Potential role of the OPG/RANK/RANKL axis in prostate cancer invasion and bone metastasis

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Abstract. Receptor activator of NF-κB (RANK), RANK ligand (RANKL) and osteoprotegerin (OPG) are key regulators of bone metabolism under both normal and pathological conditions, including prostate cancer (PCa) bone metastases. However, little is known concerning the expression and function of these regulators in prostate tumor samples and PCa cells and their correlation with invasion and bone metastasis. In the present study, we determined the expression of RANK, RANKL and OPG in 3 human PCa cell lines and 40 PCa patient samples by immunohistochemistry and enzyme-linked immunosorbent assay (ELISA). As controls, samples from 20 patients with benign prostate hyperplasia (BPH) and normal prostate epithelial RWPE2 cells were also included in the analyses. The effects of soluble RANKL (sRANKL) and OPG as well as RANK knockdown on PCa invasion were examined in Transwell assays. Immunohistochemical staining detected little RANK, OPG and RANKL expression in hyperplasia prostate while the percentages of positivity were increased to 50, 45 and 52.5%, respectively, in prostate tumor tissues. OPG and sRANKL levels in the prostate tumor samples as measured by ELISA were ~10-fold that in the BPHs ($P<0.01$) and the levels were higher in aggressive tumors than non-aggressive ones ($P<0.05$). The sRANKL level in the serum of PCa patients was the same as that in the patients with BPH, yet the serum OPG levels correlated with the tissue levels ($R^2=0.620$, $P<0.01$, which both showed a 10-fold increase in PCa over BPH ($P<0.01$) with higher levels in aggressive PCa than non-aggressive ones ($P<0.05$). Consistent with the tissue analyses, expression levels of RANK mRNA and protein were detected in multiple human PCa cell lines by RT-PCR and western blotting, respectively. The treatment of PCa cells with RANKL significantly increased the number of invaded cells ($P<0.01$), which was suppressed by the decoy receptor OPG. RANK siRNA transfection dramatically dampened the stimulatory effect of RANKL on PCa cell invasion. Our findings indicate that the expression of RANK, RANKL and OPG may be used as diagnostic markers to identify patients at high risk for aggressive PCa and that the effective suppression of PCa cell migration by OPG via the blockage of RANKL activity represents a potential therapeutic strategy for interfering with prostate tumor metastasis and progression to bone.

Introduction

Prostate adenocarcinoma is the most frequently diagnosed cancer among males in the world (1). A major challenge in prostate cancer (PCa) biology is bone metastasis and the development of osteoblastic lesions in bone. Approximately 90% of patients with advanced PCa have skeletal metastasis (2). However, the molecular mechanisms underlying PCa invasion and metastasis to the skeleton remain poorly understood and it has been difficult to develop effective treatment modalities.

The RANKL/RANK/OPG network was originally characterized as a regulator of bone remodeling (3). More and more studies have demonstrated that this network also plays a critical role in osteolysis in metastatic bone diseases (4). The RANK ligand (RANKL) include two types that are either membrane-bound on the surface of cells or a soluble form of RANKL (sRANKL) (5) whereas its specific receptor activator of NF-κB (RANK) is a membrane-bound protein on the surface of cells. Osteoprotegerin (OPG) acts as a decoy receptor that exists only as a soluble form to prevent the binding of RANKL to RANK (6). Recent studies revealed that the RANKL/RANK/OPG system is dysregulated in several tumor types such as PCa, multiple myeloma, breast cancer and malignant bone tumors (7-10). In addition, RANK and RANKL have been described to be expressed in osteoclasts and their

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precursors (11). However, the expression and functional status of RANK/RANKL/OPG in prostate tumor tissues or cells and their relationship with PCa invasion remain undefined.

The purpose of the present study was to assess the potential roles of the RANK/RANKL/OPG network in PCa progression by examining their expression in patient tissue samples. Furthermore, we employed siRNA knockdown to deplete RANK in PCa cells to explore the molecular mechanisms that govern PCa invasion with the expectation that the studies will lead to the identification of new therapeutic targets for metastatic PCa treatment.

