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Cross-Talk between Bone Morphogenic Proteins and Estrogen Receptor Signaling

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Bone morphogenic proteins (BMPs) play central roles in differentiation, development, and physiological tissue remodeling. Estrogens have key roles in a variety of biological events, such as the development and maintenance of numerous target tissues. Previous studies demonstrated that estrogens suppress BMP functions by repressing BMP gene expression. Here we present a novel mechanism for the inhibitory effect of estrogens on BMP function. BMP-2-induced activation of Sma and Mad (mothers against decapentaplegic)-related protein (Smad) activity and BMP-2-mediated gene expression were suppressed by 17β-E2 in breast cancer cells and mesangial cells. E2-mediated inhibition of Smad activation was reversed by tamoxifen, an ER antagonist. We provide evidence that the inhibitory action of ER on Smad activity was due to direct physical interactions between Smads and ER, which represents a novel mechanism for the cross-talk between BMP and ER signaling pathways. (*Endocrinology* 143: 2635–2642, 2002)

BONE MORPHOGENETIC proteins (BMPs) are members of the TGFβ superfamily that have been implicated in tissue growth and remodeling (1–3). BMPs were initially identified by the ability of bone extracts to induce bone formation at extraskeletal sites (2). BMPs bind to two types of transmembrane receptors, denoted type I and type II BMPRs, which have serine/threonine kinase activity (3). Upon ligand binding, type II receptors phosphorylate the type I receptors. The activated type I receptors then phosphorylate downstream Sma and Mad (mothers against decapentaplegic)-related proteins (Smads), Smad1, Smad5, or Smad8, which are transcription factors that regulate gene expression in response to BMPs (4–6).

ER is a ligand-activated transcription factor that is a member of the nuclear receptor superfamily (7). Two types of ERs have been identified, ERα and ERβ, that appear to have overlapping, but distinct, roles in mediating estrogen action (8–10). Estrogens play important roles in the differentiation and development of various organs and the maintenance of proper cellular function in a wide variety of tissues and are also risk factors for breast and endometrial cancer (11). ERs interact with estrogen response elements in the target gene promoters and directly regulate their transcription (7). In addition, ERs interact with other signaling pathways for which DNA binding may not be necessary (12).

BMP-2 has been shown to regulate chondrocyte differentiation and extracellular matrix composition. BMP-2, like TGFβ, up-regulated α1(I)-collagen (COL1A1) mRNA expression in osteoblastic cells (13, 14). Furthermore, BMP-2-mediated transcription of COL1A1 was blocked by the expression of a dominant-negative Smad1 expression vector (15). In previous studies estrogens have been shown to inhibit BMP functions in primary oviduct cells and osteoblasts by repressing BMP production (16, 17). Estrogen administration has also been shown to reduce collagen deposition in the aorta of hypertensive and hypercholesterolemic animals and to reduce collagen synthesis by vascular smooth muscle cells *in vitro* (18).

In this study we demonstrate a novel molecular mechanism for the inhibitory actions of estrogens on BMP-2 function. There are direct physical and functional interactions between Smad and ER. These findings provide insights into the cross-regulation between the estrogen and BMP-2 signaling pathways that may have implications in reproductive physiology and the process of chondrogenesis.

Materials and Methods

Reagents and antibodies

Human recombinant BMP-2 was purchased from Strathmann Biotech GmbH (Hamburg, Germany). 17β-E2 and tamoxifen were purchased from Wako Chemicals (Osaka, Japan). Expression vectors, FLAG-tagged Smad1, Smad5, BMPR-IA(QD), and 12xGCCG-luciferase (LUC), were provided by Drs. M. Kawabata and K. Miyazono (The Cancer Institute of JFCR, Tokyo, Japan) (19, 20). Human ERα (HEG0), ERαL-536P (HEG0L536P) (21), human ERβ (22), and human α2(I)-collagen (COL1A2) (23) were provided by Dr. P. Chambon (Institut de Chimie Biologique, Strasbourg, France), Dr. J. H. White (McGill University, Montréal, Canada), Dr. J. A. Gustaffson (Karolinska Institute, Stockholm), and Dr. H. Ihn (Tokyo University, Tokyo, Japan), respectively. ERα mutants were generated by PCR methods and sequenced (primer sequences are available upon request). Antihemagglutinin (anti-HA), anti-Myc, anti-ERα antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-FLAG M2 antibody was purchased from Upstate Biotechnology, Inc. (Lake Placid, NY).

