

## Tie2 Signaling Regulates Osteoclastogenesis and Osteolytic Bone Invasion of Breast Cancer

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### Abstract

Breast to bone metastasis is a common occurrence in the majority of patients with advanced breast cancer. The metastases are often incurable and are associated with bone destruction and high rates of morbidity. Understanding the underlying mechanisms of how metastatic tumor cells induce bone destruction is critically important. We previously reported that Tie2, a receptor tyrosine kinase, is significantly increased in human breast cancer tissues compared with normal and benign breast tumors and regulates tumor angiogenesis. In this study, we identify a new function of Tie2 in osteoclastogenesis and osteolytic bone invasion of breast cancer. Tie2 is present in hematopoietic stem/precursor cells. Genetic deletion of Tie2 or neutralization of Tie2 function using soluble Tie2 receptor impaired osteoclastogenesis in an embryonic stem cell differentiation assay. In contrast, deletion of Tie2 has no effect on osteoblastogenesis. As CD11b myeloid cells have the potential to become osteoclasts and Tie2 is present in a certain population of these cells, we isolated Tie2<sup>+</sup> and Tie2<sup>-</sup> myeloid cells. We observed a significant reduction of osteoclastogenesis in Tie2<sup>-</sup> compared with Tie2<sup>+</sup> CD11b cells. Consistently, neutralization of Tie2 activity *in vivo* significantly inhibited osteolytic bone invasion and tumor growth in a mammary tumor model, which correlated with a significant reduction of osteoclasts and tumor angiogenesis. Collectively, these data reveal a direct and novel role of Tie2 signaling in osteoclast differentiation. These findings identify Tie2 as a therapeutic target for controlling not only tumor angiogenesis but also osteolytic bone metastasis in breast cancer. *Cancer Res*; 70(7): 2819–28. ©2010 AACR.

### Introduction

Bone metastasis is a serious medical problem, which is characterized as osteolytic or osteoblastic, and most patients with breast cancer have predominantly osteolytic lesions. In osteolytic metastasis, tumor cells produce factors that promote the differentiation and activation of the cells responsible for osteolysis (i.e., the osteoclasts). In turn, bone resorption by osteoclasts releases growth factors from the bone matrix that enable tumor cells to grow and progress in the bone microenvironment. This reciprocal interaction between breast cancer cells and the bone microenvironment forms a vicious cycle that increases both bone destruction and the tumor burden (1). In addition, osteoclast hyperactivation in metastatic sites results in destructive and painful lesions that are accompanied by pathologic fracture, spinal compression, and hypercalcemia. Therefore, targeting osteo-

clast differentiation and breaking the vicious cycle offers an effective means to combat bone metastasis.

The vascular endothelium plays important roles in metastasis because tumor cells are transported through blood and lymphatic vascular systems and direct interaction between tumor cells and the endothelium is essential for metastasis as tumor cells have to intravasate and extravasate during metastasis. Emerging evidence also indicates a connection between the endothelium and osteoclastogenesis. For example, angiogenesis is closely associated with increased osteoclast formation in multiple myeloma and other cancers, as well as inflammatory conditions, such as rheumatoid arthritis (2–4). Tie2 is a receptor tyrosine kinase expressed predominantly in vascular endothelium and regulates angiogenesis. We have reported a significant elevation of Tie2 in human breast cancer tissues compared with normal breast tissues (5). Neutralization of Tie2 function using a soluble Tie2 receptor significantly inhibited tumor angiogenesis and tumor growth in mammary tumors (6, 7).

Osteoclasts are derived from hematopoietic stem cells (8, 9). Because hematopoietic cells share a common progenitor with endothelial cells, it is no surprise that these two cell types often share common mediators. In addition to endothelium, Tie2 is also expressed in hematopoietic stem/progenitor cells, and the interaction of Tie2 in hematopoietic stem cells with angiopoietin-1, a ligand for Tie2, from osteoblasts controls stem cell quiescence and survival in the bone marrow niche (10). Furthermore, overexpression of

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angiopoietin-1 in transgenic mice results in an increase of bone volume and bone parameters compared with wild-type littermates (11). We have shown that blocking Tie2 activation prevented bone destruction in arthritis (12). These data support a role of Tie2 signaling in osteoclast differentiation and function.

In this study, we report a direct role of Tie2 signaling in osteoclastogenesis. Accordingly, neutralization of Tie2 function inhibited osteolytic bone invasion, tumor angiogenesis, and tumor progression of breast cancer.

## Materials and Methods

**Materials.** Eight-week-old female BALB/c mice were purchased from The Jackson Laboratory. The animals were housed in pathogen-free units at the Vanderbilt University Medical Center in compliance with Institutional Animal Care and Use Committee regulations. Adenoviral vectors directing the expression of green fluorescent protein (AdGFP) and the soluble Tie2 inhibitor ExTek (AdExTek; ref. 7) were used. The viral vectors were propagated in 293 cells and purified by CsCl gradients as described (7). The murine mammary tumor line 4T1 was stably transfected with a luciferase construct (4T1/Luc) and used in the study.

**In vitro osteoclastogenesis and osteoblastogenesis assays.** Wild-type mouse embryonic stem (ES) cells (TL1) and Tie2-knockout ES cells were kindly provided by the Vanderbilt Stem Cell Biology Core and Dr. Daniel Dumont at the Sunnybrook and Women's Research Institute (Toronto, Ontario, Canada), respectively. The cells are maintained in the medium in the presence of leukemia inhibitory factor (1,000 units/mL; Life Technologies, Inc.) and passaged every other day as described (13). For induction of osteoclast differentiation, we used a published protocol (14). Recombinant ExTek protein (100 ng/mL) was used to neutralize Tie2 activation in TL1 ES cell differentiation. Three weeks after induction of cell differentiation, tartrate-resistant acid phosphatase (TRAP) staining was done to detect activated osteoclasts according to the manufacturer's instructions (Sigma) and multinucleated TRAP<sup>+</sup> cells (two or more nuclei per cell) were counted in randomly selected fields under a microscope. The experiment was done in triplicate and repeated three times.

