

Repulsive guidance molecules, novel bone morphogenetic protein co-receptors, are key regulators of the growth and aggressiveness of prostate cancer cells

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Abstract. Repulsive guidance molecule (RGM) family members RGMA, RGMB and RGMC are GPI-linked membrane proteins recently identified as co-receptor of bone morphogenetic proteins (BMPs). BMPs are a group of proteins enriched in bone and play important roles in prostate cancer. The current study aimed to investigate roles played by RGMs in prostate cancer. Expression of RGMs was examined in prostate cancer cell lines and prostate cancer tissues using RT-PCR and immunohistochemical staining. Knockdown of each RGM in prostate cancer cells was performed using the respective anti-RGMA, RGMB and RGMC transgenes. A variety of *in vitro* function tests were employed to analyze the influence on cancer cell functions by RGM knockdown. The implications of RGM knockdown in BMP signalling were also examined using both Western blot and real-time quantitative PCR. Knockdown of RGMA had no effect on cell growth, migration and invasion, but promoted cell-matrix adhesion. Knockdown of RGMB and RGMC increased growth and adhesion, but only RGMB knockdown increased capacities of migration and invasion in PC-3 cells. Further investigations showed an increase in Smad-3 activation and reduced levels of Smad-1 in PC-3 cells by RGMB and RGMC knockdown, and also an up-regulation of ID1, a BMP target gene particularly in exposure to BMP7. RGMs play inhibitory roles in prostate cancer by suppressing cell growth, adhesion, migration and invasion. RGMs can coordinate Smad-dependent signalling of BMPs in prostate cancer cells.

Introduction

Bone morphogenetic proteins (BMPs) have been indicated in the disease progression of prostate cancer, particularly in the

disease specific bone metastasis (1). A group of novel BMP co-receptors, namely repulsive guidance molecules (RGMs)-A, -B and -C were recently identified. RGMs are GPI-linked membrane proteins, sharing 50-60% sequence homology (2-4). RGMA mediates repulsive axonal guidance and neural tube closure, and RGMC is mutated in juvenile hemochromatosis. RGMB, also known as Dragon, is a myelin-derived inhibitor of axon growth in the central nerve system (5) and regulates the patterning of the developing nervous system (6). As BMP co-receptors, RGMA can increase binding of BMP ligands to ActRIIA, a type II receptor, and lead to an enhanced signalling by BMP-2 and BMP-4 (7). RGMA enhances signalling of BMP-2 and BMP-4, leading to activation of Smad dependent pathway and up-regulation of a target gene, ID1 (2). RGMA is also crucial as a co-receptor for Heparin expression induced by BMP-2 and BMP-4, thus to maintain systemic iron homeostasis (8). RGMB directly interacts with BMP receptors, enhancing binding to their ligands, such as BMP-2 and BMP-4, but not BMP-7 (6). RGMB can negatively regulate IL-6 expression in immune cells through Smad-independent signalling by BMPs, including p38 and ERK pathways (9). On the other hand, upon binding to its ligand neogenin, RGMA may also mediate growth cone collapse in neurites which has been shown as independent from BMPs signalling (10). A recent study also revealed that secreted von Willbrand factor type D domain of RGMB suppressed Smad-dependent signal transduction by BMPs, in which unknown receptors were involved (11). Similarly, upon binding to its receptor neogenin, RGMC is stabilized by anchoring on the cell membrane, which could compromise its inhibition on BMP signalling (12).

BMPs play profound roles in prostate cancer and the disease specific bone metastasis by regulating proliferation, apoptosis and motility of prostate cancer cells, and also tumour associated angiogenesis. For example, BMP-7 and BMP-9 inhibit the proliferation of PC-3 prostate cancer cells via inducing apoptosis through BMP receptors and Smad-dependent pathway (13,14), while BMP-10 inhibits the growth of prostate cancer cells via Smad-independent signalling in which XIAP and ERK1/2 are involved (15). Therefore as BMP co-receptors, RGMs might also be involved in the prostate cancer development and metastasis through the participation in BMP signalling pathway. To date, there is only one study investigated the implication of RGMs in cancer, in which an epigenetic down-regulation of

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RGMA was revealed in colorectal carcinomas, adenomas and colorectal cancer cell lines (16). However, the role played by RGMs in prostate cancer remains unknown. The present study aimed to examine the expression of RGMs in prostate cancer and their functions in prostate cancer cells.