Cell culture, transfections, and luciferase assays

The human embryonic kidney carcinoma cell line, 293T, was maintained in DMEM containing 10% FCS and transfected in DMEM containing 1% FCS by the standard calcium precipitation protocol. Human renal mesangial cells were obtained from Clonetics (East Rutherford, NJ) and cultured in MsGM (Clonetics) containing 5% FCS according to the

Abbreviations: BMP, Bone morphogenic protein; BMPR, bone morphogenic protein receptor; COL1A1, α1(I)-collagen; COL1A2, α2(I)-collagen; HA, hemagglutinin; LUC, luciferase; Mad, mothers against decapentaplegic; MH1, NH_2-terminal Mad homology 1; Smad, Sma and Mad-related protein; VDR, 1α,25-dihydroxyvitamin D_3 receptor.

manufacturer's instructions. Before stimulation, cells were cultured for 12 h in MsGM containing 1% FCS, followed by treatment with BMP-2 and/or E2. The human breast cancer cell line MCF-7 was a gift from Cell Resource Center for Biomedical Research Instruments, Inc. (Tohoku University, Sendai, Japan) maintained in DMEM containing 10% FCS (24). Before stimulation, the cells were cultured for 24 h in DMEM containing 1% FCS, followed by treatment with BMP-2 and/or E2 (24, 25). MCF-7 cells (2–2.5 $\times$ 10^5 in a 6-cm dish) were transfected using Lipofectamine Plus (Life Technologies, Inc., Carlsbad, CA) following the manufacturer's instructions. The luciferase assay was performed as previously described (26). The cells were harvested 48 h after transfection and lysed in 100 μl PicaGene Reporter Lysis Buffer (Toyo, Inc., Tokyo, Japan) and assayed for luciferase and β-galactosidase activities according to the manufacturer's instructions. Luciferase activities were normalized to β-galactosidase activities. Three or more independent experiments were carried out for each panel presented.

Immunoprecipitation and Western analysis

Immunoprecipitation and Western blotting were performed as described previously (26). Cells were harvested and lysed in lysis buffer [50 mM Tris-HCl (pH 7.4), 0.15 M NaCl, containing 0.5% Nonidet P-40, 1 μM sodium orthovanadate, 1 μM phenylmethylsulfonylfluoride, and 10 μg/ml each of aprotinin, pepstatin, and leupeptin]. The immunoprecipitates from cell lysates were resolved on 5–20% SDS-PAGE and transferred to Immobilon filters (Millipore Corp., Bedford, MA), which were then probed with each antibody. Immunoreactive proteins were visualized using an enhanced chemiluminescence detection system (Amersham Pharmacia Biotech, Arlington Heights, IL).

Northern blot analysis

Human renal mesangial cells were maintained as described above. After 12 h of incubation in 1% FCS, cells were treated with BMP-2 (50 ng/ml) and/or E2 (10^{-8} M) for 24 h. Total RNA was prepared using Iso-Gen (Nippon Gene, Tokyo, Japan) and was used in Northern analysis according to established procedures. A nylon membrane (Hybond N^+, Amersham Pharmacia Biotech) and radiolabeled cDNA probes were used where indicated.

Results

Estrogens inhibit BMP-2-induced Smad activation

To examine whether estrogens have any effect on BMP-2-induced transcriptional activation of cellular genes, we carried out Northern analysis on RNA samples prepared from human renal mesangial cells that were induced by BMP-2 and/or E2. As a cellular target for BMP-2, we analyzed the expression of COL1A2 that codes for a major structural component of the extracellular matrix (27) and is up-regulated by BMP-2 treatment (13, 14). As shown in Fig. 1A, BMP-2 treatment induced COL1A2 expression in human renal mesangial cells by 10-fold, and this activation was decreased by 60% in the presence of E2, whereas E2 alone slightly increased basal levels of COL1A2 expression. These data show that E2 inhibits BMP-2-induced gene expression in human renal mesangial cells.

