Breast Cancer-derived Factors Stimulate Osteoclastogenesis through the Ca\(^{2+}\)/Protein Kinase C and Transforming Growth Factor-\(\beta\)/MAPK Signaling Pathways \(^5\)

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Breast cancer commonly metastasizes to bone where its growth depends on the action of bone-resorbing osteoclasts. We have previously shown that breast cancer cells secrete factors able to directly stimulate osteoclastogenesis from receptor activator of nuclear factor \(\kappa\)B ligand (RANKL)-primed precursors and that transforming growth factor-\(\beta\) (TGF\(\beta\)) plays a permissive role in this process. Now, we evaluate the signaling events triggered in osteoclast precursors by soluble factors produced by MDA-MB-231 human breast carcinoma cells. In mouse bone marrow cultures and RAW 264.7 murine monocytic cells, MDA-MB-231-derived factors increased osteoclast number, size, and nucleation. These factors failed to induce Smad2 phosphorylation, and short interfering RNAs against Smad4 did not affect their ability to induce osteoclastogenesis. In contrast, MDA-MB-231 factors induced phosphorylation of p38 and ERK1/2, and pharmacological inhibitors against p38 (SB203580) and MEK1/2 (PD98059) impeded the osteoclastogenic effects of cancer-derived factors. Neutralizing antibodies against TGF\(\beta\) attenuated p38 activation, whereas activation of ERK1/2 was shortened in duration, but not decreased in amplitude. ERK1/2 phosphorylation induced by cancer-derived factors was blocked by MEK1/2 inhibitor, but not by Ras (manumycin A) or Raf (GW5074) inhibitors. Inhibition of protein kinase Ca using Gö6976 prevented both ERK1/2 phosphorylation and osteoclast formation in response to MDA-MB-231-derived factors. Using microspectrofluorimetry of fura-2-AM-loaded osteoclast precursors, we have found that cancer-derived factors, similar to RANKL, induced sustained oscillations in cytosolic free calcium. The calcium chelator BAPTA prevented calcium elevations and osteoclast formation in response to MDA-MB-231-derived factors. Thus, we have shown that breast cancer-derived factors induce osteoclastogenesis through the activation of calcium/protein kinase Ca and TGF\(\beta\)-dependent ERK1/2 and p38 signaling pathways.

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Breast cancer exhibits a high propensity to metastasize to bone causing bone pain, pathological fractures, hypercalcemia, spinal cord compression, and immobility (1, 2). Breast cancer cells do not resorb bone; instead they rely on stimulation of osteoclasts, cells physiologically responsible for bone destruction (1–4). Breast cancer cells can stimulate osteoclasts indirectly, by producing factors, such as parathyroid hormone-related peptide, interleukin-1, -6, and -11, which act on bone-forming osteoblasts to increase the production of an essential osteoclast stimulator, receptor activator of nuclear factor \(\kappa\)B (RANK)\(^4\) ligand (RANKL) (1, 5–11).

We have found that soluble factors produced by human or mouse breast cancer cells can directly stimulate osteoclast formation from late human or mouse osteoclast precursors (12). These effects depended on the permissive action of TGF\(\beta\), and we observed that TGF\(\beta\) type I receptor expression (T\(\beta\)RI) was up-regulated in late osteoclast precursors (12). The expression of TGF\(\beta\) and T\(\beta\)RI increases at the interface between tumor and bone in vivo (4), and interference with T\(\beta\)RI or TGF\(\beta\) 1 and 3 impairs breast cancer bone metastases in vivo (13–15). T\(\beta\)RI signals through the canonical Smad-dependent (16) or Smad-independent mechanisms (17). In the Smad pathway, T\(\beta\)RI phosphorylates Smad2 and Smad3, which complex with Smad4 and translocate into the nucleus, acting as transcriptional modulators. TGF\(\beta\) also initiates non-canonical signaling, including the mitogen-activated protein kinases (MAPKs) pathway (18). TGF\(\beta\)-activated kinase 1 is a MAPK kinase that signals through MAPK kinase (MKK) 3/6, to activate p38 and through MKK4/7 to activate JNK (19). The TGF\(\beta\)-activated kinase 1/MKK6/p38 pathway was shown to be important in osteoclastogenesis (19–21). Signaling by RANK/RANKL in osteoclasts also involves MAPKs, in particular p38 and ERK (22–25).

