The Effect of Transforming Growth Factor \( \beta \) on Human Neuroendocrine Tumor BON Cell Proliferation and Differentiation Is Mediated through Somatostatin Signaling

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Abstract
The dual effect of the ubiquitous inflammatory cytokine transforming growth factor \( \beta \) (TGF\( \beta \)) on cellular proliferation and tumor metastasis is intriguing but complex. In epithelial cell– and neural cell–derived tumors, TGF\( \beta \) serves as a growth inhibitor at the beginning of tumor development but later becomes a growth accelerator for transformed tumors. The somatostatin (SST) signaling pathway is a well-established antiproliferation signal, and in this report, we explore the interplay between the SST and TGF\( \beta \) signaling pathways in the human neuroendocrine tumor cell line BON. We defined the SST signaling pathway as a determinant for neuroendocrine tumor BON cells in responding to TGF\( \beta \) as a growth inhibitor. We also determined that TGF\( \beta \) induces the production of SST and potentially activates the negative growth autocrine loop of SST, which leads to the downstream induction of multiple growth inhibitory effectors: protein tyrosine phosphatases (i.e., SHPTP1 and SHPTP2), p21\(^{Waf1/Cip1}\), and p27\(^{Kip1}\). Concurrently, TGF\( \beta \) down-regulates the growth accelerator c-Myc protein and, collectively, they establish a firm antiproliferation effect on BON cells. Additionally, any disruption in the activation of either the TGF\( \beta \) or SST signaling pathway in BON leads to “reversible” neuroendocrine-mesenchymal transition, which is characterized by the loss of neuroendocrine markers (i.e., chromogranin A and PGP 9.5), as well as the altered expression of mesenchymal proteins (i.e., elevated vimentin and Twist and decreased E-cadherin), which has previously been associated with elevated metastatic potential. In summary, TGF\( \beta \)-dependent growth inhibition and differentiation is mediated by the SST signaling pathway. Therefore, any disruption of this TGF\( \beta \)-SST connection allows BON cells to respond to TGF\( \beta \) as a growth accelerator instead of a growth suppressor. This model can potentially apply to other cell types that exhibit a similar interaction of these pathways.
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Introduction
Accurate cellular communication in responding to environmental cues is vital for cell function, development, and survival. Transforming growth factor \( \beta \) (TGF\( \beta \)) and somatostatin (SST) are cytokines that play an important role in providing control for cell proliferation and metastasis (1-4). Intriguingly, TGF\( \beta \) activation regulates growth and differentiation in multiple cell types (4, 5) by asserting a dual and seemingly paradoxical effect on cell growth and metastasis: accelerating proliferation in some cell types, such as fibroblast cells, while suppressing proliferation in epithelial-, immune-, and neural-derived cells. Additionally, in the early stages of epithelial cell– or neural cell–derived tumor development, TGF\( \beta \) works as a suppressor for tumor proliferation and metastasis and later works as an accelerator (1, 6, 7).

Recently, TGF\( \beta \) has been shown to be a potent growth inhibitory cytokine in neuroendocrine tumor cells from the gastroenteropancreatic track (6). Neuroendocrine tumor cells are known to respond to SST signaling as a growth-suppressive signal and, thus, the SST analogue octreotide has been used for over two decades as the main treatment against neuroendocrine tumors (8, 9). SST is a neuropeptide hormone, primarily consisting of two forms (14- or 28-amino-acid peptide), and it is posttranslationally processed from preproSST. SST can directly inhibit the growth of cells and block the release of growth hormones and other small peptides in a wide array of endocrine, exocrine, neuronal, and immune cell targets (10-12). The 14-amino-acid form of SST is most prevalent and is seemingly ubiquitous in the human body but is short-lived with a half-life of only a few minutes (13). SST exerts its prominent growth-suppressive effect on the neuroendocrine tumor by acting on a family of inhibitory seven-transmembrane G protein–coupled receptors, named SST receptor subtypes 1 to 5 (SSTR1-5; ref. 14). This pronounced growth-suppressive effect exerted by SST has long been applied to treat neuroendocrine tumors. Treatments using long-acting SST analogues, such as octreotide, render neuroendocrine tumors to be static in growth, partly due to apoptosis and partly due
to cell cycle arrest at G1 (15, 16). This growth-suppressive effect of SST signaling activation is similar to the central biological response for TGFβ signaling activation. Therefore, in this report we examine if both cytokines share common growth inhibitory effectors that have previously been reported to be inducible by TGFβ and/or SST signaling activation, such as SHPTP1 and SHPTP2 (17-21), p21Waf1/Cip1 (6, 22, 23), and p27Kip1 (21, 24).

Although chronic octreotide treatment could keep neuroendocrine tumor cells static for an extended period of time, eventually a small population of tumors escape this SST-mediated growth control and ultimately metastasize (8, 25). In this study, we also examine if this process is similar to epithelial-derived tumor progression mediated via TGFβ, which is termed epithelial-to-mesenchymal transition (EMT). EMT is a process in which TGFβ works as a growth suppressor during the early stages of epithelial-derived tumor but later becomes a growth accelerator (1, 26, 27). Post-EMT tumors are mesenchymal-like with molecular changes such as an elevated expression of vimentin (28) and the metastasis marker Twist (29), as well as a decreased expression of E-cadherin on the cell membrane surface (30). This observed EMT-like process in BON cells is seemingly reversible, and therefore, we termed it “reversible” neuroendocrine-to-mesenchymal transition (NMT).

The goal of this study is to clarify the role of SST signaling in TGFβ-elicted growth suppression. Immortalized cell lines with both TGFβ and SST pathways intact are uncommon because both are considered to be barriers to tumor emergence and progression (2, 4). BON cells harbor both endogenous intact TGFβ and SST signaling pathways and are functionally responsive, and thus, these cells serve as a good model for examining the interplay between the two pathways (6, 31, 32). Therefore, in this study we postulate that TGFβ-mediated growth suppression is controlled through an intact SST signaling pathway, and any disruption within this TGFβ-SST connection will render tumors to respond to TGFβ as a proliferative and metastatic accelerator.

Results

Neuroendocrine Tumor BON Cells Are Sensitive to TGFβ as a Growth Suppressor in the Growth Media

TGFβ signaling can lead to tumorigenesis and ultimately to metastasis, such as in pancreatic cancer (33-35), glioblastoma (7), small bowel carcinoids (33, 36), and neuroendocrine tumors (6). BON cells have an intact TGFβ signaling pathway and thus they respond to TGFβ activation as a growth-suppressive signal (6). We designed BON cell growth media containing 10% fetal bovine serum (FBS) to promote cellular growth in an in vitro environment simulating an in vivo microenvironment. We explored whether 10% FBS cell growth medium has enough TGFβ to suppress cell growth. Initially, we measured the level of exogenous TGFβ in the cell growth media to determine if this amount of TGFβ could inhibit BON cell growth. As shown in Fig. 1A, a Western blot analysis was used to construct a TGFβ standard curve by using a known amount of recombinant proteins to determine the unknown samples (right). The amounts of TGFβ detected in 50% FBS and 25% FBS-containing cell growth media were measured to be 28.4 and 11 ng/mL, respectively. After calculation, 10% FBS growth medium was determined to contain ~5 ng/mL TGFβ [also confirmed with a similar value using a TGFβ enzyme immunoassay (EIA) kit from Promega, as seen in Fig. 2A], which is ~50 times the published half maximal effective concentration (EC50; ref. 6). These results confirm our hypothesis that 10% FBS in the growth media provided sufficient TGFβ to adequately impose growth inhibition.

