Original paper

Crosstalk between OPG/RANKL/RANK in bone marrow mesenchymal stem cells and Wnt/β-catenin pathway in prostate cancer cells regulates bone metastasis of prostate cancer

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Prostate cancer (Pca) has an exquisite tropism for the bone, and PCa with bone metastasis has a poor prognosis. The interaction between Pca cells and bone microenvironment is an important factor affecting Pca bone metastasis.

This study aimed to investigate whether the crosstalk between the osteoprotegerin (OPG)/receptor activator of nuclear factor-kB (RANK)/receptor activator of nuclear factor-kB ligand (RANKL) in bone marrow mesenchymal stem cells (BMSCs) and Wnt/β-catenin pathways in Pca cells regulates bone metastasis of PCa.

Our study showed that there was increased OPG/RANKL/RANK and β-catenin expression in the tissue of PCa and its bone metastasis. This study further showed that RANKL knockdown in BMSCs or β-catenin knockdown in PC-3s blocked the proliferation and migration of BMSCs and the proliferation, migration, and invasion of PC-3s in vitro. Conversely, RANKL overexpression in BMSCs and β-catenin overexpression in PC-3s promoted the proliferation and migration of BMSCs and the proliferation, migration, and invasion of PC-3s in vitro. These data indicate that the RANKL pathway in BMSCs promoted the PC-3s invasion and the catenin pathway in PC-3s activated BMSCs with expression of cancer-associated fibroblast markers, which promoted the bone metastasis.

This suggests that the interaction and crosstalk between BMSCs in bone microenvironment and PCa play a critical role in the exquisite tropism for Pca bone metastasis. Cancer therapies classically target tumour cells; however, based on this study, targeting BMSCs in bone microenvironment is a reasonable option for PCa therapy strategy.

Key words: OPG/RANKL/RANK, bone marrow mesenchymal stem cell, Wnt/β-catenin, prostate cancer, bone metastasis.

Introduction

Prostate cancer (PCa) is the most frequently diagnosed cancer in men worldwide and the fifth leading cause of cancer death in men. The mortality rate in the non-metastatic stage of PCa is low, but metastatic PCa has a poor prognosis (5-year survival rate of approximately 30%) [1, 2]. Prostate cancer cells have an exquisite tropism for the bone, and approximately 90% of all prostate metastases are bone me-

tastases [3], mainly causing morbidity and mortality in patients with PCa [4, 5]. One of the important factors affecting the bone metastasis of PCa is the interaction between cancer cells and the bone microenvironment.

The bone possesses a unique and conducive microenvironment for prostate metastatic cancer cells [6], facilitating a vicious circle based on the crosstalk between cancer cells and the bone microenvironment. The osteoprotegerin (OPG)/receptor activator of nuclear factor-kB (RANK)/receptor activator of nuclear factor-kB ligand (RANKL) system includes OPG, RANK, and RANKL [7]. In cancer bone metastasis, malignant cells reduced bone formation via overactivated RANKL–RANK signalling and further destroyed bone homeostasis. Conversely, OPG suppresses the RANKL binding to RANK and restores bone homeostasis [8]. Due to this effector of regulation, RANKL activated osteoclast-mediated bone resorption, which consequently released matrix growth factors, promoting the bone metastasis of the tumour cells. Subsequently, inhibition of RANKL signalling could ameliorate bone metastasis [8]. Bone marrow mesenchymal stem cells (BMSCs) are pluripotent stem cells in the bone that can differentiate into osteocytes, chondrocytes, and adipocytes [9] and promote the growth and metastasis of PCa [10, 11]. Studies indicate that interaction between BMSCs and PCa in the bone microenvironment promotes the chemotactic motility of PCa cells [10]. Additionally, Wnt/β-catenin signalling pathway activation promotes PCa growth and bone metastasis [12], and a cross talk occurs between OPG/RANKL/RANK signal and Wnt/β-catenin pathway in PCa [13, 14]. This study aimed to investigate whether the cross talk between OPG/RANKL/RANK in BMSCs and Wnt/β-catenin pathways in PCa cells regulate the bone metastasis of PCa.

Material and methods

Subject and tissue collection

We collected PCa and metastatic bone tissues of 20 patients with PCa and bone metastasis during surgery in the Mindong Hospital Affiliated with Fujian Medical University from January 2020 to December 2022. The digested adjacent tissues without PCa served as the control samples (para-prostate cancer as control – PaPC). All PCa cases were diagnosed by the pathologic examination. A part of each sample was snap frozen with liquid nitrogen and stored at –80°C for Western blotting, and the other part was fixed with 4% paraformaldehyde (PFA) for immunostaining. The study protocol (No. NMEE [2020]0309-4) was reviewed and approved by the institutional review board of Mindong Hospital Affiliated with Fujian Medical University and carried out in accordance with the Helsinki Declaration. All the participants were informed, and written informed consent was obtained.

Western blotting

Tissue specimens or treated cells were lysed in RIPA buffer (Beyotime, China), and 20 μg of protein from each sample was loaded and separated on SDS-PAGE. The gel was then transferred to a PVDF membrane (Millipore, USA), which was incubated with indicated primary antibodies and then with a secondary antibody. Protein bands were visualised using ECL substrates (Millipore, USA), and quantification was performed with ImageJ software. The antibodies were as follows: vimentin polyclonal antibody (10366-1-AP, Proteintech, China); anti-α smooth muscle actin (α-SMA) antibody (ab124964, Abcam); OPG antibody (40938, active motif); RANKL monoclonal antibody (66610-1-Ig, Proteintech); RANK antibody (40917, Active motif); Mouse anti-β-actin (1 : 5000, Cat: 3700, CST, USA); Goat Anti-Mouse IgG H&L-HRP (1 : 5000, Cat: ab6789, Abcam, USA); and Goat Anti-Rabbit IgG H&L-HRP (1:5000, Cat: ab6721, Abcam, USA) for one hour at RT.

Immunochemistry

Tissues were fixed with 4% PFA for 24 hours at room temperature, and the tissue blocks were then embedded in paraffin. The paraffined samples were cut into 8-micron-thick sections with a microtome, affixed onto slides, and incubated with rabbit anti-Ki-67 antibody (1 : 250; 27309-1-AP; Proteintech), Rabbit anti-PSA antibody (1 : 200; 10679-1-AP; Proteintech), and mouse anti-ganglioside disialoganglioside (GD2) (1: 250; ab68456, Abcam) for 24 hours at 4°C. After incubation with a second antibody, immunoreactivity was visualised using an avidin-biotin complex peroxidase/3,3′-diaminobenzidine (DAB) reaction with DAB horseradish peroxidase colour development kit (P0202, Beyotime, China). The density of each immunostaining was quantified with ImageJ software.

Cloning of shRNA and overexpression vectors

A shRNA targeting human RANKL and β-catenin was designed and inserted in the lentivirus vector pLVX-shRNA2-Puro (Zolgene Company, China). The sequences (5′ to 3′) were as follows:

Sh-RANKL: 5′-CACCGCACCATTGCTCAGGA TTATGCGAACATAATCCTGA GCAATGGTGC. sh-β-Catenin: 5′-CACCGCACCATTGCTCAGGAT TAT GCGAACATAATCCTGAGCAATGGTGC. The full-length sequence of RANKL and β-catenin was custom synthesised and inserted between the

BamHI and XhoI sites of pCDNA3.1 (Thermofisher, USA).

