Activin and estrogen crosstalk regulates transcription in human breast cancer cells

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Abstract

Activin is a member of the transforming growth factor β superfamily that regulates mammary cell function during development, lactation, and in cancer. Activin slows the growth of breast cancer cells by inducing G0/G1 cell cycle arrest. Estrogen is a steroid hormone that stimulates the proliferation of mammary epithelial cells in development and oncogenesis. The crosstalk between estrogen and activin that regulates activin ligand expression, activin and estrogen signal transduction, and cell cycle arrest was investigated in this study. Estrogen antagonized activin-dependent production of plasminogen activator inhibitor 1 (PAI-1) mRNA, while activin repressed estrogen-dependent transcription of trefoil factor 1. The repression of estrogen signaling by activin was recapitulated using a simple estrogen response element-luciferase construct and was enhanced in the presence of overexpressed estrogen receptor α (ERα). In contrast, estrogen-mediated repression of activin signaling could not be recapitulated on a simple CAGA Smad-binding element but did inhibit the short PAI-1 promoter, p3TP-luciferase, especially when ERα was overexpressed. Repression of both estrogen- and activin-regulated transcription was found to be ligand induced and Smad3 dependent. In addition to transcriptional repression, estrogen also reduced the amount of activin B mRNA and protein produced by MCF7 breast cancer cells. These studies demonstrate the importance of activin and estrogen crosstalk during mammary cell growth and cancer initiation.

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Introduction

Mammary epithelial cell growth and differentiation is influenced by the changing roles of factors such as steroid and peptide hormones, growth factors, and cytokines (Nicholson et al. 1999, 2005, Sabnis et al. 2005). Activin is a member of the transforming growth factor β (TGF-β) superfamily of growth factors and regulates mammary gland development, differentiation during lactation, and becomes deregulated as breast cancer increases in pathological grade (Reis et al. 2004). Estrogen is a steroid hormone that also regulates aspects of mammary gland development, lactogenesis, and mammary cancer (Katzellenbogen & Frasor 2004, Imamov et al. 2005). Breast cancer-derived cells tend to express high levels of estrogen receptor α (ERα) in the early stages of tumor development and lower levels of this receptor as tumor grade increases (Anderson 2002). Because loss of hormone responsiveness is linked to tumor progression, understanding how ERα influences growth factor cell signaling is vitally important to new methods of treatment for hormone-sensitive cancers. In accordance, investigating how estrogen signaling is regulated or altered by cytokine growth factors may provide insight into breast cancer cell proliferation and tumor development.

Estrogen regulates a variety of biological properties in mammary cells including developmental proliferation, differentiation, and regulation of cancer cells. Estrogen signals by binding to its receptor, which comes in two isoforms, ERα and ERβ. Upon binding a ligand, both form homo- or heterodimers that translocate into the nucleus and function as transcription factors by either binding directly to an estrogen response element (ERE) in the DNA or through fos, jun, and stimulating protein 1.
(Sp1) protein interactions to activating protein 1 (AP1) or GC-rich regions within the DNA (Martini & Katzenellenbogen 2003). Coactivators and corepressors bind to ERs in different complexes to alter the responsiveness of specific genes in a tissue-specific manner (Smith & O’Malley 2004). The regulation of signal transduction can therefore be managed by such coregulators to alter estrogen signaling and control a variety of responses in the breast. Breast cancer is diagnosed and treated as either hormone sensitive or insensitive based on the presence of ERs (Clarke et al. 2003). Therefore, investigating growth factors that alter estrogen responsiveness is critical to understanding mechanisms underlying breast cancer progression (Ariazi et al. 2006).

