

# Hepatocyte growth factor up-regulates the expression of the bone morphogenetic protein (BMP) receptors, BMPR-IB and BMPR-II, in human prostate cancer cells

LIN YE, JONATHAN M. LEWIS-RUSSELL, GAYNOR DAVIES,  
ANDREW J. SANDERS, HOWARD KYNASTON and WEN G. JIANG

Metastasis and Angiogenesis Research Group, Department of Surgery,  
Wales College of Medicine, Heath Park, Cardiff, CF14 4XN, UK

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**Abstract.** Hepatocyte growth factor (HGF) plays multiple roles in cancer, by acting as a motility, invasion and angiogenesis stimulating factor, which promotes metastasis and tumour growth. Bone morphogenetic proteins (BMPs) are members of the TGF- $\beta$  superfamily. The effects of BMPs are mediated by two subgroups of receptors, type I and type II. Recent studies have shown that some BMPs, via their signaling pathways, affect the growth of prostate cancer cells. BMPR-IB and BMPR-II have been reported to be expressed at low levels in prostate cancer. However, little is known about the crosstalk between HGF and BMP pathways. In this study, prostate cancer cells (PC-3 and DU-145) were exposed to HGF at different concentrations (1-75 ng/ml) for 18 h, or were treated with HGF at 40 ng/ml over various time periods (up to 24 h). The effect of HGF on BMP receptor expression was further investigated in a nude mouse PC-3 xenograft model. Mice were treated with either HGF, the HGF antagonist NK4, or a combination of both. The expression of BMPR-IB and BMPR-II mRNA was up-regulated by HGF, as shown by both conventional PCR and quantitative PCR. An elevation of BMPR-IB and BMPR-II at the protein level was confirmed by both Western blot analysis and immunocytochemical staining. In a murine prostate tumour model, infusion of recombinant HGF resulted in an increase in the levels of both BMPR-IB and BMPR-II transcript in prostate tumours. Concomitant delivery of NK4, an HGF antagonist, prevented this effect. In conclusion, HGF up-regulates the expression of the bone morphogenetic protein receptors, BMPR-IB and BMPR-II, in prostate cancer cells, both *in vitro* and *in vivo*. This may have important implications in the development of bone metastasis in prostate cancer.

## Introduction

Hepatocyte growth factor (HGF) is also known as scatter factor (SF). It was discovered approximately two decades ago (1-4), and is known to regulate many biological activities in different types of cells and tissues; for example, motility, mitogenesis, morphogenesis and angiogenesis. The complex formed by HGF and its receptor c-Met plays an important role in cancer. HGF has been demonstrated to promote tumour growth, invasion and metastasis, *in vitro* and *in vivo*. Recent evidence has demonstrated that blocking the effects of HGF by using either neutralising antibodies, the HGF antagonist NK4, or knocking out its receptor c-Met may reduce the invasion and proliferation of cancer cells *in vitro* and tumour growth *in vivo* (5-10).

Bone morphogenetic proteins (BMPs) belong to the TGF- $\beta$  superfamily, which was first named by Dr Marshall Urist (11). More than twenty members of this group have been identified in humans, since its discovery in the late 1980s (12-16). BMPs exert their effects through a heteromeric receptor complex, which consists of two types of serine-threonine kinase transmembrane receptors. These are Type-I including BMP receptor type IA (BMPR-IA), type IB (BMPR-IB) and activin A receptor type I (ActRI); and Type-II including BMP receptor type II (BMPR-II), activin A receptor type IIA (ActRII) and activin A receptor type IIB (ActRIIB). Once BMPs' homodimer or heterodimer has bound to at least one homodimer of each type receptor, the Type-II receptor then phosphorylates the Type-I receptor. This leads to recruitment of the pathway-restricted Smads (R-Smads, Smad1, 5 and 8). Smad4 is then responsible for translocation of the signal complex into the nucleus, and triggers the transcription of target genes. This is known as the Smad-dependent pathway, and Smad6 and 7 can inhibit this process. Smad is derived from both Sma (small family member, identified in *Caenorhabditis elegans*) and MAD (mothers against decapentaplegic homolog) (17). The other pathway is known as the Smad-independent pathway, in which the Map kinase pathway, the RAS pathway, or Erk kinase pathway may be involved (18).

BMPs and their receptors play an important role in bone formation and morphogenesis. Recently, their role in cancer has also been investigated, especially in the development of

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*Correspondence to:* Dr L. Ye, Metastasis and Angiogenesis Research Group, Department of Surgery, Wales College of Medicine, Heath Park, Cardiff, CF14 4XN, UK  
E-mail: yel@cf.ac.uk

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Table I. Primers used for RT-PCR or Q-PCR.

