C-Src Tyrosine Kinase Activity Is Associated with Tumor Colonization in Bone and Lung in an Animal Model of Human Breast Cancer Metastasis

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ABSTRACT

The proto-oncogene, c-src, has been implicated in the tumorigenesis in breast cancer. However, the relationship of c-src with distant metastasis is unclear. Moreover, the role of c-src in organ-preferential metastasis of breast cancer is unknown. Because breast cancer has a strong predilection for metastasizing to bone, we examined the role of c-src in bone metastases using an animal model in which inoculation of the MDA-231 human breast cancer cells into the left cardiac ventricle preferentially developed osteolytic bone metastases in female nude mice. A clone of the MDA-231 with the increased capacity of bone metastasis exhibited elevated c-src tyrosine kinase (TK) activity compared with parental cells. MDAsrc527 cells caused significantly increased size of the osteolytic bone metastases with increased number of osteoclasts and mitotic cancer cells compared with MDA-231 or MDAsrcWT. In contrast, MDAsrc295 cells caused impaired metastases to bone. Of note, mice inoculated with MDAsrc295 cells via tail vein developed reduced lung metastases and prolonged survival compared with mice with MDA-231EV cells, suggesting that c-src TK is unlikely to play a specific role in bone metastases. The growth in vitro and in vivo and production of parathyroid hormone-related protein, a key cytokine in the pathogenesis of osteolytic bone metastases in breast cancer, were promoted in MDAsrc527 and diminished in MDAsrc295. These results suggest that c-src TK is associated with the capacity of breast cancer to metastasize to bone through regulating cell growth and parathyroid hormone-related protein production. Our results together with the fact that c-src TK activity modulates the capacity of MDA-231 cells to develop bone metastases in breast cancer using a well-characterized animal model of experimental bone metastasis (13–17). We introduced WT c-src c-src and its mutants that exhibit different levels of TK activity into the MDA-231 human breast cancer cells and examined the ability of these transfected MDA-231 cells to develop bone metastases. We also examined whether the manipulation of c-src TK activity affects lung metastasis of MDA-231 cells using a different animal model. Our results show that MDA-231 cells overexpressing a constitutively active c-src TK develop increased osteolytic bone metastases. In contrast, MDA-231 cells with a kinase-dead c-src TK caused reduced bone metastases compared with control MDA-231 cells. Furthermore, mice inoculated with MDA-231 cells with a kinase-dead c-src TK developed diminished lung metastases and exhibited prolonged survival. These results demonstrate that the c-src TK activity modulates the capacity of MDA-231 cells to spread to distant organs, including bone and lung.

INTRODUCTION

The proto-oncogene c-src is the cellular homologue of v-src that was initially found in the Rous sarcoma virus that induces tumors in chickens and transformation in a variety of mammalian cells (1, 2). c-src encodes an M₆, 60,000 cytoplasmic protein with intrinsic TK activity. It consists of an NH₂-terminus-containing myristylation site for the binding to inner cell membrane, nonconserved unique domain for each src family member, a SH3 domain that binds to proline-rich sequences, a SH2 domain that binds to phosphotyrosine, a TK domain, and a short COOH-terminal tail in its primary structure (1, 3, 4). In chicken c-src, there are one lysine residue at 295, one tyrosine residue at 416 in the kinase domain, and one tyrosine residue at 527 in the COOH-terminal tail, all of which play critical roles in the regulation of c-src TK activity. Lysine 295 is the binding site for ATP that is the source of phosphates necessary for phosphorylation of c-src, tyrosine 416 is the autophosphorylation site, and tyrosine 527 is the negatively regulatory phosphorylation site of c-src TK activity (1, 3, 4). Point mutation of these sites markedly changes c-src TK activity.