We have also shown that breast cancer-derived factors sustained the activation of the osteoclastogenic transcription factor, nuclear factor of activated T cells (NFAT) c1 (12). NFAT transcription factors are controlled by the Ca\(^{2+}\)/calmodulin-

\(^4\)The abbreviations used are: RANK, receptor activator of nuclear factor \(\kappa\)B; RANKL, RANK ligand; BAPTA, 1,2-bis(\(\beta\)-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid; [Ca\(^{2+}\)]\(_i\), cytosolic free Ca\(^{2+}\) concentration; CM, conditioned medium; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; MEK, MAPK/ERK kinase; NFAT, nuclear factor of activated T cells; OC, osteoclast; PKC, protein kinase C; T\(\beta\)RI, TGF\(\beta\) type I receptor; MKK, MAPK kinase; DMEM, Dulbecco’s modified Eagle’s medium; GST, glutathione S-transferase; TRAP, tartrate-resistant acid phosphatase.
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dependent phosphatase, calcineurin (26, 27). Hyperphosphorylated NFAT is restricted to the cytosol. An increase in the cytosolic free Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_{i}\)) activates calcineurin, which dephosphorylates NFAT, exposing the nuclear localization signal and leading to NFAT translocation to the nuclei (28). In a majority of mature osteoclasts, treatment with RANKL results in a global elevation of [Ca\(^{2+}\)]\(_{i}\), (29), whereas, in osteoclast precursors, RANKL induces Ca\(^{2+}\) oscillations (30). Both RANKL-induced calcium signaling and activation of NFATc1 are essential for osteoclastogenesis (30–33). In addition to the calcineurin/NFATc1 pathway, Ca\(^{2+}\) is also linked to other pathways important in osteoclasts, such as protein kinase Ca (PKCa) signaling (34–37). Interestingly, it has been recently shown that PKCa can also activate ERK1/2 (34, 38, 39).

In the present study, we examine the mechanisms underlying the responsiveness of osteoclast precursors to factors released by breast cancer cells. We employed mouse bone marrow cultures and RAW 264.7 murine monocytic cells for osteoclast formation, human MDA-MB-231 breast carcinoma cells, which cause bone osteolytic lesions in vivo, as a source for factors produced by the breast cancer cells, and confluent human mammary epithelial cells MCF10a as a control.

**EXPERIMENTAL PROCEDURES**

**Cell Cultures**—MDA-MB-231 and MCF10a cells were kindly provided by Dr. J. Massagué (Memorial Sloan-Kettering Cancer Center, New York). MDA-MB-231 were cultured in the incubation medium (DMEM with 1.5 g/liter sodium bicarbonate, glutamine (319-020-CL, Wisent Inc.), 1 mM pyruvate (600-110-EL, Wisent Inc.), 100 units/ml penicillin, 100 \(\mu\)g/ml streptomycin (450-201-EL, Wisent Inc.), 10% fetal bovine serum (080-150, Wisent Inc.), and conditioned media (CM) was harvested after 24-h incubation. MCF10a were cultured to confluence by breast cancer cells. Cells were incubated with Ca\(^{2+}\) chelator BAPTA-acetoxyethyl ester (Invitrogen, B6769) for 10 min as described (40), washed, and treated with 10% MDA-MB-231 CM. Pan-specific TGF\(\beta\) antibody (15 \(\mu\)g/ml AB-100-NA, R&D System Inc.) at a concentration identified by the supplier and previous publications (41), was incubated with MDA-MB-231 CM for 30 min before adding to osteoclast precursors.

