Review

Multistep tumorigenesis and the microenvironment
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Abstract

Early-stage cancers have long been considered to be less aggressive than late-stage cancers because it is assumed that they have accumulated fewer of the mutations that are required for full metastatic potential. For breast cancer, recent gene expression profiling data have challenged this paradigm by identifying early-stage cancers with similar gene expression profiles to fully metastatic cancers. In this review, multistep carcinogenesis is reconsidered in light of these new data. The concept that the tumor stroma plays a key role in determining whether a metastatic tumor cell will remain dormant or become invasive is discussed. Recent studies demonstrating the feasibility of targeting tumor stroma for cancer prevention and treatment are presented.

Keywords: breast cancer, metastasis, microenvironment, multistep carcinogenesis, reactive stroma

Introduction

The prevailing hypothesis for the metastatic spread of cancer is that metastasis is the end stage of a progressive disease [1]. Specifically, cancer cells acquire the hallmarks of malignancy as they accumulate multiple, rate-limiting mutations. Furthermore, this progression occurs over an extended period of time, resulting in metastasis being a rare event with long latency. Importantly, the primary tenet behind early cancer detection and prevention strategies is that carcinogenesis can be suppressed during the post-initiation stages of the disease, specifically because of its progressive nature and long latency [2]. Recently, however, the concept of cancer as a multistep progression, with metastatic potential arising late within a rare cell, has been challenged [3–5]. In this review we discuss this controversy within the context of the tumor microenvironment and address its implications for future prevention and treatment research.

Requirement for multiple mutations

There is a tremendous body of literature that supports the hypothesis that cancer results from the slow accumulation of mutations that eventually give rise to rare variant cells with metastatic potential. For example, for most epithelial cancers there is evidence of a histologic progression of the disease from benign through malignant stages. In breast cancer this progression consists of atypical ductal hyperplasia, preinvasive ductal carcinoma in situ (DCIS), and invasive ductal carcinoma. For familial colorectal cancer the histopathologic progression, as well as the progression of genetic alterations, has been defined [6]. More recently, the histopathologic progression with corresponding genetic mutation profiles have been described for oral leukoplakia [7] and Barrett’s esophageal cancer [8]. Experimental evidence supporting the hypothesis that multiple genetic lesions drive the histopathologic progression of transformed epithelial cells is also very convincing. In order for human epithelial cells to form colonies in soft agar or tumors in immunocompromised mice, transformation with two or more known oncogenes is required [9]. More recently, Brugge and coworkers [10] demonstrated that transfection with a combination of oncogenes — one that causes constitutive proliferation cotransfected with one that inhibits apoptosis — are required for lumen filling in a three-dimensional culture model for breast cancer. A single oncogene capable of activating both proliferation

CAF = carcinoma-associated fibroblasts; DCIS = ductal carcinoma in situ; ECM = extracellular matrix; MMP = matrix metalloproteinase.
and antiapoptotic pathways, such as ErbB2, was also found to mimic DCIS in culture. In further support of multi-step carcinogenesis, these DCIS models did not exhibit motile or invasive phenotypes, implicating the requirement for additional mutational events [10].

**Long latency consistent with multistep carcinogenesis**

Considerable data indicate that cancer progression from premalignancy to malignancy is slow, which is consistent with a natural selection model in which multiple mutations are required in order to reach full metastatic potential. The best clinical data demonstrating a long latency for tumorigenesis comes from studies with familial cancer syndromes. Individuals from Li Fraumeni families who have an inactive p53 gene due to a germ-line mutation are essentially born with ‘initiated’ cells, and 90% of these individuals develop cancer by age 70 years. However, malignant transformation is limited to certain organs and latency, which varies depending on organ site, is relatively long in every case [11]. The average age of onset is 16 years for sarcomas, 37 years for breast cancer, and 50 years for lung cancer. Similarly, BRCA1 mutation carriers have a 55–58% lifetime risk of developing breast cancer (and much lower for ovarian cancer). Moreover, the risk for breast cancer remains age related, beginning in the 30s. This delay in latency may be due to two additional events required: inactivation of the other BRCA1 allele and a gain-of-function mutation that supports survival of cells with no BRCA1. Something akin to this may also be necessary for tumor development in Li Fraumeni patients. Additional support for long latency being required for manifestation of aggressive tumors is evident in cervical cancer, where it is possible to identify readily early-stage lesions. Progression of untreated cervical cancer from carcinoma in situ to invasive cancer has been reported to take between 8 and 30 years [12,13].

