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13. ABSTRACT *(Maximum 200 Words)*

The neuropeptide somatostatin is an important regulatory hormone that is widely distributed throughout the body. Somatostatin's actions are primarily inhibitory, and recently, it has been utilized as an antiproliferative agent against several tumor types, including breast neoplasms. Experimentally and clinically, somatostatin can inhibit breast cancer cell growth, possibly by inhibiting the secretion of growth factors, or by acting directly on the cells themselves to induce programmed cell death, or apoptosis. Despite increased clinical use, the mechanism(s) by which somatostatin acts to control breast cancer cell growth remain largely unknown. In this final report, I describe studies performed to survey of numerous breast cancer cell lines to predict their usefulness as models to study somatostatin action, which comprised Aim 1 of the proposal. One cell line, MDA231, shows a robust response to somatostatin with regard to growth factor stimulation of the mitogen activated protein kinase, ERK1/2 (or MAPK). Specifically, the somatostatin analog BIM23014 caused a marked dimunition of EGF-stimulated MAPK activity, likely mediated by the type 2 somatostatin receptor. Furthermore, I show data indicating that another signaling pathway, the jun n-terminal kinase (JNK) pathway may be activated in response to somatostatin. Together, these results begin to explain somatostatin's ability to inhibit cell growth and induce apoptosis in breast cancer. Identification of these target pathways defines functional assays by which the efficacy of future anti-cancer drugs can be tested.

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Introduction

The neuropeptide, somatostatin is an important regulatory hormone that is widely distributed throughout the body. For years it was recognized as the main negative regulator of growth hormone secretion from the anterior pituitary gland. Recently, however, it has become apparent that somatostatin can act at many tissues and organs, and in addition to its role as an inhibitor of hormone secretion, it is also capable of inhibiting cell growth and proliferation (1,2). As such, somatostatin is currently being investigated for its potential clinical use as an anti-cancer agent, as inhibition of cell growth is an important feature of most anti-cancer therapies. Indeed, stable somatostatin analogs, such as BIM23014 (Lanreotide) and SMS201-995 (Octreotide), are capable of inhibiting the growth of several types of tumors including pituitary adenomas, neuroblastomas, pancreatic cancer and breast cancer (2). At the cellular level, the actions of somatostatin are mediated by a family of G protein-coupled plasma membrane receptors termed ssts. To date, five distinct sst receptor subtypes have been identified, and these are named sst1-5 (3). Subtype 2 (sst2) is the most common somatostatin receptor found on tumor tissue, followed by sst5 (1, 4).

There is currently little information available regarding somatostatin action, especially as it pertains to breast neoplasms, and thus the objective of these studies is to define the functional role of somatostatin in several breast cancer cell lines as it impinges on growth factor-stimulated signaling pathways. Growth factors, such as EGF, FGF, and IGF-1, have been implicated in breast cancer tumorigenesis and progression (5), and therefore I plan to determine if these pathways are affected by somatostatin in a somatostatin-responsive breast cancer cell line. My underlying hypothesis is that somatostatin exerts its antiproliferative effects on a wide variety of tumors by interfering with multiple signaling pathways which lead to cell division.

Thus, my initial goals were to: 1) develop a somatostatin-responsive breast cancer cell model system, 2) to determine whether somatostatin inhibits growth factor- or UV light-stimulated Raf kinase, MAP kinase, Jun kinase, Elk and Jun biochemical activities, and 3) to determine the functional consequences of somatostatin action on these same components by determining whether somatostatin inhibits growth factor- or UV light-stimulated Elk-, Jun-, and Ets-mediated transcription.

Elucidation of the mechanisms involved in somatostatin action will not only further our understanding of the regulation of cell growth, and hence tumorigenesis, but may also lead to improved drug design for the treatment of breast cancer.

Body

NOTE: Although most of the data described in this report has been submitted for publication and is currently being reviewed, it has yet to be formally published. As such I would like to limit the distribution of these findings.

In this section of the report, my research results are organized according to the Specific Aims set out by my original proposal. In addition to reporting on progress made on the Specific Aims of my proposal, I also describe the future directions that my research interest is moving towards as I attempt to carve my own niche in the breast cancer research field, and establish myself as an independent researcher.

Aim 1*: Develop a somatostatin-responsive breast cancer cell model system.*

Somatostatin receptors are present in very low numbers on most cell types, making analysis of signaling events difficult to measure by current techniques. As noted in the previous annual reports, we initially proposed an approach that has been used by several investigators to elucidate signaling pathways from numerous types of receptors. The strategy was to make stable breast cancer cell lines that would overexpress sst2 and thereby amplify signaling events of this receptor in a physiologically-relevant cell type. We initially proposed to overexpress sst2 as it is believed to be the most prevalent subtype expressed in breast cancer tumors, and it is also the primary target of most of the commercially-available somatostatin analogs. However, for reasons stated in previous reports, we decided to pursue a different approach suggested by colleagues and reviewers, which was to identify a breast cancer cell line that shows a robust response to somatostatin via its endogenous somatostatin receptors. To accomplish this, several breast cancer cell lines were obtained from the University of Colorado Cell Culture Core Facility, which differed in estrogen receptor (ER) and EGF receptor status. They included the MCF-7, T47D, MDA 231 and ZR75.1 cell lines. The current literature contains few studies that have assessed somatostatin receptor expression in breast cancer cell lines, not all of the cell lines we have chosen have been examined, and furthermore, variations may exist in the "same" cell lines due to clonal differences at different sites. Therefore, we felt the best approach was to perform an analysis of sst expression in these cell lines ourselves. A reverse transcriptase-polymerase chain reaction (RT-PCR) approach was chosen because, as mentioned above, ssts are present in very low abundance and they cannot be readily detected by Northern blot or RNAse protection assays. Total RNA was prepared from the four cell lines, reverse transcribed to cDNA and then amplified by PCR with oligonucleotides specific for each of the five sst subtypes. The quantity of PCR product for each reaction was normalized to an internal control (GAPDH) in an identical reaction mix. The results indicated that each of the four cell lines examined displayed a relatively unique expression pattern of ssts, and some cell lines express certain receptors to a higher degree than others (Table 1). The information gained from this RT-PCR study led us to focus on the MDA231 cell line as our model system. MDA 231 cells expressed a relatively high amount of sst2 and sst5, and importantly, show a strong response to the somatostatin analog BIM23014, with respect to the growth factor-regulated signaling pathways that we are interested in studying.

We chose to utilize the somatostatin analog, BIM 23014 (also known as Lanreotide, Biomeasure Inc.) in our studies as it is a potent, long-acting agent that is used clinically for breast cancer treatment. We next wanted to identify which somatostatin receptor subtype was mediating the effects in our experiments. According to some reports, BIM 23014 specifically activates the sst2 receptor subtype. However, there are discrepancies in the literature with regard to the specificity so we performed studies to determine empirically the specificity of the somatostatin analog BIM23014 for each of the somatostatin receptor subtypes. Briefly, plasmids encoding somatostatin receptor subtypes 1-5 were individually transfected into Chinese hamster ovary (CHO) cells, which lack endogenous ssts. We measured inhibition of forskolin-stimulated cAMP accumulation indirectly via the highly cAMP-dependent alpha glycoprotein subunit promoter. Inhibition of cAMP is a well-established downstream effect of all five somatostatin receptors. The results of this study, shown in Fig. 1, reveal that the BIM analog selectively activates only sst2 and sst5 to inhibit cAMP, while native somatostatin activated all five receptors to inhibit cAMP, indicating that all five receptors were expressed in these cells (Fig. 1). Since sst2 and sst5 are abundantly expressed in MDA 231 cells, we feel that these cells, in combination with the relatively selective BIM analog, provide a very good model system to study the mechanisms of somatostatin's antiproliferative effects in breast cancer.

