Down-Regulation of Activin, Activin Receptors, and Smads in High-Grade Breast Cancer

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ABSTRACT

Activin and transforming growth factor (TGF)-β members of the TGF-β superfamily of growth factors, have been implicated in both mammary gland development and breast carcinogenesis. TGF-β is thought to be involved in the maintenance of mammary gland ductal architecture and postlactational involution. TGF-β acts as both a tumor suppressor and has oncogenic capacities in breast cancer tissue. Activin is associated with growth modulation in glandular organs, and its receptors and signaling proteins are present and regulated during postnatal mammary gland development, primarily during the lactational phase. The presence of the major components of the activin signal transduction pathway in different pathologic grades of breast cancer tissue has not been described thoroughly, despite evidence from in vitro studies suggesting that activin can inhibit proliferation in breast cancer-derived cells. On the basis of the growth regulatory capacity of activin, we hypothesized that the components of this signal transduction system would be deregulated as breast cancer becomes more aggressive. To test this hypothesis, breast cancer samples were stratified by pathologic grade, a known prognostic marker for breast cancer, and then examined for the presence and cellular localization of activin ligand subunits (βA- and βB-), receptors (ActRIIA, ActRIIB, and ActRIB), and signaling proteins, Smads 2, 3, and 4, by immunohistochemistry and immunofluorescent analysis. Breast tissue from healthy patients undergoing reduction mammoplasty was also studied. The activin βA-subunit was present in all of the tissues examined, whereas the βB-subunit, activin type II receptors, and Smads were less evident in high-grade cancers. Significant correlations were made in breast cancer specimens between a decrease in nuclear Smad 3 abundance and high tumor grade, high architectural grade, larger tumor size, and hormone receptor negativity. Thus, activin signal transduction components are present in normal tissue and grade 1 cancer but down-regulated in high-grade cancer. The deregulation of this signal transduction system may be relevant to advancing oncogenic progression.

INTRODUCTION

Members of the TGF-β superfamily and their associated downstream signaling components have been implicated in several aspects of breast cancer onset and progression (1). There are >40 members of this superfamily, including activin and its structural homologue, inhibin, and the TGF-β isoforms 1–3 (2). These growth factors contribute to a wide array of cellular processes including proliferation, differentiation, motility, adhesion, and apoptosis (3). Whereas a significant amount of research has focused on the role of TGF-β in breast carcinogenesis, less is known about the role of activin in this complex disease. Activin was first identified as a protein produced by the granulosa cells of the ovary, capable of stimulating follicle-stimulating hormone release from the pituitary (4). In addition, activin functions to control development, growth, and differentiation throughout the body axis (5, 6). Mature activin dimers are formed through the assembly of β-subunits to produce βA-βA, βB-βB, or βA-βB ligands. Inhibin, a protein closely related to activin, is composed of heterodimers of either βA or βB and a unique α subunit, to produce inhibin A or inhibin B. Inhibin is an activin antagonist, and exerts its primary endocrine action through the down-regulation of pituitary follicle-stimulating hormone (4).

The activin and TGF-β ligands bind to ligand-specific receptors, yet the actions of both growth factors are mediated through the same cytoplasmic signaling components, the Smads (7–10). Activins signal through a heteromeric receptor complex comprised of a type II receptor isoform (ActRIIA or ActRIIB), and one type I receptor (ActRIIB). The TGF-β ligands signal through a receptor complex consisting of TβRII and TβRII receptors (3). Assembly and activation of these heteromeric receptor complexes result in the phosphorylation of two possible receptor-regulated cytoplasmic coactivators (R-Smad 2 and R-Smad 3), which then interact with the common Smad (co-Smad 4; Refs. 5, 6, 11, 12). The phosphorylated, activated Smad 2/4 or 3/4 complex translocates to the cell nucleus to modulate the transcription of activin or TGF-β-responsive genes (11). Inhibins bind the activin type II receptors, and to an accessory protein, betaglycan, to functionally antagonize the actions of activin (2, 13). Inhibitory Smads (I-Smads 6 and 7) block phosphorylation of the R-Smads by preventing their association with the type I receptor in the cell cytoplasm, to inhibit additional Smad signaling (3, 11). As activin and TGF-β converge on the same cytoplasmic signaling molecules to execute their actions, organ-specific ligand, receptor, or cytoplasmic and nuclear cofactor expression may ultimately confer specificity to these two different growth factors (3).