**Protein Extraction, Immunoblotting, and Immunofluorescence**—Cell lysates were extracted in RIPA lysis buffer containing 50 mM Tris, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 1 mM EDTA, 1 mg/ml aprotinin, 2 mg/ml leupeptin, 0.1 mM phenylmethylsulfonyl fluoride, 20 mM sodium fluoride, 0.5 mM sodium orthovanadate and centrifuged at 12,000 \(\times\) g for 10 min at 4 °C. Supernatant was collected, and protein was measured using a Quant-iT\textsuperscript{TM} protein assay kit (Invitrogen). 20–40 \(\mu\)g of lysates was separated on a 10% SDS-PAGE and transferred to a nitrocellulose membrane (0.45 \(\mu\)m, 162-0115, Bio-Rad) using 10 mM sodium borate buffer. The membranes were blocked in 5% milk or, for p38 and p-p38, in 5% ECL advanced blocking agent (RPN418, Amersham Biosciences) in TBST buffer (10 mM Tris-hydrochloride, pH 7.5, 150 mM NaCl, 0.05% Tween 20) for 1 h at room temperature followed by overnight incubation at 4 °C with primary antibodies: p-Smad2 (1:1000, 3101, Cell Signaling), Smad2/3 (1:1000, 3102, Cell Signaling), Smad4 (1:100, sc-7966, Santa Cruz Biotechnology), \(\alpha\)-tubulin (1:5000, T9026, Sigma), p-JNK (1:200, sc-6254, Santa Cruz Biotechnology), JNK (1:100, sc-81468, Santa Cruz Biotechnology), p-p38 (1:250, 9216, Cell Signaling), p38 (1:500, 9217, Cell Signaling), p-ERK1/2 (1:500, 9101, Cell Signaling), ERK1/2 (1:500, 9102, Cell Signaling), and p-PKC\(\alpha\)/\(\beta\)II (1:1000, 9375, Cell Signaling). The blots were washed, incubated with horseradish peroxidase-conjugated secondary antibodies (anti-mouse, 170-5047; anti-rabbit, 170-5046; Bio-Rad) and visualized with a chemiluminescence sys-
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RESULTS

Breast Cancer-derived Factors Promote Osteoclast Fusion and Growth from RANKL-Primed Precursors—Osteoclastogenesis was studied using primary mouse bone marrow cells and RAW 264.7 cells. When mouse bone marrow cells were treated with RANKL (100 ng/ml) for the first 3 days only (RANKL-primed), and then cultured untreated for additional 2 days, only a few small osteoclasts were observed (Fig. 1A). In contrast, supplementation of RANKL-primed precursors with RANKL (positive control, Fig. 1B) or MDA-MB-231 CM (Fig. 1C) induced marked osteoclastogenesis. In RAW 264.7 cultures, osteoclastogenesis occurs faster (within 3 or 4 days) and requires only treatment with RANKL (50 ng/ml) (42). Similar to...
primary cultures, when RAW 264.7 cells were treated with RANKL for the first 1 or 2 days only (RANKL-primed), and then cultured untreated for additional 2 days, osteoclastogenesis was incomplete (Fig. 1D). Supplementation of RANKL-primed RAW 264.7 cultures with RANKL (Fig. 1E) or MDA-MB-231 CM (Fig. 1F) induced formation of many large multinucleated osteoclasts. In primary (Fig. 1G) and RAW 264.7 (Fig. 1H) cultures treatment of RANKL-primed precursors with MDA-MB-231 CM resulted in significant, 2.6- to 6.5-fold increases in osteoclast number compared with negative control. Medium conditioned by confluent normal breast cells MCF10a did not affect osteoclast formation from RANKL-primed RAW 264.7 (Fig. 1I). In addition, in MDA-MB-231 CM-treated cultures, osteoclast size (estimated as cell planar area) was increased 4- to 5-fold (Fig. 1, I and J), and the number of nuclei per osteoclast was increased 3-fold (Fig. 1K) compared with negative control. In MDA-MB-231 CM-treated RAW 264.7 osteoclasts, but not primary osteoclasts, the ratio of cell area/nucleus increased 2.7-fold compared with negative control (Fig. 1L). Thus, cancer-derived factors induced osteoclastogenesis from RANKL-primed precursors of bone marrow or RAW 264.7 origin.

