ORIGINAL ARTICLE

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Direct Crosstalk Between Cancer and Osteoblast Lineage Cells Fuels Metastatic Growth in Bone via Auto-Amplification of IL-6 and RANKL Signaling Pathways

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ABSTRACT

The bone microenvironment and its modification by cancer and host cell interactions is a key driver of skeletal metastatic growth. Interleukin-6 (IL-6) stimulates receptor activator of NF-κB ligand (RANKL) expression in bone cells, and serum IL-6 levels are associated with poor clinical outcomes in cancer patients. We investigated the effects of RANKL on cancer cells and the role of tumor-derived IL-6 within the bone microenvironment. Using human breast cancer cell lines to induce tumors in the bone of immune-deficient mice, we first determined whether RANKL released by cells of the osteoblast lineage directly promotes IL-6 expression by cancer cells in vitro and in vivo. We then disrupted of IL-6 signaling in vivo either via knockdown of IL-6 in tumor cells or through treatment with specific anti-human or anti-mouse IL-6 receptor antibodies to investigate the tumor effect. Finally, we tested the effect of RANK knockdown in cancer cells on cancer growth. We demonstrate that osteoblast lineage-derived RANKL upregulates secretion of IL-6 by breast cancers in vivo and in vitro. IL-6, in turn, induces expression of RANK by cancer cells, which sensitizes the tumor to RANKL and significantly enhances cancer IL-6 release. Disruption in vivo of this auto-amplifying crosstalk by knockdown of IL-6 or RANK in cancer cells, or via treatment with anti-IL-6 receptor antibodies, significantly reduces tumor growth in bone but not in soft tissues. RANKL and IL-6 mediate direct paracrine-autocrine signaling between cells of the osteoblast lineage and cancer cells, significantly enhancing the growth of metastatic breast cancers within bone. © 2014 American Society for Bone and Mineral Research.

KEY WORDS: BONE METASTASIS; BREAST CANCER; OSTEOBLAST; INTERLEUKIN-6; RANKL

Introduction

At advanced stages, breast cancer exhibits a strong propensity to metastasize to the skeleton.[1] Bone metastases cause considerable morbidity and are associated with poor clinical outcomes.[2] Prior studies have reported significant associations between bone remodeling rates and the incidence of skeletal-related events (SRE), whereas, conversely, treatment-induced reductions in bone turnover are associated with improved clinical outcomes and patient survival.[3,4] Bone turnover is, therefore, a major determinant of skeletal tumor progression in humans. Experimental models of bone metastasis have provided a mechanistic rationale for these clinical observations in that cancer cells within bone stimulate bone turnover,[5] which in turn promotes cancer growth.[6–9] Equally, suppression of bone remodeling strongly reduces intraskeletal cancer growth.[10–12]

This complex interplay between bone microenvironment and metastatic tumor cells has been described by Mundy and colleagues as the "vicious cycle."[13] In this model, tumor-derived factors, such as parathyroid hormone-related protein (PTHrP) and interleukins (IL) 6 and 8, upregulate expression of receptor activator of NF-κB ligand (RANKL) by osteoblast lineage cells (ie, osteoblast precursors, osteoblasts, and osteocytes). RANKL then binds to the RANK on osteoclasts and osteoclast precursors to increase osteoclast recruitment and formation and to activate bone resorption. Accelerated bone resorption triggers the release of growth factors embedded in bone matrix, which, in turn, act on cancer cells to promote their further growth.[13–15]

Several studies have demonstrated that high circulating levels of interleukin-6 (IL-6) correlate with disease extent and

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progression, predicting poor clinical outcomes in patients with metastatic breast cancer.[16,17] Others have shown that IL-6 promotes malignant features in cancer cells[18] and may induce epithelial-mesenchymal phenotype transition in human breast cancer.[19] In bone, IL-6 is well known to stimulate RANKL expression in osteoblasts/osteoblast lineage cells.[13,20] Previous studies using neuroblastoma or breast cancer cell lines demonstrated that cells of the osteoblast lineage produce cytokines such as IL-6, which can either promote tumor growth or induce bone resorption to allow for further tumor growth in bone.[21–24] Furthermore, many tumors express RANK, and a high concordance between RANK expression in the primary cancer and its skeletal secondaries has been reported.[25] Finally, human recombinant RANKL induces migration of RANK-expressing breast cancer and melanoma cells in vitro.[26] These data suggest a role of RANKL and RANK in bone metastasis. However, the effects of RANKL on cancer cells and the role of tumor-expressed RANK within the bone microenvironment remain to be investigated.

In the present study, we demonstrate that osteoblasts directly communicate with cancer cells in bone via two distinct but complementary signaling pathways to increase IL-6, RANK, and RANKL levels within bone. This direct crosstalk functions as a self-amplifying feed-forward loop, which operates in parallel to the "classical" vicious cycle and significantly enhances tumor growth in bone.

Materials and Methods

Antibodies and reagents

Osteoprotegerin (OPG) was provided by Amgen Inc. (Thousand Oaks, CA, USA). Recombinant human cytokines RANKL (390-TN/CF), IL-6 (206-1L), and anti-RANK monoclonal antibodies (Clone 80707) were from R&D Systems (Minneapolis, MN, USA). Tocilizumab, a humanized anti-human IL-6R monoclonal antibody, was purchased from Roche Pharmaceuticals (Roche Pharma, Grenzach-Wyhlen, Germany). MR16-1, a rat-anti mouse IL-6R antibody, was provided by Chugai Pharmaceutical (Tokyo, Japan).

Cell culture and real-time RT-PCR

The estrogen-independent human breast cancer cell line MDA-MB-231 (provided by Dr Yoneda, Japan)[27] and the mouse preosteoblastic cell line Kusa O (provided by Dr Martin, Melbourne)[28] were cultured in Dulbecco's modified Eagle medium (DMEM), supplemented with 10% fetal calf serum (FCS; JRH Biosciences, Lenexa, KS, USA) and 1% penicillin-streptomycin. Unless otherwise stated, tissue culture media and supplements were from Invitrogen (Carlsbad, CA, USA). Cell growth assays were performed as described previously.[11] A Matrigel Invasion Chamber Kit (BD Biosciences, San Jose, CA, USA) was used to assess invasive properties of cancer cells. mRNA levels for gene expression of human GADPH, IL-6, IL-6R, RANK, PTHrP, and MMP-2,3,9,13 were assessed by real-time RT-PCR using species-specific primers (Supplemental Table S1).

Biochemical assays

IL-6 protein secreted by cancer cells in vitro was measured by ELISA (BD Biosciences Pharmingen) after plating 10,000 cells/well onto 96-well plates for 24 hours. Serum levels of tartrate-resistant acid phosphatase 5b (TRAcP5b) and procollagen type I amino-terminal propeptide (PINP) were measured by ELISA.[11]

IL-6 pretreatment, RANKL treatment, and human IL-6 ELISA

Cancer cells (5×10^4 cells/well) were seeded onto 24-well plates in 10% FCS DMEM. After overnight culture, the cells were treated with IL-6 using concentrations of 12.5, 25, 50, 100 ng/mL for 4 hours under serum-free conditions (0.1% BSA DMEM), after which the cells were thoroughly washed and then treated with RANKL (50 ng/mL). After 4 and 24 hours, media were collected for measurement of human IL-6 by ELISA (see above).

