Activin A Mediates Growth Inhibition and Cell Cycle Arrest through Smads in Human Breast Cancer Cells

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Abstract

The transforming growth factor-β (TGF-β) superfamily of growth factors is responsible for a variety of physiologic actions, including cell cycle regulation. Activin is a member of the TGF-β superfamily that inhibits the proliferation of breast cancer cells. Activin functions by interacting with its type I and type II receptors to induce phosphorylation of intracellular signaling molecules known as Smads. Smads regulate transcription of many genes in a cell- and tissue-specific manner. In this study, the role of activin A in growth regulation of breast cancer cells was investigated. Activin stimulated the Smad-responsive promoter, p3TP, 2-fold over control in T47D breast cancer cells. Activin inhibited cellular proliferation of T47D breast cancer cells after 72 hours, an effect that could be abrogated by incubation with the activin type I receptor inhibitor, SB431542. Activin arrested T47D cells in the G_0-G_1 cell cycle phase. Smad2 and Smad3 were phosphorylated in response to activin and accumulated in the nucleus of treated T47D cells. Infection of T47D cells with adenoviral Smad3 resulted in cell cycle arrest and activation of p3TP-luciferase, whereas a adenoviral dominant-negative Smad3 blocked activin-mediated cell cycle arrest and gene transcription. Activin maintained expression of p21 and p27 cyclin-dependent kinase inhibitors involved in cell cycle control, enhanced expression of p15, reduced cyclin A expression, and reduced phosphorylation of the retinoblastoma (Rb) protein. Smad3 overexpression recapitulated activin-induced p15 expression and repression of cyclin A and Rb phosphorylation. These data indicate that activin A inhibits breast cancer cellular proliferation and activates Smads responsible for initiating cell cycle arrest. (Cancer Res 2005; 65(17): 7968-75)

Introduction

Activin is a member of the transforming growth factor-β (TGF-β) superfamily of growth factors and was initially isolated in 1986 based on its ability to stimulate follicle-stimulating hormone secretion from the anterior pituitary. Activin, like other members of the TGF-β superfamily, controls multiple aspects of cellular growth and developmental differentiation. The growth-regulatory effects of ligands within this superfamily involve tumor suppression; however, as cell growth becomes deregulated, these same ligands drive oncogenic progression and metastasis (1). This work focuses on activin signal transduction in breast carcinogenesis.

Three activin ligands exist: homodimers of the βA and βB protein subunits compose activin A and activin B, respectively, and a heterodimer of βA and βB protein subunits forms activin AB. These ligands signal by binding to specific serine/threonine kinase type II (ActRIIA and ActRIIB) receptors. Following recruitment of the type I receptor (Alk4) by the type II receptor, cytoplasmic signaling molecules Smad2 or Smad3 are phosphorylated. After phosphorylation, these receptor Smads join with the co-Smad, Smad4, and translocate to the nucleus where they facilitate gene transcription. The activin βA subunit is expressed in all grades of breast cancer, whereas βB subunit expression is attenuated in higher-grade cancers (2). Activin βB subunit knockout animals do not have properly elongated mammary ducts or matured alveolar buds, indicating that βB isoforms are critical for mammary development (3). A clear elucidation of how activin causes growth arrest remains to be determined and is addressed by the present study.

Activin signaling has been associated with mammary cell growth inhibition as well as regulation of mammary glandular development (4, 5). Activin and its receptors are expressed during postnatal mouse mammary development and glandular formation during the lactating phase (6). Activin receptors and signaling molecules are present in human breast cancer cell lines, and the loss or reduction of activin-signaling components, specifically nuclear localization or phosphorylation of Smad2 and Smad3, has been documented as breast cancer becomes more aggressive (2, 7, 8). Elevated activin A levels were detected from breast cancer effluents in postmenopausal women (9). Mutations in activin receptors have also been associated with pancreatic and pituitary tumors (10, 11). Activin has been reported to inhibit growth of breast cancer cells by blocking the p38 mitogen-activated protein kinase (MAPK) pathway (12). Based on these findings, mechanisms by which activin acts to maintain cell cycle progression were explored.

Activin inhibits the proliferation of breast cancer cells by activating both Smad proteins and the p38 MAPK pathway. The mechanism by which TGF-β and activin exert their growth inhibition involves modulation of key cell cycle–regulatory protein. The cell cycle is closely regulated by cyclin-dependent kinases (cdk) that can be activated by cyclins or inhibited by cdk inhibitors. Extracellular signals can trigger the transition or inhibition of the G_1-S phase by altering the phosphorylation state of the retinoblastoma (Rb) protein: phosphorylated Rb results in cell cycle progression, whereas hypophosphorylation inhibits the cell cycle. Smad3, in concert with additional cofactors, induces transcription of cdk inhibitors, p15 and p21, and facilitates cell cycle arrest in early G_1 (13, 14). As a consequence of this cell cycle repression, the Rb protein remains unphosphorylated and thus unable to cause

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entry of cells into S phase (14). Smad3 knockout fibroblasts are less growth inhibited by TGF-β than wild-type cells and have reduced cdk inhibitor p15 levels (15).

In the present study, we investigated the role of activin A and Smad3 in the redistribution of the cell cycle. In addition, we characterized the regulation of cdk inhibitors in response to activin A and their ability to hinder phosphorylation of the Rb protein. Furthermore, overexpression of Smad3 can induce cell cycle arrest, and a dominant-negative Smad3 (DN-Smad3) can prevent the ability of activin to alter the cell cycle. Taken together, these data indicate that activin stimulation of Smad proteins plays a critical role in the growth regulation of breast cancer cells.

Materials and Methods

Cell culture and materials. T47D and MCF7 breast cancer epithelial cells (American Type Culture Collection, Manassas, VA) were cultured in phenol red–free RPMI (Life Technologies, Inc., Gaithersburg, MD) supplemented with 10% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA) and 1% antimycotics/antibiotics (Invitrogen) and incubated at 37°C, 5% CO_2. Activin A and follistatin were prepared at Northwestern University in a buffer of 0.15 mol/L NaCl and 0.05 mol/L Tris (pH 7.5). The Alk4/Alk5/Alk7–specific inhibitor SB431542 was purchased from Tocris (Ellisville, MO). All other reagents were purchased from Sigma (St. Louis, MO).