Osteoclastogenesis Induced by Breast Cancer-derived Factors Does Not Depend on the Activation of the Smad Signaling Pathway—TGFβ acts as a permissive factor for MDA-MB-231 CM-induced osteoclastogenesis (12). We explored if the Smad pathway is involved in MDA-MB-231 CM-induced signaling in osteoclast precursors. RAW 264.7 cells were primed with RANKL (50 ng/ml), and cell lysates were collected before and 30 min after the medium was replaced with fresh medium without further additions (negative control), RANKL (positive control), or 10% MDA-MB-231 CM. No difference was observed in the levels of total Smad2/3 or phosphorylated Smad2 (supplemental Fig. S1A). Next, we silenced Smad signaling using short interfering RNA against the common mediator Smad4 (supplemental Fig. S1B). Smad4 short interfering RNA did not inhibit MDA-MB-231 CM-induced osteoclastogenesis (supplemental Fig. S1C), suggesting that the Smad pathway does not mediate this effect.

Breast Cancer-derived Factors Induce Osteoclastogenesis in RAW 264.7 Cells by Activating ERK and p38—We next assessed the role of ERK, JNK, and p38 in MDA-MB-231 CM-induced osteoclastogenesis. RAW 264.7 cells were primed with RANKL (50 ng/ml), then the medium was replaced with fresh medium without further additions (negative control), RANKL (50 ng/ml, positive control), MDA-MB-231 CM (10%), or MDA-MB-231 CM pretreated with pan-specific anti-TGFβ antibodies (15 μg/ml). Cell lysates were collected after 30- and 60-min incubation and immunoblotted against p-JNK, p-p38, or p-ERK1/2 (Fig. 2). Total JNK, p38, ERK1/2, and α-tubulin were used as internal and loading controls. We have found that JNK phosphorylation or total levels were not affected by any of the treatments. In contrast, MDA-MB-231 CM induced p38 phosphorylation at 30 min, which was attenuated by neutralizing TGFβ. The total level of p38 was not affected. Treatment with MDA-MB-231 CM induced ERK1/2 phosphorylation as early as 7.5 min after stimulation (data not shown). The profound ERK1/2 phosphorylation reached maximum after 15 min (data not shown) and was sustained 30 and 60 min after the addition of MDA-MB-231 CM (Fig. 2). Neutralizing TGFβ did not affect p-ERK1/2 levels 30 min after stimulation but attenuated ERK1/2 phosphorylation at 60 min (Fig. 2). Total levels of ERK1/2 were not affected by the treatments. Thus, activation of p38 and ERK1/2, but not JNK, was induced by breast cancer-derived factors in a partially TGFβ-dependent manner.

