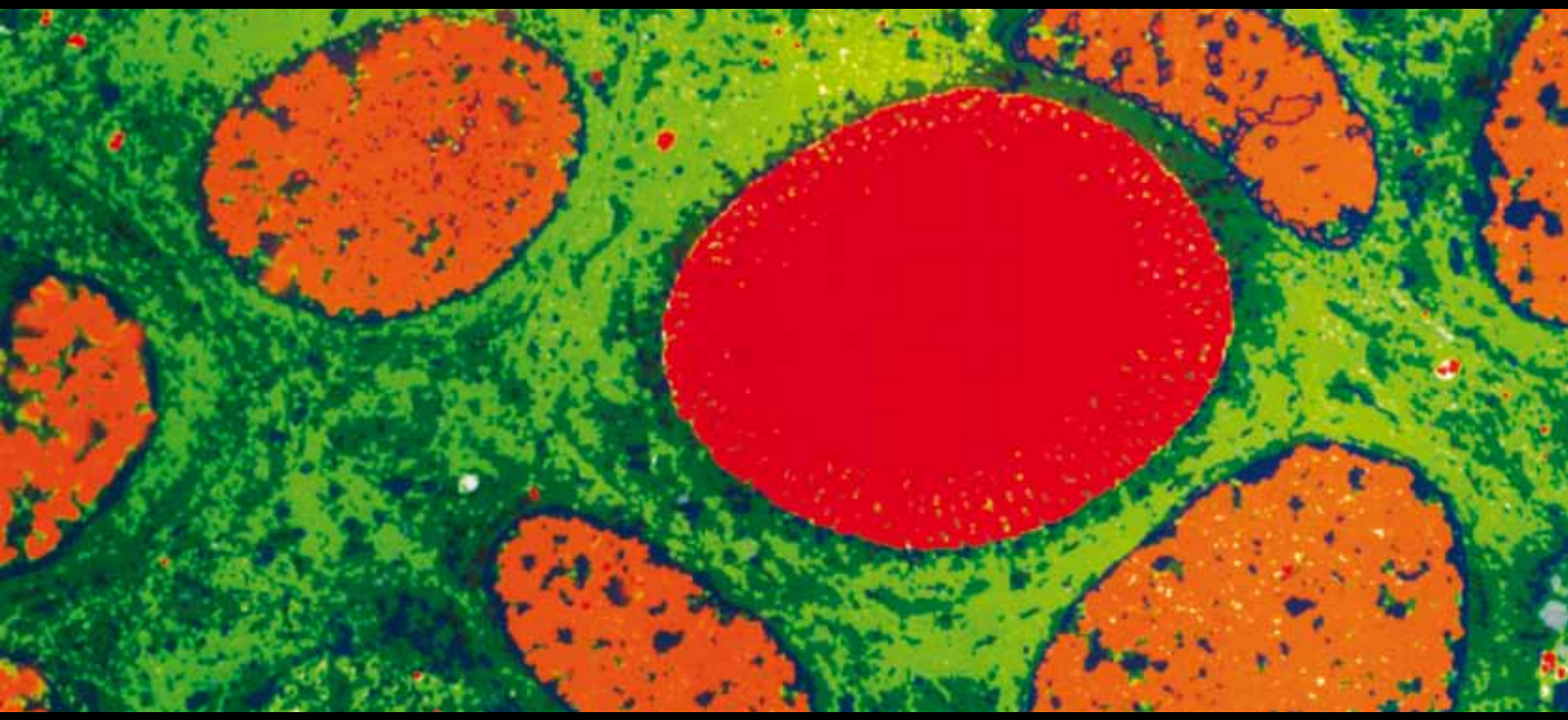
International Journal of Breast Cancer

# The Influence of the Cancer Microenvironment on the Process of Metastasis

Guest Editors: Andra R. Frost, Douglas R. Hurst, Lalita A. Shevde, and Rajeev S. Samant



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# Editorial Board

# Contents

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*Editorial*

# The Influence of the Cancer Microenvironment on the Process of Metastasis

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Metastasis of breast cancer is a multistep process that requires cancer cells to invade stroma at the primary site, gain access to vasculature, survive in the circulation, extravasate into the parenchyma of the secondary site, and survive and proliferate at the secondary site. During each of these steps, the microenvironment surrounding the cancer cells is believed to be an active participant. The cancer microenvironment also varies during the metastatic process. At the primary tumor in the breast, invasive cancer cells are surrounded by fibroblasts, extracellular matrix (ECM), cellular constituents of the vasculature, inflammatory/immune cells, and adipose tissue. Metastasizing cancer cells are exposed to a completely different microenvironment within the circulatory system. The cancer microenvironment at the secondary site is, again, very different from the microenvironment of the breast and varies depending on the sites of metastasis. At the primary and metastatic sites, the interaction between tumor cells and their surrounding milieu is reciprocal; the tumor cells influence the stroma and vice versa, ultimately fueling tumor progression. The papers in this issue discuss the dynamics of the interactions of tumor cells and their microenvironment, detailing how tumor cells manipulate their milieu and, conversely, how the reactive tumor microenvironment influences tumor cell plasticity, invasion, metastasis, and cancer therapy.

Z. I. Khamis et al. provide a thorough summary of the roles of the tumor stroma and tumor microenvironment in the various steps involved in the metastatic process, as well as in the development of breast cancer in their paper *"Active roles of tumor stroma in breast cancer metastasis."* The authors discuss research findings related to the contribution of various constituents of the tumor microenvironment, including inflammatory cells, fibroblasts, extracellular matrix, and blood vessels, in the metastatic process. They also include a discussion of the signaling pathways utilized by cancer cells to modify the stroma and ECM. This review serves as an excellent overview for this issue.

Two papers in this issue discuss the cancer cells themselves and how characteristics or functions of the cancer cells influence the tumor microenvironment. Just as the microenvironment signals to the cancer cells, the cancer cells alter the microenvironment to promote tumor progression and metastasis. J. E. Chu and A. L. Allan in their paper *"The role of cancer stem cells in the organ tropism of breast cancer metastasis: a mechanistic balance between the "seed" and the "soil?""* have exhaustively summarized the role of the cancer stem cells in determining the organ tropism exhibited by breast cancer cells. Given the fact that metastasis is an inefficient process, the authors make a compelling case for cancer stem cells to be the rare population that is equipped with the necessary armamentarium of traits to successfully metastasize. The paper summarizes the hierarchical role of cancer stem cells within the various subtypes of breast cancer and the phenotypic and functional signatures of breast cancer stem cells. It also puts into perspective the origin of cancer stem cells and their role in conditioning the premetastatic niche. The authors also provide a detailed analysis of the microenvironment of the various metastatic niches encountered by metastatic breast cancer cells, specifically the bone, brain, lungs, liver, and lymph nodes. The paper

concludes with a stimulating discussion on the contribution of cancer stem cells to therapeutic resistance taking into account the interactions of the cancer stem cells with the microenvironment.

The review by J. Alsarraj and K. W. Hunter, "*Bromodomain-containing protein 4: a dynamic regulator of breast cancer metastasis through modulation of the extracellular matrix*", is focused on the activity of bromodomain-containing protein 4 (BRD4) in breast cancer cells. BRD4 functions as an inherited susceptibility gene for breast cancer progression and metastasis and regulates the transcription of select genes through epigenetic mechanisms. Multiple ECM genes are regulated by BRD4 that may lead to changes in the overall structure of the surrounding environment or alter the cell-matrix interactions to promote breast cancer invasion and metastasis.

The roles of estrogen receptor (ER) signaling and signaling through Toll-like receptors (TLR) in the crosstalk and interactions of breast cancer cells with the tumor microenvironment are the topics of another two papers in this issue. Hormones play a critical role in directing breast cancer progression. Specifically, ER signaling is one of the critical and complex determinants of breast cancer metastasis. S. S. Roy and R. K. Vadlamudi have provided an integrated picture of this specific signaling in the paper "*Role of estrogen receptor signaling in breast cancer metastasis*". They emphasize the importance of ER-coregulatory proteins and their misexpression in promoting metastasis of ER-positive breast cancer cells. They have discussed possible therapeutic targets to block ER-driven metastasis. Most significantly, this paper brings to notice the importance of defining alternative signaling pathways. Specifically, multiple signaling pathways in addition to estrogen signaling are involved in activating ERs. Hence, combination therapies using both endocrine and nonendocrine agents that block these different pathways may have better therapeutic effects and may delay metastasis.

D. Bhattacharya and N. Yusuf discuss the data regarding TLR expression in breast cancer and its role in inflammation and cell survival in the tumor microenvironment in "*Expression of Toll-like receptors on breast tumors: taking a Toll on tumor microenvironment.*" The immune system is intricately involved in the process of tumor progression and metastasis and can play key roles in both tumor promotion and tumor suppression. TLRs are critical for innate and adaptive immunity and are expressed on inflammatory cells surrounding the tumor. Recent studies have identified many TLRs expressed by tumor cells that may promote growth and immune evasion. This has led to the emergence of TLR signaling as a potential target for the treatment of various tumors.

One of the most common sites for the metastasis of breast cancer is to bone. In accordance with this, four papers focus on breast cancer metastasis to bone. B. Y. Reddy et al. put into perspective the role of the microenvironment of the bone in breast cancer metastasis in "*The microenvironmental effect in the progression, metastasis, and dormancy of breast cancer: a model system within bone marrow.*" The heterogeneous composition of the bone microenvironment not only facilitates the growth of breast cancer cells but also supports and protects the tumor cells. There is a bidirectional crosstalk between the cells comprising the bone microenvironment and the metastatic breast cancer cells. While modulation of macrophage function can cause immune suppression, the release of inflammatory cytokines by adipocytes can stimulate tumor cell invasion, and the expression of SDF-1 by the myofibroblasts accelerates tumor cell growth. The contribution of mechanical stress in impacting tumor cell survival, elicitation of angiogenesis, and influencing drug delivery is elegantly summarized. This paper also discusses the role of microenvironment-derived cytokines, chemokines, and miRNA in inducing epithelial-mesenchymal changes and influencing cancer cell quiescence.

D. M. Sosnoski et al. present their findings on the influence of metastases on the levels of a variety of cytokines and growth factors in the bone in their research article "*Changes in cytokines of the bone microenvironment during breast cancer metastasis.*" Using a xenograft model of breast cancer metastases to bone, they demonstrate that the presence of the breast cancer cells in bone changes the normal levels of specific cytokines. Cytokines are important for bone remodeling, hematopoietic processes, and homeostatic balance in the bone. Therefore, by altering cytokine levels in the bone, metastatic breast cancer manipulates the bone microenvironment.

A complementary perspective on the dynamic dialogue between the stroma and the tumor cells, which impacts metastasis of tumor cells to bone, is provided by E. Bevilacqua et al. in "*RKIP suppresses breast cancer metastasis to the bone by regulating stroma-associated genes.*" This focuses on the metastasis suppressor, Raf Kinase Inhibitory Protein (RKIP), and its ability to influence the tumor microenvironment in the bone. RKIP inhibits breast cancer invasion, intravasation, and bone metastasis via the induction of miRNA let-7, resulting in suppression of the chromatin-remodeling factor HMGA2 and modulation of epithelial to mesenchymal plasticity. The use of a savvy, interdisciplinary approach involving expression arrays from breast cancer patients yielded a deeper understanding of key regulators of genes that form the bone metastasis signature of cancer cells, putting the spotlight on RKIP as a critical regulator of the tumor milieu and impacting the ability of tumor cells to establish bone metastases.

The bone microenvironment is a fertile soil for metastasis with multiple regulatory molecules affecting growth. Accumulating evidence supports the notion that hedgehog signaling plays a role in breast cancer metastasis to bone. In "*The hedgehog pathway conditions the bone microenvironment for osteolytic metastasis of breast cancer,*" S. Das et al. discuss our current understanding of how the hedgehog signaling pathway alters the bone microenvironment to promote metastatic breast cancer growth. Hedgehog inhibitors may be a viable option for the treatment and/or prevention of breast cancer metastasis to bone.

Finally, J. W. Rostas and D. L. Dyess have provided the surgeon's perspective of current surgical management of breast cancer in "*Current operative management of breast cancer: an age of smaller resections and bigger cures.*" Consideration of the tumor microenvironment is an emerging frontier

in the treatment of breast cancer. The surgeon's primary focus with breast-conserving surgery is obtaining tumor-free surgical margins, but there is a question as to whether residual stromal changes in the breast may affect local recurrence. The authors emphasize that surgical intervention is currently the best hope for definitive cure of breast cancer; however, advances in the treatment of breast cancer as a systemic disease are needed to facilitate long-term cures. Patient-specific molecular diagnosis and the development of targeted chemotherapeutic agents are future hopes for improved survival and will offer the surgeon an opportunity to be more focused and allow easier management of the disease.

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*Review Article*

# Active Roles of Tumor Stroma in Breast Cancer Metastasis

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Metastasis is the major cause of death for breast cancer patients. Tumors are heterogenous cellular entities composed of cancer cells and cells of the microenvironment in which they reside. A reciprocal dynamic interaction occurs between the tumor cells and their surrounding stroma under physiological and pathological conditions. This tumor-host communication interface mediates the escape of tumor cells at the primary site, survival of circulating cancer cells in the vasculature, and growth of metastatic cancer at secondary site. Each step of the metastatic process is accompanied by recruitment of stromal cells from the microenvironment and production of unique array of growth factors and chemokines. Stromal microenvironment may play active roles in breast cancer metastasis. Elucidating the types of cells recruited and signal pathways involved in the crosstalk between tumor cells and stromal cells will help identify novel strategies for cotargeting cancer cells and tumor stromal cells to suppress metastasis and improve patient outcome.

## 1. Introduction

Breast cancer is the most common malignancy and the second major cause of mortality and morbidity in Western women [1]. The systemic outgrowth and spread of the cancer cells through a process known as metastasis is the main cause of deaths in these patients. Recently, disease-related mortality and metastasis have declined as a result of early diagnosis and application of adjuvant therapy. Mammographic screening, surgery, radiotherapy, chemotherapy, antibody therapy, and endocrine therapy facilitate the suppression of the metastatic dissemination of local tumor [2]. However, these treatments target the tumor cells and disregard the auxiliary cells present in the surrounding microenvironment that is also referred to as the stromal cells. These auxiliary cells, including myoepithelial cells, fibroblasts, myofibroblasts, endothelial cells, inflammatory cells, and bone-marrow-derived cells (BMDCs) such as macrophages, mast cells, neutrophils, and lymphocytes, are widely recognized to collaborate with cancerous cells and other host cells to create a tumor-permissive microenvironment capable of providing continuous support for tumor growth, progression, angiogenesis, invasion, and metastasis [3, 4].

Metastasis is the systemic dissemination of tumor cells at sites distinct from the primary lesion. It is a multistep process that involves detachment of cells from the primary tumor, followed by survival in the blood vessels or lymphatic system and finally development of secondary tumor. It is a poorly understood aspect of carcinogenesis that requires the clarification of the underlying cellular and molecular events that control the metastatic cascade from onset to colonization [5]. It is undisputed that metastasis of tumor cells is mediated by the reciprocal interplay between tumor cells and stromal cells and the extracellular matrix (ECM).

In this paper, we discuss the different steps of breast cancer progression and delineate the importance of the myoepithelial cell layer disruption for invasion of tumor cells. We also address the tumor promoting effect of the stromal cells in each step of the metastatic cascade of breast cancer.

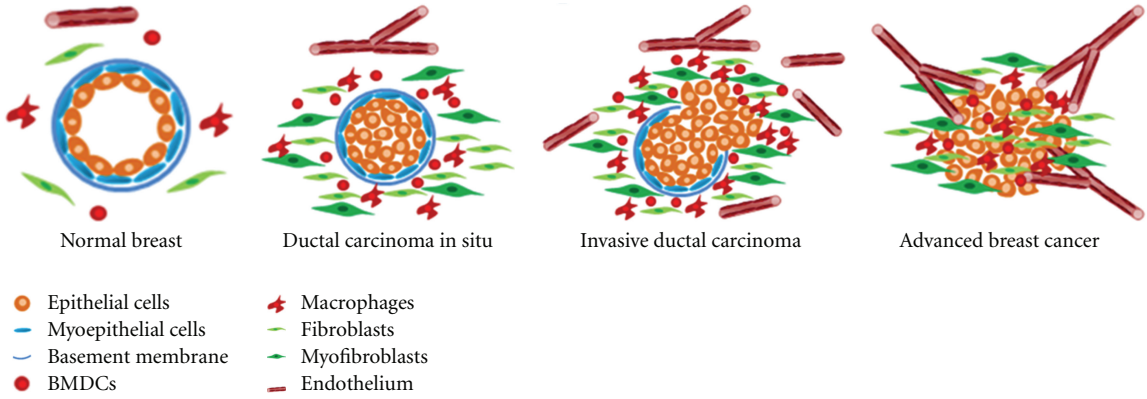


Figure 1: Schematic presentation of breast cancer progression accompanied with stromal cells. Normal breast duct is composed of a layer of epithelial cells and a layer of myoepithelial cells separated from the stroma by a basement membrane. Stromal cells include fibroblasts, BMDCs, endothelial cells, and other cells. Ductal carcinoma in situ (DCIS) is associated with luminal epithelial cells proliferation, and recruitment and expansion of stromal cells. In invasive ductal carcinoma, the myoepithelial cell layer is degraded with the underlying basement membrane and cancerous cells invade the surrounding microenvironment. Advanced breast cancer is associated with complete loss of myoepithelial cell layer and basement membrane, invasion of epithelial cells, proliferation of stromal cells, and angiogenesis.

## 2. Evolution of Breast Cancer

The development of breast cancer involves the progression via a series of intermediate hyperplastic lesions with and without atypia (atypical ductal hyperplasia, atypical lobular hyperplasia, and usual ductal hyperplasia) followed by subsequent evolution into in situ carcinoma, for example, ductal carcinoma in situ (DCIS) and lobular carcinoma in situ (LCIS), invasive carcinomas, and metastatic cancers (Figure 1) [6–9]. In atypical hyperplasia, the breast cells are abnormal in number, size, shape, appearance, and growth pattern that may be seen as an excessive growth of cells of the ducts (atypical ductal hyperplasia) or the cells of the lobules (atypical lobular hyperplasia). In usual ductal hyperplasia, the breast tissue has an increased number of benign cells within the duct. DCIS is thought to be a precursor of invasive ductal carcinoma, in which tumor cells are confined to the lumen of the mammary duct. Lobular carcinoma in situ consists of a noninvasive increase in the cells of the milk-producing lobules of the breast. Normal breast ducts are composed of a layer of epithelial cells physically separated from the normal microenvironment by a basement membrane and myoepithelial cell layer [10, 11]. In situ carcinoma is characterized by intact myoepithelial cell layer and basement membrane, and proliferation of epithelial cells [10, 11]. When the breast tissue undergoes focal disruption of the myoepithelial cell layer and degradation of the underlying basement membrane, tumor cells invade surrounding tissues and migrate to distant organs, eventually leading to metastasis [10–12]. Despite the dramatic improvement in our ability to detect carcinomas in situ (DCIS), our understanding of the pathophysiology of this disease and factors involved in its progression to invasive carcinoma lags far behind.

## 3. Myoepithelial Cells at a Glance

The normal breast tissue is comprised of two major compartments, the epithelium and the stroma. Myoepithelial cells together with luminal cells constitute the epithelium of the ducts and of the lobule of the mammary gland. The anatomical position of myoepithelial cells between the stroma and the luminal epithelial cells from which cancer arises facilitates proper communication between both compartments. They express a number of tumor suppressor proteins (maspin), ECM structural proteins (fibronectin, collagen), proteinase inhibitors (tissue inhibitor of metallopreoteinase-1, TIMP-1), and angiogenic inhibitors (thrombospondin-1) [13, 14]. They also downregulate the expression of matrix metalloproteinases (MMPs) in fibroblasts and tumor cells [15], contribute significantly to basement membrane production, and accumulate ECM rather than degrade it [14, 16, 17]. The aforementioned functions suggest that the normal myoepithelial cell layer is a natural paracrine tumor suppressor that physically and functionally inhibits tumor growth, invasion, and angiogenesis. This tumor suppressive phenotype was identified based on the ability of myoepithelial cells to secrete paracrine factors (such as bFGF, TGF-$\alpha$, and IL-6) that inhibit the growth and invasion of breast cancer cells in coculture assays *in vitro* [18–20]. Collective evidence suggests that myoepithelial cells also function as autocrine tumor suppressor that is supported by their resistance to transformation and their tendency to transform to tumors of low malignancy [19, 21]. Due to their tumor suppressor potential, myoepithelial cells have been referred to as the "Cinderella" of the breast [22]. Myoepithelial cells surround both normal ducts and precancerous lesions of the breast, for example, DCIS. However, DCIS myoepithelial cells differ from their normal counterparts in their ability to polarize luminal cells in three-dimensional collagen assays [16, 23], which indicates that tumor-derived myoepithelial cells are unable to transmit the necessary and correct signals to luminal cells. Moreover, myoepithelial cells isolated from normal tissue have distinct gene expression pattern as compared to DCIS myoepithelial cells. The former express high levels of laminin, tenascin, thrombospondin, cytokeratins, oxytocin receptor and tropomyosin, whereas DCIS myoepithelial cells

show overexpression of proteases (cathepsins, MMP-2, and PRSS11), protease inhibitors (thrombospondin 2, SERPING1, cystatin C, and TIMP3), and collagens [24]. A common diagnostic feature of breast cancer progression from in situ to invasive tumor is the aberration of the fully differentiated myoepithelial cell layer suggesting that dissolution of the myoepithelial cell layer is an absolute prerequisite for tumor invasion. However, it is unknown what mechanisms lead to focal myoepithelial cell layer disruption and its contribution to tumor progression. Studies by Man and Sang revealed that focal myoepithelial cell layer disruptions are associated with higher leukocyte infiltration supporting the release model which is proposed by Polyak el al. to describe the role of stromal and myoepithelial cells in invasion onset [11].

## 4. Microenvironmental Influences on Breast Cancer Development

The tumor microenvironment or the stroma is composed of extracellular and cellular tissue network that surrounds and interacts with tumor cells. The cellular part includes fibroblasts, myofibroblasts, endothelial cells, adipocytes, and various immune cells [25, 26]. These cells are surrounded by an ECM that is a dynamic three-dimensional structure composed of many components including collagens, laminin, and fibronectin. The ECM is also a rich source of matrix metalloproteinases (MMPs) and soluble growth factors that affect neoplastic dissemination [27]. A specialized ECM, called basement membrane, is made of several glycoproteins and proteoglycans and separates the epithelial and endothelial cell layers from the surrounding microenvironment [28]. A well-organized basement membrane acts as a gatekeeper of invasive phenotype providing a physical support, a signaling intermediate between different compartments and a regulator of cell behavior. During tumor development, cancer cells become in direct contact with a remodeled stroma that was long considered to be a passive responder to the malignant transformation [4]. The significance of the tumor microenvironment as an active contributor in promoting and initiating breast cancer development is proposed. The difference in molecular signatures between stromal cells from tumors and normal tissues bear witness that stromal cells provide cues for tumorigenesis.

In contrast to normal fibroblasts, cancer-associated fibroblasts (CAFs) [29] enhance tumor growth and metastasis through the production of growth factors and ECM proteins and modulating immune polarization [30]. They also have different gene signatures related to paracrine signaling, transcriptional regulation, extracellular matrix, cell-cell interaction and cell adhesion/migration such as wnt1 inducible signaling pathway protein 1 (WISP1), kruppel like factor 4 (KLF4), TGF$\beta$2, fibulin1 (FBLN1), plasminogen activator inhibitor 2 (PAI2), and tissue plasminogen activator (PLAT) [29, 31]. Recently, Tyan et al. found that human breast cancer cells dramatically affected surrounding fibroblasts. They induced hepatocyte growth factor (HGF) production by fibroblasts to support their own growth and progression [32]. It is noteworthy to mention that a model which delineates the role of tumor microenvironment in breast cancer initiation and progression has been proposed. This model or Reverse Warburg effect suggested that tumor cells can induce an oxidative stress on neighboring fibroblasts which promotes stromal autophagy associated with Caveolin-1 loss and elevated cytokine production. This, in turn, results in production of nutrients that can nourish anabolic tumor cells [33, 34].

Tumor stroma also includes myofibroblasts, which are activated fibroblasts with $\alpha$-smooth muscle actin ($\alpha$-SMA) expression. In human tissue sections with invasive breast cancer, higher proportion of myofibroblasts were associated with higher-grade upregulation of Ki-67, VEGF, and bFGF, and shorter overall survival and relapse-free survival [35]. Tumor invasion and angiogenesis was shown to be promoted by $\alpha$-SMA-positive myofibroblasts and not by $\alpha$-SMA-negative fibroblasts [36]. Stromal myofibroblasts promote tumorigenesis of oral squamous cell carcinomas, for example, by secreting activin A [37]. In the tumor-stroma interactive microenvironment transforming growth factor-beta 1 (TGF-beta 1) promotes stromal fibroblast-to-myofibroblast transdifferentiation by modulating phenotypic and functional genes. For example, MiR-21 was recently shown to participate in TGF-b1-induced myofibroblast transdifferentiation by targeting and downregulating programmed cell death 4 (PDCD4) gene [38, 39]. Furthermore, cancer-cell-derived TGF-b release proangiogenic vascular endothelial growth factor A (VEGFA) from the myofibroblasts in esophageal squamous cell to regulate angiogenesis [40].

Implicated with angiogenesis, endothelial cells are recruited to the tumor microenvironment where they enhance neovascularization and metastasis. Adipose tissue, composed of adipocytes, has long been associated with cancer development. Coculture of adipocytes with cancer cells resulted in increased invasiveness of cancer cells and modified phenotype of the adipocytes characterized by lower lipid accumulation, decreased expression of adipocyte markers, and overexpression of proteases (MMP-11) and proinflammatory cytokines (IL-6, IL-1$\beta$) [41]. Il-6 depletion from adipocytes inhibited the invasion and migration of breast tumor cells [42].

The stromal compartment also contains various bone-marrow-derived cells (BMDCs) such as macrophages, mast cells, neutrophils, and lymphocytes that are recruited by the primary tumor cells to increase tumor cell migration, angiogenesis, and invasion [3]. At sites of focal disruptions of the myoepithelial cell layer in breast tissues, immune infiltrates were observed at the invasive front suggesting a potential role of these cells in malignancy and subsequent metastatic spread [10, 11].

To metastasize, tumor cells are required to escape the primary tumor, intravasate the blood stream or lymphatic circulation, survive in the vasculature, extrude from the blood vessels or lymphatic system, arrest at distant sites, and develop into secondary mass. At each step of the metastatic cascade, stromal cells appear to be crucial players in the transition from benign to invasive and finally metastatic disease (Figure 2) [5, 43]. The metastatic cascade is a quite inefficient process meaning that failure to complete any

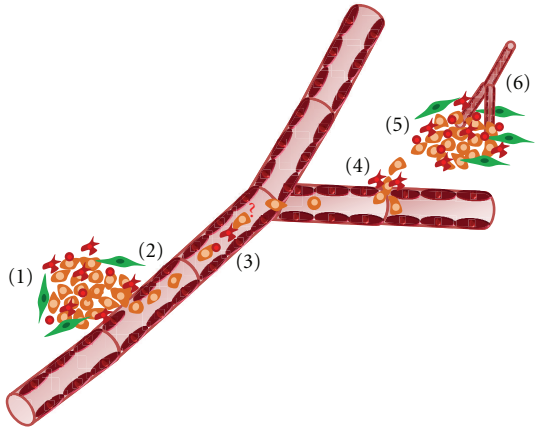


Figure 2: Stromal cells involved in metastatic cascade. (1) Myofibroblasts, fibroblasts, and macrophages and other BMDCs play a major role in promoting primary tumor growth. (2) Intravasation is enhanced by the paracrine interactions between tumor cells and macrophages. (3) The fusion of tumor cells and macrophages is questionable (?) and may promote the survival of the tumor cells in vasculature. (4) During extravasation, direct interaction of tumor cells and macrophages enhance tumor cell egress of the vessels. (5) BMDCs and myofibroblasts stimulate tumor cell metastatic dissemination. (6) The recruitment of endothelial cells, myofibroblasts, and BMDCs to the tumor site increases vascularization.

step will quench the whole process. Until now, it is unclear which step is the key rate-limiting one that contributes to the inefficiency of the metastatic lesion. Metastatic colonization has been suggested as a major rate-limiting step because intravenous injection of cancer cells resulted in about 90% arrest and extravasation with only 0.1% of cells growing at the secondary site [44, 45]. Extravasation was also thought to be a key rate-limiting step in metastasis with highly metastatic cells extravasating faster than poorly metastatic ones. However, a study by Koop et al. showed that extravasation is independent of the metastatic ability and highly metastatic ras-transformed cells extravasate at the same rate of control fibroblasts [46]. Similar to extravasation, intravasation was proposed to be a major rate-limiting step. *In vivo* data showed that intravasation can be a major rate-limiting step in the metastatic cascade. Wyckoff et al. showed that metastatic cells exhibited a faster entry into the vasculature than poorly metastatic cells. Moreover, Zijlstra et al. quantitatively evaluated the rate limiting steps in HEp-3 and HT-1080 human tumorigenic cells. The authors found that HEp-3 cells had higher metastatic rate than HT-1080 cells due to the lower efficiency of the latter cells in intravasation and metastatic colonization [47]. Collectively, intravasation and growth at secondary sites represent major rate-limiting steps in the metastatic cascade. However, the rate-limiting step may vary depending on the tumor type [45].

*4.1. Primary Tumor Growth.* Under normal physiological conditions, the surrounding microenvironment imposes proper tissue architecture maintained by basement membrane alignment and intercellular communication [3]. This interplay between epithelial cells and surrounding stroma maintains organ homeostasis that serves as a protective constraint against malignant transformation. During tumor development, cancerous cells circumvent the normal controls regulating the activity of ECM proteases. In response to these proteolytic enzymes, the basement membrane undergoes gradual degradation and structural changes causing violation of normal tissue boundaries and conduits for malignant cell egress. To invade, tumor cells should lose cell-cell and cell-ECM interactions mediated by integrins and cadherins. Invasion is also accompanied by proteolytic degradation of surrounding tissue mediated by proteases, motility of tumor cells mediated by chemokines and growth factors, and recruitment of stromal cells.

Fibroblasts play an important role in cancer progression. They are primarily responsible for the synthesis, deposition, and remodeling of the basement membrane and ECM through the production of collagen (I, III, IV, and V), fibronectin, and laminin. They are also an important source of paracrine growth factors (HGF, EGF, FGF2, and TGF$\beta$), proteolytic enzymes (MMP-1, MMP-7), and cytokines (IL6, CXCL12) that can affect cell proliferation, survival, morphology, and death [48, 49]. During tumorigenesis, fibroblasts maintain an activated phenotype characterized by the expression of $\alpha$-smooth muscle actin ($\alpha$-SMA) and are referred to as carcinoma-associated fibroblasts (CAFs) or myofibroblasts [49]. In breast carcinomas, about 80% of stromal fibroblasts acquired the CAF-activated phenotype [48]. During tumor development, CAFs exhibit a higher proliferation index and become the predominant cell population in the stroma. Once the basement membrane is degraded, CAFs are accumulated causing expansion of tumor stroma and increased deposition of ECM through expression of stress fibers and $\alpha$-SMA. This phenotype is termed desmoplasia and is associated with recruitment of inflammatory cells and activation of angiogenesis [50]. Several studies demonstrated a direct involvement of fibroblasts in initiation of cancer. In a xenograft mouse model, Kuperwasser et al. showed that the upregulation of transforming growth factor-$\beta$ (TGF$\beta$) or hepatocyte growth factor (HGF) in mouse fibroblasts stimulated the initiation of benign and malignant lesions in the breast epithelium [51]. In addition, a gene expression profiling study of all cell types in normal and neoplastic breast demonstrated that overexpression of CXCL12 in myofibroblasts was correlated with epithelial cell proliferation and invasion [23]. Another study of xenograft mouse model coinjected with MCF-7 breast cancer cells and CAFs or normal fibroblasts showed that xenografts infused with CAFs had enhanced growth than xenografts injected with normal fibroblasts [52]. To reconcile, these data reveal the major role of CAFs in tumor initiation and progression.

An initial reaction of the host to the tumor development is the recruitment of leukocytes and subsequent local inflammation [53]. As a physiological response to tissue injury, inflammatory cells are recruited to the injured site to support tissue repair and remodeling through the production of growth factors and cytokines such as TNF-$\alpha$, CCL2, CXCL8, CCL5, TGF-$\beta$ and so forth. In normal conditions, the inflammatory response is resolved once the tissue is repaired

[54, 55]. However, the tight regulation of inflammation is overridden during malignant transformation recalling the historic view of tumors as "wounds that never heal" [56]. Persistent inflammatory response characterized by activated leukocytes and secretion of several cytokines and chemokines (including tumor necrosis factor (TNF), interleukins, and interferons) elaborates the formation of tumor-promoting microenvironment. Such protumor stroma is incentive not only for primary tumor development but also for metastatic dissemination into systemic circulation.

Neoplastic transformation is regulated by dynamic reciprocity between epithelial cells, activated stromal cells and ECM components. If the changes in the microenvironment occur prior or concomitant with epithelial cell changes is still debatable. Recently, a gene expression profiling study on laser captured breast stroma and epithelia showed 90% change in the stromal gene expression at the transition from normal to DCIS, and only 10% stromal alterations in DCIS compared to invasive disease [57, 58]. Another study used mRNA in situ hybridization of breast tissue with different stages of cancer to examine the expression of angiogenic factors and stromal components. A similar expression profile was observed in carcinoma in situ, invasive cancer, and metastatic disease [59]. These studies suggest that stromal changes induced by the emerging epithelial lesions precede invasion and that cancer cells invade into an abnormal breast microenvironment with growth-promoting effects.

*4.2. Intravasation.* Intravasation or penetration of tumor cells into the vasculature involves the movement of cancer cells through the ECM, the basement membrane, and finally through the endothelium of the blood vessel or lymphatic duct. The underlying molecular mechanisms governing intravasation are not clear as all studies have focused on later steps in the metastatic cascade. Detailed evaluation of all steps in tumor metastasis is crucial to understand the cellular mechanisms controlling neoplastic dissemination. In a murine breast cancer model, Yang et al. found the transcription factor, Twist, as a key regulator of metastasis [60]. It augments epithelial-to-mesenchymal transitions and promotes the rate of hematogenous intravasation. Another possible mechanism that executes the migration of tumor cells across the vessel wall was shown by intravital imaging studies of experimental mammary carcinomas [61, 62]. These studies showed that carcinoma cells intravasate through the blood vessels due to chemoattractive gradients generated by perivascular macrophages that are recruited by the tumor cells to the injured site [61]. In breast carcinomas, macrophages and cancerous cells form a paracrine loop involving epidermal growth factor (EGF) and colony stimulating factor-1 (CSF-1) to augment chemotaxis and intravasation [63]. EGF produced by macrophages promotes migration of neoplastic cells into hematogenous vasculature through its interaction with EGF receptor expressed on breast cancer cells. Tumor cells, in turn, express CSF-1 which acts as a potent chemoattractant for CSF-1 receptor positive macrophages [64]. This crosstalk lends credence to the collaborative work between tumor microenvironment and neoplastic cells at the site of intravasation.

*4.3. Survival in Vasculature.* Once malignant cells have invaded the angiogenic vasculature, they are subject to harsh microenvironment characterized by hemodynamic shear forces, surveillance of immune cells, and lack of substratum [43]. To bypass these perils, tumor cells use platelets as a shield. Through their tissue factor, tumor cells bind coagulation factors (VIIA and X) on the platelets creating an embolus that arrests in the capillaries [65, 66]. These aggregates protect the cancerous cells from immune-cell-mediated lysis and decrease the shear forces of the blood circulation, thus increasing their survival, arrest, and extravasation [67]. Whether macrophages can infer protective effects on neoplastic cells in the bloodstream as they do during invasion and intravasation is not yet known. To explain the metastatic phenotype, Pawelek and Chakraborty proposed the fusion of macrophages or other BMDCs with cancer cells forming a hybrid capable of surviving in the circulation and homing to secondary sites [68]. Should the fusion theory be accepted in human cancers, BMDCs would have a beneficial role in the survival of tumor cells in the vasculature.

