# Somatotropinomas, But Not Nonfunctioning Pituitary Adenomas, Maintain a Functional Apoptotic RET/Pit1/ARF/p53 Pathway That Is Blocked by Excess GDNF

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Acromegaly is caused by somatotroph cell adenomas (somatotropinomas [ACROs]), which secrete GH. Human and rodent somatotroph cells express the RET receptor. In rodents, when normal somatotrophs are deprived of the RET ligand, GDNF (Glial Cell Derived Neurotrophic Factor), RET is processed intracellularly to induce overexpression of Pit1 [Transcription factor (gene : POUF1) essential for transcription of Pituitary hormones GH, PRL and TSHb], which in turn leads to p19Arf/p53-dependent apoptosis. Our purpose was to ascertain whether human ACROs maintain the RET/Pit1/p14ARF/p53/apoptosis pathway, relative to nonfunctioning pituitary adenomas (NFPAs). Apoptosis in the absence and presence of GDNF was studied in primary cultures of 8 ACROs and 3 NFPAs. Parallel protein extracts were analyzed for expression of RET, Pit1, p19Arf, p53, and phospho-Akt. When GDNF deprived, ACRO cells, but not NFPAs, presented marked level of apoptosis that was prevented in the presence of GDNF. Apoptosis was accompanied by RET processing, Pit1 accumulation, and p14ARF and p53 induction. GDNF prevented all these effects via activation of phospho-AKT. Overexpression of human Pit1 (hPit1) directly induced p19Arf/p53 and apoptosis in a pituitary cell line. Using in silico studies, 2 CCAAT/enhancer binding protein alpha (cEBP$\alpha$) consensus-binding sites were found to be 100% conserved in mouse, rat, and hPit1 promoters. Deletion of 1 cEBP$\alpha$ site prevented the RET-induced increase in hPit1 promoter expression. TaqMan qRT-PCR (real time RT-PCR) for RET, Pit1, Arf, TP53, GDNF, steroidogenic factor 1, and GH was performed in RNA from whole ACRO and NFPA tumors. ACRO but not NFPA adenomas express RET and Pit1. GDNF expression in the tumors was positively correlated with RET and negatively correlated with p53. In conclusion, ACROs maintain an active RET/Pit1/p14Arf/p53/apoptosis pathway that is inhibited by GDNF. Disruption of GDNF's survival function might constitute a new therapeutic route in acromegaly. **(*Endocrinology* 155: 4329–4340, 2014)**

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Abbreviations: ACRO, somatotropinoma; aGSU, glycoprotein hormones, common alpha subunit (CGA gene); cEBPa, CCAAT/enhancer binding protein alpha, cEBP$\alpha$; FBS, fetal bovine serum; GDNF, Glial Cell Derived Neurotrophic Factor; hPit1, human Pit1; NFPA, nonfunctioning pituitary adenoma; OIA, oncogene-induced apoptosis; OIS, oncogene-induced senescence; p-AKT, phosphorylated AKT; rPit1, rat Pit1; SF-1, steroidogenic factor 1; siRNA, small interfering RNA.

Pituitary adenomas are tumors that arise from secretory cells. Pituitary adenomas originating in somatotrophs (known as somatotropinomas [ACROs] or GH adenomas) cause acromegaly, due to hypersecretion of GH, which in turn leads to disproportionate growth, insulin resistance, weight gain, and metabolic disease as well as increase in incidence of secondary tumors (1). Nonfunctioning pituitary adenomas (NFPAs), by contrast, are unable to secrete functional hormones, so patients lack the clinical symptoms of hypersecretion.

Due to the pituitary's small size and difficult access, surgery has to be conservative and is usually endoscopic to avoid injury and consequential hypopituitarism. This makes pharmacological approaches an essential component of adenoma treatment. Somatostatin and dopamine analogs are the current adjuvant therapy in acromegaly (1). However, their effects are often only partial, meaning that excess GH is difficult to control in many patients (2). Recently, a modified human GH antagonist at the GH receptor, pegvisomant, has been approved for use in acromegaly, although it does not arrest pituitary growth and has some adverse secondary effects, such as liver injury (3). In 20%–40% of acromegalic patients, GH levels are not controlled by current treatments. This constitutes a significant problem, because excess GH is directly related to mortality (4–6). Thus, there is still a need for the development of new treatments to improve patient outcome in acromegaly.

Humans and rodents somatotrophs from normal pituitaries express the RET receptor, its GDNF family receptor alpha 1 coreceptor, and their ligand Glial Cell Derived Neurotrophic Factor (GDNF) (7, 8). We have previously described a pathway in rodents (rat and mouse), in which RET regulates somatotroph number. Thus, in the absence of sufficient GDNF, RET is intracellularly processed by caspases, and the intracellular fragment activates Pit1 expression. Excess Pit1 directly activates the p19Arf promoter, leading to p53 accumulation and apoptosis (9, 10). In contrast, in somatotrophs exposed to sufficient GDNF, RET dimmerizes and cross-activates its tyrosine-kinase activity resulting in Akt signaling. Akt restricts Pit1 levels, thus promoting cell survival (9, 10). Because Pit1 is the main transcription factor for somatotroph proliferation and phenotype, including expression of the GH gene, the RET/Pit1/Arf/p53 pathway for apoptosis is considered to be a type of oncogene-induced apoptosis (OIA).

We have previously studied a series of 50 human pituitary adenomas using semiquantitative immunohistochemistry. Similar to normal somatotrophs, all GH adenomas showed RET and GDNF family receptor alpha 1 expression, whereas NFPAs and prolactinomas (secreting PRL) did not express RET (8). Functional studies of the RET/Pit1/Arf/p53 apoptotic pathway in adenomas are lacking, however, and presence of RET does not indicate functionality of the pathway. Some studies have sequenced RET exons from the DNA of pituitary adenomas, but none has found a pathological mutation such as has been described in other endocrine cancers, eg, Multiple Endocrine Neoplasia type 2 and isolated Medullary Thyroid Carcinoma (11–13). Neither has a germline mutation been found in any RET exon in families affected by familial isolated pituitary adenoma (14). In the colon, however, where RET also seems to act as a tumor suppressor, it has recently been found that carcinomas frequently have increased methylation of RET, in comparison with normal mucosal tissue, which contributes to a decrease in its expression at both mRNA and protein levels (15).

It is important to know whether the RET apoptotic pathway is active in pituitary tumors, because its antitumorigenic potential has been demonstrated in vivo in rodent models. We have previously shown that stereotaxic injection of a RET-expressing retrovirus into the pituitary of mice blocks estrogen-induced hyperplasia and restores the pituitary to its normal size. Conversely, the RET knockout mouse has pituitary hyperplasia (9). In the current study, we focused on the functional response of human pituitary ACRO and NFPA cells to GDNF withdrawal, to investigate whether the antitumorigenic RET/Pit1/Arf/p53/apoptosis pathway is maintained in these human adenomas and how the adenomas are resistant to the pathway.

