Tumor and Stem Cell Biology

Breast Cancer Stem Cells Are Regulated by Mesenchymal Stem Cells through Cytokine Networks

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Abstract

We have used in vitro and mouse xenograft models to examine the interaction between breast cancer stem cells (CSC) and bone marrow–derived mesenchymal stem cells (MSC). We show that both of these cell populations are organized in a cellular hierarchy in which primitive aldehyde dehydrogenase expressing mesenchymal cells regulate breast CSCs through cytokine loops involving IL6 and CXCL7. In NOD/SCID mice, labeled MSCs introduced into the tibia traffic to sites of growing breast tumor xenografts where they accelerated tumor growth by increasing the breast CSC population. With immunochemistry, we identified MSC–CSC niches in these tumor xenografts as well as in frozen sections from primary human breast cancers. Bone marrow–derived MSCs may accelerate human breast tumor growth by generating cytokine networks that regulate the CSC population. Cancer Res; 71(2); 614–24. ©2011 AACR.

Introduction

Many human cancers, including breast cancer, may be driven by a population of cells that display stem cell properties. These properties include self-renewal, which drives tumorigenesis, and differentiation, which contributes to cancer cell heterogeneity. There is increasing evidence that these cancer stem cells (CSC) mediate tumor metastasis and, by virtue of their relative resistance to chemotherapy and radiation therapy, may contribute to treatment resistance and relapse following therapy (1).

Self-renewal and cell fate determination of normal stem cells are regulated by both cell intrinsic and cell extrinsic pathways. The dysregulation of these pathways resulting in stem cell expansion may be a key event initiating carcinogenesis. Developmental pathways such as Notch, Hedgehog, and Wnt play an important role in normal stem cell function and are frequently deranged in cancers (2–5). Extrinsic signals that regulate stem cell behavior originate in the stem cell microenvironment or niche. This niche contains extracellular components as well as multiple cell types.

Although there is little information on the composition and function of CSC niches, it is clear that tumor growth and metastasis is highly dependent on the tumor microenvironment. This microenvironment is composed of tumor-associated fibroblasts, endothelial cells, adipocytes, and immune cells, all of which have been shown to play a role in tumor growth and metastasis (6). Mesenchymal stem cells (MSC), which can be defined as multipotent mesenchymal stromal cells, are a heterogeneous subset of stromal stem cells that can be isolated from many adult tissues; proliferate as adherent cells; have fibroblast-like morphology; form colonies in vitro; and can differentiate into adipocytes, osteocytes, and chondrocytes (7). Recently, through the use of mouse breast cancer models, it has been shown that bone marrow–derived MSCs may be recruited to the sites of developing tumors, thus, influencing their metastatic potential (8). It has been shown that MSCs can produce IL6 (9, 10) and can stimulate tumor growth through the paracrine production of secreted IL6 (11). Both IL6 and IL8 have been implicated in the regulation of CSCs (12, 13).

We have previously shown that both normal and malignant mammary stem cells can be isolated by virtue of their increased expression of aldehyde dehydrogenase (ALDH), as assessed by the ALDEFLUOR assay. We have used this method to isolate functional stem cells from primary breast xenografts and established human breast cancer cell (BCC) lines and shown that these cells mediate tumor invasion and metastasis (14). In the present study, we examined the interaction between bone marrow–derived MSCs and CSCs with in vitro systems and mouse models. We show that mesenchymal cells (MC), like CSCs, are organized in a cellular hierarchy and that ALDEFLUOR-positive MCs regulate CSC self-renewal.
Interaction between these cell types is mediated by a cytokine network involving CXCL7 and IL6. Furthermore, we show that labeled human bone marrow MCs traffic from the bone marrow to accelerate the growth of human breast cancer xenografts at distant sites by expanding the CSC population. These studies suggest that MSCs form an important component of the CSC niche in that they regulate the self-renewal of breast CSCs.