Activin and TGF-β have been associated with both normal mammary gland development and with breast carcinogenesis (14–19). Activin and TGF-β signal transduction components have been identified in normal and breast cancer cell lines, and benign and malignant mammary tissue (14, 20–23). Moreover, activin, its receptors, and Smads are expressed in mammary tissue during postnatal development, and the regulation of these signaling proteins is correlated with the glandular changes that occur during the lactational phase (24). The TGF-β isoforms have been strongly implicated in the maintenance of normal spatial patterning of the mammary ductal tree, and TGFβ3 has been identified as a mediator of the primary phase of mammary gland involution (25–26).

Initial studies linking activin and TGF-β to growth regulatory control in breast cancer examined ligand-dependent growth inhibition in ER+ and ER− cell lines (20). ER+ and ER− cell lines were not consistently responsive to TGF-β. Yet, activin inhibited proliferation in ER+ cell lines but not ER− cell lines. The resistant breast cancer-derived cells became sensitive to TGF-β or activin after transfection of TGF-β, or activin-specific receptors or Smad 4. This data suggests that as breast cancer cells become less differentiated and lose ER expression, they may also be losing other important growth-
modulating signal transduction proteins (27, 28). Furthermore, a dichotomous role for TGF-β in breast cancer as both a tumor suppressor in early stage disease and a tumor promoter in advanced disease has been described (29). Despite these observations, other studies have shown that the TGF-β type II receptor is lost in aggressive breast cancers (30). Finally, a large tissue microarray study confirmed that Smad 2, activated phosphorylated Smad 2 (Smad 2P), and Smad 4 were present in breast cancer specimens. In this study, the loss of Smad 2P and Smad 4 correlated with poor prognosis (23).

This work attempts to more clearly define the role of activin and its associated signal transduction components in breast tumorigenesis. To this end, the cellular compartmentalization and abundance of activin and inhibitin α-, βA-, and βB-subunits; ActRIIA, ActRIIB, and ActRIB receptors; and Smad 2, Smad 3, and Smad 4 were characterized in mammary tissue from healthy patients undergoing reduction mammoplasty surgery and in patients with grades 1, 2, and 3 breast cancer. Breast cancers were stratified by pathologic grade, a known cytologic prognostic marker for breast cancer, as well as other known clinical and pathologic markers for breast cancer (31). The central hypothesis of this research is that activin is necessary to normal mammary cell function, and when activin signaling is disrupted or lost, malignant progression is potentiated. A global decrease in abundance of activin, its receptors, and Smads was measured in high-grade breast cancer. Changes in cytoplasmic and nuclear abundance, and locality of Smad 3, in relation to more aggressive breast cancer phenotype, were also observed.

MATERIALS AND METHODS

Breast Cancer Tissue. A total of 22 grade 1, 18 grade 2, and 19 grade 3 cases of infiltrating ductal carcinoma, and 20 reduction mammoplasty paraffin-embedded blocks were obtained with Institutional Review Board approval from the Evanston Northwestern Healthcare Tumor Bank and the Department of Pathology (Evanston, IL). Case-matched H&E-stained slides were also obtained. Pathologic tumor grade was verified using the Scarff-Bloom-Richardson method (grade 1 cancer, well differentiated; grade 2 cancer, moderately differentiated; and grade 3 cancer, poorly differentiated) by a cytopathologist (C. D. S.; Ref. 32). Peritumoral benign ductal tissue was defined as tissue taken from within 2 cm of the histologic edge of the carcinomas.

