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NF-κB in breast cancer cells promotes osteolytic bone metastasis by inducing osteoclastogenesis via GM-CSF

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Advanced breast cancers frequently metastasize to bone, resulting in osteolytic lesions, yet the underlying mechanisms are poorly understood. Here we report that nuclear factor–κB (NF-κB) plays a crucial role in the osteolytic bone metastasis of breast cancer by stimulating osteoclastogenesis. Using an *in vivo* bone metastasis model, we found that constitutive NF-κB activity in breast cancer cells is crucial for the bone resorption characteristic of osteolytic bone metastasis. We identified the gene encoding granulocyte macrophage–colony stimulating factor (GM-CSF) as a key target of NF-κB and found that it mediates osteolytic bone metastasis of breast cancer by stimulating osteoclast development. Moreover, we observed that the expression of GM-CSF correlated with NF-κB activation in bone-metastatic tumor tissues from individuals with breast cancer. These results uncover a new and specific role of NF-κB in osteolytic bone metastasis through GM-CSF induction, suggesting that NF-κB is a potential target for the treatment of breast cancer and the prevention of skeletal metastasis.

Breast cancer is the most common cancer affecting women in the United States and other western countries. In individuals with breast cancer, the frequency of bone metastasis is much higher than that of lung and liver metastases[1–4]. In contrast to prostate cancer, which forms osteoblastic lesions, skeletal metastasis of breast cancer typically leads to osteolysis, which is often accompanied by severe pain, pathological fracture and hypercalcemia[4,5]. Although the molecular mechanism underlying the preferential metastasis of breast cancer to bone is yet to be elucidated, it is believed that osteoclasts activated by breast cancer cells mediate osteolysis. Osteoclasts have an initiating and integral role in stimulating bone-metastatic tumor growth in the marrow cavity. Whereas bone microenvironments allow circulating breast cancer cells to preferentially arrest, colonize and survive, bone-seeking cancer cells may have an intrinsic ability to promote osteoclast formation and activation[1–5]. In bone metastasis of breast cancer, there is a vicious cycle wherein bone-residing tumor cells stimulate osteoclast-mediated bone destruction, and bone-stored growth factors released from resorbed bone promote tumor growth. Therefore, the recruitment, formation and activation of osteoclasts mediated by breast cancer cells are crucial for initiating the vicious cycle of osteolytic bone metastasis of breast cancer[1,4,6].

Genes associated with tumor invasion and metastasis, including those encoding matrix metalloproteinases and interleukin-8 (IL-8), are regulated by NF-κB, suggesting that NF-κB plays a crucial role in metastasis in general[7–10]. It is not surprising that blocking NF-κB activity suppresses tumor growth and metastasis *in vitro* and *in vivo*. However, these findings were from studies that examined how NF-κB affects metastasis to the lungs, liver and lymph nodes of various human cancers, including breast cancers[9,10]. As breast cancer frequently metastasizes to bone, these findings may be irrelevant to the potential molecular mechanism of osteolytic bone metastasis. Thus, it is crucial to determine whether NF-κB controls the bone metastasis of breast cancer, using highly relevant *in vivo* models. In the present study, we examined the role of NF-κB in osteolytic bone metastasis and the progression of breast cancer, using a well-established model of osteolytic bone metastasis[11–13]. We found that NF-κB initiated osteoclastic bone destruction and bone metastasis of breast cancer. We identified the gene encoding GM-CSF (*CSF2*) as a key target of NF-κB, and GM-CSF is secreted by breast cancer cells and promotes osteolytic bone metastasis. This finding provides a new molecular mechanism by which breast cancers induce osteolysis in skeletal metastasis.

RESULTS

NF-κB promotes breast cancer bone metastasis

Constitutive NF-κB activities have been found in human breast cancer cell lines and in primary breast cancer tissues[10,14–16]. Using electrophoretic mobility shift assay (EMSA), we found strong constitutive κB-binding activities in the highly bone-metastatic breast cancer cell line MDA-MB-231 (refs. 12,13) compared with several breast cancer cell lines that are poorly metastatic (**Supplementary Fig. 1** online). We confirmed the specificity of κB-binding activities in MDA-MB-231

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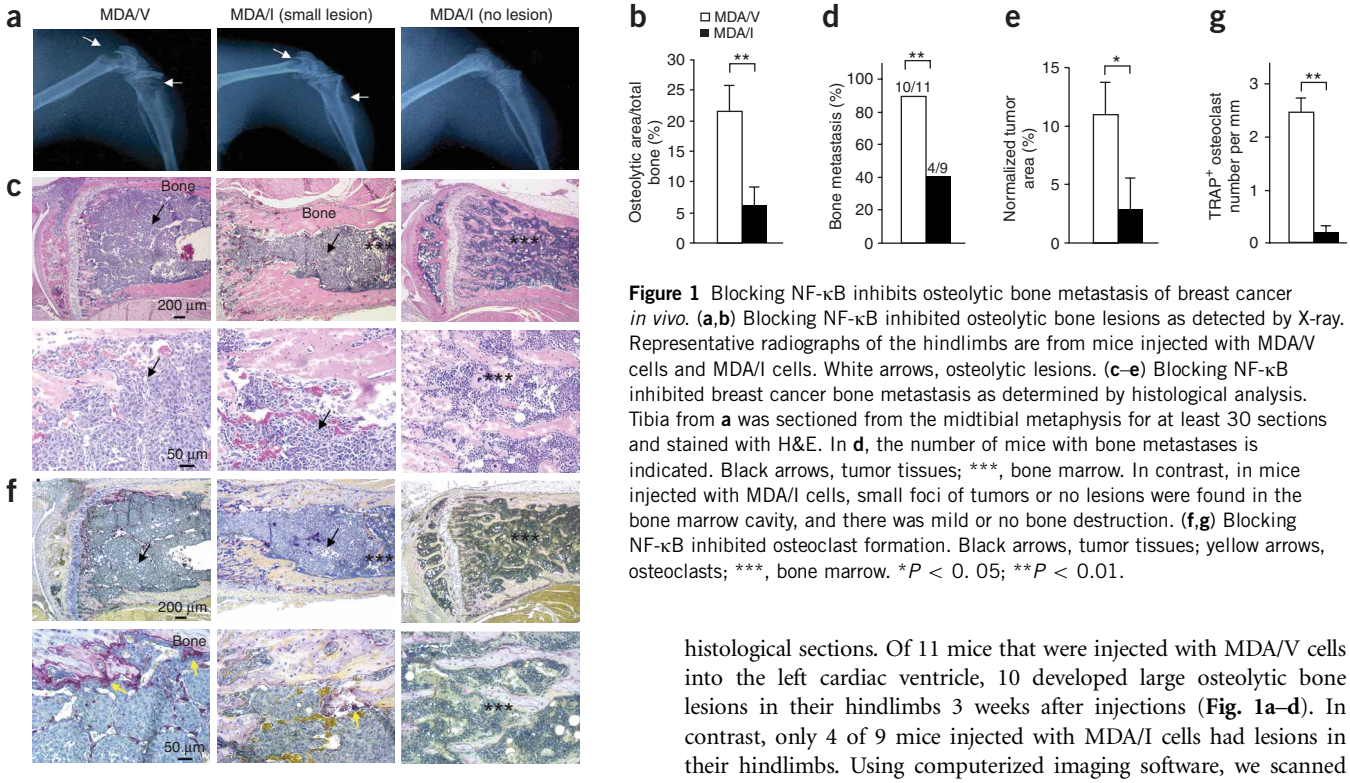

