Hallmarks of Metastasis

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Abstract Metastasis is rarely due to accidental sloughing off of cancerous cells from a nonmalignant tumor and colonizing elsewhere; on the contrary, it is an active process requiring genetic and/or epigenetic mechanisms leading to the formation of a cell capable of responding to certain chemotactic signals that direct motility, interacting with other cells to be co-translocated, implanting in foreign locations, avoiding immune response, being refractory to growth inhibitory signals, and proliferating independently of growth factors for sustained cell division. The complexity of these processes necessitates a detailed understanding of the molecular and cellular mechanisms behind each of these steps. In this chapter, we will discuss hallmarks of metastatic process, along with theories proposed, genes involved, techniques to monitor, and therapeutic implications.

Introduction

Cancer is a general term describing hundreds of diseases in which cells aggressively proliferate without regard for normal growth limits of the original tissue or organ site and then invade surrounding and adjoining tissues. Most cancers are diseases of the epithelial tissue [1], where in late stages the cancerous cells invade the mesoderm and the endodermal layers. Metastasis, the subsequent spread of these invasive cells throughout the body to other organs, accounts for 90% of human cancer deaths [2]. Interestingly, 5-year survival of stage IV patients is a dismal 3%. By contrast, 5-year survival of early-stage cancers is 49% [3]. Thus, in addition to early detection efforts, understanding the mechanisms of metastasis and halting its course are imperative to the treatment of cancer.

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Traditionally, metastasis has been characterized as a late-stage phenomenon in cancer. Pathologic staging describes first the size and local invasion of a primary tumor, then metastasis to lymph nodes, and finally distal metastases. Gene expression profiling studies [4] suggest that metastatic potential is intrinsic to all tumor cells and that metastatic spread could be an early event in tumorigenesis. However, there is some evidence for the existence of a small population of cells within a tumor, which exclusively are capable of metastasis – the so-called cancer stem cells.

Regardless of which cells are capable of metastasizing, metastasis is an extremely complex process and, thankfully, highly inefficient as only a small fraction of tumor cells are actually able to fully metastasize. The process involves migration of a cancerous cell out of the original location, overcoming barriers to implantation in a foreign location, subsequently dividing uncontrolled, and/or metastasizing further. Whereas in most cancers the cell cycle checkpoint arrest is overcome by at least one transformative event early in cancer development in the traditional model of metastasis, a second genetic or epigenetic event is usually necessary for transition of a non-metastatic tumor to metastatic. Metastasis is rarely due to accidental sloughing off of cancerous cells from a nonmalignant tumor and colonizing elsewhere; on the contrary, it is an active process requiring genetic and/or epigenetic mechanisms leading to the formation of a cell capable of responding to certain chemotactic signals that direct motility, interacting with other cells to be co-translocated, implanting in foreign locations, avoiding immune response, being refractory to growth inhibitory signals, and proliferating independently of growth factors for sustained cell division. The complexity of these processes necessitates a detailed understanding of the molecular and cellular mechanisms behind each of these steps.

The Metastatic Process

Cancer metastasis involves several interrelated steps, each of which can be rate limiting in that failure to achieve any state can shut down the entire metastatic process. Moreover, only certain cells within a heterogeneous tumor population are capable of achieving these steps. Metastasis consists of (1) detachment of epithelial cells from the extracellular matrix (ECM), (2) survival within the bloodstream, and (3) growth at the metastatic site (Fig. 1).

As metastasis tends to be the lethal aspect of cancer, dissecting its biological basis is of utmost importance in pinpointing therapeutic targets to prevent and cure it. The existence of lymph node metastases in a cancer is a strong indicator of survival in patients as well as a prognosticator of whether other distal metastases will develop. In some cancers, lymph node metastases are better indicators of distant metastasis than in other cancers. In head and neck cancer, for example, the correlation is strong – the presence of lymph node metastases in the neck halves the survival rate in patients [5]. Moreover, only 7% of