## Materials and Methods

### Patient recruitment

The study was carried out in accordance with the Declaration of Helsinki and approved by Ethics Committees from every Hospital participating in this research. Written informed consent was obtained from each patient.

### Primary culture of pituitary adenomas

Reagents were from Sigma unless otherwise indicated. Tumor dispersions and cultures were performed as previously described (16). For immunofluorescence, apoptosis, and protein studies, 20 000, 50 000, or 150 000 cells/well or coverslip were seeded in fully supplemented medium (DMEM). Every 24 hours, medium was changed according to one of the following options as appropriate to the experiment: fully supplemented DMEM, DMEM supplemented with 0.5% fetal bovine serum (FBS) only, or DMEM supplemented with 0.5% FBS and 100-ng/mL human GDNF (Calbiochem).

### Immunofluorescence and real time RT-PCR studies

Immunofluorescence was performed as described (17). Antibodies and dilutions are listed in Table 1-2. For quantitative

mRNA expression studies, commercial TaqMan assays were obtained from Applied Biosystems and used according to the manufacturer's guidelines to measure mRNA expression in extracts from frozen adenoma fragments.

### Single-cell immunotyping

For immunoblot cell experiments, we followed the protocol previously reported (18). Quantification of halo intensity and area was assessed by ImageJ software (http://rsbweb.nih.gov/ij/) and expressed as the OD per pixel and in square micrometers, respectively.

### Somatotroph cell line culture, transfection, and immunodetection

Rat GH4C1 pituitary cells were cultured in DMEM containing 10% FBS. Transfection was performed by Nucleofection (Amaxa) as previously described (10). Cells were seeded in full medium. The day after, cells were washed and cultured in DMEM + 0.1% BSA for 24–48 hours with or without rat GDNF (100 ng/mL; Calbiochem) in PBS. Apoptosis was detected by Hoechst staining as described (9, 10). Cell extracts were prepared, and Western blotting was performed as previously described (9, 10, 19). Detailed procedures and a list of plasmid, vectors, siRNAs (small interfering RNA), and antibodies are detailed in Supplemental Materials and Methods 1.

### Promoter analysis

Using Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/) and PROMO (ALGGEN) (http://alggen.lsi.upc.es/), we compared −2000 bp from the ATG of the following genes: *Homo sapiens* NCBI RefSeq NG_008225.1, *Mus musculus* NCBI NC_000082.6, and *Rattus norvegicus* NC_005110.3.

### Statistical analysis

All cell-culture experiments were performed as 3 or more replicates; experiments using cell lines were also carried out at least 4 times. Nonparametric *t* tests (Mann-Whitney) were used for statistical analysis, and $P < .05$ was considered significant.

Spearman's rank correlations were performed with data from human tumors using one-tail nonparametric significance test (GraphPad).

## Results

### Establishment of relevant pituitary cultures from human adenomas

We prepared primary cultures from a series of surgically resected pituitary ACROs and NFPAs and studied the RET apoptotic pathway. All tumors were macroadenomas. Patients' clinical and pathological data are shown in Supplemental Table 1.

A portion of each tumor was obtained, dispersed, and seeded. To evaluate the percentages of somatotroph and RET-positive cells in each cell dispersion, GH, aGSU (glycoprotein hormones, common alpha subunit [CGA gene]), or RET immunofluorescent expression tests were performed in replicate wells seeded with ACRO and NFPA cells after 48 hours in culture just before starting the experiments (Figure 1 and Supplemental Table 2). ACRO tumor cell wells presented a majority of GH+ve cells, ranging from 42% to 95% of the cells per well. By comparison, RET+ve cells showed a constant positivity of around 70% of GH values. By contrast, aGSU positivity was scarce/undetectable (<6%) in ACRO-5 or ACRO-7 cells and in ACRO-6 was low but detectable (30%). In NFPA cultures, on the other hand, more than 65% of cells were aGSU+ve, but fewer than 2% were GH+ve or RET+ve. Cell secretion content was also assessed by single-cell immunotyping, in replicate cultures seeded on immobilon membranes (Supplemental Table 2). Thus, cell dispersions from NFPA were shown to be mainly positive for aGSU, whereas those from ACRO were mainly positive for GH. A few isolated cells from other pituitary types (ACTH-positive) were also present. ACRO-2 was +ve for other hormones in accordance to its plurihormonal pathology.

### GDNF deprivation induced apoptosis in ACRO, but not in NFPA, mediated by RET/Pit1/Arf/p53 activation.

The RET ligand GDNF is normally present in serum used in cell culture and is secreted by somatotrophs. In our experiments, we compared basal apoptosis in the presence

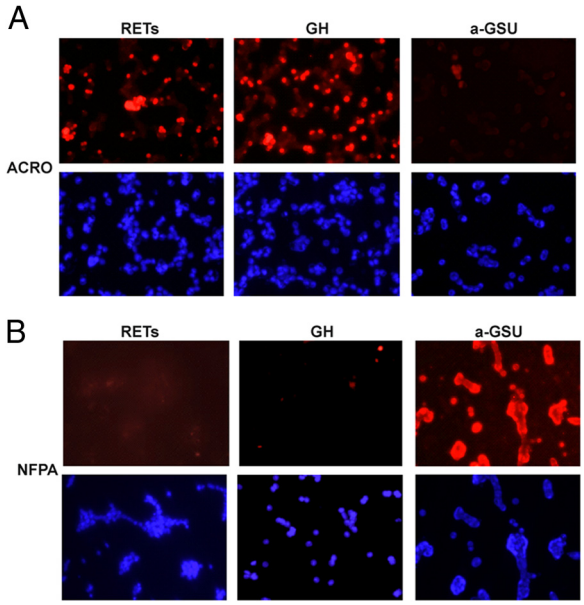



**Figure 1.** Primary cultures of human pituitary adenomas maintained their characteristic phenotypic expression. A, Immunofluorescence for RET (short isoform, RET-S) and GH shows that a majority of cells in somatotroph adenomas (ACROs) were positive for both proteins but mainly negative for aGSU. B, Nonfunctional pituitary adenomas (NFPAs) did not express RET or GH but were positive for aGSU. Nuclei are stained with DAPI (blue). (magnification, ×[400). Corresponding quantitative data are shown in Supplemental Table 2.

of 10% FBS with GDNF-deprived conditions in the presence of 0.5% FBS. Because serum deprivation per se can cause apoptosis, to determine RET-dependent apoptosis, we also measured apoptosis in the presence of GDNF (0.5% FBS + 100-ng hGDNF) (Figures 2A and 3A). ACRO and NFPA tumors showed similar basal apoptotic levels of between 5 and 25% of cells. When deprived of GDNF, all ACRO tumors showed a marked increase in apoptosis with percentages rising to 31%–62% of cells (Figure 2A). Addition of GDNF to these cultures returned apoptosis to basal levels, showing that GDNF deprivation caused RET-dependent apoptosis.