Figure 1 Blocking NF-κB inhibits osteolytic bone metastasis of breast cancer *in vivo*. (**a**,**b**) Blocking NF-κB inhibited osteolytic bone lesions as detected by X-ray. Representative radiographs of the hindlimbs are from mice injected with MDA/V cells and MDA/I cells. White arrows, osteolytic lesions. (**c**–**e**) Blocking NF-κB inhibited breast cancer bone metastasis as determined by histological analysis. Tibia from **a** was sectioned from the midtibial metaphysis for at least 30 sections and stained with H&E. In **d**, the number of mice with bone metastases is indicated. Black arrows, tumor tissues; ***, bone marrow. In contrast, in mice injected with MDA/I cells, small foci of tumors or no lesions were found in the bone marrow cavity, and there was mild or no bone destruction. (**f**,**g**) Blocking NF-κB inhibited osteoclast formation. Black arrows, tumor tissues; yellow arrows, osteoclasts; ***, bone marrow. $^{*}P < 0.05$; $^{**}P < 0.01$.

cells using the supershift assay. MDA-MB-231 cells were originally isolated from pleural effusion of a breast cancer patient with metastasis[17]. When MDA-MB-231 cells are injected into the left cardiac ventricle of immunodeficient mice, they reproducibly cause osteolytic bone lesions in the hindlimbs of these mice, which resemble the osteolytic lesions and tumor growth observed in humans with metastatic breast cancer[12,13]. To examine whether NF-κB is involved in the bone metastasis of breast cancer in mice, we retrovirally transduced MDA-MB-231 cells with the specific NF-κB inhibitor super-repressor of IκBα (SR-IκBα), a nondegradable version of IκBα containing two substitutions at serine residues targeted by the IκB kinase complex (IKK)[18–20]. MDA-MB-231 cells stably expressing SR-IκBα (MDA/I) and control cells containing empty vector (MDA/V) were generated as determined by western blot analysis. SR-IκBα suppressed the phosphorylation and degradation of IκBα induced by tumor-necrosis factor (TNF). Whereas the control cells (MDA/V) and parental cells showed constitutive nuclear κB-binding activities, no such activity was detectable in MDA/I cells. Furthermore, SR-IκBα also suppressed TNF-induced nuclear translocation of NF-κB and NF-κB-dependent transcription, as determined by a κB-dependent luciferase reporter assay (**Supplementary Fig. 1**). Whereas both MDA/V and MDA/I cells gave rise to similar numbers of tumors when they were subcutaneously implanted into mice, MDA/I cells led to smaller tumors than did MDA/V cells (**Supplementary Fig. 1**), suggesting that NF-κB activity is important for breast tumor growth *in vivo*.

As the bone microenvironment is different from the subcutaneous environment or a metastatic site in soft tissue, we examined whether blocking NF-κB affected the development of osteolytic bone metastasis. We injected MDA/V or MDA/I cells into the left cardiac ventricle of mice to allow cancer cells to circulate in the blood stream. To evaluate bone metastasis, we analyzed osteolytic lesions by microradiograph and further confirmed our results by examining histological sections. Of 11 mice that were injected with MDA/V cells into the left cardiac ventricle, 10 developed large osteolytic bone lesions in their hindlimbs 3 weeks after injections (**Fig. 1a–d**). In contrast, only 4 of 9 mice injected with MDA/I cells had lesions in their hindlimbs. Using computerized imaging software, we scanned and measured the osteolytic lesions observed by radiograph. The lesion area was significantly ($P < 0.01$) larger in mice injected with MDA/V cells than in mice injected with MDA/I cells (**Fig. 1b**). In MDA/V-injected mice, histological sections showed that both trabecular and cortical bone of the proximal tibial metaphysis had been destroyed and that the marrow cavity had been replaced by tumor tissues. In contrast, in those MDA/I-injected mice that had osteolytic lesions observable by radiograph, relatively small tumors were detected in the marrow space and bone destruction was less extensive (**Fig. 1c**). In MDA/I-injected mice without lesions observable by radiograph, no tumors were found and both trabecular and cortical bones were intact (**Fig. 1a**). Histomorphometric analysis confirmed that blocking NF-κB also significantly ($P < 0.05$) reduced the sizes of the metastatic tumors (**Fig. 1e**). As osteoclasts are crucial for osteolytic bone metastasis, we also examined whether metastatic breast cancer cells stimulated osteoclast formation, by using tartrate-resistant acid phosphatase (TRAP) staining to detect osteoclasts[6]. The number of TRAP-positive, multinuclear osteoclasts in the bone-tumor interface from mice injected with MDA/V cells were significantly ($P < 0.01$) greater than those from mice injected with MDA/I cells (**Fig. 1f**,**g**). We also noticed that numerous, small TRAP-positive osteoclasts were present within the tumor masses in MDA/V cell–injected mice, suggesting that NF-κB activities in breast cancer cells may act to recruit osteoclast precursors and to stimulate osteoclast formation.

We examined whether blocking NF-κB by IKK inhibitors could suppress the metastasis of breast cancer cells to bone. We found that the water-soluble IKK2 inhibitor VI (ref. 21) completely abolished the endogenous κB-binding activities in MDA-MB-231 cells and partially suppressed TNF-induced nuclear translocation of NF-κB (**Fig. 2a**). The IKK2 inhibitor VI also suppressed TNF-induced degradation and phosphorylation (**Fig. 2a**). Consistently, the endogenous expression of *IL8* and *BIRC3*, two well-known target genes of NF-κB, was suppressed by the IKK2 inhibitor VI, as determined by northern blot analysis (**Fig. 2b**). Like SR-IκBα, the IKK2 inhibitor VI also partially

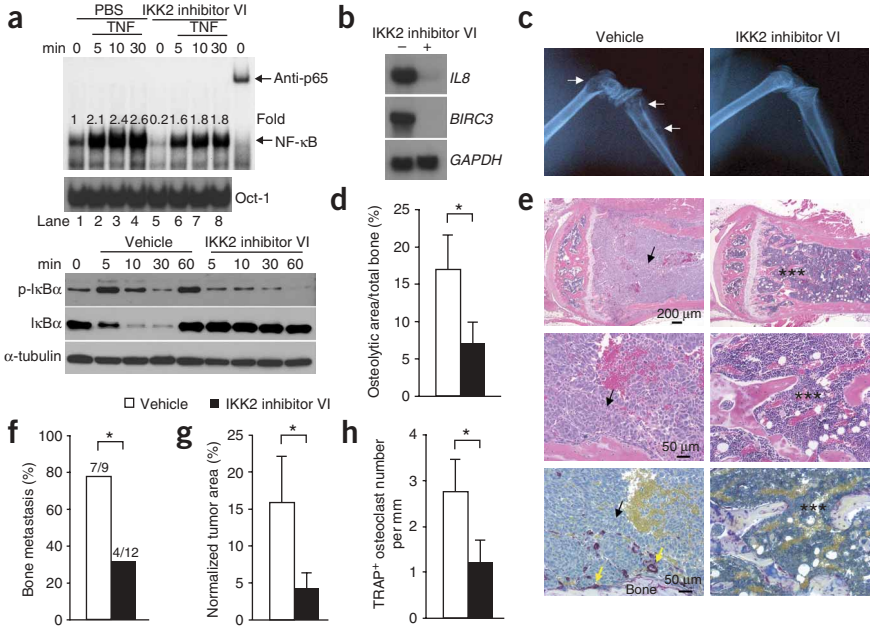

Figure 2 Inhibition of breast cancer bone metastasis by IKK2 inhibitors *in vivo*. (**a**) The IKK2 inhibitor VI suppressed NF-κB in breast cancer cells. (**b**) The IKK2 inhibitor VI suppressed the expression of *IL8* and *BIRC3*. (**c**,**d**) The IKK2 inhibitor VI inhibited osteolytic lesion formation as detected radiographically. (**e**–**g**) The IKK2 inhibitor VI suppressed breast cancer bone metastasis as detected by histological analysis. Tibias were sectioned and tumor areas were measured as described in **Figure 1**. ***, bone marrow; black arrows, tumor tissues. (**h**) The IKK inhibitor VI blocked osteoclast formation. The TRAP-positive numbers were counted as described in **Figure 1**. ***, bone marrow; black arrows, tumor tissues; yellow arrows, osteoclasts. *$P < 0.05$.