Activin slows cell proliferation of breast epithelial cells in vitro and in vivo and is lost in human cancers corresponding with an increase in tumor size. Activins A, B, and AB all bind to ActRII receptors and stimulate the phosphorylation of the type I receptor, Alk4. Alk4 phosphorylates Smads 2 and 3, which then bind to the coregulatory Smad4, and translocate into the nucleus where they regulate gene transcription. Activin was first identified as a regulator in the mammary gland based on the phenotype of the activin βB knockout mouse, which displayed developmentally deficient ductal glands and lobuloalveolar buds (Robinson & Hennighausen 1997). Unfortunately, activin βA knockout mice do not provide information regarding control of mammary cells because palette malformations result in death before the mammary gland develops (Matzuk et al. 1995). Activin A in vitro inhibits cell growth by stimulating the phosphorylation of Smad3 and this causes a redistribution of the cell cycle into G0/G1 arrest (Liu et al. 1996, Ying & Zhang 1996, Cocolakis et al. 2001, Burdette et al. 2005). Activins A and B reduce proliferation of cultured rat acini isolated during different stages of mammary gland morphogenesis and differentiation (Bussmann et al. 2004). Consistent with a role in tumor suppression, the loss of activin, activin receptors, and nuclear localization of Smad3 correlate strongly with an increase in breast cancer grade (Jeruss et al. 2003). Studies have also found that differential expression of activin and inhibin subunits correlates with human breast cancer and may serve as a marker of cancer (Di Loreto et al. 1999, Reis et al. 2004, Mylonas et al. 2005, Leto et al. 2006). In addition, activin signaling receptors are found on the leading edge of tumors generated in mouse models suggesting a role in invasion (Landis et al. 2005). These data suggest that activins control mammary gland elongation and growth inhibition in normal mouse or rat epithelium and in well-differentiated breast cancer cells.

Estrogen and activin both signal during mammary development and in cancer and therefore may serve as modulators in each pathway, thereby providing a tissue-specific signal. Furthermore, these two pathways may intersect and modulate their individual signaling cascades. Evidence supporting the signal interaction includes repression of activin B mRNA measured in a microarray analysis after treatment of MCF7 cells with estrogen (Frasor et al. 2003). Smad3 and ERs interact intracellularly in response to estrogen and TGF-β to enhance estrogen signaling and repress TGF-β signaling (Matsuda et al. 2001). Smad4 also interacts with ERs and acts as a corepressor of estrogen-regulated signaling in breast cancer cells (Wu et al. 2003). Additionally, the presence of ERs in breast cancer cells positively influences whether these cells are growth inhibited by activin (Kalkhoven et al. 1995). These studies suggest a strong link between the cytokine activin and the hormone-responsive state of breast cancer cells. Therefore, the purpose of this study was to investigate whether activin directly represses estrogen action in breast cancer cells. Also under investigation is the role that estrogen plays in diminishing activin-induced signal transduction leading to cell cycle arrest. The complexity of potential interactions between these two pathways may explain the puzzling disparity in response of cancers exposed to different hormonal milieus.

Materials and methods

Cell culture and materials

T47D and MCF7 breast cancer epithelial cells were acquired from ATCC (American Type Culture Collection, Manassas, VA, USA). All media and supplements were obtained from Life Technologies (Gaithersburg, MD, USA). T47D and MCF7 were cultured in phenol red-free RPMI 1640 supplemented with 10% FBS and 1% antibiotic. All cells were incubated at 37 °C, 5% CO₂. Activin A was prepared at Northwestern University in a buffer of 0.15 M NaCl and 0.05 M Tris (pH 7.5; Pangas & Woodruff 2002). All other reagents were purchased from Sigma unless otherwise noted.

Transient transfections

Cells were plated 1 day before transfection in 24-well plates and transiently transfected in Opti-MEM (Invitrogen) with p3TP-luciferase, CAGA (12)-lux, or ERE (3X)-luciferase (250 ng/well) alone or in
combination with expression plasmids (25 ng/well) for 12 h using LipoFectamine 2000 (Invitrogen). The ERE (3X)-luciferase reporter was a gift from Dr J Larry Jameson (Northwestern University, Evanston, IL, USA). The CAGA-luciferase plasmid was a gift from Dr Aris Moustakas (Ludwig Institute for Cancer Research, Uppsala, Sweden). Empty pcDNA3 vector was used to balance DNA when necessary. Cells were then treated with serum free media, activin (100 ng/ml), estradiol (1 nM), or tamoxifen (100 nM) for 24 h. To measure luciferase production, cells were lysed in GME buffer (25 mM glycylglycine (pH 7.8), 15 mM MgSO₄, 4 mM EGTA, 1 mM dithiothreitol, and 1% Triton X-100), and lysates were added to assay buffer (GME buffer, 16.5 mM KPO₄, 2.2 mM ATP, and 1.1 mM dithiothreitol). Luciferase activity was measured for 30 s using an AutoLumat (Berthold Technologies Co., Oak Ridge, TN, USA). Protein measurements were determined using the BCA method and did not differ significantly between transfected samples after 24 h. At least three independent experiments where performed for each experiment. Data represent the average from quadruplicate replicates ± S.D. from the representative experiment.