Recent human and animal studies suggest the association between c-src TK and the development, progression, and metastasis of breast cancer (5–11). It is demonstrated that c-src TK activity is profoundly increased in human breast cancer tissues compared with benign breast tumors or adjacent normal breast tissues (5–7, 11) and that elevated c-src TK activity is correlated with poor metastasis-free survival (8). Transgenic mice with polyoma middle T antigen under the control of mouse mammary tumor virus promoter were found to develop highly metastatic mammary tumors with increased c-src TK activity (9). Moreover, when these mice were cross-bred with c-src-deficient mice, the resulting chimeric mice no longer developed mammary tumors (9). In addition, it is described that mice overexpressing the new oncogene develop highly metastatic mammary tumors with elevated c-src TK activity (10). Taken together, these results strongly indicate that the c-src TK plays an important role in the development and progression of breast cancer. However, the relationship of c-src TK in distant metastasis of breast cancer is still largely unknown. In addition, whether c-src TK contributes to the organ preference of breast cancer metastasis is unknown. It has been long and widely recognized that breast cancer has a strong predilection for metastasizing to bone (12). Here, we investigated the role of c-src TK in the development of bone metastases in breast cancer using a well-characterized animal model of experimental bone metastasis (13–17). We introduced WT chicken c-src and its mutants that exhibit different levels of TK activity into the MDA-231 human breast cancer cells and examined the ability of these transfected MDA-231 cells to develop bone metastases. We also examined whether the manipulation of c-src TK activity affects lung metastasis of MDA-231 cells using a different animal model. Our results show that MDA-231 cells overexpressing a constitutively active c-src TK develop increased osteolytic bone metastases. In contrast, MDA-231 cells with a kinase-dead c-src TK caused reduced bone metastases compared with control MDA-231 cells. Furthermore, mice inoculated with MDA-231 cells with a kinase-dead c-src TK developed diminished lung metastases and exhibited prolonged survival. These results demonstrate that the c-src TK activity modulates the capacity of MDA-231 cells to spread to distant organs, including bone and lung.
CA). Antiphosphotyrosine antibody was described previously (18).

Herbimycin A and tyrphostin were purchased from Life Technologies, Inc. (Rockville, MD).

MDA-MB-231 Human Breast Cancer Cells. The estrogen-independent human breast cancer cell line MDA-231 (American Type Culture Collection, Rockville, MD) was cultured in DMEM (Life Technologies, Inc.) supplemented with 10% fetal bovine serum (HyClone Laboratories, Logan, UT). A bone-seeking clone of MDA-231 cells (MDA-231BO cells) was established as described (19). All cells were routinely tested for Mycoplasma contamination.

c-src Plasmids. Plasmids containing chicken c-src gene and its mutants were kind gifts from Dr. David Shalloway (20, 21). It is shown that chicken c-src is >95% homologous to human c-src at amino acid levels, that the 295 ATP-binding site and 527 tyrosine phosphorylation site are well conserved between chicken and human, and that an introduction of chicken c-src into human cells causes expected effects (1–4). The plasmid pM5HHB5 encoding the cDNA for WT chicken c-src was inserted into an expression vector pEVX. The plasmid, pCR295, has the cDNA encoding the c-src with a point mutation at the ATP-binding site (lys295 to arg295). This mutation was shown to cause complete loss of c-src TK activity and inhibit endogenous c-src TK in a dominant-negative fashion in chicken embryo fibroblasts (22). The plasmid psrc527 contains the c-src gene with a point mutation at the COOH-terminal inhibitory residue (Tyr527 to Phe527), resulting in a constitutive activation of c-src TK (18, 23, 24) by causing a conformational change (25–27).

Transfection. Transfection of MDA-231 cells was performed using LipofectAMINE (Life Technologies, Inc.) with cotransfection of pSVneo2 vector containing the neomycin resistant gene in the presence of G418 (0.75 μg/ml).

Immunoblotting and Immunoprecipitation. Cell lysates were prepared, and immunoblotting and immunoprecipitation were performed as described (18). In brief, the lysates were separated on 7.5% SDS-polyacrylamide gel and transferred onto nitrocellulose membrane. The membranes were blocked with 50 mg/ml BSA (Sigma, St. Louis, MO) in TBS (pH 7.4, 10 mM Tris-Cl and 150 mM NaCl) for 2 h at room temperature, incubated with a primary antibody in BSA-TBS for 2 h, washed with TBS containing 0.1% Triton X-100, and incubated with horse radish peroxidase-conjugated antimouse IgG (Cappel, Durham, NC) or horse radish peroxidase-conjugated protein A (Kirkegaard & Perry Laboratories, Gaithersburg, MD) in 50 mg/ml dry milk-TBS for 1 h. The signals were visualized with enhanced chemiluminescence detection system (DuPont NEN, Boston, MA).