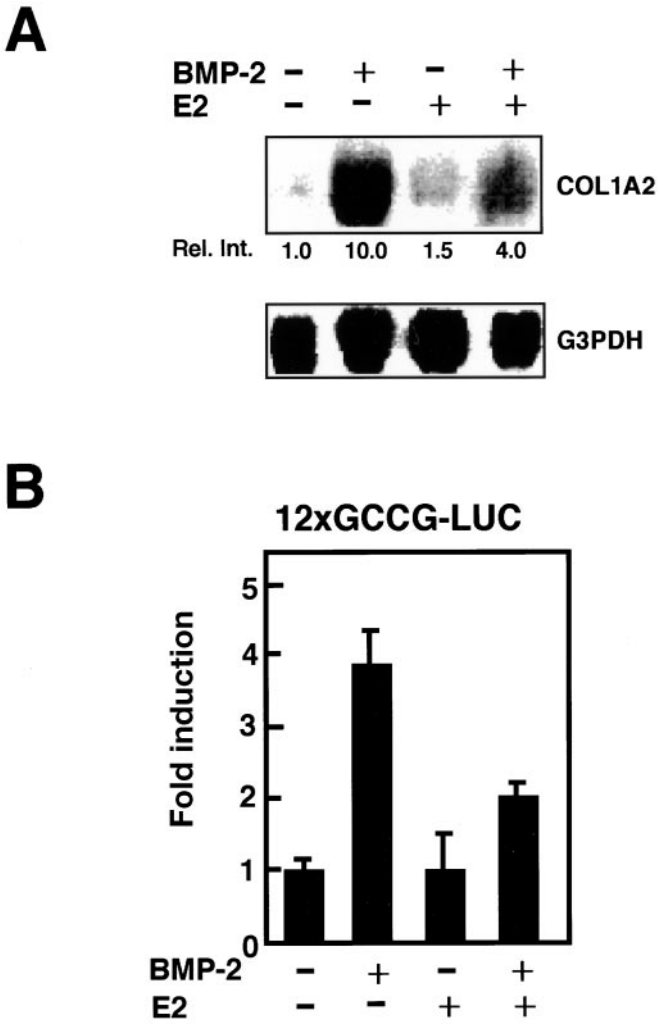
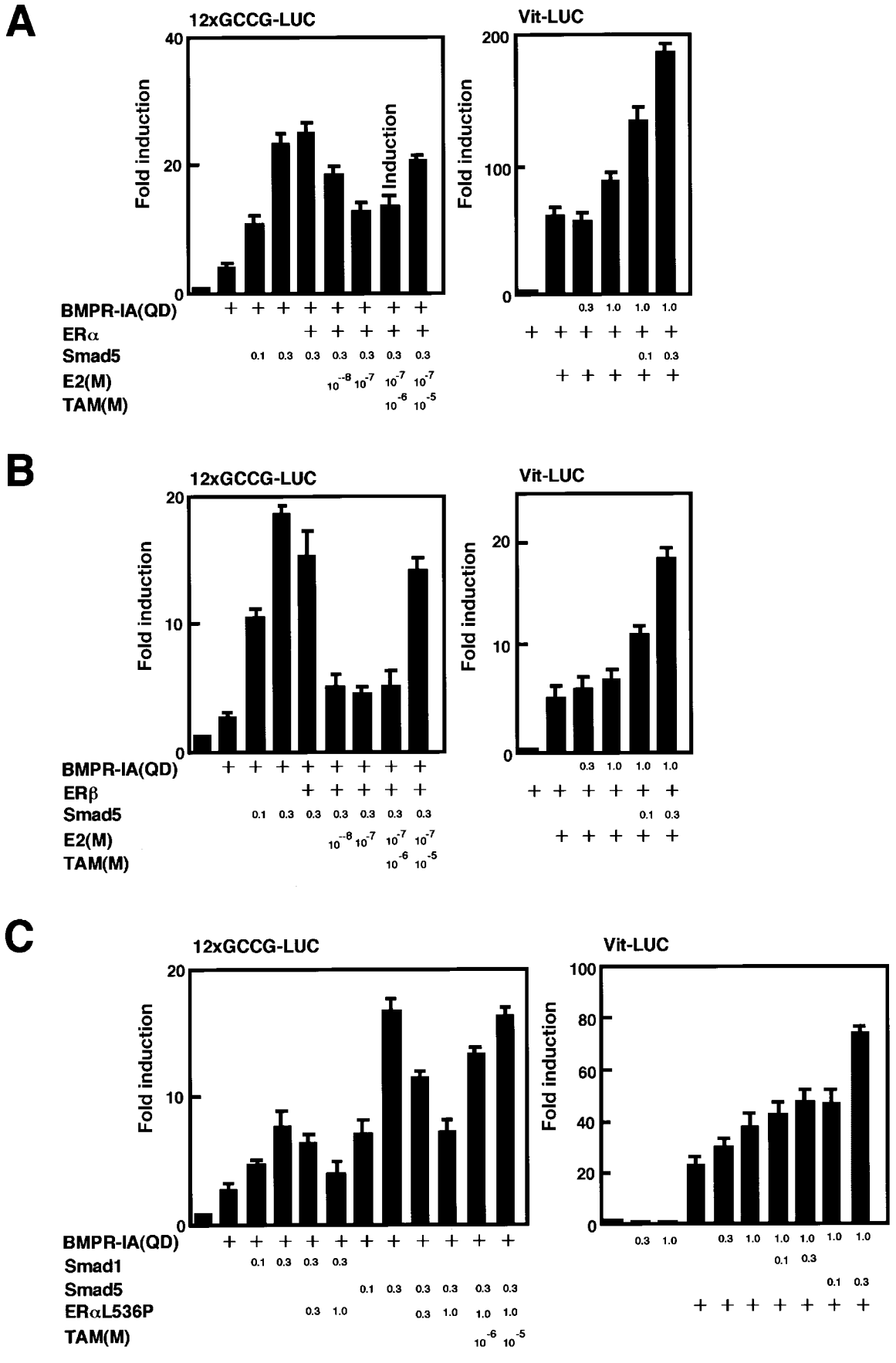
