Published in final edited form as:

Biochimie. 2014 November ; 0: 24–32. doi:10.1016/j.biochi.2014.07.017.

MAPK11 in breast cancer cells enhances osteoclastogenesis and bone resorption

Zhimin He, PhD^{1,*}, Jin He, MS^{2,*}, Zhiqiang Liu, PhD², Jingda Xu, PhD², Sofia F. Yi², Huan Liu, PhD², and Jing Yang, PhD^{1,2}

¹Cancer Research Institute and Cancer Hospital, Guangzhou Medical University, Guangzhou, China

²Department of Lymphoma/Myeloma, Division of Cancer Medicine, The University of Texas MD Anderson Cancer Center, Houston, Texas 77030, USA

Abstract

Breast cancer cells frequently metastasize to bone and induce osteolytic bone destruction in patients. These metastases cause severe bone pain, high risk of fractures and hypercalcemia, and are essentially incurable and fatal. Recent studies show that breast cancer cells in bone activate osteoclastogenesis and bone resorption. However the underlying mechanism is poorly understood. This study shows that the p38 MAPK (p38) isoform MAPK11 (p38 β) is expressed in breast cancer cells. By using specific small hairpin RNAs for MAPK11, we demonstrated that p38 β -mediated p38 activity in breast cancer cells is responsible for breast cancer-induced osteolytic bone destruction. The addition of conditioned media from breast cancer cell lines MDA-MB-231 and MDA-MB-468, which have high expression of p38 β , induced osteoclast differentiation and bone resorption. In contrast, knockdown of p38 β in breast cancer cells reduced osteoclast differentiation *in vitro* and reduced bone destruction in severe combined immunodeficiency (SCID) mouse models. The knockdown of p38 β did not affect tumor growth or survival or the ability of cancer cells to home to bone. Furthermore, our results showed that p38 β upregulated the expression and secretion of monocyte chemotactic protein-1 (MCP-1) in breast cancer cells, and upregulated MCP-1 activates osteoclast differentiation and activity. This study elucidates a novel molecular mechanism of breast cancer cell-induced osteolytic bone destruction. This study also indicates that targeting breast cancer cell p38 β and its product MCP-1 may be a viable approach to treat or prevent bone destruction in patients with bone-metastatic breast cancer.

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Corresponding Author: Jing Yang, Ph. D., Department of Lymphoma/Myeloma, Division of Cancer Medicine and Center for Cancer Immunology Research, The University of Texas MD Anderson Cancer Center, 1515 Holcombe Boulevard, Unit 0903, Houston, Texas 77030. Tel: +1 713 563 0357; fax: +1 713 563 3424., jiyang@mdanderson.org.

*These authors contributed equally to the study.

Conflict of interest statement

No potential conflicts of interest were disclosed.

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Keywords

breast cancer; MAPK11; osteoclasts; bone resorption; MCP-1

1. Introduction

Bone metastasis is present in more than 70% of patients with advanced breast cancer and often induces osteolytic bone destruction.[1] The metastatic disease causes severe bone pain, high risk of fractures and hypercalcemia.[2] Currently, this disease is considered incurable. [3] In patients with bone metastatic breast cancer, tumor cells in bone marrow induce bone resorption by activating osteoclastogenesis.[4, 5] Previous studies show that breast cancer cells secrete numbers of soluble factors, and activate osteoclastogenesis via these factors.[4] For example, breast cancer-derived parathyroid hormone-related protein upregulates the expression and secretion of receptor activator of nuclear factor κ B ligand (RANKL), an activator important for osteoclast differentiation and bone resorption activity,[6] and downregulates the levels of osteoprotegerin (OPG), an antagonist of RANKL.[7] In addition, breast cancer-derived interleukin (IL)-6[8], IL-8[9], and IL-11[10] stimulate stromal cell production of osteoclastogenesis activators. However, the mechanism by which breast cancer cells regulate the production of these factors remains unclear.