To further test this theory, we perturbed the cellular growth environment by gradually decreasing the amount of FBS in the growth media to determine if the TGFβ in FBS could have a...
direct effect on cell growth. To do this, cells were grown in 10% FBS media for 48 hours and allowed to reach their growth phase. Cell growth was then monitored once every 24 hours for a duration of 96 hours. Cell growth was then measured by MTS cell proliferation assay (Promega). Results from the MTS assay were compared with the results from the flow cytometry cell counting assay (Viacount assay using Guava PCA96) and found to be comparable. BON cells responded to decreasing FBS in the growth medium with increased cellular proliferation, which is potentially due to the proportionate decrease of TGFβ in its growth medium. This is evidenced by the decrease in growth doubling time as FBS decreased in growth media: 52 ± 1.3 hours in 20% FBS; 49 ± 3 hours in 10% FBS; 46 ± 0.5 hours in 5% FBS; 37 ± 2.1 hours in 2.5% FBS; and down to 29 ± 1 hours in 0% FBS. This growth suppression by FBS reached its maximum at ~10% FBS growth medium (Fig. 1B).

TGFβ-Dependent Growth Inhibition Is Mediated through SST Up-Regulation

To examine if SST signaling activation could enhance FBS-induced growth suppression caused by TGFβ, we grew BON cells in the following treatments as shown in the Table 1: A first set of cells were grown in 10% FBS, with no treatment (vehicle), 100 nmol/L octreotide (SST analogue), or 10 ng/mL TGFβ; a second set of cells were grown in 0% FBS, with no treatment, 100 nmol/L octreotide, or 10 ng/mL TGFβ. For BON cells to successfully proliferate through their growth phase in FBS-free growth media and reach confluence in tissue culture, the cells were initially passed in 10% FBS growth media at 7% to 10% confluence (i.e., 850,000 to 1.2 million cells in a 10-cm Falcon tissue culture plate or 2,000-2,500 cells per well in 96-well flat-bottomed plates) and allowed to grow for 24 or 48 hours. Next, once the BON cell population reached ~10% to 20% confluence, the cells were able to proliferate up to 100% confluence independent of FBS. If the cells were not acclimated with the above-mentioned steps, then they would appear to be quiescent from the start.

At saturating dosages of 100 nmol/L octreotide or 10 ng/mL TGFβ added in FBS-free cell growth media, both octreotide and TGFβ were shown to independently exert growth suppression on BON cells, as indicated by the increase in growth doubling time from 30 ± 3 hours for untreated BON cells to 46 ± 4 and 48 ± 3 hours for octreotide- and TGFβ-treated cells, respectively. Interestingly, this growth suppression resembles BON cell growth in 10% FBS cell growth media. No significant growth doubling time difference was measured in BON cells grown in 10% FBS growth medium (48 ± 4 hours) with either octreotide (50 ± 4 hours) or TGFβ treatment (49 ± 4 hours).

FBS-induced BON cell growth suppression is a result of TGFβ suppression, but it could also be the result of SST (6, 37). However, because the SST peptide has a very short half life (2-3 minutes in plasma), no immunogenic SST was detected in FBS (measured using SST EIA from Promega with half life (2-3 minutes in plasma), no immunogenic SST was detected in FBS (measured using SST EIA from Promega with detection limit of detection; data not shown). Because SST is both short-lived and absent from FBS, exogenous SST is unlikely the direct source for FBS-induced cell growth suppression.

To delineate which inhibitory cytokine is chiefly responsible for FBS growth inhibition, neutralizing antibodies against both SST and TGFβ were used to preabsorb each respective cytokine (1, 6, 14). As seen in Fig. 2A, a significant BON cell growth rescue was observed using either anti-SST or anti-TGFβ neutralizing antibodies, 158% and 143%, respectively. Cellular growth suppression imposed by endogenous TGFβ is evidenced by the addition of neutralizing TGFβ antibodies, which rescued cell growth well above 100% at 143%.
secreted by BON cells with 10 ng/mL TGF. Furthermore, as seen in Fig. 2C, when we treated the increase of TGF reached its maximum of 3 to 20 hours posttreatment, and semiquantitative RT-PCR results indicate that SST mRNA increased in response to 

As illustrated in Fig. 2B, reverse transcription-PCR (RT-PCR) results indicate that SST mRNA increased in response to elicit SST signaling. Interestingly, this overshoot in rescue activity is also evident when the neutralizing SST antibody was added, which rescued cell growth well above 100% at 158% (Fig. 2A), suggesting that the effect of TGFβ on BON cell growth suppression may involve SST signaling.

Next, we asked whether this saturating amount of TGFβ detected in FBS could have induced the expression of SST and thus fortify its effect on growth suppression. Here, we examined this by increasing TGFβ (0, 0.1, 0.5, 1, and 10 ng/mL) in FBS-free cell growth media and subsequently eliciting a TGFβ dose–dependent induction of SST mRNA. As illustrated in Fig. 2B, reverse transcription-PCR (RT-PCR) results indicate that SST mRNA increased in response to increasing TGFβ added in a dose-responsive manner and reached its maximum of ~100% at a TGFβ concentration of 1 ng/mL. Furthermore, as seen in Fig. 2C, when we treated BON cells with 10 ng/mL TGFβ, the level of SST increased dramatically from 7.1 to 37.2 attomoles/cell. Additionally, the amount of SST released into the media under the same TGFβ treatment was also measured and found to increase significantly from 3 to 19 nmol/L in 10 mL of growth medium secreted by ~12 million BON cells.

TGFβ-Dependent Growth Suppression Is Also Mediated by SSTRs

To further examine the participation of the SST signaling pathway in TGFβ-dependent growth inhibition, we used the SST antagonist cyclo-SST to directly block SST pathway activation elicited by either SSTR analogue octreotide or TGFβ. As seen in Fig. 4, cyclo-SST was effective in blocking octreotide-mediated (Fig. 4A, left, triangle symbol) or TGFβ-mediated (Fig. 4A, right, triangle symbol) growth suppression. The reversal of the antiproliferative effect caused by octreotide or TGFβ on BON cells with the addition of SSTR antagonist cyclo-SST illustrates the convergence of the SST and TGFβ pathways. This is consistent with results obtained using the SST-neutralizing antibody, which also disrupted the SST signaling pathway by absorbing most of the free and available SST in the growth media (Fig. 2).

