

Bone Morphogenetic Protein 7 Protects Prostate Cancer Cells from Stress-Induced Apoptosis via Both Smad and c-Jun NH₂-Terminal Kinase Pathways

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Abstract

We reported earlier that exposure to exogenous bone morphogenetic protein 7 (BMP7) could strongly inhibit serum starvation-induced apoptosis to C4-2B cell line, a variant of the LNCaP human prostate cancer cell line with propensity for bone metastasis. Whereas serum starvation suppressed the expression of survivin, a member of the inhibitor of apoptosis protein family, its expression was sustained in the presence of BMP7. In this study, we present evidence that BMP7 exposure up-regulated survivin promoter activity, an effect that was associated with activation of Smad, and could be repressed by dominant-negative Smad5. Additionally, serum starvation-induced suppression of c-jun NH₂-terminal kinase (JNK) activity in C4-2B cells could be mostly restored by BMP7, and a JNK inhibitor could counteract the antiapoptotic effect of BMP7, without a significant effect on the level of survivin expression. Thus, we identified JNK pathway as another signaling mode for the antiapoptotic function of BMP7. To test the effect of endogenous up-regulation of BMP7, we genetically modulated the C4-2B cell line to overexpress BMP7 protein. Not only was this altered cell line resistant to serum starvation-induced apoptosis but it also exhibited patterns of Smad activation, survivin up-regulation, and JNK activation similar to those of the parental C4-2B cells exposed to exogenous BMP7. Consistent with these *in vitro* findings of BMP7 action, we acquired correlative results of Smad activation, survivin expression, and JNK activation in the progression of prostate cancer in the conditional *Pten* deletion mouse model, in which we first obtained the evidence of BMP7 over-expression. (Cancer Res 2006; 66(8): 4285-90)

Introduction

Previously, we reported that BMP7, a member of the bone morphogenetic protein family (BMP), was strikingly up-regulated (1) during the development of primary prostatic adenocarcinoma in the conditional *Pten* deletion mouse model (2, 3), which, for brevity, could be referred to as *cPten*^{-/-} model (4). We also described that exposure to BMP7 could inhibit stress-induced

apoptosis in the human prostate cancer cell line LNCaP and more significantly in its variant C4-2B, and identified survivin, a member of the inhibitor of apoptosis family of proteins, as one of the targets that contribute to the protection of C4-2B by BMP7 (1). Thus, we became interested in dissecting the signaling pathways induced by BMP7 that prompted survival to C4-2B cells. Although the canonical pathway induced by BMPs involves Smad signaling, there are reports that BMPs can also activate mitogen-activated protein kinase (MAPK), including extracellular signal-regulated kinase (ERK), c-jun NH₂-terminal kinase (JNK), and p38, and in many circumstances, both Smad and MAPK pathways are activated by BMPs simultaneously (5, 6). After the demonstration that BMP7 could induce strong Smad activation in C4-2B cells (1), we specifically wished to examine whether Smad-dependent or Smad-independent signaling pathways or both were involved in the prosurvival effect of BMP7.

Materials and Methods

Cell lines and BMP7 protein. C4-2B and LNCaP cells were cultured as previously described (1). Human recombinant BMP7 protein (BMP7), a gift from Dr. T.K. Sampath (Creative Biomolecules, Hopkinton, MA), was used at a concentration of 50 ng/mL in all experiments for the analysis of signaling from BMP7 exposure.

Semiquantitative reverse transcription-PCR. The primers and PCR conditions for the measurement of survivin transcripts were the same as described (7). By varying PCR from 18 to 40 cycles in increments of 3 cycles, 32 cycles were determined to be optimal for survivin quantification and 21 cycles for *GAPDH* as internal control.

Cell cycle assay and apoptosis analysis (terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling). These analyses were carried out as previously described (1). After staining with propidium iodide/RNase solution (Phoenix Flow Systems, Inc., San Diego, CA), cells were analyzed by a flow cytometer for cell cycle distributions. Cellular apoptosis was assayed using APO-BRDU kit (Phoenix Flow Systems).

Transient transfection and luciferase/ β -galactosidase expression assays. Cells were transiently transfected by Tfx-20 Reagent (Promega, Madison, WI) following the instructions of the manufacturer. Briefly, cells were seeded in a six-well plate at 5×10^5 per well. Before transfection, cells were serum starved for 24 hours in 0.1% serum medium with or without BMP7 treatment. One microgram of pLuc1430 and 0.5 μ g of pSV- β -Galactosidase control vector (Promega) were cotransfected in each well. The Myc-tagged dominant-negative Smad5 construct was used as described before (8). After transfection, cells were kept in 0.1% serum medium with or without BMP7. For the control groups, cells were always incubated in 10% serum medium. Two days after transfection, cells were lysed in 1 \times lysis buffer (Promega) and used for either luciferase assay or β -galactosidase assay as described (9). The luciferase activity under the various conditions tested was normalized to the value of β -galactosidase activity.