Monocyte chemotactic protein-1 (MCP-1), also known as the chemokine (C-C motif) ligand 2, is a member of the C-C chemokine family. Although monocytes and macrophages are the major source of MCP-1, many other cell types, such as fibroblasts, endothelial cells, epithelial cells, and astrocytic cells, can also produce this chemokine.[11] MCP-1 is a potent chemotactic factor for monocytes, and it is induced by tissue injury or infection. It also recruits immune cells such as memory T cells, dendritic cells, and natural killer cells to the sites of inflammation.[12] Previous studies have shown that MCP-1 participates in the pathogenesis of many diseases, such as multiple sclerosis, rheumatoid arthritis, atherosclerosis, and diabetes. In the bone, MCP-1 is found at sites of bone degradation, and in prostate cancer patients with lytic bone lesions, the levels of MCP-1 are increased.[13] Even in the absence of RANKL, MCP-1 induces the formation of osteoclast-like cells from monocytes in vitro, although these osteoclast-like cells do not have bone resorption activity, [14] which suggests a potential role of MCP-1 in bone metastasis. However, the mechanism of how MCP-1 is regulated in bone-metastatic breast cancer remains unclear.

P38 mitogen-activated protein kinase (MAPK) is composed of four isoforms: MAPK14 (p38 α), MAPK11 (p38 β), MAPK12 (p38 γ) and MAPK13 (p38 δ). Among them, p38 α and p38 β are the predominant form. Previous studies showed that breast cancer cells with inhibition of p38 α reduced cancer metastasis to bones.[15, 16] However, the role of other isoforms of p38 in bone remains to be investigated. In this study, we found that p38 was constitutively active in metastatic breast cancer cells. Breast cancer cells expressed both p38 α and p38 β , but little or no other isoforms. We therefore hypothesized that besides p38 α , breast cancer cells with p38 β may play a role in osteolytic bone destruction. Our results show that application of small hairpin RNAs (shRNAs) specific for p38 β impaired the phosphorylation of p38 in breast cancer cells, reduced osteoclast differentiation and activity, and downregulated the secretion of MCP-1. Our study at the first time demonstrated the

ability of p38 β in breast cancer-induced osteoclastogenesis, providing strong evidence that targeting p38 β and MCP-1 may be a novel and effective therapeutic approach to treat bone destruction in breast cancer patients.

2. Materials and Methods

2.1. Breast cancer cell culture and conditioned media

Human breast cancer cell lines were purchased from American Type Culture Collection (Rockville, MD) and authenticated by DNA (STR) profiling for this study. To collect conditioned media, the breast cancer cells were cultured at a density of 1×10^5 cells/mL in serum-free DMEM for 24 hours, and the culture supernatants were collected, pooled and stored at 4 °C for up to 1 week before use. The study was approved by the Institutional Review Board at The University of Texas MD Anderson Cancer Center.

2.2. Plasmids and reagents

Specific shRNAs for p38 α or p38 β (Santa Cruz Biotechnology, Santa Cruz, CA) were packed into the retroviral vector pSIREN-RetroQ (Clontech Laboratories, Inc., Mountain View, CA). Control shRNA plasmid with scramble shRNA sequence (Santa Cruz) served as non-target shRNA control (scramble sh). Retroviral infections were performed according to the manufacturer's instructions (Santa Cruz). Recombinant human M-CSF, RANKL, and MCP-1 and neutralizing antibodies against MCP-1 and isotype control antibodies were purchased from R&D Systems (Minneapolis, MN). Other chemical reagents were purchased from Sigma (St. Louis, MO).

2.3. Real-time PCR

Total RNA was extracted from cells using an RNeasy kit from Qiagen (Valencia, CA). The total RNA (1 μ g) was subjected to reverse transcription using a SuperScript II reverse transcriptase PCR kit (Invitrogen); 1 μ L of the final cDNA was used for real-time PCR amplification with SYBRGreen using a StepOnePlus real-time PCR system (Life Technologies).

2.4. Tissue microarray

A tissue array containing 50 cases total of breast carcinoma and matched metastatic carcinoma, and 4 cases of matched non-malignant breast tissues, were purchased from Us Biomax Inc. (Rockville, MD). Immunohistochemistry staining was performed according to the manufacturer's instructions (Dako, Carpinteria, CA). In brief, the paraffin-embedded slides were deparaffinized with xylene and rehydrated through a graded alcohol series to water. Endogenous peroxidase activity was quenched with 3% hydrogen peroxide. The levels of pp38 were detected by using anti-phosphorylated p38 antibody (Cell Signaling Technology, Inc., Beverly, MA). Signal was detected using secondary biotinylated antibodies and streptavidin/horseradish peroxidase. Chromagen 3,3-diaminobenzidine/H₂O₂ (Dako) was used, and slides were counterstained with hematoxylin. All slides were observed under light microscopy, and images were captured with a SPOT RT camera (Diagnostic Instruments, Burlingame, CA).

