Smad4 as a Transcription Corepressor for Estrogen Receptor α*

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Antiestrogen compounds exhibit a variety of different effects in different tissues and are widely used for the treatment of osteoporosis, breast cancer, and other diseases. Upon examining the molecular mechanisms, we found that Smad4, a common signal transducer in the bone morphogenetic protein (BMP)/transforming growth factor-β (TGF-β) signaling pathway, functions as a transcription corepressor for human estrogen receptor α (ERα). Endogenous ERα was co-immunoprecipitated with Smad4, and the interaction was induced by antiestrogen ligands such as tamoxifen, raloxifene, and droloxifen, which was confirmed in chromatin immunoprecipitation assays. Smad4 and ERα form a complex when ERα binds to the estrogen-responsive element within the estrogen target gene promoter. Importantly, the expression of Smad4 inhibits both antiestrogen-induced luciferase activity and estrogen downstream target gene transcription in breast cancer cells. Mapping of the interaction domains indicates that the activation function 1 (AF1) domain of ERα is essential for its interaction with Smad4, while the MH1 domain and linker region of Smad4 are essential for the interaction. Our findings represent a novel mechanism that TGF-β may regulate cell fate through Smad4-mediated cross-talk with estrogen.

Estrogen regulates cell proliferation, differentiation, motility, and apoptosis in a variety of cell types (1, 2). The effects of estrogen, including breast cancer, are mediated through its binding to estrogen receptors (3). The estrogen receptor (ER)1 of which two isoforms (α and β) have been identified, are members of the steroid/thyroid hormone superfamily of nuclear receptors (4–8). ERα and ERβ display distinct expression pattern and biological functions in different tissues (9). Both of the two receptors are ligand-dependent transcriptional activators and regulate gene transcription either by binding directly to the estrogen-responsive element with a consensus sequence of 5′-GGTCAANNTGACC-3′ (10) or interacting with other transcription factors such as Sp1 and AP1 (10). Estrogen receptors recruit coactivators and corepressors in the regulation of gene transcription. Numerous coactivators have been shown to be associated with activated ER, such as CBP/p300 (11–13), SRC-1 (14, 15), TIF2 (16), GRIP1 (17, 18), TIF1 (19, 20), and RIP140 (21, 22). Fewer corepressors have been reported to date, among which SMRT (23, 24) and N-CoR (25, 26) are the important ones. These cofactors may function as molecular gates to enable integration of diverse signal transduction pathways at nuclear receptor-regulated promoters (27).

A relatively new class of synthetic drugs known as selective estrogen receptor modulators or SERMs are currently in use for treatment of osteoporosis, breast cancer, and other hormone-dependent medical disorders (3). SERMs exhibit a wide range of estrogen-like and antiestrogen actions based on the target tissue being studied (28). For example, tamoxifen is an ER antagonist in breast tissue (29) but an ER agonist in bone (30) and uterus (31) tissue. Raloxifene, a compound related to tamoxifen, is also an ER antagonist in breast tissue; however, it exerts agonist activity in bone but not uterine tissue (28). It has been believed that novel and as yet unidentified cofactors bind to the antiestrogen-modulated ER and are responsible for the observed tissue-specific activity (10).

In contrast to estrogen promotion of the development of breast cancer, transforming growth factor β (TGF-β) is known to inhibit the growth of mammary epithelial cells and may play a protective role in mammary carcinogenesis (32). TGF-β is the prototypic autocrine or paracrine inhibitor of cell cycle progression in epithelial cells and appears to directly antagonize the effects of many different mitogenic growth factors (33, 34). Loss of responsiveness to TGF-β is believed to be a major factor in tumor formation (35–43). Specific defects in TGF-β receptors (e.g. TGF-β-related signal transduction/gene activation) and TGF-β-regulated cell cycle proteins have been implicated in oncogenesis (35–43). Alterations of TGF-β signal components have occurred in some breast cancer cell lines, and these may contribute to tumor formation and proliferation (44–47). Additionally, inhibition of breast cancer cell growth by tamoxifen is mediated by TGF-β (44–48). TGF-β inhibition of ERα transcription expression occurs within 3 h in MCF-7 breast cancer cells (49). These data suggest that cross-talk between estrogen receptors and TGF-β signaling is critical in breast cancer development.