Materials and methods

Patient samples. The present study included 40 patients (aged 49-82 years) diagnosed with localized (n=23) or metastatic (n=17) prostate carcinomas between January 2005 and August 2009 at our institution (Department of Urology at the Second Hospital of Lanzhou University, China). None of the patients had received treatment at the time of sampling. Detailed clinical data regarding these patients are provided in Table I. Tumors were staged using the updated staging system by the American Joint Committee on Cancer (AJCC) and the Union Internationale Contre le Cancer (UICC) (2009 UICC) on cancer TNM classification and graded by Gleason scores. Tumors with a Gleason score of ≥7, distant-stage diseases (including T3, T4, N1 or M1) or pretreatment prostate-specific antigen (PSA) score of ≥20 ng/ml were defined as aggressive PCa, while all others were classified as non-aggressive. Twenty hyperplasia prostate patients served as controls. The Ethics Committee of the Lanzhou University approved the present study. Prior to the study, written informed consent was obtained from all individuals.

Table I. Correlation between RANKL, RANK and OPG expression and clinicopathological parameters of the prostate cancer patients.

		No. of positive cases		
Parameters	No.	RANK	RANKL	OPG
Total	40			
Age (years)				
≤65	12	5	7	5
>65	28	15	14	13
Tumor stage				
Localized	8	1	2	1
Locally advanced	15	7[a]	6[a]	5[a]
Metastatic	17	12[a,b]	13[a,b]	12[a,b]
Lymph node metastasis	6	3[a,b]	4[a,b]	3[a,b]
Bone metastasis	11	9[a,b]	9[a,b]	9[a,b]
Gleason score				
Low (<7)	23	7	8	6
High (≥7)	17	13[c]	13[c]	13[c]
Preoperative PSA (ng/ml)				
Low (<20)	11	2	3	2
High (≥20)	29	20[c]	21[c]	19[c]

RANK, receptor activator of NF-κB; RANKL, RANK ligand; OPG, osteoprotegerin; PSA, prostate-specific antigen. [a]P<0.05 and [b]P<0.05, compared to localized and locally advanced group, respectively. [c]P<0.05, compared to the group with low Gleason score or low PSA level.

Cell culture. The human prostate adenocarcinoma cancer cell lines (PC3, DU145 and LNCaP) and the normal prostate epithelial cell line RWPE2 were obtained from Shanghai Institute of Cell Biology, Chinese Academy of Sciences. The cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA, USA) at 37˚C in a humidified atmosphere with 5% CO_2.

Tissue samples and immunohistochemical staining. For the immunohistochemical staining, 4-μm paraffin sections of the tissue samples were autoclaved in citrate buffer at 121˚C for 15 min for antigen retrieval, and then incubated with the anti-RANK, -OPG or -RANKL antibody (Santa Cruz) (1:50 dilution in signal stain antibody diluent) at 4˚C overnight. Sections were incubated with the biotinylated secondary antibody and HPR-streptavidin for 30 min at room temperature, respectively, followed by a 3,3'-diaminobenzidine plus reaction (Zhongshan Golden Bridge Biotechnology, Co.). Finally, the sections were counterstained with hematoxylin. Appropriate positive controls (human breast cancer) and negative controls (human normal gastric mucosa) were run concurrently. As another negative control, sections processed in an identical fashion except for the replacement of the primary antibody with normal rabbit serum were also included. Immunoreactive cells were scored as follows: 0, positive cell staining ≤1%; 1, positive cell staining >1-25%; 2, positive cell staining >25-50%; 3, positive cell staining >50-75%; 4, positive cell staining >75% (Fig. 1). Low- and high-intensity signals were scored based on the staining intensity (negative=0; weak=1; moderate=2; and strong=3). The immunoreactive score was calculated by multiplying the percentage of immunoreactive cells by the staining intensity. Scores were considered negative (0-1) and positive (≥1).

