Cancer Cell
Article

Latent Bone Metastasis in Breast Cancer Tied to Src-Dependent Survival Signals

Xiang H.-F. Zhang,[1,6] Qiongqing Wang,[1,6] William Gerald,[2] Clifford A. Hudis,[3] Larry Norton,[3] Marcel Smid,[4] John A. Foekens,[4] and Joan Massagué[1,5,*]
[1]Cancer Biology and Genetics Program
[2]Department of Pathology
[3]Department of Medicine
Memorial Sloan-Kettering Cancer Center, 1275 York Avenue, New York, NY 10021, USA
[4]Department of Medical Oncology, Erasmus Medical Center Rotterdam, Josephine Nefkens Institute and Cancer Genomics Centre, 3015 GE Rotterdam, the Netherlands
[5]Howard Hughes Medical Institute, Chevy Chase, MD 20815-6789, USA
[6]These authors contributed equally to this work
*Correspondence: massaguj@mskcc.org
DOI 10.1016/j.ccr.2009.05.017

SUMMARY

Metastasis may arise years after removal of a primary tumor. The mechanisms allowing latent disseminated cancer cells to survive are unknown. We report that a gene expression signature of Src activation is associated with late-onset bone metastasis in breast cancer. This link is independent of hormone receptor status or breast cancer subtype. In breast cancer cells, Src is dispensable for homing to the bones or lungs but is critical for the survival and outgrowth of these cells in the bone marrow. Src mediates AKT regulation and cancer cell survival responses to CXCL12 and TNF-related apoptosis-inducing ligand (TRAIL), factors that are distinctively expressed in the bone metastasis microenvironment. Breast cancer cells that lodge in the bone marrow succumb in this environment when deprived of Src activity.

INTRODUCTION

Distant metastasis is the final and most threatening stage of malignant tumor progression, but its clinical course and biological features vary extensively depending on the type of cancer. One of the most striking variables in metastasis is its latency. In some types of cancer, metastasis occurs soon after a primary tumor develops, whereas in others it emerges years or even decades after the primary tumor is removed (Karrison et al., 1999; Schmidt-Kittler et al., 2003; Weckermann et al., 2001). During such long latency periods the disseminated cancer cells survive maladapted to the host organ microenvironment, either in a dormant state or as indolent micrometastases, until they eventually meet all the requirements for metastatic outgrowth. The long-term permanence of latent metastatic disease implies the existence of mechanisms that keep the disseminated cancer cells alive. The identification of mechanisms that support the survival of disseminated cancer cells in their host microenvironments has clear implications for understanding the biology and improving the treatment of latent metastatic disease.

Latent metastasis leading to late-onset relapse occurs frequently in breast cancer. Nearly one-third of cases of relapse emerge after 3 years of breast cancer diagnosis, with some cases emerging several decades later (Karrison et al., 1999; Schmidt-Kittler et al., 2003). A majority of these late-onset metastases arise in bone and may subsequently appear in other organs. Bone metastases from breast cancer are typically osteolytic, involving the mobilization of osteoclasts that cause pathological bone resorption, with intense pain, bone fractures, nerve compression, and hypercalcemia (Mundy, 2002). The development and osteolytic nature of these lesions are based on complex interactions between cancer cells and bone marrow

SIGNIFICANCE

We sought to identify signaling pathways that support the survival of disseminated breast cancer cells and thereby extend the period during which metastasis may emerge after the diagnosis and removal of a breast tumor. Based on bioinformatics analyses, we discovered a strong association between late-onset bone metastasis and Src activity in a cohort of over 600 breast cancer patients. We further demonstrated that Src supports cancer cell survival in the bone marrow microenvironment by facilitating CXCL12-CXCR4-AKT signaling and by conferring resistance to TRAIL. The link between Src-dependent signaling and metastatic cell survival provides mechanistic insights into metastasis latency, and suggests strategies to hasten the attrition of disseminated breast cancer cells.

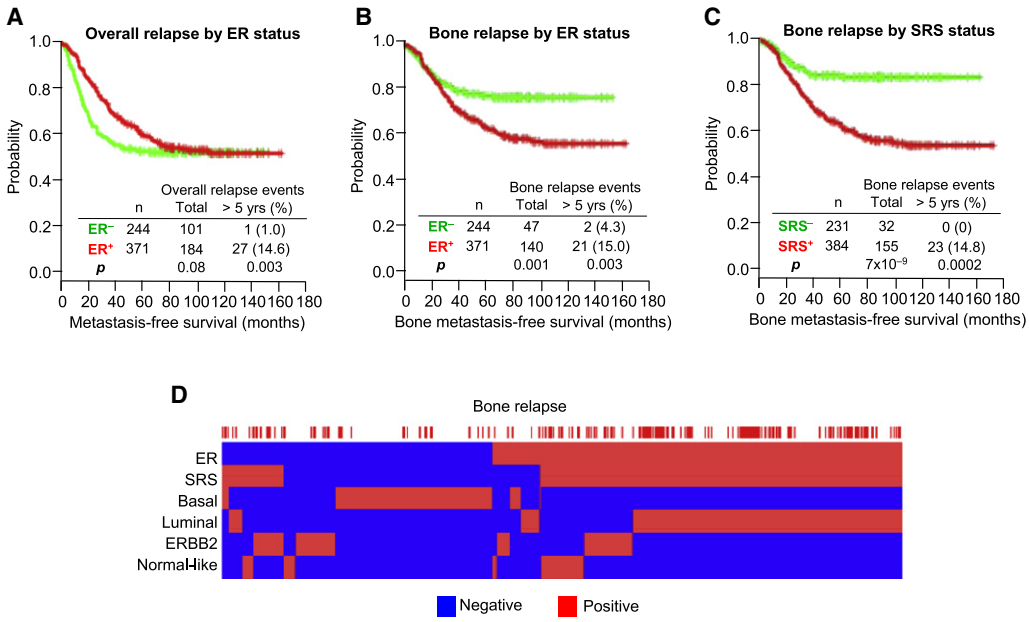

Figure 1. Src Responsive Signature in Breast Tumors Is Associated with Bone Metastasis

(A) Kaplan-Meier representation of the probability of cumulative overall metastasis-free survival in 615 breast cancer cases according to the estrogen receptor α (ER) status. The numbers of tumors in each category, the total metastasis events, the late-onset (>5 yrs) metastasis events, and the corresponding p values (log rank test) are shown in the embedded table.

(B) Same as in (A), but bone metastasis-free survival.

(C) Same as in (B), but cases are categorized according to their SRS status.

(D) Hierarchical clustering of 615 primary tumors with known bone metastasis outcomes by ER status, SRS status, and molecular subtypes. Red marks above the heat map indicate tumors that develop bone metastasis.

stroma in a cycle of bone destruction and tumor expansion. The complexity of cellular interactions and molecular components implicated in bone metastasis has hindered a mechanistic elucidation of key biological features of this process, in particular the basis for long-term survival of metastatic cells in the bone marrow (Husemann et al., 2008; Stoecklein et al., 2008).

Src is the prototypic member of a nonreceptor tyrosine kinase family. Its action on mammalian cells is broadly pleiotropic, including effects on cell morphology, adhesion, migration, invasiveness, proliferation, differentiation, and survival. Src participates in the activation of various downstream pathways through molecular interactions with growth factor receptors (EGFR, HER2), integrin cell adhesion receptors, steroid hormone receptors, G protein-coupled receptors, focal adhesion kinase, and cytoskeleton components (Bromann et al., 2004; Ishizawar and Parsons, 2004). Aberrant expression and activation of Src occurs in several tumor types and has been correlated with poor outcome, which has stimulated interest in Src kinase inhibitors as therapeutic agents in cancer, some of which have entered clinical trials (Finn, 2008; Rucci et al., 2008; Summy and Gallick, 2006). This knowledge notwithstanding, studies to date have not pinned specific roles of Src in particular aspects of tumor development in humans. Gene expression profiling techniques that can register the activity of signaling pathways provide new tools to search for links between specific pathways and defined steps of tumor progression (Bild et al., 2006; Klapholz-Brown et al., 2007; Padua et al., 2008). Using this approach, we are reporting here on an association between Src pathway activity and late-onset bone metastasis.

RESULTS

Src Activity in Breast Tumors Is Associated with Late-Onset Bone Metastasis

To investigate the association of pathway-specific gene expression signatures with breast cancer outcome we assembled a combined cohort of 615 breast tumors for which genome-wide gene expression data was available. Approximately two-thirds of these cases were lymph node negative at diagnosis, and were not subject to adjuvant therapy after primary tumor resection (Wang et al., 2005; Yu et al., 2007). The rest of cases were lymph node positive or received adjuvant hormonal therapy and/or chemotherapy (Table S1). Among the 615 cases, 43% relapsed, and the median metastasis-free survival time was 22.1 months. ER^+ cases had a longer disease-free interval and a lower rate of recurrence during the first 20 months compared with the ER^- cases (Figure 1A). The rate of late-onset metastasis (defined as relapse >5 years after cancer diagnosis) was significantly higher in ER^+ cases. The overall incidence of metastasis in ER^+ and ER^- cases merged over time. These characteristics fit with the typical course of breast cancer progression (EBCTCG, 2005; Hess et al., 2003).

