

# SOM230 Inhibits Insulin-Like Growth Factor-I Action in Mammary Gland Development by Pituitary Independent Mechanism: Mediated through Somatostatin Subtype Receptor 3?

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**A**LTHOUGH SOMATOSTATIN receptors are widely distributed throughout the body, the only well-established physiological role for somatostatin in inhibiting IGF-I, and its action, is in the control of GH secretion from the pituitary (1). In fact, the entire effect of somatostatin analog (SA) treatment in acromegaly on IGF-I action is thought to be due to this mechanism. SAs reduce pituitary GH secretion, which in turn lowers IGF-I, thus removing the pathological effects of IGF-I systemically and peripherally. Several lines of past indirect and direct evidence suggest that these medications might have been working at other sites as well. Octreotide and lanreotide rarely suppress serum GH completely. They often remain at levels consistent with continued signs and symptoms

Abbreviations: bGH, Bovine GH; E<sub>2</sub>, estradiol; IGFBP, IGF binding protein; IRS, insulin receptor substrate; SA, somatostatin analog; sstr, somatostatin subtype receptor; TEB, terminal end bud; TUNEL, terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labeling.

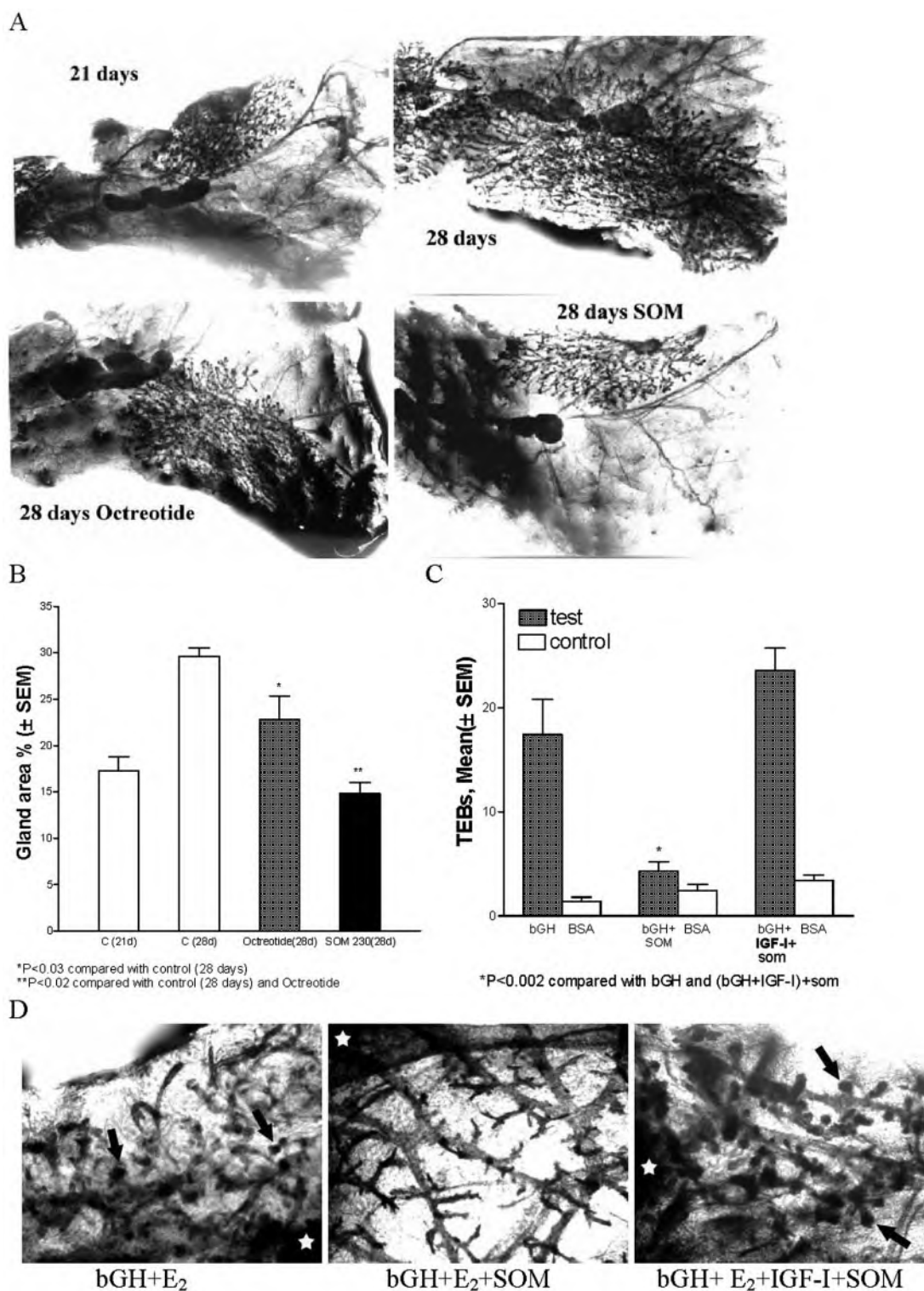
of acromegaly (2), but the medication can be fully effective even with the GH elevation. This, together with discordance between the effect of SOM230 on GH and IGF-I levels in blood (3), and the recent demonstration that octreotide and somatostatin can inhibit GH-stimulated hepatic production of IGF-I mRNA (4), suggested that SAs might act peripherally as well as centrally.

Together with the possibility that one or another form of SA might have beneficial effects in treating cancers in organs whose development is controlled by IGF-I, such as breast and prostate, we thought it would be important to determine whether SOM230 might have a peripheral inhibitory effect on IGF-I action, using mammary gland development as a model of an organ controlled by IGF-I.

## RESULTS

### Effect of SOM230 and Octreotide on Mammary Gland Development in Intact and Hypophysectomized Rats

Mammary development is controlled by estrogen and IGF-I but does not occur in the absence of IGF-I (5, 6).



**Fig. 1.** Somatostatin Analogs Inhibit Mammary Development in Intact and Hypophysectomized Female Rats

A, Shows whole mounts of lumbar mammary glands from a 21-d-old control (the time that experiments are initiated—*upper left panel*), after 7 d of treatment with octreotide (*lower left panel*) or SOM230 (*lower right panel*), compared with the control (*upper right panel*). Magnification,  $\times 1.6$ . Note that the area of glandular development is much smaller in the SOM230-treated animal than in the control and somewhat smaller in the octreotide animal. B, Represents the mean percentage of gland area before the experiment began (*far left bar*) and in six control animals (*second from left bar*) vs. six animals treated with octreotide (*second bar from right*) and SOM230 (*far right bar*). C, Results of the effects of bGH (10  $\mu$ g) administered into the substance of right lumbar (Legend continues on next page.)