Materials and methods

Materials. Antibodies to RGMA, RGMB, RGMC, phosphorylated Smad-1, and Smad-3 were obtained from Santa Cruz Biotechnology, CA. Human recombinant protein BMP-7 (rh-BMP7) was purchased from R&D System, Inc. (Abingdon, UK). All the primers were synthesized by Invitrogen (Paisley, UK), as shown in Table I.

PC-3 cell line was purchased from European Collection of Animal Cell Culture, Salisbury, UK; DU-145, LNCaP, CA-HPV-10 and PZ-HPV-7 from American Type Culture Collection. PNT-1A and PNT-2C2 were kindly provided by Professor Norman Maitland (University of York, UK). The cells were routinely maintained in DMEM supplemented with 10% fetal calf serum and antibiotics.

Prostate tissue samples were snap-frozen in liquid nitrogen immediately after radical prostatectomy, transurethral prostatectomy or prostate biopsy. All protocols were reviewed and approved by the local ethics committee and all patients gave written informed consent.

Immunohistochemical (IHC) staining procedure for frozen prostate tissue. Frozen specimens of prostate tumours (n=8) and normal prostate tissues (n=13) were cut at a thickness of 6 μm using a cryostat, and verified by two pathologists. IHC was performed using respective primary antibody and Vectastain Universal Elite ABC kit (Vector Laboratories, Peterborough, UK). The density of the IHC staining was quantified using ImageJ (<http://rsbweb.nih.gov/>).

Generation of RGM ribozyme transgenes. Specific hammer head ribozymes targeting human RGMA, RGMB and RGMC were designed and synthesised following previously described procedure (17). The ribozymes were cloned into pEF6/His TOPO mammalian expression plasmid vectors (Invitrogen Inc., Paisley, UK). Control empty plasmid vectors and the ribozyme transgenes were then transfected into PC-3 cells, followed by two weeks of selection using 5 $\mu\text{g}/\text{ml}$ blasticidin. Knockdown of specific RGM was then confirmed before further use.

RNA isolation, reverse transcription and polymerase chain reaction (PCR). RNA isolation was carried out using TRI reagent from Sigma. RNA (0.5 μg) was converted into cDNA using the iScript™ cDNA Synthesis kit (Bio-Rad, Hemel Hemstead, UK).

Real-time quantitative PCR. The real-time quantitative PCR was carried out to determine the levels of RGM transcripts and ID1 in the control and RGM knockdown cells. The assay was based on the Amplifluor technology and primers were designed using Beacon Designer software which included complementary sequence to universal Z probe (Intergen Inc., Oxford, UK), as previously reported (18,19). The primers used for RGMs

and ID1 quantification and housekeeping GAPDH are listed in Table I.

Protein extraction, SDS-PAGE and Western blot analysis. Following cell lysis, the protein concentration was quantified using the DC Protein Assay kit (Bio-Rad, USA) and a spectrophotometer (Bio-Tek, ELx800). Equal amounts of protein from each sample were loaded onto 8% polyacrylamide gel and following SDS-PAGE, the proteins were transferred onto nitrocellulose membranes subjected to blocking, and probing with the specific primary (1:200), and the corresponding peroxidase-conjugated secondary antibodies (1:2000). The protein bands were eventually visualized using the Supersignal™ West Dura system (Pierce Biotechnology, USA).

In vitro cell growth assay. The cells were seeded into a 96-well plate at a concentration of 3,000 cells per well, and incubated for periods of up to 5 days. The cells were then stained with crystal violet, and measured the absorbance. The results are shown as growth rate which was calculated by normalization against the absorbance measurements at day 0.

In vitro cell-matrix adhesion assay. 45,000 cells were seeded onto the 96-well plate coated with Matrigel basement membrane in 200 μl of normal medium and incubated for 40 min. After washing, adherent cells were fixed and visualized under the microscope under x40 objective magnification and random fields counted.

In vitro cell invasion assay. Transwell inserts (upper chamber) with an 8 μm pore size were coated with 50 $\mu\text{g}/\text{insert}$ of Matrigel and air-dried, before being rehydrated with normal medium. Then, 2×10^4 cells were added to each well. After 72 h, cells that had migrated to the other side of the insert through the matrix were fixed and stained with 0.5% (w/v) crystal violet. The invaded cells stained with crystal violet were counted under a microscope.

