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Bone Morphogenetic Protein 6 Inhibit Stress-Induced Breast Cancer Cells Apoptosis Via Both Smad and P38 Pathways

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Abstract Breast carcinoma is one of the most common malignant tumors and has become a more common cancer in women. BMP6 was abnormally expressed in breast cancer specimens and cell lines. However, the contribution of BMP6 in promoting breast cancer progression remains unknown. The purpose of our study was to establish whether expression of BMP6 in breast cancer cells affect their proliferation or apoptosis and the mechanism. We found that BMP6 inhibited proliferation of MDA-MB-231 cells and blocked cell cycle at G_0/G_1 stage. BMP6 also inhibited serum deprivation induced apoptosis in MDA-MB-231 cells. At the 4 days of serum starvation, BMP6 reduced the percentage of caspase-3 positive cells from 49% to 21%, BMP6 also reduced sub-G_1 peak induced by serum starvation. In contrast, BMP6 significantly enhanced survivin expression both at mRNA and protein levels. Dominant negative-survivin and Antisense-survivin impaired BMP6 induced antiapoptotic effect. BMP6 enhanced survivin expression at the transcription level in a Smad-dependent manner. BMP6 also played its antiapoptotic effect through activation p38 MAPK signal pathway, independent of smad/survivin pathway. These results suggested that BMP6 induced cell cycle arrest in estrogen-insensitive breast cancer cells. BMP6 inhibits stress-induced apoptosis via both Smad and p38 signal pathways. J. Cell. Biochem. 103: 1584–1597, 2008.

Key words: BMP6; breast cancer; apoptosis; proliferation; survivin; p38

Each year, approximately 200,000 women are diagnosed with breast cancer in the USA, and almost 800,000 are diagnosed worldwide [Parkin et al., 1999; Rosen et al., 2003]. Breast cancer, the third most common cancer in the world, along with other cancers including lung and prostate cancer, preferentially metastasize to bone [Coleman, 1997]. The growth of breast cancer cells is regulated by a variety of steroid hormones. In the late stage of hormone-dependent breast cancer cells, the cells become hormone-independent. MDA-MB-231 cells were originally derived from a metastasized human breast carcinoma and are used as a cell model to investigate the role of BMP6 in cell apoptosis in breast cancer cells.

BMP6 belongs to the 60A subgroup of BMP family. BMPs are members of the transforming growth factor β (TGF-β) super family. They were found not only to be potent inducers of endochondral bone formation and key regulators of bone metabolism [Urist, 1965; Wozney et al., 1988]; but also have pleiotrophic roles in cell growth, differentiation, migration, and apoptosis and are critical in embryogenesis and organogenesis [Celeste et al., 1990; Ducy and Karsenty, 2000]. BMP6 signals through heterodimerized type I and type II serine-threonine kinase receptors, which lead to the phosphorylation of the Smads. The receptor activated Smad proteins then form complexes with the co-Smad (Smad4) and are translocated into the nucleus where they regulate downstream target genes [Waite and Eng, 2003; Chen et al., 2004]. In addition to this Smad pathway, BMP6 is also known to activate and cross-talk with other pathways, such as the MAPK pathway [Derynck and Zhang, 2003].

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In addition to its effect on inducing new bone formation, BMP6 has been shown to be involved in numerous biological processes. BMP-6 may mediate the inductive interactions between mesenchymal and epithelial cells [Hogan, 1996]. BMP6 may also be involved in developmental processes of embryonic kidney and urinary systems [Bitgood and Mcmahon, 1995], differentiation of keratinocytes [Drozdoff et al., 1994], neural development [Jones et al., 1991], and liver growth and differentiation [Knittel et al., 1997]. BMP6 also affects cell proliferation and apoptosis in different cell types. BMP6 was found to inhibit cell proliferation in keratinocytes [Gosselet et al., 2007], prostate cancer cells [Haudenschild et al., 2004] and myeloma. Lories reported that BMP6 inhibits NO-induced apoptosis in fibroblast-like synoviocyte [Lories et al., 2003]. Our own work demonstrates that BMP6 inhibits neuron cell apoptosis via the MAPK pathway.

It has been reported that abnormal expression of BMP6 may be related to the tumor development and malignance. BMP6 is abnormally expressed in breast cancer [Clement et al., 1999; Akiyoshi et al., 2004], prostate cancer [Dai et al., 2005] and neoplasm of salivary gland tissues [Hatakeyama et al., 1997]. The facts demonstrate there is relativity between BMP6 expression and development of tumor. Tateyama et al. [2001] proposed BMP-6 expression may increase in complex adenomas and benign mixed tumors in canine mammary glands. It is reported that BMP6 expressed in most tumor specimens [Clement et al., 1999] and in many breast cancer cell lines [Arnold et al., 1999]. BMP-6 expression is serum-dependent and could be stimulated by EGF and other EGFR ligands under serum-free conditions [Clement et al., 1999]. However, only a limited amount of information is available on the effect of BMP6 on proliferation or apoptosis behavior of breast cancer cells.

Survivin is a member of the inhibitor of apoptosis (IAP) family. It is involved in both control of cell division and inhibition of apoptosis [Ambrosini et al., 1997; Adams and Cory, 1998]. Its anti-apoptotic function is related to the ability to inhibit caspase activity directly or indirectly [Tamm et al., 1998]. It is undetectable in most normal adult tissues but highly expressed in human cancers, such as breast cancer, prostate cancer [Li, 2003]. P53 could induce apoptosis through down-regulate survivin [Zhou et al., 2002]. Survivin down-regulation under serum-starvation related enhanced apoptosis [Yang et al., 2005].

The Smad pathway may not be viewed at a unique mean for BMP6 regulate cellular functions, as other signaling pathways including the mitogen-activated protein kinase (MAPK), the NF-κB pathways, can either be induced by BMP6, or can modulate the outcome of BMP6-induced Smad signaling [Javelaud and Mauviel, 2005]. So we focus our sight on the two pathways. Besides this, among the regulators of apoptosis that may participate in cancer, interest has been recently focused on Survivin because of its predominantly cancer-specific expression in adult human organ tissue. And survivin also was reported associated with BMP signal pathway in prostate cancer [Yang et al., 2005]. So we focus our sight on these pathways.

We initiated the studies described in this report to better understand the effect and mechanisms of BMP6 on breast cancer cells. In our experiment, we used MDA-MB-231 cells, which are estrogen-independent, maintain the responsiveness to EGF. Across our experiments, we found BMP6 inhibited proliferation of MDA-MB-231 cells and induced G_0–G_1 stage arrest. Corresponding, BMP6 decreased apoptosis induced by serum-starvation in MDA-MB-231 cells. The antiapoptoic effect was due to increase of transcription of survivin on Smad dependant manner and activation of p38 MAPK signal pathway. The two different mechanisms were not interacting.

