CCN3 promotes prostate cancer bone metastasis by modulating the tumor–bone microenvironment through RANKL-dependent pathway

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Bone metastasis in patient with advanced-stage prostate cancer, the most commonly diagnosed malignancy in Western countries, increases the risk of intractable bone pain. The nephroblastoma overexpressed (NOV/CCN3) gene, a member of the CCN gene family, is responsible for the secretion of CCN3, a matrix-associated protein involved in many cellular functions. However, the role of CCN3 in prostate cancer metastasis to bone is poorly understood. CCN3 was found to be highly expressed in bone metastasis patients and positively correlated with malignancy in human prostate cancer cells. Prostate cancer conditioned medium-induced osteoclast differentiation was inhibited by neutralizing antibody against CCN3. Specifically, CCN3 was found to induce osteoclastogenesis through the receptor activator of NF-κB ligand (RANKL)-dependent pathway, and the focal adhesion kinase/Akt/p38/NF-κB signal pathway was found to be involved in CCN3-mediated receptor activator of NF-κB expression and RANKL-dependent osteoclastogenesis. In contrast, osteoblasts were observed to play an important role in osteoclast differentiation by paracrine manner, with treatment of osteoblasts with CCN3 found to change the RANKL (osteoclastogenesis):OPG (antiosteoclastogenesis) ratio. Compared with parental PC3 cells, highly invasive PC3-I3 cells markedly enhanced osteoclast activity and bone metastasis in vivo. These results indicate that CCN3 can be used as a novel therapeutic target in the prevention of bone metastasis of prostate cancer.

Introduction
Prostate cancer is the most commonly diagnosed malignancy in the USA and other Western countries (1). Bone metastasis is a common complication associated with advanced prostate cancer, with ~70–80% of prostate cancer patients developing skeletal metastases (2). Prostate cancer metastases are most commonly diagnosed malignancy in the USA and other Western countries (1). Bone metastasis is a common complication associated with advanced prostate cancer, with ~70–80% of prostate cancer patients developing skeletal metastases (2). Prostate cancer metastases are most often characterized as osteoblastic lesions (3), which are high osteoblast activity with bisphosphonates has been found to reduce osteolytic bone resorption and improve disease-free survival (7,8), bisphosphonates (9), and receptor activator of NF-κB ligand (RANKL)-neutralizing antibody have been used in cancer patients and animal models to block tumor development in bone (7–9). However, subsequent identification of CCL2, IL-6 and IL-8 as novel mediators in prostate cancer-induced bone metastasis (12,13) indicates that several soluble factors other than RANKL may mediate tumor-induced osteoclast activity.

Materials and methods

Abbreviations: CM, conditioned medium; FAK, focal adhesion kinase; FBS, fetal bovine serum; M-CSF, Macrophage colony-stimulating factor; PBS, phosphate-buffered saline; qRT-PCR, quantitative real-time PCR; RANKL, receptor activator of NF-κB ligand; siRNA, small interfering RNA; SEM, standard error of the mean; TRAP, tartrate-resistant acid phosphatase.

The human prostate cancer cell lines PC3, DU145 and LNCaP were purchased from American Type Culture Collection (Manassas, VA). Cells were maintained at 37°C in an atmosphere of 5% CO₂ in an RPMI-1640 medium supplemented with 20 mM hydroxyethyl pipеразинеетансульфоновая acid, 10% heat-inactivated fetal bovine serum (FBS), 2mM glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin (Invitrogen, Carlsbad, CA). Murine RAW 264.7 cells, a macrophage-like cell line, were cultured in RPMI-1640 medium supplemented with 10% FBS, 2mM glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin (Invitrogen, Carlsbad, CA).
mouse macrophage cell line, were purchased from American Type Culture Collection and grown in Dulbecco’s modified Eagle’s medium supplemented with 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin. MG63 cells were purchased from American Type Culture Collection and grown in αMEM (catalog no. 001008-3DJ; Invitrogen) containing 10% FBS, 100 units/ml penicillin and 100 μg/ml streptomycin. The media were changed every 48 h.

Rat bone marrow-derived cell cultures
Rat bone marrow cells were prepared by flushing out the bone marrow cavity of the femurs of 6- to 8-week-old Sprague–Dawley rats with Dulbecco’s modified Eagle’s medium supplemented with 20 mM hydroxyethylpiperazine-N,N’-bis(2-ethanesulfonic acid), 10% heat-inactivated FBS, 2 mM glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. Non-adherent (hematopoietic) cells were collected after 24 h and used as osteoclast precursors.