**Real-time PCR**

Total RNA was isolated using Qiagen RNeasy columns with on column DNase added according to the manufacture (Qiagen). RNA samples (2 µg) were then primed with random hexamers and reverse transcribed with M-MLV reverse transcriptase (Promega) according to manufacturer’s instructions. From the original RT reaction, 1 µl was subjected to PCR amplification in a 25 µl volume with Taqman Universal PCR Master Mix (Applied Biosystems, Foster City, CA, USA) under the following conditions: 50 °C hold for 2 min, 95 °C hold for 10 min, the 40 cycles of 95 °C for 15 s, and 60 °C for 1 min. Primer and probe sets were FAM (6-carboxy-fluorescein) labeled and acquired from Applied Biosystems Assays on demand and designed to span intron/exon borders for PAI-1 (Hs00167155), trefoil factor 1 (Hs00907239), and activin B (Hs00173582). Data were normalized with VIC-labeled actin as the internal control (ΔCt) and then untreated samples were subtracted from activin (100 ng/ml), estradiol (10 nM), or both (ΔΔCt). Standard error from the mean from replicates are represented as 2^−ΔΔCt.

**Western blotting and ELISA**

The activin B ELISA was a gift from Diagnostic Systems Laboratory, Webster, TX, USA. All assays were performed according to manufacturer’s protocol utilizing standards produced in our laboratory as previously described (Kenny et al. 2002). The data represent the average from three independent replicates.

**Statistical analysis**

Statistical variation was determined by performing one-way ANOVA followed by Tukey’s post hoc test with significance reported for P values < 0.05.

**Results**

**Estrogen and activin antagonize each other in full-length endogenous promoter gene transcription assays**

In order to investigate possible transcriptional alteration of estrogen signaling in the presence of activin, T47D breast cancer cells were treated with vehicle, estrogen, activin, or the combination. Regulation of the estrogen-responsive trefoil factor 1 (TFF1 or pS2) mRNA was studied using real-time PCR 24 and 48 h after treatment (Fig. 1A). The estrogen-responsive TFF1 mRNA was induced by estrogen (10 nM) treatment 1.5-fold after 24 h and 10-fold after 48 h. Activin alone did not significantly alter expression of TFF1. However, activin in combination with estrogen significantly reduced TFF1 transcription to 0.5-fold after 24 h and 4-fold after 48 h. Thus, activin inhibits estrogen-induced gene transcription of TFF1.

Next, the influence of estrogen on activin-regulated gene expression was studied in T47D cells. The plasminogen activator inhibitor protein 1 (PAI-1) gene promoter has both Smad and estrogen-responsive elements (EREs) and therefore may be differentially regulated in the presence of activin or estrogen (Levenson et al. 1998b). mRNA was collected from treated T47D cells at both 24 and 48 h (Fig. 1B). Estrogen had only a modest effect on gene transcription after 24 h. As expected, activin increased PAI-1 mRNA production 10-fold over untreated cells after 24 h and 18-fold after 48 h. When estrogen and activin were combined, PAI-1 mRNA accumulation was reduced to 5.3- and 11.9-fold at 24 and 48 h respectively compared with activin alone. Thus, estrogen inhibited activin-induced gene transcription in vitro.

**Activin antagonizes estrogen transcription of the simple ERE promoter**

To assess whether activin had any direct regulatory properties on a simple ERE when compared with the
TFF1 complex promoter, signaling experiments were conducted by transiently transfecting a simple 3X ERE-luciferase plasmid into T47D cells (Fig. 2A). The cells were then untreated or stimulated with estrogen, activin, or the combination. As predicted, estrogen induced an 18-fold increase in luciferase accumulation when compared with untreated cells. *Indicates significant difference between activin and estrogen compared with only estrogen (P < 0.05). T47D cells were stimulated with ligands for 24 or 48 h and total RNA was measured using real-time PCR to determine the fold change in transcription when compared with untreated cells. *Indicates activin and estrogen treatment compared with activin treatment (P < 0.05). Data are plotted as the mean ± s.d. of triplicate experiments and normalized to actin.

