Bone morphogenetic protein 6 (BMP6) and BMP7 inhibit estrogen-induced proliferation of breast cancer cells by suppressing p38 mitogen-activated protein kinase activation

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Abstract

Estrogen is involved in the development and progression of breast cancer. Here, we investigated the effects of bone morphogenetic proteins (BMPs) on breast cancer cell proliferation caused by estrogen using human breast cancer MCF-7 cells. MCF-7 cells express estrogen receptors (ESR1 and ESR2), BMP receptors, and SMAD signaling molecules. Estradiol and membrane-impermeable estradiol stimulated MCF-7 cell proliferation. Estradiol also reduced mRNA levels of ESR1, aromatase, and steroid sulfatase. Treatment with BMPs and activin had no effects on MCF-7 cell proliferation. However, BMP2, BMP4, BMP6, BMP7, and activin suppressed estradiol-induced cell mitosis, with the effects of BMP6, BMP7, and activin being more prominent than those of BMP2 and BMP4. Activin decreased *ESR1* mRNA expression, while BMP6 and BMP7 impaired steroid sulfatase expression in MCF-7 cells. Interestingly, SMAD1,5,8 activation elicited by BMP6 and BMP7, but not by BMP2 and BMP4, was preserved even under the exposure of a high concentration of estradiol. The difference of BMP responsiveness was likely due to the differential

modulation of BMP receptor expression induced by estradiol. In this regard, estradiol decreased the expression levels of BMPR1A, BMPR1B, ACVR2A, and ACVR2B but did not affect ACVR1 and BMPRII, leading to the sustained effects of BMP6 and BMP7 in estrogen-treated MCF-7 cells. Estradiol rapidly activated MAPK phosphorylation including extracellular signal-regulated kinase 1/2, p38, and stress-activated protein kinase/c-Jun NH2-terminal kinase pathways and BMP6, BMP7, and activin preferentially inhibited estradiol-induced p38 phosphorylation. SB203580, a selective p38 MAPK inhibitor effectively suppressed estradiol-induced cell mitosis, suggesting that p38 MAPK plays a key role in estrogen-sensitive breast cancer cell proliferation. Thus, a novel interrelationship between estrogen and the breast cancer BMP system was uncovered, in which inhibitory effects of BMP6 and BMP7 on p38 signaling and steroid sulfatase expression were functionally involved in the suppression of estrogen-induced mitosis of breast cancer cells.

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Introduction

The involvement of estrogen in the development and progression of breast cancer is well known (Henderson *et al.* 1988, Helzlsouer & Couzi 1995, Colditz 1998, Keen & Davidson 2003). Most premenopausal and postmenopausal breast cancers are recognized to be initially estrogen dependent. Prolonged exposure to cycling estrogen such as early menarche, late menopause, and nulliparity is correlated with an increased risk of developing breast carcinoma, while the removal of endogenous estrogen by oophorectomy decreases the risk of the development of breast cancer (Keen & Davidson 2003). In postmenopausal women, the major source of estrogen is androgenic precursors derived from the adrenal glands that are converted into estrogen by the aromatase enzyme in adipose tissues (Foster 2008). Increased estrogen exposure via a variety of mechanisms is critical for the development of breast cancer, in

which the effects of estrogen are mediated via two estrogen receptor (ER) subtypes, ESR1 and ESR2. Estrogen and ER complex mediate the activation of proto-oncogenes and oncogenes, nuclear proteins, and other target genes. However, at present, there is no definite information of a direct effect of estrogen in the development of breast cancer.

There has been accumulating evidence that breast cancer tissue expresses all enzymes required for the local biosynthesis of estrogen from circulating precursors (Foster 2008). Two principal pathways are implicated in the last steps of estradiol formation in breast cancer tissues, including the aromatase pathway that transforms androgens into estrogens and the sulfatase pathway that converts estrone sulfate into estrone. The final step of the estrogen steroidogenesis is conversion of weak estrone to biologically active estradiol by actions of a reductive 17 β -hydroxysteroid dehydrogenase type 1. A recent study has suggested that estrone sulfate via sulfatase is a much

more likely precursor for estradiol than androgens via aromatase in human breast tumors (James *et al.* 2001, Pasqualini & Chetrite 2005, Stanway *et al.* 2007). It is also well established that steroid sulfo-transferases, which convert biologically active estrogens into their inactive sulfates, are also present in breast cancer tissues (Falany & Falany 1996). Therefore, therapeutic targets for breast cancers include not only the binding of estrogen to ER but also the activity of estrogenic enzymes in the tumor tissues (Pasqualini 2004, Sasano *et al.* 2006, Subramanian *et al.* 2008).

