

# Evidence for Growth Hormone (GH) Autoregulation in Pituitary Somatotrophs in GH Antagonist-Transgenic Mice and GH Receptor-Deficient Mice

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**Growth hormone (GH) modulates the hypothalamic release of somatostatin and GH-releasing hormone; however, there has been no evidence of GH autoregulation on the pituitary somatotroph. To determine the effects of GH on its own regulation, we examined the pituitaries of giant transgenic mice expressing a GH agonist (E117L), dwarf transgenic mice expressing a GH antagonist (G119K), and dwarf mice devoid of the GH receptor/binding protein (GHR/BP). In the E117L transgenic mice, the number and distribution of pituitary GH-immunoreactive cells were unchanged from nontransgenic littermate controls; an ultrastructural examination revealed typical, densely granulated somatotrophs. In contrast, the pituitaries of the G119K mice contained both moderately granulated somatotrophs and a sparsely granulated (SG) population with well-developed synthetic organelles and a distinct juxtannuclear globular GH-staining pattern. GHR/BP-deficient mice exhibited a marked reduction in the intensity of cytoplasmic GH immunoreactivity; however, prominent GH staining in the juxtannuclear Golgi was seen. GH-immunoreactive cells were increased in number, and the reticulin network pattern was distorted; stains for proliferating cell nuclear antigen confirmed mild hyperplasia. Electron microscopy showed that the somatotrophs were hyperactive SG cells with prominent endoplasmic reticulum membranes, large Golgi complexes, and numerous mitochondria. These findings are consistent with synthetic and secretory hyperactivity in pituitary somatotrophs due to the reduced GH feedback regulation. The changes are most striking in animals that are devoid of GHR/BP and less marked in animals expressing a GH antagonist; both models had reduced insulin-like growth factor-I levels, but the more dramatic change in the GHR/BP animals can be explained by abrogated GH signaling. This represents**

**the first evidence of direct GH feedback inhibition on pituitary somatotrophs, which may have implications for the use of GH analogs in different clinical settings. (Am J Pathol 2000, 156:1009–1015)**

Growth hormone (GH) secretion is under the complex control of the hypothalamus with predominant stimulation by GH-releasing hormone (GHRH) and GH-related peptide (GHRP) and inhibition by somatostatin (SRIH).<sup>1</sup> These effects are modulated by peripheral negative feedback signals, including the target growth factor of GH insulin-like growth factor-I (IGF-I), certain amino acids and nutrient metabolites, and other hormones, including glucocorticoids, that act at the level of the adenohypophysis and the hypothalamus.<sup>1</sup> GH itself can alter its own regulation at the level of the hypothalamus, where it modulates the release of GHRH and SRIH.<sup>1</sup> Thus far, however, there has been little evidence for a more direct role for GH in the autoregulation of the pituitary somatotroph.<sup>2</sup>

To determine whether GH participates in an autocrine feedback mechanism at the level of the pituitary somatotroph, we studied the pituitaries of giant transgenic mice expressing a GH agonist (E117L), dwarf transgenic mice expressing a GH antagonist (G119K), and dwarf mice that were devoid of the GH receptor/binding protein (GHR/BP).

## Materials and Methods

### Transgenic and GH Receptor-Deficient Mice

Production and characterization of transgenic mice expressing either *bGH-E117L* (GH agonist) or *bGH-G119K* (GH antagonist) genes have been described in detail.<sup>3,4</sup> The serum from the G119K mice contained approximately 2 µg/ml of the GH antagonist, whereas bGH levels in the E117L mice were approximately 0.55 µg/ml. The IGF-1 levels of the E117L animals were approximately 800 ng/ml, whereas the G119K animals possessed levels of approximately 150 µg/ml. The control animals had IGF-1 levels of 350 ng/ml.<sup>5</sup>

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**Table 1.** Physiological Features of Animals Studied

GH Agonist-Transgenic Mice	GH-Antagonist Transgenic Mice	GHR/BP <sup>-/-</sup> Mice
Giant phenotype; body weight 150% of control	Proportionate dwarf phenotype; body weight 60% of control	Proportionate dwarf phenotype; body weight 45% of control
Pituitary weight 145% of control	Pituitary weight 58% of control	Pituitary weight 58% of control
GH ↑ (agonist bGH)	GH ↑ (antagonist bGH)	GH ↑ (endogenous 30× control) <sup>6</sup>
IGF-1 ↑ (226% of control) <sup>5</sup>	IGF-1 ↓ (42% of control) <sup>5</sup>	IGF-1 ↓ (10% of control) <sup>6</sup>

The production of mice with a disrupted *GHR/BP* gene has been described.<sup>6</sup> The GHR/BP<sup>-/-</sup> mice were genotyped by polymerase chain reaction as reported.<sup>7</sup> The homozygous animals had decreased levels of IGF-1 and elevated serum GH concentrations.