**Figure 1** Activin blocks estrogen-mediated TFF1 (pS2) expression and estrogen blocks activin-mediated PAI-1 mRNA expression. (A) T47D cells were stimulated with ligands for 24 or 48 h and total RNA was measured using real-time PCR to determine the fold change in transcription when compared with untreated cells. *Indicates significant difference between activin and estrogen compared with only estrogen (P < 0.05). (B) T47D cells were stimulated with ligands for 24 or 48 h and total RNA was measured using real-time PCR to determine the fold change in PAI-1 transcription as compared with untreated cells. *Indicates activin and estrogen treatment compared with activin treatment (P < 0.05). Data are plotted as the mean ± s.d. of triplicate experiments and normalized to actin.

Activin was overexpressed in T47D cells to investigate whether activin could antagonize estrogen signaling in the presence of excess receptor (Fig. 2A). Overexpression of the receptor caused a ligand-independent induction of the ERE promoter to 8.5-fold when compared with untreated cells. This induction was significantly enhanced in the presence of estrogen by 34-fold. In cells overexpressing ERα, activin repressed the promoter 63% when compared with untreated cells.

**Figure 2** Activin blocks estrogen regulation of an estrogen-responsive element, and estrogen blocks regulation of an activin-responsive promoter. (A) The plasmid encoding the estrogen response element (3X ERE) was transfected or cotransfected with ERα cDNA. Cells were stimulated with solvent control, estrogen (1 nM), activin (100 ng/ml), or the combination for 24 h. *Indicates ERα transfected different than untransfected (P < 0.05). ‘a, b, or c’ indicates ligand treatments differ significantly (P < 0.05). (B) A luciferase reporter plasmid encoding for the short form of PAI-1 (p3TP) was cotransfected into T47D cells with ERα and treated with ligands for 24 h. *Indicates ERα transfected different than untransfected (P < 0.05). ‘a’ indicates ligand stimulation significantly different than all other ligand treatment (P < 0.05). (C) A luciferase reporter encoding for 12 copies of the CAGA canonical Smad DNA-binding sequence was transfected compared with cotransfected cells with ERα. *Indicates ligand stimulation significantly different (P < 0.05). The data represent the mean relative light units ± s.d.
When activin was combined with estrogen in the ERα-transfected cells, it significantly repressed transcription below estrogen-stimulated levels to 42%. Thus, activin signaling represses estrogen-induced expression of the ERE promoter in the presence of endogenous and overexpressed ERα protein levels.

**Estrogen antagonism of a simple PAI-1 promoter is ERα dependent**

In order to further investigate possible transcriptional regulation of activin signaling by estrogen on a simple artificial promoter, transient transfection of the Smad-responsive partial PAI-1 promoter, known as p3TP-luciferase, was performed in T47D breast cancer cells (Fig. 2B). Estrogen (1 nM) alone did not alter p3TP-driven luciferase production. Activin significantly stimulated the p3TP promoter 1.7-fold over untreated cells. Estrogen did antagonize activin by 17%, but the ligand-induced effect did not reach significance.

Estrogen-induced repression of activin on the p3TP promoter was then further investigated by transiently overexpressing ERα (Fig. 2B). Overexpression of ERα in the absence of exogenous estrogen significantly reduced p3TP induction to 50% when compared with cells not overexpressing the receptor. The reduction in baseline expression was predicted based on the high levels of activin A secreted by T47D cells that could be repressed by overexpression of ERα (Burdette et al. 2005). The addition of estradiol in combination with overexpressed ERα also significantly inhibited p3TP-induced activation when compared with estrogen treatment alone. When exogenous activin was used to further drive promoter expression, the increase in luciferase production was 64% lower than cells not transfected with ERα. This suggests that overexpression of the ER has a significant inhibitory effect on activin signaling. Therefore, the presence of ERα significantly repressed the p3TP promoter.

To test whether estrogen-induced repression of activin signaling was through direct interaction of the receptor on Smads bound to the specific binding element of DNA, a CAGA (12)-luciferase construct was transiently transfected into T47D cells that were untreated or stimulated with activin, estrogen, or the combination (Fig. 2C). Under these conditions, the addition of estrogen was unable to repress activin-induced signaling indicating that the ER does not directly repress activin stimulation of a simple canonical Smad-binding element. When ERα was overexpressed, background expression was not diminished as in the case of p3TP. CAGA-luciferase activation was slightly inhibited when ERα was overexpressed, but this was not significantly different. These data indicate that estrogen repression of activin signaling is promoter specific and not solely dependent on the ERα interfering with Smads binding to their canonical DNA-binding sequence. Negative control vectors that did not contain either CAGA or ERE elements did not demonstrate significant changes in gene transcription from activin, estrogen, or the combination demonstrating specificity of interactions (data not shown).