Materials and Methods

Cell culture
BCC lines (SUM159 and SUM149) obtained from Dr. Stephen Ethier have been extensively characterized (http://www.asterand.com/Asterand/human_tissues/hubcelllines.htm.15). The MCF-7 cell line was purchased from American Type Culture Collection. The cell lines were grown using the recommended culture conditions as described previously (14). Human bone marrow–derived MCs, which were cryopreserved at passage 1, were purchased from ScienCell Research Laboratories and grown and passaged in the recommended medium. These MCs were characterized by the expression of the MC markers CD29, CD90, CD44, and CD105 but not CD45, CD34, and CD11b at passages 2 (Supplementary Fig. S1A) and 10 (Supplementary Fig. S1B). All experiments were done with subconfluent cells in the exponential phase of growth. The primary MCs from bone marrow were purchased from Texas A&M HSC COM and cultured in the recommended culture medium.

Virus infection
A highly efficient lentiviral expression system (pLentiLox 3.7; http://www.med.umich.edu/vcore/) was used to generate DsRed-, GFP-, or luciferase-expressing lentiviruses in the UM Vector Core Facility. The cell lines were infected with the lentiviruses as described previously (3).

ALDEFLUOR assay and flow cytometry
The ALDEFLUOR kit (StemCell Technologies Inc.) was used as described previously (16). The antibodies for IL6R and gp130 were purchased from Immunotech and Pharmingen, and flow cytometry with antibody staining was done as described previously (16). To assess cell viability, 0.5 μg/mL 4′,6-diamidino-2-phenylindole (DAPI; Sigma) was used.

In vitro differentiation
In vitro differentiation of hMSC-bm (unseparated population, ALDEFLUOR-positive population, and ALDEFLUOR-negative population) was evaluated in triplicates, and the detailed procedure for the differentiation assay is described in the Supplementary Material.

RNA extraction
Total RNA was isolated using the RNeasy Micro Kit according to the manufacturer’s instructions (Qiagen).

Gene expression profiling with DNA microarrays
Affymetrix human U133 Plus 2.0 was used. Preparation of the cRNA, hybridizations, washes, and detection were done as recommended by the supplier (http://www.affymetrix.com/index.affx). Expression data were analyzed by the RMA (Robust Multichip Average) method in R with Bioconductor and associated packages (17).

Real-time quantitative RT-PCR
One nanogram of total RNA from the mammospheres or differentiated cells on collagen-coated plates was used for real-time quantitative RT-PCR (qRT-PCR), as described previously (18).

Conditioned medium, antibody array, and Luminex bead assay
To prepare conditioned media, BCC lines (SUM159 or SUM149) alone, MSC alone, or the coculture of BCC and MSC (1:1 mixture) were plated in 100-mm tissue culture dishes in the mixture of IHM and MSCM (1:1 mixture). The detailed procedure is described in the Supplementary Material.

Invasion assay
Assays were done in triplicate in invasion chambers precoated with reduced growth factor matrix from BD Biosciences. Cells were added to the upper chamber in 200 μL of serum-free medium. For the invasion assay, 20,000 MCF-7 cells were seeded on the coated chamber, and the lower chamber was filled with 600 μL of medium (Cambrex) with or without 100 ng/mL IL6; 100 μg/mL IL6 blocking antibody; and 20,000 preseeded SUM159, SUM149, or MCF-7 cells. After 27 hours of incubation, the cells on the underside of the upper chambers were stained with the blue stain in the Cell Invasion Assay Kit (Chemicon; cat. #ECM550) and counted using light microscopy.

Tumorigenicity in NOD/SCID mice
Six-week-old female NOD/SCID mice were purchased from Jackson Laboratories. Tumorigenicity of 1,000 of ALDEFLUOR-positive, -negative, and unseparated SUM159-DsRed in the absence or presence of an equal number of MSCs in the mammary fat pads of NOD/SCID mice was assessed. Six mice were assayed for each group. The animals were euthanized when the tumors were approximately 1–1.5 cm at their largest diameter, in compliance with regulations for the use of vertebrate animals in research. A portion of each fat pad was fixed in formalin and embedded in paraffin for histologic analysis. Another portion was analyzed by the ALDEFLUOR assay. In addition to the established cell lines, MC1 primary xenografts were developed and used as previously described (16).

Intratibial injection
The mouse preparation and the intramedullary injection into the tibia shaft was carried out according to previously published methods (19, 20). Briefly, a few hours before transplantation, mice were irradiated with 300 cGy from an X-ray irradiator (Mark I, Model 25; J.L. Shepherd). All procedures were approved by the Animal Care Committee of the University of Michigan. The detailed procedure is described in the Supplementary Material.
**Bioluminescence imaging**

Baseline bioluminescence was assessed before inoculation and each week thereafter. For photon flux counting, we used a charge-coupled device camera system (Xenogen) with a nosecone isofluorane delivery system and heated stage for maintaining body temperature. Results were analyzed after 2–12 minutes of exposure using the Living Image software provided with the Xenogen imaging system.