Gene	Name of primer	Sequence of primers	Optimal annealing temperature
Smad1	SMAD1F1	5'-tcaactgatccttccaacaat	55°C
	SMAD1ZR1	5'-actgaacctgaccgtacacctgggttttcaatagtgg	
Smad2	SMAD2F1	5'-gaagccgtctatcagctaac	55°C
	SMAD2ZR1	5'-actgaacctgaccgtacagcaaggagtactgttaccg	
Smad3	SMAD3F1	5'-agtctcccaactgtaaccag	55°C
	SMAD3ZR1	5'-actgaacctgaccgtacagaactcctggtgtgaaga	
Smad4	SMAD4F1	5'-ttttgttgggtcaactctc	55°C
	SMAD4ZR1	5'-actgaacctgaccgtacacaaacatcaccttcacctt	
Smad5	SMAD5F1	5'-agctcacaaaatgtgtacc	55°C
	SMAD5ZR1	5'-actgaacctgaccgtacatgaagatgaatcctcaatcca	
Smad6	SMAD6F1	5'-aaccctaccacttcagc	55°C
	SMAD6ZR1	5'-actgaacctgaccgtacacagatccagtggtgtact	
Smad7	SMAD7F1	5'-aacagggggaacgaattat	55°C
	SMAD7ZR1	5'-actgaacctgaccgtacaccactctctctctc	
Smad8	SMAD8F1	5'-cgtgtatgaactgaccaaga	55°C
	SMAD8ZR1	5'-actgaacctgaccgtacagatgaatcctcaatccagcag	
c-Met	METZF	5'-actgaacctgaccgtacagagccaaagtccttcat	55°C
	METR8	5'-atcgaatgcaatggatgat	
HGF	HGFF10	5'-tactgcagaccaatgtgcta	55°C
	HGFZR10	5'-actgaacctgaccgtacagcattgtttctcgtttat	
HGFA	HGFAF8	5'-gacacaagtgccagattg	55°C
	HGFAZR	5'-actgaacctgaccgtacaggactgcagtcgaagta	
HAI-I	HAI1SNABF	5'-gattacgtatgcctcgcatccaac	55°C
	HAI1ECORVR	5'-gatgatctcagaggggcccgggtggtgt	
BMPR-IB	BMPR1BF1	5'-atggaactgtctgtattgct	55°C
	BMPR1BZR	5'-actgaacctgaccgtacacaaactcgagtgttaggtggt	
BMPR-II	BMPR2F1	5'-tttggaaagaacaatct	55°C
	BMPR2ZR	5'-actgaacctgaccgtacatggataaggaccaattttg	
$\beta$ -actin	BACTF	5'-atgatatcgccgcgctcg	55°C
	BACTR	5'-cgctcggtaggatcttca	
	BACTINZF	5'-ggacctgactgactacctca	55°C
	BACTINZR	5'-actgaacctgaccgtacaagcttctccttaatgtcagc	

bone metastasis. 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 implicated in the progression of prostate cancer (19-21). The inhibitory effect of BMPs on tumour growth, mediated through BMPR-II, has been illustrated in an *in vivo* murine tumour model using the BMPR-II knock-out prostate cancer cell line (PC3M) (20). The expression of BMPR-IB can be regulated by androgen hormones in the androgen-sensitive prostate cancer cell line LNCaP (22).

Despite the fact that both the HGF/c-MET complex and BMP/BMPR complex are involved in cancer and cancer progression, little is known about their mutual regulation and whether there is any cross-talk between the two. A recent study has provided some interesting leads regarding their potential interactions (23). Imai *et al* have shown that following bone fracture, HGF is activated at the fracture site and may be involved in the up-regulation of BMP receptors in mesenchymal cells. In our study, we have investigated the effect of HGF on the expression of BMPR-IB and BMPR-II in prostate cancer cells.

## Materials and methods

**Reagents and antibodies.** Polyclonal goat anti-BMPR-IB IgG (SC-5679), polyclonal goat anti-BMPR-II IgG (SC-5683), and monoclonal mouse anti-Actin (SC-8432) were obtained from Santa Cruz Biotechnology (Santa Cruz, California, USA). Western blotting luminol reagent Peroxidase-conjugated anti-goat and anti-mouse IgG for Western blotting were purchased from Sigma-Aldrich Ltd (Poole, Dorset, UK).

**Cell lines.** PC-3 was acquired from the ECACC (European Collection of Animal Cell Culture, Salisbury, UK). DU-145, LNCaP-FGC, CA-HPV10 and PZ-HPV-7 were supplied by the ATCC (American Type Culture Collection). PNT-1A and PNT-2C2 were generous gifts provided by Professor Norman Maitland (University of York, UK). The cells were routinely maintained in DMEM-F12 medium supplemented with 10% foetal calf serum and antibiotics.

**Exposure of prostate cancer cells to rh-HGF.** PC-3 or DU-145 cells were treated with HGF at different concentrations for 18 h. Alternatively, cells were treated with HGF at 40 ng/ml for different time periods, up to 24 h.

**RNA extraction, reverse transcription PCR and quantitative PCR.** RNA was isolated from the cells using a Total RNA isolation reagent (ABgene, Epsom, UK). cDNA was synthesized by reverse transcription using 0.25  $\mu$ g RNA in a 20- $\mu$ l-reaction mixture as described in the protocol of the DuraScript™ RT-PCR kit (Sigma-Aldrich, Inc., Poole, Dorset). PCR was undertaken using a REDTaq™ ReadyMix PCR reaction mix (Sigma-Aldrich, Inc.). Cycling conditions for the 12- $\mu$ l-reaction mixture were 94°C for 5 min, followed by 36 cycles of 94°C for 30 sec, 55°C for 30 sec, and 72°C for 40 sec. This was followed by a final extension of 10 min at 72°C. The products were visualized on a 2% agarose gel and stained with ethidium bromide after electrophoresis. The PCR primers used are listed in Table I.

