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Prostate cancer bone metastases acquire resistance to androgen deprivation via WNT5A-mediated BMP-6 induction

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Background: Androgen ablation is the first-line therapy for patients with metastatic prostate cancer (CaP). However, castration resistance will eventually emerge. In the present study, we have investigated the role of bone morphogenetic protein-6 (BMP-6) in the development of castration-resistant prostate cancer (CRPC) in the context of bone metastases.

Methods: We initially investigated the clinical course of 158 men with advanced CaP who were treated with primary androgen deprivation therapy. To elucidate the underlying mechanism of CRPC in the context of bone metastases, we examined the impact of bone stromal cells on CaP in the absence of androgens using a co-culture model.

Results: In the 158 patients, we found that the median time to prostate-specific antigen progression was significantly shorter when bone metastases were present (14 months (95% CI, 10.2–17.8 months) *vs* 57 months (95% CI, 19.4–94.6 months)). These results suggest that bone–tumour interactions may accelerate castration resistance. Consistent with this hypothesis, *in vitro* co-cultures demonstrated that CaP cells proliferated under an androgen-depleted condition when incubated with bone stromal cells. Mechanistically, gene expression analysis using quantitative polymerase chain reaction arrays showed a dramatic induction of BMP-6 by CaP cell lines in the presence of bone stromal cells. Further studies revealed that WNT5A derived from bone stromal cells induced the expression of BMP-6 by CaP cells; BMP-6 in turn stimulated cellular proliferation of CaP cells in an androgen-deprived media via a physical interaction between Smad5 and β-catenin. Intracellularly, WNT5A increased BMP-6 expression via protein kinase C/NF-κB pathway in CaP cell lines.

Conclusions: These observations suggest that bone–CaP interaction leads to castration resistance via WNT5A/BMP-6 loop.

In the United States, prostate cancer (CaP) is the second most common cause of cancer-related deaths in men (Siegel *et al*, 2012). In the era of prostate-specific antigen (PSA), overwhelming majority of CaP cases is detected early. Yet, after a definitive treatment, ~30% of men with clinically localised CaP relapse within 5 years. For men with recurrent and advanced CaP, androgen ablation is the most effective treatment. Inevitably though, castration resistance emerges with a median time of

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Novelty and impact statement: In patients with metastatic prostate cancer, timing of androgen deprivation is still controversial. In the present study, we report that the median time to emergence of CRPC following primary androgen deprivation therapy was significantly shorter in men with bone metastases. Subsequent studies revealed that bone stromal cells render prostate cancer cells relatively resistant to castration via WNT5A/BMP6 loop. These results suggest that castration should be initiated prior to the development of bone metastases.

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18 months (Crawford *et al*, 1989; Schellhammer, 1996; Caubet *et al*, 1997). Once castration-resistant prostate cancer (CRPC) develops, secondary androgen manipulation, immunotherapy, and chemotherapy have been modestly effective. Currently, the precise mechanism underlying the emergence of CRPC remains unclear although enhanced androgen sensitivity and ligand-independent activation of androgen receptor (AR) signalling pathways have been proposed (Titus *et al*, 2005).

In men with CRPC, the most common sites of metastases are pelvic lymph nodes and bone. Indeed, recent publication has reported that the median time to development of skeletal metastasis in CRPC patients is 25.2 months (Smith *et al*, 2012). Simultaneously, autopsy studies have shown that ~80% of patients who die from CaP have bone metastases (Blaszczyk *et al*, 2004; Logothetis and Lin, 2005). As tumour microenvironment has a critical role in tumour aggressiveness and androgen sensitivity is a key feature of CaP cells, it is plausible that bone metastases contribute to the emergence of CRPC.

Bone morphogenetic proteins (BMPs) make up the largest subfamily within the transforming growth factor-β superfamily (Massague, 1998). Bone morphogenetic protein signals through a heteromeric complex of type I and type II transmembrane serine/threonine kinase receptors. To date, three distinct type I receptors, such as activin receptor-like kinase 2 (ALK2), BMP receptor type IA (BMPR-IA/ALK3), and BMP receptor type IB (BMPR-IB/ALK6), have been identified. Likewise, three type II receptors, such as BMP receptor type II (BMPR-II), activin receptor type IIA (ActR-IIA), and activin receptor type IIB (ActR-IIB), have been described (ten Dijke *et al*, 1994; Rosenzweig *et al*, 1995). In CaP, elevated levels of BMPs have been correlated with bone metastases (Autzen *et al*, 1998). Among the members of the BMP family, BMP-6 is the most frequently elevated subtype in CaP (Bentley *et al*, 1992; Barnes *et al*, 1995; Hamdy *et al*, 1997; Autzen *et al*, 1998), although increased levels of BMP-7 have also been observed (Masuda *et al*, 2003). Functionally, the most commonly reported effect of BMPs on CaP cell lines is growth inhibition. For example, studies using LNCaP human CaP cell line showed that BMP-2 and -4 inhibited cellular proliferation (Ide *et al*, 1997; Haudenschild *et al*, 2004). Similarly, BMP-6 inhibited CaP cell lines independent of androgen sensitivity (Haudenschild *et al*, 2004; Kim *et al*, 2004), whereas BMP-7 decreased the proliferation of PC3 human CaP cell line (Haudenschild *et al*, 2004).

The WNT family of proteins comprises a set of extracellular ligands rich in cysteine residues. WNTs bind to the seven-pass transmembrane receptors of the Frizzled family (Yang-Snyder *et al*, 1996) to activate the Dishevelled phosphoproteins in the cytoplasm (Noordermeer *et al*, 1994). Subsequently, WNT signalling is transduced via both β-catenin-dependent (canonical) and β-catenin-independent (non-canonical) pathway. Among the members of the WNT family, WNT5A signalling utilises the non-canonical pathway. In malignancies, elevated levels of WNT5A have been reported in melanomas, lung cancer, breast cancer, CaP, and gastric cancer (Weeraratna *et al*, 2002; Huang *et al*, 2005; Pukrop and Krischer, 2005; Kurayoshi *et al*, 2006; Yamamoto *et al*, 2009, 2010).

In the present study, we have investigated the CaP–bone stromal cells interaction. We report that WNT5A secreted by bone stromal cells increases BMP-6 expression in CaP, thereby leading to resistance to androgen deprivation via protein kinase C (PKC)/NF-κB (p52) signalling.

MATERIALS AND METHODS

Patients. Charts of 158 patients with confirmed diagnosis of CaP who were treated with primary androgen deprivation were reviewed retrospectively. Approvals from all participating institutions' Institutional Review Board were obtained: Inje University Busan Paik Hospital (Busan, Korea), Dong-A University Hospital (Busan, Korea), Ulsan University Hospital (Ulsan, Korea), Kosin University Gospel Hospital (Busan, Korea), and Ewha Womans University Mokdong Hospital (Seoul, Korea). Reasons for primary castration therapy were significant co-morbidities, advanced disease at diagnosis, or patient preference.