To explore if ERK1/2 and p38 mediate MDA-MB-231-induced osteoclastogenesis, we used pharmacological inhibitors against MEK1/2, PD98059, and against p38, SB203580. RAW 264.7 cells were primed with RANKL (50 ng/ml), then the medium was replaced with fresh medium without further additions (negative control), with RANKL (50 ng/ml, positive control), MDA-MB-231 CM (10%) alone or combined with PD98059 (100 μM), SB203580 (1 μM), PD98059 and SB203580 together, or an inactive analog of the p38 inhibitor SB202474 (1 μM). The cells were cultured for additional 2 days, fixed, stained for TRAP, and the number, size, and nucleation of osteoclasts were assessed (Fig. 3). Whereas PD98059 alone did not affect osteoclast number (Fig. 3, A and P), the p38 inhibitor SB203580 significantly reduced the number of osteoclasts formed in the presence of MDA-MB-231 CM (Fig. 3, A and S). Combination of the two inhibitors further decreased osteoclast number compared with the treatment with a single inhibitor (Fig. 3A, P+S). Even though the number of osteoclasts induced by MDA-MB-231 CM was not affected by PD98059, it induced a significant 60% reduction in osteoclast size. Inhibition of p38 reduced osteoclast size by 90%, with a decrease by 95% in the presence of a combination of ERK and p38 inhibitors (Fig. 3B). Both inhibitors, alone or in combination, reduced the osteoclast nucleation by 70–95% (Fig. 3C). Interestingly, the ratio of cell area per nucleus was increased in the presence of MEK1/2 inhibitor (Fig. 3D) but reduced in the presence of p38 inhibitor. The inactive analogue of p38 inhibitor, SB202474, did not affect osteoclast number, size, or nucleation (Fig. 3, A–D). Thus, p38...
TGF-ERK1/2 phosphorylation was only partially dependent on MEK1/2-dependent but Ras/Raf-independent osteoclast precursors. Stages of osteoclastogenesis, which become non-critical in late stages of osteoclastogenesis, are likely to represent a point where the synergy occurs and ERK1/2 mediate cancer-induced osteoclast fusion and growth.

To investigate a potential role of NFκB in breast cancer-induced osteoclastogenesis, we used NFκB peptide inhibitor SN50. SN50 blocked RANKL-induced nuclear translocation of p65 and RANKL-induced osteoclastogenesis (supplemental Fig. S2). In contrast, SN50 was ineffective in inhibiting MDA-MB-231 CM-induced osteoclast formation (supplemental Fig. S2C), indicating that the NFκB pathway likely affects early stages of osteoclastogenesis, which become non-critical in late osteoclast precursors.

Breast Cancer Factor-induced Phosphorylation of ERK1/2 Is MEK1/2-dependent but Ras/Raf-independent—Because ERK1/2 phosphorylation was only partially dependent on TGFβ, it is likely to represent a point where the synergy occurs between TGFβ and other factors produced by breast cancer cells. To analyze the signaling events leading to ERK activation, we first considered the classic Ras/Raf/MEK1/2/ERK1/2 pathway. RAW 264.7 cells were primed with RANKL (50 ng/ml) and treated with MDA-MB-231 CM alone or in combination with MEK1/2 inhibitor PD98059 (100 μM), Ras inhibitor manumycin A (3 μM), or Raf1 inhibitor GW5074 (3 μM) for 30–60 min before cell lysates were collected and immunoblotted against p-ERK1/2. As a control, RANKL-primed cells were treated with vehicle (DMSO) or MEK1/2 inhibitor (PD98059, 3 μM). PD98059 abolished ERK1/2 phosphorylation both at 30 and 60 min after exposure to MDA-MB-231 CM (Fig. 4A). However, manumycin A and GW5074 did not inhibit MDA-MB-231 CM-induced ERK1/2 phosphorylation (Fig. 4B), suggesting that breast cancer factors act through a Ras/Raf-independent pathway. Both Ras and Raf1 kinase inhibitors were effective in decreasing phosphorylation of ERK1/2 in response to RANKL (Fig. 4C).

Breast Cancer-derived Factors Employ PKCa to Activate ERK and Induce Osteoclastogenesis—It has been recently shown that ERK1/2 can also be activated by PKCa (34, 43, 44). To examine if PKCa is involved in MDA-MB-231 CM-induced ERK1/2 phosphorylation, RAW 264.7 cells were primed with RANKL (50 ng/ml) and treated with MDA-MB-231 CM alone or in combination with vehicle (DMSO), or PKCa inhibitor Gö6976 (1 μM). After 7.5, 30, and 60 min cell lysates were collected and immunoblotted against p-PKCα and p-ERK1/2. Both Ras and Raf1 kinase inhibitors decreased RANKL-induced phosphorylation of ERK1/2. Gö6976 decreased ERK1/2 phosphorylation at concentrations as low as 100 nM (Fig. 5B). Gö6976 also reduced nuclear accumulation of p-ERK1/2, nuclear RANKL-induced phosphorylation of ERK1/2, and ERK1/2 phosphorylation at 30 min after exposure to MDA-MB-231 CM (Fig. 5C–F), profoundly decreased osteoclast size and nucleation even at the lowest concentration of an inhibitor (Fig. 5D and E), and diminished the ratio of cell area per nucleus (Fig. 5F), suggesting that breast cancer factors act via...
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**PKCα to activate ERK and to induce osteoclast fusion and growth both in ERK-dependent and ERK-independent manner.**