We were able to analyze protein extracts from parallel replicates of 5 cultures (ACRO-2–ACRO-6) (Figure 2B). As expected, ACRO adenoma cultures contained much GH but no aGSU or ACTH except ACRO-6, where aGSU was detected. GDNF deprivation induced a doubling of expression of Pit1 that was associated with the increase in apoptosis (Figure 2B); p14Arf and p53 levels were also correspondingly elevated. Treatment with GDNF prevented these (Pit1, p14Arf, and p53) accumulations (Figure 2B). The RET receptor appeared as a thick band or double band of 170–150 kDa in the presence of GDNF as seen in the control Western blotting (Supplemental Figure

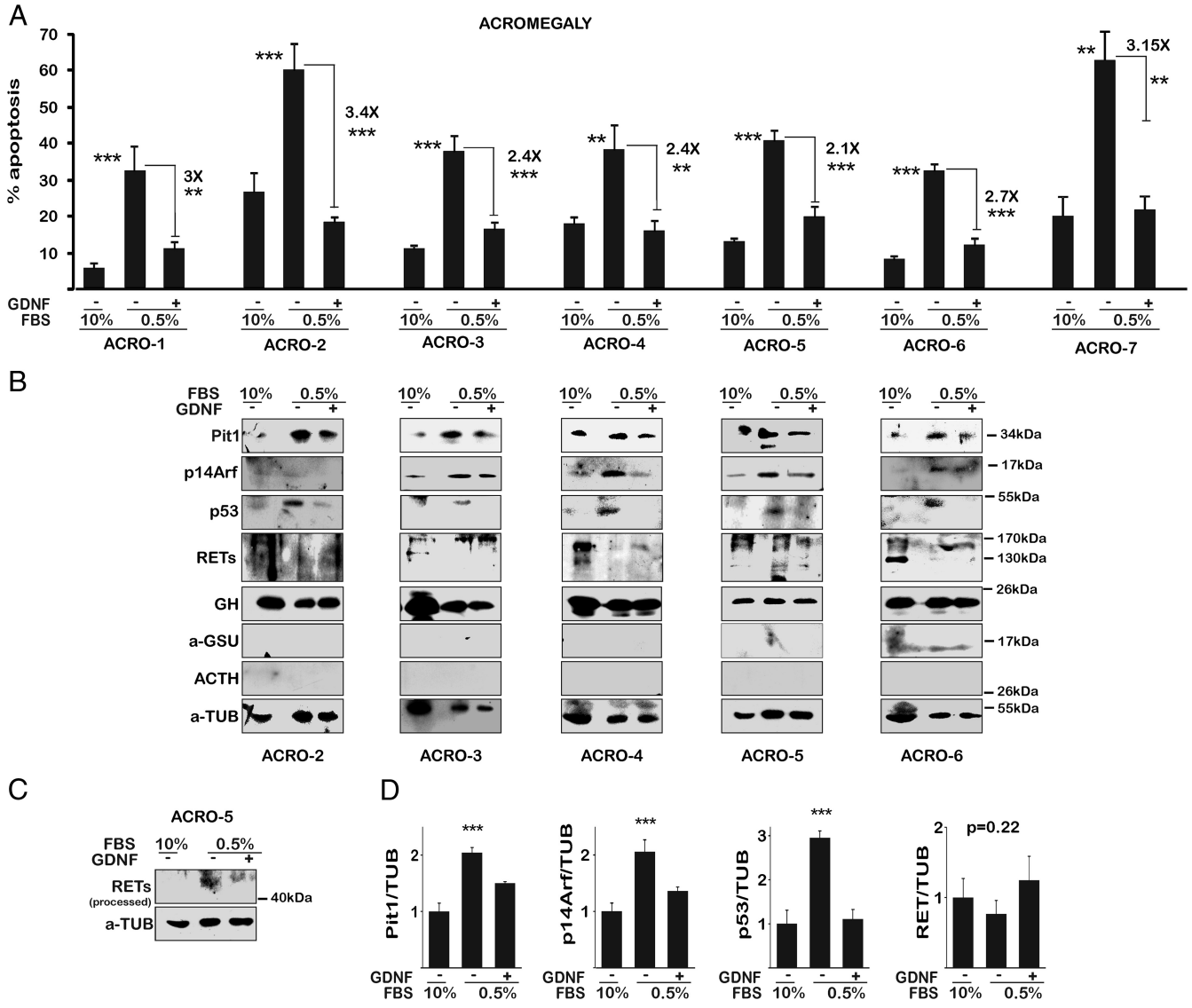



**Figure 2.** The RET/Pit1/p14Arf/p53/apoptosis pathway is conserved and active in human somatotroph adenomas (ACROs). A, Primary cultures from human ACROs were maintained in fully supplemented medium (with 10% FBS), or serum-deprived medium (0.5% FBS), with or without GDNF (100 ng/mL). Apoptosis was measured after 24 hours and protein extracted from replicates (B). Western blottings corresponding to columns in A. Increases in protein expression of Pit1, p14Arf, and p53 correlated with apoptosis and were blocked by GDNF, preventing cell death. Expression of the full-length RET receptor (170 kDa) decreased with apoptosis. C, Detection of IC-RET (45 kDa) band in GDNF-deprived extracts of ACRO-5 in relation with intracellular processing and apoptosis. GDNF prevents RET processing. D, Mean relative Pit1, p14Arf, and p53 protein expression in relation to tubulin in all ACRO cultures.