2.5. Western blotting

Cells were harvested and lysed with lysis buffer (50 mM Tris, pH 7.5, 140 mM NaCl, 5 mM EDTA, 5 mM NaN₃, 1% Triton X-100, 1% Nonidet P-40, 1 × protease inhibitor cocktail). Cell lysates were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to a polyvinylidene difluoride membrane, and immunoblotted with antibodies against phosphorylated or non-phosphorylated kinases of p38, p38 α , p38 β (Cell Signaling Technology) or MCP-1 (Sigma). The membrane was stripped and re-probed with β -actin antibody (Sigma) to ensure equal protein loading. Secondary antibodies conjugated to horseradish peroxidase were used for detection, followed by enhanced chemiluminescence treatment (Pierce Biotechnology, Rockford, IL) and autoradiography.

2.6. In vitro osteoclast formation and bone pit analysis

The method for generating osteoclasts has been described previously.[17] Primary blood mononuclear cells were cultured in 24-well plates in α -MEM supplemented with 10% fetal bovine serum, antibiotics, and human macrophage colony-stimulating factor (25 ng/ml M-CSF) for 5 to 7 days to obtain osteoclast precursors (preOCs). PreOCs were cultured with M-CSF and RANKL (50 ng/ml) for another 7 days to develop mature osteoclasts. In some experiments, preOCs were cultured in medium without RANKL and addition of conditioned media from scramble sh- or p38 β shRNA-MDA-MB-231 or MDA-MB-468 cells for 7 days. Mature osteoclasts were detected by tartrate-resistant acid phosphatase (TRAP) staining (Sigma), and cells with more than 3 nuclei were characterized as TRAP-positive multinuclear (>3) large cells. To examine osteoclast-mediated bone resorption, we performed a bone resorption assay according to the manufacturer's instructions (Osteogenic Core Technologies Inc., Choongan, Korea). In brief, osteoclasts were directly cultured in wells coated with mineralized matrices for 7 days in medium with RANKL or without RANKL but with conditioned media from the cell cultures specified above. Resorbed bone pits were detected by light microscopy and counted.

2.7. Cytokine array analysis

The Human Cytokine Antibody Array (RayBiotech, Norcross, GA) consists of antibodies specific for 48 inflammatory cytokines or chemokines. Supernatants from 48-hour cultures of various cells were collected for analysis. Detection of cytokines in supernatants was performed according to the manufacturer's instructions. Mean intensities of dots on the membranes represented relative protein levels of cytokines and were quantified with ImageQuant TL software (Amersham Biosciences, Inc., Piscataway, NJ). The data after background subtraction were normalized according to positive control densities.

2.8. Measurements of human MCP-1 in culture medium and mouse serum by ELISA

Supernatants from cultured MDA-MB-231 or MDA-MB-468 cells were collected after 48 hours. Mouse serum was collected weekly during the observation period. Briefly, a small nick was made in the mouse tail vein with a scalpel blade, and 50 μ l of whole blood was collected into an eppendorf tube. After a 5 min centrifugation at 5000 rpm, serum was collected. Human MCP-1 was quantified in the supernatants or mouse serum using an ELISA kit (human MCP-1 DuoSet, R&D Systems).

2.9. Mouse models, radiographic, μ -computed tomography and TRAP staining

CB.17 SCID mice were purchased from Harlan (Indianapolis, IN). All mice were maintained in American Association of Laboratory Animal Care-accredited facilities, and the studies were approved by the Institutional Animal Care and Use Committee of The University of Texas MD Anderson Cancer Center. Six- to 8-week-old mice were inoculated i.v. with 1×10^6 tumor cells. To measure the size of lytic bone lesions, we scanned radiographs with a Faxitron X-ray cabinet. We used μ -computed tomography (CT) to scan the trabecular bone of the distal femur (μ -CT-40, Scanco Medical, Wayne, PA) and then determined the level of osteolysis using a computerized image analysis system. Moreover, murine femurs were removed and fixed. Bone sections were stained and analyzed for TRAP activity using a leukocyte acid phosphatase staining kit according to the manufacturer's instructions (Sigma). The number of TRAP-positive, multinuclear (> 3) osteoclasts per millimeter of bone at the bone-tumor interface was calculated using a computerized image analysis system.