**Activin and estrogen-regulated transcriptional crosstalk is mediated through Alk4 receptor activation**

Experiments were next designed to understand whether the ligand-induced repression of estrogen signaling by activin was mediated directly through the activin type I receptor (Fig. 3A). In order to test this hypothesis,
constitutively activated activin type I receptor (Alk4) was overexpressed in cells transfected with the ERE-luciferase construct. Overexpression of Alk4 alone did not alter transcription of the ERE. When Alk4 was overexpressed in the presence of estrogen, it significantly blocked estrogen-induced transcription. Overexpression of ERα in combination with Alk4 and stimulated with estrogen could not reverse the Alk4 repression suggesting that activin signaling through its type I receptor blocks ERα signaling.

Constitutively activated Alk4 receptors were transiently transfected into T47D cells to investigate whether estrogen could inhibit Alk4 receptor-mediated activation of the p3TP promoter (Fig. 3B). The addition of estrogen did not significantly alter constitutively activated Alk4. However, overexpression of ERα significantly repressed p3TP activation by Alk4 in both the presence and absence of estrogen, indicating that ERs inhibit activation from activin type I receptors.

**ERα and Smad3 regulate transcriptional repression of p3TP and ERE expression**

In order to further characterize activin-induced repression of estrogen signaling, Smad2 and Smad3 proteins were overexpressed (Fig. 4A). Smad2 protein overexpression did not reduce estrogen-induced expression of the ERE promoter. However, activin in combination with estrogen did repress estrogen signaling to levels comparable with cells not transfected with Smad2 protein. When ERα was overexpressed in combination with Smad2 and stimulated with both estrogen and activin, ERα partially reversed the activin-induced repression of the ERE promoter. These data help to substantiate that activin inhibition of the ERE promoter is not enhanced by additional Smad2 protein.

To examine the role of Smad3 in activin repression of estrogen signaling, transient transfection experiments were performed in T47D cells (Fig. 4A). Smad3 significantly inhibited ERE-luciferase production to 42% compared when estrogen treatment alone. Activin alone had no effect; but in combination with estrogen and overexpressed Smad3, it significantly repressed ERE transcription 53% as compared to Smad3 estrogen-treated cells. Overexpression of ERα in combination with Smad3 partially reversed the activin-induced repression of the promoter, but it remained significantly lower than cells treated with the same ligands and overexpressing ERα but not Smad3. These data indicate that ERα-mediated transcription of...
the ERE promoter is repressed by activin signaling through Smad3.

To verify the importance and specificity of activin and estrogen crosstalk through ERα, the ER negative MDA-MB-231 cell line was used to recapitulate transcriptional repression demonstrated in T47D cells (Fig. 4B). When the MDA-MB-231 cells were transfected with ERE-luciferase and treated with the ligands, no transcription was detected due to a lack of ERα. When ERα was overexpressed, estrogen activity resumed albeit lower levels than in T47D cells. Activin in combination with estrogen did not inhibit signaling; however, when Smad3 was also overexpressed, activin-stimulated Smad3 and again antagonized estrogen signaling as it had in T47D cells. These data suggest that the activin signal is not transduced efficiently in the ERα-negative MDA-MB-231 cell line, but when components are reintroduced, the same transcriptional repression of ERα can be achieved on EREs. These data also suggest that activin antagonism of estrogen signaling is possible in cell lines that are not dependent on estrogen for cell growth.