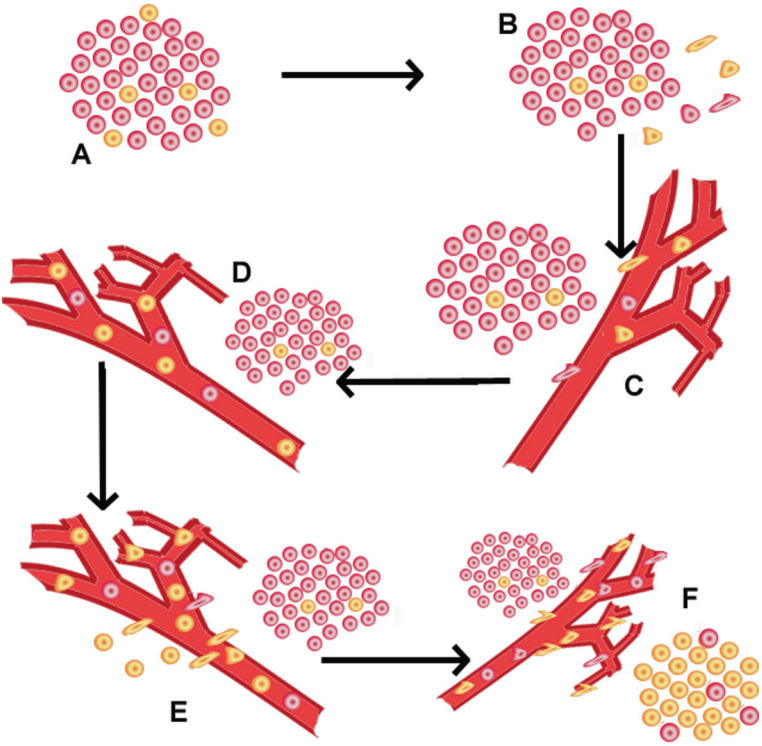

Fig. 1 Stages of metastasis. (**A**) Tumor; (**B**) detachment of tumor cells from the ECM; (**C**) intravasation of tumor cells into the bloodstream; (**D**) transport of tumor cells through the bloodstream; (**E**) extravasation of tumor cells at distal site; and (**F**) growth of metastatic lesion

patients whose necks are free of lymph node metastases develop distant metastases. This strong correlation seems to indicate that metastasis somehow involves infiltrating the lymphatic system and using it to migrate through the body. In other cancers such as breast cancer, however, 20–30% of patients whose axillary lymph nodes are free of disease still develop distant metastases. Thus, there seems to be another distinct pathway of metastatic cell dissemination that is independent of the lymphatic system. This second pathway has been shown to utilize hematogenous routes in the vascular system. Even so, the presence of axillary lymph node metastasis continues to serve as a good indicator of whether the disease will spread [6].

All cells have the default fate of apoptosis or programmed cell death. Most cells depend on extracellular signals to keep the trigger for apoptotic cascade of proteolysis at bay. Cancer cells, in general, are refractory to apoptotic signals, or their mitotic division is independent of stimulation by growth factors. As the potential for developing a tumor is directly correlated with resistance to

apoptosis, so is resistance to apoptosis correlated with the metastatic potential of a tumor. The reason behind this correlation is somewhat unclear. It is possible that certain rare cells having the ability to undergo mitosis independently of growth factors preferentially become metastatic; alternatively, the same molecular events that give rise to metastatic transformation of a tumor cell cause it to be resistant to apoptosis. Therefore, anoikis (cell death by disruption of cell adhesion and cell–ECM interactions) and amorphosis (cell death by loss of cytoskeletal structure) are vital to preventing metastasis. Normally anoikis and amorphosis are triggered by detachment of usually adherent cells from the ECM and through disruption of the actin cytoskeleton [7], which is consistent with the general observation that specific cell–cell and/or cell–matrix contact and ligand-mediated signaling are necessary to keep the apoptotic cascade from being activated. Abrogation of the need for signaling through contact for suppressing apoptosis might, therefore, lead to both immortalization and cell detachment. Alternatively, the two processes might be unrelated.

Theories of Metastasis

It is currently unclear whether any given cell within a tumor once transformed into the metastatic stage can migrate and form a secondary tumor or whether a special group of cells within a solid tumor, cancer stem cells, a rare tumor cell type with indefinite self-renewal capability, is the only cell type capable of migrating and colonizing secondary tissues and organs. In the traditional model of cancer metastasis, every malignant tumor cell supposedly possesses metastatic potential. A normal cell accumulates random mutations eventually leading to cancer, and these neoplastic cells continue to accrue mutations until some become metastatic by chance. Nevertheless, small populations of cells in many malignant tumors, including acute myeloblastic leukemia (AML) [8], glioblastoma [9], small cell lung cancer [10], non-small cell lung cancer [11], malignant melanoma [12], and breast cancer [13], display properties reminiscent of stem cells, the cell group that indefinitely retains the property of self-renewal by mitosis [14]. It is conceivable that migration of these cells could in principle lead to metastasis and successful colonization at a distant site, and this may explain why successful primary metastasis is a relatively rare event. Strong evidence implicating these "cancer stem cells" (see Chapter 3) in metastasis is provided by the observation of overlap between the genes and signaling pathways necessary for normal stem cell motility and those for metastatic cancer cells [15]. Invasive metastasis appears, for a number of cancer types, to be a property of a subpopulation of tumor cells which appear to have stem cell-like properties. Since differentiated cells rarely reenter the somatic stem cell state, progeny of previously differentiated cells should rarely metastasize.