*4.4. Extravasation.* After survival and arrest in the circulation, tumor cells must escape out of the blood and lymphatic vessels in a process known as extravasation. To do so, tumor cells induce disruptions in the endothelial junctions that allow tumor cells to bind to the subendothelial ECM and extrude into target organs. Vascular permeability is mediated by activated Src kinases in endothelial cells, which once exposed to vascular endothelial growth factor (VEGF) from tumor cells promotes endothelial retraction, resulting in movement of cancer cells towards surrounding tissues [69]. To identify the role of macrophages in metastasis, Qian et al. used an animal model of breast cancer metastasis and an intact *ex vivo* lung imaging system to show that modified host macrophages are required for proper metastatic seeding and growth [70]. The authors found that macrophage ablation dramatically decreased the number of tumor cells observable in the lungs [70]. Moreover, lung-resident macrophages were visualized to physically interact with tumor cells as soon as they extravasate through the vessel walls, thus promoting the rate of extravasation and metastatic dissemination [70]. Immunophenotyping of metastasis-associated macrophages showed distinct profile from lung resident macrophages. The prometastatic macrophages are characterized by cell surface expression of CSF1R, CD11b, F4/80, with high levels of CCR2, CX3CR1, and VEGFR, absence of Gr1 and low CD11c [70, 71].

*4.5. Metastatic Site.* Metastasis is regulated not only by changes in tumor cells but also by reciprocal interactions with the surrounding microenvironment. In his "seed and soil" hypothesis, Paget proposed that tumor cells or "seeds" can only colonize microenvironments or "soils" that are compatible with their growths [72]. For example, breast cancers metastasize to lungs, bone, liver, and brain, whereas advanced prostate cancers colonize the bone as the predominant site [45]. Since the circulatory patterns provide only partial explanation for the tissue tropism aspect of

metastasis, several molecular and cellular mechanisms have been proposed. An emerging paradigm suggests that primary tumor cells may secrete factors capable of inducing a fertile microenvironment, termed premetastatic niches, that favors the seeding and proliferation of metastatic cells at unique sites [73]. For example, (ADAMTS1) and matrix metalloproteinase-1 (MMP1) participate in a paracrine signaling cascade that includes the release of metastasis membrane-bound epidermal-growth-factor (EGF-) like growth factors, amphiregulin (AREG), heparin-binding EGF (HB-EGF), and transforming growth factor alpha (TGF alpha) from tumor cells resulting in a downregulation of osteoprotegerin expression in osteoblasts and therefore modulating brain microenvironment in favor of osteoclastogenesis and bone metastasis [74]. Recent work have identified the following mediators of extravasation and brain colonization: the cyclooxygenase COX2, the epidermal growth factor receptor (EGFR) ligand, HBEGF, and the alpha 2,6-sialyltransferase, ST6GALNAC5, which were found to enhance breast cancer passage through the blood brain barrier and to facilitate their adhesion to brain endothelial cells [75]. EGFR ligands and COX2 are also linked to breast cancer infiltration of the lungs [76, 77]. Recently, breast cancer cells infiltrating the lungs were shown to support their own metastasis-initiating ability by expressing tenascin C (TNC). TNC is an extracellular matrix protein of stem cell niches that promotes the survival and outgrowth of pulmonary micrometastases via upregulation of stem cell signaling components, musashi homolog 1 (MSI1), and leucine-rich repeat-containing G protein-coupled receptor 5 (LGR5) until the tumor stroma takes over as a source of TNC [78]. Through the production of growth factors (VEGFA) and chemokines (S100A8, S100A9), tumor cells induce the recruitment of BMDCs, and endothelial progenitor cells to the premetastatic niche [79]. The bone marrow progenitors are VEGF receptor-1 (VEGFR1) positive and can migrate and proliferate in response to tumor-derived VEGF [79]. These VEGFR1$^+$ cells also express integrin VLA-4 and tend to form clusters induced by integrin-fibronectin interactions, the latter of which is synthesized by resident fibroblasts [73]. Several other molecules have been implicated in preparing the premetastatic niche and increasing metastasis. Matrix metalloproteinase-9 (MMP-9) secreted by BMDCs degrades the basement membrane and liberates the matrix-sequestered VEGFR1 ligand, the VEGFA, promoting the homing of more VEGFR1$^+$ cells into the niche [80]. Through the production of VEGFA, TGF$\beta$, and tumor necrosis factor-$\alpha$ (TNF$\alpha$), tumor cells enhance the expression of the chemoattractants S100A8 and S100A9 in lung endothelium and myeloid cells, which in turn promote tumor cell homing and adhesion to the metastatic site [81, 82]. The role of activated fibroblasts in metastasis has been revealed in some studies. Fibroblast-specific protein-1 (FSP-1/S100A4), a fibroblast-specific marker, is highly expressed on tumor-associated fibroblasts and is released upon stimulation of fibroblasts by tumor cells. Mice deficient in FSP-1 exhibited a significant reduction in tumor growth and metastasis [83]. Injection of FSP-1-positive fibroblasts into these mice restored the ability of mammary adenocarcinoma cells to develop tumors and generate metastasis suggesting a potential role of tumor-associated fibroblasts in the metastatic dissemination. Another study found that only metastasized melanoma cells were affected by fibroblasts suggesting that fibroblasts might be important in creating the permissive soil that supports tumor cell growth at distant sites [84]. Recently, O'Connell and colleagues found that S100A4$^+$ fibroblasts provide the proper metastatic niche to support metastatic colonization. Through production of VEGFA and tenascin-C, fibroblasts can promote angiogenesis as well as provide protection against apoptosis, respectively [85].

Following the implantation of tumor cells, persistent growth of metastasis is maintained by the establishment of sufficient blood supply capable of providing the necessary oxygen, growth factors, nutrients, and metabolites. Blood vessels are composed of vascular basement membrane, endothelial cells and specialized smooth muscle cells, the pericytes [28]. The induction of a tumor vasculature termed the angiogenic switch requires basement membrane assembly, recruitment and proliferation of endothelial precursors, and pericytes attachment [86]. Initially, the vascular basement membrane is degraded by several MMPs produced by stromal cells, endothelial cells, or tumor cells [87]. This basement membrane degradation causes the release of endothelial cells to migrate and proliferate, the liberation of matrix-sequestered growth factors such as VEGF, basic fibroblast growth factor (bFGF), and platelet-derived growth factor (PDGF) and the disassembly of the pericytes that line the blood vessels [87]. In response to VEGF, VEGFR2$^+$ endothelial progenitor cells are recruited to the metastatic site through VEGFA signaling to contribute to vessel formation [88]. Analysis of these progenitor cells shows upregulation of several angiogenic molecules (VEGF, FGF, PDGF, CXCL1, etc.) that further bolster local angiogenesis and subsequent metastatic colonization [86]. Moreover, VEGFR1$^+$ BMDCs has been shown to produce several angiogenic factors and are required to provide stability to the neovessels [89]. Inhibition of VEGFR1$^+$ BMDCs either during primary tumor or after the formation of premetastatic niche caused the prevention of endothelial cell migration and metastasis [79]. Thus, the recruitment of VEGFR2$^+$ endothelial progenitor cells into vessels requires the incorporation of VEGFR1$^+$ BMDCs to support neovascularization [60, 73]. Tumor angiogenesis is also regulated by several immune cells [90]. Macrophages, for example, are a good source of angiogenic factors such as VEGF and MMP-9 [91]. In a mouse model of highly aggressive metastatic mammary carcinoma, Lin and Pollard found that tumor-associated macrophages may provide essential cues to press the angiogenic switch [91]. Colony stimulating factor-1 (CSF-1) deletion caused failure in macrophage homing to the malignant stroma that was associated with attenuated angiogenic responses, decreased neoplastic progression and inhibition of pulmonary metastasis. Moreover, CAFs have been shown to be actively involved in boosting tumor angiogenesis. The coinjection of CAFs and MCF-7 breast cancer cells into nude mice resulted in the recruitment of bone-marrow-derived endothelial progenitors in response to CAF-derived stromal cell derived factor (SDF1/CXCL12) stimulating angiogenesis and tumor formation [52]. Another

proangiogenic mechanism of CAFs involves the release of several factors such as VEGF and FGF which can positively contribute to vascularization [48].

## 5. Perspectives

It is evident that metastasis is a multistep process where each stage requires an intricate interplay between cancerous cells and cells of the microenvironment. This tumor-host crosstalk supports the notion that cotargeting cancer cells and tumor stromal cells will be a viable approach for mammary cancer prevention and treatment. Researchers are dedicated to explore the stromal cells as an effective target for anticancer therapeutics. Such host-targeted therapies should be directed towards BMDCs, fibroblasts, and endothelial cells that home to the metastatic site to support tumor dissemination and outgrowth. It is important to inhibit the mobilization and proliferation of the stromal cells and to disrupt tumor-stroma interactions mediated by paracrine factors. To achieve these goals, several agents have been investigated and they fall into several categories including protease inhibitors (e.g., MMP inhibitors), antiadhesive molecules (e.g., anti-integrin peptides or antibodies), signal pathway modulators (e.g., tyrosine kinase pathway inhibitors), antifibrotic drugs (e.g., pirfenidone), and antiangiogenic molecules (e.g., VEGF and bFGF antagonists) [92, 93]. Clinical trials using MMP inhibitors (MMPIs) were disappointing for several reasons. The trials were conducted only on patients with advanced disease, and MMPIs used were broad spectrum MMPIs which can block bad MMPs as well as good ones. Avastin/bevacizumab, a monoclonal antibody targeted against all isoforms of VEGF-A, has recently been withdrawn from the FDA list for the treatment of breast cancer [94]. Unlike trastuzumab which is a HER-2-targeted antibody, avastin delayed tumor progression with no improvement in overall survival. This was accompanied by adverse side effects including hypertension, neuropathy, and infection [95]. Recently, a variety of studies have been conducted to target the tumor microenvironment. Wu et al. have shown that targeting Galectin-1 to significantly inhibit CAF-conditioned medium-induced tumor cell migration and invasion in oral squamous cancer cells (OSCCs) resulting in a reduced metastasis *in vivo* [96]. It is followed that Galectin-1 down-regulation reduces the production of monocyte chemotactic protein-1 (MCP-1/CCL2) which promotes the migration of OSCCs by binding to CCR2 receptor. Blocking the interaction between MCP-1 and CCR2 abolishes migration. Moreover, Kim et al. have proposed to target myofibroblasts overexpressing laminin-332 which caused the formation of the dense fibrosis via desmoplastic reaction during epithelial to mesenchymal transition (EMT) [97]. This alteration of the tumor microenvironment preceded tumor invasion and was found in invasive ductal carcinoma. Targeting of laminin-332 overexpressing myofibroblasts was supposed to prevent the formation of the dense fibrosis, thus inhibiting the invasion-friendly stromal alteration. It is important to note that Angiotensin-(1–7), an endogenous 7-amino acid peptide hormone of the renin-angiotensin system, has been shown to target the tumor microenvironment to inhibit CAF growth and tumor fibrosis [98]. Additionally, Liu et al. proposed the targeting of the coagulation cascade that is activated in the tumor microenvironment and presented preclinical data targeting tissue factor (TF), an enzyme cofactor in activating coagulation that plays a critical role in tumor growth [99]. TF inhibition by TF:FVIIa inhibitor led to growth retardation in tumor models. Coenegrachts et al. demonstrated that the selective neutralization of host-derived bone-derived placental growth factor (PlGF) by anti-mouse alphaPlGF reduced the engraftment of tumor cells in the bone, inhibited their interaction with matrix components, reduced the incidence, number, and size of bone metastases, and preserved bone therefore inhibiting both the progression of metastasis and the settlement of tumor in the bone [100]. Truitt et al. have shown the role that Eph receptor tyrosine kinase EphB6 plays in suppressing cancer invasiveness through c-Cbl-dependent signaling, morphologic changes, and cell attachment and that its targeting might enable the regulation of both cell attachment and migration [101]. These stroma-targeted therapies combined with antitumor approaches will be translated into a double-edged sword that cancerous cells will not easily survive. These therapeutic approaches require a full understanding of the cellular and molecular mechanisms governing the tumor-host interactions, accompanied with the development of new mouse models and intravital imaging techniques. Once accomplished, cancer patients will experience better survival rates and quality of life.

## Conflict of Interests

The authors declare that no conflict of interests exists.

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*Review Article*

# RKIP Suppresses Breast Cancer Metastasis to the Bone by Regulating Stroma-Associated Genes

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In the past decade cancer research has recognized the importance of tumorstroma interactions for the progression of primary tumors to an aggressive and invasive phenotype and for colonization of new organs in the context of metastasis. The dialogue between tumor cells and the surrounding stroma is a complex and dynamic phenomenon, as many cell types and soluble factors are involved. While the function of many of the players involved in this cross talk have been studied, the regulatory mechanisms and signaling pathways that control their expression haven't been investigated in depth. By using a novel, interdisciplinary approach applied to the mechanism of action of the metastasis suppressor, Raf kinase inhibitory protein (RKIP), we identified a signaling pathway that suppresses invasion and metastasis through regulation of stroma-associated genes. Conceptually, the approach we developed uses a master regulator and expression arrays from breast cancer patients to formulate hypotheses based on clinical data. Experimental validation is followed by further bioinformatic analysis to establish the clinical significance of discoveries. Using RKIP as an example we show here that this multi-step approach can be used to identify gene regulatory mechanisms that affect tumor-stroma interactions that in turn influence metastasis to the bone or other organs.

## 1. Introduction

Under normal physiological conditions, the stromal compartment of epithelial tissue regulates homeostasis by maintaining the proper architecture and nutrient levels required for epithelial function. It also serves as an important barrier to cell transformation. However in response to lesions (i.e., wounding) the stromal compartment undergoes changes including the recruitment and activation of fibroblasts, immune, and endothelial cells that in turn provide growth, and matrix remodeling factors, as well as a new blood supply. Similar changes in the stromal compartment have been shown to occur during tumor growth and the importance of the stromal compartment, called "the tumor microenvironment," in modulating and driving cancer progression has become increasingly evident [1]. The tumor microenvironment has become the subject of intense therapeutic and prognostic interest as its phenotypic and molecular characteristics have been correlated with disease-free survival in multiple tumor types [2].

It is believed that during the first phase of carcinogenesis the tumor microenvironment initially reacts to suppress malignant transformation by maintaining tissue architecture and differentiation. As cancer progresses, however, the local stromal compartment shifts to an activated, growth-promoting state, in many ways similar to an inflammatory state, which is initiated and maintained by continuous paracrine communication between stromal and tumor cells. Stromal components engage in a dynamic signaling circuit with primary tumor cells and coevolve with tumor cells to promote tumor progression to an invasive phenotype [3]. Various stromal components, including vascular cells, pericytes, fibroblasts, inflammatory cells, and extracellular matrix components participate in this cycle [4, 5]. A large number of activated myofibroblasts, characterized by the expression of

$\alpha$-smooth muscle actin ($\alpha$-SMA), are frequently found in the stroma of human breast carcinoma and are referred to as carcinoma-associated fibroblasts (CAFs). The precise cellular origin of these activated myofibroblasts is not clear but it has been shown that when inoculated with carcinoma cells, CAFs can promote tumor growth in mouse xenograft models [6]. CAFs secrete high levels of stromal cell-derived factor-1 (SDF-1 or CXCL12), a chemokine that can activate its cognate receptor, CXCR4, which is expressed by many carcinoma cells, and stimulate their proliferation. On the other hand SDF-1 can mediate recruitment of endothelial progenitor cells thus promoting angiogenesis, and it has also been implicated in an autocrine signaling loop that promotes differentiation of normal stromal fibroblasts into myofibroblasts [7].

A number of other cytokines, chemokines, and growth factors secreted by cancer cells themselves or by tumor-associated stromal cells have been shown to sustain tumor cell proliferation and progression through different mechanisms. The list of these autocrine/paracrine factors is constantly growing and includes vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), transforming growth factor-$\beta$ (TGF-$\beta$), hepatocyte growth factor (HGF), interleukin-6 (IL-6), and osteopontin (OPN) [8]. An important component of this signaling loop is the recruitment and activation of bone marrow-derived myeloid cells (BMDCs), including macrophages, monocytes, mast cells, and neutrophilis. BMDCs have been shown to play a major role in the development and growth of the primary tumor and also in the subsequent hematological dissemination [9]. BMDCs can in fact contribute to the induction of angiogenesis by activating endothelial cells and are recognized as major determinants of tumor invasion. Secretion of different classes of proteases (matrix metalloproteinases, cathepsins, and serine proteases), produced by stromal and/or tumor cells, has been shown to facilitate cancer cell migration by disrupting cell-cell junctions and promoting invasion of the surrounding tissues by proteolytic degradation of the extracellular matrix (ECM) and the basement membrane.

Metastasis is the primary cause of mortality in breast cancer patients and can emerge many years after the removal of the primary tumor. Metastatic progression is a complicated multistep process which includes at least three discrete stages: (1) epithelial-mesenchymal transition (EMT) leading to migration, invasion, and intravasation; (2) circulation, transportation, and extravasation of cells, which then undergo mesenchymal-epithelial transition (MET); (3) colonization of tumor cells within distal tissues including bone and lung [10]. The efficiency of each of these steps on the way to metastasis is highly affected by interactions with a distinct local microenvironment. Cancer cells interact with an activated stroma during the initial phases of invasion and intravasation, with the bloodstream during hematological dissemination, and finally with the metastatic sites during extravasation and colonization. It is generally believed that each of these stages is highly inefficient, and, in particular, only a very small percentage of the tumor cells that enter the circulatory system are able to colonize and form a tumor at distal sites. This concept highlights the fact that healthy tissues exert a protective function toward invading cancer cells and ensure that order is preserved within the tissue through homeostatic mechanisms. Cancer cells that escape this protective function and are able to modify the surrounding stroma to their own advantage are the ones that will eventually succeed in colonizing new organs.

Many studies have highlighted the concept of tissue tropism: although the blood flow pattern certainly contributes to preferred metastatic sites of specific carcinomas, the complex molecular mechanism of homing metastatic cells is also determined by interactions with the microenvironment at target organs. A number of molecular mediators of this interaction have been revealed by recent publications, and gene expression profiling studies have generated distinct gene expression signatures for organ-specific metastatic variants [10–13]. A major role in the tropism of metastatic cells to different organs is exerted by chemokines and their cognate receptors [14]. Local expression in target tissues is believed to guide metastatic cells to specific destinations as a result of local chemotaxis in combination with induction of invasive properties. As a homing mechanism, metastatic breast cancer cells specifically express functional CXCR4 and CCR7 receptors that induce actin polymerization, formation of pseudopodia, and chemotaxis for directional migration [14]. Interestingly, their respective ligands SDF-1 and CCL21 are mainly distributed in organs that represent the main site of breast cancer metastasis, in particular bone.

Breast cancers metastasize to lung, liver, bone, and brain. Bone metastasis is very common among late-stage breast cancer patients but current treatment methods for bone metastasis are mainly palliative, and more effective disease-modifying therapies are needed. Breast cancer frequently generates osteolytic bone metastasis by secreting a series of growth factors that influence bone matrix and bone stromal cells, tipping the balance to osteolytic bone destruction. In this context tumor-derived factors include angiogenic factors (FGF and VEGF), mediators of immune cell recruitment and activation (TGF$\beta$ and TNF$\alpha$), and mediators of fibroblasts activation (FGF and TGF$\beta$). Moreover cancer cells promote bone degradation by direct secretion of metalloproteinases (such as MMP1) and collagenase I or through indirect mechanisms by activating osteoclasts. Other tumor-derived cytokines and cell surface/ECM proteins like bone morphogenetic protein (BMP), interleukin-11 (IL-11), osteopontin (OPN), and endothelin-1 participate and feed this vicious cycle. In this scenario bone reabsorption by osteoclasts releases a number of growth factors embedded in the bone matrix including insulin-like growth factors (IGFs), TGF-$\beta$, platelet-derived growth factor (PDGF), and BMP which become part of this signaling circuit that push osteolytic lesions.

Gene expression profiling of a bone-tropic subpopulation of the breast cancer cell line MDA-MB-231 has revealed a "bone metastasis signature" (BMS) [11]. As expected, the most highly overexpressed genes in the BMS encode mostly cell surface and secreted proteins that alter the bone microenvironment in order to facilitate growth of metastases and formation of osteolytic bone lesions as described above. The BMS includes OPN, connective tissue growth factor (CTGF), fibroblast growth factor 5 (FGF5), the osteoclast-activating

cytokine IL-11, CXCR4, and MMP1 as well as many other genes. Expression of these genes in the primary tumor has multiple functions including: (i) targeting cells specifically to the bone microenvironment via homing factor CXCR4; (ii) facilitating colonization of the bone via expression of bone extracellular matrix degrading enzymes (MMP1, ADAMTS1); (iii) activating osteoclasts and favoring adhesion to the bone surface through OPN [15]. Overexpression of individual genes in the signature led to only a marginal increase in bone metastasis, whereas coexpression of multiple genes dramatically increases both the rate and incidence of bone metastasis [11]. This concept implies that these genes cooperate to push the metastatic phenotype and may not be highly effective if isolated from their signaling context. This observation also highlights the importance of understanding the master molecular mechanisms that regulate expression of genes in order to develop target therapies that affect their combined expression rather than an isolated component.

## 2. RKIP Defines Ways to Suppress Invasion and Metastasis

To understand the mechanisms by which metastasis is regulated, we have focused on identifying key signaling pathways that can inhibit breast cancer metastasis to the bone. Metastasis suppressors define a class of proteins that do not affect primary tumor growth but instead regulate one or more steps in the process leading to metastasis: invasion, intravasation, circulation, extravasation, and colonization of the secondary site [16]. Raf kinase inhibitory protein (RKIP) was initially shown to function as a metastasis suppressor in a prostate xenograft mouse model [17]. More recently, we have shown that RKIP also suppresses metastatic progression to bone in breast tumor xenografts [18]. Furthermore, we demonstrated that RKIP inhibits breast cancer invasion, intravasation, and bone metastasis via a signaling pathway involving induction of the microRNA let-7. Specifically, inhibition of the Raf/MEK/MAP kinase cascade by RKIP leads to inhibition of Myc activation. Myc is a transcriptional activator of LIN28, which in turn inhibits let-7 maturation. Consistent with the role of this signaling cascade, LIN28 has been implicated in breast cancer progression and let-7 functions as an inhibitor of breast tumor formation [19]. We also showed that let-7 inhibits invasion in part via suppression of the chromatin remodeling factor high mobility group AT-hook 2 (HMGA2). HMGA2 in turn activates Snail, a transcription factor that promotes the epithelial-mesenchymal transition (EMT), a process that favors the acquisition of an invasive phenotype. To understand how this upstream signaling cascade regulates genes that are involved in the crosstalk with the tumor microenvironment, thus affecting breast cancer metastasis to the bone, we sought to identify relevant metastatic genes that function downstream of the RKIP/let-7 axis.

As a means of identifying signaling pathways downstream of a key metastasis regulator in cancer, the Rosner and Minn groups developed a novel interdisciplinary approach that utilizes clinical data from breast tumors to generate and test hypotheses [20]. The basic idea is to determine whether a discrete set of genes are targets of inhibition by a metastasis suppressor, in this case RKIP. If RKIP inhibits expression of these genes, then their expression levels in breast tumors should inversely correlate with RKIP expression. Once we identified genes that inversely correlate with RKIP in patients' tumors, we tested them experimentally *in vitro* using breast tumor cell lines and *in vivo* using a xenograft mouse model. Finally, having determined which genes regulate metastasis in experimental breast tumor models, we validated their clinical significance by further bioinfomatic analysis using independent breast tumor data.

Using this approach, we identified a number of RKIP-regulated let-7 targets including HMGA2 and a novel target, BTB-and-CNC homology 1 (BACH1). A leucine zipper transcription factor, BACH1, has been linked previously to senescence and heme oxidation but has never been correlated to cancer progression [21]. Experimental validation using a xenograft mouse model confirmed that RKIP and let-7 suppress BACH1 and HMGA2 expression and showed that BACH1 promotes invasion, intravasation, and bone metastasis of breast cancer cells.

To test the hypothesis that RKIP is a potential regulator of genes implicated in the development of bone metastasis, we performed a similar bioinformatic analysis. We initially determined whether RKIP expression inversely correlates to the expression of bone metastasis signature (BMS) genes [11]. We assembled several cohorts of primary breast tumor expression array data and performed gene set analysis (GSA) correlating the expression levels of the set of BMS genes to RKIP expression. As expected, we found a negative correlation between RKIP and BMS genes in two independent gene expression data sets of 443 and 871 breast cancer patients [20]. Thus, when RKIP is expressed, BMS genes show low expression levels and vice versa. Having found a significant correlation, we experimentally tested five genes that were previously implicated as promoting breast tumor bone metastasis by regulating interactions of cancer cells with the stroma. Of these, we were able to demonstrate that RKIP inhibits expression of MMP-1, CXCR4, and OPN thus affecting the ability of metastatic cells to create an osteolytic bone environment via crosstalk with stromal cellular and noncellular components.

Finally, we determined experimentally the relationship between RKIP, let-7, the two let-7 targets, HMGA2 and BACH1, and the three BMS genes. Interestingly, knockdown of BACH1 suppressed the BMS genes MMP1 and CXCR4 but not OPN while HMGA2 knockdown suppressed CXCR4, and OPN but not MMP1. Additionally, we could partially reverse the effects of HMGA2 and BACH1 knockdown on invasion and metastasis by overexpressing their target BMS genes MMP1, CXCR4 and OPN. The simultaneous overexpression of the three BMS genes together showed a more profound effect on the metastatic phenotype compared to the overexpression of a single gene. These results suggest that the coordinate regulation of genes with different metastasis-promoting functions is a prerequisite for efficient metastatic spread.

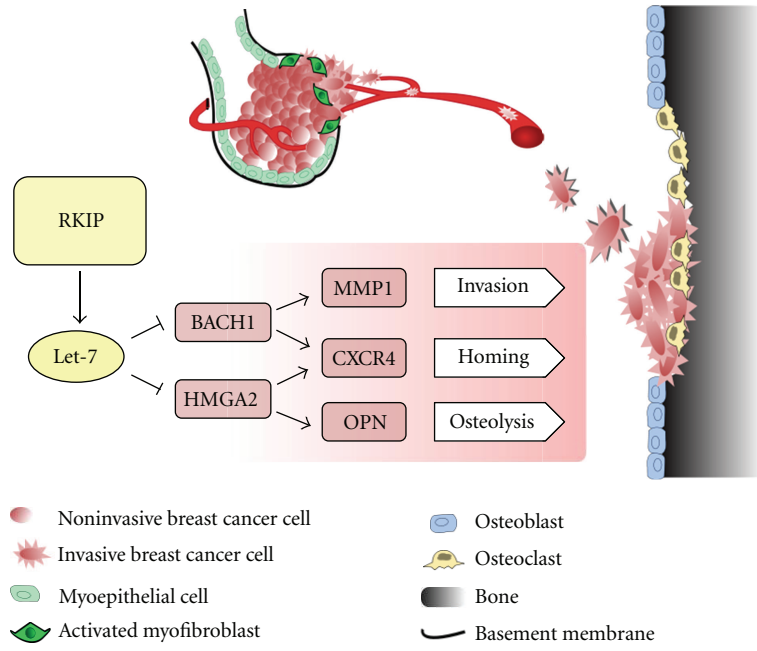


Figure 1: Schematic representation of the RKIP signaling pathway and its effects on metastatic progression to the bone.

Having determined which genes regulate metastasis in experimental breast tumor models, we defined a signaling pathway signature termed the RKIP pathway metastasis signature (RPMS) that we could use to further validate the clinical significance of our findings [20]. While typical gene expression signatures do not implicate any regulatory relationship between the genes in the signatures, the RPMS is based upon experimentally validated regulatory relationships between the components of the pathway. Bioinfomatic analysis using breast tumor data showed that the complete RPMS can predict greater risk for metastasis in patients. By contrast, the individual genes in the RPMS pathway were unable to predict metastasis-free survival. Taken together, these results highlight the importance of evaluating both regulators of tumor metastasis as well as genes that interact with the cellular signaling environment in order to be able to predict metastatic risk.

## 3. Significance

The results described here reveal a novel regulatory mechanism, controlled by the RKIP signaling pathway, that modulates the dialogue between breast tumor cells and the microenvironment and affects metastatic progression to the bone (Figure 1). Specifically, recent studies demonstrate that BACH1 and HMGA2 are key targets for inhibition by the RKIP signaling pathway via a let-7-dependent mechanism. Furthermore, BACH1 and HMGA2 promote the development of bone metastasis by inducing expression of genes (MMP1, CXCR4, and OPN) that regulate properties of the stromal compartment at the target organ site. Finally, since OPN is regulated exclusively by HMGA2 and MMP1 by BACH1, the signaling pathways downstream of RKIP exhibit a degree of specificity. While the function of these genes has been studied extensively in the past in the context of metastasis, the regulatory mechanisms and signaling pathways that control their expression were thus far incompletely investigated. The ability to manipulate a set of bone metastasis genes through a common upstream regulator such as RKIP reveals potential therapeutic targets that could have a profound impact on prevention of metastasis in breast cancer patients.

## Authors' Contribution

E. Bevilacqua and C. A. Frankenberger contributed equally to this work.

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*Review Article*

# The Microenvironmental Effect in the Progression, Metastasis, and Dormancy of Breast Cancer: A Model System within Bone Marrow

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Despite diagnostic advances, breast cancer remains the most prevalent cancer among women in the United States. The armamentarium of treatment options for metastatic disease is limited and mostly ineffective with regards to eradicating cancer. However, there have been novel findings in the recent literature that substantiate the function of the microenvironment in breast cancer progression and the support of metastasis to tertiary sites such as bone marrow. The uncovered significance of the microenvironment in the pathophysiology of breast cancer metastasis has served to challenge previously widespread theories and introduce new perspectives for the future research to eradicate breast cancer. This paper delineates the current understanding of the molecular mechanisms involved in the interactions between breast cancer cells and the microenvironment in progression, metastasis, and dormancy. The information, in addition to other mechanisms described in bone marrow, is discussed in the paper.

## 1. Introduction

The ability to invade and metastasize allows cancer cells to leave sites of primary tumor formation and recolonize in new tissues. This offers immediate metastasis to distant sites as well as the establishment of dormancy. Metastases are responsible for approximately 90% of human cancer deaths [1]. The previously established theory on metastasis described the phenomenon as a process alike to the Darwinian evolution [2]. In that perspective, cancer cells undergo a process of natural selection which favors rare cells within a tumor capable of invading and growing at sites of metastasis. The natural selection was believed to involve the development of stable genetic alterations which proffer the potential for successful metastasis. However, advances in technology, especially the development of high-throughput microarray expression profiling and *in vivo* imaging, have served to challenge this perspective of cancer metastasis [2]. Research suggests that metastatic ability is gained at earlier stages of tumor expansion than predicted by the previous model, and that this ability is acquired through transient changes in gene expression. A new tumor microenvironment invasion model reconciles the Darwinian perspective with recent discoveries. The tumor microenvironment consists of surrounding stroma, which is composed of extracellular matrix and various cell types including endothelial cells, fibroblasts, and infiltrative leukocytes.

The microenvironment, in addition to providing a scaffold for the organ, has been found to play a significant role in breast cell function through paracrine, mechanical, and hormonal interactions [3]. In the tumor microenvironment invasion model, stable genetic changes in primary tumor cells induce the microenvironment to initiate transient changes in gene expression which promote invasiveness and metastasis. Hence, the tumor microenvironment invasion model predicts that selected mutations within primary cancer cells drive the microenvironment to induce transient and epigenetic changes required of metastasis [2, 4]. This model

is supported by *in vivo* imaging of mammary tumors, which demonstrates the following regarding motile tumor cells: they represent only a small percentage of tumor cells, they are distributed throughout the tumor, and they are found most commonly localized to precise areas within the tumor [5]. Furthermore, genes associated with metastasis are expressed early and are found in tumor cells throughout the tumor [2]. Also in support of the model is the observation that micrometastases are commonly genetically heterogeneous, indicating that the invasiveness and migration are not limited to stable gene alterations.

Dormant cancer cells can remain quiescent for >10 years. Cancer can resurge and metastasize to tertiary organs. However, similar dormancy can occur in other organs. This paper will discuss on the bone marrow biology and describe how cancer cells could take advantage of the bone marrow microenvironment to adapt a dormant phenotype. Dormancy is defined as a state of fully transformed cells with nontumorigenic property that resists anticancer agents. Clinical dormancy has been defined as the time (5–25 yrs) between removing the primary tumor and relapse [6]. We expand this definition by proposing that dormant breast cancer cells exist in bone marrow and other organs long before clinical detection of the tumor [7].

We focus on bone marrow mostly due to its implication as the source of tumor-initiating cells in a large number of breast cancer resurgence [8, 9]. Also, prognosis is worse when breast cancer cells micrometastasize to the bone marrow [10]. An understanding of the mechanisms by which the bone marrow microenvironment facilitates a dormant phenotype of breast cancer cells is significant for strategies to target dormant breast cancer cells with minimum toxicity.

Bone marrow stromal cells, which are located close to the endosteum, support breast cancer cell quiescence as well as resurgence [11–15]. Quiescence is partly explained by the production of cytokines from stroma and gap junctional intercellular communication between the cancer cells and stroma [13, 16, 17]. Gap junction facilitates the passage of microRNA (miRNA) between the cancer cells and stroma [16]. Among these miRNAs are those that target CXCL12, which pass from stroma to breast cancer cells [16, 17].

Although the idea of crosstalk between the tumor and the microenvironment to promote growth and metastasis is now generally accepted in the field of cancer biology, the mechanisms underlying the interactions has not been well established. For example, in the primary site, the quantities and components of the microenvironment vary among tumors [18]. Though tumors require stroma for maintenance and growth, the malignant potential of a tumor does not correlate with the amount of surrounding stroma; both highly and less malignant cancer cells can have abundant or scarce surrounding stroma [18]. Rather, the microenvironmental effects on tumor progression are attributable to complex and dynamic epigenetic and phenotypic alterations. In addition to contributing to cancer progression and metastasis, the microenvironment may also play a pivotal role in protecting cancer cells from immune surveillance and response. In this paper, we delineate the current understanding of the microenvironemental involvement in breast cancer progression, metastasis, and dormancy in the mammary gland and then extrapolate the results to dormancy in bone marrow.

## 2. Composition of the Microenvironment

Stromal-epithelial interactions have been implicated in breast cancer progression [19, 20]. The composition of the tumor stroma is different from bone marrow stroma. The whole bone marrow-supporting stroma are mostly fibroblasts, in other organs, the tumor stroma consists of a heterogeneous population of cells, including pericytes, tumor-associated macrophages, epithelial cells, endothelial cells, fibroblasts, myeloid-derived suppressor cells, and adipocytes [21, 22]. Although each component might serve a unique role in facilitating the growth of breast cancer at the primary site, the stromal components are likely to interact to support and protect the tumor. Interestingly, these same cellular elements can be located at sites of distant metastasis, where they serve to provide a supportive niche. Reciprocal interactions between breast cancer cells and tumor stroma at the primary site govern the behavior of cancer [23]. This is explained by the report showing the secretion of soluble factors from the cancer cells to activate the surrounding stromal cells. Consequently, the stromal cells respond to promote invasiveness of the breast cancer cells [24].

Tumor-associated macrophages constitute a major immune cell population within the tumor microenvironment and play an important role in chronic inflammation during cancer progression [25]. Within the tumor-associated macrophage population, there is a high level of plasticity in terms of function [25, 26]. Primarily, the macrophages stimulate the formation of new blood vessels in the tumor bed via the production of vascular endothelial growth factor [27]. In addition, the macrophage can also induce a state of local immunosuppression, which can provide the tumor with an advantage to survive within the immune system [25]. The role of macrophages is complex since these cells can also promote the invasiveness of cancer via matrix remodeling through the secretion of matrix metalloproteases MMP7 and MMP9 [28]. Remodeling of the tumor stroma can also occur through the production of CCL18 from tumor-associated macrophages, which accelerates the invasive properties of breast cancer [29].