ER^+ breast tumors relapse most prominently to the bones over a protracted period (Hess et al., 2003). Indeed, the overall rate of bone metastasis and the rate of late-onset bone metastasis in the 615 breast cancer patients were significantly higher in ER^+ cases than in ER^- cases (Figure 1B). Reasoning that this metastatic latency may depend on a particular cell survival pathway, we

Table 1. Association of Pathway Gene Expression Signatures with Metastasis in Breast Cancer

	Bone	Lung	Brain	All
Src (SRS)	**<1e-6**	0.98	0.54	0.62
H-Ras	**0.04**	**0.03**	0.674	**0.05**
E2F3	0.09	0.52	0.84	***0.04***
β-Catenin	0.06	0.98	0.22	0.32
Myc	0.42	0.1	0.07	0.36
TGFβ	0.27	**0.008***	0.80	**0.02**
TCF4/WNT	0.19	0.11	**0.02**	0.15

The activity of seven signaling pathways was assessed in a cohort of 615 human breast tumors based on previously defined gene expression signatures (Bild et al., 2006; Padua et al., 2008; van de Wetering et al., 2002). Correlations with site of metastasis were determined using a Cox hazard ratio regression model or by log rank test. Bold numbers indicate correlations that reach statistical significance. Italic numbers indicate a negative correlation (better prognosis).

*The TGFβ response signature (TBRS) is associated with lung relapse specifically in ER$^-$ tumors (p = 0.002 within the ER$^-$ subset in this cohort; Padua et al., 2008).

employed gene expression bioinformatics classifiers that register the activity of specific pathways, including the Src, H-Ras, E2F3, Myc, β-catenin (Bild et al., 2006), TCF/Wnt (van de Wetering et al., 2002), and TGFβ pathways (Padua et al., 2008). Notably, a gene expression signature that denotes Src activity (designated as Src-responsive signature, SRS) was tightly associated with overall bone metastasis and late-onset of bone metastasis (Figure 1C). Bone relapse was more closely associated with SRS$^+$ status than with ER$^+$ status ($p = 7 \times 10^{-9}$ versus p = 0.001 for overall bone relapse; p = 0.0002 versus p = 0.003 for late-onset metastasis, log rank test). When we repeated the analysis after excluding patients who died from other metastases, SRS status was still strongly associated with overall bone metastasis ($p = 6.4 \times 10^{-6}$) and late-onset bone metastasis (p = 0.0037). The only other pathway that showed an association with bone relapse was the H-Ras pathway, but this association was marginal (p = 0.04; Table 1). The TGFβ response signature in primary tumors was associated with lung but not bone relapse (Table 1), as reported (Padua et al., 2008). Breast tumors comprise a heterogeneous mix that includes several molecularly distinct subtypes (Sorlie et al., 2003). All of these subtypes were represented among the SRS$^+$ tumors (Figure 1D).

A majority (88.4%) of ER$^+$ tumors in this cohort scored as SRS$^+$ (Figure 1C; Table S2). Indeed, Src and ERα engage in molecular interactions that lead to nontranscriptional effects (Collins and Webb, 1999; Ishizawar and Parsons, 2004). However, 23.0% of ER$^-$ tumors also scored as SRS$^+$, providing us with an opportunity to discern whether Src activity is specifically linked to bone metastasis independent of ER$^+$ status. The ER$^-$ status of these tumors was confirmed based on microarray ERα probe signal (Figure S1). Because the vast majority of bone relapses in ER$^-$ cases occur before 5 years, we decided to use a less stringent >3-year cutoff to define ER$^-$ bone metastases that occur relatively late. Indeed, a multivariate analysis based on the Cox proportional hazard model indicated that the hazard ratio of SRS$^+$ patient starts to dramatically increase 3 years after tumor resection, and remains at comparable levels at 4-year and 5-year cutoffs (Figure 2A). We therefore decided to use 3 years as the cutoff for late-onset bone metastasis in the particular case of ER$^-$ patients. Compared with ER$^-$SRS$^-$ tumors, the ER$^-$SRS$^+$ tumors showed a higher incidence of bone metastasis and late-onset bone metastasis (p = 0.003, Figures 2B and 2C, log rank test). ER$^-$SRS$^+$ cases developed bone metastasis with rate and kinetics that were similar to those of ER$^+$SRS$^+$ cases (Figures 2B and 2C). Conversely, when the analysis was restricted within the SRS$^+$ or SRS$^-$ subsets, ER status was no longer correlated with bone relapse (p = 0.33 and p = 0.69 for SRS$^-$ and SRS$^+$ groups, respectively; Figure 2B).

Multivariate analyses showed that the association of SRS$^+$ status with bone relapse and/or late-onset bone relapse was independent of progesterone receptor (PR) status, HER2 status, molecular subtypes, genomic grade index, and differentiation (Sotiriou et al., 2006; Tables S3–S5). A subset of the patients was subject to adjuvant therapies (Table S6), but a multivariate analysis showed that SRS associates with bone metastasis independently of either hormonal or chemotherapy (Table S7).

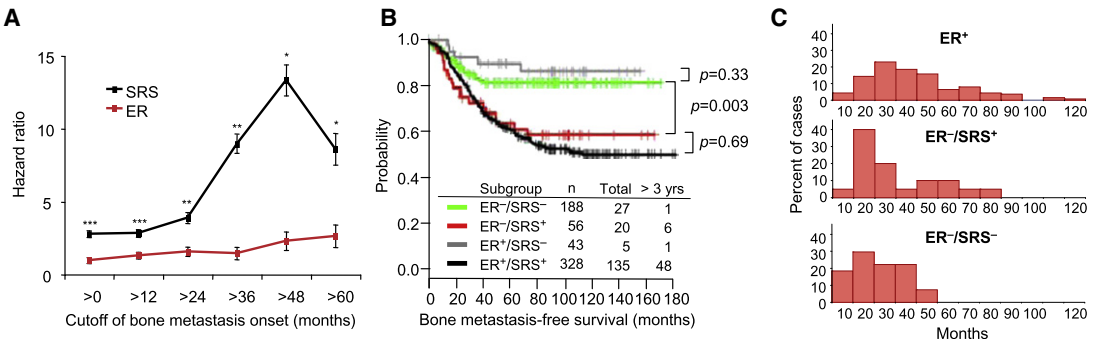

Figure 2. SRS Defines a Subset of ER$^-$ Patients that Develop Delayed Bone Metastases

(A) Hazard ratios of SRS status and ER status on late-onset bone metastasis when these parameters are fit into a bivariate Cox proportional hazard regression model. Various cutoffs from 0 to 5 years were used to define late-onset bone metastasis. Error bars and standard error as determined by the statistical model. p values assess the significance of the hazard ratio's difference from 1 (no prognostic value). *p < 0.05; **p < 0.001; ***$p < 10^{-5}$.

(B) Kaplan-Meier representation of bone metastasis-free survival. Tumors are categorized according to both ER status and SRS status. p values based log rank tests.

(C) Histograms of bone metastasis onsets in the indicated categories of tumors.

No correlation was observed between SRS status and lung or brain relapse (Table S8). An association between SRS status and liver metastasis was of marginal significance ($p = 0.035$) and limited to ER^+ patients. For some patients, metastases occurred to multiple organs, which were diagnosed at the same time. These patients were scored for each of the corresponding sites in our analyses. This potentially confounding factor made little impact to the current study, because fewer than 20% of the patients with late-onset bone metastases also developed metastases to other sites. Collectively, these observations suggest a specific link between SRS^+ status and late-onset relapse to bone in breast cancer.

Src Selectively Promotes Bone Metastasis

Because ER^-SRS^+ breast tumors indicated an ER-independent association between Src activity and bone metastasis, we sought to identify a suitable ER^-SRS^+ metastatic model system to further investigate this link. MDA-MB-231 (MDA231 for short) is a cell line derived from the pleural fluid of an ER^- breast cancer patient with disseminated disease. MDA231 is a basal subtype, triple-negative (ER^-, PR^-, $HER2^-$) cell line (Neve et al., 2006). By in-vivo selection, we previously isolated MDA231 cell populations with distinct metastatic organ tropisms in mice (Kang et al., 2003; Minn et al., 2005). Gene expression profiles from these derivatives were subjected to SRS analysis. The parental MDA231 population as well as various bone metastatic and adrenal gland metastatic derivatives showed higher SRS scores than did the lung metastatic and brain metastatic derivatives (Figure 3A; Figure S2). The level of activated (Tyr416-phosphorylated) Src (Thomas and Brugge, 1997) was higher in representative bone and adrenal metastatic MDA231 derivatives than in lung and brain metastatic derivatives (Figure 3B).

To test the role of Src activity in bone metastasis, we stably reduced the level of endogenous Src using a shRNA vector in the MDA231 bone metastatic derivative BoM-1833 (Kang et al., 2003). shRNA-mediated knockdown reduced the level of endogenous Src and of activated Src by >90% (Figure 3C). We also generated a Src-rescued BoM-1833 line expressing this shRNA and the chicken Src gene, which is resistant to the shRNA. On injection of 3×10^4 cells into the left ventricle, all mice in the BoM-1833 control group were dead with bone metastasis within 70 days. Knockdown of Src extended survival by ~25 days, whereas Src rescue reversed this gain (Figure 3D).

We monitored the kinetics of emergence of hind-limb bone lesions by quantitative bioluminescence imaging (BLI) of luciferase activity from a stably integrated vector. The control, Src knockdown, and Src-rescued BoM-1833 cells localized to the bones and initiated outgrowth at similar rates for up to 14 days (Figures 3E and 3F). However, from day 21 after injection, the rate of outgrowth of the bone lesions in the Src knockdown group decreased, becoming 10-fold lower than that of the control groups (Figure 3F). This difference was accompanied with a sharp reduction in the extent of the hind-limb osteolytic lesions, as determined by X-ray imaging and histological examination (Figure 3E). Knockdown of two other Src family kinases, Fyn and Yes, did not inhibit the bone metastatic activity in BoM-1833 cells (Figure S3).