Bone morphogenetic proteins (BMPs) that belong to transforming growth factor (TGF)- β superfamily have been widely recognized as important molecules involved in tissue differentiation and functional modulation of various endocrine systems (Simic & Vukicevic 2005). BMPs were originally identified as the active components in bone extracts capable of inducing bone formation at ectopic sites. Recently, a variety of physiological BMP actions in many endocrine tissues including the ovary (Otsuka *et al.* 2000, Shimasaki *et al.* 2004), pituitary (Otsuka & Shimasaki 2002), thyroid (Suzuki *et al.* 2005), and adrenal (Suzuki *et al.* 2004, Kano *et al.* 2005, Inagaki *et al.* 2006) have been clarified. BMPs have been paid attention for the possible link with tumorigenesis, considering the BMP action as regulators of cell proliferation and differentiation (Otani *et al.* 2007). Since BMPs constitute an important signaling pathway in the bone, the possible link with establishment of metastatic lesion in the bone has been postulated.

Recent studies have demonstrated the presence of the TGF- β signaling pathway in mammary cells and its importance in maintaining the growth state of these cells. There have been several reports showing the expression of some of TGF- β superfamily proteins, such as BMP2 (Arnold *et al.* 1999, Raida *et al.* 2005), BMP6 (Clement *et al.* 1999), and BMP7 (Schwalbe *et al.* 2003, Alarmo *et al.* 2006), in breast cancer cells, in which their possible role in breast cancer development and involvement in bone metastasis has been discussed (Pouliot & Labrie 2002, Bobinac *et al.* 2005, Clement *et al.* 2005). Furthermore, the involvement of BMP-SMAD activation in the progression and dedifferentiation of ER-positive breast cancer was recently reported (Helms *et al.* 2005). However, the pathophysiologic significance of BMP system in breast cancer remains poorly understood. In the present study, we investigated the effects of BMPs on breast cancer cell proliferation evoked by estrogen using a human breast cancer cell line. Here, we uncovered the antagonistic effects of BMP on estrogen-induced mitosis of breast cancer cells through the functional interrelationship between ER and BMP signaling.

Materials and Methods

Reagents and supplies

Dulbecco's Modified Eagle's Medium (DMEM), penicillin-streptomycin solution, 17 β -estradiol, BSA-conjugated 17 β -estradiol (estradiol-BSA), and human recombinant activin

A were purchased from Sigma-Aldrich Co. Ltd. Recombinant human BMP2, BMP4, BMP6, and BMP7 were purchased from R&D Systems, Inc. (Minneapolis, MN, USA), and U0126, SB203580, and LY294002 were from Promega Corp. SP600125 was from Biomol Lab. Inc. (Plymouth Meeting, PA, USA) and SH-5 was from Calbiochem (San Diego, CA, USA). Plasmids of *Id-1-Luc* were kindly provided from Drs Tetsuro Watabe and Kohei Miyazono, Tokyo University, Japan.

Breast cancer cell culture

The human breast cancer cell line, MCF-7, was from American Type Culture Collection (Manassas, VA, USA). MCF-7 cells were cultured in DMEM supplemented with 10% fetal calf serum (FCS) and penicillin-streptomycin solution at 37 °C under a humid atmosphere of 95% air/5% CO₂. In some experiments, cell numbers were counted by culturing MCF-7 cells in 12-well plates (1×10^5 viable cells) with serum-free DMEM for 24 h. The cells were then washed with PBS, trypsinized, and applied for a coulter counter (Beckman Coulter Inc., Fullerton, CA, USA). Changes in cell morphology and cell viability were monitored using an inverted microscope.

RNA extraction, RT-PCR, and quantitative real-time PCR analysis

To prepare total cellular RNA, MCF-7 cells were cultured in 12-well plate (5×10^5 viable cells) and treated with indicated concentrations of estradiol and growth factors including BMPs in serum-free DMEM. After 24-h culture, the medium was removed, and total cellular RNA was extracted using TRIzol (Invitrogen Corp.), quantified by measuring absorbance at 260 nm, and stored at -80 °C until assay. The expression of BMP receptors, follistatin, SMADs, *ESR1*, *ESR2*, aromatase, and steroid sulfatase mRNAs were detected by RT-PCR analysis. The extracted RNA (1.0 μ g) was subjected to an RT reaction using the First-strand cDNA synthesis system (Invitrogen Corp.) with random hexamer (2 ng/ μ l), reverse transcriptase (200 U), and deoxynucleotide triphosphate (0.5 mM) at 42 °C for 50 min and 70 °C for 10 min. Subsequently, hot-start PCR was performed using MgCl₂ (1.5 mM), deoxynucleotide triphosphate (0.2 mM), and 2.5 U of Taq DNA polymerase (Invitrogen Corp). Oligonucleotides used for RT-PCR were custom-ordered from Invitrogen Corp. PCR primer pairs were selected from different exons of the corresponding genes as follows: *ESR1*, 1393–1413 and 1632–1652 (from GenBank accession #NM_000125); *ESR2*, 395–415 and 589–609 (from AB006590); aromatase, 914–934 and 1235–1256 (from M22246); steroid sulfatase, 513–533 and 693–713 (from NM_000351); and a house-keeping gene, ribosomal protein L19 (RPL19; Szabo *et al.* 2004), 401–420 and 571–590 (from NM_000981). PCR primer pairs for ACVR1, BMPR1A, ACVR1B, BMPR1B, activin type II receptor (ACVR2A), ACVR2B, BMP type II receptor (BMPRII),