The signaling responses to TGF-βs are mediated by their intracellular substrates, the Smad proteins (34, 50). Upon ligand binding, the activated receptors directly phosphorylate regulatory Smads (R-Smads). Phosphorylated R-Smads interact with Smad4, and the complex translocates into the nucleus to regulate gene transcription. Smad4 is the common signaling

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§ The abbreviations used are: ER, estrogen receptor; EBAG, ER binding fragment-associated antigen 9; TGF-β, transforming growth factor-β; SERM, selective estrogen receptor modulator; ERE, estrogen-response element; Tam, tamoxifen; Ral, raloxifene; Dro, droloxifen; E2, β-estradiol; AF1, activation function 1; DBD, DNA-binding domain; MH1, Mad homology 1; R-Smad, regulatory Smad; HA, hemagglutinin; CATD, cathepsin D.

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molecule shared in the TGF-\(\beta\) superfamily. Smad4/DPC4 is also known as a tumor suppressor (37), mutations of which are frequently found in human cancers. Smad4 mutations account for half of pancreas cancers (37, 38) and more than 30% of invasive metastatic colorectal cancers (39) and other tumors. Oncogene Jab1 mediates the degradation of Smad4 (51). It has been shown that Smad1, Smad2, Smad3, and Smad4 all have physical interactions with ER\(\alpha\) and might be important for TGF-\(\beta\) regulation of ER\(\alpha\) signaling (52, 53). Among these Smads, Smad4 and Smad3 have also been found in another signaling system, the estrogen receptor, and androgen receptor-mediated signaling and important functions have been suggested (54). However, the possible significance of Smad4 in estrogen signaling has not been well assessed, and the detailed mechanism of the cross-talk is not clear.

In this study, we examined the potential role of Smad4 in estrogen signaling. We demonstrate that Smad4 interacts with ER\(\alpha\) in vivo and represses estrogen-induced transcriptional activity mediated through the ERE. Smad4 is incorporated in the ERE-DNA complex and might recruit other corepressors to the complex. Finally, we show that Smad4 strongly inhibits the transcription of various downstream genes of estrogen signaling. These findings suggest that TGF-\(\beta\) may regulate cell fate through Smad4-mediated cross-talk with estrogen and may provide a molecular basis to explain the wide range of SERM-mediated, tissue-selective effects in breast cancer patients.

**EXPERIMENTAL PROCEDURES**

**Constructs and Cell Culture**—In the yeast two-hybrid assay, we cloned the full-length Smad4 coding sequence into pGBT9 (Clontech) to generate the pGBT9-Smad4 bait plasmid in which Smad4 is fused with the GAL4 DNA binding domain. Likewise, the human ER\(\alpha\) was cloned into pACT2 (Clontech) and fused with the VP16 activation domain (pACT2-hER\(\alpha\)). The HA-tagged human ER\(\alpha\) expression plasmid was cloned into a pCDNA3 vector linked with HA at the amino terminus by PCR using pRST7-ER\(\alpha\) (a gift from the National Institutes of Health) as the template. The AF1 (E2), AF1 + DNA binding domain (DBD) (E1), DBD + AF2 (E3), and AF2 (E4) domains of ER\(\alpha\) were cloned into the same pCDNA3 vector by PCR and restriction enzyme digestion in BamHI and XhoI sites. An estrogen-response element-containing reporter (3x ERE-TATA) was a gift from Dr. Valerie Clack (Duke University Medical Center, Durham, NC). The FLAG-tagged Smad4 expression plasmid was cloned into a pCDNA3 vector linked with HA at the amino terminus by PCR using pRST7-ER\(\alpha\) (a gift from the National Institutes of Health) as the template. The AF1 (E2), AF1 + DNA binding domain (DBD) (E1), DBD + AF2 (E3), and AF2 (E4) domains of ER\(\alpha\) were cloned into the same pCDNA3 vector by PCR and restriction enzyme digestion in BamHI and XhoI sites. An estrogen-response element-containing reporter (3x ERE-TATA) was a gift from Dr. Valerie Clack (Duke University Medical Center, Durham, NC).

