Tumorigenesis and Neoplastic Progression

CCN3 Impairs Osteoblast and Stimulates Osteoclast Differentiation to Favor Breast Cancer Metastasis to Bone

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Bone is a preferred site for breast cancer metastasis, causing pain, fractures, spinal cord compressions, and hypercalcemia, all of which can significantly diminish the patient's quality of life. We identified CCN3 as a novel factor that is highly expressed in bone metastatic breast cancer cells from a xenograft mouse model and in bone metastatic lesions from patients with breast cancer. We demonstrate that CCN3 overexpression enhances the ability of weakly bone metastatic breast cancer cells to colonize and grow in the bone without altering their growth in the mammary fat pad. We further demonstrated that human recombinant CCN3 inhibits osteoblast differentiation from primary bone marrow cultures, leading to a higher receptor activator of NF- κ B ligand (RANKL)/osteoprotegerin (OPG) ratio. In conjunction with its ability to impair osteoblast differentiation, we uncovered a novel role for CCN3 in promoting osteoclast differentiation from RANKL-primed monocyte precursors. CCN3 exerts its pro-osteoclastogenic effects by promoting calcium oscillations and nuclear factor of activated T cells c1 (NFATc1) nuclear translocation. Together, these results demonstrate that CCN3 regulates the differentiation of bone resident cells to create a resorptive environment that promotes the formation of osteolytic breast cancer metastases. (*Am J Pathol* 2011, 178:2377–2388; DOI: 10.1016/j.ajpath.2011.01.033)

all survival relative to patients with visceral metastases, their quality of life can be significantly diminished due to pain, fractures, spinal cord compressions, and hypercalcemia.³ Given the pivotal role of the osteoclast in bone breakdown associated with osteolytic lesions, bisphosphonates (a class of drugs that inhibit osteoclast-dependent bone resorption) are routinely given to patients with breast cancer bone metastases.⁴ However, significant research efforts are now focused on the identification and development of targeted therapeutics to further enhance the management of breast cancer metastasis.^{5,6}

The CCN family derives its name from the three founding members: cysteine-rich protein 61 (*Cyr61/CCN1*), connective tissue growth factor (*CTGF/CCN2*), and nephroblastoma overexpressed (*NOV/CCN3*).^{7,8} The family also includes Wnt-inducible secreted protein-1 (*WISP1/CCN4*), *WISP2/CCN5*, and *WISP3/CCN6*.⁹ These matricellular proteins are grouped together based on the similarity of four modular domains, which include an insulin-like growth factor-binding domain, a von Willebrand factor type C domain (VWC), a thrombospondin type-1 repeat domain, and a cysteine-knot-containing C-terminal domain.^{10–12} Although

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Bone is the preferred site for breast cancer metastasis.^{1,2} Although patients with bone metastasis display better over-

the CCN proteins were originally identified as growth factors themselves, they are now thought to modify mitogenic responses by virtue of their ability to interact with and modulate the function of growth factors and cell surface receptors.^{11,12}

CCN3 exerts diverse and dichotomous functions in cancer cells. Whereas some studies suggest that CCN3 exerts growth suppressive effects on glioma, melanoma, and breast cancer cells,^{13–15} others support a prometastatic role for this protein in melanoma.¹⁶ CCN3 can impair the proliferation of Ewing's sarcoma cells but promote their migration and invasion,¹⁷ supporting the observed association between high CCN3 levels and metastatic disease in this cancer type.^{18,19} Finally, high CCN3 levels correlate with poor outcome in patients with osteosarcoma or prostate cancer.^{20,21} Together, these studies demonstrate that the effects of CCN3 are context dependent; however, the role of CCN3 in breast cancer progression to metastasis remains unclear.

We previously used 4T1 murine breast cancer cells to isolate subpopulations with enhanced bone metastasis ability.²² Using this system, we identified targets relevant to breast cancer progression and bone metastasis.^{22,23} We now describe the identification of three members of the CCN family as differentially expressed in weakly versus aggressively bone metastatic breast cancer cells. The present results indicate that CCN3 functions to promote the formation of osteolytic breast cancer metastases by modulating the bone microenvironment to favor osteoclast differentiation.

Materials and Methods

DNA Constructs

A pCMV82 vector²⁴ containing the full-length human CCN3 cDNA (GeneBank Accession number X96584) was kindly provided by Dr. Laure Rittié (University of Michigan, Ann Arbor, MI). PCR was performed using the following primers: CCN3, 5'-TGGATCCACCATGCAGAGTGTGCAGAG-3' (sense) and 5'-GGAATTCTTACATTTCCCTCTGGTAG-3' (antisense), and the PCR product was digested with EcoRI and BamHI and ligated into the EcoRI and BglII sites of pMSCVpuro vector (Clontech Laboratories, Mountain View, CA). Viruses were packaged in 293 vesicular stomatitis virus cells (kindly provided by Dr. Alain Nepveu, McGill University, Montreal, Canada), and 66cl4 cells were infected and selected to generate stable populations expressing human CCN3. Stable cell lines were maintained under 4 μ g/mL puromycin antibiotic selection.

Cell Culture

The 66cl4 murine mammary carcinoma cell lines were obtained from American Type Culture Collection (Rockville, MD). Cell lines were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 10 mmol/L HEPES, 1 mmol/L sodium pyruvate, and 1.5 g/L sodium bicarbonate. The RAW 264.7 murine monocytic cell line was also obtained from American Type Culture Collection and grown in DMEM supplemented with 10% fetal bovine serum, 1.5 g/L sodium

bicarbonate, 4.5 g/L glucose, and 1% sodium pyruvate (Wisent Inc., St-Bruno, Canada).

Microarray Analysis

Agilent gene expression data previously generated in our laboratory²² was subjected to additional analysis (parametric test, 5% false discovery rate) using GeneSpring (Agilent, Santa Clara, CA) comparing the 4T1 parental cell line with bone metastatic subpopulations (590, 592, and 593). Differentially expressed genes were identified using a parametric test combined with a false discovery rate of 0.05. *ccn1*, *ccn2*, and *ccn3* expression, derived from the Agilent data, is shown in Supplemental Figure S1 (available at <http://ajp.amjpathol.org>).