folistatin, SMAD1–8, and BMP6 and BMP7 were individually selected for regular PCR and real-time PCR, as previously reported (Takeda *et al.* 2003, Miyoshi *et al.* 2006, Inagaki *et al.* 2007). Aliquots of PCR products were electrophoresed on 1.5% agarose gels and visualized after ethidium bromide staining. For the quantification of ESR1, ESR2, aromatase, steroid sulfatase, *ACVR1*, *BMPR1A*, *BMPR1B*, *ACVR2A*, *ACVR2B*, *BMPRII*, *BMP6*, and *BMP7* mRNA levels, real-time PCR was performed using LightCycler-FastStart DNA Master SYBR Green I system (Roche Diagnostic Co.) under conditions of annealing at 60–62 °C with 4 mM MgCl₂, following the manufacturer's protocol. Accumulated levels of fluorescence were analyzed by the second derivative method after the melting curve analysis for each PCR product (Roche Diagnostic), and then the expression levels of target genes were standardized by RPL19 level in each sample.

Thymidine incorporation assay

MCF-7 cells (1×10^4 viable cells) were precultured in 12-well plates with DMEM containing 10% FCS for 24 h. After preculture medium was replaced with fresh serum-free medium and indicated combinations of estradiol, estradiol-BSA, mitogen-activated protein kinase (MAPK) inhibitors, phosphatidylinositol 3-kinases (PI3K)/AKT inhibitors, and growth factors including BMPs and activin were added to the culture medium. For the experiments using estradiol-BSA, the preparation of estradiol-BSA free of estradiol was performed as follows: 4 ml estradiol-BSA (1.25 mM in estrogen dissolved in 50 mM Tris-HCl (pH 8.5)) was added to a centrifugal Amicon Ultra filter unit with a MW cut-off of 5000 (Millipore, Bedford, MA, USA) and centrifuged at 4000 *g* until 50 µl retentate remained. The retentate was washed with Tris buffer and final volume was adjusted to 5 ml (1 mM). After 24-h culture, 0.5 µCi/ml [*methyl*-³H]thymidine (Amersham Pharmacia) was added, and incubated for 3 h at 37 °C. The incorporated thymidine was detected, as we previously reported (Takeda *et al.* 2004). Cells were then washed with PBS, incubated with 10% ice-cold trichloroacetic acid for 60 min at 4 °C, and solubilized in 0.5 M NaOH, and radioactivity was determined with a liquid scintillation counter (TRI-CARB 2300TR, Packard Co., Meriden, CT, USA).

Western immunoblot analysis

Cells (1×10^5 viable cells) were precultured in 12-well plates in DMEM containing 10% FCS for 24 h. After preculture, medium was replaced with serum-free fresh medium, and then indicated concentrations of estradiol and growth factors including BMPs and activin were added to the culture medium. After stimulation with hormones or growth factors for indicated periods, cells were solubilized in 100 µl RIPA lysis buffer (Upstate Biotechnology, Inc., Lake Placid, NY, USA) containing 1 mM Na₃VO₄, 1 mM sodium fluoride, 2% SDS, and 4% β-mercaptoethanol. The cell lysates were then

subjected to SDS-PAGE/immunoblotting analysis as we reported earlier (Otani *et al.* 2007), using anti-phospho- and anti-total-extracellular signal-regulated kinase (ERK) 1/2 MAPK antibody (Cell Signaling Technology, Inc., Beverly, MA, USA), anti-phospho- and anti-total-p38 MAPK antibody (Cell Signaling Technology, Inc.), anti-phospho- and anti-total-stress-activated protein kinase/c-Jun NH₂-terminal kinase (SAPK/JNK) MAPK antibody (Cell Signaling Technology, Inc.), anti-phospho-SMAD1,5,8 (pSMAD1,5,8) antibody (Cell Signaling Technology, Inc.), anti-steroid sulfatase antibody (Abcam Inc., Cambridge, MA, USA), and anti-actin antibody (Sigma-Aldrich Co. Ltd).