For immunoprecipitation, the cell lysates were incubated with antibodies for 4 h at 4°C, precipitated with protein G-agarose (Boehringer Mannheim, Indianapolis, IN), separated by SDS-PAGE, transferred to nitrocellulose membrane, and immunoblotted with corresponding antibodies.

In Vitro Kinase Assay. c-src protein in the cell lysates was immunoprecipitated using anti-src antibody (residues 2–17) and protein G-agarose (Boehringer Mannheim). Because the expression level of the c-src protein varied in each clone, the starting amounts of the cell lysates were adjusted according to the expression level of the c-src protein to include the same amounts of the c-src protein in the reaction mixture. The immunocomplexes were incubated with 5 μg of cdadenurated rabbit muscle enolase (Boehringer Mannheim) and 1 μl of 1.7 μM [γ-32P]ATP (6000 Ci/mmol; DuPont NEN) for 5 min at 30°C in kinase reaction buffer containing 10 mM Tris (pH 7.4), 5 mM MgCl2, 100 μM sodium orthovanadate, and 1 μM unlabelled ATP as described (28). Samples were separated by SDS-PAGE and visualized by autoradiography. Radioactivity of [32P]enolase in excised bands was determined by scintillation counting. Data were expressed as proportions to the radioactivity of [32P]enolase for MDA-231EV cells, which represent relative activities of c-src TK per unit c-src protein.

Anchorage-independent Growth in Soft Agar. Anchorage-independent growth of the transfecteds was determined by colony formation in soft agar as described (14). Colonies > 200 μm in diameter were manually counted under an inverted microscope.

PHT-P Assay. PHT-P concentration in the serum-free, 48-h conditioned media of MDA-231 cells was measured using a two-site IRMA (Nichols Institute, San Juan Capistrano, CA) that uses two polyclonal antibodies specific for the NH2-terminal (1–40) and (60–72) portions of PHT-P. Data were normalized for cell number and analyzed using Prism (GraphPAD Software for Science, San Diego, CA) on an IBM-compatible computer.

PHT-P Promoter Activity. Cells were transiently transfected with a reporter construct consisting of the PHT-P promoter hooked with the firefly luciferase gene. Luciferase enzymatic activity was measured by Luciferase Assay System (Promega, Madison, WI) using luminometer (Turner Designs luminometer Model TD-2020; Promega). Data were corrected with cell number at the end of culture period or protein concentration of the lysates.

Orthotopic Tumor Formation. MDA-231 cells (3 × 105 cells) in 0.1 ml of 50% Matrigel (Collaborative Research, Bedford, MA) or PBS were inoculated into the mammary fat pad of 5-week-old female Fox CHASE C.B-17/lcr-scid Jcl mice (Clea Japan, Tokyo, Japan). Tumors formed were excised and weighed at 3 weeks.

Bone Metastasis. Intracardiac injection was performed according to the technique described previously (13–17). Quantitative assessment for osteolytic bone metastases was described previously (13–17). Radiographs were carefully evaluated for the area of osteolytic bone metastases in long bones of hindlimbs by three individuals (P. J. W., T. Y., Mark Dallas) who had no information about the experimental design using a computer-assisted Jandral Video Analysis image analysis system (Jandral Scientific, Corte Madera, CA) as described (15).

The experiment was conducted twice using a different transfectant in each of the experiments (6 mice for each group/experiment for MDAsrcWT, 8 mice for each group/experiment for MDA231P, and 9 mice for each group/experiment for MDA231pK). Total animal numbers for each group for MDAsrcWT, MDA231P, and MDA231pK were 6 mice × two transfectants = 12 mice, 8 mice × two transfectants = 16 mice, and 9 mice × two transfectants = 18 mice, respectively.