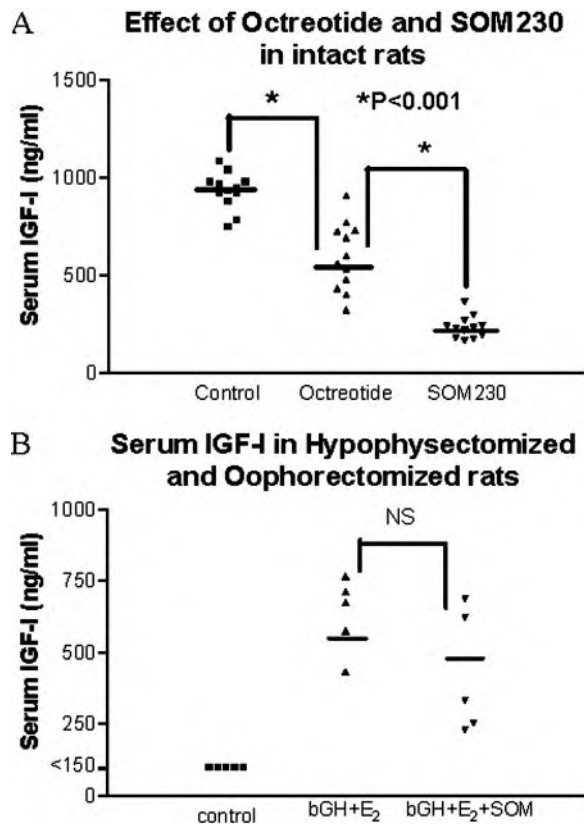
We employed mammary development in female rats as a model of peripheral action of IGF-I to ascertain whether SOM230 would affect it. We found that SOM230 and octreotide treatment, both in doses of 10  $\mu\text{g}/\text{h}\cdot\text{kg}$ , prevented mammary development in intact rats, but the effect of octreotide was significantly less than that of SOM230 (Fig. 1, A and B). At the start of the experiment, mammary development had already begun, so that 17.3% of the fat pad was filled with glands. Mean serum IGF-I in intact control and treated animals is shown in Fig. 2A.

To determine whether SOM230 would also have an effect on mammary development in the absence of the pituitary gland, we carried out experiments in hypophysectomized, oophorectomized animals. After hypophysectomy and oophorectomy, mammary development regressed so that ductal elements remained, whereas terminal end buds (TEBs) and more mature glandular elements disappeared. From previous experience, we anticipated that mammary development would occur surrounding a pellet of bovine GH (bGH) in one lumbar mammary gland but not in the contralateral one containing a pellet of BSA (5). Estradiol ( $\text{E}_2$ ) was given systemically. Indeed, bGH stimulated a significant increase in TEB number around the bGH-containing pellet but not on the other side, which held a pellet containing BSA. Treatment with SOM230 completely inhibited TEB formation that should have been stimulated by the combination of local bGH and systemic  $\text{E}_2$  (Fig. 1C). These effects on TEB development can be seen in representative photomicrographs in Fig. 1D. That an SA can inhibit mammary development in hypophysectomized animals indicates that the pituitary was not involved. Serum levels of IGF-I in the control animals and animals treated with bGH +  $\text{E}_2$  with or without SOM230 are shown in Fig. 2B. Although there was a reduction in mean serum IGF-I in the animals receiving bGH,  $\text{E}_2$ , and SOM230, it was not significant compared with levels in animals receiving only bGH and  $\text{E}_2$ .

To determine whether the effect of SOM230 was an IGF-I inhibitory one, we added IGF-I to bGH, SOM230 and  $\text{E}_2$ , and found that it completely reversed the inhibitory effect of SOM230 ( $P < 0.002$ ; Fig. 1C).

#### Effect of SOM230 on Cell Division, Apoptosis, and Phosphorylation of Insulin Receptor Substrate (IRS)-1

Phosphorylation of IRS-1 (a product of IGF-I action) in the mammary glands of intact rats was impaired



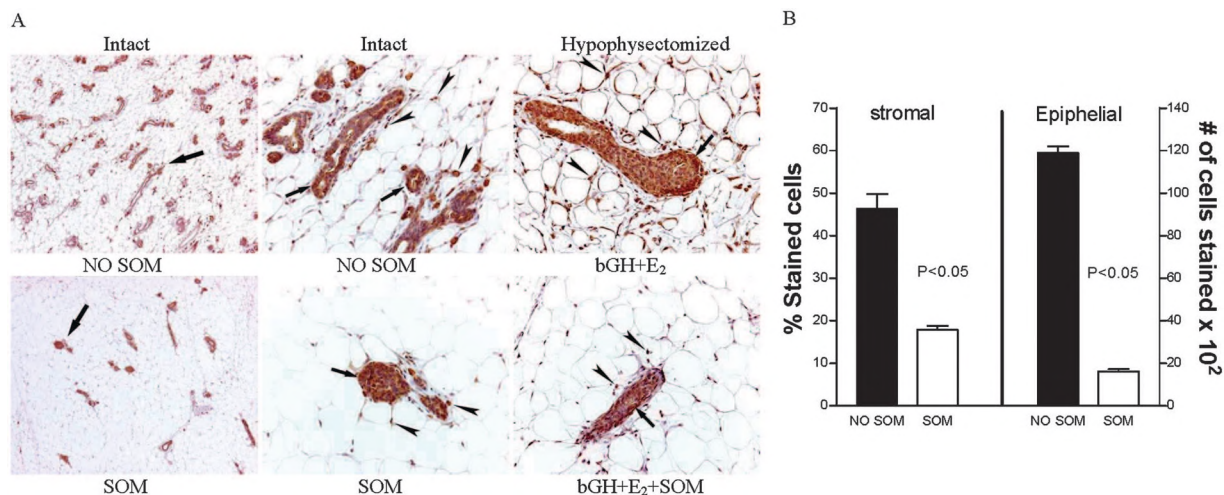
**Fig. 2.** Effect of Somatostatin Analogs on Serum IGF-I in Intact and Hypophysectomized Rats