Src knockdown did not decrease the intrinsic proliferative activity of BoM-1833 cells in culture (Figure S4), or their growth as tumors in mammary glands (Figure 3G). Src knockdown had no effect on the limited lung-metastasis activity of these orthotopic BoM-1833 tumors (Figure 3G). Using the highly lung-metastatic MDA231 derivative line LM2-4175 (Minn et al., 2005) we additionally tested the effect of Src knockdown using tail-vein inoculation assays, which test for lung colonization activity (Figure 3H). The knockdown of Src had no effect on lung tumor burden. These observations pointed to a selective and specific role of Src in bone metastasis.

Src Enhances Metastatic Outgrowth in the Bone Marrow Microenvironment

The kinetics of BoM-1833 bone colonization suggested a role of Src in the sustained outgrowth of these colonies. Indeed, when directly inoculated into the bone marrow cavity of tibiae and tracked for tumor outgrowth, the Src-depleted cells were approximately 10-fold less active than control cells, and this inhibition was rescued by Src expression (Figures 4A and 4B). Dasatinib (BMS-354825) is a pharmacological inhibitor of Src family tyrosine kinases (Shah et al., 2004). Administration of dasatinib at 10 mg/kg daily by oral gavage (Kamath et al., 2008) after inoculation of BoM-1833 cells in mice significantly prevented the formation of osteolytic metastases (Figure 4C). An inhibitory effect was observed when dasatinib treatment was started 7 days or 14 days after cancer cell inoculation. Dasatinib had no effect on the lung colonizing activity of LM2-4175 cells (Figure S5).

Dasatinib inhibits various Src family kinases, and Src is functionally important in various cell types in the bone marrow stroma (Abram and Lowell, 2008). To establish the specificity of the dasatinib effect on bone metastasis, we stably expressed a dasatinib-resistant mutant form of Src (Du et al., 2009) in Src knockdown BoM-1833 cells. The bone metastatic activity of BoM-1833 could be inhibited by dasatinib when the Src knockdown was rescued with the wild-type Src but not when it was rescued with the dasatinib-resistant Src mutant (Figure 4D). Thus, genetic and pharmacological evidence argued that Src activity is dispensable for bone marrow seeding by MDA231 cells but is rate limiting for the sustained growth of these colonies.

Src has been implicated in cancer cell proliferation and survival, and in osteoclast differentiation (Horne et al., 2005; Lowell et al., 1996). Lesions formed by Src knockdown BoM-1833 cells contained a high proportion of apoptotic (TUNEL+) cells compared with bone lesions formed by control BoM-1833 cells, whereas this proportion in Src-rescued BoM-1833 lesions was low and comparable to that in the controls (Figure 4E; Figure S6). Administration of dasatinib also induced a high level of TUNEL+ cells. By contrast, Src knockdown and dasatinib treatment had no effect on the proportion of proliferating (Ki67+) cancer cells (Figure 4F), or of tartrate-resistant acid phosphatase (TRAP+) osteoclasts located at the tumor-bone matrix interface (Figures 4G and 4H). These results suggest that Src supports the aggressive outgrowth of BoM-1833 cells by promoting their survival in the bone marrow microenvironment.

Src Supports Survival of Indolent Breast Cancer Cells in Bone Marrow

Because BoM-1833 cells are a model of acute bone metastatic colonization, we also tested the role of Src in a more indolent

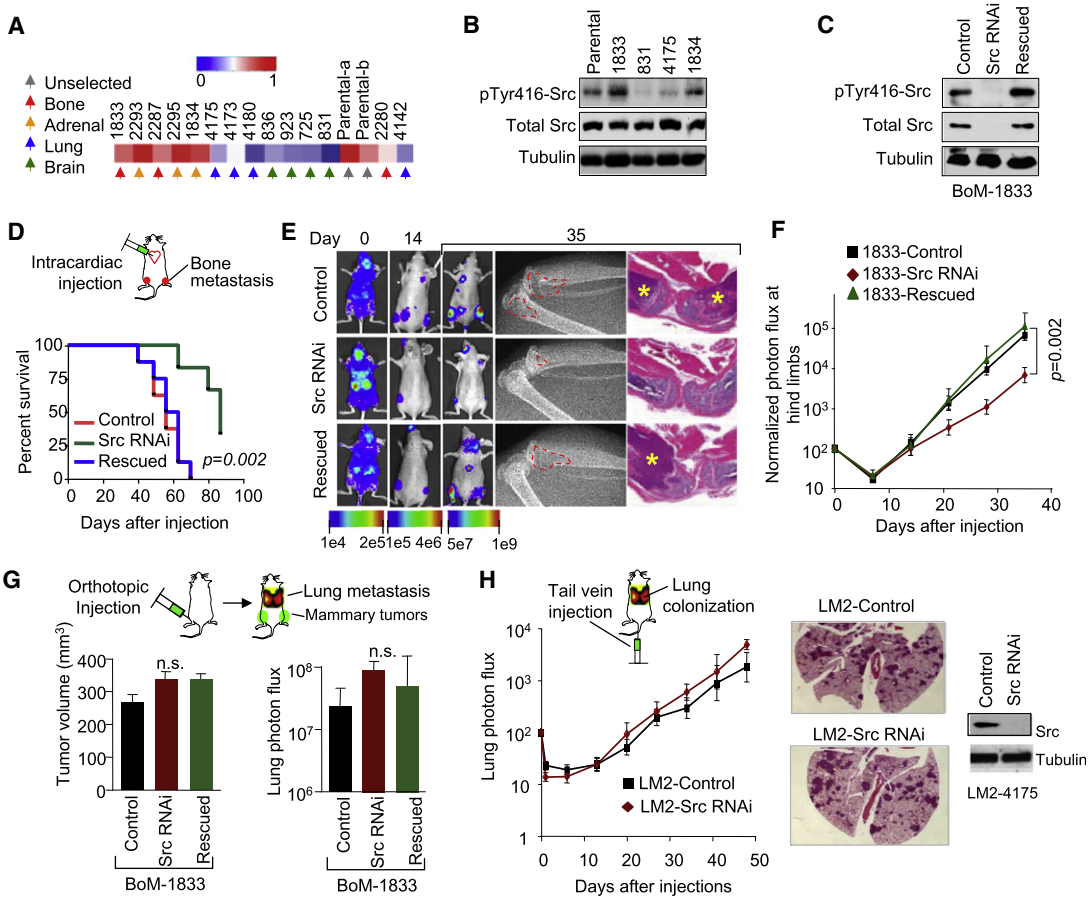

Figure 3. Src Selectively Promotes Bone Metastasis

(A) Metastasis organ-tropism and SRS activity of in vivo-selected MDA231 derivatives cell lines. The blue-white-red shows a single value score of SRS activity determined by principal component analysis of the expression of the 159 genes that constitute the SRS. Cell lines were ordered by unsupervised hierarchical clustering with the SRS genes (refer to Figure S2).

(B) Representative MDA231 derivatives were subjected to western immunoblotting with the indicated antibodies. Organ tropism: 1833, bone; 831, brain; 4175, lung; and 1834, adrenal.

(C) Knockdown of Src in BoM-1833 as confirmed by western immunoblotting analysis.

(D) Survival of mice after intracardiac injection (3×10^4 cells) with BoM-1833 transduced with a control vector (Control), a Src shRNA vector (Src RNAi), or Src shRNA and shRNA-resistant Src expression vectors (Rescued). (n = 15–20 per group).

(E) Bioluminescent, radiographic, and H&E analysis of bone lesions from representative mice in each group at the indicated times after inoculation. In the X-ray images, areas of bone lysis are indicated by dotted lines. In the H&E staining, asterisks indicate tumor.

(F) Normalized bioluminescence signal of bone metastases in the hind limbs of mice inoculated with the indicated cell lines. The signal intensities were normalized to day 0, which was set arbitrarily as 100. Data are averages ± SEM. p value calculated using Student's t test with Welch correction.

(G) Left panel: The indicated cell lines (5×10^5 cells) were injected into the cleared fourth mammary fat pad of mice. Tumor sizes were measured at day 35 (n = 7–10 in each group). Right panel: Quantification of lung metastasis burden originated from the orthotopic mammary tumors. Data are averages ± SEM.

(H) LM2-4175 cells (2×10^5 cells) expressing a control vector (LM2 control) or a Src shRNA vector control (LM2-Src RNAi) were injected into the tail vein of mice. Lung colonization was assayed by weekly bioluminescence imaging. Plots show normalized photon flux in the lung over time (n = 5 per group). Representative H&E stained lungs 5 weeks after xenografting are shown. Src knockdown was confirmed by western immunoblotting. Data are averages ± SEM.

model of bone metastatic disease. From the pleural fluid of a breast cancer patient we isolated a malignant cell population, termed CN34, which was ER^- (Figure S7A). CN34 cells transduced with a luciferase/GFP vector and a puromycin-resistant marker yielded one bone lesion after 9 weeks of intracardial inoculation in five mice. Extraction of these cells and expansion in culture, followed by two additional cycles of selection as bone lesions, yielded CN34-BoM2 derivatives. Compared with a CN34-BrM2c derivative that is metastatic to brain but not bone (data not shown), CN34-BoM2 cells showed a higher level of activated Src (Figure 5A). However, compared with BoM-1833 cells, CN34-BoM2 cells grow in the bone marrow at a 100-fold lower rate (Figure 5B). CN34-BoM2 cells are also metastatic to lymph nodes (Figure 5C; Figure S7). Src knockdown CN34-BoM2 cells (refer to Figure 7A) were almost devoid of bone metastatic activity, whereas the Src-rescued counterparts recovered the lost activity (Figure 5B). By contrast, Src knockdown did not inhibit lymph node metastasis (Figure 5C; Figure S7).