**Transfection and Luciferase Assay**—To identify the function of Smad4 on estrogen response element, 2x ERE-TATA was used as the reporter plasmid. MCF-7 cells were split and plated at 5 x 10\(^6\) cells per 100-mm dish and starved with RPMI 1640 phenol red-free medium supplemented with 10% charcoal-stripped fetal bovine serum (Cellgro) for 24 h. The cells were transfected using Tfx-50 with 0.2 \(\mu\)g of luciferase reporter and 20 ng of the hER\(\alpha\) expression plasmid. 50 ng of Smad4 expression plasmid or empty vector was also cotransfected. Twenty-four hours after transfection, the aliquots of cells were treated with one of the ligands, i.e. E2, Tam, Ral, Dro, and TGF-\(\beta\) or vehicle. Luciferase activities were assayed 48 h after transfection using the Dual-Luciferase\(\textsuperscript{TM}\) assay kit (Promega) according to the manufacturer’s instructions. Firefly luciferase activity was assayed and normalized against Renilla luciferase activity. Luciferase values shown in the figure are representative of transfection experiments performed in triplicate from at least three independent experiments. The standard deviations are shown in vertical lines.

**Gel-shift Assay**—The expression plasmids of human ER\(\alpha\) and Smad4 were cotransfected into COS-1 cells. Forty-eight hours after the transfection, the transfected cell lysate and an aliquot of non-transfected cell lysate were collected and incubated with radiolabeled ERE, which is the estrogen-response element. The radio-labeled DNA oligomers were labeled by a kinase reaction with a DNA polymerase I (Klenow) fragment and \(\alpha\)-\[^{32}P\]dCTP. Binding reactions were preincubated for 20 min at 22 \(^\circ\)C with indicated lysates in 75 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 10 mM Tris-HCl, pH 7.5, 6% of bovine serum albumin, and 25 ng of poly(I/dC) in a volume of 19 \(\mu\)L. One microliter of DNA probe (0.5 ng, 50,000–100,000 cpm) was added to each of the reactions. The reactions were subjected to nondenaturing electrophoresis on a 4% polyacrylamide gel.

**Chromatin Immunoprecipitation (ChIP) Assay**—MCF-7 cells were grown to 95% confluences in phenol red-free Dulbecco’s modified Eagle medium supplemented with 10% charcoal-dextran-stripped fetal bovine serum for at least 5 days. Following the starvation for 3 h, cells were washed and lysed according to the protocol from Dr. Myles Brown (Harvard Medical School, Boston, MA). Immunoprecipitation was performed overnight at 4 \(^\circ\)C with specific antibodies against ER\(\alpha\) or Smad4. The precipitates were extracted and heated as described (55). For PCR, (the sequences of specific primers were given as a gift from Dr. Myles Brown) 1 \(\mu\)L from a 50-\(\mu\)L DNA extraction and 21–25 cycles of amplification were used.

