Regulation of prostate cancer cell migration toward bone marrow stromal cell-conditioned medium by Wnt5a signaling

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Abstract. Bone is a major site of metastasis for several types of malignant tumor. Specific interactions between tumor cells and the bone microenvironment contribute to the tendency of tumors to metastasize to bone. Furthermore, Wnt5a participates in the progression of several types of malignant tumor. This study investigates the role of Wnt5a in the migration of the prostate cancer (PCa) cell line PC3 toward bone marrow stromal cell (BMSC)-conditioned medium (CM). The expression of 22 genes associated with bone metastasis was measured in three PCa cell lines (LNCaP, PC3 and DU145). Subsequently, the proliferation and migration capacities of PC3 cells treated either with small interfering RNA (siRNA) against Wnt5a or with recombinant mouse (rm) Wnt5a were analyzed with alamarBlue and transwell assays. BMSC-CM was collected to evaluate its effect on PC3 cell migration. Also, the expression of Wnt5a in BMSCs was knocked down prior to collection of the CM to evaluate its effects on the migration of PC3 cells. Significantly higher levels of Wnt5a mRNA expression were identified in the PC3 cells, compared with those in LNCaP and DU145 cells. Silencing Wnt5a expression with siRNA reduced the migration capacity of PC3 cells by 50%. The addition of rmWnt5a improved the migration capacity of PC3 cells in a concentration-dependent manner. PC3 cells preferred to migrate toward BMSC-CM than toward the control. CM from Wnt5a siRNA-treated BMSCs significantly reduced PC3 cell migration. Wnt5a promotes PC3 cell migration toward BMSC-CM, indicating that Wnt5a is a potential therapeutic target for the treatment of advanced PCa.

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Introduction

Prostate cancer (PCa) is the most frequently diagnosed cancer in men and it was estimated that new cases of PCa would account for 29% of all cancers in men in the United States during 2012 (1). Moreover, the estimated number of mortalities caused by PCa is up to 9% of the total number of cancer-associated mortalities in males, which is second only to lung and bronchus cancers (1). Bone is a major site of metastasis and the incidence of bone metastasis by PCa is 68% (2). Bone metastasis is associated with severe pain, hypercalcemia and pathological fractures. Although numerous methods, including surgical management and nonsurgical modalities, have been proposed (3), bone metastasis is associated with increased morbidity and a poor outcome for patients. However, the detailed mechanisms of bone-specific metastasis remain unclear. Clarification of the molecular mechanisms underlying bone metastasis is of primary importance for targeted therapeutic strategies in patients with PCa (4).

Bone metastasis of PCa requires a series of specific interactions between cancer and host cells, such as human bone marrow stromal cells (hBMSCs), at metastatic sites. The well-accepted 'seed and soil hypothesis' proposes that the bone matrix and abundant growth factors secreted by the bone marrow result in the bone microenvironment being fertile 'soil' for cancer cell ingrowth (5). In addition, there are chemotactic factors in the bone microenvironment that attract PCa cells. However, the mechanisms underlying PCa cell metastasis to bone remain unknown.

Wnt proteins constitute a large family of at least 19 secreted glycoproteins that are important during development and in cell fate, growth and migration (6). Wnt signaling occurs via canonical and non-canonical pathways. The canonical pathway is known as the β-catenin-dependent pathway and it promotes β-catenin accumulation and translocation to the nucleus for the stimulation of target gene expression. The non-canonical pathway activates the β-catenin-independent pathway through planar cell polarity and the Ca^{2+} signaling pathway. Wnt5a is an important member of the Wnt family and acts as a tumor suppressor or promoter (7-9). Moreover, Wnt5a is a regulator of structural plasticity and cell motility in PCa (10). The present study analyzed the ability of Wnt5a to regulate the migration of PCa cells toward hBMSC-conditioned medium (CM).