To test the effect of Src on survival under even more-indolent conditions, we inoculated mice with a low number (5×10^4) of

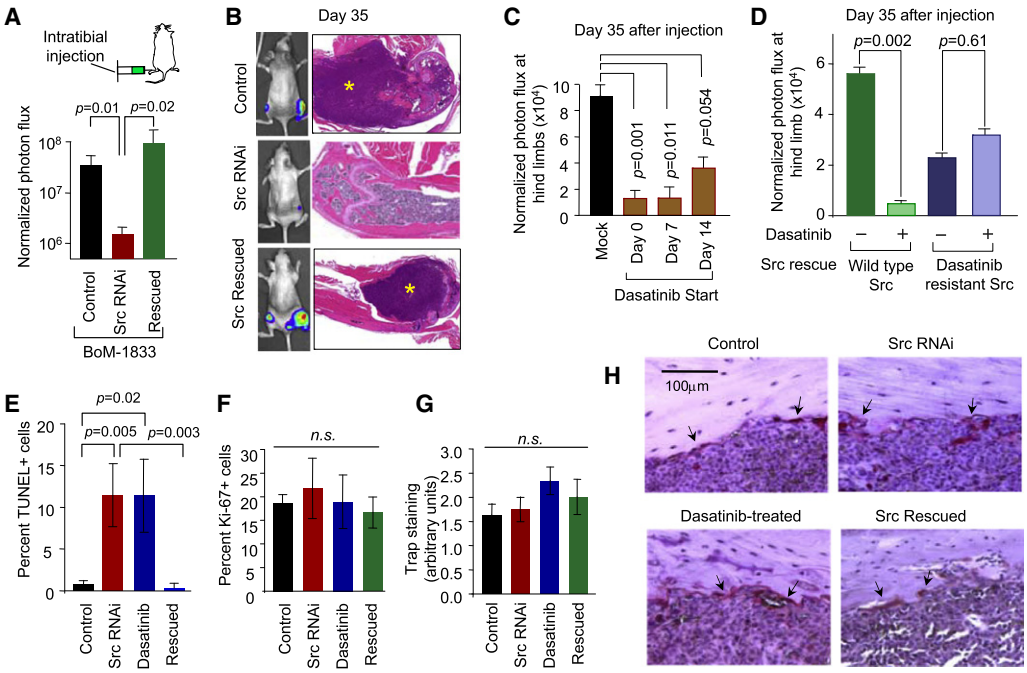

Figure 4. Src Enhances Metastasis Survival and Outgrowth in Bone Marrow

(A) Growth of the indicated cell lines after direct implantation into the marrow of tibia. Quantitative bioluminescence was done at day 35. Averages ± SEM (n = 10 per group).

(B) Representative bioluminescence imaging and H&E staining of bone lesions of each experimental group in (A) at day 35.

(C) BoM-1833 cells were injected into left cardiac ventricle of mice, and animals were treated with vehicle control (mock) or dasatinib (10 mg/kg) daily starting on days 0, 7, or 14 after inoculation. The plot shows quantitative bioluminescence values of the hind limb region at day 35. Data are averages ± SEM (n = 10 per group).

(D) Vectors encoding wild-type Src or dasatinib-resistant mutant Src were used to rescue the expression of Src in BoM-1833 cells that also expressed Src shRNA. On day 7 after intraventricular inoculation, animals were treated with vehicle control or dasatinib. Day 35 average bioluminescence ± SEM (n = 10 per group).

(E) Size-matched femoral metastases from mice inoculated with control, Src knockdown, or Src-rescued BoM-1833 cells, or with BoM-1833 cells and treated with dasatinib (starting on day 7), were extracted on day 35 (control and Src-rescued groups) or day 56 (dasatinib and Src RNAi groups). Samples were subjected to TUNEL staining. Four or more randomly picked fields were quantified, and the percent of TUNEL-positive cells ± SEM is plotted.

(F) The samples in (E) were subjected to staining and quantification of Ki-67 proliferation marker. n.s., not significant. Data are averages ± SEM.

(G) The samples in (E) were TRAP stained to identify presumptive osteoclasts at the tumor-bone matrix interface. Arbitrary units were assigned to represent the proportion of red TRAP-staining cells. n.s., not significant. Data are averages ± SEM.

(H) Representative TRAP staining. Arrows indicate positive staining.

control or Src knockdown CN34-BoM2 cells. No BLI signal developed above background (<10^4 photons per second) in 35 days. The bone marrow was flushed from the femurs of these mice and placed in culture under puromycin selection (Figure 5D). The resulting colonies expressed GFP and were stained positive for human vimentin, confirming that these were the human tumor-derived cells (Figure 5E). Compared with mice inoculated with control CN34-BoM2, mice inoculated with Src knockdown CN34-BoM2 yielded 9-fold fewer bone marrow-derived human cell colonies (Figure 5F). Thus, Src was required for the survival of these indolent breast cancer cells in the bone marrow.

Cell-Survival Factors in the Bone Metastasis Microenvironment

In light of these results, we postulated that Src may be required for the response of breast cancer cells to survival factors provided by the bone marrow microenvironment. To identify relevant factors, we conducted microarray gene expression analysis on a cohort of metastasis samples that were surgically removed from breast cancer patients at Memorial Sloan-Kettering Cancer Center (MSKCC), including 16 metastases from bone, 18 from lung, 19 from brain, and 5 from liver. Analysis of SRS status showed that 62.5% (10/16) of bone metastases were SRS$^+$ (Table S9), in agreement with a report that the majority of breast cancer cells in bone metastases immunostain positive for activated Src (Planas-Silva et al., 2006). Only 28.6% (12/42) of metastases to other sites scored SRS$^+$. Of 260 cytokines and chemokines (compiled by Gene Ontology database, ID GO:0005125) in these samples, we focused on those that are expressed in bone metastases at a higher level (>2-fold) than in metastases from other sites. Seventeen genes met these criteria (Figure 6A). These genes include the chemokine CXCL12/SDF1, which is produced by bone marrow mesenchymal cells and acts as an attractant and survival factor for cells expressing the receptor CXCR4 (Muller et al., 2001). Also included are insulin-like growth factors (IGFs), bone morphogenetic protein-2 (BMP2), and transforming growth factors β (TGFβs), which are stored in the bone matrix and released during osteolysis (Guise et al., 2006; Mundy, 2002). IGFs stimulate survival in various cell types (Stewart and Rotwein,

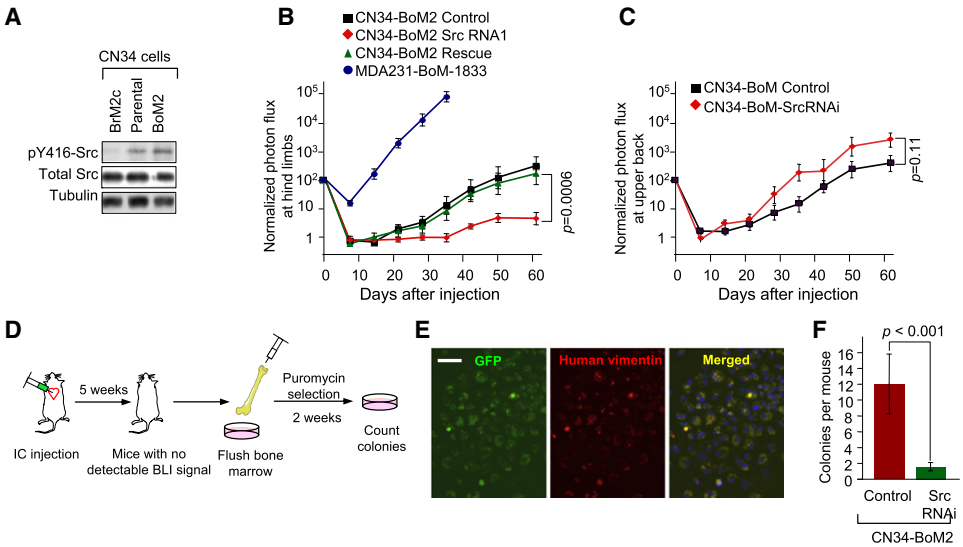

Figure 5. Src Supports Survival of Indolent Breast Cancer Cells in Bone Marrow

(A) Src and activated (Y416 phosphorylated) Src protein levels in parental, bone-tropic (BoM2), and brain-tropic derivatives (BrM2c) of CN34 cells, as determined by western immunoblotting of cell lysates.

(B) Normalized bioluminescence signal intensity at the hind limbs of mice that were intracardially inoculated with the indicated cell lines (1×10^5 cells). Data are averages ± SEM (n = 8–10 per group).

(C) Normalized bioluminescence signal intensity at the upper back region of the same mice, to capture lymph node metastasis signal. Data are averages ± SEM.

(D) Schematic of assay to determine the survival of breast cancer cells in the bone marrow.

(E) Samples (5×10^4 cells) were intracardially injected in 7-week-old mice. Surviving tumor cells were extracted and grown as shown in (D). Images show immunofluorescence staining of representative colonies with the indicated antibodies. Scale bar, 50 μm.

(F) Quantification of latency-derived human breast cancer cell colonies. Data are averages ± SEM (n = 3 per group).