RNA Extraction, cDNA Synthesis, and Quantitative PCR

RNA extraction and cDNA synthesis were performed as previously described.²² Labeling was performed using SYBR Green PCR Master Mix (Hoffmann-La Roche Ltd., Mississauga, Canada) in a final volume of 25 μ L, which contained 5 μ L of diluted cDNA template and 10 pg of the necessary primers. PCR reactions were performed as described by the manufacturer. The following primers were used for PCR amplification: for *ccn1*, 5'-GAAAAGGCAGCTCACTGAAG-3' (sense) and 5'-GGGTTGTCATTGGTAACCTCG-3' (antisense); for *ccn2*, 5'-AGCTGGGAGAACTGTGTACG-3' (sense) and 5'-TTGGTATTGTCAGCTGCTTT-3' (antisense); for *ccn3*, 5'-AGACTCGTCTCTGCATCGTT-3' (sense) and 5'-CTCGAACTGTAGGTGGATGG-3' (antisense); and for glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*), 5'-CAAGTATGATGACATCAAGAAGGTGG-3' (sense) and 5'-GGAAGAGTGGGAGTTGCTGTTG-3' (antisense). Experiments were performed in triplicate using three different cDNA preparations. Quantification of gene expression was performed using the Pfaffl analysis method.²⁵ In each experiment, the 4T1 sample served as the reference sample and *Gapdh* was used as the control. For gene expression analysis from primary cell cultures, we used the TaqMan Universal PCR Master Mix (Applied Biosystems, Streetsville, Canada) and the following TaqMan gene expression assays: β -actin (Mm00607939_s1), *RANKL* (Mm00441908_m1), and *Opg* (Mm01205928_m1). The $\Delta\Delta C_t$ method was used to obtain the expression ratio using bovine serum albumin (BSA) as the control. Real-time PCR was performed using an Applied Biosystems 7500 instrument (Applied Biosystems).

Immunoblotting

Cells were grown to 80% confluence and were lysed in TNE lysis buffer as previously described.²⁶ Unless otherwise indicated, 30 μ g of total protein or 30 μ L of conditioned media was loaded on 10% to 12% SDS-polyacrylamide gels and was transferred to polyvinylidene difluoride membranes. Membranes were blocked in 5% milk-Tris-buffered saline Tween-20 and were incubated

overnight at 4°C with the appropriate primary antibodies. Antibodies specific to CCN1 (N19; 1:800 dilution), CCN2 (K28T; 1:800 dilution), and CCN3 (K19M; 1:1500 dilution) have been described previously,^{24,27,28} and the α -tubulin antibody was purchased from Sigma-Aldrich (St. Louis, MO) (1:10,000 dilution; catalog number T6199).

Activation of cell signaling pathways was assessed by first plating 5×10^4 RAW 264.7 cells into individual wells of a six-well plate. Cells were primed for 48 hours with 50 ng/mL receptor activator of NF- κ B ligand (RANKL) and subsequently were stimulated for 15, 30, or 60 minutes with DMEM alone or DMEM containing either 50 ng/mL of mouse recombinant RANKL (rRANKL) resuspended in 0.1% BSA (catalog number 315-11; Peprotech, Rocky Hill, NJ) or 600 ng/mL of human recombinant CCN3 (rCCN3) resuspended in 0.1% BSA (catalog number 120-26; Peprotech). CCN3 cells were washed twice in PBS, and protein lysates were extracted using radioimmunoprecipitation assay buffer as previously described.²⁹ Membranes were blocked in 5% BSA-Tris-buffered saline Tween-20 and were incubated with ERK (1:1000 dilution; catalog number 9102), p-ERK (1:1000 dilution; catalog number 9101), JNK (1:1000 dilution; catalog number 9252), p-JNK (1:1000 dilution; catalog number 9251), PKC α (1:1000 dilution; catalog number 2056), or p-PKC α/β (1:1000 dilution; catalog number 9375) antibodies, all of which were purchased from Cell Signaling Technology (Danvers, MA).

The appropriate horseradish peroxidase-conjugated anti-rabbit secondary antibodies (catalog number 711-035-152; Jackson ImmunoResearch Laboratories, West Grove, PA) were used at a dilution of 1:5000, and membranes were visualized using an enhanced chemiluminescence system (catalog number 34080; Pierce Biotechnology, Nepean, Canada).

ELISA

To generate the conditioned media, cells were plated in 100-mm dishes in complete medium. The following day, the medium was replaced with 5 mL of serum-free medium and was allowed to condition for 48 hours. We used the mouse nov/ccn3 DuoSet (catalog number DY1976; R&D Systems, Minneapolis, MN) enzyme-linked immunosorbent assay (ELISA) kit to detect ccn3 in the conditioned medium harvested from the 4T1 derivatives and the human NOV/CCN3 DuoSet (catalog number DY1640; R&D Systems) ELISA kit from 66cl4 cells engineered to express human CCN3. Conditioned media was diluted (1:2 for 4T1 derivatives and 1:10 for 66cl4 cells) into the reagent diluent concentrate 2 (catalog number DY995; R&D Systems). Experiments were performed in duplicate on two independent harvests of conditioned media from 66cl4 cells and 4T1 derivative populations. Data are expressed as picograms per milliliter of CCN3 per microgram of total protein per 48 hours.

Immunohistochemical Analysis

FFPE blocks of bone metastatic lesions were obtained from patients with breast cancer who had previously undergone orthopedic surgery at Mount Sinai Hospital (Toronto, Canada) for pathologic fractures. Immunostaining was per-

formed using a polyclonal rabbit anti-CCN3 antibody (K19M antibody; 1:400 dilution). The staining intensity was evaluated by two different observers (including one pathologist, D.T.-T.), incorporating the percentage positivity and strength (negative, weak, moderate, and strong) of the observed staining. Local research ethics board (Mount Sinai Hospital) approval was obtained for the tissue banking and testing of samples used in this study.

In Vitro Proliferation and in Vivo Tumorigenesis/Metastasis Assays

Cell proliferation assays were performed by plating 8×10^3 cells from infected pools of 66cl4 cells harboring an empty vector (VC) or overexpressing human CCN3 into 12-well plates. Cells were counted every day for 4 days using an automated cell counter (Cellometer; Nexcelom Bioscience, Lawrence, MA). Two independent experiments were performed, and triplicate wells were counted at each time point.