MATERIALS AND METHODS

Cell Lines and BMP6

MDA-MB-231 cells (purchased from China Center for Type Culture Collection) were cultured as ATCC described. Human recombinant BMP6 protein (BMP6) was bought from R&D, Inc. One hundred nanograms per milliliter BMP6 was used in all experiments for the analysis of signaling from BMP6 exposure.

Cell Proliferation Assay

Cells (0.5×10^5) were plated in each well of the six-well plate in triplicates in the absence or presence of 100 ng/ml BMP6. Cells were counted using CCK-8 (Dojindo Molecular Technologies, Inc.) every 2 days. The culture medium was changed and BMP6 replenished every 2 days.

Cell Cycle Assay

Cells were typsinized then washed twice with PBS. Cells was fixed in 70% ethanol on ice for 30 min then stored at −20°C overnight. After cells were stained with propidium iodide/RNase A solution (Sigma–Aldrich Co.) in the dark for 30 min at room temperature, samples were then analyzed by a Flow Cytometry system.

RNA Preparation and Quantitative PCR

Total RNAs were extracted by TRIzol reagent (Invitrogen, Inc.) following the protocol recommended by the manufacturer. The RNA (2 μg) was reverse transcribed by oligo(dT)20 and M-MLV reverse transcriptase (Promega, Inc.) in a volume of 25 μl. The synthesized cDNA was subjected to PCR with the primers described in Table I. Real-time PCR was performed using EVER Green Supermix (Biotium, CA) according to the instructions of the manufacturer. GAPDH cDNA amplification was used as Internal Positive Control.

APOPTOSIS ANALYSIS

DNA Assay

Cells were trypsinized and washed with phosphate-buffered saline (PBS). The cells were fixed in 70% ethanol for 30 min at 0°C, centrifuged at 1,500*g* for 4 min, washed with PBS containing 0.5 mM EDTA and resuspended in PBS. For nuclear staining with propidium iodide, the cells were treated with 50 μl of 1 mg/ml RNase A (Sigma) followed by 100 μl of 100 μg/ml propidium iodide. The cells were kept at −20°C for 18–24 h before they were analyzed by flow cytometry on FAScalibur (Becton Dickinson Immunocytometry Systems, San Jose, CA). Cells were illuminated with 200 mW of light at 488 nm produced by an argonion laser and the fluorescence was read using a 630/22 nm band-pass filter. Data were analyzed for 20,000 viable cells as determined by forward and right angle light scatter and were stored as frequency histograms and subsequently analyzed by MODFIT software (Verity, Topsham, ME).

Caspase-3 Activity Assay

Apoptosis extent of cells was assayed using PE-conjugated monoclonal active-Caspase-3 antibody apoptosis Kit (BD Pharmingen™) following the instructions of the manufacturer.

Western Blot Analysis

To prepare the whole cell lysates, cells were lysed in ice-cold buffer containing 1% Triton X-100, 150 mmol/L NaCl, 10 mmol/L Tris-HCl (pH 7.4), 1 mmol/L EGTA, 1 mmol/L EDTA, and 0.5% NP40 with freshly added proteinase inhibitor (Roche) and phosphatase inhibitor cocktails (Sigma–Aldrich Co.). After 30 min on ice, cell debris was removed by centrifugation at 14,000*g* for 10 min. The antibodies used in the Western blots were anti-Survivin (Santa Cruz Biotechnology), anti-ERK, anti-phospho-ERK, anti-JNK, anti-phospho-JNK, anti-p38, anti-phospho-p38, (BD Pharmingen™), and anti-actin (Sigma–Aldrich Co.).

Plasmids Construction

Fragments of the 5′flanking region of the survivin gene were generated by PCR. All fragments share the same 3′ending site at +58 of the survivin transcription start sites. The survivin fragments were inserted into PGL4-basic plasmid in the forward orientation upstream of a luciferase reporter gene to generate different length constructs named pLuc-1703, pLuc-1174, pLuc-757, pLuc-600, pLuc-300, and pLuc-180.

Transient Transfection and Luciferase Assays

Briefly, 1×10^7 MDA-MB-231 cells in exponential growth were mixed with the corresponding survivin promoter-luciferase construct plus different doses of smad5 or DN-smad5 expression vectors and electroporated at 360 V, 950 μF using a Gene Pulser II System (Bio-Rad, Hercules, CA). Transfected cells were resuspended in 10 ml of DMEM medium containing 20% FBS then cells were seeded in a six-well plate. Cells were treated with 100 ng/ml BMP6 after cells adhering to plate. At 48 h post-transfection, cell

TABLE I. Primers Used in Experiment

	Forward primer	Reverse primer
Bax	TTTCATCCAGGATCGAGC	GCCTTGAGCACCAGTTTG
Bcl-2	ATGCCAAGGGGGAAACACC	GCCAGGAGAAATCAAACAGA
Survivin	CCACTTCCAGGGTTTATTC	GCCAGACGCTTCCTATCA
GAPDH	ACCACAGTCCATGCCATCAC	TCCACCACCCTGTTGCTGTA

extracts were prepared with lysis buffer (Promega), and then 20 μl aliquots of the supernatant were mixed with 100 μl of luciferase assay reagent (Promega) and analyzed on a microplate luminometer. Luciferase activity was normalized to Renilla activity as an internal control.

Statistical Analysis

All experiments were done in triplicates and repeated at least three times. Statistical analyses were preformed by the Student t-test and analysis of variance (ANOVA) where $P < 0.05$ was considered significant (*).

RESULTS

BMP6 Inhibit Proliferation of MDA-MB-231 Cells

We first examined the effect of BMP6 on the growth of breast cancer cells in culture. MDA-MB-231 cells were selected for the study. BMP6 (100 ng/ml) significantly inhibited MDA-MB-231 cells proliferation, up to 30% at 144 h time point (Fig. 1a). The growth inhibitory activity of BMP6 in MDA-MB-231 cells was dose dependent at the range of 50–200 ng/ml (Fig. 1b). To test whether this growth inhibition reflects attenuation of the cell cycle, MDA-MB-231 cells which were treated with 100 ng/ml BMP6 for 144 h were subjected to cell cycle analysis. As shown in Figure 1c, BMP6 significantly reduced the percentage of cells in the S-G_2-M phases of the cell cycle, indicating BMP6 blocks cells at the G_0–G_1 phase.