Immunofluorescence microscopy

Cells were precultured in serum-free DMEM using chamber slides (Nalge Nunc Int., Naperville, IL, USA) for 24 h. Cells at ~40% confluency were pretreated with estradiol (100 nM) for 1 h and then treated with BMP6 and BMP7 (100 ng/ml) for 1 h. After the stimulation, cells were fixed with 4% formaldehyde in PBS, permeabilized with 0.5% Triton X-100 in PBS at room temperature, and washed three times with PBS. The cells were then incubated with anti-phospho-SMAD1,5,8 antibody for 1 h and washed three times with PBS, then with AlexaFluor-488 anti-rabbit IgG (Invitrogen Corp.) in humidified chamber for 1 h and washed with PBS, followed by application of the counter medium containing 4',6'-diaminodino-2-phenylindole (Invitrogen Corp.), and then stained cells were visualized under a fluorescent microscope.

Transient transfection and luciferase assay

Cells (1×10^4 viable cells) were precultured in 12-well plates in DMEM with 10% FCS for 24 h. The cells were then transiently transfected with 500 ng *Id-1*-Luc reporter plasmid and 50 ng cytomegalovirus-β-galactosidase (β-gal) plasmid (pCMV-β-gal) using FuGENE 6 (Roche Molecular Biochemicals) for 24 h. The cells were then treated with BMP2, BMP4, BMP6, and BMP7 in combination with estradiol in serum-free fresh medium for 24 h. The cells were washed with PBS and lysed with Cell Culture Lysis Reagent (Toyobo, Osaka, Japan). Luciferase activity and β-gal activity of the cell lysate were measured by luminescencer-PSN (ATTO, Tokyo, Japan), as we previously reported (Miyoshi *et al.* 2006). The data were shown as the ratio of luciferase to β-gal activity.

cDNA array analysis

Oligo GEArray system (SuperArray Bioscience Corp., Frederick, MD, USA) that includes 113 genes of human TGF-β and BMP signaling pathway was used for analyzing the expression pattern of BMP signaling system in MCF-7 cells. As we previously reported (Miyoshi *et al.* 2006, Yamashita *et al.* 2008), extracted total RNAs (2.0 µg) were used as templates to generate biotin-16-dUTP-labeled cDNA probes according to the manufacture's instruction. The cDNA probes were

denatured and hybridized at 60 °C with the cDNA array membranes, which were washed and exposed to X-ray films with use of chemiluminescent substrates. To analyze the array results, we scanned the X-ray film and the image was inverted as grayscale TIFF files. The spots were digitized and analyzed using GEArray analyzer software (SuperArray Bioscience Corp.) and the data were normalized by subtraction of the background as the average intensity levels of three spots containing plasmid DNA of pUC18. The spots of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and cyclophilin A (PPIA) were used as positive controls to compare the membranes. Using these standardized data, we compared the signal intensity of the membranes using the GEArray analyzer program (SuperArray Bioscience Corp.).

Statistical analysis

All results are shown as mean $\pm$ S.E.M. of data from at least three separate experiments, each performed with triplicate samples. Differences between groups were analyzed for statistical significance using ANOVA with Fisher's protected least significant difference test (StatView 5.0 software, Abacus Concepts, Inc., Berkeley, CA, USA). *P* values < 0.05 were accepted as statistically significant.

Results

We first examined the mRNA expression of the BMP type I and type II receptors, ERs, and steroidogenic enzymes for estrogen production in MCF-7 cells by RT-PCR. As shown in Fig. 1A, the BMP type I receptors including ACVR1 (also called ACTRIA), BMPR1A, ACVR1B (ACTRIB), BMPR1B, BMP type II receptors including BMPRII, ACVR2A, and ACVR2B, and a binding protein follistatin were clearly expressed in MCF-7 cells. In addition, mRNA expression of SMAD signaling molecules including SMAD1–5,8, and inhibitory SMAD6,7 was also detected (Fig. 1A). The expression of two subtypes of ER including ESR1 and ESR2, and key estrogenic enzymes such as aromatase and steroid sulfatase mRNA was also confirmed by RT-PCR in MCF-7 cells (Fig. 1B).