**Metastasis is a rare event, consistent with end-stage disease**

Finally, clinical evidence demonstrating that metastasis is indeed a rare event in humans was definitively obtained by Tarin and colleagues [14]. In their study, patients who had metastatic spread of a variety of primary cancers to the peritoneal cavity were fitted with peritoneovenous shunts. With the shunt, abdominal pressure was alleviated by delivering the ascites fluid, with viable cancer cells, directly into the jugular vein. These metastatic tumor cells would be anticipated to have a higher probability of successfully colonizing distant organs than primary tumor cells of similar metastatic potential. This is because initial barriers to metastasis that primary tumor cells encounter are circumvented, namely the need to disrupt cell–cell and cell–matrix interactions within the primary tumor, invade through the local tissue, and gain access to the local vasculature or lymphatics [15]. Further, in that study these ascites fluid cells were already successful metastatic cells. Based on blood samples taken from 16 patients, it was estimated that on average $2 \times 10^7$ viable cells were present per 20 ml blood, of which approximately 1 in $10^4$ were clonogenic in soft agarose [14]. The average duration of exposure was 40 weeks. Most of the patients succumbed to complications of ascites fluid. At autopsy, tissues were collected for semiquantitation of macro- and micrometastases. Surprisingly, metastatic events were rare, even in lung, which was the first capillary bed encountered by the tumor cells (Table 1).

Data obtained from animal models, in which cells, either transformed with known oncogenes or isolated from metastatic tumors, were injected directly into the vasculature, corroborate the human data. In these animal experiments, formation of secondary lesions occurs only rarely, with less than 0.001% of cells with metastatic potential contributing to metastatic lesions [12,16,17]. One interpretation of these data is that, even in a population of seemingly identical metastatic cells, additional epigenetic selection is required for cells to expand and successfully form a tumor. Another interesting example of metastatic inefficiency is demonstrated by the presence of micrometastatic disease in bone marrow. Of patients with primary esophageal and pancreatic carcinoma, 20% have presumptive cancer cells in their marrow, even though bone and bone marrow are rare sites of metastatic growth [18,19]. In breast cancer, positive marrow has been associated with a somewhat worse prognosis in stages I–III, but not IV [20]. In total, the evidence is mixed and demonstrates that marrow micrometastasis is clearly not a strong prognostic factor.

Cumulatively, these clinical and experimental data support the conclusions that cancer is a progressive disease with metastasis being a rare event with a long latency. These observations indicate that a cell must accumulate numerous, independent mutations in order to overcome multiple, rate-limiting barriers to metastasis.

**Metastatic genotype acquired early: clinical evidence**

It is the recent expression profiling data obtained from microarray analyses that have revitalized the question of whether the metastatic phenotype represents end stage of a lengthy progressive disease. Nagging doubts existed before the advent of microarray technology. For example, although a clear molecular progression profile corresponding to histopathologic progression can be identified in some cancers, in fact only a small percentage of these cancers present with the ‘classic’ genetic mutation profile. Furthermore, a relationship between progressive increases in mutations and increasing histologic aggressiveness has not been identified for other common cancers such as breast, lung, or prostate. These observa-
tions indicate that mutations that result in a metastatic phenotype are not necessarily late events, and rather that it is the net cumulative effect of mutations that is more important than the order in which they are acquired.

Further doubt that metastasis is, by definition, an attribute acquired late in cancer progression has been obtained from mammography screening. Surprisingly, population scale screening has not decreased breast cancer mortality rates as dramatically as was expected, suggesting that early-stage breast cancers are not necessarily less aggressive than later stage. In fact, between 22% and 33% of stage I breast cancers progress, indicating that these early stage cancers have already spread at time of detection [21].