BMP6 Protect MDA-MB-231 Cell Against Serum Deprivation-Induced Apoptosis

We then investigated the effect of BMP6 on serum deprivation-induced apoptosis. Cells were cultured in medium containing 0.1% serum in the absence or presence of 100 ng/ml BMP6 for 4 days. Serum-starved MDA-MB-231 cells appeared mostly rounded up and feeble. In contrast, the BMP6-pretreated, serum-starved cells appeared more robust. To test whether BMP6-treatment inhibited apoptosis of serum-starved cells; caspase-3 activity assay was performed by Flow Cytometry. As shown in Figure 2a, along with serum-starvation extended, the percentage of caspase-3 positive cells slightly increased, and treatment with BMP6 (100 ng/ml) markedly decreased caspase-3 activity and increased cell survival. At the 96 h after serum starvation, the percentage of caspase-3 positive cells was reduced from 49% to 28% in the presence of BMP6. In addition to caspase-3 activity assay, we also examined apoptosis by DNA degradation assay. We found that 100 ng/ml BMP-6 obviously decreased the sub-G_1 diploid peak induced by serum starvation in MDA-MB-231 cells (Fig. 2b). We also tested the expression of some pro-apoptotic and anti-apoptotic genes via Real-time PCR. We

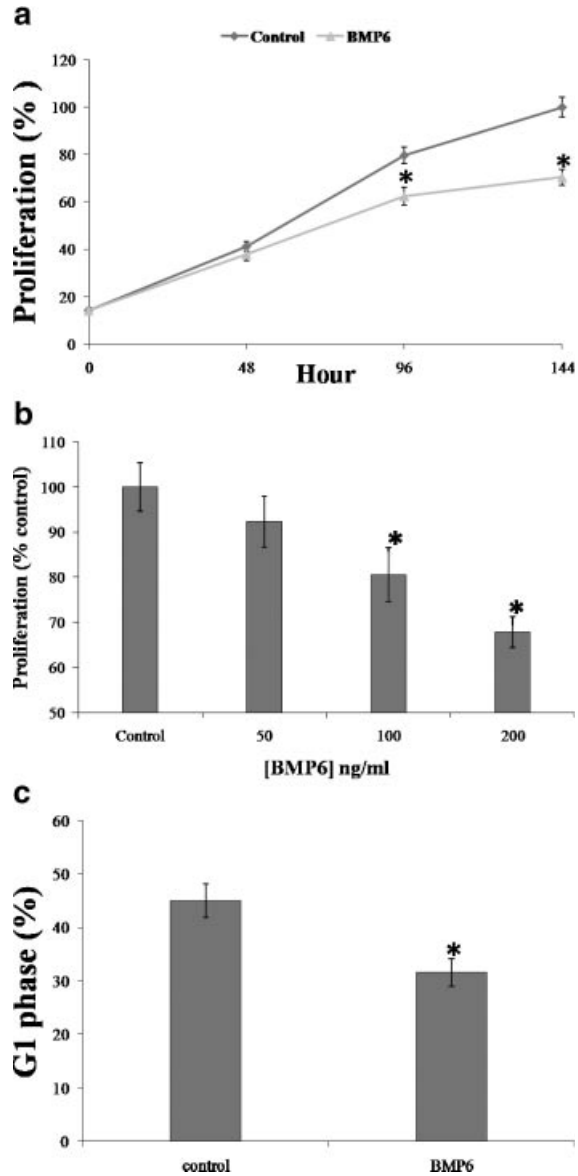


Fig. 1. Inhibition of MDA-MB-231 cells proliferation by BMP6. **a**: MDA-MB-231 cell growth in medium containing 0.1% serum was determined by counting the cell number every 48 h in the absence or presence of 100 ng/ml BMP6 protein. **b**: Anti-proliferation effect of exposure to various levels of BMP6 (50, 100, and 200 ng/ml) at the 96 h of treatment was determined. **c**: Cell cycle assay was done to examine the percentage of cell number at G_1 phases in MDA-MB-231 cells treated with 100 ng/ml BMP6 for 96 h relative to the control untreated cells.

examined expression of Bcl-2, Bax, and survivin using Q-PCR. There were no changes in Bcl-2 and Bax expression levels (data not shown). In contrast, there was a progressive decline in the level of survivin in the serum-starved control MDA-MB-231 cells (Fig. 2c). Treatment with BMP6 significantly reversed survivin reduction induced by serum-starvation (Fig. 2c).

BMP6 Up-Regulates Survivin Transcription in Serum-Starved MDA-MB-231 Cells

Since BMP6 pretreatment prevents the reduction of survivin mRNA induced by serum starvation, we next examined whether BMP6 increases survivin protein level. The result demonstrated that BMP6 rescued survivin expression both at mRNA and protein level in serum-starved MDA-MB-231 cells (Fig. 3a). The enhancement of survivin mRNA expression might occur at a post-transcriptional level such as stabilization of survivin messenger or by stimulation of transcriptional activity of survivin promoter. So survivin mRNA levels affected by BMP6 was examined in the presence of actinomycin D and cycloheximide, transcriptional, and translational inhibitor. When serum-starved MDA-MB-231 was incubated with optimal dose of BMP6 and 5 μg/ml actinomycin D for 48 h, the rescue of survivin mRNA level by BMP6 in previous experiment was inhibited, but 10 μg/ml cycloheximide did not affect this phenomenon (Fig. 3b). These results suggest that BMP6 might regulate survivin gene at transcriptional level. Because survivin expression was cell cycle regulated, it was possible that BMP6 might influence survivin expression by affecting the cell cycles. So we also examined whether cell cycle of MDA-MB-231 cells was affected by BMP6 at serum-starved conditions. We found percentage of G_1 cells increased from 50% to 69% when serum-starvation for 2 days, but there was no significant difference between control and BMP6 treatment (Fig. 3c).

Effects of Antisense Survivin and Dominant-Negative Mutant Survivin Expression on Protection of BMP6

BMP6 protected MDA-MB-231 cell against serum-deprivation-induced apoptosis might be related to the increase of survivin expression. We next tested the effects of two plasmids designed to inhibit survivin expression or function on the effect of BMP6. One plasmid, an antisense survivin expression plasmid, was expected to block the translation of endogenous survivin messenger RNA transcripts and, consequently, survivin expression. The other plasmid, an expression plasmid encoding a dominant-negative mutant form of survivin, was expected to block endogenous survivin function by competitively binding to survivin effectors. We used the two plasmids to investigate the role of survivin in BMP-induced antiapoptotic effect. We found both plasmids obviously abrogated the inhibitory effect of BMP6 on serum starvation-induced cell apoptosis (Fig. 4). The results are consistent with our findings that BMP6 protected MDA-MB-231 cell apoptosis.