2.10. Statistical analyses

All data are shown as means $\pm$ standard deviation. The Student's *t* test was used to compare various experimental groups; significance was set at *P* less than .05.

3. Results

3.1. P38 is constitutively active in breast cancer cells

Using immunohistochemistry staining, the levels of phosphorylated p38 (pp38) were detected in a tissue array that contained the biopsy specimens of breast carcinoma (invasive ductal carcinoma), matched metastatic carcinoma (metastatic invasive ductal carcinoma of the breast) patients, and specimens from adjacent normal breast tissues (US Biomax, Inc, Rockville, MD). As shown in Figure 1A, 14 of the 25 breast carcinomas and 23 of the 25 metastatic breast cancers were positive for pp38, while all of tested normal breast tissues showed little or no staining of pp38. Western blot results showed high pp38 and unchanged p38 in all tested breast cancer cells (Figure 1B).

3.2. Breast cancer cells express p38 α and p38 β isoforms

We examined the expression of p38 α , p38 β , p38 γ and p38 δ in 6 breast cancer cell lines. As shown in Figure 2A, p38 α and p38 β were the predominant isoforms that were expressed in all breast cancer cells. Of the examined cells, MDA-MB-231 and MDA-MB-468 cells showed strong expression of p38 β , combined with their ability to cause osteolytic lesions[18], therefore, our study focused on those two cell lines. By using specific shRNAs, we knocked down p38 α and/or p38 β expression in MDA-MB-231 and MDA-MB-468 cells. Our results showed that p38 α shRNA-cells had reduced levels of p38 α protein and that p38 β shRNA-expressing cells had reduced levels of p38 β protein, and the cells expressing both p38 α and p38 β shRNAs expressed less p38 α and p38 β proteins simultaneously. The levels of phosphorylated p38 (pp38), which reflect p38 activity, were partially inhibited in either p38 α shRNA-cells or p38 β shRNA-cells, and pp38 levels were almost eliminated in cells expressing both p38 α and p38 β shRNAs. The cells with scramble shRNAs had no such

effects (Figure 2B and 2C). These results indicate that besides p38 α , p38 β can also regulate p38 activity in breast cancer cells.

3.3. Breast cancer cell p38 β induces osteoclast differentiation and bone resorption

RANKL is a cytokine that is important for promoting osteoclast differentiation. Culturing preOCs in medium with added RANKL induces mature osteoclast formation, whereas culturing them in medium without RANKL does not.[19] Previous studies have shown that co-culture of preOCs with breast cancer cells in medium without added RANKL can also induce mature osteoclast formation.[20] We wondered whether knockdown of p38 β in breast cancers had a reduced effect on osteoclast differentiation. PreOCs were cultured in medium without supplementary RANKL and with conditioned media from scramble shRNA- or p38 β shRNA-cells. Our results showed that adding either RANKL or conditioned media from scramble shRNA-cells, but not conditioned media from p38 shRNA-cells, could induce osteoclast formation (Figure 3A). Addition of conditioned media from p38 shRNA-cells induced fewer amounts of osteoclasts with smaller cellular size and fewer amounts of nuclei in each osteoclast than addition of conditioned media from scramble shRNA-cells (Figures 3A, 3B). To examine the effects of breast cancer cell p38 β on osteoclast-mediated bone resorption, we seeded preOCs in mineralized matrix-coated plates and cultured the cells in medium without RANKL and with conditioned media from scramble shRNA- or p38 shRNA-cells. Compared to the cultures with RANKL or conditioned media from scramble shRNA-cancer cells, the cultures with conditioned media from p38 shRNA-cells formed smaller resorbed areas (Figure 3C) and had fewer amounts of resorbed pits (Figure 3D).