Note: Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

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Western blot analysis. The whole-cell lysates, conditioned media, and tissue lysates were prepared as described (1). The antibodies used in the Western blots were anti-BMP7 (R&D Systems, Minneapolis, MN), anti-Smad1, anti-Smad5, anti-phospho-Smad1,5,8, anti-ERK, anti-phospho-ERK, anti-JNK, anti-phospho-JNK, anti-p38, anti-phospho-p38, anti-Myc-Tag (Cell Signaling Technology, Beverly, MA), anti-phospho-survivin (Thr³⁴), and anti-actin (Santa Cruz Biotechnology, Santa Cruz, CA).

Construction of recombinant lentivirus, infection, and sorting of infected C4-2B cells. Human *BMP7* cDNA was PCR amplified with *Xba*I and *Rsr*II linkers and inserted into the polycloning site of the transducing lentivirus vector pSIN-green fluorescent protein (GFP). Lentiviruses were produced by the three-plasmid system as described (10, 11). Briefly, human 293T cells in T75 flasks at ~80% confluency were transfected with 7.5 µg of the vesicular stomatitis virus *env*-coding plasmid, pMD.G; 15 µg of the packaging plasmid, pCMVΔ8.91; and 15 µg of either the control vector pSIN-GFP or the transgene vector pSIN-BMP7. The pseudo-typed lentiviruses were collected 48 to 72 hours later. The C4-2B cells at 80% confluency in six-well plates were incubated with 1 mL of the conditioned medium containing lentiviruses in the presence of 5 µg/mL polybrene for 6 hours, washed with PBS twice, and grown in complete medium. The procedure was repeated several times until ~5% of the cells had shown green fluorescence under the microscope. Cells were then sorted by flow cytometry on the basis of GFP fluorescence.

Statistical analysis. All experiments were done in triplicates and repeated at least twice. Statistical comparisons were made using an unpaired two-tailed *t* test.

Results

BMP7 up-regulates survivin transcription after serum starvation without affecting survivin phosphorylation level or G₂-M phase in C4-2B cells. Previously, we showed that BMP7 was able to rescue the survivin protein expression that was suppressed by serum starvation. We proposed two possibilities for this effect: transcriptional regulation and posttranslational regulation (phosphorylation at Thr³⁴) by BMP7 signaling (1). Because survivin expression is cell cycle regulated, it is also possible that, by affecting the cell cycle, BMP7 might influence survivin levels. To test the possibility of posttranslational regulation by BMP7, we used Western blot analyses to examine the level of phospho-survivin (Thr³⁴) in C4-2B cells, with or without BMP7 treatment after serum starvation, at different time points. The pattern of phospho-survivin turned out to be very similar to what we previously found (1) for total level of survivin (Fig. 1A), and the ratio between phospho-survivin and total level of survivin was not significantly changed by either serum starvation or BMP7 treatment (Fig. 1B). These results suggested that BMP7 did not affect the phosphorylation of survivin in C4-2B cells. We then tested the possibility of transcriptional regulation by BMP7 by using semiquantitative reverse transcription-PCR (RT-PCR) to determine the survivin mRNA level in C4-2B cells. Whereas serum starvation suppressed survivin mRNA expression, BMP7 significantly up-regulated the mRNA level (Fig. 1C). We also examined whether cell cycle in C4-2B was affected by BMP7 treatment. Serum starvation suppressed G₂-M cell percentage from 40% to 15% but there was no significant difference between BMP7-treated and nontreated C4-2B cells (Fig. 1D).