Antibodies. Goat polyclonal antibodies against ActRIIA, ActRIIB, and ActRIB (R&D Systems, Minneapolis, MN) were used for immunohistochemical analysis at final concentrations of 20 µg/ml (ActRIIA), 20 µg/ml (ActRIIB), and 10 µg/ml (ActRIB). Rabbit polyclonal antibodies against α, βA, and βB subunits of activin and inhibitin were a gift from Dr. W. K. Vale and Dr. Joan V. Vaughn (Salk Institute, La Jolla, CA), and used at a concentration of 3 µg/ml (anti-α), 3 µg/ml (anti-βA), and 6 µg/ml (anti-βB). Rabbit polyclonal antibodies against Smad 2 and Smad 3 (Zymed Laboratories, San Francisco, CA) were used at concentrations of 2.5 µg/ml (anti-Smad 2) and 1.25 µg/ml (anti-Smad 3). Goat polyclonal antibodies against Smad 4 (Santa Cruz Biotechnology, Santa Cruz, CA) were used at concentrations of 20 µg/ml (anti-Smad 4). Biotinylated rabbit antigoat and goat antirabbit secondary antibodies (Vector Laboratories Inc., Burlingame, CA) were used at a final dilution of 1:300 in 3% BSA-TBS for immunohistochemistry, and at a final dilution of 1:400 in 0.3% Triton X-TBS for immunofluorescence.

Immunohistochemistry. The immunohistochemical methods were performed as described previously (27). For immunohistochemistry using the Smad 3 antibody, all of the cases were processed using the Ventana Medical Systems ES/Nexes Module (Ventana Medical Systems, Inc., Tucson, AZ) at Evanston Northwestern Healthcare. For this system, slides were deparaffinized and hydrated with descending strengths of alcohols to distilled water and then blocked in 3% peroxide for 10 min. Subsequently, slides were washed in distilled water, and then pretreated with citrate buffer solution (pH 6.0) in a microwave at high power for 12 min. After cooling for 5 min, slides were washed and labeled. They were then mounted into a carousel inside the staining module, and the system was run to completion. The Ventana DAB Basic Detection kit (Ventana Medical Systems, Inc.) was used, which includes a universal biotinylated IgG secondary antibody (antimouse and antirabbit antibodies), avidin horseradish peroxidase, and DAB. After staining, slides were removed from the carousel, washed, and counter stained with Mayer’s Hematoxylin.

Immunofluorescence. Immunofluorescence was performed using the TSA Plus Fluorescence System (NEN Life Sciences Products, Inc., Boston, MA) via deposition of a fluorophore-labeled tyramide proximal to the horseradish peroxidase enzyme site. Antibodies used in immunofluorescence experiments were diluted in 0.3% Triton X-TBS. All of the immunofluorescent tissues were container-stained using Vectorshield with 4’,6-diamidino-2-phenylindole (Vector Laboratories). Specificity of immunostaining was determined by incubation of slides with buffer in place of the primary antibody. All of the images were acquired using a Spot RT monochrome digital camera (Diagnostic Instruments, Sterling Heights, MI), and percentage of green/blue fluorescent overlay and percentage of green fluorescence alone were determined using Metamorph Imaging Software (Version 4.6; Universal Imaging, Downingtown, PA).

Slide Interpretation and Statistical Analysis. Immunoreactivity was scored on an ordinal scale of 0 (absent), 1 (low), 2 (moderate), and 3 (very intense staining) for each of 9 proteins evaluated (ActRIIA, ActRIIB, ActRIB, α, βA, βB, Smads 2, 3, and 4). In an attempt to diminish interobserver variability, all of the cases were scored in a blinded fashion by investigator (J. S., J.) and one board-certified anatomical pathologist (C. D. S.). To additionally examine Smad 3 immunoreactivity 20 reduction mammoplasty, 57 peritumoral (2 cases lacking peritumoral tissue on the study slide were not included), and 50 cancer cases (9 cases lacking cancer on the study slide were not included) were examined via automated immunohistochemical staining technology. Immunoreactivity scoring was collapsed into two groups: 0, 1 (no or low immunoreactivity) and 2, 3 (moderate or high immunoreactivity), for all of the statistical tests evaluating Smad 3. Fisher’s exact test was used to compare staining intensity across pathologic grade categories. The Cochran-Armitage test was used to test for two-tailed trend analysis in percentages across grade categories. McNemar’s test was used to compare staining in tumor margins to staining in the tumors. Clinical variables were related to Smad 3 staining using both the Fisher’s exact test and the independent sample t test. All of the activin and inhibitin subunit immunohistochemical cases were repeated five times for each grade, and all of the activin receptor and Smad 2 and 4 immunohistochemical cases were repeated four times for each grade. Normal cases for these proteins were repeated 10 times. Immunofluorescence was repeated three times for all of the cases.