Cloning of adenoviral Smad3 and adenoviral dominant-negative Smad3. The adenoviral transfer plasmid (16) was a kind gift from Dr. J. Larry Jameson (Northwestern University Feinberg School of Medicine). *Eco*RI segment of Smad3 cDNA was ligated into adenoviral transfer plasmid. DN-Smad3 was PCR amplified from *Cla*I and *Xba*I segments of DN-Smad3 plasmid and cloned into adenoviral transfer plasmid. After confirmation of sequences, these adenoviral transfer plasmids were used to generate recombinant adenoviral vectors (Ad-Smad3 and Ad-DN-Smad3) as described previously (17). Adenoviral β-galactosidase (Ad-β-gal), which contains β-gal driven by cytomegalovirus (CMV) promoter, was used as a control.

β-Galactosidase staining of T47D cells. Cells were plated at 1×10^6 per T25 flasks. The next day, cells were infected with Ad-β-gal and incubated at 37°C for 7 hours. The medium was changed. Twenty-four hours later, cells were fixed in 1% gluteraldehyde for 10 minutes, washed with PBS, and incubated with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) substrate solution (10 mmol/L potassium ferricyanide, 1 mmol/L $MgCl_2$, 0.1% X-Gal in PBS) at 37°C for 2 hours. Cells were then imaged using an inverted microscope (Nikon, Huntley, IL) using a SpotRT monochrome digital camera (Diagnostic Instruments, Sterling Heights, MI) and MetaMorph imaging software (version 4.6, Universal Imaging, Downingtown, PA).

Transient transfections and adenoviral transduction. Cells were plated 1 day before transfection in 24-well plates and transiently transfected in Opti-MEM (Life Technologies) with p3TP-luciferase or (CAGA)12-lux alone or in combination with expression plasmids encoding Smad3 and DN-Smad3 for 12 hours using LipofectAMINE 2000 (Invitrogen). The (CAGA)12-lux reporter was a kind 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 medium or activin (100 ng/mL), follistatin (400 ng/mL), and TGF-β (10 ng/mL) for 24 hours. To determine the efficacy of the Ad-Smad3 and Ad-DN-Smad3, T47D cells were infected for 7 hours with the adenoviral vectors. Cells were then allowed to recover overnight and, the following day, transiently transfected with the p3TP-luciferase reporter. After an overnight transfection, cells were then treated for 24 hours with serum-free medium (control) or activin. To measure luciferase production, cells were lysed in GME buffer [25 mmol/L glycylglycine (pH 7.8), 15 mmol/L $MgSO_4$, 4 mmol/L EGTA, 1 mmol/L DTT, 1% Triton X-100] and lysates were added to assay buffer (GME buffer, 16.5 mmol/L KPO_4, 2.2 mmol/L ATP, 1.1 mmol/L DTT). Luciferase activity was measured for 30 seconds using an AutoLumat (Berthold Technologies Co., Oak Ridge, TN).

Proliferation assays. Cells were seeded into 96-well plates at 6×10^3 per 100 μL in RPMI supplemented with 1% FBS. The next day, fresh 1% serum containing medium with activin A (100 ng/mL) was added to plates and the cells were allowed to grow for 72 hours. Proliferation was measured with CellTiter 96 Aqueous One Solution (Promega, Madison, WI) according to the manufacturer. Spectrophotometric analysis was completed using a BioTek EL312e microplate reader (Fisher BioTek, Pittsburgh, PA). All conditions were tested in six replicates in triplicate experiments.

Flow cytometry. T47D cells were plated into T25 flasks 1 day before treatment. For experiments with ligand, the following day, serum-free medium containing activin (100 ng/mL), follistatin (400 ng/mL), or activin and follistatin was added to the cells and incubated for 48 hours. For adenoviral infection, the following day, cells were treated with Ad-β-gal, Ad-Smad3, or Ad-DN-Smad3 in serum-rich medium for 7 hours. Cells were then treated with either serum-free medium or activin (10 ng/mL) for 48 hours. In both cases, following treatment, cells were then collected through trypsinization, washed in PBS, and then thoroughly resuspended in 500 μL PBS. These cells were then fixed in 4 mL of 70% ethanol and stored at −20°C overnight. The fixed cells were then washed with PBS and initially stained with 500 μL propidium iodide (PI) staining solution [50 μg/mL PI, 90 units RNase A, 0.1% Triton X-100, 4 mmol/L citrate buffer, 10 mmol/L polyethylene glycol (PEG) 4000]. Cells were incubated in this solution for 20 minutes at 37°C. Cells were then treated with 500 μL PI salt solution (1 mg/mL PI, 0.1 mL of 10% Triton X-100, 4 mol/L NaCl solution, 10 mmol/L PEG 4000). Flow cytometric analysis was done on a Beckman Coulter Elite ESP (Miami, FL) for at least 30,000 individual events per reaction. Data for the cell cycle analysis were done using Mod-fit software (Verity Software House, Inc., Topsham, ME). Means ± SD of means were calculated for cell populations from triplicate data.

Reverse transcription-PCR. Total RNA was isolated using the TRIzol reagent (Invitrogen). The samples were then treated with RQ1 RNase-free DNase (Promega) and phenol-chloroform extracted. RNA samples (5 μg) were then primed with random hexamers and reverse transcribed with Moloney murine leukemia virus (MMLV) reverse transcriptase (Promega) according to the manufacturer's instructions. From the original reverse transcription reaction, 4 μL were subjected to PCR amplification for 35 cycles under the following conditions: 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 90 seconds. Negative controls were run using water and RNA that had not been reverse transcribed with MMLV. Positive controls were based on PCR amplification from plasmids containing the activin or inhibin subunits.