BMP7 up-regulates survivin promoter activity after serum starvation in a Smad-dependent manner. Following the finding that BMP7 could up-regulate survivin mRNA expression in C4-2B cells after serum starvation, we investigated this transcriptional regulation further. A luciferase reporter construct, pLuc1430, which contained 1,430-bp DNA sequence upstream of *survivin* gene, was shown to exhibit the maximal survivin promoter activity (12). By

transient transfection of pLuc1430 into C4-2B cells, we found that serum starvation suppressed survivin promoter activity to ~47% of the normal level, which could be fully rescued by BMP7 (Fig. 2A). Because we showed that BMP7 induced strong Smad activation in C4-2B cells (1), it was important to determine whether the effect of BMP7 on up-regulation of survivin promoter activity was through Smad signaling. To this end, we attempted to interrupt Smad activation by cotransfecting pLuc1430 with a dominant-negative Smad5 construct. Indeed, the up-regulation of survivin promoter activity by BMP7 could be counteracted by the dominant-negative Smad5 in a dose-dependent manner (Fig. 2A). A control vector, TK-Luc, which is not responsive to BMP7 signaling, was used as a marker for normalizing transfection efficiency (Fig. 2B). In LNCaP cells, consistent with what we previously found that BMP7 could not rescue survivin expression suppressed by serum starvation,

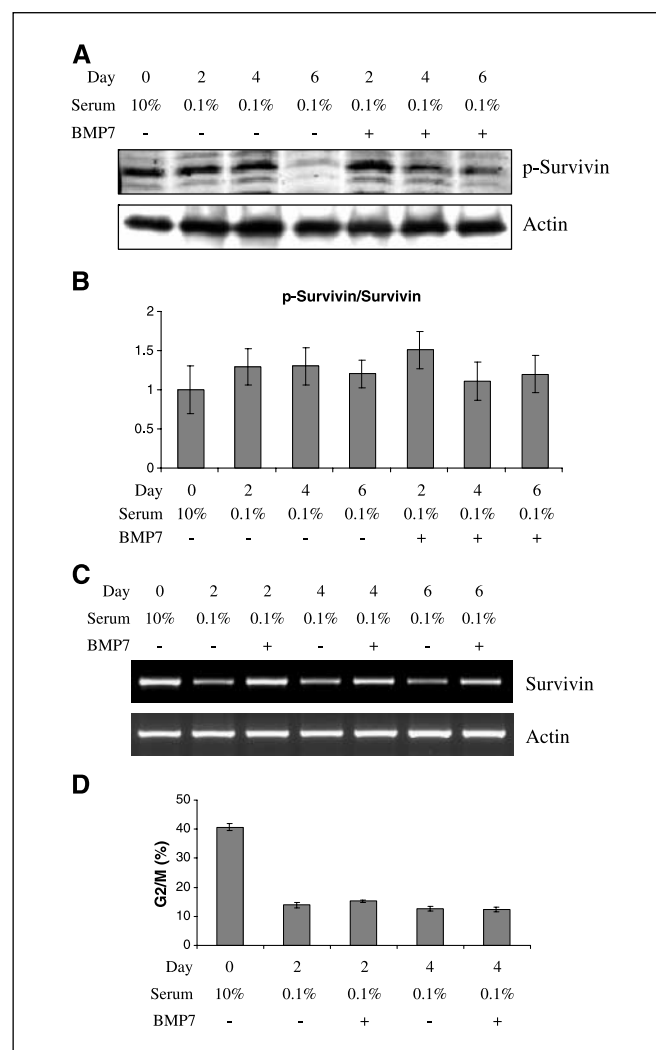


Figure 1. Regulation of survivin by BMP7. **A**, Western blot analysis for phospho-survivin level in C4-2B cells cultured in 10% serum medium (normal condition) or 0.1% serum medium (serum starvation) with (+) or without (-) BMP7 at the indicated different time points. **B**, determination of the ratio of total phospho-survivin and total survivin expression in C4-2B cells. The data for total survivin expression in the same extracts were from our previous report (1). **C**, semiquantitative RT-PCR was used to compare the mRNA level of survivin in C4-2B cells cultured in 10% serum medium and 0.1% serum medium with or without BMP7 at different time points. **D**, cell cycle assay was done to determine the percentage of C4-2B cells at G₂-M before and after 2 or 4 days of serum starvation in the presence (+) or absence (-) of BMP7.