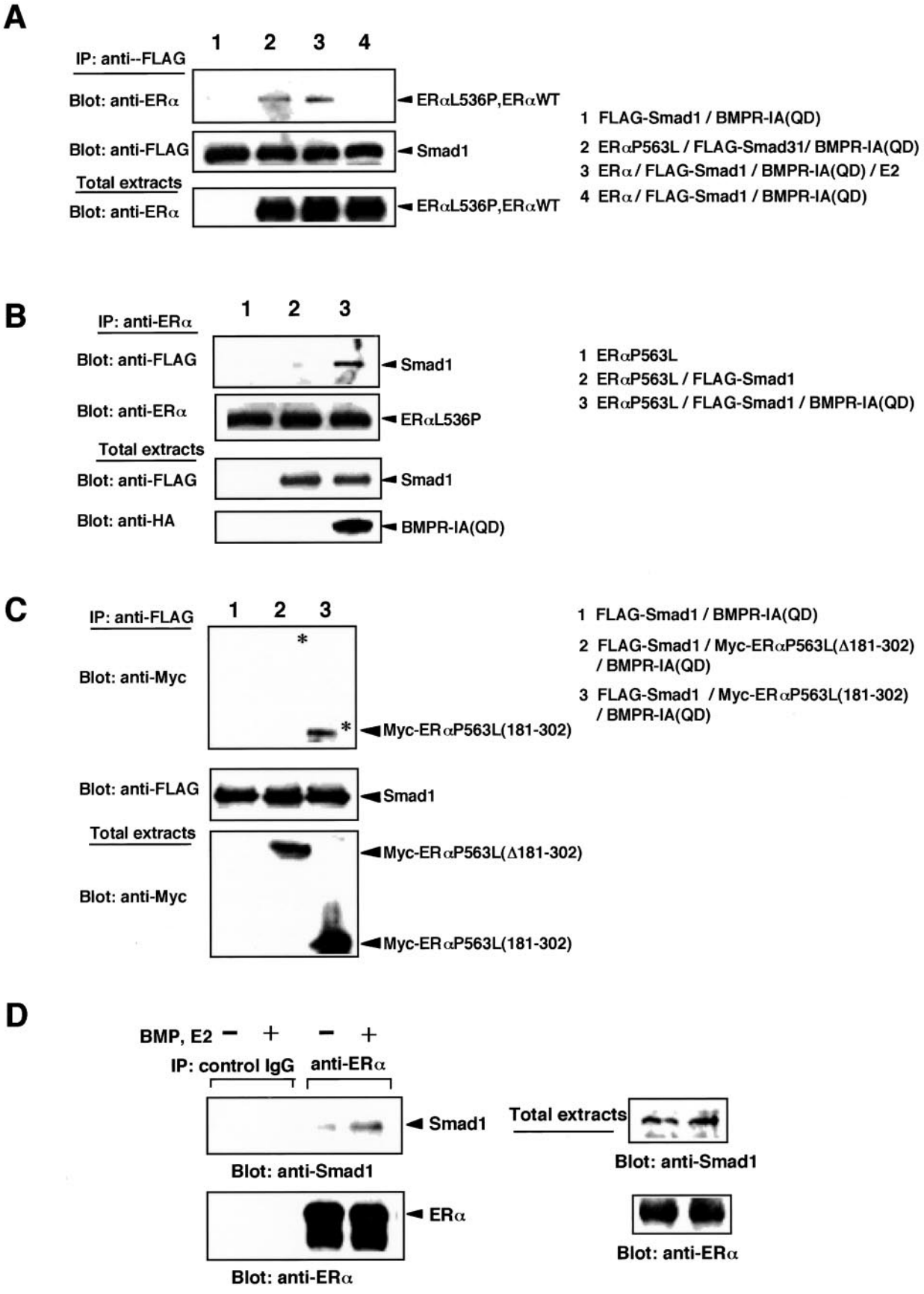