Next, Smad proteins were overexpressed to investigate whether ERα repression of activin p3TP signaling was due to the inactivation of a signal at the level of the Smads (Fig. 4C). Only Smad3 was transiently transfected into T47D cells to study its role in estrogen-induced repression of the p3TP promoter due to the fact that p3TP is more responsive to Smad3 expression when compared with Smad2 (Dennler et al. 1998). Smad3 transfection results in a ligand-independent activation of the promoter resulting in a 30% increase when compared with control (Dennler et al. 1998, Burdette et al. 2005). Estrogen alone had no alteration on this activation, while activin further stimulated luciferase production. Combining activin and estrogen ligands in the presence of overexpressed Smad3 resulted in repression of the activin stimulation of p3TP promoter that mimicked the situation when Smad3 was not overexpressed. These results suggest that Smad3 and activin-dependent activation of the p3TP promoter are inhibited by estrogen and ERs. Furthermore, overexpression of ERα and Smad3 together in the presence of all ligand treatments significantly inhibited p3TP promoter activation when compared with overexpression of only Smad3. Estrogen in combination with activin further repressed activin signaling, indicating that ligand occupied ERs can inhibit activin and Smad3-induced expression of the p3TP promoter.

**Estrogen blocks activin B synthesis**

Another possible mechanism for estrogen antagonism of activin signaling may be through modulating signaling pathway components. Previously, microarray results have reported that estrogen reduced activin B subunit expression in MCF7 cells (Frasor et al. 2003). To confirm and extend these studies, MCF7 cells were treated with estrogen and total RNA was extracted. Real-time PCR results confirmed that estrogen represses activin B mRNA expression after 24 and 48 h with no effects after 96 h (Fig. 5A). The anti-estrogen tamoxifen did not alter the expression of activin B subunit. Because activin is secreted as a dimer, conditioned media from estrogen and tamoxifen-treated cells were used to quantify activin protein from MCF7 cells (Fig. 5B). Estrogen treatment resulted in virtually no production of activin B after 24 h and this repression continued for 96 h. Tamoxifen again had no effect on activin B production.

**Figure 5** Estrogen blocks activin B synthesis. (A) MCF7 cells were stimulated with estrogen or tamoxifen for 24, 48, and 96 h. Total RNA was reverse transcribed and real-time PCR was performed to quantify the amount of activin B mRNA produced when compared with unstimulated cells (dashed line). Actin was used to normalize between samples. (B) Conditioned media from treated cells were analyzed for total content using an activin B ELISA. Data represent average ± s.o. activin B (ng/ml) secreted into the media at 24, 48, and 96 h. *Indicates P<0.05 for samples when compared with untreated.
Discussion

Breast cancer cells integrate signals from peptide and steroid hormones to instruct specific gene regulation and cellular responses. Activin reduced expression of the estrogen-regulated TFF1 mRNA while estrogen inhibited activin-induced PAI-1 expression. Furthermore, breast cancer cells produced less ERE-dependent luciferase when a functional activin signal was transduced. Estrogen treatment diminished activin signal transduction by blocking Smad3 signaling from Alk4. In addition, estrogen reduced the amount of activin B mRNA and protein limiting one potential source of cell cycle control in breast cancer cells. The inhibition of activin transcriptional activity by estrogen was found to be promoter specific such that the PAI-1 endogenous gene was specifically activated by activin and antagonized in the presence of estradiol likely due to the presence of other estrogen-regulated elements in the promoter such as AP1 and GC-rich regions. The presence of ERs was not found to be a requirement for activin-mediated cell growth inhibition in breast cancer cells. These experiments suggest that activin and estrogen intracellular signaling molecules act to repress gene expression in a cell-specific manner.

The interaction between hormone receptors and Smads provides tissue-dependent modulation of the Smad signal and may confer molecular specificity. Many studies have demonstrated that the Smad proteins physically interact with steroid receptors and alter transcription through direct complex formation, first reported using the vitamin D receptor (Yanagisawa et al. 1999). In the current study, the interaction of Smads with ERα resulted in the blockade of activin signaling by estrogen. In accordance, previous studies demonstrated that estrogen signaling repressed transcription of TGF-β and bone morphogenetic protein (BMP) signals (Matsuda et al. 2001, Yamamoto et al. 2002, Paez-Pereda et al. 2003, Helms et al. 2005). Smads and ERα formed complexes in previous studies that could be co-immunoprecipitated (Hayes et al. 2001, Matsuda et al. 2001, Yamamoto et al. 2002, Wu et al. 2003). Androgen receptors were shown to interact with and inhibit TGF-β-induced Smad signaling in prostate cancer cells supporting the transcriptional repression found in these studies with activin and ERs (Hayes et al. 2001). Recent data demonstrate that estrogen signaling diminishes TGF-β-induced cell migration due to Smad and ER interactions (Malek et al. 2006). Thus, activin signaling is reduced as tumors increase in grade and the presence of an intact estrogen signal may block Smad transactivation (Jeruss et al. 2003). However, in this study, overexpression of ERs had a negative effect on activin-regulated transcription even in the absence of ligand. The ligand unoccupied receptor as a negative regulator of activin signaling is a critical future direction to determine whether receptors can block Smad activation or uphold an activin signal through specific interactions irregardless of ligand.