These clinical observations are consistent with the idea that within each stage there are more aggressive tumors and less aggressive tumors. Furthermore, these data suggest that, early on, cancers may be separable into those with high metastatic potential and those without, independent of stage at diagnosis. The recent microarray data detailing the expression profiles of primary tumors and corresponding metastatic lesions support this concept [5,22–24].

**Metastatic genotype acquired early: molecular evidence**

In the microarray studies the identification of gene expression profiles in stage I breast cancers was found to be predictive of patient outcome [5,22–24]. Specifically, a gene signature correlating with metastatic progression could be found in a subpopulation of primary tumors. Because microarray technology is not sensitive enough to identify rare variant cells within the tumor mass, these studies show that the propensity to metastasize is not a rare event that occurs in only a few cells within the primary tumor. Furthermore, microarray data have demonstrated that expression profiles can be similar between early-stage DCIS lesions and invasive breast tumors. Two additional points can be made from these observations. First, tumors can be segregated into those with and those without metastatic potential at a clinical stage corresponding to histologically premetastatic lesions [25]. Second, for tumors with metastatic potential, there appear to be few gene expression differences between preinvasive and invasive lesions.

Although the potential implications of these data for identifying at-risk patients are clear, the question of whether metastasis is indeed the end stage of a progressive disease, and whether metastasis-specific genes exist at all, becomes less clear. In fact, Bernards and Weinberg [3] recently argued that the same classes of genes involved in cancer initiation (i.e. the classic oncogenes and tumor suppressor genes that contribute to autocrine growth factor production, resistance to cell death signals, and genomic instability) may be the elusive metastasis genes (or the upstream regulators of metastasis genes). Support for this concept is evident in the aggressive phenotype observed in immortalized human mammary epithelial MCF12A cells transfected with oncogenic V12-Ras (Fig. 1). Introduction of the single oncogenic Ras gene results in loss of the normal polarized mammary epithelial phenotype and gain of an aggressive motile phenotype consistent with a full epithelial-to-mesenchymal transition.

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**Table 1**

**Post-intravasation models for metastatic efficiency**

| Site of primary tumor | Average number of viable cells per 20 ml blood (delivered on average for 40 weeks) | Average clonogenic efficiency in soft agar | Metastatic events in patients at autopsy |
|-----------------------|-----------------------------------------------------------------------------------|-------------------------------------------|----------------------------------------|
| Ovary, breast, pancreas bronchus, and colon | $1 \times 10^7$ | $1 \times 10^4$ | 8/15: no viable cells 7/15: large number of single viable tumor cells and micrometastases in lung only |

**Tail injection [16,17]**

| Cell types | Gene expressed | Number of cells per injection | Lung colony efficiency |
|------------|----------------|-----------------------------|------------------------|
| NIH-3T3 [16] | c-H-ras | $3 \times 10^5$ | $1 \times 10^4$ |
| MDA-MB-231 [17] | v-src | $3 \times 10^5$ | $2 \times 10^5$ |
| MDA-MB-435 [17] | – | $1.0 \times 10^6$ | <1 in 10^5 |

Data from references Tarin and coworkers [14], Egan and coworkers [16], and Price and coworkers [17].
In total, clinical and experimental evidence are compelling for both models of metastasis and suggest that some tumors advance through classic progression stages and acquire metastatic potential rather late, whereas other tumors acquire this potential early on, possibly as early as initiation.

Metastatic potential is not sufficient for metastasis

When it became obvious that metastasis was a rare event, even for fully transformed cells delivered directly into the vasculature, it was hypothesized that most metastatic tumor cells die in the vasculature because the environment is hostile [12,26]. More recently, using in vivo video microscopy, Chambers and coworkers determined that survival of tumor cells in the circulation, arrest of cells in capillary beds within secondary organs, and extravasation out of the capillary bed into the local tissue are in fact highly efficient events [27]. Those researchers determined that approximately 80% of the original tumor cell inoculums arrested at a secondary site, and rather it was the abilities of these arrested cells to develop into micrometastases and then progress to vascularized lesions that were highly inefficient. These live cell imaging data, combined with the new gene profiling data, demonstrate that having metastatic potential is not sufficient to guarantee a successful metastatic event. So what determines whether a cell will manifest its malignant phenotype and colonize a distant organ successfully?