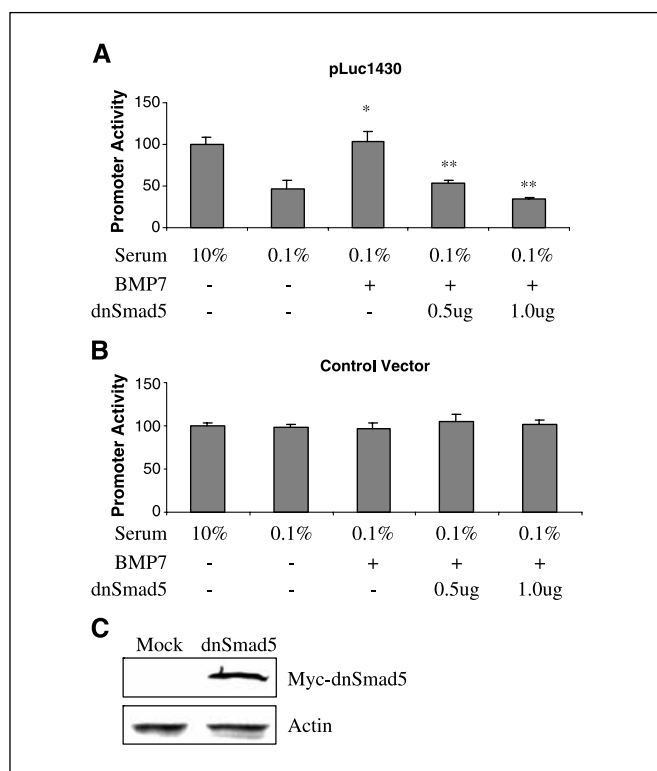


Figure 2. Luciferase reporter activity measured in C4-2B cells 48 hours after transfection with various vectors. *A*, cells were transfected with the survivin promoter reporter construct pLuc1430 (1.0 μg/well) alone or cotransfected with 0.5 μg or 1.0 μg/well of dominant-negative Smad5 (*dnSmad5*) construct. *, $P < 0.01$, significant difference between serum-starved cells with or without BMP7. **, $P < 0.01$, dominant-negative Smad5 significantly suppressed the promoter activity in BMP7-treated cells after serum starvation. *B*, cells were transfected with a control vector (1.0 μg/well) to determine transfection efficiency. *A* and *B*, all values were normalized by β-galactosidase activity and in each experiment, the value for cells cultured in 10% serum medium was adjusted to 100 as control. *C*, Western blot detection of expression of dominant-negative Smad5 protein by using Myc-Tag antibody in the Myc-tagged dominant-negative Smad5-transfected cells but not in the control mock-transfected cells.

there was no significant difference of survivin promoter activity between BMP7-treated and control LNCaP cells (Supplementary Fig. S1).

Both survivin expression and Smad activation increase with the growth of prostate tumors. After observing that in C4-2B cells BMP7 could up-regulate survivin expression through Smad signaling, we went back to the *cPten*^{-/-} mouse prostate tumor model to evaluate the levels of survivin expression and Smad activation in the course of the disease. As shown by Western blot analyses, the growth of primary prostate tumors in the anterior lobe of the *cPten*^{-/-} mice was associated with progressively increased expression of survivin as well as the level of phospho-Smad1,5,8 over an age range of 1.6 to 11 months (Fig. 3A). From an 11-month-old *cPten*^{-/-} mouse and its littermate control, we extracted proteins from different lobes, including anterior lobe, ventral lobe, and dorsolateral lobe. Western blot analysis showed that in tumors of all lobes, there was overexpression of survivin and increased Smad activation compared with the corresponding tissues from the littermate control (Fig. 3B). Interestingly, Smad5 expression level in anterior lobe was also increased in the tumored tissues at the advanced age of 11 months.

BMP7 also activates JNK pathway, independent of Smad/survivin pathway, to promote cell survival. We examined the

influence of serum starvation and BMP7 treatment on the activities of three major MAPKs (i.e., JNK, ERK, and p38) by Western blot analyses. Although the patterns of ERK and p38 activities between the control and BMP7-treated cells were not significantly affected, we found that JNK activity was suppressed by serum starvation whereas BMP7 strongly up-regulated its activity (Fig. 4A). By using JNK-specific inhibitor SP600125 to block JNK activation (Fig. 4B), we found that the antiapoptotic effect of BMP7 was counteracted by JNK inhibitor in a dose-dependent manner (Fig. 4C). As serum starvation of C4-2B cells resulted in 96% apoptosis and to only 21% in the presence of BMP7, 51% or 79% of apoptosis was induced when 10 or 20 μmol/L SP600125 was present, respectively, along with BMP7. To test whether the JNK pathway could also regulate survivin expression, we first compared the survivin promoter activity in C4-2B cells treated with BMP7 in the presence or absence of 20 μmol/L SP600125. As shown in Fig. 5A, JNK inhibitor could not suppress the survivin promoter activity. We then examined the survivin protein expression by Western blot analyses and found that 20 μmol/L SP600125 could not suppress the survivin protein level, which was sustained by BMP7 treatment after serum starvation (Fig. 5B and C). These results indicated that JNK pathway was involved in apoptosis regulation but not in survivin regulation. Recalling that LNCaP cells were not very