Culture and cell transfection

The human BMSC cell line (Cat: CP-H166) and human PCa cell line PC-3s (Cat: CL-0185) were purchased from Procell Company in China. All cell lines were validated by STR profiling and tested negative for mycoplasma. The cells were cultured in a DMEM/F12 medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% foetal bovine serum (PAN biotech, Germany) and 1% penicillin–streptomycin solution (Solarbio, China) at 37°C in a humidified 5% CO_2 atmosphere. Cell transfection with ShRNA and overexpression plasmids was performed using Lipofectamine 3000 reagent (Life Technologies, USA). The interaction of BMSCS and PC-3s cells was investigated in a cell line cultured in the Transwell insert, and another cell line was cultured in wells sharing the common media.

Wound healing assay

A sterile pipette tip (200 μl) was used for making a wound. Wound closing was measured at 0 and 24 hours post-wounding with a microscope, and the wound healing rate was determined by the ratio of healing area to total wound area.

Transwell invasion assay

For the Transwell invasion assay, 15,000 cells were seeded and allowed to adhere in Transwell inserts (Fisher Scientific, USA) with Corning Matrigel matrix for 24 hours. The cells inside the Transwell membrane were ripped off, and the cells on the lower surface were fixed with 4% PFA, stained with 0.1% crystal violet, and imaged and counted with ImageJ software from 5 randomly selected microscopic fields.

Enzyme-linked immunosorbent assay

The treated BMSCs were lysed and processed for the detection of the protein expression levels of IL-6, IL-8, IFG-1, and fibroblast growth factor (FGF)-2 with enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturer's instructions (Elabscience, China)

Statistical analysis

All experiments were performed 3 times, and data were presented as mean ± SD. Statistical analysis was performed with GraphPad Prism version 7.0 (GraphPad Software, USA). Multiple groups or 2 groups were analysed using Student's *t*-test or one-way ANOVA with Tukey's post hoc test, respectively. A 2-sided $p < 0.05$ was considered statistically significant.

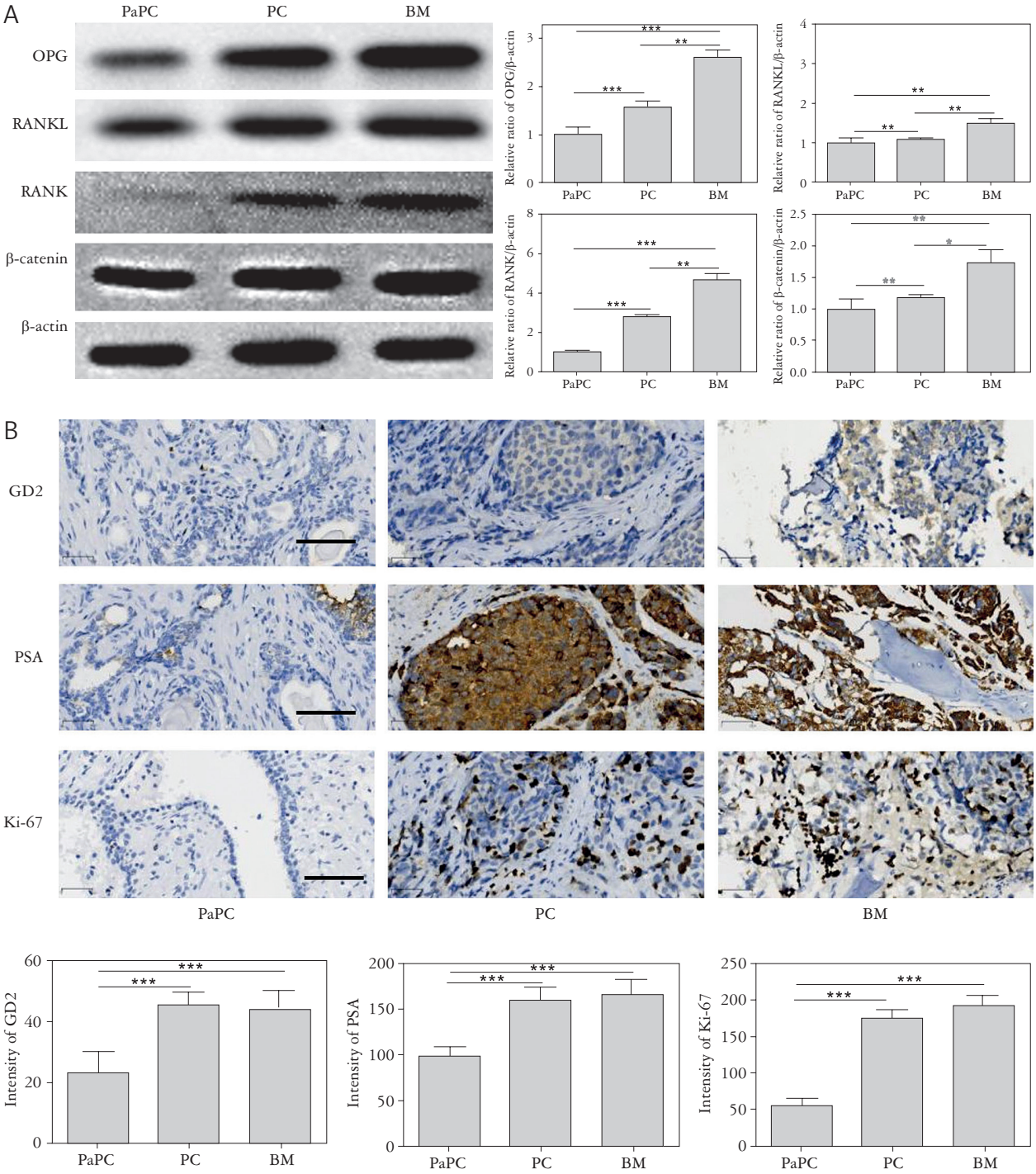
Results

Increased OPG/RANKL/RANK and β-catenin expression in prostate cancer and its bone metastasis

The protein level of OPG/RANKL/RANK and β-catenin increased in the PCa and bone metastasis (BM) groups compared with the PaPC group and showed that both OPG and RANKL were increased (Fig. 1A). Immunohistochemistry demonstrated increased GD2, prostate-specific antigen (PSA), and antigen Kiel 67 (Ki-67) staining in PC and BM compared with PaCa. GD2, PSA, and Ki-67 were markers for PCa (Fig. 1B).