Role of the microenvironment in determining the metastatic phenotype

In fact, two steps in the metastatic cascade have been described as rate limiting: gaining access to the vasculature (or lymphatics) at the site of the primary tumor [28] and tumor formation at the secondary site [29]. These observations suggest that a permissive tumor microenvironment is required for successful metastasis [27]. Specifically, the microenvironment of the primary tumor needs to support tumor cell dissemination, motility, and local invasion into the vasculature (intravasation), whereas the microenvironment at the secondary site needs to support cell adhesion, proliferation, and neovascularization. The identification of these two rate-limiting steps to metastasis is consistent with the ‘seed and soil’ hypothesis originally put forth by Dr Stephen Paget in 1889 and whose seminal work was recently reviewed [30]. Briefly, Paget proposed that the metastatic cell (the seed) requires an appropriate environment (the soil) for successful growth at the secondary site [31]. One key role for the microenvironment that has gained prominent recognition is neovascularization. Tumors simply cannot progress without concomitant growth and organization of the stromal endothelial cells [32]. However, the role played by the microenvironment in tumor development extends far beyond the contribution of a blood supply [33]. Additional mechanisms by which the microenvironment influences metastatic behavior of tumor cells are varied and include the following: changes in extracellular matrix (ECM) glycoprotein composition, which can alter cell adhesion, motility, proliferation, and apoptotic rates; altered ECM-degrading proteinase activities within the stroma, which presumably facilitate movement of tumorigenic cells by disrupting stromal barriers; and release of bioactive ECM fragments and/or growth factors that can promote or suppress neoplastic progression of both stromal and tumor cells.

Pathological changes to the microenvironment and tumor progression

It has become increasingly evident that pathologic changes in the tissue microenvironment can enhance
tumor cell progression [34–37]. In one study, newly hatched chicks injected with Rous sarcoma virus only developed tumors at the site of injection, even though virus was found circulating in the blood. Furthermore, if a wound was made away from the primary tumor, a tumor developed at the site of wounding. Investigators verified that secondary tumor development was dependent on wound induced inflammation [38] – an observation consistent with a significant body of clinical data demonstrating that pathology-induced inflammation appears to be an etiologic factor in numerous epithelial cancers. The clinical association between inflammation and cancer progression is sufficiently strong to justify a myriad of ongoing anti-inflammation based cancer chemoprevention and treatment trials [39].

Importantly, tumor cells themselves can induce a reactive stroma (i.e. desmoplasia), which can contribute to disease progression. In prostate and breast cancers, normal stromal fibroblasts are replaced by smooth muscle reactive myofibroblasts, which here also referred to as carcinoma-associated fibroblasts (CAFs) [40–42]. These CAFs alter ECM composition, elevate cytokine production, and induce infiltration of inflammatory cells. Evidence for oncogenic function of these reactive stromal cells has been obtained from studies in which immortalized but non-tumorigenic epithelial cells were combined with CAFs and engrafted into athymic nude mice. Using this strategy, both prostate and keratinocyte tumorigenic conversion by CAFs has been documented [43,44]. Similarly, irradiation of an epithelial-stripped rodent mammary gland results in a reactive stroma characterized by elevated transforming growth factor-β. Subsequent injection of nontumorigenic mammary epithelial cells into this stroma resulted in neoplastic progression [45]. These and other studies demonstrate that local injury or tumor cells themselves, via paracrine signaling to stromal cells, are capable of inducing a pro-oncogenic microenvironment that is critical for tumor cell progression. Remarkably, these experiments demonstrate that the pathologic changes in tumor microenvironment can be as important for disease progression as the mutational profile of the tumor cell itself. That is, without a permissive environment, tumor progression may not occur.

