Bone Morphogenetic Protein-9 Induces Apoptosis in Prostate Cancer Cells, the Role of Prostate Apoptosis Response-4

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Abstract
Bone morphogenetic proteins (BMP) have been implicated in the development of bone metastases in prostate cancer. In this study, we investigated the role which BMP-9 played in prostate cancer and found that the expression of BMP-9 was decreased or absent in prostate cancer, particularly in the foci of higher grade disease. We further investigated the influence of BMP-9 on the biological behaviors of prostate cancer cells. The forced overexpression of BMP-9 prevented the in vitro growth, cell-matrix adhesion, invasion, and migration of prostate cancer cells. We also elucidated that BMP-9 induced apoptosis in PC-3 cells through the up-regulation of prostate apoptosis response-4. Among the receptors which have been implicated in the signaling of BMP-9, BMPR-IB and BMPR-II have also been implicated in the development and progression of prostate cancer. Knockdown of BMPR-IB or BMPR-II using respective hammerhead ribozyme transgenes could promote cell growth in vitro. We also found that BMPR-II is indispensable for the Smad-dependent signal transduction by BMP-9 in PC-3 cells, in which Smad-1 was phosphorylated and translocated from the cytoplasm into the nuclei. Taken together, BMP-9 inhibits the growth of prostate cancer cells due to the induced apoptosis, which is related to an up-regulation of prostate apoptosis response-4 through a Smad-dependent pathway. BMP-9 could also prevent the migration and invasiveness of prostate cancer. This suggests that BMP-9 may function as a tumor suppressor and apoptosis regulator in prostate cancer. (Mol Cancer Res 2008;6(10):1594–606)

Introduction
Bone is the most common metastatic site for advanced prostate cancer. These metastases often result in bone pain, pathologic fractures, and cause spinal cord compression. The interaction between prostatic tumor cells and bone tissue may have contributed to this organ tropism and the predominantly osteoblastic characteristics of metastatic lesions from the prostate cancer. Bone morphogenetic proteins (BMP), a group of the most powerful bone inductive factors enriched in the bone matrix, have been indicated in bone metastasis in prostate cancer since the early 1990s.

BMP belongs to the transforming growth factor-β superfamily. BMPs exert their effects through a heteromeric receptor complex, which consists of two types of serine-threonine kinase transmembrane receptors. The type I receptors include BMP receptor type IA (BMPR-IA), type IB (BMPR-IB), activin receptor–like kinase-1 (ALK-1), ALK-4, ALK-5, and activin A receptor type I (ActRI). The type II receptors include BMP receptor type II (BMPR-II), activin A receptor type IIA (ActRII), and activin A receptor type IIB (ActRIIB). Upon binding to the ligands (BMPs), the type II receptors then phosphorylate the type I receptors. This leads to the recruitment of the pathway-restricted Smads (R-Smads, Smad-1, Smad-5, and Smad-8) to the complex. The intracellular signaling complex of R-Smads is then translocated into the nucleus, which then regulates the transcription of BMP-responsive genes. The translocation to the nucleus is orchestrated by Smad-4. This pathway is known as the Smad-dependent pathway, in which Smad-6 and Smad-7 act as inhibitory regulators to the signaling. The other pathway is known as the Smad-independent pathway, in which the Map kinase pathway, the RAS pathway, or the Erk kinase pathway may be involved (1).

BMPs and their receptors play an important role in bone formation and morphogenesis, and have been implicated in prostate cancer, particularly in disease-specific bone metastases (2). BMPs have demonstrable effects on the growth of prostate cancer cells. Loss of the expression of BMPR-IA, BMPR-IB, and especially BMPR-II, in both prostate cancer tissues and cancer cell lines, has been shown to have an association with the progression of prostate cancer (3-5). The inhibitory effect of BMPs on tumor growth, mediated through BMPR-II, has been illustrated in an in vivo murine tumor model using a BMPR-II knockout prostate cancer cell line (PC3M; ref. 4). Collectively, BMPs and their receptors play important roles during the development and progression of prostate cancer, particularly in disease-specific bone metastases (6-17).

BMP-9, also known as growth differentiation factor 2, was originally cloned from a fetal mouse liver cDNA library (18). The immature and unprocessed human BMP-9 precursor protein has 429 amino acids, the molecular weight of which is predicted to be ~47 kDa. As with other BMPs, it is divided into a pro-region and a mature region (13 kDa) after
posttranslational cleavage. Once secreted, BMP-9 may exist as dimers of mature regions or as BMP-9 pro-region complexes in which the pro-region of BMP-9 remains tightly associated with mature ligand dimer after secretion. The BMP-9 pro-region complexes have equivalent biological activities as BMP-9 mature ligand dimers (19).

BMP-9 has been shown as a pleiotropic cytokine, which is implicated in a number of physiologic events. These include bone morphogenesis, functions of hepatic reticuloendothelial system, hematopoiesis, neuronal differentiation, glucose homeostasis, iron homeostasis, and angiogenesis (18, 20-25). It is interesting to note that although the expression of BMP-9 was initially thought to be restricted to the liver (18, 26), recent studies have found BMP-9 mRNA expression to occur in a number of cells and tissues, including the central nervous system and bone in recent studies (22, 27). The nonparenchymal cells of the liver (endothelial, Kupffer, and stellate cells), rather than hepatocytes, are thought to be the major source of BMP-9 in the body (28).

Although BMP-9 has been shown to be crucial during the fetal or postnatal development of certain tissues, the roles played by this cytokine in cancer are largely unknown. In the current study, the expression of BMP-9 was examined in prostate cancer specimens and prostate cancer cell lines. The biological function of this molecule was investigated in cells that overexpress this molecule in order to establish the functional role of BMP-9 in prostate cancer cells.

