representative blots shown. (C) Western blot analysis of hNCC stably infected with lentivirus expressing *shp53 RNA* or scramble (Scr) *shRNA* and untreated (C) or treated with GH (500 ng/mL). (D) Western blot analysis of cytoplasmic and nuclear fractions of hNCC untreated (C) or treated with GH (500 ng/mL) for 24 h. Experiments were each performed twice and representative results are shown. (E) Assay of canonical Wnt pathway signaling through activation of a TCF/LEF luciferase reporter construct (TOPFlash) or a control reporter (FOPFlash). hNCC were nucleofected with reporter constructs and treated with GH (500 ng/mL) for 24 h. Fold-induction was calculated as normalized relative light units of TOPFlash divided by those of FOPFlash. Results are mean \pm SEM of triplicate measurements; * $P < 0.05$. The experiment was repeated four times with similar results and representative results shown. (F) Real-time PCR assessment of β -catenin target genes MMP2 and MMP9 expression in hNCC treated with GH (500 ng/mL) for 24 h. Normalized PCR results are expressed as fold-change vs. control taken as 1. Results are depicted as mean \pm SEM of triplicates measurements. Two independent experiments were performed, and representative results depicted; * $P < 0.05$. (G) Western blot analysis of c-myc in hNCC treated with GH (500 ng/mL) for 24 h. In E and G, the differences between groups were analyzed using two-tailed unpaired Student t test.



Testing β -catenin transcriptional activity after treatment of hNCC with GH showed a modest but consistent increase in canonical Wnt pathway activity as measured by assays of β -catenin-mediated transcription through T-cell factor/lymphoid enhancer binding factor (TCF/LEF) binding sites (Fig. 5E). β -Catenin targets, including matrix metalloproteinase 2 (MMP2) and MMP9 mRNA, as well as c-myc protein levels, were up-regulated after GH exposure (Fig. 5F and G), possibly as a result of β -catenin nuclear translocation.

GH suppresses PTEN and inhibits apoptosis. In both HCT116 and hNCC, we observed that PTEN, a transcriptional target of p53, was down-regulated in response to GH, likely as a result of p53 suppression (Fig. 6A and B). PTEN is a critical mediator of mitochondria-dependent apoptosis (61). Consistent with this function, we observed that, in both hNCC and HCT116 cells, GH treatment for 24 h suppressed cleaved caspase 3 expression, thereby decreasing apoptosis (Fig. 6C).

GH enhances cell motility. We further explored consequences of GH excess on markers of epithelial-mesenchymal transition (EMT) (62). The GH-induced EMT transcription factor Snail1, which activates mesenchymal markers, was induced in HCT116 cells, whereas in hNCC, Twist2 levels were also dose-dependently up-regulated (Fig. 6A and B). Induction of these transcription factors reflects EMT activation in response to GH treatment, permitting a permissive tumor microenvironment toward a mesenchymal phenotype promoting cell motility and invasion. Accordingly, consistent with Western blotting results, we observed enhanced migration of hNCC and HCT116 cells (50% and 40%, respectively) 24 h after GH treatment (Fig. 6D and Fig. S7).

Because GH expression was detected in human colon adenocarcinoma specimen stromal cells, we cocultured HCT116 epithelial tumor cells with human colon fibroblasts (hCF) infected with lentivirus expressing human GH. Using a GH-specific ELISA, we confirmed that GH-expressing fibroblasts secreted GH in culture (338 ng/mL), whereas in cultured fibroblasts expressing empty vector, GH was undetectable. In the presence of GH-secreting fibroblasts, HCT116 migration was enhanced twofold (Fig. 6E).

When anchorage-independent growth was assessed, we found that nontransformed hNCC did not form colonies in soft agar, but HCT116 cells cultured in the presence of GH-expressing

fibroblasts formed fourfold more colonies, and colony size was 4.3 times larger than in cells cultured with control fibroblasts expressing empty vector (Fig. 6F). Thus, high GH levels appear to support protumorigenic cell properties, as reflected by decreased apoptosis, enhanced EMT transcription factors, and increased cell motility and colony formation.