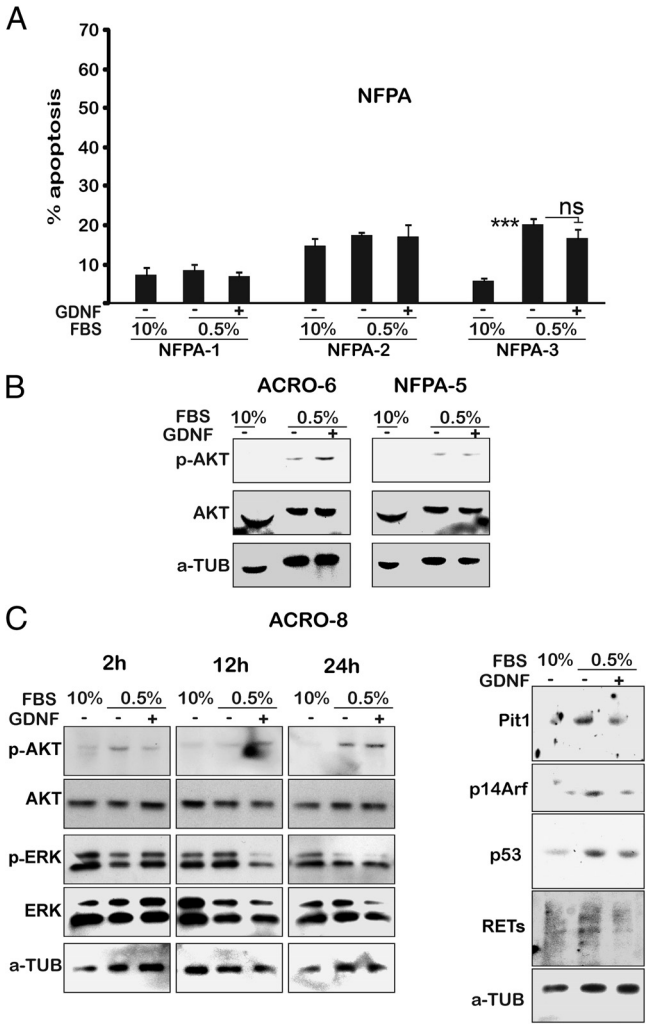



**Figure 3.** GDNF activates Akt in somatotroph adenomas (ACROs). However, neither the RET pathway nor the GDNF action is present in NFPA. A, Primary cultures of NFPAs did not show RET-dependent apoptosis. Deprivation-induced apoptosis in culture NFPA-3 was not reversed by addition of GDNF. B, In ACROs, p-AKT was markedly increased by GDNF at 24 hours. Serum deprivation induced some basal degree of AKT phosphorylation. Levels of p-AKT were unaltered in NFPAs. C, Time course of p-ERK and p-AKT activation after deprivation (0.5% FBS) with or without GDNF in comparison with full medium (10% FBS). Deprivation induces opposing oscillating activation of p-AKT (2 h high/12 h low/24 h high) and p-ERK (2 h low/12 h high/24 h low). GDNF induces a slow but steady and maintained increase in p-AKT (2 h low/12 h high/24 h highest), whereas p-ERK is progressively down-regulated (2 h high/12 h low/24 h low).

1A). When cells were deprived of GDNF, the RET bands either were reduced in intensity or fragmented in parallel with the increase in Pit1, suggesting that intracellular processing was taking place. After reintroduction of GDNF to the cultures, the higher RET band was the main one detected, suggesting that phosphorylation of RET prevented it from being processed and maintained Pit1 at basal levels. We also detected a RET band of over 45 kDa, probably attributable to the receptor being processed intracellularly (Figure 2C). This band was intense in deprived conditions and reduced in the presence of GDNF. This intracytoplasmically processed RET fragment has previously been implicated in apoptosis in human cell lines and rodent cells (9, 20). Indeed, in rat pituitary somatotrophs, this intracellular fragment per se is able to induce overexpression of Pit1 and apoptosis (9, 10). We quantified and statistically analyzed Pit1, p14Arf, p53, and RET protein expression in all Western blottings in relation to the loading control $\alpha$-tubulin (Figure 2D). Thus, when GDNF was deprived, the increase in Pit1, Arf, and p53 was significant in relation to both $\alpha$-tubulin and GH, but serum-deprived cells in the presence of GDNF expressed levels of Pit1 protein that were the same as in the presence of serum. Opposite, RET intensity was decreased in the absence of GDNF, although significance only reached $P = .22$ ($P = .14$ in relation to GH). Similar results were obtained when GH was used as the control (Supplemental Figure 1B).

We carried out parallel experiments in replicate cultures from 3 NFPA tumors (Figure 3A). Two of these (NFPA-1 and NFPA-2) did not show an increase in apoptosis in serum deprivation. NFPA-3 did show an increase in apoptosis, but this was not prevented by GDNF, indicating independence from the RET pathway. Neither GH nor ACTH was detectable in NFPA cells protein extracts (NFPA-1, NFPA-2, and NFPA-5), but all expressed aGSU (Supplemental Figure 2). No NFPA had detectable Pit1. p14Arf, p53, or RET were absent, or faintly detected but without changes upon GDNF treatment or deprivation.

## GDNF signals survival through phosphorylated AKT (p-AKT)

We have previously found that, in normal rat somatotrophs, GDNF blocks the serum deprivation-induced increase in Pit1 and apoptosis through Akt activation (9, 10). Therefore, we compared levels of p-AKT in ACRO-6 and NFPA-5 extracts (Figure 3B). Deprivation induced detectable p-AKT in both types of tumor. However, in the ACRO but not in the NFPA culture, addition of GDNF markedly increased p-AKT without altering total AKT levels.

For signal transduction through a specific pathway, there are 3 important characteristics that define the message into the cell: 1) the intensity of the phosphorylation status of the key enzyme; 2) the duration of the phosphorylation/activation of said enzyme (short vs lasting); and 3) the pattern of activation (phasic or spicky vs tonic or constant). Thus, the cells will understand different messages (survival, proliferation, secretion) from an intense but short pathway activation than from a less intense but lasting pathway activation. Albeit with different intensities, p-AKT was detected both in the absence and in the presence of GDNF and in ACROs or NFPAs.

To study the pattern of signal transduction, we performed

in another acromegaly (ACRO-8) a time course for p-AKT and p-ERK, the 2 main enzymes related to GDNF (Figure 3C) (21). Every time point after deprivation (0.5% FBS) with or without GDNF was compared with a similar replicate maintained in full medium (10% FBS). Two hours after deprivation, pERK levels drop in parallel to an increase in p-AKT. But in the presence of GDNF, p-AKTs have a slight increase, whereas p-ERK levels are maintained. Twelve hours after deprivation, p-AKT levels drop to basal and p-ERK levels rebound and raise above control levels. Opposite, in the presence of GDNF, the p-AKT activation was maintained and increased in parallel to reduced p-ERK levels. Twenty-four hours after deprivation (similar time shown for ACRO-6 and NFPA-5 in Figure 3B), p-AKTs remain high in GDNF-treated cells while start to rise again in nontreated deprived cells. At this point, p-ERKs are down-regulated in deprived cells independently of GDNF. In summary, our studies indicate that in human neoplastic somatotrophs, GDNF signals survival through a slow but steady increase in p-AKT activation.

### Human Pit1 (hPit1) can be considered an oncogene responsible for OIA

In the rat GH4C1 somatotroph cell line and in primary cultured rat pituitary somatotrophs, an increase in Pit1 above a certain threshold level was sufficient for its direct binding to and activation of p19Arf gene transcription, leading to p53 accumulation and apoptosis (10). Indeed, we proposed that rat Pit1 (rPit1) could be a type of oncogene whose presence not only is required for cell specification (GH expression) and proliferation (22) but leads to cell death in specific environments (eg, serum or GDNF deprivation) (10, 23). The latter can be considered an example of OIA. To test an intrinsic capacity for Pit1 to induce this pathway, we compared hPit1 and rPit1, by transfecting each form together with the human RET receptor (RET-S) into the GH4C1 cell line. As outcome measures, we studied apoptosis and expression of proteins in the pathway. We found that hPit1 induced apoptosis as strongly as rPit1 did (Figure 4A) and that apoptosis correlated with Pit1, p19Arf (rodent form), and p53 accumulation (Figure 4B). As expected, GDNF was only able to block the RET-induced Pit1 increase and apoptosis in RET-transfected cells and not in hPit1- or rPit1-overexpressing cells (Figure 4, A and B).