As shown in Fig. 2A, 17 β -estradiol (1–100 nM) stimulated thymidine incorporation of MCF-7 cells in a concentration-dependent manner, with the effects of estradiol saturating at 30 nM. We next examined whether nongenomic effects of estrogen were involved in the mitogenesis of MCF-7 cells. Cells were treated with BSA-conjugated estradiol (estradiol-BSA), which cannot penetrate cell membrane (Taguchi *et al.* 2004), and the mitotic activation was evaluated by thymidine uptake assay. Estradiol-BSA also induced MCF-7 cell mitosis although the effects were less potent compared with those induced by 17 β -estradiol (Fig. 2A). Taken together, nongenomic effects of estrogen is also functionally involved in the estrogen-induced cell proliferation.

Figure 1 Expression of BMP system molecules, estrogen receptors, and estrogenic enzymes in MCF-7 cells. The expression of mRNAs encoding BMP type I and II receptors, SMADs and follistatin, ESR1, ESR2, aromatase, and steroid sulfatase were examined in total cellular RNAs extracted from MCF-7 cells by RT-PCR analysis. Results were shown as representative of those obtained from three independent experiments. MM indicates molecular weight marker.

As shown in Fig. 2B, BMP2, BMP4, BMP6, BMP7, and activin A (10–300 ng/ml) alone had no significant effects on MCF-7 cell proliferation. However, the mitotic effects elicited by estradiol were suppressed by BMP2, BMP4, BMP6, BMP7, and activin A (10–100 ng/ml) in a concentration-dependent manner (Fig. 2B), suggesting that BMPs and activin antagonize estrogen actions in MCF-7 cells. It was also notable that BMP6, BMP7, and activin A were more efficacious in the inhibition of estradiol-induced cell mitosis than the effects of BMP2 and BMP4.

We next investigated the expression changes of ER in MCF-7 cells under the influence of estradiol and BMP/activin. Estradiol exposure (1–100 nM) for 24 h reduced mRNA levels of *ESR1* in MCF-7 cells (Fig. 3A), but had no significant effects on *ESR2* expression (Fig. 3B). Interestingly, activin A (100 ng/ml) significantly decreased *ESR1* expression regardless of the presence or absence of estradiol (100 nM), although BMPs did not affect either mRNA levels of *ESR1* or *ESR2*. Thus, the suppressive effect of activin A on estradiol-induced cell proliferation is likely due to the reduction of *ESR1* expression.

Figure 2 Effects of estrogen and BMP/activin on mitotic action by MCF-7 cells. Cells (1×10^4 viable cells) were treated with indicated concentrations of (A) estradiol and (B) BMP2, BMP4, BMP6, BMP7, activin A, or the combination for 24 h, and then thymidine uptake assay was performed. Results are shown as mean $\pm$ S.E.M. of data from at least three separate experiments, each performed with triplicate samples. * $P < 0.05$ and ** $P < 0.01$ versus control and between the indicated groups. Estradiol-BSA indicates estradiol conjugated with BSA.

To elucidate the effect of BMP/activin on endogenous estrogenic property in MCF-7 cells, mRNA levels of key steroidogenic enzymes, aromatase, and steroid sulfatase were determined. Estradiol exposure (1–100 nM) caused down-regulation of both aromatase (Fig. 4A) and steroid sulfatase (Fig. 4B). BMP/activin had no significant effects on aromatase mRNA levels. However, among BMP/activin ligands examined, BMP6 and BMP7 reduced the steroid sulfatase mRNA level regardless of the presence or absence of estradiol (Fig. 4B). To confirm the effects of BMP6 and BMP7 on steroid sulfatase protein expression, MCF-7 cells were treated with estradiol, BMP6, and BMP7 for 24 h, and then the cell lysates were subjected to SDS-PAGE/immunoblot analysis using steroid sulfatase-specific antibody. As shown in Fig. 5, BMP6 and BMP7 decreased the protein expression of steroid sulfatase. By contrast, estradiol had only minimal effects on steroid sulfatase expression in MCF-7 cells.

To compare the BMP signaling intensities in the presence of estrogen, the activation of BMP-induced SMAD1,5,8 signaling was quantified by luciferase analysis using the promoter activity of a BMP target gene *Id-1*. As shown in Fig. 6A, BMP2, BMP4, BMP6, and BMP7 (100 ng/ml) readily increased *Id-1*-Luc activity. Importantly, in the presence of estradiol (10–100 nM), the *Id-1* transcription induced by BMP2 and BMP4 was significantly impaired. *Id-1* activity induced by BMP6 and BMP7, however, was not influenced by estradiol treatment (Fig. 6A). Thus, the effects of estradiol on BMP2/4 signaling were clearly different from the effects on BMP6/7 signaling. The nuclear localization of the phosphorylated SMAD1,5,8 evoked by BMP6 and BMP7 was likewise not effected by the presence of estrogen (100 nM) action (Fig. 6B).