AIM 2: ***Determine whether somatostatin inhibits growth factor- or UV light-stimulated Raf kinase, MAP kinase, Jun kinase, biochemical activities.***

Somatostatin analogs have been shown to inhibit the growth rate of breast cancer cells in both cell culture and animal models (2, 9, 13). We have also shown that the somatostatin analog, BIM23014 significantly inhibits the growth rate of MDA231 breast cancer cells (data not shown). However, the mechanism(s) behind somatostatin's antiproliferative actions is not clearly understood. The anti-proliferative effect of BIM23014 could be due to either cell cycle arrest and/or due to increased apoptosis. To determine the contribution of apoptosis mediated by BIM23014, MDA231 cells were treated with this analog in the presence and absence of estrogen (17-β estradiol), as this hormone has been shown to upregulate somatostatin receptors in breast cancer cells (4). Determination of apoptosis was performed by the propidium iodide/Hoescht 33342 staining method. This method utilizes DNA-binding fluorescent dyes for determining the percentage of cells undergoing apoptosis and/or dying in a given population. Thus this method is both a quantitative and morphologic assay. The results of this experiment are shown in Fig. 2. In the top panel, which shows the results in the absence of estrogen, indicates that BIM23014 at 10 nM or at the 100 nM level, BIM23104 induces apoptosis to a level that is roughly equivalent to the known apoptotic agent, mitomycin C. Pre-treatment of the cells with EGF had only a partially protective effect against BIM-induced apoptosis. Interestingly, the addition of estrogen (Fig. 2, bottom panel) did not lead to a higher degree of BIM-mediated apoptosis, indicating that either MDA 231 cells are estrogen resistant or that the maximal degree of apoptosis has been achieved.

The next goal was to determine which signaling pathways were involved in the BIM-induced apoptosis observed in Fig. 2. We initially focused on the p44/42 MAP kinase (MAPK or ERK) pathway. The growth factor-stimulated MAPK pathway plays a central role in cell proliferation, and we have shown that this pathway is inhibited by sst2 in

pituitary tumor cells (6). Inhibition of the MAPK pathway is associated with increased apoptosis. Additionally, recent reports have implicated the importance of the MAPK pathway in breast tumorigenesis, as it has been shown to be deregulated and MAPK is hyperexpressed in breast carcinomas (7,8). To determine if somatostatin analogs can block growth factor stimulation of this pathway we initially sought to determine the optimal time frame for somatostatin addition to EGF treated cells. Figure 3 shows a time course experiment of EGF-stimulated MAPK activation. Briefly, MDA 231 cells were serum-starved to lower basal MAPK activity, and then treated with EGF (25nM), and harvested at various time points post-treatment. Equal protein amounts of whole cell extract were Western blotted and probed with an antibody specific to the activated (phosphorylated) form of MAPK. Phospho-specific antibodies are now commonly used to determine the activity of various signaling components, including kinases, and we have shown comparable results between this approach and the traditional kinase assays, without having to use high amounts of radioactivity associated with kinase assays. As seen in Fig. 3, EGF causes a rapid but transient, fifteen-fold increase in MAPK activity, beginning at 2 mins. post-treatment, peaking at 5 and 10 mins. and returning to baseline after 15 mins.

Therefore, we chose to look at the effects of BIM23014 on MAPK activity at the 5 min. time point, when MAPK activity is highest and not yet diminishing. Figure 4 shows the results of a Western blot analysis of equal amounts of MDA 231 whole cell extracts after various treatments, using an antibody that is specific for the active, phosphorylated form of MAPK (phospho-MAPK). Lane 1 in Fig. 4 is a positive control phosphorylated p44 MAPK protein purchased from New England Biolabs. Lane 2 is a negative control p44 MAPK protein that is not phosphorylated. As expected, the phospho-specific antibody detected the phosphorylated MAPK but did not cross react with a non-phosphorylated MAPK. Lane 3 is extract from MDA 231 cells that were cultured in DMEM plus 10% fetal calf serum (FCS). It is interesting to note that these cells show a very high level of phospho-MAPK. I have observed that this cell line grows very rapidly in culture, with a doubling time of 24 hours, and it was derived from an aggressive tumor, therefore, there may be a correlation between it's rapid growth characteristics and the degree of phosphorylation of MAPK (7,8). Since the level of phospho-MAPK is high in cycling MDA 231cells in complete media, the remaining samples (lanes 4-12) represent MDA 231 cells that were grown in media devoid of FCS. This strategy is commonly employed to increase the signal to noise ratio, and thus make it easier to detect changes in phospho-MAPK levels in response to somatostatin. Lane 4 represents cells that are cultured in media without FCS. As expected, cells cultured under these conditions have very low levels of phospho-MAPK that are undetectable with the phospho-specific MAPK antibody. However, addition of 25 nM EGF for 10 minutes leads to an approximately 20-fold increase in MAPK phosphorylation (Fig. 4, lanes 5 and 6) when compared to quiescent cells (lane 4). Since MDA 231 cells showed a strong sst5 signal by RT-PCR (Table 1), I used the somatostatin 28 (SRIF 28), which preferentially targets this receptor subtype, in lanes 7-9. In lane 7, SRIF 28 alone was added, and this had no further effects on basal phospho-MAPK levels. When SRIF 28 was added 5 minutes prior to EGF, it had no effect on MAPK phosphorylation when compared to EGF alone (lanes 5 and 6). MDA 231 cells also express sst2, and therefore I used the sst2-selective agonist, BIM23014 in lanes 10-12. Again, BIM alone failed to repress steady-state phospho-MAPK (lane 7). However, in

contrast to SRIF 28, BIM23014 almost completely abolished the ability of EGF to stimulate MAPK phosphorylation. Therefore, it appears that sst2 in these cells is preferentially coupled to the inhibition of MAPK phosphorylation (lanes 11,12).

Additionally, we performed experiments to determine if BIM23014 activates the Jun n-terminal kinase (JNK) pathway. Previously, our lab and others have shown that somatostatin can induce apoptosis in breast cancer cells, and that this may be a major mechanism for its anti-cancer effects (9). The JNK pathway is one of several potential routes in the apoptotic process, and in conjunction with an inhibition of MAPK provides an attractive model for somatostatin's inhibition of cell growth. In this experiment, T47D breast cancer cells were treated with and subjected to Western blot analysis and probed with an antibody specific for phospho-JNK. The results are shown in Fig. 5. A time course treatment shows a modest (approximately 2-fold) and transient increase in JNK phosphorylation, peaking at 5 minutes (Fig 5, lane 3), and then rapidly diminishing to control levels by 15 minutes. In the past year, we have repeated this study in the MDA 231 cell line. The results are essentially the same as those seen in the T47D cell line, in that JNK activity is modestly elevated in MDA 231 cells after treatment with BIM23014 (data not shown). However, we have been unable to consistently observe a greater than 2-fold increase in JNK phosphorylation. Therefore, it is difficult at this point to assess the significance of a two-fold increase in JNK activity. It is possible that the modest increase in JNK activity is relevant in these cells and this may become apparent when we assay the effects of somatostatin on Jun-mediated transcription.

AIM 3: ***Determine the effects of somatostatin on growth factor- or UV light-stimulated Elk-, Jun-, and Ets-mediated transcription.***

In order to accomplish this Aim, we utilized a transient transfection approach using constructs that code for fusion proteins of Elk, Jun, and Ets transactivation domains fused to the yeast Gal4 DNA binding domain. This allows us to focus on the transactivation potential of these factors, independent of DNA binding and complex heterodimerization systems. The reporter construct, 5X UAS TK-luc, contains five copies of a Gal4 binding site upstream of a minimal TK promoter and luciferase reporter gene.
Thus far, we have performed experiments with Elk-1 and Ets-1. The data for the Elk-1 experiments are shown in Fig 7. Briefly, MDA 231 cells were transfected by electroporation with the Gal4-Elk-1, 5X UAS-luc, and CMV- β gal plasmids (the latter is used to control for transfection efficiency). The cells were then quiesced in DMEM plus 1% fetal calf serum (FCS) for 18 hrs. Cells were treated with vehicle or 10nM BIM23014 for 30 mins. We have determined through optimization experiments that this is the time point required for BIM to activate it's receptor(s) and transmit it's inhibitory signal. After 30 mins. the cells were then stimulated with either serum, to a final concentration of 15% v/v, or EGF (25nM), and returned to the incubator for 6 hrs. The cells were lysed, extracts prepared and luciferase activity was measured and corrected for β gal activity (expressed as mean relative light units). As shown in Fig 7, the 5X UAS construct has very little activity in low serum conditions. However, as expected, serum and EGF lead to dramatic increased in Elk activity to approximately 18-and 17-fold of control, respectively. In cells pretreated with BIM23014, the serum challenge led to an increase that was approximately 59% of serum alone levels (~40% decrease in activity compared to serum alone). EGF

alone stimulated Elk-1 to levels fairly similar to serum, but in this case, pretreatment with BIM caused a much greater decrease in Elk activity, approximately 20% of EGF alone (80% decrease). Thus, BIM23014 is capable of diminishing Elk-1 transcriptional activity stimulated by serum or EGF, but to a much greater extent with EGF. The reason for this is perhaps that serum contains a complex mixture of growth factors and other stimulators of Elk-1, only a few of which may be inhibited by somatostatin.