**Quantitative PCR was performed on the Icyler IQ system (Bio-Rad).** This unit incorporates a gradient thermocycler and a 96-channel optical unit. The  $\beta$ -actin cDNA standards and cDNA from PC-3 and DU-145 cells were simultaneously assayed in duplicate using an in-house Q-Mastermix formulation. QPCR procedure was optimized at: 95 for 4 min, followed by 54 cycles at 95°C for 15 sec, 55°C for 1 min and 72°C for 20 sec. The QPCR primers for BMPR-IB and BMPR-II were identical to those used for the conventional PCR, with the addition of the Z sequence (5'-actgaacctgacctgaca-3'). The detection system in the quantitative analysis was the Uniprimer™ system (TCS Biologicals Ltd, Oxford, UK), as others have described (24,25).

**SDS-PAGE and Western blot procedure.** Following treatment with rh-HGF, PC-3 cells were lysed in HCMF buffer containing 1% Triton, 0.1% SDS, 2 mM CaCl<sub>2</sub>, 100  $\mu$ g/ml phenylmethylsulfonyl fluoride, 1  $\mu$ g/ml leupeptin, and 1  $\mu$ g/ml aprotinin, for 45 min at 4°C. This is followed by centrifugation at 13,000 x g for 15 min and collection of the protein lysate. Protein concentrations were measured using the DC protein

assay kit (Bio-Rad), and were quantified by using a spectrophotometer (Bio-Tek, ELx800). Equal amounts of protein from each cell sample (10 or 25  $\mu$ g/lane) were loaded onto a 10% polyacrylamide gel. After electrophoresis, proteins were blotted onto nitrocellulose sheets and blocked in 10% skimmed milk for 60 min before probing with the polyclonal goat anti-BMPR-IB or anti-BMPR-II antibody and peroxidase-conjugated secondary antibodies. A molecular weight marker mixture (SDS-6H; Sigma Chemical Co.) was used to determine the protein size. Protein band signal was visualized with the Supersignal™ West Dura system (Pierce Biotechnology), and images obtained using a UVITech imager (UVITech, Inc., Cambridge, UK). The protein bands were then quantified by using UViband software (UVITEC, Cambridge, UK). The relative change of band volume from cells in response to rh-HGF exposure was calculated by  $(V_s - V_c)/V_{cx}100$ , where  $V_s$  is the volume of each sample and  $V_c$  is the volume of the control. The results were normalized against the actin band volume of the respective sample.

**Immunocytochemical staining.** Cells in glass chamber slides were fixed after each experiment, and then permeabilized with 0.1% Triton for 5 min in TBS. Following a blocking with horse serum in a Super Sensitive™ Wash Buffer (BioGenex, USA), anti-BMPR-IB or anti-BMPR-II antibody was added to the cells for 60 min. After extensive washing, a bionylated secondary antibody and ABC solution was added, each separated by extensive washings. The staining was visualised using the DAB kit (VECTASTAIN® ABC system, Vector Laboratories, Inc., Nottingham, England, UK), and intensity of the staining was quantified by using the area morphometry of the Optimas image analysis software (Optimas version 6.0, OPTIMAS, Washington, USA).

**Murine tumour model.** Previous reports have validated the PC-3 xenograft model of prostate cancer in athymic nude mice (5,26). It has also been shown that these tumours responded to HGF in a manner consistent with clinical conditions (5). We have used this model to assess whether the biological effects noted in our experiments were also seen under *in vivo* conditions. Athymic female nude mice (Nude CD-1) aged 4-6 weeks old were obtained from Charles River Laboratories (Margate, Kent, UK) and maintained in sterile filter-topped units under controlled 12-h dark/light alternating conditions according to UK Home Office and United Kingdom Coordinating Committee on Cancer Research (UKCCCR) guidelines. Twenty female nude mice were divided into 4 groups. Each group was ear-coded at the beginning of the experiment. Alzet osmotic minipumps (model 2004; Alza, Palo Alto, CA) were surgically implanted in the left scapula area of each mouse. Each pump was loaded with one of the following reagents: BSA buffer (control group), rh-HGF, rh-NK4 or a combination of both rh-HGF and rh-NK4. The minipumps provide a continual infusion of rh-HGF (release rate 40  $\mu$ g/kg/day) and rh-NK4 (release rate 400  $\mu$ g/kg/day) for up to 28 days. In the prostate tumor model, PC-3 cells were subcutaneously injected into the left scapula area (i.e., near the vicinity of the osmotic minipumps). The cells ( $1 \times 10^6$  cells/mouse) were suspended in 100  $\mu$ l of Matrigel (0.5 mg/ml). Tumor sizes and animal weights were recorded in all groups over a 4-week

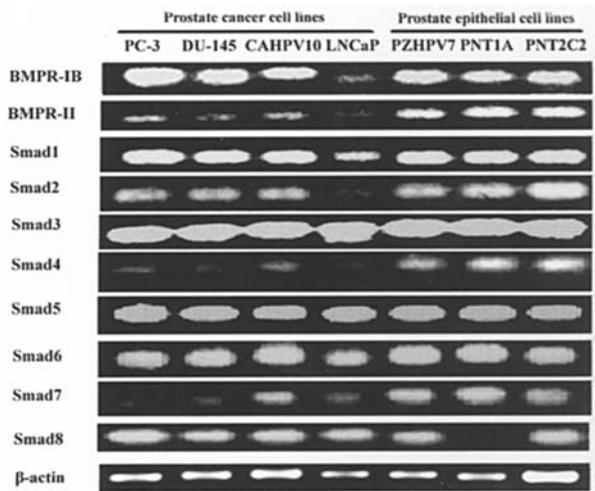


Figure 1. mRNA levels of BMPR-IB, BMPR-II and Smad family in seven prostatic cell lines were revealed by conventional RT-PCR. Relatively higher expression revealed in BMPR-IB, Smad1, 2, 3, 5, 6 and 8.