For induction of osteoblast differentiation, we followed a published protocol (15). Recombinant ExTek protein (100 ng/mL) was used to neutralize Tie2 activation in TL1 ES cell differentiation. The complete program of differentiation usually takes place in 25 to 30 d. At the end of differentiation, mineralization is observed by Alizarin Red (Sigma) staining (15). Positively stained cells were counted in randomly selected fields under a microscope. All experiments were done in triplicate and repeated twice.

Osteoclastogenesis assays were also done using myeloid cells from 8-wk-old BALB/c mice. CD11b<sup>+</sup> cells were purified from mouse bone marrow using anti-CD11b magnetic beads per manufacturer's instructions (Miltenyi Biotech). CD11b<sup>+</sup> cells were subsequently stained with phycoerythrin (PE)-labeled mouse anti-Tie2 antibody (eBioscience). Tie2<sup>+</sup> and

Tie2<sup>-</sup> myeloid cells were sorted with a FACStarPlus flow cytometer (Becton Dickinson). CD11b<sup>total</sup>, CD11b<sup>Tie2-</sup>, and CD11b<sup>Tie2+</sup> cells were seeded ( $5 \times 10^5$ ) into eight-well chamber slides (Labtek) and exposed to receptor activator of NF- $\kappa$ B ligand (50 ng/mL; R&D Systems) and macrophage colony-stimulating factor (25 ng/mL; R&D Systems) in 10% serum-containing  $\alpha$ -MEM for 14 d with change of medium every 2 to 3 d. For actin ring staining of osteoclasts, cells were fixed with 100% methanol at  $-20^\circ\text{C}$  for 10 min and incubated with phalloidin (Invitrogen) for 1 h at room temperature. Total multinucleated cells (with three or more nuclei per cell) were counted in each well and graphed. The experiment was done in triplicate and repeated three times. Osteoclast differentiation ability was calculated as the fraction of plated cells that undergo osteoclast differentiation (output/input).

**Cell proliferation assay.** ES cells were incubated with recombinant ExTek protein or bovine serum albumin (BSA) control (100 ng/mL) for 24 h. Cell proliferation was examined by the WST-1 (water-soluble tetrazolium salt) assay per manufacturer's protocol (Roche) followed by incubation with WST-1 reagent for 1, 2, and 4 h. Absorbance at 450 nm was measured on a microplate reader (Bio-Rad). The experiment was done in triplicate and repeated twice.

**Tumor cell-mediated osteolytic bone invasion model.** 4T1/Luc cells at  $1 \times 10^5/50 \mu\text{L}$  were directly injected into the right tibia of 8-wk-old BALB/c mice as described (16). One week later, the animals were divided into two groups (10 mice per group) and received i.v. injection of AdExTek or AdGFP at  $1 \times 10^9$  plaque-forming units (pfu), respectively, as previously described (7). Bioluminescent activity was imaged in a cryogenically cooled IVIS-100 system (Xenogen) at 1 and 2 wk after the treatment, and photon counts were collected to reflect live tumor volume. At each time point, radiographs of the lower extremity were obtained using an LX-60 cabinet digital X-ray system (Faxitron). An exposure time of 5 s at 30 kVp was used. The percent of cortical destruction in the upper half of the tibia was calculated from the radiographic images using Image. For each tibia, the length was measured and the midpoint of the bone was marked. The linear extent of preserved cortex was measured (using the line tool) for both the anterior and posterior portions of the cortex and the two values were averaged together and the percent of cortical destruction was calculated from that average.

**Histology and immunohistochemistry.** Animals were sacrificed at the end of the experiment. Hind limb bones were removed from mice and fixed in 10% formalin, decalcified in Immunocal (Decal Chemical) for 2 wk, and then embedded in paraffin. Tissue sections from size-matched tumors of two series experiments ( $n = 10$  mice per group) were stained with H&E, and immunohistochemistry using anti-CD31 antibody (Pharmingen) was done for vascular density measurement. Tissue sections from size-matched tumors were also assessed for TRAP activity, a marker for osteoclasts, as previously described (17). Positive staining was counted in 10 randomly selected high-power fields under a microscope and graphed.

**Statistics.** Results are reported as mean  $\pm$  SE for each group. Statistical analyses were done using two-tailed

Student's *t* test for two-group comparison. Data were calculated with Statview 5.0 (Abacus Concepts) statistical software. All tests of significance were two sided, and differences were considered statistically significant at  $P < 0.05$ .