Cell culture. LNCaP, 22Rv1 (androgen-responsive human CaP cell lines), WPMY-1 (human normal prostate stromal cell line derived from 54 years old normal Caucasian male), and HS-5 (human bone marrow stromal cell line derived from normal 30 years old Caucasian male) were purchased from the American Type Culture Collection (ATCC) (Manassas, VA, USA) and routinely maintained in RPMI 1640 containing 10% fetal bovine serum (FBS) and penicillin/streptomycin (Invitrogen, Carlsbad, CA, USA). To recapitulate androgen-deprived condition, RPMI-1640 supplemented with 1% charcoal-stripped FBS (cFBS) and 10 μM bicalutamide was used for LNCaP cells. As for 22Rv1, culture condition for androgen depletion was identical except for 25 μM bicalutamide. Unless indicated, all co-culture experiments were carried out for 48 h.

Reagents and plasmid. To generate the reporter plasmid BMP6-Lux, pGL3 basic was used as the backbone. Each of the BMP-6 promoter fragments was amplified using polymerase chain reaction (PCR). Polymerase chain reaction primer sequences are shown in Supplementary Table 1. KpnI and XhoI restriction enzyme sites were used for subcloning BMP-6 promoter fragments into pGL3. Recombinant BMP-6 and WNT5A were purchased from R&D systems (Minneapolis, MN, USA). Sources and concentrations used of the small molecule inhibitors were as follows: 100 nM Calphostin C (Calp C) (Merck, Whitehouse Station, NJ, USA), 10 μM BAY 11-7082 (EMD Millipore, Billerica, MA, USA), 20 μM U0126 (Cell Signaling Technology, Danvers, MA, USA), 20 μM SP600125 (EMD Millipore), 10 μg ml^{-1} cycloheximide (Cyclo) (EMD Millipore), and 5 μg ml^{-1} actinomycin D (ActD) (Sigma Aldrich, St Louis, MO, USA).

RT–PCR. Total RNA was extracted with Trizol (Invitrogen) and RT–PCR was carried out using a commercially available kit (One-step SuperScript RT-PCR kit, Invitrogen). Reverse transcription was carried out using the manufacturer's recommended protocol. Polymerase chain reaction condition was as follows: 94 °C for 30 s, 55 °C for 30 s, and 68 °C for 1 min for 35 cycles. Primer sequences are shown in Supplementary Table 1.

Immunoblot. Cells were rinsed with PBS and harvested using Cell Lysis Buffer (Cell Signaling Technology). After centrifugation, supernatant was transferred to a new tube and protein concentration was measured. With 30 μg of protein, electrophoresis was carried out using 12% SDS–PAGE gel. Protein was transferred to a nitrocellulose membrane (Bio-Rad, Hercules, CA, USA) and analysed with an enhanced chemiluminescence kit (Thermo Scientific, Rockford, IL, USA).

Antibodies. The following antibodies were used in this study: anti-β-actin antibody (Sigma), human BMP-6 blocking antibody (R&D), anti-human BMP-6 detection antibody (Abcam, Cambridge, MA, USA), anti-human p65 antibody (Sigma), anti-human p50 antibody, anti-human p52 antibody (Cell Signaling), anti-Smad 5 antibody (Cell Signaling), and anti-phospho Smad 5 antibody (Cell Signaling).

Luciferase assay. Luciferase assay was performed using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA). LNCaP and 22Rv1 cells were plated onto six-well plates and transfected with BMP6-Luc and CMV-Renilla luciferase plasmids using Lipofectamine LTX (Invitrogen). Cells were treated with indicated concentrations of WNT5A for 18 h. After cell lysis, the

lysates were centrifuged and the supernatant was used to measure luciferase activity. The firefly luciferase activity was normalised using the *Renilla* luciferase activity. All experiments were repeated at least three times and similar results were obtained each time.

ChIP assay. The EZ-ChIP kit (Millipore) was used. Cells were fixed and harvested using the manufacturer's protocol. After incubating with the indicated antibodies, DNA was sonicated and purified with spin column and analysed with PCR.

Statistical analysis. For all analyses, Student's *t*-test and Pearson correlation were performed. Clinical data were evaluated using Kaplan–Meier and multivariate Cox analysis. A *P-value* of <0.05 was considered statistically significant.

RESULTS

Presence of bone metastases correlates with a significantly shortened time to emergence of CRPC. Initially, we analysed the clinical course of 158 CaP patients from five institutions who were treated with primary androgen deprivation therapy. In this cohort, 57 had bone metastases as documented by bone scan. Clinical characteristics of the patients are shown in Supplementary Figure 1A. The median follow-up was 24 months (range 6–92 months). As PSA remained detectable in many of the patients, PSA progression was defined as a rise in PSA on three consecutive measurements while on androgen deprivation therapy (Bubley *et al*, 1999). The results demonstrated that the median time to the emergence of PSA progression was significantly shorter in men with bone metastases than those without skeletal disease (median time to PSA progression 14 months (95% CI, 10.2–17.8 months) *vs* 57 months (95% CI, 19.4–94.6 months), $P<0.001$) (Supplementary Figure 1B). More importantly, when patients were stratified by tumour stage, the presence of bone metastases was associated with an earlier emergence of PSA progression in both localised (T1 and T2) and locally advanced (T3 and T4) CaP (Supplementary Figure 1C). Multivariate Cox regression analysis revealed that the bone metastasis (HR, 3.291; 95% CI, 1.703–6.361; $P<0.001$) was an independent predictor of PSA progression (Supplementary Table 2).

CaP–bone stromal cell interaction in tissue culture recapitulates castration resistance. Above clinical data suggested that the skeletal microenvironment might render CaP cells resistant to castration. To test this hypothesis, we co-cultured the human CaP cell lines LNCaP and 22Rv1 with the bone marrow stromal cell line HS-5 in Boyden chambers. To establish a culture condition that mimics androgen depletion, LNCaP and 22Rv1 were cultured in RPMI-1640 supplemented with 1% cFBS and varying concentrations of the AR antagonist bicalutamide. In LNCaP cells, 10 μM bicalutamide in RPMI-1640/1% cFBS completely arrested cellular proliferation. As for 22Rv1, 25 μM bicalutamide in RPMI-1640/1% cFBS was the optimal condition (Supplementary Figure 2). Next, we generated the androgen-responsive reporter plasmid containing PSA promoter and luciferase (PSA-Lux). When LNCaP was transfected with PSA-Lux and cultured under androgen-deprived condition, increased luciferase activity of $\sim$2.5-fold was observed in the presence of bone marrow (HS-5) but not normal prostate stromal cell line (WPMY-1) (Figure 1A). Simultaneously, the cell count increased to $>250\%$ when co-cultured with HS-5 but not with WPMY-1 cells (Figure 1B). When the second androgen-responsive human CaP cell line 22Rv1 was used, a more modest but statistically significant increase in cell number was observed again in the presence of HS-5 bone stromal cells (Supplementary Figure 3A). To identify factors that potentially enhanced the ability of CaP cells to proliferate when incubated with bone stromal cells under castrate levels of androgens, quantitative PCR (Q-PCR) array was carried out using RNA harvested from LNCaP cells after co-culturing with HS-5 cells under the androgen-depleted condition. LNCaP/WPMY-1 co-culture was used as the control (data not shown). Among the factors whose expression levels increased, we focused on BMP-6 based on the published data that showed that BMPs regulate androgen sensitivity in CaP cells (Ide *et al*, 1997)