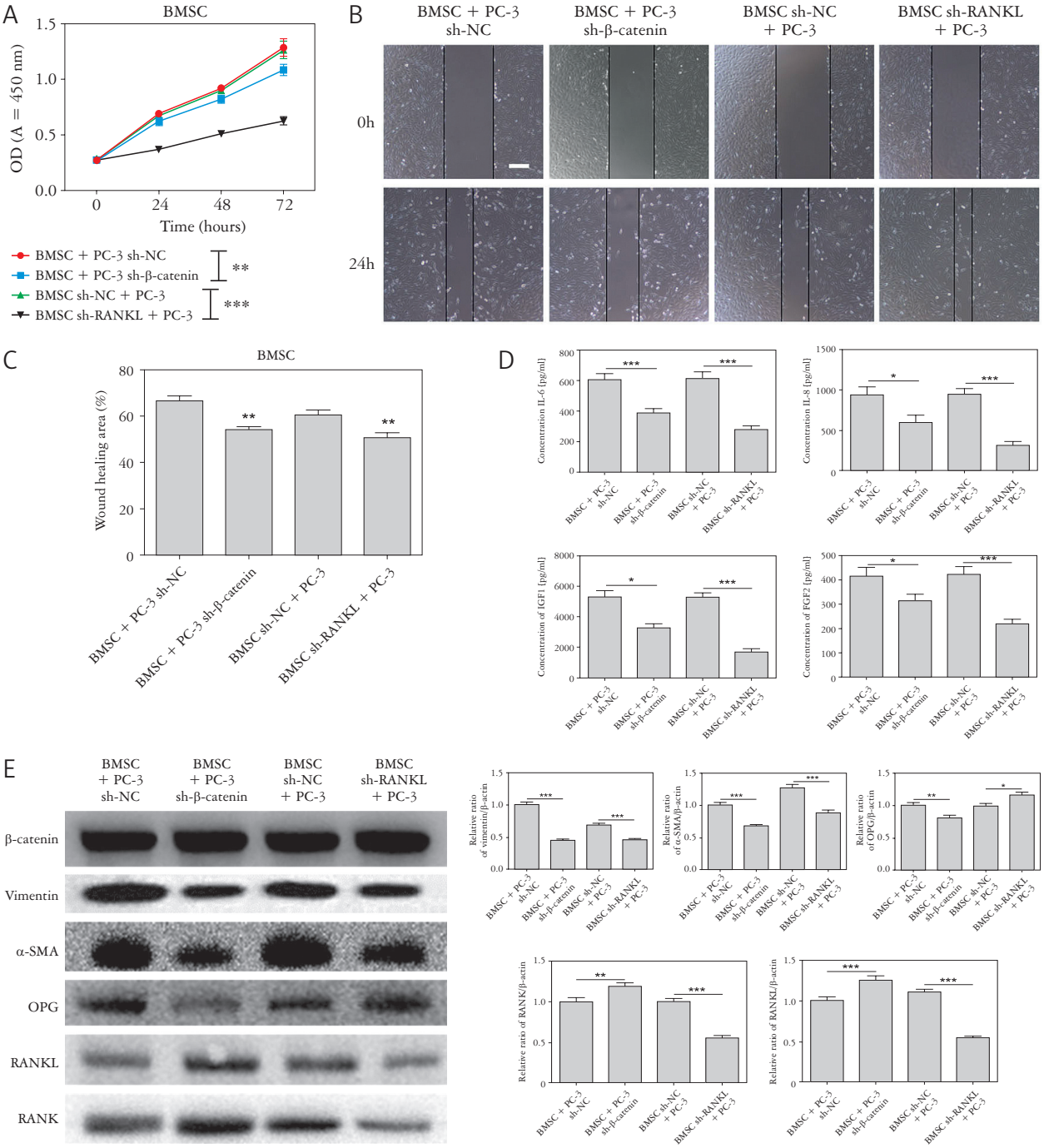
RANKL knockdown in bone marrow mesenchymal stem cells or β-catenin knockdown in PC-3s blocked the proliferation and migration of bone marrow mesenchymal stem cells

To investigate the effect of the catenin pathway in PC-3s on the RANKL system in BMSC, we transfected PC-3s with sh-NC or sh-β-catenin in a Transwell insert. Bone marrow mesenchymal stem cells were transfected with sh-NC or sh-RANKL in wells where they had no direct contact but shared the common media. The CCK8 assay showed that the RANKL knockdown in BMSCs and β-catenin knockdown in PC-3s blocked the proliferation of BMSCs (Fig. 2A). The results of wound healing assay showed that RANKL knockdown in BMSCs blocked the wound healing of BMSCs and β-catenin knockdown in PC-3s blocked the migration of BMSCs (Figs. 2B, C). The study showed that the PC-3s educated BMSC secreted IL-6, IL-8, IFG-1, and FGF-2; modified PC-3s functional pro-inflammatory cytokines; and modified its activity. The results of ELISA assay showed that the RANKL knockdown in BMSCs and β-catenin knockdown in PC-3s reduced the expression levels of IL-6, IL-8, IFG-1, and FGF-2 in the BMSCs (Fig. 2D). Bone marrow mesenchymal stem cells are cancer-associated fibroblast (CAF) precursors that can promote tumour proliferation and migration, and vimentin and α-SMA are CAF markers [15, 16]. RANKL knockdown in BMSCs reduced the protein levels of vimentin, α-SMA, RANKL, and RANK but increased the OPG level (Fig. 2E). The β-catenin knockdown in PC-3s decreased the expression levels of vimentin, α-SMA, and OPG while increasing the RANKL and RANK levels in the BMSCs (Fig. 2E). These findings indicated that RANKL knockdown in BMSCs and β-catenin knockdown in PC-3s blocked the proliferation and migration of BMSCs as the markers of CAF increased.



BM – bone metastasis of prostate cancer, GD2 – disialoganglioside, Ki-67 – antigen Kiel 67, OPG – osteoprotegerin, PaPC – para-prostate cancer as control, PC – prostate cancer, PSA – prostate-specific antigen, RANK – receptor activator of nuclear factor-kB, RANKL – receptor activator of nuclear factor-kB ligand The intensity of each immunostaining was quantified with ImageJ, n = 6

Fig. 1. Increased OPG/RANKL/RANK and β-catenin expression in prostate cancer (PC) and its bone metastasis. A) The tissues of para-prostate cancer as control (PaPC), PC, and bone metastasis of prostate cancer (BM) from patients were processed by Western blotting with osteoprotegerin, receptor activator of nuclear factor-kB, receptor activator of nuclear factor-kB, and β-catenin antibody with quantification, $n = 3$. B) The patient sample of PaPC, PC, and BM were processed by immunohistochemistry with disialoganglioside, prostate-specific antigen, and Ki-67 with quantification (scale bar = 150 μm)