The Gal4-Ets plasmid construct was a generous gift from Dr. Bohdan Wasylyk (INSERM, Strasbourg, France), and consists of the Ets-1 transactivation domain I and regulatory domain II fused to the yeast Gal4 DBD. The results of the Ets-1 experiments are given in Fig 6. These experiments were performed in essentially the same manner as those for Elk-1, described above, except that we used fibroblast growth factor-2 (FGF-2) as the growth factor stimulus. Our laboratory has shown that FGFs are important components of signaling pathways that culminate in the activation of Ets factors (11). FGFs are also critical growth factors in breast cancer that are often found to be overexpressed in breast tumors (5). Again, the level of activity of the Gal4-Ets/5XUAS combination is very low in 1% serum conditions. And, as expected, 15 % serum and FGF-2 (2 ng/ml) caused increased Ets activity to approximately 20- and 16-fold versus control, respectively (Fig 6), However, BIM pretreatment caused a much smaller dimunition of these stimuli than for Elk-1. Thus we conclude from the studies that we have performed to date that the antiproliferative effects of the somatostatin analog BIM23014 are possibly the result of decreased transcriptional activity of the Ets factor, Elk-1, rather than Ets-1. Of course other transcription factors are likely to be involved, and one in particular that we are interested in investigating is the Ets factor, ESX.

Other studies stemming from this proposal

At the time this proposal was originally submitted, somatostatin analogs were generating much excitement in the medical and scientific communities, as they were viewed as novel, well-tolerated, and potentially powerful tools in the fight against breast cancer (2, 9, 13). Indeed, experimental models in animals and cell culture showed it was effective in slowing tumor growth and as a result, several clinical studies were initiated to determine its efficacy in humans. Unfortunately, some of this excitement has waned as the results of human clinical trials have recently been revealed. In two separate Phase II clinical trials, somatostatin analog treatment of women with metastatic breast carcinoma failed to show any observable effects on tumor growth and progression (14, 15). This was true whether somatostatin was given alone or in conjunction with tamoxifen. It should be noted that these studies were performed in patients with advanced, metastatic disease with poor prognosis. Therefore, it is conceivable that somatostatin analogs will be a useful anticancer therapy in early stage, less aggressive breast tumors. Certainly, research and clinical trials will continue in this area, as somatostatin analogs are proven anticancer agents for other types of tumors, such as those of the pancreas and pituitary.

As a postdoctoral fellow, one of the main goals of my training is to eventually obtain a position as an independent investigator. Towards this end, my mentor, Dr. Gutierrez-Hartmann, has encouraged me to explore novel projects related to the control of

breast cancer cell growth that may provide the basis for future funding opportunities, and also to have a project that is my own to take with me into my own laboratory. Below, I briefly describe some recent research directions that our laboratory is exploring. I believe these studies are complementary to the goals of this proposal and the mission statement of the DoD Breast Cancer Research Project and furthermore, will allow me to remain in the field of breast cancer research.

The results described above indicate that somatostatin can inhibit the transcriptional activity of at least two members of the Ets family of transcription factors, Ets-1 and Elk-1. Ets factors have important roles in proliferation and differentiation of cells and in oncogenic transformation (10). Recently, a novel epitheial-specific human Ets factor, ESX (or ESE-1) has been identified. ESX mRNA is upregulated in certain breast cancers, and a positive feedback loop may exist between the HER2/neu proto-oncogene and ESX in human breast cancer cell lines, such that ESX modulates HER2/neu expression and HER2/neu in turn positively regulates ESX(12). We have isolated the ESX cDNA from T47D human breast cancer cells by RT-PCR, and have cloned the gene into a mammalian expression vector with an HA-epitope tag. As an extension of the current studies, we would like to determine the role ESX has in breast cancer cell growth and transformation. To accomplish this, the immortalized but nontransformed human breast epithelial cell line MCF-12A was used to evaluate the transforming capabilities of HA-ESX, HAVP16-ESX (a super-active form of ESX using the viral VP16 transactivation domain), Ets-2, and V12-Ras in foci formation assays. MCF-12A cells were transfected with the empty vector (pCGN2) or the various Ets constructs, and plated at a low density in complete Ham's F12/DME media. Figure 8A shows results from a representative experiment performed in duplicate revealing that empty vector results in a negligible number of small foci, whereas the positive V12Ras control results in a large number of moderately-sized foci. In this same assay, HA-VP16-ESX also resulted in a large number of moderately-sized foci, and these foci appear to be qualitatively similar to those generated by oncogenic V12-Ras.

In order to obtain a more complete and quantitative analysis of the foci-forming ability of the various Ets constructs, Fig. 8B shows the results of a similar study performed in triplicate using pCGN2, pCGN2-HA-ESX, pCGN2-HA-VP16-ESX, pCGN2-HA-Ets-2 and pSV Ras. Again, the negative pCGN2 control formed a minimal number of small foci, averaging 3 foci/plate, and the positive oncogenic V12 Ras control resulted in a higher number (~18 foci/plate) of moderately-sized foci (Fig. 8B). The HA-ESX and HA-VP16-ESX resulted in ~9 and ~15 foci/plate, respectively, which is about 3- and 5-fold greater than the vector clone results (Fig. 8B). As with the previous study shown in Fig. 8A, the ability of HA-VP16-ESX and V12 Ras to form foci were quite similar (~15 vs ~18 foci/plate). Finally, HA-Ets-2 revealed a robust ability to form foci in MCF-12A cells, resulting in ~50 foci/plate, which is about 2.5-fold greater than oncogenic V12 Ras (fig. 8B and Table 1). Indeed, HA-Ets-2 appeared very competent to efficiently induce foci formation that although large in number, were also generally smaller.

Because of the ability of HA-ESX and HA-VP16-ESX to induce foci formation in MCF-12A cells, we chose to test the role of endogenous ESX in foci formation in transformed T47D human breast cancer cells, since this cell line expresses easily detectable levels of ESX protein (data not shown). To this end, we transfected T47D cells

with pGFP and either anti-sense-ESX or a dominant-negative HA-ESX DBD (containing only the ESX DNA-binding domain) and selecting for the transfected population by FACS for GFP fluorescence. This enriched population of transfected cells was then plated in various concentrations of serum (0.15, 1% and 5%), and foci formation was quantitated 13 days later. As shown in Fig. 9, 20 μg of empty vector, pCGN2-HA-anti-ESX, or pCGN2-HA-ESX DBD was transfected into T47D cells along with 2 μg of a vector containing green fluorescent protein. Cells were sorted by FACS analysis and 2,000 sorted, glowing cells were plated in complete media (12.5% horse serum, 2.5% fetal calf serum). After 24 hours media was replaced with fresh media containing 0.1%, 1%, or 5% fetal calf serum. Cells were fixed, stained, photographed, and manually scored after a 13 day incubation during which they were fed regularly with media containing one of the three concentrations of serum (Fig. 8A,B). When cells were fed with media supplemented 0.1% serum, foci formation was reduced by 78% in the cells expressing HA-antisense-ESX and 83% in those expressing the HA-ESX DBD construct when compared to the empty vector controls. Foci formation was also greatly reduced from that seen in the empty vector controls when HA-antisense-ESX and HA-ESX DBD cells were fed with media supplemented with only 1% serum, 73% and 78% respectively. Finally, when transfected cells were treated with 5% serum supplemented media, a reduced but still significant effect was seen in the cells transfected with HA-antisense-ESX and HA-ESX DBD (61% and 55% reductions respectively) when compared to the cells transfected with vector controls. Taken together these data indicate that ESX may play a critical role in breast cell oncogenic transformation. A better understanding of the molecular basis of its action will be the focus of future studies.