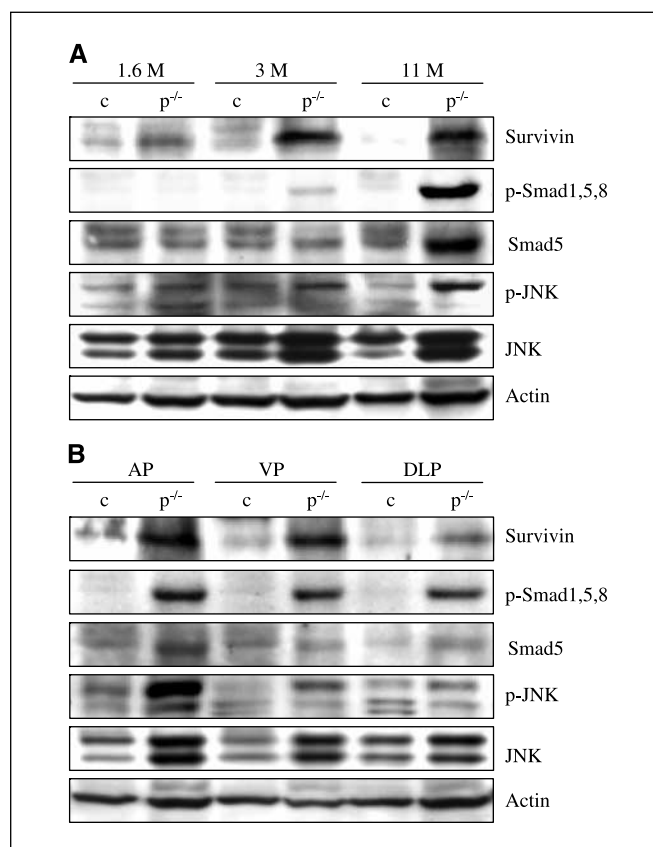


Figure 3. Western blot assays for survivin, activated Smad, and activated JNK in the prostate tissues from the *cPten*^{-/-} mice relative to the littermate control tissues. *A*, proteins from anterior lobe of the animals at different ages as indicated in months (*M*). *B*, proteins from individual lobes, anterior lobe (AP), ventral lobe (VP), and dorsolateral lobe (DLP) from an 11-month-old pair. *A* and *B*, c, littermate control, homozygous for the floxed *Pten* allele; p^{-/-}, Cre-mediated homozygous deletion of the floxed *Pten* allele. Western blot analyses of Smad5 and JNK were used as control for Smad and JNK activity, respectively, and actin was used as loading control.