A, Serum IGF-I levels in intact control rats and those treated with octreotide and SOM230. B, Serum IGF-I levels in hypophysectomized, oophorectomized controls, bGH +  $\text{E}_2$  treatment and bGH +  $\text{E}_2$  + SOM230 treatment.

by SOM230 (Fig. 3). Staining for pYIRS-1 was noted in both glandular epithelial elements and in loose connective tissue surrounding the glandular organelles. SOM230 significantly reduced the number of pYIRS-1 stained cells in the stromal compartment surrounding glands. Although staining for phosphorylated IRS-1 was similar in epithelial elements (92.8% and 95%; SOM230-treated vs. controls), there were many fewer glands in the SOM230-treated animals compared with controls (mean number of stained epithelial cells per slide 1612 vs. 11,928;  $P < 0.05$ ) (Fig. 3B). When the effect of SOM230 was evaluated in hypophysectomized oophorectomized rats, the expression of phosphorylated IRS-1 was lowered by SOM230 (Fig. 3A, right

mammary gland of seven female rats that were hypophysectomized and oophorectomized at 21 d of age, compared with the effect of BSA in the contralateral left mammary gland (*left pair of bars*). The *middle pair of bars* shows the same experiment except that SOM230 was administered as well. Note the inhibitory effect of SOM230. The *right pair of bars* shows effect of pellets of bGH and IGF-I in SOM230-treated animals. The IGF-I overcame the inhibitory effect of SOM230. D, Higher power photomicrographs of representative mammary glands from hypophysectomized, oophorectomized rats treated with bGH +  $\text{E}_2$  (*left panel*), bGH +  $\text{E}_2$  + SOM230 (*middle*) and bGH +  $\text{E}_2$  + IGF-I + SOM230 (magnification,  $\times 4.4$ ). The *star* identifies the pellets, *arrows* point to TEBs. Note that TEBs are found in the *left and right panels* but not in the middle, the one exposed to bGH +  $\text{E}_2$  + SOM230.





**Fig. 3.** SOM230 Reduced the Expression of Phosphorylated IRS-1 in Both Stromal and Epithelial Compartments of the Mammary Gland

A representative photomicrograph of a lumbar mammary gland stained for phosphorylated IRS-1 shows a high degree of mammary development in an intact control animal with many ducts stained *brown* for phosphorylated IRS-1 (*left upper*) compared with much reduced development in the SOM230-treated animal (*left lower*). *Arrows* point to glandular elements. Magnification,  $\times 40$ . The *middle panel* shows a higher power of the same slide ( $\times 200$ ) showing staining in both gland (*arrow*) and stromal compartments (*arrowhead*) in the intact animal (*upper*) and reduction of stained stromal elements (*lower*). The overall effect of SOM230 on phosphorylated IRS-1 staining in stromal and epithelial compartments in intact animals is shown in the graph (Fig. 2B). It shows the differences in the percent of cells stained for IRS-1 in the stroma (*left*) and the number of epithelial elements that are stained (*right*). The *right panel* (Fig. 2A) shows a high power photomicrograph of mammary glands from a hypophysectomized, oophorectomized female rat control treated with bGH and E<sub>2</sub> (*upper*) vs. SOM230 treatment (*lower*). Note that glandular elements are smaller and there is less staining within the stromal compartment of the SOM230-treated animal. *Arrowheads* point to stromal cells stained for phosphorylated IRS-1.

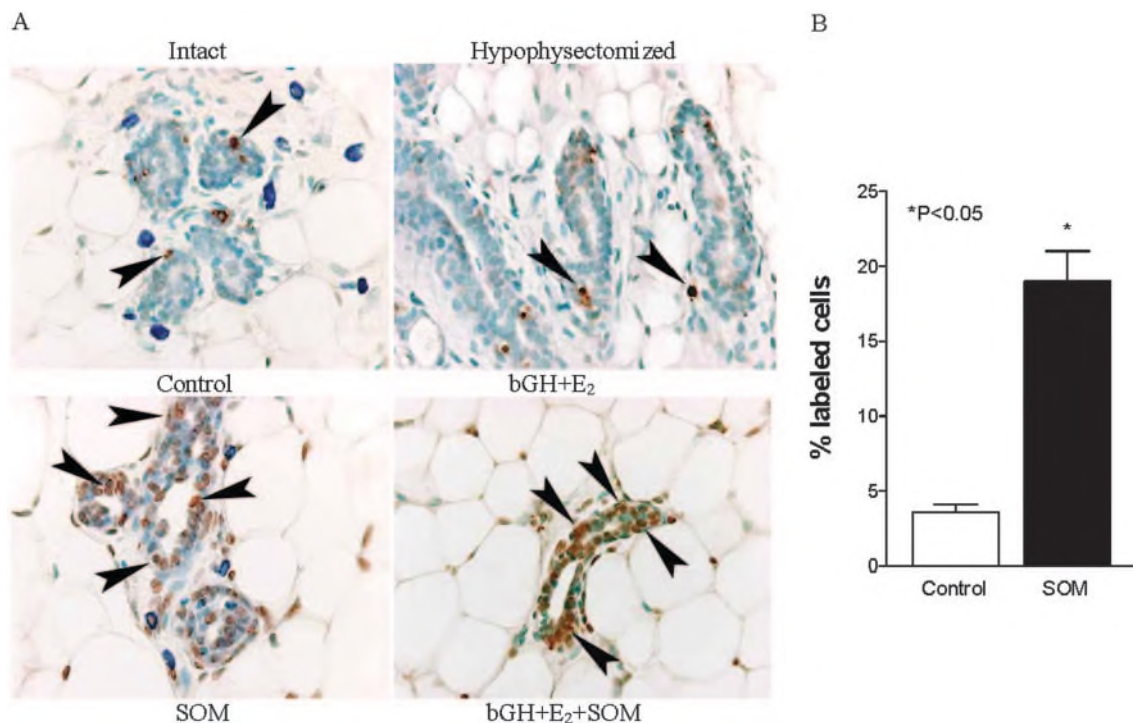
*pane(s)*, further supporting the hypothesis that the inhibitory effect of SOM230 on IGF-I action was not mediated via an action of the pituitary gland in these animals.