1996), whereas BMPs and TGFβs are implicated in the generation of osteoblasts and osteoclasts, respectively (Chen et al., 2004). Other factors of interest that were selectively expressed in bone metastasis tissues include platelet-derived growth factor-A (PDGF-A), placenta growth factor (PGF), and vascular-endothelial growth factor-C (VEGF-C), which are potential survival and microenvironment-modifying factors, and the proapoptotic member of the tumor necrosis factor family TRAIL (TNF-related apoptosis-inducing ligand; encoded by *TNFSF10*) (Ashkenazi, 2002).

To determine whether a similar profile of cytokines is present in our experimental model, BoM-1833 cells were injected in high

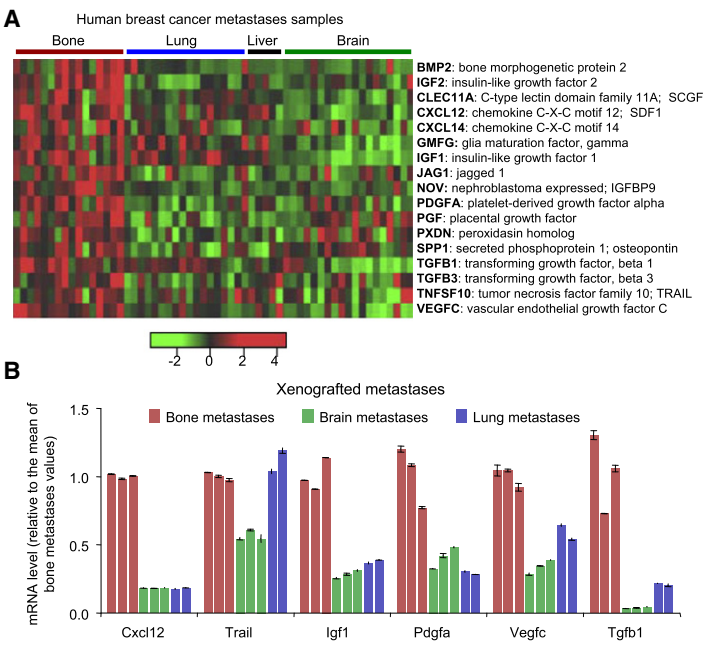

Figure 6. Cytokines of the Bone Metastasis Microenvironment

(A) Microarray gene expression analysis of 58 human breast cancer metastasis samples revealed 17 cytokines whose expression was upregulated in a majority of bone metastases but not in a majority of metastases to lung, liver, or brain.

(B) Cytokine expression in metastases from mice inoculated with MDA231 cells that are metastatic to bones, brain, or lungs. Lesions were extracted from mice on day 28 and qRT-PCR was performed for mouse genes encoding Cxcl12, Trail, Igf1, Pegfa, Vegfc, and Tgfb1. Expression levels relative to the mean of the values in bone metastasis are shown for each sample. Data are averages ± SEM.

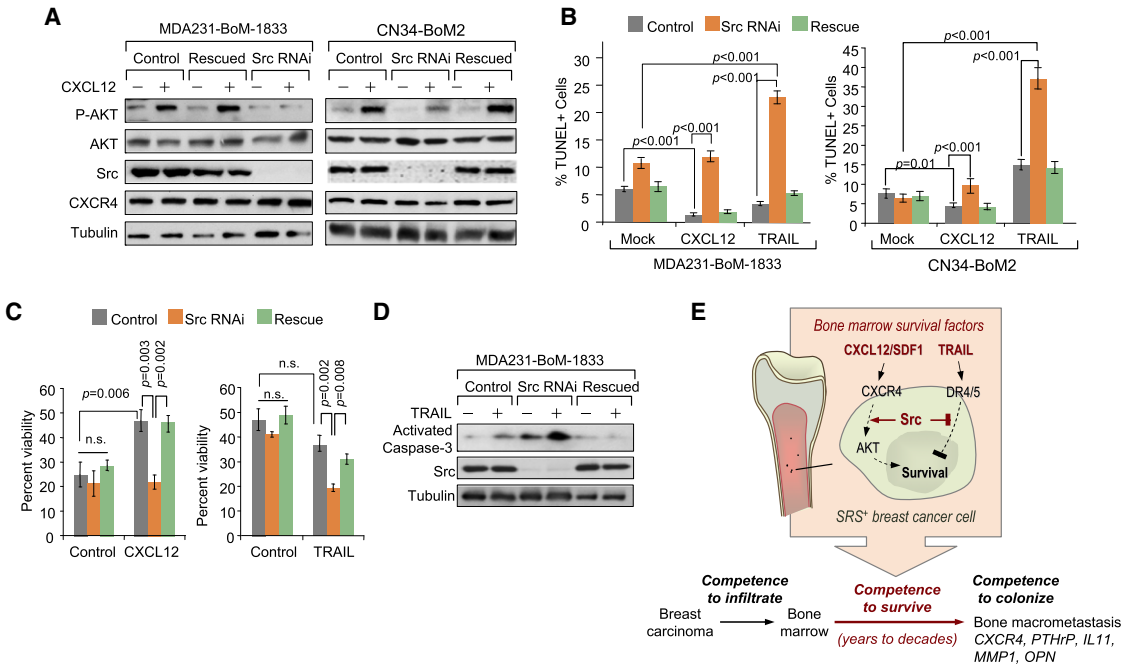

Figure 7. Src Mediates CXCL12-Dependent Survival and Resistance to TRAIL Death Signals in Breast Cancer Cells

(A) The indicated cell lines were incubated with or without CXCL12 for 30 min. Immunoblots using the indicated antibodies were performed on whole-cell extracts. P-AKT, phosphorylated (activated) AKT.

(B) TUNEL assays were performed 3 days after culturing the indicated cell lines in serum-free medium supplemented with CXCL12 or TRAIL. Data are averages ± SEM (n = 6).

(C) Cell viability assays were performed by culturing BoM-1833 cells in serum-free medium supplemented with CXCL12 (for 5 days) or TRAIL (for 3 days). Data are averages ± SEM (n = 6).

(D) Immunoblots using the indicated antibodies were performed on whole-cell extracts from the indicated BoM-1833 derivatives after incubation with or without TRAIL in the media.

(E) Upper panel: Schematic representation of the role of Src in the survival of breast cancer cells that infiltrate the bone marrow. Breast tumors that disseminate Src-activated cancer cells have an advantage for long-term survival in the bone marrow microenvironment. Src influences the responsiveness of breast cancer cells to specific bone metastasis microenvironment factors, CXCL12, and TRAIL. CXCL12 (also known as SDF1) binding to its receptor CXCR4 triggers AKT activation, and we show that Src is required for this activation and its associated prosurvival effects. Src activity is also required for the resistance of breast cancer cells to the cell-death effect of TRAIL. Lower panel: Schematic representation of the course of breast cancer metastasis. After disseminated from the primary tumors, cancer cells may infiltrate different organs. Disseminated cancer cells may survive in the form of latent disease for decades before eventually gaining competence to outgrow and colonize the host tissue through the production of the osteoclastogenic factors.

numbers (5×10^5 cells) to exploit their residual lung and brain metastatic activity of this cell line in addition to the bone metastatic activity. qRT-PCR assays of bone, brain, and lung metastases formed by BoM-1833 xenografted in mice confirmed that CXCL12, IGF1, PDGF-A, VEGF-C, and TGFβ1 were more abundantly expressed in bone metastases than in metastasis to brain or lungs (Figure 6B). TRAIL was expressed at a higher level in bone and lung metastases than in brain metastases.

Src Mediates CXCL12-Dependent Survival and Resistance to TRAIL Death Signals

CXCL12 signaling through CXCR4 activates several signaling pathways, including AKT (Epstein, 2004). Src has been independently implicated in the activation of the cell-survival AKT kinase (Lu et al., 2003). CXCR4 is consistently expressed at a high level in our cell line systems and in a majority of human bone metastases (Kang et al., 2003). Indeed, the addition of CXCL12 to BoM-1833 and CN34-BoM cells under serum-free conditions caused AKT activation, and this activation was diminished by Src knockdown cells and restored by rescuing Src expression (Figure 7A). When cultured in serum-free medium, BoM-1833 and CN34-BoM cells undergo apoptosis after 2 days (BoM-1833) or 5 days (CN34-BoM). CXCL12 significantly reduced apoptosis under these conditions, whereas Src depletion prevented this prosurvival effect (Figure 7B). Concordantly, CXCL12 enhanced cell viability in a Src-dependent manner (Figure 7C). Among the other cytokines enriched in the bone metastasis samples, IGF1 induced Src-independent AKT activation (Figure S8) but Src-dependent survival (data not shown), and TGFβ1, BMP2, PDGF-A, and VEGF-C had little or no effect on either AKT activation or cell survival (Figure S8; data not shown).

Because the proapoptotic cytokine TRAIL was abundantly expressed in bone metastasis tissue, we tested whether Src plays a role in protecting the bone metastatic cancer cells from TRAIL-induced cell death. TRAIL induces caspase activation and apoptosis by binding to cell death receptor DR4 and DR5 (Ashkenazi, 2002). DR5 is expressed in our cell line systems and in bone metastases (Affymetrix HG-U133A raw intensity range 300–600 after MAS5.0 normalization with global scaling

factor set to 500). TRAIL addition caused a small increase in the level of activated caspase-3 (Figure 7D) and apoptosis in BoM-1833 cells (Figure 7B), and a small decrease in cell viability (Figure 7C). Notably, the knockdown of Src strongly increased the sensitivity of BoM-1833 cells to these proapoptotic effects of TRAIL, and this increase was reversed by the rescue of Src expression (Figures 7B–7D). These results argue that in bone metastatic breast cancer cells, Src is required for AKT activation and cell survival in response to CXCL12, and for resistance to the proapoptotic effect of TRAIL.