BMP-6 is the mediator of cellular proliferation of CaP cells induced by bone stromal cells under androgen-depleted condition. To confirm the results of the Q-PCR array, RT–PCR was carried out after harvesting RNA from LNCaP and 22Rv1 cells co-cultured with HS-5 in the absence of androgens. Among these BMP subtypes, only the induction of BMP-6 expression was observed (Figure 1C). RT–PCR and ELISA for BMP-6 in HS-5 cells was negative, confirming that BMP-6 in our experimental context is solely derived from the CaP cell lines (data not shown). However, it cannot be ruled out the undetectable level expression in HS-5 cells. This upregulation of BMP-6 in CaP cells by bone stromal cells was confirmed at the protein level using immunoblot and ELISA (Figures 1D and E, respectively). When LNCaP was treated directly with BMP-6 in the androgen-depleted media, cell count increased by 2–3-fold in a concentration-dependent manner (Figure 1F); similar result was also obtained in 22RV1 cells (Supplementary Figure 3B). To determine whether BMP-6 is the endogenous factor that stimulates the proliferation of CaP cells in the presence of bone stromal cells under the androgen-deprived culture condition, LNCaP/HS-5 and 22Rv1/HS-5 co-cultures were treated with BMP-6 neutralising antibodies. The results demonstrated a significant reversal of the cellular proliferation of both LNCaP and 22Rv1 when BMP-6 was blocked (Figure 1G and Supplementary Figure 3C, respectively).

BMP-6-induced cellular proliferation of CaP cells in androgen-depleted media requires BMP-RII, ALK2, Smad5, and β-catenin. As BMP signalling requires a heterotetrameric combination of types I and II receptors (Miyazono *et al*, 2005), each of the type I and II BMP receptors were overexpressed in LNCaP in an attempt to investigate the mechanism by which BMP-6 enhances the proliferation of CaP cells under androgen-depleted condition. The results demonstrated that the overexpression of BMP-RII increased the cellular proliferation of LNCaP cells in the absence of androgens (Figure 2A). As a complementary experiment, each of the type II BMP receptors was knocked down using the siRNA approach that was previously published by our group (Kwon *et al*, 2009; Lee *et al*, 2010). As expected, the increase in LNCaP's cell count induced by BMP-6 under the androgen-deprived condition was blocked by shBMPRII (Figure 2B). Control experiments demonstrating the specific knockdown of the target protein following transfection with siRNA is shown in Supplementary Figure 4 (left panel). With respect to type I receptors, constitutively active mutants were used. Increase in LNCaP cell count was observed when constitutively active ALK2 (CA-ALK2) was used (Figure 2C). Knock down of ALK-2 using siRNA blocked the increase in cell count following stimulation with BMP-6 (Figure 2D). Immunoblot confirming the specificity of siRNA in knocking down the target type I receptor protein is shown in Supplementary Figure 4 (middle panel). Interestingly, knockdown of ALK3 and ALK6 also reversed the BMP-6-induced cellular proliferation. Notwithstanding, these results collectively demonstrate that ALK2 and BMP-RII are the optimal types I and II receptors that mediate cellular proliferation of CaP cells in the absence of androgens.

Following the activation of receptors, BMP signalling requires a receptor-activated Smad (R-Smad)/Co-Smad heteromeric complex. To date, three R-Smads specific for BMP signalling have been identified: Smads 1, 5, and 8 (Miyazono *et al*, 2005). In contrast, there is only one co-Smad, Smad4. When each of the R-Smads was

