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

T. Lee 1, D I Kang 1,2, Y-S Ha 1, Y S Jung 1, J Chung 2, K Min 2, T H Kim 3, K H Moon 4, J M Chung 5, D H Lee 6, W-J Kim 7 and I Y Kim *,1

1Section of Urologic Oncology, Rutgers Cancer Institute of New Jersey and Rutgers, The State University of New Jersey, 195 Little Albany Street no. 4560, New Brunswick, NJ 08901, USA; 2Department of Urology, Inje University College of Medicine, Busan, Korea; 3Department of Urology, Dong-A University, College of Medicine, Busan, Korea; 4Department of Urology, Ulsan University, College of Medicine, Ulsan, Korea; 5Department of Urology, Kosin University, College of Medicine, Busan, Korea; 6Department of Urology, Ehwa Womans University, College of Medicine, Seoul, Korea and 7Department of Urology, Chungbuk National University College of Medicine, Cheongju, Korea

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...
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 (Blasszczyk 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).

**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 varied cell 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 ~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 was overexpressed in LNCaP in an attempt to investigate the mechanism by which BMP-6 enhances the proliferation of CaP cells under androgen-deprived 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
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 bicalutamide (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. 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 Smad5 increased the TOPFlash luciferase activity most significantly (Figure 3C). The reverse experiment knocking down β-catenin involved 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).

www.bjcancer.com | DOI:10.1038/bjc.2014.23

1637
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 stimulate the proliferation of CaP cells in vitro (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%FBS/10 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.
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 blocked 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).
BMP-6 and WNT5A in CRPC

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 with 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 deprivation. 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 mediates 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 ~40 kDa proteins rich in cysteine residues and have 19 genes that are subdivided into 12 subfamilies in humans (Clevens and Nusse, 2012). In malignancies, Wnt1 has been implicated in breast and CaP while abnormal expression of WNT5A has been reported in 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.

ACKNOWLEDGEMENTS

This work was supported in part by the Tanzman Foundation, Mr John Shein, and Mr Mal Wernik.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

REFERENCES

Autzen P, Robson CN, Bjartell A, Malcolm AJ, Johnson MI, Neal DE, Hamdy FC (1998) Bone morphogenetic protein 6 in skeletal metastases from prostate cancer and other common human malignancies. Br J Cancer 78(9): 1219–1223.

Barnes J, Anthony CT, Wall N, Steiner MS (1995) Bone morphogenetic protein-6 expression in normal and malignant prostate. World J Urol 13(6): 337–343.

Bentley H, Hamdy FC, Hart KA, Seid JM, Williams JL, Johnstone D, Russell RG (1992) Expression of bone morphogenetic proteins in human
prostatic adenocarcinoma and benign prostatic hyperplasia. Br J Cancer 66(6): 1159–1163.

Blaszczyk N, Maxi BA, Mawji NR, Ueda T, Alcmineden G, Duncan CP, Sadar MD (2004) Osteoblast-derived factors induce androgen-independent proliferation and expression of prostate-specific antigen in human prostate cancer cells. Clin Cancer Res 10(5): 1860–1869.

Bubley GJ, Carducci M, Dahut W, Dawson N, Daliani D, Eisenberger M, Figg WD, Freidlin B, Halabi S, Hudes G, Hussain M, Kaplan R, Myers C, Oh W, Petrylak DP, Reed E, Roth B, Sartor O, Scher H, Simons J, Sinibaldi V, Small EJ, Smith MR, Trump DL, Wilding G et al. (1999) Eligibility and response guidelines for phase II clinical trials in androgen-independent prostate cancer: recommendations from the Prostate-Specific Antigen Working Group. J Clin Oncol 17(11): 3461–3467.

Caubet JF, Tosteson TD, Dong EW, Naylon EM, Whiting GW, Ernstoff MS, Ross SD (1997) Maximum androgen blockade in advanced prostate cancer: a meta-analysis of published randomized controlled trials using nonsteroidal antiandroges. Urology 49(1): 71–78.

Chen G, Shukeir N, Potti A, Siracik K, Aprikian A, Gottlieb D, Rabbani SA (2004) Up-regulation of Wnt-1 and beta-catenin production in patients with advanced metastatic prostate carcinoma: potential pathogenetic and prognostic implications. Cancer 101(6): 1345–1356.

Clevers H, Nusse R (2012) Wnt/beta-catenin signaling and disease. Cell 149(6): 1192–1205.