Western blotting and ELISA. Whole cell lysates were collected from six-well plates following incubation with activin (100 ng/mL), follistatin (400 ng/mL), or both after 48 hours. Western blots with adenovirus were taken from lysates infected with adenovirus for 7 hours. Cells then recovered overnight before a 48-hour treatment with activin (100 ng/mL) in serum-free medium. Cells were lysed in 400 μL lysis buffer [150 mmol/L NaCl, 1% NP40, 0.5% deoxycholate, 0.1% SDS, 10 mmol/L Tris (pH 7.5)] supplemented with Complete Mini protease inhibitor cocktail tablets (Roche, Indianapolis, IN) and phosphatase inhibitor cocktail II (Sigma). Equal protein concentrations were confirmed through a BCA assay (Pierce, Rockford, IL). Samples were run under reduced conditions in a MES buffer using 4% to 12% denaturing NuPage gel (Invitrogen) according to the manufacturer's protocol. All protein gels were transferred to a nitrocellulose membrane for 2 hours at 25 V, 100 mA. Membranes were blocked for 1 hour at room temperature or overnight at 4°C in 5% nonfat milk in TBS with 0.1% Tween. Cyclin A (554174) antibody was purchased from BD Bioscience (Bedford, MA). p27 (sc528) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The p15(Ink4) (NA33) and p21 (Ab-1) mouse monoclonal (OP64-100) antibodies were purchased from Calbiochem (San Diego, CA). The phosphorylated Rb (9308), phosphorylated Smad2 (3101), phosphorylated Smad3 (9514), phosphorylated p38 (9211), and p38 (9212) antibodies were acquired from Cell Signaling Technology (Beverly, MA). Smad2 (51-1300) and Smad3 (51-1500) antibodies were purchased from Zymed Laboratories (San Francisco, CA). Activin A antibody was a kind gift from Dr. Wylie Vale (Salk Institute, La Jolla, CA). The secondary antibodies were a donkey anti-rabbit horseradish peroxidase (HRP; Amersham Life Science, Little Chalfont, United Kingdom) and a goat anti-mouse HRP (Bio-Rad, Hercules CA). Proteins were visualized using the enhanced chemiluminescence Western

blotting detection system from Amersham International (Little Chalfont, United Kingdom). Blots were done in triplicate, and densitometry analysis was done using the NIH software. The activin A ELISA was a kind gift from Diagnostic Systems Laboratory (Webster, TX). All assays were done according to the manufacturer's protocol using standards produced in our laboratory as described previously (18).

Statistical analysis. All values are expressed as the means ± SD. Student's *t* test was used to assess differences between treatment groups and control samples. *P*s < 0.05 were considered statistically significant.

Results

T47D cells are responsive to activin A. To characterize T47D breast cancer cells response to activin, cells were transiently transfected with an activin and TGF-β-responsive reporter gene construct, plasminogen activator inhibitor-1, ligated to a luciferase reporter (p3TP-luciferase) and treated with activin (100 ng/mL), TGF-β (10 ng/mL), or follistatin (400 ng/mL). Treatment with activin for 24 hours up-regulated luciferase activity, indicating the presence of an intact activin-signaling pathway in T47D cells. T47D cells responded to activin by increasing luciferase activity 60% over untreated cells, whereas TGF-β stimulated no significant increase in luciferase response compared with basal (Fig. 1*A*). Previous studies indicated that T47D cells lack the TGF-β RII receptor, rendering them unresponsive to TGF-β (19). MCF7 cells were also transfected with p3TP-luciferase and treated with activin and TGF-β. Activin (48%) and TGF-β (93%) both significantly up-regulated the promoter, providing evidence of an intact activin and TGF pathway in another breast cancer line (data not shown). To confirm an intact signaling pathway, the Smad-binding element (CAGA) repeated 12 times and ligated to the luciferase reporter gene was also transfected into T47D cells. Activin significantly up-regulated the CAGA promoter, providing further evidence in support of activin responsiveness in breast cancer cells (Fig. 1*B*). Cells were treated with the activin-binding protein, follistatin, to examine autocrine production of, and activin action on, p3TP-luciferase activation. Follistatin in excess to activin was added and abrogated activin p3TP-luciferase transcription in T47D and MCF7 cells, demonstrating that the luciferase response is activin specific. Treatment with follistatin caused a 50% decrease in luciferase activity below basal levels in T47D cells, but not MCF7 cells, suggesting that T47D cells may produce endogenous activin.

T47D cells are growth inhibited by activin A. To investigate whether the activin could regulate cell growth in breast cancer cells, proliferation assays were done. Cells were plated in 1% serum and treated with activin (100 ng/mL). T47D (Fig. 1*C*) and MCF7 (data not shown) cell proliferation was significantly mitigated by activin treatment. To determine if the growth arrest was specific to activin-mediated signaling, T47D cells were also incubated in the presence of either an Alk4/Alk5/Alk7 inhibitor, SB431542, alone or in combination with activin. Inhibition of proliferation by activin was reversed in the presence of SB431542 (Fig. 1*D*). SB431542, a competitive ATP-binding site kinase inhibitor, does not increase proliferation compared with untreated cells likely because of nonspecific inhibition of Alk5 and Alk7 as well as other cellular kinases. Cell lysates were investigated for the presence of phosphorylated p38 protein using Western blots with phosphospecific antibodies. Under our experimental conditions, p38 did not undergo obvious changes in phosphorylation in response to activin A (Fig. 1*E*).

Activin causes a redistribution of cell cycle. Because activin slows proliferation of cells, we investigated the mechanism by which activin exerts these growth-regulatory effects. Cells treated with either serum-free medium or activin for 48 hours were stained