3.4. MCP-1 mediates breast cancer cell p38 β -induced osteoclast activation

Since MDA-MB-231 and MDA-MB-468 cells do not express RANKL (data not shown), a cytokine that activates osteoclast differentiation and bone resorption activity [6], we wondered how p38 β induced osteoclast activation. A cytokine array that contained specific antibodies for 48 cytokines was used, with majority of which are known regulators for osteoclast differentiation and activity. As shown in Figure 4A, the MDA-MB-231 cells produced a number of cytokines. Among them, the levels of MCP-1 were significantly downregulated in the conditioned media from p38 β shRNA-cells. By using real-time PCR (Figure 4B) and ELISA (Figure 4C), we further detected that p38 β shRNA-cells had lower expression (Figure 4B) and secretion (Figure 4C) of MCP-1 than that of scramble shRNA-cells. Next, we examined whether breast cancer-derived MCP-1 played a role in p38 β -induced osteoclast activation. We added anti-MCP-1 antibodies to the cultures in addition of the conditioned media from scramble shRNA-cells, and found that addition of neutralizing antibodies reduced osteoclast formation (Figure 4D). Addition of control IgG had no such effects. Alternatively, addition of exogenous MCP-1 to the cultures in addition of conditioned media from p38 β shRNA-cells restored osteoclast formation (Figure 4E).

3.5. Breast cancer cell p38 β induces osteolytic bone destruction in vivo

To determine whether breast cancer cell p38 β induces bone destruction *in vivo*, SCID mice were intravenously (iv) injected with either scramble shRNA- or p38 β shRNA-MDA-MB-231 or MDA-MB-468 cells. Using immunohistochemistry staining, we observed that

the levels of p38 were high in scramble shRNA-cells, but significantly reduced in p38 shRNA-cells in the bone marrow (Figure 5A). Radiographic examination further showed that at 8 weeks after tumor injection, the mice injected with scramble shRNA-cells displayed severe focal lesions in the femurs, while the mice injected with p38 shRNA-cells had fewer lesions (Figure 5B). In the period of observation, 7 of 10 mice injected with scramble shRNA-cells but only 3 of 10 mice injected with p38 shRNA-cells developed osteolysis. μ -CT scanning also showed that the ratio of bone volume to total volume (BV/TV; Figure 5C), trabecular bone density (Tb.N; Figure 5D), and average trabecular thickness (Tb.Th; Figure 5E), all of which reflect trabecular bone volumes, of the mice injected with p38 shRNA-cells were more than those of the mice injected with scramble shRNA-cells.

3.6. Breast cancer cell p38 increases osteoclastogenesis *in vivo*

To examine whether breast cancer cell p38 induced osteoclastogenesis *in vivo*, mouse femurs were inspected for osteoclasts on the trabecular bone surface by TRAP staining. We found a reduced percentage of osteoclast-eroded bone surface (ES/BS; Figure 6A), percentage of bone surface covered with osteoclasts (Oc.S/BS; Figure 6B), number of osteoclasts per eroded surface (N.Oc./ES; Figure 6C), and bone surface in contact with osteoclasts (Oc.S; Figure 6D), all of which reflect osteoclast bone resorption activity,[14] in the mice injected with p38 shRNA-cells than in the mice injected with scramble shRNA-cells. Furthermore, we examined the levels of human MCP-1 in mouse serum by ELISA, and found before tumor cells injection, the serum levels of human MCP-1 were undetectable, and after tumor cells injection, the levels were increased in the mice injected with scramble shRNA-cells but significantly reduced in the mice injected with p38 shRNA-cells (Figure 6E).

3.7. Disruption of breast cancer cell p38 does not affect the ability of bone homing, tumor growth, or survival

We wanted to exclude the possibility that the reduced ability of p38 shRNA-cancer cells to destroy bone resulted from their reduced ability to home to bone. Thus, SCID mice were intravenously injected with either scramble shRNA- or p38 shRNA-MDA-MB-231 cells. At various time points after tumor injection, mice were killed, and their organs and tissues were removed. The numbers of infiltrating cancer cells that expressed human leukocyte antigen (HLA)-ABC were quantified by flow cytometry. We found no difference between the numbers of HLA-ABC-positive cells in the observed organs and tissues of the mice injected with either scramble shRNA- or p38 shRNA-cells at 8 weeks (Figure 7A) or at the longer period of observation (data not shown). In addition, we examined *in vivo* apoptosis of breast cancer cells in bone marrow using the TUNEL assay, and found no difference in the numbers of HLA-ABC-positive apoptotic tumor cells in the mice injected with scramble shRNA- or p38 shRNA-cells (Figure 7B). To examine the effects of p38 on breast cancer cell growth *in vivo*, we subcutaneously injected the cells into mice and measured the tumor sizes,[21, 22] and found there is no difference between the tumor volumes of mice injected with scramble shRNA-cells and the mice injected with p38 shRNA-cells (Figure 7C).