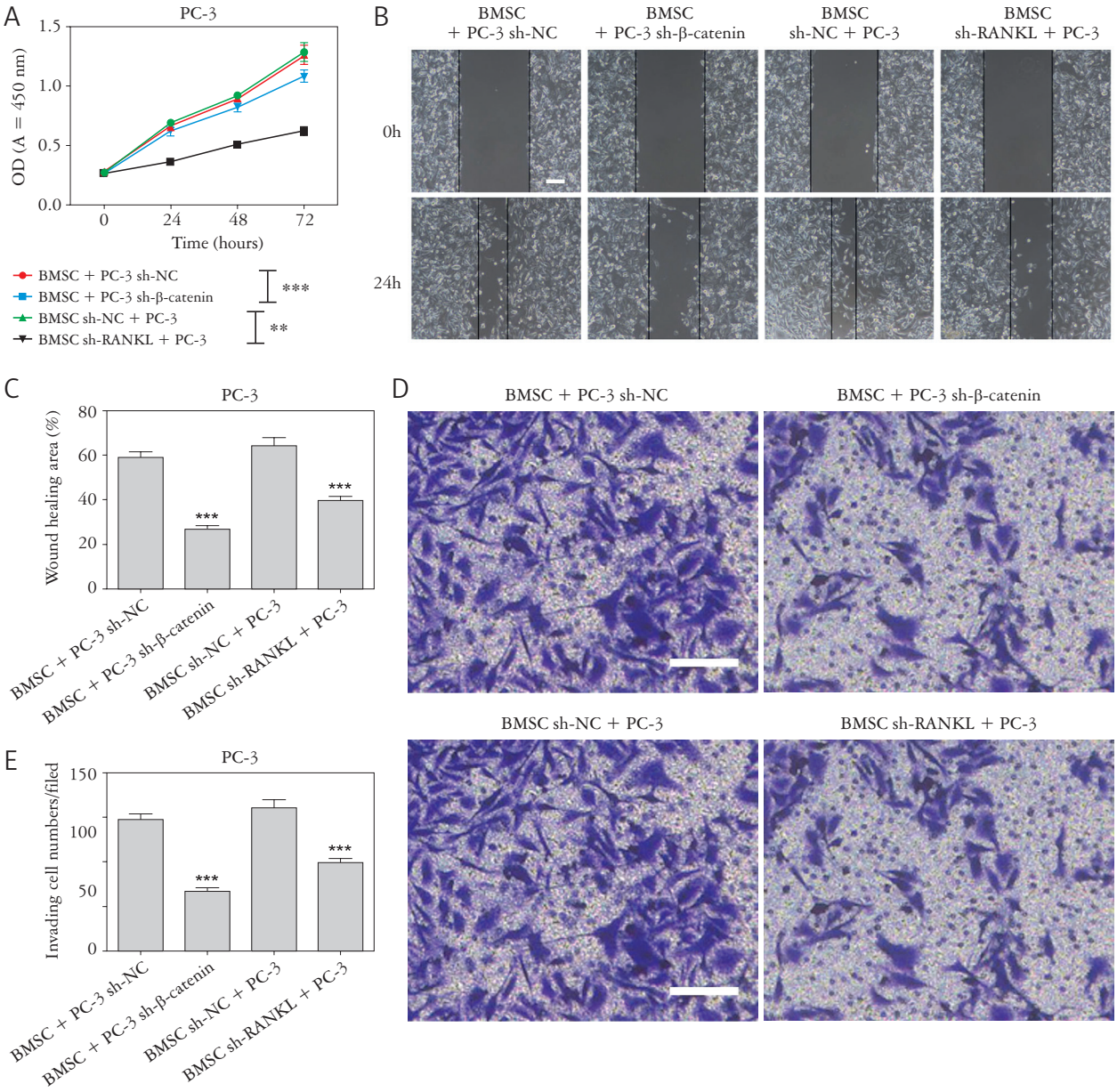


BMSCs – bone marrow mesenchymal stem cells, OPG – osteoprotegerin, PC – prostate cancer, RANK – receptor activator of nuclear factor-kB, RANKL – receptor activator of nuclear factor-kB ligand, α-SMA – smooth muscle actin

Fig. 2. Receptor activator of nuclear factor-kB ligand (RANKL) knockdown in bone marrow mesenchymal stem cells (BMSCs) or β-catenin knockdown in prostate cancer (PC)-3s blocked the proliferation and migration of BMSCs. PC-3s cells were cultured and transfected with sh-NC or sh-β-catenin in the Transwell insert, and BMSCs were cultured and transfected with sh-NC or sh-RANKL in wells for 24 hours, and the BMSCs were collected. A) CCK8 assay to detect the effect of RANKL knockdown in BMSCs and β-catenin knockdown in PC-3s on the proliferation of BMSCs. B) Wound healing assay to detect the effect of RANKL knockdown in BMSCs and β-catenin knockdown in PC-3s on the migration of BMSCs ($n = 3$, scale bar = 200 μm). C) Quantification of wound healing assay in B. D) ELISA assay for detecting the IL-6, IL-8, IFG-1, and FGF-2 expression in BMSCs affected by RANKL knockdown in BMSCs and β-catenin knockdown in PC-3s. E) Western blotting to detect the protein expression of vimentin, α-SMA, osteoprotegerin, RANKL, and receptor activator of nuclear factor-kB in BMSCs affected by RANKL knockdown in BMSCs and β-catenin knockdown in PC-3s with quantification ($n = 3$)

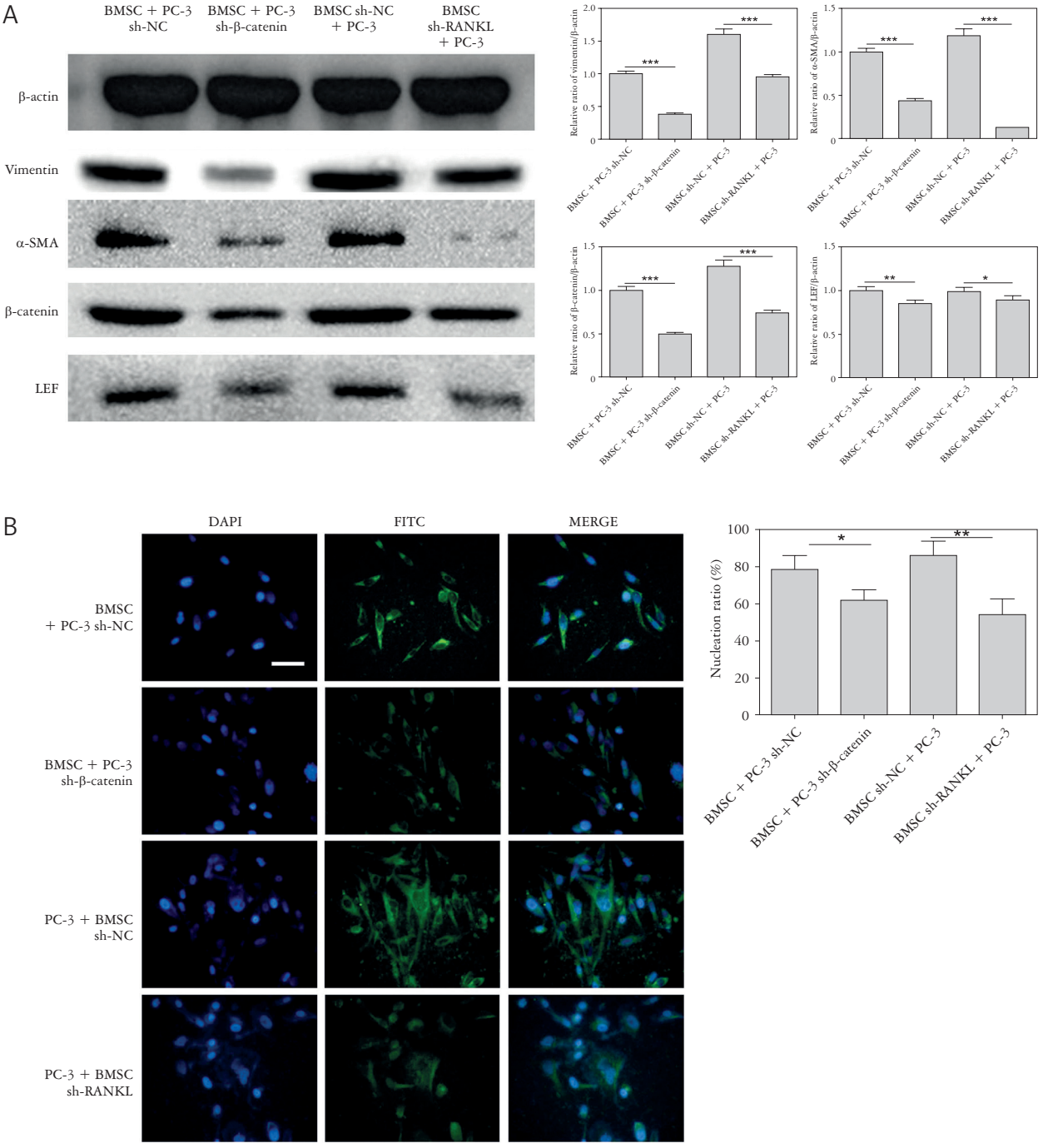
RANKL knockdown in bone marrow mesenchymal stem cells or β-catenin knockdown in PC-3s blocked the proliferation, migration, and invasion of PC-3