Materials and methods

Cell isolation and culture. hBMSCs were isolated and expanded as described previously by Li *et al* (11). Once ethical approval from the ethics committee of Shanghai Jiaotong University School of Medicine (Shanghai, China) and written informed consent from the donors was obtained, bone marrow aspirates were acquired from healthy donors during routine orthopedic surgical procedures. Approximately 10 ml volumes of the bone marrow were harvested through a bone marrow biopsy needle inserted through the iliac crest. The bone marrow aspirates were immediately seeded onto 100-mm culture dishes and cultured in complete medium consisting of α-modified minimum essential medium (HyClone, Logan, UT, USA) with 10% fetal bovine serum (HyClone, Tauranga, New Zealand), 100 IU/ml penicillin and 100 mg/ml streptomycin (HyClone, Logan, UT, USA) in a humidified 37˚C/5% CO_2 incubator. After three days, non-adherent cells were discarded by three washes with phosphate-buffered saline (PBS), and the adherent cells were cultured further until 80-90% confluence with medium changes every three days. The obtained hBMSCs were digested with trypsin (0.25%; HyClone, Logan, UT, USA) and third passage cells were used in the subsequent experiments.

Three PCa cell lines derived from different metastatic sites were analyzed in this study, namely PC3 (derived from bone), LNCaP (derived from lymph nodes) and DU145 (derived from the brain). These cell lines were purchased from the Chinese Academy of Sciences Cell Bank (Shanghai, China) and cultured in the complete medium in a humidified 37˚C/5% CO_2 incubator.

Conditioned medium preparation. The PC3 cells and hBMSCs were cultured separately in 100-mm culture dishes in complete medium, as described above, until confluence. Subsequently, cells were rinsed with PBS and incubated in 10 ml of serum-free (SF) medium. After 24 h, the CM was harvested and centrifuged at 0.3 x g for 5 min to remove cell debris. The CM was stored at -80˚C until use and was combined with 10% FBS prior to use.

RNA interference. Small interfering RNA (siRNA) and DharmaFECT 2 transfection reagent were obtained from Dharmacon, Inc. (Lafayette, CO, USA). Wnt5a expression was knocked down in the PC3 cells and hBMSCs according to the manufacturer's instructions. Confluent cells (40%) were seeded onto 96-well plates for proliferation analysis and into six-well plates for other studies. A final concentration of 50 nM siRNA and DharmaFECT 2 transfection reagent were used for *in vitro* transfection. Non-targeting siRNA (siScramble) was used as a siRNA control. Wnt5a gene expression levels were determined by quantitative PCR (qPCR) at 24 h post-transfection and the protein levels were detected by western blotting at 72 h post-transfection. To prepare the CM, the medium was replaced with SF medium at 48 h post-transfection and collected following a further 24 h.

Cell proliferation. For cell proliferation analysis, cells were seeded in 96-well plates in complete medium with or without recombinant mouse Wnt5a (rmWnt5a; R&D Systems, Minneapolis, MN, USA). An alamarBlue assay® (Biosource, Camarillo, CA, USA) was carried out at 24, 48, 72 and 96 h in accordance with the manufacturer's instructions. The absorbance of the culture medium containing alamarBlue was monitored with a spectrophotometer (ELx800; BioTek, Winooski, VT, USA) at 570 and 600 nm.

Migration assay. Cell migration assays were performed in 24-well transwell chambers with 8-μm pore polycarbonate membranes (Corning Inc., Lowell, MA, USA). PC3 cells were suspended at a density of $1x10^5$ cells/ml in SF medium, and then 100 μl of the cell suspension was added to the upper chamber of the transwell chambers. The lower chamber contained 500 μl of SF medium or hBMSC-CM with various concentrations of rmWnt5a (0.1, 0.2, 0.3 and 0.5 μg/ml). Following 16 h of culture, the cells were fixed with 4% paraformaldehyde and washed under flowing water. Cells on the upper surface of the membrane were scraped off with a cotton swab and cells on the lower surface were stained with crystal violet. The cells were then counted in five fields of each well under a light microscope (IX71; Leica, Wetzlar, Germany). Experiments were performed in triplicate.

qPCR. Total RNA was isolated from cells using TRIzol® reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. Equal amounts of RNA (1 μg) were converted into cDNA with a PrimeScript™ RT reagent kit (Takara, Dalian, China). Subsequently, qPCR was performed with SYBR® Premix Ex Taq™ (Takara) using an ABI 7500 Real-Time PCR system (Applied Biosystems, Foster City, CA, USA). PCR conditions were as follows: 40 cycles at 94˚C for 5 sec and 60˚C for 34 sec. The glyceraldehyde-3-phosphate dehydrogenase gene was used as an internal control. The primer sequences used in this study are shown in Table I.