In vitro cell invasion assays. *In vitro* Matrigel invasion assays were performed in Transwell permeable supports with porous filters (8-μm pore size) according to the manufacturer's instructions (Corning, USA). Briefly, the upper surface of the filter was coated with 500 ng/ml Matrigel (Sigma). Cells with or without siRNA of RANK suspended in the medium were added onto the upper surface of the Transwell ($2x10^5$). RANKL and/or OPG (R&D Systems) were added to the lower chamber at final concentrations between 0 and 50 μg/ml. After a 24-h incubation in medium containing chemokines, the filter was removal from the chamber. Cells that has passed through the Matrigel and attached to the lower surface of the filter were fixed with methanol and stained with H&E. Cells in 15 randomly selected microscopic fields (x200) per filter were counted.

Reverse transcription and PCR. Total RNA extracted using TRIzol reagent (Invitrogen) from the PCa cell lines was reverse

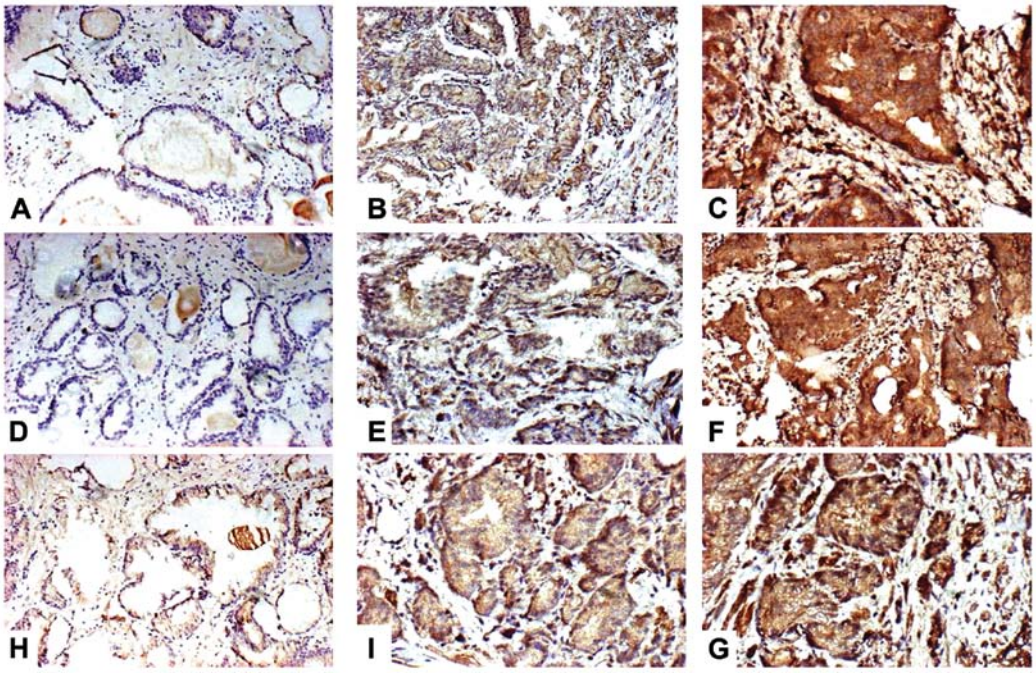

Figure 1. Expression of RANKL, its receptor RANK and OPG in malignant human prostate tumors. (A, D and H) Respective OPG, RANKL and RANK expression in hyperplasia prostate tissues; (B, E and I) Respective OPG, RANKL and RANK expression in local prostate tumor tissues with weak positive signaling. (C, F and G) OPG, RANKL and RANK expression in metastatic prostate tumor tissues with strong positive signaling. Magnification, x200. RANKL, RANK ligand; RANK, receptor activator of NF-κB; OPG, osteoprotegerin.

transcribed to cDNA using the Omniscript RT kit with the oligo(dT) primer (Qiagen, USA). Subsequently, PCR reactions were performed using a PCR kit (Bio-Rad, USA) as follows: denaturation at 95˚C for 10 min, 30 cycles consisting of 95˚C for 30 sec and 68˚C for 1 min and an extra incubation at 68˚C for 1 min. The following primers were used to amplify RANK and β-actin: RANK forward, 5'-CAG GGA TCG ATC GGT ACA GT-3' and reverse, 3'-GTT TGA GAC CAG GCT GGG TA-5'; β-actin forward, 5'-GTG GGG CGC CCC AGG CAC CA-3' and reverse, 3'-CTC CTT AAT GTC ACG CAC GTA TTC-5'. The amplified PCR products were analyzed by electrophoresis on a 2.5% agarose gel containing ethidium bromide.