Figure 3 Effects of estrogen and BMP/activin on estrogen receptor mRNA expression in MCF-7 cells. Cells were treated with estradiol (1–100 nM), BMP2, BMP4, BMP6, BMP7, activin A (100 ng/ml), or the combination for 24 h. Total cellular RNA was extracted and (A) *ESR1* and (B) *ESR2* mRNA levels were examined by real-time RT-PCR. The expression levels of target genes were standardized by RPL19 level in each sample. Results are shown as mean $\pm$ S.E.M. of data from at least three separate experiments, each performed with triplicate samples. * $P < 0.05$ and ** $P < 0.01$ versus control and between the indicated groups.

Figure 4 Effects of estrogen and BMP/activin on aromatase and steroid sulfatase mRNA expression in MCF-7 cells. Cells were treated with estradiol (1–100 nM), BMP2, BMP4, BMP6, BMP7, activin A (100 ng/ml), or the combination for 24 h. Total cellular RNA was extracted and (A) aromatase and (B) steroid sulfatase mRNA levels were examined by real-time RT-PCR. The expression levels of target genes were standardized by RPL19 level in each sample. Results are shown as mean $\pm$ S.E.M. of data from at least three separate experiments, each performed with triplicate samples. * $P < 0.05$ and ** $P < 0.01$ versus control and between the indicated groups.

Estradiol effects on the expression patterns of BMP receptors were further examined. As shown in Fig. 7A, mRNA levels of *BMPR1A*, *BMPR1B*, *ACVR2A*, and *ACVR2B* were reduced by estradiol treatment (100 nM). In addition, cDNA array analysis revealed that the expression levels of BMP/activin ligands, including BMP6, BMP7, inhibin α , activin β A, and activin β B subunits, were relatively

Figure 5 Effects of estrogen and BMP on steroid sulfatase protein expression in MCF-7 cells. Cells were treated with estradiol (100 nM), BMP6, or BMP7 (100 ng/ml) for 24 h. Cells were then lysed and subjected to SDS-PAGE/immunoblotting analysis using anti-steroid sulfatase antibody, which detects a specific band with 64 kDa, and anti-Actin antibody as a control. The results shown are representative of those obtained from three independent experiments.

Figure 6 Effects of estrogen on BMP-SMAD1,5,8 signaling in MCF-7 cells. (A) Cells (1×10^4 viable cells) were transiently transfected with *Id-1*-Luc reporter plasmid (500 ng) and pCMV- β -gal. After 24-h treatment with BMP2, BMP4, BMP6, and BMP7 (100 ng/ml) in the presence or absence of estradiol (10–100 nM), cells were lysed and the luciferase activity was measured. The data were analyzed as the ratio of luciferase to β -galactosidase (β -gal) activity. Results are shown as mean $\pm$ S.E.M. of data from at least three separate experiments, each performed with triplicate samples. * $P < 0.05$ versus corresponding control. (B) Cells were precultured for 24 h and stimulated with BMP6 and BMP7 (100 ng/ml) in the presence or absence of estradiol (100 nM). Immunofluorescent (IF) studies were performed using anti-phospho-SMAD1,5,8 antibody on BMP-treated cells for 1 h. DAPI indicates counterstaining with 4',6'-diamidino-2-phenylindole. Bars indicate 20 μ m in size.

abundant (Fig. 7B), in which BMP6, BMP7, and inhibin/activin subunits were downregulated by estradiol exposure (10 and 100 nM) in MCF-7 cells. The reduction of *BMP6* and *BMP7* mRNA by estradiol treatment was confirmed by real-time PCR analysis (Fig. 7C). Thus, estrogen induces a differential pattern of key BMP signaling molecules, suggesting the existence of a possible functional link between estrogen and breast cancer BMP system. The estrogen-induced reduction of *BMPR1A*, *BMPR1B*, *ACVR2A*, and *ACVR2B* expression was most likely reflected in the decreased *Id-1* activation by BMP2 and BMP4 (Fig. 6A).

Figure 7 Effects of estrogen on the expression of BMP systems in MCF-7 cells. (A) After preculture, the cells were treated with or without estradiol (100 nM) for 24 h. Total cellular RNA was extracted and *ACVR1*, *BMPR1A*, *BMPR1B*, *ACVR2A*, *ACVR2B*, and *BMPRII* mRNA levels were examined by real-time RT-PCR. The expression levels of target genes were standardized by RPL19 level in each sample. Results are shown as mean $\pm$ S.E.M. of data from at least three separate experiments, each performed with triplicate samples. * $P < 0.05$ versus each control. (B) Total cellular RNAs were extracted from MCF-7 cells cultured for 24 h in the absence (control) or presence of estradiol (10 and 100 nM). Extracted RNAs (2.0 μ g) were used as templates for GEArray analysis (SuperArray Bioscience Corp.). The signal intensities of the spots on the membranes obtained from two separate experiments were analyzed using the GEArray analyzer program (SuperArray Bioscience Corp.) after subtraction of the background levels of pUC18 DNA. GAPDH is used as a positive control to compare the membranes. (C) Cells were treated with or without estradiol (10 and 100 nM) for 24 h. Total cellular RNA was extracted and changes of *BMP6* and *BMP7* mRNA levels were confirmed by real-time RT-PCR. The expression levels of target genes were standardized by RPL19 level in each sample. Results are shown as mean $\pm$ S.E.M. of data from at least three separate experiments, each performed with triplicate samples. * $P < 0.05$ versus each control.