Preparation of conditioned media
PC3, DU145 and LNCaP cells (2 × 10⁶) were grown overnight in 100 mm culture dishes in cell culture medium. After two washes with phosphate-buffered saline (PBS), cells were incubated in 1% FBS in RPMI medium for 48 h before collection of the conditioned medium (CM). To normalize for differences in cell density due to proliferation during the culture period, cells from each plate were collected and total DNA content/plate was determined (spectrophotometric absorbance, 260 nm). The CM was then normalized for DNA content between samples by adding RPMI medium.

Osteoclast differentiation from rat bone marrow cells and RAW 264.7 cells
Osteoclast differentiation assay was performed by culturing either 1 × 10⁶ cells/well of adherent rat bone marrow cells or 1 × 10⁷ cells/well of RAW 264.7 cells in a 24-well plate in Dulbecco’s modified Eagle’s medium supplemented with 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin for 7 days. All cells were incubated with Macrophage colony-stimulating factor (M-CSF) (20 ng/ml) and RANKL (50 ng/ml) and exposed to various treatments consisting of CCN3 (0–100 ng/ml) or 10% CM from prostate cancer cells with or without 1 μg/ml of CCN3-neutralizing antibody. The culture medium was replaced every 3 days, and the osteoclast number was counted after 6 days. Osteoclast differentiation was measured by determining the number of cells that had been positively stained by tartrate-resistant acid phosphatase (TRAP; Acid Phosphatase Kit 387-A; Sigma–Aldrich) according to the manufacturer's instructions. Osteoclasts were identified as TRAP-positive-stained multinuclear (≥3 nuclei) cells using light microscopy and the number of TRAP-positive cells in each well was counted, with positively stained cells that contained three or more nuclei scored as osteoclast-like multinucleated cells (24).

Bone resorption assay
RAW 264.7 cells were cultured in a Corning Osteo Assay Surface 24-well plate (Corning Life Sciences, Lowell, MA). Media and treatments were changed every 3 days during the 12 day culture period, after which the wells were washed with PBS and incubated in 10% sodium hypochlorite for 5 min to remove cells. Digital images were captured and the resorbed area/well was analyzed using ImageJ software. Three wells were assessed per treatment in three different experiments.

Small interfering RNA transfection
ON-TARGETplus siRNA were purchased from Thermo Fisher Scientific (Waltham, MA). Cells were transfected with small interfering RNAs (siRNAs) (0.4 nmol) using RNAiFECT 1 Transfection Reagent (Thermo Fisher Scientific).

Flow cytometric analysis
After differentiated osteoclast cells had been grown in 6-well plates, they were washed with PBS and detached using a cell scraper. Cells were fixed for 10 min in PBS containing 3.7% paraformaldehyde, rinsed in PBS and incubated with rabbit anti-human–RANK or rabbit anti-human–integrin αvβ3 (BD Biosciences, San Jose, CA) at a 1:100 concentration for 1 h at room temperature. Cells were then washed in PBS and incubated with fluorescein isothiocyanate-conjugated goat anti-rabbit secondary immunoglobulin G at a 1:100 concentration (Leinco Technologies, St Louis, MO) for 45 min at room temperature. After a final rinse, cells were analyzed using a FACScalibur flow cytometer and CellQuest software (BD Biosciences).

Western blot analysis
Cellular lysates were prepared and proteins were resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. After proteins had been transferred to Immobilon polyvinylidene fluoride membranes (EMD Millipore Corporation, Billerica, MA), blots were blocked with 4% bovine serum albumin for 1 h at room temperature and probed with rabbit anti-human antibodies against p-FAK, FAK, p-Akt, p-p38, p-IKK, IKK, p-IB2R, IB2R, p-65, p65, RANK, CCN3 or β-actin (Santa Cruz Biotechnology) at a 1:1000 concentration for 1 h at room temperature. After three washes, blots were incubated with peroxidase-conjugated donkey anti-rabbit secondary antibody at a 1:1000 concentration for 1 h at room temperature before being visualized with enhanced chemiluminescence using X-Omat LS film (Eastman Kodak, Rochester, NY).