Western blotting. To detect RANK protein expression in the PCa cells, equal amounts of protein (50 μg) obtained from the cell extracts were separated on 10% SDS-PAGE under non-reducing conditions. The separated proteins were electroblotted onto PVDF membranes and blocked for 1 h at room temperature with Tris-buffered saline containing 5% non-fat milk. The membranes were then probed with a 1:500 dilution of the purified primary antibodies at room temperature for 2 h, followed by incubation with secondary antibodies for 1 h at room temperature. Antibody-protein complexes were detected by the ECL-Plus chemiluminescent system according to the manufacturer's instructions.

Enzyme-linked immunosorbent assay (ELISA). OPG and sRANKL levels in the prostate tissues and serum were measured using the ELISA kit (R&D Systems) according to the manufacturer's instructions.

Small interfering RNA (siRNA) transfections. To deplete RANK expression in the PCa cells, we designed 3 different RANK siRNA (siRNA1, siRNA2 and siRNA3) duplexes with BLOCK-iT™ RNAi Designer software and synthesized them through a service provided by Invitrogen. PCa cells were transfected with those siRNAs using Lipofectamine 2000 following the manufacturer's instruction. siRNA duplexes used in the present study had the following sequences: siRNA1 sense, (5'-3') CCUGGACCAACUGUACCUU and antisense, (5'-3') AAGGUACAGUUGGUCCAGG; siRNA2 sense, (5'-3') GGAGAAGACAUUUCCAGAA and antisense, (5'-3') UUCU GGAAAUGUCUUCUCC; siRNA3 sense, (5'-3') GCAAAUC CACACCUCCUUU and antisense, (5'-3') AAAGGAGGUGU GGAUUUGC; control sense, (5'-3') UAGCGACUAAACACA UCAAUU and antisense, (5'-3') UUAUCGCUGAUUUGUGU AGUU.

Statistical analyses. The data are presented as means ± SEM of the indicated number of experiment. Statistical analyses were performed by Student's t-test and analysis of variance (ANOVA). Chi-square and Fisher exact tests were performed to determine the correlation between RANKL/RANK/OPG expression and clinicopathological parameters. Pearson's test and line regression were used for correlation analysis. The value of $P<0.05$ was considered to indicate a statistically significant result.

Results

Morphometric analysis of RANK, OPG and RANKL expression in PCa lesions. The morphometric analysis of the immunohistochemical staining (Fig. 1) showed that RANK, OPG and RANKL were not significantly expressed in hyperplasia prostate, while their expression levels were increased to 50, 45 and 52.5%, respectively in the PCa tissues. As shown in

Table II. Correlation between tissue sRANKL and OPG levels, and clinicopathological parameters of the prostate cancer patients.

Parameters	No.	RANKL (ng/ml)	OPG (ng/ml)
BPH	20	0.142±0.023	0.159±0.035
PCa	40	1.415±0.501[a]	1.842±0.550[a]
Age (years)			
≤65	12	1.304±0.436	1.672±0.515
>65	28	1.414±0.531	1.906±0.432
Tumor stage			
Localized	8	0.897±0.147[a,c]	1.225±0.301[a,c]
Locally advanced	15	1.313±0.253[a,b]	1.733±0.281[a-c]
Metastasis	17	1.749±0.533[a-c]	2.228±0.519[a-c]
Lymph node	6	1.396±0.191[a,b]	1.970±0.397[a,b]
Bone	11	2.395±0.246[a-c]	2.700±0.362[a-c]
Gleason score			
Low (<7)	23	1.168±0.298	1.557±0.375
High (≥7)	17	1.749±0.533[d]	2.228±0.519[d]
Preoperative PSA (ng/ml)			
Low (<20)	11	0.935±0.172	1.327±0.313
High (≥20)	29	1.597±0.463[d]	2.068±0.493[d]

sRANKL, soluble RANKL; RANKL, RANK ligand; PSA, prostate-specific antigen. [a]P<0.05, compared to BPH. [b]P<0.05 and [c]P<0.05, compared to localized and locally advanced groups. [d]P<0.05, compared to group with low Gleason score or low PSA level.