In vitro cell motility assay using cytodex-2 beads. Cellular motility was assessed using a cytodex-2 bead motility assay. Cells (5×10^5) for each cell type were incubated in 10 ml of growth medium containing 100 μl of cytodex-2 microcarrier beads (GE Healthcare, Cardiff, UK) for 3.5 h to allow the cells to adhere to the beads. After washing the beads were resuspended in growth medium, seeded into a 24-well plate and then incubated overnight at 37°C. Cells that had migrated from the beads and adhered to the base of the well were fixed in 4% formaldehyde (v/v), stained with 0.5% crystal violet (w/v) and counted under x40 objective magnification.

ECIS-based attachment and migration assay. We used the electric cell-substrate impedance sensing (ECIS) system (Applied Biophysics, Inc., Troy, NY) to conduct the attachment and wounding assay. PC-3 cells were cultured in 8W1E ECIS arrays (Applied Biophysics). We seeded PC-3 cells at a density of 70,000 cells/well in the arrays. The cells were incubated for 4 h and the resistance was recorded every 15 sec. Once the cells fully attached, the monolayer was electrically wounded by applying an elevated voltage pulse with a frequency of 30 kHz,

Table I. Primers used in this study.

| Gene | Sense (5'-3') | Anti-sense (5'-3') |
|---------------|---|---|
| RGMA | TCGACAATAATTACCTGAACG | ACTGAACCTGACCGTACACCTGGAAGTTCTTGAAGATG |
| RGMB | TTCAGGTTCAAGTGACAAACG | ACTGAACCTGACCGTACATCATCTGTACAGCTTGGTA |
| RGMC | AATGACTTCCTCTTTGTCCA | ACTGAACCTGACCGTACACATTCCTGCATGTTCTTAAA |
| RGMA ribozyme | CTGCAGGCGCACCCAGATGGTGGTGCCC TGATGAGTCCGTGAGGA | ACTAGTGGAGATCCAGGCCAAGTACATTTTCGT CCTCACGGACT |
| RGMB ribozyme | CTGCAGGTCATCTGTACAGCTTGGTA CTGATGAGTCCGTGAGGA | ACTAGTGTACAGATCAGAAAGTTTCGTCTCCTCACGGACT |
| RGMC ribozyme | CTGCAGATCCCCAGGTCCGGTCACCTCC ATTCTGATGAGTCCGTGAGGA | ACTAGTCCTGTAGCCTTTGAAGATGGTTCTATTTTCGTC CTCACGGACT |
| ID1 | TCAACGGCGAGATCAG | ACTGAACCTGACCGTACAGATCGTCCGCAGGAA |
| GAPDH | AAGGTCATCCATGACAACCTT | ACTGAACCTGACCGTACAGCCATCCACAGTCTTCTG |

amplitude of 4 V for 10 sec. Cell migration was assessed by continuous resistance measurements for 30 h.

Statistical analysis. Normally distributed data were analyzed using the two sample t-test while non-normally distributed data were analyzed using the Mann-Whitney and Kruskal-Wallis tests.

Results

Expression of RGMs in prostate cancer. The expression of RGMs was examined in prostate cancer cell lines and prostate cancer tissues using RT-PCR. RGMs were expressed in most of the prostate cancer cell lines. No remarked difference was seen in RGM expression between prostate cancer cells and the immortalised prostatic epithelial cells. RGMB was not expressed in CAHPV-10 cells, and RGMC was barely detectable in LNCaP cells. Among the RGMs, RGMA mRNA was hardly detectable in the two prostate cancer tissues examined (Fig. 1).

To further investigate the expression of RGMs in prostate tissues, we examined their protein levels using immunohistochemical staining. RGM protein was mainly confined to the cytoplasm of cancer cells and some of the mesenchymal cells on the periphery of the tumour area. The immunochemical staining of RGMB and RGMC protein revealed subtle stronger staining in prostate cancer cells compared to prostate epithelial cells, while staining of RGMA is much weaker in both prostatic epithelia and cancer cells (Fig. 2A). The intensity of the staining in the cells was measured and calculated based on the background using ImageJ. The quantification of the IHC staining shows the levels of RGMB and RGMC in prostate tumours were significantly higher, compared to background tissues ($P < 0.05$) (Fig. 2D).