Dissemination from the Primary Tumor

The first stage of cancer metastasis is signaled by the detachment of cancer cells from the ECM before entering into the bloodstream. Changes in cell motility are major factors in enabling separation from the primary tumor, and changes in cell fate associated with cytoskeletal reorganization are needed for transition to the invading cell type. Cells display two major types of morphogenesis correlated with early metastatic ability. In the more common version – epithelial–mesenchymal transition (EMT) – the cell elongates, secretes extracellular enzymes to locally degrade the ECM, and migrates out (see Chapter 4 for discussion on EMT). While TGFβ in cooperation with the Ras-GTPase signaling pathways can induce EMT, it is unclear whether cells that are so transformed indeed constitute metastatic cells [16]. The other, more aggressive, type of motility is amoeboid transcription: the elongated cells take on a spherical morphology, and these spheroidal cells deform through pre-existing gaps in the ECM to disseminate into the bloodstream (Fig. 2). There is also a third, rarer, form of cellular motility called collective migration that involves simultaneous mesenchymal motion of a cluster of cells.

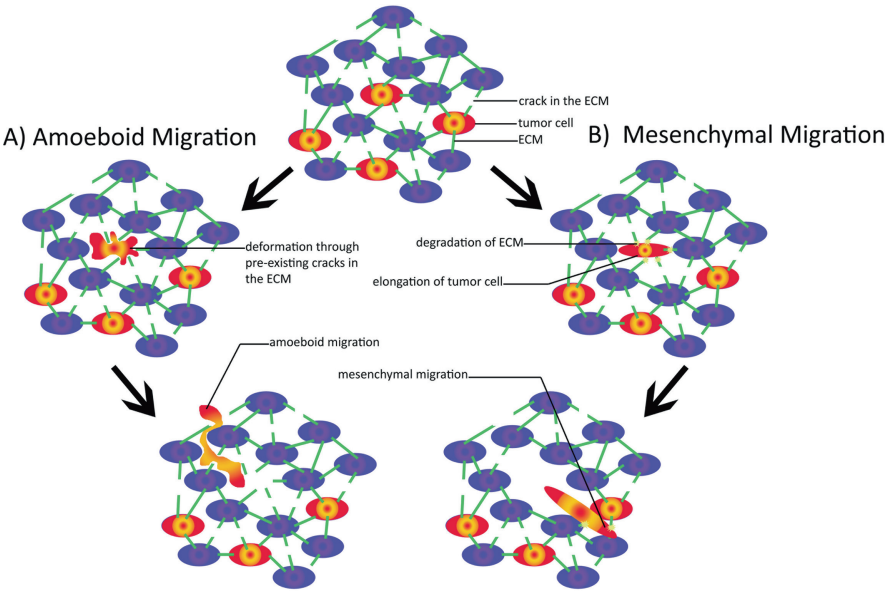

Fig. 2 (**A**) Amoeboid motility. (1) Epithelial tumor cells in the ECM. (2) Deformation through pre-existing gaps in the ECM. (3) Amoeboid migration through cracks in the ECM. (**B**) Mesenchymal motility. (1) Epithelial tumor cells in the ECM. (2) Elongation of tumor cell and degradation of ECM. (3) Degradation of ECM and mesenchymal migration

Intra- and intercellular signaling mechanisms enable these morphogenetic processes, and cell–cell cooperation is likely involved in controlling swarm-like behavior of rare metastasizing cells. Cells, however, appear to be capable of switching between different forms of motility, which renders therapeutics that target genes or proteins associated with distinct aspects of mobility somewhat refractory [17]. There is, nonetheless, evidence suggesting that certain transcriptional regulators may control entire sets of motility genes. For example, AP-1 transcription factor activity is correlated with expression of cell motility genes [18]. Twist, Six-1, and BRMS1, all transcriptional regulators, have also been implicated. Other genes involved in cell–cell signaling such as ErbB1, encoding epidermal growth factor receptor (EGFR), are implicated in cancer cell motility but not necessarily in growth of the primary tumor. Genes or proteins that are specifically implicated in cell motility in metastatic transformation are potential drug targets.

The significance of EMT lies in that disseminating metastatic cells must be able to survive without normal matrix components and evade anoikis. This survival is important in metastasis because intra/extravasating tumor cells either do not adhere to a matrix at all or encounter foreign matrices along the way [19]. Overexpression of BCL2 increases the metastatic potential of breast cancer epithelial cells by inhibiting matrix-degradation-induced apoptosis but does not affect primary tumor growth or cell motility [20, 21].