**Principle of Real Time PCR**—A fluorescence signal from each real time quantitative PCR reaction (Applied Biosystems, Foster City, CA) is collected as normalized values plotted versus the cycle number. Reactions are characterized by comparing threshold cycle (C(t)) values. The threshold cycle is a value defined in a competitive PCR reaction in which the sample fluorescence signal passes a fixed threshold above base. Quantitative values are obtained from the C(t) numbers at which the increase in signal associated with an exponential growth of PCR product starts to be detected (Applied Biosystems) according to the manufacturer’s manual. The \(\beta\)-actin gene is used as a control for the endogenous RNA transcripts, and each mRNA is normalized to \(\beta\)-actin content. The final result is expressed as relative fold by comparing the amount of RNA of target gene to the \(\beta\)-actin gene, which is determined by the equation 2^(-DeltaCt/Tar/get-DeltaCt/\(\beta\)-actin).

**Oligonucleotide Primers Design**—The target cDNA sequence was evaluated using Primer3 (www.genome.wi.mit.edu/cgi-bin/primer/ primer3_www.cgi). The sequences of primers are shown in Table 2. The sequences of primers are shown in Table 2.
The amplification reactions were performed in 25 μl containing 1/100 M MgCl₂ when both ER and Smad4 were cloned into the pGBT9 plasmid. The forward and reverse primers were designed to flank at least one intron to prevent amplification of genomic DNA. The forward primer for ERα was 5′-TCG CTT CAC TCC TGG-3′ and the reverse primer was 5′-TTA ATT TCC GTC CTC TGC-3′. The forward and reverse primers were designed to flank at least one intron to prevent amplification of genomic DNA. The forward primer for ERα was 5′-TCG CTT CAC TCC TGG-3′ and the reverse primer was 5′-TTA ATT TCC GTC CTC TGC-3′. The forward primer for ERα was 5′-TCG CTT CAC TCC TGG-3′ and the reverse primer was 5′-TTA ATT TCC GTC CTC TGC-3′.

**Results**

**Smad4 Interacts with ERα in Vivo**—To study the potential cross-talk between estrogen receptor and Smads, we first examined the interaction between Smad4 and ERα in a yeast two-hybrid system because Smad4/Endogenesin DPC4 has been identified as a candidate tumor suppressor (37). Human ERα was cloned into the pACT2 plasmid, and human Smad4 was cloned into the pGAT9 plasmid. β-galactosidase activity was stimulated by coexpression of both ERα and Smad4 (Fig. 1A), indicating Smad4 interaction with ERα in yeast. To examine the physical interaction in mammalian cells, we performed a coimmunoprecipitation experiment in COS-1 cells. HA-tagged ERα and FLAG-tagged Smad4 were cotransfected into COS-1 cells treated with E₂ (lane 4), Tam (lane 5), Ral (lane 6), Dro (lane 7) or ethanol (EtOH) vehicle (control) (lanes 1–3). Cell lysates were immunoprecipitated by anti-FLAG M2 monoclonal antibody 48 h after transfection. The precipitates were then immunoblotted with anti-HA polyclonal antibody. The result was confirmed in panel C by immunoprecipitation with anti-HA polyclonal antibody and immunoblotting with anti-FLAG monoclonal antibody.

