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Functional interaction of fibroblast growth factor-8, bone morphogenetic protein and estrogen receptor in breast cancer cell proliferation

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ABSTRACT

Estrogen is involved in the development and progression of breast cancer. Here we investigated the effect of fibroblast growth factor (FGF)-8 on breast cancer cell proliferation caused by estrogen using human breast cancer MCF-7 cells. MCF-7 cells express estrogen receptor (ER)α, ERβ, FGF receptors, and Smad signaling molecules. Estradiol stimulated MCF-7 cell proliferation in a concentration-responsive manner, whereas BSA-bound estradiol had a weak effect on MCF-7 cell mitosis compared with the effect of free estradiol. It is notable that estrogen-induced cell proliferation was enhanced in the presence of FGF-8 and that the combined effects were reversed in the presence of an FGF-receptor kinase inhibitor or an ER antagonist. It was also revealed that FGF-8 increased the expression levels of ERα, ERβ and aromatase mRNAs, while estradiol reduced the expression levels of ERs, aromatase and steroid sulfatase in MCF-7 cells. FGF-8-induced phosphorylation of FGF receptors was augmented by estradiol, which was reversed by an ER antagonist. FGF-8-induced activation of MAPKs and AKT signaling was also upregulated in the presence of estrogen. On the other hand, FGF-8 suppressed BMP-7 actions that are linked to mitotic inhibition by activating the cell cycle regulator cdc2. FGF-8 was revealed to inhibit BMP receptor actions including Id-1 promoter activity and Smad1/5/8 phosphorylation by suppressing expression of BMP type-II receptors and by increasing expression of inhibitory Smads. Collectively, the results indicate that FGF-8 acts to facilitate cell proliferation by upregulating endogenous estrogenic actions as well as by suppressing BMP receptor signaling in ER-expressing breast cancer cells.

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1. Introduction

The involvement of estrogen in the development and progression of breast cancer is well known (Colditz, 1998; Keen and Davidson, 2003; Fortunati et al., 2010). 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, ERα and ERβ. Estrogen and ER complex mediate the activation of protooncogenes and oncogenes, nuclear proteins, and other target genes. However, there is no clear explanation regarding the direct effect of estrogen in the development of breast cancer.

Recent studies have demonstrated the presence of the transforming growth factor (TGF)-β signaling pathway in mammary cells and its importance in maintaining the growth state of these cells. There have been several studies showing the expression of some TGF-β superfamily proteins, such as bone morphogenetic protein (BMP)-2, -6 and -7, in breast cancer cells (Ye et al., 2009; Alarmo and Kallioniemi, 2010), and their possible roles in breast cancer development and in bone metastasis have been suggested. BMPs were originally identified as active components in bone extracts capable of inducing bone formation at ectopic sites. Recently, a variety of physiological BMP actions in endocrine tissues including the ovary, pituitary, thyroid, and adrenal have been clarified (Shimasaki et al., 2004; Otsuka, 2010; Otsuka et al., 2011). Attention has been paid to BMPs for their possible link with

Abbreviations: ActRI and ActRII, activin type-I and -II receptor; ALK, activin receptor-like kinase; Arom, aromatase; BMP, bone morphogenetic protein; BMPRI and BMPRII, BMP type-I and -II receptor; ER, estrogen receptor; ERK, extracellular signal-regulated kinase; FGF, fibroblast growth factor; MAPK, mitogen-activated protein kinase; SAPK/JNK, stress-activated protein kinase/c-Jun NH_2-terminal kinase; TGF-β, transforming growth factor-β.

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tumorigenesis, considering the actions of BMPs as regulators of cell proliferation and differentiation. The involvement of BMP-Smad activation in the progression and dedifferentiation of ER-positive breast cancer has also recently been reported (Helms et al., 2005). In this regard, we have reported that BMPs have inhibitory effects on estrogen-induced mitosis of MCF-7 breast cancer cells by inhibiting MAPK pathways and estrogenic enzyme expression (Takahashi et al., 2008). In that study, we found that BMP-6 and -7 antagonize estrogen-induced breast cancer cell proliferation through inhibiting p38 phosphorylation as well as estrogenic enzyme expression. On the other hand, estrogen altered the Smad signaling for BMP-2 and -4 by downregulating specific BMP receptor expression in breast cancer cells. These results suggest the existence of a functional crosstalk between the BMP system and ER actions in breast cancer cells.

Since the first FGF molecule was discovered as a mitogen for cultured fibroblasts, at least 23 distinct FGFs have been identified. FGFs play a critical role in morphogenesis by regulating cell proliferation, differentiation and cell migration during gastrulation and early differentiation of the brain, cranium, pharynx, heart, kidneys and limbs (Heikinheimo et al., 1994; Meyers et al., 1998; Sun et al., 1999; Eswarakumar et al., 2005). During embryonic development, FGF-8 is widely expressed in a temporally and spatially regulated manner (Heikinheimo et al., 1994; Crossley and Martin, 1995). In adult tissues, FGF-8 is expressed at low levels in limited tissues such as ovary and testis (Mattila and Harkonen, 2007); however, human breast, prostate and ovarian tumors can express FGF-8 (Dorkin et al., 1999; Marsh et al., 1999). The mechanism by which FGF-8 is expressed in hormone-responsive tissues including the breast and prostate has yet to be elucidated.