Results

Expression Pattern of BMP-9 in Prostate Cancer Cells and Prostate Tissues

Expression of BMP-9 was examined in the prostate cell lines using conventional reverse transcription-PCR (RT-PCR). PC-3, DU-145, and CA-HPV-10 are prostate cancer cell lines. PC-3 was derived from bone metastasis, DU-145 was isolated from brain metastasis, whereas CA-HPV-10 was from primary tumor. PZ-HPV-7 cells are immortalized prostatic epithelial cells. BMP-9 was expressed, at varying levels, in these cell lines. BMP-9 transcript in PC-3 cells was relatively low in comparison with DU-145 and CA-HPV-10, whereas it was undetectable in LNCaP and PZ-HPV-7 cells (Fig. 1A).

In prostate specimens, BMP-9 staining was seen in normal prostatic epithelia, but was greatly reduced or undetectable in prostate cancer cells, particularly at the loci of high grade disease in which the gland structures were completely disrupted (Fig. 1B). Positive staining was significantly different between normal tissues and tumor tissues [13 of 18 (72.2%) in normal versus 1 of 8 (12.5%) in tumor; \( P = 0.0093 \) by Fisher’s exact test].

We also examined the expression of three BMP receptors and the R-Smads (Smad-1, Smad-5, and Smad-8) using conventional RT-PCR. Figure 1A illustrates the subtle variations in the level of the mRNA transcripts of BMPR-IB and BMPR-II. Both receptors were detectable in the five prostate cell lines examined. BMPR-IB was expressed at a relatively

![FIGURE 1. The expression pattern of BMP-9 in prostate cancer cells and tissues.](image-url)
higher level compared with BMPR-II. ALK-1, one of the BMP-9 type I receptors, was not detected in these prostate cell lines. Smad-1, Smad-5, and Smad-8 were clearly expressed in all cell lines.

The Influence of BMP-9 Overexpression on Cellular Biological Behaviors in Prostate Cancer Cells

PC-3 cells represent an aggressive prostate cancer, which was derived from bone metastasis of prostate cancer. They also have lower mRNA levels of BMP-9 (Fig. 1A). Therefore, it was chosen to overexpress BMP-9 in the current study. PC-3 cells were transfected with BMP-9 transgenes and control plasmids, respectively. BMP-9 was successfully up-regulated in PC-3BMP-9exp cells following the transfection of PC-3 cells with the BMP-9 transgenes. The overexpression of BMP-9 in PC-3BMP-9exp cells, compared with the PC-3 wild-type (PC-3WT) and control plasmid (PC-3pEF/His) was shown at the mRNA level (Fig. 2A). To investigate whether the

![Figure 2](image-url)

**FIGURE 2.** The influences of BMP-9 overexpression on cellular biological behaviors of prostate cancer cells. A, Verification of the overexpression of BMP-9 in PC-3 cells using RT-PCR (1) and Western blotting (2). The expression of BMP-9 in PC-3 was significantly elevated after the transfection, and the BMP-9 transcripts were increased in PC-3BMP-9exp cells compared with PC-3WT and PC-3pEF/His controls (1). An increasing protein level was revealed in the PC-3BMP-9exp cells. The three bands on the Western blot under reduced conditions are BMP-9. The band of ~60 kDa is the monomer of the BMP-9 precursor protein, the bands of ~14 kDa represent monomers of matured ligands (2). B1. Overexpression of BMP-9 inhibits the growth of PC-3 cells using an in vitro cell growth assay. The growth of the cells was reduced by the overexpression of BMP-9 after 3 days of incubation. *, P < 0.05 and **, P < 0.01, vs. both PC-3WT and PC-3pEF/His. Columns, mean; bars, SE. B2. The influence of BMP-9 overexpression on the invasiveness of prostate cancer cells using the in vitro invasion assay. Overexpression of BMP-9 decreases the invasiveness of prostate cancer in vitro. **, P < 0.01 vs. PC-3WT and PC-3pEF/His. Each test was conducted in triplicate with three fields being counted for each sample. Three independent experiments were done. Bars, SD. B3. Overexpression of BMP-9 influences the cell-matrix adhesion of PC-3 cells using the in vitro cell-matrix adhesion assay. BMP-9 significantly reduces the adhesion of the prostate cancer cells in vitro. Bars, SD. Eight wells were set for each cell line, three independent experiments were done. 4. Overexpression of BMP-9 inhibited the migration of PC-3 cells using the in vitro wounding assay. *, P < 0.05 vs. both PC-3WT and PC-3pEF/His. Statistical results of three independent experiments. Columns, mean; bars, SE.
transfected cells also produced more BMP-9 protein, we determined the protein level of BMP-9 in the cells using Western blot analysis. Overexpression of the BMP-9 protein in PC-3BMP-9exp cells was confirmed with increasing protein production of BMP-9 precursor protein (60 kDa) and monomers (14 kDa) of BMP-9 mature ligands (Fig. 2A).

To assess whether overexpression of BMP-9 would influence the biological function of prostate cancer cells, we first determined the effect of this overexpression on in vitro cell growth. BMP-9 overexpression significantly reduced the growth of PC-3 cells (PC-3BMP-9exp) in vitro (Fig. 2B). The absorbance of PC-3BMP-9exp cells after a 3-day incubation period was 1.082 ± 0.052, P < 0.05 versus PC-3WT (1.186 ± 0.091) and P < 0.01 versus PC-3pEF/His (1.437 ± 0.021). A more marked reduction of cell growth was seen after a 5-day incubation period, the absorbance of PC-3BMP-9exp cells being 2.099 ± 0.222, P < 0.01 versus both PC-3WT (2.701 ± 0.156) and PC-3pEF/His (2.698 ± 0.082).