Western blot analysis. Cells were lysed for 30 min on ice in radioimmunoprecipitation assay lysis buffer containing 1% phenylmethylsulfonyl fluoride. Proteins were fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and then electrotransferred onto nitrocellulose membranes. Blocked membranes were incubated overnight with antibodies against Wnt5a (Abcam, Cambridge, UK) and β-actin (Cell Signaling Technology, Inc., Danvers, MA, USA). Following incubation with the appropriate secondary antibodies (IRDye 800CW-Conjugated Goat Anti-Rabbit IgG; LI-COR Biosciences, Lincoln, NE, USA) for 1 h, the membranes were scanned with an Odyssey® CLx system (LI-COR Biosciences).

Statistical analysis. The results are expressed as the mean ± standard deviation. An unpaired t-test was used to compare single groups. Analysis of variance was used to test for significant differences between >2 groups. The significance level for all tests was $P<0.05$.

Results

Expression profiles of bone metastasis-associated genes in three PCa cell lines. Three PCa cell lines (PC3, LNCaP, and DU145) were analyzed in this study. The expression of

Table I. Primer oligonucleotide sequences used for qPCR.

Gene	Forward primer (5'-3')	Reverse primer (5'-3')	Product size (bp)
BMP2	ACCCGCTGTCTTCTAGCGT	TTTCAGGCCGAACATGCTGAG	180
BMP4	AAAGTCGCCGAGATTCAGGG	GACGGCACTCTTGCTAGGC	135
BMP7	TCGGCACCCATGTTCATGC	GAGGAAATGGCTATCTTGCAGG	150
CXCR4	ACTACACCGAGGAAATGGGCT	TTCTTCACGGAAACAGGGTTC	65
IGF2	GGAGACGTACTGTGCTACCC	CTGCTTCCAGGTGTCATATTGG	124
IGF1	GCTCTTCAGTTCGTGTGTGGA	CGACTGCTGGAGCCATACC	71
IL11	CGAGCGGACCTACTGTCCTA	GCCCAGTCAAGTGTCAGGTG	272
MMP1	GGGGCTTTGATGTACCCTAGC	TGTCACACGCTTTTGGGGTTT	142
MMP7	GAGTGAGCTACAGTGGGAACA	CTATGACGCGGGAGTTTAACAT	158
OPG	GCGCTCGTGTTTCTGGACA	AGTATAGACACTCGTCACTGGTG	226
OPN	CTCCATTGACTCGAACGACTC	CAGGTCTGCGAAACTTCTTAGAT	230
RANKL	CAACATATCGTTGGATCACAGCA	GACAGACTCACTTTATGGGAACC	161
TGFB2	CAGCACACTCGATATGGACCA	CCTCGGGCTCAGGATAGTCT	113
WNT10B	CATCCAGGCACGAATGCGA	CGGTTGTGGGTATCAATGAAGA	204
WNT2	ATGTGCGATAATGTGCCAGG	AGATTCCCGACTACTTCGGAG	207
WNT3A	CCTGGCTTTGGAATGCTC	CCTCTGCGAAGTCCCTGT	172
WNT5A	TTGGTGGTCGCTAGGTATGAA	AGTGGCACAGTTTCTT	120
WNT7B	GAAGCAGGGCTACTACAACCA	CGGCCTCATTGTTATGCAGGT	155
bFGF	AGAAGAGCGACCCTCACATCA	CGGTTAGCACACACTCCTTTG	82
IGF1R	AGGATATTGGGCTTTACAACCTG	ACAGAGGTCAGCATTTTTCTCAA	74
MMP9	TGTACCGCTATGGTTACACTCG	GGCAGGGACAGTTGCTTCT	97
VEGF	CGCAGCTACTGCCATCCAAT	GTGAGGTTTGATCCGCATAATCT	192
GAPDH	TCACCATCTTCCAGGAGCGA	CACAATGCCGAAGTGGTCGT	293