Key Research Accomplishments

* Performed comprehensive assessment of the expression levels of all five somatostatin receptor subtypes in several breast cancer cell lines.

*Determined that the antiproliferative effects of somatostatin on breast cancer cells are due, at least in part, to the induction of programmed cell death, or apoptosis.

*Demonstrated that the MAPK signaling pathway, which is overexpressed in certain aggressive breast cancers, is strongly inhibited by the somatostatin analog BIM23014, most likely via the type 2 somatostatin receptor (sst2).

* Identified N-terminal Jun kinase (JNK) pathway as a potential mechanism by which somatostatin exerts its pro-apoptotic effects in cancer cells.

*Demonstrated that transcription potency of factors downstream of the Ras/Raf/MAPK mitogenic pathway are inhibited by BIM23014. Specifically, EGF and FGF-stimulated Ets-1 and Elk-1 activity.

*Extended current studies to include an examination of the role of the Ets factor, ESX in breast cancer cell transformation.

Reportable Outcomes

Abstracts and Manuscripts

*Abstract: **Tentler, J.J.,** Eckel, K.E., Gutierrez-Hartmann, A. "The somatostatin analog, BIM23014 inhibits the growth factor/MAPK/Elk-1 Pathway in MDA231 breast cancer cells". University of Colorado Annual Research Forum, Department of Medicine. May 10, 2000

*Abstract: **Tentler, J.J.,** Eckel, K.E., Gutierrez-Hartmann, A. "Somatostatin Inhibits Breast Cancer Cell Growth via Multiple Signaling Pathways". DoD Era of Hope Annual Meeting, Atlanta, Ga. June 8-12, 2000.

*Oral Presentation: **Tentler, J.J.,** Bradford, A.P., Gutierrez-Hartmann, A. "Stable expression of dominant-negative Ets inhibits endogenous prolactin gene expression". Presented at the 2000 Meeting of the Endocrine Society, June 21-24, 2000 Toronto, Ontario Canada.

*Abstract: Gutierrez-Hartmann, A., **Tentler, J.J.,** Diamond, S.E., Duval, D., Schweppe, R. A precise molecular code of Pit-1 and Ets factor isoforms regulates prolactin promoter activity. International Congress of Endocrinology, Sydney, Australia. 11/2000

*Manuscript: **Tentler, JJ**, Diamond, SE, Gutierrez-Hartmann, A. Control of Pituitary Gene Expression. In: *Pituitary Diseases:Diagnosis and Treatment.* JD Baxter, S Melmed and N New, Eds. Humana Press, Totowa NY In press, 2000.

* Manuscript submitted: **Tentler, J.J**., Eckel, K., and Gutierrez-Hartmann, A. "Somatostatin Inhibits Breast Cancer Cell Growth via Multiple Signaling Pathways". Submitted to the journal, Breast.

*Manuscript submitted: **Tentler, J.J.,** Bradford, A.P., Schweppe, R.E and Gutierrez-Hartmann, A. "Selective repression of the prolactin gene by stable expression of dominant-negative Ets". Submitted to Journal of Biological Chemistry.

*Manuscript submitted: **Tentler, J.J**., Hadcock, J.R., Eckel, K., and Gutierrez-Hartmann, A "Signaling via the type 2 somatostatin receptor inhibits the growth factor-stimulated MAP kinase pathway". Submitted to the journal, Neuroendocrinology

Awards, Funding, and Employment Opportunities

*Selected to receive a 2000 Endocrine Society Travel Grant Award , for oral presentation of research findings at the 2000 Meeting of the Endocrine Society June 21-24, 2000, Toronto, Ontario, Canada

* Funding support for 1 year on UCHSC Endocrine Division NIH Training Grant

*Promotion in UCHSC Division of Endocrinology from Postdoctoral fellow to Instructor

*Currently applying for faculty research positions at several institutions and scientist positions at several biotech and pharmaceutical companies.

Conclusions

In this final report, I describe the progress made on the elucidation of the signaling components mediating the anti-proliferative effects of somatostatin and its analogs in breast cancer cells. After testing several breast cancer cell lines for somatostatin receptor expression and response to somatostatin analogs, we have chosen the MDA 231 cell line for our studies. This cell line expresses relatively high levels of the sst2 and sst5 receptor subtypes, which we have empirically determined to be the primary targets for the long-acting somatostatin analog, BIM23014 used in our experiments. Using this system, we have shown that the growth factor-stimulated MAPK pathway is strongly inhibited by the somatostatin analog, BIM23014. We have also shown that in these cells, activation of sst2, but not sst5, leads to inhibitory signals that block the EGF stimulation of the MAPK pathway. Furthermore, we have shown that the transactivation potential of two members of the Ets family of transcription factors, Elk-1 and Ets-1 are inhibited by BIM23014, The effects on Elk-1 in response to EGF are the most dramatic. Since EGF and the MAPK pathway have been shown to be critical in breast cancer tumorigenesis, these findings offer significant insights into the antiproliferative actions of somatostatin. Additionally, some preliminary data in T47D and MDA231cells indicates a possible role of the JNK pathway in the control of cell proliferation as well. Future studies include mapping the precise point(s) in the MAPK pathway where sst2 and sst5 signals act, and investigating the potential role of phosphatases, including MAPK phosphatase and PTP1B in somatostatin's inhibition of MAPK. I will be completing studies on raf kinase activity (Aim 2) and Jun transactivation activity (Aim 3) when technical details of these experiments and problem areas are worked out. Also, some of the experiments presented in this report will need to be repeated before publishing the results. Finally, I also plan to assess the effects of somatostatin on various cell cycle regulating factors, which may be critical effectors of the somatostatin inhibitory signal. In related studies I will also be exploring the possible effects of somatostatin on the expression of a novel Ets factor implicated in breast cancer progression, ESX. Furthermore, I would like to determine the protein partners of ESX that may be responsible for mediating the oncogenic transformation of this factor. To accomplish this, I have begun studies that will utilize an ESX antibody column as an affinity purification strategy that will hopefully isolate an intact ESX transcriptional complex. The proteins in this complex will then be identified using state of the art proteomics approaches, including MALDI-TOF and MS/LCQ sequencing.

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Somatostatin Receptor Subtype Expression in Human Breast Cancer Cell Lines: Results of an RT-PCR Study

		Somatostatin Receptors				
Cell Line	**Background**	**sst1**	**sst2**	**sst3**	**sst4**	**sst5**
MCF-7	Human tumorigenic adenocarcinoma ER+, EGFR-	-	+ +	-	+ +	-
MDA 231	Human tumorigenic carcinoma ER-, EGFR+	-	+ +	-	-	+++
ZR 75.1	Human tumorigenic carcinoma ER+, EGFR-	-	+	-	-	-
T47D	Human tumorigenic carcinoma ER+, EGFR+	-	+	-	+ +	-

Table 1: Results of RT-PCR study of several breast cancer cell lines. ER=estrogen receptor, EGFR=epidermal growth factor receptor, sst1-5 =somatostatin receptor subtypes 1-5. - = no receptor detected. + = sst receptor positive.

