Abstract. Bone morphogenetic protein-6 (BMP-6) is a multifunctional molecule with distinct abilities in embryogenesis and organogenesis. In the present study, our results showed that the rate of BMP-6-negative expression was 30.56% in breast cancer tissues, but was 9.58% in normal tissues by immunohistochemical staining. This implied that BMP-6 expression is absent in breast cancer tissues and may suppress breast cancer metastasis. In addition, stable overexpression of BMP-6 in MDA-MB-231 cells was established to analyze the metastatic ability. The Boyden chamber assay showed that BMP-6 inhibited the migration and invasion of MDA-MB-231 cells. Moreover, real-time PCR analysis showed that BMP-6 markedly downregulated matrix metalloproteinase-1 (MMP-1) expression at both the mRNA and protein levels in the MDA-MB-231 cells. Importantly, the results of luciferase and CHIP assays revealed that BMP-6 inhibited MMP-1 promoter activity through the AP-1 response element. In MDA-MB-231 cells treated with BMP-6, a significant decrease in the recruitment of AP-1 components, c-Jun/c-Fos, to the endogenous MMP-1 promoter was noted. We also demonstrated that BMP-6 inhibited the invasion of MDA-MB-231 cells, and this effect was significantly attenuated by overexpression of MMP-1. In contrast, MMP-1 knockdown by RNA interference or MMP-1 inhibitor exhibited an opposite effect. These observations suggest a novel role of BMP-6 in the inhibition of breast cancer metastasis by regulating secretion of MMPs in the tumor microenvironment.

Introduction

Breast cancer, a pathologically and clinically heterogeneous disease, is the most frequent malignancy diagnosed in women (1). Invasion and metastasis of breast cancer are the primary causes of treatment failure and mortality in women (2). Moreover, invasion and metastasis of cancer cells are complex multistep processes that involve cell adhesion, proteolytic enzyme degradation of the extracellular matrix (ECM) and production of growth factors that influence cell migration (3). Degradation of the basement membrane mainly by matrix metalloproteinases (MMPs) is considered to be a factor of crucial importance for breast cancer cell invasion and metastasis (4-6).

MMPs comprise a large family of zinc-dependent endopeptidases that have the capacity to cleave ECM. Owing to their matrix-degrading abilities and high expression in advanced tumors, the activity of MMPs has been shown to be required for breast cancer cell invasion and angiogenesis (4,7,8). Among the MMPs, MMP-1 has been reported to be upregulated in breast cancer cell lines with an enhanced ability of tumor growth, invasion and distant metastasis (9,10). Induction of MMP-1 by transcription factors, such as δ-crystallin enhancer factor 1 (δEF1) has been reported to contribute to enhanced cell migration and invasion (10). Therefore, factors with the ability to block MMP-1 activation may have therapeutic potential for the treatment human cancer.

Bone morphogenetic protein-6 (BMP-6) belongs to the TGF-β superfamily. The traditional BMP-6 signaling pathway is Smad-dependent (11). In addition to the Smad pathway, BMP-6 is also known to activate and crosstalk with other pathways, such as the MAPK pathway (12). In addition to its effect on inducing endochondral bone formation, BMP-6 has been shown to be involved in numerous biological processes. In our previous studies, we found that BMP-6 induced E-cadherin and microRNA-21 expression in MDA-MB-231 breast cancer cells, which are critical genes involved in breast cancer invasion and metastasis (13,14). Moreover, we confirmed that BMP-6 inhibits MMP-9 expression by regulating heme oxygenase-1 in MCF-7 breast cancer cells (15). MMP-9 is particularly known to play a critical role in breast cancer invasion and distant metastasis (16). Taken together, these results suggest that BMP-6 may play an important role in breast cancer invasion and metastasis.
In the present study, we demonstrated that the expression of BMP-6 was absent in breast cancer tissues. Moreover, BMP-6 inhibited MMP-1 expression in MDA-MB-231 cells at the transcriptional level, an effect that was mediated via reduction of the recruitment of the AP-1 components, c-Jun/c-Fos to the endogenous MMP-1 promoter. BMP-6 also blocked MDA-MB-231 cell invasion by inhibiting MMP-1 expression.