GH effects in colon mucosa of 3D primary human colon organoids. We tested GH actions in induced pluripotent 3D intestinal organoid cultures that recapitulate cellular heterogeneity of human colon mucosa. Human fibroblast cell lines derived from healthy individuals were reprogrammed to generate iPSCs using a non-integrating reprogramming system (63). Cells were induced to form hindgut and, ultimately, intestinal organoids. Unlike colon tumor cell lines, intestinal organoids are polarized and comprise several intestinal epithelial subtypes, including enterocytes, goblet cells, and enteroendocrine cells (64) (Fig. S8). Treatment of organoids for 48 h with GH (500 ng/mL) suppressed p53, CDX2, and APC. E-cadherin, responsible for cell-to-cell adhesion, was also decreased, whereas N-cadherin, which mediates cell migration and invasion (65), was induced, reflecting pro-neoplastic effects of GH in colon tissue (Fig. 7A).

High GH suppresses murine colon p53 in vivo. To translate our findings to an in vivo model, nine nude mice were injected with HCT116 cells stably infected with lentivirus expressing murine GH (lentimGH) or empty vector (lentiV), recapitulating systemic GH increase. All mice developed xenografted tumors and were killed 5 wk after injections; colon and liver tissue collected and analyzed. Western blot analysis of p53 and p21 expression in colon tissue (Fig. 7B and Fig. S9A) with ImageJ measurements showed that five of nine mice bearing GH-secreting tumors exhibited decreased p53 expression ($21 \pm 2.6\%$) and seven of nine mice exhibited decreased p21 expression ($31 \pm 4.7\%$) compared with controls bearing lentiV xenografts (Fig. 7B), confirming the suppressive effects of circulating GH on colon p53 expression.

Circulating murine GH levels were increased (Fig. S9B), and GH activity was evidenced by increased circulating and liver IGF1, but not colon IGF1, in mice bearing GH-expressing tumors (Fig. S9B and C). Hepatic p53 expression was not altered in experimental mice (Fig. S9C), underscoring cell-specific effects of GH