**Because PKCα is a Ca^{2+}-dependent isoform, we investigated if MDA-MB-231 CM induc changes in Ca^{2+} in osteoclast precursors.** RANKL-primed RAW 264.7 cells were loaded with fura-2-AM. Changes in [Ca^{2+}], were monitored within 2 min and 30–60 min after bath addition of DMEM, 50 ng/ml RANKL, 5–10% MDA-MB-231 CM, or 5–10% of MCF10a CM. No striking acute changes in [Ca^{2+}], were observed immediately upon addition of DMEM, RANKL, or CM from both cell types. However, as early as 2 min after addition of MDA-MB-231 CM, 20 ± 3% of osteoclast precursors exhibited elevations of [Ca^{2+}], compared with 11 ± 3% in cultures exposed to DMEM. Although the difference did not reach statistical significance (p = 0.07), this trend was augmented at the later time points (30 and 60 min). At 30 min, significantly more cells exhibited elevations of [Ca^{2+}], in cultures treated with MDA-MB-231 CM and RANKL, compared with cultures treated with DMEM or MCF10a CM (Fig. 6, A and C). Neutralizing TGFβ did not reduce the number of cells exhibiting Ca^{2+} elevations. Cells treated with MDA-MB-231 CM or RANKL dem-

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**We have shown that breast cancer-derived factors employ MAPKs and calcium/PKC pathways to stimulate formation of osteoclasts from RANKL-primed precursors.** We have previously exposed TGFβ as a permissive but indispensable factor for these effects, and now we demonstrate that TGFβ-induced signaling does not involve the Smad pathway but acts instead through activation of MAPKs, p38 and, partially, ERK1/2. We have found that inhibition of TGFβ shortened the duration of ERK1/2 activation but did not decrease its amplitude. In contrast, inhibition of calcium-dependent PKCα resulted in suppression of cancer factor-induced phosphorylation of ERK1/2. We have found that breast cancer-derived factors, similar to RANKL, induced sustained oscillations in [Ca^{2+}], in osteoclast precursors. Thus, we conclude that breast cancer-derived factors induce osteoclast formation by joined activation of calcium/PKCα and TGFβ/MAPK pathways.