Knockdown of RGMA, RGMB and RGMC in PC-3 cells. To test the potential role of RGMs in prostate cancer cells, knockdown of RGMA, RGMB and RGMC in PC-3 cells were performed using the respective anti-RGM transgenes. The knockdown of

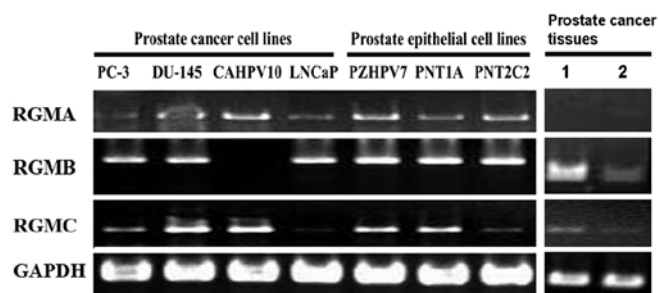


Figure 1. RT-PCR shows expression pattern of RGMs in prostate cancer cell lines. RGMs were expressed in most the prostate cancer cell lines and the immortalized prostatic epithelial cell lines. In the two prostate cancer tissue samples examined in current study, RGMB was expressed at relatively higher levels.

RGMs mRNA and protein were then verified using both real-time quantitative PCR and Western blotting (Fig. 3).

Effect on in vitro growth of prostate cancer cells by RGMs knock-down. Following the verification of knockdown, we examined effect on functions of prostate cancer cell by RGMs knockdown. Firstly, we assessed the influence on cell growth. Compared to control cells, PC-3^{ARGMA} cells did not show difference in cell growth ($p = 0.07$). In contrast, PC-3^{ARGMB} and PC-3^{ARGMC} cells showed increase in cell growth, both $p < 0.05$ compared to PC-3^{pEF} control cells (Fig. 4A).

Influence on cell-matrix adhesion by knockdown of RGMs in prostate cancer cells. In the adhesion assay, reduced expression of RGMs (RGMA, B and C) in PC-3 cells increased cell adhesion, compared to the control cells. The number of cells adhered to matrix for PC-3^{ARGMA} (26.93 ± 13.23), PC-3^{ARGMB} (30.67 ± 12.43), PC-3^{ARGMC} (30 ± 12.43) were increased, $p < 0.05$ compared to PC-3^{pEF} control cells (16.67 ± 8.09) (Fig. 4B).

Effect on motility and invasiveness of prostate cancer cells by RGMs knockdown. Furthermore, RGM knockdown cells