FIG. 1. Estrogens inhibit BMP-2-induced gene activation *in vivo*. A, Human renal mesangial cells (HRMC) were either left untreated or treated with BMP-2 (50 ng/ml) and/or E2 (10^{-8} M). COL1A2 expression was monitored by Northern blot analysis of 15 μg total RNA for each treatment. The same blot was probed with glyceraldehyde-3-phosphate dehydrogenase (G3PDH) cDNA as the control (*lower panel*). The relative intensities (Rel. Int.) of the bands shown below the autoradiograms were determined by densitometric analysis. B, MCF-7 cells were grown in a 6-cm dish and transfected with 12xGCCG-LUC, then stimulated with BMP-2 (50 ng/ml) and/or E2 (10^{-7} M) as indicated. Forty-eight hours after transfection, cells were stimulated for an additional 12 h. Cells were harvested, and relative luciferase activities were measured. The results are presented as the fold induction of luciferase activity from triplicate experiments, and the error bars represent the SDs. There were no significant changes in basal activity for the different treatments (data not shown).

FIG. 2. Details of the cross-talk between BMP-2 and ER signaling in 293T cells. A, 293T cells were transfected with 12xGCCG-LUC or Vit-LUC (1 μg each) together with ERα (1.0 μg), and/or 1.0 μg or the indicated amounts of BMPR-IA(QD) (0.1–1.0 μg) and Smad5 (0.1–0.3 μg). Forty-eight hours after transfection, cells were stimulated for an additional 12 h with or without the indicated doses (10^{-6}–10^{-5} M) of tamoxifen in the presence or absence of E2 (10^{-8} or 10^{-7} M), and LUC activities were determined. B, 293T cells were transfected with 12xGCCG-LUC or Vit-LUC (1 μg each) together with ERβ (1.0 μg), and/or 1.0 μg or the indicated amounts of BMPR-IA(QD) (0.1–1.0 μg) and Smad5 (0.1–0.3 μg). Forty-eight hours after transfection, cells were stimulated for an additional 12 h with or without the indicated doses (10^{-6}–10^{-5} M) of tamoxifen in the presence or absence of E2 (10^{-8} or 10^{-7} M), and LUC activities were determined. C, 293T cells were transfected with 12xGCCG-LUC or Vit-LUC (1 μg each) together with ERα-L536P (1.0 μg), and/or 1.0 μg or the indicated amounts of BMPR-IA(QD) (0.1–1.0 μg) and Smad1 or Smad5 (0.1–0.3 μg). Forty-eight hours after transfection, cells were stimulated for an additional 12 h with or without various doses (10^{-6}–10^{-5} M) of tamoxifen in the presence or absence of E2 (10^{-8} or 10^{-7} M), and LUC activities were determined. There were no significant changes in basal activity for the different treatments (data not shown).





To further examine the molecular basis of the cross-talk between BMP-2 and estrogen signaling pathways, we used a BMP-2-responsive, ER-positive breast cancer cell line, MCF-7 (24, 25), and the transient transfection assay. The BMP-2-mediated transcriptional responses were measured using 12xGCCG-LUC, which is a reporter construct that directly detects Smad phosphorylation, and therefore activation, by BMPRs (20). MCF-7 cells were transfected with 12xGCCG-LUC and treated with BMP-2 and/or E2, and LUC activities were determined. As shown in Fig. 1B, BMP-2 stimulated 12xGCCG-LUC activity approximately 4-fold, whereas E2 alone did not have an effect. When cells were treated with both BMP-2 and E2, 12xGCCG-LUC expression was decreased by 50% compared with the activation by BMP-2 alone.