**Calcium signaling has been shown before to be a critical intermediary for RANKL-induced osteoclastogenic signaling (30). Interestingly, in osteoclast precursors RANKL was shown to induce delayed calcium oscillations of comparatively low amplitude (30), whereas in mature osteoclasts, RANKL induces acute global elevation of [Ca^{2+}], (40). In keeping with previous reports, we have found that priming with RANKL induced oscillations in ~40% of RAW264.7 cells. However, when RANKL was withdrawn from the media, the number of oscillating cells declined to ~10%. Breast cancer-derived factors were capable of supporting the [Ca^{2+}], oscillations in osteoclast precursors even in the absence of RANKL. This effect of breast cancer factors on calcium signaling mirrors its effect on NFATc1 activation, which we described in the previous study (12). In addition to the calcium/calcineurin/NFATc1 pathway, we have now demonstrated that breast cancer-derived factors also stimulate calcium-dependent PKC signaling, likely acting through PKCα. We demonstrate that PKC-dependent signals cooperate with TGFβ-induced signals to stimulate ERK1/2 phosphorylation. We have found that PKCα stimulates ERK1/2 phosphorylation in a Ras and Raf-independent manner. Although not common, this pathway of ERK activation has been previously described in osteoclasts and other cell types (34, 39, 43). On the other hand, we have found that TGFβ signaling acts to prolong ERK activation for > 1 h, so that together actions of PKC and TGFβ result in a substantial and sustained
large multinucleated osteoclasts. Osteoclast differentiation is associated with increased expression of proteins directly needed for osteoclastic resorptive activity, such as proteolytic enzymes cathepsin K and matrix metalloproteinase 9 (48). We have previously shown that osteoclasts formed in the presence of breast cancer-derived factors express appropriate levels of cathepsin K and matrix metalloproteinase 9 and are capable of bone resorption. In addition, several steps aiming at producing a giant polycarbonate can be distinguished during osteoclastogenesis. First, monocyte precursors fuse to form multinucleated cells. Second, the volume of the cytoplasm increases, both due to cytoplasm gain through fusion and due to post-fusion increase in osteoclast membrane and cytoplasm volume. And third, multinucleated osteoclasts can fuse with other osteoclasts to form even bigger cells. Osteoclast size significantly affects the effectiveness of resorption (49). Mice lacking DC-STAMP, the protein critical for osteoclast fusion, have small mononuclear osteoclasts, which are capable of bone resorption, but at a greatly reduced activity (50–52). Moreover, bigger osteoclasts exhibit increased resorptive activity (53–55) and are associated with pathological bone destruction (56, 57).

We have found that p38, ERK1/2, and PKCa pathways contribute to different steps of osteoclast formation in a distinct manner. Inhibition of p38 led to a 2-fold decrease in osteoclast number, ~7-fold decrease in osteoclast size and the number of nuclei per osteoclast, and 2-fold decrease in the cell area/nucleus that reflects the post-fusion osteoclast growth, demonstrating that p38 is important for monocyte fusion, osteoclast growth, and osteoclast fusion. In contrast, inhibition of ERK did not affect osteoclast number, suggesting that ERK signaling is not involved in monocyte fusion. However, ERK inhibition significantly decreased osteoclast size and nucleation while significantly increasing the cell area/nucleus. These data suggest that the ERK pathway plays an inhibitory role in the regulation of osteoclast growth while positively regulating the fusion of osteoclasts. Interestingly, the combination of inhibitors for ERK and p38 resulted in an additional decrease in osteoclast number. It has been shown previously that inhibition of ERK can induce compensatory stimulation of p38 (58), which can offset its potential inhibitory effects. However, we could not detect a
noticeable increase in p-p38 in samples treated with MEK1/2 inhibitor (data not shown), likely suggesting that even subtle changes in balance between p38 and ERK may have significant downstream effects. We have found that chelation of Ca^{2+} as well as inhibition of PKCa significantly decreased osteoclast number and size. Although ERK phosphorylation was drastically reduced in the presence of PKCa inhibitor, the effects of ERK and PKCa inhibition are different, suggesting that some of osteoclastogenic effects of PKC are induced in an ERK-independent manner. For all the inhibitors, the effect on osteoclast size and nucleation was more dramatic than the effect on osteoclast number, suggesting that the initial fusion of monocytes is regulated prior to, or differently from expansion of osteoclast size either by fusion of osteoclasts or by cell growth.

Thus, our data reveal the mechanism underlying the direct stimulatory effect of breast cancer cells on osteoclast formation. This process is complementary to other known effects of breast cancer cells on different cell types present in the bone microenvironment, such as cancer-induced stimulation of RANKL and other paracrine mediators by osteoblasts (1, 6, 8, 9). Breast cancer metastases are known to preferentially establish in the skeletal sites undergoing active bone turnover (59–61), where the numbers of osteoclast precursors primed by physiological stimuli is increased. The signaling pathways allowing osteoclast precursors to respond to the stimulation by breast cancer cells represent potential targets for the development of new therapies for osteolytic bone metastases.

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