inhibited subcutaneous tumor growth but did not decrease tumor incidence (**Supplementary Fig. 2** online). To determine whether the IKK2 inhibitor VI suppressed osteolytic bone metastasis, we injected mice with MDA-MB-231 cells and subsequently treated them with the IKK2 inhibitor VI or vehicle control for 2 weeks. X-ray and histological analysis revealed that the IKK2 inhibitor VI significantly ($P < 0.05$) decreased the size and number of osteolytic bone lesions in mice, compared to vehicle control (**Fig. 2c**–**g**). Moreover, TRAP staining revealed that the number of osteoclasts induced by tumor cells was also significantly ($P < 0.01$) reduced (**Fig. 2e**,**h**).

CSF2 is a key target gene of NF-κB in bone metastasis

Blocking NF-κB activities substantially reduced the frequency of bone metastasis of breast cancer. Although MDA/V and MDA/I cells proliferated at similar rates *in vitro* (**Supplementary Fig. 3** online), we could not rule out the possibility that blocking NF-κB also inhibited primary tumor growth and/or tumorigenicity *in vivo*[22,23]. Currently, there are no *in vivo* model systems that can directly address this crucial question. We used an *in vitro* Matrigel invasion assay to show that blocking NF-κB strongly suppressed cell invasion (**Supplementary Fig. 3**), supporting the idea that NF-κB is important for metastatic invasion. However, this finding does not confirm the hypothesis that NF-κB specifically promotes skeletal metastasis. Therefore, we used gene expression profiling to identify the target genes of NF-κB that may be associated with osteoclastic bone destruction. Together with this information, gain- and loss-of-function experiments could help to determine whether these NF-κB–inducible genes are crucial for osteolytic bone metastasis, thereby providing direct evidence to verify the functional role of NF-κB in bone-metastatic breast cancer. We compared the gene expression profiles of both MDA/V and MDA/I cells using human Affymetrix microarrays. As expected, we found that several well-known NF-κB–dependent and tumor growth–associated genes, including *IL8* and *BIRC3* (refs. 9,19), were highly expressed in MDA/V cells compared with MDA/I cells. We were surprised to find that *CSF2*, which is currently being tested for use in adjuvant therapy in several human cancers[24], was highly expressed in MDA/V cells but had low levels of expression in MDA/I cells. Moreover, the signal intensities of *CSF2* transcripts were markedly higher than those of *IL8* and other target genes of NF-κB in MDA/V cells. The expression of transcripts for parathyroid hormone–related protein (*PTHLH*) and activator of NF-κB ligand (*TNFSF11*), which promote osteolytic bone lesions[25,26], was not affected by blocking NF-κB (data not shown), suggesting that NF-κB may promote bone metastasis of breast cancer independent of these genes. Northern blot analysis confirmed that *CSF2* was highly expressed in MDA/V cells but not in MDA/I cells (**Fig. 3a**). Moreover, ELISA also revealed that the expression level of GM-CSF in serum-free culture media from MDA/V cells was higher than that from MDA/I cells (**Fig. 3b**).

GM-CSF is an important pro-inflammatory cytokine in macrophage recruitment in various inflammatory diseases[27–32]. Notably, we did not detect GM-CSF in breast cancer cell lines that cause little bone metastasis, including MDA-MB-468, SK-BR-3, MCF-7 and MDA-MB-157 (**Fig. 3a**). To determine whether *CSF2* was a key target of NF-κB for promoting osteolytic bone metastasis, we stably restored GM-CSF expression in NF-κB–deficient MDA/I cells (**Fig. 3c**) and injected both MDA/I cells expressing GM-CSF (MDA/I/GM-CSF) and control cells (MDA/I/C) into the left cardiac ventricle of mice. Whereas MDA/I/C cells resulted in no lesions or few, small osteolytic lesions in mice, MDA/I/GM-CSF cells generated significantly ($P < 0.05$) more and larger osteolytic bone lesions, as determined by both X-ray and histological analysis (**Fig. 3d**–**g**). TRAP staining revealed an increased number of osteoclasts in osteolytic lesions from mice injected with MDA/I/GM-CSF compared with mice injected with MDA/I/C cells (**Fig. 3d**,**h**). GM-CSF binds to its receptor to activate MAPK or PI3 kinase/Akt in hemapoietic cells[27]. However, we found that there were no differences either in the phosphorylation of Akt and ERK or in the activity of NF-κB between MDA/I/GM-CSF and MDA/I/C cells. These cells also did not express *CSF2RB* (**Supplementary Fig. 4** online), suggesting that GM-CSF did not promote bone metastasis through an autocrine mechanism.

To further determine whether NF-κB and GM-CSF are associated with bone metastasis of breast cancer, we examined whether GM-CSF could be detected in bone-metastatic breast tumors in humans. Immunostaining revealed GM-CSF in 75% of bone-metastatic tumor samples ($n = 32$). Using p65-specific monoclonal antibodies, which detect the active NF-κB subunit p65 in the nucleus, we found nuclear localization of NF-κB in 75% of samples. Moreover, statistical analysis revealed that GM-CSF expression was correlated with the