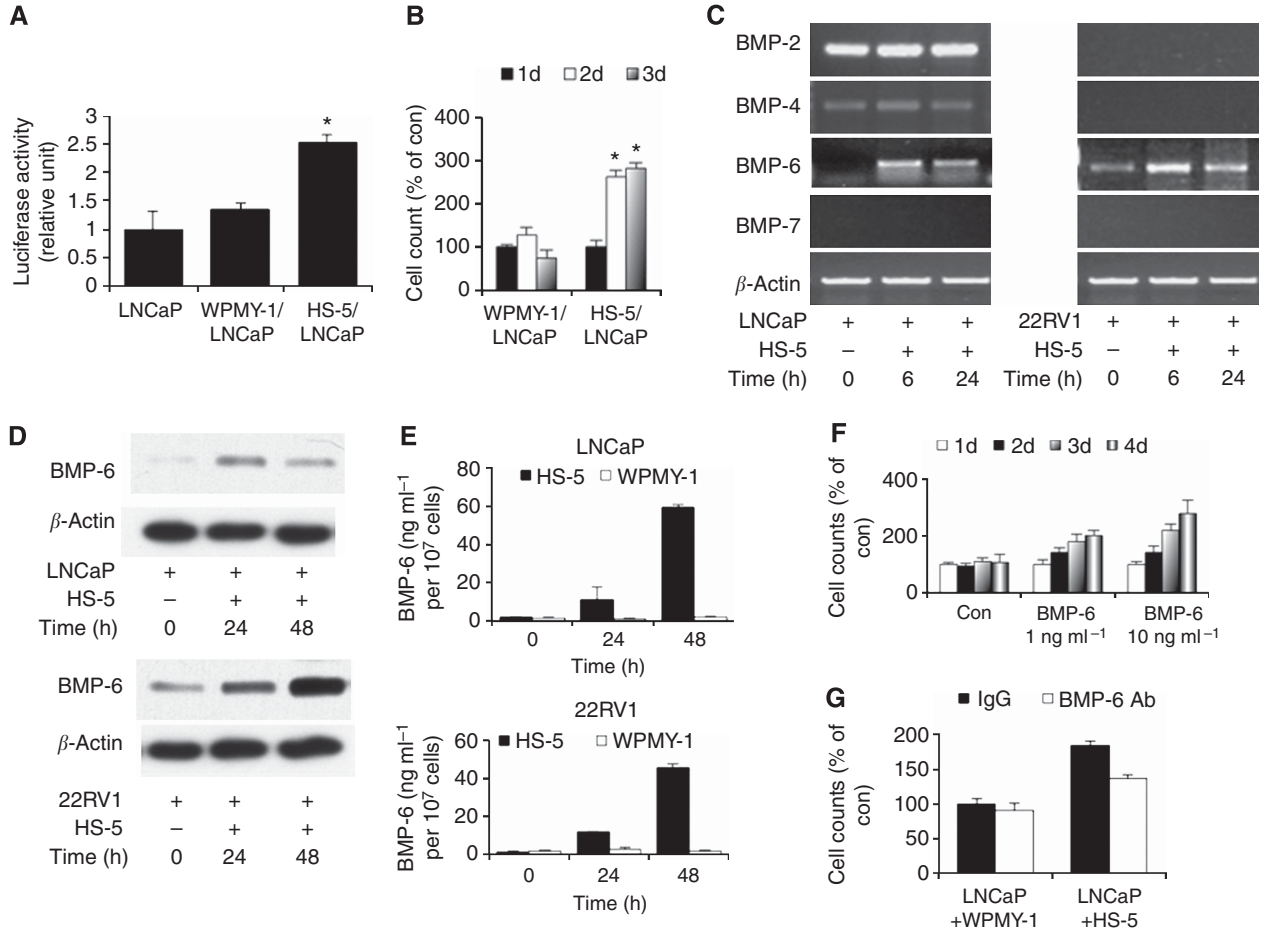

Figure 1. Recapitulation of bone and CaP interaction *in vitro* and expression of BMP-6. (**A**) LNCaP, an androgen-sensitive human CaP cell line, was transiently transfected with the androgen-responsive reporter, PSA-Lux and co-cultured with either WPMY-1 (human prostate stromal cell line) or HS-5 (human bone stromal cell line). Luciferase activity was significantly higher in LNCaP/HS-5 than in LNCaP/WPMY-1 when cultured under androgen-deprived condition (RPMI-1640 with 1% charcoal-stripped serum and 10 μM bicalutamide) ($P<0.05$). (**B**) Cell count also increased when LNCaP and 22Rv1 were co-cultured with HS-5 but not WPMY-1 in androgen-depleted condition. (**C**) Semi-quantitative RT–PCR for BMP-2, -4, -6, and -7 in androgen-sensitive human CaP cell lines, LNCaP and 22RV1, after co-culturing with HS-5. Only BMP-6 expression was induced in both LNCaP and 22RV1 cells. (**D**) Immunoblot analysis for BMP-6. Again, co-culturing with HS-5 increased the expression of BMP-6 in LNCaP and 22RV1. (**E**). ELISA demonstrated a dramatic increase in BMP-6 in conditioned media of LNCaP and 22Rv1 co-cultured with HS-5. (**F**). Direct effect of BMP-6 on cell growth under androgen-deprived condition in LNCaP. Bone morphogenetic protein-6 increased cellular proliferation of LNCaP cells in androgen-deprived condition in a time- and concentration-dependent manner. (**G**). Neutralisation of BMP-6 in LNCaP/HS-5 co-culture. LNCaP/HS-5 was co-cultured in the presence of BMP-6 neutralising antibody. The increase in LNCaP cell count was partially abrogated with BMP-6 neutralising antibody. *, statistically significant ($P<0.01$).

co-transfected with Smad4, only the Smad5/Smad4 combination resulted in an increased cell count when treated with BMP-6 in RPMI-1640/1% cFBS/10 μM bicalutmide (Figure 2E). Again, using our previously reported siRNA approach targeting Smads (Lee *et al*, 2010), the knock down of Smad5 blocked the BMP-6-induced proliferation of LNCaP cells under androgen-depleted condition (Figure 2F). Again, Supplementary Figure 4 (right panel) shows the efficacy of siRNA in knocking down each of the R-Smads. In addition, a more modest but a statistically significant decrease in cell count was observed when Smad 1 and 8 were knocked down. Each type of siRNA had little bit affected to the cells. However, it is not statistically significant.

To further elucidate the mechanism of BMP-6-induced proliferation of CaP cells in the absence of androgens, yeast two-hybrid screening using Smad5 as the bait was carried out. The results identified β-catenin as a potential binding partner of Smad5 (Figure 3A). Nidogen and collagen were used as negative and positive controls, respectively. The interaction between endogenous Smad5 and β-catenin was then confirmed using immunoprecipitation in LNCaP cells (Figure 3B). On the basis of these preliminary results, each R-Smad expression vector was co-transfected with the β-catenin luciferase expression vector (8 × TOPFlash (TCF/LEF binding site)) into the HEK293 cells. Subsequently, luciferase assay demonstrated that the overexpression of Smad5 increased the TOPFlash luciferase activity most significantly (Figure 3C). The reverse experiment knocking down β-catenin via shRNA blocked the BMP-6-induced proliferation of LNCaP and 22Rv1cells in RPMI-1640/1% cFBS (Figure 3D). Finally, immunofluorescence microscopy demonstrated that both phosphorylated Smad5 (pSmad5) and β-catenin translocated to the nucleus in LNCaP cells when stimulated with BMP-6 under androgen-deprived condition (Figure 3E). As one potential mechanism of castration resistance mediated by β-catenin involves the upregulation of AR (Yang *et al*, 2006), we next used Q-PCR to measure the mRNA level of AR in LNCaP and 22Rv1 cells when co-cultured with HS-5 or WPMY-1 cells. The results demonstrated 3.95- and 2.66-fold increases in AR mRNA in the presence of HS-5 in LNCaP and 22Rv1, respectively (Figure 3F).

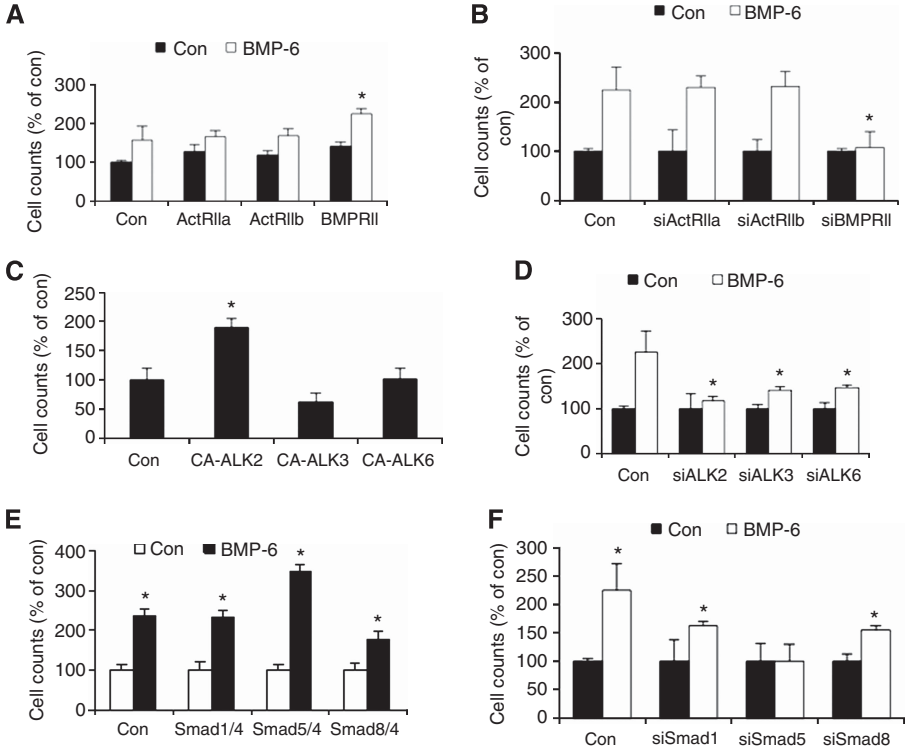

Figure 2. Role of BMP receptors and Smads under androgen-deprived condition. (**A**) LNCaP cells were transiently transfected with each of the type II BMP receptors and treated with 10 ng ml^{-1} BMP-6 for 24 h. Only the overexpression of BMP-RII significantly increased LNCaP cell count upon exposure to BMP-6 in RPMI-1640/1%cFBS/10 μM bicalutamide. (**B**) Each of the BMP type II receptors was knocked down using siRNA. Loss of BMP-RII expression resulted in blocking the cellular proliferation of LNCaP induced by BMP-6 under androgen-deprived condition. Control experiments demonstrating the specificity of knock down are shown in Supplementary Figure 3. (**C**) Constitutively active BMP type I receptors were transfected into LNCaP cells and incubated with BMP-6 in androgen-depleted media. Constitutively active ALK2 (CA-ALK2) induced higher cell count in LNCaP in the presence of BMP-6. (**D**). Bone morphogenetic protein type I receptors were knocked down using siRNA. Knockdown of ALK2 blocked BMP-6-mediated proliferation of LNCaP under androgen deprivation. Control experiments demonstrating the specificity of knockdown are shown in Supplementary Figure 3. (**E**) Overexpression of R-Smads 1, 5, and 8 along with the co-Smad Smad4. Only when Smad5 and Smad4 were co-transfected, BMP-6 further increased the cell count of LNCaP in the absence of androgen. (**F**) When siRNA targeting each of the R-Smads was transfected into LNCaP cells, only the knockdown of Smad5 blocked the BMP-6-induced cellular proliferation of LNCaP cells. Control experiments demonstrating the specificity of knockdown are shown in Supplementary Figure 3. *, statistically significant ($P<0.01$).