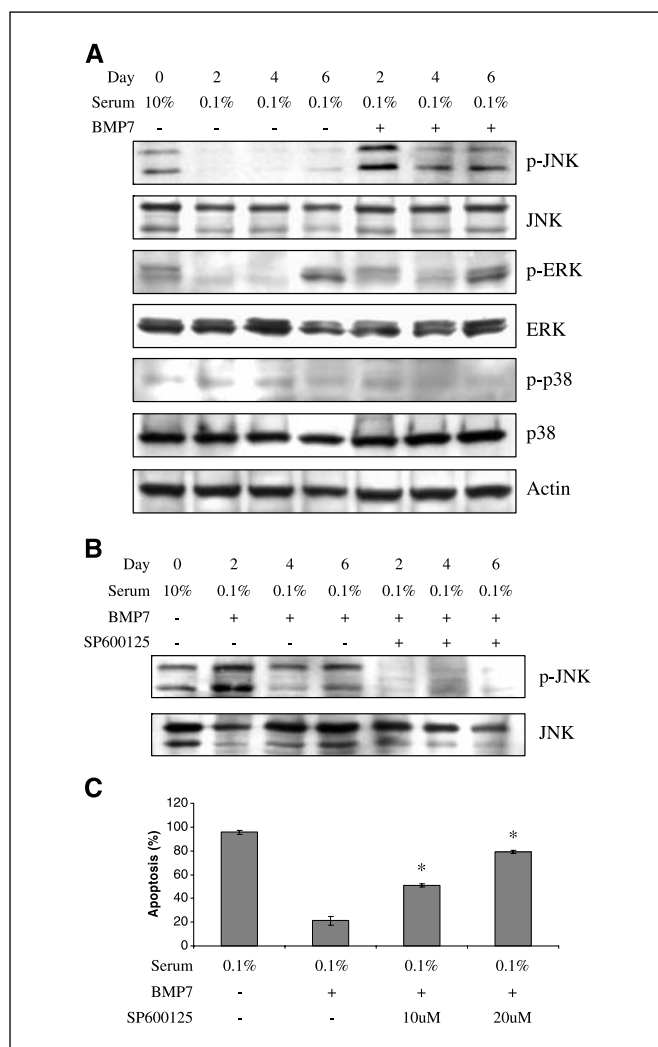


Figure 4. BMP7 regulation of JNK activity and its effect on cell survival. *A*, Western blots for the activities of JNK, ERK, and p38 in C4-2B cells, cultured in 10% serum medium or in 0.1% serum medium with or without BMP7 treatment for the indicated time periods. *B*, JNK-specific inhibitor SP600125 (20 μ mol/L), with or without BMP7, was used to treat C4-2B cells after serum starvation, and the JNK activity was determined by Western blot. *C*, determination of the percentage of apoptotic cells in serum-starved (6 days) C4-2B cells, grown without BMP7, with BMP7 alone, or with BMP7 and two different doses of SP600125. *, $P < 0.001$, significant increases in apoptosis when serum-starved cells were treated with BMP7 in the presence of SP600125.

sensitive to serum starvation (only 35% of apoptosis was induced by serum starvation; ref. 1), we found that there was no suppression of JNK activity by serum starvation in LNCaP cells and BMP7 did not exhibit a significant effect on JNK activation (Supplementary Fig. S2A). Although JNK inhibitor SP600125 (20 μ mol/L) could induce more apoptosis after serum starvation, it did not counteract the antiapoptotic effect of BMP7 in LNCaP cells as the extent of the apoptosis inhibition by BMP7 with or without SP600125 was very similar (Supplementary Fig. S2B).

In the analysis of prostate tissues from the *cPten*^{-/-} mice, we found that like BMP7, activated Smad, and survivin, there was an increase in the level of activated JNK with the growth of the tumors (Fig. 3A and B). Besides phospho-JNK, the level of JNK also seemed to increase in the tumored tissues, especially in those collected at the advanced age of 11 months.

Endogenous overexpression of BMP7 protects C4-2B cells from stress-induced apoptosis through similar mechanisms as exogenous BMP7. We created a BMP7-overexpressing C4-2B cell line (C4-2B/BMP7) and also a control GFP-expressing C4-2B cell line (C4-2B/GFP) by lentivirus-mediated gene transduction. The overexpression of BMP7 protein was confirmed by Western blot analysis with the conditioned medium. There was >6-fold increase in secreted BMP7 protein in C4-2B/BMP7 cells compared with C4-2B parental cells or C4-2B/GFP control cells (Fig. 6A). By testing the response of C4-2B/BMP7 and C4-2B/GFP cells to serum starvation, we found that, similar to the observations with the C4-2B parental cells that were treated with exogenous BMP7 protein, C4-2B/BMP7 exhibited much more resistance to serum starvation-induced apoptosis. As shown in Fig. 6B, 6 days of serum starvation induced 68% apoptosis in C4-2B/GFP cells but only 18% in C4-2B/BMP7 cells. We then compared the JNK and Smad activities and survivin expression in these two cell lines after 6 days of serum starvation by Western blot. In C4-2B/GFP cells, serum starvation suppressed JNK activity whereas in C4-2B/BMP7, a robust JNK activity was maintained (Fig. 6C). Although there was no detectable Smad phosphorylation in C4-2B/GFP cells cultured in 10% or 0.1% serum medium, there was strong Smad activation in C4-2B/BMP7 cultured in 10% serum medium and less but still significant Smad activation in C4-2B/BMP7 after 6 days of serum starvation. Finally, we examined the expression level of survivin protein in these two cell lines. In C4-2B/GFP cells, serum starvation suppressed the