Next, the involvement of MAPK activation in estrogen-induced cell proliferation was examined. Estradiol (100 nM) induced ERK1/ERK2, p38 MAPK, and SAPK/JNK phosphorylation, whereas BMP2, BMP4, BMP6, BMP7, and activin (100 ng/ml) did not affect these MAPK pathways (Fig. 8). By immunoblotting, the phosphorylated ERK1/ERK2, p38, and SAPK/JNK were rapidly observed 10 min after treatment with estradiol. Notably, treatment with

Figure 8 Effects of BMP/activin on estrogen-induced MAPK activation in MCF-7 cells. Cells (1×10^4 viable cells) were precultured for 24 h and stimulated with BMP2, BMP4, BMP6, BMP7, and activin A (100 ng/ml) in the absence or presence of estradiol (100 nM). After 10-min culture, cells were lysed and subjected to SDS-PAGE/immunoblotting analysis using anti-phospho-ERK1/2 (pERK1/2), anti-total-ERK1/2 (tERK1/2), anti-phospho-p38 (pp38), anti-total-p38 (tp38), anti-phospho-SAPK/JNK (pSAPK/JNK), anti-total-SAPK/JNK (tSAPK/JNK), anti-phospho-SMAD1,5,8 (pSMAD1,5,8), and anti-actin antibodies. The results shown are representative of those obtained from three independent experiments.

BMP6, BMP7, and activin specifically inhibited estradiol-induced phosphorylation of p38 MAPK (Fig. 8). In the same cell lysate, the presence of BMP signaling induced by each BMP ligand was confirmed by immunoblotting using anti-phospho-SMAD1,5,8 antibody.

To examine whether MAPK is functionally involved in MCF-7 cell mitosis, cells were treated with specific MAPK inhibitors including U0126, SB203580, and SP600125 (1–10 μ M) that suppresses ERK1/2, p38, and SAPK/JNK phosphorylation respectively. In addition, functional roles of PI3K–AKT signaling in MCF-7 cell proliferation were also examined using an AKT inhibitor, SH-5, and a PI3K inhibitor, LY294002. As shown in Fig. 9, treatments with SB203580 showed potent reduction of estradiol-induced cellular DNA synthesis in a concentration-dependent manner, compared with the effects of U0126 and SP600125. In contrast, the MAPK inhibitors showed equivalent reduction of basal mitotic activity of MCF-7 cells (Fig. 9). These findings suggest that p38 MAPK activation is functionally linked to cellular mitosis, in particular mitosis stimulated by estradiol. Treatment with SH-5 and LY294002 independently inhibited basal MCF-7 cell mitosis more potently than the MAPK inhibitors. The decreasing effects of SH-5 and LY294002 on basal cell mitosis were in parallel with those on estrogen-induced cell mitosis (Fig. 9), suggesting that PI3K–AKT pathway plays a key role

by transcriptional effects of nuclear ER (Nadal *et al.* 2001, Song *et al.* 2002, Gaben *et al.* 2004). Based on the rapid activation of estrogen-induced MAPK phosphorylation including ERK, p38, and SAPK/JNK pathways, nongenomic effects should be involved in the signal activation in MCF-7 cells. BSA-conjugated estradiol, which cannot bind to nuclear ER (Taguchi *et al.* 2004), also induced MCF-7 cell mitosis although the effects were less potent than those induced by unbound estradiol. Taken together, nongenomic effects of estradiol are also involved, at least in part, in the estrogen induction of cell proliferation. Immunoreactive ER antigen was reportedly detected on the surface of both naturally ER-positive cells and in cells transfected with ER expression constructs (Watson *et al.* 2002). ER-transfected cells also resulted in detectable membrane ER, in which estradiol mediates MAPK action, activation of the cAMP, and inositol-triphosphate pathways (Razandi *et al.* 1999). Considering that BMPs have also been shown to signal via the p38 class of MAPK in some cell types (Iwasaki *et al.* 1999, Lee *et al.* 2002), a functional crosstalk may exist between the breast cancer BMP system, ER signal, and MAPK pathway. In this regard, Yamamoto *et al.* (2002) reported that estrogen inhibition on BMP2-induced SMAD signaling was due to the direct physical interaction between SMADs and ER in MCF-7 cells. Further study would be needed to elucidate the molecular mechanism by which BMPs antagonize the MAPK activation through genomic and/or nongenomic actions induced by estrogen.