Reconstitution of the cross-talk between BMP and ER signaling pathways in 293T cells

To further delineate the mechanisms of cross-talk between BMP and ER signaling pathways, we carried out transient transfection experiments in 293T cells using the respective receptors and the downstream activators for the BMP signaling, Smad1 and Smad5. In addition, in some of these experiments a constitutively active form of BMP type IA receptor, BMPR-IA(QD), was used (19).

When 293T cells were transfected with 12xGCCG-LUC together with an expression vector for BMPR-IA(QD), LUC expression increased by 3- to 4-fold (Fig. 2A). Additional expression of Smad5 augmented 12xGCCG-LUC expression by 20-fold (Fig. 2A). We then examined the effect of E2 on BMP signaling in this model system. 293T cells were transfected with an expression vector for ERα, BMPR-IA(QD), Smad5, and 12xGCCG-LUC and were either left untreated or were treated with E2. As shown in Fig. 2A, E2 suppressed BMPR-IA(QD)/Smad5-induced 12xGCCG-LUC expression by approximately 50% in a dose-dependent manner. This inhibition was largely reversed in the presence of the antiestrogen tamoxifen (Fig. 2A). These results indicate that the inhibitory effects of E2 on BMPR-IA(QD)/Smad5-induced transcriptional activity are mediated by ERα and can be reconstituted in 293T cells similar to those observed in MCF-7 cells.

We then assessed the reverse situation for the possible effect of BMP signaling on ERα activity using the reporter gene Vit-LUC, in which two copies of an estrogen response element drive expression of the LUC gene. In the presence of ERα, E2 treatment resulted in a 50-fold increase in Vit-LUC activity (Fig. 2A). Surprisingly, this activation was augmented by BMPR-IA(QD)/Smad5 expression in a dose-dependent manner, up to approximately 3-fold more than by E2 alone, although BMPR-IA(QD)/Smad5 alone did not affect reporter activity (data not shown). These results suggest that in contrast with the inhibitory effects of ERα on BMP signaling, activation of the BMP pathway has a stimulatory role in ERα signaling in 293T cells.

We next examined whether the other major ER isoform, ERβ (8, 9), has similar inhibitory effects on TGFβ signaling in an analogous experiment. As shown in Fig. 2B, BMPR-IA(QD)/Smad5-induced 12xGCCG-LUC activity was inhibited by ERβ in the presence of E2 similar to that observed with ERα, and this inhibitory effect was reversed by tamoxifen. ERβ-induced Vit-LUC activation was augmented by BMPR-IA(QD) and Smad5, similar to that observed for ERα. These data suggest that both ER isotypes may be involved in the cross-talk of ER signaling with the BMP pathway.

To examine the interactions between the BMP and ER signaling in greater detail, we used a constitutively active form of ERα, ERα-L536P (21). 293T cells were transfected with 12xGCCG-LUC, an expression vector for ERα-L536P, and/or increasing amounts of an expression vector for BMPR-IA(QD) and/or Smad1 or Smad5, and the LUC activities were measured. As shown in Fig. 2C, BMPR-IA(QD)-plus Smad1- or Smad5-induced 12xGCCG-LUC activity was inhibited by ERα-L536P in a dose-dependent manner. This inhibition was reversed in the presence of tamoxifen, indicating that it is mediated directly by ERα-L536P (Fig. 2C and data not shown).

In contrast, the expression of BMPR-IA(QD) in the presence of either Smad1 or Smad5 resulted in further enhancement of ERα-L536P-induced Vit-LUC activation. These results are consistent with the data presented in Fig. 2A and clearly document the two-way cross-talk between BMP and ER signaling in 293T cells.

Physical interactions between ER and Smads in vivo

One of the possible mechanisms that would be consistent with the data described above is that there are direct physical interactions between ERs and Smad1. We tested this possibility by coimmunoprecipitation experiments. 293T cells were transfected with expression vectors encoding ERα-L536P or wild-type ERα together with FLAG-tagged Smad1 and BMPR-IA(QD). Cells that were transfected with ERα