**Physiologic changes to the microenvironment and tumor progression**

More recently, physiologic changes in stroma have also been implicated in tumor progression, opening up an entirely new area for investigation. Normal human senescent fibroblasts stimulate premalignant and malignant cells to proliferate in culture and form tumors in mice [46]. Krtolica and Campisi [47] proposed that, as the number of senescent fibroblasts increases with age, a pro-oncogenic tissue environment is created that drives the rise in cancer incidence that occurs with age.

In our laboratory we have focused on characterizing changes in the mammary gland microenvironment induced as a result of pregnancy. We have found that mammary gland ECM isolated from glands regressing after pregnancy has attributes of reactive stroma, namely elevated matrix metalloproteinase (MMP) activity and bioreactive matrix fragments [48]. Furthermore, in comparison with ECM obtained from quiescent glands, involution ECM significantly enhanced tumor cell motility and invasion in vitro [49]. Our data demonstrate that reproductive state alters mammary ECM composition, which influences tumor cell metastatic potential. These in vitro observations may shed light on both pregnancy-associated breast cancer and the 'dual effect' pregnancy has on breast cancer risk. Specifically, we propose that the period of active tissue remodeling that occurs during gland regression may create a microenvironment that is permissive for tumor cell dissemination. Thus, women with occult disease at the time of gland involution may be at increased risk for metastatic spread, and older women would be at greatest risk because they are more likely to have occult disease than younger women.

**The microenvironment and tumor suppression**

The question of whether the tumor microenvironment can actively inhibit metastasis is less well studied. However, the fact that tumor cells can reside for decades in a dormant state, combined with experiments that have determined that tumor cells arrive at secondary sites at high rates but fail to thrive in the new environment, effectively demonstrate that the microenvironment can exert a significant protective effect. Importantly, experimental data demonstrate that even fully malignant cells can undergo phenotypic reversion, given the appropriate microenvironment.

In an elegant concept paper, Pierce and Speers [50] proposed that this reversion occurs because of tissue interactions similar to those that determine cell fate during embryogenesis. The fact that the connective tissue of an organ can dictate epithelial cell form and function is common knowledge in the field of developmental biology, where stromal–epithelial interactions have been studied for well over a century [51]. Recently, the idea that the microenvironment can be manipulated to induce tumor cell reversion was experimentally verified. Disrupting tumor cell ECM via integrin blocking antibodies resulted in phenotypic reversion in a breast cancer model [52]. With antibody treatment, these aggressive, disorganized malignant breast tumor cells reverted to well organized, polarized cells that formed ascini in culture. Of potential in vivo relevance, it has been reported that morphologically normal tissue, adjacent to breast carcinomas, displays loss of heterozygosity similar to that displayed within the tumor tissue [53]. One interpretation of these observations is that progression of these adjacent cells to an invasive phenotype is suppressed by their microenvi-
environment. The observation that stromal–tumor cell interactions can actually inhibit disease progression brings us to the following question; what is the role of the stromal sheath that is often found encapsulating primary tumors?

**Re-evaluating the tumor stroma**

There is a fibrous tunic or sheath that encapsulates many epithelial tumors (Fig. 2). The question of whether the stromal sheath is a reaction by the host to contain the tumor or whether it is the result of tumor–stromal interactions that promote tumorigenesis has been considered for decades. Although the majority of studies indicate a promotional role for stroma in tumor progression, this may be due to an inclination for disease-based research to focus on causation. Furthermore, the focus has been on the target cell (i.e. the epithelial cell) rather than on the cell and its environment. For example, MMPs have long been implicated in metastasis. This is because it is apparent that in order to metastasize successfully, tumor cells must invade local tissues. Thus, enzymes that are capable of disrupting stromal barriers, such as the MMPs, are key candidate mediators of invasion. Importantly, tumor homogenates contain elevated MMP activity, and transfection of tumor cells with MMP expression constructs convincingly increases their tumorigenicity and metastatic potential in both *in vitro* and *in vivo* models. Based on these studies numerous inhibitors of MMPs were designed, which were very effective at blocking tumorigenesis of MMP over-expressing tumor cells in mouse models. However, clinical trials evaluating the efficacy of MMP inhibitors in human cancers were disappointing, and in some cases MMP inhibition corresponded with tumor progression.