DISCUSSION

The present work provides clinical and experimental evidence for a role of Src as a critical mediator of survival signals in breast cancer cells that infiltrate the bone marrow. Src mediates survival responses of breast cancer cells to CXCL12 and TRAIL—factors that are prominently expressed in the bone metastasis microenvironment (Figure 7E). Strategies to suppress the survival of disseminated cancer cells can be envisioned based on these results.

Src Activity Linked Late-Onset Bone Metastasis in Breast Cancer

Using gene expression signatures that denote activation of specific pathways we show that expression of a Src response signature (SRS) in primary breast tumors is associated with late-onset bone metastasis. Among 615 cases that we analyzed, nearly 15% of bone relapses from SRS^+ tumors occurred 5 years after diagnosis, versus none in SRS^- tumors. This association occurs in breast tumors of different subtypes and independently of regional spread or adjuvant therapy. Although most ER^+ breast tumors are SRS^+, and estrogen receptors can interact with Src (Ishizawar and Parsons, 2004), the link of SRS status with bone metastasis is independent of ER status. Nearly one quarter of all ER^- tumors in this cohort are SRS^+, and this subset also shows a high incidence of late bone metastasis. The association of SRS^+ status with bone metastasis is more striking than the association of ER^+ status with bone metastasis, and the same applies to the association with late-onset bone metastasis.

Our evidence suggests a more robust and sustained ability of SRS^+ breast cancer cells to develop bone metastases. A majority of breast cancer bone metastases show activated Src by immunostaining (Planas-Silva et al., 2006), and we show that Src hyperactivity endows breast cancer cells with a superior ability to persist in the bone marrow microenvironment. Although Src has well-characterized effects on cell motility and invasion (Dehio et al., 1995), and the Src knockdown BoM-1833 cells showed decrease in these activities in vitro, no effect of Src on bone metastasis seeding was manifest in vivo. That most ER^+ tumors are SRS^+, and thus may derive a survival advantage in the bone marrow microenvironment, is consistent with previous evidence that interactions with estrogen receptor-α activate Src (Migliaccio et al., 2002). The mechanism for Src activation in ER^- breast cancer cells remains unknown but could involve receptor tyrosine kinases, G protein-coupled receptors, integrins, and focal adhesion kinase (Bromann et al., 2004; Guo and Giancotti, 2004; Ishizawar and Parsons, 2004; Mitra and Schlaepfer, 2006).

Src-Dependent CXCL12 Survival Signals in the Bone Marrow Microenvironment

Our results suggest a cell-autonomous, prosurvival role of Src in breast cancer cells in the bone marrow microenvironment. Analysis of human breast cancer metastasis tissues revealed a group of 17 secreted factors that are selectively expressed in bone metastases but not in lung, liver, or brain metastases. We show that the prosurvival effect of one of these factors, CXCL12, on metastatic breast cancer cells requires Src. CXCL12 signals through the heterotrimeric G protein-coupled receptor CXCR4 (Epstein, 2004), which is clinically and experimentally implicated in breast cancer metastasis to bone (Kang et al., 2003; Liang et al., 2005; Muller et al., 2001). CXCR4 is highly expressed in BoM-1833 and other bone metastatic MDA231 derivatives (Kang et al., 2003) and in clinical bone metastasis samples. CXCR4 (Chinni et al., 2006) and Src (Lu et al., 2003) have been implicated in the activation of the AKT cell survival pathway. Our finding that Src is required for CXCL12 activation of AKT and cell survival in bone metastatic breast cancer cells provides a common thread for these various observations. We additionally show that Src activity is required for resistance of metastatic breast cancer cells to the proapoptotic effects of TRAIL. Through the death receptors and activation of the extrinsic proapoptosis pathway, TRAIL mediates immune surveillance and antitumor cytotoxicity by dendritic cells, monocytes, NK cells, and effector T cells (Ashkenazi and Herbst, 2008). By mediating CXCL12 survival signals and protecting against TRAIL cell death signals, Src provides a cell-autonomous prosurvival function in cancer cells that infiltrate bone marrow.

Bone Marrow Infiltration, Cancer Cell Survival, and Metastatic Colonization

Breast tumors are competent for early dissemination and infiltration of distant organs (Husemann et al., 2008; Stoecklein et al., 2008) but, unlike other types of tumors (e.g., lung adenocarcinoma, pancreatic carcinoma), the disseminated breast cancer cells often lack the competence for immediate metastatic outgrowth (Figure 7E). Years may pass before aggressive bone colonization occurs through the expression of osteoclastogenic factors and other mediators (Guise et al., 2006; Kang et al., 2003; Mundy, 2002; Yin et al., 1999). We suggest that by enhancing the responsiveness of breast cancer cells to bone marrow survival factors, Src extends the endurance of disseminated cancer cells, thereby increasing the probability of acquisition of full metastatic competence (Figure 7E). Other survival mechanisms may support latency in other organs (e.g., the lungs) or in other late-onset metastasis cancers (e.g., prostate carcinoma), although bone-derived secondary metastases to other organs are also possible.

Despite effective systemic treatments, including chemotherapy, hormone therapy, and monoclonal antibody therapy, high-risk subsets of patients continue to have unacceptable rates of relapse in the bone and other organs (Hayes et al., 2007; Hudis, 2007). High-dose chemotherapy in combination with autologous hematopoietic stem-cell support has not shown a benefit in high-risk breast cancer patients compared with conventional standard-dose chemotherapy (Zander et al., 2004). At present, the major clinical benefits from postoperative adjuvant drug therapies are observed in the first few years after

treatment, suggesting that latent cancer cells are at least partially resistant to conventional therapy (Berry et al., 2006). Disseminated cancer cell pools may act as reservoirs that are resistant to such treatments but potentially susceptible to treatments targeting mediators of latent cancer cell survival. Our data raise the possibility that we might be able to use such agents to attack reservoirs of disseminated latent cancer cells, including circulating tumor cells or mitotically dormant tumor cells in various organs. Consistent with this is the fact that current chemotherapy and hormone-directed drugs do not specifically target Src. Recently, however, several agents that target Src pathways have become available for clinical testing (Finn, 2008; Rucci et al., 2008; Summy and Gallick, 2006). This approach could involve the use of Src inhibitors, TRAIL-receptor agonists (Ashkenazi and Herbst, 2008), or other latency-targeted agents either simultaneously with adjuvant drug regimens or afterwards in order to achieve long-term maintenance of disease-free status.

EXPERIMENTAL PROCEDURES

Additional methods for cell culture assays; quantitative real-time PCR; immunoblotting; generation knockdown and overexpression cell lines; details of tumor xenografting; histological analyses; long-term survival assays; and statistical analyses are provided in Supplemental Data.

Primary Tumor and Metastasis Tissue Samples

We compiled a microarray dataset of 615 patients from Memorial Sloan-Kettering Cancer Center (MSKCC) and Erasmus Medical Center (EMC344, EMC189, and MSK82; GEO accession numbers GEO2603, GSE5327, GSE2034, and GSE12276). These datasets were all normalized using MAS5.0, and each microarray was centered to the median of all probes. For each patient, metastasis-free survival (MFS) is defined as the time interval between the surgery and the diagnosis of metastasis.

Archival human breast carcinoma metastasis specimens were obtained and processed in compliance with protocols approved by the MSKCC Institutional Review Board. Samples were snap frozen in liquid nitrogen and stored at −80°C. Each sample was examined histologically using hematoxylin and eosin-stained cryostat sections. Regions were manually dissected from the frozen block to provide consistent tumor cell content of greater than 70% in tissues used for analysis. RNA was extracted from frozen tissues by homogenization in TRIzol reagent (Gibco-BRL, Carlsbad, CA) and evaluated for integrity. Complementary DNA was synthesized from total RNA using a T7-promoter-tagged-dT primer. RNA target was synthesized by in vitro transcription and labeled with biotinylated nucleotides (Enzo Biochem, Farmingdale, NY). Labeled target was assessed by hybridization to Test3 arrays (Affymetrix, Santa Clara, CA). All gene expression analysis was carried out using HG-U133A (for 36 samples) or HG-U133plus2 (for 29 samples) GeneChips. Seven samples were profiled on both platforms, and the data were averaged for the common probes. Gene expression was quantitated using GCOS.

Molecular Pathway Gene Expression Signatures

To predict pathway activation from microarray gene expression data, we used gene expression profiles derived from the overexpression of Src, H-Ras, β-catenin, E2F3, and Myc in quiescent mammary epithelial cells (Bild et al., 2006). We derived a gene signature-expression classifier for each of these pathways using a false discovery rate of 0.05 and a fold change of 1.5 as criteria. The 605 genes that met these thresholds in the Src responsive gene set were filtered by using the EMC-344 data set to eliminate noninformative genes. After eliminating genes that were either expressed at a low level (raw intensity <64 or 2^6 in more than 25% tumors) or nonvariable across samples (SD < 0.8 for log2 intensity, equivalent to the median of all genes), 159 genes remained and constituted the Src responsive signature (SRS) used here.

The SRS was applied to EMC-344 and MSK-82 data sets, which are based on HG-U133A and were combined, and to EMC-189 data set, which is based on HG-U133plus2 and was processed separately. To search for breast cancers with SRS expression pattern similar to the Src-activated mammary epithelial cells (Bild et al., 2006) we performed unsupervised clustering (using "heatmap.2" function in gplots package of R statistical software). Two clusters were consistently revealed by such procedure (R index = 0.85) (McShane et al., 2002). One cluster was identified as SRS^+ based on the criteria that it exhibits gene expression similarity to Src-activated mammary epithelial cells as gauged by positive Pearson's correlation coefficients, "metagene" scores (Bild et al., 2006), and that it enriches ER^+ tumors (Collins and Webb, 1999; Ishizawar and Parsons, 2004). The other cluster showed the opposite characteristics and was denoted as SRS^-.