Enzyme-linked immunosorbent assay
Osteoblast-like MG63 cells (2 × 10⁵) were cultured in 24-well culture plates and incubated in CCN3 (0–100 ng/ml) for 24 h at 37°C. After incubation, the medium was removed and stored at −80°C until assay of RANKL and OPG using enzyme immunoassay kits according to the procedure described by the manufacturer (Biocompare, San Jose, CA).

Quantitative real-time PCR
Total RNA was extracted from prostate cancer cells using a TRIzol kit (MDBio, Taipei, Taiwan) and reverse transcription was performed using 1 μg of total RNA. Quantitative real-time PCR (qRT-PCR) was conducted using a TaqMan One-step PCR Master Mix Kit (Applied Biosystems, Foster City, CA). Total cDNA (100 ng) was added to each 25 μl reaction with sequence-specific primers and TaqMan probes. All target gene primers and probes were purchased commercially, including those for glyceraldehyde 3-phosphate dehydrogenase (Applied Biosystems), which was used as an internal control. qRT-PCR was conducted in triplicate using the StepOnePlus Sequence Detection System (Applied Biosystems).

Selection of invasive cells in Transwell invasion chamber
Subpopulations of PC3 cells were selected according to analysis of differential invasive ability using Transwell invasion assay. After coating the polycarbonate membranes of the Transwell inserts with BD Matrigel (BD Biosciences), cells were resuspended in RPMI containing 1% FBS (Invitrogen) and seeded into the wells in the upper layer, with the lower layer supplied by RPMI containing 10% FBS. Following incubation for 72 h at 37°C, the inserts were removed, and cells that had migrated through the membranes and become attached to the lower-chamber compartments were trypsinized and expanded for second-round selection. After three rounds of selection, the subclone cell line derived from the PC3 cells was designated the PC3-13 cell line.

Intratibial injection of PC3 cells in SCIID mice and measurement of osteolytic lesion area
PC3-13 and PC3 cells that had been cultured with fresh culture medium for 24 h after intratibial injection were harvested and maintained at 4°C before injection. After deep anesthesia using trichloracetaldehyde monohydrate (0.4 mg body weight; KANTO Chemical Co., Tokyo, Japan), 150 μl of cell suspension containing 2 × 10⁶ cells was injected into the bone marrow cavity of the tibia of 4-week-old male CB17-SCIID mice. After visualization of a tumor mass around the proximal tibia 28 days post-injection, radiography was performed using a soft X-ray generating unit to ensure bone osteolysis. Animals were deeply anesthetized using trichloracetaldehyde monohydrate, placed in a prone position on Kodak Scientific Imaging film (13 × 18 cm) and exposed to X-rays (45 kV) for 5 s for measurement of the area of osteolytic lesions using an image analysis system (ImageJ software) and expressed as a percentage of the total tissue area.

Histopathology and bone histomorphometry
For histological observation of bone, tibiae specimens were removed, fixed using 4% paraformaldehyde in PBS for more than 72 h, decalcified in a 14% ethylenediaminetetraacetic acid solution for 14 days, dehydrated in increasing concentrations of ethanol and embedded in paraffin. Serial histological sections of 5 μm thickness were cut longitudinally and stained with Mayer’s hematoxylin and eosin solution to assess histology or used to perform TRAP staining (Acid Phosphatase Kit, Model 387-A; Sigma Diagnostics, St Louis, MO). TRAP staining was performed by staining the specimens with acid phosphatase and tartrate solution for 1 h at 37°C before selecting three random regions of interest within each tibia for examination and determination of the number of TRAP-positive osteoclasts.

Immunohistochemistry
Human prostate cancer tissue array (T195a and PR56) was purchased from Biomax (Odenton, MD) in the form of 5 μm sections of paraaffin-embedded tissue on glass slides. After rehydration and incubation in 3% hydrogen peroxide to block endogenous peroxidase activity, sections were blocked by incubation in 3% bovine serum albumin in PBS. The primary antibody, monoclonal mouse anti-human CCN3 antibody, was applied to the slides at a dilution of 1:50 and incubated at 4°C overnight. After three washes in PBS, the samples were treated with goat anti-mouse immunoglobulin G biotin-labeled secondary antibodies at a dilution of 1:50 and bound antibodies were detected using an ABC Kit (Vector Laboratories, Burlingame, CA). The slides were stained with chromogen diaminobenzidine, washed, counterstained with Delafield’s hematoxylin, dehydrated, treated with xylene and mounted.
Statistical analysis

Data are presented as mean ± standard error of the mean (SEM). Statistical analysis between two samples was performed using the Student’s t-test. Statistical comparisons of more than two groups were performed using one-way analysis of variance with Bonferroni’s post hoc test. In all cases, a $P < 0.05$ was considered an indication of statistical significance.