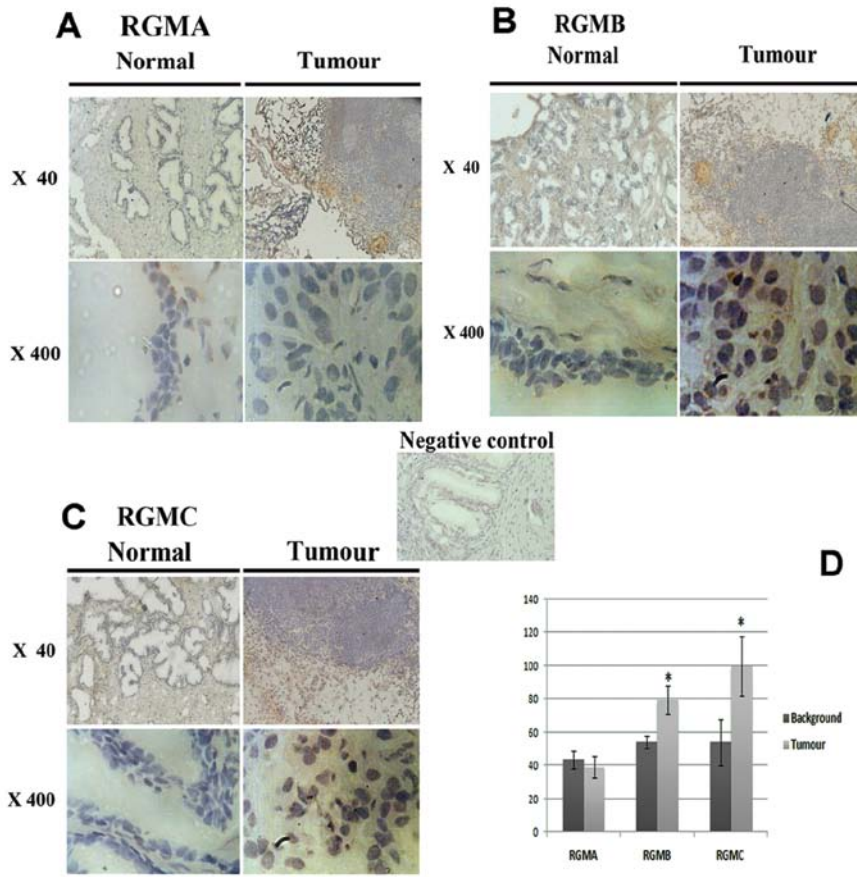


Figure 2. IHC staining of RGMs in human prostate cancer. (A-C) Immunochemical staining of RGMA, RGMB and RGMC in prostate cancer specimens, reduced from x40 and x400, respectively. (D) There is no significant difference of the staining of RGMA, RGMB and RGMC seen in the prostate cancer tissues when compared with background tissues using semi-quantification of the staining using ImageJ software. For negative control only secondary antibody was applied during the probing procedure. *P<0.05 vs normal prostate tissue.

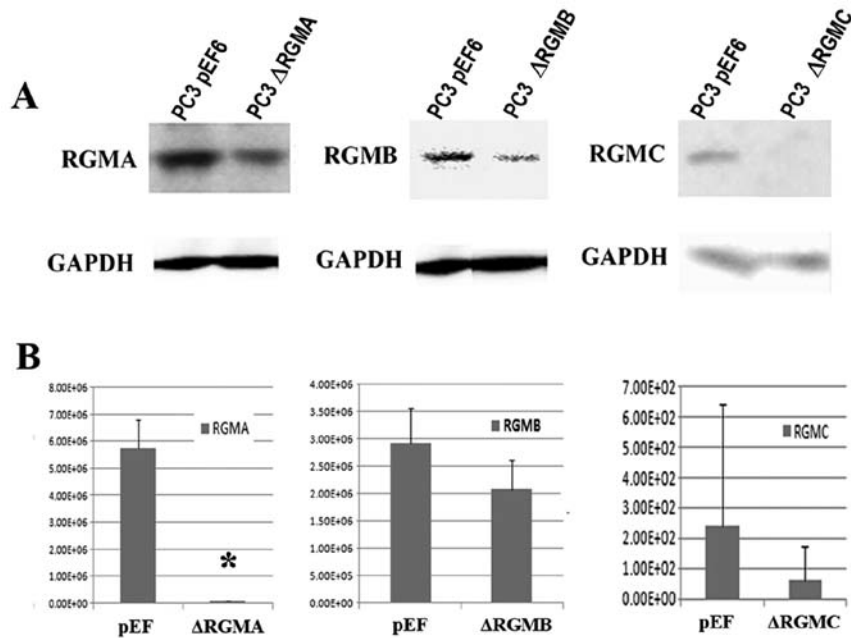


Figure 3. Knockdown of RGMs in PC-3 cells. (A) Reduced protein expression of RGMs was verified in PC-3^{ΔRGMA}, PC-3^{ΔRGMB} and PC-3^{ΔRGMC} cells, in comparison with PC-3^{pEF} cells using Western blotting. (B) Real-time quantitative PCR revealed reduced transcript level of RGMs by ribozyme transgenes. A significant reduction of RGMA transcripts was seen in PC-3^{ΔRGMA} cells ($1.05 \times 10^6 \pm 1.20 \times 10^4$), $p=0.007$ compared with PC-3^{pEF} control cells ($5.76 \times 10^6 \pm 1.05 \times 10^6$). Although reduced levels of RGMB and RGMC transcripts were seen in the corresponding cells, but there was no significant difference according the statistical results. *P<0.05 vs control.