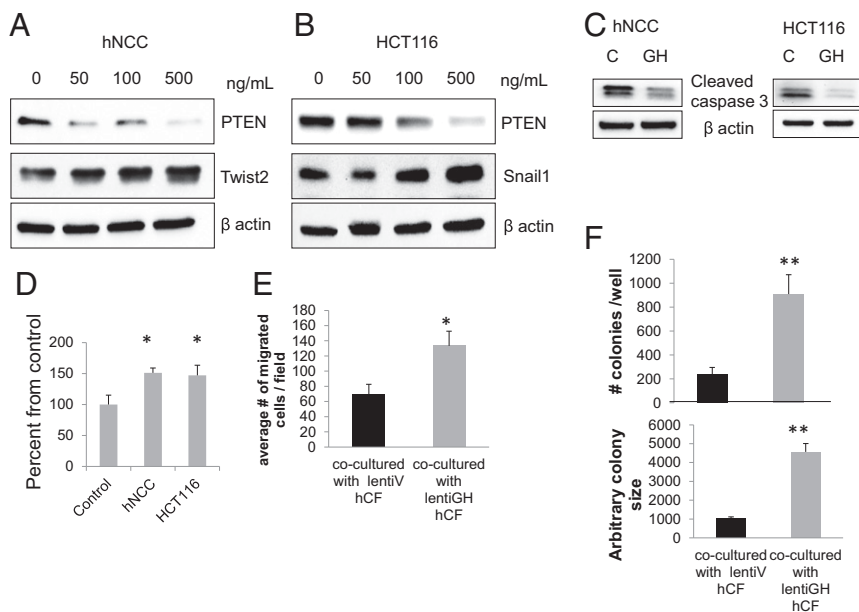


Fig. 6. GH induces EMT, suppresses apoptosis, and increases motility. Western blot analysis of PTEN and EMT factors in (A) hNCC and (B) HCT116 cells treated with indicated doses of GH for 24 h. (C) Western blot analysis of cleaved caspase 3 in cells treated with GH (500 ng/mL) for 24 h. Experiments were performed at least three times, and representative results shown. (D) Migration of hNCC and HCT116 cells treated with GH (500 ng/mL) and harvested 48 h after plating. (E) Migration of HCT116 cocultured for 48 h with hCF infected with lentivector or lentiGH. In D and E, for quantification, the number of migrated cells per 1,000 cells in five randomly chosen fields in each duplicate transwell were counted and means calculated. Results are presented as mean \pm SEM of three independent experiments; $*P < 0.05$. (F) Number of colonies and arbitrary colony size formed in soft agar by HCT116 cells cocultured with hCF infected with lentivector or lentiGH, and tested for anchorage independent growth. Colony size was determined using ImageJ software. Results are presented as mean \pm SEM of duplicates from two independent experiments; $**P < 0.01$ vs. control. In D–F, the differences between groups were analyzed using two-tailed unpaired Student t test.

and confirming our *in vitro* observations that effects of GH in the colon may be independent of IGF1 in this experimental model.

Discussion

We demonstrate novel mechanisms underlying the relationship between GH and specific tissue growth (21, 45, 66, 67). Our results suggest that the known protective effect of GH signaling deficiency on neoplastic tissue growth is mediated, at least partially, by regulating p53 expression. In contrast, GH may act as a tumor promoter in colon tissue by suppressing p53, PTEN, and APC.

In skin fibroblasts derived from *GHR^{mut}* Laron and from normal subjects, baseline p53 were higher in *GHR^{mut}* cells. Reconstituting the WT GHR in mutant fibroblasts resulted in dose-dependent p53 attenuation, whereas suppressing GH signaling in WT human fibroblasts led to p53 accumulation. Comparable results were obtained in mice with GH signaling deficiency. Colon tissue derived from *Prop1^{-/-}* dwarf mice exhibited markedly increased p53 and p21 levels associated with restrained cell proliferation. These specific observed changes are likely responsible for the striking decrease in intestinal tumor number and size, as well as in the rate of colon tumors observed in doubly mutant *APC^{min/+}Prop1^{-/-}* animals that express high colon p53 levels.

We cannot exclude that tumor protective effects observed in *Prop1^{-/-}* mice may also be attributed to other hormone deficiencies, as these animals may also lack thyroid stimulating hormone and prolactin, and may have low gonadotropin levels (68). However, *GHR^{-/-}* mice exhibit increased colon p53 expression as well, and direct *in vitro* GH suppression in hNCC and HCT116 colon cells also leads to p53 induction. Importantly, blocking of colon cell GHR by pegvisomant *in vitro*, and in acromegaly patients, increased p53 expression. These results suggest that tumor-protective effects of GH signaling deficiency are, at least in part, mediated by inducing p53. Other growth factors, such as epidermal growth factor, may also be involved in these effects (69).

In contrast, high GH dose-dependently suppresses both p53 and its transcriptional target p21. GH increases p53 ubiquitination by inducing two E3 ligases, Pirh2 and TRIM29. Indeed, when Pirh2 was suppressed, normal colon cells did not respond to GH with p53 degradation. Induced TRIM29 does not ubiquitinate p53 per se, but binds p53, resulting in nuclear export and subsequent ubiquitination by other E3 ligases (55). TRIM29 abundance was

strongly induced by GH. Because TRIM29 suppression by specific shRNA resulted in p53 stabilization, our results suggest that TRIM29 likely translocates p53 to the cytoplasm for ubiquitination. Thus, both Pirh2 and TRIM29 overexpression may constrain p53 stability in response to GH. Although p53 is a short-lived protein, prominent p53 suppression in our experiments was observed 24 h after GH treatment, likely because of the involvement of these E3 ligases.