Apoptosis was increased within the glandular elements of the mammary gland in SOM230-treated animals in comparison to those not treated with SOM230, a further indication of inhibition of IGF-I action (Fig. 4A, *left panel*, and B). Also consistent with inhibition of IGF-I action, cell division was lower in animals treated with SOM230 than in those not receiving SOM230 (Fig. 5A, *left panels*, and B). Again similar findings were noted in mammary glands from oophorectomized, hypophysectomized rats (Figs. 4A, *right panels*, and 5A, *right panels*).

#### Do Changes in IGF-I Production, IGF Binding Protein (IGFBP)-1, or IGFBP-5 Mediate SOM230 Action?

To find possible mechanisms by which SOM230 acts to inhibit mammary development by inhibiting production of IGF-I in mammary gland, we used two quantitative RT-PCR approaches to determine the relative levels of IGF-I and  $\beta$ -actin mRNAs in tissues isolated from SOM230 and vehicle-treated rats. SOM230 was without significant effect on IGF-I mRNA production, suggesting that the effects of SOM230 were indepen-

dent of IGF-I mRNA levels. Due to technical problems inherent in measurement of IGF-I in mammary gland homogenates, it was not possible to determine whether SOM230 affected the protein. We then attempted to determine whether IGFBP-1 might have mediated the action of SOM230. This was assessed because we had previously found that IGFBP-1 inhibited mammary development (Ruan, W., and D. L. Kleinberg, unpublished data) (7). However, no binding protein 1 was detected in the mammary glands of SOM230-treated or untreated animals. We then assessed the effect of SOM230 on IGFBP5, a binding protein known to inhibit IGF-I action during lactational involution (8, 9). Mean intensity of staining for IGFBP-5 in epithelial cells was more than twice as dense in mammary glands from SOM230-treated animals than in those not exposed to SOM230 (Fig. 6, A and B). The total amount of IGFBP-5 measured by Western blotting was approximately double in homogenates of mammary glands from control animals vs. that in mammary glands having been exposed to SOM230 (Fig. 6C). However, there were more than 7.5 times the number of epithelial cells that stained for IGFBP-5 in control glands than in SOM230-treated ones. Therefore, we estimate that there was 3.75-fold the amount of IGFBP-5 in the mammary glands exposed to SOM230 compared with controls. To assess whether



**Fig. 4.** SOM230 Increased Apoptosis in Mammary Glands

A, Sections of mammary gland from intact animals stained for TUNEL reveals an increase in apoptosis in SOM230-treated animals (*lower left*) compared with intact animals (*upper left*). The brown-stained nuclei in epithelial cells are positive for staining for apoptosis. Similar findings are seen in mammary glands from hypophysectomized, oophorectomized rats treated with bGH + E<sub>2</sub> (*upper right*) vs. bGH + E<sub>2</sub> + SOM230 (*lower right*). Magnification,  $\times 400$ . B, Although there were many more epithelial cells in the mammary gland not seeing SOM230 the percent of cells stained for TUNEL in the SOM230-treated glands was greater than in the animals not seeing SOM230.

IGFBP-5 was capable of inhibiting GH-induced mammary development in hypophysectomized, oophorectomized female rats, we implanted bGH. Half of the animals also received IGFBP-5. Pellets containing bGH (10  $\mu$ g) with or without IGFBP-5 (200  $\mu$ g) were implanted into right lumbar mammary glands for a period of 5 d. E<sub>2</sub> was given systemically. IGFBP-5 was found to significantly inhibit mammary development ( $P < 0.001$ ) (Fig. 7, A and B;  $P < 0.002$ ).

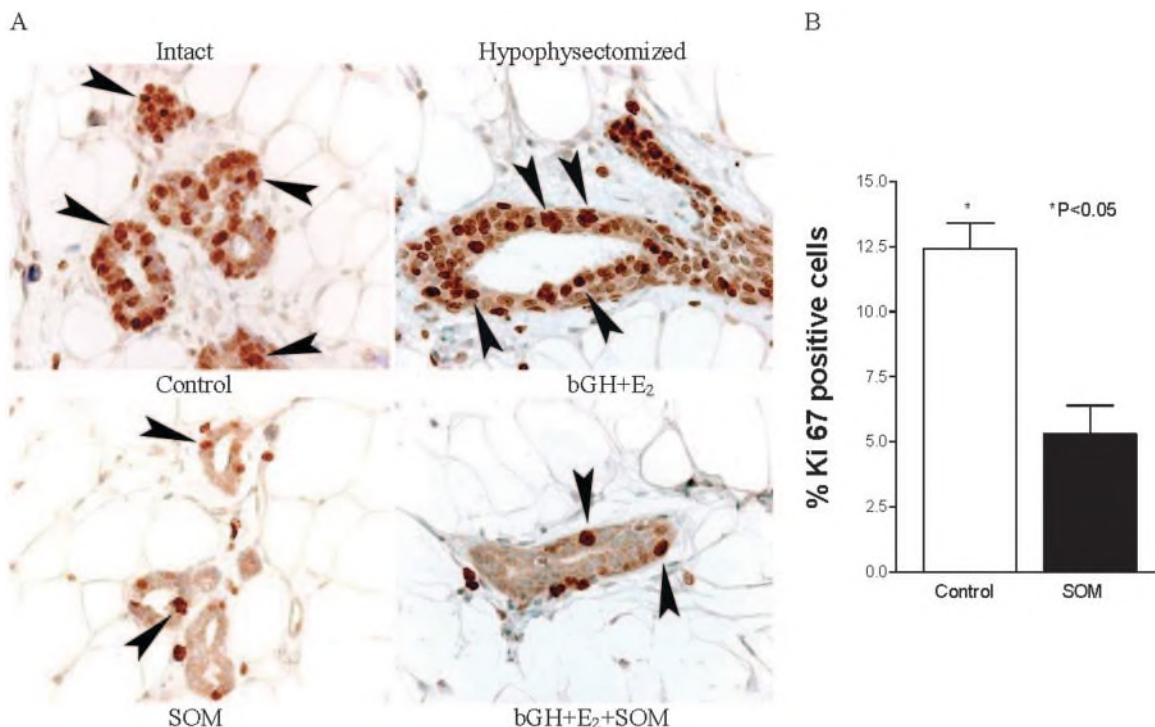
#### Which Somatostatin Subtype Receptors Mediate the Action of SOM230 and Octreotide?