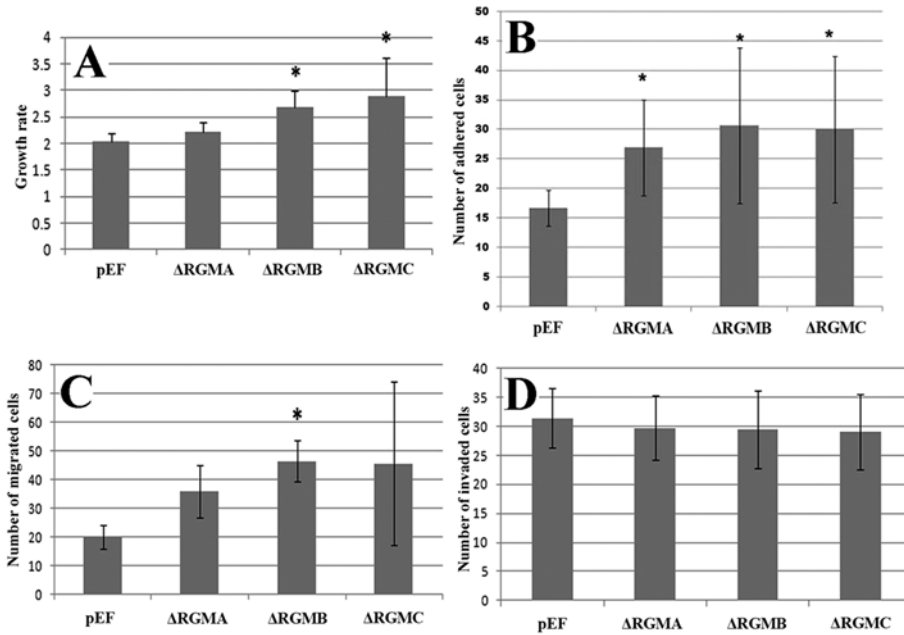


Figure 4. Effects on *in vitro* functions of PC-3 cells by RGMs knockdown. (A) Shown is the absorbance of day 3 which represented the cells number of each cell lines. The reduction of RGMB and C in PC-3 significantly promoted growth rate. (B) Knockdown of RGMs promoted cell-matrix adhesion of prostate cancer cells. (C) Knockdown of RGMB and RGMC resulted in elevated motility in PC-3 cells. (D) The invasiveness of PC-3 cells had not been altered by knockdown of RGMs. Minimum three independent experiments were conducted and a representative example is shown. *P<0.05 vs PC-3^{pEF/His}.

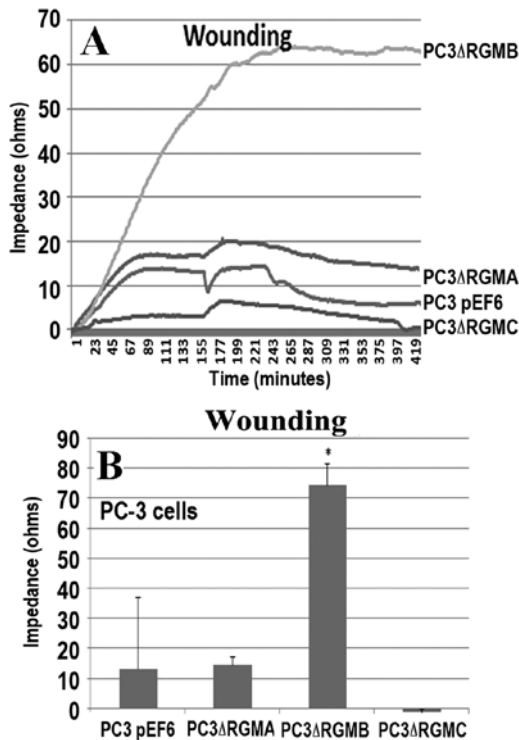


Figure 5. Effect on cell migration by knockdown of RGMs was also determined using the ECIS assay. (A) PC-3^{ΔRGMB} cells showed marked increase in migration after wounding. (B) The mean of increased impedance (ohms) at 1 h after wounding was calculated based on three individual experiments. Error bars presents standard error. *P<0.05 vs PC-3^{pEF/His}.

displayed increased motility, (Fig. 4C). The number of migrated PC-3^{ΔRGMA} cells was 36±9.2, being 46.4±7.06 and 45.54±28.46 for PC-3^{ΔRGMB} and PC-3^{ΔRGMC} cells, respectively. However, only

the increase in PC-3^{ΔRGMB} cells was statistically significant (p<0.01) compared with PC-3^{pEF} control cells (20.2±4.06).