The role of adipocytes in the primary tumor microenvironment has been studied recently in an effort to determine the effects of obesity on cancer progression. Coculture of adipocytes with breast cancer cells resulted in adipocyte activation and secretion of MMP11, as well as proinflammatory cytokines IL-6 and IL-1$\beta$ [24]. The increased production of IL-6 from cancer-associated adipocytes promotes breast cancer cell invasion [24]. Since obesity results in poor prognosis of breast cancer [30] and adipose tissues are a source of mesenchymal stem cells [31], studies on adipose cells are relevant to the well-established interaction between mesenchymal stem cells and breast cancer cells [32]. Mesenchymal stem cells, through the production of interleukin-6, can enhance breast tumor growth [23].

Additional role of mesenchymal stem cells are included in this section. The role of fibroblasts within the breast tumor microenvironment as cellular support for cancer cells is not mutually exclusive of mesenchymal stem cells. Soluble factors from tumors are thought to differentiate mesenchymal stem cells into myofibroblast, which produce stromal cell-derived factor-1 (SDF-1) to accelerate breast cancer growth [33]. The mechanisms underlying this interaction have been determined to be hepatoma-derived growth factor and cyclophilin B from the tumor-conditioned media [34]. In addition, carcinoma-associated fibroblasts can alter the local T-cell balance by polarizing towards a Th2-type response, and this resulted in the loss of the antitumor Th1 effects [35]. This immune switch is not only limited to the differentiated mesenchymal stem cells. Studies with bone marrow mesenchymal stem cells showed similar findings, in addition to increases in regulatory T cells and reduced production of granzyme B to induce cytotoxicity [36].

The myeloid-derived suppressor cells can also protect the tumors from the immune system [37]. Myeloid suppressor cells are a heterogeneous collection of immune cells with immune-inhibitory properties [38]. Their numbers are increased in the circulation of patients with breast cancer as compared to healthy controls [39]. Although the studies on myeloid-derived suppressor cells in breast cancer are relatively limited, this area is a rapidly expanding area of cancer research. Recent findings demonstrate that the myeloid suppressor cells are capable of interfering with the activation of antitumor T-cell responses. Interestingly, interluekin-12, with antitumor activity [40], has been shown to decrease the number of myeloid-derived suppressor cells in the tumor microenvironment [38], underscoring another mechanism by which cells within the tumor microenvironment can protect the cancer cells from the immune response.

Overall, this section provides an overview of the tumor microenvironment at the primary site, with a diverse group of cells that promote and protect tumors. The majority of cells, however, appear to play key roles in breast cancer growth at the primary sites. The bidirectional crosstalk between breast cancer cells and microenvironmental components cannot be overlooked, since cellular interactions *in vivo* have a strong influence on the biological behavior of cancer cells. The significance of these findings points to an important role for stromal-epithelial interactions in overall breast cancer progression and metastasis. A recent review paper describes that a shift in the microenvironment can lead to the tumor and how this information can be explored for clinical intervention [20].

## 3. Mechanical Interactions

Although the interactions between tumor cells and stroma through cytokines and other soluble factors has received significant attention in the literature, the less familiar topic of mechanical interactions is also important to cancer progression and metastasis. Cells within tissue are under constant physical forces from neighboring cells and surrounding extracellular matrix (ECM), and these forces can be in the form of shear stress, compression, or tension. These forces from the microenvironment can serve to initiate mechanical signaling pathways after being perceived by mechanically responsive sensors present throughout the cell [18]. This signaling can subsequently induce changes at the molecular levels which promote cell survival, division, and motility. For example, an important family of mechanotransducers is the integrins, plasma membrane proteins which interact externally with ECM and internally with components of the cytoskeleton [18]. Integrins can undergo force-dependent activation resulting in the formation of focal adhesions, which can serve to induce growth and migration [41]. During the development of breast cancer, tension homeostasis is significantly perturbed [18]. There are amplified compression forces secondary to pressure from the progressively enlarging mass, matrix tightening from desmoplastic changes, and elevated interstitial pressure from leaky vasculature and compromised lymphatic drainage [18]. This state of abnormal force leads to the disruption of cell-cell junctions and polarity, and these changes collectively promote anchorage-independent survival and invasion. Also, the compression stress can lead to tumor angiogenesis directly through increasing VEGF-A expression or indirectly by generating hypoxic conditions through disrupting existing vasculature around the tumor, which also ultimately leads to increased VEGF-A expression [18]. Furthermore, exceeding compression force significantly reduces surrounding interstitial space, which allows for abnormal accumulation of fluid from leaky vasculature and blocked lymphatic drainage. This fluid tends to contain concentrations of cytokines and growth factors much greater than physiologic levels, promoting aggressive tumor expansion and migration. In addition, the overwhelming interstitial pressure can also serve to obstruct access of chemotherapeutic medications to the tumor. In summary, the mechanical influences of the microenvironment are extremely important to carcinogenesis and metastasis, and hence this topic warrants further investigation.

## 4. Epithelial-to-Mesenchymal Transition (EMT)

EMT is a complex phenomenon that is believed to play a role in dormancy and metastasis. EMT is a normal physiologic process during embryogenesis, wound healing and repair, and tissue remodeling [42]. EMT is characterized by the loss of epithelial polarity and the subsequent development of a fibroblast-like phenotype (Figure 1) [43]. The precise mechanisms of EMT in breast cancer remains uncertain, but it is believed to involve diverse changes at the genetic and molecular levels. Phenotypically, EMT involves the loss of epithelial cell markers such as E-cadherin, $\gamma$-catenin, zonula occludens-1 (Zo-1), and the acquisition of mesenchymal markers, such as vimentin, fibronectin, and N-cadherin [43]. The role of N-cadherin in promoting invasion, and migration of cancer cell has been established [44]. Moreover, the upregulation of EMT markers is correlated with poor prognosis [44]. An examination of the cell qualities of

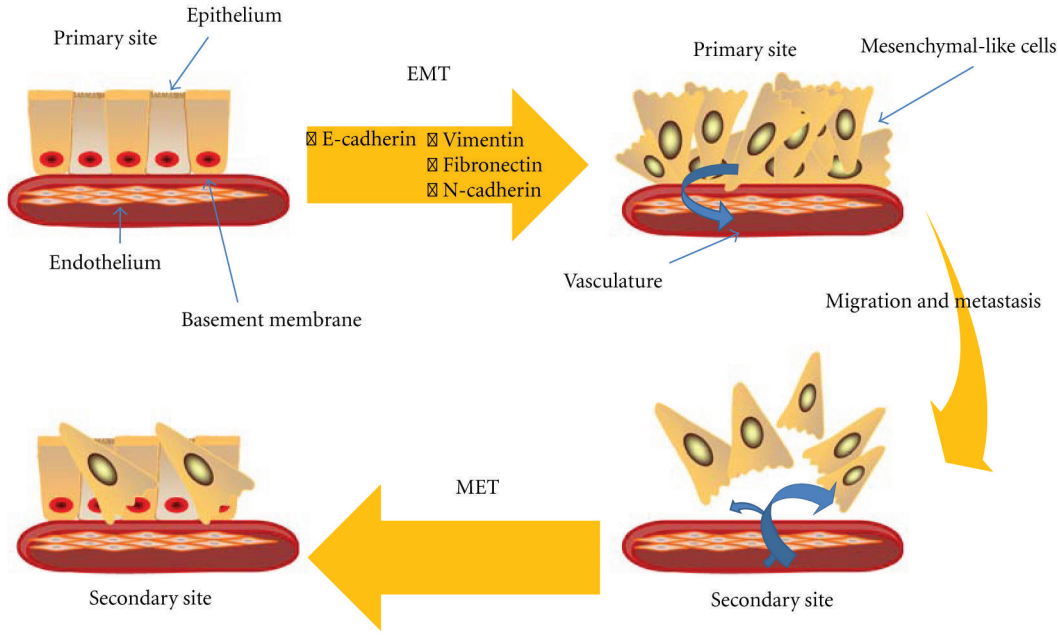


Figure 1: The epithelial-to-mesenchymal transition (EMT) is a physiological process by which an epithelial cell loses polarity and assumes a mesenchymal phenotype. While EMT can occur naturally in gastrulation and wound repair, it is involved as a route of metastasis in cancer. Through molecular changes, such as the loss of E-cadherin, the epithelial cell undergoes remodeling and loosens its attachments from the basement membrane and adjoining cells to enter the vasculature. Once mobile, the malignant cells can take up residence at secondary sites, reverting to an epithelial cell type or remaining dormant.

epithelial and mesenchymal cells demonstrates how EMT promotes cancer metastasis. Epithelial cells are organized tightly together to form a continuous layer above a basement membrane, while mesenchymal cells are loosely anchored and have the capability of becoming motile [45].

The microenvironment can trigger EMT through induction via upregulation of specific cytokines and growth factors. TGF-$\beta$ is known to be a potent inducer of EMT, particularly during the early stages of carcinogenesis [43]. Also, phorbol myristate acetate (PMA) can initiate EMT through the activation of protein kinase C [46]. Furthermore, the microenvironment can influence EMT through facilitating inflammation and accompanying leukocyte migration. Inflammation-associated EMT involves epigenetic changes induced by the increased expression of NF-$\kappa\beta$, Src, microRNAs, and IL-6 [3]. The mechanism through which CD8+ T cells can induce EMT involves the induction of CD44+/CD24− stem cell-like phenotype in breast cancer cells, which promotes invasiveness and metastasis, along with resistance to chemotherapy [3].

EMT is a particularly important area of microenvironment-breast cancer crosstalk because it is a process that can be potentially inhibited by therapeutic intervention. Several agents have shown promise with regards to inhibition of cancer progression associated with EMT. For example, Withaferin-A, a biologically active inhibitor of vimentin, has been found to suppress the mesenchymal phenotype through the induction of apoptosis, while preventing angiogenesis [47]. Also, Klf4, a well-known activator of E-cadherin, has also been found to inhibit EMT and associated invasive potential of transformed BCCs [43]. Inhibitors of the phosphatidylinositol 3-kinase (PI3K)/Akt/mTOR signaling, such as phosphatidylinositol ether lipid analogs and rapamycin, have been also found effective in suppressing EMT [48]. Hence, these preliminary findings demonstrate the promising therapeutic potential of EMT modulators.

## 5. Dormancy

A significant challenge of breast cancer treatment is the transition of cancer cells to a dormant phenotype. The literature supports that breast cancer relapses from bone marrow years after remission, suggesting a preferential niche in the bone marrow microenvironment for circulating tumor cells [49]. Dormant cells are arrested at the G1 phase of cell cycling. Quiescence proffers cancer cells with survival advantage through resistance to chemotherapeutic agents, which are designed to target proliferating cells [49]. Experimental evidence suggests that dormant cancer cells exist in the bone marrow near the endosteum, where they form gap junctional intercellular communication (GJIC) with hematopoietic-supporting cells and stroma (Figure 2) [50]. Connexin 43 (Cx43) is involved in the formation of GJIC between breast cancer cells and stroma [16]. An important factor of the breast cancer cell-stroma crosstalk in the bone marrow is CXCL12, a chemokine that interacts with CXCR4 and CXCR7 [31]. CXCL12 is normally constitutively generated by stroma, but it is downregulated when breast cancer

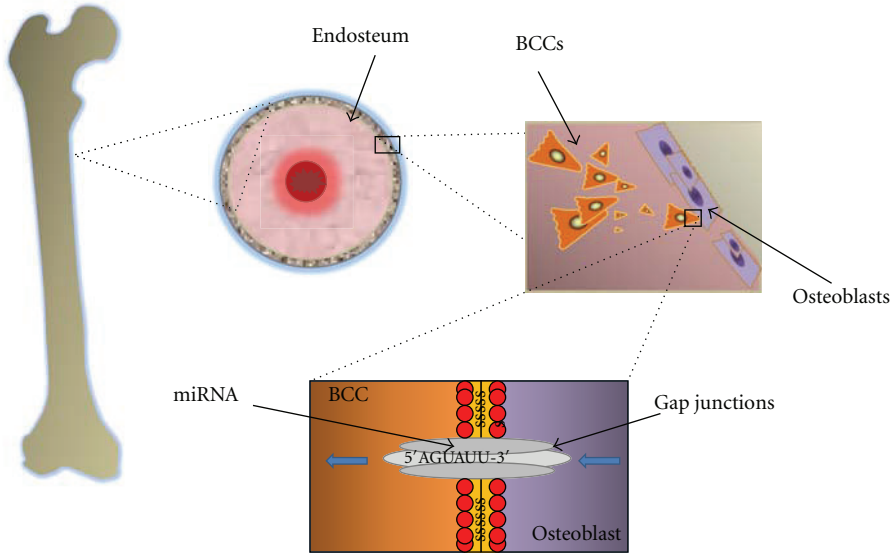


Figure 2: Stromal cells in the endosteal region of the bone marrow produce the chemokine, CXCL12, a known regulator in hematopoiesis. Through an interaction between CXCL12 and CXCR4 (a receptor on the BCC), malignant cells are drawn from circulation to the stromal niche. There, BCCs may form gap junctions with osteoblasts, which facilitates the intercellular transfer of small molecules such as miRNAs. Experimental evidence demonstrates that micro-RNAs can traverse gap junctions and induce dormancy of BCCs.

cells contact stroma [17]. A decline in CXCL12 production correlates with decreased breast cancer cell proliferation [17]. A recent study identified certain microRNAs (miRNAs) which cross GJICs between breast cancer cells and stroma and specifically reduce CXCL12 levels [16]. In this study, 4 miRNAs were found to traverse GJICs and transition BCCs to the $G_0$ phase of the cell cycle [16]. These novel findings suggest that microRNAs may play an integral role in breast cancer dormancy in the bone marrow. Furthermore, these data offer significant promise for developing treatment options targeting dormant cancer cells. Currently, there is an ongoing phase I clinical trial using siRNA to treat patients with solid cancers; hence, targeting miRNAs may also be a plausible treatment strategy in the near future [51].

The interaction between mesenchymal stem cells (MSCs) and BCCs in the bone marrow microenvironment is also implicated in dormancy. It has been found that BCCs interact with MSCs through CXCL12-CXCR4 upon traversing blood vessels in the bone marrow [49]. The mechanism through which MSCs offer protection to BCCs is hypothesized to involve the immunosuppressive properties of MSCs [42]. MSCs have been found to induce the production of regulatory T cells ($T_{regs}$) when cocultured with BCCs, which allows BCCs to evade immune response [52]. This concept of MSCs preventing the eradication of cancer cells from physiologic antitumor immune responses is termed oncoprotection [42]. The involvement of MSCs in breast cancer and other cancers is rapidly expanding area of basic science research, which is bound to lead to promising discoveries. The development of therapies aimed at eliminating MSC-related oncoprotection will be challenging, given the ubiquitous existence of MSCs and their relevance to many important biological functions. However, if further research uncovers specific distinctions in MSCs involved in oncoprotection, compared to normal MSCs, then the potential for therapy will certainly be more promising.

## 6. Conclusion

Studies on the microenvironment of breast cancer are rapidly growing. Novel findings in the recent literature demonstrate the significance of the microenvironment in the progression, metastasis, and dormancy of breast cancer. The objective for scientists, going forward, is transforming the data gained from basic science research into effective therapeutic options. However, the precise mechanisms through which the microenvironment induces molecular alterations in cancer cells remain yet to be elucidated. Also, the parallels of pathologic microenvironmental interactions and physiologic roles pose significant challenges to developing treatment strategies free of adverse side effects. Therefore, further investigations aimed at deciphering the intricacies of the microenvironment need to be performed to optimize therapeutic development.

## Abbreviations

BCC: Breast cancer cell
ECM: Extracellular matrix
VEGF: Human leukocyte antigen
EMT: Epithelial-to-mesenchymal transition
GJIC: Gap junctional intercellular communication.

## Conflict of Interests

The authors declare that there is no conflict of interests.

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*Research Article*

# Changes in Cytokines of the Bone Microenvironment during Breast Cancer Metastasis

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It is commonly accepted that cancer cells interact with host cells to create a microenvironment favoring malignant colonization. The complex bone microenvironment produces an ever changing array of cytokines and growth factors. In this study, we examined levels of MCP-1, IL-6, KC, MIP-2, VEGF, MIG, and eotaxin in femurs of athymic nude mice inoculated via intracardiac injection with MDA-MB-231$^{GFP}$ human metastatic breast cancer cells, MDA-MB-231BRMS1$^{GFP}$, a metastasis suppressed variant, or PBS. Animals were euthanized (day 3, 11, 19, 27 after injection) to examine femoral cytokine levels at various stages of cancer cell colonization. The epiphysis contained significantly more cytokines than the diaphysis except for MIG which was similar throughout the bone. Variation among femurs was evident within all groups. By day 27, MCP-1, MIG, VEGF and eotaxin levels were significantly greater in femurs of cancer cell-inoculated mice. These pro-osteoclastic and angiogenic cytokines may manipulate the bone microenvironment to enhance cancer cell colonization.

## 1. Introduction

The colonization and growth of cancer metastases in the bone depends on a cooperative interaction of the cancer cells with the host cells in the bone microenvironment. This microenvironment includes the resident osteoblasts, osteoclasts, endothelial cells, bone-lining cells, stromal cells, hematopoietic stem cells, and transient cells such as macrophages, lymphocytes, neutrophils, and other blood cells. While cell-cell contacts are established between cancer cells and bone cells via adhesion molecules, a wider network of communication occurs through secreted cytokines and growth factors. These soluble molecules play a critical role in the normal bone remodeling process as well as in cancer cell colonization of the bone marrow.

The interplay of the cancer cells with the cells of the bone marrow cavity has been described in terms of a vicious cycle [1]. In brief, cytokines or growth factors secreted by invading cancer cells (e.g., parathyroid hormone-related protein, PTHrP) act to stimulate osteoblasts to produce more receptor activator of nuclear factor kappa-B ligand (RANKL) and less osteoprotegerin (OPG), a decoy receptor for RANKL. The RANKL binds to RANK on osteoclast precursors leading to differentiation and activation of osteoclasts. Activated osteoclasts degrade bone matrix releasing growth factors such as transforming growth factor beta (TGF-$\beta$) and insulin-like growth factor (IGF). These molecules, in turn, stimulate further cancer cell growth. This series of events provides an explanation of the osteolytic outcome of breast cancer metastasis in bone; that is, an increase in osteoclast activation leads to excess bone breakdown and further stimulation of cancer cells. Drugs targeted to osteoclasts slow down formation of bone lesions. However, by and large, the lesions do not heal. In our previous research, we found that metastatic breast cancer cells also inhibit the differentiation of osteoblasts, thereby diminishing bone formation. The combination of increased bone degradation and decreased bone rebuilding has a net outcome of bone loss.

Through cell culture studies, we discovered that metastatic breast cancer cells induce an osteoblast inflammatory

response. When conditioned medium from metastatic human breast cancer cells, MDA-MB-231, was added to human osteoblasts (hFOB1.19) or murine (MC3T3-E1) or primary osteoblasts, the osteoblasts increased their secretion of interleukin-6 (IL-6), interleukin-8 (IL-8), and monocyte chemoattractant protein-1 (MCP-1). Under these conditions, the osteoblasts did not differentiate in culture; that is, they did not produce characteristic osteoblast differentiation proteins such as alkaline phosphatase, osteocalcin, or bone sialoprotein [2, 3].

These observations were followed with *in vivo* experiments. By using green fluorescent protein (GFP) expressing cancer cells in a xenograft model, we were able to monitor the progress of cancer colonization in the femurs [4]. We saw that the cancer cells appeared throughout the bone but cleared quickly from the diaphysis. Some cells localized in the ends of the femur where they developed into large colonies. For the most part, the cancer cells were associated with the endosteal surface of the bone marrow compartment. As part of an *ex vivo* study, we examined the cytokines produced by the bone cells in the presence of cancer cells [5]. Mice received intracardiac injections of MDA-MB-231$^{GFP}$ cells and were sacrificed three weeks later. In this study, the marrow was removed from the femurs, the bones separated into diaphysis (shaft) and epiphyses (ends), crushed and incubated in culture medium for 24 hr. Species-specific antibodies were used to distinguish between host (murine) and cancer (human) cytokines. We found that murine IL-6, MCP-1, macrophage inflammatory protein-2 (MIP-2) (human IL-8), vascular endothelial growth factor (VEGF), and keratinocyte chemoattractant (KC) (human growth-regulated oncogene-alpha, GRO-$\alpha$) were greater in ends of the bone compared to shafts and were increased in cancer-bearing mice. This *ex vivo* assay confirmed the *in vitro* findings that host cytokines in the bone microenvironment increase in the presence of cancer cells.

These initial findings led us to investigate how the cytokine profile of the bone microenvironment changed over time following the appearance of cancer cells in the bone marrow. We designed an experiment to ask how cytokines changed over time in the femurs of mice inoculated with metastatic MDA-MB-231$^{GFP}$ cells. Concurrently, we wished to investigate whether or not the cytokine profile of the bone microenvironment differed when the mice were injected with the highly metastatic MDA-MB-231 line or the metastasis-suppressed variant MDA-MB-231BRMS1 which traffics to the bone but does not grow there [6]. In order to examine the bone microenvironment in its entirety, the bone marrow was left intact. Femurs were separated into shafts and ends, crushed and incubated for 24 hours in serum-free medium. An initial assay panel of 32 mouse cytokines revealed two cytokines, eotaxin and monkine induced by interferon gamma (MIG), in addition to IL-6, MCP-1, MIP-2, KC, and VEGF that merited further investigation. In the final experiment, athymic nude mice were injected in the left cardiac ventricle with either MDA-MB-231$^{GFP}$ cells, MDA-MB-231BRMS1$^{GFP}$ cells, or PBS. Four days were chosen for sacrifice (3, 11, 19, and 27 days after injection) to represent early, middle, and late stage metastasis. Culture supernatants from femoral shafts and ends were analyzed for MCP-1, IL-6, KC, MIP-2, VEGF, MIG, and eotaxin. Changes in cytokine levels were compared over time as well as between injection groups.

## 2. Materials and Methods

*2.1. Cell Lines.* The human metastatic breast cancer cell line MDA-MB-231$^{GFP}$ (231) and the metastasis-suppressed derivative MDA-MB-231BRMS1$^{GFP}$ (BRMS1) were obtained from Danny Welch, University of Alabama, Birmingham and cultured in DMEM (Mediatech, Herndon, VA), 5% fetal bovine serum (PAA Laboratories, Etobicoke, ON, Canada), and 1X nonessential amino acids (Mediatech). Antibiotics were not used to culture cells for a minimum of two weeks prior to injection. For intracardiac injection, cells were detached with trypsin-EDTA solution, centrifuged and washed twice with sterile phosphate-buffered saline (PBS, Hyclone, Logan, Utah). Cells were resuspended at a concentration of $1.5 \times 10^6$ cells/mL in sterile PBS and held on ice until injection.

*2.2. Intracardiac Inoculation.* Six-week-old female athymic nude mice were obtained from Charles River Laboratories and were housed and handled in strict accordance with IACUC regulations (Penn State IACUC Protocol 28631). On the day of inoculation, mice were anesthetized with 120 mg/kg body weight of ketamine and 16 mg/kg of xylazine. When animals were completely anesthetized, 200 $\mu$L of PBS or cancer cell suspension ($3 \times 10^5$ cells) were injected directly into the left ventricle of the heart. For the pilot experiment to screen for relevant cytokines, 3 mice were injected with either PBS, 231, or BRMS1-expressing cells and kept for a period of 3 weeks before sacrifice. For the primary experiment, 8 mice were inoculated with either PBS, 231, or BRMS1-expressing cells for each of the four time points. After recovery from the procedure, mice were returned to sterilized cages with air filters and observed daily for signs of illness or distress. On days 3, 11, 19, and 27 after injection, mice were euthanized by $CO_2$ inhalation. Both femurs were removed from each mouse, cleaned of exterior tissue, and placed in PBS on ice prior to processing.

*2.3. Fluorescence Stereomicroscopy and Metastasis Detection.* Femurs were examined by fluorescence stereomicroscopy (40x magnification) with a Nikon SMZ 1500 Fluorescence Stereoscope (Nikon Instruments, Inc., Melville, NY) with GFP long bandpass fluorescence filter (excitation = 488 nm; emission = 515 nm, Chroma Technology Corporation, Rockingham, VT). Images were captured using a Nikon Coolpix 8400 digital camera (Nikon Instruments, Inc.).

*2.4. Femur Cultures.* Proximal and distal ends of each femur were separated from the shaft of the bone. The ends were cut so that they contained the epiphyseal plates and the metaphyses. The ends were placed together in a 2 cm$^2$ tissue culture well. The shaft was placed in a separate well. Bone samples were crushed with a small glass pestle, and the

fragments were cultured in 1 mL of $\alpha$ MEM (MediaTech) for 24 hours. Culture supernatants were then collected, centrifuged to remove cells and bone fragments, and frozen at $-80^{\circ}$C.

*2.5. Cytokine Assays.* To determine which cytokines might play an important role in the metastasis of cancer to bone, we first assayed bone culture supernatants of femurs of mice that had been injected with either PBS, 231, or BRMS1-expressing cells 3 weeks prior to sacrifice. For this determination, we used a Milliplex 32-plex mouse cytokine array (Millipore Corporation, Billerica, MA) which allowed for the simultaneous quantitation of 32 mouse cytokines. After relevant cytokines were established for the main experiment, eotaxin, KC, MIG, MIP-2, and VEGF were assayed using a Milliplex 5-plex mouse cytokine array. Two other cytokines, IL-6 and MCP-1 were assayed by standard sandwich ELISA techniques as previously described [7].

*2.6. Statistical Analysis.* A statistical model was fit to allow for correlations within a mouse and within batches of mice using random effects in order to compare each cytokine across each group, that is, PBS, MDA-MB-231, and MDA-MB-231BRMS1. Prior to statistical analyses, assumptions for linear statistics were verified and $\log_{10}$ transformations were used and rechecked to assure statistical validity of the analyses. For the shaft versus ends comparison, pg/mL values were analyzed with a two-way ANOVA analysis with a Bonferroni post hoc correction. To compare each cytokine among injection groups and over time, a two-way ANOVA test with a Fisher's LSD post hoc analysis was used. Shown in bar graphs is the mean $\pm$ standard error (SE). Box plots display mean, 25–75 percentile range and max/min values. Significance was set at $P \leq 0.05$.

## 3. Results

*3.1. Selection of Cytokines.* In previous experiments, we had access to a limited panel of cytokines available in a multiplex format. In preparation for this current study, we carried out a more extensive screen with a 32-plex cytokine array to identify more cytokines of interest. Femurs were harvested from 9 mice (3 per group) three weeks following intracardiac inoculation with MDA-MB-231$^{GFP}$ cells, MDA-MB-231BRMS1$^{GFP}$ cells, or PBS. Two femurs from each mouse were incubated as described in the methods section and the culture supernatants were tested. The patterns of cytokines were similar in all three groups but generally highest in the culture supernatants from MDA-MB-231 cells. Of the 32 murine cytokines tested (Table 1), nine were below the levels of detection, approximately 3.2 pg/mL. Another four (IL-2, IL-17, M-CSF, and RANTES) were present in very small amounts, $\leq$10 pg/mL. Thirteen ranged in concentrations from 10 to 100 pg/mL. Six (G-CSF, IL-6, KC, MCP-1, MIG, and VEGF) were present at >100 pg/mL. We had previously reported that IL-6, KC, MCP-1, VEGF, and MIP-2 were secreted by osteoblasts and increased in the presence of breast cancer cells *in vitro* and *in vivo* [5].

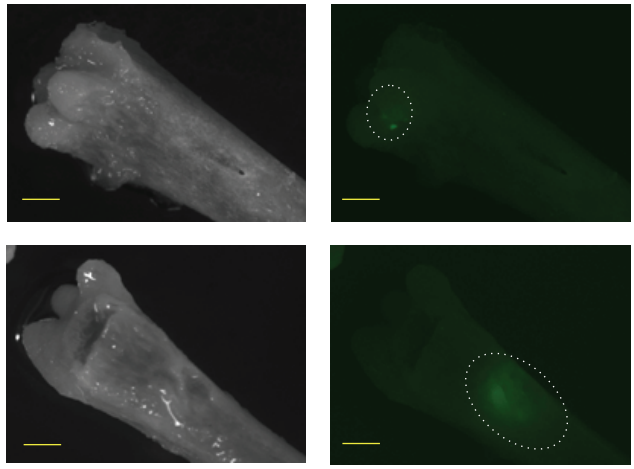
Table 1: Summary of 32-plex mouse cytokine array.

| None detected | | 1–10 pg/mL | 10–100 pg/mL | | >100 pg/mL |
|---|---|---|---|---|---|
| IFN-$\gamma$ | IL-15 | IL-2 | Eotaxin | IP-10 | G-CSF |
| IL-3 | TNF-$\alpha$ | IL-17 | GM-CSF | LIF | IL-6 |
| IL-4 | IL-7 | M-CSF | IL-1$\alpha$ | LIX | KC |
| IL-5 | IL-10 | RANTES | IL-1$\beta$ | MIP-1$\alpha$ | MCP-1 |
| IL-12p40 | | | IL-9 | MIP-1$\beta$ | MIG |
| | | | IL-12p70 | MIP-2 | VEGF |
| | | | IL-13 | | |

We choose to assay for these five cytokines plus MIG which ranged in concentration from 100–1000 pg/mL. MIG is a target gene of RANKL and is involved in osteoclast activation [8]. We also selected eotaxin (10–40 pg/mL) because of its reported roles in angiogenesis in breast cancer metastasis patients [9] and in multiple myeloma [10]. The multiplex cytokine array for the remainder of the study included IL-6, KC, MCP-1, VEGF, MIG, MIP-2, and eotaxin. At the conclusion of the study, it was found that MIP-2 levels were negligible for most of the samples assayed and were not considered in further analyses.

*3.2. Detection of Femur Metastases.* Prior to crushing the femurs for incubation, they were examined with a fluorescence stereomicroscope. We detected GFP in some of the femurs of mice inoculated with MDA-MB-231$^{GFP}$ or MDA-MB-BRMS1$^{GFP}$ cells taken at various times (Figure 1). However, the sensitivity of the microscope and the location of the cells combined with the thickness of the bone made it likely that not all metastatic cells were detected by microscopy. There were not enough femurs with GFP detectable colonies to be examined as a group for cytokines separate from the other femurs. For the most part, the GFP-expressing metastases appeared much larger in the femurs of mice inoculated with MDA-MB-231$^{GFP}$ than those with MDA-MB-231BRMS1$^{GFP}$ (Figure 1).

*3.3. Cytokines in the Diaphysis versus the Epiphysis of the Femurs.* We first compared the levels of MCP-1, IL-6, MIG, KC, VEGF, and eotaxin in the diaphysis (shaft) versus the epiphyses (end) of each bone. The epiphyseal end of the femur contains the metaphysis, the region of bone remodeling rich in cytokines and growth factors, in addition to the epiphyseal growth plate. In contrast, the function of the diaphysis is to provide support and is less metabolically active. As expected, the ends of long bone were a much richer source of cytokines than the shaft (Figure 2). The exception was MIG which was found distributed equally in both areas. Eotaxin was present in less than 10 pg/mL in the shaft supernatant but was 5-6- fold higher in the ends. MCP-1 was also present in low concentrations but ends contained about 10-fold more. Little to no KC was found in the shaft but approximately 200 to 600 pg/mL were detected in the ends depending on the group of mice. VEGF and IL-6 were present in the shafts at about half of the concentration in the ends of the bone which was approximately 200 and



(a)

| Time from inoculation | Mice w/detectable GFP cells | |
|---|---|---|
| | 231 | BRMS1 |
| Day 3 | 6/8 | 1/7 |
| Day 11 | 1/8 | 3/8 |
| Day 19 | 1/8 | 1/8 |
| Day 27 | 2/8 | 1/8 |

(b)

Figure 1: Breast cancer metastases in femoral bone. Female athymic nude mice were inoculated in the left cardiac ventricle with $3 \times 10^5$ MDA-MB-231$^{GFP}$ or MDA-MB-231BRMS1$^{GFP}$ cells, eight per group. Animals were euthanized at day 3, 11, 19, or 27 after injection. Femurs were removed, placed in PBS, and imaged with light and fluorescence stereomicroscopy at a 40x magnification to detect GFP-labeled cancer cell metastases. Images are shown for 27 day metastases (a) of MDA-MB-231BRMS1 (top) and MDA-MB-231 (bottom). Note colony size difference between the two variants. Scale bar = 1 mm. Table (b) summarizes the incidence of detectable GFP-expressing cells for each injection group.

600 pg/mL, respectively. The cytokine found in the greatest concentration was MIG registering 500–2000 pg/mL for both shafts and ends.

*3.4. Comparison of Cytokines in Three Groups of Mice.* The cytokines from femurs of animals inoculated with MDA-MB-231$^{GFP}$ cells, with MDA-MB-231BRMS1$^{GFP}$ cells, or with PBS were compared at four times, day 3, 11, 19, and 27. The values presented (Figure 3) are the cytokine concentrations from the ends of the bone. At the earliest time (day 3), most of the cytokine concentrations were similar in all groups except for VEGF. The femurs of mice inoculated with MDA-MB-231$^{GFP}$ showed significantly greater amounts of VEGF than the mice inoculated with MDA-MB-231BRMS1$^{GFP}$ cells. However, neither group was different than PBS. At day 11, IL-6 was greater in the femurs of mice with MDA-MB-231$^{GFP}$ than in the femurs of the PBS group. Interestingly, at this time, the femurs of the mice inoculated with BRMS1-expressing cells had a greater concentration of VEGF and MIG than the mice with 231 cells. The measurements on day 19 showed few differences among the groups except for MIG. MIG was significantly less in the animals injected with cancer cells than those injected with PBS. By day 27, the differences among groups were most pronounced. MCP-1, MIG, eotaxin, and VEGF were all significantly greater in the cancer-inoculated mice than in those inoculated with PBS. Mice bearing MDA-MB-231$^{GFP}$ showed less IL-6 than those with PBS or MDA-MB-231BRMS1$^{GFP}$ cells. No differences were apparent among the groups for KC at any of the times tested.

*3.5. Changes in Cytokines over Time.* One of the original objectives of this study was to examine the pattern of cytokine changes over time. We found that there was considerable variation from femur to femur even within the same animal. In the animals treated with PBS, there were increases and decreases over time in 5 cytokines tested (Figure 4). Since these animals did not harbor tumor cells, these differences likely reflect normal physiological variation over time. For the mice inoculated with MDA-MB-231$^{GFP}$ cells, neither VEGF nor eotaxin showed significant increases or decreases over the experimental time frame (Figure 4). In contrast, MCP-1, MIG, and IL-6 exhibited a significant decrease on day 19 when compared to day 3. While IL-6 and MIG levels rose moderately on day 27, the level of MCP-1 was substantially elevated. In animals injected with the metastasis suppressed variant, MDA-MB-231BRMS1$^{GFP}$, the expression pattern for MCP-1, MIG, and IL-6 was similar to results obtained for the metastatic cells. Most notably, MCP-1 levels were significantly elevated by day 27. Interestingly, the BRMS1-expressing cells elicited a variable expression pattern for VEGF and eotaxin that closely resembled the control PBS injection, suggesting that these two cytokines may be implicated in tumor cell colonization. KC was excluded from this analysis due to the lack of change among groups.