Table III. Correlation between serum sRANKL and OPG levels and clinicopathological parameters of prostate cancer patients.

	No.	RANKL (ng/ml)	OPG (ng/ml)
BPH	20	0.254±0.079	0.276±0.075
PCa	40	0.956±0.283[a,b]	2.763±0.936[a]
Age (years)			
≤65	12	0.926±0.206	2.931±0.932
>65	28	0.973±0.320	2.672±0.943
Tumor stage			
Localized	8	1.103±0.341	1.741±0.435[a,c]
Locally advanced	15	1.024±0.256	2.534±0.500[a,b]
Metastasis	17	0.828±0.234	3.446±0.875[a-c]
Lymph node	6	0.920±0.254	2.810±0.536[a-c]
Bone	11	0.777±0.218	3.793±0.841[a-c]
Gleason score			
Low (<7)	23	1.051±0.283	2.258±0.606
High (≥7)	17	0.828±0.234	3.446±0.875[d]
Preoperative PSA (ng/ml)			
Low (<20)	11	1.112±0.300	1.960±0.572
High (≥20)	29	0.897±0.258	3.068±0.868[d]

sRANKL, soluble RANKL; RANKL, RANK ligand; OPG, osteoprotegerin. [a]P<0.01, compared to BPH. [b]P<0.05 and [c]P<0.05, compared to localized and locally advanced groups, respectively. [d]P<0.05, compared to the group with low Gleason score or low PSA level.

Table I, there was a positive correlation between the expression of RANK, RANKL and OPG and the aggressiveness of tumors, based on Gleason score (≥7), distant-stage disease or pretreatment PSA score (≥20 ng/ml). The expression frequency of RANK, RANKL and OPG in the metastatic PCa cases was significantly higher than that in the localized and locally advanced cases. We also observed that the expression of RANK, OPG and RANKL in the tissues of PCa patients with bone metastasis was more intense than that in the patients with lymph node metastasis, suggesting a potential selective involvement of these molecules in bone metastasis.

Levels of sRANKL and OPG in the prostate tumor tissue samples. RANKL exists in both sRANKL and membrane-bound forms. OPG exists only in soluble form. In order to determine if sRANKL and OPG have any effect on prostate tumor metastasis, we next quantified the amounts of sRANKL and OPG by ELISA in the human PCa tissue samples. As shown in Table II, OPG and sRANKL levels in the prostate tumor tissues were ~10-fold of the levels in the BPHs (P<0.01). The levels of sRANKL and OPG in aggressive and metastatic tumors were increased 2.01- and 1.8-fold compared with levels in the non-aggressive and localized/locally advanced tissues, respectively. Consistent with the immunohistochemical studies, ELISA assays detected higher levels of OPG and sRANKL in the tissues of PCa patients with bone metastasis than the levels in patients with lymph node metastasis (Table II).

Level of RANKL and OPG in the serum of PCa patients. To further assess the role of sRANKL and OPG in prostate tumor metastasis, we detected sRANKL and OPG levels in serum by ELISA. As shown in Table III, serum sRANKL levels in PCa patients were the same as that in the BPH cases. However, the serum levels of OPG showed a 10-fold increase in PCa over BPH. A significant increase in OPG levels was also observed in aggressive PCa samples and those of bone metastasis.

Change of RANKL and OPG expression levels in tumor tissues and serum of PCa patients. As shown in Fig. 2, a positive correlation was observed between serum and tissue OPG level. However, the same analyses did not reveal a correlation between serum and tissue RANKL.