BMP6 Enhances Survivin Expression in a Smad5-Dependent Mechanism

BMP signaling is mediated by type I and type II serine-threonine kinase receptors. Once the type I/type II receptor-ligand complex is assembled, the intrinsically active kinase of the type II receptor phosphorylates the intracellular domain of the type I receptor, thereby activating its kinase activity. The activated type I receptor propagates the signal by phosphorylating Smad proteins. In order to clarify whether BMP6-induced survivin expression in serum-starved MDA-MB-231 cells is Smad-dependent, wild-type and dominant-negative (DN) Smad5 was transfected into MDA-MB-231 cells. As shown in Figure 5a,b, although Smad5 has minor effect on BMP6-induced survivin expression, DN-Smad5 significantly inhibited BMP6-induced survivin mRNA and protein expression. We then constructed a luciferase reporter construct named pLuc1703. The plasmid contained 1,703-bp DNA sequence upstream of survivin gene from transcription start site. By transient transfection of pLuc1703 plasmid into MDA-MB-231 cells, we found that serum starvation suppressed survivin promoter activity by 42% and this effect can be reversed by addition of BMP6 (Fig. 5c). We also determined the effect of Smad5 on pLuc1703 promoter activity. Indeed, BMP6-induced up-regulation of survivin promoter activity was counteracted by the expression of dominant-negative Smad5 in a dose-dependent manner (Fig. 5c). Figure 5d demonstrated that Smad5 and DN-Smad5 protein was expressed in these cells. Our findings indicate that BMP6 enhances survivin expression in a Smad-dependent manner. We

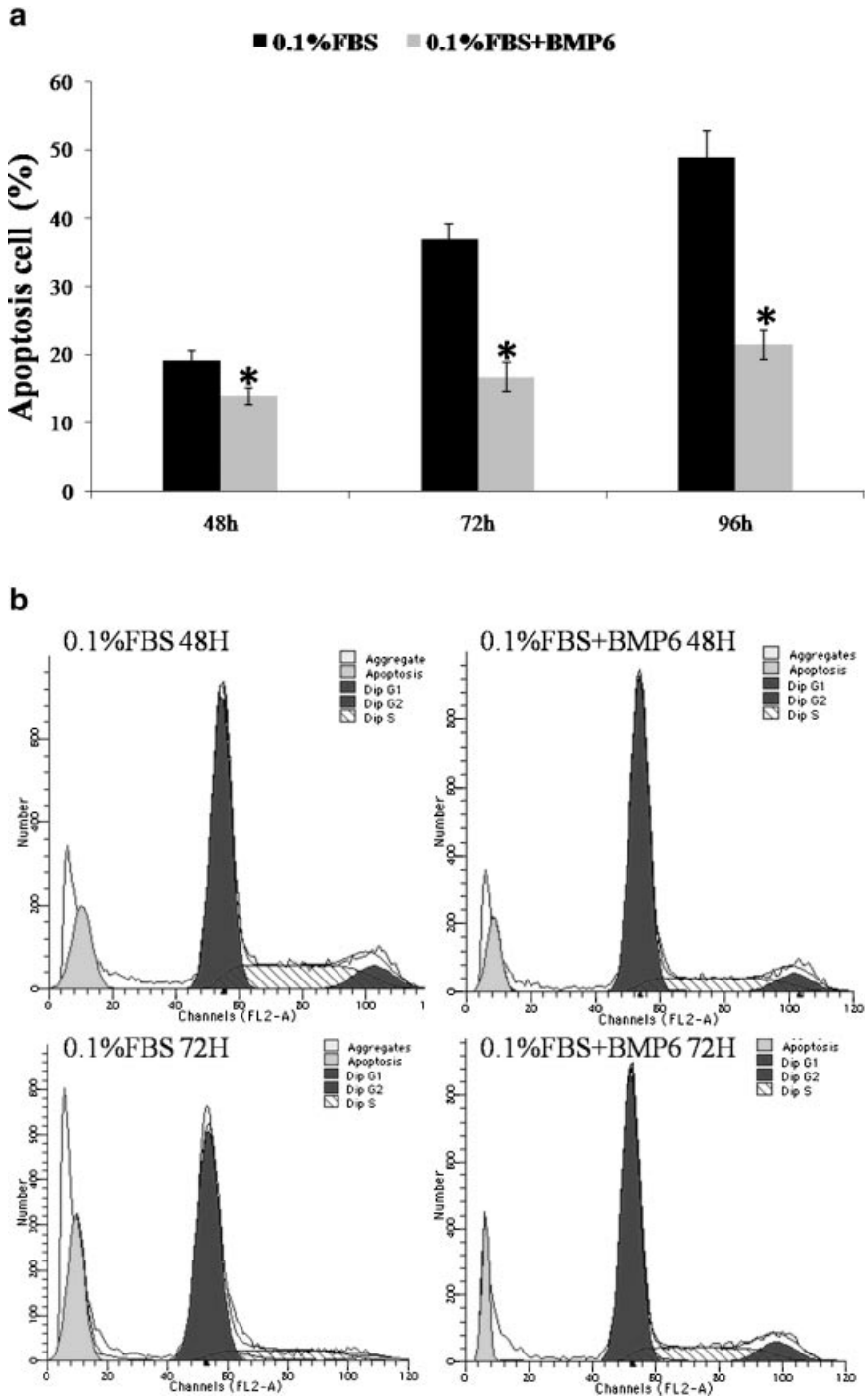


Fig. 2. BMP6 inhibited serum starvation–induced apoptosis in MDA-MB-231 cells. Serum-starved MDA-MB-231cells were treated with or without 100 ng/ml BMP6. **a**: At different time point, cells were collected and the caspase-3 activity was examined by Flow Cytometry. **b**: At different time point, cells were collected and the DNA degradation were assayed by Flow Cytometry. **c–e**: Levels of expression of Bcl-2, Bax and survivin at different time points after serum starvation were determined by Real-time PCR.