We further examined the influence of BMP-9 on the invasiveness of prostate cancer cells. BMP-9 overexpression markedly reduced the invasiveness of PC-3 cells, the number of invading cells which moved across the matrix barrier for PC-3BMP-9exp being 3.7 ± 2.1, P < 0.01 versus both PC-3WT cells (38.2 ± 6.7) and PC-3pEF/His cells (34.1 ± 5.4; Fig. 2B3).

Adhesion to the extracellular matrix is an important property and process of cancer cells during metastasis. To investigate the effect of BMP-9 on the ability of prostate cancer cells to adhere to the extracellular matrix, we used a previously established in vitro cell-matrix adhesion assay. Overexpression of BMP-9 markedly reduced cell-matrix adhesion of PC-3 cells. The number of adherent PC-3BMP-9exp cells was 7 ± 2.4, P < 0.01 versus both PC-3WT cells (30 ± 6.7) and PC-3pEF/His (37 ± 10.3), as shown in Fig. 2B4.

We also examined the effect of BMP-9 overexpression on cellular motility using an in vitro migration/wounding assay. PC-3BMP-9exp cells showed a significantly reduced cellular migration, compared with controls. A significant reduction of distance migrated was seen in these cells, 60 minutes after wounding. The average distance migrated over 90 minutes for PC-3BMP-9exp cells was 17.22 ± 3.61 μm, P < 0.05 versus both PC-3WT (32.47 ± 2.98 μm) and PC-3pEF/His (30.26 ± 2.43 μm; Fig. 2B1).

**BMP-9 Induces Apoptosis in PC-3 Cells via Par-4**

To investigate whether apoptosis is involved in the inhibitory effect of BMP-9 on the growth of prostate cancer cells, we determined the proportion of apoptotic cells. As shown in FIGURE 3. BMP-9 induces apoptosis in PC-3 cells using flow cytometry. The percentage of the apoptotic cells in PC-3BMP-9exp cells was 29.4% (top), which is markedly higher than the 0.45% of PC-3pEF/His cells (middle), and 5.00% of PC-3WT (bottom).
Fig. 3A, there was a marked shift of cell population to apoptosis in the BMP-9–overexpressing cells, which is 29.4% in the PC-3BMP-9exp cells, in comparison with PC-3WT (5.0%) and PC-3pEF/His (0.45%).

Prostate apoptosis response-4 (Par-4) has been shown as being up-regulated in androgen-independent prostate cancer cells which were induced to undergo apoptosis (29-31). The PC-3 cells used in this study are androgen-insensitive, and were derived from bone metastases of prostate cancers. Therefore, we hypothesized that Par-4 may be involved in apoptosis induced by BMP-9. We examined the levels of Par-4 mRNA in the PC-3BMP-9exp cells using conventional RT-PCR, in comparison with the wild-type and control plasmid transfectants. Figure 3B shows an up-regulation in the mRNA level of Par-4 in PC-3BMP-9exp cells. We further verified BMP-9–induced expression of Par-4 at the protein level in PC-3 cells using Western blot analysis and immunochemical staining. An elevated protein level of Par-4 was shown in the PC-3BMP-9exp cells (Fig. 3B, bottom). The immunocytochemical staining also showed an increased Par-4 protein in PC-3BMP-9exp cells which appeared to be more condensed in the nuclei of the cells (Fig. 3C, top). This phenomenon was further elucidated in the immunofluorescent staining of Par-4 (Fig. 3C, bottom). The up-regulation of Par-4 mRNA and protein were also seen in PC-3 cells when exposed to recombinant human BMP-9 (rh-BMP-9; 40 ng/mL) over a time period up to 24 hours (Fig. 3D). The earliest increase of Par-4 transcripts was seen 5 minutes after exposure to BMP-9.

To further investigate whether the proapoptotic effect of BMP-9 is Par-4–dependent, we constructed ribozyme transgenes specifically targeting the Par-4 transcript and transfected PC-3 cells. The knockdown of Par-4 was seen at both mRNA and protein levels in PC-3Δpar-4 cells compared with PC-3WT and PC-3pEF/His controls (Fig. 4A and B). The proapoptotic effect of BMP-9 was abolished in the PC-3Δpar-4 cells exposed to rh-BMP-9 (40 ng/mL, for 48 hours), in clear contrast with both PC-3WT and PC-3pEF/His controls (Fig. 4C).

Both BMPR-IB and BMPR-II Mediate Inhibitory Effects on the In vitro Growth of Prostate Cancer Cells

Both BMPR-IB and BMPR-II were detectable in PC-3 cells (Fig. 1A). In the current study, we transfected the ribozyme

![FIGURE 4.](image-url) The Par-4 dependency of BMP-9 induced apoptosis. A. Knockdown of Par-4 transcripts was seen in PC-3Δpar-4 cells compared with PC-3WT and PC-3pEF/His controls using RT-PCR. B. A reduction of Par-4 protein was also verified in the PC-3Δpar-4 cells compared with both controls using Western blot analysis. C. The apoptotic population in PC-3 cells was analyzed using flow cytometry after exposure to rh-BMP-9 (40 ng/mL) for 48 h. A markedly reduced response to BMP-9 was seen in the PC-3Δpar-4 cells compared with PC-3WT and PC-3pEF/His controls.
transgenes into PC-3 cells targeting either BMPR-IB or BMPR-II. The expression of BMPR-IB mRNA was completely eliminated from PC-3 cells by the ribozyme transgenes (top). The PCR reactions were done in 30 cycles. The mRNA level of BMPR-II was also markedly reduced in the PC-3\textsuperscript{ΔBMPR-II} compared with the PC-3\textsuperscript{WT} and PC-3\textsuperscript{pEF/His} (bottom). A substantial decrease of the BMPR-II transcripts was shown in PC-3\textsuperscript{ΔBMPR-II} cells, compared with both PC-3\textsuperscript{WT} and PC-3\textsuperscript{pEF/His} cells (Fig. 5A, bottom). Knockdown in the mRNA of both receptors was reflected at the protein level. As shown in Fig. 5B, there was a significant reduction of BMPR-IB protein (45 kDa) in PC-3\textsuperscript{ΔBMPR-IB} cells, in comparison to the PC-3\textsuperscript{WT} and PC-3\textsuperscript{pEF/His} cells (Fig. 5B, top). In fact, the BMPR-IB protein was not detectable in PC-3\textsuperscript{ΔBMPR-IB} cells. The protein yields of BMPR-II (both mature protein, 70-80 kDa; and pro-protein, 115 kDa) were also decreased after the knockdown of BMPR-II transcripts in PC-3\textsuperscript{ΔBMPR-II} cells (Fig. 5B, bottom).