RANK expression in human prostate carcinoma cell lines. RANK is the only receptor of RANKL and the central player of the RANKL/RANKL/OPG signaling network. Since

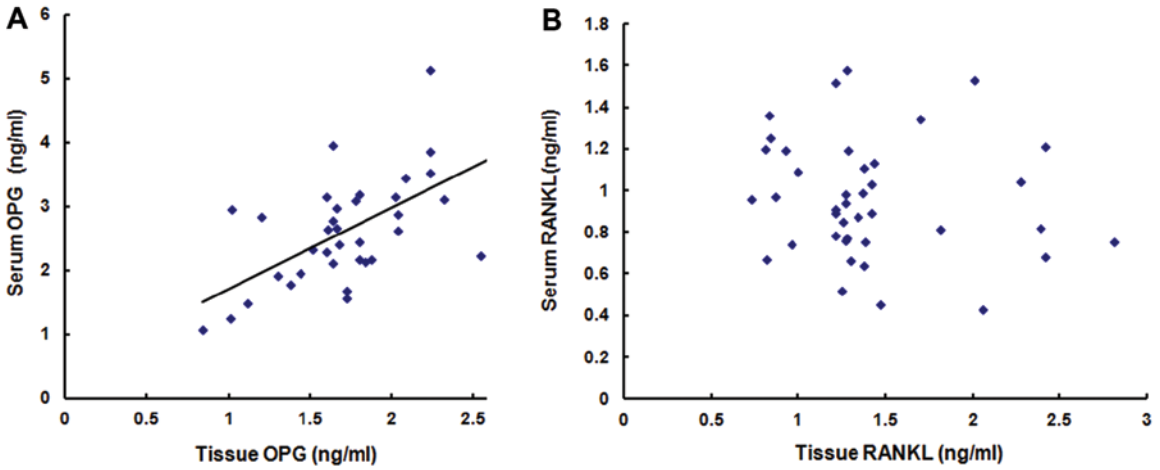

Figure 2. Association between tissue and serum RANKL and OPG expression levels in the PCa patients. The results were obtained by using the calibration curves for (A) OPG and (B) RANKL. PCa, prostate cancer; RANKL, RANK ligand; OPG, osteoprotegerin.

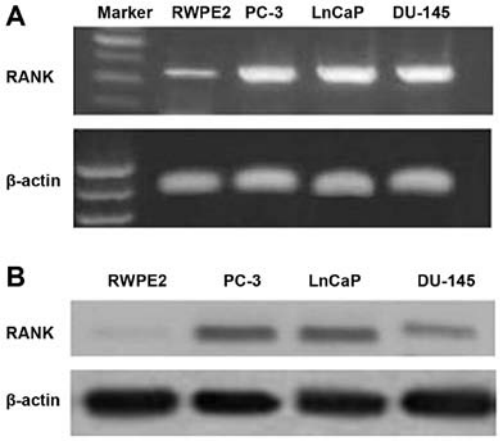

Figure 3. Expression of RANK is increased in malignant human PCa cell lines. (A) RANK mRNA and (B) protein were detected by RT-PCR and western blotting, respectively. The data are representative of three independent experiments.

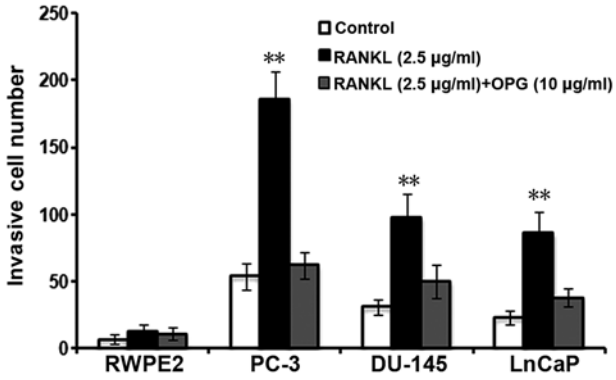

Figure 4. RANKL promotes PCa invasion which is inhibited by OPG pre-treatment. Confluent monolayer of human PCa cells seeded on a Transwell membrane was treated with RANKL and/or OPG. Cells that invaded through the Matrigel and stayed on the opposite surface of the membrane were counted. The experiment was repeated five times. RANKL, RANK ligand; PCa, prostate cancer; OPG, osteoprotegerin.