Lung Metastasis. MDA-231 cells (1 × 106 cells/mouse) were inoculated via the lateral tail vein of 5-week-old, female Fox CHASE C.B-17/lcr-scid Jcl mice (Clea Japan). Lungs were harvested 7 weeks after tumor cell inoculation, fixed, and stained with Bouni’s solution, and the number of metastatic foci was counted as described previously (29).

Histology and Determination of Mitotic Index. Hind limbs were fixed with 10% natural buffered formalin for 48 h, decalcified in EDTA (pH 7.2) for 2 weeks, and processed for conventional paraffin-embedded H&E staining. Mitotic index was determined by manually counting mitotic figures in 10 randomly selected high-power fields of the metastatic tumors in bone on histological sections.

Statistical Analysis. All results are expressed as the mean ± SE. Data were analyzed by Student’s t test or repeated measures ANOVA followed by Tukey-Kramer post test. Survival curves were estimated by Kaplan-Meier method, and the difference was analyzed by generalized Wilcoxon test. Ps of <0.05 were considered significant.

RESULTS

Relationship between the Capacity of Bone Metastasis and c-src TK Activity in MDA-231 Cells. To examine the relationship between c-src TK activity and the capacity of bone metastasis, we compared c-src TK activity between parental MDA-231 cells (MDA-231P) and a clone of MDA-231 cells, which show increased capacity of bone metastasis (MDA-231BO; Ref. 19). Western analysis demonstrated that the MDA-231BO cells expressed elevated c-src protein (Fig. 1a) and tyrosine phosphorylation of c-src (Fig. 1b, bottom) and several unidentified cellular proteins (Fig. 1b, top) compared with MDA-231P cells. Moreover, c-src kinase activity was also higher in MDA-231BO than MDA-231P cells (Fig. 1c). These results suggest that c-src TK activity correlates with the capacity of bone metastasis of MDA-231 cells.

c-src TK Activity and Bone Metastasis in MDA-231 Cells. To investigate the effects of c-src TK activity on the development of bone metastasis in MDA-231 cells, we examined the capacity bone metastasis of MDA-231 cells that were stably transfected with EV, WT c-src (pM5HHB5), kinase-dead c-src (pCR295), and constitutively active c-src (pcsr527). These transfecteds were found to stably express WT, kinase-dead, and constitutively active c-src, respectively (Fig. 2a) and designated as MDAsrcWT, MDAsrc295, and MDAsrc527, respectively. In vitro kinase assays demonstrated that kinase activity in MDAsrcWT was higher than MDA-231EV (Fig. 2,
The anti-v-src antibody (clone 327, 1:400 dilution) was used to detect c-src TK activity in parental MDA-231 cells (MDA-231P cells) and MDA-231BO cells (MDA-231B cells). Western blot analysis on whole cell lysates of MDA-231P and MDA-231BO cells using anti-v-src antibody (clone 327, top) and anti-β-actin antibody (bottom). Expression levels of c-src and β-actin protein were semiquantified using an image analyzer, and the ratio of c-src/β-actin was calculated. The number shown is calculated by dividing c-src (BO) by c-src (P). b, tyrosine-phosphorylation of cellular proteins in whole cell lysates (top) and auto-phosphorylation of immunoprecipitated c-src (bottom) in MDA-231P and MDA-231BO cells using antiphosphotyrosine antibody. c, in vitro kinase assay. Immunoprecipitated endogenous c-src from lysates of MDA-231 cells and enolase was used as a source of c-Src TK and a substrate, respectively. Top band, auto-phosphorylation of c-src; bottom band, TK activity of c-src on enolase.

Fig. 2. Establishment of MDA-231 cells overexpressing c-src with different kinase activity. a, Western blotting for c-src using an anti-v-src antibody (clone 327, 1:400 dilution) in the cell lysates of MDA-231EV, MDAsrcWT, MDAsrc295, or MDAsrc527. b, in vitro kinase assay. The lysates of c-src-transfected MDA-231 cells immunoprecipitated with an anti-v-src antibody (residues 2-17) and enolase were used as a source of c-Src TK and a substrate, respectively. Top band, auto-phosphorylation of c-src; bottom band, TK activity of c-src on enolase. c, specific activity of c-src kinase. The bottom bands shown in b were excised and measured for their radioactivity in a scintillation counter. Values on Y axis are relative radioactivity to that of MDA-231EV cells.

b and c). On the other hand, MDAsrc527 showed markedly higher kinase activity than others, and kinase activity in MDAsrc295 was abolished (Fig. 2, b and c).