Bone marrow mesenchymal stem cells with sh-NC or sh-RANKL were cultured in Transwell insert wells, and PC-3s cells were transfected with sh-NC or sh-β-catenin in wells. The CCK8 assay results showed that the RANKL knockdown in BMSCs and catenin knockdown in PC-3s blocked the proliferation of PC-3s cells (Fig. 3A). The wound healing assay also showed that both the RANKL knockdown in BMSCs and catenin knockdown in PC-3s blocked the migration of PC-3s (Figs. 3B, C). Transwell invasion assay



BMSCs – bone marrow mesenchymal stem cells, PC – prostate cancer, RANKL – receptor activator of nuclear factor-kB ligand

Fig. 3. Receptor activator of nuclear factor-kB ligand (RANKL) knockdown in bone marrow mesenchymal stem cells (BMSCs) or β-catenin knockdown in prostate cancer (PC)-3s blocked the proliferation, migration, and invasion of PC-3s. PC-3s cells were cultured and transfected with sh-NC or sh-β-catenin in the Transwell insert, and BMSCs were cultured and transfected with sh-NC or sh-RANKL in wells for 24 hours, and PC-3s were collected. A) Processed for CCK8 assay detects the effect of RANKL knockdown in BMSCs and β-catenin knockdown in PC-3s on the proliferation of PC-3s. B) Wound healing assay to detect the effect of RANKL knockdown in BMSCs and β-catenin knockdown in PC-3s on the migration of PC-3s (scale bar = 200 μm). C) Quantification of wound healing assay in B (n = 3). D) Transwell invasion assay to detect the effect of RANKL knockdown in BMSCs or catenin knockdown in PC-3s on the invasion of PC-3s(scale bar = 50 μm). E) Quantification of Transwell invasion assay in D (n = 3)



LEF –, α-SMA – anti-α smooth muscle actin
Quantification presented n = 3

Fig. 4. Receptor activator of nuclear factor-kB ligand (RANKL) knockdown in bone marrow mesenchymal stem cells (BMSCs) or β-catenin knockdown in prostate cancer (PC)-3s blocked the fibrosis protein and β-catenin activity of PC-3. PC-3s cells were cultured and transfected with sh-NC or sh-β-catenin in the Transwell insert and BMSCs were cultured and transfected with sh-NC or sh-RANKL in wells for 24 hours and PC-3s were collected. A) Western blotting with quantification to detect the protein expression of vimentin, α-SMA, β-catenin, and LEF in PC-3s affected by RANKL knockdown in BMSCs and β-catenin knockdown in PC-3s ($n = 3$). B) Immunofluorescence staining with anti-β-catenin antibody and DAPI staining on PC-3s to detect the nucleus translocation of β-catenin affected by RANKL knockdown in BMSCs and β-catenin knockdown in PC-3s (scale bar = 50 μm)

indicated that the RANKL knockdown in the BMSCs or catenin knockdown in PC-3s blocked the invasion of PC-3s (Figs. 3D, E). RANKL knockdown in BMSCs reduced the protein levels of vimentin, α-SMA, catenin, and LEF in PC-3s (Fig. 4A). The β-catenin knockdown in PC-3s decreased the expression levels of vimentin, α-SMA, catenin, and LEF in PC-3s (Fig. 4A). These results indicated that RANKL knockdown in BMSCs and β-catenin knockdown in PC-3s blocked the proliferation, migration, and invasion of PC-3s with reduced CAF markers and catenin. The results further showed that RANKL knockdown in the BMSCs or catenin knockdown in PC-3s blocked catenin translocation to the nucleus, a maker of catenin activity in PC-3s.

RANKL overexpression in bone marrow mesenchymal stem cells and β-catenin expression in PC-3s promoted the proliferation and migration of bone marrow mesenchymal stem cells

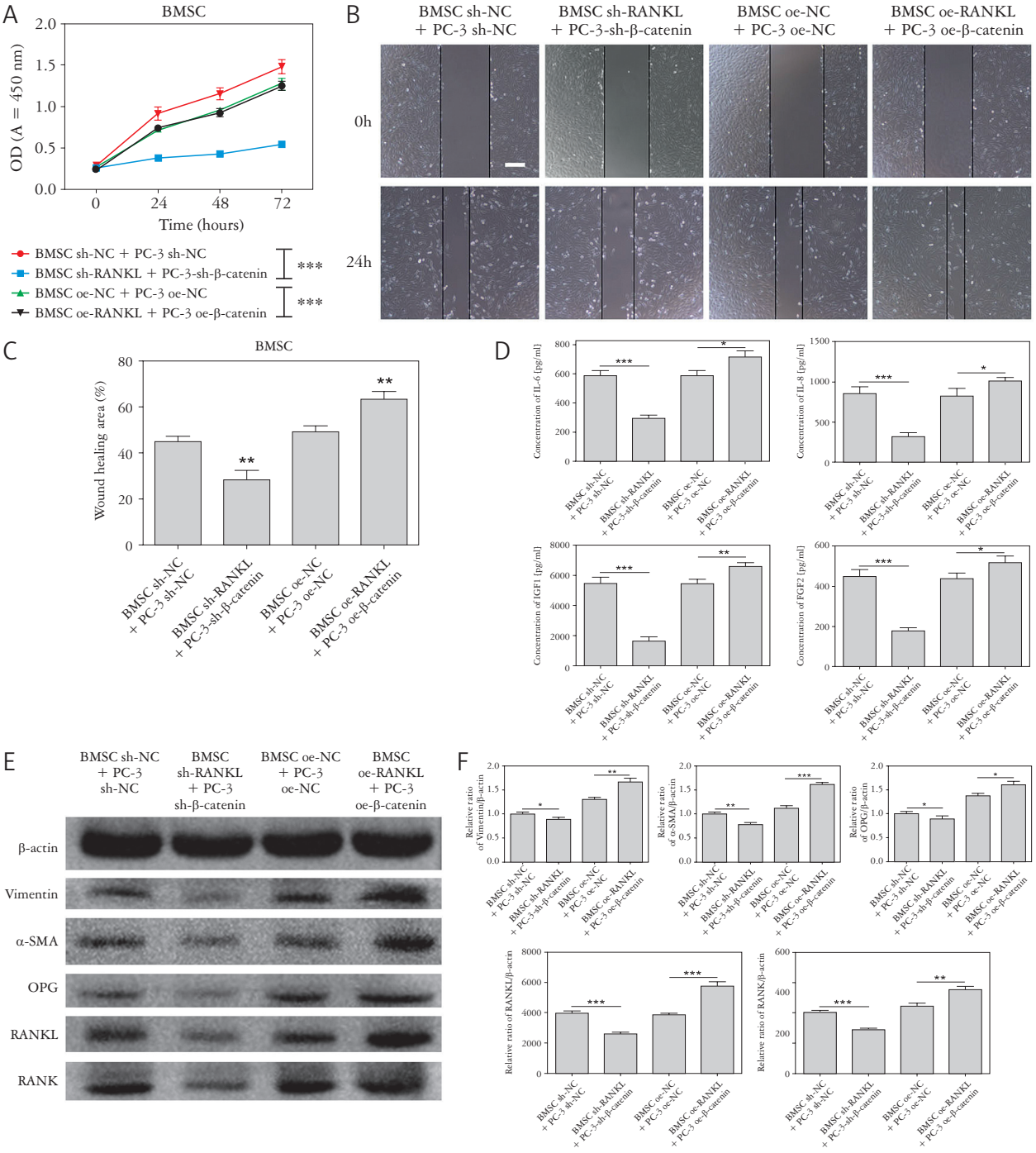
The CCK8 assay showed that the RANKL knockdown in BMSCs and β-catenin knockdown in PC-3s blocked the proliferation of BMSCs, and RANKL overexpression in BMSCs and β-catenin overexpression in PC-3s promoted the proliferation of BMSCs (Fig. 5A). The wound healing assay showed that the RANKL knockdown in BMSCs and β-catenin knockdown in PC-3s blocked the migration of BMSCs, and RANKL overexpression in BMSCs and β-catenin overexpression in PC-3s promoted the migration of BMSCs (Figs. 5B, C). ELISA assay indicated that the RANKL knockdown in BMSCs and β-catenin knockdown in PC-3s reduced the expression of IL-6, IL-8, IFG-1, and FGF-2 of BMSCs (Fig. 5D) while RANKL overexpression in BMSCs and β-catenin overexpression in PC-3s increased the expression levels of IL-6, IL-8, IFG-1, and FGF-2 in the BMSCs. Western blotting indicated that RANKL knockdown in BMSCs and β-catenin knockdown in PC-3s reduced the protein levels of vimentin, α-SMA, OPG, RANKL, and RANK in the BMSCs (Fig. 5E). The RANKL overexpression in BMSCs and β-catenin overexpression in PC-3s increased the protein levels of vimentin, α-SMA, OPG, RANKL, and RANK in the BMSCs (Fig. 5E). These data indicated that RANKL overexpression in the BMSCs and β-catenin overexpression in PC-3s promoted the proliferation and migration of BMSCs with increased CAF markers.