Figure 1

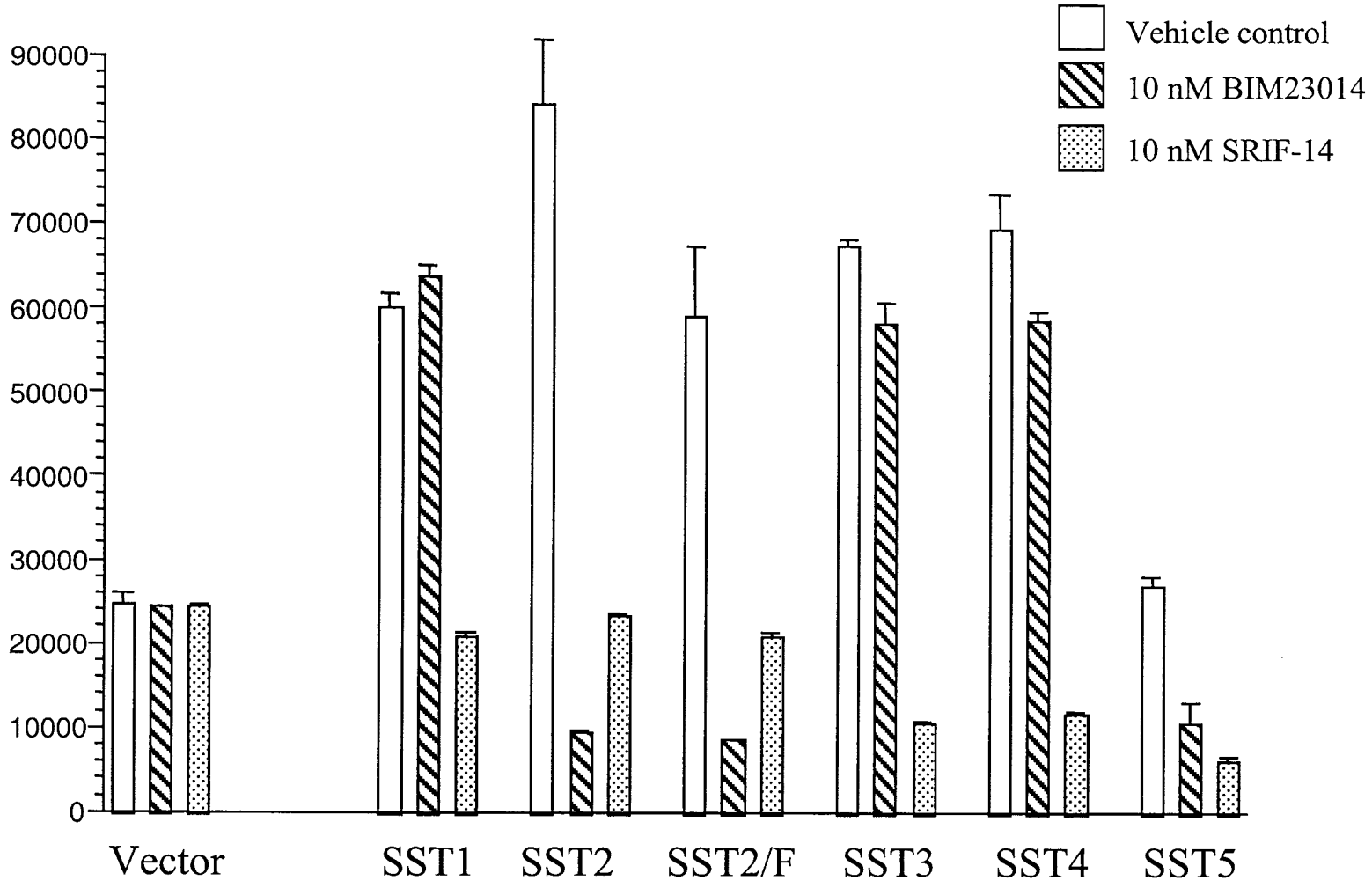


Fig. 1: Somatostatin receptor subtype selectivity for BIM23014 and native somatostatin-14. Somatostatin receptor subtype inhibition of the cyclic AMP/CREB-responsive a-glycoprotein subunit promoter. CHO cells, lacking endogenous sst's were co-transfected with each of the sst subtypes (sst 1-5), and the α-glycoprotein subunit promoter fused to the luciferase reporter gene. Cells were pretreated with either vehicle (DMEM) or BIM23014 or the native ligand, somatostatin 14 (SRIF-14). Light units corrected for total protein are shown. sst2/F is a FLAG epitope-tagged version of sst2

Figure 2

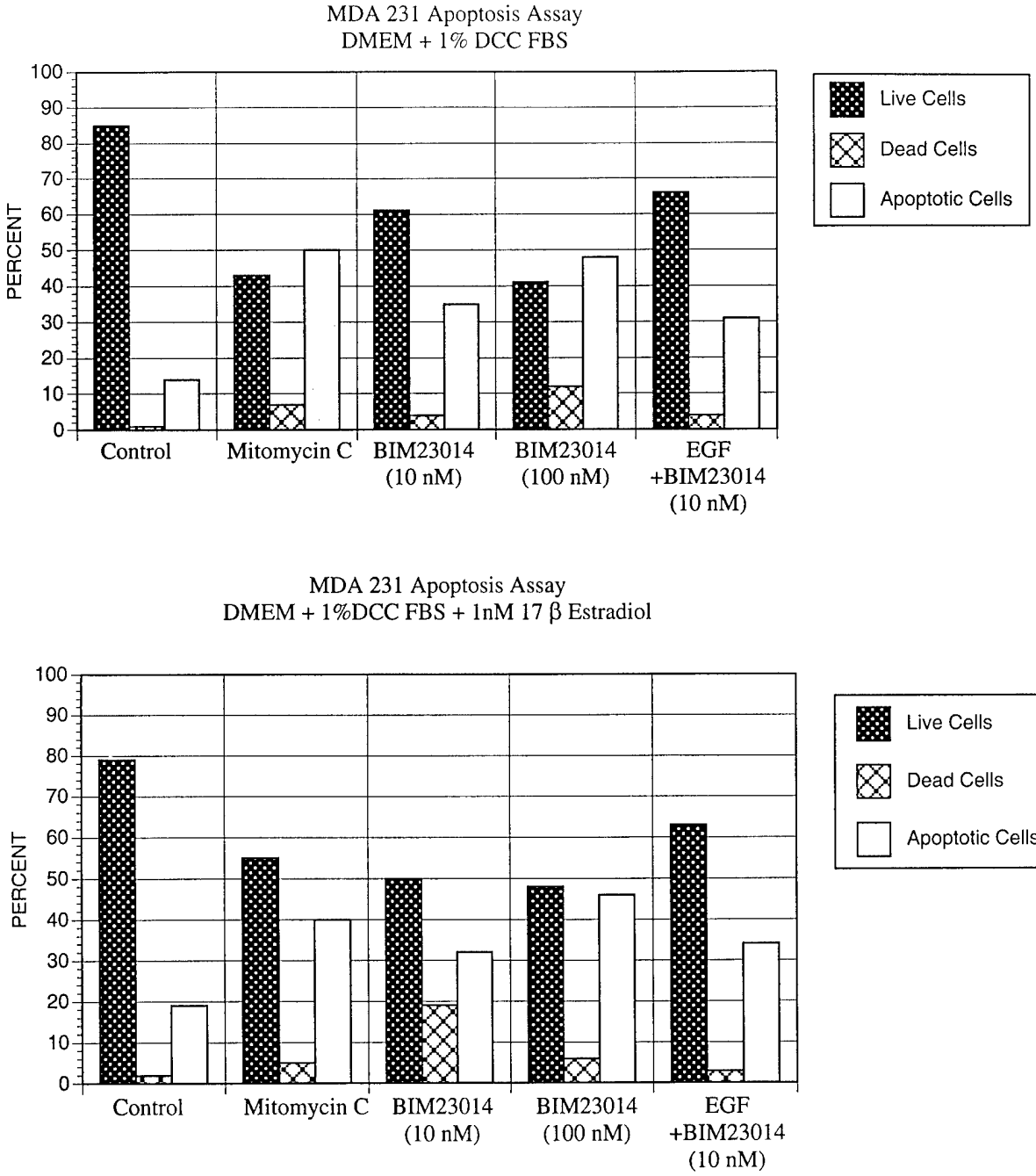


Fig. 2 BIM23014-induced apoptosis in the absence and presence of estradiol. Approximately 1x10^6 MDA231 cells were plated in triplicate in DMEM plus 10% charcoal stripped FCS and treated with 10 nM BIM 23014 or BIM 23014 plus estradiol vehicle for 24 hours. Cells were washed, harvested, and pelleted by low speed centrifugation. Five (5) µl of propidium iodide/Hoechst staining mixture was added to each pellet. Ten µl of the cell/Hoescht stain mixture were spotted onto a microscope slide and analyzed using a Leitz fluorescent microscope. Several fields were analyzed and 100 cells/slide were counted. Data were converted to percent of total cells.