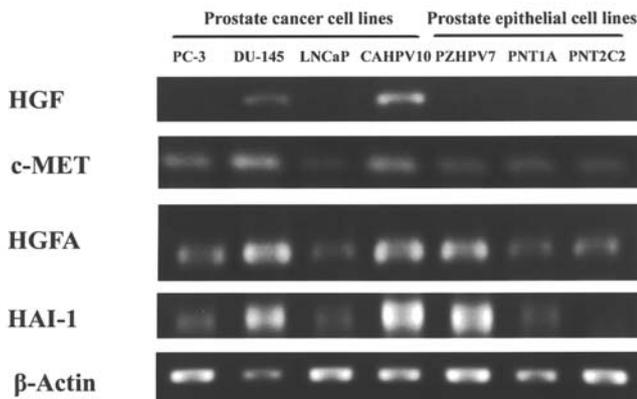


Figure 2. Expression of HGF, c-Met, HGFA and HAI-1 was revealed in seven prostatic epithelial cell lines by using conventional RT-PCR.

period using digital callipers and a top pan balance. Nude mice were terminated at the end of each 4-week experiment. The primary tumours were dissected, weighed and frozen at  $-80^{\circ}\text{C}$ . Total RNA was isolated, and the level of messenger RNA for each receptor was assessed using reverse transcription and quantitative PCR.

Statistical analysis was performed using the Minitab statistical software package (version 10). Non-normally distributed data was assessed using the Mann-Whitney test, while the two sample t-test was used for normally distributed data.

## Results

*The expression of BMPR-IB, BMPR-II and downstream signalling molecules in prostate cell lines.* PC-3, DU-145, LNCaP and CA-HPV-10 are cancer cell lines, whereas PZHPV-7, PNT-1A and PNT2-C2 are immortalised prostatic epithelial cells. We screened these cell lines to assess for the expression of the two BMP receptors and the intracellular signal molecules using conventional RT-PCR. Fig. 1 illustrates

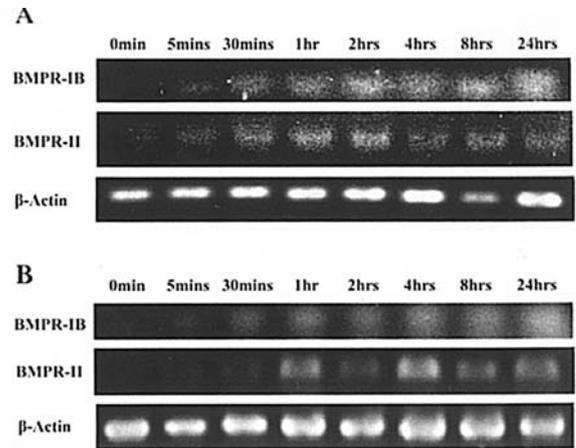


Figure 3. Up-regulations of both BMPR-IB and BMPR-II by HGF (40 ng/ml) in a treating time related manner in PC-3 and DU-145 were revealed by using conventional RT-PCR, as shown in panels A and B respectively.

the subtle variations in the level of expression. Both receptors were detectable at some level in all seven prostate epithelial cell lines examined. BMPR-IB was expressed at a relatively higher level compared with BMPR-II, and BMPR-II has a lower expression in prostate cancer cell lines compared with the prostate epithelial cell lines (Fig. 1). Eight Smads were detected, Smad1, 2, 3, 5, 6 and 8 were clearly expressed in all cell lines. Smad4 and 7 were barely seen in the three more aggressive prostate cancer cell lines, PC-3 (derived from bone metastasis), DU-145 (derived from brain metastasis) and LNCaP (derived from metastatic lymph node), but were apparently expressed in the prostatic epithelial cell lines and the less aggressive prostate cancer cell line CA-HPV-10. Smad4 is not only an important molecule for translocation of Smad complex during signal transduction of BMP, and it has also been reported as an onco-suppressor gene.

*Expression of c-Met, HGFA, and HAI-1 in prostate cancer cell lines.* To evaluate the HGF system in prostate cancer cells, we examined the mRNA level for HGF itself, its receptor (c-Met), the activating factor (hepatocyte growth factor activator, HGFA), and an inhibitor of HGFA-hepatocyte growth factor activator inhibitor-1 (HAI-1) (Fig. 2). mRNA for HGF was detectable in two prostate cancer cell lines, DU-145 and CA-HPV-10. c-Met, HGFA and HAI-1 were detected in all seven prostate epithelial cell lines, with a suggestion of higher levels of expression of HAI-1 in DU-145, CA-HPV-10, and PZ-HPV-7.

*Effect of HGF exposure on the level of receptor mRNA.* Having broadly established that BMP receptors BMPR-IB and BMPR-II are present in all these cell lines (Fig. 1), we investigated whether HGF would exert any influence over their expression. Using PC-3 and DU-145, two of the aggressive phenotypes, we exposed the cells to recombinant human HGF (rh-HGF) and then assessed the level of receptor mRNA, and whether this was influenced by the duration of exposure. By optimising the conventional PCR parameters, we were able to qualitatively detect an up-regulation in the level of receptor mRNA on exposure to HGF (Fig. 3). The level of BMPR-IB