We then studied MDA-231EV, two clones of MDAsrcWT (A and B), two clones of MDAsrc527 (A and B), and two clones of MDAsrc295 (A and B) for the capacity to develop bone metastases using a well-characterized animal model. As shown in Fig. 3, a and b, MDAsrc527 (A) developed increased size of osteolytic bone metastases compared with MDA-231EV cells and MDAsrcWT (A) cells on radiographs. Quantification of these osteolytic lesions using computerized image analysis system revealed that the area of osteolytic lesion was significantly greater in mice inoculated with MDAsrc527 (A) cells than mice inoculated with MDA-231EV or MDAsrcWT (A) and (B) cells (Fig. 3b). MDAsrc527 (B) also developed larger bone metastases. In contrast, mice inoculated with MDAsrc295 (A and B) cells developed significantly smaller osteolytic bone metastases than mice inoculated with MDA-231EV cells (Fig. 3, a and b).

Histological examination revealed MDAsrcWT colonization in bone with osteoclastic bone resorption (Fig. 4, a and d). MDA-src295 developed markedly reduced tumor in bone (Fig. 4, b and e). In contrast, MDAsrc527 caused profoundly increased bone metastases with increased number and size of osteoclasts (Fig. 4, c and f). MDA-231EV developed equivalent bone metastases to MDAsrcWT (data not shown). These data demonstrate that elevated c-src TK activity promotes the development of osteolytic bone metastases of MDA-231 cells and that decreased c-src TK activity impairs bone metastases of MDA-231 cells. The results are consistent with a notion that c-src TK modulates the capacity of breast cancer colonization in bone.
c-src TK Activity and Lung Metastasis. To examine whether c-src TK specifically affects bone metastases, the capacity of MDAsrc295 cells to spread to lung after tail vein inoculation was assessed compared with MDA-231EV cells. We found that lung metastases of MDAsrc295 were significantly decreased (Fig. 5a). As a consequence of this, survival of mice bearing MDAsrc295 cells was significantly prolonged (Fig. 5b). Thus, c-src TK affects not only bone metastases but also lung metastases.

Effects of c-src TK on MDA-231 Cell Growth in Vivo and in Vitro. Because c-src plays a critical role in cell growth (30), we next examined the effects of c-src TK on the growth of MDA-231 cells metastasized in bone by manually counting the number of mitotic cells on the histological sections. Mitotic index in MDAsrc295 cells in bone was significantly greater than MDA-231EV, MDAsrcWT, and MDAsrc295 cells (Fig. 6a). Consistent with these results, MDAsrc527 cells exhibited larger tumor formation than MDAsrcEV in the orthotopic mammary fat pad (Fig. 6b).

We subsequently determined the relationship between the c-src TK activity and anchorage-independent growth in MDA-231 cells by assessing for the colony formation in soft agar. Colony formation of MDAsrc295 cells was significantly greater than other src-transfected MDA-231 cells (Fig. 6c). Collectively, these data show that elevated c-src TK activity promotes MDA-231 cell growth in vitro and in vivo.

PTH-rP Production in c-src-transfected MDA-231 Cells. We have reported that overexpression of PTH-rP cDNA increased osteolytic bone metastases and a neutralizing antibody against PTH-rP inhibited bone metastases in MDA-231 cells in the same animal model of metastasis as described here (15, 17). In addition, it was also reported that v-src-transfected fibroblasts produced increased levels of PTH-rP (31). We, therefore, determined the effects of c-src TK on the PTH-rP production in MDA-231 cells. As demonstrated in Fig. 7a, MDAsrc527 cells produced significantly greater amounts of PTH-rP than MDA-231EV cells. On the other hand, MDAsrc295 showed significantly reduced PTH-rP production compared with MDA-231EV cells. It is likely that this decrease in PTH-rP production, in part, contributes to the reduced bone metastases in MDAsrc295 despite that the mitosis (Fig. 6a) and anchorage-independent growth (Fig. 6c) of MDAsrc295 were equivalent to MDA-231EV. In addition, herbimycin A, an inhibitor of c-src TK (32), significantly reduced elevated PTH-rP production in MDAsrc527 cells (Fig. 7b), suggesting an involvement of c-src TK in the regulation of PTH-rP production.