To further confirm the effect on cell motility by RGMs knockdown, we examined the cell migration using ECIS. The migration rate was much higher of PC-3^{ΔRGMB} cells compared to control, while the RGMA and RGMC knockdown has no significant effect on the migration (Fig. 5A). One hour after the wounding, the average impedance in PC-3^{ΔRGMB} cells was significantly higher than the p^{EF} control cells (p=0.009). However, the RGMA and RGMC knockdown cells did not show such significant effect on migration in the ECIS wounding assay (Fig. 5B). This is thus consistent with the observations using the afore-mentioned motility assay. We further demonstrated that knockdown of RGMs had no effect on invasion of prostate cancer cells (Fig. 4D).

Potential roles of RGMs in BMP Smad-dependent signalling and regulation of ID1 expression. To investigate effect on BMP signalling by RGMs knockdown in PC-3 cells, we analyzed levels of phosphorylated Smad-1 and Smad-3 in the cells, and also transcript levels of ID1. RGMs knockdown affected BMP signalling in PC-3 cells, the BMP downstream signalling molecules (Smads) activation profile was examined, which might be associated with ID1 expression in these transfected cells.

RGMB and RGMC knockdown in PC-3 cells significantly increased Smad-3 activation, but not in PC-3^{ΔRGMA} cells, compared to the control cells (Fig. 6A and B). The increased activation was enhanced by stimulation of rh-BMP7 (40 ng/ml, 30 min), which indicated RGMB and RGMC knockdown can enhance the Smad-3 dependent pathway activated by BMP7 (Fig. 6A and B). In contrast to the effect on Smad-3 phosphorylation, reduced levels of activated Smad-1 were seen in PC-3^{ΔRGMB} and PC-3^{ΔRGMC} cells, but not in PC-3^{ΔRGMA} cells (Fig. 6A).