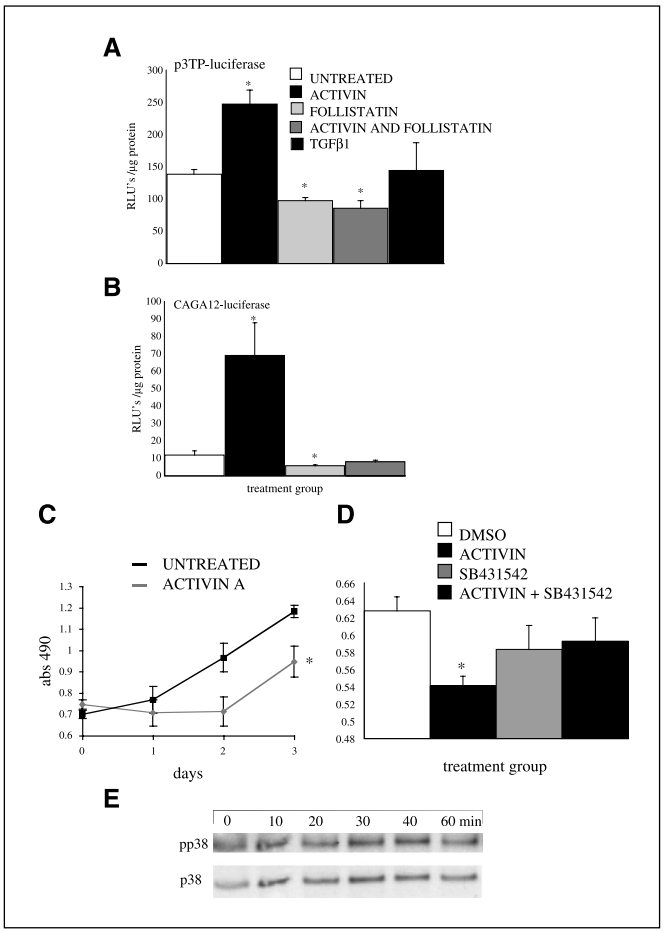

Figure 1. Activin inhibits proliferation of T47D breast cancer cells. T47D cells transfected with the activin and TGF-β-responsive promoters (*A*) p3TP and (*B*) (CAGA)12 driving luciferase production were stimulated with activin A (100 ng/mL), follistatin (400 ng/mL), TGF-β (10 ng/mL), and activin (100 ng/mL) in combination with follistatin (400 ng/mL) for 24 hours. *Columns,* mean relative light units (*RLU*)/μg protein of triplicates from a representative experiment; *bars,* SD. *C,* T47D cells were grown in 1% serum for 72 hours in the presence and absence of activin (100 ng/mL). Cell proliferation was assessed by the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt colorimetric assay in six replicates. *D,* T47D cells were grown in 1% serum in the presence and absence of activin (100 ng/mL), an Alk4/Alk5/Alk7 inhibitor SB431542 (10 μmol/L), or both for 72 hours. *Columns,* mean of triplicate experiments in arbitrary absorbance units; *bars,* SD. *, significant difference from untreated controls. *E,* T47D cells were treated with activin and cell lysates were analyzed by Western blot using a specific antibody to phosphorylated p38 (*pp38*) protein. Protein loading was normalized based on total p38.

with PI and analyzed by flow cytometry. T47D cells grown in serum-free medium have a normal cell distribution with 70 ± 1% of the cells in G_1. However, activin caused a redistribution of the cell cycle in T47D cells resulting in an accumulation of 83.7 ± 1.6% of the cells into the G_1 phase (Table 1). Follistatin in combination with activin inhibited the activin-mediated growth arrest. Follistatin alone caused further cell progression and accumulation of cells in S phase when compared with control cells. Previous reports indicated induction of growth arrest in T47D cells due to phosphorylation of the p38 protein. To assess the effect p38 played in the redistribution of the cell cycle, the chemical inhibitor SB203580 was incubated with T47D cells alone and in combination with activin. The p38 inhibitor SB203580 did not alter the cell cycle and did not reduce the G_0-G_1 redistribution induced by activin A (data not shown).

Table 1. Activin redistributes T47D cells into G_0-G_1 cell cycle arrest

T47D	G_0-G_1	S	G_2
Untreated	70.2 ± 1.1	18.3 ± 0.6	11.5 ± 0.7
Activin A	83.7 ± 1.6	7.8 ± 0.9*	8.5 ± 0.7*
Follistatin	60.6 ± 3.6	26.2 ± 2.1*	13.2 ± 1.5
Activin and follistatin	58.2 ± 3.0	28.1 ± 1.9*	13.6 ± 1.2*

NOTE: T47D cells were treated with activin A (100 ng/mL), follistatin (400 ng/mL), activin and follistatin combined, or TGF-β (10 ng/mL) for 48 hours in serum-free conditions and then stained with PI. DNA content was then analyzed by flow cytometry. Distribution of cells in the three phases of the cell cycle is represented by percentages. Representative means ± SD from three replicates of a representative experiment.
*Significant difference from untreated controls.

Activin secretion from T47D cells. The follistatin-mediated release from growth inhibition and cell cycle arrest in untreated cells suggested that T47D cells secrete activin. mRNA from T47D cells was isolated to investigate if the cells produced endogenous activin. Although T47D cells are unable to produce inhibin α and activin βB subunit mRNA, they do make the activin βA subunit (data not shown). Western blots done with an antibody directed against the βA subunit revealed an intense band at ~52 kDa (data not shown). This molecular weight correlates to the reduced prepro-activin βA subunit revealing that T47D cells produce endogenous activin protein. An ELISA-directed against activin A indicated that T47D cells secrete between 40 and 50 ng/mL of activin into medium within 48 hours.

Activin stimulates a redistribution of cell cycle through cyclin-dependent kinase inhibitors and retinoblastoma. We investigated the ability of activin to alter proteins involved in transition from G_0-G_1 into the S phase of the cell cycle. Candidate proteins chosen for analysis were cyclins A and D, the cdk inhibitor proteins p21(waf1/cip1), p15(Ink4), and p27, and the Rb protein. The S-phase cyclin A was reduced by 50% in Western blots following treatment with activin A after 48 hours in T47D lysates (Fig. 2*A* and *B*). Treatment with follistatin alone and in combination with activin resulted in equal levels of cyclin A protein compared with untreated cells. Activin treatment was able to marginally increase p15 and p21 expression levels. Although activin did not further increase p27, incubation with follistatin alone or in combination with activin down-regulated p15, p21, and p27. These results suggest that endogenous activin regulates tonic p15, p21, and p27 levels. An alteration in c-myc protein was not detected after 24 or 48 hours of treatment (data not shown). Finally, the Rb protein was hypophosphorylated in response to activin. These data indicate that activin is able to alter cell cycle progression by down-regulating cyclin A, maintaining expression of cdk inhibitors, and ultimately reducing phosphorylation of the Rb protein.

Smad2 and Smad3 as a regulatory molecule of activin signaling. To investigate if Smad2 and Smad3 were activated by activin, T47D cells were treated with activin and harvested at varying time points. The lysates were analyzed using an antibody directed against the phosphorylated form of Smad2 and showed a time-dependent increase in Smad2 phosphorylation. These same lysates were then probed with a phosphospecific antibody directed against Smad3. These data show that activin regulates gene expression through Smad2 and Smad3 in T47D cells (Fig. 3*A*). To extend and confirm these results, nuclear location of Smad2 and Smad3 was measured after activin treatment. Cells were serum starved for 24 hours and then pretreated with follistatin (400 ng/mL) for 16 hours before activin addition. The pretreatment step was necessary to bioneutralize endogenous activin and permit the Smads to redistribute to the cytoplasm (data not shown). Nuclear preparations were made from activin-treated and untreated cells. Smad2 and Smad3 were predominately located in the cytoplasm of untreated cells. Smad2 and Smad3 were found in the nucleus of activin-treated cells (Fig. 3*B*). These results confirm that Smad2 and Smad3 were phosphorylated by activin and translocated into the nucleus to function as transcription factors.