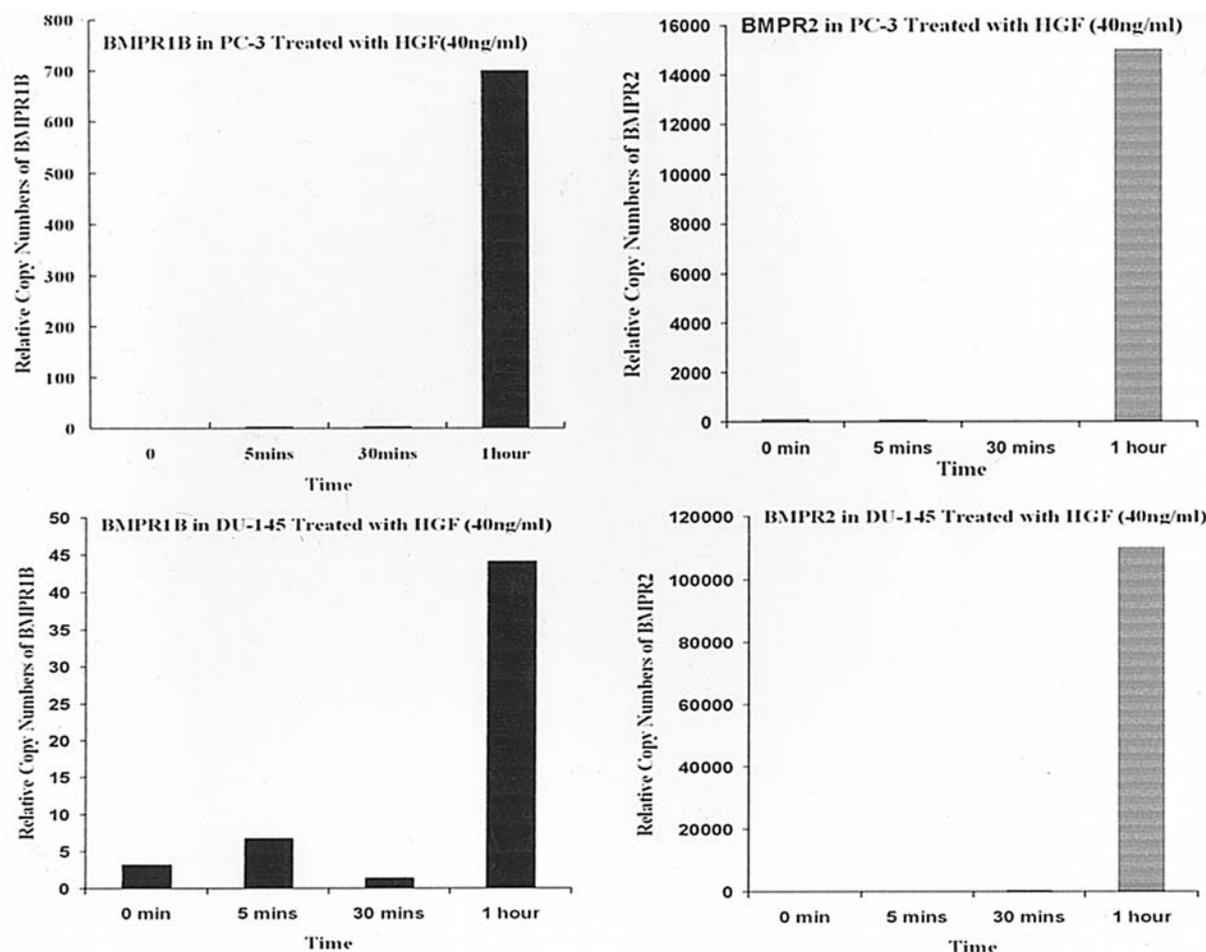


Figure 4. mRNA of both BMPR-IB and BMPR-II were apparently up-regulated over 60-min exposure of HGF (40 ng/ml), as revealed by using quantitative PCR.

mRNA was elevated by exposure to rh-HGF (40 ng/ml) in PC-3 and DU-145. The effect became apparent after 1 h, but there was an indication of an earlier response. In a similar fashion, BMPR-II mRNA was also detectable after 60-min exposure of rh-HGF in both cell lines. The elevation remained high indicating that the change of the level was sustained over time.

In order to further characterise this response, the tests were repeated and the level of mRNA transcript for BMPR-IB and BMPR-II was analysed by quantitative PCR. PC-3 and DU-145 cells were exposed to rh-HGF at a concentration of 40 ng/ml for up to 60 min (Fig. 4). The results were almost identical to that of conventional PCR, in that there was an elevation in the copy number of mRNA transcript after 60 min of HGF exposure for both receptors in each of the cell lines.

*HGF increases protein levels of both BMP receptors.* To determine whether these changes in mRNA were reflected at the protein level, Western blot analysis and immunocytochemistry were employed. PC-3 cells were exposed to rh-HGF at a concentration of 40 ng/ml for a specific duration of time. The protein levels of each receptor were determined by Western blot analysis (Fig. 5). There was an initial surprisingly rapid increase in the level of both receptors over the first hour. BMPR-IB rose to a level of approximately 40% above

the baseline over the first hour, and then fell before rising again over an 8- to 24-h time frame. Following the initial rapid rise, the level of BMPR-II peaked at 4 h at ~140% above the baseline level of protein production, and then gradually fell back to near the pre-treatment level by 24 h.

Immunocytochemistry revealed a similar response (Fig. 6). PC-3 cells exposed to rh-HGF at a concentration of 40 ng/ml exhibited an increased level of BMPR-IB protein. This was again maintained over 24 h although the specific response pattern seen by Western blot analysis was not duplicated by this method. Similarly, the BMPR-II protein level was also elevated in response to rh-HGF and maintained over 24 h. This confirms independently that the level of expression and deployment of these receptors is responsive to rh-HGF under *in vitro* conditions.