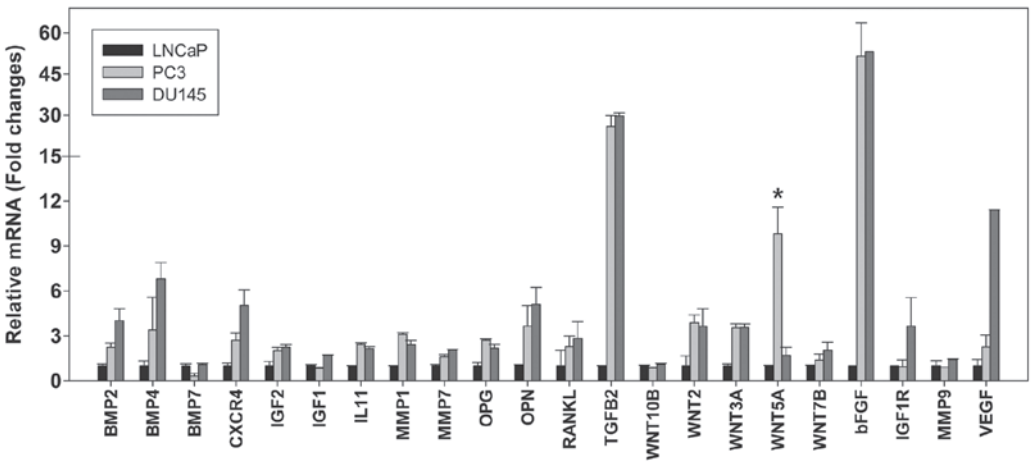

Figure 1. Expression levels of 22 genes associated with bone metastasis measured by quantitative PCR in three PCa cell lines (PC3, LNCaP and DU 145). The results were expressed as fold-changes relative to LNCaP cells. Data are shown as the mean ± S.D. *P<0.01.

22 genes associated with bone metastasis was measured by qPCR (Fig. 1). Significantly higher levels of Wnt5a mRNA expression were observed in the PC3 cells, at 10- and 6-fold higher than those in the LNCaP (P<0.01) and DU145 cells (P<0.01), respectively.

Proliferation and migration of PC3 cells transfected with siRNA against Wnt5a. siRNA against Wnt5a expression was employed to investigate the role of Wnt5a in the proliferation and migration of PC3 cells. Wnt5a siRNA reduced the levels of Wnt5a mRNA by ~75% compared with those in the control (Fig. 2A). The alamarBlue assays indicated that Wnt5a siRNA significantly decreased the proliferation rate of cells cultured for 72 and 96 h (P<0.05 and P<0.01, respectively; Fig. 2B). Transwell chambers were used to assess cell migration. Representative images of migrated cells stained with

