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Lung-Derived Factors Mediate Breast Cancer Cell Migration through CD44 Receptor-Ligand Interactions in a Novel *Ex Vivo* System for Analysis of Organ-Specific Soluble Proteins[1,2]

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Abstract

Breast cancer preferentially metastasizes to lung, lymph node, liver, bone, and brain. However, it is unclear whether properties of cancer cells, properties of organ microenvironments, or a combination of both is responsible for this observed organ tropism. We hypothesized that breast cancer cells exhibit distinctive migration/growth patterns in organ microenvironments that mirror common clinical sites of breast cancer metastasis and that receptor-ligand interactions between breast cancer cells and soluble organ-derived factors mediate this behavior. Using an *ex vivo* model system composed of organ-conditioned media (CM), human breast cancer cells (MDA-MB-231, MDA-MB-468, SUM149, and SUM159) displayed cell line–specific and organ-specific patterns of migration/proliferation that corresponded to their *in vivo* metastatic behavior. Notably, exposure to lung-CM increased migration of all cell lines and increased proliferation in two of four lines ($P < .05$). Several cluster of differentiation (CD) 44 ligands including osteopontin (OPN) and L-selectin (SELL) were identified in lung-CM by protein arrays. Immunodepletion of SELL decreased migration of MDA-MB-231 cells, whereas depletion of OPN decreased both migration and proliferation. Pretreatment of cells with a CD44-blocking antibody abrogated migration effects ($P < .05$). "Stemlike" breast cancer cells with high aldehyde dehydrogenase and CD44 ($ALDH^{hi}CD44^{+}$) responded in a distinct chemotactic manner toward organ-CM, preferentially migrating toward lung-CM through CD44 receptor-ligand interactions ($P < .05$). In contrast, organ-specific changes in migration were not observed for $ALDH^{low}CD44^{-}$ cells. Our data suggest that interactions between $CD44^{+}$ breast cancer cells and soluble factors present in the lung microenvironment may play an important role in determining organotropic metastatic behavior.

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Abbreviations: ALDH, aldehyde dehydrogenase; ANOVA, analysis of variance; AP, alkaline phosphatase; bFGF, basic fibroblast growth factor; BM, bone marrow; BMSC, bone marrow stromal cell; BrdU, bromodeoxyuridine; CD, cluster of differentiation; CM, conditioned media; COX-2, cyclooxygenase 2; DAPI, 4′,6-diamidino-2-phenylindole; DEAB, diethylaminobenzaldehyde; DMEM, Dulbecco's modified Eagle's medium; ECM, extracellular matrix; ER, estrogen receptor; EREG, epiregulin; FITC, fluorescein isothiocyanate; HPF, high-powered field; LN, lymph node; LNSC, lymph node stromal cell; NIH, National Institutes of Health; OPN, osteopontin; PR, progesterone receptor; RT, room temperature; SELE, E-selectin; SELL, L-selectin; SELP, P-selectin; uPA, urokinase-type plasminogen activator; VCAM-1, vascular cell adhesion molecule 1; VEGFA, vascular endothelial growth factor A

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[2]This article refers to supplementary materials, which are designated by Tables W1 and W2 and Figures W1 to W5 and are available online at www.neoplasia.com.
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Introduction

Breast cancer remains a leading cause of morbidity and mortality in women [1], mainly due to the propensity of primary breast tumors to metastasize to distant sites and the failure of most therapies in the metastatic setting. Further insight into the biology of metastasis is therefore essential to gain a greater understanding of this process and to develop better cancer therapies.

Metastasis is a complex process, and tumor cells must successfully negotiate a series of sequential steps to establish clinically relevant macrometastases. These steps include dissemination from the primary tumor through blood or lymphatic systems, survival within the circulation, extravasation into secondary sites, initiation of growth into micrometastases, and maintenance of growth as vascularized macrometastases [2]. Clinical observations indicate that many cancers show an organ-specific pattern of metastasis, termed *organ tropism*, and it is well established that breast cancer favors metastasis to the lung, liver, bone, lymph node (LN), and brain [2–4]. In the 1920s, James Ewing first proposed that blood flow patterns alone were sufficient to account for both physical delivery of tumor cells to secondary organs and for patterns of organ-specific metastasis [5]. However, several theories have challenged this idea by proposing that there are additional, molecular-level mechanisms that explain why and how cancer cells can travel to and grow in "favorite" metastatic sites. Among these is Paget's seminal "seed and soil" hypothesis, first proposed in 1889 [6]. This predicts that a cancer cell ("seed") can survive and proliferate only in secondary sites ("soil") that produce appropriate molecular factors. A meta-analysis of published autopsy data [7] demonstrated that, in some cases, metastases detected at autopsy were in proportion to blood flow from the primary tumor to the secondary organ. However, in many cases, more or fewer metastases than would be expected by blood flow alone were detected, indicating that the microenvironment is likely very important for metastatic dissemination and growth.