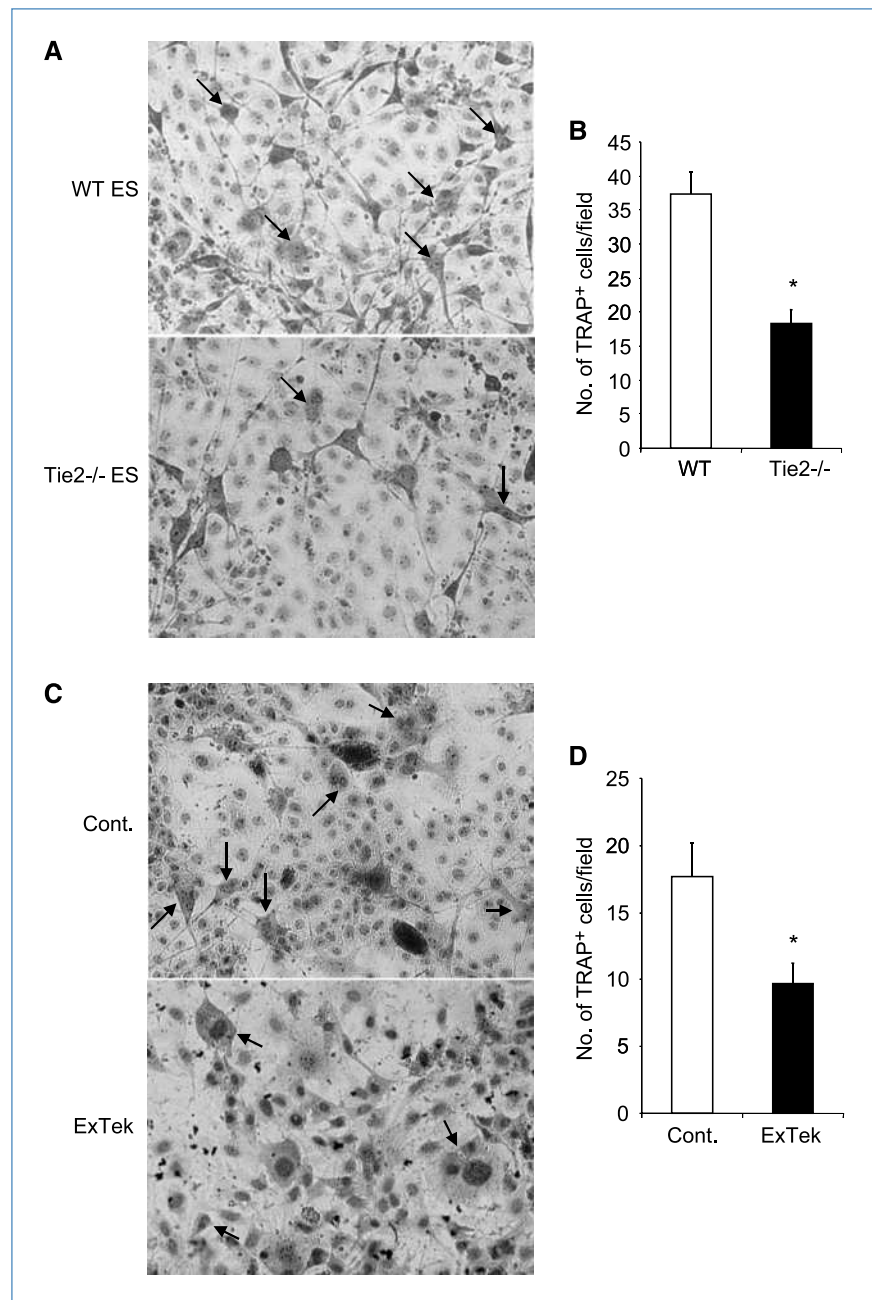
## Results

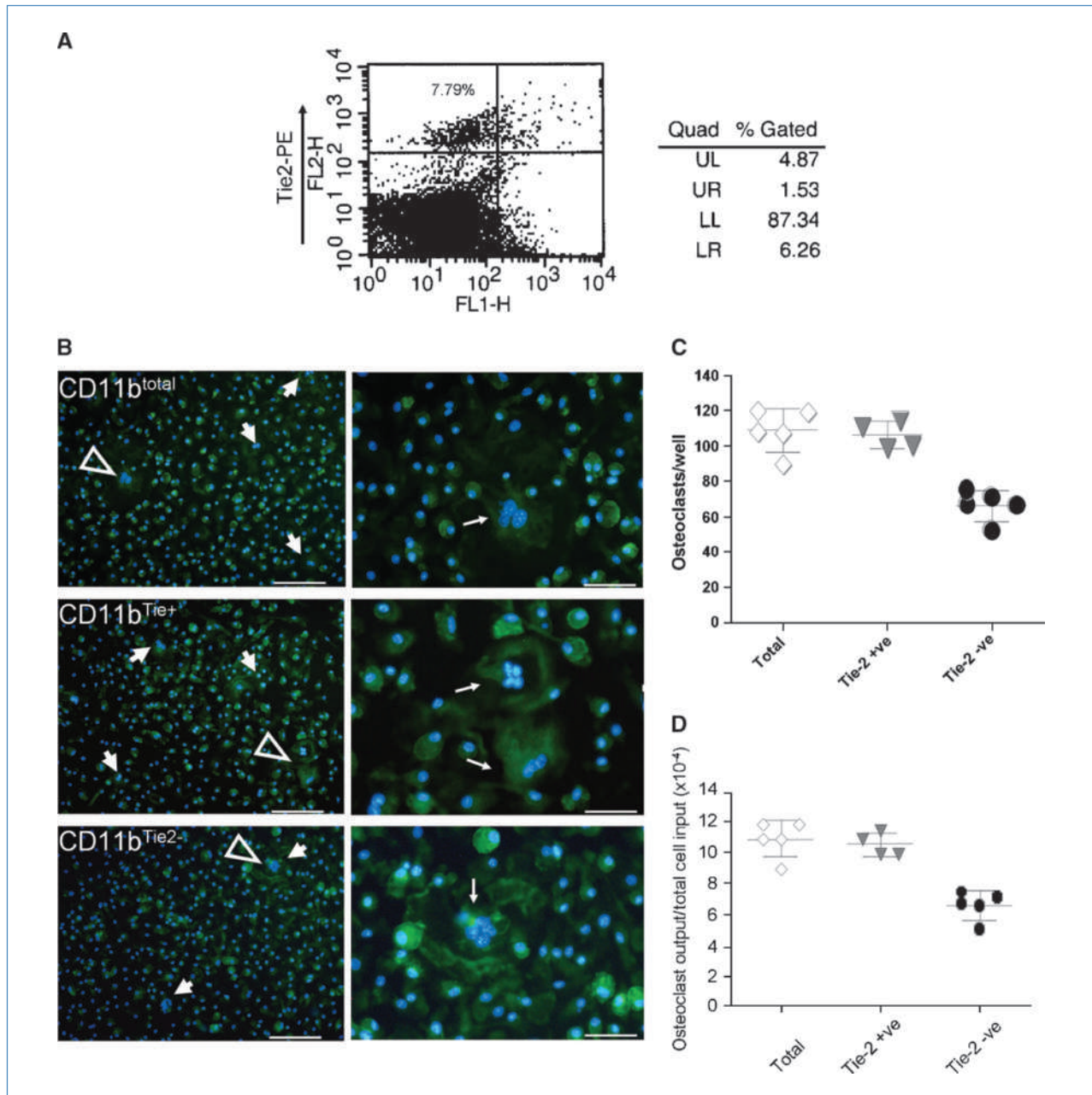
### *Tie2 signaling directly regulates osteoclastogenesis.*