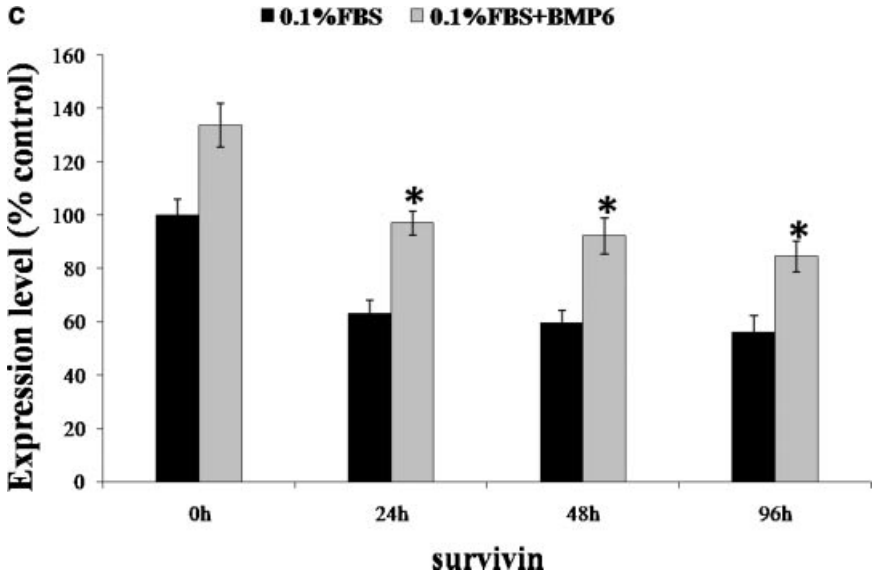
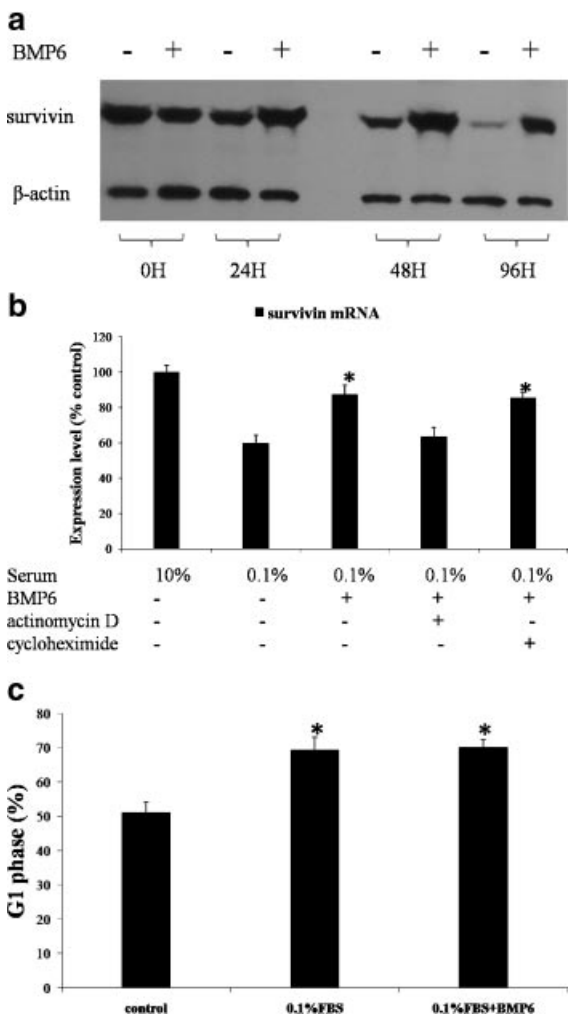


Fig. 2. (*Continued*)



also performed sequential deletion analysis of survivin promoter by constructing five survivin promoter deletion constructs (pLuc1174, pLuc757, pLuc600, pLuc300, and pLuc180). BMP6 stimulated the promoter activity of pLuc1174, pLuc757, and pLuc600 but lost its stimulatory effect on pLuc300 and pLuc180 (Fig. 5e). These results suggest that BMP6 responsive element may be located in the −600/−300 region of the survivin promoter.

BMP6 Inhibits MDA-MB-231 Apoptosis Through Enhancing p38 Activity

In addition to Smad-dependent mechanism, BMPs also play their roles in a Smad-independent manner. BMPs have been shown to activate ERK, p38 and JNK MAPKs in numerous cell types [Wakefield et al., 2001; Derynck and Zhang, 2003]. Therefore, we also examined the influence of serum starvation or BMP6 treatment on MAPK signal pathway by Western blot analysis. BMP6 had no effect on ERK activity but activated JNK activity. The effect of BMP6 on JNK activity was declined to the control level 48 h after serum starvation. BMP6 enhanced p38 activity which was not affected by the serum starvation (Fig. 6a). To examine whether

Fig. 3. Survivin was regulated by BMP6. **a**: Western blot analysis indicated survivin level affected by BMP6 in serum-starved MDA-MB-231 at the indicated different time points. **b**: Actinomycin D and cycloheximide affect survivin mRNA level enhancement induced by BMP6. **c**: Cell cycle assay was done to determine the percentage of MDA-MB-231 cells at G_1 before and after 2 days of serum starvation in the presence or absence of BMP6.

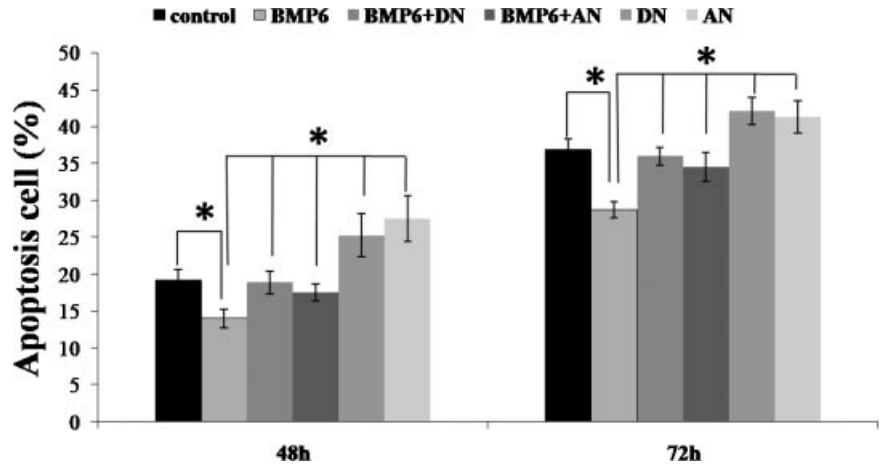


Fig. 4. Effect of Antisense Survivin and Dominant-Negative mutant survivin expression on protective effect of BMP6 at the indicated different time points.

BMP6-induced p38 is affected by the cell survival, we detected caspase-3 activity by Flow Cytometry. We found that the antiapoptotic effect of BMP6 was inhibited by p38 inhibitor SB203580 in a dose-dependent manner although the inhibitor alone at the same concentration did not affect cell apoptosis (Fig. 6b). Ninety-six hours serum starvation induced 53% cells apoptosis but only 21% cells apoptosis was achieved in the presence of BMP6. When 5 and 10 μM of SB203580 was added, the percentage of apoptotic cells reversed to 32% and 42%, respectively.

Survivin Expression did not Affect by BMP6-Induced p38 Activity

To test whether the p38 pathway could also regulate survivin expression, we first compared the survivin promoter activity in MDA-MB-231 cells treated with BMP6 in the presence or absence of 10 nM SB203580. As shown in Figure 7a, p38 inhibitor did not suppress the survivin promoter activity. Subsequently, we detected the survivin protein expression by Western blot analyses and found that 10 nM SB203580 did not affect the survivin protein level, which was enhanced by BMP6 treatment after serum starvation (Fig. 7b). These results indicated that p38 pathway was involved in BMP6 apoptosis regulation but not in survivin regulation.