Coculture of osteoblasts and MDA-MB-231cells

Kusa O preosteoblasts were differentiated under osteogenic conditions (50 μg/mL ascorbic acid and 10 mM β-glycerophosphate) in a 24-well plate. On day 11 after differentiation, MDA-MB-231 cells were seeded (3×10^4 cells per well) on the top of Kusa O cells and cocultured for 24, 48, or 72 hours. Media were collected and IL-6 levels assayed by human (see above) and mouse IL-6–specific (BD Biosciences Pharmingen) ELISA. For primary osteoblasts, mouse calvaria cells were isolated by sequential enzyme digestion as per a previous study.[29] Populations 3 and 4 were cultured and differentiated in a similar manner to KUSA O preosteoblasts in a 24-well plate. On day 10 after differentiation, MDA-MB-231 were seeded (5×10^4 cells per well) on top of differentiated mouse primary calvaria osteoblasts and cocultured for 24, 48, and 72 hours. To evaluate the role of RANKL in the coculture system, OPG was added at a concentration of 1 ng/mL to the media every 24 hours. The conditioned media were collected, and IL-6 level was determined by human and mouse IL-6 ELISA as above.

Knockdown of IL-6 and RANK expression in cancer cells

IL-6 and RANK expression was silenced in MDA-MB-231 cells via a lentiviral-based expression system driving the production of short hairpin RNA species (shRNAs, Sigma, St. Louis, MO, USA), according to the manufacturer's protocol. The clones selected were IL-6 (TRCN0000059205), RANK (TRCN0000003350), and nontarget control (SHC002V).

Animal models, histology, and histomorphometry

Murine xenograft models were used to study breast cancer cell growth in bone and, orthotopically, in mammary fat pads or in subcutaneous tissues, in accordance with Animal Welfare Guidelines and approved protocols. Technical details of these models as well as the in vivo histology and histomorphometry techniques used for analysis are described in the Supplemental Data.

Statistical analysis

All statistical analyses were conducted using one-way ANOVA followed by Bonferroni's adjustment, where there were multiple comparisons by means of SPSS 17.0 for Windows (SPSS Inc., Chicago, IL, USA). Significance was accepted where $p < 0.05$.

Results

Suppressed and accelerated bone resorption cause corresponding changes in tumor IL-6 expression

To examine the effects of bone turnover on cytokine expression by cancer cells, MDA-MB-231 cells were implanted intratibially into nude mice fed a diet normal or low in calcium content,

resulting in normal or accelerated bone turnover, respectively. As previously demonstrated,[8] increased bone turnover resulting from high PTH levels in low-calcium-fed mice was associated with larger tumors and bone lesions compared with controls (Fig. 1*A, B*). Tumors in mice with accelerated bone turnover contained a higher proportion of IL-6-positive cells than those from animals with normal bone turnover (Fig. 1*C*), consistent with changes in human IL-6 mRNA expression (Fig. 1*D*). Simultaneously, serum RANKL levels were significantly higher in mice with accelerated bone turnover compared with those with normal bone remodeling rates (59.4 ± 1.5 pg/mL versus 35.8 ± 9.4 pg/mL, $p < 0.05$) (Fig. 1*E*). In a corresponding experiment testing the effects of reduced bone turnover on cytokine expression, nude mice implanted intratibially with MDA-MB-231 cells were treated with OPG (1 mg/kg/d), ibandronate (IBN; 160 μg/kg/d), or saline for 7 days, commencing on day 10 postimplantation. Compared with control mice, treatment with OPG or IBN similarly inhibited development of lytic bone lesions (Fig. 1*F, G*). Interestingly, however, only treatment with OPG was associated with a reduction in human IL-6 mRNA expression levels (Fig. 1*H*).

Taken together, these data demonstrate that in vivo, low and high levels of bone turnover are associated with corresponding changes in tumor IL-6 expression. Because OPG inhibits osteoclast differentiation and function by neutralizing RANKL, whereas bisphosphonates such as IBN inactivate osteoclasts through direct cytotoxic effects, the differential effects of OPG and IBN on tumor IL-6 expression indicate that locally available RANKL rather than osteoclastic bone resorption determines tumor IL-6 expression.

Recombinant and cell-derived RANKL promote IL-6 expression in cancer cells in vitro

To further explore the above hypothesis, we tested whether treatment of MDA-MB-231 cells with recombinant human RANKL (rhRANKL) impacted on tumor IL-6 expression in vitro. As shown in Fig. 2*A*, rhRANKL increased IL-6 expression in cancer cells in a concentration-dependent manner, with a maximum response at 50 ng/mL. There was little difference in IL-6 expression between 50 and 100 ng/mL rhRANKL, suggesting signaling pathway saturation at these concentrations. Prolonged treatment of MDA-MB-231 cells with 50 ng/mL rhRANKL for up to 24 hours resulted in sustained increases in tumor IL-6 expression (Fig. 2*B*). In addition, rhRANKL transcriptionally regulated IL-6 expression in MDA-MB-231 cells using IL-6 promoter constructs (Supplemental Data, Supplemental Fig. S1*A, B*).

Osteoblast-generated RANKL promotes IL-6 expression in cocultured cancer cells

Because locally produced RANKL is primarily derived from the cells of osteoblast lineage,[30,31] we next employed an in vitro coculture model to investigate whether RANKL generated by osteoblasts increases IL-6 secretion in cancer cells. To this aim, murine Kusa O osteoblast-like cells were differentiated for 11 days (Supplemental Data, Supplemental Fig. S2*A*), at which time they release high levels of RANKL (Fig. 2*C*). These cells were then cocultured with MDA-MB-231 cells for 72 hours. Compared with MDA-MB-231 cells cultured alone, coculture with RANKL-secreting osteoblast-like cells increased IL-6 production by MDA-MB-231 cells more than 10-fold (Fig. 2*D*), with no effects on IL-6 secretion from Kusa O cells (Supplemental Data, Supplemental Fig. S2*B*). In addition, we further demonstrated that primary osteoblasts isolated from mouse calvaria expressed high levels of RANKL at day 11 when differentiated (Supplemental Data, Supplemental Fig. S2*C*). Coculture of MDA-MB-231 cells and primary osteoblasts yielded results similar to those obtained with Kusa O cells (Fig. 2*E*). Furthermore, OPG reversed the effect of RANKL in the coculture system (Fig. 2*E*).

IL-6 induces RANK expression in cancer cells in vitro

The above experiments suggest that RANKL regulates tumor IL-6 expression in MDA-MB-231 breast cancer cells. Tumor-derived IL-6 is well known to increase RANKL expression in osteoblasts and other cells of the osteoblast lineage.[32,33] Because MDA-MB-231 cells do not express RANKL[34] (Supplemental Fig. S2*D*), we first tested whether IL-6 affects expression of RANK by cancer cells. As shown in Fig. 2*F*, treatment of MDA-MB-231 cells with IL-6 significantly increased RANK mRNA expression in vitro. These data suggest that tumor cells may be autosensitized to the action of osteoblast-derived RANKL via an IL-6-mediated, autocrine mechanism that increases RANK expression.

To further investigate this possibility, MDA-MB-231 cells were pretreated with IL-6 (from 12.5 to 100 ng/mL) for 4 hours to enhance RANK expression. After a thorough wash, cells were then exposed to RANKL (50 ng/mL) for 4 to 24 hours. As shown in Fig. 2*G, H*, the amount of IL-6 released in response to RANKL was significantly greater in cancer cells pretreated with IL-6, with concentrations as low as 12.5 ng/mL being effective. Thus, although rhRANKL alone has relatively modest effects on IL-6 expression by native tumor cells (Fig. 2*A, B*), pre-exposure of such cells to IL-6 greatly amplifies the effects of RANKL on tumor IL-6 expression.