The modulation of ER expression may also be involved in the mechanism by which estrogen stimulates breast cancer cell proliferation. In the present study, we found that ESR1 expression was not only autoregulated by estradiol but also controlled by activin. Inhibitory effect of activin on MCF-7 cell mitosis could be partly due to the reduction of ESR1 expression. It was also notable that activin inhibited estradiol-induced phosphorylation of p38 MAPK signaling, which was similar to the effects elicited by BMP6 and BMP7. Thus, activin possibly plays an inhibitory role in estrogen-induced MCF-7 cell mitosis by reducing ESR1 expression as well as by inhibiting estrogen-induced MAPK phosphorylation. Estrogen exerts biologic actions, including broad changes in gene expression, through nuclear proteins called ER, which now include two subtypes, ESR1 and ESR2 (Nilsson *et al.* 2001). ESR1 is detectable in 40–70% of breast tumors and the presence of ESR1 protein has been a standard criterion for adjuvant therapy with antiestrogens that antagonize ER function and/or aromatase inhibitors (Shupnik 2007). In contrast, the roles of ESR2 have been obscure in the breast cancer tumorigenesis. ESR1 and ESR2 are structurally similar having high homology within the DNA and hormone-binding domains at 95 and 53% respectively (Keen & Davidson 2003). Although this domain-specific homology suggests that ESR1 and ESR2 are likely to share similar DNA and ligand-binding function, the low overall homology at 30% may indicate the global difference of their function. In the present study, the change of *ESR2* mRNA was not clearly detected by BMP/activin treatments regardless of estrogen action. Given the finding that the presence of ESR2 can be an

indicator for therapeutic responses in ESR1-positive tumors (Lin *et al.* 2007), *ESR2* may act by antagonizing *ESR1* on a specific subset of estrogen-stimulated genes, leading to the evasion of ESR1-stimulated cell growth.

In addition to the control of ER signaling, the regulation of estrogenic enzymes is effective to control the growth of breast cancer (Subramanian *et al.* 2008, Foster 2008). It has been widely accepted that breast tumors can synthesize 17 β -estradiol from adrenal androgen precursors. This occurs through the aromatization of androstenedione to estrone by aromatase, followed by the conversion of estrone to 17 β -estradiol by 17 β -hydroxysteroid dehydrogenase type 1 (Pasqualini 2004, Foster 2008). Breast cancer cells express both steroid sulfotransferase and steroid sulfatase activities, the latter of which can release estrone from estrone sulfate peripherally synthesized in adipose tissues. Pasqualini & Chetrite (2001) estimated that steroid sulfatase activity is by far greater than aromatase activity in both premenopausal and postmenopausal breast tumors. In our study, estrogen exposure downregulated not only ESR1 expression but also reduced steroid sulfatase expression in MCF-7 cells. Since steroid sulfatase activity contributes to accumulation of intra-tumor 17 β -estradiol, the expression level of steroid sulfatase can be a critical factor to estimate the growth activity of breast cancer. In the present study, BMP6 and BMP7 efficaciously suppressed steroid sulfatase expression in MCF-7 cells at mRNA and protein levels. However, the reduction of steroid sulfatase expression by BMPs was not directly reflected to the basal MCF-7 cell proliferation in serum-free conditions. Given that steroid sulfatase inhibitors are effective for inhibiting MCF-7 cell proliferation induced by estrone sulfate, i.e. a substrate for steroid sulfatase (Selcer *et al.* 1997, Rasmussen *et al.* 2007), the effects of BMP6 and BMP7 on steroid sulfatase expression may also contribute to the inhibition of estrogen-sensitive breast cancer cell proliferation.

Collectively, BMP6 and BMP7 antagonize estrogen-induced breast cancer cell proliferation through inhibiting p38 phosphorylation as well as estrogenic enzyme expression. These results suggest the existence of a novel functional crosstalk between the BMP system and estrogen signaling through MAPK in breast cancer cells. On the other hand, estrogen altered the BMP–SMAD signaling by downregulating specific BMP receptor expression in breast cancer cells. The inhibitory effects of BMP/activin on MAPK pathway and/or the expression of ER and estrogenic enzymes are most likely involved in the suppression of estrogen-induced mitosis of breast cancer cells.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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