We further examined the effect on cell growth after loss of BMPR-IB and BMPR-II in PC-3 cells. Cell growth was facilitated by the knockdown of BMPR-II, the rate of cell growth at day 4 was significantly increased in PC-3\textsuperscript{ΔBMPR-II} (543.8 ± 119.1%), P < 0.01 versus PC-3\textsuperscript{WT} (239.3 ± 30.7%) and P < 0.05 versus PC-3\textsuperscript{pEF/His} (209.9 ± 26.9%). There was also a marked increase of cell growth in PC-3\textsuperscript{ΔBMPR-IB} cells (423.4 ± 57.1% of PC-3\textsuperscript{WT} cells and 543.8 ± 119.1% of PC-3\textsuperscript{ΔBMPR-II} cells). The growth rate was calculated as a percentage using the absorbance of day 1 as a baseline. Bars, SD (n = 6). Three independent experiments were done.

To evaluate the influence on the invasive capacity of PC-3 cells by knockdown of BMPR-IB and BMPR-II, we assessed the invasion using an in vitro invasion assay. Each cell line was tested in triplicate, three independent experiments were done. Bars, SD.
BMP-9 Signals through a Smad-Dependent Pathway in PC-3 Cells

To investigate the involvement of BMPR-IB and BMPR-II in the signal transduction of BMP-9 in PC-3, we used the aforementioned PC-3 cells which had lost or reduced the expression of these receptors. Both receptors (Fig. 6C, top and middle) were phosphorylated in the PC-3 wild-type cells (PC-3WT) upon exposure to rh-BMP-9 (Fig. 6A). The activation of BMPR-II was more apparent in comparison with that of BMPR-IB. The phosphorylation of BMPR-IB by BMP-9 was prevented by the knockdown of BMPR-II. Similarly, a blockage of the phosphorylation of BMPR-II was seen in the BMPR-IB knockdown cells. This suggests that the loss of either receptor could reduce the activation of another in PC-3 cells.

To elucidate the Smad-dependent signaling by BMP-9 in prostate cancer cells, we examined the activation of Smad-1, Smad-5, and Smad-8 (Fig. 6C, bottom), which are the R-Smads involved in the signaling of the BMPs. BMP-9 induced a remarkable phosphorylation of Smad-1, and weak phosphorylation of Smad-5 and Smad-8. The loss of BMPR-II significantly diminished the phosphorylation of Smad-1, Smad-5, and Smad-8. In contrast, the loss of BMPR-IB only reduced the phosphorylation of BMPR-II, but had no effect on the activation of Smads except Smad-5. This indicated that BMP-9 was unable to induce the phosphorylation of BMPR-II without BMPR-IB. Immunofluorescent staining of phosphorylated Smad-1 further revealed an activation and nuclear translocation of Smad-1, as the result of exposure to rh-BMP-9 in PC-3 cells (Fig. 6B). The phosphorylation and translocation of Smad-1 was remarkably reduced by the knockdown of BMPR-II, but not by the loss of BMPR-IB. Interestingly, rhBMP-7 (20 ng/mL), a member of the BMP family failed to show a similar pattern of activation in BMPR1B, BMPR-II, and the R-Smads.

Discussion

BMP-9 is a pleiotropic cytokine, and has been implicated in bone morphogenesis, hematopoiesis, neuronal differentiation, glucose homeostasis, iron homeostasis, angiogenesis, and the hepatic reticuloendothelial system (18, 20-25). In the current study, we first showed the presence of BMP-9 in the human prostate at both mRNA and protein levels. BMP-9 mRNA was detectable in most of the currently available prostate cell lines used in our laboratory (Fig. 1A). However, the variable mRNA

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FIGURE 6. BMP-9 signals through a Smad-dependent pathway in PC-3 cells. A. rh-BMP-9 used in the study, as shown by Western blotting. B. Immunofluorescent staining of phosphorylated Smad-1 by BMP-9 (20 ng/mL) in PC-3 cells. C. BMP-9 signal through Smad-dependent pathways in PC-3 cells. The role of BMPR-IB (top) and BMPR-II (middle) in BMP-9 signaling in PC-3 cells. Phosphorylated R-Smads are involved in signal transduction of BMP-9 using immunoprecipitation and Western blot analysis (bottom).
level of BMP-9 in these cell lines might be due to immortalization by virus transfection and in vitro culture condition such as the different hormone levels and growth factors and cytokines. The immunohistochemical staining revealed an expression of BMP-9 protein in normal prostatic epithelia, which is decreased in prostate cancer cells as shown in our pilot study using human prostate specimens (Fig. 1B). This suggests that BMP-9 is widely expressed in prostate tissues and may play an important role in prostate cancer. Presently, we are expanding the evaluation in a larger prospective study using real-time quantitative PCR and immunohistochemical staining of BMP-9, in order to reveal its clinical value in prostate cancer.