Taken together, the above in vitro data suggest the presence of two signaling pathways that support and amplify direct crosstalk between cancer and bone cells: First, RANKL presented by osteoblasts in response to tumor-derived IL-6 directly promotes the release of IL-6 by cancer cells, which contributes to the maintenance of RANKL-induced osteoclastic bone resorption. This effect is then amplified through a second autocrine pathway where tumor-derived IL-6 upregulates RANK expression within cancer cells, thus sensitizing the tumor to RANKL, further increasing its IL-6 output. We next examined whether disrupting these signaling pathways at various levels would alter skeletal cancer growth in vivo.

Knockdown of IL-6 expression in MDA-MB-231 cells reduces circulating RANKL levels and tumor growth in bone but has no effect on orthotopic tumor growth

IL-6 expression was knocked down in MDA-MB-231 cells via a lentiviral-based expression system driving the production of short hairpin RNA species. A nontarget sequence was used as control (Fig. 3*A, B*). In vitro characterization of nontarget (NT) versus IL-6 knockdown MDA-MB-231 (MDA-IL6-KD) cells demonstrated that IL-6 knockdown significantly reduced MMP-2, 3, 9, and 13 mRNA expression, as well as tumor cell invasiveness (9% versus 21%, $p < 0.05$) (Fig. 3*C, D*). Importantly, however, IL-6 knockdown had no effect on cell growth in vitro (Fig. 3*E*).

To investigate the effects of disrupted IL-6 signaling on tumor growth in vivo, nontarget control or IL-6 knockdown MDA-MB-231 cells were injected into nude mice fed a low-calcium diet to increase bone turnover. Compared with NT controls, IL-6 knockdown MDA-MB-231 cells produced significantly smaller osteolytic lesions ($p < 0.05$ for all time points; Fig. 3*F, G*). Similarly, total tumor area at endpoint was significantly smaller in mice

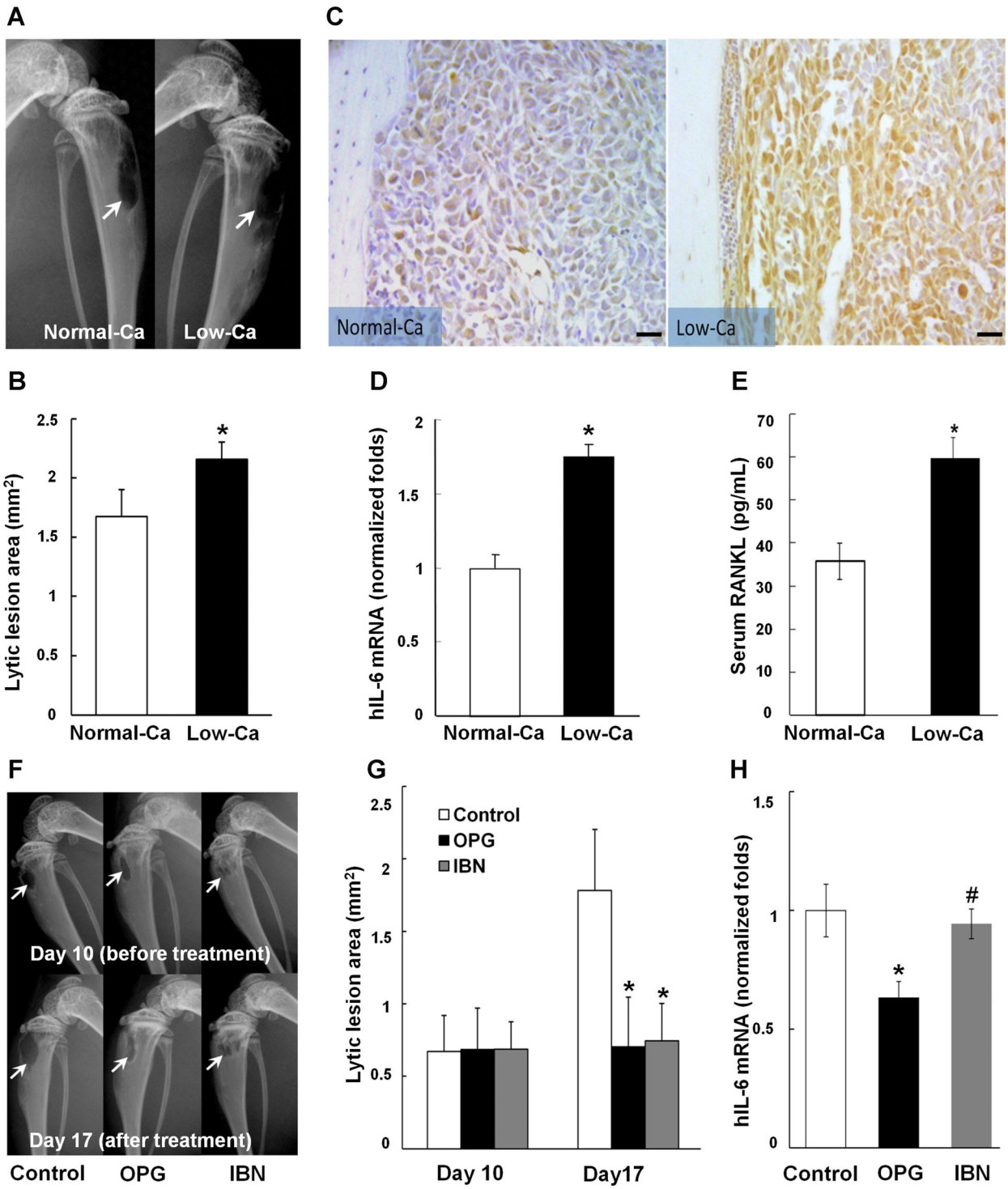

Fig. 1. Low and high bone turnover are associated with corresponding changes in tumor IL-6 expression *in vivo*. (*A, B*) Compared with controls (Normal-Ca), a low-calcium diet (Low-Ca) promoted the development of lytic bone lesions. (*A*) Representative radiographs showing lytic lesions (arrows). (*B*) Quantification of lytic lesion area ($n = 5$). (*C*) Immunohistochemistry revealed a higher proportion of IL-6-positive cancer cells in bone in the Low-Ca compared with the Normal-Ca group (scale bar = 20 μm). (*D*) Human IL-6 mRNA expression levels, relative to tumor-derived GAPDH (using human-specific primers), were increased in the Low-Ca group compared with controls ($n = 5$). (*E*) Serum murine RANKL levels were significantly higher in mice with accelerated bone turnover (Low-Ca) compared with those with normal bone remodeling rates (Normal-Ca) ($n = 5$). (*F, G*) Compared with controls, treatment with osteoprotegerin (OPG) or ibandonate (IBN) significantly reduced the development of radiographic lytic bone lesions (*F*, arrows), with no quantitative difference between the two treatment regimens (*G*). (*H*) Treatment of MDA-MB-231 cells with OPG reduced IL-6 mRNA expression levels compared with both untreated controls and IBN-treated cells. MDA-MD-231 IL-6 mRNA expression levels were similar in untreated controls and IBN-treated cells. Data are mean ± SEM. *Significantly different from vehicle-treated group ($p < 0.05$). #Significantly different from OPG treatment ($p < 0.05$).