In addition to suppressing p53, high GH attenuates expression of the tumor-suppressor APC. Most colorectal tumors contain an inactivating APC mutation (34), and APC restoration drives tumor-cell differentiation and regression (70). GH was shown to induce ERK phosphorylation (71, 72), which, in turn, suppresses CDX2. In our experiments, pERK was indeed up-regulated in cells treated with GH, resulting in decreased CDX2 and, subsequently, APC. p53 loss induces cell proliferation by activating Raf/Mek/ERK signaling in a Ras-independent manner (73), whereas p53 activation contributes to cellular senescence through ERK1/2 dephosphorylation (74). Based on our observations, it appears that GH-induced p53 suppression leads to increased ERK phosphorylation and decreased APC expression. It is not clear whether APC expression is p53-dependent (75, 76). Our results showing decreased APC in cells where p53 is suppressed suggest that, in colon cells, APC is at least partially dependent on intact p53.

Low APC expression results in β -catenin nuclear accumulation and increased Wnt signaling through activation of proliferative genes (59, 77). In our experiments, in nontumorous colon cells, GH induced β -catenin nuclear translocation with subsequent activation of β -catenin-mediated transcriptional activity. p53 and APC suppression may therefore underlie the progrowth potential of GH. Furthermore, by suppressing both p53 and PTEN, GH abrogated apoptosis thereby favoring cell survival (78). We also show GH-induced cell migration and anchorage-independent growth with expression of transcription factors Snail1 and Twist 2, early steps in EMT activation (62).

Because GH is induced in CAFs surrounding colon adenocarcinoma, and GHR is abundantly expressed in colon cancer cells, our results suggest that CAF-derived GH may enhance cell transformation as evidenced by increased anchorage-independent growth of HCT116 cells cocultured with GH-expressing fibroblasts. These results are consistent with observations by others that intracellular