The developmental signaling pathways Wnt, Notch, and Hedgehog have also been linked to EMT [19]. Wnt signaling is of particular interest as it has been associated with collective migration and is aberrantly activated in lung cancers [22]. With regard to metastasis, loss of signaling by Wnt1, the first of the Wnt proteins to be discovered, has been shown to reduce the size of lung metastases in mice [23].

As stated earlier, most motile cells normally move mesenchymally (Fig. 2). After tumor cells undergo EMT, they migrate by polarizing and extending pseudopodia-like projections (lamellipods) on their anterior ends, binding specific cell surface or extracellular matrix ligands, pushing themselves forward through actin-based contractions of the cell body, and then releasing the adhesive bonds at the rear. Adhesion to the ECM substrate is mediated through interactions of beta-integrins, a major group of cell surface receptor ligands. Subsequently, signaling by the integrins, as well as integrins themselves, cooperate with and recruit cell surface proteases (such as matrix metalloproteinases, MMPs) to locally degrade the ECM. MMPs break down collagen in the ECM (collagenolysis). Mesenchymal motion is proteolytic and path generating: gaps in the ECM through which the cell ultimately passes are created by the cell itself [24]. Actin filaments are the dominant structural component of lamellipods [25]. H-, N-, and K-Ras are small GTPases that promote mesenchymal lamellipod extension by regulating *PtdIns(3,4,5)P3* levels. Cdc42 and Rac1 are also small GTPases that promote formation of actin-rich protrusions. Unfortunately for patients, near-total inhibition of cell surface proteases by protease inhibitor

treatment induces conversion of mesenchymal cells to spherical morphology and virtually no change in migration rates [24].

Amoeboid motion – where spherically shaped cells deform and slip through pre-existing cracks in the ECM – is protease independent and path finding (in contrast to the proteolytic path-generating nature of mesenchymal locomotion) (Fig. 2). RhoA (a small GTPase) activates the ROCK protein, which phosphorylates MLC2, a myosin light chain protein, which in turn activates a signaling cascade implicated in the development of spheroid structure and induction of cellular locomotion. The Smurf1 protein, an ubiquitin ligase, is responsible for targeting RhoA for degradation. When Smurf1 is activated, RhoA activity is depleted due to ubiquitin-mediated proteolysis, possibly involving the proteasome, and cells form lamellipods that aid in mesenchymal cell movement. On the other hand, when Smurf1 activity is downregulated, the RhoA cascade is activated and induces amoeboid cell invasion, which is actually more aggressive than mesenchymal motion [26].

The signaling cascades leading to different types of motion ultimately influence cytoskeletal elements to reorganize and the molecular motors to generate force that leads to cellular motion. Altered MLC organization is related to the amoeboid tumor cells' ability to generate sufficient mechanical force to deform the extracellular mesh of collagen fibers and to enable the cell to push through the ECM [27]. ROCK regulates MLC phosphorylation, and inhibition of ROCK (but not of MMPs) reduces in vivo cancer cell motility. The protein ezrin is localized in the direction of cell movement in amoeboid cells [28]. Ezrin provides a functional link between the plasma membrane and the cortical actin cytoskeleton of the cell. Forced ezrin expression induces a highly metastatic state in certain poorly metastatic tumor cell lines [27]. Combined blockade of extracellular proteases and ROCK prevents tumor cells from switching between types of motility and also blocks cell invasion.

In a study to identify a gene expression signature associated with the propensity for metastasis, invasive breast cancer tumor cells were collected in vitro by virtue of their chemotactic ability (migration toward a source of EGF), and their mRNA expression levels were assayed in relation to their less invasive counterparts [17]. Genes associated with motility were most strikingly differentially regulated in invasive cells compared to those in non-invasive cells. For example, cofilin, Arp2/3 complex, and capping protein, all involved in lamellipod protrusion, extension, and tail retraction, were coordinately upregulated in the invasive cells. Genes encoding Rho and ROCK were significantly upregulated. Upregulation of cofilin, Arp2/3, and capping protein results in increased protrusion velocities of up to 10-fold higher than those in cells with lower levels of expression of these proteins. By contrast, the ZBP1 gene is strongly downregulated in invasive breast carcinoma cells. ZBP1 binds to beta-actin mRNA and localizes the mRNA to the leading edge of cells. Beta-actin is the most common form of actin which is polymerized as filaments within the lamellipod and is acted on by cofilin, capping protein, and the Arp2/3 pathways. The site of sub-cellular localization of the ZBP1 protein likely determines, by controlling

localization of the beta-actin mRNA (thus its site of translation), the site at which these pathways converge. Downregulation of ZBP1-mediated beta-actin mRNA targeting, which is associated with inhibition of lamellipod formation in mesenchymal cells, results in the formation of highly invasive amoeboid cells and increased chemotaxis [4].