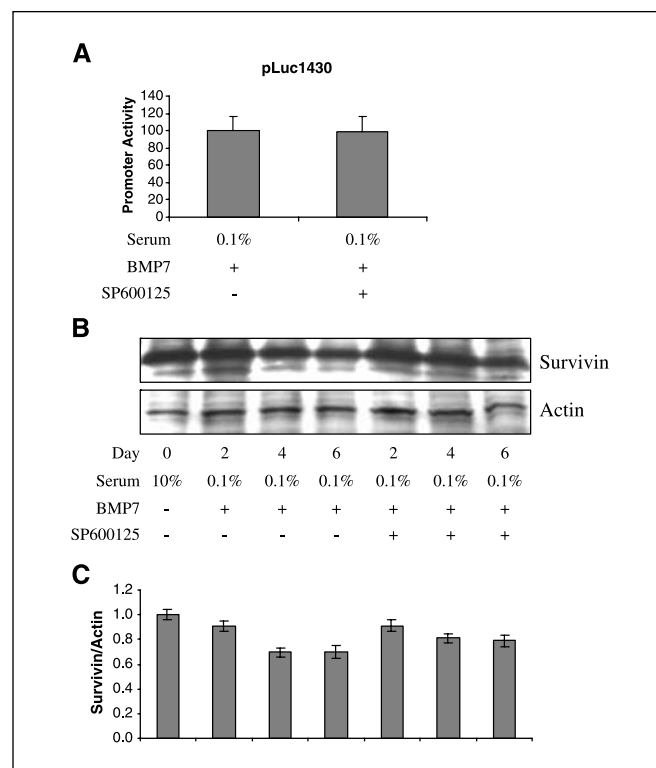


Figure 5. Inhibition of JNK activity has no significant effect on survivin promoter activity or survivin protein expression in C4-2B cells. *A*, luciferase assay was done to determine the survivin promoter activity in BMP7-treated cells with or without JNK-specific inhibitor SP600125 (20 μ mol/L) under 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 BMP7 alone or cotreated with BMP7 and 20 μ mol/L SP600125 at different time points as indicated. *C*, estimation of changes in survivin protein levels at the various conditions as in (B).

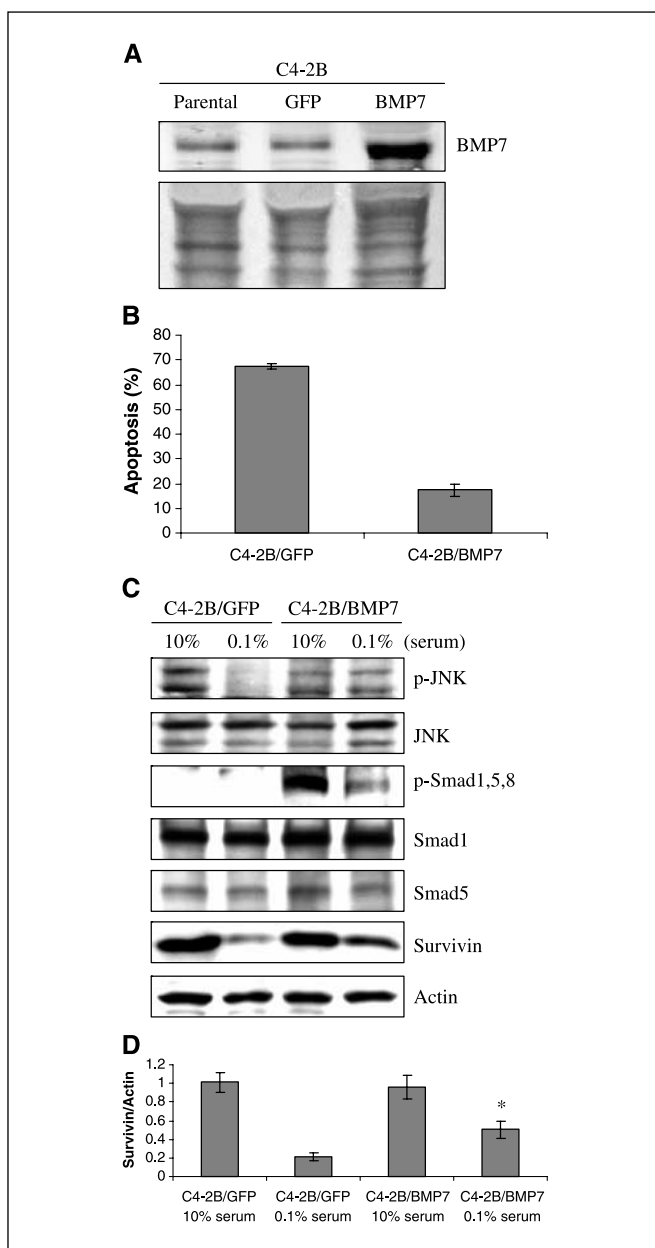


Figure 6. Stable endogenous overexpression of BMP7 protects C4-2B cells against serum starvation-induced apoptosis similar to the exogenous BMP7 treatment. *A*, determination of secreted BMP7 protein level by Western blots of conditioned medium from C4-2B parental cells, C4-2B/GFP cells, and C4-2B/BMP7 cells (*top*). Coomassie blue staining was used to assess loading in each lane (*bottom*). *B*, the percentage of apoptotic cells in C4-2B/GFP cells compared with C4-2B/BMP7 cells after 6 days of serum starvation ($P < 0.001$). *C*, Western blot analyses of the JNK and Smad activities and survivin expression in C4-2B/GFP or C4-2B/BMP7 cells before and after 6 days of serum starvation. *D*, estimation of survivin protein levels in (*C*). *, $P < 0.01$, significant difference in survivin expression between C4-2B/GFP and C4-2B/BMP7 cells after serum starvation.