Next, to quantify the cell proliferation rescuing by cyclo-SST through blocking of SST signaling activation caused by either octreotide or TGFβ, data collected from the growth curves, as seen in Fig. 4A, were further processed to derive the cell doubling time and used to construct the growth suppression (%) bar chart as seen in Fig. 4B. Octreotide has high affinity for SSTR2 and SSTR5, whereas it has very low or no affinity for other SSTR subtypes (41). Octreotide extended the growth doubling time of BON cells from 30.2 ± 1.9 to 46 ± 2.1 hours, whereas TGFβ extended cell doubling time to 48 ± 2.4 hours. As seen in Fig. 4A, both cytokines produced significant growth suppression and can be reversed almost completely by adding SSTR antagonist cyclo-SST.
with the fact that SSTR2 is inducible via TGFβ and octreotide, as seen in Fig. 3, collectively, this evidence suggests that SSTR2 is potentially the major receptor subtype that mediates TGFβ-induced SST growth inhibition.

**SST and TGFβ Signaling Pathway Activation Affects Similar Growth Effectors**

We examined if known SST downstream growth effectors could also be influenced by TGFβ signaling activation. Either SST analogue octreotide or TGFβ was added, and Western blot analysis was used to examine the increase in the expression of common growth effectors shown in Fig. 5A to E. Western blot results for BON cell samples treated with 10 ng/mL TGFβ, 100 nmol/L octreotide, and 10% FBS were detected for changes in the following cellular growth inhibitors: SHPTP1, SHPTP2 (17-20), p21Waf1/Cip1 (22, 23), and p27Kip1 (21). A decrease in the expression of the growth accelerator c-Myc protein was also detected (Fig. 5E). We further examined if the induction of SHPTP1 and SHPTP2 expressions seen in Fig. 5A and B Western blots correlates to

**FIGURE 3.** Human SSTR2 expression can be up-regulated by either TGFβ-induced SST— or SST analogue octreotide—treated BON cells. A. In the DNA agarose gel (top), BON cells were treated with either 10 ng/mL TGFβ (left) or 100 nmol/L octreotide (right), mRNA was extracted and reverse transcribed to cDNA for RT-PCR analysis. Next SSTR1, SSTR2, SSTR3, SSTR5, and β-actin messages were detected by RT-PCR and were qualitatively compared for the expression of these receptors between treated and untreated samples. β-Actin RT-PCR result serves as an indicator for technical consistency. Both treatment results were further processed for their SSTR mRNA response and plotted as bar graphs in comparison with untreated BON cells. B. Subtype-specific anti-SSTR2 and anti-SSTR5 polyclonal rabbit antibodies were used to detect SSTR2 and SSTR5 expressions on the cellular membrane surface using FACS. Mean fluorescence intensity was detected and plotted as a bar graph: Mean fluorescence intensity fold of change is plotted on the Y-axis and the types of treatment are indicated on the X-axis. SSTR2 expression was inducible with the addition of 10% FBS, 100 nmol/L octreotide (Oct.), or 10 ng/mL TGFβ; SSTR5 expression was not inducible by any of the above-mentioned treatments. C. SST analogue octreotide (black rectangular box) was used to activate SST signaling modulated through SSTR2 and SSTR5 in shSSTR5-BON cells (depicted as an oval with gray outline). On tetracycline treatment (+), shSSTR5-BON cell lines showed a dramatic down-regulation of SSTR5 mRNA as shown here by RT-PCR and visualized by DNA agarose gel electrophoresis; in the presence of tetracycline, SSTR2 is the most abundant receptor subtype in shSSTR5-BON cells that can be activated by octreotide. Bottom, when shSSTR5-BON is under tetracycline control with down-regulated SSTR5, both SST and SSTR2 mRNA are still inducible by octreotide, as seen in the DNA agarose gel. β-Actin was also examined in all RT-PCR reactions and served as negative controls.
any increase in phosphatase activities. We used the immunoprecipitation capture assay kits specifically designed to detect SHPTP1 and SHPTP2 activities (DuoSet 1C from R&D Systems), and the results are plotted as bar graphs in Fig. 5A and B, respectively.

Figure 4. SSTR antagonist cyclo-SST blocks both TGFβ- and SST-elicted growth suppression. BON cells were grown in FBS-free growth media and treated once every 24 h. MTS assay was used to measure cell proliferation once every 24 h for a span of 72 h. The addition of SSTR antagonist cyclo-SST blocked cell growth suppression caused by either octreotide (left) or TGFβ (right). Negative controls with either vehicle (×) or 200 ng/mL cyclo-SST only (●) yielded no growth difference. Octreotide 100 nmol/L–treated (left) or TGFβ 10 ng/mL–treated (right) BON cells were significantly slowed in their growth (∙), but this growth delay was reversed by adding 200 ng/mL cyclo-SST (●). Points, mean from four separate experimental samples (n = 4), all with R2 > 0.95; bars, SD. Box with the gray dashed outline (Control, cyclo-SST and cyclo-SST + octreotide or cyclo-SST + TGFβ), P < 0.01, versus the corresponding value of octreotide (left) or TGFβ (right) treated BON cells. B. Data from growth curve presented in A were further processed to calculate the growth doubling time of BON cells and presented below as a growth suppression bar graph. Horizontal columns, mean from four separate experimental samples (n = 4); bars, SD. *P < 0.01, versus the corresponding value of control or cyclo-SST–treated BON cells. ◆, P < 0.01, versus the corresponding value of cyclo-SST + octreotide or cyclo-SST + TGFβ–treated BON cells.

Broken TGFβ-SST Connection Diminishes E-cadherin Expression and Disrupts Cell Clusters

To further investigate the significance of the TGFβ-SST connection in metastasis, we used phase-contrast microscopy any increase in phosphatase activities. We used the immunoprecipitation capture assay kits specifically designed to detect SHPTP1 and SHPTP2 activities (DuoSet 1C from R&D Systems), and the results are plotted as bar graphs in Fig. 5A and B, respectively. The basal level was measured to be 1.8 fmol of phosphate release per cell (plotted in the graph as 1-fold) for SHPTP1 and 2.0 fmol of phosphate release per cell for SHPTP2. After treatment with 10% FBS, phosphatase activity increased dramatically to 2.8-fold for SHPTP1 and 5.5-fold for SHPTP2, which was reverted to 0.9- and 0.6-fold, respectively, when neutralizing anti-TGFβ antibodies were added. Furthermore, when BON cells were treated with 10 ng/mL TGFβ, SHPTP1 activity was increased by 2.7-fold and SHPTP2 activity was increased by 4.6-fold and reverted to as low as 0.8- and 0.7-fold, respectively, when neutralizing anti-SST antibodies were added. BON cells treated with 100 nmol/L octreotide also displayed a significant 2.7-fold increase in SHPTP1 activity and a

2.6-fold increase in SHPTP2 activity. Results from Fig. 5A and B indicate that the increase in phosphatase activity triggered by FBS was caused by TGFβ and ultimately dependent on SST. Together, the increase in SHPTP1 and SHPTP2 expressions and activities is dependent on the TGFβ-SST connection.