### Morphological Methods

The 5-month-old male mice of each group and equal numbers of the age-matched littermate controls were sacrificed by decapitation. At autopsy, the pituitaries were removed and weighed, and the other organs were carefully inspected, weighed, and measured.

For light microscopy, sections of the autopsied tissues were fixed in buffered formalin and embedded in paraffin; 4- to 5- $\mu$ m-thick sections were stained with hematoxylin and eosin. The pituitaries were also stained with the Gordon-Sweet silver method to demonstrate the reticulin fiber network.

Immunocytochemical stains to localize adenohypophysial hormones were performed using the streptavidin-biotin-peroxidase complex technique. Primary polyclonal antisera directed against rat pituitary hormones were used at the specified dilutions: GH, 1:2500; prolactin, 1:2500;  $\beta$ -thyroid-stimulating hormone ( $\beta$ -TSH), 1:3000;  $\beta$ -follicle-stimulating hormone ( $\beta$ -FSH), 1:600;  $\beta$ -luteinizing hormone ( $\beta$ -LH), 1:2500 (National Hormone and Pituitary Program, Rockville, MD); and adrenocorticotropin prediluted preparation, which was further diluted 1:20 (Dako, Carpinteria, CA). To evaluate cell proliferation, a monoclonal antibody directed against proliferating cell nuclear antigen (PCNA) from Novocastra (Newcastle-On-Tyne, UK) was applied at 1:4000. The entire adenohypophysial area of the immunostained sections was analyzed, and the percentage of immunoreactive cells was determined for each hormone and for PCNA staining.

Double staining was performed to colocalize PCNA and the various pituitary hormones as described previously.<sup>8</sup> For these stains, the cytoplasmic hormone reactivity identified by polyclonal antisera was detected with streptavidin-biotin-peroxidase (Autoprobe III detection system; Biomeda, Foster City, CA) and visualized with the chromogen NovaRED Substrate (Vector Laboratories, Burlingame, CA). The nuclear-PCNA reactivity identified by the monoclonal antibody was localized with the Vectastain elite detection system (Vector Laboratories) and revealed using cobalt 3,3'-diaminobenzidine.

For electron microscopy, small pieces of adenohypophysial tissue were fixed in 2.5% glutaraldehyde, post-fixed in 1% osmium tetroxide, dehydrated in graded

ethanols, processed through propylene oxide, and embedded in epoxy resin. Semithin sections were stained with toluidine blue, and appropriate areas were selected for the fine structural study. Ultrathin sections were stained with uranyl acetate and lead citrate and investigated with a Philips CM 100 electron microscope.

### Results

#### Gross Appearance

The transgenic mice expressing the E117L agonist were large and resembled animals that overexpress wild-type GH, as described previously.<sup>3</sup> The animals weighed 150% of controls. The pituitaries were proportionately larger than those of the controls (145% of control) but anatomically normal.