RESULTS

Characterization of Clinical Samples. Breast cancer cases were subclassified by pathologic grade. Case-matched benign peritumoral tissue was also examined for every cancer case. As a control, breast tissue from healthy women undergoing reduction mammoplasty surgery was studied. Activin subunit, receptor, and Smad protein was measured in the three different breast tissue groups (normal, peritumoral, and cancer) to characterize the abundance and cellular localization of these components in breast tissue from healthy women, and those patients with low-, moderate-, and high-grade breast cancer. Benign peritumoral tissue from patients with breast cancer was evaluated separately to determine the relatedness of this tissue to that collected from noncancerous patients.

Activin βB-Subunit and Activin Receptors Decrease as Tumor Grade Increases. The activin βA-subunit was detected in the cytoplasm of ductal epithelial cells, and intense staining was detected in cancers of all three of the grades (Fig. 1, A–C), in benign peritumoral ductal tissue (Fig. 1, D–F), and in normal tissue (Fig. 1G). The activin βB-subunit was also present in the cell cytoplasm. Moderate to low staining levels were found in all of the grades of breast cancer (Fig. 2, A–C), and moderate to intense levels were detected in benign peritumoral ductal tissue (Fig. 2, D–F) and in normal tissue (Fig. 2G). The α-subunit was low to undetectable in normal tissue, benign ducts adjacent to the cancers, and the cancers of all three of the grades (data
When comparing cytoplasmic intensity of Smad 3 in normal and benign peritumoral ducts, cytoplasmic Smad 3 was more frequently intense in normal tissue (10 of 20; 50%) than it was in peritumoral tissue [3 of 22 (14%) grade 1; 4 of 17 (24%) grade 2; 8 of 18 (44%) grade 3; \( P = 0.042 \); Table 1]. Smad 3 cytoplasmic intensity in peritumoral tissues increased as tumor grade increased (\( P \) for trend = 0.029). In the cancer tissue, cytoplasmic staining for Smad 3 appeared to trend upward as pathologic grade increased [4 of 18 (22%) grade 1; 7 of 16 (44%) grade 2; and 8 of 16 (50%) grade 3], but was not found to be statistically significant. No significance was found between peritumoral tissue and cancers regarding cytoplasmic staining intensity.

**Smad 2, Smad 3, and Smad 4 Immunofluorescent Imaging.** Smads 2 and 4 were not easily detected in the nucleus by DAB staining. Therefore, immunofluorescent imaging using a signal amplification method was performed in normal, benign peritumoral, and cancer tissue to characterize the nuclear and cytoplasmic abundance of Smads 2, 3, and 4. Nuclei were stained blue. In normal and benign peritumoral tissue, an overlay of Smad 2 and 4 immunofluorescent images (green/blue overlay) indicated that nuclear colocalization of these two coregulators was low to moderate, whereas cytoplasmic Smad 2 and 4 levels (green) were moderate to high (data not shown). Consistent with the results described above, Smad 3 nuclear colocalization was moderate to high in normal and peritumoral tissue, whereas cytoplasmic Smad 3 abundance was low to moderate in these tissues (data not shown). Nuclear colocalization of all of the Smads was moderate to high in grades 1 and 2 cancers (Figs. 5 and 6; Fig. 7, A and D), and lower in grade 3 cancer (Figs. 5 and 6; Fig. 7G). Concomitantly, Smad 2, 3, and 4 cytoplasmic levels increased as tumor grade increased (Figs. 5 and 6; Fig. 7, B, E, and H).