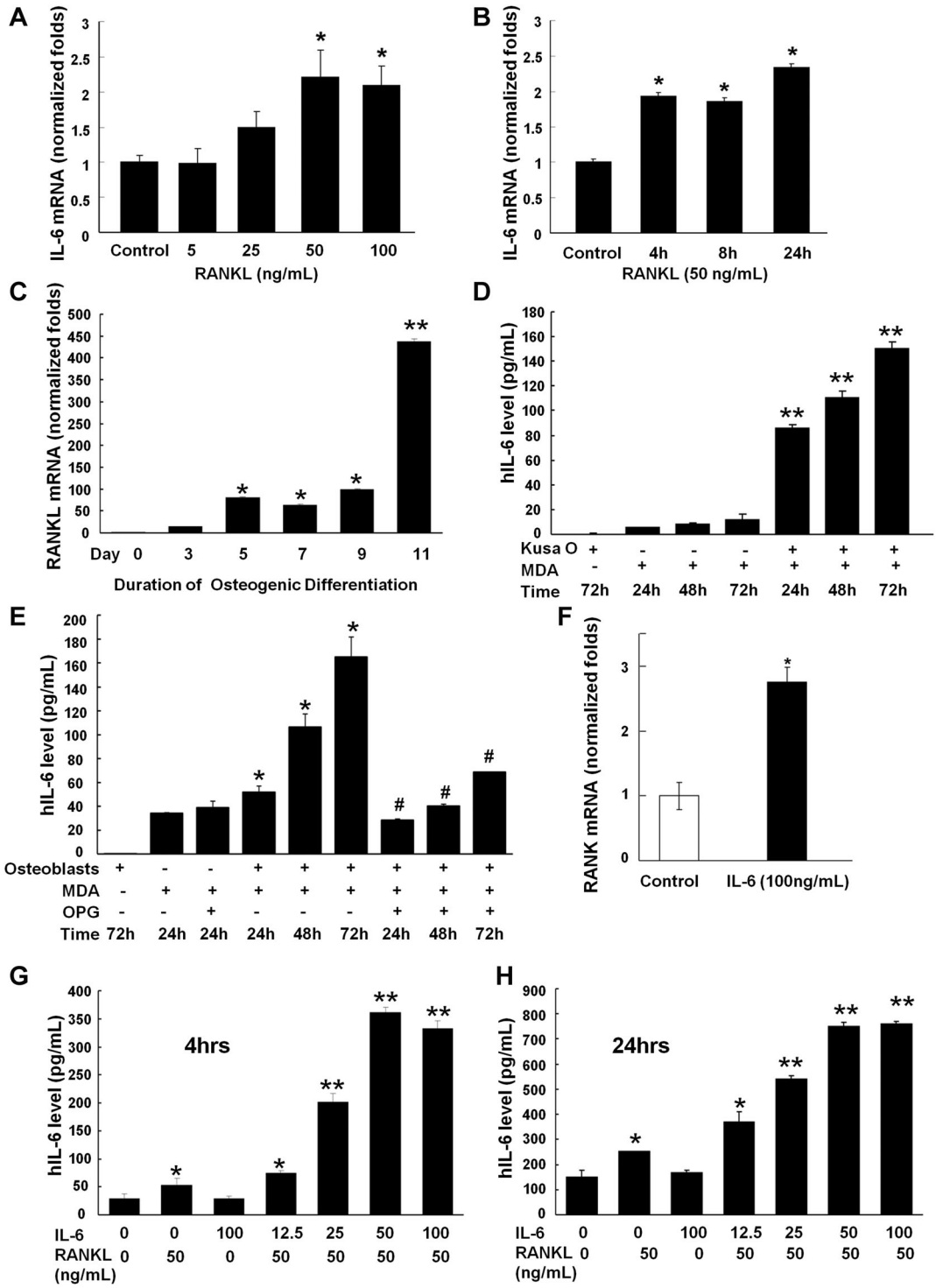

Fig. 2. RANKL regulates IL-6 expression, whereas IL-6 induces RANK expression in MDA-MB-231 cells in vitro. (*A*) Treatment of human breast cancer cells with RANKL for 4 hours dose-dependently increased IL-6 expression in MDA-MB-231 cells. (*B*) Treatment of MDA-MB-231 cells with RANKL (50 ng/mL) for 4, 8, and 24 hours resulted in a sustained increase in IL-6 expression levels. (*C*) Differentiated Kusa O cells had increased levels of RANKL expression by day 11. (*D*) Coculture of MDA-MB-231 cells with differentiated Kusa O cells expressing high levels of RANKL (day 11) promoted human IL-6 production in media in a time-dependent manner. No such effect was seen when MDA-MB 231 cells were cultured alone. (*E*) Coculture of MDA-MB-231 cells and the primary osteoblasts yielded a similar result as shown with Kusa O cell line. Furthermore, OPG was able to reverse the effect of RANKL in the coculture system (#$p < 0.05$ compared with the corresponding time points coculture without OPG treatment). (*F*) Treatment of MDA-MB-231 cells with recombinant IL-6 (100 ng/mL for 24 hours) induced RANK mRNA expression in vitro. (*G, H*) Pretreatment of MDA-MB-231 with IL-6 (12.5 to 100 ng/mL) for 4 hours increased RANK expression by cancer cells. Cells were then treated with RANKL 50 ng/mL. From 4 (*G*) or 24 (*H*) hours, RANKL treatment significantly boosted IL-6 production in pretreated cells compared with controls (ie, untreated cells or cells treated with IL-6 only). Data are mean ± SEM. Asterisks denote significantly different from controls ($^{*}p < 0.05$; $^{**}p < 0.01$). In vitro experiments were repeated 3 × ; results shown are from a representative experiment.