T47D cells are transducible with adenoviral β-galactosidase. Adenoviral vectors containing β-gal (control-Ad-β-gal), Smad3 (Ad-Smad3), or DN-Smad3 (Ad-DN-Smad3) were developed to determine what effects overexpression of an Ad-Smad3 or Ad-DN-Smad3 might have on the cell cycle of T47D cells. To determine transduction efficiency of adenoviral vectors, T47D cells were infected with increasing 25, 50, and 75 plaque-forming units (pfu)/cell of Ad-β-gal. After allowing 24 hours for protein expression, the

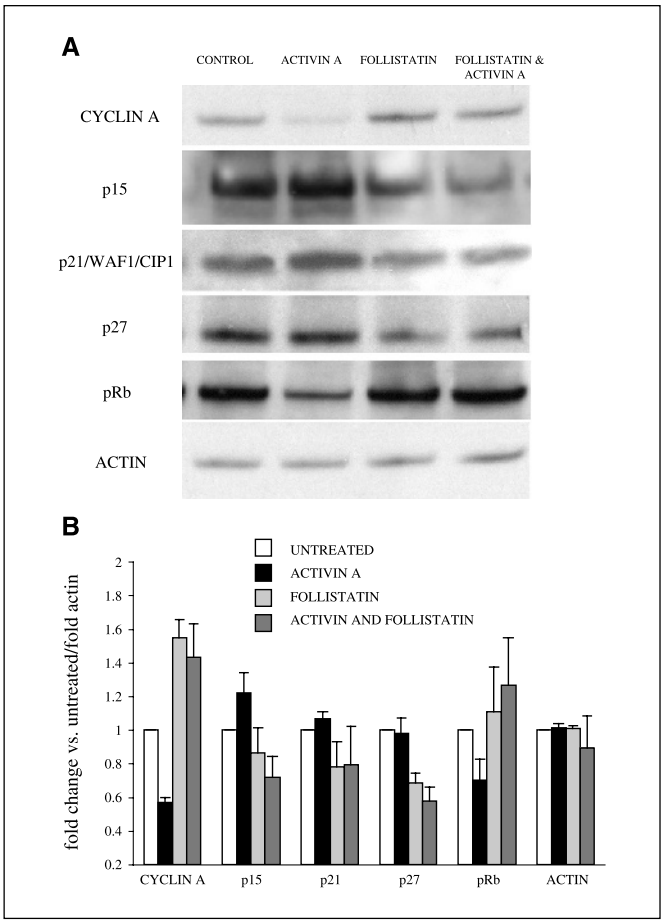

Figure 2. Activin regulates expression of cell cycle proteins in T47D cells. *A,* T47D cells were treated with activin, follistatin, or activin and follistatin for 48 hours. Cell lysates were analyzed by Western blot using a specific antibody to cyclin A, p15, p21, p27, and phosphorylated Rb (*pRb*) protein. Each antibody was used on the same membrane in at least three independent experiments. Protein loading was normalized based on actin. *B,* densitometry was done on all blots using NIH software. *Columns,* mean of triplicate experiments; *bars,* SD.

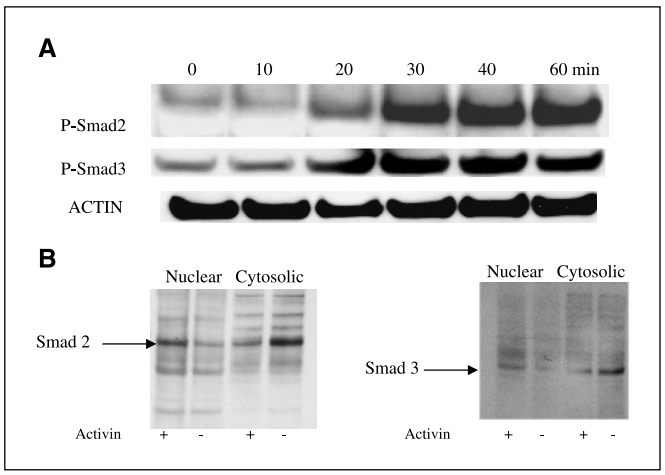

Figure 3. Activin induces Smad2 and Smad3 phosphorylation and nuclear localization in T47D cells. *A,* T47D cells were pretreated with follistatin, washed, and treated with activin (100 ng/mL) for 0, 10, 20, 30, 40, and 60 minutes. Lysates were analyzed using phosphospecific Smad2 (*P-Smad2*) and phosphospecific Smad3 (*P-Smad3*) antibodies. Actin was used as a loading control. *B,* T47D cells were pretreated with follistatin, washed, and treated with activin A (100 ng/mL) for 30 minutes. Nuclear and cytosolic fractions of cells from activin-treated and untreated cells were then analyzed with a specific antibody for Smad2 and Smad3. Protein concentrations were predetermined using a BCA assay and protein (30 μg) was loaded for each sample due to differences with actin in nuclear and cytosolic preparations.

cells were stained for β-gal. Cells infected at 25 pfu/cell had ~60% transduction, whereas those cells treated with 50 and 75 pfu/cell had 90% to 100% infection efficiency (Fig. 4*A*). Based on these findings, 75 pfu/cell were chosen for further adenoviral experimentation. Uninfected cells did not stain positive for β-gal (Fig. 4*A*).

Adenoviral Smad3 and dominant-negative Smad3 are functional in T47D cells. Cells were then transfected with p3TP-luciferase and infected with Ad-Smad3 to discern the efficacy of the transgene. Cells treated with the Ad-β-gal responded with increased luciferase expression in response to activin (Fig. 4*B*). Further, infection of T47D cells with Ad-Smad3 had a comparable luciferase response to that of T47D cells treated with activin alone. This ligand-independent action of overexpression of Smad3 has been reported previously and indicates that Smad3 is capable of simulating activin-responsive genes in T47D cells. The ligand-independent effect may also be enhanced by the endogenous activin A produced in these cells. Treatment of Ad-Smad3-transfected cells with activin resulted in an ~2-fold increase in gene response. Together, these findings illustrate that Ad-Smad3 is functional in T47D cells and is capable of activating activin-responsive genes and that these cells are responsive to activin.

We sought to determine if the truncated Ad-DN-Smad3 would abrogate activin and Smad3 activation of the p3TP-luciferase reporter in T47D cells. The Ad-DN-Smad3 construct inhibits signaling by deletion of three serines that would typically be phosphorylated in response to activin. Therefore, the truncated Smad3 (DN) remains at the cell surface, is unphosphorylated, and prevents intracellular signaling. Activin-stimulated activity was significantly inhibited in the presence of Ad-DN-Smad3 (Fig. 4*B*). This finding suggests that activin signaling through Smads can be inhibited by overexpression of DN-Smad3. Taken together, these results further confirm that the activin pathway is functional in this breast cancer cell line and that overexpression of Ad-Smad3 or the Ad-DN-Smad3 can alter activin-responsive genes.