Figure 3

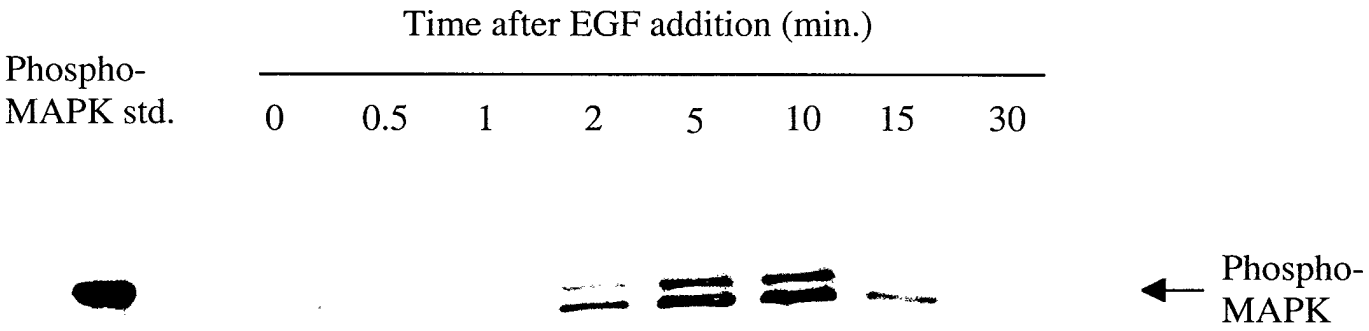


Fig. 3: Time course of EGF-induced MAPK phosphorylation in MDA 231 breast cancer cells. MDA 231 cells were cultured in DMEM with 1% fetal calf serum for 16 hours to lower basal MAPK activity. Cells were then treated with EGF (25nM) for the indicated times. Western blot analysis was performed using a phospho-specific p44/42 MAPK antibody. Peak phosphorylation is observed at 5 and 10 minutes after EGF treatment. Subsequent experiments utilizing the BIM analog were performed at the 10 minute time point.

Figure 4

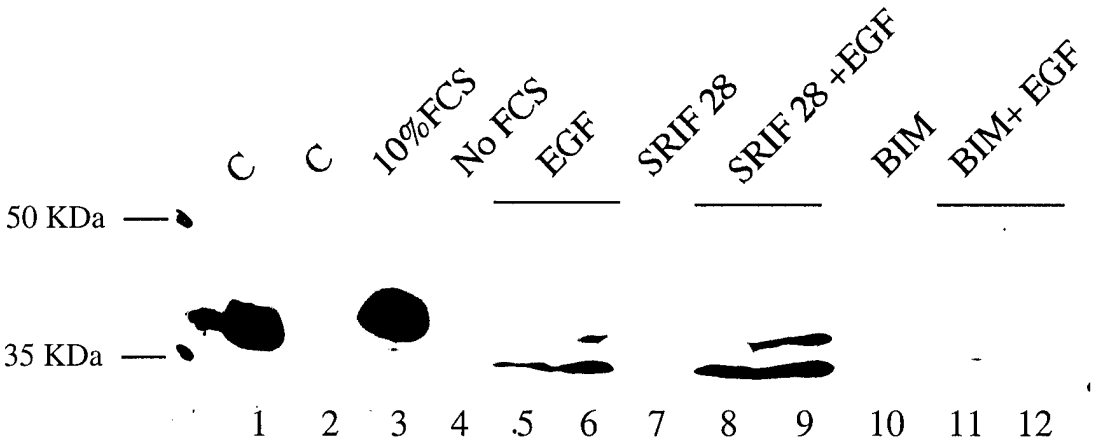


Figure 4: Western blot analysis of MDA231 breast cancer cell extracts treated as shown above and as described in Body text. Equal protein amounts (50 μg) were separated on 12% SDS-PAGE, blotted onto Immobilon and probed with an antibody specific for the phosphorylated form of MAPK.

Figure 5.

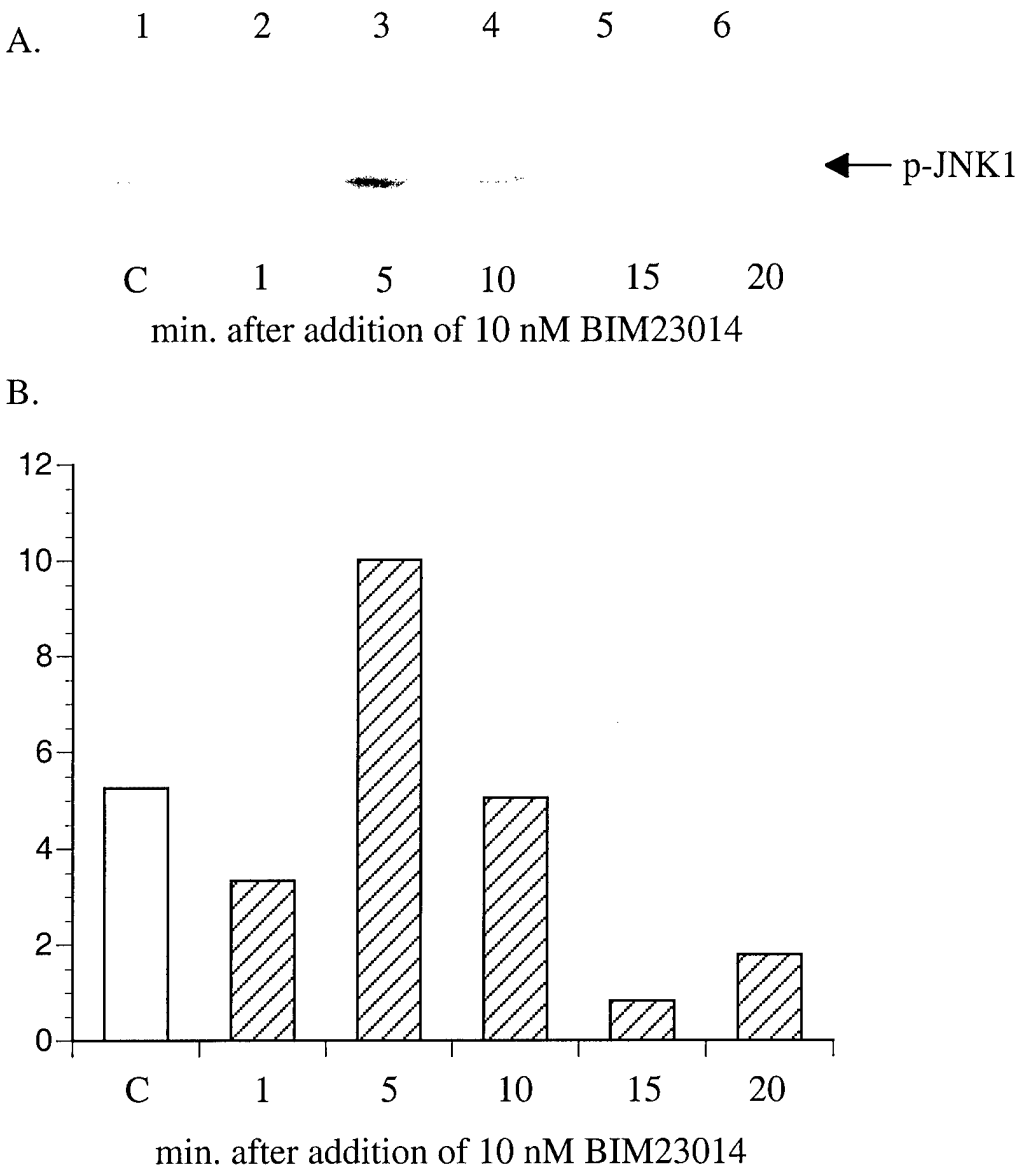


Figure 5. The somatostatin analog BIM23014 induces a transient increase in JNK phosphorylation. A. Western blot analysis of T47D breast cancer cells treated with vehicle (C) or 10nM BIM23014 for the indicated time points, and as described in the Body text. Extracts were prepared, and equal protein amounts (50 μg) were separated on a 12%SDS-PAGE, transferred to Immobilon and probed with anatibody specific for phosphorylated JNK. **B**. Densitometric analysis of the blot shown in **A**. Values are corrected for loading differences.