**Immunostaining**

For ALDH1, DsRed, GFP, and DAPI quadruple fluorescent staining, paraffin-embedded sections of breast tumors from xenografts were deparaffinized in xylene and rehydrated in graded alcohol. Antigen enhancement was done by incubating the sections in citrate buffer pH 6.0 (Dakocytomation) as recommended. ALDH1 antibody (BD biosciences), DsRed antibody (Santa Cruz), and GFP antibody (Neomarker) were used at a 1:25 dilution and incubated for 1 hour. PE-, FITC-, and Cy5-labeled secondary antibodies (Jackson Labs) were used at a 1:250 dilution and incubated for 20 minutes. PE-conjugated secondary antibody (red color in the staining) was used to detect the ALDH1 primary antibody, FITC-conjugated secondary antibody (green color in the staining) was used to detect the DsRed primary antibody, and Cy5-conjugated secondary antibody (purple color in the staining) was used to detect the GFP primary antibody. Nuclei were counterstained with DAPI/antifade (Invitrogen; blue color in the staining) and cover-slipped. Sections were examined with a fluorescent microscope (Olympus FV-500 Confocal).

**Statistical analysis**

Results are presented as the mean ± standard deviation (STDEV) for at least 3 repeated individual experiments for each group. Mean and STDEV was determined on the basis of an analysis of at least 3 replicates using Microsoft Excel. Statistical differences were determined by using ANOVA and Student’s t test for independent samples. A value of P < 0.05 was considered statistically significant.

**Results**

**Bone marrow–derived MSCs can expand the breast cancer stem cell population**

To assess the ability of bone marrow MSCs to affect breast CSC functionality, we cocultured DsRed-labeled SUM159 BCCs with human bone marrow–derived MCs. Following coculture, cell populations were separated by flow cytometry, and ALDH1-expressing populations were assessed by the ALDEFLUOR assay. SUM159 cells cultured alone contained approximately 4% ALDEFLUOR-positive cells. Coculture with MCs increased the proportion of ALDEFLUOR-positive cells over 3-fold to 14% without affecting the total cell numbers as determined by MTT assay (Fig. 1A, right), suggesting that this interaction leads to an increase in CSC self-renewal (Fig. 1A). To find whether this increase required contact between cancer cells and MCs, each of these cell types were cultured in transwells, which precluded direct cell–cell contact but allowed communication via soluble factors. As shown in Figure 1A, transwell culture recapitulated the effects of direct cell–cell contact. Furthermore, expansion of the CSC population was reproduced by the addition of conditioned medium obtained from either coculture or transwell culture of both cell compartments but not by conditioned medium obtained from a culture of MCs alone (Fig. 1A). Similar results were seen in 2 other BCC lines, SUM149 and MCF-7, representing basal and luminal subtypes (Supplementary Fig. S2B and C). The coculture of tumor cells with MCs also increased the percentage of tumor cells expressing the breast CSC markers CD24+CD44+ (ref. 21; Supplementary Fig. S2). This suggests that the CSC compartment is regulated by soluble factors that are generated as a result of the interaction between MCs and cancer cells.