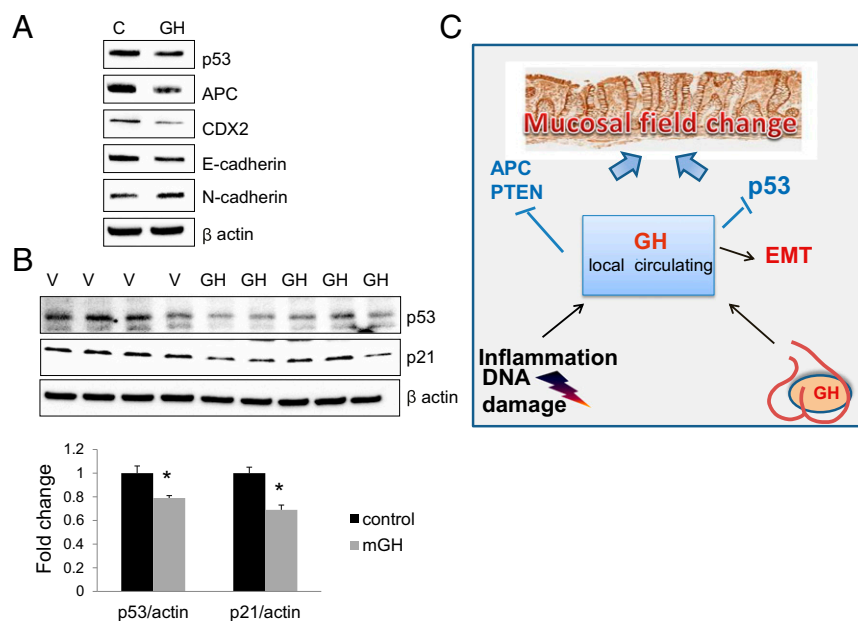


Fig. 7. GH suppresses p53 in human intestinal organoids and in murine colon. (A) Western blot analysis of intestinal organoids treated with GH (500 ng/mL) for 48 h. (B) Athymic nude mice were injected subcutaneously with 500,000 HCT116 cells stably infected with lentimGH or lentiV and killed 5 wk after injection. (Upper) Western blot analysis of representative colon tissues derived from nude mice bearing mGH-secreting xenograft tumors. Four control and five experimental mice are shown here, and the rest of the animals are shown in Fig. S9A. (Lower) Intensities of protein bands from Western blot quantified, normalized to β -actin, and depicted as fold-change relative to control animals taken as 1. $n = 9$ mice/group, * $P < 0.05$. Differences between groups were analyzed using two-tailed unpaired Student t test. (C) A proposed model for colon p53-GH interaction. Local GH is induced as a result of DNA damage or inflammation. Circulating GH is induced as a result of acromegaly. High GH (circulating or local) suppresses p53, APC, PTEN, and apoptosis, and stimulates EMT, enabling a pro-growth mucosal field change.

GH promotes breast cancer cell transformation (79, 80) and induces an invasive phenotype by triggering EMT (81).

GH treatment of intestinal organoids closely recapitulating normal human intestinal and colon mucosa resulted in suppression of p53 and APC. Moreover, GH treatment led to down-regulation of E-cadherin, which maintains cell adhesion (82), whereas N-cadherin, indicative of a mesenchymal phenotype (62), was induced, underscoring the role of colon GH in EMT.

Five of nine mice responded to high circulating GH concentrations with suppressed colon p53, and seven of nine mice responded with p21 suppression, validating our in vitro results. The response of colon tissue to the circulating GH was not universal, which may reflect individual sensitivity to high GH.

Several studies show that GH acts independently of IGF1 in multiple tissues (7, 22, 83), and in our studies IGF1 suppression does not alter suppressive effects of GH on p53. Effects of IGF1 on p53 appear to be tissue specific as IGF1 induces p53/21 in human skin fibroblasts and primary liver cells (84–86), while reducing p53 in rat myocytes (87). However, we cannot exclude that our observed in vivo GH effects may be mediated, at least in part, by IGF1, as high circulating GH increased IGF1 blood levels and liver expression. Circulating IGF1 may induce murine colon cancer growth (88), whereas in humans, the colon cancer risk for high IGF1 is modest compared with that seen in acromegaly (89). GH overexpression in transgenic mice is associated with development of liver cancers, but transgenic IGF1-overexpressing mice do not exhibit liver pathology (22, 23). As p53 suppresses IGF1R expression, down-regulated p53 in our experiments may result in IGF1R up-regulation, thereby further activating cell-survival mechanisms by suppressing apoptosis and potentiating transformation (90). Nevertheless, our results provide further innovative insights for understanding direct GH actions (91, 92). GH effects appear to be cell-specific, as GH decreased colon but not liver p53. Transgenic mice overexpressing circulating bovine GH were also shown to exhibit induced p53 in white adipose tissue (93).

The important finding of this study is that both circulating and local GH, acting through the GHR, attenuate p53 stability, and blocking GH signaling results in p53 induction. High circulating GH levels can be caused by a GH-secreting pituitary tumor, and local colon GH can be induced as a result of DNA damage (51) or inflammation as within the pituitary gland (94). Regardless of

the origin, high GH appears to exhibit properties of a component of the field change milieu affecting normal colon cells. We provide biological and clinical evidence to support the hypothesis that GH excess suppresses p53 levels, with subsequent decreased p21 expression, suppressed PTEN, APC, and apoptosis, and enhanced EMT transcription factors, thereby enabling cell survival and motility. Through these mechanisms, GH excess might therefore be permissive to polyp and colon cancer development observed in patients with UC and acromegaly (Fig. 7C). Because we also show abundant GH expression in colon CAFs, we propose that GH is a component of the tumor microenvironment sustaining neoplastic growth. Our results support the hypothesis that protective effects of GH signaling deficiency against neoplasms are associated with increased abundance of tumor suppressor proteins p53, p21, and APC. Our study provides a rationale for targeting GH signaling pathways as a potential treatment of colon neoplasms.