To further confirm the endogenous protein interaction between Smad4 and ERα in breast cancer cells, co-immunoprecipitation assays were performed with MCF-7 cells and T47D cells. The aliquot of cells was treated with one of the ligands as indicated in Fig. 2. The whole cell lysates were immunoprecipitated with anti-Smad4 monoclonal antibody, and the precipitates were immunoblotted with an anti-ERα polyclonal antibody. The endogenous ERα and Smad4 were coimmunoprecipitated in MCF-7 cells as expected (Fig. 2A, lane 2), indicating the interaction between these two endogenous proteins. Moreover, the interaction was enhanced to different levels with the treatment of different antiestrogens (Fig. 2A, lanes 4–6). Unlike the case in MCF-7 cells, however, the endogenous interaction was not detectable in T47D cells even with E₂ treatment (Fig. 2B, lanes 3–4), which can be due to the lower expression level of endogenous Smad4 proteins in T47D cells. Interestingly, only antiestrogen ligands induced the interaction (Fig. 2B, lanes 5–7). Taken together, these results suggest that the interaction between endogenous Smad4 and ERα proteins are differentially regulated by SERMs.
Smad4 Represses Transactivation by Estrogen—To examine the function of the interaction between Smad4 and ERα, a luciferase reporter plasmid bearing two repeats of ERE (2×ERE-TATA) was cotransfected into MCF-7 cells with ERα. The aliquots of cells were treated with 17β-estradiol (E2), TGF-β1, or both. Fig. 3A shows that TGF-β1 inhibited E2-induced transcription activity as expected. The ectopic expression of Smad4 exhibited a stronger inhibition of E2-induced luciferase activity in comparison with TGF-β1. The inhibition was further demonstrated in a dose-dependent manner (Fig. 3B). Because different SERMs exhibit tissue-specific transcription activity, the antiestrogen compounds tamoxifen, raloxifene, and droloxifen were also examined. As expected, binding of these ligands to ERα did not result in luciferase activation. In the presence of Smad4, however, the transcription levels were suppressed by these antiestrogen ligands (Fig. 3C). Raloxifene appears to be the most potent inhibitor of luciferase activity, exhibiting 3-fold higher levels of inhibition than the control vehicle. Consistent with previous data that SERMs can affect the interaction between Smad4 and ERα, these results indicate that Smad4 acts as an ERα transcription corepressor, and its repression activity is differentially regulated by different SERMs. Similar results were obtained in T47D cells (data not shown).

Previous reports showed that ER-mediated transcriptional activation can be enhanced by Smad3 (52, 53) by physical interaction between Smad3 and ERα. Because TGF-β induces the formation of a Smad3 and Smad4 complex (58), we examined the effect of Smad4 on Smad3-enhanced ERα transactivation in MCF-7 cells. As shown in Fig. 3D, Smad3 increased E2 activity on ERα transcription in the presence of TGF-β1. The addition of Smad4, however, can repress the Smad3-enhanced ERα transactivation in a dose-dependent manner. It is also noteworthy that the amounts of Smad4 used in this assay are significantly lower than that of Smad3. Together, the results from luciferase assays indicate that Smad4 is a corepressor of ERα and suppresses Smad3-mediated ERα transactivation.

Smad4 Forms a Complex with ERα on DNA Element—Because ERα can regulate gene transcription via direct binding to the ERE on the target gene promoter, we tested whether Smad4 and ERα form a complex on ERE using gel-shift assays. Smad4 and ERα expression plasmids were cotransfected into COS-1 cells, and the complex of Smad4-ERα formed a shift band (Fig. 4A, lane 3) when compared with non-transfected COS-1 cell lysate (Fig. 4A, lane 2). The addition of antibodies against ERα or Smad4 supershifted the complex (Fig. 4A, lanes 4 and 5), confirming that the complex contains Smad4 and ERα. This result argues against the idea that Smad4 interferes with the DNA binding of ERα. Genes regulated through direct ER binding include cathepsin D (CATD) (59) and ER binding fragment-associated antigen 9 (EBAG9) (50, 60). To confirm Smad4-ERα-DNA complex formation in vivo, we applied a chromatin immunoprecipitation assay (55) with the promoter region of CATD containing ERE (55). MCF-7 cells were grown in the absence of estrogen for at least 3 days followed by treatment with vehicle or different ligands. The endogenous transcription complex present on the ERE region of the promoter was determined by immunoprecipitation using antibodies against Smad4 or ERα, followed by semi-quantitative reverse transcriptions PCR with specific pairs of primers spanning the ERE region. As shown in Fig. 4B, the occupancy by both ERα and Smad4 indicates the formation of the Smad4-ERα complex on the CATD gene promoter. Treatment with antiestrogen ligands increased the occupancy, which is consistent with our previous finding that SERMs regulate Smad4 interaction with ERα and the repression activity of Smad4 on estrogen signaling.