Fig. 1. CCN3 is highly expressed in human bone metastases of patients with prostate cancer. (A) Representative immunohistochemical images of CCN3 expression in paraffin-embedded sections of human prostate cancer bone metastases. Extent of CCN3 staining is slight in tissue of normal and early stage prostate cancer patients (top), moderate in tissue of late-stage prostate cancer patients (bottom, left) and strong in bone metastatic lesions of patients with prostate cancer (bottom, right). (B) Quantification of CCN3 staining of prostate cancer immunohistochemical results. (C–E) Total mRNA, protein secreted in medium and total protein were prepared from PC3, DU145 and LNCaP cells, and the CCN3 expression was examined by qRT-PCR, enzyme-linked immunosorbent assay and western blot analysis. Results are expressed as mean ± SEM of triplicate samples. *$P < 0.05$ compared with LNCaP group.
Results

CCN3 is highly expressed in bone metastasis patients with prostate cancer

We had previously observed that CCN3 increases cell migration and metastasis in human prostate cancer cells (23). To elucidate the relationship between CCN3 expression and bone metastasis in prostate cancer patients, the expression level of CCN3 in 10 samples of normal human prostate tissue, 47 samples of localized human prostate tumor tissue and 3 samples of human prostate bone metastasis tissue was examined by immunohistochemistry using a CCN3-specific antibody, and the staining intensity was scored from 1 to 5. Higher CCN3 expression was found to be associated with higher clinical pathologic stages, with CCN3 expression observed to be stronger in all prostate bone metastasis samples compared with the localized tumor samples (Figure 1A and B). Detection of CCN3 level in the three human prostate cancer cell lines PC3, DU145 and LNCaP revealed that the aggressive cell line PC3 expresses higher levels of CCN3 mRNA and secretes more protein compared with the less aggressive cell line LNCaP (Figure 1C–E). These results suggest that CCN3 is associated with prostate cancer progression and bone metastasis.

Fig. 2. CCN3-neutralizing antibody inhibits prostate cancer CM-induced osteoclast formation. (A and B) RAW 264.7 cells were cultured in a 24-well plate (1 × 10^3 per well) and incubation with 10% CM collected from PC3, DU145 or LNCaP cells. (C) RAW 264.7 cells were cultured in a 24-well plate and incubation with 10% PC3 CM in the presence of neutralizing antibodies against CCN3 (1 μg/ml) and immunoglobulin G (1 μg/ml) or transfection with CCN3 siRNA and negative siRNA. After 7 days, the osteoclast-like cells were identified by TRAP staining. Positively stained cells that contained three or more nuclei were scored as osteoclast-like multinucleated cells. (A and C) Representative microscopy graphs of TRAP staining of RAW 264.7 cell cultures. (B and D) Quantification of number of osteoclast-like multinucleated cells per well. Results are expressed as mean ± SEM of triplicate samples. *P < 0.05 compared with PC3 CM-treated group.
CCN3 mediates prostate cancer-induced bone resorption

Neutralizing antibody for CCN3 inhibits prostate cancer CM-induced osteoclast formation

Previous studies have shown that many soluble factors in the CM can induce osteoclastogenesis and promote bone metastasis (12,25). To determine the role of CCN3 in prostate cancer CM-induced osteoclastogenesis, murine osteoclast precursor RAW 264.7 cells were cultured with CM collected from PC3, DU145 or LNCaP cells. After 7 days, the CM from each cell line was observed to have induced TRAP-positive