Methods

Human Subjects. Seven acromegaly patients in whom pegvisomant treatment was indicated were recruited for the clinical trial (<https://clinicaltrials.gov/ct2/show/NCT01261000>) and underwent screening evaluation for inclusion criteria (see Table S1 and SI Methods for inclusion and exclusion criteria).

Before initiating pegvisomant treatment, participants underwent colonoscopy, during which pretreatment colon mucosal biopsies were obtained. Patients then received a loading dose of 40 mg pegvisomant by subcutaneous injection, followed by a daily subcutaneous pegvisomant injection of 20 mg/d for 8 wk.

Following 8 wk of pegvisomant treatment, patients underwent sigmoidoscopy, during which posttreatment colon mucosal biopsies were obtained. Patients were evaluated for signs and symptoms of acromegaly at this time and IGF1 levels were again measured. Patients were monitored for potential side effects for the duration of the protocol, and continued to receive daily subcutaneous pegvisomant treatment for 6 mo after the study.

Human colon tissue arrays, which include pathology diagnosis, were purchased from US Biomax.

Mice. *Prop1*^{-/-} (*Oca2 Prop1*^{df/J}) and *APC*^{min/+} breeding pairs were purchased from Jackson Laboratory. Both *Prop1*^{-/-} and *APC*^{min/+} mice were backcrossed to the parental strains at least four times. WT and *Prop1*^{-/-} mice were obtained by breeding *Prop1*^{+/-} females and males. *APC*^{min/+} *Prop1*^{-/-} mice were obtained by breeding *APC*^{min/+} males to *Prop1*^{+/-} females. *APC*^{min/+} mice usually do not develop colon tumors unless they are fed with a high-fat diet. Therefore, mice were placed on a high-fat diet starting from age 2 mo.

Cells. Human skin fibroblasts derived from a 21-y-old short-stature male carrying a GHR mutation and from his 23-y-old normal height male sibling (both passage 4) were a generous gift from Ron Rosenfeld (Oregon Health Science University, Portland, OR). Human fibroblasts were cultured in α MEM medium (Invitrogen) supplemented with 10% (vol/vol) FBS.

Human colon carcinoma HCT116 cells were obtained from American Type Culture Collection (ATCC) and cultured in McCoy's 5A medium (Invitrogen) and 10% (vol/vol) FBS. Primary hNCC were purchased and cultured in plates pre-treated with Applied Cell Extracellular Matrix in PriGrow III Media (both from Applied Biological Materials) supplemented with 5% (vol/vol) FBS. Cells from passage ≤ 4 were used for experiments.

Normal hCF were purchased and cultured in full fibroblasts media (both from Cell Biologicals). Cells from passage 3 were used for experiments.

All cell lines tested negative for mycoplasma.

Constructs and Transfections. For constructs and transfections, see *SI Methods*.

Three-Dimensional Intestinal Organoids. Healthy control fibroblasts were obtained from the Coriell Institute for Medical Research or derived from healthy human volunteer donors at Cedars-Sinai (IRB# 00027264). Three-dimensional intestinal organoids were generated from a control fibroblast "83i" iPSC line generated using an episomal plasmid reprogramming system, which has been extensively characterized elsewhere (63). To induce definitive endoderm formation, all iPSCs were cultured with a high dose of Activin A (100 ng/mL; R&D Systems) with increasing concentrations of FBS over time [0%, 0.2%, and 2% (vol/vol) on days 1, 2, and 3, respectively]. Wnt3A (25 ng/mL; R&D Systems) was also added on the first day of endoderm differentiation. To induce hindgut formation, cells were cultured in Advanced DMEM/F12 with 2% (vol/vol) FBS along with CHIR 99021 (2 μ M; Tocris) and FGF4 (500 ng/mL; R&D Systems). After 3–4 d, free-floating epithelial spheres and loosely attached epithelial tubes became visible and were harvested. These epithelial structures were subsequently suspended in Matrigel and then overlaid in intestinal medium containing CHIR99021 (2 μ M; Tocris), noggin, and EGF (both 100 ng/mL; all R&D Systems) and B27 (1 \times ; Invitrogen). Organoids were passaged every 7–10 d thereafter.