By quantitative real-time PCR, we assessed presence or absence of receptors in RNA extracted from mammary glands of the following targets: somatostatin subtype receptors, sstr<sub>1</sub>, sstr<sub>2</sub>, sstr<sub>3</sub>, sstr<sub>4</sub>, sstr<sub>5</sub>, and rRNA 18S. As seen in Table 1 and Fig. 8, sstr<sub>3</sub> was present in the highest concentration in mammary glands from both intact and hypophysectomized and oophorectomized animals. mRNA for receptor subtypes 4 and 5 were present in much lower amounts, and no sstr<sub>1</sub> or sstr<sub>2</sub> was detected. There were lower amounts of the three receptors identified in mammary glands from intact control animals than from hypophysectomized, oophorectomized ones, but the differences were significant

only in regard to sstr<sub>4</sub>. SOM230 did not affect receptor mRNA expression.

#### DISCUSSION

These studies indicate that in addition to reducing serum IGF-I by inhibiting pituitary GH secretion, SOM230 can inhibit IGF-I action via a pituitary independent mechanism. SOM230 was found to inhibit bGH- and E<sub>2</sub>-stimulated mammary development in hypophysectomized, oophorectomized female rats. We know from previous experiments that GH is the pituitary hormone essential for mammary development (10, 11), and that the entire effect of GH-induced mammary development is mediated by local production of IGF-I (5, 6, 12). Therefore, the inhibitory effect of SOM230 could be on the actions of GH, IGF-I, E<sub>2</sub>, or a combination. That E<sub>2</sub> has no independent stimulatory effect on the early phases of mammary development (13) suggests that the inhibitory effect of SOM230 is on the GH-IGF-I cascade or the combined actions of GH-IGF-I and E<sub>2</sub>. Well-known effects of IGF-I, without or with E<sub>2</sub>, include formation of TEBs and duct development, branching and extension during mammary development (14), stimulation of cell



**Fig. 5.** SOM230 Inhibited Cell Division in Mammary Glands

A, This figure shows an increase in cell division, as measured by staining for Ki67, in the mammary glands not exposed to SOM230. There was greater staining for Ki67, as determined by the *brown* nuclear staining, in the mammary gland from an intact animal (*left upper*) and a gland from a hypophysectomized animal treated with bGH + E<sub>2</sub> (*right upper*). In contrast, mammary glands from both an intact rat treated with SOM230 (*left lower*) and a gland from a hypophysectomized, oophorectomized rat treated with bGH + E<sub>2</sub> + SOM230 (*right lower*) had less evidence of cell division. B, These data in intact rats are shown graphically in this figure, again comparing the percentage of cells staining positively for Ki67 in controls vs. SOM230-treated animals.

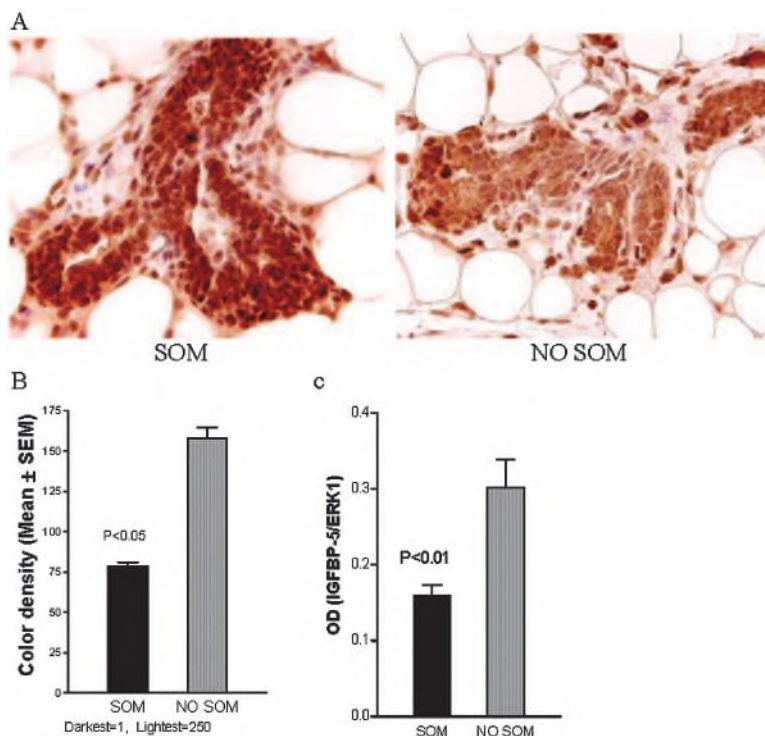
division (15), expression of phosphorylated IRS-1 (16), and inhibition of apoptosis (17, 18). Each of these was reversed by SOM230. These observations, together with our finding that IGF-I overcame the inhibitory effect of SOM230, indicates that a major effect of SOM230 was to antagonize the effects of IGF-I.

To assess the mechanism by which SOM230 inhibits IGF-I action, we looked at several possibilities. Studies on the effect of SOM230 on IGF-I mRNA in mammary gland did not show an inhibitory effect. We were unable to measure IGF-I in mammary gland homogenates, and thus, cannot be certain that there was not a reduction in the IGF-I concentration. Such an effect might have been expected because of the known effect of octreotide on liver *ex vivo* (4). However, there was no significant reduction in serum IGF-I in bGH-treated hypophysectomized, oophorectomized rats when SOM230 was given, suggesting that the major effect of SOM230 is not via reduction in liver IGF-I production in our model. Because IGFBPs regulate IGF-I activity and bioavailability, we investigated whether the effect of SOM230 was mediated by altering the concentration of an IGFBP. One possibility was that IGFBP-1 was intermediary in this process, but we were unable to detect IGFBP-1 in the mammary glands. However, IGFBP-5 had previously been found

in the mammary epithelium and in milk during lactational involution. That this binding protein was acting via an increase in apoptosis was suggested by the finding that there was an increase in apoptosis in mammary glands of IGFBP5 transgenic mice (9, 19). IGFBP5 has also been shown to be up-regulated in involuting prostate and thyroid glands (20). We found that SOM230 increased the amount of IGFBP-5 in mammary glands. Using Western blotting and immunostaining, we estimated that mammary glands exposed to SOM230 had 3.75 times the amount of IGFBP-5 compared with control mammary glands relative to the degree of development. That direct administration of IGFBP-5 inhibited GH-induced mammary development was also shown in these experiments.