**Correlation of Nuclear Smad 3 with Clinical and Biological Markers of Breast Cancer.** The presence of nuclear Smad 3 represents functional and activated activin or TGF-\( \beta \) cellular signaling. Once in the nucleus, Smad 3 acts through regulation of gene tran-

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**Fig. 1.** \( \beta \)A-subunit immunostaining in cancerous, peritumoral, and normal tissue. Tissue stained brown is positive for immunoreactivity. Nuclei are stained blue. A, grade 1 cancer. B, grade 2 cancer. C, grade 3 cancer. D, peritumoral tissue from grade 1 cancer. E, peritumoral tissue from grade 2 cancer. F, peritumoral tissue from grade 3 cancer. G, normal tissue. H, normal tissue control treated with secondary antirabbit IgG only. I, peritumoral tissue control treated with secondary antirabbit IgG only. J, grade 3 cancer tissue control treated with secondary antirabbit IgG only. Magnification is \( \times 40 \) for all panels. Scale bar in J, 500 \( \mu \)m. Arrows denote epithelial cell staining.

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**Fig. 2.** \( \beta \)B-subunit immunostaining in cancerous, peritumoral, and normal tissue. Tissue immunoreactivity, magnification, scale bar, and arrow are the same as described in Fig. 1 with exception of J. J, grade 1 cancer tissue control treated with secondary antirabbit IgG only.
The frequency of nuclear and cytoplasmic Smad 3 staining intensity was correlated with tumor stage, tumor grade, architectural grade alone, lymph node status, tumor size, the presence of lymphatic and vascular invasion, HER-2/neu expression, patient age, family history, and ER/PR status to better understand the relatedness of activated nuclear Smad 3 to known tumor markers (currently, average length of follow-up for all of the patients was 3 years; thus, survival data will be the subject of future long-term studies). Of those variables investigated, tumor grade, architectural grade, tumor size, and hormone receptor status significantly correlated to the presence and intensity of nuclear Smad 3 staining (Table 1).

Architectural grade, a subcomponent of overall tumor grade, reflects the percentage of cancer tissue that maintains a glandular phenotype, and this variable was considered independently. Architectural grade 1 cancers correlated with intense nuclear Smad 3 staining, (10 of 13; 77%). Architectural grade 2 cancers (11 of 17; 65%) and architectural grade 3 cancers (8 of 20; 40%) had less intense nuclear Smad 3 staining (P for trend/H11005 0.030). The abundance of cytoplasmic Smad 3 appeared to trend upward in cancer tissue as architectural grade increased but was not found to be significant. Conversely, patients with architecture grade 1 tumors had no intense cytoplasmic Smad 3 staining in their benign peritumoral tissue (0 of 14; 0%); whereas in architecture grade 2 patients (6 of 21; 29%) and architecture grade 3 patients (9 of 22; 41%), there was an increased frequency of intense cytoplasmic Smad 3 staining in the benign peritumoral tissue (P for trend = 0.008). Smad 3 nuclear staining in peritumoral tissue was abundant and did not change in conjunction with architectural grade.

Additionally, mean tumor size in 29 patients with intense nuclear Smad 3 in their cancer tissue was 1.34 cm, whereas the 21 patients with low or no nuclear Smad 3 staining in their tumors had a mean tumor size of 2.80 cm (P = 0.007). Lastly, those patients with ER+ tumors (27 of 35; 77%) showed a preponderance of intense nuclear Smad 3 staining when compared with ER− patients (2 of 15; 13%; P = <0.001). These findings were similar for PR+ patients where 22 of 29 (76%) had intense nuclear Smad 3 staining and only 7 of 21 (33%) PR− patients had intense nuclear Smad 3 staining (P = 0.004). No other clinical variables correlated significantly with Smad 3 staining intensity or distribution.

**DISCUSSION**

The goal of this study was to examine the presence and abundance of components of the activin signal transduction system in breast
cancer tissue, to understand the potential physiological role of activin in low, moderate, and high grade cancers. Correlations between the activin signal transduction proteins and known clinical and prognostic markers for breast cancer were also established. Tissue from healthy patients undergoing reduction mammoplasty and tissues from patients with breast cancer were examined. Benign ductal tissues were taken from within 2 cm of the histological edge of the carcinomas and were considered separately from infiltrating ductal cancers. Distinctions in activin signal transduction component localization and abundance were then made in normal tissues, nonmalignant peritumoral tissues, and carcinomas. Activin is known to have a growth inhibitory effect on normal and cancerous mammary epithelial cells, and a growth modulatory role in glandular organs formed through branching morphogenesis (33, 34). Consequently, we hypothesized that activin signal transduction, when disrupted or lost, would contribute to malignant progression.