Osteoclasts are derived from hematopoietic progenitor cells and Tie2 is present in these progenitor cells and important in hematopoiesis (10). In a previous study, we showed that neu-

tralization of Tie2 signaling inhibited bone erosion in arthritis, suggesting a role of Tie2 in osteoclast differentiation (12). To test this hypothesis, we used an *in vitro* osteoclast differentiation assay from mouse ES cells because genetic manipulations of ES cells have been well established and a variety of knockout ES cells are available. After an induction of osteoclast differentiation from ES cells, we found a significant reduction of TRAP-positive multinucleated osteoclasts developed from the Tie2-null cells compared with wild-type cells (Fig. 1A and B). To verify this finding, we used a biochemical approach, in which we induced osteoclast differentiation

**Figure 1.** Tie2 signaling directly regulates osteoclastogenesis *in vitro*. TL1 ES cells and Tie2-null ES cells were induced to differentiate into osteoclasts for approximately 3 wk *in vitro*. TRAP staining was done to detect mature multinuclear osteoclasts as marked by arrows (A). The number of TRAP-positive multinuclear osteoclasts was counted in randomly selected fields under a microscope and graphed between wild-type and Tie2-null ES cells (B). The experiment was done in triplicate and repeated three times. Columns, mean; bars, SE. \*,  $P < 0.05$ , calculated with Statview 5. TL1 ES cells were induced to differentiate into osteoclasts for approximately 3 wk in the presence of recombinant ExTek at 100 ng/mL or control protein. The media were replaced twice a week. TRAP staining was applied to detect mature osteoclasts as marked by arrows (C). The number of TRAP-positive cells was counted in randomly selected fields under a microscope and graphed between the two groups (D). The experiment was done in triplicate and repeated three times. Columns, mean; bars, SE. \*,  $P < 0.05$ , calculated with Statview 5.





**Figure 2.** Tie2<sup>+</sup> myeloid cells give rise to osteoclasts *in vitro*. CD11b-positive myeloid cells were isolated from bone marrow of wild-type mice by magnetic cell sorting. The cells were then stained with an anti-Tie2-PE antibody and analyzed by flow cytometry for Tie2<sup>+</sup> myeloid cells (A). Tie2<sup>+</sup> and Tie2<sup>-</sup> myeloid cell populations were isolated from CD11b<sup>+</sup> myeloid cells by fluorescence-activated cell sorting. These cells were subjected to induction of osteoclast differentiation for 3 wk, followed by actin ring staining. Actin ring-positive (green) multinucleated (blue) osteoclasts were identified (arrow) in the CD11b<sup>total</sup>, CD11b<sup>Tie2-</sup>, and CD11b<sup>Tie2+</sup> groups. Open arrows (B, left) indicate areas of higher magnification (B, right). The number of multinucleated osteoclasts (three or more nuclei per cell) was counted in each well under a microscope and graphed (C). These experiments were done in triplicate and repeated on three independent occasions. \*,  $P < 0.05$ , CD11b<sup>Tie2-</sup> versus CD11b<sup>Tie2+</sup> or CD11b<sup>total</sup>. The osteoclast differentiation abilities of CD11b<sup>Tie2-</sup>, CD11b<sup>Tie2+</sup>, and CD11b<sup>total</sup> myeloid cells were calculated and graphed (D). These experiments were done in triplicate and repeated on three independent occasions. \*,  $P < 0.05$ , CD11b<sup>Tie2-</sup> versus CD11b<sup>Tie2+</sup> or CD11b<sup>total</sup>.

from wild-type ES cells in the presence of recombinant soluble Tie2 protein (ExTek) to block Tie2 activation. Consistent with the genetic data, there was a significant inhibition of osteoclast differentiation in the ExTek-treated group com-

pared with controls (Fig. 1C and D). In addition, we did not observe any effect of ExTek on ES cell proliferation (Supplementary Fig. S1), which argues against toxicity playing a role in ES cell differentiation. Together, these results reveal a

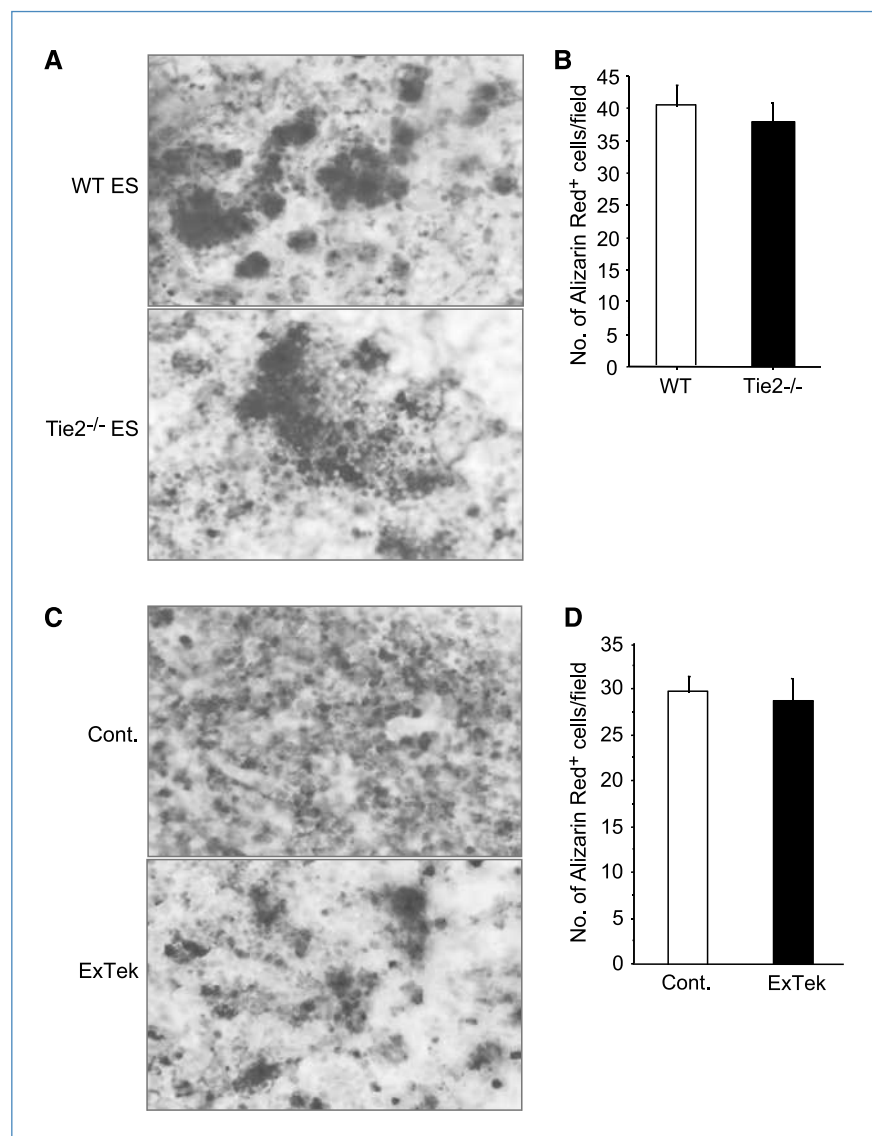
direct role of Tie2 signaling in osteoclast differentiation from progenitors.