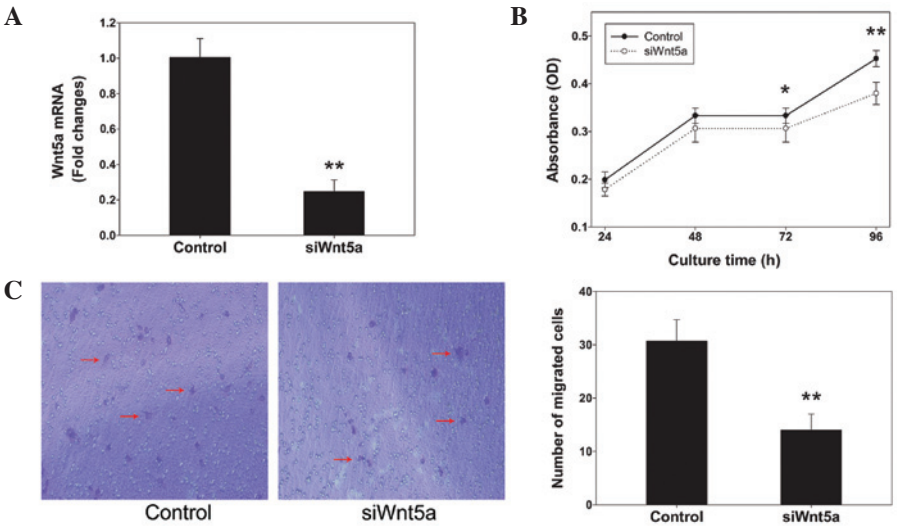

Figure 2. Effects of siRNA against Wnt5a on the proliferation and migration of PC3 cells. (A) Wnt5a siRNA reduced the Wnt5a mRNA level compared with that in the control. Cells transfected with a scrambled sequence served as a control. (B) Wnt5a siRNA significantly decreased the proliferation rate of cells cultured using the alamarBlue assays. (C) Inhibition of Wnt5a expression by siRNA knockdown significantly reduced PC3 cell migration. Representative images of migrated cells (red arrows) stained with crystal violet are shown (magnification, x200). The number of migrated cells was counted. Data are shown as the mean ± S.D. *P<0.05, **P<0.01. siRNA, small interfering RNA.

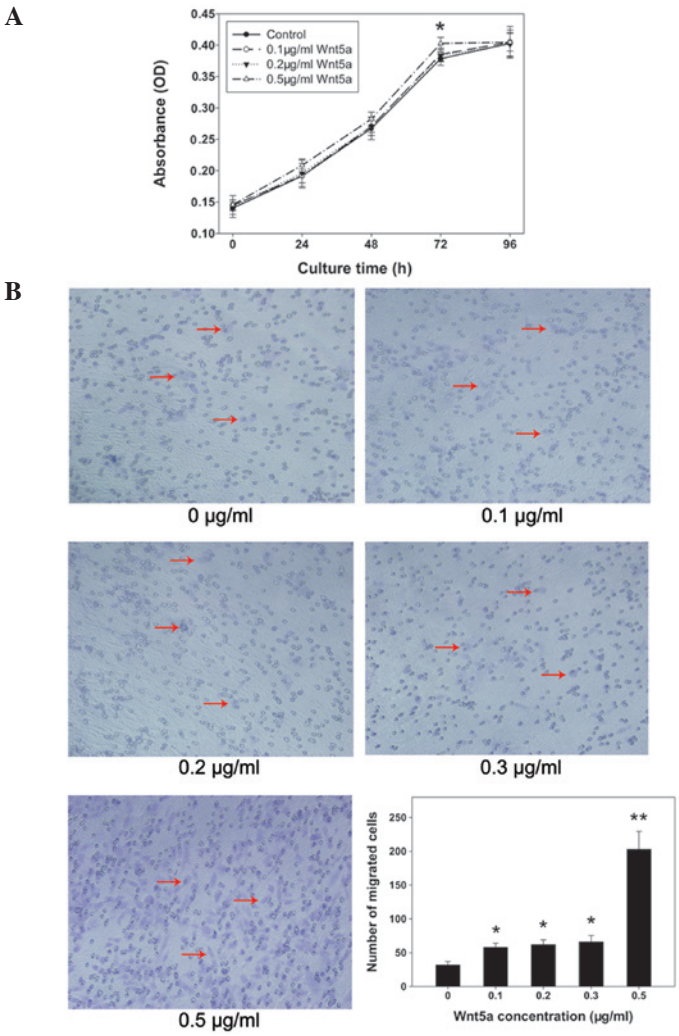

Figure 3. Role of rmWnt5a in the proliferation and migration of PC3 cells. (A) Proliferation of PC3 cells was evaluated by alamarBlue assays in the presence of various concentrations of rmWnt5a (0.1, 0.2, 0.3 and 0.5 μg/ml). The culture medium without rmWnt5a served as a control. (B) Migration of PC3 cells was assessed with transwell assays. Various concentrations of rmWnt5a (0.1, 0.2, 0.3 and 0.5 μg/ml) were applied to the lower chamber. Representative images of migrated cells (red arrows) stained with crystal violet are shown (magnification, x200). The number of migrated cells was counted. Data are shown as the mean ± S.D. *P<0.05, **P<0.01. rmWnt5a, recombinant mouse Wnt5a.