To assess tumor growth *in vivo*, 66cl4 mammary carcinoma cells were harvested from subconfluent plates and were washed once with PBS, and 1×10^5 cells were resuspended in 50 μ L of a 50:50 solution of Matrigel (BD Biosciences, Mississauga, Canada) and PBS. Cell suspensions were injected into the right and left abdominal mammary fat pads of BALB/c mice ($n = 5$ per group) (Charles River Laboratories, St-Constant, Canada), and weekly tumor measurements were obtained. Tumor volumes were calculated using the following formula: $\pi LW^2/6$, where L is the length and W is the width of the tumor.

To evaluate the capacity of the cells to form bone metastases, we injected the 66cl4 mammary carcinoma cells (10^5 cells) into the left cardiac ventricle of 4- to 5-week-old BALB/c mice as previously described.²² To visualize bone metastases, mice were anesthetized, and digital X-rays were taken using an XPRT80 specimen radiography system (KUB Technologies, Milford, CT) on day 7 after injection and before sacrifice (day 14). Paraformaldehyde-fixed tissues were paraffin embedded, sectioned, and subjected to H&E staining. The number of mice possessing at least one histologically verified lesion was divided by the total number of animals in each cohort to determine the percentage of mice developing bone metastases. The number of lesions over the total number of bones (femur and tibia) analyzed in each cohort was also determined. To quantify lesion area/bone area, a distance of 5 mm was measured from the head of the femur or tibia (at the joint) down the shaft of the bone. The area of this region of bone was calculated by tracing the outline of the femur or tibia, ending at the 5-mm point, using ImageScope (Aperio Technologies, Vista, CA). This allowed a standard tissue area to be calculated across all samples. For bones possessing a lesion, the area of the metastases was circumscribed and quantified using a positive pixel count algorithm (ImageScope). The percentage of lesion area/bone area was then calculated.

Osteoblast Differentiation Assay

Mouse-derived bone marrow cells were collected from mouse tibia and femora as described previously.²⁹ One day after plating, 50 ng/mL ascorbic acid (AA) (A5960; Sigma-Aldrich Canada Ltd, Oakville, ON, Canada) was added to induce osteoblast differentiation. The indicated treatments, including 600 ng/mL BSA (BioShop Canada Inc., Burlington, Canada) and 600 ng/mL rCCN3 (Peprotech) were freshly added on days 1, 3, 5, and 7. On day 9, cell cultures were fixed using 4% paraformaldehyde for 10 minutes, washed with PBS, and stained for alkaline phosphatase (Fast Red, catalog number F4381; Sigma-Aldrich). Images were analyzed using BIOQUANT software (BIOQUANT Image Analysis Corp, Nashville, TN) and are expressed as the average percentage coverage over the well.

Osteoclast Differentiation Assay

Osteoclast differentiation assays were performed as previously described.³⁰ Briefly, 5×10^3 RAW 264.7 cells were plated in each well of a 48-well plate. On day 1 and day 3, fresh media containing the specified factors was applied to the cultures (rRANKL, 50 ng/mL; rCCN3, 600 ng/mL). When specified, cells were incubated with Ca^{2+} chelator BAPTA-acetoxymethyl ester (catalog number B-6769; Invitrogen, Carlsbad, CA) on day 3 for 10 minutes and were washed before treatment with recombinant protein as previously described.³¹ Cells were fixed in 4% paraformaldehyde and were stained for tartrate-resistant acid phosphatase (catalog number 387A; Sigma-Aldrich).

Microspectrofluorometry

Ca^{2+} imaging was performed as previously described.³⁰ Raw 264.7 cells were plated on glass-bottom 35-mm dishes (MatTek Corp., Ashland, MA). rRANKL-primed cells were washed twice and loaded at room temperature for 40 minutes with fura-2-AM (catalog number F1221; Invitrogen). $[\text{Ca}^{2+}]_i$ was calculated using a fura-2 calcium imaging calibration kit (catalog number F-6774; Invitrogen). After recording a baseline DMEM, 50 ng/mL rRANKL or 600 ng/mL rCCN3 was added to the plate and the calcium concentration was recorded for 120 seconds. A cell was considered as oscillating when two or more peaks were recorded.

Immunofluorescence

To examine ccn3 localization or nuclear factor of activated T cells c1 (nfatc1) nuclear translocation, cells were plated onto glass coverslips that were placed on the bottom of a 24-well dish. ccn3 immunofluorescence was performed as previously described³¹ using an Alexa-488-conjugated goat anti-rabbit antibody (catalog number A11034; Invitrogen). For nfatc1 immunofluorescence, RAW cells that were left unprimed or primed for 48 hours with 50 ng/mL rRANKL were stimulated with DMEM alone or DMEM containing either rRANKL (50 ng/mL) or rCCN3 (600 ng/mL) for 15, 30, and 60 minutes. nfatc1 immuno-

fluorescence was performed as previously described³² using a mouse anti-nfatc1 antibody (catalog number 7294; Santa Cruz Biotechnology, Santa Cruz, CA) and Alexa-488-conjugated goat anti-mouse antibody (catalog number A11029; Invitrogen). Four to eight random fields were quantified for each condition. Cells were considered positive for nfatc1 nuclear localization if the fluorescence intensity of nuclei exceeded that of cytoplasm.

Statistical Analysis

Statistical analyses (parametric *t*-test) were performed using VassarStats software (<http://faculty.vassar.edu/lowry/VassarStats.html>). *P* < 0.05 was considered significant.