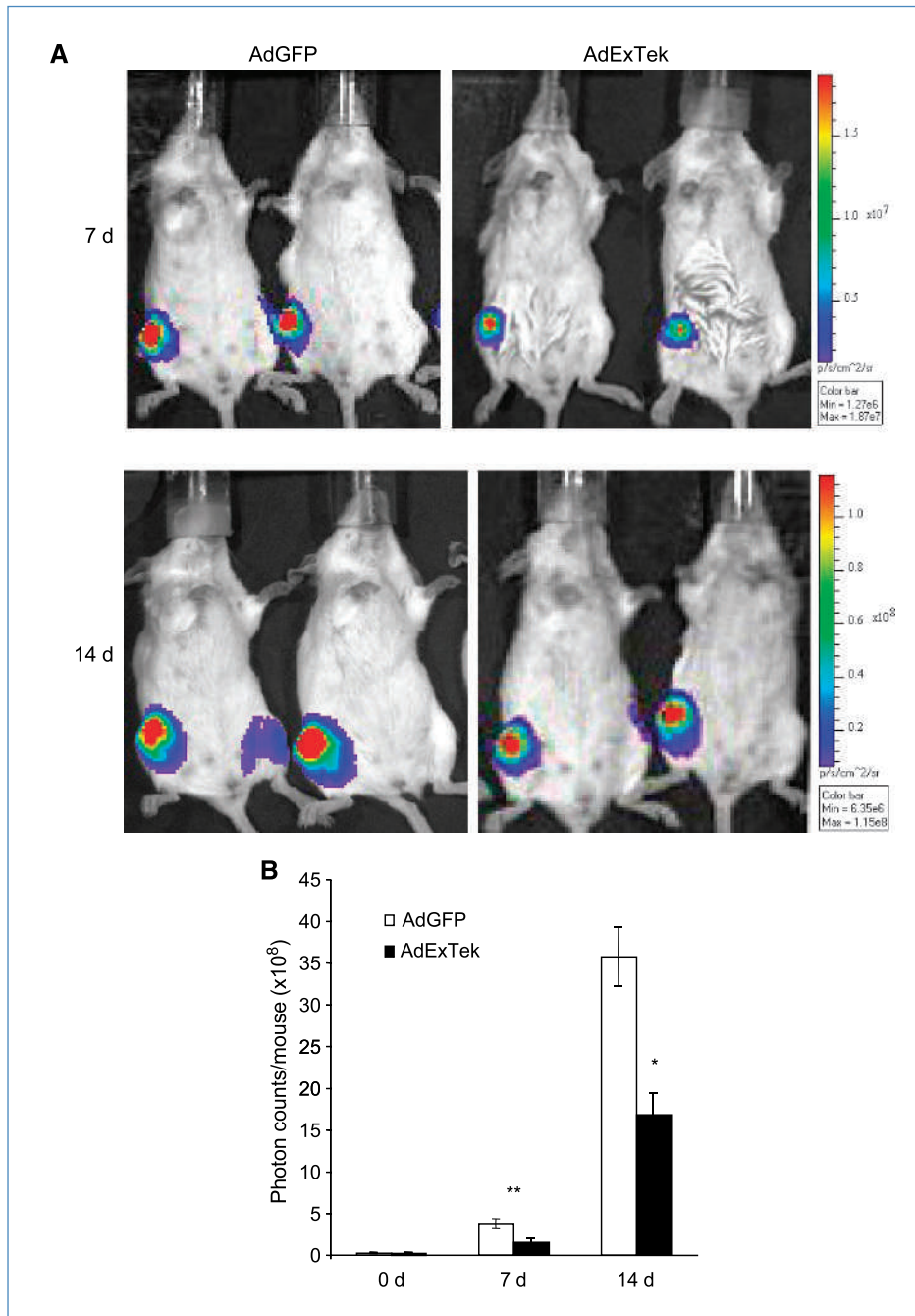
These results were further corroborated using purified myeloid precursors from bone marrow. CD11b<sup>+</sup> myeloid cells have the potential to differentiate into osteoclasts and they are commonly used in osteoclastogenesis assays. CD11b<sup>+</sup> myeloid cells are heterogeneous with respect to the expression of Tie2 (18). Therefore, we isolated CD11b<sup>+</sup> myeloid cells from BALB/c mouse bone marrow using magnetic cell sorting. CD11b<sup>+</sup> cells (CD11b<sup>total</sup>) were further separated by flow cytometry into Tie2-positive (Tie2<sup>+</sup>) and Tie2-negative (Tie2<sup>-</sup>) cells. Approximately 7.79% CD11b<sup>+</sup> myeloid cells were Tie2 positive (Fig. 2A). We achieved >98% purity of Tie2<sup>+</sup> and Tie2<sup>-</sup> cells after cell sorting (data not shown), which were then used to induce osteoclast differentiation *in vitro*. After counting multinucleated cells (with three or more nuclei

per cell), we found that Tie2-negative myeloid cells (CD11b<sup>+</sup> Tie2<sup>-</sup>) form significantly fewer osteoclasts compared with Tie2<sup>+</sup> myeloid cells (CD11b<sup>+</sup> Tie2<sup>+</sup>) and total CD11b myeloid cells (Fig. 2B and C). The osteoclast differentiation abilities (fraction of plated cells that undergo differentiation) of Tie2<sup>+</sup> and Tie2<sup>-</sup> CD11b myeloid cells are  $1.1 \times 10^{-3}$  and  $0.6 \times 10^{-3}$ , respectively. These data are consistent with early results and show a direct function of Tie2 signaling in osteoclastogenesis.