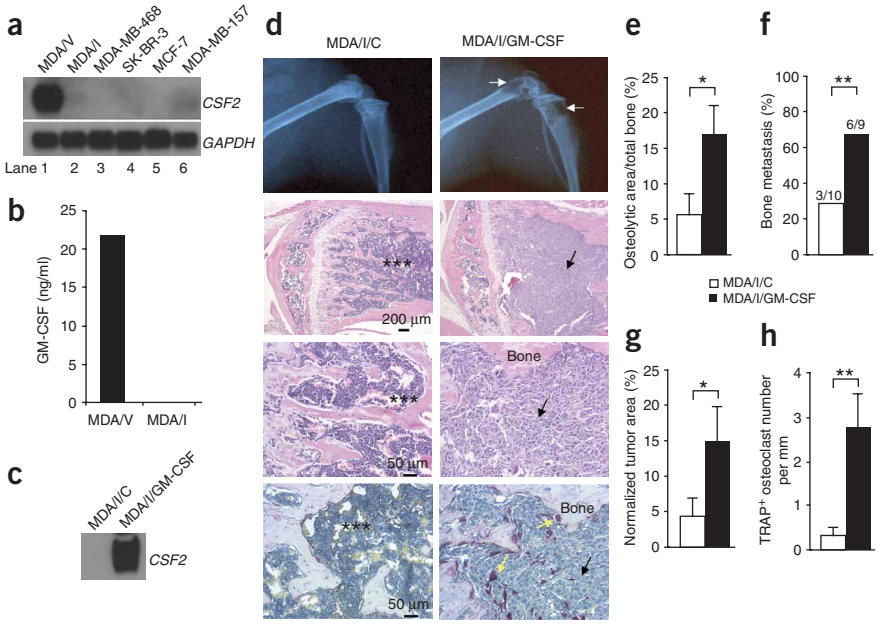

Figure 3 NF-κB-induced *CSF2* in breast cancer cells promotes breast cancer bone metastasis *in vivo*. (**a**) *CSF2* was induced by constitutive NF-κB in breast cancer cells. (**b**) Confirmation of GM-CSF expression by ELISA. (**c**) The restoration of GM-CSF in NF-κB-deficient MDA/I cells. (**d**,**e**) The restoration of GM-CSF in NF-κB-deficient breast cancer cells induced osteolytic lesion formation as detected by X-ray and histology. The injection and radiographs were performed as in **Figure 1**. White arrows, osteolytic lesions. (**f**,**g**) The histological analysis and tumor size measurement. Black arrows, tumor tissues; ***, bone marrow. (**h**) The restoration of GM-CSF in NF-κB-deficient breast cancer cells promoted osteoclast formation. The multinuclear, $TRAP^+$ osteoclasts were counted as in **Figure 1**. ***, bone marrow; black arrows, tumor tissues; yellow arrows, osteoclasts. $^*P < 0.05$; $^{**}P < 0.01$.

nuclear localization of NF-κB in bone-metastatic tumor samples (Pearson correlation coefficient $r = 0.88$; $P < 0.02$; **Supplementary Fig. 5** online).

Critical role of GM-CSF in osteolytic bone metastasis

To further test whether GM-CSF is a specific factor that mediates NF-κB–dependent bone metastasis of breast cancer, we used short hairpin RNA (shRNA) to knockdown *CSF2* in MDA-MD-231 cells. MDA-MD-231 cells were stably transduced with retroviruses expressing shRNAs for GM-CSF or luciferase. Northern blot analysis showed that expression of GM-CSF shRNA almost completely inhibited *CSF2* expression in MDA-MB-231 cells and ELISA also confirmed that GM-CSF protein levels were reduced by 95% (**Fig. 4a**). The knockdown of GM-CSF did not affect tumor cell proliferation *in vitro* or subcutaneous tumor growth *in vivo* (**Supplementary Fig. 6** online). In contrast, we found that the knockdown of GM-CSF significantly reduced ($P < 0.05$) osteolytic bone metastases and tumor burdens, as determined by both X-ray and histological analysis (**Fig. 4b**,**c**). TRAP staining revealed that osteoclast numbers were significantly ($P < 0.01$) reduced in tumors from MDA/shRNA-GM-CSF cells compared with tumors from MDA/shRNA-Luc cells. To rule out the nonspecific effects of shRNA, we also examined whether the inhibition of GM-CSF function by GM-CSF–specific neutralizing antibodies suppressed bone metastasis. Of note, GM-CSF–specific antibodies did not affect cell growth *in vitro* (**Supplementary Fig. 6**). Compared with control IgG, GM-CSF–specific antibodies also strongly inhibited the metastasis of tumor cells to bone and osteoclastic bone destruction *in vivo* (**Fig. 4d**,**e**; **Supplementary Fig. 7** online).

Next, we determined the role of NF-κB–dependent production of GM-CSF by breast cancer cells in osteoclastogenesis from primary bone marrow cells. As a positive control, RANKL treatment induced osteoclast formation from primary bone marrow cells (**Fig. 5a**). Whereas the conditioned media from MDA/I cells had minimal effect

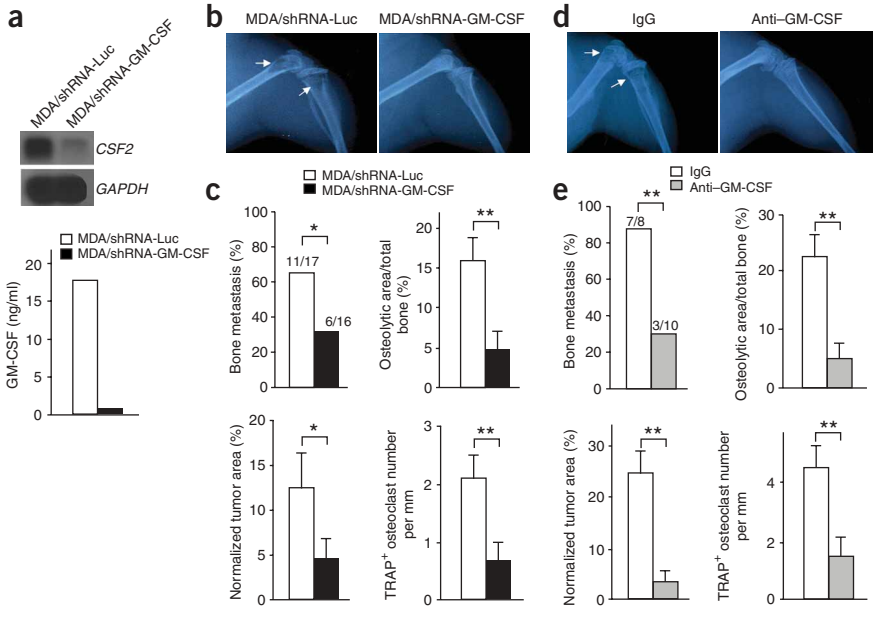

Figure 4 GM-CSF is crucial for breast cancer bone metastasis induced by NF-κB *in vivo*. (**a**) The knock-down of GM-CSF expression in breast cancer cells by shRNA for *CSF2*. (**b**) The knock-down of GM-CSF inhibited osteolytic lesions as determined by X-ray. Injection and radiographs were carried out as described in **Figure 1**. Arrows, osetolytic lesions. (**c**) The knock-down of GM-CSF inhibited breast cancer bone metastasis as determined by histological analysis. The histological analysis and tumor size measurement were performed as described in **Figure 1**. $^*P < 0.05$; $^{**}P < 0.01$. (**d**) GM-CSF-specific antibodies inhibited osteolytic lesion formation as determined by X-ray. Arrows, osetolytic lesions. (**e**) GM-CSF-specific antibodies inhibited breast cancer bone metastasis and osteoclast formation as determined by histological analysis. $^{**}P < 0.01$.