It is interesting to see that almost all of the above-mentioned growth suppressors increased significantly in the presence of treatments that activate SST signaling. This increase in the expression of growth suppressors was coupled with a decrease in the growth accelerator c-Myc, which effectively suppressed cellular proliferation. As evidenced here, when BON cells were treated with either FBS or octreotide, changes in downstream growth effectors were similar to when BON cells were treated with TGFβ. Therefore, these results further support the notion that SST signaling activation is required for TGFβ-imposed cellular growth suppression.

The Intact TGFβ-SST Connection Mediates Reversible NMT

To address the possible role of the TGFβ-SST connection in cellular differentiated state and metastasis-associated gene expression, we examined the effect of SST pathway inactivation on the expression of neuroendocrine markers chromogranin A and PGP 9.5 in BON cells (Fig. 6A and B). We also examined the effect of SST pathway inactivation on the expression of tumor metastasis markers in BON cells, mainly the mesenchymal-related protein vimentin and metastasis markers E-cadherin and Twist (Fig. 6C-E).

Western blot analyses in Fig. 6A and B show that failure to activate the SST signaling pathway in BON cells in untreated samples resulted in a significant decrease of neuroendocrine markers chromogranin A and PGP 9.5 by ~5- and 4-fold, respectively, when compared with treated samples. This loss in the neuroendocrine marker in BON cells was rescued by adding 100 nmol/L octreotide, 10 ng/mL TGFβ, or 10% FBS to activate the SST signaling pathway. This display of plasticity by the neuroendocrine cell as a neuronal characteristic is remarkable. To further understand this plasticity, the mesenchymal marker vimentin was examined. Vimentin is an intermediate filament protein expressed highly in mesenchymal cells, and it showed an ~2- to 3-fold increase in the absence of treatments when compared with treated samples as seen in Fig. 6C. In the absence of any treatment, BON cells showed an ~4-fold decrease in expression of the cell-cell adhesion protein E-cadherin. Aberrant expression of E-cadherin is associated with the progression of tumor metastasis (30). The presence of E-cadherin on the cell membrane surface is further examined in Fig. 7. Lastly, the Twist protein is commonly associated with morphogenesis and has been linked to tumor metastasis (29, 42); on further examination, we found that the Twist protein is expressed at a higher level in untreated cells, an ~2- to 4-fold increase when compared with treated cells (Fig. 6E).
and immunofluorescence microscopy to visualize cellular morphologic changes and E-cadherin presence on the cellular membrane. BON cells were grown in the presence or absence of FBS and with various treatments to either disrupt or induce the SST signaling pathway. E-cadherin immunofluorescence microscopy results are seen in red by using Qdot 655 goat anti-rabbit IgG as the reporter and shown in the insets of Fig. 7A to H. BON cells were either treated with FBS (Fig. 7A) or with rabbit IgG (Fig. 7C) as controls, and the results here indicate that E-cadherin is well represented on the BON cell membrane surface. When treated with FBS, BON cells appeared to be rounder, and cell-cell boundaries became difficult to distinguish. In addition, less light was able to pass through the cell-cell junctions and, collectively, BON cells appeared to be granular (Fig. 7A and C). When anti-TGFβ (Fig. 7E) or anti-SST (Fig. 7G) neutralizing antibodies were added to the FBS-containing media to block SST signaling activation, cells began to lose their cell-cell contact and allowed more light to pass through the junctions. Simultaneously, E-cadherin on the membrane surface decreased (insets). Furthermore, BON cells appeared more spread out and seemingly fibroblastic, resembling BON cells grown in FBS-free media (Fig. 7B). Next, 10 ng/mL TGFβ (Fig. 7D) or 100 nmol/L octreotide (Fig. 7F) was added to BON cells grown in FBS-free media. Evidenced here, morphologic changes in BON cells deprived of FBS were reversible with either TGFβ or octreotide treatment. To further test this reversible NMT process, BON cells grown in FBS media were treated with the SSTR antagonist cyclo-SST and dosed daily for 48 hours (Fig. 7H). Consequently, the observed morphology is similar to that of cells grown in FBS-free media, and the E-cadherin on the membrane is also lost. Together, results from Figs. 6 and 7 suggest that the TGFβ-SST connection enables reversible NMT in BON cells.

**FIGURE 5.** SST and TGFβ up-regulate similar downstream intracellular growth suppressors and down-regulate the growth accelerator c-Myc. BON cells were treated with TGFβ, octreotide, or FBS and analyzed by Western blot analysis for their growth effector expression change. When 10 ng/mL TGFβ, 100 nmol/L octreotide, or 10% FBS was added to BON cells once every 24 h for a span of 48 h, Western blot analysis revealed that common TGFβ- and SST-mediated downstream growth suppressors SHPTP1 (63 kDa; A) and SHPTP2 (70 kDa; B), p21Waf1/Cip1 (21 kDa; C), and p27Kip1 (27 kDa; D) were induced, whereas the expression of the growth accelerator c-Myc (64 kDa; E) was reduced. Anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody was used to detect glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and to ensure technical integrity of the experiment. A and B, right, results of the SHPTP1 and SHPTP2 activity assays, respectively. Here immunoprecipitation capture assay kits (DuoSet 1C kits from R&D Systems) were used to determine the phosphatase activity by detecting free phosphate level (spectrophotometer reading at absorbance 620 nm) in BON cells 48 h after the following treatments: 10% FBS, 10% FBS + 5 μg/mL neutralizing anti-TGFβ antibody, 10 ng/mL TGFβ, 10 ng/mL TGFβ + 5 μg/mL neutralizing anti-SST antibody, and 100 nmol/L octreotide.
Discussion

TGFβ signaling manages homeostasis in different cells and tumors by playing paradoxical roles in proliferation and is critical for the development and survival of organisms (1, 43, 44). Tumors that are further along in their progression may transition to respond to TGFβ as a growth accelerator and develop a greater potential for metastasis. This tumor transition is an unwelcome event and can be deadly. To date, the mechanism(s) that cells use to decipher TGFβ signaling is unclear. This report is the first to examine and reveal an intact endogenous human TGFβ-SST connection. We propose that SST signaling pathway activation is a potential determinant for mediating TGFβ responsive cellular growth suppression in neuroendocrine tumor BON cells. Additionally, we investigate and confirm the importance of this intact TGFβ-SST connection in tumor transitioning in the neuroendocrine tumor cell system in tumor proliferation and differentiation transition.