**Fig. 5. c-src TK and lung metastasis.** a. number of metastatic foci in lung 7 weeks after i.v. inoculation of MDA-231 cells (1 × 10⁶ cells in 0.1 ml of PBS). The experiment was conducted twice using 10 mice for each group. Values represent mean ± SE. b. overall survival curves estimated by Kaplan-Meier method for the mice inoculated with MDA-231 cells via tail vein. *, P < 0.001 versus MDA-231EV.

**Fig. 6. c-src TK activity and MDA-231 cell growth.** a. mitotic index in metastatic MDA-231 cells in bone. There are numeral mitotic cells in metastatic tumors in bone (Fig. 4). b. tumorigenicity of MDA-231 cells in the mammary fat pad. c. anchorage-independent growth of MDA-231 cells. Five-hundred MDA-231 cells suspended in 0.45% agarose gel were inoculated onto 35-mm dishes and grown for 2 weeks. Colonies > 200 μm in diameter were manually counted under an inverted microscope. Data represent the mean ± SE (n = 6) from two separate experiments using different transfectants. *, P < 0.001 versus other groups.
and treated with TK inhibitors at indicated concentrations (luciferase gene driven by PTH-rP promoter were cultured in serum-free media for 18 h after transfection, luciferase activity of the cell lysates was determined. Values represent mean ± SE (n = 6). *P < 0.001 versus MDA-231EV. In b, MDAsrc295 cells were treated with a c-src TK selective inhibitor, herbimycin A at 1 µg/ml as described in the text, and PTH-rP concentration of the conditioned media was determined by ELISA. Values represent mean ± SE (n = 3). †P < 0.05 versus MDA-231EV. 1, P < 0.05 versus MDAsrc295 without Herbimycin A. In c, PTH-rP promoter hooked with luciferase gene was transiently transfected into MDA-231EV, MDAsrc295, and MDAsrc527 cells. Twenty-four h after transfection, luciferase activity of the cell lysates was determined. Values represent mean ± SE of luciferase activity corrected by cell number at the end of the culture period (n = 4). *P < 0.001 versus MDA-231EV. In d, MDA-231 cells stably transfected with luciferase gene driven by PTH-rP promoter were cultured in serum-free media for 18 h and treated with TK inhibitors at indicated concentrations (µg/ml) for 6 h. Data represent mean ± SE of luciferase activity corrected by protein concentration (n = 3). *P < 0.001 versus untreated.

Finally, we examined whether c-src TK regulated PTH-rP gene transcription using the reporter construct described in “Materials and Methods.” Transactivation of PTH-rP promoter was significantly less in MDAsrc295 cells than MDA-231EV cells (Fig. 7c). In contrast, MDAsrc527 cells showed up-regulated PTH-rP promoter activity. Furthermore, herbimycin A suppressed PTH-rP promoter activity in a dose-dependent manner (Fig. 7d), whereas tyrphostin, an inhibitor of epidermal growth factor receptor TK, showed no effect. These data suggest that c-src TK regulates PTH-rP production in MDA-231 cells at transcriptional levels.

DISCUSSION

Bone is one of the commonest target sites of distant metastasis in breast cancer (12). However, the molecular mechanism by which breast cancer preferentially spreads to bone is poorly understood. In the present study, we examined whether c-src TK plays a specific role in bone metastasis in breast cancer. Our results demonstrate that the mutant c-src with constitutively active TK (src527) enhances and the mutant c-src with little TK activity (src295) decreases the development of osteolytic bone metastases in MDA-231 cells, respectively. Furthermore, we also found that a clone of MDA-231 cells that exclusively and aggressively metastasized to bone (MDA-231BO cells) exhibited increased c-src TK activity compared with parental MDA-231 cells. These results suggest that c-src TK activity is positively correlated with the capacity to develop bone metastases in the MDA-231 human breast cancer cells. However, because our data also show that MDAsrc295 cells develop reduced lung metastases compared with MDAsrcEV cells, it is likely that c-src TK activity does not play a specific role in bone metastases but affects nonbone metastases as well.