We further demonstrated that these two BMP receptors in PC-3 cells responded to exposure to rh-HGF over a wide range of concentrations. At the higher concentration range of 40-50 ng/ml, which was utilised in the previous experiments, there was a massive increase in protein levels of both BMPR-IB and BMPR-II. However, as can be seen from Fig. 7, a much lower concentration of rh-HGF (1-5 ng/ml) also induced a significant response. The lower concentration of rh-HGF used here mimics the serum levels of HGF seen in patients with prostate cancer (0.4-3.2 ng/ml) (27).

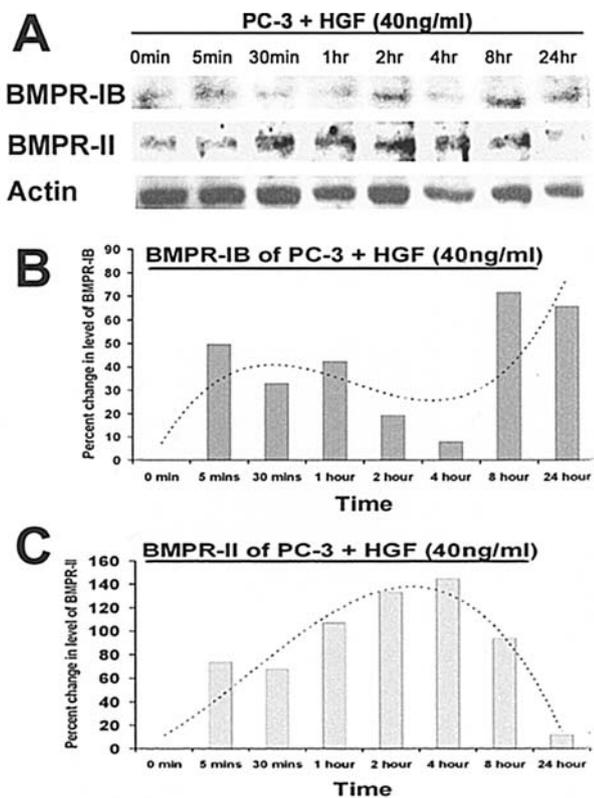


Figure 5. Protein levels of BMPR-IB and BMPR-II as determined by Western blot analysis and UVI Band analysis. PC-3 cells were exposed to rh-HGF (40 ng/ml) for up to 24 h. (A) Western blotting of protein bands: BMPR-IB (50 kDa) and BMPR-II (~100 kDa). (B) BMPR-IB showed an initial rapid rise followed by a second delayed rise over 8-24 h. (C) BMPR-II rose rapidly following rh-HGF exposure with the maximum level of protein produced at ~4 h.

*BMPR-IB and BMPR-II are up-regulated by HGF, in vivo.* The infusion of recombinant HGF in the *in vivo* prostate tumour model resulted in an increase in the level of BMPR-IB transcript ( $38.9 \pm 22.9$  copies) in prostate tumours, compared with the control ( $22.5 \pm 17.1$ ) (Fig. 8). This change was in line with the *in vitro* data, although it did not reach statistical significance. NK4 is a variant of HGF which retained the receptor binding and all four kringle domains and acts as an HGF antagonist. When NK4 was delivered to block the influence of rh-HGF, the expression of BMPR-IB mRNA was reduced to below control levels ( $1.4 \pm 5.6$  HGF+NK4,  $p < 0.05$  vs HGF only). NK4 administered alone also reduced the BMPR-IB mRNA level compared to the treatment group ( $1.9 \pm 7.6$ ,  $p < 0.05$  vs HGF). This may be due to the fact that NK4 also antagonised endogenous murine HGF (5).

There was a similar rise in the level of BMPR-II induced by rh-HGF *in vivo* ( $2.71 \pm 0.58$  copies,  $p = 0.001$  vs control,  $0.60 \pm 0.54$ ). Administration of NK4 alone, or in combination with HGF increased the expression of BMPR-II from the control but not to a statistically significant level.

## Discussion

HGF is a growth factor which is intimately involved in the progression of many solid tumours, including prostate cancer. The major sources of HGF in the body are fibroblasts. Lipocytes are largely used as the main storage cells for HGF

in the body (7). However, it can also be secreted by some cancer cells (2) and some peripheral blood cells including leukaemia cells (28-30), therefore HGF acts as both an autocrine and a paracrine factor in cells including cancer cells.

HGF is elevated in both serum and tumor tissues in clinical prostate cancer. A higher plasma level of HGF in men with prostate cancer is associated with an advanced stage of malignancy and reduced patient survival (27,31). In prostate tumours, HGF is not only highly expressed in prostate stromal cells, overexpression was also revealed in some carcinoma foci. This feature is associated with progression from an androgen-dependent to an androgen-independent state. There is an adaptive shift from paracrine to autocrine secretion of HGF in prostate cancer cells which supports this progression (32,33). A vicious cycle can be achieved through induction or promotion of HGF secretion in prostate stromal cells by the cancer cells. This is mediated by interleukin-1 $\beta$  (IL-1 $\beta$ ),  $\beta$ -fibroblast growth factor ( $\beta$ -FGF) and platelet-derived growth factor (PDGF). This does not occur in benign prostatic epithelial cells (34).

The HGF receptor, c-Met, is most commonly overexpressed in poorly differentiated and locally advanced prostate cancers (35). It was also found with increased frequency in bone metastases and lymph node metastases (36).