Recent studies have shown that BMP-9 plays some critical roles during fetal or postnatal development, particularly during the morphogenesis of certain tissues. Thus far, BMP-9 is also one of the most effective BMPs in bone formation (21, 32-34). However, its role in cancer remains unknown, particularly in the area of bone metastasis. To investigate the biological functions of BMP-9 in prostate cancer cells, we overexpressed human BMP-9 in PC-3 cells, which have a low level of expression and is also a more aggressive prostate cancer cell line derived from bone metastasis (Fig. 1A). Previously, Song et al. has shown that BMP-9 could stimulate the proliferation of both human HepG2 liver tumors cells and rat hepatocytes (26). However, most BMPs thus far investigated inhibit the proliferation of prostate cancer cells. For example, BMP-2 and BMP-4 inhibit the growth of LNCaP cells, an androgen-sensitive prostate cancer cell line (35, 36), whereas BMP-6 and BMP-7 are likely to inhibit the proliferation of both androgen-sensitive and androgen-insensitive prostate cancer cells (37, 38). The present study reveals an inhibitory effect of BMP-9 on cell growth of prostate cancer cells, which is in line with the decreased expression of BMP-9 in prostate cancer and the documented effects of other members of the BMP family in prostate cancer. The current study also reveals an inhibitory effect of BMP-9 on the motility of prostate cancer cells. Most interestingly, both migration and invasion of PC-3 cells were reduced after overexpression of BMP-9, which are somewhat very different from the effects on motility by other known BMPs, for example, BMP-2, BMP-4, BMP-6, and BMP-7 can stimulate the migration and invasion of prostate cancer cells (17, 39, 40). The effects of BMP proteins on cancer cells have been a topic of discussion and the results from the literature (17, 39, 40). The effects of BMP proteins on cancer cells have been shown to inhibit apoptosis (45). However, a role of BMPs in apoptosis in prostate cancer cells is still not clear. It has been shown that BMP-7 can stabilize the level of survivin in prostate cancer cells (LNCaP and C4-2B), and restore the activity of c-Jun NH2-terminal kinase, both of which contribute to the antiapoptotic activity of BMP-7 (44, 45). In the present study, we have shown that BMP-9 inhibited in vitro cell growth of prostate cancer cells. This was not related to necrotic cell death, as observed during the study. This has lead us to the discovery that apoptosis is the potential underlying mechanism for the reduced rate of cell growth induced by BMP-9. Here, we have provided evidence for the very first time that one of the critical mechanisms underlying BMP-9–induced apoptosis in prostate cancer cells is the induction of Par-4. Par-4 is a proapoptotic protein which was originally identified in prostate cancer cells undergoing apoptosis in response to ionomycin (30). Par-4 is involved in the apoptosis induced by tumor necrosis factor, doxorubicin, etoposide, UV irradiation, growth factor deprivation, and ionizing radiation (46, 47). In the current study, we found that the apoptosis induced by BMP-9 was due to an up-regulation of Par-4. An elevated and condensed immunofluorescent nuclear staining of Par-4 was seen in BMP-9–overexpressed PC-3 cells. The phosphorylation of its T155 residue and nuclear translocation of Par-4 are essential for the apoptosis mediated by Par-4 (48). The nuclear translocation of Par-4 was also revealed in the BMP-9–overexpressed PC-3 cells using immunofluorescent staining of Par-4. The critical role of Par-4 was further supported by the Par-4 knockdown experiments, in which removal of Par-4 from the cells abolished BMP-9–induced apoptosis. It is concluded that Par-4 is involved in apoptosis induced by BMP-9 in the PC-3 androgen–insensitive prostate cancer cells (PC-3).

Loss of the expressions of BMP receptors, type I (BMPR-IA, BMPR-IB), and type II (BMPR-II), especially BMPR-II in prostate cancer, has been implicated in the progression of prostate cancer (3-5). Furthermore, the expression of these receptors can be regulated by hormones (49) and cytokines such as hepatocyte growth factors (50). This inhibitory effect on tumor growth mediated through BMPR-II had been highlighted in an in vitro study, by using a nude mice model in which a BMPR-II knockout prostate cancer cell line (PC3M) was inoculated s.c. (4). In another study, PC-3 cells were engineered to constitutively express an active BMPR-IB in a tetracycline-regulated manner. Tetracycline/doxycycline-regulated the expression of the constitutively active BMPR-IB results in the inhibition of both in vitro cell proliferation and tumor growth in vivo (38). In the current study, knockdown of both receptors using the respective ribozyme transgenes can promote the in vitro cell growth of PC-3 cells (Fig. 4). This is consistent with the documented effects mediated by these receptors in prostate cancer (4, 38). Collectively, this suggests Apoptosis is the key event for physiologic growth control and regulation of tissue homeostasis. Aberration of apoptosis plays a crucial role during oncogenesis and subsequent progression. BMPs are able to regulate the apoptosis of malignant cells, for example, BMP-2 induces apoptosis in medulloblastoma cells and colonic epithelial cells (41, 42), but prevents apoptosis in breast cancer cells (MCF-7; ref. 43). The role of BMPs in apoptosis in prostate cancer cells is still not clear. It has been shown that BMP-7 can stabilize the level of survivin in prostate cancer cells (LNCaP and C4-2B), and restore the activity of c-Jun NH2-terminal kinase, both of which contribute to the antiapoptotic activity of BMP-7 (44, 45). In the present study, we have shown that BMP-9 inhibited in vitro cell growth of prostate cancer cells. This was not related to necrotic cell death, as observed during the study. This has lead us to the discovery that apoptosis is the potential underlying mechanism for the reduced rate of cell growth induced by BMP-9. Here, we have provided evidence for the very first time that one of the critical mechanisms underlying BMP-9–induced apoptosis in prostate cancer cells is the induction of Par-4. Par-4 is a proapoptotic protein which was originally identified in prostate cancer cells undergoing apoptosis in response to ionomycin (30). Par-4 is involved in the apoptosis induced by tumor necrosis factor, doxorubicin, etoposide, UV irradiation, growth factor deprivation, and ionizing radiation (46, 47). In the current study, we found that the apoptosis induced by BMP-9 was due to an up-regulation of Par-4. An elevated and condensed immunofluorescent nuclear staining of Par-4 was seen in BMP-9–overexpressed PC-3 cells. The phosphorylation of its T155 residue and nuclear translocation of Par-4 are essential for the apoptosis mediated by Par-4 (48). The nuclear translocation of Par-4 was also revealed in the BMP-9–overexpressed PC-3 cells using immunofluorescent staining of Par-4. The critical role of Par-4 was further supported by the Par-4 knockdown experiments, in which removal of Par-4 from the cells abolished BMP-9–induced apoptosis. It is concluded that Par-4 is involved in apoptosis induced by BMP-9 in the PC-3 androgen–insensitive prostate cancer cells (PC-3).