Results

CCN3 Expression Is Higher in Bone Metastatic Breast Cancer Cell Populations

We previously described the isolation of 4T1 breast cancer cells that are aggressively metastatic to the bone.²² Additional analysis of differentially expressed genes in weakly (4T1 and 511) versus aggressively (590, 592, and 593) bone metastatic breast cancer cells identified three members of the CCN family: *ccn1*, *ccn2*, and *ccn3*. Agilent microarray data revealed that *ccn1* and *ccn2* transcript levels are higher in weakly bone metastatic populations, whereas *ccn3* is overexpressed in breast cancer cells that aggressively metastasize to bone (see Supplemental Figure S1A at <http://ajp.amjpathol.org>). Quantitative PCR analyses confirmed that *ccn1* and *ccn2* transcripts were, indeed, reduced and that *ccn3* mRNA was elevated in aggressively bone metastatic breast cancer cells compared with their weakly bone metastatic counterparts (Figure 1A). Finally, we performed immunoblot analyses on whole-cell lysates and conditioned media from the 4T1-derived subpopulations. Whereas *ccn1* expression was reduced in the aggressively bone metastatic populations compared with in the weakly bone metastatic cells, *ccn2* levels were similar across all subpopulations examined (see Supplemental Figure S1, B and C, at <http://ajp.amjpathol.org>). Neither CCN1 nor CCN2 was found in the conditioned media of the cells (data not shown). In contrast, a higher level of secreted CCN3 was observed in the aggressively bone metastatic populations compared with their weakly metastatic counterparts. This corresponded with a concomitant reduction in *ccn3* expression levels in whole-cell lysates from aggressively bone metastatic breast cancer populations (Figure 1B). We further quantified the amount of secreted ccn3 in conditioned media of aggressively and weakly bone metastatic cells by ELISA. On average, the aggressively metastatic breast 4T1-derived cancer cells secreted approximately twice the amount of ccn3 compared with the weakly bone metastatic cells (Figure 1C). Finally, we performed immunofluorescence staining to determine whether *ccn3* localization varied between the *in vivo* selected versus

Figure 1. *ccn3* expression is elevated in highly bone metastatic 4T1 populations. **A:** *ccn1* (left), *ccn2* (middle), and *ccn3* (right) mRNA expression normalized to *gapdh* and expressed relative to 4T1 parental cells was determined using quantitative real-time PCR (qRT-PCR). The data are representative of three independent experiments, each performed in triplicate. **B:** Immunoblot analysis of *ccn3* protein expression in the conditioned media (CM; top) or whole-cell lysate (WCL; middle) of weakly (4T1 and 511) and highly (590, 592, and 593) bone metastatic cell populations. An immunoblot for α -tubulin (bottom) served as loading control. **C:** The concentration of secreted *ccn3* protein in CM was determined by ELISA. Error bars represent SD.

parental cell populations. We observed the accumulation of *ccn3* in cellular protrusions specifically in the highly bone metastatic subpopulations (592) compared with parental 4T1 cells (see Supplemental Figure S2 at <http://ajp.amjpathol.org>). This observation is in agreement with previously published data demonstrating that CCN3 overexpressed in MDA-MB-231 cells also localizes to the leading edge of actin-rich cellular protrusions.³³ Together, these results demonstrate that CCN3 is highly expressed in bone metastatic breast cancer cells and raises the possibility that it may play a functional role in this process.

CCN3 Is Expressed in Bone Metastases from Patients with Breast Cancer

To extend these observations from a murine model of breast cancer bone metastasis to the human disease, we performed immunohistochemical staining for CCN3 on paraffin-embedded bone metastatic lesions from human patients with breast cancer. Most of these samples were characterized as luminal tumors based on estrogen receptor/progesterone receptor positivity (see Supplemental Table S1 at <http://ajp.amjpathol.org>). Staining intensity and the extent of positivity were scored, and we found that of the 14 bone metastasis samples available for analysis, half possess moderate to strong staining in most of the tumor epithelium (Figure 2). However, moderate to strong CCN3 expression in the stromal compartment was present in most of the bone metastases analyzed (11 of 14) (Figure 2). These results demonstrate that strong CCN3 expression in the tumor epithelium or stroma is a common feature of human breast cancer bone metastases.

CCN3 Overexpression Increases Bone Metastasis

To investigate a role for CCN3 in promoting breast cancer metastasis to bone, we used the 66cl4 breast cancer cell

line, which was derived from the same primary tumor as 4T1 cells but lacks robust bone metastatic ability.^{34,35} We infected 66cl4 cells with virus carrying a VC or an expression vector containing the full-length human CCN3 cDNA. The CCN3-infected pool displayed elevated levels of CCN3 in whole-cell lysates and conditioned media compared with 66cl4 VC control cells (Figure 3, A and B). Previous studies have demonstrated that CCN3 can either enhance or reduce the growth of various cell types.¹² To investigate this possibility in the present system, we established *in vitro* growth curves for VC control and CCN3-expressing 66cl4 cells and found that CCN3 expression has little effect on the growth of these breast cancer cells (Figure 3C). We extended these analyses *in vivo* and determined that CCN3 expression did not affect the tumor formation or outgrowth of 66cl4 cells in BALB/c mice (Figure 3D).

We next determined whether CCN3 could affect the ability of breast cancer cells to form osteolytic bone metastases. VC control and CCN3-expressing 66cl4 cells were injected into the left cardiac ventricle, and mice were sacrificed 14 days later. Hind limb sections were H&E stained and examined for the presence of bone metastases. We found that 14% of mice (2 of 14) injected with 66cl4-VC control cells developed metastases compared with 31% of animals (4 of 13) injected with CCN3-expressing cells (Figure 4A, left). Moreover, when the number of lesions per bone (femur or tibia) was scored, we observed that 4% of bones (2 of 56) contained 66cl4-VC-derived lesions compared with 17% of bones (9 of 52) with 66cl4-CCN3-expressing lesions ($P = 0.025$; Figure 4A, middle). We next quantified the total tumor burden in bone lesions formed by VC control and CCN3-expressing breast cancer cells. We observed that the average percentage of tumor area/tissue area in lesions formed from control cells was 1.7 times smaller than that in lesions arising from CCN3-expressing cells [Figure 4, A (right) and B]. These results demonstrate that CCN3 specifically enhances the formation of bone metastases without affecting mammary tumor outgrowth in the primary site.

Figure 2. CCN3 is expressed in human bone metastatic lesions from patients with breast cancer. **A:** Quantification of CCN3 staining in epithelial (blue) and stromal (black) compartments of breast cancer bone metastases. **B:** Representative images of human bone metastatic lesions from patients with breast cancer with negative epithelial and stromal CCN3 staining (**top**), strong epithelial CCN3 staining (**middle**), and strong stromal CCN3 staining (**bottom**). Scale bar = 2 μ m.