Fig. 3. CCN3 synergizes with RANKL to promote osteoclast formation in RAW 264.7 cell cultures and rat bone marrow cultures. (A) RAW 264.7 cells were cultured in a 24-well plate (1 × 10³ per well) before incubation with different doses of CCN3 (0–100 ng/ml) in the presence 20 ng/ml of M-CSF and 50 ng/ml of RANKL for 7 days. Number of osteoclast-like multinucleated cells per well was quantified by TRAP staining. (B) Non-adherent rat bone marrow cells were cultured in a 24-well plate (1 × 10⁶ per well) and treated as described in (A). Number of osteoclast-like multinucleated cells per well was quantified by TRAP staining. (C) Representative microscopy graphs of TRAP staining of non-adherent rat bone marrow cell cultures. (D) RAW 264.7 cells were seeded onto a Corning Osteo Assay Surface 24-well plate (1 × 10³ per well) and treated as described in (A). After 12 days, cells were removed and area of resorption was determined by microscopy using ImageJ software. (E) Representative images of resorption pits on the plate surface described in (D). Results are expressed as mean ± SEM of triplicate samples. *P < 0.05 compared with CCN3-untreated group.
multinuclear cell formation and osteoclast formation, both of which could be correlated with the CCN3 expression level in the respective prostate cancer cell line (Figures 1A&B and 2A&B). In addition, pretreatment with the different neutralizing antibodies for CCN3 was found to have significantly decreased PC3-CM-induced osteoclast formation (Figure 2C&D). Moreover, transfection of PC3 cells with CCN3 siRNA reduced PC3-CM-induced osteoclast formation (Figure 2C&D). These data provide strong evidence that prostate cancer produced CCN3 plays a key role in tumor-induced osteoclast formation in vitro.

**CCN3 promotes RANKL-dependent osteoclastogenesis**

It has been reported that prostate cancer CM induces osteoclast formation in either a RANKL-dependent or RANKL-independent manner (12,26). To examine the role of CCN3 in driving osteoclastogenesis, RAW 264.7 cells were cultured with increasing amounts of CCN3 (0, 10, 30 and 100 ng/ml) in either the absence or presence of M-CSF (20 ng/ml) and RANKL (50 ng/ml) for 7 days. Although no obvious osteoclastogenesis was observed in RAW 264.7 cells treated with CCN3 (10–100 ng/ml) alone (data not shown), a significantly larger number of osteoclasts were observed in RAW 264.7 cultures and Sprague–Dawley rat bone marrow cells that had been treated with CCN3 and RANKL simultaneously (Figure 3A–C). The results of qRT-PCR revealed that the mRNA levels of common osteoclast markers (Trap, MMP9, integrin α and integrin β) had been upregulated after CCN3 treatment in a dose-dependent manner and to a greater extent compared with RANKL alone treatment (Supplementary Figure S1, available at Carcinogenesis.

**Fig. 4.** CCN3 increases RANK mRNA and protein expression in RAW 264.7 cells. (A) RAW 264.7 cells were stimulated with CCN3 for 24 h and RANK expression was examined by qRT-PCR. (B) RAW 264.7 cells were stimulated with CCN3 for 24 h and RANK expression on cell surface was detected with specific antibodies using flow cytometry. (C) RAW 264.7 cells were incubated with CCN3 for 24 h and RANK expression was examined by western blot analysis. (D) RAW 264.7 cells were incubated with different doses of CCN3 (0–100 ng/ml) for 1 h and phosphorylation of FAK, Akt, p38, IKKα/β, IκBα and p65 was determined by western blot analysis. (E and F) RAW 264.7 cells were pretreated with FAKi (10 μM), Akti (1 μM), SB203580 (1 μM), PDTC (10 μM) and TPCK (1 μM) for 30 min or transfected with FAK, Akti, p38 and p65 siRNA for 24 h, followed by stimulation with CCN3 (30 ng/ml) for 24 h. Total mRNA was extracted and RANK expression was examined by qRT-PCR. (G and H) RAW 264.7 cells (1 x 10⁶/well) were cultured in a 24-well plate and pretreated as described in (E and F) for 30 min or 24 h. Cells were then treated with 30 ng/ml of CCN3 in the presence of 20 ng/ml of M-CSF and 50 ng/ml of RANKL for 7 days before the number of osteoclast-like multinucleated cells per well was quantified by TRAP staining. Results are expressed as mean ± SEM of triplicate samples.

*P < 0.05 compared with CCN3-untreated group; **P < 0.05 compared with CCN3 treatment.
Online). To further evaluate the role of CCN3 in RANKL-dependent bone resorption, RAW 264.7 cells were seeded onto a Corning Osteo Assay Surface 24-well plate before incubation with different doses of CCN3 (0–100 ng/ml) in the presence of 20 ng/ml of M-CSF and 50 ng/ml of RANKL for 12 days for bone resorption pit assay. After treatment by CCN3 plus M-CSF and RANKL for 12 days, the digital images in the microscope were captured and the resorbed area/well was measured (Figure 3D and E), along with the results described above, indicates that CCN3 promotes the formation of functional osteoclasts through RANKL-dependent osteoclastogenesis.