In addition, we also examined the potential role of Tie2 in osteoblastogenesis using ES cells. After a few weeks of induction of ES cell differentiation, we did not observe any significant difference in Alizarin Red-positive osteoblasts between wild-type ES cells and Tie2-null ES cells (Fig. 3A and B). Additionally, inhibition of Tie2 function using recombinant ExTek protein had no effects on osteoblast differentiation from wild-type ES cells compared with the control protein-treated

**Figure 3.** Genetic deletion of Tie2 has no effect on osteoblastogenesis *in vitro*. TL1 ES cells and Tie2-null ES cells were induced to differentiate into osteoblasts for approximately 4 wk *in vitro*. At the end of the experiments, cells were stained with Alizarin Red for osteoblasts (A). The number of Alizarin Red-positive cells was counted and graphed between wild-type and Tie2-null ES cells (B). Columns, mean from three separate experiments; bars, SE. TL1 ES cells were induced to differentiate into osteoblasts in the presence of recombinant ExTek at 100 ng/mL or control BSA protein (C). The number of Alizarin Red-positive cells was counted and graphed between the two groups (D). Columns, mean from three separate experiments; bars, SE.





**Figure 4.** Neutralization of Tie2 function inhibits tumor growth in tibia bone. 4T1/Luc cells at  $1 \times 10^6$ /50  $\mu$ L/mouse were directly injected into the right tibia bone of 8-wk-old BALB/c mice. Seven days after tumor implantation, the mice were randomly divided into two groups and received either AdExTek or control AdGFP injection ( $10^9$  pfu/mouse) via tail vein. Bioluminescence imaging was done before the treatment and 1 and 2 wk after the viral injection. Representative photos of mice over a 2-wk period of time are shown (A). Photon counts were collected at each time point in each group and graphed (B).  $n = 10$  mice per group. The experiment was repeated once. Columns, mean; bars, SE. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ .

group (Fig. 3C and D). These findings point to a specific role of Tie2 signaling in osteoclastogenesis, but not in osteoblastogenesis.

**Neutralization of Tie2 function inhibited osteoclast formation and osteolytic bone invasion of mammary tumors in vivo.** Because osteoclasts are critical for osteolytic bone metastasis and Tie2 signaling regulates osteoclast differentiation, we postulated that Tie2 signaling would be crucial for osteoclastogenesis in the mammary tumor-bone micro-

environment. To address this question, we developed a 4T1 breast cancer cell line stably expressing luciferase (4T1/Luc) for noninvasive imaging. The 4T1 mouse mammary tumor cell line developed from BALB/c mice has several key advantages: (a) it is one of only a few breast cancer models with the capacity to metastasize efficiently to sites affected in human breast cancer; (b) it resembles advanced breast cancer in humans; and (c) it is done in an immunocompetent mouse.

We directly injected 4T1/Luc cells into the right tibia of 8-week-old female BALB/c mice. On establishment of skeletal metastasis 1 week after tumor injection, the mice were randomly divided into two groups and received an injection of either AdExTek or control AdGFP through tail vein, respectively. Tumor growth was imaged by bioluminescent imaging at 1 and 2 weeks after the treatment (Fig. 4A). There was a significant reduction of tumor volume as reflected in the photon counts in animals that have received the ExTek treatment compared with controls (Fig. 4B;  $n = 10$  mice per group,  $P < 0.05$ ).

At each time point, we also measured osteolytic bone destruction in live animals using digital X-ray radiography. At 1 week, the control tibias showed an average of 41.7% of the cortex of the upper tibia destroyed, whereas the treatment group showed an average of 15.0% of the cortex destroyed (Fig. 5A and B;  $n = 10$  mice per group;  $P < 0.05$ ). At 2 weeks, the control group showed an average of 61.8% of the cortex destroyed and the treatment group showed an average of 40.2% of the cortex destroyed (Fig. 5C and D;  $n = 10$  mice per group;  $P < 0.05$ ). Reduction of osteolytic bone destruction is consistent with the observation that Tie2 signaling plays a role in osteoclast differentiation.

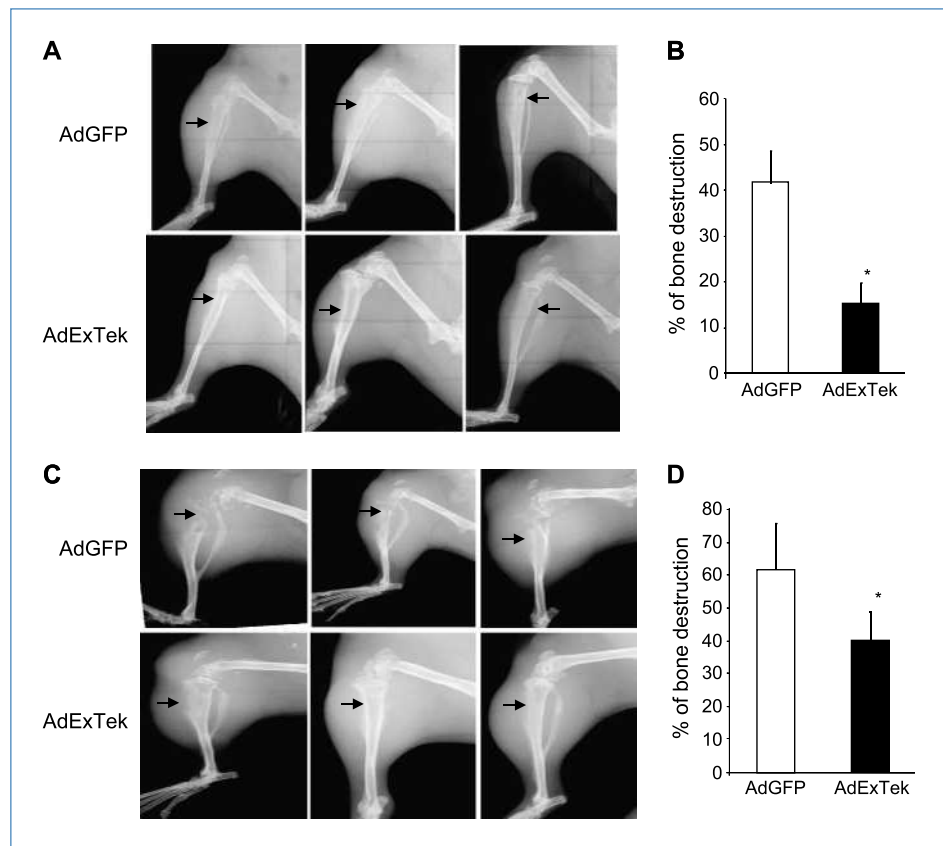
Finally, we performed histologic examination of tumor tissues in bone. We selected tissues from mice with similar-sized tumors from each group to minimize/eliminate the potential effect of tumor burden on data analysis. We found that