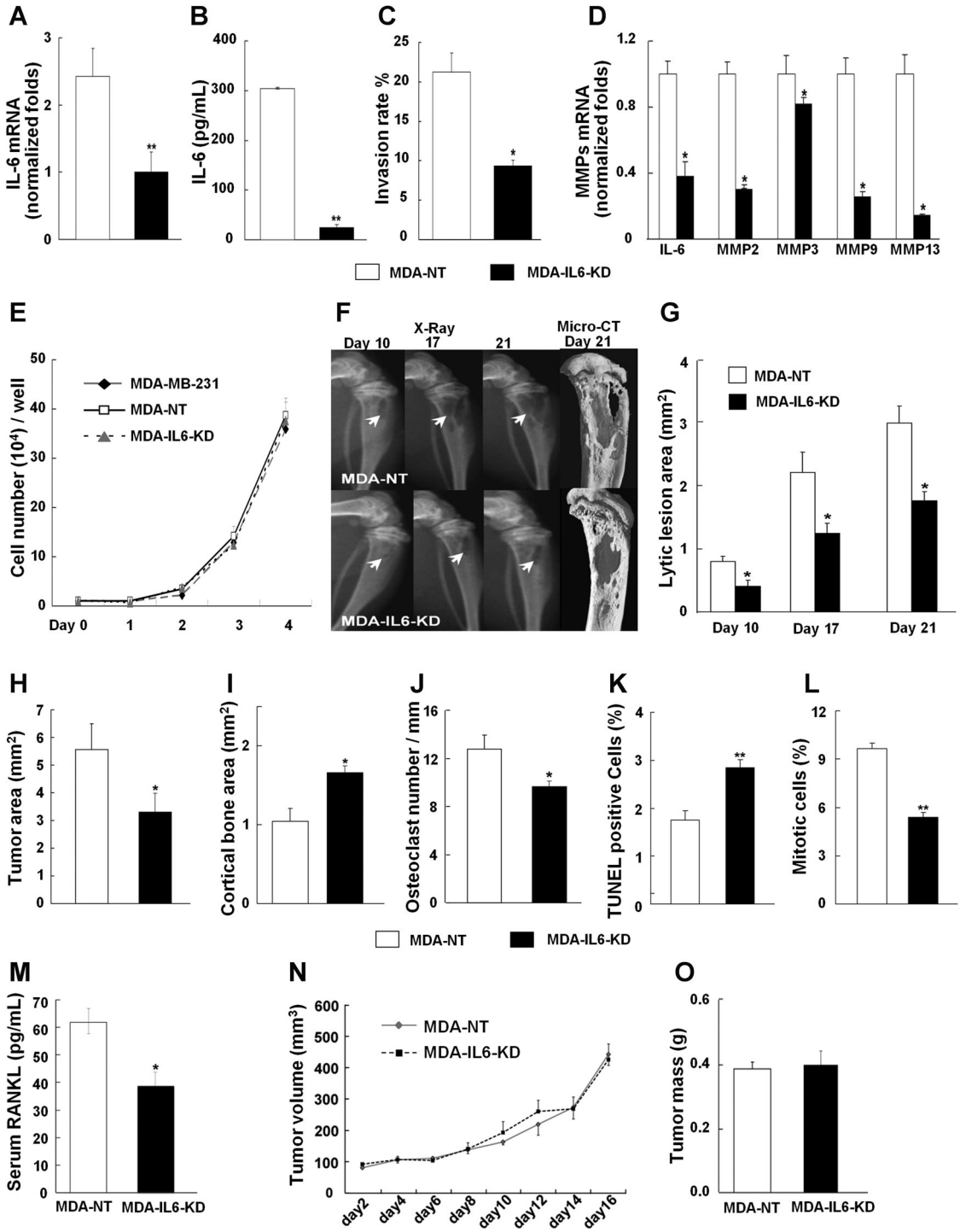

Fig. 3. Knockdown of IL-6 expression in MDA-MB-231 cells reduces circulating RANKL levels and tumor growth in bone but has no effect on orthotopic tumor growth. (*A, B*) Compared with nontarget (NT) control cells, knockdown efficacy was 60% for mRNA (real-time RT-PCR) and 80% for protein (ELISA). (*C, D*) Knockdown of IL-6 expression in MDA-MB-231 cells significantly reduced cell invasiveness and mRNA levels of MMP2, 3, 9, and 13. (*E*) Knockdown of IL-6 expression has no effect on cell growth in vitro. (*F, G*) Compared with controls, intratibial implantation of IL-6 knockdown cells resulted in significantly reduced osteolytic lesions (arrows) on days 10, 17, and 21 ($n = 8$). (*H, I*) Compared with controls, intratibial implantation of IL-6 knockdown cells resulted in significantly reduced total tumor area (*H*) and preservation of cortical bone area (*I*) on day 21. (*J*) Osteoclast numbers at the tumor-bone interface were significantly higher in mice implanted with NT control cells than with IL-6 knockdown cells. (*K, L*) Lower mitotic activity and increased cell apoptosis were observed in tumors derived from IL-6 knockdown cells compared with controls. (*M*) Significantly lower serum murine RANKL levels were measured in mice injected with IL-6 knockdown cells compared with controls. (*N, O*) IL-6 knockdown and NT control cells showed similar growth rates when implanted into the mammary fat pad. Data are mean ± SEM. *Significantly different from vehicle-treated group ($p < 0.05$). In vitro experiments were repeated 3 ×; results shown are from a representative experiment.

injected with IL-6 knockdown than NT cells (3.30 ± 0.68 mm^2 versus 5.57 ± 0.98 mm^2, $p < 0.05$) (Fig. 3*H*). Likewise, cortical bone destruction was reduced in tumors derived from IL-6 knockdown cells, concurrent with reduced osteoclast-lined bone surfaces at the bone/tumor interface (9.7 ± 0.5/mm versus 12.8 ± 1.2/mm, $p < 0.05$) (Fig. 3*I, J*). Tumors derived from IL-6 knockdown cells were characterized by lower mitotic activity (–44%) and higher rates of apoptosis (+62%) compared with NT cell-derived tumors (Fig. 3*K, L*, Supplemental Fig. S2*E*). Compared with controls, serum RANKL levels were significantly lower in mice injected with IL-6 knockdown cells (61.6 ± 10.2 pg/mL versus 37.8 ± 12.4 pg/mL, $p < 0.05$) (Fig. 3*M*).

Growth of MDA-MB-231 cells implanted orthotopically into the mammary fat pad was similar in animals injected with IL-6 knockdown or control cells (Fig. 3*N, O*). These results contrast the observations made in bone, indicating that the bone environment and the signaling molecules expressed by its cells are pivotal for the action of IL-6 on metastatic tumor growth in bone.

Disruption of IL-6 signaling via IL-6 receptor blockade reduces tumor progression in bone and circulating RANKL levels

To further distinguish the contribution of tumor cell–derived IL-6 from host, ie, bone cell–derived IL-6, on cancer growth in bone, we took advantage of the differing specificities of the anti-human IL-6R antibody, tocilizumab, and the anti-mouse IL-6R antibody, MR16-1 (Fig. 4*A–C*). Human IL-6 can bind and activate both human and murine IL-6R, but murine IL-6 only binds and activates the murine IL-6 receptor.[35] In our experimental setting, the cancer cells introduced into mice are of human origin. IL-6 produced by these cells will signal through both the human (tumor) and murine IL-6R. In contrast, murine IL-6 only interacts with mouse IL-6R and thus affects only cells of the murine bone environment. Because tocilizumab binds exclusively to hIL-6R[36] present on the implanted cancer cells, it will only prevent signaling by human, ie, cancer-derived IL-6. Tocilizumab does not affect signaling by human or mouse IL-6 through the mouse IL-6R on host cells.[35] In contrast, the mouse-specific IL-6R antibody, MR16-1, inhibits human and murine IL-6 signaling in mouse cells only.[35,37]

In the intratibial xenograft model performed in mice fed a normal diet/chow, we found that both tocilizumab and MR16-1 significantly inhibit MDA-MB-231 cell growth in bone. Thus, compared with controls, both antibodies reduced osteolytic area (1.53 ± 0.24 mm^2 [tocilizumab] and 1.49 ± 0.20 mm^2 [MR16-1] versus 1.98 ± 0.23 mm^2 [controls], $p < 0.05$ each; Fig. 4*D, E*) and total tumor area (2.17 ± 0.27 mm^2 and 2.67 ± 0.38 mm^2 versus 5.62 ± 0.67 mm^2, $p < 0.01$ each; Fig. 4*F*), prevented cortical bone destruction (1.86 ± 0.15 mm^2 and 1.83 ± 0.15 mm^2 versus 1.19 ± 0.16 mm^2, $p < 0.05$ each; Fig. 4*G*), decreased proliferation of MDA-MB-231 cells within the tumor by 60% and 64% ($p < 0.001$ versus control) (Fig. 4*H*), and increased cell apoptosis by 107% and 70%, respectively ($p < 0.01$ versus control) (Fig. 4*I*).