DISCUSSION

We known breast cancer is the most common malignancy cancer among women. In developed countries, it is the second overall cause of death. Be similar to prostate cancer, breast cancer is prone to metastasis [Cicek and Oursler, 2006]. Breast cancer cells often metastasize to bone presumably because of a favorable growth-promoting environment provided by the bone where various growth factors are expressed in abundance [Massague, 1998; Pluijm et al., 2000].

Be similar as other members of BMP family, BMP6 is widely expressed both in bone and in breast cancer cells. It is not only a regulator of bone metabolism, but also works in tumor cell growth, differentiation, migration, and apoptosis [Lories et al., 2003; Haudenschild et al., 2004; Gosselet et al., 2007]. Although there are many to be understood about the complexity of BMP signaling in cancer, however, previous studies emerge evidence from a variety of tumor systems that the effects of BMPs are cell specific and could be either protumorigenic or antitumorigenic [Ten et al., 2003; Waite and Eng, 2003]. In our study, we try to investigate role of BMP6 in breast cancer cell proliferation and apoptosis.

We first investigated whether BMP6 affect breast cancer cells proliferation. We found BMP6 inhibited proliferation in MDA-MB-231 cells; a cell line is prone to metastasis to bone. The effect was dose-dependent. BMP6 inhibit MDA-MB-231 proliferation through inducing cell cycle arrest. The concretely mechanism of inhibit proliferation needed further research. Based on previous researches and our study, we supposed it may related with (a) induce expression of cyclin kinase inhibitor p21 and hypophosphorylation of retinoblastoma protein

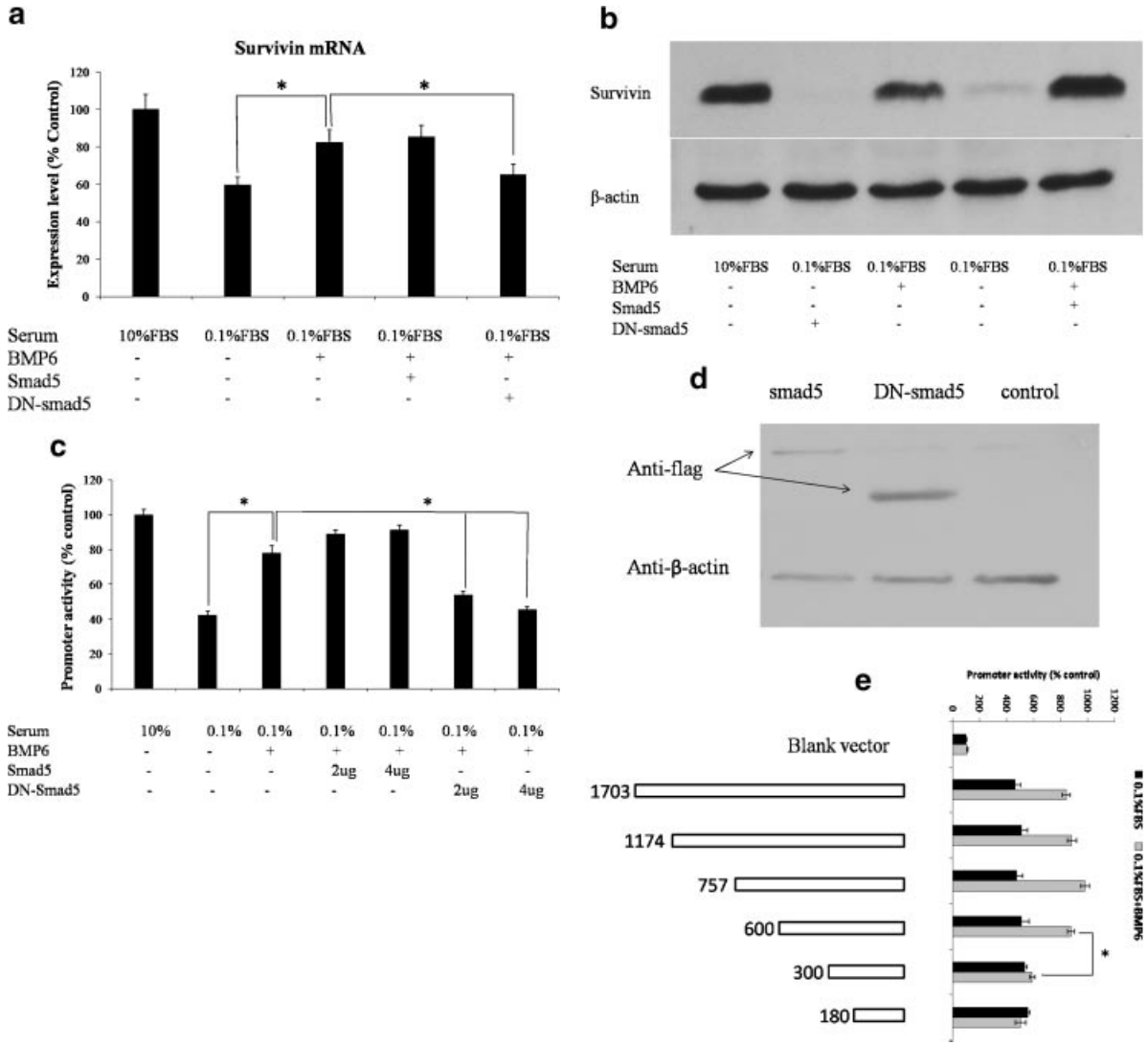


Fig. 5. **a**: Survivin mRNA level in MDA-MB-231 cells measured 48 h after transfection. **b**: Survivin protein level in MDA-MB-231 cells measured 96 h after transfection. **c**: Luciferase reporter activity measured in MDA-MB-231 cells 48 h after transfection with various vectors. **d**: Western blot was used to detect expression of Smad5 and dominant-negative Smad5 protein. Both proteins were tagged with flag-tag. **e**: Identification of BMP-responsive region on survivin promoter, survivin promoter was sequential deleted, promoter activity was assayed after serum-deprivation with or without BMP6 treatment.

[Ghosh-Choudhury et al., 2000a]; (b) up-regulate Id-1 expression, which were considered the major target genes for Smad-signaling [Kersten et al., 2005]; (c) affect some factors related with cell proliferation and cell cycle. This observation was unexpected, as it was logical to predict that BMP6, which is present in the microenvironment of bone, may not have any effect, or it may support growth of these cancer cells present in the microenvironment of bone. However, BMP6 is present in bone matrix at a very low concentration, whereas other growth factor such as TGF-beta is present at a high concentration. Thus it is possible that the concentration of BMP6 in the bone microenvironment may not be sufficient to inhibit the breast cancer cell growth. The result was consistent with previous reports about BMP2 [Ghosh-Choudhury et al., 2000a,b] and BMP6 in other type cells [Gosselet et al., 2007; Haudenschild et al., 2004].