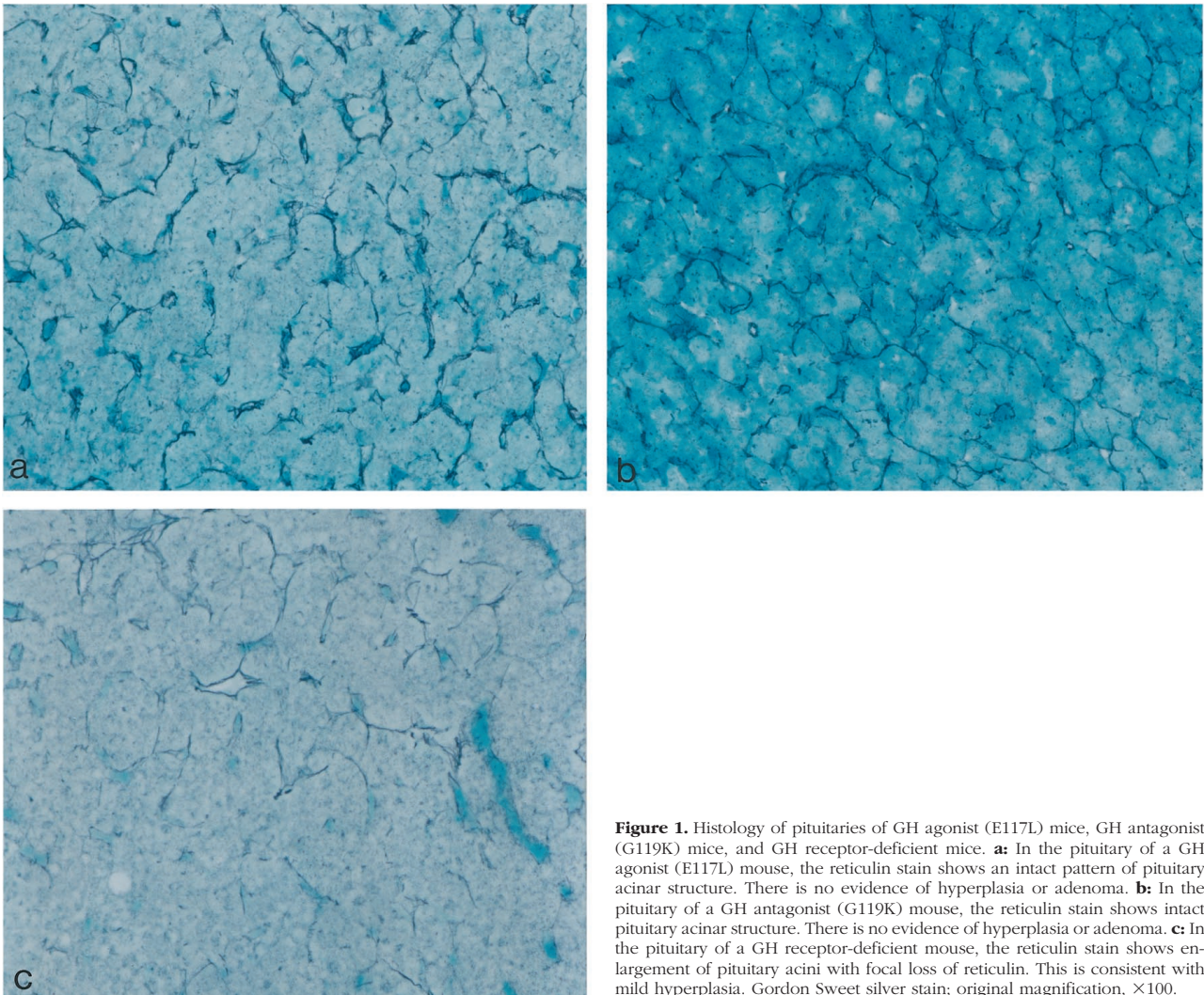
The transgenic mice expressing the G119K antagonist exhibited visible somatic-growth suppression (weights 60% of controls) as previously documented.<sup>9</sup> These pituitaries were proportionately smaller than those of the controls (58% of controls) but again were anatomically normal for the size of the animal.

The mice that were deficient in the GH-receptor also exhibited visible somatic-growth suppression (weights 45% of controls) as previously documented.<sup>6,9</sup> Their pituitaries were smaller than those of the controls (58% of controls), but in this model the reduction of the pituitary was less than that of the overall weight.

The physiological features of these mice, including size, GH, IGF-1, and GHR/BP levels, are detailed in Table 1.

#### Morphological Findings

In the E117L transgenic mice, all organs were proportionately large but exhibited a relatively normal morphology. In the adenohypophysis, the architecture was normal with an intact reticulin-fiber network delineating acini of a normal and consistent size (Figure 1a). The distribution of pituitary GH-immunoreactive cells (64%–68%) was unchanged from the controls (62%–69%). GH immunoreactivity was found as diffuse cytoplasmic staining in the somatotrophs of the controls (Figure 2a) and the transgenic mice (Figure 2b). Electron microscopy demonstrated the presence of somatotrophs with relatively scant profiles of the rough endoplasmic reticulum, small Golgi complexes, and moderate numbers of large, round, evenly electron-dense secretory granules (Figure 3a). Lactotrophs, corticotrophs, thyrotrophs, and gonadotrophs were all identified in normal proportions: lactotrophs, 15% to 20%; corticotrophs, 5% to 7%; thyro-