Figure 6

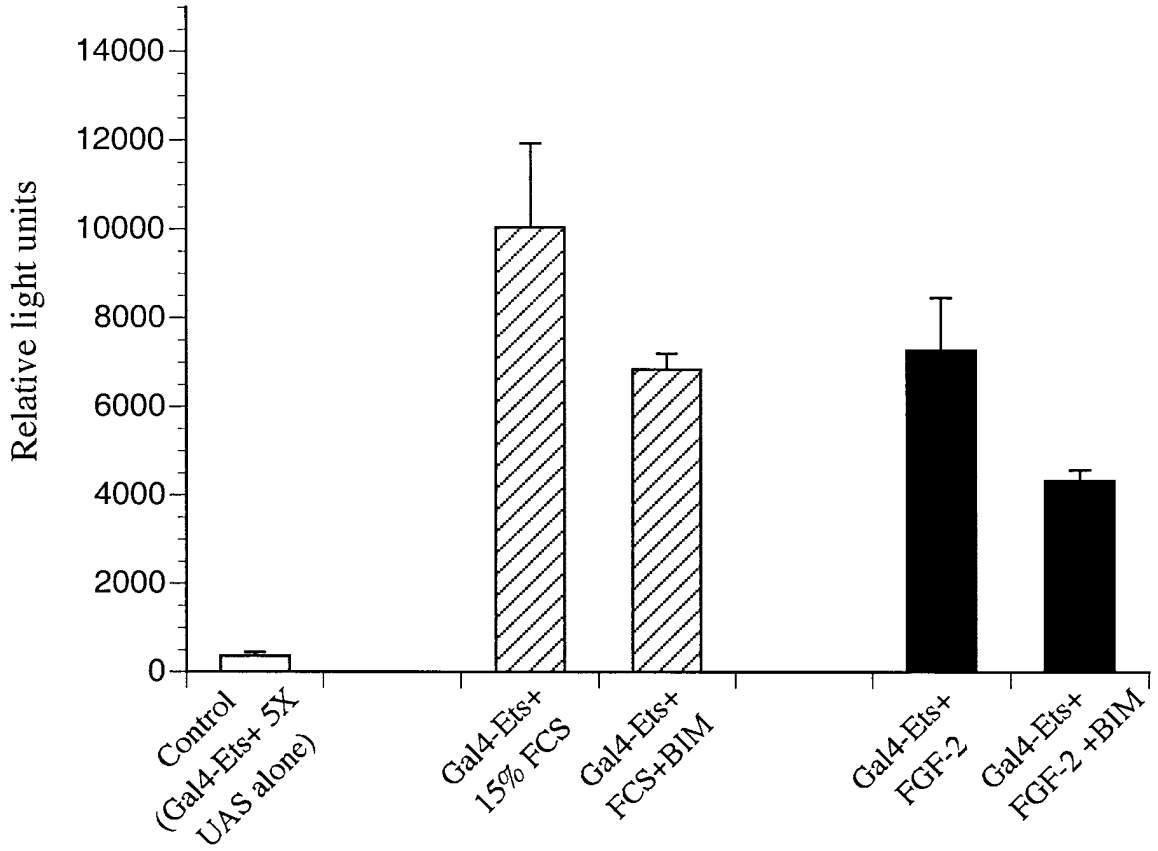


Figure 6: Effects of BIM 23014 on serum and FGF-2-stimulated Ets-1 transcriptional activity. MDA 231 cells were transiently transfected by electroporation with a Gal4-Ets-1 fusion and a 5X UAS construct, and then incubated for 18 hrs. in DMEM with 1% FCS. Some cells were pretreated with 10nM BIM23014 for 30 mins., then stimulated with FCS (15% v/v final concentration) or FGF-2 (2 ng/ml) and incubated for 6 hrs. Values represent mean relative light units +/- SD. n=3 per experimental group.

Figure 7

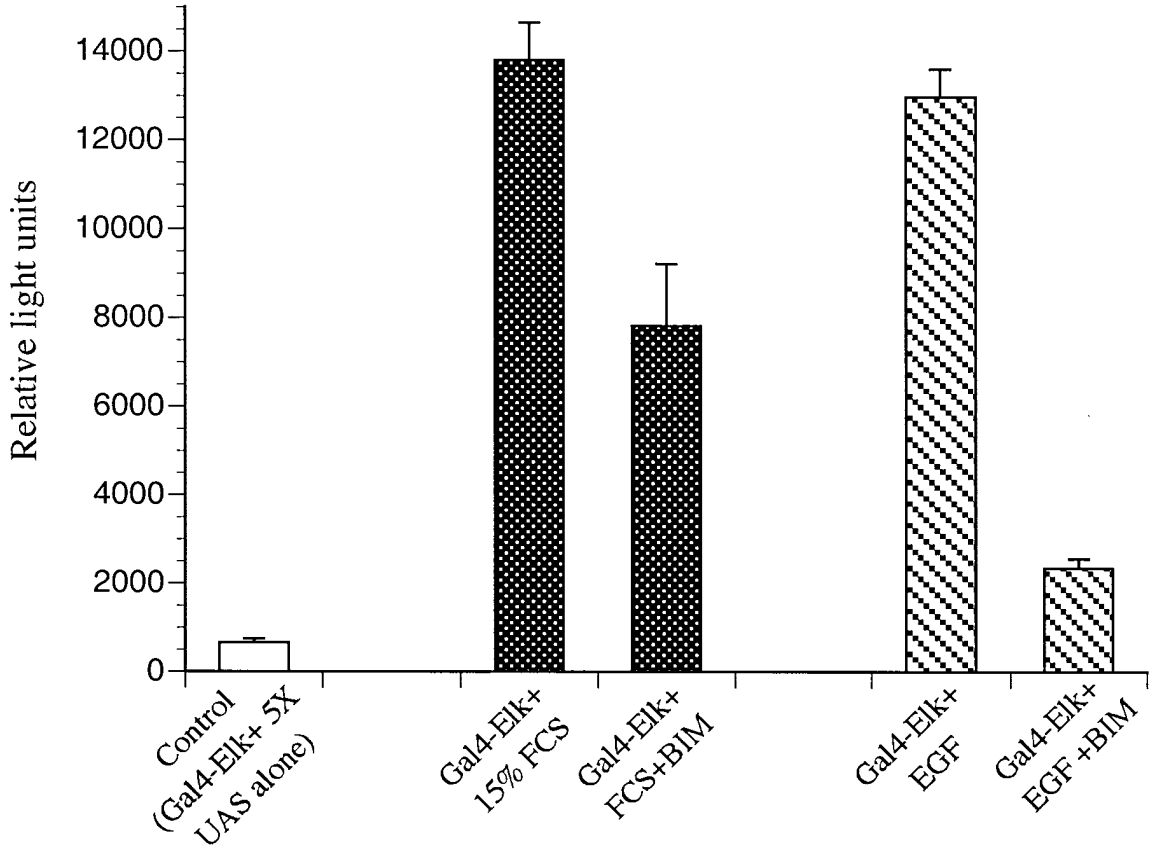


Figure 7: Effects of BIM 23014 on serum and EGF-stimulated Elk-1 transcriptional activity. MDA 231 cells were transiently transfected by electroporation with a Gal4-Elk-1 fusion and a 5X UAS construct, and then incubated for 18 hrs. in DMEM with 1% FCS. Some cells were pretreated with 10nM BIM23014 for 30 mins., then stimulated with FCS (15% v/v final concentration) or EGF(25nM) and incubated for 6 hrs. Values represent mean relative light units +/- SD. n=6 per experimental group.