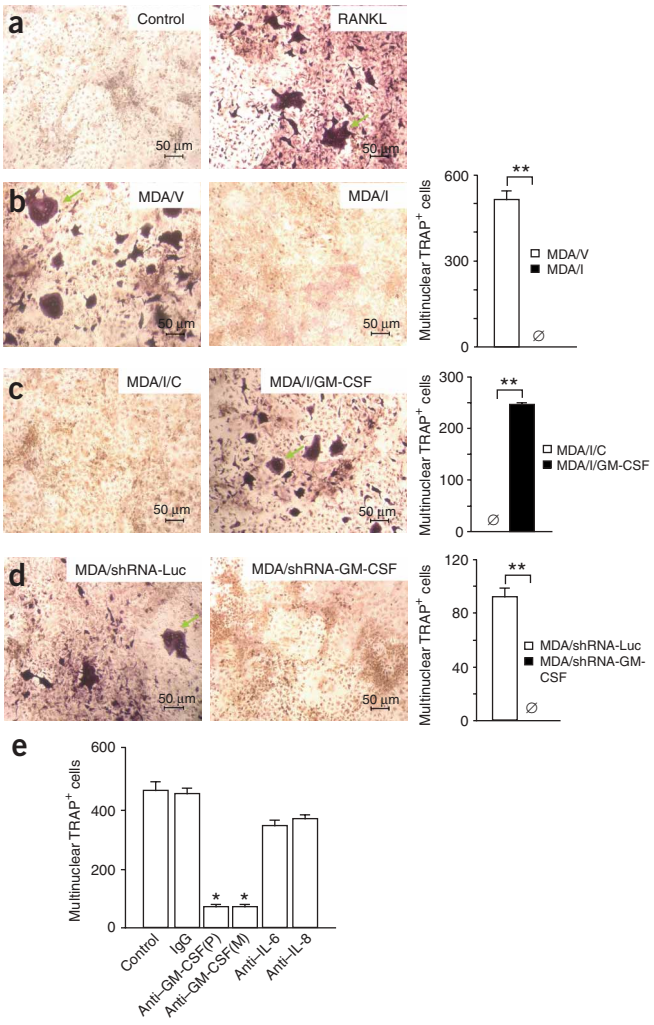

Figure 5 Constitutive NF-κB activities in breast cancer cells stimulated osteoclast formation through GM-CSF *in vitro*. (**a**) The induction of osteoclast formation by RANKL. (**b**) Constitutive NF-κB in breast cancer cells stimulated osteoclast formation *in vitro*. The results represent average values from two independent experiments. (**c**) The restoration of GM-CSF in NF-κB-deficient breast cancer cells stimulated osteoclast formation *in vitro*. (**d**) The knockdown of GM-CSF abolished osteoclast formation induced by constitutive NF-κB activities in breast cancer cells. Green arrow, osteoclasts. $^{**}P < 0.01$. (**e**) GM-CSF-specific neutralizing antibodies inhibited osteoclast formation induced by conditioned media from tumor cells. The results represent average values ± s.d. from two experiments. $^{*}P < 0.001$. M, monoclonal antibodies; P, polyclonal antibodies; ϕ, too few cells to plot.

on osteoclast formation, that from MDA/V cells significantly ($P < 0.01$) enhanced osteoclast formation from primary bone marrow cells (**Fig. 5b**). In contrast, conditioned media from MDV/I/GM-CSF cells significantly ($P < 0.01$) increased osteoclast formation (**Fig. 5c**). Moreover, the conditioned media from MDA/shRNA-GM-CSF cells did not induce osteoclast formation, in contrast to the conditioned media from MDA/shRNA-Luc (**Fig. 5d**). Finally, two different GM-CSF–specific neutralizing antibodies also potently inhibited osteoclast formation induced by conditioned media from tumor cells, whereas IL-8– or IL-6–specific neutralizing antibodies only had a modest effect on osteoclast formation (**Fig. 5e**).

Metastasis is a complex multistep process[7,33,34]. To target bone, breast cancer cells need to circulate in the bloodstream, extravasate and adhere to extracellular matrix[1,35,36]. It is possible that blocking GM-CSF function might also interfere with these processes and thereby inhibit bone metastasis indirectly. Although NF-κB may facilitate these metastatic processes, we hypothesize that GM-CSF may induce osteolytic bone lesions by stimulating osteoclast formation *in vivo*; to test this, we performed direct intratibial injections of MDA/shRNA-GM-CSF or MDA/shRNA-Luc cells in mice. Mice injected with MDA/shRNA-Luc cells rapidly developed large osteolytic bone lesions in their tibias 3 weeks after injection, whereas mice injected with MDA/shRNA-GM-CSF formed smaller or no lesions, as determined by X-ray analysis (**Fig. 6a,b**). Histological staining revealed extensive trabecular and cortical bone destruction in tumors derived from MDA/shRNA-Luc cells compared with those derived from MDA/shRNA-GM-CSF (**Fig. 6c–e**). Moreover, we detected an increased number of TRAP-positive and multinuclear osteoclasts lining the bone surface area in tumors derived from MDA/shRNA-Luc cells compared with tumors derived from MDA/shRNA-GM-CSF (**Fig. 6f,g**). Finally, we found that GM-CSF–specific neutralizing antibodies also blocked osteolytic tumor growth and osteoclast formation compared with control IgG treatment (**Fig. 6h**).

DISCUSSION

Our results demonstrate that the IKK/NF-κB signaling pathway is important in osteolytic bone metastasis and the progression of breast cancer. Using both biological and chemical inhibitors, we found that blocking NF-κB suppressed osteolytic lesion development through osteoclastic bone resorption *in vivo*. Notably, we identified *CSF2* as a key target of NF-κB, and found that it mediates osteoclastic bone destruction and metastatic tumor growth. Our *in vitro* cell culture assay further revealed that constitutive NF-κB activity in breast cancer cells stimulated osteoclastogenesis by upregulating GM-CSF. Consistent with these findings, we found that the expression of GM-CSF was correlated with NF-κB activity in bone-metastatic tumor tissues from humans with breast cancer. Taken together, our experiments identify a new connection between osteoclastic bone destruction and bone metastasis through the IKK/NF-κB signaling pathway.

GM-CSF was originally defined by its ability to induce both granulocyte and macrophage colonies from precursor cells in the bone marrow. GM-CSF is a pro-inflammatory cytokine that is highly expressed in arthritis and other inflammatory diseases. It helps to recruit macrophages to inflammatory sites and to enhance inflammatory mediator production[27]. Osteoclasts and macrophages are derived and differentiated from the same granulocyte macrophage colony–forming progenitor cells. NF-κB is important in bone resorption and osteoclast development. Osteoclasts can be considered as specialized macrophages that resorb bone matrix in inflammatory bone diseases[6,37,38]. Notably, we found that GM-CSF along with active NF-κB was expressed in bone-metastatic breast tumor tissues. In human bone marrow culture systems, numerous studies have demonstrated that GM-CSF potently induces osteoclast formation[28–31]. The inhibition of GM-CSF by neutralizing antibodies or small molecule inhibitors suppresses osteoclast formation[32]. These studies support the idea that *CSF2* is a crucial target of NF-κB and that GM-CSF mediates osteolytic bone metastasis of breast cancer. However, it should be pointed out that there are some controversies about the role of GM-CSF in osteoclast differentiation. These differences may be due to the cells or culture system used for osteoclast differentiation *in vitro*. For example, one *in vitro* study showed that breast cancer cells promote osteoclast formation by stimulating IL-11 and inhibiting GM-CSF (ref. 39), but the MDA-MB-231 cells used do not metastasize