Vascular Transport of Metastatic Cells

Even though both mesenchymal and amoeboid cells can migrate toward and intravasate into blood vessels, amoeboid cells are better suited to survive within the vascular system. A mesenchymal to amoeboid transformation is important during entry into blood vessels because elongated mesenchymal cells tend to shatter, or undergo amorphosis, in response to the force of blood flow (hemodynamic shearing). The spheroid morphology characteristic of amoeboid cells can better withstand high shear stress and hence these cells survive better in the bloodstream [29]. Thus, solitary cancer cells in circulation are sensitive to apoptosis, particularly to that induced by mechanical stress and immune-mediated destruction. Potentially metastatic cells that have entered the bloodstream are destroyed either by mechanical stress or, supposedly, by immune-directed cell death.

Once disseminated into the bloodstream, tumor cells are able to circulate throughout the body. Oftentimes cancer patients have significant quantities of these cells both in blood and in bone marrow long after removal of the primary tumor and before any sign of metastasis occurs [30]. Are each of these dormant cells capable of producing their own clonal metastases or do only a fraction possess the pluripotency to form the seed of metastatic malignancy? To answer this question, a distinction between the tumor cells circulating in peripheral blood (circulating tumor cells – CTCs) as opposed to those aggregated in the bone marrow (disseminated tumor cells – DTCs) must be established.

DTCs have been documented in the bone marrow for most types of epithelial cancers [30]. While a number of studies have revealed correlation between the presence of DTCs and postoperative metastatic relapse, viable use of DTCs as prognosticators of recurrence has yet to be unequivocally demonstrated. A point of interest is that presence of DTCs is associated not only with bone metastases but also with distal tumor development in lung, brain, and liver; thus, it is likely that DTCs accumulating in the bone marrow eventually reenter the vasculature to travel throughout the body [31]. Incidentally, the processes by which tumor cells disseminate appear to vary by cancer. In early-stage breast cancer, DTCs are heterogeneous and do not possess the same changes as the tumor; however, in late stages the DTC genotypes are largely homogeneous. This observation seems to indicate that dissemination of DTCs is an early event after which the cells accumulate other mutations, some of which eventually overwhelmingly favor metastasis. In prostate cancer, however, CTCs appear

genotypically homogeneous and similar to cells in the primary tumor. This observation suggests that as the tumor develops, cells with metastatic potential eventually arise directly from the primary tumor and spread forth throughout the body [30].

At present, only limited data exist correlating DTCs with concurrent CTCs, and the significance of CTCs in peripheral blood is as yet unclear [30]. In most cancers, patients appear to present with a higher fraction of DTCs than CTCs, suggesting that bone marrow may provide better conditions for tumor cell homing and survival [30]. Another hypothesis of note is the speculation as to whether surgery itself can dislodge tumor cells from the primary malignancy, thereby allowing these cells to become CTCs. Bone marrow possibly forms a pre-metastatic niche and that it offers a site for dormancy is evidenced by the presence of DTCs in colorectal carcinomas, a cancer in which bone metastases are rare [31].

It is usually assumed that invasive tumor cells entering blood vessels and foreign tissues will be recognized and targeted by the immune system. Thomas and Burnet [32, 33] suggested that immunosurveillance was responsible for the targeted elimination of cancerous cells, particularly in that immune response is often associated with advanced carcinogenesis (for discussion on immunesurveilance and tumor progression see Chapter 6). Indeed, immune surveillance may contribute to dormancy in DTCs. The nature of dormancy is variable in that in some cases it is characterized by a balance between apoptosis and proliferation whereas in other cases it describes either non- or slowly proliferating cells. It has been shown in murine models that upon depletion of CD4 + and CD8 + leukocytes, progressive growth is initiated in previously dormant tumor cells [30]. In lung cancer, increased presence of CD8 + cytotoxic lymphocytes has been observed, but this escalation in immune response does not appear to correlate with outcome. Tumor cells appear to evade the immune system because, as native cells, they are poorly immunogenic. Moreover, tumor cells also downregulate antigens by interfering with antigen-presenting cells and secrete cytokines that may aid in both immune tolerance and suppression. Tumor cells are often also resistant to cytotoxic T-lymphocytes as evidenced by the cells' failure to undergo apoptosis upon attack. Nonetheless, reduction of malignant tumor has been observed alongside bacterial infection, leading to hope of an anti-tumor vaccine created from dead bacteria. Although no conclusively successful anti-tumor vaccines have yet been reported, a number of such vaccines have entered clinical trials [34].