we found that the levels of sRANKL and OPG in prostate tumor tissues were significantly correlated with malignancy, we investigated the RANK expression in different human prostate carcinoma cell lines. Our RT-PCR and western blot analyses detected RANK expression at both the mRNA and protein levels in all 3 human prostate carcinoma cell lines: LNCaP, DU145 and PC3. In contrast, low levels of RANK RNA and protein were detected in the normal prostate epithelial RWPE2 cell line (Fig. 3). The data are consistent with the tumor sample analyses, suggesting that increased RANK signaling may play an important role in PCa development and progression.

Effect of RANKL and OPG on PCa cell invasiveness. To investigate the effect of sRANKL and OPG on PCa invasion, PCa cells which express RANK were seeded on Matrigel-coated Transwell and treated with different concentrations of sRANKL with or without OPG (2.5, 10 or 50 μg/ml, respectively) for 24 h. The concentrations were chosen according to our preliminary study in which we found that 2.5 μg/ml sRANKL increased PCa invasion (data not shown). As shown in Fig. 4, treatment with 2.5 μg/ml RANKL significantly increased the number of invaded cells when compared to the PBS group ($P<0.01$). This was particularly true in the PC3 cells. Furthermore, the RANKL effect was suppressed by the decoy receptor OPG with a maximum effect observed at 10 μg/ml. In contrast, this phenomenon was not detected in the normal prostate epithelial RWPE2 cells (Fig. 4).

Silencing of RANK suppresses RANKL-induced PCa cell invasion. RANK plays important roles in tumor invasion and metastasis. To evaluate the effect of direct knockdown of RANK expression on RANKL-induced PCa cell invasion, we examined cell invasion after transfection of RANK siRNA in DU-145, PC-3 and LnCaP cells. We found that siRNA2 caused an 80% decrease in RANK protein expression as compared to siRNA1 and siRNA3 (Fig. 5). Consistently, RANK-siRNA-2 decreased cell invasion induced by RANKL by 60, 34 and 52%, respectively, in the PC-3, DU-145 and LNCaP cells (Fig. 5B).