**Mesenchymal cells are organized in a cellular hierarchy in which ALDEFLUOR-positive MCs regulate CSC expansion**

To determine whether MCs are organized in a cellular hierarchy, we used the ALDEFLUOR assay to isolate ALDH1-expressing subpopulations from human bone marrow–derived MCs. As indicated in Figure 1B and Supplementary Figure S1, MCs contain approximately 5%–6% ALDEFLUOR-positive cells. In both coculture and transwell culture, this ALDEFLUOR-positive subfraction is unaffected by the presence of SUM159 cells (Fig. 1B). Defining characteristics of stem cells include their ability to self-renew and to undergo multilineage differentiation. When placed in an appropriate induction medium, ALDEFLUOR-positive MCs (mesenchymal stem cells) displayed adipogenic or osteogenic differentiation, whereas ALDEFLUOR-negative MCs did not (Fig. 1C). Furthermore, we found that ALDEFLUOR-positive MCs can regenerate both ALDEFLUOR-positive and ALDEFLUOR-negative MCs, but ALDEFLUOR-negative MCs cannot regenerate ALDEFLUOR-positive MCs (Supplementary Fig. S3A)—a property that also applies to the ALDEFLUOR-positive and -negative BCCs (Supplementary Fig. S3B). This indicates that MCs are organized in a hierarchy in which ALDEFLUOR-positive MCs can undergo self-renewal and multilineage differentiation. To find whether the differentiation of MCs affected their ability to regulate breast CSCs, we determined the effect of ALDEFLUOR-positive and ALDEFLUOR-negative MCs on SUM159 cells in coculture. As shown in Figure 1D, the effect of MCs on the SUM159 CSC population was mediated by the ALDEFLUOR-positive MC population whereas ALDEFLUOR-negative MCs had no effect. These results were confirmed by freshly isolated and well-characterized MCs from bone marrow (Supplementary Fig. S4). These experiments show that interactions between MSC and CSC populations regulate the proportion of CSCs.

**A cytokine network mediates interaction between BCCs and MCs**

To determine the molecular mechanisms mediating the interaction between BCCs and MCs, we examined the effect of coculture on the global gene expression profile of each cell population, as assessed by an Affymetrix microarray. We compared gene expression patterns of SUM159 cells and...
MCs cultured alone or in coculture conditions. Among the gene families induced by coculture were cytokine genes. As shown in Supplementary Table 1A, coculture induced mRNA expression of CXCL5 (ENA78), CXCL6 (GCP2), and CXCL1 (Gro-a), as well as IL6 and IL8 in both the SUM159 cells and in MCs. These results were confirmed by a real-time RT-PCR in the SUM159 cell line (Supplementary Table 1B) as well as in the primary xenograft MC1 (Supplementary Fig. S15). In addition, we detected a 6-fold increase in CXCL7 (NAP2) expression only in MCs induced by coculture with BCCs. Both antibody arrays and the Luminex bead assay were used to quantitatively assess the effects of coculture on cytokine protein expression. As shown in Figure 2 and Supplementary Figure S5A, the levels of CXCL1, CXCL5, CXCL6, and CXCL7 were all substantially increased by coculture. In addition, coculture substantially increased IL6 and IL8 production. Similar results were seen in other BCC lines: SUM149 (Supplementary Figs. S5B and S6) and MCF-7 (Supplementary Figure S5C). To determine which of the cytokines induced by coculture was responsible for affecting the CSC population, we used the ALDEFLUOR assay to assess the effect of each of these cytokines on the proportion of CSCs. Dose–response curves were obtained for each cytokine (Supplementary Fig. S7). The effect of optimized concentrations of each cytokine on the ALDEFLUOR-positive population of SUM159 cells is shown in Figure 3A. IL6 and IL8 induced substantial increases in the ALDEFLUOR-positive SUM159 cells, consistent with previous reports (14, 24, 25). CXCL1 had no significant effect, and CXCL5 and CXCL6 generated smaller but statistically significant increases in ALDEFLUOR-positive SUM159 cells. In contrast, the addition of CXCL7 produced almost a 3-fold increase in ALDEFLUOR-positive SUM159 cells—a level comparable with that produced by coculture with MCs. Similar results were seen in other BCC lines: SUM149 (Supplementary Fig. S8A) and MCF-7 (Supplementary Fig. S8C).

To determine the contribution of these cytokines in mediating the interaction of MSCs with CSCs, we used cytokine-blocking antibodies. Dose–response curves for antibody-blocking were obtained (Supplementary Fig. S7). The
concentration of antibody producing maximal inhibition was used and are shown in Figure 3B. The interaction of CSCs with MSCs was partially inhibited by anti-IL6 and, more completely, by anti-CXCL7, suggesting an important role for these 2 cytokines in mediating MSC–CSC interactions. Similar results were seen in other BCC lines: SUM149 (Supplementary Fig. S7B) and MCF-7 (Supplementary Fig. S8D).