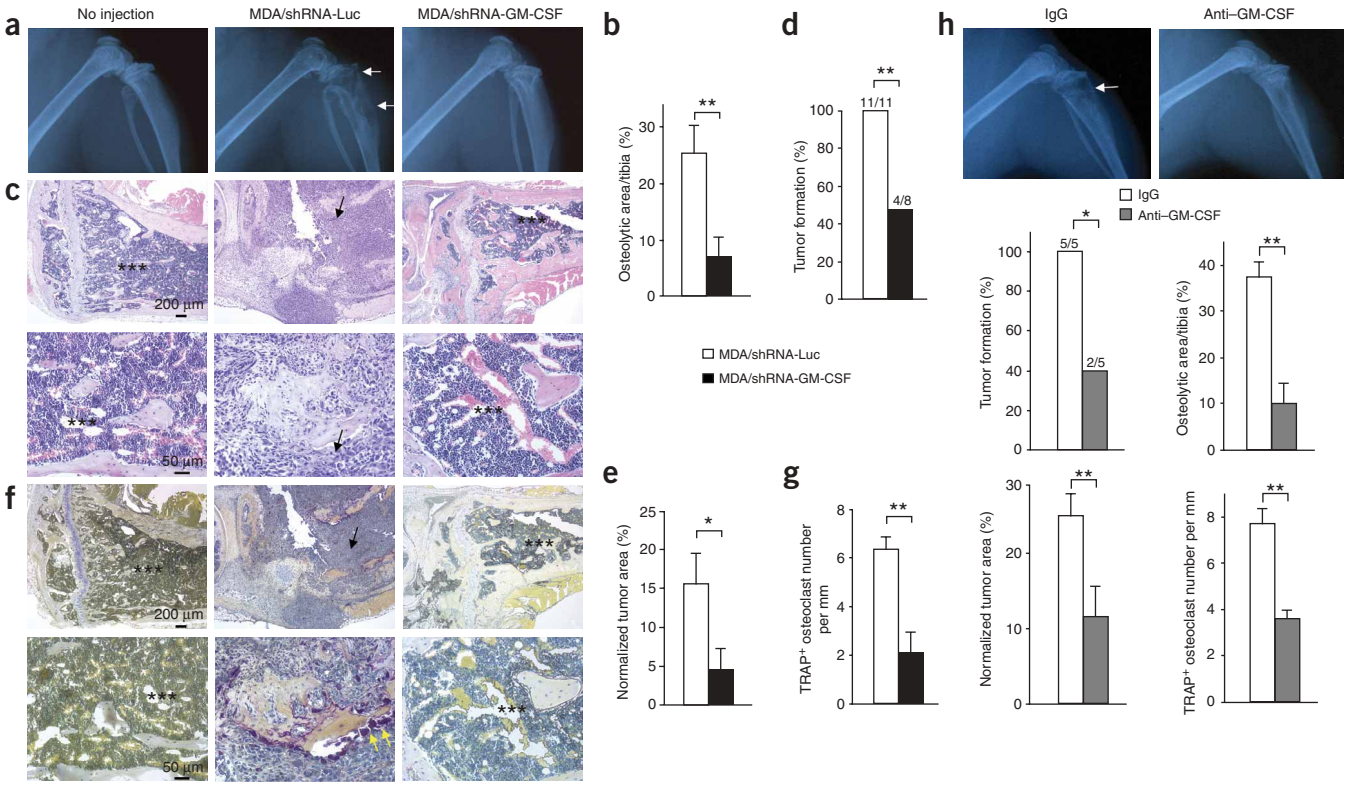

Figure 6 NF-κB directly stimulates osteoclast formation and promotes bone-metastatic tumor growth *in vivo* through GM-CSF. (**a**,**b**) GM-CSF was essential for osteolytic lesion formation induced by constitutive NF-κB activities in breast cancer cells. White arrows, osteolytic lesions. (**c**–**e**) GM-CSF was essential for osteolytic tumor growth. Black arrows, tumor tissues; ***, bone marrow. (**f**,**g**) GM-CSF was essential for osteoclast formation induced by constitutive NF-κB activities in breast cancer cells. The TRAP staining was performed as described in **Figure 2**. $*P < 0.05$; $**P < 0.01$. Black arrow, tumor tissues; yellow arrows, osteoclasts; ***, bone marrow. (**h**) The inhibition of GM-CSF function by GM-CSF-specific neutralizing antibodies suppressed osteolytic tumor growth as determined by X-ray and histology. White arrows, osetolytic lesions. The histological analysis and the TRAP staining were performed as described in **Figure 1**. $**P < 0.01$; $*P < 0.05$.

to bone, and the authors did not provide *in vivo* data to confirm their *in vitro* findings. In contrast, we used primary mouse bone marrow cells isolated from the long bones. Our gain- and loss-of-function studies demonstrated that NF-κB–induced GM-CSF was essential for osteoclast formation *in vivo* and *in vitro*, as (i) the depletion of GM-CSF abolished osteoclast formation induced by conditioned media from tumor cells; (ii) the inhibition of GM-CSF by two different neutralizing antibodies suppressed osteoclast formation; and (iii) the inhibition of GM-CSF function by shRNA or GM-CSF–specific neutralizing antibodies substantially inhibited osteolytic tumor growth and osteoclast formation *in vivo* (in two different tumor models). Supporting our findings, *in vivo* experiments[31] have demonstrated that GM-CSF corrects osteopetrosis in mice by stimulating osteoclast formation. It is commonly believed that GM-CSF stimulates proliferation of osteoclast precursors and induces the development, differentiation and survival of mononuclear osteoclasts[28,30,31]. In keeping with this, we observed that the number of mononuclear osteoclasts was markedly increased at bone-metastatic sites, suggesting that GM-CSF helps to recruit osteoclast progenitors and to enhance the early stage of osteoclast formation during the development of bone metastasis. Finally, several pro-inflammatory cytokines such as IL-8 and IL-6 are also regulated by NF-κB. It is possible that GM-CSF may collaborate with these cytokines to stimulate osteoclast differentiation.

Recently, genetic studies[23,40] demonstrated that inflammation and inflammatory cells associated with the tumor microenvironment promote tumor growth and metastasis. Also, it was reported that GM-CSF recruits neutrophils and promotes tumor cell transmigration through the endothelial barrier[41], and macrophages were found to be critical in breast cancer growth and progression[42]. Inflammatory cells frequently infiltrate breast cancer tissues and may promote tumor angiogenesis and growth. Paradoxically, some studies also found that inflammatory cytokines may function to inhibit tumor growth and development. GM-CSF has been used as an adjuvant therapy or a tumor vaccine to promote the production of immune cells such as dendritic cells, macrophages and inflammatory cytokines[24]. Although clinical trials of a GM-CSF–based vaccine showed some promising results in metastatic melanoma, some patients were withdrawn early owing to rapid disease progression[24]. It will be important to determine whether overexpression of GM-CSF has an adverse effect on disease progression. According to our results and recent genetic studies, using GM-CSF as adjuvant therapy to induce inflammatory cells and cytokines may increase bone metastasis and other distant-site metastasis[23,40]. Moreover, due to the nature of advanced or metastatic breast cancer, these cancer cells may be resistant to immunoregulation mediated by the GM-CSF–based tumor vaccine[43].

In conclusion, given the fact that NF-κB is associated with tumor progression and cancer therapy resistance, NF-κB is under intense investigation as a potential therapeutic target[18,20,40,44,45]. Here we revealed a specific role of NF-κB in osteolytic bone metastasis. Therefore, in addition to sensitizing cancer cells to apoptosis, inhibiting NF-κB signaling in breast cancer may also have important

implications for treating or preventing bone metastasis of breast cancer.

METHODS

Cell culture, retroviral infection and reagents. MDA-MB-231 cells were cultured in DMEM supplemented with 10% FBS (Invitrogen). Other breast cancer cell lines were obtained from American Type Culture Collection. The full-length cDNA clone for human *CSF2* was purchased from ATCC and subcloned into pBabe-puro retroviral vectors. The retroviral vector pSIREN-RetroQ was purchased from BD Biosciences Clontech, and the shRNA oligonucleotide for *CSF2* (targeting sequence: 5′-AACCTGAGTAGAGA CACTG-3′) was designed according to the manufacturer's instructions. Retroviral infections were performed as described previously[46].