**Materials and methods**

**Immunohistochemical analysis.** Formalin-embedded breast cancer samples were obtained from the Department of Surgical Pathology of Tangshan People’s Hospital. Immunohistochemical analysis was performed on paraffin-embedded sections using the EnVision kit (Dako, Glostrup, Denmark) following the manufacturer’s protocols. Sections were boiled in retrieval solutions to expose the antigens. Polyclonal anti-BMP-6 (ab134723) and control primary antibodies were applied to the sections at a dilution of 1:50. The section-affixed slides were counterstained with hematoxylin, dehydrated and mounted.

**Cell culture.** Human breast cancer cell line MDA-MB-231 was maintained in Dulbecco’s modified Eagle’s medium (DMEM)-high glucose medium (Gibco) supplemented with 10% fetal bovine serum (FBS) (HyClone), penicillin and streptomycin. MDA-MB-231 cells were plated at a density of 2x10^4 cells/well into 24-well plates for use in the luciferase assays, and at a density of 8x10^4 into 6-well plates for western blotting and quantitative CHIP assays.

**Plasmid construction and transfection.** The constitutive vectors, pcDNA6B-BMP-6 (17), hMMP-1-WT and hMMP-1-mAP1 (10) were previously described. The full-length MMP-1 CDS was PCR amplified using the following primers: forward, 5'-AGCAAGCTTAGATGCACA GTTTTCCTCCAC-3' and reverse, 5'-AGTCTCGAGTCAAT TTTCCTGACGTTGAA-3'. The HindIII/XhoI site-tagged potential target sequence was then cloned into the HindIII/XhoI site of the pcDNA-6B vector.

The MDA-MB-231 cells were plated into 6-well plates and transfected with pcDNA6B-BMP-6 or pcDNA6B using Lipofectamine 2000 (Invitrogen). Transfected cells were resuspended in 10 ml of DMEM containing 10% FBS and seeded in a 10-cm-dish. Bacterial-resistant clones were isolated over a period of 3–4 weeks. Overexpression of BMP-6 was confirmed by quantitative RT-PCR and western blotting.

**Preparation of small interfering RNAs.** The siRNA target sequences of human BMP-6 and MMP-1 were, 5’-GGCGACAC CACAAAGAGTTCTT-3’ and 5’-GAGTACAACTTACATCG TG-3’ as previously reported (9,18). Sense and antisense oligonucleotides with the internal loop were synthesized (Takara). These were annealed and ligated into the BamHI and HindIII sites of pSilencer 4.1-CMV neo (Ambion) to construct the BMP-6- and MMP-1-specific siRNA expression plasmids, according to the manufacturer’s instructions. pSilencer 4.1-CMV neo expressing scrambled siRNAs (Ambion) were used as controls.

**Scratch-wound assay.** 231-control and 231-BMP-6 cells were seeded into a 6-well plate. For the scratch-wound healing assay, linear scratches were made in cell sheets with a pipette tip. After 24 and 48 h of growth, images were captured of the cells using a microscope.

**Migration and invasion assays.** Transwell 24-well chambers with 8-µm pore size (Costar) were used as recommended by the manufacturer. The membranes were precoated with collagen matrix (Sigma), which was reconstituted by adding 0.5 ml serum-free medium to the well for 2 h. To assess the invasive ability of the cells, 2.5x10^4 cells in 0.5 ml medium containing 1% FBS were placed into the upper compartment of the wells and 0.75 ml of medium containing 10% FBS was placed in the lower compartment. The Transwell chambers were incubated for 16 h at 37°C in a 5% CO₂ incubator. Cell penetration through the membrane was detected by staining the cells on the porous membrane with 0.25% crystal violet. To quantify the data, we washed the chamber twice with phosphate-buffered saline (PBS), and then used 33% acetic acid to wash off the excess crystal violet. Crystal violet remaining on the membranes was measured on a spectrophotometer at A570.