CCN3 Inhibits Osteoblast Differentiation and Promotes Osteoclastogenesis in rRANKL-Primed Precursors

Breast cancer cells must interact productively with bone resident cells, such as osteoblasts and osteoclasts, to establish metastases in the bone.^{6,36} Recently, a role for ccn3 as an inhibitor of osteoblast differentiation has been revealed.^{37–40} To confirm this, we established an *in vitro* osteoblast differentiation assay using primary cultures of bone marrow cells. Ascorbic acid was used as an inducer of osteoblast differentiation, which was measured by the degree of alkaline phosphatase staining (Figure 5A). Human rCCN3 impaired ascorbic acid-induced osteoblast differentiation from murine primary bone marrow cells in a dose-dependent manner, causing a 19% decrease in alkaline phosphatase staining at a concentration of 300 ng/mL ($P = 0.05$) and a 44% decrease at 600 ng/mL

($P = 0.001$). Thus, CCN3 is a potent inhibitor of osteoblast differentiation.

The RANKL/osteoprotegerin (OPG) system is critical for modulating the cross talk between osteoblasts and osteoclasts.^{41–43} It is known that RANKL expression is highest in osteoblast precursors and diminishes during osteoblast differentiation. Conversely, OPG levels are low in osteoblast precursors and its expression increases as osteoblasts differentiate.⁴⁴ To determine whether CCN3 influences the levels of RANKL and OPG during osteoblast differentiation, we examined their expression in ascorbic acid-treated primary bone marrow cultures that were cultured in the absence or presence of 600 ng/mL rCCN3. We demonstrate that rCCN3-treated cells expressed higher levels of RANKL and lower levels of OPG compared with untreated control cultures (Figure 5B, top). The increased RANKL/OPG ratio (Figure 5B, bottom) induced by rCCN3 is presumably mediated through an indirect effect on osteoblast differentiation.

Although these observations suggest that CCN3 could indirectly influence osteoclast formation through impaired osteoblast differentiation, we asked whether CCN3 might have a direct role in modulating osteoclast differentiation. To address this question, we used RAW 264.7 cells, a mouse monocytic cell line that expresses macrophage colony-stimulating factor and its receptor and can differentiate into osteoclast-like cells after treatment with rRANKL.^{45,46} First, we assessed whether addition of rCCN3 could substitute for rRANKL to induce osteoclast formation from monocytes. However, we did not observe

Figure 3. CCN3 overexpression does not promote mammary tumorigenesis. **A:** Immunoblot of CCN3 expression in the conditioned media (CM) and whole-cell lysate (WCL) of 66cl4 cells harboring a VC or an infected pool engineered to overexpress human CCN3. An immunoblot for α -tubulin serves as loading control. **B:** Secreted CCN3 protein in the CM was quantified by ELISA. Concentrations are expressed as picograms per milliliter of CCN3 present in media conditioned for 48 hours that has been normalized to micrograms of total lysate. **C:** *In vitro* proliferation assays for 66cl4 cells harboring a VC and an infected pool overexpressing human CCN3. Data are representative of two independent experiments, counted in triplicate for each time point. **D:** *In vivo* mammary outgrowth for 66cl4 cells overexpressing human CCN3 compared with VC control cells. The average tumor volume was calculated from eight individual VC and seven independent CCN3-overexpressing mammary tumors. Error bars represent SD.

Figure 4. CCN3 expression enhances the formation of bone metastases. **A:** Cardiac injections were performed with 66cl4 cells harboring a VC or those overexpressing human CCN3. Mice were sacrificed after 2 weeks, and the percentage of mice developing histologically verified lesions (**left**), the percentage of bones (femur or tibia) possessing lesions (**middle**), and the percentage tumor area/tissue area (**right**) were determined. * $P = 0.025$. **B:** Representative images of H&E-stained hind limbs are shown from mice injected with VC (**left**) or CCN3-expressing 66cl4 (**right**). Dotted lines delimit the region of hind limbs with 66cl4-derived bone metastatic lesions.

osteoclast formation in cultures treated only with rCCN3 (see Supplemental Figure S3, CC, at <http://ajp.amjpathol.org>), which resembled the undifferentiated monocytes present in RAW 264.7 cells cultured in DMEM alone (see Supplemental Figure S3, DD, at <http://ajp.amjpathol.org>). We previously showed that soluble factors produced by breast cancer cells can induce osteoclast formation from monocytes primed for a short time with rRANKL^{30,32}; therefore, we considered whether rCCN3 could be effec-

tive in inducing osteoclastogenesis from rRANKL-primed precursors. RAW 264.7 cells were primed for 2 days with rRANKL, after which the media was replaced by DMEM alone or DMEM containing rCCN3 (600 ng/mL). We observed the cultures for an additional 2 days and stained them for tartrate-resistant alkaline phosphatase, a marker of osteoclast differentiation. We found that rCCN3 induced an eightfold increase in the number of osteoclasts that formed in rRANKL-primed cultures compared with rRANKL-primed cells treated with DMEM (Figure 5, C and D). In addition, rRANKL-primed cells (2 days) subsequently treated with a combination of rRANKL and rCCN3 (3 days; see Supplemental Figure S3, RR + C, at <http://ajp.amjpathol.org>) repeatedly displayed higher numbers of osteoclasts compared with cultures subjected to 5 days of rRANKL treatment (see Supplemental Figure S3, RR, at <http://ajp.amjpathol.org>); however, this trend was not significant. This is the first evidence that CCN3 can directly enhance the differentiation of osteoclast precursors that have been primed with rRANKL.