Activin responsiveness can also be measured by the phosphorylation of Smad molecules. To determine Smad phosphorylation changes in response to T47D infections with Ad-β-gal, Ad-Smad3, or Ad-DN-Smad3, Western blots were done using phosphospecific antibodies and cells infected with a virus either with or without activin stimulation (Fig. 4*C*). Phosphorylated Smad2 increased in response to activin, whereas endogenous phosphorylated Smad3 was not detectable due to the elevated signal in Ad-Smad3-infected lanes. Infection with Ad-Smad3 increased phosphorylation, which was further stimulated by the addition of exogenous activin. Overexpression of Ad-Smad3 blocked Smad2 phosphorylation. The Ad-DN-Smad3 construct was able to block phosphorylation of both Smad2 and Smad3.

Smad3 induced a redistribution of the cell cycle to the G_0-G_1 phase in T47D cells. To determine if T47D cells infected with Ad-Smad3 or Ad-DN-Smad3 and treated with activin would undergo redistribution of the cell cycle, cells were infected with Ad-β-gal, Ad-Smad3, or Ad-DN-Smad3. The percentage of control Ad-β-gal-infected cells in G_0-G_1 was 54 ± 0.5% and this number significantly increased to 63 ± 0.4% in activin-treated cells. The percentage of

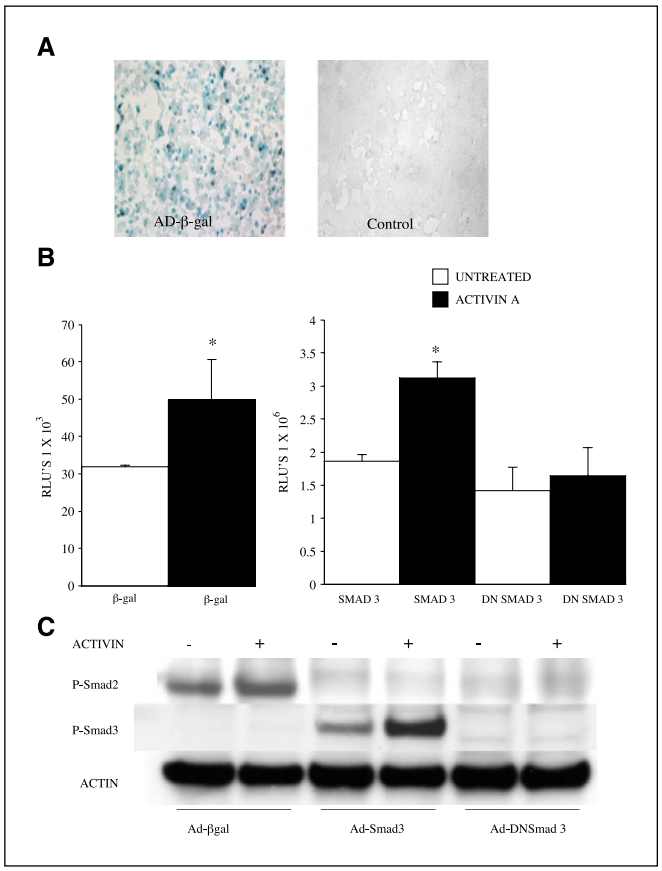

Figure 4. Adenoviral constructs infect T47D cells and function in promoter activation. *A,* an adenovirus containing the β-gal gene driven by a CMV promoter was used to infect T47D cells at 75 pfu/cell and stained with X-gal solution. *B,* T47D cells were infected with an Ad-β-gal, Ad-Smad3, or Ad-DN-Smad3 virus for 7 hours. Following the viral infection, cells were transfected with an activin-responsive p3TP promoter driving luciferase production and stimulated with or without activin (100 ng/mL) for 24 hours. *Columns,* mean RLU of triplicates from a representative experiment; *bars,* SD. *, significant difference from untreated controls. *C,* T47D cells were infected with Ad-β-gal, Ad-Smad3, or Ad-DN-Smad3 virus for 7 hours, allowed to recover overnight, and stimulated with or without activin (100 ng/mL) for 48 hours. Lysates were analyzed using phosphospecific Smad2 and Smad3 antibodies.

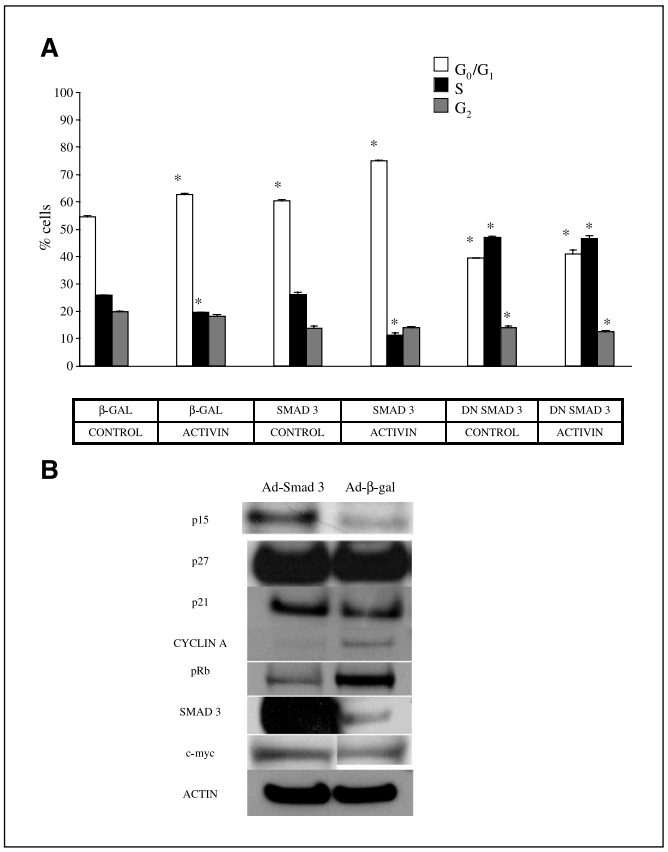

Figure 5. Smad3 redistributes T47D cells into G_0-G_1 growth arrest. *A,* T47D cells were infected with Ad-β-gal, Ad-Smad3, or Ad-DN-Smad3 for 7 hours and then treated with or without activin (100 ng/mL) for 48 hours. DNA content was then analyzed by flow cytometry. Distributions of cells in the three phases of the cell cycle are represented by percentages. *Columns,* mean from three replicates of a representative experiment; *bars,* SD. *, significant difference from untreated controls. *B,* T47D cells were infected with Ad-Smad3 or an Ad-β-gal construct for 7 hours and allowed to grow in serum-free medium for 48 hours. Cell lysates were analyzed by Western blot using a specific antibody to cyclin A, c-myc, p15, p21, p27, and phosphorylated Rb protein. Protein loading was normalized based on actin.