The physical interaction of ERα and Smads may also inhibit estrogen-dependent gene transcription in the presence of activin-stimulated Smad3. The evidence supporting that activated Smads render estrogen signaling deficient differs from TGF-β signaling which reportedly increases production of the vitamin D receptor that contains EREs (Yanagisawa et al. 1999). However, other studies have demonstrated that Smad4 co-immunoprecipitates with ERα in breast cancer studies where it, along with TGF-β stimulation, acts as a repressor of ERα activation of a 3XERE reporter (Wu et al. 2003, Li et al. 2005). In addition, Smad3 suppresses androgen receptor-mediated transcription in prostate cells through direct interactions (Hayes et al. 2001). In our studies, repressive competition was confirmed on the endogenous estrogen-responsive promoter, TFF1 in T47D cells.

One method through which estrogen and activin oppose one another is by repressing transcription of promoters from each respective ligand. The repression of activin signaling by estrogen appeared to be specific to each promoter as the p3TP promoter and endogenous PAI-1 gene were inhibited by treatment with estrogen but the synthetic CAGA promoter was not inhibited by estrogen. Estrogen represses urokinase-type plasminogen activator and PAI-1 (Levenson et al. 1998a,b), while activin-responsive Smads 3 and 4 directly bind to the PAI-1 promoter and induce its expression (Dennler et al. 1998). Induction of PAI-1 in breast cancer strongly correlates with poor prognosis where invasive cell systems are characterized as having high PAI-1 expression but lacking ERα (Tong et al. 1999, Corte et al. 2005). Previous reports have indicated that breast cancer cells have less PAI-1 expression when ERs are expressed confirming the relevance of crosstalk for extracellular matrix degradation (Levenson et al. 1998b). The role for both signaling pathways to mitigate one another implies that ER-positive cells are likely less invasive due to repression of invasion signals such as PAI-1. When breast cancer cells lose ERα and become more metastatic, the production of PAI-1 by activin may resurface due to loss of the corepressor and enhance cancer cell production of invasion factors. Future experiments will test the role of activin and estrogen crosstalk in governing cell motility.

Estrogen signaling may repress activin B mRNA and protein as a mechanism to escape cell growth inhibition. Menopausal women had higher amounts of activin in their breast tissue when compared with cycling control women undergoing mammoplasty.
providing some in vivo evidence of estrogen-dependent repression of activin in the breast (Reis et al. 2002). The regulation of activin B by estrogen may occur directly through promoter regulation or through alternative signaling pathways. Previous studies have tested the influence on estrogen signaling in MCF7 by performing microarray analysis (Frasor et al. 2003). In the current study, the downregulation of activin B mRNA and protein was confirmed while tamoxifen did not alter activin B production. This is dissimilar to TGF-β where tamoxifen increases its production and also enhances growth inhibitory stimuli in breast cancer cells (Knabbe et al. 1987, Malet et al. 2001, Brandt et al. 2003). When breast cancer cells become resistant to the growth inhibitory effects of TGF-β, they also gain partial resistance to tamoxifen (Herman & Katzenellenbogen 1996, Arteaga et al. 1999, Buck et al. 2004). Because activins do not seem to be altered through the same pathway, resistance and growth inhibition from tamoxifen likely do not involve activin B production. However, ER-positive tumors stimulated by endogenous estradiol may hamper the activin-induced cell cycle arrest by preventing its production.

Estrogen and activin signaling cascades intersect and act in a dual repressive manner to hamper the downstream transcription in each pathway. The activin signal is diminished by estrogen in a promoter-specific manner. Estrogen also downregulates breast cancer cell production of activin B subunits thereby eliminating one potential cytokine responsible for growth arrest. The interaction of these pathways could therefore allow for estrogen-responsive breast cancer cells to reduce cell control, but after ERs are lost, activin may resurface and signal to activate genes such as the PAI protein responsible for encouraging invasion. In this way, these two pathways intersect to alter transcription and control breast cancer cell growth and behavior.

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References