4. Discussion

Our study demonstrates the important role of p38 β in osteolytic bone destruction induced by breast cancer cells and elucidates a novel mechanism by which p38 β upregulates the expression and secretion of MCP-1, which leads to enhanced osteoclast differentiation and bone resorption. Our findings thus provide a rationale for p38 β as a novel potential target in clinical trials to improve the current treatment efficacy in patients with bone metastatic-breast cancer.

Bone metastasis may be asymptomatic at first, but it can lead to bone pain, hypercalcemia, pathological fractures, and anemia in advanced breast cancer patients. Currently, bisphosphonates are the treatment of choice for breast cancer patients with osteolytic bone lesions. Zoledonic acid, sold commercially as Zometa, is a newer generation, nitrogen-containing bisphosphonate. Clinical studies have shown that zoledonic acid inhibits osteoclast-mediated bone resorption in cancer patients with osteolytic bone lesions. Compared to first-generation bisphosphonates, such as pamidronate, zoledonic acid is more effective and better tolerated as a treatment for hypercalcemia associated with advanced breast cancer and skeletal complications of metastatic bone disease. [23] However, zoledronic acid's potential as a standard of care against bone destruction is not yet fully supported, because it still offers only moderate benefits in the patients.[24] Development of more effective therapies is important for those patients with osteolytic bone destruction.

Recent studies show that p38 signaling in breast cancer cells regulates cancer cell migration[25, 26] and in preOCs regulates RANKL-induced osteoclast differentiation.[27] Recent studies also report that p38 α is critical for arthritic bone loss[28], and involves in bone metastasis in breast cancer.[15] However, the role of other isoforms of p38 in osteoclast activation and bone destruction is unknown. Our study clearly showed that breast cancer cells express both p38 α and p38 β isoforms, and besides p38 α , p38 β is also a regulator for p38 activity in breast cancer cells and for osteoclast activation and bone resorption induced by breast cancer cells. Our results showed that p38 β shRNAs disrupted p38 activity in breast cancer cells and reduced osteoclastogenesis and bone destruction. Our results showed that p38 β is a novel contributor to osteoclast-mediated bone resorption, which indicates that targeting p38 β may improve the efficacy of current therapies for bone-metastatic breast cancer.

Previous studies show that soluble factors released by breast cancer cells to bone marrow microenvironment mediate osteoclast activation.[29] In addition, p38 signaling pathway mediates the production of inflammatory cytokines, such as TNF α [30] and soluble RANKL[31], from bone marrow stromal cells. Inhibition of p38 suppresses inflammatory, angiogenic, and pro-apoptotic cytokine production in bone marrow stromal cells.[32] MCP-1 is a chemokine that can recruit monocytes, T cells, and dendritic cells to sites of infection or tissue injury,[12] and the expression of MCP-1 is increased during osteoclast maturation.[33] Although MCP-1 is mainly released from bone marrow stromal cells[34], our results showed that breast cancer cells also secreted MCP-1, and p38 β in breast cancer cells upregulated the expression and secretion of MCP-1. Knockdown of p38 β expression significantly abrogated breast cancer cell expression and secretion of MCP-1. However, it is

necessary to further elucidate the mechanism of the expression of MCP-1 in breast cancer cells as regulated by p38 β . Furthermore, recent studies have demonstrated that MCP-1 activates osteoclast differentiation and bone resorption in many tumors.[13, 35] In inflammatory osteoporosis, addition of MCP-1 enhances the fusion of hematopoietic precursors and the formation of mature osteoclasts via binding to its receptor CCR2.[14] Our results showed that neutralizing MCP-1 by the antibodies abrogated p38 β -induced osteoclast differentiation, whereas the addition of MCP-1 enhanced it, suggesting that inhibition of breast cancer-derived MCP-1 may be a novel and effective approach to treat patients with bone metastatic breast cancer. However, the mechanism underlying the regulation of osteoclastogenesis by MCP-1-mediated signaling pathways requires further investigation.