Crawford ED, Eisenberger MA, McLeod DG, Benson R, Bubley GJ, Carducci M, Dahut W, Dawson N, Daliani D, Eisenberger MA, McLeod DG, Spaulding JT, Benson R, Huggins C (1942) Effect of orchiectomy and irradiation on cancer of the prostate. Science 96(2484): 346–347.

Kwon SJ, Lee GT, Lee JH, Kim WJ, Kim IY (2009) Bone morphogenetic protein-6 signaling and BMP antagonist network. Mol Cancer 8: 176–185.

Kim IY, Lee DH, Lee DK, Ahn HJ, Kim MM, Kim SJ, Morton RA (2004) BMP-2 and BMP antagonist noggin in prostate cancer. Cancer Res 64(22): 8276–8284.

Huang CL, Liu D, Nakano J, Ishikawa S, Kontani K, Yokomise H, Ueno M (2005) Wnt5a expression is associated with the tumor proliferation and the stromal vascular endothelial growth factor—an expression in non-small-cell lung cancer. J Clin Oncol 23(34): 8765–8773.

Huggins C (1942) Effect of orchietomy and irradiation on cancer of the prostate. Ann Surg 115(6): 1192–1200.

Ide H, Yoshida T, Matsumoto N, Aoki K, Osada Y, Sugimura T, Terada M (1997) Growth regulation of human prostate cancer cells by bone morphogenetic protein-2. Cancer Res 57(22): 5022–5027.

Iozzo RV, Eichstetter I, Danielson KG (1995) Aberrant expression of the growth factor Wnt-5a in human malignancy. Cancer Res 55(16): 3495–3499.

Katoh M, Katoh M (2007) WNT signaling pathway and stem cell signaling network. Clin Cancer Res 13(14): 4042–4045.

Kim JY, Lee DH, Lee DK, Ahn HJ, Kim MM, Kim SJ, Morton RA (2004) Loss of expression of bone morphogenetic protein receptor type II in human prostate cancer cells. Oncogene 23(6): 7651–7657.

Kurayoshi M, Oue N, Yamamoto H, Kishida M, Inoue A, Asahara T, Kikuchi A (2006) Expression of Wnt-5a is correlated with aggressiveness of gastric cancer by stimulating cell migration and invasion. Cancer Res 66(21): 10349–10448.

Kwon SJ, Lee GT, Lee JH, Kim WJ, Kim IY (2009) Bone morphogenetic protein-6 induces the expression of inducible nitric oxide synthase in macrophages. Immunology 128(1 Suppl): e758–e765.

Lee GT, Kwon SJ, Lee JH, Jeon SS, Jang KT, Choi HY, Kim IY (2010) Induction of interleukin-6 expression by bone morphogenetic protein-6 in macrophages requires both SMAD and p38 signaling pathways. J Biol Chem 285(50): 39401–39408.

Lee J, Son MJ, Woollard K, Donin NM, Li A, Cheng CH, Fine HA (2008) Epigenetic-modulated dysfunction of the bone morphogenetic protein pathway inhibits differentiation of glioblastoma-initiating cells. Cancer Cell 13(1): 69–80.

Logothetis CJ, Lin SH (2005) Osteoblasts in prostate cancer metastasis to bone. Nat Rev Cancer 5(1): 21–28.

Masiello D, Chen SY, Xu Y, Verhoeven MC, Choi E, Hollenberg AN, Balk SP (2004) Recruitment of beta-catenin by wild-type or mutant androgen receptors correlates with ligand-stimulated growth of prostate cancer cells. Mol Endocrinol 18(10): 2388–2401.

Massague J (1998) TGF-beta signal transduction. Annu Rev Biochem 67: 753–791.

Masuda H, Fukabori Y, Nakano K, Takezawa Y, Cuzikui T, Yamanaka H (2003) Increased expression of bone morphogenetic protein-7 in bone metastatic prostate cancer. Prostate 54(4): 268–274.

Messing EM, Manola J, Yao J, Kieman M, Crawford D, Wilding G, Trump D (2006) Immediate versus deferred androgen deprivation treatment in patients with node-positive prostate cancer after radical prostatectomy and pelvic lymphadenectomy. Lancet Oncol 7(6): 472–479.

Miyazono K, Maeda S, Imamura T (2005) BMP receptor signaling: transcriptional targets, regulation of signals, and signaling cross-talk. Cytokine Growth Factor Rev 16(3): 251–263.

Noroozme J, Klingensmith J, Perrimon N, Nusse R (1994) Dishevelled and armadillo act in the wingless signalling pathway in Drosophila. Nature 367(6458): 80–83.