Loss of the expressions of BMP receptors, type I (BMPR-IA, BMPR-IB), and type II (BMPR-II), especially BMPR-II in prostate cancer, has been implicated in the progression of prostate cancer (3-5). Furthermore, the expression of these receptors can be regulated by hormones (49) and cytokines such as hepatocyte growth factors (50). This inhibitory effect on tumor growth mediated through BMPR-II had been highlighted in an in vitro study, by using a nude mice model in which a BMPR-II knockout prostate cancer cell line (PC3M) was inoculated s.c. (4). In another study, PC-3 cells were engineered to constitutively express an active BMPR-IB in a tetracycline-regulated manner. Tetracycline/doxycycline-regulated the expression of the constitutively active BMPR-IB results in the inhibition of both in vitro cell proliferation and tumor growth in vivo (38). In the current study, knockdown of both receptors using the respective ribozyme transgenes can promote the in vitro cell growth of PC-3 cells (Fig. 4). This is consistent with the documented effects mediated by these receptors in prostate cancer (4, 38). Collectively, this suggests
that both BMPR-IB and BMPR-II play crucial roles in the control of the cellular behavior of prostate cancer cells. It has been shown that ALK-1, ALK-2, and BMPR-IB (ALK-6) are the putative type I receptors for BMP-9 signal transduction, whereas BMPR-II and ActR-IIA are the type II receptors with relatively higher affinity to BMP-9 (19, 25, 51). However, whether these two receptors are involved in the signal transduction of BMP-9 in prostate cancer warrants further investigation.

The involvement of ALK-1 in the signaling of BMP-9 in PC-3 cells can be excluded, as the transcript of ALK-1 was not detectable in the prostate cell lines examined (Fig. 1A). In the present study, we have further elucidated the signal transduction of BMP-9 in PC-3 cells, and the involvement of BMPR-IB and BMPR-II. Upon binding of BMP-9 to the receptors, both BMPR-IB and BMPR-II were phosphorylated. Knockdown of either receptor could reduce the activation of another. Following the activation of both BMPR-II and BMPR-IB, the R-Smads were then stimulated and translocated into the nuclei, leading to the transcriptional regulation of the responsive genes. Although Smad-1, Smad-5, and Smad-8 were phosphorylated by BMP-9 in PC-3 cells, activation of Smad-1 was the strongest one. This would suggest that Smad-1 is the major R Smad responsible for the intracellular signaling of BMP-9 in PC-3 cells. Loss of BMPR-II significantly diminished the phosphorylation of both BMPR-IB and R-Smads. In contrast, loss of BMPR-IB only reduced the phosphorylation of BMPR-II by BMP-9, with little or no effect on the activation of Smads. This suggests that BMPR-II is an indispensable type II receptor for BMP-9 to signal through the Smad-dependent pathway, whereas the phosphorylation of its serine/threonine domain may not be necessary (Fig. 7). This may also be due to the involvement of other receptors, such as ALK-2 and ActR-IIA, which needs further investigations.

In conclusion, BMP-9 can inhibit cell growth, adhesion, invasion, and migration of prostate cancer cells in vitro. BMP-9 induces apoptosis in PC-3 cells via the up-regulation and activation of Par-4, which contributes to its inhibitory effect on cell growth. BMP-9 up-regulates Par-4 through the Smad-dependent pathway, in which activation and nuclear translocation of Smad-1 lead to the transcriptional regulation of BMP responsive genes (Fig. 7). BMPR-II is essential for BMP-9 to trigger this signaling pathway. Collectively, BMP-9 is a putative tumor suppressor in prostate cancer. It has profound
potential in the prognosis and therapy of prostate cancer, in which further investigation may lead to a novel therapeutic approach of prostate cancer, particularly in disease-specific bone metastases.

Materials and Methods

Materials and Cell Culture

PC-3 (European Collection of Animal Cell Cultures), DU-145, LNCapFGC, CA-HPV-10, and PZ-HPV-7 (American Type Culture Collection) were routinely maintained in DMEM-F12 medium supplemented with 10% FCS and antibiotics. Polyclonal goat anti-BMP-9, monoclonal mouse antiactin, polyclonal goat anti–phosphorylated Smad-1, and monoclonal mouse anti–Par-4 were obtained from Santa Cruz Biotechnology. Recombinant human BMP-7 was from R&D Systems Europe. Other reagents or kits were obtained from Sigma-Aldrich.