Acknowledgments

We thank Michael Starbuck for technical support with TRAP and toluidine blue staining, Brian Dawson for technical support with μ -CT scanning and analysis, and our Departmental Myeloma Tissue Bank for providing patient samples. We also thank Ms. Zachary S Bohannon and Kathryn Carnes for providing editorial assistance. This work was supported by National Cancer Institute K99/R00 CA137158 (J. Yang) and American Society of Hematology (J. Yang).

Abbreviations

HLA	human leukocyte antigen
IL	interleukin
MAPK	mitogen-activated protein kinase
MCP-1	monocyte chemotactic protein-1
M-CSF	macrophage colony-stimulating factor
OC	osteoclast
OPG	osteoprotegerin
p38α	MAPK14
p38β	MAPK11
p38γ	MAPK12
p38δ	MAPK13
pp38	phosphorylated p38
preOC	osteoclast precursor
RANKL	nuclear factor κ B ligand
shRNA	small hairpin RNA
TRAP	tartrate-resistant acid phosphatase

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Highlights

- MAPK11 is highly expressed in the metastatic breast cancer patients and in the breast cancer cell lines;
- MAPK11 is responsible to breast cancer-induced osteolytic bone destruction;
- Upregulated MCP-1 activates osteoclast differentiation and activity;
- Novel molecular mechanism of breast cancer cell-induced osteolytic bone destruction.

Fig. 1. Constitutive activation of p38 in breast cancer cells. **(A)** Representative images of immunohistochemistry staining for pp38 in 1 out of 4 biopsy specimens of normal breast tissues (N) and 3 (Pt1–Pt3) out of 50 biopsy specimens of breast carcinoma and matched metastatic carcinoma from patients in the tissue arrays. Magnification, upper panels: $\times 100$; lower panels: $\times 400$. **(B)** Western blot analysis showing the levels of phosphorylated (pp38) and non-phosphorylated p38 (p38) in 6 human breast cancer-derived cell lines. The levels of β -actin are shown as loading controls. Representative results of three independent experiments are shown.

Fig. 2.

Expression of p38 isoforms in breast cancer cells. (A) RT-PCR showing the mRNA levels of p38 α , p38 β , p38 γ , p38 δ in 6 breast cancer-derived cell lines. The mRNA levels of GAPDH served as loading controls. (B and C) Western blot analysis showing the protein levels of p38 α , p38 β , total p38 and the levels of phosphorylated p38 (pp38) in MDA-MB-231 (B) and MDA-MB-468 (C) cells infected with the retroviruses containing p38 α and/or p38 β shRNAs compared with the cells infected with scramble shRNA control retroviruses. The levels of β -actin are shown as loading controls. Representative results of four independent experiments are shown.

Fig. 3.

Disruption of p38 β in breast cancer cells inhibits osteoclast differentiation and bone resorption *in vitro*. (A) Representative images of TRAP staining for TRAP-positive multinuclear osteoclasts in the cultures in medium with or without addition of RANKL, or in medium without RANKL and with conditioned media from scramble shRNA-(scramble sh-conditioned media) or p38 β shRNA-treated MDA-MB-231 (p38 β sh-conditioned media) cells. Mature osteoclasts are defined as TRAP-positive multinuclear cells. (B) Representative images of bone resorbed pits showing large numbers and total areas of resorbed pits on mineralized matrices induced by treatment with mature osteoclasts generated in the cultures with or without RANKL or in the cultures without RANKL and with conditioned medium from scramble shRNA-treated or p38 β shRNA-treated MDA-MB-231 cells. Summarized data showing the numbers of (C) mature osteoclasts (TRAP-positive cells) and (D) resorbed pits on mineralized matrices in the cultures with or without

RANKL and with conditioned medium from scramble shRNA-treated or p38 β shRNA-treated MDA-MB-231 and MDA-MB-468 cells. Representative results of five experiments are shown. ** $P < 0.01$.

Fig. 4.

Identification of MCP-1 as a mediator of breast cancer cell p38 β -induced osteoclastogenesis.