All this being said, our understanding of the role immunosurveillance plays in maintaining dormancy is murky, at best. In most cases, tumor development appears to be similar in normal and in immunocompromised animal models, and any systematic correlation between immune deficiency and human cancer has yet to be demonstrated. Thus, it is unlikely that immune surveillance offers much protection against anything but pathogen-associated cancers [35].

What then of the heightened immune cell activity at primary tumor and metastatic sites? Macrophages have been observed to produce matrix metalloproteinases that promote tumor cell mobility by degrading the ECM. These

tumor-associated macrophages (TAMs) express EGF and promote EGFR-dependent cell invasion (for more detailed discussion of TAMs and other immune cells see Chapter 11). TAMs tend to aggregate along blood vessels and tumor margins and create an EGF gradient responsible for directing chemotaxis. This gradient likely promotes intra- and extravasation. In fact, prior to extravasation, leukocytes are recruited when tumor cells attach to blood vessel walls, and the leukocytes are thought to extravasate ahead of the cancer cells, in essence by ushering them out [36].

Proliferative Ability at Site of Metastasis

Metastasis has been directly correlated, on a single animal basis, with the blood burden of tumor cells. A high concentration of CTCs in the bloodstream is related to a higher likelihood of metastasis [17]. Nevertheless, most cells die rapidly after extravasation [37, 38], which is likely why DTCs seem to correlate better with prognosis than CTCs. In as far back as 1889, Stephen Paget posited the "seed and soil" hypothesis [39] – that metastatic ability was dependent upon cross-communication between specific tumorigenic cells (seeds) and the distal organ microenvironment (soil). The more tumor "seeds" that are available in distal organs, the more likely it is that some will take root and metastasize. The actual site of metastasis is, incidentally, not directly linked to blood profusion through that specific organ. This is because certain markers at the secondary tumor site genetically predispose tumor cells to attach and develop into micro-metastases – the importance of "soil."

Organs with dense capillary beds (bone, liver, and lung) are common metastatic sites probably because CTCs are mechanically arrested in small vessels [7]. That being said, certain cancers are predisposed to metastasize to certain organs and there is no systematic correlation between blood burden and metastasis on an organ level. Instead, organs with high metastatic potential in a specific instance tend to exhibit predisposition toward angiogenesis. For example, cells of these tissues express high levels of growth factors such as VEGF, HGF, FGF, and EGF. These growth factors stimulate macrophages to produce MMPs that locally degrade the ECM and allow tumor cells to take root and proliferate. CD44 has been implicated in tumor cell adhesion and is important for endowing the expressing cells with the ability to form micrometastases at distal site. Disruption of the CD44–ECM interaction tends to induce apoptosis and prevent metastasis [40].

Without a blood supply, tumor cells adhered at a secondary site constitute foci of dormant micrometastases, and although they may remain dormant for years, they undergo rapid proliferation once angiogenesis occurs. While dormant, these cells are largely quiescent or, in some cases, proliferate at extremely slow rates. Once growth factor-induced angiogenesis takes place, however, the cells begin to rapidly divide. Blood supply provides oxygen, growth factors, and nutrients vital to the proliferation of metastatic cells. Angiogenesis is triggered

when angiogenic inducers (mainly growth factors) are favored over inhibitors (for further discussion on angiogenic diversity see Chapters 7 and 8). The inhibitors tend to be ECM proteins or protein fragments such as thrombospondin and endostatin [31]. Inhibition of angiogenesis appears to stymie metastatic spread and has thus received much attention of late with regard to targeted therapeutics.

Genes Involved in Metastasis

Are metastatic cancer cells genetically different from non-metastatic cancer cells? The genetic variability usually associated with most solid tumor cells provides a window into the mechanism of metastatic spread. The spectrum of genetic variability among secondary tumors of diverse locations resembles more closely those present in cells of the primary tumors in the same individual than either is to the same cancer type from different individuals [41]. These results are consistent with the idea that metastatic cells are clonally derived from primary tumor cells but do not necessarily signify that metastatic tumors, or tumors in general from different individuals, are genetically heterogeneous. On the contrary, the question of whether the same or similar genetic or epigenetic changes are necessary for all metastatic cells remains open.