**CCN3 upregulates RANK expression in murine osteoclast precursors in RAW 264.7 cells to promote RANKL-dependent osteoclastogenesis**

It has been well discussed that the expression of RANK and signaling through this receptor is pivotal in osteoclast differentiation (27). To investigate whether RANK is involved in CCN3-promoted RANKL-dependent osteoclastogenesis, the relative RANK mRNA expression level of RAW 264.7 cells that had been stimulated with increasing amounts of CCN3 (0, 10, 30 and 100 ng/ml) for 24 h was determined by qRT-PCR. The results showed that CCN3 treatment had increased RANK mRNA expression and that maximal induction had occurred with treatment of 30 ng/ml of CCN3 (Figure 4A). The results of flow cytometry and western blotting performed to detect protein expression confirmed that CCN3 treatment had upregulated RANK protein expression and that maximal induction had occurred with 30 ng/ml of CCN3 (Figure 4B and C).

As previous studies have shown that the CCN family activates signal pathways by binding to integrin receptors on the cell surface (16,28), identification of the signal pathways that are regulated by integrin and could be involved in RANK upregulation was the next research aim. To do so, increasing levels of phosphorylated FAK, Akt, p38, IKKα/β, IκBα and NF-κB subunit p65 were added in a dose-dependent manner following CCN3 stimulation (Figure 4D). CCN3-induced RANK expression and osteoclastogenesis were reduced by pretreatment with FAK inhibitor (FAKi, 10 μM), Akt inhibitor (Akti, 1 μM), p38 inhibitor (SB203580, 1 μM), NF-κB inhibitor (PDTC, 10 μM) or IκB protease inhibitor (TPCK, 1 μM) (Figure 4E and G). Moreover, transfection of RAW 264.7 cells with FAK, Akt, p38 and p65 siRNAs also inhibited CCN3-increased RANK expression and osteoclast formation (Figure 4F and H). These results provide clear evidence that CCN3 promotes RANKL-dependent osteoclastogenesis and that RANK expression is mediated by the FAK/Akt/p38/NF-κB signaling pathway.

**CCN3 changes RANKL:OPG ratio in osteoblasts**

Prostate cancer cells must interact with osteoblasts and osteoclasts, the main cells, in the bone microenvironment to establish metastases in the bone (3,6,29). The secretion of RANKL and OPG in osteoblasts is critical for osteoclastogenesis by paracrine manner...
Fig. 6. CCN3 overexpression in PC3 cells increases osteoclast formation and bone metastasis in vivo. (A) *In vitro* migration of PC3 and PC3-I3 cells was measured using Transwell assay. (B) Total mRNA was extracted from PC3 and PC3-I3 cells and CCN3 expression level was detected by qRT-PCR analysis. (C) SCID mice were treated by intratibial injection of PC3 and PC3-I3 cells in left leg and radiographed after 28 days (left). Tumor-induced bone osteolysis was evaluated by X-ray radiography, hematoxylin and eosin staining and TRAP staining. On radiograph, arrows point to tumor-induced osteolysis. On image of hematoxylin and eosin and TRAP staining, T represents tumor cells and arrows point to osteoclasts in the tumor–bone interface. (D) Quantification of tumor weight following intratibial injection. (E) Quantification of bone resorption area following intratibial injection. (F) Quantification of osteoclast number per field in tumor–bone interface as determined by TRAP staining in three random regions. Results are expressed as mean ± SEM. *P < 0.05 compared with PC3.
CCN3 mediates prostate cancer-induced bone resorption

To investigate whether CCN3 affects the expression ratio of RANKL:OPG in osteoblasts, RANKL:OPG mRNA expression and protein secretion in osteoblast-like MG63 cells were detected after CCN3 treatment. The results showed that CCN3-treated osteoblasts expressed higher levels of RANKL and lower levels of OPG compared with untreated cells (Figure 5A–D), indicating that CCN3 had changed the bone microenvironment by affecting the RANKL:OPG expression ratio in osteoblasts and indirectly regulating osteoclast formation.