**Interaction Domains of Smad4 and ERα**—We mapped the interaction domain(s) of both Smad4 and ERα. ERα has A to F domains from the NHL terminus to COOH terminus containing two important activation function (AF) domains, AF1 and AF2 (61–63). A series of truncated ERα constructs in HA-tagged pCDNA3 vector have been cloned, with deletion of either the AF1 or the AF2 domain as shown in Fig. 5A. HA-tagged ERα (E) or one of its four deletion mutants was cotransfected into COS-1 cells with FLAG-tagged Smad4. The immunoprecipitation was performed with either anti-FLAG antibody or anti-HA antibody, followed by immunoblotting with anti-HA or anti-FLAG antibody, respectively. It has been shown in Fig. 5B that the mutants E3 and E4 in which AF1 domain is deleted were unable to bind Smad4, whereas E1 and E2 lacking either AF2 or AF2 + DBD retained the interaction. These results indicate that AF1 domain is essential for the interaction of ERα with Smad4, whereas the AF2 domain or the DBD is not required.

We next determined the domain(s) of Smad4 which mediate(s) the interaction with ERα, using MH1, linker (L), MH1 + linker (m1), MH2 + linker (m2), or MH2 (m3) truncated fragments of Smad4 (Fig. 6A). FLAG-tagged Smad4 (S4) or one of its five fragments was cotransfected into COS-1 cells with ERα. Cells were lysed and subjected to immunoprecipitation with anti-FLAG antibody, followed by immunoblotting with anti-HA antibody. As indicated in Fig. 6B, the MH1 domain and linker region were both sufficient to interact with ERα, whereas the MH2 domain was unable to bind ERα. As expected, the MH2 domain could not inhibit E2-induced transacti

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**Fig. 2. Smad4 interacts with ERα in breast cancer cells.** T47D cells (A), or MCF-7 cells (B), were treated with E2 (lane 4), Tam (lane 5), Ral (lane 6), Dro (lane 7) or vehicle (lanes 1–3). Cell lysates were immunoprecipitated by an anti-Smad4 monoclonal antibody (Smad4) (lanes 1 and 3–7) or pre-immunized antibody (Pre) (lane 2) (control) 48 h after transfection. The precipitates were then immunoblotted with an anti-hERα polyclonal antibody.
FIG. 3. Effect of Smad4 on ERα-mediated transcriptional activity. A luciferase reporter plasmid containing estrogen response element (2×ERE-TATA) was cotransfected with human ERα into the MCF-7 cells. A, the effect of Smad4 on ERα transactivation by estrogen was examined. The aliquot of cells was treated with E2, TGF-β1, E2, and TGF-β1 or ethanol vehicle (control). The values represent artificial units of luciferase activity after normalization. B, increased amounts of Smad4 were cotransfected, and luciferase activity was assayed. C, the effect of Smad4 on ligand-mediated ERα transactivation was examined. The MCF-7 cells were cotransfected with 2×ERE-TATA reporter, ERα(open bar), ERα and Smad4(closed bar). The aliquot of cells was treated with one of the four ligands, i.e. E2, Tam, Ral, Dro or vehicle. Luciferase activity was assayed and compared. D, the effect of Smad4 on Smad3-enhanced ERα transactivation was examined. Increased amounts of Smad4 were cotransfected with fixed amount of Smad3. The aliquot of cells was treated with E2 and TGF-β1. Luciferase activity was assayed and analyzed as described.

FIG. 4. Smad4 forms a complex with ERα on DNA. A, Smad4 and ERα were cotransfected into COS-1 cells, and the cell lysates were collected and incubated with radiolabeled ERE as a probe in the presence or absence of anti-hERα(lane 4) and/or anti-Smad4(lane 5) antibodies as indicated. The Smad4/hERα complexes are indicated in lanes 3–5. B, MCF-7 cells were starved for 3 days and treated with different ligands (E2, Tam, and Ral) for 3 h. The cell lysates were collected and immunoprecipitated with anti-ERα and anti-Smad4 monoclonal antibodies, respectively. Semi-quantitative reverse transcription PCR was performed with the precipitates and specific primers for the CATD promoter region as indicated.
MH1 domain and linker region retained the ability of inhibition (Fig. 6C).