Western blot, northern blot and EMSA. The experiments were performed as described previously[46]. The primary antibodies were from the following sources: IκBα-specific and GM-CSF–specific polyclonal antibodies for immunostaining from Santa Cruz Biotechnology; GM-CSF–specific polyclonal and monoclonal antibodies for neutralization from R&D systems; phospho-IκBα–specific antibodies from Cell Signaling; and α-tubulin–specific antibodies from Sigma-Aldrich.

Human Affymetrix microarray. Total RNAs were extracted with Trizol reagents. To eliminate contaminated genomic DNA, total RNAs were cleaned with an RNeasy kit (Qiagen). 10-μg aliquots of total RNA from each sample were utilized for microarray analysis as described previously[46]. Briefly, RNAs were transcribed to double-stranded complementary DNA (cDNA) using SuperScript II RT (Invitrogen) with an oligo-dT primer that has a T7 RNA polymerase site on the 5′ end. Then, the cDNAs were used in an *in vitro* transcription reaction in the presence of biotin-modified ribonucleotides to generate single stranded RNAs. The biotin-labeled RNAs were fragmented and hybridized with an Affymetrix human U133A gene chip. The arrays were scanned with the GeneArray scanner (Affymetrix). Signal intensity was calculated using the one-step Tukey's Biweight Estimate. Affymetrix® Microarray Suite (MAS) 5.0 was used for data analysis. All microarray data have been submitted to the Gene Expression Omnibus (GEO) under accession number GSE6324.

Immunohistochemistry. The use of human breast cancer tumor samples was approved by the University of Michigan and Henry Ford Health System Institutional Review Board with waiver of consent from subjects. Serial tissue sections from bone-metastatic tumors from humans with breast cancer were deparaffinized, and antigen retrieval was performed by pressure-cooking in a Decloaking chamber (Biocare Medical) in citrate buffer (2.1 g/l citric acid, pH 6.0) at 120 °C for 20 min. Serial tissue sections were preincubated with peroxide-blocking solution for 10 min and then incubated with human GM-CSF–specific polyclonal antibodies (Santa Cruz; 1:100) or p65-specific monoclonal antibodies (Rockland; 1:100) at 4 °C overnight. The sections were then incubated with HRP-labeled polymer for 30 min and the immunocomplexes were detected with 3-amino-9-ethylcarbazole (AEC^+) chromogen (Dako EnVision System) and counterstained with hematoxylin as described previously[46].

***In vivo* mouse model of osteolytic bone metastasis, radiographic and histological analysis.** Animal experiments were approved by the University of Michigan Committee on Use and Care of Animals (UCUCA). Breast cancer cells (1×10^5) were injected into the left cardiac ventricle in 5-week-old immunodeficient mice (Taconic). For treatment with the IKK2 VI inhibitor, mice were injected with breast cancer cells (1×10^5), and then given injections of the IKK2 VI inhibitor (Calbiochem; 1 mg per kg body weight) via the tail vein twice per day for 3 weeks. For antibody treatment, mice were injected with GM-CSF–specific neutralizing antibodies (R&D systems; 5 mg/kg) or control IgG intraperioneally once per day for 20 d. Three weeks after injection, radiographs were taken using a Faxitron MX-20 X-ray machine (Faxitron X-ray). For intratibial injection, the right tibia of 8-week-old mice was injected with breast cancer cells (1×10^5) in 20 μl of the cell suspension. To measure the size of osteolytic lesions, radiographs were scanned. All specimens were fixed in 10% formalin for 24 h and decalcified in 10% EDTA (pH 7.4) for 14 d at 4 °C. Then serial sections were prepared and stained with hematoxylin and eosin. Lesions observed by radiograph were confirmed with histological examination. Quantification of osteolytic lesion areas and tumor sizes per total bone area (tibia and femur) was carried out by computer-assisted histomorphometry using ImagePro Plus 4.5 (Media Cybernetics) and SPOT 4.0 software (Diagnostic Instruments).

To quantify the number of TRAP-stained osteoclasts at the bone-tumor interface, histological sections were deparaffinized at 60 °C for 1 h and rehydrated through decreasing alcohol. The sections were stained with TRAP activities using a leukocyte acid phosphatase staining kit according to the manufacturer's instruction (Sigma). The number of TRAP-positive, multinuclear (>3) osteoclasts per millimeter of bone was calculated using a computerized image analysis system as described above.

***In vitro* osteoclast formation assay.** Long bones (femurs and tibias) were removed from 3-week-old mice, dissected free of soft tissue, and washed with PBS. Bone marrow cells were collected by injecting PBS (using a 1-ml syringe) throughout the entire marrow cavity several times, and then gently triturated 10 times with a transfer pipette. Bone marrow cells were plated in MEM-α medium containing 15% heat-inactivated FBS. Then 1×10^4 cells were plated in 24-well plates and stimulated with the conditioned media from each cell type for 10 d. For neutralization, cells were treated with polyclonal (1 μg/ml) or monoclonal (10 μg/ml) antibodies to GM-CSF, polyclonal antibodies to IL-6 and IL-8 (1 μg/ml), or control IgG (10 μg/ml). Cells were washed in PBS and fixed in fixative solution (1.56 ml citrate solution, 4.06 ml acetone and 0.5 ml of 37% formaldehyde) for 30 s. Cells were stained for TRAP activities. Multinuclear, TRAP-positive cells were counted and compared in each group. Three independent experiments were performed.

Statistics. Data are present as the mean ± s.d. The significance of the difference between groups was evaluated with the Student's *t*-test or χ^2 test. $P < 0.05$ was considered significant.

Note: Supplementary information is available on the Nature Medicine website.

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AUTHOR CONTRIBUTIONS

B.K.P., S.C. and C.-Y.W. designed and organized the experiments, performed the animal studies, analyzed the data; generated the figures and wrote the manuscript. H.Z. performed the subcloning. Q.Z., T.G., K.G., V.S., L.P. and R.J.Z. conducted the histological analysis. J.D., S.S., E.T.K. and L.M. performed the animal studies and wrote the manuscript.

COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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1. Roodman, G.D. Mechanisms of bone metastasis. *N. Engl. J. Med.* **350**, 1655–1664 (2004).
2. Mundy, G.R. Metastasis to bone: causes, consequences and therapeutic opportunities. *Nat. Rev. Cancer* **2**, 584–593 (2002).
3. Javed, A. *et al.* Impaired intranuclear trafficking of Runx2 (AML3/CBFA1) transcription factors in breast cancer cells inhibits osteolysis *in vivo*. *Proc. Natl. Acad. Sci. USA* **102**, 1454–1459 (2005).
4. Kozlow, W. & Guise, T.A. Breast cancer metastasis to bone: mechanisms of osteolysis and implications for therapy. *J. Mammary Gland Biol. Neoplasia* **10**, 169–180 (2005).
5. Zhang, J. *et al.* Osteoprotegerin inhibits prostate cancer–induced osteoclastogenesis and prevents prostate tumor growth in the bone. *J. Clin. Invest.* **107**, 1235–1244 (2001).
6. Martin, T.J. Paracrine regulation of osteoclast formation and activity: milestones in discovery. *J. Musculoskelet. Neuronal Interact.* **4**, 243–253 (2004).