Some of the other regulatory factors of HGF are also altered in prostate cancer, changes which support progression of disease. Serum HGFA tends to be higher in patients with more advanced stage prostate cancer (37). The role of the HGFA inhibitors in prostate cancer remain to be elucidated.

HGF plays an important role in promoting proliferation and invasion, and is active throughout the process of metastasis, via paracrine and autocrine control loops. HGF derived from prostate stromal cells enhances the growth, invasion and metastasis of androgen-independent prostate cancer cells through a paracrine mechanism mediated by the c-Met receptor (38-40). These effects can be reduced by use of the antagonist, NK4, or a ribozyme transgene targeted at c-Met (10,41). HGF produced by osteoblasts induces migration of cancer cells from sinusoidal capillaries into the bone marrow space and stimulates growth of cancer cells within the bone microenvironment. Thus, osteoblasts appear to promote the establishment of bone metastasis in some cancers via HGF-c-Met signaling (42). These studies collectively support a role for HGF and its receptor in progression of the disease and the development of metastases in prostate cancer.

BMPs are osteogenic factors. They are essential during embryogenesis and organogenesis and to cell proliferation, differentiation, migration and apoptosis. The expression of BMPs (BMP-2, -4, -5, -6 and -7) has been detected in normal and malignant prostate tissue. BMP-4, -6 and -7 were detected in bone metastases from prostate cancer primaries and, significantly, the level of BMP-7 mRNA in bone metastatic lesions was much higher than that in the surrounding bone microenvironment itself (43). Of the three Type-I receptors and three Type-II receptors for BMP signal transduction, the expression of BMPR-IA, BMPR-IB, and BMPR-II in human prostate cancer tissues has been examined in relation to tumour grade. Frequent loss of the expression of these three receptors in high-grade prostate cancer has been noted. The loss of expression of BMPR-II correlates with poor prognosis in

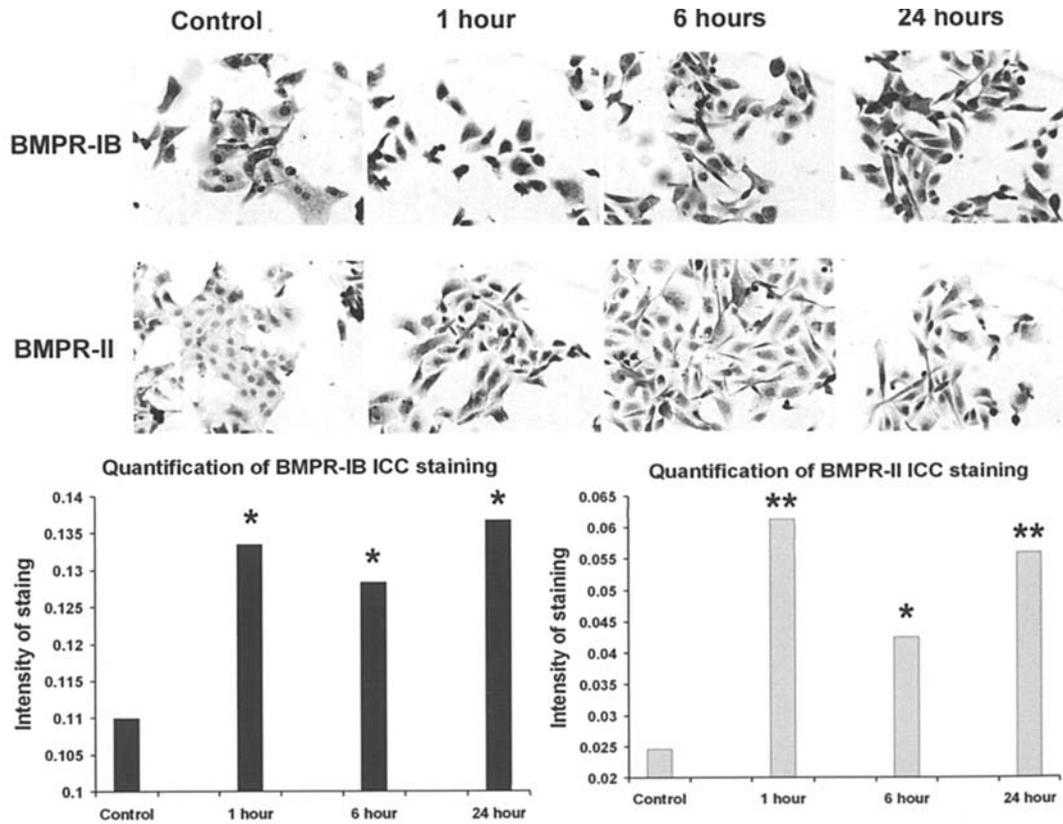


Figure 6. BMPR-IB and BMPR-II were immunocytochemically stained in PC-3 cells which had been treated with HGF 40 ng/ml for 0, 1, 6 and 24 h respectively. The staining was determined using the Optimas 6.0 image analysis software. Protein levels of BMPR-IB and BMPR-II were up-regulated by HGF after 1-h treatment. \* $p < 0.05$  vs control (0 h); \*\* $p < 0.01$  vs control (0 h).