The same approach was applied for β-catenin, E2F3, H-Ras, and c-Myc pathways. TGFβ pathway was gauged as previously described (Padua et al., 2008). For TCF/Wnt pathway, we performed unsupervised clustering using the dominant negative TCF4 signature (van de Wetering et al., 2002). One of the two clusters significantly overexpresses the vast majority of the dnTCF4 genes, and tumors in this cluster are therefore defined as TCF/Wnt+.

To examine the prognostic value of SRS in different subsets of breast cancers, we divided the breast cancer samples based on their ER status or molecular subtypes. For ER status, we used either published pathological annotations (for GSE2063, GSE5327, and GSE2034) or the intensity of probe "205225_at" (ESR1) on the Affymetrix chip when the pathological status was not available (for GSE12276). We used raw intensity of 1000 as the cutoff to define ER^- versus ER^+. It has been established that this is an appropriate cutoff when the data is normalized with MAS5.0 and the global scaling is set to 600 (Foekens et al., 2006). Molecular subtype classification was done as previously described (Smid et al., 2008) according to published classifiers (Perou et al., 1999; Sorlie et al., 2003). Of note, a nontrivial proportion of luminal tumors cannot be unambiguously determined between luminal A and luminal B subtypes. We therefore merged the two subtypes in some analyses.

Survival analyses were carried out using the "survival" package of *R*. P values were calculated by the "survdiff" command in the package, which is based on log rank tests. When sample size is small, we also performed Fisher's exact test. Kaplan-Meier curves were drawn with the "survfit" command in the same package. The Cox proportion hazard regression analyses were performed using the "coxph" method in the same package.

Cytokine Gene Expression Analysis

We compiled a list of 260 cytokine genes using GO database (cytokine activity entry: GO:0005125). These cytokines were mapped to 404 probes on the Affymetrix HG-U133A platform. We screened these probes in each tissue sample for those whose intensity value was greater than the median of all genes, and statistically overexpressed in bone metastasis samples compared with other metastasis (t test with Welch's correction, $p < 0.05$ with correction for multiple tests).

Tumor Xenografts and Analysis

All procedures involving mice were approved by the MSKCC Institutional Animal Care and Use Committee. Details are provided in Supplemental Data.

Statistical Analysis

Bone metastasis assay in BoM-1833 line was repeated twice (n = 7–10 for each cohort at each time). The results were pooled and shown as Figures 3D–3F. The same result was reproduced independently later in two additional experiments. For the bone metastasis assay of CN34-BoM2 (Figure 5), lung colonization assay (Figure 3H), orthotopic proliferation (Figure 3G), intratibial growth (Figure 4A), and dasatinib treatment (Figures 4C and 4D), one experiment was performed with n = 10–15 in each cohort. Results are reported as mean ± SEM, as indicated in the figure legends. Comparisons between Kaplan-Meier curves were performed using the log rank test. Other comparisons were performed using unpaired two-sided t test without equal variance assumption unless otherwise specified.

ACCESSION NUMBERS

The raw and normalized data of breast cancer metastases have been deposited in the Gene Expression Ominbus (GEO) database under accession number GSE14020.

SUPPLEMENTAL DATA

Supplemental Data include Supplemental Experimental Procedures, eight figures, and nine tables and can be found with this article online at http://www.cell.com/supplemental/S1535-6108(09)00179-2.

ACKNOWLEDGMENTS

This work is dedicated to the memory of our esteemed colleague and coauthor William Gerald. We thank M. Resh, H. Varmus, D. Nguyen, P. Bos, D. Padua, S. Tavazoie, Q. Chen, S. Gao, and Q. Xi for helpful discussions and technical suggestions; T. Faye and M. Ladanyi for clinical annotations; and M. Donepudi and R. Somwar for reagents. This work was supported by grants from the National Institutes of Health (U54 CA126518); the Alan and Sandra Gerry Metastasis Initiative and the Hearst Foundation (J.M.); and the Netherlands Genomics Initiative (NGI)/Netherlands Organization for Scientific Research (M.S. and J.A.F.). J.M. is an investigator at the Howard Hughes Medical Institute.

Received: December 3, 2008
Revised: March 20, 2009
Accepted: May 4, 2009
Published: July 6, 2009

REFERENCES

Abram, C.L., and Lowell, C.A. (2008). The diverse functions of Src family kinases in macrophages. Front. Biosci. *13*, 4426–4450.

Ashkenazi, A. (2002). Targeting death and decoy receptors of the tumour-necrosis factor superfamily. Nat. Rev. Cancer *2*, 420–430.

Ashkenazi, A., and Herbst, R.S. (2008). To kill a tumor cell: the potential of proapoptotic receptor agonists. J. Clin. Invest. *118*, 1979–1990.

Berry, D.A., Cirrincione, C., Henderson, I.C., Citron, M.L., Budman, D.R., Goldstein, L.J., Martino, S., Perez, E.A., Muss, H.B., Norton, L., et al. (2006). Estrogen-receptor status and outcomes of modern chemotherapy for patients with node-positive breast cancer. JAMA *295*, 1658–1667.

Bild, A.H., Yao, G., Chang, J.T., Wang, Q., Potti, A., Chasse, D., Joshi, M.B., Harpole, D., Lancaster, J.M., Berchuck, A., et al. (2006). Oncogenic pathway signatures in human cancers as a guide to targeted therapies. Nature *439*, 353–357.

Bromann, P.A., Korkaya, H., and Courtneidge, S.A. (2004). The interplay between Src family kinases and receptor tyrosine kinases. Oncogene *23*, 7957–7968.

Chen, D., Zhao, M., and Mundy, G.R. (2004). Bone morphogenetic proteins. Growth Factors *22*, 233–241.

Chinni, S.R., Sivalogan, S., Dong, Z., Filho, J.C., Deng, X., Bonfil, R.D., and Cher, M.L. (2006). CXCL12/CXCR4 signaling activates Akt-1 and MMP-9 expression in prostate cancer cells: the role of bone microenvironment-associated CXCL12. Prostate *66*, 32–48.

Collins, P., and Webb, C. (1999). Estrogen hits the surface. Nat. Med. *5*, 1130–1131.

Dehio, C., Prevost, M.C., and Sansonetti, P.J. (1995). Invasion of epithelial cells by Shigella flexneri induces tyrosine phosphorylation of cortactin by a pp60c-src-mediated signalling pathway. EMBO J. *14*, 2471–2482.

Du, J., Bernasconi, P., Clauser, K.R., Mani, D.R., Finn, S.P., Beroukhim, R., Burns, M., Julian, B., Peng, X.P., Hieronymus, H., et al. (2009). Bead-based profiling of tyrosine kinase phosphorylation identifies SRC as a potential target for glioblastoma therapy. Nat. Biotechnol. *27*, 77–83.

EBCTCG. (2005). Effects of chemotherapy and hormonal therapy for early breast cancer on recurrence and 15-year survival: an overview of the randomised trials. Lancet *365*, 1687–1717.

Epstein, R.J. (2004). The CXCL12-CXCR4 chemotactic pathway as a target of adjuvant breast cancer therapies. Nat. Rev. Cancer *4*, 901–909.

Finn, R.S. (2008). Targeting Src in breast cancer. Ann. Oncol. *19*, 1379–1386.

Foekens, J.A., Atkins, D., Zhang, Y., Sweep, F.C.G.J., Harbeck, N., Paradiso, A., Cufer, T., Sieuwerts, A.M., Talantov, D., Span, P.N., et al. (2006). Multicenter validation of a gene expression-based prognostic signature in lymph node-negative primary breast cancer. J. Clin. Oncol. *24*, 1665–1671.

Guise, T.A., Mohammad, K.S., Clines, G., Stebbins, E.G., Wong, D.H., Higgins, L.S., Vessella, R., Corey, E., Padalecki, S., Suva, L., and Chirgwin, J.M. (2006). Basic mechanisms responsible for osteolytic and osteoblastic bone metastases. Clin. Cancer Res. *12*, 6213s–6216s.

Guo, W., and Giancotti, F.G. (2004). Integrin signalling during tumour progression. Nat. Rev. Mol. Cell Biol. *5*, 816–826.

Hayes, D.F., Thor, A.D., Dressler, L.G., Weaver, D., Edgerton, S., Cowan, D., Broadwater, G., Goldstein, L.J., Martino, S., Ingle, J.N., et al. (2007). HER2 and response to paclitaxel in node-positive breast cancer. N. Engl. J. Med. *357*, 1496–1506.

Hess, K.R., Pusztai, L., Buzdar, A.U., and Hortobagyi, G.N. (2003). Estrogen receptors and distinct patterns of breast cancer relapse. Breast Cancer Res. Treat. *78*, 105–118.

Horne, W.C., Sanjay, A., Bruzzaniti, A., and Baron, R. (2005). The role(s) of Src kinase and Cbl proteins in the regulation of osteoclast differentiation and function. Immunol. Rev. *208*, 106–125.

Hudis, C.A. (2007). Trastuzumab–mechanism of action and use in clinical practice. N. Engl. J. Med. *357*, 39–51.

Husemann, Y., Geigl, J.B., Schubert, F., Musiani, P., Meyer, M., Burghart, E., Forni, G., Eils, R., Fehm, T., Riethmuller, G., and Klein, C.A. (2008). Systemic spread is an early step in breast cancer. Cancer Cell *13*, 58–68.