We know that somatostatin receptors are widespread (21) in normal and tumor tissues, but the only well-established physiological action of somatostatin on inhibiting IGF-I action is the inhibition of pituitary GH secretion. To our knowledge, this is the first report showing that somatostatin analogs can inhibit peripheral IGF-I action. If this effect of SOM230 in the mammary gland is more widespread, such an effect of SOM230 or other somatostatins could occur at other sites where GH-induced IGF-I has effects such as





**Fig. 6.** SOM230 Increased Staining Intensity of IGFBP-5 in Mammary Epithelial Elements

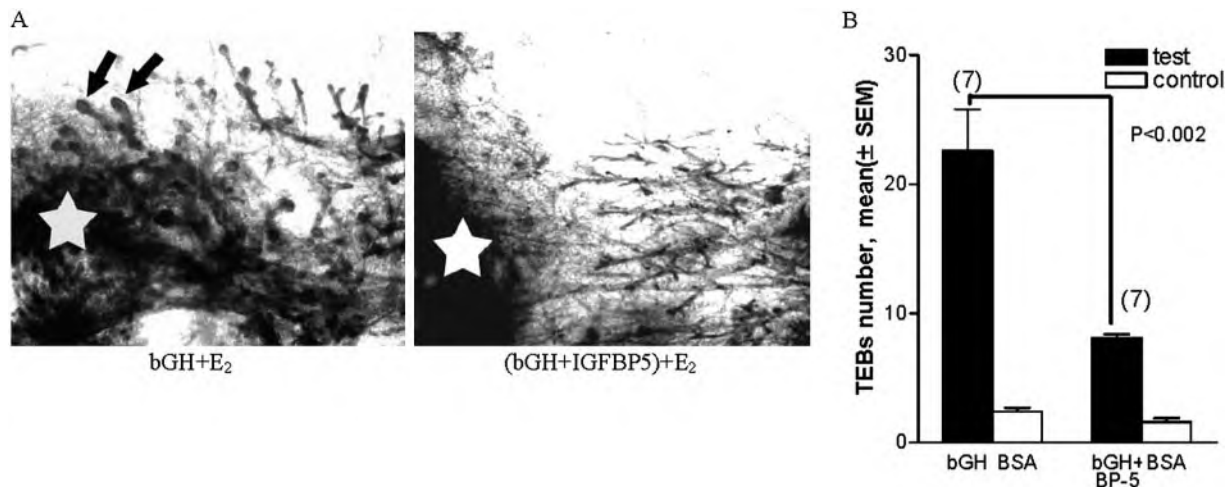
A, The photomicrographs show mammary gland sections from one animal having received SOM230 immunostained for IGFBP5 (*left panel*) and the other not having seen SOM230 (*right panel*). Note the increased intensity of the *brown* staining on the left glandular epithelial structure compared with the right. Magnification,  $\times 400$ . B, This graph shows measurement of mean color density of immunostaining for IGFBP-5 in mammary glands exposed to SOM230 (*left*) or control (*right*). Note that the lower the number the higher the density. C, This graph shows OD of a mean of four Western blots of mammary gland homogenates four lumbar mammary glands from four different animals showing binding protein 5 concentration in the mammary glands with SOM (*left*) and without SOM (*right*).

epiphyseal growth (22), prostate development (23), and muscle growth.

Our findings show that IGFBP-5 can inhibit the actions of IGF-I on mammary gland. Therefore, we believe that IGFBP-5, which is up-regulated by SOM230, could be a mediator of SOM230 action in the mammary gland. SOM230 might also affect IGF-I action via a direct effect not mediated by a binding protein. However, this is speculative because we do not know whether SOM230 is acting directly on the mammary gland. Analysis of *sstr* receptor message shows that receptors 1 and 2 are not present but that receptors 3–5 are. That *sstr*<sub>2</sub> is not present in these glands indicates that this receptor is not the mediator of the actions of SOM230 and octreotide. Also unlikely is that *sstr*<sub>4</sub> is the mediating receptor because neither octreotide nor SOM230 have high enough binding affinity to this receptor (24). Likewise, the binding affinity for *sstr*<sub>1</sub> is low and there is no evidence that those receptors are present in mammary gland. Thus, the only remaining candidates for mediating the actions of octreotide and SOM230 are *sstr*<sub>3</sub> and *sstr*<sub>5</sub>. That octreotide has only 1/40th the binding affinity for *sstr*<sub>5</sub> of SOM230 reduces the likelihood that this receptor is the or a mediator for inhibiting IGF-I action because SOM230 was only approximately twice as potent as

octreotide in inhibiting mammary development. However, *sstr*<sub>5</sub> may, nevertheless, play a role. Because octreotide has 4- to 5-fold less binding affinity for *sstr*<sub>3</sub> than SOM230, it seems more likely that this receptor is mediating the action of those compounds at least in part. If we are correct in assuming that *sstr*<sub>3</sub> is mediating these actions, it would be the first such demonstration *in vivo*. An effect on apoptosis of this receptor has been previously shown (3, 25), however. Our studies also show that mRNAs for receptors *sstr*<sub>3</sub> and *sstr*<sub>4</sub> go up after hypophysectomy and oophorectomy. Although we might speculate that lower GH or IGF-I or estrogen may be the cause for this observation, the mechanism of action will have to await proof by further studies.

Several studies have demonstrated an epidemiological relationship between increased serum IGF-I concentrations and increased prevalence of breast and/or prostate cancer (26–28). In addition, *in vitro* tissue culture studies have shown that IGF-I and IGFBP-5 can modulate cellular functions that are related to the development of breast cancer such as cell attachment, migration, and replication (27, 29–31). In addition, treatment of breast and/or prostate cancer cells with known inhibitors of tumor cell growth such as antiestrogens results in the induction of IGFBP-3 or IGFBP-5 in several model systems (32, 33). Addition-



**Fig. 7.** IGFBP-5 Inhibited Mammary Development in Female Rats

A, The *left panel* is a photomicrograph of a representative mammary gland whole mount from a hypophysectomized, oophorectomized rat having been treated with bGH + E<sub>2</sub> for 5 d. The *white star* overlies the pellet through which bGH was administered and the *arrows* point to TEBs. The *left panel* contains a photomicrograph from an animal treated with bGH and E<sub>2</sub>. The *right panel* represents treatment with the same hormone combination but with the addition of IGFBP5 in the pellet. Note that the IGFBP5 inhibited TEB formation (magnification, ×2.8). B, This graph reflects the effect of IGFBP-5 on the ability of bGH with E<sub>2</sub> to stimulate mammary development as assessed by formation of TEBs. Bovine GH + E<sub>2</sub> stimulated development of a mean of 22.6 TEBs per gland in seven animals in the mammary gland with the pellet of bGH compared with a mean of 2.4 TEBs in the contralateral lumbar mammary gland (*left*). The *right panel* shows the effect of IGFBP-5 on this process. TEB formation was decreased so that only a mean of 8.1 TEBs formed.