## 4. Discussion

Previously, we have reported changes in the inflammatory cytokines IL-6, MCP-1, VEGF, KC, and MIP-2 in the culture supernatants from femurs of athymic mice three weeks after intracardiac injection of MDA-MB-231 cancer cells [5]. We sought to verify and expand these findings to answer several key questions. What other cytokines and growth factors may be involved in the metastatic process? How does the inclusion of the marrow affect the assay of cytokine expression in

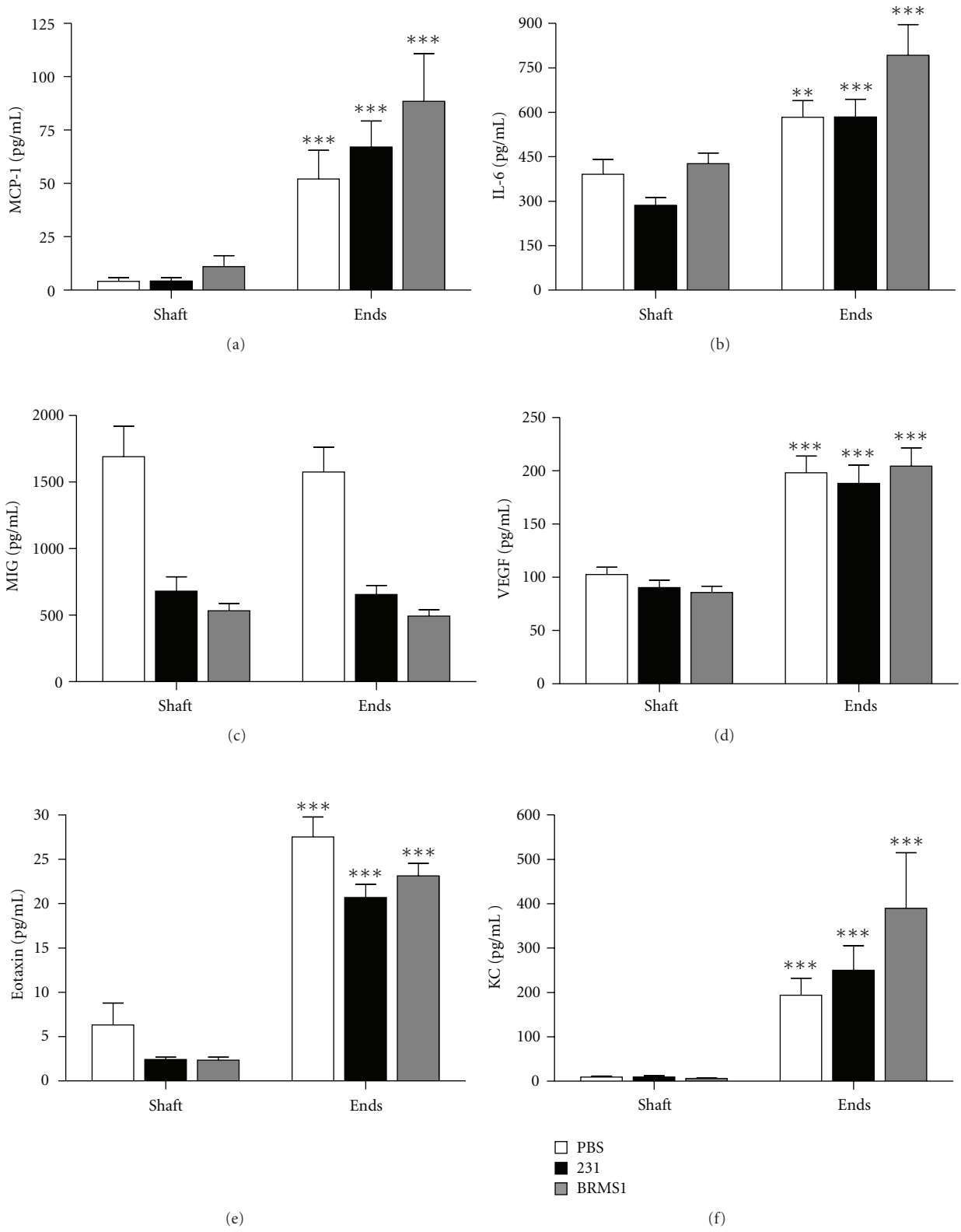



Figure 2: Cytokine levels in bone diaphysis versus bone epiphysis. Athymic nude mice received intracardiac inoculations of either PBS, MDA-MB-231$^{GFP}$, or MDA-MB-231BRMS1$^{GFP}$ cells. Femurs were harvested at days 3, 11, 19, and 27 after inoculation and separated into shafts and ends. Shown here are the results from day 19, but the results were similar for the other days. Bone sections were crushed and cultured in serum-free medium for 24 hours. Resulting supernatants were assayed for MCP-1 (a), IL-6 (b), MIG (c), VEGF (d), eotaxin (e) and KC (f). MIP-2 values were very small or below the level of detection and were not included. With the exception of MIG, the cytokine levels were significantly higher in the ends of the femur than in the shaft. $^{***}P < 0.001$; $^{**}P = 0.01$–$0.001$. $n = 8$ for each group.

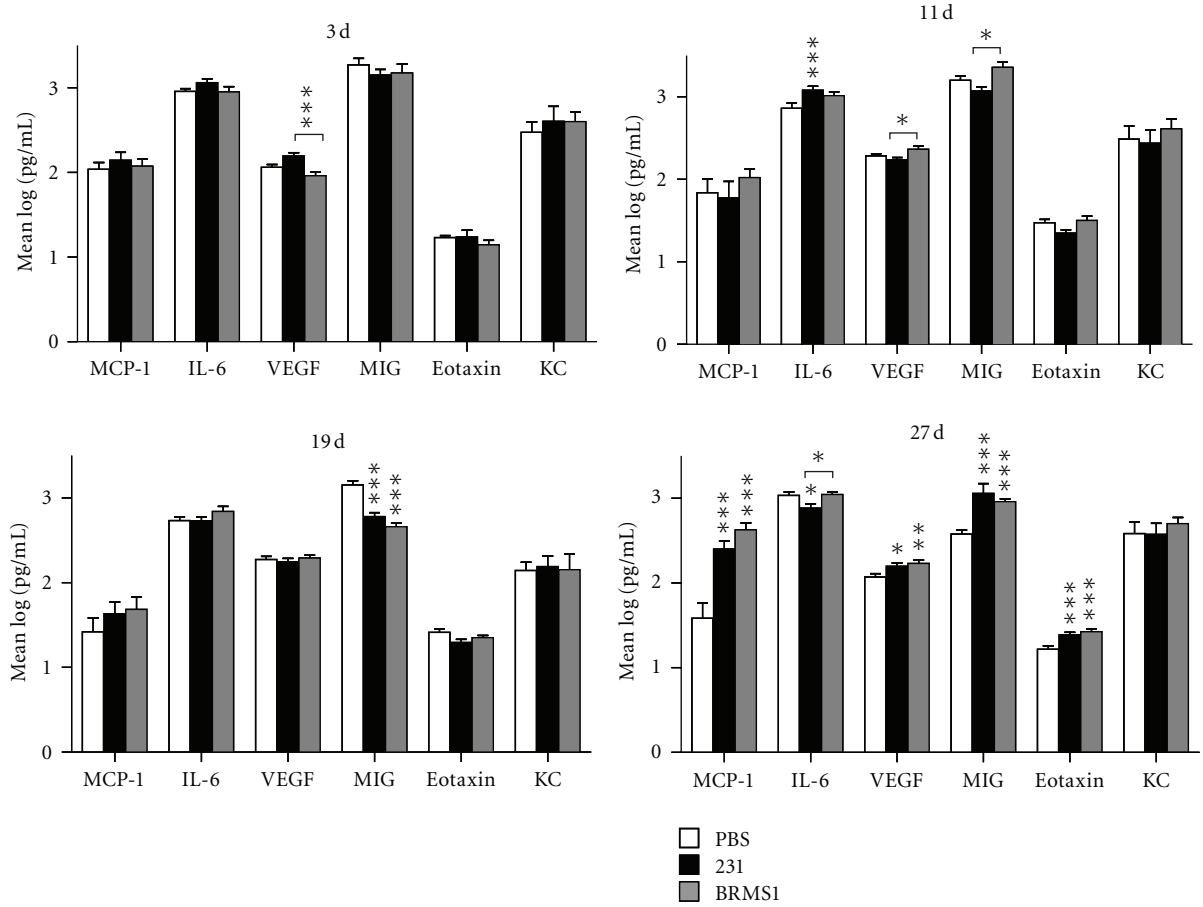



Figure 3: Comparison of bone cytokine levels in mice inoculated with PBS, MDA-MB-231$^{GFP}$ or MDA-MB-231BRMS1$^{GFP}$,cells. Mice were inoculated and femurs processed as described in Section 2. Cytokine values were $\log_{10}$ transformed for analysis and graphic comparison of each postinjection group. Statistical significance is shown for comparison to PBS unless otherwise noted with a bracket. The only significant difference shown on day 3 post injection was for VEGF which was higher in 231-injected mice than in BRMS1. On day 11, VEGF and MIG were slightly higher in BRMS1 injected mice than in 231, while IL-6 values were higher in 231 injected mice than in PBS. On day 19, the only cytokine that varied significantly was MIG, with higher values for 231 and BRMS1 mice than in the control animals. In later stage metastasis (day 27), the levels for 4 (MCP-1, VEGF, MIG, and eotaxin) of the 6 cytokines were significantly higher in both the 231-and the BRMS1-injected mice when compared to PBS. IL-6 levels were lower in animals injected with 231 cells. KC levels did not vary between groups at any of the time points. $^{***}P < 0.001$; $^{**}P = 0.01$–$0.001$; $^{*}P = 0.05$–$0.01$. $n = 8$.

the presence of metastatic cancer? Does the cytokine profile of the bone microenvironment change over time after the introduction of cancer cells? Does the presence of metastasis-suppressed breast cancer cells elicit a bone cytokine profile that differs from the profile generated by metastatic cancer cells?.

Cytokine analysis of bone culture supernatants with an expanded 32-plex array revealed the presence of several cytokines in addition to the five (IL-6, MCP-1, VEGF, KC, and MIP-2) previously reported. IL-2, IL-17, M-CSF, and RANTES were detected but only in small amounts; due to cost constraint, we elected not to include them in the panel. MIG and eotaxin were found to be expressed in the mouse femurs and appeared to vary with the presence of MDA-MB-231. MIG is a target for RANKL [8] and as such is involved in osteoclast activation. Eotaxin is believed to play a key role in angiogenesis [11]. Because osteolysis and tumor angiogenesis are intimately tied to cancer metastasis in bone, MIG and eotaxin were included in the cytokine analysis panel.

The epiphyses is a favored site of breast cancer metastasis to bone [12]. Unlike the bone shaft, the ends of the long bones are areas of high bone turnover and are comprised of a specialized arrangement of osteoblasts, osteoclasts, stromal cells, hematopoietic cells, and endothelial cells. In order to examine the cytokine profile of the total bone microenvironment, we left the bone marrow intact when culturing the bones. One obvious outcome of this study was that the cytokine concentrations in the ends of the bones were significantly higher than in the shaft. The exception was MIG. MIG is a product of T cells and endothelial cells. It has also been reported to be produced by osteoblasts [13]. Because the femurs are from athymic mice, the sources of MIG in these experiments are likely osteoblasts and bone endothelial cells. Eotaxin is also a product of T cells and

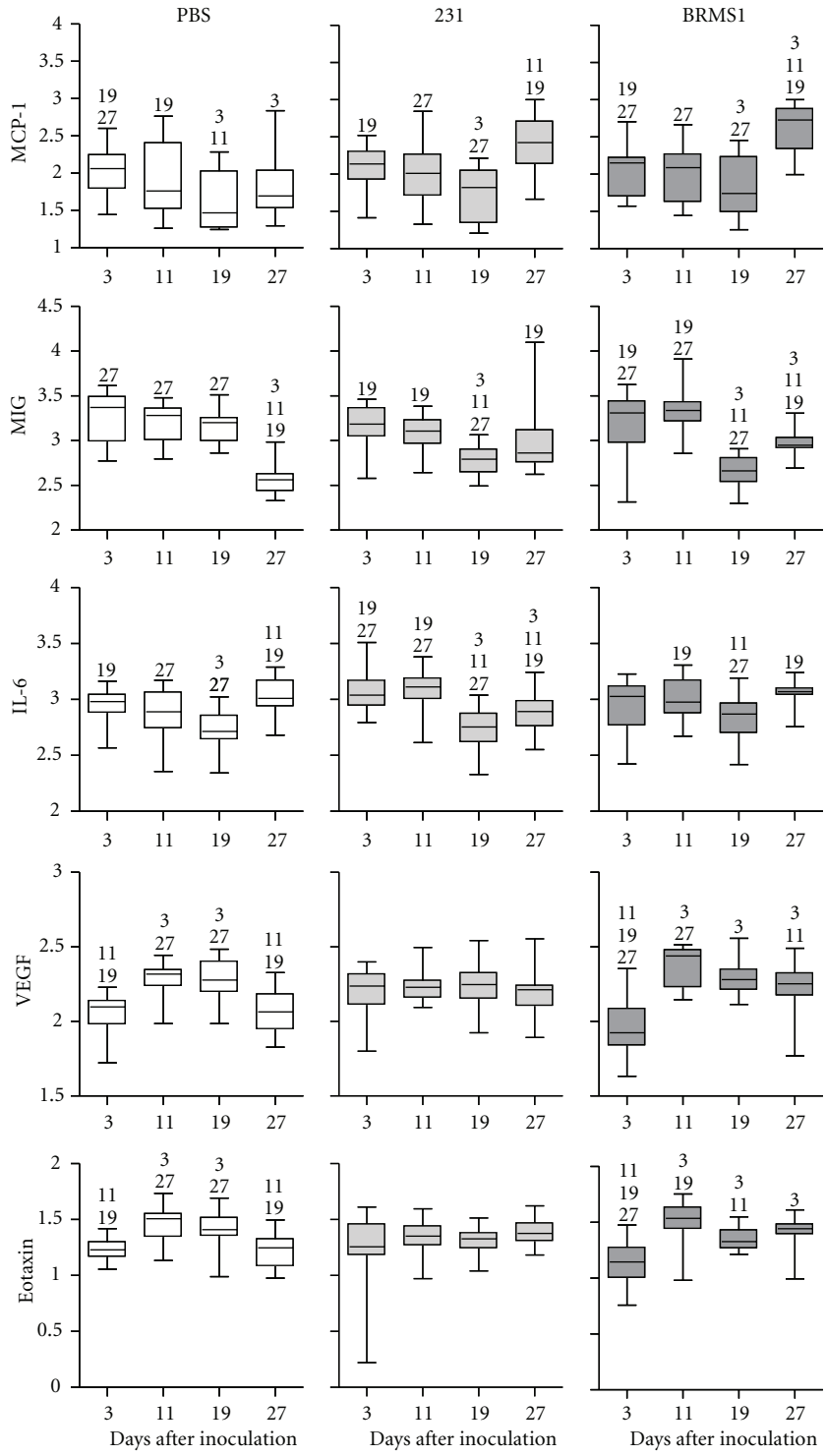



FIGURE 4: Changes in bone cytokine levels over time. Cytokine values were obtained as described in Section 2. Results were $\log_{10}$ transformed for analysis and graphic comparison of each injection group over the 4 points of the study. Each box plot graph represents the change in a particular cytokine level for one injection group over time. The box represents the 75–25% range of values; the horizontal line within the box denotes the mean. Bars above and below the box mark the maximum and minimum values. Numbers above each box denote a significant difference ($P < 0.05$) between time points. $n = 8$.

endothelial cells, but not osteoblasts [14, 15]; thus, its likely origin is the bone vascular endothelium. Athymic mice lack T cells but retain much of the innate immune system. We cannot rule out that cytokines may also be due to transient monocytes, or to hematopoietic stem cells. Using 8 mice per injection group per time point, we noticed a great deal of variation in cytokine levels from mouse to mouse within the same time and injection group. In many cases, two femurs from the same mouse yielded very different results. Some of this variation may be due to imprecise sectioning of the ends and shaft of the bones. In addition, transient cells in the marrow such as monocytes or granulocytes and the general health of the animal independent of the presence of cancer metastasis could also account for this wide variation. In the case of animals injected with cancer cells, only the femurs were examined for the presence of metastases. If the cancer cells had colonized another locus in the body, it is possible that the tumor may have had a more widespread effect on the cytokine levels in general.

In order to examine the changes in MCP-1 IL-6, KC, MIP-2, VEGF, MIG, and eotaxin levels over time, we chose to sacrifice animals at 3, 11, 19, and 27 days after injection of MDA-MB-231$^{GFP}$, MDA-MB-231BRMS1$^{GFP}$, or PBS. These times represent early, middle, and late stage metastasis. Unfortunately, this experimental design did not allow us to sample the same animal over time. For this study, two cytokines, MIP-2 and KC, were omitted from final analysis. MIP-2 was not detected at all in a large number of the samples and KC showed no significant changes over time in any of the injection groups. At day 3, 11, and 19, there were some statistically significant changes in MCP-1, MIG, VEGF, eotaxin, and IL-6. Since many of these changes also occurred in mice injected with PBS, the variation can likely be attributed to cyclic expression of cytokines in the bone, possibly due to age. Originally, we postulated that if a particular cytokine was elevated early in the metastatic process, it could be acting as a chemoattractant for cancer cells or a catalyst for cancer cell colonization. However, there is insufficient evidence from this experiment to pinpoint such a cytokine from the seven cytokines assayed. The most striking results were observed at day 27 when levels of MCP-1, MIG, VEGF, and eotaxin were significantly higher in mice injected with either breast cancer cell variant than in mice injected with PBS. IL-6, MCP-1, VEGF, and MIG have all been implicated in osteoclastogenesis [8, 16]. An increase in these molecules in the microenvironment in response to cancer cells correlates with increased osteoclast differentiation and activation and thus bone resorption. Osteoblasts have been reported to display an "inflammatory cytokine stress response" to titanium in joint replacements [17] and to bacteria in osteomyelitis [18]. The same cytokine response occurs when breast cancer and likely other epithelial cells invade the marrow cavity. Because several of these cytokines are also expressed by osteoblasts during their normal differentiation and during the bone remodeling process, it is easy to see how the introduction of cancer cells to the bone microenvironment can disrupt both of these important functions. Additionally, VEGF and eotaxin are known promoters of angiogenesis [11, 19] and may be responsible for the vascularization of a newly formed metastatic tumor.

In comparing the cytokine profiles of animals injected with metastatic MDA-MB-231$^{GFP}$ to metastasis-suppressed MDA-MB-231BRMS1$^{GFP}$ cells, we observed that at day 27 both cell types elicited significant elevations in MCP-1, MIG, VEGF, and eotaxin levels. These data indicate that these four cytokines are not likely to be responsible for the inability of the BRMS1-expressing cells to colonize the bone. However, we were intrigued by the difference in cytokine expression patterns over time for VEGF and eotaxin. While VEGF and eotaxin levels remained unchanged in animals injected with 231 cells, the expression levels for PBS- and BRMS1-injected animals showed a similar pattern of significant variation over time (i.e., reduced expression levels at day 3). One possible interpretation of these data is that higher sustained levels of VEGF and eotaxin are enabling the metastatic cancer cells to colonize and thrive in the bone environment.

It is interesting to note that MDA-MB-231 and MDA-MB-231BRMS1 themselves secrete IL-6, VEGF, IL-8, and GRO-$\alpha$ (the human homologues of MIP-2 and KC, resp.) [5]. MCP-1 is made in small amounts and MIG is reported to be absent from the 231 cancer cells [20]. In this study, human cytokines generated by the cancer cells present in the bone were not measured. In addition, the cancer cells have been reported to express receptors to IL-6 [21], MIP-2 [22], KC [22], VEGF [23], MCP-1 [22], and MIG [24]. The mRNA for the receptor for eotaxin was not detected in MDA-MB-231 cells [22]. In a recent publication, MIG was reported to be produced by bone marrow mesenchymal stem cells and enhanced the invasion and motility of MDA-MB-231 cells [24]. In the cross-species xenograft model for breast cancer utilized in this experiment, mouse cytokines can activate human receptors with the exception of IL-6 [25]. Thus the cytokine changes that occur in the microenvironment as a consequence of the cancer cells may also be responsible for the progression of the metastatic tumor.

In summary, cytokines in the bone microenvironment are critical components for bone remodeling and hematopoietic processes. The presence of cancer cells changes the normal levels of these cytokines which in turn disrupts the homeostatic balance in the bone. Abnormal cytokine levels may also serve to fuel the propagation and further metastasis of breast cancer cells. Whether these changes are limited to the immediate location of the cancer cells or are the result of a systemic effect has yet to be determined.

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*Review Article*

# Role of Estrogen Receptor Signaling in Breast Cancer Metastasis

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Metastatic breast cancer is a life-threatening stage of cancer and is the leading cause of death in advanced breast cancer patients. Estrogen signaling and the estrogen receptor (ER) are implicated in breast cancer progression, and the majority of the human breast cancers start out as estrogen dependent. Accumulating evidence suggests that ER signaling is complex, involving coregulatory proteins and extranuclear actions. ER-coregualtory proteins are tightly regulated under normal conditions with miss expression primarily reported in cancer. Deregulation of ER coregualtors or ER extranuclear signaling has potential to promote metastasis in ER-positive breast cancer cells. This review summarizes the emerging role of ER signaling in promoting metastasis of breast cancer cells, discusses the molecular mechanisms by which ER signaling contributes to metastasis, and explores possible therapeutic targets to block ER-driven metastasis.

## 1. Introduction

The steroid hormone, estradiol, plays an important role in the progression of breast cancer, and a majority of the human breast cancers start out as estrogen dependent and express the estrogen receptor (ER). The biological effects of estrogen are mediated by its binding to one of the structurally and functionally distinct ERs (ER$\alpha$ and ER$\beta$) [1]. Endocrine therapy using Tamoxifen, a selective estrogen receptor modulator [2], and aromatase inhibitors, which ablate peripheral estrogen synthesis, has been shown to substantially improve disease-free survival [3]. Endocrine therapy has also been shown to have a positive effect on the treatment of ER-positive breast cancer [4]. Despite these positive effects, initial or acquired resistance to endocrine therapies frequently occurs with tumors recurring as metastatic. Tumor metastasis comprises a series of discrete biological processes that moves tumor cells from the primary neoplasm to a distant location [5] and involves a multi-step cascade of coordinated cell adhesion and contractility as well as proteolytic remodeling of the extracellular matrix (ECM) [6, 7]. Even though substantial information is available on the process of metastasis, the molecular basis of breast cancer progression to metastasis and the role of ER$\alpha$ signaling in this process remain poorly understood. A few early studies suggested a negative effect of ER$\alpha$ signaling on motility and invasion of cells [8, 9], while several recent studies showed a positive effect of ER signaling on motility [10–14]. In this review, we summarized the emerging evidence for the role of ER$\alpha$ signaling in breast cancer progression to metastasis and discuss the possibility of targeting ER$\alpha$ signaling crosstalk with cytosolic kinases as a possible additional therapeutic target for treating/preventing ER-positive metastatic breast cancer.

## 2. ER$\alpha$ Signaling Mechanisms

ER$\alpha$ is the major ER subtype in the mammary epithelium and plays a critical role in mammary gland biology as well as in breast cancer progression [15, 16]. The ER$\alpha$ comprises an N-terminal AF1 domain, a DNA-binding domain, and a C-terminal ligand-binding region that contains an AF2 domain [17]. Upon the binding of estrogen to ER$\alpha$, the ligand-activated ER$\alpha$ translocates to the nucleus, binds to the responsive element in the target gene promoter, and stimulates gene transcription (genomic/nuclear signaling) [18, 19]. Emerging evidence suggests that ER signaling is

complex, involving coregulatory proteins and also genomic actions and extranuclear actions [20, 21].

Multiprotein complexes containing coregulators assemble in response to hormone binding and activate ER-mediated transcription [18]. The ER$\alpha$ transcriptional outcome is regulated by dynamic chromatin modifications of the histone tails, and the ligand-bound ER$\alpha$ facilitates these modifications via coregulator recruitment [22]. For example, coactivators like SRC-1, amplified in breast cancer (AIB1), and CBP have been shown to possess histone acetyltransferase activity, whereas corepressors, such as NCOR and MTA1, are associated with histone deacetylases [20, 23]. It is generally accepted that some of the diverse functions of E2 depend on differential recruitment of coregulators to the E2-ER complex [24]. Even though coregulators modulate ER functions, each coregulator protein appears to play an important but not overlapping function *in vivo* [25–27].

Emerging findings suggest that ER-coregulatory proteins have potential to be differentially expressed in malignant tumors and that their functions may be altered, leading to tumor progression [28]. *In vivo* studies using wild type (WT) and SRC3/AIB1$^{-/-}$ mice harboring the mouse mammary tumor virus-polyomavirus middle T (PyMT) transgene (Tg) revealed that AIB1 knock down significantly reduces lung metastasis but not mammary tumorigenesis. Compared with *WT*/*PyMT* mice, Tg *SRC*-1$^{-/-}$/*PyMT* mice had intravasation of mammary tumor cells. In addition, the frequency and extent of lung metastasis were drastically lower in the Tg mice than in the WT mice [29]. Another study using Tg *SRC*-1$^{-/-}$ mice reported that deficiency of SRC-1 coregulator increases MMTV-neu-mediated tumor latency and differentiation-specific gene expression and decreases metastasis [30]. Collectively, these emerging findings implicate the role of the ER$\alpha$-coregulator-associated activities/functions in breast cancer metastasis.

## 3. ER$\alpha$ Genomic Actions and Metastasis

Within the last decade, research has provided substantial data to suggest that alteration in cellular concentration or genetic dysfunction of coregulators can contribute to a pathologic outcome by modulating ER genomic actions and has potential to drive cancer cell proliferation and metastasis [31]. Loss of the epithelial adhesion molecule E-cadherin is implicated with a critical role in metastasis by disrupting intercellular contacts, an early step in metastatic dissemination [32]. Functional or transcriptional loss is commonly associated with an invasive and poorly differentiated phenotype [33]. Deregulation of ER-coregulator signaling can lead to aberrant expression of Snail, resulting in the loss of expression of E-cadherin and invasive growth. For example, MTA1, a commonly deregulated coregulator in breast cancer, promotes transcriptional repression of ER, leading to metastatic progression [34]. The ER$\alpha$ coregulator (AIB1) amplified in breast cancer has been shown to promote breast cancer metastasis by activation of PEA3-mediated matrix metalloproteinase 2 (MMP2) and MMP9 expression [35]. SRC-1, another ER coregulator, has also been shown to promote breast cancer invasiveness and metastasis by coactivating PEA3-mediated Twist expression [36]. Recent studies have found deregulation of the ER coregulator PELP1 in invasive and metastatic breast tumors [37, 38]. Recent studies using PELP1 overexpression and knockdown demonstrated that PELP1 plays an important role in ER$\alpha$-positive metastasis [10]. Collectively, these studies indicate that ER$\alpha$ and ER coregulators modulate expression of genes involved in metastasis.

## 4. ER$\alpha$ Extranuclear Actions and Metastasis

Emerging evidence suggests that the ER$\alpha$ participates in extranuclear signaling [39]. ER$\alpha$ activation, by E2, induces key features of motile cells including rapid cytoskeletal reorganization and the development of specialized structures including fillopodia and ruffles [37]. To establish the role of E2-mediated extranuclear actions, researchers developed E2-Dendrimers (EDCs), which are nanoparticles coated with estrogen. These EDCs uniquely localize in the membrane and cytoplasm, preferably activating ER$\alpha$ extranuclear signaling. Using these EDCs, researchers have demonstrated that ER$\alpha$ extranuclear pathways have distinct biological outcomes [40]. Our laboratory using EDCs provided further evidence that ER$\alpha$ extranuclear signaling has the potential to contribute to the breast cancer cell motility (Figure 1) [10]. ER$\alpha$ extranuclear signaling promotes stimulation of the Src kinase, mitogen-activated protein kinase (MAPK), phosphatidylinositol 3-kinase (PI3K), and protein kinase C pathways in the cytosol (10, 11). Recent studies identified PELP1 as one of the components of the ER$\alpha$ signalosome in the cytoplasm, and estrogen-mediated extranuclear signaling promotes cytoskeleton reorganization via ER-Src-PELP1-PI3K-ILK1 pathway [10]. Many of the kinases activated by ER$\alpha$ extra-nuclear signaling are implicated in breast cancer metastasis. For example, ERK and protein kinase B (AKT) phosphorylation play important roles in breast cancer cell migration [14], and Src and ILK1 kinases play critical roles in invasion and metastasis of breast cancer cells [41, 42].

In addition to ER$\alpha$ interactions with cytosolic kinases, few other mechanisms by which the ER$\alpha$ activates extranuclear signaling have been reported. Membrane-bound ER$\alpha$ has been reported to be associated with growth factor receptors such as IGF-1R, EGFR, and HER2; such interactions play a role in cytoskeleton reorganization [43]. Dysregulation of HER2 in breast cancer cells enhances the expression of an isoform of MTA1 (MTA1s), which promotes the cytoplasmic sequestration of ER$\alpha$ leading to constitutive activation of MAPK. These study findings implicate the regulation of the cellular localization of ER$\alpha$ by MTA1s as a mechanism for enhancing ER$\alpha$ extranuclear actions by nuclear exclusion [44]. Recent studies also found that the ER$\alpha$ was methylated via posttranslational modifications, and methylated ER$\alpha$ was predominantly present in the cytoplasm, suggesting that deregulation of arginine methylases may have consequences in activation of ER$\alpha$ extranuclear actions [45]. Collectively, these emerging results suggest that ER extranuclear signaling has the potential to promote breast cancer cell migration and metastasis.

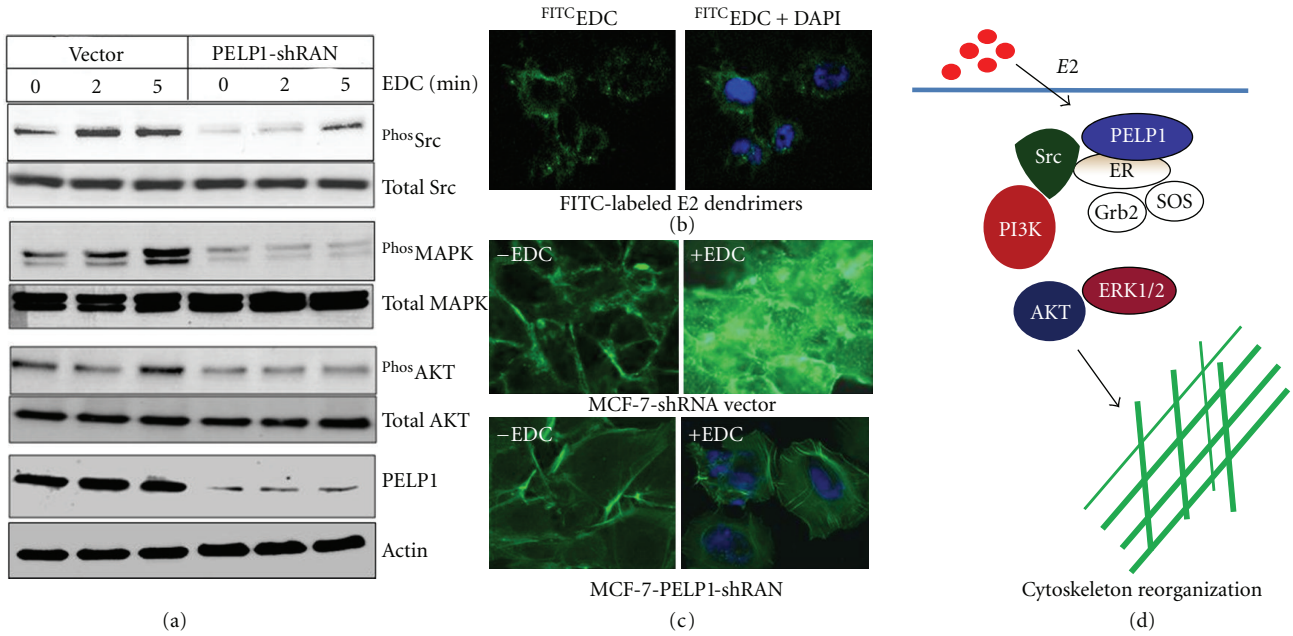


Figure 1: ER-extranuclear signaling promotes actin reorganization via ER coregulator PELP1. (a) MCF7 shRNA vector control and MCF7-PELP1-shRNA cells were cultured in 5% DCC serum containing medium treated with or without estrogen dendrimers (EDC). The activation of signaling pathways was analyzed by Western blotting of total protein lysates with phospho-specific antibodies. (b) MCF7 cells were treated with FITC-labeled EDC and localization of EDC was analyzed by confocal microscopy. *Green; EDC; Blue, DAPI.* (c) MCF7 or MCF7-PELP1-shRNA cells were treated either with E2 or EDC and the F-actin status was analyzed by phalloidin staining and visualized by confocal microscopy. (d) Schematic representation of estrogen-mediated extranuclear signaling. Adapted from [10].

## 5. ERα Regulation of Metastasis

Metastases spawned by malignant tumors that have acquired increased invasiveness are responsible for almost all breast-cancer-related morbidity and mortality. The majority of ERα-positive cells retain their ERα and respond positively to initial endocrine therapy for the treatment of advanced metastatic disease. Several recent studies have detected the presence of ERα expression in metastatic tumors [46–48]. A correlation between ERα-positive tumors and the development of bone metastasis has been observed clinically [49, 50]. Many metastatic tumors retain ERα. If primary tumors are ERα positive, greater than 80% of the lymph node metastases, and 65–70% of distant metastases retain ERα [46, 47]. A clinical correlation has also been reported between ERα-positive tumors and the development of bone metastasis [49, 50]. ERα signaling has also been shown to enhance lung metastasis [51]. In addition, ERα-mediated signaling has enhanced lung metastasis by promoting host-compartment response [51]. These emerging findings suggest that ERα signaling plays a role in metastasis.

## 6. ERβ Regulation of Cell Migration and Metastasis

ERβ, similar to ERα, also functions as a transcription factor that mediates different physiological responses to estrogen signaling. However, the physiological consequences of ERβ-mediated transcriptional regulation are distinct from those of ERα [1]. A number of recent studies suggest that an increase in ERβ expression decreases cell proliferation and that ERβ has antiproliferative (tumor suppressor) functions [52–54]. Reduced expression of ERβ was reported in invasive breast cancer [55], and ERβ expression is associated with less invasive and proliferating tumors [56]. Downregulation of ERβ is shown to promote epithelial-to-mesenchymal transition (EMT) in prostate cancer cells [57]. A recent study using breast cancer model cells provided evidence that ERβ expression was associated with less cell migration. Mechanistic studies indicated that ERβ affects integrin expression and clustering and consequently modulates adhesion and migration of breast cancer cells [58]. Collectively, the emerging evidence in various model cells (including ovary and prostrate) suggests that ERβ signaling may promote antimigratory and anti-invasive responses; however, future studies using breast models are needed to further validate these findings.

## 7. Estrogen Regulation of EMT

EMT constitutes the loss of hallmark structures and physiologic properties associated with the epithelia and the gain of new properties, including migratory and invasive growth patterns [59]. Loss of E-cadherin is a key initial step in the transdifferentiation of epithelial cells to a mesenchymal phenotype, which occurs when tumor epithelial cells invade the surrounding tissues [60]. Evolving evidence suggests that estrogen signaling can influence EMT and ERα signaling

crosstalk with several EMT regulators such as Snail and Slug. ER$\alpha$ directly binds to and regulates the promoter of metastasis tumor antigen (MTA) 3 that suppresses *Snail*, a gene implicated in EMT transition [61]. ER$\alpha$ downregulates *Slug* transcription by the formation of a corepressor complex involving HDAC1 (histone deacetylase 1) and N-CoR (nuclear receptor co-repressor) [62]. Estrogen promotes down-regulation of E-cadherin via transcriptional regulation by recruitment of corepressors such as scaffold attachment factor B [63]. Estrogen plays an important role in cytoskeletal rearrangements mediated by delocalization of E-cadherin [64]. Furthermore, a recent study found that E2 promotes reversible EMT-like transition as well as collective motility in ER$\alpha$-positive cells [65]. Estrogen-regulated EMT is complex and is dependent on temporal expression patterns of MTA family members, cell-adhesion-essential regulators, and ER coregulators [66]. ER$\alpha$ signaling negatively regulates EMT by modulating MTA3 expression and thus promotes differentiation [61]. Collectively, these findings implicate that estrogen-mediated EMT depends on the cellular repertoire of ER$\alpha$ coregulators and EMT regulators and that their cross talk has potential to differentially affect breast cancer progression, leading to metastasis via EMT changes.