Further characterization of bone specimens obtained from tumor-bearing mice demonstrated that the number of osteoclasts at the tumor-bone interface was significantly lower in tocilizumab-treated animals compared with controls (8.52 ± 0.47 versus 11.05 ± 0.63 osteoclasts/mm, $p = 0.05$). A similar though statistically nonsignificant trend was observed in MR16-1-treated mice (9.73 ± 0.75 versus 11.05 ± 0.63 osteoclasts/mm, $p = 0.10$) (Fig. 4*J*).

Serum biochemistry revealed no significant differences between any of the groups in PINP levels (a marker of bone formation; data not shown), whereas circulating levels of the bone resorption marker TRAcP5b were significantly lower in both tocilizumab and MR16-1 treated mice compared with controls (4.77 ± 0.54 U/L and 4.85 ± 0.47U/L versus 7.35 ± 0.94 U/L, $p < 0.05$) (Fig. 4*K*). These results are consistent with the changes in osteoclast number at the tumor-bone interface (Fig. 4*J*). Furthermore, compared with controls, serum levels of RANKL were significantly lower in tocilizumab and MR16-1–treated animals (23.31 ± 5.80 pg/mL and 24.83 ± 5.81 pg/mL versus 44.83 ± 6.21 pg/mL, $p < 0.05$) (Fig. 4*L*). In addition, RANK expression measured in MDA-MB-231 tumor sections obtained from tocilizumab-treated mice was significantly reduced compared with controls (Supplemental Fig. S2*F*).

Our results demonstrate that both IL-6 knockdown in tumor cells and disruption of IL-6 receptor signaling in either the tumor or the host (bone) cells have significant effects on cancer cell behavior and growth and, in addition, reduce circulating RANKL levels. To further substantiate these observations, we next tested whether knockdown of the cognate receptor for RANKL, RANK, in human breast cancer cells would reduce tumor growth in bone.

Knockdown of RANK expression in MDA-MB-231 cells: effects on tumor growth in bone and nonbone tissues

Using a lentiviral-based shRNA strategy, RANK expression was knocked down in MDA-MB-231 cells, with a nontarget sequence used as control. Knockdown efficacy was 80% by real-time RT-PCR and Western blot (Fig. 5*A, B*) with no increased IL-6 expression after stimulation of RANK-knockdown cells with RANKL when compared with cells transfected with a nontarget sequence (Fig. 5*C*).

When tested in vitro, knockdown of RANK expression in MDA-MB-231 cells had no effect on cell growth (Fig. 5*D*). In contrast, intratibial injection performed in mice fed a normal diet/chow with RANK knockdown MDA-MB-231 cells in vivo resulted in significantly smaller osteolytic lesions compared with mice injected with NT cells ($p < 0.05$ for all time points) (Fig. 5*E, F*). Consistent with these radiographic results, total tumor area measured histologically at endpoint was significantly smaller in mice injected with RANK knockdown cells than that in NT cell controls (3.59 ± 0.42 mm^2 versus 1.19 ± 0.14 mm^2, $p < 0.05$) (Fig. 5*G*). Furthermore, cortical bone destruction was reduced in tumors derived from RANK knockdown cells, concurrent with reduced osteoclast-lined bone surfaces at the bone/tumor interface (11.09 ± 0.4/mm versus 13.21 ± 0.5/mm, $p < 0.05$) (Fig. 5*H, I*). Tumors derived from RANK knockdown cells were characterized by lower mitotic activity (–49%) and higher rates of apoptosis (+67%) compared with NT cell–derived tumors (Fig. 5*J, K*).

When RANK knockdown or control MDA-MB-231 cells were implanted orthotopically into the mammary fat pad, tumors progressed at a similar growth rate (Fig. 5*M, N*), indicating that the effect of RANK knockdown on tumor growth in bone is entirely dependent on the bone microenvironment. Interestingly, in the intratibial model, serum RANKL concentrations were significantly lower in animals injected with RANK knockdown cells compared with NT controls (20.32 ± 1.99 pg/mL versus 37.3 ± 2.37 pg/mL, $p < 0.05$), whereas there was no difference in circulating RANKL levels between the two groups in the subcutaneous model (Fig. 5*L*). These findings support the concept that the functionality of the RANKL-RANK-IL-6 signaling pathway is dependent on the bone environment and its cells.