WNT5A is the bone marrow stromal cell line-derived factor that induces BMP-6 expression by CaP cells following androgen-deprivation. The clinical and co-culture data presented in Figures 1–4 suggest the presence of a bone stroma-derived factor that stimulates the proliferation of CaP cells when castrated. As BMP-6 is an autocrine factor in our experimental context, we hypothesised that bone stromal cells secrete factor(s) that induces the expression of BMP-6 in CaP cells under androgen-depleted condition. As an initial attempt to identify this bone marrow stromal cell-derived product that induces the expression of BMP-6 in CaP cells, we focused on WNTs because they have been previously shown to induce BMP expression, with WNT5A in particular being tied to BMP-6 (Dai *et al*, 2008). To determine whether WNTs are expressed in our CaP/bone stromal cell co-culture system, semi-quantitative RT–PCR was carried out using RNA harvested from HS-5 cells. The results demonstrated the induction of WNT5A only when HS-5 bone stromal cells were co-cultured with either LNCaP or 22Rv1 cells (Figure 4A and Supplementary Figure 5). In LNCaP and 22Rv1 cells also expressed very low level of WNT5A. HS-5 cell expressed >100-fold of WNT5A (data not shown). However, it cannot be ruled out the small amount of WNT5A from prostate cancer cells also. The induction of WNT5A was also reproduced at the protein level when HS-5 was co-cultured with either LNCaP or 22Rv1 cells, although the response in the presence of LNCaP was significantly more robust (Figure 4B). Under the androgen-depleted condition, WNT5A increased the cellular proliferation of LNCaP and 22Rv1 cells in a concentration-dependent manner (Figures 4C and D, respectively). More importantly, co-culturing either LNCaP or 22Rv1 cells with HS-5 in RPMI-1640/1%cFBS/10 or 25 μM bicalutamide significantly decreased the proliferation of the CaP cells when the respective co-culture was treated with a WNT5A neutralising antibody (Figures 4E and F, respectively).

Bone stromal cell-derived WNT5A induces BMP-6 expression through the NF-κB non-canonical WNT pathway. To investigate whether WNT5A induces BMP-6 expression in CaP cells, LNCaP was treated with WNT5A and BMP-6 expression levels were measured using Q-PCR. The results demonstrated that WNT5A induced BMP-6 expression at the mRNA level both in a concentration- and time-dependent manner (Figures 5A and B, respectively). When the co-cultures LNCaP/HS-5 and 22Rv1/HS-5 were pre-treated with BMP-6 neutralising antibody, the previously observed CaP cellular proliferation under androgen-depleted condition was no longer detected (Figures 5C and D, respectively). These results suggest that the induction of BMP-6 by WNT5A is one mechanism that underlies, in part, the proliferation of CaP cells elicited by bone stromal cells.

Next, LNCaP cells were treated with ActD and Cyclo to investigate the mechanism of BMP-6 induction by WNT5A.

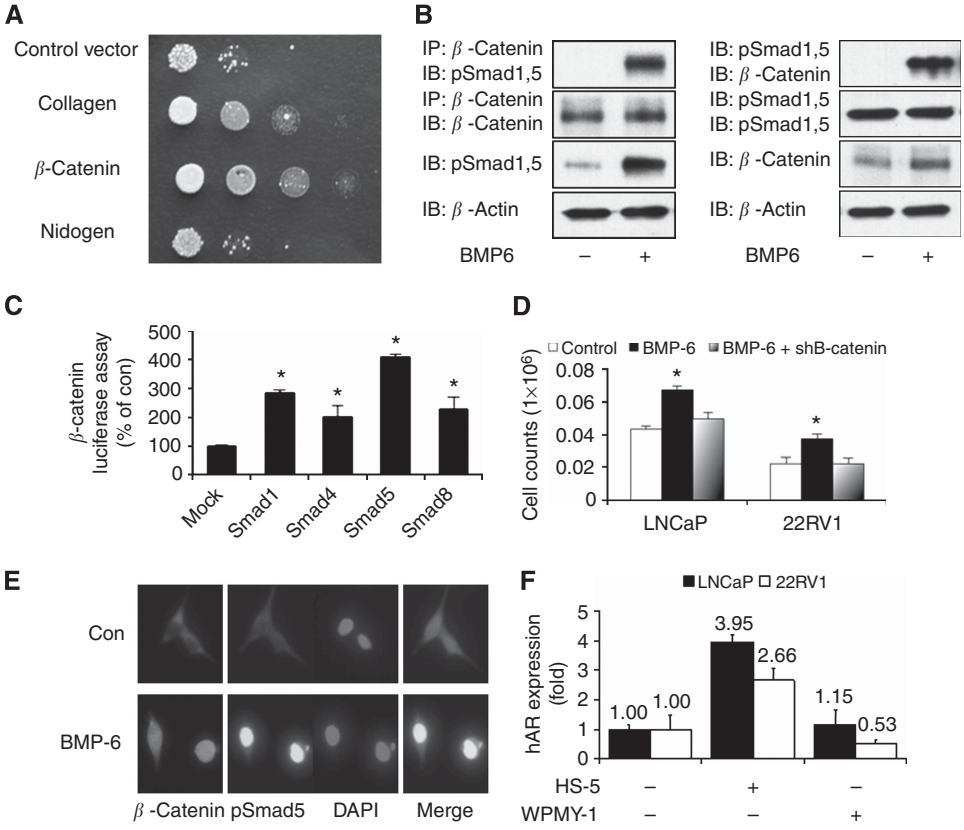

Figure 3. (A) Yeast-two-hybrid for identification of Smad5-interacting proteins. The results demonstrated a strong interaction between β-catenin and Smad5. Nidogen = neg control. (**B**) Interaction between Smad5 and B-catenin endogenously was confirmed using immunoprecipitation. Because antibody specific for phosphorylated Smad5 (pSmad5) is not available, antibody that targets both pSmad1 and 5 was used. When LNCaP was treated with BMP-6 under an androgen-deprived condition, immunoprecipitation demonstrated that pSmad1/5 co-precipitated with β-catenin (left panel). The reverse experiment showed that β-catenin was pulled down when pSmad1/5 was immunoprecipitated (right panel). As expected, immunoblot confirmed that BMP-6 increased the protein levels of both pSmad1/5 and β-catenin. (**C**) Effect of R-Smads on β-catenin target gene (8 × TOPFlash-lux). When each of the R-Smad was overexpressed along with TOPFlash-lux in HEK293 cells, only Smad5 transfection increased luciferase activity. (**D**) Effect of β-catenin knockdown on cellular proliferation initiated by BMP-6 in the absence of androgen in LNCaP. Transfection with β-catenin shRNA inhibited the increased cellular proliferation induced by BMP-6. (**E**) Nuclear translocation of Smad5 and β-catenin by BMP-6 in LNCaP. After treatment with 100 ng ml^{-1} BMP-6 in androgen-depleted media, Smad 5 and β-catenin translocated simultaneously into the nucleus. (**F**) Q-PCR revealed that only in the presence of HS-5 bone stroma cells, AR mRNA levels increased in both LNCaP and 22Rv1 under androgen-depleted culture condition. *, statistically significant ($P<0.01$).