Given the complexity of the metastatic process, genetic mechanisms behind it are likely to be complex. The motivations for studying genes involved in metastasis are 2-fold: understanding the biological basis of the process and finding a molecular signature of metastasis for better diagnosis, prognosis, and therapeutic intervention to restrict it. Both motivations are well served by studies that aim to identify the predominant genetic factors correlated with metastasis. A groundbreaking step in understanding the genetic basis of metastasis was taken by Ramaswamy et al. [42] when the authors measured genome-wide gene expression profiles of 12 samples of confirmed metastatic adenocarcinomas of diverse origins (breast, lung, prostate, uterine, and ovarian cancers) and compared them with those obtained from 64 confirmed non-metastasizing cancers of the same types. The comparison yielded a best descriptor transcript set of 128 genes, of which 64 were overexpressed and 64 were underexpressed in the metastatic cancer samples. The descriptor gene set did not provide any obvious set of genes with related function. In fact, some genes that are underexpressed are unexpected (e.g., *MLC2*) and others that are overexpressed are of unknown significance for metastasis (e.g., glucose phosphate isomerase). From this larger gene set, the authors derived a core gene expression signature with a refined set of 17 metastasis markers (8 overexpressed and 9 underexpressed). These 17 genes performed well as predictors of metastasis on other unrelated tumors with or without metastasis. This refined core set of genes also included several genes whose expression signatures defy simple logical expectations: e.g., actin gamma 2, myosin heavy chain 11, and myosin light chain kinase genes are underexpressed, whereas lamin B and type 1 collagens $\alpha 1$ and $\alpha 2$ are

overexpressed. Thus, despite the obvious utility of this core set of gene expression signature markers for more accurate prognosis, a biological understanding of their basis was not forthcoming.

The results of Ramaswamy et al. [42] can in principle be interpreted to mean that a majority of cells in solid tumors, which carry a signature set of gene expression values defining potential for metastasis, are able to metastasize. This conclusion could be drawn because a minority contribution of rare metastatic cells to the overall mRNA levels would have gone undetected in their experiments and could give credence to the traditional theory of metastatic progression. Alternatively, it might also mean that a small population of cancer stem cells in these tumors are actually capable of metastasis, yet by cell division, they give rise to two cell types: one along the linear stem cell line that maintains a constant cell number and the another that proliferates to differentiate into non-stem cell character but retains the epigenetic signature of the original metastatic stem cells. The reason for not detecting this core gene expression signature in non-metastatic tumors might just be that the stem cell populations in these tumors are below a critical number or that there is a reversal of epigenetic signatures among some of their progeny.

A biologically insightful understanding of metastasis has come from identifying genes that suppress tumor metastasis using several different in vivo metastasis assays [43]. Metastasis suppressor genes, or MSGs, are a special class of genes that are turned off in metastatic cells but, when re-expressed, inhibit metastasis without affecting tumorigenicity. At least 12 suppressor genes have been identified to date, beginning with the discovery of NM23 in 1988 [44]. Other MSGs include *NME1* – a member of the nucleoside diphosphate kinase family of proteins implicated in cell cycle regulation, *KISS1* – a regulator of metalloproteases and a ligand of a G-protein-coupled receptor [45], a mitogen-activated protein kinase gene (*MKK4*), and *BRMS1* which functions in gap junctions and reduces motility. Each of these genes provides interesting anchor to the spectrum of events thought to be responsible for distinct cellular stages of metastasis. In lung cancer, the invasion suppressor CRMP1 (collapsin response-mediator protein 1) has been identified as an invasion suppressor, but its efficacy as an MSG has only been demonstrated in vitro and thus has yet to be validated as a true MSG [44]. More work is needed in this direction to understand the detailed molecular pathways that integrate functions of MSGs in gene regulatory and signaling networks.

Techniques for Monitoring Metastasis

Because only a small fraction of malignant cells eventually metastasize, and these are difficult to identify early in a heterogeneous tumor cell population, studying metastasis has proven difficult until only recently. With the advent of refined optical imaging techniques, on both a whole-body and microscopic

scale, and by the identification of cellular and molecular markers to define the metastatic stage, a better understanding of metastasis is now possible. Whole-body imaging allows non-invasive study of the tumorigenic and metastatic processes and their development within a single organism. Microscopic techniques allow morphological analysis on a cellular and sub-cellular level [46]. Currently, most in vivo techniques for studying metastasis are usable only in animal models.

Goodale et al. developed a flow cytometry method to quantify CTCs in mice and further adapted this technique along with laser scanning cytometry methods to study both bone marrow and lymph node dissemination of tumor cells [47]. Essentially, mice were injected with metastatic human breast cancer cells and at progressive time points, the animals were sacrificed for harvest of peripheral blood, lymph nodes, and bone marrow. These samples were then fluorescently labeled and studied using cytometric techniques. Unfortunately, since this method requires sacrifice of the animal model, it is not translatable to human research.

Multiphoton confocal microscopy has also been used to study metastatic cells in vivo by tagging these cells with green fluorescent protein and tracking their motion but again, this technology is not approved for use in clinic patients [46]. Sipkins and colleagues have used dynamic intravital confocal imaging to demonstrate unique regions within bone marrow to which metastatic leukemia cells may home [48]. A group at the University of Pennsylvania employed GFP tagging to study apoptosis in potentially metastatic melanomas and found that propensity to apoptose after arrest in pulmonary vasculature was a distinguishing factor between metastatic and non-metastatic cells [49].