survivin expression to only 20% of the normal level. In contrast, there was still 51% of survivin expression in C4-2B/BMP7 after serum starvation (Fig. 6D).

Discussion

The fact that survivin is overexpressed in almost all human cancers has drawn much attention to cancer research (13). There is a

large body of studies showing the association between survivin expression and prostate carcinoma and the antiapoptotic role of survivin in prostate cancer cells (14–16). Because the protein stability of survivin could be increased by the phosphorylation at Thr³⁴, it is possible that BMP7 signaling leads to posttranslational regulation of survivin by increasing its phosphorylation level. Because survivin expression is cell cycle dependent and mostly expressed in G₂-M phase, another possibility would be that BMP7 might cause a shift to G₂-M phase, thereby promoting survivin expression indirectly. However, these possibilities are now ruled out in C4-2B cells, as BMP7 has no major effect on either the phosphorylation of survivin or on the cell cycle. On the other hand, it is clearly shown that BMP7 can induce transcriptional up-regulation of survivin through promoter activation in these cells grown under serum starvation. Because dominant-negative Smad5 is able to counteract the up-regulation of survivin promoter activity by BMP7 in a dose-dependent manner, we conclude that this effect is Smad dependent. Unlike the C4-2B cells, in LNCaP cells BMP7 cannot rescue the survivin promoter activity that is suppressed by serum starvation, a point consistent with what we previously found that BMP7 did not sustain the survivin protein level after serum starvation in these cells.

The finding that BMP7/Smad signaling is able to up-regulate survivin expression in C4-2B cells led us to go back to the *cPten*^{-/-} tumor model in which we first observed the overexpression of BMP7. Conceivably, increased BMP7 availability should result in increased Smad activation, and possibly, increasing Smad activation could result in progressively more survivin expression. In this regard, both elevated Smad activity and overexpression of survivin are now documented in the model. This provides the first evidence to suggest that the BMP/Smad pathway is most likely to be one of the pathways that up-regulate survivin expression in the prostate tumorigenesis *in vivo*.

Another point of interest is that BMP7 activates not only the Smad pathway but also the JNK pathway. Whereas most studies show that JNK expression or activation is increased in neoplastic cells, the role of JNK in apoptosis is context dependent; as in different types of cells or even under different conditions in the same type of cells, JNK can be either prodeath or prolife (17, 18). In our study, serum starvation suppresses JNK activity whereas BMP7 rescues it, and JNK activity must be important for cell survival because JNK inhibitor could counteract the prosurvival effect of BMP7. These results indicate that BMP7 also uses the JNK pathway to protect C4-2B cells from apoptosis. Through a BMP7-overexpressing cell line, C4-2B/BMP7, we showed that endogenous BMP7 can also protect C4-2B cells against stress-induced apoptosis via both Smad/survivin and JNK pathways, similar to what we found when C4-2B cells were treated with exogenous BMP7. In LNCaP cells, JNK activity was not suppressed by serum starvation, which partially explains why LNCaP cells are less sensitive to serum starvation-induced apoptosis. Consistent with increased BMP7 expression in the tumors of *cPten*^{-/-} mice, however, a correlation exists with JNK activity with the tumor growth.

In summary, for the first time we showed the relationship between BMP/Smad signaling and survivin expression in the prostate cancer cell line C4-2B. We also identified JNK activation as another pathway that contributes to the prosurvival effect of BMP7. These results also correlate well with the BMP7 up-regulation, Smad activation, survivin overexpression, and JNK activation that we observed in tumor progression in the prostate tumor mouse model examined. Further work is necessary to understand the mechanisms by which Smad signaling regulates

survivin promoter activity, and what other major prosurvival molecules that are induced by BMP7 may be regulated by Smad or JNK signaling in prostate cancer cells. It would also be important now to define the role of BMP/Smad or BMP/JNK signaling in prostate cancer progression and metastasis and to test the potential of these pathways as therapeutic targets for prostate cancer.

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