In this study, we determined that the amount of TGFβ in 10% FBS cell growth media is excessive and can cause potent antiproliferative actions in BON cells (Fig. 1). Therefore, when BON cells were grown in the presence of FBS, cells were irresponsive to either TGFβ or octreotide treatment (Table 1). This was best illustrated when anti-TGFβ or anti-SST neutralizing antibodies were added to FBS-containing cell growth media to relieve FBS-induced growth suppression (Fig. 2A). Additionally, both SST (Fig. 2B and C) and SSTR2 (Fig. 3A and B) protein expressions were shown to be inducible by the stimulation of SST signaling activation by either cytokine, which suggests a cross-activation of SST by TGFβ that ultimately leads to the activation of the endogenous SST autocrine loop. Further study using shSSTR5-BON cells showed that SST and SSTR2 mRNA induction by octreotide was not affected when SSTR5 was down-regulated by adding tetracycline, which supports the fact that SST-SSTR2 autocrine loop activation is independent of SSTR5 (Fig. 3C). Our observation of this endogenous SST-SSTR2 autocrine loop is consistent with the previously reported mouse recombinant system built using SSTR2-transfected mouse fibroblast NIH-3T3 cells (39). This SST-TGFβ connection was further examined by directly blocking SST with the antagonist cyclo-SST, which effectively reversed both octreotide- and TGFβ-mediated antiproliferative actions (Fig. 4).

The scheme shown in Fig. 8 is a working model showing how the TGFβ-SST connection could work in neuroendocrine tumors. The scheme shows that TGFβ signaling activation (depicted in Fig. 8, 1) induces SST expression, and consequently, a SST-SSTR2 negative growth autocrine loop is forged (depicted in Fig. 8, 2-4). This induction of SST may also

![Figure 6](link-to-image)
indiscriminately activate other SSTR subtypes and ultimately lead to growth and differentiation changes (depicted in Fig. 8, 3'). Next, induced expression and increased overall activity of downstream protein tyrosine phosphatases, SHPTP1 (Fig. 5A) and SHPTP2 (Fig. 5B), occur and, together with the induction of cycline-dependent kinase inhibitors p21Waf1/Cip1 (Fig. 5C) and p27Kip1 (Fig. 5D; depicted in Fig. 8, 5), provide an effective means of antiproliferation. Concurrently, the activation of the TGFβ-SST connection prevents cells from undergoing the process of reversible NMT, as evidenced by the loss of neuroendocrine cell markers PGP 9.5 (Fig. 6A) and chromagranin A (Fig. 6B), together with the increase of the mesenchymal cell marker vimentin and the metastasis markers E-cadherin (Fig. 6D) and Twist (Fig. 6E; depicted in Fig. 8, 6). Changes in molecular markers can be seen in phase-contrast and immunofluorescence microscopy images (Fig. 7), visible by a decrease in BON cell clustering that is coupled with the loss of visible E-cadherin on BON cell surface.

The TGFβ and SST signaling pathways are known barriers for tumorigenesis, and therefore, alterations in their signaling components are common for tumors in gaining a growth advantage (4, 9). The role of TGFβ in carcinogenesis and development has been well documented (4, 27). In some tumors, any breach in TGFβ signaling integrity, such as alterations in TGFβ receptor II, can lead to gobliostoma, gastric, and colorectal cancers (7, 45, 46), and alterations in Smad2 can lead to colorectal and lung cancers (46). It has been shown that among neuroendocrine tumors, such as pituitary, parathyroid, chromaffin, and enterochromaffin cell–derived tumors, a change in SST signaling may have been overlooked in that, in the case of pancreatic cancer, the signaling pathways identified as a tumor suppressor (50, 51). Therefore, it seems that, in the case of pancreatic cancer, the signaling pathways for both TGFβ and SST are essential in preventing tumorigenesis or keeping tumor growth under control.

A potential molecular mechanism for activating the TGFβ-SST connection is the role of Smad4 as a Sstr2 gene transcription activator (52). This was shown by using the luciferase reporter assay, which determined that the SP-1 and CAGA-box nucleotide sequences in the mouse SSTR2 promoter are required for Smad4 to work as a transcriptional activator, as shown in human pancreatic cancer (i.e., Panc1) and mouse pituitary (i.e., AtT20) cell lines (52). Interestingly, more than 50% of the Smad4 genes has been found to be either mutated or deleted in pancreatic cancer (34), whereas SSTR2 is often not expressed (52). Furthermore, when the Smad4 gene was reintroduced into a human pancreatic cancer cell line (BxPC-3), SSTR2 expression was restored (52). Although the TGFβ effector Smad4 seems to have a direct role in the amplification of SST signaling, the role of other known SSTR2 transcription factor(s) is not so clear. For example, the role(s) of the known mouse SSTR2 promoter transcription factors, such as SEF-2 and MIBP1, and their interplay with Smad4 have yet to be determined (53).

The TGFβ downstream effector Smad4 is a potent transcription factor, which is capable of eliciting more than 500 known transcriptional responses (54). The results of our study show the existence of an endogenous human TGFβ-SST connection in the neuroendocrine cell type. However, our study does not exclude the possibility that TGFβ inhibition of cellular growth could be independent of SST signaling in other cell types. Although we identified the TGFβ-SST connection to be a major cytostatic force in neuroendocrine tumor BON cells, we do not claim it to be the only one. However, we believe that the involvement of SST signaling may have been overlooked in

![FIGURE 7](image-url)

**FIGURE 7.** Blocked SST signaling diminishes E-cadherin expression and disrupts cell clustering. Phase-contrast microscopy was used to examine E-cadherin expression on the BON cell membrane surface. Insets, immunofluorescence microscopy results. Cells were treated in the presence or absence of FBS with the following treatments: 10% FBS (A); without FBS (B); 10 µg/mL rabbit IgG with 10% FBS (C); 10 ng/mL TGFβ without FBS (D); 10 µg/mL anti-TGFβ neutralizing antibody with 10% FBS (E); 100 nmol/L octreotide without FBS (F); 10 µg/mL of anti-SST antibody with 10% FBS (G); and 200 nmol/L cyclo-SST with 10% FBS (H).
FIGURE 8. A working model on how an intact TGFβ-SST connection controls cell proliferation and allows reversible NMT. 1, the TGFβ signaling pathway is activated on the cell surface by first binding to its receptor TGFβ-R2 and recruiting TGFβ-R1 to the cell surface, subsequently phosphorylating the intracellular effectors Smad2 and Smad3. Next, phosphorylated Smad2 and Smad3 form a complex with Smad4 and they are translocated into the nucleus to elicit a wide array of transcriptional activities (58-60). 2, one of these transcriptional activities resulted in the induction of SST. 3, next, TGFβ-induced-SST signaling up-regulates both SST and SST-R2 expressions and forges a SST-SST-R2 negative growth autocrine loop as depicted in 4. This induced expression of SST can also exert a negative growth effect and possibly reversible NMT on BON cells through other SSTR subtypes (subtype 1, 3, or 5) as depicted in 3’. In 5, the SST-SST-R2 autocrine loop is firmly established and asserts its growth-suppressive effect (38, 39). This growth suppression was established by eliciting the induction of SHPPT1, SHPPT2, and cyclin-dependent kinase inhibitors p21Waf1/Cip1 and p27Kip1; concurrently, the expression of the growth accelerator c-Myc is down-regulated (Fig. 5). Collectively, these changes in the expression of downstream growth effectors provide maximal cellular growth suppression (20, 61, 62). 6, concurrently, the activated TGFβ-SST connection aids BON cells in retaining their neuroendocrine characteristics [abundant chromogranin A (CgA) and PGP 9.5] while showing only minimal mesenchymal-like characteristics (high level of E-cadherin with low amount of vimentin and Twist; Fig. 6). However, any disruption within this TGFβ-SST connection will reverse the above-mentioned outcome and result in rapid cellular proliferation, a decrease in neuroendocrine characteristics, and an increase in metastasis characteristics.