A thought provoking synthesis of the MMP experimental studies and clinical trials was recently reported by Matrisian and colleagues [54]. In fact, in human cancers most MMPs are synthesized by stromal cells rather than by the tumor cells. Furthermore, some matrix proteolytic fragments have been identified that appear to act as tumor suppressors rather than tumor promoters. For example, angiotatin (a 38-kDa internal proteolytic fragment of plasminogen) and endostatin (a 20-kDa carboxyl-terminal fragment of collagen XVIII) are both endogenous inhibitors of angiogenesis, and suppress tumor growth in animal models [55,56]. Recently, the protease responsible for the production of angiotatin in the Lewis lung carcinoma model was identified as MMP-2 [57]. Thus, the ying–yang of MMP activity in tumorigenesis becomes apparent. We suggest that further study of the stromal sheath and the desmoplastic response of tumors will identify a wealth of additional stromal factors that will either promote or suppress epithelial tumor cells, depending on their stage of transformation.

**Targeting the stroma for cancer prevention and treatment**

An important question to be answered is whether the microenvironment can be targeted for therapeutic or preventive strategies. Because the relationship between epithelial cells and the microenvironment is complex, the success of these strategies will undoubtedly be dependent on stage of epithelial cell transformation. For example, in a rodent model, hypoxia induced by angiogenesis inhibition resulted in stimulation of tumor cell invasion rather than inhibition, as anticipated [58]. This unintended effect – promotion of metastasis – indicates that the ‘genomic’ flux of the tumor cell must be taken into account when developing antiangiogenic therapies [59].

However, data from two chemoprevention studies provide proof of concept that the microenvironment can be targeted to inhibit mammary tumor progression. We demonstrated that chemopreventive doses of difluoromethylornithine and retinoids inhibit progression of chemically induced rat mammary tumors [60]. Treated animals exhibited reduced mammary epithelial complexity, similar to that observed in mammotrophic hormone depleted mammary glands. Furthermore, changes in mammary gland stroma, consistent with a desmoplastic reaction, were apparent. Cumulatively, our data are consistent with these chemopreventive agents causing a disruption of epithelial cell–ECM interactions, resulting in epithelial cell loss by apoptosis and subsequent protection of the

![Figure 2](image_url)

Ductal carcinoma of the breast surrounded by a fibrous stromal sheath. Depicted is a single duct containing an intraductal carcinoma. White arrows show lumen-like structures; however, normal epithelial cell polarity is lost. Black arrows show delineation between tumorigenic mammary epithelial cells and stromal tissue. The asterisk shows stromal sheath encapsulating the ductal carcinoma *in situ*. Magnification: 400×. From University of Connecticut’s Virtual Pathology Museum (http://pathweb.uchc.edu).
mammary gland from tumor progression [48,60,61]. Similarly, mammary glands of rats treated with protective doses of conjugated linoleic acid exhibited a desmoplastic stromal reaction, decreased epithelial cell proliferation, and a decrease in angiogenesis [62]. Given the importance of stromal–epithelial interactions in determining normal mammary gland development, tissue homeostasis, and tumor progression, the observation that preventive agents can target stroma is not surprising. The challenge remains to gain a full understanding of these interactions in order to maximize treatment efficacy for each stage of transformation.

**Conclusion**

In summary, epithelial cell transformation is necessary but not sufficient for metastasis. The microenvironment is as rate-limiting with respect to metastatic success as is the genotype of the tumor cell itself. Stromal cells contribute to both tumor cell suppression and progression, with current data suggesting that the ratio of stromal inhibitors to promoters determines tumor cell fate. Finally, even fully malignant cells appear to be able to undergo phenotypic reversion in the appropriate environment. A model depicting the permissive and suppressive functions that the microenvironment can impart on tumor cell phenotype is summarized in Fig. 3. Given the critical role of the environment to metastatic success, it is likely that control of metastasis will only be accomplished by investing in stroma research to the same degree as is focused on the tumor cell itself.

**Competing interests**

None declared.

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