ally, some whole animal studies have shown that castration that is associated with prostate cancer epithelial cell regression is also associated with induction of IGFBP-5 (34), and oophorectomy in mice is associated with induction of IGFBP-5 and breast tumor regression (35). Therefore, it appears that there is an association between increasing tissue concentrations of IGFBP-5 and breast or prostate tumor regression. Both breast and prostate rely on IGF-I for development (23). This study and that of Allen *et al.* (36) are the first papers to demonstrate that direct injection of IGFBP-5 into a whole animal model of breast development results in decreased epithelial cell development and proliferation. The findings strongly suggest that induction of IGFBP-5 by agents such as SOM230 may be useful in limiting IGF-I-dependent breast carcinoma epithelial cell proliferation. Prior studies on the effect of somatostatin analogs in breast cancer have shown an inhibitory effect in cell line tumors in nude mice (37) but have not been found highly effective in humans with tumors (38). Despite the failure of these medications that act mainly through sstr<sub>2</sub> receptors, different medications with more efficient capacity to bind to sstr<sub>3</sub> and sstr<sub>5</sub> might prove more efficacious.

## MATERIALS AND METHODS

### Animals

Intact female CD rats (Charles River, Wilmington, MA) were treated with SOM230 (10 μg/kg-h), octreotide (10 μg/kg-h), or vehicle for 7 d in Alzet pumps (Model 2001; Alza Corp.)

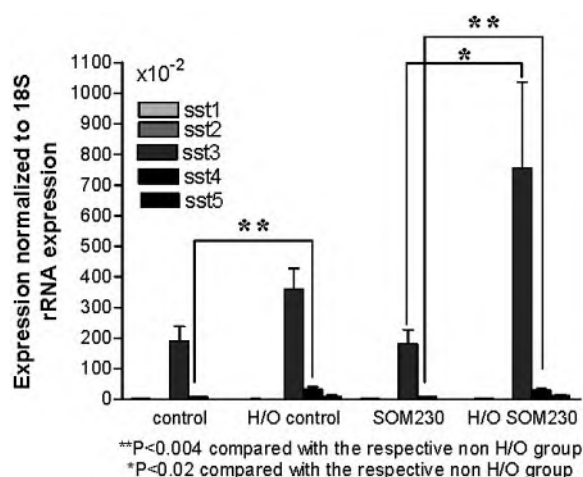
implanted sc on their backs. Another group of CD rats were hypophysectomized and oophorectomized at 21 d of age. These animals received replacement doses of levothyroxine (1 μg/d) and dexamethasone (1.5 μg/d) as previously described (5, 39). The bGH 10 μg (Monsanto, Torrance, CA) and bGH with Des(1-3)IGF-I 42 μg or with IGFBP5 200 μg were administered by Elvax P40 (ethylene-vinyl acetate copolymer; DuPont Chemical Co., Universal City, CA) pellets embedded into the substance of the right lumbar mammary gland. The contralateral left mammary gland was implanted with control pellets containing BSA to provide an internal control. The E<sub>2</sub> in SILASTIC brand capsules (Dow Corning, Midland, MI) and the SOM230 or octreotide containing pumps were placed on the back for 5 d. Anesthesia consisted of Ketamine (90 mg/kg body weight) and Xylazine (10 mg/kg body weight) ip. Pellet implantation has been described (10).

After 5 or 7 d, animals were decapitated under anesthesia and mammary glands were removed and either prepared for whole mount analysis by fixing them in 10% formalin, and staining with iron-hematoxylin (40, 41) or embedded. Mammary development and differentiation were assessed quantitatively by examining the whole mounts under a dissecting microscope (Nikon SMZ-U, Melville, NY) at a magnification of ×15. TEBs were counted and the percentage of the mammary fat pad occupied by glandular structures was determined as previously described (42).

### Immunohistochemistry and Terminal Deoxynucleotidyl Transferase-Mediated Deoxyuridine Triphosphate Nick End Labeling (TUNEL) Staining

Rat mammary glands were sagittally embedded in paraffin. For antigen retrieval, sections (4 μm thick) were heated in 0.01 M sodium citrate buffer (pH 6.0) in a microwave oven (700 W) for 15 min. To inactivate endogenous peroxidase activity, slides incubated in 3% H<sub>2</sub>O<sub>2</sub> for 10 min and blocked with 10% normal goat serum for 1 h at room temperature, and incubated overnight at 4 C or 1 h at room temperature





**Fig. 8.** Expression of mRNA of SST1–5 in mRNA Extracted from Mammary Glands Five Animals from Each of Four Variables

The first two come from intact controls vs. hypophysectomized, oophorectomized rats treated with bGH + E<sub>2</sub> (*left*), the two groups on the *right* are intact controls treated with SOM230 vs. hypophysectomized, oophorectomized rats treated with bGH + E<sub>2</sub> + SOM230. There was a significant increase in expression of SST4 mRNA between the intact control and treated control after hypophysectomy and oophorectomy, and a significant increase in both SST3 mRNA and SST4 mRNA when animals were hypophysectomized and oophorectomized.

with primary antibodies. The primary antibodies used were anti-IRS-1 pY896 (1:100 dilution; Biosource, Camarillo, CA), anti-IGFBP5 (1:1000 Upstate Biotechnology, Lake Placid, NY) and anti-Ki67 (1:1000 Novocastra Lab Ltd., Burlingame, CA). Slides were then incubated for 1 h at room temperature with goat antirabbit immunoglobulins, horseradish peroxidase (1:100 dilution; Dako, Carpinteria, CA). Sections were developed with a diaminobenzidine solution and counterstained with hematoxylin or 0.5% methyl green. Negative controls for IRS-1 identification were incubated with primary antibody preabsorbed with 200-fold molar excess of the IRS-1 (pY896) phosphopeptide. Normal rabbit IgG was used instead of primary antibody as a negative control for IGFBP-5 and Ki67 staining. Apoptotic cells were detected by TUNEL staining with ApopTag peroxidase *in situ* apoptosis detection kit, according to the manufacturer's instruction (Chemicon, Temecula, CA).