Semi-quantitative RT–PCR demonstrated that the transcription inhibitor ActD but not the translation inhibitor Cyclo blocked the induction of BMP-6 mRNA by WNT5A (Figure 6A). These observations suggest that WNT5A directly regulates the expression of BMP-6 at the transcription level.

WNTs signal through two types of pathways: the canonical β-catenin pathway and non-canonical pathways involving PKC and jun-activated kinase (JNK). When LNCaP was pre-incubated with the PKC inhibitor Calp C or JNK inhibitor SP600125, Q-PCR revealed that the induction of BMP-6 mRNA was blocked only when PKC was neutralised (Figure 6B). Similar results were obtained also with 22Rv1 cells (Supplementary Figure 6). Because NF-κB and ERK are downstream of PKC, LNCaP cells were subsequently treated with Bay 11-7082 (NF-κB inhibitor) and U0126 (ERK inhibitor) and BMP-6 expression was measured. As shown in Figure 6C, only Bay 11-7082 completely inhibited the WNT5A-mediated BMP-6 expression in LNCaP cells.

To further study the mechanism of WNT5A-mediated BMP-6 induction in CaP cells, we cloned the 1.5 kb BMP-6 promoter from LNCaP into the pGL3-Basic luciferase reporter vector (BMP6-Lux). When LNCaP was transfected with BMP6-Lux, WNT5A increased the luciferase activity by more than two-fold (Figure 6D). Next, deletion constructs of BMP-6 promoters were cloned using PCR into pGL3-Basic. Transfection of these various BMP-6 promoter deletion reporter plasmids into LNCaP and 22Rv1 cells localised WNT5A response element from −0.6 kb to −0.9 kb region of BMP-6 promoter (Figure 6E and Supplementary Figure 7, respectively). We then analysed this 300 bp region of the BMP-6 promoter that contains the WNT5A response element using the TESS computer simulation. This analysis revealed multiple potential NF-κB binding sites. To confirm the interaction between various NF-κB subtypes (p50, p52, and p65) and the WNT5A responsive region of BMP-6 promoter, chromatin immunoprecipitation (ChIP) assay was finally carried out. The results revealed that only p52 interacted with the BMP-6 promoter (Figure 6F).

The model that summarises our findings concerning CRPC and bone metastases is shown in Supplementary Figure 8. Once CaP cells metastasise to the bone, bone stromal cells induce CaP cells to express BMP-6 via the WNT5A-stimulated PKC/NF-κB pathway. Bone morphogenetic protein6, then, activates BMP-RII and ALK2 in CaP cells that leads to the phosphorylation of Smad5. Finally, Smad5 interacts with β-catenin and induces the expression of target genes, including AR.

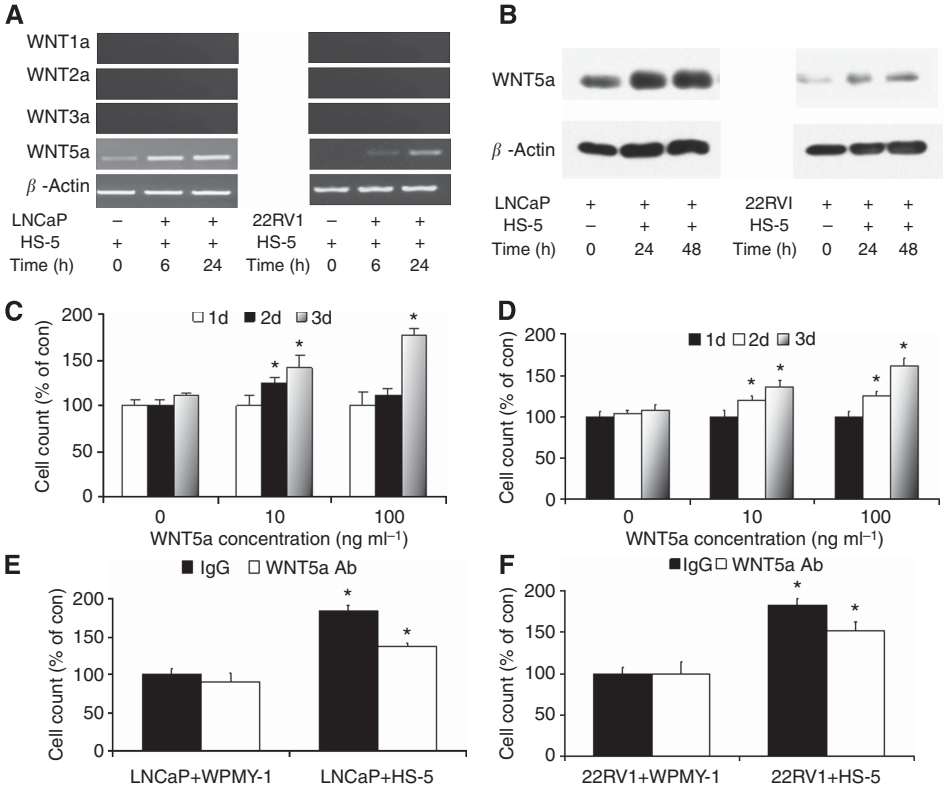

Figure 4. WNT5A detection in bone marrow stromal cells after co-culture with CaP cells. (**A**) Semi-quantitative RT–PCR for WNT1, 2, 3A, and 5A in HS-5 after co-culture with LNCaP and 22RV1 demonstrated an increase in Wnt5A mRNA. (**B**) Immunoblot analysis revealed the induction of WNT5A in HS-5 when co-cultured with either LNCaP or 22Rv1 cells. (**C**) Effect of WNT5A on cellular proliferation in androgen-depleted media. When LNCaP cells were cultured under an androgen-deprived condition, WNT5A increased the cell count in a concentration-dependent manner. (**D**) As with LNCaP, 22Rv1 also proliferated in response to WNT5A stimulation in a concentration-dependent manner. (**E** and **F**) Neutralising antibodies against WNT5A partially blocked the HS-5-mediated cellular proliferation under androgen-deprived condition in both LNCaP and 22Rv1 cells. *, statistically significant ($P<0.01$).