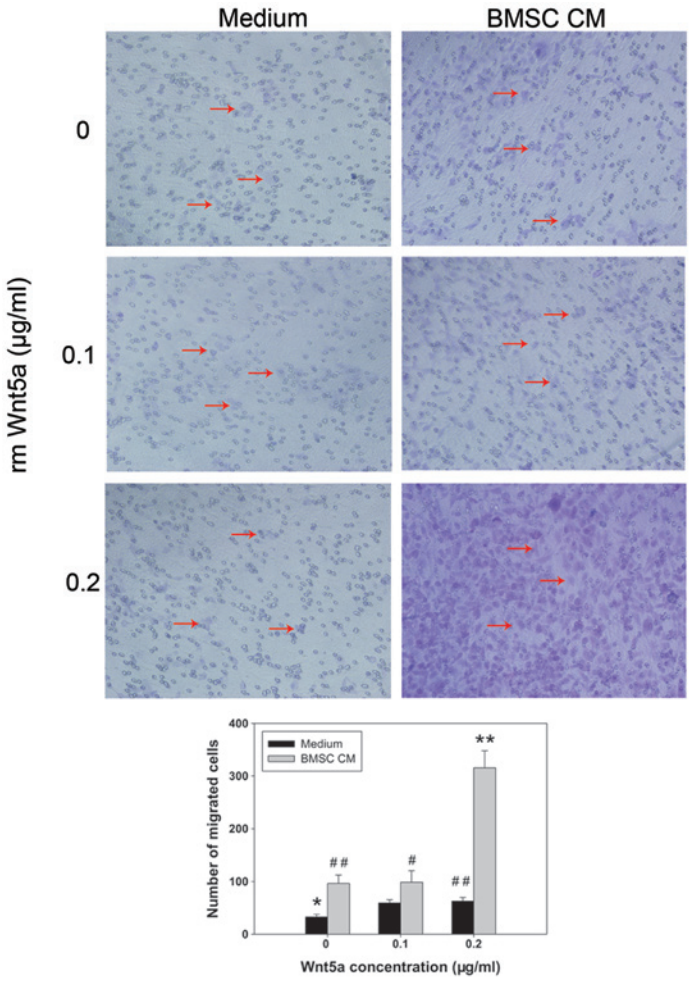

Figure 4. Enhancement of PC3 cell migration toward BMSC-CM, as compared with culture medium with various concentrations of rmWnt5a (0, 0.1 and 0.2 μg/ml) in the lower chambers. Representative images of migrated cells (red arrows) that were stained with crystal violet are shown (magnification, x200). The number of migrated cells was counted. Data are shown as the mean ± S.D. *Significant difference among different concentrations of rmWnt5a in the same medium (*P<0.05, **P<0.01). $^{\#}$Significant difference between culture medium and BMSC CM containing the same concentration of rmWnt5a ($^{\#}$P<0.05, $^{\#\#}$P<0.01). BMSC-CM, bone marrow stromal cell-conditioned medium; rmWnt5a, recombinant mouse Wnt5a.