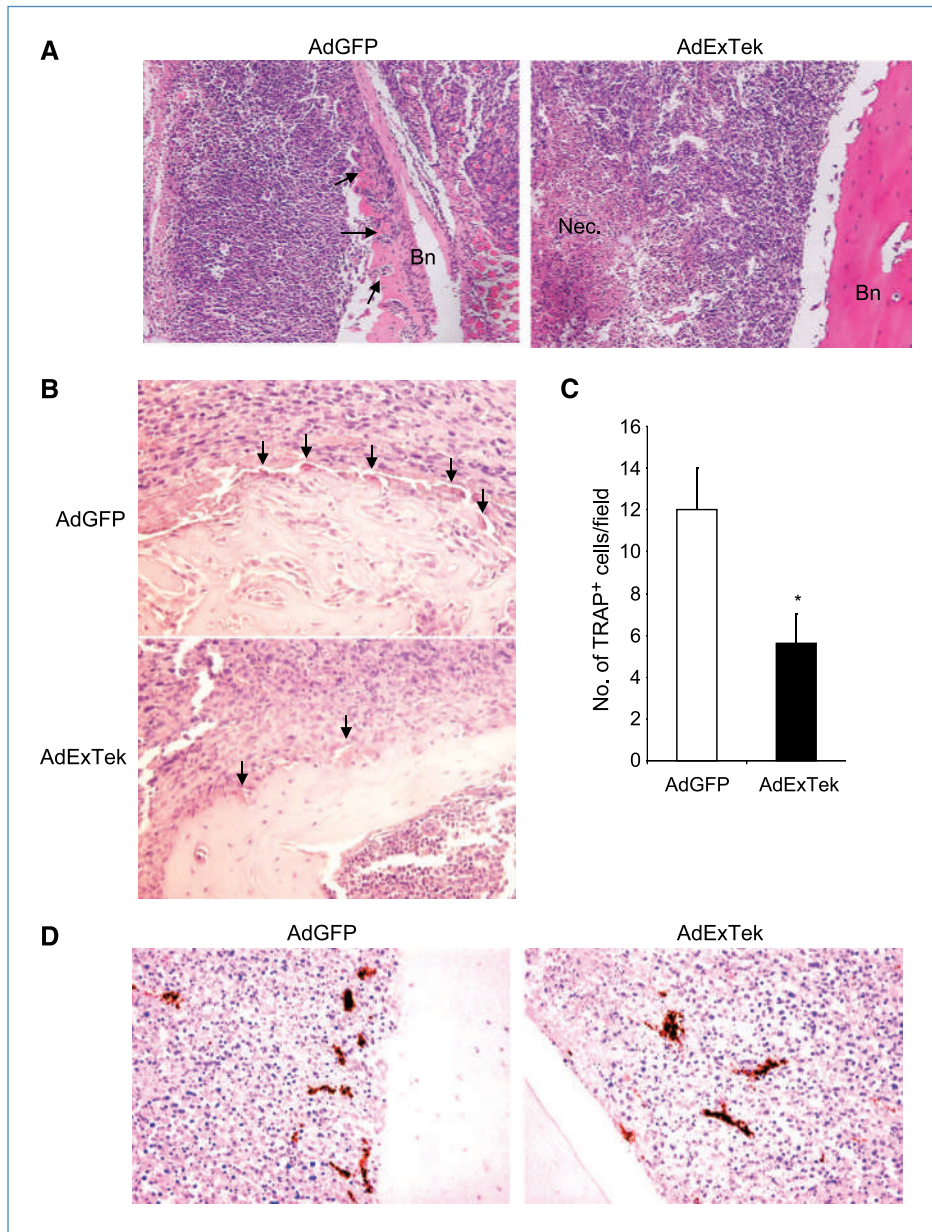
the cortical bone in the proximal tibias was largely destroyed and 4T1 cells were noted in the space between the remaining proximal and distal ends of the tibias. In contrast, there was limited cortical bone destruction in the ExTek treatment group (Fig. 6A). Consistently, TRAP staining revealed significantly fewer osteoclasts at the bone/tumor interface from ExTek-treated animals compared with control-treated animals (Fig. 6B and C). These findings are consistent with the assessment that Tie2 plays a role in osteoclast differentiation, and neutralizing Tie2 function inhibits osteoclast differentiation and osteolytic bone erosion. In addition, we also examined tumor angiogenesis between the groups. CD31 staining showed a significant reduction of tumor vascular density when Tie2 function was blocked (Fig. 6D; Supplementary Fig. S2), which correlated with increased necrosis in tumor section when compared with controls (Fig. 6A). Taken together, these results support a role of Tie2 signaling in osteoclast differentiation and osteolytic bone invasion, in addition to tumor angiogenesis.