To further define the role of CXCL7 and IL6 in mediating the interaction of MSCs with CSCs, we examined the effects of CXCL7 and IL6 on cytokine production by each cellular subcomponent. The addition of CXCL7 to SUM159 cells induced a cytokine expression pattern similar to that observed in coculture (Fig. 3C). Furthermore, the addition of CXCL7-blocking antibody to the coculture completely blocked the expression of induced cytokines CXCL1, CXCL5, CXCL6, IL6, and IL8 (Fig. 3C). These experiments suggest that cytokine production by SUM159 cells in coculture is largely due to CXCL7 produced by MSCs. Similar results were seen in another BCC line: SUM149 (Supplementary Fig. S9).

CXCL7 production by MSCs is regulated by IL6

The previous experiments suggest a critical role for MSC-derived CXCL7 in mediating signaling between the epithelial and mesenchymal components of tumors. To find how this cytokine was regulated, we examined the effect of individual cytokines on CXCL7 production by MSCs. As shown in Figure 4A, the addition of IL6 induced a >10-fold increase in CXCL7 production by MCs. The effects of IL6 are mediated by a receptor complex composed of the IL6 receptor IL6R and gp130. We assessed the expression of these receptors on ALDEFLUOR-positive and ALDEFLUOR-negative MCs. We separated the ALDEFLUOR-positive and ALDEFLUOR-negative MCs by flow cytometry, immunostained the separated populations with IL6R and gp130 antibodies, and reanalyzed the cells by flow cytometry. As indicated in Figure 4B, both the IL6R and gp130 were primarily expressed in ALDEFLUOR-positive MCs (MSCs). Furthermore, although the CXCL7 mRNA level was undetectable in the MC control group, the addition of 100 ng/mL IL6 dramatically increased the expression of CXCL7 mRNA in ALDEFLUOR-positive MCs (Fig. 4C).

IL6 mediates MSCs chemotaxis

To address the functional significance of increased IL6R expression in MSCs, we examined whether this cytokine mediated the chemotaxis of these cells. Recombinant IL6 mediated chemotaxis of ALDEFLUOR-positive but not ALDEFLUOR-negative MCs, an effect inhibited by the IL6-blocking antibody (Fig. 4D). In addition, ALDEFLUOR-positive but not ALDEFLUOR-negative MCs were chemotactic toward breast tumor cells SUM159, SUM149, and MCF-7, an effect that was substantially inhibited by the IL6-blocking antibody (Fig. 4D). These results suggest that the chemotaxis of MSCs toward BCCs is primarily mediated by IL6.

These experiments suggest the existence of a cytokine network that mediates the interaction between MCs and cancer cells in which IL6 produced by cancer cells interacts with IL6R/gp130 expressed on MSCs, which produce CXCL7 in response to this IL6 stimulation. CXCL7, in turn, induces the secretion of a number of cytokines from both SUM159 and MCs, including IL6, IL8, CXCL16, and CXCL5. All of these cytokines are capable of expanding the ALDEFLUOR-positive CSC population. Furthermore, increased IL6 interacts with MSCs, forming a positive feedback loop. This model of cytokine networks mediating the interaction between MCs, BCCs, and breast CSCs is illustrated in Figure 4E.

MSCs stimulate the growth of breast tumor xenografts in NOD/SCID mice by affecting the CSC population

We have previously shown that ALDEFLUOR-positive but not ALDEFLUOR-negative SUM159 cells are tumorigenic in NOD/SCID mice (14). To assess the contribution of different subpopulations of MCs on tumor growth, ALDEFLUOR-positive, ALDEFLUOR-negative, and unsorted MCs mixed in a ratio of 1:1 with SUM159 cells were orthotopically implanted in...
Figure 3. CXCL7 and IL6 play key roles in mediating the interaction of MCs and SUM159 cells. A, recombinant cytokines increase the ALDEFLUOR-positive population of SUM159 cells in the order of CXCL7 > IL6 > IL8 > CXCL6 > CXCL5. B, blocking antibodies decrease the ALDEFLUOR-positive population of SUM159 cells in the coculture in the order of anti-CXCL7 > anti-IL6 > anti-IL8 > anti-CXCL6 > anti-CXCL5. NT, “no treatments.” The P values refer to the significant difference of sample groups compared with the NT group. C, the addition of CXCL7 to SUM159 cells induces a cytokine expression pattern, which recapitulated that of the coculture. Alternatively, the addition of CXCL7 blocking antibody to the coculture completely blocked the expression of induced cytokines. *, P < 0.05 refers to the significant difference of the individual cytokine from the “coculture treated with 20 µg/ml anti-CXCL7” group compared with the corresponding cytokine from the coculture group or the significant difference of the individual cytokine from the “SUM159 treated with 10 ng/mL CXCL7” group compared with the corresponding cytokine from “SUM159-alone” group. Error bar, SD.
NOD/SCID mice. Unsorted MCs alone were implanted as control. The addition of ALDEFLUOR-positive MCs greatly accelerated tumor growth, whereas ALDEFLUOR-negative MCs had no effect on the growth of SUM159 tumors. Unsorted MCs were intermediate in their ability to stimulate tumor growth (Fig. 5A and Supplementary Fig. S11A). MCs alone were not tumorigenic (Fig. 5A). The introduction of the GFP label into MCs and the DsRed label into SUM159 cells allowed for the separation of these cells from established tumors and the assessment of the effects of added MCs on the CSC population in vivo.