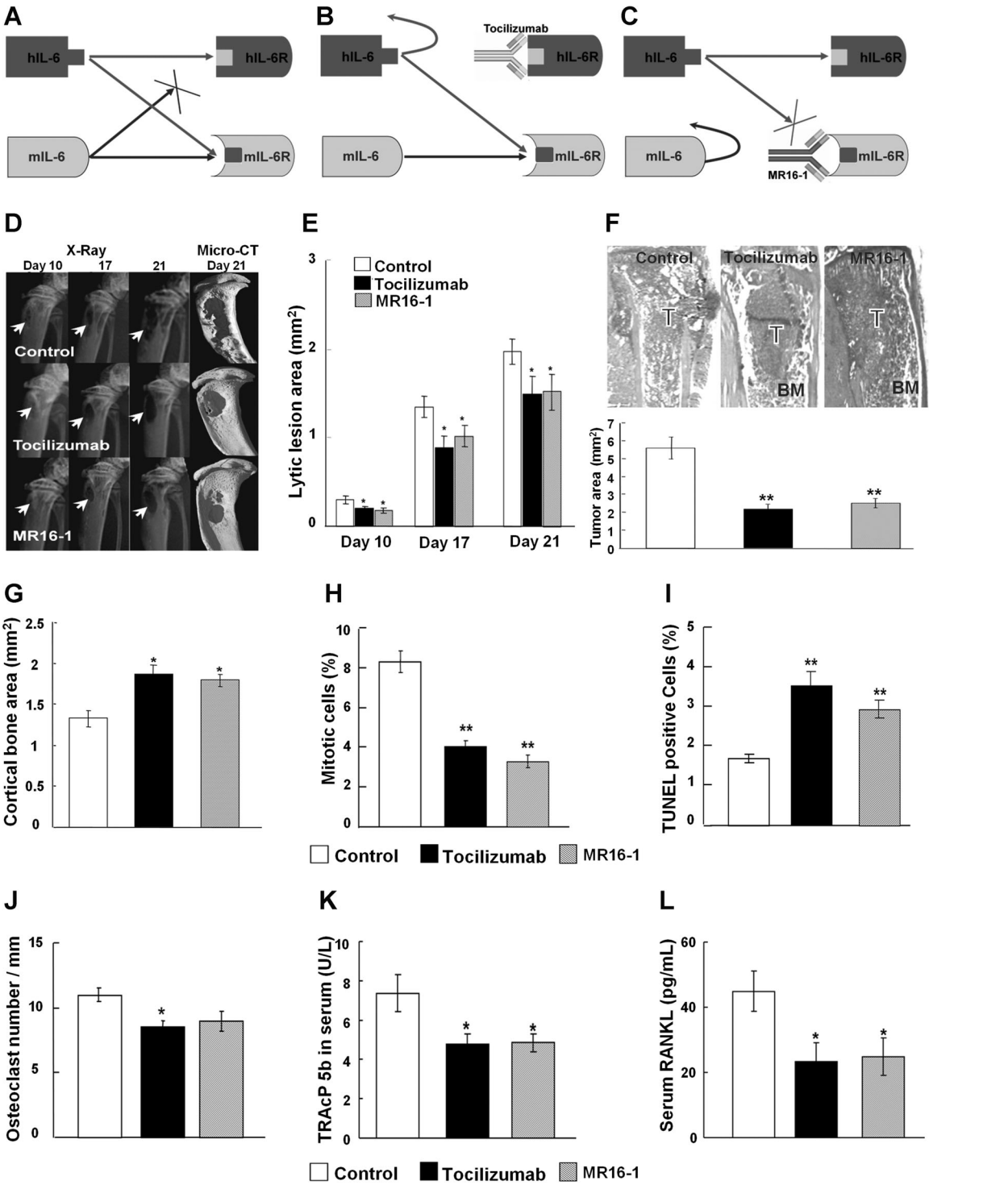

Fig. 4. Disruption of IL-6 signaling via IL-6 receptor antibodies reduces MDA-MB-231 tumor progression in bone and circulating RANKL levels. (*A–C*) Schematic representation of IL-6/IL-6 receptor interactions in human (tumor) and mouse (bone) cells. (*A*) Tumor-derived human IL-6 (hIL-6) binds to both the human hIL-6R and the mouse IL-6R (mIL-6R). In contrast, bone cell–derived mouse IL-6 (mIL-6) binds only to the mIL-6R. Blocking the hIL-6R with tocilizumab only inhibits hIL-6 action on cancer but not on bone cells (*B*). Blocking the mouse IL-6R with MR16-1 inhibits endogenous mIL-6 and hIL-6 signaling in the host (murine bone) cells only (*C*). (*D–H*) Compared with controls, treatment with tocilizumab or MR16-1 reduced osteolysis (*D, E*, X-ray) and total tumor areas (*F*, histology; T = tumor, BM = bone marrow), and prevented cortical bone destruction (*G*) ($^*p < 0.05$) ($n = 11$ to 13). (*H, I*) Compared with controls, treatment with tocilizumab or MR16-1 resulted in lower proliferation and higher apoptosis rates within the tumor ($^*p < 0.01$). (*J*) Osteoclast number at the tumor-bone interface was significantly lower in tocilizumab-treated animals ($p = 0.05$ versus control). A trend toward lower osteoclast number was also observed in MR16-1-treated mice ($p = 0.10$ versus control). (*K, L*) Serum TRAcP5b (*K*) and murine RANKL levels (*L*) were significantly lower in tocilizumab and MR16-1-treated mice compared with controls ($^*p < 0.05$). Data are mean ± SEM.

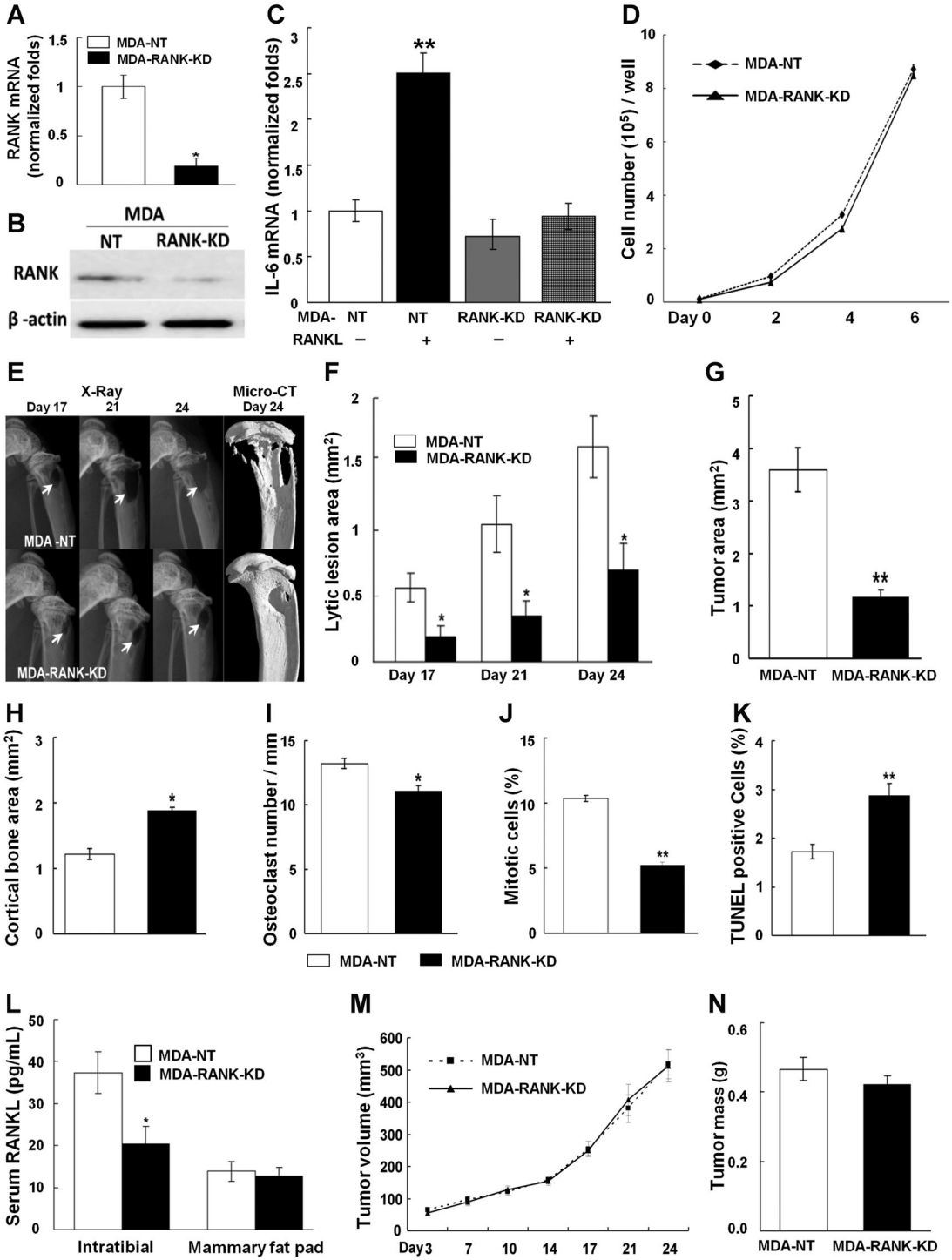

Fig. 5. Knockdown of RANK expression in MDA-MB-231 cells reduces tumor growth in bone but has no effect on orthotopic tumor growth. (*A, B*) RANK expression was knocked down in MDA-MB-231 cells by 80%. (*A*) Real-time RT-PCR, (*B*) Western blot, and (*C*) RANKL stimulation of MDA-MB-231 cells increased IL-6 expression in NT controls with no effect in RANK knockdown cells. (*D*) Knockdown of RANK expression did not affect cell growth of MDA-MB-231 cells in vitro. (*E–G*) Compared with controls, intratibial injection with RANK knockdown cells resulted in significantly reduced osteolysis (*E, F*, radiographic analysis) and tumor burden on day 24 (*G*, histologic analysis) ($n = 10$). (*H, I*) Compared with controls, cortical bone destruction was reduced in tumors derived from RANK knockdown cells, concurrent with reduced osteoclast-lined bone surfaces at the bone/tumor interface. (*J, K*) Tumors derived from RANK knockdown cells demonstrated lower mitotic activity and higher rates of apoptosis compared with NT cell–derived tumors. (*L*) In the intratibial model, serum levels of murine RANKL were significantly lower in animals injected with RANK knockdown cells compared with controls. No such differences were found in the subcutaneous model. (*M, N*) Growth of tumor cells implanted orthotopically into the mammary fat pad were similar in animals injected with either RANK knockdown or control MDA-MB-231 cells. Data are mean ± SEM. Asterisks denote significantly different from controls ($^*p < 0.05$; $^{**}p < 0.01$).