Ishizawar, R., and Parsons, S.J. (2004). c-Src and cooperating partners in human cancer. Cancer Cell *6*, 209–214.

Kamath, A.V., Wang, J., Lee, F.Y., and Marathe, P.H. (2008). Preclinical pharmacokinetics and in vitro metabolism of dasatinib (BMS-354825): a potent oral multi-targeted kinase inhibitor against SRC and BCR-ABL. Cancer Chemother. Pharmacol. *61*, 365–376.

Kang, Y., Siegel, P.M., Shu, W., Drobnjak, M., Kakonen, S.M., Cordon-Cardo, C., Guise, T.A., and Massagué, J. (2003). A multigenic program mediating breast cancer metastasis to bone. Cancer Cell *3*, 537–549.

Karrison, T.G., Ferguson, D.J., and Meier, P. (1999). Dormancy of mammary carcinoma after mastectomy. J. Natl. Cancer Inst. *91*, 80–85.

Klapholz-Brown, Z., Walmsley, G.G., Nusse, Y.M., Nusse, R., and Brown, P.O. (2007). Transcriptional program induced by Wnt protein in human fibroblasts suggests mechanisms for cell cooperativity in defining tissue microenvironments. PLoS ONE *2*, e945.

Liang, Z., Yoon, Y., Votaw, J., Goodman, M.M., Williams, L., and Shim, H. (2005). Silencing of CXCR4 blocks breast cancer metastasis. Cancer Res. *65*, 967–971.

Lowell, C.A., Niwa, M., Soriano, P., and Varmus, H.E. (1996). Deficiency of the Hck and Src tyrosine kinases results in extreme levels of extramedullary hematopoiesis. Blood *87*, 1780–1792.

Lu, Y., Yu, Q., Liu, J.H., Zhang, J., Wang, H., Koul, D., McMurray, J.S., Fang, X., Yung, W.K., Siminovitch, K.A., and Mills, G.B. (2003). Src family protein-tyrosine kinases alter the function of PTEN to regulate phosphatidylinositol 3-kinase/AKT cascades. J. Biol. Chem. *278*, 40057–40066.

McShane, L.M., Radmacher, M.D., Freidlin, B., Yu, R., Li, M.C., and Simon, R. (2002). Methods for assessing reproducibility of clustering patterns observed in analyses of microarray data. Bioinformatics *18*, 1462–1469.

Migliaccio, A., Castoria, G., Di Domenico, M., De Falco, A., Bilancio, A., and Auricchio, F. (2002). Src is an initial target of sex steroid hormone action. Ann. N Y Acad. Sci. *963*, 185–190.

Minn, A.J., Gupta, G.P., Siegel, P.M., Bos, P.D., Shu, W., Giri, D.D., Viale, A., Olshen, A.B., Gerald, W.L., and Massagué, J. (2005). Genes that mediate breast cancer metastasis to lung. Nature *436*, 518–524.

Mitra, S.K., and Schlaepfer, D.D. (2006). Integrin-regulated FAK-Src signaling in normal and cancer cells. Curr. Opin. Cell Biol. *18*, 516–523.

Muller, A., Homey, B., Soto, H., Ge, N., Catron, D., Buchanan, M.E., McClanahan, T., Murphy, E., Yuan, W., Wagner, S.N., et al. (2001). Involvement of chemokine receptors in breast cancer metastasis. Nature *410*, 50–56.

Mundy, G.R. (2002). Metastasis to bone: causes, consequences and therapeutic opportunities. Nat. Rev. Cancer *2*, 584–593.

Neve, R.M., Chin, K., Fridlyand, J., Yeh, J., Baehner, F.L., Fevr, T., Clark, L., Bayani, N., Coppe, J.P., Tong, F., et al. (2006). A collection of breast cancer cell lines for the study of functionally distinct cancer subtypes. Cancer Cell *10*, 515–527.

Padua, D., Zhang, X.H., Wang, Q., Nadal, C., Gerald, W.L., Gomis, R.R., and Massagué, J. (2008). TGFbeta primes breast tumors for lung metastasis seeding through angiopoietin-like 4. Cell *133*, 66–77.

Perou, C.M., Jeffrey, S.S., van de Rijn, M., Rees, C.A., Eisen, M.B., Ross, D.T., Pergamenschikov, A., Williams, C.F., Zhu, S.X., Lee, J.C., et al. (1999). Distinctive gene expression patterns in human mammary epithelial cells and breast cancers. Proc. Natl. Acad. Sci. U.S.A. *96*, 9212–9217.

Planas-Silva, M.D., Bruggeman, R.D., Grenko, R.T., and Stanley Smith, J. (2006). Role of c-Src and focal adhesion kinase in progression and metastasis of estrogen receptor-positive breast cancer. Biochem. Biophys. Res. Commun. *341*, 73–81.

Rucci, N., Susa, M., and Teti, A. (2008). Inhibition of protein kinase c-Src as a therapeutic approach for cancer and bone metastases. Anticancer Agents Med. Chem. *8*, 342–349.

Schmidt-Kittler, O., Ragg, T., Daskalakis, A., Granzow, M., Ahr, A., Blankenstein, T.J., Kaufmann, M., Diebold, J., Arnholdt, H., Muller, P., et al. (2003). From latent disseminated cells to overt metastasis: genetic analysis of systemic breast cancer progression. Proc. Natl. Acad. Sci. U.S.A. *100*, 7737–7742.

Shah, N.P., Tran, C., Lee, F.Y., Chen, P., Norris, D., and Sawyers, C.L. (2004). Overriding imatinib resistance with a novel ABL kinase inhibitor. Science *305*, 399–401.

Smid, M., Wang, Y., Zhang, Y., Sieuwerts, A.M., Yu, J., Klijn, J.G., Foekens, J.A., and Martens, J.W. (2008). Subtypes of breast cancer show preferential site of relapse. Cancer Res. *68*, 3108–3114.

Sorlie, T., Tibshirani, R., Parker, J., Hastie, T., Marron, J.S., Nobel, A., Deng, S., Johnsen, H., Pesich, R., Geisler, S., et al. (2003). Repeated observation of breast tumor subtypes in independent gene expression data sets. Proc. Natl. Acad. Sci. U.S.A. *100*, 8418–8423.

Sotiriou, C., Wirapati, P., Loi, S., Harris, A., Fox, S., Smeds, J., Nordgren, H., Farmer, P., Praz, V., Haibe-Kains, B., et al. (2006). Gene expression profiling in breast cancer: understanding the molecular basis of histologic grade to improve prognosis. J. Natl. Cancer Inst. *98*, 262–272.

Stewart, C.E., and Rotwein, P. (1996). Growth, differentiation, and survival: multiple physiological functions for insulin-like growth factors. Physiol. Rev. *76*, 1005–1026.

Stoecklein, N.H., Hosch, S.B., Bezler, M., Stern, F., Hartmann, C.H., Vay, C., Siegmund, A., Scheunemann, P., Schurr, P., Knoefel, W.T., et al. (2008). Direct genetic analysis of single disseminated cancer cells for prediction of outcome and therapy selection in esophageal cancer. Cancer Cell *13*, 441–453.

Summy, J.M., and Gallick, G.E. (2006). Treatment for advanced tumors: SRC reclaims center stage. Clin. Cancer Res. *12*, 1398–1401.

Thomas, S.M., and Brugge, J.S. (1997). Cellular functions regulated by Src family kinases. Annu. Rev. Cell Dev. Biol. *13*, 513–609.

van de Wetering, M., Sancho, E., Verweij, C., de Lau, W., Oving, I., Hurlstone, A., van der Horn, K., Batlle, E., Coudreuse, D., Haramis, A.P., et al. (2002). The beta-catenin/TCF-4 complex imposes a crypt progenitor phenotype on colorectal cancer cells. Cell *111*, 241–250.

Wang, Y., Klijn, J.G., Zhang, Y., Sieuwerts, A.M., Look, M.P., Yang, F., Talantov, D., Timmermans, M., Meijer-van Gelder, M.E., Yu, J., et al. (2005). Gene-expression profiles to predict distant metastasis of lymph-node-negative primary breast cancer. Lancet *365*, 671–679.

Weckermann, D., Muller, P., Wawroschek, F., Harzmann, R., Riethmuller, G., and Schlimok, G. (2001). Disseminated cytokeratin positive tumor cells in the bone marrow of patients with prostate cancer: detection and prognostic value. J. Urol. *166*, 699–703.

Yin, J.J., Selander, K., Chirgwin, J.M., Dallas, M., Grubbs, B.G., Wieser, R., Massagué, J., Mundy, G.R., and Guise, T.A. (1999). TGF-beta signaling blockade inhibits PTHrP secretion by breast cancer cells and bone metastases development. J. Clin. Invest. *103*, 197–206.

Yu, J.X., Sieuwerts, A.M., Zhang, Y., Martens, J.W., Smid, M., Klijn, J.G., Wang, Y., and Foekens, J.A. (2007). Pathway analysis of gene signatures predicting metastasis of node-negative primary breast cancer. BMC Cancer *7*, 182.

Zander, A.R., Kroger, N., Schmoor, C., Kruger, W., Mobus, V., Frickhofen, N., Metzner, B., Schultze, W., Berdel, W.E., Koenigsmann, M., et al. (2004). High-dose chemotherapy with autologous hematopoietic stem-cell support compared with standard-dose chemotherapy in breast cancer patients with 10 or more positive lymph nodes: first results of a randomized trial. J. Clin. Oncol. *22*, 2273–2283.