Because of rapid growth of tumor cells, the nutrition always is in short supply. It is important that viability under jejune environment. But it is uncertain which genes are involved in this process. In order to determine the role of BMP6 expression in breast cancer

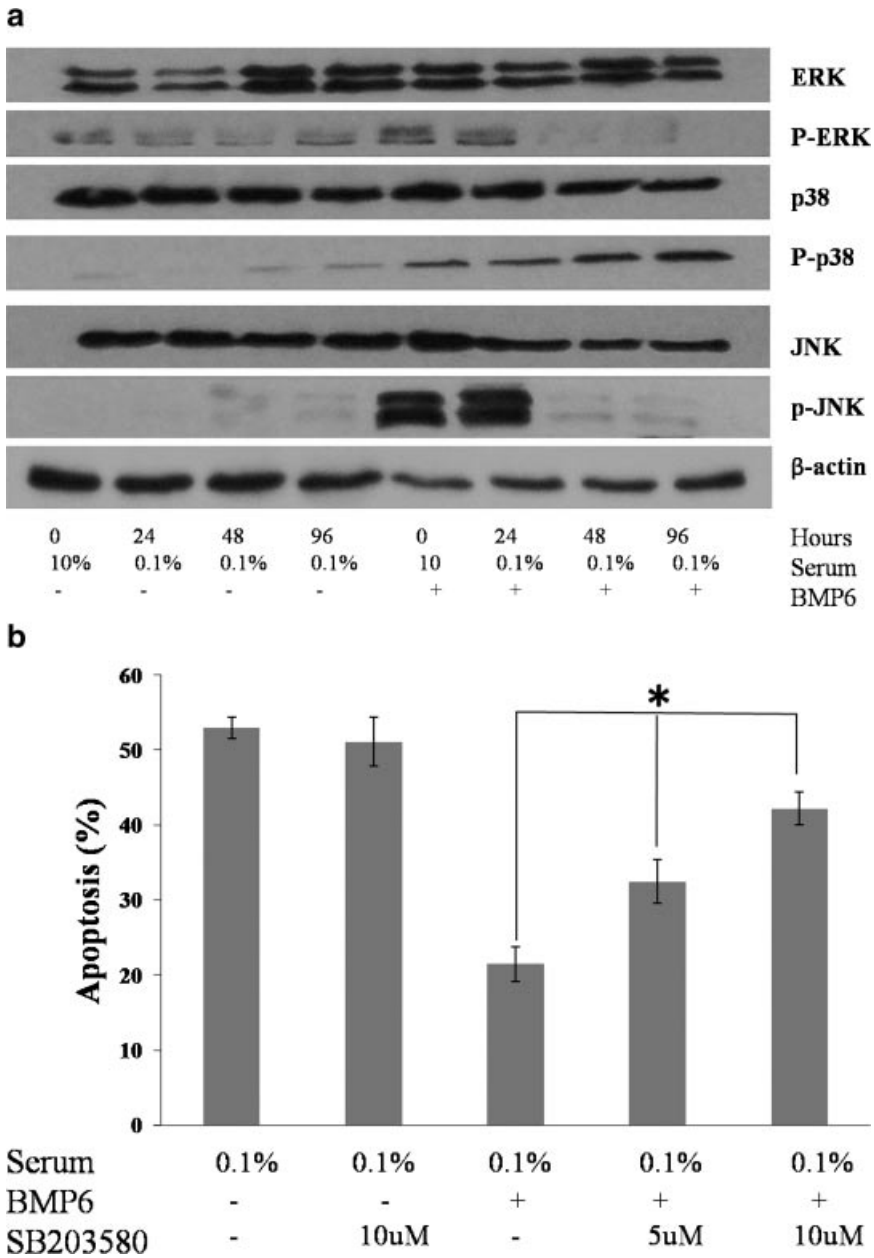


Fig. 6. Regulated of p38 activity by BMP6 and its effect on cell survival. **a**: Western blots for the activities of JNK, ERK, and p38 in MDA-MB-231 cells, cultured in 10% serum medium or in 0.1% serum medium with or without BMP6 treatment for the indicated time periods. **b**: Determination of the percentage of apoptotic cells in serum-starved (96 h) MDA-MB-231 cells, grown without BMP6, with BMP6 alone, or with BMP6 and two different doses of SB203580. Significant increases in apoptosis when serum-starved cells were treated with BMP6 in the presence of SB203580.

cells, we examined whether BMP6 affect stress induced apoptosis in breast cancer. The MDA-MB-231 cell is an aggressive estrogen-insensitive cell line which tends to metastasis to bone. We used serum starvation to induce apoptosis, which was extensive at day 4 of culture. Fist we used caspase-3 activity as an apoptotic marker. It is indicated that antiapoptotic effect of BMP6 was pronounced in MDA-MB-231 cells (percentage of caspase-3 positive cells was declined from 49% in controls vs. to 21% with BMP6). Besides, apoptotic cells can also be recognized by their diminished stain ability with DNA specific fluorochromes, such as propidium iodide (PI). After apoptosis set in, DNA became degraded and subsequently leaked from the cells. When