RANKL overexpression in bone marrow mesenchymal stem cells and catenin overexpression in PC-3s promoted the proliferation, migration, and invasion of PC-3s

The CCK8 assay showed that RANKL knockdown in BMSCs and β-catenin knockdown in PC-3s blocked the proliferation of BMSCs while RANKL overexpression in BMSCs and β-catenin overexpression in PC-3s promoted the proliferation of PC-3s (Fig. 6A). Wound healing assay showed that the RANKL knockdown in the BMSCs and β-catenin knockdown in PC-3s blocked the migration of PC-3s while the RANKL overexpression in BMSCs and β-catenin overexpression in PC-3s promoted the migration of PC-3s (Figs. 6B, C). Transwell invasion assay indicated that the RANKL knockdown in BMSCs and β-catenin knockdown in PC-3s blocked the invasion of PC-3s while RANKL overexpression in BMSCs and β-catenin overexpression in PC-3s promoted the invasion of PC-3s (Figs. 6D, E). Western blotting showed that RANKL knockdown in BMSCs and β-catenin knockdown in PC-3s reduced the protein level of vimentin, α-SMA, catenin, and LEF in PC-3s (Fig. 7A). RANKL overexpression in BMSCs and β-catenin overexpression in PC-3s increased the protein level of vimentin, α-SMA, catenin, and LEF in PC-3s (Fig. 7A). Catenin translocation assay showed that RANKL knockdown in BMSCs or catenin knockdown in PC-3s blocked catenin translocation to the nucleus in PC-3s (Fig. 7B) while RANKL overexpression in BMSCs and β-catenin overexpression in PC-3s increased catenin translocation to the nucleus in PC-3s (Fig. 7B). These data indicated that RANKL overexpression in BMSCs and β-catenin overexpression in PC-3s promoted the proliferation, migration, and invasion of PC-3s with increased CAF markers and protein level and activity of catenin.