cells treated with Ad-Smad3 and serum-free medium in G_0-G_1 was 60 ± 0.4% and this was also stimulated significantly in response to activin to 75 ± 0.04%. The percentage of cells treated with Ad-DN-Smad3 and serum-free medium in G_0-G_1 dropped to 39 ± 0.1% and 41 ± 1% in activin-untreated and treated cells, respectively (Fig. 5*A*). Transduction of cells with Ad-Smad3 alone contributes to the redistribution of cells in the cell cycle to the G_0-G_1 population, and transduction with Ad-DN-Smad3 induced a cell population shift to the S phase.

Overexpression of adenoviral Smad3 alters expression of cell cycle–regulatory proteins. To determine if the redistribution of the cell cycle by Ad-Smad3 into G_0-G_1 arrest is due to the same cell cycle–regulatory protein changes induced by activin, T47D cells were infected with Ad-β-gal and Ad-Smad3 viruses. After 7 hours of infection and overnight recovery, the cells were allowed to incubate in serum-free medium 48 hours before lysates were collected (Fig. 5*B*). T47D cells infected with Ad-Smad3 highly expressed Smad3 protein compared with the amount of endogenous Smad3 in Ad-β-gal-infected cells, indicating that the overexpression of Smad3 produced viable protein. Similar to activin treatment, overexpression of Smad3 resulted in a reduction in the amount of cyclin A protein compared with control virus-infected cells. The amount of p27 and p21 expression was not significantly altered in response to the Smad3 overexpression, suggesting that activin may have additional effects on the cell cycle independent of Smad3 signaling. Smad3 increased production of p15 protein similar to activin stimulation. An alteration in c-myc was not detected from Ad-Smad3 infection. Finally, overexpression of Smad3 strongly inhibited phosphorylation of the Rb protein, indicating the importance of this molecule in dictating the transition from G_0-G_1 into S phase.

Discussion

Activin inhibits cellular proliferation in breast cancer cell lines (20). However, the mechanism by which activin halts cell growth had not been fully elucidated. In the mouse mammary gland, characterization of activin and its receptors during lactation indicated a keen role for activin as a molecule capable of both stimulating and inhibiting growth within the mammary gland (6). Dysregulation of the signaling molecules in the activin pathway has also been correlated to an increase in mammary cancer grade as well as abundant secretion of activin subunits in breast cystic fluid (2, 21, 22). To understand the pathways through which activin controls cellular growth and proliferation and the likely factors altered in cancer cells, an investigation of activin-signaling pathways in breast cancer cells was done. In these experiments, activin up-regulated p3TP-luciferase and CAGA-luciferase constructs and this transcriptional activation could be blocked in the presence of follistatin. Activin slowed the proliferation of T47D and MCF7 cells grown in culture. Smads are known downstream targets of the activin-signaling pathway and have been reported to up-regulate a variety of genes important in cell cycle control (23). Although previous experiments reported a functional and activated Smad2 response in T47D cells treated with activin, the importance of Smad3 and its control of the cell cycle was not investigated (12). These data indicate that Smad2 and Smad3 are both phosphorylated in response to activin and translocate to the nucleus. Activin redistributed the cell population such that a higher abundance of the cells were in G_0-G_1 phase. Again, the activin-binding protein, follistatin, inhibited the G_0-G_1 cell cycle arrest from activin, indicating the specificity of activin in controlling this cell cycle checkpoint. Smad3 overexpression induced cell cycle arrest, inhibited cyclin A, and induced Rb hypophosphorylation phenocopying the activin effect. An overexpressed Ad-DN-Smad3 abolished both Smad3- and activin-induced cell cycle arrest demonstrating the importance of activin-dependent Smad3 activation for maintaining growth inhibition. The presence of activin and activin-signaling receptors in mouse mammary glands, human mammary glands, and human breast cells suggests that they play an important role in maintaining control over cellular proliferation during both normal development and in cancer. These experiments suggest that the regulation of proliferation is partially due to the arrest of the cell cycle by activin in the breast and offers a potential mechanistic explanation for down-regulation of the ligands and signaling components in the activin pathway during cancer progression.

Because activin was able to arrest the cell cycle by inhibiting the phosphorylation of the Rb protein, it most likely functions by blocking cdk4 activity. Cyclin D is the primary regulator of cdk4 activity during G_0-G_1, yet we were unable to detect changes in the amounts of cyclin D in T47D cells in response to activin. Cyclin D is dysregulated in 40% of breast cancers and it may be this overexpression in T47D cells that prevents activin regulation of the gene (24). Although activin was able to alter cyclin D levels in plasmacytic cells, overexpression of cyclin D only partially blocked

the ability of activin to hypophosphorylate the Rb protein suggesting cooperation with other molecules (25). In T47D cells, the up-regulation of p21 may be sufficient to alter the phosphorylation state of the Rb without changing cyclin D levels. TGF-β alters Rb by acting through both p15 and p21; however, p15 was not altered in hybridoma cells treated with activin (25). Hepatoma cells also respond to activin by up-regulating the expression of the p21 and p15 protein (26, 27). Activin stimulation of p15 protein expression in T47D cells has previously not been detected, although an increase in p15 mRNA was found following activin treatment (12). Here, we were able to detect an increase in p15 protein expression from activin treatment and Ad-Smad3 overexpression. The data found in these studies indicate that p21 and p15 are partially responsible for altering the phosphorylation state of the Rb protein during cell cycle regulation in T47D cells.