**RNA extraction and quantitative RT-PCR.** Total RNA was extracted from MDA-MB-231 cells that were treated with BMP-6 protein using the TRIzol reagent (Invitrogen). Total RNA (0.5 µg) from each sample was used for first-strand cDNA synthesis (M-MLV reverse transcriptase; Promega, Madison, WI, USA). Specific products of human MMP-1 (10), MMP-2 (10), MMP-9 (10), MMP-14 (19), TIMP-1 (20) and TIMP-2 (20) were amplified by quantitative PCR using the primers as reported. GAPDH was used as an internal control. Verification of the expression levels of genes was performed by quantitative RT-PCR using EvaGreen (Botium).

**ELISA.** MMP-1 protein expression levels were measured with an MMP-1 ELISA kit (cat. #QIA55; Calbiochem) according to the manufacturer’s instructions. To prepare samples for ELISA, cells were grown to 80% confluency and transfected with MMP-1-pcDNA6B, si-MMP-1 or treated with 2 µM MMP-1 inhibitor (cat. # 444250; EMD Chemicals, Gibbstown, NJ, USA) for 24 h; supernatants from the 48-h incubation were collected and concentrated 10-fold, using Amicon Ultra-4 centrifugal filters (Merck Millipore, Billerica, MA, USA). MMP-1 amounts were calculated as ng/ml protein.

**Western blotting and antibodies.** Preparation of total cell extracts and western blotting with the appropriate antibodies was performed as previously described (13). The following antibodies were used: rabbit polyclonal Ab against MMP-1 (sc-30069; Santa Cruz), mouse monoclonal Ab against actin (A-4700; Sigma), rabbit polyclonal Ab against c-Fos (sc-52), and rabbit polyclonal Ab against c-Jun (sc-1694) (both from Santa Cruz).

**Luciferase assay.** MDA-MB-231 cells were co-transfected with wild-type or mutant human MMP-1 promoter constructs into 24-well plates using Lipofectamine 2000. Cells were treated with TPA (100 ng/ml), curcumin (20 µM) or BMP-6 (100 ng/µl) for 6 h after transfection. Lysates were prepared
and the luciferase activity was then measured using the Dual-Luciferase Reporter Assay system (Promega) according to the manufacturer's instructions. Luciferase activity was normalized using the Renilla luciferase activity.

Quantitative chromatin immunoprecipitation (q-CHIP). ChIP assays were performed using reagents commercially obtained from Upstate Biotechnology (Lake Placid, NY, USA), essentially according to the manufacturer's instructions. The antibodies and primers used in these experiments were previously reported (10).

Results

BMP-6 expression is absent in a certain percentage of breast cancer tissues. To better understand the expression of BMP-6 in breast cancer, we collected 36 cases of advanced breast tumor specimens with lymph node metastasis, and 32 cases of paired or unpaired normal tissues. We detected the BMP-6 expression level in breast cancer and normal breast tissues by immunohistochemical staining. The expression of BMP-6 was represented by the numbers or percentages of negative cases. The results showed that the rate of BMP-6-negative expression was 30.56% in breast cancer, but was 9.58% in the normal tissues (Table I and Fig. 1). These results from clinical samples suggest that the absence of BMP-6 expression in a certain percentage of the breast cancer tissues may be closely related with the metastasis of certain breast cancer cases.

BMP-6 inhibits the migration and invasion of MDA-MB-231 cells. To confirm the potential role of BMP-6 in cell migration and invasion in vivo, MDA-MB-231 cells were stably transfected with the BMP-6 expression plasmid (231-BMP-6) or the empty vector (231-control). The overexpression of BMP-6 was confirmed by quantitative RT-PCR and western blotting (Fig. 2A). The morphology of 231-control and 231-BMP-6 breast cancer cells was detected under a light microscope. As shown in Fig. 2B, the 231-BMP-6 cells were arranged more closely than that of the 231-control cells. The results of the wound healing test showed that the motile ability of the 231-BMP cells was weaker than that of the 231-control cells (Fig. 2C). Furthermore, we examined the effect of BMP-6 on the ability of breast cancer cells to migrate through an artificial basal membrane using the Boyden chamber assay. The results of cell migration and invasion assays indicated that MDA-MB-231 cells overexpressing BMP-6 presented a distinct decrease in the number of migrating and invading cells in comparison to the empty-vector control (Fig. 2D).