### Conserved promoter elements maintain the pathway in mammals. cEBP$\alpha$ (CCAAT/enhancer binding protein alpha, cEBPa), the key transcription factor for Pit 1 overexpression and apoptosis

The pathway by which the RET receptor induces Pit1 expression in rat somatotrophs involves Caspase-3, protein kinase C delta, and JNK (protein kinase products of the gene MAPK8), with JNK recruiting cAMP responsive element binding protein and cEBPa transcription factors to the promoter (9). Although there are 2 cEBPa consensus sites in the rPit1 promoter, the only DNA element found to be essential to the response is the −459/−447 cEBPa site. Deletion of this element abolishes the RET-dependent induction of rPit1 expression (9). We searched the promoters of the hPit1, mouse Pit1, and rPit1 genes for conserved sequences, comparing them by direct alignment and in silico using PROMO. We also compared the hPit1, rPit1, and mouse Pit1 promoters using CLUSTAL Q, to assess sequence conservation (Figure 4C and Supplemental Figure 3). Thus, we found few conserved consensus elements, including one c-Jun, 2 cEBPa, 1 RXR (retinoid X receptor), and 4 Pit1-binding sites. Of the 2 putative cEBPa elements, 1 was distal, located at −430 to −422 bp, and 1 proximal at −209 to −203 bp. Both of these were completely conserved between the 3 species. Interestingly, the distal site at −430 to −423 is the 8-bp consensus TAGCAAAA that has been found to be essential in the Pit1 response to RET using the rat promoter (Figure 4D) (9).

We studied activity of a human −1.5-kb Pit1 promoter and of 2 progressive deletions, −728 and −226 bp, in the presence of RET-S and its ligand GDNF, using the rat GH4C1 somatotroph cell line (Figure 4D). After serum deprivation, cells cotransfected with RET showed a significant increase in activity of the −1.5-kb promoter that increased with time (compare 24 and 48 h). The presence of the rat GDNF ligand blocked this effect of RET on Pit1 induction. Either the stimulatory effect of RET or the inhibitory effect of GDNF was maintained in the −728-bp promoter, which still contained 2 cEBPa sites, but was lost in the −226-bp promoter, which retained only the proximal cEBPa element. These results confirm the importance of the TAGCAAA-cEBPa-binding site in the hPit1 promoter.

To study the role of cEBPa in the RET/Pit1 pathway, we performed interference siRNA experiments. cEBPa siRNA was as efficient as Pit-1 siRNA (9) to block RET-S-induced apoptosis, because both siRNAs prevented Pit 1 protein increase above the tolerated threshold level (Figure 4, E and F). Consequently, cEBPa siRNA also blocked RET-S induction of −1.5-kb Pit 1 promoter seen in Figure 4D (data not shown). It is well known that Pit-1 is a gene autoregulated in a positive feedback loop (24–26). And our "in silico" study (Supplemental Figure 3) demonstrate that the 4 Pit-1-binding elements within the Pit-1 promoter are conserved between species (human, rat, and mouse). Intriguingly, both cEBPa and Pit 1 siRNAs prevented Pit-1-induced apoptosis, because they were also able to block the positive feedback for Pit 1 autoregulation (Figure 4, E and F). Altogether, these data suggest that