**Figure 1.** Histology of pituitaries of GH agonist (E117L) mice, GH antagonist (G119K) mice, and GH receptor-deficient mice. **a:** In the pituitary of a GH agonist (E117L) mouse, the reticulin stain shows an intact pattern of pituitary acinar structure. There is no evidence of hyperplasia or adenoma. **b:** In the pituitary of a GH antagonist (G119K) mouse, the reticulin stain shows intact pituitary acinar structure. There is no evidence of hyperplasia or adenoma. **c:** In the pituitary of a GH receptor-deficient mouse, the reticulin stain shows enlargement of pituitary acini with focal loss of reticulin. This is consistent with mild hyperplasia. Gordon Sweet silver stain; original magnification,  $\times 100$ .

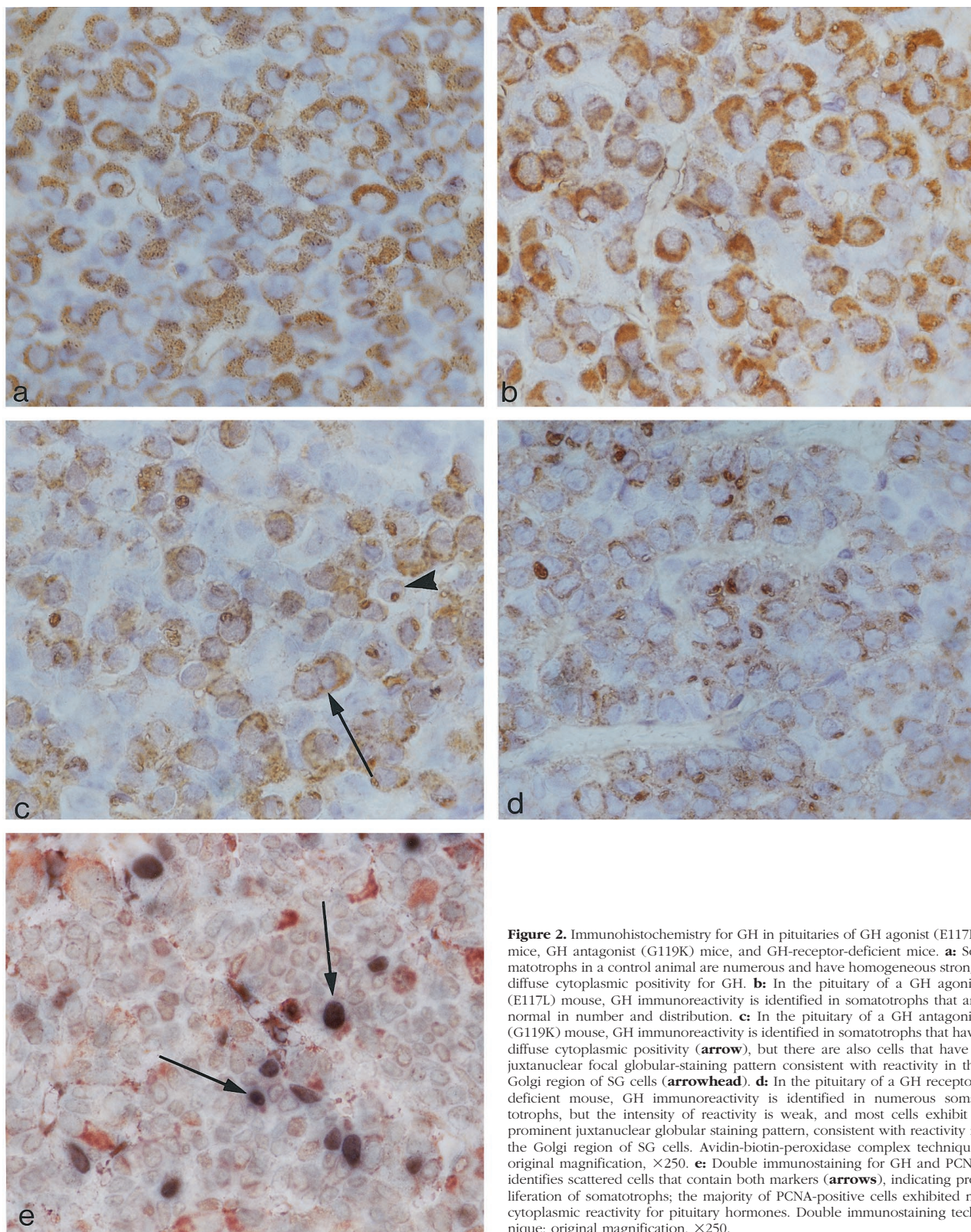
trophs, approximately 3%; and gonadotrophs, 10% to 15% of the adenohypophysial cell population. These cells exhibited normal morphology at the light and electron microscopic level (not shown). In general, gonadotrophs were relatively inactive.