Smad4 Inhibits Downstream Gene Expression of Estrogen Signaling—To further confirm the repression effects of Smad4 on ER transactivation, we applied real time PCR to examine the mRNA expression level of the ER target gene CATD and EBAG9. T47D cells were transfected with Smad4 expression plasmids or empty vector as a control. The aliquot of cells was treated with 100 nM E2 for 24 h before RNA extraction and PCR assays. As Fig. 7 shows, E2 can induce the mRNA expression of both CATD (Fig. 7A) and EBAG9 (Fig. 7B). Ectopic Smad4 decreases the E2 induction of both genes without changing their basal expression levels, confirming that Smad4 can suppress estrogen signaling in breast cancer cells.

DISCUSSION

Estrogen regulates cell proliferation, differentiation, motility, and apoptosis in a variety of different cell types. It plays important roles not only in normal mammary gland cells but also in breast cancer cells. Recent studies indicate that various ligands, including SERMs, induce distinct conformational changes in the ER (64, 65). These changes in ER may, in turn, alter the interactions of the receptor with cell- and tissue-specific coactivating or corepressing transcription factors (4, 10). Here we have identified Smad4 as an ERα corepressor and have found that antiestrogens enhance the endogenous interactions between Smad4 and ERα. This finding provides further evidence to support the belief that ER conformational changes regulate the interaction of ER with its cofactors and may also help to explain the diversity of estrogen biological activity.

Smad4 is a common partner for the signaling molecules in the TGF-β pathway. Identification of Smad4 as an ERα corepressor provides a mechanism of cross-talk between TGF-β and estrogen. Consistent with the previous observation (52), our data also demonstrated that Smad3 enhanced ER-mediated transactivation. Interestingly, Smad4 can reverse Smad3-enhanced gene transcription. Both Smad3 and Smad4 can interact with ERα independently. It appears that Smad3 alone activates ER-mediated gene transcription, whereas TGF-β or the Smad3 and Smad4 complex inhibit ER-mediated transcription. As we know, TGF-β induces Smad3 phosphorylation, which forms a complex with Smad4 and translocates into the nucleus in regulation of gene transcription. So our results indicate that, in the presence of Smad4, TGF-β inhibits ER-induced transactivation. When Smad4 is mutated or deleted in cancer cells, TGF-β is no longer able to inhibit ER-induced gene transcription and likely activates gene transcription instead. If the findings are confirmed to be true in clinical samples, Smad4 may be used for a therapeutic purpose.

As our data have shown, antiestrogens can induce the interaction between endogenous Smad4 and ERα in T47D cells, which have a low abundance of Smad3 mRNA (45), suggesting that Smad4 and ERα interaction is not likely to be dependent on Smad3. The MH2 domain of Smad proteins are known for their interactions with other proteins (67–69). However, our mapping data show that the Smad4 MH2 domain is not involved in binding with ERα. Because the MH2 domain of Smad3 is required for efficient physical and functional interaction with ERα (52,53), and the MH2 domains mediate homomeric and heteromeric complex formation (67–69), it is possible that the interaction of Smad3 with ERα is mediated through
Smad4. Furthermore, Smad4 could also disrupt the interaction between Smad3 and ERα by competing with the Smad3 MH2 domain binding site of ERα. In their recent work, Kang et al. (54) have also demonstrated that Smad4 can decrease the AR-Smad3 interaction and repress the Smad3-enhanced AR transactivation. Nevertheless, there are many important questions that remain to be addressed relevant to other R-Smads such as Smad2 and Smad1 in bone morphogenetic protein (BMP) signaling.