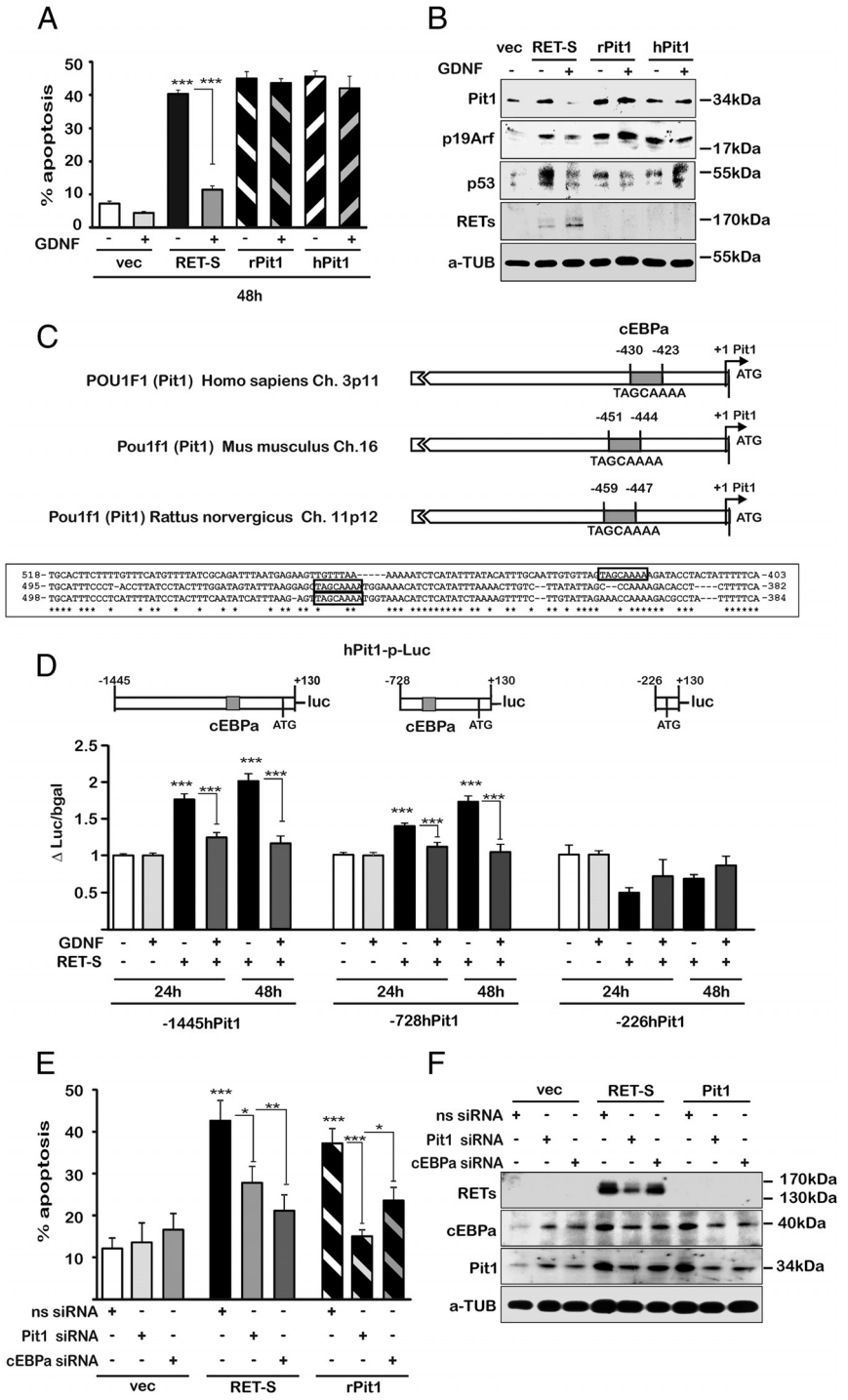
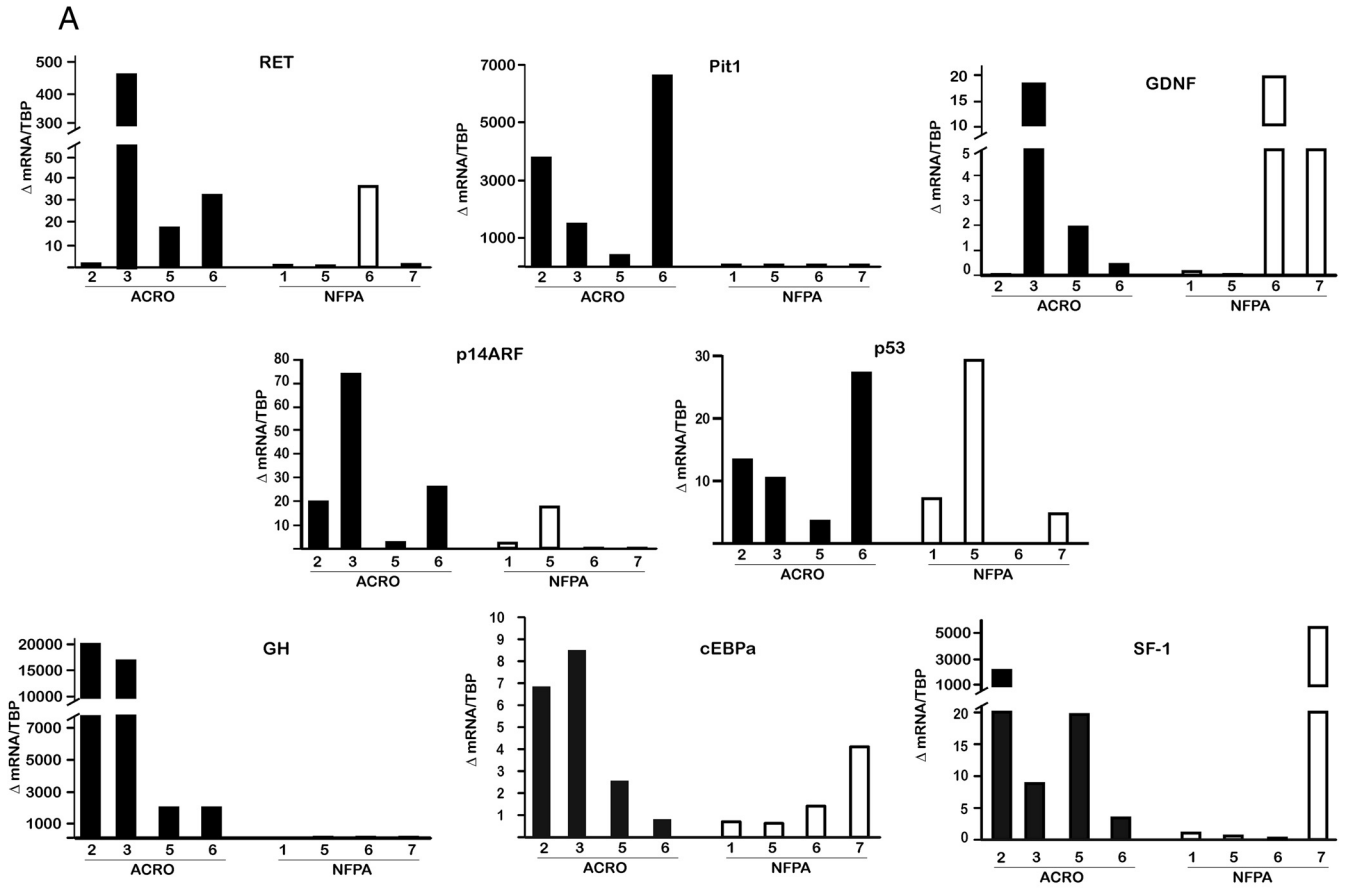



**Figure 4.** Overexpression of hPit1 in rat pituitary cells can directly activate Arf, p53, and apoptosis. A fully conserved cEBPa-binding site regulates RET-dependent hPit1 expression. cEBPa is essential for Pit-1 overexpression and apoptosis induced by RET-S or Pit-1. A, Direct induction of apoptosis by hPit1 is at similar levels to those obtained with rPit1 or RET transfection. Although GDNF prevents RET-induced apoptosis, it does not affect induction of apoptosis by hPit1 or rPit1. B, hPit1 induces rat p19Arf and p53 expression as well as rPit1 and RET. Rc, control empty vector. C, In silico studies revealed 3 elements completely conserved between the human, mouse, and rPit1 promoters, 1 c-Jun site, and 2 cEBPa sites. In the first cEBPa site, a sequence of 8 bases between −450 and −430 bp is fully conserved in the 3 species. D, Transfection of hPit1 promoter with progressive deletions, alone and together with the RET receptor, in rat GH4C1 cells. RET induces −1450 hPit1 promoter expression, which increases with time. In the presence of GDNF, this promoter is not activated by RET. Deletion of the −450-bp cEBPa site prevents RET induction. E, Both cEBPa siRNA and Pit-1 siRNA block RET-S- or Pit-1-induced apoptosis. F, cEBPa siRNA and Pit-1 siRNA prevent overexpression of the Pit-1 protein over the tolerated threshold levels.

cEBPa is implicated in the Pit 1 positive feedback loop, because cEBPa is able to reduce Pit 1 increase mediated by Pit 1 transfection, blocking Pit 1-induced apoptosis.

## Correlation between functional protein studies and whole mRNA expression in the pituitary adenomas

Our results showed that the pathway previously discovered in rodent somatotroph cells was fully functional in human ACROs, suggesting that RET is a good candidate for future therapeutic development in acromegaly. Such studies will require larger series of tumors and quantification of responses to the presence of RET and Pit1. We therefore explored the feasibility of quantitative TaqMan RT-PCR for analyzing mRNA expression of the genes implicated in the pathway in the current set of acromegalies, for which we also had analyzed protein extracts from primary cultures and had functional measurements of apoptosis. We were able to obtain total tumor RNA from 4 of the current series of ACROs (ACRO-2, ACRO-3, ACRO-5, and ACRO-6) and from 4 NFPAs, 2 from the current series (NFPA-1 and NFPA-5) and 2 from our bank (NFPA-6 and NFPA-7) (Figure 5A). Interpretation of the results must be always careful, because RNA and protein expression are not always correlated. Furthermore, tumor samples obtained from surgery, and pituitary adenomas are not an exception, have not only tumor tissue but also in more or less degree periphery remnants of the normal organ. Moreover, in pituitary adenomas, it is frequently described expression of other hormones corresponding to cells intermixed with the principal cellular component. Thus, we added a detailed immunohistochemistry analysis to help in the interpretation of the results (Figure 5B).