Modulation of distant metastases of MDA-231 cells by c-src TK activity is largely attributable to changes in the cell growth. Histological examination revealed increased mitosis in MDAsrc527 cells compared with MDA-231EV, MDAsrcWT, and MDAsrc295 cells in bone metastases. MDAsrc527 cells formed much larger tumors than MDAsrc295 in the orthotopic mammary fat pad. Furthermore, MDAsrc527 cells showed increased anchorage-independent growth compared with other MDA-231 cells in soft agar. Consistent with our data, previous studies have reported that microinjection of a neutralizing anti-Src/Fyn/Yes antibody into mouse fibroblasts at G2 phase blocks subsequent cell division (30) and that the expression of antisense cDNA for c-src decreases the growth of colon cancer cells in vitro and in vivo (33). Chk, which down-regulates c-src TK activity, has been shown to inhibit the growth of the human breast cancer cell line MCF-7 in vitro and in vivo (34). Taken together, it is suggested that increased osteolytic bone metastases of MDAsrc527 cells are attributable to the stimulation of cell proliferation in bone. Conversely, reduced lung metastases in MDAsrc295 cells are likely attributable to decreased cell proliferation.

Elevated production of PTH-rP, which is a powerful stimulator of osteoclastic bone resorption, also significantly contributes to increased bone metastases in MDAsrc527 cells. Accumulating clinical (35–37) and experimental data, including ours (15, 17, 38), has shown that PTH-rP plays an important role in the development of osteolytic bone metastases in breast cancer. Our results show increased PTH-rP production in MDA-231src 527 cells and reduced PTH-rP production in MDAsrc295 cells. Furthermore, our data also demonstrate that activation of PTH-rP gene promoter is controlled with c-src TK activity, which is consistent with an earlier report (31). Taken together, it is suggested that c-src TK stimulates PTH-rP production at transcriptional levels in MDA-231 breast cancer cells, which in turn activates osteoclastic bone resorption, leading to the progression of osteolytic bone metastases.

The mechanism by which c-src TK promotes PTH-rP production is currently unknown. Recent studies have reported that PTH-rP production in MDA-231 cells is increased by transforming growth factor-β through an activation of cytoplasmic signaling pathways, including Smad and MAP kinases (39), and that there is a cross-talk between c-src and MAP kinase (40). It is, therefore, possible that c-src TK stimulates PTH-rP production in MDA-231 cells through establishing interactions with MAP kinase signaling pathways.

The substrates for c-src TK that are involved in bone metastasis of MDA-231 cells were not determined in this study. It is notable that amplified expression of a cytoskeletal protein cortactin/EMS1, which is a relatively specific substrate for c-src TK (41–43), has been reported to be correlated with increased metastases (44) and poor survival in breast cancer patients (45, 46). Moreover, cortactin has been recently found to increase bone metastasis of MDA-231 cells in the same animal model as described here (47). Thus, cortactin/EMS1 is a likely candidate for a substrate for c-src TK that plays a role in bone metastasis in breast cancer.

It should be noted that c-src TK is known to be essential for osteoclasts to resorb bone (48, 49). Osteoclasts play a central role in the development and progression of osteolytic bone metastases. These facts and our result that c-src TK activity is critical to bone metastasis in breast cancer cells collectively raise the possibility that inhibition of c-src TK in both osteoclasts and breast cancer cells suppresses bone metastases in an additive fashion (32, 50). Our results provide a support for this notion.

In conclusion, we have shown that c-src TK activity modulates the capacity of MDA-231 human breast cancer cells to colonize in bone at transcriptional levels.
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and lung. Although extensive clinical studies are needed to clarify whether this is also the case in metastases in breast cancer patients, our results suggest that c-src TK is a potential molecular target for designing specific therapeutic interventions for the treatment of distant metastases, particularly bone metastases, in breast cancer.

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