Smad2 and Smad3 were phosphorylated in response to activin in T47D breast cells. To investigate if Smad signaling was integral to the cell cycle arrest orchestrated by activin, adenoviral gene delivery was necessary for gene transduction. A high transfection efficiency is necessary to accurately measure changes from exogenous genes using flow cytometry. Adenovirus was able to infect cells at 99% efficiency and was therefore deemed appropriate. Use of adenoviral vectors indicated that Smad3 was able to halt cell cycle progression similar to activin; however, the addition of activin provided even further control of the cell cycle. Interestingly, the overexpression of Ad-DN-Smad3 increased the background luciferase response compared with Ad-β-gal-infected cells. The overexpression of Smad molecules has been reported to aberrantly increase nuclear localization (28). The Ad-DN-Smad3 may increase background p3TP-luciferase transcriptional activity by improperly entering the nucleus; however, it completely abrogates activin cell surface receptor-mediated transcription, which seems to be integral for cell cycle regulation. The Ad-DN-Smad3 construct blocked activin-mediated cell cycle control as well as produced a significant increase in the S-phase population that did not resemble that of follistatin-treated cells. Therefore, it is possible that Smad3 will mediate a second set of cell cycle regulators important during S phase, which a dominant negative was capable of disrupting. Further studies are planned to investigate this possibility.

The adenoviruses used in these studies were made using Smad3 cDNA. Previous reports indicate that the mechanism through which the truncated Smad exerts its dominant-negative effect is by blocking phosphorylation, thereby trapping the Smad at the receptor. This lack of specificity also renders Smad2 inactive because the receptor complex cannot phosphorylate Smad2 in the presence of the DN-Smad3 (29). Therefore, these data suggest that Smad3 is important in regulating cell cycle control but do not rule out the importance of Smad2 in regulating these events, consistent with published results (30). In fact, previous data suggest that both Smad2 and Smad3 participate in regulating mammary gland growth and differentiation (31). However, overexpression of Smad3 mitigated phosphorylation of Smad2, indicating that the expression of Smad3 under our experimental conditions might limit the role of Smad2 in controlling cell cycle. Smad2 is inactivated by mutation in tumors, whereas Smad3 is rarely mutated in solid tumors. The loss of Smad2 or lack of phosphorylation correlates to poor survival outcome for patients with head and neck squamous cell carcinoma, breast, and colorectal cancers (8, 32, 33). Several Smads exhibited decreased protein expression in a chemical carcinogenesis model of skin cancer, with Smad3 demonstrating the most profound loss (34). In addition, a loss of Smad3 in tandem with alterations in cdk inhibitors was implicated in T-cell leukemogenesis (35). Several other possible mechanisms for inactivation of Smad2/Smad3 have been identified in breast cancer cells, such as overexpression of ras, c-ski, c-sno, and evi-1 (36–40). The loss of Smad3 function by repression has frequently been reported to be important in the progression of gastric cancer (41). Overexpression of DN-Smad3 in nontransformed breast cells was capable of enhancing the malignancy of xenografted tumors and cells but inhibited lung metastasis (30). Finally, experiments using antisense molecules have indicated that the genes important in regulating TGF-β cell cycle arrest are primarily activated by Smad3 (42). Consequently, it may be more appropriate to consider the loss of Smad3 by either decreased production, increased degradation, or deranged signaling cofactors in cancer pathology as opposed to mutations. These data indicate that activin also uses Smad3 to stimulate a redistribution of the cell cycle and activin ultimately results in heightened expression of cdk inhibitors p15, p21, and p27.

Although the overexpression of Smad3 was capable of inducing cell cycle arrest in T47D cells, Smad3 was not as proficient as activin at increasing and maintaining the expression of the cdk inhibitors p21 and p27. The experiments with follistatin and activin in combination illustrated the importance of the endogenous activin in T47D cells to maintain expression of these cdk inhibitors. Therefore, overexpression of Smad3 may not increase their production but simply block their degradation. Interestingly, Smad3 was able to alter the phosphorylation state of the Rb and expression of p15 proteins, suggesting that Smad3 alone plays a critical role in altering the cell cycle. Some of the repression of Rb protein phosphorylation in response to Smad3 overexpression may come from competition for cdk2 and cdk4 phosphorylation, as both Rb and Smad3 are substrates for these kinases (43). Those data are also confirmed by the flow cytometry data where overexpression of Smad3 was able to redistribute the cell cycle into G_0-G_1. The flow cytometry data indicated that this growth arrest could be further amplified with additional activin, perhaps through the regulation of p15, p21, and p27 by activin, through Smad2- or a Smad-independent pathway. Promoter studies using Smad overexpression to investigate regulation of the *p21* and *p15* genes have illustrated that Smad2, Smad3, and Smad4 all contribute to transcriptional activation (44–46). The inhibition of Smad2 phosphorylation by Ad-Smad3 overexpression may explain how activin signaling was able to increase expression of p21 and p27 more effectively than Smad3 alone.

The activation of the p38 MAPK pathway has been reported to be an important target of activin signaling in T47D breast cancer cell lines (12). Inhibition of this pathway by small-molecule chemical inhibitors was reported previously to abolish the activin-induced inhibition of proliferation. Under our experimental conditions, activin A did not phosphorylate p38 in T47D cells and chemical inhibitors of this pathway did not alter the activin-initiated cell cycle arrest. T47D cells in our laboratory produced large quantities of activin A, and this abundance of endogenous signal likely blocks the detection of phosphorylation changes in p38. The overexpression of Smad3 was capable of redistributing the cell into G_0-G_1 arrest, an effect that could be augmented by the addition of exogenous activin. These data indicate that Smad3 alone can redistribute T47D cells into G_0-G_1 cell cycle arrest, and other pathways, such as the p38 pathway, activated by activin stimulation, may augment this affect. However, the complete abrogation of cell cycle arrest by overexpression of Ad-DN-Smad3 indicates that the lack of a Smad3 response renders T47D cells unable to initiate growth arrest. Although T47D cells are cancerous, they responded to activin by undergoing growth

inhibition. Thus, the process by which mammary tissues become cancerous must involve a multistep component where other genes and pathways are dysregulated resulting in malignancy.

In summary, these data indicate that activin A inhibits cellular proliferation and cell cycle progression in breast cancer cells. Activin mediates these responses by signaling through the Smad2 and Smad3 molecules. Smad3 redistributes the T47D cell cycle independent of activin signaling. Finally, activin reduces cyclin A, up-regulates p15, p21, and p27, and reduces phosphorylation of the Rb protein leading to growth arrest. Therefore, activin as well as Smad molecules are important in the control of cellular growth and possibly tumor suppression in breast cancer cells.

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Activin A Mediates Growth Inhibition and Cell Cycle Arrest through Smads in Human Breast Cancer Cells

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