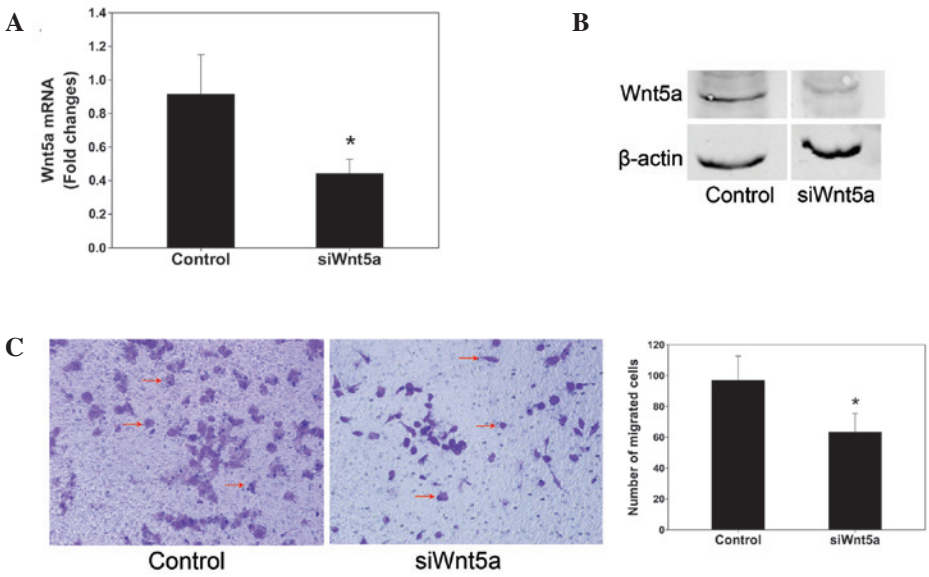

Figure 5. PC3 cell migration decreased toward CM of Wnt5a siRNA-transfected BMSCs. (A) Wnt5a gene expression of BMSCs was confirmed to be reduced by Wnt5a siRNA after 24 h using quantitative PCR. Cells transfected with a scrambled sequence served as a control. (B) Western blot analysis of Wnt5a expression of PC3 cells treated with siRNA. (C) Migration of PC3 cells was assessed with transwell systems. The CM of BMSCs treated with siRNA was applied to the lower chamber. Representative images of migrated cells (red arrows) stained with crystal violet are shown (magnification, x200). The number of migrated cells was counted. Data are shown as the mean ± S.D. *P<0.05. siRNA, small interfering RNA; BMSC, bone marrow stromal cell; CM, conditioned medium.

crystal violet are shown in Fig. 2C. The inhibition of Wnt5a expression by siRNA knockdown significantly reduced the PC3 cell migration by 50% compared with that in the control (P<0.01).

Proliferation and migration of PC3 cells treated with rmWnt5a. PC3 cells were treated with various concentrations of rmWnt5a and then alamarBlue assays were performed. Proliferation rates were equal among groups with the exception of 0.5 μg/ml rmWnt5a, which showed a higher cell proliferation rate than that in the other groups at 72 h (P<0.05) (Fig. 3A). Results from the transwell assays demonstrated that the migration of PC3 cells was significantly promoted by increasing concentrations of rmWnt5a (P<0.05) (Fig. 3B). The number of cells treated with 0.5 μg/ml rmWnt5a that migrated was ~6-fold higher than that in the control (P<0.01). In addition, the numbers of migrated cells in the other groups (0.1, 0.2 and 0.3 μg/ml rmWnt5a) were nearly two-fold higher than that in the control (P<0.05).

Enhancement of PC3 cell migration in hBMSC-CM. Bone metastasis depends on complex interactions between tumor cells and cells in the bone microenvironment, such as hBMSCs. Thus, hBMSC-CM was prepared to investigate the indirect interactions of PC3 cells with hBMSCs *in vitro*. The results from the transwell assays demonstrated that the migration of PC3 cells was significantly promoted in hBMSC-CM compared with that in SF medium (P<0.01) (Fig. 4). When rmWnt5a was added to the hBMSC-CM, the number of migrated cells increased further. The number of migrated cells in hBMSC-CM containing 0.2 μg/ml rmWnt5a was three-fold higher than that in hBMSC-CM without rmWnt5a (P<0.01) and five-fold higher than that in SF medium containing 0.2 μg/ml rmWnt5a (P<0.01).

PC3 cell migration decreases in CM of Wnt5a siRNA-transfected hBMSCs. To obtain further evidence that Wnt5a is important in the interaction between PC3 cells and hBMSCs, Wnt5a gene expression in hBMSCs was knocked down using siRNA prior to collection of CM. Subsequently, the CM obtained from these cells was applied to PC3 cells in transwell assays. The results from qPCR and western blot analyses demonstrated that Wnt5a gene expression levels were reduced by 50% (Fig. 5A and B). Fig. 5C shows representative crystal violet staining of migrated cells in transwell assays. CM from Wnt5a siRNA-transfected hBMSCs significantly reduced PC3 cell migration compared with that of CM from siScramble-transfected hBMSCs (P<0.05). These results suggest that the migration of PC3 cells toward hBMSCs is, at least in part, dependent on Wnt5a secreted from hBMSCs.