BMP-6 inhibits MMP-1 expression at the transcriptional level. To understand the molecular changes, we performed quantitative RT-PCR to examine the effect of BMP-6 on MMPs and TIMPs in the MDA-MB-231 cells. MMP-1 has been described as a mediator that controls the ability of breast cancer growth, invasion and distant metastasis (9,10). In the present study, MDA-MB-231 cells were treated with different doses (50, 100 and 200 ng/ml) of BMP-6 protein. Total RNAs were collected at 24 h. As shown in Fig. 3A, BMP-6 downregulated MMP-1 expression in a dose-dependent manner. Then, MDA-MB-231 cells were treated with BMP-6 (100 ng/ml). Total RNAs were collected at 0, 12, 24 and 48 h. In the MDA-MB-231 cells treated with BMP-6 (100 ng/ml) for 12 h, an up to 40% decrease in the expression of MMP-1 mRNA was noted when compared to the basal level (Fig. 3B). The inhibition was further decreased to ~60% between 24 and 48 h (Fig. 3B). The expression of MMP-1 was significantly lower in the 231-BMP-6 cells compared with the level in the control group at both the mRNA and protein levels (Figs. 3C, 5A and B). Additionally, we examined BMP-6-regulated expression levels of another three MMP members, MMP-2, MMP-9 and MMP-14 in the MDA-MB-231 cells. However, as shown in Fig. 3D-F, BMP-6 slightly downregulated MMP-2, MMP-9 and MMP-14 expression at the mRNA level. The MMP system is counterbalanced by a group of four tissue inhibitors of metalloproteinases (TIMPs) that have varying specificities for individual MMPs (21). Thus, we further assessed the potential effect of BMP-6 on TIMP-1, TIMP-2, TIMP-3 and TIMP-4. The results of quantitative RT-PCR showed that BMP-6 only slightly upregulated the mRNA levels of TIMP-1 and TIMP-2 (Fig. 3G and H). BMP-6 resulted in an up to 3-fold increase in the expression of TIMP-1 mRNA at 48 h, compared to the basal level (Fig. 3G). TIMP-3 and TIMP-4 mRNA levels were not significantly affected (data not shown). The above observations suggest there may be a dual effect of BMP-6 in promoting MDA-MB-231 cell migration, through concurrently downregulating MMPs and upregulating TIMP-1.
and TIMP-2. However, inhibition of MMP-1, which was specifically and significantly mediated by BMP-6, contributed to induce the metastasis of breast cancer.

*BMP-6 inhibits MMP-1 activity through AP-1.* Having found that BMP-6 inhibits MMP-1 expression in MDA-MB-231 cells, we next assessed whether BMP-6 is a true suppressor of MMP-1 transcription using reporter gene assays. As shown in Fig. 4B, a reporter gene assay showed that BMP-6 significantly inhibited human MMP-1 promoter activity of the wild-type -159/+19 reporter gene. The inhibition was >50% following BMP-6 treatment, compared to that of the control. However, knockdown of BMP-6 using RNA interference resulted in increased luciferase activity of the human MMP-1 promoter, compared to that of the si-control (Fig. 4B). In order to clarify the regulatory mechanism by which BMP-6 affects MMP-1
expression, we analyzed the MMP-1 promoter using the online tools TRANSFAC (http://www.generegulation.com) and thTESS (http://www.cbil.upenn.edu/cgi-bin/tess/tess). Based on our online analysis, we found that there was an AP-1 binding site (CATGAGTCAG) at the position -70/-60 of the MMP-1 promoter (Fig. 4A). Our previous results indicated that BMP-6 inhibited the expression of microRNA-21 via reduction in the expression of δEF1 and c-Fos/c-Jun (22). In the present study, luciferase assay results indicated that TPA (activator of the AP-1 signaling) upregulated the promoter activity...
of MMP-1, whereas curcumin (inhibitor of AP-1 signaling) repressed its activity in the MDA-MB-231 cells (Fig. 4C), suggesting a potential effect of the AP-1 pathway to mediate MMP-1 transcription. Thus, the AP-1 element on the human MMP-1 promoter was mutated to generate the MMP-1-mAP-1 construct (10). The results of the luciferase assay demonstrated that the mutation of the AP-1 element on human MMP-1 promoter totally depleted its response to TPA, curcumin and BMP-6.
BMP-6 (Fig. 4D). We investigated whether BMP-6 affects c-Fos or c-Jun expression at the protein level. Western blotting experiments demonstrated that both c-Fos and c-Jun expression was obviously inhibited in the 231-BMP-6 cells (Fig. 4E). Importantly, the ChIP assays indicated that c-Fos/c-Jun was able to bind to the MMP-1 promoter during basal conditions in an AP-1 site-dependent manner (Fig. 4F). The result of the q-CHIP assay showed that the binding of c-Fos/c-Jun to the MMP-1 promoter was decreased by BMP-6 induction (Fig. 4G).