some studies of TGFβ-modulated growth suppression. For example, in the case of glioblastoma, it has been reported that among 36 human glioblastoma specimens analyzed, 100% showed the presence of at least one SSTR subtype mRNA and with 70% SSTR2 presence (55). Additionally, glioblastoma responds to SST signaling activation as an antiproliferative agent, except when downstream SST effector(s) are compromised, such as protein tyrosine phosphatase (55). Interestingly, it also has been shown that TGFβ signaling can act directly as a cytostatic agent in glioblastoma through the Smad-FoxO complex serving as a transcriptional activator of the p21Waf1/Cip1 promoter (7). However, in the above-mentioned study, it took nearly 20 hours before luciferase reporter activity was recorded (7). Therefore, this lag time for detecting p21Waf1/Cip1 promoter reporter activity, combined with the known SST cytostatic effect, provides the possibility that SST signaling could serve as a determinant for TGFβ-elicted growth suppression in glioblastoma (7, 55).

Human neuroendocrine tumor cell lines are extremely difficult to immortalize in cell cultures, even with the help of a potent oncogenic factor such as SV40.2 Additionally, many of the immortalized neuroendocrine tumor cell lines that exist today are probably not accurate representations of the true genotype of tumor. We acknowledge that this is also likely to be the case for our BON cell model. However, because the TGFβ and SST signaling pathway connection is disadvantageous to tumor growth, it is unlikely that BON cells would subsequently acquire this negative growth connection. This assumption is further supported by studies of pancreatic cancers (33-35, 50), glioblastoma (7), and small bowl carcinoids (36), which all suggest that the disruption in either the TGFβ (i.e., Smad alteration) or SST (i.e., SSTR2 deletion) signaling pathway may be the reason for tumorigenesis and/or enhancement of tumor growth. Therefore, we believe that this TGFβ-SST connection found in BON is physiologically relevant.

Our report does not claim that the existence of the TGFβ-SST connection is universal, rather we believe that this connection is likely to be cell type dependent and/or developmentally specific. TGFβ is seemingly ubiquitous, and others have reported the presence of an intact SST signaling pathway in many different cell types (46, 56, 57). Therefore, by elucidating the role of SST signaling in TGFβ-dependent growth suppression, a connection between these two signaling pathways was made. In the scheme of cancer progression, the antiproliferative effect exerted by this connection could be stage specific and may eventually be lost in favor of cancer proliferation and metastasis. For example, some tumors that are slow-growing are also commonly resistant to chemotherapy, such as carcinoids, which bear a real metastasis threat for patients (25). Because TGFβ has a paradoxical effect on growth in different cell types and at various stages of tumor development, this makes the TGFβ pathway a difficult target for therapy. Therefore, the identification and further examination of this TGFβ-SST connection in the human neuroendocrine cell will allow us to gain a better understanding of the biology behind the TGFβ and SST pathways and facilitate the discovery of novel therapeutic targets. However, the universality and the finer mechanisms of this TGFβ-SST connection remain to be examined and elucidated.

**Experimental Procedures**

**Tissue Culture**

BON cells were derived from a human functional neuroendocrine pancreatic tumor as reported by Arany et al. (31). The BON cells were a gift to our laboratory at the Verto Institute from Dr. Kjell Öberg’s laboratory (Department of Endocrine Oncology, University Hospital, Uppsala, Sweden). BON cells were grown in Ham’s F12 (Invitrogen)/DMEM (50:50; Invitrogen) supplemented with Glutamax (cell growth media; Invitrogen) in 10% certified FBS (Invitrogen). To prepare cells for experiments, cell growth was synchronized before each experiment by alternating overnight treatments of 10% FBS and 0% FBS.
FBS–containing media with 0% FBS media. All cells were kept at 95% air and 5% CO₂ at 37°C in an incubator. Cell number and viability were monitored periodically using the Guava Technologies personal flow cytometer PCA96 and its reagent Viacount Flex.

Cell Growth Assays

CellTiter 96 Aqueous One Solution Cell Proliferation Assay or MTS Cell Proliferation Assay (Promega). The assay measures dehydrogenase enzyme activity found in metabolically active cells converting soluble substrate tetrazolium salt, MTS, to formazan. The production of formazan is proportional to the number of living cells, and the intensity of the produced color is directly proportional to the viability of the cells. This correlation was independently verified using Viacount Flex assay and reading using Guava Technologies PCA96 flow cytometer. The absorbance at 490 nm is directly proportional to the number of live cells. Victor 3V plate reader (PerkinElmer) was used to determine the absorbance of each well at 490-nm wavelength. In a general MTS cell proliferation assay, cells were plated in 96-well culture dishes (Costar) at a density of 2,500 per well in 10% FBS growth media and allowed to attach and grow for 48 h in 100 μL of cell culture media. Next, the growth media were switched to growth media containing 0% FBS. The cell growth curves were constructed by measuring once every 24 h for a span of 72 or 96 h. Data were collected in the presence or absence of the following treatments (for details, see Results and figure legends): octreotide (Sigma-Aldrich), FBS (Invitrogen), TGFβ (BioSource), rabbit IgG (Vector labs), anti-SST antibody (Biomedia), anti-TGFβ antibody (Santa Cruz), and cyclo-SST (Sigma-Aldrich).

Guava Technologies Personal Flow Cytometry, Viacount Flex. Cells were plated in six-well plates and each well started out at a density of ~250,000 cells per well in 2 mL of cell growth medium containing 10% FBS to allow cells to attach and grow overnight. The cells were then switched to 0% FBS growth medium and allowed to grow for another 24 h before treatment. Samples were collected and counted in accordance with Guava Technologies Viacount Flex instructions. At the end of each treatment, cells were dissociated from the plate using 0.5× or 1× trypsin. Dissociated cells were collected and trypsin was quickly quenched with 10 mL of 10% FBS cell medium in 15-mL Falcon tubes. Next, PBS was used as an exchange buffer by tabletop centrifugation at 900 rpm (Beckman). After each centrifugation, supernatant was discarded and the cell pellet was resuspended with PBS using the appropriate volume to achieve 100 to 500 cells/μL.

Preparation of Total RNA and cDNA for RT-PCR

Total RNA was isolated from cells in six-well plates using RNAeasy Mini Kit (Qiagen) and genomic DNA was cleaned up using RNase-Free Dnase Set (Qiagen) per manufacturers’ instructions. For conversion of total RNA to cDNA, a 25-μL reaction mixture was prepared containing 1× reverse transcriptase buffer, 0.5 mmol/L deoxynucleotide triphosphates, 1 μmol/L random hexamers, 200 units of MMLV-Rtase (Promega), and 2 μg of total RNA. The reaction was incubated for 1 h at 37°C. cDNA was stored at −20°C until use. Total RNA and DNA were determined using a spectrophotometer (Beckman-Coulter Spectrophotometer 640 DU) by reading the absorbance at wavelength in the UV range of 260 nm over 280 nm.