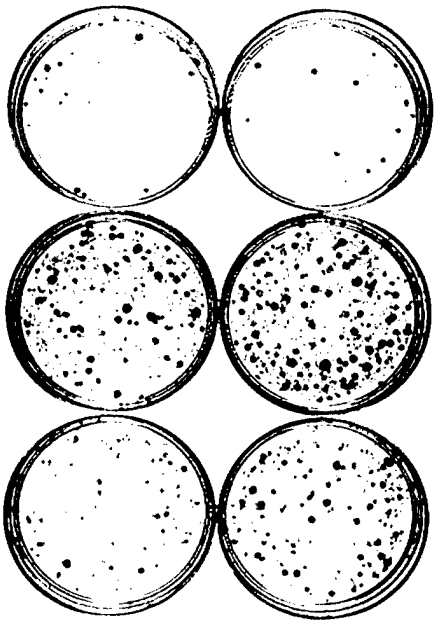
Figure 8

A

Empty Vector

V12-Ras

HA-VP16-ESX



B

Empty Vector

HA-ESX

HA-VP16-ESX

HA-Ets-2

V12-Ras

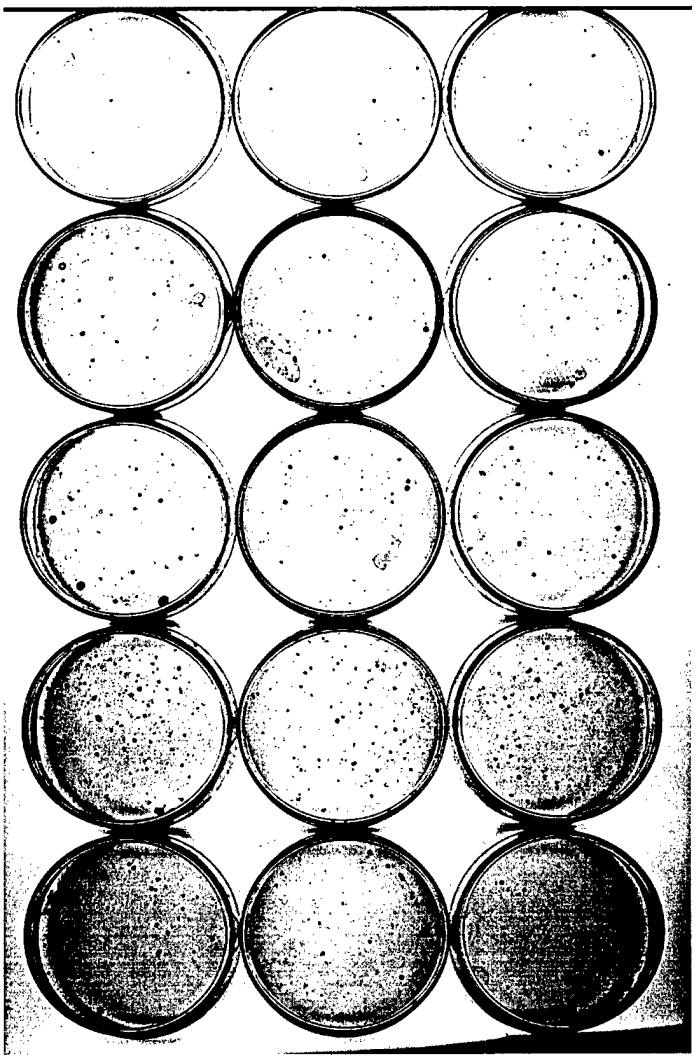
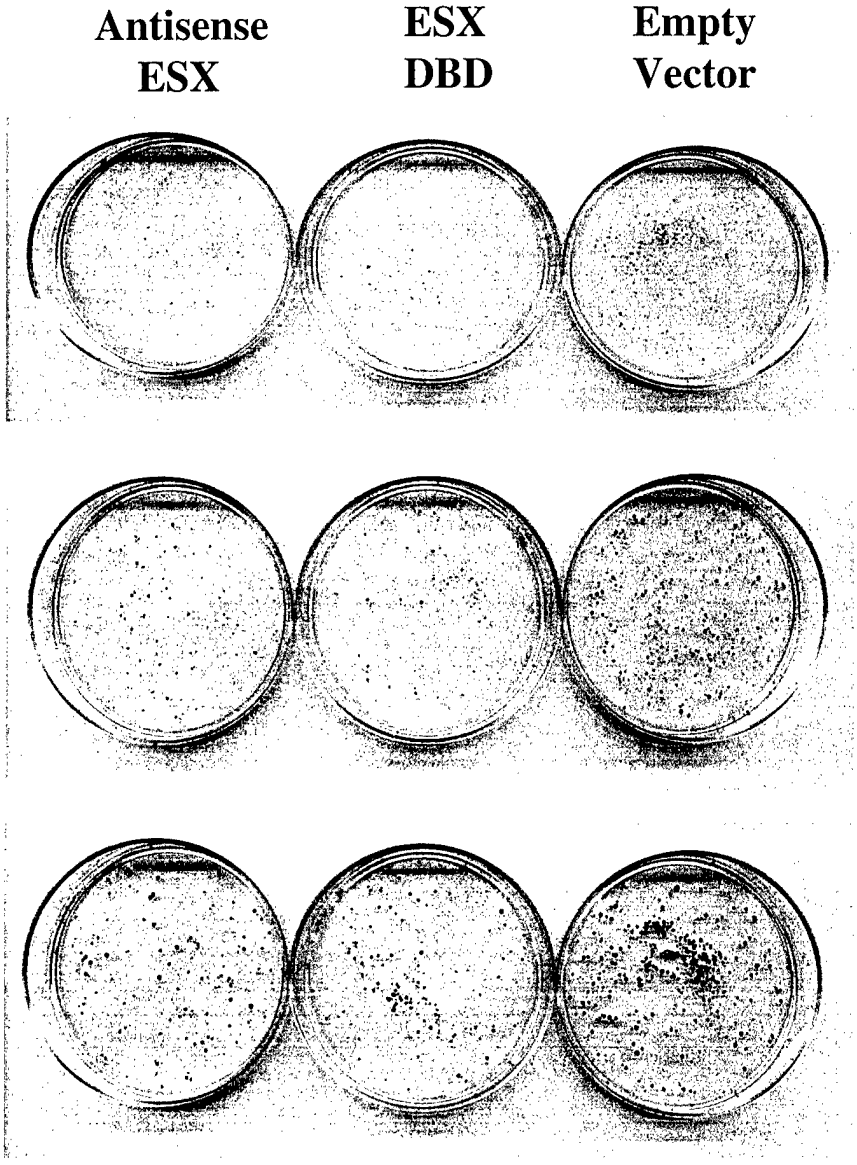


Fig. 8. Foci formation in MCF-12A cell line. MCF 12A epthelial breast cells were transfected with either emptyvector, HA-ESX, HA-VP16-ESX, HA-Ets-2 or oncogenic V12-Ras. Human MCF-12A breast cells were transfected by electroporation with 10 μg of DNA and 1000 cells were plated per plate. Plates were incubated for 13 days. Media was changed every 3 days, and following incubation, cells were fixed, stained, and photographed. **A**. Pilot experiment performed in duplicate. **B**. Experiment similar to that shown in A., performed in triplicate with additional vectors, as indicated.

Figure 9



Foci number

%Fetal Calf Serum	Antisense ESX	ESX DBD	Empty Vector
0.1%	67	52	303
1%	82	67	301
5%	126	146	326

Fig. 9. Antisense-ESX and HA-ESX DBD inhibit foci formation of T47D cells. T47D cells were co-transfected with a plasmid encoding green fluorescent protein (GFP) and plasmids encoding inhibitory ESX effectors (antisense-ESX or HA-ESX DBD) or with empty vector. Cells were sorted by FACS based on GFP fluorescence and 2,000 sorted cells were plated in 60 mm dishes in DMEM plus the indicated concentration of fetal calf serum. Cells were allowed to incubate and form foci for 13 days. Colonies were fixed and stained (A) and then manually counted (B).