## Discussion

Breast to bone metastases are predominately osteolytic in nature. While the metastasizing tumor cells invade the bone, osteoclasts in the area are stimulated to resorb bone and release growth factors that enable tumor cells to grow and

**Figure 5.** Neutralization of Tie2 function impairs osteolytic bone destruction *in vivo*. Bone destruction after tumor implantation was imaged using a Faxitron LX-60 digital X-ray system in live animals 1 wk (A) and 2 wk (C) after AdExTek and AdGFP injection. Representative radiographs of osteolytic lesions in hind limbs of BALB/c mice are shown. The percent of cortical bone destruction in the upper half of the tibia was calculated from the radiographic images using Image at 1 wk (B) and 2 wk (D) after the treatment.  $n = 10$  mice per group. The experiment was repeated once. Columns, mean; bars, SE. \*,  $P < 0.05$ .





**Figure 6.** Histologic evaluation of tumor-induced bone resorption. Tibia bones from the size-matched control AdGFP- and AdExTek-treated groups were harvested 3 wk after tumor implantation. Tissue sections were stained with H&E to reflect histology and bone resorption. Representative images are shown. Bn, bone; Nec, necrosis. Arrows, tumor cell invasion and eroded bone (A). Tibia metastatic bone tissues from the size-matched AdGFP- and AdExTek-treated groups were subjected to TRAP staining for osteoclast cells. Representative images of TRAP-positive cells in tibia metastatic bone tissues are shown. Arrows, osteoclasts (B). TRAP-positive cells were counted in 10 randomly selected fields under a microscope and graphed (C). \*,  $P < 0.05$ . Tibia metastatic bone tissues were incubated with anti-CD31 antibody for tumor vasculature (D). Representative images were shown.

progress in the local microenvironment. This reciprocal interaction between cancer cells and osteoclasts forms a vicious cycle that increases both bone destruction and the tumor burden (1). Therefore, targeting osteoclast differentiation and breaking the vicious cycle offers an effective means to control bone metastasis. In this study, we reveal a critical role of Tie2, a mediator important in breast cancer angiogenesis, in osteoclastogenesis and osteolytic bone invasion. Neutralization of Tie2 activity inhibited the formation of osteolytic bone metastases and the tumor growth of breast cancer. An understanding of molecular mechanisms underlying breast cancer bone metastases enhances the development of regimens to treat this disease.

Emerging evidence supports a close association of angiogenesis with osteoclast formation and activity, and that endothelial cells stimulate osteoclastogenesis (19). In addition to the paracrine interaction of endothelial cells with osteoclasts, angiogenic mediators may directly regulate osteoclastogenesis and function. Osteoclasts are derived from hematopoietic stem cells and these cells are known to express certain endothelium specific receptors, such as vascular endothelial growth factor receptor (VEGFR) and Tie2. Studies have shown that VEGFR-1 plays a predominant role in osteoclastogenesis and the maintenance of bone marrow functions (20). In this study, we show a direct function of Tie2 signaling specifically in osteoclastogenesis using both



genetic and biochemical approaches. Deletion of Tie2 or inhibition of Tie2 activity impaired osteoclast development using an ES cell osteoclast differentiation assay but had no effect on osteoblastogenesis. In addition, ExTek had no effects on ES cell proliferation. These data suggest a specific function of Tie2 in osteoclast differentiation and argue against toxicity of ExTek affecting ES cell differentiation. In addition, we applied a commonly used osteoclastogenesis assay from bone marrow–derived CD11b<sup>+</sup> myeloid cells. Consistently, we found a significant reduction of osteoclastogenesis in Tie2<sup>-</sup> CD11b<sup>+</sup> myeloid cells compared with Tie2<sup>+</sup> myeloid cells. These data collectively reveal a direct function of Tie2 signaling in osteoclast differentiation.

Interestingly, although Tie2<sup>+</sup> cells only account for less than 10% of total CD11b<sup>+</sup> myeloid cells, the difference in osteoclastogenesis between total CD11b myeloid cells and Tie2<sup>+</sup> myeloid cells is relatively small. This discrepancy may likely come from differences in the levels of cytokines, such as angiopoietin, in culture conditions. It is likely that Tie2<sup>+</sup> myeloid cells give rise to osteoclasts, and Tie2<sup>-</sup> cells are a major supplier for the ligand. There exist multiple ligands for Tie2, and soluble Tie2 receptor likely binds all these factors. Based on the fact that angiopoietin-1 transgenic mice display increased bone formation (11), it is reasonable to assume that soluble Tie2 inhibits bone resorption likely through depletion of angiopoietin-1.

In agreement with a role of Tie2 in osteoclast differentiation, blocking Tie2 activity using a soluble Tie2 receptor inhibited osteolytic bone invasion of breast cancer cells *in vivo*. We observed a 2.8-fold reduction of bone destruction 1 week after the injection of adenoviral vector expressing ExTek compared with controls, and the difference decreased to 1.5-fold at 2 weeks after the viral injection. This decrease of therapeutic index is likely due to a reduction of ExTek protein *in vivo*, as ExTek expression lasts for about 1 week to 10 days after the viral injection in mice (7).

Moreover, we performed histologic examination of tissue sections harvested from mice with similar-sized tumors to minimize the effect of tumor burden. We found that blocking

Tie2 function using ExTek resulted in a significant reduction of osteoclasts in addition to reduced tumor angiogenesis compared with controls. Accordingly, there was less bone destruction and more necrosis in the treated group than in controls. Considering that Tie2 is present in both endothelial cells and osteoclast precursors and Tie2 signaling regulates both angiogenesis and osteoclast differentiation, blocking Tie2 function could simultaneously inhibit tumor angiogenesis as well as osteoclast-mediated osteolytic bone destruction.

In conclusion, this study identifies a novel function of Tie2 in osteoclastogenesis and osteolytic bone invasion of breast cancer. Taken together with previous findings, we would like to argue that Tie2-based inhibitors may have therapeutic benefits over the current treatment regimens for patients with metastatic breast cancer (i.e., bisphosphonates) because Tie2 signaling regulates not only angiogenesis but also osteoclastogenesis. Neutralization of Tie2 function targets the stromal cells of the tumor microenvironment on multiple fronts, including tumor vascular development, inflammation, and osteolytic bone metastasis. Tie2 is an important therapeutic target deserving further investigation.

### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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