Pawlofski IE, Erelt JR, Allen MP, Xu M, Butler C, Wilson EM, Wierman ME (2002) Liganded androgen receptor interaction with beta-catenin: nuclear co-localization and modulation of transcriptional activity in neuronal cells. J Biol Chem 277(23): 20702–20710.

Planas Morin J, Morote Robles J (2012) Skeletal complications of ADT: disease burden and treatment options. Asian J Androl 14(3): 670–675.

Pukrop R, Krischer M (2005) Changing views about personality disorders: comment about the prospective studies CIC, CLPS, and MSAD. J Pers Disord 19(5): 563–572; discussion 594–566.

Rosenweig BL, Imamura T, Okadome T, Cox GN, Yamashita H, ten Dijke P, Miyazono K (1995) Cloning and characterization of a human type II receptor for bone morphogenetic proteins. Proc Natl Acad Sci USA 92(17): 7632–7636.

Saylor PJ, Smith MR (2013) Metabolic complications of androgen deprivation therapy for prostate cancer. J Urol 189(1 Suppl): S34–S42; discussion S43–34.

Schellhammer PF (1996) Combined androgen blockade for the treatment of metastatic cancer of the prostate. Urology 47(5): 622–628.

Siegel R, Naishadham D, Jemal A (2012) Cancer statistics, 2012. CA Cancer J Clin 62(1): 10–29.

Smith MR, Saad F, Coleman R, Shore N, Fizazi K, Tombal B, Goess C (2012) Denosumab and bone metastasis-free survival in men with castration-resistant prostate cancer: results of a phase 3, randomised, placebo-controlled trial. Lancet 379(9810): 39–46.

Song LN, Herrell R, Byers S, Shah S, Wilson EM, Gelmann EP (2003) Beta-catenin binds to the activation function 2 region of the androgen receptor and modulates the effects of the N-terminal domain and TIF2 on ligand-dependent transcription. Mol Cell Biol 23(5): 1674–1687.

ten Dijke P, Yamashita H, Ichiho J, Franzen P, Laiho M, Miyazono K, Heldin CH (1994) Characterization of type I receptors for transforming growth factor-beta and activin. Science 264(5155): 101–104.

Titus MA, Schell MJ, Lih FB, Tomer KB, Mohler JL (2005) Testosterone and dihydrotestosterone tissue levels in recurrent prostate cancer. Clin Cancer Res 11(13): 4635–4637.

Truica CJ, Byers S, Gelmann EP (2000) Beta-catenin affects androgen receptor transcriptional activity and ligand specificity. Cancer Res 60(17): 4709–4713.

Tsuchimoto AS, Grosschedl R, Guzman RC, Parlow T, Varmus HE (1988) Expression of the int-1 gene in transgenic mice is associated with mammary gland hyperplasia and adenocarcinomas in male and female mice. Cell 55(4): 619–625.

Weeraratna AT, Jiang Y, Hostetter G, Rosenblatt K, Duray P, Bittner M, Trent JM (2002) Wnt5a signaling directly affects cell motility and invasion of metastatic melanoma. Cancer Cell 1(3): 279–288.

Yamamoto H, Kitadai Y, Oue N, Ohdan H, Yasui W, Kikuchi A (2009) Expression of Wnt-5a is correlated with aggressiveness of gastric cancer by stimulating cell migration and invasion. Cancer Res 69(22): 5873–5879.

Yamamoto H, Oue N, Sato A, Hasegawa Y, Matsubara A, Yasui W, Kikuchi A, Heldin CH (1994) Characterization of type I receptors for transforming growth factor-beta and activin. Science 264(5155): 101–104.
Yang F, Li X, Sharma M, Sasaki CY, Longo DL, Lim B, Sun Z (2002) Linking beta-catenin to androgen-signaling pathway. *J Biol Chem* 277(13): 11336–11344.

Yang X, Chen MW, Terry S, Vacherot F, Bemis DL, Capodice J, Buttyan R (2006) Complex regulation of human androgen receptor expression by Wnt signaling in prostate cancer cells. *Oncogene* 25(24): 3436–3444.

Yu PB, Deng DY, Beppu H, Hong CC, Lai C, Hoyng SA, Bloch KD (2008) Bone morphogenetic protein (BMP) type II receptor is required for BMP-mediated growth arrest and differentiation in pulmonary artery smooth muscle cells. *J Biol Chem* 283(7): 3877–3888.

This work is published under the standard license to publish agreement. After 12 months the work will become freely available and the license terms will switch to a Creative Commons Attribution-NonCommercial-Share Alike 3.0 Unported License.

Supplementary Information accompanies this paper on British Journal of Cancer website (http://www.nature.com/bjc)