Figure 4. IL6 regulates CXCL7 production by MSCs and mediates MSC chemotaxis, and the interaction between mesenchymal cells and tumor cells is regulated by a positive feedback loop including CXCL7 and IL6. A, the effects of the optimized concentration of cytokine on the induction of CXCL7 by MCs. IL6 induces more than a 10-fold increase of CXCL7 production by MCs. NT, “no treatments.” The \( P \) value refers to the significant difference of the sample group compared with the NT group. B, both the IL6R and GP130 were primarily expressed in the ALDEFLUOR-positive populations of MCs. The \( P \) values refer to the significant difference of the ALDH\(^+\) group compared with the ALDH\(^-\) group. C, MCs were treated with vehicle control or 100 ng/mL of IL6 for 4 days; both ALDEFLUOR-positive and ALDEFLUOR-negative cells were sorted out from both groups, and total RNA was isolated. Real-time RT-PCR showed that CXCL7 mRNA level was undetectable in the MC control group but elevated in ALDEFLUOR-positive MCs with IL6 treatment. The \( P \) value refers to the significant difference of the ALDH\(^+\) cells compared with the ALDH\(^-\)/C0 cells in the same group. D, ALDEFLUOR-positive MCs, but not ALDEFLUOR-negative MCs, have chemotactic capability, which is increased by IL6. Chemotaxis of ALDEFLUOR-positive MCs toward tumor cells is inhibited by IL6 blocking antibody. The \( P \) values refer to the significant difference of sample groups compared with the control group. E, a model of cytokine networks mediating the interaction between MSCs, BCCs, and breast CSCs. Error bar, SD.
MSCs Regulate Breast Cancer Stem Cells

Figure 5. ALDEFLUOR-positive MCs induce CSCs stimulating breast tumor growth in NOD/SCID mice. A, ALDEFLUOR-positive (ALDH+) but not ALDEFLUOR-negative (ALDH-) MCs stimulate tumor growth of SUM159 cells in NOD/SCID mice. *, P < 0.05 refers to the significant difference of the sample groups compared with the “SUM159-DsRed ALDH+ alone (1k)” group. B, ALDEFLUOR-positive but not ALDEFLUOR-negative MCs increase the ALDEFLUOR-positive population of SUM159 cells in NOD/SCID mice. The P values refer to the significant difference of sample groups compared with the SUM159-alone group. C, 4-color immunofluorescence staining reveals close apposition between ALDH1-positive MCs and ALDH1-positive SUM159 cells. To quantitate this association, we counted 9 fields to determine the frequency of association of ALDH-positive CSCs and MSCs. There are 14 CSC-MSC direct-contacts out of a total of 38 MSCs and 35 CSCs. This statistically significant positive association shows the nonrandom distribution of these 2 cell types in tumor xenografts. SUM159-DsRed: DsRed-labeled SUM159; MC-GFP: GFP-labeled MC; blue: DAPI staining; green: DsRed staining; purple: GFP staining; red: ALDH1 staining. White arrow, ALDH1+ 2 cell types in tumor xenografts. SUM159; white triangle, ALDH1+ MC. D, serial dilutions of tumor cells isolated from primary tumors of SUM159-alone or SUM159 mixed with MCs were engrafted into secondary NOD/SCID mice. Tumor latency was reduced, and tumor growth was increased in the tumors generated from primary tumors of SUM159 mixed with MCs. *, P < 0.05 refers to the significant difference of the “SUM159-DsRed from mix” compared with the “SUM159-DsRed from alone” group injected with the same number of cells. Error bar, SD.