**CCN3 expression is higher in highly invasive PC3 cells and increases osteoclastogenesis and bone metastasis in vivo**

It has been reported that CCN3 improves the capacity for invasion, an initial step of metastasis, in tumor cells (20). To investigate the role of CCN3 in promoting prostate cancer metastasis to bone, Transwell invasion chamber was used to select highly invasive populations from the PC3 cell line for expansion to produce the PC3-I3 clonal cell population, a highly invasive prostate cancer cell line. The results of migration assay revealed that the PC3-I3 cells had higher migration ability than the PC3 cells (Figure 6A). When qRT-PCR was then performed to determine whether CCN3 mRNA expression was elevated in the PC3-I3 cell line, the results showed that CCN3 expression had been upregulated in PC3-I3 cells (Figure 6B).

Osteoclastic bone resorption has been implicated in cancer bone metastasis (32). To examine the effect of CCN3 on osteoclastic bone resorption and prostate cancer bone metastasis in vivo, PC3 and PC3-I3 cells (2×10⁷) were locally injected into the bone marrow cavity of the left tibia of 4-week-old SCID mice. To analyze bone ostkolysis, mice were killed 28 days post-injection for radiography using a soft X-ray generating unit, histomorphometric analysis and TRAP staining. Although all three forms of measurement confirmed that injection of both PC3 and PC3-I3 cells had resulted in the development of osteolytic lesions in the tibia, radiography revealed that the size of the osteolytic lesions was significantly larger in the tibia tissue injected with PC3-I3 cells, which have higher CCN3 expression (Figure 6C, left). In addition, TRAP staining at the tumor–bone interface demonstrated that tumor-induced osteoclastic activity was significantly greater in the tibia tissue that had been injected with PC3-I3 cells compared with PC3 cells (Figure 6C, three photos on the right). Quantitative assessment of tumor weight, osteolytic lesion and osteoclast number in the tumor–bone interface confirmed that overexpression of CCN3 had increased both tumor growth and osteolytic lesion formation (Figure 6D–F). These results demonstrate that CCN3 expression is correlated with invasion ability and enhanced prostate cancer bone metastasis in vivo.

**Discussion**

In recent years, a correlation between CCN3 expression and tumorigenesis has been identified in a variety of cancers, but the mechanism of CCN3 activity in tumorigenesis remains unclear (17,18,33). The mechanism behind the correlation between prostate cancer and CCN3 that has been previously reported (22) is hypothesized to be increased migration in prostate cancer cells due to CCN3-induced transcriptional upregulation of ICAM-1 expression (23). Despite the fact that bone metastasis is the primary cause of mortality for patients with prostate cancer, its mechanism had not been investigated prior to this study. This study thus filled a significant research gap by revealing, for the first time, that CCN3 is highly expressed in bone metastasis patients with prostate cancer. Specifically, CCN3 increases prostate cancer-induced, RANKL-dependent osteoclastogenesis via upregulation of RANK expression in osteoclast precursors by activating the FAK/Akt/p38/NF-κB signaling pathway (Figure 6G).

The results of this study confirmed the existence of a correlation between CCN3 expression and prostate cancer bone metastasis. In clinical tumor specimens, CCN3 levels were correlated with tumor stage of prostate cancer, with higher CCN3 expression associated with later clinical pathologic stages, suggesting that CCN3 may be associated with prostate cancer progression. Importantly, CCN3 expression levels were observed to be higher in the bone metastases than in the localized tumors of prostate cancer patients (Figure 1A and B). Among the prostate cancer cell lines, the PC3 cell line, which has a relatively aggressive phenotype, secreted greater levels of CCN3 compared with the LNCaP cell line, a less aggressive tumor cell line (Figure 1C and D). These results reveal that CCN3 could be used as a biomarker of tumor progression, specifically for bone metastases.

It is well established that osteoclastogenesis and bone resorption are critical steps leading to the development of skeletal metastases and are essential for prostate cancer establishment in the bone (34,35). There are multiple factors, including RANKL, that act as important mediators of bone remodeling (26,36,37). But there are other soluble factors that could contribute to cancer-induced osteoclast activity. CCN3, an extracellular matrix-associated protein, was identified as a factor promoting osteoclastogenesis and osteoclast activity for the first time in this study. Specifically, prostate cancer CM-induced osteoclastogenesis and PC3 CM-induced osteoclast formation were found to be inhibited by treatment with a neutralizing antibody against CCN3 or transfection of PC3 cells with CCN3 siRNA (Figure 2), whereas RANKL-dependent osteoclastogenesis was observed to be increased, both in terms of osteoclast number and activity, by treatment of RAW cell cultures and rat bone marrow cells with both CCN3 and RANKL (Figure 3). These findings were confirmed by measurement of increased osteoclast-marker expression upregulation by CCN3 treatment (Supplementary Figure S1, available at Carcinogenesis Online). All these findings indicate that CCN3 may play a key role in the osteoclastogenesis resulting from the interaction between tumor cells and osteoclasts in the bone microenvironment.