**Discussion**

Breast cancer is a leading cause of cancer-related mortality among women worldwide, and is the second most common metastatic cancer, frequently metastasizing to the bone, lung, liver and brain (23). Moreover, distant metastases account for most incidences of breast cancer recurrence and are often the cause of death in breast cancer patients (24,25). Due to clinical importance, the detection and treatment of breast cancer metastasis have been urgently researched. We previously uncovered that BMP-6 exhibits a potential inhibitory effect on breast cancer invasion and metastasis (13,26). However, little is known concerning the mechanisms of BMP-6 in breast cancer metastasis. In the present study, we provided novel evidence that BMP-6 suppressed breast cancer metastasis by downregulating MMP-1 expression in MDA-MB-231 cells. We further demonstrated that this effect was mediated through decreasing the binding of c-Fos/c-Jun to the MMP-1 promoter. Since MMP-1 has been confirmed as a factor that facilitates breast cancer metastasis, our research provides a novel function of BMP-6, namely, acting as an MMP-1 inhibitor in breast cancer progression. This regulatory mechanism by BMP-6 has clinical significance in breast cancer progression and metastasis research.

It has been known that MMPs are expressed in nearly all tumors, where they facilitate tumor growth, invasion and metastasis (7,8). In breast cancer, short hairpin RNA-mediated
stable knockdown of MMP-1 or induction of MMP-1 by over-expression of δ-crystallin enhancer factor 1 (δEF1) was found to significantly regulate the invasive ability of MDA-MB-231 cells (9,10). These findings are consistent with our present study that inhibition of MMP-1 by BMP-6 exhibited an antitumorigenic effect to inhibit MDA-MB-231 cell invasion in the Boyden chamber assay. Collectively, these observations suggest that MMP-1 inhibitors, such as BMP-6, may play roles as agents that inhibit tumor invasion and metastasis, with the potential for further therapeutic development.

Recently, a number of studies have shown that δEF1 is closely associated with the malignant progression of breast cancer (10,27,28). We previously reported that δEF1 functions primarily as an inducer of EMT, and δEF1 promotes osteolytic metastasis of MDA-MB-231 breast cancer cells by regulating MMP-1 expression (10,13,28). Moreover, BMP-6 has been implicated as an antimetastatic factor, involving the transcriptional repression of δEF1 in MDA-MB-231 cells (22). In the present study, we demonstrated that BMP-6 inhibited the metastasis of MDA-MB-231 cells by downregulating MMP-1 expression. Our results collectively indicate a mechanism of the BMP-6/δEF1/MMP-1 cascade involved in the regulation of breast cancer metastasis. Factors that regulate δEF1 or BMP-6 levels would subsequently change the expression of MMP-1, thus altering the metastatic ability of breast cancer cells.

In conclusion, we provide novel findings of a potential mechanism for BMP-6-regulated metastasis of breast cancer, which in effect is mediated through the reduction in MMP-1 expression in a paracrine manner. Thus, BMP-6 activation may decrease the metastatic ability of breast cancer cells. However, the interactions among cellular factors occurring in the metastasis of breast cancer are far more complex in vivo. Further investigation in vivo is required to elucidate the exact role of BMP-6 in breast cancer metastasis.

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