Supplementary Fig. S11B). To provide functional data confirming the ALDEFLUOR results, we examined the ability of serial dilutions of cells obtained from primary tumors to form secondary tumors in NOD/SCID mice. As shown in Figure 5D, cells obtained from primary tumors grown in the presence of MCs had significantly greater tumor-generating capacity in secondary mice than cells obtained from primary tumors grown in the absence of MCs. These results confirm the ALDEFLUOR data, suggesting that MSCs have the capacity to increase the breast CSC population. Furthermore, the percentage of CSCs assessed by the ALDEFLUOR assay or by the expression of CD24+CD44+ in the secondary tumors was similar in secondary tumors generated from MC-supplemented or control primary tumors. This suggests that the accelerated growth of primary tumors by MCs results from an increase in CSCs rather than from an alteration of the biological properties of these cells (Supplementary Fig. S11C). Similar in vivo results were in other BCC lines: SUM149 (Supplementary Fig. S12) and MCF-7 (Supplementary Fig. S13). To show that the effect of MSCs on breast CSCs was not limited to established cell line–generated xenografts, we used MC1, a breast xenograft established directly from a human breast tumor tissue. We have previously shown that ALDEFLUOR-positive cells in this tumor display CSC properties (14). As was the case with established cell lines, the addition of MSCs accelerated tumor growth by increasing the proportion of ALDEFLUOR-positive tumor cells in this model (Supplementary Fig. S14).

To document physical interactions between MSCs and CSCs in growing tumors, we used 4-color fluorescence in which SUM159 cells were identified by DsRed expression (green), MCs by GFP expression (purple), and stem cells (MSCs and CSCs) by the expression of the stem cell marker by ALDH1 expression (red). Nuclei were identified by DAPI staining (blue). We have previously reported that the ALDEFLUOR-positive SUM159 cells can be identified in situ by using an ALDH1 monoclonal antibody (14). Four-color fluorescence revealed close apposition between ALDH1-positive MCs (MSCs) and ALDH1-positive SUM159 cells (CSCs; Fig. 5C).
MSCs traffic from bone marrow to primary breast tumor sites in xenografts and are detected in primary human breast cancers.

To determine whether bone marrow MSCs are capable of trafficking to primary breast tumor sites, we labeled MCs with luciferase and DsRed. The growth of implanted MCs at the tibial site was shown by bioluminescence (Fig. 6A, left). One week after the MC tibial implantation, we implanted SUM159 cells in the mammary fat pads. Tumor size was monitored weekly. As shown in Figure 6A (right), breast tumor growth was significantly accelerated by human MCs introduced into the mouse tibia. Five weeks after tumor implantation, animals were sacrificed, and the presence of MSCs and CSCs was assessed by immunochemistry and immunofluorescence. As shown in Figure 6B, DsRed immunochemistry revealed the presence of MCs in tumors grown in animals with tibial MC inoculation but not in controls. Immunofluorescence detects DsRed-labeled MC (green), ALDH1-positive MC (yellow), and ALDH1-positive SUM159 cells (red) in the merged image. C, immunolocalization of ALDH1-positive tumor cells, MCs, and endothelial cells in breast cancer clinical specimens. We counted 6 fields to determine the frequency of association of ALDH-positive CSCs and MSCs. There are 21 CSC–MSC direct-contacts out of a total of 115 MSCs and 33 CSCs. Stain ALDH1 (a stem/progenitor marker), CD105 (a MSC marker and endothelial cell marker), CD31 (an endothelial cell marker), and Pan-CK AE1/AE3 (Pan-CK, an epithelial cell marker) in consecutive serial sections from a primary breast tumor. One representative sample from 3 independent samples is shown. Red arrow, CSCs (ALDH1+/CD105–/CD31–/Pan-CK+); yellow arrows, MSCs (ALDH1+/CD105+/CD31+/Pan-CK–); green arrows, endothelial progenitors (ALDH1+/CD105–/CD31–/Pan-CK–). Error bar, SD.

Figure 6. MSCs traffic from bone marrow to primary breast tumor sites in xenografts and are detected in primary human breast cancers. A, MCs labeled with luciferase and DsRed were introduced via intratibial injection. The tumor growth was facilitated by introducing MCs into mouse tibia. *, P < 0.05 refers to the significant difference of the “with MC intratibial injection” group compared with the “without MC intratibial injection” group.