Discussion

Besides the well-established "vicious cycle" in the bone metastasis,[13–15] we have integrated two novel signaling pathways capable of significantly promoting cancer growth in bone (Fig. S3). First, in addition to its known effects on osteoclasts, RANKL directly stimulates IL-6 expression in cancer cells. As shown previously by others, IL-6 then promotes RANKL expression in osteoblasts and other cells of the osteoblast lineage.[13,20] This signaling pathway establishes, therefore, a direct feed-forward loop between these osteoblasts/osteoblast lineage cells and the tumor. Second, cancer cell–derived IL-6 induces the expression of RANK by tumor cells via an autocrine mechanism, thus sensitizing the cancer to the action of RANKL. These interdependent, self-amplifying signaling pathways act synergistically to increase cytokine output within the bone microenvironment and stimulate metastatic tumor growth in bone (Fig. S3).

RANKL is present at high levels in bone and is derived mainly from cells of the osteoblast lineage[30,31] as well as T cells.[38] Because athymic nude mice lack T cells and because the tumor cells used in our experiments do not express RANKL,[34] we conclude that in our studies RANKL is derived from cells of the osteoblast lineage. In this context, it is important to note that low and high bone turnover—and hence lower or higher amounts of available RANKL[30,31]—are associated with corresponding changes in tumor IL-6 expression in vivo. Using breast cancer or neuroblastoma cell lines, previous studies have established that osteoblast-derived cytokines, such as IL-6, IL-8, or monocyte chemoattractant protein-1 (MCP-1), can promote metastatic cancer growth in bone either through activation of osteoclastic bone resorption or direct stimulation of tumor cell proliferation.[21–24] We here demonstrate that within the bone microenvironment, cells of the osteoblast lineage interact directly with tumor cells via RANKL signaling.

High circulating levels of IL-6 have been shown to be associated with adverse clinical outcomes in patients with metastatic breast cancer. For example, survival rates are significantly shorter in cancer patients with persistently high serum IL-6 levels.[16,17] Stable knockdown of IL-6 expression in MDA-MB-231 cells significantly reduced the tumor growth in bone but not in the mammary fat pad, indicating that tumor-derived IL-6 is an important determinant of MDA-MB-231 cell growth in bone. This would be consistent with a dependence on host cell expression of RANKL, which is abundant in bone but not mammary tissue of nonpregnant/nonlactating mice.[39]

Tocilizumab is a monoclonal anti-human IL-6R antibody approved for the treatment of inflammatory autoimmune conditions. Human studies have demonstrated tocilizumab to be effective in the treatment of IL-6–related disorders such as rheumatoid arthritis or Crohn's disease.[40] Furthermore, targeting the IL-6R may be of clinical benefit in certain malignancies such as multiple myeloma.[41] So far, tocilizumab has not been studied in human metastatic bone disease. Using the species-specific features of the anti-human IL-6R antibody, tocilizumab, and the anti-mouse IL-6R antibody MR16-1, we established that both antibodies inhibit breast cancer growth in murine bone to a similar extent, indicating that IL-6 signaling to both cancer and host cells promotes tumor growth. However, because of its specificity for the human IL-6R, tocilizumab in our model can only inhibit IL-6 signaling between human (ie, cancer) cells, while permitting human IL-6 to activate the murine IL-6R on host (ie, bone) cells[35] (see also Fig. 4*A*–*C*).

We have observed that tocilizumab treatment strongly inhibits tumor growth within the murine bone environment, suggestive of an autocrine IL-6 signaling pathway in and between cancer cells. Of note, MR16-1 also inhibited skeletal tumor growth, most likely because the mouse-specific anti-IL-6R antibody prevents activation of the murine IL-6 receptor by human IL-6 (Fig. 4*C*). This interaction of MR16-1 with cells of the host bone environment would reduce RANKL expression by cells of the osteoblast lineage, thereby slowing tumor growth. It was also shown that MR16-1 dose-dependently inhibited osteoclast-like multinucleated cell formation,[42] which could have additional effects on tumor. Furthermore, MR16-1 prevents human IL-6 activation of the murine IL-6R on cells within the bone environment. The finding that serum RANKL levels were significantly lower in tumor-bearing mice treated with either tocilizumab or MR16-1 provides further evidence that cells of the osteoblast lineage are able to directly communicate with cancer cells via a RANKL/IL-6–dependent pathway distinct from the osteoclast-mediated signaling pathway of the established vicious cycle.[13–15]

We further demonstrated that in addition to its known effects on osteoblasts, tumor-derived IL-6 also acts via an autocrine mechanism to stimulate RANK expression in cancer cells. This sensitizes the metastatic tumor to RANKL and, as shown above, significantly increases its IL-6 output (Fig. 2). We propose that this local, autocrine feed-forward loop within the tumor is critical to amplify the IL-6/RANKL–driven crosstalk between osteoblastic and cancer cells. This second element may be of particular relevance during early stages of metastasis, when local cell populations and cytokine levels are still low. Accordingly, activation or disruption of IL-6 signaling induced corresponding changes in tumor RANK expression, whereas knockdown of RANK expression in breast cancer cells reduced tumor growth in bone. These results confirm that the direct crosstalk between metastatic tumor cells and cells of the osteoblast lineage is indeed via RANKL-RANK-IL-6, and support clinical findings that high RANK expression in the primary cancer and its skeletal secondaries are correlated with poor clinical prognosis.[25]

Jones and colleagues demonstrated that RANKL triggers migration of RANK-expressing human epithelial cancer cells and melanoma cells.[26] In the same study, OPG was also found to reduce tumor burden in bones but not in other organs in a mouse model of melanoma metastasis. These data pointed to that local RANKL may have an important role in cell migration and the tissue-specific metastatic behavior of cancer cells.[26] The interplay of RANKL-RANK-IL-6 from our results further consolidates the role of interaction between osteoblasts and cancer cells in the bone microenvironment.

The additional signaling pathways identified in this study act by amplifying but not replacing components of the classic vicious cycle.[13] The effects of intervention in any of the elements of the new signaling pathways are dependent on the effects on the other elements, and our observations are consistent with changes in bone resorption being the critical mediator influencing tumor growth.[5,14] For example, ibandronate treatment blocks bone resorption without altering IL-6 expression by the tumor but has similar effects on tumor growth in bone to inhibiting bone resorption with OPG, which inhibits both bone resorption and the increase in IL-6 expression by the tumor. As demonstrated in our study, the lack of activity of any of these treatments in soft tissues emphasizes the importance of the bone-specific actions of RANKL, RANK, and IL-6. Similarly, the

relevance of other elements, such as the release of growth factors by bone or proresorptive PTHrP[43] by the tumor, have been demonstrated by others. Thus, there appears to be strong interdependence between the elements of the cycles with bone resorption being the common denominator and final mediator of tumor growth.

In conclusion, the novel signaling pathways identified in the current study drive metastatic tumor growth through self-amplification, which explains the rapid growth and spread of cancer cells within the bone microenvironment, particularly during early stages of metastasis. Our results add credence to the concept that disruption of RANKL-RANK-IL-6 signaling pathways at different levels may be beneficial in the treatment of patients with breast cancer bone metastases.

Disclosures

All authors state that they have no conflicts of interest.

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