The G119K transgenic mice had proportionately small organs; however, their microscopic appearance was not detectably different from the controls with the exception of the adenohypophysis. The histological appearance of the gland was not different from that of the normal control animals, and the reticulin pattern indicated preservation of the acinar structure (Figure 1b). The pituitaries contained immunoreactive GH cells that were not detectably increased in number (65–68% of cells) and were normally distributed, but they exhibited unusual immunohistochemical features. Unlike the control tissues with numerous diffusely and strongly immunoreactive, densely granulated cells (cf Figure 2a), the somatotrophs comprised a mixed population of diffusely immunoreactive, moderately granulated cells and sparsely granulated (SG) cells with a distinctly juxtannuclear globular-staining pattern for GH (Figure 2c). Electron microscopy confirmed the presence of moderately granulated soma-

trophs with well-developed endoplasmic reticulum and large Golgi regions, as well as an SG population with well-developed synthetic organelles and a distinct juxtannuclear globular Golgi complex (Figure 3b). Other adenohypophysial cell types were present in normal numbers (as above) and exhibited a normal ultrastructural morphology.

The GHR/BP-deficient mice exhibited an obvious dwarf phenotype not different from the G119K transgenic mice; they also had smaller organs but with no discrete pathology. In the pituitaries of these animals that lack the GH receptor binding, there was obvious disruption of the reticulin-fiber network with expansion of acini with loss of reticulin (Figure 1c); this change was diffuse throughout the gland, and no focal lesion was identified. There was an increase in the proportion of GH-immunoreactive cells (76% to 79%, compared with controls, 61% to 67%) and a marked reduction in the intensity of cytoplasmic GH immunoreactivity in individual cells; the numerous somatotrophs exhibited prominent, juxtannuclear Golgi positivity (Figure 2d). The immunoreactivity for other pituitary hormones was retained in a normal distribution, but the percentages of the immunoreactive cells for each hor-