In an investigation of the reportedly important role played by RANK in osteoclastogenesis (38), we found that CCN3 upregulates RANK expression in osteoclast precursor cells (RAW 264.7 cells) to increase osteoclastogenesis (Figure 4A–C). This finding, which clarifies that CCN3 induces osteoclast formation via RANKL-dependent osteoclastogenesis, strongly suggests that CCN3 stimulates osteoclastogenesis via two serial mechanisms; first, by directly acting on osteoclast precursors and inducing RANK expression and, second, by stimulating RANKL expression in osteoblasts to promote osteoclastogenesis.

As previous studies have shown that CCN3 elicits cell-signaling pathways by binding to integrin receptors on the cell surface (16,23,28), the effect of CCN3 treatment on the signaling pathways downstream of integrin receptors was examined. CCN3 treatment was observed to increase FAK/Akt/p38/NF-κB pathway (IκB, IKKβ and p65) phosphorylation in a dose-dependent manner (Figure 4D), whereas other MAPK elements (JNK and MEK) were not increased (data not shown), and pretreatment with inhibitors or transfection with siRNAs of these signaling proteins was found to reduce the effects of CCN3 on RANK expression and osteoclast formation (Figure 4E–H). Taken together, these results provide evidence that CCN3 upregulates RANK expression via the FAK/Akt/p38/NF-κB signaling pathway in osteoclast precursor cells.

Prostate cancer preferentially metastasizes to bone, leading to mortality at a late stage. It has been well documented that the interaction between cancer cells and the bone microenvironment results in a ‘vicious cycle’ that increases both bone destruction and tumor growth (32,39). The establishment of the vicious cycle is complex, with cells residing in the tumor–bone microenvironment...
(cancer cells, osteoblasts and osteoclasts) interacting in a manner that leads to the development and progression of bone metastases. In this environment, RANKL plays a critical role in promoting osteoclastogenesis, whereas OPG serves as a soluble decoy receptor for RANKL that blocks osteoclastogenesis by inhibiting RANKL binding to RANK, its receptor (40,41). Our results demonstrate that alteration of CCN3 expression could change the RANKL:OPG ratio in a manner that promotes upregulation of RANKL and downregulation of OPG (Figure 5). Previous observation that CCN3 may affect bone formation by inhibiting osteoblast differentiation through BMP and Wnt family signaling (42,43) indicates that CCN3 may serve as an important cytokine that presents in the bone microenvironment and regulates bone metastasis in prostate cancer.

Previous studies have shown that increased CCN3 expression can increase the capacity to invade, an initial step in metastasis, in many cancers (20). Based on this finding, it was hypothesized that highly invasive prostate cancer cells increase CCN3 expression, thus enhancing bone metastasis. To test this hypothesis by determining the correlation among CCN3 expression, prostate cancer invasion and bone metastasis, the highly invasive cell line PC3-13, which had been subcloned from PC3 cells and overexpressed CCN3, was injected into SCID mice. As expected, bone histomorphometry revealed that injection with PC3-13 cells had significantly increased tumor growth in vivo. Combining these findings with the results of this study, we are confident that CCN3 will not only be a target of clinical therapy in the future. Used as a prognostic biomarker for prostate cancer development but also as a target of clinical therapy in the future.

In this environment, RANKL plays a critical role in promoting osteoclastogenesis and excessive bone resorption is a key feature of bone metastasis in prostate cancer. The presence of a high RANKL:OPG ratio in most bone metastatic lesions and the development of osteolytic bone metastases have been associated with increased CCN3 expression, thus suggesting a potential role for CCN3 in the development of bone metastases. These findings lead to the conclusion that secretion of CCN3 by prostate cancer cells increases the RANKL:OPG ratio in bone, which in turn increases osteoclast differentiation and promotes tumor-induced osteoclasts. These findings further suggest that CCN3 may be a target for the prevention and treatment of bone metastases in prostate cancer patients.

Supplementary Information
Supplementary Figure S1 can be found at http://carcin.oxfordjournals.org/

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References

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