7. Fidler, I.J. Critical determinants of metastasis. *Semin. Cancer Biol.* **12**, 89–96 (2002).
8. Chambers, A.F., Groom, A.C. & MacDonald, I.C. Dissemination and growth of cancer cells in metastatic sites. *Nat. Rev. Cancer* **2**, 563–572 (2002).
9. Huang, S., Pettaway, C.A., Uehara, H., Bucana, C.D. & Fidler, I.J. Blockade of NF-κB activity in human prostate cancer cells is associated with suppression of angiogenesis, invasion, and metastasis. *Oncogene* **20**, 4188–4197 (2001).
10. Huber, M.A. *et al.* NF-κB is essential for epithelial-mesenchymal transition and metastasis in a model of breast cancer progression. *J. Clin. Invest.* **114**, 569–581 (2004).
11. Kang, Y. *et al.* A multigenic program mediating breast cancer metastasis to bone. *Cancer Cell* **3**, 537–549 (2003).
12. Guise, T.A. *et al.* Evidence for a causal role of parathyroid hormone-related protein in the pathogenesis of human breast cancer-mediated osteolysis. *J. Clin. Invest.* **98**, 1544–1549 (1996).
13. Yin, J.J. *et al.* TGF-β signaling blockade inhibits PTHrP secretion by breast cancer cells and bone metastases development. *J. Clin. Invest.* **103**, 197–206 (1999).
14. Sovak, M.A. *et al.* Aberrant nuclear factor-κB/Rel expression and the pathogenesis of breast cancer. *J. Clin. Invest.* **100**, 2952–2960 (1997).
15. Hu, M.C. *et al.* IκB kinase promotes tumorigenesis through inhibition of forkhead FOXO3a. *Cell* **117**, 225–237 (2004).
16. Eddy, S.F. *et al.* Inducible IκB kinase/IκB kinase ε expression is induced by CK2 and promotes aberrant nuclear factor-κB activation in breast cancer cells. *Cancer Res.* **65**, 11375–11383 (2005).
17. Cailleau, R., Young, R., Olive, M. & Reeves, W.J., Jr. Breast tumor cell lines from pleural effusions. *J. Natl. Cancer Inst.* **53**, 661–674 (1974).
18. Wang, C.-Y., Mayo, M.W. & Baldwin, A.S. TNF- and cancer therapy-induced apoptosis: potentiation by inhibition of NF-κB. *Science* **274**, 784–787 (1996).
19. Wang, C.-Y., Mayo, M.W., Korneluk, R.C., Goeddel, D.V. & Baldwin, A.S. NF-κB antiapoptosis: induction of TRAF1 and TRAF2 and c-IAP1 and c-IAP2 to suppress caspase-8 activation. *Science* **281**, 1680–1683 (1998).
20. Wang, C.-Y., Cusack, J., Liu, R. & Baldwin, A.S. Control of inducible chemoresistance: enhanced anti-tumor therapy via increased apoptosis through inhibition of NF-κB. *Nat. Med.* **5**, 412–417 (1999).
21. Baxter, A. *et al.* Hit-to-lead studies: the discovery of potent, orally active, thiophene-carboxamide IKK-2 inhibitors. *Bioorg. Med. Chem. Lett.* **14**, 2817–2822 (2004).
22. Mayo, M.W. & Baldwin, A.S. The transcription factor NF-κB: control of oncogenesis and cancer therapy resistance. *Biochim. Biophys. Acta* **1470**, M55–M62 (2000).
23. Greten, F.R. *et al.* IKKβ links inflammation and tumorigenesis in a mouse model of colitis-associated cancer. *Cell* **118**, 285–296 (2004).
24. Dranoff, G. Cytokines in cancer pathogenesis and cancer therapy. *Nat. Rev. Cancer* **4**, 11–22 (2004).
25. Yoneda, T. & Hiraga, T. Crosstalk between cancer cells and bone microenvironment in bone metastasis. *Biochem. BioPhys. Res. Commun.* **328**, 679–687 (2005).
26. Jones, D.H. *et al.* Regulation of cancer cell migration and bone metastasis by RANKL. *Nature* **440**, 692–696 (2006).
27. Hamilton, J.A. GM-CSF in inflammation and autoimmunity. *Trends Immunol.* **23**, 403–408 (2002).
28. Hodge, J.M. *et al.* Osteoclastic potential of human CFU-GM: biphasic effect of GM-CSF. *J. Bone Miner. Res.* **19**, 190–199 (2004).
29. MacDonald, B.R. *et al.* Effects of human recombinant CSF-GM and highly purified CSF-1 on the formation of multinucleated cells with osteoclast characteristics in long-term bone marrow cultures. *J. Bone Miner. Res.* **1**, 227–233 (1986).
30. Menaa, C. *et al.* Annexin II increases osteoclast formation by stimulating the proliferation of osteoclast precursors in human marrow cultures. *J. Clin. Invest.* **103**, 1605–1613 (1999).
31. Myint, Y.Y. *et al.* Granulocyte/macrophage colony-stimulating factor and interleukin-3 correct osteopetrosis in mice with osteopetrosis mutation. *Am. J. Pathol.* **154**, 553–566 (1999).
32. Li, F. *et al.* Annexin II stimulates RANKL expression through MAPK. *J. Bone Miner. Res.* **20**, 1161–1167 (2005).
33. Coussens, L.M. & Werb, Z. Inflammation and cancer. *Nature* **420**, 860–867 (2002).
34. Hanahan, D. & Weinberg, R.A. The hallmarks of cancer. *Cell* **100**, 57–70 (2000).
35. Clark, E.A., Golub, T.R., Lander, E.S. & Hynes, R.O. Genomic analysis of metastasis reveals an essential role for RhoC. *Nature* **406**, 532–535 (2000).
36. Horak, C.E. & Steeg, P.S. Metastasis gets site specific. *Cancer Cell* **8**, 93–95 (2005).
37. Jimi, E. *et al.* Selective inhibition of NF-κB blocks osteoclastogenesis and prevents inflammatory bone destruction *in vivo*. *Nat. Med.* **10**, 617–624 (2004).
38. Ruocco, M.G. *et al.* IκB kinase (IKK)β, but not IKKα, is a critical mediator of osteoclast survival and is required for inflammation-induced bone loss. *J. Exp. Med.* **201**, 1677–1687 (2005).
39. Morgan, H., Tumber, A. & Hill, P.A. Breast cancer cells induce osteoclast formation by stimulating host IL-11 production and downregulating granulocyte/macrophage colony-stimulating factor. *Int. J. Cancer* **109**, 653–660 (2004).
40. Luo, J.L., Maeda, S., Hsu, L.C., Yagita, H. & Karin, M. Inhibition of NF-κB in cancer cells converts inflammation-induced tumor growth mediated by TNFα to TRAIL-mediated tumor regression. *Cancer Cell* **6**, 297–305 (2004).
41. Queen, M.M., Ryan, R.E., Holzer, R.G., Keller-Peck, C.R. & Jorcyk, C.L. Breast cancer cells stimulate neutrophils to produce oncostatin M: potential implications for tumor progression. *Cancer Res.* **65**, 8896–8904 (2005).
42. Lin, A. & Karin, M. NF-κB in cancer: a marked target. *Semin. Cancer Biol.* **13**, 107–114 (2003).
43. Condeelis, J. & Pollard, J.W. Macrophages: obligate partners for tumor cell migration, invasion, and metastasis. *Cell* **124**, 263–266 (2006).
44. Li, Q. & Verma, I.M. NF-κB regulation in the immune system. *Nat. Rev. Immunol.* **2**, 725–734 (2002).
45. Chen, Z.J. Ubiquitin signalling in the NF-κB pathway. *Nat. Cell Biol.* **7**, 758–765 (2005).
46. Zeng, Q. *et al.* Crosstalk between tumor and endothelial cells promotes angiogenesis through MAPK activation of Notch. *Cancer Cell* **8**, 13–23 (2005).