(A) Densitometric data of a cytokine array showing the profile of cytokine expression in conditioned medium from scramble shRNA- and p38 β shRNA-treated (p38 β sh) MDA-MB-231 cells. MDA-MB-231 cells were cultured in serum-free DMEM medium for 24 hours, and the supernatants (conditioned media) were collected. The cytokine array was incubated with conditioned media from the different cells. The value from scans was adjusted based on the intensity of positive control spots on each membrane. Summarized results from the array showing the reduced levels of cytokines in the conditioned medium from p38 β shRNA-treated MDA-MB-231 cells compared with those in conditioned medium from scramble shRNA-treated MDA-MB-231 cells. Representative results of three experiments are shown. (B) Detection of mRNA expression using real-time PCR and (C) soluble levels of MCP-1 using ELISA in conditioned media from scramble shRNA- and p38 β shRNA treated MDA-MB-231 and MDA-MB-468 cells. Shown are representative results of five experiments. (D) Shown are the effects of neutralizing antibodies against MCP-1 on osteoclast differentiation in the cultures in medium with addition of conditioned media from scramble shRNA-treated MDA-MB-231 and MDA-MB-468 cells (scramble shRNA-conditioned media), and (E) the effects of exogenous MCP-1 on osteoclast differentiation in the cultures with added conditioned media from p38 β shRNA-treated MDA-MB-231 and MDA-MB-468 cells. Mature osteoclasts were determined by TRAP staining. TRAP-positive multinuclear cells were counted. Representative results of five experiments are shown. * P 0.05; ** P 0.01.

Fig. 5.

Reduced breast cell p38 β impairs breast cancer cell-induced osteolytic bone destruction *in vivo*. (A) Immunohistochemical staining shows the reduced p38 levels and (B) x-ray shows the reduced osteolytic bone lesions in mice injected with p38 β shRNA-treated MDA-MB-231 cells (p38 β sh) in comparison to the lesions in mice injected with scramble shRNA-treated MDA-MB-231 cells. μ -CT scanning shows higher (C) bone volume/total volume (BV/TV), (D) trabecular bone density (Tb.N), and (E) average trabecular thickness (Tb.Th) in the femurs of the mice injected with p38 β shRNA-treated MDA-MB-231 and MDA-MB-468 cells (p38 β sh) than those in the mice injected with scramble shRNA-treated cancer cells. Representative results of five experiments are shown. * P 0.05; ** P 0.01

Fig. 6.

P38β in breast cancer cells enhances osteoclastogenesis *in vivo*. At 8 weeks after the injection of scramble shRNA- or p38β shRNA-treated (p38βsh) MDA-MB-231 cell, the femurs of the mice were removed. Bone sections were performed with TRAP staining to detect mature osteoclasts in the trabecular bones. TRAP staining showing (A) reduced levels of osteoclast-mediated bone erosion (ES/BS), (B) a lower percentage of trabecular bone surface covered with osteoclasts (Oc. S/BS), (C) fewer osteoclasts per erosion surface (N.Oc/ES), and (D) less bone surface in contact with osteoclasts (Oc.S) in the bone marrow of the mice injected with p38β shRNA-treated MDA-MB-231 cells than in the bone marrow of the mice injected with scramble shRNA-treated cells. (E) ELISA showing the serum levels of human MCP-1 in the mice injected with stable scramble shRNA- or p38β shRNA-treated (p38βsh) MDA-MB-231 cells at 8 weeks after tumor injection. ** $P < 0.01$. Representative results of five experiments are shown.

Fig. 7.

Knocking down breast cancer cell p38 β does not affect tumor growth and tumor cell homing to bone. **(A)** Flow cytometry showing tissue-infiltrating human HLA-ABC⁺ breast cancer cells in the mice iv injected with scramble shRNA- or p38 β shRNA-treated (p38 β sh) MDA-MB-231 cells at 4 weeks after tumor injection. **(B)** *In situ* TUNEL assay showing the percentage of apoptotic tumor cells in the bone marrow (BM) of the mice injected with scramble shRNA- or p38 β shRNA-treated (p38 β sh) MDA-MB-231 cells at 4 weeks after tumor injection. **(C)** Shown are s.c. tumor volumes of the mice injected with scramble shRNA- or p38 β shRNA-treated (p38 β sh) MDA-MB-231 cells at weeks 0, 2, 4, and 6 after tumor injection. The results represent the average value from three independent experiments of five mice per group.