Mammary gland sections from three different levels of each gland were examined by taking photographs using a Nikon E400 microscope at  $\times 200$  magnification attached to a Nikon digital camera DXM1200 running software Nikon

ACT-1 on a PC system. pY IRS-1 896 and IGFBP-5-positive staining and labeled cells for Ki67 and TUNEL staining in 10 random fields of each sample (per field of glands at  $\times 200$  magnification) were quantified and analyzed using a semiautomatic computer system (Image-Pro plus, Version 4.5, Media Cybernetics (Silver Spring, MD). Morphometry was carried out by identifying positive brown staining cells in the sections. All stained stromal and epithelial cells were counted for pY IRS-1 antibody. Ki67 and TUNEL staining were expressed as the percentage of labeled cells by total number of epithelial cells. At least 2000–2500 cells/sections of a mammary gland were counted in the fields. IGFBP5 color density (mean) was carried out by identifying positive brown staining area of the whole section (10–15 fields at  $\times 40$  magnification of whole section), and then submitted to analysis by a semiautomatic computer system. The darkest is 1, the lightest is 250.

#### RNA Isolation and Quantification of IGF-I mRNA and SST<sub>1-5</sub>

Total RNA was isolated from mammary gland and liver samples using the Trizol reagent (Invitrogen, Basel, Switzerland) for IGF-I mRNA and RNA for sstr receptors was isolated from mammary glands using an RLT lysis buffer (RNeasy minikit from QIAGEN, Basel, Switzerland) according to the manufacturer's instructions. Total RNA (1  $\mu$ g) was reverse transcribed into cDNA using the Superscript First-Strand Synthesis System (Invitrogen) and random primers (Roche Diagnostics, Basel, Switzerland). Reactions lacking reverse transcriptase were used as controls. IGF-I mRNA was quantified relative to  $\beta$ -actin mRNA using two approaches. The first approach was coamplification of IGF-I and  $\beta$ -actin in the cDNA samples using quantitative radiolabeled PCR as previously described by us (31). The second approach was to use rapid cycle real-time quantitative PCR with the Lightcycler (Roche). For real-time PCR, specific amplification primers and oligonucleotide hybridization probes for dual color detection were designed by Idaho Biochem Inc. (Salt Lake City, UT). The sequences of the amplification and hybridization oligonucleotides for IGF-I mRNA are shown in Table 1. The LightCycler PCR conditions were 95 C for 10 min followed by 45 cycles of denaturation (95 C for 10 sec), annealing (52 C for 15 sec) and amplification (72 C for 15 sec). A postamplification melting curve analysis was conducted to ensure specificity of the amplified product. The sequences for primers and probes for sstr<sub>1-5</sub> are also found in Table 2. Those for sstr<sub>1</sub>, and sstr<sub>3-5</sub> and for rRNA 18S were provided by assays-by-design (Applied Biosystems, Foster City, CA), and those for sstr<sub>2</sub> by assays-on-demand (No. Rn\_00571116\_m1). For rRNA 18S evaluation, the Taqman rRNA control reagents were used according to the manufacturer's instructions (Applied Biosystems, Foster City, CA).

#### Statistics

Statistics were done employing three to seven animals in each group, depending on the experiment. The Mann-Whitney *U* two-tailed test was employed between two groups.

**Table 1.** Expression Normalized to 18S rRNA Expression (A.U.)

	sstr1	sstr2	sstr3	sstr4	sstr5
Control	1.12 $\pm$ 0.14	0.08 $\pm$ 0.02	189.65 $\pm$ 48.64	6.99 $\pm$ 0.45	0.13 $\pm$ 0.11
SOM230	1.07 $\pm$ 0.30	0.10 $\pm$ 0.02	180.97 $\pm$ 45.85	8.44 $\pm$ 0.91	0.24 $\pm$ 0.15
H/O control	0.65 $\pm$ 0.14	0.11 $\pm$ 0.01	359.02 $\pm$ 68.58	32.31 $\pm$ 8.67 <sup>a</sup>	7.54 $\pm$ 7.19
H/O SOM230	1.25 $\pm$ 0.10	0.10 $\pm$ 0.04	754.43 $\pm$ 281.23 <sup>b</sup>	28.70 $\pm$ 6.06 <sup>a</sup>	10.67 $\pm$ 4.32

H/O, Hypophysectomized, oophorectomized; A.U., arbitrary units.

Data are presented as mean  $\pm$  SE ( $10^{-2}$ ).

<sup>a</sup> *P* < 0.004 compared with control and SOM230, respectively.

<sup>b</sup> *P* < 0.02 compared with control and SOM230, respectively.

**Table 2.** Sequences of Primers Used for PCR and Real-Time PCR

Primer	Sequence (5'-3')
IGF-I forward	CTTCAGTTCGTGTGTGGA
IGF-I reverse	AGATCACAGCTCCGGAAG
B-ACT forward	AATGCTTCTAGCGGACT
B-ACT reverse	ACTCCCAGGGAGACCAA
IGF-I Fluor	AGCATTTCGGAGGGCACC-FITC
IGF-I 640	LCR640-AGACGGGCATTTGTGGATGAGT-P
B-ACT Fluor	CGGAGACGGGGTACC-FITC
B-ACT 705	LCR705-CACTGTGCCCATCTATGAGGGTTACG-P
sstr1 forward	AGAGCGGCTACCAGACTGT
sstr1 reverse	GGAGCTGGGCTTACTTGACA
sstr1 probe	CCAGTTACCGCTCCCC
sstr3 forward	GTCCCTTCCACCTTTGCA
sstr3 reverse	GAGGATCAGTTCAGCAACTAG
sstr3 probe	CAACCCCGTAAGGTTTG
sstr4 forward	GGATCGCTATGTGGCTGTAGTG
sstr4 reverse	GGCCGCCGGTAGGT
sstr4 probe	CCCTCTGCGAGCTGC
sstr5 forward	CGGCTTTCTCTCGGACAACCT
sstr5 reverse	CTCTACGTAGGCACAGAACCTT
sstr5 probe	TCCGCCAGAGCTTC

B-ACT,  $\beta$ -Actin; Fluor, fluorescent labeled.

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