In Vitro Treatments. Recombinant human GH (Bio Vision or R&D Systems) was reconstituted in culture medium containing 0.1% BSA. Cells were placed in culture medium free of serum containing 0.1% BSA, GH was added, and cells were harvested 24 h later. If treatment was extended to 48 h, GH was added daily. MG-132 (Sigma-Aldrich; 10 mM in DMSO) was added for 24 h. Control cells were treated with DMSO.

Protein Analysis. Cells were homogenized and lysed in RIPA buffer (Sigma-Aldrich) with 10 μ M protease inhibitors (Sigma-Aldrich). For Western blot analysis, proteins were separated by SDS/PAGE, electroblotted onto Trans-Blot Turbo Transfer Pack 0.2- μ m PVDF membrane (Bio-Rad), and incubated overnight with antibodies, followed by corresponding secondary antibodies (Sigma-Aldrich). See *SI Methods* for antibody information.

For immunohistochemical analysis of human tissue, we used antibodies to GH followed by secondary antibodies conjugated with chicken anti-rabbit

AlexaFluor 488 or donkey anti-goat Alexa Fluor568 (Invitrogen). Antigen retrieval was performed in 10 mM sodium citrate, and control reactions were devoid of primary antibodies or stained with blocking antibodies. Staining GHR was performed using antibodies from LSBio (#LS-C171952). Samples were imaged with a Leica TCS/SP spectral confocal scanner (Leica Microsystems) in dual-emission mode to distinguish autofluorescence from specific staining.

NE-PER Nuclear and cytoplasmic Extraction Reagents (Thermo Scientific, #78833) were used to isolate cytoplasmic and nuclear fractions in hNCC.

Immunoprecipitation and real-time PCR, SA- β -galactosidase Activity, cell proliferation assay and motility assay were performed as previously described (51, 95). For details, see *SI Methods*.

TOPFlash Reporter Assay. hNCC cells were nucleofected with 1 μ g DNA of the standardized pair of Super16 \times TOPFlash and Super16 \times FOPFlash plasmids (# 12456, #12457 Addgene), each with a *Renilla* control luciferase plasmid applied for signal normalization. Luciferase assays were performed using a Dual-Luciferase Reporter Assay Kit (Promega), and fold-induction was calculated as normalized relative light units of TOPFlash divided by those of FOPFlash.

Mouse Xenograft Model. HCT116 cells stably infected with lenti-murine GH (5 $\times 10^5$ cells in 0.05 mL PBS) were mixed (1:1) with Matrigel (Corning) and injected subcutaneously into the right flank of nine athymic nude female mice (Jackson Laboratory) to establish a model of excess systemic GH. Control mice were injected with HCT116 cells infected with lentiV. All nine mice developed xenograft tumors and were killed 5 wk after injection. Blood, liver, and colon tissues were collected and analyzed.

Hormone Measurements. Plasma GH was measured using mouse/rat GH ELISA from Millipore and plasma IGF1 was measured using mouse/rat IGF1 ELISA from ALPCO Diagnostics according to the manufacturer's instructions.

Statistics. Differences between groups were analyzed using two-tailed unpaired Student *t* test. Differences in average tumor counts and size between groups by genotype were tested by way of ANOVA followed by Tukey test to adjust for multiple comparisons. All testing was done two-sided. Probability of $P < 0.05$ was considered significant.

Study Approval. The pegvisomant clinical trial protocol (<https://clinicaltrials.gov/ct2/show/NCT01261000>) was approved by the Cedars-Sinai Institutional Review Board and informed consent for use of biopsy tissue or paraffin slides for research was obtained from each patient before study procedure. Experiments performed on mice were approved by the Cedars-Sinai Institutional Animal Care and Use Committee.

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