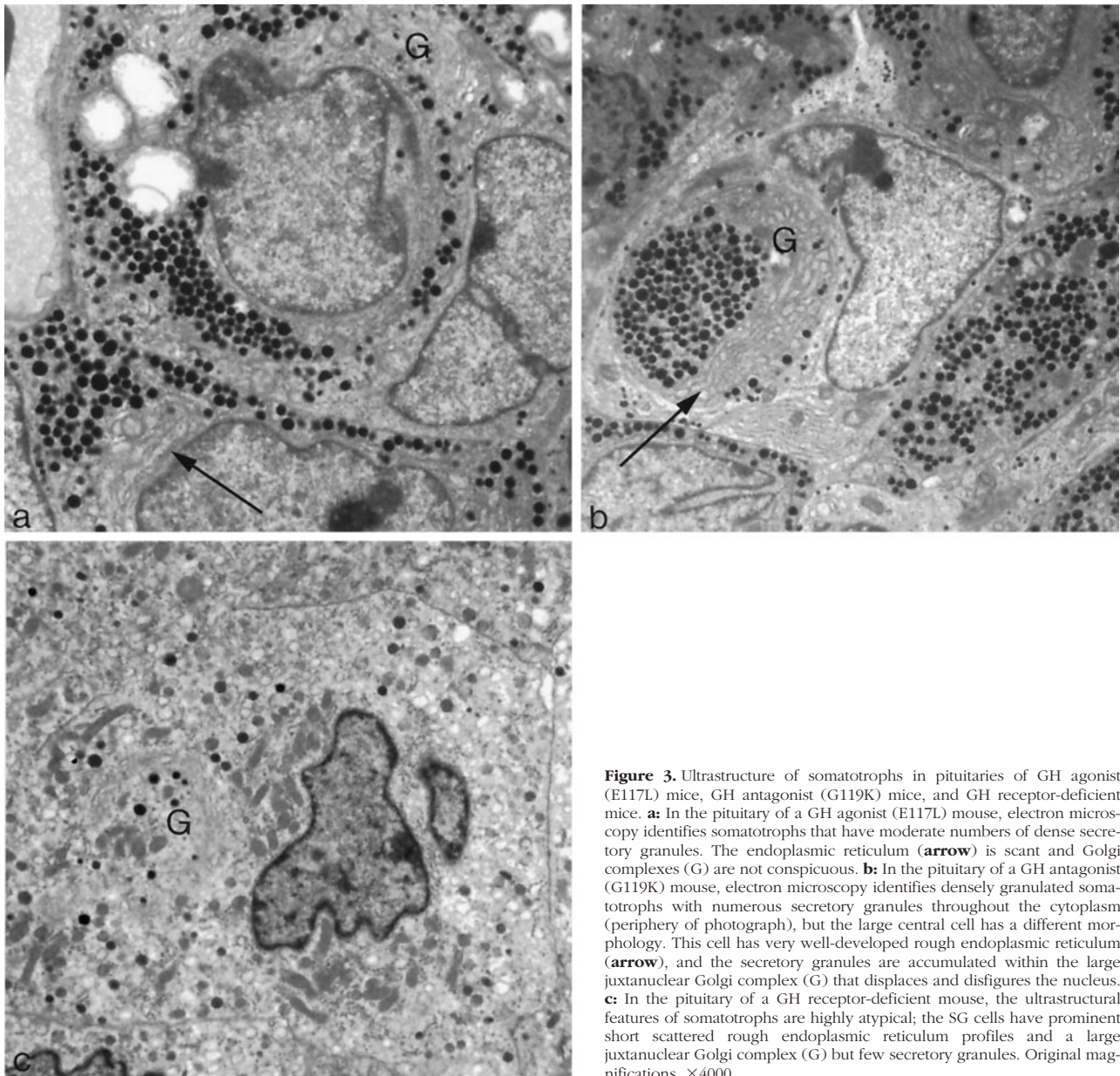


**Figure 2.** Immunohistochemistry for GH in pituitaries of GH agonist (E117L) mice, GH antagonist (G119K) mice, and GH-receptor-deficient mice. **a:** Somatotrophs in a control animal are numerous and have homogeneous strong, diffuse cytoplasmic positivity for GH. **b:** In the pituitary of a GH agonist (E117L) mouse, GH immunoreactivity is identified in somatotrophs that are normal in number and distribution. **c:** In the pituitary of a GH antagonist (G119K) mouse, GH immunoreactivity is identified in somatotrophs that have diffuse cytoplasmic positivity (**arrow**), but there are also cells that have a juxtannuclear focal globular-staining pattern consistent with reactivity in the Golgi region of SG cells (**arrowhead**). **d:** In the pituitary of a GH receptor-deficient mouse, GH immunoreactivity is identified in numerous somatotrophs, but the intensity of reactivity is weak, and most cells exhibit a prominent juxtannuclear globular staining pattern, consistent with reactivity in the Golgi region of SG cells. Avidin-biotin-peroxidase complex technique; original magnification,  $\times 250$ . **e:** Double immunostaining for GH and PCNA identifies scattered cells that contain both markers (**arrows**), indicating proliferation of somatotrophs; the majority of PCNA-positive cells exhibited no cytoplasmic reactivity for pituitary hormones. Double immunostaining technique; original magnification,  $\times 250$ .

lactotrophs, 10% to 15%; corticotrophs, 3% to 4%; thyrotrophs, approximately 2%; and gonadotrophs, 6% to 8% of the adenohypophysial cell population. The gonadotrophs were

characterized by weak hormonal reactivity. These features were consistent with a subtle and diffuse hyperplasia of the hyperactive somatotrophs. By electron microscopy, typical somatotrophs were not recognized; instead,