Fig. 3. Physical interactions between Smad3 and ER. A, 293T cells (1×10^7) were transfected with ERα or ERα-L536P (7.5 μg) and FLAG-tagged Smad1 (10 μg) together with BMPR-IA(QD) (3 μg). Forty-eight hours after transfection, cells were starved for 12 h, followed by treatment with or without E2 (10^{-8} M) for 12 h. Cell lysates were then immunoprecipitated with an anti-FLAG antibody, and immunoblotted with an anti-ERα antibody (*upper panel*) or an anti-FLAG antibody (*middle panel*). Total cell lysates (20 μg) were blotted with an anti-ERα antibody (*lower panel*). B, Cells (1×10^7) were transfected with ERα-L536P (7.5 μg) and/or FLAG-tagged Smad1 (10 μg) in the presence or absence of HA-tagged BMPR-IA(QD) (3 μg). Cell lysates were then immunoprecipitated with an anti-ERα antibody, and immunoblotted with either anti-FLAG antibody (*upper panel*) or anti-ERα antibody (*middle panel*). Total cell lysates (20 μg) were blotted with anti-FLAG antibody or anti-HA antibody as indicated (*lower panel*). C, Mapping the Smad1 interaction domain of ERα. 293T cells (1×10^7) were transfected with ERα-L536P(Δ181–302) or ERα-L536P(181–302) (10 μg) and FLAG-tagged Smad1 (10 μg) together with BMPR-IA(QD) (3 μg). Forty-eight hours after transfection, cells were lysed, immunoprecipitated with an anti-ERα antibody, and immunoblotted with anti-Myc antibody (*upper panel*) or anti-FLAG antibody (*middle panel*). Total cell lysates (20 μg) were blotted with anti-Myc antibody (*lower panel*). The *asterisks* indicate the migration position of ERα-L536P (Δ181–302) or ERα-L536P(181−302). D, MCF-7 cells (5×10^7cells) were maintained in DMEM containing 1% FCS for 12 h before stimulation. After 1 h of stimulation with or without BMP-2 (50 ng/ml) and E2 (10^{-8} M), cells were lysed, immunoprecipitated, and immunoblotted with control IgG or anti-ERα or anti-Smad1 antibody as indicated. Total cell lysates (20 μg) were blotted with anti-ERα or anti-Smad1 antibody.

were either left untreated or were treated with E2, whereas cells that were transfected with ERα-L536P were left untreated during the course of the experiment. The cells were then lysed and subjected to immunoprecipitation with an anti-FLAG antibody. Immunoprecipitates were used in Western analysis with an antiserum against ERα. As shown in Fig. 3A, the constitutively active ERα-L536P and Smad1 were found to be in a complex in 293T cells. Furthermore, consistent with the fact that ERα inhibits BMP-2 signaling only in the presence of E2, ERα-Smad1 interactions were only detected in E2-treated cells (Fig. 3A).

We next tested whether BMP affects ERα-Smad1 interactions. 293T cells were transfected with ERα-L536P together with FLAG-tagged Smad1 in the presence or absence of BMPR-IA(QD), and immunoprecipitation and Western analysis were carried out as described above. As shown in Fig. 3B, ERα-L536P interacted with Smad1 only in the presence of BMPR-IA(QD), suggesting that stimulation of the BMP signaling pathway is a prerequisite for ERα-Smad1 interactions.

We next determined the domains of ERα that mediate interactions with Smad1, using deletion mutants of ERα-L536P (21). In a previous study 1α,25-dihydroxyvitamin D_3 receptor (VDR) was shown to interact with Smad3 through a region in the ligand binding domain (28). We therefore used two deletion mutants of ERα-L536P in which either the DNA binding domain was removed [ERα-L536P(Δ181–302)] or only the DNA binding domain was present [ERα-L536P(181–302)]. Expression vectors encoding FLAG-tagged Smad1 and/or Myc-tagged ERα-L536P(Δ181–302) or ERα-L536P(181–302) were transiently transfected into 293T cells in the presence of BMPR-IA(QD). Cells were lysed and subjected to immunoprecipitation with an anti-FLAG antibody. Immunoprecipitates were then used in Western blot analysis with an anti-Myc antibody. As shown in Fig. 3C, whereas the DNA binding domain alone, ERα-L536P(181–302), interacted with Smad1, ERα-L536P(Δ181–302), which lacks the DNA binding domain, was unable to bind Smad1. These results indicate that in contrast to the VDR-Smad3 interactions (28), efficient ERα-Smad1 interactions require the DNA binding domain of ERα.

To examine the cross-talk between BMP-2 and estrogen signaling pathways under more physiological conditions, we used a BMP-2-responsive, ER-positive breast cancer cell line, MCF-7 (24, 25). In parallel with the data in Fig. 1B, coimmunoprecipitation experiments were performed using cell lysates obtained from MCF-7 cells that were either left untreated or were treated with BMP-2 and E2. Similar to the results obtained in transfected 293T cells (Fig. 3, A and B), ERα coimmunoprecipitated from MCF-7 cells as a complex with Smad1, and this interaction was dependent on the presence of BMP-2 and E2 (Fig. 3D).