Most human models of metastasis involve ex vivo analysis of cells purified from either blood or surgically resected samples [46]. Such methodology, however, limits the insight gained regarding the initial process of metastasis away from the original tumor. In terms of monitoring metastases in patients, PET and CT scans are used to regularly check cancer patients for new lesions and pathologists use traditional observation and staining methods to determine whether these lesions are new primary cancers or secondary or tertiary metastases.

Therapeutics and Future Directions

Since, as mentioned earlier, metastasis accounts for nearly 90% of cancer-related deaths, recognizing and preemptively treating carcinomas with high metastatic potential is vital to reducing disease mortality. Identifying and targeting the so-called cancer stem cells is a key step in such early treatment. However, to do so requires definite identification of such cancer stem cells as well as therapeutic regimens that demonstrably target only cancer stem cells but not normal stem cells [50].

Apoptosis resistance has been shown to be a key feature both in tumorigenesis and in metastatic spread but the direct correlation with metastasis has yet to be illuminated due to limited models [7]. Moreover, a cell's intrinsic survival properties likely play a role in its ability to survive in a distal microenvironment, and these properties must be elucidated to better target highly metastatic cells.

Total blood and bone marrow burdens of disseminated cells seem to play a role in the likelihood of cancer recurrence, so early detection of these disseminated tumor cells could perhaps determine whether patients should undergo systemic therapies adjuvant to surgical resection. Although all such therapies do target disease relapse, there is currently little selection in place to determine which patients are at greater statistical relapse than others, leading to toxic and unpleasant overtreatment of patients [51]. For example, currently less than 25% of breast cancer patients lacking overt lymph node metastases suffer from relapse within 10 years after operation, but greater than 90% receive chemotherapy [52]. In non-small cell lung cancer (NSCLC), patients presenting with early-stage disease generally forgo adjuvant therapy post-resection, but some of these patients, those who suffer relapse within 5 years of operation, need to be selectively identified for therapies complementing surgery [53].

Inhibition of cellular motility is also an important target, particularly in managing early-stage disease [17]. As early detection becomes more commonplace, targeted therapies limiting dissemination of cancers that have not yet undergone micrometastases become more important. Unfortunately, evaluating the effectiveness of therapies based on limiting invasiveness of tumor cells has proven difficult because cellular motility cannot be assessed in patients and histological analysis has thus far proven unreliable. In fact, this difficulty in assessing the efficacy of motility-targeting therapeutics was a likely cause for the failure of clinical trials using MMP inhibitors [54].

A major area of current study on therapeutic directions focuses upon the targeting of angiogenesis. Without its own blood supply, a distal micrometastasis is unable to continue proliferation. Angiogenesis appears to be closely linked to the presence of a variety of growth factors and their receptors, in particular basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF) [55], and epidermal growth factor receptor (EGFR) [56]. These factors have been shown to be of importance for tumor growth and invasiveness. A majority of lifetime non-smoking NSCLC patients, females of Caucasian and Asian descent in particular, present with an EGFR mutation treatable by the targeted small molecule EGFR inhibitor erlotinib (Tarceva) [57]. In recent years, various solid tumors have been treated with a reasonable degree of success by targeted monoclonal antibodies (MAbs) against EGFR and VEGF. Such therapies include cetuximab, panitumumab, and bevacizumab [56]. The two former antibodies target EGFR whereas bevacizumab is a humanized IgG1-type MAb directed against soluble VEGF. Bevacizumab, in particular, has become part of the standard first-line chemotherapy regimen for NSCLC patients [57].

In conclusion, the metastatic cascade is grossly implicated in the lethality of cancer, lung cancer included, and dissecting the process is essential to treating the disease. Medical scientists are faced with a number of key questions in metastasis to tackle: Is metastatic spread a capability intrinsic to all cells or to only a select few cancer stem cells? Which are the genes responsible for EMT? What genes control all subtypes of cellular locomotion? Are cell detachment from the ECM and the bypass of apoptosis separate or linked processes? How do DTCs and CTCs relate to each other, and what are their prognostic and mechanistic roles with regard to metastasis? What role does immunosurveillance play in the spread of cancer? What factors cause metastatic cells to "home" in on specific organs? How do dormant disseminated cancer cells begin to rapidly proliferate? By what biology do metastatic prognosticator genes actually aid and abet metastasis? How can MSGs be better characterized, and how can they be reactivated in later stage cancers? Although the processes leading up to cellular metastasis are still poorly understood, a great deal has been learned in recent years. As the chain of events leading to metastasis is better elucidated, our ability to medically target various parts of the process will in turn be enhanced.

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