Fluorescence-Activated Cell Sorting Analysis for the Detection of SSTR Surface Expression Profiles on BON Cells

In Fig. 3B, BON cells were plated in six-well plates at 100,000 per well in FBS-containing media. Cells were allowed to grow to 85% confluence and then the media were changed to FBS-free or 10% FBS–containing media. With the exception of the negative controls, all experimental wells were treated with either 20 ng/mL human TGFβ (BioSource) or 100 mmol/L octreotide (Sigma-Aldrich). Next, SSTR2 and SSTR5 were detected with biotinylated SSTR2 and SSTR5 subtype–specific polyclonal rabbit antibodies via Guava Express (Guava Technologies) per manufacturer’s instructions. Samples were incubated overnight in the incubator. On the following day, BON cells were harvested for experimentation by using 1× trypsin (Invitrogen) and incubated for 3 min, and immediately thereafter, trypsin was quenched by adding 10% FBS. Next, BON cells were resuspended in the fluorescence-activated cell sorting (FACS) buffer [1× PBS (Invitrogen) supplemented with 3% FBS (Invitrogen) and with 0.01% NaN₃ (Sigma-Aldrich)] at 250,000/mL and cells were plated into appropriately labeled wells of a 96-well round-bottomed plate at ~50,000 per well. All 96-well plates were subjected to centrifugation at 1,000 × g to pellet cells; supernatants were aspirated and discarded; and the cell pellet was resuspended. Each of the polyclonal human SSTR2 and SSTR5 subtype–specific antibodies was made against an epitope on the extracellular region of seven-transmembrane receptors,1 and antiserum was further subjected to Protein A column chromatography (Bio-Rad) IgG purification. Next, cell pellets were suspended and 1.5 μg of biotinylated human SSTR subtype–specific primary antibodies (Synpep) were then added to each sample; in particular, subtype 2, 3, or 5 in 100 μL of FACS buffer per sample. Next, samples were incubated for 40 min at 4°C. After incubation, the plates were washed twice in FACS buffer and the supernatants were aspirated. Next, 1 μg of strepavidin-phycocerythrin (Vector Laboratories) was added to the resuspended cells in 100 μL of FACS buffer per well. Samples were incubated for 30 min at 4°C in the dark. Immediately following incubation, plates were washed thrice in FACS buffer and supernatants were aspirated. Pellets were resuspended in 200 μL of FACS buffer and acquired on FACS (Guava Technologies PCA96).

Construction of Stable SSTR5 Knockdown BON Cells Using a Recombinant pSUPERIOR.puro Vector

BON cells were stably transfected with a recombinant pSUPERIOR.puro vector (OligoEngine) containing a short oligonucleotide that forms a hairpin RNA (shSSTR5). When transcribed, shSSTR5 RNA degrades the SSTR5 mRNA. This oligonucleotide was designed in part using an online siRNA program from the IDTDNA website, http://www.idtdna.com. shSSTR5 RNA was effective in down-regulating SSTR5 gene expression. The short oligonucleotide has 5′ BglII and 3′
**HindIII restriction enzyme sites designed for cloning purposes.** Their sequence information is as follows: sense strand, GATCTCGGTCTGTCAGTTCGTTAAGAGAAGACGGCGACATGGCAGAGACTTTTTA; antisense strand, AGCCTTAAAAGTCTCTGATGCTGTGCTGTCTCTGGAACAGGCCATGACAGACAGGGA. We took 1 μg/mL of the recombinant plasmid, shSSTR5, and stably cotransfected it with 4 to 6 μg/mL of pcDNA6/TR (Invitrogen) using FuGENE Transfection Reagent (Roche Applied Science). After 7 to 10 d of selection pressures using puromycin (10 μg/mL) and blasticidin (5 μg/mL), individual colonies were selected using a sterile cloning disc (Science Lab) and were allowed to grow in 24-well plates (Corning). shSSTR5-BON cells were grown and maintained in Ham’s F12/DMEM in 10% FBS supplemented with 10 μg/mL puromycin and 5 μg/mL blasticidin. On addition of 2 μg/mL tetracycline to shSSTR5-BON cells, the transcription of shSSTR5 oligonucleotide was derepressed and, consequently, SSTR5 was down-regulated.

**Phase-Contrast Microscopy and Immunofluorescence Detection Using Pseudoconfocal Microscopy**

BON cells were plated at 10% confluence (300,000 cells) in 10% FBS cell growth media and allowed to attach to the plate and grow for 24 h before exchanging their growth media to 0% FBS. Cells were then allowed to grow under different treatments on the coverslips in six-well plates for the next 48 h before visualizing by microscopy. Zeiss Axiovert 35 microscope was used for phase-contrast microscopy, whereas Zeiss Axiovert 200M with Apotome noise reduction was used for pseudoconfocal fluorescence microscopy. For treatment details, see Fig. 7 legend and/or the article text. After treating the cells for 48 h (when their confluence reached ~50-70%), phase-contrast microscopy was done with the Zeiss Axiovert 35 microscope with 20× objective water lens to record cell morphologic alterations in the presence or absence of each treatment. All phase-contrast images were taken at 20-ms exposure time. Furthermore, some treated samples were also subjected to immunofluorescence microscopy for the detection of E-cadherin on the membrane surface of live cells. This was done with E-cadherin rabbit polyclonal antibody (Santa Cruz) as the primary antibody (1:100). Immediately after cell fixation with 3.7% formaldehyde at 37°C for 10 min, the secondary antibody Qdot 655 goat anti-rabbit IgG (Invitrogen) was added (1:50) for visualization using the pseudoconfocal fluorescence microscope Zeiss Axiovert 200M with Apotome noise reduction. The microscope oil lens objective was set at 63×/1.4 for imaging. All fluorescent images captured for Q-dot 655 fluorescence were captured with Cy 3.5 filter with 2.5-s exposure time, and Axiovision 4.4 software was used for settings and image processing.

**Visualization and Quantification of DNA Products from RT-PCR on Agarose Gels**

PCR was done in a volume of 25 μL using 1 μL of the template strand cDNA, 0.2 μmol/L of each primer, and 1× PCR Master Mix (Promega). PCR conditions consisted of a denaturing step at 95°C for 2 min for one cycle, followed by 25 cycles at 95°C for 45 s, 60°C for human SST, 55°C for human SSTR subtypes for 1 min, and 72°C for 45 seconds, and then an extension step at 72°C for 7 min. The reaction was electrophoresed on 1.2% agarose gels. The DNA bands on the gels were stained with ethidium bromide (Sigma-Aldrich) and a picture was obtained under UV light at 365 nm. Ethidium bromide–stained DNA images were captured using the Alpha Innotech FluorChem 9900 imaging system and results were quantified using AlphaEase FC software version 3.3.