Prostate tissue samples were available following collection from the patients at the Department of Urology, University Hospital of Wales. All samples were snap-frozen in liquid nitrogen immediately after radical prostatectomy, transurethral prostatectomy, or transurethral resection of prostate tissue (prostatectomy, transurethral prostatectomy, or transurethral resection of prostate tissue). All protocols were reviewed and approved by the local ethical committee and all patients gave written informed consent.

Immunohistochemical Staining Procedure for Frozen Prostate Tissue

Frozen specimens of prostate tumors (n = 8) and normal prostate tissues (n = 18) were cut at a thickness of 6 μm. After fixation, the sections were blocked with horse serum and were then incubated with or without polyclonal goat antibody against BMP-9 for 1 h. The secondary biotinylated antibody and the avidin-biotin complex were used to detect BMP-9 expression in accordance with the Vectastain Universal Elite ABC kit protocol (Vector Laboratories). Staining procedures were done under standardized conditions and the sections were counterstained with Gill’s hematoxylin. The intensity of staining was independently assessed by the authors.

Construction of BMP-9 Expression Vectors and Transfection

The first-strand cDNA was synthesized from RNA isolated from normal human tissues using a DuraScript RT-PCR kit. PCR was then used to amplify the coding sequence of full-length human BMP-9 using the Extensor Hi-Fidelity PCR master mix (Abgene, Ltd.). The sequences of primers are shown in Table 1. The verified BMP-9 insert was cloned into a mammalian expression plasmid vector (pEF/His TOPO TA plasmid vector; Invitrogen, Inc.). The recombinant plasmid sequence was cloned into the pEF/His TOPO TA plasmid vector and purified. rh-BMP-9 and BMPR-9 transgenes were then transfected into PC-3 cells individually using an Easjet Plus electroporator (EquiBio, Ltd.). After up to 3 weeks of selection with blasticidin, the transfectants were used in certain studies.

Construction and Purification of rh-BMP-9

A pair of primers was used to amplify the coding sequence of full-length human BMP-9 (with a deletion of the stop codon to allow the addition of a polyhistidine tag). The coding sequence was cloned into the pEF/His TOPO TA plasmid vector and purified. rh-BMP-9 transgenes were then transfected into the murine fibroblast 3T3 cells (American Type Culture Collection). Following verification, the transfectants carrying rh-BMP-9 transgenes were grown to sufficient amounts. The cells were subsequently lysed using lysis buffer in the absence of SDS. The recombinant proteins were then purified using metal-chelating affinity chromatography.

Knockdown of Par-4, BMPR-IB, and BMPR-II in PC-3 Cells Using the Respectively Prepared Ribozyme Transgenes

Antihuman Par-4, BMPR-IB, and antihuman BMPR-II hammerhead ribozyme targeting was designed based on the

<table>
<thead>
<tr>
<th>Primer Sequences for PCR</th>
<th>Forward</th>
<th>Reverse</th>
<th>PCR products (bp)</th>
<th>Annealing temperature (°C)</th>
</tr>
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<tbody>
<tr>
<td>hBMP-9</td>
<td>5'-CAGTACACGAGGAGGACAC</td>
<td>5'-GATGTCCTGGAAGATTTACC</td>
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<td>hBMPR-IB</td>
<td>5'-ATGAAATGCTGTTATGCT</td>
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<tr>
<td>hBMPR-II</td>
<td>5'-TTTGGAAGAGAAACAAATCT</td>
<td>5'-TGAGTAAGGCAAAATTTTG</td>
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<td>55</td>
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<tr>
<td>hALK-1</td>
<td>5'-GGGGATGTTGCTGTTGGA</td>
<td>5'-GCTGGAAGGTCAGGTAAT</td>
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<td>55</td>
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<tr>
<td>hSmad-1</td>
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<td>5'-CCTCTGTTTCTAAATTTG</td>
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<tr>
<td>hSmad-5</td>
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<td>5'-TGAAGATGAAATCTCAATCA</td>
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<tr>
<td>hSmad-8</td>
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<td>55</td>
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<tr>
<td>hPar-4</td>
<td>5'-GATCTTATCCCTCCCTTACCC</td>
<td>5'-ATGGCAAGGAGGACACCTC</td>
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<td>55</td>
</tr>
<tr>
<td>BMPR-9 ribozyme</td>
<td>5'-ATGGTCTCTGGGGACCTGT</td>
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<td>66</td>
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<tr>
<td>rh-BMP-9</td>
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<td>5'-CCTGACACCCACACCTCTG</td>
<td>1,287</td>
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<td>BMPR-IB ribozyme</td>
<td>5'-CTGGAGTCTGGGGACCTTCA</td>
<td>5'-ACTAGTCCCATTCTATCAA</td>
<td>AGAAGATTCTGTCCTACGACT</td>
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<tr>
<td>BMPR-II ribozyme</td>
<td>5'-CTGGAGTCTGGGGACCTTCA</td>
<td>5'-ACTAGTCCCATTCTATCAA</td>
<td>AGAAGATTCTGTCCTACGACT</td>
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<tr>
<td>Par-4 ribozyme</td>
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<td>5'-ACTAGTCCCATTCTATCAA</td>
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<td></td>
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<tr>
<td>hβ-actin</td>
<td>5'-ATGGGATGACGAGGCTGAGG</td>
<td>5'-CGGCAAGAGCGGAGGCTGAGG</td>
<td>503</td>
<td>55</td>
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</table>
secondary structure of the respective gene generated using Zuker’s RNA mFold program (ref. 52; refer to Table 1 for sequence). The ribozymes were individually cloned into a mammalian expression pEF6/VS-His-TOP plasmid vector (Invitrogen). Ribozyme transgenes and control plasmid vectors were then transfected into PC-3 cells, respectively. After up to 3 weeks selection using blasticidin, the transfectants were used in the current study.