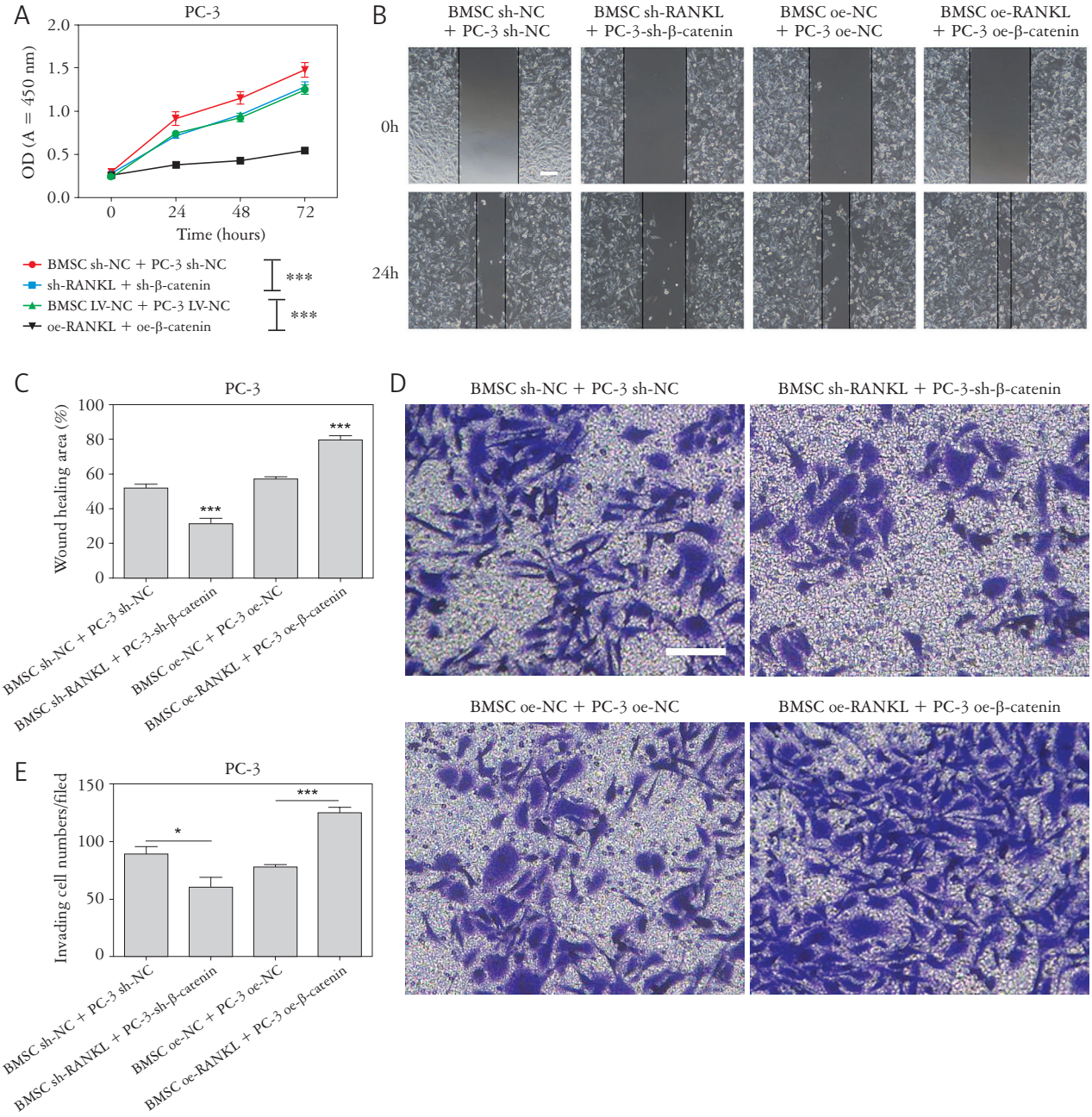
Discussion

This study showed that OPG/RANKL/RANK and β-catenin expression levels increased in PCa and metastatic bone tissues from patients with PCa. Both RANKL and OPG increased in PCa and bone, metastatic PCa. In cancer bone metastasis, RANKL–RANK signalling destroyed bone homeostasis, and OPG suppressed the RANKL binding to RANK. Our study showed that the OPG/RANKL/RANK system and the ratio of RANKL/OPG were increased in PCa and bone metastatic PCa and indicated that RANKL played more prominent role than that of OPG. RANKL knockdown in BMSCs or β-catenin knockdown in PC-3s blocked the proliferation and migration of BMSCs and the proliferation, migration, and invasion of PC-3s *in vitro*. Overexpression of RANKL in BMSCs and overexpression of β-catenin in PC-3s promoted the proliferation and migration of BMSCs and the proliferation, migration, and invasion of PC-3s *in vitro*. These results indicated that the RANKL pathway in BMSCs promoted PC-3s invasion, and the catenin pathway in PC-3s activated BMSCs with increased expression of fibroblast markers, which promoted bone metastasis of PCa. This



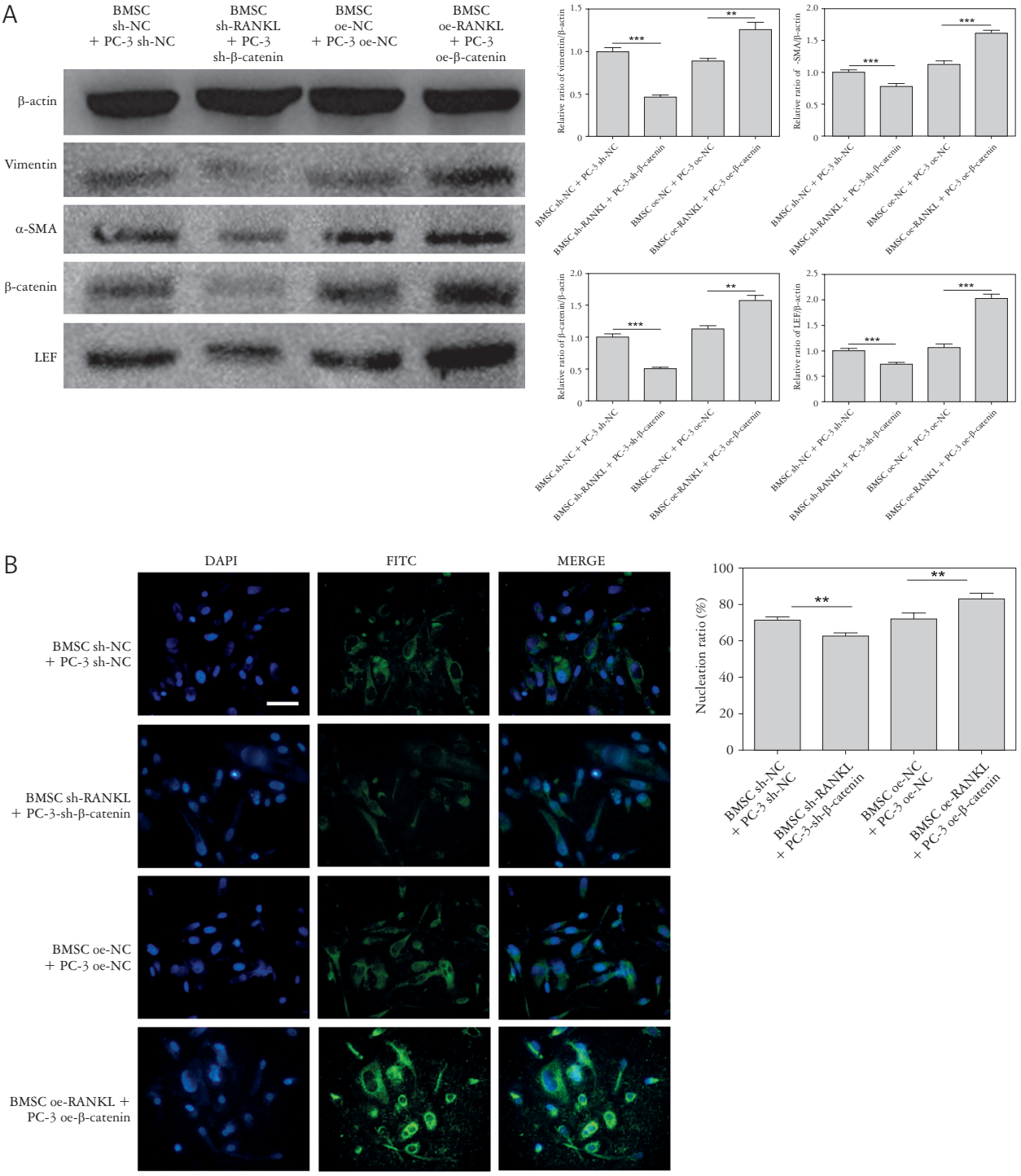
BMSCs – bone marrow mesenchymal stem cells, oe-NC – overexpression of control, oe-RANKL overexpression of receptor activator of nuclear factor-kB ligand, OPG – osteoprotegerin, PC – prostate cancer, RANK – receptor activator of nuclear factor-kB, RANKL – receptor activator of nuclear factor-kB ligand, α-SMA – smooth muscle actin

Fig. 5. Receptor activator of nuclear factor-kB ligand (RANKL) overexpression in bone marrow mesenchymal stem cells (BMSCs) and β-catenin overexpression in prostate cancer (PC)-3s promoted the proliferation and migration of BMSCs. BMSCs were cultured and transfected with sh-NC or sh-RANKL and overexpression of control (oe-NC) or RANKL (oe-RANKL) in the Transwell insert and PC-3s were cultured and transfected with sh-NC or sh-β-catenin and overexpression of control (oe-NC) or β-catenin (β-catenin) in wells for 24 hours, and BMSCs were collected. A) Processed for CCK8 assay to detect the effect of RANKL knockdown or overexpression in BMSCs and β-catenin knockdown or overexpression in PC-3s on the proliferation of BMSCs. B) Wound healing assay to detect the effect of RANKL knockdown or overexpression in BMSCs and β-catenin knockdown or overexpression in PC-3s on the migration of BMSCs (scale bar = 200 μm). C) Quantification of wound healing assay in B (n = 3). D) ELISA assay for detecting IL-6, IL-8, IFG-1, and FGF-2 expression in BMSCs affected by RANKL knockdown or overexpression in BMSCs and β-catenin knockdown or overexpression in PC-3s. E) Western blotting to detect the protein expression of vimentin, a-SMA, OPG, RANKL, and RANK in BMSCS affected by RANKL knockdown or overexpression in BMSCs and β-catenin knockdown or overexpression in PC-3s. F) Quantification of western blotting in (E) with ImageJ software (n = 3)