B

| Staining | ACRO 2 | ACRO 3 | ACRO 5 | ACRO 6 | NFPA 1 | NFPA 5 | NFPA 6 | NFPA 7 |
|---|---|---|---|---|---|---|---|---|
| GH | +++ | +++ | ++ | ++ | - | - | - | - |
| aGSU | ++ | ++ | - | ++ | -/+ | +++ | -/+ | + |
| PRL | -/+ | ++ | + | + | - | - | - | - |
| TSH | - | - | - | ++ | - | - | - | - |
| FSH | + | - | - | - | -/+ | - | -/+ | + |
| LH | ++ | - | - | - | -/+ | - | -/+ | ++ |
| ACTH | - | - | - | - | -/+ | - | - | - |
| H.E nAP% | 5 | 10 | 0 | 0 | 0 | 0 | 0 | 0 |
| Oncoc% | 0 | 0 | 0 | 0 | 0 | 0 | >50 | 0 |

C

| Correlation | r2(Spearman) | P Value |
|---|---|---|
| Pit1 vs. Arf | 0.690 | 0.035* |
| Pit1 vs. p53 | 0.405 | 0.163 |
| Arf vs. p53 | 0.667 | 0.042* |
| **RET vs. GDNF** | **0.690** | **0.035*** |
| **RET vs. Pit1** | **0.333** | **n.s.** |
| RET vs. cEBPa | 0.571 | 0.069 |
| cEBPa vs. Pit1 | 0.476 | n.s. |
| **GDNF vs. p53** | **-0.690** | **0.035*** |

**Figure 5.** In the acromegaly tumors, excess ligand GDNF is preventing RET-induced apoptosis and correlates positively with RET but negatively with p53. A, Quantitative TaqMan RT-PCR detection in whole pituitary adenomas. Four acromegalies (ACRO-2, ACRO-3, ACRO-5, and ACRO-6) were compared with 4 NFPAs. Because we only had frozen tissue from NFPA-1 and NFPA-5, we added 2 other frozen NFPAs obtained during the same period (NFPA-6 and NFPA-7). Data were obtained in relation to gene expression of the control TBP and are expressed in relation to median of the NFPA tumors (taken as value 1). RET is highly expressed in GH-secreting adenomas and is poorly expressed in NFPAs. However, in the ACRO-2 tumors, RET expression is not much bigger than in NFPAs. However, ACRO-2 primary cultures presented a strong RET-dependent apoptosis and expressed RET protein (Figure 2). On the other hand, NFPA-6 presented RET levels comparable with acromegaly. GH and Pit1 mRNA expression were present in ACROs and not in NFPAs as expected. SF-1 is a transcription factor specific for gonadotropin expression (aGSU, LH, and FSH). SF-1 levels were markedly high precisely in ACRO-2 and NFPA-7. p14Arf, p53 and GDNF were abundantly expressed in adenomas. B, Detailed immunohistochemical studies in the 8 adenomas. H.E. % nAP is the percentage of normal adenopituitary tissue present at the periphery of the tumor sample in the hematoxylin eosin staining. Immunohistochemistry was quantified and refers exclusively to the tumor adenoma area. ACRO-2 was the only acromegaly that presented FSH and LH expression in correlation to SF-1 levels. NFPA-7 also presented LH and FSH positivity not unusual in NFPAs. The only difference in NFPA-6 was the presence of more than 50% of oncocytic cells. This data could suggest that mitochondrial alterations can be related to paradoxical RET expression in NFPAs. C, Spearman's rank correlation coefficients for mRNA data. RET and GDNF were significantly positively correlated. Pit-1/Arf and Arf/p53 were also positively correlated. However, GDNF was inversely correlated with p53 possibly explaining the absence of significant correlation between RET and Pit-1.

Three of the 4 ACROs (ACRO-3, ACRO-5, and ACRO-6), as expected, had a high level of RET mRNA expression, between 20 and 500 times higher than the median level of NFPA. Also as expected, 3 of the 4 NFPA cultures (NFPA-1, NFPA-5, and NFPA-7) showed very low RET mRNA expression. Despite the low, but detect-

able, level of RET mRNA expression, ACRO-2 cultures exhibited a good degree of GDNF-preventable apoptosis (Figure 2). NFPA-6, on the other hand, presented a level of RET expression compatible with acromegaly. This appeared to be an anomalous result, because all other NFPA in which we have analyzed protein expression, both in the current and previous studies, have been negative for RET expression (8). Whether this could be related to the heterogeneity of NFPA is unclear at present. In any event, GDNF seemed to follow a pattern related to RET expression. Pit1 and GH mRNAs were highly expressed in the 4 ACRO samples and difficult to detect in the NFPA samples. p14Arf and TP53 were also highly expressed in ACRO and less in NFPA samples. The gonadotroph-specific SF-1 (steroidogenic factor 1) transcription factor was low in ACRO-3, ACRO-5, and ACRO-6 but very high in ACRO-2 in a pattern inversely mirroring RET expression in this group. Using immunohistochemistry, we could see that ACRO-2 contained LH+ and FSH+ cells, which could explain the relative reduction of RET expression compared with total RNA obtained from the tumor (Figure 5B). In agreement with this, SF-1 mRNA expression was also high in NFPA-7, in which LH+ and FSH+ cells were also detected. There are a number of reports in the literature that describe SF-1 detection in ACROs by RT-PCR and immunohistochemistry (27–31). SF-1 is a transcription factor required for transcription of the aGSU gene (32, 33). However, SF-1 is only one of a combination of transcription factors required to activate aGSU promoter, the other being DAX-1 (transcription factor product of the gene NR0B1) and nuclear receptor correpressor (34). In vivo deletion of SF-1 markedly reduces the presence of aGSU cells in the pituitary (35, 36). But the elements in the mouse SF-1 gene that regulate expression in the pituitary "in vivo" are different than the ones that regulate expression in the hypothalamus, gonads, and adrenals, the difference being the "methylation" status of a pituitary-specific enhancer (37, 38).

We are not aware of any study regarding the methylation status of the human SF-1 gene pituitary-specific enhancer in pituitary adenomas. It could be interesting to know whether a demethylation occurs in some acromegaly tumors. On the other hand, the possibility of a quadruple positive (SF-1+Pit-1+aGSU+GH) precursor cell as the origin for some pituitary adenomas has not been definitely ruled out.