CCN3 Induces Intracellular Calcium Oscillations in rRANKL-Primed RAW 264.7 Cells

Based on these results, we investigated how CCN3 could potentiate osteoclast differentiation in RANKL-primed precursors. Previous studies demonstrated that CCN3 could induce intracellular calcium release and the uptake of extracellular calcium in glioblastoma cells.^{47,48} Moreover, it has been demonstrated that Ca^{2+} signaling is critical for osteoclast differentiation.⁴⁹ Based on these observations, we evaluated whether CCN3 could influence $[Ca^{2+}]_i$ in rRANKL-primed RAW 264.7 cells. RAW 264.7 cells were primed for 48 hours with rRANKL, and the cells were subse-

Figure 5. CCN3 impairs osteoblast differentiation while stimulating osteoclast differentiation. **A:** Ascorbic acid (AA)-induced osteoblast differentiation from primary bone marrow cultures was quantified after treatment with BSA or rCCN3 by measuring the average surface area of alkaline phosphatase (ALP) staining (**left**). MEM indicates cultures that were not stimulated with AA. Data are the average of six independent experiments performed in duplicate. Error bars represent SE. * $P = 0.05$, *** $P \leq 0.001$. Representative image depicting ALP staining of primary mouse bone marrow cells in response to the indicated treatments (**right**). **B:** Quantitative RT-PCR was performed to monitor expression of RANKL and OPG mRNA (normalized to β -actin) in primary bone marrow cells after 9 days of treatment with AA alone or combined with 600 ng/mL rCCN3 (**top**). Ratio of RANKL/OPG expression in AA- and rCCN3-treated primary bone marrow cultures (**bottom**). Data represent the average of three independent experiments performed in triplicate. Error bar represents SE. **C:** Osteoclast numbers observed in rRANKL-primed cultures treated with rCCN3 (600 ng/mL) were normalized to the numbers observed in rRANKL-primed cultures treated with DMEM. Data are the average of four independent experiments performed in duplicate. Error bars represent SE. * $P < 0.001$. **D:** Representative images of tartrate-resistant alkaline phosphatase-stained RAW 264.7 mouse monocytic cells primed with 50 ng/mL rRANKL for 48 hours followed by addition of DMEM alone (**left**) or DMEM containing human rCCN3 (600 ng/mL) (**right**) for 48 hours.

Figure 6. CCN3 induces calcium signaling in rRANKL-primed RAW 264.7 cells. **A:** Raw 264.7 cells were primed with RANKL for 48 hours and then loaded with a calcium-sensitive dye, fura-2-AM. Baseline $[Ca^{2+}]_i$ was recorded, then DMEM alone or combined with rRANKL (50 ng/mL) or human rCCN3 (600 ng/mL) was added and the cells were imaged for 2 minutes. Representative $[Ca^{2+}]_i$ traces in three different cells after addition of DMEM (**left**), rRANKL (**middle**), or rCCN3 (**right**). Data are from one of four independent experiments. **B:** Representative images of pseudocolors varying from blue-green (low $[Ca^{2+}]_i$) to red-white (high $[Ca^{2+}]_i$), before (**top**) and after (**bottom**) addition of media alone (**left**) or media containing rRANKL (**middle**) or rCCN3 (**right**). **C:** Numbers of active cells (≥ 2 oscillations) after addition of DMEM alone or DMEM containing rCCN3 were normalized to the numbers of active cells after addition of DMEM containing rRANKL. Data represent the average ratio of four independent experiments. Error bars represent SE. * $P < 0.002$.

quently loaded with fura-2-AM. The $[Ca^{2+}]_i$ was measured over 2 minutes after addition of DMEM, rRANKL (50 ng/mL), or rCCN3 (600 ng/mL). Cells were considered as oscillating when at least two peaks of calcium increase were observed during the recording time. As expected, DMEM addition [Figure 6, A and B (left)] induced no changes in $[Ca^{2+}]_i$, whereas rRANKL-stimulated cells [Figure 6, A and B (middle)] displayed calcium oscillations. We found that rCCN3 treatment of RAW 264.7 cells also induced rapid oscillatory changes in $[Ca^{2+}]_i$ [Figure 6, A and B (right)]. Moreover, the percentage of cells in a field undergoing active oscillations in $[Ca^{2+}]_i$ was significantly higher in rCCN3-stimulated cultures compared with cells treated with DMEM or rRANKL (Figure 6C). However, we did not observe additive or synergistic effects when cells

were treated with both rRANKL and rCCN3 (see Supplemental Figure S4 at <http://ajp.amjpathol.org>).

To determine whether these observed calcium oscillations were important for CCN3-induced osteoclastogenesis of rRANKL-primed RAW 264.7 cells, we incubated cells with a Ca^{2+} chelator (BAPTA-acetoxymethyl ester) on day 3 before treatment with either DMEM alone or DMEM containing rCCN3 or rRANKL. We determined that treatment with the chelator resulted in 50% and 25% decreases in the formation of osteoclasts by rCCN3 and rRANKL, respectively (Figure 7). Taken together, these results indicate that CCN3 is capable of inducing the mobilization of $[Ca^{2+}]_i$, which plays an important role in promoting CCN3-induced osteoclastogenesis of rRANKL-primed RAW 264.7 cells.

Figure 7. CCN3-mediated induction of osteoclast differentiation of rRANKL-primed monocytes relies on calcium signaling. The number of osteoclasts that differentiated in response to DMEM alone (DD) or human rCCN3 alone (CC) (600 ng/mL) or after priming with rRANKL and subsequent stimulation with DMEM (RD), human rCCN3 (RC; 600 ng/mL), or rRANKL (RR; 50 ng/mL) is shown in the graph. In addition, rRANKL-primed RAW264.7 cells were pretreated with a calcium chelator (BAPTA) before stimulation with rCCN3 (RC + B) or rRANKL (RR + B). Data are from one of two independent experiments, each performed in triplicate. Error bars represent SD. * $P < 0.02$; ** $P < 0.04$. ns, not significant. Representative images of tartrate-resistant alkaline phosphatase-stained RAW 264.7 mouse monocytic cells treated with DMEM alone (DD) or human rCCN3 alone (CC) (600 ng/mL) or after priming with rRANKL (50 ng/mL) for 2 days and subsequent stimulation with DMEM (RD), human rCCN3 (RC; 600 ng/mL), or rRANKL alone (RR; 50 ng/mL) or pretreated with BAPTA before stimulation with rCCN3 (RC + B) or rRANKL (RR + B). **Arrows** indicate an osteoclast. Scale bar = 250 μ m.