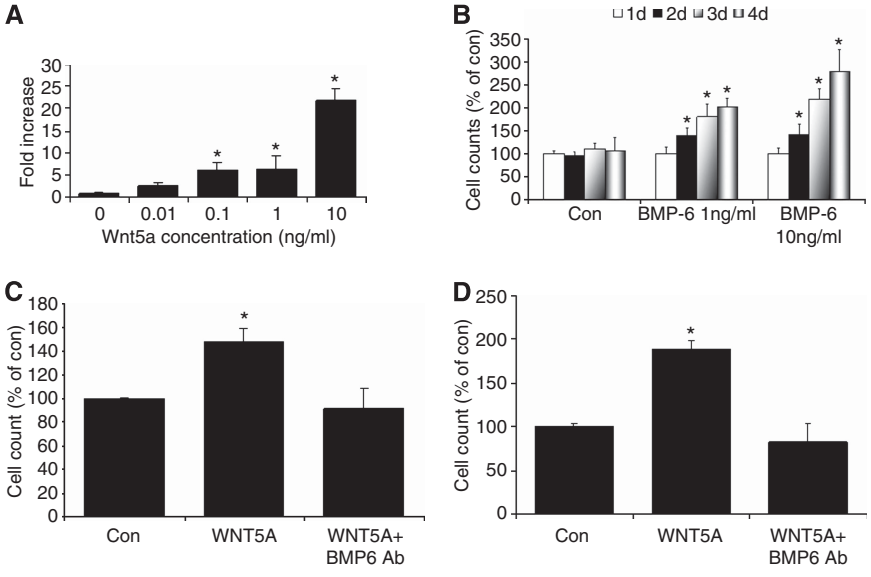

Figure 5. Bone morphogenetic protein-6 induction by WNT5A in androgen-depleted media. (**A**) Q-PCR demonstrated that WNT5A increased BMP-6 mRNA in LNCaP cells in a concentration-dependent manner when cultured under androgen-deprived condition. (**B**) Q-PCR demonstrated that 1 ng ml^{-1} of WNT5A increased BMP-6 mRNA in a time-dependent manner. (**C**) Bone morphogenetic protein-6 neutralising antibody blocked WNT5A-induced cellular proliferation of LNCaP cells when cultured in an androgen-deprived condition. (**D**) Bone morphogenetic protein-6 neutralising antibody blocked WNT5A-induced cellular proliferation of 22RV1 cells when cultured in an androgen-deprived condition. *, statistically significant ($P<0.01$).

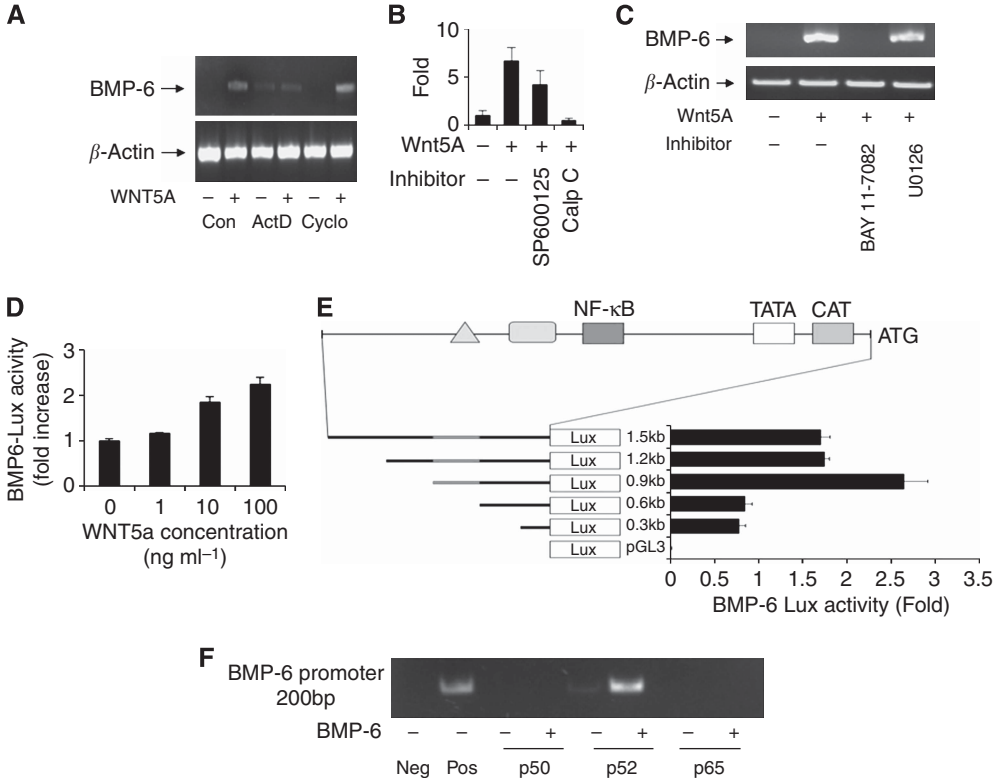

Figure 6. Mechanism of induction of BMP-6 by WNT5A in androgen-deprived culture. (**A**) Induction of BMP-6 by WNT5A in LNCaP under androgen deprivation was blocked by ActD but not by Cyclo. (**B**) LNCaP was incubated in androgen-depleted media with 1 ng ml^{-1} WNT5A and either the PKC inhibitor Calp C (100 nM) or JNK inhibitor SP600125 (20 μM). Q-PCR revealed that the induction of BMP-6 mRNA was blocked only when PKC was neutralised (Calp C). (**C**) In addition to 1 ng ml^{-1} WNT5A, LNCaP was treated with Bay 11-7082 (NF-κB inhibitor) (10 μM) or U0126 (ERK inhibitor) (20 μM). Semi-quantitative RT–PCR revealed that only blocking the NF-κB pathway inhibited BMP-6 mRNA induction. (**D**) The reporter plasmid BMP6-Lux (contains 1.5 kb BMP-6 promoter and luciferase) was transfected transiently into LNCaP cells. Following treatment with 1 ng ml^{-1} WNT5A, luciferase activity increased to more than two-fold. (**E**) Serial deletion constructs of BMP-6 promoter were generated using PCR and subcloned into a luciferase reporter plasmid. WNT5A-response element within the BMP-6 promoter was localised to a region between 0.6 and 0.9 kb. (**F**) ChIP revealed that NK-κB p52 interacted with the WNT5A-response region within BMP-6 promoter.

DISCUSSION

In the present study, we have demonstrated that when CaP patients are treated with primary androgen deprivation therapy, the time to the emergence of castration resistance in men with bone metastasis is significantly shorter than those with only a loco-regional disease. When the interaction between CaP cells and bone microenvironment was recapitulated in tissue culture using co-cultures of CaP and bone stromal cell lines, CaP cells became resistant to androgen deprivation. Further studies revealed that CaP cells attain the capacity to proliferate under androgen-depleted condition by activating the WNT5A/BMP-6 loop that involves bone stromal cells. Taken together, these results suggest that the bone microenvironment may render CaP metastases resistant to castration and suggest a novel target of intervention in men with CRPC and bone metastases.

Since the seminal report by Huggins in 1942, castration has remained the centerpiece of various treatment regimens for men with metastatic CaP (Huggins, 1942). Androgen deprivation, though, is not curative and CRPC inevitably emerges with a median time of 18–24 months (Crawford *et al*, 1989; Schellhammer, 1996; Caubet *et al*, 1997). This limited clinical effectiveness along with the complications such as bone loss and metabolic syndrome (Planas Morin and Morote Robles, 2012; Saylor and Smith, 2013) suggests that there is a theoretical advantage of delaying the initiation of castration as long as possible in men with metastatic CaP. Notwithstanding, a randomised prospective clinical trial has demonstrated that immediate rather than delayed androgen deprivation in patients with lymph node-positive metastases following radical prostatectomy results in an increased median survival of 2.6 years with a mean follow-up of 11.9 years (Messing *et al*, 2006). Although the biological explanation for the advantage of early castration remains unclear, results of the present study suggest that androgen ablation is less effective in the presence of bone metastases. Specifically, the median time to the emergence of PSA progression was 14 months in men with bone metastases who were treated with primary androgen deprivation. In contrast, PSA progression emerged with a median time of 57 months in men who were without bone metastases. Therefore, androgen deprivation therapy is more effective when initiated early prior to the development of metastatic disease in the bone.