**Figure 3.** Ultrastructure of somatotrophs in pituitaries of GH agonist (E117L) mice, GH antagonist (G119K) mice, and GH receptor-deficient mice. **a:** In the pituitary of a GH agonist (E117L) mouse, electron microscopy identifies somatotrophs that have moderate numbers of dense secretory granules. The endoplasmic reticulum (**arrow**) is scant and Golgi complexes (G) are not conspicuous. **b:** In the pituitary of a GH antagonist (G119K) mouse, electron microscopy identifies densely granulated somatotrophs with numerous secretory granules throughout the cytoplasm (periphery of photograph), but the large central cell has a different morphology. This cell has very well-developed rough endoplasmic reticulum (**arrow**), and the secretory granules are accumulated within the large juxtannuclear Golgi complex (G) that displaces and disfigures the nucleus. **c:** In the pituitary of a GH receptor-deficient mouse, the ultrastructural features of somatotrophs are highly atypical; the SG cells have prominent short scattered rough endoplasmic reticulum profiles and a large juxtannuclear Golgi complex (G) but few secretory granules. Original magnifications,  $\times 4000$ .

there was a prominent population of hyperactive SG cells with prominent endoplasmic reticulum membranes, large Golgi complexes, and numerous mitochondria (Figure 3c). Other adenohypophysial cell types exhibited normal ultrastructural morphology, but gonadotrophs were relatively inactive.

In the pituitaries of the control animals and the G119K animals, PCNA labeled 3% to 6% of the adenohypophysial cells. In the pituitaries of the E117L animals, PCNA labeled 5% to 9% of the adenohypophysial cells, and in the GHR/BP-deficient mice, there was an increased PCNA index from 10% to 14% of the adenohypophysial cells, consistent with mild hyperplasia. Colocalization studies identified PCNA-nuclear reactivity in scattered GH-positive cells (Figure 2e), but the majority of PCNA-positive nuclei were found in cells that harbored no detectable hormone staining.

## Discussion

The role of GH in regulating its own expression and secretion is governed by a multilevel system of endocrine, autocrine, and paracrine interactions between GH and its putative regulators. These include central factors such as GHRH, GHRP, and SRIF, as well as peripheral factors, including IGF-I and GH itself. GHRH and GHRP favor GH secretion, whereas SRIF inhibits GH secretion as is evidenced by *in vitro* and *in vivo* studies.<sup>1</sup> *In vivo* GH administration reduces GH responsiveness to GHRH stimulation in humans.<sup>10</sup> This rapid inhibitory effect occurs prior to a corresponding rise in plasma IGF-I. These findings have been cited as evidence that reduces the likelihood that IGF-1 is responsible for the feedback observed.<sup>11</sup> The role of central GHRH, SRIF, and GH is further complicated by the reciprocal interactions be-

tween the GHRH and SRIF regulation within the hypothalamus. GHRH and SRIF expression are closely coupled with evidence that SRIF neurons from the periventricular nuclei synapse on GHRH neurons in the arcuate nucleus.<sup>12</sup> These findings are suggestive of hypothalamic control of an inverse relationship between GHRH and SRIF. Moreover, GH appears to also suppress the hypothalamic expression of the GH receptor.<sup>13</sup> The intracerebroventricular administration of an antisense GH receptor oligonucleotide augments GH secretion while reducing the hypothalamic SRIF expression in the rat.<sup>14</sup> These findings suggest a role for the GH receptor, as well as SRIF, in mediating the inhibition of GH-induced negative feedback. Additionally, GH has been suggested to decrease GHRP receptor expression,<sup>15</sup> further adding to the central indirect mechanisms by which GH can modulate itself. Peripheral negative feedback inhibition by IGF-I on the somatotroph has been well characterized. The *in vivo* as well as *in vitro* administration of IGF-I has been shown to reduce GH secretion and gene transcription.<sup>1</sup>

The third  $\alpha$ -helix of GH is a critical domain involved in GH receptor signaling and biological properties.<sup>4,9,16,17</sup> The substitution of Gly-119 with a variety of amino acids results in GH antagonists, ie, GH analogs, that inhibit GH-receptor signaling systems.<sup>3</sup> Indeed, we have shown that animals transgenic for this substitution are dwarfs<sup>3</sup> and that these animals demonstrate diminished liver and kidney GH-binding activity as well as circulating plasma IGF-I. Thus, this or similar analogs may represent a new pharmacologic approach to the manipulation of the GH/IGF-I axis in various medical conditions. For example, acromegaly is a disease that is characterized by GH and IGF-I excess due to a somatotroph adenoma in which peripheral GH antagonism represents a potential targeted treatment strategy.<sup>18</sup> However, in such patients, the loss of negative feedback inhibition due to the GH antagonists may, in concert with the associated reduction in IGF-I, be envisioned to result in somatotroph hyperplasia and pituitary enlargement.