BMSCs – bone marrow mesenchymal stem cells, oe-NC – overexpression of control, oe-RANKL overexpression of receptor activator of nuclear factor-kB ligand, PC – prostate cancer, RANKL – receptor activator of nuclear factor-kB ligand

Fig. 6. Receptor activator of nuclear factor-kB ligand (RANKL) overexpression in bone marrow mesenchymal stem cells (BMSCs) and β-catenin overexpression in prostate cancer (PC)-3s promoted the proliferation, migration, and invasion of PC-3s. BMSCs were cultured and transfected with sh-NC or sh-RANKL and overexpression of control (oe-NC) or RANKL in the Transwell insert and PC-3s were cultured and transfected with sh-NC or sh-β-catenin and oe-NC or β-catenin in wells for 24 hours, and PC-3s were collected. A) Processed for CCK8 assay to detect the effect of RANKL knockdown or overexpression in BMSCs and β-catenin knockdown or overexpression in PC-3s on the proliferation of PC-3s. B) Wound healing assay to detect the effect of RANKL knockdown or overexpression in BMSCS and β-catenin knockdown) or overexpression in PC-3s on the migration of Pc-3 (scale bar = 200 μm). C) Quantification of wound healing assay in B. D) Transwell invasion assay to detect the effect of RANKL knockdown or overexpression in BMSCS or catenin knockdown or overexpression in PC-3s on the invasion of PC-3s (scale bar = 50 μm). E) Quantification of Transwell invasion assay in D (n = 3)



BM – bone metastasis of prostate cancer, GD2 – disialoganglioside, Ki-67 – antigen Kiel 67, OPG – osteoprotegerin, PaPC – para-prostate cancer as control, PC – prostate cancer, PSA – prostate-specific antigen, RANK – receptor activator of nuclear factor-kB, RANKL – receptor activator of nuclear factor-kB ligand The intensity of each immunostaining was quantified with ImageJ, n = 6

Fig. 7. Receptor activator of nuclear factor-kB ligand (RANKL) overexpression in bone marrow mesenchymal stem cells (BMSCs) and β-catenin overexpression in prostate cancer (PC)-3s promoted the fibrosis protein and β-catenin activity of PC-3. BMSCs were cultured and transfected with sh-NC or sh-RANKL and overexpression of control (oe-NC) or RANKL in the Transwell insert, and PC-3s were cultured and transfected with sh-NC or sh-β-catenin and overexpression of control (oe-NC) or β-catenin (β-catenin) in wells for 24 hours, and PC-3s were collected. A) Processed for Western blotting with quantification to detect the protein expression of vimentin, a-SMA, β-catenin, and LEF in PC-3s affected by RANKL knockdown or overexpression in BMSCs and β-catenin knockdown or overexpression in PC-3s ($n = 3$). B) Immunofluorescence staining with anti-β-catenin antibody and DAPI staining on PC-3s to detect the nucleus translocation of β-catenin affected by RANKL knockdown or overexpression in BMSCs and β-catenin knockdown or overexpression in PC-3s (scale bar = 50 μm)

interaction and cross talk between BMSCs and PCa play a critical role in the exquisite tropism for the bone metastasis of PCa.

The interaction and cross talk between tumour cells and the bone microenvironment are key factors for PCa bone metastasis. Bone marrow mesenchymal stem cells in the bone microenvironment play a role in tumour growth and metastasis of numerous types of cancer, including PCa [17]. Bone marrow mesenchymal stem cells stimulate the proliferation, migration, and invasion of the PCa cell line PC-3s *in vitro* via transforming growth factor β [18]. This study also showed the RANKL knockdown by siRNA in BMSCs can promote the proliferation, migration, and invasion of PC-3 [8]. Our study demonstrated that RANLKL knockdown in BMSCs decreased the β-catenin protein level and activity (nucleus translocation) in PC-3s and reduced the proliferation, migration, and invasion of PC-3s, and RANKL overexpression in BMSCs had the opposite effect on PC-3. Bone marrow mesenchymal stem cells exposed to co-cultured PC-3s were induced to secret the pro-tumourigenic factors IL-6, IL-8, IFG-1, and FGF-2, consistent with other studies indicating that IL-8, OPN, and FGF-2 were increased in BMSCs induced by conditional media from PC-3s [11]. This result indicated that educated BMSCs from metastatic PCa considerably increased the chemoattraction for PCa cells by activating BMSCs that secreted pro-tumourigenic factors. The RANKL-RANK system activated the NF-kB pathway, which promoted the release of pro-tumourigenic factors including inflammatory and angiogenetic growth factors [19]. The RANKL pathway promoted osteoclast differentiation and local osteolysis, which released more growth factors such as insulin-like growth factors and FGFs in the bone matrix [20]. The metastatic bone microenvironment, which is a site of inflammation triggered by tumour, stimulates BMSCs to migrate close to the tumour site and interact with tumour cells to further promote tumour progression and metastasis [20, 21].

In addition, our study showed that the β-catenin knockdown in PC-3s resulted in reduced expression of IL-6, IL-8, IFG-1, and FGF-2 of BMSCs and reduced the protein levels of vimentin, α-SMA, RANKL, and RANK, while increasing the OPG level in the BMSCs. The Wnt/β-catenin signalling pathway in PCa can activate BMSCs in the metastatic microenvironment. The activated BMSCs with increased CAF markers, including vimentin and α-SMA, could promote tumour growth and metastatic progression [21]. The Wnt/β-catenin signalling pathway promotes tumour cell invasion and metastasis by interacting with the tumour microenvironment (TME) [22]. The extracellular matrix, inflammatory factors, and growth factors, which are target genes of the activated Wnt/β-catenin pathway in cancer cells, were indicated to play critical roles in the regulation of the TME [23]. Studies showed that the Wnt pathway in cancer cells regulates the TME through the fine cross talk with infiltrating immune cells and CAFs through co-regulation of TGF-β and Wnt signals in a Smad2/3-Dvl2/3-dependent manner [24]. Under a TME with chronic inflammation, tumour cells were activated by Wnt signalling pathway and released pro-inflammatory cytokines and chemokines, such as IL-6, IL-8, CD105, MCP-1, and MIF. These released cytokines and chemokines stimulated BMSC to become CAFs and further stimulated tumour growth and its invasive capacity [10, 25].

Conclusions

Our study indicated that the RANKL pathway in BMSCs promoted invasion by PC-3s, and the catenin pathway in PC-3s activated BMSCs in the bone microenvironment, which promoted bone metastasis of PCa. This cross talk formed a vicious cycle that promoted bone metastasis of PCa and contributed to the exquisite tropism for the bone metastasis of PCa. Targeting BMSCs in the bone microenvironment is a reasonable option for cancer therapy strategy.

Disclosures

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2. Assistance with the article: None.
3. Financial support and sponsorship: None.
4. Conflicts of interest: None.

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