Immunoreactivity was moderate to intense for the activin βA-subunit in normal tissue, peritumoral benign ducts, and cancer tissue of all three of the grades. βB-subunit immunoreactivity decreased as cancer grade increased. These findings indicate that the βA-βA ligand is likely to be abundant in all of the mammary tissues including cancer. Additionally, the abundance of βA-βB and βB-βB ligands may decrease as cancer becomes more aggressive. The relative roles of the different activin ligands in the maintenance of normal mammary gland integrity has yet to be determined, and assays for detecting dimeric βA-βB and βB-βB are being developed currently. Recently, Reis et al. (35) reported the presence of dimeric βA-βA in breast cancers and normal tissues, with elevated levels being produced in by cancer tissue. Yet, theoretically, if βA-βB or βB-βB were the dominant isoform mediating activin action in the mammary gland, a relative decrease in this ligand could be responsible for the apparent abrogation of Smad nuclear localization that is observed in the high-grade cancers. Low to undetectable levels of the α-subunit in all of the cancer tissues recapitulates the earlier findings of Di Loreto et al. (14); however, we did not observe the intense α-staining in normal tissue described in that work. We conclude from our findings that locally derived inhibins are not the primary mediators of activin inhibition in the normal or cancerous breast.

No tissue in our study showed complete absence of the activin receptors. Overall, type II activin receptor abundance was low in grade 3 cancers. ActRIB receptor expression was consistently moderate in normal and margin tissue, and present but decreased in cancerous tissue. Moderate receptor immunoreactivity in normal tissue, and grade 1 and 2 cancers correlates well to frequencies of Smad 3 nuclear intensity: 60% for the normal tissue, 78% for grade 1 cancers, and 63% for grade 2 cancers. Likewise, low amounts of activin receptor in high-grade cancer correlated well to a decrease in nuclear Smad 3 intensity found in grade 3 cancers (31%). The concomitant decrease in activin receptor levels and nuclear Smad 3 in grade 3 cancers may be indicative of a breakdown in activin signal transduction in these cancers. Accordingly, Gobbi et al. (30) has shown a correlation between loss of the TGF-β II receptor and high tumor grade in breast cancer. Also, recent work by Inman et al. (36) has described a system by which levels of activated nuclear Smads

### Table 1: Correlations for intense nuclear Smad 3 and clinical and pathological markers of breast cancer.

<table>
<thead>
<tr>
<th>Smad 3 data</th>
<th>Normal tissue</th>
<th>Grade 1 tissue</th>
<th>Grade 2 tissue</th>
<th>Grade 3 tissue</th>
<th>P differences</th>
<th>P trend</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intense nuclear Smad 3/peritumoral tissue</td>
<td>86%</td>
<td>100%</td>
<td>89%</td>
<td>0.361</td>
<td>0.72</td>
<td></td>
</tr>
<tr>
<td>Intense cytoplasmic Smad 3/peritumoral tissue</td>
<td>14%</td>
<td>24%</td>
<td>44%</td>
<td>0.1</td>
<td>0.059</td>
<td></td>
</tr>
<tr>
<td>Intense nuclear Smad 3/cancer</td>
<td>78%</td>
<td>63%</td>
<td>31%</td>
<td>0.023*</td>
<td>0.006*</td>
<td></td>
</tr>
<tr>
<td>Intense cytoplasmic Smad 3/cancer</td>
<td>22%</td>
<td>44%</td>
<td>50%</td>
<td>0.27</td>
<td>0.09</td>
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*Denotes P < 0.05 and is considered statistically significant.
correlate to the amounts of activated TGF-β receptor in the cell cytoplasm. Moreover, the duration of the activated Smad within the nucleus has been shown to be reflective of signal strength of the ligand (36). Pancreatic tumor cells with low levels of TGF-β receptor expression, and, thus, transient nuclear Smad activation, are resistant to TGF-β growth arrest, whereas cells with high levels of receptor and normal duration of nuclear Smad activation retained their ligand sensitivity (36). Therefore, the decrease in activin receptor level and decrease in nuclear Smad 3 detected in grade 3 breast cancers implies a potential insensitivity to the growth suppressant action of activin (or TGF-β) in this high-grade cancer.