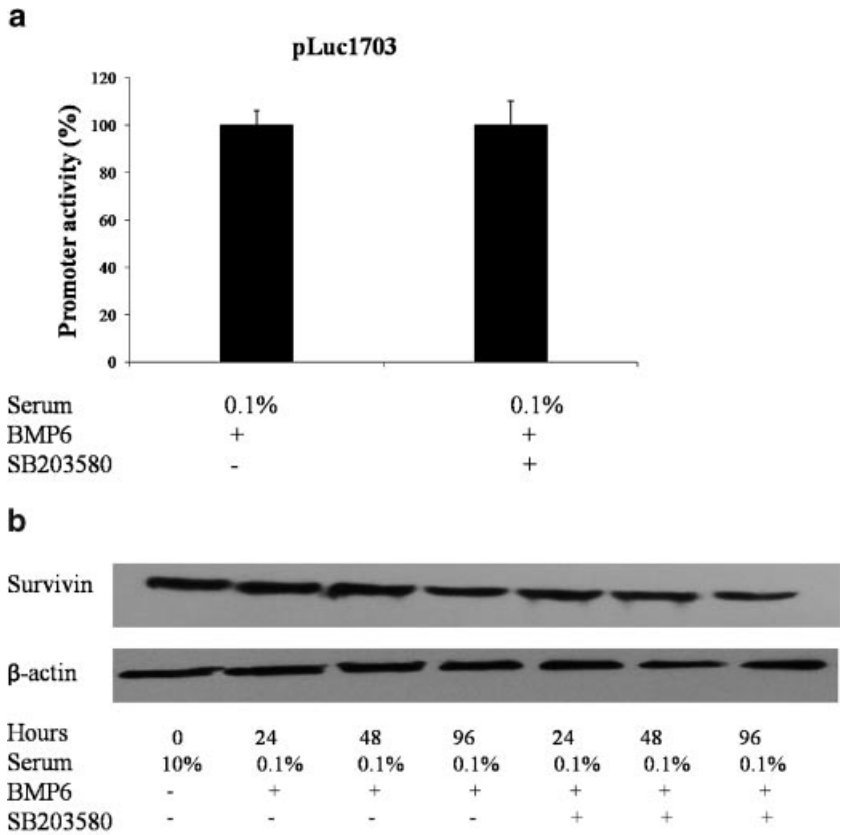


Fig. 7. Inhibition of p38 activity has no significant effect on survivin promoter activity or survivin protein expression in MDA-MB-231 cells. **a**: Luciferase assay was done to determine the survivin promoter activity in BMP6-treated cells with or without p38-specific inhibitor SB203580 (10 nmol/L) under serum starvation. **b**: Western blots were done to examine survivin expression in cells cultured in 10% serum medium and 0.1% serum medium treated with BMP6 alone or cotreated with BMP6 and 10 nmol/L SB203580 at different time points as indicated.

compared with normal cells, a sub-G_1 diploid peak appeared on cellular DNA content frequency histograms when analyzed by flow cytometry. So we subsequently did DNA degradation assay by Flow Cytometry. The experiment results also consistent with caspase-3 activity assay. BMP6 commonly exerts its biological role as a smad-dependent manner. Smad4 and smad5 were both expressed in MDA-MB-231 cells in our previous results, so we tested whether smad signal pathway was activated in the BMP6-induced antiapoptotic process using smad5. We found that transient transfection a truncated construct of smad5 obviously impaired protective effect of BMP6.

Besides confirming smad dependent manner, we also attempted to detect the expression pattern of a series of proapoptotic and antiapoptotic genes as that may be altered by exposure to BMPs. we tested the mRNA expression levels for Bcl-2, Bax, or survivin at different time points under serum starvation with or without BMP6. In our experiment, we did not find BMP6 changed Bcl-2 and Bax expression level. But survivin, a unique member of the IAP family [Kaur et al., 2004; Schimmer, 2004] might be a player in the protection. There was a remarkable conservation of survivin level at the end point (day 4) in the presence of BMP6 in the MDA-MB-231 cells. Two plasmids designed to inhibit survivin expression or function proved BMP6 protected MDA-MB-231 cells was dependent on survivin enhancement. The fact that survivin is over expressed in almost all human cancers has drawn much attention to cancer research [Li, 2003]. There are many studies showing the association between survivin expression and breast cancer and the antiapoptotic role of survivin in breast cancer cells [Tanaka et al.,

2000]. So we focused our sight on association between BMP6 and survivin.

We found BMP6 enhanced survivin expression level, and then we tried to investigate the mechanism. We supposed there were three possibilities. First, BMP6 signaling might transcriptionally up-regulated survivin expression despite the cell cycle arrest. Second, a post-translational stabilization of survivin mRNA by BMP6 signaling was also possible. Third, the survivin expression is cell cycle regulated and mostly expressed at G_2/M stage [Li et al., 1998; Altieri, 2003], the decline of survivin expression level in the control cultures may caused by to cell cycle arrest induced by stress. BMP6 might affect the cell cycle. We used actinomycin D and cycloheximide, transcriptional and translational inhibitor, to investigate the work manner of BMP6. We found actinomycin D inhibited BMP6 effect, but not cycloheximide. These results suggested that BMP6 might increase survivin expression at transcriptional level. We also examined whether cell cycle of MDA-MB-231 cells under serum-starvation was affected by BMP6 treatment. Serum-starvation for 2 days increased percentage of G_0/G_1 cells from 50% to 69% but there was no significant difference between BMP6 presence and absence. So we concluded that BMP6 could induce transcriptional up-regulation of survivin through promoter activation in these cells grown under serum starvation.

We supposed BMP6 activated survivin promoter in a smad-dependent manner. Because dominant-negative smad5 was able to counteract the up-regulation of survivin promoter activity rescued by BMP6 in a dose-dependent manner. And smad5, although weakly, exerted opposite effect. To identify the BMP-responsive element of survivin promoter, we constructed a series sequential deletion constructs of survivin promoter for Luciferase experiment. We found that BMP reactivity diminished when the fragment between −300 and −600 bp of survivin promoter was deleted. We supposed there may be smad binding sites. We found 3 GTCT motifs also termed as SBE in this sequence. Although smad proteins have an intrinsic transcriptional activation domain located in their carboxyl termini [Liu et al., 1996] and reporter genes with multimerized SBEs can respond to BMP signaling, Smad binding to DNA alone is generally believed to be too weak for Smads to function alone as effective and highly specific DNA binding proteins in vivo [Kusanagi et al., 2000]. Thus, additional DNA-binding partners are thought to be required for efficient DNA binding of smad. So we need further work to clarify idiographic interaction between BMP and survivin promoter.

Besides smad pathway, BMP6 might exert its effect through other signal pathway, such as MAPK, Wnt. In our experiment, we found BMP6 treatment activated p38 and JNK signals, but JNK activity declined went with stress persist. Be different with JNK, p38 activity sustained in the serum-starvation. Whereas many studies show that p38 expression or activation is increased in different cell types under stress, the role of p38 in apoptosis is context dependent; as in different types of cells or even under different conditions in the same type of cells, p38 can be either prodeath or prolife [Lennmyr et al., 2003; Nishimura et al., 2003]. In our study, BMP6 activated it, and it might be important for cell survival because p38 inhibitor could counteract the prosurvival effect of BMP6. These results indicate that BMP6 also protected MDA-MB-231 cell from apoptosis through the p38 pathway. And we found p38 inhibitor did not affect survivin promoter activity and protein level enhanced by BMP6, so the two antiapoptotic pathways found in our experiment were independently. The results indicated BMP6 may protect MDA-MB-231 cell apoptosis from apoptosis via two different pathways which had no interact.

In summary, our data suggest that BMP-6 inhibit breast cancer cell lines proliferation and induced cell cycle arrest. BMP6 also protected MDA-MB-231 cell from apoptosis via smad/survivin pathway and p38 MAPK pathway.

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