Discussion

Recent studies have identified interactions between TGFβ and steroid receptor signaling pathways. It was reported that Smad3 enhanced VDR transcriptional activity by physically interacting with ligand-induced VDR in complex with SRC-1/TIF2 (28). In prostate cancer cells AR stimulated TGF-β signaling via direct binding to Smad3 (29), whereas Smad3 repressed AR-mediated transcription (30). However, interaction between GR and Smad3 suppressed TGFβ signaling in hepatoma cells (31). In the case of ER, ER-mediated transcriptional activation was enhanced by TGFβ signaling, whereas ER suppressed Smad3 activity (32).

In contrast, the possible interactions between the BMPs and steroid receptor signaling pathways have not been studied in detail. Recent findings demonstrated that antiestrogens specifically up-regulated BMP4 promoter activity (16), and estrogen opposed the apoptotic effects of BMP7 on tissue remodeling (17). Repression of BMP expression by estrogens may be one of the inhibitory mechanisms that regulate BMP signaling. We here demonstrated an alternative inhibitory pathway, which was due to the direct interaction between components of the two signaling pathways. The findings we present in this paper provide an additional molecular mechanism for at least some of these previous observations. This is also the first time that interactions between BMP-regulated Smads, Smads 1, 5, and 8, with a member of the steroid receptor family has been documented. It would be expected that repression of BMP expression in addition to inhibition of Smad activity may bring a more accentuated repressed state of the BMP pathway than with one mechanism alone. Further work is required to determine whether these two pathways are active simultaneously in the same cell type.

In the interaction between VDR and Smad3, the NH_2-terminal Mad homology 1 (MH1) region of Smad3 and the middle region of the ligand binding domain (E domain) of VDR were shown to be required for the interaction (28). We had demonstrated that the MH2 domain of Smad3 is required for the cross-talk between ERα and TGFβ signaling in both directions (32). The MH2 domain is known as an important region that interacts with other coactivators, such as p300 and CBP (33, 34). ER may compete with p300/CBP in Smad binding as well as the Smad corepressor c-Ski (35).

In contrast, in this study we found that the middle region of ER containing the DNA binding domain was required for the interaction between ERs and Smads. At present we do not know whether these interactions are direct or mediated by other cofactors. Future interaction studies performed *in vitro* and more detailed mapping of the domains involved should provide more precise information regarding the detailed molecular mechanisms involved.

Interestingly, the cross-talk between ER and BMPs that we have documented is a mirror image of that observed between AR and TGFβ, but is similar to that between VDR and TGFβ. It would be of interest to delineate the mechanism of these similarities and differences, because all of the steroid receptors involved share significant similarity of structure and function.

E2 was previously shown to antagonize TGFβ1-stimulated type IV collagen synthesis at the level of transcription in murine mesangial cells, and this effect may be mediated by interactions with the transcription factor Sp1 (36). Other transcriptional cofactors similar to Sp1 may also be involved in the interaction of BMP-regulated Smads with ER. Further work is required to assess this possibility.

We found that the tamoxifen concentration necessary for reversing the E2 effects is higher in our experiments, con-

sistent with our recent findings on E2-TGFβ signaling cross-talk (32), compared with that previously found in similar experiments involving other reporters and signaling pathways (37). We do not know the reason for this difference in the level of tamoxifen required for the various signaling pathways, but it could be due to changes in the expression level of the factors or the absence/decreased levels of a specific cofactor that is involved in these activities in the cell lines under study. Further work is needed to assess these possibilities.

BMPs are also known to induce differentiation of multipotent mesenchymal cells to the osteoblastic (38, 39) and chondroblastic (40) lineages and thus may play a role in bone remodeling and fracture healing. Estrogens have direct effects on osteoblasts (41, 42) and osteoclasts (43) by acting through the ER. It has been suggested that many of the estrogen effects on inhibition of osteoclastic activity may be mediated by paracrine action of bone-active cytokines secreted by osteoblasts, including IL-1 and IL-6 (44, 45), TNFα (44), TGFβ (46), and BMP-6 (47). The cross-talk between the BMPs and ERs that we present in this study may be responsible for these important biological outcomes. Further delineation of the interactions between BMP-regulated Smads and ER will not only provide critical information on bone remodeling and kidney biology, but may also be instrumental in the development of new treatment strategies in related diseases.

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