## 8. Tumor Microenvironment Regulation of ER Signaling

The metastasis signaling cascade is orchestrated through the activation of biochemical pathways that involve the tumor microenvironment. Stromal cells (fibroblasts, inflammatory cells, and endovascular cells) play important roles to create a supportive environment for tumor cell growth [67, 68]. Chemokines produced by stromal cells have potential to influence ER$\alpha$-positive breast cancer progression to metastasis. The chemokine CXCL12/SDF-1 and its G-protein-coupled receptor CXCR4-mediated signaling pathways play important roles in the migration and invasion of breast cancer cells. Some evidence suggests that HER2-mediated breast tumor metastasis may involve HER2 and CXCR4 signaling pathway cross talk [69]. CXCR4 overexpression correlated with worse prognosis in patients and constitutive activation of CXCR4 in poorly metastatic ER-positive MCF7 cells led to enhanced tumor growth and metastasis. The results from this study showed that enhanced CXCR4 signaling is sufficient to drive ER$\alpha$-positive breast cancers to a metastatic and endocrine-therapy-resistant phenotype via increases in MAPK signaling [70].

The intratumoral levels of estrogens and growth factors are regulated by the tumor-stromal interactions in the tumor microenvironment [71]. Cross talk between the tumor and stromal cells promote expression of aromatase, a key enzyme in E2 biosynthesis, resulting in intra-tumoral estrogen production in postmenopausal breast tumors [72]. Tumor-stromal cross talk regulates aromatase gene expression via the production of various factors such as COX2, tumor necrosis factor-$\alpha$, interleukin-6, and interleukin-11 [71]. Tumor-stromal interactions also contribute to the expression of growth factors such as EGF and IGF-1, which activate the ER$\alpha$ through growth factor receptor cross talk, leading to ER$\alpha$-positive breast cancer progression [73].

## 9. ER Signaling Components as Potential Biomarkers for Predicting Metastasis

ER$\alpha$ status is routinely used in the clinic for treatment selection; however, additional markers are urgently needed to predict metastasis. Considering the evolving significance of ER$\alpha$ coregulators (SRC family members such as SRC-3/AIB1) in mammary tumor invasion and metastasis [74], SRC-3 status could be used as a diagnostic biomarker. Similarly, expression of the ER coregulator PELP1 is deregulated in metastatic breast tumors [37], and PELP1 protein expression is an independent prognostic predictor of breast cancer-specific survival and disease-free survival [38]. Since PELP1 plays a critical role in estrogen-mediated extranuclear signaling, these findings suggest that PELP1 could be used as a potential biomarker for predicting ER-driven metastasis. Several studies using various Src kinase inhibitors and dominant-negative mutants demonstrated that inhibiting c-Src activity decreased the metastatic potential of breast cancer cells [75]. Given the role of Src kinase in ER signaling, phosphor-c-Src is an attractive biomarker for predicting breast cancer metastasis in conjunction with other prognostic factors. Few recent preclinical studies using Src inhibitors confirmed the downstream target of Phos-Src and -FAK and could be possible diagnostic markers [76]. Because AKT signaling is implicated in invasive ductal carcinoma of the breast and implicated in ER$\alpha$-mediated extranuclear actions leading migration/invasion, Phospho AKT (pAKT) status could be a potential biomarker in the prediction of therapeutic response in invasive ductal carcinoma of the breast [74]. Even though these emerging findings suggest ER$\alpha$-signaling molecules as potential biomarkers, additional studies using a large set of human tumor samples are needed to clearly establish them as prognostic markers.

## 10. Therapeutic Targeting of ER$\alpha$ Signaling for Blocking Metastasis

The emerging significance of the ER$\alpha$ in the metastatic cascade indicates novel possibilities for therapeutic targeting of specific ER$\alpha$ signaling components that mediate migration, invasion, and EMT. A large portion of metastases retain their ER$\alpha$ when the primary tumors are ER$\alpha$ positive. Several recent studies detected the presence of ER$\alpha$ and aromatase expression in metastatic tumors [46–48]. We envision that the therapies targeting ER signaling axis leading to metastasis are more suitable for early stage patients who have tumors that are amenable to biopsy and IHC analysis. Potential markers of ER$\alpha$ signaling that are implicated in metastasis (including kinases such as Src, AKT, and PI3K and coregulators such as PELP1, AIB1, and SRC-1) could be used in addition to traditional ER$\alpha$ status to identify this subset of patients.

Aromatase is recognized as a potent target in endocrine therapy for the treatment of postmenopausal breast cancers

[73]. Because some metastases retain their ERα signaling, screening of patients with advanced breast cancer for expression of ERα, ER-coregulators, and aromatase may provide a rationale for the development of customized treatment of a subset of patients with ERα-positive and aromatase-positive cancer. These patients could be treated with an aromatase inhibitor (Letrozole) that ablates peripheral estrogen synthesis and ERα degraders/signaling blockers for their ERα-positive metastatic tumors.

Because ERα and ERβ have different physiological functions and have ligand-binding properties that differ enough to be selective in their ligand binding, opportunities now exist for testing of novel ER subtype-specific, selective ER modulators [77]. Several synthetic or novel natural compounds derived from plant materials have the potential to function as ERβ agonists [54, 78], and these compounds may have utility in augmenting ERβ tumor suppressive functions.

If ERβ can hamper the regulation of ERα and inhibit the proliferation as well as affect the crosstalk with growth factors and their receptors, testing of ERβ agonist in combination with other endocrine therapies will provide a novel means to target ERα-driven metastasis. Recent studies found a therapeutic efficacy using ERβ agonists in combination with aromatase inhibitors, and this strategy may be useful in treating aromatase-inhibitor-(AI-) resistant metastatic breast cancer [79].

ERα-positive metastasis has been associated with chemokine signaling through SDF-1-CXCR4. Therefore, CXCR4 signaling is a rational therapeutic target for the treatment of ER-positive advanced breast carcinomas [70]. Integrin-linked kinase (ILK) is a nodal molecule in many molecular pathways that are implicated in cancer metastasis. Recent evidence suggests that ER extranuclear signaling utilizes the ILK axis [10]; therefore, ILK inhibitors such as QLT-0267 could be used to curb motility of breast cancer cells [80]. Since arginine methylation is implicated in ERα extranuclear signaling, blocking arginine methylases could be a possible therapeutic target. Compounds such as guanidine-nitrogen-substituted peptides or the thioglycolic amide RM65 may be useful to block this pathway [81, 82]. SRC3/AIB1 is frequently amplified or overexpressed in human breast cancer and is implicated in breast cancer progression to advanced ERα-positive tumors. Mechanistic studies showed AIB1 overexpression activates the mammalian target of rapamycin (mTOR), and activation of mTOR pathway is critical for AIB1-driven tumorigenesis [83]. Recent studies suggest that mTOR inhibition and ER-targeted endocrine therapy may improve the outcome of the subset of patients with ER-positive breast cancers overexpressing AIB1 [84].

Emerging evidence suggest that Src participates in ERα extranuclear actions and its wide deregulation in breast tumors suggests that it could be a potential candidate for treating ERα-positive metastasis [85]. The fact that Src can mediate interactions between the ERα and growth-factor-signaling pathways is of particular importance because cross talk between these pathways is implicated in activation of ERα extranuclear signaling leading to cell migration and invasion [10]. Further, the ability of the Src axis to promote local estrogen synthesis via aromatase activation has potential to form an autocrine loop of ERα signaling leading to tumor cell proliferation and metastasis [86]. Thus, blocking the Src axis could block ERα signaling at multiple fronts and thus reducing the ability of the ERα to promote metastasis. Recent studies found that inhibition of the Src family tyrosine kinases using inhibitors such as dasatinib can block ERα-mediated extranuclear actions leading to cell migration and invasion [10]. Therefore, it is tempting to speculate that combination of hormonal therapy with dasatinib, an orally available inhibitor of Src family tyrosine kinases that is currently approved for clinical trials to treat solid tumors [87–89], may be useful in curbing breast cancer metastases.

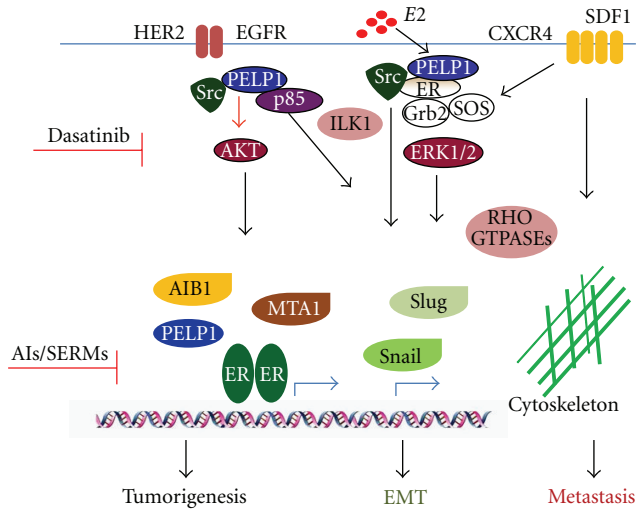


Figure 2: Schematic representation of hormonal regulation of metastasis. ERα-mediated signaling involves nuclear as well as extranuclear actions and growth factor signaling cross talk. Estrogen signaling has the potential to activate extranuclear signaling that activates several kinase cascades, which have potential to alter cytoskeleton, EMT and enhance cell migration. Deregulation of ERα-mediated signaling crosstalk will have implications in estrogen-mediated tumor progression to metastasis.

## 11. Conclusions/Significance

The most deadly aspect of breast cancer is its ability to spread or metastasize. Recent mechanistic studies have increased our understanding and highlight a role of estrogen-induced rapid ERα extranuclear signaling in facilitating the metastatic process. This signaling pathway thus provides new targets for therapeutic intervention. During progression from tumorigenesis to invasion, tumor cells trigger signals that activate ERα-extranuclear-signaling pathways, leading to enhanced cell migratory functions and metastasis, thus ER extranuclear signaling represents an important target for metastatic control of ERα-positive tumors (Figure 2). Since multiple signaling pathways in addition to estrogen are involved in activating ERs, combination therapies using both endocrine and nonendocrine agents that block different pathways may have better therapeutic effects and

may delay the development of estrogen-driven metastasis. Future studies identifying the molecular mechanisms of ER$\alpha$ signaling contributing to ER$\alpha$-driven metastasis as well as examining the prognostic/diagnostic significance of ER$\alpha$ signaling components using a larger sample size of tumors is warranted. Further, elucidation of the pathologic roles of ER$\alpha$ extranuclear signaling in metastasis will have important implications for development of novel breast cancer therapeutics and in the development of the next generation of selective ER modulators.

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*Review Article*

# Current Operative Management of Breast Cancer: An Age of Smaller Resections and Bigger Cures

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Surgical resection was the first effective treatment for breast cancer and remains the most important treatment modality for curative intent. Refinements in operative techniques along with the use of adjuvant radiotherapy and advanced chemotherapeutic agents have facilitated increasingly focused breast cancer operations. Surgical management of breast cancer has shifted from extensive and highly morbid procedures, to the modern concept obtaining the best possible cosmetic result in tandem with the appropriate oncological resection. An ever-growing comprehension of breast cancer biology has led to substantial advances in molecular diagnosis and targeted therapies. An emerging frontier involves the breast cancer microenvironment, as a thorough understanding, while currently lacking, represents a critical opportunity for diagnosis and treatment. Collectively, these improvements will continue to push all therapeutic interventions, including operative, toward the goal of becoming more focused, targeted, and less morbid.

## 1. Introduction

Breast cancer is the most frequently diagnosed nondermatological malignancy in women and ranks second only to lung in cancer-related deaths [1]. While the incidence has increased over the past decade, (Figures 1(a) and 1(b)) the mortality rate of breast cancer has gradually declined [2, 3] (Figure 2). This improved survival may stem from earlier detection as well as improved therapies [2, 3].

Surgical resection was one of the first effective treatments for breast cancer and continues to play a critical role in the treatment of this disease. A multidisciplinary approach is now standard of care, involving a coordinated effort with the surgeon working in concert with the medical and radiation oncologist to achieve the best possible outcome for each individual. Improvements in both the quality and quantity of life for victims of breast cancer can be attributed to the advances made in each of these disciplines. As with all cancers, earlier stage disease is more readily manageable than after significant advancement. It is these early-stage cancers in which the most significant improvements in the operative management has occurred. Adoption of breast conservation surgery has allowed an increased focus on the cosmetic outcome, during a time that has also witnessed improved survival. This represents a clear victory for breast cancer patients, which needs to be extended to breast cancer of all stages.

## 2. Historical Progression of the Surgical Therapy of Breast Cancer

The Greek physician Galen is considered to be one of the earliest advocates of surgical treatment, recommending wide excision of breast tumors nearly 2000 years ago. Galen, like his predecessor Hippocrates, also recognized that breast cancer should be considered a systemic disease. Hippocrates proposed that cancers were the result of an imbalance of the four basic humours-blood, phlegm, and yellow and black bile. He attributed an excess of black bile for postmenopausal women having a greater incidence of breast cancer, as premenopausal women were relieved of this excess black bile with regular menstruation [4]. Although primitive, this concept can be extended to the current breast cancer treatment paradigm:

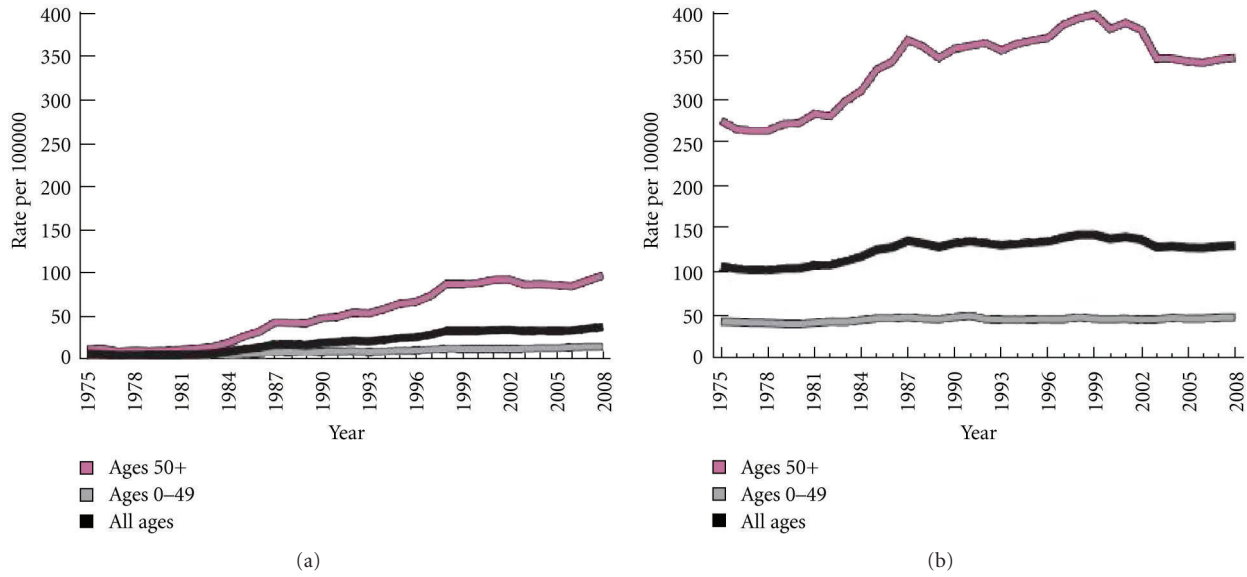


Figure 1: Incidence rates of In situ (a) and Invasive (b) female breast cancer in the United States (1975–2008). American Cancer Society. *Breast Cancer Facts and Figures 2011-2012*. Atlanta: American Cancer Society, Inc.

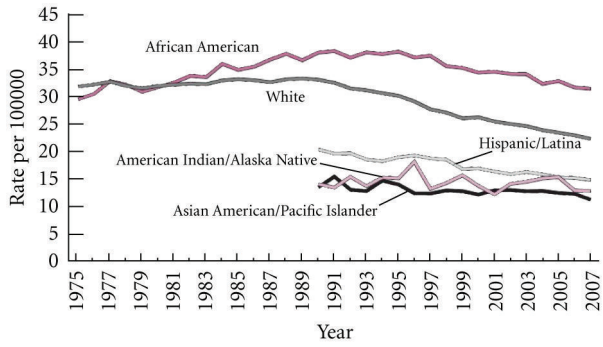


Figure 2: Mortality rate of female breast cancer, by race and ethnicity (1975–2007). American Cancer Society. *Breast Cancer Facts and Figures 2011-2012*. Atlanta: American Cancer Society, Inc.

systemic control of the disease at a molecular level, with local control by surgical intervention. While we now know that "black bile" does not result in breast cancer, the most effective breast cancer management embodies this concept of breast cancer as a systemic disease.

Early operations to treat breast cancer were primitive and brutal. These procedures consisted of amputation followed by cauterization, performed as rapidly as possible to minimize hemorrhage. Unfortunately, patients surviving the initial surgical procedure would all too frequently die of fulminant sepsis. In the late 19th to early 20th century, the advances of general anesthesia and antiseptic techniques facilitated more extensive procedures. Some of the most dramatic changes in surgical therapy for breast cancer were pioneered by William Steward Halstead [5]. His approach to the mastectomy helped change the surgical therapy of the breast from a simple amputation to a formal procedure. His technique, now termed the "radical mastectomy," involved en bloc resection of the breast, the pectoralis muscle, and the axillary contents. This procedure was as effective at initial local tumor control as any early technique, with the significant advancement of a dramatically decreased rates of recurrence that plagued Halstead's predecessors [5].

During Halstead's era, prior to any understanding or capacity for early diagnosis, initial presentation of profoundly advanced tumors was the norm. Accepting Halstead's basic principles, surgeons attempted progressively extensive resections for cure of widely disseminated tumors. This evolved into dissection of the neck, abdomen, and even the mediastinum to remove diseased lymph nodes. Around the same period, early methods for surgical staging were developed, yielding a basic classification of patients with tumors in which radical mastectomy was potentially curative and those with disseminated cancer not appropriate for attempted resection. However, it would not be until the 1940s when evidence from preoperative staging brought the futility of what had become "superradical mastectomies" into question [5].

Initial deviation from the tenets of Halstead began in the late 1930s with an initial push for preservation of the nondiseased breast tissue during cancer resection. Shortly thereafter, postoperative radiotherapy was added for control of local tumor recurrence, laying the groundwork for breast conservation therapy (BCT) as we know it today [6]. Although BCT was not significantly implemented in clinical practice until the 1980s, the stage was set for the current surgical treatment of breast tumors utilizing either BCT or mastectomy.

## 3. Current Operative Management of Breast Cancer

Optimal management of a patient with breast cancer includes establishing a pathologic diagnosis prior to any

definitive operative intervention. Formal surgical excision in the operating room is rarely required to establish the diagnosis of breast cancer, as there are many alternative techniques to obtain tissue for diagnosis. For example, much pathologic information can be gained from small, 1-2 mm "core" samples, allowing precise recommendations for treatment. The diagnosis of breast cancer is confirmed by histological evaluation, and the tumor is assessed for grade as well as human epidermal growth factor receptor 2 (HER2), estrogen, and progesterone receptor status [7]. This information is critical for optimal decision making regarding treatment options, most importantly allowing for coordination of care for those patients that will benefit from neoadjuvant chemotherapy prior to operative intervention [7].

After the diagnosis of breast cancer is established, patients are evaluated to determine the extent of the disease. Standard of care includes bilateral mammography to identify any suspicious areas in either breast that will impact surgical management. Laboratory values that will assist in treatment recommendations include complete blood count, liver function tests, and alkaline phosphatase. There are not established tumor markers for breast cancers, although cancer antigen (CA) 15-3 and CA 27-29 may be helpful when elevated. Additional imaging studies to evaluate for metastatic disease are obtained depending on signs and symptoms of the patient, as well as the clinical stage at presentation. A bone scan is indicated if the patient has localized bony pain or elevated alkaline phosphatase, chest imaging is indicated for pulmonary symptoms, and abdominal imaging by computerized tomography is indicated for abnormal liver functional tests or abdominal symptoms. A review of the acquired data, including pathology, laboratory assessment, and imaging, allows the multidisciplinary team to make recommendations for definitive management of the patient with breast cancer. Those patients with evidence of advanced disease are typically managed medically with preoperative chemotherapy, prior to any definitive surgical management.

Locoregional (operative) control of breast cancer remains the mainstay of treatment. Surgical treatment should allow the patient to be involved in the decision-making process, with the surgeon providing information about all surgical options available. Definitive surgical management typically involves breast conservation (BCT) or mastectomy. Local excision alone is at times acceptable, usually in the setting of elderly or otherwise debilitated patients without adjuvant radiation. This decision must be carefully weighed and based on evaluation of tumor aggressiveness and comorbid conditions of the patient.

There are two required components for BCT. First, tumors must be resectable with a pathologically clear margin, that is, a surrounding margin of breast parenchyma without disease. Secondly, patients undergoing partial mastectomy typically receive whole breast irradiation to achieve local control in the breast. Tumor size must be sufficiently small relative to the entire breast, such that the appearance of the breast is cosmetically acceptable following partial mastectomy. Additionally, all suspicious findings on imaging must be resectable with the partial mastectomy. The presence of diffuse highly concerning microcalcifications on mammography is a contraindication to BCT. Pregnancy and a history of previous chest irradiation do not allow BCT, as they are contraindications to the requisite postoperative radiotherapy. Positive margins after BCT require a repeat attempt at excision or completion mastectomy to achieve clear margins. Findings of involved margins with partial mastectomy significantly increase the chance of disease recurrence [8].

Mastectomy is indicated for the curative resection of tumors (i.e., absence of metastatic disease) not amenable to BCT, and for those patients that do not want to consider conservation even though they meet criteria. The modern version of this procedure is termed the "modified radical mastectomy," which entails removal of the breast, its underlying pectoralis fascia, and axillary contents, performed for more extensive disease.

In addition to resection of the primary tumor, all invasive breast cancers require assessment of axillary lymph nodes for tumor invasion. The ipsilateral axillary lymph nodes are theoretically the first site that breast cancer is expected to spread, with the sentinel nodes representing the first group of nodes at risk for invasion. Assessment of the axillary nodes includes sentinel lymph node biopsy (SLNB) during lumpectomy, or at the time of mastectomy. The SLNB represents another hallmark of targeted surgical therapy. Injection of a dye and/or radio-isotope into the breast allows the surgeon to identify the first ("sentinel") lymph node draining the tumor basin. Involvement of axillary nodes is considered regional disease (not metastatic) and is usually followed by complete axillary node resection [8]. Nodal status provides critical staging information necessary for the proper selection of adjuvant therapy. Furthermore, negative findings after a properly performed SLNB allow a patient to avoid the potential for significant morbidity after axillary dissection. An all too common and often debilitating complication of this procedure is upper extremity lymphedema [9].

In situ breast cancer is a neoplasm that is completely contained within its basement membrane. This early neoplasm can be derived from a duct or lobule and is, therefore, referred to as lobule carcinoma in situ (LCIS) or ductal carcinoma in situ (DCIS). LCIS of the breast requires special consideration, as it is considered a marker for the future development of invasive breast cancer. The risk of developing invasive cancer is low, and if it occurs, histology tends to be favorable. For this group of women, LCIS is managed by appropriate monitoring without additional intervention. Alternatively, hormonal therapy can be administered for the purpose of breast cancer prevention. The potential adverse reactions of these medications must be considered and balanced with the presumed risk reduction.

In contrast to LCIS, the diagnosis of DCIS requires treatment for local control at the time of diagnosis. With the development of techniques for the earlier diagnosis of breast cancer, DCIS is the only diagnosis in approximately 15% of newly diagnosed breast cancer patients. This finding must be addressed, as the survival rates for treated DCIS are near 100%, but the development of invasive disease occurs in up to 30% of patients with untreated DCIS [10]. Treatment options include breast conservation with partial mastectomy and radiation, or total mastectomy. Although DCIS is often

found in conjunction with an invasive carcinoma, treatment for the invasive component takes precedence and dictates both surgical and medical management. In contrast to management of invasive disease, those patients with DCIS usually do not require axillary dissection, as axillary nodal involvement in patients with pure DCIS is unusual. As a small number will have axillary involvement, sentinel node evaluation should be performed if mastectomy is the chosen operation for local control [11].

## 4. Breast Cancer Surgery and Chemotherapy

Starting in the mid-twentieth century, most notably in the lab of Bernard Fisher, early chemotherapeutic agents were being analyzed for use in the preoperative setting. The use of neoadjuvant chemotherapy (NACT) prior to an attempted surgical resection represents a dramatic improvement in breast cancer therapy, addressing the systemic aspect of this disease. NACT is indicated for locally advanced tumors or inflammatory breast cancer. Locally advanced breast cancer entails large tumors or those that invade the chest wall or skin (T4) or have spread to the axillary nodes (N2 or N3) [12].

An excellent response to chemotherapy merits reassessment of the patient to ensure a concomitant clinical and radiological response. Eradication of all tumor after neoadjuvant chemotherapy is termed pathological complete response (pCR), strictly defined as the absence of invasive cancer from the breast and axilla on pathological assessment in response to chemotherapy [13]. While achieving pCR has been found to increase long-term survival [14], a wide range of local recurrence rates (2.6–22.6%) after BCT following neoadjuvant therapy has been noted [15]. One recent study indicates that Her2 positive and positive axillary lymphadenopathy may predict this recurrence after pCR [15]. While high risk populations certainly merit close postoperative surveillance for recurrent disease. Appropriately placing those patients achieving excellent response to chemotherapy into the algorithm for the surgical management of breast cancer requires further assessment. Improved methods are needed to predict those tumors best amenable to downstaging to BCT, as certain patients may in fact be better candidates for mastectomy. Furthermore, strict criterion defining the medical management of successful pCR is also needed. Molecular tests such as the 21 gene (oncotype DX) and 70 gene (mammaPrint) assay [7], that provide tumor-specific scores reflecting risk of recurrence, may become useful in this scenario.

The effectiveness of NACT for locally advanced disease eventually led to the use of pre-operative treatment in an attempt to "downstage" even more advanced cancer to a scope amenable to treatment by mastectomy [12]. A recent extension of these principles is the use of chemotherapy to downstage tumors, in order to avoid mastectomy altogether in lieu of BCT. NACT is indicated for tumors meeting all criteria for breast conservation (see above) except for tumor size. An excellent response in this scenario has now allowed the option for BCT in a patient who would have required a mastectomy.

## 5. Recent Advances in the Surgical Therapy of Breast Cancer

Most of the recent advances in the surgical management of breast cancer follow the basic template of ever more conservative surgical resections. The first involves operative breast cancer therapy with a concomitant focus on breast reconstruction, known as oncoplastic breast surgery [16]. This trend represents another advancement made possible by the refinements in the use of postoperative radiation, the same concept that led to the advent of BCT. Oncoplastic surgery entails the use of plastic surgery techniques to restore cosmesis and natural symmetry, ideally during cancer resection [16]. Plastic surgery techniques utilized include breast augmentation and reduction, flaps, implants, and expanders, on both the diseased and the normal breast if necessary to achieve the desired symmetry. Indications are still widely debated, but appropriate candidates are those that have sufficient residual breast after the oncological resection to facilitate the necessary reconstruction [6].

One of the most recent advances in surgical therapy involves management of the positive sentinel lymph node biopsy (SLNB). Traditionally, a positive SLNB represents an absolute mandate for a complete axillary dissection. Substantial morbidity, not unlike that which was seen in the days of Halstead, all too often follows. However, a recent study has demonstrated that high-risk patients with small tumors (T1-T2) and limited lymph node spread, who are able to receive radiotherapy, do not benefit further from complete axillary lymph node removal [17]. Simply stated, survival for small breast tumors with limited spread does not improve after axillary dissection in older individuals or those with significant medical problems. This early work has found that this subset of patients suffer more from the complications of the procedure than benefit. The adjuvant radiotherapy therapy given for this early-invasive disease seems to provide most of the survival benefit.

A recent trend including surgery as cancer prevention has gained wide acceptance. Contralateral prophylactic mastectomy (CPM) has been found to decrease the risk of development of a cancer in the disease-free breast in women at high risk. Those women harboring a BRCA mutation or a strong family history of breast cancer may be considered candidates for prophylactic bilateral mastectomy. As mentioned previously, with the diagnosis of LCIS, the risk of developing an invasive breast cancer is equal in both breasts, such that bilateral mastectomy may be necessary for true risk reduction. Many women, in an otherwise low-risk category, also opt for CPM after a newly diagnosed breast cancer. This usually involves fear of developing disease in the contralateral breast. While recent data suggests an increased overall as well as disease-free survival after CPM [18], the debate is ongoing regarding the appropriate indications for CPM. This is in fact an extensive operation with the potential for significant morbidity. The decision to take such a measure is formidable. Similarly, the quest to identify the population benefiting the most from this intervention must be equally rigorous.

## 6. Surgery and Breast Cancer Metastasis

The most successful operative management of metastasis is prophylactic: appropriate screening for detection of suspicious lesions of the breast, followed by appropriate local control to minimize the potential for metastatic dissemination. This is reflected in recent trends showing improved survival of breast cancer patients, as screening and early intervention has translated into improved outcomes. After the diagnosis and completion of treatment of a primary breast cancer, surveillance for recurrence or metastatic spread ensues. Follow-up entails focused clinical and laboratory assessment, and mammography to detect new or recurrent lesions.

The discovery of metastatic disease at any point merits a complete reevaluation. Traditionally, surgical intervention was avoided in the patient harboring metastatic disease, due to a perceived lack of benefit. Only those patients with extremely limited metastatic lesions were considered for therapeutic resection. For the most part, patients found to have metastatic disease were deferred to induction chemotherapy in the hopes of an excellent response and prolongation of life.

Most traditional use of surgical intervention in the setting of metastatic disease was for palliative purposes, at either the primary tumor site or any distant metastatic lesions. For example, resection of the primary tumor was considered for persistent infection, bleeding, or general difficulty maintaining cleanliness. However, many recent studies have been able to challenge this practice of avoiding intervention on the primary tumor in the setting of metastatic disease. Early studies indicate that resection of a primary breast lesion may increase survival in the setting of limited metastasis. This effect probably stems from more effective and specific chemotherapy, but randomized trials are needed to define both the optimal candidates and indications for this intervention. However, the significance of the early findings of reduced need for chemotherapy, improved quality of life, and even long-term cures with the concomitant resection of (limited) metastatic lesions cannot be overstated [19].

Operative intervention for metastasic lesions is typically palliative, involving the treatment of a symptomatic mass. This may entail bypassing an obstructing metastatic lesion in the bowels, utilizing a normal segment of bowel to allow free flow of intestinal contents. However, aggressive resection of metastatic lesions for curative intent has gained favor in recent years. The best studied is the resection of metastatic lesions to the lung, in which long-term success and even some cures have been reported. The patients most amenable to metastasectomy are those with limited metastatic burden (oligometastases) with hormonally responsive tumors. Operative characteristics include smaller lesions in a location that facilitates complete removal [20].

It is a well-known fact that the most common site of breast cancer metastasis is the bone, with breast cancer being the leading cause of bone metastasis of any cancer in women. The lung, liver, and brain are other common sites of metastasis. However, it has recently been demonstrated that the basic breast cancer subtypes (Luminal A, luminal B, HER-2 positive, and basal) differentially target certain sites for metastasis. For example, the HER-2 positive and triple negative subtypes have been shown to preferentially metastasize to the brain over the other subtypes [21]. While this represents an interesting finding, further investigation is needed to translate this data into clinical practice. For example, knowledge of the presence of a basal phenotype in a high risk patient may merit more aggressive, organ-specific followup.

## 7. Surgery and the Breast Cancer Microenvironment

Surgical resection of breast cancer is absolutely curative if performed while the primary tumor is contained. Escape of tumor cells from the primary lesion completely changes therapeutic management, expectations, as well as outcomes. Chemotherapy becomes the primary hope for cure as opposed to surgical intervention. Interestingly, some early stage tumors, all of which were previously assumed to be self-contained, have been shown to harbor the capacity for systemic tumor dissemination. While there is no method to accurately predict which tumors have this devastating capacity, certain factors such as large tumors, younger age at diagnosis, vascular invasion, and nodal involvement have been found to be associated with a high risk of developing distal metastasis after appropriate treatment [22, 23].

The best treatment option currently available is effective loco-regional control of the primary tumor. The surgeon's primary focus at the time of resection is obtaining clear margins. Most studies have found that obtaining at least a 2 millimeter margin for invasive and in situ breast cancer best minimizes the chance of local recurrence [24, 25]. This threshold has consistently led to reduced local recurrence rates, while balancing the potential for an overly aggressive resection. Effective local control removes the nidus for both local and distant recurrence, emphasizing the management of the primary tumor on the systemic aspect of the disease. This effect is exemplified by the significant increase in distant metastasis rates and subsequent survival with the development of a local recurrence of a resected breast tumor [22–26].

Further evidence that breast cancer, even at its early stages, can be a systemic disease can be found in animal studies and early analysis in cancer patients. Utilizing PCR and immunohistochemistry, increased cancer-related cells have been demonstrated in the systemic circulation due to surgical manipulation [27–30]. Needle biopsies of primary tumors have even been found to result in increased rates of nodal metastasis [31, 32]. Tumor cells that break off from the primary site and enter the systemic circulation are referred to as circulating tumor cells (CTCs). While the CTCs were first described over a century ago, the technology for their detection has only recently become reliable. Current methods allow for the enrichment of CTCs by antibody-mediated targeting of the epithelial cell adhesion marker (EpCAM). While the clinical usefulness of CTC assessment is controversial, some consider that greater than five CTCs is the breaking point for a poor prognosis in breast cancer [33, 34].

Detection of CTC has been used to demonstrate significant shedding of putative tumor cells into the systemic circulation during surgical manipulation [35]. While this

shedding is known to occur in both breast and lung cancers [36], the functional result and ability of these cells to successfully migrate and seed distant sites is not known [37]. Furthermore, some hypothesize that the tissue trauma resultant from needle or operative manipulation may lead to the expression of an invasive or metastatic phenotype [38]. This alteration may lead to cancer progression or the release of CTCs, respectively [39]. Pathways implicated in these effects are normal and appropriate wound healing responses, such as those involved in inflammation and angiogenesis [38]. With the continued technological improvement for the detection of CTCs, determining the clinical relevance of these effects may become possible. The assessment of CTCs could one day provide the basis for highly specific real-time biopsies, yielding a strong potential for the modification of surgical techniques and traditional indications. The capacity to harvest and analyze CTCs could become a key feature of individual tumor profiling, allowing for patient-specific therapies to further reduce the current complication profile of today's interventions [8, 40].

## 8. Conclusion

Surgical intervention is currently the best hope for definitive cure of breast cancer. Even so, recent advances represent significant steps away from the extensive resections performed by Halstead and his predecessors. While these early attempts successfully decreased local recurrence rates, advances in the treatment of breast cancer as a systemic disease were needed to facilitate long-term cures. Continued improvements in early diagnosis via breast imaging, advanced prognostic tests, patient-specific molecular diagnosis, and the development of targeted chemotherapeutic agents provide hope for improved survival rates. By doing so, breast cancer therapy will become more focused, increasing efficacy and reducing complications of all the treatment disciplines. This will move the bar closer to the ultimate goal of transforming breast cancer into an easily targeted, readily manageable disease.

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*Review Article*

# The Hedgehog Pathway Conditions the Bone Microenvironment for Osteolytic Metastasis of Breast Cancer

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The microenvironment at the site of tumor metastasis plays a key role in determining the fate of the metastasizing tumor cells. This ultimately has a direct impact on the progression of cancer. Bone is the preferred site of metastasis of breast cancer. Painful, debilitating osteolytic lesions are formed as a result of crosstalk between breast cancer cells and cells in the bone, predominantly the osteoblasts and osteoclasts. In this paper, we have discussed the temporal and spatial role of hedgehog (Hh) signaling in influencing the fate of metastatic breast cancer cells in bone. By virtue of its secreted ligands, the Hh pathway is capable of homotypic and heterotypic signaling and consequently altering the microenvironment in the bone. We also have put into perspective the therapeutic implications of using Hh inhibitors to prevent and/or treat bone metastases of breast cancer.