Discussion

Tumor metastasis indicates a poor prognosis and bone is a prime target for PCa metastasis. When PCa metastasizes to bone, the five-year survival rate drops to ~30% from virtually 100% for PCa that remains confined to the prostate (12). However, the mechanisms of PCa bone metastasis are unclear. Thus, the expression of 22 genes associated with bone metastasis was analyzed in PCa cell lines. The present study identified that Wnt5a was highly expressed in PC3 cells that were derived from a bone metastasis site. Therefore, the study focused on this potential candidate gene.

Wnt5a regulates a variety of cellular functions such as adhesion, proliferation, differentiation and migration (13,14). A number of important roles of Wnt5a have been demonstrated in organ development (15). In addition, Wnt5a participates in tumor progression. Wnt5a has either a tumor-suppressing or -promoting function depending on the type of cancer (16). A number of studies have indicated increased expression levels of Wnt5a in melanoma (9,17,18), breast cancer cells (13), gastric cancer (19), pancreatic cancer (20) and non-small cell lung cancer (16). Wnt5a expression is correlated with aggressiveness and a poor prognosis of gastric cancer (19,21). It is a mediator of chemoresistance in ovarian cancer (22) and correlates with the clinical grade (23). Although there is firm evidence that Wnt5a has an oncogenic role, certain studies have indicated that Wnt5a has a suppressive role in tumors arising from a variety of tissues. Wnt5a is downregulated in certain malignancies, including colorectal cancer (24), neuroblastoma (25), invasive ductal breast carcinomas (26) and leukemias (27), indicating a tumor-suppressive effect (24).

In PCa, Wang *et al* identified that the Wnt5a protein levels are increased compared with those in benign tissue (10). However, Syed Khaja *et al* demonstrated that elevated levels of Wnt5a protein in patients with localized PCa predict a favorable outcome following surgery (28). In the present study, the addition of rmWnt5a enhanced PC3 cell migration in a concentration-dependent manner. Furthermore, when Wnt5a gene expression was silenced by siRNA, PC3 cell migration was reduced by 50%. Yamamoto *et al* have also demonstrated that knockdown of Wnt5a in human PCa cell lines reduces the cells' invasive activities, indicating that Wnt5a promotes the aggressiveness of PCa and is involved in relapses following prostatectomy (29).

The role of Wnt5a in PCa cell proliferation was also assessed. Although decreased proliferation rates were observed when PC3 cells were cultured for 72 and 96 h following Wnt5a gene knockdown, the addition of rmWnt5a did not significantly affect PC3 cell proliferation. Thus, the role of Wnt5a in PCa cells is mainly associated with cell migration. Wnt5a activates the Wnt/Ca^{2+} pathway in PCa cells, which causes a major reorganization of the cytoskeleton in cancer cells by decreasing the length and frequency of fine filopodia-like actin structures and results in an increase in cell motility (10).

The role of Wnt5a in mediating PCa bone metastasis is unclear. Bone metastasis is a multistep process. The 'seed and soil' hypothesis suggests that there are chemotactic factors in the bone microenvironment that attract PCa cells (2,5). Cell-cell interactions between PCa cells and cells in the bone microenvironment are important and contribute to metastatic cell behavior (30,31). Previous studies have shown that BMSCs are significant in PCa cell metastasis (32,33). Therefore, in the present study hBMSC-CM was collected for further study and it was demonstrated that the number of PC3 cells that migrated toward hBMSC-CM was ~3-fold higher than that toward SF medium. To determine whether the enhanced migration of PC3 cells toward hBMSC-CM was due to Wnt5a protein expression in hBMSCs, Wnt5a expression in hBMSCs was knocked down and PC3 cell

migration toward hBMSC-CM was reduced by 30%. The migration of PC3 cells toward hBMSC-CM was confirmed to be at least partly dependent on Wnt5a expression in hBMSCs. In addition to Wnt5a, other factors participate in PCa cell migration toward hBMSCs. The interaction between the stromal-derived factor-1 and the CXCR4 ligand-receptor system is the most studied and is also involved in the activation of PCa cell migration (34,35). Overall, the findings of the present study implicate Wnt5a in the stimulation of PCa cell migration toward BMSC-CM. Consequently, the inhibition of Wnt5a signaling may be an attractive therapeutic target for the treatment of advanced PCa.

Acknowledgements

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