Pituitary somatotrophs are a remarkably stable cell population in the adenohypophysis. Their number, immunoreactivity, and ultrastructure remain unchanged in various pathological conditions. They are characteristically densely granulated cells with large homogeneous secretory granules. Whereas there may be an increase in the amount of rough endoplasmic reticulum and a prominence of the Golgi region in situations of chronic hyperstimulation by GHRH,<sup>19–21</sup> the functionally suppressed somatotroph cannot be distinguished from a somatotroph that is actively synthesizing and secreting hormone in the normal state. This is evident in the somatotrophs of patients with idiopathic GH deficiency; their somatotrophs are clearly in a resting state and can be stimulated by GHRH,<sup>22</sup> but they are morphologically indistinguishable from normal somatotrophs.<sup>23</sup> It is also true of adenomatous somatotrophs that are exposed to somatostatin analogs.<sup>24</sup> The only morphologically altered somatotroph is the SG variant, a cell type that has been identified exclusively in human pituitary adenomas<sup>25</sup> and as a transient phenomenon in the human fetal pituitary during midgestation, when circulating GH levels reach acromegalic

proportions.<sup>26</sup> These SG somatotrophs are characterized by a significant reduction in the number and size of secretory granules, a large Golgi complex, and a unique collection of keratin filaments that form a juxtannuclear whorl, the so-called "fibrous body." The pathogenesis of this cellular alteration has been unclear, because the administration of GHRH is unable to cause a transition of densely-granulated somatotrophs to this phenotype,<sup>21</sup> and somatostatin is unable to reverse the morphology even when GH release is suppressed.<sup>24</sup>

Our data indicate that, at least in the mouse model, marked somatotroph hyperplasia and neoplasia are not significant features of GH antagonism because there is no gross enlargement of the pituitaries in these animals. In GHR/BP-deficient animals, reticulin staining, GH, and PCNA labeling provided evidence of mild somatotroph hyperplasia. The lack of somatotroph hyperplasia in GH-antagonist mice and the relatively subtle changes in GHR/BP-deficient animals contrast with the striking proliferative effect that GHRH stimulation has on these cells.<sup>20,27,28</sup> The subcellular changes, however, indicate that endogenous intracellular GH production may be accelerated in the setting of reduced GH signaling. Well-developed rough endoplasmic reticulum and large juxtannuclear Golgi complexes in SG cells are found in GH antagonist-transgenic mice and moreso in GH receptor-deficient mice; these morphological characteristics resemble the features of stimulated lactotrophs in rodents and humans and of SG somatotroph adenomas in humans. The lack of keratin filaments is the only difference between these stimulated somatotrophs and the cells of SG somatotroph adenomas. This is not a surprising or unexpected difference because the accumulation of keratin filaments in adenohypophysial cells is known to be a species-specific phenomenon; Crooke's hyalinization in glucocorticoid-suppressed corticotrophs is unique to humans and is not seen in rodent models of glucocorticoid excess.

The SG morphology of somatotrophs is most striking and there is acinar enlargement of the reticulin-fiber network of the gland in the animals that lack GHR/BP, where the GH feedback to the somatotroph would be totally reduced. Because both types of dwarf mice examined in this study have reduced IGF-1 levels, the differences between the somatotrophs in the two models can be explained by direct GH autoregulation that would be completely absent in the GHR/BP-deficient mice. A role for IGF-1 and/or GHRH as mediators of this phenomenon cannot be excluded.

The reverse does not appear to lead to major alterations in the somatotroph. The expression of the potent GH agonist E117L did not alter the subcellular morphology, and there was no evidence of involution or reduction of somatotrophs. This lack of evidence of feedback suppression is consistent with the lack of morphological evidence of involution in the nontumorous adenohypophysis of patients with acromegaly. It seems that this cell type provides morphological evidence of hormonal activity only in a hyperstimulated situation.

In this report we have demonstrated synthetic and secretory hyperactivity in pituitary somatotrophs due to

reduced GH feedback regulation. These changes are most striking in animals devoid of GHR/BP, in which there is even mild hyperplasia, and these changes are less marked in animals expressing a GH antagonist. Our findings represent the first evidence of direct GH feedback inhibition on pituitary somatotrophs, which may have implications for the use of GH analogs in different clinical settings.

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