B, in situ localization of ALDH1+ MCs and ALDH1+ SUM159 cells identifies a CSC niche. Light imaging reveals the presence of DsRed-labeled MC in mice with intratibial MC inoculation but not in controls. Brown, DsRed. Immunofluorescence detects DsRed-labeled MC (green), ALDH1-positive MC (yellow), and ALDH1-positive SUM159 cells (red) in the merged image.

C, immunolocalization of ALDH1-positive tumor cells, MCs, and endothelial cells in breast cancer clinical specimens. We counted 6 fields to determine the frequency of association of ALDH-positive CSCs and MSCs. There are 21 CSC–MSC direct-contacts out of a total of 115 MSCs and 33 CSCs. Stain ALDH1 (a stem/progenitor marker), CD105 (a MSC marker and endothelial cell marker), CD31 (an endothelial cell marker), and Pan-CK AE1/AE3 (Pan-CK, an epithelial cell marker) in consecutive serial sections from a primary breast tumor. One representative sample from 3 independent samples is shown. Red arrow, CSCs (ALDH1+/CD105–/CD31–/Pan-CK+); yellow arrows, MSCs (ALDH1+/CD105+/CD31+/Pan-CK–); green arrows, endothelial progenitors (ALDH1+/CD105–/CD31–/Pan-CK–). Error bar, SD.

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consistent with the previously shown increased invasive transfection increased the invasive capacity of BCCs (28), increased in breast carcinomas (28). Furthermore, CXCL7 expression of CXCL7 and its receptor CXCR2 has been shown to be consistent with previous reports (27). The expression of CXCL7, in turn, interacts with cancer cells through the CXCR2 receptor (27), where it induces the synthesis of a number of cytokines, including IL6 and IL8. These pleiotropic effects of CXCL7, in turn, interacts with cancer cells triggering their self-renewal and invasive properties (14, 30). IL6 has also been reported to be capable of regulating breast stem cells (22) and colon CSCs (23). In addition to regulating CSCs, IL6 produced by cancer cells interacts with MSCs, further increasing their CXCL7 production, generating a positive feedback loop (Fig. 5A). We confirmed the functional importance of these interactions by showing that MSCs accelerate breast tumor growth in NOD/SCID mice. Furthermore, as was the case in vitro, in this mouse model, this effect was mediated by ALDEFLUOR-positive MSCs, which were capable of increasing the CSC population in vivo. The close apposition of ALDH1+ tumor cells and MSCs was also shown in frozen sections of primary human breast cancers.

It is clear that the tumor microenvironment plays an important role in tumor growth and metastasis (8). Previous elegant studies have suggested a role for MSCs in tumor metastasis, which is mediated by CCL5 (8). In addition, researchers reported that IL1α produced by MSCs mediates similar effects (31) and that MSCs promote LTIC-IC expansion (32). Our studies extend these previous findings by demonstrating that MSCs regulate cancer cell behavior through their effect on CSCs. On the other hand, MSCs have been shown to actually inhibit tumor growth in some models (33). This suggests that the effects of MSCs on tumor growth are complex and may be context-dependent.

The homing of bone marrow–derived MSCs to sites of tumor growth may rely on similar mechanisms for the homing of these cells to sites of tissue injury. Developing tumors may recruit MSCs from the bone marrow where they interact with and regenerate CSCs. If this is the case, then the development of strategies aimed at interfering with these pathways may provide a means of targeting CSCs. Since these cells may mediate tumor growth and metastasis as well as contribute to treatment resistance, these strategies may lead to improved clinical outcomes for patients with advanced breast cancers.

**Disclosure of Potential Conflicts of Interest**

M. Wicha has financial holdings in OncoMed Pharmaceuticals, which has applied for a patent on CSC technologies. The other authors disclosed no potential conflicts of interest.

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References

Correction: Breast Cancer Stem Cells Are Regulated by Mesenchymal Stem Cells through Cytokine Networks

In this article (Cancer Res 2011;71:614–24), which was published in the January 15, 2011 issue of Cancer Research (1), an incorrect version of Figure 4E was published. The correct version of the figure is provided below.

Reference


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Breast Cancer Stem Cells Are Regulated by Mesenchymal Stem Cells through Cytokine Networks

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