Figure 8. CCN3 promotes nfatc1 nuclear localization in RANKL-primed RAW 264.7 cells. **A:** RAW 264.7 cells were primed with rRANKL (50 ng/mL) for 48 hours, and then media alone or media containing rRANKL (50 ng/mL) or rCCN3 (600 ng/mL) was added for 15, 30, or 60 minutes. Cells were fixed and stained for nfatc1. nfatc1 was visualized by immunofluorescence, and a minimum of four fields per condition were used to determine the percentage of cells exhibiting nuclear nfatc1 over the total number of cells present in the field. The data represents three independent experiments performed in duplicate, and two observers scored the percentage of cells with nuclear nfatc1 localization. * $P < 0.10$; ** $P < 0.05$. **B:** Representatives images of nfatc1 immunofluorescence on the RAW 264.7 cells after 60 minutes of treatment with DMEM only (**top**), rRANKL (50 ng/mL) (**middle**), or rCCN3 (600 ng/mL) (**bottom**). **White arrows** indicate a nuclear localization of nfatc1. Scale bar = 10 μ m and applies to all panels.

CCN3 Induces Nuclear nfatc1 Localization in rRANKL-Primed RAW 264.7 Cells

It has been demonstrated that Ca^{2+} signaling is critical for osteoclast differentiation in part through its ability to induce nuclear localization of nfatc1, a transcription factor important in osteoclastogenesis.⁴⁹ We performed immunofluorescence experiments to determine whether CCN3 could also affect the nuclear translocation of nfatc1. We observed that treatment with rCCN3 (600 ng/mL), similar to rRANKL stimulation (50 ng/mL), resulted in a significant increase in the percentage of cells displaying nfatc1 nuclear localization, which was sustained for at least 60 minutes after stimulation (Figure 8). Moreover, rCCN3-stimulated cultures displayed a higher percentage of cells with nuclear nfatc1 compared with cells treated with rRANKL (Figure 8), which is in agree-

ment with the percentage of cells displaying calcium oscillations in response to these stimuli (Figure 6C). Together, these results demonstrate that rCCN3 can induce nfatc1 nuclear localization, which is important for osteoclast differentiation.

Signaling through JNK and PKC Is Involved in CCN3-Induced Osteoclast Differentiation

It has previously been shown that breast cancer–derived factors involve MAPKs and calcium/PKC pathways to form osteoclasts from rRANKL-primed precursors.³⁰ Thus, we sought evidence of whether CCN3 played a role in activating these signaling pathways in rRANKL-primed RAW 264.7 cells. We observed that CCN3 treatment resulted in an increase in phosphorylation of JNK and PKC but not p38 (data not shown) or ERK1/2 compared with the control treatment (DMEM) (see Supplemental Figure S5 at <http://ajp.amjpathol.org>). In fact, CCN3 stimulation resulted in reduced levels of pERK1/2 compared with DMEM-treated controls. Together, these results argue that CCN3-dependent activation of JNK and PKC signaling pathways may be involved in rCCN3-induced osteoclastogenesis of rRANKL-primed precursors.

Discussion

Previous studies have examined the relationship between *CCN1/Cyr61*, *CCN2/CTGF*, and *CCN3/Nov* expression and clinical outcome in breast cancer. A positive association exists between high CCN1 expression and larger tumor size, lymph node involvement, and metastasis.^{50–52} Whereas some studies suggest that CCN2/CTGF is underexpressed in breast tumors relative to normal tissue,⁵¹ others demonstrate that high CCN2/CTGF levels are critical for the bone metastatic phenotype of breast cancer cells.^{53,54} Several studies have also examined the relationship between CCN3 levels and clinical outcome in patients with breast cancer. One study found no correlation between CCN3 mRNA levels and clinical outcome.⁵² Paradoxically, although high CCN3 expression in breast tumors is associated with good patient outcome,⁵¹ it also predicts resistance to endocrine therapies and/or cross-resistance in breast tumor samples and cell lines.⁵⁵ Indeed, *CCN3* is an estrogen-regulated gene, which may contribute to these contradictory biological outcomes.⁵⁶ The present data extend these observations and demonstrate a role for CCN3 in promoting breast cancer metastasis to bone.

CCN3 was first identified as a gene overexpressed in myeloblastosis-associated virus-induced nephroblastoma in chickens, giving rise to the designation of *Nov* for nephroblastoma overexpressed.⁵⁷ CCN3 effects on tumor cell growth are complex and context dependent. Indeed, some studies suggest that CCN3 suppresses breast cancer cell proliferation,³³ although we do not observe any effect of CCN3 on the proliferation of 66cl4 breast cancer cells *in vitro* or on mammary tumor outgrowth in the orthotopic site. Thus, CCN3 does not consistently regulate primary tumor cell growth, suggesting that it may elicit varying responses de-

pending on the cell type and the interacting partners present in these cells.^{12,58}

Conversely, additional studies link high *CCN3* expression to poor clinical outcomes in prostate²⁰ and renal cell carcinomas.^{58,59} These observations are in agreement with a recent study demonstrating that *CCN3* increases the metastatic potential of melanoma cells without affecting subcutaneous growth rates.¹⁶ *CCN3* can impair the proliferation of Ewing's sarcoma cells and diminish their tumorigenicity *in vivo* while enhancing the migratory and invasive phenotype of Ewing's sarcoma cells.¹⁷ Moreover, high *CCN3* levels are associated with increases in the incidence of metastatic disease in patients with Ewing's sarcoma.¹⁸ We demonstrate a novel role for *CCN3* in the bone microenvironment in facilitating the formation of osteolytic bone metastases. The present results reveal that *CCN3* is expressed in breast cancer cells in bone metastases, which is in agreement with a previous study revealing that *CCN3/Nov* is highly expressed in breast cancer bone metastases compared with visceral metastases, such as lung, liver, and brain.⁶⁰ We demonstrate that *CCN3* also enhances breast cancer growth in the bone microenvironment. These data argue that *CCN3* expression may not affect the aggressiveness of breast cancer cells in the primary site but may play a role in the ability of breast cancer cells to grow in the bone microenvironment.