RNA Isolation and RT-PCR
RNA was isolated using Total RNA Isolation Reagent (ABgene). Reverse transcription was done using the DuraScript RT-PCR kit, followed by PCR using a REDTaq ReadyMix PCR reaction mix (primer sequences shown in Table 1). Cycling conditions were 94°C for 5 min, followed by 36 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 40 s. This was followed by a final 10-min extension period at 72°C. The products were visualized on 2% agarose gel stained with ethidium bromide.

Immunoprecipitation and Western Blot Analysis
The protein concentration in cell lysates were determined using the DC Protein Assay kit (Bio-Rad) and an ELX800 spectrophotometer (Bio-Tek). Equal amounts of proteins were separated by SDS-PAGE and blotted onto nitrocellulose sheets. Proteins were then probed with the anti–BMP-9 antibody and peroxidase-conjugated secondary antibodies. Protein bands were visualized using the Supersignal West Dura system (Pierce Biotechnology, Inc.), and photographed using a UVITech imager (UVITech, Inc.).

In the case of immunoprecipitation and Western blotting of phosphorylated R-Smads, following 2 h of incubation in serum-free DMEM, the cells were then exposed to rh-BMP-9 (20 ng/mL), or serum-free DMEM alone for 1 h. The immunofluorescent staining of phosphorylated Smad-1 was done using anti–phosphorylated serine/threonine were then immunoprecipitated using an antiphosphoserine/phosphothreonine antibody (Abcam plc). The resulting immunoprecipitates and whole cell lysates were loaded onto the SDS-PAGE gel. Following Western blotting, proteins were then probed using goat polyclonal anti-BMPR–IB IgG, goat polyclonal anti–BMPR-II IgG, mouse monoclonal anti–Smad-1 IgG, goat polyclonal anti–Smad-5 IgG, and rabbit polyclonal anti–Smad-8 IgG (Santa Cruz Biotechnology).

In vitro Cell Growth Assay
Cells were plated onto a 96-well plate (2,500 cells/well). Cell growth was assessed after a period of incubation (up to 5 days). Crystal violet was used to stain cells, and absorbance was determined at a wavelength of 540 nm using a spectrophotometer (Bio-Tek, ELx800).

In vitro Invasion Assay
Transwell inserts with 8 μm pore size were coated with 50 μg of Matrigel (BD Matrigel Basement Membrane Matrix) and air-dried. The Matrigel was rehydrated before use. Twenty thousand cells were added to each well. After 96 h, cells that had migrated through the matrix to the other side of the insert were fixed in 4% formalin, stained with 0.5% (weight/volume) crystal violet, and counted under a microscope.

Wounding Assay
Fifty thousand cells were seeded into each well of a 24-well plate and allowed to reach near-confluency. The layer of cells was then scraped with a fine-gauge needle. The movement of cells to close the wound was recorded using a time-lapse video recorder and analyzed using the Optimas 6.0 motion analysis.

Cell-Matrix Adhesion Assay
Forty thousand cells were added in each well of a 96-well plate, previously coated with Matrigel (5 μg/well). After 40 min of incubation, nonadherent cells were washed off using a balanced salt solution buffer. The remaining adherent cells were then fixed and stained with crystal violet. The number of adherent cells was then counted.

Flow Cytometric Analysis of Apoptosis
All cells including those floating in the culture medium were harvested after a period of incubation. Cells were washed in cold PBS and resuspended in 1× annexin-binding buffer at a density of 1 × 10⁶ cells/mL after centrifugation. Five microliters of FITC annexin V and 1 μL of the propidium iodide working solution (100 μg/mL; Molecular Probes) were added to 100 μL of the cell suspension. After a 15-min incubation at room temperature, 400 μL of 1× annexin-binding buffer was added, mixed gently and the samples were kept on ice. The stained cells were immediately analyzed using the flow cytometer and FlowMax software package.

Immunocytochemical Staining of Par-4
Cells were fixed and permeabilized with 0.1% Triton for 5 min in tris-buffered saline. Following blocking with horse serum, the cells were probed with anti–Par-4 antibody, labeled with biotinylated secondary antibody and visualized using the Vectastain ABC system (Vector Laboratories, Inc.).

Immunofluorescent Staining of Par-4 and pSmad-1
Cells were fixed in ice-cold ethanol and then rehydrated and permeabilized before staining. Immunofluorescent staining of Par-4 was done using anti–Par-4 antibody (1:100 from original), and the FITC labeled anti-mouse IgG was used at 1:100. The staining was visualized under Olympus BX51 fluorescent microscope, and photographed using a cooled digital C4742-80 camera (Hamamatsu Photonics, Ltd.).

To stain the phosphorylated Smad-1, PC-3 cells were seeded on a glass chamber slide and done in duplicate. Following 2 h of incubation in serum-free DMEM, the cells were exposed to rh-BMP-9 (20 ng/mL), or serum-free medium alone for 1 h. The immunofluorescent staining of phosphorylated Smad-1 was done using anti–phosphorylated Smad-1 antibody at 1:50 from the original, and the TRITC-labeled anti-goat IgG was used at 1:200, and then photographed.

Statistical Analysis
Statistical analysis was done using the Minitab statistical software package (version 14). Non-normally distributed data was assessed using the Mann-Whitney test, whereas the two-

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sample t test was used for normally distributed data. Analysis on positivity of immunohistochemical staining in prostate tissues used Fisher’s exact test. Differences were considered to be statistically significant at P < 0.05.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

References
Bone Morphogenetic Protein-9 Induces Apoptosis in Prostate Cancer Cells, the Role of Prostate Apoptosis Response-4

Lin Ye, Howard Kynaston and Wen G. Jiang


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