In summary, for the characterization in our series, SF-1 was high in those tumors with clear-cut positive immunostaining for gonadotropins (LH and FSH), ie, NFPA-7 (gonadotropic adenoma) and ACRO-2 (plurihormonal adenoma). SF-1 was very low in those tumors with (near) negative immunostaining for gonadotropins (NFPA-1, NFPA-5, and NFPA-6). And in ACRO-3, ACRO-4, and ACRO-6, there is the doubt of whether the GH adenomas are usually able to coexpress SF-1/aGSU or whether the expression is due to a partial contamination with normal pituitary tissue (although the possibility had been microscopically ruled out for ACRO-5 and ACRO-6).

NFPA-6, by contrast, did not contain any GH+, or Pit1+ cells that could have explained its high RET mRNA expression. The only difference that we could find between NFPA-6 and the other NFPA was that it contained more than 50% oncocytic cells. Because oncocytic changes in pituitary tumors originate from DNA mutations in somatic complex I of the mitochondrial genome (39), we think that apoptosis could already be affected in this tumor, thus allowing aberrant RET expression.

The mRNA results were analyzed using the Spearman's rank correlation test analysis (Figure 5C). As expected, Pit-1 was directly correlated with p14Arf and Arf with p53 mRNA expression. However, against expected, RET was not significantly correlated with Pit-1 expression. This is opposite to what was previously found in normal rat somatotrophs and contradicts the results found in the primary cultures of ACRO adenomas (Figure 2). Notably, RET was significantly and directly correlated with GDNF expression ($r^2 = 0.69$, $P = .035$), and when we studied the correlation of GDNF with p53, we found a significant negative correlation ($r^2 = -0.69$, $P = .035$). Both results indicate that in the pituitary ACRO adenomas, the constant production of GDNF restrains the action of RET onto the Pit-1 gene, functionally blocking the apoptotic RET/Pit1/Arf/p53 pathway.

## Discussion

Acromegaly is caused by a tumor of somatotroph origin, in which the cells maintain their GH-production and secretion phenotype through expression of the transcription factor Pit1 (40). Indeed, Pit1 is essential for maintenance of differentiation of somatotrophs, because expression of both GH and the receptor for the trophic hypothalamic factor GHRH (GHRHR) is absolutely dependent on Pit1 expression (41, 42). In conjunction with other transcription factors, Pit1 is also required for proliferation of somatotrophs (22, 43). A third function for Pit1 has previously been described by our group, in rodent somatotrophs, that Pit1 can act as a key inductor of apoptosis (9).

In the current study, we show that the RET/Pit1 apoptotic pathway is both active and regulated in human somatotroph adenomas, at the tissue and cell level. Although the number of tumors and cultures was relatively small in

our study, acromegaly is a rare disease, and very few, if any, previous studies have combined clinical data with tissue, cell, and functional studies. We have also shown that a cEBPa site is fully conserved between the hPit1, rPit1, and mouse Pit1 promoter. This is important because it is the main site of regulation of Pit1 by RET. In fact, silencing of cEBPa blocks RET-induced apoptosis, preventing the rise of Pit-1 above the tolerated threshold levels. Chromatin immunoprecipitation (ChIP) studies in human somatotrophs are lacking due to technical constraints. However, in rat somatotrophs, such studies show that there is strong cEBPa binding to the Pit1 promoter and, further, that this is dependent on RET intracellular processing and blocked by GDNF, Caspase-3 inhibitors, rottlerin, and the dominant-negative mutant killer-cAMP responsive element binding protein (9). cEBPa is also known for its differentiation-inducing capability (44). Studies in rat pituitary cell lines have demonstrated that cEBPa and Pit1 colocalize and interact in the nucleus of lactotroph cells (45) and are able to regulate GH and PRL expression (23). Other cEBP family members have also been implicated in growth and secretion of lactotrophs (46).

The RET receptor provides an important link between differentiation and apoptotic pathways in somatotrophs. The relationship between proliferation, differentiation, and apoptosis is complex, but several oncogenes are known to function in more than 1 of these processes. For example, although Ras and cMyc are both known for their tumorigenic effects through promotion of cell cycle, both oncogenes can induce OIS or OIS oncogene-induced apoptosis or senescence in specific situations. Ras can augment an epithelial phenotype when overexpressed in noncancerous epithelial cells (47, 48), whereas cMyc is able to induce differentiation in skin epithelial stem cells (reviewed in Ref. 49). Indeed, the concept of oncogene-induced differentiation should arguably be added to the repertoire.

Because Pit1 is essential for somatotroph differentiation, proliferation and, as we have shown, apoptosis, it can arguably be considered as an oncogene in the same group as Ras and cMyc. On the other hand, the distinctness of Pit1 in relation to these other oncogenes could reside in its ability to participate more in differentiation and apoptosis and being only permissive for cell growth. Indeed, the growing evidence that Pit1-dependent (maintenance of) differentiation and apoptosis pathways are so intertwined might provide a clue as to how somatotroph tumors remain benign in terms of growth rather than evolving into carcinomas.

RET protein and mRNA are both expressed by normal somatotrophs and in somatotrophic tumors, as shown previously (8) and in the current study. Because our mRNA data were not quantitatively consistent with protein expression, however, we believe that detection of mRNA expression in whole tumors should be supported by protein and functional studies.

Our results also suggest a potential new avenue for the treatment of human acromegaly. Thus, although in the absence of GDNF RET is clearly acting as a dependence receptor, being intracellularly processed and activating the Pit1/p14Arf/p53/apoptotic pathway, we show that tumors overexpress the ligand GDNF inducing RET tyrosine kinase when dimmerization and p-AKT, and promoting cell survival by blocking p53 accumulation. pAKT has been described as a constant pathway activated in pituitary adenomas when compared with the normal pituitary tissue (50). In 2 different series, activating point mutations or amplifications of the catalytic subunit of the phosphatidylinositol-4,5-bisphosphate 3-kinase (PIK3CA) have been found in some (10%–25%) pituitary adenomas (51, 52). Moreover, down-regulation of phosphate and tensin homolog (inhibitor of pAKT) through microRNA in acromegaly has recently been proven in a subset of aggressive tumors (53).

Taken together, these data strongly suggest that ACROs are absolutely dependent on a GDNF-rich environment to survive. We therefore believe that in our current culture experiments, we have observed a rupturing into the GDNF-RET somatotroph survival loop and that pharmacological tools might accordingly be developed to take advantage of this phenomenon (Supplemental Figure 4). The potential therapeutic implications of such tools at least merit further investigation into alternatives to dopamine and somatostatin analogs, and pegvisomant, for future treatments of resistant acromegalies. Given that there are currently clinical trials of new RET kinase inhibitors in several endocrine tumors (21), our work provides mechanistic insight and scientific support to future preclinical studies of RET inhibitor therapy in aggressive acromegaly.

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