## 1. Introduction

The overwhelming numbers of cancer patients ($\geq$90%) that die due to the dissemination of cancer cells rather than the primary tumor throw the process of metastasis to the centre stage of clinical management of cancer [1]. However, even as we embark on this review, the most poorly understood aspect of the pathogenesis and progression of cancer is the process of metastasis of the tumor.

Evolving literature supports that metastasis is a second disease imposed on the primary tumor. The outcome of metastasis is determined by the interplay between the subpopulation of metastatic cells and host homeostatic factors in the specific organ microenvironment [2]. The metastatic cascade can be conceptually organized and simplified into two major phases: (i) physical translocation of a cancer cell from the primary tumor to the microenvironment of a distant tissue (Figure 1) and (ii) colonization of secondary site (Figure 2) [3].

The metastasizing tumor cells hijack many of the pathways that play major roles during normal development. Many of the embryonic developmental signaling pathways, such as the Wnt, Hedgehog (Hh), and Notch pathways, affect the survival of tumor stem cells and orchestrate a complex microenvironment that promotes tumor survival and progression. In this review, we will highlight the significance of the Hh pathway in developmental biology and our present understanding of its role in regulating breast cancer metastasis to bone. We will elaborate how a pathway that is so critical in normal development of the embryo is usurped by the breast cancer cells to serve their own purpose of invading the tissue of its origin, extravasation, survival during translocation, and adaptation at the distant site to bring about proliferation and colonization.

## 2. The Hh Pathway in Normal Development

The Hh pathway plays a central role in embryonic development and maintenance of stem or progenitor cells in many adult tissues [4]. The Hh family of secreted proteins signal through both autocrine and paracrine mechanisms to control cell proliferation, differentiation, and morphology [5]. The ligands comprise desert hedgehog (DHH), Indian hedgehog (IHH), and Sonic hedgehog (SHH). Hh signaling in mammalian cells is mediated by the GLI family of zinc finger transcription factors comprising GLI1, GLI2, and GLI3. GLI1 is a strong transcriptional activator; GLI2 can function as an activator or a repressor in a context-dependent manner; GLI3 is mostly a repressor [6]. In its classical form,

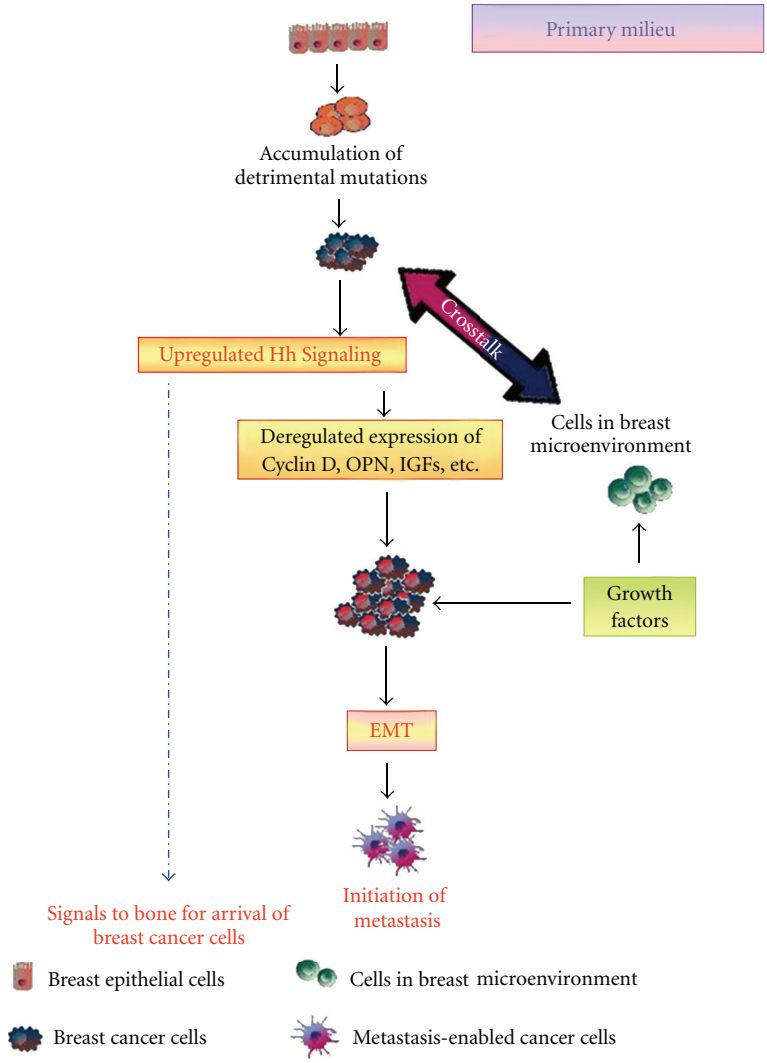


Figure 1: Hh signaling conditions the milieu to support metastasis of breast cancer cells to the bone. Depicted here is the first of the two microenvironments, the milieu of the primary tumor. Hh signaling in the tumor cells impacts the stromal cells in the environment, which in turn amplify paracrine Hh signaling by producing growth factors that propel epithelial-mesenchymal transition. Concomitantly, secreted, soluble proteins produced by the primary tumor contribute towards conditioning the secondary site for the arrival of the tumor cells.

in the absence of the ligand, the Hh-signaling pathway is inactive, GLI1 is sequestered in the cytoplasm and repressed for its transcription activity. Binding of the Hh ligands to the receptor, a 12-pass transmembrane protein called patched-1 or patched-2 (PTCH1 or -2), releases the inhibitory affect of PTCH on a serpentine protein called Smoothened (SMO) [7]. SMO gets hyperphosphorylated and localizes to primary cilia where [8] GLI1 is activated by release from a large protein complex and translocates to the nucleus to function as a transcriptional activator [9] of several target genes, including PTCH, insulin-like growth factor-binding protein and cyclin D2 [10].

The involvement of the Hh pathway, in particular the ligand SHH, with the skeletal system begins with embryonic development, where SHH is expressed in the notochord, the floorplate of the neural tube, the brain, the zone of polarizing activity in the developing limbs, and the gut [11, 12]. SHH specifically functions in many different ways to contribute to the patterning of a developing embryo in a concentration-dependent manner along a target range [13]. A variety of embryonic defects and diseases result from mutations in the Hh pathway [14]. The long-range morphogenic properties of SHH signaling are also evident in the development of the CNS [15]. Thus, temporal and spatial regulation of SHH signaling is key to proper organogenesis. However, in the adults, this pathway is mainly inactive [16] and may play a role in the maintenance and renewal of normal stem cell population in the nervous system [17]. Moreover, Lavine et al. reported that the Hh signaling is essential for cardiac function at the level of the coronary vasculature [18].

## 3. The Hedgehog Pathway in Cancer

The Hh pathway is required for normal proliferation of human melanocytes *in vitro* and for proliferation and survival

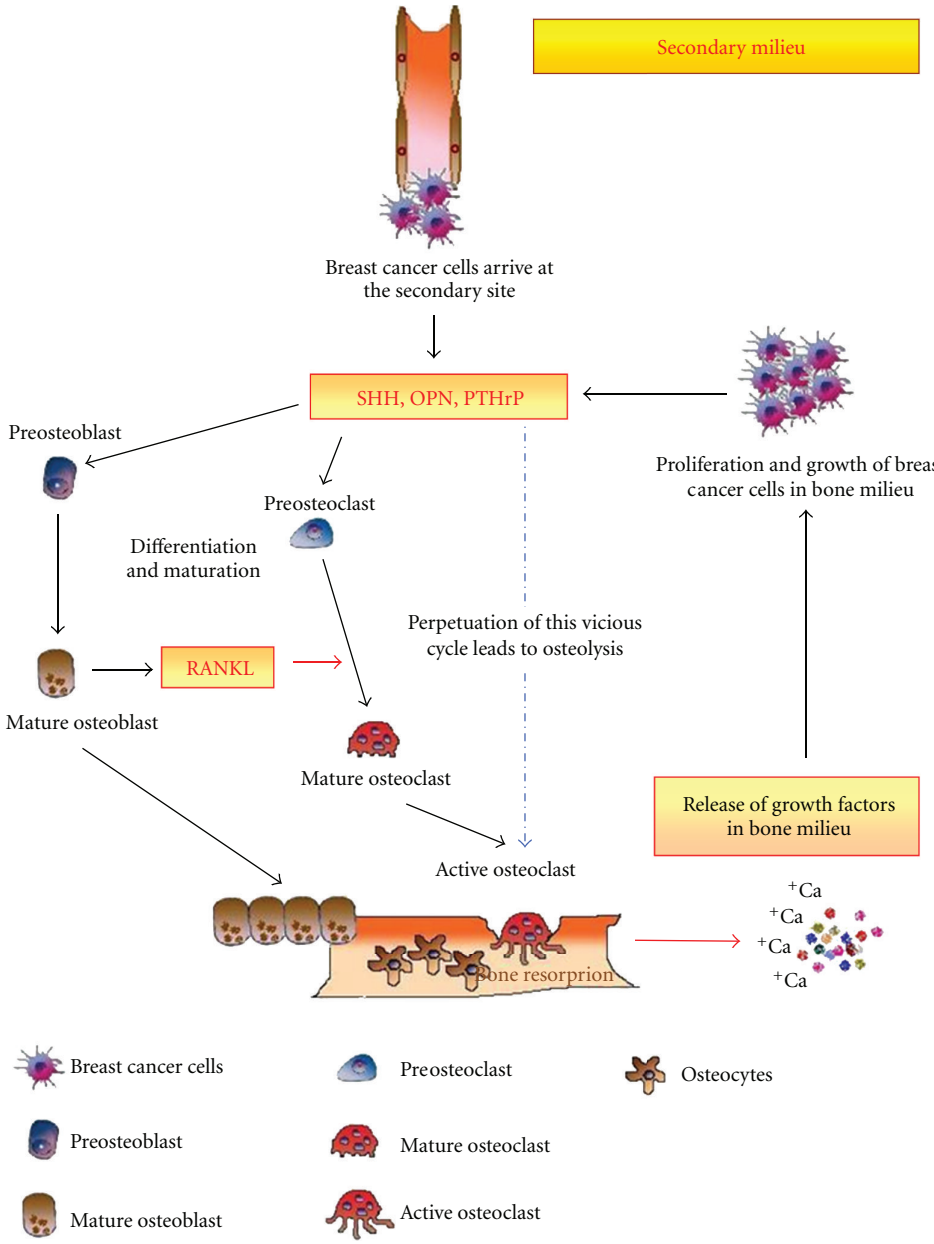


Figure 2: Breast cancer cells armed with Hh signaling disrupt the dynamic equilibrium in the bone to serve its purpose of self propagation and subsequent osteolysis. Breast cancer cells engane in a crosstalk with osteoblasts and osteoclasts. This cumulatively results in the differentiation and activation of osteoclasts and eventually leads to enhancing osteolysis and growth of breast tumor cells in the bone. Overall, this figure addresses the role of Hh signaling in the vicious cycle of osteolytic metastasis of breast cancer.

of human melanoma *in vivo* [19, 20]. In esophageal squamous cell carcinoma, GLI1 expression has been associated with lymphatic metastasis [21], while in breast cancer, strong nuclear GLI staining was observed [22]. Li et al. have recently reported that pancreatic cancer stem cells express high levels of SHH [23]. This is interesting given the implications for SHH in adult stem cell renewal, in pancreatic ductal progenitor cells, and also in adult hair follicle stem cells [24]. SHH is misregulated in pancreatic adenocarcinoma, prostate adenocarcinoma, esophageal and stomach cancer, and nonsmall cell carcinoma [14]. As such, Hh signaling has been shown to be active in multiple cancer types [22, 25–48] (Table 1).

Active Hh signaling is also found to influence the tumor stromal microenvironment [27] and supports stem cells in the tumor in an undifferentiated, proliferative state [26, 50]. SHH is not only a mediator of angiogenesis but has also been shown to induce vessel formation in endothelial cells [51] and activate expression of angiopoietins I and II, and VEGF-signaling proteins from mesenchymal cells, highlighting the significance of tumor-associated fibroblasts in combination with canonical Hh signaling to mediate

Table 1: Cancers with aberrant activation of Hh signaling.

| Milieu | Hh Signaling caused by | Molecule(s) involved | Type of cancer | Reference |
|---|---|---|---|---|
| I | Overexpression | GLI1 | Glioblastoma | [30] |
| | Mutations | PTCH | Basal cell carcinoma (BCC) | [31, 32] |
| | | SMO | Basal cell carcinoma | [31, 32] |
| | | PTCH | Medulloblastoma | [33] |
| | | PTCH | Rhabdomyosarcoma | [34] |
| | | PTCH1 | Gorlin syndrome BCC | [35, 36] |
| | | SMO & PTCH1 | Nonfamilial BCC | [37] |
| II | Ligand-dependent autocrine | | Breast | [22] |
| | | | Pancreatic | [38] |
| | | | Lung cancer | [39] |
| | | | Oesophagal | [40] |
| | | | Prostate | [41] |
| | | | Gastric adenocarcinoma | [42] |
| | | | Colorectal | [43] |
| | | | Hepatocellular adenocarcinoma | [44] |
| | | | Ovarian carcinoma | [45, 49] |
| | Ligand-dependent paracrine | | Pancreatic | [46–48] |

Milieu I represents the microenvironment of the primary tumor; Milieu II represents the microenvironment at the metastatic site.

blood vessel formation [52]. Cancer cells utilize abnormal Hh signaling (both autocrine and paracrine) to influence proliferation and differentiation of their surrounding environment.

The role of Hh signaling in cancer has been revealed by studies that have manipulated the expression of the GLI transcription factors or the ligands or upon treatment with pharmacologic inhibitors that restrict Hh signaling. In pancreatic cancer cell lines, disruption of Hh signaling by the inhibitor cyclopamine, inhibited epithelial-mesenchymal-transition (EMT) [53, 54]. Tumor burden and metastasis in both prostate and pancreatic adenocarinomas were also reduced as a result of Hh signaling inhibition [53, 55]. In contrast, enforced expression of GLI1 induced the expression of Snail [56], an EMT marker. Conversely, we observed loss of mesenchymal markers upon abrogation of GLI1 expression [19]. Overall, GLI1 silencing had a pronounced effect on tumor malignancy *in vivo* by reducing metastasis. We also reported that signaling via the Hh pathway transcriptionally upregulates OPN [19]. OPN is a secreted protein that influences multiple downstream signaling events that allow cancer cells to resist apoptosis, invade through extracellular matrix, evade host immunity [57], and influence growth of indolent tumors [58, 59]. OPN constitutes a component of the secretome of several melanoma-derived cell lines [60, 61] and is also expressed in metastatic breast cancer cell lines [62]. It is highly probable that active Hh signaling in a subset of cancer cells can be propagated in a paracrine manner by OPN secreted into the tumor microenvironment. OPN, by virtue of its ability to signal through multiple receptors, can promote malignant behavior in neighboring cancer cells, regardless of the status of the Hh pathway, thereby propagating paracrine Hh signaling. Thus, at the site of origin, the breast tumor cells not only potentiate their own aggressiveness by influencing the neighboring cells, but also send signals to the secondary target organ to condition for relocalization [58, 63, 64].

For the purpose of this review, we have focused the remainder of the article on discussing the role of Hh signaling in impacting breast cancer metastasis to the bone. This complication of breast cancer continues to present a challenge to oncologists and reduces the chances of survival for breast cancer patients. Among breast cancers that become aggressive, metastasis to bone marrow is common. Detection of bone metastasis often signals the onset of the life-threatening phase of breast cancer. The 5-year survival rate is 98% for breast cancer when detected early; this precipitously drops to 83% for patients initially diagnosed with regional spread and to 26% for those with distant metastases. In the following sections, we will discuss the role of Hh signaling in mediating a crosstalk between breast cancer cells and cells in the bone and the overall impact on the ability of breast cancer cells to sculpt the bone microenvironment and cause osteolysis (Figures 1 and 2).

## 4. The Bone Microenvironment

The bone microenvironment comprises osteoblasts, osteoclasts, mineralized bone matrix, and other cell types, such

as the osteocytes embedded within bone. Of these, the most important ones (from the perspective of this article) are the bone-resorbing osteoclasts and bone-forming osteoblasts.

Osteoblasts are derived from mesenchymal stem cells, which can also give rise to chondrocytes, fibroblasts, myocytes, or adipocytes [65]. Formation of new bone and the regulation of osteoclastogenesis through expression of RANKL and OPG are two main functions of the osteoblasts. Various growth factors and hormones like BMPs, PTHrP, TGF$\beta$, and so forth are known to take part in the differentiation of preosteoblasts into mature osteoblasts. Eventually, mature, mineralizing osteoblasts become embedded in the newly secreted bone matrix and undergo terminal differentiation to form osteocytes. Although the osteocytes have much reduced activity as compared to osteoblasts, their long processes allow them to connect the entire matrix via a series of canaliculi. It is understood that the osteocytes ensure communication between sites deep in the bone and the extraosseous world; they create an enormous increase in mineral surface exposed to extracellular fluid and cellular activity and function as mechanosensory cells of bone, involved in the transduction of mechanical loads into biochemical signals [66].

Osteoclasts, on the other hand, are large multinucleated terminally differentiated cells with a unique ability for bone resorption [67]. They are derived from hematopoietic stem cells. The cells undergo proliferation in response to M-CSF. The precursor cells flaunt receptor activator of nuclear factor $\kappa$B (RANK) on the surface, while the ligand RANKL is expressed by the bone marrow stromal cells and osteoblasts. Binding of the ligand to the receptor commits the precursor cells to the osteoclast lineage. The same interaction is also critical for osteoclast formation and can also promote osteoclast activity, since RANK is also present on the surface of terminally differentiated osteoclasts. The fusion of osteoclast precursor cells results in the formation of large multinucleated active osteoclasts.

Osteoprotegerin (OPG) is a soluble decoy receptor and a competitor of RANKL in its binding with RANK and thus can inhibit osteoclastogenesis. Therefore, the balance of RANKL and OPG is critical for osteoclast formation and activity. Osteoclasts attach to the bone surface via actin-rich podosomes enabling them to form sealed zones with ruffled borders. Proteolytic enzymes such as CTSK (Cathepsin K) and MMPs are secreted into this isolated environment, resulting in degradation of the bone matrix, dissolution of the bone mineral, and resorption of the bone [68]. Evidently behind its outward rigidity, bone is a highly dynamic organ where homeostasis is tightly controlled and largely dependent upon cellular communication between osteoclasts and osteoblasts. This tight coupling between bone resorption and bone formation is essential for the correct function and maintenance of the skeletal system, repairing microscopic skeletal damage, and replacing aged bone. Any deviation from this homeostasis results in a range of pathologic diseases, including osteoporosis and cancer-induced bone disease.

## 5. The Metastasis of Breast Cancer Cells to the Bone

The vertebral venous system is the most common mode of transport of breast cancer cells from the breast to bone [69]. This allows breast cancer cells to come into contact with the axial skeleton, including the ribs, spine, pelvis, and proximal humerus and femur, which is the main distribution of bone metastases in breast cancer patients [70]. Tumor cells, even at their site of origin, send signals to their preferred secondary site [64] of metastasis. This modulates the microenvironment of that region. It is likely that the Hh ligands and secreted factors such as IGFs and OPN may impact this "homing" mechanism. It can be speculated that the factors secreted by breast cancer cells create a "premetastatic niche" as termed by Lyden and colleagues [64, 71]. The role of chemokines and cytokines as well as the homing mechanism has also been elaborately discussed in a review by Bussard et al. [72]. Our findings show that expression and secretion of Hh ligands by the breast cancer cells augments these processes (Figure 1). Once malignant cells have migrated to the bone, their ability to colonize is facilitated by the bone microenvironment. MMPs, chemokine receptor 4 (CXCR4), VEGF, and connective tissue growth factors supposedly target metastatic tumor cells to bone and facilitate their survival within the bone microenvironment [73, 74]. Physical factors within the bone microenvironment, including hypoxia, acidic pH, and extracellular calcium, and bone-derived growth factors, such as TGF-$\beta$ and insulin-like growth factors activate tumor expression of VEGF, PDGF, and endothelin (ET-1) [75]. Factors such as PTHrP, TGF-$\beta$, and IL-11 produced by breast cancer cells favor osteoclast maturation and osteolysis, leading to the release of growth factors that stimulate malignant tumor growth [76]. In fact, expression of IL-11 and OPN by breast cancer cells has been found to be critical for the osteolytic activity of breast cancer cells [74]. Thus, signals from the breast cancer cells at their primary site might trigger a cascade of events involving the osteoblast-mediated initiation of osteoclastogenesis which releases a plethora of growth factors in the bone milieu which not may only act as chemoattractants for the "metastasis-enabled" breast cancer cells but also favor the latter's establishment and further proliferation once they have migrated to the bone. This would in turn tilt the balance in favor of osteoclastogenesis as more favorable factors are then readily available to the osteoclasts in the bone milieu itself and thus would lead to a self-perpetuating *vicious cycle* of events (Figure 2).

## 6. Hh Signaling in the Bone Microenvironment

Hh-signaling-activated GLI2 transcription mediates osteoblast differentiation [77]. This is likely due to the regulated expression of bone morphogenetic protein-2, BMP-2, that is involved in osteogenic differentiation by promoting commitment of mesenchymal stem cells to the osteoblast lineage. GLI2 transcriptionally activates BMP-2 expression and also synergizes with BMP-2 in osteoblasts [78]. These contentions are contradicted by Plaisant et al. who have

reported that Hh signaling causes a decrease in the expression of Runx2, a key transcription factor that regulates osteoblast differentiation [79]. It is proposed that Hh signaling may be regulating different aspects of bone formation in rodent and human systems.

OPN is one of the abundant noncollagenous proteins in bone. It promotes osteoclast function and is consistently overexpressed in highly metastatic cells. OPN accumulates at cement lines in remodeling bone [80] and is localized to cell-matrix and matrix-matrix interfaces in mineralized tissue, where it is deposited by actively resorbing osteoclasts. OPN positively impacts osteoclast formation, migration, and resorptive activity [81, 82]. We recently reported that OPN is regulated, in part, by the Hh pathway [19]. We have also shown that breast cancer cells express Hh ligands and engage in a crosstalk with osteoblasts and osteoclasts [83]. Our recent studies (communicated to Breast Cancer Research) have shown that the Hh pathway plays a role in initial osteoblasts maturation, especially in the presence of breast cancer cells (Figure 2). Following an initial accelerated differentiation process, characterized by the expression of alkaline phosphatase and expression of collagenous and noncollagenous matrix proteins such as BSP and OPN and osteoclast-maturation proteins including RANKL and PTHrP, the osteoblasts appear to undergo apoptosis.

The Hh ligands also mediate a direct dialogue between breast cancer cells and preosteoclasts and induce changes in preosteoclasts that influence the production of OPN and essential bone-resorbing proteases, CTSK, and MMP9 by osteoclasts [83]. Thus, Hh ligands produced by the metastasizing breast cancer cells are instrumental in initiating a crosstalk directly with osteoclasts and promote osteoclast differentiation and resorption activity (Figure 2). Breast cancer cells also express PTHrP as a result of Hh signaling, further amplify paracrine Hh signaling in the bone microenvironment, and add to the overall osteolytic conditions [84].

Thus, the vicious cycle of bone metastasis involves a complex crosstalk between the metastasizing breast tumor cells and the bone microenvironment through multiple extracellular factors and signaling pathways with the Hh pathway playing an essential role. Based on our findings, we would like to propose that the newly arrived breast tumor cells induce initial osteoblast differentiation which stimulates osteoclast differentiation. Soon, the situation is overwhelmed by osteoclast differentiation followed by intense bone resorption leading to the local release of generous amounts of growth factors that not only encourage their growth but also alter their phenotype, making them (cancer cells) resistant to standard cytotoxic antitumor treatments see the appendix [85, 86].

## 7. Conclusion

The bone microenvironment with ongoing bone resorption almost resembles sites of wound healing. The bone stroma is almost guaranteed to provide hospitable sites for disseminating colonization-competent breast cancer cells [61]. This ensures the successful proliferation and ultimate colonization of the bone by metastasizing breast tumor cells. The crosstalk between the metastasizing breast cancer cells and the bone cells, namely, the osteoblasts and the osteoclasts occurs in a fashion that not only favors proliferation of the newly arrived tumor cells in the bone milieu but also ultimately the complete subjugation of the resident (bone) pathways to serve the purpose of establishment and well-being of the tumor cells with concurrent destruction of the host environment. Therefore, it is essential to understand the interactions between tumor and bone and identify microenvironment-selective agents to halt tumor growth and bone metastasis thereby reducing the morbidity of skeletal related events [62]. Thus, given the fact that breast cancer cells express Hh ligands and that Hh signaling propels breast cancer progression, it is likely that administration of pharmacological Hh inhibitors can inhibit Hh signaling in both breast cancer cells and osteoclasts and may reduce breast-cancer-mediated bone loss in metastatic disease. This strategy targets the tumor cells as well as the bone and its microenvironment and can reduce tumor burden and tumor-derived bone lesions.

## Appendix

*Some of the Key Players in Osteolytic Metastasis of Breast Cancer*

BMP: bone morphogenetic protein, a group of cytokines responsible for the tissue architecture throughout the body.

IGF: insulin-like growth factors are responsible for cell proliferation and form the IGF axis.

PDGF: platelet derived growth factor, a secreted molecule that regulates growth and cell division.

PTHrP: parathyroid hormone-related protein is a hormone that regulates endochondral bone development and also regulates epithelial mesenchymal interactions in mammary gland formation. It is secreted by several cancer cells.

MMPs: matrix metalloproteases are zinc-dependent endopeptidases, capable of degrading all kinds of extracellular matrix proteins and processing a number of bioactive molecules. They play a major role on cell proliferation, migration (adhesion/dispersion), differentiation, angiogenesis, apoptosis, and host defense.

OPG: osteoprotegerin (OPG), also known as osteoclastogenesis inhibitory factor (OCIF), or tumor necrosis factor receptor superfamily member 11B (TNFRSF11B), is a basic glycoprotein that is a decoy receptor for the receptor activator of nuclear factor kappa B ligand (RANKL) and can inhibit osteoclastogenesis.

RANK: receptor activator of nuclear factor $\kappa$B (RANK), also known as TRANCE Receptor, is a type I membrane protein expressed on the surface of osteoclasts and is involved in their activation upon ligand binding.

RANKL: receptor activator of nuclear factor kappa B ligand, also known as tumor necrosis factor ligand superfamily member 11 (TNFSF11), TNF-related activation-induced cytokine (TRANCE), osteoprotegerin ligand (OPGL), and osteoclast differentiation factor (ODF). It functions as a key factor for osteoclast differentiation and activation.

TGF-$\beta$: transforming growth factor beta is an antiproliferative factor protein that controls proliferation, cellular differentiation, and other functions in most cells.

VEGF: vascular endothelial growth factor is a signal protein produced by cells that stimulates vasculogenesis and angiogenesis.

## Abbreviations

BMP: Bone morphogenetic protein
CTSK: Cathepsin K
CXCR4: Chemokine receptor 1
DHH: Desert hedgehog
EMT: Epithelial-Mesenchymal transition
ET-1: Endothelin-1
GLI: Glioma-associated oncogene
Hh: Hh pathway
IHH: Indian Hedgehog
IL-11: Interleukin-11
M-CSF: Macrophage colony-stimulating factor
MMP9: Matrix metalloprotease 9
OPG: Osteoprotegerin
OPN: Osteopontin
PTCH: Patched
PDGF: Platelet-derived growth factor
PTHrP: Parathyroid Hormone-related protein
RANK: Receptor activator of NF-$\kappa$B
RANKL: Receptor activator of NF-$\kappa$B ligand
SHH: Sonic hedgehog
SMO: Smoothened
TGF-$\beta$: Transforming growth factor-$\beta$
VEGF: Vascular endothelial growth factor.

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*Review Article*

# Expression of Toll-Like Receptors on Breast Tumors: Taking a Toll on Tumor Microenvironment

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Breast cancer remains a major cause of death in women in the developed world. As Toll-like receptors (TLRs) are widely expressed on tumor cells and play important roles in the initiation and progression of cancer, they may thus serve as important targets and have an effective perspective on breast cancer treatment. Expression of TLRs on breast cancer cells and mononuclear inflammatory cells can promote inflammation and cell survival in the tumor microenvironment. Inflammation and cancer are related. It is well known that persistent inflammatory conditions can induce cancer formation, due to production of cytokines and chemokines, which play a crucial role in promoting angiogenesis, metastasis, and subversion of adaptive immunity. TLR signaling in tumor cells can mediate tumor cell immune escape and tumor progression, and it is regarded as one of the mechanisms for chronic inflammation in tumorigenesis and progression. This paper delineates the expression of various TLRs in promotion of inflammation and development of mammary tumors. Understanding the mechanisms through which TLRs on breast cancer cells and inflammatory cells regulate growth, survival, and metastatic progression can make them potential targets for breast cancer therapy.

## 1. Introduction

Breast cancer is the most common cancer among American women, except for skin cancers. The chance of developing invasive breast cancer at some time in a woman's life is a little less than 12%. It is the second leading cause of cancer death in women, exceeded only by lung cancer. The chance that breast cancer will be responsible for a woman's death is about 3% [1]. Although clinical signs of disseminated disease occur in fewer than 10% of women at the time of diagnosis, the disease relapses in the form of metastasis within 5 years of surgery in about half of apparently localized tumors. It is difficult to predict the occurrence of distant metastases since breast cancer is a heterogeneous disease encompassing complex pathologic entities [2]. Thus, there is a need for new and effective breast cancer therapies.

A dynamic interaction between tumors and the immune system is essential for tumor survival, growth, and metastasis [3]. Tumors are infiltrated with large number of immune cells that constitute a major cell population in the tumor microenvironment. Tumor cells depend on their microenvironment to provide signals for growth, anti-apoptosis, angiogenesis, and metastasis [4]. However, tumor cells are also under the surveillance due to their recognition by immune cells as foreign. Therefore, tumors have to overcome such immune surveillance to progress. Analysis of the interactions between tumor cells and the host's immune system has led to the realization that tumor cells have devised multiple strategies to evade immune attack. Development of an invasive cancer, however, is not only a result of the genetic changes in the tumor cell but also the result of genetic and epigenetic changes within the host. Host cells, including inflammatory cells, endothelial cells, and fibroblasts, are recruited and activated in the microenvironment of transformed cells. The acute inflammatory response might succeed in eliminating the malignant cells, but if not, a chronic inflammatory process develops in conjunction with

the dying tumor cells. The subsequent reciprocal interactions between these responding normal host cells and genetically altered cells result in the development of an invasive cancer. There is a constant interplay between the innate and adaptive immune systems, which leads to a protective immune response against pathogens and transformed cells and contributes effectively to discrimination between self and nonself. Persistent protumor immune responses (inflammation), now generally accepted as initiating primary tumor development, are also being recognized as mediators of cancer metastasis. Thus, novel anticancer therapeutic strategies targeting molecular and/or cellular mechanisms regulating these collaborative interactions may provide efficacious relief for metastatic disease [5].

Both infection and sterile tissue injury generate strong immune responses. This paradox was first resolved by Matzinger in 1994, who proposed that our immune system is designed to combat danger, rather than mediate recognition of nonself over self [6]. Pathogen-associated molecular patterns (PAMPs) and endogenous molecules created upon tissue injury, since called damage-associated molecular patterns (DAMPs), signal the threat of either infection or injury to the organism, independently of their nonself- or self-identity [7–10]. Damage-associated molecular patterns (DAMPs) include endogenous intracellular molecules released by activated or necrotic cells and extracellular matrix (ECM) molecules that are upregulated upon injury or degraded following tissue damage. Among the cellular receptors that sense these danger signals, Toll-like receptors (TLRs) represent a key molecular link between tissue injury, infection, and inflammation. TLRs are critical in bridging innate and adaptive immune responses and play a significant role in cancer immunosurveillance [5]. Innate immune cells including natural killer (NK), natural killer T (NKT), and $\gamma\delta$T cells play a critical role in protecting the host against cancer [5]. Macrophages and dendritic cells (DCs), in particular, function as major sensors of invading pathogens and transformed cells via the TLRs. Adaptive immunity is crucial to the elimination of pathogens and tumor cells in the late phase of host defense responses and generates more specific tumor immunity and immunological memory [11]. TLRs are known to regulate cancer immunity and tolerance by controlling the suppressive function of regulatory T (Treg) cell and through innate immune responses mediated by other immune cells [11–13]. TLR signaling, critical for innate and adaptive immune responses, has been thought to be restricted to immune cells [14]. However, many studies suggest that tumor cells bear TLRs and that TLR signaling promotes tumor growth and immune evasion [15–17]. TLR activation by DAMPs may initiate positive feedback loops where increasing tissue damage enhances proinflammatory responses leading to chronic inflammation. As TLRs are widely expressed on tumor cells and immune cells and play important roles in the initiation and progression of cancer, they may thus serve as an important target and have an effective perspective on breast cancer treatment.

Currently, 13 mammalian-TLR analogs have been identified. TLRs 1, 2, 4, 5, and 6 are expressed on the cell surface; TLRs 3, 7, 8, and 9 are found almost exclusively within endosomes. Different TLRs exhibit specificity for pathogen-derived ligands; TLRs 2, 3, 4, 5, 7, and 9 recognize bacterial lipoproteins, double-stranded RNA/poly (I : C), lipopolysaccharides (LPS), flagellin, single-stranded RNA, and CpG-containing DNA, respectively [18–23]. The ligands for TLRs 10, 12, and 13 remain unidentified. TLR10 is expressed in humans but not in mice, TLR8 is not functional in mice and TLRs 11, 12, and 13 are expressed in mice but not in humans.

There are several studies which suggest that DAMP-mediated inflammation plays a vital role. Necrotic cells were found to induce proinflammatory and tissue repair gene synthesis and cause DC maturation in a TLR2-dependent manner, as a result of the release of their intracellular contents. Other intracellular molecules such as heat shock proteins including HSP70, Gp96, HSP22, and HSP72 and high-mobility group box-1 protein (HMGB1) as well as ECM molecules such as biglycan, tenascin-C, versican, and fragments of ECM molecules including oligosaccharides of hyaluronic acid (HA) and heparan sulfate (HS) have been shown to activate TLRs. TLR1, along with TLR2, was found to be important for the activation of professional antigen-presenting cells by $\beta$-defensin-3, a host-derived antimicrobial peptide. Self-nucleic acids have also been described as endogenous danger signals, namely, mRNA recognized by TLR3, single-stranded RNA (ssRNA) sensed by TLR7 and 8, and IgG-chromatin complexes recognized by TLR9. TLR2, 4, 7, and 8 were shown to be activated by antiphospholipid antibodies (APL) isolated from patients with APL syndrome [24].

The signaling pathways utilized by various TLRs differ, which results in varied cellular responses. For example, TLR3, the receptor for double-stranded RNA couples to the adaptor protein TRIF. In contrast, other TLRs couple to the adapter myeloid differentiation primary response gene 88 (MyD88) [25, 26]. The MyD88-adapter protein recruits IRAKs and TRAF6. The TRAF6 in turn activates TAK1 that phosphorylates and activates the IKK complex resulting in the release and translocation of NF-$\kappa$B to the nucleus. TAK1 also activates stress-activated protein kinase (SAPK) pathways and activates c-Jun-NH2-kinases (JNK) and p38. The MyD88-coupled TLRs induce the synthesis of cytokines such as TNF-$\alpha$, IL-6, and IL-1, key mediators of the inflammatory response [27, 28]. TLR4, the receptor for LPS, is unique in that it activates both MyD88-dependent and TRIF-dependent pathways [28].