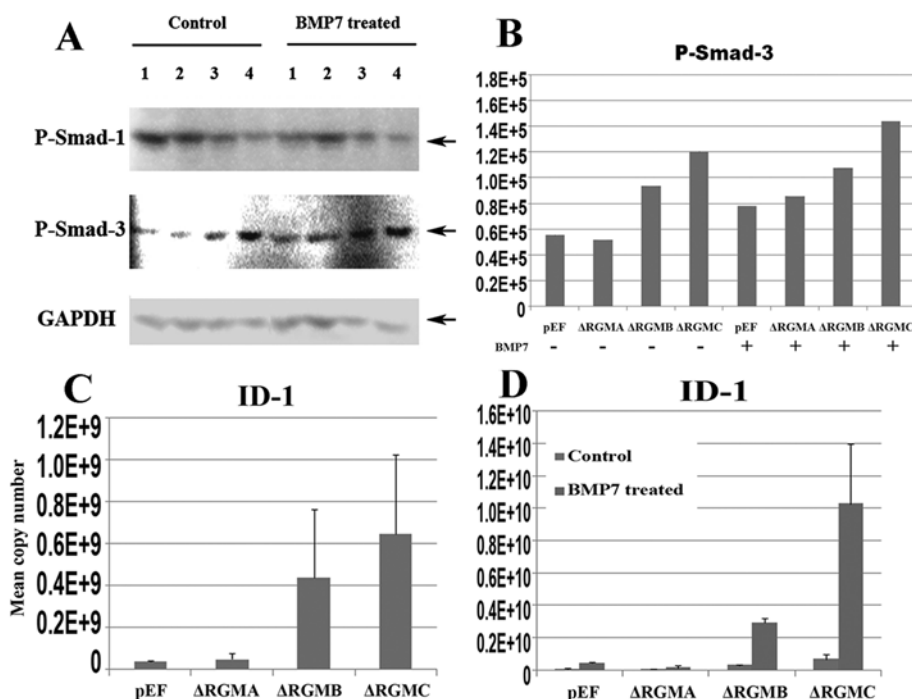


Figure 6. Influence on Smad dependent signal transduction by RGM knockdown. (A) Activated Smad-3 expression was up-regulated after RGMB and RGMC were knocked down and this is enhanced by stimulation of BMP7 (40 ng/ml, 30 mins), while there is no significant change in phosphorylated Smad-1 levels. (B) Shown is the band volume of P-Smad-3 being normalized against corresponding GAPDH band volume. (C) ID1 transcript levels in the transfected cells were determined using real-time quantitative PCR. ID1 transcripts in RGMA, RGMB and RGMC knockdown cells were upregulated, being $4.93 \times 10^7 \pm 2.83 \times 10^7$, $4.4 \times 10^8 \pm 3.24 \times 10^8$ and $6.49 \times 10^8 \pm 3.77 \times 10^8$ respectively), $p=0.52$, 0.09 and 0.05 compared to PC-3^{pEF} control cells ($3.76 \times 10^7 \pm 0.28 \times 10^7$). (D) The ID1 level was elevated in RGM knockdown cells in exposure to BMP7 treatment (40 ng/ml, 30 min) and this effect was strong in RGMB and RGMC. Shown is the copy numbers of each RGM normalised against corresponding GAPDH.

Expression of ID1, a BMP responsive gene in these cells was then determined using real-time quantitative PCR. In line with the observation of activated Smad-3 in PC-3^{ARGMB} and PC-3^{ARGMC} cells, increased transcript levels of ID1 were evident in these cells and also in the cells exposed to BMP-7. Similarly, the up-regulation of ID1 was not seen in the PC-3^{ARGMA} cells (Fig. 6C and D).

Discussion

In the present study, roles played by RGMs in prostate cancer were investigated. The expression of RGMA, RGMB and RGMC was evident in most examined prostate cancer cell lines, and also in the prostate cancer tissues. Although stronger staining of RGMB and RGMC was seen in prostate cancer cells in comparison to prostatic epithelia of normal prostate tissues, the association with disease development and progression requires further investigation in a large cohort and an animal model.

Knockdown of RGMs using hammerhead ribozyme transgenes resulted in accelerated cell growth and enhanced adhesion *in vitro*. Interestingly, the effects on cellular behaviour were most significant in RGMB knockdown cells. The knockdown of RGMB significantly enhanced the prostate cancer cell capacity, namely increased growth, adhesive, motility and mobility. RGMC knockdown did not affect cell motility, however, had an effect on cell growth and adhesion. In comparison to RGMB and RGMC, knockdown of RGMA only affected cell-matrix

adhesion, but not growth, migration and invasion. It suggests that RGMs, particularly RGMB and RGMC trigger or mediate inhibitory effect on growth, adhesion and motility of prostate cancer cells.

To reveal what mechanism(s) is involved in the regulation of prostate cancer cells by RGMs, we investigated the effect of RGM knockdown on Smad-dependent signal transduction. An increased activation of Smad-3 was evident in the PC-3 cells by knockdown of RGMB and RGMC, while activation of Smad-1 was suppressed in these cells. It suggests RGMs, particularly RGMB and RGMC coordinate signal transduction of BMPs and divert the signal to be relayed via a different Smad.

DNA-binding protein inhibitor (ID1) proteins belong to the helix-loop-helix (HLH) transcriptional regulator family, and functions as an antagonists of basic HLH transcription factors by inhibiting their ability to bind specific DNA sequences within target gene promoters, thereby negatively regulate cell differentiation (20). Additionally, they have also been revealed to participate in tumour cell growth and promote cell survival in prostate cancer (21). ID1 is a direct downstream target gene of BMPs being induced by the BMP Smad-dependent signalling pathway (22,23), promoting cell growth and inhibiting cell differentiation in breast cancer and prostate cancer (24,25). In the current study, up-regulation of ID1 expression was seen in PC-3 cells by knockdown of RGMB and RGMC, which were enhanced by addition of BMP7. The increased level of ID1 in RGM knockdown cells could at least partially be responsible

for their effects (promotion) on cell proliferation, adhesion and motility. However, the regulation of ID1 expression may be mediated via Smad-3-dependent pathway, rather than the Smad-1 pathway as previously reported (26-29). Collectively, it was indicated that RGMb and RGMc exert their regulation both on endogenous and exogenous BMPs. Interestingly, the three RGM family members in prostate cancer seem not functionally complementary and associated, as after knockdown of each one the effect will not be substituted by the other two.

In conclusion, RGMs play inhibitory roles in prostate cancer by suppressing cell growth, adhesion, migration and invasion. RGMs can coordinate Smad-dependent signalling by BMPs in prostate cancer cells. The current study indicates the interesting role of RGMs in prostate cancer and a potential therapy target in the future.

Acknowledgements

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