Tumor cells must interact with cells present in the metastatic microenvironment to grow and survive in a distant site. Bone is composed of two main types of cells, osteoblasts and osteoclasts, which are responsible for bone formation and resorption, respectively. The coordinated differentiation of these cell types is critical to maintain normal bone turnover and tissue homeostasis. However, the arrival of tumor cells in the bone microenvironment can disrupt this interplay and tip the balance to a resorptive state. This creates a vicious cycle whereby factors released from the bone microenvironment through osteoclast-mediated bone resorption can promote tumor cell growth, which further potentiates bone destruction.³ Two proteins, RANKL and OPG, are critical for maintaining the coordinated action of osteoblasts and osteoclasts. RANKL induces osteoclastogenesis and is expressed by osteoblast precursors, whereas OPG is a soluble decoy receptor that binds and neutralizes RANKL.^{42,43} We demonstrate that *CCN3* can impair osteoblast differentiation in a primary culture of bone marrow cells. In doing so, *CCN3* alters the RANKL/OPG ratio to favor increased concentrations of RANKL and lower concentrations of OPG, a soluble decoy receptor for RANKL. This observation is in agreement with published results demonstrating that rCCN3 can impair osteoblast differentiation of established cell models by interacting directly with and activating Notch, a known inhibitor of osteoblast differentiation.^{38,61} The C-terminal domain of *CCN3* has been implicated in the activation of Notch signaling. The *CCN3* C-terminal domain induces transcription of *hey1* and *hes1*, two downstream targets of the cleaved Notch intracellular domain, whereas a mutant *CCN3* protein lacking this domain (*Ccn3delCT*) fails to induce these targets.³⁸ However, we performed immunofluorescence on rCCN3-treated primary culture of bone marrow cells and

did not observe significant nuclear translocation of the Notch intracellular domain or induction of *Hes1* or *Hey1* transcripts (data not shown). Furthermore, *CCN3* can also interact with and antagonizes bone morphogenetic protein 2 signaling, which can also lead to the inhibition of osteoblast differentiation.^{38–40,61,62}

The importance of *CCN3* in regulating bone formation has also been revealed through the characterization of transgenic mouse models. Overexpression of *CCN3*, driven by the osteocalcin promoter, resulted in a decrease in bone formation in one transgenic line and osteopenia, secondary to an increase in bone resorption, in a second transgenic line.⁴⁰ The present results are in agreement with these observations and suggest that breast cancer cells can subvert the normal role played by *CCN3* to favor bone destruction that characterizes osteolytic breast cancer bone metastases. In another transgenic mouse model, targeting of the third exon encoding the von Willebrand factor type C domain (*NOV^{del3}–/–*) resulted in mice that did not express full-length *CCN3* and displayed very low levels of a *CCN3* protein lacking the von Willebrand factor type C domain. These mice displayed numerous phenotypes, including enlarged vertebrae, elongated long bones/digits, and a delay in ossification.³⁷

Another novel finding in the present study was the demonstration that rCCN3 can support osteoclast differentiation of rRANKL-primed, but not naive, osteoclast precursors. This observation is interesting in light of the results demonstrating that rCCN3 impairs osteoblast differentiation, leading to elevated levels of RANKL and decreased OPG expression. Thus, breast cancer cells that express high levels of *CCN3* would be equipped to alter the functions of osteoblasts and osteoclasts to favor bone destruction. Indeed, most breast cancer bone metastases that we analyzed demonstrated strong *CCN3* staining in the tumor epithelium or stroma. The present observations, coupled with published data suggesting that *CCN3/NOV* is highly expressed specifically in bone metastases,⁶⁰ suggest that *CCN3* expression may be up-regulated once breast tumor cells reach the bone.

The present results suggest that *CCN3* promotes osteoclast differentiation of rRANKL-primed precursors through its ability to induce calcium oscillations and subsequent nfatc1 nuclear localization. A role for *CCN3* in calcium signaling has not been extensively studied; however, previous studies have shown that *CCN3* can induce calcium mobilization in different cell types.^{47,48} In the osteoclast precursor, nuclear localization of the transcription factor nfatc1 is essential for the expression of tartrate-resistant acid phosphatase, calcitonin receptor, cathepsin K, and $\beta 3$ integrin, which leads to osteoclastogenesis.^{49,63–65} RANKL contributes to the expression of nfatc1^{63,65} and allows nfatc1 to undergo autoamplification after a positive feedback regulation.⁶⁶ We demonstrate that rCCN3, in rRANKL-primed cells, can induce calcium mobilization to a greater degree compared with rRANKL itself, resulting in the robust nuclear translocation of nfatc1. These observations are interesting in light of the results indicating that rCCN3-mediated effects on osteoclast differentiation seem to be more sensitive to a calcium chelator com-

pared with rRANKL stimulation. These data extend previous studies implicating CCN3 in the control of calcium mobilization and is the first to demonstrate such a role in osteoclast differentiation.

The present results argue that CCN3-mediated effects on osteoclast differentiation may be influenced by its ability to activate JNK and PKC α/β phosphorylation. Moreover, CCN3 stimulation reduced pERK1/2 levels while having no effect on p38 phosphorylation (data not shown). We previously demonstrated that soluble factors secreted from human breast cancers favor osteoclastogenesis of rRANKL-primed monocytes by inducing calcium oscillations in these cells. In these studies, activation of the p38, ERK, and PKC pathways were observed after treatment with conditioned media from breast cancer cells.^{30,32} Thus, CCN3 seems to engage different signaling pathways to promote osteoclastogenesis of rRANKL-primed precursors. The present results are consistent with previous observations indicating that CCN3 can impair ERK phosphorylation in chronic myeloid leukemia.⁶⁷ Moreover, CCN3 has recently been shown to differentially rely on JNK or p38 signaling in astrocytes, which is believed to be dictated by the particular integrin receptors engaged by CCN3.⁶⁸

We revealed a functional role for CCN3 in promoting breast cancer metastasis to bone. This effect is likely to stem from the ability of CCN3 to impair osteoblast differentiation while simultaneously promoting osteoclastogenesis from rRANKL-primed precursors. In this way, CCN3 influences both of the main cell types in bone to create a pro-resorptive environment characteristic of osteolytic breast cancer bone metastases.

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