Other mechanisms may be responsible for the decrease in nuclear Smads that occur in high-grade cancer in this study. Although Smad complexes bind DNA directly, primarily through Smad 4, Smad transcriptional activity depends on cooperation with cytoplasmic and DNA cofactors that determine tissue specificity of activin and TGF-β responses (6, 11). Several coactivators and corepressors of Smad signaling have been identified, and these coregulatory molecules may be intimately associated with the actions of the TGF-β superfamily in breast cancer. Oncogenic Ras interferes with the nuclear activation of Smad 2 and 3 through extracellular signal-regulated kinase mitogen-activated protein kinases in mammary epithelial cells (37, 38). The oncoproteins Ski and SnoN modulate Smad function through a transcriptional corepressor N-CoR (39, 40). The oncoprotein Evi-1 interacts with the nuclear Smad3/4 complex to repress Smad 3 interaction with its cognate Smad binding element (41). Moreover, cytoplasmic Smad 3 cofactors E2F4/5 and p107 act in concert with Smad 4 to bind a Smad-E2F site on c-myc. This transcription complex represses the cell cycle mitogen, c-myc, suggesting that disruption or loss of any components within this complex could lead to the loss of cell cycle control detected in aggressive cancers (42, 43). Increased amounts of oncogenic Ras have been shown to correlate to high tumor grade (38, 44). Possibly, elevated levels of oncogenic Ras in the grade 3 tumors in our study are responsible for the lower levels of activated nuclear Smads in those cases. Lastly I-Smad 6 and 7 overexpression in high-grade breast cancer patients has not been examined (38). The description of the I-Smads and Smad cofactors in breast cancer will be the focus of additional investigation in our laboratory.

The activins and TGF-β have a role in the maintenance of the branching phenotype in glandular organs. In Drosophila, the TGF-β signaling pathway aids in the regulation of the branched tracheal pattern (45). Activins play a dynamic role in the branching morphogenesis of several glandular organs including the mammary gland, kidney, lung, and prostate (33). In the prostate, in vitro evidence points toward a role for activin as an inhibitor of branching morphogenesis in a complex developmental process that requires the interplay of several hormones and growth factors (46). We have described previously the presence of the activin signaling components in the mammary gland during the lactational phase, which may enable the additional development of lobuloalveoli while preventing hyperproliferative changes in this tissue (24). In the current study, nuclear Smad 3 immunoreactivity was less intense as pathologic tumor grade and architectural grade increased. Breast cancers change dramatically from a tubular phenotype, reminiscent of the normal ductal branched pattern, in lower grade lesions, to a sheet-like phenotype, devoid of ductal structures, as cancers become histologically more aggressive. It is possible that a decrease in or insensitivity to activin signaling in high-grade cancer contributes to the morphological change seen in more dedifferentiated malignancy. Theoretically, morphological changes that diverge from the normal ductal phenotype in cancer might involve negative alterations in cell-cell interactions, including contact inhibition. Additionally, cytoplasmic Smad 3 staining increased in peritumoral tissue as cancer architectural grade increased, whereas these tissues also maintained the highest abundance of nuclear Smad 3 intensity. Separate evaluation of the subcomponents of the Scarff-Bloom-Richardson tumor grading system showed that survival was significantly better in tumors with a score of 1 or 2 for tubule formation (47). More aggressive tumors may influence ligand action and Smad 3 trafficking in peritumoral tissue, and consequently alter the antiproliferative action of activin in these cells. Compromise of the surrounding peritumoral tissue might have additional implications regarding metastatic spread, and thus survival.
In addition to their effects on branching morphogenesis, activin and TGF-β have effects on general growth control. Activin has been associated with the inhibition of growth in breast cancer cells and of epithelial branching (15, 33, 34, 46). TGF-β has been associated with growth arrest and apoptosis in breast cancer-derived MCF-7 cells, mediated through TNF-α in an autocrine fashion (48, 49). Additionally, the TGF-β ligand has a primary role in the apoptosis of early involution in the mouse mammary gland (26, 50). In our study, mean tumor size in patients with moderate to intense nuclear Smad 3 in their cancer tissue was 1.46 cm smaller than those patients with low or no nuclear Smad 3 staining in their tumors. This correlation between nuclear Smad 3 and smaller tumor size implicates activated nuclear Smad 3, by activin or TGF-β, in the control of tumor cell proliferation. Tumor size is one of the most powerful predictors of breast cancer prognosis, with smaller tumors having a more favorable outcome (51, 52). Nuclear Smad 3 intensity showed an independently significant correlation with smaller tumor size in this study, and, so, may also have favorable prognostic significance.