## 2. Inflammation and Cancer Metastasis

The link between inflammation and cancer is well documented [29, 30]. Several inflammatory diseases, including inflammatory bowel disease, increase the risk of cancer. Conversely, in tumors that are epidemiologically unrelated to overt inflammatory conditions (such as breast cancer), the activation of oncogenes can trigger the production of inflammatory molecules and the recruitment of inflammatory cells. In the tumor microenvironment, inflammatory cells and molecules influence almost every aspect of cancer

progress, including the metastatic ability of tumor cells [29]. There is biological heterogeneity among tumors with regard to cellular infiltrates, identifying subsets of mononuclear inflammatory cells both at the tumor centre and at the invasive front, which seem to be associated with the occurrence of distant metastasis. Intratumour leucocytes from peripheral blood penetrate the tumor architecture after their phenotypic modification, from the invasive front to the tumor centre. This seems to be a dynamic process in which inflammatory cells and immunomodulatory mediators present in the tumor microenvironment polarize the host immune response towards specific phenotypes impacting on tumor progression [31]. Previously, there were six recognized hallmarks of cancer, namely, unlimited replicative potential, self-sufficiency in growth signals, insensitivity to growth inhibitors, evasion of programmed cell death, ability to develop blood vessels, and tissue invasion and metastasis [4]. Cancer-related inflammation has now emerged as the seventh hallmark of cancer. A group of cytokine proteins, including IL-1, IL-6, TNF-$\alpha$, and RANKL, activate inflammation and are known to augment tumor cells' ability to metastasize by affecting several steps in the cells' dissemination and implantation at secondary sites [29, 32, 33]. Inflammatory cytokines lie downstream of the "master" gene transcription factor NF-$\kappa$B, for promoting inflammation which is itself activated by them [29]. There is strong evidence that the tumor microenvironment is inflammatory and that activation of the innate immune system plays a role in the progression of cancer [34, 35]. A major source of inflammatory cytokines in the tumor microenvironment, are specialized white blood cells called macrophages. Tumor-associated macrophages assist the malignant behaviour of tumor cells, not only by producing cytokines, but also by secreting growth factors and matrix-degrading enzymes [36–38]. It has long been suggested that there may be common pathways of inflammation shared by responses to infection and to malignancy. Recent evidence indicates that TLRs on macrophages may be critical elements in these common pathways. MyD88 has been reported to activate not only AP-1 and NF-$\kappa$B subunit p65 and p50, but also c-Rel, C/EBP$\beta$, and C/EBP$\delta$. In case of LPS signaling through TLR4, where NF-$\kappa$B and AP-1 activities are relatively preserved in MyD88-deficient macrophages, the specific defect in c-Rel and the profound defect in C/EBP$\beta/\delta$ activation likely accounts for the reduction of IL-12 p40, IL-6, and TNF$\alpha$. The absence of both C/EBP$\beta/\delta$ specifically in TLR signaling impairs key proinflammatory cytokines without affecting other NF-$\kappa$B-dependent genes such as I$\kappa$B$\alpha$ [39].

## 3. Toll-Like Receptors in Inflammation-Induced Breast Cancer

Toll-like receptors are expressed on cells of the immune system but there is growing evidence that TLRs are also expressed on tumor cells, where they may influence tumor growth and host immune responses [15]. Activation of TLRs expressed on tumor cells may have profound consequences for tumor growth by factors released after TLR activation. Tumor immune evasion may be facilitated by inhibitory cytokines, inflammatory factors, proteinases, and other small molecules such as nitric oxide [40]. Recent evidence suggests that TLRs also contribute to tumor-cell resistance to apoptosis and increased invasiveness. The human breast cancer cell line MDA-MB-231 was found to express TLR1-TLR10 at both the mRNA and protein levels. TLR4 was found to be the highest expressed TLR in MDA-MB-231. Knockdown of *TLR4* gene in MDA-MB-231 resulted in a dramatic reduction of breast cancer cell viability and inhibition of IL-6 and IL-8 cytokines compared with vector control [41]. Another study highlights the role of TLR9 in highly invasive MDA-MB-231 breast cancer cell line which when activated promotes MDA-MB-231 cell invasion by increasing the activity of matrix metalloproteinase 13 (MMP13), but not MMP8 [42]. Samples of mammary carcinomas with recurrence have also exhibited a significant increase in the mRNA levels of TLR3, TLR4, and TLR9. A significant percentage of tumors also showed TLR4 expression by mononuclear inflammatory cells (21.6%) and TLR9 expression by fibroblast-like cells (57.5%). Tumors with high TLR3 expression by tumor cell or with high TLR4 expression by mononuclear inflammatory cells (MICs), but not TLR9 high fibroblast like cells were significantly associated with higher probability of metastasis [2]. This study highlights the importance of the tumor stromal cells in tumor behavior, and how TLR-induced inflammation on inflammatory cells drives metastatic cascade.

Synthetic TLR9-ligands (CpG-sequence containing oligonucleotides) stimulated TLR9 expressed on cancer cells as well as various normal cells, including mesenchymal stem cells and stimulated their invasion *in vitro*. This invasion was mediated via downregulation of tissue inhibitor of matrix metalloproteinase-3 (TIMP-3) and through matrix metalloproteinase-13 (MMP-13) activation. Expression of TLR9 isoforms A and B have been detected in clinical breast cancer specimens. Expression of TLR9 and its invasive effects on breast cancer cells has been found to be regulated by estrogen receptor-$\alpha$ (ER$\alpha$) and sex steroid hormones. TLR9 expression was also found to be affected by commonly used hormonal cancer therapy bicalutamide [43].

Activation of TLR signaling on tumor cells by their ligands can also trigger apoptosis and may have therapeutic effects. For example, in a randomized clinical trial for the efficacy of poly (A : U) dsRNA, therapeutic effect was mediated through TLR3 expressed on tumor cells, and could therefore represent an effective targeted treatment in patients with TLR3-positive cancers. The predictive value of TLR3 expression by tumor cells for the efficacy of Poly (A : U) dsRNA was determined in 194 breast cancer patients enrolled in a randomized clinical trial. However, conventional chemotherapy or *in vivo* injection of poly (A : U), alone or in combination, failed to reduce tumor growth unless an immune-chemotherapeutic regimen of vaccination against tumor antigens was included [44].

Recently, TLR5 has been found to be highly expressed in breast carcinomas and activation of TLR5-signaling pathway was found to be overly responsive in breast cancer cells by inhibiting cell proliferation and an anchorage-independent

growth. In addition, the secretion of soluble factors induced by flagellin, was found to the growth-inhibition of breast cancer cells in an autocrine fashion. This inhibitory activity was further confirmed *in vivo* using mouse xenografts models of human breast cancer cells [45]. Sites of chronic inflammation are often associated with the establishment and growth of various malignancies including breast cancer. Enhanced neutrophilic and granulocytic infiltration in lungs and bone of the proarthritic and arthritic mice and subsequent increase in circulating levels of proinflammatory cytokines, such as macrophage colony stimulating factor (M-CSF), interleukin-17 (IL-17), interleukin-6 (IL-6), vascular endothelial growth factor (VEGF), and tumor necrosis factor-alpha (TNF-alpha) were found to contribute to the increased metastasis. Breast cancer-associated secondary metastasis was found to be significantly increased in pro-arthritic and arthritic conditions. Breast cancer metastasis was found to be significantly reduced by blocking the IL-17 and COX-2 pathway [46]. Inflammatory TLR signaling has also been shown to promote the attraction and generation of Th17 cells induced by tumor cells and tumor-derived fibroblasts. Enhanced migration of Th17 cells to tumor sites was reported to be due to the expression of chemokines and tumor-derived fibroblasts [47].

Therapeutic development targeting TLRs is at early clinical stages. There are currently approximately twenty drugs in preclinical development, with a further dozen or so in clinical trials [48]. There are clearly many options for the targeting of TLRs, because the key function of TLRs is to induce cytokines, which are well validated in these diseases and are successfully being targeted in the clinic. TLRs occur early in pathways and so inhibiting them might be more potent than inhibiting their downstream cytokine targets. Different approaches are being taken to target TLRs. Neutralizing antibodies to TLRs are a feasible option, but only for those on the cell surface, such as TLR2, TLR4, and TLR5. Small-molecule antagonists (e.g., eritoran against TLR4 or ODN-based inhibitors of TLR7) might be a better prospect, but it is hard to predict their off-target effects and efficacy. Because there are kinases on the signaling pathways, these might also be sensitive to inhibition. One major concern here, however, is that such inhibitors might block multiple TLRs and therefore give rise to unwanted immunosuppression. Monotherapies against a specific TLR might not have this problem. Studies on knockout mouse indicate that there is less redundancy in TLRs in relation to inflammation. TLR-based adjuvants also have the potential to yield new agents. Imiquimod is already approved for its antiviral effects, whereas MPL is approved as a vaccine adjuvant. In terms of antagonism, effects of TLR inhibitor, eritoran have been found to be significant but somewhat marginal [49].

To further develop more effective TLR therapeutic targeting strategy, there are a few more tasks: further identifying and determining the pathogenesis of challenging medical conditions like cancer; analysis of genetic sequence, molecular structure, epigenetic observations, and functional activities on both animal model and human clinical studies; design of clinical study based on study indication, dosing regimens, drug delivery route or format consideration, and pharmacokinetics; timely and objective assessment of adverse events with details. Targeting TLRs will therefore in all likelihood prevent the induction of many immune and inflammatory proteins. The wide tissue distribution of TLRs, however, may make it difficult to determine whether an agonist or an antagonist will be most effective therapeutically.

## 4. Conclusions

Metastasis is regulated not only by intrinsic genetic changes in malignant cells, but also by the microenvironment. Several studies have demonstrated that sites of chronic inflammation are often associated with the establishment and growth of various malignancies. Toll-like receptors (TLRs) have emerged as sensors that can detect a variety of invading pathogens and malignant cells. Since their discovery a decade ago, TLRs have been shown to be critical for efficient innate and adaptive immunity and the framework of TLR-mediated signaling pathway has been explained. However, TLR activation may be a two-edged sword, with both antitumor and pro-tumor consequences. The general expression of functionally active TLRs by tumor cells and inflammatory cells in the stroma by putative endogenous ligands suggests that TLR signaling may be continually activated and may contribute to tumor progression and metastasis. Understanding TLR function in tumor biology may lead to discovery of new therapeutic targets in cancer therapy.

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*Review Article*

# The Role of Cancer Stem Cells in the Organ Tropism of Breast Cancer Metastasis: A Mechanistic Balance between the "Seed" and the "Soil"?

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Breast cancer is a prevalent disease worldwide, and the majority of deaths occur due to metastatic disease. Clinical studies have identified a specific pattern for the metastatic spread of breast cancer, termed organ tropism; where preferential secondary sites include lymph node, bone, brain, lung, and liver. A rare subpopulation of tumor cells, the cancer stem cells (CSCs), has been hypothesized to be responsible for metastatic disease and therapy resistance. Current treatments are highly ineffective against metastatic breast cancer, likely due to the innate therapy resistance of CSCs and the complex interactions that occur between cancer cells and their metastatic microenvironments. A better understanding of these interactions is essential for the development of novel therapeutic targets for metastatic disease. This paper summarizes the characteristics of breast CSCs and their potential metastatic microenvironments. Furthermore, it raises the question of the existence of a CSC niche and highlights areas for future investigation.

## 1. Introduction

Due to the expanding and aging global population, it is no surprise that cancer incidence and mortality are increasing despite ongoing research in the areas of cancer treatment and prevention. In North American women, breast cancer represents the most commonly diagnosed and the second highest cause of cancer-related deaths [1, 2]. Although the collection of exact global cancer statistics is difficult due to differences in healthcare infrastructure and data collection methods, the GLOBOCAN study ranks breast cancer as the most frequently diagnosed and the most prevalent cause of cancer-related death among women globally [3]. In the past, breast cancer has been a higher burden in developed countries, likely due to more risk factors associated with lifestyle such as postponement of pregnancy until after 30, less breast-feeding, smaller families, and a less active workplace [4]. It is predicted that as developing countries improve their economic conditions and adopt a more "westernized" lifestyle, incidence rates will increase [5]. The challenge then presents itself: what is the best way to target this lethal disease in developed countries while also counteracting the predicted increase in mortality in developing countries? The answer lies in the understanding of metastatic disease, the most lethal aspect of breast cancer.

## 2. Metastasis

Even though advances have been made in prevention, detection, and treatment, the mortality rate associated with breast cancer has remained high [3]. Primary breast tumors originate within the lobule or duct of the breast, and

therapies are highly efficient if the neoplasm is detected while localized within the original structure (*in situ*) or even still localized within the breast itself [6]. Therapeutic efficacy is greatly reduced once the cancer acquires invasive and metastatic properties. Therefore, metastatic disease represents the aspect of breast cancer responsible for the majority of breast-cancer-related mortalities.

Following successful angiogenesis at the primary tumor site, the stepwise process of metastasis has been clearly defined. During the initial stage, cells escape from the primary tumor into the blood and/or lymphatic system via a process called intravasation. Once in the circulation, these cells must survive until they reach a secondary site where they arrest and enter the tissue (extravasation). Tumor cells able to initiate and maintain colony growth in these secondary sites form micrometastases which, following angiogenesis, grow into clinically detectable macrometastases [7–9].

## 3. Metastatic Theories

Clinical observations highlight that different cancers exhibit characteristic sites for secondary metastases that are dependent on the origin of the primary tumor, a phenomenon termed organ tropism [10, 11]. For example, breast cancer preferentially spreads to the lymph nodes, lung, liver, bone, and brain, while other primary cancers have different preferential sites of metastasis (i.e., prostate cancer and colorectal cancer spread to bone and liver, respectively) [11]. While there are many theories concerning the mechanisms of metastasis (eloquently summarized by Hunter et al. [12]), only a few sufficiently account for the organ tropism phenomenon. Two of the main theories that have been proposed to explain this organ tropism of cancer metastasis include the "seed and soil" theory, first documented by Stephen Paget in 1889, and Ewing's mechanical arrest theory [13, 14]. Paget postulated that organ-specific patterns could be accounted for by the needs of the cancer cell (the seed) for a specific environment (the soil) in order to initiate and maintain growth [13]. Ewing's theory, proposed thirty years later, postulates that organ tropism can be accounted for by circulatory patterns within the body and that cells are mechanically arrested in the first capillary bed they encounter [14]. It is likely that these two theories are not mutually exclusive, but rather that they work in concert to produce successful metastases: cells arrest due to mechanical obstruction and/or specific chemical signals and then require a suitable microenvironment for initiation and maintenance of secondary tumor growth.

An autopsy study by Dr. Leonard Weiss [10] addressed the differences between the "seed and soil" and mechanical arrest theories by not only investigating the incidence of metastatic lesions at secondary sites, but by also taking into account the innate blood flow to each of the sites. This study used these two parameters to generate a "metastatic efficiency index" (MEI) that was used to rank pairs of primary and secondary sites as either accounted for by blood flow alone, or as "friendly" (more incidence than suggested by blood flow patterns alone) versus "hostile" (less incidence than dictated by blood flow) interactions. Interestingly, 66% of the pairs could be attributed to blood flow due to the sheer number of cancer cells delivered to the sites in arterial blood (i.e., mechanical arrest), while 20% of pairs were ranked as "friendly" and 14% of pairs were deemed to be due to "hostile" interactions. Of note, prostate and breast cancer were seen to exhibit a "friendly" interaction with bone; while ovarian, prostate, stomach, and urinary bladder cancers were seen to have a "hostile" interaction with the brain [10] (Table 1). This study suggests that some site-specific metastases can be attributed to blood flow patterns, but that there is also a distinct seed and soil effect for others. The question of whether the properties of the secondary organ or the properties of the cancer cell are more important in mediating the organ tropism of breast cancer remains to be answered.

## 4. Metastatic Inefficiency

Although often lethal when successful, the multistep nature of the metastatic process lends itself to a high degree of inefficiency. In an experimental mouse model, Luzzi et al. used *in vivo* videomicroscopy to demonstrate that only 0.02% of melanoma cells injected intraportally to target the liver could successfully complete the entire metastatic process [15]. Interestingly, the authors noted that not all metastatic stages are equally inefficient, but rather that the main inefficiencies occur during the initiation and maintenance of metastatic lesions in the secondary organ. Many tumor cells are capable of extravasating into the secondary site, but may become dormant due to lack of external growth signals [16], and/or may fail to colonize the site due to a lack of ability to recruit sufficient blood supply to support the formation of a clinically relevant lesion.

This inefficiency appears to be mirrored in humans as, in a limited study of palliative ovarian cancer patients, ascites fluid full of tumor cells that was shunted directly into the venous circulation via peritoneovenous shunts did not always cause secondary lesions. Some but not all of these cases resulted in pulmonary metastases, although these lesions were clinically irrelevant as patient mortality resulted first from primary tumor progression. Other cases did not develop detectable metastatic lesions within the timeframe of the study (up to 27 months) before they too succumbed to their original tumor [17]. Both murine and human studies suggest that only a rare subpopulation of primary tumor cells can successfully complete the metastatic process, and likely the outcome also depends on the secondary organ microenvironment. Our group and others hypothesize this rare subpopulation of tumor cells to be cancer stem cells (CSCs) [18–21].

## 5. Cancer Stem Cells

The composition of primary breast tumors has been shown to be heterogeneous with respect to both molecular subtype (luminal A, luminal B, basal-like, HER2-overexpressing, normal breast-like, and claudin-low) [22, 23] and cellular function, even within the same tumor [24, 25]. This heterogeneity can be accounted for by the CSC hypothesis,

Table 1: Interactions between primary cancer site and target organ based on metastatic efficiency indexes.

| Primary cancer site | Target organ | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Kidney | Brain | Bone | Skeletal muscle | Skin | Heart | Thyroid | Adrenal |
| Bone | — | — | — | — | — | / | — | ↑ |
| Breast | — | — | ↑ | — | — | — | ↑ | ↑ |
| Cervix | — | — | — | — | ↓ | / | ↑ | ↑ |
| Colorectal | — | — | — | ↓ | — | — | — | ↑ |
| Esophagus | — | — | — | — | ↓ | / | ↑ | ↑ |
| Kidney | — | — | — | ↓ | — | — | ↑ | ↑ |
| Lung | — | — | — | / | / | — | — | ↑ |
| Lung(SCC) | — | — | — | / | / | / | — | ↑ |
| Osteosarcoma | ↓ | ↓ | — | ↓ | / | ↓ | — | ↓ |
| Ovary | ↓ | ↓ | — | / | — | — | ↑ | ↑ |
| Ovary* | — | ↓ | — | — | — | / | — | ↑ |
| Pancreas | — | — | — | — | — | — | — | ↑ |
| Prostate | — | — | ↑ | — | ↓ | ↓ | — | ↑ |
| Prostate* | — | ↓ | ↑ | / | ↓ | — | — | ↑ |
| Stomach | — | ↓ | — | ↓ | — | / | — | ↑ |
| Testis | — | — | — | ↓ | — | / | — | ↑ |
| Thyroid | — | — | — | — | — | / | — | ↑ |
| Urinary Bladder | — | ↓ | — | — | — | / | ↑ | ↑ |
| Uterus | — | — | — | — | ↓ | / | ↑ | ↑ |

Adapted from Weiss (1992) [10].
↑ Friendly (Increased incidence) (MEI > 0.100).
↓ Hostile (Decreased incidence) (MEI < 0.009).
— Neutral (0.010 < MEI < 0.099).
/ Not reported.
SCC: small cell carcinoma.
*Duplicate sites due to different autopsy studies used.

also known as the hierarchy theory, which posits that there is a small, phenotypically identifiable subpopulation of cancer cells with stem cell-like characteristics [26]. These CSCs sit at the top of this functional hierarchy and are postulated to be capable of tumor propagation and maintenance due to their ability to self-renew and to differentiate into the cells comprising the bulk of the tumor. Conversely, the terminally differentiated non-CSCs are not capable of producing large amounts of progeny or of tumor propagation [25, 27, 28].

The first identification of CSCs in solid tumors came from the seminal work of Dr. Michael Clarke's group [29] following the lead of Dr. John Dick and colleagues in the leukemia field [30]. Working with cells isolated from the pleural effusions and primary tumors of breast cancer patients, Al-Hajj et al. [29] isolated distinct subpopulations of tumor cells using fluorescence-activated cell sorting. The epithelial-specific antigen positive ($ESA^+$) $CD44^+$ $CD24^{-/low}$ lineage negative ($Lin^-$) subpopulation was capable of forming tumors when as few as 100 cells were injected into the mammary fat pad of nonobese diabetic/severe combined immune deficiency (NOD/SCID) mice, whereas tens of thousands of cells from other subpopulations were nontumorigenic. Ginestier et al. [31] further purified this breast CSC subpopulation by adding in the criteria of high aldehyde dehydrogenase activity ($ALDH^{hi}$). $ALDH^{hi}$ $CD44^+$ $CD24^-$ breast tumor cells were capable of tumor initiation when as few as 20 cells were injected into NOD/SCID mice. These tumors exhibited the same phenotypic heterogeneity as the initial tumors, exhibiting both tumorigenic and nontumorigenic subpopulations. Furthermore, this tumor formation and heterogenic recapitulation could be replicated upon serial passaging in naïve NOD/SCID mice of the $ALDH^{hi}$ $CD44^+$ $CD24^-$ cells isolated from tumors derived from the initial CSC injection, demonstrating the CSCs' differentiation and self-renewal potential [31].

Breast CSCs demonstrate an increased metastatic propensity *in vitro* [18, 32, 33], *in vivo* [18, 21, 34], and in clinical observation [20, 35]. Although their metastatic role is not fully understood, many theories have attempted to explain the contribution of CSCs to breast cancer metastasis. The most common site of breast cancer metastasis is to the bone, but metastatic lesions are also found in the lungs, brain, and liver [11]. The high level of CD44 expression by CSCs has been highlighted as one possible contributor, as both hyaluronan and osteopontin (OPN), common ligands for CD44, are expressed in the bone and other common sites of metastasis [36], suggesting a possible adhesive

interaction for circulating tumor cell arrest. *In vitro*, the CD44-hyaluronan interaction has been shown to mediate the attachment of metastatic breast cancer cells to human bone marrow endothelial cells [37]. Moreover, this interaction could be abrogated through the depletion of CD44 expression using RNA interference and induced by the transfection of a $CD44^{low}$ breast cancer cell line with CD44 expression vectors [37]. Additionally, breast cancer cell lines exhibit different levels of Chemokine (C-X-C motif) Receptor 4 (CXCR4), which appears to positively correlate with both CSC proportions and the propensity of breast cancer cell lines to metastasize [18, 38]. Similar observations were made in pancreatic cancer, where within the identified $CD133^+$ CSC population, there existed two subpopulations based on CXCR4 expression, and only the $CXCR4^+$ population was capable of metastasizing [39]. Although the mechanisms have not yet been elucidated, there is evidence to suggest that CSCs are not only tumor-initiating cells, but also metastasis-initiating cells (M-ICs). The role of CSCs in driving organ tropism of breast cancer remains to be determined.

Recent work has also highlighted that CSCs isolated from tumors originating in the breast and other tissues exhibit resistance to chemotherapy and radiation [40–43]. A study of human leukemia revealed that the chemoresistance of leukemic CSCs arises from the quiescent nature of these cells, as they are stationary in the $G_0$ phase, which limits the effectiveness of chemotherapeutics that target actively replicating cells [44]. In humans, an increase in the proportion of $CD44^+$ $CD24^-$ breast cancer cells has been observed after neoadjuvant chemotherapy, indicating likely CSC therapy resistance *in vivo* [19]. Possible mechanisms for this include the expression of cell surface drug efflux pumps, such as breast cancer resistance protein-1 (BCRP1; ABCG2), which are capable of expelling chemotherapeutic drugs [45]. Interestingly, BCRP1 is also highly expressed in normal hematopoietic stem cells [46]. Additionally, the presence and activity of ALDH, an enzyme that is capable of metabolizing and inactivating cytotoxics such as cyclophosphamide [47], is likely playing a key role in the observed chemoresistance. Other factors potentially prolonging the lifespan of CSCs include the increased expression of antiapoptotic molecules such as Bcl-2 and survivin [48, 49]. It remains unclear whether this observed metastatic ability and resistance to therapy is a property attributable only to the CSCs (i.e., innate therapy resistance), or whether these specialized cells also receive signals from their microenvironment in the secondary organ that enhance their survival and resilience in the face of cytotoxic treatment. New therapeutic targets may therefore emerge as we gain a greater understanding of the organ-specific interactions between tumor cells (the "seeds") and secondary organ sites (the "soil").

## 6. CSCs and the Metastatic Microenvironment

There are two prevailing schools of thought as to the origin of the CSC: either (1) a CSC may originate from a normal tissue stem cell (SC) that has acquired tumorigenic mutations; or (2) a CSC may originate from a more differentiated progenitor/mature cell that has dedifferentiated and adapted a stem-like phenotype. Both theories remain under investigation. Recent work by Gupta et al. supports the latter theory by demonstrating that subpopulations within the SUM149 and SUM159 breast cancer cell lines are capable of interconversion between stem-like, basal, and luminal populations. They demonstrate that a phenotypic equilibrium is consistently reached over time both *in vitro* and *in vivo*, although the *in vivo* growth requires coinjection of basal or luminal cells with irradiated carrier cells to allow for these two subtypes to persist long enough to give rise to stem-like cells [50]. The rate at which this interconversion occurs depends only on the current subpopulation of a cell and is not influenced by the history of the cell. In support of this, Scaffidi and Misteli successfully generated CSC-like and non-CSC-like cells after oncogenic reprogramming of differentiated fibroblasts. They observed a stochastic emergence of a small population of CSC-like cells expressing stage-specific embryonic antigen 1 (SSEA-1), a marker that did not arise in any of their control lines, suggesting that the CSC phenotype may occur spontaneously after the main oncogenic events have occurred [51]. Further work that supports this "dedifferentiation" of non-CSCs into CSCs demonstrates the possibility that IL-6 may be a key mediator of the process [52] and highlights the need for further investigation into the origin of CSCs and the effects of their microenvironment on regulating this cellular plasticity.

Regardless of their origin, the functional similarities between CSCs and normal SCs are striking. Normally, the SC niche provides signals that either maintain SC quiescence, promote symmetrical division leading to self-renewal, or promote asymmetrical division leading to differentiation and progression down the lineage [53]. Interactions between SCs and their niche are highly dynamic and essential for proper function [54]. As SCs depend on the surrounding microenvironment for important signals, it is not unreasonable to hypothesize that CSCs may also rely on their microenvironment to maintain their tumor-initiating and metastasis-initiating capacity and that a "metastatic niche" may exist in those organs in which these cells are more likely to create metastatic lesions. This niche may play an important role in the organ tropism observed in breast and other cancers. Additionally, signals from the metastatic niche may cause the interconversion of non-CSCs that have arrived from the primary tumor into more metastatic CSCs.

## 7. Seed and Soil Interactions in the Metastatic Niche

In the bone marrow, there are functionally different hematopoietic stem cell niches depending on physical location [53, 55]. Synonymously, the metastatic niches around the body may vary, thus dictating what types of cancer cells will be successful in various secondary organs and contributing to the observed organ tropism of different cancer types. The next part of this paper summarizes what is currently known about the metastatic microenvironments provided by

common sites of breast cancer metastasis, including bone, brain, lung, liver, and lymph node (Figure 1).

*7.1. Bone.* Bone is one of the main sites of metastasis for breast cancer, and many groups postulate that this is due to the rich nature of the niche, as it is already optimized for support of normal hematopoiesis [60, 61]. Bone cells express high amounts of stromal-derived factor 1 (SDF-1), which may allow for breast cancer cell migration in a $CXCR4^+$-dependent manner [62]. Additionally, the bone microenvironment is rich in ligands such as OPN, which may further support CSC recruitment to the bone through interactions between tumor cell-surface receptors such as CD44 [36, 55]. When a breast cancer cell line variant was selected *in vivo* for increased metastatic capacity for bone, genotypic analysis revealed the upregulation of many genes relative to those expressed by an adrenal medulla seeking variant of the same cell line, including CXCR4, fibroblast growth factor-5 (FGF-5), connective tissue-derived growth factor, interleukin-11 (IL-11), and matrix metalloproteinase 1 (MMP1). This suggests that these cells have innate capabilities to interact with the bone microenvironment, including promotion of both angiogenesis and osteolysis through the differentiation of osteoclasts or cleavage of collagen [57].

Once in the bone, tumor cells exert a profound effect on the bone microenvironment, known as the "vicious cycle" [61]. Normally, the bone is a highly dynamic structure, constantly undergoing remodeling in a carefully regulated balance of osteoblast-mediated bone formation and osteoclast-mediated bone resorption. Breast cancer commonly causes osteolytic bone metastases, indicating the balance has shifted in favor of bone degradation. In a clinical study of breast cancer metastases to the bone, 92% of bone metastases scored high by immunohistochemistry for parathyroid-hormone-related protein (PTHrP) compared to 17% in nonbone sites [63], an observation that was further supported by similar *in situ* hybridization results [64]. It is thought that PTHrP plays an important role in mediating osteolytic bone metastases [65]. The secretion of PTHrP causes osteoblasts to increase their expression of the membrane protein receptor activator of nuclear factor $\kappa$B (RANK) ligand (RANKL), which promotes osteoclast precursor differentiation and activation through RANK activation [66]. Degradation of the bone matrix causes the release of growth factors, including transforming growth factor-$\beta$ (TGF-$\beta$), insulin-like growth factors I and II (IGF-I and II), platelet-derived growth factor (PDGF), FGF-1 and -2, and bone morphogenic proteins (BMP), all of which have effects on both osteoblasts and tumor cells [67], causing an increase in tumor cell secretion of PTHrP and the propagation of the vicious cycle. Additionally, these growth factors enter the systemic circulation where they have potential to stimulate cells at distant sites, potentially creating additional metastatic niches and permitting tumor spread. Interestingly, in a large prospective study involving 526 patients afflicted with operable breast cancer, Henderson et al. found that positive PTHrP staining in the primary tumor correlated with an improved survival in 79% of cases, contrary to expected results [68]. These results highlight the need for further investigation of the interaction between breast cancer cells and the bone microenvironment as it appears to be more complex than originally thought.

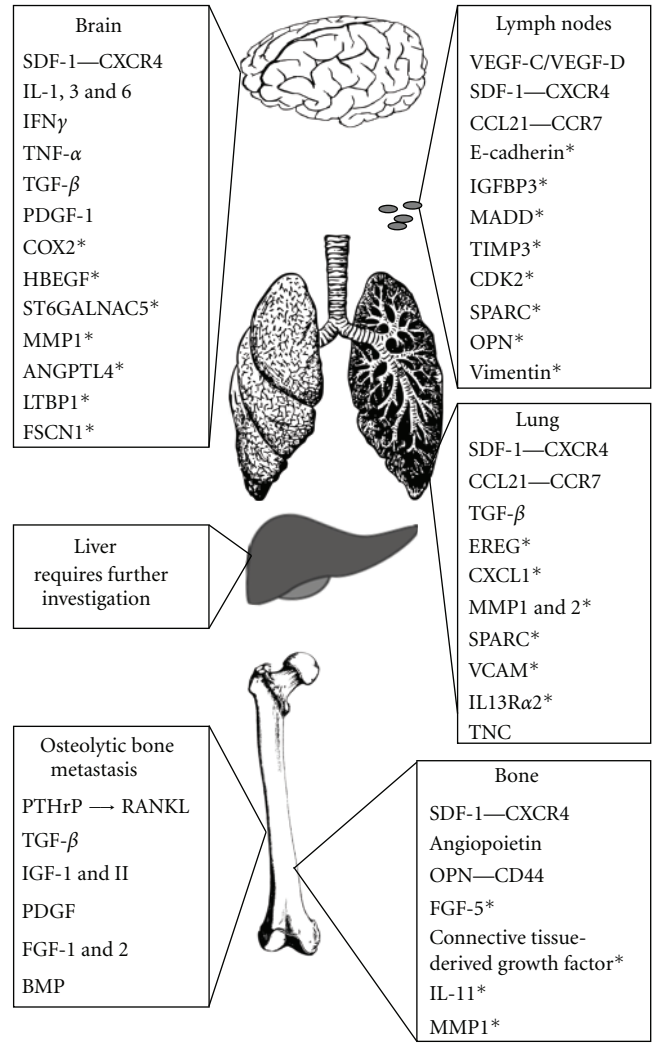


Figure 1: Potential factors involved in the organ-specific metastasis of breast cancer to the brain, liver, lymph nodes, lung, and bone. Brain, lung, and liver images were acquired with thanks to Creative Commons Licensing (CC0 1.0, Public Domain Dedication). Bone image from Gray's Anatomy (1918, Public Domain, copyright expired). Underlining indicates tumor-derived factors. *Italics* indicate organ-derived factors. *indicates factor identified by microarray analysis of organ-specific metastatic cell line variants [56–59].

*7.2. Brain.* The brain represents a unique metastatic niche. It is judiciously guarded by the blood-brain barrier (BBB), a continuous sheet of nonfenestrated endothelium joined by tight junctions and supported by a basement membrane, pericytes and astrocytes [69]. These endothelial cells are armed with ATP-binding cassette C1 (ABCC1) and P-glycoprotein (PGP/ABCB1) and are thus capable of active efflux of most chemotherapeutic drugs from the brain parenchyma [70]. The mechanism by which tumor cells

traverse the BBB is poorly understood, but it is postulated that tumor cells adhere to the endothelium and promote endothelial retraction to expose the basement membrane and allow for tumor cell invasion [71].

Brain metastases are associated with later stages of disease progression and often only occur secondary to other metastatic lesions in the bone, lung, and/or liver [72]. Thus, brain metastases may potentially represent the manifestation of the true metastatic cascade, or metastasis of metastases [10]. This theory suggests that primary tumor cells first colonize a visceral organ or regional lymph node before acquiring the phenotype necessary to successfully traverse the BBB and interact with the brain microenvironment. Once inside the brain parenchyma, tumor cells encounter a rich microenvironment of cytokines and growth factors, predominantly produced by astrocytes (i.e., SDF-1$\alpha$ [73], IL-1, IL-3, IL-6, interferon-$\gamma$ (IFN-$\gamma$), tumor necrosis factor-$\alpha$ (TNF-$\alpha$), TGF-$\beta$, and PDGF-1 [74]), which the tumor cells usurp to promote survival, growth, and potentially organ-specific metastasis [62]. Furthermore, astrocytes have been shown to exert a tumor-protective effect from chemotherapeutics via direct cell-cell contact [75]. It is likely that a combination of these factors contributes to the highly resistant nature of brain metastases to therapeutics and must be taken into account for the development of new therapeutics.

Further insight into the interactions between tumor cells and the brain microenvironment has been elegantly demonstrated by isolation of a brain-specific metastatic variant of the MDA-MB-231 human breast cancer cell line through repeated selection *in vivo* by Bos and colleagues [56]. Genetic comparison with the parental line highlighted increased expression in the brain variant of cyclooxygenase-2 (COX2), heparin-binding EGF (HBEGF), and sialyltransferase ST6 (alpha-N-acetyl-neuraminyl-2,3-beta-galactosyl-1,3)-N-acetylgalactosaminide alpha-2,6-sialyltransferase 5 (ST6GALNAC5) as potential facilitators of tumor-cell passage through the BBB. Additionally, the authors highlighted collagenase-1 (MMP1), angiopoietin-like 4 (ANGPTL4), latent TGF-$\beta$-binding protein (LTBP1), and fascin-1 (FSCN1) as genes that were upregulated in the brain-seeking population and thus, potential mediators of brain metastasis, providing more insight into possible tumor-specific therapeutic targets.

*7.3. Lung.* The physical characteristics of the lung make it an ideal site for colonization and eventual outgrowth of tumor cells. The combination of immense surface area and numerous capillaries make it likely that tumor cells will lodge in the vasculature by sheer mechanical forces. The CXCR4/SDF-1 and chemokine (C-C motif) receptor 7/chemokine (C-C motif) ligand 21 (CCR7/CCL21) interactions may play key roles in accentuating the adhesion of tumor cells as the lung endothelium expresses a high level of SDF-1 and CCL21 to complement tumor cell expression of CXCR4 and CCR7 [62, 76]. Additionally, the growth factor transferrin has been suggested to have protumor effects on cells that have the potential to metastasize to the lung but not to their nonmetastatic counterparts [77]. In a Neu-induced transgenic mouse model of breast cancer, TGF-$\beta$ functioned to promote lung metastases [78], in agreement with the well-established multifunctionality of TGF-$\beta$ as being a tumor suppressor in the early stages of cancer, but a metastatic promoter in late stages [79].