FGF-8 was originally cloned and characterized from androgen-dependent mouse mammary carcinoma cells (Tanaka et al., 1992). FGF-8 has been shown to induce cancer cell proliferation and tumor growth in cell culture (Tanaka et al., 1995; Mattila et al., 2001; Ruohola et al., 2001) and transgenic animal models (Daphna-Iken et al., 1998). Since overexpression of FGF-8 to breast cancer cells provides increased growth potential *in vitro* and *in vivo*, FGF-8 has also been categorized as an oncogene having transforming ability (Mattila and Harkonen, 2007).

During development, there are several organs in which BMP and FGF signals cooperate to regulate cell differentiation. During calvarial suture osteogenesis, FGF-2 augments BMP-4 signaling by suppressing the expression of a BMP antagonist, noggin (Warren et al., 2003). FGF-18 facilitates BMP-2 signaling by suppressing noggin mRNA expression in chondrogenesis (Reinhold et al., 2004). Other examples of cooperation between BMP and FGF signaling have been reported in the nervous system and in ectopic bone formation (Hayashi et al., 2003; Nakamura et al., 2005; Marchal et al., 2009). On the basis of results showing that FGFs and BMPs regulate cell differentiation cooperatively in a cell/tissue-dependent manner, we attempted to clarify the underlying mechanism of ER-sensitive breast cancer cell proliferation through the interrelationship between FGF-8 and the BMP system.

2. Materials and methods

2.1. Reagents and supplies

Dulbecco's Modified Eagle's Medium (DMEM), penicillin–streptomycin solution, 17β-estradiol, BSA-conjugated 17β-estradiol (estradiol-BSA) and ICI-182780 (also called fulvestrant) were purchased from Sigma–Aldrich Co., Ltd. (St. Louis, MO). Recombinant human BMP-7 and mouse FGF-8b were purchased from R&D Systems, Inc. (Minneapolis, MN) and SU5402 was purchased from Calbiochem (Gibbstown, NJ). Plasmids of Id-1-Luc and the expression plasmid for Smad4 (pcDEF3-Flag(N)-Smad4) were kindly provided by Drs. Tetsuro Watabe and Kohei Miyazono, Tokyo University, Japan.

2.2. Breast cancer cell culture

The human breast cancer cell line MCF-7 was from American Type Culture Collection (Manassas, VA). 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% CO_2. 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 phosphate-buffered saline (PBS), trypsinized, and counted using a coulter counter (Beckman Coulter Inc., Fullerton, CA). Changes in cell morphology and cell viability were monitored using an inverted microscope.

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

To prepare total cellular RNA, MCF-7 cells were cultured in 12-well plates (5×10^5 viable cells) and treated with indicated concentrations of estradiol and growth factors including BMP-7 and FGF-8 in serum-free DMEM. In the indicated experiments, 500 ng of an expression plasmid encoding wild-type Smad4 DNA or an empty vector was transfected using FuGENE 6 (Roche Molecular Biochemicals, Indianapolis, IN) for 24 h, and then cells were treated with BMP-7 and FGF-8 in serum-free conditions. After 24-h culture, the medium was removed, and total cellular RNA was extracted using TRIzol® (Invitrogen Corp., Carlsbad, CA), quantified by measuring absorbance at 260 nm, and stored at −80 °C until assay. The extracted RNA (1.0 μg) was subjected to an RT reaction using the First-Strand cDNA synthesis system® (Invitrogen Corp.) with random hexamer (2 ng/μl), reverse transcriptase (200 U), and deoxynucleotide triphosphate (0.5 mM) at 42 °C for 50 min and at 70 °C for 10 min. 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: ERα, 1393–1413 and 1632–1652 (from GenBank Accession #NM_000125); ERβ, 395–415 and 589–609 (from AB006590); aromatase (Arom), 914–934 and 1235–1256 (from M22246); steroid sulfatase (STS), 513–533 and 693–713 (from NM_000351); Id-1, 218–240 and 357–377 (from NM_012797) and a house-keeping gene, ribosomal protein L19 (RPL19), 401–420 and 571–590 (from NM_000981). PCR primer pairs for ALK-2, -3, -6, activin type-II receptor (ActRII), ActRIIB, BMP type-II receptor (BMPRII), Smad6 and Smad7 were individually selected for regular PCR and real-time PCR as we previously reported (Takahashi et al., 2008). Aliquots of PCR products were electrophoresed on 1.5% agarose gels and visualized after ethidium bromide staining. For the quantification of ERα, ERβ, Arom, steroid sulfatase, Id-1, ALK-2, -3, -6, ActRII, ActRIIB, BMPRII, Smad6 and Smad7 mRNA levels, real-time PCR was performed using LightCycler-FastStart DNA Master SYBR Green I system® (Roche Diagnostic Co., Tokyo, Japan) under conditions of annealing at 60–62 °C with 4 mM $MgCl_2$, following the manufacturer's protocol. Accumulated levels of fluorescence for each product were analyzed by the second derivative method after melting-curve analysis (Roche Diagnostic Co.), and then, following assay validation by calculating each amplification efficiency, the expression levels of target genes were quantified on the basis of standard curve analysis for each product. For each transcript, all treatment groups were quantified simultaneously in a single LightCycler run. To correct for differences in RNA quality and quantity between samples, the expression levels of target gene mRNA were normalized by dividing the quantity of the target gene by the quantity of RPL19 in each sample. The raw data of each target

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