Patients with ER+/PR+ tumors showed a preponderance of intense nuclear Smad 3 staining when compared with ER−/PR− patients. Hormone receptor status in breast cancer is a short-term prognostic factor, appearing to have clinical significance during the first 5 years of survival (53, 54). ER+ breast cancer cells commonly show resistance to TGF-β, and this resistance has been attributed to the loss or lack of expression of TGFβRII (55). Reintroduction of TGF-β receptors in these cells was shown to lead to reversion of malignancy (56). As the preponderance of ER+ cancers in this study were found to have activin receptor immunoreactivity, activated nuclear Smad 3 activity, and ER+ cancers are thought to be insensitive to TGF-β, it follows that activin may be of greater importance for Smad 3 signaling actions in ER+ breast cancer cells than TGF-β. In contrast, ER− breast cancers show significant disparity in their responsiveness to both activin and TGF-β. This has been attributed to deregulation of both TGFβ and activin at the receptor and Smad level. It follows that the ER− higher grade breast cancers in this study had significantly lower activated nuclear Smad activity.

A large study examined recently the presence of Smad 2, Smad 2P, and Smad 4 proteins in breast cancer (23). All of the cases studied were positive for cytoplasmic Smad 2. Smad 2P was absent in 6.6% of cases and correlated to a worsened prognosis in stage II breast cancer patients. Two percent of cases lacked expression of Smad 4, and this correlated to the presence of lymph node metastasis. Those cases that lacked Smad 2P were additionally examined and found to have no TGF-β receptor mutations. In these cases, failure to activate Smad 2 may be secondary to the down-regulation of activin βB-subunit expression, decreased activin receptor expression, or deregulation of Smad cofactors. This previous work supports the idea that derangement of Smad signaling enhances breast cancer progression, as lack of R-Smad 2 activation had negative implications on survival in stage II patients. Our work additionally supports this hypothesis in that modulation of activin signaling component abundance and abrogation of nuclear R-Smad 3 correlated to high grade breast cancer, high architectural grade cancer, increased tumor size, and ER/PR negativity.

This study describes the presence of activin ligand subunits, receptors, and Smads in normal, peritumoral, and cancerous epithelial cells, indicating that activin is capable of transducing a signal in these tissues. Whereas βA−βA, βB−βB, or βA−βB may have a role in the initiation of activin-dependent gene regulation, the independent significance of relative increases or decreases in these ligands in mammary physiology has yet to be elucidated. Activin receptors were present in all of the tissue studied, yet these receptors were down-regulated in grade 3 breast cancer. Decreased receptor abundance may be associated with the attenuated level of activated nuclear Smads that occurred in high-grade cancers. Furthermore, the decrease in activated nuclear Smad 3 in high-grade cancers may impact tumor growth, as tumors with no or low nuclear Smad 3 were significantly larger than those of which the Smad 3 activation was intact. Other studies have shown ER+ tumors to be largely resistant to TGF-β, whereas abundant amounts of nuclear Smad 3 were found in the ER+ tumors included in this study. Thus, it is likely that activin has an independent growth regulatory role in ER+ breast cancers. Finally, activated nuclear Smad 3 may be important for the maintenance of less aggressive phenotypes of breast cancer, which could have additional implications on tumor cell-cell interactions, as well as tumor interactions with surrounding breast tissue. Future studies will aim to rescue activin function in high-grade breast cancer tissues to determine how this crucial signaling pathway acts in breast cancer onset and progression.

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