Using CaP/bone stromal cell line co-culture models, the mechanism underlying the castration resistance induced by bone metastases was found to involve BMP-6 induction by WNT5A. To date, the most commonly reported direct effect of BMPs in CaP cell lines is growth inhibition. Indeed, we have previously reported that BMP-6 inhibits the proliferation of human CaP cell lines LNCaP, PC3, and DU145 (Ide *et al*, 1997; Kim *et al*, 2004). Yet, in the context of androgen-deprived condition and bone stromal cells, BMP-6 appears to promote cellular proliferation of CaP cells. Such condition-dependent effects of BMPs have also been reported with BMP-2 as Ide *et al* have reported that BMP-2 inhibits and

stimulates the cellular growth of LNCaP cells in the presence and absence of androgens, respectively (Ide *et al*, 1997). Therefore, the ultimate effect of BMPs in CaP may be dictated by the tumour microenvironment.

BMP signalling requires a heterotetrameric complex composed of BMP receptors type II and I, which then activates both the Smad-dependent and -independent pathways. To date, three each of type I and II receptors as well as three R-Smads have been identified (Miyazono *et al*, 2005). Although these BMP receptors and R-Smads are promiscuous and signal for multiple BMP subtypes, published data suggest that there is an optimal receptor combination for each BMP (Yu *et al*, 2008). In this regard, the current study suggests that BMP-RII and ALK2 are the optimal receptors for mediating resistance to androgen depletion. Downstream of BMP-RII and ALK2, Smad5 is required. The precise mechanism underlying the activation of Smad5 by ALK2 in the present experiments remains unclear. One possibility is that ALK2 preferentially activates Smad5. Alternatively, ALK2 may phosphorylate all R-Smads equally efficiently but that the expression levels of R-Smads vary with different culture conditions. Under this circumstance, the expression profile of R-Smad dictates the signalling specificity. Additional studies are underway to investigate these possibilities.

To the growing list of dysregulated factors that have been shown to contribute to the emergence of CRPC, BMP-6 signalling pathway involving Smad5 and β-catenin should be added. As Smad5 mediated the BMP-6-induced resistance of CaP cells to androgen deprivation, yeast-two-hybrid was carried out to identify the potential binding proteins of Smad5 that may provide a reasonable mechanistic explanation. This led to the identification of β-catenin. Previously, it has been reported that β-catenin interacts with AR to increase its transcriptional activity as measured by androgen-induced reporter gene constructs (Truica *et al*, 2000; Pawlowski *et al*, 2002; Yang *et al*, 2002; Song *et al*, 2003; Masiello *et al*, 2004). Simultaneously, β-catenin pathway has been shown to increase the transcript level of AR (Yang *et al*, 2006). In the present study, we have confirmed that AR mRNA level is indeed upregulated in CaP cells in the presence of bone stromal cells. Nevertheless, the possibility remains that Smad5/β-catenin interaction may enhance the activity of AR.

Results of the present study also give a glimpse of a new mechanism concerning the regulation of BMP-6 in CaP. Autzen *et al* in 1998 reported the frequent overexpression of BMP-6 in skeletal metastases of CaP (Autzen *et al*, 1998). Now, we have demonstrated that WNT5A derived from bone stromal cells induce the expression of BMP-6. Wnts are $\sim$40 kDa proteins rich in cysteine residues and have 19 genes that are subdivided into 12 subfamilies in humans (Clevers and Nusse, 2012). In malignancies, Wnt1 has been implicated in breast and CaP while abnormal expression of WNT5A has been reported in CaP (Tsukamoto *et al*, 1988; Iozzo *et al*, 1995; Chen *et al*, 2004). More relevant to the current study, Wnt signalling has been implicated in bone–CaP interactions (Dai *et al*, 2008). Among the members of the WNT family, WNT5A signalling utilises the non-canonical pathway that is independent of β-catenin (Katoh and Katoh, 2007). Consistent with this concept, we have also observed that WNT5A induces BMP-6 expression in CaP cells via PKC/NF-κB pathway.

The observation that WNT5A is not detected in HS-5 cells in the absence of CaP cells suggests that there is a CaP-derived factor that stimulates the induction of WNT5A. Currently, the identity of this factor that induces WNT5A expression in bone stromal cells has not been elucidated. Future studies will focus on clarifying the initiating factors that activate the WNT5A/BMP-6 loop in CaP bone metastases.

It should be noted that there are few discrepancies in the current study. First, it has previously been reported that BMP-RIA mediates growth stimulation in LNCaP cells in the absence of androgens (Ide *et al*, 1997). In contrast, our data suggest that ALK2 (Act-RIA) signals for cellular proliferation in androgen-deprived condition. The precise reason for this difference is unclear. However, as the stoichiometry of BMP receptors has been shown to impact the ultimate effects of BMPs (Yu *et al*, 2008), it is entirely possible that the BMP receptors expression profile changes significantly in the presence of bone stromal cells. Second, Dai *et al* have reported that WNT5A upregulates BMP-6 expression via JNK (Dai *et al*, 2008). In the present study, though, PKC/NF-κB pathway, but not JNK, was involved in WNT5A-induced BMP-6 expression. It is likely that the different culture conditions have contributed to this difference. Third, advanced human primary CaP tissues frequently have a loss of expression of BMP receptor type II (Kim *et al*, 2004). As the mechanism elucidated in the present study involves an autocrine effect of BMP-6, the clinical implication of the present study may be limited. However, as the decreased expression of BMP receptors in CaP may be due to epigenetic mechanism (Lee *et al*, 2008), it is possible that the selection pressure of the bone microenvironment may reverse the loss of expression of BMP-RII in CaP skeletal metastases. Lastly, we have observed that only the overexpression of ALK2 increased the proliferation of CaP cells. The complementary study though, demonstrated that the knockdown of all three BMP type I receptors blocked the BMP-6-mediated increase in cell count. Although this difference between the overexpression and knockdown studies on ALK3 and ALK6 are difficult to explain at the present time, we propose that ALK3 and ALK6 are necessary but not sufficient for the induction of cellular proliferation by BMP-6. Recently, we have begun developing ALK2, 3, and 6 knockout CaP cell lines to test this hypothesis.

In conclusion, we have herein demonstrated that the bone–CaP interaction results in resistance to androgen deprivation. At the cellular and molecular levels, this castration resistance is mediated by bone stroma-derived WNT5A that leads to the induction of BMP-6; BMP-6, in turn, permits CaP cells to proliferate in the absence of androgens by interacting with β-catenin. Taken together, these results suggest that castration should be initiated prior to the development of bone metastases for the optimal clinical benefit in patients with advanced CaP. Simultaneously, targeting WNT5A/BMP-6/β-catenin may be a viable therapeutic option in men with CaP skeletal metastases and CRPC.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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