**Western Blot Analysis**

BON cells were plated at 10% confluence (~1.2 million cells) in 10-cm tissue culture plates with 10% FBS cell growth media. After 48 h, 0% FBS cell growth media replaced the initial 10% FBS cell growth media with the following treatments: 10% FBS, 100 nmol/L octreotide, and 10 ng/mL TGFβ. Another 48 h later, cells were harvested for Western blot analysis. Cells were harvested for proteins using M-PER lysis buffer (Pierce), and 80 μg of protein for each sample were solubilized in sample buffer containing 62.5 mmol/L Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, and 50 mmol/L DTT. Next, these solubilized samples were loaded onto 12% Ready Gel Tris-HCl gels (Bio-Rad) per lane and fractionated by electrophoresis. They were transferred by electrophoresis onto nitrocellulose membranes in a transfer buffer containing 0.025 mol/L Tris, 0.2 mol/L glycine, and 15% methanol. For the downstream growth effectors examined in Fig. 5, the blots were probed with the following rabbit polyclonal antibodies at 1:500 dilution from Santa Cruz Biologicals, unless otherwise indicated: SHPPT1, SHPTP2, p21Waf1/Cip1, p27Kip1, and c-Myc. The membranes were also probed with neuroendocrine markers as seen in Fig. 5A and B: PGP 9.5 from Abcam and chromagranin A. Additionally, membranes were probed with EMT markers such as vimentin, E-cadherin, and Twist with respective polyclonal rabbit antibodies. In all samples examined, glyceraldehyde-3-phosphate dehydrogenase antibody was used as the control sample to normalize experimental sample values. The Visualizer Western blot detection kit, rabbit chemiluminescent system (Upstate Biological) was used for the detection of the primary antibody bound to the antigen on the membrane. The kit uses a goat anti-rabbit IgG conjugated to horse radish peroxidase, which we used at a ratio of 1:10,000 for detection. Chemiluminescent bands were captured using Alpha Innotech FluorChem 9900 imaging system and results were quantified using AlphaEase FC software version 3.3. The detected and quantified chemiluminescent bands were expressed in area density in arbitrary units. Various treatments will cause differential cellular growth and thus yield different amounts of protein per cell. Therefore, to compare between treatments, next to the Western blots (Fig. 6A-E) are bar graphs plotted by first normalizing the sample protein signals against the glyceraldehyde-3-phosphate dehydrogenase protein band signal and then further averaged for each cell.

Additionally, Western blot analysis was used in Fig. 1A to determine the level of TGFβ in FBS using TGFβ antibody (Santa Cruz). BON cells were grown in 0% FBS cell growth media for 48 h and harvested at 80% confluence (~10 million cells) in a 10-cm tissue culture plate. Next, harvested media and BON cells were used for Western blot analysis to determine TGFβ concentration in samples. The Western blot procedure is
similar to what was described above. Increasing concentrations of TGF\(\beta\) (Invitrogen), 0.5, 1, 2, 5, and 10 ng/mL of recombinant proteins at 40 \(\mu\)L per lane, were loaded as samples for the standard curve. Normalized arbitrary units were determined for each band as described under “Western Blot Analysis” and plotted as the Y-axis versus the logarithmic value of the TGF\(\beta\) concentration as the X-axis. This standard curve is then used to determine the amount of TGF\(\beta\) in FBS, whole cells, and cell growth media.

**SHPTP1 and SHPTP2 Phosphatase Activity Assays**

BON cells were grown and treated as described in “Western Blot Analysis.” In particular, the following treatments were used: 10% FBS, 10% FBS + 5 \(\mu\)g/mL neutralizing anti-TGF\(\beta\) antibody, 10 ng/mL TGF\(\beta\), 10 ng/mL TGF\(\beta\) + 5 \(\mu\)g/mL neutralizing anti-SST antibody, and 100 mmol/L octreotide. Next, 6 to 10 million BON cells were collected (10 million/mL) and used in accordance with the procedures described in the kits used, DuoSet 1C SHPTP1 and DuoSet 1C SHPTP2 (R&D Systems). Briefly, the DuoSet 1C kit is an immunoprecipitation capture assay using an agarose-immobilized antibody, which is specific for SHPTP and binds both active and inactive SHPTP. After washing away unbound material, a synthetic phosphopeptide substrate is used and is dephosphorylated by active SHPTP to generate free phosphate and unphosphorylated peptides. The agarose beads are pelleted by centrifugation and the supernatant is transferred to a 96-well microplate. The amount of free phosphate in the supernatant is determined by a sensitive dye-binding assay using malachite green and molybdcic acid. The DuoSet 1C kit is a spectrophotometric assay that can detect the phosphate release for each sample at \(A_{620}\) nm using the microplate reader Victor 3V (Perkin-Elmer). The activity of SHPTP in the BON cell lysate is determined based on a standard curve derived from free phosphate concentrations of 0, 1.5, 3, 6, 12, 25, 50, and 100 mmol/L. See the DuoSet 1C manual for details and assay protocol.

**TGF\(\beta\) and SST EIA Assays**

BON cells were plated at 10% confluence (~1.2 million cells) in 10-cm tissue culture plates with 10% FBS cell growth media. After 48 h at 20% confluence, we replaced the initial 10% FBS cell growth media with 0% FBS cell growth media to allow both TGF\(\beta\) and SST to accumulate in the cell growth media for another 48 h. Subsequently, the ELISA immunoassay kits were used to determine the amounts of TGF\(\beta\) and SST in the whole-cell lysate and in the growth media. Additionally, FBS was also examined for TGF\(\beta\) and SST levels. TGF\(\beta\) EIA (Promega; ref. 40) and SST EIA (Phoenix Pharmaceutical) were used according to the manufacturers’ instructions.

**Data Calculation**

Growth response (%) in Fig. 2A is calculated using the following equation: \([\text{treated cells} - \text{rabbit IgG}-\text{treated cells}] / \text{rabbit IgG}-\text{treated cells}] \times 100\%\).

mRNA response for the SST/\(\beta\)-actin (%) bar graph in Fig. 2B is calculated using the following equation: \([\{\text{SST/}\beta\text{-actin of treated cells} - \text{SST/}\beta\text{-actin of untreated cells}] / \text{SST/}\beta\text{-actin of untreated cells}] \times 100\%\).

Mean fluorescence intensity fold change in Fig. 3B is calculated using the following equation: (mean fluorescence intensity of cells treated) / (mean fluorescence intensity of cells untreated).

Phosphate fold increase in Fig. 5A and B bar graphs is calculated using the following equation: (free phosphate measured in treated cells / free phosphate measured in cells at basal level).

**Statistical Analysis**

The effect of treatments was analyzed via one-way ANOVA using Microsoft Excel 2002 software. \(P < 0.01\) was considered significant. Additionally, values were calculated and plotted using the Microsoft Excel 2002 software and presented as mean ± SD.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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**References**


The Effect of Transforming Growth Factor β on Human Neuroendocrine Tumor BON Cell Proliferation and Differentiation Is Mediated through Somatostatin Signaling

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