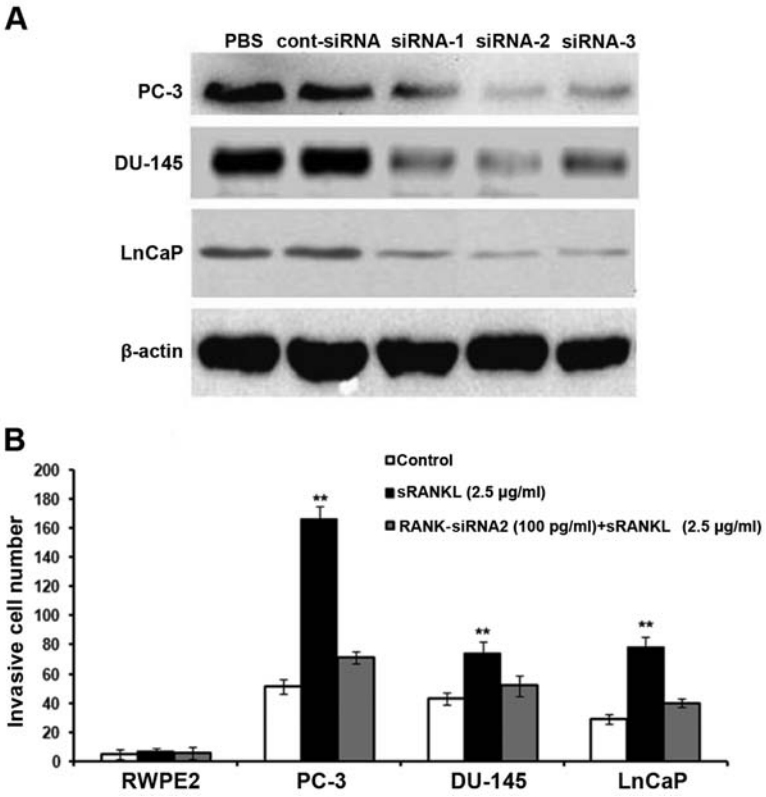

Figure 5. RANK siRNA transfection decreases its mRNA expression in PCa cells and reduces the stimulatory effect on PC-3 invasion by RANKL. (A) RANK mRNA expression in cells transfected with control or RANK siRNAs (siRNA1, siRNA2 and siRNA3). (B) Invasion of cells transfected with control, sRANKL, or RANK siRNA2 and sRANKL. The images are representative of three independent experiments. $^{*}P<0.05$. RANK, receptor activator of NF-κB; PCa, prostate cancer; RANKL, RANK ligand.

Discussion

The RANKL/RANK/OPG axis plays a critical role in promoting osteoclast differentiation and activation, leading to bone resorption (11,12). More and more studies indicate that the RANKL/RANK/OPG signaling network is also involved in tumorigenesis and tissue-specific metastasis in numerous cancer types (13). Our results with clinical samples showed that higher tissue levels of sRANKL and OPG were significantly correlated with more aggressiveness and the increased metastasis of PCa to bone. However, it remains to be determined whether and how RANKL/RANK/OPG regulates PCa invasion to the bone marrow.

Our data are the first to show a generally high level of RANK/RANKL/OPG expression in metastatic prostate tumor tissues. These results suggest that high RANK/RANKL/OPG levels may facilitate PCa metastasis, an idea consistent with what was reported in previous studies, that signaling through the RANK/RANKL/OPG axis is related to bone metastases of solid tumors (14-16). Notably, we demonstrated that the levels of OPG in the serum of PCa patients were associated with the progression of the disease. Moreover, the serum and tissue OPG levels were positively correlated. These data suggest that OPG may be produced by PCa cells, which is then released to the serum. Consistent with our data, it was demonstrated previously that OPG, by binding to RANKL, inhibits osteoclastogenesis necessary for PCa cells to settle in the bone environment and thus the development of bone metastasis (13,17,18).

As a critical osteoclast differentiation factor, RANKL can trigger cytoskeletal changes and promote the invasion of several types of human epithelial tumor cells that express RANK. Our data showed that RANKL bound to RANK-positive PCa cells enhanced cell migration *in vitro*. To determine whether sRANKL plays a critical role in regulating human PCa cell invasion via RANK, we reduced RANK expression by siRNA and tested its effect in *in vitro* invasion assays. We found that RANK siRNA blocked the RANKL-induced PCa cell invasion, either completely or partly dependent on the cell type. Since the direct downregulation of RANK by siRNA was effective in reducing the effect of sRANKL on cell invasion, the effect of sRANKL was mediated through RANK.

Recent findings suggest that OPG and soluble RANK-Fc block RANKL activity. Research suggests that the inhibition of tumor cell proliferation by OPG may be mediated through the bone marrow microenvironment rather than a direct effect on cancer cells (18). The present data showed that the inhibition of RANK/RANKL signaling by OPG significantly reduced the invasiveness of PCa cells, whereas OPG expression was positively correlated with PCa bone metastases. We suggest that the high levels of OPG in serum and tissue may represent a mechanism used by the patient's body to contain tumor invasion.

Overall, our results demonstrate that high levels of RANK/RANKL/OPG expression can help to identify PCa patients at high risk of developing bone metastasis. Since RANK is expressed in PCa cells and OPG can effectively reduce cell invasion via the blockage of RANKL activity, the inhibition of RANK/RANKL actions by OPG may offer a promising

therapeutic strategy to suppress PCa metastasis and progression to bones. Further studies are needed to define the exact mechanisms underlying the promotion of PCa invasion by the RANK/RANKL/OPG axis and its crosstalk with other signaling pathways.

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