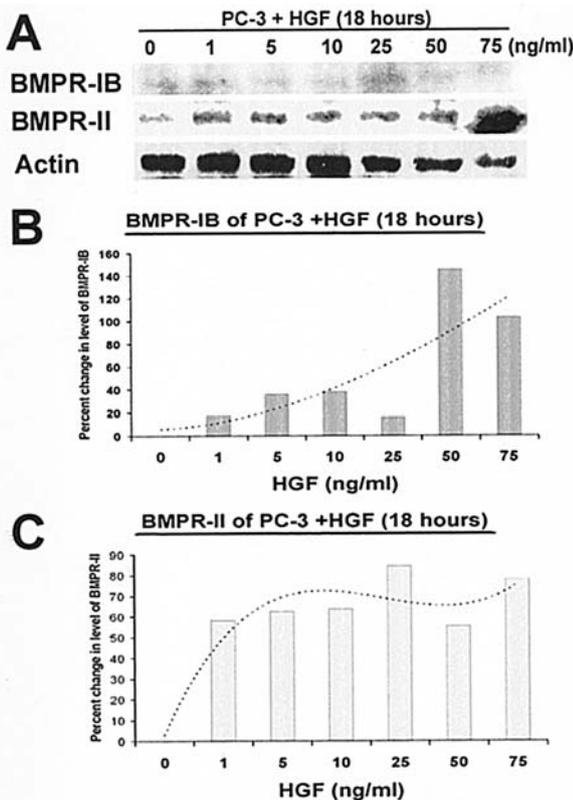


Figure 7. Up-regulation of BMPR-IB and BMPR-II in PC-3 by HGF in a concentration-dependent manner as revealed by Western blot analysis. This response in PC-3 was seen over a wide range of concentrations of HGF, from 1 ng/ml to 75 ng/ml.

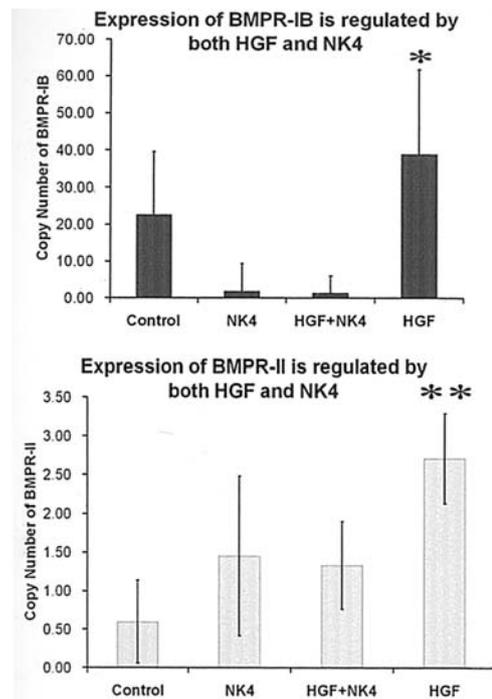


Figure 8. Up-regulation of BMPR-IB and BMPR-II by HGF *in vivo* using quantitative PCR. Administration of recombinant human-HGF in the *in vivo* model resulted in an increase in the levels of BMPR-IB in prostate tumours. Delivery of the HGF antagonist, NK4, significantly reduced the levels of BMPR-IB in both control group and the combination group. Similar observation was revealed with BMPR-II (shown are log transformed data). The relative copy numbers of BMPR-IB were determined with quantitative PCR. \* $p < 0.05$  vs HGF+NK4; \*\* $p < 0.01$  vs HGF+NK4.

prostate cancer patients (19,20). BMPR-IB and BMPR-II are specifically involved in the signal transduction of BMPs, especially BMP-2, -4, -6 and -7 (44). The role of BMPs and the BMP receptors in prostate cancer is diverse and remains to be further elucidated. Some BMPs and receptors appear to be inhibitory to the proliferation of prostate cancer cells, whereas others promote invasion, motility and migration. The effects are probably dependant on the individual BMP and the particular cell type involved. For example, BMP-2 has an androgen-dependant inhibitory effect on cell proliferation only in LNCaP cells (an androgen-sensitive prostate cell line). On the other hand, BMP-6 can inhibit the growth of DU-145 cells, an effect which can be blocked by Noggin, an antagonist of BMP-6 (45). But BMP-6 has other biological functions which favour the progression of tumours. It promotes the development of bone metastases through the dual mechanisms of promoting invasive ability and osteoblastic activity, but has no direct effect on the growth of an implanted primary tumour (46).

The up-regulation of BMP receptor expression demonstrated by the present study may have a profound impact in clinical prostate cancer. The commonly seen overexpression of the HGF receptor in prostate cancer cells and overexpression of HGF itself in prostate stromal cells may result in prostate cancer cells being at a 'super' active status in their response to HGF. In addition, the resultant up-regulation of the BMP receptors, in response to HGF, may enable cells to be better adapted to survive and proliferate in the bone microenvironment, thus facilitating the formation of metastatic deposits. The data presented in this current study shows up-regulation of the BMP receptors at both protein and mRNA levels in response to HGF, suggesting that the regulation occurs at pre-mRNA levels. Although mutations of and changes in the level of the BMP receptors have been reported, their regulation by factors, particularly physiological factors such as cytokines, has not been commonly recognised. We concur with Imai *et al* (23) that HGF is a significant regulator of BMP receptor expression. The exact transcriptional regulation of the BMP receptors is not yet clear. Further research is indicated to investigate if the HGF/cMET responsive genes, such as the cyclins, are also implicated here.

We have demonstrated that BMPR-IB and BMPR-II are up-regulated by HGF under both *in vitro* and *in vivo* conditions. This contribution furthers our understanding of the extensive role which HGF plays in the progression of a primary tumour and the development of secondaries, particularly bone metastases.

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