Genetic analysis of a lung-specific metastatic variant of the MDA-MB-231 human breast tumor cell line has identified several genes that appear to mediate successful lung metastasis. Minn et al. [58] highlight a combination of secretory and receptor proteins including EGF family member, epiregulin (EREG), CXCL1, MMP1 and 2, cell adhesion molecules secreted protein acidic and rich in cysteine (SPARC; osteonectin) and vascular cell adhesion molecule 1 (VCAM1), and the IL-13 decoy receptor IL13R$\alpha$2. Further analysis of this lung-targeting variant has highlighted the increased expression of tenascin C (TNC) when compared to the parental MDA-MB-231 line. TNC is a component of the extracellular matrix, and the authors suggest that tumor-secreted TNC plays an important role in determining the metastasis initiating capacity of a cell [59]. While there is some overlap between gene expression profiles of organ-specific variants of the same cell line, enough of a discrepancy exists that there are clear lung, bone, and brain metastasis signatures.

*7.4. Liver.* The prevalence of liver metastases in colon cancer far exceeds that of breast cancer, which has resulted in more research being done on the former. Consequently, identified interactions between colon cancers and hepatic metastases may not apply to breast cancers. However, hints about the metastatic mechanisms of breast cancer do arise in the observation of liver colonization by breast cancer cells. In a study by Stessels et al., 43 out of 45 breast cancer cases examined with liver metastases exhibited what is known as replacement growth, where tumor cells displace hepatocytes to coopt the sinusoidal blood vessels while preserving liver architecture [80]. This method of colonization allows for tumor growth independent of angiogenesis. To date, liver-targeting breast cancer cell line variants have not been established, but once selected for, genetic comparison between the organ-specific variants mentioned above will provide invaluable insight into the mechanisms driving liver-specific metastatic disease.

*7.5. Lymph Nodes.* In addition to hematogenous dissemination, breast cancer cells may also metastasize via the lymphatic system. Metastatic tumor cells may either stimulate lymphangiogenesis and enter the nascent vessels or may invade into preexisting lymphatic vasculature. Important primary tumor-derived signals may stem from the VEGF-C/VEGF-D activation of lymphatic endothelial VEGFR-3, which stimulates lymphangiogenesis toward the primary tumor and allows for cellular dissemination [81]. Conversely, molecules proposed to be direct mediators of lymphatic colonization include CCL21 and SDF-1 interacting with their tumor-expressed receptors, CCR7 and CXCR4, respectively. These pairs play important roles in the physiologic homing of lymphoid or hematopoietic cells, and their ligands are highly expressed in the lymph nodes. Additionally, blocking of the CXCR4-SDF-1 interaction with a neutralizing antibody in an

*in vivo* model of breast cancer metastasis successfully blocked metastases to the axillary lymph nodes [62].

A lymph node specific variant (468LN) of the MDA-MB-468 breast cancer cell line has been isolated and its mRNA expression compared to a variant of low lymphatic metastatic capacity (468GFP) [82]. When genes identified by differential expression were further compared to gene sets identified through clinical observations to ensure relevance, pathways associated with cell survival and growth in foreign environments were highlighted. Of note, E-cadherin, insulin-like growth factor binding protein 3 (IGFBP3), MAP-kinase activating death domain (MADD), and tissue inhibitor of metalloproteinase 3 (TIMP3) were downregulated, while cyclin-dependent kinase 2 (CDK2), SPARC, OPN, and vimentin were all upregulated. Additionally, the 468LN line harbored a larger $CD44^+$ $CD24^-$ population (96.4%) than the 468GFP line (6.3%) suggesting a role for breast CSCs in mediating this metastatic capacity.

The factors that have been discussed above for the various metastatic niches represent a brief summary of what is known and are not exhaustive. The diversity of the potential interactions between seed and soil highlights the need for further research. In particular, the question of whether the presence of the primary tumor can influence microenvironmental changes in distant organs prior to tumor cell arrival and metastatic colonization is intriguing.

## 8. Prepping the "Soil": The Premetastatic Niche

Recent work has shown that primary tumors may play an important role in creating a "premetastatic niche" prior to cancer cell arrival at secondary sites. Work by Kaplan et al. [83] highlighted the role of vascular endothelial growth factor receptor-1 positive ($VEGFR1^+$) hematopoietic progenitor cells (HPCs) in the creation of this niche. When signals from the primary tumor tip the normal balance between pro- and antiangiogenic signals in favor of angiogenesis, the angiogenic switch is triggered, causing the recruitment of new vessels to the tumor site [84]. During this process, HPCs are mobilized and migrate towards the tumor-specific premetastatic niche where they form clusters. Characterization of these cells revealed conserved progenitor markers of CD133, CD34, CD117 (c-Kit) in addition to expression of very late antigen-4 (VLA-4; integrin $\alpha 4\beta 1$), suggesting a VLA-4-fibronectin interaction between migrating HPCs and the new microenvironment. Additionally, MMP9 was expressed by the premetastatic clusters, potentially due to integrin-dependent activation of $VEGFR1^+$ HPCs, thereby altering the microenvironment through the breakdown of basement membranes and resultant release of soluble Kit-ligand. This study further showed that the $VEGFR1^+$ cells supported tumor cell adherence and growth and that metastasis could be abrogated upon the treatment with an anti-VEGFR1 antibody, highlighting the importance of these clusters in the creation of the premetastatic niche [83].

Another method that tumors use to condition the metastatic niche relies on microvesicular (MV) deposition of factors. Tumor-derived MVs, or exosomes, are derived from the inner membranes of the late endosomes and range from 40 to 100 nm in diameter. Release into the surrounding tissue or bloodstream occurs when the endosomes fuse with the cellular membrane [85]. Although the underlying mechanism is not fully understood, MVs may stimulate target cellular receptors directly, transfer surface receptors from cell to cell, deliver proteins [86], or may even cause epigenetic reprogramming of cells [87]. Additionally, MVs have been found to harbor immunosuppressive molecules [88]. Thus, exosomes may provide important signals to the tumor cells once they arrive in the metastatic niche, in addition to sculpting the stromal and immune cells systemically.

A recent concern arising from the revelation that exosomes are functional moieties and not just carriers of cellular waste arises from the potential for horizontal gene transfer between tumor cells and bone-marrow-derived cells (BMDCs) recruited to the premetastatic niche. Lyden and colleagues call this phenomenon "tumor exosome-driven education" of BMDCs [89]. This process likely promotes the progrowth and survival environment of the niche and may potentiate the metastatic process. Given their multifunctionality, it is likely that tumor-derived exosomes contribute to the creation of the premetastatic niche. Therefore, although the immunosuppressive effects of exosomes must first be negated, exosomes may represent a novel cell-free source of tumor antigens that can be utilized in the creation of an anticancer immunization to enhance the anti-tumor immune response [90].

## 9. The Cancer Stem Cell Niche: Does It Exist?

To date, published literature has used whole cell populations of organ-specific metastatic variants of human tumor cell lines as a model to investigate the organ tropism of metastasis [56–58]. However, these studies have overlooked the involvement of CSCs in this process. Further characterization of the distinct subpopulation of CSCs within these metastatic variants is needed to see if more refined genetic signatures can be obtained, possibly dictating a more specific niche for metastasis. If CSCs are indeed the initiators of metastasis, it is important to determine if these cells also exhibit organ-specific behaviors or if they are innately more metastatic to all sites in a nonspecific manner. Further investigations could also include murine models of spontaneous metastasis utilizing CSC and non-CSC subpopulations to elucidate if both subpopulations equally recruit the $VEGFR1^+$ population observed by Kaplan et al. [83] to the premetastatic niche, or if this capacity resides within one subpopulation. Our lab has observed increased tumorigenicity and metastatic ability to the lung of stem-like $ALDH^{hi}$ $CD44^+$ stem-like breast cancer cells relative to nonstem-like $ALDH^{low}$ $CD44^-$ cells [18]. This observed metastatic proficiency of CSCs may be partially attributed to their ability to create the premetastatic niche, in addition to their ability to form significant primary tumors. However, the exact mechanism behind this increased metastatic potential remains unknown. Additional characterization of the cell surface molecules expressed by CSCs may also provide further insight into their roles in metastatic organ tropism. For example, CSC expression of receptors such as CXCR4 would confer specific

targeting to areas where SDF-1 is highly expressed, such as bone, lung, lymph node, and brain [62, 73, 76], where the cells would then receive additional signals to support colonization. Additionally, CSCs may express higher levels of cell-surface receptors than their non-CSC counterparts so that they may fully harness the soluble growth factors present at secondary sites, conferring a growth advantage and permitting successful colonization.

## 10. Therapeutic Implications/Conclusions

A better understanding of the mechanism underlying the metastatic process is needed in order to increase the efficacy of treatments against this lethal process of disease progression. Metastatic lesions are often highly resistant to therapies, possibly due to the resident CSCs. In breast cancer, it would appear that the purported CSC subpopulation also encompasses the metastasis-initiating population. A better understanding of the interactions between CSCs and host organs may therefore lead to the identification of new targets that may allow for the abrogation of metastatic growth signals and consequently successful targeting of metastatic disease. Conversely, innate inhibitory factors may be found in the hostile secondary organs that may also be harnessed for therapeutic purposes. The definition of the microenvironment has evolved to include soluble factors, extracellular matrix, cell surface molecules, chemokines, hormones, and now exosomes, widening the scope of interactions that must be investigated.

There is no question that the clinically observed patterns of metastasis are relevant for cancer therapy, as there must be specific organ-cancer cell interactions contributing to the viewed success and failures of cancer cells to colonize specific secondary sites. In addition to targeting tumor-secreted factors, research is needed to identify key innate factors providing attractive and/or growth signals for the arriving cancer cells, so that inhibitors or specific targeting molecules may be developed against these factors. Furthermore, elucidation of the role of CSCs in this metastatic organ-tropism is also important, as new therapies are required to target this innately therapeutic resistant subpopulation. In light of the potential for interconversion between non-CSCs and CSCs, new therapies must target both populations of cells to be effective.

Further understanding of the role of CSCs in metastasis can be acquired with the characterization of circulating tumor cells (CTCs). Research in the CTC field is rapidly developing, and innovative techniques for the capture and characterization of CTCs are rapidly evolving. The many platforms to date (eloquently reviewed by Lowes et al. [91] and Yu et al. [92]) allow researchers to choose their method of capture based on either molecular cellular characteristics such as epithelial cell adhesion molecule $(EpCAM)^+CD45^-$Cytokeratin 8, 18, and $19^+$ (CellSearch; Veridex), $EpCAM^+$ (microfluidic CTC-chip [93, 94]), or markers of the researcher's choice (Fiber-optic array scanning technology [95, 96]), or physical cell size (filter-based platforms [97, 98]). Regardless of the platform, these techniques will allow for the further characterization of CTCs providing insight into the mechanisms driving organ tropism and whether CSCs are involved. Additionally, CTC data will offer distinct benefits for individualized therapy, as physicians could tailor therapy to the characteristics of the CTCs.

As the world's population ages, the incidence of cancer is projected to increase, making more effective treatments vital to help combat this growing world-wide burden. Although methods for early detection are in place for more developed countries, these capacities are not readily available in developing countries. Thus, cancers in these areas will often be detected during the later stages of disease progression, when metastasis has likely already occurred. Novel, more effective metastatic treatments may be the only option for this new group of cancer patients and are already desperately required for those in developed countries burdened with metastatic breast cancer. In addition to further understanding the characteristics of cancer stem cells, future research should focus on the interactions between CSCs and the secondary organs of metastasis, as we believe this to be where new metastatic targets will arise.

## Abbreviations

| | |
|---|---|
| ABCB1: | ATP binding cassette subfamily B member 1 |
| ABCC1: | ATP binding cassette subfamily C member 1 |
| ABCG2: | ATP binding cassette subfamily G member 2 |
| ALDH: | Aldehyde dehydrogenase |
| ANGPTL4: | Angiopoietin-like 4 |
| BBB: | Blood-brain barrier |
| BCRP1: | Breast cancer resistance protein-1 |
| BMDC: | Bone-marrow-derived cells |
| BMP: | Bone morphogenic protein |
| CCL21: | Chemokine (C-C motif) ligand 21 |
| CCR7: | Chemokine (C-C motif) receptor type 7 |
| CD: | Cluster of differentiation |
| CDK2: | Cyclin-dependent kinase 2 |
| COX2: | Cyclooxygenase-2 |
| CSC: | Cancer stem cell |
| CXCR4: | Chemokine (C-X-C motif) receptor 4 |
| EREG: | Epiregulin |
| EpCAM: | Epithelial cell adhesion molecule |
| ESA: | Epithelial-specific antigen |
| FGF: | Fibroblast growth factor |
| FSCN1: | Fascin-1 |
| HBEGF: | Heparin-binding EGF |
| HPC: | Hematopoietic progenitor cell |
| IFN-$\gamma$: | Interferon-$\gamma$ |
| IGF: | Insulin-like growth factor |
| IGFBP3: | Insulin-like growth factor binding protein 3 |
| IL: | Interleukin |
| Lin: | Lineage |

LTBP1: Latent TGF-$\beta$-binding protein
M-IC: Metastasis-initiating cell
MADD: MAP-kinase activating death domain
MEI: Metastatic efficiency index
MMP: Matrix metalloproteinase
MV: Microvesicle
NOD/SCID: Nonobese diabetic severe combined immune deficiency
OPN: Osteopontin
PDGF: Platelet-derived growth factor
PGP: P-glycoprotein
PTHrP: Parathyroid hormone-related protein
RANK: Receptor activator of nuclear factor $\kappa$B
RANKL: Receptor activator of nuclear factor $\kappa$B ligand
SC: Stem cell
SDF-1: Stromal-derived factor-1
SPARC: Secreted protein acidic and rich in cysteine
SSEA-1: Stage-specific embryonic antigen 1
ST6GALNAC5: ST6 (alpha-N-acetyl-neuraminyl-2,3-beta-galactosyl-1,3)-N-acetylgalactosaminide alpha-2,6-sialyltransferase 5
TIMP3: Tissue inhibitor of metalloproteinase 3
TGF-$\beta$: Transforming growth factor-$\beta$
TNC: Tenascin C
TNF-$\alpha$: Tumor necrosis factor-$\alpha$
VCAM: Vascular cell adhesion molecule
VEGF: Vascular endothelial growth factor
VEGFR: Vascular endothelial growth factor receptor
VLA-4: Very late antigen-4.

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*Review Article*

# Bromodomain-Containing Protein 4: A Dynamic Regulator of Breast Cancer Metastasis through Modulation of the Extracellular Matrix

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Metastasis is an extremely complex process that accounts for most cancer-related deaths. Malignant primary tumors can be removed surgically, but the cells that migrate, invade, and proliferate at distant organs are often the cells that prove most difficult to target therapeutically. There is growing evidence that host factors outside of the primary tumors are of major importance in the development of metastasis. Recently, we have shown that the bromodomain-containing protein 4 or bromodomain 4 (*Brd4*) functions as an inherited susceptibility gene for breast cancer progression and metastasis. In this paper, we will discuss that host genetic background on which a tumor arises can significantly alter the biology of the subsequent metastatic disease, and we will focus on the role of *Brd4* in regulating metastasis susceptibility.

## 1. Introduction

Breast cancer is the most common cancer diagnosed in women worldwide. In the United States the estimates for 2010 were 209,060 new cases of invasive breast cancer and 40,230 deaths [1]. The main cause of breast cancer-related deaths is metastatic disease. The overall 5-year relative survival of patients with metastatic breast cancer is 23%, while the relative survival of breast cancer patients with nonmetastatic tumors is 98% [2]. Patients who have no evidence of tumor dissemination at the time of diagnosis are still at risk of metastatic disease. Approximately one-third of women who are sentinel lymph node negative at the time of surgical resection of the breast primary tumor will eventually develop clinically detectable secondary tumors [3]. Therefore, understanding the mechanisms governing tumor dissemination and developing new strategies to control or effectively treat patients with or at risk of metastatic disease would significantly improve the overall outcome of the disease.

Metastasis is a multistep complex process that involves the detachment of tumor cells from the primary tumor, migration and invasion through the surrounding tissues and basement membranes, intravasation and survival in the small blood vessels or lymphatic channels, and colonization in a distant target organ. These steps are usually followed by extravasation into the surrounding tissue, survival in the foreign microenvironment, proliferation, and induction of angiogenesis (Figure 1). It has become apparent that the vast majority of tumor cells within the primary tumor and also the disseminated tumor cells will not form distant metastases, either because they die or remain dormant [4]. The dormancy phenomenon probably explains what is seen in the clinic in which some cancer patients remain free of clinical evidence of metastatic disease for years or even decades after primary tumor resection, and after this prolonged period of time these patients show signs of tumor relapse. The development of the primary tumor microenvironment is also an important determinant of tumor dissemination; this tumor microenvironment may influence the release of cancer cells into the blood and the lymphatic systems and subsequently promote continued survival and proliferation at the secondary site. It has been well known that the interaction between tumor cells and their microenvironment is important for establishing metastatic colonies and for

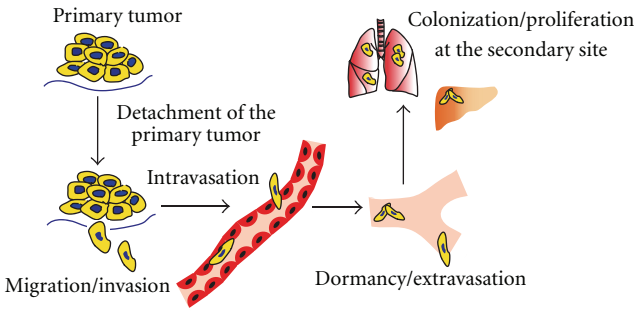


Figure 1: The steps of the metastatic cascade.

defining the balance between dormancy and malignant growth [5, 6]. Furthermore, the microenvironment of metastatic tumor cells has recently been thought to play a major role in tumor progression. Although tumor cells may be continually released from the primary site, relatively few of them are able to efficiently form macrometastasis. What are the factors controlling this important step?

In this paper we will discuss a more global view of host-tumor interactions in which the metastatic potential of tumors is an inherent component of cells forming the primary tumor mass at an early time in tumor progression. We will also discuss the association of two genes, *Brd4* and *Sipa1* (signal-induced proliferation-associated 1), with mammary tumor progression in both the mouse and the human. Here, we will focus on the role that the metastasis susceptibility gene *Brd4* plays in the regulation of extracellular matrix (ECM) gene expression and subsequently metastatic progression.

## 2. Genetic Background Plays an Important Role in Metastasis

Studies from our laboratory have demonstrated that the inherited polymorphism, or the genetic background on which a tumor arises, plays an important role in determining the probability that the given tumor will progress to metastatic disease. These findings are based on a series of genetic mapping studies using the highly metastatic polyoma middle-T (PyMT) transgenic mammary mouse model. This mouse expresses the mouse polyoma virus middle-T antigen in the mammary epithelium of FVB/N inbred mice [7] from an early age [8], which results in the development of highly aggressive mammary tumors that metastasize to the lung with high frequency [7]. Specifically, when the male PyMT transgenic mouse was bred to different inbred strains, the F1 progeny showed significant variation in tumor characteristics, such as tumor latency, growth kinetics, and metastatic capacity [9]. It is important to point out that these tumors were all induced by the same oncogenic event, the activation of the PyMT transgene. Subsequent analysis identified several interacting quantitative trait loci (QTL), some of which were found to map to homologous regions associated with loss of heterozygosity in human breast cancer [8, 10, 11]. Together, these findings suggest that inherited germline polymorphisms may contribute to the age of onset in human breast cancer and also the ability of tumor cells to metastasize. Further investigation of these earlier observations identified the first known polymorphic metastasis susceptibility gene, the Rap-GTPase activating protein (GAP) SIPA1 [12]. Experimental manipulation of cellular *Sipa1* mRNA levels in a highly metastatic mouse mammary tumor cell line showed that subtle differences in *Sipa1* levels significantly affected the ability of the cells to colonize to the lungs, while not impacting primary tumor kinetics [12]. Studies of human breast cancer have suggested that *SIPA1* germline polymorphisms are associated with aggressive disease behavior and with indicators of poor prognosis [13, 14], suggesting that *Sipa1* may play an important role in establishing metastatic susceptibility in humans as well as in mice.

## 3. Inherited Polymorphisms and ECM Gene Expression Profile

We have shown so far that hereditary polymorphisms modulate metastatic potential. To further study whether genetic polymorphisms could be an important factor in the induction of prognostic signature profiles, previously published metastasis-predictive gene expression signatures were examined both in the mouse and in humans. ECM genes were found to be common components of the metastasis-predictive gene signature in both human breast tumors [15–17] and in PyMT-induced mouse mammary tumors [18, 19], suggesting an important association of these genes with breast cancer progression. Briefly, the ECM components constitute a structure that is not only essential for the maintenance of tissue integrity but is also important for regulating cell migration. Historically, tumor interaction with the basement membrane was defined as the critical event in tumor invasion that signals the initiation of the metastatic cascade. Many steps in metastasis formation require specific interactions with the ECM [20]. The nature and degree of this interaction will change from step to step during the metastatic process. However, the type of specific interactions between tumor cells and the ECM might be influenced by the type of tumor cells and the type of matrix in which they reside. For example, tumor cells may respond differently to various extracellular matrices and stromal cells that are encountered during metastasis formation, and this might result in the emphasis of some steps over others at particular points in the metastatic cascade (reviewed in [21]).

A study by Bergamaschi and colleagues has portrayed the tumor-stroma composition of invasive breast carcinomas by characterizing the ECM components [22]. Differential expression of ECM-related genes identified four distinct groups. The ECM classification was recapitulated in a set of early-stage primary breast carcinomas [22, 23]. Survival analysis on the early-stage breast carcinoma dataset showed significant differences in clinical outcome among the various ECM subclasses [22]. Several studies that explored gene expression differences of primary breast and metastatic lymph node tumors have shown that genes involved in

changes in extracellular matrix stability are critical for the early stages of the metastatic process [24–27]. Furthermore, ECM gene dysregulation has been shown to be a very prominent feature of metastatic progression and may well explain why highly metastatic mouse mammary tumor cell lines are typically more adhesive, invasive, and migratory than the less metastatic lines [28]. To determine whether the ECM dysregulation is under germline control, the AKXD recombinant inbred mice (RI) [29] were used to define ECM expression quantitative trait loci (eQTL). An eQTL is a genetically defined genomic locus associated with variation of gene expression, in this case ECM gene expression [30]. We chose the AKXD RI mice because they are considered a useful tool for the study of germline-encoded metastatic propensity since they are derived from a highly metastatic strain, AKR/J, and a weakly metastatic strain, DBA/2J [9]. We found that the most significant eQTL in these mice is located on proximal mouse chromosome 17. This eQTL colocalizes to the peak region of linkage of a metastasis susceptibility QTL [31]. Both of these eQTL and metastasis loci colocalize and reside in a genomic region that contains the gene *Brd4*, suggesting that *Brd4* modulates ECM gene expression.

## 4. *Brd4* Is a Potential Metastasis Susceptibility Gene

*BRD4* is the mammalian member of the BET (bromodomain and extra-terminal) family [32, 33], whose members carry two tandem bromodomains [34, 35]. BRD4 has been shown to regulate cell growth by acting at different stages of the cell cycle and also to interact with acetylated chromatin through its two bromodomains [32, 33]. Given the apparent modulation of ECM gene expression, we further investigated the possibility that *Brd4* might be a metastasis susceptibility gene. Indeed, we found that ectopic expression of *Brd4* in a highly metastatic mouse mammary tumor cell line reduces both primary tumor growth and metastatic capacity in our mouse model [36]. *In vitro* analyses showed that *Brd4* ectopic expression reduces both cell invasion and cell migration and also reduces cellular growth in three-dimensional cultures [36]. These data are consistent with our previous findings that *Brd4* modulates ECM gene expression. Microarray gene expression analysis of the cell lines ectopically expressing *Brd4*, further confirmed that *Brd4* is a regulator of at least some of the ECM gene family members [36]. Some of the ECM genes that were altered by ectopic expression of *Brd4* are the collagen genes *Col1a1*, *Col5a3*, *Col6a2*, the fibrillin gene *Fbn1* and *Serping1*, indicating that *Brd4* is a causative factor in the transcriptional regulation of these genes [36].

*4.1. Brd4 and Sipa1 Interaction and Metastatic Progression.* BRD4 has been previously found to interact *in vitro* and *in vivo* with the metastasis modifier SIPA1 [37]. This interaction modulates the enzymatic activity of SIPA1 by increasing its RAP-GAP activity. The N-terminus bromodomain II of BRD4 was shown to be the domain where BRD4 and SIPA1 interact [37]. Deletion of bromodomain II resulted in further suppression of primary tumor growth and lung metastasis mediated by *Brd4* and also induced a conversion to a more epithelial state [38]. These results are consistent with our previous findings that SIPA1 is associated with greater malignancy [12]. It is important to mention here that BRD4 and SIPA1 were shown to regulate each other's subcellular localization, with BRD4 being redirected from the nucleus to the cytoplasm [37]. It is possible that the interaction between these two proteins contributes to tumor progression, and also the activity of *Brd4* might be modulated by compartmentalization; however, the mechanism by which this occurs has yet to be explored. One possibility could be that there is a balance between BRD4 and SIPA1 within the cell. Under normal conditions BRD4 and SIPA1 interact in the nucleus while the cytoplasmic SIPA1 does not take part in this interaction. Upon *Brd4* overexpression, SIPA1 accumulates in the perinuclear region and in some cases in the nucleus near the nuclear membrane [37]. However, when *Sipa1* is overexpressed, a large fraction of BRD4 gets moved to the cytoplasm leading to a more malignant phenotype. Our results suggest that the loss of the ability of SIPA1 to relocalize or sequester the bromodomain II mutant to the cytoplasm would increase the nuclear concentration of BRD4, leading to a more differentiated state and a less malignant phenotype. At this point it is not known whether the BRD4-SIPA1 interaction influences the small GTPase RAP1 levels within the tumor cell. RAP1 activity has been shown to play an important role in tumor formation and progression to malignancy [39, 40]. Further investigations of the BRD4-SIPA1 relationship and the influence that it could have on RAP1 levels might reveal a novel mechanism associated with malignant progression.

*4.2. Brd4 and Regulation of Epithelial-to-Mesenchymal Transition (EMT).* BRD4 is known to be a transcriptional regulator. As mentioned earlier BRD4 contains two bromodomains that bind acetylated histones [32]. A recent report has shown that the extraterminal (ET) domain of BRD4 is an important transcriptional regulatory domain [41]. The C-terminal domain contains a single defined domain that binds the transcriptional elongation factor P-TEFb [42]. BRD4 also contains regions of high serine, proline, and glutamine content of unknown function. Indeed, microarray gene expression analysis of the cell lines that ectopically express *Brd4* has revealed that *Brd4* modulates the expression of genes involved in processes such as cellular proliferation, cell cycle progression, and chromatin remodeling [36]. Other processes that are critical for metastasis, such as cytoskeletal remodeling, cell adhesion, and as mentioned earlier ECM expression regulation, were also regulated by *Brd4* [36]. Furthermore, microarray gene expression analysis of cell lines that express a C-terminal deletion of *Brd4* show modulation of other classes of genes involved in EMT and stem cell conversion processes [38].

EMT is a multigstep process in which the cells acquire molecular changes that lead to a loss of cell-cell junctions, dysfunctional cell-cell adhesion, and rearrangement of the cytoskeleton, leading to a loss of polarity and the acquisition of a more spindle-shape morphology [43–48]. These alterations might eventually promote cancer cell progression and invasion through the basement membrane and into the

surrounding tissues. Indeed, several studies have associated EMT with cancer progression and metastasis [49–52]. For example, EMT markers have been found to be present in invasive breast cancer especially in the invasion-metastasis cascade [47, 53]. Recently, a concept of the "migratory cancer stem cell" has been described [54], in which a tumor cell possesses both stemness and motility properties. It is suggested that cancer stem cells that have undergone EMT can disseminate, and those that retain stem-cell functionality can form metastatic colonies [54]. More recently the EMT process has also been linked to the ability of self-renewal [55]. Current thinking suggests that disseminated cancer cells may need to acquire self-renewal properties similar to those exhibited by the stem cells, in order to achieve formation of macroscopic metastases [55]. The role of EMT in tumor-initiating cells has also been described in human specimens. Breast cancer tumor-initiating cells and mesenchymal claudin-low-subtype cells show an association based on gene expression pattern [56]. Furthermore, higher expression of mesenchymal genes was detected in breast cancer tumors before and after treatment with letrozole, indicating that the epithelial cancer cells have undergone EMT [56].

Ectopic expression of the C-terminal deletion mutant of *Brd4* (ΔC) in a highly metastatic cell line induced significant morphological and physiological changes reminiscent of EMT-like and cancer stem cell-like properties [38]. Microarray gene expression analysis of these cell lines demonstrated that ectopic expression of the ΔC mutant modulated the expression of some previously described EMT markers and stem cell markers. It is important to point out here that this mutant still contains the P-TEFb-binding domain suggesting that EMT-like and stem cell-like changes appear to be mediated by this P-TEFb-binding region. The mechanism on how this might occur is currently under investigation.

*4.3. Brd4 Isoforms and Metastasis Regulation.* *Brd4* has two alternatively spliced variants that differ in the coding region and have a distinct 3′ UTR. Both isoforms have the same N-terminal region containing the chromatin-binding bromodomains and the serine-rich domain; however, the C-terminal proline-rich and P-TEFb-binding domains are absent in the shorter isoform. We have found that ectopic expression of the short isoform enhances metastatic colonization [38], as opposed to that seen by ectopic expression of the longer isoform [36]. This would suggest that the *Brd4* short isoform might be a competitive inhibitor of the longer isoform and that this inhibition would increase the ability of tumors to progress to metastatic disease. This also suggests that metastatic susceptibility might be encoded by a ratio between the two isoforms. The above data also suggest that the carboxy terminal half of the full-length isoform mediates the ability of *Brd4* to suppress progression and metastasis. This was confirmed by the finding that expression of the C-terminal ΔC mutant of *Brd4* increased lung colonization [38]. This increased malignancy is consistent with the *in vitro* data that cells expressing this mutant possess EMT- and stem cell-like properties. It is not known at this point whether the ratio between the two *Brd4* isoforms influences the expression of *Sipa1* or vice versa. It would also be highly interesting to determine whether the BRD4 short isoform and SIPA1 could change each other's subcellular localization as seen with the longer isoform. The ratio between these three proteins and their cellular localization could be critical for malignant progression.

It is important to mention here that, in rare midline carcinomas, a highly malignant form of human squamous carcinoma, the *BRD4* short isoform is frequently fused to the *NUT* (nuclear protein in testis) oncogene via an intronic translocation [57–60]. The major oncogenic effect of BRD4-NUT fusion protein appears to lie in its ability to arrest the differentiation of the so-called NUT-midline carcinoma cells [59]. This is consistent with our findings that the shorter *Brd4* isoform promotes metastatic capacity and also that the competitive inhibition of the longer *Brd4* isoform would increase the ability of tumors to progress to metastatic disease [38].

*4.4. Brd4 Isoforms Expression and Gene Expression Signatures.* Several studies have demonstrated that primary tumors with a higher propensity to metastasize exhibit gene expression patterns that predict the likelihood of metastatic potential [15–17]. As mentioned earlier, *Brd4* is responsible, at least partially, for the presence of ECM components in the metastatic-predictive gene signatures [36], suggesting that *Brd4* itself might be a predictive of survival. We have found that the *Brd4* long isoform induces a gene expression signature that predicts good outcome in human breast cancer datasets. This suggests that *Brd4* activation is an important determinant in the overall likelihood of relapse and/or survival [36]. The *Brd4* gene expression signature was also able to stratify breast cancer patients with lymph-node-negative and estrogen-receptor-positive at presentation into high- and low-risk patients [36]. The gene expression signature induced by the *Brd4* short isoform, however, predicted poor outcome in these human breast cancer datasets [38], confirming that the shorter isoform might be a competitive inhibitor of the longer isoform. Additionally, the *Brd4* long- and short-isoform gene expression signatures were compared to a 19-gene signature that was defined by correlating tumor growth expression, histological grade, and survival [61]. We found that the *Brd4* longer isoform signature matches low-grade G1 breast cancer tumors while the shorter isoform matches high-grade G3 tumors [36, 38]. These observations were completely consistent with our *in vivo* data. The outcome prediction and the signature convergence might be of potential importance in the clinic where it could improve the stratification of patients into different subtypes and in turn enable clinicians to tailor treatments for individual patients.

## 5. *Brd4* as a Therapeutic Target

Selective inhibitors of the BET family members have been recently developed [62–65]. A competitive binding of the small molecule inhibitor JQ1, for example, was shown to displace the BRD4 fusion oncoprotein from chromatin, promoting squamous differentiation and specific anti-proliferative effects [63]. These effects were seen in BRD4-dependent cell lines and patient-derived xenograft models

[63]. In another study, Zuber and colleagues studied acute myeloid leukemia (AML), which is an aggressive hematopoietic malignancy that is often associated with aberrant chromatin states [65]. Suppression of *Brd4* by shRNA or by JQ1 compound led to robust antileukemic effects both *in vivo* and *in vitro*. *Brd4* inhibition also led to myeloid differentiation and leukemia stem-cell depletion [65]. At this point it is not known whether the small-molecule inhibition of *Brd4* would have any effect on breast cancer and metastatic progression. However, the recent findings establish *Brd4* as a promising target for therapeutic intervention.

## 6. Conclusions

It is clear that the genetic background is an important determinant of tumor progression. The genetic background impacts not only the primary tumor but all of the tissues, which play a role in the establishment of the microenvironment in both primary and metastatic tumor cells. This would suggest that an earlier prognosis in nontumor tissues should be possible even before cancer develops. This is only possible if a sufficient fraction of metastatic risk is encoded by germline polymorphisms, rather than autonomous somatic events within the tumor.

Our recent data suggest that the metastasis susceptibility gene *BRD4* appears to play a significant role in establishing transcriptional programs that predict breast cancer outcome via a balance between the tumor- and metastasis-suppressive long isoform and the metastasis-promoting short isoform. Given the fact that *BRD4* regulates important intermediates and processes within the metastatic cascade suggests that *BRD4*, and possibly other metastatic susceptibility genes, may be altering the risk of developing distant metastases by predisposing the tumors of high-risk patients to undergo conversion to a more dedifferentiated or primitive state. Finally, the *Brd4* gene expression signature identified could be applied as a useful predictive tool by identifying those patients with low risk of relapse at presentation. This combined with the traditional clinical variables such as lymph node-negative and ER-positive patients would facilitate the identification and the initiation of new treatment protocols that could be applied for individual patients.

## Glossary

*Invasion.* A process that initiates metastasis and consists of changes in tumor cell adherence to the extracellular matrix, proteolysis of the extracellular matrix and the surrounding tissues and migration through these tissues.

*Intravasation.* The entry of tumor cells into the bloodstream.

*Extravasation.* The escape of tumor cells from the circulation into the parenchyma of an organ.

*Colonization.* A process by which disseminated tumor cells grow to form clinically detectable metastatic lesions.

*Angiogenesis.* The formation of new blood vessels that are needed for the growth of the primary tumor and metastases.

*Dormancy.* A period in which the cells are in a non-dividing state.

*Polymorphism.* A variation within a gene where two or more alleles exist at a frequency of at least 1% in the general population.

*Extracellular Matrix.* The matrix that is laid down by cells in which they adhere and move.

*Expression Quantitative Trait Locus (eQTL).* A genetically defined genomic locus associated with variation of expression of the genes that underlie the trait in question.

*Epithelial-to-Mesenchymal Transition (EMT).* A potential mechanism in tumor progression by which some cancer cells acquire the ability to convert from polarized epithelial cells to mesenchymal motile cells facilitating metastasis at distant sites.

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