The AF1 and AF2 domains are two important activation functional domains of ER. AF1, which is localized in the NH2-terminal A/B region, is believed to be constitutive in a cell- and promoter-specific manner (72), whereas AF2 resides in the COOH-terminal ligand-binding domain (LBD) (region E) and
exerts estrogen-dependent transcriptional activity by recruiting coactivators. These two domains have been tested frequently in studying the regulation of ER function. In mapping the ERα interaction domains with Smad4, our data demonstrate that ERα N-terminal AF1 is sufficient to mediate ERα interaction with Smad4. Several lines of evidence indicate that ERα AF1 domain is under negative regulation. Corepressors such as N-CoR bind to the tamoxifen-ligated ERα and inhibit AF1 activity in which the mechanism remains unknown (25, 26). It is also known that SERM agonist effects stem from ERα AF1, which directly contacts coactivators. Interestingly, tamoxifen-ligated ERα enhances the activity of the progesterone receptor AF1, presumably by sequestration of active repressor complex, and overexpression of N-CoR reverses this effect. It is clear that SERMs inhibit ERα action both by blocking coactivator recruitment and promoting corepressor recruitment. Interaction of Smad4 with the ERα AF1 domain provides evidence to explain SERM-ligated ERα transcription activity. The SERMs do not behave the same in the presence of ERβ (9), however. ERβ also inhibits ERα-dependent estrogen and tamoxifen responses in heterodimers, and it is believed that ERβ homodimers respond to SERMs differently.

We have examined potential interaction between ERβ and Smad4 in similar co-immunoprecipitation and gel shift assays. In comparison to ERα, the interaction was not detectable, and Smad4 could not form a complex with ERβ on DNA (data not shown). ERβ appears unresponsive to Smad4-mediated transcription repression induced by tamoxifen (data not shown). Tamoxifen produces objective tumor shrinkage in advanced breast cancer and reduces the risk of relapse in women treated for invasive breast cancer (71). Clinically, breast cancer patients with ERα tumors will initially benefit from tamoxifen treatment. However, most of the patients eventually develop tamoxifen resistance, the mechanism of which is not well understood, although several possible mechanisms have been proposed (66, 70). It has been shown that ERβ mRNA expression in primary breast tumors is up-regulated in tamoxifen-resistant breast cancer patients (57). Clinical studies show that the beneficial response of breast cancer to tamoxifen is related to ERα, and ERβ-positive cells are associated to elevated levels of the proliferation markers Ki67 and cyclin A (56). Because the resistance of breast cancer to tamoxifen therapy is correlated to the presence of ERβ (56, 57), it is likely that tamoxifen specifically induces the interaction of ERα, instead of ERβ, with some tumor suppressors such as Smad4. Smad4 is able to regulate ERβ-mediated transcription activity by interacting with the other partner (i.e., ERα) of the heterodimer. However, with the increased ratio of expression level of ERβ/ERα, the effect of Smad4 on ER could be decreased; thus, the tamoxifen effect on transcription repression would also be abolished. Likewise, the effects of Smad4 on antiestrogen-mediated transcription can also be variable in cells with a different ratio of ERα/ERβ.

Taken together, we demonstrated an in vivo interaction between Smad4 and ERα and identified the function of Smad4 as a transcriptional corepressor for ERα. It would be a future focus to investigate how the R-Smads and Smad4 are assembled in the physiological context with mutually exclusive function of coactivation and corepression. More detailed understanding of the regulation by different SERMs may also be helpful in the design of novel selective estrogen receptor modulators for the treatment of hormone-dependent medical disorders such as breast cancer, osteoporosis, and other pathological conditions.

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Cross-talk of TGF-β and Estrogen Signaling through Smad4

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Smad4 as a Transcription Corepressor for Estrogen Receptor α
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