For the past several years, elegant work by Joan Massagué and colleagues has focused on defining specific genes that mediate organ-specific metastasis in breast cancer [4,8–10]. Using *in vivo* selection and genetic analysis of the MDA-MB-231 human breast cancer cell line, this group demonstrated that particular genes can mediate experimental breast cancer metastasis in an organ-specific manner to lung [10], bone [9], and brain [8] and validated that these genes reflect organ-specific metastatic disease in patients with breast cancer. Although these studies contribute valuable knowledge regarding the contribution of the cancer cell ("seed") to organ tropism of breast cancer, the factors contributed by the metastatic microenvironment ("soil") still remain poorly understood. In addition, these studies do not take into account the concepts of tumor cell heterogeneity and the cancer stem cell hypothesis.

Despite the deadly nature of metastasis, it is an inherently inefficient process [2,11]. This suggests that only a small subset of cells can successfully navigate the metastatic cascade. We believe that these metastasis-initiating cells may in fact be cells with "stemlike" properties [12]. In breast cancer, tumor-initiating cells have been isolated from primary tumors and pleural effusions on the basis of a cluster of differentiation (CD) 44-positive–CD24-negative ($CD44^+CD24^-$) phenotype [13] and/or high aldehyde dehydrogenase (ALDH) activity [14]. Our group and others have demonstrated that breast cancer cells with an $ALDH^{hi}CD44^+$ phenotype show enhanced metastatic behavior *in vitro* and *in vivo* compared to their $ALDH^{low}CD44^-$ counterparts [15–17]. However, the role of such cells in mediating organ-specific metastasis has not been investigated.

In the current study, we hypothesized that breast cancer cells exhibit distinctive growth and migration patterns in organ microenvironments that mirror common clinical sites of breast cancer metastasis and that receptor-ligand interactions between breast cancer cells and specific soluble organ-derived factors can mediate this behavior. We first developed and validated a comprehensive *ex vivo* model system for investigating the influence of organ-specific soluble factors on metastatic behavior of human breast cancer cells. Our results indicate that human breast cancer cells with varying genetic backgrounds exhibit differential migration and growth patterns toward specific organ conditions. Notably, these patterns reflect the known metastatic dissemination patterns of these cell lines *in vivo* and highlight the lung as an important source of soluble factors that mediate metastatic behavior. Furthermore, our results suggest for the first time that interactions between subpopulations of CD44-expressing breast cancer cells (including $ALDH^{hi}CD44^+$ cells) and soluble ligands present in the lung microenvironment may play an important role in determining organotropic metastatic behavior.

Materials and Methods

Cell Culture and Reagents

MDA-MB-231 cells [18] were obtained from American Type Culture Collection (Manassas, VA) and maintained in Dulbecco's modified Eagle's medium (DMEM)/F12 + 10% FBS. SUM159 and SUM149 cells [19] were obtained from Asterand Inc (Detroit, MI) and maintained in HAMS:F12 + 5% FBS + 5 μg/ml insulin + 1 μg/ml hydrocortisone + 10 mM Hepes. MDA-MB-468 cells were obtained from Dr Janet Price (MD Anderson Cancer Center, Houston, TX [20]) and maintained in α minimum essential medium + 10% FBS. Cell lines were authenticated through third-party testing (CellCheck; IDEXX RADIL, Columbia, MO) in January 2012. All Media/supplements were from Invitrogen (Carlsbad, CA); FBS was from Sigma-Aldrich (St Louis, MO).

Organ-Conditioned Media

Healthy female nude mice (Hsd:Athymic Nude-*Foxn1*^{nu}; Harlan Sprague-Dawley, Indianapolis, IN) were maintained as per the Canadian Council of Animal Care under a protocol approved by the Western University Animal Use Subcommittee (No. 2009-064). Mice (6-12 weeks old) were euthanized, and individual organs (lung, liver, and brain), femurs, and axillary/brachial/inguinal LNs were aseptically removed, washed, and cut into ~1-mm^3 fragments. Liver-conditioned media (CM) were isolated in the presence of 1X Halt protease inhibitor (aprotinin, bestatin, E-64, leupeptin, NaF, Na_3VO_4, $Na_4P_2O_7$, and β-glycerophosphate) (Pierce, Nepean, Ontario). Lung, liver, and brain tissues were weight normalized by resuspending in 4:1 media to tissue (vol/wt) ratio in DMEM/F12 + 1X MITO+ (BD Biosciences, Mississauga, Ontario) + penicillin (50 U/ml)/streptomycin (50 μg/ml) (pen/strep; Invitrogen). Organs were cultured for 24 hours before collecting CM for storage at −20°C.

Due to the smaller cellular content of LN and bone marrow (BM) relative to other organs, a different approach was taken to generate CM from these tissues. LNs were mechanically dissociated, washed, and plated at a density of 5×10^6 cells per well in six-well dishes in Roswell Park Memorial Institute Medium 1640 + 10% FBS + pen/strep + 5×10^{-5} M β-mercaptoethanol (BioShop, Burlington, Ontario) as previously described [21]. BM was collected by flushing femur cavities as